



Drought stress responses in tolerant and sensitive varieties of black pepper (*Piper nigrum* Linn.)

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Abstract

Stress responses towards drought was studied in drought tolerant (Panniyur 5) and sensitive (Panniyur 1) varieties of black pepper. On exposure to 15 days (incipient wilting) of drought, Panniyur 1 (P1) showed low RWC of leaves, highly reduced ψ_s and decrease in the capacity to synthesize sugars and proline as compared to Panniyur 5 (P5). These features indicate the reduced potential of P1 to tolerate long periods of drought as compared to P5. The malondialdehyde content was found to be significantly higher in P1 when compared to P5 during drought. The activity of antioxidant enzymes (guaiacol peroxidase and superoxide dismutase) were found to be low in P1 as compared to P5 at different stages of drought stress, indicating that P1 is comparatively less shielded than P5 from the deleterious effects of reactive oxygen species generated as a result of drought exposure. The photosystems and mitochondria of P1 were more sensitive to drought as compared to that of P5. The effective control of lipid peroxidation and better osmotic adjustments in cells of tolerant variety (P5) may be the prime factors for the increased tolerance of photosystem and mitochondrial systems towards drought stress.

Keywords: Antioxidation, mitochondria, osmotic potential, photosystems, proline

Introduction

Black pepper, the king of spices is grown in India as a vegetatively propagated plantation crop. It has a greater export potential for its berries. The plant is grown as a crop which prefers an average rainfall of 2000-3000 mm for its optimum growth (Gopakumar, 2011). Changes in the rainfall pattern in pepper-growing belt of India are severely affecting the crop productivity. Among the various abiotic stresses, drought stress is the most severe stress for black pepper (Anuradha, 2004). To combat drought stress, plants operate different strategies which includes various biochemical and physiological responses.

Drought stress usually causes oxidative damage to plants by inducing the accumulation of reactive oxygen species (ROS) (Apel and Hirt, 2004). Plants protect their cells from ROS induced damages by their own antioxidative defense mechanisms, which

include enzymatic and non-enzymatic systems (Mittler, 2002). Enzymes of this system include peroxidases, superoxide dismutase (SOD), catalase and enzymes of ascorbate glutathione cycle (Shan *et al.*, 2011). Non-enzymatic antioxidants include the major cellular redox buffers, ascorbate, glutathione, carotenoids and tocopherols (Apel and Hirt, 2004). Mehler reaction in chloroplasts, over-reduction of the electron transport chain in mitochondria and lipid catabolism in microbodies, all lead to the generation of ROS (Mittler *et al.*, 2004). The extent of oxidative stress in a cell is determined by the accumulation of ROS, such as superoxide, H_2O_2 and hydroxyl radicals. SOD is the major scavenger of oxygen radicals and its action results in H_2O_2 formation, which is subsequently eliminated by catalase and several classes of peroxidases. Rather than ROS, drought also induces osmotic stress in plant cells which disrupts normal cellular activities (Chae *et al.*, 2007).

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Though antioxidant activities and stress related metabolism of black pepper has been studied earlier (Krishnamurthy *et al.*, 2000; Thankamani and Asokan 2002, 2004a,b), the photosynthetic and mitochondrial responses of the crop encountering drought stress has not been well studied in detail so far. Hence, our study focuses, on drought stress related metabolism and emphasizes on the photosynthetic and mitochondrial responses of the crop during drought stress. Understanding the water stress related metabolism in drought tolerant and sensitive varieties of black pepper is imperative for crop improvement, through approaches like genetic engineering for drought tolerance.

Materials and methods

Plant material

Two black pepper varieties Panniyur 1 (P1) and Panniyur 5 (P5) were selected for the study. P1 is the first black pepper hybrid in the world and is reported to have the highest yield of dry pepper among all the released varieties. P1 prefers open condition whereas P5 can tolerate shaded condition (Vanaja *et al.* 2006). Though P1 is considered as more productive, it is sensitive to drought. On the other hand P5 is relatively more tolerant to drought compared to P1 (Thankamani *et al.*, 2003; Vijayakumari and Puthur, 2012).

Sixty plantlets of each variety were raised in poly bags of size 30×28 cm filled with potting mixture, consisting of soil, sand and farm yard manure in 1:1:1 proportion, during the month of June, 2011 and 2012. The growing pepper shoots (9-10 month old) were trained on split bamboo poles for support. Plants were maintained in green house of Calicut University at a relative humidity of 60±5 per cent and temperature of 28±2 °C. Drought stress was imposed on half the number of the plants raised in the month of April (2011 and 2012), by withholding irrigation and the stress treatment continued till the plants showed symptoms of permanent wilting. Youngest, fully unfolded mature leaves were taken for analysis. Various observations were recorded on 0, 6, 12 and 15 days, wherein the soil moisture content recorded was 30, 18, 12, and 8 per cent respectively.

Enzyme assay

Estimation of guaiacol peroxidase activity was carried out according to Gasper *et al.* (1975). SOD

was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according Giannopolitis and Ries (1977).

Measurements of osmotic potential (ψ_s) and RWC

The ψ_s of leaf was determined using a vapour pressure osmometer (Wescor 5520, USA). Ten leaf discs of diameter five mm collected from the middle portion of the leaves of five plants were tested for ψ_s , after the calibrating the instrument. Calibration of the chamber was done using 290 and 1000 mmol kg⁻¹ standard solutions (Wescor, USA)

Relative water content (RWC) of the leaves were measured as per Weatherley (1950). Soil moisture content was determined by gravimetric method (Black, 1965).

Estimation of metabolites

Proline content was estimated according to Bates *et al.* (1973). Total sugar was estimated according to Montgomery (1957). The malondialdehyde (MDA) content estimation was done according to Heath and Packer (1968).

Estimation of photosynthetic pigments and analysis of photosystem I and II activity

Chlorophyll and carotenoid estimation was carried out by the method of Arnon (1949). Thylakoids from leaves were isolated according to Puthur (2000). Photochemical activities of isolated thylakoids were assayed polarographically with a Clark-type oxygen electrode (DW1/AD, Hansatech, Norflok, UK) which was connected to a digital control box (OXYG1, Hansatech, Norflok, UK). The light dependent O₂ uptake/evolution was measured by irradiating the sample with saturating intensity of white light (~1800 μmol photons m⁻² s⁻¹), provided by a 100W halogen lamp (LS2, Hansatech, Norflok, UK). The activity of PS I and PS II was expressed in terms of μmol of O₂ consumed/evolved min⁻¹ mg⁻¹ chlorophyll.

Analysis of mitochondrial activity

Isolation of mitochondria was done according to Kollofel (1967) and mitochondrial activity was measured polarographically with a Clark-type oxygen electrode at 25 °C as per Schmitt and Dizengremel (1989). The mitochondrial activity

was calculated in terms of $\text{nmol O}_2 \text{ consumed min}^{-1} \text{ mg}^{-1} \text{ protein}$.

Results and discussion

In black pepper, drought stress causes various physiological and biochemical changes (Krishnamurthy *et al.*, 2000). In our study, tolerant (P5) and sensitive (P1) varieties showed differential responses during exposure to drought stress. RWC decreased in both the varieties (Table 1). The reduction in RWC was more in P1 (51% on 12 d of stress treatment to 72% on 15 d of stress) when compared to P5 (34% on 12 d and 53% on 15 d). RWC is considered as one of the indices for

screening water stress tolerant varieties in black pepper (Thankamani and Asokan, 2004 b). Krishnamurthy and Saji (2006) have reported that RWC decreases in the entire *Piper* species subjected to drought stress and the rate of decrease varied among the different species.

The ψ_s in leaves of P1 reduced with increase in severity of drought stress, but the decrease

Table 1. RWC in leaves of Panniyur 1 and Panniyur 5 varieties of black pepper subjected to drought stress.

Days	Panniyur 1	Panniyur 5
0 d	93.51 \pm 3.95 (0)	92.31 \pm 2.97 (0)
6 d	61.21 \pm 2.11 (35)	88.23 \pm 3.33 (5)
12 d	45.78 \pm 2.17 (51)	60.84 \pm 2.45 (34)
15 d	26.22 \pm 1.21 (72)	43.22 \pm 1.84 (53)

Control values for P1=93.76 \pm 3.81 and P5=92.01 \pm 3.11. The values in parenthesis denote the percentage decrease over the control. The data is an average of recordings from three independent experiments each with three replicates (*i.e.* n=9). The data represent mean \pm standard error

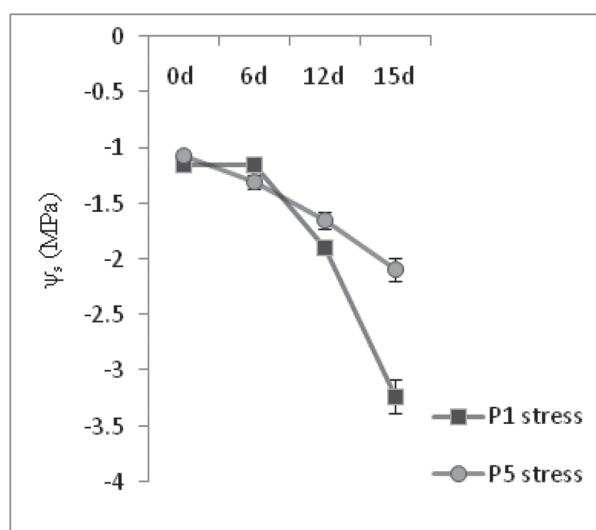


Fig. 1. ψ_s of leaves in P1 and P5 varieties subjected to increasing drought condition (0-15 d). The vertical bars represent SE of the mean value of recordings from 3 independent experiments each with a minimum of 3 replicates

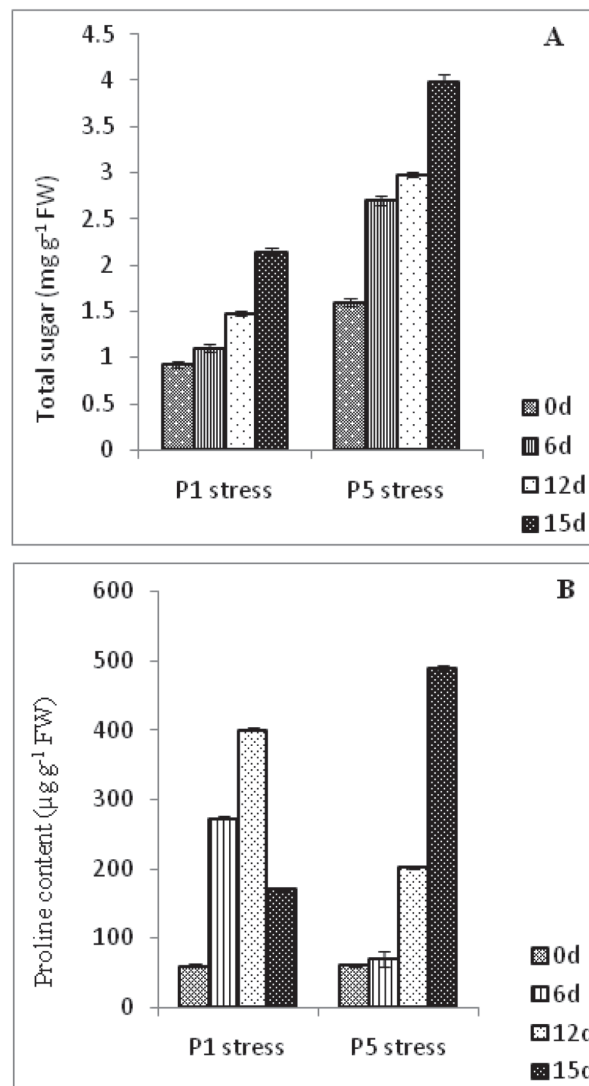


Fig. 2. (A) Total sugar (control values: P1= 0.83 \pm 0.02 and P5 = 1.40 \pm 0.04) and (B) Proline content (control values: P1= 52.42 \pm 2.6 and P5 = 55.0 \pm 1.7) in leaves of P1 and P5 varieties subjected to increasing drought condition (0-15 d). The vertical bars represent SE of the mean value of recordings from 3 independent experiments each with a minimum of 3 replicates

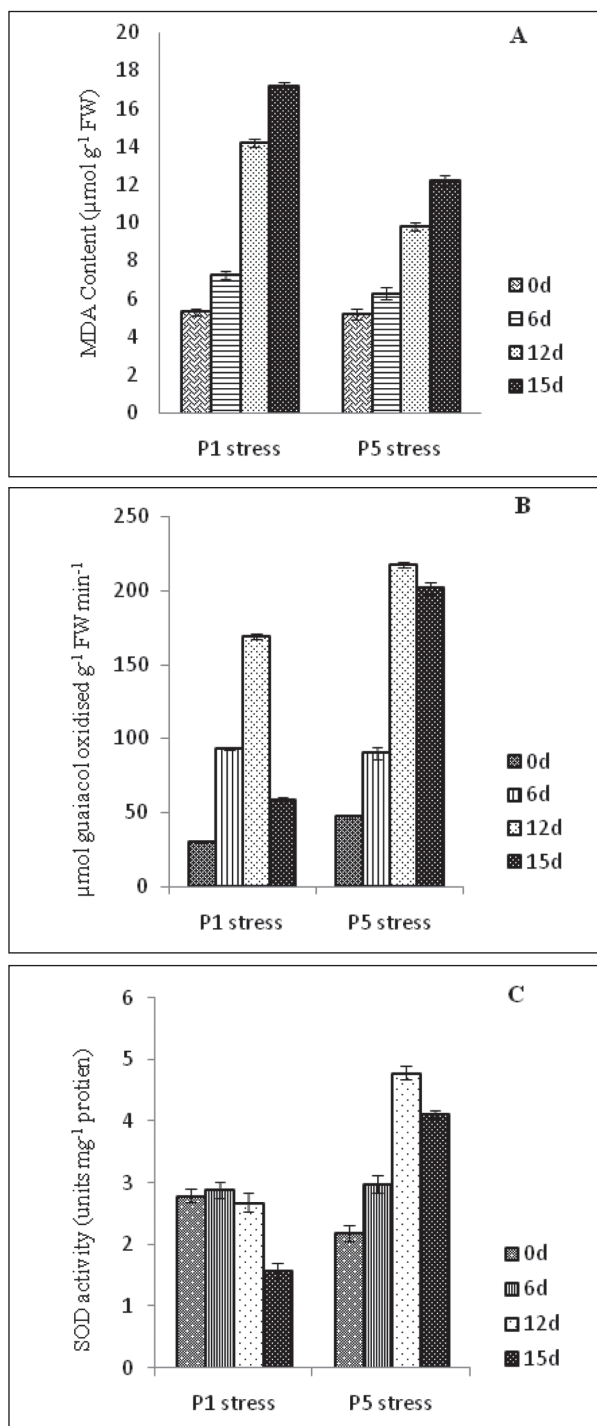


Fig. 3. (A) level of MDA Content (control values: P1= 6.74 ± 0.31 ; P5= 5.67 ± 0.23) (B) Guaiacol peroxidase activity (control values: P1= 24.4 ± 0.75 ; P5= 40.45 ± 2.03) and (C) SOD activity (control values: P1= 3.01 ± 0.06 ; P5= 2.14 ± 0.08) in leaves of P1 and P5 varieties subjected to increasing drought condition (0-15 d). The vertical bars represent SE of the mean value of recordings from 3 independent experiments each with a minimum of 3 replicates

appeared to be gradual and less in P5 (Fig. 1). It was interesting to observe that although the ψ_s was significantly more negative in P1 on 15 d of stress, the total sugar content of P1 was lower at any stage of stress as compared to P5 (Fig. 2 A). Therefore, ψ_s can provide a good index for evaluating plant responses to water stress (Souza *et al.*, 2012). Earlier report shows that there is a significant increase in reducing sugars on imposition of drought stress in *Piper nigrum* and increase was proportional to the severity of stress (Anuradha, 2004). In our observations it was clear that sugars had no significant role in contributing to the reduction of ψ_s in P1. Therefore, it was of interest to observe whether any osmosolutes other than sugars contributed to the reduced ψ_s in P1 with increase in period of drought stress.

On analyzing the proline content, it was observed that the drought sensitive variety could not keep up a sustained level of accumulation of this compatible solute to encounter the stress on 15 d. Though the proline accumulation was faster in P1 variety after the stress (6 d), the rate of proline accumulation slowed down with increase in period of stress (15 d), but proline content increased slowly and reached to the highest level in the tolerant variety (P5) on 15 d of drought (Fig. 2B). It can be concluded that proline too has no role to contribute towards the reduced ψ_s of P1. Though, the relationship between the ability to accumulate proline and stress tolerance is still not clear (Kanawapee *et al.*, 2012), some authors suggest that proline accumulation is related to the degree of stress tolerance (Zhani *et al.*, 2012). Proline is attributed with multiple functions; osmotic adjustment, maintenance of protein stability and as storage of N and C to overcome the unfavourable conditions resulting from stress (Netto *et al.*, 2005; 2010).

The level of lipid peroxidation estimated in terms of malondialdehyde content, was found to be significantly higher in P1 when compared to P5. The increase was 185 and 211 per cent in P1 and 73 and 111 per cent increase in P5 after 12 and 15 d of stress, respectively (Fig. 3A). Lipid peroxidation and the resulting membrane damage leads to cell leakiness and ultimately to the death of cells (Montillet, 2005).

Guaiacol peroxidase activity was highest for the tolerant variety P5 on 12 d of stress treatment.

Table 2. Chlorophyll and carotenoid content (mg g⁻¹ FW) in leaves of Panniyur 1 and Panniyur 5 varieties of black pepper subjected to drought stress.

Day	Chl <i>a</i>		Chl <i>b</i>		Total Chl		Carotenoids		Chl <i>a/b</i>		Car/Total Chl	
	P1	P5	P1	P5	P1	P5	P1	P5	P1	P5	P1	P5
0 d	1.34±0.05	1.06±0.05	0.35±0.01	0.31±0.01	1.64±0.07	1.38±0.05	0.74±0.03	0.55±0.02	3.83±0.13	3.42±0.14	0.45±0.01	0.39±0.01
6 d	1.63±0.07	0.96±0.04	0.36±0.01	0.27±0.01	1.99±0.08	1.23±0.06	0.79±0.03	0.65±0.03	4.53±0.21	3.56±0.14	0.39±0.00	0.53±0.00
12 d	0.83±0.03	0.76±0.03	0.26±0.01	0.26±0.01	1.09±0.04	1.02±0.04	1.35±0.06	0.58±0.01	3.23±0.09	2.92±0.10	1.24±0.03	0.57±0.00
15 d	0.72±0.02	0.64±0.03	0.25±0.01	0.21±0.01	0.97±0.03	0.86±0.03	1.09±0.05	0.32±0.01	2.88±0.13	3.05±0.13	1.12±0.02	0.37±0.01

Control values: Chl *a* - P1=1.39±0.06 and P5=1.09±0.05; Chl *b* - P1=0.30±0.03 and P5=0.31±0.02; Total Chl - P1=1.61±0.07 and P5=1.33±0.06; Carotenoids - P1=0.75±0.04 and P5=0.52±0.03. The data is an average of recordings from three independent experiments each with three replicates (*i.e.* n=9). The data represent mean±standard error

The activity of this enzyme was found to be low and sustained only for a short duration (between 6 and 12 d of stress) and thereafter activity declined in P1. Peroxidase in P5 variety showed a sustained activity during the entire drought stress period and the activity was high at 12 and 15 d of stress (Fig. 3B). In the case of SOD, activity was higher under normal condition in P1 and it did not increase further during drought stress. At severe stress, the SOD activity declined below control level in P1. But in P5, SOD was found to be more active during stress and it increased steadily with increase in stress upto 12 d (Fig. 3C). Krishnamurthy and Saji (2006) have reported increased peroxidase and SOD activity during water stress in some species of *Piper*. They further found that SOD activity had a positive correlation with days taken for wilting.

It was interesting to observe that drought sensitive variety P1 showed higher PS I and PS II activity when compared to P5 plants in the absence of any stress (Fig. 4A). Moreover, chlorophyll and carotenoid content in P1 was higher than in P5 (Table 2). P1 even showed a slight increase of total chlorophyll and carotenoid content during moderate stress (6 d). This result denotes the high photosynthetic rate in P1 and it very well correlates with the high potential yield of dry pepper of P1 over P5 variety (Vanaja *et al.*, 2006). The percentage reduction of chlorophyll was faster and higher in P1 (51%) when compared to P5 (37%) on 15 d of stress. The carotenoid to chlorophyll ratio was also found to be significantly higher in the P1 variety during the entire period of drought stress. The higher carotenoid content in P1 even during the period of stress shows the increased role

of carotenoids in antioxidation function in P1. Carotenoids are known to be effective antioxidants (Strzalka *et al.*, 2003).

The photosystems of P1 were more sensitive to drought as compared to that of P5. The reduction of PS II activity of P1 was 70 per cent and 78 per cent as compared to their control on 12 d and 15 d of stress whereas in P5, it was only 44 per cent and 52 per cent respectively. Both in P1 and P5, PS I activity also showed a decrease with increasing drought period (Fig. 4A, B). The data shows that the PSII reaction centers of P1 are highly vulnerable as compared to that of P5. The vulnerability could be due to faster rate of degradation of oxygen evolving complex (OEC) on exposure to drought stress. Moreover, D1 polypeptide of PSII has a faster turnover rate and the depleted levels of D1 are not replenished by fresh synthesis after exposure to stresses (Giardi *et al.*, 1997).

On the contrary to the photochemical activity, the mitochondrial O₂ uptake was more in the variety P5 as compared to P1, in absence of stress. The mitochondrial activity decreased at a faster rate in P1 as compared to P5 on imposing drought. On induction of drought stress, decrease of mitochondrial activity over control in P1 was 50 and 77 per cent respectively on 12 d and 15 d, whereas in P5 variety, decrease was 10 and 43 per cent respectively on 12 d and 15 d (Fig. 4C). Though studies of photosynthetic function in plants were undertaken by many researchers, mitochondrial activity was poorly studied in plants under stress. The final step of ascorbate synthesis in plants occurs on the inner mitochondrial membrane, indicating that

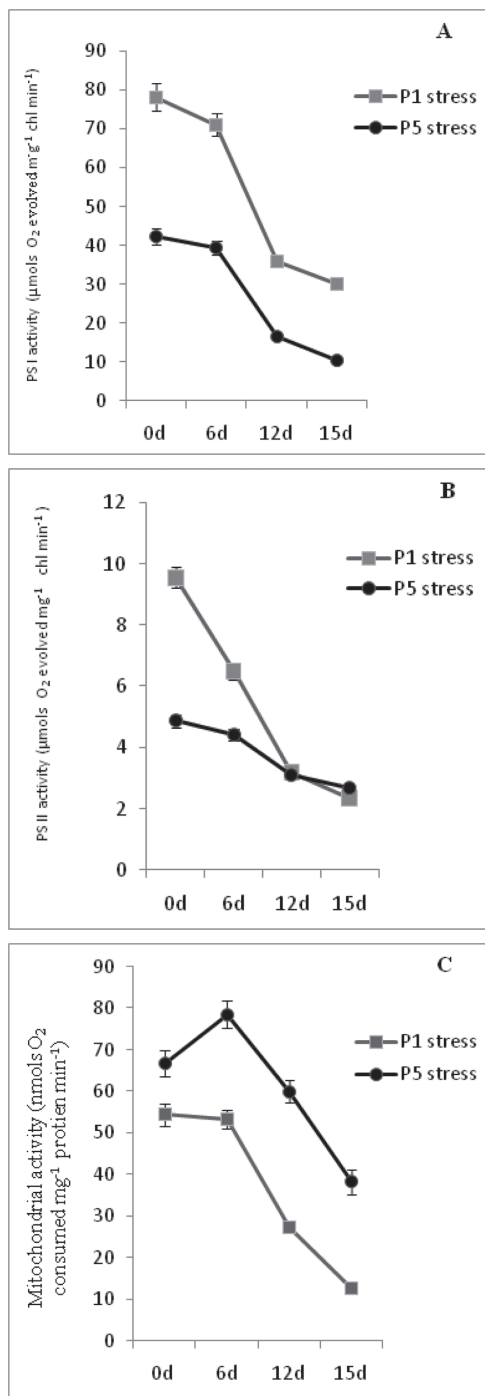


Fig. 4. (A) PS I (control values: P1= 78.13 ± 1.56; P5 = 47.84 ± 1.71) (B) PS II (control values: P1= 10.03 ± 0.39; P5 = 5.67 ± 0.25) (C) Mitochondrial activities (control values: P1= 54.39 ± 1.16; P5 = 66.71 ± 1.04) in leaves of P1 and P5 varieties subjected to increasing drought condition (0-15 d). The vertical bars represent SE of the mean value of recordings from 3 independent experiments each with a minimum of 3 replicates

mitochondrial function is vital for cellular antioxidant defense (Bartoli *et al.*, 2000). Pastore *et al.* (2007) have proposed that mitochondria may also play a key role in adaptation to drought by acting against drought induced oxidative stress. The decrease in the mitochondrial activity may be due to the fact that when cells experience oxidative stress, the important plant mitochondrial enzymes involved in the electron transport chain are damaged due to ROS action (Millar and Leaver, 2000).

In conclusion, our investigation reveals that drought tolerance in black pepper is attained through osmotic adjustment and better ROS scavenging machinery, functioning through different antioxidant enzymes. In drought tolerant Panniyur 5 variety, better osmotic adjustment is achieved through the maintenance of comparatively low ψ_s in cells by the adequate accumulation of compatible solutes like proline and sugars during drought stress. Likewise, the activities of antioxidant enzymes like SOD and peroxidase becoming higher during stress in tolerant variety. The effective control of lipid peroxidation and better osmotic adjustments in cells of the tolerant variety may be the prime factors for the increased tolerance of photosystem and mitochondrial systems towards drought stress.

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