EFFECTS OF DIFFERENT CONCENTRATION OF BOTH NAPHTHALENEACETIC ACID AND 6-BENZYLAMINOPURINE IN CALLUS INDUCTION OF *CAPSICUM FRUTESCENS*

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Abstract

Capsicum frutescens var. bird's eye chili belongs to *Solanaceae* family is known to have economic properties due to its secondary metabolites such as capsaicin that can be used to treat various diseases. In tissue culture, this plant is often been associated with recalcitrant which inability to regenerate in *in –vitro* environment. Therefore in order to overcome this challenge, callus culture becomes one of the important approaches to gain result in many tissue culture studies. Callus induction of *C. frutescens* can provide many tremendous benefits such as somatic embryogenesis and genetic engineering. In this study, the effect of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in different concentration becomes subject of interest as to obtain optimum result in term of growth formation, earliest initiation and morphology of callus that may become the key factor for successful future studies that related with *C. frutescens*. The foliar explants of *C. frutescens* were chosen as explant sample and further been sterilized by using 15% NaOCI and ethanol 70%. The explants were then been cultured in MS media with seven different treatments contained NAA and BAP (0.0 + 2.5, 2.5+0.0, 2.5+2.5, 2.5+5.0, 5.0+2.5, 2.5+10.0, and 10.0+2.5 mg/L) for one month. The result had shown that, treatments with concentration of NAA (2.5 mg/L) and BAP (5.0 mg/L) had gained optimum level for earliest callus initiation in day 5 with highest callus growth. The result also has the best callus morphology which was pure white, friable and soft texture which suitable to be used in future studies.

Keywords: *Capsicum frutescens,* callus induction, plant hormone, naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP)

Introduction

Capsicum belongs to *Solanaceae* family is also known as "chilies" can be further classified into a division of Magnoliophyta, class of Magnoliopsida and order of Solanates (Heiser & Smith, 1953). *Capsicum frutescens* var. bird's eye chili possesses valuable economic status as a commercial crop that mainly as spices and vegetables. However, *Capsicum* spp. plants also have very high medicinal value due to its special secondary metabolite contain such as capsaicin, capsaicinoids, capsinoids, quercetin, and luteolin (Materska & Perucka, 2005). These compounds have been used as medicine to treat many types of diseases such as to prevent joint pain, improved metabolism and also act as anticancer and antioxidant (Luo et al., 2011). It is also used in clinical as medicine for various degenerative diseases such as heart disease and osteoporosis (Ruanma et al., 2010).

Plant calluses culture can be defined as a group of undifferentiated cells that growth due to the effect of plant hormone secretion. In a natural environment, the callus growth may occur due to

secretion of its plant natural hormone such as auxin and cytokinin. However, this phenomenon can be considered quite rare and may due to a certain environmental stress and special occurrence. In recent years, the benefits of callus have been noticed to trigger many plant studies especially somatic embryogenesis and plant regeneration (Evans et al., 1981). Therefore, many recent studies were done in callus induction of *Capsicum* spp. and have been established by using the role of plant hormone especially in *Capsicum annum* such as in hypocotyl, cotyledon and leaf explants, with single hormone or combination hormone such as indole-3-acetic acid (IAA), 2, 4-Dichlorophenoxyacetic acid(2, 4-D), and 6-Benzylaminopurine(BAP) (Rodeva et al., 2006; Ashrafuzzaman et al. , 2009; Aniel Kumar et al., 2010; Andrzej and Tomasz, 2011; Srinath and Prathiba, 2015). However, the study that uses foliar explant of *C. frutescens* with Naphthaleneacetic acid (NAA) and BAP still inadequate as most of the trend recent research focusing the single hormone effect which either auxin or cytokinin. Different concentration of hormone combination also may lead to different callus morphology that become key factor to determine successfully result in future related study in which some recent study do lack to emphasize this subject matter . As a result there are more data tend to focus more on growth rate and scales of callus but not viability of callus (El Kaaby et al., 2015; Ikhajiagbe et al., 2016; Swet Nisha et al., 2018).

The synergistic effect of both hormones auxin (NAA) and cytokinin (BAP) became the main objective of study in this research in order to gain related data not only toward high callus growth scales but also initiation day and viability of the callus that optimum to be used to enhance further research study in the future. This is because, callus morphology condition relatively has high impact on plant regeneration of *Capsicum* spp. which is known to be challenging due to recalcitrant nature which caused by inability of plant cells, tissues and organs to respond to *in-vitro* culture (Kothari *et al.,* 2010). Therefore, the successfully induce callus not only useful for plant regeneration but also provide clean stock that is free from contamination and plant disease. These obtained calluses can become very useful tools for many related studies such as micropropagation, somatic embryogenesis, protoplast fusion, plant hybrid from mixed callus culture and organogenesis (Castellar et al., 2011**).**

Materials and Methods

Explant samples preparation and sterilization procedures

About one week old of young foliar explants had been taken from natural plant of *C. frutescens* var bird eyes chili and were cut approximately 5mm^2. The cut explants were sterilized by using 15% sodium hypochlorite (NaOCI) with 2-3 drops tween 20 between 80 for 5 minutes. Then, the explants were rinsed with distilled water 3 times before continued to be sterile with ethanol 70% for 2 minutes. The final rinses of foliar explant with distilled water were done five times afterwards.

Media preparations

MS (Murashinge & Skoong, 1962) media were used as cultured medium and were prepared by adding 4.4g/L MS medium powder, 30g/L of sucrose, 1g/L myo-inositol, 4g/L gelrite into distilled water. The media pH were adjusted in between 5.7 to 6.0 after plant hormone were added respectively with seven different concentration treatments of NAA + BAP (0.0+2.5, 2.5+0.0, 2.5+2.5, 2.5+5.0, 5.0+2.5, 2.5+10.0, and 10.0+2.5 mg/L).

Callus culturing procedure and data collection

Three sterilized foliar explants were then cultured in one petri dish contain treatment media. Each treatment consist 20 replications were all incubated with 16 hours light and 8 hours night photoperiod. The cultures were observed under 25ºC for one month (30 days). The earliest days of first callus to induce were calculating in mean and the changes of both callus and explants morphology were observed. The growths of callus were scaled based on macroscopic observation.

Result and Discussion

In this experiment, *C. frutescens* foliar explants in MS media culture treated with NAA and BAP plant hormones were successfully inducing calluses during one month (30 days) of observation period except for MS control treatment. The plant hormone treatments consist of single treatment of NAA, BAP and also combination of NAA and BAP with different concentrations were able to induce different morphology varieties of calluses which clearly shown in the result (**Table 1** and **Figure 2**).

Low concentration of a single cytokinin hormone which was BAP (2.5 mg/L) successfully induced high callus growth. However in this treatment, the callus morphology became excessively brownish and required longer days (day 13) for callus to initiate (**Table 1** and **Figure 2a**). BAP commonly use as synthetic cytokinin plant hormone for shoot induction formation in many herbaceous plant (Gunay & Rao, 1978). However, in recent year many synthetic cytokinin including BAP and kinetin has been used as tool to induce not only shoot formation but callus induction in *Capsicum* species (El Kaaby et al., 2015; Sanjeev Kumar et al., 2017). Meanwhile, similar amount of a single auxin hormone NAA (2.5 mg/L) was able to produce better callus morphology with yellowish callus with early callus initiation days (day 8) (**Table 1** and **Figure 2b**). However, callus growth was very poor as compared to single hormone BAP. The presence of auxin in optimum concentration is usually related toward activation of expansion enzyme. Auxin may say to trigger phenomenon of loosening the explant cell wall leading to increase in initial growth of explant (Sanjeev Kumar et al., 2017; Swet Nisha et al., 2018). It's also stated that, the optimal amount of auxin such as NAA, 2, 4-D may increase rRNA transcription and propagation that causing the enzyme activity to accelerate the production of callus (Sanjeev Kumar et al., 2017).

However, some plant may require more than one hormone combination to gain desirable callus culture result and some plant do greatly depends on the balance between auxins and cytokinin in the cells. The treatment was later proceeding with the combination of both NAA and BAP, to enhance synergistic effect of both hormone compare to the use of single hormone concentration. Each treatment continuously added with the fix increment of 2.5 mg/L of either NAA or BAP in order to gain the optimum amount for positive synergistic effect of both hormone combination that shows relatively in callus initiation day, callus growth and morphology viability of callus.

The result shows that, treatments with same amount of NAA and BAP combination which was 2.5 mg/L each was successfully able to induce better callus morphology with pure whitish colour, soft and friable texture (**Figure 2d**). However, the callus has slightly moderate callus growth on day 9 as compared to single hormone NAA or BAP (**Table 1**). These combinations of growth regulators had shown synergistic effects compare to single hormone treatment which also shown similar result in studies of callus induction of *Capsicum annum* using cotyledon explant (Yang et al., 2000) and hypocotyl explant (Sanjeev Kumar et al., 2017) by using both NAA and BAP hormone combination. Double increase concentrations of BAP (5.0 mg/L) over NAA (2.5 mg/L)

in hormone combination not only able to produce the highest callus growth scale in shortest time (day 5) but it also successfully induce pure white, friable and soft callus texture (**Table 1** and **Figure 2e**). High amount of BAP hormone was required to trigger cell to undergo rapid cell division instead of cell differentiation process. Since, the BAP hormone is known to have very low toxicity (Roderick, 1992), therefore double concentration of BAP had shown high compatibility in inducing callus of *C. frutescens* with a few sign of necrosis or cell death. High concentration of BAP which was 10.0 mg/L and low concentration of NAA (2.5 mg/L) also able to initiate callus earlier which in day 6 (**Figure 1**). However, the morphology of callus growth show less formidable with transparent in colour with soft and friable texture. This shows that excessive concentration of BAP had started to affect the cell growth and may become toxic by acting as inhibitor rather than cofactor in inducing the calluses (**Figure 2g**).

The result was different with NAA, as the foliar explant cultured with additional concentration of auxin hormone had cause the opposite effect towards successful callus growth. Both high concentration of auxin which treatment 5.0 mg/L NAA +10.0 mg/L BAP and 10.0 mg/L NAA + 2.5 mg/L BAP respectively had held the callus from growing efficiently. The callus that successfully been induced was hard and non-friable which make it difficult to use for further plant studies (**Figure 2f** and **2h**). The hardened callus may lead toward difficulty of callus sub-culture process during micropropagation transfer. Furthermore it may contain high percentage of lack totipotency cell which has no longer viability to regenerate or propagate (Kothari et al., 2010).

Apart from that, high concentrations of NAA also increased the time to induce callus which happen in third week (**Figure 1**). This shows that the high concentration of NAA may cause this plant hormone to act as an inhibitor towards callus growth rather than to induce it. The result also shows that NAA may not compatible towards *C. frutescens*. NAA may resulting toxicity inside cell, which include the blocking the cell channel, expanding cell wall or trigger cell to release toxic content that may lead to cell death or inhibit cell growth which commonly display by inhibitor (Laura et al, 2012).

Conclusions

The treatment combination of NAA (2.5mg/L) and BAP (5.0 mg/L) by using foliar explants of *C. frutescens* was able to gain successful optimum level for callus induction in term of the best quality of callus morphology and growth in the fastest inducing time. The role of hormone combination between NAA and BAP were essential to produce high and efficient amount of callus in the earliest day as compared to the used of single hormone NAA and BAP. It is also able to produce white, friable and soft callus that are more suitable for plant studies such as plant regeneration, organogenesis, somatic embryogenesis and other related studies in the future. It is also recommended that more further studies on other relevant factors that may also contribute to increase the callus induction such as pH, light and dark condition, media type and explant types instead of plant hormones can be carried out. The use of other types of plant hormone to replace NAA or BAP may also affect callus growth and morphology variation for callus induction of *C. frutescens*.

Table 1 Growth scales and morphology of Callus induction of *C. frutescens* foliar explant in different concentration of NAA and BAP after 30 days (one month).

NAA + BAP (mg/L) concentration	Callus growth scales	Callus morphology characteristic
Control	-	-
0.0 + 2.5	+++	Yellowish white with friable and soft texture
2.5 + 0.0	+	Brownish white with friable and soft texture
2.5 + 2.5	++	Pure white with friable and soft texture
2.5 + 5.0	++++	Pure white with friable and soft texture
5.0 + 2.5	+	Brownish white with non-friable and hard texture
2.5 +10.0	++	Transparent white with friable and soft texture
10.0 + 2.5	+	Brownish white with non-friable and hard texture

*(-) =none, (+) = poor, (++) =moderate, (+++) = excessive and (++++) = highly excessive

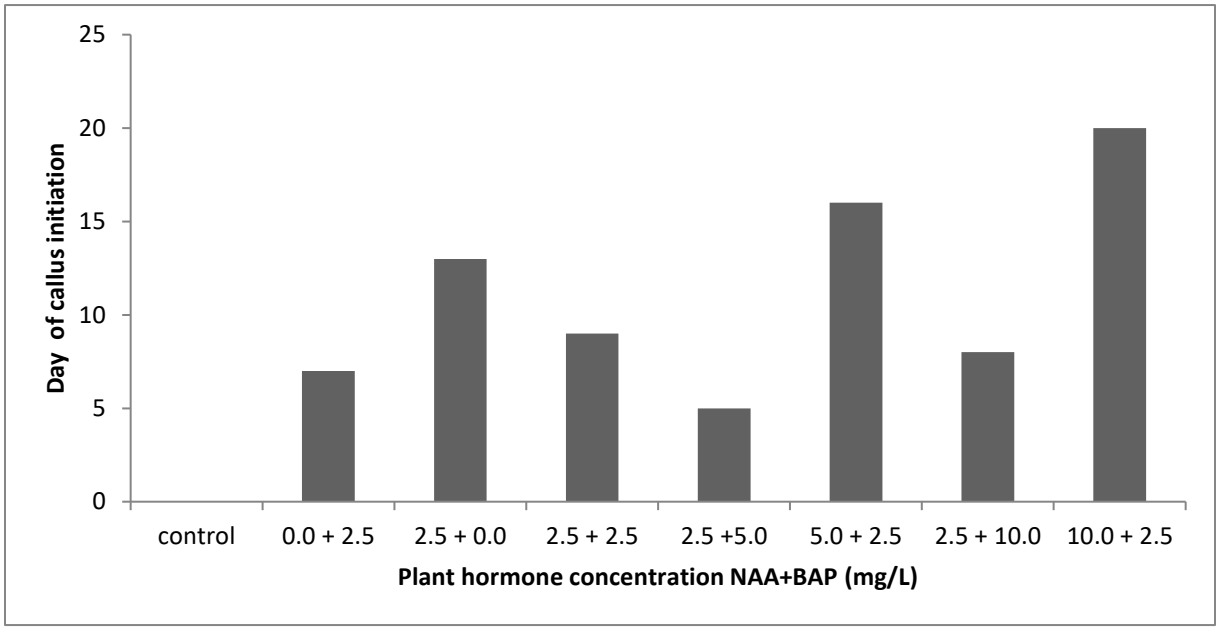

Figure 1 Effect on different concentration of plant hormones (NAA and BAP) in callus initiation for one month (30 days) duration.

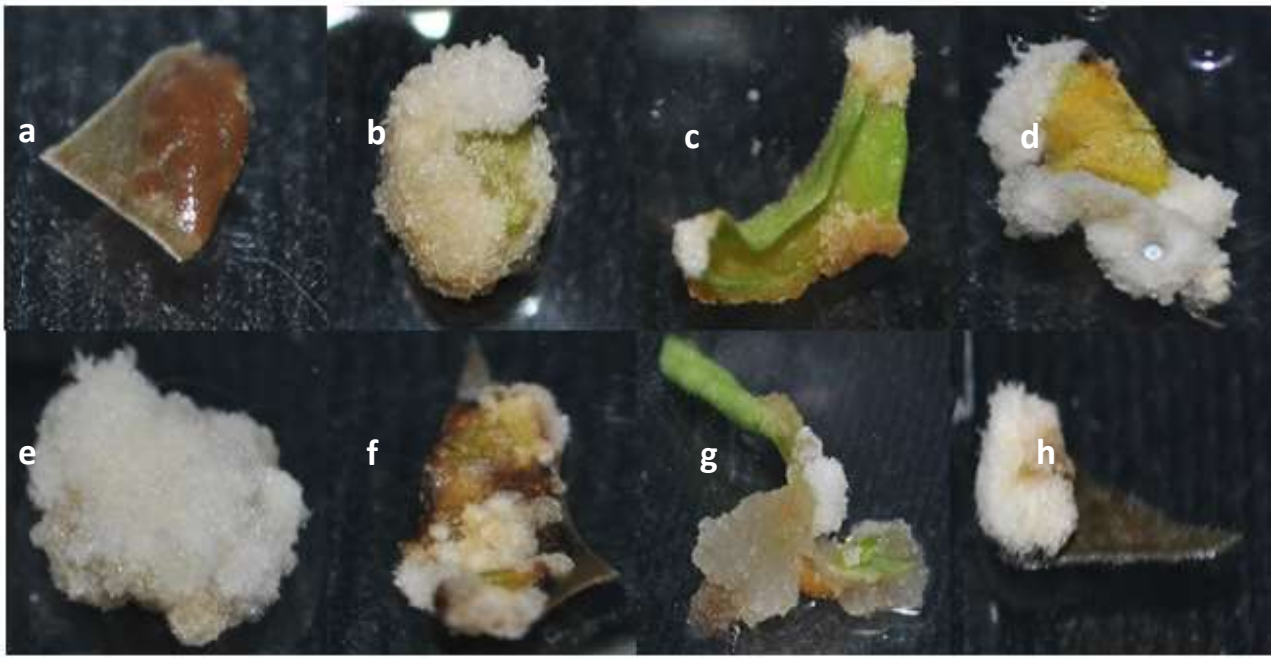

Figure 2 Callus morphology of *C. frutescens* by using foliar explant in different concentration treatments (mg/L) of NAA and BAP (a) control, (b) 0.0 + 2.5, (c) 2.5 + 0.0, (d) 2.5 + 2.5, (e) 2.5 +5.0, (f) 5.0 + 2.5, (g) 2.5 + 10.0, and (h) 10.0 + 2.5

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Conflict of interest

This study declared has no conflict of interest in any eligible or potential research or individual that related toward plant tissue culture field that focus in callus induction. This research study was carried out as complementary data during post graduated study which takes place in UiTM.

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