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Callus cell and explants regeneration, glucose, mineral, antioxidant and flavonoid content development using broccoli root tip and leaf cutting *in vitro*

ABM Sharif Hossain[1*], Hassan A. Alrudayni[1], Faouzi Haouala[1], Mohammad Saad Aleissa[1], Danial Kahrizi[2], Abu Saleh Ahmed[3]

[1] Department of Biology, College of Science, Imam Muhammad Ibn Saud Islamic University, Riyadh, Saudi Arabia
[2] Agricultural Biotechnology Department, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
[3] School of Engineering and Technology, University of Technology Sarawak, Malaysia

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ABSTRACT

The study was conducted to evaluate the root, shoot and leaf callus cell regeneration and its biochemical properties like antioxidant, carbohydrate, pigment and mineral content from broccoli root, shoot and leaf cutting *in vitro*. An *in vitro* factorial experiment was carried out based on a Completely Randomized Design (CRD) with 5 replicates in tissue culture applying different IBA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/l) and BAP (1 mg/l) concentrations using broccoli root tip and leaf cutting. The results showed that a higher callus weight was found in the cultured leaf cutting than in root tip cutting in the concentration of 1.0, 1.5 & 2.0 mg/l IBA + 1.0 mg/l BAP combination. The highest callus weight was found in the cultured leaf cutting than root tips cutting at the concentration of 1.5mg/l IBA+1.0 mg/l BAP. Furthermore, the highest inverted sugar and glucose, chlorophyll and nutrient content (K+, NO_3- & Ca++), total phenol, flavonoid and total antioxidant were found in the concentration of 1.5mg/l IBA+1.0 mg/l BAP combination in both broccoli leaf and root cutting. The results seemed that it was best to use the combination of the IBA and BAP in the concentration of 1.0-2.0 mg/l and 1mg/l to regenerate root, leaf and callus cell proliferation of broccoli from the root tip and leaf cutting.

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Introduction

The field of plant cell and tissue culture biotechnology has successfully regenerated cell or tissue cultures from different organs such as stems, leaves, roots, crowns, stems or embryos. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant called 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. Modern plant tissue culture is performed under aseptic conditions under filtered air (2). Millions of ornamental, vegetable, agronomic or fruit plants like pineapple, grapes, and peach explants can be produced by tissue culture from root, leaves, crown or stem per year (1). Propagation of plant can be gained in vitro treated with BAP alone (3) a mixture of hormones like BAP and naphthalene acetic acid (NAA) (4) indole butyric acid (IBA) (5) indole acetic acid (IAA) (6) and 2,4-dichlorophenoxy acetic acid (2,4-D) (7) combination of BAP and two auxins as NAA and IAA (8), IAA and IBA (9) and IBA (10). Application of BAP alone was cost-effective and could be useful over a combination of two and three hormones. Moreover, the optimum concentration of BAP was not yet recommended extensively. BAP at the concentration of 1.0 (3), 2.0 (11, 12), 2.5 (13,14), 3.0 (4) and 4.0 mg/l (15) were recommended for the multiplication of plantlet. It was (14) stated that the use of a wider concentration range in caster beans increased the castor proliferation rate five times higher. There are no available literatures found on the present research except few information. Therefore, the following objectives were undertaken:

1. To observe the effect of the different concentrations of IBA and BAP on the roots, leaf, callus cell formation and weight from the broccoli root tip and leaf cutting.

2. To investigate the biochemical and antioxidant properties of explants regenerated from broccoli root and leaf cutting.

Materials and Methods

Preparation of MS Media

The Murashige and Skoog (16) (MS) media were used for the preparation of cultural media by following the standard procedures for MS powder (4.4g) form. MS powder was added to a beaker filled with 800 ml of distilled water. Then it was followed up with 30 g of sucrose by adjusting the pH (5.8) and adding 2.8 phyta gels.

Media in Autoclave

MS media was prepared by adjusting the pH to 5.8 using 1 N HCL and 1 M NaOH. Then, the media was autoclaved at 15 psi and 121˚C for 20 minutes. After that, the sterilized media were cooled and kept in a culture room under dark conditions. Preparation of media was done a

* Corresponding author. Email: hossainsharif41@gmail.com, ashossain@imamu.edu.sa

week before use.

Seed sterilization and germination in MS Media

Broccoli seeds were washed using 70% ethanol for 5 minutes and then rinsed in 15% chlorox for 15 minutes. The seeds were brought into the laminar flow and continued to rinse with sterile DH20. Then, the sterile seeds germinated in MS basal media for 7 days. This process was carried out under aseptic conditions in the laminar flow. The seeds were exposed to light-cool white fluorescent tubes for a photoperiod of 16 hours in the incubation room at 25˚C (12).

MS basal media with IBA and BAP

The MS media with IBA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 & 3.5 mg/l) and BAP (1 mg/l) were used as rooting media and added in a beaker filled with 800 ml distilled water and 30 g of sucrose was added. Then, the hormones with specific concentrations from the stock solution were added. The media with hormones prepared for five replicates of each hormone concentration.

Root cutting culture on MS Supplemented with IBA and BAP

The roots were collected from seedlings and root tips were cut and put into the media with the hormone concentrations of IBA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 & 3.5 mg/l) and BAP (1 mg/l). Five replications were used per treatment.

Leaf-cutting slice culture on MS Supplemented with IBA and BAP

After one week of germination, seven-day seedlings were selected as the source of explants. The explants (leaf slice) were transferred into media without auxin (control) and media with varying levels of IBA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 & 3.5 mg/l) and BAP (1 mg/l). Five replications were used for each concentration.

Data observation

Callus cell or tissue formation, root, shoot and leaf proliferation were observed and callus weight was measured.

Leaf explant extraction

Leaf samples at different concentrations of IBA and BAP were collected. Then it was ground with a motor and pestle and filtered the extract and finally extracted sample was separated and stored in the freezer.

Data analysis

Biochemical (sugar, antioxidants, etc.) content was determined.

Glucose content

Glucose was investigated by using a glucose refractometer. Three drops of leaf extract sample were placed on the disc of the meter and data were observed and documented.

Inverted sugar

Inverted sugar was investigated by using an inverted sugar refractometer. Three drops of leaf extract sample were placed on the disc of the meter and data were observed and recorded.

Chlorophyll content

Total chlorophyll was determined from leaves samples according to the methods of Lichtenthaler and Wellburn (17).

Total antioxidant

1mM trolox standard solution was used. Water was poured into each well to make the volume to 100 µL. Leaf extracts were directly added to the wells. For small molecule TAC, samples were diluted at a 1:1 ratio with Protein Mask. 20 µL of the sample was used in wells. Distilled water was put in preparing the volume of 100 µL. 100 µL of Cu2+ working solution was added to all standard and sample wells and mixed properly using a horizontal shaker and the reaction was incubated for 90 minutes at room temperature. The plate was protected from light at the time of incubation and finally measured the absorbance at 570 nm.

Flavonoid

Total flavonoid content (FC) was investigated with aluminum chloride colorimetric assay, using catechin as a standard.

Nutrient or Mineral content

Nutrient content (Ca++, NO3-- and K+) was determined by using Horiba Scientific Ca, NO3 and K meters (Japan). 3 drops of extract sample were put on the disc sensor of the meter using a small dropper and data were displayed and recorded.

Statistical Analysis

Statistical analysis of the data was carried out by using analysis of variance (ANOVA) and differences among treatment means were compared by using the Least Significance Difference (LSD) Test at a 5% probability level.

Results

Callus, root, shoot and leaf formation

Root formation from the root tip and leaf cutting was higher in the concentration of 1.5 & 2.0 of IBA + BAP (1.0 mg/l) than in the concentration of 0.25, 0.5, 1.0, 2.5, 3.0 and 3.5 mg/l of IBA + BAP (1.0 mg/l) (Table 1). The observation of callus cell formation was positive in the case of all concentrations except 0.25 and 0.5 mg/l for both root tip and leaf cutting explant (Table 2).

In the case of all concentration of leaf-cutting, callus cell was initiated and formed except 0.25 and 0.5 mg/l of concentration, however, in the root tip, callus cell was initiated and formed in the concentration of 1.0, 1.5, and 2.0 mg/l IBA+BAP (1mg/l). Leaf proliferation was initiated in the case of 2.5, 3 and 3.5 mg/l of concentration of IBA + BAP (1.0 mg/l) in the root tip, however in leaf-cutting explants, it was initiated in all concentrations except 0.25 and 0.5mg/l (Table 3). The highest callus cell weight was found in the cultured from leaf cutting compared to the rooted cutting in the concentration of IBA+BAP, 1.5+ 1.0 mg/l combination (Table 4).

In the case of leaf-cutting, it was found 3.9g per callus weight than 3.5g per callus in the root tip in the concentration of IBA+BAP, 1.5+ 1.0 mg/l combination. Moreover, callus weight was observed higher in 1.5 and 2.0 mg/l of concentration than the others in the case of both root tip

Table 1. Effects of IBA and BAP on root formation from the broccoli root tip and leaf cutting.

BAP	IBA	Root formation from root tip cutting	Root formation from leaf cutting
1.0	0.25	0.6 ± 0.02	1.05 ± 0.29
1.0	0.5	0.55± 0.03	1.25 ± 0.25
1.0	1.0	1.6± 0.01	1.5 ± 0.65
1.0	1.5	1.75± 0.02	1.8± 0.62
1.0	2.0	1.8± 0.02	2.0± 0.71
1.0	3.0	1.7± 0.03	1.5± 0.29
1.0	3.5	0.5 ± 0.03	1.5 ± 0.65

Mean ± SE (n= 5).

Table 2. Effects of IBA and BAP on callus formation from the broccoli root tip and leaf cutting.

IBA	BAP	Callus from root tip	Callus from leaf cutting
0.25	1.0	-	-
0.5	1.0	-	-
1.0	1.0	Callus formed	Callus formed
1.5	1.0	Callus formed	Callus formed
2.0	1.0	Green and whitish	Green and whitish
2.5	1.0	--	Green and whitish
3.0	1.0	--	Compact and globular
3.5	1.0	--	Compact and globular

*P <0.05 is significantly different for each treatment obtained from the One Way ANOVA test.

Table 3. Effects of IBA and BAP on the leaf proliferation from the broccoli root tip and leaf cutting.

BAP	IBA	Leaf proliferation from root tip	Leaf proliferation from leaf cutting
1.0	0.25	-	-
1.0	0.5	-	-
1.0	1.0	-	+
1.0	1.5	-	+
1.0	2.0	-	+
1.0	2.5	+	+
1.0	3.0	+	+
1.0	3.5	+	+

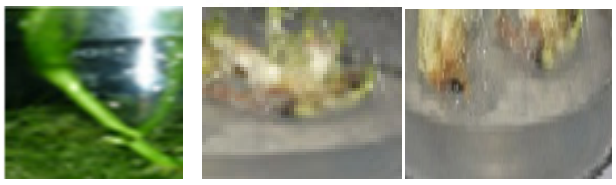
Mean ± SE of 5 replicates. + = organ (leaf) formation was indicated. - no indication of organ formation. *P <0.05 is significantly different for each treatment obtained from the One Way ANOVA test.

and leaf cutting explants. In the case of explant produced from leaf cutting, callus weight was found for all concentrations (Table 4).

Glucose, chlorophyll, mineral content and antioxidant

Inverted sugar and glucose content was found higher at the concentration of 1.0, 1.5 and 2.0 mg/l IBA+ 1.0mg/l BAP than the other concentrations of both root tip and leaf cutting explant (Table 5). In addition, the highest chlorophyll content was exhibited at the concentration of 1.5 mg/l IBA+ 1.0mg/l BAP in the case of the root tip (3.5 µg/g) and leaf cutting (5.1µg/g) explant. Nutrient content (K^+, $NO3^-$, and Ca^{++}) was found higher in root tip and leaf cutting explant in the concentration of IBA 1.0 and 1.5 mg/l IBA+ 1.0 mg/l BAP than the concentration of others (Table 6).

However, the highest nutrient content was found in the concentration of 1.5 mg/l IBA+ 1.0 mg/l BAP in both root tip and leaf cutting explant. Moreover, antioxidant and flavonoids exhibited higher in the concentration of 1.0, 1.5 mg/l IBA +1.0 mg/l BAP combination than in the concentration of 2.0, 2.5, 3.0 and 3.5 mg/l IBA + 1.0 mg/l BAP in the case of leaf-cutting explant (Table 7). Figure 1 shows the image of the broccoli culture procedure.



a. Broccoli seedling b. Callus proliferation from leaf slice and root tip

Figure 1. The rooting, callus, shoot and leaf proliferation formation.

Discussion

From the above-mentioned results, it has been discussed that the leaf-cutting showed higher callus formation represented as callus weight, root and leaf explants carbohydrate represented as inverted sugar and glucose, chlorophyll and nutrient content (K+, NO_3- and Ca++ than in different concentrations of IBA 1, 1.5, 2.0 mg/l IBA + 1.0 mg/l BAP combination compared to the root tip cutting. These concentrations might not be suitable for root proliferation and callus formation. It was reported (18,19)

Table 4. Effects of a different combination of the hormone on fresh weight of callus produced from the broccoli root tip and leaf cutting.

Sources IBA + BAP (mg/l)	Callus cell weight (g) Root tip	Leaf cutting
0.25+1.0	--	-
0.5+1.0	--	-
1.0+1.0	1.3± 0.2c	1.40± 0.1d
1.5+1.0	3.5± 0.1a	3.9± 0.3a
2.0+1.0	2.3± 0.3b	2.35± 0.2b
2.5+1.0	-	1.80± 0.3dc
3.0+1.0	--	2.15± 0.4c
3.5+1.0	--	2.18± 0.5c

The means followed by the common letters are not significantly different at the 5%level by the Least Significant different test (LSDT). Mean ± SE (n= 5).

Table 5. Effects of IBA and BAP on the sugar and chlorophyll content from broccoli root and leaf cutting. The means followed by the common letters are not significantly different at the 5%level by the Least Significant different test (LSDT). Mean ± SE (n= 5).

IBA +BAP (mg/l)	Inverted sugar (mg/100g)		Glucose (mg/100g)		Chlorophyll (µg/g)	
	Root tip	Leaf cutting	Root tip	Leaf Cutting	Root tip	Leaf Cutting
0.25+1.0	0	0	0	0	0	0
0.5+1.0	0	0	0	0	0	0
1.0+1.0	2.3± 0.13a	3.4 ± 0.1	3.4 ± 0.2a	5.2± 0.2b	2.7± 0.3b	3.8 ± 0.1b
1.5+1.0	2.4 ± 0.2a	3.6± 0.2a	3.5 ± 0.2a	5.5± 0.1a	3.6 ± 0.2a	5.1± 0.2a
2.0+1.0	2.0 ± 0.1b	3.5± 0.1a	3.5 ± 0.1a	4.8± 0.3c	1.9 ± 0.1c	2.8 ± 0.2c
2.5+1.0	0	2.6 ± 0.2b	0	3.5± 0. 1d	0	2.0± 0.3d
3.0+1.0	0	2.4± 0.3ab	0	2.0± 0.1e	0	1.8± 0.2d
3.5+1.0	0	2.0± 0.2c	0	2.0± 02e	0	1.5± 0.2de

Table 6. Effects of IBA and BAP on the nutrient content from broccoli root and leaf cutting. Mean ± SE (n= 5).

IBA +BAP (mg/l)	K+ (PPM)		NO3- (PPM)		Ca++ (PPM)	
	Root tip	Leaf cutting	Root tip	Leaf cutting	Root tip	Leaf cutting
0.25+1.0	0	0	0	0	0	0
0.5+1.0	0	0	0	0	0	0
1.0+1.0	405 ± 0.1c	805 ± 0.1c	345± 0.2c	695 ± 0.2c	108± 0.4b	168± 0.5b
1.5+1.0	445± 0.2a	845± 0.2a	399± 0.4a	775 ± 0.4a	135± 0.3a	213± 0.6a
2.0+1.0	420 ± 0.3b	820 ± 0.3b	379± 0.2b	712 ± 0.2b	145± 0.5a	208± 0.4a
2.5+1.0	0	621 ± 0.1d	0	598 ± 0.3d	0	149± 0.2c
3.0+1.0	0	525 ± 0.3e	0	535 ± 0.5e	0	154± 0.3c
3.5+1.0	0	422 ± 0.2f	0	515 ± 0.2f	0	107± 0.6d

Table 7. Effects of IBA and BAP on the antioxidant and flavonoid from broccoli root and leaf cutting.

IBA +BAP (mg/l)	Total antioxidant (mg/100g)		Flavonoid (mg/100g)	
	Root tip	Leaf cutting	Root tip	Leaf cutting
0.25+1.0	0	0	0	0
0.5+1.0	0	0	0	0
1.0+1.0	255± 0.5b	350 ± 0.9b	1.70±0.3a	3.60±0.4a
1.5+1.0	290 ± 0.5a	399 ± 0.3a	1.60±0.2b	2.80±0.1b
2.0+1.0	246± 0.3b	346 ± 0.2b	1.4±0.1c	1.8±0.3c
2.5+1.0	0	241 ± 0.4bc	0	1.6±0.2cd
3.0+1.0	0	236 ± 0.3c	0	1.5±0.1d
3.5+1.0	0	235 ± 0.2 c	0	1.5±0.2d

Mean ± SE (n= 5). Callus produced per leaves explants, Average ± SE of 5 replicates.

that growth and morphogenesis of cell culture or organ were affected by genotype, substrate, environment and tissues have been used. It was reported (20) that, the genotype which had the high capability was important to be chosen to produce good regeneration in tissue culture.

It was reported (1) that the suitable part that could be cultured depended on the species and explants reaction also depended on different conditions of the mother plants. Plant tissue culture needs several organic chemicals such as nitrogen, magnesium sulphate, phosphorus, natrium and

chloride ion (20, 21). Different combinations and concentrations of hormones affect the plants growth. It was reported (22) that, the different concentrations of auxin and cytokinin are important to roots, meristem and shoots for explants from meristems tissue of tobacco, banana (23) and pineapple (1). According to the researcher (24), callus formation was obtained if the concentration of auxin and cytokinin was the same. *Brassica olerace var italica* callus was also obtained from media supplemented with different concentrations of auxin and cytokinin (25) which showed similar to the present results. In this study, the antioxidant activities of leaf extracts of broccoli were evaluated. Several different methods have been developed to evaluate the antioxidant activity of biological samples (26).

The leaf-cutting explant showed the highest sugars, total antioxidant, flavonoid, chlorophyll and nutrient content compared to the other explants in the concentration of 1.5mg/l IBA+1 mg/l BAP. This might be due to the different parts of the plant producing different compounds or different amounts of compounds due to their differential gene expression. Therefore, this particularly affects the antioxidant potential of the different parts of a given plant (27). Other factors that may increase or decrease the antioxidant compounds which include the sample condition and polarity of the extraction solvents (28). In addition to that, it is well known that red and dark green colored leafy vegetables are richer in nutrient content than lighter-colored vegetables. The naturally occurring compounds adequate for food coloring pigments, such as chlorophyll, anthocyanins, betalains (betacyanin and betaxanthin) and carotenoids are involved in leaf coloration. All of these components have been established to have antioxidant activities (29). Hence, this proved that the green color of leaves can affect or increase the antioxidant activity of leaf extracts in this study.

Conclusion

It can be concluded that the best combination of the concentration of IBA and BAP in the concentration of 1.5 mg/l IBA+ 1.0 mg/l BAP to regenerate callus cells from root, shoot and leaf cutting of broccoli. In addition, the highest sugars, total antioxidant, chlorophyll, flavonoid and nutrient content was found in the concentration of IBA and BAP (1.5mg/l and 1.0 mg/l) as compared to the IBA (1, 2 mg/l) and BAP (1.0mg/l) concentrations.

Conflict of interest

The authors have no conflict of interest.

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