

Effects of the interaction between vapor-pressure deficit and salinity on growth and photosynthesis of *Cucumis sativus* seedlings under different CO₂ concentrations

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Abstract

We studied growth and photosynthesis of cucumber (*Cucumis sativus*) seedlings under two vapor-pressure deficit levels (VPD; 0.4 and 3.0 kPa), two salinity levels (0 and 34 mM NaCl), and two CO₂ concentrations ([CO₂]; 400 and 1,000 μmol mol⁻¹). Relative growth rate (RGR) decreased with increasing VPD, but the causal factor differed between salinity levels and CO₂ concentrations. Under ambient [CO₂], RGR decreased with increasing VPD at low salinity mainly due to decreased leaf area ratio (LAR), and decreased net assimilation rate (NAR) at high salinity. The decrease in intercellular [CO₂] (C_i) with decreasing stomatal conductance caused by high VPD did not significantly limit net photosynthetic rate (P_N) at low salinity, but P_N was potentially limited by C_i at high salinity. At high [CO₂], high VPD reduced LAR, but did not affect NAR. This is because the decrease in C_i occurred where slope of P_N-C_i curve was almost flat.

Additional key words: evaporative demand; gas exchange; growth analysis; humidity; stress response; water potential.

Introduction

The vapor-pressure deficit (VPD), which creates evaporative demand, influences the water balance of plants that in turn affects their photosynthesis and growth. Controlling VPD is therefore an effective strategy for improving crop yield in protected cultivation (Lu *et al.* 2015, Zhang *et al.* 2015), because high evaporative demand can inhibit plant growth. The reduction of plant growth with increasing VPD has two main causes: reduction of photosynthesis per unit leaf area due to water stress, or reduction of the whole-plant light interception area due to decreasing leaf expansion.

There have been many reports that greater evaporative demand decreases photosynthesis and plant growth by decreasing stomatal conductance (g_s) (Raschke and Resemann 1986, Dai *et al.* 1992, van de Sanden and Veen 1992, Wong 1993, Ottosen *et al.* 2002, Ben-Asher *et al.* 2013). On the other hand, several studies have suggested that high evaporative demand may not decrease photo-

synthesis even if it reduces g_s (Carins-Murphy *et al.* 2014, Shibuya *et al.* 2016b). For the reduction of the light interception area, many researchers have reported that higher evaporative demand reduces leaf expansion (Salah and Tardieu 1997, Munns *et al.* 2000, Tardieu *et al.* 2000, 2010, Körner and Challa 2003, Zhang *et al.* 2015). This, in turn, decreases whole-plant light interception and photosynthesis, although contradictory results have also been reported (van de Sanden and Veen 1992, Wong 1993). The existence of contradictory results for the photosynthetic rate and leaf expansion indicates that one or more other factors interact with regulation of photosynthesis and light interception area to affect the responses of plants to high evaporative demand.

Here, we focused on the salinity, which affects water uptake, and the atmospheric CO₂ concentration ([CO₂]), which affects CO₂ assimilation, as potential interactive factors. The rhizosphere water status and VPD both affect

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Abbreviations: AC – ambient [CO₂]; ANOVA – analysis of variance; [CO₂] – CO₂ concentration; C_i – intercellular CO₂ concentration; DM – dry mass; EC – elevated [CO₂]; g_s – stomatal conductance; J₃₀₀ and J₁₅₀₀ – electron transport rates at photosynthetic photon flux densities of 300 and 1,500 μmol m⁻² s⁻¹, respectively; LAR – leaf area ratio; LMR – leaf mass ratio; NAR – net assimilation rate; P_N – net photosynthetic rate; Ψ_l – leaf water potential; RGR – relative growth rate; SLA – specific leaf area; V_{cmax} – maximum rate of Rubisco carboxylase activity; VPD – vapor-pressure deficit.

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plant water relations, and thus can interactively affect photosynthesis (Xue *et al.* 2004, El-Sharkawy 2011) and leaf expansion (van de Sanden and Veen 1992, Tardieu *et al.* 2000). [CO₂] and VPD, which is one of the driving forces for CO₂ and vapor diffusion, can also interact with other factors to affect photosynthetic properties (Gislerød and Nelson 1989, Wong 1993). However, most previous studies were conducted under conditions in which neither parameter was fully controlled, or growth and photosynthetic properties were not investigated simultaneously, thereby preventing a more comprehensive explanation of interacting effects. In addition, the simultaneous effects of the three factors (VPD, rhizosphere conditions, and [CO₂]) have not been investigated. This is an important omission,

Materials and methods

Growth conditions: Cucumber (*Cucumis sativus* L. cv. 'Hokushin') seeds were sown individually in vermiculite in plastic cell trays (cell size: 25-mm square) and germinated in a custom-designed growth chamber (*Ikeya Co.*, Kashiba, Japan) maintained at an air temperature of 28°C, a VPD of 0.4 kPa (relative humidity of 90%), and [CO₂] of 400 µmol mol⁻¹ until the cotyledons had expanded (6 d after seeding). Illumination was supplied by fluorescent lamps (*FPL55EX-N*; *Panasonic Corp.*, Kadoma, Japan) with a PPFD of 300 µmol m⁻² s⁻¹. The light:dark period was 16-h light/8-h dark throughout the experiment. The seedlings were transplanted into plastic bottles (250 mL) containing nutrient solution (details below) and then grown hydroponically in the growth chambers (*Ikeya Co.*, Kashiba, Japan) maintained at a VPD of 0.4 kPa (hereafter, low VPD) or 3.0 kPa (hereafter, high VPD) and at [CO₂] of 400 µmol mol⁻¹ (hereafter, ambient [CO₂], AC) or 1,000 µmol mol⁻¹ (hereafter, elevated [CO₂], EC) for 8 d. These VPD values correspond to a relative humidity of 90 and 20%, respectively, at an air temperature of 28°C. Nutrient solutions (*the A-type recipe of OAT Solution*; *OAT Agrico Co. Ltd.*, Tokyo, Japan) were supplied at two different salinity levels (0 mM and 34 mM NaCl, hereafter, the low and high salinity, respectively) as the growing medium. The composition of the standard nutrient solution in mEq L⁻¹ was: N, 18.6; P, 5.1; K, 8.6; Ca, 8.2; Mg, 3.0. The electrical conductivity was 2.6 mS cm⁻¹, and the pH value was 6.5. The high salinity treatment was created by adding NaCl (0.2% w/v) to the standard solution; this increased electrical conductivity to 5.0 mS cm⁻¹. The osmotic potential values, which were estimated from the electrical conductivity values using the equation of Richards (1954), were -0.09 and -0.18 MPa in low and high salinity, respectively. The nutrient solution was added to the bottles during the growing period to replace losses to evaporation. The seedlings were arranged with sufficient space to avoid mutual shading among neighboring seedlings.

Growth analysis: We sampled 12 seedlings in each

because it is possible that the interaction of two factors may be altered by changes in the third factor. A better understanding of these possible interactions will help us to interpret the results obtained under different environmental conditions.

In the present study, growth and photosynthetic properties of cucumber (*Cucumis sativus* L.) seedlings grown under two VPD levels, two salinity levels of the nutrient solution, and two CO₂ concentrations were determined by means of growth analysis and gas-exchange measurements to reveal the potential interactions among these factors and to determine underlying causal factor or factors that limit plant growth under high evaporative demand.

treatment group at 6 d after seeding (at the start of the treatments) and ten seedlings in each group at 14 d. This growing period seems short, but our previous research suggested that it is long enough to evaluate the initial growth of cucumber seedlings (Shibuya *et al.* 2016a). We measured the leaf area of each seedling using an image scanner and the *LIA32 for Win* image analysis software (K. Yamamoto, Nagoya University, Nagoya, Japan), and then determined the whole-plant and leaf dry masses (DM, measured after 3 d of drying at 80°C). The relative growth rate (RGR, g g⁻¹ d⁻¹), net assimilation rate (NAR, g m⁻² d⁻¹ on DM basis), leaf area ratio (LAR, m² g⁻¹), specific leaf area (SLA, m² g⁻¹), and leaf mass ratio (LMR, dimensionless) were calculated using the following equations (Radford 1967, Hunt *et al.* 2002):

$$\text{RGR} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} = \text{NAR} \times \text{LAR} \quad (1)$$

$$\text{NAR} = \frac{W_2 - W_1}{A_2 - A_1} \times \frac{\ln A_2 - \ln A_1}{t_2 - t_1} \quad (2)$$

$$\text{LAR} = \frac{A_2 - A_1}{\ln A_2 - \ln A_1} \times \frac{\ln W_2 - \ln W_1}{W_2 - W_1} = \text{SLA} \times \text{LMR} \quad (3)$$

$$\text{SLA} = \frac{A_2 - A_1}{\ln A_2 - \ln A_1} \times \frac{\ln L_2 - \ln L_1}{L_2 - L_1} \quad (4)$$

$$\text{LMR} = \frac{L_2 - L_1}{\ln L_2 - \ln L_1} \times \frac{\ln W_2 - \ln W_1}{W_2 - W_1} \quad (5)$$

where W_1 and W_2 are the whole-plant DM [g] at times t_1 and t_2 (6 and 14 d after seeding, respectively), A_1 and A_2 are the corresponding total leaf areas [m²], and L_1 and L_2 are the corresponding leaf DM values [g]. The averages of W_1 , A_1 , and L_1 (from 12 seedlings) were used as representative values at t_1 for calculation.

Photosynthetic properties and water status: We measured the net photosynthetic rate (P_N) and g_s of the first true leaf in ten seedlings 14 d after seeding using an *LI-6400* photosynthesis system (*LI-COR Inc.*, Lincoln, NE, USA) at a measurement PPFD of 300 µmol m⁻² s⁻¹ (the same as the growing conditions) or 1,500 µmol m⁻² s⁻¹ (saturating light intensity), at a leaf temperature of 28°C. Different seedlings were used for each PPFD measurement. Illumination was supplied by red and blue LEDs at

a ratio of 9:1. The measurements were conducted at [CO₂] of 70, 100, 150, 200, 400, 800, and (only for EC treatments) 1,000 μmol mol⁻¹ for a PPFD of 300 μmol m⁻² s⁻¹ and of 70, 100, 150, 200, 400, 600, and 800 μmol mol⁻¹ for saturating light. The VPD in the measuring system was maintained at approximately 1.2 or 2.6 kPa for the seedlings grown under low or high VPD, respectively. These measurement VPD are near the minimum and maximum for this equipment. Inter-cellular [CO₂] (C_i) was calculated according to the method of von Caemmerer and Farquhar (1981). The electron transport rates at PPFDs of 300 and 1,500 μmol m⁻² s⁻¹ (J₃₀₀ and J₁₅₀₀, respectively) were estimated from the P_N-C_i curves using a curve-fitting model developed by Sharkey *et al.* (2007). The maximum rate of Rubisco carboxylase activity (V_{cmax}) was estimated from the P_N-C_i curves at the saturating light intensity using the same model.

Leaf water potential (Ψ_l) of the first true leaf was measured for another five seedlings in each treatment group using a dewpoint potentiometer (WP4-T; Decagon Devices, Inc., Pullman, WA, USA) according to the manufacturer's

Results

Growth parameters: RGR tended to decrease with increasing VPD and increasing salinity, but their interaction differed between the CO₂ concentrations (Table 1). At AC, there was a significant VPD × salinity interaction for RGR, with a stronger VPD effect at high salinity; RGR at high VPD was 83 and 93% (for high and low salinity, respectively) of the corresponding values at low VPD. On the other hand, at EC, the interaction was not significant, but the effect of VPD on RGR tended to be greater at low salinity; at low salinity, RGR at high VPD was 91% of that at low VPD, but there was no significant difference between the two treatments at high salinity. The whole-plant DM showed similar results (Fig. 1A,B).

NAR was only significantly affected by VPD at high salinity under AC; NAR was lower at high VPD (Table 1).

recommendations. The measurement of Ψ_l was conducted approximately at the middle of photoperiod.

Statistical analysis: The effects of the VPD × salinity interaction on growth parameters, photosynthetic properties, and water status at each [CO₂] were determined by means of two-way analysis of variance (ANOVA). Analysis of the whole-plant DM was performed after logarithmic transformation of the data. Significant differences in these values between the treatments in each [CO₂] were then identified by means of the Tukey-Kramer's test (with significance at P<0.05). The effects of the VPD × [CO₂] interaction at each salinity level were also determined. One sample used to determine the growth parameters was excluded from the combination of low VPD, low salinity, and AC because its RGR was an outlier (based on the results of the Grubb's test at P<0.05). All analyses were performed using the Statcel 2 software (OMS Publishing Inc., Tokorozawa, Japan). The experiments were not replicated, but were conducted under a precisely controlled environment.

There was a significant VPD × salinity interaction for NAR at AC; high VPD did not significantly affect NAR at low salinity, but at high salinity, it significantly decreased NAR, to 82% of the value at low VPD. No significant interaction was observed for NAR at EC; the NAR was not significantly affected by either VPD or salinity.

There was a significant VPD × salinity interaction for LAR (Table 1). LAR tended to decrease at high VPD under both [CO₂], but only at low salinity; LAR decreased to 94 and 91% of the value at low VPD under AC and EC, respectively. SLA showed similar results to LAR, indicating that the smaller LAR under high VPD resulted from decreased SLA. LMR was not significantly affected by VPD except at high salinity under AC.

Table 1. Relative growth rate (RGR) [g g⁻¹ d⁻¹], net assimilation rate (NAR) [g m⁻² d⁻¹], leaf area ratio (LAR) [m² g⁻¹], specific leaf area (SLA) [m² g⁻¹], and leaf mass ratio (LMR) [g g⁻¹] of cucumber (*Cucumis sativus*) seedlings grown under different combinations of vapor-pressure deficit, salinity of the nutrient solution, and atmospheric CO₂ concentration ([CO₂]; ambient [CO₂], AC, and elevated [CO₂], EC) for 8 d after transplantation. Data are the average of ten replicate plants (n = 10), except for the combination of low VPD, low salinity, and ambient [CO₂] (n = 9). Mean values of a parameter at a given [CO₂] that are labelled with different letters, differed significantly (Tukey-Kramer's test, P<0.05).

Salinity	VPD	AC (400 μmol mol ⁻¹)					EC (1,000 μmol mol ⁻¹)				
		RGR	NAR	LAR	SLA	LMR	RGR	NAR	LAR	SLA	LMR
Low (0 mM NaCl)	Low (0.4 kPa)	0.309 ^a	13.7 ^a	0.0226 ^a	0.0309 ^a	0.733 ^b	0.340 ^a	15.6 ^a	0.0218 ^a	0.0300 ^a	0.730 ^b
	High (3.0 kPa)	0.288 ^b	13.5 ^a	0.0213 ^b	0.0292 ^b	0.731 ^b	0.310 ^b	15.6 ^a	0.0199 ^b	0.0264 ^b	0.747 ^{ab}
High (34 mM NaCl)	Low (0.4 kPa)	0.277 ^b	13.7 ^a	0.0204 ^b	0.0272 ^b	0.749 ^a	0.302 ^b	15.6 ^a	0.0195 ^b	0.0254 ^b	0.762 ^a
	High (3.0 kPa)	0.231 ^c	11.2 ^b	0.0206 ^b	0.0283 ^b	0.727 ^b	0.293 ^b	15.2 ^a	0.0194 ^b	0.0257 ^b	0.754 ^a
ANOVA (P value)	VPD	<0.001	<0.001	0.091	0.548	0.002	0.003	0.706	0.003	0.002	0.224
	Salinity	<0.001	0.002	<0.001	<0.001	0.081	<0.001	0.582	<0.001	<0.001	0.001
	VPD × salinity	0.017	0.002	0.021	0.003	0.005	0.089	0.634	0.008	0.003	0.066

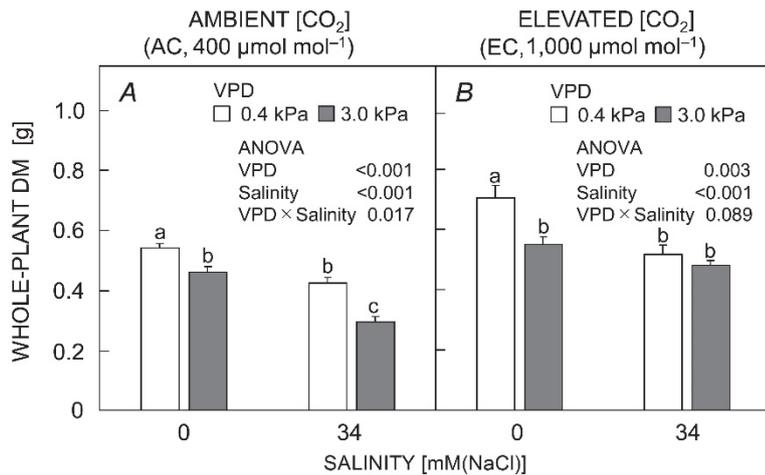


Fig. 1. Whole-plant dry mass (DM) of cucumber (*Cucumis sativus*) grown under different combinations of vapor-pressure deficit, salinity of the nutrient solution, and atmospheric CO₂ concentration ([CO₂]; [A] ambient [CO₂], AC, and [B] elevated [CO₂], EC) for 8 d after transplantation. Data are average \pm SE ($n = 10$), except for the combination of low VPD, low salinity, and ambient [CO₂] ($n = 9$). At a given [CO₂], mean values labelled with *different letters*, differed significantly between treatments (*Tukey–Kramer* test, $P < 0.05$). The statistical analyses were performed after logarithmic transformation of the DM.

Table 2. Net photosynthetic rate (P_N) [$\mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$], stomatal conductance (g_s) [$\text{mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{ s}^{-1}$], intercellular CO₂ concentration (C_i) [$\mu\text{mol mol}^{-1}$], and leaf water potential (Ψ_l) [MPa] of cucumber (*Cucumis sativus*) seedlings grown under different combinations of vapor-pressure deficit, salinity of the nutrient solution, and atmospheric CO₂ concentration ([CO₂]; ambient [CO₂], AC, and elevated [CO₂], EC) for 8 d after transplantation. The P_N , g_s , and C_i were measured at a photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and cuvette [CO₂] of 400 and 1,000 $\mu\text{mol mol}^{-1}$, respectively, for seedlings that were grown under AC and EC. Measurements were conducted at the end of treatment period (8 d after transplantation). Data are the average of five replicate plants ($n = 5$). Mean values of a parameter for a given [CO₂] that are labelled with *different letters*, differ significantly (*Tukey–Kramer's* test, $P < 0.05$).

Salinity	VPD	AC (400 $\mu\text{mol mol}^{-1}$)				EC (1,000 $\mu\text{mol mol}^{-1}$)			
		P_N	g_s	C_i	Ψ_l	P_N	g_s	C_i	Ψ_l
Low (0 mM NaCl)	Low (0.4 kPa)	15.1 ^a	2.05 ^a	375 ^a	-0.67 ^a	17.0 ^a	1.94 ^a	968 ^a	-0.65 ^a
	High (3.0 kPa)	14.8 ^a	0.75 ^b	348 ^b	-0.68 ^{ab}	16.7 ^{ab}	0.64 ^c	918 ^c	-0.75 ^{ab}
High (34 mM NaCl)	Low (0.4 kPa)	14.6 ^a	1.60 ^a	372 ^a	-0.85 ^c	16.9 ^a	1.25 ^b	961 ^b	-0.83 ^b
	High (3.0 kPa)	12.2 ^b	0.28 ^b	308 ^c	-0.84 ^{bc}	16.5 ^b	0.42 ^c	891 ^c	-0.88 ^{bc}
<i>ANOVA</i> (<i>P</i> value)	VPD	<0.001	<0.001	<0.001	0.981	0.001	<0.001	<0.001	0.020
	Salinity	<0.001	0.004	<0.001	0.001	0.144	0.004	0.007	<0.001
	VPD \times salinity	<0.001	0.938	<0.001	0.833	0.666	0.099	0.070	0.322

Photosynthetic properties and water status: There was a significant VPD \times salinity interaction for P_N under AC; high VPD did not affect P_N at low salinity, but significantly decreased P_N at high salinity, to 84% of the value at low VPD (Table 2). No significant interaction was observed under EC; VPD and salinity had little influence on P_N , although high VPD slightly decreased P_N (by about 2%). These results were similar to the results for NAR (Table 1). There was no significant VPD \times salinity interaction for g_s at either [CO₂]; g_s decreased greatly and significantly at high VPD and high salinity under both [CO₂]. C_i decreased significantly both at high VPD and at high salinity under both [CO₂]. The order of C_i values among the treatments was the same as the order of g_s at both [CO₂], though the proportional decrease was smaller for C_i .

Ψ_l was lower (more negative) at high salinity under both [CO₂], but was not significantly affected by VPD at either salinity level (Table 2).

The P_N – C_i curve measured at a PPFD of 300 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ (the same as the growth conditions) was similar at both [CO₂] conditions and did not much differ between the treatment groups (Fig. 2A,B). Table 3 summarizes the electron transport and carboxylation values. The J_{500} value, which was estimated from these curves, was not significantly affected by VPD and salinity under both [CO₂]. In addition, the V_{cmax} and J_{1500} values, which were estimated from the P_N – C_i curves at saturating light intensity (Fig. 2C,D), were also not significantly affected by VPD and salinity, although V_{cmax} tended to be lower at high salinity. These results indicate that the mesophyll photosynthetic capacity did not much differ between the treatment groups.

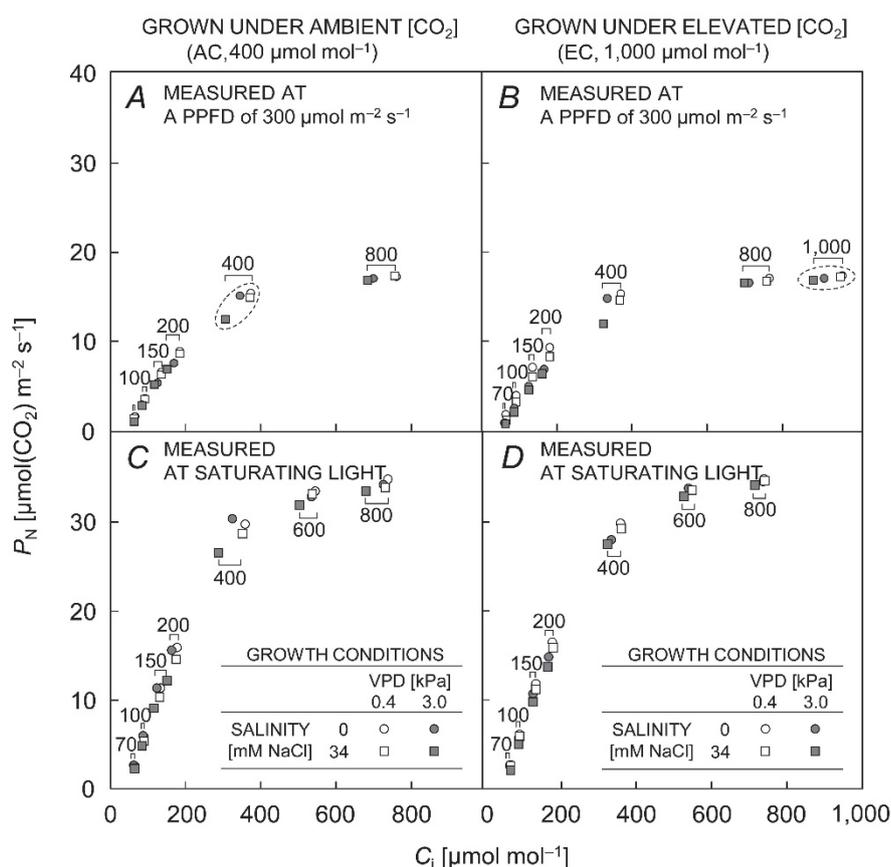


Fig. 2. Relationships between the intercellular CO₂ concentration (C_i) and the net photosynthetic rate (P_N) for cucumber (*Cucumis sativus*) seedlings grown under different combinations of vapor-pressure deficit, salinity of the nutrient solution, and atmospheric CO₂ concentration ([CO₂]; [A, C] ambient [CO₂], AC, and [B, D] elevated [CO₂], EC) for 8 d after transplantation. Photosynthesis was measured at a PPFD of (A, B) 300 and (C, D) 1,500 (saturating light) $\mu\text{mol}(\text{photon})\text{m}^{-2}\text{s}^{-1}$. The values above the symbols are the [CO₂] value for each measurement. The symbols enclosed by dashed lines indicate values measured at the same PPFD and [CO₂] as the growth conditions (the P_N and C_i values of these symbols are shown in Table 2). Measurements were conducted at the end of treatment period (8 d after transplantation). Data are the average of five replicate plants ($n = 5$).

Table 3. Electron transport rates at a PPFD of 300 $\mu\text{mol}\text{m}^{-2}\text{s}^{-1}$ (J_{300}) [$\mu\text{mol}(\text{e}^-)\text{m}^{-2}\text{s}^{-1}$] and 1,500 $\mu\text{mol}\text{m}^{-2}\text{s}^{-1}$ (J_{1500}) [$\mu\text{mol}(\text{e}^-)\text{m}^{-2}\text{s}^{-1}$], and the maximum rate of Rubisco carboxylase activity (V_{cmax}) [$\mu\text{mol}(\text{CO}_2)\text{m}^{-2}\text{s}^{-1}$] of cucumber (*Cucumis sativus*) seedlings grown under different combinations of vapor-pressure deficit, salinity of the nutrient solution, and atmospheric CO₂ concentration ([CO₂]; ambient [CO₂], AC, and elevated [CO₂], EC) for 8 d after transplantation. Values of J_{300} were estimated from the relationships between net photosynthetic rate (P_N) and the intercellular CO₂ concentration (C_i) measured at a PPFD of 300 $\mu\text{mol}\text{m}^{-2}\text{s}^{-1}$ (Fig. 2A,B). Values of J_{1500} and V_{cmax} were estimated from the relationships between P_N and C_i measured at a PPFD of 1,500 $\mu\text{mol}\text{m}^{-2}\text{s}^{-1}$ (Fig. 2C,D). Measurements were conducted at the end of treatment period (8 d after transplantation). Data are the average of five replicate plants ($n = 5$). Mean values of a parameter for a given [CO₂] that are labelled with *different letters*, differ significantly (Tukey–Kramer's test, $P < 0.05$).

Salinity	VPD	AC (400 $\mu\text{mol}\text{mol}^{-1}$)			EC (1,000 $\mu\text{mol}\text{mol}^{-1}$)		
		J_{300}	J_{1500}	V_{cmax}	J_{300}	J_{1500}	V_{cmax}
Low (0 mM NaCl)	Low (0.4 kPa)	96 ^a	170 ^a	129 ^a	90 ^a	169 ^a	128 ^a
	High (3.0 kPa)	97 ^a	168 ^a	132 ^a	96 ^a	168 ^a	131 ^a
High (34 mM NaCl)	Low (0.4 kPa)	94 ^a	168 ^a	121 ^a	93 ^a	168 ^a	124 ^a
	High (3.0 kPa)	94 ^a	165 ^a	119 ^a	93 ^a	169 ^a	127 ^a
ANOVA	VPD	0.516	0.306	0.869	0.124	0.858	0.323
	Salinity	0.262	0.207	0.077	0.794	0.706	0.207
	VPD \times salinity	0.311	0.964	0.676	0.167	0.362	0.935

Interaction between VPD and [CO₂]: There were significant VPD × [CO₂] interactions for RGR ($P=0.024$), NAR ($P=0.023$), P_N ($P<0.001$), and g_s ($P<0.001$) at high salinity, with a stronger VPD effect at AC, but insignificant interactions were observed at low salinity (RGR,

$P=0.421$; NAR, $P=0.732$, P_N , $P=0.945$; g_s , $P=0.993$). In addition, insignificant VPD × [CO₂] interaction was observed for LAR at high and low salinity ($P=0.610$ and $P=0.302$, respectively).

Discussion

Effect of salinity: In general, salinity reduces the plant growth and photosynthesis due to two phases: osmotic and ionic (Munns and Tester 2008). Chen *et al.* (2015) indicates that osmotic stress strongly affects g_s , but has no influence on photosynthetic capacity whereas ionic stress affects both. The high salinity treatment (34 mM NaCl) possibly reduces the photosynthetic capacity of cucumber through ionic effect (Drew *et al.* 1990, Chen *et al.* 2015), but did not appear to affect significantly this capacity in the present study. Thus, we consider that the salinity effects, which were observed in the present study, were mainly due to osmotic stress, although the effects of two phases could not be fully separated.

Growth analysis: The RGR is the product of NAR, which is largely the net result of carbon gain and carbon losses expressed per unit of leaf area, and LAR, which is the ratio of leaf area to whole-plant mass (*i.e.*, $RGR = NAR \times LAR$) (Poorter and Remkes 1990). The results for these parameters explain why the growth inhibition, which occurs with increasing VPD, differed between salinity and CO₂ concentrations. Under AC, high VPD significantly inhibited growth under both high and low salinity, but the underlying causal factors differed. At low salinity, the decrease in RGR with increasing VPD resulted from significantly decreased LAR, whereas the decrease in RGR at high salinity was due to significantly decreased NAR. The decrease in LAR at low salinity was mainly due to significantly reduced SLA. These results suggest that the growth inhibition caused by high evaporative demand resulted mainly from a reduction of leaf enlargement per unit of leaf DM under well-watered conditions, but resulted mainly from a reduction of net photosynthesis per unit of leaf area under water deficit conditions. This is of interest for greenhouse growers that aim to optimize environmental conditions, because, for example, CO₂ fertilization could mitigate growth inhibition caused by high VPD for situations in which such inhibition is mainly a result of a low NAR.

VPD × salinity interaction for photosynthesis: The significant VPD × salinity interaction for NAR under AC, which we observed in the present study, is similar to a previous report, in which the photosynthesis of field-grown winter wheat decreased with increased VPD at a low soil water potential but did not respond to increased VPD at a high soil water potential (Xue *et al.* 2004). This phenomenon can be explained based on the relationships between P_N and C_i obtained in the present study.

At low salinity, P_N was not reduced by the high VPD, whereas g_s decreased greatly and significantly in the same way described in our previous report (Shibuya *et al.* 2016b). In general, P_N is limited by both stomatal and nonstomatal (mesophyll) factors. The lack of significant differences in the values of J_{300} , J_{1500} , and V_{cmax} between the high and low VPD levels indicates that the mesophyll photosynthetic capacity (a nonstomatal factor) was not much altered by VPD in the present study. The P_N-C_i curve indicates that, at low salinity, the observed decrease in g_s would decrease C_i in a range where this would have only a small effect on P_N under AC (Fig. 2A). Therefore, the decreased g_s caused by higher VPD did not significantly reduce P_N by reducing C_i at low salinity. At high salinity, the observed decrease in g_s would decrease C_i in a range where this would have a considerable effect on P_N (*i.e.*, the part of the curve P_N-C_i with the steeper slopes (Fig. 2A). This difference in responses between high and low salinity may be responsible for the significant VPD × salinity interaction observed for P_N at AC and, consequently, for the significant decrease in NAR at high salinity.

The insignificant reduction of photosynthetic capacity caused by increasing VPD probably resulted from the fact that the high VPD did not decrease Ψ_1 sufficiently to affect photosynthesis although a reduction of Ψ_1 can inhibit photosynthetic capacity (Keck and Boyer 1974, Flexas and Medrano 2002, Lawlor 2002). In addition, the higher VPD greatly reduced g_s in all treatments, and this decrease would potentially maintain Ψ_1 at non-damaging levels by decreasing water loss. This probably resulted from feed-forward responses of the stomata to atmospheric moisture contents (Lange *et al.* 1971, Farquhar 1978, Grantz 1990, Saliendra *et al.* 1995, Bunce 1997, Buckley 2005, Peak and Mott 2011).

Effect of EC on photosynthesis: Under EC, there was little effect of VPD on P_N . This is due to the fact that the decrease in C_i with decreasing g_s occurred within the range of the P_N-C_i curve where the slopes were almost flat because of the higher [CO₂] (the symbols enclosed by dashed line in Fig. 2B), as reported by Wong (1993). On the other hand, at AC, higher VPD significantly decreased P_N at high salinity. This difference resulted in a significant VPD × [CO₂] interaction for P_N only at high salinity; the [CO₂] effect on P_N was greater at high VPD. This agrees with previous reports that the effect of EC on photosynthesis is greater under conditions with greater water stress (Wong 1993, Newton *et al.* 1996, Mishra *et al.* 1999,

Poorter and Pérez-Soba 2001, Pérez-López *et al.* 2013), although a contradictory result, in which EC did not reduce the sensitivity of P_N to VPD, has also been reported (Bunce 2003).

Our results seem conflict with a previous report that CO₂ fertilization had a greater effect on greenhouse-grown plants at lower VPD (Gislerød and Nelson 1989). This contradiction may be explained by the response of leaf expansion to evaporative demand. In the present study, the greatest seedling growth (RGR and DM) under EC was observed at low VPD, and this was caused by increased leaf expansion (LAR) but not by increased photosynthesis (NAR). Our results indicate that the VPD × salinity (probably osmotic potential) interaction for LAR should be considered when investigating the simultaneous effects of [CO₂] and VPD on plant growth.

VPD × salinity interaction for LAR: The reduction of SLA, which seems to be the main factor responsible for the smaller LAR, at high VPD and high salinity may be partly due to a decrease in water availability to support cell expansion (Poorter *et al.* 2009), a process that competes with transpiration (McIntyre and Boyer 1984, Westgate and Boyer 1984, Waldron and Terry 1987, Pantin *et al.* 2012). However, the reduction at high VPD cannot be fully explained by changes in the water balance, because the change in LAR was not clearly related to the change in Ψ_1 . This is probably because evaporative demand potentially controls leaf expansion without changing Ψ_1 (Salah and Tardieu 1997, Munns *et al.* 2000, Tardieu *et al.* 2000, 2010). In addition, a water deficit in the rhizosphere can directly control leaf expansion through signal transduction

pathways mediated by abscisic acid (Passioura 1988, Davies and Zhang 1991, Fricke *et al.* 2004, Liu *et al.* 2006, Zhang *et al.* 2006). The ionic stress also can induce the increase in leaf mass per area (the inverse of SLA) (Poorter *et al.* 2009). These responses are possible reasons for the reduction of LAR in response to high VPD and high salinity.

Tardieu *et al.* (2000) reported that evaporative demand and rhizosphere water potential independently affected leaf expansion, with near-additive effects. This does not agree with our results, in which the interaction between VPD and salinity (probably osmotic stress) significantly affected LAR at both [CO₂]. The significant VPD × salinity interaction for LAR, which we observed, suggests that a common underlying mechanism mediated the decreased leaf expansion caused by increasing evaporative demand and increasing salinity stress.

Conclusions: The evaporative demand and salinity interactively affected the growth and photosynthetic properties of cucumber seedlings, and these interactions differed between ambient and elevated atmospheric CO₂ concentrations. The increase in VPD reduced the seedlings' growth mainly by reducing LAR at low salinity, but by decreasing NAR at high salinity. In addition, under elevated CO₂, VPD also affected LAR, but not NAR. Such complex interactions resulted from the interactions among and individual effects of these environmental factors on photosynthesis and expansion growth in complicated ways. The underlying factors responsible for these interactions will provide important clues about the complexity of plant growth under fluctuating environments.

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