Response of combined abiotic stresses on maize (*Zea mays* L.) inbred lines and interaction among various stresses

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Abstract

In tropics, maize is largely grown as rain fed crop in marginal areas often faces drought and waterlogging simultaneously in same season that critically affects plant growth and development. The aim of the research was to study the response of combined abiotic stresses and the interaction among various stresses on maize inbred plants. In the present study, eighty maize (*Zea mays* L.) inbred lines were screened, for multiple abiotic stresses at the vegetative stage. All the traits, observed were severely affected, in drought x low-N stress compare to waterlogging x low-N stress. However in both the stress combinations (drought x low-N and waterlogging x low-N) chlorophyll content decreases significantly, low-N stress has synergistic effect on the maize plants. Hence the overall effect of stress combination was negative causing enhanced damage to plants. Whereas, interaction of drought x waterlogging stress showed the antagonized each other response that primarily enhanced growth parameters (leaf area, plant height and stem diameter) and it has improved the tolerant mechanism of maize plants. Therefore we concluded, the response of maize various plants exposed to combinations of stresses is depend on interaction of stresses.

Introduction

Climate change is threatening food production systems and therefore, food security and livelihoods of billions of people in the world. The agricultural systems, are largely dependent on prevailing weather conditions and therefore extremely vulnerable to climate change effects. Several climates modeling studies (IPCC, 2007) suggest, sharper increases in both day and night-time temperatures, in the future, which could adversely impact maize production in the tropical regions (Cairns et al, 2012). Maize is the major crop grown for food, and fodder, in tropics, it is largely grown in marginal areas in rain fed ecology during summer-rainy season that often in compression of various biotic and abiotic stresses. The major abiotic constrains that plants faces are drought, waterlogging, nutrient, high temperatures and salinity during their lifespan. Plants are sessile, they have developed several mechanism to detect environmental changes and are capable to respond and cope with different abiotic stress or combination of stresses. They respond to these abiotic stresses either, by escaping that is completing the life cycle before the onset of stress or avoidance and tolerance through, morphological alterations and by changes, in their physiological processes. Many physiological or biochemical traits, associated with, improved drought tolerance have been identified (Blum, 1989; Foulkes et al, 2007) for waterlogging tolerance, (Rathore et al, 1996; Zaidi et al, 2003) for low-Nitrogen fertilization (Cirilo et al, 2009). However, until now most of the studies on the plant-environment interaction were focused on a single stress (Mittler R, 2006). Although, tolerance to two different abiotic stresses has been emphasized on the breeding strategy for corn and some other crops (Heyne and Brunson, 1940; Jiang and Huang, 2001). Additionally, germplasm selected for tolerance to drought also shows resistance towards low-N and waterlogging stress, hence there is spillover from drought to low-N/ waterlogging tolerance in maize genotypes (Zaidi et al, 2008). Recent evidence shows that plants respond to multiple stresses differently from individual stresses (Atkinson and Urwin, 2012). Plants activate a specific and unique stress response when subjected to the combination of multiple stresses (Rizhsky et al, 2004b). They modifies their response according to multiple stress conditions and shows several unique and common responses. Therefore, the effect of combined stress factors on crop is depends on the nature of interactions between the stresses. (Pandey et al, 2015a, b; Choudhary et al, 2016; Ramu et al, 2016). To show the interactions among various abiotic and biotic stresses on plant growth and productivity a "stress matrix" was developed (Mittler, 2006; Suzuki et al, 2014) that explain the stress combinations can have negative as well as positive effects on plants. Simultaneous occurrences of abiotic stresses like drought and heat stress causes severe damage to crops than their separate occurrences at different crop growth stage (Mittler, 2006; Prasad et al, 2011). The abiotic stresses either applied singly or in combination, they affect the crop growth and development, though, in the early stages of crop no visible symptoms are detected but significant changes in plant physiology are already induced (Cramer et al, 2011) Hence, to understand the mechanisms of plant response to combined stresses is crucial. The main priority is to characterize the plant germplasm for multiple stress tolerant traits, so that sustainable germplasm could be developed for breeding programs. Therefore, the present work was undertaken to study the response of maize inbred lines to simultaneous exposure to the multiple abiotic stresses and interaction among various stresses. Also to examine, weather interaction of stresses cause severe damage to crop or it might prove to be helpful in acclimatization of the plants under stress condition.

Materials and Methods

Green house Abiotic Stress Tolerance Screening

Plant Material and Growth conditions

Maize seeds were obtained from the International Maize and Wheat Improvement Center (Spanish acronym; CIMMYT®). These identified inbred seeds having distinct difference in terms of tolerance/susceptibility to single stress, like waterlogging, drought and low-N. In the present work, eighty (80) inbred lines(Zea mays L) were screened, for multiple stresses in combination (drought x low-N and waterlogging x low-N) applied concurrently at vegetative stage. The experiment was conducted with a completely randomized design, the seeds were sown into the pots (12cm diameter) that were filled with sandy loam soil with no added organic fertilizer. Pots were kept in a naturally lit greenhouse, with air temperature 25-30°C and relative humidity 55-65% during day and night and were well-watered. In each pots four-five seeds were sown, later, they were thinned 2 plants per pot after seedling emergence with three replications of each.

Stress treatment

Low-N stress The fertilizers requirement for maize plant as per agronomic recommendation are, N 120 kg/ha (urea) P_2O_5-60kg/ha K_2O-60kg/ha and $ZnSO_4$-25kg / ha. The nutrient applied in pots, were calculated on the basis per kg soil, full dose of phosphorous, potash, zinc, were mixed in the soil before sowing. For low-N stress only 25% of the recommended amount (urea) was added (calculated on the basis of per kg soil in the pots).

Drought stress The maize plants were exposed to a mild drought stress 30 DAS (Days after sowing) by withholding water for 10 days, Soil moisture content was determined in pots before stress and the last days of the stress by 'soil drying method' Fig S1 (Supplementary) and various growth parameters were recorded, before stress (0 day) and after stress (10 day) each pot formed one replication. A minimum of three pots were sampled for each observations and data was expressed on per plant basis.

Waterlogging stress After drought x low-N stress, plants were re-watered for two days, after that pots were sealed at the bottom, with sand and cement mixture, so that the water-air drainage could be stopped and the same plants were subjected to waterlogging treatment continuously for 7 days, pots were fully flooded with water and further to maintain the standing water depth 2-3cm above soil surface pots were watered twice a day. After completion of the stress treatment holes were opened, water was drained out from the pots. Though excessive soil moisture stress continued till 10th days. All the data were recorded on the last day of stress. Each pot formed one replication. A minimum of three pots was sampled for the observation and data were expressed on a per plant basis.

Growth parameters

Plant height: measured as the distance between the soil surfaces to the flag leaf node.

Leaf area: leaf length x leaf maximum width x 0.75 (Montgomery, 1911) x total number of leaf /plant.

Stem diameter: basal stem diameter (in mm/cm) were recorded, before and after stress.

Morpho-physiological parameters

Fresh and dry weights: Fresh shoot weight, fresh root weight, and total fresh weight were measured by means of electronic balance. Root dry weight and shoot dry weight (mg/plant) was determined by means of electronic balance after drying root and shoots at 80°C in an oven, for 72hrs. Total dry weight was calculated by adding the dry weight of shoot and root.

Chlorophyll Content Analysis Chlorophyll was extracted, by a non-maceration method using, dimethyl sulphoxide (Hiscox and Israelstam, 1979). Subsequently, the absorbance was read at 663 and 645 nm in Hitachi's spectrometer (Model-2900). The amount of chlorophyll a and b in µg/ml was calculated using the formula given by Arnon, (1949). Total chlorophyll content was determined by adding chlorophyll a and b contents. The amount of chlorophyll was finally calculated in terms of mg/g fresh weight.

Leaf Relative water content Analysis The parameter has been analyzed in drought x low-N stress. On the seventh day of drought stress, the fully expanded leaves were plucked and cut into the pieces of 10cm, and immediately measures for fresh weight. (FW) This leaf was immersed, in distilled water in vials, then after 12 hours, turgid weight was determined. (TW) Finally, to measure the leaf dry weight, leaf segments were soaked, with tissue paper and kept in the oven, for drying at 70°C for 24h . The relative water content (RWC) was calculated by using the formula as

$$RWC\% = FW\text{-}DW/TW\text{-}DW \times 100\%.$$

Statistical analysis For all parameters measured each pot represented one replication. A minimum of three pots was sampled for all observation and data were expressed on a per plant basis. To verify the significance of the variations of all the parameters, One-way analysis of variance (ANOVA) followed by the post hoc Tukey test ($p < 0.01$) was used. Two and three way ANOVA was done to evaluate the interaction study between two stresses and inbred plants. All the statistical analyses were conducted using the SPSS 17.0 software package.

Results and discussion

Response of maize plants in drought x low-N stress

The combination of two stresses is regarded as the new state of stress, which may cause severe damage or enhanced tolerance of crop plants. The green house screening of the eighty maize inbred lines, showed differential response to combined drought and low-N stress, some plants were less affected, while, others were severely affected. The effect was most apparent on growth parameters like leaf area, and plant height. The leaf area in response to drought x low-N stress decreases. Fig 1 Shows leaf area before stress and after, Nesmith and Ritchie, (1992) shows under short term drought stress leaf tip emergence delayed and leaf area reduces, whereas long duration drought reduces final sizes of the leaf. In our study, drought stress does not affect the final size of leaf, though it delay the new leaf emergence. The drought stress also affects cell division and cell elongation due to low turgor pressure therefore restricting plant height, the data showed that plant height varies from highest (38.33±1.45) to lowest (7.33±0.88) among different inbred plants. Hence for a

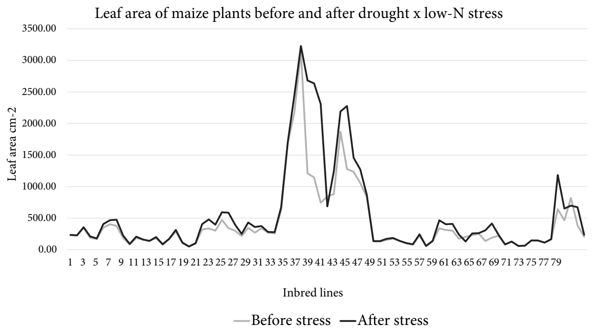

Fig. 1 - Leaf area of various maize plants screened in drought x low-N. The data represent the mean values of three replicates from each lines.

given stress, its impact varied greatly among different plants (Chen et al, 2012) . Among observable phenotypic changes induced by drought stress were leaf rolling and wilting. Based on phenotypic observation of leaf rolling in the scale of (1-5) some inbred lines selected on the scale of 1-3 for estimation of leaf relative water content. Drought stress decreased RWC of leaf tissue in maize plants as shown in Fig 2. The RWC content of the selected maize plants ranges from 92% to 38.165%

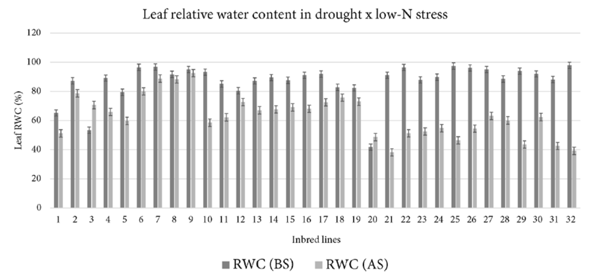

Fig. 2 - For estimation of RWC, inbred plants that shows leaf rolling scale of (1-3) were selected. (from 1 to 32 inbred lines). The mean values of three replication of each line represented here. RWC- Relative water content; BS-Before stress; AS- After stress.

and these difference were highly significant ($p<0.01$) and coherent with phenotypic visual observations. RWC shows plants cellular water status and the drought tolerance mechanisms associated with the ability of a plant to retain cellular water under stressful conditions (Erice et al, 2010). Hence this could be an important physiological measurement to differentiate between tolerant and susceptible plants under drought stress. Another trait that was notably affected was leaf chlorophyll content, it decreases significantly in all the plants. The coefficient of variation varies from 17.711 to 36.796 and critical difference before stress (0.51) and after stress (1.082) in chlorophyll content. Our results supported by various reports that showed the chlorophyll content decreases under drought stress (Bijanzadeh and Emam, 2010; Mafakheri et al, 2010; Din et al, 2011). Also nitrogen deficits reduces

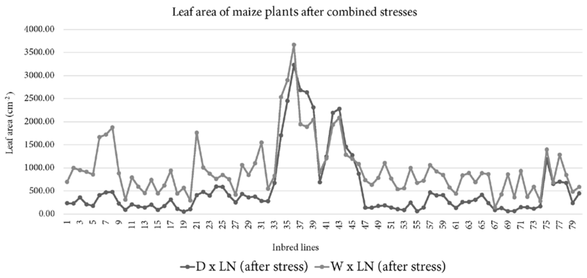

Fig. 3 - Leaf area measured in combined stress conditions. Shows significant increase in leaf area after waterlogging x low-N stress. The following terms: D x LN –Drought x low-N and W x LN- Waterlogging x low-N stress.

leaf chlorophyll content in maize (Pandey et al, 2000). Similarly, Wolfe et al, (1988) observed positive correlation between chlorophyll concentrations (leaf greenness) in maize with leaf N concentration. Henceforth, interaction of drought x low-N stress reduces leaf chlorophyll content.

Response of maize plants in waterlogging x low-N stress

Waterlogging conditions caused severe plant mortality, stunted plant growth, and reduced leaf area growth and total biomass production at all the physiological stages (Zaidi et al, 2004). However, our results showed leaf area increases of more or less in all inbred lines in waterlogging x low-N condition (Fig 3), the critical difference of leaf area before stress was (493.186) and after stress (584. 337).Whereas, Zaidi et al, (2003) reported high leaf area per plant and longer leaf area duration might be assets across the growing conditions. The increase in plant height of different genotypes ranges from (48.33±4.26) to lowest (8.0±1.00), the overall increases 8% after stress (Fig 4). Stem diameter trait is associated for lodging resistance for cereal crops (Kashiwagi et al, 2008). Waterlogging stress reduces stem diameter in most plants (Promkhambut et al, 2011). In contrast our results showed, significant increase in basal diameter of some inbred lines Fig (5), it might prove a useful trait that

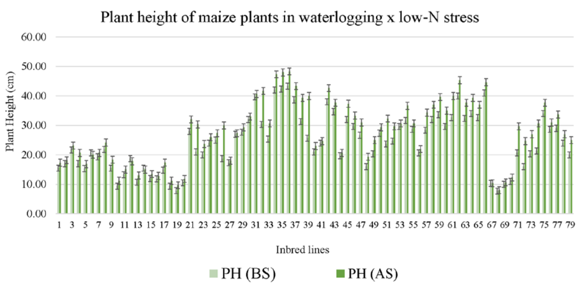

Fig. 4 - A bar graph showing plant height of various maize plants before and after stress- Plant height; BS-Before stress and AS-After stress.

Table 1: Interaction and effects of combined stresses on morpho-physiological traits

Traits	Drought x LN			Waterlogging x LN			Interaction A x B (F-calculated)	Significance
	Mean Square	C.D	SE(m)	Mean Square	C.D	SE(m)		
FSW	1,791.24	1.327	0.665	89.536	4.197	2.104	1.356	0.17408
DSW	43.613	0.222	0.079	1.971	0.703	0.249	2.725	0.00097
FRW	41.896	0.224	0.079	1.535	0.707	0.251	1.902	0.02514
DRW	8.755	0.112	0.04	0.43	0.355	0.126	4.302	0
TFW	72.253	0.399	0.142	17.399	1.262	0.448	4.939	0
TDW	4343.93	1.385	0.491	64.309	4.379	1.553	3.913	0.00001
R/S ratio	0.294	0.085	0.03	0.058	N/A	0.096	0.847	0.64638

resist lodging under waterlogging condition. Various morpho-physiological parameters like fresh shoot and fresh root weight, dry shoot, and dry root weight and total dry weight and total fresh weight enhanced in most plants,two way Anova values of some selected genotypes as shown in Table 1. Whereas dry weight of both shoots and roots significantly reduced under 6-days waterlogging treatments (Liu yong-zhong et al. 2010). Leaf chlorophyll concentration is an indicator of chloroplast development, leaf nitrogen content and involved in photosynthesis or general plant health

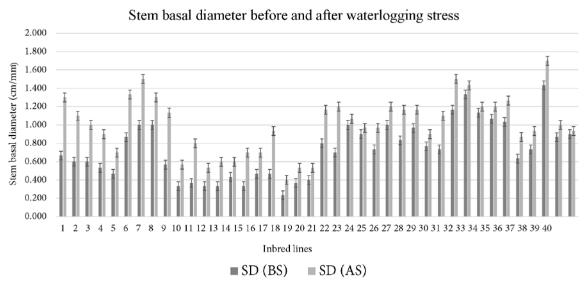

Fig. 5 - Stem basal diameter measured before and after waterlogging stress in selected maize lines. SD- Stem basal diameter; BS-Before stress and AS- After stress

(Ling et al, 2011). A significant decreased in chlorophyll content was observed in all maize lines. Zhou et al, (2004) identifies decreased in chlorophyll content a first stress symptom under waterlogging stress that may be related to nitrogen deficiency. Further, (Rathore et al, 1996) correlated this to leaching and denitrification of the soil nitrogen. However only few plants able to maintain steady chlorophyll content (< 40 % decrease) after combined stresses. Data for analysis of variance shown in Table 2. Maize plants which showed higher chlorophyll content after stress they were more vigorous in growth as compared to plants which have lower chlorophyll content.

The interaction between stresses drought low-N x waterlogging x low-N) and response of maize plants.

Different types of stress interactions their nature, severity and duration have profound influences on the plants. Like, simultaneous heat and drought occurrence may lead to more drying of soil that resulted in worse drought condition and hence more yield loss. They

Table 2: ANOVA results of total chlorophyll content of maize plants before and after stress in combined stresses.

			Before Stress			After Stress		
	Source	**df**	**SS**	**MS**	**Significance**	**SS**	**MS**	**Significance**
D x LN	Replication	2	0.235			1.076		
	Treatment	46	59.176	1.286	0	82.033	1.783	0
	Error	92	9.075	0.099		40.812	0.444	
	Total	140	68.486			123.921		
	Replication	2	0.002		0.1071	0.001	0.002	0.02574
	Treatment	19	0.017	0.001		0.033		
	Error	38	0.021	0.001		0.032	0.001	
	Total	59	0.041			0.065		
W x LN	Replication	2	0.027			0.034		
	Treatment	46	55.679	1.211	0	67.529	1.468	0
	Error	92	17.156	0.186		28.138	0.308	
	Total	140	72.88			95.882		
	Replication	2	0.001			0.002		
	Treatment	19	0.039	0.002	0.01381	0.02	0.001	
	Error	38	0.033	0.001		0.014	0	0.00315
	Total	59	0.073			0.036		

In above table total chlorophyll content estimated in 66 lines, in two set (1-46 lines and 2- 20 lines) data of 14 lines missing due to termite attack. The following terms represent: SS-Sum of square; MS-Means of square; BS-Before stress; AS- After stress; DxLN- Drought x low-N stress; WxLN- waterlogging x low-N stress.

have synergistic effects on plant physiology, causing in greater negative net impact shown as severe yield reductions (Mittler, 2006). In our study also the interaction of drought x low-N stress and waterlogging x low-N stress caused a significant decrease in chlorophyll content in maize inbred plants. Thus, interaction of two stresses interaction have synergistic effects on physiology of maize plants, the overall two stresses becomes additive resulting in enhanced damage to plants under this stress combination. Similarly, Rizhsky et al, (2002) reveals that during heat stress enhanced transpiration to cool leaf surface exacerbate the effects of concurrent drought and salinity because increased transpiration rate leads to more water loss and increased uptake of salts. However, the interaction of two stress combination (abiotic/abiotic or abiotic/ biotic) sometimes does not produce negative impact on plants. They antagonize the effect of each other,

Table 3: Interaction of stresses and effect on various traits

Treatment	**Traits**	**Before stress**			**After stress**			**Interaction A x B**	**Signific-ance**
		C.D	**SE(m)**	**C.V.**	**C.D**	**SE(m)**	**C.V.**		
DxLN	Leaf area	471.866	168.75	68.614	445.658	159.378	49.736	4.204	0.04073
WxLN		493.186	176.374	39.468	584.337	208.972	36.711		
DxLN	Plant height	5.831	2.085	21.979	8.265	2.956	26.247	1.185	0.27684
WxLN		7.267	2.599	18.768	7.537	2.696	16.859		
WxLN	Stem diameter	0.311	0.110	26.231	0.325	0.115	19.888	0.913	0.61979
DxLN	Leaf RWC	7.676	2.649	4.312	24.932	8.604	19.285	4.064	0
DxLN	T C(Set 1)	N/A	0.014	26.86	0.048	0.017	42.959	0.016	0.90097
WxLN	TC	0.049	0.017	27.546	0.032	0.011	22.384		
DxLN	TC (Set 2)	0.51	0.181	17.711	1.082	0.385	36.796	1.245	0.2653
WxLN	TC	0.701	0.249	23.485	0.901	0.32	31.158		

Three way ANOVA results show the interaction of stresses for various traits. The following terms represent: C.D- Critical difference; SE (m)- Standard errors of means; DxLN)- Drought x low-N stress; WxLN - Waterlogging x low-N stress; TC (Set 1)- total chlorophyll content in 20 lines and TC (Set 2)- total chlorophyll in 46 lines. CV- Coefficient of variation.

A- Before stress/After stress; B – Drought x low-N/ Waterlogging x low-N leading to a net neutral or positive impact on plants, or one stress may also provide endurance to plants against another stress (Pandey et al, 2017). As in our study, interaction of drought x waterlogging primarily increase in leaf area, leaf number, and plant height, fresh and dry weight of shoots and roots, and has alleviated the tolerance of maize plants. Therefore, the interaction between two stresses leads to a completely unique response that ensures the best utilization of plant energy resources. Three way analysis of variance for various traits shown in Table 3. Moreover reports (Kaddour and Fuller, 2004; Song et al, 2014; Rivero et al, 2014), were also in agreement to our results that shown the effect of two stress combination were ameliorated. According to, Bowler and Fluhr, (2000) the cellular responses to these range of environmental stresses (Abiotic) are similar, that's why plant resistant to one stress are sometimes cross tolerant to others. Moller and Chua, (1999) emphasize that various interactions and intersections are important to modulate and balance the different signaling cascades, for plants proper developmental responses. Further, simultaneous occurrence of several stress results in highly complex responses of plants and these response to combined stresses is largely controlled by different, and sometimes opposing, signaling pathways that may interact with and inhibit each other (Vile et al, 2012; Suzuki et al, 2014).Therefore, may be in our results interaction of drought x waterlogging might activates pathways that have antagonized each other effects and when plants exposed to second stressor it leads to increase in various growth parameters. Maize plantsto the combined stresses.Thus, help to acclimatize the plants to combined stresses.

Conclusions

Based on the hypothesis, that a combination of two stresses may cause severe damage or enhanced tolerance of crop plant, there is interaction of stresses. The overall response of plants to stress combination is apparently governed by the more severe stress. The interaction of drought x low-N and waterlogging x low-N, was damaging, and it decreases chlorophyll content significantly. Whereas, the interaction between two stresses sometimes primarily produce unique response that ensures best utilization of plant energy resources. Therefore, in our study also, drought x waterlogging stress causes enhancement in growth parameters. Interaction of these two stresses (drought x waterlogging) was positive and it enhances the tolerance mechanism of maize plants.

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