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Combined effect of osmotic stress induced by polyethylene glycol-6000 and alcohol on *in vitro* growth of *Vigna aconitifolia*

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Abstract

The present study wass conducted to determine the reliability of in vitro screening method. In vitro screening method proves to be an ideal method for screening large set of germplasm with less efforts accurately and cost effective. A set of 12 genotypes of moth bean were evaluated for desiccation tolerance at seedling stage against two chemical (PEG and alcohol) to check their response. Differential response was observed at 15% PEG and 1% ethanol on seedling level respectively. The twelve varieties were classified as tolerant, moderatly tolerant and sensitive based on number of seedlings showing wilting symptoms at seedling stage at 15% PEG and 1% alcohol. Variety RMO-435, RMM-12-Single, RMM-12-Poly and CZM-105 were considered tolerant showing 14 to 19% wilted seedlings. Varieties RMM-12-Zero, RMM-12-Double, RM0-423, CZM-18 were considered moderate tolerant with 19 to 42% wilted seedlings. Varieties showing more than 72% wilted seedlings (RMO-225, RM0-40, RMO-257, and CZM-04-01) were considered sensitive.

Key words: Genotypes, osmoticum, *Vigna aconitifolia,* drought stress.

Water deficit is the most limiting factor on plant growth, development and yield. As a result of global climate change, the negative impact of drought on yield is expected to increase in future, especially in combination with a rise in temperature (Marchese *et al*., 2010). Therefore, there is an urgent need to make plants more tolerant to such conditions. Moth bean (*Vigna aconitifolia* (Jacq.) (Marechal) is an important grain-legume, grown in rain-fed areas of hot desert regions of Thar, under scorching sun rays with minimum supply of water. It adapts to extreme ecological niches particularly, in areas receiving scanty rainfall with erratic distribution. Due to its harsh natural habitat it has evolved few morphological and physiological features imparting tolerance to extreme drought stress (Kumar and Singh, 2002). Hence, the present investigation was emphasized on screening of genetically diverse moth bean genotypes based on their response under water deficit.

Materials and Methods

Plant material and chemicals

The experimental material for the present investigation consisted of 12 (RMO-423, RMO-435, RMO-40, RMO-225, RMO-257, RMM-12-Single, RMM-12-Double, RMM-12-Poly, RMM-12-Zero, CZM-105, CZM-04-01 and CZM-18) moth bean genotypes, which were collected from Agricultural Research Station, Swami Keshwanand Rajasthan Agricultural University, Bikaner.

Preparation of PEG-6000 and ethanol solutions

Fifteen per cent PEG-6000 solutions were prepared by 15 g PEG-6000 dissolved in 100 ml distilled water. 1 per cent ethanol solutions were prepared by 1 ml ethanol dissolved in 99 ml distilled water. Borosil grade glasswares were used for all the experiments. Oven dried (250°C)

Erlenmeyer flasks, pipettes, petridishes, beakers, volumetric flasks and measuring cylinders (10, 25, 50, 100, 500 and 1000 ml) were used for media preparations.

For sterilization, the culture medium and PEG solution was poured in flasks and plugged with cotton and wrapped with aluminum foil. Autoclaving was done at 15-16 psi (1.06 kg/cm^2) for 18 min and stored at 27 ± 2°C.

Nutrient media

Throughout the course of investigation, Murashige and Skoog (1962) medium was used for plant tissue culture. Stock solutions of various MS medium constituents were prepared separately and stored in refrigerator. To prepare a given quantity of medium, stock solutions were mixed proportionately and the final volume was made after dissolving the sugar and agar. pH of MS medium was adjusted to 5.8-6.0 using 1N NaOH or 0.1N HCl.

Determination of appropriate concentration of PEG-6000 and alcohol for Screening of germplasm

PEG-6000 and alcohol was used to induce water stress under laboratory conditions. For *in vitro* screening, seeds were first surface sterilized using 0.1% $HgCl_2$. Then seeds were grown for 7 days to obtain seedlings on 1/4 MS (Murashige and Skoog, 1962) liquid media. In order to evaluate for tolerance level at seedling stage these seedlings were grown for 7d under normal condition and were shifted to different combination of PEG-6000 (5%, 10%, 15% and 20%) and ethanol (0.5%, 1% and 1.5%) concentration for creating drought conditions.

After 7d of treatment, control and treated plants were used for various measurements. All plant materials were cultured in growth chambers at 26°C, 70 % humidity, 16/8 h light/ dark photoperiod and 2500-3000 lux light intensity.

Nine different sets of experiments were performed to check the reproducibility of results. Data were recorded for root length, shoot length, fresh and dry weight of shoot and root. In each experiment, rank was assigned to the genotypes as selection criteria to pick tolerant and susceptible varieties for gene expression studies.

Measurement of growth

The shoot and root lengths (cm) were measured on seedlings after 7 days of PEG treatment with the help of meter scale. Fresh weight of the root and shoot samples was taken with the help of weighing balance (Mettler Toledo, USA) immediately after taking them out of test tubes and wiping out the moisture with paper towel. The roots and shoots were oven-dried at 80°C for 48 h in order to take their dry weight.

Results

Treatment of 20% PEG to seven days old seedlings inhibited growth, induced chlorosis, wilting, burning of roots and leaves in all twelve varieties however, at different periods of treatment. Differential visible effect of 15% PEG was considered suitable for further growth and metabolic studies. Leaf shrinking, yellowing of root tips, leaf curling, diffused growth of roots were the main symptoms observed after seven days of 15% PEG treatment (Fig. 1). There was no effect of 0.5% ethanol at plant growth. At 1.5% ethanol most of plants showed wilting, at 1% ethanol differential visible effect was found suitable for further growth and metabolic studies (Table 1). There was no significant change observed after 1 day of stress treatment, while on 3rd day some seedlings started leaf curling, yellowing of leaves etc. On 5th day browning of roots was observed in RMM-12- double while RMO-423 showed blackening of roots. On 7th day of 15% PEG + 1% ethanol treatment, most plants showed differential visible effect so optimized for screening. More wilting was shown on 9th day by all the varieties (Table 2). Symptom development rate was high and appeared on lower concentrations of PEG and alcohol in susceptible genotypes in comparison to tolerant. The twelve varieties were classified as tolerant, moderately tolerant and sensitive based on visual observations. Variety RMO-435, RMM-12-Single, RMM-12-Poly and CZM-105 were considered tolerant showing 14 to 19% wilted seedlings. Varieties RMM-12-Zero, RMM-12-

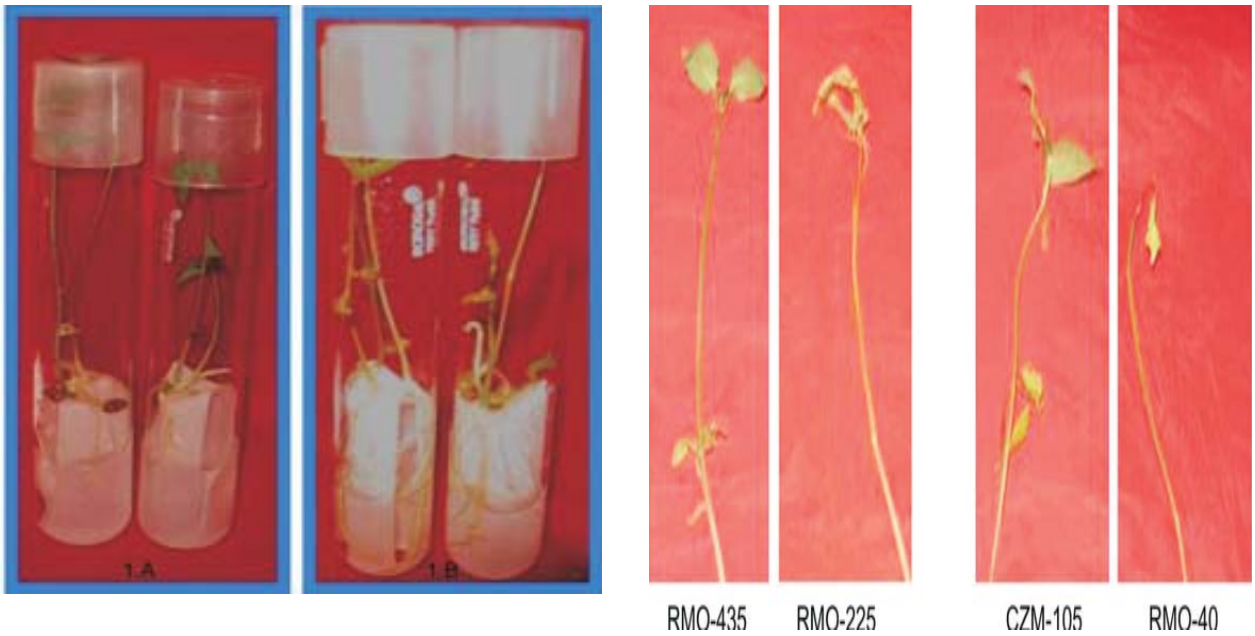

Fig 1. Varietal specific differential effect on moth bean seedlings after 7 days of treatment of 15% PEG+1% ethanol. A-RMM-12-Single, B-RMO-257

Double, RM0-423, CZM- 18 were considered moderate tolerant with 19 to 42% wilted seedlings. Varieties showing more than 72% wilted seedlings (RMO-225, RM0-40, RMO-257, and CZM-04-01) were considered sensitive.

Drought stress reduced most of parameters like root length, shoot length, root fresh weight, shoot fresh weight, root dry weight and shoot dry weight by 43, 36, 36, 38, 45 and 30%, respectively, in *Vigna aconotifolia* plants (Table 3).

Discussion

Drought is one of the most common environmental stresses that affects growth and development of plants (Aslam *et al.*, 2006). The selection of tolerant genotypes having high yield in drought stress conditions is important to enhance yield in crops (Richards *et al.*, 2002). Moth bean is a major crop of arid zone with high tolerance against drought. Water stress often occurs during crop establishment and seedling growth either directly due to low availability of soil moisture or from osmotic effects associated with drought. Studies on this crop are therefore expected to improve our understanding of the effect of water and drought stresses during germination and seedling growth. Generally, evaluation and identification systems of drought-tolerant plants include field testing, controlled drought stress and natural desiccation method (Oya *et al.*, 2004). Field based screening is simple and reliable but is restricted by the season, labor-demand and needs controlled drought stress. However, chemical identification method with polyethylene glycol (PEG-6000) to make osmotic stress overcomes the short coming of field screening. Various research groups have been using high osmotic solutions such as PEG-6000 to screen and evaluate the genotypes. PEG-6000 is a non-ionic water soluble polymer, which does not penetrate to intact plant tissues rapidly and is widely used to induce water stress in higher plants (Nepomuceno *et al.*, 1998). It is known to stimulate drought stress in a way similar to soil drying (Larher *et al.*, 1993) and several authors have reported use of PEG-6000 for *in vitro* drought screening in crop plants (Gopal and Iwama, 2007).

Additionally, intracellular water activity is also reduced by chaotropic compounds, such as ethanol that decreases strength of hydrogen bonding and other electrostatic interactions thereby perturbing the interaction and function

Table 1. Effect of different concentration of PEG-6000 (5-20%) and 1% ethanol at seedling level on the growth and development of mothbean after 7d of treatment

Genotypes	5% A+1% B	10% A+1% B	15% A+1% B	20% A+1% B
RMO-435	No effect	No effect	Leaf shrinking, Initial symptoms of growth retardation	Chlorosis, Growth retardation
RMM-12-Single	No effect	No effect	Leaf curling	Drooping
CZM-105	No effect	No effect	Chlorosis	Complete yellowing
RMM-12-Poly leaves	No effect	No effect	Burning of root tips	Buring of root and
RMM-12-Double	No effect	No effect	Diffused growth of roots	Wilting
RMM-12-Zero	No effect	No effect	Shrinking of leaves	Inhibited growth
RMO-423	No effect	No effect	Leaf curling	Leaf shrinked
CZM-18	No effect	Initial stage of chlorosis	Yellowing of leaves	Wilting
RMO-225	No effect	No effect	Yellowing of root tips	Inhibited growth
RMO-40	No effect	No effect	Growth retardation	Drooping
RMO-257	No effect	Leaf curling	Browning of roots	Growth retardation
CZM-04-01	No effect	Leaf curling	Browning of roots	Growth retardation

Table 2. Day-wise effect of 15% PEG-6000 and 1% ethanol, on growth and development of 7d old seedlings of moth bean genotypes

Genotypes	1std	3rdd	5thd	7thd	9thd (% Wilting)
RMO-435	No effect	No effect	No effect	Chlorotic leaves	40%
RMM-12-Single	No effect	No effect	Shrinking of leaves	Yellowing of leaves, shrinking of leaves	45%
CZM-105	No effect	No effect	No effect	burning of root tips	50%
RMM-12-Poly	No effect	No effect	Burning of root tips	Drooping	60%
RMM-12-Double	No effect	No effect	Browning of roots	Chlorotic leaves	60%
RMM-12- Zero	No effect	No effect	Burning of leaf tips	Chlorotic leaves	80%
RMO-423	No effect	Burning of tips	Blackening of roots	Leaf curling	85%
CZM-18	No effect	No effect	No effect	Yellowing of leaves	65%
RMO-225	No effect	Yellowing of leaves	Chlorosis	Drooping, growth retardation	98%
RMO-40	No effect	Leaf curling	Burning of tips	Wilting	100%
RMO-257	No effect	No effect	yellowing of tips and blackening	Shrinking of leaves, retarding growth of roots	
CZM-04-01	No effect	Shrinking of leaves	Drooping	Yellowing of leaf tips	97%

Table 3 Effect of PEG-6000 and 1% ethanol treatments on root and shoot length, dry and fresh weight of shoot and root after 7d of seedlings of genotypes of mothbean

Parameter	Mean	Range		SD	Mean	Range		SD	Range of % increase/decrease due to treatment	
		Min	Max			Min	Max		Min	Max
RL	10.7	8.3	13.7	1.6	9.2	6.1	12.9	1.3	-42.6	4.9
SL	10.8	7.3	15.3	0.9	8.9	5.3	14.1	0.9	-35.5	-4.0
RFW	296.1	170	533.3	10.4	254.8	136.3	480	10.9	-35.6	-2.4
SFW	694.9	613.3	806.7	11.3	611.3	440	730	10.4	-38.1	4.4
RDW	71.4	62	94.7	6.2	93.4	36.7	181.3	11.1	-45.3	132.1
SDW	223.6	170	306	10.1	290.6	123.3	553.3	9.6	-30.2	102.6

of hydrated macromolecules such as nucleic acid, protein and lipids.in our study, we termed 15% PEG+1% ethanol mimics cellular stress of drought.

In crops like chickpea (Mbarek *et al.,* 2013) and bread wheat (Geravandi *et al.,* 2010) researchers have attempted to identify the lines which adapt to drought at germination and at seedling stage (Yagmur *et al.,* 2008) under variable stress conditions. This study sheds light on the relationship between drought stress and its effect on morpho-physiological (root length, shoot length, fresh and dry weights of shoots and roots) parameters of *Vigna aconitifolia*.

All the genotypes show reduction in growth in response to PEG and ethanol induced stress. Though, root and shoot length were reduced in all the genotypes the length under unstressed condition was more and reduction under stressed condition was comparatively less in tolerant genotypes identified on the basis of wilting percentage except CZM-18 which showed greater reduction in both root and shoot length although it was selected as moderately tolerant genotype. Water deficit is referred to reduce plant growth under drought stress in pearl millet (Kusaka *et al*, 2005). Drought stress decreased the root length in various plant species such as wheat and maize (Nayar and Gupta, 2006). The basal level of root length was also high in tolerant and moderately tolerant genotypes as compared to sensitive genotypes except in RMO-40 implying that root length is important for a plant to exploit the available water. Root length is known important trait in selection of drought resistant variety. According to the Imanparast and Hassanpanah, (2009) genotypes that had good coleoptiles length had excess germination percentage too and seeds had good root growth could have better settlement and had high yield under insufficient environmental condition. Growth parameters like fresh and dry weights are known to have a profound effect on water limited conditions. In the present study, a reduction in root and shoot fresh weight was recorded in stressed conditions in all the genotypes although a marginal increase in root fresh matter (5.26%) was reported in var. RMO-423. However, basal (unstressed control) level of fresh mass was more in tolerant varieties as compared to sensitive varieties. Most of the tolerant genotypes accumulated more dry matter under control that too increased under stress (15% PEG). However, moderately tolerant or susceptible genotypes recorded comparatively less increase or even decrease in dry weight of roots and shoots. Moreover, the ratio of dry matter over fresh weight also increased in most genotypes increase being much higher in tolerant genotypes both for root and shoot.

All the growth parameters *viz.* root length, shoot length, root fresh weight, shoot fresh weight, root dry weight, shoot dry weight, were significantly correlated with each other and with wilting resistant percentage recorded for stress condition. Similar results were obtained for unstressed condition also.

Thus, it is evident that *in vitro* screening can be used as an efficient tool to screen a large number of accessions or breeding lines for their drought tolerance. The results of the present study form an excellent guideline for *in vitro* screening of moth bean cultivars for abiotic stress like drought tolerance.

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