International Journal of Agricultural Research
ISSN 1816-4897
Callus Induction from Leaf Explants of *Ficus deltoidea* Jack

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**Abstract:** Murashige and Skoog (MS) nutrient media supplemented with auxins 2,4-dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IBA) and 4-amino-3, 5, 6-trichloro picolinic acid (picloram) each at the concentrations of 1, 3 and 5 mg L\(^{-1}\) were evaluated for their effects on the callus induction from the leaf explants of *Ficus deltoidea*. The best calli with healthy morphology and growth was obtained from explants cultured on MS media supplemented with 3 mg L\(^{-1}\) picloram. Frable calli white, greenish or yellowish colour were obtained after 10 days upon culturing. 2, 4-D and IBA induced morphologically unhealthy calli, whereas cultured explants gave a negative response to MS media supplemented with various levels of NAA. For callus maintenance, the study indicated that media supplemented with 1 mg L\(^{-1}\) picloram gave the best growth rate of calli and was the most suitable callus maintenance medium to be used.

**Key words:** Callus, *Ficus deltoidea*, tissue culture, medicinal plant, organogenesis

**INTRODUCTION**

*Ficus deltoidea* Jack, better known as mistletoe fig or Mas Cotek in Malay term, is from Moraceae, the Mulberry family (Delto, 2004). Even though *F. deltoidea* is indigenous to the southern Philippines, southward and westward to Southeast Asia, Malaysia and Indonesia, it has been cultivated in various parts of the world as a houseplant or as an ornamental shrub (Starr *et al.*, 2003). It is an evergreen shrub or small tree with aerial roots and often begins its life as an epiphyte but it is not a banyan (Starr *et al.*, 2003). Usually *F. deltoidea* is seen as a large cascading epiphytic shrub on large trees, thus one of the common names of mistletoe fig (Yaacob and Mamat, 2005). Different appearances can be observed between the male and female plants of *F. deltoidea* based on the leaf shapes. The male plant has leaves that are small, long and thin, with red spots on the lower surface of its leaves (Yaacob and Mamat, 2005). For the female plant however, the leaves are bigger in size, thicker and have oval or round shape as compared to the male plant. The spots on the lower surface of the leaves are black in colour instead of red in colour (Osman, 2004).

*F. deltoidea* has begun to gain more and more attention among the groups practicing traditional medicines. The increased popularity of *F. deltoidea* is owing to its nutritional values in treating various diseases and for healthcare maintenance (Yaacob and Mamat, 2005). Different traditional practitioners suggest different ways of using or consuming products from this plant such as in the form of decoction, oil or ground product either from the leaf, stem, fruit and root or in combination of these parts (Zaki, 2005).
F. deltoides is popularly known as the female Viagra (Yvonne, 2003). It is also served as a health tonic which can help to harmonise the body. Traditionally, F. deltoides is a well-known herb in which its leaves are taken by female after childbirth to constrict the womb, to improve blood circulation and to treat problem of menstrual cycle (Yaacob and Mamat, 2005). Besides the various usages, F. deltoides is claimed to have the potential in preventing and curing diseases namely watery lung, diabetes, kidney problem, high blood pressure, gout and diarrhoea (Yvonne, 2003; Osman, 2004). It is also believed that the leaf decoctions are able to emulsify the fats and reduce the excessive cholesterol in artery and prevent the patients from migraine (Seman, 2005). The research done by scientist showed that the roots or leaves of this species can be used for various treatments such as skin disease, cough, fever, toothache, stomach ache, headache and wounds (Osman, 2004).

F. deltoides is a valuable medicinal plant that is good for healthcare and possesses a wide variety of medicinal properties, which however, has not yet known by many. Besides, information about this plant species is still quite limited and so far, there is a lack of studies especially the tissue culture study on this plant species. In view of this, the present study was carried out with the aims to investigate the effects of different auxins at various concentrations on the induction of callus from leaf explants of F. deltoides as well as to determine the suitable auxin and its concentration for callus maintenance.

MATERIALS AND METHODS

Plant Materials
Young leaf tissues from the male plants of F. deltoides obtained between June to December 2005 were used as the explants in this study.

Surface Sterilization
The leaf explants were washed under running tap water for 30 min and transferred to laminar flow to be surface-sterilized in 20% Clorox® solution containing three drops of Tween 20 (Amresco, USA), with a continuous shaking for 10 min. The explants were then rinsed three times with sterile distilled water for 5, 10 and 15 min, respectively.

Culture Medium
MS medium was used as the basal medium in all the cultures. To test the effect of growth regulators, MS medium w as supplemented with 2,4-D (Sigma, USA), NAA (Duchefa, Netherlands), IBA (Duchefa, Netherlands) and pelorin (Duchefa, Netherlands) at the concentrations of 1, 3 and 5 mg L⁻¹. A total of 3% of sucrose was added and the pH was adjusted using 0.1 M sodium hydroxide (NaOH) or 0.1M hydrochloric acid (HCl) to 5.7±0.1 with a pH meter (Mettler Toledo). The medium was solidified with 3 g L⁻¹ gelrite (Duchefa, Netherlands) and was placed in the autoclave machine (Hinyama, Japan) for autoclaving at 121°C for 15 min.

Callus Induction
The leaf explants of F. deltoides were cut into squares (7×7 mm) and cultured in the MS medium. A total of 5 explants were placed on each of the culture medium with different treatments of Plant Growth Regulators (PGRs) and concentrations. Each treatment, which consists of 5 explants, was repeated at least 3 times. The percentage and day of callus formation, morphology of callus and intensity of callus growth were observed weekly. Callus induction percentage was determined after 8 weeks of culture. The calli were transferred to fresh medium every 6 weeks.
Callus Maintenance

The calli being induced in the best callus induction medium were separated from the explants and transferred to fresh MS media supplemented with 1, 3 and 5 mg L\(^{-1}\) picloram. The morphological changes of the calli were observed weekly. The best callus maintenance medium that gave the healthiest and best callus growth was determined after 4 weeks of culture. The subsequent subculture of callus was done every 6 weeks in the fresh best maintenance medium.

Culture Conditions

In this study, all the cultures were maintained under a photoperiod of 16 h light and 8 h darkness at 26±2°C with a light intensity of 1000 lux provided by white fluorescent tubes.

RESULTS AND DISCUSSION

Callus Induction

Different types of auxins were found to have different effects on callus induction. In this study, calli were successfully being induced from the leaf explants of *F. deltoides* cultured in MS media supplemented with picloram, IBA and 2,4-D.

It was observed that all the concentrations of picloram tested (1, 3 and 5 mg L\(^{-1}\)) were capable of inducing calli, with the highest percentage (45.5%) being obtained from 3 mg L\(^{-1}\) picloram supplemented MS media (Table 1). This was followed by 1 and 5 mg L\(^{-1}\) picloram, with an induction percentage of 29.4 and 25.0%, respectively. In terms of days of callus formation, the callus was observed to be induced from the explants with in two weeks time in all the three concentrations of picloram supplemented MS media, among which 3 mg L\(^{-1}\) picloram was found to induce calli in the shortest period, which was 10 days after culture (Table 1). On the other hand, 1 and 5 mg L\(^{-1}\) picloram supplemented MS media induced calli on the 14th day upon culture. Among the three concentrations of picloram, however, only 1 and 3 mg L\(^{-1}\) picloram produced healthy and friable calli with profuse growth, which appeared yellowish, greenish or white in colour (Fig. 1b and 1c). Meanwhile, unhealthy watery calli were obtained in MS medium supplemented with 5 mg L\(^{-1}\) picloram (Fig. 1d). The positive effect of picloram on callus induction was also reported for other monocotyledons plant (Kedra and Bach, 2005). The higher efficiency of picloram as compared to other auxins was also observed in callus culture of *Zea mays*. The study on the plant of *Rollinia mucosa*.

Table 1: Callus induction from the leaf explants of *F. deltoides* cultured in MS media supplemented with picloram, IBA and 2,4-D at the concentrations of 1, 3 and 5 mg L\(^{-1}\)

<table>
<thead>
<tr>
<th>Plant growth regulators</th>
<th>Concentrations (mg L(^{-1}))</th>
<th>Percentage of callus formation (%)</th>
<th>Days of Callus Formation</th>
<th>Callus Colour/Morphology</th>
<th>Degree of callus formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picloram</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>29.4</td>
<td>14</td>
<td>Yellowish, greenish, white, friable</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>45.5</td>
<td>10</td>
<td>Yellowish, greenish, white, friable</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25.0</td>
<td>14</td>
<td>Yellow-brown, watery</td>
<td>-</td>
</tr>
<tr>
<td>IBA</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.1</td>
<td>18</td>
<td>Yellow-brown</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.1</td>
<td>18</td>
<td>Brownish</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.1</td>
<td>49</td>
<td>Brownish</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.4</td>
<td>49</td>
<td>Brownish</td>
<td>+</td>
</tr>
</tbody>
</table>

Callus growth rating value: (+) poor, (+++) moderate, (++++) profuse and (-) no callus formation. Note: NAA supplemented MS media: no callus was observed.

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Fig. 1: Callus induction from the leaf explants of *F. deltoidea* after 4 weeks of culture in MS medium supplemented with different concentrations of plolemum. (a) control, (b) 1 mg L$^{-1}$, (c) 3 mg L$^{-1}$, (d) 5 mg L$^{-1}$

Fig. 2: Callus induction from the leaf explants of *F. deltoidea* after 4 weeks of culture in MS medium supplemented with different concentrations of IBA. (a) 1 mg L$^{-1}$, (b) 3 mg L$^{-1}$
Fig. 3: Leaf-derived calli of *P. deltoidea* maintained in MS medium supplemented with different concentrations of picloram after 5 weeks of subculture. (a) 1 mg L\(^{-1}\), (b) 3 mg L\(^{-1}\), (c) 5 mg L\(^{-1}\).

Gave a similar result, in which the presence of picloram induced friable calli which were visible within 10 days and possessed a high growth rate (Figueiredo et al., 2003).

Auxin IBA, although managed to cause callusing, was found to be able to induce callus only at the concentrations of 1 and 3 mg L\(^{-1}\). Both of these concentrations were, however, managed to induce callus with only a low induction percentage (11.1%), which was 34.4% lower than that obtained in 3 mg L\(^{-1}\) picloram that gave the highest percentage of callus induction (Table 1). No callus induction was observed with an increase in IBA concentration to 5 mg L\(^{-1}\). This could be due to the fact that synthetic auxins may possess herbicidal property at high concentrations which inhibit callus formation (Evans et al., 2003). It was noticed that both 1 and 3 mg L\(^{-1}\) IBA supplemented MS media took a period of 18 days to induce callus. Brownish and yellow-brown calli were obtained from explants cultured in the respective 1 and 3 mg L\(^{-1}\) IBA supplemented MS media (Fig. 2). Besides, the intensity of callus growth was found to be poor.

2, 4-D was generally claimed to be one of the most effective auxins in the induction and growth of callus (Dodds and Roberts, 1995). However, this study gave a contradictory result to this statement. In this study, it was found that a low concentration of 2, 4-D (1 mg L\(^{-1}\)) did not stimulate any callus induction. However, an increase in concentration from 1 to 3 mg L\(^{-1}\) resulted in 7.1% of callus induction (Table 1). A further increase in concentration from 3 to 5 mg L\(^{-1}\) increased the percentage of induction to 15.4%, resulting in a further increment of 8.3%. This result was in agreement with the findings by Cucco and Jaume (2000) in the study of callogenesis for the different explants of the
Runner variety. It was noticed that explants cultured in 2, 4-D supplemented media took the longest period (49 days) to show callusing, which was approximately 5 times slower than that with 3 mg L\(^{-1}\) picloram treatment. Calli produced in these media also exhibited poor growth and brown in colour, which eventually died after a few weeks of culture. This was corresponding to the callus induction in Carob (Ceratonia siliqua), in which 2,4-D allowed the formation of small calli which turned brown and stopped to grow (Carini et al., 1997).

Among the auxins tested, only explants cultured on MS media supplemented with NAA did not show any signs of callusing. Instead, browning of the explants was observed at about two weeks after culture. Browning of the surface of plant tissues accompanied by darkening of culture medium due to the oxidation of phenolic compounds resulted in the formation of quinines which are highly reactive and toxic to plant tissue (Chawla, 2004). This phenomenon was undesirable because it affected the growth and establishment of explants, which in turn, affected the results in obtaining callus formation. In the experiment, frequent subculturing of the explants to fresh media was tried out to overcome the browning effect. It has also been suggested that thorough rinsing after sterilisation can remove phenolic compounds and thus decrease the damage to some plant tissues (Hartmann et al., 2002). Meanwhile, the most effective methods were the addition of activated charcoal in the media and incubation in the dark. However, higher levels of growth regulators would be needed to compensate for the inactivating properties of activated charcoal (Rao et al., 2006). Apart from that, other treatments that tend to minimise this problem include treating the explants with an antioxidant such as citric acid or ascorbic acid (Hartmann et al., 2002). The oxidation of phenolic compounds was shown to be controlled in banana clones using L-cysteine HCl at 40 mg L\(^{-1}\) incorporated in the media (Khatri et al., 1997).

Similarly, explants cultured on the control media, with no treatment of any auxins, did not show any callus formation as well (Fig. 1a). As suggested, most excised tissues had exogenous requirements of one or more growth regulators in order to initiate callus formation (Dodds and Roberts, 1995). In contrast to this, the study on the morphogenesis of Lilium martagon reported that the growth regulator-free medium also stimulated the formation of callus on the explants, albeit less frequently (Kedra and Bach, 2005).

**Callus Maintenance**

The MS media supplemented with 1, 3 and 5 mg L\(^{-1}\) picloram were tested to determine the best maintenance medium for maintaining as well as multiplying the calli. It was found that 5 mg L\(^{-1}\) picloram supplemented MS media failed to maintain healthy calli. Instead, the calli obtained were watery in appearance and gradually turned to brown in colour as time proceeded (Fig. 3). From the observation, it was noticed that calli maintained in 1 and 3 mg L\(^{-1}\) picloram supplemented MS media grew almost equally healthy, but in overall, 1 mg L\(^{-1}\) picloram seemed to give higher amounts of healthy calli with a faster growth rate as compared to the one maintained in MS media supplemented with 3 mg L\(^{-1}\) picloram. Thus, a change in the exogenous requirement of callus for picloram from a concentration of 3 mg L\(^{-1}\) during induction to 1 mg L\(^{-1}\) in the subsequent callus maintenance was noted in this study. This may be a result of the habituation phenomenon, in which the callus culture no longer require or require less growth regulators after a period of time. Franklin and Dixon (1994) have also claimed that callus growth may often require lower levels of auxin than needed for callus induction. This was suggested to be due to the ability of some cultured tissues to develop auxin biosynthetic pathways (Dodds and Roberts, 1995). However, the actual mechanism underlying habituation was not understood (Street, 1977). It was realized that the results of this study was in accordance with the observations obtained for the rye species, in which the requirement for picloram had been reduced from 2.5 to 1.5 mg L\(^{-1}\) in the callus maintenance medium (Anon, 2006). The
phenomenon of habituation was also observed in the callus tissues of *Scolzonera hispanica*, which initially had a requirement for auxin for growth, occasionally developed sectors which had the capacity to grow indefinitely on a medium lacking auxin (Street, 1977).

Seeing that the use of leaf explants on *in vitro* propagation opens an alternative way for enhancing the plant propagation efficiency, plant regeneration from the leaf-derived callus for rapid and economical production of *F. deltoidea* for the use in pharmaceutical industries is proved to be encouraging for the future research. In addition, as the study on the secondary metabolites of *F. deltoidea* is still limited and since it is evident that callus tissues are an alternative source of secondary metabolites, further experiment can be carried out to produce and study secondary metabolites in *in vitro* culture of *F. deltoidea*, with the aim to further understand and discover the medicinal roles and properties of this plant species.

**ACKNOWLEDGMENTS**

This research was supported by Universiti Tunku Abdul Rahman (UTAR), Malaysia.

**REFERENCES**


