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Induction and Maintenance of Callus from Leaf Explants of *Mirabilis jalapa* L.

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ABSTRACT

Mirabilis jalapa L., commonly known as 'four o'clock plant' produces a strong, sweet smelling fragrance after the flowers open at late afternoon. It is a well known ornamental plant as the flowers of different colours can be found simultaneously on the same plant or an individual flower can be splashed with different colours. The colour-changing phenomenon is one of the unique characteristics of *M. jalapa* as it can display flowers with different colour when it matures. Apart from its ornamental value, it has also earned its place in herbal medicine practices around the world. Its array of biological activities continues to support its use worldwide for control of viruses, fungi and yeast. In this study, callus culture was initiated from the leaf explants of *M. jalapa*. The suitable strength of MS (Murashige and Skoog) nutrient media was determined and the effects of different types of auxins [4-amino-3,5,6-trichloro picolinic acid (picloram), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA)] and cytokinin [6-benzyl amino purine (BAP)] at 0.0 2.5, 5.0, 7.5, 10.0, and 20.0 μM were investigated in order to determine the suitable callus induction and maintenance media. The establishment of callus culture was greatly influenced by the strength of MS medium, type as well as the concentration of plant growth regulators (PGRs) used. The best callus induction response was obtained on half strength (½) MS media supplemented with 20.0 μM picloram which produced healthy and friable callus. Meanwhile, ½ MS supplemented with NAA or BAP as well as PGR-free medium did not produce any callus; rather, explants became necrotic after 3 to 4 weeks of culture. Calli were successfully maintained in ½ MS supplemented with 10.0 μM picloram. Callus in maintenance medium showed a sigmoid growth pattern and reached a maximum growth rate between weeks 1 and 3.

Keywords: browning effects, callus cultures, growth curve, medicinal plant
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BAP**, 6-benzylamino purine; **MS**, Murashige and Skoog medium; **NAA**, α-naphthaleneacetic acid; **picloram**, 4-amino-3,5,6-trichloro picolinic acid; **PGR**, plant growth regulator

INTRODUCTION

Mirabilis jalapa L. also known as the "four o'clock plant", belongs to the Nyctaginaceae family. It is a tropical herb commonly cultivated in North America, a perennial in the south, warm west and an annual in the north. Long before the Spanish Conquest of Mexico, it was cultivated by the Aztecs for its medicinal properties and for its showy fragrant flowers (Le Duc 1986). In the traditional popular medicine of the South American continent, particularly in Brazil, the roots, shoots, leaves, fruits, and seeds of *M. jalapa* L. are employed for different affections (Lorenzi 1991; Lorenzi and Souza 1995). For instance, traditionally healers in Brazil use a paste of the root applied as a poultice to treat scabies and muscular swellings; the juice of the leaves is used in the treatment of diarrhoea, indigestion and fevers (Diggs *et al.* 1999).

Conventionally, *M. jalapa* reproduces via viable seeds but the low percentage of seed viability limits its natural propagation since the hawk-moth population are the only species that act as its pollinator (Martinez and Búrquez 1996). Hence, alternative techniques like plant tissue culture were established to propagate a large number of *in vitro* plants. A rapid and efficient *in vitro* regeneration system has been developed from nodal segments (Zaccai *et al.* 2007) and cotyledons (Xu *et al*. 2005) of *M. jalapa*. By using plant tissue culture technique, genetically similar plantlets can be produced in relatively short time. Besides, by maintaining genetic stability, tissue cultured plants are also valuable in speeding up conventional breeding and propagation, reducing space and labour requirement and achieving manipulative goals that cannot be carried out via *in vivo* conditions. For instance, with the advances in plant biotechnology, the extract of *M. jalapa* cultured cells and its precipitate fraction with 90% saturated ammonium sulfate was found to show an anti-plant viral activity comparable to that of the roots and leaves of the original plant (Ikeda *et al*. 1987). In view of these facts, the present study was conducted to determine and examine the suitable strength of MS media, and the effects of different auxins and cytokinin at various concentrations on callus induction from leaf explants. In addition, the best maintenance medium and the growth curve of leaf-derived callus of *M. jalapa* L. was also determined.

MATERIALS AND METHODS

Plant materials

The seeds of *M. jalapa* were obtained from Sabah, Malaysia. The black coloured mature seeds were germinated on the soil with the composition of nitrogen, phosphate, potassium at the ratio of 1: 3: 2. The seeds germinated easily (with 96% of germination percentage) within 3 to 7 days. The leaves were collected from one-month old healthy seedlings.

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Surface sterilization

One-month old leaves were collected and washed under running tap water for 30 min then initially surface sterilized in 0.2% (w/v) fungicide (Ancom Thiram, USA) for 30 min followed by a dip in 70% (v/v) ethanol for 1 min. Leaves were then immersed for 25 min in 15% (v/v) Clorox® containing 2 to 3 drops of Tween-20 (Amresco, USA) with gentle and continuous shaking. The leaves were then rinsed three times with sterile distilled water for 5, 10, and 15 min, respectively.

Culture media

Standard MS (Murashige and Skoog 1962) solid media was used as the basal medium. To screen the suitable strength of MS media on callus induction of *M. jalapa*, full- and half-strength (½) MS media supplemented with various plant growth regulators (PGRs) at different concentrations were prepared. Meanwhile, medium without any PGR was used as the control. For the preparation of MS media, macronutrient, micronutrient, vitamin, FeNaEDTA were added and fortified with 3% (w/v) sucrose; PGRs were added before autoclaving. The pH of the medium was adjusted to 5.7 ± 0.1 using 0.1 M NaOH or 0.1 M HCl and solidified with 0.8% (w/v) agar. The media were then autoclaved at 121°C for 15 min. Approximately 25 mL of the sterile medium were distributed into each 100 mm × 15 mm sterile disposable Petri dish (Labchem, Malaysia).

Callus initiation

The surface-sterilized leaves were cut into approximately 5 mm^2 explants and cultured on either full- or ½ MS solid media supplemented with different PGRs – picloram (Duchefa, Netherlands), 2,4-D (Sigma, U.S.A), NAA (Duchefa, Netherlands), and BAP (Duchefa, Netherlands) at 0.0, 2.5, 5.0, 7.5, 10.0, and 20.0 µM. A total of 5 explants were used for each treatment and all the experiments were performed with three replicates and repeated twice.

Culture conditions

All the cultures were incubated and maintained at 26 ± 2°C under a light intensity of approximately 2000 Lux provided by cool white fluorescent lamps under a 16-h photoperiod. Analysis of the percentage of callus induction, days of callus formation, morphology and colour of the callus, intensity of callus growth, and root initiation were observed every 5 days.

Callus maintenance

After 18 days of culture, young, friable, and healthy callus with high frequency of formation was chosen and transferred onto the best induction media (½ MS supplemented with 20.0 µM picloram) and subcultured at two weeks interval for at least three times. In order to determine the best maintenance medium, callus was also transferred to ½ MS supplied with 0.0, 10.0, 20.0 and 30.0 µM picloram.

Growth curve measurement

Subsequently, the best treatment or maintenance medium (½ MS + 10.0 µM picloram) was subjected to growth curve analysis with the initial fresh weight of 0.01 g. The growth of leaf-derived callus was evaluated by measuring the fresh weight at one-week interval continuously for four weeks. The experiments were conducted with three replicates and repeated twice.

Statistics analysis

The data collected in this study were subjected to the statistical analysis. One way ANOVA and Tukey's Honestly Significant Difference (Tukey's HSD) test ($p<0.05$) were used to determine the differences in mean number of all tested parameters. Statistical analysis was performed using SPSS software (version 11.5) (SPSS Inc. USA).

RESULTS AND DISCUSSION

Effects of medium strength on callus induction

Different strengths of MS media produced different effects on callus induction (**Table 1**). The results were evaluated in terms of the day of callus induction, the intensity of callus growth as well as the morphology of the callus formed. It was observed that all the full- and ½ MS medium supplemented with 2, 4-D (2.5, 5.0, 7.5, 10.0, and 20.0 µM) successfully induced the callus from the leaf explants of *M. jalapa* within 4 weeks of culture. In terms of days of callus formation, explants cultured in ½ MS medium supplemented with 7.5 µM 2, 4-D required the shortest period which was 7 days of culture for callus formation. This treatment produced profuse growth of friable and whitish callus (**Fig. 1A**). On the other hand, full MS medium supplemented with 7.5 µM 2,4-D induced the callus after 10 days of culture and the unhealthy yellowish watery callus (**Fig. 1B**) were observed in this treatment.

From the results, it indicated that the callus induction from the leaf explants of *M. jalapa* was influenced by the strength of the MS medium. The explant supplemented with same component of concentration of 2,4-D (7.5 µM) but different strength of MS medium showed different results in morphology of callus and days of callus initiation in which it could be determined that ½ MS medium was more suitable for callus induction. This phenomenon is in accordance with the *Taxus* species whereby the callus was successfully induced on ½ MS media but showed poor sign of callusing when cultured on full MS media (Chang and Yang 1995).

Table 1 Effect of full- and ½ MS medium supplemented with 2,4-D at various concentrations on the callus induction and characteristics of callus derived from the leaf explant of *Mirabilis jalapa* L.

MS media strength	2,4-D concentration (µM)	Days of callus initiation*	Degree of callus formation	Morphology of callus	Colour of callus	Root initiation
½ MS	0.0	-	-	-	-	Negative
	2.5	12 b	+	Friable	WB	Positive
	5.0	9 bc	+++	Friable	W	Positive
	7.5	7 c	+++	Friable	W	Negative
	10.0	10 bc	++	Friable	WY	Negative
	20.0	19 ab	+	Compact	WY	Negative
MS	0.0	-	-	-	-	Negative
	2.5	17 b	+	Friable	WB	Positive
	5.0	15 b	++	Watery	WY	Positive
	7.5	10 bc	+++	Watery	WY	Positive
	10.0	20 a	+	Watery	WY	Negative
	20.0	26 a	+	Compact	WB	Negative

* Values followed by the same letter are not significantly different at the $p < 0.05$ level, according to the Tukey's HSD test.
- indicates no callus formation
Callus growth scoring value = +: Poor, ++: Moderate, +++: Profuse
Colour of callus formed = W: White, WY: Whitish Yellow, WB: Whitish Brown
Root formation rating = Positive: Roots formation, Negative: No root formation

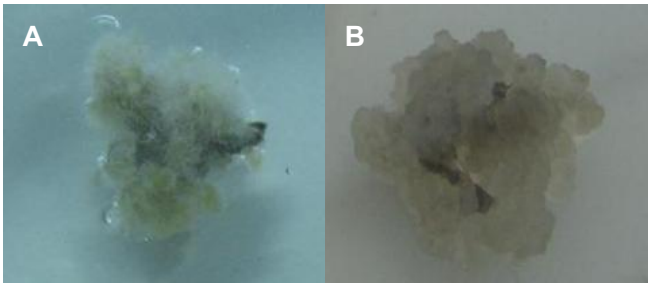

Fig. 1 Effects of different strength of MS medium supplemented with 7.5 µM 2,4-D on the morphology of the callus formed from leaf explants of *M. jalapa*. (**A**) Friable and whitish callus formed in full MS medium. (**B**) Yellowish water callus formed in ½ MS medium.

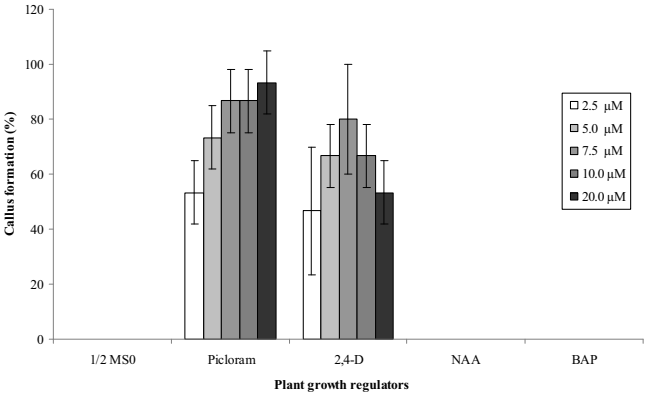

Fig. 2 Percentage of callus induction from the leaf explants of *Mirabilis jalapa* L. after 3 weeks of culture on ½ MS media supplemented with different plant growth regulators at various concentrations. Bar indicates the mean ± standard deviation.

Similar finding was reported by Islam *et al*. (2004) in which the MS medium significantly affected the callus induction in *Oryza sativa* L. Other than this, Kester and Hesse (1995) also reported the production of friable and whitish callus when cultured the leaf explants of *Brassica* on ½ MS medium while full MS medium inhibited the growth of callus in which the callus subsequently turned brown and died after 4 weeks of culture. From the fact that ½ MS medium contains lower concentration of salts than the full MS medium, it might hypothesize that a high concentration of salts may inhibit the callus induction from the leaf explants of *M. jalapa*.

Effects of plant growth regulators on callus induction

The potential of different PGRs in the induction of callus has been well documented in different plants in which generally, it was essential for the initiation of callus *in vitro* (Qi *et al.* 2005). Similarly, in this study the concentration of plant growth regulators gave a remarkable effect on callus induction from the leaf explant of *M. jalapa*. Leaf explants that cultured on the PGR-free medium (control) did not produce any callus and the explants eventually turned to brown in colour and become necrosis after 3 weeks of culture. This observation was in accordance with the results obtained by Chen and Chang (2001) who reported that there was no sign of callus formation when leaf explants of *Oncidium* were cultured on PGR-free medium and subsequently the explants turned dark and died after several weeks of culture.

Leaf explants treated with picloram exhibited callus formation at the cut ends and gradually extended over the entire surface of the explant. As shown in **Table 2**, different types of auxins have different effects on the callus induction from the leaf explant of *M. jalapa*. It was found that 20 µM picloram gave the best callus induction response (93.3%) (**Fig. 2**). The leaf explants treated with 20 µM picloram showed friable, profuse and whitish healthy callus (**Fig. 3A**) and the callusing was observed after 6 days of culture. This was in agreement with the observation reported by Figueiredo *et al.* (2003) who studied the *Rollinia* plant and found that with the presence of picloram, whitish callus were induced and visible within 10 days. Further decreased in the concentration of picloram was deleterious for callus induction. At lower concentration (2.5 µM), the callus induction percentage was decreased from 94.0 to 53.3% (**Fig. 2**). Poor and brownish callus were observed at this concentration after 3 weeks of culture (**Fig. 3B**). The observation obtained in this study agreed with the findings by Hill (1996), in which the callus formation for stem explants of *Onvolvulus arvensis* L. were inhibited at the lower concentration (0.5-2 µM) of picloram. In addition, Chernova *et al*. (2005) proved that a high concentration of picloram produced beneficial effects in the callus culture of wheat and onions. However, very high concentration of picloram may cause cytotoxic to the cell and retarded the somatic embryo formation.

According to da Silva *et al*. (2005), 2,4-D is the main synthetic auxin used to induce the callogenesis. This is due to one of its main characteristics that capable of simulating the cell division in tissues of several plants. In the present study, 2,4-D also managed to induce callus in all the concentrations tested (2.5, 5.0, 7.5, 10.0, and 20.0 µM). However, a relatively low callus induction response was shown as compared to picloram treatments (**Table 2**). Instead of

Table 2 Effect of ½ MS media supplemented with Picloram and 2,4-D at the concentrations of 0.0, 2.5, 5.0, 7.5, 10.0, and 20.0 µM on induction of callus and characteristics of callus derived from the leaf explant of *Mirabilis jalapa* L.

Treatments	Concentration (µM)	Days of callus initiation*	Degree of callus formation	Morphology of callus	Colour of callus	Initiation of root
Picloram	0.0	-	-	-	-	Negative
	2.5	10 b	+	Friable	WB	Negative
	5.0	9 b	++	Friable	WB	Negative
	7.5	7 c	+++	Friable	WY	Negative
	10.0	5 c	+++	Friable	W	Negative
	20.0	6 c	+++	Friable	W	Negative
2,4-D	0.0	-	-	-	-	Negative
	2.5	13 b	+	Friable	WB	Positive
	5.0	10 b	+++	Friable	W	Positive
	7.5	8 c	+++	Friable	W	Positive
	10.0	11 b	++	Friable	WY	Negative
	20.0	18 a	+	Compact	WB	Negative

* Values followed by the same letter are not significantly different at the $p < 0.05$ level, according to the Tukey's HSD test.
**No callus formation was obtained in ½ MS media supplemented with NAA and BAP at the concentration of 0.0, 2.5, 5.0, 7.5, 10.0, and 20.0 µM.
- indicates no callus formation
Callus growth scoring value = +: Poor, ++: Moderate, +++: Profuse
Colour of callus formed = W: White, WY: Whitish Yellow, WB: Whitish Brown
Root formation rating = Positive: Roots formation, Negative: No root formation

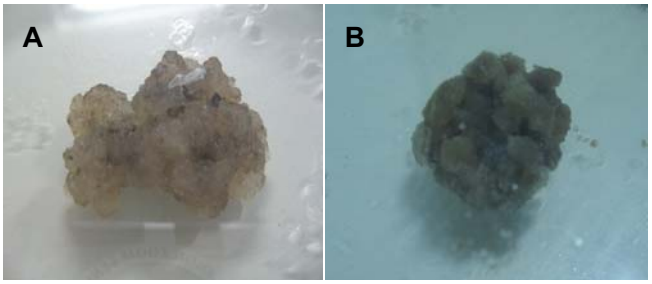

Fig. 3 Callus induction from the leaf explant of *Mirabilis jalapa* L. after 3 weeks of culture on ½ MS medium supplemented with different concentrations of picloram. (**A**) Friable and whitish callus formed in 20.0 µM picloram. (**B**) Brownish callus formed in 2.5 µM picloram.

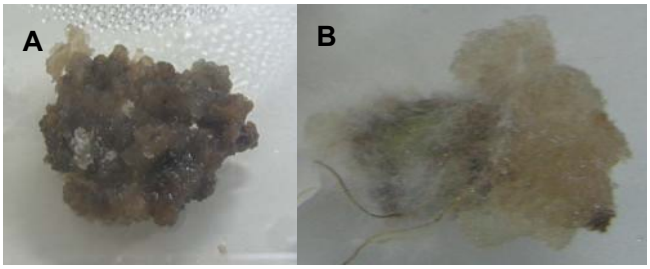

Fig. 4 Effects of ½ MS medium supplemented with different concentrations of 2,4-D on leaf explants of *M. jalapa*. (**A**) Brownish callus was formed in 20.0 µM 2,4-D. (**B**) arrow shows the formation of adventitious roots with hair in 5.0 µM 2,4-D.

2,4-D, Fitch and Moore (1990) reported that the media supplemented with picloram was better to induce the profuse, green and friable callus cultures of sugarcane. Within the 2,4-D treatments, the highest percentage of callus induction (80.0%) from the leaf explants was obtained in ½ MS medium supplemented with 7.5 µM 2,4-D (**Fig. 2**) and the callus was observed after 8 days of culture. The observation obtained in this study was similar with the findings by Chernova *et al*. (2005), in which the best callusing response on legumes tissue was in treatments with 5.5 to 7.5 µM of picloram. Low or high level of 2,4-D eventually affected and inhibited the callus induction from the leaf explant of *M. jalapa*. For example, decrease in concentration (7.5 to 2.5 µM) or further increase the concentration from 7.5 to 20.0 µM 2,4-D, both resulted a reduction response on callus induction which decreased from 80.0 to 46.7 and 53.3%, respectively (**Fig. 2**) whilst poor, brownish callus was observed in these cultures. Similar observation was demonstrated by Bajaj and Pierik (1994) on the vegetative propagation of *Freesia* through callus cultures, whereby the growth of callus were retarded in low concentrations of 2,4-D. On the other hand, Banerjee and Gupta (1996) reported a high concentration of 2,4-D affected the callus texture and morphology of *Nigella sativa* leaf explant.

Apart from the callus induction, it was noticed that adventitious roots with hair (**Fig. 4**) were induced in the explants cultured with relatively low concentration of 2,4-D (2.5, 5.0, 7.5 µM). Likewise, in other plant species such as *Morus latifolia* and *Morus alba*, rooting of explants in the presence of 2,4-D was observed (Chitra and Padmaja 1999; Lu 2002). Although 2,4-D managed to show high callus initiation percentage after one week of culture, adventitious roots began to form from the callus at day 12 and the growth of calli was subsequently retarded and eventually ceased at the fourth week. This study discovered that the roots formation were undesirable phenomenon in the callus study as the roots that induced will compete nutrient and space with callus hence repressed growth of callus (Leroux 1997).

Synthetic auxin, NAA is known to induce good-quality callus and somatic embryogenesis or organogenesis in a number of medicinal plants (Arditti and Ernst 2008). Ziv *et al*. (1997) also reported that callus culture of *Gladiolus* responded well in NAA-supplemented media. Similarly, NAA was found to be effective in stimulating the callogenesis response in *Narcissus* (daffodil) species (Seabrook and Cumming 1998). However, in this study, contradictory results were obtained. This varied effect of NAA might be due to the fact that the effect of PGRs is plant specific. Thus, different plant species responded differently to the same type of PGR. NAA failed to induce the callus from leaf explants of *M. jalapa*, regardless of the concentrations tested. The explants turned brown after 2 weeks of culture and became necrotic within the third and fourth week. This phenomenon could have resulted from the browning effects due to the oxidation of phenolic compounds. In fact, the accumulation of phenolics is a response to either microbial infection or physiological stress such as wounding of tissues, which is a part of plant defense mechanisms whereby phenolic compound production serves to limit the microbial invasion into cells (Rahman and Punja 2005). As observed in this study, the wounding of leaf explants promoted the damage of tissues or cells at the cutting area to release phenolic compounds in response to the cutting stress condition. Therefore, explants eventually turned brown or dark during the period of observation. Besides that, the browning effects on the plant tissue could also be due to the exposure of stress exerted by the detrimental effects of NAA, which also acts as auxinic herbicide (Aslam *et al*. 2005). The browning effect is an undesirable phenomenon in plant tissue culture studies as it is one of the factors that could limit proper tissue multiplication and maintenance (Alemanno *et al*. 2003). Similar observations were obtained in *Pinus sylvestris* L. in which Anderson and Levinsh (2002) indicated that the explants eventually turned brown and became necrotic after several weeks of culture due to phenolic oxidation.

Cytokinin can promote cell enlargement and cell division in certain tissues (Rayle *et al.* 1982; Ross and Rayle 1982). Nevertheless, there was no sign of callusing in the ½ MS medium incorporated with BAP as a source of cytokinin and the explants of *M. jalapa* turned yellowish and brown after the third and fourth week of culture. A similar observation was obtained when *Begonia hiemalis* was treated with BAP (Welander 2002). Tanaka and Sakanishi (1998) suggested that higher concentrations of BAP inhibited callus proliferation. Other than that, Kaul and Sabharwal (2002) also reported that *Haworthia* cultured in MS media with low concentrations of BAP failed to induce any callus.

Callus maintenance

After callus induction, it is essential to maintain the callus in a suitable maintenance medium to ensure the viability of callus for a long period of time. Callus culture was initially isolated from the parent explant and transferred to a fresh medium, usually consisting of the same composition with the best induction medium, and subcultured at least three times (Samantaray *et al*. 1995). As callus is an undifferentiated clump of cells, the nutrients required to grow them is extremely high. Moreover, repeated subcultures on fresh agar medium may improve the amount and friability of the callus (Smith 1992).

In order to determine the best maintenance medium, leaf-derived calli were also subcultured onto fresh ½ MS media supplemented with 0.0, 10.0, 20.0 or 30.0 µM picloram. Calli maintained on 10.0 µM picloram gave the best growth (**Fig. 5**) and the calli developed were the healthiest and exhibited the highest callus growth rate while calli maintained on fresh medium containing 0.0, 20.0 and 30.0 µM picloram showed browning, retarded growth and were desiccated. A change in the exogenous requirement of callus for picloram from a concentration of 20.0 µM (best induction medium) to 10.0 µM in the subsequent callus maintenance may due to the habituation phenomenon in which callus and cell systems lose their requirement for specific

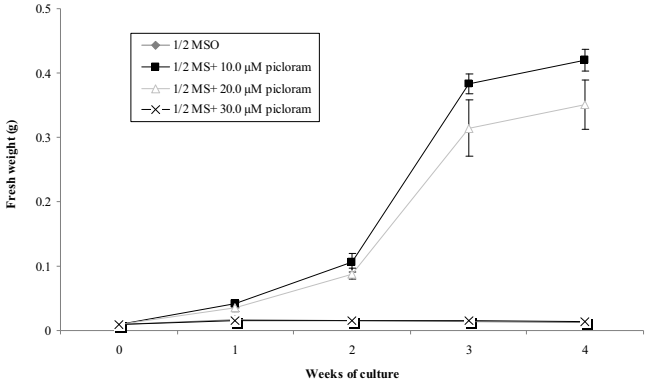

Fig. 5 Growth of leaf-derived callus maintained on ½ MS media supplemented with picloram at various concentrations (0.0, 10.0, 20.0, 30.0 µM). Bar indicates the mean ± standard deviation.

PGRs after a period of time (Mein and Binns 1998). According to Pierik *et al*. (1999), the growth of *Gerbera* species callus required a lower level of auxin for maintenance as compared to the induction. This study was in accordance with the results obtained by Meyer (1996), in which the requirement for picloram in *Paeonia* species has been reduced from 25.0 µM (induction) to 10.0 µM in the callus maintenance medium.

Determination of growth curve

The leaf-derived callus of *M. jalapa* cultured in ½ MS medium supplemented with 10 µM picloram over a period of 4 weeks exhibited a sigmoid growth pattern. It was found that within 4 weeks of culture, the calli underwent a lag phase, followed by an exponential phase and an early stationary phase (**Fig. 6**). It was observed that the lag phase occurred during first week of culture, in which the fresh weight of the callus increased slowly and slightly from 0.041 to 0.105 g. The growth of callus did not begin immediately when the callus was newly introduced to a medium since it required time to adapt to the new environment such as media pH, temperature, and culture medium. Thus, the metabolism of callus mainly is used to make them suitable to grow in the new medium (Scragg and Allan 1993). According to Astarita and Guerra (2000), this phase is the adaptive and energy producing period. Hence, it showed a very slow growth rate. The exponential phase took place from first week up to third week. This was relatively similar to other plant species such as *Taxus* that has an exponential growth phase between 13 to 20 days (Mihaljevic *et al*. 2002). During the second to third week, the calli showed a 3- to 4-fold increment in the fresh weight, from 0.105 to 0.383 g at week 3. The increase in callus fresh weight was in accordance with an increase in the callus size. According to da Silva *et al*. (2005), the exponential phase is the period of maximum cellular division and the greatest growth rate of callus. It also represents active cells division, caused the cells to proliferate rapidly and reached the maximum growth rate. The period of decelerating growth of callus and began to remain essentially constant was observed between third and fourth week whereby the growth rate was gradually reduced and the fresh weight gained was 0.419 g at week 4. The occurrence of a stationary phase could be due to the depletion of nutrients, drying of agar, accumulation of toxic byproducts and reduction of the oxygen amounts inside the cells (Smith 1992). According to Liu *et al*. (2001), growth on the same medium for an extended period will lead to the depletion of essential nutrients and to a desiccation of the gelling agent.

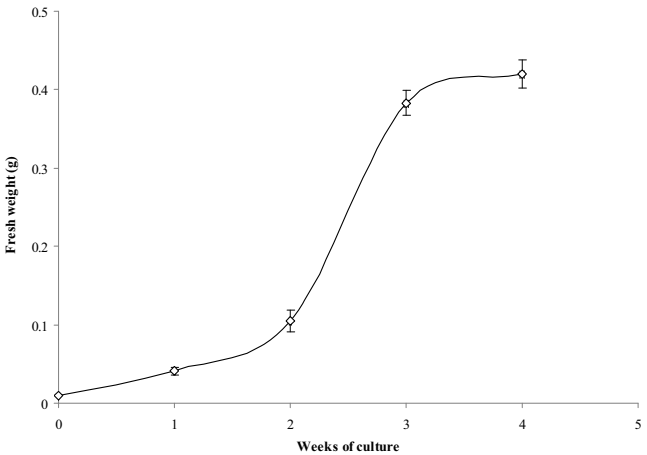

Fig. 6 Growth curve of the leaf-derived callus maintained on ½ MS media supplemented with 10.0 µM picloram. Bar indicates the mean ± standard deviation.

CONCLUSIONS

Since friable calli have great potential in suspension cell cultures, it is suggested that further study of *in vitro* regeneration should be carried out from the leaf-derived callus in order to enhance the propagation efficiency of *M. jalapa* as a high multiplication rate could be achieved using micropropagation rather than traditional vegetative propagation methods. In addition, as the study on the secondary metabolites of *M. jalapa* is still very limited, and since it is evident that callus tissues are an alternative source of secondary metabolites, further experiments can be carried out to identify as well as to produce secondary metabolites from the leaf-derived callus of *M. jalapa*.

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