PHOTOSYNTHETICA
International Journal for Photosynthesis Research

DOI 10.32615/ps.2024.030 PHOTOSYNTHETICA 62 (3): 292-301, 2024

Morphophysiological responses of black pepper to recurrent water deficit

T.R. FERREIRA*, V.P. SALLIN***, B. CERRI NETO***, J. CRASQUE***, A. PIRES*, P.S. RODRIGUES*, H. CHISTÉ*, A.B.P. LIMA*, J.A. MACHADO FILHO**, L.O. ARANTES**, J.M.S. LIRA#, A.R. FALQUETO*, and S. DOUSSEAU-ARANTES**,+

*Department of Agrarian and Biological Sciences, North Espírito Santo University Center, Federal University of Espírito Santo, BR 101 North, Km 60, Litorâneo District, 29932-540 São Mateus, Espírito Santo, Brazil**
*Capixaba Institute for Research, Technical Assistance and Rural Extension, BR 101 North, Km 151, P.O. Box 62, Linhares, Espírito Santo, Brazil***
*Department of Biological Sciences, Center for Human and Natural Sciences, Federal University of Espírito Santo, Avenida Fernando Ferrari 514, Goiabeiras, 29075-910 Vitória, Espírito Santo, Brazil****
Federal University of Alfenas, Rua Gabriel Monteiro da Silva 700 – Center, 37048-395 Alfenas, Minas Gerais, Brazil#

Abstract

This study investigated the effects of recurrent water deficit on drought tolerance traits in black pepper (*Piper nigrum* L.) 'Bragantina'. Plants were subjected to three cycles of water deficit followed by recovery periods. Water deficit reduced stomatal conductance, photosynthesis, transpiration, and water potential while increasing water-use efficiency. In addition, intercellular CO_2 concentration, leaf temperature, root starch, and adaptive morphological characteristics in leaves and roots increased. Despite these adaptations, plants did not recover vegetative growth after rehydration. The primary tolerance mechanisms observed included increased abaxial epidermis thickness, stomatal density, fine roots, periderm thickness, and starch accumulation in roots. Although gas exchange and leaf water potential were restored, vegetative growth did not fully recover. This study highlights the response of black pepper to recurrent water stress and the underlying mechanisms of its drought tolerance.

Keywords: anatomy; black pepper; drought cycles; gas exchange; vegetative growth.

Highlights

- Recurrent water deficit cycles alter the morphophysiological parameters of 'Bragantina'
- With each water deficit cycle, 'Bragantina' extended its tolerance to drought stress
- Tolerance mechanisms: increased abaxial epidermis, stomatal density, and fine roots

Received 31 January 2024
Accepted 12 August 2024

+Corresponding author
e-mail: saradousseau@gmail.com

Abbreviations: ABA – abscisic acid; CE – carboxylation efficiency; C_i – intercellular CO_2 concentration; Cor Thi – cortex thickness; E – transpiration; Ep Ab – abaxial epidermis; FAA 70% – formaldehyde, acetic acid, and 70% ethanol; FM – fresh mass; FTE – fritted trace elements; g_s – stomatal conductance; LA – leaf area; LDM – leaf dry mass; LED – light-emitting diode; LMA – leaf mass area; LVB – length of vascular bundles; MT – thickness of the central vein; Nº VB – vascular bundle number; Nº XV – number of xylem vessels; Per Thi – periderm thickness; PL – phloem length; P_N – net photosynthetic rate; RDM – root dry mass; RF – root fineness; RS – reducing sugars; RV – root volume; RWC – relative water content; Sd – stomatal density; SDM – shoot dry mass; SI – sclerophyll index; SLA – specific leaf area; T_{LEAF} – leaf temperature; TM – turgid mass; TRL/SDM – the ratio of total root length to shoot dry mass; TSS – total soluble sugars; WUE – water-use efficiency; Ψ_w – leaf water potential.

Acknowledgments: We thank the State Secretariat for Agriculture, Supply, Aquaculture, and Fisheries (SEAG) and the Espírito Santo State Research Support Foundation (FAPES) SE-AG/FAPES (Edict: DI 004/2022) FAPES nº: 232/2022-P 2022-RGG9N – Project Bank – Phase II and the National Council for Scientific and Technological Development (CNPq) for financing the project: Process: 421159/2018-9 – Liaison institution: Instituto Capixaba de Pesquisa, Assistência Técnica e Exsistência Rural/INCAPER-ES - Call: MCTIC/CNPq Call nº 28/2018 – Universal. This study was partly financed by CAPES/FAPES Cooperation – Postgraduate Development Program – PDPG.

Conflict of interest: The authors declare that they have no conflict of interest.

Introduction

Drought reduces growth and productivity, making it one of the most restrictive abiotic factors for crops worldwide (Salehi-Lisar and Bakhshayeshan-Agdam 2016). Recurrent water deficits occur in both natural and agricultural environments; however, most knowledge about the effects of drought comes from studies treating this stress as a singular event (Menezes-Silva *et al*. 2017). Studies by Galle *et al*. (2011), Menezes-Silva *et al*. (2017), Yan *et al*. (2017), Tombesi *et al*. (2018), Li *et al*. (2019), and Mantoan *et al*. (2020) have shown that exposure to drought alters plant responses to stress. Therefore, studies characterizing the effects of recurrent water deficit can better reflect the acclimatization state of plants in the field and help identify promising genetic materials, and establish efficient management strategies.

The plant's ability to recover from stress is considered a key drought resistance strategy, involving the resumption of important metabolic processes such as transpiration and photosynthesis. Restoring photosynthetic capacity provides a higher yield by reestablishing Calvin cycle activities and the electron transport chain, thereby reducing oxidative damage and restoring assimilated production (Chaves *et al*. 2009, Rivas *et al*. 2016). Restoring transpiration is vital for resuming leaf thermal balance and carbon dioxide absorption (Ergo *et al*. 2018, Shukla *et al*. 2018).

To understand the mechanisms of response to recurrent water deficit, this study utilized black pepper cv. 'Bragantina' (Schmildt *et al*. 2018). Black pepper, known as the King of Spices, is the most popular spices in the world (Joshi *et al*. 2018). It is used in a myriad of applications, from food to the pharmaceutical industry, due to the presence of secondary metabolites throughout the plant, primarily in its berries (Takooree *et al*. 2019).

Furthermore, 'Bragantina' is one of the earliest genetically improved cultivars and therefore the most cultivated worldwide (Schmildt *et al*. 2018). However, decreasing production has been observed in the last two decades due to reduced precipitation (Hamdin *et al*. 2020). Brazil, one of the main producers of this spice, faces prolonged periods of drought, making water deficit the main challenge to production (Ambrozim *et al*. 2022). Therefore, studies simulating recurrent water deficits can offer a more realistic approach to understanding the effects of drought in the field.

Numerous studies have examined the effect of drought on black pepper; however, they all consider only a singular drought event (George *et al*. 2017, Negi *et al*. 2021, Cruz *et al*. 2022). Krishnamurthy *et al*. (2016) reported that the response of black pepper to drought varies among genotypes, resulting in a reduced photosynthetic rate, stomatal conductance, transpiration, and increased leaf temperature. They emphasized how drought affects leaf area and plant height, with water-stressed plants accumulating more reducing sugars. Sugar accumulation offers an advantage to plants that have suffered water deficit, reducing the osmotic potential and maintaining the turgor pressure of plant cells (Al-Huqail *et al*. 2020).

The 'Bragantina' cultivar was considered drought-sensitive due to the severe decrease in leaf water potential under water deficit conditions (Krishnamurthy *et al*. 2016). Recently, Ambrozim *et al*. (2022) confirmed the susceptibility to water restriction of 'Bragantina' and observed a low recovery capacity after stress, noting reductions in stomatal conductance, water potential, and relative leaf water content. However, no study has addressed the responses of black pepper to recurrent water deficit. Therefore, this study aimed to evaluate the effects of recurrent water deficit on the morphophysiological characteristics of both the aboveground part and root system, as well as to identify which characteristics are related to drought tolerance in 'Bragantina' black pepper.

Materials and methods

Study location and sample preparation: The experiment was conducted at the experimental farm of the Capixaba Institute for Research, Technical Assistance, and Rural Extension (Incaper), located at 19°25'00.1"S, 40°04'35.3"W, in the Linhares municipality, the northern region of the state of Espírito Santo. The seedlings were propagated from stem cuttings using the method described by Ambrozim *et al*. (2017). They were grown in tubes with a capacity of 280 cm^3, filled with *Bioplant*® substrate, and supplemented with 3 g of slow-release fertilizer (NPK 19-6-10) for six months.

Once the aboveground length reached the commercial standard of 30 cm (90 d after staking), the seedlings were transplanted into 8-L pots filled with soil. The soil was previously analyzed at the 'Laboratory of Agronomic Analysis and Consulting' and corrected according to Prezotti *et al*. (2007). Specifically, for every 6 m^3 of soil, 13 kg of limestone, 30 kg of *Super Simples*, 2 kg of potassium chloride (KCl), and 350 g of fritted trace elements (FTE), and 12 bags of chicken manure were applied. Additionally, each pot received 5 g of slow-release fertilizer (NPK 19-6-10) aged for 8 months. After transplantation, the plants remained in full sun for 12 months, receiving regular nutritional and water management to foster vegetative growth. Subsequently, the plants were transferred to a greenhouse for a 15-d acclimatization period.

Experimental design: The recurrent water deficit experiment comprised three cycles, each including a dehydration phase (irrigation suspension) followed by a rehydration phase (irrigation resumption). The maximum period of water restriction was determined based on the ability of the plants to maintain stomatal conductance around 0–1 μmol m^{-2} s^{-1}, indicating maximum stress, as defined by Cerri Neto *et al*. (2023). Plant recovery was determined when gas-exchange assessments were similar to those of the control group. The drought and recovery periods varied within the cycles.

The experiment was conducted in a randomized complete block design, with three blocks, each containing ten plants per plot. This design was chosen to mitigate environmental variations within the greenhouse, such as wind direction and intensity, as well as the distribution of humidity from the cooling system. The blocks were arranged perpendicular to the fans and the greenhouse cooling wall to minimize the interference of these factors on the treatments.

During the experiment, the temperature and relative humidity inside the greenhouse were measured using the digital thermometer-hygrometer model *Incoterm 7666.02.0.00*. Readings were taken twice, at 8:00 and 17:00 h (Fig. 1S, *supplement*).

Analysis of gas-exchange variables: Gas-exchange variables were evaluated over time under drought treatments, where plants were subjected to drought episodes, and control treatments, where plants were irrigated regularly. Additionally, during the evaluation of the third cycle, plants were separated into three treatments: (T0) plants before initiation of the third cycle of water deficit, (3WD) plants on the last day of water deficit in the third cycle, and (Recovery) plants evaluated after 72 h of rehydration. Gas-exchange measurements were conducted using the *LI-COR 6400* Infrared Gas Analyzer – IRGA (*LI-COR Inc*., Lincoln, NE, USA).

The *LI-COR 6400* IRGA camera has an analysis area of 6 cm^2, which emits red-blue light through light-emitting diodes (LED). PAR was set at 1,200 µmol(photon) m^{-2} s^{-1}, temperature at 25°C, CO_2 flow at 500 µmol s^{-1}, and reference CO_2 at 400 µmol mol^{-1} (Sulok *et al*. 2019). In each plot, two plants were evaluated, with measurements taken from fully expanded leaves located on the second or third node from the apex of the plagiotropic branch.

The assessments were conducted between 8:00 and 11:00 h, evaluating the following characteristics: net photosynthetic rate (P_N), transpiration (E), intercellular CO_2 concentration (C_i), leaf temperature (T_{LEAF}), and stomatal conductance (g_s). Instantaneous water-use efficiency (WUE) was calculated as the ratio of P_N to E, and carboxylation efficiency (CE) was calculated as the ratio of P_N to C_i (Cerri Neto *et al*. 2023).

Leaf water potential (Ψ_w) was measured from plants exposed to three drought and rehydration cycles (3WD), plants before drought exposure (T0), and control plants (not subjected to drought). Measurements were taken at 5:00 h using a Scholander pressure chamber (*Model 1000*, *PMS Instrument Co*., Albany, OR, USA) on healthy, fully expanded leaves collected from the middle third of the plants.

Carbohydrate assessment: Carbohydrate allocation was assessed by quantifying reducing sugars (RS), total soluble sugars (TSS), and starch in the leaves, stem, and root that underwent three cycles of drought and recovery (3WD + Recovery) and control plants. Dried plant organs were ground in a *STAR FT-50* mill and stored in a freezer at –18°C. Sugar extraction followed the method by Zanandrea *et al*. (2010). The dried sample (0.2 g) was homogenized in 5 mL of 0.1 M potassium phosphate buffer (pH 7.0), incubated in a water bath at 40°C for 30 min, and centrifuged using an *NI 1811-A* model at 5,000 rpm for 20 min. The supernatant was collected, and the precipitate was resuspended twice in 5 mL of the same potassium phosphate buffer. The combined supernatants were frozen for RS and TSS quantification, and the precipitate was frozen for starch extraction. The protocol used for RS quantification was the Miller (1959) dinitro-salicylic acid method, whereas the TSS quantification followed a modified Yemm and Willis (1954) anthrone method, using 2 mL of 0.1% anthrone solution in 93.33% sulfuric acid, plus 1 mL of the plant extract, placed in a water bath at 100°C for 3 min.

Starch extraction: For starch extraction, the precipitate was resuspended in 8 mL of 0.2 M potassium acetate buffer (pH 4.8) and then placed in a water bath at 100°C for 5 min. After this process, 61.5 µL of the enzyme amyloglucosidase was added. The mixture was incubated again in a water bath at 40°C for 2 h, followed by centrifugation at 5,000 rpm for 30 min. 5 mL of the supernatant was collected, and 10 mL of Thivend extraction buffer (Thivend *et al*. 1965, MacRae and Armstrong 1968) was added. Starch was quantified using the anthrone method described for TSS.

Fully expanded leaves were removed from the third branch, and root fragments were collected 5 cm above the root cap for anatomical analysis. The materials were fixed in FAA 70% (formaldehyde, acetic acid, and 70% ethanol) for 24 h and subsequently stored in 70% alcohol (Johansen 1940). Transverse sections were made in the root 1 cm below the collar and the midrib of the leaves using a sharp blade. The sections were clarified with sodium hypochlorite and stained with safrablue (Kraus and Arduin 1997). Paradermal sections of the leaves were made using the epidermal impression technique with universal instant adhesive (*Super Glue*®) as described in Segatto *et al*. (2004). After sectioning, histological slides were prepared (Kraus and Arduin 1997). All sections were analyzed under a bright-field microscope (*Euromex*), and images were captured with a micro-camera (*CMEX 5*).

Biometric assessment: Biometric measurements were conducted using the *ImageFocus 4.0* software. The following variables were obtained from the longitudinal view of the leaf: abaxial epidermis thickness (Ep Ab), number of vascular bundles (Nº VB), length of vascular bundles (LVB), number of xylem vessels (Nº XV), and midrib thickness (MT) (Nascimento and Potiguara 1999, Gogosz *et al*. 2012). In the front view of the leaf, only stomatal density (Sd) was assessed (Segatto *et al*. 2004). In the root, phloem length (PL), cortex thickness (Cor Thi), and periderm thickness (Per Thi) were evaluated (Nascimento and Potiguara 1999, Gogosz *et al*. 2012).

Leaf attributes were determined and adapted according to Barrs and Weatherley (1962). Ten leaf discs, each measuring 0.219 mm, were removed from plants that underwent three cycles of drought and recovery (3WD + Recovery) and from control plants. The leaf discs were weighed using an analytical balance to obtain fresh mass (FM), then hydrated with distilled water for 24 h to obtain turgid mass (TM), expressed in grams

(Barrs and Weatherley 1962). After 24 h, the leaf discs were weighed again, placed in paper bags, and dried in an oven at 60°C until a constant dry mass was achieved, yielding the leaf dry mass (LDM), expressed in g (Barrs and Weatherley 1962, Witkowski and Lamont 1991). Additional measurements included the leaf mass per unit leaf area (the ratio of leaf dry mass to leaf area; LMA) and the specific leaf area (the ratio of leaf area to leaf dry mass; SLA) (Witkowski and Lamont 1991), the relative water content (RWC) calculated as the difference between FM and LDM divided by the difference between TM and LDM, multiplied by 100 (Barrs and Weatherley 1962), and the sclerophyll index (SI) calculated as (SI) = dry mass [g]/2 × leaf area [cm^2] (Rizzini 1976).

The number of leaves and plant height were quantified at three stages: before the third cycle of water deficit (T0), after undergoing the three cycles of water deficit and recovery (3WD + Recovery), and in control plants. Leaf count was performed manually, while plant height was measured using a measuring tape from the stem to the apex. Allocation of dry mass was assessed at the end of the rehydration phase of the third cycle. Root dry mass (RDM) and shoot dry mass (SDM) were obtained by sectioning the plant and drying it in a forced-air circulation oven at 65°C until constant mass, followed by weighing on an analytical balance. Leaf area (LA) was determined using the *LI-COR 3100* meter, and root volume (RV) was measured based on water displacement in a beaker. Root fineness (RF) was calculated as the ratio of total root length to root volume and, finally, as the ratio of total root length to shoot dry mass (TRL/SDM).

Statistical analysis: Gas-exchange data were analyzed descriptively using the standard error of the mean with *SIGMA PLOT Software 11.0*. For variables including water potential, carbohydrates, anatomy, leaf attributes, biomass, height, and number of leaves, an analysis of variance (*ANOVA*) was conducted. The means were compared using *Tukey*'s test at a 5% probability level ($p<0.05$), using the *SISVAR* computational program *version 5.8* (Ferreira 2011). *SISVAR* is a statistical software developed by *Borland Turbo Pascal 3.0* for *ANOVA* in linear statistical models, multiple comparisons, and regression analysis for quantitative factors. Its features include analyzing interactions and nested effects between fixed factors, as well as applying multiple comparison procedures and regression analysis for quantitative factors (Ferreira 2011).

Results

During the drought episodes, there was a significant decrease in g_s, P_N, E, and CE, accompanied by an increase in C_i (Fig. 1*A–C*,*G*). The T_{LEAF} initially decreased during the first cycle of water deficit but stabilized at control levels by the end of the second and third cycles (days 16 and 48) (Fig. 1*E*). WUE remained consistent during the last day (9 d) of drought in the first cycle, but declined in subsequent cycles (day 16 and 48) (Fig. 1*F*).

Following the 3WD, there was a reduction in the mean g_s, P_N, E, and CE. However, these variables recovered after a 72-h rehydration period, with no statistical difference observed compared to T0 plants, except for g_s (Table 1). C_i levels were higher in 3WD but decreased after rehydration, although no statistical difference was observed compared to the T0 plants (Table 1). The T_{LEAF} increased in 3WD and during recovery compared to T0. Finally, WUE did not show statistical differences between treatments (Table 1). Leaf water potential was reduced with water deficit but recovered after rehydration to values similar to the T0 treatment (Table 1).

The RS and TSS contents in the leaves, stems, and roots of the 3WD group were similar to those of the control group (Fig. 2*A*,*B*). However, in the 3WD group, plants accumulated more starch in the roots (Fig. 2*C*). Additionally, in the leaves of the 3WD group, there was an increase in the Ep Ab, Nº VB, LVB, MT, and Sd, while Nº XV decreased. In the roots of the 3WD group, the PL and Per Thi increased in comparison to the control group, while the Cor Thi decreased (Table 2).

Regarding the leaf attributes of the 3WD group, the TM, LDM, LMA, and SI increased, while the SLA decreased (Table 3). Biomass analysis of the 3WD treatment revealed a reduction in the RDM, LA, and RV, while RF and TRL/SDM increased (Table 4). Plant height growth was halted, and even with rehydration, it did not surpass the means of the T0 and control treatments (Fig. 3). In addition, the recovered plants exhibited a reduced number of leaves compared to the control plants.

Discussion

The exposure of 'Bragantina' to recurrent drought cycles significantly altered the physiological and morphological parameters studied, suggesting a readjustment in the morphophysiological mechanisms that were likely responsible for the plant's ability to withstand prolonged stressful events (Tombesi *et al.* 2018).

In this study, it was expected that the P_N of the third cycle at maximum water stress would be higher than that of previous cycles. This expectation was based on observations from previous studies on recurrent water deficit using the sugarcane variety IAC SP94-2094 and coffee clones 109 and 120 (Menezes-Silva *et al.* 2017, Marcos *et al.* 2018). It was anticipated that an improvement in P_N would extend the duration of drought tolerance. The results revealed that after nine days under drought stress in the third cycle, corresponding to 44 d (Fig. 1), 'Bragantina' exhibited higher mean P_N, g_s, E, and CE compared to the same period in the first cycle, consistent with findings by Menezes-Silva *et al.* (2017) and Marcos *et al.* (2018). This suggests that prior exposure to stress improved the subsequent plant responses, indicating the incorporation of information from previous stressful events to enhance the plant's performance under low water availability (Alves *et al.* 2020).

However, after 48 d of water limitation, the mean g_s, P_N, E, and CE decreased (Fig. 1*A–C*,*G*); nevertheless, the plants extended the duration of stress with each successive drought event. The first cycle lasted 9 d, the second cycle for 11 d (from day 17 to 27), and the third

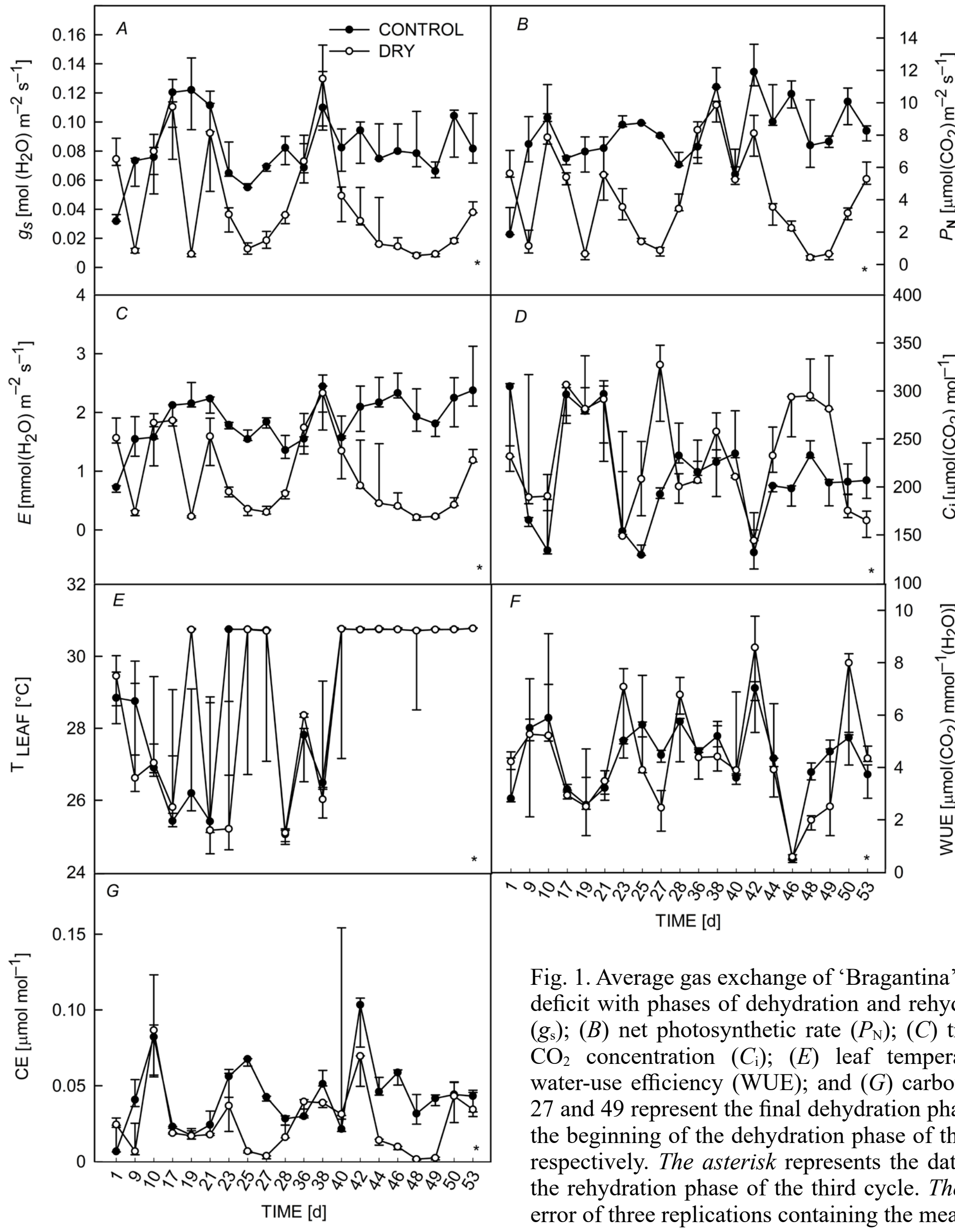

Fig. 1. Average gas exchange of 'Bragantina' subjected to three cycles of water deficit with phases of dehydration and rehydration. (*A*) *S*tomatal conductance (g_s); (*B*) net photosynthetic rate (P_N); (*C*) transpiration (*E*); (*D*) intercellular CO$_2$ concentration (C_i); (*E*) leaf temperature (T$_{\text{LEAF}}$); (*F*) instantaneous water-use efficiency (WUE); and (*G*) carboxylation efficiency (CE). Days 9, 27 and 49 represent the final dehydration phase. Days 17 and 36 correspond to the beginning of the dehydration phase of the second and third drought cycle, respectively. *The asterisk* represents the data collected from the third day of the rehydration phase of the third cycle. *The bar* corresponds to the standard error of three replications containing the mean of two plants per plot.

Table 1. Average stomatal conductance (g_s), net photosynthetic rate (P_N), transpiration (*E*), intercellular CO$_2$ concentration (C_i), leaf temperature (T$_{\text{LEAF}}$), instantaneous water-use efficiency (WUE), and carboxylation efficiency (CE) before dawn (Ψ_w) of the third water deficit cycle of the 'Bragantina' cultivar. (T0) plants before being subjected to the third water deficit cycle, (3WD) third water deficit cycle and (Recovery) 72 h after rehydration. Values are means ± SE, *n* = 3. Means followed by *the same letter* do not differ from each other according to *Tukey*'s test ($p<0.05$).

Parameter	T0	3WD	Recovery
g_s [mol(H$_2$O) m^{-2} s^{-1}]	0.07 ± 0.01^a	0.01 ± 0.00^b	0.04 ± 0.00^{ab}
P_N [μmol (CO$_2$) m^{-2} s^{-1}]	7.53 ± 1.28^a	0.48 ± 0.19^b	5.64 ± 0.69^a
E [mmol(H$_2$O) m^{-2} s^{-1}]	1.63 ± 0.34^a	0.22 ± 0.02^b	1.26 ± 0.11^a
C_i [μmol(CO$_2$) mol^{-1}]	215.46 ± 11.22^{ab}	308.60 ± 27.97^a	161.05 ± 13.70^b
T$_{\text{LEAF}}$ [°C]	28.35 ± 0.05^b	30.75 ± 0.02^a	30.78 ± 0.01^a
WUE [μmol(CO$_2$) mmol^{-1}(H$_2$O)]	4.88 ± 0.26^a	3.05 ± 1.66^a	4.51 ± 0.29^a
CE [μmol·mol^{-1}]	0.03 ± 0.01^a	0.00 ± 0.00^b	0.03 ± 0.01^a
Ψ_w [MPa]	0.26 ± 0.04^a	1.79 ± 0.32^b	0.26 ± 0.03^a

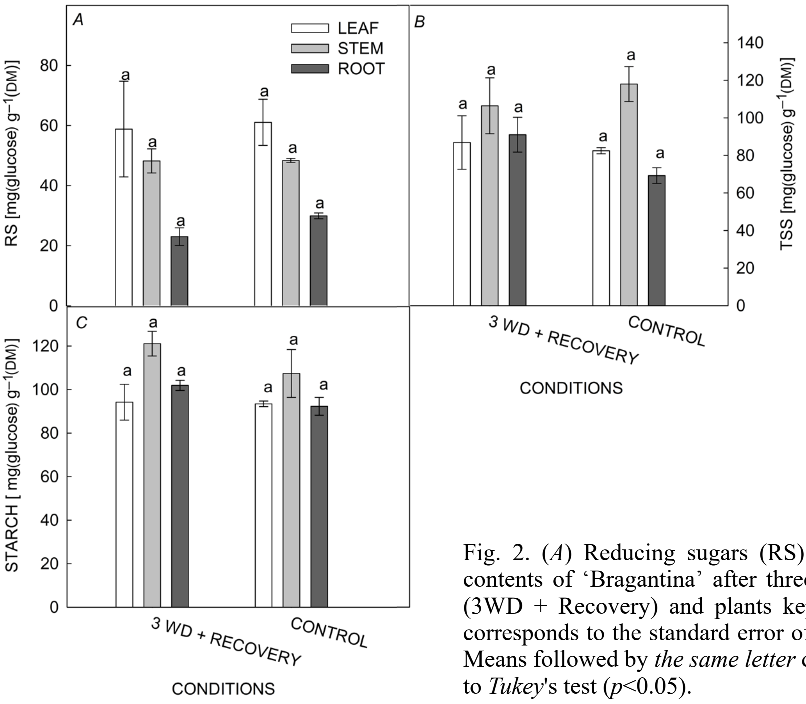

Fig. 2. (*A*) Reducing sugars (RS); (*B*) total soluble sugars (TSS); and (*C*) starch contents of 'Bragantina' after three cycles of water deficit followed by rehydration (3WD + Recovery) and plants kept irrigated (Control). DM – dry mass. *The bar* corresponds to the standard error of three replicates consisting of five plants per plot. Means followed by *the same letter* do not differ statistically from each other according to *Tukey*'s test ($p<0.05$).

Table 2. Anatomy of 'Bragantina' leaves and roots after three cycles of water deficit and rehydration (3WD + Recovery) and plants kept irrigated (Control). Leaf variables: abaxial epidermis (Ep Ab), number of vascular bundles (Nº VB), length of the vascular bundle (LVB), number of xylem vessels (Nº XV), midrib thickness (MT), and stomatal density (Sd). Root variables: phloem length (PL), cortex thickness (Cor Thi), and periderm thickness (Per Thi). Values are means ± SE, $n = 3$. Means followed by *the same letter* on the row do not differ statistically from each other according to *Tukey*'s test ($p<0.05$).

Parameter	3WD + Recovery	Control
Leaf anatomy		
Ep Ab [mm]	0.017 ± 0.003^a	0.013 ± 0.000^b
Nº VB	4.733 ± 0.657^a	1.000 ± 0.000^b
LVB [mm]	0.286 ± 0.032^a	0.244 ± 0.003^b
Nº VX	28.400 ± 4.300^b	43.060 ± 0.593^a
MT [mm]	0.634 ± 0.006^a	0.503 ± 0.003^b
Sd [mm^{-2}]	92.258 ± 3.048^a	69.892 ± 4.285^b
Root anatomy		
PL [mm]	0.401 ± 0.076^a	0.309 ± 0.050^b
Cor Thi [mm]	0.354 ± 0.009^b	0.425 ± 0.009^a
Per Thi [mm]	0.055 ± 0.003^a	0.077 ± 0.003^b

cycle for 14 d (from day 36 to 49) (Fig. 1). This indicates that the plants were able to improve their gas-exchange means up to a certain period, after which they declined. The prolonged duration under water deficit was evidenced by the decrease in P_N, g_s, E, and CE together with the changes in the in water potential, starch content, leaf and root anatomy, and leaf attributes, ultimately impacting the plant's biomass and vegetative growth.

Table 3. Leaf attributes of 'Bragantina'after three cycles of water deficit and rehydration (3WD + Recovery) and plants kept irrigated (Control). Turgid mass (TM), leaf dry mass (LDM), leaf mass by area (LMA), specific leaf area (SLA), sclerophyll index (SI), and relative water content (RWC). Values are means ± SE, $n = 3$. Means followed by *the same letter* on the row do not differ statistically from each other according to *Tukey*'s test ($p<0.05$).

Parameter	3WD + Recovery	Control
TM [g]	0.026 ± 0.002^a	0.024 ± 0.001^b
LDM [g]	0.007 ± 0.000^a	0.006 ± 0.001^b
LMA [g m^{-2}]	84.713 ± 4.648^a	69.344 ± 8.411^b
SLA [cm^2 g^{-1}]	118.946 ± 6.107^b	150.441 ± 19.690^a
SI [g mm^{-2}]	0.298 ± 0.016^a	0.244 ± 0.030^b
RWC [%]	79.167 ± 5.763^a	84.237 ± 3.899^a

One of the main physiological processes affected by the water deficit was the g_s. When soil water availability became critical, g_s values decreased (Table 1). Stomatal closure is induced by hydraulic signals and increased leaf abscisic acid (ABA) contents (Brunetti *et al.* 2019). Roots act as sensors of water deficit, sending signals (ABA) to the shoots, where it is detected by stomatal guard cells, leading to either complete or partial stomatal closure (Fathi and Tari 2016). With partial stomatal closure, there was a decrease in E, restricting water loss and improving WUE (Table 1).

As depicted in Fig. 1, P_N and plant g_s during the drought decreased and C_i increased. In the 3WD treatment, the same behavior was observed for P_N, g_s, and C_i (Table 1). The data suggest that the main factor in the decline in P_N during water deficit is associated with mesophilic resistance, as there was an increase in C_i in plants that

Table 4. Fresh and dry biomass of 'Bragantina' after three cycles of water deficit and rehydration (3WD + Recovery) and plants kept irrigated (Control). Leaf area (LA), root volume (RV), root dry matter (RDM), Shoot dry matter (SDM), root fineness (RF), and total root length/shoot dry matter ratio (TRL/SDM). Values are means ± SE, $n = 3$. Means followed by *the same letter* on the row do not differ statistically from each other according to *Tukey*'s test ($p<0.05$).

Parameter	3WD + Recovery	Control
LA [cm^2]	3,263.199 ± 373.130^b	4,357.550 ± 163.313^a
VR [cm^3]	66.000 ± 3.055^b	109.333 ± 8.743^a
RDM [g]	10.719 ± 0.605^b	15.219 ± 1.337^a
SDM [g]	75.114 ± 4.876^b	98.050 ± 2.721^a
RF [cm cm^{-3}]	1.097 ± 0.041^a	0.623 ± 0.026^b
TRL/SDM [cm g^{-1}]	1.093 ± 0.036^a	0.773 ± 0.028^b

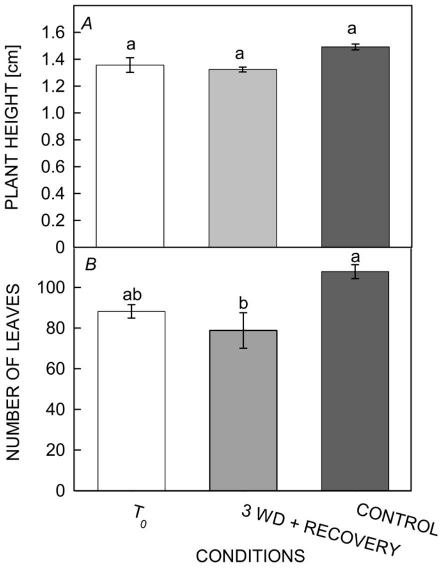

Fig. 3. (*A*) Height and (*B*) number of leaves of the 'Bragantina' cultivar. (T0) before the imposition of the third water deficit, (3WD + Recovery) plants that suffered three cycles of water deficit and rehydration, and control plants (Control) maintained at field capacity that did not suffer from water stress. *The bar* corresponds to the standard error of three replications containing the mean of two plants per plot. Means followed by *the same letter* do not differ statistically from each other according to *Tukey*'s test ($p<0.05$).

suffered from water deficit (Pompelli *et al.* 2022). CO_2 probably suffered resistance to reach the carboxylation sites of the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), causing a decrease in CE.

The decrease in CE under water deficit can also be attributed to changes in biochemical reactions and thylakoid membrane composition caused by water deficiency, compromising metabolic and enzymatic processes (Aires *et al.* 2022). Aires *et al.* (2022), in their study on the foliar application of salicylic acid to mitigate water deficit in tomatoes, noted a similar decrease in CE and increase in C_i among plants subjected to water deficit but not treated with salicylic acid.

The C_i of the control treatment ranged from approximately 300–150 µmol(CO_2) mol^{-1} over time (Fig. 1*D*). Interestingly, when C_i was high, g_s, P_N, E, and CE decreased, a behavior observed only on the first day of water deficit (Fig. 1*A–C,G*). However, on subsequent days, regardless of C_i levels, the g_s, P_N, E, and CE remained similar or higher in the control plants compared to the drought treatment (Fig. 1*A–C,G*).

Despite being adequately irrigated and the greenhouse having fans and a cooling system, data from the first day of water deficit indicate that the control treatment plants were under thermal stress. This stress was likely due to high temperature and humidity, which were around 29°C and 67%, respectively (Fig. 1S). According to Monda *et al.* (2021), under high temperature and humidity conditions, plants tend to reduce transpiration, and this reduction compromises gas exchange and plant thermal regulation. High temperatures deactivate Rubisco, which leads to increased C_i and decreased CE (Fathi and Tari 2016).

The reduction in water potential and the accumulation of higher transient starch content in the roots are seen as tolerance mechanisms. Since the accumulation of osmotically active solutes has been reported in many crops, an increase in solute concentration can contribute to a reduction in osmotic potential, thus maintaining turgor potential in cells (Blaya-Ros *et al.* 2021). These mechanisms allowed 'Bragantina' to minimize water loss and survive under water deficit for up to 14 d in the 3WD treatment.

Furthermore, the maintenance of plant water status can be attributed to the increased number of finer roots (Table 3). Fine roots constitute a significant portion of the root system, thereby increasing surface area and maximizing water absorption (Wasaya *et al.*, 2018). The increase in Ep Ab thickness (Table 2) concurrent with the reduction in LA (Table 3), coupled with the decrease in E (Table 1), reflects characteristics aimed at avoiding water loss. Additionally, the decrease in the number of leaves (Fig. 3) further reduces transpiring surface area. Consequently, plants capable of maintaining a balance between water uptake by the root system and water loss through transpiration are more tolerant to water deficit (He *et al.* 2017, Tardieu *et al.* 2017).

Anatomical changes in leaf tissues serve as vital indicators of species acclimation to limited water availability, playing a crucial role in mitigating water loss (Souza *et al.* 2018). The increase in Ep Ab correlates with the increased Sd (Table 2), a characteristic consistent with the hypostomatic nature of black pepper stomata (Sulok *et al.* 2019). Previous studies, such as those by Zhao *et al.* (2015), Scholasch and Rienth (2019), and Bertolino *et al.* (2019) have shown an increase in Sd following long dry periods.

Modifications in leaf anatomy have been interpreted as an adaptation to drought, as the increase in stomata also increases the supply of CO_2 in the leaves (Conti *et al.* 2019). Furthermore, the increase in Sd in 'Bragantina' may be associated with faster opening and closing of stomata, thus allowing for more efficient regulation of stomatal conductance (Zhao *et al.* 2015). Since stomata play a crucial role in regulating water use and carbon assimilation in plants, they represent an important target for improving WUE. Therefore, the increase in the number of stomata along with the increase in Ep Ab reflects another adaptive response by 'Bragantina' to water stress.

Furthermore, changes in the root are considered adaptive mechanisms for plants to withstand stress conditions (Zulfiqar *et al.* 2020). Root anatomy analysis revealed that 'Bragantina' increased the thickness of the periderm in the plants subjected to drought cycles (Table 2). According to Campilho *et al.* (2020), the thickness of the periderm provides plants with greater resistance to water loss from the root surface to the environment. In addition to this, water deficit altered the cortical area (Table 2). The cortical parenchyma is the main region for water storage in plants, thus any alteration can affect water conservation and storage (Zulfiqar *et al.* 2020).

Small changes in root phloem size can significantly affect root growth as well as water transport and utilization during drought (Strock and Lynch 2020). Thus, the increase in PL (Table 2) may have influenced the root allocation of resources between shoot and root growth, representing an important mechanism in response to drought. Since there was an increase in starch concentration in the roots (Fig. 2) and the TRL/SDM ratio (Table 3), these results suggest that roots act as the primary sink for photoassimilates produced during stress, despite no increase in root volume and dry mass (Table 3).

Cell growth is a physiological process sensitive to water deficiency (Anjorin *et al.* 2016). Under dry conditions, cell wall size decreases due to reduced turgor pressure, causing it to loosen and inhibit cell division (Fathi and Tari 2016). Consequently, the observed decrease in Nº VX (Table 2) in plants subjected to water deficit may be due to a reduction in the number of divisions in cambial cell activity (Mickky *et al.* 2018).

Recurrent water deficit also impacted vegetative growth, as indicated by reductions in SLA, LA, and SDM observed in 'Bragantina' following the drought and rehydration cycles, indicating that plant growth was impaired under drought. The decrease in water-use efficiency by the plant, coupled with low photosynthetic activity during periods of water limitation, led to an increase in SI, reducing the LA development (Silva *et al.* 2022). Consequently, these changes were reflected in the lower values of SLA and SDM.

Thus, 'Bragantina' plants could recover g_s, P_N, E, CE, RWC, and hydraulic potential after 72 h of rehydration. However, recurrent water deficit halted vegetative growth, and after the final rehydration, the plants were unable to exhibit average vegetative growth equal to or greater than the control, which may be attributed to the cultivar's inability to invest carbohydrates in growth when the plants were rehydrated (Dong and Beckles 2019).

Conclusion: Recurrent water deficit reduced g_s, P_N, E, CE, hydraulic potential, Nº VX, SLA, LA, and NF in 'Bragantina' plants. Despite these challenges, the cultivar exhibited an adaptive response, progressively extending its tolerance to drought stress with each new cycle of water deficit. Similarly, gas exchanges and hydraulic potential were effectively recovered after 72 h of rehydration.

Recurrent water deficit also impaired root volume and dry mass, but it led to a greater accumulation of starch, total root length concerning shoot dry mass, and a higher quantity of fine roots.

The main mechanisms involved in tolerance responses of 'Bragantina' to water deficit included increased thickness of the abaxial leaf epidermis along with increased stomatal density, greater presence of fine roots, thicker periderm, and starch accumulation in the roots. Despite these adaptations, the plants were unable to resume vegetative growth after rehydration.

References

Aires E.S., Ferraz A.K.L., Carvalho B.L. *et al.*: Foliar application of salicylic acid to mitigate water stress in tomato. – Plants-Basel **11**: 1175, 2022.

Al-Huqail A., El-Dakak R.M., Sanad M.N. *et al.*: Effects of climate temperature and water stress on plant growth and accumulation of antioxidant compounds in sweet basil (*Ocimum basilicum* L.) leafy vegetable. – Scientifica **4**: 3808909, 2020.

Alves R.D.F.B., Menezes-Silva P.E., Sousa L.F. *et al.*: Evidence of drought memory in *Dipteryx alata* indicates differential acclimation of plants to savannah conditions. – Sci. Rep.-UK **10**: 16455, 2020.

Ambrozim C.S., Furtado J.G., Valani R.S. *et al.*: [Propagation of black pepper in different concentrations of indolebutyric acid.] – Rev. Ifes Ciênc. **3**: 17-28, 2017. [In Portuguese]

Ambrozim C.S., Medici L.O., Cruz E.S.D. *et al.*: Physiological response of black pepper (*Piper nigrum* L.) to deficit irrigation. – Rev. Cienc. Agron. **53**: e20207348, 2022.

Anjorin F.B., Adejumo S.A., Agboola L., Samuel Y.D.: Proline, soluble sugar, leaf starch and relative water contents of four maize varieties in response to different watering regimes. – Cercet. Agron. Mold. **XLIX**: 51-62, 2016.

Barrs H.D., Weatherley P.E.: A re-examination of the relative turgidity technique for estimating water deficit in leaves. – Aust. J. Biol. Sci. **15**: 413-428, 1962.

Bertolino L.T., Caine R.S., Gray J.E.: Impact of stomatal density and morphology on water-use efficiency in a changing world. – Front. Plant. Sci. **10**: 225, 2019.

Blaya-Ros P.J., Blanco V., Torres-Sánchez R., Domingo R.: Drought-adaptive mechanisms of young sweet cherry trees in response to withholding and resuming irrigation cycles. – Agronomy **11**: 1812, 2021.

Brunetti C., Gori A., Marino G. *et al.*: Dynamic changes in ABA content in water-stressed *Populus nigra*: effects on carbon fixation and soluble carbohydrates. – Ann. Bot.-London **124**: 627-643, 2019.

Campilho A., Nieminen K., Ragni L.: The development of the periderm: the final boundary between a plant and its environment. – Curr. Opin. Plant Biol. **53**: 10-14, 2020.

Cerri Neto B., Silva F.R.N., Ferreira T.R. *et al.*: Responses of wild *Piper* species to drought and rehydration cycles considering stomatal closure as a marker of the alarm phase. – Photosynthetica **61**: 363-376, 2023.

Chaves M.M., Flexas J., Pinheiro C.: Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. – Ann. Bot.-London **103**: 551-560, 2009.

Conti V., Mareri L., Faleri C. *et al.*: Drought stress affects the response of Italian local tomato (*Solanum lycopersicum* L.) varieties in a genotype-dependent manner. – Plants-Basel **8**: 336, 2019.

Cruz E.S.D., Medici L.O., Leles P.S.D.S. *et al.*: Growth of black pepper plantlets under different substrates and irrigation levels. – Sci. Agric. **79**: e20200094, 2022.

Dong S., Beckles D.M.: Dynamic changes in the starch-sugar interconversion within plant source and sink tissues promote a better abiotic stress response. – J. Plant. Physiol. **234-235**: 80-93, 2019.

Ergo V.V., Lascano R., Vega C.R.C. *et al.*: Heat and water stressed field-grown soybean: A multivariate study on the relationship between physiological-biochemical traits and yield. – Environ. Exp. Bot. **148**: 1-11, 2018.

Fathi A., Tari D.B.: Effect of drought stress and its mechanism in plants. – Int. J. Life Sci. **10**: 1-6, 2016.

Ferreira D.F.: Sisvar: a computer statistical analysis system. – Ciênc. Agrotec. **35**: 1039-1042, 2011.

Galle A., Florez-Sarasa I., El Aououad H., Flexas J.: The Mediterranean evergreen *Quercus ilex* and the semi-deciduous *Cistus albidus* differ in their leaf gas exchange regulation and acclimation to repeated drought and re-watering cycles. – J. Exp. Bot. **62**: 5207-5216, 2011.

George K.J., Malik N., Vijesh Kumar I.P., Krishnamurthy K.S.: Gene expression analysis in drought tolerant and susceptible black pepper (*Piper nigrum* L.) in response to water deficit stress. – Acta. Physiol. Plant. **39**: 104, 2017.

Gogosz A.M., Boeger M.R.T., Negrelle R.R.B., Bergo C.: [Comparative leaf anatomy of nine species of the genus Piper (*Piperaceae*).] – Rodriguésia **63**: 405-417, 2012. [In Portuguese]

Hamdin N.E., Hussain H., Chong N.F.-M.: Identification of stress-related proteins during the growth and development of *Piper nigrum* L. – In: IOP Conf. Ser.: Earth Environ. Sci. **549**: 012072, 2020.

He J., Du Y.L., Wang T. *et al.*: Conserved water use improves the yield performance of soybean (*Glycine max* (L.) Merr.) under drought. – Agr. Water Manage. **179**: 236-245, 2017.

Johansen D.A.: Plant Microtechnique. Pp. 523. McGraw-Hill Book Company, New York-London 1940.

Joshi D.R., Shrestha A.C., Adhikari A.: A review on diversified use of the king of spices: *Piper nigrum* (black pepper). – Int. J. Pharm. Sci. Res. **9**: 4089-4101, 2018.

Kraus J.E., Arduin M.: [Basic manual of methods in plant morphology.] Pp. 198. EDUR, Seropédica 1997. [In Portuguese]

Krishnamurthy K.S., Ankegowda S.J., Umadevi P., George J.K.: Black pepper and water stress. – In: Rao N., Shivashankara K., Laxman R. (ed.): Abiotic Stress Physiology of Horticultural Crops. Pp. 321-332. Springer, New Delhi 2016.

Li P., Yang H., Wang L. *et al.*: Physiological and transcriptome analyses reveal short-term responses and formation of memory under drought stress in rice. – Front. Genet. **10**: 55, 2019.

MacRae J.C., Armstrong D.G.: Enzymatic method for determination of α-linked glucose polymers in biological materials. – J. Sci. Food Agr. **19**: 578-581, 1968.

Mantoan L.P.B., Corrêa C.V., Rainho C.A., de Almeida L.F.R.: Rapid dehydration induces long-term water deficit memory in sorghum seedlings: advantages and consequences. – Environ. Exp. Bot. **180**: 104252, 2020.

Marcos F.C.C., Silveira N.M., Mokochinski J.B. *et al.*: Drought tolerance of sugarcane is improved by previous exposure to water deficit. – J. Plant. Physiol. **223**: 9-18, 2018.

Menezes-Silva P.E., Sanglard L.M.V.P., Ávila R.T. *et al.*: Photosynthetic and metabolic acclimation to repeated drought events play key roles in drought tolerance in coffee. – J. Exp. Bot. **68**: 4309-4322, 2017.

Mickky B.M., Abbas M.A., El-Shhaby O.A.: Alterations in photosynthetic capacity and morpho-histological features of leaf in alfalfa plants subjected to water deficit-stress in different soil types. – Indian J. Plant. Physiol. **23**: 426-443, 2018.

Miller G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. – Anal. Chem. **31**: 426-428, 1959.

Monda K., Mabuchi A., Negi J., Iba K.: Cuticle permeability is an important parameter for the trade-off strategy between drought tolerance and CO_2 uptake in land plants. – Plant Signal. Behav. **16**: 1908692, 2021.

Nascimento M.E.., Potiguara R.C.V.: [Anatomical aspects of the vegetative organs of *Piper hispidinervium* C.DC. (Piperaceae) and their secretory structures.] – Boletim do Museu Paraense Emílio Goeldi **15**: 39-104, 1999. [In Portuguese]

Negi A., Kokkat J.G., Jasrotia R.S. *et al.*: Drought responsiveness in black pepper (*Piper nigrum* L.): Genes associated and development of a web-genomic resource. – Physiol. Plantarum **172**: 669-683, 2021.

Pompelli M.F., Espitia-Romero C.A., Jaraba-Navas J.D. *et al.*: *Stevia rebaudiana* under a CO_2 enrichment atmosphere: Can CO_2 enrichment overcome stomatic, mesophilic and biochemical barriers that limit photosynthesis? – Sustainability **14**: 14269, 2022.

Prezotti L.C., Gomes J.A., Dadalto G.G., Oliveira J.A.: [Liming and fertilization recommendation manual for the State of Espírito Santo.] Pp. 289. SEEA, INCAPER, CEDAGRO Vitória 2007. [In Portuguese]

Rivas R., Falcão H.M., Ribeiro R.V. *et al.*: Drought tolerance in cowpea species is driven by less sensitivity of leaf gas exchange to water deficit and rapid recovery of photosynthesis after rehydration. – S. Afr. J. Bot. **103**: 101-107, 2016.

Rizzini C.T.: [Treatise of Phytogeography of Brazil.] Pp. 374. Editora de Humanismo, Ciência e Tecnologia, São Paulo 1976. [In Portuguese]

Salehi-Lisar S.Y., Bakhshayeshan-Agdam H.: Drought stress in plants: causes, consequences, and tolerance. – In: Hossain M., Wani S., Bhattacharjee S. *et al.* (ed.): Drought Stress Tolerance in Plants. Vol. 1. Pp. 1-16. Springer, Cham 2016.

Schmildt E.R., Arantes L.O., Hell L.H. *et al.*: [Black pepper varieties.] – In: Silva M.B., Da Vitória E.L., Campanharo A. (ed.): [Black Pepper Culture.] Pp. 19-39. Araçá, São Mateus 2018. [In Portuguese]

Scholasch T., Rienth M.: Review of water deficit mediated changes in vine and berry physiology; Consequences for the optimization of irrigation strategies. – OENO One **53**: 423-444, 2019.

Segatto F.B., Bisognin D.A., Benedetti M. *et al.*: [A technique for the anatomical study of potato leaf epidermis.] – Ciênc. Rural

34: 1597-1601, 2004. [In Portuguese]

Shukla P.S., Shotton K., Norman E. *et al.*: Seaweed extract improve drought tolerance of soybean by regulating stress-response genes. – AoB Plants **10**: plx051, 2018.

Silva T.I., Dias M.G., Grossi J.A.S. *et al.*: Application of phytohormones as attenuators of salt stress in *Tropaeolum majus* L. (Tropaeolaceae). – Acta. Bot. Croat. **81**: 51-60, 2022.

Souza P.U., Lima L.K.S., Soares T.L. *et al.*: Biometric, physiological and anatomical responses of *Passiflora* spp. to controlled water deficit. – Sci. Hortic.-Amsterdam **229**: 77-90, 2018.

Strock C.F., Lynch J.P.: Secondary root growth: an unexplored component of soil resource acquisition. – Ann. Bot.-London **126**: 205-218, 2020.

Sulok K.M.T.A., Haruna A.O., Shang C.Y. *et al.*: Leaf stomatal density and distribution in black pepper under field conditions. – J. Trop. Plant Physiol. **11**: 24-31, 2019.

Takooree H., Aumeeruddy M.Z., Rengasamy K.R.R. *et al.*: A systematic review on black pepper (*Piper nigrum* L.): from folk uses to pharmacological applications. – Crit. Rev. Food Sci. Nutr. **59**: S210-S243, 2019.

Tardieu F., Draye X., Javaux M.: Root water uptake and ideotypes of the root system: whole-plant controls matter. – Vadose Zone J. **16**: 1-10, 2017.

Thivend P., Mercier C., Guilbot A.: Dosage de l'amidon dans les milieux complexes. – Ann. Biol. Anim. Bioch. Biophys. **5**: 513-526, 1965. [In French]

Tombesi S., Frioni T., Poni S., Palliotti A.: Effect of water stress "memory" on plant behavior during subsequent drought stress. – Environ. Exp. Bot. **150**: 106-114, 2018.

Wasaya A., Zhang X., Fang Q., Yan Z.: Root phenotyping for drought tolerance: a review. – Agronomy **8**: 241, 2018.

Witkowski E.T.F., Lamont B.B.: Leaf specific mass confounds leaf density and thickness. – Oecologia **88**: 486-493, 1991.

Yan W., Zhong Y., Shangguan Z.: Rapid response of the carbon balance strategy in *Robinia pseudoacacia* and *Amorpha fruticosa* to recurrent drought. – Environ. Exp. Bot. **138**: 46-56, 2017.

Yemm E.W., Willis A.J.: The estimation of carbohydrates in plant extracts by anthrone. – Biochem. J. **57**: 508-514, 1954.

Zanandrea I., Alves J.D., Deuner S. *et al.*: Tolerance of *Sesbania virgata* plants to flooding. – Aust. J. Bot. **57**: 661-669, 2010.

Zhao W., Sun Y., Kjelgren R., Liu X.: Response of stomatal density and bound gas exchange in leaves of maize to soil water deficit. – Acta Physiol. Plant. **37**: 1704, 2015.

Zulfiqar F., Younis A., Riaz A *et al.*: Morpho-anatomical adaptations of two *Tagetes erecta* L. cultivars with contrasting response to drought stress. – Pak. J. Bot. **52**: 801-810, 2020.