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Establishment of a rapid screening method for drought tolerance of small cardamom by using polyethylene glycol-6000

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Abstract
Small cardamom is a premier spices crop of India. Drought is the chief abiotic stress causing up to 50-80% crop loss in small cardamom. Lack of accurate screening techniques is a limiting factor to develop small cardamom cultivars tolerant to drought, which is the most important constraint in small cardamom productivity. In order to optimize the osmotic stress induced by different concentrations of PEG-6000 for moisture stress tolerance screening in small cardamom, a laboratory experiment was conducted in a factorial randomized complete design with four replications. Four different concentrations of PEG-6000 (5, 10, 15 and 20 per cent) along with a control were used in small cardamom cv. Appangala-1. PEG-6000 concentration above 15% has reduced per cent seedling survival almost by 50%. However, at 15% PEG-6000, a significant increase in proline, phenolic content and scavenging enzyme activity and decreased chlorophyll fluorescence were recorded. Hence, 15% PEG-6000 appears to be an ideal concentration for screening of small cardamom genotypes for moisture stress tolerance.

Keywords: Small cardamom, drought stress, rapid screening, PEG 6000, phenolics, Antioxidant enzymes

Introduction
Drought is one of major abiotic stresses constraining crop productivity worldwide; it has negative effect on carbon assimilation through reduction in photosynthesis rate, which results in plant growth reduction and yield (Singh *et al*., 2014). Severe drought stress also inhibits the photosynthesis of plants by causing changes in the chlorophyll content and damaging the photosynthetic apparatus (Dalton *et al*., 1998) [8]. Generally, drought stress induces the accumulation of reactive oxygen species (ROS) in plants and breaks cellular physiological homeostasis (Xu *et al*., 2010 and Fernandez-Ocana *et al*., 2011) [32, 11]. Higher plants have active oxygen-scavenging systems, such as the antioxidant enzymes superoxide dismutase (SOD), Ascorbate Peroxidase (APX), Catalase (CAT) and Polyphenol oxidase (PPO) protect membranes from the deleterious effects of Reactive oxygen species (ROS) (Foyer and Noctor 2005) [12]. Drought stress induces the accumulation of compatible osmolytes like proline in drought tolerant plants and controls osmotic regulation and alleviates stress in cell membranes. It also acts as a protective agent for enzymes function and as a free radical scavenger (Kishor and Sreenivasulu, 2014) [20]. Abiotic stress condition results the metabolic processes shifted towards biosynthesis of highly reduced phenolics in the plants for tolerance and prevent the oxidative damage of the cells (Alagupalamuthirsolai *et al*., 2018) [1]. Drought stress inhibits that the light harvesting efficiency, photochemical conversion and photosynthetic electron transport, which must affect the yield and quality of crops (Zhang *et al*., 2017) [34].
Small cardamom (*Elettaria cardamomum* (L.) Maton, Zingiberaceae), a shade-loving herbaceous shrub, popularly called "Queen of spices. It is cultivated extensively in the altitude ranging from 500-1500 m above MSL with an average annual rainfall between 1500 to 5000 mm and annual lowest and highest temperature varying from 10 ºC to 36 ºC. Cardamom is grown in India with a production of 20,650 MT (Spices Board, 2017-18). Cardamom is a high value (second costliest crop in the world after saffron) and high income crop, sensitive to both biotic and abiotic stresses (Murugan *et al*., 2008) [27].

Reduced soil water content can decrease the yield of cardamom significantly during summer in the months of April and May (Murugan *et al.*, 2008) [27]. The sharp decline in area under cardamom was noticed across the cardamom tracts of Western Ghats in recent decades. The failure of showers during this period results in affects panicle initiation, subsequent growth and crop yield. The unprecedented drought in summer 1983 adversely affected the growth and yield of cardamom in south India and resulted in as high as 50% mortality in some of the estates. There is a close relationship between cardamom production and rainfall distribution during summer. Summer rain during March, 2008 have benefited the cardamom crop of 2008-2009 up to 20-30%. Therefore, summer rains have positive influence on cardamom. There exists a strong relationship between the water deficit during summer and cardamom production (Prasada Rao, 2016) [29].

The analysis of plant growth, photosynthetic traits, secondary metabolites and antioxidant capacity in plants exposed to drought stress appears to be a promising approach to identify the deleterious effects of water deficit in small cardamom.

Polyethylene glycol (PEG) has been used as osmoticum to induce water stress on plant tissues (Meneses *et al.*, 2011) [24]. The PEG molecules are too large to be absorbed by plant roots, thus increased PEG concentration in the surrounding medium causes outward movement of water from the plant cells (Mohammadkhani *et al.*, 2008) [25]. Thus plant cells undergo situations of water stress (Hamayun *et al.*, 2010) [15]. Under PEG induced water stress, resistant lines have been reported in tomato (Claussen *et al.*, 2005) [6], and soybean (Sakthivelu *et al.*, 2008) [30].

This study was conducted to assess the effects of PEG-induced drought on seedlings survival and physio-chemical characters of small cardamom; to distinguish the treatment that is most suitable for classifying the small cardamom on the basis of tolerance to early-imposed drought; as well as to distinguish populations which could be used for breeding tolerant varieties.

Materials and methods

The 45 days old small cardamom seedlings (cv. Appangala-1) were obtained from ICAR-Indian Institute of Spices Research, Regional station, Madikeri. Good quality of plastic containers with size 28×21.5×5cm were selected to take nutrient solution. Slightly large sized thermocol sheet of size 35×25.5×4cm having holes of 5×5mm were placed above the thermocol sheet. The roots of the seedlings were inserted through the holes of the sheet in to the nutrient solution taken in the container. The piece of foam sheet was tied around the color region on the sheet to provide a mechanical support to the seedlings. The sheets were placed in such a way that only the roots become in touch with the nutrient solution. Four trays for each treatment were taken and 1500 ml of Modified Hoagland solution (Epstein, E. (1972) [9] was added to all the treatment trays. The seedling of the small cardamom were subjected to osmotic stress at seedling stage induced by PEG-6000 at different concentrations viz., 5, 10, 15, 20 per cent in 4 replications. For control, modified Hoagland solution was applied without PEG-6000 solution.

Random sampling was followed by collecting a minimum of four plants from the trays of each treatment at intervals of 15 days from the day of treatment. The collected plants were thoroughly washed in tap water, blotted to remove water adhered on it and separated into root and leaves. All these samples were used separately for various studies. The observations on seedling survival and physio-chemical constituents were recorded 15 days after treatment incubation.

Total chlorophyll and soluble protein measurement

All biochemical measurements were carried out on four fresh fully expanded leaves each from the four plants.

Chlorophyll 'a', chlorophyll 'b, and total chlorophyll content were estimated by adopting the method of Yang *et al*. (2014) [33] and expressed as mg g^{-1} of fresh weight. Soluble protein content was estimated with TCA extract of leaves sample following the method of Lowry *et al.* (1957) and expressed in mg g^{-1} fresh weight.

Total phenols and proline measurement

The total phenolic content of the leaves extract was determined by the Folin–Ciocalteu method (Kaur and Kapoor, 2002) [19]. The total phenolic content was calculated from the calibration curve and expressed as mg g^{-1} of fresh weight. Proline content of the leaf sample was estimated by the method of Bates *et al.* (1973) [2] and expressed as µg g^{-1} of fresh weight.

Analysis antioxidant enzyme activities

Assay of superoxide dismutase (SOD)

The enzyme extract was prepared by homogenizing 1g tissue of leaf in 2ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 15,000 g at 4°C for 30min The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT (Giannospolitis and Ries, 1977) [14]. The assay mixture (3ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin. 0.1 mM EDTA and 100µl of the enzyme extract and the riboflavin was added at the end. Tubes were shaken and placed under a 40-W fluorescent lamp at 25°C. The reaction was initiated and terminated by turning the light on and off respectively. The absorbance at 560 nm was measured against identical non-illuminated in parallel to the sample tubes for blank. Each extract was subtracted from the blank and mathematical difference was then divided by blank and multiplied by 100 to obtain the percentage inhibition of NBT photo-reduction. The SOD activity was expressed in SOD units g^{-1} tissue (50% NBT inhibition = 1 unit) (Belid El-Moshaty *et al.*, 1993) [3].

Assay of catalase (CAT)

CAT activity was assayed spectrophotometrically as described by Chaparro-Giraldo *et al.* (2000) [4] using 3 ml assay mixture containing 100 mM potassium phosphate buffer (pH 7.5) and 2.5 mM H_2O_2 prepared immediately before use and 100 µl enzyme extract. The activity was measured by monitoring the degradation of H_2O_2 using UV-Visible Spectrophotometer (Varian Cary 50) at 240 nm over 1 min, against a plant extract-free blank. The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, activity was calculated using the extinction coefficient (ε_{240nm} = 40 mM^{-1} cm^{-1}) for H_2O_2 and expressed in µmol min^{-1} mg^{-1} of plant tissue.

Assay on peroxidase (PO)

Assay of PO activity was carried out as per the procedure described by Hammerschmidt *et al*. (1982) [16]. The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1 ml)

was added to initiate the reaction, which was followed colorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme was used as blank. Activity was expressed as the increase in absorbance at 420 nm min^{-1} mg^{-1} of plant tissue.

Assay of polyphenoloxidase (PPO)

One gram of sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 20,000 g for 15 min at 4°C. The supernatant served as enzyme source and polyphenoloxidase activity was determined as per the procedure given by Mayer *et al.* (1965) [22]. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as change in absorbance at 470 nm $min^{-1}mg^{-1}$ of plant tissue.

Statistical analysis

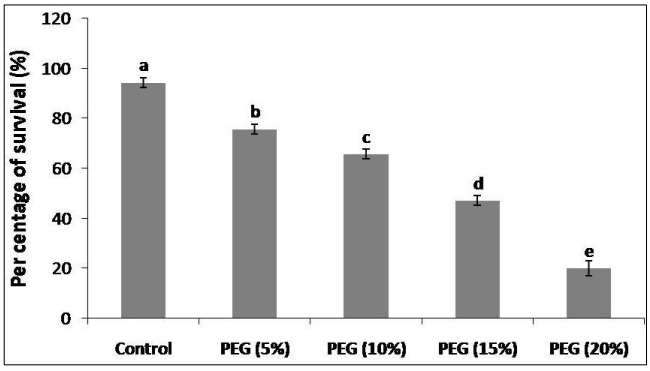
Data was subjected to analysis of variance and Duncan's multiple range tests was used to differentiate means as described by Duncan (1955). Values were considered at a significance level of 95% ($p < 0.05$). Statistical analyses were performed using WASP-Web Agri Stat Package 2.0.

Result and discussion

A fast screening method would be helpful in selecting valuable genotypes with defined growth strategies that translate to drought tolerance and are suitable for experiment and/or breeding (Meher *et al.*, 2018) [23]. The attainment of a drought tolerance screening method depends on identifying a critical level of stress induced by a particular concentration of an agent capable of inducing moisture stress (Yohannes *et al.*, 2014; Harish Babu and Gobu, 2016) [33, 17]. In this present investigation, the plants were short-term drought stressed by PEG-6000 with various concentrations for inducing variable degrees of osmotic stress. Analysis of variance revealed significant for different traits in small cardamom cv. Appangala-1 at various concentrations of PEG-6000.

Growth characters

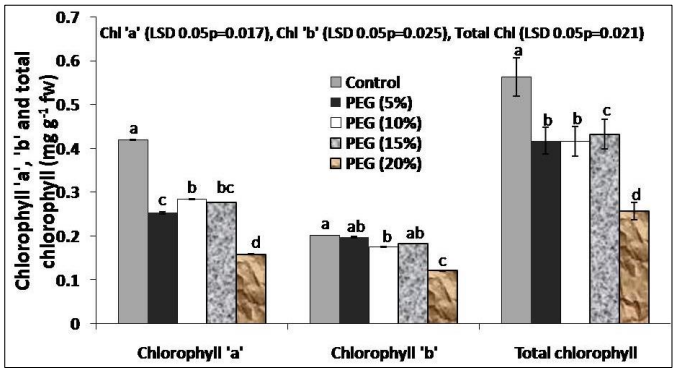
The per cent of survival in small cardamom differed from 30 to 100 at various concentrations of PEG-6000 and it was 100 per cent in case of control (0% PEG-6000) (Fig.1). Reduction in per cent survival (50% and 70%) were recorded at 15 and 20 % PEG-6000 concentration respectively compared to control as PEG 6000 is known to induce osmotic stress which affects per cent germination and survival in many crop plants at varying concentrations (Harish Babu and Gobu, 2016) [17].



Physio-chemical constituents

Photosynthetic pigments

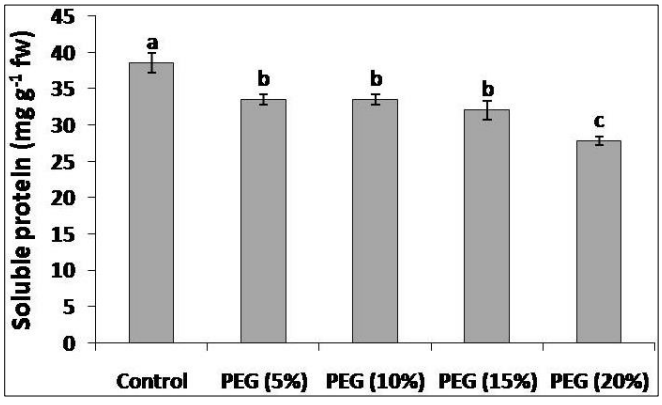
The reduction of photosynthesis driven by lower chlorophyll contents has been observed in numerous plants in response to drought stress (Chaves *et al.* 2002) [5]. In our present report it was observed that PEG-6000 treatment caused a decline in the contents of Chlorophyll 'a', 'b', and total Chlorophyll (Fig. 2). The reduction in chlorophyll content due to drought by producing reactive oxygen species (ROS) such as O_2^- and H_2O_2, can led to lipid peroxidation and consequently, chlorophyll destruction also, with decreasing chlorophyll content (Farooq *et al*. 2009 and Meher *et al*., 2018) [10, 23].

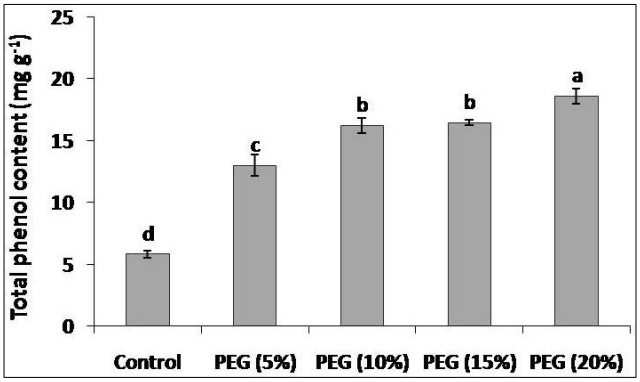


Total soluble protein and total phenol concentrations

Similar to chlorophyll content, the highest total soluble protein was observed in control treatment (Fig. 3). *In vitro* drought stress by PEG-6000 significantly reduces total soluble protein in leaves of treated plants and as the concentration decreases, small cardamom showed lower concentration of total soluble protein at PEG 20% and similar findings have been reported by Mohd. Aminur Faiz Suis *et al.*, (2015) [26].

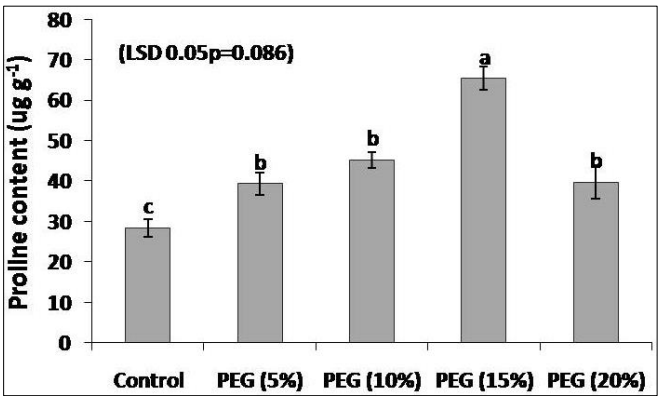
The total phenolic content was significantly higher under PEG-6000 induced drought stressed plant than the control. The treatment PEG 20% (18.4 mg/g FW) showed the highest increase in phenolic contents when exposed to drought conditions compared to the control (5.69 mg/g FW) (Fig. 4). In small cardamom under drought induced drought stress related metabolic alterations and free radical production. In this condition small cardamom need more supply of phenolics for tolerance and prevent the oxidative damage of the cells. As a result, the metabolic processes are shifted towards biosynthesis of highly reduced phenolic in the stressed plant for better adaptation and similar findings have been reported by Natesan and Subramanian (2017) [28].





Proline content

The proline accumulation has the linearity to osmotic stress (Ghorbanli *et al.*, 2012) [13]. In our study, proline content increased gradually, but the extent of this increase was significantly different ($p < 0.05$) in the treatments, and the maximum proline accumulation was observed in PEG 15% (65.4 µg g^{-1} FW), whereas the lowest was in control (28.4 µg g^{-1} FW) (Fig. 5). Elevated proline content under drought stress is proposed to cell water level and thus preventing plants from damages. The increased proline contents were observed in our study and similar findings have been reported by Javed and Ikram *et al.*, 2008 [18].

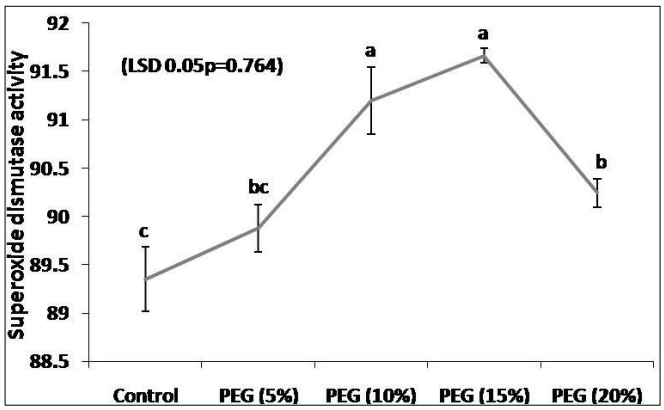


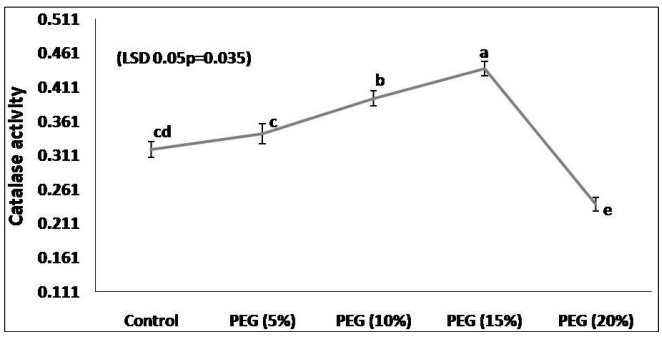
Antioxidant enzyme activities

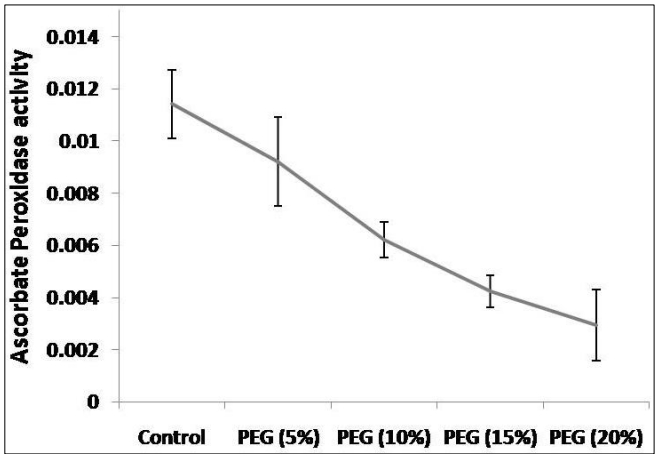
The activity of enzymes (SOD, CAT, APX and PPO) involved in ROS scavenging varied significantly ($p < 0.05$) upon drought treatment (Fig. 6-9). Antioxidant activities of small cardamom leaves showed noticeable impact when PEG-6000 was treated in hydroponic solution. Based on Fig. 6, leaves in control plants showed relatively low total SOD activities. The SOD activities of leaves treated with 10 and 15% PEG-6000 were then significantly induced more production of ROS. SOD is the first line of plant defense system against uncontrolled oxidation during unfavorable conditions. Cruz de Carvalho (2008) [7] reported that scavenging activity of SOD includes the conversion of highly reactive singlet oxygen molecules into more stable hydrogen peroxide. In the present investigation, SOD activity was significantly the highest in drought stressed plant as compared to control (Fig. 6).

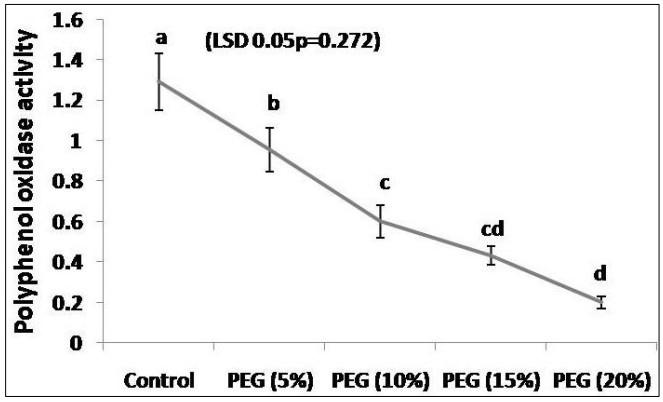
Similar pattern of antioxidant activity was also exhibited by CAT activity (Fig. 7). As concentration of PEG-6000 increases, CAT activity of treated plant leaves increases. Nevertheless, control, 5%, 10 % and 15% PEG treated plant leaves produced higher CAT activity compared to 20% PEG 6000 treated plant leaves. Perhaps this might be due to the low affinity of CAT towards hydrogen peroxide. Cruz de Carvalho (2008) [7] stated that the enzyme CAT has low affinity for hydrogen peroxide and effective only in high concentration of hydrogen peroxide. In fact, high concentration of PEG 6000 in hydroponic medium induced excessive production of hydrogen peroxide in leaves which was favorable to the enzyme CAT and similar findings have been reported by Mohd. Aminur Faiz Suis *et al.* (2015) [26].

Shigeoka *et al*. (2002) [31] claimed that the APX exist as isoenzymes and has an important role in metabolism of hydrogen peroxide in higher plants. Lower APX activity of leaves at 10%, 15% and 20% PEG 6000 (Fig. 8) could be due to the impact of drastic degradation of chlorophyll. Besides that, present study also implied that the activity of APX and PPO were observed highest in control leaves (Fig. 8). Unfortunately, activity of APX and PPO decreased as the concentration of PEG-6000 increases (Fig. 8&9). These enzymes played a major role in maintaining the optimum level of reactive oxygen species which were involved in plant signaling and defense mechanism (Cruz de Carvalho 2008) [7].



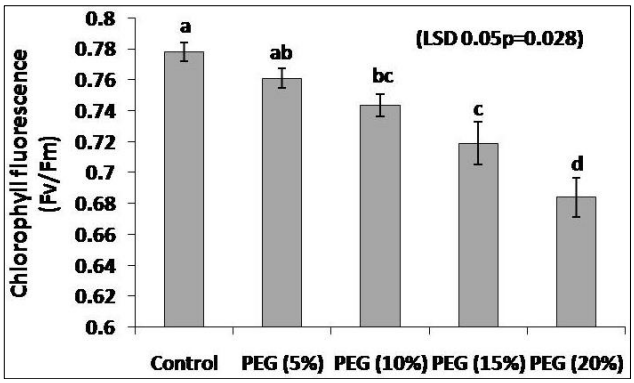






Chlorophyll fluorescence

The chlorophyll fluorescence parameter characterized by the maximal photochemical efficiency PSII (Fv/Fm) is widely used to determine the photosynthetic activity or efficiency of a plant (Dalton *et al.*, 1998) [8]. PEG-6000 treatment decreased the Fv/Fm values indicate a lower photosynthetic efficiency, with damages to the enzymatic apparatus of both photosystems I and II (Živčák *et al.* 2008) [35]. In present study, a significant decrease in the Fv/Fm ratio was observed in all the treatments during the drought stress, suggesting a possible inhibition of PSII photochemistry by an insufficient energy transfer from the light harvesting chlorophyll complex to the reaction center (Fig. 10).



Conclusion

Based on the findings of the present investigation, the PEG-6000 at 15% concentration (equivalent to -2.95 bars) can be used for screening a large number of germplasm collections of small cardamom in a short time under in vitro conditions.

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