IJIRR

International Journal of Information Research and Review
Vol. 02, Issue, 12, pp. 1496-1499 December, 2015

Research Article

# ACCUMULATION OF INDIGO IN TISSUE CULTURES OF *STROBILANTHES CUSIA* (NEES) KUNTZE

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**ARTICLE INFO**

***Article History:***

Received 09th, September 2015
Received in revised form
11th, October 2015
Accepted 30th, November 2015
Published online 30th, December 2015

***Keywords:***

Strobilanthes cusia (Nees) Kuntze,
callus; indigo,
Micropropagation,
Spectrophotometry.

**ABSTRACT**

The present study reports for the first time the assessment of indigo content of in *vitro* regenerated plantlets and callus tissues of *Strobilanthes* cusia (Nees) Kuntze. Maximum shoot multiplication from shoot- tip explants was observed from explants cultured on Murashige and Skoog (MS) medium supplemented with 1mg/L naphthalene acetic acid (NAA) in combination with 1mg/L kinetin (Kin) followed by MS medium supplemented with 0.5 mg/L NAA alone and the shoot buds also showed rooting in these media. Rooted plantlets were successfully established in the soil. Callus was initiated from leaf explants of in *vitro* regenerated plantlets on medium supplemented with 3mg/L NAA, 0.2mg/L 6-benzylaminopurine (BAP), 0.2mg/L Kin and 0.2mg/L 2, 4- dichlorophenoxyacetic acid (2,4-D). Leaves of in *vitro* regenerated plantlets and callus tissues showed the accumulation of indigo dye although the amount was lesser compared to leaves of field-grown plants.



## INTRODUCTION

Indigo dye is a prevalent natural dye obtained commonly from many plant species such as *Strobilanthes cusia* (Nees.) Kuntze., *Indigofera suffruticosa* Mill, *Polygonum tinctorium* Palette, *Isatis indigotica*, Fort, etc. and is used in textile dyeing and printing (Clark *et al*., 1993). In Manipur, *Strobilanthes cusia* (Nees.) Kuntze., locally known as "Kum", plays an important role in handloom industries as the source of indigo colour called "kum dye" for dyeing of cotton and silk threads used for weaving traditional attires (Akimpou *et al*., 2005, Sharma *et al*., 2005, Singh *et al*., 2009, Ningombam *et al*., 2012; Potsangbam *et al*., 2008). Traditionally, the "kum dye" is obtained from the leaves of *S. cusia* (Nees.) Kuntze. by fermentation followed by calcination which is further used to obtain different shades of colour by mixing with different plant species (Potsangbam *et al*., 2008 and Rajendro *et al*., 2009). During the fermentation process, indigo is formed from indican which readily hydrolyzes to release β-D-glucose and indoxyl followed by air oxidation of indoxyl (Laitonjam and Wangkheirakpam, 2011; Schorlemmer and Carl, 1874). The leaves of *S. cusia* (Nees.) Kuntze. used in traditional dyeing are usually collected from plants cultivated in kitchen gardens or from the surrounding hills. Since the plants are cultivated in small scale and the leaves are harvested in a particular season only, the production is very low and hardly meets the demand.

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Therefore, there is an urgent necessity to develop a method for increased production of the dye. Since plant tissue cultures provide an efficient method for rapid mass propagation and production of useful secondary metabolites, micropropagation and *in vitro* production of the dye by cultured cells will provide an alternative method for large scale multiplication and production of indigo. It will also minimize the over-exploitation and from nature. However, there has been few reports on micropropagation (Deb and Arenmongla, 2011; Mangkita *et al*., 2011) of the plant no study has been conducted to estimate the indigo production potential of *in vitro* cultures of the plant. Hence, the study was undertaken to assess the accumulation of indigo in *in vitro* cultures of *S. cusia* (Nees.) Kuntze.

## MATERIALS AND METHODS

### Micropropagation

The excised shoot tips of *S. cusia* (Nees.) Kuntze. collected from local cultivation gardens were surface sterilized and cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins (0.5, 1 and 3 mg/L BAP, 0.5 mg/L NAA or combination of 1mg/L NAA with 1mg/L Kin) for shoot bud multiplication. *In vitro* raised shoots were transferred to MS medium supplemented with 0.5 and 1 mg/L of NAA, Indole-3-acetic acid (IAA) or Indole-3- butyric acid (IBA) for induction of rhizogenesis. The rooted plantlets were then transplanted to plastic cups

containing sand: soil mixture (1:1) which were frequently watered and kept covered with perforated clear polyethylene bags to maintain humidity. The plants were then transferred to field condition after two months.

**Callus induction**

For callus induction, leaves excised from four-week old *in vitro* multiplied plantlets were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins (1 and 3 mg/L IAA, NAA or IBA with 1 and 3 mg/L of BAP or Kin; combination of 1mg/L NAA with 1mg/L BAP and 0.2mg/L 2,4-D; or combination of 3mg/L NAA with 0.2 mg/L BAP, 0.2 mg/L Kin and 0.2 mg/L 2,4-D). All cultures were incubated in a growth chamber at 25 ± 2°C and 16-h photoperiod except for callus induction where the cultures were incubated under dark condition. Each treatment for shoot multiplication, root induction and callus induction had ten replicates and all the experiments were repeated thrice. Data for shoot multiplication were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

**Indigo estimation**

Young leaves from field grown plants, leaves from six-week old *in vitro* propagated plantlets and callus tissues were used as explants for indigo dye extraction with water following the methods described by Laitonjam and Wangkheirakpam (2011) in two ways i.e., with and without $Ca(OH)_2$. The concentrations of indigo present in the samples were estimated by measuring their absorbance at 611 nm with the help of a spectrophotometer and using calibration curve prepared by measuring various concentrations of standard indigo (Sigma).

## RESULTS AND DISCUSSION

The shoot-tip explants proliferated multiple shoot buds on bud induction medium after four weeks of culture (Table 1). A significantly higher number of shoot buds (11.5) was obtained from explants cultured on medium containing 1 mg/L NAA in combination with 1mg/L Kin (Fig. 1 a) followed by medium supplemented with 0.5 mg//l NAA alone (10.13 shoot buds). The shoot buds cultured on these media also showed the induction of rooting and further multiplication was achieved by subculturing the buds on these media after every four weeks.

Earlier studies on micropropagation of *Strobilanthes cusia* (Nees) Kuntze. (Mangkita *et al*., 2011; Deb and Arenmongla, 2011) reported that BAP showed higher effectiveness in inducing shoot multiplication. However, in the present study, shoot-tip explants cultured on medium supplemented with BAP alone formed rosettes and the number of healthy shoot buds regenerated were lesser in these media (Fig.1b). Among the different auxins used for root induction, the best rooting and elongation occurred on medium containing 0.5 mg/L NAA, where the plantlets produced a significantly higher number of roots with abundant branches and root hairs this medium followed by medium supplemented with 1 mg/L NAA.

**Table 1. Effect of growth regulators on multiple shoot bud induction from shoot-tip explants of Strobilanthes cusia (Nees) Kuntze**

| NAA (mg/L) | Kin (mg/L) | BAP (mg/L) | No. of shoots (mean± S.E.) | Rooting (+/-) |
|---|---|---|---|---|
| 1 | 1 | _ | $11.43^{a}$ ±0.49 | +ve |
| 0.5 | _ | _ | $10.13^{b}$ ±0.36 | +ve |
| _ | _ | 0.5 | $6.43^{c}$ ± 0.25 | -ve |
| _ | _ | 1 | $7.16^{c}$ ± 0.26 | -ve |
| _ | _ | 3 | $5.93^{c}$ ± 0.15 | -ve |

The effectiveness of NAA in inducing rhizogenesis in *Strobilanthes flaccidifolious* (Nees.) has been reported earlier by Deb and Arenmongla, 2011. Earlier, Mangkita *et al*., (2011) reported the production of maximum number of roots (4.8) from buds of *Strobilanthes cusia* (Nees) on medium supplemented with 5 mg/l IAA. However, in our study, the rooting efficiency of the shoot buds cultured on medium supplemented with IAA or IBA was less as the buds cultured on these media produced fewer numbers of roots which were thin and without much root hair. The i*n vitro* regenerated plantlets showed 70- 80 % survival during transplantation (Fig.1c). The leaf explants cultured on callus induction medium formed callus within four weeks of culture (Table 2). The leaf explants produced callus with the best morphological and growth characteristics suitable for subculture on medium supplemented with 3mg/L NAA, 0.2mg/L BAP, 0.2mg/L Kin and 0.2mg/L 2,4-D (Fig.1d) while the explants cultured on medium supplemented with different concentrations of IAA, IBA or NAA in combination with different concentrations of BAP or Kin only produced compact calli generally on the peripheral regions of the explants.

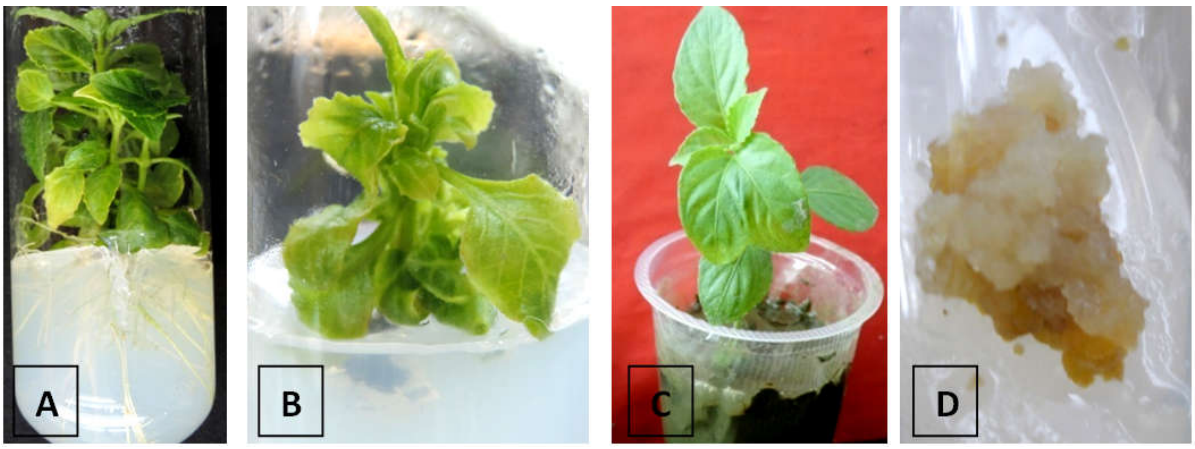


**Figure 1. *In vitro* culture of *Strobilanthes cusia* (Nees) Kuntze. A) proliferation of shoot buds along with rooting from shoot-tip explants cultured on MS medium supplemented with 1 mg/l Kin and 1 mg/l NAA; B) rosette formation on buds cultured on medium supplemented with 1 mg/l BAP; C) Transplanted plantlet; and D) Callus induced from leave explants cultured on medium supplemented with 3mg/L NAA, 0.2mg/L BAP, 0.2mg/L Kin and 0.2mg/L 2,4-D**

**Table 2. Effects of growth regulators in callus induction from leave explants of Strobilanthes cusia (Nees) Kuntze**

| NAA (mg/L) | IBA (mg/L) | IAA (mg/L) | Kin (mg/L) | BAP (mg/L) | 2,4-D (mg/L) | Percent (%) Response | Morphology and extent of callus |
|---|---|---|---|---|---|---|---|
| _ | 1 | _ | _ | 1 | _ | 13.5 | Creamy, compact, and spread on the margins. |
| _ | 3 | _ | _ | 3 | _ | 20.0 | Creamy, compact, and spread on the margins. |
| _ | 1 | _ | 1 | _ | _ | 15.3 | Creamy, compact, and spread on the margins. |
| _ | 3 | _ | 3 | _ | _ | 07.0 | Creamy, compact, and spread on the margins. |
| _ | _ | 1 | 1 | _ | _ | 30.0 | Creamy, compact, and spread on the margins. |
| _ | _ | 3 | 3 | _ | _ | 45.0 | Creamy, compact, and spread on the margins. |
| _ | _ | 1 | _ | 1 | _ | 75.0 | White, compact, and spread all over |
| _ | _ | 3 | _ | 3 | _ | 60.0 | White, compact, and spread all over |
| 1 | _ | _ | _ | 1 | _ | 87.0 | White, compact, and spread all over |
| 3 | _ | _ | _ | 3 | _ | 85.5 | Creamy, compact, and spread all over |
| 1 | _ | _ | _ | 1 | 0.2 | 25.0 | White, friable, and and spread all over |
| 3 | _ | _ | 0.2 | 0.2 | 0.2 | 55.0 | White, friable, and and spread all over |

**Table 3. Yield of indigo dye from ex vitro leaves, in vitro leaves and callus of Strobilanthes cusia (Nees) Kuntze**

| Sample | Water extract | | $Ca(OH)_2$ extract | |
|---|---|---|---|---|
| | Paste ( mg/g) fresh weight | Indigo dye (mg/g) mean ± S.E | Paste (mg/g) fresh weight | Indigo dye (mg/g) mean ± S.E |
| *Ex vitro* leaves | 645.30 | 2.07 ± 0.31 | 477.3 | 1.50 ± 0.19 |
| *In vitro* leaves | 611.30 | 1.30 ± 0.16 | 392 | 0.80 ± 0.04 |
| callus | 506.66 | 0.90 ± 0.10 | 390 | 0.70 ± 0.09 |

The effectiveness of the combination of NAA, BAP, Kin and 2,4-D in callus induction is similar to earlier reports of callus induction in other members of acanthaceae where combinations of IAA, BA, Kin, and 2, 4-D or NAA, BAP, Kin, and Zeatin were found to be effective for successful callus induction from leaves of *Adhatoda vasica* (Maurya *et al*., 2010) and *Justicia betonica* (Yaacob *et al*., 2013) respectively.

The leaves of *in vitro* regenerated plantlets and callus tissues showed the presence of indigo although the content was lesser compared to leaves collected from field samples (Table 3). The yield of indigo paste and indigo content of all the three explants were more in the water extract compared to the extract obtained using $Ca(OH)_2$ and this finding is in line with earlier studies (Laitonjam and Wangkheirakpam, 2011; Chanayath *et al*., 2002) where indigo extraction from leaves of plant samples with water only were shown to be more effective than with the use of $Ca(OH)_2$.

Thus, the present study presents the preliminary report on the establishment of an efficient *in vitro* propagation and callus induction method as well as the measurement of indigo accumulation in cultures of *S. cusia*. Further work is being carried out to optimize *in vitro* indigo production by cell suspension cultures of the plant.

**Acknowledgement**

The authors are thankful to the Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India for financial support (Grant No. SR/FT/LS-104/2010).

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