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RESEARCH ARTICLE

Assessment of cold tolerance in chickpea (*Cicer* spp.) grown under cold/freezing weather conditions of North-Western Himalayas of Jammu and Kashmir, India

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Abstract Chickpea is one of the most important grain legume crops in the world. India is the largest producer, consumer as well as importer of chickpea. Cold stress (temperature < 15 °C) is one of the important abiotic stresses limiting chickpea production by hampering its growth and vigor at all phenological stages. This study was aimed to characterize a diverse set of 366 chickpea genotypes for cold tolerance and identify most promising cold tolerant chickpea genotypes in the Western-Himalayas of Jammu and Kashmir, India. The 366 genotypes used during the present study including genotypes belonging to cultivated, primary and secondary gene pools of chickpea. Two important approaches were used including visual screening under field conditions and screening under controlled conditions by measuring cell membrane stability through electrolyte leakage tests. The analysis of trait data collected through both the approaches led to the identification of five most promising/candidate cold tolerant chickpea genotypes including one wild genotype "Ortan-066" from secondary gene pool species (C. echinospermum), one wild genotype "Cudi 1-022" from primary gene pool species (C. reticulatum) and three genotypes (IC 116783, ICC 15200 and AGBLG 170004) from the cultivated species (*Cicer arietinum*). Wild genotype "Ortan-066" was found best cold tolerance source with the mean Cold Tolerance Rating (CTR) of 2 and Electrolyte Leakage Index (ELI) of 10.82%, followed by wild genotype "Cudi 1-022" (CTR = 3, ELI = 18.89%), and three cultivated genotypes viz., IC 116783, ICC 15200 and AGBL-G-170004, with the mean CTR of 3 and an estimated mean ELI of 21.26%, 21.58% and 21.94%, respectively. The promising, candidate cold tolerant genotypes identified during the present study could be used in chickpea breeding programs aimed at improving cold tolerance of cultivated chickpea worldwide. The candidate lines can be also used for developing bi-parental mapping populations, wild × cultivated introgression lines, transcriptomics and for differential expression analysis of cold tolerant genes in chickpea.

Keywords Chickpea · Gene Pools · Cold Stress · Freezing Stress · Western Himalayas · Promising Cold/Freezing Tolerant Lines

Introduction

Chickpea is the second dominant cool season food legume in the world after dry beans (Mallikarjuna et al. 2017). India is the largest producer, consumer as well as importer of chickpeas in the world, reflecting the importance of chickpea as a protein source in the diet of people in India (Mallikarjuna et al. 2017; Gaur et al. 2019). However, chickpea production is not fully realized owing to several biotic and abiotic stresses, restricting chickpea productivity to ~ 1 ton per hectare (t ha^{-1}) (Chaturvedi et al. 2018; Roorkiwal et al. 2018). The optimum tempeature for chickpea growth and reproduction has been suggested to be

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21–26 degrees Celcius (°C) day and 18–21 °C night temperatures and an annual rainfall of 600–1000 mm (Duke 1981; Berger et al. 2012). Among abiotic stresses that affect chickpea production, drought/heat, cold and salinity stresses are considered as major constraints. Cold stress adversely affects plant growth and development (Janska et al. 2010), membrane structure (Ruelland et al. 2009) and photosynthetic activity (Kalaji et al. 2016; Arslan et al. 2018). Low temperature stress is an important issue for winter-sown chickpea in the countries surrounding the Mediterranean Sea, the tropical highlands, and temperate growing areas. The most affected regions are northern south Asia and parts of Australia, where chickpea faces low-temperature stress (< 15 °C) which limits chickpea growth and vigor at all phenological stages but particularly during vegetative and reproductive stages leading to chlorosis and necrosis of leaf tips, substantial loss of flowers, and pod abortion, reduced pollen viability and pollen tube growth and thus, reduced seed quality and yield potential by 30–40% (Rani et al. 2020). Over time, its sensitivity to low temperature has further increased, possibly due to the use and reuse of a limited number of germplasm/donor parents, with has led to more adverse effects on growth and yield (Muehlbauer and Sarker 2017). Significant economic losses due to drought/heat (1.3 billion US dollars), cold (186 million US dollars) and salinity (354 million US dollars) have raised major concerns among the chickpea-growing countries (Jha et al. 2014). This situation is exacerbated by climate change which may cause higher intensity and frequency of abiotic stress like cold spills in temperate regions of the world, thereby necessitating the identification and development of climate-resilient chickpea cultivars having region specific traits, which can perform well under stress.

Being a predominantly winter crop in the Indian subcontinent, chickpea is exposed to chilling/freezing temperatures and decreasing photoperiod during germination, vegetative and reproductive phases, especially in the northern parts of India (Srinivasan et al. 1999; Berger et al. 2006). Some chickpea accessions have shown to survive at temperatures as low as − 8 °C and − 12 °C, at early growth stages (Wery 1990; Croser et al. 2003), suggesting its potential to select for cold tolerant cultivars at germination and during seedling growth from the existing chickpea germplasm. A rating scale of 1–9 has been used for measuring cold stress injury during the early vegetative stage or seedling stage in earlier studies (Singh et al. 1989). Cold stress also results in membrane integrity leading to ice formation in plant tissues, which leads to solute leakage. Electrolyte leakage is widely used as a test for the stress-induced injury of plant tissues (Demidchik et al. 2014). Nayyar et al. (2005) found an increase in electrolyte leakage and triphenyl tetrazolium (TTC) content was decreased at 4 °C. Chickpea wild relatives have been recommended as sources of cold tolerance, a study reported that wild species have significantly higher levels of cold tolerance than the best cold-tolerant cultivars (Toker 2005). Also, alleles for winter hardiness and a vernalization requirement exist and are prevalent in chickpea's wild progenitor, *C. reticulatum* (Abbo et al. 2002). Berger et al. 2012 suggested that wild relatives of chickpea in the primary and secondary gene pool (*C. reticulatum* and *C. echinospermum*) are cold tolerant and are crossable with the cultigens and can be ideal sources for introgression of cold tolerance in cultivated chickpea for the development of winter hardy cultivars. Therefore, screening of the commonly cultivated species and their wild relatives to identify chilling tolerant accessions is a prerequisite.

Keeping this in view, we initially conducted a preliminary study and evaluated a set of only 30 genotypes for one year (year 2017–18) in field/under controlled conditions. The study helped us to identify some cold tolerant genotypes (Mir et al. 2019). However, to validate the results of this preliminary study involving only a few genotypes (30 genotypes), we extended this study by involving large diverse germplasm (366 genotypes) from different national/international sources and evaluated the bigger set in the field and under controlled conditions to better predict/understand the genotype performance and genotype cold/freezing response. Therefore a large diverse set of chickpea germplasm was characterized during the present study at sub-zero temperatures under field conditions to screen for any damage symptoms caused by cold stress and used electrolyte leakage, an indicator to test cold injury in the stressed plant tissues (leaf). During the second year (year 2018–19) only a diverse set of 192 lines were screened for cold tolerance. The comparison of different accessions could be useful in identifying the relative potential of each accession and their ultimate use in chickpea breeding programs aimed at enhancing the cold tolerance of already released chickpea cultivars.

Materials and methods

Plant material

In the present study, 366 chickpea accessions which included cultivated desi chickpea (brown, green, black seeded), Kabuli chickpea (cream and beige seeded), intermediate pea-shaped chickpea and their wild relatives from the primary gene pool (*Cicer reticulatum*), and secondary gene pool (*Cicer echinospermum*) were evaluated for cold tolerance (ESM Table 1). Among the 366 lines, a small set of 30 genotypes was earlier separately evaluated by us for cold tolerance for only one year (see Mir et al. 2019) as a preliminary study. Cold susceptible check (ICC 12968/ICCV2) and a local cultivar (Shalimar Chickpea-1) were included in the studied accessions. The seed material was procured from various national/international institutes viz., 49 lines were collected from International Crops Research Institute for Semi-arid Tropics (ICRISAT), Hyderabad, which included nutrient-rich green chickpeas, disease resistant lines (showing resistance to *Ascochyta* blight and *Fusarium* wilt), cold tolerant, extra early and early maturing lines. Around 20 chickpea lines were received from Rafi Amhad Kidwai (RAK) College of Agriculture, Rajmata Vijayaraje Schindia Krishi Vishwa Vidyalaya (RVSKV), Sehore, Madhya Pradesh. A set of 114 chickpea germplasm lines were procured from the Indian Council of Agricultural Research (ICAR)-Indian Institute of Pulse Research (IIPR), Kanpur. The remaining 182 chickpea lines were collected from ICAR- National Bureau of Plant Genetic Resources (NBPGR), New Delhi. While classifying the germplasm between local/indigenous *vs* exotic genotypes, it was noticed that 331 genotypes were indigenous while 35 were exotic genotypes (ESM Table 1).

Experimental design and layout

The accessions were sown in October during the year 2017–18 and year 2018–19, in an augmented block design (ABD) in the research field of Division of Genetics and Plant Breeding, Faculty of Agriculture (FoA), (SKUAST-K), Wadura Campus, Sopore, Jammu and Kashmir, India. The field location possesses a temperate climate (34 17 North latitude, 74° 33 East longitude, and at an altitude of 1594 m above sea level). The soil of the experimental field was a typical inceptisol with clay loam texture. Prior to planting, the experimental field was ploughed and manually leveled with the help of spades and forked jembes. Seeds were hand drilled and sown at a depth of 5 cm deep, which showed beneficial effects on the growth and yield of chickpea. The accessions were sown in rows of 4 m (m) in length with inter and intra row spacing of 45 and 10 cm (cm), respectively. For comparison, the already known cold sensitive check ICC 12968 and the two cold tolerant genotypes ICCV 96029 and ICC 96030 were repeated every 10 rows (Malhotra and Saxena 1993). Pre-sowing irrigation, pre-emergence application of herbicide, and other standard agronomic practices were followed to raise a good crop.

Weather conditions at the experimental site

The cold weather conditions in the Kashmir valley prevailing during the winter season were found ideal for the screening of 366 chickpea accessions for cold stress tolerance. During the year 2017–2018 (October–February), the average high/day temperatures ranged from 12 to 26 °C, while the range of average low/night temperature recorded was − 2 to 9 °C. The average amount of snowfall during this period ranged from 0.6 cm (December) to 10.8 cm (February). On average, plants were covered under the snow for a period of 3 days. In the fall of winter (2018–2019), the average high/day temperatures ranged from 7 to 21 °C, while a range of average low/night temperatures was − 1 to 7 °C. The average snowfall amount during this period ranged from 2.8 to 30 cm. The year 2018–19 (winter) was recorded as the coldest year in the past 28 years with the minimum temperature recording as low as − 7.6 °C in the month of December. The crop was covered under snow for a maximum period of 45 days (Fig. 1a, b) (https://www.worldweatheronline.com/).

Methodology

The set of 366 chickpea accessions was evaluated for cold tolerance screening in the field under natural conditions on a cold tolerance rating (CTR) scale of 1–9 (where, 1 = no visible leaf damage, 2 = highly tolerant, 3 = tolerant, 4 = moderately tolerant, 5 = intermediate, 6 = moderately susceptible, 7 = susceptible, 8 = and highly susceptible, and 9 = 100% leaf damage or dead), described by Singh et al. (1989). Data was recorded for cold stress tolerance in the month of March after the plants exposure to cold temperature in the fall of winter. Plants were evaluated for

Table 1 Mean ± Standard error, range and standard deviation of cold tolerance component traits of chickpea collected over different environment (year 2017–18 and year 2018–19)

Trait	Mean ± SE		Range		SD	
	Year 2017–18	Year 2018–19	Year 2017–18	Year 2018–19	Year 2017–18	Year 2018–19
CTR (scale 1–9)	6.53 ± 0.08	7.34 ± 0.12	2–9	2–9	1.58	1.73
ELI (under cold conditions)	46.05 ± 0.49	49.59 ± 0.87	10.53–74.0	11.11–83.0	9.47	12.11
ELI (at room temperature)	34.72 ± 0.36	38.47 ± 0.62	9.38–43.10	10.66–57.55	7.06	8.68

CTR, cold tolerance rating; ELI, Electrolyte Leakage Index

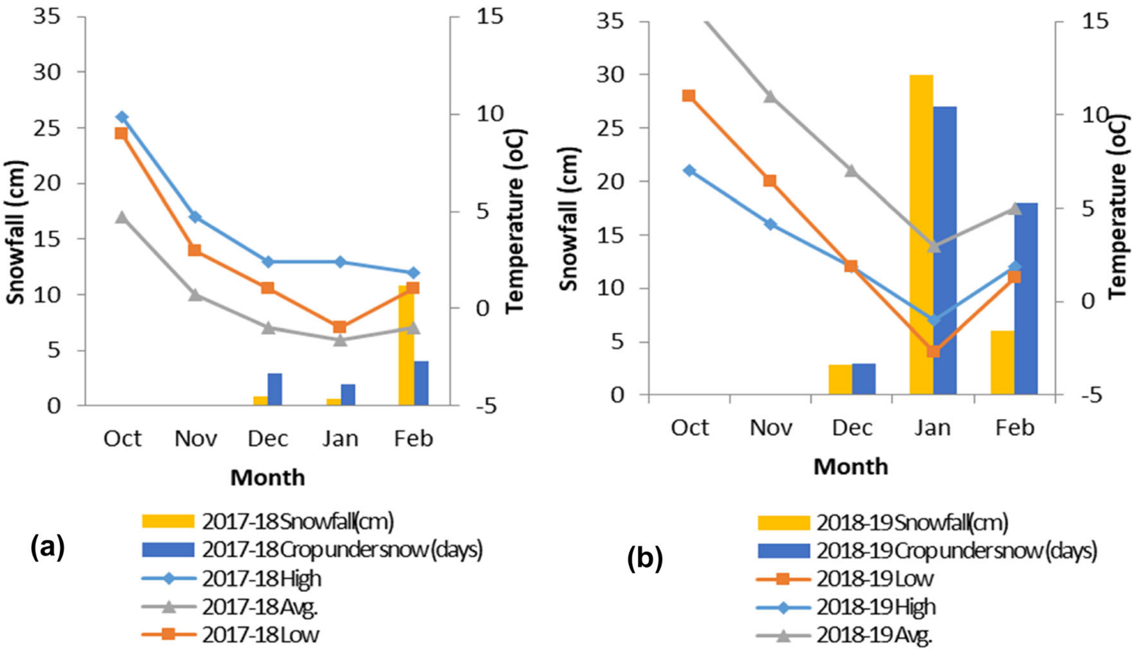


Fig. 1 **a**, **b** Month-wise variation in snowfall and temperature during chickpea cropping season (2017–18 and 2018–19), at SKUAST-K, Wadura, Jammu and Kashmir, India

cold tolerance only after the death of the susceptible check. These accessions were further assessed for the chilling injury in the laboratory with electrolyte leakage test from the leaf tissues damaged by cold stress. For comparison seeds of the same set of chickpea accessions were also grown and maintained in green house under controlled conditions at 25 °C using pink LED lights (a mix of red and blue wavelength) that emit the right shade of magenta to grow plants and a photoperiod of 16 h. Electrolyte leakage of the cold stressed plants grown under natural conditions (field) and controlled conditions (green house) was measured using young leave tissues of chickpea seedlings (Lutts et al. 1996). To remove any surface adhered electrolytes, samples (1 g) were first washed with deionized water. These were then placed in closed glass vials containing 10 ml (mL) of deionized water and were incubated at room temperature for thirty (30) minutes with a constant shaking on a rotary shaker at 150 revolutions per minute (rpm), and followed by measuring the electrical conductivity of the solution (L1) in microsiemens/centimeter (µS/cm). Each sample was then autoclaved at 120 °C for 10 min and the electrical conductivity (L2) was again obtained after shaking for thirty minutes at room temperature. The electrolyte leakage index (ELI) was calculated as ELI (%) = (L1/L2) × 100. The ELI represents the leakage of electrolytes from damaged plant tissues as the percent of the leakage from tissues completely destroyed after autoclave (100%).

Statistical analysis

The phenotypic data was analyzed separately for cold tolerance rating and electrolyte leakage index and diverse statistical parameters, including mean, range, standard deviation (SD), and Pearson's correlation coefficient (r), were calculated using SPSSv17.0 (http://www.spss.com/statistics).

Results and discussion

In the past decade, the production of cool-season legumes including chickpea has developed greatly in the temperate regions of the world. However, the yield stability in chickpea lags much behinds because of some biotic and abiotic stresses (particularly, cold stress in the temperate areas). The limited genetic diversity available to breeders requires extensive field screening to identify cold tolerant genotypes. To this end, in the present study, a range of cultivated and wild chickpea germplasm was evaluated in the field conditions under cold/freezing stresses during the years 2017–18 and 2018–19 in Kashmir valley (Western-Himalayas) using visual field screening on a scale of 1–9 and electrolyte leakage index for measuring cell membrane stability.

Table 2 Evaluation of 366 chickpea genotype for cold tolerance

Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession Number	CTR	ELI (under cold conditions)	ELI (at room temperature)
Ortan-066	2	10.53	9.38	ICC 11040	6	42.86	42.86	ICC 12411	8	51.11	38.18
Cudi 1-022	2	18.52	16.67	ICC 10943	6	42.86	30.77	EC 498825	8	51.14	43.08
ICC 15200	3	21.05	18.18	IC 555135	6	42.86	22.22	EC 555574	8	51.16	37.78
IC 116783	3	21.21	15.15	ICC 4676	6	42.86	38.46	ICC 4969	8	51.25	31.25
Bari-3-106D	3	21.43	18.18	IC 244433	6	42.86	33.33	IC 272471	8	51.25	38
IC 348481	3	22.73	18.75	IC 118913	6	42.86	35	JG 11	8	51.28	42.42
AGBL-G-170004	3	22.78	19.35	IC 408004	6	43.14	34.04	IC 305439	8	51.28	42.5
JG 74	3	26.32	21.05	ICC 8242	6	43.33	34.48	IC 567580	8	51.35	42.11
Bari-2-072	3	26.67	23.08	IC 83411	6	43.33	33.33	IC 487193	8	51.35	41.67
IC 0486625	3	26.92	23.08	IC 396753	6	43.33	27.5	IC 327868	8	51.43	37.93
KAK 2	3	27.27	20	EC 528340	6	43.33	36	Harighantas	8	51.52	40.54
ICC 13090	3	28.36	22.73	ICC 13077	6	43.37	37.04	ICC 10956	8	51.61	44.44
IC 408322	3	29.9	22.86	ICC 14706	6	43.75	31.25	IC 552170	8	51.72	41.67
IPC-09-58	3	30	26.67	IC 408075	6	43.75	38.46	ICC 14760	8	51.85	41.38
ICC 10540	3	30	28.57	IC 272340	6	43.75	23.08	ICC 12968	8	51.85	48.15
ICC 14449	4	28.38	21.82	ICC 15668	6	44	30	IC 487120	8	51.85	30.56
ICC 14843	4	31	22.47	IC 269681	6	44	36.84	IC 522130	8	52	41.86
ICC 15089	4	31.25	30.19	RVSSG 44	6	44.44	37.5	IC 376248	8	52	40
IC 552192	4	31.25	30.95	JGC1	6	44.44	33.33	ICC 11284	8	52.17	43.48
AGBL-G-170003	4	31.25	25	ICC 15894	6	44.44	37.5	IC 83959	8	52.17	36
ICC 12299	4	31.43	25.81	ICC 1304	6	44.44	37.93	ICC 10952	8	52.27	40.54
ICC 15033	4	31.58	23.68	ICC 10799	6	44.44	33.33	ICC 10946	8	52.38	34.78
IC 95076	4	31.58	21.05	IC 328046	6	44.44	37.5	IC 275637	8	52.38	40.63
IC 270707	4	31.71	29.41	IC 308562	6	44.44	33.33	IC 244352	8	52.38	40
IC 83729	4	31.82	27.27	IC 272205	6	44.44	37.21	IC 327995	8	52.5	36.73
GL12021	4	31.82	25	EC 532477	6	44.44	42.86	IC 83129	8	52.63	36.36
ICC 8243	4	32	24	Avrodhi	6	44.44	36	IC 269578	8	52.63	37.5
ICC 17660	4	32	25	RVSSG 46	6	45	30	IC 486809	8	52.78	44.44
ICC 14657	4	32	31.88	ICC-11224	6	45	35	ICC 12936	8	52.94	36
PI 489777	4	32.35	31.25	ICC 14727	6	45	33.33	ICC 12026	8	52.94	39.29
GCS 5	4	32.5	27.27	ICC 10300	6	45	37.21	ICC 10247	8	52.94	41.18
RVSSG 30	4	33.33	20	ICC 12954	6	47.5	34	IC 486965	8	52.94	40
ICC14685	4	33.33	20	IC 396751	6	53.85	42.86	IC 486954	8	52.94	38.46

Table 2 continued

Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession Number	CTR	ELI (under cold conditions)	ELI (at room temperature)
ICC 8504	4	33.33	25	IC 95077	6	66.67	39.39	IC 485688	8	52.94	41.18
ICC 4567	4	33.33	20.83	EC 548032	7	44.44	33.33	ICC 11091	4	42.86	30
ICC 10140	4	33.33	22.22	EC 555205	7	52.94	46.15	EC 382406	8	52.94	30.77
IC 506711	4	33.33	28.57	EC 555508	7	45.16	20	BGM-413	8	52.94	39.29
IC 486818	4	33.33	28.57	ICC 16349	7	41.67	37.5	RSG-2	8	53.13	41.18
IC 396762	4	33.33	25	ICC 15185	7	45.45	35.48	IC 269467	8	53.33	40
AGBL-P-S-170005	4	33.33	28.57	ICC 12922	7	45.45	40	IC 269140	8	53.33	39.29
AGBL-K-170014	4	33.33	20	ICC 12387	7	45.83	37.04	IC 209463	8	53.33	40
AGBL-D-170011	4	33.33	30.77	IC 408182	7	45.83	21.88	IC 209237	8	53.33	36.59
AGBL-D-170010	4	33.33	26.67	IC 208293	7	45.83	32.26	EC 555299	8	53.33	43.48
ICC 15041	4	34.38	28	ICC 15198	7	42.86	27.27	EC 555158	8	53.33	32.73
ICC 15201	4	34.5	30.77	IC 116384	7	46	31.71	IC 487033	8	53.49	39.02
ICC 11567	4	35	34	ICC 6306	7	46.15	46.15	ICC 92944	8	53.57	33.33
IC 269161	4	33.33	30	ICC 5810	7	46.15	46.15	ICC 15101	8	53.57	42.55
IC 486952	5	33.33	29.17	ICC 15117	7	46.15	42.86	IC 275402	8	53.57	42.11
IC 486922	5	34.78	25	IC 348453	7	46.15	37.04	ICC 6814	8	53.64	40
GSC 7	5	35.14	29.73	IC 209240	7	46.3	32.5	IC 446511	8	53.85	35.71
ICC 5238	5	35.29	34.48	IC 296163	7	46.34	35.29	IC 275853	8	53.85	42.86
EC 275474	5	35.71	23.33	IC 587382	7	46.39	42.22	IC 24415	8	53.85	29.41
IC 272459	5	36	26.09	ICC 16641	7	46.43	38.89	EC 441827	8	53.85	33.33
EC 490096	5	36	25	IC 408261	7	46.43	39.13	IC 83660	8	54.05	44.59
AGBL-D-170013	5	36	28	EC 489924	7	46.51	38.24	IC 512062	8	54.29	38.89
ICC 13464	5	36.17	29.17	BG-256	7	46.51	40	ICC 11726	8	54.55	32.35
IC 73119	5	36.36	30.77	ICC 15921	7	46.67	33.33	IC 487072	8	54.55	34.04
IC 487464	5	36.36	26.42	ICC 15850	7	46.67	40	IC 269404	8	54.84	42.42
IC 272089	5	36.36	31.82	ICC 12948	7	46.67	39.53	IPC-0935	8	55	42.55
AGBL-G-170001	5	36.36	30.77	ICC 12383	7	46.67	30	ICC 15182	8	55	40
Pusa-254	5	36.84	32.26	ICC 11564	7	46.67	40	IC 299330	8	55	42.55
IC 305487	5	36.84	35.71	ICC 11180	7	46.67	35.71	IC 275593	8	55	45.45
ICC 5727	5	36.96	32.61	ICC 10452	7	46.67	45.95	IC 269123	8	55	40

Table 2 continued

Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession Number	CTR	ELI (under cold conditions)	ELI (at room temperature)
ICC 12436	5	37.04	29.63	IC 272650	7	46.67	31.25	IC 486996	8	55.17	41.38
ICC 4672	5	37.14	32.26	IC 271940	7	46.67	40.74	IC 487002	8	55.32	42.31
RVSSG 32	5	37.5	23.08	IC 269505	7	46.67	37.5	IC 551991	8	55.56	35.29
Rau-52	5	37.5	23.53	IC 209243	7	46.67	40	IC 486365	8	55.56	35.29
PUSA-209	5	37.5	23.33	EC 555296	7	46.67	43.33	IC 487402	8	56	47.62
ICC 8245	5	37.5	25	EC 532435	7	46.67	36.67	IC 275481	8	56	31.58
ICC 13074	5	37.5	31.25	AGBL-D-170009	7	46.67	40	IC 487126	8	56.25	38.89
ICC 12424	5	37.5	35.71	IC 489883	7	46.88	33.33	IC 486114	8	56.25	31.25
ICC 11366	5	37.5	29.41	ICC 10418	7	46.94	38.64	IC 297528	8	56.25	42.11
IC 248382	5	37.5	20.69	ICCV 96029	7	47.06	35.29	IC 209355	8	56.25	31.82
IC 117730	5	37.5	28.57	ICC 1915	7	47.06	40	IC 327534	8	56.34	43.33
ICC 12354	5	37.84	33.33	ICC 1882	7	47.06	27.59	IC 328087	8	56.52	38.46
ICC 3276	5	37.93	31.08	ICC 15019	7	47.06	40	RVSSG 47	8	57.14	42.86
IC 468851	5	37.93	36.17	IC 486981	7	47.06	37.04	IC 485693	8	57.14	37.93
ICC 9239	5	38.1	28.57	IC 424324	7	47.06	30.43	IC 305597	8	57.14	29.41
ICC 14729	5	38.1	35.71	IC 272678	7	47.06	25	IC 468856	8	57.45	44.44
IC 487499	5	38.1	24	IC 272642	7	47.06	22.22	Pusa 547	8	57.5	40
RSG-931	5	38.46	25	IC 269629	7	47.06	39.29	ICC 11218	8	57.58	44.44
IC 348499	5	38.46	30.77	IC 209285	7	47.06	45.45	IC 83444	8	57.69	41.18
IC 272672	5	38.46	30	ICC 15058	7	47.14	41.67	IC 486997	8	57.89	38.89
ICC 13089	5	38.75	28.57	ICC 10400	7	47.17	38.3	IC 486862	8	57.89	39.13
Shalimar Chickpea-1	5	38.89	27.78	GMG-1488	7	47.27	38.98	IC 271922	8	57.89	37.04
ICC 8244	5	38.89	22.22	ICC 96030	7	47.37	40	IC 269413	8	57.89	41.67
ICC 11706	5	38.89	27.78	ICC 17661	7	47.37	40	AGBL-D-170012	8	57.89	37.5
IC 172324	5	38.89	29.63	ICC 15920	7	47.37	43.75	ICC 14866	8	58	34.62
ICC 11911	5	39.29	30.77	ICC 13081	7	47.37	47.06	IC 275738	8	58.06	38.46
IC 265298	5	39.29	35.71	ICC 11163	7	47.37	30	ICC 7537	8	58.33	44.12
EC 555720	5	39.29	28.57	ICC 10548	7	47.37	45.45	ICC 328286	8	58.33	30
ICC 15847	5	39.47	31.82	IC 408198	7	47.37	42.86	ICC 1165	8	58.33	44.19
GL-769	5	39.58	29.55	IC 117673	7	47.37	38.46	IC 327779	8	58.33	34.29
ICC 16012	5	40	30.77	IC 328009	7	47.5	34	IC 424251	8	58.82	32
ICC 11075	5	40	26.09	IC 487145	7	47.62	25.81	IC 327528	8	58.82	42.86

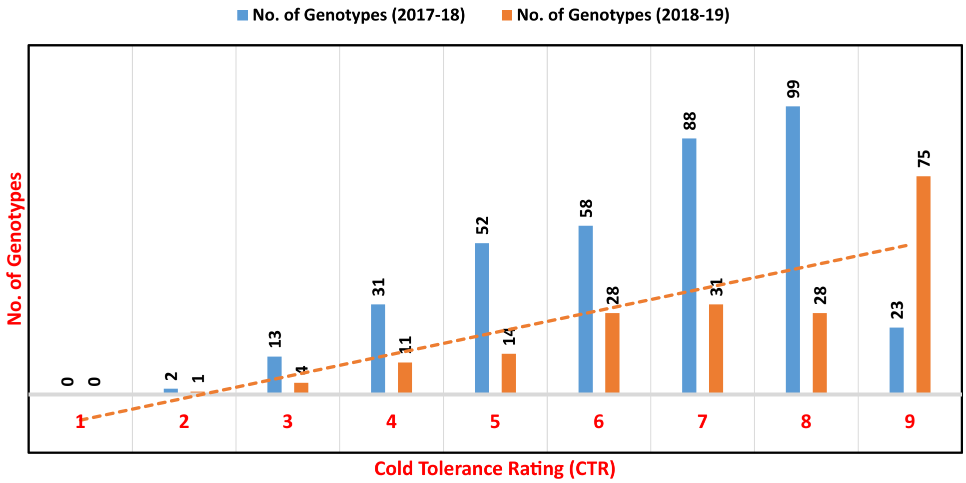
Table 2 continued

Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession number	CTR	ELI (under cold conditions)	ELI (at room temperature)	Accession Number	CTR	ELI (under cold conditions)	ELI (at room temperature)
IC 83999	5	40	27.27	AGBL-P-S-170006	7	47.76	36.96	IC 275630	8	58.82	40
IC 83362	5	40	31.58	JG-218	7	47.78	35.56	IC 486759	8	59.09	42.86
IC 458659	5	40	25	ICC 15648	7	47.83	27.03	IC 2083188	8	60	45.83
ICC 17505	5	40.91	30.43	ICC 12909	7	47.83	41.67	IC 327508	8	60	37.5
IC 117677	6	40.91	35.14	IC 415651	7	47.83	42.11	ICC 12370	9	60	37.93
EC 498756	6	40.91	23.33	IC 408262	7	47.83	39.29	IC 555550	9	60.87	44.83
AGBL-G-170002	6	41.18	33.33	EC 555544	7	47.83	43.48	IC 208305	9	60.98	38.89
ICC 10505	6	41.38	34.62	GG-2	7	48	41.67	ICC 14805	9	61.02	40.74
Pusa-261	6	41.18	37.93	IC 275563	7	48.15	38.46	ICC 12364	9	61.11	43.06
ICC 16207	6	41.18	27.78	AGBL-P-S-170007	7	48.21	41.67	IC 327654	9	61.11	31.82
ICC 10048	6	41.18	33.33	IC 468727	7	48.48	27.27	Vikas	9	61.29	44
ICC 269605	6	41.43	33.33	EC 442024	7	48.54	41.03	IC 269280	9	62.07	43.48
EC 3507	6	41.46	32.43	IC 487961	7	48.57	40.91	IC 268895	9	62.07	46.15
ICC 6426	6	41.67	35.42	IC 468644	7	48.57	31.25	EC 555589	9	62.07	44.44
ICC 13044	6	41.67	33.33	ICC I9114	7	49.09	36.84	EC 555696	9	62.16	43.48
C-235	6	41.67	30.56	ICC 11332	7	49.18	42.31	ICC 15683	9	62.5	38.24
IC 116280	6	41.94	34.78	RVSSG 37	7	50	41.67	ICC 14245	9	62.86	38.64
ICC 16684	6	42.11	36.84	PUSA372	7	50	30.95	IC 0468653	9	62.96	42.42
ICC 15663	6	42.11	32.35	ICC 8927	7	50	37.5	IC 244340	9	63.16	42.86
PUSA 391	6	42.31	30.77	IC 76623	7	50	35.9	EC442298	9	63.33	45
IC 269630	6	42.31	36.36	IC 512069	7	50	35.71	IC 275319	9	64.29	37.5
IC 348528	6	42.5	38.89	IC 486221	7	50	33.33	ICC 5064	9	65	38.98
ICC 15177	6	42.55	42.42	IC 269877	7	50	43.9	ICC 12408	9	66.67	42.86
ICC 17531	6	42.86	28.57	IC 244325	7	50	33.33	IC 327216	9	66.67	39.39
IC 468615	6	45.21	36.36	EC 538493	7	50	41.67	DCP-92-3	9	68	43.64
IC 487243	6	45	32	DEREI-070	7	50	41.67	IPC-2000133	9	70	44.44
ICC 11056	6	42.86	36.36	AGBL-P-S-170008	7	50	40	GSJ-515	9	74	43.1

The table shows the data recorded visually in the field (Cold tolerance rating: CTR) and data recorded under controlled conditions for measuring cell membrane stability through electrolyte leakage test (Electrolyte leakage index: ELI)

CTR, cold tolerance rating; ELI, Electrolyte Leakage Index

Fig 2. Cold tolerance rating (CTR) of 366 chickpea accessions for year 2017–18 and for core set of 192 chickpea accessions for the year 2018–19 on a scale of 1–9



Field evaluation under natural/field conditions

Evaluation of 366 chickpea accessions (Year 2017–18) during their growing season under natural field conditions for cold stress revealed substantial/ highly significant differences in the levels of cold tolerance between different accessions (Tables 1, 2; Fig. 2). On a cold tolerance rating (CTR) scale of 1–9, the genotypes varied significantly showing CTR of 2 to 9, with the mean CTR of 6.53 and its standard deviation was found to be 1.58 (Table 1). The cold sensitive check "ICC 12968" showed a CTR of 8 and succumbed to cold injuries. The two already released cold tolerant cultivars (ICCV 96029 and ICC 96030), also recognized as early flowering/maturing genotypes did not perform well (showed CTR of 7) in our study under field conditions. Similar results were also reported by Heidarvand et al. (2011), where ILC 8262 (cold resistant source) did not perform well in particular environmental conditions. Singh et al. (1993), in their study also reported early maturing lines suffered severe cold damage and did not produce any seed. The analysis of results revealed that the wild progenitor *Cicer* species (*C. echinospermum* and *C. reticulatum*) were the best cold tolerant sources for chickpea breeding programs. Among *C. echinospermum* and *C. reticulatum* accessions, the two most promising cold tolerant genotypes showing cold tolerant score of 2 on the scale of 1–9 were "Ortan-066" and "Cudi-1-022", respectively. The genotype "Ortan-066" belongs to the secondary gene pool while as genotype "Cudi-1-022" belongs to the primary gene pool species. These genotypes grow wild in their natural habitat in Turkey where extreme cold/freezing weather conditions similar to ours in Kashmir exists. The other two additional wild genotypes that possess good cold tolerance (having cold tolerance score of 3 on a rating scale of 1–9) are "Bari-3-106D" and "Bari-2-072". These two wild accessions belong to the primary gene pool species "*C. reticulatum*" and can be easily crossed with cultivated chickpea genotypes for transfer of cold tolerance genes. The cold tolerance of these wild genotypes has been also confirmed in our earlier field study comprising a panel of 30 different genotypes (Mir et al. 2019). On the other hand, the analysis of results of cold tolerance of cultivated germplasm revealed that 11 genotypes (AGBL-G-170004, ICC 13090, ICC 15200, KAK 2, IPC-09-58, JG 74, IC 116783, IC 348481, ICC 10540, IC 0486625 and IC 408322) possess very good cold tolerance. These 11 cultivated genotypes possessed cold tolerance score of 3 on the rating scale of 1–9. Among the 11 genotypes, two genotypes AGBL-G-170004 and ICC 13090 have been reported cold tolerant in our earlier study (Mir et al. 2019). The identification of cold tolerant genotypes in the cultivated gene pool is considered very important for the chickpea breeding community since these genotypes can be easily crossed with released cultivars to produce fertile hybrids/segregating populations without linkage drag. The genotype "AGBL-G-170004" is an advanced chickpea breeding already evaluated at multiple locations across Kashmir for its suitability for release as variety. In summary, the most tolerant wild genotypes having cold tolerance scores of 2–3 (Ortan-066, Cudi-1-022, Bari-3-106D and Bari-2-072) and eleven (11) cold tolerant cultivated genotypes showing cold tolerance score of 3 identified during the present study will prove as assets in future chickpea breeding programs. Thirty-one (31) chickpea accessions possessing moderate tolerance to cold (showing cold tolerance score of 4 on a rating scale of 1–9) have also been identified during the present study (Table 2). These cold tolerant genotypes will prove useful in chickpea breeding programs aimed at enhancing cold tolerance of modern chickpea varieties that are otherwise susceptible to cold.

During the second year (year 2018–19) under harsh and prolonged winter conditions the evaluation of only a core set of 192 lines revealed that wild *C. echinospermum* genotype “Ortan-066” followed by *C. reticulatum* genotype “Cudi 1-022” were the most promising cold tolerant species with the cold tolerance score of 2 and 3, respectively. Among the cultivated species, only three (3) genotypes “AGBL-G-170004, ICC 15200 and IC 116783” were found tolerant with the cold tolerance score of 3. A set of only eleven (11) chickpea genotypes from the core set of 192 were found to be moderately tolerant (CTR score of 4). A list of these promising genotypes consistently exhibiting same level of tolerance to cold, has been tabulated (Table 3) with their cold tolerance rating (CTR) and electrolyte leakage index (ELI%). The range of cold tolerance score in the core set varied from 2 to 9 with the mean CTR of 7.34 and its standard deviation was found to be 1.73 (Table 1). Majority of the accessions (210) for the year 2017–18 and 134 accessions in the year 2018–19 had a CTR score of 7–9 and were found cold susceptible, with many of them succumbing to cold injury (Figs. 2, 3).

Therefore, during the second year of screening, the cold tolerance of two wild genotypes “Ortan-066 and Cudi-1-022” among the four and three cultivated genotypes “AGBL-G-170004, ICC 15200 and IC 116783” among the 11 were validated /confirmed. Therefore, in total five genotypes including two wilds and three cultivated genotypes showed stable cold tolerance performance consecutively for two years. These genotypes were therefore declared as most promising/candidate genotypes for chickpea cold tolerance breeding (Table 4). The confirmation validation of only 3 cultivated genotypes among the 11 and only two wilds among the 4 identified during year 2017–18 may be due to severe harsh/prolonged winters during year 2018–19 (Fig. 1b). The findings also confirmed the effects of environment on cold tolerance in chickpea necessitating the identification of genotype × environment interactions in future. The wild and cultivated genotypes that have been validated as cold tolerant during both the years of evaluation in field are declared stable cold tolerant

Table 3 Details of 16 promising genotypes identified during the present study for cold tolerance (year 2017–18 and 2018–19)

Accession Number	Other name	Species/gene pool	Origin	Biological status	Mean CTR	Mean ELI (under cold conditions)	Mean ELI (at room temperature)
Ortan-066		*Cicer echinospermum*/2^0 *gene pool (wild)*	Turkey	Wild	2	10.82	10.02
Cudi 1-022		*Cicer reticulatum*/ 1^0 *gene pool (wild)*	Turkey	Wild	3	19.26	17.11
AGBL-G-170004		*Cicer arietinum/ cultivated/ advanced breeding line*	India	Breeding line/ improved material	3	21.94	17.95
IC 116783		*Cicer arietinum/ cultivated*	India	Landrace	3	21.32	17.57
ICC 15200		*Cicer arietinum/ cultivated*	India	Landrace	3	22.13	19.48
ICC 14449	PI 216026	*Cicer arietinum/ cultivated*	India	Landrace	4	27.19	22.77
GL 12021		*Cicer arietinum/ cultivated*		Breeding line/ improved material	4	29.46	24.49
ICC 15201		*Cicer arietinum/ cultivated*	India	Landrace	4	33.16	22.73
KAK 2	AGG43281CHIC	*Cicer arietinum/ cultivated*	India	Released Cultivar	4	29.59	22.22
Bari-3-106D		*Cicer reticulatum*/ 1^0 *gene pool (wild)*	Turkey Beri	Wild	4	26.71	20.98
ICC 14843		*Cicer arietinum/ cultivated*		Landrace	4	32.01	23.98
IC 83729		*Cicer arietinum/ cultivated*		Landrace	4	32.57	26.99
ICC 15033		*Cicer arietinum/ cultivated*		Landrace	4	32.74	23.99
ICC 8504	JM 533 Aethiopia 57/62	*Cicer arietinum/ cultivated*	Ethopia	Landrace	4	33.66	25.55
RVSSG 30		*Cicer arietinum/ cultivated*	India	Breeding line/ improved material	4	33.66	21.33
ICC 8243	NEC 2406 PI 315810	*Cicer arietinum/ cultivated*	India	Landrace	4	34.27	23.5

CTR, cold tolerance rating; ELI, Electrolyte Leakage Index

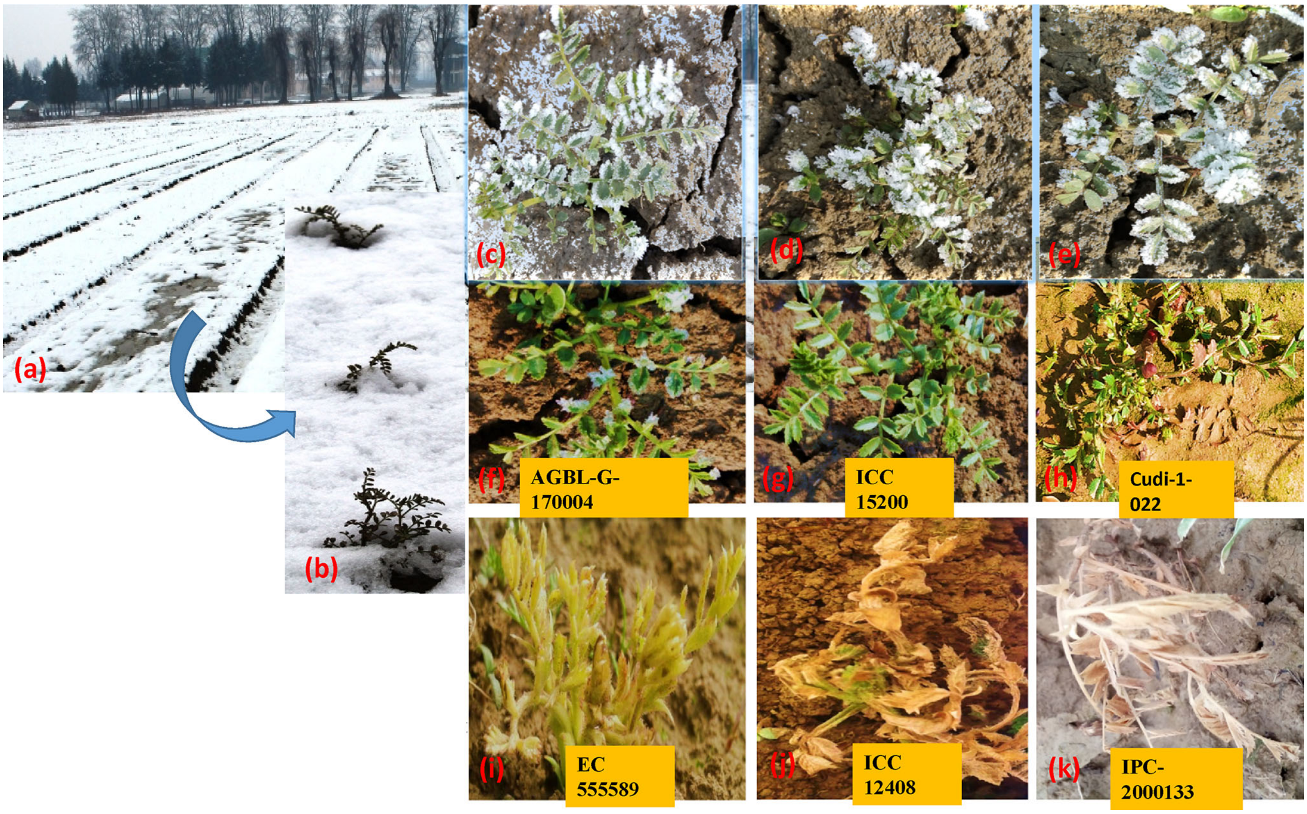


Fig 3 Field view showing snow covered chickpea fields (**a**) and a zoomed chickpea genotype after partial snow melting (**b**). The figure also shows chickpea plants covered with winter frost (**c**–**e**) and three cold tolerant genotypes (**f**–**h**) and three cold susceptible genotypes (**i**–**k**)

Table 4 A list of 5 most promising/candidate cold tolerant chickpea genotypes showing stable performance over different environments (year 2017–18 and 2018–19)

Genotype	Mean CTR	Mean ELI(%) under cold conditions	Mean ELI(%) at room temperature
Ortan-066	2	10.82	10.02
Cudi 1-022	3	19.26	17.11
IC 116783	3	21.32	17.57
ICC 15200	3	22.13	19.48
AGBL-G-170004	3	21.94	19.95
Mean	2.8	19.09	16.82
SD	0.44	4.76	3.99

The genotypes are recommended as first priority for breeding cold tolerant chickpeas

CTR, cold tolerance rating; ELI, Electrolyte Leakage Index

genotypes and can be used in chickpea breeding programs for enhancing cold tolerance of cultivated chickpea genotypes. The use of these cold tolerant chickpea genotypes will help in the development of next-generation chickpea cultivars with enhanced cold tolerance. The genotypes can be also used as parental genotypes in development of mapping populations for gene mapping for cold tolerance and in differential gene expression for identification of differentially expressed genes for cold tolerance.

Similar to our results, among the wild chickpea species *C. echinospermum* was found as the most cold tolerant, followed by *C. reticulatum* species (Clarke and Siddique 2004; Berger et al. 2012). The *C. reticulatum* and *C. echinospermum* are crossable with cultivated *C. arietinum* and previous studies have reported successful introgression of genes into the cultivated species from these two closely related species (Gaur et al. 2012; Berger et al. 2012). Likewise, Toker (2005) in his study reported that the wild

Cicer species including *C. reticulatum* were found to be highly cold tolerant (possessing cold tolerance score of 2). These results suggest that wild species of chickpea could be an important source of cold tolerance and can be exploited to introgress cold tolerance to the *C. arietinum* cultigen. Our results also confirmed earlier reports of field screening for cold tolerance at the vegetative stage, where Heidarvand et al. 2011 identified two genotypes (Sel 95Th1716 and Sel 96Th11439) as chilling tolerant that showed low scores for cold tolerance on a rating scale in comparison with ILC 8262 (released as cold tolerant). The results obtained during the present study are also in agreement with results of several earlier studies reported on cold tolerance in chickpea (Singh et al. 1990, 1995, 1998; Robertson et al. 1995; Kanouni et al. 2009; Berger et al. 2012).

Electrolyte leakage index (ELI)/cell membrane stability

Membrane stability means membrane integrity and is defined as the percent electrolyte leakiness from the cell which is the ratio of conductivity after immersion (of leaves) in water to conductivity after boiling. It reflects the damage to cellular membrane by stress and, hence membrane activity is directly related to electrolyte leakage. Wery et al. (1993) considered membrane stability, an important mechanism of cold tolerance to adjust the number of seeds according to the level of stress and osmotic adjustment, which is involved in cold tolerance.

During the present study, the ELI was used as a physiological index to identify the magnitude of injury between different genotypes after plants were exposed to cold stress conditions in the field. Leakage score of more than 50% is considered lethal to plants. More the leakage of electrolytes, the lesser is the tolerance of plants to cold stress. For comparison, the difference between the accessions in terms of the ELI was also studied under controlled conditions in green house where plants were maintained at 25 °C. The results of the ELI test under natural and controlled conditions indicated significant differences between accessions. Minimum electrolyte leakage among 366 chickpea accessions during the year 2017–18 was estimated in wild (*C. echinospermum*) genotype “Ortan-066” (10.53) and *C. reticulatum* genotypes “Cudi 1-022” (18.52) followed by Bari-3-106D (21.43) and Bari-2-072 (26.67). The results validated our earlier preliminary results using a small set of 30 genotypes for cold tolerance evaluation (Mir et al. 2019). Among the cultivated species, least electrolyte leakage under cold stress conditions was observed in genotypes ICC 15200 (21.05), IC 116783 (21.21), IC 348481 (22.73) and JG 74 (26.32). Estimated electrolyte leakage was found maximum i.e., 74.00 and 68.00 in cultivated accessions GSJ-515 and IPC-2000133, respectively (Table 2). The mean ELI under cold conditions was found to be 46.05 ± 0.49 with a standard deviation of 9.47 (Table 1). The results of electrolyte leakage index (%) obtained during the present study were in agreement with earlier published reports. For instance, Kumar et al. 2011 observed less electrolyte leakage in two chickpea accessions (ICC16348 and ICC16349) that were able to flower and set pods when subjected to cold stress conditions. Similar results were obtained by Chohan and Raina (2011) where electrolyte leakage was significantly higher in the leaves of chilling sensitive genotypes as compared to chilling tolerant genotypes. Fathi et al. (2016) reported that with the increase of cold stress intensity from 15 to 0 °Celsius, the amount of electrolyte leakage increased and reached a maximum at a temperature of about 0 degrees. Nevertheless, under controlled conditions (25 °C) a low magnitude of leakage was observed in our study for all the genotypes compared to cold stress conditions. The minimum ELI under controlled conditions was found in wild species Ortan-066 (9.38) and Cudi 1-022 (16.67) and the maximum leakage was found in genotype ICC 12968 (48.15) and maximum leakage of 44.44 and 43.10 was observed in cultivated genotypes IPC-2000133 and GSJ-515, respectively (Table 2). The mean value of ELI under controlled conditions was estimated to be 34.72 ± 0.36 and a standard deviation of 7.06 (Table 1). Similar to our results, Heidarvand et al. (2011) observed less electrolyte leakage in chickpea accessions under controlled conditions as compared to electrolyte leakage under cold stress conditions. The electrolyte leakage analysis was in agreement with the observed data for cold tolerance rating suggesting more leakage of electrolytes in the identified cold susceptible accessions. Similar results were found by analyzing the core collection of 192 accessions for electrolyte leakage under natural and controlled conditions, where minimum electrolyte leakage was found in wild accession Ortan-066 (11.11) and Cudi 1-022 (20.00), followed by cultivated genotypes AGBL-G-170004 (21.11), IC 116783 (21.43), ICC 15200 (23.22), ICC 14449 (26.0) and GL 12021 (27.01). Whereas, maximum electrolyte leakage of 83.00 and 81.00 was found in cultivated genotype IC 587382 and IC 424251. The mean value of ELI under cold stress and controlled conditions was estimated to be 49.59 ± 0.87 and 38.47 ± 0.62, respectively (Table 1). A significantly positive correlation was found between electrolyte leakage index (ELI%) and cold tolerance rating (CTR) ($r = 0.84$, $p < 0.01$), implying more leakage of electrolytes in genotypes with increased cold tolerance rating (Fig. 4). The wild genotypes (Ortan-066, Cudi 1-022) and cultivated genotypes (AGBL-G-170004, IC 116783, ICC 15200) with least electrolyte leakage over the two environments and also validated in field screening

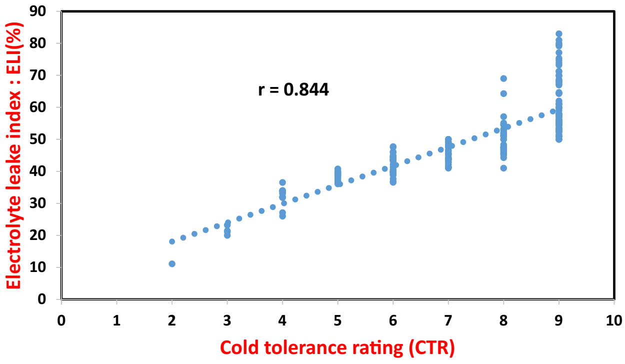


Fig 4 Correlation coefficient between mean cold tolerance rating (CTR) and electrolyte leakage test (ELI%)

techniques for year 2017–18 and year 2018–19 had a mean CTR value of 2.8 and mean ELI(%) of 19.09 under cold conditions and 16.82 at room temperature (Table 4). These five promising genotypes identified in our study were found stable across environments and declared as highly cold tolerant genotypes and will prove useful world-wide in breeding chickpea for cold tolerance through a variety of approaches.

Conclusion

Cold tolerance screening in the field for two years (winter-sown; 2017–18 and 2018–19) as well as for cell membrane stability using electrolyte leakage index (ELI %) leads to identification of promising cold tolerance chickpea genotypes. The response of studied accessions to cold in field was consistent and showed a strong relationship with performance of genotypes under controlled conditions through electrolyte leakage test. A significant and positive correlation was found between CTR and ELI% and, thus can be used as reliable indicators for cold tolerance in chickpea. The overall results revealed that significant variation exists in the chickpea germplasm for told tolerance. Out of 366 accessions evaluated for cold tolerance (2017–18), 320 accessions did not contribute to cold tolerance. Among the 46 accessions that were found highly cold tolerant to moderately cold tolerant in the first year of screening (2017–18) (Table 2), only 16 chickpea accessions were validated in the second year of screening showing consistent level of tolerance to cold (with mean CTR 2–4, mean ELI $\leq$ 35%), both under field screening and in independent laboratory experiments by studying membrane permeability (Table 3). The cold tolerant accessions identified in the two years study can be used in chickpea breeding programs, however we recommend only five candidate chickpea genotypes which include one *Cicer echinospermum* accession (Ortan-066), one *Cicer reticulatum* accession (Cudi 1-022) and three cultivated genotypes (AGBL-G-170004, IC 116783 and ICC 15200) which were found highly cold tolerant showing cold tolerant score 2–3 on CTR scale and ELI $\leq$ 22%.. These five genotypes reported in our study were found most promising cold tolerant and thus can be exploited in breeding programs for developing superior varieties in more efficient way and in short time period with enhanced/improved cold tolerance. These candidate lines can also be used as important genetic resources for developing bi-parental mapping populations, transcriptomics and for differential expression analysis of cold tolerant genes in chickpea.

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