Hot Water and Sulfuric Acid Solution for Increasing The Viability and Vigor of Indigofera (Indigofera zollingeriana)

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Abstract. Indigofera (Indigofera zollingeriana) is a multi-benefit plant, two of them are for natural dyes and livestock feed supplement. Indigofera seeds are hard and thick-coated so that the germination percentage is low and not grow in simultaneously. This study aimed to obtain an effective and practical method of increasing seed viability and vigor was carried out from March to May 2019 at the BBTP Central Java. The research used a completely randomized design, consisted 9 treatments (control, soaked in hot water by 80 and 100°C then leave it for 24 hours, and soaked in a solution of H2SO4 95% for 10, 20, 30, 40, 50 and 60 minutes) and 4 replications. The germination method used the top of paper with filter paper media. The results showed that soaking the seeds in hot water and sulfuric acid solutions significantly increased germination, maximum growth potential, vigor index, and growth rate. Soaking seeds in a 95% H2SO4 solution for 10 minutes increased the germination of the seeds (84%), maximum growth potential (91%), vigor index (69%), and growth rate by (13.81% etnal1). The finding concludes that the best treatment was soaking seed in H2SO4 solution for 10 minutes, and longer soaking time (> 50 minutes) will decrease all parameters.

1. Introduction
Indigofera (Indigofera zollingeriana) is the third-largest genus in Leguminosae, this genus consists of 750 species as much as 75% spread in Africa and Madagascar [1]. Indigofera originating from tropical Africa, Asia, Australia, North, and South America then distributed throughout the dry zone of Africa, Asia and began to enter Indonesia around the 1900s brought by invaders from Europe. In Asia, the distribution of indigoferaincludes Myanmar, China, Taiwan, Vietnam, and Indonesia [2].

Indigofera is a multi-benefit plant including natural dyes and livestock feed supplement. Indigofera species that used as natural dyes is I. tinctoria. The tannin content in indigofera is used for roasting milk fish (Chanos chanos). Milk fish is marinated with 25% indigofera solution before roasting to produce the best quality of milk fish [3]. I. zollingeriana is very suitable to be used as livestock feed related to the dry matter content of leaves and stems, 29.9 and 25%, and crude protein 23.1 and 17.6% respectively. Whereas neutral detergent fiber (NDF) and acid detergent fiber (ADF) indigofera leaves were 35.9 and 25.1% respectively [4].

Based on indigofera farmers information, the main challenges in nursery is low seed germination and not growing in uniform. Fresh seed of indigofera do not germinate immediately due to dormancy. Dormancy in the indigofera was believed to be due to hard seed coat [5]. Breaking the thick and hard-coated seed dormancy could be physical, chemical, and mechanical. Soaking Malva sylvestris seed...
(freshly harvested) in sulfuric acid 95% for 240 and 270 minutes were able to increase germination from 0% (control) to 73.3%, while six month later the highest germination was achieved with 120 minutes (93.3%) [6]. In Convolvulus arvensis seed, soaking with sulfuric acid 80% for 15 to 60 minutes or sulfuric acid 98% for 15 to 30 minutes had germination rate above 80% and soaking seeds in hot water (70°C) for 4 to 16 minutes or in boiling water for 5 to 20 second were effective in breaking seed dormancy [7]. Pre treatment Sunflower seed with hot water (70°C) for 15 minutes followed by one day dry effectively increased the percentage of germination by 39.4% than control (6.4%) [8]. Soaking Gleditsia triacanthos seed in boiling water treatment for 15 minutes have the highest germination percentage (79.0%) followed by acid treatment using sulfuric acid for 45 minutes (75.4%) compare than control (39.9%) [9]. The maximum germination percentages of the Gaborone seed were recorded in hot water treatment (85%), boiling water in 1 minute (87%) and sulfuric acid for 45 minutes (87%)[10].

Information about breaking dormancy in legumes is well documented, but for species Indigofera zollingeriana has not been done much. The purpose of this study was to obtain technology to increase seed viability and vigor of indigofera seed.

2. Materials and Method
The research conducted at seed laboratory of Balai Benih Tanaman Perkebunan in Salatiga, Central Java Province (7° 20'56" S 110° 29'20" E). The research carried out for 3 months, March 1st to May 31st 2019. Indigofera seeds obtained from the Experimental Garden of the Jonggol Animal Husbandry Education and Research Unit of IPB in August 2018. The seed was brownish-green with a shiny skin surface and irregular round shape.

Seeds are germinated by the top of paper method using filter paper and sterile petri dish. The seeds placed in a petri dish with moistened filter paper pads (3 sheets). Then the closed petri dish is put into the eco germinator in the position according to the experimental design. If the filter paper looks dry during observation, moisture returned by adding distilled water. Seeds that have germinated normally immediately taken from the petri dish, as well as moldy seeds which are removed immediately so that no contamination occurs. Moldy growing media immediately replace with new filter paper. One treatment used 100 seeds which divided into 4 replications. Observations were made every day until 15 DAP (days after planting). The normal germination criterion used was the radicle length of 1.0 cm.

The experimental design used was completely randomized design (CRD) with a single factor. Soaking treatment factors consist of 9 levels, namely: control (without treatment), the seeds soaked in hot water (2 treatments) and seed soaked in H2SO4 solution 95% (6 treatments).

Experiment 1: hot water treatment, seeds were soaked in an initial temperature of 80 and 100°C left to cool for 24 hours. Experiment 2: sulfuric acid treatment, the seeds soaked in H2SO4 solution 95% for 10, 20, 30, 40, 50 and 60 minutes. The seeds were soaked in hot water then drained and dried. While the seeds soaked in H2SO4 solution rinsed with running water for 5 minutes then drained and ready to be planted in a petri dish.

Data obtained from the results of the study were analyzed using analysis of variance (F test) using SPSS. If there any significant effect on the level of α = 0.05, the statistical test continued with further tests using Duncan's Multiple Range Test at a 95% confidence level.

3. Results and Discussion
3.1 Germination percentage (GP)
Seed germination varies between treatments from 3-5 DAP, the fastest germination (3 DAP) seed is soaked in hot water (80 and 100°C), and the latest in control at 5 DAP. At 3 days after planting, the soaked seeds in hot water at 100°C gave the highest percentage of seed germination at 8%, followed by soaking of seeds in hot water at 80°C at 4%. At the beginning of germination, control and soaking in the H2SO4 solution give a low yield of 0-1%. Starting from 5-15 DAP, soaking seeds in H2SO4 solution has higher seed germination compared to control and soaking seeds in hot water (Table 1).
At 15 DAP, the highest percentage of seed germination achieved by soaking the seeds in 95\% H\textsubscript{2}SO\textsubscript{4} solution for 10 minutes by 84\%, not significantly different with the soaking of the seeds in 95\% H\textsubscript{2}SO\textsubscript{4} solution for 20-50 minutes (72-74\%), but different significantly with soaking in hot water (80\^{\circ}C}=62\%; 100\^{\circ}C}=59\%) and also control (36\%). Soaking of hot water up to 100\^{\circ}C increases germination percentage as well as soaking of seeds in 95\% H\textsubscript{2}SO\textsubscript{4} solution up to a time limit of 50 minutes. The longer soaking time for seeds in H\textsubscript{2}SO\textsubscript{4} solution (> 50 minutes) decreases the percentage of seed germination.

**Table 1.** Effect of indigofera seed soaking in hot water and sulfuric acid solution on the germination of seeds

| Treatment                  | GP3 DAP (%) | GP5 DAP (%) | GP7 DAP (%) | GP9 DAP (%) | GP11 DAP (%) | GP13 DAP (%) | GP15 DAP (%) |
|----------------------------|-------------|-------------|-------------|-------------|--------------|--------------|--------------|
| Control                    | 0\textsuperscript{a} | 9\textsuperscript{a} | 17\textsuperscript{a} | 24\textsuperscript{a} | 28\textsuperscript{a} | 32\textsuperscript{a} | 36\textsuperscript{a} |
| Hot water 80\^{\circ}C     | 4\textsuperscript{b} | 22\textsuperscript{b} | 37\textsuperscript{b} | 46\textsuperscript{b} | 53\textsuperscript{b} | 55\textsuperscript{b} | 62\textsuperscript{b} |
| Hot water 100\^{\circ}C    | 8\textsuperscript{c} | 25\textsuperscript{c} | 45\textsuperscript{c} | 49\textsuperscript{c} | 55\textsuperscript{c} | 57\textsuperscript{c} | 59\textsuperscript{c} |
| H\textsubscript{2}SO\textsubscript{4} 10 minutes | 0\textsuperscript{d} | 33\textsuperscript{d} | 69\textsuperscript{d} | 77\textsuperscript{d} | 82\textsuperscript{d} | 84\textsuperscript{d} | 84\textsuperscript{d} |
| H\textsubscript{2}SO\textsubscript{4} 20 minutes | 0\textsuperscript{e} | 32\textsuperscript{e} | 61\textsuperscript{e} | 66\textsuperscript{e} | 70\textsuperscript{e} | 73\textsuperscript{e} | 73\textsuperscript{e} |
| H\textsubscript{2}SO\textsubscript{4} 30 minutes | 1\textsuperscript{f} | 39\textsuperscript{f} | 63\textsuperscript{f} | 70\textsuperscript{f} | 73\textsuperscript{f} | 73\textsuperscript{f} | 74\textsuperscript{f} |
| H\textsubscript{2}SO\textsubscript{4} 40 minutes | 0\textsuperscript{g} | 20\textsuperscript{g} | 47\textsuperscript{g} | 66\textsuperscript{g} | 72\textsuperscript{g} | 72\textsuperscript{g} | 72\textsuperscript{g} |
| H\textsubscript{2}SO\textsubscript{4} 50 minutes | 0\textsuperscript{h} | 25\textsuperscript{h} | 49\textsuperscript{h} | 66\textsuperscript{h} | 71\textsuperscript{h} | 72\textsuperscript{h} | 72\textsuperscript{h} |
| H\textsubscript{2}SO\textsubscript{4} 60 minutes | 1\textsuperscript{i} | 22\textsuperscript{a} | 42\textsuperscript{a} | 52\textsuperscript{a} | 61\textsuperscript{a} | 63\textsuperscript{a} | 63\textsuperscript{a} |

Note: The means followed by the same letter in the same column is not significantly different according to Duncan's multiple tests of 5\%. GP: germination percentage, DAP: days after planting

At the beginning of growth (3 DAP), soaking the seeds in hot water at 80 and 100\^{\circ}C gives higher seed germination and significantly different from the control and soaking in the H\textsubscript{2}SO\textsubscript{4} solution. This explained because in the soaking temperature range the seed coat is cracked, so the imbibition process takes place faster. The following research [12] on the seeds of *Acacia kempeana* that soaking the seeds in hot water can cause the cuticles to lift and swell. The following process will uncover the seed strophiole so that water infiltrates the seed (imbibition), then the seeds germinate.

At the beginning of germination, soaking the seeds in hot water at temperature of 80 and 100\^{\circ}C the seed germination percentage is higher than the control and soaking in H\textsubscript{2}SO\textsubscript{4}. Based on SEM (Scanning Electron Microscope) observations, it can be seen that the control (Figure 1a) of the smooth seed surface had no skin cracks. On soaking the seeds in hot water (80 and 100\^{\circ}C left to cool for 24 hours) could break the seed coat so that the endosperm appears, but it does not occur in all seeds (Figure 1b). While soaking the seeds in a solution of H\textsubscript{2}SO\textsubscript{4} can erode the seed coat almost all over the seed surface, even though the endosperm is not visible (Figure 1c). This results in early growth, seeds soaked in hot water(80and 100\^{\circ}C) germinate earlier than control and soaking the seeds in 95\% H\textsubscript{2}SO\textsubscript{4} solution for 10-60 minutes. Seeds soaked in H\textsubscript{2}SO\textsubscript{4} solution begin to germinate maximally after 4 DAP until the end of the observation period.

This result is supported by [13] that the pre treatment seed with a sulfuric acid for 5 minutes gives the maximum germination of the *Innula racemosa* (97.2\%) followed by *Rheum webbianum* (95.1\%) *Carum carvi* (93.4\%) and *Saussurea lappa* (90.0\%) and *Bunium persicum* (81.4\%). Soaking the seeds in a solution of sulfuric acid for a longer time will disrupt seed germination.

Application of seed soaking in sulfuric acid concentration for 30 minutes induces germination of *Leucaena leucocephala*, *Erythrostemon giliesii*, *Styphnolobium japonicum*, and *Acacia dealbata* with respective final germination of 100\%, 95\%, 100\% and 82.5\%. A prolonged duration of 90 minutes of pre treatment was very fatal for *L. leucocephala*, *A. dealbata*, and *Brachychiton populneus*. Sulfuric
acid treatment of seed removes waxy layer of the seed coat by chemical decomposition of seed coat component which is similar to breakdown process that occurs during microbial attack [14].

Figure 1. Micrograph SEM seed surface. *Zollingeriana* (a) control, (b) soaking the seeds in hot water 100°C, and (c) soaking the seeds in 95% H$_2$SO$_4$ solution for 10 minutes (50x magnification).

3.2 Maximum Growth Potential (MGP)

The maximum growth potential is the sum of normal and abnormal germination during the test period (15 DAP). Based on the results of various treatments significantly affected the maximum growth potential. The highest MGP (91%) obtained at the soaking in H$_2$SO$_4$ solution for 10 minutes was not significantly different from the soaking of the seeds in the H$_2$SO$_4$ solution for 20-50 minutes (83-87%). Soaking the seeds in a longer H$_2$SO$_4$ solution (60 minutes) decreases MGP by up to 79%. While soaking the seeds in hot water (80°C and 100°C) and allowed to cool for 24 hours increases MGP compared to controls, but the value is below soaking in H$_2$SO$_4$ solution.

| Treatment                  | Maximum Growth Potential (%) |
|---------------------------|------------------------------|
| Control                   | 48$^a$                       |
| Hot water 80°C            | 67$^b$                       |
| Hot water 100°C           | 63$^b$                       |
| H$_2$SO$_4$ 10 minutes    | 91$^d$                       |
| H$_2$SO$_4$ 20 minutes    | 83$^{cd}$                    |
| H$_2$SO$_4$ 30 minutes    | 83$^{cd}$                    |
| H$_2$SO$_4$ 40 minutes    | 87$^{cd}$                    |
| H$_2$SO$_4$ 50 minutes    | 85$^{cd}$                    |
| H$_2$SO$_4$ 60 minutes    | 79$^c$                       |

Note: The means followed by the same letter in the same column is not significantly different according to Duncan's multiple tests of 5%.
Increasing the duration of soaking of acacia seeds in sulfuric acid (from 60 minutes to 120 minutes) will increase the percentage of *Acacia cyanophylla* and *A. farnesiana* seed germination (98% and 99%) while increasing the soaking time harms *A. decurrens* germination. At 60 minutes of immersion, the percentage of germination was 97% and decreased to 43% at the time of soaking 120 minutes [15].

### 3.3 Vigor Index

Vigor index is measured based on the percentage of the normal number of sprouts on the first day of observation, 7 DAP. Vigor seeds show high speed in their growth process if the conditions around them are sub-optimum for growth, and their metabolic processes are not inhibited [16].

**Table 3.** Effect of indigofera seed soaking in hot water and sulfuric acid solution on the vigor index

| Treatment                  | Vigor index (%) |
|----------------------------|-----------------|
| Control                    | 17<sup>a</sup>  |
| Hot water80°C              | 37<sup>b</sup>  |
| Hot water 100°C            | 45<sup>bc</sup> |
| H<sub>2</sub>SO<sub>4</sub> 10 minutes | 69<sup>e</sup>  |
| H<sub>2</sub>SO<sub>4</sub> 20 minutes | 61<sup>cde</sup> |
| H<sub>2</sub>SO<sub>4</sub> 30 minutes | 63<sup>de</sup> |
| H<sub>2</sub>SO<sub>4</sub> 40 minutes | 47<sup>bcd</sup> |
| H<sub>2</sub>SO<sub>4</sub> 50 minutes | 49<sup>bcd</sup> |
| H<sub>2</sub>SO<sub>4</sub> 60 minutes | 42<sup>b</sup>  |

Note: The means followed by the same letter in the same column is not significantly different according to Duncan’s multiple tests of 5%.

Soaking the seeds in 95% H<sub>2</sub>SO<sub>4</sub> solution for 10 minutes gave the highest vigor index value (69%), but not significantly different with soaking in 95% H<sub>2</sub>SO<sub>4</sub> solution for 20 and 30 minutes. The longer soaking time in the H<sub>2</sub>SO<sub>4</sub> solution tends to decrease the vigor index. While the soaking of seeds in hot water 80 and 100°C, the value of the vigor index is not significantly different and tends to be lower than the soaking of the seeds in H<sub>2</sub>SO<sub>4</sub> solution.

Research on *Desmodium trifolium* seeds with a shelf life of 8 months soaked in the concentrated sulfuric acid solution for 10 minutes will increase the germination of seeds up to 86% better than the control of 22% [17]. *Harpagophytum procumbens* seeds soaked in sulfuric acid solution gave a percentage of seed germination of 17% higher compared to controls (5.3%). Sulfuric acid can damage the seed coat so that it will be easier for water and oxygen diffuse on the seeds and the germination process occurs. Higher concentrations of sulfuric acid are corrosive compared to lower concentrations [18]. Pre treatment with concentrated sulfuric acid @ 200 ml/kg for duration 5 minutes recorded the highest germination (91%), speed of germination (5.0) and vigour index (2879) as compared to control which lowers germination (65%), speed of germination (3.0) and vigor index (1447) [19].

### 3.4 Growth Rate

Soaking the seeds in hot water and H<sub>2</sub>SO<sub>4</sub> solution has a real effect on increasing the growth rate. The highest yield obtained by soaking of seeds in the H<sub>2</sub>SO<sub>4</sub> solution for 30 minutes (13.85% et mal<sup>1</sup>), not significantly different from soaking for 10, 20, 40 and 50 minutes (11.67-13.81% et mal<sup>1</sup>) (Table 4). The longer soaking time for seeds in H<sub>2</sub>SO<sub>4</sub> solution tends to decrease the growth rate, conversely increasing the soaking temperature to 100°C increases the growth rate.
Table 4. Effect of indigofera seed soaking in hot water and sulfuric acid solution on growth rate

| Treatment                  | Growth rate (% etmal) |
|----------------------------|-----------------------|
| Control                    | 4.88^a                |
| Hot water 80°C             | 9.88^b                |
| Hot water 100°C            | 10.83^bc              |
| H₂SO₄ 10 minutes           | 13.81^d               |
| H₂SO₄ 20 minutes           | 13.10^ed              |
| H₂SO₄ 30 minutes           | 13.85^ed              |
| H₂SO₄ 40 minutes           | 11.67^bcd             |
| H₂SO₄ 50 minutes           | 12.13^bcd             |
| H₂SO₄ 60 minutes           | 10.54^bc              |

Note: The means followed by the same letter in the same column is not significantly different according to Duncan's multiple tests of 5%.

According to [20], pre treatment of Leucaena leucocephala seed with immersion in concentrated sulfuric acid for 20 minutes was the most efficient in overcoming seed dormancy, showing higher values of first germination count (88.0%), germination percentage (93.0%), germination speed index (89.3%) and average time of germination (6 days). In Flemingia semialata, seed scarified in concentrated sulfuric acid for 1 minute give the best result at germination rate (7.32) compared with control (3.48) [21].

4. Conclusion

Indigofera seed soaking in hot water and H₂SO₄ solution significantly increased the germination of seeds, maximum growth potential, vigor index, and growth rate. Soaking indigofera seeds in a 95% H₂SO₄ solution for 10 minutes was able to increase seed germination (84%), maximum growth potential (91%), vigor index (69%), and growth rate of 13.81% etmal⁻¹. The longer soaking time for seeds in the H₂SO₄ solution was reduced seed germination, maximum growth potential, vigor index, and growth rate. The finding concludes that the best treatment was soaking seed in H₂SO₄ solution for 10 minutes, longer soaking time (> 50 minutes) will decrease all parameters. Indigofera seed soaking technology in hot water is relatively practical and inexpensive, but soaking in sulfuric acid is relatively effective and easy to approach for higher technology for farmers.

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