



## Effects of light intensity and co-inoculation of arbuscular mycorrhizal fungi and rhizobium on root growth and nodulation of *Indigofera tinctoria*

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### ABSTRACT

*Indigofera tinctoria* is a legume that is cultivated as a source of natural indigo dyes. As a legume, *Indigofera tinctoria* is capable of symbiosis with soil microbes. This study evaluates the effects of light intensity and microbial inoculation on root growth and nodulation. The study used a complete randomized block design with a split-plot pattern. Light intensity was the main plot with four levels of light intensity 100%, 50%, 25%, and 10%. Microbial inoculation was a subplot with four levels without inoculation, mycorrhizae inoculation, rhizobium inoculation, and double inoculation with both mycorrhizae and rhizobium. The results obtained show that light intensity and microbial inoculation affected root length, root fresh weight, root biomass, and the number of nodules. 50% light intensity was optimum for root length, while 100% light intensity was optimum for root fresh weight, root biomass, and a number of nodules. Root growth and nodulation were further increased with double inoculation. The combination of light intensity and microbial inoculation affected root biomass and nodulation. The combination of 100% light intensity and double inoculation resulted in the highest root biomass and nodule numbers. Mycorrhizae and rhizobium have a synergistic relationship to nodulation and root growth. Double inoculation with mycorrhizae and rhizobium efficiently increased root biomass and the number of nodules under low or high light intensity.

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## 1. Introduction

Synthetic dyes used in the batik industry in Indonesia contribute to environmental pollution by producing waste which causes groundwater pollution (Kristijanto et al., 2011; Handayani et al., 2019). The environmental impact caused by synthetic dye waste must be reduced; one option is the use of natural dyes. *Indigofera tinctoria* is a family of legume that contains an indigo pigment that produces a natural blue color suitable to replace synthetic dyes in the batik industry (Hariri, Chikmawati, & Hartana, 2017).

Indigo pigment production is very responsive to light (Angelini, Tozzi, & Nassi O Di Nasso, 2004). This is because the indigo precursor is a metabolite compound that contains nitrogen and is produced through the shikimic acid pathway. In the shikimic acid pathway, precursors derived from glycolysis and pentose phosphate are converted to aromatic amino acids (Taiz & Zeiger, 2006). Nitrogen containing metabolites alkaloids, glucosinolates, and cyanogen

glycosides are increased with reduced light intensity (Coelho et al., 2007), while growth and plant biomass are optimum at high light intensities (Wu et al., 2017). Plant growth and yield are largely determined by light intensity and nutrient availability; the supply of nutrients needed to optimize the growth and yield of *Indigofera tinctoria* varies based on light intensity.

Nutrient requirements can be met through the incorporation of mycorrhizae and rhizobium. *Indigofera tinctoria* is a legume that can form a symbiotic tripartite association with rhizobium which induces the production of nodules and mycorrhizae which plays a key role in phosphate and nitrogen efficiency (Xavier & Germida, 2003). The roots of *Indigofera tinctoria* plants are symbiotic with rhizobium in the nitrogen cycle, while mycorrhizae infect the root system of the host plant producing external hyphal tissue that grows rapidly and penetrates the sub soil layer, thereby increasing

root capacity for nutrient and water absorption (Smith, Grace, & Smith, 2009). Mycorrhizae and Rhizobium can increase plant growth by regulating the balance of nutrients and hormones; as growth regulators, they dissolve nutrients and induce resistance to plant pathogens. In addition, these microbes also show synergistic interactions with other microbes in the soil environment (Nadeem, Ahmad, Zahir, Javaid, & Ashraf, 2014).

Double inoculation with mycorrhizae and rhizobium significantly increases root growth and yields of *Glycine max*, *Phaseolus mungo*, *Vigna radiate*, *Cicer arietinum*, *Lens culinaris*, and *Pisum sativum* under low or high light intensity (Shukla et al., 2018). Light intensity significantly affects rhizobium and arbuscular mycorrhizal fungal (AMF) activities (Kumar et al., 2007; Houx et al., 2009; Shukla et al., 2009; Sarr & Yamakawa, 2015). The study of Sindhu et al. (2016) found that the combination of organic fertilizer and mycorrhizae can increase the growth of *Indigofera tinctoria*, indican content, and nitrogen and potassium content in the soil. Few studies have been conducted on the combination of light intensity treatment with microbial inoculation on the root growth and nodulation of *Indigofera tinctoria*. The novelty of this study is that it combines both light intensity and microbial inoculation in *Indigofera tinctoria* in tropical climates to assess the effects of mycorrhizal and rhizobium inoculation under several light intensities on root growth and nodulation.

## 2. Materials and Methods

The study was conducted from April to November 2019, in Puron Village, Bulu District, Sukoharjo, Central Java, Indonesia. The research location is at 110°51'49.44"E and 7°48'54.3"S. Based on the results of soil analysis, the soil had a neutral pH of 7.31, 0.36% nitrogen (N), 15.72 ppm phosphorous (P), and 0.42 me 100 g of soil potassium (K). C-organic was low at 1.52%, and organic matter was low at 2.62%. Materials used in this study included green seeds of *I. tinctoria*, mycorrhizae, and rhizobium obtained from the Laboratory of Microbiology, Faculty of Agriculture, Gadjah Mada University. The instruments were paranets with various densities to produce different light intensity and a lux meter used to measure light intensity.

This study used a complete randomized block design arranged in a split-plot pattern. The levels of light intensity for the main plot consisted of A1 = 100% light intensity (6232.8 cd m<sup>-2</sup>), A2 = 50% light intensity (3013.05 cd m<sup>-2</sup>), A3 = 25% light intensity (1605.9 cd m<sup>-2</sup>), and A4 = 10% light intensity (623.99 cd m<sup>-2</sup>). Microbial inoculations as subplots consisted of: B1 = without inoculants, B2 = rhizobium 1 g plant<sup>-1</sup>, B3 = mycorrhizal 10 g plant<sup>-1</sup>, B4 = mycorrhizal 10 g plant<sup>-1</sup> and rhizobium 1 g plant<sup>-1</sup>. Each unit of the experiment was repeated three times so that there were 48 total experimental units, and in a single unit, there were six plants. *Rhizobium* sp. was applied while the plants were in the nursery; mycorrhizal inoculation was applied during transplant into the study field.

Observation variables included the number of nodules, root length, root fresh weight, and root biomass measured at the maximum vegetative phase 8 weeks after planting. Research data were analyzed using analysis of variance with

α 5% test level 95% confidence level. If it had a significant effect, further analysis was done using Duncan's Multiple Range Test (DMRT).

## 3. Results

Light intensity affects root length, root fresh weight, root biomass, and the number of nodules (Table 1). The result of the light intensity of 50% on root length was not significantly different from light intensities of 100% and 25%. The light intensity of 10% showed the shortest root fresh weight, root biomass, and the number of nodules. The light intensity of 100% showed the highest root fresh weight of 12.15 g and root biomass of 6.13 g. On the number of nodules, a light intensity of 100% was not significantly different from a light intensity of 25%.

**Table 1.** Effect of light intensity on root and nodule growth

Light Intensity (%)	Root length (cm)	Root fresh weight (g)	Root Biomass (g)	Number of nodules
100	20.92 ab	12.15 c	6.13 c	24.75 c
50	23.17 b	7.93 b	3.54 b	21.25 b
25	22.33 b	9.46 b	4.50 b	23.59 bc
10	18.74 a	4.78 a	2.37 a	17.58 a

Note: Numbers followed by the same letters in the same column showed no significant differences based on the DMRT level of 5%.

**Table 2.** Effect of microbial inoculation on root fresh weight, biomass, length, and nodulation

Microbial Inoculation	Root length (cm)	Root fresh weight (g)	Root Biomass (g)	Number of nodules
Without inoculation	13.83 a	4.06 a	1.48 a	13.17 a
Mycorrhizae	25.58 c	6.64 b	3.06 b	18.92 b
Rhizobium	19.17 b	9.71 bc	4.89 c	22.17 c
Mycorrhizae + rhizobium	26.58 c	13.89 c	7.11 d	32.83 d

Note: Numbers followed by the same letters in the same column showed no significant differences based on the DMRT level of 5%.

**Table 3.** Combination of light intensity and microbial inoculation on root biomass (g)

Light Intensity (%)	Microbial Inoculation			
	WI	M	R	M+R
100	1.59 ab	4.19 abcd	7.67 e	11.09 f
50	1.18 ab	2.22 abc	5.03 cde	5.74 de
25	2.32 abc	3.82 abcd	4.35 bcd	7.51 e
10	0.80 a	2.04 abc	2.52 abcd	4.11 abcd

Note: WI: Without inoculation, M: Mycorrhizae, R: Rhizobium, M+R: Mycorrhizae + rhizobium. Numbers followed by the same letters showed no significant differences based on the DMRT level of 5%.

**Table 4.** Effect of combining light intensity and microbial inoculation on the number of nodules

Light Intensity (%)	Microbial Inoculation			
	WI	M	R	M+R
100	12.67 a	19.00 abcd	23.00 cde	39.33 f
50	14.33 ab	18.00 abcd	23.67 de	39.30 f
25	13.67 a	24.00 de	22.33 bcde	29.00 e
10	12.00 a	14.67 abc	19.67 abcd	24.00 de

Note: WI: Without inoculation, M: Mycorrhizae, R: Rhizobium, M+R: Mycorrhizae + rhizobium. Numbers followed by the same letters showed no significant differences based on the DMRT level of 5%.

**Table 5.** Correlation between root length, root fresh weight, root biomass, and a number of nodules

	Root length	Root fresh weight	Root biomass	Number of nodules
Root length	1	.403**	.432**	.544**
Root fresh weight		1	.947**	.771**
Root biomass			1	.800**
Number of nodules				1

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

Based on an analysis of the variances, microbe inoculation significantly affects root length, root fresh weight, root biomass, and the number of nodules (Table 2). Double inoculation with both mycorrhizae and rhizobium increased root length 92% compared to without inoculation. Root length with double inoculation was not significantly different from mycorrhizae inoculation alone. Root fresh weight on double inoculation was not significantly different than rhizobium inoculation alone. Without inoculation showed the lowest root fresh weight and the results were significantly different from all treatments. The highest increases in root biomass and a number of nodules were seen with double inoculation.

The combination of light intensity and microbial inoculation significantly affected root biomass (Table 3). The highest root biomass, 11.09 g, was found in the combination of 100% light intensity with double inoculation. The combination of light intensity and microbial inoculation significantly affected the number of nodules (Table 4). Double inoculation increased the number of nodules compared to without inoculation at several levels of light intensity. The number of nodules with a combination of double inoculation and 100% light intensity was not significantly different from the combination of double inoculation and 50% light intensity. Table 5 shows that root length positively correlated to root fresh weight, root biomass, and the number of nodules; increased root length predicts an increase in root fresh weight, root biomass, and the number of nodules.

#### 4. Discussion

The combination of light intensity and microbial inoculation significantly affected root biomass (Table 3). Double inoculation increased root biomass compared to

without inoculation at several levels of light intensity. Double inoculation with mycorrhizae and rhizobium efficiently increased yields under shade or not under shade (Shukla et al., 2018). In addition, double inoculation with mycorrhizae and rhizobium increased root length by 92% compared to without inoculation (Table 2). These results are presumably due to the formation of a mutualistic symbiotic relationship between the roots of *Indigofera tinctoria* with mycorrhizae and rhizobium microorganisms in the soil. The synergistic effect of mycorrhizal fungi and rhizobium on legume roots increased growth, nutrient uptake, and nitrogen fixation (Xavier & Germida, 2003). The association with AMF and rhizobia directly improved nutritional status and growth (Li et al., 2012). Rhizobium and AMF were found to be interdependent and engaged in mutual promotion (Haro, Sanon, Le Roux, Duponnois, & Traoré, 2018). AMF inoculation promoted root nodule formation and rhizobium inoculation increased the percentage of AMF infections (Xu et al., 2016). Besides, the double inoculation with mycorrhizae and rhizobium helped plants maintain beneficial liquid water paths (LWP) for the access of root water and accumulation of sugar levels in the roots (Chaudhary, 2019). Thus, the highest root fresh weight and root biomass were also found in double inoculation (Table 2). Root fresh weight and root biomass with double inoculation increased by 242% and 380%, respectively, compared to without inoculation. This is because double inoculation increased nutrient absorption, thereby encouraging optimum plant growth and biomass increases (Hemmat Jou and Besalatpour, 2018).

Mycorrhizae inoculation coupled with 10% light intensity decreased root biomass 105% when compared to 100% light intensity (Table 3). This result is expected as AMF colonization is reduced under low light intensity (Lau et al., 2012). The light intensity of 10% showed the shortest root biomass (Table 1) because low light intensity reduces the accumulation of net photosynthetic products and compounds needed for normal growth (Sauvadet et al., 2019). Decreased biomass of roots, stems, and leaves in low light correlates to a reduced rate of photosynthesis, transpiration, and conductance of stomata water vapor under low light (Mielke and Schaffer, 2010). Consistent with the correlations, (Table 5), root biomass decreased as a result of shorter root length. This result shows that root lengths are very short on 10% light intensity. This result was expected as the very low light inhibits the allocation of carbon to root growth (Gommers, Visser, Onge, Voesenek, & Pierik, 2013). Lower light intensity caused a significant decrease in the root fresh weight and biomass (Table 3). These results are consistent with Yang et al. (2008), which found that that root fresh weight and root biomass decreased at low light intensities and are the lowest at a light intensity of 16.7%. This is because growth depends on carbon, and carbon is greatly influenced by light conditions; the addition of carbon in roots comes from the stem through the phloem, while the main loss of root carbon occurs through respiration associated with ion growth and absorption (Li et al., 2014). As a result, the root shoot ratio was higher in plants with high levels of light treatment compared to plants with low levels of light treatment.

*Indigofera tinctoria* is a member of the Leguminosae family. Plants of the Leguminosae family form a symbiosis with some rhizobial bacteria and develop nodules in the roots. The combination of light intensity and microbial inoculation significantly affects the number of nodules (Table 4). Double inoculation increased the number of nodules compared to without inoculation at several levels of light intensity. This is in accord with Shukla et al. (2018) who found that double inoculation with mycorrhizae and rhizobium increases nodulation whether under shade or not under shade. Double inoculation increased the number of nodules by 149% compared to without inoculation (Tabel 2). AMF inoculation promoted root nodule formation while rhizobium inoculation increased the percentage of AMF infections (Gage, 2004). These results were expected; there is a known symbiotic relationship between mycorrhizae and rhizobium in *Phaseolus vulgaris* (Mortimer et al., 2012) and *Vicia faba* (Abd-Alla, El-Enany, Nafady, Khalaf, & Morsy, 2014); plants with double inoculation show higher nodulation, growth, and yield when compared to those inoculated with only mycorrhizae or only rhizobium. Nodulation requires P (Mortimer et al., 2008), and the development of AMF structures requires substantial N (Johnson, Wilson, Wilson, Miller, & Bowker, 2015). Rhizobium and mycorrhizae, when combined, facilitate the absorption of both N and P in legumes (Mortimer et al., 2008).

Single inoculation with mycorrhizae increased the number of nodules by 43% compared to without inoculation (Table 2). Due to the introduction of AMF, more nodules are effective in attracting P and N and subsequent water absorption in legume plants (Andrade, Abreu, De Abreu, & Silveira, 2004). Mycorrhizae can increase rhizosphere soil phosphatase activity which further increases phosphate. Phosphate is an essential ingredient of plants for nodulation and growth (Sánchez-Díaz, Pardo, Antolín, Peña, & Aguirreolea, 1990). In addition, rhizobium inoculation increased the number of nodules by 68% compared to without inoculation (Table 2). These results are in line with Denton et al. (2017) where single inoculation or double inoculation with mycorrhizae and rhizobium significantly increased root growth and nodulation. This is presumably because rhizobium is able to increase nodulation and enzyme activity (Tilak, Ranganayaki, & Manoharachari, 2006). Rhizobium symbiosis with legumes affects the hydraulic characteristics of plant roots; rhizobium symbiosis causes a decreased osmotic potential of xylem sap so that the root osmotic water flow increases, then rhizobium inoculation increases plant growth (Franzini, Azcón, Ruiz-Lozano, & Aroca, 2019).

The combination of 10% light intensity with double inoculation and single inoculation yielded a low number of nodules. This is due to the fact that light intensity has a significant effect on rhizobium and AMF (Kumar et al., 2007; Houx III et al., 2009). Light is an important signal that controls the growth, development, and behavior of many organisms. All multicellular organisms have various sensor systems to detect light. Bacteria have both photosynthesis tools that convert light into chemical energy and photoreceptor proteins. One of the bacteria that play a role in N fixation is rhizobium. Rhizobium bacterium in the soil infects the root

hairs of legumes and induces nodule formation and nitrogen-fixing. Light influences the reproduction of these bacteria; brighter light can increase the number of nodules per plant (Bonomi et al., 2012) and improve nitrogen nodulation and fixation in legumes (Rinnan et al., 2005). In this study, light intensity affected the number of nodules (Table 1); the lowest number of nodules was at a light intensity of 10%. Reductions in the number of nodules and dry weight of nodules with low light intensity have also been reported (Sarr & Yamakawa, 2015).

## 5. Conclusion

The light intensity and microbial inoculation individually and together affect root biomass and nodulation in *Indigofera tinctoria*. Double inoculation with mycorrhizae and rhizobium efficiently increases root biomass and the number of nodules independent of light intensity. With double inoculation, mycorrhizae and rhizobium have a synergistic relationship that produces even greater nodulation and root growth.

## Declaration of Competing Interest

The authors declare no competing financial or personal interests that may appear and influence the work reported in this paper.

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