



## **Callus cell weight, antioxidant, carbohydrate, pigment and nutritional properties from broccoli explants in vitro: A nutritional vegetable**

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### **Abstract**

The purpose of the study was to evaluate the callus cell weight, antioxidant, carbohydrate, pigment and nutritional properties from broccoli explants as nutritious vegetable. The results showed that the higher callus weight was found in the cultured leaf cutting than root and shoot tips cutting in the concentration of IBA 1.0 + 2.0 mg/l BAP, IBA 2.0 + 2.0 mg/l BAP, and IBA 3.0 + 2.0 mg/l BAP combination. The highest callus weight was found in the cultured leaf cutting than root and shoot tips cutting at the concentration of IBA 2.0 + 2.0 mg/l BAP. Carbohydrate (as inverted sugar and glucose), chlorophyll and nutrient content (K<sup>+</sup>, NO<sub>3</sub><sup>-</sup>, Ca<sup>++</sup> and Na<sup>+</sup>), total phenol, antioxidant (DPPH), flavonoid and total antioxidant were found in the concentration of IBA 2.0 + 2.0 mg/l BAP combination from broccoli leaf cutting. The results seemed that it was better for nutrition and growth to use the combination of the IBA and BAP in the concentration of 1.0-3.0 mg/l to regenerate root, shoot, leaf and callus cell proliferation and nutrition of broccoli from root tip, shoot tip and leaf cutting.

**Keywords:** root, shoot, callus cell, broccoli, antioxidant

### **Introduction**

Cell or tissue culture as micro-propagation from stem, leaves, root, crown, sucker or embryo has been successfully done in plant tissue culture Biotechnology. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant called as totipotency <sup>[1]</sup>. 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 <sup>[2]</sup>. Millions of ornamental, vegetable or fruit plant like pineapple explants can be produced by tissue culture from root, leaves, crown or stem per year <sup>[1]</sup>. Propagation of plant can be gained in vitro treated with BAP alone <sup>[3]</sup> mixture of hormones like BAP and naphthalene acetic acid (NAA) <sup>[4]</sup> indole butyric acid (IBA) <sup>[5]</sup> indole acetic acid (IAA) <sup>[6]</sup> and 2, 4-dichlorophenoxy acetic acid (2,4-D) <sup>[7]</sup>, combination of BAP and two auxins as NAA and IAA <sup>[8]</sup>, IAA and IBA <sup>[9]</sup> and IBA <sup>[10]</sup>. Application of BAP alone was cost effective and could be useful over combination of two and three hormones. Moreover, the optimum concentration of BAP was not yet recommended extensively. BAP at the concentration of 1.0<sup>3</sup>, 2.0<sup>11</sup>, 2.5<sup>12</sup>, 3.0<sup>4</sup> and 4.0 mg/l<sup>13</sup> were recommended for multiplication of plantlet. Sujatha and Reddy <sup>[14]</sup> reported that the use of wider concentration range in castor bean increased castor proliferation rate five times higher. The present study was done using the broccoli root, shoot tip and leaf cutting using IBA and BAP at different concentrations. There are not available literatures found on the present research. Therefore the following objectives were undertaken:

1. To find out the effect of the concentration of IBA and BAP (1 and 2 mg/l) on the roots, shoot and leaf callus cell

formation (weight) from broccoli root, shoot tip and leaf cutting.

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

### **Materials and Methods**

#### **Preparation of MS Basal Media**

The Murashige and Skoog <sup>[15]</sup> (MS) basal media were used as control and seed germination was prepared following the standard procedures for MS powder (4.4g) form preparation. MS powder form was added in a beaker filled with 800 ml 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 basal media 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 culture room under dark condition. Preparation of media was done a week before use.

#### **Seed Sterilization and Germination in the MS Media**

Seeds of broccoli were used to culture on MS <sup>[5]</sup> basal medium collected from nursery. The seeds were washed using 70% ethanol for 5 minutes, and then rinsed in 15% chlorox for 15 minutes. The seeds were brought into laminar flow and continued rinsed with sterile DH20 only for a few seconds. Then, the sterile seeds were germinated in MS basal media for 7 days. This process was carried out under aseptic condition 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.

#### **MS basal media with IBA and BAP (2nd time media preparation)**

The MS media with IBA and BAP 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 concentration from stock solution were added. The media with hormones prepared for five replicates of each hormone concentration. IBA and BAP concentrations were 1.0 and 2.0 mg/l.

#### **Root Culture on MS Supplemented with IBA (1, 2, & 3mg/l) and BAP (2 mg/l)**

The roots were collected from seedling and root tips were cut and put into the media with the hormone concentrations of IBA and BAP. There was per treatment was consisted of five replications.

#### **Shoot tip Culture on MS Supplemented with IBA (1, 2 & 3mg/l) and BAP (2 mg/l)**

After one week of germination, seven days seedlings were selected as source of explants. The hypocotyls explants shoot tip were cut and transferred into media with different concentrations of IBA and BAP. Each treatment was consisted of five replications.

#### **Leaf cutting slice Culture on MS Supplemented with IBA (1, 2 & 3mg/l) and BAP (2 mg/l)**

After one week of germination, seven days seedlings were selected as source of explants. The hypocotyls explants (leaf slice) transferred into media without auxin (control) and media with varying levels of IBA and BAP. Each treatment was consisted of five replications.

#### **Leaf extraction preparation**

The leaf samples at different concentration (IBA 1, 2 and 3 mg/l) and BAP 2mg/l) were ground with motor and pestle and filtered the extract and finally extracted sample was separated and stored in the freezer.

#### **Data analysis**

Biochemical (total sugar, antioxidants) content was determined.

#### **Glucose content**

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

#### **Inverted sugar**

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

#### **Pigment (Chlorophyll a & b) content**

Total chlorophyll was determined according to the methods of Lichtenthaler and Wellburn <sup>[16]</sup>.

#### **Total antioxidant**

1mM trolox standard solution was used. Water was poured to 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 1:1 ratio with Protein Mask. 20 µL of sample was used into wells. Distilled water was put in preparing the volume of 100 µL. 100 µL of Cu<sup>2+</sup> 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 made the measurement of the absorbance at 570 nm.

#### **Flavonoid**

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

#### **Total phenols**

Total phenolic content was determined using the Folin-Ciocalteu assay <sup>[17]</sup>. Folin-Ciocalteu (FC) colorimetry was consisted of a chemical reduction of reagent, tungsten mixture and molybdenum oxides. 1ml of leaf extract, gallic acid calibration standards, folin-Ciocalteu (FC) reagent were stored in the dark and separated until reagent had appeared as green, Sodium carbonate solution (100ml) was utilized in the volumetric flask. Spectrophotometer was placed to 765 nm, having 1cm, 2ml plastic or glass cuvettes. 1ml of extract was poured to 25 ml of volumetric flask, having 9 ml of distilled water. 1 ml of Folin-Ciocalteu's phenol reagent was poured to the mixture. The solution was diluted with distilled water and mixed well. At last, the solution was incubated at room temperature. Absorbance was determined against reagent blank at 750 nm using an UV Spectrophotometer.

#### **Nutrient or Mineral content**

Nutrient content (NO<sub>3</sub><sup>-</sup> and K<sup>+</sup>) was determined by using Horiba Scientific NO<sub>3</sub> and K meters (Japan). 3 drops of extract sample were put on the disc sensor of the meter using small dropper and data were displayed and recorded.

#### **Antioxidant as DPPH activity**

The DPPH free-radical scavenging activity was determined as described in Yang *et al* <sup>[18]</sup>.

#### **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 Least Significance Difference (LSD) Test at 5% probability level.

#### **Results**

##### **Callus formation**

The highest callus weight was found in the cultured from shoot and leaf cutting than root cutting in the concentration of IBA 2.0 + 1.0 mg/l BAP combination (Table 1).

##### **Antioxidant, carbohydrate, pigment and mineral properties**

Carbohydrate represented as inverted sugar and glucose, chlorophyll and nutrient content (K<sup>+</sup>, NO<sub>3</sub><sup>-</sup>, Ca<sup>++</sup> and Na<sup>+</sup>) were

found higher in the concentration of IBA 2.0+2.0 mg/l BAP combination from broccoli leaf cutting (Table 2). Moreover, total phenol, antioxidant (DPPH free radical scavenging activity), flavonoid and total antioxidant exhibited higher in the concentration of IBA 2.0+2.0 mg/l BAP combination than in the concentration of IBA 1.0 + 2.0 mg/l BAP and IBA 3.0 + 2.0 mg/l BAP combinations (Table 3). Figure 1 shows the image of the broccoli culture procedure.

### Discussion

From the above mentioned results, it can be discussed that the leaf and shoot cutting showed higher callus formation represented as callus weight, carbohydrate represented as inverted sugar and glucose, chlorophyll and nutrient content (K<sup>+</sup>, NO<sub>3</sub><sup>-</sup>, Ca<sup>++</sup> and Na<sup>+</sup>) than in different concentration of IBA 2.0 + 2.0 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 [19] 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<sup>1</sup> that the suitable part could be cultured depended on the species and explants reaction also depended on different condition of the mother plants. Plant tissue culture needs several organic chemical such as nitrogen, magnesium sulphate, phosphorus, sodium and chloride ion [21]. Different combination and concentration of hormone affects the plants growth. It was reported [22] that, the different concentration 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 George and Sherrington (1984) [19], callus formation was obtained if the concentration of auxin and cytokinin was same. For *Brassica olerace var italica* callus also obtained from media supplemented with different concentration of auxin and cytokinin [24] 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 [25].

The leaf extracts showed the highest free radical scavenging potential, sugars, total antioxidant, total phenol, chlorophyll, and nutrient content than other extracts in the concentration which are IBA and BAP (2 mg/l and 2 mg/l). This is due to the different parts of the plant produce different compounds or different amount of compounds due to their differential gene expression. Therefore, this particularly affects the antioxidant potential of the different parts of a given plant [26]. De-coloration due to reaction of antioxidants in extracts with the stable free DPPH radical was measured by spectrophotometer. 2, 2-diphenyl-2-picrylhydrazyl (DPPH) assay evaluates the ability of antioxidant to scavenge free radicals. Other factors that can increase or decrease the antioxidant compounds include samples condition and polarity of the extraction solvents [27].

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 the chlorophyll, anthocyanins, betalains (betacyanin and betaxanthin) and carotenoids are involved in leaf coloration. All of these components have been established to have antioxidant activities [27]. Hence, this proved that green color of leaves can affect or increase the antioxidant activity of leaf extracts in this study.

**Table 1:** Effects of different combination of hormone on fresh weight of callus produced from broccoli root tip, shoot tip and leaf cutting. Means followed by the common letters are not significantly different at the 5% level by Least Significant different test (LSDT). Mean  $\pm$  SE (n= 5).

Sources	IBA (1mg/l) + BAP (2mg/l)	IBA (2mg/l) + BAP (2mg/l)	IBA (3mg/l) + BAP (2mg/l)
Root tip cutting	2.30 $\pm$ 0.25a	2.4 $\pm$ 0.2a	1.8 $\pm$ 0.04a
Shoot tip cutting	2.35 $\pm$ 0.23a	2.37 $\pm$ 0.12b	1.85 $\pm$ 0.04b
Leaf cutting	2.40 $\pm$ 0.23a	2.47 $\pm$ 0.19a	2.35 $\pm$ 0.10a

**Table 2:** Effects of IBA and BAP on the sugar, chlorophyll and nutrient content from broccoli leaf cutting. Means followed by the common letters are not significantly different at the 5% level by Least Significant different test (LSDT). Mean  $\pm$  SE (n= 5)

IBA +BAP (mg/l)	Inverted sugar (mg/100g)	Glucose (mg/100g)	Chlorophyll ( $\mu$ g/g)	K+(PPM)	NO <sub>3</sub> - (PPM)	Ca <sup>++</sup> (PPM)	Na <sup>+</sup> (PPM)
1.0+2.0	3.3 $\pm$ 0.1ab	5.2 $\pm$ 0.2b	3.8 $\pm$ 0.1b	805 $\pm$ 0.1c	710 $\pm$ 0.2b	168 $\pm$ 0.6c	69 $\pm$ 0.6b
2.0+2.0	3.8 $\pm$ 0.2a	6.1 $\pm$ 0.1a	5.1 $\pm$ 0.2a	845 $\pm$ 0.2a	773 $\pm$ 0.4a	211 $\pm$ 0.6a	82 $\pm$ 0.4a
3.0+2.0	2.6 $\pm$ 0.2b	5.1 $\pm$ 0.3b	2.8 $\pm$ 0.2c	820 $\pm$ 0.3b	690 $\pm$ 0.2c	175 $\pm$ 0.6b	66 $\pm$ 0.7b

**Table 3:** Effects of IBA and BAP on the total phenol, antioxidant (DPPH) and total antioxidant from broccoli leaf cutting. Mean  $\pm$  SE (n= 5)

IBA +BAP (mg/l)	Total antioxidant (mg/100g)	DPPH activity (mg/100g)	Total Phenol (GAE/100g)	Flavonoid (mg/100g)
1.0+2.0	350 $\pm$ 0.9	9.2 $\pm$ 0.2	356 $\pm$ 0.6	3.70 $\pm$ 0.4
2.0+2.0	399 $\pm$ 0.4	15.2 $\pm$ 0.1	433 $\pm$ 0.9	2.90 $\pm$ 0.1
3.0+2.0	346 $\pm$ 0.2	11.3 $\pm$ 0.2	367 $\pm$ 0.3	1.9 $\pm$ 0.3

Callus produced per leaves explants, Average  $\pm$  SE of 5 replicates.



a. Broccoli growth b. Shoot and leaf cutting c. Callus proliferation from Shoot and leaf

**Fig1:** Photo shows the rooting, callus shoot and leaf proliferation formation

### Conclusion

It can be concluded that the best combination of the IBA and BAP in the concentration of 2.0 mg/l (IBA and BAP) to regenerate callus cell from root, shoot and leaf cutting of broccoli. In addition, the highest sugars, total antioxidant, total phenol, chlorophyll, DPPH and nutrient content was found in the concentration of IBA and BAP (2 mg/l and 2 mg/l) than IBA and BAP (1 mg/l and 2 mg/l) and IBA and BAP (3 mg/l and 2 mg/l).

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### Significant statement

Broccoli root, shoot and leaf slice cutting can be used as small scale. IBA and BAP can be used as low concentration (1-3 mg/l) which would be a cost effective in the experiment.

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