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Research Article

In Vitro Regeneration of Soybean Genotypes and Induction of Drought Stress *In Vitro* Condition and Influence on Secondary Metabolites in Soybean Callus Culture

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Abstract

Soybean (*Glycine max L* Leguminosae) is an important grain legume that is not only a valuable oil seed crop but also used as feed for livestock and aquaculture. Soybean genotypes viz., CO-1 and JS 335 were used for the study of drought-induced variations in growth. The present investigation was undertaken to study the effect of two concentrations of PEG on callus induction of soybean genotypes. Seeds of soybean were inoculated on MS medium supplemented with two different concentrations of PEG (2% and 4%) cultures were incubated at 26 ± 2°C under 16h photo periods. The effect of different concentration of PEG on callus induction was investigated. *In vitro* callus cultures of both genotypes (CO-1and JS 335) showed a reduction in callus growth during PEG treatment as compared with the control. The presence of PEG in the medium elevated dry matter content in all treatments compared with the control. Similarly flavonoid levels and phenolic contents were higher in the PEG treatments in comparison to control. Our results can be used for *in vitro* screening and manipulations of soybean cultivars for improvement of drought tolerance.

Keywords: *Glycine max*; Flavonoid; Total Phenolic; Polyethylene Glycol (PEG); Relative Growth Rate; Dry Matter Content; Drought Tolerance

Introduction

Drought is one of the difficult ecological variables influencing development and yield of soybean in bone-dry and semi-dry zones of the world. This issue is exaggerated by the conceivable world-wide environmental change situations (IPCC 2001). For example, Simon [1] reported that returning drought because of lack of rain-fall in semi-dry and dry sub-humid zones of the country is one of the significant reasons of reduced production of sorghum. Plant tissue culture is one of the useful tool for the study stress tolerances mechanism under the *in vitro* condition as well as providing proficient method for understanding plant genetic procedures in brief time frame in a controlled environment [2]. Wani., *et al.* [3] reported that *in vitro* culture of tissue and plant cell has obtained significant interest over years because it not only provides the way to study plant physiological characteristics and genetic processes but also help in the breeding of upgraded cultivars by increasing genetic variability. *In vitro* culture systems reduce environmental variants because of defined nutrient media, controlled conditions and homogeneity of stress application. For *in vitro* drought stress enlistment, a standout amongst the most prevalent methodologies

is to utilize high molecular weight osmotic substances, like PEG. Muhammad., *et al.* [4] reported that PEG 6000 a nontoxic osmotic and non-penetrable substances which is used to lower the water capability of the culture medium and it has been used to pretend drought stress in cultured plant tissues. The main objectives were to design an *in vitro* regeneration system for soybean genotypes used. Therefore, the present work was conducted to find the effect of PEG on the cellular phenolic and flavonoids levels so that the ability of callus to produce higher content of their metabolites could be established. This study has implication in selecting the calli for regenerating the plants for higher content of secondary metabolites and also for drought resistance.

Material and Methods

Explants preparation

The seeds of the soybean genotypes (JS-335 and CO-1) were provided by GKVK Bangalore, Matured seeds of soybean cultivars were surface sterilized with distilled water for three to four times followed by 10 min in 1% Bavistin and rinsed with sterile water for four to six times. Wash with 70% ethanol for 2 min followed by 2 min in 0.1% mercuric chloride and rinsed with sterile distilled water for five to six times.

Sterilization of culture media and culture condition

MS medium (Murashige and Skoog 1962) with sucrose (30g l^{-1}) was used as the basal medium and was supplemented with different concentration and combination of plant growth regulators for different types of responses as per the requirement. The pH of the medium was set to 5.6-5.8 (Mann., *et al.* 1982) using a pH meter. For solid medium, tissue culture grade agar (Hi-medium) at 0.8%(w/v) was added as a gelling agent, boiled and dispensed into 150ml flask or tissue culture bottles and sterilized at 121°c with a pressure of 15lb for 20 min [5]. Hormones or plant growth regulators that are added to the sterilized medium under sterile condition in a laminar air flow chamber. The cultures were maintained in a 16/8h (light/drak) photoperiod using the flurosecent light (Philips India Ltd., Mumbai, India) at 24 ± 2°C.

Callus induction

Matured seeds of soybean cultivars (JS-335 and CO-1) were surface sterilized with distilled water for three to four times followed by 10 min in 1% bavastin and rinsed with sterile water for four to six times. Wash with 70% ethanol for 2 min followed by 2min in 0.1% mercuric chloride and rinsed with sterile distilled water for five to six times. Disinfected seeds were kept for callus induction in MS [6] basal medium (pH 5.7) with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 2mg l^{-1} 2,4 D and 0.5 mg l^{-1} kinetin.

Effect of PEG and explant on in vitro regeneration

Matured seeds of soybean cultivars (JS-335 and CO-1) were surface sterilized with distilled water for three to four times followed by 10 min in 1% bavastin and rinsed with sterile water for four to six times. Wash with 70% ethanol for 2 min followed by 2 min in 0.1% mercuric chloride and rinsed with sterile distilled water for five to six times. Disinfected seeds were kept for callus induction in MS [6] basal medium (pH 5.7) with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 2mg l^{-1} 2,4 D and 0.5mg l^{-1} kinetin. All the cultures were maintained at 23 ± 1 ° C under 16 h illuminations. Drought was simulated by the addition of polyethylene glycol (molecular weight 6000) at concentrations of 2% and 4% (w/v) to the media. The cultures were kept for seven weeks to study their growth potential, percentage of dry matter of soybean callus, phenolic content and flavonoid content.

Relative growth rate and dry matter percentage

After seven weeks, the samples were analyzed for their relative growth rate (RGR) and dry matter percentage (DM). Five samples were analyzed for each treatment and the mean values were recorded. Callus RGR was calculated according to the following formula: RGR = (FW2 - FW1)/Number of days, where, FW1 is the fresh weight of the callus at the beginning of the test period and FW2 is the fresh weight of the callus at the end of the test period. The percentage of dry matter in callus tissues was estimated by the formula, DM = (DW2/FW2) X 100, where DW2 is the dry weight of the callus at the end of test period.

Preparation of the extracts for phenolic and flavonoid analyses

Callus cultures were dried in hot air oven at 70°C till constant weight was obtained. The dried material was powdered and weighed (1 gm) followed by extraction in 80% methanol. The extract was separated and evaporated in a crucible in oven and the same was stored in refrigerator until use. At the time of analyses the extract was redissolved in 80% methanol and made up to 1 ml to proceed with analytical procedure for phenolic or flavonoid.

Determination of total flavonoid content

Total flavonoid content was measured with the aluminium chloride colorimetric assay. 1ml of aliquots and 1ml standard quercetin solution (100,200,400,600,800,1000 µg ml^{-1}) was placed into test tubes and 4ml of distilled water and 0.3 ml of 5% sodium nitrite solution was added into each. After 5 minutes, 0.3 ml of 10% aluminium chloride was added. Finally, volume was adjusted up to 10ml with distilled water and mixed well. Orange yellowish color was developed. The absorbance was measured at 510 nm using UV-visible spectrophotometer. Distilled water was set as a blank. Quercetin was used as standard. The samples were performed in triplicates. The calibration was plotted using standard quercetin [7].

Determination of total phenol content

The total phenolic content was determined as described by Velioglu., *et al.* [8] with slight modifications. An aliquot (200 mL) of extract was mixed with 1.6 ml of FC reagent and 0.8 ml of 7.5% sodium carbonate. It was incubated for 120 min at 37°c the absorbance was measured at 740 nm against distilled water as blank. Total phenolic content was expressed as gallic acid equivalents (mg GAE/100g of soybean) through standard calibration curve of freshly prepared gallic acid. All measurements were conducted in triplicate.

Preparation of standard solutions

The standard stock solution (1000 µg ml^{-1}) of quercetin and Gallic acid (100 µg ml^{-1}) were prepared by dilution in absolute alcohol and water respectively. The dilute standard solutions of concentration (100-1000 µg ml^{-1} of quercetin and 20-100 µg ml^{-1} of gallic acid) were prepared from above stock solution.

Results and Discussion

In vitro regeneration of soybean genotypes

The cotyledons of soybean inoculated on MS basal media containing different level of Kinetin (1 mg l^{-1}, 2 mg l^{-1}, 3 mg l^{-1}) resulted in shoot multiplication in Table 1 (Figure 1) and activated charcoal media for root induction (Figure 2).

Callus culture

For callus induction hypocotyl explants from aseptically germinated seedlings of varieties (JS-335, CO-1) were placed on MS basal medium supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin

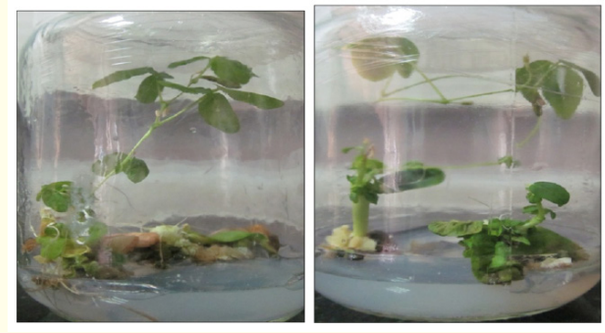

Figure 1: *In vitro* multiplication of soybean variety (JS-335 and CO-1) with shooting stage.

Figure 2: *In vitro* multiplication of soybean variety (JS-335 and CO-1) with rooting stage.

Explant No	No. of multiple shoots at different levels of kinetin					
	JS-335			CO-1		
	1mg l^{-1}	2 mg l^{-1}	3 mg l^{-1}	1 mg l^{-1}	2 mg l^{-1}	3 mg l^{-1}
Average no. of shoots/ explants	1.7 ± 0.67	2.8 ± 1.03	1.8 ± 0.78	1.5 ± 0.70	2 ± 0.94	1.8 ± 0.78

Table 1: *In vitro* multiple shoot induction in soybean genotypes at different levels of Kinetin.

Treatment	No of explants inoculated	JS-335			CO-1		
		No of plant responded	Plant height	No of shoot/ plant	No of plant responded	Plant height	No of shoot/ plant
Control	40	35	11.92±2.5	3.2±0.78	28	7.98±1.27	2.3±0.48
2%PEG	40	24	7.92±1.11	1.7±0.48	14	5.26±1.28	1.4±0.51
4%PEG	40	16	2.92±1.00	1.1±0.32	11	1.4±0.33	1.2±0.46

Table 2: Effect of PEG 6000 on shoot bud induction from cotyledonary nodes* of cv. JS-335 and cv. CO-1

(Figure 3 and 4). All the cultures were maintained at 26±2°C under 16 h photo periods. Observations were recorded weekly for 4 weeks.

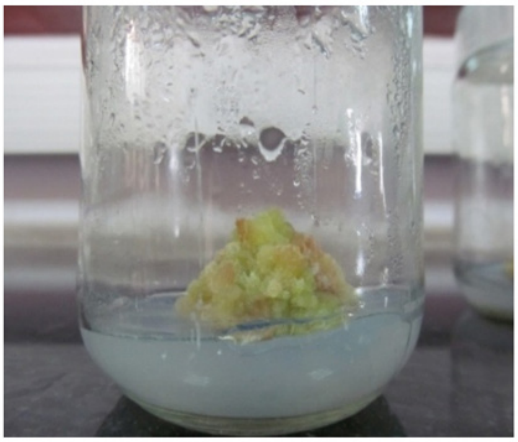

Figure 3: Callus culture of soybean (JS335).

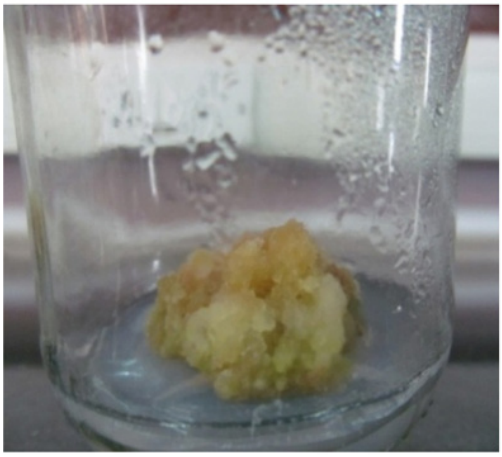

Figure 4: Callus culture of soybean (CO-1).

Selection of PEG tolerant genotypes

The callus cultures of JS-335 and CO-1 variety of soybean from MS medium with 2 mg l^{-1} 2, 4 D were transferred to 2% and 4% PEG 6000, and the resistant calli which tolerated the treatment (Figure 5 and 6). The PEG resistant calli are being now tried for relative growth rate and dry matter percentage of stress and non-stress callus culture.

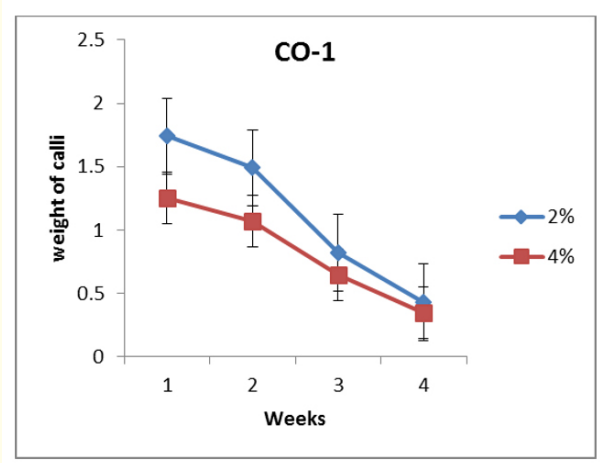

Figure 5: Response of soybean (JS-335 and CO-1) callus to different levels of PEG.

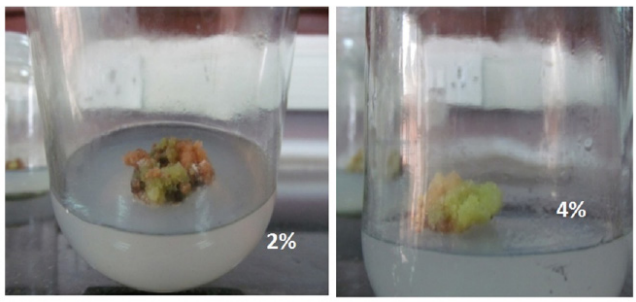

Figure 6: Callus inductions as differed across PEG treatment level.

Relative growth rate and dry matter percentage

The relative growth rate and dry matter percentage of stressed and non-stressed callus cultures of both genotypes are précised in figure 7 and 8. A significant decrease in the RGR of callus cultures was observed in genotype CO-1 with increasing PEG concentrations compared to the control (Figure 5). In both the PEG treat-

ments, CO-1 showed expressively lower RGR than JS-335. Higher percentage of dry matter (DM) was observed in JS-335 genotypes under non-stressed (control) culture conditions (Figure 7 and 8) whereas, when subjected to PEG treatments, both cultivars showed decrease DM percentage at PEG level compared to the control.

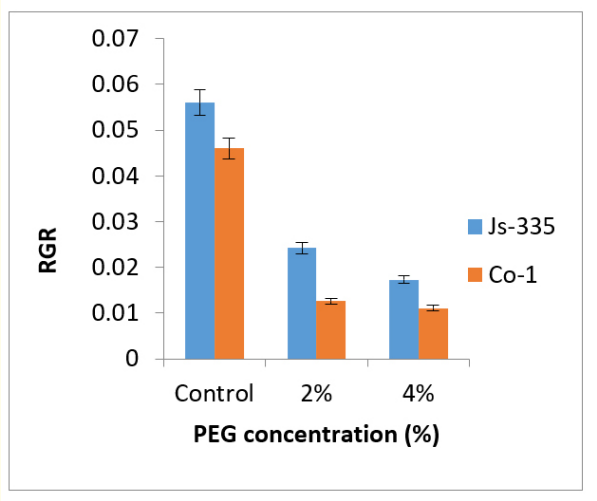

Figure 7: Relative growth rate.

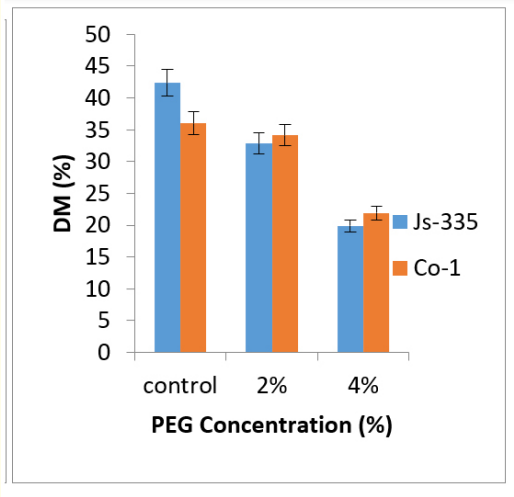

Figure 8: Dry Matter Percentage.

In vitro regeneration with PEG treatment

Disinfected seeds were kept for *in vitro* regeneration in MS (Murashige and Skoog, 1962) basal medium (pH 5.7) with 30 g l^{-1} sucrose, 8 g l^{-1} agar, 2 mg l^{-1} and 1% activated charcoal. All the cultures were maintained at 23 ± 1 ° C under 16 h illuminations. Drought was pretended by the addition of polyethylene glycol (Molecular weight 6000) at concentrations of 2% and 4% (w/v) to the media. This study shown differences in plant performance between the physiological and agronomical characteristics of variety of soybean JS-335 and CO-1 under the *in vitro* condition. The data in table 2, clearly shows that both the concentration of PEG resulted in significant decrease in shoot length and overall growth of soybean plants. (Figure 9).

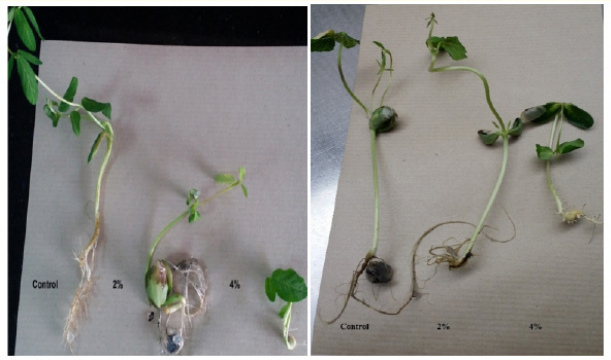

Figure 9: Effect of PEG on plant growth of cv. JS-335 and cv. CO-1.

Phenolic content in control and treated calli

The total phenolic content of methanol extracts of control and treated was presented in table 3 and 4 It is expressed in terms of GAE using the standard curve equation y = 0.008x + 0.0006, R^2 = 0.998. The total phenolic content was highest in treated PEG of both soybean genotypes compared to untreated soybean genotypes (Table 3 and 4).

Flavonoid levels in control and treated calli

The total flavonoid content of methanol extracts was expressed in terms of quercetin using the standard curve equation is y = 0.0665x + 0.002, R^2 = 0.990. The total flavonoid content was highest in treated PEG genotypes of soybean compared to untreated soybean genotypes (Table 5 and 6).

Soybean callus (JS-335) treated with PEG	TPC (mg of GAE/g)
*Control	17.6 ± 0.006
2%	22.7 ± 0.010
4%	18 ± 0.014

Table 3: Effect of PEG on total phenol content on soybean genotype.

(*without treatment of PEG).

Soybean callus (CO-1) treated with PEG	TPC (mg of GAE/g)
*Control	15 ± 0.012
2%	19.7 ±0.014
4%	16.2 ± 0.01

Table 4: Effect of PEG on total phenol content on soybean genotype.

(* without treatment of PEG).

Soybean callus (JS-335) treated with PEG	Total flavonoid (mg of Qurecitin /g)
*Control	0.44 ± 0.0031
2%	0.582 ± 0.0017
4%	0.599 ± 0.0051

Table 5: Effect of PEG on total flavonoid content on soybean genotype.

Soybean callus (CO-1) treated with PEG	Total flavonoid (mg of Qurecitin/g)
*Control	0.36 ± 0.0041
2 %	0.456 ± 0.0051
4 %	0.442 ± 0.0017

Table 6: Effect of PEG on total flavonoid content on soybean genotype.

Discussion

The present investigation was undertaken to study of Drought-induced variation in growth of soybean cultivars with different concentrations of polyethylene glycol (2% and 4%). Gopal and Iwama [9] has reported that the accumulation of PEG to the MS medium decreased the water potential of the media inducing water stress that negatively affected the callus growth of the soybean cultivars. Matheka., *et al.* [10], Biswas., *et al.* [11] and Abdel-Ghany., *et al.* [12] studied *In vitro* selection and characterization of drought tolerant somaclones of tropical maize they have found that callus induction efficiency decreased significantly in genotypes under higher PEG levels. Decrease in callus induction efficiency is a typical response of explants of crop genotypes when subjected to PEG stress. Sakthivelu., *et al.* [13] studied Drought-induced alterations in growth, osmotic potential and *in vitro* regeneration of soybean cultivars. The response of callus of soybean genotypes subjected to PEG with increase in phenolics and flavonoids indicate that the plants adopt to drought stress with the enhanced production of their secondary metabolites. JS335 had higher phenolics and flavonoids in addition to higher growth rate. Hence invitro screening of germinating of soybean under PEG will be an indicator of drought tolerance. This technique may be applicable in future to select efficient drought tolerant line of agriculture importance.

Conclusion

In conclusion, the soybean cv. JS 335 showed superior resistance towards PEG-induced water stress compared to cv. CO-1 hence; it is obvious that *in vitro* screening can be used as a resourceful tool to screen a large number of successions or breeding lines for their drought tolerance. Our results can be used for *in vitro* screening and controls of soybean cultivars for development of drought tolerance.

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