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Organogenesis of *Brassica oleracea* var. *italica* Through *In Vitro* Culture

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Abstract: ***Brassica oleracea* var. *italica* or commonly known as broccoli is an edible plant grown in Cameron Highland, Malaysia. This research aims to study organogenesis of broccoli through *in vitro* culture. The seeds were serially sterilized using 70%, 50%, 20% and 10% sodium hypochlorite. Subsequently, the seeds were sterilized using 70% alcohol. Sterilized broccoli seeds were cultured onto Murashige and Skoog, 1962 (MS) basal medium. *In vitro* response of explant growth was observed. Seed germination was observed within day 12 of seed culture. Elongation of shoots was observed after three to four week of culture. Complete plantlets were observed after 6 weeks of culture. Further organogenesis response was observed when shoot, stem and root explants of eight-week-old aseptic seedlings were then transferred onto Murashige and Skoog medium added with combination of Naphthalene Acetic Acid (NAA) and Benzylaminopurine (BAP). Complete plant regeneration was successfully achieved when root, stem and leaf aseptic explant was cultured on MS medium supplemented with NAA and BAP plant growth regulators. The experimental design used in this study was Completely Randomized Design (CRD). MS medium supplemented with 1.0 mg/L IBA + 0.5 mg/L BAP found to be the optimum medium for shoot regeneration with 5.367±0.768 shoot per explant. Meanwhile 2.0 mg/L NAA shows the optimum medium for leaf and root explant for root induction producing 7.567±0.498 and 8.767±0.707 roots per explant. Micropopagation of *Brassica oleracea var. Italica* was successfully achieved through plant tissue culture process and proven to be a good alternative to conventional planting.**

Keywords: **organogenesis, *in vitro*, Murashige and Skoog medium, seedlings, plantlets.**

I. INTRODUCTION

B*rassica oleracea var. italica* or commonly known as Broccoli is derived from Brassica family. Broccoli has has a lot of nutrition that are good for human body [1]. According to Ong (2008) [2], by taking broccoli regularly, risk of breast, colon and lung cancer can be lowered. Broccoli has high content of antioxidant such as sulforus, beta-carotene, and indole. By taking food that contain antioxidant regularly, the percentage of oesophagus and stomach cancer can be reduced. Besides, antioxidant helps to prevent the harmful effects from the by-product of oxidation [2]. Carotenes, vitamin C and E are the food that contains major antioxidant that are beneficial to human health. This showed that broccoli has high important value in daily intake.

Broccoli are highly vulnerable to high temperature and these vegetable crops grow best in cool climate [3]. In order to propagate broccoli in low land area, plant tissue culture or organogenesis technique can be used as a platform. Plant tissue culture also referred to as *in vitro*, axenic or sterile culture [4]. Organogenesis has been widely reported as the most popular method for the formation of *Brassica* crops. Organogenesis is a technique to produce a plant using explants in sterile or aseptic media and environment. According to Darus (2010), organogenesis can be used to overcome lack of seedling problems [5]. By organogenesis, plants that are free from diseases can be produced. Besides, organogenesis also can enhance the growth of plant that is difficult to growth in normal climate. There are many benefits of broccoli that consumers find very nutritious.

Organogenesis are the alternative technique that can be used to propagate Broccoli. Hormones are used as a growth regulator in organogenesis. This is proven by Widiyanto and Erytrina (2001), which they have carried the *in vitro* technique for clonal propagation of Broccoli. The aims of their research were to investigate how growth regulator influences axillary shoot induction and root formation. Benzyl adenine (BA) and Kinetin (KIN) were combined with Naphthalenacetic Acid (NAA) to promotes shoot reproduction. Meanwhile for root formation, NAA and Indole Acetic Acid (IAA) were applied. Their research showed that BA induces more adventitious shoot compare to other cytokinin. While for root formation, NAA, IAA and Indole Butyric acid (IBA) at 0.1-10.0 µM can induce root formation effectively compare to other concentration. At the end of their research, the plantlets were efficiently transferred to a soil sand mixture medium (1:1) and they were naturally grown in green house. The survival rate of plant regeneration was 90 -95%.

Previous studies have shown many shoot regenerations from many varieties of *B. oleracea* were done but, there are generally low in rate of shoot production and number of shoot per explant [6]. Studies carried out by them were aimed to identify whether peduncles explant are suitable to get high level of shoot regeneration of *B. oleracea* varieties. The results showed that peduncles explant can be used for micropropagation and multiplication

Other researchers who used organogenesis technique for *B. oleracea* var. *italica* were Ravanvar et.al., 2014. Their main objectives were to examine the effect of Thidiazuron (TDZ) either alone or in combination with NAA on *in vitro* adventitious shoot formation from cotyledon explants [7]. Studies showed by using TDZ with or without NAA, adventitious shoot formation was influenced. The addition of 0.1 mg/L TDZ with 0.1 mg/L NAA to the culture medium was the best combination to produce shoot formation which produced 76.66% shoot formation and 0.9 shoot per explants.

Hussain et al., 2012 stated that, tissue culture technique has been widely applied for plant multiplication in large scale and it is considered to be the most effective technique for the improvement of crop productivity [8]. Tissue culture is also important in the propagation of plant, elimination of plant disease, improvement of plant diseases, improvement of plant production, and secondary metabolite production. Through this technique, small cutting of plant which is known as explant can produce hundreds and thousands of plants in short time. This shows that plant tissue culture or commonly known as micropropagation helps to produce plant in a very effective and efficient ways.

II. MATERIAL AND METHOD

A. Source of Seeds

The seeds of *B. oleracea* var. *italica* obtained from Green World Genetics Sdn. Bhd. from Kepong, Kuala Lumpur, Malaysia.

B. Preparation of MS (Murashige and Skoog, 1962) Medium

The basic medium used in this study was Murashige and Skoog (MS). This medium was chosen based on the previous study by other researchers. According to Hasbullah (2010), thirty g/L of sucrose was added to 800mL of distilled water [9], followed by adding 8.0g/L agar into the solution, and 4.0g/L of Murashige and Skoog medium (1962). Next, distilled water was added into the solution as to reach a total amount of 1L. The pH was then adjusted to approximately 5.8 by adding hydrochloric acid to reduce the pH value or sodium hydroxide to rise the pH value. Subsequently, solution was stirred and heated for a period of time to ensure that the agar was entirely dissolved. The medium was then autoclaved at a temperature 121°C, 15 psi (103 kPa) for 21 minutes prior to being transferred and cooled in the laminar flow cabinet until the media is in a solid state.

C. Sterilization of Seeds

Seeds of *B. oleracea* var. *italica* have been surface sterilized prior to being inoculated onto the medium agar in order to avoid contamination from occurring. The purpose of surface sterilization process is because microorganisms always naturally contaminate the living plant materials from the environment on the surface and sometimes on the internals. Chemical solutions such as alcohol or bleach were required to carry out surface sterilization process [10]. Surface sterilization of the seeds was carried out by using 70%, 50%, 20%, and 10% sodium hypochlorite for 5 minutes respectively. Lastly, the seeds were cleaned using distilled water. All of this surface sterilization process was done outside the laminar flow. The next process took place in the sterilized laminar flow chamber. Laminar flow was sterilized by turning on the UV lamp about one hour. In the laminar flow, 70% ethanol was applied to the seeds for 30 seconds and rinsed with autoclaved distilled water. By using autoclaved filter paper, the seeds were dried before they were cultures on

the MS basal medium (Murashige and Skoog, 1962). Three to four seeds were transferred into each jar containing the media. In order to prevent contamination, the jars were tightly capped using parafilm. The cultures were incubated at temperature 25±1°C under a 16 hours lights and 8 hours dark photoperiod for germination.

D. Preparation of Explant

In this study, sterile mature seeds and three types of explants comprising leaf disc, stem, and root segments of *B. oleracea* var. *italica* were used. The explants obtained from *in vitro* seedling were excised approximately 1-5 mm in length and cultured on MS medium with additional of different concentrations of plant growth regulators such as NAA, BAP, IBA, IAA, 2,4-D, and Zeatin. All cultures were examined for *in vitro* propagation response under the same condition of *in vitro*.

E. Screening for Suitable NAA and BAP for Shoot and Root Regenerations

Experiment was performed to examine the effect of various concentrations of growth regulators, BAP and NAA, on micropropagation of *B. oleracea* var. *italica*. After being cultured for 5 weeks, *in vitro* plantlets were excised into small pieces and then transferred into the MS medium with different plant growth regulator for additional *in vitro* propagation. In this case, basal MS medium functions as control. The following list entails the media with supplemented with various concentrations of plant growth regulators:

1 MS medium + 0.5 mg/L BAP + 0.5 mg/L NAA
2 MS medium + 0.5 mg/L BAP + 1.0 mg/L NAA
3 MS medium + 0.5 mg/L BAP + 1.5 mg/L NAA
4 MS medium + 0.5 mg/L BAP + 2.0 mg/L NAA
5 MS medium + 1.0 mg/L BAP + 0.5 mg/l NAA
6 MS medium + 1.0 mg/L BAP + 1.0 mg/L NAA
7 MS medium + 1.0 mg/L BAP + 1.5 mg/L NAA
8 MS medium + 1.0 mg/L BAP+ 2.0 mg/L NAA
9 MS medium + 1.5 mg/L BAP + 0.5 mg/L NAA
10 MS medium + 1.5 mg/L BAP + 1.0 mg/L NAA
11 MS medium + 1.5 mg/L BAP + 1.5 mg/L NAA
12 MS medium + 1.5 mg/L BAP + 2.0 mg/L NAA
13 MS medium + 2.0 mg/L BAP + 0.5 mg/L NAA
14 MS medium + 2.0 mg/L BAP + 1.0 mg/L NAA
15 MS medium + 2.0 mg/L BAP + 1.5 mg/L NAA
16 MS medium + 2.0 mg/L BAP + 2.0 mg/L NAA
17 MS medium + 0.0 mg/L NAA + 0.0 mg/L BAP (control)

III. DATA ANALYSIS

All data was analyzed statistically. Analysis of variance (ANNOVA) using Duncan's Multiple Range Test (DMRT) was performed in all data obtained. Mean with different letters in similar column has a significant difference at p=0.05.

IV. RESULT AND DISCUSSION

A. Identification of Shoot Regeneration Media on MS Supplemented with NAA and BAP

In this study, stem, leaf and root were used as source of explant. Different concentration of NAA and BAP were added into MS media for shoot regeneration. Based on the data, only two explants gave respond which were stem and leaf. Root did not respond to the shoot regeneration. As for stem explants, nine treatments gave respond for shoot regeneration. MS medium comprising 1.0 mg/L NAA + 1.5 mg/L BAP gave optimum respond which was 3.000±0.434 shoots per explant (Fig. 1a), followed by 1.5 mg/L NAA + 0.5 mg/L BAP with 1.400±1.400 shoots per explant. However, there were eight treatments that did not responded to shoot development in stem explant.

Based on table 1, shoot only emerged at two treatments for leaf explant which was when MS medium were supplemented with 0.5 mg/L NAA + 1.0 mg/L BAP and 1.0 mg/L NAA + 0.5 mg/L BAP. Optimum respond was achieved when explants were cultured on MS medium supplemented with 0.5 mg/L NAA + 1.0 mg/L BAP with 0.600 ± 0.189 shoots per explant (Fig. 1b).

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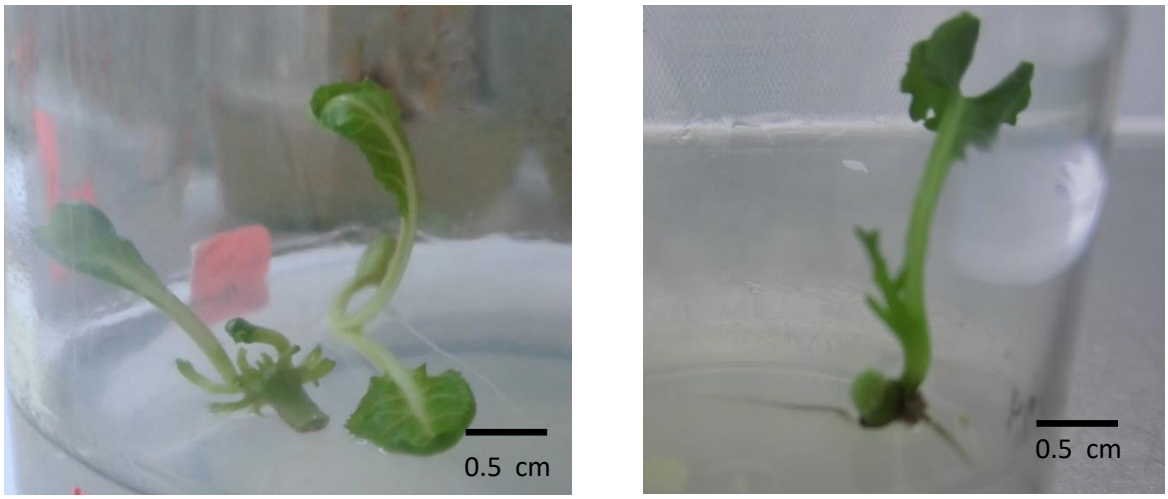
Table 1: The Effect of Different Concentration and Combinations of NAA and BAP on Stem and Leaf Explants Cultured on MS Media for Regeneration of Shoots at 25±1°C with 16 Hours Light and 8 Hours Darkness.

No	Treatments	Explants (Mean ± SE)	
		Stem	Leaf
1	0.5 mg/L NAA + 0.5 mg/L BAP	nr	nr
2	0.5 mg/L NAA + 1.0 mg/L BAP	$1.100\pm0.211_{b}$	$0.600 \pm 0.189_{a}$
3	0.5 mg/L NAA + 1.5 mg/L BAP	$0.200\pm0.139_{c}$	nr
4	0.5 mg/L NAA + 2.0 mg/L BAP	nr	nr
5	1.0 mg/L NAA + 0.5 mg/L BAP	nr	$0.200 \pm 0.074_{b}$
6	1.0 mg/L NAA + 1.0 mg/L BAP	nr	nr
7	1.0 mg/L NAA + 1.5 mg/L BAP	$3.000\pm0.434_{a}$	nr
8	1.0 mg/L NAA + 2.0 mg/L BAP	nr	nr
9	1.5 mg/L NAA + 0.5 mg/L BAP	$1.400\pm1.400_{b}$	nr
10	1.5 mg/L NAA + 1.0 mg/L BAP	$0.400\pm0.123_{c}$	nr
11	1.5 mg/L NAA + 1.5 mg/L BAP	$1.100\pm0.268_{b}$	nr
12	1.5 mg/L NAA + 2.0 mg/L BAP	nr	nr
13	2.0 mg/L NAA + 0.5 mg/L BAP	nr	nr
14	2.0 mg/L NAA + 1.0 mg/L BAP	$1.067\pm1.852_{b}$	nr
15	2.0 mg/L NAA + 1.5 mg/L BAP	nr	nr
16	2.0 mg/L NAA + 2.0 mg/L BAP	$0.067\pm0.463_{c}$	nr
17	0.0 mg/L NAA + 0.0 mg/L BAP	$1.333\pm0.586_{b}$	nr

Mean ± SE, n=30. Mean with Different Letters in the Same Column Differ Significantly at p=0.05.

*nr: non-responsive

In shoot regeneration of MS media supplemented with NAA and BAP, optimum respond for the shoot formation from stem explant were MS medium supplemented with 1.0 mg/L NAA + 1.5 mg/L BAP with 3.000±0.434 shoot per explant. Meanwhile optimum respond for shoot formation in leaf explant were MS medium supplemented with 0.5 mg/L NAA + 1.0 mg/L BAP with 0.600±0.189 shoots per explant. This is supported by Panjaitan et al., 2007 where the highest mean number of shoot formation per explant can be obtained with the mixture of 0.05 mg/L NAA with 1.0 mg/L BAP [11]. Shoots can be induced from explant when auxin concentration is lower than cytokinine [12]. This is proven in this experiment when shoot formation for NAA and BAP supplemented hormone optimally achieved when auxin concentration which is NAA is lower that BAP concentration. This showed that the function of BAP hormone was essential in the formation of shoots [13]. Teo (1992) stated that one of the cytokinin function were stimulation root formation [14].



(a) (b)

Fig 1. Identification of Shoot Regeneration Media of *Brassica oleracea* var. *italica.* (a) Shoot regeneration from Stem Explant for Concentration 1.0 mg/L NAA + 1.5 mg/L BAP; (b)) Shoot regeneration from Leaf Explant for Concentration 0.5 mg/L NAA + 1.0 mg/L BAP

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B. Identification of Root Induction Media on MS Supplemented with NAA and BAP

Table 2 described rooting effect shown by explant tested with MS media supplemented with various concentration of NAA and BAP. Shoot, leaf and root were used as explant for this treatment. For stem explant all treatment gave respond towards treatment given. Treatment with 0.5 mg/L NAA + 1.0 mg/L BAP showed optimum respond with 7.767 ± 0.752 roots per explant (Fig. 2a), followed by 0.5 mg/L NAA + 0.5 mg/L BAP with 6.100 ± 0.734 roots per explant and 1.0 mg/L NAA + 1.5 mg/L BAP with 5.600 ± 0.514 roots per explant.

For leaf explant, explants responded to all treatments. Optimum treatment for rooting in leaf explant was treatment 2.0 mg/L NAA + 0.5 mg/L BAP with 7.567 ± 0.498 roots per explant (Fig. 2b), followed by 0.5 mg/L NAA + 0.5 mg/L BAP treatment and 0.5 mg/L NAA + 1.0 mg/L BAP. Meanwhile for root explant, MS medium supplemented with 2.0 mg/L NAA + 0.5 mg/L BAP was identified as the optimum medium for root with 8.767 ± 0.707 roots per explant (Fig. 2c), followed by 1.5 mg/L NAA + 1.5 mg/L BAP with 7.633 ± 0.497 roots per explant and 2.0 mg/L NAA + 1.0 mg/L BAP with 7.233 ± 0.527 roots per explant.

Table 2: The Effect of Different Concentration and Combinations of NAA and BAP on Stem, Leaf and Root Explants Cultured on MS Media for Regeneration of Root at 25±1ºC with 16 Hours Light and 8 Hours Dark.

No	Treatments	Explants (Mean ± SE)		
		Stem	Leaf	Root
1	0.5 mg/L NAA + 0.5 mg/L BAP	$6.100 \pm 0.734_{b}$	$6.233 \pm 0.678_{b}$	$6.500 \pm 0.851_{c}$
2	0.5 mg/L NAA + 1.0 mg/L BAP	$7.767 \pm 0.752_{a}$	$6.133 \pm 0.535_{c}$	$3.100 \pm 0.533_{g}$
3	0.5 mg/L NAA + 1.5 mg/L BAP	$0.200 \pm 0.743_{i}$	$5.000 \pm 1.037_{g}$	$1.900 \pm 0.366_{h}$
4	0.5 mg/L NAA + 2.0 mg/L BAP	$4.400 \pm 0.768_{e}$	$4.600 \pm 0.400_{g}$	$3.333 \pm 0.388_{g}$
5	1.0 mg/L NAA + 0.5 mg/L BAP	$4.267 \pm 0.540_{f}$	$5.133 \pm 0.476_{ef}$	$3.667 \pm 0.375_{c}$
6	1.0 mg/L NAA + 1.0 mg/L BAP	$4.300 \pm 0.350_{ef}$	$3.467 \pm 0.345_{g}$	$4.300 \pm 0.350_{e}$
7	1.0 mg/L NAA + 1.5 mg/L BAP	$5.600 \pm 0.514_{c}$	$5.233 \pm 0.354_{de}$	$4.700 \pm 0.767_{e}$
8	1.0 mg/L NAA + 2.0 mg/L BAP	$4.067 \pm 0.514_{f}$	$4.533 \pm 0.367_{g}$	$1.400 \pm 0.367_{h}$
9	1.5 mg/L NAA + 0.5 mg/L BAP	$4.000 \pm 0.258_{f}$	$5.933 \pm 0.555_{d}$	$2.300 \pm 0.284_{h}$
10	1.5 mg/L NAA + 1.0 mg/L BAP	$2.833 \pm 0.225_{h}$	$3.500 \pm 0.266_{g}$	$4.200 \pm 0.539_{e}$
11	1.5 mg/L NAA + 1.5 mg/L BAP	$5.100 \pm 0.413_{d}$	$4.400 \pm 0.373_{g}$	$7.633 \pm 0.497_{b}$
12	1.5 mg/L NAA + 2.0 mg/L BAP	$4.267 \pm 0.531_{f}$	$3.900 \pm 0.629_{g}$	$2.300 \pm 0.254_{h}$
13	2.0 mg/L NAA + 0.5 mg/L BAP	$5.133 \pm 5.133_{d}$	$7.567 \pm 0.498_{a}$	$8.767 \pm 0.707_{a}$
14	2.0 mg/L NAA + 1.0 mg/L BAP	$3.667 \pm 0.308_{fg}$	$4.600 \pm 0.388_{g}$	$7.233 \pm 0.527_{b}$
15	2.0 mg/L NAA + 1.5 mg/L BAP	$2.533 \pm 0.520_{h}$	$4.800 \pm 0.408_{g}$	$6.700 \pm 0.607_{c}$
16	2.0 mg/L NAA + 2.0 mg/L BAP	$3.467 \pm 0.274_{g}$	$4.200 \pm 0.330_{g}$	$5.300 \pm 0.510_{d}$
17	0.0 mg/L NAA + 0.0 mg/L BAP	$1.800 \pm 0.133_{h}$	$2.000 \pm 0.166_{h}$	$2.267 \pm 0.166_{h}$

Mean ± SE, n=30. Mean with Different Letters in the Same Column Differ Significantly at p=0.05

In the present study, all explant gave respond towards MS media supplemented with various concentration NAA and BAP hormone for root initiation. Addition of 2.0 mg/L NAA induced root formation from leaf and stem explant. The effect of 2.0 mg/L NAA showed the highest root formation for leaf explant with 7.567±0.498 per explant and 8.767±0.707 per explant for root explant. Meanwhile for stem explant, MS medium supplemented with 0.5 mg/L NAA + 1.0 mg/L BAP give optimum response toward root initiation with 7.767±0.752 per explant. According to Widiyanto and Erytrina (2001) high concentration of NAA can enhance roots to actively induced [15].

The experiment with the use of auxin hormones conducted by Sevik and Guney (2013) concluded that auxin group hormones (IAA, IBA and NAA) did not affect directly to the rooting percentage, but these hormones were detected affecting the morphological characteristics on the newly generated plants specifically on the root generation [16]. Ogunsola and Ilori (2008) stated that MS medium supplemented with 1.0 – 2.0 mg/L IBA and 0.1 mg/L BAP induced better rooting [17].

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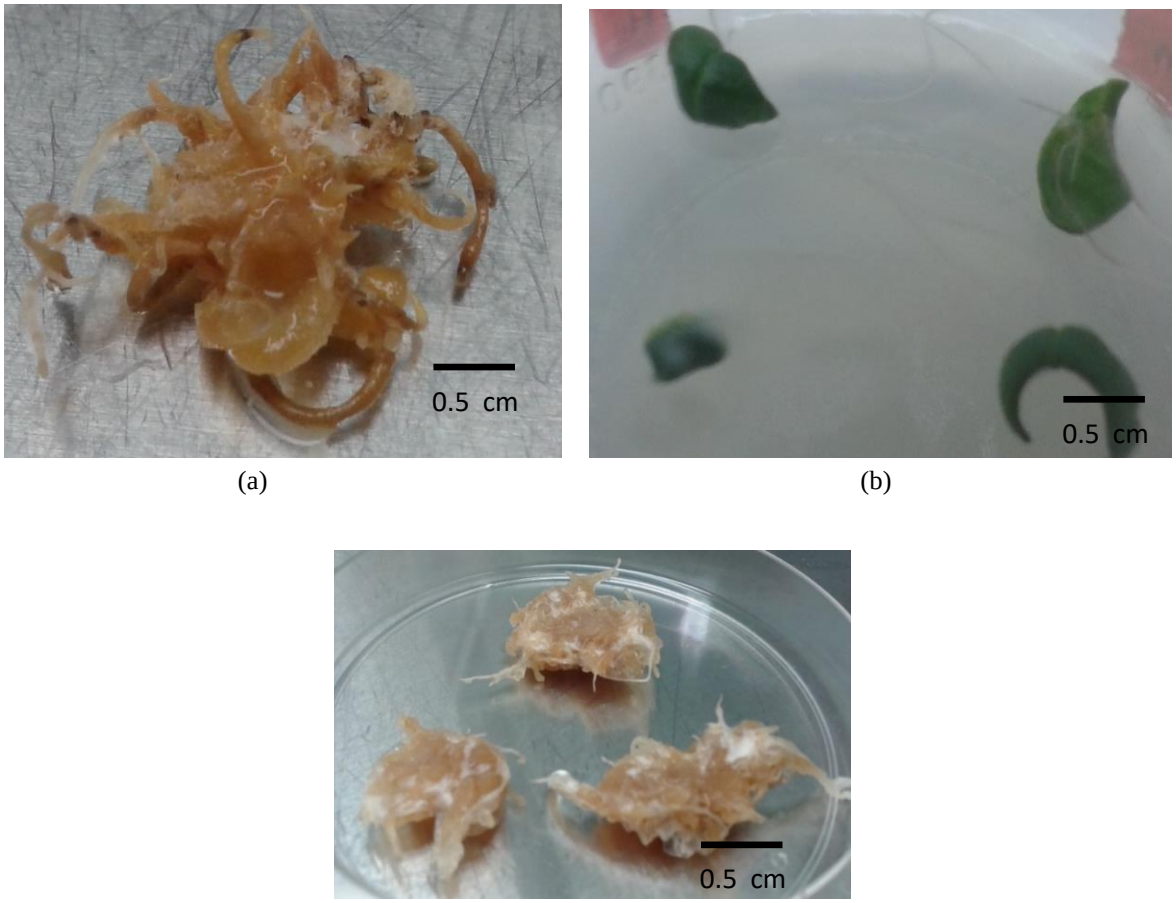

(a) (b)

(c)

Fig 2. Identification of Root Regeneration Media of *Brassica oleracea* var. *italica.* (a) Regeneration of root from stem explant cultured on MS medium supplemented with 0.5 mg/L NAA + 1.0 mg/L BAP; (b)) Regeneration of root from leaf explant cultured on MS medium supplemented with treatment 2.0 mg/L NAA + 0.5 mg/L BAP; (c) Regeneration of root from root explant cultured on MS medium supplemented with 2.0 mg/L NAA + 0.5 mg/L BAP

V. CONCLUSION AND RECOMMENDATION

As a conclusion, *in vitro* growth respond of *B. oleracea* var. *italica* through tissue culture system was successfully achieved. The work on organogenesis of *B. oleracea* var. *italica* through tissue culture system were reportedly very limited. In the future, research on tissue culture of broccoli could be more developed, so that useful information about this plant species could be provided to many people especially people who are very keen to explore this field. The interesting findings on these studies could add to the present knowledge and information so that it can be used with other people in the same area of study.

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