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The Effect of BAP and NAA Treatment on Micropropagation of *Cucumis sativus.L*

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Abstract: ***Japanese cucumber is one of the cucumber varieties that become popular among people through out the world as salad. Recently it become popular among Malaysian. There is an increasing activities in growing this plant among farmers. One of the obstacle arises in growing this plant by farmers in Malaysia is the lack of planting materials. Seeds have to be imported from Takii company in Japan. If these F1 seeds are germinated, grow, flowered, and pollinated among each other, the offsprings at the second filial generation will not be identical to its parent plant. Micropropagation can be an alternative approach to produce offsprings that is identical to its parent plant. Therefore this experiment is carried out to micropropagate Japanese cucumber F1 hybrid (Cucumis sativus L) using shoot tip explants. These explants are cultured in MS medium containing different concentration of BAP(6-Benzylamino purine)(1, 2.5, 5, and 10mg/L) for shoot multiplication. Shoots are rooted in a medium containing NAA (Naphthalene Acetic Acid) at 0.1, 0.5, 1.0, and 2.5mg/L All the experiments are conducted using Complete Randomized Design(CRD) . Data are analyzed using analysis of Variance (ANOVA) and Duncan New Multiple Range Test. It is expected that certain concentration of BAP and NAA can maximize shoot regeneration and rooting in C.sativus F1 hybrid and the protocol for micropropagation of this crop be developed.***

Keywords: *Cucumis sativus.L* **,** BAP & NAA, Plant Growth Regulator (PGR), Micropropagation

1. Introduction

Japanese cucumber is a popular vegetable species throughout the world. Botanically it belongs to the class magnoliopsida, order cucurbitales and family cucurbitaceae. Its botanical name is *Cucumis sativus L.* It is a monoecious plant with it's stalk length reaches 1.5 m long, orbicular, orbicular-cut or cut, prostrate, branching, with simple short tendrils, with varying degrees of pubescence. Bushes with stalk's length of 20-30 cm are formed. The root is a rod and ramified, its great bulk is in an arable layer of soil. It's leaves are formed alternately, five-bladed (blades sharp) and less often integral (oval). It's length can be short with an average of 12-15 cm, or long with an average of more than 15 cm. It's flowers are auxiliary, diclinous and rarely bisexual (Plader et al, 2007). The male flowers are single or in 3-7 floral cluster on the main stalk and axils of the first order, while female flowers are solitary or in pairs on branches of the second and the subsequent orders. The corolla is yellow in colour and funnel-shaped. The ovary is long and pubescence.Its fruits are various in form, size and color. Fruits in technical ripeness are dark green, green or light green, equally-colored or with white spots and strips and weighed from 50 – 400g (Tabei et al,1998) . Their surface may be smooth, fine-tubercular, large-tubercular, furrowed, with thorns of different coloring. It's length is from 5 - 30 cm and more (Doijode, 2001). The net production rate of cucumber in Malaysia for 2002 was 43389 metric ton. Overall, 32.73% of national cucumber production for 2002 came from Johor.. The size of local markets for cucumbers in Malaysia is estimated at 37.74 metric ton in 2003. Of this amount only Malaysia dominate 0.098% of the world market size of 38,321,636 metric ton in 2003. (Kementerian Pertanian Dan Industri Asas Tani, 2010). According to international estimates of market size in 2003, Japan was the country most needs the supply of cucumber with the quantities of 685 metric ton. In terms of exports, the most importing countries were Singapore with the quantities of 22 metric ton worth U.S. $ 2804, followed by Brunei 20 MT with a value of U.S. Dollar $ 13 and Indonesia 53 metric ton worth U.S. Dollar $ 13 in 2003. (Kementerian Pertanian Dan Industri Asas Tani, 2010)The problem start to arise when the main Japanese Cucumber seed producer, the Takii Seed Corporation, whose headquarter is at Japan, cannot supply sufficient seed to Japanese Cucumber growers in Malaysia. Therefore, an alternative must be find to overcome this problem. Having interrupted supply issue with the seeds, some growers tend to produce the seed from the F1 hybrid cucumber plants. These seed if allowed to germinate, will produce plants which differ in terms of characteristics and traits from its mother plant. Therefore, vegetative propagation via tissue culture technique might be an alternative solution for this problem.

The objectives of this research are :

1)To determine the best BAP concentration combined with 0. 5 mg/L NAA in inducing shoot multiplication

*2)*To determine the highest level of concentration of BAP that can be tolerated in *Cucumis sativus.L.*

2. Literature Review

In vitro technique is a method of producing small pieces of tissue or organs from the parent plant under sterile condition. (George,2000) The plant parts or organs that usually used for culturing are seeds, pollen, shoot tip, embryo, callus, single cell, stem, and root tip. (Hartmann *et al*, 2007) The basic of tissue culture refers to the theory of cell (Stephen, 2003). Every cell is a living unit. This means that the cell that have experienced differentiation in a multicellular organism still containing its genetic information from its previous form. This is associated with the totipotency concept which stated

that each cell in plant tissue carry the same genetic materials and it can expand and differentiate into a perfect organism and is identical to its parent cell if the requirements are met and under suitable environmental conditions (Hall,1999). The first success in using tissue culture technique was archived by White in 1943 by culturing the root tip of tomato plant in a medium and promoting the growth of the root for 28 years and have produced 1600 subculture (Razdan,2005).

Recently, the shoot tip culture have been used widely in producing more than 300 ornamental species. In addition, the crops and fruits can produce new generation using the *in vitro* culture (Bonga and Aderkas, 1992). In general, the production of good plant can be obtained using tissue culture technique. This technique is suitable for mass production because of easy management and small space needed (Jain et al, 2006). *In vitro* culture also assist in producing free pathogen plants. *In vitro* culture consist of several stages, which is induction, shoot multiplication, rooting and finally the stage of transferring the seedling into the field (Razdan, 2005) .

2.1 Plant Growth Regulator

Plant growth regulator is an organic substance required by the plant in small amount to regulate the growth of a plant (Chen et al,2008). It is also known as plant hormone. George (2000) stated that hormone can be found naturally or synthetically. They play roles in promoting cell differentiation, growth and cell division. There are 5 main classes of plant hormones. These 5 classes are auxin, cytokinin, gibberellins, abscisic acid, and ethylene. Auxin and cytokinins are the most widely used plant growth regulators in plant tissue culture. The ratio of auxin to cytokinin determine the type of culture established or regeneration type (Herman, 2006). The ratio usually differ according to genera and species in terms of plant sensitivity to the hormones.Razdan (2005) was involve in making references of application of hormone in critical stages in meristem culture and shoot tip. The ratio of auxin and cytokinins is a determinant in the formation of meristem and the balance of hormone proven its ability to stimulate the type of shoot formation. The hormone levels in the plant itself, which affect the intake of hormones from the outside and is also dependent on genera, species and cultivars.

2.2 Cytokinin

Cytokinins are hormones that play a central role in the regulation of the plant cell cycle and numerous developmental processes. Cytokinins were discovered by Skoog and Miller during the 1950s as factors that promote cell division (cytokinesis). The first cytokinin discovered was an adenine (aminopurine) derivative named kinetin (6-furfuryl- aminopurine), which was isolated as a DNA degradation product. The first common natural cytokinin identified was purified from immature maize kernels and named zeatin (6-(4- hydroxy-3-methylbut-2-enylamino) purine. Cytokinins are present in all plant tissues. They are abundant in the root tip, the shoot apex and immature seeds. Their endogenous concentration is in the low mg/L range (Hartmann et al, 2007). Although there are several species that did not need exogenous hormone in initial culture, but most of the plants need little amount of cytokinin to support the meristem growth(Dixon and Gonzales, 1995). The cytokinin that mostly used in plant tissue culture are BAP (6-Benzylamino purine). This is because BAP is more stable than other cytokinin, harder to oxidize by light, cheaper and easily obtained (Koning, 1994). BAP is also more effective because it has more ability in promoting other hormonal synthesis (Uranbey, 2004). The use of BAP in plants usually range from 1.0 to 3.0 mg/L. If the concentration is too high, it can cause shoot bud retardation rendering the plant growth stunted but the effect will be vary with different species (George, 2000).

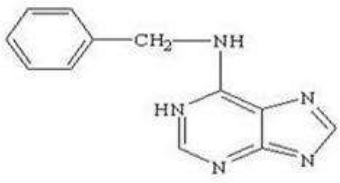

Figure 2: BAP (6-Benzylamino purine) chemical structure

2.3 Auxin

Auxin is a hormone that is involved in root elongation and the formation of adventitious root. (Ameena and Khaleed, 2009) In other way, auxin also cause fruit and leaf senescence, inhibiting lateral bud growth, and inhibiting shoot multiplication. In tissue culture, auxin is mainly used in promoting the growth of plant root and adventitious root (Razdan, 2005). Like other hormone, its different concentration also resulting in different result depending on plants species and genera. In the initial stage of culturing, auxin usually is not needed if compared to cytokinin but if added in small amount, combined with the application of cytokinin, can produce positive result (Hall, 1999). This is because although auxin did not stimulate shoot proliferation, but it promotes the growth of root which plays vital role in absorbing nutrient (Filipecki et al, 2005). Better rooting system will provide the plant with more ability to absorb nutrients.As stated before, auxin can cause fruit and leaf senescence and inhibit lateral bud growth. This is because high concentration of auxin can stimulate the production of ethylene, which cause all the symptoms above (Jaafar et al, 1999). In plant tissue culture, the mostly used auxin is IAA(Indole-3-acetic acid) and NAA(α-naphthalene acetic acid). IAA is the native auxin that is naturally produced while NAA is the synthetically produced auxin. However, IAA is more unstable because its chemical structure tend to easily altered. George (2000) stated that NAA is generally used in plant cell culture at a concentration range of 0.01-1.0 mg/L to induce rooting. Above this concentration, rooting can be inhibited due to excessive callus formation.

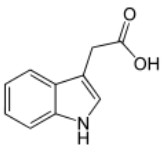

Figure 3: IAA(Indole-3-acetic acid) chemical structure

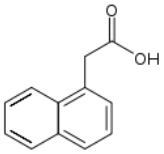

Figure 4: NAA (α-naphthalene acetic acid) chemical structure

3. Materials and Method

3.1 Experiment location

The research was conducted in *In Vitro* Laboratory, Department of Agriculture Technology, Faculty of Agriculture, University Putra Malaysia.

3.2 Explant source

The explants used in this research is the shoot tip of germinated F1 hybrid *Cucumis sativus L* plants.

3.3 Explant preparation

The seeds were obtained from an agricultural material shop located at Sri Serdang. The seeds were washed in running tap water for 5 minutes. The seeds were then sterilized in 70% ethanol solution for 2 minutes followed by sterilization in 20% Clorox + 3 drops of Tween 20 and shaked the whole time for 15 minutes. Then the seeds were rinsed in sterile distilled water 3 times and then cultured in the prepared media. The explants were excised from 14 day old seedlings. Each media are cultured with 5 seeds.

Figure 5: F1 Hybrid Japanese Cucumber seeds

Figure 6: Seeds were washed in running tap water

Figure 7: Seeds were Sterilized in 70% ethanol solution.

Figure 8: Seeds were Sterilized in 20% Clorox with Tween 20

Figure 9: Seeds were Rinse in sterile distilled water

Figure 10: Cultured seeds in prepared media

Figure 11: Sterile jar, sterile distilled water, clorox, tween 20 and media used for seed sterilization

Plate 1(Figure 5-11): Step by step procedure of seed sterilization

The shoot tip explants were derived from the 14 days germinated seedlings. This to ensure that the explants used were large enough for culturing. George (2000) stated that large explants have advantages over smaller ones for initiating shoot culture in terms of better survival rates when transferred, more rapidly commence growth, and more axillary buds. The plate below show step by step procedure in excising shoot tip and culture it in treatment media.

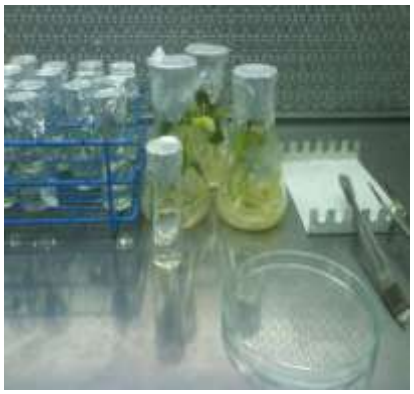

Figure 12: Media, cultured plants, petri dish. Forceps and knife used for shoot tip culturing

Figure 13: Germinated F1 hybrid *Cucumis sativus L* seed

Figure 14: Plant placed on petri dish for excision

Figure 15: The explant ready to be cultured

Figure 16: Vial mouth flamed as part of aseptic manipulation

Figure 17: Cultured explant in media

Plate 2 (Figure 12 – 17): Step by step shoot tip culturing procedure

3.4 Media preparation

In this research, the medium used is Murashige and Skoog (1962) medium which contains 30g/L sucrose, 0.1g/L myo-inositol, 25mL macro nutrient, 5mL micro nutrient, 5 mL vitamin, 5 mL iron, and 4.5g/L Gelrite agar, and different concentration of BAP and NAA according to treatment desired. After that the medium's pH was adjusted to 5.7-5.8. The pH was adjusted using 1M NaOH that function to increase the pH and 1M HCl to decrease the pH. After that, the medium was placed into the microwave oven for 15 minutes.Next, the media solution was poured into vials and closed using aluminium foil. Finally the vials were autoclaved at a temperature of 121 degree celcius under 103 kPa pressure for 20 minutes.

3.5 Aseptic manipulation

Aseptic manipulation is done to ensure that the explants were cultured in a condition that is always sterile. This is to prevent any microorganism intrusion into the container that can cause contamination, Before culturing is done, the laminar air flow cabinet is sterilized using 70% ethanol solution and left for 30 minutes. Next, all the materials that need to be put into the laminar air flow cabinet were sterilized first using 70% ethanol solution. All the culturing tools such as forceps and knife were dipped into 95% ethanol solution and burned for a few seconds every time before and after inoculation. Another important things that were given attention is the researcher's dress and cleanliness. The researcher must wore gloves and sprayed his hands with 70% ethanol solution before and after doing culturing. The vial's mouth also was flamed for a few seconds before closed back using aluminium foil to prevent contamination.

Figure 18: Autoclave machine as part of aseptic manipulation

3.6 Incubation

All the cultures were brought to the incubation room for storage. The temperature of this room was set at 27 ± 2°C with the relative humidity at 50 – 70%. The light source used is the fluorescent lamp and the light was controlled using timer at 16 hours per day with light intensity of 65 µE/m^2 /s.

3.7 Treatments

The objective of this research is to determine the best concentrations of BAP in combination with 0.5 mg/L NAA in promoting optimum shoot multiplication from shoot tip explant of Japanese Cucumber. Treatments were replicated 10 times.

Table 1: Different combination of BAP and 0.5 mg/L NAA for promoting shoot multiplication

Treatment (T)	BAP concentration (mg/L)	NAA concentration (mg/L)
T0 (Control)	0	0.5
T1	0.5	0.5
T2	1.0	0.5
T3	1.5	0.5
T4	2.5	0.5
T5	5.0	0.5

3.8 Parameter

Observation and data collection were taken at week 10 after initial culture. The parameters of this experiment were:
a) Number of shoot per explant
b) The shoot length per explant

3.9 Experimental Design

This experiment was conducted using Complete Randomized Design (CRD). The data collected were analyzed using Analysis Of Variance (ANOVA). The differences between treatment were analyzed using Duncan's New Multiple Range Test (DNMRT) at 5% level.

4. Results and Discussion

From the table 2, all of the treatments showed significance difference on mean number of shoots formed per explant compared with the control treatment. The explant of *Cucumis sativus L* respond to the T0, T1, T2, T3, and T4 treatments. The treatments also accelerate the time it takes for the first shoot of each explant to appeared. The T3 (1.5 mg/L BAP + 0.5 mg/L NAA) explant was observed to produce the fastest early shoot. It takes about 5 days for the first shoot to appeared, followed by T2 (1.0 mg/L BAP + 0.5 mg/L NAA) treatment which produce the first shoot at day 7. Treatment 3 (1.5 mg/L BAP + 0.5 mg/L NAA) was observed as the best treatment in increasing shoot multiplication. George (2000) stated that BAP is commonly used for shoot multiplication within the concentration range of 1.0 – 3.0 mg/L. Higher concentration of BAP can caused shoot bud retardation because excess hormone can become toxic to the plant. Treatment 5 containing highest concentration of BAP (5.0 mg/L BAP + 0.5 mg/L NAA) did not trigger any shoot multiplication. This might be because the concentration used was too high and might have cause shoot bud retardation resulting in zero shoot multiplication. In fact, abnormal growth had been observed on the explant. It had been observed that treatment 3 produce the best result because the BAP concentration used in the treatment was in the range suggested by George (2000). A lower BAP concentration combined with 0.5 mg/L NAA as was shown in treatment 3 also resulted in explant giving better root system resulting in

better ability in absorbing nutrients. The formation of root was also observed in this experiment due to the presence of NAA in the media. Most of the root formation was observed abundance in Treatment 0 and Treatment 1. According to Murashige (1990), the root formation was induced by low level of cytokinin in the media combined with the auxin.Treatment 4 showed low mean number of shoots formed per explant . This indicate that the formation of shoot starts decreasing if the concentration of BAP was higher than 2.0 mg/L. While T2 treatment had been observed to produce second best result. It is assumed that *Cucumis sativus L* effective exogenous BAP concentration is at 1.0-1.5 mg/L. Treatment 3 (1.5 mg/L BAP + 0.5 mg/L NAA) was observed to produce optimum result in increasing the shoot length, followed by Treatment 2 (1.0 mg/L BAP + 0.5 mg/L NAA). This might be due to the BAP concentration used in the treatment was in the range suggested by George (2000). Treatment 5 (5.0 mg/L BAP + 0.5 mg/L NAA) was observed not to produce any shoot. George (2000) stated that BAP is commonly used for shoot multiplication within the concentration range of 1.0 – 3.0 mg/L. Higher concentration can cause shoot bud retardation because excess hormone can become toxic to the plant. The BAP concentration used in T5 treatment was 5.0 mg/L which was a lot higher than suggested by George (2000). Treatment 4 was observed to produce a reduced shoot length because the BAP concentration used in this treatment was not in the range suggested by George (2000). This indicate that the shoots length starts decreasing if the concentration of BAP was higher than 2.0 mg/L.

Table 2: The effect of different concentration of BAP combined with 0.5 mg/L NAA on number of shoots formed per explant and shoot length(cm).(Data were collected 10 week after initial culture.)

Treatment	BAP concentration (mg/L)	NAA concentration (mg/L)	Mean number of shoots per explant	Mean shoot length (cm)
T0	0.0	0.5	1.80^{e}	1.43^{e}
T1	0.5	0.5	3.90^{c}	2.74^{c}
T2	1.0	0.5	6.98^{b}	3.56^{b}
T3	1.5	05	8.91^{a}	5.58^{a}
T4	2.5	0.5	2.90^{d}	2.25^{d}
T5	5.0	0.5	0.00^{f}	0.00^{f}

Mean with different letter within the same column indicate there is significant difference at 5% level based on Duncan's New Multiple Range Test.

Figure 19: T0 (0.0 mg/L BAP and 0.5 mg/L NAA) Control. Explant observed to produce little number of shoots

Figure 20: T1 (0.5 mg/L BAP and 0.5 mg/L NAA) Explant observed to produce little number of shoots.

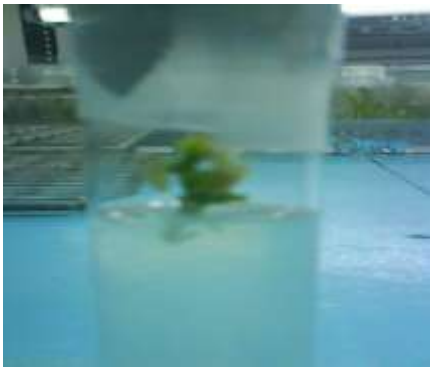

Figure 21: T2 (1.0 mg/L BAP and 0.5 mg/L NAA) Explant observed to produce a few number of shoots.

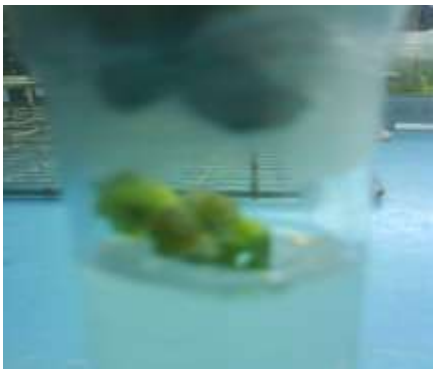

Figure 22: T3 (1.5 mg/L BAP and 0.5 mg/L NAA) Explant observed to produce a lot of shoots.

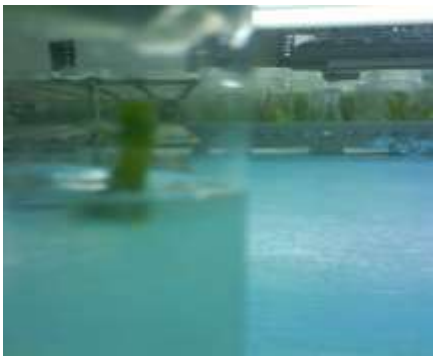

Figure 23: T4 (2 .5 mg/L BAP and 0.5 mg/L NAA) Explant observed to produce little amount of shoots.

Figure 24: T5 (5.0 mg/L BAP and 0.5 mg/L NAA) Explant observed resulted in abnormal growth .

Plate 3 (figure 19-24) : The effect of different combination of BAP and NAA on number of shoots formed per explant and shoots length.

5. Conclusion

From the experimental study it can be concluded that BAP at 1.5 mg/L combined with NAA at 0.5 mg/L is the best treatment in triggering shoot multiplication with a mean of 8.91 shoots formed per explant. The shoot tip of *Cucumis sativus L* did not respond to the BAP at higher concentration. It maybe because there are level of concentrations that plant could tolerate the substance before it could become toxic. The optimum shoot length of explant was observed at Treatment 3 with the mean shoot length of 5.58 cm. Treatment 5 had been observed to produce abnormal growth of callus. Further study should be done in order to determine this phenomenon.

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Author Profile

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