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

Siti MAESAROH¹*, Çiğdem Alev ÖZEL²

¹Department of Field Crops, Faculty of Agriculture, Ankara University, 06110, Dıskapı, Ankara, Turkey
²Department of Biology Education, Faculty of Education, Gazi University, 06500, Teknikokullar, Ankara, Turkey

Geliş Tarihi (Received Date): 19.02.2019
Kabul Tarihi (Accepted Date): 26.08.2019

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 GA₃ 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 tohumlarının *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 dormansısı olduğunu göstermektedir. Bu çalışmada farklı ön muameleler ile yüksek...
tohum dormansisini kırılması ve in vitro koşullar altında tohum çimlenmesinin gelişirmesini amaçlamıştır. Yapılan denemelerde hem mekanik hem de kimyasal muamelesinin birlikte uygulanması sonrası 0.11 mg/L GA₃ uygulamasının tohum çimlenmesinde önemli etkisi olduğu sonucuna varılmıştır. Çimlenen tohumlar MS besin ortamına alnarak büyümeye bırakılmıştır. Bu şekilde çimlenirilen tohumlar sadece zimpara ya da asit muamelesi uygulaması ile çimlenirilen 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 4th days with germination below 35% after 2 months of storage period [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%
for 10 minutes. Subsequently, they were rinsed $3 \times 5$ 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 arcsine 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 lose 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 flasks on horizontal shakers with aim to optimize the best effective GA$_3$ concentration. The GA$_3$ concentrations other than 0.11 mg/L GA$_3$ were 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 GA₃ treated seeds on MS medium.

![Germination Percentage of I. zollingeriana](chart1.png)

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

![Commercial bleach treated germinated seedlings with increased blue or violet pigments in the culture medium.](chart2.png)

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

![Germination Percentage of I. zollingeriana](chart3.png)

**Fig. 3.** Effects of variable concentration of gibberellic acid (GA₃) 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 GA3 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 pigmention 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 GA3 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% H2SO4 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 GA3 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 GA3 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% GA3 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 GA3 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 GA3. 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 GA3 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 H₂SO₄) scarification for 5 min, followed by seedling sprouting in 0.11 mg/L GA₃ 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 GA₃. 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 H₂SO₄ scarification on germination percentage of *I. zollingeriana* stored seeds for 13, 15, 17 months.

| Scarification treatment | Type of treatment | Percentage (%) of germination on storage |
|------------------------|-------------------|-----------------------------------------|
| Sandpaper + 98% H₂SO₄ for 5 min | 3 days treatment with 0.11 mg/L GA₃ by continuous shaking | 90.00aA 85.00aB 85.00aB |
| No scarification (control treatment 1) | No treatment | 5.00bA 5.00bA 5.00cA |
| Sandpaper + 98% H₂SO₄ for 5 min (control treatment 2) | 3 days treatment with sterile water by continuous shaking | 88.33aA 83.33bA 81.67bB |

¹Means not followed by same letter within a column differ significantly at 0.05 level of significance using LSD test.

²Means 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 integrity 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 \( \text{GA}_3 \) 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 \( \text{GA}_3 \). Longer periods of scarification were hazardous even if the seeds were treated with \( \text{GA}_3 \).

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 \( \text{Indigofera} \) (\( I. \ cryptantha, I. \ brevicalyx, I. \ arrecta, I. \ spicata, I. \ trita \) and \( I. \ spicata \)). In another study Hassen et al. [54] improved germination of \( \text{Ziziphus mucronata} \) (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 with 13.2% up to 85.3% and \( \text{Lessertia frutescens} \) 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 \( \text{Indigofera} \) that successfully improved germination seed of \( \text{in I. cryptantha} \) and \( I. \ spicata \) by sandpaper scarification [53].

\( \text{H}_2\text{SO}_4 \) 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., \( \text{Medicago polymorpha} \) L. and \( \text{Mycrotyloma daltoni} \) Webb. Verde, \( \text{Medicago rigudula} \) L. and Astragallus seeds in their studies. Ersin et al. [58] reported that highest germination rate in \( \text{Medicago polymorpha} \) L., \( \text{Trifolium lappaceum} \) 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 \( \text{Trifolium resupinatum} \) 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 micropylar 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 GA₃ 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 GA₃ 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 + GA₃ 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.

Acknowledgements

The authors are grateful to Prof. Luki Abdullah, Bogor Agricultural University, for help in give of seeds.

References

[1] Skerman, P.J., *Tropical forage legumes*, Food and Agricultural Organization, Rome, (1982).
[2] Hassen, A., Rethman, N.F.G., Van Niekerk, W.A., and Tjelele, T.J., Influence of season/year and species on chemical composition and *in vitro* digestibility of five Indigofera accessions, *Animal Feed Science Technology*, 136, 312-322, (2007).
[3] Bakasso, S.A., Lamien-Meda, C.E., Lamien, M., Kiendrebeogo, J., Millogo, A.G., and Ouedraogo Nacoulma, O.G., Polyphenol contents and antioxidant
activities of five *Indigofera* species (*Fabaceae*) from Burkina Faso, *Pakistan Journal of Biological Sciences*, 11, 1429-1435, (2008).

[4] Wiradiadina, H., *Indigofera L. (Papilionaceae) di Indonesia* In Ginting, S.P., Prawiradipura, B.R., and Purwantari, N.D., *Indigofera sebagai Pakan Ternak*, IAARD Press, 9-24, Jakarta, (2012). [in Indonesian Language]

[5] Abdullah, L., and Suharlina, Herbage yield and quality of two vegetative parts of indigofera at different times of first regrowth defoliation, *Media Peternakan*, 33, 1, 44-49, (2010).

[6] Herdiawan, I., and Sutedi, E., Produktivitas tanaman pakan *Indigofera* sp. pada tingkat kekeringan dan interval pemangkasan berbeda, *Indonesian Journal of Animal and Veterinary Science*, 17, 2, 161-167, (2012).

[7] Herdiawan, I., Abdullah, L., Sopandie, D., Karti, P.D.M.H., and Hidayati, N., Respon fisiologis tanaman pakan *Indigofera zollingeriana* pada berbagai tingkat kekeringan dan interval pemangkasan, *Indonesian Journal of Animal and Veterinary Science*, 18, 1, 54-62, (2013).

[8] Akbarillah, T., Kususiyah., Kaharuddin, D., and Hidayat, Tepung daun Indigofera sebagai suplementasi pakan terhadap produksi dan warna yolk puyuh (*Coturnix coturnix japonica*), *Jurnal Sain Peternakan Indonesia*, 3, 1, 20-23, (2008).

[9] Abdullah, L., *Prospektif agronomi dan ekofisiologi Indigofera sebagai tanaman pakan berkualitas tinggi* In Ginting, S.P., Prawiradipura, B.R., and Purwantari, N.D., *Indigofera sebagai Pakan Ternak*, IAARD Press, 47-58., Jakarta, (2012). [in Indonesian Language]

[10] Girsang, R.C., Viability of *Indigofera* (*Indigofera zollingeriana*) seed after carbon dioxide (CO\textsubscript{2}) injection and storing, (2017). http://repository.ipb.ac.id/jspui/bitstream/123456789/54853/8/D12rcg.pdf, (26.06.2018).

[11] Abdullah, L., Girsang, R.C., Putra, N.P., Wiryawan, K.G., and Permana, I.G., Viabilitas, intensitas kontaminasi jamur, dan tinggi hipokotil sebagai respon terhadap modifikasi atmosfer dengan injeksi CO\textsubscript{2} selama penyimpanan benih *Indigofera zollingeriana* In Abdullah, L., Astuti, D.A., and Suharlina, *Bunga Rampai Hasil Riset dan Pengembangan Indigofera zollingeriana*, IPB Press, 6-13, Bogor, (2016) [in Indonesian language]

[12] Pradhan, B.K., and Badola, H.K., Seed germination response of populations of *Swertia chirayita* following periodical storage, *Seed Technology*, 30, 1, 63-69, (2008).

[13] Murashige, T., and Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiologia Plantarum*, 15, 3, 473-497, (1962).

[14] Snedecor, G.W., and Cochran, W.G., *Statistical methods*. 6th ed. Oxford and IBH Publishing, New Delhi, (1976).

[15] Abdullah, L., Prospektif agronomi dan ekofisiologi *Indigofera zollingeriana* sebagai tanaman penghasil hijauan pakan berkualitas tinggi, *Pastura*, 3, 2, 79-83, (2014).

[16] Van Hezewijk, M.J., Van Beem, A.P., Verkleij, J.A.C., and Pieterse, A.H., Germination of *Orobanche crenata* seeds, as influenced by conditioning temperature and period. *Canadian Journal of Botany*, 71, 6, 786-792, (1993).

[17] Müller, E., Cooper, E.J., and Alsos, I.G., Germinability of arctic plants is high in perceived optimal conditions but low in the field, *Botany*, 89, 5, 337-348, (2011).
[18] Roberts, E.H., Predicting the storage life of seeds, Seed Science and Technology, 1, 499-514, (1973).
[19] Vertucci, C.W., and Roos, E.E., Theoretical basis of protocols for seed storage. Plant Physiology, 9, 3, 1019-1023, (1990).
[20] Vertucci, C.W., and Roos, E.E., Seed moisture content, storage, viability and vigour. Seed Science Research, 1, 277-279, (1991).
[21] Ellis, R.H., Hong, T.D., and Roberts, E.H., Seed moisture content, storage, viability and vigour, Seed Science Research, 1, 275-277, (1991).
[22] Ellis, R.H., Hong, T.D., and Roberts, E.H., The low-moisture-content limit to the negative logarithmic relation between seed longevity and moisture content in three subspecies of rice, Annals of Botany, 69, 1, 53-58, (1992).
[23] Butola, J.S., and Badola, H.K., Effect of pre-sowing treatment on seed germination and seedling vigour in Angelica glauca, a threatened medicinal herb, Current Science, 87, 6, 796-799, (2004a).
[24] Butola, J.S., and Badola, H.K., Seed germination improvement using chemicals in Heracleum candicans wall, a threatened medicinal herb of Himalaya, Indian Forester, 130, 5, 565-572, (2004b).
[25] Yang, Q.H., Ye, W.H., Deng, X., Cao, H.L., Zhang, Y., Xu, K.Y., Seed germination eco-physiology of Mikania micrantha H.B.K, Botanical Bulletin. Of Academia Sinica, 46, 4, 293-299, (2005).
[26] Onyekwelu, J.C., and Fayose, O.J., Effect of storage methods on the germination and proximate composition of Treculia africana seeds [Internet]. Tropentag: University of Kassel-Witzenhausen and University of Göttingen. 2007-[cited 2017 Dec 11].
[27] Bewley, J.D., and Black, M., Seeds physiology of development and germination, 2nd ed, Plenum Publishing Corporation, Boston, (1994).
[28] Baskin, J.M., Nan, X., and Baskin, C.C., A comparative study of seed dormancy and germination in an annual and a perennial species of Senna (Fabaceae), Seed Science Research, 8, 501-512, (1998).
[29] Baskin, J.M., Baskin, C.C., and Li, X., Taxonomy, ecology, and evolution of physical dormancy in seeds, Plant Species Biology, 15, 139-152, (2000).
[30] Baskin, J.M., and Baskin, C.C., Classification, biogeography, and phylogenetic relationships of seed dormancy In Pritchard, H., Seed Conservation: Turning Science into Practice, The Royal Botanic Gardens Press, Kew, (2004).
[31] Ren, C., and Kermode, A.R., Analyses to determine the role of the megagametophyte and other seed tissues in dormancy maintenance of yellow cedar (Chamaecyparis nootkatensis) seeds: morphological, cellular and physiological changes following moist chilling and during germination, Journal of Experimental Botany, 50, 1403-1419, (1999).
[32] Ren, C., and Kermode, A.R., An increase in pectin methyl esterase activity accompanies dormancy breakage and germination of yellow cedar seeds, Plant Physiology, 124, 231-242, (2000).
[33] Schmitz, N., Abrams, S.R., and Kermode, A.R., Changes in abscisic acid content and embryo sensitivity to (+)-abscisic acid during dormancy termination of yellow-cedar seeds, Journal of Experimental Botany, 51, 1159-1162, (2000).
[34] Schmitz, N., Abrams, S.R., Kermode, A.R., Changes in ABA turnover and sensitivity that accompany dormancy termination of yellow-cedar...
(Chamaecyparis nootkatensis) seeds, *Journal of Experimental Botany*, 53, 89-101, (2002).

[35] Terskikh, V.V., Feurtado, J.A., Ren, C., Abrams, S.R., Kermode, A.R., Water uptake and oil distribution during imbibition of seeds of Western white pine (*Pinus monticola* Dougl. Ex D. Don) monitored *in vivo* using magnetic resonance imaging, *Planta*, 221, 17-27, (2005).

[36] Roberts, E.H., *Seed ageing-the genome and its expression* In Nooden, D., and Leopold, A.C., *Senescence and Ageing in Plants*, Academic Press, 465-598, New York, (1988).

[37] Adkins, S.W., Loewen, M., Symons, S.J., Variation within pure lines of wild oats (*Avena fatua*) in relation to degree of primary dormancy, *Weed Sciences*, 34, 6, 859-864, (1986).

[38] FAO, *Seed toolkit: Seed quality assurance*, 111, The Food and Agriculture Organization of the United Nations and AfrikaSeeds, Rome, (2018).

[39] Stefanie Kramer, Relationship between tetrazolium and germination test, (2010). https://www.seedtest.org/upload/cms/user/Relationshipbetweentetrazoliumandgerminationtests_StefanieKramer.pdf, (12.01.2019).

[40] Coimbra, M.C., Castro, A.H.F., Different methods for surface sterilization of *Pyrostegia venusta* (Ker Gawl.) Miers (Bignoniaceae) leaf explants, *Plant Cell Culture and Micropropagation*, 12, 2, 34-38, (2016).

[41] Khawar, K.M., and Ozcan, S., High frequency shoot regeneration from cotyledonary node explants of different lentil (*Lens culinaris* Medik) genotypes and *in vitro* micrografting, *Biotechnology and Biotechnological Equipment*, 16, 1, 12-17, (2002).

[42] Nwachukwu, C.U., and Edeoga, H.O., Tannins, starch grains and crystals in some species of *Indigofera*. Leguminosae-Papilionoideae), *International Journal of Botany*, 2, 159-162, (2006).

[43] Patel, R.G., and Mankad, A.U., Effect of gibberellins on seed germination of *Tithonia rotundifolia* Blake, *International Journal of Innovative Research in Science, Engineering and Technology*, 3, 3, 10680-10684, (2014).

[44] Desai, A., Panchal, B., Trivedi, A., and Prajapati, D., Studies on seed germination and seedling growth of papaya (*Carica papaya* L.) CV. madhubindu as influenced by media, GA₃ and cow urine under net house condition, *Journal of Pharmacognosy and Phytochemistry*, 6, 4, 1448-1451, (2017).

[45] Kermode, A.R., Regulatory mechanisms involved in the transition from seed development to germination, *Critical Reviews in Plant Sciences*, 9, 155-195, (1990).

[46] Kermode, A.R., *Regulatory mechanisms in the transition from seed development to germination: interactions between the embryo and the seed environment* In Kigel, J., and Galili, G., *Seed development and germination*, Marcel Dekker Inc, 273-332, New York, (1995).

[47] Meinke, D.W., Molecular genetics of plant embryogenesis, *Annual Review of Plant Physiology and Plant Molecular Biology*, 46, 369-394, (1995).

[48] Bewley, J.D., Seed germination and dormancy, *Plant Cell*, 9, 1055-1066, (1997).

[49] Taylor, I.B., Sonneveld, T., Bugg, T.D.H., and Thompson, A.J., Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors, *Journal of Plant Growth Regulation*, 24, 253-273, (2005).
[50] Xu, N., and Bewley, J.D., Sensitivity to abscisic acid and osmoticum changes during embryogenesis of alfalfa (*Medicago sativa*), *Journal of Experimental Botany*, 42, 821-826, (1991).

[51] Jiang, L., Abrams, S., Kermode, A.R., Vicilin and napin storageprotein gene promoters are responsive to abscisic acid in developing transgenic tobacco seed but lose sensitivity following premature desiccation, *Plant Physiology*, 110, 1135-1144, (1996).

[52] Hutchison, J.M., and Ashton, F.M., Effect of desiccation and scarification on the permeability and structure of the seed coat of *Cuscuta campestris*, *American Journal of Botany*, 66, 40-46, (1979).

[53] Hassen, A., Pieterse, P.A., Rethman, N.F.G., Effect of pre-planting seed treatment on dormancy breaking and germination of *Indigofera* accessions, *Tropical Grasslands*, 38, 154-157, (2004).

[54] Hassen, A., Rethman, N.F.G., Van Niekerk, W.A., Effect of different seed treatment options on dormancy breaking germination and emergence of *Ziziphus mucronata* (Buffalo thorn) seed, *Tropical Grassland*, 39, 124-128, (2005).

[55] Hari, N., Warrier, K.C.S., and Gopalakrishnan, P.K., Pre-sowing treatments to promote seed germination in *Indigofera tinctoria* Linn, (2002). https://www.researchgate.net/publication/281405050_Pre-sowing_Treatments_to_Promote_Seed_Germination_in_Indigofera_tinctoria_Linn, (27.06.2018).

[56] Balouchi, H.R., and Sanavy, S.A.M.M., Effect of gibberellic acid, pre-chilling, sulfuric acid and potassium nitrate on seed germination and dormancy of annual medics, *Pakistan Journal of Biological Sciences*, 9, 2875-2880, (2006).

[57] Dilaver, Z., Mirzapour, M., and Kendir, H., Breaking seed dormancy and micropropagation of perennial Vulneraria Milkvetch (*Astragalus vulnerariae* Dc.). *ACTA Scientiarum Polonorum Horticulture*, 16, 4, 79-88, (2017).

[58] Ersin, C., Nafiz, C., Rustu, H., and Suleyman, A., Breaking seed dormancy of some annual Medicago and Trifolium species by different treatments, *Turkish Journal of Field Crops*, 14, 2, 72-78, (2009).

[59] Asl, M.B., Sharivivash, R., and Rahbari, A., Effect of different treatments on seed germination of Honey Locust (*Gleditschia triacanthos*), *Modern Applied Science*, 5, 1, 200-204, (2011).

[60] Williams, H.A., Bewley, J.D., Greenwood, J.S., Bourgault, R., and Mo, B., The cell walls of the endosperm of *Asparagus officinalis* seeds during development and following germination, *Seed Science Research*, 11, 305-315, (2001).

[61] Silva, E.A.A., Toorop, P.E., Van Aelst, A.C., and Hilhorst, H.W.M., Abscisic acid controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination, *Planta*, 220, 251-261, (2005).

[62] Medeiros Filho, S., Franca, E.A., and Innecco, R., Germinacao de sementes de *Operculina macrocarpa* (L.) Farwel de *Operculina alata* (Ham) Urban, *Revista Brasileriana Sementes*, 24, 2, 102-107, (2002).