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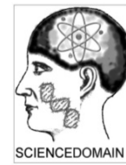

Cell Proliferation Using Broccoli Leaf Cutting *in vitro* Culture: Its Biochemical and Antioxidant Properties

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Authors' contributions

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

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ABSTRACT

Significance of the study: Tissue or cell culture keeps a significant role in micro-propagation in the plant production industry. Nowadays it has been successfully performed from many species in plant. Millions of explants can be produced by tissue or cell culture per year in any plant production industry.
Aim: The study was conducted to investigate the root, shoot and leaf formation from the leaf cutting *in vitro* culture of broccoli *(Brassica oleracea* var italic*)*.
Methodology: Different BAP (6-Benzylaminopurine) and IBA (Indole-butaric-acid) hormone (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 was used to culture leaf cutting *in vitro*.

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Results: The results showed that there was no shoot formed, but a few leaves and bulky callus proliferation were appeared. The highest number (6.75) of root proliferation was found in the concentration of 2.0 mg/l IBA + 0.25 mg/l BAP combination. 2,2-diphenyl-2-picrylhydarzyl (DPPH) free radical scavenging potential was higher (70%) in leaves extract than in callus extracts (46%) at concentration of 10 mg/ml.
Conclusion: The present results conclude that it is better to use the combination of BAP and IBA at different concentration to produce root proliferation, leaf initiation and callus formation in broccoli.

Keywords: Cell and tissue culture; leaf tip; IBA; BAP; explants.

1. INTRODUCTION

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 tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant called as totipotency [1]. Single cells, 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 or fruit plant like pineapple propagule can be produced by tissue culture from root, leaves, crown or stem per year [1]. Multiplication and total number of plantlets production were reported and recommended by using plant growth regulators. A total number of plantlets production ranging from 5000 [3], 40000 [4], 100000 [5], from single explant per year. Propagation of plant can be gained *in vitro* treated with BAP alone [6], mixture of hormones like BAP and naphthalene acetic acid (NAA) [7], indole butyric acid (IBA) [8], indole acetic acid (IAA) [9] and 2,4-dichlorophenoxy acetic acid (2,4-D) [4], combination of BAP and two auxins as NAA and IAA [10], IAA and IBA [11] and IBA [12]. 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 [6], 2.0 [13], 2.5 [14], 3.0 [7], and 4.0 mg/l [15], were recommended for multiplication of plantlet. Sujatha and Reddy [16] reported that the using wider concentration range in caster bean increased castor proliferation rate five times higher. They also reported that wider concentration range and mixture of hormones were not recommended now-a-days by Environmental biologists although it showed better shoot formation but, due to the pollution and not cost effective, it may be avoided. This study was done using the broccoli leaf cutting slice. There are not available literatures found on the present research. Therefore the following objectives were undertaken.

To regenerate broccoli plants from explants of the broccoli from leaf cutting via organogenesis.

To investigate the effect of the different concentration of auxin on the leaf, roots and callus formation from broccoli leaves cutting.

To investigate the antioxidant activity by using explant from *in vitro* culturing by observing their free radical scavenging activity.

2. MATERIALS AND METHODS

2.1 Preparation of Hormones Stock Solution

The hormones stock solution was prepared separately in 50 ml each. For IBA, 100 mg of IBA were dissolved in a few drops of 95% ethanol and make up final volume to 100 ml with distilled water.

2.2 Media Preparation

MS basal and MS modified with auxin prepared by adjusted the pH to 5.8 by using 1 N HCL and 1 M NaOH. Then, the media was fractional in 30 ml into jam jars 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 done a week before use to reduce water condensation in jam jars and to make sure the media completely sterilized.

2.3 Preparation of Murashige and Skoog Basal Media

The MS [17] basal media were used as control and seed germination was prepared follow the

standard procedures for MS powder form preparation (Table 1). MS powder form was added in a beaker filled with 800 ml distilled water. Then followed up with 30 g of sucrose, adjusted the pH and added 2.8 phyta gels. The media was made up until 1000 ml.

2.4 Preparation of MS basal Supplemented with IBA and BAP

The MS modified media with IBA and BAP were used as media, MS powder was 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 by using micropipette. Adjusted the pH and 2.8 g phyta gel was added and made the media until 1000 ml. The media with hormones prepared for five replicates of each hormone concentration. IBA and BAP concentrations were 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/l.

Table 1. Standard procedures for MS media preparation and hormone BAP

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

2.5 Seed Sterilization and Germination

Seeds of *I. aquatica* were obtained from the nursery. A total of 300 seeds were used to culture on MS [17] basal medium. About 60 jam jars were used to culture the seeds and about five seeds were germinated on every jam jars. The seeds were washed in 70% ethanol about 5 minute, and then rinsed in 15% chlorox about 15 minutes. The seeds were bringing 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 25-28℃.

2.6 Leaf Cutting Culture on MS Supplemented with IBA and BAP

After one week of germination, seven days seedlings were selected as source of explants. The hypocotyls explants with root transferred into media without auxin (control) and media with varying levels of IBA and BAP 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/l. Each treatment was consisted of five replications. Murashige and Skoog (MS) [17] basal and the modified MS media with auxin were used in this experiment. MS basal was used as control for rooting media and germination media, while MS modified with BAP and IBA were used as media. The rooting number was analyzed using statistical analysis. After that, *in vitro* roots cultures on MS basal and MS modified were used in cytological and antioxidants studies.

2.7 Antioxidant Activity of Broccoli

The antioxidant potential of crude petroleum which was methanol extracts from B.oleracea var. italica 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 taken at 515 nm using spectrometer. Using the following formula for vitamin c as the use of the percentage of free radical scavenging activity was done. Vitamin C = [(A control- A sample)/A control] x 100.

2.8 Statistical Analysis

Statistical analysis like standard error (SE) of the data was done. Least significance test (LSD) test was employed at 5% level of significance.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Root, leaf proliferation and callus formation

After two weeks of sub-culture, the roots have shown as the positive results. It was mostly produced roots on media with hormone and shown negative results for the formation of shoots. There was higher formation of root observed from the leaf cutting in different concentration of IBA and BAP (Table 2). Also the

formation of callus from leaf cutting mostly gave the best responds in media with combination of IBA and BAP at different concentrations. The root formation was found higher at 0.25 mg/l IBA + 1.5 mg/l BAP, 1.0 mg/l IBA + 3.5 mg/l BAP, 2 mg/l IBA + 0.25 mg/l BAP, 2.0 mg/l IBA + 1.5 mg/l BAP and 2.5 mg/l IBA + 1.5 mg/l BAP than other concentrations of hormone IBA and BAP. It was found that the highest root formation was occurred at the concentration of 2 mg/l IBA + 0.25 mg/l BAP (Table 2). Mostly the initial leaf proliferation, callus formation, then green and whitish callus as well as compact and globular callus formation later was found at the concentration of BAP 1.0-2.0, IBA 1-3.5 mg/l combination (Table 2). Callus weight was found higher at the concentration of 1.0 BAP+1.5 IBA (3.9), 2.0 BAP+0.25 IBA and 1.5 BAP+0.5 IBA, than other concentrations (Table 3). The second highest (2.74) was found at the concentration of 2.0 BAP and 0.25 IBA. However, the lowest (0.6) was found at the concentration of 1.0 BAP, 0.25 IBA (Table 3). Fig. 1 shows the germinated plant, root shoot formation from seed and callus proliferation from broccoli leaf tip.

These results were based on the neutralization of DPPH radical of samples in the free radical scavenging activity assay. BHT (butylated hydroxyl toluene) and Vitamin C (Ascorbic acid) were used as the positive control. The results (Fig. 2) showed the free radical scavenging potential of broccoli leaves extract (70%) is significantly higher than callus extracts (46%) at concentration of 10 mg/ml. At concentration of 20 mg/ml both extract showed lower antioxidant activity than at concentration 10 mg/ml which were leaves (53%) and callus (37%). Besides that both extracts also had significantly lower DPPH free radical scavenging activity compared to the positive control, vitamin C (91%) and BHT (86%).

Table 2. Effects of IBA and BAP in combination with IBA on roots and callus formation from broccoli leaves

IBA	BAP	No. of root formation	Observation of callus	Leaf proliferation
0	0	0.0±0.0	-	-
0	0.25	0.25±0.25	-	-
0	0.5	0.0±0.0	-	-
0	1.0	0.0±0.0	-	-
0	1.5	0.0±0.0	-	-
0	2.0	0.0±0.0	-	-
0	2.5	0.0±0.0	-	-
0	3.0	0.0±0.0	-	-
0	3.0	0.0±0.0	-	-
0.25	0.25	1.0±0.41	-	-
0.25	0.5	1.75±0.25	-	-
0.25	1.0	2.0±0.41	-	-
0.25	1.5	3.5±0.65	-	-
0.25	2.0	2.5±0.29	-	-
0.25	2.5	2.0±0.41	-	-
0.25	3.0	2.25±0.25	-	-
0.25	3.5	2.25±0.25	-	-
0.5	0.25	1.5±0.29	-	-
0.5	0.5	1.25±0.25	-	-
0.5	1.0	1.5±0.65	-	-
0.5	1.5	1.75±0.48	-	-
0.5	2.0	2.0±0.71	-	-
0.5	2.5	1.75±0.48	-	-
0.5	3.0	1.5±0.29	-	-
0.5	3.5	1.25±0.25	-	-
1.0	0.25	1.75±0.49	-	-
1.0	0.5	1.25±0.49	-	-
1.0	1.0	1.5±0.29	Callus formed	+
1.0	1.5	1.5±0.29	Greenand whitish callus	+
1.0	2.0	1.75±0.48	Compact and globular callus	+
1.0	2.5	1.5±0.29	-	-

IBA	BAP	No. of root formation	Observation of callus	Leaf proliferation
1.0	3.0	2.0±0.41	-	-
1.0	3.5	3.25±0.75	-	-
1.5	0.25	2.75±0.48	-	-
1.5	0.5	3.5±0.65		+
1.5	1.0	2.5±0.65	Callus formed	+
1.5	1.5	2.75±0.48	Green and whitish callus	+
1.5	2.0	1.5±0.65	Compact and globular callus	-
1.5	2.5	2.25±0.48	-	-
1.5	3.0	2.25±0.25	-	-
1.5	3.5	2.0±0.41	-	-
2.0	0.25	6.75±0.49	-	-
2.0	0.5	3.5±0.65	-	+
2.0	1.0	2.5±0.29	Callus formed	+
2.0	1.5	3.5±0.65	Green and whitish callus	+
2.0	2.0	2.5±0.29	Compact and globular callus	-
2.0	2.5	3.0±0.41	-	-
2.0	3.0	2.5±0.29	-	-
2.0	3.5	2.75±0.25	-	-
2.5	0.25	2.5±0.65	-	-
2.5	0.5	2.25±0.25	-	+
2.5	1.0	2.5±0.29	Callus formed	+
2.5	1.5	4.0±0.41	Green and whitish callus	+
2.5	2.0	2.0±0.41	Compact and globular callus	-
2.5	2.5	3.0±0.71	-	-
2.5	3.0	2.5±0.29	-	-
2.5	3.5	2.5±0.29	-	-
3.0	0.25	2.0±0.41	-	-
3.0	0.5	4.0±0.41	-	+
3.0	1.0	2.5±0.29	Callus formed	+
3.0	1.5	2.75±0.25	G Green and whitish callus	+
3.0	2.0	3.5±0.65	Compact and globular callus	-
3.0	2.5	3.0±0.41	-	-
3.0	3.0	2.5±0.29	-	-
3.0	3.5	2.75±0.63		-
3.5	0.25	2.75±0.48	-	-
3.5	0.5	4.0±0.41	-	+
3.5	1.0	3.25±0.48	Callus formed	+
3.5	1.5	2.5±0.29	Green and whitish callus	+
3.5	2.0	2.75±0.48	Compact and globular callus	
3.5	2.5	2.5±0.29	-	
3.5	3.0	3.0±0.41	-	
3.5	3.5	2.5±0.29	-	

Mean ± SE of 10 replicates. + = organ (leaf) formation was indicated. - no-indication of organ formation

Seed germination

Shoot and leaves growth

Callus and leaf formation from leaf cutting

Fig. 1. Photo shows the callus and explants from leaf cutting of broccoli

Table 3. Effects of different combination of hormone on fresh weight of callus produced from leaves

BAP	IBA	Callus weight
1.0	0.25	0.6±0.15
	0.5	1.02±0.06
	1.0	1.4±0.09
	1.5	3.9±0.15
	2.0	2.35±0.23
	2.5	1.8±0.11
	3.0	2.15±0.07
1.5	3.5	2.18±0.4
	0.25	2.7±0.15
	0.5	2.4±0.04
	1.0	2.37±0.12
	1.5	2.45±0.10
	2.0	2.3±0.09
	2.5	2.35±0.06
	3.0	2.0±0.17
2.0	3.5	2.22±0.10
	0.25	2.47±0.19
	0.5	1.4±0.15
	1.0	1.85±0.04
	1.5	2.15±0.06
	2.0	2.15±0.06
	2.5	2.35±0.12
	3.0	2.3±0.04

Callus produced per leaves explant, Mean ± SE of 10 replicates

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

Samples	OD reading (515 nm)
Control (ethanol 95%)	0.058
Vitamin C	0.005
BHT	0.008
Leaves extracts (10 mg/ml)	0.017
Callus extracts (10 mg/ml)	0.031
Leaves extracts (20 mg/ml)	0.027
Callus extracts (20 mg/ml)	0.036

3.2 Discussion

Our results were found the optimization of the cell culture and regenerate the root and callus proliferation. For the formation of roots the best medium to give the highest average number for root was medium supplemented with 0.25 mg/l BAP and 2.0 mg/l IBA. It showed significant difference with respect to the average number of roots produced per leaf cutting compared to other treatments. Root formation occurred within 10-20 days after transferring shoots and leaf to the rooting treatments.

Callus was formed from leaf explants which were leaf tips. The duration to form callus for each explant after two weeks of sub-cultured. But after the four weeks the leaf explants produced bigger callus and small leaf proliferation in diameter and was heavy based on the fresh weight (g). Leaf explants produced callus with the green and whitish color with the weight 6.9 g. Callus was initiated from the margin along the cut surface. Callus also developed from the surface of the leaf in contact with the culture medium. Vigorous callus proliferation was observed from the basal part of the explants. However, general morphology of the callus was similar in case.

These concentrations might not be suitable for root proliferation and callus formation. George and Sherrington [18] reported that growth and morphogenesis of cell culture or organ were affected by genotype, substrate, environment and tissues have been used. Koa and Michayluk [19] reported that, the genotype which had the high capability was important to be chosen to produce good regeneration in tissue culture. For optimum success, explants were obtained from healthy vigorous plants. Practically, any part of the plant could be successfully cultured *in vitro* and can regenerate plantlets provided the explants were obtained at the proper physiologically stage and development. The age of the explants could be very important. The young explants have the high potential to divide rapidly compared to matured explants. Alsaif et al. [1] reported that the suitable part to be cultured depends to the species and explants reaction also depends to different condition of the mother plants such as grow condition and age of the explants. Besides that, soft woody plant or non woody plant was easy to culture compared to woody plants.

The media which have been used in this experiment was Murashige and Skoog [17] as based medium which can give the best grow to all types of plants. This media contained organic salts, plant growth regulator, vitamin, amino acid, carbohydrate, and matric medium which enough to complete plant growth. Plant tissue culture needs several organic chemical such as nitrogen, magnesium sulphate, phosphorus, natrium and chloride ion [20].

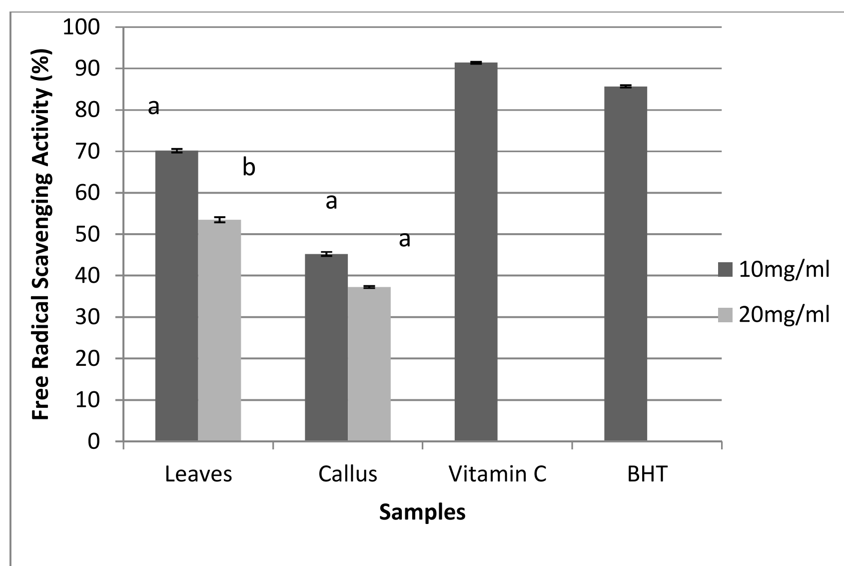

Fig. 2. The antioxidant activity of the selected parts (leaves and callus) of *Brassica oleracea* var italica at concentration of 10 and 20 mg/ml. Same letters in column are represented as not significant by LSD at 5% level of significance

The effects of pH, temperature, light and oxygen are all parameters that must be examined in the studied of plant tissue culture. The pH media used in this study was adjusted to 5.8, which is optimal pH for the growth of plant. If the pH was below 5.5, the agar will not gel properly and above 6.0, the gel may be too firmed. Usually, pH of media drop 0.6-1.3 units after autoclaving [21]. Cultures of some plant tissue cause a pH drop over time that is attributed to the production of organic acids or nitrogen utilization. Media pH is influenced by the inorganic salts, carbohydrate source, gelling agents and medium storage method [21]. Light also influences the success of *in vitro* cultures. Light is an important components in photosynthesis. But sucrose was provided in *in vitro* cultures as energy sources, light is not so important for photosynthesis but it plays a role in process called photo-morphogenesis which develops organ such as shoot and roots. Several studies have demonstrated that light plays an important role in inducing organogenesis.

Different combination and concentration of hormone affects the plants growth. Fujita [22] reported 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].

Results showed the effects of hormone on the callus growth. Callus formation was obtained from leaf tips in media supplemented with different combinations and concentrations of hormone. According to George and Sherrington [18], callus formation was obtained if the concentration of auxin and cytokinin was same. But, actually this statements was suitable only for certain species. 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 callus and leaf extracts of broccoli were evaluated. There are many different antioxidant components in plants it is relatively difficult to measure each antioxidant components separately. Therefore, several different methods have been developed to evaluate the antioxidant activity of biological samples [25].

Results showed the free radical scavenging potential of broccoli leaf extract (70%) was significantly higher than callus extracts (46%) at concentration of 10 mg/ml. At concentration of 20 mg/ml both extract showed lower antioxidant activity than at concentration 10 mg/ml which are leaves (53%) and callus (37%). Besides that both extracts also had significantly lower DPPH free radical scavenging activity compared to the positive control, vitamin C (91%) and BHT (86%). Based on the results, the leaf extracts showed the highest free radical scavenging potential than callus extract in both different concentration

which are 10 mg/ml and 20 mg/ml. This is because 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-picrylhydarzyl (DPPH) assay evaluates the ability of antioxidant to scavenge free radicals. The DPPH molecule characterized as stable-free radical by virtue of the delocalization of the spare electron over the molecule; delocalization gives rise to a deep violet color, characterized by an absorption band in methanol solution centre at 515 nm. When DPPH methanol solution mixed with antioxidant that act as a hydrogen donator, DPPH free radicals will reduced to diphenylpicrylhydrazine form, with the loss of violet color. Antioxidant in any test sample may be influenced by several factors such as extraction methods by environmental factors such as climatic growth conditions, growth, ripening stage, temperature and duration of storage. 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. However, most of the leafy vegetables are green. Color is the most important trait used in consumer evaluation of product quality, playing a decisive role in the acceptability of the product color may be considered an index for estimating the antioxidant properties of the leafy vegetables. The naturally occurring compounds adequate for food colouring 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, it was proved that green color of leaves affected or increased the antioxidant activity of leaf extracts.

4. CONCLUSION

The best medium for callus proliferation of this vegetable crop was MS basal medium supplemented with 1-1.5 mg/l IAA, BAP and IBA from root tips. For roots formation, the best medium was MS basal medium supplemented with 1-2.5 mg/l IAA, BAP and IBA. Free radical scavenging potential of broccoli leaves extract (70%) was significantly higher than callus extracts (46%) at concentration of 10 mg/ml. Besides that both extracts also had significantly lower DPPH free radical scavenging activity compared to the positive control, vitamin C (91%) and BHT (86%).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Al-Saif A, Hossain ABMS, Taha RM. Effect of benzylamino purine and naphthalene acetic acid on proliferation and shoot growth *in vitro*. African J Biotechnol. 2011; 10:5291-5.
2. Sathyanarayana BN. plant tissue culture: practices and new experimental protocols. I.K. International. 2007;106. ISBN 978-81-89866-11-2.
3. Zepeda C, Sagawa Y. *In vitro* propagation of pineapple. Hortic. Sci. 1981;16:495.
4. Liu LJ, Rosa-Marquez E, Lazardi E. Smooth leaf (spineless) red Spanish pineapple (*Ananas comosus* (L.) Merr) propagated *in vitro*. J Agric Univ Puerto Rico. 1989;73:301-11.
5. Sripaoraya S, Marchant R, Power JB, Davey MR. Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L.). *In vitro* cell development. Biol Plant. 2003;39:450-4.
6. Be LV, Debergh PC. Potential low cost micropropagation of pineapple (*Ananas comosus*). S Afr J Bot. 2006;72:191-4.
7. Firoozabady E, Gutterson N. Cost effective *in vitro* propagation methods for pineapple. Plant Cell Rept. 2003;21:844-50.
8. Boxus P, Terzi JM, Lieves C, Pylyser M, Ngaboyamahina P, Duhem K. Improvement and perspectives of micropropagation techniques applied to

some hot climate plants. Acta Hortic. 1991;289:55-9.

9. Hamad AM, Taha RM. The effect of different hormones and incubation periods on *in vitro* proliferation of pineapple (*Ananas comosus* L.) Merr cv. Smooth Cayenne) shoot-tip culture. Pak J Biol Sci. 2008;11:386-91.
10. Mathews HV, Rangan TS. Multiple plantlets in lateral bud and leaf explant *in vitro* cultures of pineapple. Sci Hortic. 1979;11:319–28.
11. Teixeira SL, Ribeeiro JM, Teixeira MT. Influence of NaClO on nutrient medium sterilization and on pineapple (*Ananas comosus* cv. Smooth cayenne) behavior. Plant Cell Tiss Organ Cult. 2006;86:375-8.
12. Soneji JR, Rao PS, Mhatre M. Somaclonal variation in micropropagated dormant axillary buds of pineapple (*Ananas comosus* L. Merr.). J Hort Sci Biotechnol. 2002;77:28-32.
13. Bhatia P, Ashwath N. Development of rapid method for micropropagation of a new pineapple (*Ananas comosus* (L.) Merr. clone Yeppoon gold. Acta Hortic. 2002; 575:125-31.
14. Smith MK, Ko HL, Hamill SD, Sanewski GM. Pineapple transformation: Mangging somaclonal variation. Acta Hortic. 2002; 575:69-74.
15. Omokoio ND, Tita MA, Niemenak N. Direct *in vitro* regeneration of *Ananas comosus* L. Merr. Var. Cayenne from crowns cultivated in a liquid medium. Fruits. 2001;56:415-21.
16. Sujatha M, Reddy TP. Differental cytokinin effects on the stimulation of *in vitro* shoot proliferation from meristematic explants of castor (*Ricinus communis* L.). Plant Cell Rept. 1998;17:561-6.
17. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15:473-97.
18. George EF, Sherrington PD. Plant propagation by tissue culture. Exegetics Ltd., Eversley, England; 1984.
19. Kao KM, Michayluk MR. Embryoid formation in alfalfa cell suspension cultures from different plants. *In vitro*. 1981;17: 645–8.
20. Hossain ABMS, Mizutani F. Growth regulatory effect of cytokinin in peach trees as affected by ringed bark strip by using bioassay *in vitro*. Can J Pure Appl Sci. 2009;3(1):655-9.
21. Gautheret RJ. Factors affecting differentiation in plant tissue grown *in vitro*. In: Cell differentiation and morphogenesis. Beerman W, editor. North Holland Publ. Co. 1966;55-95.
22. Fujita Y. The production of industrial compounds. In: Plant Tissue Culture: Application and Limitations. Bhojwani SS, editor. 1990;259-73.
23. Wirakarnain S, Hossain ABMS, Chandran S. Plantlet production through development of competent multiple meristem cultures from Male Inflorescence of Banana, Musa acuminta cv. Pisang Mas (AA). Am J Biochem Biotechnol. 2008; 4(4):325-8.
24. Ying Q, Hong-Ling L, Yang-Dong G. High-frequency embryogenesis, regeneration of broccoli (*Brassica oleracea* var. italica) and analysis of genetic stability by RAPD. Sci Hortic. 2006;111:203–8.
25. Chang CT, Hseueh YL, Shung HY. Purification and properties of chitinase from cabbage stems with roots. Biochem Mol Biol Int. 1996;40:417–25.
26. Arash R, Koshy P, Sakeran M. Antioxidant potential and content of phenolic compounds in ethanolic extracts of selected parts of *Andrographis paniculata*. J Med Plant Res. 2010;4:197-202.
27. Ali MB, Laila K, Shinya O. Comparative study on functional components, antioxidant activity and color parameters of selected colored leafy vegetables as affected by photoperiods. J Food Agr Environ. 2009;7:392-8.

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