HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

Analysis of the transcriptomic responses of cucumbers to waterlogging stress and identification of markers, regulators, and effectors of tolerance

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1. INTRODUCTION

Both in nature and in agriculture, plants often encounter environmental tensions. How soil and climate restrict the distribution of plant species is determined by stress. The term stress is usually used to describe a negative effect on plant's physiological functions, which is triggered by a sudden change in the environment's optimal state where equilibrium is maintained to a suboptimal one, causing the plant's physiology to change. In most cases, stress limits crop productivity or primary assimilation processes, all of which are related to overall growth decline. Being stationary, plants are incapable of overcoming environmental challenges by simply moving to a more conducive environment. Instead, plants have honed their ability to handle stressful situations by altering their physiological and developmental routines to sustain growth and reproduction to their greatest extent (Redondo et al. 2013).

Cucumber is one of the most consumed traditional vegetables worldwide. It is commercially grown in open fields and greenhouses. Because of extensive breeding efforts, marketed hybrids are ideally suited to the specific growth systems in which they are designed to grow. Molecular investigations indicate a wide range of possible cucumber genotypes (Han et al. 2022). Cucumbers are often thought to be susceptible to waterlogging (WL). Intraspecies variability in waterlogging tolerance has been detected in cucumber varieties (Xu et al. 2016). Slicing cucumbers are typically produced under sheltered conditions with intense fertigation, frequently on soilless substrates. In this instance, low rhizosphere volume and intensive irrigation/fertigation predispose roots to WL stress and thus hypoxia. Under these conditions, intensive slicing hybrids remain productive. This is most likely due to the features of thoughtful breeding efforts, which have allowed roots to adapt to hypoxia. WL tolerance thresholds can differ among plant species (Tyagi et al. 2023). Soil waterlogging occurs when excess water saturates the soil pores. Because gas diffusion in water is several times slower than that in air, the oxygen content in waterlogged soils drops rapidly, causing a cascade of conditions that are harmful to the growth of most plant species (Colmer and Greenway 2011). This has been connected to features such as aerenchyma production, the development of adventitious roots, and/or changes in sensory and metabolic pathways (Irfan et al. 2010; Fukao et al. 2019; Jethva et al. 2022). The molecular basis for these features has been intensively explored and partially grasped. Adventitious roots alleviate hypoxia in root tissues by delivering oxygen internally, reducing the requirement for conversion to anaerobic metabolic pathways to sustain energy production (Evans et al. 2004). Aside from morphological adaptations, hypoxia causes plant physiological responses at the molecular level. Group VII ethylene response factors (ERFs) are among the elements that contribute to the upregulation of genes involved in anaerobic metabolism and adaptation to hypoxia (Mustroph et al. 2010; Giuntoli et al. 2018).

Plant cysteine oxidases (PCOs) are another class of genes associated with hypoxia stress adaptation; their products are known as O_2-sensing enzymes. PCOs can catalyze the oxidation of cysteine to Cys-sulfinic acid in O_2-dependent processes, which initiates, and it leads to degradation/deactivation of Group VII ethylene response factors (ERFs) (Taylor et al. 2022; Dalle et al. 2023). PCOs may be expressed constitutively, but they are upregulated by ERFVIIs, therefore they can be proposed as markers for the extent of plants hypoxic responses in waterlogging (White et al. 2020). By promoting the expression of anoxia-related genes, certain important transcription factors, such as those belonging to the group VII ethylene response factors (ERF-VIIs), contribute to the low oxygen response (Gibbs et al. 2011; Bailey-Serres et al. 2012; Gasch et al. 2016). Although the central components of plant hypoxia adaptation and anaerobic metabolism have been extensively studied over the last decade, identifying the mechanisms of low-oxygen response activation remains an active research topic (Eysholdt et al. 2023). The development of adventitious roots (ARs), which reduce the distance for oxygen transport and increase gas diffusivity, is another important adaptation strategy to waterlogging (Sauter, 2013). The submerged area of the basal stem or hypocotyls is typically where ARs form, and they can take the place of the major roots that are degrading (Bailey-Serres et al. 2012; Sauter, 2013). Thus, for survival in damp/waterlogged soil the adaptive responses of AR generation to waterlogging may be more crucial than those of the main roots (Li et al. 2009; Yamauchi et al. 2014).

This study involved young cucumber seedlings. WL is used for selected genotypes in a semi-hydroponic growing environment based on perlite. Hybrids of the open field and greenhouse varieties were tested by flooding their root systems with nutritional solution. Research has sought to uncover hybrid-specific reactions to WL and to pinpoint the morphological, physiological, and molecular characteristics that are connected to the applied stress. The quantity of adventitious roots (ARs) was assessed in multiple cultivars of the two main types of commercial cucumber hybrids to provide a more comprehensive understanding of the frequency of AR development. The experiments presented in this thesis aimed to evaluate the potential contribution of cysteine oxidase genes to regulation of WL stress responses in cucumber and to compare the physiological characteristics of two commercial F1 hybrid cultivars subjected to these treatments.

The experimental approach used in this study is directly related to commercial growth conditions in cucumber production and has practical application potential.

1.1. Objectives

The main objectives of this work is to uncover morphological, physiological and genetic differences between representatives of the two major cultivation types of cucumber, greenhouse vs open field grown hybrids under waterlogging conditions.

Specific Objectives

- observe AR formation in several hybrids of both cultivation types in normal growth conditions to find potential differences in this important characteristic
- measure growth and AR numbers in two representative hybrids in WL for a detailed analysis of their morphological response
- determine changes in oxidative stress marker status (malondialdehyde level) and anaerobic metabolism (alcohol dehydrogenase activity) in both hybrids to compare responses
- measure expression level for a class of signaling components (cysteine oxidase genes) in order to establish their induction kinetics and potential differences between the hybrids

2. LITERATURE REVIEW

2.1. Cucumber (*Cucumis sativus L.*) Botany and Origin

In the *Cucurbitaceae* family of gourds, cucumber (*Cucumis sativus*) is a commonly produced creeping vine plant that produces spherical to cylindrical fruits (Fig. 1), that are eaten as culinary vegetables (Britannica, 1993). Cucumbers are available in three kinds (Silvertown, 1985): slicing, pickling, and seedless, each with a distinct group of cultivars. Originally from Asia, they could be found in China, India, Bangladesh, Nepal, and Northern Thailand, cucumbers are now grown on most continents. Many different species of cucumbers are grown commercially and sold around the world (Chomicki et al. 2020; Weng, 2021; Malepszy, 1988; Bisht et al. 2004). Cucumbers have many small, edible seeds embedded in a gel-like matrix within their crisp, tasty flesh. (Mariod et al. 2017). Cucumbers were later brought and grown in

America by Spanish settlers (Valcárcel et al. 2018). Cucumber is thus one of the oldest vegetables grown by humans.

2.2. Nutritional Profile and Aroma

95% of the raw cucumber (with peel) is water, 4% is carbohydrate, 1% is protein, and very little fat is present. A reference serving 100 grams (3+1⁄2-ounces) has 65 kilojoules (16 kilocalories) of food energy. vitamin K It has a low micronutrient content, with vitamin K accounting for only 16%. Certain cucumber types have a subtle melon flavor and scent, which can be attributed in part to unsaturated aldehydes such as (E, Z)-nona-2,6-dienal and the cis- and trans-isomers of 2-nonenal. The slightly bitter flavor of cucumber rinds is caused by cucurbitacins (Schieberle et al. 1990; Shang et al. 2014).

2.3. Scientific Classification

Kingdom: Plantae
Clade: Tracheophytes
Clade: Angiosperms
Clade: Eudicots
Clade: Rosids
Order: Cucurbitales
Family: Cucurbitaceae
Genus: Cucumis
Species: Cucumis sativus
Binomial name: Cucumis sativus L.

Figure 1: Fruiting cucumber plants with several lateral branches.

2.4. Production

China is the world's leading producer of cucumbers, according to data from the Food and Agriculture Organization (FAO) of the United Nations, as of January 2022. China produces many agricultural products, and its varied environment makes it possible to grow cucumbers in many parts of the country. FAO offers statistical information on the output of agricultural products worldwide, including cucumbers (FAOSTAT" 18 March 2024). Although China is the world leader in cucumber production, the United States, Turkey, Iran, and Russia also make significant contributions to the world's cucumber supply. In many areas, year-round production of cucumbers is possible due to the cultivation of vegetables in both open fields and controlled systems such as greenhouses. However, production numbers can change from year to year depending on factors such as market demand, weather, and farming methods (Fig. 2a and b).

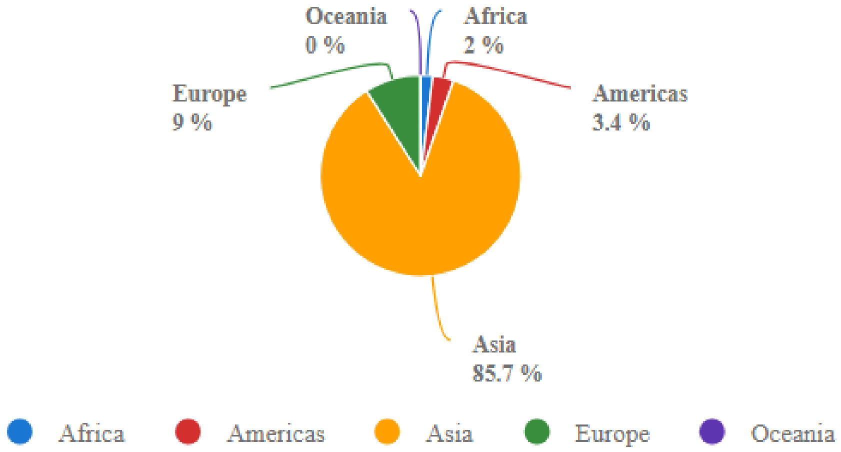

Figure 2a. Proportion of cucumber and gherkin production by region (1994-2022).

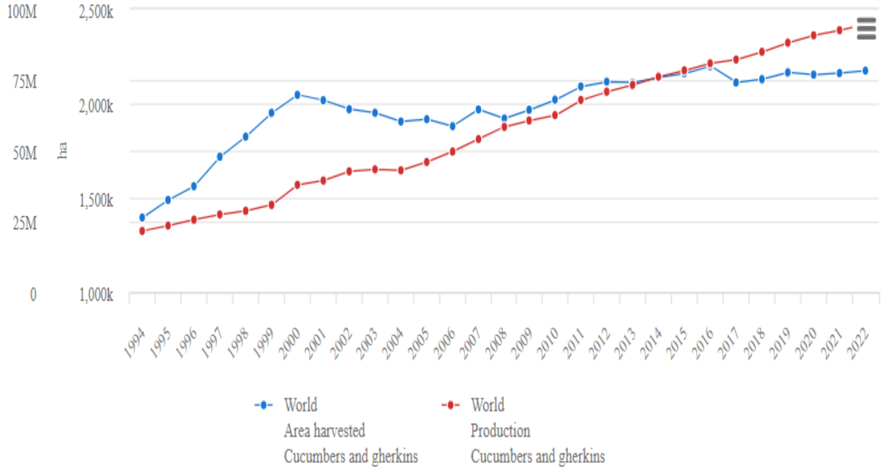

Figure 2b. Production/yield quantities of cucumber and gherkins worldwide (total), 1994-2022.

2.5. Genetics

Cucumis sativus L., or cucumber, is a major vegetable crop grown all over the world. Cucumber is the only species in the genus with 2n = 2x = 14 chromosomes out of about 66 species in the genus Cucumis. The remaining species have multiples of 12 chromosomes or 2n = 2x = 24 chromosomes, including its sister species, *Cucumis hystrix*. Except for cucumber chromosome 7, which remained mostly intact throughout the entire evolution of Cucumis, many chromosome rearrangement events (inversions, fusions, and translocations) were involved in the diploid chromosome reduction that allowed cucumber to evolve from its extinct 2n = 24 ancestor.

Cucumis sativus comprises four botanical varieties that are cross-compatible: the semiwild Xishuangbanna cucumber (*Cucumis sativus* var. *xishuangbannesis*), the Sikkim cucumber (*Cucumis sativus* var. *sikkimensis*), and the cultivated cucumber (*Cucumis sativus* var. *sativus)* (Weng et al. 2021).

2.6. Economic Importance of Cucumber

According to Golabadi et al. (2012), cucumbers are often grown in fields throughout the spring and summer months or in separate greenhouse seasons because they are thermophiles and susceptible to cold. One of the vegetable plants with significant economic value is cucumber, which is widely consumed by both raw and processed consumers (Innark et al. 2014). In Western Europe, the crop ranks second to tomatoes as the most important vegetable crop, but in Asia, it ranks fourth most important after tomatoes, cabbage, and onions (Eifedyi, 2010). The cucumber fruit can reach a maximum length of 62 centimeters and a maximum diameter of 10 centimeters (Zhang et al. 2019). Much like tomatoes and squash, cucumber is often consumed fresh (Staub et al. 2008).

2.7. Greenhouse Types

Compared with that in an open field culture, cucumber productivity in a covered culture can be four times greater (Singh et al. 2019). Numerous published studies have examined the impact of various nitrogen (N) application rates on greenhouse hydroponic production systems (Guler et al. 2006; Zhang et al. 2011). When mineral N fertilizer was used together with other N sources to increase greenhouse tomato yield, relatively little extra mineral N was applied, a high percentage of the total available N was recovered, and there was less risk of N loss to the environment (Gallardo et al. 2019).

Cucumber plants grown in greenhouses develop slowly initially but swiftly during the vegetative and reproductive phases, according to Singh et al. (2019). In addition, they generate roots into their growth substrate and absorb large amounts of water and nutrients. Irrigation has been shown to have a significant impact on the biomass and morphology of cucumbers growing in greenhouses (Wang et al. 2019). The cucumber fruits reach their maximum length and weight when nitrogen is applied more heavily. According to Arshad et al. (2014), fertigation with 150 mg N L^{-1} produced an enormous number of leaves, leaf area, and fresh and dry weight of the roots and shoots as well as an increase in fruit production of cucumbers.

2.8. Open Field Types

The productivity rate of cucumber cultivated in open fields is relatively low. According to Smitha and Sunil (2016), open field cucumbers are cultivated in warm, rainy seasons, with 20 to 30 degrees Celsius being the best temperature for growth and development (Dhakal et al. 2019). These fruits are frequently available in grocery shops and are also grown for the pickling industry. They require many nutrients, like those produced in greenhouses. If the soil exhibits indications of nutrient deficits, it will perform poorly in the field, leading to lower yields, deformity, and poor flavor (Muslat and Alrahman, 2018). Cucumber cultivation soils need moderate to high levels of nutrients to produce high yields; thus, standardized integrated nutrient management strategies are necessary for cucumber in open field situations to increase early yield, productivity, and quality (Hafeez et al. 2013). Several abiotic stress variables, such as low temperature, mechanical stress, and salinity, appear to reduce cucumber seedling performance in open-field environments (Demir and Mavi, 2008).

2.9. Waterlogging Stress

Waterlogging is a common abiotic stressor for plants. During waterlogging, the suppression of aerobic respiration reduces energy metabolism and a wide range of developmental processes, including seed germination, vegetative growth, and subsequent reproductive growth are affected. Plants achieve regular growth by absorbing water through their roots and transpiring through their leaves. Transport of water and minerals require energy. During waterlogging stress, root respiration is inhibited, and toxic chemicals accumulate, which has a negative impact on both vegetative and reproductive growth, eventually leading to yield loss or even harvest failure (Ashraf et al. 2012). Plants respond to waterlogging stress by modifying their morphological structure, energy metabolism, endogenous hormone production, and signalling pathways Figure 3, (Pan et al. 2021).

Unfavorable weather events increase due to global warming, which continues to be a threat to world agriculture. Droughts and floods are examples of extreme weather that compromises food safety by changing the availability of water (FAO, 2017). From 2006 to 2016, more than two-thirds of crop losses and damage were caused by floods alone, having a billion-dollar economic effect (Conforti et al., 2017). To maintain successful farming and encourage suitable climate change responses, it is essential to explore plant waterlogging tolerance and its mechanisms, since forecasts of greater and/or extended rainfall and frequent floods are being followed by global warming.

Changes in precipitation and flooding incidents brought on by global climate change are closely linked to climate change. Flooding stress puts the plant under low-oxygen conditions, influencing the phases of plant development throughout its life cycle. According to Zhou et al. (2020), plants control fluctuations in their energy metabolism, photosynthesis, respiration, endogenous phytohormone production, and architecture.

The rhizosphere experiences hypoxia upon flooding due to the submergence and rises of ground water level. According to Fukao et al. (2019), hypoxia in the rhizosphere produces anaerobic conditions that prevent oxygen absorption and ultimately result in plant death.

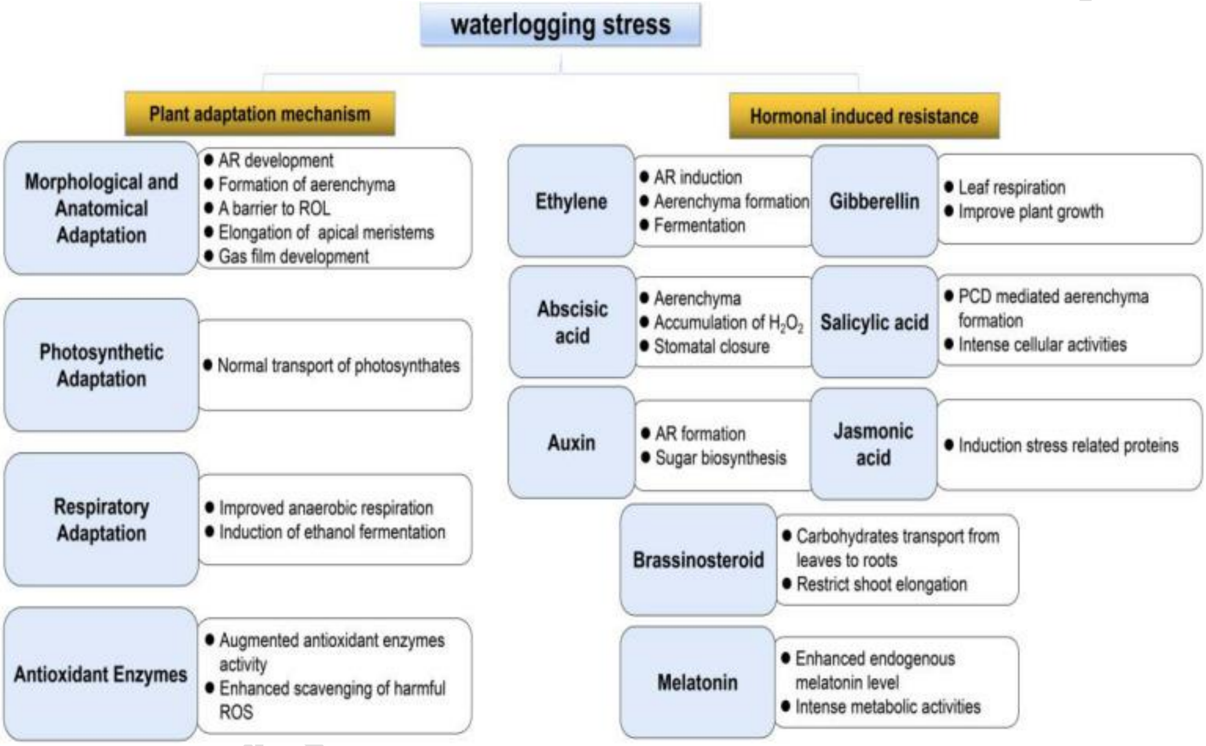

Figure 3: Plant responses to waterlogging stress, including hormonal effects (Pan et al. 2021).

2.10. Alterations in Gene Expression

In general, waterlogging alters plant's genes expression, which results in morphological and physiological changes. Studying grapevines under flooding resulted in transcriptional and metabolic reprogramming during and after waterlogging exposure as well as metabolic differentiations in root (Ruperti et al. 2019).

Under severe waterlogging conditions, the gene expression levels of *AOX1A* (Alternative Oxidase 1A), *CYP81D8* (Cytochrome P450 81D8), and putative PFP (Phosphofructokinase) genes were examined in commercial maize hybrids when morphological changes have been observed (Panozzo et al. 2019). In waterlogged hybrids, the transcript of the *AOX1A* gene, which encodes a protein associated with the mitochondrial respiratory electron transport chain, was markedly downregulated. However, the observed deficits in shoot and root growth were only partially correlated with the expression of these genes during severe waterlogging (Panozzo et al. 2019). CYP81D8 is a gene that encodes cytochrome P450, and its role in waterlogging stress tolerance has been documented. The expression of PFP, which is involved in glycolytic processes (Dwivedi, 2015), was significantly reduced by submergence in all the maize lines tested by Campbell et al. (2015), although in investigation of PFP transcript abundance was higher in maize hybrids more tolerant to heavy waterlogging (Panozzo et al. 2019).

Abscisic acid (ABA) is a very important hormone in plants. It governs plant growth and development and is involved in biotic and abiotic stress responses. Pyrabactin resistance 1-like (PYR/PYL) proteins play an important function in ABA signal transduction. Cucumber's six chromosomes had fourteen PYL genes, and their encoded proteins were projected to be distributed in the cytoplasm and nucleus. The qRT-PCR results revealed that most PYL gene expression levels were up-regulated in abiotic stresses (including NaCl, PEG treatments). (Zhang et al. 2022). Furthermore, orthologous genes in other crops have been reported, such as 13 PYLs in rice, 13 PYLs in maize, 27 PYLs in cotton, 14 PYLs in tomato, and 8 PYLs in grape (Yadav et al. 2020). PYLs may be essential components of abiotic stress signaling pathways because they are ABA receptors.The PYL family of receptors, which is the biggest family of plant hormone receptors, perceives ABA. The ABA signaling pathway, which is negatively regulated by type 2C protein phosphatase (PP2C), is crucial for the transduction of stress signals in plants. The results obtained indicate that different expression patterns for the CsPP2C family genes were seen in response to ABA, drought, salt, and cold treatment (Zhang et al. 2022).

Peptide-signaling networks like CLE (CLAVATA3/Embryo surrounding region-related) are essential for many biological processes in plants, including cell-to-cell communication, cellular proliferation and differentiation, cellular determination of self-incompatibility, and defensive responses. Chinese long "9930" cucumbers were found to contain 26 distinct CLE genes. These cucumbers were shown to be closely related to melon and Arabidopsis, with melon and Arabidopsis having 15 and seven orthologous CLE genes, respectively (Qin et al. 2021). The short peptides encoded by the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION-RELATED (CLE) gene family are involved in vascular patterning, environmental reactions, and plant meristem maintenance. The morphology of the root tips was examined in relation to the impact of SlCLEs on tomato roots. Not only was there a decrease in root length but also in root diameter and columella length in three treatments (SlCLE15, SlCLE19, and SlCLE24) (Carbonnel et al. 2022).

Chalcone synthase (CHS) is well-known as the gatekeeper of the anthocyanin pathway (Dao et al., 2011). Furthermore, it was discovered that the *CsCHS* genes exhibited distinct expression patterns in response to hormonal treatments and salinity stress. *CsCHS* genes are essential for plant defense against insect pests, as revealed by the qRT-PCR study of these genes during an aphid infestation. Only *CsCHS3* showed high expression in response to naphthalene acetic acid (NAA) and ethylene (ETH) under conditions of waterlogging stress. Additionally, it was found that *CsERF1* and *CsERF3* may interact with *CsCHS2* to control how the cucumber's immune system reacts to certain stresses. Differential expressions in response to heat and salinity were also noted, highlighting the critical function of *CsCHS* genes. Aphid feeding increased CsCHS mRNA levels and might have triggered downstream gene transcription to further customize cucumber response. Furthermore, it was found that *CsERF1* and *CsERF3* may interact with *CsCHS2* to control how the cucumber's immune system reacts to certain stressors. Therefore, *CsCHS2* may be essential for producing cucumber lines that can withstand a range of environmental challenges. (Ahmad et al. 2023).

Waterlogging stress first affects plant roots, and it is well established that aerenchyma formation is essential for long-distance oxygen supply from shoots to roots in waterlogged soil (Xu et al. 2023). The study of the cross-sectional area of root tissues in three crop species (wheat, maize, and rice) under aerated and deoxygenated conditions revealed that a high cortex-to-stele ratio paired with a wide root diameter increases oxygen transmission from shoot to root tips (Yamauchi et al. 2019). The cortex-to-stele ratio (CSR) and aerenchyma-to-cortex ratio (ACR), which are associated with gas spaces, were much greater in rice roots than in wheat and maize roots, showing that these structural characteristics are essential for a high capacity for oxygen transport along roots.

Flooding has several effects, including reducing nitrogen turnover in plants; increasing the formation of ethylene, abscisic acid, and ethylene precursors; stimulating partial stomatal closure, epinasty, and abscission of leaves; partially blocking enzymes; degrading cellular membrane systems; and disassembling mitochondria and microbodies (Pooja et al. 2020). Flooding can reduce nitrogen uptake in legume crops by limiting symbiotic N_2 fixation (Pucciariello et al. 2019). The mechanisms underlying symbiotic processes and their functions during waterlogging have been investigated, with a focus on the oxygen-sensing systems of the host plant and its symbiotic partner (Pucciariello et al. 2019).

The evaluations of ethylene synthesis, alcohol dehydrogenase (ADH) activity, pyruvate decarboxylase (PDC) activity, and ethanol generation all matched the proteomic findings (Xu et al. 2016).

2.11. Changes Caused by Waterlogging in Physiological Mechanisms

The primary response of plants to waterlogging is to reduce stomatal conductance (Dat et al. 2006). Plants stressed by floods exhibit increased resistance to water absorption as lack of energy and changing pH impairs water transport and leads to internal water deficit (Parent et al. 2008). Furthermore, lower root permeability caused by low O_2 levels may diminish hydraulic conductivity (Else et al. 2001). According to Ashraf et al. (2011), when there is a lack of oxygen, the net photosynthetic rate often decreases dramatically.

Decreases in transpiration and photosynthesis are caused by stomatal closure (Ashraf and Arfan, 2005). Low photosynthetic rates are also related to other factors, such as decreased chlorophyll levels, leaf senescence, and decreased leaf area (Malik et al. 2001). Yordanova et al. (2005) reported that the stomata of barley plants quickly close in response to flooding conditions. Similarly, pea plants exhibit rapid stomatal closure when exposed to flooding conditions (Zang and Zang, 1994).

2.12. Oxidative Damage Caused by Reactive Oxygen Species

Although oxygen is required for most life forms on Earth. Reduction in oxygen supply may result in the production of reactive oxygen species (ROS), which could affect the metabolic processes of a plants at the cellular level (Ashraf, 2009; Ashraf et al. 2010). Reactive oxygen species include superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). ROS include singlet oxygen, which is formed when oxygen interacts with excited chlorophyll (Ashraf and Akram, 2009). These reactive oxygen species (ROS) harm a variety of biological components and metabolites, including proteins, lipids, pigments, and DNA (Ashraf, 2009).

Reactive oxygen species are produced by various cellular organelles, including mitochondria, chloroplasts, and peroxisomes. Their unstable structure leads to interaction with other biological components, producing more free radicals and/or oxidized products (Foyer and Halliwell, 1976; Hideg, 1997).

2.13. Antioxidant Defense Mechanism in Waterlogged Conditions

Plants can ameliorate the detrimental effects of reactive oxygen species (ROS) by producing a variety of antioxidants. Antioxidants are categorized as enzymatic or nonenzymatic. Enzymatic antioxidants include ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), whereas nonenzymatic antioxidants include ascorbic acid, glutathione, tocopherols, and carotenoids (Moussa et al. 2019).

For example, when mung bean plants were subjected to waterlogging stress, the activities of many antioxidant enzymes, including glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), were dramatically reduced (Ahmed et al. 2002). Moreover, it has been claimed that oxidative damage was not the cause of the degradation of the photosynthetic machinery in plants when they were exposed to water. Although antioxidant enzymes are essential for reducing oxidative stress, other elements might also play a part in a plant's overall ability to withstand harsh environments. Similarly, waterlogging has been shown to lower the activity of SOD, some oxygen-processing enzyme in corn (Yan et al. 1996).

In contrast, when maize seedlings were exposed to varying degrees of waterlogging stress, the activities of several antioxidant enzymes increased (Bin et al. 2010). Pigeon pea genotypes subjected to waterlogging stress showed considerable increases in superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activity (Kumutha et al. 2009).

2.14. Photosynthesis and Energy Metabolism in Plants during Waterlogging

Carbon dioxide and light are required for photosynthesis, which provides photoautotrophic plants with energy and carbon for growth and development. To withstand submersion, certain plant species generate a gas coating on their hydrophobic cuticles. This gas film facilitates gas exchange during floods, increasing the rate of photosynthesis (Colmer and Pedersen, 2008). Plant waterlogging stress is mediated through phytohormone pathways. Plant hormones regulate seed germination and abiotic stress responses (Shu et al. 2016). According to Grichko and Glick (2001); Hattori et al. (2009), and other researchers, ethylene is one of the most important phytohormones for plant responses to waterlogging.

2.15. Transcriptional Activation of Genes in WL stress

This molecular reaction to waterlogging stress is regulated by members of the ethylene responsive transcription factor (ERF) family, notably Group VII. The involvement of five *Arabidopsis thaliana* ERF-VII transcription factors in the anaerobic response hierarchy was confirmed (Bui et al. 2015). Based on a set of hypoxia-responsive promoters, the transcription factors *RAP2.2*, *RAP2.3*, and *RAP2.12* were found to be the most effective activators. In transactivation tests using Arabidopsis protoplasts, *RAP2.12* was further dissected to identify the regions responsible for transcriptional activation. A single C-terminal motif is sufficient to stimulate gene transcription. Finally, they validated that *RAP2.2* and *RAP2.12* have a significant influence on the anaerobic response using real-time RT–PCR in single and double mutants for the corresponding genes (Bui et al. 2015). Group VII ET-response factors (ERF-VIIs) strongly influence responses to waterlogging and ethylene response signal transduction (Gasch et al. 2016). *ZmEREB180*, a member of the maize ERF-VII gene family, positively regulates ROS levels as well as the generation and growth of ARs; overexpression of *ZmEREB180* in maize also increases the proportion of plants that withstand long-term waterlogging stress (Yu et al. 2019).

2.16. Plant Cysteine Oxidases

Cysteine (Cys) oxidation is an important posttranslational modification (PTM) that influences protein half-life and function (Chung et al. 2013). According to research, plant cysteine oxidation can facilitate ROS-mediated hormone signaling. This process is associated with root growth, pollen tube elongation, and a range of abiotic stress responses (Romero et al. 2014). Plant cysteine oxidases (PCOs) catalyze the conversion of N-terminal cysteine residues to sulfinic acid, that may be followed by subsequent Nt-arginylation facilitating proteasomal degradation of the protein (Figure 4) (Heo et al. 2021). This may happen on target proteins such as ERF VII TFs, as well as on other substrates eg. ZPR2 (protein LITTLE ZIPPER 2); or VRN2 (VERNALIZATION 2) (Gibbs et al. 2018; Weits et al. 2019), (White et al. 2017; Weits et al. 2014). Moreover, ERF-VIIs are known to govern hypoxia-regulated transcriptional reprogramming to adapt to environmental changes (Licausi et al. 2010; Hattori et al. 2009; Hinz et al. 2010).

PCO can function as a plant O_2 sensor, playing an important role in controlling ERF-VII stability since it is sensitive to physiologically relevant fluctuations in O_2 availability (White et al. 2018). Plant cysteine oxidases (PCOs) are enzymes that act as direct links between environmental signals and their molecular biological consequences (Taylor et al. 2022). In Arabidopsis, AtPCOs catalyze a process that results in cotranslational methionine cleavage, exposing the N-terminal Cys to oxidation at the N-terminus of target proteins. This process transforms cysteine into Cys-sulfinic acid (White et al. 2017; Weits et al. 2014; White et al. 2018). Disrupting the N-degron pathway of ERF-VIIs in barley alters seed germination and increases yield under waterlogging stress because PCOs regulate ERF-VIIs, and ERF-VIIs activate the anaerobic gene expression of alcohol dehydrogenase (ADH), pyruvate decarboxylase (*PDC1*), and hypoxia responsive attenuator 1 (*HRA1*). Thus, characterizing PCO function is crucial for understanding hypoxic responses in plant stress tolerance and development (Taylor et al. 2022).

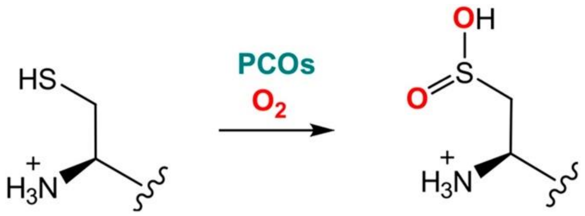

Figure 4: Plant cysteine oxidases catalyze the dioxygenation of conserved cysteine residues found at the N terminus of group VII ethylene response factors. Cys-sulfinic acid is formed by incorporating both molecular O_2 (red) atoms into the product.

2.17. Cysteine Oxidase’s Role in Adaptive Flood Strategies

All aerobic organisms require oxygen to survive. O_2 is the terminal electron acceptor in oxidative phosphorylation, allowing for efficient ATP synthesis and glucose consumption. If there is inadequate O_2, there is a decrease in ATP generation, resulting in an energy crisis, as well as increased mitochondrial reactive oxygen species (ROS) generation, which can cause cell damage and death. Plants and animals can detect hypoxia and respond by reducing demand or increasing the supply of oxygen (Hammarlund et al. 2020; Holdsworth and Gibbs, 2020).

Plants experience acute hypoxia during flood-induced submergence due to lower O_2 diffusion through water relative to air (Bailey-Serres et al. 2012; Holdsworth and Gibbs, 2020). Adaptive physiological traits include 'escape' strategies triggered by ethylene accumulation, such as elongation of petioles and root aeration via aerenchyma formation (Loreti et al. 2016; Voesenek and Bailey-Serres, 2015), and energy-saving 'quiescence', whereby a metabolic switch to starch catabolism and anaerobic metabolism maintains sufficient ATP production for survival while growth is arrested (Bailey-Serres et al. 2012).

Transcription factors belonging to the Group VII ethylene response factor (ERF-VII) family are responsible for several physiological changes. *RAP2.12* may be bound to the cell membrane under non stressful conditions while in hypoxic environments, *RAP2.12* release is initiated by a reduction in ATP availability (Licausi et al. 2011; Schmidt et al. 2018). As a result, *RAP2.12* can translocate to the nucleus, and a core group of hypoxia response genes, of which many are involved in anaerobic metabolism, can be expressed (Lee et al. 2011; Mustroph et al. 2009).

Like the adaptive response to hypoxia in animals, which is initiated by (a decrease in) enzyme-catalyzed hydroxylation of prolyl residues via hypoxia-inducible factor (HIF), the adaptive response to hypoxia in plants is mediated by PCO-catalyzed ERF-VII oxidation (Figure 5). Human prolyl hydroxylases have also been extensively studied as O_2 sensors (Ehrismann et al. 2007; Myllyharju, 2013; Tarhonskaya et al. 2014).

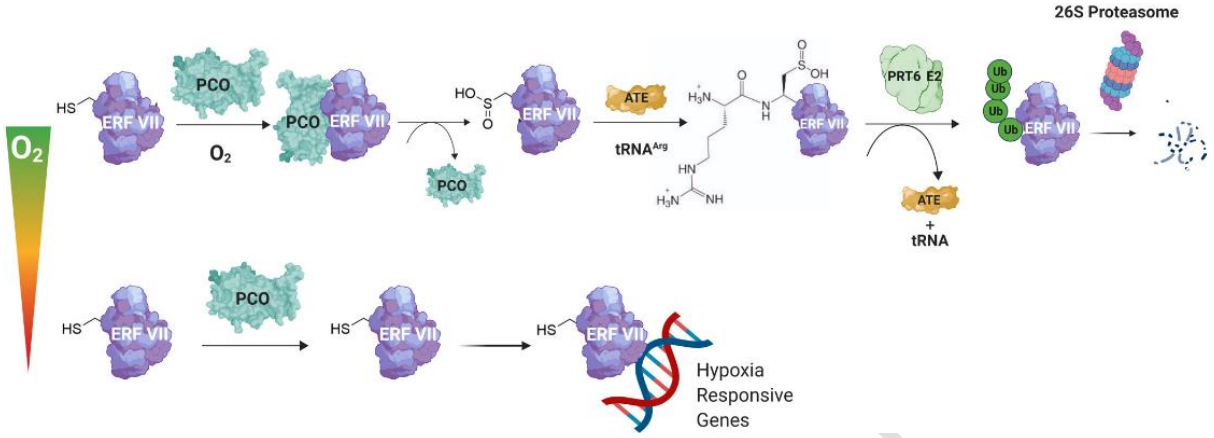

Fig 5. PCO-mediated regulation of the stability of ERF-VII transcription factors via the N-degron pathway. At normal O_2 concentrations (top), PCO enzymes catalyze the oxidation of ERF-VII N-terminal cysteine residues to cysteine-sulfinic acid; subsequent ATE-catalyzed arginylation renders the ERF-VIIs substrates for the ubiquitin ligase PRT6, resulting in proteasomal degradation. Under hypoxia (bottom), e.g., upon submergence, insufficient O_2 prevents PCO activity, rendering ERF-VIIs stable to initiate the expression of genes that help plants adapt to hypoxic conditions. PCO, plant cysteine oxidase (PDB ID: 6S7E; White *et al*., 2020); ATE, arginyl transferase; PRT6 (PDB ID: 6LHN; Kim *et al*., 2020); Ub, ubiquitin.

3. MATERIALS & METHODS

3.1. Plant Materials and Growth Conditions

This study investigated and employed four open-field-grown cucumber F1 cultivars ('Joker', 'Dirigent', 'Harmony', and 'Promissa') as well as four hybrids bred primarily for greenhouses ('Oitol', 'Forami', 'Diapason', and 'Grafito'). The cucumber seeds were purchased from Royal Sluis Magrovet Kft (Kecskemét, Hungary), except for the 'Oitol' hybrid, which was purchased from Semillas Fito Co. (Barcelona, Spain).

Cucumber seeds were soaked for twenty-four hours at 25 °C in 100 milliliters of distilled water before starting the experiment. The experiment took place in a growth chamber (FitoClima 600, Rio de Mouro, Portugal) using a semi-hydroponic technique that resembles commercial production practices using soilless media. The seeds were planted one by one in 10-cm-diameter pots filled with perlite. The plants were grown at 26 ± 1 °C with a 16-hour photoperiod, 160–180 $\mu mol\ m^{-2}\ s^{-1}$ light intensity at the culture level, and 75–80% relative humidity. The plants were watered with distilled water for the first week. Each pot was then treated with 150 ml of nutrient solution every other day EC: 1.25 µS/cm) for 14 days (Table 1).

Table 1. Composition of the applied fertigation solutions.

KNO_3	**4 mM**
$MgSO_4$	0.5 mM
NaFe-EDTA	5 µM
KH_2PO_4	2 mM
H_3BO_3	5 µM
Na_2MoO_4	0.1 µM
$ZnSO_4$	0.5µM
$MnCl_2$	1 µM
$CuSO_4$	0.25 µM
$CoCl_2$	0.2 µM
$NiSO_4$	0.1µM
$CaSO_4$	1.5 mM
MES	0.5 Mm

The adventitious roots above the perlite level were counted at one-week intervals. Waterlogging treatment was applied to 'Joker' and 'Oitol' plants, which represent the two principal cucumber types: greenhouse and open field types. The plants were cultivated as described above until the second leaf was fully extended (approximately two weeks after planting). Waterlogging treatment was performed by immersing the pots in larger containers loaded with full strength nutritional solution. The surface of the nutrient solution was kept at the top layer of the perlite. The solution's O_2 concentration (DOI) was measured using an oxygen meter (Voltcraft DO-101, Conrad Electric SE, Germany).

The nutrient solution was replaced once after 7 days of treatment with a new solution and was set to the same DOI as the solution to be replaced. Every other day, the plants in both groups received 150 ml of the same nutritional solution. After 14 days of waterlogging, leaf samples were taken and photographed, and the total leaf area per plant was estimated using ImageJ software. The number of ARs above the perlite level was counted, and an adequate number of leaf and root samples were frozen in liquid nitrogen for RNA extraction and physiological analyses. The fresh weight (total foliar mass) of each intact plant was determined using a laboratory scale, and the leaves of individual plants were detached from the stem, placed in paper bags, and dried in an oven at 70 °C for 72 hours to determine the dry leaf weight.

3.2. Alcohol Dehydrogenase (ADH) Activity

To determine ADH enzyme activity a spectrophotometry assay was used according to the method described by Kang et al. (2009) by measuring NADH oxidation at 340 nm. Root samples (100 mg) were homogenized in a mortar with 500 µl of extraction buffer (50 mM Tris-HCl (pH 6.8), 5 mM $MgCl_2$, 5 mM mercaptoethanol, 15% (v/v) glycerol, 1 mM EDTA, and 0.1 mM PMSF). To measure ADH activity, a 1-ml reaction mixture comprising 50 mM TES (pH 7.5), 0.17 mM NADH, 0.2% (v/v) acetaldehyde, and 100 µl of enzyme extract was used. The enzyme activity was measured using the molar extinction coefficient for NADH (6.22 mM^{-1} cm^{-1}) and represented as µmol NADH min^{-1} g FW^{-1}.

3.3. Malondialdehyde (MDA) Level and Guaiacol Peroxidase (POD) Enzyme Activity

The MDA level of the plants was determined using the thiobarbituric acid (TBA) method of Hodges et al. (1999), with minor modifications. MDA was extracted using 0.1% (w/v) TCA. The reaction of 0.5% (w/v) TBA in 20% (w/v) TCA was carried out at 100 °C for 30 minutes. The samples were cooled on ice before being tested for absorbance at 532, 600, and 440 nm. The results were estimated with a molar absorption coefficient of 1.55 mM^{-1} cm^{-1} and are reported as MDA nmol g FW^{-1}.

The activity of the guaiacol peroxidase enzyme was determined using the method described by Jócsák et al. (2020), with minor modifications. Plant leaf samples (100 mg) were homogenized in 1 ml of isolation solution (10 mM phosphate buffer, pH 7.2, 1 mM EDTA, 2 mM DTT). The crude extract (20 µl) was diluted with 136 µl of distilled water in the wells of a 96-well plate. The reaction was started by adding 200 µl of substrate solution (150 µl of 0.1 M phosphate (pH 6.0), 14 µl of 0.015 M H_2O_2, and 36 µl of 0.02 M guaiacol). The absorbance was measured at 470 nm every 60 seconds for 3 minutes. The data were reported in mol tetraguaiacol min^{-1} g FW-1 using the molar absorption coefficient of tetraguaiacol (26.6 mM^{-1} cm^{-1}).
These assays provide valuable information regarding oxidative stress and antioxidant defense mechanisms in plants, contributing to our understanding of plant responses to environmental stresses and potential strategies for enhancing stress tolerance.

3.4. RNA isolation, cDNA synthesis, and qRT–PCR

Total RNA was isolated from deep frozen root samples of control and waterlogged 'Joker' and 'Oitol' plants (taken from three different plants in different pots) after grinding in liquid N_2 with a sterile mortar and pestle. A modified CTAB-based procedure (Jaakola et al. 2001) was utilized to produce intact RNAs, which were then visualized on a 1% agarose gel stained with ethidium bromide. The RNA concentration was standardized with a NanoDrop 1000 spectrophotometer.

The integrity of the RNA was checked again on an agarose gel after DNase I (Thermo Scientific, Waltham, MA, USA) treatment and before reverse transcription. To synthesize first-strand cDNA, 5 µg of total RNA was utilized in a 100 µl reaction volume with the Maxima H Minus Reverse Transcriptase kit (Thermo Scientific) and oligo(dT)20 primers following the manufacturer's instructions. The cysteine oxidase gene primers and a control *Actin* gene (Table 2) were evaluated for RT-PCR amplification using GO Taq G2 DNA polymerase (Promega, Madison, WI, USA). The PCR products were subjected to gel electrophoresis in a T100™ Thermal Cycler (Bio-Rad, Germany) after 3 min at 95 °C and 26 cycles of 30 s at 95 °C, 20 s at 58 °C, 20 s at 72 °C, and a final extension for 7 min at 72 °C.

Real-time PCR was carried out in a CFX 96 Real-Time PCR System (Bio-Rad, USA) using the SsoAdvanced Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad) for fluorescence detection in a 96-well optical plate. Each PCR reaction had a total volume of 10 µL, including 1 µL of 10 times diluted cDNA, 4 µL of super mix, 0.5 µL (100 µM) of forward and reverse primers, and 4 µL of PCR-grade water. Amplification began with polymerase activation and DNA denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95

°C for 10 s, annealing, and extension at 60 °C for 30 seconds. Melting curve analysis (65-95 °C) was used to confirm the specificity of the PCR products. The PCR efficiency and stability of the internal standard gene (*Actin*) were examined and confirmed in accordance with Oszlányi et al., 2020. Fold changes in relative gene expression were assessed using the Bio-Rad CFX Maestro software's built-in $2^{-\Delta\Delta Ct}$ technique.

Table 2. List the target genes, oligonucleotide primers for qRT–PCR, and predicted amplicon sizes (bp).

Gene	**Accession No**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**bp** cDNA	**bp** gDNA
CysOx 1	CsaV3_6G015320	GCAAAGCTGCATTGTCTCCT	GGAATGATTGTCGTCTCCCA	171	1926
CysOx 2	CsaV3_3G041620	CCTTCAGGTGTCATTCCAC	GCTGTGAAGTCTGCATCTAC	206	837
CysOx 3	CsaV3_4G012050	GCTGTCTTCACTTCACCC	CATCCATAACCCTCACCC	227	855
CysOx 4	CsaV3_1G003120	GGTTGGCTAAGTTAGCCG	GCAGGACCAACATACATTCC	277	384
ACTIN	CsaV3_2G01809	TCGTGCTTGACTCTGGTGATGG	ACAACCACTGCCGAACGGGAAA	171	171

3.5. Statistical Analysis

All the data were statistically evaluated using SPSS software (IBM SPSS Statistics Version 27.0, IBM Corp., Armonk, NY, USA), and the results are presented as the mean values with standard deviations for at least two biological replicates. Before conducting further analyses, the normality of the data distribution was assessed using Shapiro–Wilk's test. This test is commonly employed to determine whether a dataset follows a normal distribution. Following the confirmation of normality, the homogeneity of variances was evaluated using Levene's test. This test assesses whether the variance among different groups is similar, which is an assumption underlying many statistical analyses.

To investigate the mean differences among various experimental groups, one-way analysis of variance (ANOVA) was performed. ANOVA is suitable for comparing means across multiple groups simultaneously. Subsequently, Tukey's post hoc honestly significant difference (HSD) test was employed to identify specific group differences while controlling for the familywise error rate. The significance level for all analyses was set at $p < 0.05$, indicating that the observed differences were considered statistically significant if the probability of their occurrence by chance alone was less than 5%.

Furthermore, Microsoft Excel 2017 was utilized for graphical representation of the data. Excel is widely used for creating charts and graphs to visually depict trends and patterns in the data, providing a clear and intuitive representation of the results for better interpretation and communication.

4. RESULTS

4.1. Adventitious Roots (ARs) of Various F1 Hybrids under Normoxia

Cucumber cultivars could be classified based on adventitious root formation under normal growth conditions. ARs were more common in hybrids produced for greenhouse cultivations. On average, plants with 2-3 leaves produced seven to fifteen ARs (Table 3). The number of ARs of the open field cucumber types were markedly less when compared to their counterparts. For example, there was no occurrence of AR in the 'Dirigent', 'Promissa', or 'Joker' plants after three weeks of growth in perlite (Table 4).

These observations suggest that genetic factors may influence the development of adventitious roots in cucumber varieties. The greater prevalence of ARs in greenhouse-grown hybrids than in open field types implies that breeding predisposed hybrids for relevant cultivation practices. In greenhouse cultivation environmental conditions, such as hypoxic root conditions might be alleviated by frequent AR development.

Table 3. shows the number of adventitious roots in four cucumber hybrids grown in greenhouses and four in open fields under controlled conditions.

Cultivar		Number of adventitious roots
Greenhouse type hybrids	Oitol	9.13 ± 7.0 bc
	Diapason	8.44 ± 4.0 bc
	Grafito	14.13 ± 8.7 c
	Forami	7.75 ± 3.4 b
Open field type hybrids	Harmony	1.88 ± 1.7 a
	Dirigent	0.0 a
	Promissa	0.0 a
	Joker	0.0 a

Different letters indicate significantly different values (Tukey's, $p < 0.05$). *n*=8

4.2. AR counts of 'Joker' and 'Oitol' plants during WL treatments

The production of adventitious roots was measured after the first and second weeks of flooding the roots of WL-treated plants (Table 4). 'Joker' and 'Oitol' plants show AR development in both the control and WL environments. In the first week after the WL treatment, the 'Oitol' plants exhibited more intensive AR growth than did the similarly treated 'Joker' plants. ARs increased 20-fold on the stems of control 'Oitol' plants and 8-fold on stressed 'Oitol' plants. AR formation began on the 'Joker' plants during the second week of flooding (Table 4). At the end of the experiment, the amount of ARs in the control and waterlogged 'Oitol' plant stems was three times greater than that in the Joker plant stems, which was also a significant increase. These observations indicate that compared with 'Joker' plants, 'Oitol' plants exhibited a more pronounced response in terms of AR growth under both control and waterlogged conditions. Additionally, compared with 'Joker' plants, 'Oitol' plants showed earlier initiation of AR formation and maintained greater ARs throughout the trial period. This suggests that 'Oitol' plants may possess enhanced adaptability or tolerance to flooding stress, as reflected in their vigorous adventitious root growth.

Table 4. Number of adventitious roots of two cucumber hybrid lines under control and waterlogged conditions.

Cultivar	Treatment	Number of adventitious roots	
		1 week after WL	2 weeks after WL
Oitol	Control	17.3 ± 4.5 b	40.6 ± 3.2 c
	Waterlogged	31 ± 13.5 c	60.1 ± 5.8 d
Joker	Control	0.86 ± 0.9 a	12.4 ± 2.3 a
	Waterlogged	3.8 ± 2.9 a	19.5 ± 5.2 b

Different letters in each column indicate significantly different values according to Tukey's post hoc test ($p < 0.05$). n=8

4.3. Growth responses and redox balance factors in 'Joker' and 'Oitol' plants under WL stress

'Oitol' and 'Joker' plants were treated with WL at the two-true leaf stage, ensuring that the nutrient solution reached the whole root zone. Every other day, the amount of dissolved oxygen (O_2) in the WL solution was measured to monitor the progress of hypoxia. The dissolved oxygen level decreased to approximately 2 mg/L by the end of the two-week treatment (Figure 6B).

Under both control and waterlogging conditions, the total leaf area and shoot fresh weight of the greenhouse-type 'Oitol' plants were considerably ($p<0.05$) greater than those of the open field 'Joker' plants. The overall leaf area and shoot fresh weight increased considerably in WL-treated 'Oitol'. Plants in both groups grew by more than 30% (Figure 1A, C-D). Leaf dry weight increased by 24.5% in treated 'Oitol' plants (Figure 1E). Under waterlogging conditions, 'Joker' plants showed no significant phenotypic differences from the controls (Figure 6A, C-E).

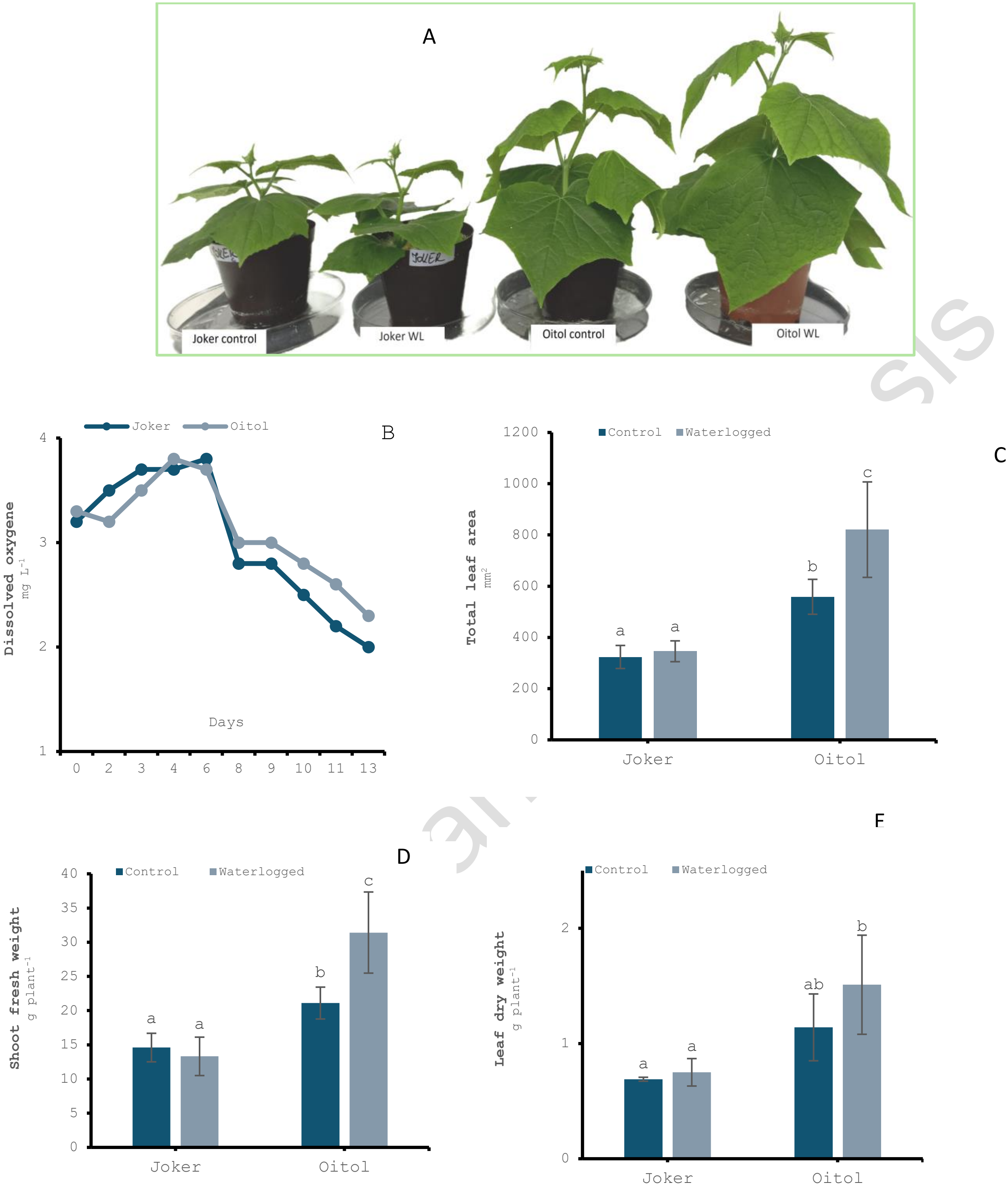

Figure 6. The morphological responses of the 'Joker' and 'Oitol' cucumber cultivars to waterlogging and control treatments after 14 days (A). Changes in dissolved oxygen level (B), total leaf area (C), shoot fresh weight (D), and shoot dry weight (E) for the 'Joker' and 'Oitol' cucumber hybrid lines under control and waterlogged conditions. Tukey's test ($p < 0.05$) revealed significant differences in values between letters. The data are presented as the means ± SDs (n = 6-10). WL: Waterlogged.

Increased malondialdehyde (MDA) levels are among the most prominent indicators of free radical-induced lipid peroxidation and membrane damage. In response to waterlogging, the MDA levels in the 'Joker' and 'Oitol' leaves declined dramatically compared to those in the control plants (Figure 7A). Joker plants had significantly increased guaiacol peroxidase (POD) activity, although waterlogging had no statistically significant effect on POD activity between control and waterlogged plants of both hybrids (Figure 7B).

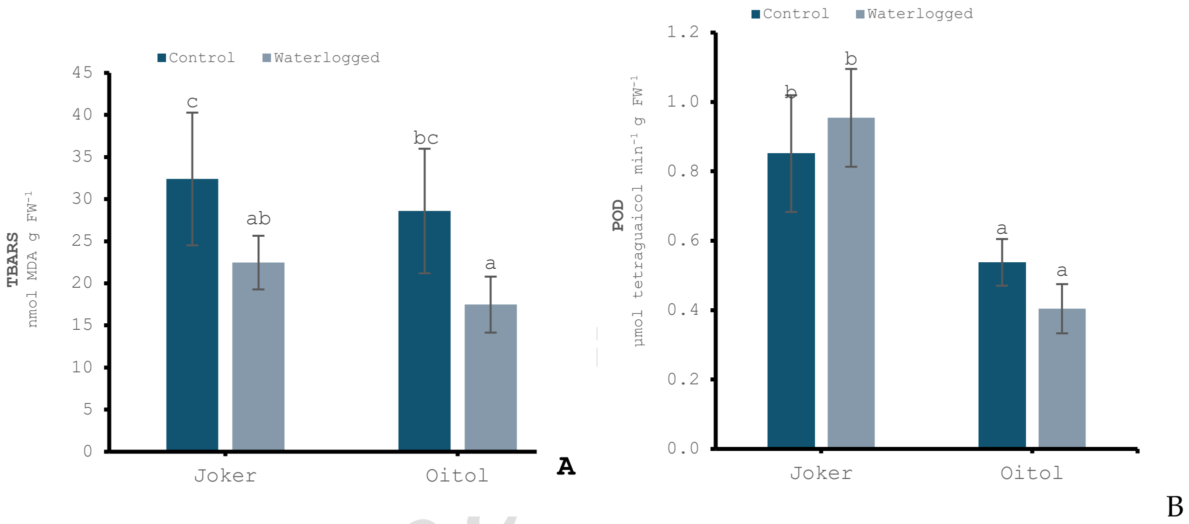

Figure 7. The malondialdehyde content (A) and guaiacol peroxidase enzyme activity (B) of cucumber leaves from the 'Joker' and 'Oitol' plants after waterlogging. The values are averages ± SDs, with various letters above the bars indicating significant differences between treatments (Tukey's test, $p < 0.05$). n = 6

4.4. Alcohol dehydrogenase activity in roots of ‘Joker’ and ‘Oitol’ plants under WL treatments

The activity of alcohol dehydrogenase (ADH) enzymes enables glycolysis to continue for a longer period of time under hypoxia. ADH activity was determined in the root samples of ‘Joker’ and ‘Oitol’ plants under WL stress. Due to waterlogging, the activity of ADH increased significantly ($p < 0.05$) in the roots of both hybrids (‘Joker’ and ‘Oitol’), as shown in Figure 3. ADH activity was greater (12.59 µmol min^{-}1 FW^{-1}) in 'Joker' than in the nonstressed control (0.85 µmol min^{-}1 g FW^{-1}). The ‘Oitol’ plants showed significantly lower ADH activity than did the ‘Joker’ plants under both the control and WL conditions (Figure 8).

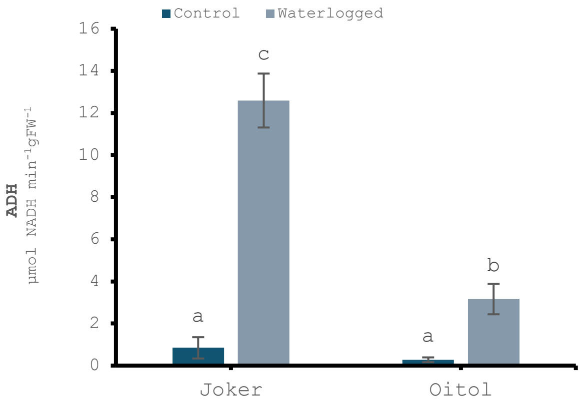

Figure 8. Activity of alcohol dehydrogenases in roots of the ‘Joker’ and ‘Oitol’ hybrids at the end of the waterlogging treatment. The data shown are the means ± SDs. The different letters indicate significant differences between the control and waterlogged plants (Tukey’s test, $p < 0.05$). $n=6$

4.5. Expression of cysteine oxidase genes (PCOs) in cucumber roots under WL treatment

Figure 9. shows the relative gene expression analysis of the four cysteine oxidase genes (PCOs) identified in the cucumber genome. The product specificity of the amplified fragments was validated by melting curve analysis using real-time PCR. Waterlogging clearly increased the expression of all cucumber PCOs in 'Joker' plant roots (Figure 9). When comparing the mRNA levels in the waterlogged 'Joker' plants to those in the nonstresses samples, *CysOx1* and *CysOx4* exhibited the greatest increases, with increases of approximately 17- and 5-fold, respectively. The expression of *CysOx1* and *CysOx4* in the roots of 'Oitol' plants did not change; however, *CysOx2* and *CysOx3* expression increased somewhat (less than twofold) in both hybrids (Figure 9). The results indicate that waterlogging-derived hypoxia induces cysteine oxidase gene expression in cucumbers to varying degrees.

Real-time PCR was used to analyze the relative gene expression levels of four cysteine oxidase genes (PCOs) annotated in the cucumber genome. The specificity of the amplified fragments was confirmed through melting curve analysis. Plants of two cucumber hybrids, 'Joker' and 'Oitol', were subjected to waterlogging stress, and root samples were collected for gene expression analysis. These results indicate the differential response of cucumber PCOs to waterlogging-induced hypoxia. The significant upregulation of *CysOx1* and *CysOx4* in 'Joker' plants suggested their crucial role in responding to hypoxic stress. The contrasting response in 'Oitol' plants suggests possible genotype-specific differences in the regulation of PCOs under waterlogging conditions.

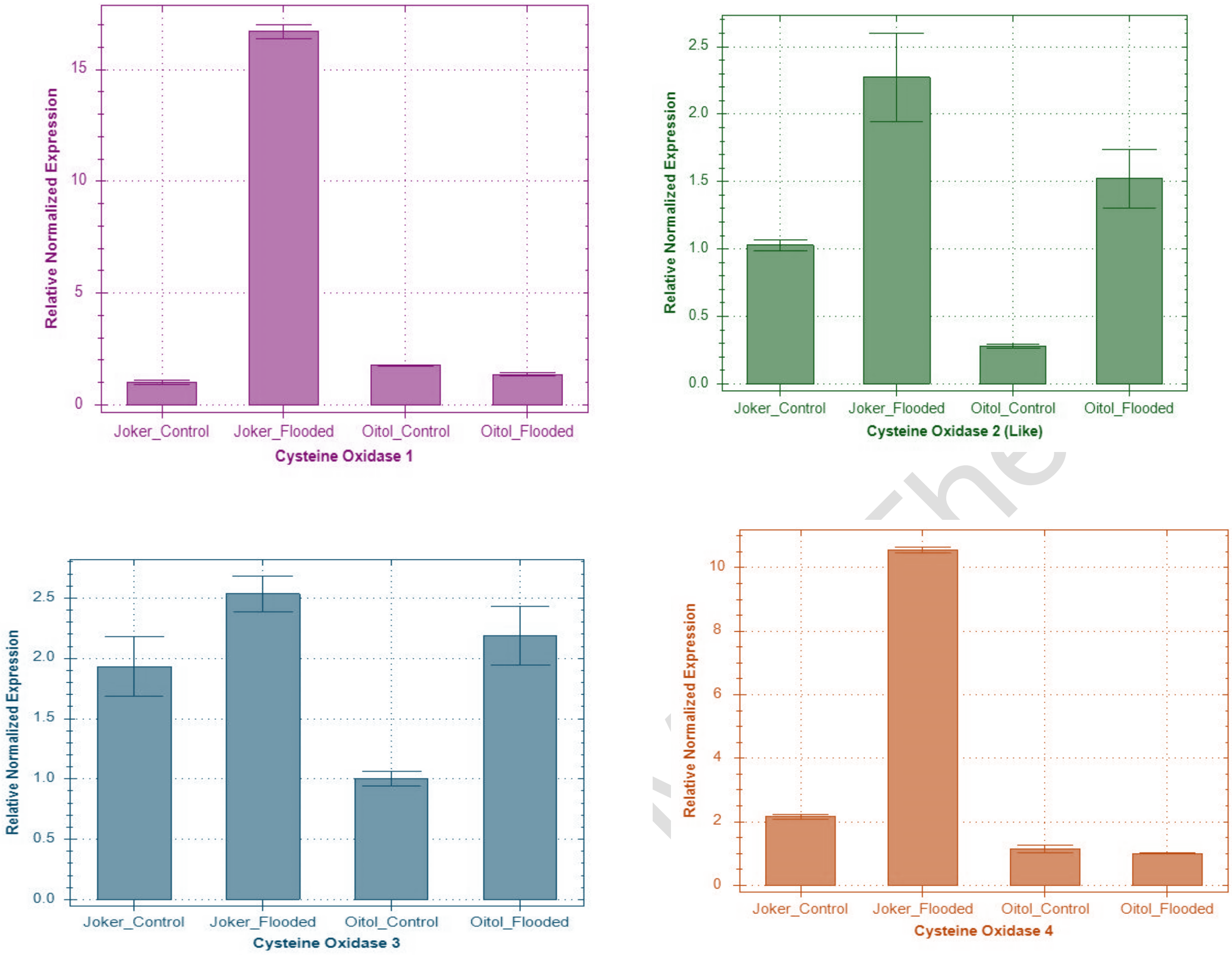

Figure 9. The results of real-time PCR (qRT–PCR) analysis of *CysOx 1–4* genes in two different cucumber cultivars ('Oitol' and 'Joker') under control and WL conditions.

5. DISCUSSION

Modern cucumber F1 hybrids are the result of intense breeding and are suitable for either greenhouse or open field production. Greenhouse-type hybrids are commonly produced in soilless media, where irrigation/fertigation generates a waterlogged (WL) environment for the root system in a small volume compartment. To identify traits that may mitigate the effects of WL stress, adventitious root (AR) development in various hybrids of both cultivation types was examined. ARs were more common in hybrids designated for greenhouse cultivation, even in the absence of any stressors. The relationship between the type of hybrid and the frequency of AR formation suggested that internal aeration supported by ARs is especially important for hybrid adaptation to vigorous fertigation under protected conditions. ARs may provide these hybrids with additional growth potential under typical growing conditions. This outcome highlights the importance of AR formation in the productive commercial growth of cucumber in greenhouses. This study analyzed a pair of cucumber genotypes that represented two major hybrid types: greenhouse-grown 'Oitol' and open-field-optimized 'Joker'. The experimental conditions were designed to mimic commercial farming procedures as closely as possible. The root system was flooded with nutrient solution, and rockwool cubes in perlite were utilized as a medium. No precautions were taken to prevent oxygen from passing through the surface into the nutrient solution.

Waterlogging treatment caused a steady decrease in the dissolved oxygen concentration in the media to approximately 2 mg/L^{-1} (Figure 6B). The use of perlite as part of growth medium could account for the slow depletion of dissolved oxygen. The air content of the perlite may have buffered the reduced external supply of oxygen to the roots; thus, the oxygen content of the media did not decrease rapidly (Figure 6B). The diffusion of air across the surface of the liquid media may have also helped to replenish the oxygen levels. The slow buildup of hypoxia and the high nutritional level of the media may have allowed the WL-treated plants to acclimate. Cucumber adapts well to WL conditions (Keska et al. 2021). The previously mentioned parameters, alone or in combination, are hypothesized to explain the absence of growth retardation in 'Joker' and the extent of the growth response in 'Oitol' under WL treatment. Redox measurements suggested that the plants did not undergo significant oxidative stress (see the MDA concentration of leaves in Figure 2A). The lower basal level of MDA in 'Oitol' leaves may be due to the hybrid's high antioxidant capacity, as reported in an earlier study (Hesari et al. 2023). The treatment reduced the MDA levels in both hybrids, resulting in enhanced antioxidant defense, which was most likely part of the acclimation process. Redox changes did not affect guaiacol peroxidase activity. In other studied, increasing nutritional (particularly nitrogen) input resulted in a sustained redox balance in WL (Men et al. 2020).

By the second week of treatment, the oxygen levels in the media clearly suggested hypoxic conditions around the plant roots. This resulted in the induction of root ADH activity in both hybrids. The significant role of ADH in fermentation processes in WL-induced hypoxia in cucumber has been extensively described (Xuewen et al. 2014). The lower ADH activity of ‘Oitol’ roots indicated a smaller need for fermentation in this hybrid. Cysteine oxidases play an important role in the hypoxia sensing system by modifying ERF-VII transcription factors and directing their action toward the activation of genes relevant for hypoxia tolerance (Cho et al. 2021). On the other hand, cysteine oxidases are regulated by the same transcription factors (Weits et al. 2014).

Therefore, we used cucumber cysteine oxidases as genetic markers to estimate the level of hypoxia sensed in the roots. The abundance of cysteine oxidases increased in both hybrids. *CysOx1* and *CysOx4* genes were strongly stimulated, but only in 'Joker' roots, not in 'Oitol' roots. These two genes are consequently proposed as genetic indicators for hypoxia in cucumber under WL conditions. *CysOx2* and *CysOx3* was elevated in both genotypes, with 'Joker' showing the highest expression levels. Higher induction of ADH enzyme activity and greater CysOx gene expression suggested that the 'Joker' roots experienced more hypoxia, but 'Oitol' effectively alleviated this stress.

Despite low DOI values in the media and clear evidence of hypoxia reactions in both hybrids' roots, neither hybrid suffered growth retardation. The absence of growth inhibition by WL was most likely due to the above-mentioned acclimatization process, which was caused by the delayed build-up of hypoxia and the high nutritional level of our treatment media. According to Manik et al. (2019), a high nutritional supply may help to mitigate WL stress.

It's possible that the plants went through an adaptation phase in reaction to the continuous development of hypoxia. Through this mechanism, plants gradually adjust to low oxygen levels, which might lessen the negative impact of hypoxia on growth. plants may have been able to adapt and activate mechanisms to deal with the stress without suffering from appreciable growth retardation due to the treatment's gradual increase in hypoxia levels.

Suberin and lignin are both components of the plant cell wall. These barriers may also play a role in waterlogging tolerance by regulating the movement of gases and nutrients within the root system. Understanding the composition and integrity of these barriers in cucumber roots under waterlogged conditions could provide insights into their role in tolerance. Tissue porosity refers to the presence of air spaces within plant tissues, which can affect the movement of gases such as oxygen and carbon dioxide. Roots with higher porosity may facilitate gas exchange, potentially enhancing tolerance to waterlogging. Schizogenous tissue development involves the formation of air spaces within tissues through cell separation. Although aerenchyma formation may not be typical in cucumber roots, the development of schizogenous tissue in the cortex could serve a similar function by promoting root aeration. While 'Joker' shoot growth remained constant compared to that of the control, 'Oitol' had a growth-promoting effect after two weeks of WL treatment in nutrient solution. The increased growth response of 'Oitol' under WL conditions was especially striking.

Accelerated shoot growth manifested primarily as elongation, while the increase in leaf dry weight did not reach a significant level, whereas the leaf surface area increased. This may resemble rice's low oxygen escape syndrome (Kuroha et al. 2018), although in this case, growth induction appears to be aided by efficient hypoxia avoidance. Extra growth may be accompanied by frequent adventitious (AR) development in the hybrid 'Oitol'. AR production occurred even in the absence of stress treatment and increased significantly in response to WL. In response to waterlogging (WL) stress, plants generate ARs to avoid root hypoxia as much as possible (Qi et al. 2019). AR formation is a feature of WL tolerance in various plant species, and it has been shown to be cultivar specific in some circumstances (Armstrong et al. 1994; Sasidharan et al. 2015). ARs play a critical role in agricultural WL tolerance (Yamauchi et al. 2018). In our study, we discovered that frequent AR production was associated with restrained induction of ADH activity in WL-stressed 'Oitol' roots. This finding is consistent with the suggested involvement of ARs in oxygen delivery. AR formation was identified as a key characteristic that may have contributed to the increased development of the greenhouse-optimized hybrid 'Oitol' under WL conditions by relieving hypoxic stress. When grown under usual conditions, the other greenhouse types evaluated may benefit from increased growth potential due to internal oxygenation of the root system via ARs. Aside from ARs as a primary feature, other potential variables of WL tolerance, such as the suberin/lignin barrier and tissue porosity in roots, require additional exploration in our system. Although aerenchyma production has not been definitively observed in cucumber, schizogenous tissue development in the cortex may promote root aeration, as has rarely been described for this species (Ma et al. 2016). These additional factors deserve further examination.

6. SUMMARY

Cucumber (*Cucumis sativus* L.) F1 cultivars from greenhouses and open fields were tested for differential waterlogging (WL) tolerance. Hypoxic condition developed because of flooding the root system, as evidenced by the decreased oxygen content in the root zone of the investigated plants. The applied WL did not significantly reduce the plant canopy or biomass output. Even improved performance was observed in the greenhouse-type F1 hybrid, as evidenced by the total leaf area and fresh and dry weight of vegetative tissues obtained following the 2-week waterlogging. High nutrient supply of the flooding media has been put forward as a potential explanation for the observations. A much higher rate of established adventitious root (AR) production was associated with the observed adaptations, which was particularly prominent in greenhouse varieties. Such a greater AR development rate was also observed in a type-specific way among the six investigated types under normal growing conditions.

The induction pattern of cysteine oxidase (CysOx) genes, considered to be part of the hypoxia sensing system, revealed that the severity of oxygen deprivation was more noticeable in the roots of open-field cucumber ('Joker') plants. The discovery of diverse features, such as growth parameters and variable gene expression, between hybrid lines of a single species might provide a new opportunity to better understand the molecular mechanisms of stress response and adaptation physiology in plants. To increase crop resilience and agricultural productivity, it is essential to understand how plants react to and adapt to environmental stresses. Hypoxia is a serious stressor that has a substantial effect on the growth and development of plants. The hypoxia sensing system of plants includes cysteine oxidase (CysOx) genes, which are essential for controlling plant reactions to oxygen deprivation. The CysOx genes have become significant players in this process, and the degree of oxygen deprivation that plants undergo is reflected in the patterns of their expression. Studies have shown that CysOx genes are upregulated under hypoxic conditions, indicating their involvement in the plant's adaptive response to oxygen deficiency. In conclusion, the induction pattern of CysOx genes in cucumber varieties, particularly exemplified by the 'Joker' cultivar, highlights the importance of hypoxia sensing in the plant stress response. The distinctive traits observed between greenhouse and open-field crops provide a unique opportunity to unravel the molecular mechanisms underlying stress adaptation in plants. By elucidating these mechanisms, researchers can develop strategies to enhance crop resilience and productivity in the face of environmental challengesindicating a potential involvement for these genes in the plant's adaptive reaction to oxygen scarcity.

In conclusion, the importance of hypoxia sensing in the plant stress response is highlighted by the induction pattern of CysOx genes in cucumber cultivars, especially the 'Joker' cultivar. The distinguishing characteristics between open-field and greenhouse varieties offer a rare chance to understand the molecular processes underlying plant stress tolerance. Researchers can create ways to improve agricultural productivity and resilience in the face of environmental difficulties by clarifying these systems. Cysteine oxidase (CysOx) genes, which are known components of the hypoxia sensing system, exhibited less marked overexpression, further indicating that the roots of greenhouse-type (or "Oitol") plants were not exposed to as much hypoxic stress. One could consider the relationship between biochemical and genetic markers of root hypoxia, WL-stressed hybrids with different cultivation growth parameters, and AR formation as a novel opportunity to better understand the molecular mechanisms of stress response and adaptation physiology in plants.

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DECLARATION

We (István Papp PhD and Iman Mirmazloum PhD)

As a consultants, we declare that we have reviewed the final [✓ thesis]/thesis/dissertation/portfolio and that I have informed the student of the requirements, legal and ethical rules for the correct handling of literary sources.

We [✓ recommend]/do not recommend the final [✓ thesis]/dissertation/portfolio to be defended in the final examination.

The thesis contains a state or official secret: yes [✓ no]*

Date: 2024 year 04 month 22 day

Insider consultant

DECLARATION

Me, as the undersigned Imran Khan (Code-Neptun: A3N459) declares, that the Diploma Thesis entitled ‘’Analysis of the transcriptomic responses of cucumbers to waterlogging stress and identification of markers, regulators, and effectors of tolerance’’submitted in 2024 is my own intellectual property.

I hereby acknowledge that the presentation of my thesis in the Dean's Office according to the schedule does not mean at the same time the acceptance of my dissertation from professional and content-related aspects.

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