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COMPARATIVE CULTURE RESPONSE OF THREE *Coleus blumei* Benth. VARIETIES AS BASIS FOR EXPLANT SELECTION FOR CALLUS INDUCTION

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ABSTRACT – Callus was induced from three locally available *Coleus blumei* Benth. varieties; 'Defiance,' 'Shocking Pink,' and 'Small Leaf.' The objective is to determine the most suitable variety to use as explant source for *in vitro* studies of *Coleus* species for rosmarinic acid production. Ease of explant sterilization, culture responsiveness, as well as initial rosmarinic acid content were the key factors considered. Based on thin layer chromatography, 'Small Leaf' has the highest amount of rosmarinic acid. When leaf explants were inoculated on culture media for callus induction, 'Small Leaf" had the highest percent of contamination at 81.4% compared to - 'Defiance' at 75.0%, and 'Shocking Pink' at 20.0%. Morpho-anatomical observations of the leaf of each variety showed 'Defiance' to have the longest trichomes (0.254 mm) while 'Small Leaf' has the highest density of trichomes (200 trichomes per mm^2). Trichome characteristics could be one of the contributing factors affecting the effectiveness of explant sterilization method. All explants were surface sterilized using water and detergent, dipped in 95% ethanol for 30 sec. and immersed in 5% calcium hypochlorite with surfactant for 5-10 mins. In terms of culture response, 'Small Leaf' explant grown on MS medium with 1.0 mgl^{-1} BAP and 6.0 mgl^{-1} 2,4-D, was the most responsive; producing white friable calli on green explant tissue at three weeks after inoculation. 'Shocking Pink' produced brown friable calli with extensive tissue browning due to phenolic oxidation 4 weeks after inoculation. 'Defiance' did not produce any callus in culture even at 8 weeks after inoculation. Although explant sterilization was a problem due to high density of trichomes, 'Small Leaf' proved to be the most promising to use as source of explant for *C. blumei* tissue culture for rosmarinic acid production.

Keywords: callus induction, Coleus blumei, culture response, rosmarinic acid

INTRODUCTION

Coleus blumei Benth**.,** locally known as 'Mayana' is an ornamental plant which is widely cultivated and collected in the country for its several varieties of colorful foliage. Traditionally, it is used as a remedy for bruises and boils. The reported biologically active secondary products from this plant include a sterol mixture, which contains β-sitosterol and stigmasterol as major components, and the caffeic acid ester rosmarinic acid (Alfermann and Petersen, 1988; Rahayu, 1999; Petersen and Simmonds, 2003).

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C. blumei is of particular interest in the study of secondary products *in vitro*. Manipulation of the growth conditions in the cell suspension cultures of this plant species can lead to an increase in secondary metabolite production. Ellis and Towers (1970), Zenk et al. (1977), and Petersen et al. (1994), showed that the production of rosmarinic acid in *in vitro* cultures of *C. blumei* can be increased up to 19% dry weight by increasing the amount of sucrose in the nutrient media. This information is beneficial for large-scale rosmarinic acid production which is economically important because of its antioxidant, anti-inflammatory and antimicrobial activities (Zelic et al., 2005).

Recent studies have used suspension cultures of *C. blumei* to identify important enzymes for rosmarinic acid biosynthesis. The cDNA encoding for some of these enzymes have been isolated and functionally expressed in *E. coli* (Petersen and Simmonds, 2003; Petersen et al., 2009).

The experiments by Dr. B. Ulbrich of Nattermann and Cie Company, Germany used pre-induced cell-lines of *C. blumei* from the callus cultures induced in 1979/80 (Ellis and Towers, 1970; Szabo, 1994; Meinhard, 1995). Although rosmarinic acid can be synthesized, the biological system such as in *Coleus* is a good model system to study elicitation of secondary metabolites by understanding its biochemical pathway. Unfortunately, most of these previous studies using cell suspension did not indicate the specific variety used as source of explant.

One of the major considerations in plant *in vitro* studies is the culture responsiveness of an explant source (Evans et al., 2003). This involves ease of sterilization, length of time required for callus induction, and the quality of callus and its ability to produce the target secondary metabolite. Within a species, some have explants that do not form callus (Dougall, 1972). As such, variations in the biosynthetic capability of different explants are also expected. Some may not be able to yield target secondary metabolites under *in vitro* conditions.

This study was conducted to determine the variations in the *in vitro* response of three varieties of *C. blumei* with the synthesis of rosmarinic acid as ultimate goal. The desired explant source for callus induction is one with high culture responsiveness and high rosmarinic acid content. The three *C. blumei* varieties: 'Defiance', 'Shocking Pink', and 'Small Leaf' were compared based on morphological and phytochemical characteristics pertinent to callus induction and levels of rosmarinic acid content. The three varieties were chosen for this study based on the availability of the samples. The plants were procured in Bay, Laguna and were identified based on the website www.coleusfinder.org.

MATERIALS AND METHODS

Morpho-anatomy of Explant Source

Dimensions of the mother plants such as plant height, leaf length and width, for each variety of *C. blumei*: 'Defiance', 'Shocking Pink', and 'Small Leaf' were determined based from the average measurements of 20 samples. The trichome length and density were observed from the underside or abaxial surface of the young leaf located at the second to third position from the shoot tip. These young leaves served as explant source for tissue culture studies. Measurements were made using Image J version 1.24® computer program developed by the National Institutes of Health, USA (2004). Average trichome length was determined by measuring 50 trichomes per variety. Trichome density was computed by counting the number of trichomes present in an area of 0.05 mm^2. Twenty-five leaf areas along the leaf blade were designated randomly and the values per area served as basis for computing the number of trichomes per mm^2.

Data gathered for the trichome length and density were analyzed using the one way analysis of variance (ANOVA) at 5% degrees of freedom.

Thin Layer Chromatography: ***Test for rosmarinic acid***

Leaves from the different varieties were oven dried at 70°C for eight hours and were ground using mortar and pestle. One gram powdered leaves was extracted by adding 5ml methanol. The solution was placed in a water bath at 60°C for 5 minutes with continuous shaking. From the methanolic leaf extracts, 100µl aliquots were loaded on TLC plates (Silica gel 60 F_{254}, Merck$^{\rightarrow}$) using capillary tubes. The developing solvent system recommended for rosmarinic acid was 5ml toluene: 4ml ethyl fomiate: 1ml formic acid.

For the detection of rosmarinic acid, the plates were sprayed with 10 ml of 1% methanolic diphenyboric acid-β-ethylamino ester (NP), followed by 8 ml of 5% ethanolic polyethylene glycol-4000 (PEG 4000). After drying, the plates were viewed under UV_{365} for fluorescence (Wagner and Bladt, 1996). The Rf values of the fluorescing bands were measured and compared with those of rosemary plant, *Rosmarinus officinalis* L, that served as reference material. In rosemary chromatogram, rosmarinic acid had an Rf value of 0.30. Rosmarinic acid forms a bluish white fluorescing band under UV_{365} (Wagner and Bladt, 1996).

Induction of Callus

Without taking the height of mature plant in consideration, young leaves at the second position from the shoot apex was used as explant source. Leaf samples from each variety were surface sterilized in water and Teepol® solution (a blend of linear alkyl benzene sulfonate, sodium salt, sodium lauryl ether sulfate, and cocamidopropyl betaine); and dipped in 95% ethanol for 30 seconds. The materials were then immersed in 5% calcium hypochlorite added with surfactant for 5 to 10 minutes and rinsed four times. Leaf explants were cut into square sections measuring 3 x 3 mm^2. Twenty test tubes per variety and treatment were inoculated with surface sterilized leaf explants.

The basic culture medium used was MS (Murashige and Skoog, 1962). The different combinations of plant growth regulators added into MS medium constitute the treatments as follows; 0.5 mgl^{-1} BAP and 1.0 mgl^{-1} 2,4-D; 1.0 mgl^{-1} BAP and 1.0 mgl^{-1} 2,4-D; 1.0 mgl^{-1} BAP and 3.0 mgl^{-1} 2,4-D; and 1.0 mgl^{-1} BAP and 6.0 mgl^{-1} 2,4-D. The pH of the media was adjusted to 5.8 prior to autoclaving. The cultures were maintained at 25°C (+/- 2) with a 16 hr photoperiod.

The cultures were observed weekly for 2 months for significant morphological changes, i.e. explant expansion and swelling, and for callus formation. A leaf explant tissue producing at least 1 mm of unorganized cell clusters is scored for callus formation (Bradley et al., 2001).

RESULTS AND DISCUSSION

Morpho-Anatomy of Explant Source

Coleus blumei var. **'**Defiance' reaches a height of 60-65 cm (Fig. 1). It has crimson red velvety soft foliage fringed with yellow along the crenate margin. The leaf measures 9-13 cm L x 6-8 cm W at maturity. 'Shocking Pink' exhibits yellow-green velvety foliage that changes color to pink at maturity. It reaches 35-60 cm in height with leaves that measure 7-11 cm L x 5-8 cm W. 'Small Leaf' has a creeping habit that reaches a height of 7-15 cm. It has green leaves with red violet center. The leaves measure 0.4-2.5 cm L x 0.3-1.5 cm W.

Examinations of the abaxial leaf surface of the varieties: ‘Defiance’, ‘Shocking Pink’, and ‘Small Leaf’ showed differences in trichome density and length (Fig. 2). The trichomes observed were multicellular and mostly non-glandular. ‘Small Leaf’ variety showed abundant trichomes compared to ‘Defiance’ and ‘Shocking Pink’ (Table 1). Trichomes were mainly found at the midrib and vein area, rather than on the leaf blade. The three varieties differed in trichome length with ‘Defiance’ showing the longest, with average length of 0.254 mm, followed by ‘Shocking Pink’ at 0.101 mm, and ‘Small Leaf’ at 0.097 mm. Analysis of variance (ANOVA) at 5% degrees of freedom showed that varietal differences in trichome length and density were significant.

Figure 1. Varieties of C. blumei used: 1. ‘Defiance’, 2. ‘Shocking Pink’, and 3. ‘Small Leaf’.

Table 1. Trichome length (mm) and density (no. per mm^2) of the three *Coleus blumei* varieties: ‘Defiance,’ ‘Shocking Pink,’ and ‘Small leaf'.

VARIETIES	Trichome Length (mm)	Trichome Density (no. per mm^2)
‘Defiance’	0.23	200
‘Shocking Pink’	0.10	140
‘Small Leaf’	0.09	320

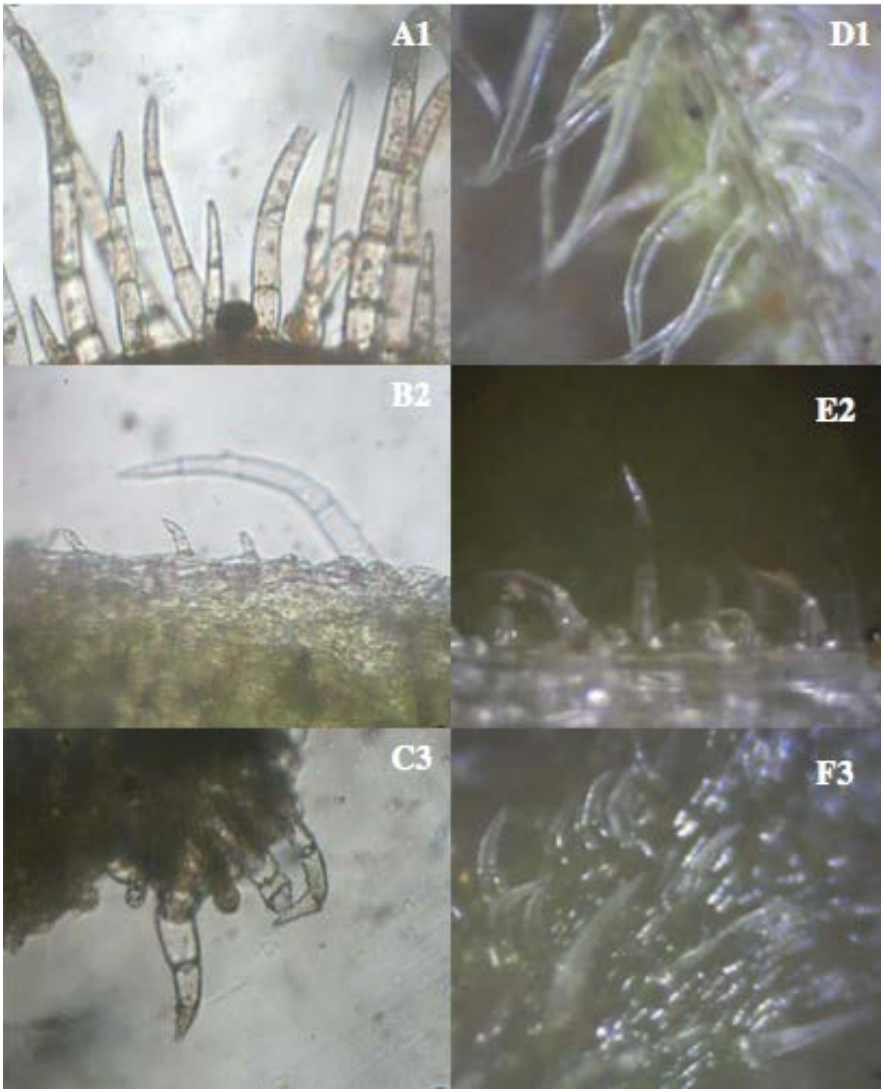

Figure 2. Trichomes of leaf cross sections viewed under the photomicroscope at 100X (A-C) and on the leaf surface viewed with Micron cam at 120X (D-F) of *C. blumei* varieties: 'Defiance' (1), 'Shocking Pink' (2), and 'Small Leaf' (3).

Thin Layer Chromatography: ***Test for rosmarinic acid***

Each of the *C. blumei* varieties tested had a fluorescing bluish white band at the same Rf value of rosmarinic acid in the reference rosemary chromatogram, which is at Rf = 0.30 (Fig. 3; Wagner and Bladt, 1996). It can, thus, be inferred that the three varieties tested also contained rosmarinic acid. The difference in the intensity of the fluorescing band may also indicate a corresponding difference in the level of rosmarinic acid present in the different varieties. In this study, 'Small leaf' variety showed the most intense fluorescence, followed by 'Shocking Pink' and 'Defiance.' This may suggest high rosmarinic acid content of 'Small leaf' variety compared to Defiance' and 'Shocking Pink'.

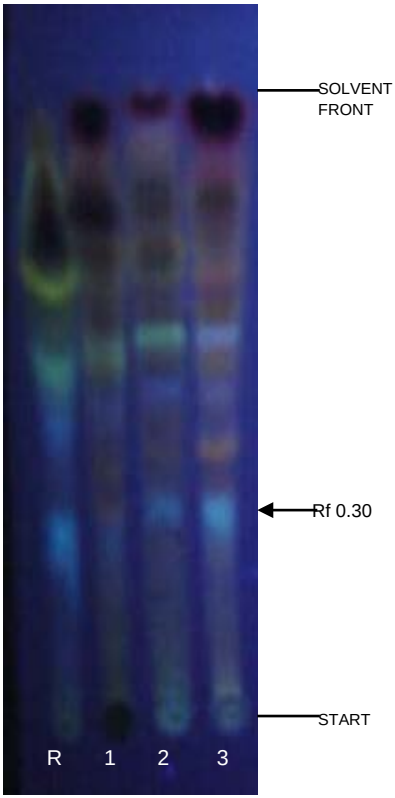

Figure 3. Thin Layer Chromatogram for the presence of rosmarinic acid from leaf extracts of Rosemary (R), *C. blumei* varieties: 'Defiance' (1), 'Shocking Pink' (2), and 'Small Leaf' (3) under UV_{365} (B).

Induction of Callus

Sterilization of Plant Material

One problem encountered during the establishment of tissue culture was the high occurrence of contamination. The same standard surface sterilization procedure was used for all three *C. blumei* varieties. However, there was variation in their response to the surface sterilization method. 'Shocking Pink' leaf explants were effectively sterilized showing only 20.0% contamination. On the contrary, 'Defiance' and 'Small leaf' were difficult to decontaminate as shown in the high percentage of contamination of 75.0% and 81.4%, respectively.

For the three varieties, the high contamination occurrence could be in related to certain factors such as the trichome length and density, the size of leaf and its proximity to the ground from the actual plant where the explant were taken. The trichomes could be the cause of contamination because the higher the density and the finer or longer the trichomes, the more surface area is available for the attachment of bacteria and fungi along with their infective spores. Morpho-anatomical studies showed that 'Defiance' has the longest trichome while 'Small Leaf' has the shortest trichomes but the most number of trichomes per unit area. Most probably, these were the reasons for the occurrence of high contamination in the cultures of these two varieties. The height of the plant could have increased the risk of contamination. 'Small leaf' is a dwarf variety that grows 7-15 cm from the soil. It is constantly exposed to soil particles that contain contaminants that may not be fully removed during surface sterilization. The leaf explant size is the same for the three varieties but the effective surface areas differed due to the trichomes.

Choice of Growth Regulators

'Defiance' variety did not show any morphological changes leading to callus formation in all treatments of varying growth regulators. Callus formation was only observed in varieties 'Shocking Pink' and 'Small Leaf' having treatments with higher 2,4-D concentrations; 3.0 mgl^{-1} and 6.0 mgl^{-1}, respectively. With the increase in the 2,4-D concentration, the time for callus induction was shortened from 60 days to 30 days for 'Shocking Pink' and 30 days to 24 days for 'Small leaf'.

Quality of Callus induced

'Small Leaf' explant remained green during the culture and produced white friable callus after 24 days of culture. 'Shocking Pink' produced brownish calli indicative of high phenolic content and onset of necrosis (Ozyigit et al., 2007). This phenomenon is due to the accumulation of phenolic compounds within the explant tissue (Arnaldos et al., 2001). For tissue culture studies, phenolic compounds generally have negative effects on *in vitro* calli proliferation. The detrimental effects of phenolics occur when these compounds are oxidized and inhibit enzyme functions resulting to inactivation of growth, darkening or browning of the media and lethal browning of the explant (Laukkanen et al., 1999).

At higher 2,4-D levels, the three varieties exhibited variable leat explant response. 'Small Leaf' was less affected by tissue browning and remained in culture compared to the other two varieties. This may have significantly contributed to the initiation of callus formation.

Table 2 summarizes the characteristics evaluated to select the most suitable variety for callus induction. With emphasis on the rosmarinic acid content and culture responsiveness, the variety 'Small Leaf' is the most promising.

Selection of the most culture responsive explant for callus induction is necessary for *in vitro* production of secondary metabolites. According to previous investigations, increase of secondary metabolites synthesis is possible through a two-stage culture system. The cells are first cultured in a medium suitable for maximum biomass production and then transferred to growth-limiting medium to elicit maximum secondary metabolite production (Oksman-Caldentey and Barz, 2002).

Table 2. Summary of the characteristics considered for the most suitable variety to use as explant in callus induction in terms of culture responsiveness and rosmarinic acid content.

VARIETIES	% Callus Formation	Days to Callus Formation	Browning of Tissues	ROSMARINIC ACID CONTENT*
'Defiance'	0	0	-	1
'Shocking Pink'	100	30	+++	2
'Small Leaf'	100	24	-	3

CONCLUSION AND RECOMMENDATIONS

Among the varieties tested, 'Small Leaf' emerged to have a greatest potential as a source of explant material for callus induction. It was the most responsive in culture since callus formation occurred within 24 days after inoculation in MS medium supplemented with 1.0 mgl^{-1} BAP and 6.0 mgl^{-1} 2,4-D. It produced white friable callus while maintaining a green tissue explant. This means no or minimal phenolic oxidation was occurring in its tissue culture. The only disadvantage of using 'Small Leaf' as explant was its high percentage of contamination. If the protocols for surface sterilization and hormone combinations were optimized, further studies of *C. blumei* tissue culture for rosmarinic acid production could be pursued using the variety 'Small Leaf' as explant source.

High percentage of contamination compromises the whole culture system. The use of sonication and vacuum during surface sterilization may be more efficient in removing contaminants. It is recommended that surfaced sterilized seeds germinated in sterile conditions be used as source of explants for cultures. It would be more advantageous if the newly germinated seedlings were used as explants since they have lower concentrations of phenolics and are of actively dividing cells, which will be favorable for callus initiation.

It is further recommended that purification and verification for the presence of rosmarinic acid be done from cultures *in vitro* using high performance liquid chromatography (HPLC) to fully determine the capacity of the plant to produce this secondary metabolite.

STATEMENT OF AUTHORSHIP

The first author conducted the literature search, collected the plant materials, performed the laboratory work, formulated recommendations, and prepared the write-up for publication. The second author initiated the concept, identified some issues, formulated recommendations, and reviewed the paper.

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