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C_4 Photosynthetic Enzymes Engaged in the Habituation of *Allium cepa* L. Seedlings to Drought Stress

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Abstract

Drought is one of the major stress which affects agricultural crop productivity around the world. In this study the role of Phos phoenol pyruvate carboxylase (PEPC, EC 4.1.1.31), NADP-malic enzyme (NADP-ME, EC 1.1.1.40), and pyruvate, phosphate dikinase (PPDK, EC 2.7.9.1) in different onion cultivars (*Allium cepa* L.) under drought stress was investigated.An elevated specific activity of these three enzymes was monitored in onion cultivars after 8 h of drought stress. With the increasing concentration of drought there was an increase in C_4 enzyme activities. PEPC showed 3.5-fold increase after 24 h, NADP-ME 3.9-fold, and PPDK 3.2-fold increase was observed in Arkalalima (AL)onion after 48 h when compared to control. Statistical analysis including regression analysis suggested significant results to onion drought resistant cultivars under drought stress condition. The results revealed that the variety AL exhibited best response followed by Arkakirthaman (AK) and Bellary(BL). Therefore PEPC, PPDK and NADP-ME participate in the drought response of onion, a C_3 Plant. Future research will allow further understanding of the drought stress mechanism of adaptation of the onion germplasm to drought.

Key words: Drought stress, PEG-6000, PEPC, NADP-ME, PPDK, Onion cultivars,C_4 enzymes.

Introduction

Globally 90% of cultivated land is affected by stressful conditions at a certain point or throughout the development cycle of crops (1).Drought affects the morphological, physiological and biochemical processes in crops leading to reductions in crop growth rate and biomass formation. This is because of reduction in the rate of cell division and expansion, leaf size, stem elongation and root proliferation, and disturbed stomatal oscillations, plant water and nutrient relations with diminished crop productivity. Many workers have worked at physiological, biochemical, agronomic and molecular levels in crops to understand the mechanism of abiotic stress(2, 3). Plants sense climatic changes and adapt and adjust their metabolism depending on their capacity. Plants grow by maintaining metabolic homeostasis and tolerate a particular stress without undergoing stress induced damages. Sensitive plants die due to their inability in establishing metabolic homeostasis which results in growth reduction (4). Various studies have been carried out on response mechanisms to abiotic stress for crop improvement like drought; salt (5, 6),low temperature(7),flooding(8).

The effect of stress depends on the duration and hardness of the stress in addition to the growth stage of the plant. Usually, seedling stage and seed germination are treated as the most sensitive and the critical stage in the plants life cycle. Hence when seedlings are exposed to drought stress the affect is observednot only on seed germination (9), but also in the increase in germination mean duration in plants (10).

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Plants in xeric conditions with less water availability and high light intensities perform C_4 photosynthesis and adapt to extreme conditions (11). All the C_3 plants have C_4 enzymes in non photosynthetic tissues, which may become active under stressful environment (12). Phosphoenol-pyruvate carboxylase (PEPC, EC 4.1.1.31), NADP-malic enzyme (NADP-ME, EC 1.1.1.40), and pyruvate, phosphatedikinase (PPDK, EC 2.7.9.1) are C_4 photosynthetic enzymes. The activities of the C_4nonphotosynthetic enzymes increased in tobacco, a C_3 plant under both biotic stress and abiotic stress induced by drought (13, 14).Some of the protective reactions of plants to abiotic and biotic stress are similar, like activation of antioxidant system, accumulation of osmolytes, production of protective compounds etc. under drought and salt stress.

The individual non-photosynthetic Hatch-Slack enzymes were found to be involved in other stressor-plant interactions, too. PEPC provides carbon skeletons for ammonium assimilation and functions in C/N partitioning. For example, PEPC is involved in biosynthesis of organic acids in the conditions of phosphorous deficiency (15) and iron deficiency (16), ozone stress (17) and aluminum toxicity (18). PEPC is also related to drought (19),chilling stress (20), salt stress (21) where it is supposed to synthesize L-malate (in a cooperation with NAD-malate dehydrogenase), which serves as an osmolyte and as an additional sink for the carbon assimilation and NADPH (19). The non-photosynthetic NADP-ME isoform supplies reduced equivalents of NADPH for biosynthetic metabolic pathways, maintains intracellular pH and, together with PEPC, supplies intermediates for other pathways. NADP-ME is associated with the plant metabolic response to drought (22), salt (23), biotic stress (24)and cadmium (25).PPDK catalyses a reversible reaction converting ATP, Pi, and pyruvate to PEP, AMP, and PPi. All types of water stress (drought, high salt, and mannitol treatment) as well as cold and low-oxygen stress induce PPDK protein in roots of rice seedlings (26) biotic stress (27). The isoforms of non-photosynthetic Hatch-Slack enzymes were found to express in different stress. Hence these enzymes cooperate during stress response as a part of defense strategy.

Several authors have reported that, use of Poly Ethylene Glycol (PEG) is one of the appropriate procedures to induce a drought condition for screening the germplasmfor several drought tolerance indices such as plant height, root length and biomass to discriminate the genotypes for drought and salt stress (9, 28, 29). Many investigations reported that the PEG induced drought stress is a dependable approach for the in vitro screening and selection of germplasm by studying plant germination indices under stress.At the whole plant level, all abiotic stresses induce a cascade of physiological and molecular events resulting, in some cases similar responses. Drought, high salinity and cold can all be exhibited as a physiological dehydration at the cellular level (30). Drought stress takes place when soil and atmospheric humidity is low and the ambient air temperature is high. This condition is the result of an imbalance between the evapotranspiration.

It is essential to understand how different onion cultivars react to drought and adjust to the stress. The aim of the study was to characterize the physiological and biochemical response of seedlings of onion germplasm at different time-intervals of drought stress. The detailed investigation of physiological parameters of plant against to short term drought stress presented here will not only provide the information about response of plants to stress but also may be helpful in selection of drought-tolerant and drought sensitive germplasm. Hence present study was conducted to study the response of different cultivars of onions non-photosynthetic Hatch-Slack enzymes to drought stress.

Materials and methods

Collection, Surface sterilization and germination of onion seeds : Six cultivars of onion seeds were collected such as Agrifound Rose, Prema-178, Nasik Red (National

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Horticultural Research and Development Foundation, Nasik, Maharastra state), Arkakirthiman, ArkaLalima (Indian Institute of Horticultural Research, Banglore, Karnataka state) and Bellary (local farmers). Collected seeds were washed with distilled water then with 70 % ethanol and then with 2 % sodium hypochlorite followed by washing with sterile distilled water. The sterilized onion seeds were allowed to germinate for 10 days at 25/23 ^{o}C day/night temperature in plant growth chamber under16 h day/8 h night photoperiod at a light intensity of 130 µmol $m^{-2}s^{-1}$.

Stress treatment : All seedlings were treated with 0, 25, 50, 75 and 100 g/l PEG-6000 solution for different time intervals viz. 0, 4, 8, 24 and 48 h. The harvested onion tissue was frozen in liquid nitrogen and stored at -80ºC until required for analysis.

Enzymes extraction : Approximately 0.5 g of onion seedling tissue was homogenized with 1.5 mL of extraction buffer containing 100 mMTris-HCl pH 7.8;5 mM $MgCl_2$; 1 mM EDTA; 1 mMdithiothreitol; 1% (v/v) protease inhibitor cocktail for tissue and plant extracts (Sigma, USA). Then 0.02 g of PVP was added and homogenate was centrifuged at 600g at 4 ^{o}C for 15 min. Supernatant was collected to determine the specific activity of enzymes (31).

Enzyme activity assays of phosphoenol-pyruvate carboxylase (PEPC), NADP-dependent malic enzyme (NADP-ME) and pyruvate, phosphate dikinase (PPDK)

To determine the specific activity of PEPC, NADP-ME and PPDK with specific pHwith activator was added to assay mixture as follows.

PEPC : Assay mixture (1 ml) contained 100 mMTris-HCl buffer (pH 8.1), 5 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM PEP, and 0.2 mM NADH (32).

NADP-ME : Assay mixture (1 ml) contained 100 mMTris-HCl buffer (pH 7.4), 10 mM malate, 2 mM $MgCl_2$ and 0.2 mM $NADP^+$(33).

PPDK : Assay mixture (1 ml) contained 100 mMTris-HCl buffer (pH8.1), 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 2 mM pyruvate, 2 mM K_2HPO_4, 1 mM ATP and 0.2 mM NADH (34).

All the activities were determined by UV-Vis spectrophotometre (Thermoscientific Evolution 401) at 25 ^{0}C at 340 nm and activities were calculated as [µmol (substrate/product). $min^{-1}µg^{-1}$ (fresh weight)].

Statistical analysis : Each treatment was replicated thrice and triplicate results were used to construct graphs through Graph pad PRISM 5.0 software. The mean values for various parameters of the plants were subjected to statistical analysis following the standard procedure described by (35). The means were compared in One-way ANOVA analysis by means of Dunnet's multiple comparison tests for significant difference in order to study the significanceata P≤" 0.001 level of probability. Graph pad PRISM 5.0 software was used for multiple linear regression analysis of enzyme specific activity and different PEG concentration to determine the R^2 and regression equation.

Results and Discussion

Effect of drought stress on PEPC activity : With increasing concentration of PEG-6000 at different time interval, there was increase in enzyme activity in all the cultivars (Fig.1). The activity was maximum in AL followed by other cultivars this was similarly seen in transgenic rice (36), wheat (37), soybean (38) and tobacco (13). After 4 h interval of drought treatment the enzyme activity in AL was 1.5 folds at 25g/l, 1.9 folds at 50 g/l, 2.0 folds at 75g/l, and 2.2 folds at 100g/l followed by BL, NR, PR, AF and AK (Fig. 1B). After 8hrs of drought stress the PEPCactivity at pH 8.1 increased in AL. At 25g/l concentration 1.9 folds, 2.7 folds at 50 g/l, 2.9 folds at 75g/l, and 2.0 folds at 100g/l, followed by BL, NR, AK, PR (Fig. 1C). After 24h of drought stress PEPC at pH 8.1 activities increased rapidly in cultivar AL. At 25g/l PEG 2.3 folds increase, 2.7 folds at 50 g/l, 2.9 folds at 75g/l, and 3.5 folds at 100g/l, followed by

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BL, AK, NR, PR and AF(Fig 1D). The drought tolerant mechanism may be due to calcium signal cascade. Ca^{2+} dependent increased expression of transcription factors NAC6 and bZIP60 and protein kinase genes (CPK9) which enhanced the activities of PEPC,hence conferring drought tolerance in plants (36).There was no significant increase in PEPC activity in all the cultivars after 48 h of drought treatment (Fig. 1E). The transgenic rice plants overexpressed with C_4 photosynthetic enzymes exhibited significant increase in drought tolerance (39).

Effect of drought stress on NADP-malic enzyme activity : The strongest response to drought stress was observed in the case of NADP-ME,where the increasing intensity of stress with different time intervals NADP-ME activity increased in all the cultivars (Fig. 2). Similar results were reported in tobacco and barley under drought stress (13)(40). During the different time intervals the NADP-ME activity increased in AL followed by other cultivars (Fig. 2 B, C, and D). After 4 hrs of drought treatment there was an increase in enzyme activity in AL (1.9 folds at 25g/l, 2.3 folds at 50 g/l, 2.4 folds at 75g/l, and 2.6 folds at 100g/lFig. 2B). After 8 hrs of drought treatmentthe enzyme activity was 2.8 folds at 50 g/l, and 3.4 folds at 100g/l PEG-6000 was observed in AL (Fig. 2C). After 24h of drought stress NADP-ME activity in AL was 2.5 folds at 25g/l concentration at 50 g/l, 2.9 folds 3.2 folds at 75g/l, and 3.5 folds at 100g/l, followed by BL, AK, NR, AF, PR (Fig. 2D). At 48 h AL shows highest response at 25g/l concentration AL showed 2.9 folds, 3.4 folds at 50 g/l, 3.6 folds at 75g/l, and 3.9 folds at 100g/l (Fig. 2E). This cultivar is followed by AK, BL, NR, PR, and AF (AL>AK>BL>NR>PR>AF). In C_3 plants NADP-ME located in guard cells, plays a vital role in stomatal movement. During day time under water-deficit conditions stomata closes by degradation

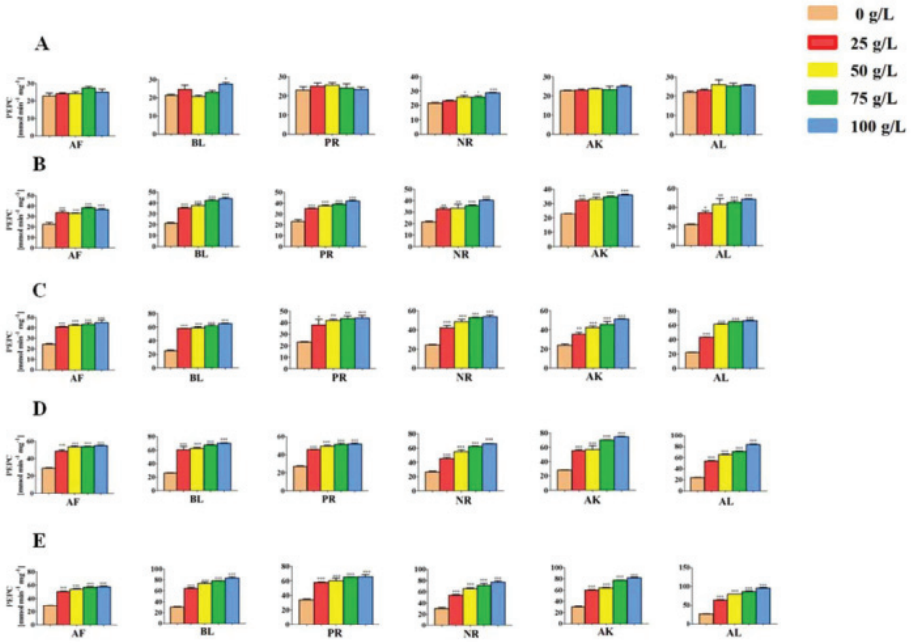

Fig.1. Specific activity of PEPCin onion seedlings exposed todrought stress at different time interval stress (A) 0 h (B) 4 h (C) 8 h (D) 24 h (E) 48 h. The specific activity wasmeasured in at least 3 samples, S.D. are shown. Statistical analysis was done using ONE WAY ANOVA t-test. *Denotes significant difference of drought-stressed samples from controls ones at$P < 0.001$.

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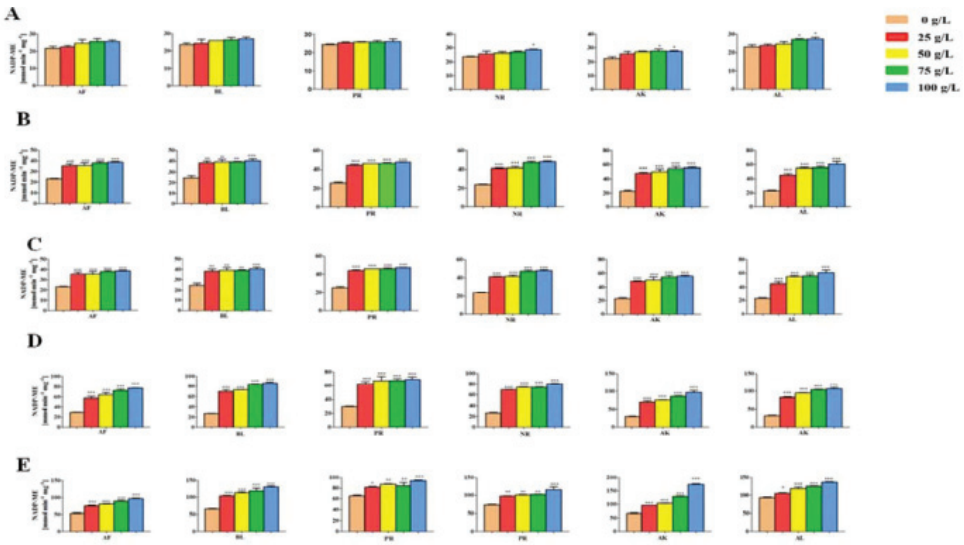

Fig. 2. Specific activities ofNADP-ME in onion seedlings exposed todrought stress at different time interval stress(A) 0 h (B) 4 h (C) 8 h (D) 24 h (E) 48 h. The specific activity wasmeasured in at least 3 samples, S.D. are shown. Statistical analysis was done using ONE WAY ANOVA t-test. *Denotes significant difference of drought-stressed samples from controls ones atP < 0.001.

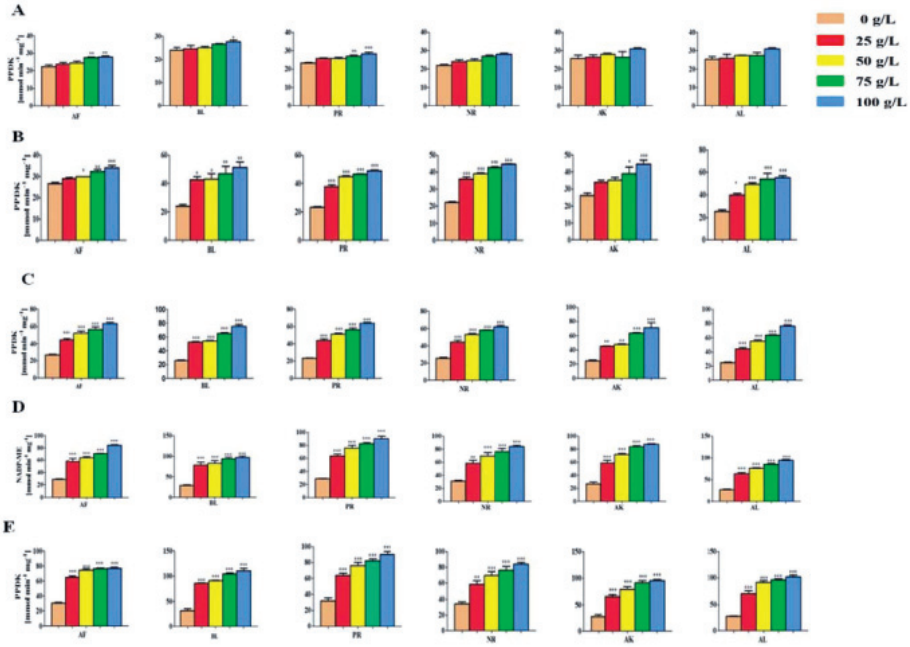

Fig.3. Specific activities of PPDKin onion seedlings exposed todrought stress at different time interval stress(A) 0 h (B) 4 h (C) 8 h (D) 24 h (E) 48 h. The specific activity wasmeasured in at least 3 samples, S.D. are shown. Statistical analysis was done using ONE WAY ANOVA t-test. *Denotes significant difference of drought-stressed samples from controls ones atP < 0.001.

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of malate. Therefore regulation of NADPME expression has been proposed for drought tolerance (41).

Effect of drought stress on PPDK activity : Under stressful periods there is a demand on ATP, as the production by mitochondria is limited, PPDK catalyses the yield of ATP and Pi and maintain the bioenergetic balance (42). After 4 h of drought induction the increase in enzyme activity was seen in cultivar AL 1.5 folds at 25g/l concentration, 1.9 folds at 50 g/l, 2.1 folds at 75g/l, and 2.18 folds at 100g/l (Fig. 3B).PPDK activity in AL was 1.7, 2.2, 2.5, and 3.06 at respective concentrations followed by BL, AK, PR, NR, and AF after 8h of drought stress. 2 fold enhanced PPDK activity at 4hand 3 fold enhanced activity at 100g/l PEG after 8h of stress was observed (Fig. 3C). After 24h of drought stress PPDK activity in AL at 25g/l concentration showed 2.2 folds, 2.6 folds at 50 g/l, 3.0 folds at 75g/l, and 3.2 folds at 100g/l, followed by BL, AK, PR, NR, and AF (Fig. 3D). This is similar to the PPDK activity in tobacco during stress (13). PPDK enzyme may also involve in some abiotic stress caused by drought (43) and biotic stress induced by viral infection (44). After 48 h drought stress PPDK activity of AL shows 2.4, 3.2, 3.3, and 3.2 at respective concentrations followed by BL, AK, PR, NR, and AF (Fig. 3E).Osmotic adjustment, antioxidant activities, and altered growth regulators are among the major physiological adaptations of plants under drought stress. Increased accumulation of osmoprotectants such as proline, glycine betaine, amino acid, and sugars are involved in osmoregulation. Riazunnisa and Sai (45) reported higher proline content in AL onion cultivar under drought stress.

Relationship between PEG concentration and enzyme activity : The linear regression analysis of all cultivar showed linear relationship (R^2= 0.9) at 0 h but from 4 to 48 h onwards the cultivars at different PEG concentration shows positive

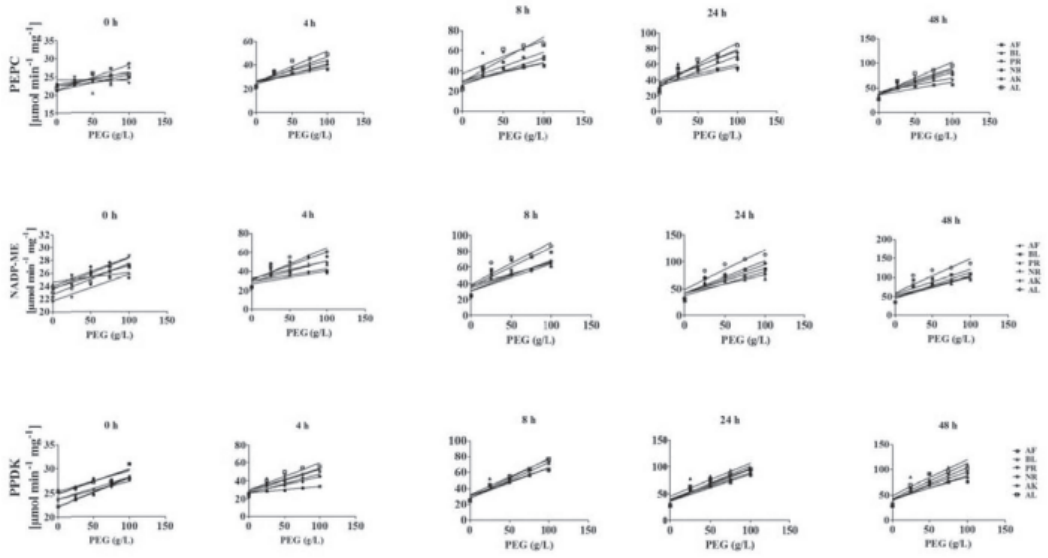

Fig. 4. Relationship between drought stress at different time intervals (0, 4 h, 8 h, 24 h and 48 h) and enzyme activity by multiple linear regression analysis of PEPC, NADP-ME and PPDK.

Table 1 Multiple linear regression analysis of PEPC, NADP-ME and PPDK enzyme specific activities in different onion cultivars.

S. No.	Culti vars	0 h		4 h		8 h		24 h		48 h	
		Y intercept	R^2	Y intercept	R^2	Y intercept	R^2	Y intercept	R^2	Y intercept	R^2
PEPC											
1	AF	0.0320x+23	0.2192	0.1307x+26.33	0.6458	0.1760x+30.33	0.6315	0.2280x+36.53	0.6755	0.2560x+36.67	0.7187
2	BL	0.044x+21.27	0.2526	0.2093x+25.67	0.8202	0.3347x+37.13	0.6563	0.3840x+38.20	0.6787	0.4840x+41.53	0.7961
3	PR	0.00133x+24.27	0.0003	0.1687x+26.93	0.7784	0.1907x+28.60	0.5908	0.2227x+34.00	0.696	0.2827x+42.33	0.6905
4	NR	0.06933x+21.40	0.7788	0.1640x+24.47	0.7337	0.2813x+30.13	0.7817	0.3867x+31.53	0.8963	0.4453x+37.47	0.8703
5	AK	0.0200x+22.53	0.2223	0.1173x+25.80	0.7184	0.2600x+26.60	0.8841	0.4293x+35.40	0.8321	0.4827x+38.60	0.8798
6	AL	0.03867x+22.47	0.306	0.2573x+25.87	0.7641	0.4427x+29.40	0.847	0.5507x+32.33	0.9083	0.6333x+38.53	0.8731
NADP-ME											
1	AF	0.04133x+21.73	0.3191	0.1320x+27.33	0.6062	0.3347x+30.80	0.7955	0.4387x+37.47	0.8318	0.5613x+45.60	0.795
2	BL	0.03467x +23.73	0.2899	0.1293x+29.73	0.5121	0.4893x+36.07	0.8299	0.5320x+41.40	0.7585	0.6440x+56.13	0.6849
3	PR	0.01467x+24.67	0.1576	0.1813x+32.73	0.6066	0.2800x+37.60	0.6136	0.3333x+42.27	0.5623	0.4800x+52.53	0.6256
4	NR	0.04533x+24	0.559	0.2160x+29.53	0.7611	0.3180 x+34.80	0.7088	0.4547x+42.07	0.6701	0.5520 x+48.73	0.6953
5	AK	0.0480 x+23.60	0.4408	0.2893x+31.67	0.6624	0.3713x+31.27	0.7809	0.5947x+42.07	0.8261	0.6733x+45.47	0.8464
6	AL	0.0480x+22.73	0.5859	0.3440x+30.67	0.7673	0.5280 x+38.4	0.7712	0.7347x+48.67	0.8209	0.8907x+59.27	0.7643
PPDK											
1	AF	0.06133x+22.20	0.7757	0.0720x+26.77	0.8362	0.3387x+ 31.73	0.8723	0.4973x+ 36.33	0.8733	0.4200x+ 43.33	0.6932
2	BL	0.03733x+23.73	0.5065	0.2360x+ 29.80	0.6159	0.4453x+ 32.20	0.8909	0.6040x+ 45.53	0.6979	0.7013x+ 49.20	0.7787
3	PR	0.04533x+ 23.73	0.7136	0.2413x+ 28.37	0.8302	0.3720x+ 28.93	0.8911	0.5627x+ 40.07	0.8299	0.5360x + 42.07	0.8336
4	NR	0.0600 x+ 22.13	0.7694	0.2080x+26.47	0.8297	0.3480x+ 31.27	0.8824	0.4960x+ 39.07	0.8243	0.4693x+ 41.07	0.8274
5	AK	0.04267x+25.33	0.222	0.1699x+27.25	0.7304	0.4440x+28.27	0.871	0.5853x+ 36.53	0.8714	0.6387x+39.80	0.8387
6	AL	0.05067x+ 24.87	0.4093	0.2973x+29.87	0.7997	0.4907x+28.47	0.965	0.6320x+37.67	0.8804	0.6907x+42.93	0.8028

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correlation. But the R^2 value decreased at 48 h when compared to 24 h (Table 1). AL cultivar presented more R^2 value of about (R^2= 0.9) in linear regression with PPDK and PEPC and (R^2= 0.8) for NADP-ME. This is followed by AK, BL, AF, PR, and NR. Hence by linear regression analysis AL cultivar may be exploited for breeding programme (Fig. 4).

Conclusion

With increasing global warming leading to climate change with escalating emissions ofgreenhouse gases, the frequency of drought has been predicted to further increase in the near future. The present study was carried out to evaluate the onion germplasm under PEG induced drought stress. In conclusion, the AL cultivar exhibited enhanced activities of PEPC, NADP-ME and PPDK suggesting that this cultivar may adjust better to drought. C_4 enzymes are involved in the adaptation of C_3 plants under drought stress conditions. The evaluated onion germplasm showed correlation between drought stress and enzyme specific activity. Among all the cultivars, AL cultivar exhibited best response during time interval drought stress at seedling stage followed by AK, BL, PR, NR and AF.

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Conflict of interest

There is no conflict of interests declared regarding this research publication.

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