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RESEARCH ARTICLE

Evaluation of Different Salinity Tolerance of Aeluropus and Rice Based on the Variation in Structure and Expression of their Catalase Enzyme

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ABSTRACT: There is an imbalance between increase rate in demand for agricultural products and the growth rate of agricultural production. Much of this production deficit is attributed to abiotic stresses. These stresses reduce the yield of crops by more than 50%. Obviously, it worth studying any idea which may lead to reducing the damages of them. In the present research, the transcription level of Catalase in root and shoot of *Oryza sativa* var. IR64 and *Aeluropus littoralis* using qRT-PCR and the Aromatic and Instability indices based on amino acid composition were evaluated. The samples were taken at short-term, mid-term and long-term stress span. Analysis of the results showed significant differences in the both gene expression and studied biochemical aspects. The expression of catalase gene in Aeluropus roots was periodic and showed a twice increase and then a decrease however in roots of rice there was just a rise in its expression. In the all sampling of the rice shoots, *CAT* gene expression levels were either lower or without any significant different in contrast to the control samples. Meanwhile, the rates of expression in most of stressed Aeluropus shoots were significantly higher than control samples. Comparison of the biochemical indices showed that Aeluropus has a relative superiority over rice in terms of amino acid sequences. Based on the evaluated indices, the differences of response to salinity stress in the studied plants could be attributed to the differences in the promoter and nucleotide sequences of their genes.

KEYWORDS: Aromatic Index, Instability Index, Oxidative Stress, Salt Stress.

INTRODUCTION

One of the most serious problems in the current agricultural macroeconomics is the imbalance between high rate of increase in demand for agricultural products and the current growth rate of agricultural production. The demand is increasing at least due to two factors; Population growth and increase in the new variety of consumption, such as biofuels. The human population is estimated to reach 9 billion by 2050, with 44 million tons of additional food production that are required to meet agricultural demand. In contrast, the current rate of growth in agricultural production is about 32 million tons per year [1,2] which means a 38% lack of the required rate. Much of this production deficit is often attributed to abiotic stresses [3]. Abiotic stresses such as drought, salinity, extreme temperatures and the abuse of chemical agents are seen as increasing threats to agricultural security. These stresses cause a series of adverse molecular, biochemical, physiological and morphological changes that reduce the yield of many important crops by more than 50 percent [3]. While considering the salient role of ROS in triggering the signaling system and observing their wide variations in response to salinity

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stress [4], the aim of this study was to explore how the leaves and roots of rice plants (*Oryza sativa* var. IR64) and Aeluropus (*Aeluropus littoralis*) response to oxidative damages caused by salinity stress. The transcription level of the Catalase gene and the Aromatic and Instability indices based on amino acid composition were evaluated in the two plants with contrasting features [5,6]. Among the 134 introduced indices, the instability and aromatic indices are two of the factors that affect the stability of the protein in an experimental environment. So, based on our evaluation goal, these two indices were chosen to compare differences of catalase (*CAT*) gene properties between rice and Aeluropus while salt concentration increased. The instability index is a reliable index determined based on the amino acid content of a polypeptide's sequence which have been normalized based on its length. In fact, this index, have been calculated based on statistical tests on a number of stable and unstable proteins and actually indicates the stability or half-life of a polypeptide strand outside the cellular environment [7]. The critical value of stability and instability in this index is considered to be 40. values smaller than 40 suggest greater stability, whereas values greater than 40 indicate intense instability [7].

In other words, it could be a determinant of sensitivity to changes in environmental conditions. The current study was being tried to assess what effects the amino acid composition and expression of the catalase gene may have in the ability of rice and Aeluropus to tolerate salinity stress.

MATERIALS AND METHODS

Preparation of plant materials

Seeds of *A. littoralis* were purchased from *Center for Research of Agricultural Science and Natural Resources* (CRASN) of Isfahan and the seeds of *O. sativa* var IR64 were kindly provided by the CRASN of Amol. Rice (*O. sativa* var IR64) is relatively susceptible to salinity and was released by IRRI [8] The seeds were sown in sand as a culture medium. After 21 days, the seedlings that had grown uniformly were transferred to 8-liter pots containing Yoshida nutrient solution with a pH of about 5.5 that was constantly aerated by an air pump.

Preliminary experiment

In a preliminary experiment, optimal NaCl concentrations and the number of evaluations for the two plant species were determined while relevant specifications from the literature were taken into account. The specifications were based on physiological responses and the main experiment was carried out accordingly. Three weeks after transferring the seedlings to the hydroponic culture medium, the salinity stress was applied. The salinity concentration was increased gradually (every 48 hours) until the desired concentration was reached. In the case of Aeluropus, 100 mM NaCl was added to the culture medium every 48 hours until the concentration reached 600 mM. Samples of the Aeluropus group were harvested at 1, 2, 4, 6, 8, 12, 24 and 48 hours at salinity concentrations of 100, 200, 300, 400, 500 and 600 mM. In the case of rice, the salinity concentrations were increased every 48 hours and the plants were sampled every 2, 4, 8, 24 and 48 hours at salinity concentrations of 30, 60, 100 and 150 mM. The control plants were grown simultaneously in pure Yoshida culture medium. For each time duration/salinity concentration-dependent sampling point, three replications were taken. The main characteristics evaluated were fresh and dry weight, sodium and potassium concentrations, and electrolyte leakage index (data not shown). Finally, reliable sampling points were revealed and chosen for use in the main experiment. Regarding Aeluropus, the elite sampling points were 6 h / 100 mM, 6 h / 200 mM, 6 h / 300 mM, 48 h / 300 mM, 144 h / 300 mM and 264 h / 300 mM. the elite sampling timepoint of rice, were 6 h / 30 mM, 6 h / 60 mM, 6 h / 100 mM, 48 hours / 100 mM, 144 hours / 100 mM and 264 hours / 100 mM.

Gene expression

The RNA extraction was specifically performed on the shoots and roots of Aeluropus (*A. littoralis*) and rice (*O. sativa*) using Dena Zist Company kit (# S-1020, Iran), according to instructions in the manual guide. To eliminate the possibility of contamination by genomic DNA, 10 µl Thermo® DNase I, was added to each 5000 ng of total RNA. The first strand of complementary DNAs (cDNAs) were synthesized using a Thermo® kit according to the manual guide. qRT-PCR reactions were performed using AMPLIQON's RealQ Plus mixture in Bioer Real-Time thermo cycler. In order to normalize the data, as internal control, *UBQ5* and *elf* genes were used in the case of rice, whereas β*act* and *elf* genes were used for Aeluropus. The primers used in this experiment which were designed using Primer Premier© v5 are shown in Table 1. In addition to three biological replications, two or three technical replications were considered for each

Table 1. Primer list for qRT-PCR analysis of rice and Aeluropus CAT gene.

Plant	Gene	sequences (5'–3')	size (bp)	Accession
Rice	*CAT*	ggacgaggaggtggactact tgcttgtgtatcgtcgcctt	117	NM_001401748.1
	ubq	gcggaagtaaggaaggaggag cagaggtgatgctaaggtgttc	116	XM_015766305.2
	elf	acggtgatgctggtatgg ggttgggtccttcttctcc	155	XM_015774317.2
Aeluropus	*CAT*	cggcgtcaacacctacacc gaagtttcctgctgcgatgg	181	HQ389206.1
	βact	ttgctggccgagaccttac ggcgagcttttccttgatg	113	FJ603097.1
	elf	accttctctgaataccctcctctg cttctccacactcttgatgactcc	90	XM_004962214.4

sample in order to improve experiment accuracy [9]. In the current research, relative quantification and $2^{-\Delta\Delta Ct}$ methods were used for analyzing the results of quantitative polymerase chain reactions [10].

Gene structure

In studying the structure of genes, effective in oxidative stress, their sequences were extracted with the maximum possible length. The primers were designed using Primer Premier V5 and, their binding status checked using the Primer BLAST tool. Details of the designed primers are given in Table 1. For reducing mistakes in the replication process, amplifying the sequences of the genes was done using Suprime HF DNA Polymerase enzyme (GenetBio Co.), which had editing ability during replication. The pTZ57R vector in *Escherichia coli* (DH5α strain) was used for cloning gene fragments. Recombinant plasmids were extracted and sent to MacroGen for sequencing their gene fragments. In order to increase accuracy, paired-end sequencing was performed.

The results were analyzed using NCBI database, and BioEdit V2.2 and Mega V6 softwares. Nucleotide and amino acid sequences that resulted from the studied genes were identified from three aspects of genetic distance, similarity coefficient and closest relative. biochemical features of the resultant proteins, such as Isoelectric points, Calculated Weight, instability indices and aromatic index, were analyzed Insilco using CLC Genomics Workbench 12 and Geneious Prime 2019.

RESULTS

CAT gene expression in IR64 and Aeluropus roots

The expression of catalase gene in Aeluropus roots (Fig.1) was periodic and showed a twice increase and then a

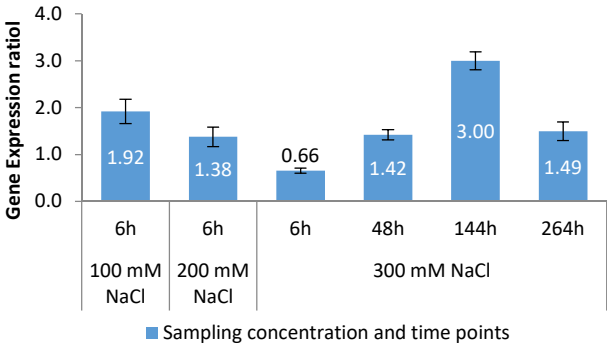


Figure 1. expression ratio of CAT in root of treated Aeluropus to control.

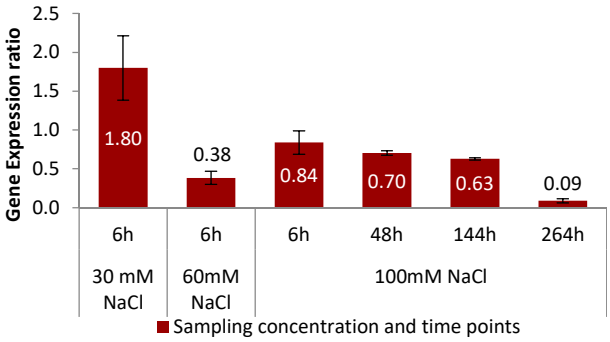


Figure 2. expression ratio of CAT in root of treated IR64 to control.

decrease through the timespan of the experiment. From there, and with the onset of salinity stress, CAT expression increased to about 1.9 times, compared to that of the control samples. Despite an increase in salinity concentration and exposure time, mRNA expression decreased, so that after six hours of 300 mM exposure, the expression rate was 30% lower than in the corresponding control samples. A recurrent increase in the expression of this gene was observed after 144 h of root exposure to 300 mM salinity. The highest mRNA concentration in the present study was about 3 times that of the control samples and was observed at this time duration/salinity concentration-dependent sampling time (Fig. 1). A long-term exposure (11 days) of the Aeluropus roots to the salinity and the stress thereof did not result in a significant increase in gene expression when compared to control samples (based on comparison of mean values using the t-test).

In rice roots, expression of the CAT gene was not similar to the case of Aeluropus. According to the data, six hours after the exposure of rice roots to NaCl solution, there was a rise (1.8 times) in CAT expression (Fig. 2). Contrary to expectations, however, increasing the concentration and duration of stress caused a continuous decrease of CAT

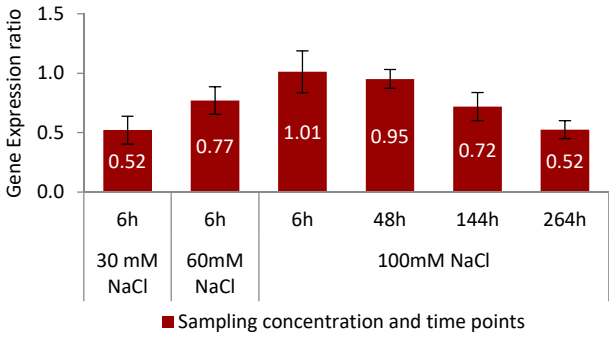


Figure 3. expression ratio of CAT in Shoot of treated IR64 to control.

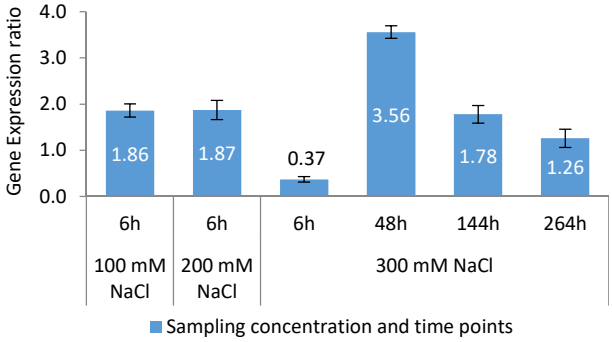
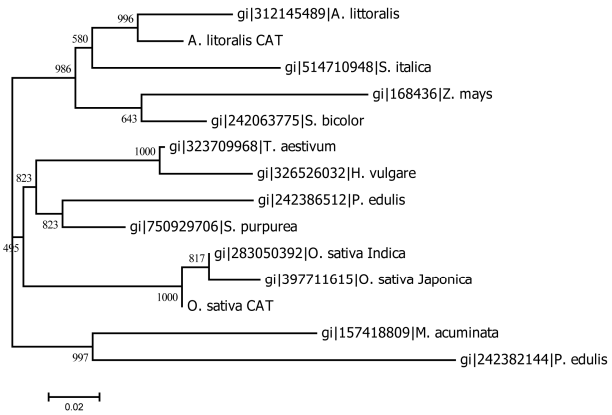
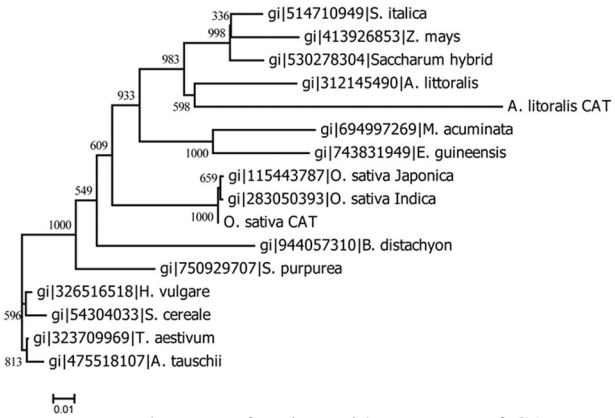


Figure 4. expression ratio of CAT in Shoot of treated Aeluropus to control.



Figuer 5. Dendrogram of nucleotide sequences of CAT gene using n-joining method.



Figuer 6. Dendrogram of amino acid sequences of CAT gene using n-joining method.

mRNA in IR64 roots. Under 100 mM NaCl, there was a stable trend of decrease in mRNA, so that after 11 days the expression of this gene was suppressed to one tenth of the control samples. Unlike Aeluropus roots, there was no secondary additive response in rice roots.

CAT gene expression in shoots

In the case of all-time duration/salinity concentration-dependent sampling times, CAT gene expression levels were lower or even no different in contrast to the control samples in the IR64 shoots (Fig. 3). Meanwhile, Aeluropus shoots responded differently. Apart from samples that were exposed to 300 mM NaCl for 6 and 264 hours, the rates of expression in other stressed samples were higher than in the control samples (Fig. 4). The highest increase in expression (about 3.5 times that of the control sample) was observed after 48 hours of exposure to 300 mM salinity, whereas the lowest expression (about 0.4 of the control sample) was observed after 6 hours in response to the same salinity concentration. As the duration of stress increased, the expression of CAT decreased in the photosynthetic tissues of Aeluropus (Fig.4).

Bioinformatically analysis of the CAT sequences Similarity

The study of genetic distance in the catalase gene of the studied plants showed that the nucleotide sequence of the catalase gene in IR64 have similarity with Indica type of *O. sativa* (GI: 283050392), Japonica type of *O. sativa* (GI: 397711615) and *Hordeum vulgare* by 99%, 99% and 87%, respectively (Fig. 5). The Aeluropus catalase gene was similar to those of *A. littoralis*, *Setaria italica* and *Sorgom bicolor* sequences by 94%, 87% and 86%, respectively. Comparing IR64 and Aeluropus, the sequence similarity of this gene was 81% (Fig. 5). The amino acid sequence of the catalase gene of IR64 was fully consistent with its corresponding sequence in the Japonica rice. Thereafter, the most similar sample was the sequence of Indica rice with 99% similarity coefficient, which is registered in the NCBI database as GI 283050392. The closest sequence after that was the sequence of *Stipa purpurea* with 83% similarity (Fig. 6). The sequences of two species in this study (IR64 and Aeluropus) showed only 49% similarity to each other in terms of the amino acid sequence of the catalase gene. Meanwhile, *S. purpurea* is a species that has good tolerance to abiotic stresses such as drought and cold,

Table 2. Some biochemical and biophysical parameters of rice and Aeluropus's CAT genes.

Protein Parameters	*O. sativa (IR64)*	*A. litoralis*
Calculated Weight (kDa)	56.01	40.57
Isoelectric point	6.19	8.45
Aromatic index	0.123	0.110
Instability index	39.28	35.87

whereby a better performance of enzymes reportedly exists in response to oxidative stress [11].

Biochemical indices

A comparison of the instability index and aromatic index showed that Aeluropus has a relative superiority over IR64 in terms of amino acid sequences (Table 2). Of all the amino acid sequences studied in this research, the sequences of the catalase gene showed the greatest difference between the two species. The ORF of catalase in Aeluropus was shorter in length than that of the sequence in IR64.

DISCUSSION

Any change in the amino acid sequence of a polypeptide strand can affect the physicochemical structure of the polypeptide strand. So far, 134 properties have been identified for a polypeptide strand, the modification of which can affect its function, stability, and structure [7,12,13]. Although the value of each is different in terms of the final change, so far, no credible reference has scaled them in order of importance. In the present study, in addition to evaluation of the similarity of nucleotide and amino acid sequences, 4 of the mentioned properties were evaluated and compared. In addition, the reoccurrence of similarity in different genetic sequences with some species such as *S. italica*, which have multiple sequences registered in NCBI databases, could facilitate future studies on *A. littoralis*, of which very limited gene sequence information has been recorded so far. In the present study, as is summarized in Table 3, the instability index of CAT protein in Aeluropus is lower than that of the corresponding sequence in IR64. This difference may indicate that the enzyme structure in Aeluropus cells is more stable in response to salinity stress-related changes, such as changes in ion concentrations, than in rice cells. For instance, previous research on *E. coli* showed that the protein D-amino acid oxidase with an instability index of 34.4 had a half-life of 118 hours, while HSP70 protein with instability index of 44.1 had a half-life of only 1 to 2 hours [14].

Aromatic index is also a measure of the abundance of aromatic amino acids in a polypeptide strand. The most important of these amino acids are Phe, Trp and Tyr. The aromatic ring of these amino acids usually play an important role in the rate and accuracy of secondary structure formation [15,16]. The significance of this index is also taken into account in recent protein engineering studies. For instance, the presence of sulfur groups attached to aromatic rings reportedly caused an increase in the folding rate of RNase A by up to 23 times *in vitro* [17]. The current research also showed that this index is higher in Aeluropus CAT enzyme, compared to the IR64 index in polypeptide strand of its CAT. This difference can be cited as an indication of the relative superiority of Aeluropus over IR64 regarding the amino acid sequences of the enzymes involved in oxidative stress response. The replacement of Tyr with Phe reportedly has a negligible effect on the function of the oxytocin hormone, while its replacement with Ser, which lacks an aromatic ring in its structure, reduced the function of the hormone to such an extent that its activity was undetectable [7]. In calculating this index, thus, the prevalence of these amino acids is emphasized more than their type, so that the value coefficient of one is considered equal to another [18]. The feasibility or impossibility of a genetic research process is determined by three factors: identification of the desired gene, an appropriate method of gene transfer, and the use of an appropriate promoter for regulated expression of the transferred gene. These markers actually emphasize differences in the quality of proteins that are themselves derived from nucleotide composition and, along with the rate of expression, affect their durability under stress conditions. In applied biotechnology-based breeding researches, this index can be used for selecting a gene or its donor [19–21].

Indeed, catalase, as an enzyme, is responsible for the majority of H_2O_2 degradation (detoxification); therefore, reducing its function (duo to diminution of its concentration or activity) may result in excessive H_2O_2 accumulation [22]. H_2O_2 accumulation and a decreased level of CAT concentration in older tissues than in young or ABA-treated tissues have also been demonstrated in many reported researches [23]. Inactivation of CAT with aminotriazole also increased the internal concentration of H_2O_2 and the cellular damage associated with it. It was previously reported that after the RNAi method significantly reduced the level of CAT production in

tobacco (*Nicotiana tabacum* cv. 'Petit Havana SR'), the H_2O_2 concentration increased rapidly in response to increasing light intensity, as well as a hypersensitivity reaction and associated symptoms such as increased ethylene and salicylic acid production [22]. an important problem that occurs from increasing concentration of H_2O_2 is its ability to convert to hydroxyl free radical. Gondim et al., (2012) showed that the main factor in reducing CAT concentration was a decrease in gene expression at the mRNA level in tobacco. According to the results, the lower level of CAT expression and irregular changes in its expression in rice plants compared to Aeluropus resulted in H_2O_2 accumulation. This can be attributed to the higher sensitivity of IR64 to salinity stress, compared to the response staged by Aeluropus.

While the final product of superoxide dismutase (SOD) activities is the production of H_2O_2 and based on the function of H_2O_2 and the role of CAT in its scavenging, researchers have discovered important clues in outlining the patterns of signaling and imposing damages by monitoring changes in H_2O_2 concentrations in comparisons between halophytes and glycophytes [25–27]. For instance, a comparison was made between *Cakile maritime* as a halophyte and *Arabidopsis taliana* as a glycophyte [5]. It was found that the concentration of H_2O_2 in *C. maritime* during the first 4 hours of salinity stress reached a maximum and then decreased rapidly, whereas in *A. taliana* the H_2O_2 concentration increased gradually throughout the study period (for 72 h from the initiation of stress). This difference clearly indicates that the halophyte plant experienced immediate stress signaling in the form of an increase in H_2O_2 concentration. Following the completion of signaling, the H_2O_2 concentration was adjusted and under the control of an efficient antioxidant system.

In the present study, the average expression of CAT gene in the roots and shoots of Aeluropus was higher than in rice, especially in response to increasing the salinity concentration and extending the exposure time of stress. CAT activity and a decrease in H_2O_2 concentration can help increase plant tolerance to oxidative stress not only by removing the risk of ROS production, but also by enhancing antioxidant system activities in the cells [27–29]. Also, the role of H_2O_2 signaling and its importance in inducing and stimulating the antioxidant system has been proven through artificial treatments of plants with H_2O_2. When different tissues of salinity-sensitive rice (roots) and corn (roots and stems) were treated with low concentrations of hydrogen peroxide (10 mM), with simultaneous exposure to salinity stress, the stress-induced damage was reduced, and the shoots fresh and dry weight, as well as CAT activity, were increased, while a new isoform of CAT was created [24]. They attributed the decrease in CAT activity by salinity stress to the lack of mRNA production, even as low concentrations of H_2O_2 improved CAT gene function and expression. In the presence of intense light stress, foliar application of tobacco leaves (*N. tabacum*) with low concentrations of H_2O_2 reduced oxidative stress in plants [30]. Treatment of *Cucumis sativus* leaves with diluted H_2O_2 also reduced lipid peroxidation and preserved the chloroplast structure in cells [24]. Nonetheless, studying the effect of ultraviolet light on bread wheat (*Triticum aestivum*) showed that it led to signs of aging by increasing the concentration of H_2O_2, possibly due to lower CAT activity [31]. Finally, according to the available references and the observed results, it can be concluded that in rice tissues the CAT expression is lower and H_2O_2 accumulation is higher than in Aeluropus. In fact, the severity of stress symptoms can be attributed to the balance between CAT expression and H_2O_2 accumulation. Since traits such as tolerance to abiotic stresses are evident examples of quantitative traits that range in expression by several indices, a detailed examination of possible indices can offer apt prospects in plant breeding

CONCLUSION

In the present research, the difference in catalase efficiency was an indicator that, compared to Aeluropus, rice is more sensitive to salinity stress and to the chain of negative effects it has. Based on these findings, it is suggested that parental lines with faster and longer durations of catalase expression get used as donor parents in breeding programs. In order to improve salinity-sensitive rice cultivars through breeding programs, catalase inefficiency can be compensated by genes or promoters that associate with rapid and durable catalase expression and function. Aeluropus as an indigenous plant from Poaceae with studied and known genetical information could be a good candidate for this goal. In addition to CAT activity, other enzymes such as APX are also involved in controlling the concentration of H2O2, which can be studied in future research while comparing plants with or without optimal tolerance to environmental stresses.

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ارزیابی تفاوت تحمل به تنش شوری درگیاهان آلوروپوس و برنج بر اساس تفاوت ساختار و بیان ژن کاتالاز آنها

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چکیده

در حال حاضر بین نرخ رشد نیاز به محصولات کشاورزی و نرخ رشد تولید آنها عدم تعادلی وجود دارد که گزارش‌های موجود سهم زیادی از این کمبود را به تنش‌های غیر زنده نسبت می‌دهند. این گروه از تنش‌ها، عملکرد گیاهان زراعی را تا ۵۰ درصد کاهش می‌دهند بر همین اساس به نظر می‌رسد هر ایده‌ای که بتواند به کاهش میزان آسیب این تنش‌ها منتهی شود، ارزش مطالعه را خواهد داشت. به همین منظور در پژوهش حاضر، تفاوت شاخص آروماتیکی و ناپایداری آنزیم کاتالاز بر اساس ترکیب آمینو اسیدی و سطح بیان ژن آن در ریشه و اندام هوایی آلوروپوس و واریته IR64 برنج در شرایط تنش شوری مورد ارزیابی قرار گرفتند. با هدف بررسی بهتر اثرات شوری بر گیاهان مورد مطالعه، نمونه برداری از آنها در شرایط تنش در بازه‌های زمانی کوتاه مدت، میان مدت و بلند مدت صورت گرفت. تحلیل نتایج حاصل نیز وجود تفاوت معنادار بین پاسخ و پتانسیل‌های این دو گیاه در برابر تنش شوری را نشان داد. طی بررسی‌ها بیان ژن کاتالاز در ریشه گیاه آلوروپوس به صورت دوره‌ای با دو مرتبه افزایش خود را نشان داد در حالی که پاسخ این ژن در گیاه برنج تنها با یک مرتبه افزایش بیان در کل دوره تنش همراه بود. بیان این ژن در اندام هوایی برنج نیز در مقایسه با شاهد یا کمتر و یا بدون تفاوت معنادار بود در حالی که در تمام مراحل تنش در اندام هوایی آلوروپوس به طور معناداری بیشتر از نمونه‌های شاهد آن بود. مقایسه شاخص‌های بیوشیمیایی ترکیب آمینو اسیدی کاتالاز نیز برتری نسبی آنها را در آلوروپوس نشان داد. به عنوان نتیجه گیری کلی از ارزیابی‌های صورت گرفته می‌توان به این مطلب اشاره کرد که بخشی از تفاوت تحمل و پاسخ دو گیاه به تنش شوری از تفاوت چیدمان نوکلئوتیدهای بخش بیانی و پروموتور ژن کاتالاز این دو گیاه منشاء گرفته است. در نتیجه انتخاب، استخراج و انتقال ژن و پروموتور از آلوروپوس به گیاهی حساس به شوری همچون برنج قابل پیشنهاد است.

کلمات کلیدی: شاخص آروماتیک، شاخص ناپایداری، تنش اکسیداتیو، تنش شوری