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Full Length Research Paper

Characterization of callus formation in leaf of *Euphorbia helioscopia*

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The callus was induced on Murashige and Skoog's (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or 6-Benzylaminopurine (BAP) from the Chinese medicinal herb *Euphorbia helioscopia* in the family Euphorbiaceae. The highest frequencies of callus induction were observed on MS medium supplemented with 3.0 mg l^{-1} 2,4-D. Callus (yellow, loose, granular) was chosen as research focus in the long-term callus maintenance, the proliferation coefficient could still hold about 10 after subcultured 5 turns. Histological analysis was done to reveal the developmental pattern of callus formation. Detailed analysis revealed induction of leaf yielded many endogenous eumeristematic cells which were still embedded in the parenchymatic tissue.

Key words: *Euphorbia helioscopia*, callus induction, callus maintenance, histological, developmental pattern.

INTRODUCTION

Euphorbia helioscopia is an important Chinese medicinal herb species in the family Euphorbiaceae. Plants of *Euphorbia* have been used in the traditional medicine for treatment of cancers, tumors and warts for hundreds of years (Madureira et al., 2004). It is well known that they contain irritant and tumor-promoting constituents. Quite a number of species are used in folk medicine as drugs and raw materials for pharmaceutical preparations (Hecker, 1977). Early reports revealed that plants of Euphorbiaceae contain many kinds of secondary metabolites, such as triterpenoids (Teresa et al., 1987; Biesboer et al., 1982), diterpenoids (Yamamura et al., 1989; Sahai et al., 1981; Madureira et al., 2004; Öksüz et al., 1995), steroids (Biesboer et al., 1982), lipids (Teresa et al., 1987; Biesboer et al., 1982).

Some of these compounds had significant effects on certain tumours (Yamamura et al., 1989; Hata et al., 2003; Madureira et al., 2004), some could relieve cardiovascular activity (Barla et al., 2006). As in other members of the genus, plants of *E. helioscopia* secreted copious amounts of milky-white latex from non-articulated laticifers (Biesboer et al., 1982), and contained a number of important chemistry constituents such as diterpenoid (Yamamura et al., 1989; Zhang and Guo, 2006), triterpenoid and steroid (Barla et al., 2006). But no report on callus formation was found in *E. helioscopia*. The aim of this study is to develop a protocol for callus induction from leaf of *E. helioscopia* and understand the origination of callus by histological analysis.

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Abbreviations: 2,4-D; 2,4-dichlorophenoxyacetic acid, **BAP**; 6-Benzylaminopurine, **MS**; Murashige and Skoog's medium.

MATERIALS AND METHODS

Plant material and culture conditions

Plants of *E. helioscopia* L. (Euphorbiaceae) were collected in March 2005 from Nanchong, China and were cultivated in greenhouse (27±2℃). The specimen was identified by Prof. Zisheng Qin (A voucher specimen was deposited in the Herbarium of China West Normal University). The mature leaf discs were sterilized by immersion in 70% ethanol for 20 s and then in 2% (v/v) sodium hypochlorite with 3 drops of Tween-20 for 20 min. The sterilized explants were washed several times with sterile, distilled water and then were inoculated on flasks containing culture medium (2 explants per flask). All media were supplemented with 2.5% sucrose and 0.8% agar (w/v) (Sigma), the pH value was adjusted to 5.8 before autoclaving (121℃, 20 min).

Table 1. Effects of different concentrations of 2,4-D or BAP on callus induction of *E. helioscopia*.

Growth regulator		Callus formation (%) (mean ± SE) *	Callus morphotype**
2,4-D(mg l^{-1})	BAP(mg l^{-1})		
0		0	
1.0		44.81±2.89	yc; lg
2.0		55.19±2.89	yc; lg
3.0		75.93±3.03	yc; lg
4.0		56.67±3.33	yc(bc); lg
	0.5	0	
	1.0	0	
	1.5	10.37±0.37	gc; cc
	2.0	13.33±3.33	pg; mf

*Each treatment was comprised of three replications, with 10 explants per replication. The data are presented as mean±SE (n = 3).
**bc, brownish callus; cc, compact callus; gc, green color; lg, loose granular callus; mf, more friable callus; pg, pale green color; yc, yellow color.

Callus induction

Leaf discs were cultivated horizontally on MS medium (Murashige and Skoog, 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 1.0, 2.0, 3.0, and 4.0 mg l^{-1}) or with 6-Benzylaminopurine (BAP; 0.5, 1.0, 1.5 and 2.0 mg l^{-1}). Cultures were maintained at 25±2°C, under a 16-h photoperiod (the photon fluency of 36 umol·m^{-2}·s^{-1}). The experimental design had three replications, with 10 leaf discs per replication.

Callus maintenance

Callus (yellow, loose, granular) were selected and subcultured. The subcultures were conducted on MS media supplemented with 2,4-D (1.0; 2.0; 3.0 and 4.0 mg l^{-1}). For each treatment, 500 mg fresh weight callus was equally divided into 10 masses and incubated in a flask containing 20 ml medium as a replication. Each treatment had five replications. All treatments were cultured in dark environment for 14 days. The morphological differences were observed at the end of each subculture.

Morphological and histological investigations

The callus morphotypes were determined according to the description of various phenotypic characters (Jheng et al., 2006) which allowed simple identification under a stereomicroscope (Leica MZFLIII, Germany). Samples were collected at intervals of 5-day during whole experiment for histological observations. Each sample was fixed in FAA solution (70% ethanol, 5% glacial acetic acid, and 5% formaldehyde) for 24 h in room temperature. Fixed materials were dehydrated with a series of graded ethanol and butanol solutions and then were embedded in paraffin (Li, 1987). Serial sections (10 µm) were prepared in a rotary microtome (Leica RM2235, Germany) with a steel knife. The sections were floated in water drops, dried on a hot plate (37°C), stained with acid fuchsin (0.1%), then counter stained with toluidine blue (0.05%) for structural observations under a light microscope (Motic BA300, China).

Statistical analysis

All data were analyzed using one way ANOVA with Duncan analysis test by SPSS11.5 software.

RESULTS

Callus induction

Preliminary cultures procured different types of calli from leaf discs on MS medium containing different concentrations of 2,4-D or BAP (Table 1). The highest frequencies of callus induction were observed on MS medium supplemented with 3.0 mg l^{-1} 2,4-D (75.92±3.03%). As the level of 2,4-D rose (0 to 3.0 mg l^{-1}), the frequency of callus induction increased significantly. These calli were loose granuliform and yellow in color (Figure 1A). Their cells were close, small, high in nucleus/cytoplasm ratio, and abundant in cell contents by histological analysis (Figure 2D). Callus was brown when the concentration of 2,4-D rose to 4.0 mg l^{-1}. On the other hand, Callus induced on MS medium supplemented with BAP was compact and green in color. These calli were organogenic, whose cells were relatively large, loose, low in nucleus/cytoplasm ratio, and lacked of cell contents.

Callus maintenance

Callus (yellow, loose, granular) induced from leaf was chosen as research focus in the long-term callus maintenance. After the first subculture, optimal proliferation efficiency of callus was observed when cultured on MS medium supplemented with 3.0 mg l^{-1} 2,4-D. The proliferation coefficient could still hold about 10 after subcultured several turns (Table 2). Furthermore, the morphology of these calli still kept yellow in color, loose and granular in shape (Figure 1B). When subcultured with 4.0 mg l^{-1} 2,4-D, callus presented brownish.

Callus formation developmental pattern

When leaf discs were induced for 5 days, cells on cut edge began to dedifferentiate (Figure 1A). The ordered

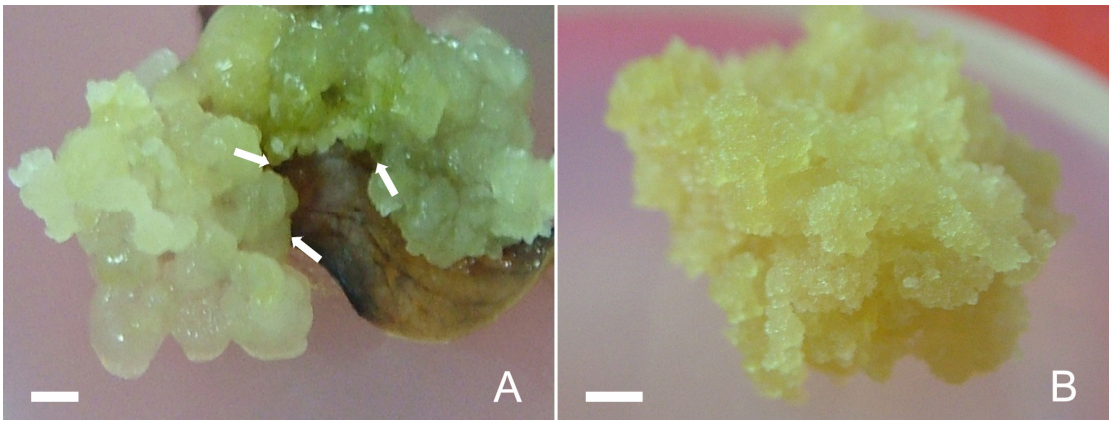

Figure 1. Callus formation of *E. helioscopia*.
(A) Callus formation from the cut (arrows) of leaf discs. (B) The maintained callus. (Bars = 1.0 mm)

Table 2. Effects of different combinations of 2,4-D on the maintenance of callus.

Subculture turn	2,4-D concentration (mg l^{-1})	Fresh weight increase (mean±SE) ** (mg)	Proliferation coefficient***	Callus morphotype****
The first subculture*	1.0	204.48±6.28d	5.06±0.12c	yc; lg
	2.0	339.04±6.10c	7.80±0.10b	yc; lg
	3.0	479.70±4.52a	10.39±0.20a	yc; lg
	4.0	373.04±6.44b	8.20±0.17b	yc; lg
The third subculture *	1.0	212.52±6.08d	5.18±0.14d	yc; lg
	2.0	350.94±5.28c	7.83±0.10c	yc; lg
	3.0	484.90±4.71a	10.48±0.19a	yc; lg
	4.0	376.26±9.21b	8.26±0.11b	yc(bc); lg
The fifth subculture*	1.0	249.14±11.16c	5.76±0.31c	yc; lg
	2.0	345.92±9.88b	7.75±0.13b	yc; lg
	3.0	481.96±7.84a	10.38±0.20a	yc; lg
	4.0	355.54±4.20b	7.94±0.06b	yc(bc); lg

* In each turn of subculture, 500 mg fresh weight callus was divided into 10 masses and incubated in a flask containing 20 ml medium as a replication. All treatments were cultured in dark environment.
** The mean number of callus proliferation in five replications at the end of each 14-day subculture interval, the different letters are significantly different at $P < 0.05$ with One-Way ANOVA Duncan's multiple range test.

$$*** \text{ Proliferation coefficient} = \frac{\text{Fresh weight}_{(\text{after 14-day subculture})}}{\text{Fresh weight}_{(\text{before 14-day subculture})}}$$

**** yc, yellow color; bc, brownish callus; lg, loose granular callus.

structure (Figure 2A) of epidermis, mesophyll and vein were destructed and disappeared, series changes including retraction of the protoplast, reduction of the vacuole volume and cytoplasm fragmentation appeared in their cells. The indeterminate eumeristematic masses emerged from parenchymatic perivascular cells surrounded by the mesophyll parenchyma (Figure 2B). Sections prepared after having been cultured 10 days exhibited many endogenous eumeristematic cells that still embedded in the parenchymatic tissue. Cells of these masses were organized in three distinct regions (Figure 2C):

a) Vacuolated cells bearing several vacuoles and dense cytoplasm.
b) Eumeristematic cells with dense cytoplasm and absence of vacuoles.
c) Differentiated cells bearing loose cytoplasm filled with starch grains, which were vacuolizated to a certain degree. Leaf discs were finally induced to callus in 25 days (Figure 2D).

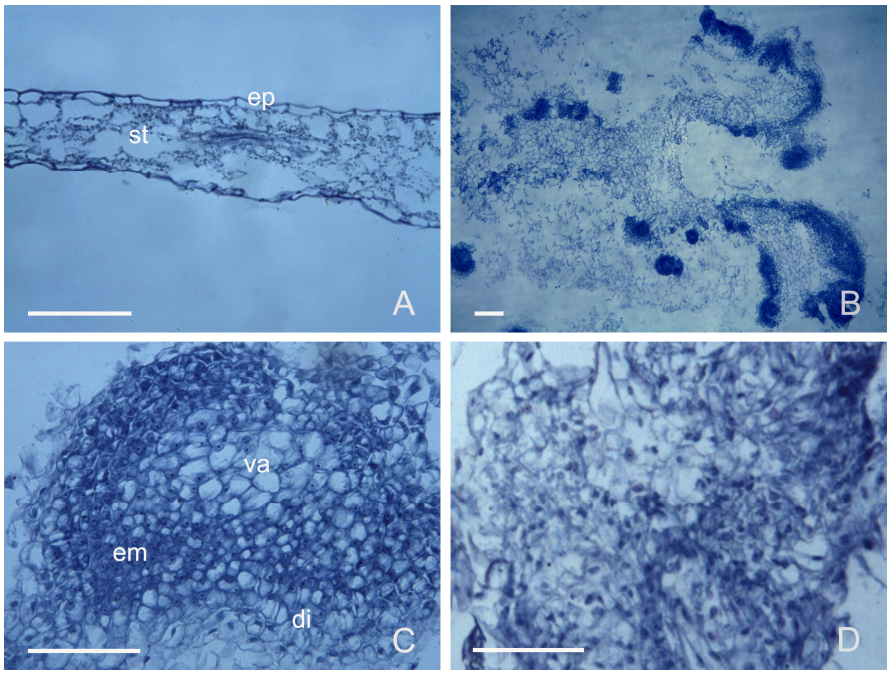

Figure 2. Histological observations of callus formation from leaf discs of *E. helioscopia*. (A)The transverse section of leaf disc. (B) Leaf disc during the course of callus-transformation. (C) Regions of endogenous eumeristematic cells embedded in the parenchymatic tissue. (D) The callus from leaf disc. (ep, epicuticula; st, spongy tissues; di, differentiating cells; em, eumeristematic cells; va, vacuolated cells) (Bars = 100 mm in B; 200 mm in A and C-D).

DISCUSSION

The power of plant hormones to promote development in tissue culture has been amazed and baffled plant scientists for many years (Che et al., 2002). Growth regulators, as one kind of signal molecule, have been shown to play an important role during the callus formation (Dodeman et al., 1997; Skoog and Miller, 1957; Sugiyama and Imamura, 2006). In our experiments, we use different concentration of 2,4-D or BAP to induce callus of *E. helioscopia* and finally found the highest frequencies of callus inducement were on MS medium supplemented with 3.0 mg l^{-1} 2,4-D (75.92±3.03%). Experiments with combinations of different growth regulators' concentration are going to do next step to obtain optimal conditions of Callus inducement in *E. helioscopia*.

Juvenile explants are frequently used for the initiation of *in vitro* cultures primarily because of their high morphogenetic potential and low contamination rates (Muñoz et al., 1999). *In vitro* culture of plant using mature tissues as explants could not be frequently accomplished, mainly due to the high level of contamination, the reduced or absence of morphogenetic ability and poor rooting of the regenerated shoots (Almeida et al., 2003).

However, in our study, *in vitro* culture of mature leaf discs of *E. helioscopia* which were cultivated on certain media showed strong ability to dedifferentiate (Table 1). The callus (yellow, loose, granular) exhibited higher proliferation efficiency and finer morphology than other types of callus in short-term subculture experiments. The maintenance of *E. helioscopia* callus is easy and proliferation coefficient could still hold about 10 after subcultured several turns. The character of *E. helioscopia* callus maintenance provide us much convenient for the further studies in which *E. helioscopia* callus could be widely used. Moreover, organogenic calli could be induced on MS medium only supplemented with BAP. Same result had also been obtained in *Rosa hybrida* (Li et al., 2002). And the mechanism is worth further research.

In view of the difficulty to identify the original cells actually involved in the developmental processes of *in vitro* culture, few studies concerning the initial stages of callus development have been carried out (Dodeman et al., 1997). The anatomical analysis conducted here revealed the callus formation pattern by using leaf of *E. helioscopia* as explants. Callus induced from leaf discs for 10 days possessed many endogenous proembryogenic masses which still embedded in the parenchymatic tissue. These pro-embryogenic masses originated from perivascular cells or from indeterminate meristematic masses appearing in the vascular tissue (Tarré et al., 2004).

In synthesis, the protocol described here displayed callus induction with high efficiency in *E. helioscopia*. It would be very useful in widespread application of plant biotechnology for tissue culture. Our histological results clearly delineated the origination of callus. The developmental pattern

described here would possible play a useful role in future researches of higher plants.

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