

ORIGINAL PAPER

Free-proline and total flavonoid responses in leaves of *Fagus crenata* current-year seedlings to short-term soil drought stress

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ABSTRACT

To examine effects of soil drought stress on the production of potentially stress-related compounds in current-year seedlings of *Fagus crenata*, we collected seeds from two sites in the northern region of mainland in Japan. We then germinated and grew them in well-watered (watered once every two days) or water-stressed (no watering) conditions in an environmental chamber for ca. two weeks. The concentrations of biochemical compounds in leaves were estimated to be in the range 0.81–3.44 $\mu\text{mol/gDW}$ and 2.70–5.73 mg/gDW for free-proline and total flavonoids, respectively. Negative associations were found between the seedlings' height at the start of the experiment and concentrations of free-proline and total flavonoids in leaves. After accounting for the effect of height, there was no significant difference between the two sites with respect to the concentrations of free-proline and total flavonoids in leaves of seedlings. The concentrations of free-proline in leaves of seedlings from both sites were higher under water-stressed conditions than in well-watered, whereas there were no differences between the two conditions for total flavonoids, irrespective of sampling site. These results suggest that the concentrations in leaves vary depending on biochemical compounds examined as well as properties of individual plants relating to their metabolism, and that free-proline participates, at least in part, in a rapid acclimation response to short-term drought stress in current-year beech seedlings.

KEY WORDS

Fagus crenata seedling, free-proline, growth experiment, soil drought, total flavonoids

Introduction

Japanese beech *Fagus crenata* Blume (*Fagaceae*) is the dominant tree species of the deciduous broadleaved forests that are widely distributed across the cool-temperate zone of Japan. However, based on future climate scenarios, it is predicted that beech forests will greatly decline in Japan because conditions will become less suitable for them (Matsui *et al.*, 2004). Global climate

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warming is a probable agent for the decline of beech forests; it causes evaporation of soil moisture and leads to soil desiccation (Mouri and Shinoda, 2005), imposing physiological stress on plants (hereafter referred to as ‘drought stress’). Given that seedlings are the life stage most vulnerable to environmental stress (Leck *et al.*, 2008), it is essential to examine how they respond to drought stress in order to understand species’ physiological relevance to future climates. A recent global climate trend is the increased frequency of serious environmental fluctuations and/or rapid environmental changes (Hoffmann and Sgro, 2011). Such phenomena are occurring in a much shorter period (*i.e.*, much shorter than one life history stage within a tree generation) than natural selection based on genetic variation (Darwinian evolution, Rando and Verstrepen, 2007) occurs over generations (Hoffmann and Sgro, 2011). Therefore, there is an urgent need to understand how beech seedlings rapidly respond and/or acclimate to short-term adverse environmental changes.

Producing osmoprotectants, such as free-proline, glycine betaine, and polyols is a major acclimation response to drought stress in plants (Williamson *et al.*, 2002; Kishor *et al.*, 2005; Ashraf and Foolad, 2007). Accumulation of osmoprotectants in the cells increases cellular osmolality that drives an influx of water or reduces its efflux, thus maintaining cell turgor pressure (Seleiman *et al.*, 2021). In plants, free-proline is a well-documented osmoprotectant and is accumulated in leaves exposed to drought stress (Sofa *et al.*, 2004; Aranda *et al.*, 2018). Another plant acclimation response to drought stress is to scavenge excessive amounts of reactive oxygen species (ROS) that react vigorously with compounds such as proteins and lipids, causing cell damage (Karuppanapandian *et al.*, 2011). Besides the primary metabolite of free-proline, flavonoids are major secondary metabolites that exhibit antioxidant functions; it has been reported that flavonoids are accumulated in leaves under drought stress (Shojaie *et al.*, 2016; Jafarnia *et al.*, 2018). While these different aspects of functions serve as plant acclimation responses to drought stress, experimental studies have reported that the responses to such stress (*e.g.*, changes and/or increased/decreased concentrations) vary depending on different biochemical compounds and species (Jafarnia *et al.*, 2018; Amrutha *et al.*, 2021). In addition, even in individuals of the same origin (*e.g.*, maternal families), concentrations vary with plant size (Cao *et al.*, 2018; Padilha *et al.*, 2022). Therefore, examining multiple biochemical compounds as well as their associations with plant size would provide a better understanding of the species-specific physiological responses to drought stress.

In this paper, in order to better understand species’ physiological properties, especially potential rapid response to short-term adverse environmental changes, we performed a growth experiment under different watering regimes and investigated the concentrations of two potentially drought-related biochemical compounds (free-proline and total flavonoids) in leaves of beech seedlings. The specific objectives of our study were (*i*) to examine how plant size affects the concentrations of these biochemical compounds, (*ii*) to compare concentrations of these compounds between different watering regimes.

Materials and methods

SAMPLING AND GROWTH EXPERIMENTS. In September 2020, seeds were collected from an adult beech tree at two sites in a natural forest in Aomori Prefecture, Japan (Supplementary materials Fig. S1): Shiroiwa Forest Park (40°34' N, 140°39' E, elevation 285.0 m; hereinafter Shiroiwa) and Mt. Takakuramori (40°54' N, 140°16' E, elevation 829.3 m; hereinafter Takakuramori). On 23 March 2021, three seeds were sown per polypot (12–13 cm in diameter×10–11 cm in height), each of which was filled with planting mix [vermiculite:compost (palm fiber, baked soil, bark compost,

humus, *etc.*)=1:1]. The seedlings were well-watered until the start of the experiment described below. In late April, we randomly selected twenty-four seedlings (twelve in each site) with two true leaves completely unfurled. We measured the heights of these seedlings and confirmed that there were no significant differences between treatments (see below) and sampling sites at the start of the experiment (Table S1). Then, in an environmental experimental chamber (LP-1.9P-S, Nippon Medical & Chemical Instruments Co., Ltd., Japan), we applied two watering regimes as follows. We continued to water (100 ml) once every 2 days per pot in the well-watered (WW) seedlings, whereas we ceased watering in the water-stressed (WS) seedlings (six seedlings in each combination of watering regime and site). The positions of pots were randomly changed every day to mitigate positional effects in the chamber. Environmental conditions in the chamber were: 25°C from 6:00 a.m. to 6:00 p.m. and 15°C from 6:00 p.m. to 6:00 a.m. the next day, photoperiod of 14-h (6:00 a.m. to 8:00 p.m.) light/10-h darkness (8:00 p.m. to 6:00 a.m.), 135–190 $\mu\text{mol}/\text{m}^2\text{s}$ photosynthetic photon flux density (PPFD) for the light period. Measurements of relative soil moisture content in the WW and WS treatment pots were obtained using a soil moisture meter (Lutron PMS-714, Taiwan) at a depth of 5 cm from the surface once every two days; the experiments ran until the relative soil moisture content in SW was about one third of that at the beginning of the experiment (18 days for seedlings from Shiroiwa and 15 days for those from Takakuramori). Our two-week pilot experiment showed that gravimetric soil water content (mean \pm SE) was $69.5 \pm 3.5\%$ ($n=5$) and $51.5 \pm 4.2\%$ ($n=5$) in the WW and WS groups, respectively, indicating a significant difference between them (t -test, $t=-7.389$, $P=8.789 \times 10^{-5}$). At around 1:00 to 3:00 p.m. on the last day of the experiment, we collected the two true leaves that had completed unfurled at the beginning of the experiment. These leaves were immediately frozen in liquid nitrogen and stored at -85°C until they were served for biochemical analyses.

BIOCHEMICAL ANALYSES. The concentrations of free-proline and total flavonoids in the leaves of beech seedlings were estimated by the following method. First, the fresh samples (*i.e.*, two true leaves) were powdered under liquid nitrogen, then extracted twice with 80% ethanol and once with 50% ethanol. The concentration of free-proline in the leaves was estimated by the method of Bates *et al.* (1973), where the extracts were colored using the ninhydrin method and absorbance was measured at 520 nm using a spectrophotometer (ASV11D, AsOne, Tokyo). Proline solutions (0.5 mM, 1 mM, 2 mM) and distilled water (0 mM) were used to generate calibration curves. Similarly, to determine the concentration of total flavonoids in the leaves, extracts were colored using the aluminum chloride method (Kiranmai *et al.*, 2011) and absorbance at a wavelength of 415 nm was measured. Calibration curves were prepared using quercetin solutions (25 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$) and distilled water (0 $\mu\text{g}/\text{ml}$). The concentrations of the target compounds in the extracts were measured three times per sample, and the average of these values was used as the representative value for the extract from individual seedlings. The concentrations of free-proline and total flavonoids in 1 g fresh weight (FW) of leaf tissue were estimated. Finally, to estimate the concentrations of the two biochemical compounds relative to dry weight (per 1 g of leaf DW), which can be deduced from the ratio of flesh/dry weight of leaves (*e.g.*, Forlani *et al.*, 2019), we harvested five seedlings each from the WW and WS groups and recorded the fresh weights of their completely unfurled true leaves. These leaves were then dried for 72 h at 80°C and the dry weights recorded (Table S2).

DATA ANALYSES. To analyze the effect of drought treatment on the amount of free-proline and total flavonoids in leaves of beech seedlings, we used a generalized linear model (GLM) with

a gamma distribution and logarithmic link function (f). The expected value of biochemical concentration (per 1g leaf DW) $[E(Y)]$ of the response variable can be expressed as follow:

$$f[E(Y)] = \beta_{\text{Intercept}} + \beta_{\text{Height}} \times \log(\text{Seedling height at the start of the experiment}) + \\ + \beta_{\text{Treatment}} \times \text{Treatment} + \beta_{\text{Site}} \times \text{Site}$$

where the explanatory variables were the treatment (WW=0, WS=1), the site (Shiroiwa=0, Takakurumori=1). Standardized values of seedling height at the start of the experiment were input into the GLMs. Because our pilot analyses showed there were no significant coefficients for the interactions between treatments and sites in the models for free-proline and total flavonoid (see $\beta_{\text{Interaction}}$ in Table S3), we excluded the interaction terms in the GLMs. Bayesian estimation of the coefficients of the explanatory variables was performed using Markov Chain Monte Carlo (MCMC) sampling (five independent chains, 5000 steps in each chain, 3000 steps of burn-in, and every two steps for thinning). If the 95% credible interval (CI) of coefficients did not contain 0, it was assumed that there was a significant difference (e.g., Torimaru *et al.*, 2015) due to the drought treatment or the sampling site. In addition, to check for a convergence of the posterior distribution, \hat{R} (Gelman and Rubin, 1992) was calculated and confirmed to be less than 1.1 (Burkner, 2017). These analyses were performed using R 4.1.1 (R Development Core Team, 2021), with the aid of R packages RStan (Stan Development Team, 2020).

Results and discussion

The average concentrations of free-proline in leaves (mean \pm SE) per experimental category ranged between 0.50 and 0.80 $\mu\text{mol/gFW}$ (Table 1). These values were comparable with results for *Fagus sylvatica*, in which the four-year-old seedlings were reported to have concentrations of free-proline in leaves ranging between 0.18 and 2.34 $\mu\text{mol/gFW}$ (Schraml and Rennenberg, 2000, 2002). After accounting for dry-to-fresh ratio of leaf weight (Table S2), estimates of concentration of free-proline in leaves ranged between 0.81 and 3.44 $\mu\text{mol/gDW}$ (Fig. 1), which clearly overlapped the range of ca. 0.02-2.01 $\mu\text{mol/gDW}$ per family in current-year seedlings of *F. sylvatica* (Aranda *et al.*, 2018). On the other hand, the average concentrations of total flavonoids in leaves per experimental category ranged between 1.72 and 1.94 mg/gFW (Table 1). Furthermore, the estimates of total flavonoids ranged between 2.70 and 5.73 mg/gDW (Fig. 1), which is around lower limit but still within the range for *F. sylvatica* trees of varying ages (ca. 3-13 mg/gDW , Formato *et al.*, 2022). These comparisons with available studies of the same genus suggest that the meas-

Table 1.

Summary of the concentrations (mean \pm SE) of free-proline (a) and total flavonoids (b) in 1g leaf fresh weight in current-year beech seedlings

(a)

Site	Free-proline [$\mu\text{mol/gFW}$]	
	Well-watered	Water-stressed
Shiroiwa	0.58 \pm 0.05 ($n=6$)	0.80 \pm 0.16 ($n=6$)
Takakurumori	0.50 \pm 0.04 ($n=6$)	0.67 \pm 0.10 ($n=6$)

(b)

Site	Total flavonoids [mg/gFW]	
	Well-watered	Water-stressed
Shiroiwa	1.82 \pm 0.22 ($n=6$)	1.91 \pm 0.17 ($n=6$)
Takakurumori	1.72 \pm 0.16 ($n=6$)	1.94 \pm 0.14 ($n=6$)

urements and estimates of the concentrations of free-proline and total flavonoids in the present study are credible.

The GLM analyses showed significant negative effects of seedling height on the concentrations of free-proline and total flavonoids in leaves: taller seedlings at the beginning of the treatment accumulated less of these compounds per unit dry mass of leaves at the end of the experiment (Table 2). Padilha *et al.* (2022) found a negative relationship between the concentration of free-proline in leaves and hypocotyl length of seedlings in *Phaseolus vulgaris* L. (common bean). Similarly, in the medicinal tree *Cyclocarya paliurus* (Batalin) Iljinsk., tree heights were negatively correlated with the contents of two flavonoid species (quercetin and kaempferol) in the leaves (Cao *et al.*, 2018). While respiratory costs of maintaining existing plant biomass increase with plant size (Amthor, 1984), high metabolic energy costs are also required to synthesize the

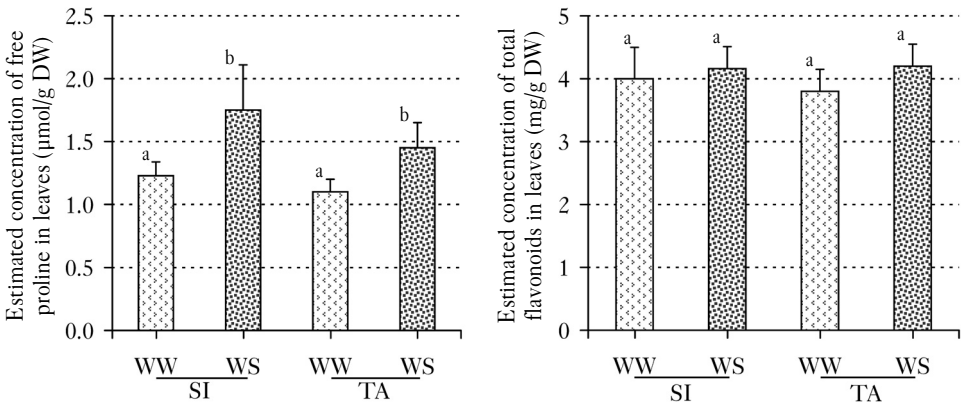


Fig. 1.

Estimates of concentrations of free-proline (left) and total flavonoids (right) in 1g leaf dry weight in current-year beech seedlings. White and gray bars represent well-watered (WW) and water-stressed (WS) groups, respectively (six seedlings in each combination of watering regime and site). SI and TA represent Shiroiwa Forest Park and Mt. Takakuramori, respectively. Error bars represent standard errors. In the panels, different letters indicate significant differences between categories (i.e., treatment-site combinations)

Table 2.

Summary of coefficients in GLMs with free-proline (a) and total flavonoid (b) concentrations per 1g leaf dry weight as response variables

(a)

Coefficients	Estimates	95% CI (lower, upper)	\hat{R}^2
$\beta_{\text{Intercept}}$	0.15	(-0.05, 0.37)	1.00
β_{Height}	-0.19	(-0.31, -0.05)	1.00
$\beta_{\text{Treatment}}$	0.40	(0.15, 0.63)	1.00
β_{Site}	-0.07	(-0.32, 0.16)	1.00

(b)

Coefficients	Estimates	95% CI (lower, upper)	\hat{R}^2
$\beta_{\text{Intercept}}$	1.30	(1.14, 1.46)	1.00
β_{Height}	-0.11	(-0.21, -0.01)	1.00
$\beta_{\text{Treatment}}$	0.15	(-0.04, 0.33)	1.00
β_{Site}	0.04	(-0.14, 0.22)	1.00

amino acid (Kaur and Asthir, 2015) and secondary metabolites (Gershenzon, 1994). Our results suggest the presence of a trade-off between the production of metabolites and plant size in the beech seedlings. In addition, shorter individuals may have had less developed root systems than the taller ones and may, therefore, have been unable to access soil moisture at greater depths (Lansac *et al.*, 1994), resulting in more severe drought stress for the former. Given the substantial increase in amounts of free-proline in the leaves of seedlings subject to drought stress (Fig. 1), it is possible that variations in root system sizes influenced the concentrations of free-proline among the beech seedlings.

Whilst there was no significant difference in the concentrations of free-proline and total flavonoids in leaves of seedlings between the two sites, watering regimes did have significant effects on the concentrations of free-proline per unit dry mass of leaves: the concentrations were estimated to be about 1.49 times [=exp (0.40)] higher in WS than in WW, regardless of site, when the effect of tree height was held constant (Fig. 1, Table 2). Significant increases in free-proline have been demonstrated in studies exposing *F. sylvatica* trees to drought stress for periods longer than our present experiment (Aranda *et al.*, 2018; Schraml and Rennenberg, 2000, 2002). In addition, the present result is also in accordance with studies of tomato (Heuer, 1999) and two-year-old olive trees (Sofa *et al.*, 2004), where the plants were exposed to drought stress for short periods (two weeks and eight days, respectively). The latter study pointed out, apart from the osmoprotectant function, the importance of free-proline and its metabolism for the restoration of cellular homeostasis during recovery from osmotic stress (see also Verbruggen *et al.*, 1996). Furthermore, studies have shown that free-prolines exhibit antioxidant activity (Alia *et al.*, 2001), metabolism protection [*e.g.*, through stabilization of proteins (Bandurska, 1993) and cytosol pH regulation (Venekamp, 1989)] under drought stress. Given the multifunctionality of free-prolines, the rapid accumulation found in the present study may have, at least in part, played a role in acclimation to drought stress in the leaves of the beech seedlings. Studies have also shown that proline is one of the main biochemical markers found in studies on the impact of drought on European tree species including *F. sylvatica* (Rennenberg *et al.*, 2006; Coccozza *et al.*, 2016).

In contrast, there was no significant difference in the concentration of total flavonoids in leaves between the WW and WS groups, irrespective of sampling site (Fig. 1, Table 2), indicating that the concentration of total flavonoids is unlikely to be a marker for a rapid response to drought stress. In a study of *F. sylvatica* current-year seedlings, while the concentrations of some kinds of flavonoid species (kaempferol and quercetin) in leaves were unaffected by the drought treatment, it was also demonstrated that the concentrations of other species (epicatechin and catechin) in leaves decreased significantly in response to drought stress (Aranda *et al.*, 2018). Thus, further studies should focus on which flavonoid compounds exhibit changes in concentration in response to short-term drought stress of *F. crenata* seedlings.

Conclusions

It is probable that concentrations in leaves vary depending on which biochemical compounds are examined as well as properties of individual plants relating their metabolism, and that free-proline may participate in a rapid acclimation response to short-term drought stress in current-year beech seedlings. Thus, free-proline is a promising candidate biochemical marker to assess the response to drought stress of *F. crenata*.

Authors' contributions

T.T. and H.A. conceived the study; T.T., H.A., Y.A., and S.A. designed the field sampling and completed the sample collection; T.T., H.A., Y.M., and H.K. performed the growth chamber

experiment; H.A. conducted the lab experiments and the analyses, under T.T.'s direction and supervision; T.T. and H.A. wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interests

No conflict of interest declared.

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Supplementary materials

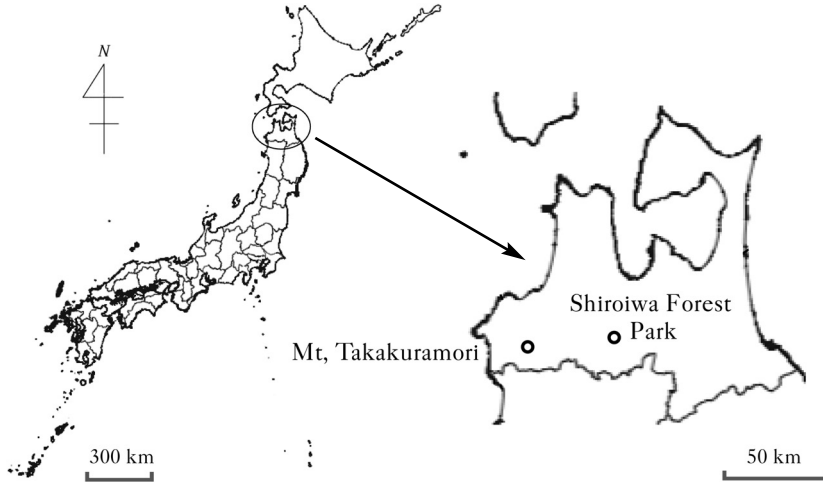


Fig. S1.
Sampling locations

Table S1.

Height (mean \pm SD) of beech seedlings at the start of the experiment

Site	Seedling height* [cm]	
	Well-watered	Water-stressed
Shiroiwa	6.8 \pm 1.4 (n=6)	7.3 \pm 1.9 (n=6)
Takakuramori	7.1 \pm 1.3 (n=6)	8.6 \pm 1.5 (n=6)

*There were no significant differences in the seedling height between experimental groups within sites (*t*-test, Shiroiwa: *t*=-0.489, *P*=0.636, Takakuramori: *t*=1.789, *P*=0.105) or between sites within the same watering regimes (*t*-test, well-watered: *t*=-0.335, *P*=0.745, water-stressed: *t*=-1.308, *P*=0.221)

Table S2.

Dry-to-fresh leaf weight ratio (mean \pm SE) of current-year beech seedlings in well-watered and water-stressed treatments

	Well-watered	Water-stressed
Dry-to-fresh leaf weight ratio	0.456 \pm 0.007 (n=5)	0.460 \pm 0.016 (n=5)

Table S3.

Summary of coefficients in GLMs with free-proline (a) and total flavonoid (b) concentrations per 1g leaf dry weight as response variables when the interaction term was taken into account

(a)

Coefficients	Estimates	95% CI (lower, upper)	\hat{R}
$\beta_{\text{Intercept}}$	0.16	(-0.07, 0.41)	1.00
β_{Height}	-0.19	(-0.32, -0.05)	1.00
$\beta_{\text{Treatment}}$	0.37	(0.04, 0.70)	1.00
β_{Site}	-0.09	(-0.43, 0.26)	1.00
$\beta_{\text{Interaction}}$	0.05	(-0.42, 0.51)	1.00

(b)

Coefficients	Estimates	95% CI (lower, upper)	\hat{R}
$\beta_{\text{Intercept}}$	1.32	(1.14, 1.51)	1.00
β_{Height}	-0.11	(-0.22, -0.02)	1.00
$\beta_{\text{Treatment}}$	0.10	(-0.16, 0.37)	1.00
β_{Site}	-0.01	(-0.26, 0.25)	1.00
$\beta_{\text{Interaction}}$	0.10	(-0.27, 0.47)	1.00

STRESZCZENIE

Reakcja wolnej proliny i flawonoidów w liściach siewek *Fagus crenata* na krótkotrwałą stres suszy glebowej

Buk karbowany *Fagus crenata* Blume (*Fagaceae*) jest gatunkiem szeroko rozpowszechnionym i dominującym w lasach liściastych chłodnej i umiarkowanej strefy Japonii. Jednakże na podstawie scenariuszy klimatycznych przewiduje się, że w przyszłości udział lasów bukowych w Japonii znacznie się zmniejszy – ze względu na niekorzystną dla tego gatunku zmianę warunków. Biorąc pod uwagę, że najbardziej podatnym na stres środowiskowy stadium rozwojowym jest siewka, istnieje pilna potrzeba zrozumienia, jak szybko siewki buka reagują i/lub aklimatyzują się do krótkotrwałych niekorzystnych zmian środowiskowych. W celu lepszego zrozumienia właściwości fizjologicznych tego gatunku, zwłaszcza potencjalnej szybkiej reakcji na krótkotrwałe niekorzystne zmiany środowiskowe, przeprowadzono eksperyment wzrostu w różnych wariantach nawadniania i zbadano stężenie 2 potencjalnie związanych z suszą związków biochemicznych (wolnej proliny i flawonoidów) w liściach siewek buka.

Nasiona zebrano z dojrzałych drzewostanów bukowych z 2 lokalizacji Aomori Prefecture w Japonii (ryc. S1): Shiroiwa Forest Park (40°34' N, 140°39' E, wysokość 285,0 m; dalej Shiroiwa) i Mt. Takakuramori (40°54' N, 140°16' E, wysokość 829,3 m; dalej Takakuramori). Po wykiełkowaniu wybrano losowo 24 siewki (12 z każdej lokalizacji) z 2 całkowicie rozwiniętymi liśćmi. Zmierzono wysokość siewek i potwierdzono, że nie było istotnych różnic między zabiegami (dane poniżej) i miejscami pobierania próbek na początku eksperymentu (tab. S1). Następnie w środowiskowej komorze doświadczalnej zastosowano 2 warianty nawadniania: w pierwszym kontynuowano podlewanie (100 ml) raz na 2 dni (siewki dobrze nawodnione – WW), w drugim wariantcie zaprzestano podlewania i poddano siewki stresowi wodnemu (WS) (6 siewek w każdej kombinacji

wariantu nawadniania i miejsca). Eksperymenty prowadzono przez 18 dni dla siewek z Shiroiwa i 15 dni dla siewek z Takakuramori. Ostatniego dnia eksperymentu pobrano próbki, które stanowiły 2 liścienie, będące całkowicie rozwinięte już na początku eksperymentu. Próbki były ekstrahowane etanolem w celu pomiaru stężenia wolnej proliny i flawonoidów (na 1 g suchej masy liścia). Dodatkowo zebrano po 5 siewek z grup WW oraz WS i ważono świeżą masę ich całkowicie rozwiniętych liścieni. Zmierzone suchą masę i obliczono stosunek miąższu do suchej masy liści (tab. S2), aby oszacować stężenie 2 związków biochemicznych w stosunku do suchej masy (na 1 g suchej masy liścia). Po potwierdzeniu braku istotnych korelacji pomiędzy zabiegami i stanowiskami w modelach statystycznych (tab. S3) przeanalizowano wpływ zabiegów i stanowisk na stężenie 2 związków biochemicznych.

Średnie stężenie wolnej proliny w liścieniach (średnia \pm błąd statystyczny) dla każdej kategorii doświadczenia wynosiło od 0,50 do 0,80 $\mu\text{mol/gFW}$ (tab. 1). Po uwzględnieniu stosunku suchej do świeżej masy liścieni szacunkowe stężenie wolnej proliny w liścieniach wahało się od 0,81 do 3,44 $\mu\text{mol/gDW}$ (ryc. 1). Z kolei średnie stężenie flawonoidów w liścieniach mieściło się w poszczególnych wariantach doświadczenia w przedziale od 1,72 do 1,94 mg/gFW (tab. 1). Szacunkowe wartości flawonoidów wahały się od 2,70 do 5,73 mg/gDW (ryc. 1). Wartości te były podobne do podawanych w innych badaniach nad *Fagus sylvatica* L., co sugeruje wiarygodne pomiary i szacunki stężeń wolnej proliny i flawonoidów w niniejszym badaniu.

Stwierdzono istotny negatywny wpływ wysokości siewek na stężenie wolnej proliny i flawonoidów w liścieniach: wyższe siewki na początku doświadczenia gromadziły mniej tych związków na jednostkę suchej masy liścieni niż pod koniec doświadczenia (tab. 2). Ponadto sposób nawadniania miał istotny wpływ na stężenie wolnej proliny na jednostkę suchej masy liścieni. Oszacowano, że stężenie to było około 1,49 raza [=exp (0,40)] wyższe w WS niż w WW, niezależnie od stanowiska, gdy efekt wysokości drzew był utrzymywany na stałym poziomie (ryc. 1; tab. 2). Nie wykazano natomiast istotnej różnicy w stężeniu flawonoidów w liścieniach pomiędzy grupami WW i WS, niezależnie od miejsca pobierania prób (ryc. 1; tab. 2). Wyniki te sugerują, że stężenie w liścieniach różni się w zależności od badanego związku biochemicznego, jak również od właściwości poszczególnych roślin związanych z ich metabolizmem oraz że wolna prolina uczestniczy, przynajmniej częściowo, w szybkiej odpowiedzi aklimatyzacyjnej na krótkotrwały stres suszy u jednorocznych siewek buka.