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Morphological and Histochemical Characterization of Callus from Leaf Explant of *Tagetes lucida* Cav. (Asteraceae)

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ABSTRACT

This is the first attempt to characterized callus of *Tagetes lucida* that has been successfully induced using leaf as explant and MS with NAA (3ppm) and KI (0.5ppm) as culture medium. Morphological examinations revealed that callus cells have no definite shape and size and mostly transparent with slight yellowish to greenish appearance. Viability test showed that all cells examined are alive which indicates the capacity of the cells to proliferate or differentiate. Alkaloids and terpenes are present in the callus of *T. lucida* based on the results of histochemical analysis. Qualitative detection of these compounds can be used for future quantitative analysis using this plant. Thus, callus cultures of this species can be one of the sources of these compounds for various purposes.

Key words: callus, morphology, histochemistry, leaf explant.

INTRODUCTION

Tagetes lucida Cav., commonly known as Mexican tarragon, is an aromatic herb of family Asteraceae (APG, 2016). Traditionally, this plant is used as spice, as antiseptic, to control diseases which can either be infectious, emotional or of spiritual origin. In Latin-American population, it is used for the treatment of gastrointestinal disorders (Giron et al., 1991; Damian Badillo et al., 2008). This species has been cultivated nowadays considering its various importance.

Several studies suggest that biological extract of this plant exhibit nematicidal, fungicidal and insecticidal activities. Olivero-Verbel et al. (2010) reported that the plant contains essential oils that have antioxidant activity. This is also confirmed by Regalado et al. (2011) wherein antioxidant capacity of the essential oils is measured and significant activities are evident. The main component of the essential oils of *T. lucida* extract is methyl chavicol (estragole) which constitute 95.7%. Other components include linalool, anethole, eugenol and methyl eugenol (Hethelyi et al., 1987; Bicchi et al., 1997). Some major plant secondary products detected from this

species include alkaloids, terpenes, and coumarin. However, there is no available information whether these compounds are also detected in callus cultures.

Considering the bulk of published information on callus cultures of several plant species as well as various compounds detected from these plants, it is interesting to note that there is limited information on the characteristics of *T. lucida* callus cultures. The only available information on this aspect is presented in the study of Rocha-Mendoza et al. (2013) wherein callus from *T. lucida* flower contains flavonoid which is twice higher than the ligule.

This study provides baseline information of morphological and histochemical characteristics of callus from leaf explant of *T. lucida*. Histochemical characterization has been proven to qualitatively detect the presence of some important plant secondary products. A wide array of studies has elaborated the potentials of callus cultures in the production of economically important plant secondary metabolites. Results of this study provides a venue to highlight the potentials of callus culture to produce abovementioned compounds known to this species.

MATERIALS AND METHODS

Media Preparation

Preparation of growth medium was based on the composition of Murashige and Skoog' basal medium (1962) (Table 1). Prepared macro- and microelement stock solutions were mixed considering the accurate volume of each solution based on the protocol. Hormones added to the medium include NAA (3ppm) and KI (0.5ppm). The pH of the growth medium was measured and was set to 5.8. Agar (Gelrite) was then added to the growth medium and was cooked using a hot plate/stirrer. In each test tubes, 4 mL growth medium was dispensed and covered with cotton plugs. Growth medium, together with other materials needed for inoculation, was sterilized.

Table 1. Composition of Stock Solutions for Murashige and Skoog's Basal Medium (1962).

Constituent	Recommended Values (mg/L)	Stock Concentration	Vol./ g needed in 1000mL Growth Medium
Macronutrient			
KNO_3	1900		50 mL
NH_4NO_3	1650		50 mL
$CaCl_3$	440	20X	50 mL
$MgSO_4$	370		50 mL
KH_2PO_3	170		50 mL
Micronutrients			
$MnSO_4.4H_2O$	15.6		
$ZnSO_4.7H_2O$	8.6		
H_2BO_3	6.2		
KI	0.83	200X	5 mL
$CuSO_4.5H_2O$	0.025		
$NaMoO4.2H_2O$	0.25		
$CoCl_2.6H_2O$	0.025		
Fe-EDTA			
$FeSO_4$	27.8	200X	5 mL
Na_2EDTA	37.3		
Vitamins	0.5	200X	5 mL
Hormones			
Auxin: NAA		100 ppm (3ppm)	30 mL
Cytokinin: Ki		100 ppm (0.5ppm)	5 mL
Myo-inositol	100		0.1 g
Sugar: Sucrose	3000		30 g
Agar: Gelrite			2 g
pH- 5.7-5.8			

Explant Preparation and Sterilization

Young leaves, specifically near the tip, of *T. lucida* were used as explants. Surface sterilization procedures for leaves were adopted from cf. Setz et al., 1985 with modifications. Samples were washed under running water for 10 minutes. Samples were then soaked in 95% ethanol for 1 minute and washed with sterile water right after, also for 1 minute. Disinfection was done through the use of 2% chlorox. Samples were soaked in chlorox (2%) for 5 minutes. It was then washed with sterile water for three (3) times with 1-minute duration per washing.

Callus Initiation and Maintenance

Cutting of explant from sterile plant material and inoculation was done under sterile condition in an inoculation chamber. Portions of the lamina near the leaf base were selected as explant. In each species, however, sizes of the explant differ due to the variation in leaf sizes. The explant sizes ranges from 4-5 mm^2. Brown portions of sterile plant material were not included as part of the inoculum. Explants of each species were placed on the growth medium. The cultures were placed in a culture room with specific temperature and light irradiance. Each culture was observed per week for callus growth. After four weeks, calli were sub-cultured in a new medium with the same composition as the growth medium used in callus initiation. Large bottles were used for sub-culturing and maintenance. Sub-cultured calli (Fig. 1) were inoculated for 4 weeks prior to analysis.

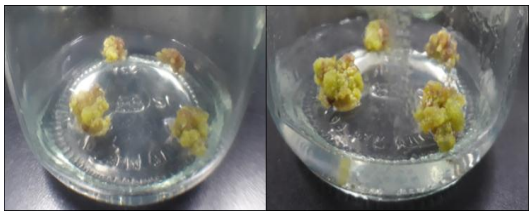

Fig. 1. Sub-cultured calli of *Tagetes lucida.*

Morphological and Histochemical Characterization

Morphological and histochemical analysis of *T. lucida* callus was done under light compound binocular microscope. Callus cells were mounted in glass slide using distilled water for morphological characterization. This also serves as a control. Viability of callus cells were also tested using neutral red. Qualitative histochemical analysis was done by using specific reagents to detect major group of phytochemical compounds. Histochemical test for alkaloids and terpenes were carried out. A summary of reagents used and positive reactions for each of the tested metabolite is provided in Table 2. Cross sections of the leaf were also subjected to histochemical analysis for comparison. Photomicrographs were taken on each test.

Table 2. Major compounds, stains and indicators used for the histochemical test.

Compound Group	Stains/Reagents	Positive Indicator
Alkaloids	Dragendorff's Reagent	Chocolate brown precipitate (Furr & Mahlberg, 1981)
Terpenes	Conc. H_2SO_4	Light violet/ yellow coloration (Cappelletti et al., 1986)

RESULTS AND DISCUSSION

Morphological Characterization

It was observed that callus cells of *Tagetes lucida* were transparent with slight yellowish and greenish coloration as shown in Fig. 2. It was also observed that some callus cells occurred in clusters. Rounded, elongated, rod-shape and isodiametric cells of varying sizes were observed in all sections examined.

Cell Viability

Callus cells of *T. lucida* were alive based on the results of viability test as shown in Fig. 3. Cells showed affinity to the stain giving red color. In addition, cytoplasmic contents of some callus cells were darkly stained. One interesting observation in one of the sections is the presence of scalariform-like structure. This can probably be a differentiated vascular cell; however, further investigation is needed to be certain on the identity of this structure.

Histochemical Characterization

Test for Alkaloid

It was observed that alkaloids are present in callus cells of *T. lucida* (Fig. 4). Most of the cells exhibit chocolate brown precipitate which indicates the presence of alkaloids. Cells on the leaf cross section also showed positive result which can confirm the presence of alkaloid in callus cells.

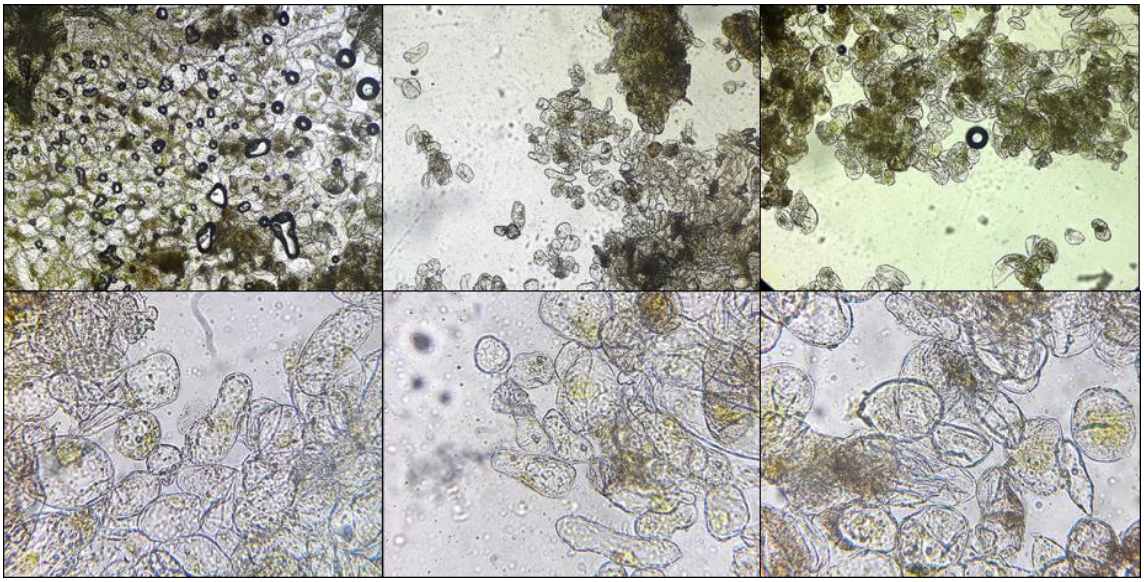

Fig. 2. Callus cells of *T. lucida*. Cells are transparent with slight yellowish and greenish coloration. Cells have various cells shape. (*Above- Mag.= 100X; Below- Mag= 400X*).

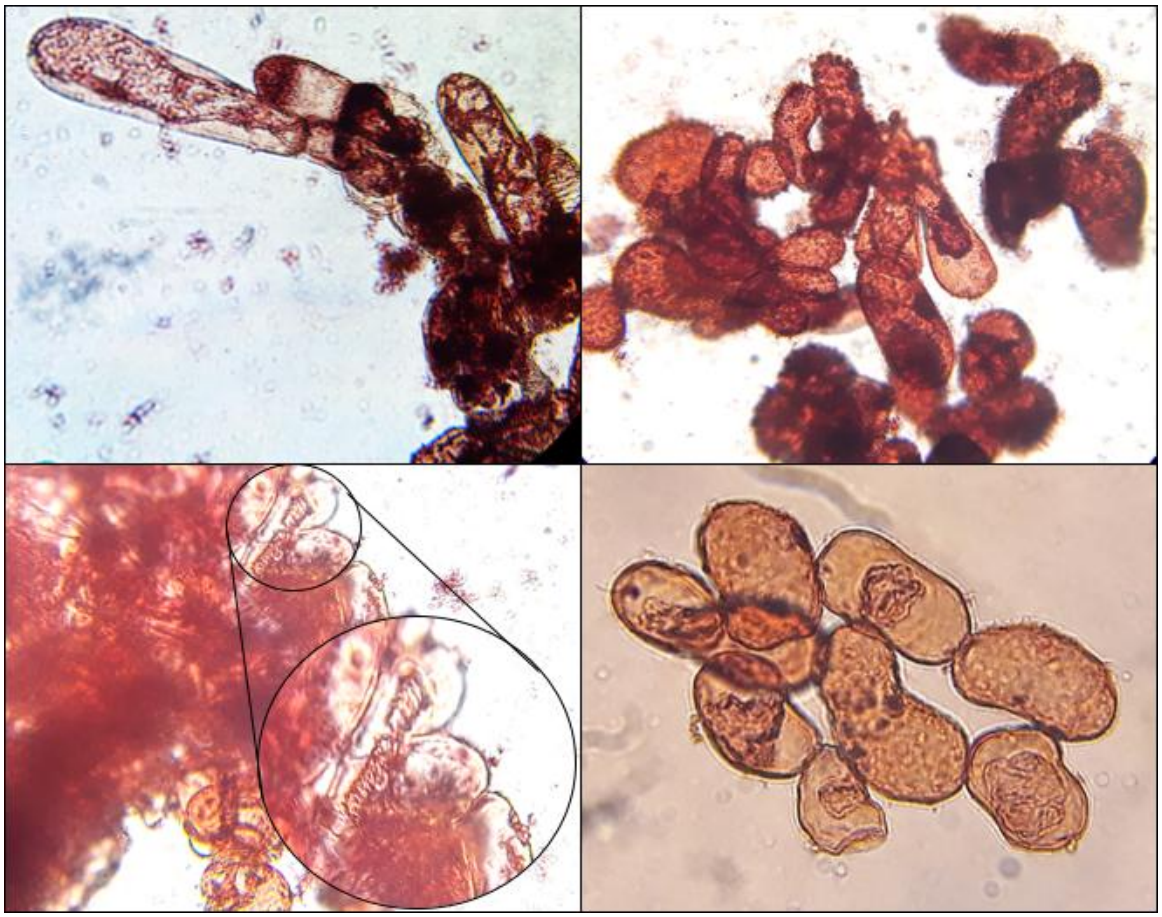

Fig. 3. Result of viability test showing viable callus cells of *Tagetes lucida*. One section showing a scalariform-like structure (magnified). (Mag.= 400X).

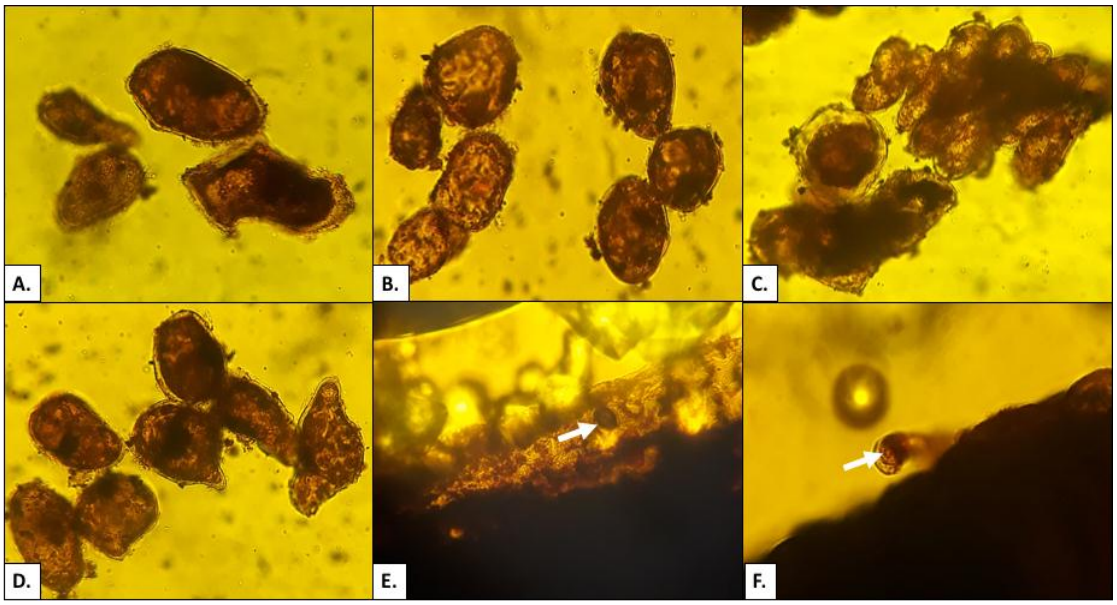

Fig. 4. Callus cells (A-D) and leaf cross sections (E-F) of showing positive result for alkaloid test. Chocolate brown precipitate was observed in both callus cells and leaf cross sections (white arrows).

Test for Terpenes

Not all callus cells showed positive result for terpenes. Most of the cells showed brown coloration (Fig. 5). This coloration indicates the presence of sugars. However, there were few cells showed positive result wherein light -yellow coloration was observed. Leaf cross sections also showed light-yellow coloration which can possibly indicate the reaction of conc. H_2SO_4 with terpenes. However, uncertainty remains since the light-yellow coloration leached out the cells.

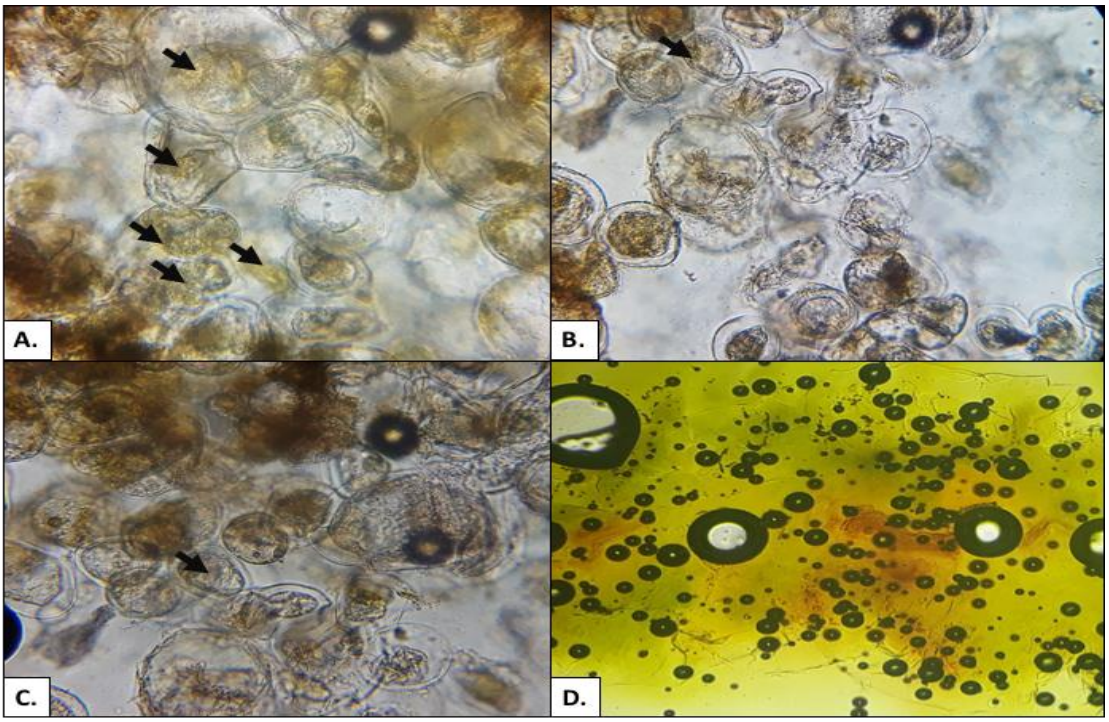

Fig 5. Callus cells (A-C) and leaf cross section (D) of *T. lucida* showing cells with light-yellow coloration (arrows) indicating positive result for terpenes. (*Mag.= 400X).*

As observed, callus cells of *Tagetes lucida* have no definite shape and size. Most of the observed cells were rounded, elongated, rod-shape and isodiametric. Generally, callus tissues are extremely heterogeneous which is composed of small cells with dense cytoplasm and large cells with vacuolated cytoplasm. Also, it is a common observation that the cells within the callus varies from spherical to markedly elongated. Larger elongated cells are considered non-dividing cells

having large vacuoles while small cells are actively dividing with reduced vacuole size (Falco et al., 1996).

Calli were also observed to have greenish appearance which may indicate the presence of chloroplast. In most literature, green callus is termed as chlorophyllus callus. The development of chloroplast, or simply greening of the callus can also be associated on the type of hormone used for callus induction and maintenance. Both NAA (3ppm) and KI (0.5ppm) are used in this study. In the study of Sharma et al. (2011), callus grown in culture medium with NAA showed yellowish green appearance and noted to be fast growing. These characteristics were also observed in the callus of *T. lucida*. In addition, KI (cytokinin) also induced the greening of callus. The greening of non-green callus tissue of tobacco is one of the effects of kinetin (cytokinin) (Kaul & Sabharwal, 1971). This can also be associated to the function of cytokinins as retardants of chlorophyll and protein degradation of senescing leaves.

Callus cells of *T. lucida* are viable and alive. Other indicator of viability is cytoplasmic streaming for regular observation. Cell viability provides information on the capacity of the callus cells to proliferation and to differentiate. With regards to differentiation, a scalariform-like structure was observed in one of the sections for viability test. There is a high probability that this is a cell which has differentiated into tracheary element. This claim can be supported by the fact that elongated cells within the callus tissue may differentiate into lignified xylem tracheids or phloem-like cells (Aloni, 1980). Some cells may have been differentiated considering the age of the callus after subculture prior to analysis.

Both alkaloids and terpenes are detected in the callus cells of *T. lucida*. Alkaloids are not commonly known to be present in *Tagetes*. However, Kulbi et al. (2013) detected alkaloids from *Tagetes erecta.* It was noted by Sutfeld et al. (1985) that *T. lucida* contains non-alkaloid compound, Salvanorin A, and the plant has no alkaloid. This claim was disputed by the study of Guadarrama-Cruz et al. (2012) wherein major metabolites detected from extracts of *T. lucida* aerial parts include alkaloids, terpenes, and phenolic compounds. Alkaloids present in this plant are known to produce antidepressant-like effect on rats. On the other hand, several species of *Tagetes* are known to produce essential oils (Gupta & Vasudeva, 2012).

Based on the studies of Aquino et al. (2002), Ciccio (2005) and Cespedes et al. (2006), one of the major group of secondary metabolites obtained from *T. lucida* are terpenes. Other species known to produce terpenes include *Tagetes minuta, T. erecta, T. patula, T. gladulifera, T. filifolia* and *T. tenuifolia.* Terpenes like α-pinene, β-pinene, dipentene, methol and geraniol are known to be present in *T. erecta*. Essential oils examined from the same species are composed of acyclic monoterpene ketones (Baslas & Singh, 1980). In addition, terpene components of *Tagetes* like limonene, caryophyllene and ocimene are now being studied for the development of new insecticide. This possibility was based on the fact that these terpenes have larvicidal activity against *Aedes aegypti* (Mejia-Barajas et al., 2012). The presence of alkaloids and terpenes on callus cells of *T. lucida* can be validated by these information since these compounds are produced by the plant. The positive results indicate the ability of the callus cultures to produce alkaloids and terpenes.

CONCLUSION

Callus of *Tagetes lucida* can grow and proliferate in MS medium with NAA (3ppm) and KI (0.5ppm). One of the observed effect of hormone composition is the production yellowish to greenish calli. As observed, callus cells have no definite shape and size, transparent with slight yellowish to greenish, and are viable. Alkaloids and terpenes are present in callus cells, thus, callus culture of *Tagetes lucida* can be used as source of these compounds for various purposes. Further investigation such as optimization of medium components can be done in order to enhance the production of these compound for callus cultures of *T. lucida.*

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