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RESEARCH ARTICLE

CALLUS INDUCTION AND CELL LINE ESTABLISHMENT FROM VARIOUS EXPLANTS OF *Kaempferia galanga*

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ABSTRACT

Kaempferia galanga or also known as *cekur* is a valuable traditional herb for folk medication. The secondary metabolites of this plant have various pharmaceutical properties such as vasorelaxant and antinociceptive effect. The conventional planting method of this plant is insufficient to fulfill the increasing demand of this plant for the production of secondary metabolites thus *in vitro* culture technique was used. This project focused on selection of elite cell lines of *K. galanga* to be used for the production of medicinally important bioactive compounds in large scale by using bioreactors. The callus derived from the root, leaf sheath and rhizome of this plant made up a total of 26 cell lines. These calli were induced and maintained on Murashige and Skoog (1962) medium supplemented with 1.0 mg/L 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 30 g/L sucrose, solidified with 7.7 g/L agar, and subcultured every 21 days for six subculture cycles. From these 26 cell lines, only eight cell lines could be selected as stable lines and categorized into fast, intermediate and slow growing lines based on their Growth Index. Among the explants, leaf sheath gave the most stable and fast growing lines. This is an effective tool for conservation of this important medicinal plant and to make the source of active compounds available throughout the year. The selected cell lines can be used as the material source for future preparation of cell suspension culture of *K. galangal.*

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INTRODUCTION

Kaempferia galanga, a perennial rhizomatous plant, belongs to family Zingiberaceae. *K. galanga* is native to India and distributed throughout tropics and sub-tropics of Asia and Africa (Chithra *et al.,* 2005). It is an important medicinal plant, commonly known as Cekur in Malaysia. The rhizomes of *K. galanga* are used in the treatments of indigestion, cold, pectoral and abdominal pains, headache, toothache, cholera, contusions and chest pains (Mustafa *et al*., 1996; Sirirugsa, 1997; Kanjanapothi *et al*., 2004) and malaria (Chithra *et al.,* 2005). The rhizome juice is used as a carminative and expectorant (Othman *et al*., 2006). Stem of this plant is used for menstrual stimulation and treatment of dyspepsia, whereas the leaves and flowers are used for the treatment of skin disease, eye diseases and seizures (Pongboonrod, 1976). Various researchers reported that *K. galanga* possess various pharmacological and biological activities such as antibacterial, antimicrobial, anticancer, larvicidal, amebicidal, antifungal, antiviral, vasorelaxant and anti-inflammatory (George and Pandalai, 1949; Gupta and Banerjee, 1976), Kosuge *et al*., 1985; Kiuchi *et al*., 1988; Pitasawat *et al*., 1998; Chu *et al*., 1998; Vimala *et al*., 1999; Mustafa *et al*., 1996; Othman *et al*., 2006).

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The use of rhizomes is a principle method of vegetative propagation of *K. galanga* and large quantities of rhizomes are often required because the efficiency of vegetative propagation is low. Conventional propagation of *K. galanga* by rhizome cutting is season dependent and requires long time to build up for the commercial quantities and involves high cost of production and disease susceptibility (Chithra *et al.*, 2005). Besides this, the species show poor natural regeneration by rhizomes thus reaching the verge of being recognized as an endangered species (Shirin *et al*., 2000). Tissue culture techniques are recognized as excellent tools for propagation of medicinal plants, allowing the production of contaminant-free plants under controlled conditions with independence of climatic factors. Cell suspension cultures are capable to produce medicinal compounds at similar rate or higher than intact plants due to optimization of the cultural conditions, selection of high-producing strains and precursor feeding without producing the whole plant (Vanisree *et al*., 2004). Besides the use for large-scale metabolite production, cell suspension culture technique may be employed for studying important biosynthetic pathways. Previous studies reported that *in vitro* plant regeneration of *K. galanga* have been developed by using axillary buds and rhizomes as explants (Vincent *et al*., 1992; Shirin *et al*., 2000). But to date no information is available on establishment of cell suspension culture of *K. galanga*. Therefore, in the present work, we

established different lines of callus and cell suspension cultures, which can foster studies to improve *in vitro* production of secondary metabolites. The detailed objectives of the study are (i) to determine the effect of subculturing on variation of *K. galanga* (ii) to categorize the callus line of *K. galanga* into fast, intermediate and slow growing line according to their growth performance and (iii) to select stable callus culture based on Growth Index.

MATERIALS AND METHODS

Callus line selection

The callus of *K. galanga* was induced from the root, leaf sheath and rhizome explants of *K. galanga* cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30g/L sucrose and 7.5g/L agar and supplemented with 1.0 mg/L 2, 4-Dichlorophenoxyacetic acid (2, 4-D). The callus was maintained on the same medium and 0.5g of callus derived from explants was inoculated separately into 300ml culture bottle containing 30ml of the callus proliferating medium. The cultures were maintained in the culture room at 25 ± 2°C with continuous light exposure of 32.5 µE m^{-2} s^{-1}. The fresh callus biomass was determined after four weeks by using digital balance (Mettler Toledo Dragon 602). Subsequent sub-culturing was carried out every four weeks interval. The fresh callus biomass was determined at each subculture cycle. The growth index was then determined based on the fresh biomass as:

$$Growth\ index = \frac{Final\ weight - Initial\ weight}{Initial\ weight}$$

Evaluate the growth pattern of callus culture of *K. galanga*

Each callus type that was established from the root, leaf sheath and rhizome explants were sub-cultured and categorized into various lines. A total of 9 lines were derived from root callus, 10 lines from leaf sheath and 9 lines from rhizome tissue. The callus (1.5 g) was used for each line and for this study the subculture cycle was fixed at every 21 days until sixth subculture cycles. The cultures were maintained in the culture room at 25 ± 2°C with continuous light exposure of 32.5 µE m^{-2} s^{-1}. The fresh weight of the callus was recorded after each subculture cycle. The other morphological characters of the callus such as color and texture were also observed and recorded.

Categorization of the selected cell lines

Based on the Growth Index, the selected stable cell lines were categorized into fast, intermediate and slow growing cell lines. The fast growing lines were selected based on mean Growth Index above 2.0. The intermediate growing lines have mean Growth Index from 1.5 to 2.0 while slow growing lines have mean Growth Index of lesser then 1.5.

RESULT

Callus was induced in all explants of *K. galanga* was in MS medium supplemented with 1.0 mg/L 2,4 Dichlorophenoxyacetic acid (2, 4-D) except the control

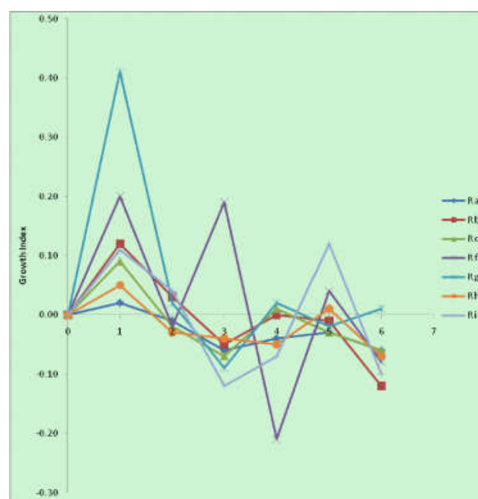

Fig. 4.1: Growth pattern of root derived callus of *Kaempferia galanga* over 6 subculture cycles on MS supplemented with 1mg/L 2, 4-D

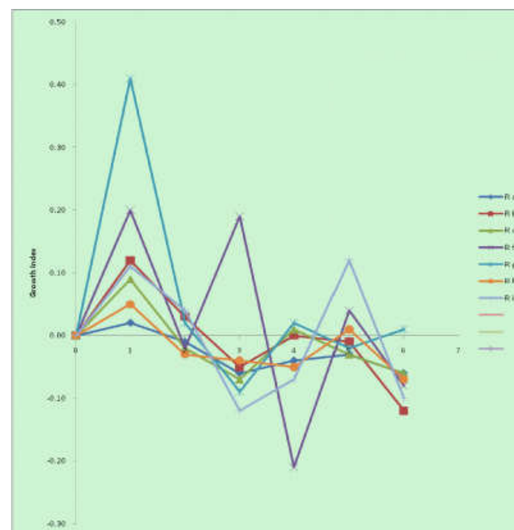

Fig. 4.2. Growth pattern of leaf sheath derived callus of *Kaempferia galanga* over 6 subculture cycles on MS supplemented with 1mg/L 2, 4-D.

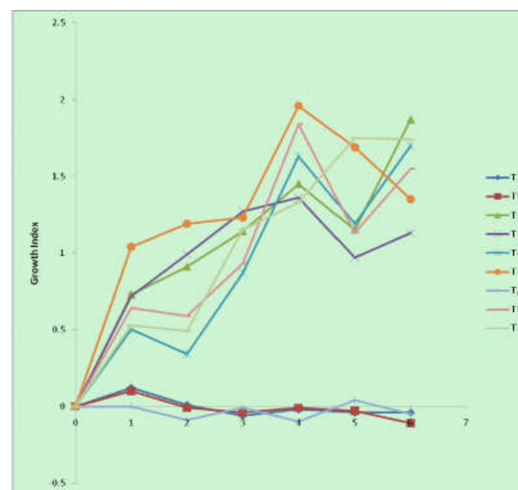

Figure 4.3: Growth pattern of rhizome derived callus of *Kaempferia galanga* over 6 subculture cycles on MS supplemented with 1mg/L 2, 4-D.

Table 4.1: Growth Index of stable cell lines of *K. galanga* for 6 subculture cycles

Cell lines	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6
Sg	0.23	1.30	1.36	2.91	1.53	2.12
Sh	1.58	2.43	2.31	3.81	3.10	3.65
Si	0.69	2.90	2.46	3.77	1.94	1.67
Tc	0.73	0.91	1.14	1.44	1.13	1.87
Td	0.72	0.99	1.27	1.36	0.97	1.12
Te	0.50	0.34	0.87	1.63	1.19	1.70
Th	0.64	0.59	0.93	1.84	1.13	1.55
Ti	0.53	0.49	1.15	1.33	1.75	1.74

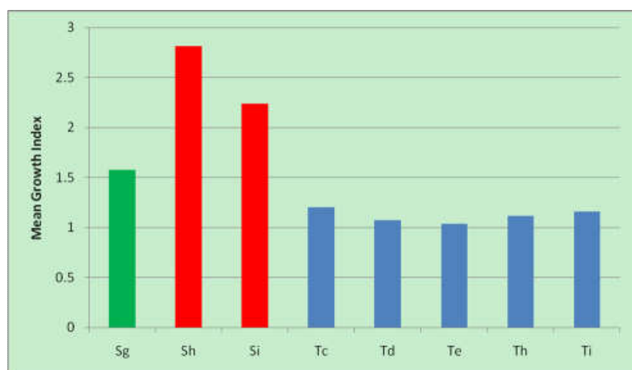

Figure 4.4: Mean Growth Index of stable cell lines of *Kaempferia galanga*. (Red: Fast growing lines; Green: Intermediate growing line; Blue: Slow growing lines)

(growth regulator-free). The callus appearance and callus color are explant types dependent. Two types of callus were obtained, friable with light yellowish color obtained from the root explants and leaf sheath explants and rough with brownish yellow color callus observed on rhizome explants.
Although, the root derived callus showed poor growth, but it can be differentiated into nine cell lines. Out of the nine cell lines, two lines (Rc and Re) were discarded due to contamination during the subculture process. After six subculture cycles, the Growth Index of the callus culture ranged from -0.21 to 0.41 on (Figure 4.1). This indicated that the root derived callus did not proliferate on MS medium supplemented with 1.0 mg/L 2,4-D. Based on the growth pattern, all the callus cell lines derived from the root explant did not show a stable and consistent growth.

Cali obtained from the leaf sheath explants lead to the development of 10 cell lines. Among these cell lines, six lines (Sa, Sb, Sc, Sd, Se and Sf) showed poor growth and four lines (Sg, Sh, Si and Sj) showed promising growth. Our result showed that, the leaf sheath cell line Sj showed sudden decrease in Growth Index at the 5th and 6th subculture cycle. This caused the cell line Sj to be considered as unstable line. After six subculture cycles, the Growth Indexes of the cell line Sg, Sh and Si indicated that they had consistent increase in biomass, therefore they were selected as stable cell lines (Figure 4.2). The nine cell lines derived from the rough brownish yellow callus of the rhizome explant were aggregated into a hard mass. After six subculture cycles, cell lines Ta, Tb and Tg showed poor growth. The lines Tc, Td, Te, Tf, Th and Ti on the other hand showed good growth. This indicated that cell lines Tc, Td, Te, Tf, Th and Ti proliferated well on MS medium supplemented with 1.0 mg/L 2, 4-D, while cell lines Ta, Tb and Tg did not. Thus, cell lines Tc, Td, Te, Th and Ti were considered as stable cell lines because of the trend of increase in their Growth Index (Figure 4.3). The Growth Index values for the six subculture cycles allowed eight cell lines to be selected as stable cell lines (Table 4.1). These eight lines were then divided into three categories; fast, intermediate and slow growing lines. Based on the mean Growth Index, Sh and Si were categorized as fast growing lines with an average Growth Index of 2.81 and 2.42 respectively. Cell line Sg was the only intermediate growing cell line with an average of 1.58. The cell lines Tc, Td, Te, Th and Ti were categorized as slow growing cell lines with average Growth Index ranged from 1.04 to 1.20 (Figure 4.4).

DISCUSSION

Cell cultures of medicinal plants have been used for the production of various groups of secondary metabolites (Alfermann and Petersen, 1995). Callus induction and subsequent cell line differentiation are the prime steps for the production of secondary metabolites (Wewetzer, 1998; Kuruvilla *et al*., 1999; Prakash *et al*., 2002). As plant cells are bio-synthetically totipotent; that is, cells in culture retain complete genetic information, consequently, they are capable of producing metabolites found in the mother plant (Rao and Ravishankar, 2002). Heterogeneity in biochemical activity within a population of cells derived from the same plant species or even various explants from the same plant can be exploited to obtain highly productive cell lines (Evans et al., 1984). Here in this study a total of 9 cell lines from the root derived callus, 10 cell lines from the leaf sheath derived callus and 9 cell lines from the rhizome derived callus were established for this purpose. These callus cultures were induced and maintained using MS supplemented with 1.0 mg/L 2, 4-D. Many researchers observed 2, 4- D as the best auxin for callus induction as common in monocot and even in dicot (Evans *et al*., 1984; Ho and Vasil, 1983; Jaiswal and Naryan, 1985; Chee, 1990; Mamun *et al*., 1996). Moreover, Tan *et al*. (2010) reported that the explants cultured on medium supplied with 1.0-2.5 mg/l of 2, 4-D gave very similar results. Therefore from an economic point of view, one should choose 1.0 mg/l 2, 4- D for callus induction. Primary role of secondary metabolites which are released due to defense responses to protect plant but due to its medicinal value it attracts attention of researcher (Sharma *et al*., 2011). One of the major constrains in producing theses metabolites through cell suspensions cultures that often, suspension and callus cultures produce a desired compound initially, but after a few passages the capacity to synthesize the product decreases considerably (Chattopadhyay *et al*., 2002). Therefore, callus lines need to be screened in order to evaluate the productivity of each cell line so that the best performing lines can be taken to cell suspensions (Yesil-Celiktas *et al*., 2010). Here in this study 26 lines were screened based on the mean of Growth Index, two cell lines (Sh and Si) were selected as stable fast growing lines in order to be used in secondary metabolites production through cell suspensions cultures.

CONCLUSION

Since the leaf sheath callus of the *K. galanga* contains most of the secondary metabolites (alkaloids, steroids and carbohydrates) present in the *in vivo* plants, the rapidly growing leaf callus may be used for the production of medicinally important drugs in large scale by using bioreactors provides a commercially realistic alternative to

whole plant for the important chemicals or drugs and minimizes the exploitation of this species. This is an effective tool for conservation of the species and to make the source of drugs available throughout the year.

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