

EVALUATION ON THE EFFECTS OF *P. OSTREATUS* SPENT MUSHROOM COMPOST AND BAP HORMONE TOWARDS *C. NUTANS* IN VITRO CULTURE

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ABSTRACT

Realization in minimizing production cost for in vitro culture had brought to a study on application of *P. ostreatus* spent mushroom compost (SMC). Sterile nodal explants was inoculated on different treatments with 15 replicates each. Treatments were MS medium supplemented with different concentrations of SMC (1 and 2 %) and Benzylaminopurine (BAP) at 0.01, 0.1 and 1.0 mg/L respectively. Observation on plant growth parameters were done and data was recorded on week eight. It can be concluded that high concentration of BAP (1.0 mg/L) with greater SMC concentrations (2%) had showed highest shoots regeneration (4.59 ± 0.76), shoot length (1.6 ± 0.36 cm) and number of leaves (10.33 ± 0.58). This study proved that the interaction between SMC and BAP had formed and influenced the regeneration performance of *C. nutans*.

Keywords: spent mushroom compost; Benzylaminopurine; *Clinacanthus nutans*; growth performance; waste.

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1. INTRODUCTION

Nowadays, medicinal plant has become well identified and research on it had been widened up by the scientist with several discoveries. This is due to medicinal plant implies it important as a therapeutic agent and more [1]. One of the medicinal plant which can give benefits in medicine line through proven research is *Clinacanthus nutans*, as it can treat several diseases including cancer [2] without giving signs of toxicity and morbidity [3]. Other than that, this plants also were generally used in Indonesia and Thailand for treating skin rashes, insects and snake bites, diabetes mellitus, fever, diuretics and dysuria [4]. It also been demonstrated to have heat and stasis reducing effects, liver cleansing and also used in regulate women menstruation [5].

In order to fulfill high demand towards this plant, tissue culture technique is applied to highly regenerate the explant. Tissue culture is one of the techniques, which is powerful for conservation and rapid multiplication of plant species [6]. It is a useful propagation and conservation in faster rate for valuable plant that is really required by pharmaceutical line for medicine production especially from plant herbs extract to treat diseases.

In order to conduct the propagation process, few environmental factors need to be controlled to make sure explant grow perfectly. The factors involved for optimum growth of plant includes sterilization, pH and light temperature. As for example, pH is maintained at 5.8 throughout the plant tissue culture before being autoclaved [7]. Then, for growing temperature in the growth chamber is at 25°C [6].

Medium is one of the important parts of tissue culture. The types of medium use in tissue culture depends on the plant demands to grow with adequate amount of nutrients. The medium is usually added with sucrose and gelled with agar. Sucrose functions to supply carbon sources and plant also depends on the hormone that been supplemented to control its physiological growth.

Healthy and vigorous plant growth can be obtained by taking up large amount of some inorganic element by the intact plant. This includes ions of nitrogen, potassium, calcium, phosphorus, magnesium and sulphur. They are macronutrients that need most by plant. Plant also needs small quantity of other element. It is known as micronutrient. Micronutrients include iron, nickel, chlorine, manganese, zinc, boron, copper and molybdenum. This entire

requirement usually needs to be present in the medium for the necessity of the plant tissue culture.

Different in characteristics of elementary compound is used in order for different species to adapt with the medium promoted. Murashige and Skoog medium is one of the medium use as the basal medium for shoot and root proliferation [8].

Hormone is plant growth regulating substance which control growth, development and movement of plants. Plant growth regulator includes plant hormones and non-nutrient chemicals. For hormones, they can be categorized as naturally and synthetically produced hormones. Examples of naturally produced hormones are indoleacetic acid (IAA) and zeatin. Examples of synthetic hormones are 2, 4-dichlorophenoxyacetic acid (2, 4-D) and benzylaminopurine (BAP). In this study, the main hormone used as a plant growth regulator is cytokinins. Cytokinins functionally act to regulate cell divisions in shoots and delay leaf senescence. Explants are implanted on media supplemented with cytokinin in adequate concentration. The effect of benzylaminopurine (BAP) which is example of cytokinin has been demonstrated in many medicinal plants, for example *Santolina canescens*, *Bupleurum fruticosum* and turmeric [9]. It also stated that the most effective medium for axillary bud proliferation was MS medium fortified with benzylaminopurine (BAP). In [10] also reported the similar observation which BAP was the most effective hormone for plant regeneration compared to other. This shows that BAP have high stimulating effect on bud break and multiple shoot formation [11].

This study focuses on BAP hormone influenced on *C. nutans* shoot proliferation. There was several studies reported on *C. nutans* for callus induction by applying cytokinin hormone. For example study done by [12] that successfully induced callus of *C. nutans* leaf explants with Dicamba hormone. However, there is lack of documentation for direct regeneration from nodal explant by using 6-benzylaminopurine (BAP) hormone. Thus, BAP was selected in order to measure its capability in elicits plant growth and development responses.

BAP also can give synergistic effect to the culture when combine with other hormone. For example, kinetin which is also a plant hormone combines with BAP had gave high multiple shoot induction [13]. However, in realization towards reducing consumption of expensive plant hormone, agricultural waste also was applied as additional supplement or as hormone

replacement in this research.

SMC is known as an agricultural waste from edible mushroom and in [14] had discovered that SMC is potentially useful source of sulphur, potassium, calcium and magnesium. The benefits content within the waste had led to a study on its effect in plant tissue culture and how it will react in combination with exogenous hormone in growth media. The objective in combining the hormone and SMC in *C. nutans* regeneration would help in increasing more shoots proliferation and lead to an idea of recycling this waste material into a beneficiary product. The finding will also give benefits to the economy and environment as the waste would be recycled. In the same time, the usage of this inexpensive material would minimized the consumption of exogenous hormone. Thus, this study focused on the effect of BAP hormone combination with SMC towards *C. nutans* shoots growth.

2. METHODOLOGY

2.1. Preparation of *Pleurotus ostreatus* Spent Mushroom Compost

The *P. ostreatus* spent mushroom compost was grounded and sieved into particular size of 150 μm . The powder form of *P. ostreatus* spent mushroom compost was added into the MS media to be tested its effect on the plant growth.

2.2. Surface Sterilization

The apparatus applied were sterilized by using autoclave machine at standard condition (121°C under 15 lbs for 15 minutes) prior to usage. Stems from the naturally planted *C. nutans* were excised at the nodal segments with 1.0 cm size and washed for 5 minutes with tap water to remove any debris and foreign particles that may cause contamination. Once completed, the nodal segments of *C. nutans* were soaked in 30 % ethanol for 5 minutes followed by 70 % chlorox. The nodal segments were further rinsed using sterile distilled water and air dried before inoculated into each media

2.3. Media Preparation

Murashige and Skoog (MS) was selected as germination medium, added with 3 % sucrose and 2.2 % (w/v) of gelrite. Nine different treatments were prepared. Each was supplemented with different concentrations of BAP (0.01, 0.1 and 1.0 mg/L) and *P. ostreatus* spent mushroom compost (1 % and 2 %). MS media in absence of BAP was used as control. The

pH of media was stabilize 5.8 prior autoclave. Then, the autoclaved media were poured in jam jar at 100 ml.

2.4. Shoot induction

The sterile nodal segments were then cultured in each media. Three nodal segments were cultured in each jam jar and the steps repeated five times. Then, cultures were maintained in incubator at $25 \pm 2^\circ\text{C}$ under cool white fluorescent light with 16 hours of light and 8 hours of dark photoperiod.

2.5. Subculture

The explant was sub cultured in MS medium every 3 weeks for further growth. On week eight, number of shoots and leaves that successfully regenerate were measured and recorded.

2.6. Statistical Analysis

Data recorded were subjected to the step of analyzing using one-way analysis of variance (ANOVA). Software of SPSS Statistics version 20 for Window 7 was used to express the value in mean \pm standard error (SE).

3. RESULTS AND DISCUSSION

3.1. Growth Morphology

The in vitro studies on propagation of *C. nutans* had been conducted to develop protocol for plant generation from nodal segments. This technique used Murashige and Skoog as growth medium added with different concentrations of BAP. The formation of shoots and leaves can be seen within two weeks after inoculation. Fig. 1 shows in vitro growth *C. nutans* on three different periods. All explants grown in MS media were all survived with 100% survival rate. After a week the shoots already started to form and leaves grew at the tip of each shoots. The grown leaves morphology (Fig. 1c) shows pale green color, simple, opposite narrowly elliptic oblong with acute apex size at 1-1.5cm long and 0.3-0.2 wide. The stem is straight green with white internodes and vertical strips can be seen throughout entire stem. Similar findings by [15] was also reported on the similar new propagated plantlet morphology.

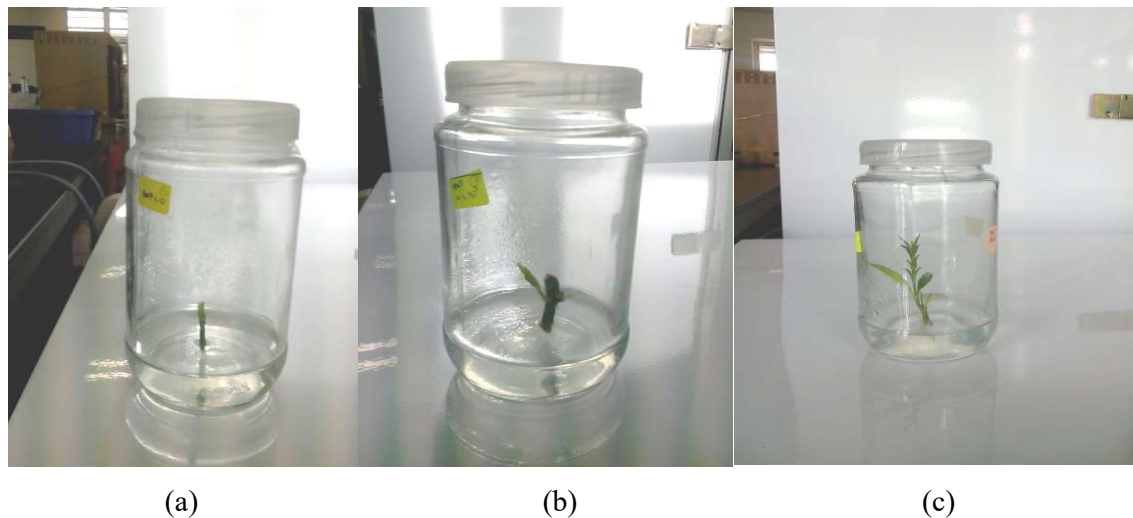


Fig.1. In vitro propagation of *C. nutans* on (a) first day (b) second week (c) fifth week

Tissue culture gives grower a means to produce plants with identical traits. It is important that the regenerated plantlet maintain its physical morphology and chemical content as the plant contain with many medicinal values. From the result, it shows that the propagation technique in MS media do not cause changes on its physical structure. The grown explant is having similar stem, leaves and roots structure with wild grown plant. Therefore, this will benefits and fulfil demand towards this plant especially for its pharmaceutical value as the shoots could multiply within weeks in a correct growth condition.

3.2.Survival Percentage

In this experiment, MS media was added with BAP hormone to study the effects of plant growth regulator concentrations on the explant. To measure the explant growth, survival percentage was firstly taken. The survival percentage were firstly measured after 2 weeks by using formula given.

The data measured will explain the relation between BAP concentration applied and the explant survival. Number of explant that successfully grown were taken and contaminated explant were isolate. The contamination can be confirmed by the presence of growing white fungus on the media and at the explant surface. It is important to do the isolation process so that the contamination will not prolong and invade other explant.

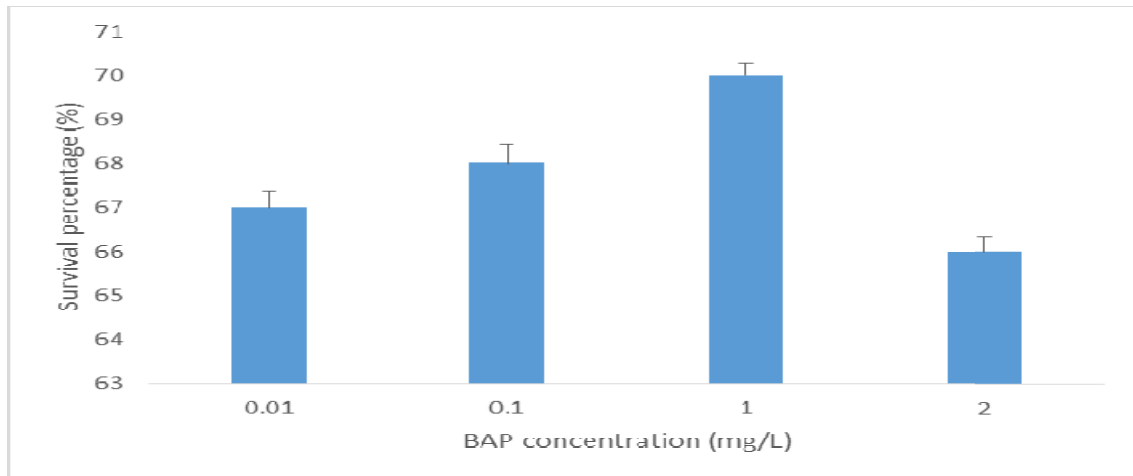


Fig.2. Percentage of explants survived in treatment with different BAP concentrations

Based on Fig. 2, the survival percentage of *C. nutans* explant in the media are in the range of 66-70% with no significant difference ($P>0.05$). Result documented that *C. nutans* could survive with addition of BAP hormone in the growth media at concentration as minimum at 0.01 mg/L and up to 2 mg/L. The successful in vitro propagation of *C. nutans* was supported with findings from some other researcher. In [16] had efficiently propagated explant from *Acanthacea* family through in vitro culture on MS media supplemented with BAP. Same result by [11] sample studied survived in growth medium with BAP.

3.3.Shoot Regeneration

BAP hormone was added to the media growth culture at different concentrations to observe the effect of hormone on shoot growth. The number of shoots is one of parameter used to measure successful *C. nutans* propagation within media. Results of shoots development from initial explants are stated in figure below.

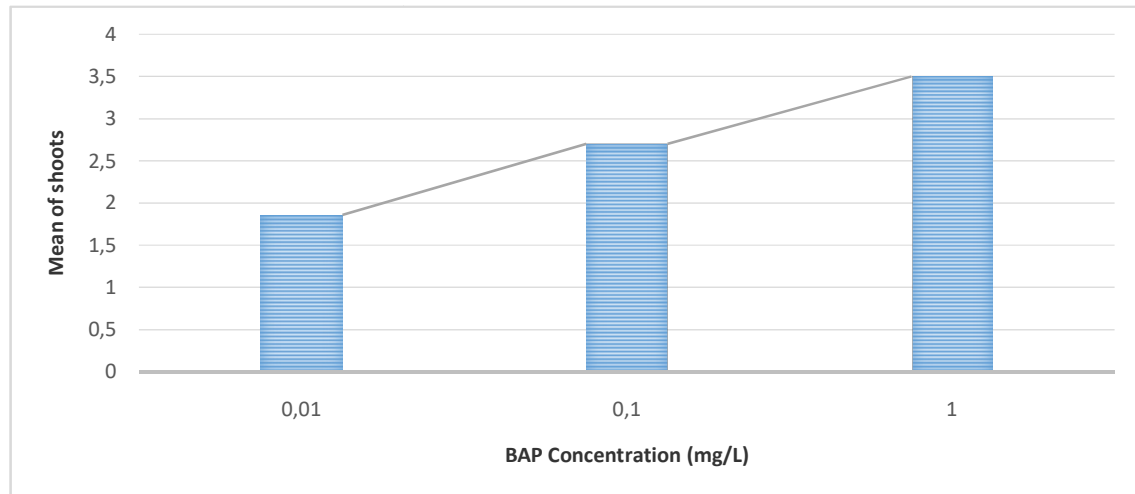


Fig.3. Shoots mean in treatment with different BAP concentrations

After 8 weeks in controlled growth condition, data shows that the nodal explant had successfully initiate bud and further grown into shoots and leaves. These described that a rapid and efficient in vitro multiplication and regeneration system of *C. nutans* was developed using sterile nodal explants.

To study the effect of BAP hormone, Fig. 3 documented that there is increment in mean of shoots from 1.86 ± 0.38 up till 3.50 ± 0.93 by increasing of BAP concentration at 0.01, 0.1 and 1 mg/L. This defined that the addition of BAP hormone was essential for adventitious of shoot initiation and development. Highest application of BAP concentration (1 mg/L) had successfully stimulate large multiple shoot formation by initiate bud formation from explants. This observation is consistent with finding by [17] on *Momordica balsamina* shoots proliferation from bud explant in MS media supplemented with 1.0 mg/L BAP.

Then study continued by using BAP hormone together with SMC to observe the shoots growth efficiency after using this waste material in growth media. This is because according to several study, BAP had synergistic effect on the regeneration of culture when combine with other hormone. As for example, BAP with NAA had successfully induced high multiple number of shoots in *Arachis hypogaea* L. [18].

SMC was combined at 1% and 2% with BAP hormone at three different concentrations (0.01, 0.1 and 1 mg/L). After eight week, it has been identified that addition of SMC together with BAP had gave better response on the number of shoots in general instead media with BAP hormone only.

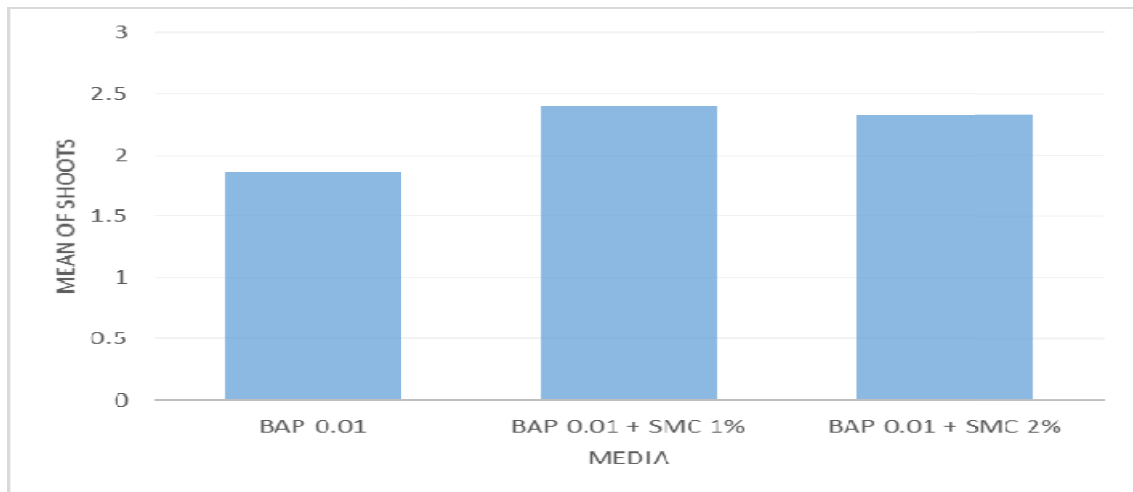


Fig.4. Shoots mean in treatments of SMC with BAP hormone at 0.01 mg/L

In three different medium studied, shoot's number increased with combination of SMC and 0.01 mg/L BAP hormone. Increment in concentration of SMC from 0 % to 2 % had given unalike results in shoots number (Fig. 4). It was found that combination of 0.01 mg/L BAP and 1% SMC had gave optimum reading in the number of shoots (2.40 ± 1.52). This indicated that SMC did influenced in shoots formation of *C. nutans* even with low concentration of BAP hormone.

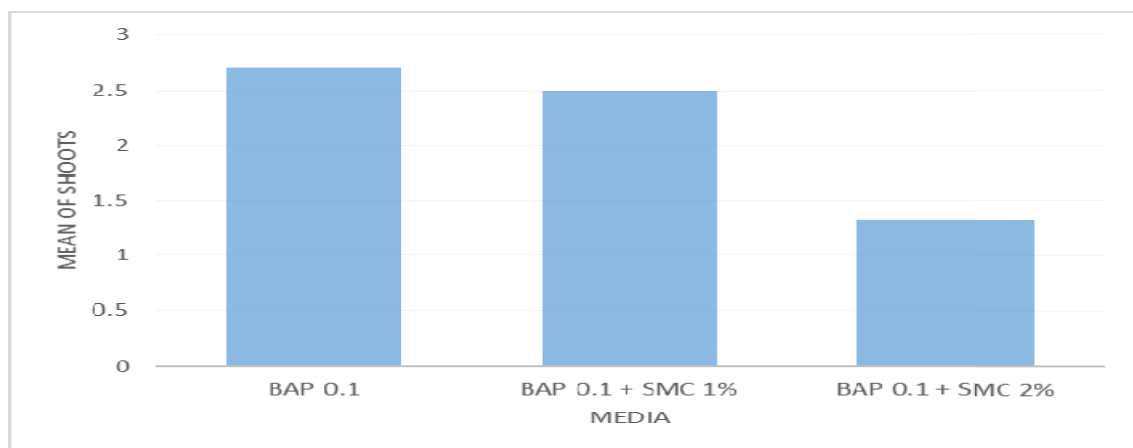


Fig.5. Shoots mean in treatments of SMC with BAP hormone at 0.1 mg/L

However, result of shoots number in MS media added with 0.1 mg/L BAP and different percentages of SMC (Fig. 5) is giving a dissimilar pattern with Fig. 4. According to the result, there was a decrement in the number of shoots by increasing of SMC percentage. This finding come up with an idea that combination of SMC with BAP hormone at 0.1 mg/L is not suitable for the explant growth.

Then the result was further analyzed in media combined with SMC (0, 1 and 2 %) and BAP at high concentration (Fig. 6). Result documented that media with 2% SMC and 1 mg/L BAP had successfully regenerated significantly highest number of shoots (4.59 ± 0.76). The result explains that the mean number of shoots could be maximized with increment of BAP and SMC concentrations. The combination of BAP and SMC together were perfectly effective for shoot regeneration.

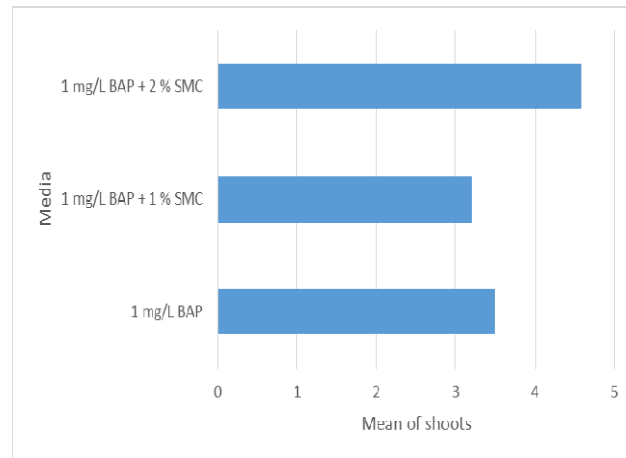


Fig.6. Shoots mean in treatment of SMC with BAP hormone at 1 mg/L

From this study, interaction between the SMC (additional supplement) that come from agricultural waste had successfully interact with the BAP hormone. As mentioned by [19], the SMC consists with high beneficiary values such as enzymes, extra nutrients and minerals (sulphur, potassium, calcium and magnesium). Thus, results had successfully indicated that hormone-waste interactions applied was significantly effective for the shoots formation in *C. nutans* nodal explant especially at high concentration.

A comparison of the relative effectiveness of different medium for shoot regeneration were revealed in order of effectiveness: SMC and 1 mg/L BAP > SMC and 0.1 mg/L BAP > SMC and 0.01 mg/L BAP > BAP only

3.4. Shoot Length

3.4.1. Shoot Length with Presence of BAP

Study continued on measuring the effect of BAP hormone towards the shoot length. The regenerated shoots were measured in centimeters and the mean was calculated. Fig. 7 is the summarization of shoots length for *C. nutans* in medium with different concentrations of BAP. From the result, different BAP concentrations had gave variety in the shoots growth.

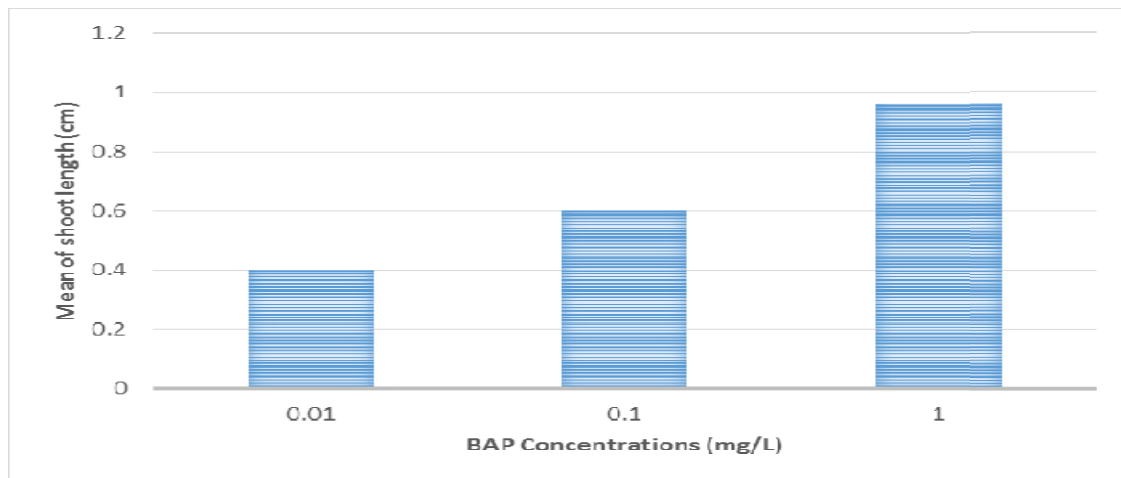


Fig.7. Shoots length in treatments with BAP hormone

Data shows that there is a linear increase in the shoot length. The highest mean length of shoots is at 1.0 mg/L BAP (0.9625 ± 0.4005), followed by 0.1 mg/L BAP (0.6 ± 0.3123) and 0.01 mg/L BAP (0.4 ± 0.3247). Statistical analysis documented that there are significant different ($P < 0.05$) on the mean of the shoots length for all three medium studied. Thus, the optimum concentration for greater length of shoots is 1.0 mg/L BAP. Research conducted by [20] on *Acanthacea* plant also give a similar result that increasing in BAP concentration causes for higher mean length of shoots (95cm).

3.4.2. Shoot Length in Combination of BAP and SMC

Media with 2% SMC and 1 mg/L BAP had successfully initiate buds formation that eventually grew into shoots. Thus study continued on the effects of this waste towards *C. nutans* shoot length. After several weeks, data was measured and mean were calculated. Each of shoots proliferated indicates that *C. nutans* is highly adaptable with SMC in supplying nutrient. Fig. 8 shows the summarization on the mean length of shoots with different concentrations of BAP and SMC.

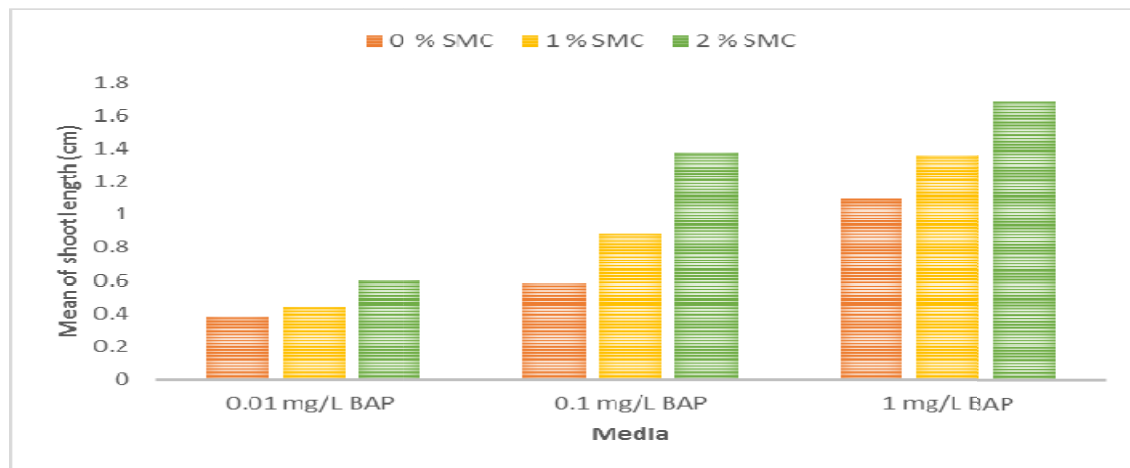


Fig.8. Shoots length in treatments of SMC and BAP hormone

In media with BAP hormone only, 1 mg/L BAP was the best media for shoot growth. However, with combination of *P. ostreatus* spent mushroom compost (SMC) and BAP giving more effect as the shoot length highly increased. By comparing media 0.01 mg/L BAP and SMC at 1-2%, 2% SMC gives more shoot growth.

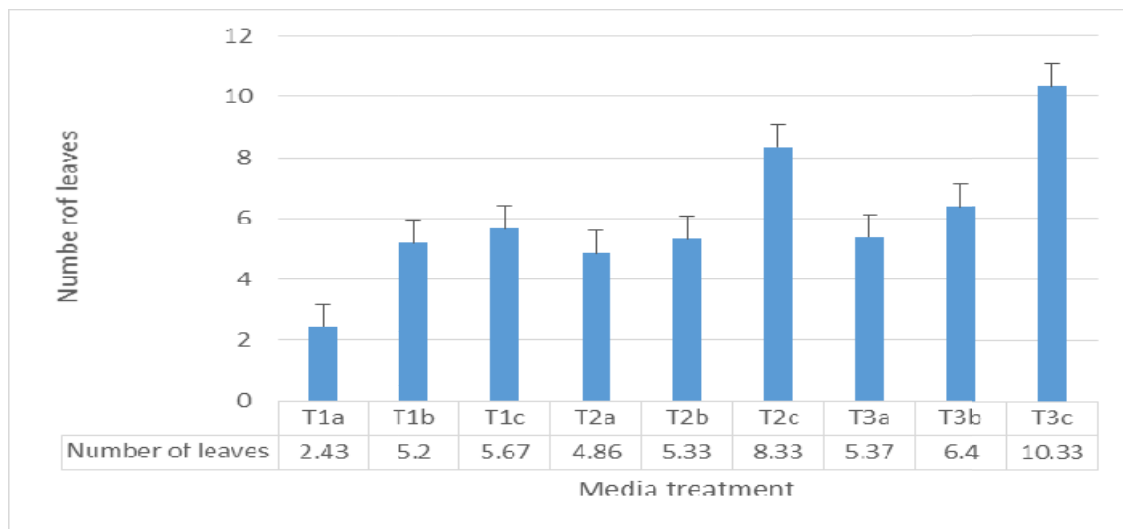
Further study was conducted on seeing the effects of BAP concentration and SMC percentage. BAP concentration tested was at 0.1 and 1 mg/L and SMC at 1 and 2%. It was found that by increasing the concentration of BAP together with SMC percentage had generated longer shoots of *C. nutans*. According to Fig. 8, the most optimum media for shoots growth is media treated with 1 mg/L BAP and 2% SMC as it increase the shoot length up to 1.6 ± 0.36 cm. The increment on shoot length is supported by [21-22] in their studies that propose SMC is a material that can supply extra nutrients and minerals. Thus, this further explained on the result of shoots growth obtained.

3.5. Leaves Formation

The commercial benefits of the *C. nutans* leaves in pharmaceutical and health products [23] had led to numerous study focuses on high production of leaves via in vitro culture. Thus, the number of leaves in each successfully regenerated explant is also one of the parameter that is being measured.

Table 1. Media treatment

Media Treatment	Code
BAP 0.01 mg/L	T1a
BAP 0.01 mg/L + SMC 1%	T1b
BAP 0.01 mg/L + SMC 2%	T1c
BAP 0.1 mg/L	T2a
BAP 0.1 mg/L + SMC 1%	T2b
BAP 0.1 mg/L + SMC 2%	T2c
BAP 1 mg/L	T3a
BAP 1 mg/L + SMC 1 %	T3b
BAP 1 mg/L + SMC 2 %	T3c

**Fig.9.** Number of leaves grown in culture treated with BAP hormone and SMC

After 8 weeks which is the same week result of shoot formation is obtained, the number of leaves were measured and results were recorded. Fig. 9 summarized the mean of leaves number.

From observation of all nine different medium studied, the hormone was not only increased the shoot proliferation but also induced the formation of leaves. In media with BAP only, increment of BAP concentration had increased number of leaves. There was a linear increase in number of leaves with increasing of BAP concentrations in T1a, T2a and T3a medium.

It was recorded that media with highest concentration of BAP hormone (T3a) gave the highest

number of leaves formation (5.37 ± 0.99). The efficiency of BAP in leaves development was recorded having similarity with study done by [24]. The study found that BAP hormone is significantly produced highest mean number of leaves and explant height at highest concentration on *C. nutans* explant.

Study was further conducted by comparing leaves number in media combined with different concentrations of BAP and percentage of SMC. Fig. 9 shows that T3c was the most optimum media to be applied for high leaves formation. In [25] have done a slightly similar research on *C. nutans* by using SMC only in the growth media. The result found that SMC only did give significant high number of leaves at average 7.

However, in this research, SMC was combined together with BAP hormone to investigate the hormone-waste interactions effect on *C. nutans* leaves formation. After 8 weeks in the same growth condition, it was documented that treatment with combination of BAP and SMC at highest percentage (T3c) showed the highest reading on leaves formation (10.33 ± 0.58). Based on one-way analysis of variance (ANOVA) obtained from the SPSS statistical analysis, there is significant difference ($P \leq 0.05$) between medium on leaves production.

The result indicated that the use of BAP hormone and SMC is relevant in manipulating the number of leaves in plant regeneration study. Thus, combination of SMC with BAP showed better result as increased more number of leaves with average of 10.

4. CONCLUSION

The study on the influence of SMC in presence of BAP was conducted and it can be concluded that BAP is best to be used with SMC .at highest concentration (BAP 1 mg/L and 2% SMC) for shoot regeneration and leaves formation. Application of SMC in tissue culture technique not only improves plant shoot regeneration and leaves formation but also had convert the agricultural waste into an additional supplement product for tissue culture. Thus, the study result is in-line with green technology development and zero waste concept. It is recommended that further studied to be done in seeing the combination effects between SMC and other exogenous hormone.

5. ACKNOWLEDGEMENTS

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