Photosynthetic Properties and Response to Drought in Cucumber Seedlings Acclimatized to Different Vapor-pressure-deficit Levels

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Hardening with a high vapor-pressure-deficit (VPD) can decrease conductance (g_s) and thereby enhance drought tolerance by reducing transpiration, which is particularly useful during transplant establishment. However, high-VPD hardening may decrease photosynthetic performance due to stomatal (diffusion) and non-stomatal (mesophyll) limitation. If g_s can be lowered without significantly reducing photosynthesis, water-use efficiency (WUE = CO_2 assimilation/transpiration), which is an important criterion for transplant quality, would improve. We investigated the photosynthetic properties of cucumber (*Cucumis sativus* L.) leaves acclimatized to different VPD levels (0.4 and 3.2 kPa at 28°C), and determined whether photosynthesis was limited by stomatal or non-stomatal factors at high VPD. The net photosynthetic rate (P_n) and g_s were measured at a VPD of 0.8 kPa and a leaf temperature of 28°C under saturating light. The photosynthetic response to the intercellular CO_2 concentration (C_i) was used to quantify the effects of VPD acclimatization on carboxylation efficiency. P_n did not differ significantly between acclimatization VPDs, but g_s was much lower ($\times 0.36$) in high-VPD leaves at an ambient CO_2 concentration of $400 \mu\text{mol}\cdot\text{mol}^{-1}$. Thus, the intrinsic WUE ($= P_n/g_s$) of the high-VPD leaves was much higher ($\times 2.85$). The C_i of high-VPD seedlings was lower than that of low-VPD seedlings, but, this did not cause any significant reduction in P_n in the high-VPD treatments because the decrease in C_i occurred within a range in which photosynthesis was not limited by ribulose-1,5-bisphosphate carboxylation in this experiment. The P_n – C_i curve did not differ between the VPD treatments, indicating that carboxylation efficiency was not affected. When VPD-acclimatized seedlings experienced a limited water supply, the low-VPD leaves showed lower leaf water potential and more severe wilting than the high-VPD leaves 30 min after water limitation began. The g_s of the high-VPD leaves was significantly lower (approximately $\times 0.5$ in average) than that of the low-VPD leaves throughout the water-supply-limitation treatment. The lower g_s maintained the water status of the high-VPD leaves at less-wilting levels by decreasing transpiration. These results indicate that high-VPD hardening could enhance the tolerance to short-term drought without stomatal or non-stomatal limitation of photosynthesis when controlling g_s effectively.

Key Words: carboxylase activity, hardening, intercellular CO_2 concentration, stomatal conductance, water-use efficiency.

Introduction

The atmospheric moisture content influences leaf gas-exchange properties via its effects on stomatal dynamics in many species (Buckley, 2005; El-Sharkawy et al., 1985). Hardening with a high vapor-pressure-deficit (VPD) can decrease stomatal conductance (g_s)

and thereby enhance drought tolerance by reducing transpiration, which is particularly useful during transplant establishment in horticultural production (Bañon et al., 2006; Sánchez-Blanco et al., 2004; Shibuya et al., 2003, 2006). If the net photosynthetic rate (P_n) is not affected, acclimatization at high VPD can improve water-use efficiency (WUE), which is an important criterion for transplant quality: carbohydrate accumulation combined with efficient water use is advantageous when transplanted seedlings experience a water deficit. However, high-VPD acclimatization may limit photosynthesis through both stomatal (diffusion) and non-stomatal (mesophyll) factors (Dai et al., 1992; Flexas and Medrano, 2002; Raschke and Resemann, 1986). In the

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former case, high VPD may decrease g_s and thereby limit CO_2 assimilation by decreasing the intercellular CO_2 concentration (C_i) due to decreased gas exchange through the stomata. In the latter case, the higher evaporative demand may decrease the leaf's water potential (Ψ_l) and consequently inhibit carboxylation activity in the chloroplasts (Keck and Boyer, 1974; Lawlor, 2002).

Despite these potential limitations, Carins Murphy et al. (2014) reported that high VPD did not decrease photosynthesis by *Toona ciliata* when g_s decreased to maintain transpiration, implying that neither stomatal nor non-stomatal factors limited photosynthesis even under high-VPD conditions. Two hypotheses may explain this observation. The first is that the decrease in C_i caused by a reduction in g_s would not significantly decrease CO_2 assimilation when the decrease occurred within a range in which photosynthesis was mainly limited by ribulose-1,5-bisphosphate (RuBP) regeneration, but was not limited by RuBP carboxylation; in the latter reaction, photosynthesis would be strongly influenced by C_i . The g_s and C_i may be altered by VPD via changes in stomatal size and distribution (Carins Murphy et al., 2014; Hovenden et al., 2012) in addition to the stomatal aperture. The second hypothesis is that the high-VPD acclimatization does not lower Ψ_l when the water supply is sufficient and transpiration is suppressed sufficiently by stomatal closure to prevent the development of water stress (Carins Murphy et al., 2014; Itagaki et al., 2014). In this case, high-VPD acclimatization can only weakly inhibit carboxylation activity.

In the present study, we investigated the photosynthetic properties including gas exchange (P_n , g_s , and C_i) and carboxylation activity of cucumber (*Cucumis sativus* L.) leaves acclimatized to different VPD levels, and determined whether photosynthesis was limited by stomatal or non-stomatal factors at high VPD. In addition, the short-term response to a water deficit in the acclimatized seedlings was evaluated by measuring changes in stomatal conductance and water status during water-supply limitation.

Materials and Methods

Plant growth conditions

'Hokushin' cucumber seeds were sown in vermiculite in plastic pots (60 mm in diameter, 55 mm in depth) and germinated at 28°C, 90% relative humidity (RH), a photosynthetic photon flux (PPF) of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a photoperiod of 16:8-h L:D. The seedlings were maintained in growth chambers (custom model; Ikeya Co., Kashiba, Japan) at 28°C under a high VPD (3.2 kPa) or a low VPD (0.4 kPa), which were equivalent to 15% and 90% RH, respectively, immediately after germination. Illumination was supplied by fluorescent lamps (FPL55EX-N; Panasonic Corp., Kadoma, Japan). The pots were placed in nutrient solution (the A-type recipe of OAT Solution; OAT Agrio Co. Ltd.,

Tokyo, Japan) to a depth of 10 to 20 mm. At high VPD, the surface of the vermiculite was covered by plastic film to minimize evaporation. The moisture level was almost saturated in the growing medium of all treatments.

Measurements of photosynthetic properties and leaf water status

When the first true leaves had expanded and the second leaves had begun to emerge (10 days after seeding in the high-VPD treatment, 8 days after seeding in the low-VPD treatment), P_n and g_s of the first true leaf (approximately 36 cm^2 for the first true leaf in both treatments) in 10 seedlings were measured using an LI-6400 photosynthesis system (LI-COR Inc., Lincoln, NE, USA). A comparison between plants at the same developmental stage but of different ages was preferred when the rate of plant development was different between the treatments. The measurements were conducted at ambient CO_2 concentrations (C_a) of 50, 100, 150, 200, 400, and 800 $\mu\text{mol}\cdot\text{mol}^{-1}$ to determine P_n - C_i curves. Illumination was supplied at a saturating PPF of 2000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ by red and blue LEDs at a ratio of 9:1. The measurements began after reaching an equilibrium between P_n and g_s under the specified light intensity, which typically occurred within 10 min. In the leaf chamber of the measuring system, the leaf temperature and VPD were maintained at 28°C and 0.8 kPa (80% RH), respectively. During the measurements, C_a was progressively decreased from 400 $\mu\text{mol}\cdot\text{mol}^{-1}$ to the lower values, and then increased to 800 $\mu\text{mol}\cdot\text{mol}^{-1}$. C_i was calculated according to the method of von Caemmerer and Farquhar (1981). The maximum rate of Rubisco carboxylase activity (V_{cmax}) and the electron transport rate (J) were estimated from P_n - C_i curves using a curve-fitting model developed by Sharkey et al. (2007). The intrinsic WUE (WUE_i) was calculated by dividing P_n by g_s (Chaves et al., 2004). Ψ_l was measured for another five seedlings in each treatment group using a dewpoint potentiometer (WP4-T; Decagon Devices, Inc., Pullman, WA, USA) immediately after VPD acclimatization.

Microscopic observation of the leaf surface

Imprints of the adaxial and abaxial surfaces of another five first true leaves were obtained by spreading a liquid adhesive (Ekiban A; Taihei Yakuin Co., Ltd., Tokyo, Japan) onto the leaf surface, allowing it to dry, and then peeling the adhesive from the leaf. The imprints were observed using a VHX-1000 digital microscope (Keyence Corp., Osaka, Japan) at 1000× magnification, and the numbers of stomata and epidermal cells in three randomly selected fields of view in each leaf were counted. The average for these fields of view was then calculated. The stomatal lengths were measured using image-processing software provided with the microscope (VHX-H1M1; Keyence Corp.).

The stomatal and epidermal densities were calculated by dividing the number of stomata and epidermal cells, respectively, by the area of the field. The stomatal length was measured using the image-processing software, and the stomatal index was defined as the number of stomata in a given area divided by the total number of stomata plus epidermal cells in that area (Casson et al., 2009).

Effect of acclimatization VPD on short-term response to drought

Five seedlings that had been acclimatized to high VPD for 10 days and five that had been acclimatized to low VPD for 8 days were exposed to a limited water supply immediately after VPD acclimatization to evaluate the effect of acclimatization VPD on the short-term (< 30 min) response to a water deficit. The roots of the seedlings were cleaned by quickly washing away the vermiculite growing medium, and were then exposed to saturated vapor conditions by inserting the roots in a closed plastic bottle containing a wetted paper towel. The shoots of the seedlings were then maintained in the growth chamber at 28°C under a VPD of 1.9 kPa (approximately 50% RH). Illumination was supplied by fluorescent lamps at a PPF of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The adaxial and abaxial g_s of the first true leaf in each seedling were measured for 30 min at 3-min intervals using a SC-1 porometer (Decagon Devices, Inc.). The porometer was used instead of the LI-6400 for quick measurements. The Ψ_l of the plants ($n=5$) in each treatment group was measured using the dewpoint potentiometer 30 min after starting the water supply limitation.

Statistical analysis

Significant differences between treatment means were identified using the Student's *t*-test. Statistical analyses were performed using the Statcel 2 software program (OMS Publishing Inc., Tokorozawa, Japan). The measurements of photosynthetic properties were repeated twice at different times. The other measurements were not repeated, but were conducted under a precisely controlled environment.

Results

Photosynthetic properties and water status

There was no significant difference in P_n between acclimatization VPDs, but g_s was much lower ($\times 0.36$) in the high-VPD leaves at C_a of 400 $\mu\text{mol}\cdot\text{mol}^{-1}$ (Table 1). C_i of high-VPD seedlings was lower ($\times 0.91$) than that of low-VPD seedlings (Table 1). The WUE_i of the high-VPD leaves was 2.85 times that of the low-VPD leaves (Table 1). The P_n – C_i curve did not differ between the VPD treatments (Fig. 1). The V_{cmax} and J did not differ significantly between the two treatments (Table 1). In addition, Ψ_l did not differ significantly between the acclimatization VPDs (Table 1). Similar results of photosynthesis were obtained after repeating the experiment at different times (data not shown).

Microscopic observation of the leaf surface

The adaxial and abaxial stomatal densities were sig-

Fig. 1. Relationships between the intercellular CO_2 concentration (C_i) and the net photosynthetic rate (P_n) for *Cucumis sativus* leaves acclimatized to a high (3.2 kPa) or low (0.4 kPa) vapor-pressure-deficit (VPD). Photosynthesis was measured at a PPF of 2000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, ambient CO_2 concentrations (C_a) of 50, 100, 150, 200, 400, and 800 $\mu\text{mol}\cdot\text{mol}^{-1}$, a leaf temperature of 28°C, and a VPD of 0.8 kPa (80% relative humidity). Values are the means of 10 replicate plants. The values at the right side of the plots are C_a for measurement. The P_n and g_s measured at C_a of 400 $\mu\text{mol}\cdot\text{mol}^{-1}$ are the same data as in Table 1.

Table 1. Net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), intrinsic water-use efficiency ($\text{WUE}_i = P_n/g_s$), maximum rate of Rubisco carboxylase activity (V_{cmax}), electron transport rate (J), and leaf water potential (Ψ_l) of first true leaves of *Cucumis sativus* acclimatized to high (3.2 kPa) or low (0.4 kPa) vapor-pressure-deficit (VPD).

Acclimatization VPD	P_n ($\mu\text{molCO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	g_s ($\text{molH}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	C_i ($\mu\text{mol}\cdot\text{mol}^{-1}$)	WUE_i ($\mu\text{molCO}_2\cdot\text{mol H}_2\text{O}^{-1}$)	V_{cmax} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	J ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Ψ_l (MPa)
High	30.3±0.5	1.00±0.09	327±6	32.8±3.4	165±4	177±4	−1.05±0.01
Low	31.7±0.6	2.80±0.09	361±1	11.5±0.5	157±8	175±4	−1.00±0.02
Significance	NS	***	***	***	NS	NS	NS

Photosynthesis was measured at a PPF of 2000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, an ambient CO_2 concentration of 400 $\mu\text{mol}\cdot\text{mol}^{-1}$, a leaf temperature of 28°C, and a VPD of 0.8 kPa (80% RH). The P_n and g_s are the same data as in Figure 1. Values are means±SE (photosynthetic properties, $n=10$; Ψ_l , $n=5$). Significance (Student's *t*-test): NS, non-significant ($P>0.05$), ***, $P<0.001$.

Table 2. Stomatal and epidermal cell density, stomatal index, and stomatal length for first true leaves of *Cucumis sativus* acclimatized to high (3.2 kPa) or low (0.4 kPa) vapor-pressure-deficit (VPD).

Acclimatization VPD	Stomatal density (stomata·mm ⁻²)		Epidermal cell density (cells·mm ⁻²)		Stomatal index (%)		Stomatal length (μm)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
High	542±36	820±28	2548±137	2381±63	17.5±0.3	25.6±0.4	9.9±0.3	9.8±0.3
Low	371±14	581±17	1721±355	1781±27	17.7±0.2	24.7±0.3	16.8±0.3	15.4±0.2
Significance	**	***	**	***	NS	NS	***	***

Values are means±SE (n=5). Significance (Student's *t*-test): NS, non-significant ($P>0.05$); **, $P<0.01$; ***, $P<0.001$. The stomatal index is defined as the ratio of the number of stomata in a given area divided by the total number of stomata plus other epidermal cells in that area (Casson et al., 2009).

Fig. 2. Microscopic images of the adaxial and abaxial surfaces of first true leaves of *Cucumis sativus* acclimatized to a high (3.2 kPa) or low (0.4 kPa) vapor-pressure-deficit (VPD). Scale bars represent 50 μm. The photographs were trimmed and re-touched to improve contrast from the originals.

nificantly higher for high-VPD leaves: 1.46 times the number in low-VPD leaves on the adaxial side versus 1.41 times on the abaxial side (Table 2). The epidermal cell density was also higher in the high-VPD leaves: 1.48 and 1.33 times the density on the adaxial and abaxial sides, respectively, of the low-VPD leaves (Table 2). The stomatal index did not differ significantly between the VPD treatments (Table 2). The stomatal lengths of the low-VPD leaves were significantly greater (1.70 and 1.57 times on the adaxial and abaxial sides, respectively) than those of the high-VPD leaves (Table 2; Fig. 2). The epidermal cells also tended to be larger in the low-VPD leaves than in the high-VPD leaves (Fig. 2).

Effect of acclimatization VPD on short-term response to drought

The g_s was greater at the start of the water-supply limitation in the low-VPD leaves (approximately 2 times that in the high-VPD leaves) (Fig. 3). g_s in both VPD leaves decreased, and then reached approximately 50% of initial levels 30 min after the treatment started.

Fig. 3. Changes in stomatal conductance (g_s) of the first true leaves of *Cucumis sativus* that had been acclimatized to a high (3.2 kPa) or low (0.4 kPa) vapor-pressure-deficit (VPD), followed by water-supply limitation for up to 30 min. Values represent the sums of adaxial and abaxial g_s . Leaf water potential (Ψ_l) 30 min after limiting the water supply is shown at the right side of the graph. The water-supply limitation was conducted by exposing roots to saturated vapor conditions. The shoots of the seedlings were maintained at 28°C under a VPD of 1.9 kPa (approximately 50% RH). Values are means ± SE (n = 5). Significance: *, $P<0.05$; **, $P<0.01$ (Student's *t*-test). The g_s values cannot be directly compared with those in Table 1 because of the different measurement devices.

The values of g_s in this experiment cannot be directly compared with those in the photosynthesis measurements (Table 1) because of the different measuring devices. g_s of the high-VPD leaves was significantly lower ($\times 0.53$ at the start of the treatment; $\times 0.51$ 30 min after starting the treatment) than that of the low-VPD leaves throughout the water-supply-limitation treatment (Fig. 3). Ψ_l of the low-VPD leaves was significantly lower than that of the high-VPD leaves 30 min after starting the water-supply limitation (Fig. 3). As a result, wilting appeared to be more severe in the low-VPD leaves than in the high-VPD leaves (Fig. 4).

Discussion

The lack of a significant difference in the P_n-C_i curves, V_{cmax} , and J between the VPD treatments indicates that photosynthesis of the high-VPD leaves was

Fig. 4. Photographs of a *Cucumis sativus* seedling that had been acclimatized to a high (3.2 kPa) or low (0.4 kPa) vapor-pressure-deficit (VPD), at the start of (upper), and 30 min after (lower) water-supply limitation.

not limited by non-stomatal factors (i.e., carboxylation efficiency) in this experiment. This probably resulted from the fact that the high-VPD treatment did not significantly decrease Ψ_1 compared with the value in the low-VPD treatment (Table 1); decreased Ψ_1 may cause inhibition of carboxylation and thereby reduce photosynthetic efficiency (Keck and Boyer, 1974; Lawlor, 2002). This is probably because the water content of the medium was maintained at a sufficiently high level during the treatments, and the distance from the roots to the leaves was relatively short in the young plants. In addition, the high-VPD leaves had significantly reduced stomatal conductance (Table 1), which could maintain Ψ_1 at non-damaging levels by suppressing transpiration. This probably resulted from feed-forward responses of the stomata to atmospheric moisture levels: stomata decrease transpiration in response to increasing evaporative demand (Buckley, 2005; Farquhar, 1978; Grantz, 1990; Lange et al., 1971; Peak and Mott, 2011). From the P_n - C_i curve analysis, the decrease in C_i with decreasing g_s occurred within the range in which photosynthesis is mainly limited by RuBP regeneration rather than by RuBP carboxylation; in the latter reaction, photosynthesis is strongly related to C_i . Therefore, the decreased g_s in the high-VPD leaves did not significantly limit P_n via a reduction in C_i (i.e., stomatal factors) in the present study.

The leaf surface properties differed greatly between the treatments, even though the leaf areas were similar. The higher stomatal density and smaller stomatal size in the high-VPD leaves agrees with previous reports, in which stomatal density increased, but stomatal size decreased, under water stress in several species (Bosabalidis and Kofidis, 2002; Boughalleb and Hajlaoui, 2011; Hovenden et al., 2012). The higher stomatal density and smaller stomatal size influence g_s an-

tagonistically and compensate for each other. Therefore, the difference in g_s between the treatments probably depends on the degree of stomatal aperture and thus represents a plastic response. This hypothesis is supported by a previous study (Shibuya et al., 2003), in which a similar VPD acclimatization was applied to the same species; in the previous study, it was found that the decreased g_s in high-VPD leaves could easily recover to the same level as that in low-VPD leaves after a 24-h low-VPD treatment following high-VPD acclimatization. The stomatal index did not differ significantly between the VPD treatments (Table 2), indicating that high VPD did not accelerate differentiation of stomata from epidermal cells. This agrees with a previous report (Hovenden et al., 2012), in which the stomatal index was not significantly affected by changes in ambient humidity. Thus, the higher stomatal density and smaller stomatal size in high-VPD leaves probably resulted from a significantly higher epidermal-cell density (Table 2), which may be caused by the limitation of epidermal-cell expansion in response to greater evaporative demand (Alves and Setter, 2004; Lecoeur et al., 1995; Tardieu et al., 2000).

The seedlings in the high-VPD treatment took 2 days longer than the low-VPD seedlings (10 and 8 days after seeding, respectively) to reach the same developmental stage based on the size of the first true leaves, indicating that the seedlings' growth was suppressed at high VPD. In the present study, the photosynthesis on a leaf-area basis was not altered by the VPD treatments, despite the growth differences between treatments. These results sound contradictory, but probably resulted from the suppressed leaf enlargement under high-VPD conditions, which decreases photosynthesis on a whole-plant basis, thereby limiting whole-plant growth even though photosynthesis on a leaf-area basis was not suppressed. This suggests that high VPD can suppress plant growth not only through stomatal and non-stomatal photosynthetic limitation, but also through a limitation of leaf enlargement in response to greater evaporative demand; the latter is probably the main factor that contributed to growth inhibition under high VPD in the present study.

In the water-supply limitation experiment, g_s of the low-VPD leaves was greater than that of the high-VPD leaves after the start of water-supply limitation, but the low-VPD leaves showed a lower (more negative) Ψ_1 and appeared to show more severe damage (wilting) 30 min after the treatment started, indicating that the water status of the high-VPD leaves was maintained at less damaging levels by decreasing transpiration after VPD acclimatization. This result agrees with the previous reports in which high-VPD hardening reduced the transpiration of seedlings after transplantation and thereby enhanced their drought tolerance (Bañón et al., 2006; Sánchez-Blanco et al., 2004; Shibuya et al., 2003, 2006). In addition to these results, the present study demonstrated that high-VPD hardening could enhance

the tolerance to short-term (< 30 min) drought without stomatal or non-stomatal limitation of photosynthesis when controlling g_s effectively.

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