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Waterlogging Impairs Quality and Flower Longevity in *Zinnia elegans* Jacq. cv. 'Dreamland'

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ABSTRACT

Common causes of waterlogging are intense rainfall, excessive irrigati on, and poor drainage. In Iran, notwithstanding the northern parts of the country with high precipitation, waterlogging may occur due to over-irrigation or inadequate drainage, especially in the case of landscape ornamentals. In this study, zinnia plants (*Zinnia elegans* Jac q.) were exposed to waterlogging at three growth stages (i.e. four-leaf stage, full growth, and flowering) and the effects of waterlogging were monitored at different durations of the episode (0, 6, 12, and 24 h) on flowering induction, plant growth, and external quality. Waterlogging generally downgraded visually-perceived quality (e.g. s tem length, flower size, leaf coloration), increased the risk of buckling (i.e. lower stem strength) and shortened flower bud longevity. Waterlogging adversely affected biomass accumulation by decreasing light capture (leaf area) and photosynthesis. While water relations were distorted by waterlogging, waterlogged plants underwent oxida tive damage as indicated by reduced chlorophyll content and elevated lipid peroxidation level. Waterlogging was further associated with increased activity of antioxidant enzymes (i.e. ascorbate peroxidase and peroxidase). The waterlogging-induced effects were generally more prominent as waterlogging duration increased. The plants were more susceptible to waterlogging in their early growth stages. At the four-leaf stage, waterlogging for 24 h caused the most negative effects , making plant dry weight decrease by 75% of that in the control. In conclusion, the results offered a quantitative analysis of how the growth stage and duration of exposure can shape waterlogging-induc ed injury in zinnia.

Introduction

When moisture content exceeds field capacity, soil pores become saturated with excess water at the expense of air deprivation (Najeeb et al., 2015). This condition is termed waterlogging and is associated with intense rainfall or excessive irrigation events (Manik et al., 2019; Tian et al., 2021). With climate change, where precipitation extremes can become more frequent and intense, the risk of waterlogging episodes is rising (Ploschuk et al., 2018). Waterlogging occurrence is generally reinforced by weak soil drainage (Najeeb et al., 2015; Manik et al., 2019). Poor drainage may be caused by a large amount of clay in the soil and the excessive use of agricultural machinery (Najeeb et al., 2015; Ploschuk et al., 2018). Waterlogging is highly relevant in over 12% of arable land around the globe (Tian et al., 2021). Waterlogging adversely affects plant growth and development, and as a result, limits yield (Arguello et al., 2016; Anee et al., 2019). Alleviating the negative effects of waterlogging on yield remains a challenging task, necessitating knowledge of the underlying aspects.

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The negative effect of waterlogging on yield varies depending on the growth stage, at which waterlogging occurs, and the duration of the event (Ren et al., 2016; Anee et al., 2019). To maintain productivity in sites, where waterlogging periodically occurs, the most sensitive growth stages ought to be identified (Ren et al., 2016; Anee et al., 2019). Relevant knowledge is essentially lacking for several horticultural species, including zinnia (*Zinnia elegans* Jacq). The waterlogging-induced yield loss is triggered by a multitude of factors. For instance, it reduces carbon assimilation via a decrease in both leaf area (thus light interception) and chlorophyll content (Ahmed et al., 2002; Pompeiano et al., 2019). Stomatal factors have also been suggested to contribute to the noted decline in carbon exchange rates (Pan et al., 2021). In addition, the equilibrium between the production and detoxification of reactive oxygen species is often disturbed by waterlogging (Ahmed et al., 2002; Anee et al., 2019). Excessive accumulation of reactive oxygen species triggers a range of harmful effects, such as lipid peroxidation (Fanourakis et al., 2022). As carotenoids are strong antioxidant agents, they actively contribute to antioxidant defense (Ahmadi-Majd et al., 2021a, b; Chen et al., 2021). This defense is further supported by specific enzymes. For instance, superoxide is catalyzed into H2O2 via superoxide dismutase, which is then eventually reduced to H2O by peroxidase, catalase, or glutathione reductase (Ahmadi-Majd et al., 2021a, b; Chen et al., 2021). Ornamental plants such as *Cistus* × *hybridus*, *Lavandula angustifolia*, *Salvia officinalis*, and *Stachys byzantina* responded to waterlogging by increasing the production of lateral roots close to the surface, thereby enabling them to acclimate to subsequent anoxia. This response greatly increased their chances of survival (Cameron et al., 2010). Short-term waterlogging in herbaceous peony (*Paeonia lactiflora* Pall.) caused the leaves to turn yellow or red and the roots to turn black. Waterlogging significantly decreased the root-shoot ratio. The activity of antioxidant enzymes and the content of osmotic regulators increased under waterlogging (Liu et al., 2021).

Zinnia (*Zinnia elegans* Jacq.) is of the Asteraceae family with colorful flowers and is native to Mexico (Goma et al., 2019). Zinnia is very popular both as a specialty cut flower, and a landscape plant (Sharif et al., 2019; Kalinowski et al., 2022). It is mostly used as a garden plant in Iran. Transplanting zinnia plants is difficult due to the severe interruption in growth. It is usually recommended to plant the seeds directly in their permanent home. Therefore, there is a knowledge gap in the available literature regarding a quantitative analysis of how growth stage and duration of exposure determine waterlogging-induced injury in zinnia.

Although most cases of research are reportedly focused on drought stress, waterlogging can also occur in conditions of floods, heavy or prolonged rainfall, excessive irrigation, and poor drainage (Najeeb et al., 2015; Manik et al., 2019; Tian et al., 2021). The objectives of this study were, for the first time, to evaluate the dual effects of growth stage, at which waterlogging is encountered, and the duration of the episode on zinnia growth and productivity. Evaluations included important aspects of external quality (i.e. flower size, stem length, stem strength, leaf greenness) (Fanourakis et al., 2016; Mladenović et al., 2020). A more fundamental basis for the noted effects was sought by evaluating photosynthetic efficiency, stomatal anatomical features, lipid peroxidation, and two critical H_2O_2 scavenging enzymes.

Materials and Methods

Plant materials and growth conditions

Zinnia seeds (*Z. elegans*) (cv. 'Dreamland') were obtained from a commercial source (Taki Company, Chiba, Japan). This cultivar was selected based on popularity among growers in Lorestan province, Iran. Seeds were sanitized (15 min) in sodium hypochlorite solution (1%; v/v), and then rinsed (2 min) with distilled water. Four days before planting, the growth medium was disinfected using a systemic fungicide, thiophanate-methyl (Topsin – M; .5 g L^{-1}). Pots (2.5 L; dimensions of 15 × 15 cm) were filled with soil, sand (particle size of 0.9–2 mm), and composted cow manure (1:1:1, w/w). To facilitate drainage, 50 g of pebbles (particle size of 8–20 mm) were placed at the bottom of each pot. To implement sand burial, 50 g of sand was also placed on the center of the medium surface. Seeds were manually sown. The sowing depth was kept constant at 1 cm, and then the seeds were covered with sand immediately. Three seeds were sown at the center of each pot. At the cotyledon stage (i.e. fully open cotyledons and before the appearance of the first leaf), seedlings were thinned by keeping the most vigorous one in each pot. Then, the pots were randomly allocated in a greenhouse compartment. The greenhouse was situated in the central-west part of Iran (Khorramabad, 33° N). Plant density was set at 20 pots m^{-2}. During cultivation, plants were exposed to naturally fluctuating conditions of light intensity, temperature, and relative air humidity. The mean air temperature was 21.2±1.6 °C (range 14.3–

28.1), while the mean relative air humidity was 62±3% (range: 50–74). The average daily light integral was 10.7±0.3 mol m^{-2} day^{-1} (range 7.8–13.6) (LI-250A, LI-COR, Lincoln, NE). Substrate water content was maintained close to retention capacity by regular irrigation.

The experiment consisted of 12 treatments (i.e. 4 waterlogging durations × 3 growth stages) with four replications. The experiment was arranged as factorial based on a completely randomized design. Waterlogging was performed one time in each treatment by placing the pot in a container filled with water for a certain period (0, 6, 12, and 24 h). In the course of waterlogging, the underground parts of the plants (i.e. under the root-to-shoot junction) were submerged. This treatment was applied at different growth stages (four-leaf stage, full vegetative growth, and flowering, corresponding to 38, 48, and 62 days of cultivation).

Measurements were conducted on each plant and leaf. In the case of leaf-level measurements, the sampled leaves were fully expanded. In all cases, the time between sampling and the start of the evaluation did not exceed 15 min. When this was not possible, samples were placed in vials, flash-frozen in liquid nitrogen, and transferred to a freezer (−80°C) for storage. Replicate flowers and leaves were collected from separate plants.

Initiation of flowering and flower bud opening were recorded during cultivation. Plant growth, morphology, and biomass allocation were assessed 73 days after sowing. All the remaining measurements were made 1–3 days earlier. Unless indicated otherwise (i.e. gas exchange features, water status), sampling was conducted at the onset of the light period (07:00–08:00 h; 17–19 °C).

Plant growth, morphology, and biomass allocation

Seventy-three days after sowing, plant growth, morphology, and biomass allocation were evaluated. Determinations included the number of lateral branches, lateral branch length, main stem length (from the root-to-shoot junction to the apical inflorescence end), main stem diameter (assessed midway along its length), flower diameter (the mean of the largest diameter and the one perpendicular to it), the number of leaves, and leaf area. For leaf area assessment, leaves were scanned (HP Scanjet G4010, Irvine, CA, USA) and then evaluated by using the free UTHSCSA IMAGE TOOL program (University of Texas Health Science Centre at San Antonio, TX).

Before the assessment of root traits, each pot was placed in a container filled with water for 1 h. Following the removal of the substrate from the roots by gentle washing, root volume was measured by employing a volume-displacement technique (Javadi Asayesh et al., 2021). Plant roots were suspended in a cylinder filled with water. Root volume was then determined by measuring the volume of water displaced by the plant roots. Root diameter corresponded to the diameter of the largest sphere, which fitted into the root and contained it. Root length was regarded as the length from the shoot-to-root junction to the tip of the primary root.

The main stem, lateral branch, leaf, flower, and root (fresh and dry) masses were also recorded (± 0.001 g; Mettler ME303TE, Giessen, Germany). For measuring dry weight, samples were placed in a forced-air drying oven for 72 h at 80 °C (Javadi Asayesh et al., 2021). By using dry mass, specific leaf area (leaf area/leaf mass), flower mass ratio (flower mass/plant mass), leaf mass ratio (leaf mass/plant mass), and root-to-shoot ratio (root mass/aboveground mass) were calculated. The main stem strength (mass per unit length) and tissue density (mass per unit volume) were also computed, as potential indicators of its sensitivity to bending (Fanourakis et al., 2016). All the measurements were conducted on 4 plants per treatment.

Flower induction and longevity

The treatments affected the initiation of flowering, flower bud opening, and intact flower bud longevity on the plants. The time to flowering (also referred to as the time to visible bud) was recorded as the period from sowing to the appearance of the flower bud (i.e. flower bud length ≈ 0.5 cm). Intact flower bud longevity (on plant) was computed as the period between opening and wilting (i.e. petal turgor loss) of the flower bud. The former was reached when the outer petals were fully expanded and one row of florets had opened (Kalinowski et al., 2022). Four replicates were assessed.

Gas exchange features

Gas exchange features were evaluated in situ on the attached leaves. Measurements were taken by a portable photosynthesis system (CI-340; CID, Inc., Camas, WA, USA). Leaf chamber (6.25 cm^2) conditions were rendered specific: 22°C air temperature, (50%) relative air humidity, and an incoming air CO_2 concentration of 400 µmol mol^{-1}. Light intensity was set at 200 µmol m^{-2} s^{-1}. Evaluations took place 2 h following the onset of the light period to assure a steady state of stomatal conductance (Taheri-Garavand et al., 2021). Four replicates were assessed.

Stomatal and epidermal cell features

Stomatal and epidermal cell features were determined. The sampling area (1 × 1 cm) was located midway between the leaf base and tip, as well as between the midrib and lateral margin (Sørensen et al., 2020; Seif et al., 2021). The abaxial leaf surface was coated by a thin layer of nail polish. After 10 min, a strip of transparent sticky tape was used for taking off the dried polish. Sticky tapes with dried polish were mounted on the microscopic slides, and observations took place by a camera-attached light microscope. Images were taken by the Omax software (ver. 3.2, Omax Corp.). Stomatal dimensions were determined on 10 randomly selected stomata (magnification × 100), while stomatal and epidermal cell densities (i.e. number per unit leaf area) were counted on five non-overlapping interveinal fields of view per leaf (Fanourakis et al., 2015). Stomatal index (i.e. stomatal number per total (stomatal and epidermal cell) number) was computed (Fanourakis et al., 2015). The stomatal size was defined as stomatal length (i.e. longest diameter) multiplied by stomatal width (i.e. shortest diameter) (Fanourakis et al., 2015; Fanourakis et al., 2022). Stomatal area per leaf area was computed (stomatal size × stomatal density). Accordingly, epidermal cell area per leaf area was computed (106 – stomatal area per leaf area). Mean epidermal cell size was then computed by dividing the epidermal cell area per leaf by epidermal cell density. Image processing was performed with the UTHSCSA IMAGE TOOL program (University of Texas Health Science Centre at San Antonio, TX). Four replicates were assessed per treatment.

Chlorophyll and carotenoid content

Samples were processed immediately after collection (Paschalidis et al., 2021). Following fine chopping, portions weighing 0.1 g were homogenized with the addition of 10 mL of (100%) acetone. The extract was then centrifuged (14000 g for 20 min), and the supernatant was collected. Since chlorophyll is light-sensitive, the extraction took place in a dark room (Ahmadi-Majd et al., 2021a, b). The obtained extract was subjected to reading on a spectrophotometer (Mapada UV-1800; Shanghai Mapada Instruments Co., Ltd., Shanghai, China). Total chlorophyll and carotenoid contents were calculated according to Lichtenthaler and Wellburn (1983).

Water status

Leaf water status was assessed by measuring relative water content (RWC, also referred to as relative turgidity). Samples were collected 3 h following the onset of the photoperiod (Seif et al., 2021). Following excision, fresh weight was gravimetrically obtained (±0.0001 g; Mettler AE 200, Giessen, Germany). Immediately then, the samples were floated on distilled water inside a Petri dish, covered with a lid. After 24 h of incubation, the weight was recorded and expressed as turgid (saturated) weight. Then, the dry weight (48 h at 80°C) was determined. RWC was calculated according to a method in the literature (Taheri-Garavand et al., 2021). Four replicates were assessed per treatment.

Lipid peroxidation

Malondialdehyde (MDA) content, taken as an indication of lipid peroxidation level, was evaluated by employing the thiobarbituric acid reactive substance assay (Ahmadi-Majd et al., 2021a, b). Freshly cut leaf discs (0.1 g) were homogenized, and then added to 5 mL of (20%) (w/v) trichloroacetic acid and (0.5%) (w/v) thiobarbituric acid. The suspension was centrifuged (6000g for 15min) and then heated (100°C for 25min). After equilibration at 25°C, the suspension was centrifuged (6000g for 5 min). The amount of MDA was calculated from the value of absorbance at 532 nm after subtracting the non-specific absorption at 450 and 600 nm (Mapada UV-1800; Shanghai Mapada Instruments Co., Ltd., Shanghai, China). The extinction coefficient of 156 mmol MDA L^{-1} cm^{-1} was used (Ahmadi-Majd et al., 2021a, b). Four discs were assessed per replicate sample. Four replicates were assessed per treatment.

Enzymatic activity

Ascorbate peroxidase activity was assessed using a method described by (Ahmadi-Majd et al., 2021). Fresh frozen leaf segments (0.1g) were ground in liquid nitrogen, homogenized with 1 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and (1%) polyvinylpyrrolidone (PVP), and centrifuged (14000g) for 20 min at 4 °C. Ascorbate peroxidase activity in the supernatant was assessed following a decrease in absorbance at 290 nm for 2 min (10 s intervals) in a reaction mixture containing sodium phosphate buffer, ascorbic acid, and H_2O_2. An extinction coefficient of 2.8 mM^{-1} cm^{-1} was used. Ascorbate peroxidase activity was expressed as μmol of ascorbate oxidized min^{-1} g^{-1} tissue. Peroxidase activity was assayed as described in the literature (Ahmadi-Majd et al., 2021). Fresh frozen leaf segments (0.3 g) were ground in liquid nitrogen, homogenized with 1.5

mL of 50 mM potassium phosphate buffer (pH 7.0), and centrifuged (14000g) and 4 °C for 20 min. Peroxidase activity in the supernatant was assessed following a decrease in absorbance at 470 nm for 2 min (10 s intervals) in a reaction mixture containing potassium phosphate buffer, guaiacol, and H_2O_2. An extinction coefficient of 26.6 mM^{-1} cm^{-1} was used. Peroxidase activity was expressed as μmol of reduced H2O2 min^{-1} g^{-1} tissue.

Statistical analysis

Data were subjected to an analysis of variance using SPSS 23 (SPSS Inc., Chicago, IL, USA). A factorial experiment was based on a completely randomized design and consisted of 12 treatments (4 waterlogging durations × 3 growth stages) with four replications. Subsequently, estimated values of least significant differences (LSD) among the treatment groups were determined ($P \leq 0.05$).

For the twelve experimental units, eigenvalues were extracted and the most contributing variables for each dimension were computed and identified. The first two eigenvalues contributed to more than 66% of the total variance and were retained to produce the principal components. A principal component analysis (PCA) was produced to depict correlations between the developmental stages – at which waterlogging was encountered – and waterlogging durations as principal components. Individuals were grouped (by discrete colour) and variables by their contribution to the principal components (gradient colours). A correlation plot was also computed to depict positive and negative associations across the variables inquired. The "corrplot", "FactoMineR", "factoextra" and "readxl" libraries were used under the R-studio integrated development environment (RStudio suite V 1.2.5033).

Results

Plant growth, morphology, and biomass allocation

As the waterlogging duration increased, the values of all traits decreased. These included traits such as lateral branches (count, length), main stem (length, diameter), leaves (number, area), flower (diameter) and root (length, diameter, volume). This effect was more pronounced at early growth stages (Table 1).

At the four-leaf stage and full growth stage, 24 h of waterlogging tended to decrease stem strength (mass per unit length) (Table 1). In stem tissue density (mass per unit volume), this effect was evident at the full growth stage (Table 1). As the waterlogging duration increased, plant dry weight decreased (Table 2). This effect was similar to the case of plants at the four-leaf stage and full growth stage, whereas a less prominent effect was noted at the start of the flowering stage (Table 2). The individual weights of different organs (i.e. main stem, lateral branches, leaf, flower, and root) were also recorded (Table 2). Waterlogging decreased the dry weight of different organs. The negative effect of waterlogging on dry weight was generally more prominent as waterlogging duration increased and at early growth stages. Plants that were waterlogged at the four-leaf stage for 24 h showed the most negative effects and their plant dry weight was reduced to 75% of that in the control (Table 2).

Across the 12 treatments, larger plants exhibited larger leaf areas and increased weights of individual organs (i.e. main stem, lateral branches, leaf, flower, and root) (Figs. 1,4A-4F). Higher flower mass was not translated to wider flower diameter (Figs. 1, 5). The root traits (i.e. length, diameter, and volume) effectively reflected changes in the root dry weight (Figs. 1, 6A-6C). As the waterlogging duration increased, leaf thickness tended to increase (i.e. a decrease in specific leaf area) (Table 4). At full growth and the start of the flowering stage, 24 h waterlogging tended to increase the root-to-shoot ratio (Table 1). At the four-leaf stage, waterlogging tended to decrease biomass allocation to leaves, and instead caused biomass allocation to the flower (Table 1). Across the 12 treatments, larger plants tended to have thinner leaves (i.e. a higher specific leaf area), with increased biomass allocation (Figs. 1; 7B, 7C). The relation between root-to-shoot ratio and plant weight was inconsistent (figs. 1, 7A). Mass allocation to flowers was clearly not associated with plant weight (Figs. 1,7D).

Flower induction and intact flower bud longevity

As the waterlogging duration increased, there was a decrease in the time to flowering, time to flower opening, time to flower wilting, and intact flower bud longevity at the four-leaf stage (Table 3). Among these traits, the time to flower opening was the least affected (Table 3). For instance, as the waterlogging duration increased from 0 to 24 h, the time to flowering, time to flower opening, time to flower wilting, and intact flower bud longevity decreased by 5, 1, 4, and 2 d, respectively. In the remaining two stages (full growth, start of flowering), waterlogging duration had no significant effect on these three traits (Table 3).

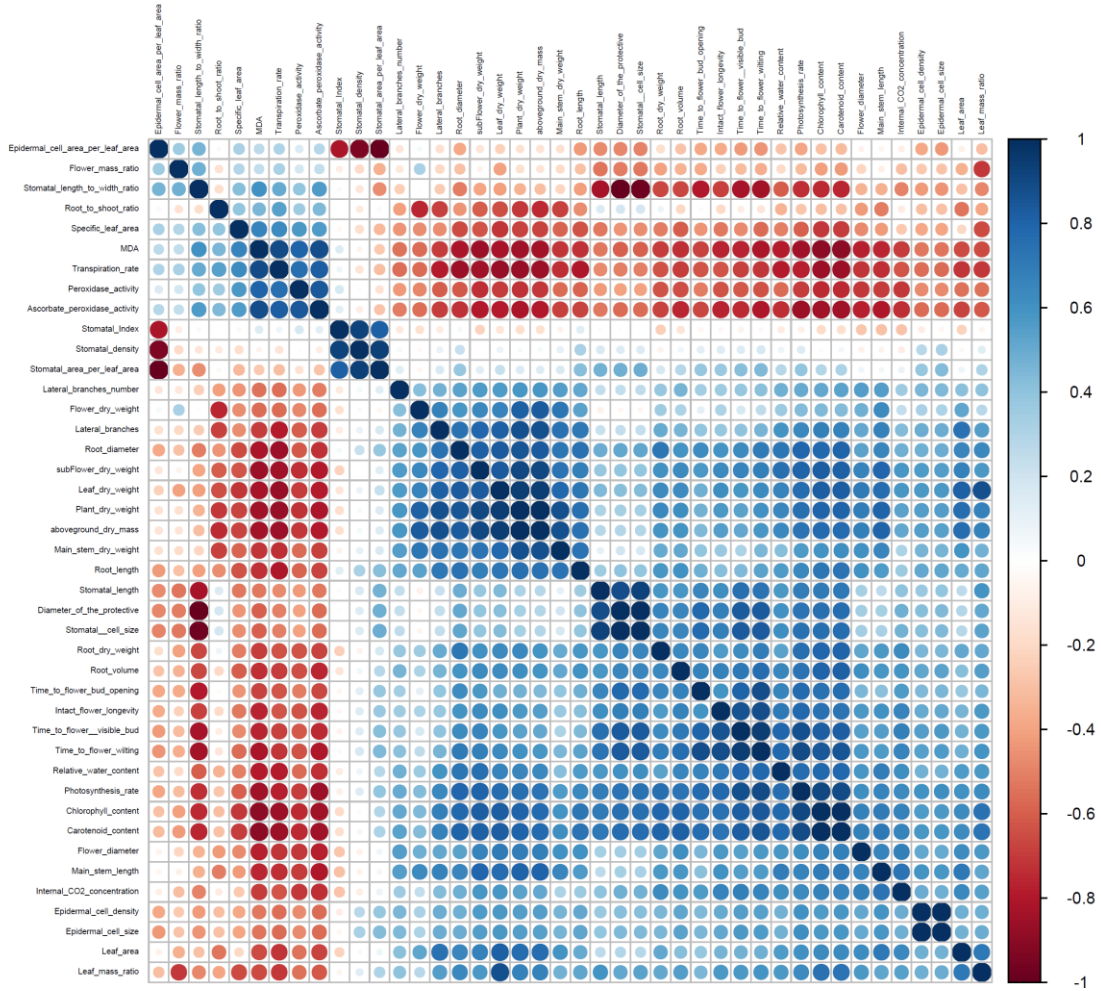

Fig. 1. Positive, neutral, and negative affinities across morpho-physiological traits in *Zinnia elegans* cv. 'Dreamland'. Plants were cultivated under different waterlogging durations (0, 6, 12, and 24 h). The treatment was applied once (a single time) at three developmental stages (i.e. four-leaf stage, full growth, and flowering initiation). Positive correlations are portrayed by blue circles, while negative associations are indicated by red circles. The intensity of color corresponds to the correlation coefficient (r) ranging from –1 to 1 (scale). The larger size of circles indicates statistically significant values (non-significant, $p = 0.05$, $p = 0.01$, $p = 0.001$ respectively).

Table 1. Effect of waterlogging duration applied once (a single time) at three developmental stages on several morphological features of Zinnia elegans cv. 'Dreamland'

Stage	Waterlogging duration (h)	Lateral branches		Main stem				Internode	Leaf		Flower	Root		
		number	length (cm)	length (cm)	diameter (mm)	strength (g cm^{-1})	tissue density (g cm^{-3})	length (cm)	number	area (cm^2)	diameter (mm)	length (cm)	diameter (cm)	volume (cm^3)
Four-leaf stage	0	4.25a	35.9a	24.8a	4.10ab	0.011ab	0.083de	4.94a	30.0a	215.0a	71.0a	25.1a	3.65ab	2.90a
	6	3.43abc	30.7b	23.9cd	3.91bc	0.011ab	0.091bcd	4.78cd	28.3bcd	188.4abc	69.0bc	23.8abcd	3.35bcd	2.75ab
	12	3.25bc	24.7d	23.2ef	3.62d	0.011bc	0.103ab	4.64fg	26.5e	161.7cde	67.9cde	22.6cdef	3.05de	2.50b
	24	3.00c	22.5d	22.9f	3.50d	0.010c	0.105a	4.59g	26.5e	154.0de	66.5e	20.9f	2.70f	2.12c
Full growth	0	4.00ab	35.5a	24.7a	3.91bc	0.011a	0.095abc	4.97a	29.5ab	205.3ab	69.9ab	24.7ab	3.67a	2.95a
	6	3.62abc	29.5bc	24.2bc	3.91bc	0.010bc	0.087cde	4.83bc	28.8abc	196.6ab	69.6b	23.1bcde	3.28cde	2.85a
	12	3.25bc	26.0cd	23.8cd	3.86c	0.010c	0.085cde	4.76cde	28.5bc	180.4bcde	68.6bcd	22.1def	3.05de	2.75ab
	24	3.00c	23.0d	23.0f	3.83c	0.009d	0.077e	4.60g	27.0de	157.7e	67.3de	21.3ef	2.98ef	2.65ab
Start flowering	0	4.25a	36.0a	24.6ab	4.17a	0.012a	0.085cde	4.91ab	29.5ab	190.5abc	69.6b	25.3a	3.70a	2.90a
	6	4.00ab	32.9ab	23.8cd	4.10ab	0.012a	0.088cde	4.77cde	28.0cd	189.0abc	69.1bc	24.9ab	3.60ab	2.86a
	12	3.81abc	31.3b	23.6de	4.02abc	0.011ab	0.087cde	4.72def	27.5cde	185.7bcd	68.8bc	24.8ab	3.56abc	2.79ab
	24	3.81abc	31.2b	23.5de	3.96bc	0.011ab	0.089cd	4.69ef	27.5cde	181.1bcde	68.6bcd	24.5abc	3.52abc	2.77ab

Means followed by different letters indicate significant differences ($P \leq 0.05$). Data are mean values of four replications.

Table 2. Effect of waterlogging duration applied once (a single time) at three developmental stages on plant growth, biomass allocation, and morphology of *Zinnia elegans* (cv. 'Dreamland')

Stage	Waterlogging duration (h)	dry weight (g)						Root-to-shoot ratio (g g^{-1})	Specific leaf area (cm^2 g^{-1})	Leaf mass ratio (g g^{-1})	Flower mass ratio (g g^{-1})
		Main stem	Lateral branches	Leaf	Flower	Root	Plant				
Four-leaf stage	0	0.27ab	0.18 ab	0.58a	0.47 a	0.20 a	2.08a	0.11cd	359bc	0.29a	0.41cd
	6	0.26abc	0.17 ab	0.50c	0.46 a	0.19 ab	1.94bc	0.11cd	343de	0.26bc	0.42abc
	12	0.24cd	0.15 cd	0.42de	0.45 ab	0.18 bc	1.75de	0.11cd	316f	0.25c	0.43ab
	24	0.23d	0.14 d	0.33f	0.41 bc	0.16 c	1.55f	0.11bc	302g	0.22d	0.44a
Full growth	0	0.28a	0.18a	0.54abc	0.49 a	0.21 a	2.12a	0.11cd	367b	0.26bc	0.42abc
	6	0.25bcd	0.17ab	0.49c	0.47 a	0.20 ab	1.93c	0.11cd	348cd	0.26bc	0.42abc
	12	0.24cd	0.14d	0.42de	0.39 cd	0.19 ab	1.7e	0.13ab	337de	0.25bc	0.42bcd
	24	0.20e	0.13d	0.38ef	0.34 d	0.19 ab	1.57f	0.14a	331e	0.25bc	0.41bcd
Start flowering	0	0.28a	0.18 ab	0.57ab	0.50 a	0.20 a	2.14a	0.10d	394a	0.27ab	0.43abc
	6	0.27a	0.18 ab	0.54abc	0.49 a	0.20 ab	2.05ab	0.11cd	383a	0.27ab	0.42bcd
	12	0.26abc	0.17 ab	0.50bc	0.46 a	0.19 ab	1.94bc	0.11cd	367b	0.27bc	0.42bcd
	24	0.25abc	0.16 bc	0.48cd	0.40 c	0.19 ab	1.83cd	0.12bc	336de	0.27ab	0.4d

Means followed by different letters indicate significant differences ($P \leq 0.05$). Data are mean values of four replications.

Table 3. Effect of waterlogging duration applied once (a single time) at three developmental stages on time to flowering (also referred to as the time to visible bud), time to flower opening (i.e. when the outer petals were reflexed and one row of florets had opened), time to wilting (i.e. petal turgor loss), and intact flower bud longevity (on plants) [(time to wilting) – (time to open flower)] of *Zinnia elegans* cv. 'Dreamland'

Stage	Waterlogging duration (h)	Time to			Intact flower bud longevity (d)
		flowering (d)	open flower (d)	flower wilting (d)	
Four-leaf stage	0	42.2a	51.8a	72.1ab	20.3a
	6	40.1b	51.1bc	70.4d	19.3cd
	12	38.1c	50.7c	69.5e	18.8d
	24	37.1d	49.8d	67.8f	18.0e
Full growth	0	42.1a	52.0a	72.3a	20.3a
	6	41.7a	51.9a	71.8abc	19.9abc
	12	41.7a	51.7ab	71.5bc	19.9abc
	24	41.6a	51.5ab	71.3c	19.7abc
Start flowering	0	42.1a	51.9a	72.0ab	20.1ab
	6	41.7a	51.5ab	72.0ab	19.9abc
	12	41.6a	52.0a	71.8abc	19.8abc
	24	41.5a	52.1a	71.7abc	19.6bc

Means followed by different letters indicate significant differences (P = 0.05; comparison in columns). Values are the mean of four replications.

Gas Exchange Features

As waterlogging duration increased, both transpiration and stomatal conductance increased (Table 4). This increase was more prominent, as the growth stage was less advanced (Table 4). For instance, as waterlogging duration increased from 0 to 24h, transpiration rate increased by 83 and 12% for four-leaf stage and start flowering stages, respectively.

By contrast, as the waterlogging duration increased, both internal CO_2 concentration and photosynthesis decreased (Table 4). This decrease was stronger at earlier growth stages (Table 4). For instance, as the waterlogging duration increased from 0 to 24 h, the photosynthesis rate decreased by 52 and 18% at the four-leaf stage and at the start of the flowering stage, respectively.

A decrease in photosynthesis was associated with a higher stomatal conductance and a lower internal CO_2 concentration (Table 4, Fig. S1).

Table 4. Effect of waterlogging duration applied once (a single time) at three developmental stages on gas exchange features of Zinnia elegans cv. 'Dreamland'

Stage	Waterlogging duration (h)	Transpiration rate (mmol m^{-2} s^{-1})	Stomatal conductance (mol m^{-2} s^{-1})	Internal CO_2 concentration (ppm)	Photosynthesis rate (µmol m^{-2} s^{-1})
Four-leaf stage	0	1.64 g	0.39 h	391a	19.33bc
	6	1.87 ef	0.41 g	320f	14.10g
	12	2.44 b	0.51 b	298h	12.63g
	24	3.00 a	0.54 a	276i	9.20h
Full growth	0	1.59 g	0.42 fg	365b	20.43ab
	6	1.72 fg	0.46 d	346c	17.40de
	12	1.85 f	0.49 c	333d	17.31de
	24	2.1 cd	0.51 b	328de	15.69f
Start flowering	0	2.05 de	0.42 g	321ef	20.93a
	6	2.12 cd	0.43 efg	316fg	20.00abc
	12	2.22 cd	0.43 ef	312g	18.72cd
	24	2.3 bc	0.44 e	311g	17.10ef

Means followed by different letters indicate significant differences ($P \leq 0.05$). Data are mean values of four replications.

Stomatal and epidermal cell anatomical features

Waterlogging was associated with minor effects on stomatal and epidermal cell densities, and thus on the stomatal index (Table 5). Waterlogging decreased stomatal size when applied at the four-leaf stage and increased it when applied in the remaining two stages (full growth and flowering initiation) (Table 5). This effect was mostly mediated by changes in stomatal width and, secondarily, by alterations in stomatal length (Table 5). When waterlogging was applied at the four-leaf stage, minor effects were noted in stomatal shape (length-to-width ratio) and epidermal cell size (Table 5).

Water status

At the four-leaf stage and full growth stage, waterlogging for 12 and 24 h slightly impaired leaf hydration status (RWC) (Table 6).

Chlorophyll and carotenoid content

As the waterlogging duration increased, chlorophyll and carotenoid contents decreased (Table 6). This decrease was more prominent at earlier growth stages (Table 6). A decrease in photosynthesis was associated with lower chlorophyll content (Figs. 1, 3A).

Lipid peroxidation

As the waterlogging duration increased, the MDA content as an index of lipid peroxidation increased (Table 6). This increase was more prominent at earlier growth stages (Table 6).

Table 5. Effect of waterlogging duration applied once (a single time) at three developmental stages on epidermal cell and stomatal anatomical features of *Zinnia elegans* cv. 'Dreamland'

Stage	Waterlogging duration (h)	Epidermal cell			Stomatal						
		density (mm^{-2})	size (μm^2)	area per leaf area (μm^2 mm^{-2})	density (mm^{-2})	index (%)	length (μm)	width (μm)	size (μm^2)	length-to-width ratio	area per leaf area (μm^2 mm^{-2})
Four-leaf stage	0	25.1a	25.6a	978819bcde	7.90abc	24.0bc	71.3bc	18.80c	2681b	3.80d	21181abcd
	6	24.4ab	24.8ab	981016ab	7.26cd	23.0cd	71.0cd	18.39d	2613c	3.87c	18984de
	12	23.9bc	24.4bc	980703ab	7.55bcd	24.0bc	70.8d	18.03e	2553d	3.93b	19297de
	24	23.3c	23.8c	981335a	7.68abcd	24.7abc	70.0e	17.35f	2430e	4.04a	18665e
Full growth	0	25.1a	25.7a	979103abcd	7.77abc	23.6bc	71.2c	18.87bc	2687b	3.78de	20897bcde
	6	24.8ab	25.3ab	981348a	6.87d	21.7d	71.3bc	19.02abc	2712ab	3.75de	18652e
	12	24.5ab	25.0ab	979934abc	7.33cd	23.0cd	71.5ab	19.12ab	2735a	3.74de	20066cde
	24	24.3abc	24.8abc	978895bcde	7.69abcd	24.1abc	71.6a	19.15a	2744a	3.75de	21105abcd
Start flowering	0	25.2a	25.7a	979391abcd	7.68abcd	23.4bcd	71.3bc	18.80c	2682b	3.8d	20609bcde
	6	24.8ab	25.4a	978146cde	8.01abc	24.4abc	71.5ab	19.07ab	2729a	3.76de	21854abc
	12	24.6ab	25.1ab	977410de	8.25ab	25.1ab	71.6ab	19.13a	2738a	3.75de	22590ab
	24	24.3abc	24.9ab	976635e	8.50a	25.9a	71.6a	19.19a	2749a	3.74e	23364.6a

Means followed by different letters indicate significant differences ($P \leq 0.05$). Data are mean values of four replications.

Table 6. Effect of waterlogging duration applied once (a single time) at three developmental stages on the relative water content, chlorophyll, carotenoids, and malondialdehyde (MDA) contents, as well as the peroxidase and ascorbate peroxidase activity of *Zinnia elegans* cv. 'Dreamland'

Stage	Waterlogging duration (h)	Relative water content (%)	Chlorophyll content (mg g^{-1} FW)	Carotenoid content (mg g^{-1} FW)	MDA content (µmol g^{-1} FW)	Peroxidase activity (µmol min^{-1} g^{-1} FW)	Ascorbate peroxidase activity (µmol min^{-1} g^{-1} FW)
Four-leaf stage	0	75.8a	11.30ab	2.07a	1.01e	0.019f	0.058gh
	6	75.2abc	10.75f	1.98c	1.09cd	0.019def	0.069c
	12	73.8d	10.14h	1.87e	1.23b	0.022b	0.074b
	24	73.3d	9.20i	1.70f	1.36a	0.024a	0.083a
Full growth	0	75.7a	11.28ab	2.07a	1.01e	0.019ef	0.056h
	6	75.3abc	11.03cd	2.03b	1.08cd	0.020cdef	0.062ef
	12	74.9bc	10.72f	1.97cd	1.14c	0.021bcd	0.066cde
	24	74.7c	10.55g	1.93d	1.22b	0.022b	0.076b
Start flowering	0	75.7a	11.40a	2.09a	1.01e	0.019def	0.061fg
	6	75.5ab	11.19bc	2.05ab	1.06de	0.020cde	0.064def
	12	75.3abc	11.01de	2.02b	1.09cd	0.021bcd	0.065def
	24	75.2abc	10.86ef	2.02b	1.13cd	0.021bc	0.067cd

Means followed by different letters indicate significant differences ($P \leq 0.05$). Data are mean values of four replications. FW, fresh weight.

Enzymatic activity

As the waterlogging duration increased, the activity of antioxidant enzymes increased (ascorbate peroxidase, and peroxidase) (Table 6). This increase was more prominent at earlier growth stages (Table 6).

Principal component analysis

PCA was conducted to identify and quantify the components that regulate the connections between the developmental stages and waterlogging episode durations (Figs. 2A-2B). Eigenvalues were examined to determine the number of principal components. The first two dimensions explained more than 66% of the total variance (Fig. 8). The level of a significant contribution of morphological traits to the PCA was estimated by the cos_2 index (Fig. 9). Among these descriptors, chlorophyll and carotenoid pigments, as well as plant weight and leaf dry weight had profound imprints in the categorization of the experimental units. PCA was based on the first two components and revealed the complex relationships among the treatments (Fig. 2A). The first axis revealed that the most significant discrimination was based on waterlogging, especially at the four-leaf stage. It appears that as waterlogging was prolonged (from 6 to 24 h), the morpho-physiological responses became more intense. This phenomenon is further augmented at the four-leaf stage, where plants responded less adequately to stress. The second axis displays the discriminating effects imposed by waterlogging applied at the full-grown stage and durations of 12 and 24 h. Interestingly, plants at the flowering stage seemed to be less affected in terms of most morpho-physiological attributes. Furthermore, positive and negative correlations across morphological and physiological traits were evident (Fig. 2B). Several indices were highly homogenous (indicating co-regulation) as were the stomatal size and pigment content (carotenoid and chlorophyll), suggesting that larger stomata can better ameliorate the adverse effects of waterlogging.

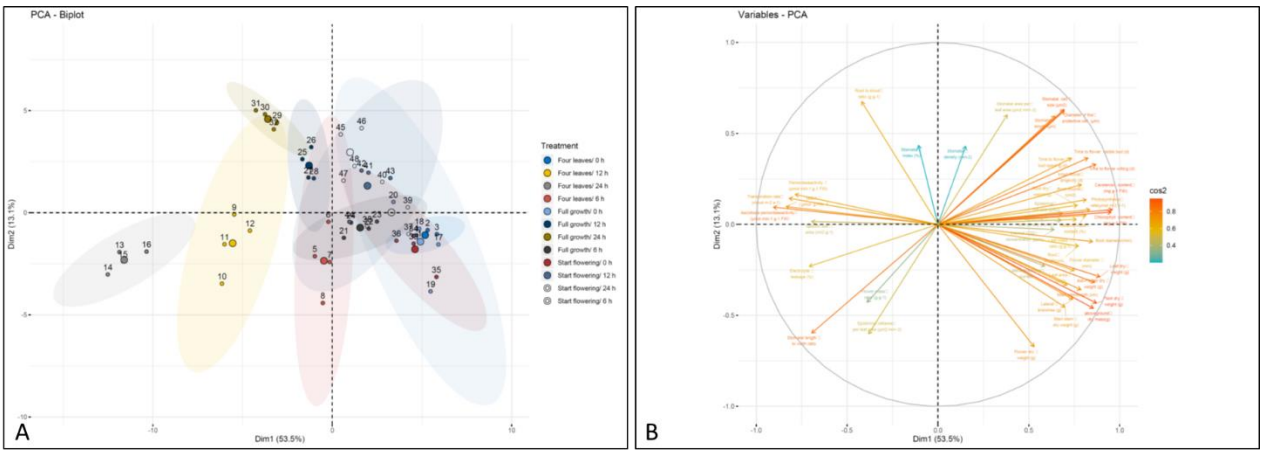

Fig. 2. A. Principal coordinate analysis between developmental stages (four-leaf stage, full-growth, flowering initiation) and waterlogging episode durations (0, 6, 12, and 24 h) in *Zinnia elegans* (Fig. 2A). Larger dots indicated mean values calculated from four discrete biological replications. B. The contribution of each trait in the two dimensions is indicated by a gradient scale and color intensity (scale). Vectors near the plot center have lower cos2 values. Narrow angles among the variables indicated affinity, whereas wide angles indicated a negative correlation.

Discussion

In this study, for the first time, the combined effects of growth stage and duration of exposure to waterlogging were evaluated on zinnia growth and productivity. The importance of this plant is traced to its use as a specialty cut flower and a landscape plant (Sharif et al., 2019; Kalinowski et al., 2022). Waterlogging decreased the main stem length and flower size (Table 1) which are key external quality aspects determining cut flower quality and price (Fanourakis et al., 2016; Mladenović et al., 2020). This negative effect was more pronounced when waterlogging duration increased, especially at earlier stages of growth (Table 1) (Fig. 2A). Additionally, the intact flower longevity was shorter when waterlogging was imposed at the four-leaf stage (Table 3). When the flowering stem remained attached to the plant, the water uptake was optimal and flower bud longevity was considerably longer than in cut flowers (Fanourakis et al., 2012). On this basis, it may be expected that the effect of waterlogging on

cut flower longevity will be more pronounced than the one recorded here. At the four-leaf stage and full growth stage, notably, waterlogging applied for 24 h was associated with lower stem strength (mass per unit length), which corresponded with a higher risk of buckling (Fanourakis et al., 2016). Taken together, these results implicate that waterlogging may adversely affect visually-perceived quality, stem bending incidence, and flower bud longevity, especially when it is experienced at an early developmental stage. Although this deterioration in inner quality (flower bud longevity) appears rather mild when zinnia is grown as a pot or landscape plant, this effect will be most likely aggravated when cultivated as a cut flower.

Leaf coloration is conventionally used as a criterion of pot and landscape plant vigor, as well as health status, across the production–distribution chain which includes nurseries, traders, landscapers, and consumers (Javadi Asayesh et al., 2021; Moosavi-Nezhad et al., 2021; Paschalidis et al., 2021). As the waterlogging duration increased, leaf chlorophyll content decreased, especially when waterlogging occurred at a less advanced growth stage (Table 6) (Fig. 2A). This decrease in chlorophyll content, as a result of waterlogging stress, was noted in the case of other species as well (Anee et al., 2019). Therefore, waterlogging deteriorates pot and landscape plant ornamental value by attenuating leaf coloration.

Biomass accumulation correlated negatively with the duration of the waterlogging episode (Table 2, Fig. 2A). A milder effect of waterlogging was documented when applied at the most advanced stage (flowering initiation) (Table 2, Fig. 2A). As waterlogging induced a decrease in leaf area (i.e. less light capture) (Table 1), the lowered chlorophyll content (Table 6) and internal CO2 concentration (Table 4) contributed to a notable decrease in plant growth (Fig. 2B). This adverse effect was generally evident at the whole-plant and individual organ levels (i.e. main stem, lateral branches, leaf, flower, and root) (Table 2, Figs. 4A-4F).

A decrease in photosynthesis was associated with a lower chlorophyll content and a lower internal CO2 concentration (Figs. 1, 3A, 3C). The lower internal CO2 concentration was not mediated by a decrease in stomatal conductance, since the latter rather increased in response to waterlogging (Table 4). As the waterlogging induced an increase in stomatal conductance (Table 4), the largely unaffected stomatal anatomical traits (Table 5) indicated that the decrease in internal CO2 concentration and the decline in photosynthesis were not related to stomatal features.

Waterlogging increased the stomatal conductance and transpiration rate (Table 4). Earlier works indicated that this effect was related to increased ethylene biosynthesis (Xiao et al., 2020). Notably, plants that underwent waterlogging generally exhibited impaired hydration status (Table 6). A decrease in leaf hydration was documented earlier in the case of plants belonging to other taxa, regarding their response to waterlogging (Anee et al., 2019). Therefore, despite ample soil water availability, the water uptake of plants by waterlogging was not sufficient to compensate for the increased transpiration rate. Besides, as a matter of disturbed water relations, waterlogged plants underwent oxidative damage as indicated by a decrease in chlorophyll content and an increase in MDA content (Table 6).

Waterlogged plants showed more enzymatic activity (i.e. ascorbate peroxidase, peroxidase) (Table 6). These antioxidant enzymes are activated to scavenge and detoxify H2O2 (Ahmadi-Majd et al., 2021a, b; Chen et al., 2021). However, a higher induction of these two enzymes was not adequate to relieve the waterlogging-induced oxidative damage, as observed from the chlorophyll and MDA contents (Table 6). Nonetheless, a broader analysis of the oxidative protection networks, including a wider range of enzymes and metabolites, is essential for a deeper understanding of the underlying processes and the identification of critical antioxidant elements in response to waterlogging.

Conclusion

Waterlogging generally impaired visually-perceived quality. It amplified the risk of stem bending (as a matter of lower stem strength) and suppressed flower bud longevity. Waterlogging decreased plant growth by reducing the amount of light capture (leaf area) and photosynthesis. Waterlogging generally exerted a limited influence on anatomical features of the stomata (i.e. their density, size, and shape). Regardless of the impaired hydration status, waterlogging further induced oxidative stress as indicated by the chlorophyll content and lipid peroxidation level. Waterlogging enhanced the activity of antioxidant enzymes (i.e. ascorbate peroxidase and peroxidase). The severity of the waterlogging-induced effect and the different growth stages made variations in the measured traits. This effect was generally stronger as the duration of waterlogging increased, especially at the earlier stages of plant growth. In conclusion, the growth stage and the duration of exposure determined the waterlogging-induced negative

effects on zinnia. In Iran, the northern parts of the country have high precipitation, whereas in the rest of the country waterlogging may occur due to over-irrigation or inadequate drainage. In clay soils, adding soil amendments such as sand can alternatively solve the problem. Further studies could be directed at plant responses at different levels, regarding their defense strategies against waterlogging and the practical methods of managing the problem in landscape areas, especially at the early stages of plant growth.

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Conflicts of Interest
All authors declare no conflict of interest.

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1 Supplementary Figures

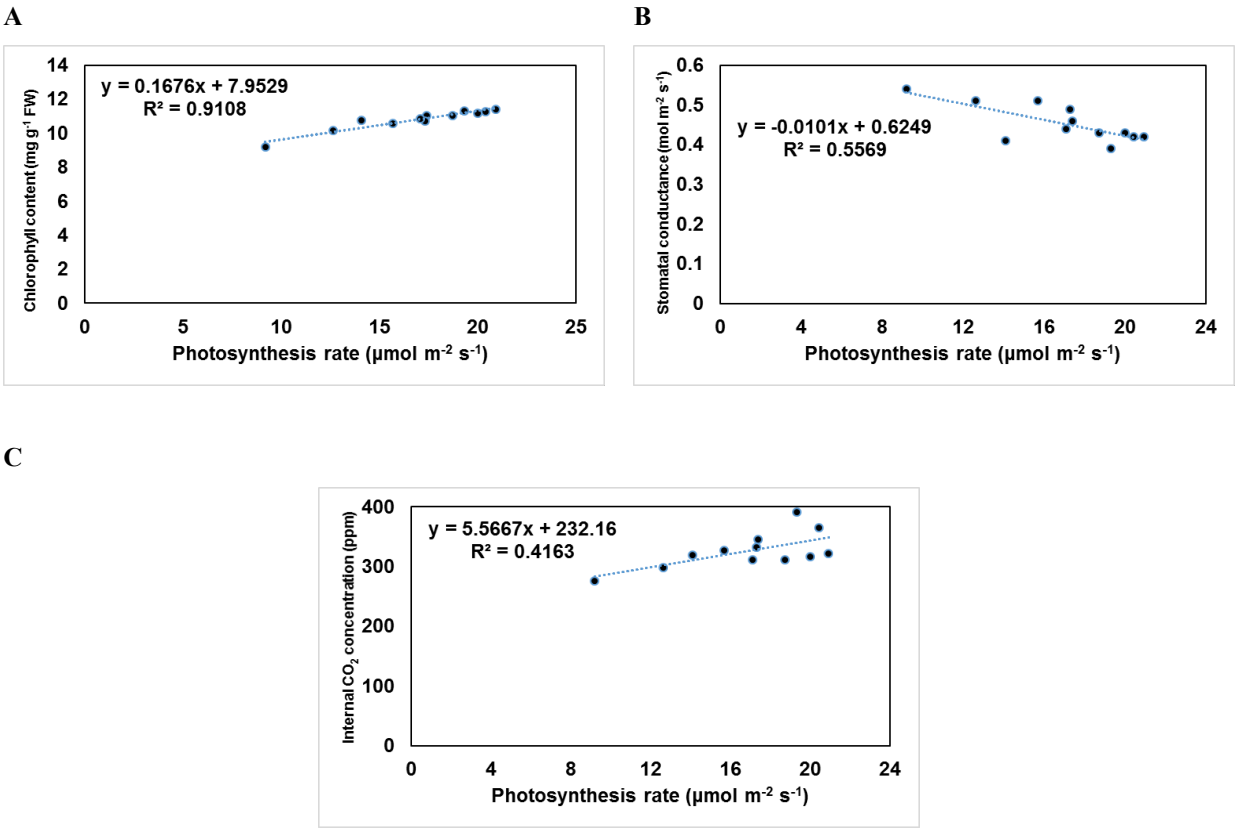

Figure S1. Chlorophyll content, stomatal conductance, and internal CO_2 concentration as a function of photosynthesis rate in *Zinnia elegans* cv. 'Dreamland'. Plants were cultivated under different waterlogging durations (0, 6, 12, and 24 h), applied once (a single time) at three developmental stages (full growth and start flowering). Individual treatment data are provided in Tables 1 and 5. Values are the mean of four replications. FW, fresh weight at the four-leaf stage.

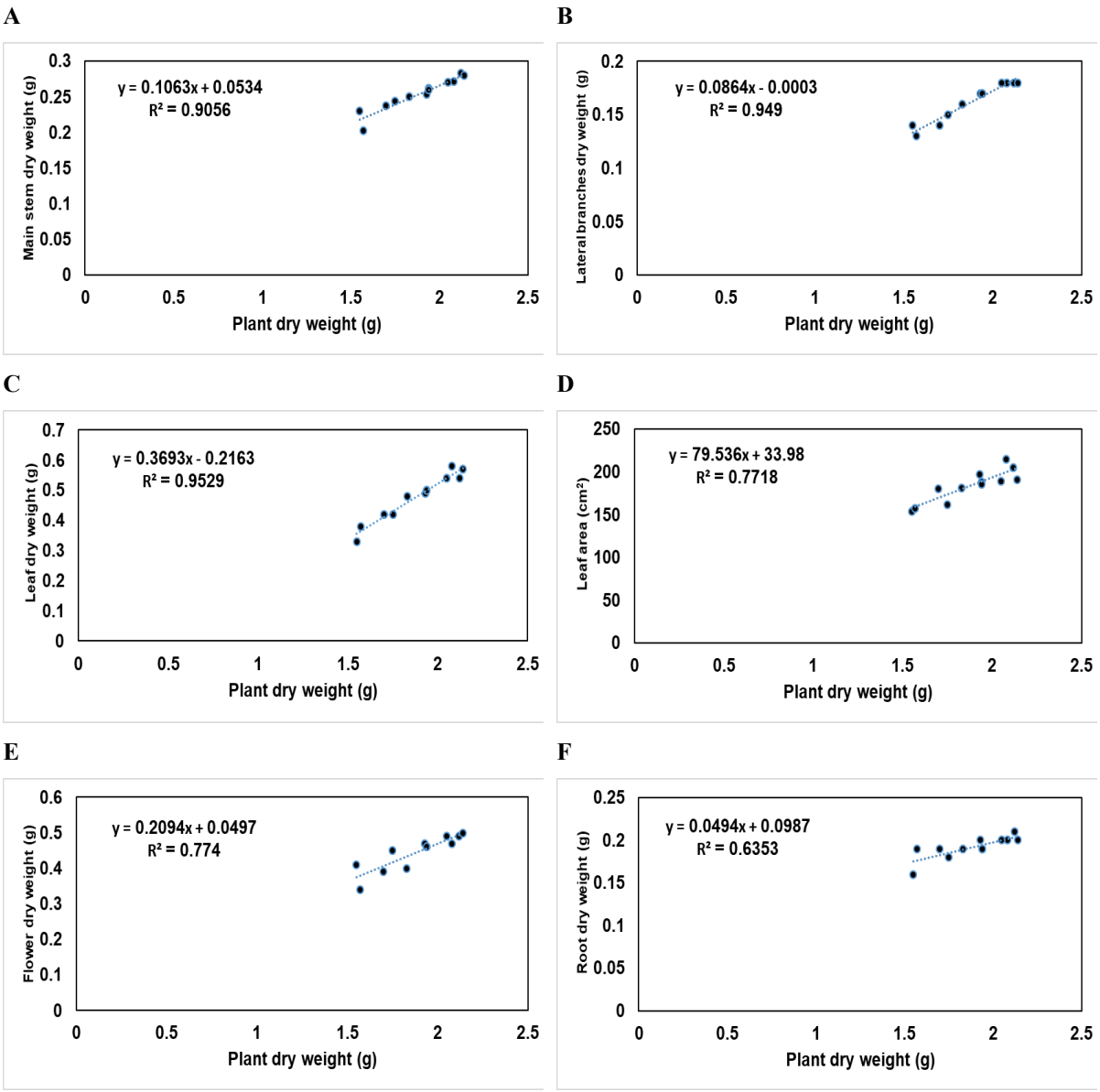

Figure S2. Individual organs (i.e. main stem, lateral branches, leaves, flowers, and root) dry weight, and leaf area as a function of plant dry weight in *Zinnia elegans* cv. 'Dreamland'. Plants were cultivated under different waterlogging durations (0, 6, 12, and 24 h), applied once (a single time) at three developmental stages (four-leaf stage, full growth, and flowering initiation). The differences in the y-axis scale are noted. Individual treatment data are provided in Tables 3 and 4. Values are the mean of four replications.

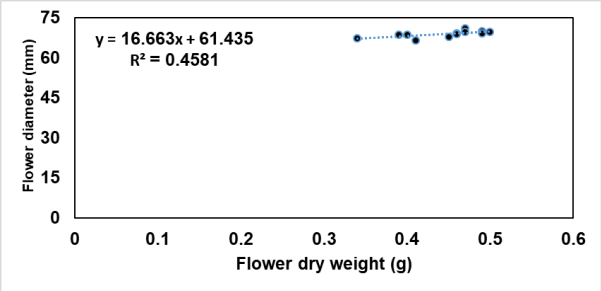

Figure S3. Flower diameter as a function of flower dry weight in *Zinnia elegans* cv. 'Dreamland'. Plants were cultivated under different waterlogging durations (0, 6, 12, and 24 h), applied once (a single time) at three developmental stages (four-leaf stage, full growth, and flowering initiation). Individual treatment data are provided in Tables 3 and 4. Values are the mean of four replications.

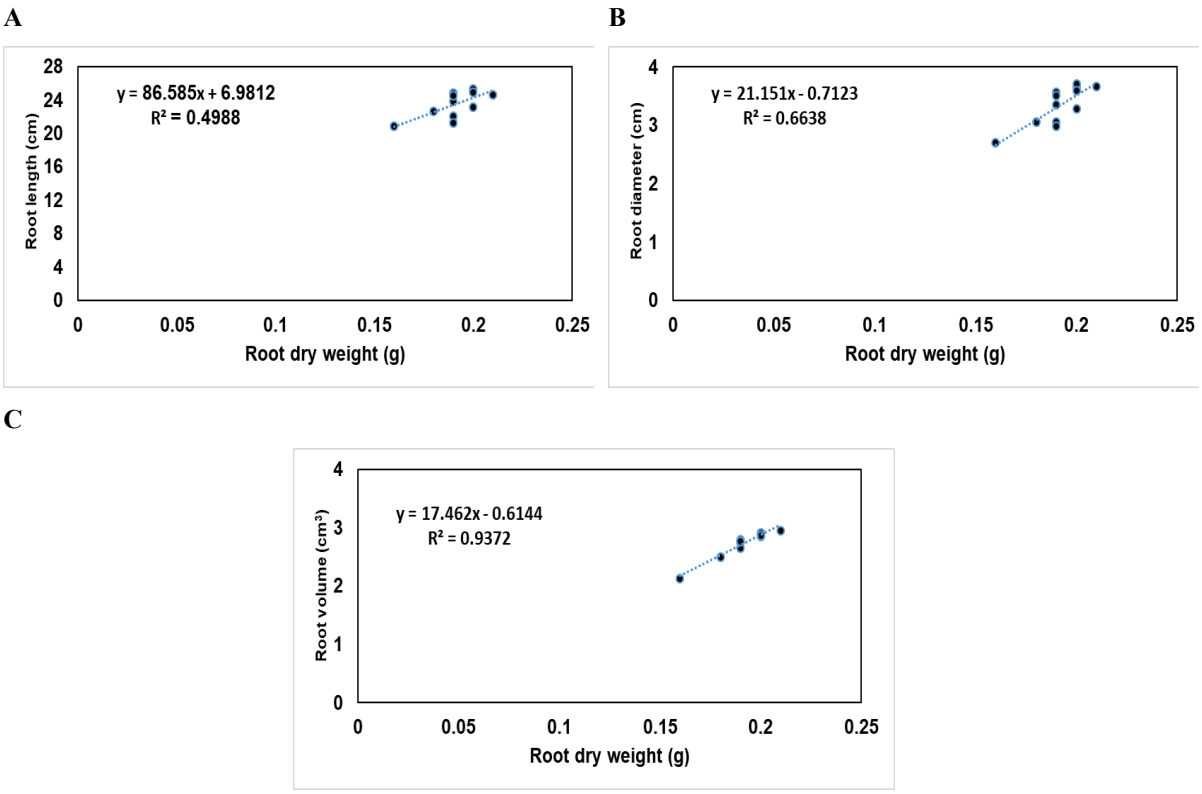

Figure S4. Root traits (length, diameter, volume) as a function of root dry weight in *Zinnia elegans* cv. 'Dreamland'. Plants were cultivated under different waterlogging durations (0, 6, 12, and 24 h), applied once (a single time) at three developmental stages (four-leaf stage, full growth, and flowering initiation). Individual treatment data are provided in Tables 3 and 4. Values are the mean of four replications.

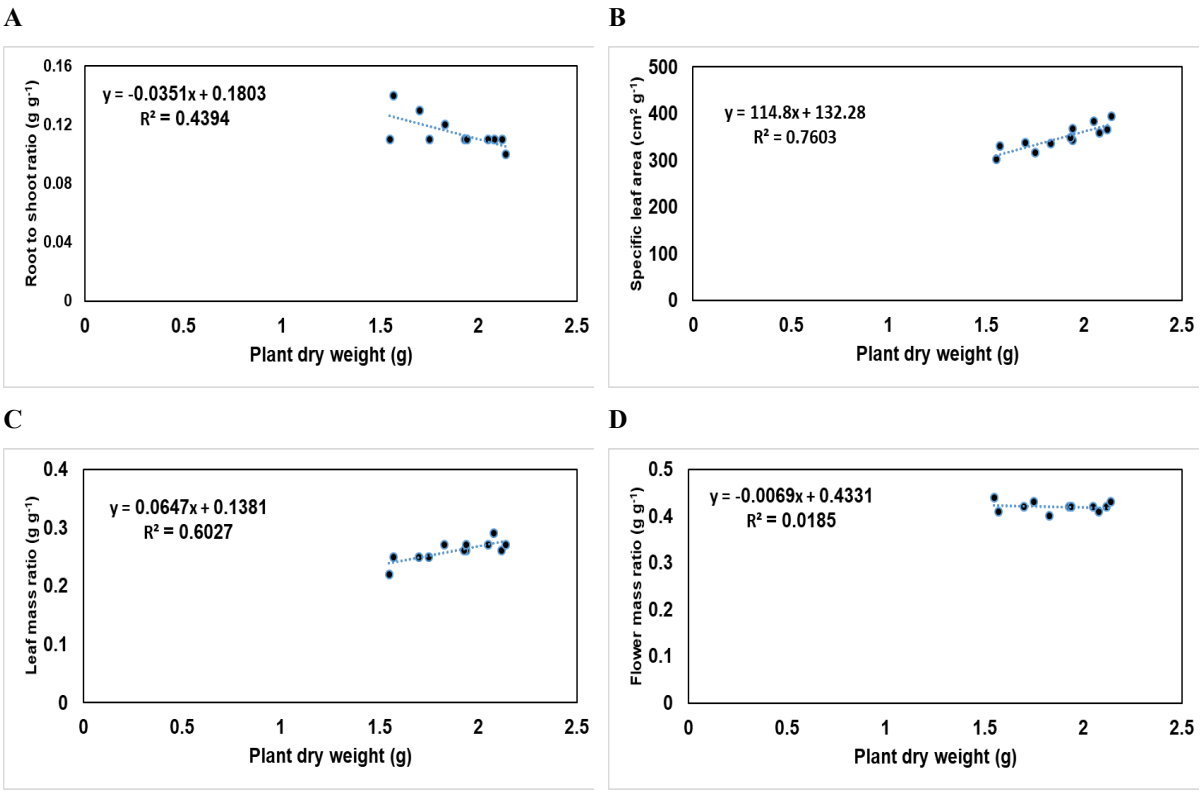

Figure S5. Root-to-shoot ratio, specific leaf area, leaf mass ratio, and flower mass ratio as functions of plant dry weight in *Zinnia elegans* cv. 'Dreamland'. Plants were cultivated under different waterlogging durations (0, 6, 12, and 24 h), applied once (a single time) at three developmental stages (four-leaf stage, full growth, and flowering initiation). The differences in the y-axis scale are noted. Individual treatment data are provided in Table 4. Values are the mean of four replications.

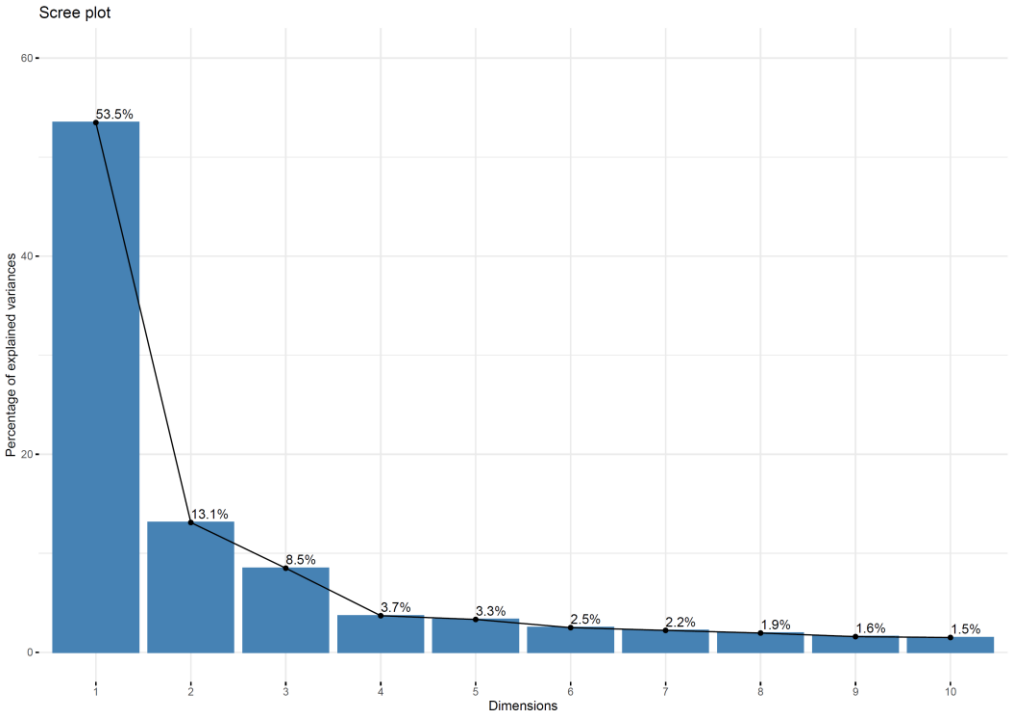

Figure S6. The first ten principal components and percentages of variations. The first two eigenvalues were used for constructing the principal component analysis biplot (accounting for 66.6 % of the cumulative percentage).

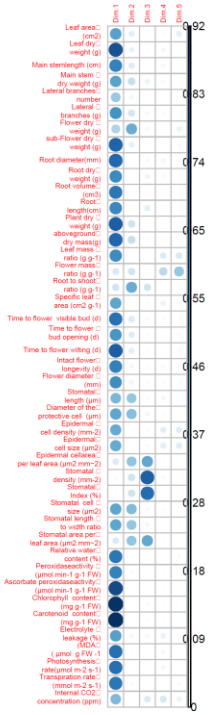

Figure S7. Quality representation (cos_2) of the variables on a factor map. Variables on the first five dimensions are displayed. Size and color intensity correlate with a better representation of specific morphological traits.