

## PRELIMINARY STUDY OF THE INDICAN PRODUCTION IN TISSUE CULTURES OF *Indigofera suffruticosa* MILL.

### ESTUDIO PRELIMINAR SOBRE LA PRODUCCIÓN DE INDICAN EN CULTIVO DE TEJIDOS DE *INDIGOFERA SUFFRUTICOSA* MILL.

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**Abstract:** Hypocotyl explants of *Indigofera suffruticosa* Mill were cultured on Murashige and Skoog medium supplemented with various concentrations of 6-benzyladenine or kinetin combined with different levels of 2,4-Diclorofenoxiacetic acid, to induce callus formation and indigo production. No calluses were obtained when growth regulators were used individually. The callus with the best morphological and growth characteristics were obtained with the combination of 2.26  $\mu$ M and 4.44  $\mu$ M of 2,4-Diclorofenoxiacetic acid and 6-benzyladenine, respectively. 6-benzyladenine proved to be better than kinetin to induce callus formation. All obtained callus showed indican accumulation. The combination of 2.26 $\mu$ M 2,4-Diclorofenoxiacetic acid and 0.44  $\mu$ M 6-benzyladenine, or 0.46  $\mu$ M kinetin gave the highest indican concentration (16.27 mg g<sup>-1</sup> dry weight).

**KEYWORDS:** 6-benzyladenine; Callus; 2,4-Diclorofenoxiacetic Acid; Indigo; Indirubin; kinetin.

**Resumen:** Explantes de hipocotilo de *Indigofera suffruticosa* Mill se cultivaron empleando medio de Murashige y Skoog suplementado con diferentes concentraciones de 6-benciladenina o de cinetina combinadas con diferentes niveles de ácido 2,4-Diclorofenoxiacético para inducir la formación de callos y la producción de índigo. El uso de reguladores de crecimiento individualmente no indujo la formación de callos. Los callos con mejores características de crecimiento y morfológicas se obtuvieron con la combinación de 2.26  $\mu$ M de ácido 2,4-Diclorofenoxiacético y 4.44  $\mu$ M de 6-benciladenina. La 6-benciladenina mostró ser mejor que la cinetina para la inducción de la formación de callos. Todos los callos obtenidos acumularon indican. La mayor concentración de indican (16.27 mg g<sup>-1</sup> de peso seco) se obtuvo con la combinación de 2.26 $\mu$ M de ácido 2,4-Diclorofenoxiacético y 0.44  $\mu$ M 6-benciladenina.

**KEYWORDS:** 6-benciladenina; Callos; ácido 2,4-Diclorofenoxiacético; Indigo; Indirubina; cinetina.

Glossary: 6-Benciladenine (BA); 2,4-Diclorofenoxiacetic Acid (2,4-D); High Performance Thin Layer Chromatography (HPTLC); Kinetin (KIN)

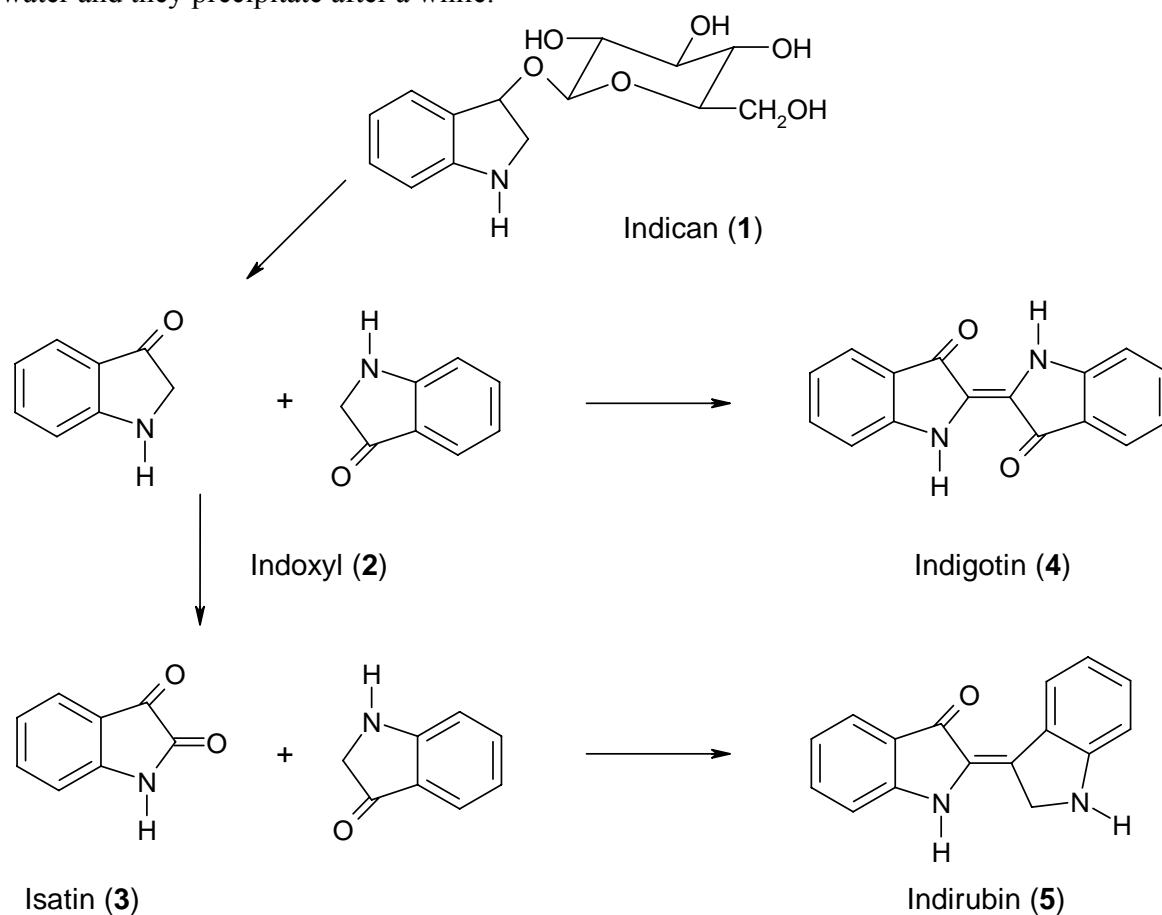
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## Introduction

*Indigofera suffruticosa* Mill is a short-lived shrub that reaches 1 to 2 m in height and 1 to 2 cm in stem diameter. This plant is found in tropical and subtropical areas and well adapted to growth in semi-arid regions and soil of low fertility [1].

The biosynthetic pathway of indigo varies among plant species, but it was found that indole is the common precursor. From indole is synthesized indican, which is the main precursor of the formation of indigo from *Indigofera* species [2]. [3, 4]. This compound is mainly present in the leaves and it is liberated in aqueous solutions during the extraction; and then indican (Figure 1, 1) is hydrolyzed to glucose and **indoxyl (2)** by the action of a specific natural  $\beta$ -glucosidase enzyme located in chloroplasts of mesophylls cells [5]. Indoxyl can follow two routes to form different compounds; in the first route, two indoxyl isomerized molecules can be condensed to yield **indigotin (4)**, while in the second route indoxyl is oxidized into **isatin (3)** which can be bound to a free indoxyl molecule becoming **indirubin (5)** [4]. The conversion of free indoxyl by air oxidation into indigo and indirubin occurs spontaneously. These molecules are not soluble in water and they precipitate after a while.



**Figure 1.** Conversion of indican into indigo and indirubin during extraction from plants [6]

Indigo, the natural oldest dye used by the mankind, is obtained by fermentation from a great variety of plants and some species of mollusks [7]. Some of the plant species that have been used for the indigo extraction are *Isatis tinctoria* L. (Woad) in the Southwest of France, *Polygonum tinctorium* Ait. (true indigo) in Asia and, *Indigofera suffruticosa* Mill. (Añil) in Central America and Mexico [8, 9]. At the end of the 19th century synthetic indigo almost completely replaced natural indigo. The synthetic indigo blue dye is constituted of pure indigo; the natural indigo dye contains variable proportions of the isomer indirubin, a common pink colored by-product of indigo extraction, which provides a distinct purple hue to natural indigo. Currently, the main use of indigo is textile industry dyeing the cotton yarn to the production of denim, but some authors had reported that indigo and indirubin could have potential medicinal applications such as antibacterial, anticancer and anti-inflammatory [10-12]. Moreover, textiles dyed with indigo have shown bactericidal activity, which was greater with natural indigo extracted from plants than synthetic indigo [13].

Recently, because of the importance of natural indigo, considerable research has been performed to replace chemical synthesis of indigo by the application of biotechnological methods based on bacteria [14], transgenic tobacco plants [15] or plant cell culture [16, 17].

Plant cell and tissue cultures have been used for the production of secondary metabolites, such as colorants, flavors and medicinal compounds. Higher metabolite yields can be obtained from cultured plants cells as compared to intact plants. Some studies have suggested the possibility of secondary metabolite production by large-scale plant cell cultures [18].

Therefore, the aim of this work was to develop a protocol for the induction of *in vitro* cultures from *Indigofera* to take alternatives for production of this compound as well as to measure indican accumulation in this system.

## ***Materials and Methods***

### ***Biological material.***

*In vitro* plant cultures of *Indigofera suffruticosa* Mill were obtained from seeds collected at Jalisco State, México; which were germinated in MS medium culture.

### ***Callus induction***

Callus cultures were started from hypocotyl explants of 5 mm long obtained from 30 days old plantules. The explants were put into glass containers with 30 mL of Murashige and Skoog medium (MS) supplemented with combinations of 2,4-Dichlorofenoxyacetic Acid (2,4-D) (0, 0.45, 2.26 and 4.53  $\mu$ M) and 6-Benzyladenine (BA) (0, 0.44, 2.22 and 4.44  $\mu$ M) or Kinetin (KIN) (0, 0.46, 2.23 and 4.65  $\mu$ M). The cultures were incubated at 25 °C, under white continuous 600 lux light to induce non-differentiated tissue formation (Callus, about 30 days of culture). Afterward, callus were separated from differentiated tissue and transferred into fresh culture medium keeping the initial plant growth regulators concentration and subcultured each 15 days.

### **Indican extraction**

In order to extract indican from callus culture, a modification of the method described by Minami et al. [5] was used. 0.5 g of callus (wet weight basis) were frozen in liquid nitrogen and broken up in a glass homogenizer (potter). The broken tissues were suspended in 5 mL of a chloroform:methanol:water (12:5:3 v/v) solution, mixed vigorously in a vortex mixer and centrifuged at 26,000 g by 15 min. The aqueous layer was saved and the lower layer was extracted twice more by the same way. The combined upper layers were mixed with 0.35 mL of chloroform and 0.5 mL of distilled water and centrifuged one more time. The final extract corresponded to the aqueous layer which was used for the quantitative analysis of indican.

### **Quantitative measurement of indicant**

Indican was quantified by thin layer chromatography using High Performance Thin Layer Chromatography (HPTLC) normal phase 5 X 10 cm chromatoplates (Merk, Kiesegel 60 PF<sub>254</sub>). 80 µL of callus extract or 5 µL of standard indican solutions (SIGMA I3750) were deposited on chromatographic plates which were eluted by 3 min using 2-morpholinoethanesulphonic acid-KOH:Acetonitrile (20:80 v/v) solution as a mobile phase. Subsequently, the plates were exposed to UV light in a transilluminator (UV Transilluminator UVP; 115 V, 60 Hz). The spots with a similar retention factor (Rf) value to that of the indican standard were signed, scraped from the plates and suspended in 1 mL of bidistilled water and then centrifuged at 5000 rpm by 15 min at 4 °C. A preliminary wavelength scan to determine the maximum indigo absorption was carried out in a UV-Vis spectrophotometer (CINTRA 5, GBC). The absorbance of the supernatant was read at 220 nm. An indican calibration curve (0 to 10 µg mL<sup>-1</sup>) was used to calculate concentrations of unknowns.

## **Results and Discussion**

### **Callus induction**

The response for callus induction and regeneration is not the same in different media concentrations. Sixteen treatments resulted from each combination of the auxin 2,4-D and one cytokinin (BA or KIN). The type and concentration of plant regulator is considered a critical factor for callus formation [19]; while auxins may regulate callus initiation and growth, and embryogenesis; cytokinins regulate cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, inhibit root formation, activate RNA synthesis, and stimulate protein and enzyme activity. In this work, no callus were obtained when auxins or cytokinins were utilized individually, at any evaluated concentration, however, induction of friable callus and the pigment production has been reported in *Mammillaria candida* Scheidweiler (cactaceae) by using only 2-4 D [20]. When combinations 2,4-D and BA were used, nine of the treatments resulted in non-differentiated tissue formation after three months. During the callus *Indigofera suffruticosa* Mill induction, at concentrations of 2,4-D 0.45 and 2.26 µM, the increase in BA concentration promotes dedifferentiation and growth. The opposite effect was observed at the highest concentration of 2,4-D. Our results show that the increase in BA concentration is favorable for callus induction. After twelve transferences (six months), only six treatments developed yellow friable callus. The callus with the best morphological and growth characteristics were obtained with the combination of 2.26 µM and 4.44 µM of 2,4-D and BA respectively.

Only, three of the 2,4-D and KIN sixteen combinations (2.26/0.46, 2.26/2.32 and 4.53/4.65 µM), resulted in compact callus formation after six months of culture. These results suggest that callus induction depends on the 2, 4-dichlorophenoxyacetic acid concentration only, and kinetin is not essential for callus induction. The

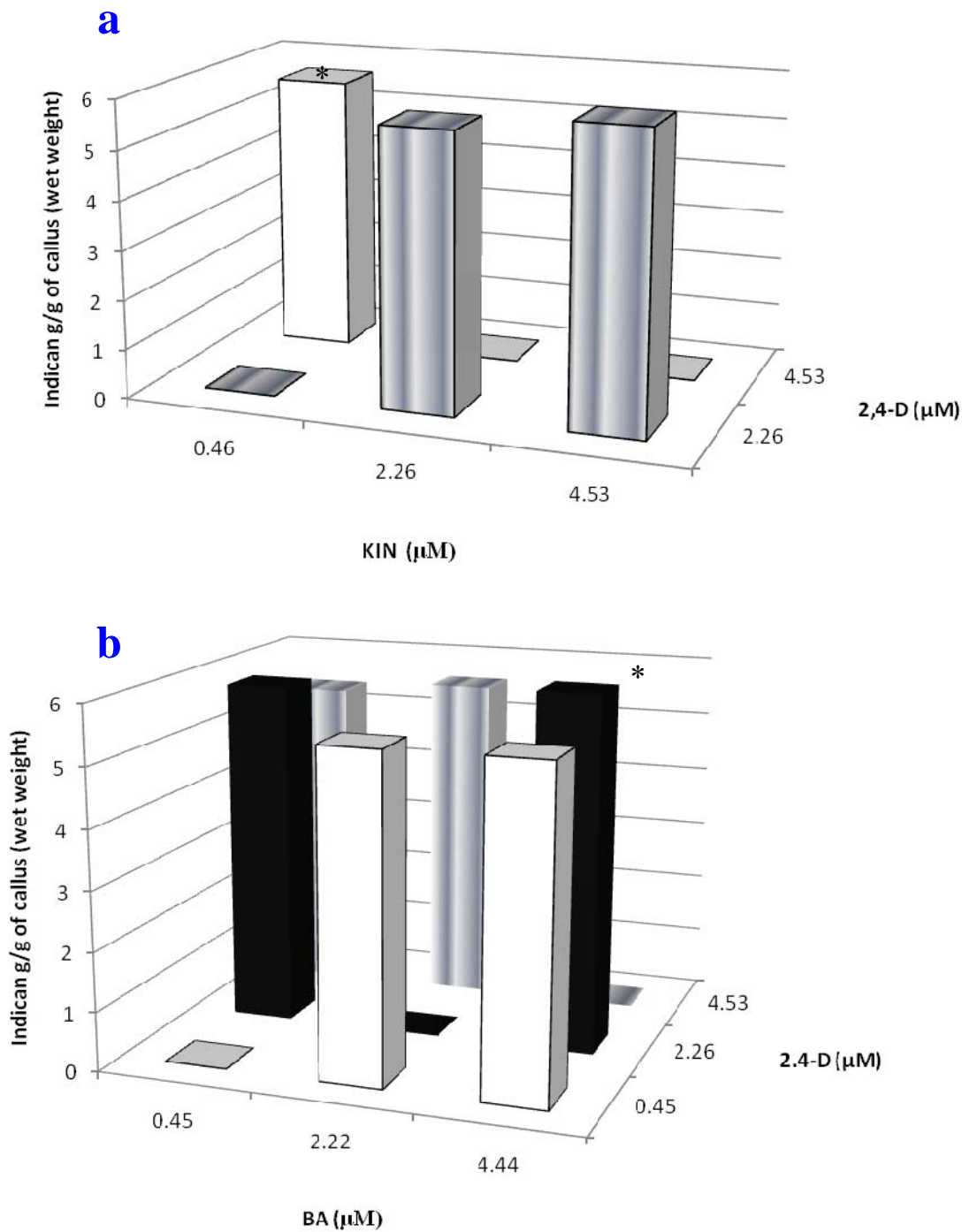
presence of kinetin in callus initiation media retarded callus initiation of *Medicago sativa* L., but enhanced division and differentiation of callus cells [21].

### ***Indican accumulation***

It is known that secondary metabolites such as pigments are produced naturally in most plants during growth [9]. For plant cell cultures, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells. Cultured plant cells often produce reduced quantities and different profiles of secondary metabolites when compared with the intact plant and these quantitative and qualitative features may change with time. The poor product expression is often attributed to a lack of differentiation in cultures. *Indigofera* and *Polygonum tinctorium* plants contain indoxyl- $\beta$ -D-glucoside (indican) which serves as starting material for indigo blue and indirubin production [22]. Minami et al. [5] reported that the etiolated leaves of indigo plants did not contain indican, and affirmed that its storage may be related to the maturation of leaves and development of chloroplasts. Marshall et al. [23] observed that on initial culturing and dedifferentiation, cell lines often fail to produce the quantity or complexity of secondary metabolites present in the explants tissue used. Besides, it has been observed that some degree of cell differentiation is often required for accumulation of secondary metabolites in cell cultures [24]. However, in this study, accumulation of indican was detected in nine of the callus cultures obtained (Figure 2(a) and (b)).

The maximum indican concentration was achieved when the combinations of 2.26  $\mu$ M 2,4-D and 4.44  $\mu$ M BA, or 2.26  $\mu$ M 2,4-D and 4.53  $\mu$ M KIN were used (Figure 2a and b respectively). Furthermore, the callus with better morphological characteristics accumulated the highest indican concentration. The results suggest that middle levels of 2,4-D (2.26  $\mu$ M) in the medium, promote higher indican accumulation than the rest of concentrations applied in these experiments with *Indigofera suffruticosa* Mill. The possibility of producing indigo and indirubin from *Polygonum* plant suspension cell cultures was reported previously [25, 26]. However, to our knowledge, any study has been carried out for dye production in *Indigofera suffruticosa* tissue cultures.

These results can be the basis to develop an efficient protocol to induce callus formation to produce indican, the precursor of indigo and indirubin. Therefore, more studies are currently being done to optimize *in vitro* indigo and indican production.



**Figure 2.** Indican accumulation in callus obtained under different treatments with 2,4-D and BA (a) or KIN (b). \* statically different ( $\alpha = 0.05$ ).

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