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RESEARCH ARTICLE

Inheritance and biochemical basis of yellowing of apical leaves: a unique trait in chickpea (*Cicer arietinum* L.)

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Abstract. A unique trait, i.e. yellowing of apical/young leaves in response to low temperature and high relative humidity was identified in a chickpea genotype, ICCX110069. To determine inheritance pattern of this trait, ICCX110069 was crossed to four other genotypes, GL14050, GL14049, GL14059 and SAGL152117, that exhibited normal green apical leaves under similar environmental conditions. The F_1, F_2, F_3, BC_1F_1 and BC_1F_2 generations were generated. A ratio of 13 normal green leaf: three yellow leaf was found to be the best fit, indicated digenic gene action with suppressor effect of normal green leaf over the expression of yellowing of apical/young leaf trait. The chlorophyll content was significantly lower, while guaiacol peroxidase activity was significantly higher in yellow leaves of ICCX110069 as compared to green leaves of the same genotype and of GL14049, indicating the competence of antioxidative defence mechanism involved with the expression of this trait.

Keywords. chickpea; inheritance; cold sensitivity; yellowing of apical leaves.

Introduction

Chickpea (*Cicer arietinum* L.) or garbanzo bean ($2n = 2x = 16$) is a temperate, autogamous annual legume from south-eastern Turkey as centre of origin (Ladizinsky 1975). It is categorized into two major types, namely '*desi*' and '*kabuli*'. *Desi* chickpeas are extensively cultivated in Indian sub-continent and Australia while *kabuli* types are grown in Middle-East and Americas. Chickpeas occupy 14.56 million ha area globally with 14.77 mt production and 1014.6 kg/ha productivity (FAO 2018, http://www.fao.org/faostat/en/#data/QC/visualize). India is the major chickpea growing country having 10.76 million ha area with 11.16 mt of production and 1037.0 kg/ha of productivity (Anonymous 2018). Like any other crop, chickpea is also facing the consequences of global climate change, experienced in the form of fluctuating temperature regimes, rainfall and frequent dry spells. Considering the effects of different biotic and abiotic stresses on productivity levels there is an urgent need to put forth serious efforts for the development of cultivars with wider adaptability and stability to achieve steady productivity levels under adverse agro climatic conditions.

Chickpea researchers have tried to develop ideal plant types by studying different morphological traits along with their effect on yield levels. Some of the traits considered for ideal plant type include; leaf type (Toker *et al*. 2012), foliage colour (Rao *et al*. 1980), growth habit (Singh *et al*. 2006; Upadhyaya *et al*. 2017), time to flower (Anbessa *et al*. 2006), flower colour (Atanasova and Mihov 2006), open flower (Srinivasan and Gaur 2012), double podded trait (Kivrak *et al*. 2020), pigmentation (Singh *et al*. 2006), seed shape (Meena and Kumar 2012), etc. Under north Indian conditions (at Ludhiana), a unique trait, i.e. yellowing of apical or young leaves has been observed consistently over the years by us in chickpea germplasm line, ICCX110069, during winter months. This character is observed to be more pronounced during the months of December and January when minimum temperature ranges from 2.8 to 10.3°C, maximum temperature from 10.3 to

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24.5°C, relative humidity ranges from 60 to 85% and sunshine vary between 4.2 and 7.9 hours. The young apical leaves of this genotype turn yellow when temperature goes down and relative humidity goes up and has the ability to revert back to normal green colour when the temperature starts rising towards beginning of February month. This is a unique trait as the yellow leaves of this genotype did not show senescence at low temperature. This is the first report in chickpea, thus the basis of this unique trait needs to be identified. Hui *et al.* (2012) reported that antioxidant defence system plays an important role in delaying leaf senescence in stay-green phenotype of wheat. Antioxidative enzymes also play an important role in enhancing chilling tolerance in chickpea by protecting plants from chilling induced oxidative stress (Turan and Ekmekci 2011). Yu *et al*. (2020) reported that the rate of returning to green stage reflects recovery growth of wheat plants with leaves turning from yellow to green depict cold tolerance behaviour of wheat. However, no such studies have been reported in chickpea so far. Therefore, there is need to decipher the mechanisms causing physiological changes and metabolic adjustments in chickpea at low temperature. One aspect of the present investigation was to evaluate enzymes of antioxidative defence system in green and yellow leaves of same plant of ICCX110069 along with green leaves of normal genotype GL14049 (control).

As this trait can be very easily identified visually, thus has the potential to be utilized as phenotypic marker if linked to some economically important traits like cold sensitivity/tolerance, disease resistance etc., thereby making the selection process easy and economical for economically important traits. The genetic control of this unique trait, i.e. yellowing of apical leaves during winter has not been reported so far. Thus, the present study was planned to untangle the inheritance of yellowing of apical leaves as well as to compare antioxidative enzymatic activity in leaves, as it can open new vistas in chickpea breeding.

Material and methods

Experimental material

The experimental materials comprised of an advance breeding line, ICCX110069 (developed from cross: Genesis836/JG130//ICC4958TM/ICCV97105) sensitive to low temperature coupled with high humidity during winter months (designated as sensitive); and four advance breeding lines (GL14050, GL14049, GL14059 and SAGL152117) whose leaves remain green normally throughout the winter season (designated as tolerant). ICCX110069 and SAGL152117 were acquired from ICRISAT, Hyderabad, while the other three lines, GL14050, GL14049 and GL14059 were developed at Punjab Agricultural University (PAU), Ludhiana.

Generation of crosses and recording of leaf colour data

To study the inheritance behaviour of the trait under question, the genotype ICCX110069 was crossed (as female parent) with GL14050, GL14049, GL14059 and SAGL152117 (as male parents) to develop four crosses, namely ICCX110069 × GL14050 (cross I), ICCX110069 × GL14049 (cross II), ICCX110069 × GL14059 (cross III) and ICCX110069 × SAGL152117 (cross IV) during rabi (winter) 2016–2017. Reciprocals of crosses I and II were also generated. The F_1s were raised during rabi 2017–2018 to get F_2 seeds. The F_1 plants of cross I (ICCX110069 × GL14050) were also backcrossed to female parent ICCX110069 to generate BC_1F_1 seeds. During rabi 2018–2019, F_2 of all the four crosses and BC_1F_1 plants of cross I were grown under field conditions and were scored as tolerant (normal green leaves) and sensitive (yellowing of apical leaves) plants. For the confirmation of results obtained from F_2 and BC_1F_1 generation, F_3 progenies of all the four crosses and BC_1F_2 population of cross I were also studied for their segregation behaviour for the trait: yellowing of apical leaves, during rabi 2019–2020. All generations were raised at the Experimental Farm of Pulses Section, Department of Plant Breeding and Genetics, PAU, Ludhiana which is situated at 30° 54'N latitude, 75° 48'E longitude, 247 m AMSL. Soil of the experimental site was loamy sand having pH of 7.8–8.2. During all the seasons/years, the crop was sown during first week of November and harvested during third week of April. Data for sensitivity to low temperature (i.e. yellowing of apical leaves) were recorded after 60 and 80 days of sowing, i.e. in the months of December and January, respectively. The phenotypic ratios, thus obtained, were tested for goodness of fit by chi-square test to find out the most fitted ratios. The average temperature and humidity levels were recorded weekly (table 1) for the period under study (2016–2017 to 2019–2020) during the months of December and January, when the yellowing of apical leaves initiated and was maximum.

Chlorophyll estimation

The chlorophyll content of the parental lines, F_1s and segregating generations was recorded using soil plant analysis development (SPAD) meter 502 Plus (Konica Minolta). SPAD values were recorded for leaf samples from apical and intercalary region of selected plants. Five leaves were taken from each plant. Individual selected leaf samples were inserted into the receptor window such that it is completely covered by the sample. The relative content of chlorophyll within the leaf sample was recorded by closing the measuring head until beep sound and display of measured value on the screen.

Extraction and estimation of antioxidative enzymatic activities

Antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOX),

Table 1. Meterorological data from 2016 to 2020 for December and January months.

Year	Week	Max temp. (°C)	Min. temp (°C)	RH (%)	Rainfall (mm)	Sunshine hour
2016–17	49	10.3	2.8	58.0	0	4.2
	50	13.6	3.5	66.0	0	0.9
	51	15.1	3.6	66.1	0	0.9
	52	15.5	5.3	67.0	0	2.2
	1	15.9	5.4	68.0	4	2.3
	2	16.0	5.4	68.0	0	2.6
	3	16.1	5.7	68.6	1.6	2.8
	4	16.2	5.9	69.0	40.4	2.8
2017–18	49	16.3	6.0	69.0	0	3.3
	50	16.3	6.1	69.0	1	3.4
	51	17.1	6.2	69.0	0	3.6
	52	17.2	6.2	70.0	0	3.6
	1	18.2	6.2	71.0	0	3.8
	2	18.3	6.3	71.0	0	4.0
	3	18.4	6.6	71.0	0	4.1
	4	19.3	6.7	73.0	18.4	4.5
2018–19	49	19.5	7.1	73.0	0	4.7
	50	19.8	7.2	73.0	0	4.8
	51	20.0	7.3	73.0	0	4.9
	52	20.3	7.4	76.0	0	5.1
	1	20.3	7.4	79.0	2	5.1
	2	20.7	7.4	79.0	2	5.5
	3	20.8	7.6	79.0	46.4	6.2
	4	20.9	7.7	80.0	15.6	6.2
2019–20	49	21.1	7.9	80.0	0	6.3
	50	21.9	8.1	81.0	46.8	6.3
	51	22.0	9.3	81.0	0	6.5
	52	22.4	9.4	81.0	0	7.1
	1	22.5	9.5	82.0	13.4	7.6
	2	22.6	9.7	83.0	20	7.6
	3	22.7	10.1	83.0	0	7.7
	4	24.5	10.3	84.5	6.4	7.9

ascorbate peroxidase (APX) and glutathione reductase (GR) were estimated. Antioxidative enzymes were extracted by homogenizing the leaves in a pre-chilled pestle and mortar with potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM ethylene diamine tetra acetic acid (EDTA,), 10 mM mercaptoethanol and 1%, polyvinylpyrrolidine. The homogenate after filtering through muslin cloth was centrifuged at 20000×g for 25 min at 4°C and clear supernatant was used to estimate enzymatic activities.

The activity of superoxide dismutase (EC 1.15.1.1) was measured according to the Marklund and Marklund (1974) method. The assay mixture consisted of 0.1 M Tris HCl buffer (pH 8.2), 6 mM EDTA. The reaction was initiated by the addition of 6 mM pyrogallol. Autoxidation of pyrogallol was measured after an interval of 30 s up to 3 min at 420 nm in the absence and presence of enzyme. One unit of enzyme activity has been represented as the amount of enzyme which causes 50% inhibition pyrogallol autoxidation. Catalase (EC 1.11.1.6) activity was recorded as a decrease in absorbance following the decomposition of H_2O_2 (ε = 0.0394 mM^{-1} cm^{-1}) at 240 nm using the Chance and Maehly (1955) method.

Peroxidase activity (EC 1.11.1.7) was estimated as an increase in absorbance due to oxidation of guaiacol (ε = 26.6 mM^{-1} cm^{-1}) in the presence of H_2O_2 at 470 nm according to the method of Shannon *et al*. (1966). Ascorbate peroxidase (EC 1.11.1.11) activity was measured by recording the decrease in absorbance after an interval of 30 s for 3 min (ε = 2.8 mM^{-1} $cm^{-1)}$ at 290 nm (Nakano and Asada 1981). Modified method of Esterbaur and Grill (1978) was used to measure glutathione reductase activity (EC 1.6.4.2). The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.5), 1.5 mM $MgCl_2$, 2 mM EDTA, 0.25 mM NADPH, enzyme extract. Oxidised glutathione (0.5 mM) was added at the end to initiate the reaction and decrease in absorbance was measured for 3 min at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). The protein content of enzyme extract was estimated by the method of Lowry *et al*. (1951). Pearson's correlation coefficients for antioxidative biochemical activity parameters were obtained using SPSS software (SPSS, Chicago, USA).

Results and discussion

Several germplasm lines were grown to evaluate various traits during rabi 2016–2017. Of these, one line ICCX110069 showed yellowing of apical or young leaves when minimum temperature dropped below 10°C, while leaves of all the other gemplasm lines remained normal green. Significant differences for chlorophyll content in leaves of line ICCX110069 and other lines were also observed. Based on this preliminary information, the line ICCX110069 and four advanced breeding lines, namely GL14050, GL14049, GL14059 and SAGL152117 were selected to generate crosses to work out the inheritance pattern. The line ICCX110069 consistently showed yellowing of apical leaves (figure 1) during the period of study, while leaves of other four lines remained normal green. This yellowing of leaves coincided with certain weather parameters. As the temperature dropped and humidity increased during 2nd fortnight of December, yellowing of apical leaves was observed in ICCX110069. On the other hand, the apical leaves of other four genotypes: GL14050, GL14049, GL14059 and SAGL152117, remained normal green under same environmental conditions. The yellowing of apical leaves in ICCX110069 was not expressed at ICRISAT, Hyderabad as the average minimum temperature remains above 12°C. For the confirmation of sensitivity to low temperature, ICCX110069 was also grown in screen house (average minimum temperature between 11°C and 20°C), where yellowing of apical leaves was not expressed. Hence, the genotype ICCX110069 was designated as sensitive while other four genotypes, GL14050, GL14049, GL14059 and SAGL152117 were designated as tolerant to low temperature coupled with high humidity. The weekly meteorological

(a) (b)

Figure 1. Chickpea germplasm line ICCX 110069 displaying (a) yellowing of apical leaves and (b) segregation for yellowing of apical leaves in BC_1F_2.

data for the months of December and January for the years 2016–2020 is presented in table 1. Perusal of meteorological data revealed that mean temperature during second fortnight of December to end of January dropped down and ranged from 7.9 to 17.0°C while relative humidity during this period went up and ranged between 58.0 and 84.5%. Earlier studies revealed that yellowing of young leaves in chickpea occurred as a result of iron deficiency (Saxena and Sheldrake 1980; Saxena *et al*. 1990) which was reverted to normal after foliar spray of 0.5% $FeSO_4$. However, in the present study, the foliar spray of 0.5% $FeSO_4$ was unable to revert yellow leaves to normal green leaves. Hence, it was assumed that yellowing of apical leaves of genotype ICCX110069 is not due to iron deficiency but is an expression in response to low temperature coupled with high humidity and might be under genetic control.

Inheritance studies

To work out the genetics of the yellowing of apical leaves trait, F_1, F_2, BC_1F_1 and BC_1F_2 generations derived from crosses involving sensitive and tolerant genotypes were grown in the field. Apical leaves of F_1 plants of all the four crosses (crosses I to IV) remained normal green as in case of their male parents throughout the growing season. The results indicated that the trait yellowing of apical leaves is under genetic control and is governed by recessive gene(s), not expressed due to the dominance nature of the normal green trait under low temperature coupled with high relative humidity conditions (table 2). The apical leaves of F_1 plants of reciprocal crosses (crosses I and II) also remained normal green indicating that there is no reciprocal cross difference and the trait under study is solely governed by nuclear gene(s). Further, segregating populations of different crosses were studied for segregation pattern of yellowing of apical leaves. The F_2 plants were found to be clearly segregating into two classes, namely normal green leaves (tolerant types) and yellow apical leaves (sensitive types) (table 3).

Table 2. Phenotypes of apical leaves of parents and F_1 hybrids in chickpea.

Parents/cross	Total no. of plants	Observed no. of plants	
		Green	Yellow
Parents : 2016–17			
ICCX110069	25	25	25
GL14050	25	25	–
GL14049	25	25	–
GL14059	25	25	–
SAGL152117	25	25	–
F_1s (cross no.): 2017–18			–
ICCX110069 × GL14050 (cross I)	9	9	–
ICCX110069 × GL14049 (cross II)	7	7	–
ICCX110069 × GL14059 (cross III)	7	7	–
ICCX110069 × SAGL152117 (cross IV)	5	5	–
GL14050 × ICCX110069 (reciprocal of cross I)	8	8	–
GL14049 × ICCX110069 (reciprocal of cross II)	5	5	–

In F_2 generation of cross I (ICCX110069 × GL14050), out of 373 plants, 294 had normal green leaves and 79 had yellow apical leaves, while in cross II (ICCX110069 × GL14049), 134 plants had normal green leaves and 38 had yellow apical leaves out of a total of 172 plants scored for the trait. Similarly, in F_2 generation of cross III (ICCX110069 × GL14059), out of 332 plants, 265 had normal green leaves and 67 had yellow apical leaves, while in cross IV (ICCX110069 × SAGL152117), 198 plants were evaluated for the trait, out of which 157 plants had normal

Table 3. Segregation pattern for green leaves vs yellowing of apical leaves in various segregating generations of different crosses in chickpea.

		Observed no. of plants		Expected no. of plants				
Cross/generation no plants	Total no. of plants	Green	Yellow	Green	Yellow	Expected ratio	χ2	*P* value
F_2 generation: 2018–19								
ICCX110069 × GL14050 (cross I)	373	294	79	303.06	69.94	13:3	1.4453	0.2293
ICCX110069 × GL14049 (cross II)	172	134	38	139.75	32.25	13:3	1.2618	0.2613
ICCX110069 × GL14059 (cross III)	332	265	67	269.75	62.25	13:3	0.4461	0.5042
ICCX110069 × SAGL152117 (cross IV)	198	157	41	160.88	37.12	13:3	0.4978	0.4805
BC_1F_1 generation: 2018–19								
(ICCX110069 × GL14050) ICCX110069	21	12	9	10.50	10.50	1:1	0.4286	0.5125
BC_1F_2 Progenies: 2019–20								
(ICCX110069 ×	9	0	9	–	–	All yellow	–	–
GL14050) ×	21	0	21	–	–	All yellow	–	–
ICCX110069	46	39	7	37.38	8.62	13:3	0.3768	0.5393
	16	4	12	4.00	12.00	1:3	0.0000	1.0000
	19	0	19	–	–	All yellow	–	–
	15	12	3	12.19	2.81	13:3	0.0154	0.9013
	14	11	3	10.50	3.50	3:1	0.0952	0.7576
	21	15	6	15.75	5.25	3:1	0.1429	0.7055
	45	0	45	–	–	All yellow	–	–
	10	3	7	2.50	7.50	1:3	0.1333	0.7150
	25	18	7	18.75	6.25	3:1	0.1200	0.7290
	31	24	7	23.25	7.75	3:1	0.0968	0.7557
	18	15	3	14.62	3.38	13:3	0.0513	0.8208
	29	22	7	21.75	7.25	3:1	0.0115	0.9146
	15	12	3	12.19	2.81	13:3	0.0154	0.9013
	18	4	14	4.50	13.50	1:3	0.0741	0.7855
	8	6	2	6.00	2.00	3:1	0.0000	1.0000
	15	0	15	–	–	All yellow	–	–
	19	15	4	15.44	3.56	13:3	0.0661	0.7971
	25	17	8	18.75	6.25	3:1	0.6533	0.4189
	8	2	6	2.00	6.00	1:3	0.0000	1.0000

green leaves and 41 had yellow apical leaves (table 3). All the four crosses gave good fit to the 13:3 ratio with χ^2 value of 1.4453 (*P* value: 0.2293), 1.2618 (*P* value: 0.2613), 0.4461 (*P* value: 0.5042) and 0.4978 (*P* value: 0.4805) respectively in crosses I, II, III and IV. Hence, confirming the digenic action with suppressor effect of one gene over the other gene for the expression of normal green or yellow apical leaf trait.

To confirm the results of F_2 generations, the segregation pattern in BC_1F_1, BC_1F_2 and $F_{2:3}$ populations of cross I (ICCX110069 × GL14050) was also investigated. In BC_1F_1, 1:1 segregation pattern for normal green leaves vs yellow apical leaves was observed with χ^2 value of 0.4286 (*P* value: 0.5125). Since a segregation ratio of 1:1 (green leaves: yellow leaves) in BC_1F_1 is also possible when trait is governed by a single gene therefore to confirm the segregation pattern, BC_1F_2 and $F_{2:3}$ populations were also studied. In BC_1F_2, of the 427 plants, 208 were observed to be tolerant (having normal green leaves) and 219 were sensitive (having yellow apical leaves) under low temperature coupled with high humidity (table 3). This segregation pattern fits well in 29:35 ratio for normal green and yellow apical leaves as expected in case of inhibitory gene action. The segregation pattern of BC_1F_2 progenies was studied by growing individual BC_1F_1 plant progenies. Of the 21 representative BC_1F_2 progenies (having sufficient plants), seven progenies segregated in 3:1, five segregated in 13:3 and four progenies segregated in 1:3 ratio for normal green leaf : yellow apical leaf plants. Five progenies did not segregate as all plants had yellow apical leaves. For the confirmation of results, $F_{2:3}$ progeny families were also studied for all the four crosses, i.e. cross I (267 progenies), cross II (80 progenies), cross III (61 progenies) and cross IV (55 progenies). The plants in $F_{2:3}$ families were found to segregate into five types, namely all green, all yellow, 3 green: 1 yellow, 13 green: 3 yellow and 1 green: 3 yellow. Thus, the segregation pattern obtained

in BC_1F_2 and $F_{2:3}$ populations ruled out the possibility of single gene control and confirmed that the trait is governed by two genes with inhibitory effect of one gene over the other gene (table 3). The inheritance of yellowing of young leaves has also been reported in some previous studies but the trait observed in our study is different from earlier reports. Gaur *et al*. (2004) reported yellowing of apical leaves in mutants but leaves remained yellow throughout until the physiological maturity of plants. They reported that yellowing is controlled by single recessive gene. However, we observed yellowing of apical leaves under low temperature coupled with high humidity that has the tendency to revert back to normal green leaves under high temperature and low humidity. Another type of leaf yellowing due to iron deficiency was reported to be under the control of either single gene (Gowda and Rao 1986; Ali *et al*. 1988) or two dominant genes (Gumber *et al*. 1997). The cold stress was observed to have adverse effect on seed yield as $F_{2:3}$ plants of sensitive progenies recorded considerably lower yield (13.5–15.0 g plant^{-1}) as compared to the normal green plants (17.5–20.8 g plant^{-1}).

In chickpea, inheritance of some other visually distinguishable morphological traits have been documented, e.g. single dominant gene for growth habit (Singh and Gumber 1995), supplementary gene action for flower colour (Kumar *et al.* 2000), duplicate epistasis for time to flowering (Anbessa *et al*. 2006), complimentary or duplicate gene action for anthocyanin pigmentation (Singh *et al*. 2006), inhibitory gene action for bushy growth habit (Sandhu *et al*. 2010), and flowering time (Mallikarjuna *et al*. 2017).

Based on the results obtained, we proposed the genotypes of parents, their F_1 hybrids and segregating generations presented in table 4. It can be explained that the normal green leaf trait is governed by a dominant gene (*GG*) that inhibits the effect of another dominant gene (*YY*), governing yellowing of apical leaves. The yellow leaf trait will express when the first gene is recessive at both the loci (*gg*) and the second gene is dominant at least at one of the loci (*Y_*), while the normal green trait will express when the first gene is dominant at least at one of the loci (*G_*) or both the genes are homozygous recessive at both the loci (*ggyy*).

Table 4. Proposed genotypes of different parents, F_1 and segregating generations for yellowing of apical leaves.

Parent/generation	Proposed genotype	Phenotype	Segregation ratio (green : yellow)
ICCX110069 Parent with yellow apical leaves	ggYY	Y	–
All parents with normal green leaves	GGyy	G	–
F_1 (direct cross)	GgYy	G	–
F_1 (reciprocal cross)	GgYy	G	
F_2	G_Y_	9(G)	13:3
	G_yy	3(G)	
	ggY_	3(Y)	
	Ggyy	1(G)	
Back cross with parent exhibiting apical yellowing (ICCX110069)			
BC_1F_1	GgY_	1(G)	1:1
	ggY_	1(Y)	
BC_1F_2	GgYY	3(G):1(Y)	29:35
	GgYy	13(G):3(Y)	
	ggYY	All yellow	
	ggYy	1(G):3(Y)	

G, normal green leaves; Y, yellow apical leaves.

Chlorophyll estimates

It is a well-known fact that yellowing of leaves is due to reduction in chlorophyll content of the leaves. In many crops, yellowing of leaves or reduction of chlorophyll content has been correlated to sensitivity to low temperature. In chickpea plants low temperature has been reported to decrease the chlorophyll content (Turan and Ekmekci 2011). This low temperature induced reduction in chlorophyll content might be due to its reduced synthesis or faster degradation or both (Mohanty *et al*. 2006). Thus, SPAD values were recorded to quantify the amount of chlorophyll present in the leaves and correlated with leaf colours in the parents, their F_1s and segregating generations. The SPAD readings of apical leaves of genotype ICCX110069 ranged from 0.5 to 2.5 while in green leaves in the intercalary region of the same plant ranged from 21.3 to 37.9. In case of other four genotypes having normal green leaves, the SPAD readings of apical leaves and intercalary region ranged from 34.2 to 44.6 and 37.4 to 56.6, respectively. This indicated that the chlorophyll content in apical leaves is significantly reduced in the genotype ICCX110069 during the months of December and January when temperature was low and relative humidity was high. The reduction in chlorophyll content seems to be responsible for the yellowing of apical leaves. The SPAD meter readings of leaves in F_2 populations for normal (green) wild type segregants ranged from 21.0 to 41.5 for apical region and from 36.2 to 53.8 for intercalary region, which was similar to their male parents. However, for the F_2 plants having yellow apical leaves, the SPAD reading ranged from 2.9 to 10.7 for apical region and from 22.5 to 53.6 for intercalary region. Based on visual observations, F_2 plants were categorized into two classes, namely yellow and green apical leaves for working out genetic ratios. It is worth mentioning here that the SPAD values of plants having yellow apical leaves was <11.0, while the SPAD values of plants having green apical leaves was ≥ 21.0.

Table 5. Activities of antioxidative enzymes in leaves of chickpea genotypes.

	Enzymes	Genotypes	Activity	Specific activity
1	SOD*	GL14049 (green leaf)	24.44±2.39[a]	0.437±0.020[a]
		ICCX110069 (yellow leaf)	28.20±0.68[a]	0.390±0.005[a]
		ICCX110069 (green leaf)	27.14±1.19[a]	0.342 ±0.013[a]
2	CAT	GL14049 (green leaf)	8556.69±800.21[a]	141.75 ±8.91[a]
		ICCX110069 (yellow leaf)	8513.37±727.66[a]	117.92 ±8.75[a]
		ICCX110069 (green leaf)	8416.69±704.92[a]	106.21±8.26[a]
3	GPOX	GL14049 (green leaf)	12.92±2.16[b]	0.223±0.09[a]
		ICCX110069 (yellow leaf)	26.80±2.58[a]	0.371±0.03[a]
		ICCX110069 (green leaf)	12.91±0.65[b]	0.162 ±0.007[a]
4	APX	GL14049 (green leaf)	71.68±15.17[b]	1.183±0.04[b]
		ICCX110069 (yellow leaf)	65.30±5.18[b]	0.904 ±0.06[c]
		ICCX110069 (green leaf)	121.57±3.19[a]	1.53±0.031[a]
5	GR	GL14049 (green leaf)	46.92±6.31[a]	0.78 ±0.08[a]
		ICCX110069 (yellow leaf)	48.99±2.88[a]	0.678±0.032[a]
		ICCX110069 (green leaf)	52.12±5.16[a]	0.657 ±0.061[a]

*Enzyme activities have been expressed as amount of enzyme required for 50% inhibition of autooxidation of pyrogallol (SOD); μmol of H_2O_2 decomposed min^{-1} g^{-1} (CAT); change in absorbance min^{-1} g^{-1} (GPOX); μmol of monodehydroascorbate formed min^{-1} g^{-1} (APX); μmol of $NADP^+$ formed min^{-1} g^{-1} (GR); μmol of $NADP^+$ formed min^{-1} g^{-1}. Specific activity refers to enzyme activity per mg of the protein. The different alphabetical letters in the superscript explain the significant differences obtained by tukey's post hoc test, among the cultivars at $P \leq 0.05$.

Table 6. Pearson's correlation coefficient among antioxidative enzymes in leaves of chickpea.

	CAT	GPOX	APX	GR
SOD	0.808	0.599	−0.441	0.872*
CAT		0.134	−0.307	0.915*
GPOX			−0.847*	0.127
APX				−0.091

*Significant at 0.05%. SOD, superoxide dismutase; CAT, catalase; GPOX, guaiacol peroxidase; GR, glutathione reductase; APX, ascorbate peroxidase.

Antioxidative enzymatic estimates

Plant cells induce cascades of changes in metabolic pathways and the regulation of gene expression to cope up with cold stress tolerance. One of the major consequence of cold stress is alteration in redox homeostasis in plants due to the accumulation of reactive oxygen species (ROS) causing oxidative stress (Awasthi *et al*. 2015). Antioxidative defence system of plants comprising enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOX) and glutathione reductase (GR) along with nonenzymatic antioxidants such as phenolics, ascorbate, carotenoids and tocopherols maintain steady state of ROS under stressed conditions by scavenging them (Apel and Hirt 2004; Gill and Tuteja 2010). The efficacy of antioxidative defence system has been correlated with stress tolerance. Enhanced activities of antioxidative enzymes confer cold stress tolerance in chickpea (Kumar *et al*. 2011).

In the present investigation, the activities and specific activities of SOD and CAT in green leaves of GL14049, yellow and green leaves of ICCX110069 were found to be comparable (table 5). It was observed that guaiacol peroxidase (GPOX) activity was found to be significantly higher in yellow leaves of ICCX110069 as compared to green leaves of the same genotype as well as GL14049. The activity of APX was found to be the highest in green leaves of ICCX10069, followed by green leaves of GL14049 and minimum in yellow leaves of ICCX110069. The GR activity was found to be similar in leaves of both genotypes. A significant negative correlation was also observed between the APX and GPOX ($r = -0.847$), while significant positive correlation was observed between SOD and GR ($r = 0.872$) and between CAT and GR ($r = 0.915$) (table 6). It appears that although leaves of ICCX110069 turned yellow but their defence mechanism is at par with green leaves that might be helping them to survive under low temperature stress.

Low temperature stress affects the vegetative and reproductive growth of chickpea thereby reducing the productivity (Croser *et al*. 2003). Low temperature induced yellowing in leaves of ICCX110069 during vegetative stage and reversion to normal green at the end of January before the onset of flowering without undergoing senescence indicating that this genotype has the ability to avoid cold stress and maintains photosynthetic ability of plant at the time of flowering (first reproductive stage), so the allocation of photosynthates remain unaffected during reproductive development. Significantly higher GPOX activity in yellow leaves of ICCX110069 compared to green leaves of the same genotype as well as green leaves of GL14049 along with sustained activities of all other antioxidative enzymes might be helpful in evading senescence.

Higher activity of peroxidase is crucial for regulating ROS under cold stress as peroxidase had higher affinity for H_2O_2 (μM range) compared with catalase (mM range) (Gill and Tuteja 2010). Apart from detoxification of H_2O_2, peroxidases are also directly involved in lignin formation as they catalyze the oxidative polymerization of lignin precursors in the presence of H_2O_2 (Ma *et al.* 2012). Although we have not estimated lignin content but relationship between peroxidases activity in the plants with cell wall hardening had already been established (Barcel 1995). Lignin is an important component of cell wall and plays an important role in biotic and abiotic stress tolerance; reduction in lignin content renders plants more susceptible to stresses (Moura *et al*. 2010). Khaledian *et al*. (2015) reported that increase in peroxidase activity during cold acclimation in chickpea plants might be responsible for increased lignin content in acclimated plants as compared to nonacclimated plants that would probably alleviate the physical, mechanical and biochemical effects of cold stress in chickpea plants. Thus, leaves of ICCX110069 in spite of their yellow colour at low temperature have the tendency to sustain induced oxidative stress.

It is suggested that the trait yellowing of apical leaves, as observed under low temperature coupled with high relative humidity conditions, can be used as phenotypic marker if it is linked to some economically important traits. The selection process can be eased specifically for oligogenic/polygenic traits where visual screening is tedious and require the intervention of molecular markers. Henceforth, linkage of the trait; yellowing of apical leaves with economically important traits such as cold sensitivity/tolerance or resistance to diseases could ease and hasten the selection process even by conventional breeding methods. Further, studies are required to map this unique trait and establish relationship with economically important traits.

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