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Physiological Characteristics of Flooding Tolerance in Wild and Domesticated Species of *Capsicum*

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Abstract. **Flooding stress severely impacts plant growth and productivity by reducing oxygen availability, disrupting photosynthesis, and inducing oxidative stress. Although extensive studies have been conducted to examine drought and heat stress in *Capsicum* species, relatively little is known about response to flooding. In this study, we evaluated 17 entries from five *Capsicum* species (*Capsicum annuum*, *Capsicum baccatum*, *Capsicum chacoense*, *Capsicum chinense*, and *Capsicum frutescens*) under controlled flooding conditions to assess physiological and morphological response. Plant height, width, pollen activity, wilt index, chlorophyll content (SPAD), and leaf temperature (T_{leaf}) were measured to determine flooding tolerance. Principal component analysis revealed distinct flooding responses, with members of *C. annuum* and *C. chinense* exhibiting greater sensitivity, whereas members of *C. frutescens* and *C. baccatum* demonstrated higher tolerance to flooding stress for 5 days. Plant height and width were generally reduced under flooding stress, although responses varied by species and season. Pollen activity remained largely unaffected, suggesting that reproductive structures may be less sensitive to short-term flooding. Seasonal differences influenced flooding responses, with greater physiological stress observed in the summer, likely due to elevated temperatures. Wilt index was the most responsive indicator, showing significant interspecific variation. T_{leaf} increased under flooding stress, correlating with reductions in chlorophyll content and growth parameters. Proline accumulation increased significantly but was more closely associated with heat stress than flooding stress. These findings provide insights into species-specific flooding responses in *Capsicum* and highlight key traits for breeding flood-tolerant pepper varieties to improve climate resilience in pepper production.**

Pepper (*Capsicum annuum*) is the most important spice and an important vegetable crop worldwide, with a substantial and continuous increase in consumer demand over the past 4 decades, especially for hot pepper (Bosland and Votava 2012). It has been estimated that peppers are consumed daily by about a quarter of the world's population (Halikowski-Smith 2015). Global production of pepper was 41.9 million tonnes on an area of 3.7 million hectares in 2022 and ∼70% of pepper is produced in Asia (FAO 2023). Climate change significantly reduces agricultural productivity (Ortiz-Bobea et al. 2021) and exposure to the effects of climate change are most severe in Asia, particularly South Asia (Byers et al. 2018), where most pepper production occurs and large populations of people, who are vulnerable to poverty, live.

The primary limitations to increased pepper productivity and quality are biotic and abiotic stresses (Gull et al. 2019). The major abiotic stresses limiting pepper productivity include high or low temperatures, water deficiency, or flooding stress (Palada and Wu 2010). Although a significant body of research on the response of pepper to high temperatures (Aloni et al. 1992; Erickson and Markhart 2002; Guo et al. 2014; López-Marín et al. 2013) and water deficit stress (Alvino et al. 1994; Delfine et al. 2001; Lim and Kim 2013; Lurie et al. 1986; Yanez et al. 1992) exists, there are a limited number of flooding stress studies in pepper. However, it has been known for more than 40 years that pepper is particularly sensitive to soil inundation (Cotter 1980; Tindall 1983). Flooding of pepper plants for 96 h significantly reduced photosynthetic rate and mean leaf size and the resulting seeds from flooded plants had lower viability and germination rates (Sundstrom and Pezeshki 1988).

Flooding stress significantly impacts plant growth and productivity by causing oxygen deficiency (hypoxia or anoxia) in the root zone, leading to a shift from aerobic to anaerobic respiration, reduced ATP production, and the accumulation of toxic by-products such as ethanol and lactic acid (Loreti et al. 2016). Photosynthetic activity is suppressed due to chlorophyll degradation, stomatal closure, and damage to the photosynthetic apparatus (Jackson and Colmer 2005), whereas nutrient imbalances arise from inhibited uptake, leaching, and ion toxicity (Striker 2012). Oxidative stress from reactive oxygen species (ROS) accumulation damages cellular components (Voesenek and Bailey-Serres 2015), further exacerbated by hormonal imbalances such as increased ethylene and abscisic acid levels, which mediate stress responses like adventitious root formation and stomatal closure (Renziehausen et al. 2024). Morphological adaptations, including aerenchyma formation and stem elongation, are common survival strategies (Sasidharan et al. 2018), but growth inhibition, carbohydrate depletion, and susceptibility to pathogens often hinder recovery. The array of physiological responses to flooding stress highlight the complexity of the plant response and underscore the need for resilient crop varieties and effective management practices to mitigate the effects of flooding stress. Screening flooding stress tolerance in crop varieties from diverse genetic backgrounds could be efficiently conducted by assessing their phenotypes using these physiological indices.

The flooding tolerance of various *Capsicum* species differs due to their unique physiological and genetic characteristics. Members of *C. annuum* generally exhibit sensitivity to waterlogging, often resulting in plant death and yield reduction, although genotypic variability exists within *C. annuum* (Gong et al. 2022). In contrast, members of *C. frutescens* have demonstrated a degree of tolerance to short-term waterlogging, with certain varieties withstanding up to 10 d of flooding without significant adverse effects (Saptiningsih

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Table 1. The pepper entries used in the study.

Entry	Other name	Species
VI037629	PBC 743	*C. annuum*
PBC 2029	17HT3	*C. annuum*
PBC 2021	14DI3	*C. annuum*
VI046870	PBC 481	*C. annuum*
AVPP9824		*C. annuum*
VI028788	PI 439372	*C. baccatum*
VI029116	PI 441596	*C. baccatum*
VI012904	PBC 2075	*C. baccatum*
VI013286	PBC 2074	*C. baccatum*
VI029119	PI 260427	*C. chacoense*
VI044312	PBC 2077	*C. chacoense*
VI057807	PBC 2078	*C. chinense*
VI044313	PI 152225	*C. chinense*
AVPP1923	PBC 1423	*C. chinense*
VI029485	PI 224431	*C. frutescens*
PBC 934	Green chuna -Acc.1	*C. frutescens*
VI037646	Toom	*C. frutescens*

et al. 2023). However, detailed studies on the flooding tolerance of other species, such as *C. baccatum*, *C. chinense*, and *C. chacoense*, are limited, necessitating further research to fully understand their responses to flooding stress (Tajima 2020). The variation in response to flooding underscore the importance of species-specific investigations to enhance flooding tolerance in peppers through targeted breeding and effective management practices.

To address this gap in knowledge, the objectives of the present study were to assess the flooding tolerance of members of *C. annuum*, *C. frutescens*, *C. baccatum*, *C. chinense*, and *C. chacoense* by evaluating their physiological and morphological responses to waterlogging stress. Specifically, we aimed to identify phenotypic traits associated with flooding tolerance. By comparing these species, we sought to contribute to the development of more resilient pepper varieties capable of withstanding the increasing challenges posed by climate change and waterlogging conditions.

Materials and Methods

Plant material. For this experiment, we evaluated 17 entries, five members of *C. annuum*, four *C. baccatum*, two *C. chacoense*, three *C. chinense*, and two *C. frutescens* (Table 1). The previously reported tolerant *C. frutescens* entry 'Toom' was used as a positive control (Palada and Wu 2008). The selection of these lines was based on preliminary experimental results, which are unpublished, and observations under open-field production conditions.

Experimental conditions. The experiment was conducted at the World Vegetable Center in Shanhua, Tainan, Taiwan (lat. 23.1°N, long. 120.3°E, elevation 12 m). The location has a warm, humid subtropical climate (Köppen *Cfa*) and a tropical wet and dry climate (Köppen *Aw*) (Kottek et al. 2006). The experiment was a complete randomized design with four plants per treatment (unflooded control and flooded). Individual plants were used as experimental units. Seeds of each entry were sown into 72-cell plastic trays filled with sterilized coconut-coir media. At the four to six true-leaf stages, the seedlings were transplanted into 7-inch pots filled with triple-autoclaved soil. The experiment was repeated two times, once during winter, with sowing on 12 Oct and transplanting on 12 Nov, and then once during the summer season, with sowing on 3 May and transplanting on 31 May. The plants were maintained in a polyhouse without climate control and managed as uniformly as possible. The plants were hand-watered twice daily, from sowing until treatment. A balanced fertilizer was applied every 2 weeks throughout the growing season and scouting for pests and diseases was done twice weekly, and appropriate pesticides were applied as needed.

Pretreatment data collection. Before the flooding treatment, measurements of plant height, plant width, flower number, leaf temperature, wilting score, chlorophyll content, proline content, pollen concentration, and pollen activity were taken as baseline measurements. Plant height was measured using a ruler from the soil line to the top of the plant at its highest point. Similarly, plant width was measured from the top of the plant canopy at the widest points. The flower number was the total number of flowers per plant. Pollen concentration and pollen activity were measured on six flowers from each replication using impedance flow cytometry, following the protocol established by Lin et al. (2022). For proline analysis, 10 leaves were collected from each of four replications at 8:00 AM to 9:00 AM 5 d after flooding. Free total proline (PRL) was determined by a colorimetric assay (Claussen 2005) with some modifications. Leaf samples were stored at −80 °C for 24 h, then the samples were lyophilized (−72 °C) in a freezer dryer (IlShinBioBase, Dongducheon, South Korea) for 3 d. Leaf samples were ground to a fine powder in liquid N_2 and 300 mg (dry weight) was taken and homogenized with 1.5 mL of 3% (wt/vol) aqueous sulfosalicylic acid solution. The homogenate was centrifuged at 14,000 rpm for 7 min. Then 1 mL of supernatant was transferred to 5 mL microtubes and 1 mL acid ninhydrin solution was added. Acid ninhydrin was prepared by adding 2.5 g ninhydrin to 100 mL solution containing glacial acetic acid, distilled water, and 6M ortho-phosphoric acid 85% at a ratio of 6:3:1. The reaction mixtures were then immediately incubated in a boiling water bath for 1 h and subsequent absorption was read at 546 nm on a spectrophotometer (EON^{TM} BioTek Instruments, Winooski, VT, USA) and the proline content was estimated using a standard curve determined with 0, 4, 8, 12, 16, and 20 $\mu g \cdot mL^{-1}$ proline.

Flooding treatment. For the flooding treatment, the pots were submerged in irrigation water to the level of $\sim$2 cm above the soil line (Supplemental Fig. 1). The submergence was accomplished by placing the pots into buckets that were then filled with water (day 0). The plants were maintained in the flooded conditions for 5 d (day 5), throughout which measurements were taken each day. Then, the plants were removed from the flooded conditions and not irrigated for 3 d to allow the plants a recovery period. After the recovery period of 3 d (day 8), measurements were taken again.

Wilt index and chlorophyll content. Data were collected on wilting (visual score), leaf temperature, plant height, canopy size, and chlorophyll content estimated by SPAD index (SPAD-502, Konica Minolta Inc., Tokyo, Japan). The wilting score was determined using a scale from 0 (normal) to 5 (severe). To measure the SPAD index, readings were taken from five leaves per plant using the SPAD device at 10:00 AM.

Statistical analysis. The data underwent analysis using R version 4.3.2 (R Core Team 2021). Given the different environmental conditions over the two growing seasons of the study (Fig. 1), which was also confirmed by the clustering by principal components analysis (PCA) (Fig. 2), the focus of the statistical analysis was on evaluating the effects of flooding stress on the growth and physiological responses of the pepper entries. Consequently, the data from the winter (cool) and summer (hot) seasons were analyzed separately, and the outcomes are presented accordingly.

To understand the underlying data structure, PCA was conducted using the winter and summer season data to evaluate the influence of species and flooding treatments on the measured variables. For multivariate analysis, Pearson's

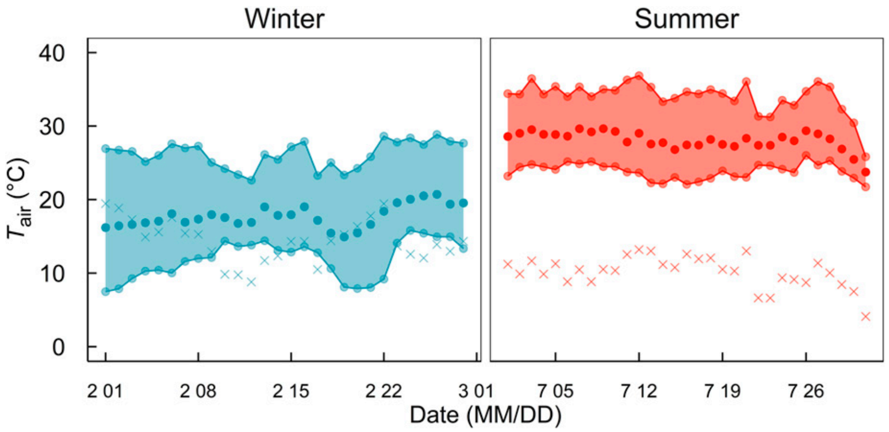

Fig. 1. Daily air temperatures (maximum, average, minimum, and difference) over the course of the study (left: winter growing season; right: summer growing season).

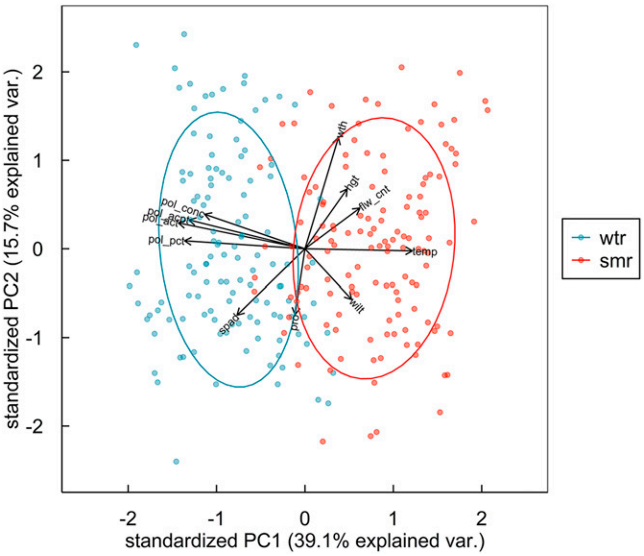

Fig. 2. Principal component analysis results separated by season (blue: winter; red: summer). Arrows indicate the loadings of each variable[i] on principal component (PC)1 (horizontal) and PC2 (vertical). [i]flw_cnt = numbers of flowers; hgt = plant height; pol_acpt = numbers of acceptable pollen; pol_act = numbers of active pollen; pol_ conc = pollen concentration; pol_pct = active pollen in percent; pro = proline content; spad = chlorophyll content in SPAD unit; temp = leaf temperature; wilt = wilt index; wth = plant width.

correlation coefficients (Pearson's r) were computed to assess the relationship between the growth and the physiology variables in both untreated control and flooding stress-treated groups, with the aim of investigating the impact of flooding stress on these correlations in the entries. Furthermore, a simple linear regression model was employed to examine the associations between variables. The regression coefficients derived from this model were then compared between untreated and flooding-stressed groups using Zou's method (Zou 2007), which is based on Fisher's z-transformation with $\alpha = 0.05$ for all analyses.

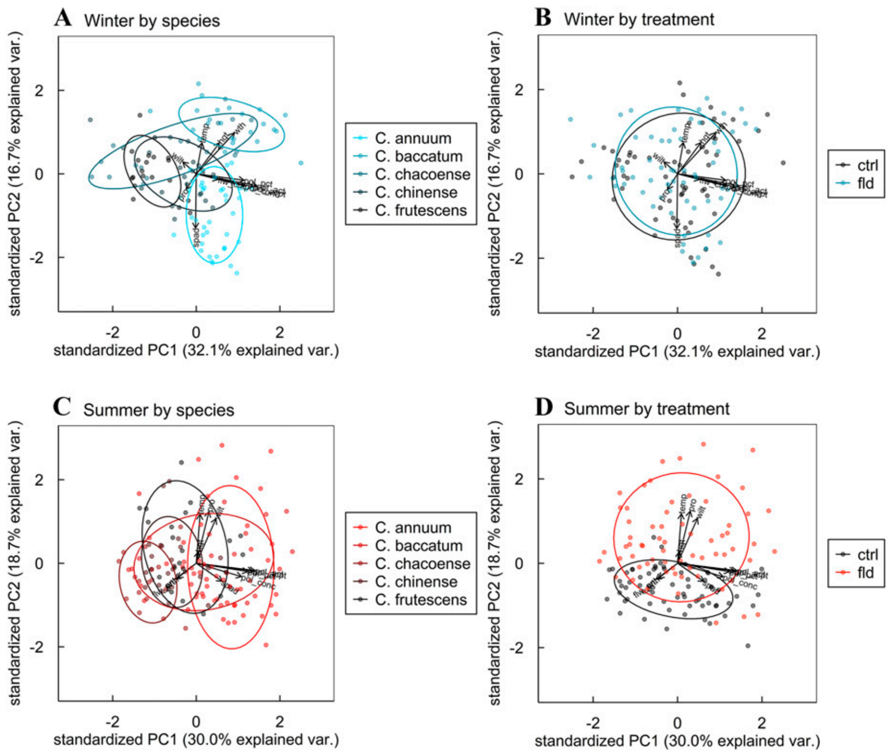

Fig. 3. Principal component analysis results, separated by species and by season (blue: winter; red: summer). Arrows indicate the loadings of each variable[i] on principal component (PC)1 (horizontal) and PC2 (vertical). [i]flw_cnt = numbers of flowers; hgt = plant height; pol_acpt = numbers of acceptable pollen; pol_act = numbers of active pollen; pol_ conc = pollen concentration; pol_pct = active pollen in percent; pro = proline content; spad = chlorophyll content in SPAD unit; temp = leaf temperature; wilt = wilt index; wth = plant width.

Finally, for the individual variable analysis, a fixed-effect three-way analysis of variance (ANOVA) model was employed, with genotype effects of the entry, stress effects of the flooding treatments, and time effects of the flooding treatments [days after flood treatment (DAT)] designated as fixed effects, and their interaction effects were integrated into the model. Following the ANOVA of the three main factors, a post hoc mean separation was conducted on the variables measured at each time point using the *t* test procedure to investigate the effects of treatment between control and treated plants.

Results

Environmental data. The mean daily average air temperature was 17.7 and 28.1 °C in the winter and the summer, respectively (Fig. 1). The mean daily maximum and minimum air temperatures were 26.2 and 11.9 °C for the winter and 34.0 and 23.8 °C for the summer. The mean solar radiation was similar between the two seasons at 5164 and 5277 MJ·m^{-2} for the winter and the summer, respectively. The mean reference evapotranspiration was greater in the winter, at 4.65 mm, than in the summer, at 3.77 mm. Notably, the differences in average temperature, and also the mean daily temperature deficits, were higher at 14.4 °C in the winter season, compared with 10.2 °C during the summer.

Principal components analysis. We identified differences in the response variables to flooding stress by season (Fig. 2). The distribution and the segregation of the response variables to flooding stress differed among the species and entries, but not as drastically as the seasonal differences. Treated groups moved toward positive principal component (PC)2 in the winter vs. negative PC2 in the summer.

PC1 and PC2 explained 39.1% and 15.7% of the variance in the data, respectively, and together explained 54.8% of the total variance (Fig. 2). There was an overlap between the winter and summer seasons. However, the seasons were largely distinct, indicating that the first two PCs captured a significant separation between these groups. The winter season tended to cluster toward the negative side of PC1, indicating higher values for the pollen variables of pollen concentration, active pollen, the proportion of active to inactive pollen (hereafter pollen activity), and SPAD values. The summer season tended to cluster toward the positive side of PC1 and PC2, suggesting higher values for wilt index, numbers of flowers, and T_{leaf}.

For the winter season, although the first two PCs explained a substantial portion of the variance, they did not fully distinguish between the different species, given the overlap in the ellipses (Fig. 3A). Members of *C. annuum* and *C. frutescens* had wider dispersions, indicating more variability within these species, whereas members of *C. chinense* and *C. chacoense* had more compact distributions, indicating less variability within these species. The variable wilt index separated some

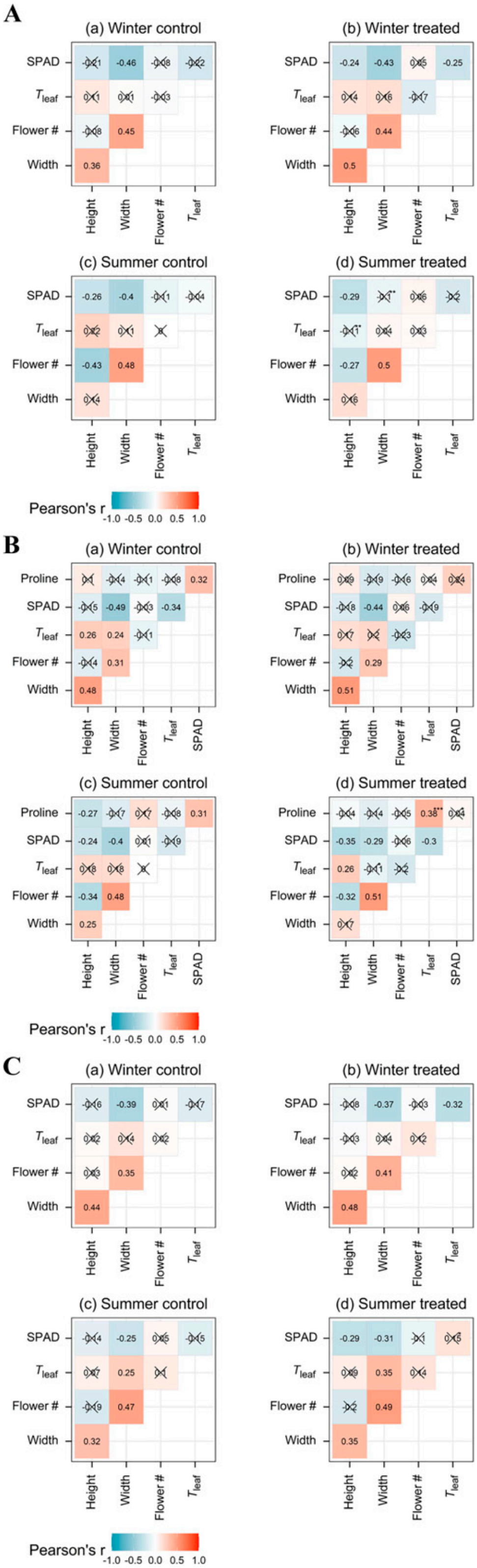

Fig. 4. Correlation matrix of measured variables at 1 d (**A**), 3 d (**B**), and 5 d (**C**) after flooding treatment separated by season. SPAD = chlorophyll content; T_{leaf} = leaf temperature.

species along PC2, whereas the pollen variables separated along PC1. The pattern in variable loadings was also observed in the PCA plot by treatments (Fig. 3B). The control group had a wider dispersion, indicating more variability within this group. The treated group showed a more compact distribution, indicating less variability within this group. The two groups (winter and summer seasons) were not fully distinguishable. Similarly, for the summer season, although the first two PCs explained a substantial portion of the variance, given the overlap in the ellipses, they did not fully distinguish between the different species (Fig. 3C). The control group had a compact distribution, whereas the treated group had a dispersed distribution of the PCs (Fig. 3D) and this pattern was opposite to the winter season. In addition, despite the overlap, subtle discrimination of the control group cluster against the treated group cluster was observed. Notably, the loading of proline content on PC2 was negative for the winter season (Fig. 3B) but positive for the summer season (Fig. 3D).

Time-course correlation analysis. The correlations between the variables (indices) changed over the time of the measurements (Fig. 4). At 1 DAT (Fig. 4A), drastic differences between treatment were found across the seasons. Growth was slightly greater in the summer compared with the winter, as indicated by greater height, width, and numbers of flowers. Importantly, there were weak correlations between all the measured variables, and as a result, the correlation coefficients were less than ± 0.5 at 3 DAT (Fig. 4B). Proline content was correlated with both the wilt index and T_{leaf} only in the summer season. At 5 DAT (Fig. 4C), T_{leaf} was lower in the summer than in the winter, with a positive correlation with wilt index.

As the time after flooding treatment progressed, the physiological indices responded at a different pace, indicated in the progression of T_{leaf}, wilt index, and SPAD. We detected changes in leaf temperature, in response to flooding treatment, at early time points (at 3 DAT) than for wilt index and SPAD. The wilt index followed the flooding stress responses; differences were found at 3 DAT to some extent, which became notable at 5 DAT. SPAD was found to be lower in treated groups by 5 DAT, compared with the control. Proline content was higher in treated groups by DAT 3, compared with the control, but little association with the other indices was found.

Height and width were positively correlated (0.32–0.48), which was greater in the winter (0.44 and 0.48 for control and treated, respectively) than in the summer (0.32 and 0.35 for control and treated, respectively). The correlation coefficients were nominally greater under flooding treatment, as compared with the control, but not significant. The number of flowers and plant width consistently correlated across the seasons and time after treatment, which differed from plant height and flower number. With the exception at 1 DAT, flower number and plant height were consistently negatively correlated. In the summer, the correlations of proline content and SPAD were reversed after treatment (both significant), compared with the winter.

ANOVA. The three-way interaction was not significant for all the variables measured except for wilt index (Table 2), whereas the two-way interactions of entry and flooding treatment and the 2-way interactions of treatment and time (DAT) all significantly contributed to the variability observed during the summer evaluation. As a follow-up, we dissected two-way interactions into main effects at each level of one factor at each time point

Table 2. Three-way analysis of variance results on the physiological parameters measured (*F*-statistics with *P* values in parentheses).

			Parameters[i]							
Season	Factor[ii]	*f*	Height	Width	Flower count	Leaf temp.	SPAD	Wilt index	Pollen activity	Proline content
Winter	VI	6	742.1	342.2	45.5	52.7	476.1	11.3	5.589	10.34
			(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
	TRT	1	0.34	7.44	1.90	156.8	21.3	448.4	0.009	14.68
			(0.561)	(0.007)	(0.169)	(<0.001)	(<0.001)	(<0.001)	(0.927)	(<0.001)
	DAT	2	97.8	45.3	23.8	481.0	1.7	155.1	—	—
			(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.193)	(<0.001)		
	VI × TRT	16	2.63	4.95	0.45	3.3	3.2	10.4	0.599	0.715
			(0.001)	(<0.001)	(0.969)	(<0.001)	(<0.001)	(<0.001)	(0.868)	(0.773)
	VI × DAT	32	1.62	1.98	1.04	3.0	3.3	4.54	—	—
			(0.022)	(0.002)	(0.407)	(<0.001)	(<0.001)	(<0.001)		
	TRT × DAT	2	1.30	2.12	0.23	95.4	30.8	145.3	—	—
			(0.273)	(0.122)	(0.973)	(<0.001)	(<0.001)	(<0.001)		
	VI × TRT × DAT	32	0.33	0.44	0.35	0.9	0.9	4.32	—	—
			(0.999)	(0.997)	(1.000)	(0.612)	(0.696)	(<0.001)		
Summer	VI	6	231.3	180.6	181.6	50.7	289.3	19.4	15.3	3.7
			(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
	TRT	1	0.08	45.7	1.67	423.9	46.4	2036	0.023	122.1
			(0.776)	(<0.001)	(0.198)	(<0.001)	(<0.001)	(<0.001)	(0.881)	(<0.001)
	DAT	2	49.8	5.72	25.3	2286	16.7	352.2	—	—
			(<0.001)	(0.004)	(<0.001)	(<0.001)	(<0.001)	(<0.001)		
	VI × TRT	16	4.46	6.06	1.43	2.5	4.4	11.8	1.08	3.02
			(<0.001)	(<0.001)	(0.127)	(0.002)	(<0.001)	(<0.001)	(0.383)	(<0.001)
	VI × DAT	32	1.21	2.47	2.74	5.7	2.6	2.26	—	—
			(0.205)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)		
	TRT × DAT	2	20.1	18.2	2.34	82.7	11.3	364.8	—	—
			(<0.001)	(<0.001)	(0.098)	(<0.001)	(<0.001)	(<0.001)		
	VI × TRT × DAT	32	0.27	0.46	0.77	0.90	1.2	2.16	—	—
			(1.000)	(0.996)	(0.810)	(0.561)	(0.188)	(<0.001)		

[i] Height, width, flower count, and chlorophyll content (SPAD) were measured 1, 3, and 5 d after flooding treatment (DAT), whereas leaf temperature was measured also on 4 DAT and wilt index 2, 4, and 8 DAT. Pollen activity and proline content were measured only on 3 DAT.

[ii] VI, entries (genotype effects); TRT, flooding treatment (stress effects); DAT, days after flooding treatment (time effects).

(i.e., DAT). Then, within-group comparisons of the means were conducted.

For T_{leaf}, treatment effects were substantial with all two-factored interactions being significant. Likewise, SPAD showed a high sensitivity to flooding treatment. Wilt index was the most responsive variable measured over the treatment period, including the recovery after the flooding event. Three-way interactions were also significant for both seasons for wilt index. Unexpectedly, pollen activity showed no significant differences by either treatment or the two-way interaction of entry by treatment and statistical differences were observed only for entries.

The difference by treatment for plant width was found only at 5 DAT for both the winter and the summer. Flower counts in the winter did not show meaningful differences, whereas in the summer, both treatment and the two-way interaction of entry by treatment were significantly different for flower count. A difference by treatment was detectable in T_{leaf} at 3 DAT from the data in the winter. The observation was consistent over at 1, 3, and 5 DAT in the winter. The summer data showed no difference by treatment at 1 DAT. At 3 DAT, all effects were significant; the entries responded differently to flooding treatment (stress effects) (TRT).

For SPAD, only at 5 DAT, a significant difference was detected from treatment in both the winter and the summer. In addition, the interaction effect of entry by treatment was also observed in the summer.

For non–time-series variables, treatment did not affect pollen activity in either the winter or summer seasons. Proline was significantly different for the flooding treatment during both seasons.

Most of the variables changed as time after flooding treatment progressed. We confirmed that the development of the symptoms detected by the variable depended on the sensitivity of the variables. Less sensitive variables could be detected at 5 DAT; more sensitive variables at 3 DAT.

Physiological indices. During the winter season, VI044312 had an increase in plant height at 1 DAT under flooding treatment (Fig. 5A). During the summer season, plant height was reduced for two entries, PBC 2029 and PBC 743 at 5 DAT, indicating these two entries were rapid in response to flooding stress.

Plant width was more responsive to flooding stress compared with plant height (Fig. 5B). The width of PBC 743 under flooding stress was significantly reduced at 5 DAT in the summer. For VI029119, a decrease in width was found on all time points at 1, 3, and 5 DAT in the winter. For the summer, two entries—PBC 934 and VI037646—had reduced plant width after flooding at 3 and 5 DAT.

As expected, flower counts for most of the entries were not significantly different between control and treated groups by flooding (Fig. 5C). However, only VI028788 and VI044312 in the summer displayed a significant difference at 5 DAT.

As flooding stress was imposed, from 1 to 3 DAT, T_{leaf} decreased in the winter and increased in the summer (Fig. 5D). The increases in T_{leaf} by flooding were distinct in the summer across all entries. Over the recovery period, from 3 to 5 DAT, T_{leaf} returned to the levels at 1 DAT. For T_{leaf}, highly significant differences were found, especially for the summer at 3 DAT (Fig. 5D). With the exception of VI046870, all the entries showed substantial increases in T_{leaf} under flooding stress. The T_{leaf} during the winter season increased for some of the entries as flooding treatment progressed to 5 DAT.

Three *C. frutescens* entries (PBC 934, VI029485, and VI037646) had a reduction in SPAD values as the flooding stress progressed (Fig. 5E), during the summer season, and no other significant differences were found for SPAD.

Clear differences between seasons and by species were found for wilt index (Fig. 5F). The impacts of the flooding treatment in the summer appeared to be greater than in the winter. In the winter, two *C. annuum* entries (AVPP9824 and VI046870), three *C. baccatum* entries (VI012904, VI013286, and VI029116), and two *C. chacoense* entries (VI029119 and VI044312) had low wilt index under flooding treatment, but with varying degrees.

A single entry, PBC 2021 had reduced pollen activity after the 3-d flooding (Fig. 6A). Most entries had significant increases in proline content only in the summer (Fig. 6B). Average pollen activity values were lower (less than 60% even for control groups) in the summer than those in the winter (~90% overall) (Fig. 6A).

After 5 d of flooding treatment, plant height decreased in all entries except for AVPP9824, VI029116, VI013286, VI057807, and VI037646, which showed no significant difference from the control (Fig. 5A). Plant canopy width was slightly smaller in the flooding treatment for all *C. pubescens* entries. Wilting did not occur quickly during

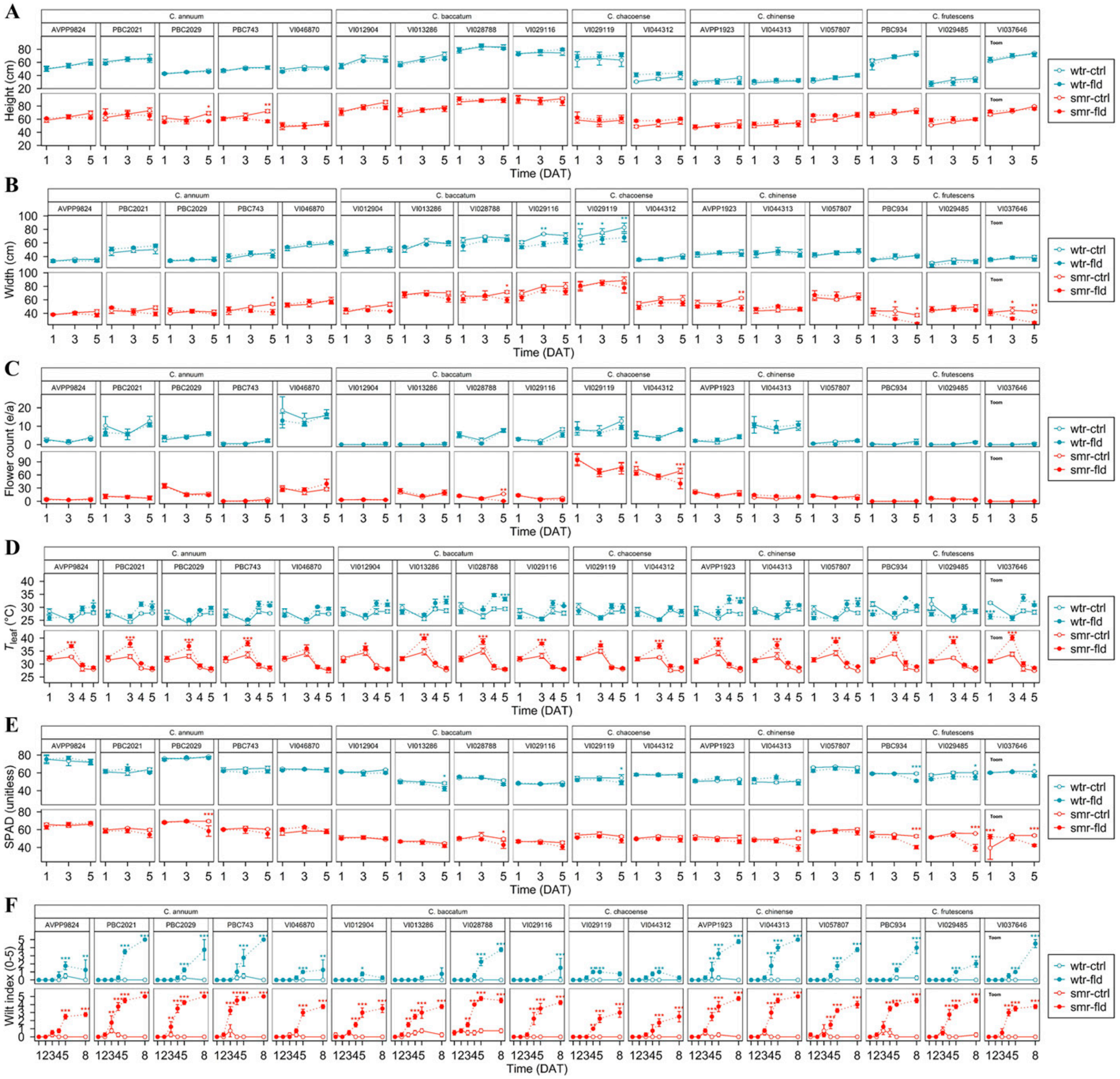

Fig. 5. Time-course responses of the measured variables—height (**A**), width (**B**), flower counts (**C**), leaf temperature (T_{leaf}) (**D**), chlorophyll content (SPAD) (**E**), and wilt index (**F**)—of 17 chili pepper entries of five *Capsicum* species—*annuum*, *baccatum*, *chacoense*, *chinense*, and *fruitescens*—grown during winter (wtr) and summer (smr) seasons. The plants were subjected to 5-d flooding stress and the subsequent 3-d recovery with drainage. Open symbols indicate the means from nonflooded control plants (ctrl) and closed symbols indicate those from flooding-treated plants (fld) with four replications. Error bars present ±1 standard error of the means. The flooding tolerance reference entry, VI037646, is annotated with *Toom*.

the winter; however, 3 d after flooding, the wilt index was severe for *C. pubescens* and *C. chinense* entries (Fig. 5F). The T_{leaf} of most entries increased at 5 d after flooding compared with the first day (Fig. 5D). However, after recovery, the wilt index of VI046870, AVPP9824, VI029116, VI012904, VI013286, VI029119, VI044312, and VI0 29485 was less than 2.5 (Fig. 5F). The SPAD index decreased in most entries except in PBC 2029, VI029116, and VI0443 12 in response to flooding (Fig. 5E). In most entries, pollen viability was reduced in the flooding treatment, but pollen viability of PBC 743, VI046870, VI029116, VI013286, VI029485, and PBC 934 was not significantly different from the nonflooded control (Fig. 6A). Flower number tended to decrease with flooding stress, but was not significantly different among the treatments, except for VI028788 and VI044312, which both were reduced (Fig. 5C). Proline content significantly increased in flooding-treated plants, and variability occurred among the entries (Fig. 6B).

Discussion

The PCA findings on species (Fig. 3A and 3C) indicate that *C. annuum* and *C. chinense* exhibit similar responses to flooding stress in both seasons. However, they were distinctly separated from *C. chacoense*, which appears more tolerant to flooding stress, particularly in the summer season. In contrast, three entries of *C. frutescens* were found to be sensitive to flooding stress (Pahlevi et al. 2019). Entries of *C. annuum* and *C. chacoense* are known to be sensitive to flooding stress, thus requiring careful water management. Compared with *C. annuum* and *C. chacoense*, *C. chinense* exhibits better flooding tolerance. Wild species tend to have a greater flooding stress resistance than domesticated species of *Capsicum* (Ou et al. 2011).

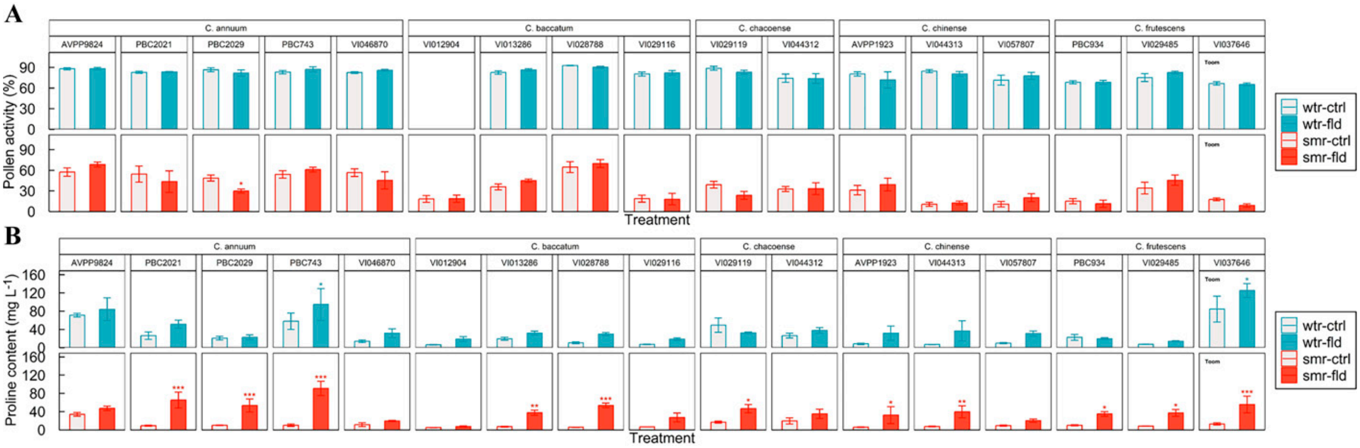

Fig. 6. Pollen activity (**A**) and proline content (**B**) of 17 chili pepper entries from five *Capsicum* species—*annuum*, *baccatum*, *chacoense*, *chinense*, and *fruitescens*—grown during winter (wtr) and summer (smr) seasons. The plants were subjected to 5-d flooding stress and the measurements were made 3 d after the flooding treatment. Open symbols indicate the means from nonflooded control plants (ctrl), whereas closed symbols indicate those from flooding-treated plants (fld) with four replications. Error bars present ±1 standard error of the means. The flooding tolerance reference entry, VI037646, is annotated with *Toom*.

Greater average daily air temperatures (Fig. 1) during the summer season likely contributed to the variations between seasons observed in the study. Distinct clustering between the two seasons in the PCA (Fig. 2) also supports the segregation of the data from the winter and the summer seasons. Increased temperatures tend to exacerbate flooding stress on the plants (Schulze et al. 1973), which aligned with the observations that higher T_{leaf} (Fig. 5D) and lower SPAD (Fig. 5E) occurred in the summer, as compared with the winter. The higher T_{leaf} and lower SPAD contributed to more distinguished clustering between the control and flooding-treated groups in the summer (Fig. 3D) than in the winter (Fig. 3B). The increased air temperature likely affected T_{leaf} (Fig. 5D), leading to damage to chlorophyll (Hu et al. 2020), supported by the decreased concentration of chlorophyll during the flooding conditions (Fig. 5E). The chlorophyll degradation of the leaves under flooding can be attributed to decrease in oxygen availability (Bailey-Serres and Colmer 2014), activation of chlorophyllase enzymes (Panda and Barik 2021), production of ROS (Guo et al. 2022), and promotion of the senescence pathway (Banga et al. 1997).

The summer experiment resulted in a greater intensity of flooding stress responses as compared with the winter season, as demonstrated by the differences in T_{leaf} and wilt index (Fig. 5D and 5F, respectively). However, biomass indices, such as height (Fig. 5A), width (Fig. 5B), and flower number (Fig. 5C), did not respond timely enough to be used as a proxy for flooding tolerance. Similarly, none of the pollen-related indices were markedly reduced by flooding treatment (Fig. 6A). Proline content seemed to be related to both the wilt index and T_{leaf} only in the summer season (Fig. 6B), indicating that the heat stress was likely more associated with proline content and not the flooding stress or a combination of both stress factors.

The 3-way ANOVA results showed the vegetative indices were more sensitive to flooding stress than the reproductive indices (Table 2). This may imply that the vegetative organs and tissues would be quickly affected by flooding due to the lack of O_2 delivery to the cells over the imposed flooding stress, while the reproductive capacity would be slowly reduced. The pollen that had already formed at the beginning of treatment could maintain its viability and activity at the time of measurement (i.e., 3 DAT), suggesting that sampling timing might have been too early to detect the differences. As for height, entry was the greatest contributor to variability observed for plant height during both the winter and summer seasons. Post hoc pairwise comparison differentiated entries on height; however, these differences only tell the general phenotypical characteristics of each entry, not providing much information about how tolerant they are against the flooding treatment.

We can divide the measured variables into three groups in relation to their sensitivity to treatment. Less responsive time-series variables were plant height, flower counts, and pollen activity, while more responsive time-series variables were plant width (notably in the summer), T_{leaf}, SPAD, and wilt index. Among these, quickly measurable physiological indices—T_{leaf} and SPAD—would have the potential to be used in screening phenotypes. Particularly, in relation to SPAD, genotypes differ in their SPAD response to flooding under higher temperatures (i.e., the summer), although the threshold critical time is at 5 DAT.

In line with these observations, T_{leaf} (Fig. 5D) was a sensitive variable that can enable us to easily detect flooding stress, while not distinguishing the indiscreet differences in tolerance to the stress. Using T_{leaf} was effective for selecting a hyper-flooding-tolerant entry. We speculate that the increased T_{leaf} (Fig. 5D) affected the leaf photosynthetic functions at 3 DAT, began to damage the photosynthetic pigments and the composition of the light-harvesting complex, and in the end, led to the decreased SPAD (Fig. 5E) (i.e., a proxy for chlorophyll content) at 5 DAT. The observation is similar to the results reported by Rho et al. (2022).

Wilt index was confirmed as the most crucial index of all the variables, associated with flooding tolerance, collected in the study (Fig. 5F). In both the winter and summer seasons, at the onset of flooding, entries did not show any differences, whereas 5 DAT appears to be the critical time to observe the wilting responses and up to at 8 DAT. Even at 3 DAT, the wilt index demonstrated significant differences for treatment and entry × treatment. The same pattern was observed at 5 DAT, with a highly significant influence of treatment. As also indicated in the three-way ANOVA results, the wilt index was the most responsive variable to detect the symptoms of flooding at the physiological level.

The entry VI044312 has an adaptive trait to elongate (etiolate) the stem (Fig. 5A) to supposedly get more oxygen during the flooded soil. This stem (or leaf) elongation can be a strategy to avoid damage of flooding stress and previous work has demonstrated that leaf or stem elongation strategy under flooding can differ even within the same species (Chen et al. 2010).

A 3-d flood was not enough to observe reductions in biomass-related responses. Along with the wilt index, T_{leaf} is a highly responsive index for evaluating flooding tolerance. The relationship between proline content and flooding tolerance was not explicit enough to draw a conclusion (Fig. 6B), although previously it was reported that flooding treatment dramatically increases proline content (Ou et al. 2011). We found that *C. pubescens* entries were highly sensitive to flooding stress, which is contradictory to the previous research. Entries of *C. annuum* (VI046870, AVPP9824), *C. baccatum* (VI029116, VI012904, VI013286), *C. chacoense* (VI029119, VI044312), and *C. frutescens* (VI029485) could be tolerant to flooding, but only observed in the summer season with heat likely influencing the results; further research is needed to confirm this.

Overall, this study provides new insights into interspecific variation in flooding tolerance among *Capsicum* species and identifies wilt index, leaf temperature, and chlorophyll content as key indicators of stress response. These findings offer a foundation for developing rapid, canopy-based phenotyping tools and support breeding efforts to enhance flooding resilience in pepper cultivars under changing climate conditions.

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