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Morphological Characterization and Germination-Based Screening for Cold Stress Response of *Vigna radiata* L.

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SLM and GRR conceived and designed the experiments. Author SLM conducted the experiments. Author GRR provided the seed source. Author KCP provided infrastructural facility for work. Authors GRR and KCP provided funding support. Authors SLM and MP analyzed the data. Author GRR wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Morphological characterization and germination based screening for cold stress response of *Vigna radiata,* was performed to identify the cold resistant genotypes.
Methodology: Augmented design experiment was performed to characterize 204 mungbean genotypes under field condition based on 24 DUS characterization. A germination-based screening at 10°C was performed in a total of 204 germplasms in a plant growth chamber to identify the cold responsive genotypes. Seeds were soaked in water for 6h in the dark under normal temperature and imposed cold stress (10^0C) for 2, 4, 6, 8 days and further transferred to control temperature (22^0C) for recovery.

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Results: On the basis of the 24 DUS characterizations of 204 mungbean genotypes are classified into three major clusters. Highest number of genotypes in cluster I (113 genotypes), followed by 81 genotypes in cluster II and least number (10) of genotypes in cluster III. Based on cold stress study and biplot analysis showed that germination percentage, germination rate index, Timson's index, mean germination time and coefficient of velocity of germination to be more influential and categorized into three groups having 11 genotypes are resistant, 135 genotypes are susceptible, and 58 genotypes are intermediate. Biochemical analysis showed that the tolerant genotype having increased chlorophyll, carotenoid and MDA content and oxidative enzyme activity as compared with susceptible and intermediate genotypes under cold stress.
Conclusion: The present study identified 11 genotypes having cold resistant and may be used for breeding program.

Keywords: Biochemical marker; cold stress; seed germination; screening Vigna radiate.

1. INTRODUCTION

Plants are forced to adapt to the prevailing environment surrounding them since they are sessile and unable to move under unfavourable climatic conditions [1]. About 64% of the entire earth acreage has average minimum temperature < 0°C [2]. Basically, plants basically require a fixed range of temperature for their optimum vegetative and reproductive and acclimatization. The optimum conditions of temperature vary from plant to plant and from species to species. Upon exposure to low temperatures (> 10–15°C), signs of injury were observed in various crops including oil seeds, legumes and vegetable [1,3]. With an unplanned growth of industrialization, population growth and climate change due to global warming are the serious concern about the crop growth and productive. The world crop production needs to be increased to fulfil the nutrient requirement of ~10 billion people [4]. Breeders are facing problems to develop improved genotypes to sustain in the stress condition particularly drought, cold and high temperature climates. In particular, cold stress is a serious threat to have sustainable crop yields [1,5]. With very few commendable exceptions, the genotypes of the tropical region are sensitive to chilling temperatures (0 to 15°C) during almost all stages of plant development, including seed germination, vegetative growth and reproduction. Cold stress induces irreversible electrolyte leakage from the cell, reduction in scavenging enzymes' activity, loss of energy in photosynthetic apparatus and stability of proteins [1].

Green gram, *Vigna radiata*, a tropical pulse crop largely grown under semi-arid and subtropical environment is very sensitive to low temperature regimes. Though green gram has a multifarious advantage, sensitivity to chilling stress can largely limit its productivity [6]. Low temperatures can hamper the seed germination [7] and delay plant growth and development leading to variability in crop maturation and reproductive ability of plants [8]. During later stages, plant growth is highly reduced with limited or no flowers and fruit. Very limited studies are reported for screening, identifying chilling tolerant genotypes that can be taken as marker for selecting cold tolerant genotype [9]. Severe susceptibility of mungbean to chilling stress and also lack of suitable resistant genotypes have thus become the reason for decrease in mungbean productivity [10]. So, it is utmost necessary to identify the cold tolerant mungbean genotypes for crop productivity in adverse climate situation. The present study is aimed at screening of 204 greengram genotypes for cold stress response during germination stage. It also includes study of the significant traits, biochemical parameters contributing to cold tolerance of the representative set of tolerant and susceptible germplasms.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Condition

A total of 200 *Vigna radiata* accessions including 127 Indian varieties, 58 Odisha landraces, 15 recombinant Inbred lines (RIL) along with 4 high yielding released varieties (OBGG-52, KAMDEV, IPM-02-3, IPM-02-14) were used in this study. All the genotypes were obtained from field grown plants with augmented design during August, 2019 at research station of Odisha University of Agriculture and Technology, Bhubaneswar, India with geographic details of 20.2650° N, 85.8117° E, 25.9 meters above the sea level. The average day/night temperature was 30.5°C /25.5°C and the relative humidity was 86% within average of

~8.1 hours (h) average day length. Irrigation was done in every 3rd-day interval. Twenty four (Anthocyanin occurrence, Time of flowering, plant growth habitat, plant habitat, stem puberscence, stem colour, leaflet lobes, leaf shapes, leaf colour, leaf vein colour, petiole colour, leaf size, flower colour, colour of premature pod, pod pubescence, pod position, plant height, pod colour, curvature of matured pod, pod length, seed colour, seed lusture, seed shape, seed size) DUS (Distinctiveness Uniformity Stability) characters in different scale for *V. radiata* were recorded as per the guidelines of Protection of Plant Varieties and Farmers' Rights Authority [11].

2.2 Screening at Seedling Stage

For screening under cold stress, the *V.radiata* seeds were soaked in water for 6h followed by germination induction under white light (WL) for 6h. The seeds were sown uniformly at 1.5 cm depth in pro-trays containing soil mixture as used by Kumar et al. [12]. Germination assay was conducted in plant growth chambers (Model No. CU36L6, Percival USA) maintained separately at 10°C (cold stress: CS) and 22°C (control condition: CC) with 70% relative humidity at National Institute of Science Education and Research (NISER), India. Light was obtained from Philips 17 W F17T8/TL741 USA Alto II technology tubes with 40% light intensity, which is equivalent to ~67.5 $\mu molm^{-2}s^{-1}$. The growth chambers were programmed to provide cycles of 8h light and 16h dark (Short Day: SD). All seedling experiments were performed in 3 replications of 2 sets having 10 plants of each accession. Scoring for germination was done on the 2nd, 4th, 6th and 8th days (d) after sowing (DAS). On 8th day the plants were shifted from 10°C to 22°C chamber and grown for 2d for acclimatization. Further, all the plants were shifted to net house for growth under natural day-night conditions.

2.3 Phenotypic Assessment for Cold Stress Tolerance

Several phenotypic parameters including germination percentage, 50% emergence time (50E), seedling height on 8th day, plant height (PH), days to 50% flowering (DFF), number of pods/plants, seeds/pod, test weight and yield/plant were taken in the net house at NISER under natural day-night conditions. Germination parameters including seedling length (SL), seedling vigour (SV), germination rate index (GRI), mean germination time (MGT), coefficient of velocity of germination (CVG) [14], mean germination rate (MGR) [13] and Timson's germination index (TI), 50% emergence (50E) were computed from the data obtained till 14th DAS. The leaf area was taken using ImageJ software (version No: 1.53k) (Table 1). Each of the parameters is the mean of 15 observations from either control or cold condition.

2.4 Statistical Analysis

Twenty-four (24) DUS characters of the 204 accessions were taken for making cluster analysis based on the hierarchical clustering approach [14]. Dendrogram was generated based on the phenotyping. Statistical significance analysis was made using one-way ANOVA (* p<0.01, ** p<0.001 and *** p<0.0001 and t-test).

Table 1. The formulas used for computing assessment of phenotyping traits

Parameter category	Parameter name	Calculation
Germination parameters	Germination rate index (GRI)	GRI = G1 1 + G2 2 + ---+ Gx x G1, G2… Gx represents the percentage of germination in first, second and xth day after sowing respectively
	Mean germination time (MGT)	MGT= Σ (ni/di) ni: number of germinated seeds, di: day of counting
	Coefficient of velocity of germination	Coefficient of velocity (CV) = (number of germinated seeds per day) CVg= ΣNi 100 x Σ (Ti x Ni)
	Mean germination rate (MGR)	1/(MGT)
	Timson's germination index	Timson germination index (TGI) = ΣG/T, where G is the percentage of seed germinated per day, and T is the germination period

Each of the parameters are the mean of 15 observations from either control or cold condition

2.5 Biochemical Analysis

Biochemical parameter such as pigment content (chlorophyll and carotenoid), malondialdehyde (MDA), total sugar content, superoxide dismutase (SOD) enzyme activity and reactive oxygen species (ROS), were estimated from the 3 selected lines (One genotype from each line i,e. resistant, susceptible and Intermediate) and each data was a mean of 30 observations from 3 biological replicates. For all these measurements, statistical significance among the genotypes at 10°C and 22°C were calculated using Graphpad Prism 8.0.1

Pigment content was estimated from ~25 mg of leaf sample (from two-leaf seedlings samples on the 8^{th}DAS) was frozen in liquid nitrogen and extraction of total chlorophyll and carotenoid was done in 3 ml 80 % acetone extracts according to Arnon (1949) with small modification [15]. Estimation of chlorophyll and carotenoids were done by measurement of absorbance (A) at 645 nm 663 nm and 480 nm by using the following formulas:

Carotenoid (µg/mg) = ((A480 + (0.114 * A663) - (0.638 * A647)) * 3000) / (1000 * FW)

Chlorophyll (µg/mg) = 20.2 (A645) + 8.02 (A663).

For malondialdehyde (MDA) assay, ~100 mg of the leaf samples (8-d-old seedlings) were grounded with 0.25 % of TBA and 10% tricholoroacetic acid (TCA). Further, it was incubated at 95°C for 30 min, centrifuged at 1000 rpm for 10 min. The absorbance of the supernatant was determined spectrophotometrically at 532 nm and 600 nm.

Total sugar content was estimated as explained by Buysse et al. (1993) and with small modification [16]. Briefly,~50 mg fresh weight of leaves were ground with 5 ml of 80% methanol and the ground extract was boiled at 95 °C for 5 minutes. The supernatant was collected in a test tube. Further, 5ml of 80% (v/v) methanol was added and boiled for 2 minutes. The supernatant of the two steps were added and measured. From the supernatant, 1ml was taken out in another test tube. Then 1ml of 18% phenol and 5ml of conc. H_2SO_4 was added to the test tube. It was mixed well in a vortex mixture for 20 minutes. Absorbance was taken at 490 nm before 1h of mixing. The sugar estimation was done using glucose standard prepared from a mixture of Glucose: Fructose: Galactose at 1:1:1 ratio.

Superoxide dismutase (SOD) enzyme activity was determined spectrophotometrically as described by Dhindsa et al. (1981)with small modification (Panigrahy et al. 2019). For these assays, 100 mg of leaf sample (1^{st} and 2^{nd} leaf 8-d-old seedling) was ground with 4 ml of extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA) and filtered through 4 layers of cheese cloth. The filtrate was transferred to centrifuge tubes and centrifuged at 15000 rpm for 20 min. The supernatant was used as the enzyme extract and 100 µl of this extract was used for each enzyme activity assay. Briefly, SOD activity was determined by measuring the decrease in the absorbance of blue coloured form zone and $O_2^{\cdot -}$ at 560 nm.

For reactive oxygen species (ROS) estimation, Nitrobluetetrazolium (NBT) staining was used. 10 ml of the NBT staining solution (0.2% NBT in 10mM Phosphate buffer at pH 7) was added to each of the leaf (8-d-old). Vacuum was provided for 3 times at 5 minutes interval using vacuum desiccators and left overnight. NBT solution was removed and 15 ml compound solution (Acetic acid: glycerol: ethanol at 1:1:3) was added and kept at 95°C for 15 min. After cooling, it was stored in storage solution (50ml glycerol in 200ml ethanol). Amount of ROS was estimated by calculating thepercentage of stained area to the total area using ImageJ software version 1.53k.

3. RESULTS

3.1 Morphological Characterization Based on DUS Characterization

Based on the 24 DUS morphological characters from the 204 genotypes grown under natural day-night conditions, similarity analysis was performed and a dendrogram was prepared (Fig. 1). Ward method followed to obtain a higher agglomerative coefficient (i.e., 0.974) indicated a sound platform of existing variability among the germplasms selected for analysis. This result indicated that there could be 3 different categories of *Vigna radiata* Indian population under natural growth condition. The population was observed as 3 clusters consisting of highest number of genotypes (i.e., 113) in cluster I, followed by 81 genotypes in cluster II and least number (i.e., 10) of genotypes in cluster III. While the cluster I could be subdivided into 10 clades, cluster II could be sub divided into 3 and cluster III could be sub divided into 7 clades.

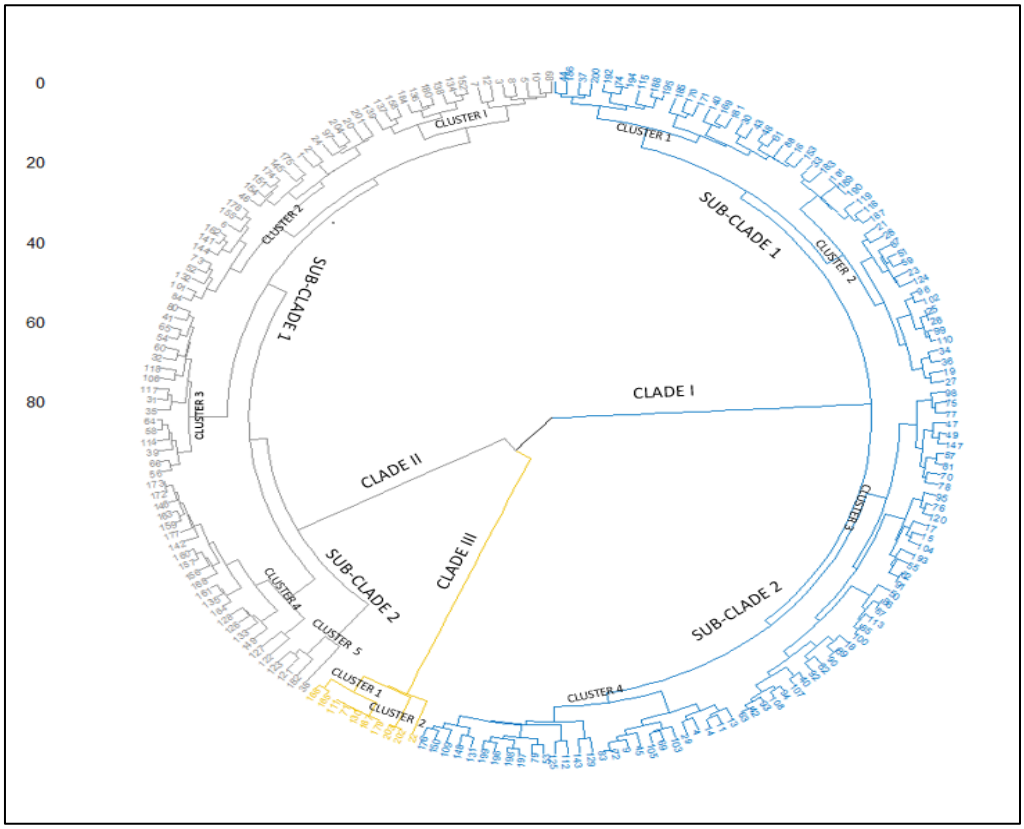

Fig. 1. Cluster analysis of 204 mungbean genotypes based on 24 DUS characters

3.2 Effect of Cold Stress on Germination and Recovery at Seedling Stage

Effect of cold stress (CS) on germination was studied at 10°C or 22°C till 8th day and grouped according to percentage of germination. Germination at control condition (CC) was 100% indicated the status of healthy and viable seeds. Under cold stress, there was a significant ($p<0.0001$) decrease in germination % median value 0.6 which was in the range of 20% to 70% compared to control when considering the inter-quartile range. Germination Index (GI) interrelates germination percentage with time. GI showed nearly 5-fold decrease (from 60 to 12.5 median value) under CS as compared to the CC (Fig. 2) indicating a severe curb on growth and establishment of the germinated seed under CS. Shoot length (SL) of the seedlings after 8-days also showed significant decrease (~7.1 fold) under CS when compared with that of CC. As, the median shoot length in the CC-grown seedlings (i.e., 12.2 cm) was lesser than of the CS-grown seedlings (i.e., 1.7 cm). These results indicated that cold stress dampened the growth and establishment of the seedlings more harshly than the germination. Population distribution according to germination % (Fig. 2) and 100% recovery (Fig. 2) was studied under CS to acquire an overall impression of resilience under cold for the genotypes in study. The check genotype IPM-02-14 fell into higher germination percentage range of 81-90% and could recover in the range of 90-100% at CS (Fig. 2).The graphs showed that the maximum no. of genotypes (i.e., 15%) fell in the interval of 61-70 % germination. Lines with higher germination % (i.e., 71-80%, 81-90% and 91-100%) could also recover at higher parentage, as these lines had recovery above 60% (Fig. 2). Lines with 41-50% and 61-70% germination under CS included the cases where >60% recovery was 46% and 29% respectively (Table 2). These results indicated that germination under CS correlated with % recovery under CC after a CS.

3.3 Effect of Cold Stress Germination on Growth at Vegetative and Reproductive Stage

Germination and initial seedling establishment under CS for 8-days impacted significantly the further growth of plant at vegetative and also at reproductive stage including several yield

parameters, when grown natural day-night condition for 25-30 days (Fig. 3). The median for plant height of CC and CS are 29.75 cm and 34.83cm respectively. Hence, there was ~20% decrease in plant height in the plants that were CS in early seedling stage. Days to 50% flowering (DFF) showed significant difference even though it did not follow a particular trend. The majority of plants which experienced CS in early seedling stage flowered earlier by ~5 ± 5 days than the control. In case of pods/plant, there was a drastic decrease of 54% in case of cold stressed plants. Seeds/pod (Fig. 3) surprisingly showed an increase of 14% in the plants that were CS in early seedling stage when compared to the control population. Considering yield per plant, the median values for control and CS plants were 1.124 g and 0.469 g, respectively indicating 58.2% decrease in yield / plant in the plants with CS in early seedling stage. The median for 100-seed weight was 4.8 g for control population and 3.6 g for test population respectively indicating 33.3% decrease in the CS plants. These results indicated that though the CS was imposed at the germination and initial seedling establishment stage, it could significantly affect the morphological parameters at vegetative and reproductive stage including some yield parameters.

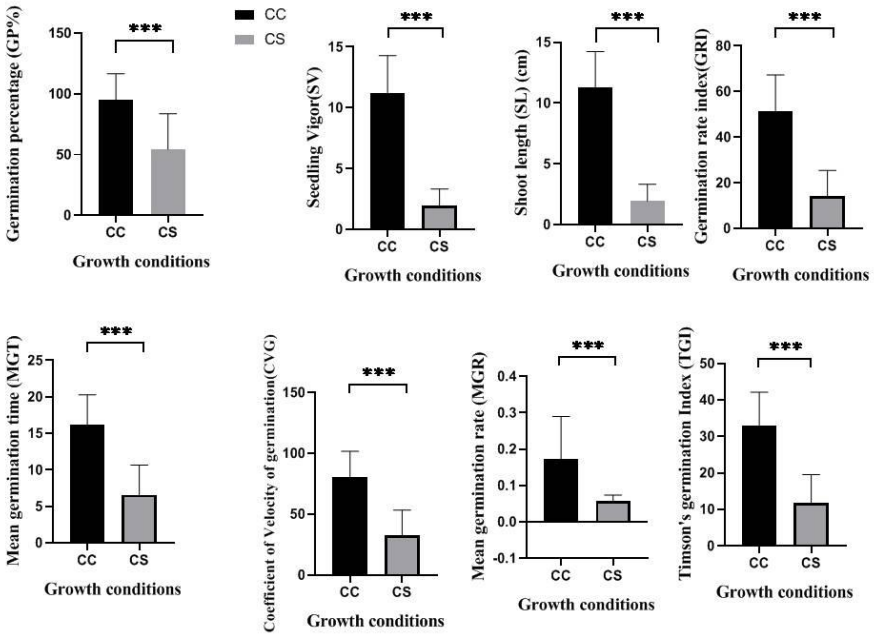

Fig. 2. Phenotyping of mungbean genotypes under cold stress (CS) at 10 ^{0}C and control condition (22 ^{0}C). Data represented were mean of 10 measurements for each with 3 biological replicates. Statistical significance using unpaired t-test indicated as *** p<0.0001, ** p<0.001 and * p<0.01

Table 2. Percentage of germination and percentage of recovery rate of 204 mungbean genotypes under 10^{0}C cold stress

Germination percentage category (10ºC)	Recovery percentage				
	<60% recovery	>60% recovery	100 % recovery	Total	Number of genotypes
0	0	0	0	12	AKM8802,MH1323,ML2056,VC3960-88,CPRBAMGP437,KVK BARAGARH-3, KVK- BOLANGIRI-

Germination percentage category (10ºC)	Recovery percentage				
	<60% recovery	>60% recovery	100 % recovery	Total	Number of genotypes
					1, KVK BOUDH-2, CPRBAMGP302, RIL19, C2-KAMDEV, C3-IPM-02-3.
0-10%	2	10	15	17	ADT3, AKM12-24, ASHAMUNG,AVMU1684, DGG8, MGG385, ML2479,ML818,PDM139, RMG62,CPRBAMGP280, CPRBAMGP280, CPRBAMGP301,CPRBAMGP334, KVK SAMBALPUR3, KVK SUNDERGARH-II-2, VBN GG2, RIL6
11-20%	7	1	2	10	ANGUL MUNG, AVMU1680, C693369, EC693356, GANGA-1, JHAIN MUNG GREEN, CPRBAMGP268,CPRBAMGP306, RIL10, RIL25,
21-30%	18	2	0	20	AVMU1683, BM-2012-9, IPM-312-20, KM2355,KPS2,MH13-25,NM94, PUSA RATNA, PUSA-BM-1, USAM1771,V1000-318-AG, VC6372(45-8-1), VGG16-055, CPRBAMGP267, CPRBAMGP289, CPRBAMGP294, CPRBAMGP321,CPRBAMGP324, CPRBAMGP 351, RIL33
31-40%	8	1	1	10	BANSAPAL, C693367,1PM512-1, OBGG102, PANTMUNG4, PDM11,PM14-11,PUSA VISHAL, VGG16-029,CPRBAMGP 287
41-50%	10	12	4	26	AVMU1678 ,BGS9, COGG13-39, LGG450,ML1907, NARENDRA MUNG1, RMG1087, RMG1092, SML1901, SML668,V100-319-AG,V101400-AG,VC6368-46-40-1, CPRBAMGP 258,CPRBAMGP 283,CPRBAMGP 287, CPRBAMGP 295,CPRBAMGP 304, KVK DHENKANAL-1, COPERGOAN, RIL28, RIL38, RIL45, RIL56,C1-OBGG-52
51-60%	21	2	2	25	AVMU1691,AVMU1698, DGVV18, GGG-1,HUM0936, IPM409-4, MH318,NDMK-16-324,PANTMUNG8, PUSA9531, SKM1504, SML1815, SVM6133, TM8134,TRCM351-2-1,VG16-064,VGG14-1, VGG16-036,

Germination percentage category (10ºC)	Recovery percentage				
	<60% recovery	>60% recovery	100 % recovery	Total	Number of genotypes
					VGG16-036,CPRBAMGP 262, CPRBAMGP305,CPRBAMGP349, RIL26, RIL34, RIL37,RIL50
61-70%	22	9	0	31	AVMU1695, GKM2016-1, HUM12, IPM205-7,IPM312-9, JAUM0936, KPS-1, MDGVV16, MGG373, ML115, NBPGR150, NVL855,PM11-25, PUSA105, PUSA1841,RMB12-07, RMG1097, RMG344,SKNM1502, TM96-2,VC6153-13-20, VG15-29,CPRBAMGP 297, CPRBAMGP329,CPRBAMGP337, KVK KENDRAPADA-1, KVK NAYAGARH-1, KVK SAMBALPUR-2, KVK-MAYURABHANJA11-1, RIL27, RIL42
71-80%	18	3	0	21	AVMU16100, AVMU1682, VC693367,HUM6, IPM410-9,IPM-99-125,MH421, NAYAGARAH LOCAL GREEN, PM14-3,PUSAM1772,PUSA1841,TARM1 , VGG16-058,CPRBAMGP299, CPRBAMGP335,CPRBAMGP339, CPRBAMG342,CPRBAMG354,CP RBAMG359, KVK JAIPUR-1, C4-IPM-02-14
81-90%	11	1	0	12	AVMU1688, AVMU1699, LGG595, VGG15-30, CPRBAMGP331,CPRBAMGP333, CPRBAMGP344, CPRBAMGP353, KVK-NAYAGARH-6, KVK-PURI, KVK-JHARSUDA-1, SUNDERGARH-11-3
91-100%	15	5	0	20	COGG912, HUM2, IGKM6-26-5, IPM02-17, IPM410-3, KM5-18,LGG807, MK15-513, OBGG56, PAU911, PUSA1641, PUSA1672, PUSA9072, CPRBAMGP290, CPRBAMGP345, CPRBAMGP348, KVK BANJANGAR-1, KVK-JAGATHSIGHPUR-1, KVK-PURI-4, KVK PURI-2.
				204 (TOTAL)	

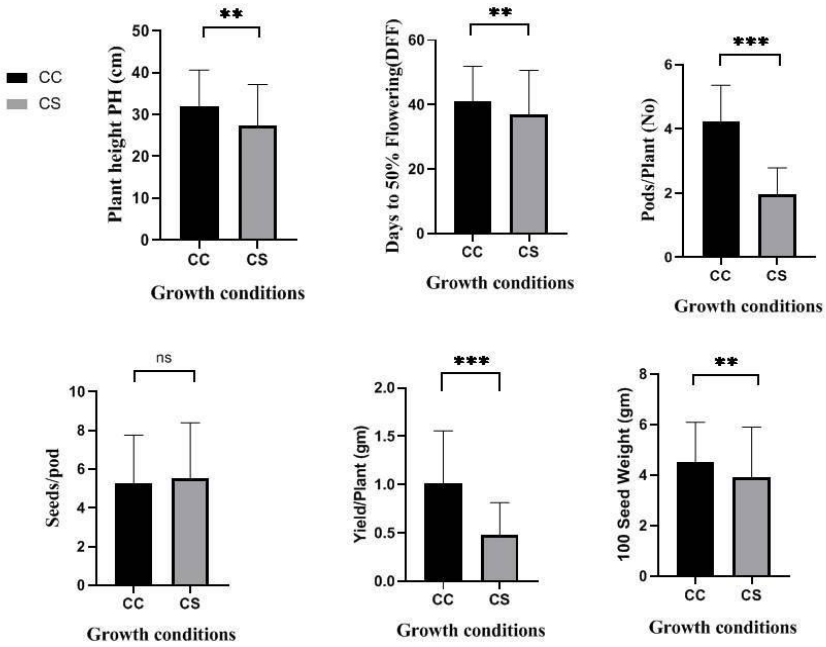

Fig. 3. Growth of the seedling at vegetative and reproductive stage under cold stress. Data represented were mean of 10 measurements for each with 3 biological replicates. Statistical significance using unpaired t-test indicated as *** p<0.0001, ** p<0.001 and * p<0.01

3.4 Seedling Phenotype of Selected Lines for Response to Cold

Based on germination percentage, recovery rate and grouping of genotypes, G77 (PAU 911), G92 (PUSA9531) were selected as the representatives for resistant and susceptible genotypes respectively in response to cold stress. G88 (PUSA1672) had inconsistent positioning in PCA-biplot and similar trend in case of stress analysis, hence taken as representative of the intermediate cold response genotype (Fig. 4). These three lines were analysed for their seedling phenotype and biochemical characteristics under cold stress. Root length and shoot length showed significant ($p<0.0001$) reduction in response to 8-days cold in all the genotypes. Whereas, in case of rootlet number and leaf area highest reduction was observed in the G92 found to be susceptible and least reduction in G77 considered as resistant in previous categorization. G88 had intermediate values in case of root length number and leaf area, which matched its previous categorization under intermediately cold resistant (Fig. 5).

3.5 Biochemical Characteristics of Selected Lines with Response to Cold

A stress of 8-days cold could lead to drastic reduction in the total chlorophyll (Chl) as well as the Chl a, b pigments in all the 3 genotypes, with the highest in the G88 (PUSA1672) under cold stress intermediate genotype. Similarly, carotenoid content (Fig. 6) was also least in G92 (PUSA9531) under control condition, and highest in G88 after CS. Total Chl content was found to be least in the G92 in CC, with reverse trend of higher Chl A than Chl B to that of the G77 (PAU 911) and G88. This result indicated the CS susceptible genotype had less chlorophyll and pale green leaves than the CS resistant and intermediate genotypes under normal growth CC. This indicted that least effect of CS on Chl and carotenoid was observed in the CS intermediate G88. Relative water content was affected only in G88 after CS, with nearly no affect in G77 and G92. Malondialdehyde (MDA) and sugar content tend to increase in all the 3 genotypes with effect to CS. The highest increase in MDA and sugar content was observed in G88 after CS. Reactive oxygen species and its detoxification during CS was assayed with the help of nitrobluetetrazolium (NBT) staining to stain the $O2^{\cdot-}$ radicals and superoxide dismutase (SOD) enzyme activity assay respectively (Fig. 6F, G). SOD enzyme actively increased with effect to CS in the 3 genotypes with the highest in G92, which was the CS susceptible genotype. The $O2^{\cdot-}$ radicals were found to be decreased under CS, and least $O2^{\cdot-}$ was also found in G92 (PUSA9531).

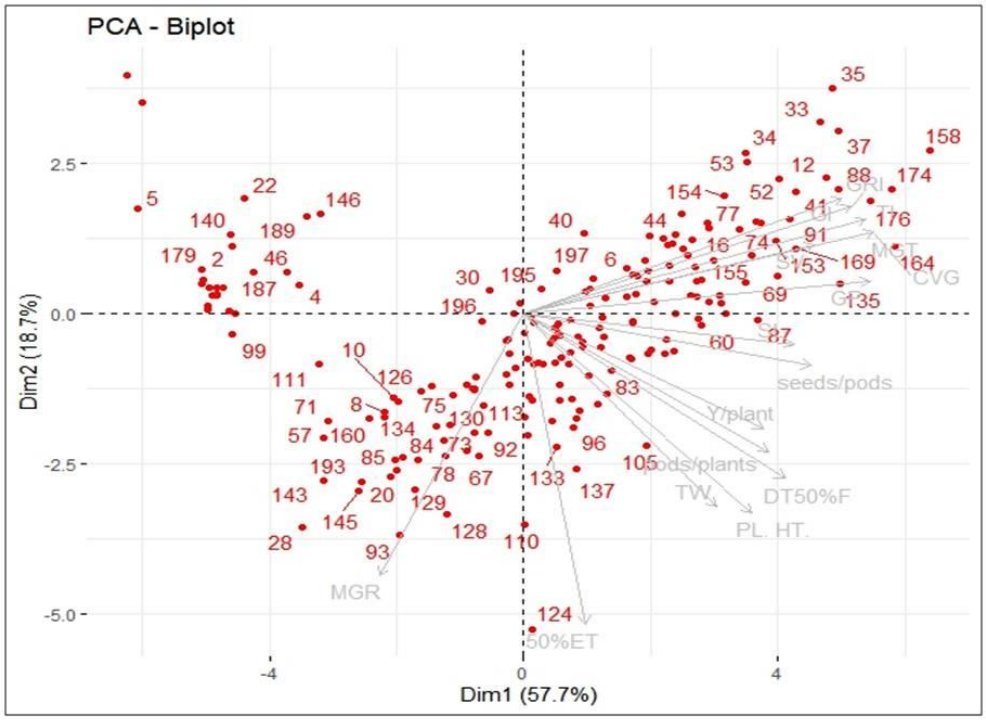

Fig. 4. PCA-biplot analysis based on germination percentage, recovery rate and grouping of genotypes on cold stress

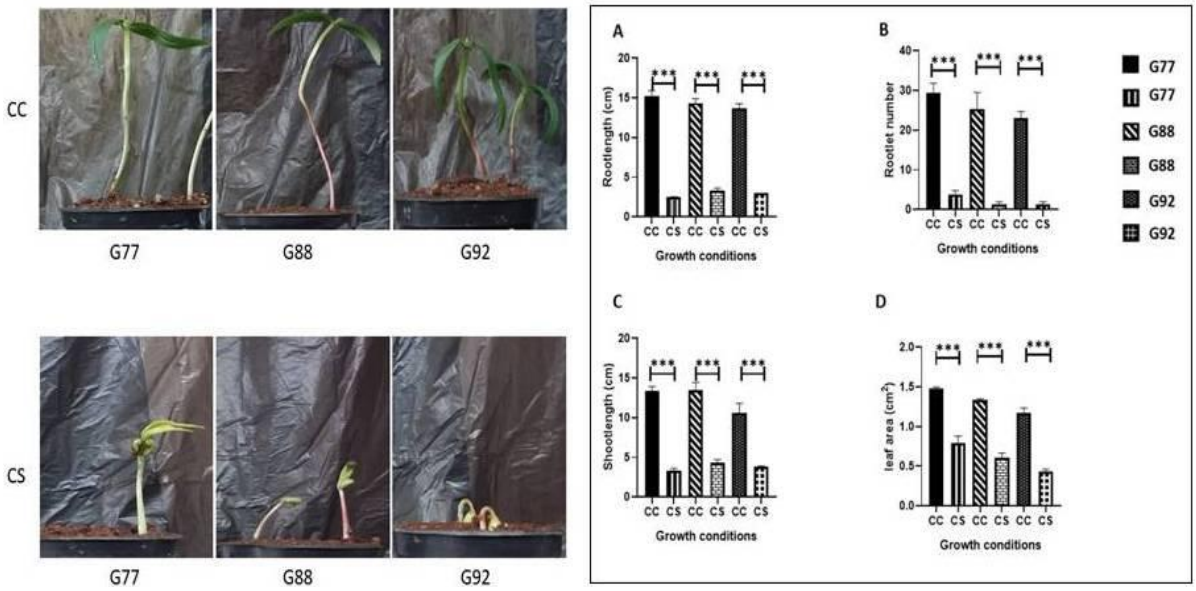

Fig. 5. Phenotyping of resistant G77 (PAU 911), Intermediate G88 (PUSA1672) and susceptible genotypes G92 ((PUSA9531) grown under cold stress and control condition. Data represented were mean of 10 measurements for each with 3 biological replicates. Statistical significance using one-way ANOVA indicated as *** p<0.0001, ** p<0.001 and * p<0.01

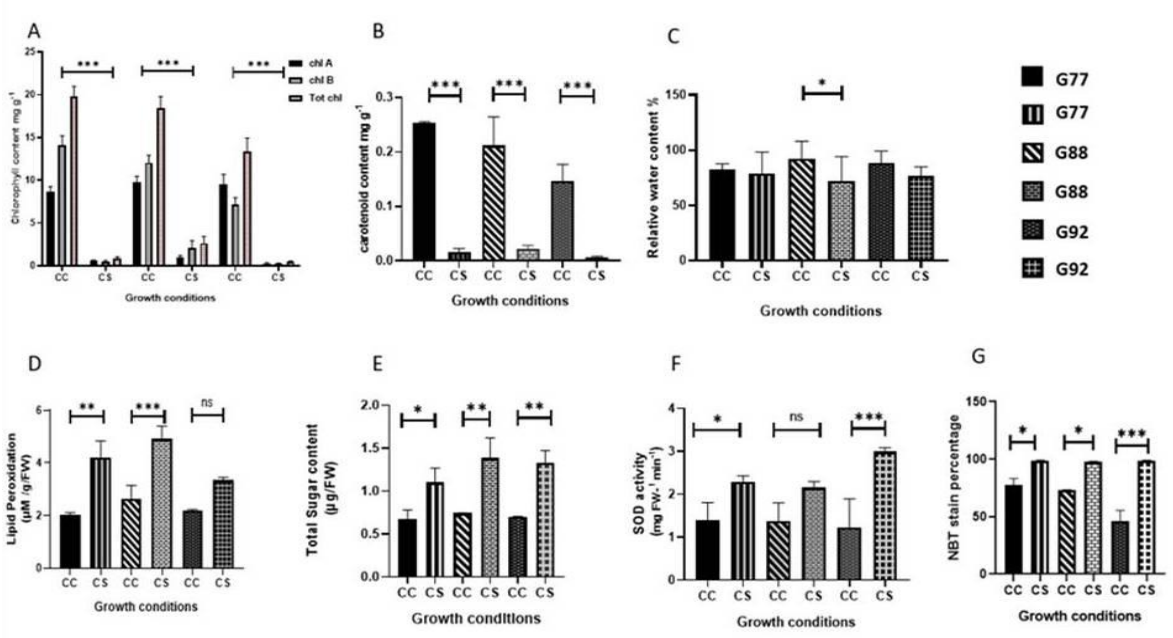

Fig. 6. Biochemical characterization of resistant G77 (PAU 911), Intermediate G88 (PUSA1672) and susceptible genotype G92 (PUSA9531) grown under cold stress (CS) and control conditions (CC). Data represented were mean of 3 measurements for each with 3 biological replicates. Statistical significance using one-way ANOVA indicated as *** p<0.0001, ** p<0.001 and * p<0.01

4. DISCUSSION

Cold stress severely affects the delaying germination, poor emergence, seedling establishment, reduced plant density, and uneven stand establishment. Our study presented an approach based on germination parameters to establish methods for screening of mungbean genotypes for cold stress response at seedlings stage, which can indicate cold tolerance at the vegetative and reproductive stage with the sustainable yield. Initial screening and scoring on the basis of 24 DUS characters represented variability in population and basis of undertaking this study. Our result showed that germination percentage (GP) along with percentage of recovery rate could be proved as a strong reliable parameter for early screening for cold tolerance during the germination stages, apart from the other parameters. Surprisingly, cold stress of the initial 8-days during seedling establishment imposed negative impact throughout the life of mung genotypes, which were reflected in different parameters of plant growth including plat height, days to 50% flowering and pods/plant as well as in the reproductive stage such as seeds/pod, yield/plant and 100-seed weight. Correlation analysis showed distinct correlation among the seedling traits and mature plant traits separately. However, the biplot analysis could bring better resolution in the grouping of genotypes. Our phenotyping revealed that the resistant and intermediate genotype had morphological and phenotypic advantage over the susceptible ones under cold stress. Among the biochemical characteristics studied, chlorophyll, carotenoid and MDA content could appropriately represent the cold response. Cold-induced photosynthetic inhibition is due to reduced chlorophyll synthesis, poor chloroplast development, diminished efficiency of photosynthetic apparatus, restricted carbohydrates transportation, limited stomatal conductivity, suppressed the Rubisco activity during carbon assimilation, disrupted electron transport chain, and decreased energy stock [17,18]. These were evident with a better survival method was observed in the resistant genotype G77 (PAU 911) and intermediate G88 (PUSA1672). It also inferred that the cold stress during germination could have considerable influence in its vegetative and reproductive fitness of the plant. Seedling phenotype reclaimed that the resistant and intermediate genotype has better stress tolerance in terms of rootlet number than the susceptible one. Biochemical analysis also revealed that similar cold tolerance responses over the susceptible

ones during CS. Biochemical analysis showed that similar advantages of the cold tolerant phenotypes over the susceptible. Prolonged exposure to cold stress results in stunted growth, diminished root-shoot surface area, leaf chlorosis, and disturbed water and nutrient relations. Such indicators lead to a significant reduction in yields and quality [19]. Cold stress induces a complex network of signalling pathways mediated by transcription factors, plant hormones, reactive oxygen species (ROS), and other primary and secondary messengers. ROS are generated as a result of aerobic metabolism in plants. Due to its unstable nature, these ROS can damage various cellular components. In order to avoid such damage, plants have developed various redox regulatory systems. Interestingly, ROS are purposefully produced to serve as messengers by plants when they are under cold stress. ROS are long known as cytotoxic molecules that damage the cellular metabolism [20]. Reactive oxygen species (ROS) have dual functions first with a loop dependent on the dosage, at a low level acting to trigger defences and developmental responses at early stages and second with a high level attacking the cell membrane for destroying the cell under breakdown of the defence barrier. ROS are produced by enzymes in plant cells and fine-tune plant signalling in growth and defence against cold stress. ROS regulate the trade-off between growth and defence, where a central transcriptional network regulates the growth and defence pathways to balance the fate of the plant. Therefore, stresses can trigger oxidative stress, and thus, limit vegetative growth and reproduction capacity [21]. On the basis of MDA content during different stages of plant development, it is evident that the adverse effects are accompanied by structural alterations in the membrane [22] which were subsequently followed by cellular leakage of electrolytes and amino acids, diversion of electron flow toward alternate pathways [23], alterations in protoplasmic streaming and re-distribution of intracellular calcium ions. These symptoms are directly correlated with injury to membrane structures of cells and changed lipid composition. Cold-induced alterations in crop plants in particular lead to decreased ATP synthase activity, followed by inhibition of Rubisco regeneration and photophosphorylation [24]. Cold-induced photo-inhibition subsequently leads to a reduction in photosynthetic activity [25]. If cold stress remained for a shorter duration, plants could recover their normal state, but such a situation is irreversible under prolonged duration. The prolonged cold stress period causes severe damage to the mitochondrial structure, slows down the flow of kinetic energy, and disrupts enzymatic activity, ultimately diminishing the respiration rate [26,27]. The multiple signalling pathways in plant response to cold stress enables access to a large network of transcriptional regulators. Fine-tuning through regulation of the cellular abundance and activities of the transcriptional factors are important for plant adaptation and survival. Cold adaptation will increase the plant's tolerance to physical and physicochemical changes when exposed to cold temperatures. [28] Plant resistance to low temperatures has a complex mechanism that involves different metabolic pathways in diverse cellular segments [29,30].

5. CONCLUSION

In conclusion, on the basis of germination-based screening and phenotyping, it was noted that the resistant and intermediate genotypes having more advantage over the susceptible ones under cold stress. The study reported that out of 204 genotypes, only 11 genotypes are cold resistant, which could be used as reliable source for breeding program.

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DATA AVAILABILITY STATEMENT

Data will available with the department. On request it will be provided.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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