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Research Article

In vitro Callus Regeneration and Chlorophyll Content Estimation in *Glycine max (L.)* variety from Uttarakhand, India

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Abstract

Soybean (*Glycine max)* is considered one of the most substantial produced crops across the globe because of its high nutritional value. It is a great alternative for lactose-intolerant patients and vegans to fulfil their daily protein requirements. Soybean has various varieties depending upon the colour of their seed coat. In the past few years, the consumption of Soybean has increased which demands higher production, better yield, and better seed quality. Conventional propagation methods fail to fulfil such demands. The alternate method of plant tissue culture ensures rapid mass propagation, better yield, and quality of the plants. However, the technique is often beset with challenges of low-field performance of tissue culture-raised plants due to defective chloroplast machinery. The present study investigates the effect of various plant growth regulators (PGRs) on *in vitro* propagation of soybean cultivars from different regions of Uttarakhand, India, and their effect on chlorophyll content in the regenerated tissues.

Keywords: Callus induction, Chlorophyll content, Carotenoids, *Glycine max*, Mass propagation

Introduction

Soybean is a major food crop belonging to East Asia. Soybean scientifically called *Glycine max (L.)* Merr. falls under the family Fabaceae and subfamily Papilionaceae. The seed coat colour of the Soybean seeds ranges from light/pale yellow to dark/black colour. The colour difference is considered because of the presence of anthocyanins, chlorophyll, and various pigments (Naresh et al., 2019).

Soybean was introduced by China in India in the tenth century past Himalayan routes as well as by Indonesia via Burma (now Myanmar). The Kumaon region of Uttarakhand, Himachal Pradesh, the Naga Hills, Eastern Bengal, Madhya Pradesh, and the Khasi Hills are known to cultivate Soybean ages on a very small scale (Hymowitz, 1990; Khoshoo, 1995; Agarwal et al., 2013). According to Parmar & Devi (2021) during the year 2018-19, India produced approximately 11 metric tons of Soybean, ranking fifth in the worldwide ranking of soybean production after China.

Earlier soybean was essentially used for its high oil content (18-22%) (Naresh et al., 2019) but now apart

from high oil content, soybean is also a high protein source (40-42%) (Naresh et al., 2019). Nowadays, vegan products are high in demand and soybean products like Soy milk, "Tofu", "Miso", and Soy sauce are in great demand for being an alternative to dairy products also, soybean and their leftovers can be utilized in multiple ways such as leftovers of soy after oil extraction are used to feed animals. Soybean oil has been used in cooking for ages, apart from that various uses of soybean oil are in cosmetics, varnishes, pharmaceuticals, paper, paints, inks, and pesticides (Pratap et al., 2012), and the oil can also be used as biodiesel, a renewable energy source in industries (Pratap et al., 2015). Soybean has high antioxidant properties, possesses anti-hypersensitive effects, aid in diabetes, maintain cholesterol levels, and prevent cancer.

Need for tissue culture of soybean

The demand for Soybean has increased rapidly in the past several decades leading it to become the fourth-largest oil crop in the world and it has been estimated that the demand will increase in the upcoming years for food, fuel, and feed (Raza et al., 2017). Several measures are being taken to improve the quality of the crop to curb the demand of rising global hunger via conventional breeding but the efforts are not as fruitful.

Plant tissue culture is one such biotechnological tool that has helped in refining the quality of Soybean plants. Rigorous research has helped in successfully establishing the tissue culture of Soybean via different explants such as seed/embryo, hypocotyl/epicotyl, cotyledons, leaf disc culture, etc.

Presence of chlorophyll in soybean callus

Chlorophyll is a green pigment found in plants that is essential for carrying out photosynthesis in plants by absorbing sunlight. Chlorophyll is found in five different forms among various organisms. Chlorophyll a and b are majorly seen in ferns, mosses, and higher plants while chlorophyll c, d, and e are found mostly in some bacteria and algae. Another pigment commonly known as carotenoid is also present in plants and fruits and imparts red, yellow, or orange colour. Carotenoids aid in passing absorbed light to chlorophyll and protect the chlorophyll from photo-oxidative destruction (Sudhakar et al., 2016). Since the plant tissue culture technique involves growing plantlets under controlled physicochemical conditions over a long period, more often the regenerated plantlets exhibit problems in chlorophyll synthesis. It, therefore, becomes important to establish a method that ensures improving chlorophyll content in regenerated plants.

The present study focuses on establishing contamination-free seed cultures of *Glycine max* varieties collected from different villages of Uttarakhand and the assessment of various pigments present in the *in vitro* generated callus.

Materials and Methods

Explant collection and treatment

Soybean seeds (Figure 1) were collected from different districts of Uttarakhand, India, and annotated as SC1 and SC2 from the Chamoli district, SP1 and SP2 from Pauri (Garhwal) district, SR1 and SR2 from Rudraprayag district, and ST1 and ST2 from Tehri district.

Figure 1: *Glycine max* seeds. Seeds with yellow coat (a), seeds with black coat (b).

Seeds were washed underneath tap water for 3-5 minutes to ensure that seeds were dust and dirt-free. Alcohol (70%) treatment was given to the seeds for 5 minutes, then rinsed with tap water 3-5 times. Liquid detergent (Teepol) treatment was given to the seeds for 10-15 minutes and then rinsed underneath tap water, seeds were then treated with Tween-20 for 15-20 minutes and rinsed thoroughly under running tap water. Fungicide (1% Bavistin) treatment was given to the seeds for 20-25 minutes and then rinsed under tap water. Seeds were surface sterilized thoroughly with 0.1% mercuric chloride ($HgCl_2$) for different durations to achieve contamination-free cultures and finally were rinsed with autoclaved distilled water.

Culture initiation and conditions

After surface sterilization, seeds cultures were established on a culture medium (MS medium) consisting of a carbon source (3% sucrose), gelling agent (6 g w/v agar), and various PGRs at different concentrations such as cytokinin (0.5-1.0 mg/l) BAP for culture establishment. The pH of the media was set at 5.8 with NaOH (1N) or HCl (1N). The nutrient medium was autoclaved at 121℃ temperature, 15 psi pressure for 15 minutes. Cultures were maintained at 25±2℃ with 60-65% relative humidity (RH) under 16/8 light-dark conditions in the culture room.

To initiate callus, *in vitro* cotyledonary leaves of generated seeds (SC1, SC2, SP1, SP2, SR1, SR2, ST1, and ST2) were inoculated on MS medium supplemented with 3% sucrose, 6g w/v agar, and different amount of PGRs. PGRs used in callus induction were BAP (0.5-2.0 mg/l), Kn (0.5-1.0 mg/l), and 2,4-D (0.5-1.0 mg/l).

Callus multiplication

Callus initiated from *in vitro* cotyledonary leaf was further multiplied by transferring it onto MS medium consisting of 3% sucrose, 6 g w/v agar, and different PGRs at various concentrations and various amalgamations. PGRs included in the media were BAP (0.5-1.0 mg/l), IAA (0.5-1.0 mg/l), Kn (0.5-1.0 mg/l), TDZ (0.5-1.5 mg/l), NAA (0.5-1.0 mg/l), and 2,4-D (0.5-1.0 mg/l).

Quantification of chlorophyll and carotenoids

Multiplied callus of the seed varieties generated *in vitro* were tested for the presence and concentration of chlorophyll a, b, and carotenoids. Chlorophyll is loosely bound to proteins and thus, can be extracted easily in the presence of organic solvents such as DMSO (Dimethyl sulfoxide), acetone, or ether. The samples were macerated and 80% acetone was added to the macerated sample so that chlorophyll would dissolve in it. For maximum absorption of chlorophyll(a) the suitable wavelength is 663nm and for chlorophyll(b) is 645 nm. The OD (optical density) of the samples was taken at these wavelengths for the estimation of chlorophyll(a) and (b) content in the samples (Arnon, 1949). For carotenoids, the suitable wavelength is 663 nm, 645 nm, and 480 nm (Price & Hendry, 1991; Kamble et al., 2015). The formulas used to estimate the content of pigment are shown in Figure 2.

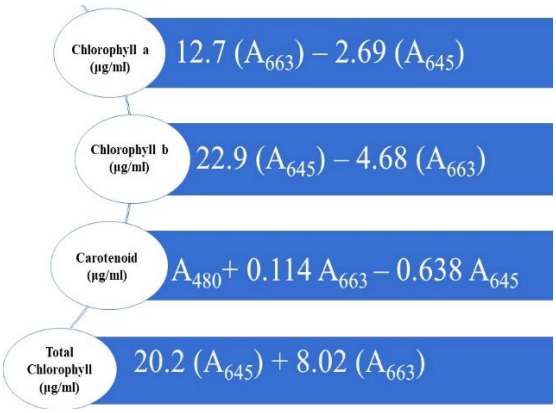

Figure 2: Formulas for the estimation of pigment content in the callus.

Results and Discussion

Callus induction

In the current study, seed samples of different varieties of Soybean were collected from various districts of Uttarakhand, India. The seed samples were inoculated on the nutrient medium to obtain cotyledonary shoots and leaves from the seeds. Surface sterilized seeds were inoculated on a nutrient medium. The best media for cotyledonary shoot induction was found to be MS + BAP (1.0 mg/l) as shown in Figure 3a, 3b and 3c. Raza et al. (2017) reported that the best seed germination medium for *Glycine max* was Gamborg B5 basal medium supplemented with BAP (1 mg/l).

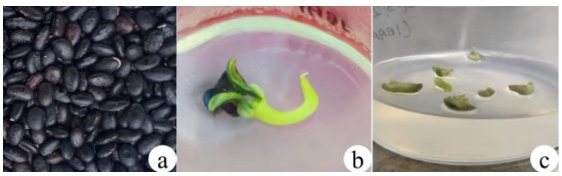

Figure 3: Cotyledonary shoot induction and inoculation for callus induction. *Glycine max* seeds (a), cotyledonary shoot formation from seed (b) and cotyledonary leaves inoculation (c).

In vitro cotyledonary leaves obtained from seed samples SC1, SP1, SP2, and ST1 were inoculated on a culture medium to induce callus. It was concluded from the present study that various PGRs have different effects on callus induction as well as on the colour of the callus.

Table 1: Different media combinations for callus induction and its properties.

BAP (mg/l)	Kn (mg/l)	TDZ (mg/l)	2,4-D (mg/l)	IAA (mg/l)	NAA (mg/l)	Degree of callus				Colour of callus			
						SC1	SP1	SP2	ST1	SC1	SP1	SP2	ST1
-	-	1	-	-	-	+				Pale green			
1	-	-	-	0.5	-	++				Light green			
-	-	-	0.5	-	-	+++				Dark green			
1	-	0.5	-	-	-	++				Light green			
1	-	1	-	-	-	+				Green			
1	-	-	1	-	-	+++				Dark green			
0.5	-	-	-	-	-		++				Light green		
1	-	-	-	-	-		+				Pale green		
1	-	-	-	0.5	-		+++				Dark green		
-	-	-	0.5	-	-		++				Light green		
1	-	1	-	-	-		+				Pale green		
-	-	0.5	-	-	-			++				Light green	
-	-	1	-	-	-			++				Light green	
0.5	-	-	-	-	-			+				Pale green	
1	-	-	-	-	-			++				Light green	
1	-	-	-	0.5	-			+				Pale green	
-	-	-	0.5	-	-			++				Light green	
1	-	1	-	-	-			+++				Dark green	
1	-	0.5	-	-	-			++				Green	
1	-	-	1	-	-			++				Green	
1	0.5	-	-	-	-				+				Pale green
1	1	-	-	-	-				+				Pale green
-	-	1	-	-	-				+				Pale green
0.5	-	-	-	-	-				+				Pale green
1	-	-	-	0.5	-				+				Green
1	-	1	-	-	-				++				Green
1	-	-	1	-	-				++				Green
1	-	-	-	-	-				+++				Dark green
1	-	1	-	-	-				+++				Dark green

For the cotyledonary shoot induction, various combinations of PGRs were tried as shown in Table 1, the best media was found to be MS medium containing BAP (1.0 mg/l) + 2,4-D (1.0 mg/l) for SC1, BAP (1.0 mg/l) + IAA (0.5 mg/l) for SP1, BAP (1.0 mg/l) + TDZ (1.0 mg/l) for SP2, and BAP (1.0 mg/l) for ST1.

The various PGRs had different effects on the colour of the callus, the callus obtained had varying shades of green colour from dark green to pale green as shown in Figure 4a, 4b and 4c. In a study conducted by Kim & LaMotte (1987), 77% of embryoids from cotyledon explant were generated in the presence of 4.5 µM Dicamba when cultured on MS medium, consisting of 2% sucrose. It was reported by Radhakrishnan & Ranjithakumari (2007) that callus induction from the half-seed of Soybean when cultured on B_5 medium consisting of a variety of PGRs such as 2, 4-D, BAP, Kn, NAA, and IAA. B_5 medium supplemented with 13.3 µM BAP+ 13.5 µM 2, 4-D was proven best to induce callus, and B_5 medium containing 13.3 µM BAP gave the best results for plant regeneration. Ether et al. (2013) reported the best callus induction in *Glycine max* from half seed explants on MS medium augmented with BAP (2 mg/l) + 2,4-D (5 mg/l). In a study conducted by Faraz et al. (2019), callus was successfully induced from the cotyledonary node and leaves of *G. max* when cultured on MS medium containing 2,4-D (0.5-4.5 mg/l) with Kn (0.2-2.5 mg/l), NAA (0.1-5 mg/l) and BAP (1-2 mg/l). Maximum callus weight was observed in 2,4-D (4 mg/l) in the leaves (0.73 g) and cotyledonary node (0.8 g). Apart from this medium supplemented with BAP + NAA at 2 mg/l and 3 mg/l concentration also resulted in the highest callus weight. Furthermore, callus culture has shown positive results in the development of somaclonal variations in the Soybean for the development of a better-quality variety that can be stress-tolerant or might have raised nutrient content (Singh et al., 2020). Callus

was multiplied by sub-culturing on MS medium after every 15 days. Cytokinin alone or in combination with auxins can be used to induce callus in *Glycine max* explants for mass propagation.

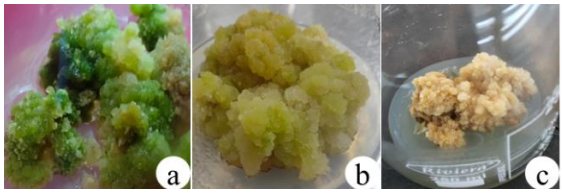

Figure 4: Callus induction from the cotyledonary explant. Dark green callus (a), light green callus (b) and pale green callus (c) of SP1, SP2 and SC samples.

Chlorophyll content

Different PGRs have different effects on the callus thus giving it a range of light to dark green color. Kresnawati (2006) reported that growth regulators play a major role in the callus colour of the explant. The colours of the different calluses obtained from various seed samples collected were quantified to calculate the concentration of chlorophyll(a) and (b) as well as carotenoids present in the seed samples. The estimation of pigments (chlorophyll a, b, and carotenoids) was done as shown in Table 2 after preparing an extract from the callus as shown in Figure 5a, 5b and 5c. The dark green and light green callus extract from each sample was taken for the estimation and pale green callus was found unsuitable for the estimation. The highest content of chlorophyll(a) (1.51 µg/ml), (b) (2.75 µg/ml), total chlorophyll (4.26 µg/ml), and carotenoids (2.03 µg/ml) were found in the sample ST1 which was collected from the Tehri region of Uttarakhand.

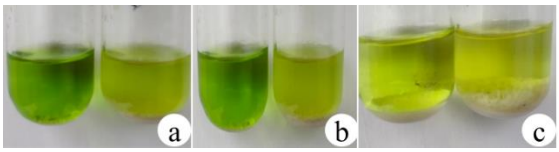

Figure 5: Extract of callus for pigment content estimation. Dark and light green of SP1, SP2 and SC samples (a, b and c).

Several studies have found a relationship between PGRs and chlorophyll content in plants. In an experiment conducted by Aldesuquy & Gaber (1993), it was found that pre-soaking soybean seeds in Kn resulted in enhanced chlorophyll(a) while soaking in other PGRs such as IAA or GA_3 did not show any significant outcome on the chlorophyll(a) content while presoaking in IAA decreased chlorophyll(b). The addition of cytokinin in the culture medium often shows a green colour because of cytokinin in the formation of chlorophylls (Widyawati, 2010). Dobránszki & Mendler-Drienyovszki (2014) reported that the type of cytokinin affects the content of chlorophyll(a) and (b). *In vitro*-raised apple leaves when treated with BA reported the highest chlorophyll content (1846-2176 µg/1g fresh weight of the leaf). Leupin et al. (2000) and Sari et al. (2018) stated high concentrations of cytokinin and light exposure play an active role in chlorophyll initiation. Sari et al. (2018) also reported that pale-coloured callus depicts the degradation of chlorophyll.

Table 2: Estimation of Chlorophyll(a), (b), total chlorophyll and carotenoids content of samples.

Callus colour	Absorbance			Chlorophyll(a) (µg/ml)	Chlorophyll(b) (µg/ml)	Total Chlorophyll (µg/ml)	Carotenoids (µg/ml)
	480 nm	645 nm	663 nm				
Light Green	0.096	0.098	0.099	0.99	1.78	2.77	0.044
Dark Green	0.144	0.145	0.145	1.45	2.64	4.13	0.068
Light Green	0.148	0.150	0.150	1.50	2.73	4.23	0.69
Dark Green	0.099	0.100	0.101	1.01	1.81	2.83	0.04
Light Green	0.025	0.023	0.025	0.25	0.40	0.66	0.01
Dark Green	0.140	0.141	0.141	1.41	2.56	3.97	0.06
Light Green	0.098	0.100	0.101	1.01	1.81	2.83	0.04
Dark Green	0.149	0.151	0.151	1.51	2.75	4.26	2.03

Conclusion

Conventionally, Soybean is propagated through seeds, and for better quality classical breeding is practised but it does not help in achieving the goal of mass production along with better quality. For this purpose, Soybean is raised via tissue culture. In the present study, various seed samples of Soybean were collected from different regions of Uttarakhand and were inoculated on MS+ BAP (1mg/l) for *in vitro* cotyledonary shoot induction and afterwards, *in vitro* leaves were inoculated on MS medium augmented with cytokinin with auxin or cytokinin alone for callus induction. PGRs also showed an effect on the colour of the callus which varied from dark green to light green in the presence

of various cytokinins and auxins. Furthermore, chlorophyll(a) and (b), total chlorophyll and carotenoid content were estimated from the different colored callus extracts of seed samples and seed sample annotated as ST1 from Tehri region of Uttarakhand showed the highest chlorophyll(a) (1.51 µg/ml), chlorophyll(b) (2.75 µg/ml), total chlorophyll (4.26 µg/ml) and carotenoid content (2.03 µg/ml).

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