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Improving *in vitro* seed sprouting on legume of *Indigofera zollingeriana* stored seed

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Abstract

Experimental evidence shows that notable Indonesian forage crop zollinger blue or Indigofera zollingeriana has high seed dormancy that hinders its reproduction on large scale. This study reports different pre-treatments to break seed dormancy and improve seed germination of I. zollingeriana seeds under in vitro conditions. Experimental evidence suggest that both mechanical and chemical scarification followed by treatment with constantly agitated liquid 0.11 mg/L GA_3 has significant effects on seed germination of the plant. The germinated seeds were cultured on MS medium to aid seedling growth. The results showed improved germination and raising of the seedlings compared to the treatments; when the seedlings were germinated using sand paper or acid scarified seeds singly. However, acid scarification for longer time affect negatively on germination especially roots.

Keywords: *Forage, gibberelic acid, legumes, mechanical scarification, chemical scarification.*

Baklagil bitkisi *Indigofera zollingeriana*'nın depolanmış tohumların *in vitro* koşullarda çimlendirilmesi ve geliştirilmesi

Özet

Deneysel çalışmalar, Endonezya'da zollinger mavisi ya da Indigofera zollingeriana olarak bilinen yem bitkisinin üretimini büyük ölçüde engelleyen faktörün yüksek tohum dormansisi olduğunu göstermektedir. Bu çalışmada farklı ön muameleler ile yüksek

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tohum dormansisinin kırılması ve in vitro koşullar altında tohum çimlendirilmesinin geliştirilmesi amaçlanmıştır. Yapılan denemelerde hem mekanik hem de kimyasal muamelesinin birlikte uygulanması sonrası 0.11 mg/L GA_3 uygulamasının tohum çimlenmesinde önemli etkisi olduğu sonucuna varılmıştır. Çimlenen tohumlar MS besin ortamına alınarak büyümeye bırakılmıştır. Bu şekilde çimlendirilen tohumlar sadece zımpara ya da asit muamelesi uygulaması ile çimlendirilen tohumlara göre daha iyi çimlenme göstermiştir. Bununla birlikte asit uygulama süresinin artması özellikle çimlenen tohumların köklerinde olumsuz etkiye sebep olmuştur.

***Anahtar kelimeler**: Yem, gibberellik asit, baklagil, mekanik aşındırma, kimyasal aşındırma.*

1. Introduction

Indigofera are leguminous plant with their tolerance to abiotic stresses like drought, light floods, acidic soils and salinity [1-2]. *Indigofera* species distribute thoroughly in tropical region especially South Asia and Indonesian island [3-4]. The utilization of *Indigofera* has been reported as sources of forage crop, natural dyes, cosmetics and pharmaceuticals.

Indigofera zollingeriana Miq. with its drought of tolerance, high herbage production and nutritional quality is one of important forage sources that grows in Indonesia [5-7]. Akbarillah et al. [8] reported Indigofera's leave meal contain 27% crude protein, 19.94% crude fiber, 9.96% crude fat, 0.22% Ca, 0.18% P and 1700 kkal/Kg energy metabolism.

It is well known as one of cultivation problem that normal seed *I. zollingeriana* germinated at 4^{th} days with germination below 35% after 2 months of storage periode [9]. Low seed germination of *I. zollingeriana* is partially related to thick skin coat and fungal invasions during storage that influence seed germination behavior of stored seeds [10-11]. Seed has important role to support breeding and quality forage production. However, high seed dormancy that increase with the passage of time in *I. zollingeriana* offer considerable challenge to expand its production.

This study aimed to offer practical solutions to solve seed dormancy under *in vitro* conditions.

2. Materials and methods

The seeds were collected from Prof. Dr. Luki Abdullah of the Department of Nutrition Science and Feed Technology, Bogor Agricultural University, Indonesia.

2.1 Tetrazolium test

Tetrazolium test was carried out following Pradhan and Badola [12] to evaluate seed viability of fresh, 9, 13, 15 and 17 months old seeds. Each sample contained 100 seeds.

2.2 Surface disinfection methods

Sixty (60) seeds each of *I. zollingeriana* were surface disinfected using 20, 40, 60, 80 and 100% concentration of commercial bleach (Domestos-Turkey, containing 5%

NaOCl) for 10 minutes. Subsequently, they were rinsed 3 × 5 min with autoclaved distilled water to remove the traces of commercial bleach. The disinfected sterilized seeds were cultured on sterile MS medium [13], pH 5.6-5.8 to optimize the best disinfection conditions.

2.3 Seed dormancy break treatments

The experiment determined effectiveness of mechanical (sand paper) and chemical (acid) scarification of the seeds singly or in combination. Thereafter, initially the 9 months old seedlings were treated/shaked with 0.0, 0.11, 0.16 and 0.21 mg/L GA_3 (Gibberellic acid) for 3 days to optimize best GA_3 concentration. Subsequently, the 13, 15 and 17 months old seedlings were treated with the optimized concentration of GA_3. The control treatments contained the seeds that were not scarified and not treated with GA_3. All seeds were cultured in growth chamber.

2.4 Statistical analysis

Each experimental treatment used 60 seeds divided into equally distributed 4 replicates; each containing 15 seeds or explants. The control treatments contained the seeds or explants that were not scarified and not treated with GA_3. The data was analyzed by comparing means using SPSS 24 program for Windows. The significant differences among the means were determined by Duncan's Multiple Range Test (DMRT). The percentage data obtained from the experiments were subjected to arsine transformation before statistical analysis [14].

~~3.~~ Results

3.1 Tetrazolium test

Tetrazolium test showed 100%, 90%, 85% and 85% seed viability on seeds stored for 9 months, 13, 15 and 17 months respectively showing that the seeds loose viability progressively with the passage of time.

3.2 Seed sterilization of I. zollingeriana and germination

No contamination was noted on any sterilization treatment after 3 weeks. Seed germination percentage ranged 5-18.33% (Fig. 1). It was noted that > 20% commercial bleach caused progressively increasing inhibition on germination of *I. zollingeriana* seeds. Seed germination decreased with each advancing concentration of commercial bleach. Moreover, these commercial bleach concentrations also had damaging effects on respective seed coat/testas that secreted increased blue or violet pigments in the culture medium. These pigments had stunting effects on growth of seedlings even after 10-12 days of culture post germination (Fig 2).

In the 2nd experiment, the sterilized seeds were sprouted using different concentrations of GA_3 in sterilized erlenmeyer fasks on horizontal shakers with aim to optimize the best effective GA_3 concentration. The GA_3 concentrations other than 0.11 mg/L GA_3were not as effective and had inclination towards gigantic growth and callusing on the germinating seedlings. A non significant increase (20%) in germination percentage was noted; with significant differences compared to the former experiment, showing radicular protrusions on the germinated seedlings using 0.11 mg/L GA_3 (Fig. 3). No inhibition was noted on these seedlings with development of comparatively longer (~3.5 cm) shoots in 10-12 days'time. Therefore, this concentration of GA_3 was used in all

subsequent experiments. No release of pigmentation was noted on any shaked liquid GA_3 treated seeds on MS medium.

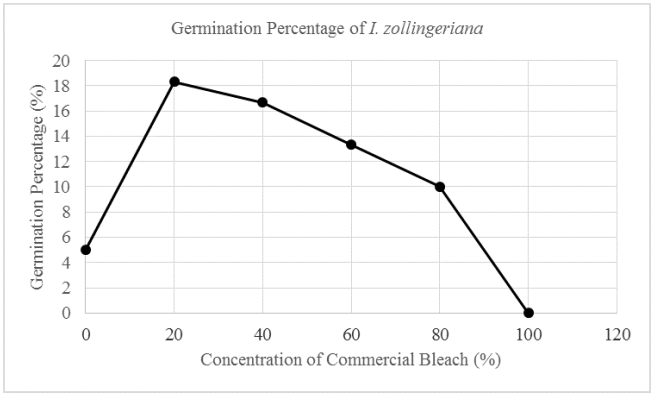

Fig. 1. Effect of variable concentration of commercial bleach on seed germination percentage of *I. zollingeriana.*

Fig. 2 Commercial bleach treated germinated seedlings with increased blue or violet pigments in the culture medium.

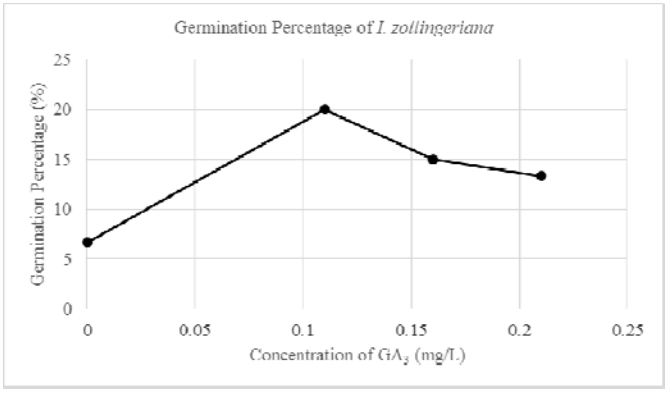

Fig. 3. Effects of variable concentration of gibberellic acid (GA_3) on seed germination percentage of Zollinger's Indigo.

3.3 Scarification treatments of 9 months old seeds

3.3.1. Sand paper scarification

Sand paper scarification on 9 months old seeds followed by treatment with liquid 0.11 mg/L GA_3 was not as effective as acid scarification strategy to germinate the 9 months old seeds and the seed germination never surpassed 50%. Sand paper scarified seeds were prone to secrete pigmentations compared to acid scarified seeds, in to the culture medium and hindered growth and development of seedlings. The germinated seedlings were subcultured three times to avoid the problem. This might have inhibited growth and germination of seedlings.

3.3.2 Sulphuric acid scarification

The seed germination percentage ranged 18.33-66.67% (Table 1) on sulphuric acid scarified seeds for 5 to 20 min followed by treatment with 0.11 mg/L GA_3 for 3 days. Acid treated seeds had bleached seed coat and 2-3 cm long shoots with leaves in 10-12 days. Minimum and maximum seed sprouting was noted on 5 and 10 min sulphuric acid scarification of seeds in the same order.

Table 1. Influence of sandpaper and 98% H_2SO_4 treatment on 9 months old seed germination of *I. zollingeriana* under *in vitro.*

Scarification treatment		Percentage of germination (%) after 3 days treatment with 0.11 mg/L GA_3 by continuous shaking before culture on agar solidified MS medium
Sand paper scarification with 2-3 scratches	Sulfuric acid scarification treatment (min)	
-	5	18.33e
-	10	73.33c
-	20	66.67d
+	5	95.00b
+	10	100.00a
+	20	100.00a

Means not followed by the same letter within a column differ significantly at 0.05 level of significance using Duncan's multiple range test.

3.4 Treating 9 months old sand paper + sulphuric acid scarified seeds after treatment with liquid GA_3 and culture on MS medium

It was noted that the seeds that were acid scarified for 5 to 20 min followed by treatment with 0.11% GA_3 induced 18.33-66.67% sprouting of the seedlings (Table 1). Contrarily, combined scarification (sand paper + 5 to 20 min acid scarification) followed by 0.11 mg/L GA_3 imbibition with continuous shaking improved the seedling sprouting percentage in range of 95-100% (Table 1). Minimum seed germination (95%) was noted on 5 min and 100% seed germination was noted on combined 10-20 min acid scarified seeds followed by treatment with liquid 0.11 mg/L GA_3. However, 10 min and 20 min scarified seeds showed burning of radicular tips on the germinated seeds. This showed that longer acid treatments are hazardous for seeds and should be avoided. Therefore, combined scarification and post treatment with GA_3 was preferred in all subsequent experiments to sprout seedlings. Combined sand paper and acid scarified seeds were the most effective and gave the highest seed germination and significant improvement in the germination percentage of the seeds.

3.5 Effect of different durations of storage on seed germination

The result showed 90, 85 and 85% seed germination on 13, 15 and 17 months old stored seeds in the same order at room temperature, using combined (sand paper and H_2SO_4) scarification for 5 min, followed by seedling sprouting in 0.11 mg/L GA_3 treatment (Table 2).

Seed germination percentage of 5, 5 and 5% was noted on 13, 15 and 17 months stored non scarified seeds that were treated without 0.11 mg/L GA_3. The seeds did not show any sprouts on agar solidified MS medium (control treatment 1). Whereas, germination percentage of 13, 15 and 17 months stored seeds was 88.33, 83.33 and 81.67% in the same order on combined scarified seeds shaked in sterile water (control treatment 2).

Table 2. Influence of sandpaper and 5 min 98% of H_2SO_4 scarification on germination percentage of *I. zollingeriana* stored seeds for 13, 15, 17 months.

Scarification treatment	Type of treatment	Percentage (%) of germination on storage		
		13 months	15 months	17 months
Sandpaper + 98% H_2SO_4 for 5 min	3 days treatment with 0.11 mg/L GA_3 by continuous shaking	90.00aA	85.00aB	85.00aB
No scarification (control treatment 1)	No treatment	5.00bA	5.00bA	5.00cA
Sandpaper + 98% H_2SO_4 for 5 min (control treatment 2)	3 days treatment with sterile water by continuous shaking	88.33aA	83.33aB	81.67bB

[1]Means not followed by same letter within a column differ significantly at 0.05 level of significance using LSD test.
[1]Means not followed by same letter within a row differ significantly at 0.05 level of significance using Duncan's multiple range test.

4. Discussion

4.1 Tetrazolium and dormancy

Tetrazolium (2, 3, 5 triphenyl tetrazolium chloride or bromide) test is the best indicator to evaluate seed viability potential that could germinate under field conditions. All living tissues respire and reduce colorless tetrazolium chloride into a non diffusible, red compound formazan by transfer of H ion transfer reactions, that transform living tissues red [12]. Tetrazolium test indicated 100% seed viability on 9 months stored seeds and variable reduction in seed viability thereafter on the seeds stored for 13, 15 and 17 months stored seeds. It is common belief and the previous studies by Abdullah [15], who suggest that storage of *I. zollingeriana* seeds under ex situ conditions for long term basis reduce seed germination. The results of this study confirmed that it is not seed viability but dormancy that affects seed germination of the stored seeds. The seeds stored longer than 9 months had reduced germination in agreement with Van Hezewijk et al. [16] and Müller et al. [17]. This reduction in germination could be due to different factors including seed moisture content, surrounding temperature, relative humidity and storage conditions that are natural during storage [12, 18-26]. Another reason of dormancy could be the development of combined embryo and seed coat dormancy [27].

It is assumed that the *I. zollingeriana* embryos used in this study were very weak or had developed enhanced level of ABA with the passage of time. Resultingly, these failed to press the seed coat to rupture it and break the related seed coat dormancy. Thus both of these dormancies coexisted and blocked seed germination in agreement with Baskin et al. [28], Baskin et al. [29] and Baskin and Baskin [30]. However, inhibition caused by seed coat was more prominent compared to the other embryo related dormancy/ies. As and when the seed coats were splitted due to combined scarification, GA_3 treatment reduced the ABA caused embryo growth blockage leading to the breaking of other types of seed dormancy [31-35].

The results further showed that the seeds did not lose seed integritiy but germination capability is gradually lost in seeds stored for a longer time [36]. This also suggests that these seeds should be exposed to appropriate germination conditions like scarification, under warm moist conditions for termination of dormancy. Adkins et al. [37] suggests that addition of GA_3 in the seed germination environment improves germination and seed sprouting.

The validity of tetrazolium test that carried out to determine the percentage of viable seeds of seed lot is noted for all species in order to the method is described in the ISTA Rules [38]. The positive correlation between tetrazolium test and germination test are noted in many cases. The low seed germination [9] can be checked with tetrazolium test due to physical damage (broken seeds and heat damage) or physiological dormancy of mature seeds) [39].

4.2 Seed sterilization of I. zollingeriana and germination

Sodium hypochlorite is a fast, simple, economic and effective method of *in vitro* sterilization [40]. Although sodium hypochlorite is the most commonly of used, sterilizing agent, it could behave variably depending on the texture of seed coat among different species [41]. Sodium hypochlorite based sterilization was effective in this case as well. It was noted that increasing concentrations of bleach induced damage to the embryos of the seeds that reduced seed germination. However, the treatment was effective in partial germination of 18.33% with protrusion of radicles only. The cultured seeds secreted blue or violet colored anthocyanin pigments into the culture medium. This was controlled by 2-3 seed subcultures after every 3-4 days to avoid damage to the germinating seeds. Nwachukwu and Edeoga [42] established that several Indigofera species including *I. zollingeriana* contain starch grains, tannins and some crystal types besides number of pigments in all parts of the plants.

4.3 GA_3 treatment

GA_3 is known to have a significant role in seed germination and elongation. This has been conformed in other studies. Patel and Mankad [43] noted maximum seed germination percentage on *Tithonia rotundifolia* Blake using 500 mg/L (ppm) of GA_3. This (0.11 mg/L GA_3) treatment was effective to induce 20% germination with protrusions of both radicles and plumules. However, the seed coats were very hard and they did not allow more germination and improved elongation. Desai et al. [44] report that application of 150 mg/L of GA_3 on *Carica papaya* L. sprouts improved elongation of sprout shoots along with germination percentage.

4.4 Germination of seeds after scarification (mechanical and acid) followed by germination with liquid GA_3 treatments

Tetrazolium test indicated seed viability of 100, 90, 85 and 85% for 9, 13, 15 and 17 months stored seeds respectively. The results showed that the seeds viability is lost gradually after a prolonged storage period. It is believed that ABA biosynthesis in the embryo and close by seed tissues continue maturation and storage proteins and lipids synthesis that suppress early embryo germination [45-46]. Generally, seed ABA level is low during early growth period that improves thereafter, and peaks around mid maturity period [27, 46-49]. Besides this other factors also aid increase or fall of ABA, that include sensitivity of ABA to seed development stage seed tissues and different thresholds to start and maintain seed development [46, 50-51]. Light, temperature and water availability can significantly affect level of ABA contents and sensitivity to seed maturity. Dormancy of *I. zollingeriana* might be caused by hard seed coat or wax layer beneath seed coat that can interfere water imbibition during germination. The results showed best results on 10 min acid scarified seeds treated with 0.11 mg/L GA_3. Longer periods of scarification were hazardous even if the seeds were treated with GA_3.

Sandpaper scarification is one of methods to soften seed coat and improve seed germination. Two three scratches of sand paper were considered as enough to crack seed coat/testa and avoid considerable damage to seed embryos making their seed coat more permeable to water with improved germination. Increased seed crushing or scratching can damage seed embryos fully or partially [52]. They reported that mature dodder seeds could be germinated by sand paper scarification. Hassen et al. [53] has reported sand paper scarification as an effective method to break seed dormancy and they improved seed germination from 41-73% compared to boiled water treatment in 6 accession of *Indigofera* (*I. cryptantha*, *I. brevicalyx*, *I. arrecta*, *I. spicata*, *I. trita* and *I. spicata*). In another study Hassen et al. [54] improved germination of *Ziziphus mucronata* (buffalo thorn) from 10.7% on non treated seeds to 65.4% after sand paper scarification, also report improvement of seed germination of forage legume from the control wıth 13.2% up to 85.3% and *Lessertia frutescens* 8% germination on control to 99.6% with mechanical scarification with sandpaper. Sandpaper can soften the hard seed coats of *I. zollingeriana*. It was similar to other small seed legumes species of *Indigofera* that successfully improved germination seed of *in I. cryptantha* and *I. spicata* by sandpaper scarification [53].

H_2SO_4 acts both as effective disinfectant and scarifying agent that helps to break seed coat and accelerate seed germination. Comparing the two types of scarification, sulphuric acid scarification was less effective compared to the combined scarification. in agreement with Hari et al. [55] and Balouchi and Sanavy [56]. Dilaver et al. [57], who confirmed increasing concentrations might have induced variable damage to the embryos of the *I. tinctoria* L.*, Medicago polymorpha* L. and *Mycrotyloma daltoni* Webb. Verde, *Medicago rigudula* L. and Astragallus seeds in their studies. Ersin et al. [58] reported that highest germination rate in *Medicago polymorpha* L., *Trifolium lappaceum* L.*, T. scabrum* L. and *T. strictum* L. was 47.5%, 90%, 12.5% and 10% using 95-97% concentrated of sulphuric acid for 5 min. The soaking of seeds in 98% concentrated of sulphuric acid for 15 min improved germination rate of *Trifolium resupinatum* L. up to 90.1%. Asl et al. [59] also confirm that acid scarification is effective to improve germination of species with hard seed coat.

The hard-seeded *I. zollingeriana* used in this study has weak embryos surrounded by a thick seed coat with strong micropylar openings that hinder entering of water into the seed structure, prevent sprouting and germination. This thickening of micropyler opening cell tissue structure propose that it offers strong resistance against water uptake and emergence of radicles [60-61]. To solve this problem seed coat hydrolysis is likely alternative to permit emergence of radicles and plumules [60] that is not possible in this case. This suggests need to give seed coat scarification treatments to overcome this issue in agreement with Bewley [48].

The results of this study shows that radicle emergence is likely dependent upon the weakening of the seed coat cell walls (by mechanical and acid scarification) and GA_3 have combined effect of weakening the seed coat and helping to increase seed germination. Medeiros Filho et al. [62] also affirmed above mentioned observations with seed coat cracks that allowed improved imbibition of water and seed germination.

The results of the study approve that combined pre scarification of seeds followed by treatment with 0.11 mg/L GA_3 were effective to enhance seed germination compared to control treatments (sterile water treated seeds). The results of this study show a significant improvement over the results of the previous studies of Abdullah [9, 16], who noted 28-35% germination of *I. zollingeriana* seeds stored for two months. Whereas, Abdullah [16] noted that storage period of more than 4 weeks decreased seed viability or increased seed dormancy resulting in decreasing germination percentage up to 24%.

5. Conclusion

The investigation demonstrate a comparison of mechanical and acid scarification used singly or both treatments combined + GA_3 treatment. Combination of all methodologies were more favorable to induce seed germination and solved the problem of unfavorable physiological impact of the seed embryos and hard seed coating with the highest seed germination rates.

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