Efficiency of Biochemical Characterization to Differentiate Chickpea Genotypes for Cold Stress

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Abstract

A factorial experiment based on a completely randomized design was conducted in three replications to differentiate susceptible and tolerant chickpea genotypes based on different biochemical traits. A combination of two factors including genotype (cold tolerant and susceptible genotype) and temperature (4 ℃ and 20 ℃) were used in this experiment. Leaf samples were sampled 12 and 72 hours after stress. Based on the results obtained, H_2O_2 content was significantly higher in the susceptible genotype under both conditions. Sequencing results of comparison showed that the proline dehydrogenase gene expression increased in the susceptible genotype. In addition, expression of an isoform of proline 5 carboxylate synthetase reduced in this genotype. Cold stress caused a significant increase in catalase activity in the tolerant genotype. Decreased expression of a homologue of ascorbate oxidase in susceptible genotype showed a positive role of this enzyme and regulation of ascorbate homeostasis in the tolerant genotype. Decreased expression of ABA hydrolase in the tolerant genotype decreased expression of ABA receptor in the susceptible genotype. Expression of different ABI5 isoforms in the tolerant genotype under stress as compared to the susceptible genotype indicated differences in ABA transduction pathway in the susceptible and tolerant genotypes. The result of this study clearly explained the differences between the biochemical responses of tolerant and susceptible chickpea genotypes in response to the cold stress, which could be an indirect screening method for identification of chickpea genotypes tolerant to cold stress conditions.

Keywords: biochemical traits, *Cicer arietinum*, chilling, gene expression

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Introduction

The main goal and an important challenge of rainfed national chickpea research in Iran is to achieve cold tolerant cultivars, in order to improve varieties for autumn sowing and to overcome the challenge of water shortage in spring sowing. Some factors like unpredictability of climatic conditions, the occurrence of untimely colds, and variations in the number of frosty days, make the selection for cold tolerance unsuccessful and inefficient. In such circumstances, using indirect

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screening methods and understanding the plant mechanisms for adaptation process are considered crucial to improve cold tolerant cultivars. Hence, identification of tolerant genotypes as well as recognition of adaptability-related traits have shown significant importance in chickpea cold tolerance breeding programs.

Effects of cold on chickpea plant are not limited to a specific phenological stage. Cold in the germination stage causes delay in germination, poor plant establishment, increased susceptibility to soil-borne pathogens, and reduced seed vigor (Croser et al., 2003). In the vegetative growth stage, it reduces the growth rate and the dry matter production and increases the chlorosis and necrosis of the old leaves, and even in more severe cases, could cause the death of plants (Croser et al., 2003). In the early stages of the reproductive phase, cold affects gamete development and function, decreases stigma receptivity and ovule viability, and could lead to increased sterile flowers in the plant (Yadav et al., 2007).

Various biochemical changes occur in plants exposed to biotic and abiotic stresses, which mitigate various destructive effects of stress on different plant cell processes. Biochemically, the presence of specific proteins, synthesis and accumulation of soluble organic matter, and the presence of a protective systems are considered as the important aspects of stress tolerance. Therefore, several traits should be used to study stress tolerance such as synthesis of specific proteins, accumulation of different osmolytes (Nayyar et al*.,* 2005), increasing the capacity of the antioxidant system (Rakei et al*.,* 2016), and reducing lipid peroxidation (Kazemi et al*.,* 2014). Each of these biochemical aspects can be used alone or in combination with each other as biochemical markers to screen susceptible and tolerant genotypes.

This study investigated the biochemical traits involved in cold tolerance through comparing tolerant and sensitive chickpea genotypes. Sequencing assay and biochemical evaluations were combined to develop a comprehensive approach providing useful information on possibility of application of tolerant-related biochemical traits for screening and breeding chickpea genotypes under cold stress conditions.

Materials and Methods

Seed planting and cold stress treatment

Seeds of cold tolerant (Saral) and susceptible (ILC533) genotypes were disinfected with benomyl fungicide and then were planted in the pots containing soil prepared from a mixture of field soil, sand, and peat in a volume ratio of 2: 1: 1, respectively. To meet the nutritional needs of the plants, the prepared soil was mixed with potassium nitrate, calcium nitrate, and superphosphate before filling the pots as described by Pouresmael et al. (2013). The pots were kept in a greenhouse with natural light at a temperature of 20±3 ℃, photoperiod of 16/8 h (night / day), and irrigated when needed until plants reached 4 to 5-leaf stage of growth.

To study the effect of cold, one group of pots was transferred to the cold chamber at 4 ℃ whereas others, the control, were transferred to another growth chamber with a temperature of 20 ℃. Except temperature, both growth chambers were under similar conditions. The experiment was performed as factorial following a completely randomized design in three replications with a combination of two genotypes, and two temperature treatments cold (4 ℃) and control (20 ℃) with two sampling, 12 and 72 hours after cold stress application.

Sampling and evaluation of biochemical traits

Plant leaves were sampled 72 hours after cold stress. The collected samples were placed in a freezer at -80 ℃ until the measurements were performed. Biochemical assays included membrane stability using electrolyte leakage method (Tripathy et al., 2000), lipid peroxidation based on measurement of malondialdehyde content (Hodges et al., 1999), hydrogen peroxide following Nakano and Asada (1981), and proline content following Bates (1973). The content of antioxidant enzymes including catalase (Aebi, 1984; Rao et al., 1996), peroxidase (Chance and Maehly, 1955) and ascorbate peroxidase (Nakano and Asada, 1980; Cakmak and Marschner, 1992)

were also evaluated and their activities were calculated using the method explained by Bergmeyer (1983). Buffers preparation, extraction method, and measurement method of all above mentioned traits were described in detail by Pouresmael et al. (2015).

Comparison of the biochemical traits-related transcripts

To compare the transcripts of plants exposed to cold stress, plant leaf sampling was performed 12 hours after application of stress (for both 4 ℃ and control treatments). Ten developed leaves from the plants under each temperature conditions were used for RNA extractions.

Total RNA was extracted from leaf samples collected from Saral and ILC533 plants under normal and cold stress conditions using RNeasy Plant kit (Qiagen) according to the manufacturer instructions. The extracted RNA samples, analyzed qualitatively and quantitatively, were sent to Novogene Bioinformatics Institute (Beijing, China) for sequencing. Sequencing by Illumina Hiseq 2500 platform led to producing 150 bp paired-end reads. After analyzing the RNA-Seq data using Tophat-Cufflinks pipeline, the differentially expressed genes were identified using the threshold of $\log_2$ (fold change) ≥1 or ≤−1 and Qvalue ≤ 0.05 based on what described in detail in recent publication by Akbari et al. (2023).

Sequencing results related to the biochemical traits described in section 2 of M&M were described. An attempt was made to combine sequencing and biochemical evaluation results to develop a comprehensive view of genotype differences and to find effective tolerance traits to be used in complementary evaluations of the related quantitative gene expression.

Results

Proline content in susceptible vs. tolerant genotypes

Evaluation of cold treatment on leaf proline content of susceptible and tolerant genotypes of chickpea showed that the proline content in the tolerant genotype was significantly higher than that in the susceptible genotype under control conditions; however, it decreased significantly in both susceptible and tolerant genotypes under cold treatment (Fig. I). This is consistent with the results reported by Kazemi et al. (2014). Although there was no significant difference between susceptible and tolerant genotypes for their proline contents under cold stress conditions (Fig. I), the differences under normal conditions can be used as a biochemical marker for indirect assay under cold tolerance conditions.

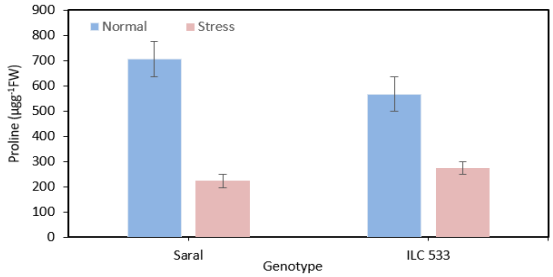

Fig. I. Leaf proline content in cold susceptible (ILC533) and tolerant (Saral) genotypes of chickpea; (Evaluations were made 72 hours after stress.

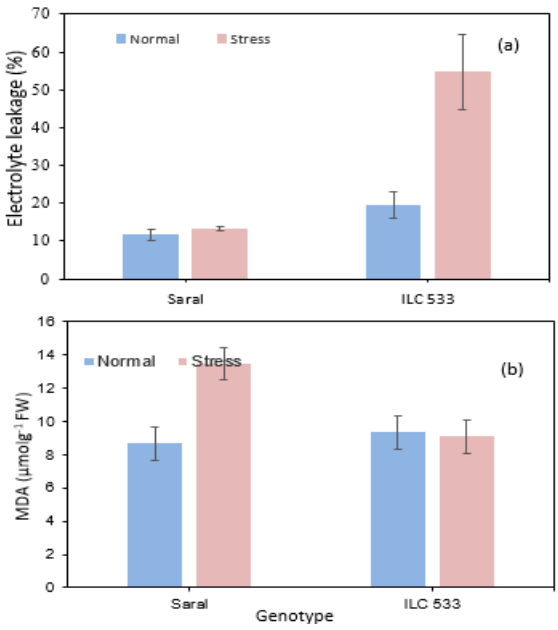

Fig. II. Electrolyte leakage percentage (a) and malondialdehyde content (b) in cold susceptible (ILC533) and tolerant (Saral) genotypes of chickpea; Evaluations were made 72 hours after stress.

The expression of an isoform of delta proline 5-carboxylate synthetase in Saral and ILC533 line increased 1.3 and 1.54 times, respectively (Table 1). However, an isoform of proline 5-carboxylate synthetase showed a decrease in expression in the

Table 1
The expression changes ratio in transcripts related to proline synthesis, degradation, transport, and action in susceptible (ILC533) and tolerant (Saral) genotypes of chickpea under cold stress conditions. Evaluations were made 12 hours after stress.

Gene ID	Annotation	Fold_Change	
		ILC533	Saral
LOC101490622	Delta-1-pyrroline-5-carboxylate synthase	1.54	1.30
LOC113784843	Delta-1-pyrroline-5-carboxylate synthase-like	-1.27	_
LOC101492274	Proline dehydrogenase	1.40	
LOC101495711	Proline-rich extensin-like protein EPR1	2.54	2.61
LOC101495711	Proline-rich extensin-like protein EPR1	2.19	1.92
LOC101502950	Proline-rich receptor-like protein kinase PERK15	1.00	1.32
LOC101492998	36.4 kDa proline-rich protein isoform X2	2.64	2.89
LOC101510818	Proline-rich receptor-like protein kinase PERK14	1.83	_
LOC101501701	Proline-rich protein	1.32	1.40
LOC101507200	Proline transporter 1-like	1.41	1.55
LOC101514935	Splicing factor, proline- and glutamine-rich	1.56	1.71
LOC101489541	Proline-rich protein	2.23	1.23
LOC101507952	Proline-rich protein 4	1.43	_
LOC101499432	Hydroxyproline O-galactosyltransferase GALT6-like	1.42	_
LOC101488919	Proline-, glutamic acid- and leucine-rich protein 1	2.68	2.12
LOC101491738	Proline-rich receptor-like protein kinase PERK1	1.85	1.61
LOC101506929	Hydroxyproline-rich glycoprotein	1.02	_
LOC101491744	Proline-rich receptor-like protein kinase PERK8	1.07	1.34
LOC101499779	Proline transporter 2-like	2.82	1.75
LOC101514641	Proline transporter 1-like isoform X1	1.15	1.31

Table 2
Variance analysis of biochemical traits of two chickpea genotypes under cold stress treatment

Source of Variation	df	Mean of Squares						
		H_2O_2	POX	CAT	APX	Proline	MDA	EL
Genotype (G)	1	908.485*	0.002	0.00006	0.00001	5748.5	10.14	1837*
Cold stress (S)	1	23.421	0.137*	0.001	0.0002	450732**	15.74	1020.8
G×S	1	5.255	0.001	0.008**	0.0016**	27301	19.15	850
Error	8	150.137	0.025	0.001	0.0001	18708	5.2	314.2

POX: Peroxidase, CAT: Catalaze, APX: Ascorbate peroxidase, MDA: malondialdehyde content, El: Electrolyte leakage; * and **: significant differences at 0.05 and 0.1 probability levels, respectively

susceptible line ILC533 (Table 1). Moreover, results showed that the expression of proline dehydrogenase gene in the susceptible and the expression of proline-rich proteins in both susceptible and tolerant genotypes increased under cold stress (Table 1). Proline transporters also increased expression in both susceptible and tolerant genotypes genotypes (Table 1).

Membrane stability of susceptible vs. tolerant genotypes

A significant increase was recorded in the electrolyte leakage in ILC533 under cold stress, which was 2.8 times higher than that in the control conditions (Fig. II. a). Malondialdehyde showed no significant change in susceptible genotype under cold treatment, but significant increases were observed in the tolerant genotype (Fig. II. b).

Antioxidant system of susceptible vs. tolerant genotypes

Based on the results obtained, hydrogen peroxide (H_2O_2) content was significantly ($P<0.05$) different between genotypes (Table 2). However, cold stress and its interaction with genotype did not have significant effect on H_2O_2 contents.

The H_2O_2 content was significantly higher in the susceptible genotype, ILC533, under both control and stress conditions (Fig. III. a)). In addition, the H_2O_2 content was not affected by stress (Table 2).

The catalase activity in the susceptible genotype showed a significant decrease 72 hours after

Table 3
The expression changes ratio in genes related to ascorbate metabolism in susceptible (ILC533) and tolerant (Saral) genotypes of chickpea under cold stress conditions; Evaluations were made 12 hours after stress.

Gene ID	Annotation	Fold_Change	
		Saral	ILC533
LOC101510634	L-ascorbate oxidase	1.95	_
LOC101489296	Monodehydroascorbate reductase	1.06	1.14
LOC101510707	L-ascorbate oxidase homolog	1.26	1.74
LOC101506817	L-ascorbate oxidase homolog	2.30	2.80
LOC101495562	L-ascorbate oxidase homolog	_	-2.74
LOC101498105	L-ascorbate oxidase homolog	_	1.51

stress (Fig. III. b). Also, ascorbate peroxidase activity increased in both Saral and ILC533 genotypes under cold stress while the increase was not statistically significant (Fig. IV. a). Comparison of the expression of enzymes related to ascorbate homeostasis in this study showed that the activity of monohydro-ascorbate reductase increased in both genotypes under chilling conditions (Table 3). While the expression of the two same isoforms and one specific isoform of ascorbate oxidase increased in both genotypes under stress, one specific homolog of this enzyme showed a decreased expression only in susceptible genotype (Table 3).

Cold stress had a significant effect on the peroxidase activity (Table 2), which decreased in both genotype (Fig. IV. b). Also, there was no significant difference between the POX activity of the two genotypes under study. Some isoforms of these enzymes showed similar changing pattern for expression in both susceptible and tolerant genotypes of chickpea under cold stress conditions (Table 4). However, the expression of peroxidase 51 and peroxidase A2 reduced in susceptible genotype due to the stress (Table 4).

ABA transduction pathway in susceptible vs. tolerant genotypes

Comparison of ABA transduction pathway in susceptible and tolerant chickpea genotypes showed that intracellular ABA concentrations increased in the tolerant genotype while the expression of ABA receptor decreased in the susceptible genotype (Table 5).

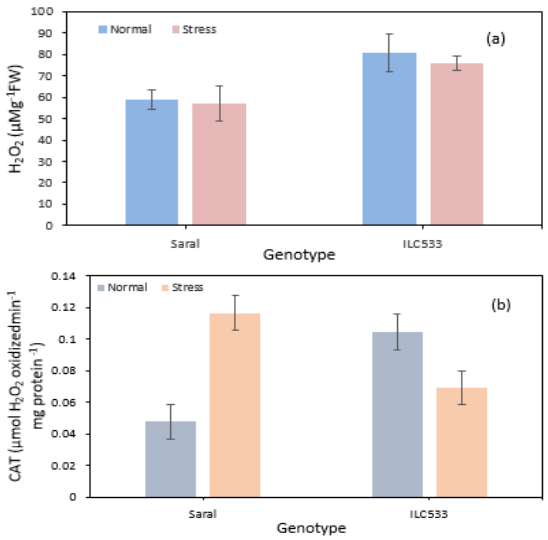

Fig. III. Content of hydrogen peroxide (a) and activity of catalase (b) enzyme in cold susceptible (ILC533) and tolerant (Saral) genotypes of chickpea; Evaluations were made 72 hours after stress.

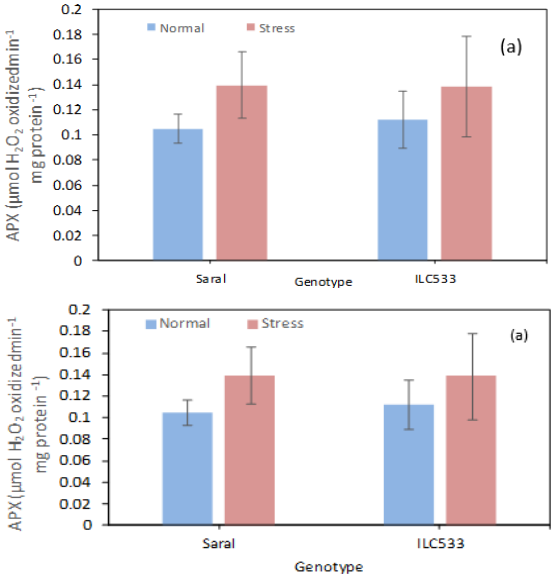

Fig. IV. Activity of ascorbate peroxidase (a) and peroxidase (b) enzymes in cold susceptible (ILC533) and tolerant (Saral) genotypes of chickpea. Evaluations were made 72 hours after stress.

Table 4
The expression changes ratio of different isoforms of peroxidase enzyme in susceptible (ILC533) and tolerant (Saral) genotypes of chickpea under the cold stress conditions; Evaluations were made 12 hours after stress.

Gene ID	Annotation	Fold_Change	
		ILC533	Saral
LOC101494255	Peroxidase A2	#NAME?*	_
LOC101494733,	Peroxidase 31	2.27	_
LOC101501013	Peroxidase P7	4.58	
LOC101498468	Peroxidase 51-like	-2.66	_
LOC101488447	Peroxidase 17-like	-1.57	-1.07
LOC101514325	Peroxidase 64-like protein	-1.53	-1.01
LOC101499357	Peroxidase 3	-3.89	-3.76
LOC101501223	Peroxidase 4-like protein	8.01	7.84
LOC101495715	Peroxidase 63-like protein	1.98	2.27
LOC101491575	Protein Over-expressor of Cationic Peroxidase 3	3.81	3.37
LOC101490947	Probable glutathione peroxidase 3, mitochondrial-like	1.08	1.10
LOC101500893	Cationic peroxidase precursor	1.12	1.18

*: #NAME? shows severe reduction expression.

Table 5
The expression changes ratio of ABA signal transduction factors and cold tolerance protein in susceptible (ILC533) and tolerant (Saral) genotypes of chickpea under cold stress conditions; Evaluations were made 12 hours after stress.

Gene ID	Annotation	Fold_Change	
		Saral	ILC533
LOC101508615	Putative abscisic acid receptor PYL4-like protein	2.39	2.54
LOC101506390	Protein Abscisic acid-insensitive 5	2.08	_
LOC101505121	Abscisic acid receptor PYL8	2.11	2.15
LOC101509736	Abscisic acid receptor PYL4-like	3.56	_
LOC101505927	Abscisic acid 8'-hydroxylase CYP707A2-like	3.57	4.67
LOC101490679	Abscisic acid 8'-hydroxylase 1	1.59	2.10
LOC101500873	Abscisic acid 8'-hydroxylase 4-like isoform X2	-1.29	_
LOC101513441	Abscisic acid-insensitive 5-like protein 2	-1.35	_
LOC101510806	Abscisic acid receptor PYL2	_	-1.49
LOC101509121	Cold tolerant protein		#NAME? *

*: #NAME? shows severe reduction expression.

Discussion

Proline content of leaves decreased significantly in both susceptible and tolerant genotypes under cold treatment. This is consistent with the results reported by Kazemi et al. (2014). On the other hand, a significant difference was found in proline contents between susceptible and tolerant genotypes under normal conditions, which can be employed as a biochemical marker for indirect assay of the genotypes' cold tolerance. It seems necessary to include more tolerant and sensitive genotypes in future studies for further confirmation. Consistent with this finding, Kaur et al. (2012) in their study of proline content in the seeds of susceptible and tolerant genotypes of chickpea showed that the level of proline in tolerant genotypes was higher than the susceptible genotype.

Proline, as an enzyme stabilizing factor, has the ability to modulate osmosis, stabilizing intracellular structures, and scavenging free radicals (Hayat et al., 2012). Proline has hydrophilic properties and replaces water molecules around nucleic acids, proteins, and membranes and prevents the interaction of destabilizing ions with these fragments (Yokota et al., 2006).

Proline 5 carboxylate synthetase is an enzyme that limits the rate of proline synthesis, which is inhibited by feedback effect of proline concentration (Hong et al., 2000). It has been

shown that in plants exposed to stress not only transcription of this enzyme increases but also the feedback inhibition reduces. Decrease in expression of this isoform in susceptible genotype confirms the decrease in free proline content in this genotype under cold stress.

The expression of proline dehydrogenase gene increased in the susceptible genotype in this study. Therefore, the increase in the proline oxidizing enzyme is considered as one of the reasons for the decrease in the free proline concentration of the susceptible line. The rate of proline degradation in the mitochondria increased during stress. Proline acts as an energy store to regulate the redox potential in the cell (Hayat et al., 2012) and its degradation improves the energy state of the cell.

Understanding the various functions of proline remains a mystery. Like other amino acids, proline is a precursor and building block of proteins, and acts as a precursor to other nitrogenous compounds (Shin et al., 2018). Under cold stress, the expression of proline-rich proteins was found to increase in both susceptible and tolerant chickpea genotypes. Moreover, proline is one of the components of cell wall proteins. These proteins are essential for maintaining the structure and function of the cell wall and show specific expression depending on the developmental stage, organ, tissue, and even cell. The role of proline and hydroxyproline-rich proteins in mechanical support of the cells and preserving cell morphology has been well described (Kavi Kishor et al., 2015).

Furthermore, proline transporters also showed increased expression in both susceptible and tolerant genotypes. The presence of both high and low affinity carriers for proline in plants and the selective transfer of proline with these carriers and their increased expression during stress indicate their vital roles in regulating proline homeostasis under stress conditions (Kavi Kishor et al., 2015).

The electrolyte leakage in ILC533 increased significantly due to cold stress in comparison with the control conditions. Consistent with this study, an increase in electrolyte leakage was reported as an indicator of membrane damage in cold treatment of chickpea by Kumar et al. (2008).

Malondialdehyde levels in susceptible genotype did not show significant change due to cold treatment but they revealed significant increases in the tolerant genotype. This finding contradicts the results of Kaur et al. (2012) and Kazemi et al. (2014) who observed lower levels of malondialdehyde in their tolerant genotypes.

In past decades, lipid peroxidation process has been described as a destructive process. The increased thiobarbituric acid in cold-stressed chickpea has also been shown to increase lipid peroxidation and membrane damage (Kumar et al., 2008; Nayar and Chander, 2004). However, it is believed now that lipid peroxidation, lipid degradation products, and lipid peroxidation initiators (ROSs) can participate in transduction cascades. Additionally, a few research has suggested the role of malondialdehyde as a messenger and regulator of gene expression (Ayala et al., 2014).

In the present study, the level of malondialdehyde in the tolerant genotype increased significantly, and it was significantly higher than the level of malondialdehyde in the susceptible genotype. This indicates that the product resulted from lipid peroxidation itself could act as a signal and activate the stress tolerance related signal transduction pathways enabling the plant to express appropriate responses. This is consistent with the study reported by Bhattacharjee (2012).

Research on cell function at different levels of lipid peroxidation products has shown that if these products are made and metabolized at the physiological level, the process of cell activity and life is properly maintained. Increasing these products in low levels triggers signal transduction cascades, gene expression, and stress-responsive responses (including increased levels of antioxidants) and preserve cell life. An increase in moderate levels causes damage to organs and proteins, leading to autophagy, aging, or the cell cycle stopping while at high levels, it causes necrosis and at very high levels even causes irreversible cell damage and programmed cell death (Ayala et al., 2014).

Despite a significant increase in electrolyte leakage under cold stress, no increase in malondialdehyde content was observed in the susceptible genotype. This finding suggests that the final product of lipid peroxidation in this genotype could not be malondialdehyde since malondialdehyde is considered as one of the products of lipid peroxidation.

Malondialdehyde is highly reactive and can bind to proteins to form products such as ethanol, propanal, hexanal, glyoxal, 4-hydroxy-2-hexenal, formaldehyde, and acetaldehyde, which are the end products of lipid peroxidation. Thus, lipid peroxidation is often used to determine the amount of lipid peroxidation by measuring the content of thiobarbituric acid; however, assays that cold measure the products of the subsequent reaction of malondialdehyde are more useful to determine the amount of lipid peroxidation since the final products resulting from these reactions are relatively stable.

Contradictory results have also been reported regarding the effects of stress treatments on malondialdehyde content of chickpea under drought stress (Mohammadi et al., 2011; Rahbarian et al., 2012; and Pouresmael et al., 2015).

Significantly higher H_2O_2 content was observed in the susceptible genotype, ILC533, under both control and stress conditions. This is consistent with the results of Karami-Moalem et al. (2018).

It was also found that the H_2O_2 content was not affected by stress in this study. Yousefi et al. (2018) reported that H_2O_2 content in ILC533 genotype was almost constant at different stress levels and did not differ significantly from that of the control. The result obtained in this research may be inconsistent with the results of some previous studies on chickpea plants (Heidarvand and Maali-Amiri, 2013 and Rakei et al., 2016); however, this inconsistency could mainly be related to temporal differences in the measurement of hydrogen peroxide content.

The increase in H_2O_2 content is a rapid response to stress to trigger signal transduction cascades and the emergence of an appropriate response to stress conditions. Therefore, it is suggested to observe the changes in H_2O_2 content; hence, sampling was performed in short intervals after stress. It appears that after 72 hours of stress (sampling time in this study), the genotypes maintained hydrogen peroxide homeostasis in cells by increasing their antioxidant capacity, including activating antioxidant systems to take cell ROS content under their control.

The amount of ROS in the cell is always maintained by a balance between the mechanisms of production and its removal in a stable state (Slesak et al., 2002). Hence, this balance is crucial for the plant survival under stress conditions. Under normal growth conditions, the production of ROS in the cell is low and the existing antioxidant defense system provides adequate protection against reactive oxygen species and free radicals. Under stress, cell homeostasis is disrupted, the production of ROS increases, and in response, the capacity of the antioxidant defense system increases (Arora et al., 2002).

Based on the results of this study, the effect of cultivar and stress was not significant on the catalase and ascorbate peroxidase activity. But their interaction effect was significant at the 1% probability level.

The initial activity level of catalase in the susceptible genotype was higher than that in tolerant cultivar; this is consistent with higher institutional level of H_2O_2 content and the role of catalase activity to make redox homeostasis of ILC533 genotype under control. Catalase is among tetrameric enzymes which is involved in the removal of H_2o_2 (Jithesh et al., 2006). Cold stress increased the activity of catalase enzyme in Saral cultivar. Consistent with these results, Yousefi et al. (2018) also reported that unlike the sensitive genotype, ILC533, catalase activity increased in all cold tolerant genotypes and the highest level of catalase activity was observed in the cultivar Saral.

The catalase activity in the susceptible genotype significantly decreased 72 hours after stress. In agreement with the results obtained in the current research, a significant decrease in catalase activity in ILC533 was reported 48 hours after stress by

Yousefi et al. (2018). Sing et al. (2017) also reported that the level of catalase activity in the wild genotype was significantly higher than that in the susceptible genotype, ILC533 under cold stress conditions. Higher activity of antioxidant enzymes and less damage due to cold stress in tolerant genotype was also indicated by Rakei et al. (2016). It was reported that the increase and/or decrease in catalase activities could be affected by the time of evaluation (Thakur et al., 2020).

Ascorbate peroxidase activity increased in both Saral and ILC533 genotypes under cold treatment 72 hours after stress, although the increase was not significant. This suggests that increasing the activity of this enzyme could be considered as a rapid response to stress. Therefore, it is suggested to observe the activity of APX sampling to be performed shortly after exposing to the stress. In fact, it seems that 72 hours after stress (sampling time in this study), genotypes use the capacity of other antioxidant enzymes to maintain cell homeostasis. This is supported by the study by Yousefi et al. (2018) who found significant increase in the APX activity in the susceptible genotype within 24 hours and then a decrease in this enzyme within 48 hours after cold stress.

Initial activity level of this enzyme was also higher in susceptible genotype than that in the tolerant one, although this difference was not statistically significant. Consistent with this result, Yousefi et al. (2018) reported that the level of ascorbate peroxidase activity in all cultivars of chickpea under cold stress was significantly higher than that in the control, and the highest activity of ascorbate peroxidase was observed in genotype ILC533.

APXs are antioxidants that perform the same function as CAT (Jithesh et al., 2006). But, their different affinity to H_2O_2, which is at the micromolar and millimolar level, respectively, indicates that they belong to two different classes of sweeping mechanisms. While APXs is responsible for the fine-modulation of reactive oxygen radicals for the transduction, catalase is responsible for the removal of excess ROS under the stress conditions (Mittler, 2002).

It was indicated that the APX activity during early weeks after drought stress could be used as an indicator for selection of drought tolerant genotypes (Pouresmael et al., 2015). It seems that increase in APX activity is also a rapid response to cold stress. But, over time other enzymes such as catalase (in Saral genotype) have been activated to balance the H_2O_2 concentration in the plant under stress.

The activity of monohydro-ascorbate reductase in this study showed an increase in both genotypes under chilling conditions, and the activity of a specific homolog of this enzyme decreased only in susceptible genotype. This result intensifies the positive role of this homolog in ascorbate homeostasis regulation in the tolerant cultivar. Recently, it was demonstrated that members of the ascorbate oxidase gene family which are regulated differently in response to abiotic stresses play an important role in stress tolerance. Hence this gene could be considered as an appropriate candidate for the development of stress-resistant transgenic plants (Batth et al., 2017).

Cold stress significantly decreased the POX activity in the genotype under study while there was no significant difference in the activity of POX between genotypes. There are usually more than one peroxidase isoforms in plants, which are generally different from catalytic and structural point of view; however, their biological roles have not yet been identified. Some of these isozymes are expressed more in a specific tissue or at a certain stage of the plant growth. In addition, environmental stresses also cause changes in a particular isozyme expression (Lüthje and Martinez-Cortes, 2018).

As expected, because of different functional roles of peroxidases families, some isoforms of POX enzymes showed similar trends for expression in both susceptible and tolerant genotypes of chickpea under cold stress conditions. On the other hand, the expression of peroxidase 51 and peroxidase A2 reduced in susceptible genotype under the cold stress (Table 4). This may suggest the role of these enzymes in negative response of susceptible genotype against the cold stress. Consistent with this result, Su et al. (2020) demonstrated that wheat mutant with high

expression of peroxidase A2 showed a higher salinity tolerance as compared with the wild type.

Based on the result of this study, decreased expressions of ABA 8'-hydroxylase 4-like isoform X2 in the tolerant cultivar increased the intracellular ABA levels resulting in triggering the ABA-dependent signal transduction cascades. ABA hydroxylases are involved in ABA catabolism (Skubacz et al., 2016). An increase in ABA receptor PYL4-like expression was observed in the tolerant cultivar. Therefore, in contrast to susceptible genotype, increased expression of ABA receptors was probably considered as one of the reasons for proper response of tolerant cultivar to cold conditions. ABA receptors are considered the starting point of ABA transmission cascade. The cold stress perceived by these receptors targeted downstream events and caused changes in stress-responsive genes expression or transcription factors controlling these genes.

Abscisic acid-insensitive 5, ABI5, is a transcription factor in the ABA signal transduction pathway. ABA causes the accumulation of ABI5 through two mechanisms: increasing its transcription or decreasing its proteolysis (Liu and Stone, 2014). Under non-stress conditions, a low level of ABI5 is maintained through its degradation by 26S proteasome. Exposure to stress or an increase in ABA content led to a decrease in proteasome activity. Accumulation of ABI5 regulates the expression of stress-responsive genes which have ABA-responsive elements in their promoter region and induces stress tolerance (Liu and Stone, 2014; Collin et al., 2021). It has been demonstrated that ABI5 target genes could cause more adaptability of the plant to abiotic stresses (Skubacz et al., 2016). Hence it has been suggested that increasing ABI5 expression could be used as an effective biotechnological tool for the development of stress-resistant cultivars (Collin et al., 2021).

Comparison of the expression of ABI5 transcripts in susceptible and tolerant genotypes in this study showed that in contrast to the susceptible genotype, the expression of different ABI5 in the chickpea tolerant cultivar was affected by the stress. The presence of two transcripts which were affected differentially under cold stress conditions in the tolerant cultivar indicates possible different roles of these transcription factors under cold adaptation conditions. Probably, one transcript increases the level of ABI5 resulting in an appropriate response to the stress, whereas the other maintains homeostasis among the occurrence of these responses. It has been reported that ABI5 activates its expression by binding to the ABI5 gene promoter. ABI5 overexpression indicates hypersensitivity to ABA (Liu and Stone, 2014). Therefore, its activity is strictly controlled by various regulators at the transcription and protein levels to ensure an accurate response to the surrounding environmental conditions. The balance of ABI5 expression plays an important role in occurrence of an appropriate response to stress. High expression of ABI5 in corn caused chlorophyll degradation and decreased activity of peroxidase and superoxide dismutase enzymes under environmental stress (Yan et al., 2012). Thus, under stress conditions, the negative feedback loop in the ABA transduction pathway ensures equilibrium in ABI5-dependent responses to achieve a balanced and adequate response depending on the intensity and type of stress (Skubacz et al., 2016). The role of ABA in increasing stress tolerance has been reported in many studies (Kumar et al., 2008; Bakht et al., 2013; Nayyar et al., 2005).

The expression of an unknown protein called cold tolerant protein was severely reduced by stress in the susceptible genotype (Table 5). Increase in transcript of this protein has been reported to be due to cold treatment in the mRNA sequencing of wild chickpea species *Cicer microphyllum*, (https://www.uniprot.org/uniprot/J9PDE1). Identifying the exact characteristics of this protein could be considered as an effective tool in identifying tolerant from susceptible chickpea genotypes, which for sure requires further investigation.

Conclusion

Exploration of cold-tolerance differentiating characteristics of chickpea germplasm, implementation of robust and high-performance screening techniques, recognition of adaptability-related traits, and hence identification of tolerant genotypes gains particular importance for the

development of autumn chickpea cultivars. Various biochemical changes occur in plants exposed to biotic and abiotic stresses, which mitigate the destructive effects of stress on various plant cell processes.

Comparison of biochemical traits of tolerant (Saral) and sensitive (ILC533) chickpea genotypes in this study showed that, leaf proline content was significantly reduced by cold treatment in both genotypes. Based on sequencing results, the expression of proline dehydrogenase gene increased in the susceptible genotype. In addition, an isoform of proline 5 carboxylate synthetase showed reduced expression in this genotype.

Membrane instability and high electrolyte leakage was observed in susceptible genotype. Electrolyte leakage in susceptible genotype under cold stress was 2.8 times higher than that in the control conditions. Malondialdehyde levels in the susceptible genotype did not show any significant changes whereas it increased significantly in the tolerant cultivar studied.

H_2O_2 content in both genotypes was not affected by stress while it was significantly higher in the susceptible genotype under both control and stress conditions. While the initial activity level of ascorbate peroxidase and catalase in the susceptible genotype were higher than those in the tolerant cultivar, cold stress caused a significant increase in catalase activity in the tolerant cultivar. Moreover, decreased expression of a homologue of ascorbate oxidase in susceptible genotype due to cold stress shows a positive role of this enzyme and regulation of ascorbate homeostasis in the tolerant cultivar.

There were not any significant differences for the level of peroxidase activity between susceptible and tolerant cultivars under control and stress conditions. However, comparison of the expression of different isoforms of peroxidase enzyme between the two susceptible and tolerant genotypes showed that peroxidase A2 was severely reduced due to stress in the susceptible genotype.

In our research, differences in ABA reception addresses the variable responses observed between tolerant and susceptible genotypes. In the tolerant genotype, intracellular ABA concentrations increased due to a decreased expression of ABA hydrolase activity while under stress conditions, the expression of ABA receptor decreased in the susceptible genotype.

Overall, based on the results obtained, high level of hydrogen peroxide content and ascorbate peroxidase activity in the susceptible genotype as well as high levels of activity of catalase enzyme in stress-tolerant genotype are the most important traits that can clearly distinguish cold-tolerant from susceptible genotypes.

The results of this study clearly explain the differences between the biochemical responses of tolerant and susceptible chickpea genotypes in response to cold stress. Each of these biochemical aspects can be used alone or in combination as a biochemical marker to screen cold tolerant chickpea genotypes at the national chickpea breeding program.

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