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B Mahadeva Prabhu
Department of Biological Sciences, Faculty of Science, Sam Higginbottom University of Agriculture and Technology Sciences, Allahabad, Uttar Pradesh, India

PW Ramteke
Department of Biological Sciences, Faculty of Science, Sam Higginbottom University of Agriculture and Technology Sciences, Allahabad, Uttar Pradesh, India

Pradeep Kumar Shukla
Department of Biological Sciences, Faculty of Science, Sam Higginbottom University of Agriculture and Technology Sciences, Allahabad, Uttar Pradesh, India

Pragathi Mishra
Department of Molecular and Cellular Engineering, Jacob Institute of Biotechnology and Bioengineering, Sam Higginbottom University of Agriculture and Technology Sciences, Allahabad, Uttar Pradesh, India

Aashu Attri
Nuclear Research Laboratory, IARI, New Delhi, India

Bupinder Singh
Nuclear Research Laboratory, IARI, New Delhi, India

Gaurav S Pagire
Department of Biological Sciences, Faculty of Science, Sam Higginbottom University of Agriculture and Technology Sciences, Allahabad, Uttar Pradesh, India

Correspondence
B Mahadeva Prabhu
Department of Biological Sciences, Faculty of Science, Sam Higginbottom University of Agriculture and Technology Sciences, Allahabad, Uttar Pradesh, India

Evaluation of response of exogenous nitric oxide on photosynthetic enzymes and pigments of C_3 and C_4 plants grown under drought stress

B Mahadeva Prabhu, PW Ramteke, Pradeep Kumar Shukla, Pragathi Mishra, Aashu Attri, Bupinder Singh and Gaurav S Pagire

Abstract
Globally cereals are considered as basic or staple diet for man, as cereals are rich in nutritive value. Cereals are severely pretentious by stressful environments such as drought, salinity, extreme high temperatures and other stresses. At present, drought stress is considered as the major factor that adversely affects crop growth and yield, generating declines in global cereal's production including wheat and maize by 3.8% and 5.5%, respectively. Nitric Oxide (NO) is a growth regulator and a potent signaling molecule for plant systems. Effect of NO on photosynthetic enzymes and pigments such as Rubisco Activity, Chlorophyll a and b, Total Chlorophyll and carotenoid content of wheat (a C_3 plant) and maize (a C_4 plant) under different levels of PEG induced drought were probed in the present research. Two genotypes, each of wheat and maize where exposed to different levels of drought [induced by 8%, 16%, 24% PEG] with and without nitric oxide. Results revealed that NO application increased rubisco activity, % rubisco in total protein, chlorophyll a and b, total chlorophyll and decreased carotenoid level. The effect of NO was more profound on heat tolerant genotype, W-7 in contrast to heat susceptible genotype PW-353. In general the effect of NO was more prominent in wheat genotypes comparison to maize genotypes however; drought didn't affect much on maize genotypes than wheat. Investigating the rubisco activity, % rubisco in total protein, chlorophyll a and b, total chlorophyll content under different levels of drought stress, maize genotypes showed better performance than wheat genotypes.

Keywords: cereals, peg, nitric oxide, photosynthetic enzymes

Introduction
Altered gene expression and disturbances in cellular metabolism, as a consequences of environmental turbulences, whether –from biotic and abiotic stresses, can trigger a wide variety of plant responses, adversely affecting growth rates and crop yields. Abiotic stress such as high salinity, drought, temperature extremes, water logging, high light intensity or mineral deficiencies are principal causes of crop failure, decreasing the average yields of most major crops by more than 50 % and threatening the sustainability of agriculture worldwide (Galmes *et al*., 2013, Ashraf and Harris., 2013) [7, 4]. The principal abiotic stress in India is drought affecting nearly two-thirds area under arid and semi arid eco systems (Grover *et al*., 2011) [9]. The term "drought" indicates an agricultural malady, where rainfall and / or irrigation systems fail to supply the adequate amount of water to meet the transpiration demand of the crop (Tuberosa., 2012) [24]. Drought implicates diverse array of plant responses at physiological, biochemical and molecular levels. Among the functional processes, affected by drought, photosynthesis is the most sensitive process. The inhibitory effects of photosynthesis may be associated with low CO_2 availability due to stomatal and non-stomatal limitations. Drought stress damages the thylakoid membrane, inhibit photochemical activities and adversely affect enzymatic activities in the Calvin cycle such as Rubisco, ultimately accounting for the reduced photosynthetic rate (Shehab *et al*., 2010, Anjum *et al*., 2011, Gonzalez-Cruz and Pastene., 2012, Amirjani and Mahdiyeh., 2013) [22, 2, 8, 1]. Drought stress reduces Chlorophyll-a, Chlorophyll-b and total chlorophyll content by the accumulation of chlorophyllase and peroxidase enzymes, thus leading to the decrease in growth yield (Arivalagan and Somasundaram., 2015, Rabert *et al*., 2015, Saeidi *et al*., 2015) [3, 17, 19]. Since the water availability is very low due to low rainfall and water pollution worldwide, we can consider Growth regulators or chemical application are the most important measures in the recent years, to overcome the drought stress. It was observed that application of Nitric Oxide (NO) donors (sodium nitroprusside, SNP) enhances plant tolerance to specific stresses (Shallan *et al*., 2012) [21].

NO is considered as a phytohormone and a key signalling molecule functioning in various physiological processes of plants. It has been observed that NO improved drought tolerance and enhanced net photosynthetic rate in wheat and rice by improving stability of membrane, enhancing activities of antioxidant enzymes and reducing H_2O_2 and MDA contents (Oz *et al*., 2015) [15]. Considering that the NO decreases the inhibitory affects caused by drought stress, this study was aimed at evaluating the response of NO on photosynthetic enzymes and pigments of C_3 and C_4 crops under different levels of drought stress.

Materials and Methods

Experimental site and materials

Experiment was conducted during late Rabi, 2016. Seeds of two varieties each of wheat (PW353 and W7) and maize (Macca 3 and P3546) were directly sown in mud points containing mixture of equal proportions of peat, clay and sand and kept inside polyhouse under natural light. After germination plants were thinned to one healthy plant per pot. For drought induction, PEG 6000 was given at 8, 16 and 24 %, the plants without PEG treatment were treated as control.

NO treatment

The foliage of control and drought stressed plants were sprinkled thrice with solutions of 100µl of NO (sodium nitroprusside served as NO donor) in different sets at after five days interval of drought induction. The nozzle of the sprayer was adjusted in such a way that it pumped out 1 ml (approximately) in single sprinkle. Therefore, each foliage of plants finally received 3 ml SNP solution prior to flowering. The plants were selected randomly in all the varieties and treatments. After 5 days of SNP treatment, fresh leaf samples were collected for further analysis.

Spectrophotometric determination of photosynthetic pigments

Estimation of chl was done by using dimethyl sulphoxide (DMSO) extraction procedure, the method given by Hiscox and Israelsham, 1979. Plant leaves were collected at random from each pot after 5 days of NO treatment and chopped into fine pieces. 50 mg samples from these chopped leaves were added in replicated tubes each containing 10 ml dimethyl sulphoxide (DMSO).The tubes containing leaf pieces and DMSO were incubated at 65^0 C for 3 hours in an oven by providing gentle shake twice. After complete extraction, clear supernatants were used for measuring the absorbance with the help of a spectrophotometer against DMSO blank. The chl a, chl b and total chl contents were calculated according to the formula given below on mg g^{-1} fresh weight of leaf tissue basis.

The Chl was calculated by following equation:

1. Chl a (mg g^{-1} fresh weight)
= 12.7 (A_{663}) – 2.63 (A_{645}) x (V/1000 x W)
2. Chl b (mg g^{-1} fresh weight)
= 22.9 (A_{645}) – 4.45 (A_{663}) x (V/1000 x W)
3. Total chl (mg g^{-1} fresh weight)
= (20.2 x A_{663}+ 8.02 x A_{645}) x (V/1000 x W)

Estimation of carotenoid was done by using the method given by Lichtenthaler & Welburn, 1983. 0.5 gm of fresh leaves was weighed and homogenized in 10 ml of acetone (80% acetone). The tube containing the sample was centrifuged at 3000 rpm for 10 min. The absorbance was recorded at 470 nm.

It was calculated by the formula,

Total carotenoids = [1000A_{470}- (3.27 Chl a + 104 Chl b)]/229

Determination of rubisco activity

Rubisco Activity was estimated essentially as described by *Fair et al. (1973)* [6]. 500 mg leaf samples were taken and crushed in pre cooled pestle and mortar in the presence of liquid nitrogen using 5 ml extraction buffer. A pinch of Polyvinylpyrrolidone was added during grinding. The homogenate was centrifuged at 9000 g for 20 mins at 4 ºC in refrigerated centrifuge (Hermle Z323K). The supernatant was used for activation in scintillation vials (Tarson). The enzyme present in 1 ml supernatant was activated by incubating the extract at room temperature (25-27 ºC) with 0.1 ml each of 10 mM $NaHCO_3$, 10mM $MgCl_2$, 5 mM glutathione, 0.1 mM EDTA an 50 mM tris HCl (pH 8.0) for 10 min.

All the components of assay medium except RuBP were added into scintillation vials. Assay medium without RuBP served as blank for each assay. The reaction was started by addition of RuBP in vials and terminated after 10 min with the addition of 0.2 ml of 6N acetic acid. The contents of vials oven dried at 65º C. The acid stable ^{14} C was subsequently counted in a liquid scintillation counter after adding 10 ml of scintillation fluid (SRL Pvt Ltd.).

Results

Regulatory effects of exogenous NO using SNP as a source on photosynthetic pigments and Rubisco in C_3 and C_4 plants grown under PEG induced drought stress were evaluated. The results obtained in the present investigation are described here under.

Rubisco activity (µmol ($^{14}co_2 kg^{-1} g^{-1}$) and % rubisco present in total protein (µmol (co_2 g^{-1} (rubisco) s^{-1})

The results given in table 1 revealed that the drought stress decreased the rubisco activity and % rubisco in total protein at different levels; however the reductions were higher in C3 plants than C4 plants at 24 % PEG. In C3 plants PW-353 showed a severe reduction in rubisco activity and % rubisco in total protein when compared to W-7 genotype, whereas in C4 plants both the genotypes showed similar reductions under different levels of drought.

Nitric oxide improved the rubisco activity and % rubisco in total protein at all the levels of drought stress. The effect of nitric oxide in improving rubisco activity and % rubisco in total protein was showed higher in C3 plants than C4 plants and the effect was found to be more efficient at 16% of drought stress. The % response of nitric oxide in improving the rubisco activity and % rubisco in total protein under different levels of drought stress is depicted in fig 1 and 2 respectively.

Chlorophyll a and Chlorophyll b (mg/g fw)

Drought stress decreased the chlorophyll a and b gradually at every level. The highest reduction was found in wheat (C_3) crop when compared with maize (C_4) crop (table 2). The response of Nitric Oxide on C_3 and C_4 crops chlorophyll a and b grown under PEG induced drought condition showed that, Nitric Oxide influenced the chlorophyll a and b positively in both the crops. The higher effects of nitric oxide were observed in C_3 plants when compared to C_4 plants which are depicted in fig 3 and 4 respectively. When the effect of Nitric Oxide was studied on both the plants subjected to drought stress, Nitric Oxide decreased the negative effect of drought

condition induced by PEG by increasing the chlorophyll a and b. When both the varieties of wheat were compared, W-7 genotype performed better and considering the maize varieties i.e. Macca-3 and P-3546, both the varieties shown almost similar responses with and without Nitric Oxide.

Chlorophyll content (mg/g fw) and carotenoid content (mg/g fw)

The amount of total chlorophyll was reduced with the increase in the level of drought stress in wheat (C_3) and Maize (C_4) and the reduction was found to be higher in C_3 crop but the drought stress inversely affected the carotenoid content by increasing it at every level gradually and it is given in table 3. Application of Nitric Oxide enhanced total chlorophyll content in both the plants; however the effects were more prominent in C_3 when compared to C4 plants. The ameliorative effect of Nitric Oxide was also studied on both the plants subjective to drought stress. Results showed that Nitric Oxide had reversed the adverse condition generated by PEG induced drought by improving total chlorophyll content under drought stress condition. Among the two varieties of wheat, W-7 performed better and among the maize varieties, both the varieties i.e. Macca-3 and P-3546 showed almost similar responses with and without Nitric Oxide. Whereas, the application of Nitric Oxide decreased the carotenoid content in both the plants and when compared to C_4 plants the decrease was more in C_3 plants. Highest reduction was observed in W-7 genotype, under wheat than PW-353 genotype. Whereas, among maize both the genotypes i.e. Macca-3 and P-3546 showed the similar responses in the presence of Nitric Oxide. The response percentage is represented in the fig 6. The reduction in total carotenoid is may be due to changes in chlorophyll a and b pigment. Similar results were observed by (Sanchez-Estudillo *et al.*, 2006) [20].

Chlorophyll a/b ratio and Total Chlorophyll/Carotenoid ratio

The response of Nitric Oxide on wheat (C_3) and maize (C_4) chlorophyll a/b and total chlorophyll/carotenoid ratio grown under PEG induced drought condition is represented in the table 4. The results showed that, Nitric Oxide influenced the chlorophyll a/b ratio positively in both the crops at 16 % drought stress and enhanced the total chlorophyll/carotenoid ratio in both the plants. The higher effects were observed in C_3 plants when compared to C_4 plants. When the effect of Nitric Oxide was studied on both the plants subjected to drought stress, Nitric Oxide did not affect positively on chlorophyll a/b ratio but affected positively on the total chlorophyll/carotenoid ratio in both the plants; and it is depicted in the fig 7 and 8 respectively. When both the varieties of wheat were compared, W-7 genotype performed better and considering the maize varieties i.e. Macca-3 and P-3546, both the varieties shown almost similar responses with and without Nitric Oxide.

Table 1: Effect of nitric oxide on rubisco activity (µmol ($^{14}co_2kg^{-1}g^{-1}$) and % rubisco present in total protein (µmol (co_2 g^{-1} (rubisco) s^{-1}) of wheat and maize under different levels of drought stress.

Treatments	Rubisco Activity (µmol($^{14}CO_2Kg^{-1}g^{-1}$)				% rubisco content in total protein (µmol(CO_2 g^{-1} (Rubisco) s^{-1})			
	Wheat (C_3)		Maize (C_4)		Wheat (C_3)		Maize (C_4)	
	PW-353	W-7	Macca-3	P-3546	PW-353	W-7	Macca-3	P-3546
Control	8.32	8.38	8.45	8.54	0.176	0.174	0.182	0.186
100 µM SNP	9.99	10.06	10.12	10.21	0.195	0.195	0.206	0.208
8 % PEG	6.78	6.85	6.91	7.00	0.169	0.167	0.169	0.173
8 % PEG with 100 µM SNP	7.92	7.99	8.05	8.14	0.208	0.208	0.213	0.222
16 % PEG	6.42	6.48	6.55	6.64	0.172	0.169	0.174	0.178
16 % PEG with 100 µM SNP	7.75	7.81	7.88	7.97	0.261	0.252	0.235	0.249
24 % PEG	6.17	6.23	6.30	6.39	0.175	0.174	0.178	0.187
24 % PEG with 100 µM SNP	7.37	7.43	7.50	7.59	0.282	0.291	0.255	0.268
C.D @ 5%	0.19336	0.20351	0.21575	0.22154	0.03196	0.03756	0.04756	0.05647

Table 2: Effect of nitric oxide on chlorophyll a and chlorophyll b (mg/g fw) of wheat and maize under different levels of drought stress.

Treatments	Chlorophyll a (mg/g fw)				Chlorophyll b (mg/g fw)			
	Wheat (C_3)		Maize (C_4)		Wheat (C_3)		Maize (C_4)	
	PW-353	W-7	Macca-3	P-3546	PW-353	W-7	Macca-3	P-3546
Control	2.35	2.373	2.103	2.083	1.03	1.18	1.00	1.00
100 µM SNP	2.461	2.463	2.173	2.153	1.13	1.21	1.08	1.06
8 % PEG	1.975	1.983	1.817	1.797	0.84	0.86	0.82	0.81
8 % PEG with 100 µM SNP	2.167	2.25	1.977	1.957	0.97	1.01	0.99	0.97
16 % PEG	1.787	1.807	1.69	1.677	0.82	0.84	0.8	0.78
16 % PEG with 100 µM SNP	2.052	2.177	1.957	1.937	0.94	0.94	0.95	0.92
24 % PEG	1.657	1.78	1.677	1.657	0.75	0.75	0.76	0.73
24 % PEG with 100 µM SNP	2.008	2.045	1.927	1.907	0.88	0.95	0.90	0.87
C.D @ 5%	0.02543	0.02345	0.02995	0.03654	0.02073	0.02984	0.03654	0.04512

Table 3: Effect of nitric oxide on total chlorophyll content (mg/g fw) and carotenoid content (mg/g fw) of wheat and maize under different levels of drought stress.

Treatments	Total Chlorophyll Content (mg/g fw				Carotenoid content (mg/g fw)			
	Wheat (C_3)		Maize (C_4)		Wheat (C_3)		Maize (C_4)	
	PW-353	W-7	Macca-3	P-3546	PW-353	W-7	Macca-3	P-3546
Control	3.38	3.55	3.11	3.08	0.52	0.48	0.45	0.46
100 µM SNP	3.59	3.67	3.25	3.21	0.50	0.47	0.44	0.45
8 % PEG	2.81	2.84	2.64	2.60	0.56	0.52	0.51	0.52

8 % PEG with 100 µM SNP	3.14	3.26	2.97	2.93	0.53	0.49	0.46	0.46
16 % PEG	2.60	2.64	2.49	2.45	0.57	0.53	0.52	0.53
16 % PEG with 100 µM SNP	2.99	3.12	2.91	2.86	0.54	0.49	0.48	0.50
% Response	+14.76	+17.86	+16.86	+16.52	-5.42	-6.41	-7.86	-5.75
24 % PEG	2.41	2.53	2.44	2.39	0.57	0.54	0.53	0.54
24 % PEG with 100 µM SNP	2.79	3.00	2.83	2.78	0.55	0.51	0.5	0.51
C.D @ 5%	0.03584	0.06541	0.09547	0.05745	0.00730	0.00954	0.01254	0.01014

Table 4: Effect of nitric oxide on Chlorophyll a/b ratio and total chlorophyll/carotenoid ratio of wheat and maize under different levels of drought stress.

Treatments	Chlorophyll a/b ratio				Total chlorophyll/carotenoid ratio			
	Wheat (C_3)		Maize (C_4)		Wheat (C_3)		Maize (C_4)	
	PW-353	W-7	Macca-3	P-3546	PW-353	W-7	Macca-3	P-3546
Control	2.36	1.98	2.05	2.08	6.97	6.58	6.48	6.49
100 µM SNP	2.17	2.08	2.02	2.04	7.57	7.30	6.99	7.02
8 % PEG	2.32	2.28	2.22	2.19	6.09	5.28	4.91	4.86
8 % PEG with 100 µM SNP	2.18	2.25	2.01	2.03	6.39	6.48	5.91	5.91
16 % PEG	2.12	2.09	2.12	2.15	5.92	4.85	4.74	4.65
16 % PEG with 100 µM SNP	2.22	2.35	2.07	2.29	6.04	6.06	5.56	5.55
24 % PEG	2.25	2.39	2.16	2.19	5.39	4.53	4.30	4.48
24 % PEG with 100 µM SNP	2.20	2.14	2.08	2.11	6.36	5.61	5.41	5.41
C.D @ 5 %	0.03043	0.05462	0.03564	0.04265	0.25774	0.29875	0.35642	0.33728

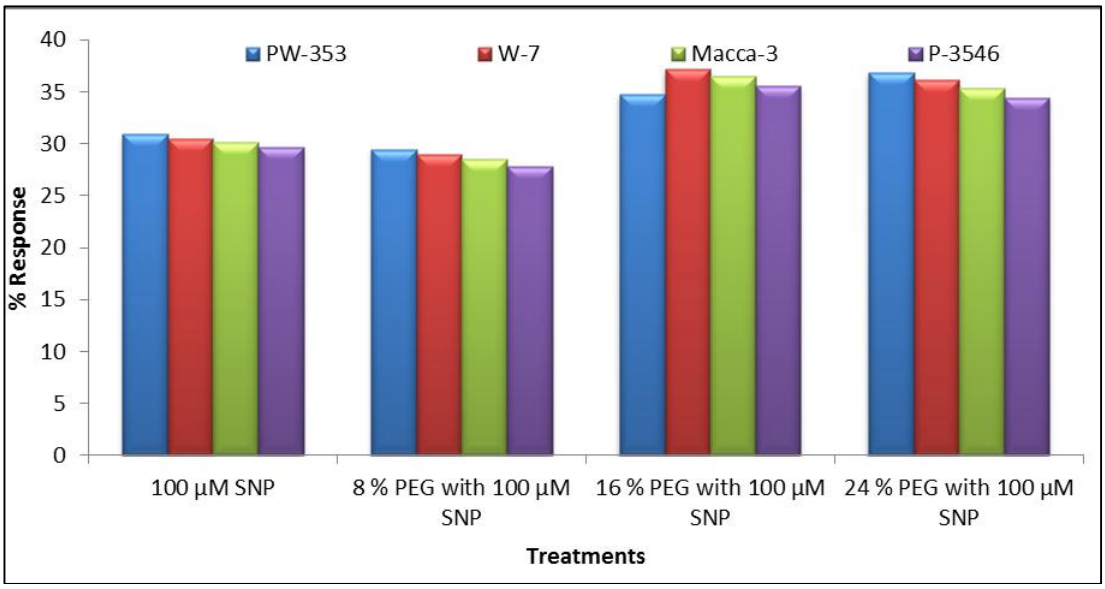

Fig 1: Response of nitric oxide on rubisco activity (µmol ($^{14}co_2kg^{-1}g^{-1}$) of wheat and maize under different levels of drought stress

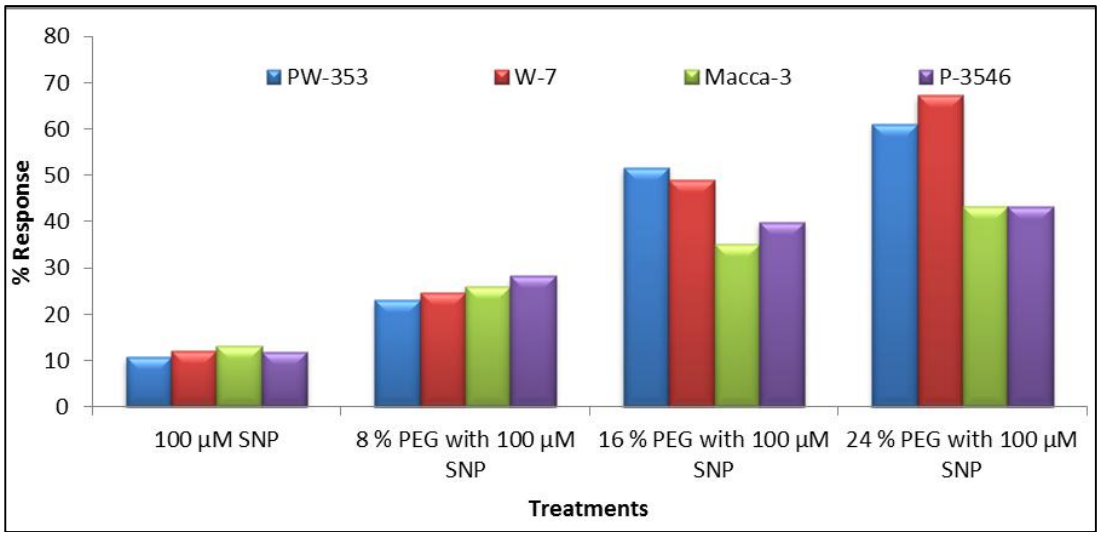

Fig 2: Response of nitric oxide on % rubisco present in total protein (µmol (co_2 g^{-1} (rubisco) s^{-1}) of wheat and maize under different levels of drought stress

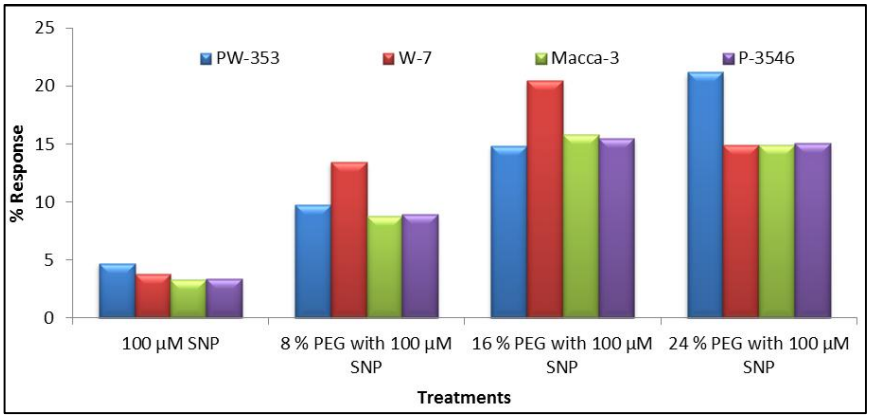

Fig 3: Response of nitric oxide on chlorophyll a (mg/g fw) of wheat and maize under different levels of drought stress

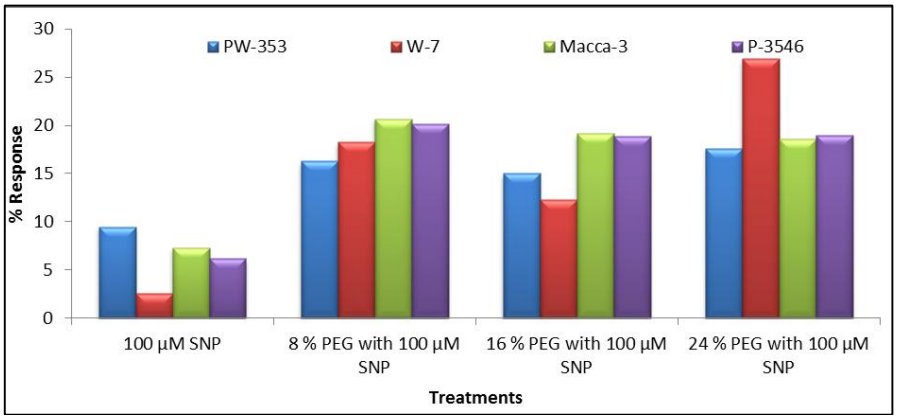

Fig 4: Response of nitric oxide on chlorophyll b (mg/g fw) of wheat and maize under different levels of drought stress

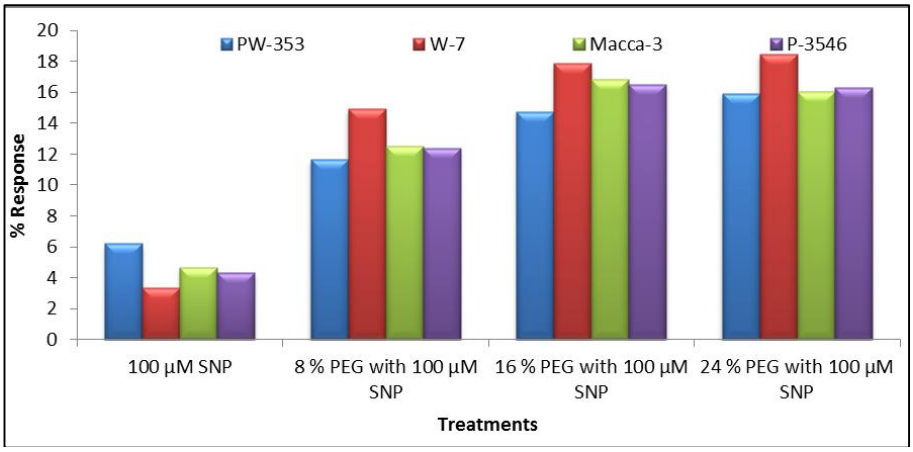

Fig 5: Response of nitric oxide on total chlorophyll content (mg/g fw) of wheat and maize under different levels of drought stress

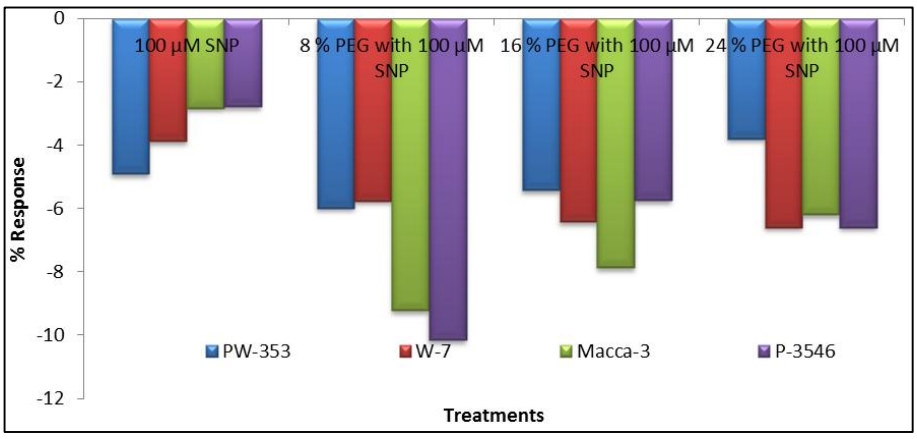

Fig 6: Response of nitric oxide on carotenoid content (mg/g fw) of wheat and maize under different levels of drought stress

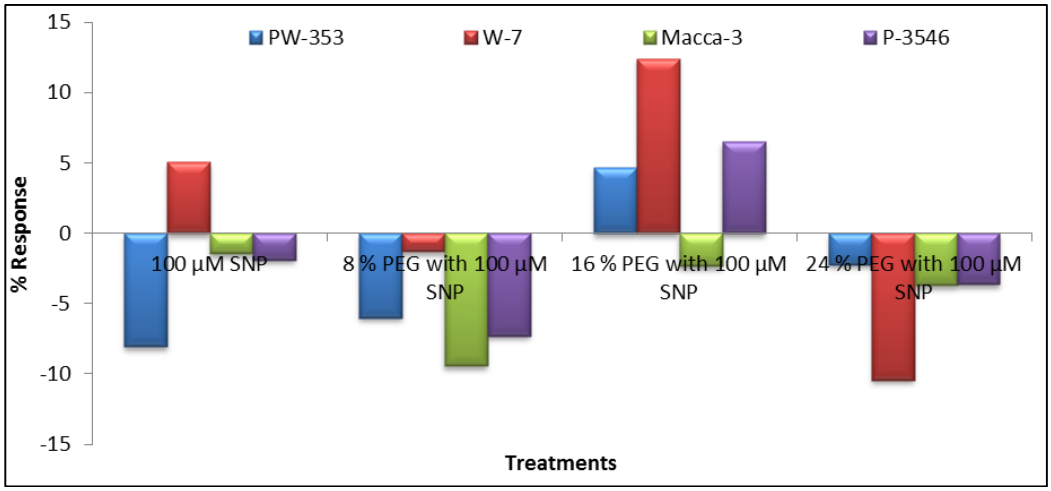

Fig 7: Response of nitric oxide on chlorophyll a/b ratio of wheat and maize under different levels of drought stress

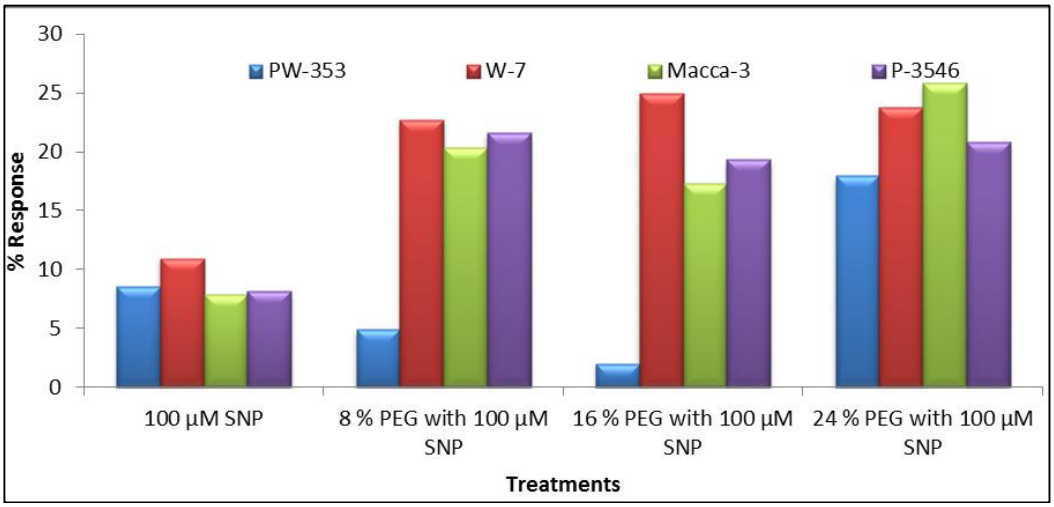

Fig 8: Response of nitric oxide on total chlorophyll/carotenoid ratio of wheat and maize under different levels of drought stress

Discussion

Drought triggers a wide variety of plant responses, ranging from cellular metabolism to changes in growth rates and crop yields and inducing several physiological responses. It mainly inhibits the photosynthesis of plants, causes changes of chlorophyll contents and components and damage to the photosynthetic apparatus. Along with that photochemical activities are inhibited and the enzyme activities in the Calvin cycle are decreased (Shehab *et al.*, 2010) [22].

The rate of photosynthesis in higher plants depends on the activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco) as well as synthesis of ribulose- 1, 5-bisphosphate (RuBP) (Parry *et al.*, 2002) [16]. The Rubisco activity and /or leaf RuBP content were drastically decreased with the amount and duration of drought *(*Bota *et al.*, 2004) [5]. Chlorophyll is one of the major chloroplast components for photosynthesis. (Rathore *et al.*, 2013) [18] determined the chlorophyll concentration of the mature leaf in selected plants (*Catharanthus roseus* (L) G. Don (C_3 plant) and *Andropogon citratus* DC (C_4 plant). The chlorophyll concentration in *Catharanthus roseus* (L) G. Don (C_3 plants) was found to be more than in *Andropogon citratus* DC (C_4 plant). Based on the plastochron index the growth rate of the C_3 plant was faster than the C_4 plants. This supports the fact that the C_3 plants are more efficient in growth in comparison to the C_4. Decreased or unchanged chlorophyll level during drought stress has been reported in many species, depending on the duration and severity of drought (Kyparissis *et al.*, 1995; Zhang and Kirkham, 1996) [12, 25].

Drought stress caused a large decline in the chlorophyll a content, the chlorophyll b content, and the total chlorophyll content in different sunflower varieties *(*Manivannan *et al.*, 2007) [14]. Loss of chlorophyll contents under water stress is considered as a main cause of inactivation of photosynthesis. Furthermore, water deficit induced reduction in chlorophyll content has been ascribed to loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets (Kaiser *et al.*, 1981*)* [11]. Low concentrations of photosynthetic pigments can directly limit photosynthetic potential and hence primary production. From a physiological perspective, leaf chlorophyll content is a parameter of significant interest in its own right. In severe drought stress condition chlorophyllase and peroxidase enzymes increased, as a result the chlorophyll content is decreased. The carotenoid content was decreased under drought condition in sorghum plant. Carotenoids participate in energy dissipation and can aid plant resistance against drought stress. In addition, carotenoids have a critical role as photoprotective compounds by quenching the triplet state of chlorophyll molecules and singlet oxygen derived from excess light energy, thus limiting membrane damage (Arivalagan and Somasundaram., 2015) [3]. 0.1 mM sodium nitroprusside (SNP), a source of NO, delayed the senescence of wheat leaves by inhibiting the degradation of chlorophyll

and soluble proteins, especially Rubisco (Tian and Lei., 2007; Shallan *et al.*, 2012) [23, 21].

Conclusion

The present investigation can be concluded that the effect of nitric oxide was found more effective on W-7 genotype which was a heat tolerant. Whereas, the effect was less on PW-353 (heat susceptible) genotype. Also the drought effect was higher on PW-353 genotype than W-7 genotype. The effect of nitric oxide was lower on maize genotypes when compared to wheat genotypes, but the drought didn't affect much on maize genotypes than wheat. Investigating the photosynthetic enzymes and pigments under different levels of drought stress, maize genotypes showed better performance than wheat genotypes.

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