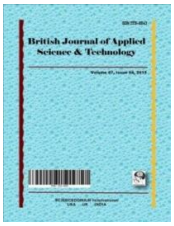

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Callus Cell Proliferation and Explants Regeneration Using Broccoli Shoot Tip *in vitro* Culture: Biochemical and Antioxidant Properties

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ABMSH and IH designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors MSA, NAI and KBR managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Significance of the Study: Nowadays cell or tissue culture has been successfully performed using different types of species like fruit, vegetable, ornamental and forest plant. Millions of explants can be produced by tissue or cell culture per year in any plant production industry. Combination of 6-Benzylaminopurine (BAP) and other plant growth regulators like 1-Naphthaleneacetic acid (NAA) or Indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was used in the most of the research in tissue culture.
Aims: The study was carried out to investigate the root, callus, shoot and leaf proliferation from the shoot tip in vitro culture using different IBA and BAP concentration.
Methodology: Shoot tip slice was used to culture in the MS (Murashige and Skoog) media.

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Different IBA and BAP (0, 0.25, 0.50, 1.0, 1.50, 2.0 2.5, 3.0 and 3.5 mg/l) concentrations in combination with MS media were used.

Results: The highest number (7.4) of shoot proliferation was observed in the concentration of IBA 0.25+BAP 2.5 mg/l combination. The maximum root proliferation was found in the concentration of IBA 0.25+ BAP 1.5. However, callus formation was observed better at the concentration of BAP 1-3.5 and IBA 1-3.5 mg/l combination than other combination of concentrations. The 2,2-diphenyl-2-picrylhydarzyl (DPPH) free radical scavenging potential was higher (70.1%) in leaves extract than in callus extracts (46.3%) at the concentration of 10 mg/ml. Besides, both extracts had lower DPPH free radical scavenging activity compared to the positive control, vitamin C and BHT.

Conclusion: The present results conclude that it is better to use the combination of the IBA and BAP concentration to produce root, shoot, leaf and callus cell proliferation in broccoli.

Keywords: Broccoli; Brassica oleracea; callus cell; in vitro culture; BAP; IBA; DPPH.

1. INTRODUCTION

In vitro Vegetable culture is an important branch of Horticultural Biotechnology. Cell or tissue culture as micro-propagation from stem, leaves, root, crown, sucker or embryo etc has been successfully done in plant tissue culture Biotechnology. Plant cell or tissue cultures have the ability to regenerate a whole plant called as totipotency [1]. A single cell, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones [2,3]. Millions of ornamentals, vegetables or fruits plant like pineapple plantlet, hibiscus plant, banana plantlet etc. can be produced by tissue culture from root, leaves, crown or stem per year [1,3]. Multiplication and total number of plantlets production were reported and recommended by using plant growth regulators [3]. A total number of plantlets production successfully done ranging 5000 [4], 40000 [5], 100000 [6] using single ex-plant per year. Propagation of plant can be gained in vitro treated with BAP alone [7], mixture of hormones, BAP, indole butyric acid (IBA) [8] indole acetic acid (IAA) [9]. It has been reported that combination of BAP [5] and auxin like NAA and IAA [10], IAA and IBA [11] and IBA [12]. Application of BAP alone could be a cost effective and useful over combination of two and three hormones. BAP at the concentration of 1.0 [7], 2.0 [13], 2.5 [14], 3.0 [15] and 4.0 mg/l [16] were recommended for multiplication of plantlet. It has been stated that the use of higher concentration range in caster bean increased castor proliferation rate five times higher [17]. This study has been investigated using the broccoli shoot tip slice. There is no available literature found on the present research. Therefore, the following objectives were undertaken.

1. To regenerate broccoli plants from explants of the broccoli from shoot tip.
2. To evaluate the effect of the different concentration of IBA and BAP on the roots, shoot, leaf and callus cell formation from broccoli shoot tip.
3. To investigate the biochemical and antioxidant activity by observing their free radical scavenging activity.

2. MATERIALS AND METHODS

2.1 Preparation of Murashige and Skoog (MS) Basal Media

The MS basal media [18] were used as control and seed germination was prepared following the standard procedures for MS powder form preparation (Table 1). MS powder form was added in a beaker filled with 800 ml distilled water to be followed by 30 g of sucrose and 2.8 phyta gels and adjustment the pH to 5.8 so that the final medium volume was 1000 ml.

2.2 Media in the Autoclave

MS basal media with auxin was prepared by adjusting the pH to 5.8 by using 1 N HCl and 1 M NaOH. Then, the media was fractional in 30 ml and was added into jam jars (7 x 4.5 cm2) and autoclaved at 15 psi and 121°C for 20 minutes. After that, the sterilized media were cooled and kept in culture room under dark condition. Preparation of media was completed a week before use to reduce water condensation in jam jars and the media was sterilized completely.

2.3 Seed Sterilization and Germination in the MS Media

Seeds of broccoli were obtained from the nursery. A total of 100 seeds were used on MS [18] basal

medium. The 20 jam jars were used to culture the seeds and five seeds were germinated on every jam jars. The seeds were washed in 70% ethanol for about 5 minute, and then rinsed in 15% chlorox for about 15 minutes. The seeds were brought into laminar flow hood and further rinsed with sterile DH20 for a few seconds. Then, the sterilized seeds were germinated on MS basal media for 7 days. This process was carried out under aseptic condition in the laminar flow. The seeds were exposed to light from cool white fluorescent tubes for a photoperiod of 16 hours in the incubation room at 25-28°C.

2.4 MS Basal Media with IAA and IBA and BAP (2nd Time Media Preparation)

The MS media with IBA and BAP were used as rooting media, MS powder form was added in a beaker filled with 800 ml distilled water and 30 g of sucrose was added. Then, the hormones with specific concentration were added. The pH was similarly adjusted and 2.8 g phyta gel was added, so that 1000 ml of medium was prepared. The media with hormones were prepared for five replicates of each hormone concentration. The BAP (as cytokinin) and IBA (as auxin) concentrations were 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3 and 3.5 mg/l.

Table 1. Standard procedures for MS media preparation

Component	Unit
MS powder form with vitamin	4.4 g
Sucrose	30 g
Phyta gel	2.8 g
pH	5.8

2.5 Shoot Tip Culture on MS Supplemented with IBA and BAP

After one week of germination, seven days seedlings were selected as a source of explants. The hypocotyls explants shoot tip were cut and transferred into media with different concentrations of IBA and BAP 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/l. Each treatment was consisted as five replications. Broccoli shoot tips were sliced in the clean bench. After that, in vitro shoot tip culture on MS basal was performed. The shoot tip cultures were put in the growth chamber in the incubation room at 25-28°C. Randomized complete block designed (RCBD) was used during sampling setting.

2.6 Antioxidant Activity of Broccoli

The antioxidant was evaluated based on the scavenging activity on the stable 2,2-diphenyl-2-picrylhydarzyl (DPPH) free radical measured by spectrophotometer. DPPH was useful reagent for investigating the free radical activities of compounds. A freshly prepared DPPH solution exhibits a deep purple color with maximum absorption at 515 nm. The DPPH test was a non-enzymatic method currently used to provide basic information on the ability of extracts to scavenge free radical. The OD reading of control, positive control (Vitamin C and BHT) and all samples were taken at 515 nm using spectrometer. The use of the percentage of free radical scavenging activity was calculated by the following formula for vitamin c.

Vitamin C = [(A control- A sample)/A control] x 100.

2.7 Data Collection

Root and callus formation, shoot and leaf proliferation were observed and data were collected after one month of treatment setting. The 2,2-diphenyl-2-picrylhydarzyl (DPPH) free radical was measured.

2.8 Design and Statistical Analysis

Randomized block designed was used during sampling setting. Standard deviation and then standard Error was made to compare the replicates. Least Significant Difference (LSD) test was used for data analysis.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Root, callus, shoot and leaves proliferation

After two weeks of sub-culture, the callus cell and roots have shown as the positive results. It was mostly produced roots on media with hormone. After three weeks the formation of shoots was found initially after four weeks the leaf formation was observed. There was higher formation of root observed from the shoot tip in different concentration of IBA and BAP (Table 2). In addition to that the formation of callus from shoot tip cutting mostly showed the best response in combination with IBA and BAP at

different concentrations. The root formation was found higher at 0.25 mg/l IBA + 0.5 mg/l BAP, 1.5 mg/l IBA + 0.25 mg/l BAP, 3 mg/l IBA + 0.5 mg/l BAP, 0.25 mg/l IBA + 1.5 mg/l BAP and 0.25 mg/l IBA + 0.25 mg/l BAP than other concentrations of hormone IBA and BAP. It was found that the higher shoot formation was occurred at the concentration of 1.5 mg/l IBA + 0.25 mg/l BAP (Table 2). Most of the initial leaf proliferation, callus formation, then green and whitish callus as well as compact and globular callus formation were found at the concentration of BAP 1.0-2.0 and IBA 1-3.5 mg/l (Table 2). However, shoot formation was found progressive after the callus formation. The shoot formation was found higher at 0.25 mg/l IBA + 0.5 mg/l BAP, 1.0 mg/l IBA + 2.5 mg/l BAP, 1 mg/l IBA + 2.0 mg/l BAP, 1.5 mg/l IBA + 2.5 mg/l BAP and 2.0 mg/l IBA + 0.25 mg/l BAP, 0.5 mg/l IBA + 1.0 mg/l BAP, 0.5 mg/l IBA + 2.5 mg/l BAP than other concentrations of hormone IBA and BAP. The highest shoot formation (4.6) was occurred at the concentration of 1 mg/l IBA + 2.0 mg/l BAP (Table 2). Leaf initiation was found in the concentration of 1.0-3.5 mg/l IBA+1.0-2.0 mg/l BAP compared to the other concentration (Table 2). Callus weight was found higher at the concentration of 1.0 BAP+1.5 IBA (1.95), 1.5 BAP+0.25 IBA and 1.5 BAP+0.3 IBA, than other concentrations (Table 3). The highest callus weight (1.95) was found at the concentration of 2.0 BAP and 0.25 IBA. However, the lowest callus weight (0.6) was found at the concentration of 1.0 BAP, 0.25 IBA (Table 3). Fig. 1 shows the germinated seedling, root, shoot and callus proliferation from broccoli leaf tip. In Table 4, the results were based on the neutralization of DPPH radical of samples in the free radical scavenging activity assay. BHT and Vitamin C (Ascorbic acid) were used as the positive control.

In Fig. 2 the Results have shown the free radical scavenging potential of broccoli leaves extract (70.1%) which was significantly higher than callus extracts (46.3%) at concentration of 10 mg/ml. At concentration of 20 mg/ml both extract showed lower antioxidant activity than the concentration of 10 mg/ml which were 53.3% in leaves and 37.1% in callus Besides that both extracts also had significantly lower DPPH free radical scavenging activity compared to the positive control, vitamin C (91.3%) and BHT (86.2%).

Table 2. Effects of IBA and BAP on the roots, callus, shoot and leaves formation from broccoli shoot tip cutting

IBA	BAP	No. of root formed	No. of shoot formed	Observation of callus	Leaf proliferation
0	0	0	0	-	-
0	0.25	1.0±0.41	1.67±0.33	-	-
0	0.5	1.25±0.25	2.67±0.33	-	-
0	1.0	1.0±0.41	2.67±0.33	-	-
0	1.5	1.1±0.65	2.33±0.33	-	-
0	2.0	1.2±0.29	2.67±0.33	-	-
0	2.5	1.0±0.41	3.33±0.33	-	-
0	3.0	1.25±0.25	1.67±0.33	-	-
0	3.5	1.25±0.25	1.33±0.33	-	-
0.25	0.25	1.75±0.48	0.67±0.33	-	-
0.25	0.5	3.25±0.25	1.0±0.58	-	-
0.25	1.0	1.5±0.29	0.67±0.33	-	-
0.25	1.5	2.75±0.25	1.0±0.58	-	-
0.25	2.0	1.5±0.29	1.33±0.33	-	-
0.25	2.5	1.5±0.29	0.67±0.33	-	-
0.25	3.0	1.75±0.49	1.33±0.33	-	-
0.25	3.5	1.5±0.29	1.0±0.58	-	-
0.5	0.25	0.75±0.48	4.0±0.32	-	-
0.5	0.5	0.75±0.48	2.4±0.36	-	-
0.5	1.0	1.0±0.58	4.4±0.36	-	-
0.5	1.5	1.0±0.41	3.2±0.41	-	-
0.5	2.0	0.25±0.25	3.0±0.50	-	-
0.5	2.5	0.0±0.0	2.6±0.36	-	-

IBA	BAP	No. of root formed	No. of shoot formed	Observation of callus	Leaf proliferation
0.5	3.0	0.25±0.25	3.8±0.41	-	-
0.5	3.5	0.0±0.0	3.6±0.36	-	-
1.0	0.25	0.75±0.49	3.6±0.57	-	-
1.0	0.5	0.5±0.29	2.0±0.22	-	-
1.0	1.0	1.25±0.63	2.4±0.36	Callus formed	+
1.0	1.5	1.25±0.48	2.8±0.26	Green, whitish callus	+
1.0	2.0	1.5±0.65	4.6±0.36	Compact , globular callus	+
1.0	2.5	1.25±0.63	3.4±0.36	-	
1.0	3.0	0.75±0.49	2.2±0.26	-	-
1.0	3.5	1.0±0.41	2.6±0.36	-	-
1.5	0.25	3.0±0.41	2.6±0.28	-	-
1.5	0.5	1.5±0.65	2.6±0.49	-	-
1.5	1.0	0.75±0.25	3.2±0.41	-	+
1.5	1.5	1.0±0.41	3.2±0.35	Callus formed	+
1.5	2.0	1.25±0.48	4.0±0.5	Green, whitish callus	+
1.5	2.5	0.5±0.29	4.0±0.32	Compact, globular callus	-
1.5	3.0	0.75±0.48	3.0±0.50	-	-
1.5	3.5	1.25±0.63	3.0±0.22	-	-
2.0	0.25	1.0±0.41	2.2±0.14	-	-
2.0	0.5	1.0±0.41	2.4±0.36	-	+
2.0	1.0	1.25±0.63	2.4±0.17	Callus formed	+
2.0	1.5	1.0±0.41	3.2±0.52	Green, whitish callus	+
2.0	2.0	1.25±0.75	3.8±0.52	Compact, globular callus	-
2.0	2.5	1.25±0.25	3.4±0.36	--	-
2.0	3.0	1.0±0.41	2.4±0.17	-	-
2.0	3.5	1.5±0.65	2.6±0.28	-	-
2.5	0.25	1.5±0.29	2.2±0.26	-	-
2.5	0.5	0.25±0.25	2.4±0.17	-	
2.5	1.0	0.0±0.0	3.0±0.32		+
2.5	1.5	0.25±0.029	3.2±0.41	Callus formed	+
2.5	2.0	0.75±0.49	3.6±0.26	Green, whitish callus	+
2.5	2.5	0.5±0.29	3.2±0.26	Compact , globular callus	-
2.5	3.0	1.0±0.41	2.4±0.36	-	-
2.5	3.5	1.0±0.41	2.0±0.22	-	-
3.0	0.25	0.75±0.25	2.0±0.22	-	-
3.0	0.5	3.12±0.48	2.2±0.26	-	-
3.0	1.0	0.0±0.0	2.8±0.26	-	+
3.0	1.5	0.0±0.0	2.6±0.17	Callus formed	+
3.0	2.0	0.25±0.025	2.6±0.36	Green, whitish callus	+
3.0	2.5	0.75±0.48	2.6±0.48	Compact, globular callus	-
3.0	3.0	0.5±0.29	2.8±0.26	-	-
3.0	3.5	0.5±0.29	2.2±0.26	-	-
3.5	0.25	0.75±0.48	2.4±0.36	-	-
3.5	0.5	0.5±0.29	2.0±0.22	-	+
3.5	1.0	0.25±0.025	4.0±0.22	Callus formed	+
3.5	1.5	0.75±0.48	3.2±0.26	Green and whitish callus	+
3.5	2.0	0.75±0.25	3.0±0.5	Compact and globular	-
3.5	2.5	0.75±0.48	4.0±0.32	callus	-
3.5	3.0	0.5±0.29	2.8±0.26	-	-
3.5	3.5	1.0±0.41	2.8±0.41	-	
				-	

Mean ± SE of 5 replicates. + = organ (leaf) formation was indicated. - no-indication of organ formation

Table 3. Effects of different combination of hormone on fresh weight of callus produced from broccoli shoot tip cutting

BAP	IBA	Callus weight (g)
1.0	0.25	0.25±0.06
	0.5	0.35±0.02
	1.0	0.43±0.02
	1.5	1.2±0.29
	2.0	1.13±0.09
	2.5	1.25±0.16
	3.0	1.95±0.06
1.5	3.5	1.4±0.22
	0.25	1.73±0.49
	0.5	1.43±0.06
	1.0	1.68±0.25
	1.5	1.35±0.13
	2.0	1.43±0.11
	2.5	1.45±0.06
	3.0	1.73±0.18
2.0	3.5	1.53±0.12
	0.25	1.63±0.49
	0.5	1.18±0.06
	1.0	1.53±0.25
	1.5	1.10±0.13
	2.0	1.23±0.11
	2.5	1.2±0.06
	3.0	1.37±0.18
	3.5	1.37±0.12

Callus produced per leaves explant, Average ± SE of 5 replicates

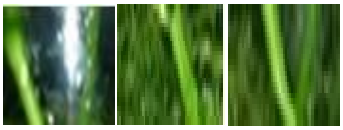
3.2 Discussion

Our results demonstrated the optimization of the cell culture and root generation, callus, shoot and leaves proliferation. In the concentration of 0.25 and 0.5 mg/l of BAP and IBA root and shoot formation occurred. In addition, at the concentration of 0, 0.25 and 0.5 mg/l of IBA and BAP, there was no callus and leaves proliferation. These might be due to the cell differentiation and division not happened to these concentrations. These concentrations might not be effective for cell differentiation and division for callus and root proliferation. These might be due to the cell differentiation and division was not occurred to these concentrations [19]. However, overall root, callus, shoot and leaves formation were occurred at the concentration of 1.0-3.5 mg/l IBA and 1.0-2.0 mg/l BAP concentrations. These concentrations might be effective for cell differentiation and division for callus, root, shoot and leaves proliferation. It has been reported that 1.0 mg/l IAA and 1.0 mg/l combined with 0.25 mg/l gibberellic acid showed better root regeneration in potato than other concentration. [13] It has been reported that 1.0 mg/l IAA and 1.0 mg/l IBA combined with 0.3 mg/l auxin (NAA) showed better root regeneration in potato than other concentration. [20] It has been reported that 1.0 mg/l IAA and 1.0 mg/l IBA combined with 1.0 mg/l auxin (NAA) showed the best root, shoot and leaf regeneration in potato.

Table 4. Measurement of the OD reading of control, positive control (Vitamin C and BHT) and all samples taken at 515nm using spectrometer

Samples	OD reading (515 nm)
Control (Ethanol 95%)	0.058
Vitamin C	0.005
BHT	0.008
Leaves extracts (10 mg/ml)	0.019
Callus extracts (10 mg/ml)	0.035
Leaves extracts (20 mg/ml)	0.029
Callus extracts (20 mg/ml)	0.039

Germinated plants



Shoot tip cutting

Callus formation and explants

Fig. 1. Photograph show the callus and explants from shoot tip of broccoli

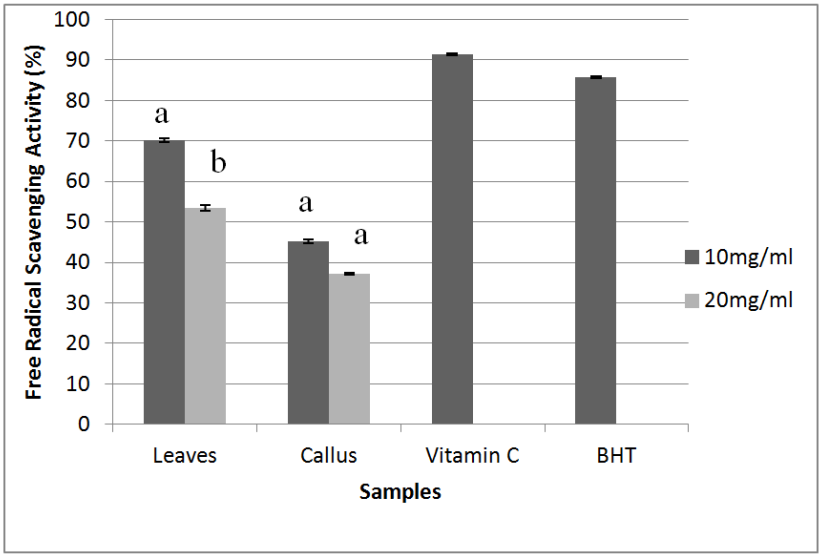


Fig. 2. The antioxidant activity of the selected parts (leaves and callus) of broccoli explants at concentration of 10 and 20 mg/ml. The same letters are not statistically different at 5% level of significance by Least Significant Difference (LSD) test

Different combinations and concentrations of hormone affect the plants growth [5]. It has been reported that the different concentrations of auxin and cytokinin are important for the roots and shoots of explants from meristemic tissues of tobacco, banana [21] and pineapple [1].

Table 2 showed the effects of hormones on the callus growth. Callus formation was obtained from root tips in media supplemented with different combinations and concentrations of hormone. According to [14,16,22,23] callus formation was obtained if the concentration of auxin and cytokinin was the similar. But, actually this statement was suitable only for certain species. It has been stated that in the case of broccoli and other medicinal plant, callus was obtained using media supplemented with different concentrations of auxin and cytokinin [6,10,17,24].

4. CONCLUSION

The best medium for callus proliferation of broccoli was MS basal medium supplemented with 1-1.5 mg/l BAP and IBA from shoot tips. For root formation, the best concentration was of 1-2.5 mg/l BAP and IBA. The concentration of 1 mg/l IBA + 2.0 mg/l BAP was the best for shoot formation. Free radical scavenging potential of broccoli leaves extract (70.1%) was significantly higher than callus extracts (46.3%) at the concentration of 10 mg/ml. In addition to that both extracts also had significantly lower DPPH free radical scavenging activity compared to the positive control, vitamin C (91.3%) and BHT (86.2%).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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