



ASSESSMENT OF GENETIC DIVERSITY AND EFFECT OF PEG INDUCED DROUGHT STRESS ON GROUNDNUT (*ARACHIS HYPOGAEA* L.) GENOTYPES

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ABSTRACT

Cultivated groundnut or peanut is one of the important legume crops of tropical and semi-arid tropical countries, where it provides a major source of edible oil and vegetable protein. Abiotic stresses directly or indirectly affects the physiological status of an organism by altering its metabolism, growth and development. The objectives of this study were to estimate the genetic diversity between eight groundnut genotypes and to evaluate the effect of Polyethylene glycol (PEG-6000) induced drought stress on germination. The variable responses of groundnut genotypes were characterized using 10 RAPD primers and tested for drought tolerance on different levels of osmotic potential of -2, -4, -6, -8, -10 and -12 bars of physiological drought initiated by PEG-6000 and the data was recorded on various seedling parameters like germination rate, root length and shoot length. The total numbers of amplification products generated were 52, and among them 34 were found to be polymorphic. Primer OPD-02 generated the maximum number of amplified products with 100% polymorphism. Among all the genotypes there is close relationship between two genotypes Narayani and Dharani and the data revealed that PEG induced drought stress showed general negative effect on seedling morphological characters indicated by root and shoot length. The study indicated the scope and usefulness of RAPD markers for diversity analysis and provided useful data in screening of drought tolerant genotypes using PEG at seedling stage.

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important, leguminous oil seed crop which is cultivated mostly in the tropical and sub-tropical regions of the world, under rainfed environments and low-input agricultural system (Bhauso *et al.*, 2014). One-third of global groundnut production is used for food and the remaining is used for oil production. Globally, groundnut is grown on an average of ~21 million hectares (ha) that produces ~33 million metric tons (Feng *et al.*, 2012). Two-third of the world's groundnut is produced by India and China. Approximately 70% of the global groundnut growing areas are located in semi-arid regions, where drought is a key environmental constraint limiting groundnut production. According to recent estimation, global groundnut productivity incurred annual loss of approximately 6 million tons due to drought alone (Bhatnagar *et al.*, 2014). Under drought stress, seed germination and seedling establishment were inhibited due to the drop of water potential, which results in the decline of water uptake (Farooq *et al.*, 2009).

Knowledge of the genetic diversity is a fundamental aspect in the improvement of a crop species. In cultivated groundnut although the molecular markers are becoming progressively important as useful tools in assessing the genetic diversity in crop breeding programs, their application in groundnut improvement is lagging behind because of the limited knowledge of the groundnut genome. Extensive variation for morphological and physiological characteristics exists in both wild and cultivated groundnut but abundant DNA polymorphism has been observed only in wild diploid *Arachis* species as compared to cultivated species (Halward *et al.*, 1993). Evaluation of genetic diversity using DNA marker technology is non-destructive, requires small amount of samples, is not affected by environmental factors, and does not require large experimental setup and equipments for measuring physiological parameters. Markers systems have been successfully used over the last several decades to construct genetic maps, access genetic diversity and locate genes of interest in a number of agriculturally important crops for the desired traits (Garcia *et al.*, 2005). Among molecular marker systems used to identify and assess the genetic diversity and phylogenetic relationships in plant genetic resources, random amplified polymorphic DNA (RAPD) technique is the fastest and simplest. Random Amplified Polymorphic DNA (RAPD)

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is a powerful technique for screening different germplasm for assessing their genetic diversities (Varshney *et al.*, 2005; Gupta *et al.*, 2010).

Drought is one of the major abiotic stresses that seriously decrease crop productivity in arid and semi-arid regions of the world (Yang *et al.*, 2010; Lipiec *et al.*, 2013). A fast screening tool would be helpful in selecting valuable genotypes with defined growth strategies those confers to drought tolerance suitable for breeding programmes. Seed germination and early seedling growth are critical stages for plant establishment (Li *et al.*, 2011), and are more sensitive to drought stress during the seedling stages. Screening drought-resistant plant genotypes is a fundamental goal targeted in arid and semi-arid regions. Nonetheless, drought cannot be easily controlled in the field because of rainfall that can impede water deficit (Muscoloa *et al.*, 2014). It is necessary to alleviate the adverse effects of drought stress for achieving good crop yields (Ashraf and Rauf, 2001). Therefore, assessing plant response to drought at early seedling stage was commonly achieved using chemical desiccators such as polyethylene glycol (PEG). Several reports have shown that in vitro screening technique using PEG is one of the dependable approaches for the selection of desirable genotypes to study in detail on water scarcity on plant germination indices (Landjeva *et al.*, 2008; Almaghrabi, 2012; Ahmad *et al.*, 2013; Jatoi *et al.*, 2014). The upsurge in concentration of PEG caused decrease in germination percentage, seedling vigour in certain crop plants (Khodarahmpour, 2011). One of the important findings is that a positive correlation between drought tolerance of the genotypes in the field and in laboratory experiments was noted (Kosturkova *et al.*, 2014). Therefore the current study was planned to assess genetic diversity among eight groundnut genotypes using RAPD markers and to find out appropriate criteria for simple and quick screening of genotypes that have higher tolerance to drought in support of breeder's crop improvement programs.

MATERIALS AND METHODS

Plant Material

The groundnut seeds of eight genotypes (Kadiri 9, Narayani, Dharani, Greeshma, Abhaya, TPT 3 JL24 and Kadiri 6) used in the present study were obtained from Regional Agricultural Research Station, ANGRAU, Tirupati, Andhra Pradesh, India.

DNA Extraction and RAPD Analysis

DNA was extracted based on the cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson (1980). The quality and quantity of DNA was assessed spectrophotometrically and also by agarose gel electrophoresis using 1% agarose. A total of ten random primers, each of 10 nucleotides (decamers) obtained from M/s Operon technologies, Almedas, USA were used for RAPD analysis (Table 1).

Polymerase Chain Reaction (PCR) was carried out in 25 µl reaction mixture containing 1X reaction buffer, 1 U *Taq* DNA polymerase (Thermoscientific), 200 µM of dNTP mix, 0.5 µM/reaction of random primer's (Sigma Aldrich, USA) and 50 ng of template DNA. The amplification reaction was performed in the Biorad Master cycler with an initial denaturation for 4 minutes at 94°C, then 35 cycles: 1 minute denaturation at 92°C, 1 minute annealing at 37°C, 2 minutes extension at 72°C. Final extension was carried out at 72°C for

5 minutes (Dwivedi, *et al.*, 2001, Rungnoi *et al.*, 2012). The RAPD-PCR amplified products were analyzed by gel electrophoresis in 1% ultrapure agarose in 1X TBE buffer stained with ethidium bromide (10mg/ml).

Statistical Analysis

For each individual RAPD primer, PCR amplified products were designated. Data were scored on the basis of the presence or absence of the amplified products. If the product is present in a genotype, it was scored as 1, if absent, it was designated as 0. Using the SPSS software (Version-20) genetic similarities between the groundnut genotypes were calculated using the simple matching coefficient and clustered by unweighted pairs group method with arithmetic average (UPGMA) based on the average linkage method of calculating distance between clusters.

Screening of Groundnut Genotypes for Drought Stress using PEG

Seeds were disinfected with 4% Sodium hypochlorite solution for 15 minutes with gentle shaking at room temperature. After the surface sterilization, the seeds were washed three times with autoclaved distilled water. The pre-soaked seeds were first air dried to eliminate the surface water. Ten seeds from each genotype were germinated on two layers of filter paper in petridishes with different osmotic potentials i.e. 0 (control), -2, -4, -6, -8, -10 and 12 bars using appropriate concentration of PEG-6000 (Michel and Kaufmann, 1973). Distilled water was used as control. The treated and controlled seeds were allowed to germinate in a biological oxygen demand (BOD) incubator at 25°C ± 2°C for seven days. Filter papers were moistened at regular intervals with the above mentioned solutions. The lid of the petriplates were opened and replaced for exchange of fresh air to the growing seedlings at regular intervals. The experiment was terminated by harvesting seedlings after seven days and germination rate, root length and shoot length data was noted.

Table 1 List of the RAPD primers used for genetic diversity analysis

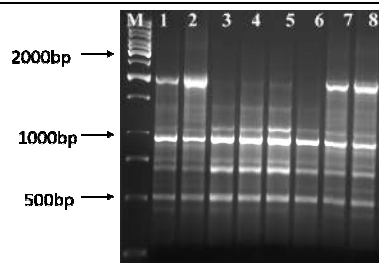
S.No	Name of the Primer	Primer Sequence (5' - 3')
1	OPA-03	AGTCAGCCAC
2	OPA-05	AGGGGTCTTG
3	OPA-10	GTGATCGCAG
4	OPA-15	TTCCGAACCC
5	OPA-19	CAAACGTCGG
6	OPD-02	GGACCCAACC
7	OPD-06	ACCTGAACGG
8	OPD-11	AGCGCCATTG
9	OPD-17	TTTCCCACGG
10	OPD-20	ACCCGGTCAC

Table 2 Number of polymorphic bands produced after PCR amplifications

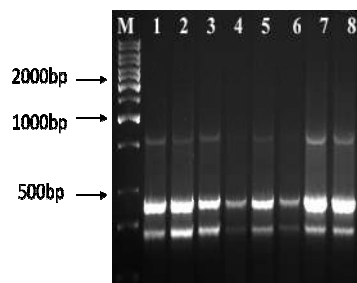
Name of the Primer	Primer Sequence (5' - 3')	Total Number of Polymorphic bands	Polymorphic bands
OPA-03	AGTCAGCCAC	14	8
OPA-05	AGGGGTCTTG	3	1
OPA-10	GTGATCGCAG	8	2
OPA-15	TTCCGAACCC	0	0
OPA-19	CAAACGTCGG	12	8
OPD-02	GGACCCAACC	15	15
OPD-06	ACCTGAACGG	0	0
OPD-11	AGCGCCATTG	4	3
OPD-17	TTTCCCACGG	0	0
OPD-20	ACCCGGTCAC	12	08

Table 3 Jaccard's similarity coefficient matrices for groundnut genotypes based on RAPD profiling

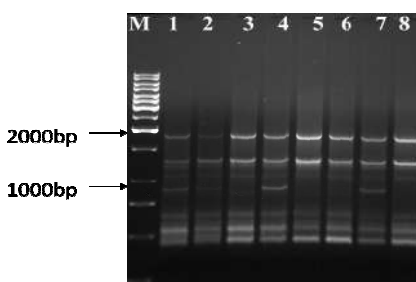
Groundnut genotypes	Similarity Matrix							
	1.Greeshma	2.Narayani	3.Kadiri-6	4.JL24	5.TPT-3	6.Kadiri-9	7.Dharani	8.Abhaya
1.Greeshma	1.000							
2.Narayani	.532	1.000						
3.Kadiri-6	.548	.489	1.000					
4.JL24	.784	.478	.605	1.000				
5.TPT-3	.523	.683	.590	.500	1.000			
6.Kadiri-9	.703	.413	.611	.735	.500	1.000		
7.Dharani	.533	.868	.524	.512	.784	.409	1.000	
8.Abhaya	.605	.651	.778	.585	.610	.550	.700	1.000



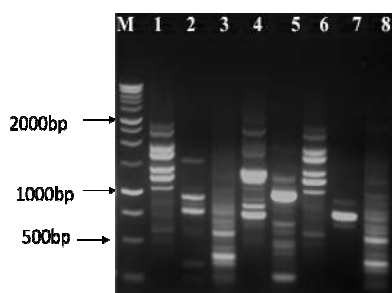
1 OPA-03



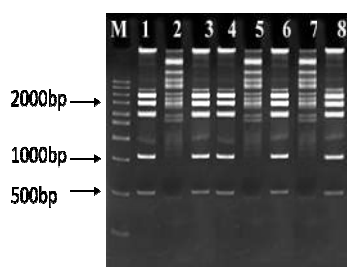
2 OPA-05



3 OPA-10



4 OPA-2



5 OPD-20

Lane M- Marker
 Lane 1- Greeshma
 Lane 2- Narayani
 Lane 3- Kadiri-6
 Lane 4- JL24
 Lane 5- TPT-3
 Lane 6- Kadiri-9
 Lane 7- Dharani
 Lane 8- Abhaya

Fig 1-5 Fig. 1-5 RAPD profiles of groundnut genotypes

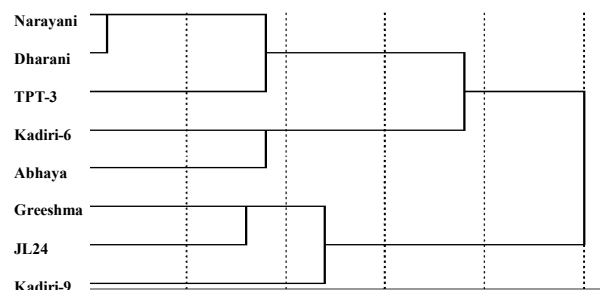


Fig 6 Dendrogram generated for groundnut genotypes using UPGMA cluster analysis

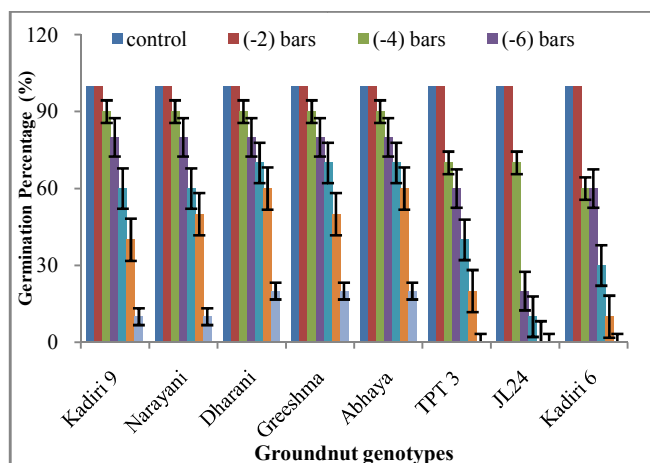


Fig 7 Germination percentage (%) of groundnut genotypes subjected to PEG-6000 induced drought stress

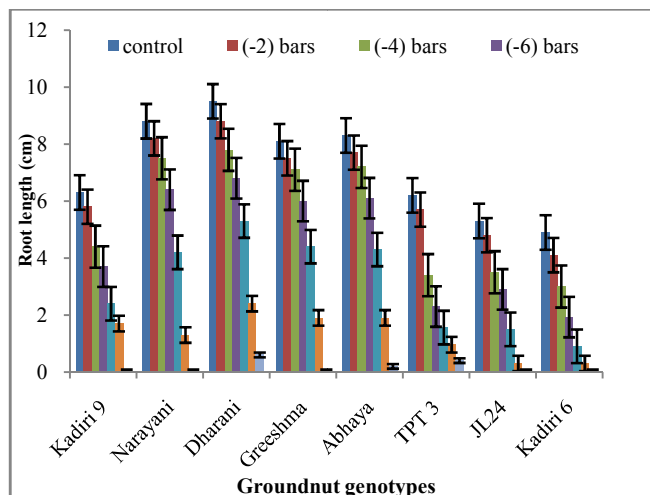


Fig. 8 Effect of PEG-6000 induced drought stress on root length of groundnut genotypes. Bars indicate the mean values ± S.E.M.

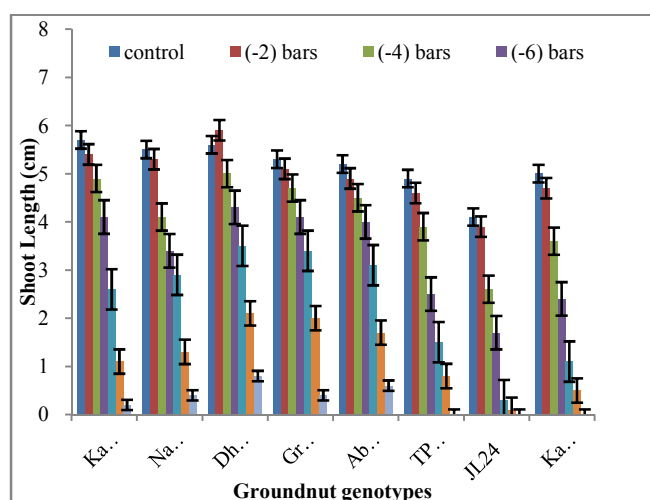


Fig 9 Effect of PEG-6000 induced osmotic stress on shoot length of groundnut genotypes. Bars indicate the mean values \pm S.E.M.

RESULTS AND DISCUSSION

Five selected primers gave total of 52 amplification products, out of which 34 were polymorphic (Table 2). Among the primers of OPA and OPD series, 5 primers produced scorable and reproducible amplifications in all the genotypes. Maximum polymorphism was showed in PCR reaction with primer OPD-02 and showed 100% polymorphism in size ranging from 1 to 2 kb. They were closely followed by OPA-03 with 60% polymorphism. The banding pattern of primers OPA-05 and OPA-10 was found to be identical with many primers and in case of primer OPD-02, total number of bands were 15 out of which 15 were polymorphic. The size of amplified products varied from 500 to 2000bp (Fig. 1-5). Banding profiles obtained with 5 random primers for 8 groundnut genotypes were analysed on the basis of presence and absence of the bands. Jaccard's similarity co-efficient between the isolates were calculated (Table 3) and the similarity matrix thus produced indicated that Dharani and Kadiri 9 were genetically distinct as they showed only 40.9% similarity followed by Kadiri-9 and Narayani with 41.3%. While the genotypes Narayani and Dharani were found to be genetically similar with 86.8% similarity followed by 78.4% similarity between JL24 and Greeshma, 77.8% between Kadiri 6 and Abhaya, 70.8% between Kadiri 9 and Greeshma. The similarity co-efficients subjected to SPSS software produced a dendrogram with two major clusters (Fig. 6). First cluster having Narayani and Dharani with first sub-cluster TPT 3 and Kadiri 6, second sub-cluster having Kadiri 6 and Abhaya. Second cluster having Greeshma and JL24 with sub-cluster Greeshma and Kadiri 9. Genotypes JL24 and TPT 3; Dharani and Kadiri 6 are distantly related and from separate branches as they were not grouped with these clusters.

Dwivedi *et al.*, (2001) and Nalini Mallikarjuna *et al.*, (2005) also reported a high level of genetic variation in groundnut using RAPD markers. In a similar study involving 12 released cultivars, Radhakrishnan *et al.*, (2004) reported wide genetic diversity among released cultivars i.e. within cultivated groundnut in contrast to earlier reports and concluded that with RAPD technique, sufficient polymorphism can be detected in cultivated groundnut which would help in genetic mapping. Vyas *et al.*, 2014 and Suneetha *et al.*, 2015 also reported a high level of genetic variation in groundnut using RAPD markers. Among all the genotypes there is close relationship between

two genotypes Narayani and Dharani. The characterization of *Arachis hypogaea* L. genotypes by RAPD had proved useful in separating all the isolates from each other. It has also provided us with markers that can be used to separate and distinguish each genotype. This possibility of distinguishing different genotypes by a rather simpler technique of genomic fingerprinting based on PCR-RAPD could be of great importance for biotechnological use, where additional more easily detectable markers are not available.

Effects of Drought stress on Germination

Germination was significantly affected by the osmotic potential. Germination percentage of groundnut markedly decreased with increase in PEG concentration (Fig. 7). Seeds germinated more often and vigorously under mild stress (0, -2 and -4 bars PEG concentrations) than heavy stress (-8, -10 and -12 bars). At -10 bars PEG concentration, a drastic reduction in germination rate was noted. A significant decline in the germination percentage was recorded at -12 bars PEG, indicating that -12 bars PEG concentration is a threshold value for the good germination of groundnut seeds. Conversely, no seeds germinated at the -14 bars PEG concentration, which indicated that -14 bars PEG is the lowest osmotic potential for groundnut germination. Dharani showed 20% germination even at the higher stress level of -14bars PEG than other genotypes.

Effects of Drought Stress on Root Length

All the genotypes showed decreased root length with the increased PEG concentration. There is a significant difference among all the genotypes screened at -2 bars PEG osmotic stress. Root length reduction was significant in all the genotypes at -4, -6, -8 bars except Dharani. Drastic decrease in root length was identified at -10 bars PEG in JL24 and Kadiri-6. The mean root length of all varieties was almost rudiment and differences were not found at -12 bar (Fig. 8). Above this concentration the germination of the all varieties was completely inhibited.

Effects of Drought Stress on Shoot Length

The effect of osmotic stress induced by PEG showed decreased shoot length with increased concentration. All the varieties showed common trend reduction rate in shoot length with increasing concentrations with exception at -2 bars PEG. The genotype Dharani has shown superior growth in shoot length at -2 PEG in comparison with control (Fig. 9). The longest shoot length was identified in Dharani (5.9 cm) at -2 bars PEG compared to other genotypes. At the concentration of -14 bars PEG all the genotypes shoot growth was completely inhibited.

Among all the genotypes screened, Dharani has low reduction in germination rate with increasing PEG concentration. Decreasing in growth rate with increasing osmotic stress was reported in several studies (Kulkarni and Deshpande, 2007; Aazami *et al.*, 2010). Under drought conditions roots are the primarily effected plant part than any other parts (Ghafoor, 2013). Dharani has shown low reduction in root length among the genotypes screened for drought stress and the genotypes TPT 3, JL24 and Kadiri 6 were highly affected at higher PEG concentration. Reduction in root and shoot length with increasing osmotic stress was identified in different crop plants (Jajarmi *et al.*, 2009, Basha *et al.*, 2015). There are significant genotypic variations in response to drought and their tolerance

levels in groundnut as reported by Azevedo Neto *et al.*, 2010; Sarkar *et al.*, 2014; Vaidhya *et al.*, 2015. On the basis of germination percentage, root and shoot length parameters under drought stress imposed by PEG-6000, the promising genotypes were Dharani, Narayani and Kadiri-9. This helps to find out the optimized concentration for quick screening of large number of groundnut genotypes. Further investigations on physiological, biochemical studies will be helpful in selecting drought tolerant genotypes.

CONCLUSION

The study has clearly indicated the scope of using RAPD markers for varietal differentiation and diversity assessment at molecular level. Genotype specific amplification profiles observed with specific primers would help in the identification of the genotypes resistant to biotic, abiotic stress and agronomically important characters. Development of tolerant genotypes is one of the important approaches to overcome the problem associated with the drought stress. The current study reveals the appropriate optimized concentration of PEG for screening the groundnut genotypes which could be useful further physiological studies. The results highlight the importance of the PEG as an artificial stress inducer for quick and efficient screening in the laboratory conditions for identification of drought tolerant genotypes for breeding programs in groundnut.

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