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# The Critical Period of Production of the Secondary Metabolite Indican in (*Indigofera tinctoria* L.) on Light Intensity

M.T.S. Budiastuti[1], D. Setyaningrum[2], D. Purnomo[1], Supriyono[1], B. Pujiasmanto[1], I.R. Manurung[1]

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## ABSTRACT

**Background:** *Indigofera tinctoria* plays the role of a natural dye source that produces indigo color and contains the secondary metabolite indican which is highly responsive to light intensity. This study aims to examine the critical period for the formation of the secondary metabolite indican on light intensity.

**Methods:** The method used was a completely randomized block design with a split-plot design. The study consisted of 2 treatment factors, namely: length of shade (main plot) and light intensity (subplot). The length of shade included 5 levels, namely early growth phase (up to 1 month after planting), mid-growth phase (up to 2 months after planting), maximum growth phase (up to 3 months after planting), 1 month before harvest and 2 months before harvest. Light intensity had 3 levels, namely 50% light intensity (38,464.3 lux), 25% (19,232.15 lux) and 10% (7,692.86 lux).

**Result:** The combination of duration of shade and light intensity affected the growth, yield and content of secondary metabolites (indican) in *Indigofera tinctoria*. The highest number of leaves, plant fresh weight and biomass was found in the combination of shade in the early growth phase (up to 1 month after planting) with 50% light intensity. The highest indican production was found in the mid-growth shading (up to 2 months after planting) with 10% light intensity, which was 843.33 ppm. The critical period of shade to increase indican production along with the number of leaves was the mid-growth phase (up to 2 months after planting).

**Key words:** Indican, Indoxyl-β-D glukosida, Light intensity, Natural dyes.

## INTRODUCTION

*Indigofera tinctoria* is a tropical plant that serves as a source of natural dye. This is because the leaves of *Indigofera tinctoria* contain the secondary metabolite indican (indoxyl β-D-glucoside) which is an indigo precursor (Wu, Komolpis and Wang, 1999; Nakai *et al.*, 2020). Indigo is the final product of the synthesis of indican which is responsible for the final blue color (Minami *et al.*, 1997). Indican is synthesized in the cytosol of leaf cells from indoxyl and UDP-glucose by catalysis of indoxyl-β-Dglucoside synthase (PtIGS), then transported into vacuoles and localized in chloroplasts. Indican synthesis occurs when *Indigofera tinctoria* leaf cells are subjected to biotic or abiotic stress (Inoue *et al.*, 2018). The quality of light in biotic stress such as excess light stress makes it essential for PHOT2 to avoid chloroplasts in order to reduce CRY1-light intensity. This is vital for the induction of transcription of excess light-responsive genes and anthocyanin biosynthesis acting through COP1 and HY5. During biotic stress, light intensity, quality and duration are of crucial importance for the activation of the full immune response in plant-pathogen interactions. Both chloroplasts and photoreceptors mediate light signals in plant-pathogen defense responses and phyA, phyB, CRY1 and PHOT2 contribute to systemic acquired resistance (Roeber *et al.*, 2021). Indigo growth and precursors are highly dependent on environmental conditions, one of which is sunlight (Stoker, Cooke and Hill, 1998; Maugard *et al.*, 2001). Indigo precursors in *Isatis tinctoria* L. and *Isatis indigotica* species are affected by light quality (Tozzi *et al.,* 2005).

[1]Department of Agrotechnology, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Postal Code 57126, Central Java, Indonesia.

[2]Doctoral Program of Agricultural Science, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Postal Code 57126, Central Java, Indonesia.

**Corresponding Author:** M.T.S. Budiastuti, Department of Agrotechnology, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Postal Code 57126, Central Java, Indonesia.
Email: mariatheresia@staff.uns.ac.id

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The indigo precursor in *I. tinctoria* is the indican content found mainly in the leaves at a level ranging from 0.2% to 0.76% (Angelini *et al.,* 2007). In contrast to primary metabolites, secondary metabolites are minor compounds in plants that occur in low concentrations because they are strongly influenced by environmental factors. Indican is a secondary metabolite that contains nitrogen and is produced through the shikimic acid pathway (Thoma *et al.*, 2020). Nitrogen-containing metabolites increase with reduced light (Coelho *et al.*, 2007). The biosynthesis and accumulation

of these secondary metabolites are mainly triggered by light. Photoreceptors are associated with signaling pathways and cause changes in gene expression when activated by photons. The combination of photoreceptor proteins and chromophore determines the nature of light absorption (Nocchi *et al*., 2020). Environmental factors such as light have been reported to play an important role in various physiological processes in plants (Wongshaya *et al*., 2020). The stimulatory and inhibitory effects of light on secondary metabolite production have been reported in many plants (Irshad *et al*., 2018; Prinsloo and Nogemane, 2018; Li *et al*., 2020). The content of shikomic acid in the shikimate acid pathway is highest at 50% light intensity. The activity of the enzymes 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase, phenylalanine ammonia lyase, cinnamate-4-hydroxylase and 4-coumarate: CoA ligase increases proportionally with light intensity (Wang *et al*., 2020). Light can be used as an abiotic elicitor in the production of the secondary metabolite indican because light affects the enzyme activity of the shikimate acid pathway.

Indigo content is affected by light and correlates with nitrogen in the leaf tissue of *Indigofera tinctoria*. Low light intensity results in high indigo content but also leads to stunted leaf growth (Setyaningrum *et al.,* 2020). Light and temperature can increase the production of indigo precursors (Campeol *et al*., 2006). Light greatly affects the biosynthesis and accumulation of various plant secondary metabolites that are important for plant quality (Siddiqui *et al.,* 2020). Secondary metabolites are mainly triggered through light. Photoreceptors are linked to signaling pathways and lead to gene expression changes when being activated by photons. The combination of a photoreceptor protein and a chromophore defines the light absorbing properties (Tilbrook *et al*., 2013; Folta and Carvalho, 2015). There are three main variables when considering light requirements in horticulture: light quality, light quantity and photoperiodism (Kozai *et al*., 2016). There is little existing research that investigates how light intensity affects *Indigofera tinctoria*'s protection and adaptation to environmental stress. This shows how plants respond to light stress. Therefore, it is necessary to examine the level of light intensity and duration of shade to optimize biomass and production of the secondary metabolite indican, in addition to discovering the critical period of sunlight for plants to produce secondary metabolites, which is essential to determine the level of plant stress to light.

## MATERIALS AND METHODS

The research was conducted at the ECODYE Natural Dye Production Center, Universitas Sebelas Maret in Batik Biru Bulu (Sub Village II, Puron, Bulu, Sukoharjo Regency, Central Java Province) from January-June 2021. The research location was situated at 1100 51′49,44″ east longitude and 70 48′54.3″ south latitude. 100% light intensity at the research site was 76928.6 lux. The study used a completely randomized block design arranged in a split-plot design with 2 treatment factors, namely: Length of shade as the main plot and light intensity as the subplot. The length of shade (as the main plot) consisted of 5 levels, namely the length of shade in the early growth phase (up to 1 month after planting), the length of shade in the mid-growth phase (up to 2 months after planting), the length of shade until the maximum growth phase (up to 3 months after planting), the length of shade in the phase 1 month before harvest and the length of shade in the phase 2 months before harvest. Light intensity (as the subplot) had 3 levels, namely 50% light intensity (38,464.3 lux), 25% (19,232.15 lux) and 10% (7,692.86 lux). This research used 3 replications. There were 12 plants/treatment, 4 plant samples/treatment, with a total sample of 180 plants and total number of trial units of 540 plants.

The tools used in the research were a lux meter, thermohydrometer, analytical balance, paranet as light intensity application and HPLC (high-performance liquid chromatography) three decimal analytical balance, weighing bottle, desiccator, digestion tube and digestion block, tube shaker, distillation apparatus, 250 ml boiling flask, 100 ml erlenmeyer, test tube, beaker, measuring flask and pipette. The material used in the research was *Indigofera tinctoria* seeds which were green in color. The indican content was analyzed using HPLC with a method based on Muzzazinah *et al.,* (2016) as follows: 0.5 g of leaves were put into a glass tube containing 2 ml of $H_2O/CH_3CN$ (75%/25%); then the tube was closed and heated at 90$^{o}$C for 2 minutes. The leaf material was then separated from the remaining mixture, cooled to 25$^{o}$C and centrifuged for 10 minutes at 6,000 rpm. The supernatant was put into a microtube and centrifuged for 10 minutes at a speed of 13,000 rpm. Then, 200 µL of the supernatant was transferred to an HPLC *vial* and 10 µL of the supernatant was injected into the HPLC-DAD for identification and quantitative analysis. An indication analysis was performed using Alliance HPLC 2695 (Waters) equipped with a 2996 photodiode detector (Waters). Material separation was carried out on a 5 µm, 150×4.6 mm Symmetry C18 column. The research data were analyzed using analysis of variance based on the F test with a test level of 5% (95% confidence level). If it had a significant effect, further analysis was carried out using duncan's multiple range test (DMRT). The variables observed were number of leaves, plant fresh weight, biomass and indican content, taken at 12 weeks after planting.

## RESULTS AND DISCUSSION

### Number of leaves

The combination of shade treatment in the early growth phase (up to 1 month after planting) with 50% light intensity (638.67 leaves) was not significantly different from 25% (502.00 leaves) and 10% light intensity (438.00 leaves) (Table 1). *Indigofera tinctoria* plant morphology and physiology are responsive to light intensity (Budiastuti *et al.,* 2021; Setyaningrum *et al.,* 2021). The number of leaves was reduced by 27% in the early growth phase shade treatment (up to 1 month after planting) with an intensity of

25% compared to the early growth phase shade treatment (up to 1 month after planting) with 50% light intensity. Long-term shading, depending on the intensity, resulted in a decrease in plant leaf growth. The reduced number of leaves was a form of plant adaptation that aims to reduce respiration in the plant body. Plants under shaded conditions receive far-red light so that the phytochromes in the leaves do not work properly and the stems become longer. Far-red light (FR) is not efficient for photosynthesis, so it requires the addition of light with a lower wavelength to be more efficient. Far-red light (FR) interferes with the ability of plants to produce leaves because the quality and quantity of light affect the morphological and physiological characteristics of plants (Azaman *et al.*, 2020).

The leaves are part of the *Indigofera tinctoria* plant that are used as a source of natural blue dye. This is because of the indican content found in the leaves. The results show that the number of leaves correlated negatively with the indican content (Table 5). This is because leaves are the main organ for plant photosynthesis and transpiration. Under normal light conditions, palisade tissue lengthens and increases the channel area of chloroplasts through $CO_2$ absorption, thereby increasing leaf thickness and strengthening photosynthetic ability (Shafiq *et al.,* 2021). Low light conditions substantially affect various agronomic traits of plants that have an impact on morphology (WU *et al.*, 2017), plant physiology and biochemistry (Li *et al.*, 2017) especially photosynthesis (Yang *et al.*, 2018). Light intensity is closely related to plant photosynthetic activity, carbon fixation, vegetative growth and dry matter accumulation, while secondary metabolites, including indicans, are formed from photosynthetic carbon (Raffo *et al.*, 2019). Production of secondary metabolites increases in the presence of environmental stress such as low light conditions. The shading period that increases indican production is the mid-growth phase (up to 2 months after planting) because the vegetative phase occurs during the mid-growth phase. The vegetative phase is crucial in plants,

**Table 1:** The effect of the combination of shade duration and light intensity on the number of leaves of *Indigofera tinctoria* at 12 weeks after planting.

| Shade length | Light intensity (%) | | | Average |
|---|---|---|---|---|
| | 50 | 25 | 10 | |
| Early growth phase (up to 1 month after planting) | 638.67 d | 502.00 cd | 438.00 abcd | 526.22 c |
| Mid-growth phase (up to 2 months after planting) | 395.67 abcd | 223.33 ab | 179.00 a | 266.00 a |
| Maximum growth phase (up to 3 months after planting) | 231.33 ab | 265.00 abc | 176.33 a | 224.22 a |
| Long shade in phase 1 month before harvest | 407.67 abcd | 331.00 abc | 465.67 bcd | 401.44 b |
| Long shade in phase 2 months before harvest | 413.00 abcd | 295.00 abc | 296.67 abc | 334.89 ab |
| Average | 417.27 b | 323.27 a | 311.13 a | |

Note: Numbers followed by the same letter in the same column and row are not significantly different based on DMRT (α=0.05).

**Table 2:** Effect of combination of shade length and light intensity on fresh weight of *Indigofera tinctoria* at 12 weeks after planting (g).

| Shade length | Light intensity (%) | | | Average |
|---|---|---|---|---|
| | 50 | 25 | 10 | |
| Early growth phase (up to 1 month after planting) | 462.33 c | 366.33 bc | 296.67 abc | 375.11 b |
| Mid-growth phase (up to 2 months after planting) | 293.00 abc | 149.67 a | 127.00 a | 189.89 a |
| Maximum growth phase (up to 3 months after planting) | 257.67 ab | 226.67 ab | 196.67 ab | 277.00 a |
| Long shade in phase 1 month before harvest | 373.00 bc | 190.33 ab | 210.00 ab | 257.78 a |
| Long shade in phase 2 months before harvest | 332.00 abc | 240.00 ab | 151.67 a | 241.22 a |
| Average | 343.60 b | 234.60 a | 196.40 a | |

Note: Numbers followed by the same letter in the same column and row are not significantly different based on DMRT (α=0.05).

**Table 3:** Effect of combination of shade duration and light intensity on plant biomass *Indigofera tinctoria* at 12 weeks after planting (g).

| Shade length | Light intensity (%) | | | Average |
|---|---|---|---|---|
| | 50 | 25 | 10 | |
| Early growth phase (up to 1 month after planting) | 144.40 c | 84.07 abc | 88.45 abc | 105.61 a |
| Mid-growth phase (up to 2 months after planting) | 120.73 bc | 51.03 ab | 50.13 ab | 73.97 a |
| Maximum growth phase (up to 3 months after planting) | 80.27 abc | 70.72 abc | 54.78 ab | 68.59 a |
| Long shade in phase 1 month before harvest | 101.67 abc | 40.90 a | 65.03 ab | 69.20 a |
| Long shade in phase 2 months before harvest | 95.52 abc | 87.80 abc | 41.95 a | 75.09 a |
| Average | 108.52 b | 66.90 a | 60.05 a | |

Note: Numbers followed by the same letter in the same column and row are not significantly different based on DMRT (α=0.05).

beginning with germination and continuing through tillering, where the growth tissues (meristems) are busy producing leaves. Sufficient leaf surface area is needed to capture sunlight and continue photosynthesis.

**Plant fresh weight**

The combinations of shade length and light intensity were found to have a significant effect on plant fresh weight (Table 2). Long shading with lower light intensity had the effect of lowering the fresh weight of higher plants. The combination of mid-growth shading (up to 2 months after planting) with 10% light intensity showed the lowest plant fresh weight. This is because the light intensity correlated positively with the photosynthesis rate and the amount of chlorophyll. The photosynthesis rate increases with increasing light intensity (Konvalinková *et al.*, 2015). The availability of carbohydrates that depend on photosynthesis and the allocation of photoassimilate are very much determined by the response of plants to light (Lugassi-Ben-Hamo *et al.*, 2010). Short shade treatment with higher light intensity has the ability to increase the fresh weight of the plants. This is supported by the total free amino acid content and the expression of several genes involved in metabolism increases with increasing light intensity. Changes in light conditions can affect plant growth and yield through photosynthesis adjustments.

**Biomass**

The combinations of treatment of duration of shade and light intensity had a significant effect on plant biomass (Table 3). The combination of shade in the initial phase of growth (up to 1 month after planting) with 50% light intensity showed the highest biomass of 144.40 g. Plant biomass correlated positively with plant fresh weight. Plant biomass decreased with longer shade and lower light intensity. This is because photosynthesis and stomata respond to changes in light intensity. Lower light intensity leads to slower photosynthetic development [in terms of increased photosynthetic maximum quantum efficiency (PSII) and non-photochemical cooling (NPQ)] compared to higher light intensity, thus resulting in lower biomass accumulation (Ghorbanzadeh *et al.*, 2021).

**Indican content**

The combinations of treatment of duration of shade and light intensity had a significant effect on indican content (Table 4). The highest indican content was found in the mid-growth shading treatment (up to 2 months after planting) with 10% light intensity, which was 843.33 ppm. Based on the research of (Muzzazinah, Chikmawati and Ariyanti, 2016), *Indigofera tinctoria* contains indican at an amount of 414 ppm. The indican content decreased by 34% in the mid-growth shading treatment (up to 2 months after planting) with 25% light intensity. These results indicate that indican content decreases with increasing light intensity. The results of this study are in line with (Budiasturi *et al.*, 2021) who explains that increasing light intensity can cause a decrease in indigo content in *Indigofera tinctoria*. Several studies have shown that indican is a secondary metabolite which is responsive to light (Stoker, *et al.* 1998; Angelini *et al.* 2004b; Angelini *et al.*, 2007; Nakai *et al.*, 2020). Low light intensity is a form of abiotic stress that can damage leaf cells, so that the indican accumulated in the vacuole is released by the indican degradation enzyme, b glucosidase which is localized in the chloroplast (Inoue *et al.*, 2017; Inoue, Morita and Minami, 2021). Indican is stored in leaves but not in other tissues, such as stems and roots. Furthermore, PtIGS protein and mRNA are also found only in leaves. In addition, PtFMO protein and mRNA occur mostly in leaves. In the indican biosynthetic pathway, PtFMO can function in the oxidation of indole to indoxyl and the supply of indoxyl to PtIGS, resulting

**Table 4:** The effect of the combination of shade duration and light intensity on the indicant content *Indigofera tinctoria* 12 weeks after planting (ppm).

| Shade length | Light intensity (%) | | | Average |
|---|---|---|---|---|
| | 50 | 25 | 10 | |
| Early growth phase (up to 1 month after planting) | 44.00 a | 31.00 a | 182.33 bcd | 85.78 a |
| Mid-growth phase (up to 2 months after planting) | 461.67 fg | 508.07 g | 843.33 h | 604.56 c |
| Maximum growth phase (up to 3 months after planting) | 348.33 ef | 241.00 cd | 255.00 de | 280.78 b |
| Long shade in phase 1 month before harvest | 400.00 fg | 104.00 abc | 219.00 cd | 241.00 b |
| Long shade in phase 2 months before harvest | 56.00 a | 85.33 ab | 97.00 ab | 79.44 a |
| Average | 261.60 b | 194.00 a | 319.33 c | |

Note: Numbers followed by the same letter in the same column and row are not significantly different based on DMRT (α= 0.05).

**Table 5:** Correlation between number of leaves, fresh weight of plants, biomass and content of indicant.

| | Number of leaves | Fresh weight of plants | Biomass | Content of indicant |
|---|---|---|---|---|
| Number of leaves | 1 | 0.594** | 0.531** | -0.336* |
| Fresh weight of plants | 0.594** | 1 | 0.846** | -0.285 |
| Biomass | 0.531** | 0.846** | 1 | -0.101 |
| Content of indicant | -0.336* | -0.285 | -0.101 | 1 |

Note: **Correlation is significant at the 0.01 level (2-tailed).

in the production of indican (Inoue *et al*., 2020). Based on Table 4, the shading period to increase indican production is the mid-growth phase (up to 2 months after planting) and subsequently receiving full light for 1 month before harvesting.

## CONCLUSION

The number of leaves, plant fresh weight and biomass was found to be highest in the treatment combination of shade in the early phase of growth (up to 1 month after planting) with 50% light intensity. The highest indican production was found in the mid-growth shading treatment (up to 2 months after planting) with 10% light intensity, which was 843.33 ppm. The indican content correlated negatively with the number of leaves. The critical period of shade to increase indican production along with the number of leaves is the mid-growth phase (up to 2 months after planting). Increasing the number of secondary metabolites in *Indigofera tinctoria* will increase protection and adaptation to environmental stress.

**Conflict of interest:** None.

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