



## **Development of Screening Method for Moisture Deficit Tolerance of Black Pepper Genotypes**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author AM designed and supervised the experiment and literature search and reviews. Authors VS and CS executed the work, analysed the data and wrote the first manuscript. Authors CKT and KSK assisted in editing the manuscript, data analysis and interpretation of results. Author KPS assisted in sample collection and laboratory work. All authors have read and approved the final version of the manuscript.*

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### **ABSTRACT**

Black pepper is basically a rainfed crop in India. Drought is the chief abiotic stress causing up to 50-80% crop loss in black pepper. Lack of precise screening methodology to develop moisture stress tolerant lines is a limiting factor in black pepper productivity. To develop a rapid screening methodology, a laboratory experiment was conducted with rooted black pepper cuttings under hydroponic culture. The experiment was designed in a completely randomized with four replications. Moisture stress was imposed with six different concentrations of Polyethylene glycol-6000 (5, 8, 10, 12, 15 and 20 per cent) along with control in black pepper cv. Panniyur-1, IISR-Thevam, IISR-Sreekara and IISR-Girimunda. The results showed that Panniyur-1, IISR-Thevam, IISR-Sreekara and IISR-Girimunda at the PEG-6000 concentrations of 8%, 10%, 10%, 10% respectively reduced per cent cuttings survival almost by 50% Lethal dose (LD<sub>50</sub>) after 10 days of PEG stress and also, a significant increase in proline was recorded up to 10% PEG-6000. Hence, 10% PEG-6000 appears to be an ideal concentration for screening black pepper genotypes for moisture stress tolerance.

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## 1. INTRODUCTION

Moisture deficit stress is one of the major abiotic stresses limiting crop productivity [1]. Severe moisture deficit stress hampers the photosynthetic rate of plants by causing damage in the photosynthetic apparatus and reduction in the chlorophyll pigments [2]. Moisture deficit stress induces the accumulation of compatible osmolytes like proline in tolerant plants and controls osmotic regulation and alleviates stress in cell membranes. It also acts as a protective agent for enzyme function and as a free radical scavenger [3]. Relative water content (RWC) is found to be better physiological characters for the characterization of drought /moisture stress tolerance [4].

Black pepper (*Piper nigrum* L) is basically rainfed crop and requires a well distributed rainfall of 2000-3000mm for better productivity. It is susceptible to moisture deficit stress. Rainfall distribution is uneven in black pepper growing areas leading to a reduction in plant growth and development and in some cases even the death of plants due to severe water deficit occurs. The yield of black pepper declines if summer showers are received and followed by a dry spell [5]. Identification of moisture deficit tolerant genotypes is essential to avoid moisture stress problems. Lack of accurate screening methodology is a limiting factor to develop black pepper cultivars tolerant to moisture deficit. The physiological and biochemical parameters contributing to moisture deficit tolerance need to be studied in detail.

Polyethylene glycol (PEG) has been used as osmoticum to induce moisture stress in plant tissues [6]. The PEG-6000 are larger molecular weight molecules, thus increased osmotic potential in the surrounding medium causes outward movement of water from the plant root cells [7]. Under PEG induced water stress, resistant lines have been reported in tomato [8], and durum wheat [9]. The advantage of hydroponic method of screening is rapid and imposing moisture stress is accurate than in other pot culture studies.

This study was conducted to identify the effective concentration of PEG-6000 for screening a large number of black pepper genotypes for moisture

deficit tolerance analysis of physiological traits conferring tolerance.

## 2. METHODOLOGY

### 2.1 Plant Material

The 75 days old black pepper (cv. Panniyur-1, IISR-Thevam, IISR-Girimunda, IISR-Sreekara) rooted cuttings were obtained from ICAR-Indian Institute of Spices Research, Kozhikode. The experiment was conducted in a completely randomized design with four replications. One litre Modified Hoagland (MH) nutrient solution [10] was taken in a plastic container (28×21.5×5cm) and a thermocol sheet (35×25.5×4cm) having holes of 5×5mm was placed above the container. The rooted cuttings were inserted through the holes of the sheet into the MH nutrient solution in the container. A piece of foam sheet was tied around the collar region of the rooted cuttings on the thermocol sheet to provide mechanical support to the cuttings and ensured the root was in contact with the nutrient solution.

### 2.2 Plant Growth and Stress Treatment

The rooted cuttings were subjected to osmotic stress induced by PEG-6000 at different concentrations. Apart from the control ( $T_0=0\%$  PEG), six different concentrations of PEG [ $T_1=5\%$  PEG (weight/volume of water);  $T_2=8\%$  PEG;  $T_3=10\%$  PEG;  $T_4=12\%$  PEG;  $T_5=15\%$  PEG;  $T_6=20\%$  PEG] were used for moisture stress induction in Hoagland solution growth medium. Growth measurement in terms of mortality of the cuttings was recorded 10 days after placing rooted cuttings in the PEG supplemented MH medium. Similarly, for relative water content and biochemical estimations 10 days old, treated cuttings were used.

### 2.3 Mortality of Rooted Cuttings

The rooted cuttings mortality (%) was calculated from the total number of killed rooted cuttings per treatment for each genotype 10 days after the moisture stress treatment as per the following formula:

$$\text{Rooted cuttings mortality (\%)} = \left( \frac{\text{Number of survived rooted cuttings}}{\text{Total number of rooted cuttings planted}} \right) \times 100$$

The mortality was plotted against the concentration of PEG to obtain dose responsive graph for various black pepper genotypes to PEG (%) treatment, which was used to calculate the 50% lethal dose (LD<sub>50</sub>).

## 2.4 Relative Water Content (RWC)

The leaves were transferred to a sealed flask, after taking fresh leaf weight (FW), it was rehydrated in 200 ml of distilled water for 5 h until fully turgid. They were reweighed after surface dried (turgid weight, TW) followed by oven-dried at 72°C for 48 h (dry weight, DW). The RWC was calculated by using the following formula [11].

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

## 2.5 Total Chlorophyll

Samples were taken from the third leaves of each plant for all biochemical estimation. Chlorophyll 'a', chlorophyll 'b', and total chlorophyll content from selected leaves (100 mg) were extracted with 80% acetone and quantified according to Arnon's method [12]. Spectral absorption was measured at 645, 663, 652 nm and the chlorophyll contents were expressed as mg g<sup>-1</sup> of fresh weight.

$$\text{Chlorophyll 'a'} = (12.7 \times \text{OD}_{663}) - (2.69 \times \text{OD}_{645}) \times V / 1000 \times W$$

$$\text{Chlorophyll 'b'} = (22.9 \times \text{OD}_{645}) - (4.68 \times \text{OD}_{663}) \times V / 1000 \times W$$

$$\text{Total chlorophyll} = (\text{OD}_{652} \times 1000 / 34.5) \times V / 1000 \times W$$

## 2.6 Proline Content

Proline content of the leaf sample was estimated by the method of Bates et al.[13]. Proline was extracted in a 3% aqueous sulfosalicylic acid solution; the sample was mixed with acid ninhydrin solution, incubated for 1 h at 95°C, cooled on ice and extracted with toluene. The absorbance of the supernatants was read at 520 nm using toluene as a blank. Pure proline was assayed in parallel to obtain a standard curve.

Proline concentration was expressed as µg g<sup>-1</sup> fresh weight.

## 2.7 Total Phenol Content

The amount of total phenolics in the leaf sample was determined according to the Folin-Ciocalteu procedure [14]. Extracted samples (1.0 ml) were poured into test tubes; 1.0 ml of Folin-Ciocalteu's reagent and 0.8 ml of sodium carbonate (8%) were added. The tubes were mixed and allowed to stand for 30 minutes. Absorption at 765 nm was measured (UV-VIS spectrophotometer). The total phenolic content was expressed as gallic acid equivalents in mg/g fresh weight.

## 2.8 Statistical Analysis

The analysis of variance was calculated from replication data and subjected to Duncan's multiple range test as described by Duncan [15]. Values were considered at 95% (p < 0.05) significant level. Statistical analyses were performed using WASP-Web Agri Stat Package 2.0.

## 3. RESULTS AND DISCUSSION

A rapid screening methodology would help select suitable genotypes with desirable physiological traits for moisture deficit tolerance and for the further breeding programme [16]. The establishment of a screening method depends on optimizing a critical level of stress induced by a particular concentration of an agent capable of inducing moisture deficit stress [17,18]. In this present study, the plants were short-term moisture stressed by PEG with various concentrations for inducing variable degrees of osmotic stress. Analysis of variance revealed significant for different traits in various black pepper genotypes at different concentrations of PEG.

### 3.1 Growth Characters

PEG is known to induce osmotic stress which affects per cent germination and survival in many crop plants at different concentrations [18,19]. In our study, although <10% cuttings mortality was noted in each genotype in T<sub>0</sub> (control), thereafter a significant change in the mortality was noted in various genotypes. The per cent mortality in rooted cuttings of black pepper significantly varied from 37.5 to 86.6 % at various

concentrations of PEG. The lowest mean cuttings mortality was noted in  $T_1$  (37.5%) followed by  $T_2$  (46.6%), while the highest was observed in  $T_6$  (86.6%). Among the four black pepper genotypes studied, Panniyur-1 showed the lowest  $LD_{50}$  value i.e., 8% PEG after 10 days of treatment. The highest  $LD_{50}$  value (10% PEG) was observed in IISR-Thevam, IISR-Sreekara and IISR-Girimunda. However, the average  $LD_{50}$  value of black pepper irrespective of genotype was calculated as 10% PEG after 10 days of treatment (Fig. 1).

### 3.2 Physiological and Biochemical Parameters

#### 3.2.1 Relative water content

The PEG induced moisture stress resulted in a significant change in the RWC. The genotypic mean value of RWC showed highest in the  $T_0$  (84.5%), which decreased subsequently as PEG concentration increased and led to the lowest in  $T_6$  (32.3%) (Fig.2). High organic osmolytes accumulation like proline maintains high water content in plant tissue [20,21]. In the present study also observed that leaf relative water content decreased gradually up to 10% PEG (when proline concentration was increased up to 10%) after that it decreased sharply up to 20% PEG.

#### 3.2.2 Photosynthetic pigments

The reduction of photosynthetic rate observed by lower photosynthetic pigments has been

observed in several plants in response to drought stress [22]. In our present study observed that PEG treatment caused a significant decline in Chlorophyll 'a', 'b', and total chlorophyll in all the genotypes (Fig. 3). The genotypic mean of Chl 'a', Chl 'b' and total chlorophyll showed higher concentration (1.329, 0.312 and 1.70  $mg\ g^{-1}$ , respectively) at 0% PEG and gradually significantly declined with an increase in PEG concentration and reached lowest at 20% PEG (0.497, 0.112 and 0.616  $mg\ g^{-1}$ , respectively). Reactive oxygen species (ROS) such as  $O_2^-$  and  $H_2O_2$  produced during the stress, can lead to cell membrane lipid peroxidation and resulting in the destruction of chlorophyll pigments [16].

#### 3.2.3 Total phenol concentrations

The total phenols in the rooted cuttings increased significantly with an increase in PEG concentration. The treatment  $T_6$  (2.86  $mg\ g^{-1}\ FW$ ) showed the highest increase in genotypic mean phenolic contents compared to the  $T_0$  (0.837  $mg\ g^{-1}\ FW$ ) (Fig. 4). Plant cells would have undergone stress related metabolic alterations and free radical production in black pepper under PEG induced moisture stress. In this condition, black pepper needs more supply of phenolics to prevent oxidative damage to the cells. Hence, there may be shifting of metabolic processes towards biosynthesis of highly reduced phenolics in the stressed plant for better adaptation, leading to higher phenolic content. Similar findings have been reported by Natesan and Subramanian [23].

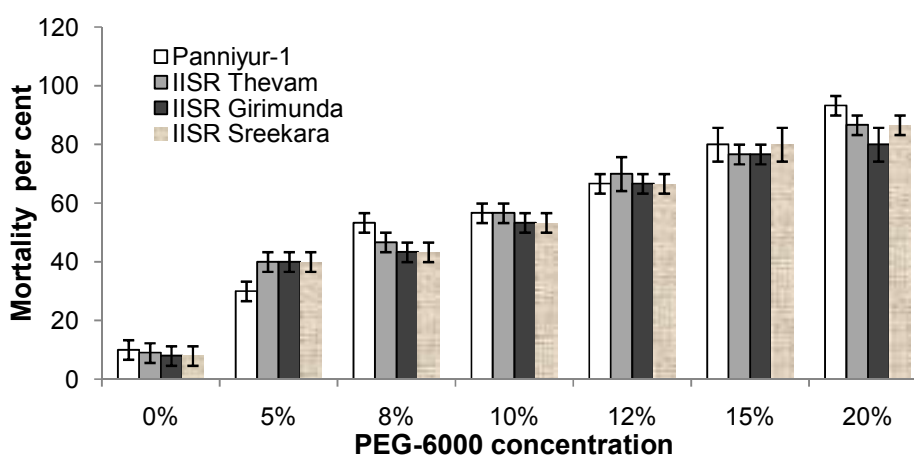


Fig.1. Effect of PEG on mortality per cent of black pepper rooted cuttings

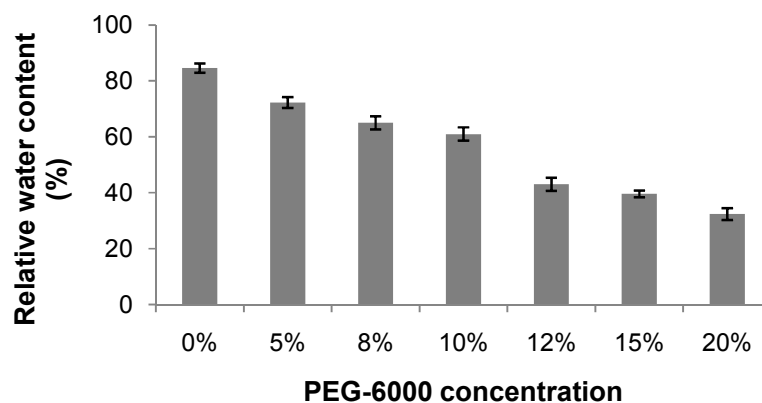


Fig. 2. Effect of PEG on the relative water content of black pepper rooted cuttings

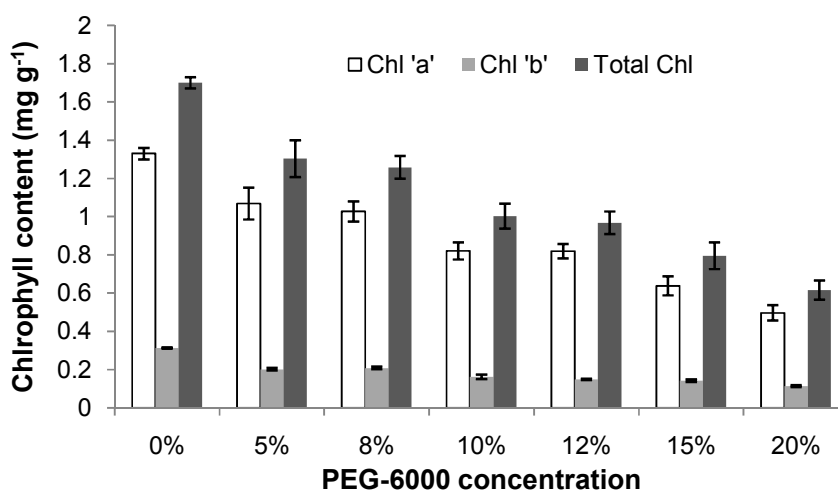


Fig. 3. Effect of PEG on the relative water content of black pepper rooted cuttings

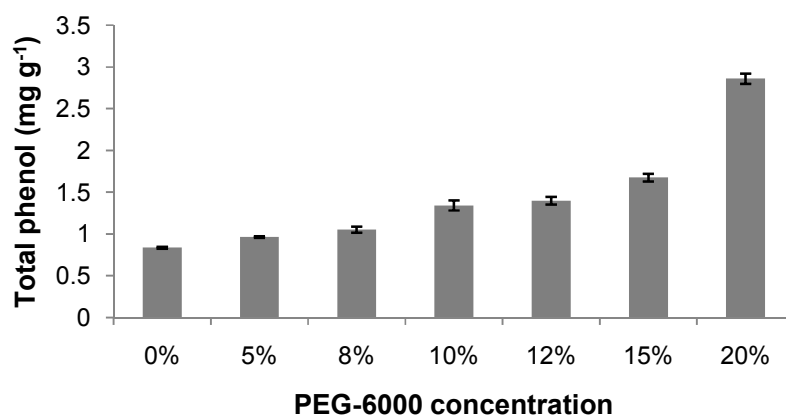
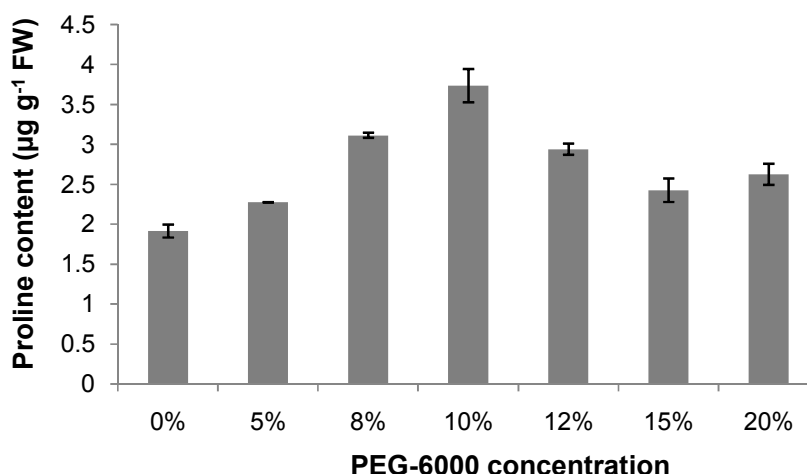


Fig. 4. Effect of PEG on total phenol content of black pepper rooted cuttings



**Fig. 5. Effect of PEG on proline content of black pepper rooted cuttings**

### 3.2.4 Proline content

Accumulation of organic solutes, such as proline has been protecting the plant from various stresses and also helps the plant to overcome stress rapidly [24]. Apart from osmotic adjustment proline has versatile roles including increase growth, membrane integrity maintenance, and protection of various proteins or enzymes, free radical scavenging activities, etc. [25]. The proline accumulates in relative amounts under osmotic stress [26]. In the present study, the accumulation of proline was found to be increased due to supplementation of PEG to the growth medium. However, this increase was concentration dependent and an increase in proline was noticed up to 10% PEG concentration. The lowest genotypic mean proline content ( $1.914 \mu\text{g g}^{-1} \text{FW}$ ) was noticed in the  $T_0$ , which followed an increasing trend with an increase in PEG concentration and the highest was noticed in 10% PEG ( $3.73 \mu\text{g g}^{-1} \text{FW}$ ) and thereafter a decreasing trend was noticed up to 20% PEG ( $2.62 \mu\text{g g}^{-1} \text{FW}$ ) (Fig. 5). Elevated proline content under drought stress is known to act as an osmolyte and helps to maintain cell turgidity and stabilizing sub-cellular structure (e.g., membranes and proteins), thus preventing further damage to the cells. Similar findings have been reported by Javed and Ikram et al. [27].

### 4. CONCLUSION

The data from this experiment revealed that the PEG-6000 at 10% concentration can be used for

screening a large number of black pepper genotypes for moisture deficit tolerance rapidly under in vitro conditions, and the tolerant genotypes thus identified can further be utilized for crop improvement.

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### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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