Full Length Research Paper

Preliminary responses of 2, 4-D and BAP on callus initiation of an important medicinal-ornamental \textit{Hymenocallis littoralis} plants

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Accepted 24 February, 2012

\textit{Hymenocallis littoralis} locally known as Melong kecil is an ornamental plant traditionally believed to have medicinal properties. This study was done to identify the best concentration and combinations of plant growth regulators (PGRs) for the initiation and production of friable callus. Four (4) weeks old plant was used in this study. Bulb was dissected and cleaned (sterilized) using standard surface sterilization technique and then cultured in semi-solid Murashige and Skoog (MS) media with combinations of 6-Benzylaminopurine (BAP) and 2, 4-D hormones at different concentrations ranging from 2.25 to 22.50 $\mu$M. The best concentration for callus induction was determined by using percentage of callus induction. The optimum concentration for callus initiation was obtained by using 13.50 $\mu$M of 2, 4-D and 4.50 $\mu$M of BAP with 93.75\% of callus induction rate. However, concentration 22.50 $\mu$M of 2, 4-D and 9.50 $\mu$M of BAP produced the lowest callus induction at 12.50\%. The earliest time period, which is needed to initiate callus from the meristematic tissue was obtained at 15 days on semi-solid MS media with 13.50 $\mu$M of 2,4-D and 4.50 $\mu$M of BAP. In contrast, culture medium with 18.00 $\mu$M of 2, 4-D and 9.00 $\mu$M of BAP took 40 days from inoculation to produce callus. Culture medium containing 9.00, 13.50 and 18.00 $\mu$M of 2,4-D together with 4.50 $\mu$M of BAP produced yellow friable callus whereas the other concentrations produced yellow compact calluses. Hence, 13.50 $\mu$M of 2,4-D and 4.50 $\mu$M of BAP concentration is the best medium for callus induction for \textit{H. littoralis}.

Key words: \textit{Hymenocallis littoralis} 2, 4-D, benzylaminopurine (BAP), bulb, callus initiation.

INTRODUCTION

Plants in the family of Amaryllidaceae have high alkaloidal contents. For many years, the alkaloids from the extracts of Amaryllidaceae plants have been widely used in chemical and pharmacological investigations. For decades, many plants from the Amaryllidaceae plant family had been used as remedy for innumerable illnesses. As reviewed by Abou-Donia et al. (2008), \textit{Hymenocallis salis}. genus was first phytochemically studied in 1920, which resulted in the isolation of lycorine. \textit{Hymenocallis littoralis} under Amaryllidaceae family is a bulbous perennial herb.

Large number of alkaloids was isolated from this plant bulb such as lycorine (dominant), narciclasine, lycoricidine (7-deoxynarciclasine) (bulb), pancratistatin (bulb and roots in small extent), trisphaeridine, Hippeastrine (bulb), littoraline, lycorenine, and haemanthamine (Lin et al., 1995). These phytochemicals have influence on various pharmacological aspects such as antiviral, anti-parasitic, anti-cancer, anti-bacterial, antioxidant, and wound healing properties (Backhaus et al., 1992; Pettit et al., 1993; Isset et al., 2001). In addition, \textit{H. littoralis} also exhibit numerous medicinal properties apart from being an ornamental plant (Backhaus et al., 1992). The bulbs of \textit{H. littoralis} were reported for the treatment of varicose veins, sores and swellings. Recently, Ocampo and Balick (2009) reported that applying the crushed bulbs in oil mixture can be used...
to treat freckles and blemishes. In vitro propagation is an important tool for rapid multiplication of medicinal plants (Atal and Kapur, 1982; Yew et al., 2010) as well as for the extraction of secondary metabolite products. Plant growth and developmental processes require the action and cross talk of phytohormones including auxins and cytokinins (Bajguz and Piotrowska 2009). Yew et al. (2010) reported the effect of different cytokinins on in vitro shoot length and multiplication of H. littorals. Auxins could regulate and influence diverse responses on a whole-plant level, such as tropisms, apical dominance and root initiation, and responses on cellular level, such as cell enlargement, division, and differentiation (Hagen and Guilfoyle, 2002). Cytokinins act at the cellular level by inducing expression of some genes, promotion mitosis and chloroplast development but also on the organ level by releasing buds from apical dominance or by inhibiting root growth (Riefier et al., 2006; Yew et al., 2010). Thus, auxins and cytokinins interact in the control of many central developmental processes in plants, particularly in apical dominance and root and shoot development. It was clearly documented that auxin may regulate cytokinin level and metabolism and vice versa (Nordström et al., 2004). By adjusting phytohormone concentration in the medium, differences in amount, rate and growth patterns of explants can be observed (Pierik, 1987; Ekiz and Konzak, 1997). Generally, high concentration of auxins and low cytokinins in the medium promote abundant cell proliferation with the formation of callus (Shah et al., 2003).

In this study, callus initiation from the meristematic tissues of H. littorals using plant growth regulators (PGRs) was carried out. The objective of the present study is to identify the best concentrations and combinations of 6-Benzylaminopurine (BAP) and 2, 4-D hormones for the initiation and production of friable callus which will be used in the next phase for the production of secondary metabolites using cell culture technology.

MATERIALS AND METHODS

Bulb preparation

In this study, the bulb of four (4) weeks H. littorals plant was used. In order to obtain the bulb of the plant, the outer polyethylene cover and the soil covering the bulb were removed. Then, the leaves and roots from the plant were cut off carefully without damaging the structure of the bulb which was around 3 to 4 cm long. The severed bulbs from the plants were then subjected to surface sterilization procedure.

Bulb surface sterilisation and dissection treatment

The bulbs had a brown colored coating that was peeled off. After that, the clean white coloured bulbs were placed in a beaker and two to three drops of Teepol (Sigma-Aldrich) solution was added. The beaker was then covered with a mesh and washed in running tap water for about 45 min to remove the surface adhering dirt, debris and soil. The bulbs were then dissected using a scalpel to obtain the inner meristematic tissues. Surface sterilisation procedures were conducted onto the meristematic tissues prior to subculturing. The meristematic tissues were first soaked in 70% ethanol for two minutes and thereafter in 50% commercial Clorox (5.50 % sodium hypochlorite solution) for 20 min. Upon completion of the sodium hypochlorite wash, subsequent steps were carried out in aseptic conditions in the laminar flow hood. Meristematic tissues were soaked in 70% ethanol for another 2 min. Then, the meristematic tissues were washed three times using sterile distilled water. Finally, the meristematic tissues were blot dried on a sterile filter paper prior to culturing it onto culture media.

In vitro culture establishment

To initiate a culture, the basal vascular portion was cultured in semi-solid MS (Murashige and Skoog, 1962) media containing different combinations and concentrations of 2.25, 4.50, 9.00, 13.50, 18.00, and 22.50 µM of 2,4-D together with either 0, 4.50 or 9.00 µM of BAP. For every concentration, four explants were cultured on the semi-solid MS medium in a single culture jar (59 mm × 66 mm). It was then repeated three times which made up to a total of 16 replicates (4 explants × 4 jars). Finally, the cultures were kept on a white wooden rack in culture room under dark condition at 25 ± 2°C. The cultures and the time taken to produce callus was monitored closely and recorded for four (4) weeks.

Callus initiation

After recording the duration to form callus, each callus were taken out from the culture jar and placed onto a sterile Petri dishes. Then, the morphology of the callus was carefully studied by observing and comparing the callus with each other. The colour (white, green and yellow) and texture of the callus (compact or friable) were recorded vigilantly. All the procedures were done under strict aseptic technique in order to prevent any forms of contaminations. After four (4) weeks of observation, the entire callus was subcultured to a culture jar containing semi-solid MS media with 13.5 µM of 2,4-D with 4.5 µM of BAP for propagation purpose.

RESULTS AND DISCUSSION

Percentage of callus induction

Bulb meristematic tissues were used to initiate callus on semi-solid MS media. The best concentration for callus induction was determined using percentage of callus induction. The formula for calculating callus induction is shown:

\[ C_p = \frac{\text{Number of explants with callus}}{\text{Total number of explants used}} \times 100\% \]

The optimum concentration for callus initiation in H. littorals was obtained by using 13.50 and 18.00 µM of 2,4-D with 4.50 µM of BAP. By using this combination and concentrations of PGRs, 93.75% of callus induction was obtained as shown in Figure 1. The 2.25 µM of 2,4-D with combination of 0 and 4.50 µM produced 60% callus induction meanwhile the 2.25 µM of 2,4-D with combination of 9.00 µM of BAP produced 70% callus...
induction. However, our results contradicted with Aftab et al. (2008), who reported that combination of 4.44 μM of BAP and 8.88 μM of 2,4-D produced highest callus induction for Gladiolus hybridus. Roy et al. (2008) reported that combination of 2,4-D with Kinetin (4.50 to 22.20 μM) resulted in the best callus initiation in Gymnema sylvestris.

Instead, 0 μM of BAP shows a depleted callus induction for all 9.00, 13.50, 18.00 and 22.50 μM of 2, 4-D. Concentration of 4.50 μM of BAP produced higher than 50% of callus induction in various concentrations of 2,4-D. Callus induction performance dropped to 69% using 9.00 μM of BAP. Media with 22.50 μM of 2,4-D and 9.00 μM of BAP produced the lowest callus induction.

Figures 3, 4 and 5 showed the initiation of callus from meristematic tissues of H. littoralis in different concentration. Turhan and Baser (2004) reported that, best callus induction percentage from the matured embryo of Triticum aestivum L. was obtained by using 4 mg/L (18.00 μM) of 2,4-D and 1 mg/L of NAA. This concentration does coincide with present study for the 2,4-D. Haliloglu (2006) supplemented MS media with 2 mg/L (9.00 μM) 2,4-D and produced 96% embryogenic callus from wheat leaf base segments.

Different concentrations of 2,4-D were also reported by Sarker and Biswas (2002) for callus induction in wheat and they found that 6 mg/L (27 μM) was the best concentration for callus induction treatment. Thus, the literature findings and current study explain that the various plant species needs different combinations of PGR and concentration for callus induction from meristematic tissue (Ault and Siqueira, 2008). There were also records of studies that has been done on various other plants which uses different other plant parts to induce callus such as leaves, nodes and buds (Mungole et al., 2009). Zeng et al. (2009) used stem and leaves from Elaeagnus angustifolia to induce callus. There are also documentations of different media being used for callus induction purpose. Manivannan et al. (2010) used N6 media to induce callus from elite Indian maize inbreeds’ immature embryo.

### Duration of callus initiation

Callus formation duration assessment was undertaken using the similar PGRs and concentrations as in callus induction percentage calculation evaluation. The earliest time period needed to initiate callus from the meristematic tissue by using semi-solid MS media was at 15 days (Figure 2) by using 13.50 μM of 2,4-D and 4.50 μM of BAP. For 4.50 μM of BAP, the longest callus initiation duration was obtained for 2.25 μM of 2,4-D at 20 days.

The shortest duration of callus initiation for 0 μM BAP was 20 days using 9.00 and 18.00 μM of 2, 4-D and the longest duration taken for callus initiation was at 23 days for 4.50 and 13.50 μM of 2, 4-D. The higher concentration which is 9.00 μM of BAP does not initiate callus as soon as the earlier concentrations as mentioned above. This is because the shortest duration for callus initiation was 25 days for 2.25 μM of 2,4-D with a combination of 9.00 μM

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**Figure 1.** Percentage of the callus induction using different combinations and concentrations of 2,4-D and BAP in H. littoralis. Different superscripts indicate statistical significant differences among the concentrations at a p-value < 0.05. Results were analysed by one way ANOVA and means were compared by using Duncan test.
Figure 2. Duration to form callus in *H. littoralis* using various combinations and concentrations of 2,4-D and BAP. Different superscripts indicate statistical significant differences among the concentrations at a *p*-value < 0.05. Results were analysed by one way ANOVA and means were compared by using Duncan test.

Figure 3. Initiation of callus from meristematic tissues of *H. littoralis* in different concentrations of 2,4-D (µM) in solid medium for 22 days. (A) 2.25 µM 2,4-D; (B) 4.5µM 2,4-D; (C) 9.0 µM 2,4-D; (D) 13.5 µM 2,4-D; (E) 18.0 µM 2,4-D; and (F) 22.5 µM 2,4-D. Scale bar = 2 cm.

of BAP. The longest duration taken for the explants to produce callus, was observed using 18.0 µM of 2,4-D and 9.00 µM of BAP at 40 days.

Similar study was demonstrated by Shah (2003) whereby callus formed in wheat (*T. aestivum*) after two weeks by using 9.00 µM of 2,4-D and 2.22 µM of BAP.
Figure 4. Initiation of callus from meristematic tissues of *H. littorallis* in different concentration of 2,4-D (µM) and BAP (µM) in solid medium for 22 days. (A) 2.25 µM 2,4-D and 4.5 µM BAP; (B) 4.5 µM 2,4-D and 4.5 µM BAP; (C) 9.0 µM 2,4-D and 4.5 µM BAP; (D) 13.5 µM 2,4-D and 4.5 µM BAP; (E) 18.0 µM 2,4-D and 4.5 µM BAP; and (F) 22.5 µM 2,4-D and 4.5 µM BAP. Scale bar = 2 cm.

Figure 5. Initiation of callus from meristematic tissues of *H. littorallis* in different concentrations of 2,4-D (µM) and BAP (µM) in solid medium for 22 days. (A) 2.25 µM 2,4-D and 9.0 µM BAP; (B) 4.5 µM 2,4-D and 9.0 µM BAP; (C) 9.0 µM 2,4-D and 9.0 µM BAP; (D) 13.5 µM 2,4-D and 9.0 µM BAP; (E) 18.0 µM 2,4-D and 9.0 µM BAP; and (F) 22.5 µM 2,4-D and 9.0 µM BAP. Scale bar = 2 cm.

(Shah et al., 2003). Another study reported that 5 mg/L (22.00 µM) of 2,4-D induced callus in 7 days in *Ocimum sanctum* (Holy basil) plant (Lim et al., 2009). Meanwhile, Hassan et al. (2009) reported that initiation of callus was
apparent as a white translucent tissue on the surface of the embryonic side of Aqab-2002, Inqalab-91, Saleem-2000, Sultan-96, Wafaq-2001, Zarlashta-99 wheat genotypes seed as soon as within 3 to 7 days after culture. Meanwhile in this study, media containing 9.00, 13.50 and 18.00 µM of 2,4-D together with 4.50 µM of BAP produced yellow friable callus whereas the other concentrations produced yellow compact calluses.

Conclusion

The highest percentage of callus induction at 93.75% was obtained by using 13.5 and 18.00 µM of 2,4-D and 4.50 µM of BAP. Earliest time period observed for the callus induction was at 15 days by using 13.50 µM of 2,4-D with 4.50 µM of BAP. Hence, this callus induction optimized protocol can be used for the establishment of callus and cell suspension culture from H. littoralis in future study.

ACKNOWLEDGEMENTS

The authors wish to thank Universiti Sains Malaysia for the Short Term grant and MOSTI for their support.

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