ABSTRACT - Winter season maize (*Zea mays* L.) has emerged a new crop in many parts of South and Southeast Asia, where the crop has to face low temperature regimes (<5°C) for few weeks during vegetative growth stage. The objective of this study was to identify the morphological and physiological traits associated with cold stress tolerance during vegetative growth period, when maximum dry matter is accumulated and floral primodia are formed. A total of 80 cultivars, including hybrids and open pollinated varieties (OPVs) from International Maize and Wheat Improvement Center (CIMMYT) and Indian maize program were evaluated in replicated trials at Indian Agricultural Research Institute (IARI), New Delhi and at Regional Research Station, Haryana Agriculture University (HAU), Uchani, Karnal, India during the winter season, where critical period of vegetative growth and floral primodia developmental stage was exposed to <10°C temperature. Data on various growth and developmental traits and key physiological traits were recorded during the low temperature regime. Except ears per plant and physiological maturity, the cold stress significantly affected all the growth and developmental traits and also physiological traits studied. However, significant genotypic variability was observed for most of the traits studied. Genotypes with relatively high leaf appearance and extension rate, less cold injury symptoms and cell membrane damage showed good level of cold tolerance in terms of reproductive behavior and eventually grain yield under cold stress. These secondary traits could be used in selection index along with days to anthesis, anthesis-silking interval (ASI) and grain yield for selection and improvement of tropical maize for low temperature adaptation.

KEYWORDS: Maize; *Zea mays*; Cold stress; Low temperature; Secondary traits.

INTRODUCTION

Maize (*Zea mays* L.) is a *thermophilic* plant species and highly sensitive to low temperature at all stages of development (MieDEMA, 1982; STAEBLER, 2001). Current trend of growing maize in non-traditional areas during winter seasons has increased the likelihood that a maize plant will spend most part of early development under suboptimal temperature conditions. In Indo-Gangetic plains (IGP) region of South Asia the winter season maize crop invariably face severe low temperature regime during winter months. The average minimum temperature may fall below 5°C or even less, especially in the North-West Plains of IGP. An example of extreme low temperature stress was experienced by maize crop in northern India during winter season of 2002-03 and again northern India and Nepal during 2009-10, which caused severe yield losses in large maize growing areas of IGP. Adaptation of maize to winter season in IGP and similar environment in tropics requires genetic improvement for cold tolerance, which implies vigorous seedling growth without suffering with cold injuries under low temperature conditions.

Being cold sensitive plant maize is prone to physiological damages during non-freezing suboptimal temperatures. Leaves of maize, which develop under low temperature conditions, are characterized by a lower photosynthetic capacity, lower quantum efficiency of CO$_2$-fixation, and lower quantum efficiency of electron transfer at PSII ($\Phi_{PSII}$) than leaves which develop under more favorable conditions (NIE *et al.*, 1992; LEIPNER *et al.*, 1999). One reason for the lower photosynthetic performance might be the perturbation of chloroplast development, specifically, the limited ability of maize leaves to develop a functional photosynthetic apparatus at low temperature (NIE and BAKER, 1991). MIEDEMA *et al.* (1987) studied the effect of low temperature on seedling growth from germination to 6$^{th}$ leaf stage, and suggested that resistance to chlorosis and rapid leaf ex-
The improvement of low-temperature tolerance has emerged as an important issue. In the present study, we attempted to identify those morphological and physiological traits that help in discriminating tolerant and susceptible genotypes, their relationship with yield under cold stress, and those traits could be used as selection criteria for further improving cold tolerance in tropical maize.

**MATERIAL AND METHODS**

**Germplasm**

A total of 80 cultivars were used in the present study, which includes 46 genotypes from CIMMYT highland maize program including hybrids, open-pollinated varieties (OPVs) and synthetics, and 32 tropical/sub-tropical hybrids from regional research station. Haryana Agriculture University (HAU), Uchandi, Karnal, India. Two tropical hybrids (Buland and Sheetal) were released from Punjab Agriculture University (PAU), Ludhiana, India as cold tolerant hybrids for cultivation during winter season in North India. Selection criteria for the improvement of low-temperature adaptation. YING et al. (2000) observed that maize hybrids differ significantly in leaf CER response to cold night temperature during flowering/grain filling period, which directly affects effectiveness of grain filling. However, genetic variation does exist for the severity of the response to low temperatures for several parameters, such as leaf CER, quantum efficiency of PSII, rate of development, and rate of dry matter accumulation (Ying et al., 2002). Several studies have been conducted on cold tolerance in temperate and highland maize growing areas, largely focusing on germination and early growth stages. However, limited information is available on cold tolerance in maize grown during winter season in tropical/sub-tropical environment. Keeping in view the current trend of maize cultivation during winter cycle South Asian tropics and other areas with similar ecology crop improvement for low-temperature tolerance has emerged as an important issue. In the present study we attempted to identify those morphological and physiological traits that help in discriminating tolerant and susceptible genotypes, their relationship with yield under cold stress, and those traits could be used as selection criteria for further improving cold tolerance in tropical maize.

**Experimental site, cultural practices and stress treatment**

Experiments were conducted during the Rabi (winter season) of 2006-07 at the maize research farm, Indian Agricultural Research Institute (IARI), New Delhi, India (28.4°N, 77.1°E, 228.1 masl) and Regional Research Station, C.C.S. Haryana Agriculture University (HAU), Uchandi, Karnal, India (29.43°N, 76.58°E, 245.0 masl). Soil of the IARI experimental station is characterized as sandy loam with a pH of 7.8, while HAU, Karnal is clay loam with a pH 7.4. At both locations planting was done during second week of November. The time of planting date was chosen on the basis of long-term (10 years) climatic data of the two locations. The maize was inoculated with maize seed strains A. brasilense strain 5004 and A. brasilense strain 5002. The experimental design was a randomized complete block with five replications and the plot size was 4 × 5 m with four rows of 2.5 m length. The time of planting date was chosen on the basis of long-term (10 years) climatic data of the two locations. The maximum, minimum temperature (°C) and mean relative humidity (%) during the winter crop season at Regional Research Station, Haryana Agriculture University, Karnal, Haryana and Indian Agricultural Research Institute, New Delhi, India.
locations in order to get vegetative stage (V6-V8 stage, tassel and ear primodia emergence stage) exposed to low temperature regimes (<10°C). Climate data recorded during experimental period showed that at both the locations minimum temperature was less than 10°C for over one month during mid-December to mid-January, and occasionally dropped down below to 2°C (Fig. 1).

All the entries were over-sown and thinned to one plant per hill at V2 growth stage to give a population density of 66000 plants ha⁻¹. Each entry was planted in one row plot, each 4.0 m long, with 20 cm spacing within and 75 cm between rows. Before planting 60 kg nitrogen (N) ha⁻¹ in the form of urea, 60 kg phosphorous ha⁻¹ as single super phosphate, 40 kg potassium ha⁻¹ as muriate of potash and 10 kg zinc as zinc sulfate were applied as a basal dressing. Second and third doses of N (each 30 kg N ha⁻¹) were side-dressed at knee-high and tassel emergence stages. Pre-emergence application of pendimethalin and atrazine (both at 0.75 kg ha⁻¹ a.i., tank mixed) were used for weed management in the experimental plots. Experiments were kept free from insect, weeds and diseases using recommended post-emergence chemical measures, and managed under optimal agronomic practices.

**Observations**

In vitro chlorophyll content in the upper-most fully expanded leaf was recorded on five plants in each plot using a Minolta SPAD-502 chlorophyll meter in each plot twice, first during 4th week of December and second during 2nd week of January, and averaged. Plant height, number of fully appeared leaves (the leaves with fully visible collar) and leaf area was recorded at weekly interval during the low temperature regime (<10°C), on five plants from each plot and averaged. During this period, starting from 4th week of December until 3rd week of January, the low temperature remained below 10°C, except very few exceptions at Karnal location (Fig. 1). Leaf length and maximum width of the leaf was calculated using the formula: length x maximum width x 0.75 (Montgomery, 1911). Leaf area of a single leaf was multiplied by total number of leaves to estimate the total area for each plant, and averaged for five plants to calculate the leaf area per plant. Increase in these traits during the one month period (15 December to 15 January) was divided by total number of days and expressed in terms of percent growth rate per day in plant height, leaf appearance and leaf area. Visual cold tolerance score (1-3 scale) was recorded by daily visual observations during late grain filling stage. At maturity, ears were harvested, excluding two plants close to alley from both end of the rows; ear number per plant was determined, and ears per plant (EPP) was calculated. Ears were dried and grain yield was recorded on shelled grain basis and adjusted to 15% moisture level. Per day yield was computed by dividing grain yield with total number of days taken to reach to physiological maturity.

Electrolyte leakage, anthocyanin concentration and total soluble sugar was measured in selected best (9) and worst (17) hybrids in terms of cold tolerance score, along with nine other randomly selected hybrids. All the three traits were measured twice, first during 4th week of December and second during 2nd week of January, and averaged. Cold-induced cell membrane injuries in leaf tissues were quantified by estimating the electrolyte leakage (%) in top most fully expanded leaf. The electrolyte leakage was measured using the standard protocol (http://www.plantstress.com/methods/index.asp). Anthocyanin pigments were extracted from the 50mm leaf disc of top-most fully expanded leaves using acidified ethanol (95% ethanol in 1.5 N HCl). Total anthocyanin concentration was measured using spectrophotometer at 546 nm wave-length (BEGGS and WELLMANN, 1985). Preserved leaf samples in ethanol (95%) were used to extract soluble sugar content through repeated boiling and extraction in 80% ethanol (Mc CREARY et al., 1950). An aliquot was used for determining the reducing sugars through Nelson’s arsenomolybdate method (NELSON, 1949) using improved copper reagent (SOMOGYI, 1952).

**RESULTS AND DISCUSSION**

All the traits, except ears per plant and physiological maturity, were significantly affected by cold stress (Table 1). Cold stress resulted in severe leaf chlorosis, which was evident in terms of highly significant (P<0.01) genotypic variability for leaf chlorophyll content. Reduced chlorophyll content in maize leaves under cold stress has also been reported by others (LEIPNER et al.; 1999; LEE et al., 2002).

Inhibition of chlorophyll accumulation at low tem-
perature was attributed to the photo-oxidation of the chlorophyll at a faster than it was being synthesized. However, it did not significantly correlate with stress sensitivity (MIEDEMA et al., 1982); probably because chlorotic plants recover at the rise of temperatures but growth remain inhibited in comparison with non-chlorotic plants. Anthocynin pigmentation in leaves showed significant variation among genotypes under cold stress, which was more pronounced when best and worst type of entries were compared (P<0.01). Maize lines capable of accumulating high amounts of anthocynin in the illuminated leaf surface may present an adaptive response to harmful conditions of low temperature associated with high light and it is not limiting to photosynthesis (PIETRINI et al., 2002). Highland maize, which usually exposed to cold stress, accumulates anthocynin pigments in stems and leaves (CHONG and BRAWN 1969). According to Gould et al. (2000), the role of anthocynin pigments is not clear and may depend on whether their location is in the vacuoles of the abaxial or adaxial leaf epidermis, in the cytosol of mesophyll cells, in roots, or in stems. Apart from visual score on cold injuries, genotypic variability for the developmental traits, such as leaf appearance and leaf area growth rate, was highly significant (P<0.01). This is in agreement with the observations made by Lee et al. (2002) on maize inbred lines, where they found that leaf appearance rate was about three times slow in cold stressed maize seedling (15/3°C; 16-h photoperiod) than grown under normal temperature (25/15°C; 16-h photoperiod). Leaf area growth was also reported to be a function of prevailing temperature regime. Tol-

### TABLE 1 - Mean, standard error and variation range of various morpho-physiological traits observed on of maize hybrids under cold stress.

<table>
<thead>
<tr>
<th>Traits</th>
<th>Mean (Best)</th>
<th>Mean (Worst)</th>
<th>SEM (Best)</th>
<th>SEM (Worst)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll (SPAD unit)</td>
<td>23.2</td>
<td>19.62</td>
<td>43.8</td>
<td>1.754</td>
</tr>
<tr>
<td>Cold tolerance score (0-3 scale)</td>
<td>1.13</td>
<td>0.74</td>
<td>2.04</td>
<td>0.206</td>
</tr>
<tr>
<td>Leaf emergence rate (% day⁻¹)</td>
<td>1.63</td>
<td>0.59</td>
<td>2.48</td>
<td>0.255</td>
</tr>
<tr>
<td>Leaf area growth rate (% day⁻¹)</td>
<td>1.22</td>
<td>1.12</td>
<td>3.96</td>
<td>0.313</td>
</tr>
<tr>
<td>Growth in plant height (% day⁻¹)</td>
<td>1.61</td>
<td>0.67</td>
<td>3.16</td>
<td>0.137</td>
</tr>
<tr>
<td>50% anthesis (days)</td>
<td>119.4</td>
<td>132.9</td>
<td>103.7</td>
<td>3.016</td>
</tr>
<tr>
<td>Anthocynin-silking interval (days)</td>
<td>4.99</td>
<td>7.63</td>
<td>2.9</td>
<td>0.707</td>
</tr>
<tr>
<td>Ears per plant</td>
<td>0.68</td>
<td>0.63</td>
<td>0.87</td>
<td>0.051</td>
</tr>
<tr>
<td>Physiological maturity (days)</td>
<td>158.9</td>
<td>166.2</td>
<td>153.5</td>
<td>6.036</td>
</tr>
<tr>
<td>Grain yield (t ha⁻¹)</td>
<td>3.97</td>
<td>2.13</td>
<td>7.16</td>
<td>0.359</td>
</tr>
<tr>
<td>Per day yield (kg ha⁻¹)</td>
<td>24.5</td>
<td>13.14</td>
<td>44.13</td>
<td>4.033</td>
</tr>
<tr>
<td>Anthocynin (OD cm⁻²)</td>
<td>1.11</td>
<td>0.46</td>
<td>2.83</td>
<td>0.064</td>
</tr>
<tr>
<td>Total soluble sugar (mg g⁻¹ fresh weight)</td>
<td>14.05</td>
<td>11.08</td>
<td>26.04</td>
<td>1.469</td>
</tr>
<tr>
<td>Electrolyte leakage (%)</td>
<td>19.57</td>
<td>28.92</td>
<td>10.87</td>
<td>2.017</td>
</tr>
</tbody>
</table>

### TABLE 2 - Mean squares (MS), percent coefficient of variation (CV) and standard error (SE) of the hybrid trial for chlorophyll content, cold tolerance score, leaf emergence rate, leaf area growth rate, plant height growth rate, per day yield and grain yield of hybrids grown under cold stress.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Chlorophyll Mean</th>
<th>CV (%)</th>
<th>SE</th>
<th>Cold tolerance score Mean</th>
<th>CV (%)</th>
<th>SE</th>
<th>Leaf emergence rate Mean</th>
<th>CV (%)</th>
<th>SE</th>
<th>Leaf area growth rate Mean</th>
<th>CV (%)</th>
<th>SE</th>
<th>Plant height growth rate Mean</th>
<th>CV (%)</th>
<th>SE</th>
<th>Per day yield Mean</th>
<th>CV (%)</th>
<th>SE</th>
<th>Grain yield Mean</th>
<th>CV (%)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>79</td>
<td>163.29**</td>
<td>18.69</td>
<td>1.45</td>
<td>2.14**</td>
<td>26.35</td>
<td>0.09</td>
<td>2.034**</td>
<td>31.16</td>
<td>0.10</td>
<td>3.104**</td>
<td>41.36</td>
<td>0.12</td>
<td>3.98*</td>
<td>41.36</td>
<td>0.16</td>
<td>143.58**</td>
<td>55.97</td>
<td>0.24</td>
<td>5.59**</td>
<td>55.97</td>
<td>0.24</td>
</tr>
<tr>
<td>Error</td>
<td>79</td>
<td>4.208</td>
<td>2.017</td>
<td>2.017</td>
<td>0.09</td>
<td>0.07</td>
<td>0.07</td>
<td>0.10</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>1.64</td>
<td>1.64</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* and ** indicate statistical significance at 0.05 and 0.01 probability levels, respectively.
 Lenaar et al. (1979) reported that the optimum temperature for leaf extension in maize was 30°C, and the extrapolated minimum 7°C. Miedema et al. (1982) also found genetic variation in leaf extension rate at day/night temperatures of 15/10, 20/15, and 25/20°C. Cold stress significantly affected days to 50% male flowering, which resulted in significant variation in anthesis-silking interval (ASI). Miedema et al. (1987) suggested that resistance to chlorosis and rapid leaf expansion at low temperature are considered major selection criteria for the improvement of low-temperature adaptation. Bechoux et al. (2000) reported that chilling significantly affected the tassel morphogenesis, and significantly reduced number of tassel branches and spikelet pairs in maize. Cold waves during winter cycle of 2002-03 in North India severely affected male flowering aspects of maize crop, including tassel development, limited growth of anther lobes and filaments and pollen shedding, which resulted in severe yield losses (Samra et al., 2005). The effect of cold stress on leaf appearance resulted in significant (P<0.05) genotypic variability in vertical growth, which was expressed in terms of significant effects on growth in plant height. Cold stress significantly reduced cell division and cell elongation, which eventually resulted in stunted growth (Miedema et al., 1982). 

Apart from morphological traits, the physiological traits including total soluble sugars in leaf and electrolyte leakage (indicator of cell membrane damage due to cold stress) also showed significant (P<0.05) genotypic variability under cold stress. Soluble sugars have been found to play an important role during cold acclimation process (YuanYuan et al., 2009). Soluble sugars exert their positive effects to protect plant cells from damage caused by cold stress through several ways, including serving as osmo-protectants, nutrient as well as interacting with the lipid bi-layer. Lukatkin (2003) observed a gradual increase in leakage of ions from the cells upon prolongation of chilling exposure, with the maximum attained by the end of 24-h chilling treatment. Farooq et al. (2008) found that reduced membrane permeability contributed towards chilling tolerance in maize hybrids. The overall effect of cold stress on various morpho-physiological traits eventually resulted in highly significant (P<0.01) genotypic variability for grain yield under stress. Though, the effect of cold stress was statistically non-significant on physiological maturity, it has significant effects on per day yield (P<0.01). The traits such as - ears per plant and physiological maturity in leaves showed non-significant genotypic variation across all genotypes, however the variation was statistically significant when the best and worst genotypes were compared (Table 1). Fast early growth and early flowering with long grain-fill duration, and extended stay-green have provided the basis for hybrids with high yield potential under low temperature stress (Frei, 2000). Analysis of variance (Table 2) of various traits showed highly significant contribution of genotype in genotypic variability under cold stress. Though coefficient of variation was on higher side for some of the traits, for example - leaf appearance, cold tolerance score and area growth rate, but it was largely because of low mean of the trials, not because of high error of the trial. 

Data indicated that there was a sharp decrease in leaf appearance rate during cold temperature regime (Fig. 2). In genotypes with poor cold tolerance it decreased significantly during 2nd week of December (86.4%) in comparison to their leaf appearance rate during last week of November. The decrease was maximum during 1st week of January (95.9%), and the low rate of leaf appearance was continued until end of January. The leaf appearance rate slowed down in case of even best cold tolerant genotypes; however, the effect was comparatively less severe, except during first week of January (80.7%), when low temperature dropped down below 2°C (Fig. 1). Similar effect of cold temperature regime was observed on rate of increase in plant height as well (Fig. 2). Vertical growth was worst af-
fected during last week of December and first week of January. With increase in temperature during end of January the recovery was comparatively faster in vertical growth than leaf appearance rate. Variation in susceptibility to low temperature on leaf appearance rate directly affected overall plant growth rates of best and worst group of genotypes (Fig. 3). The rate at which successive new leaves emerge at the stem apex in gramineae (phyllochron) is largely controlled by temperature (Wilhelm and McMaster, 1995; Van Esbroeck et al., 2008), which was found curvilinear over a range of temperature regime (Warrington and Kanemasu, 1983). After V3 stage, when temperature dropped down below 10°C, the worst group of genotypes took significantly more number of days to achieve the various growth stages in comparison to best entries for cold tolerance; for example – worst group of genotypes reached to tassel appearance (VT) growth stage about 20 days later than best group of entries. However, the differences between best and worst group of genotypes gradually reduced at later stages (after pollination), which resulted in comparatively less grain filling period for worst group of genotypes, and eventually affected final grain yield.

Phenotypic correlation and regression analysis showed significant relationship between various secondary traits with grain yield under cold stress (Table 3). However, intercept and slope of the regression curve for various traits on grain yield varied significantly. Among all the traits, leaf appearances rate showed strongest positive relationship with grain yield under cold stress ($r = 0.69^{**}$; $R^2 = 0.73^{**}$). Cold tolerance score and per day yield also showed significant relationship with grain yield under cold stress. Visible scores on cold tolerance was significantly related with total soluble sugar concentration in leaves ($R^2 = 0.56^{**}$) and electrolyte leakage from the leaf tissues ($R^2 = 0.64^{**}$) (Fig. 4), which indicated that availability of total soluble sugar and reduced ion leakage helped in reducing the cold injuries. The low availability of total soluble sugars under cold stress might be related to reduced rate of photosynthesis that resulted in low assimilates starvation, and eventually visible stress symptom on plants. Inhibition of photosynthesis is known to be an early response to low temperature (Ying et al., 2002). Regression analysis between per day yield and ratio of days of 50% anthesis and physiological
maturity (Fig. 5) showed significant negative relationship ($R^2 = 0.465^*$). This indicates that the genotypes which required more days to complete their vegetative growth had fewer days for reproductive growth, which resulted in low per day yield, and eventually low final grain yield under cold stress.

Our findings suggest that, in general, tropical maize is highly sensitive to cold stress during vegetative growth stage. However, there is considerable genotypic variability available for various traits, such as – photosynthetic pigments, growth and development traits. Presence of genetic variation for these secondary traits indicates that alleles are available in breeding materials that can improve cold tolerance. CIMMYT gene pools developed for high altitudes contain valuable material for these traits (BECK et al., unpublished data). We conclude that selection of genotypes using secondary traits, such as- leaf appearance and extension rate, visual score for cold injuries, reduced electrolyte leakage along with grain yield under cold stress may be used as a selection index for identification and further improvement of cold tolerance in tropical maize.

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