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*Full Length Research Paper*

# Establishment of the callus and cell suspension culture of *Elaeagnus angustifolia* for the production of condensed tannins

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**The objective of this work was the optimization of the conditions of callus and cell suspension culture of *Elaeagnus angustifolia* for the production of condensed tannins. The effects of different conditions on the callus growth and the production of condensed tannins were researched. The leaf tissue part of *E. angustifolia* was optimum explant of callus induction. The best callus growth and the highest condensed tannins production was obtained under the culture on MS basal medium containing 1.00 mg·L$^{-1}$ 2,4-D and 0.50 mg·L$^{-1}$ BA. The optimum time of subculture was 20 - 25 d. The results showed the contents of condensed tannins decreased gradually at the beginning of the callus differentiation. Vigorous and friable callus was used for cell suspension culture. Study on the effect of medium and the hormone combination showed that plenty of incompact and rapid growing suspension cells were obtained from cultures grown in liquid medium supplemented with 0.1 mg·L$^{-1}$ BA + 0.1 mg·L$^{-1}$ TDZ. The study provided an efficient way for *E. angustifolia* cell suspension culture to produce secondary metabolite.**

**Key words:** *Elaeagnus angustifolia* L, callus, condensed tannins, suspension culture.

## INTRODUCTION

*Elaeagnus angustifolia* L. is an Eurasian tree that had become naturalized and had invaded zones along watercourses in many arid and semiarid regions of the world. These habitats are characterized by vertical environmental gradients and hence the trees had developed much plasticity to adapt to the wide range of site conditions (Klich, 2000). The health-protective properties of condensed tannins had been increasingly appreciated by the scientific community, based on the accumulation of evidence for chemopreventive, anti-inflammatory and cardioprotective roles of these phytochemicals from teas, red wines and fruits (Prior and Gu, 2005). In recent years, condensed tannins had been shown to have a cholesterol-lowering effect (Preuss et al., 2000), cytotoxic effects on human cancer cells (Ye et al., 1999) and cardioprotective properties (Sato et al., 2001), and stimulated angiogenesis in dermal wound healing (Khanna et al., 2001) without inducing significant toxicological effects (Wren et al., 2002).

*E. angustifolia* is also well known for its rich source of condensed tannins. Shoot regeneration *in vitro* has

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**Abbreviations: PGR,** plant growth regulator; **DW,** dry weight; **FW,** fresh weight; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **BA,** 6-bezyladenine; **NAA**, α-naphthaleneacetic acid; **IAA,** Indole-3-acetic acid.

**Table 1.** Effect of plant growth regulator on the callus growth of *E. angustifolia.*

| Combination Of PGR | 2,4-D ($mg \cdot L^{-1}$) | 6-BA ($mg \cdot L^{-1}$) | NAA ($mg \cdot L^{-1}$) | TDZ ($mg \cdot L^{-1}$) | Induction rate (%) | | Callus growth state[a] | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Leaf | Stem | Leaf | Stem |
| M1 | 2.00 | 0.50 | | | 86.66 ± 2.33 | 75.00 ± 2.65 | Preferable | Preferable |
| M2 | 1.00 | 0.50 | | | 98.00 ± 1.15 | 94.75 ± 2.90 | Fine | Fine |
| M3 | 1.00 | 1.00 | | | 56.33 ± 1.76 | 51.26 ± 1.27 | Poor | Preferable |
| M4 | | 0.10 | | 0.10 | 91.62 ± 3.80 | 90.32 ± 1.20 | Fine | Fine |
| M5 | | 0.50 | | 0.05 | 52.33 ± 3.52 | 57.20 ± 5.13 | Preferable | Preferable |
| M6 | | 2.00 | 0.10 | | 69.33 ± 1.21 | 78.50 ± 5.06 | Preferable | Preferable |
| M7 | - | 1.00 | 0.10 | | 77.50 ± 2.18 | 84.28 ± 4.86 | Fine | Preferable |
| M8 | - | 1.00 | 0.30 | | 81.46 ± 0.79 | 83.25 ± 1.38 | Preferable | Poor |

[a]Callusgrowth state: fine, callus was white and compact; preferable, callus was dark yellow and soft; poor, callus was brown and dying.

already been reported for *E. angustifolia* (Economou and Maloupa, 1995; Economou and Spanoudaki, 1988; Iriondo et al., 1995; Li et al., 2004). Production of secondary metabolites with distinct and complex structures in plants by callus or cell cultures has been one of the most extensively explored areas in recent years owing to the enormous commercial value of those compounds, the scarcity of the plants in the world and the extremely low levels of such compounds in plants. However, no report has been found in the literature on plant regeneration from leaves and stem and suspension culture of *E. angustifolia*.

The purposes of the study were to (i) establish the conditions for callus induction from leaves and stem of *E. angustifolia* for the production of condensed tannins, (ii) analyse the effects of different factors, such as explants, plant growth regulators on the callus induction and accumulation of condensed tannins, and (iii) determine the optimum medium and the hormone combination for cell suspension culture of *E. angustifolia*. This paper presents the feasibility of condensed tannins production in callus and cell culture of *E. angustifolia*.

## MATERIALS AND METHODS

### Plant materials and experimental conditions

The experiments were performed on 3 years old *E. angustifolia,* which was cultivated under natural conditions in the field. Five whole plants were harvested on September and separated into leave, annual branch and bark. Materials were oven-dried at 80°C for 12 h individually and kept dry until analysis.

### Induction culture

The stems and leaves of *E. angustifolia* were washed thoroughly with tap water for 30 min and surface sterilized in 10% $Ca(ClO)_2$ for 10 min followed by rinsing them with sterile distilled water for 3 times and excised into proper sizes (stems segments approximately 8 mm in length; leaves, 5 × 5 mm sections). Every 4 explants were placed on MS basal medium supplemented with different plant growth regulators (Table 1). The cultures were incubated in a culture room with a 16/8 h day/night cycle at 25 ± 2°C under an irradiance of 60 mmol $m^{-2}$ $s^{-1}$ provided by cool-white fluorescent tubes. After 28 days inoculation, the ability of the explant to develop callus under various conditions was recorded as the callus induction rate (%) which was defined as: Callus induction rate (%) = (Total number of explants produced callus/ Total number of explants cultured) × 100%. 30 replicates were used per treatment. Analysis of variance (ANOVA) was performed on the collected data.

### Subculture

After 4 weeks incubation, 4 kinds of initial calli in good state were separated from explants and transferred to fresh medium of the same composition. The calli obtained were sub-cultured for an additional 4 weeks, which were incubated under light and darkness, respectively.

### Differentiation culture

The differentiation culture were established by planting callus on MS basic medium supplemented with 0.50 $mg \cdot L^{-1}$ 2, 4-D and 1.00 $mg \cdot L^{-1}$ BA. Controlled experiment was based on callus subcultures.

### Cell line and culture conditions

The calli for suspension culture was induced from the young leaves of *E. angustifolia*. The calli obtained were subcultured for 3 times. Then the calli was used for suspension culture in MS-NT-WPM-B5 and IS medium supplemented with 0.1 $mg \cdot L^{-1}$ BA + 0.1 $mg \cdot L^{-1}$ TDZ, respectively. According to fresh weight and condensed tannins content of the cells, the study determined the optimum suspension culture medium. Then callus were placed on the optimum basal medium supplemented with different hormone combinations (Table 2). The callus line had been in culture for 5 months by the time of this study. The suspension culture of the cell line was initiated from the callus culture on a liquid medium similar to that for the callus culture but with 30 $g \cdot L^{-1}$ sucrose and excluding the agar. The medium was adjusted to pH 5.8 and then sterilized at 121°C for 20 min. The

**Table 2.** Effect of plant growth regulator on cell suspension culture of *E. angustifolia*.

| S/N | Concentrations of TDZ and BA (mg·L$^{-1}$) | Fresh weight (g) | Condensed tannins content (mg·g$^{-1}$ DW) | Medium state | Suspension cells growth state |
|---|---|---|---|---|---|
| N1 | 6-BA 0.1 + TDZ 0.05 | 2.96 ± 0.41 | 5. 0147 ± 0.0768 | Clarification | Most of cell browning and death |
| N2 | 6-BA 0.1 + TDZ 0.1 | 5.73 ± 0.41 | 5.2027 ± 0.0067 | Yellow turbid | Yellowish green |
| N3 | 6-BA 0.2 + TDZ 0.05 | 7.93 ± 0.79 | 6.9125 ± 0.0356 | Yellow turbid | Dark green, adventitious buds forming |
| N4 | 6-BA 0.2 + TDZ 0.1 | 8.23 ± 0.85 | 11.0152 ± 0.0254 | Black turbid | Dark green, adventitious buds forming |
| N5 | 6-BA 0.2 + TDZ 0.15 | 3.80 ± 0.68 | 12.6418 ± 0.0979 | Clarification | Intermediate browning and surrounding yellow-green |
| N6 | 6-BA 0.4 + TDZ 0.1 | 3.94 ± 0.14 | 2.72420 ± 0.0119 | Black turbid | Intermediate tawny and death surrounding green callus |
| N7 | 6-BA 0.50 + 2,4-D 1.00 | 5.03 ± 0.21 | 4. 6123 ± 0.0436 | Yellow turbid | Brown callus |
| N8 | 6-BA 1.00 + 2,4-D 1.00 | 4.67 ± 0.34 | 4. 8005 ± 0.0637 | Yellow turbid | Dark brown callus |

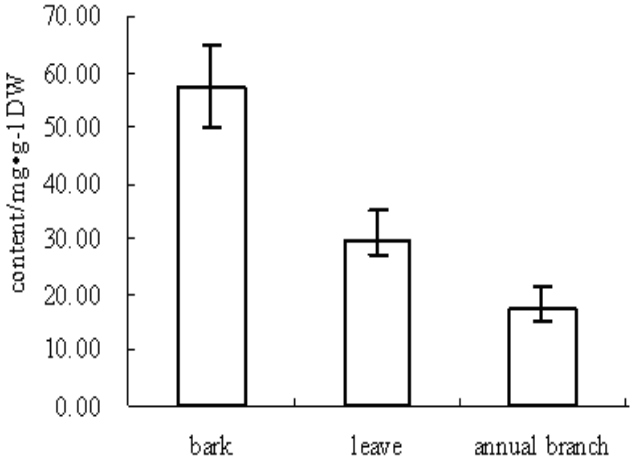


**Figure 1.** Spatial variations of condensed tannins content in *E. angustifolia*.

suspension culture was maintained in 100 mL Erlenmeyer flasks with a liquid volume of 60 mL and incubated on an orbital shaker incubator in the dark at 120 rpm and 25℃. The suspension culture was subcultured every 3 weeks.

#### Analysis of condensed tannins

The callus samples were oven-dried at 80℃ for 12 h. 0.50 g sample (powdered in a mortar) was homogenized with 5 ml of 50% aqueous methanol, sonicated for 20 min and centrifuged at 12000 × g for 10 min. HPLC method was used to determine the content of condensed tannins. Chromatographic experiments were performed on C18 column (4.6 mm × 250 mm, 5 μm), the mobile phase was acetonitrile and 1% acetic acid aqueous solution (10:90); the detection wavelength was 260 nm, column temperature was 30℃; the flow rate was 0.8 mL·min$^{-1}$.

## RESULTS AND DISCUSSION

### Condensed tannins content in *E. angustifolia*

As shown in Figure 1, there was obvious difference in condensed tannins content among different tissues of *E. angustifolia*. The content of condensed tannins in the bark was 57.12 mg·g$^{-1}$ DW, while condensed tannins content in annual branch was only 17.49 mg·g$^{-1}$ DW. The reason was probably related with the complexity of differrential expression of secondary metabolism genes, physiological states and ability of precursor synthesis in different plant tissues. The contents of condensed tannins among different tissues of *E. angustifolia* are as follows: bark>leave>annual branch. That is associated with plant defense system to radiation and pathogen as condensed tannins is characterized by absorbing ultraviolet radiation and binding protein.

### Callus induction of *E. angustifolia*

Different combination of plant growth regulators in MS basal medium were studied for callus induction of *E. angustifolia*. As shown in Table 1, among the different plant growth regulators, which were used, the highest induction rates (98.00%) was found in presence of 1.00 mg·L$^{-1}$ 2,4-D and 0.50 mg·L$^{-1}$ BA (M2). The induction rate of leaf was 94.62% in 0.10 mg·L$^{-1}$ BA and 0.10 mg·L$^{-1}$ TDZ (M4). TDZ can be substituted with adenine-type cytokinins in various culture systems, including callus and micropropagation of many woody species (Lu, 1993). TDZ has been shown to have cytokinin-like effects also on cucumber cotyledons (Visser et al., 1995). However, in the present study, callus cultures from leaves based on

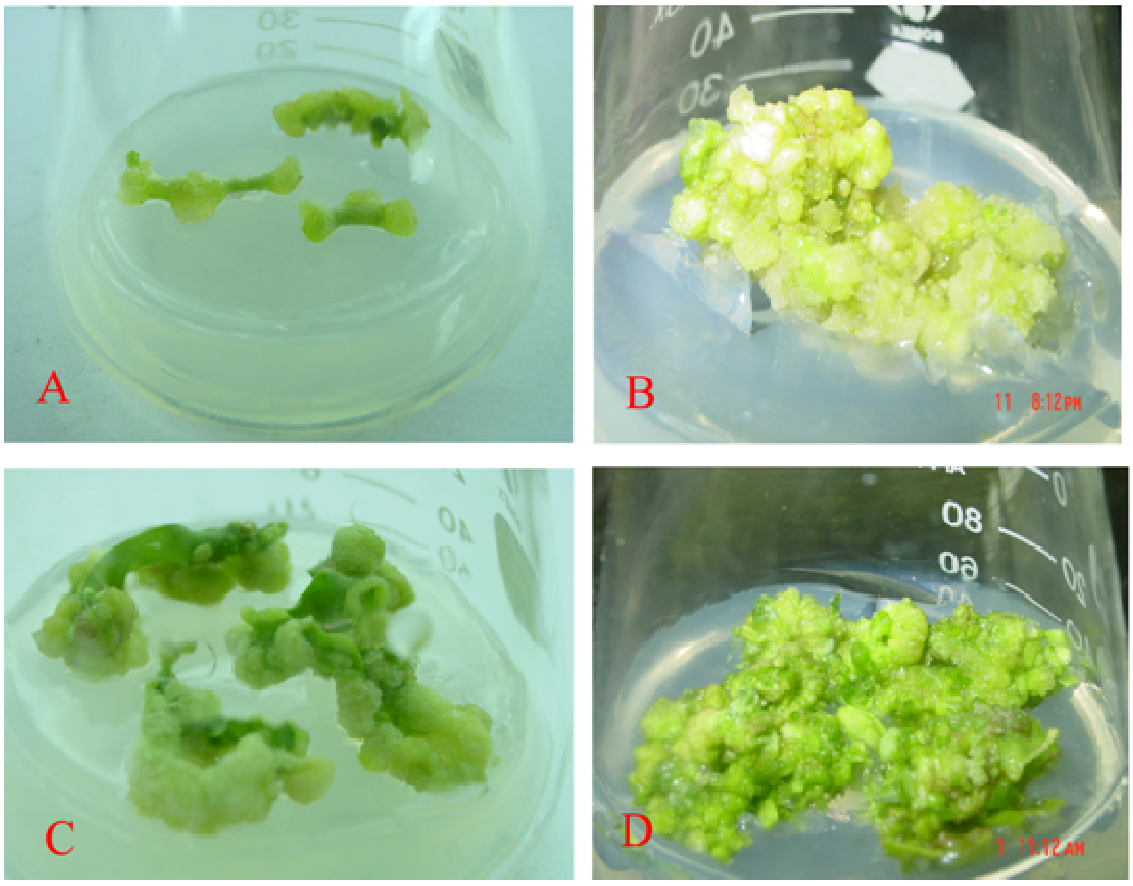


**Figure 2.** Callus of *E. angustifolia* in different conditions. A. Callus induced from stem under the culture on MS basal medium containing 1.00 mg·L$^{-1}$ 2,4-D and 0.50 mg·L$^{-1}$ BA. B. Callus induced from leaf under the culture on MS basal medium containing 1.00 mg·L$^{-1}$ 2,4-D and 0.50 mg·L$^{-1}$ BA. C. Subculture of callus from leaf under the culture on MS basal medium containing 1.00 mg·L$^{-1}$ 2,4-D and 0.50 mg·L$^{-1}$ BA. D. Shoot-forming culture of callus under the culture on MS basal medium containing 2.25 mg·L$^{-1}$ 2,4-D and 4.40 mg·L$^{-1}$ BA.

MS basal medium containing 1.00 mg·L$^{-1}$ 2, 4-D and 0.50 mg·L$^{-1}$ BA were better than that based on TDZ and BA. It was also observed that in these treatments the stem callus produced dark yellow and friable unlike the leaf callus which presented white and compact. These results suggested that leaf was better than stem in callus induction (Figure 2).

## Effect of culture medium on cell suspension culture and condensed tannins accumulation

As shown in Table 3, fresh weight of cells in B5 suspension culture medium was 3.52 ± 0.72 g, which was lower than others. Cell growth in MS and WPM suspension culture medium was higher than others, but suspension callus cells browned and were thus dying about 3 weeks after subculture. So B5, MS and WPM medium were not suitable for suspension culture. Fresh weight of cells in NT and IS suspension culture medium was 4.86 ± 0.18 and 4.92 ± 0.89, respectively. However, some of the suspension callus in IS suspension culture medium became brown after subculture. Also the content of condensed tannins was 2.7170 ± 0.9650 mg·g·DW$^{-1}$, which was lower than that in NT suspension callus cells. The content of condensed tannins of suspension callus cells in NT medium was 5.0700 mg·g·DW$^{-1}$. So NT was optimum medium for cell suspension culture of *E. angustifolia*.

## Effect of plant growth regulator on cell suspension culture and condensed tannins accumulation in *E. angustifolia*

The effect of different concentrations of TDZ and BA on cell suspension culture of *E. angustifolia* was studied. As shown in Table 2, suspension callus cells in N1, N5 and N6 treatments was dark yellow and brown in colour. The

**Table 3.** Effect of culture medium on cell suspension culture.

| Medium | Fresh weight (g) | Condensed tannins content (mg·gDW$^{-1}$) | Medium state | Cell growth state | Suitable for subculture |
|---|---|---|---|---|---|
| NT | 4.86 ± 0.18 | 5.0700 ± 0.0190 | Yellow turbid | Yellowish green | Yes |
| WPM | 5.58 ± 0.63 | 1.4990 ± 0.5650 | Black turbid | Intermediate dark brown surrounding light-green | No |
| B5 | 3.52 ± 0.72 | 1.2000 ± 0.0670 | Clarification | Light-green and a few browning callus | Yes |
| IS | 4.92 ± 0.89 | 2.7170 ± 0.9650 | Black turbid | Yellow and brown callus | Yes |
| MS | 4.11±0.27 | 8.1111±0.8870. | Black turbid | Intermediate yellow-green, surrounding have black stain callus | No |

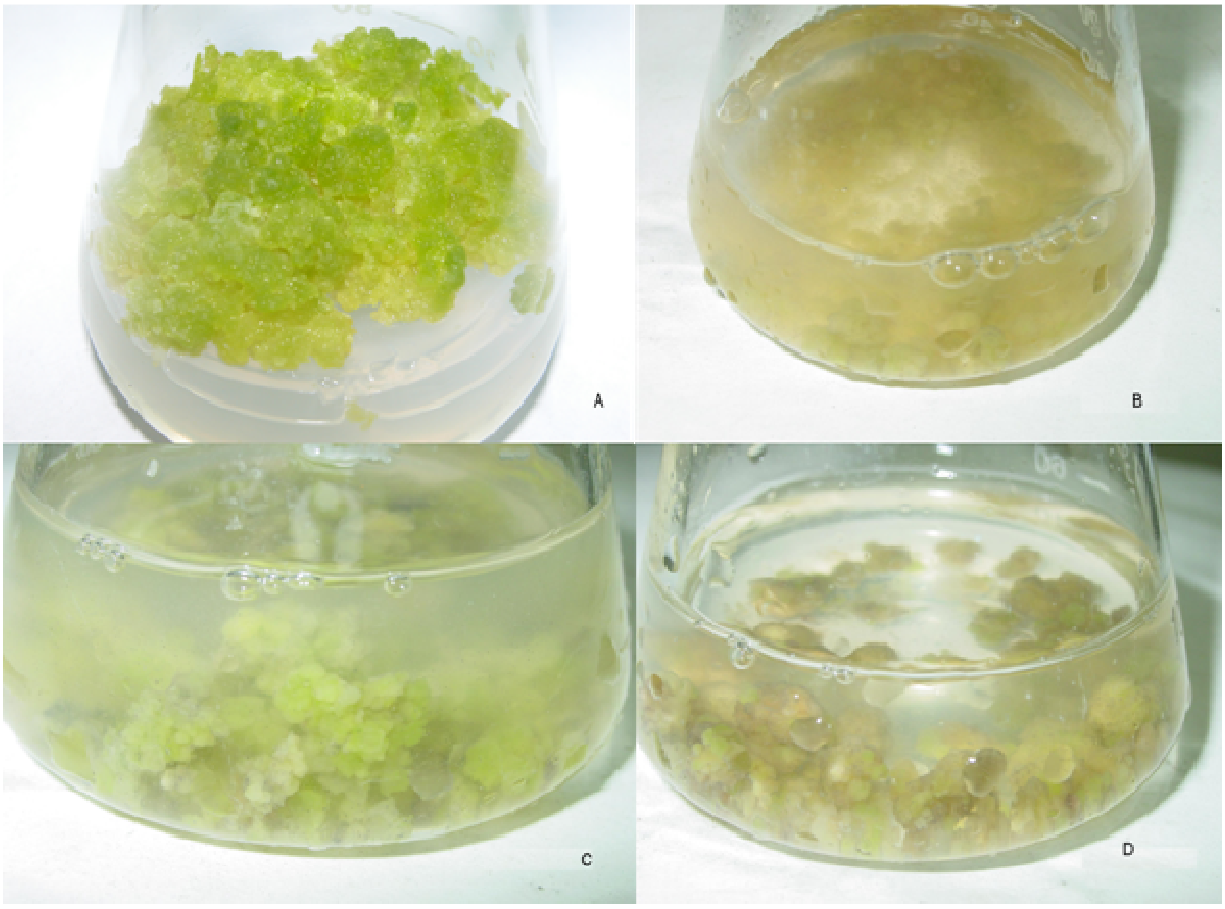


**Figure 3.** Cell suspension culture of *E. angustifolia*. A. Subcultures of callus on 0.1 mg·L$^{-1}$ BA + 0.1 mg·L$^{-1}$ TDZ. B. Yellow-green suspension cells on NT + 0.1 mg·L$^{-1}$ BA + 0.1 mg·L$^{-1}$ TDZ. C. Suspension callus with green spot that could regenerate into shoot. D. Browning and death of suspension cells.

callus cells growth was lower than other treatments. Fresh weight of suspension cells in N2, N3 and N4 increased 4 to 5 times during 10 days of culture. However, adventitious buds were formed from subcultured suspension callus cells in N3 and N4 treatments. So the present results indicated that NT medium containing 0.1 mg·L$^{-1}$ BA+0.1 mg·L$^{-1}$ TDZ (N2 treatment) was most suitable for cell suspension culture and condensed tannins accumulation in the cells of *E. angustifolia* (Figure 3).

## Conclusion

This study provides a protocol for callus and cell suspension culture of *E. angustifolia* for the production of condensed tannins. The leaf tissue part of *E. angustifolia* was optimum explant of callus induction. The best callus growth and the highest condensed tannins production was obtained under the culture on MS basal medium containing 1.00 mg·$L^{-1}$ 2,4-D and 0.50 mg·$L^{-1}$ BA. Vigorous and friable callus was used for cell suspension culture. Incompact and rapid growing suspension cells were obtained from cultures grown in liquid medium of NT supplemented with 0.1 mg·$L^{-1}$ BA + 0.1 mg·$L^{-1}$ TDZ. The paper provides a new and efficient way for *E. angustifolia* to produce secondary metabolites.

## ACKNOWLEDGMENT

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