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BIOLOGY

Ethylene responsive element binding factors contributes to waterlogging tolerance by regulating photosynthetic and physiological parameters in petunia

Dongmei Yin[1] **Yueyue Wang**[1] **Zhuqing Han**[1] **Daoyang Sun**[2*]

[1]College of Ecology, Shanghai Institute of Technology, Shanghai, China.
[2]College of Landscape Architecture and Arts, Northwest A&F University, 712100, Yangling, Shaanxi, China. E-mail: sundaoyang@nwsuaf.edu.cn.
*Corresponding author.

ABSTRACT: *Ethylene-responsive element binding factors (ERFs) are widely involved in the regulation of plant responses to different abiotic stresses. In petunia (Petunia × hybrida), PhERF2 belonging to the subfamily Ⅶ of ERF transcription factors participates in the response to waterlogging stress. In this study, we investigated waterlogging tolerance variation of WT and transgenic petunia plants with RNAi silencing and overexpression of PhERF2 through photosynthetic and physiological performance. Chlorophyll content and root vigor declined continuously in both WT and PhERF2 transgenic lines under waterlogging stress, but the extent of the fall in PhERF2-overexpressing lines was less than that in WT and PhERF2-RNAi lines. At the end of waterlogging treatment, soluble protein levels in PhERF2-overexpressing lines were significantly higher than those in WT and PhERF2-RNAi lines, while the latter showed a higher malondialdehyde content overall. Different degrees of reductions in Pn, Gs, and Tr levels occurred in both WT and PhERF2 transgenic lines upon exposure to waterlogging. The Ci levels of PhERF2-overexpressing lines decreased after 3 hours of waterlogging treatment, and the Ci levels of WT and PhERF2-RNAi lines gradually increased from 6 to 72 hours of waterlogging treatment. These data suggested that non-stomatal factors were the primary limiting factors for Pn in WT and PhERF2-RNAi lines under severe stress, while the stomatal opening was the main factor limiting Pn in PhERF2-overexpressing lines. Our results demonstrated that the contribution of PhERF2 to the waterlogging tolerance of petunia appears to depend on the regulation of physiological and photosynthetic responses. PhERF2 represents a hopeful candidate gene for enhancing waterlogging tolerance of ornamental plants.*

Key words: *petunia, waterlogging, PhERF2, photosynthesis, physiology.*

Os fatores de ligação do elemento responsivo ao etileno contribuem para a tolerância ao encharcamento, regulando os parâmetros fotossintéticos e fisiológicos na petúnia

RESUMO: *Fatores de transcrição de resposta ao etileno (ERF) estão amplamente envolvidos na regulação das respostas das plantas a diferentes estresses abióticos. Em petúnia (Petunia × hybrida), PhERF2 pertencente à subfamília Ⅶ de fatores de transcrição ERF participa da resposta ao estresse de alagamento. Este estudo teve como objetivo investigar a variação da tolerância ao alagamento de plantas WT e petúnia transgênica com silenciamento de RNAi e superexpressão de PhERF2 através do desempenho fotossintético e fisiológico. O conteúdo de clorofila e o vigor da raiz diminuíram continuamente em ambas as linhagens transgênicas WT e PhERF2 sob estresse de alagamento, mas a extensão da queda nas linhas de superexpressão de PhERF2 foi menor do que nas linhas WT e PhERF2-RNAi. No final do tratamento de alagamento, os níveis de proteína solúvel nas linhas com superexpressão de PhERF2 foram significativamente maiores do que nas linhas WT e PhERF2-RNAi, enquanto a última mostrou um teor geral de malondialdeído maior. Diferentes graus de reduções nos níveis de Pn, Gs e Tr ocorreram em ambas as linhas transgênicas WT e PhERF2 após a exposição ao alagamento. Os níveis de Ci das linhas de superexpressão de PhERF2 diminuíram após três horas de tratamento de alagamento, e os níveis de Ci das linhas de WT e PhERF2-RNAi aumentaram gradualmente de seis para 72 horas de tratamento de alagamento. Esses dados sugeriram que os fatores não estomáticos foram os principais fatores limitantes para Pn em linhas WT e PhERF2-RNAi sob estresse severo, enquanto a abertura estomática foi o principal fator limitante de Pn em linhas com superexpressão de PhERF2. Nossos resultados demonstraram que a contribuição do PhERF2 para a tolerância ao alagamento da petúnia parece depender da regulação das respostas fisiológicas e fotossintéticas. PhERF2 representa um gene candidato esperançoso no aumento da tolerância ao alagamento de plantas ornamentais.*

Palavras-chave: *petúnia, alagamento, PhERF2, fotossíntese, fisiologia.*

INTRODUCTION

Petunia, cultivated all over the world, is one of the most important Solanaceae plants utilized for ornamental purpose (GERATS & STROMMER, 2011). It is of great significance in global floriculture production, and usually planted in outdoor gardens in many countries (XIA et al., 2006). Waterlogging has become one of the main abiotic stresses restricting common planting and landscape cultivation of

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petunia. It is urgent to discover the wild excellent germplasm with stress resistance for developing anti-stress breeding by molecular technology.

The ethylene-responsive element binding factors (ERFs) are essential transcriptional regulators in response to different biotic and abiotic stresses in plants. ERFs belong to the plant-specific AP2/ERF transcription factor superfamily, which has great potential in transgenic breeding (DAOYANG et al., 2016). Previous research has reported the biological role of ERFs in the modulation of waterlogging stress. Overexpression of an ERF gene *Sub1A* in rice leads to the enhancement of waterlogging tolerance (FUKAO et al., 2006; XU et al., 2006; FUKAO & BAILEY-SERRES, 2008). *HRE1*, a hypoxia-inducible ERF gene, confers waterlogging tolerance in transgenic Arabidopsis plants (LICAUSI et al., 2010). Petunia is greatly susceptible to waterlogging; and is therefore, a promising landscape flower for studying the waterlogging responses. We have reported that *PhERF2* plays an important role in antiviral RNA silencing (DAOYANG et al., 2016), and the expression levels of *PhERF2* were significantly increased by abiotic stress such as cold, salt, and waterlogging treatments.

The changes of photosynthetic parameters Photosynthetic rate (*Pn)*, Stomatal conductance (*Gs)*, Transpiration rate (*Tr)*, and Intercellular carbon dioxide concentration (*Ci)* reflect the effects of waterlogging stress on photosynthetic characteristics of plants. Previous research has indicated that, compared with waterlogging-sensitive plants, photosynthesis was less affected in the tolerant ones. The native plants in wetland show better adaption to flooding without restraint on photosynthesis (PEREIRA et al., 2014; WARING & MARICLE, 2012; KUMAR et al., 2013; SAGREDO et al., 2014; ZHU et al., 2016; STRIKER & COLMER, 2017). The decrease in stomatal conductance (*Gs*) leads to the reduction in CO_2 availability, which is the primary negative effect of waterlogging on *Pn* during short term waterlogging stress (IRFAN et al., 2010; HERZOG et al., 2016; PÉREZ-JIMÉNEZ et al., 2018). After long-term waterlogging stress, lowered CO_2 assimilation, decreased chlorophyll content, and excessive ROS production lead to non-stomatal inhibition on photosynthesis, which is the main limiting conditions for *Pn* (ZHENG et al., 2009). Photosynthesis is increasingly suppressed by the changes in leaf wilting and sagging, and the decrease in chlorophyll levels (SMETHURST et al., 2005).

Nowadays, it has been widely characterized that waterlogging stress increases the ROS production and leaf lipid peroxidation in various plants (XIAO et al., 2016; ZHANG et al., 2016). The final product of membrane lipid peroxidation is malondialdehyde (MDA), that combines with the specific proteins on the cell membrane to inactivate or destroy the structure and function of biofilm (SONG, 2012). Soluble protein focuses on the ability of mediating osmotic adjustment, stabilizing subcellular structures, and scavenging free radicals (DELAUNEY & VERMA, 1993). Under waterlogging stress, soluble protein as an osmolyte functions to maintain plant water status and hydraulic conductivity (BARICKMAN et al., 2019). When soil waterlogging takes place, the root system is directly affected by the stress. Oxygen deprivation in the root severely inhibits the growth and development of plants due to the degradation of chlorophyll (DREW, 1997). According to previous studies, we reported that *PhERF2* plays an essential role in petunia anoxia tolerance (YIN et al., 2019). The regulatory mechanism of waterlogging response by *PhERF2* requires a further dissection in the aspects of photosynthesis and physiology. Thus, in this study, wild-type (WT) and transgenic petunia plants with RNAi silencing and overexpression of *PhERF2* were selected as experimental materials to measure the levels of leaf photosynthetic parameters, chlorophyll, MDA, soluble protein, and root vigor under waterlogging stress. Our results supported an essential role of *PhERF2* in waterlogging tolerance of petunia.

MATERIALS AND METHODS

Plant materials and treatments

Petunia × hybrida seeds of wild-type (WT) ‘Mitchell Diploid’, *PhERF2*-RNAi (1A, 1B, and 4B) and *PhERF2*-overexpressing (C, D, and I) lines were harvested in University of California Davis. Three experimental materials were taken for *PhERF2* overexpression and *PhERF2*-RNAi respectively, in order to avoid chance, reduce experimental errors, and make experimental results more accurate. Seeds were sown in the botanical garden of Shanghai Institute of Technology. Two weeks post germination, the seedlings were transferred into plastic pots (12cm in height, and 10.5cm in diameter) filled with soil mixture (vermiculite: peat: perlite, 1:1:1) and grown in an artificial climatic room (50~60% relative humidity, 23/19 ℃ day/night, and 12/12h light/dark).

Eight weeks later, uniform petunia plants were selected for waterlogging treatments to examine the impact of waterlogging on WT and homozygous T2 generation transgenic petunia lines.

Potted plants were put in plastic containers, and the containers were filled with tap water to keep the water levels at 2-3 cm above soil surface during the whole waterlogging treatments. WT, *PhERF2*-RNAi, *PhERF2*-overexpressing lines and the seven waterlogging treatments (0, 3, 6, 12, 24, 48 and 72h of waterlogging) were respectively arranged as sub-plot and main-plot. All treatments were repeated 3 times and 6 plants were used for each replication; and therefore, there were 18 plants for each treatment. The control plants were untreated with waterlogging. We hypothesized that *PhERF2* plays an essential role in improving petunia waterlogging tolerance. To test the hypothesis, the leaf and root samples were collected for measuring photosynthetic parameters and physiological indicators at 0, 3, 6, 12, 24, 48, and 72h after waterlogging stress.

Measurement of photosynthesis and related parameters

Net photosynthetic rate (*Pn*), stomatal conductance (*Gs*), transpiration rate (*Tr*) and intercellular CO_2 concentration (*Ci*) were measured on the expanded leaves by the LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE, USA). CO_2 concentration, actinic light intensity and temperature were set at 400µmol mol^{-1}, 1000µmol m^{-2} s^{-1} and 25 ℃, respectively. For each treatment, the values of *Pn*, *Gs*, *Tr* and *Ci* were recorded for six leaves at each time point.

Measurement of chlorophyll and soluble protein contents

Approximately 0.2 g of fresh leaf samples were sliced and incubated in 95% ethanol in the dark for 24h at 25 ℃, until the tissues became white. The absorbance of the extract was measured at 663 and 645nm for calculating chlorophyll content (ARNON, 1949). Soluble protein content of leaf was measured following SHI et al. (2007), bovine serum albumin was used as a standard. Specific enzyme activities were expressed as units (U) per g of protein.

Measurement of malondialdehyde

The levels of lipid peroxidation were determined in terms of MDA content according to thiobarbituric acid (TBA) reaction method (KUN et al., 2010). 0.5g of fresh leaf tissues were ground by ice rubbing and homogenized in 5 ml 5% trichloroacetic acid (TCA). After centrifugation at 4000×g for 10 min, 2 ml of supernatant was dissolved in 2 ml of 0.6% TBA. The mixture was heated to 99 ℃ for 30 min and then cooled quickly on ice. After that, the mixture was centrifuged at 3000×g for 15 min, and then the supernatant was used to measure the absorbance at 600, 532, and 450nm.

Measurement of root vigor

About 0.1g of root samples were harvested at the end of each waterlogging treatment, and extracted following the method as previously described (MIN & DE -XIAN, 2011). The material was homogenized in 5 ml of 0.4% TTC and 5 ml of 0.1mol/L phosphate buffer solution (pH 7.5), and heated to 37 ℃ for 1.5h in darkness. The reaction was ended with 2 ml of 1mol/L sulphuric acid. After that, the mixture was ground and homogenized in 10 ml of ethyl acetate. After precipitation, the absorbance of the supernatant was measured at 485nm.All the spectrophotometric analyses above were conducted on a 752 UV-Vis spectrophotometer (“GUANG PU”, Shanghai, China), and all the chemicals were purchased from Sangon Biotech.

Statistical analysis

Statistical analysis was conducted by the Excel2007 and SPSS17.0, and graphs were drawn by Origin 2017. Data were expressed as means ± errors. One-way ANOVAs followed by Duncan’s multiple range test was employed to assess the statistical significance of the treatment difference ($P \leq 0.05$).

RESULTS

Photosynthetic parameters

After 3h of waterlogging stress, *Pn* in WT and *PhERF2*-RNAi (1A, 1B, and 4B) lines reached the highest point, increasing to 1.2-, 1.4-, 1.1-, and 1.3-fold of the control levels, respectively. After that, *Pn* declined continuously by prolonged periods of waterlogging stress. In *PhERF2*-overexpressing (C, D, and I) lines, *Pn* was constantly reduced during the whole period of waterlogging treatment. Both at 12 and 48h, *Pn* in overexpressing lines was obviously higher than that in silenced lines. On day 3, a reduction in *Pn* was reported in both WT and transgenic petunia lines, but the extent of the decrease in *PhERF2*-overexpressing lines was less than that in WT and *PhERF2*-RNAi lines, decreasing to 56.28%, 62.02%, 71.73%, 1.89%, 41.2%, 49.13%, and 48.83% of the background levels, respectively. Meanwhile, *Pn* in overexpressing lines was significantly higher than that in the other lines, and the value of line I was the highest of others (Figure 1a).

As shown in figure 1b, *Gs* in *PhERF2*-RNAi (1A, 1B, and 4B) lines continued to decrease

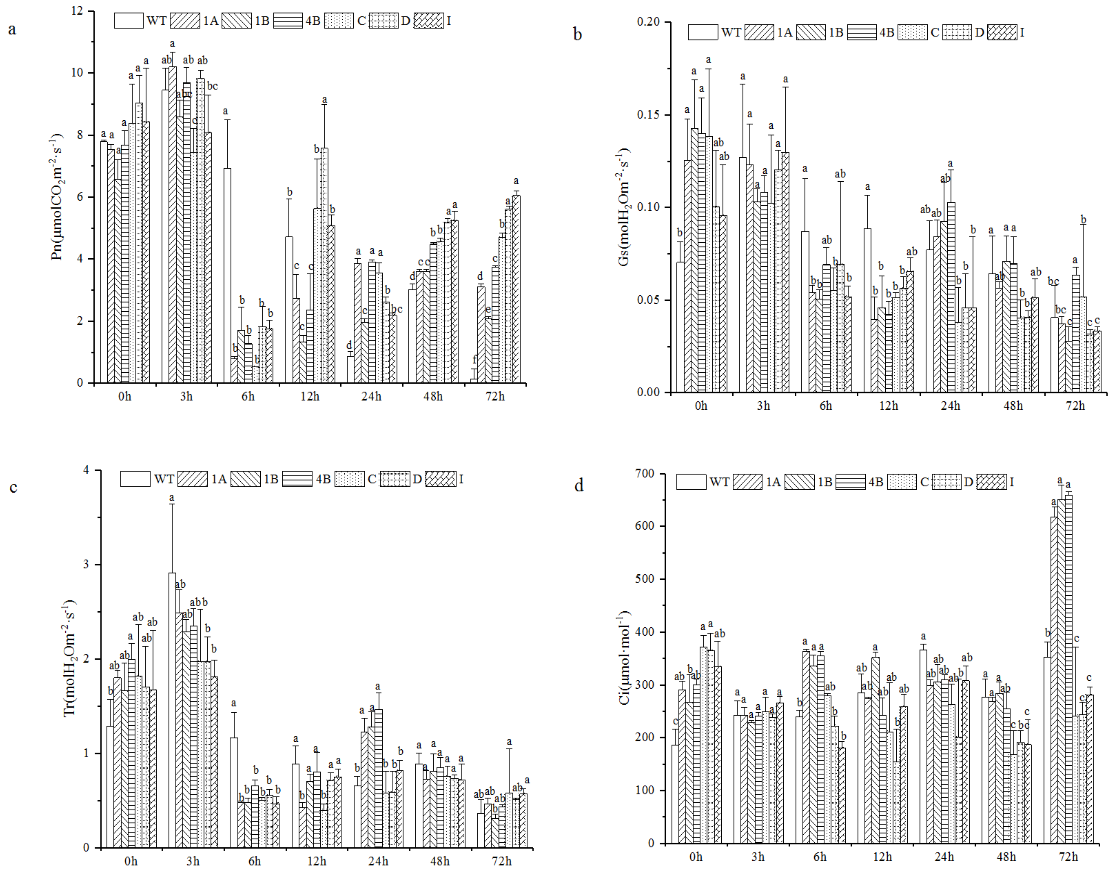

Figure 1 - The effects of waterlogging on (a) *Pn*, (b) *Gs*, (c) *Tr*, and (d) Ci levels in the leaves of eight-week-old seedlings of wild type (WT), *PhERF2*-RNAi (1A, 1B, and 4B), and *PhERF2*-overexpressing (C, D, and I) lines at 0h, 3h, 6h, 12h, 24h, 48h, and 72h, respectively. The values are means±standard errors of three replications. Different lower case letters represent significant differences based on Duncan's multiple in comparisons between WT, 1A, 1B, 4B, C, D, and I under waterlogging, at $P < 0.05$, respectively.

with the prolonged waterlogging treatment. *Gs* in WT and *PhERF2*-overexpressing (D and I) lines reached a peak at 3h of waterlogging, and the respective increases were 1.8-, 1.2-, and 1.6-fold of their non-waterlogged controls. When waterlogging stress continued for 48h, *Gs* in WT and *PhERF2*-RNAi (1B, 4B) lines was remarkably higher than that in *PhERF2*-overexpressing (C and D) lines. After 3 days of waterlogging stress, compared with the control levels, *Gs* in WT, *PhERF2*-RNAi (1A, 1B, and 4B) and *PhERF2*-overexpressing (C, D, and I) lines dropped to 31.87%, 29.73%, 36.69%, 30.12%, 37.51%, 31.51%, and 42.18%, respectively, and meanwhile the degree of the fall in line I was the least.

The levels of *Tr* increased in WT, *PhERF2*-RNAi (1A, 1B, and 4B) and *PhERF2*-overexpressing (C, D, and I) lines after 3h of waterlogging stress, and all the lines above reached the highest values, rising to 2.2-, 1.4-, 1.4-, 1.2-, 1.1-, 1.2-, and 1.1-fold of the background levels, followed by a rapid drop. After 24h of waterlogging treatment, *Tr* in *PhERF2*-RNAi lines was prominently higher than that in *PhERF2*-overexpressing lines. Three days of waterlogging reduced the *Tr* of WT and *PhERF2* transgenic lines to 28.37%, 25.88%, 26.43%, 38.31%, 26.88%, 29.86%, and 34.10% of the non-waterlogging levels, respectively (Figure 1c).

As displayed in figure 1d, the *Ci* of *PhERF2*-RNAi (1A, 1B, and 4B) lines dropped to the lowest point in response to 3h of waterlogging. After that, there was a progressive increase of *Ci*, by day 3, *Ci* in *PhERF2* silencing lines peaked at 1.4-, 2.4-, and 2.2-fold of the initial value, respectively. With the prolongation of waterlogging treatment time, the *Ci* of *PhERF2*-overexpressing (C, D, and I) lines continued to decrease, and dropped to 65.01%, 66.82%, and 83.95% of the control levels on day 3. For 6, 48, and 72h of waterlogging stress, the *Ci* of WT and *PhERF2*-RNAi lines were significantly higher than that of *PhERF2*-overexpressing lines.

Leaf chlorophyll and MDA content

As shown in figure 2a, on the whole, chlorophyll content was followed by a progressive fall in both WT and *PhERF2* transgenic lines with the extension of waterlogging treatment. After 3 days of waterlogging stress, chlorophyll content of WT plants, lines 1A, 1B, 4B, C, D, and I decreased to 57.58%, 75.95%, 63.35%, 64.91%, 75.46%, 58.21%, and 64.57% of the non-waterlogged levels, respectively. Meanwhile, the chlorophyll content in *PhERF2*-overexpressing (C, D, and I) lines was significantly higher than that in WT and *PhERF2*-RNAi (1A, 1B, and 4B) lines. Particularly, the value of line I was the highest and line 4B was the lowest.

Under waterlogging stress, the MDA content remarkably increased followed by a decline (Figure 2b). By 6h of waterlogging, MDA content in WT, *PhERF2*-RNAi and -overexpressing lines peaked with 4.6-, 4.1-, 4.4-, 5.4-, 1.3-, 1.1-, and 1.4-fold of the non-waterlogged controls, respectively. It was substantially higher in WT and RNAi silencing lines (1A, 1B, and 4B) than in overexpressing lines (C, D, and I) of *PhERF2*, and thereafter, MDA content decreased sharply. At 24 and 48h of waterlogging stress, the content of MDA in WT and *PhERF2*-RNAi lines was significantly higher than that in *PhERF2*-overexpressing lines as well. After 72h of waterlogging, in WT and RNAi silencing lines, MDA content rose to 2.3-, 1.8-, 1.7-, and 3.3-fold, while in *PhERF2*-overexpressing lines, the equivalent levels decreased to 33.33%, 45.1%, and 58.5%, respectively, compared to corresponding unstressed control.

Soluble protein content and root vigor

The soluble protein content of *PhERF2*-overexpressing lines was significantly higher than that of WT and *PhERF2*-RNAi lines during the waterlogging period from 24h to72h. Compared with other lines, silenced line 4B maintained the lowest value, and conversely, overexpressing line I stayed at the highest level. By 24h of waterlogging, soluble protein content in line I was 0.37 mg/g FW, a level about twice than that in line 4B (0.19 mg/g FW). After 48h of exposure to waterlogging stress, line I (0.55 mg/g FW) was 2.4 times that of line 4B (0.23 mg/g FW). At the end, line I was 0.2 mg/g FW; and however, line 4B decreased sharply to approach the zero (Figure 3a).

With the prolongation of waterlogging treatment time, the root vigor of WT, *PhERF2*-RNAi (1A, 1B, and 4B) and *PhERF2* overexpressing (C, D, and I) lines continued to decrease. At 72h of waterlogging treatment, root vigor in overexpressing line I was 967.2 µg TTC/g·h, which was about twice the average of the three silenced lines (488.8 µg TTC/g·h), By comparison with control lines without waterlogging stress, root vigor was decreased by 25.8% in WT plants, by 26.4%, 31.2%, and 25.1% in three silencing lines, and by 26.5%, 31.6%, and 45.8% in three overexpressing lines in response to 72h of waterlogging. In particular, the decrease of overexpressing lines was smaller than that of *PhERF2* silenced lines, among which line I had the smallest decrease and line 4B had the largest decrease (Figure 3b).

DISCUSSION

There is a large demand for petunia with the development of the landscaping industry. The ability of a plant to maintain healthy under waterlogging, drought, and salt stresses is a criterion for the choice of these horticultural plants. Sometimes, the landscaping plants with elite traits have been developed in conventional breeding programs, while these plants do not express as good phenotypes as expected. The wide application of molecular technology together with the discovery of the ERF transcription factors has provided an alternative way to improve stress tolerance for the landscaping plants. In this study, we confirmed the function of *PhERF2* in waterlogging tolerance using WT and its transgenic plants. Under waterlogging stress, hypoxia is the primary influencing factor (SHIONO et al., 2008). When tissues are anoxic, the aerobic energy-generating system declines rapidly and then affects the roots directly (ELSE et al., 2001). We reported that root vigor in WT and *PhERF2*-overexpressing lines was greater than that in *PhERF2*-RNAi lines under waterlogging treatment (Figure 3b). The result is consistent with a previous report in wheat (CHEN et al., 1998). Some scholars have shown a co-ordination between above- and below-ground parts of plant, involving signal transduction from the root to

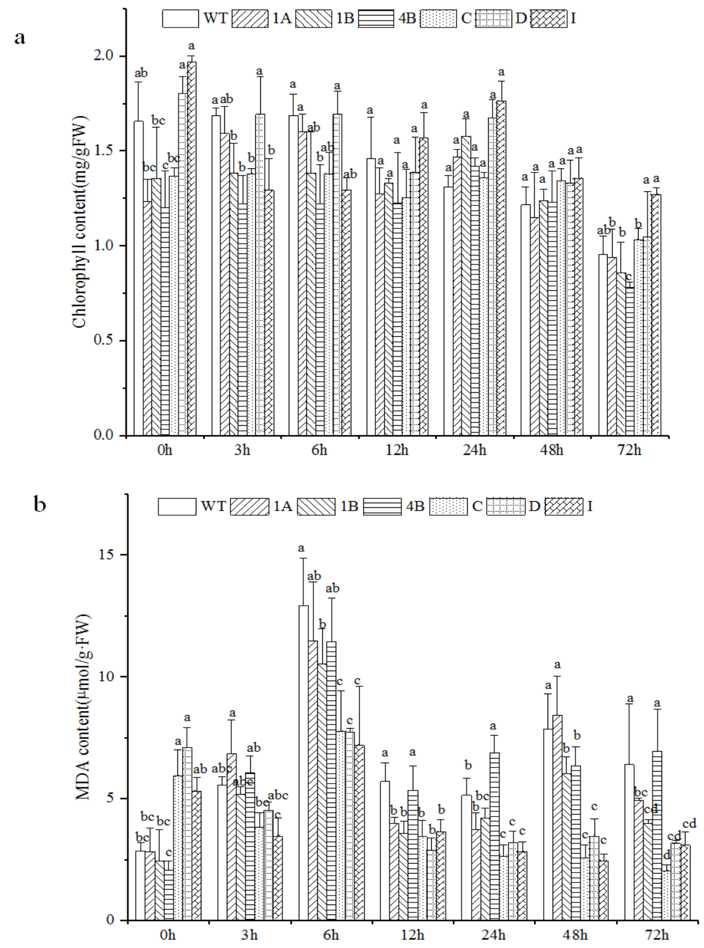

Figure 2 - Physiological response of wild type (WT), *PhERF2*-silenced (1A, 1B, and 4B), and *PhERF2*-overexpressing (C, D, and I) lines exposed to flooding treatment. (a-b) Measurements of chlorophyll content and MDA content at 0h, 3h, 6h, 12h, 24h, 48h, and 72h, respectively. The values are means±standard errors of three replications. Different lower case letters represent significant differences based on Duncan's multiple in comparisons between WT, 1A, 1B, 4B, C, D, and I under waterlogging, at $P < 0.05$, respectively.

the shoot and subsequent physiological responses. GAO et al. (2018) reported that both salt and drought treatments induced oxidative stress response in *Chrysanthemum morifolium* 'White Snow' leaves.

When plants are exposed to waterlogging stress, oxidative stress will induce a large amount of toxic active oxygen which causes damages to chlorophyll, proteins, membrane lipids, and subcellular organelles (MUNNÉ-BOSCH & ALEGRE, 2000; RONG-HUA et al., 2006). After waterlogging treatment, chloroplast ultrastructure is damaged, resulting in a decrease of chlorophyll content (AHMED et al., 2002; KUMUTHA et al., 2008). For example, leaf chlorophyll content in both waterlogging-tolerant and -susceptible plants of pigeon pea decreases under waterlogging (KUMUTHA et al., 2009). In the current study, chlorophyll content declined in both WT and all transgenic lines, but

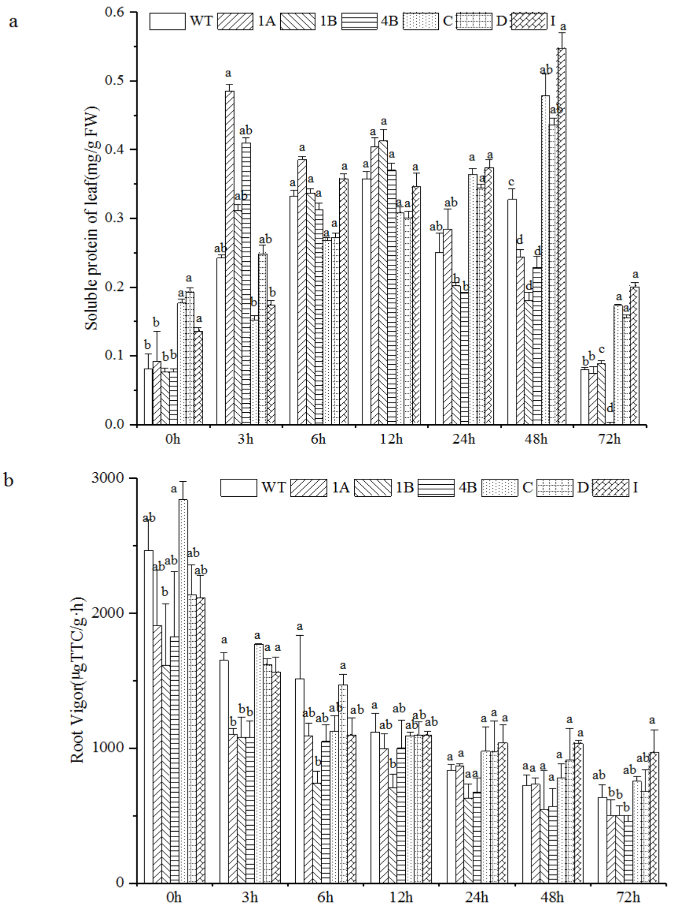

Figure 3 - Soluble protein and root vigor of wild type (WT), *PhERF2*-silenced (1A, 1B, and 4B), and *PhERF2*-overexpressing (C, D, and I) lines exposed to flooding treatment. (a-b) Measurements of soluble protein and root vigor at 0h, 3h, 6h, 12h, 24h, 48h, and 72h, respectively. The values are means±standard errors of three replications. Different lower case letters represent significant differences based on Duncan's multiple in comparisons between WT, 1A, 1B, 4B, C, D, and I under waterlogging, at P < 0.05, respectively.

the chlorophyll levels were significantly higher in *PhERF2*-overexpressing lines than in *PhERF2*-RNAi lines at the final stage of waterlogging stress (Figure 2a). It suggested that these plants probably exerted the *PhERF2*-regulated pathways to make overexpressing lines to suffer less from flooding stress than RNAi silencing lines. Furthermore, the changes in MDA and soluble protein content also concur with the physiological traits for waterlogging resistance (YIN et al., 2009; YIN et al., 2010). The greater accumulation of ROS in waterlogged tissues elevated the extent of lipid peroxidation. MDA level representing the degree of lipid peroxidation is a classic parameter to reflect oxidative injury (WU et al., 2003; YAN et al., 2013). In our study, MDA content in WT and three silencing lines was higher than three overexpressing lines throughout the waterlogging period (Figure 2b), indicating that overexpression of *PhERF2* in plants seems to reduce oxidative injury. The synthesis and degradation of soluble proteins are associated with the responses of plants to stressful conditions (GUAN et al., 2010). The results from our studies showed that soluble protein content increased first followed by a remarkable reduction. During the waterlogging phases from 24h to 72h, the content of soluble protein in *PhERF2*-overexpressing lines was remarkably higher than in WT and *PhERF2*-RNAi lines (Figure 3a). These results of physiological indicators indicated that transgenic petunia plants overexpressing *PhERF2* were more tolerant to waterlogging stress than WT and *PhERF2*-silenced plants, which is similar to the findings in transgenic tobacco plants with overexpression of the cotton *CBF* gene *GbCBF1* (GUO et al., 2011).

The decrease in *Pn* demonstrates the vulnerability of photosynthesis in Jerusalem artichoke under waterlogging. According to the collective decline of *Ci*, *Gs* and *Tr,* the waterlogging stress forced the stomata of plant leaves to close, *Gs* decreased, and the ability of leaves to absorb CO2 decreased, resulting in a decrease in *Ci*, which in turn led to a decrease in *Pn*, which further led to the stomatal limitation of photosynthesis. (YAN et al., 2018). In *PhERF2*-overexpressing lines, *Ci*, *Gs*, and *Tr* all decreased after 6h of waterlogging treatment, compared with their control levels (Figure 1), indicating that *Pn* was mainly affected by limited leaf gas exchange. The decrease in stomatal conductance is the primary limiting factor for *Pn*, while non-stomatal limitation can also affect *Pn* under severe waterlogging stress, such as the decreased chlorophyll content and increased MDA levels. Severe waterlogging causes a large amount of ROS to accumulate in plants and damage the chloroplasts, resulting in a decrease in the activity of photosynthetic enzymes, a decrease in the ability of plants to carry out photosynthesis, and a decrease in the assimilation ability of mesophyll cells to CO2, which directly leads to a decrease in *Pn* (ZHENG et al., 2009). In WT and *PhERF2*-RNAi lines, *Gs* and *Tr* decreased but *Ci* gradually increased during the periods from 6h to 72h, compared to the control plants without waterlogging (Figure 1), suggesting that non-stomatal limitation factors are the major factors for *Pn*. Previous studies have reported that stomatal closure would be helpful to keep water status in leaves by reducing the water loss from transpiration (TAKAHASHI & MURATA, 2008; GILL & TUTEJA, 2010). In both WT and transgenic lines, *Gs* declined continuously during the whole experiment, while *Gs* in WT and *PhERF2*-RNAi lines was higher than that in *PhERF2*-overexpressing lines at the late stages of waterlogging (Figure 1b), implying that overexpression of *PhERF2* would make these transgenic plants more tolerant to waterlogging stress.

CONCLUSION

In summary, a set of photosynthetic and physiological parameters were variable in *PhERF2*-silenced and -overexpressing transgenic petunia plants under waterlogging stress, compared to WT control. The overexpressing lines exhibited a better maintenance of chlorophyll content and root vigor than the WT and silenced lines upon waterlogging treatment. The waterlogging resulted in a reduction in *Pn*, *Gs*, and *Tr* levels in all lines, but an increase in *Ci* levels in *PhERF2*-silenced lines. Our data support an important positive role of *PhERF2* in the modulation of waterlogging tolerance in petunia.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

REFERENCES

AHMED, S., et al. Alterations in photosynthesis and some antioxidant enzymatic activities of mungbean subjected to waterlogging. **Plant Science**, v.163, n.1, p.117-123. 2002. Available from: <https://doi.org/10.1016/S0168-9452(02)00080-8>. Accessed: Jul. 07, 2021. doi: 10.1016/S0168-9452(02)00080-8.

ARNON, D. I. Copper enzymes in isolated chloroplasts. polyphenoloxidase in beta vulgaris. **Plant Physiology**, n.1, p.1. 1949. Available from: <https://academic.oup.com/plphys/article/24/1/1/6076157?searchresult=1>. Accessed: Jul. 07, 2021. doi: 10.1104/pp.24.1.1.

BARICKMAN, T. C., et al. Waterlogging causes early Modification in the Physiological Performance, Carotenoids, Chlorophylls, Proline, and Soluble Sugars of Cucumber Plants. **Plants**, v.8, n.6. 2019. Available from: <https://www.mdpi.com/2223-7747/8/6/160/htm>. Accessed: Jul. 07, 2021. doi: 10.3390/plants8060160.

CHEN, D. Q., et al. Comparative Physiological Effects of Genotype Difference of Wheat Resistance to Waterlogging on Stress. **Journal**

of Hub Agricultural College, v.18. 1998. Available from: <http://en.cnki.com.cn/Article_en/CJFDTOTAL-HBNX804.001.htm>. Accessed: Jul. 07, 2021. doi: CNKI:S512.101.

DAOYANG, S., et al. A petunia ethylene-responsive element binding factor, PhERF2, plays an important role in antiviral RNA silencing. **Journal of experimental botany**. 2016. Available from: <https://doi.org/10.1093/jxb/erw155>. Accessed: Jul. 07, 2021. doi: 10.1093/jxb/erw155.

DELAUNEY, A. J.; D. P. S. VERMA. Proline biosynthesis and osmoregulation in plants. **Plant Journal for Cell & Molecular Biology**, v.4, n.2, p.215-223. 1993. Available from: <https://doi.org/10.1046/j.1365-313X.1993.04020215.x>. Accessed: Jul. 07, 2021. doi: 10.1046/j.1365-313X.1993.04020215.x.

DREW, M. C. Oxygen deficiency and root metabolism: Injury and Acclimation Under Hypoxia and Anoxia. **Annu Rev Plant Physiol Plant Mol Biol**, v.48, n.1, p.223-250. 1997. Available from: <https://www.annualreviews.org/doi/abs/10.1146/annurev.arplant.48.1.223>. Accessed: Jul. 07, 2021. doi: 10.1146/annurev.arplant.48.1.223.

ELSE, et al. Decreased root hydraulic conductivity reduces leaf water potential, initiates stomatal closure and slows leaf expansion in flooded plants of castor oil (*Ricinus communis*) despite diminished delivery of ABA from the roots to shoots in xylem sap. **Physiologia Plantarum**. 2001. Available from: <https://doi.org/10.1034/j.1399-3054.2001.1110107.x>. Accessed: Jul. 07, 2021. doi: 10.1034/j.1399-3054.2001.1110107.x.

FUKAO, T.; J. BAILEY-SERRES. Submergence tolerance Conferred by Sub1A Is Mediated by SLR1 and SLRL1 Restriction of Gibberellin Responses in Rice. **Proceedings of the National Academy of Sciences of the United States of America**, v.105, n.43. 2008. Available from: <https://doi.org/10.1073/pnas.0807821105>. Accessed: Jul. 07, 2021. doi: 10.1073/pnas.0807821105.

FUKAO, T., et al. A variable Cluster of Ethylene Response Factor–Like Genes Regulates Metabolic and Developmental Acclimation Responses to Submergence in Rice W. **Plant Cell**, v.18, n.8, p.2021-2034. 2006. Available from: <https://doi.org/10.1105/tpc.106.043000>. Accessed: Jul. 07, 2021. doi: 10.2307/20076754.

GAO, et al. Overexpression of Chrysanthemum lavandulifolium ClCBF1 in *Chrysanthemum morifolium* 'White Snow' improves the level of salinity and drought tolerance. **Plant Physiol Bioch**. 2018. Available from: <https://doi.org/10.1016/j.plaphy.2018.01.004>. Accessed: Jul. 07, 2021. doi: 10.1016/j.plaphy.2018.01.004.

GERATS, T.; J. STROMMER. Petunia. Evolutionary, developmental and Physiological Genetics. **Annals of Botany**, v.107, n.4, p.: vi–vii. 2011. Available from: <https://doi.org/10.1093/aob/mcr051>. Accessed: Jul. 07, 2021. doi: 10.1007/978-0-387-84796-2.

GILL, S. S.; N. TUTEJA. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiol Biochem**, v.48, n.12, p.909-930. 2010. Available from: <https://doi.org/10.1016/j.plaphy.2010.08.016>. Accessed: Jul. 07, 2021. doi: 10.1016/j.plaphy.2010.08.016.

GUAN, Z. Y., et al. Screening of salt-tolerance concentration and comparison of salt-tolerance for chrysanthemum and its related taxa. **Chinese Journal of Ecology**. 2010. Available from: <http://en.cnki.com.cn/Article_en/CJFDTOTAL-STXZ201003010.htm>. Accessed: Jul. 07, 2021. doi: 10.1080/00949651003724790.

GUO, H. M., et al. Cloning of cotton CBF Gene and Its Cold Tolerance Expression in Transgenic Tobacco. **Acta Agronomica Sinica**, v.37, n.2, p.286-293. 2011. Available from: <https://doi.org/10.1016/S1875-2780(11)60009-6>. Accessed: Jul. 07, 2021. doi: 10.3724/SP.J.1006.2011.00286.

HERZOG, M., et al. Mechanisms of waterlogging tolerance in wheat - a review of root and shoot physiology. **Plant Cell & Environment**, v.39, n.5, p.1068-1086. 2016. Available from: <https://doi.org/10.1111/pce.12676>. Accessed: Jul. 07, 2021. doi: 10.1111/pce.12676.

IRFAN, M., et al. Physiological and biochemical changes in plants under waterlogging. **Protoplasma**, v.241, n.1-4, p.3-17. 2010. Available from: <https://link.springer.com/article/10.1007/s00709-009-0098-8>. Accessed: Jul. 07, 2021. doi: 10.1007/s00709-009-0098-8.

KUMAR, P., et al. Yield, growth and physiological responses of mung bean [*Vigna radiata* (L.) Wilczek] genotypes to waterlogging at vegetative stage. **Physiology & Molecular Biology of Plants**, v.19, n.2, p.209-220. 2013. Available from: <https://doi.org/10.1007/s12298-012-0153-3>. Accessed: Jul. 07, 2021. doi: 10.1007/s12298-012-0153-3.

KUMUTHA, D., et al. Waterlogging induced oxidative stress and antioxidant activity in pigeonpea genotypes. **Biologia Plantarum**, v.53, n.1, p.75-84. 2009. Available from: <https://doi.org/10.1007/s10535-009-0011-53>. Accessed: Jul. 07, 2021. doi: 10.1007/s10535-009-0011-5.

KUMUTHA, D., et al. Effect of waterlogging on carbohydrate metabolism in pigeon pea (*Cajanus cajan* L.): Upregulation of sucrose synthase and alcohol dehydrogenase. **Plant Science**, v.175, n.5, p.706-716. 2008. Available from: <https://doi.org/10.1016/j.plantsci.2008.07.013>. Accessed: Jul. 07, 2021. doi: 10.1016/j.plantsci.2008.07.013.

KUN, et al. Responses of photosynthesis, lipid peroxidation and antioxidant system in leaves of Quercus mongolica to elevated O3. **Environmental and Experimental Botany**. 2010. Available from: <https://doi.org/10.1016/j.envexpbot.2010.03.008>. Accessed: Jul. 07, 2021. doi: 10.1016/j.envexpbot.2010.03.008.

LICAUSI, F., et al. HRE1 and HRE2, two hypoxia-inducible ethylene response factors, affect anaerobic responses in Arabidopsis thaliana. **Plant Journal.** 2010. Available from: <https://doi.org/10.1111/j.1365-313X.2010.04149.x>. Accessed: Jul. 07, 2021. doi: 10.1111/j.1365-313x.2010.04149.x.

MIN, H. U.; H. E. DE -XIAN. Study on diurnal Fluctuations of Physiological Activity of Roots and the Optimal Time to Sample and Determine Their Activity in Wheat (*Triticum aestivum* L.). **Journal of Triticeae Crops**. 2011. Available from: <http://en.cnki.com.cn/Article_en/CJFDTOTAL-MLZW201106017.htm>. Accessed: Jul. 07, 2021. doi: CNKI:SUN:MLZW.0.2011-06-017.

MUNNÉ-BOSCH, S.; L. ALEGRE. Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in Rosmarinus officinalis plants. **Planta**, v.210, n.6, p.925-931. 2000. Available from: <https://doi.org/10.1007/s004250050699>. Accessed: Jul. 07, 2021. doi: 10.1007/s004250050699.

PEREIRA, et al. Tolerance to waterlogging in young Euterpe oleracea plants,. **Photosynthetica**: **International Journal for**

Photosynthesis Research. 2014. Available from: <https://doi.org/10.1007/s11099-014-0021-1>. Accessed: Jul. 07, 2021. doi: 10.1007/s11099-014-0021-1.

PÉREZ-JIMÉNEZ, M., et al. Are commercial sweet cherry rootstocks adapted to climate change? Short-term waterlogging and CO2 effects on sweet cherry cv. 'Burlat'. **Plant**, **Cell & Environment**, v.41, n.5. 2018. Available from: <https://doi.org/10.1111/pce.12920>. Accessed: Jul. 07, 2021. doi: 10.1111/pce.12920.

RONG-HUA, L. I., et al. Evaluation of chlorophyll Content and Fluorescence Parameters as Indicators of Drought Tolerance in Barley. **Agricultural ences in China**, v.5, n.10, p.751-757. 2006. Available from: <https://doi.org/10.1016/S1671-2927(06)60120-X>. Accessed: Jul. 07, 2021. doi: 10.1016/S1671-2927(06)60120-X.

SAGREDO, et al. Physiological and morphological responses of Prunus species with different degree of tolerance to long-term root hypoxia. **Scientia Horticulturae**. 2014. Available from: <https://doi.org/10.1016/j.scienta.2014.09.055>. Accessed: Jul. 07, 2021. doi: 10.1016/j.scienta.2014.09.055.

SHI, Y. W., et al. Effects of water Stress on Soluble Protein,Soluble Sugar and Proline Content in Tamarix hispida. **Journal of Xinjiang Agricultural University**. 2007. Available from: <https://doi.org/1007-8614.2007.02.002>. Accessed: Jul. 07, 2021. doi: 10.3969/j.issn.1007-8614.2007.02.002.

SHIONO, K., et al. Role of ethylene in acclimations to promote oxygen transport in roots of plants in waterlogged soils. **Plant Science**, v.175, n.1-2, p.52-58. 2008. Available from: <https://doi.org/10.1016/j.plantsci.2008.03.002>. Accessed: Jul. 07, 2021. doi: 10.1016/j.plantsci.2008.03.002.

SMETHURST, C. F., et al. Nutritional and chlorophyll fluorescence responses of lucerne (*Medicago sativa*) to waterlogging and subsequent recovery. **Plant and Soil**, v.270, n.1, p.31-45. 2005. Available from: <https://doi.org/10.1007/s11104-004-1082-x>. Accessed: Jul. 07, 2021. doi:10.1007/s11104-004-1082-x.

SONG, L. U. Effects of drought stress on plant growth and physiological traits. **Journal of Jiangsu Forestry Science & Technology**. 2012. Available from: <http://en.cnki.com.cn/Article_en/CJFDTOTAL-JSLY201204014.htm>. Accessed: Jul. 07, 2021. doi: 10.3969/j.issn.1001-7380.2012.04.015.

STRIKER, G. G.; T. D. COLMER. Flooding tolerance of forage legumes. **Journal of experimental botany**, v.68, n.1851, p.1851. 2017. Available from: <https://doi.org/10.1093/jxb/erw239>. Accessed: Jul. 07, 2021. doi: doi:10.2307/2997075.

TAKAHASHI, S.; N. MURATA. How do environmental stresses accelerate photoinhibition? **Trends in Plant Science**, v.13, n.4, p.178-182. 2008. Available from: <https://doi.org/10.1016/j.tplants.2008.01.005>. Accessed: Jul. 07, 2021. doi: 10.1016/j.tplants.2008.01.005.

WARING, E. F.; B. R. MARICLE. Photosynthetic variation and carbon isotope discrimination in invasive wetland grasses in response to flooding. **Environmental & Experimental Botany**, v.77, n.none, p.77-86. 2012. Available from: <https://doi.org/10.1016/j.envexpbot.2011.10.013>. Accessed: Jul. 07, 2021. doi: 10.1016/j.envexpbot.2011.10.013.

WU, F., et al. Four barley genotypes respond differently to cadmium: lipid peroxidation and activities of antioxidant capacity. **Environmental & Experimental Botany**, v.50, n.1, p.67-78. 2003. Available from: <https://doi.org/10.1016/S0098-8472(02)00113-2>. Accessed: Jul. 07, 2021. doi: 10.1016/S0098-8472(02)00113-2.

XIA, Y., et al. **The World Floriculture Industry**: **Dynamics of Production and Markets**: Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues Vol IV. 2006.

XIAO, et al. Physiological and proteomic mechanisms of waterlogging priming improves tolerance to waterlogging stress in wheat (*Triticum aestivum* L.). **Environmental & Experimental Botany**. 2016. Available from: <https://doi.org/10.1016/j.envexpbot.2016.09.003>. Accessed: Jul. 07, 2021. doi: 10.1016/j.envexpbot.2016.09.003.

XU, K., et al. Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. **Nature**, v.442, n.7103, p.705-708. 2006. Available from: <https://doi.org/10.1038/nature04920>. Accessed: Jul. 07, 2021. doi: 10.1038/nature04920.

YAN, K., et al. Physiological adaptive mechanisms of plants grown in saline soil and implications for sustainable saline agriculture in coastal zone. **Acta Physiologiae Plantarum**, v.35, n.10, p.2867-2878. 2013. Available from: <https://doi.org/10.1007/s11738-013-1325-7>. Accessed: Jul. 07, 2021. doi: 10.1007/s11738-013-1325-7.

YAN, K., et al. Vulnerability of photosynthesis and photosystem I in Jerusalem artichoke (*Helianthus tuberosus* L.) exposed to waterlogging. **Plant Physiology & Biochemistry**, v.125, p.239-246. 2018. Available from: <https://doi.org/10.1016/j.plaphy.2018.02.017>. Accessed: Jul. 07, 2021. doi: 10.1016/j.plaphy.2018.02.017.

YIN, D., et al. Morphological and physiological responses of two chrysanthemum cultivars differing in their tolerance to waterlogging. **Environmental and Experimental Botany**, v.67, n.1, p.87-93. 2009. Available from: <https://doi.org/10.1016/j.envexpbot.2009.06.006>. Accessed: Jul. 07, 2021. doi: 10.1016/j.envexpbot.2009.06.006.

YIN, D., et al. Morpho-anatomical and physiological responses of two Dendranthema species to waterlogging. **Environmental & Experimental Botany**, v.68, n.2, p.122-130. 2010. Available from: <https://doi.org/10.1016/j.envexpbot.2009.11.008>. Accessed: Jul. 07, 2021. doi: 10.1016/j.envexpbot.2009.11.008.

YIN, D., et al. PhERF2, an ethylene-responsive element binding factor, plays an essential role in waterlogging tolerance of petunia. **Horticulture Research**, v.6, n.1, p.83. 2019. Available from: <https://doi.org/10.1038/s41438-019-0165-z>. Accessed: Jul. 07, 2021. doi: 10.1038/s41438-019-0165-z.

ZHANG, Y., et al. Growth, lint yield and changes in physiological attributes of cotton under temporal waterlogging. **Field Crops Research**, p.83-93. 2016. Available from: <https://doi.org/10.1016/j.fcr.2016.05.006>. Accessed: Jul. 07, 2021. doi: 10.1016/j.fcr.2016.05.006.

ZHENG, C., et al. Effects of salt and waterlogging stresses and their combination on leaf photosynthesis, chloroplast ATP synthesis, and antioxidant capacity in wheat. **Plant Science An International Journal of Experimental Plant Biology**, v.176, n.4, p.575-582. 2009. Available from: <https://doi.org/10.1016/j.plantsci.2009.01.015>. Accessed: Jul. 07, 2021. doi: 10.1016/j.plantsci.2009.01.015.

ZHU, M. et al. Morphological and photosynthetic response of waxy corn inbred line to waterlogging. **Photosynthetica**. 2016. Available from: <https://doi.org/10.1007/s11099-016-0203-0>. Accessed: Jul. 07, 2021. doi: 10.1007/s11099-016-0203-0.