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MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL RESPONSE OF TWO PUMPKIN SPECIES TO SOIL MOISTURE GRADIENTS

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Abstract

Plants in natural ecosystems face various environmental challenges that adversely affect their morphology and physiology, ultimately reducing the productivity of food and vegetable crops. Among these, drought is one of the most damaging factors, affecting plant growth and crop yields and posing a major challenge for sustainable farming. Although many studies have examined plant responses to drought stress, no study has specifically evaluated how pumpkin species respond to varying soil moisture conditions, reflecting the increasing frequency and intensity of droughts in the Khyber Pakhtunkhwa region of Pakistan. This study examined the performance of two pumpkin species, *Cucurbita maxima* and *Cucurbita pepo*, under different soil moisture gradients. Pot culture experiments were conducted using a completely randomized design (CRD). Morpho-physiological traits, phytochemical contents, and enzyme/antioxidant activities were assessed for both species at the end of the experiment. The findings indicated that both species showed an overall decrease in plant height under low moisture conditions, although root traits including root length, specific root length (SRL), and root mass fraction (RMF) showed the opposite trend. Biomass accumulation and organ mass allocation were significantly greater in *C. pepo* compared with *C. maxima* across all treatments. In contrast, *C. maxima* produced a higher average fruit mass and a greater number of fruits per plant than *C. pepo*. Physiological traits, including net assimilation rate, transpiration rate, stomatal conductance, and specific leaf area (SLA), were also higher in *C. pepo* under high moisture conditions. Secondary metabolites and total antioxidant activity increased under low moisture conditions and decreased under medium and high moisture conditions. These findings suggest that *C. pepo* is better adapted to resist drought stress, *C. maxima* may be more productive in terms of fruit yield under varying moisture regimes. Further research is warranted to investigate both below- and above-ground traits of diverse cultivated species in natural environments. Such studies could improve our understanding of plant responses to drought and help develop strategies to enhance agricultural productivity under water-limited conditions.

Key words: Biomass allocation; Biochemical response; Physiological response; Pumpkin; Soil moisture

Introduction

Plants face significant challenges globally due to drought conditions, which affect plant growth, development, and fitness (Lin *et al*., 2006; Hasnat *et al*., 2018). Drought is a climatic phenomenon that can occur in all climatic zones, but it is particularly dominant in arid and semi-arid region (Liu *et al*., 2023). This phenomenon causes physiological damage to plants and adversely affects entire agroecosystems(Fukao *et al*., 2011). Drought stress affects plant morphology and physiology, leading to reductions in plant biomass, fruit, and seed yields (Biareh *et al.*, 2022). These reductions not only impact individual plant performance but also threaten food security by affecting crop yields and agricultural productivity (Ahmadikhah & Marufinia, 2016; Lin *et al*., 2006). Drought severity is unpredictable due to its dependence on various factors, including rainfall occurrence and distribution, evapotranspiration demands, and soil water storage capacity (Riaz *et al.*, 2013). The state of water supplies in different regions has become increasingly alarming, showing a progressive decline exacerbated by global climate change (Cook *et al.*, 2007; Riaz *et al.*, 2013). Consequently, many regions of the world are experiencing more frequent and prolonged drought events, leading to reductions in plant growth and development, seed yields, biomass production, and increased food scarcity (Thomas *et al.*, 2008). Furthermore, drought can lead to mineral deficiencies in plants, resulting in secondary effects that further compromise their health and productivity (Wang *et al.*, 2018). Although these traits are expected to integrate in response to different environmental conditions, empirical evidence remains limited on whether above- and below-ground traits respond in a coordinated manner to varying soil moisture conditions (Reich, 2014; Asefa *et al*., 2022).

Plants adjust their growth patterns in response to different soil moisture conditions, particularly by increasing root development under low moisture conditions to improve water uptake capacity (Chaves *et al.*, 2003; Chadha *et al*., 2019). This adaptation enables them to access a larger soil volume for water, which is vital during drought periods. These

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morphological changes are crucial for maintaining physiological functions, as they directly impact the plant's ability to sustain growth and development under stressful conditions (Vennam *et al*., 2023). Soil moisture content significantly affects key processes such as photosynthesis, transpiration, and gas exchange (Razi & Muneer, 2021). Under low moisture conditions, plants often reduce stomatal conductance and transpiration rates to conserve water, leading to a decrease in net photosynthesis. This reduction slows leaf development and decreases leaf area, further limiting the plant's ability to capture sunlight for photosynthesis (Buckley, 2019). Conversely, optimal soil moisture conditions promote enhanced leaf expansion and overall vegetative growth, leading to increased biomass production and reproductive success. Additionally, soil moisture influences the accumulation of secondary metabolites and antioxidants, which are vital for plant stress responses. Under high-stress conditions, plants increase the synthesis of secondary metabolites and antioxidants to combat oxidative stress caused by reactive oxygen species (ROS) (Dhanda *et al*., 2004; Rauf *et al*., 2007; Miller *et al*., 2010). This biochemical adaptation helps protect cellular structures from damage and enhances resilience to environmental stresses. Moreover, the production of secondary metabolites such as proline rises in response to water deficit, aiding in osmotic adjustment and cellular stabilization. These physiological and biochemical changes are essential for maintaining metabolic functions and supporting survival during water scarcity (Miller *et al*., 2010).

Pumpkin (squash) is an important vegetable crop that requires adequate irrigation, especially during seedling development and flowering stages. Globally, pumpkins are widely cultivated due to their significant nutritional and economic value (Paris *et al*., 2003; Li *et al*., 2021) However, in Pakistan, climate change over the past few decades has contributed to a decline in pumpkin production since 2005, particularly in the Khyber Pakhtunkhwa (KP) region. In both the northern and southern parts of KP, two pumpkin species, *Cucurbita maxima* and *Cucurbita pepo* are commonly grown and consumed. Between 2000 and 2015, the area devoted to pumpkin cultivation (winter squash) in this province has decreased significantly due to increasing water scarcity. While global pumpkin production reaches approximately 25 million tons annually, Pakistan produces only about 2,500 metric tons from an area of 29,913 hectares. Given this context, developing or introducing drought-tolerant pumpkin species and varieties is essential for improving economic efficiency and ensuring sustainable production with limited water resources (Seymen *et al*., 2019). We cultivated two pumpkin cultivars under different soil moisture gradients using a pot experiment to assess their growth performance and seed/yield production under different water availability conditions. Following questions were addressed: (1) How do *C. maxima* and *C. pepo* morphologically and physiologically adapt and respond to different soil moisture gradients, and (2) How do different soil moisture gradients affect antioxidant activity and phytohormone profiles? (3) What is the relationship between soil moisture gradients and phytohormone regulation in pumpkin plants, and do these responses differ between both the species? We hypothesize that: (1) Belowground traits will show gradient-specific responses, with the strongest responses under low moisture gradients, followed by medium and high moisture gradients. In contrast, aboveground traits-such as total biomass, shoot biomass, aboveground biomass allocation, fruit mass, and number of fruits per plant-will follow the opposite trend, reaching their highest values under high moisture, followed by medium and low moisture gradients. (2) Antioxidant activity and phytohormone levels will be closely linked across soil moisture gradients. Increases in antioxidant defenses, including total antioxidant capacity, proline, tannins, and flavonoids, will correspond with shifts in phytohormone concentrations, particularly indole-3-acetic acid (IAA) and salicylic acid (SA). (3) Soil moisture stress will significantly influence phytohormone regulation in pumpkin plants, with IAA and SA playing central roles in the physiological response to low moisture gradients. These hormonal responses are expected to differ between the two pumpkin species.

Material and Methods

Seed collection, germination and treatments: Seeds of two mature pumpkin species, *C. maxima* and *C. pepo*, were collected from fruits stored in the seed bank at the University of Agriculture, Peshawar, Khyber Pakhtunkhwa (KP) Province. The seeds were surface sterilized with a 1% potassium permanganate solution and stored at 4°C. Seeds were sown in a growth chamber at the laboratory of the Botany Department, Abdul Wali Khan University, Mardan. One week after germination, seedlings with three leaves and uniform size were transplanted into pots for further growth and experimentation. The experiment was conducted in a greenhouse using soil collected from the field where the pumpkin plants naturally grew.

We conducted three soil moisture levels to represent a range of moisture gradients: 10% (low/dry), 30% (medium/control), and 50% (high/wet). These treatments were designed to simulate the soil moisture variability typically observed in the Mardan district, where moisture levels range from approximately 10-15% during the dry season to 35-45% in the rainy season. Each pot, measuring 20 cm in diameter and 30 cm in height, was filled with soil collected from a nearby field, closely resembling the natural habitat of the pumpkin species. To stimulate the regional rainfall patterns, seedlings were watered twice a week to maintain the target soil moisture levels of 10%, 30%, and 50%. Soil moisture was monitored daily by measuring volumetric water content (VWC) using a conductivity probe (Watermark Soil Moisture Sensor, Irrometer, USA) in 8 to 10 randomly selected pots per treatment. Based on the average VWC of these pots, we calculated the amount of water required to adjust all pots to the desired moisture level. After watering, we re-evaluated moisture levels in ten randomly selected pots for each treatment to maintain the required soil moisture level. The daytime temperature fluctuated between 22 and 26°C, while the nighttime temperature ranged from 18 to 24°C.

For each treatment, 40 pots were used for both plant species, 20 pots for each species, two plant species × 3 moisture treatments × 20 replicates which gives a total of 120 pots. The study was conducted from April to July 2018, which is the optimal growing season for these species to reach the fruiting stage.

Morphological and biomass traits measurement: Morphological traits, belowground and aboveground were measured, including total root length (cm) and specific root length (SRL, m root length g^{-1} root mass), which reflect how efficiently a plant acquires water and other resources(Sher *et al.*, 2023; Tomlinson *et al.*, 2012) Aboveground traits such as plant height (cm) and stem length (cm) were also recorded. For biomass measurements, roots, stems, and leaves were oven-dried at 70°C for 48 hours. The resulting dry mass was used to calculate total plant dry mass (g, leaf +stem +root), shoot dry mass (, g, leaf+ stem), and biomass allocation traits such as root mass fraction (RMF, , g root g^{-1} total mass), stem mass fraction (SMF, , g stem g^{-1} total mass), and leaf mass fraction (LMF, g leaf g^{-1} total mass). To assess yield production under different soil moisture gradients, the average fruit mass and total number of fruits per plant at the end of the experiment were recorded.

Physiological traits and chlorophyll contents: Photosynthetic parameters were measured using a portable infrared gas analyzer (IRGA) system (LI-6400; Li-Cor Inc., Lincoln, NE, USA). To minimize discrepancies between ambient conditions and those within the gas exchange cuvette, the internal settings were standardized: CO_2 concentration at 400 μmol mol^{-1}, relative humidity at 65%, and leaf temperature at 28°C. Leaf conductance was assessed using an AP4 leaf porometer (Delta-T Devices, UK), focusing on the middle portion of the youngest fully expanded leaves. Leaves were kept in the cuvette until photosynthetic readings were stabilized, typically reaching a steady state within approximately 30 seconds due to the close match between external and cuvette conditions. Measurements were recorded once the rate of photosynthesis showed less than a 0.5% change over one minute. Gas exchange measurements were conducted outdoors (in situ) between 8:30 and 11:30 AM following a standardized protocol. As described above, environmental conditions inside the cuvette were controlled, and a minimum of 20 minutes was usually required to ensure stable reading, indicated by a variation in photosynthetic rate of less than 0.5% over one minute (De Kauwe *et al.*, 2016). During the experiment, measurements were taken from 10 plants per treatment.

We also measured specific leaf area (SLA) (cm^2 g^{-1}), which indicates the leaf surface area available for photosynthesis and gas exchange per unit of leaf biomass, by dividing leaf area by leaf dry mass(Sher *et al.*, 2023). In addition, chlorophyll content was assessed during the experimental period. Chlorophyll was extracted using 80% acetone, and the samples were centrifuged at 20,000 g for 20 minutes. The absorbance of the supernatant was then measured at 663 nm and 645 nm using a spectrophotometer. Total chlorophyll content was calculated following the method described by (Mafakheri *et al.*, 2010).

Determination of antioxidants and plant hormones

Determination of total antioxidant: To determine total antioxidant activity, 1 mL of ABTS reagent was mixed with 1 mL of ethanolic extract of the whole plant in a test tube. The mixture was thoroughly shaken and incubated in the dark at room temperature for 30 minutes. Absorbance was then measured at 734 nm using a spectrophotometer. Each sample was measured in triplicate. The percentage inhibition of ABTS radicals was calculated using the formula:

$$\%\ \text{Inhibition} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 is the absorbance of the control (ABTS without extract) and A_1 is the absorbance of ABTS with the ethanolic extract. Total antioxidant activity was calculated as:

$$\text{Total antioxidant activity} = \%\ \text{Inhibition} \times (1 / IC_{50})$$

where IC_{50} is the concentration of the extract required to inhibit 50% of ABTS radicals. The IC_{50} value was determined by plotting the percent inhibition against extract concentration. This assay reflects the free radical scavenging capacity of ethanolic extract. Antioxidant activity is expressed as the lowest IC_{50} value per gram of plant dry weight (mg/g DW), with lower IC_{50} values indicating higher antioxidant potential (Stratil *et al.*, 2006: Serpen *et al.*, 2008).

Determination of ascorbic acid: To determine the concentration of ascorbic acid, fresh plant tissues were homogenized in 5% metaphosphoric acid at a ratio of 1 g of tissue to 10 mL of solution. The homogenate was then centrifuged at 10,000 rpm for 15 minutes, and the resulting supernatant was filtered through Whatman filter paper to remove any residual particles. For the quantitative assay, 1 mL of the filtered extract was mixed with 1 mL of 2,6-dichlorophenol indophenol (DCPIP) solution, which served as the titrant. The mixture was titrated until a persistent pink color appeared, indicating the endpoint of the reaction. A standard curve was prepared using known concentrations of ascorbic acid. Absorbance was measured at 520 nm using a spectrophotometer. The concentration of ascorbic acid in the plant samples was then calculated from the standard curve and expressed as milligrams per gram of dry weight (mg/g DW).

Determination of indole acetic acid (IAA): Fresh tissue samples of whole plant parts (roots, stems, and leaves) were used to determine the concentration of indole-3-acetic acid (IAA). The tissues were homogenized in 10 mL of 80% ethanol at a ratio of 1 g tissue per 10 mL solution. The homogenate was then centrifuged at 10,000 rpm for 15 minutes, and the supernatant was collected. The IAA-containing supernatant was filtered through Whatman filter paper to remove any remaining particulates. For quantification, 1 mL of the filtered extract was transferred to a test tube and mixed with 1 mL of Salkowski's reagent. Salkowski's reagent was prepared by combining 50 mL of distilled water with 1 mL of concentrated hydrochloric acid (HCl) and 0.5 g of ferric chloride ($FeCl_3$). The mixture was incubated at room temperature for 30 minutes, during which a pink coloration developed in the presence of IAA. The absorbance of the resulting solution was measured at 530 nm using a spectrophotometer (Mohite, 2013; Goswami *et al.*, 2015).

Determination of salicylic acid (SA): Fresh tissues from the whole plant (roots, stems, and leaves) were used to determine the concentration of salicylic acid (SA). The tissues were homogenized in 80% ethanol at a ratio of 1 g of tissue per 10 mL of solution. After homogenization, the mixture was centrifuged at 10,000 rpm for 15 minutes to separate the supernatant. The supernatant was then filtered through Whatman filter paper to remove any particulate matter. To concentrate the extract, the solvent was evaporated using a low-pressure rotary evaporator. The resulting residue was re-dissolved in a fixed volume of 5 mL distilled water, for further analysis. For quantification, 1 mL of the extract was transferred to a test tube, followed by the addition of 1 mL of 1 M hydrochloric acid and 1 mL of 0.5% ferric chloride solution. The mixture was incubated at room temperature for 30 minutes for color development. The absorbance of the resulting solution was then measured at 410 nm using a spectrophotometer (Warrier *et al*., 2013).

Phytochemicals test

Determination of total flavonoids content: Total flavonoid content was determined using the aluminum chloride colorimetric method (Chang *et al*., 2002; Sarker & Oba, 2018). For the analysis, 500 µL of whole-plant dried extract was mixed with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water in a test tube. The mixture was then allowed to stand at room temperature for 30 minutes. After incubation, the absorbance was measured at 415 nm using a spectrophotometer (HITACHI, Tokyo, Japan).

Determination of total phenols: For the determination of total phenolic content, 100 mL of the plant dried extract was added to a test tube. Then, 0.5 mL of the extract was transferred to a separate test tube, followed by the addition of 0.5 mL of ethanol. The mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected, and the tube was equilibrated and centrifuged again following the same procedure. The solvent was then evaporated using a water bath. Finally, 3 mL of distilled water and 0.5 mL of Folin–Ciocalteu reagent were added to each test tube. The absorbance was measured at 415 nm using a spectrophotometer (Sánchez-Rangel *et al*., 2013; Sher *et al*., 2023).

Determination of total proline: Proline content was determined following the method described by (Bates *et al*., 1973). Approximately 0.04 g of dry plant extract was homogenized in 3% sulfosalicylic acid after 72 hours. The homogenate was then centrifuged at 3,000 g for 20 minutes. The supernatant reacted with acid ninhydrin and glacial acetic acid, and the mixture was boiled for 1 hour. After cooling, the absorbance was measured at 520 nm using a spectrophotometer. Proline content was expressed in milligrams per gram of dry leaf weight (Gharibi *et al*., 2016).

Determination of total tannins: Total tannin content was determined following the method described by Amarovich *et al*., 2004). Briefly, 0.5 mL of the plant dried extract was mixed with 3 mL of 4% vanillin solution prepared in methanol. Then, 1.5 mL of concentrated hydrochloric acid was added to the mixture. The solution was allowed to stand at room temperature for 20 minutes. After incubation, the absorbance was measured at 500 nm using a spectrophotometer. Total tannin content was expressed as milligrams of catechin equivalents per gram of dry weight (mg CE/g DW).

Statistical analysis

Statistical analyses were conducted using R version 4.0.3 (Lepik *et al*., 2012). A two-way ANOVA was used to assess the effects of soil moisture gradients and plant species on a range of plant traits, including belowground and aboveground morphological traits, biomass allocation, biomass accumulation, physiological parameters, and biochemical characteristics. Post hoc pairwise comparisons were performed using Tukey's Honest Significant Difference (HSD) test, implemented with the "glht" function from the *multcomp* package (Piepho, 2004). Additionally, principal component analysis (PCA) was conducted on the individual × trait matrix using the "rda" function from the *vegan* package (Oksanen, 2013) to explore patterns of trait variation across treatments. We further tested whether the first two PCA axes were significantly associated with the two factors, providing insights into the relationships between environmental conditions, species identity, and plant trait responses.

Results

Response of morphological traits, biomass accumulation, and fruit mass to soil moisture treatments: Soil moisture gradients had a significant effect on plant height. Both species showed increased growth under high soil moisture, while plant height decreased under low moisture. Across all treatments, *C. pepo* consistently grew taller than *C. maxima* (Table 1; Fig. 1a). Root length (RL) and specific root length (SRL) were negatively affected by low moisture, with both species showing changes in root traits. Overall, root traits were greater under low moisture, followed by medium and high moisture levels (Table 1; Fig. 1b & c). These results suggest that high soil moisture promotes overall shoot growth, whereas limited moisture stimulates root elongation as a survival strategy, particularly in *C. pepo*, which showed greater drought tolerance than *C. maxima.*

C. pepo showed significantly higher LMF and SMF than *C. maxima* across all treatments. In contrast, RMF was influenced only by soil moisture gradients. Both species had greater RMF under low moisture gradients, while there was no significant difference under medium and high moisture gradients (Table 1; Fig. 1d-f).

Shoot biomass and total plant biomass of *C. pepo* were significantly higher than *C. maxima* under high soil moisture gradients, followed by medium and low moisture gradients (Table 1; Fig. 2a & b). In contrast, *C. maxima* produced a significantly higher average fruit mass (kg) and a greater number of fruits per plant compared to *C. pepo*, but only under high moisture gradients (Table 1; Fig. 2c & d).

Table 1. Fixed-effect ANOVA of morphological and biomass traits of *C. maxima* and *C. pepo* in response to soil moisture gradients. Soil moisture and species were included as fixed factors, and their interaction (soil moisture × species) was tested to evaluate whether the effect of moisture differed between species. Data were transformed as indicated to meet assumptions of normality. Statistical significance is indicated as: *p*≤0.05; p≤0.01; *p*≤0.001.

Response variable	Predictor	df	F. ratio	P value
Plant height	Treatment (T)	2	93.72	0.000***
	Species (S)	1	10.88	0.001**
	T×S	2	12.08	0.000***
Root length	Treatment (T)	2	78.31	0.000***
	Species (S)	1	24.52	0.000***
	T×S	2	1.09	0.339
Specific root length (SRL)	Treatment (T)	2	48.03	0.000***
	Species (S)	1	1.55	0.215
	T×S	2	0.80	0.450
Leaf mass fraction (LMF)	Treatment (T)	2	7.25	0.001**
	Species (S)	1	65.68	0.000***
	T×S	2	4.54	0.012*
Root mass fraction (RMF)	Treatment (T)	2	25.61	0.000***
	Species (S)	1	1.14	0.288
	T×S	2	0.92	0.402
Stem mass fraction (SMF)	Treatment (T)	2	31.70	0.000***
	Species (S)	1	19.68	0.000***
	T×S	2	1.28	0.28
Shoot biomass	Treatment (T)	2	18.17	0.000***
	Species (S)	1	20.19	0.000***
	T×S	2	2.16	0.12
Total plant biomass	Treatment (T)	2	81.10	0.000***
	Species (S)	1	24.67	0.000***
	T×S	2	0.31	0.734
Average mass per fruit	Treatment (T)	2	37.31	0.000***
	Species (S)	1	5.80	0.018*
	T×S	2	6.72	0.001**
Fruits per plant	Treatment (T)	2	58.87	0.000***
	Species (S)	1	0.06	0.813
	T×S	2	2.05	0.134

Table 2. Fixed-effect ANOVA of physiological response variables of *C. maxima* and *C. pepo* in response to different soil moisture gradients. Soil moisture and species were included as fixed factors, and their interaction (soil moisture × species) was tested. Data were transformed as indicated to meet assumptions of normality. Statistical significance is indicated as: *p*≤0.05; p≤0.01; *p*≤0.001.

Response variable	Predictor	df	F ratio	P value
Net assimilation rate (Am)	Treatment (T)	2	62.70	0.000***
	Species (S)	1	24.06	0.000***
	T×S	2	7.30	0.001**
Transpiration rate (Vs)	Treatment (T)	2	58.88	0.000***
	Species (S)	1	6.71	0.010*
	T×S	2	0.90	0.407
Stomatal conductance (gs)	Treatment (T)	2	57.27	0.000***
	Species (S)	1	3.92	0.050
	T×S	2	6.80	0.001**
Specific leaf area (SLA)	Treatment (T)	2	50.93	0.000***
	Species (S)	1	5.04	0.027*
	T×S	2	1.46	0.235
Total chlorophyll	Treatment (T)	2	749.52	0.000***
	Species (S)	1	6.16	0.014*
	T×S	2	0.87	0.418

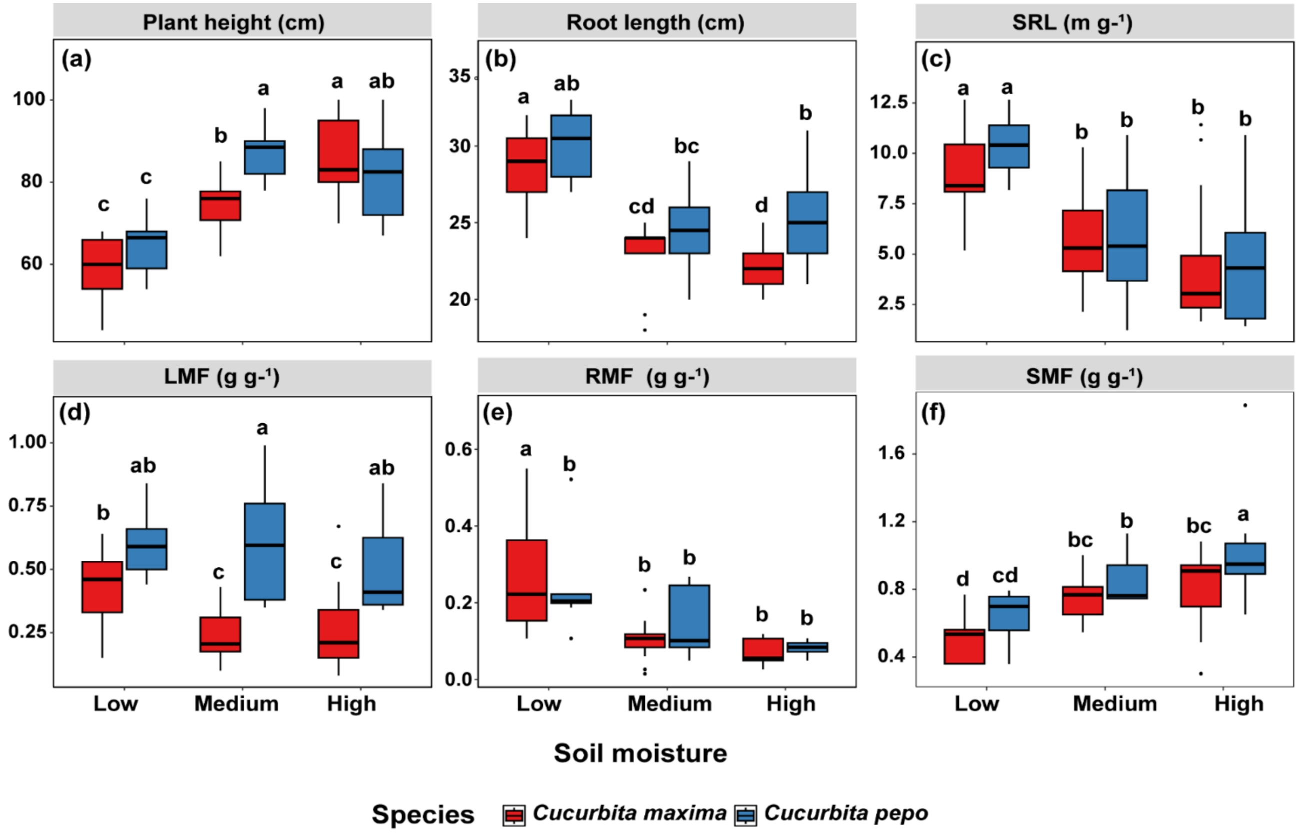

Fig. 1. Figures show (a), plant height (cm), (b) root length (cm), (c) specific root length (SRL) (m g^{-1}), (d) leaf mass fraction (LMF g g^{-1}), (e) root mass fraction (RMF g g^{-1}), and (f) stem mass fraction (SMF g g^{-1}) of *C. maxima* and *C. pepo* under different soil moisture gradients. Same letters within each panel show no statistically significant difference among the treatment pairs in that panel(based on Tukey HSD tests).

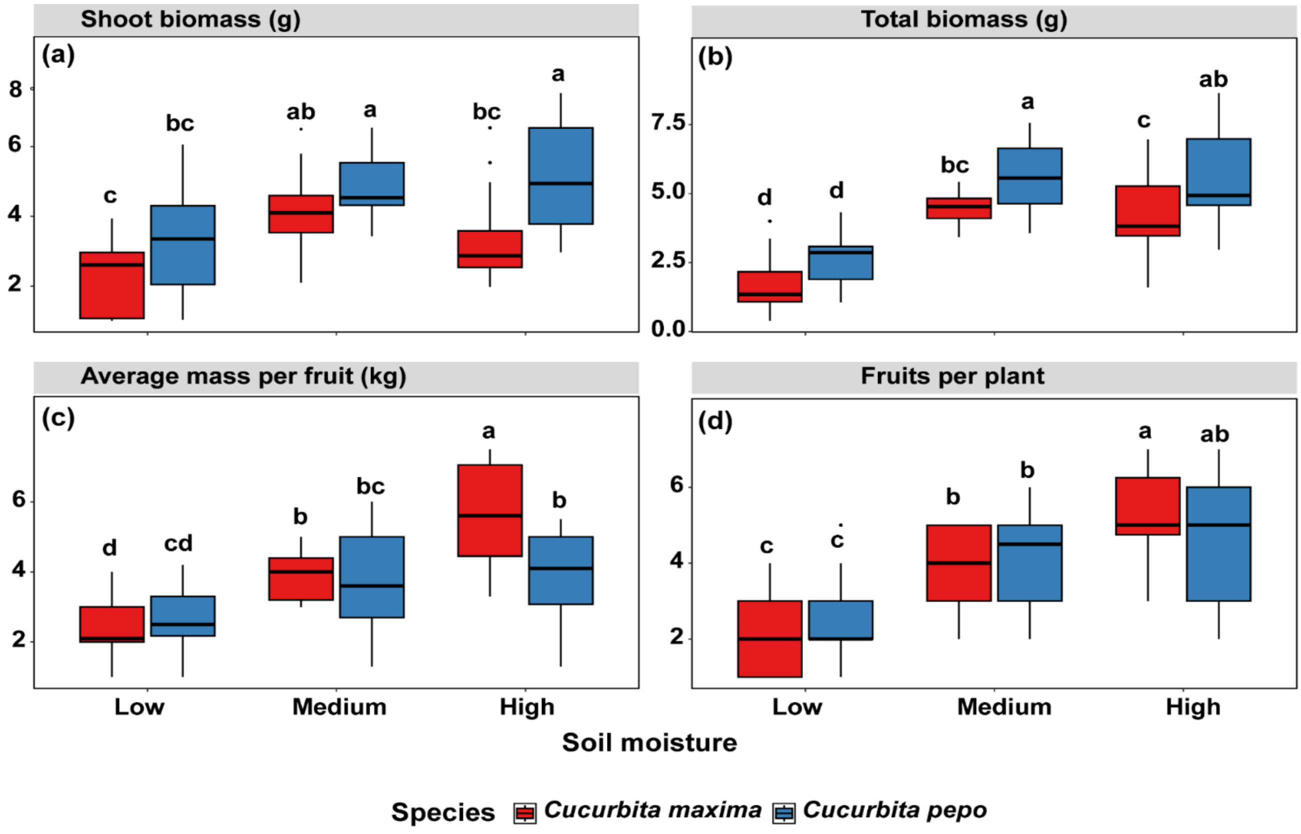

Fig. 2. Figures show (a) shoot biomass (g), (b) total biomass (g), (c) average mass per fruit (kg). (d), fruits per plant of both species *C. maxima* and *C. pepo* under different soil moisture gradients. Common letters within each panel indicate no statistically significant difference among the treatment pairs in that panel (based on Tukey HSD tests).

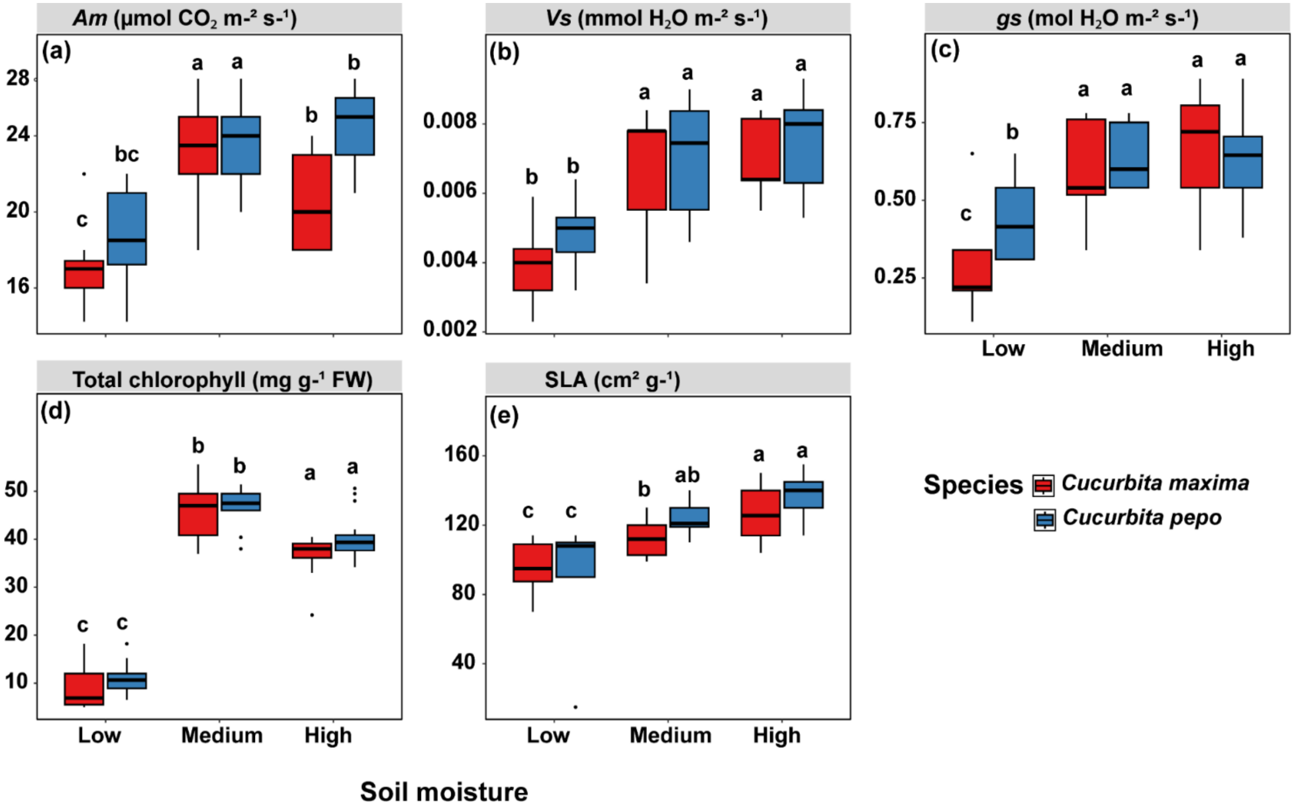

Fig. 3. Figure shows (a) net assimilation rate (*Am*, µmol CO_2 m^{-2} s^{-1}), (b) transpiration rate (*Vs*, H_2O m^{-1} s^{-2}), (c) stomatal conductance (*gs*, H_2O m^{-2} s^{-1}), (d) total chlorophyll (mg g^{-1} FW), and (e) specific leaf area (cm^{-2} g^{-1}, of *C. maxima* and *C. pepo* under different soil moisture gradients. Common letters within each panel indicate no statistically significant difference among the treatment pairs in that panel (based on Tukey HSD tests).

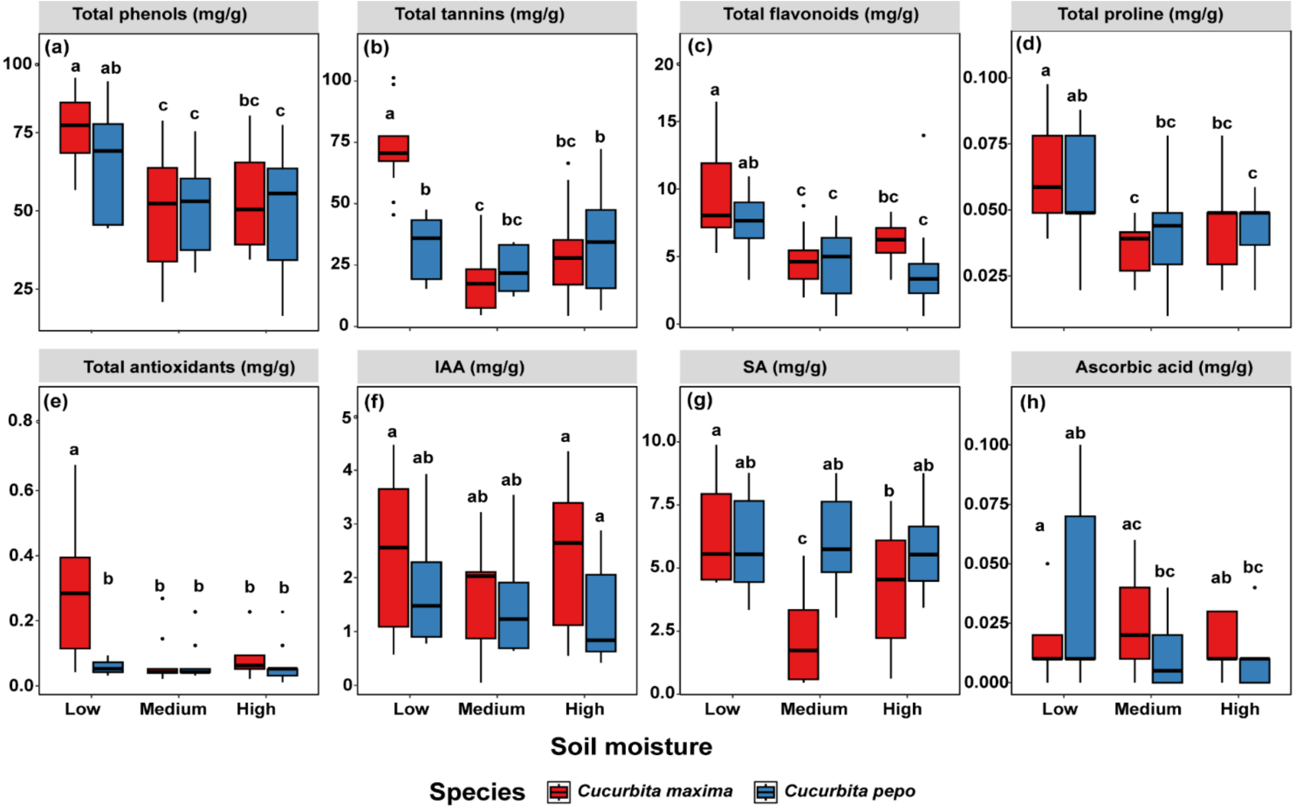

Fig. 4. Figure show (a) total phenols (mg/g), (b) total tannins (mg/g), (c) total flavonoids (mg/g), (d) total proline(mg/g), (e) total antioxidants (mg/g), (f) indole-3-acetic acid (IAA) (mg/g), (g) salicylic acid (SA, mg/g), and (h) ascorbic acid (mg/g) across different soil moisture gradients. Each panel displays the respective compound's concentration in relation to different soil moisture levels. Common letters within each panel indicate that there are no statistically significant differences among the treatment pairs (based on Tukey's HSD tests).

Table 3. Fixed-effect ANOVA of phytochemical, antioxidant, and hormone response variables of *C. maxima* and *C. pepo* in response to different soil moisture gradients. Soil moisture and species were included as fixed factors, and their interaction (soil moisture × species) was tested. Data were transformed as indicated to meet assumptions of normality. Statistical significance is indicated as: $p \leq 0.05$; $p \leq 0.01$; $p \leq 0.001$.

Response variable	Predictor	df	F ratio	P value
Total phenols	Treatment (T)	2	21.56	0.000***
	Species (S)	1	1.34	0.248
	T×S	2	1.03	0.359
Total tannins	Treatment (T)	2	46.18	0.000***
	Species (S)	1	11.70	0.000***
	T×S	2	26.95	0.000***
Total flavonoids	Treatment (T)	2	26.79	0.000***
	Species (S)	1	8.96	0.003**
	T×S	2	1.27	0.285
Total proline	Treatment (T)	2	19.27	0.000***
	Species (S)	1	0.21	0.644
	T×S	2	2.00	0.139
Total antioxidants	Treatment (T)	2	14.27	0.000***
	Species (S)	1	22.33	0.000***
	T×S	2	15.99	0.000***
Indole acetic acid (IAA)	Treatment (T)	2	2.45	0.090
	Species (S)	1	9.68	0.002**
	T×S	2	1.74	0.178
Salicylic acid (SA)	Treatment (T)	2	10.78	0.000***
	Species (S)	1	17.93	0.000***
	T×S	2	12.31	0.000***
Ascorbic acid (AA)	Treatment (T)	2	1.07	0.345
	Species (S)	1	1.41	0.236
	T×S	2	2.89	0.059

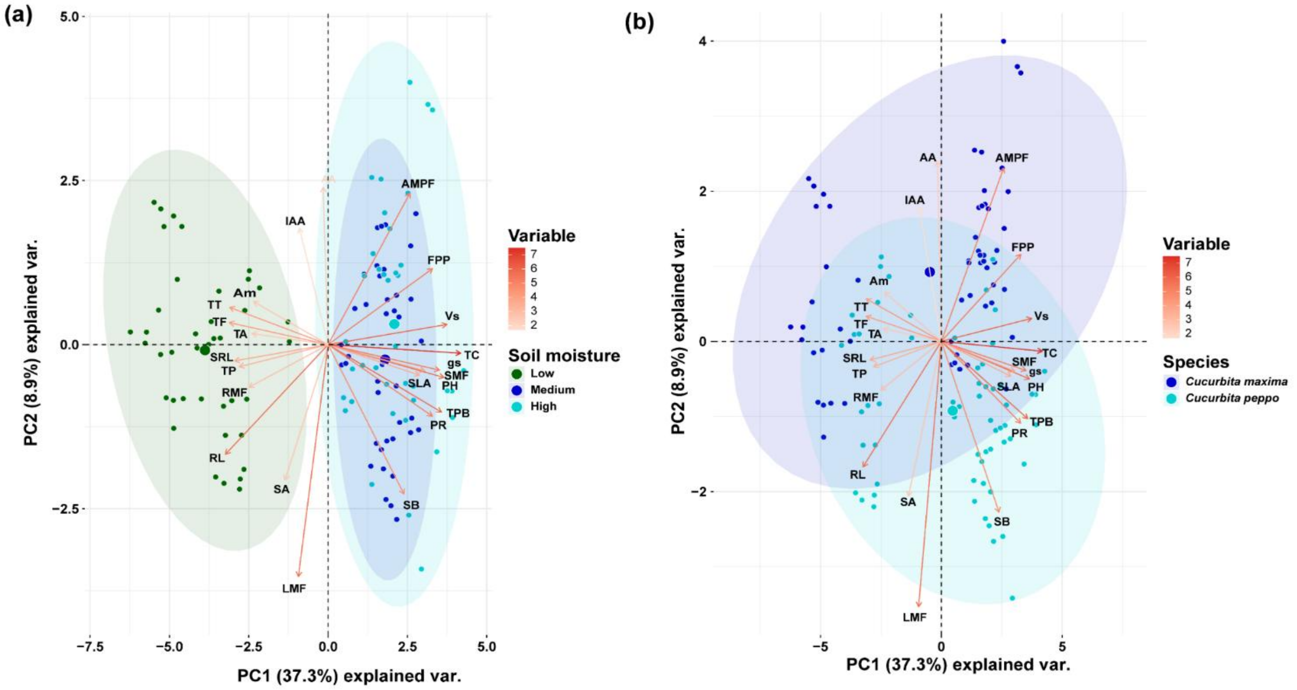

Fig. 5. Principal component analysis (PCA) of functional traits in *Cucurbita* species under soil moisture gradients and between species. Principal component biplots showing the distribution of samples and trait loadings along the first two principal components (PC1 = 37.3% and PC2 = 8.9%). (a) PCA based on three soil moisture gradients (low, medium, high). (b) PCA based on two species (*Cucurbita maxima* and *Cucurbita pepo*). Arrows represent trait loadings, and ellipses indicate 95% confidence intervals for groupings. Trait abbreviations are as follows: total plant biomass (TPB), shoot biomass (SB), root biomass (RB), root mass fraction (RMF), leaf mass fraction (LMF), plant height (PH), specific leaf area (SLA), root length (RL), specific root length (SRL), total chlorophyll (TC), stomatal conductance (*gs*), transpiration rate (*Vs*), net assimilation rate (*Am*), indole-3-acetic acid (IAA), salicylic acid (SA), ascorbic acid (AA), total phenolics (TP), total flavonoids (TF), total tannins (TT), salicylic acid (SA), total antioxidant activity (TA), average mass per fruit (AMPF), and fruits per plant (FPP).

Response of photosynthetic traits and chlorophyll contents to soil moisture gradients: Net assimilation rate (*Am*) and transpiration rate (*Vs*) were significantly affected by soil moisture gradients. Both species showed higher assimilation and transpiration rates under medium and high moisture conditions compared to low moisture gradients. *C. pepo* showed higher stomatal conductance (*gs*) than *C. maxima* under low moisture gradients, while no significant differences were observed under medium and high moisture gradients (Table 2; Fig. 3a-c).

Total chlorophyll content and specific leaf area (SLA) were significantly affected by both species and soil moisture treatments in an additive manner. Both species showed higher chlorophyll content under medium and high soil moisture levels compared to low moisture. Similarly, SLA increased with soil moisture and was higher under medium and high moisture levels relative to low moisture (Table 2; Fig 3d & e).

Response of phytochemicals, antioxidants and plant hormones to soil moisture gradients: Soil moisture gradients had a significant effect on total phenol and total proline concentrations, with the highest levels observed under low moisture, followed by medium and high moisture levels. In contrast, total tannin and total flavonoid contents were significantly influenced by both soil moisture and species. Under low moisture conditions, *C. maxima* exhibited higher tannin and flavonoid concentrations than *C. pepo*, whereas no significant differences were observed between the two species under medium and high moisture levels (Table 3; Fig. 4a–d).

Total antioxidant capacity and salicylic acid (SA) concentrations were influenced by both soil moisture levels and species, whereas indole-3-acetic acid (IAA) was affected only by species. Total antioxidant capacity was significantly higher in *C. maxima* than in *C. pepo* under low moisture, with no significant differences observed under medium and high moisture levels. IAA concentrations were generally higher in *C. maxima* across all treatments, although they did not vary significantly with soil moisture. Both species exhibited increased SA levels under low moisture, followed by medium and high moisture levels. Ascorbic acid (AA) concentrations showed no significant differences across treatments or between species (Table 3; Fig. 4e–h).

Principal component analysis (PCA): Principal Component Analysis (PCA) was performed to examine coordination among morphological, physiological, and biochemical traits in response to soil moisture gradients. The first two principal components explained 46.2% of the total variation, with PC1 accounting for 37.3% and PC2 for 8.9% (Fig. 5).

PC1 had strong positive loadings for total biomass (TB), shoot biomass (SB), root mass fraction (RMF), root length (RL), specific root length (SRL), total chlorophyll (TC), stomatal conductance (*gs*), transpiration rate (*Vs*), fruit production per plant (FPP), and plant height (PH), reflecting growth and resource acquisition. Leaf mass fraction (LMF), total tannins (TT), and salicylic acid (SA) loaded negatively, indicating a trade-off between growth and defense. PC2 was positively associated with average mass per fruit (AMPF), net assimilation rate (*Am*), indole acetic acid (IAA), ascorbic acid (AA), total proline (TP), total phenolics (TP), total flavonoids (TF), and total antioxidant activity (TA), representing physiological and biochemical responses, whereas leaf mass fraction (LMF), root length (RL), shoot biomass (SB), and salicylic acid (SA) had negative loadings, highlighting contrasting allocation between growth and stress-related traits. Overall, PCA revealed that pumpkin plants coordinate morphological, physiological, and biochemical traits under varying soil moisture, indicating that shifts in growth, resource allocation, and stress-related metabolism are key strategies for coping with water limitation.

Discussion

Response of morphological traits and biomass allocation to soil moisture gradients: Our study revealed significant differences in the morphological responses of *C. maxima* and *C. pepo* to different soil moisture gradients. In both species, plant height decreased progressively from high to low moisture conditions. However, the decrease was substantially greater in *C. maxima*, indicating higher drought sensitivity and a greater reduction in growth under water limitation (Ahmadikhah & Marufinia, 2016). This pattern is consistent with previous findings that drought stress adversely affects plant morphology, particularly plant height (Anjum *et al*., 2017). Below-ground traits were significantly influenced by both soil moisture gradients and species identity. Under low-moisture conditions, both *C. maxima* and *C. pepo* exhibited increased root length and specific root length (SRL) compared with medium- and high-moisture conditions, reflecting a common drought-avoidance strategy. Notably, *C. pepo* developed significantly longer roots and higher SRL than *C. maxima* across all moisture gradients, suggesting a stronger adaptive response to water limitation. These results indicate that, under drought stress, plants allocate more resources to root development to enhance water uptake from deeper soil layers (Schenk *et al*., 2002; Comas *et al*., 2013). The increases in below-ground traits observed in both species highlight a strategic investment in root architecture that improves soil exploration and water-absorption efficiency during drought (Sher *et al*., 2024). This response may be attributed to genetic factors promoting root elongation and the development of a more efficient root system. In contrast, *C. maxima* showed comparatively weaker root development under similar conditions, suggesting greater reliance on alternative physiological or biochemical strategies to cope with drought stress.

Biomass allocation and accumulation are key factors in cultivated plants affected by water limitation. Numerous studies conducted in both field and greenhouse conditions have demonstrated that limited water supply significantly reduces biomass accumulation (Nadeem *et al*., 2012; Tátrai *et al*., 2016). In our study, *C. pepo* consistently showed higher biomass allocation and accumulation than *C. maxima* across all soil moisture gradients, with these differences becoming more pronounced under high moisture conditions. Under low soil moisture, both species shifted their resource allocation toward root development, an adaptive response commonly associated with drought tolerance. This similar investment in belowground traits likely reduced the interspecific differences in total biomass accumulation under water-limited conditions. However, under high moisture availability, the species exhibited more distinct biomass allocation strategies, enabling each to optimize growth above-ground in response to increased water supply. These findings suggest that soil moisture not only governs total biomass production but also shapes species-specific resource allocation patterns (McConnaughay & Coleman, 1999; Brock *et al*., 2017; Shoaib *et al*., 2022). *C. pepo*, in particular, may invest more in above-ground structures such as specific leaf area (Fig. 3e) and plant height to enhance light capture and maximize growth (Lambers *et al*., 2019). In contrast, *C. maxima* appear to follow a more conservative growth strategy, leading to lower biomass accumulation compared to *C. pepo* under favorable conditions. This difference may also be linked to physiological adaptations to high-moisture environments. For instance, *C. pepo* may possess traits that enable it to utilize water more efficiently, such as enhanced water transport capacity or higher photosynthetic rates (Fig. 3a-c) (Turner, 1986; Lambers *et al*., 2019). These adaptive advantages likely contribute to the superior biomass accumulation observed in *C. pepo* compared to *C. maxima* under well-watered conditions.

Response of photosynthetic traits to soil moisture gradients: Soil moisture stress has significant impacts on plant fitness, affecting various physiological processes such as stomatal regulation and photosynthetic traits (Lang *et al*., 2018; Chaves *et al*., 2009). In many regions, drought has become more frequent and severe, posing a serious threat to plant photosynthesis (Lang *et al*., 2018). In our study, both species showed higher assimilation rates, transpiration, and stomatal conductance under medium and high soil moisture compared to low moisture gradients. This suggests that water stress limits the plant's ability to produce carbohydrates, resulting in reduced net assimilation. When water is scarce, plants tend to close their stomata to minimize water loss, which leads to lower transpiration and gas exchange rates (Lang *et al*., 2018). This stomatal closure under low moisture conditions significantly reduces stomatal conductance (Xia *et al*., 2014; Lang *et al*., 2018). Such a physiological response is vital for conserving internal water and ensuring the plant retains enough moisture for essential functions like transpiration, which supports nutrient transport and evaporative cooling. In contrast, adequate soil moisture allows stomata to stay open for longer durations, enabling greater gas exchange and photosynthetic activity. This allows plants to absorb more carbon dioxide and convert it into carbohydrates efficiently (Lang *et al*., 2018). Additionally, medium to high moisture levels help relieve water stress, allowing plants to maintain higher transpiration and assimilation rates. These conditions ultimately promote plant health, enhance growth, and increase productivity. By maintaining open stomata under favorable moisture conditions, plants can optimize carbon dioxide uptake, improve water-use efficiency, and increase biomass accumulation, leading to better overall physiological performance.

We observed that specific leaf area (SLA) decreased under low soil moisture gradients. A reduction in SLA due to drought stress negatively affected photosynthesis and overall plant growth performance (Villar *et al*., 2005). Leaves that develop under water-deficient conditions tend to exhibit lower expansion rates (Poorter *et al*., 2009). Our findings are consistent with those of (Wellstein *et al*., 2017), who also reported a significant decrease in SLA in response to drought. Lower SLA is generally associated with slower plant growth, which is commonly observed under drought conditions (Villar *et al*., 2005; Ilyas *et al*., 2021). In addition, we observed a decline in chlorophyll content under low moisture gradients. This reduction is primarily attributed to the damaging effects of reactive oxygen species on chloroplasts (Mafakheri *et al*., 2010). Our results align with those of (Duan *et al*., 2018), who also reported a significant decrease in chlorophyll content among 6 wheat cultivars under water-limited conditions. However, some studies suggest that chlorophyll levels may either decline or remain unchanged during drought stress, depending on the severity and duration of the stress (Mafakheri *et al*., 2010).

Response of phytochemicals, antioxidants and plant hormones to soil moisture gradients: In both *Cucurbita* species, the concentrations of total phenols, tannins, flavonoids, and proline were inversely related to soil moisture gradients. As soil moisture decreased from high to low, the accumulation of these secondary metabolites were progressively increased. Notably, this increase was significantly more pronounced in *C. maxima* than in *C. pepo* under the same moisture conditions. This differential biochemical response suggests that *C. maxima* may possess stronger adaptive mechanisms to cope with drought stress. The enhanced accumulation of phenolic compounds, flavonoids, tannins, and proline in *C. maxima* likely contributes to improved oxidative stress protection, better water retention, and more effective osmotic adjustment under water-limited conditions. These findings highlight the important role of secondary metabolites and compatible solutes in drought tolerance and emphasize species-specific differences in stress response strategies within the *Cucurbita* genus (Flores-Iga *et al*., 2023). The substantial increase in these metabolites in *C. maxima* may be due to several factors. First, *C. maxima* may possess a more efficient signaling pathway that regulates the biosynthesis of secondary metabolites and proline in response to drought stress (Li *et al*., 2021). This enhanced signaling capacity may allow *C. maxima* to mount a more robust biochemical defense against water deficit conditions compared to *C. pepo*. Additionally, *C. maxima* may have evolved a greater capacity for the compartmentalization and storage of these compounds, enabling higher accumulation under stress. Together, the increased production of phenolics, flavonoids, tannins, and proline in *C. maxima* likely provides superior protection against oxidative damage, enhances water conservation, and supports more effective osmoregulation. These biochemical adaptations may explain the greater drought tolerance observed in *C. maxima* compared to *C. pepo*.

We also observed that *C. maxima* exhibited higher total antioxidant capacity and indole-3-acetic acid (IAA) levels under low soil moisture compared to *C. pepo*. Both parameters showed a decreasing trend as soil moisture increased from moderate to high levels. In contrast, the levels of salicylic acid (SA) and ascorbic acid (AA) were not significantly influenced by changes in soil moisture. Under drought conditions, plants experience heightened oxidative stress due to the accumulation of reactive oxygen species (ROS). In response, *C. maxima* appear to activate its antioxidant defense system more effectively, resulting in increased accumulation of antioxidant compounds such as phenolics and flavonoids, which help mitigate oxidative damage. Previous studies have shown that *C. maxima* generally possess a higher antioxidant capacity than *C. pepo*, likely due to its greater content of bioactive compounds that efficiently scavenge ROS (Kulczyński *et al*., 2020; Miljić *et al*., 2021). Moreover, IAA, a key phytohormone involved in plant growth and stress response may be produced in higher amounts in *C. maxima* as a drought adaptation strategy. High IAA levels can promote root growth and enhance water uptake, helping plants cope with low soil moisture. This hormonal adjustment suggests that *C. maxima* have a more robust regulatory mechanism to support its growth and survival under stress. Overall, the higher antioxidant capacity and IAA levels observed in *C. maxima* under drought conditions reflect its superior ability to manage oxidative stress and maintain physiological functions compared to *C. pepo* (Jahan *et al*., 2023).

Conclusions

Our findings indicate that *C. maxima* is more water-demanding than *C. pepo*, requiring greater moisture availability throughout the experimental period. This higher water requirement makes *C. maxima* more vulnerable to drought stress, which can limit its growth and development. Low soil moisture significantly impacts plant morphology, physiology, and biomass, ultimately reducing overall plant performance. Despite this sensitivity, *C. maxima* exhibit a strong metabolic response to drought by accumulating higher levels of secondary metabolites and antioxidants compared to *C. pepo*, enhancing its ability to tolerate stress. In contrast, *C. pepo* appears better adapted to drought conditions, maintaining more stable growth under limited water availability. These results suggest a trade-off between drought tolerance and productivity: while *C. pepo* is more resilient under drought, *C. maxima* may offer higher fruit yields when sufficient water is available. Further research is needed to explore both below- and above-ground traits of different Cucurbita species under natural environmental conditions. Such studies will deepen our understanding of species-specific drought responses and contribute to the development of drought-resilient, high-yielding cultivars for sustainable agriculture under changing climate scenarios.

Conflict of Interest: The authors declare that they have no known competing financial interests or personal relationships that influence the work reported in this paper.

Author Contributions: JS designed the research project, conducted the experiments, and drafted the initial manuscript. MR and WM compiled and analyzed the data. AA and JZL critically revised the manuscript. All authors read and approved the final version of the manuscript.

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