

## Seed Germination and Dormancy Breaking of *Thalictrum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai

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**Abstract** - This study was carried out to develop an effective seed propagation method for *Thalictrum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai by analyzing seed dormancy types and germination characteristics. Seeds were collected between September to October at Gangwon province, and well-selected seeds were used while being dry-stored at 4±1 °C. The seed size ranged 4.52 × 1.58 mm and the weight of thousand seeds were 1,603.5 ± 0.02 mg. The moisture content was 7.2%. Seeds were achene type, and morphology characters showed an elliptical shape and rough texture, and light brown in color. Moist-chilling treatment was conducted for dormancy breaking because the seeds had an undeveloped embryo of liner type. The embryo had developed during a moist-chilling period, constantly, and fully developed in 10 weeks. Consequently, it seemed to be non-deep complex or intermediate complex type of morphophysiological dormancy, and embryo dormancy was broken by wet-chilling for 10 weeks. After 10 weeks of wet-chilling treatment, seed germination began. Germination percentage was higher in dark condition rather than light condition and recorded the maximum at 25 °C in the dark (16.3%). A pre-soaking treatment with a combined plant growth hormones promoted germination and shortened T<sub>50</sub>. Specifically, seed germination of 84.5% was achieved by pre-soaking of seeds with a combined solution of 500 mg/L GA<sub>3</sub> and 10 mg/L kinetin for 24 h after a wet-chilling treatment for 10 weeks. Thus the effect of plant growth hormones coupled with chilling temperature on seed breaking dormancy provide a subsequent growth of seedlings for successful plantation.

**Key words** - Dormancy breaking, Morphophysiological dormancy, Plant growth hormone, Seed propagation, *Thalictrum* species

### Introduction

Genus *Thalictrum* of Ranunculaceae has approximately 200 varieties throughout the world, mostly populated in temperate regions. *T. rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai (hereinafter *T. rochebrunianum* var. *grandisepalum*), a perennial plant, has ovules in reverse position, no petals and can be distinguished by a clear vein on the side of its achene. *T. rochebrunianum* var. *grandisepalum* is native to Korea and of particular academic and ecological interest as it is listed in as the first grade of specific plant species for the floral region (ME, 2011; 2007). It is indigenous to mountainous regions north of Seoul and grows to 70-100 cm in height. The leaflets

are 2-3 cm in length and 1.5-2.5 cm in width. They are obovate and their bottom is round or heart-shaped with three serrates at the end. A panicle with anthers, yellow filaments and a lavender calyx, of which's veins are discernable, blooms from July to August, when is summer season in Korea (Lee, 2003).

Wild *T. aquilegifolium* var. *sibiricum* has long been used as home remedy for bruises, stomach aches and neuralgia (Lee, 1981). *T. uchiyamai*, an endemic species to Korea, contains in its underground part corypalline, protothalpine and others with exceptional antiseptic attributes in roots and has been reported as third grade of phenolic compounds (Lee and Lee, 1981, 1982; Lee, 1984). Therefore, wild *Thalictrum* species can be deemed a side effects and tolerance free alternative to synthetic medicines and could be developed as

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a natural drug. However, considering that the medicinal value lies in the roots, an abundance of the plant must first be established. Rigorous research into mass propagation using seeds is first needed.

Dormant seeds that do not germinate despite of suitable environmental factors, have been categorized into five in Baskin and Baskin (2004) after a careful consideration of the morphology, physiology and biology of subject matter: physiological, morphological, morphophysiological, physical, and combinational dormancy. Seeds of the Ranunculaceae family has usually showing morphological dormancy, but in the *T. rochebrunianum* var. *grandisepalum* was reported to have also showing morphophysiological dormancy, recently (Lee *et al.*, 2015).

To break dormancy, appropriate methods such as injection of plant growth hormone or priming via mineral salts and wet-chilling can be used (Jang *et al.*, 2016; Lee *et al.*, 2000). These intentional treatments cause the grade of germination retardant and catalyst within the seed to change which promotes germination. Dormancy of *Lespedeza tomentosa* seeds were broken by chemical treatment as concentration of moisture increases in seeds (Lee *et al.*, 2016). Dormancy of peach, Persian walnut and apple seeds were broken in low temperature as germination retardants (like ABA) decrease (Lipe and Crane, 1966; Martin *et al.*, 1969; Rudnicki, 1969). In addition, dormancy of hazel, beech, *Fraxinus excelsior* and apple seeds were also broken by low temperature treatment as gibberellin's concentration increases in seeds (Frankland and Wareing, 1968; Kentzer, 2015; Ross and Bradbeer, 1968; Sinska and Lewak, 1970).

Hamilton and Carpenter (1975) used oleaster seeds to confirm that storing them at 5°C for 90 days causes germination promoting substance to be created. Furthermore, this catalysts broke physiological dormancy caused by a germination retardant in coumarin, and also Paul *et al.* (1973) reported that storing *Pinus taeda* seeds in tiers at 5°C for 42 days caused the catalysts to increase while reducing concentration of the retardant close to zero.

*T. rochebrunianum* var. *grandisepalum* has much ornamental value while holding a significant promise in natural antineoplastic, hypotensive and antiseptic research. This research was performed to find and analyze dormancy patterns of seeds

and its germination. In addition, discovery of ideal germination conditions and improvement of germination productivity were attempted to make an efficient propagation method.

## Materials and Methods

### Seed characteristics

Seeds of *T. rochebrunianum* var. *grandisepalum* for research were harvested in and around Dunnae-myeon, Hoengseong-gun, Gangwon-do from September 10th to October 6th of 2011. After harvesting, the seeds were kept at indoor temperature for a month to dry the seeds, then some well-selected seeds were used to classify dormancy types, immediately. And most seeds stored at  $4 \pm 1.0^\circ\text{C}$  in dry condition using silica gels (Mr. Keeper, Sungel, Korea) for germination test.

Seeds length, width (mm) and weight of thousand seeds (mg) were measured using digital vernier calipers (NA500-150S, Bluebird, China) and an electronic scale (IB-610S, Innotem, Korea). Each measurement was repeated 10 times. Moisture content (MC, %) was measured difference in the weight between before and after seed weight by drying in 70°C with hot air for 48 hours.

The seeds were dipped for 7 days and the ratio of MC to moisture absorption was observed. The seeds were dipped into test tubes containing 15 ml of distilled water then refrigerated at  $4 \pm 1.0^\circ\text{C}$ . Every 24 h, the seeds were taken out. Seed coats were wiped with filter paper (Advantec, Toyo, Japan), weighed then dipped in new distilled water. The interval moisture absorption rate was calculated by the accumulative MC on top of the average weight of seeds.

### Seed dormancy type

To classify dormancy types, three stage as follows were conducted. First, seeds were cut along the major or minor axis using a stainless blade (Platinum ST-300, Dorco, Korea). The cross-section was photographed by an icamscope (ICS, Sometech inc., Korea) then observed using IT Plus 4.0 software. And then, embryo length was calculated as a ratio to seed length (E:S ratio, %) (Vandelook *et al.*, 2007). Embryo development status was distinguished by criteria such as whether cotyledon (s) was differentiated or developed but not differentiated, whether embryo was immature or not existed.

Second, the seeds were submerged in distilled water for 24 hours at  $4 \pm 1.0^\circ\text{C}$  then placed on petri dishes ( $\varnothing$  8.9 mm,) using two sheets of filter paper ( $\varnothing$  90 mm, Advantec, Toyo, Japan). The petri dishes were placed in to growth chambers set up at light (continuous light of  $23 \pm 0.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) or dark condition and  $20^\circ\text{C}$ . Filter paper were wetted with sterilized water every day to avoid drying, and percent germination (%) was calculated by totaling all seeds germinated in 30 days.

Third, the seeds were dipped in water for seven days and the ratio of moisture absorption was observed. The seeds were dipped into test tubes containing 15 ml of distilled water then kept refrigerator at  $4 \pm 1.0^\circ\text{C}$ . Every 24 hours, the seeds were taken out, and seed coats were wiped with filter paper, weighed then dipped into new batches of distilled water. The interval moisture absorption rate (%) was calculated by the accumulative MC in addition of the average weight of seeds, calculated every 24 hours.

#### Breaking of MPD according to wet-chilling treatment

A wet-chilling condition was established by disinfecting the seeds with 1% (v/v) NaOCl for 10 minutes, wrapping them in gauze and putting in a sealable glass jar then sufficiently filling the jar with cotton. The jar was then wetted with sterilized water, reversed to rid run-off water then sealed with a lid. Once the wet-chilling condition was set up, the jar was kept at  $4 \pm 1.0^\circ\text{C}$ . The seeds were then weighed every two weeks then moisture absorption rate was calculated by the accumulative MC. Ten seeds were taken randomly then cross section was analyzed to observe vis-a-vis development of the embryo.

#### Seed germination according to environmental conditions and PGRs

The seeds with developed embryos by wet-chilling treatment for 10 weeks were placed upon disposable petri dishes with two sheets of filter paper placed at the bottom (top of paper method). The petri dishes were placed in the germinator at different temperatures - 15, 20, 25, and  $30^\circ\text{C}$  - with light (continuous light of  $23 \pm 0.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) or dark condition, then analyzed.

GA<sub>3</sub> was given at 100, 200 and 500 mg/L and kinetin at 10

and 20 mg/L. The seeds that had underwent wet-chilling treatment were submerged in respective solutions for 24 h. The seeds were then cleaned of the solution using distilled water three times. Analysis was carried out under the germination conditions outlined above.

#### Investigations and statistical analysis

The seeds were examined every day, and those of which's embryo had broken through the seed coat were deemed germinated, germination energy (GE, %, germination percentage at 7 days after sowing), percent germination (PG, %, number of germinated seeds / number of total seeds)  $\times$  100), and T<sub>50</sub> (days, number of days until 50% of percent germination), was calculated.

All germination experiments were repeated 4 times with 50 seeds in each experiment. SAS version 9.3 (SAS institute Inc., Cary, NC, USA) was used to compute average and standard error. Using Duncan's multiple range test, statistical significance was established at the level of  $p < 0.05$ .

## Results

#### Characteristics of seed morphology and dormancy

The seed's dimensions were  $4.52 \times 1.85$  mm while the weight of thousand seeds was  $1,603.5 \pm 0.02$  mg. The MC was 7.2%. The seeds were an achene in light brown (Fig. 1A). They were also elliptical in shape with both ends pointed. A furrow could be found along the major axis which confirmed the commonalities shared by seeds of the genus *Thalictrum* (Tamura, 1995). A study of the seed's cross section revealed that the seed case (pericarp) was fairly thick at  $0.31 \pm 0.09$  mm (Fig. 1B). An elongated and elliptical embryo of which's border coincided significantly with the endosperm was covered by a thin inner seed coat (tegman) and found near the hilum. In addition, a crack smaller consist of 10  $\mu\text{m}$  was found on the pericarp (Fig. 1C).

Seeds had a distinguishable embryo at 0.75 mm and endosperm not contained cotyledon or hypocotyl (Fig. 1B). E:S ratio of seeds were 6.9%, and direct sowing experiments of seeds led to lack of germination within 4 weeks (data not shown). An analysis of the seeds that had been submerged for 7 days revealed that, despite the thickness of achene's

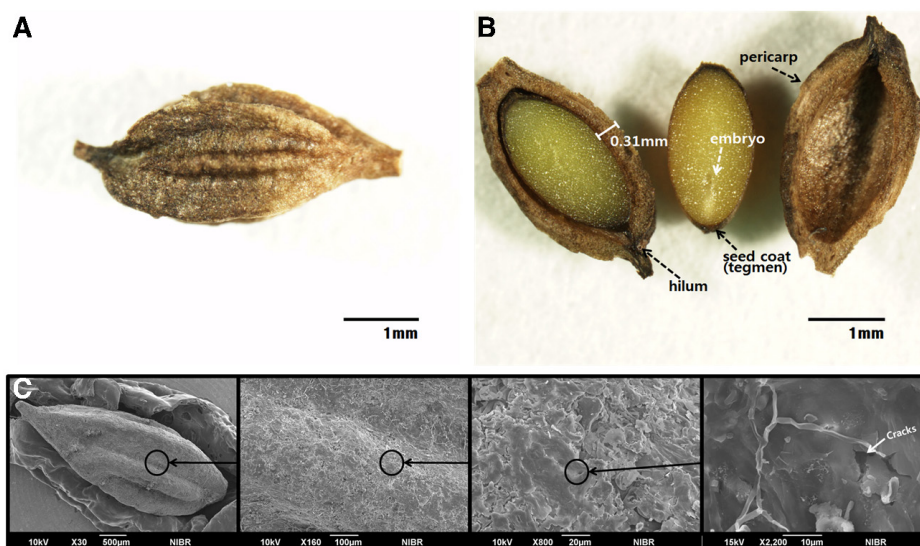


Fig. 1. Seed structure of *Thalictum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai. A, Achene exterior; B, Longitudinal section of seed; C, Scanning electron microscope pictures of seed coat surface. Circles indicate a seed coat enlargement.



Fig. 2. Embryo development of *Thalictum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai seeds during wet-chilling period at 4°C. Scale bars: 1.0 mm.

pericarp, MC rose rapidly to 61.5% in 24 h and reached the maximum MC (64.9%) after 48 h (data not shown).

#### Embryo growth and water absorption according to wet-chilling treatment

The observation of immature embryo during the wet-chilling

process found the embryo becoming distinguishable boundary under two weeks and its growth evident. The pericarp that absorbed moisture became less rough and its color turned from light brown to dark brown (Fig. 2).

Six weeks of wet-chilling treatment saw the embryo's volume, in addition to length, increasing. And the 10 week

saw the embryo's length becoming 3.3 times (2.47 mm) that before the treatment, in addition 70% of the total seed size was taken up by the embryo (Fig. 3). The 12 week saw a further development of the embryo and the form of a cotyledon could be recognized. The hilum started to crack and germination began. As germination took place, the pericarp burst along the major axis and two cotyledons as seedlings were seen (Fig. 2).

By analyzing the incremental MC under wet-chilling treatment, in the 2nd week, when the embryo development began, it reached the maximum MC of 72.8% (Fig. 4). This figure is greater than that after 7 days of dipping treatment (64.9%). The following 8 week that saw a continual development of the embryo saw the seeds maintain a similar level of

MC under both methods. The immature embryo of *T. rochebrunianum* var. *grandisepalum* seed was thought to have absorbed enough moisture for embryo growth during the 2 weeks of wet-chilling treatment.

#### Germination characteristics by temperature and light condition

Germination condition research was conducted with seeds that treated wet-chilling condition for 10 weeks. In the dark at 25°C, the seeds germinated at the rate of 16.3% (Fig. 5). The second best rate (10.5%) came with 20°C, light. Regardless of light condition, germination rate was uniformly low at 15°C and 30°C. The seeds tended to fare better in dark so it is thought that light suppressed germination.

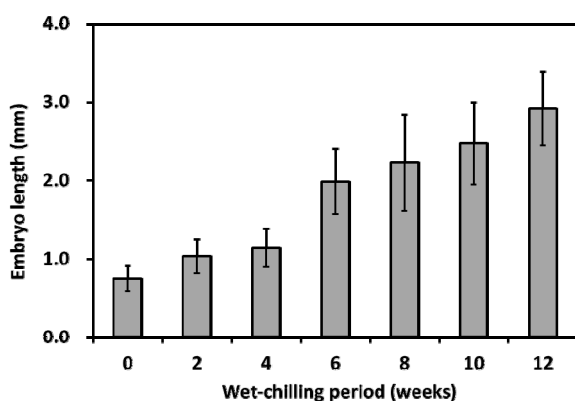


Fig. 3. Change of embryo length of *Thalictrum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai seed during wet-chilling period at 4°C. Bars represent standard errors (n=4).

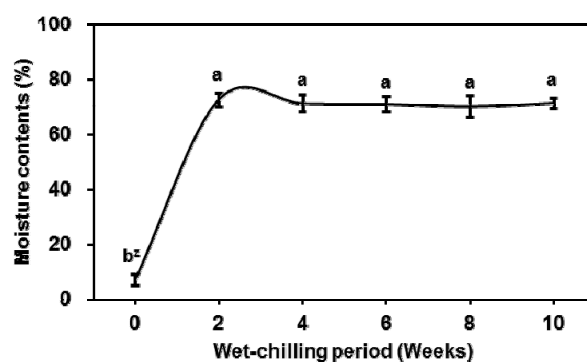


Fig. 4. Moisture content changes of *Thalictrum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai seed during wet-chilling period at 4°C. Bars represent standard errors (n=4).

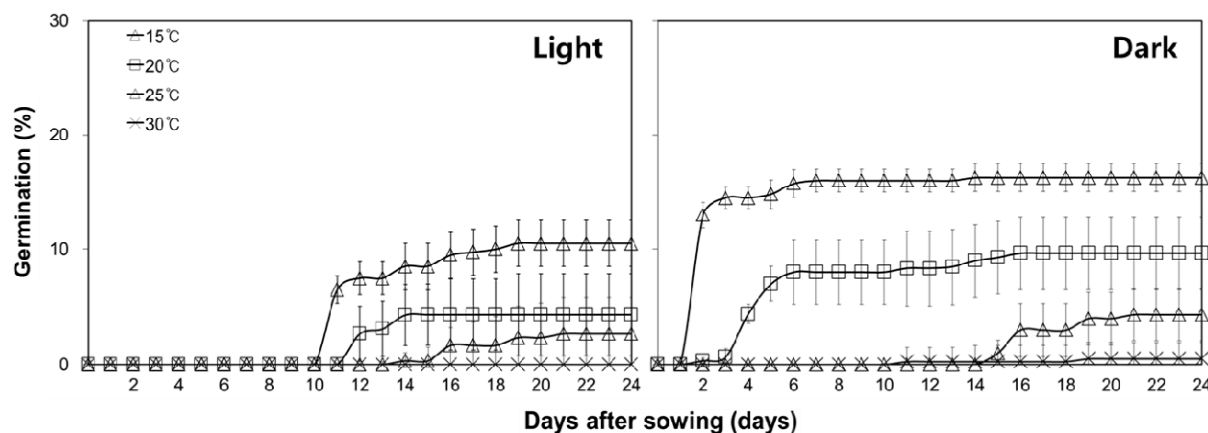


Fig. 5. Effect of light and temperature on germination rate of *Thalictrum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai seeds after wet-chilling treatment for 10 weeks. Bars represent standard errors (n=4).

Table 1. Effect of GA<sub>3</sub> and kinetin concentration on seed germination of *Thalictrum rochebrunianum* var. *grandisepalum* (H. Lev.) Nakai at 25°C, dark condition

PGRs (mg/L)		PG <sup>z</sup> (%)	GE <sup>y</sup> (%)	T <sub>50</sub> <sup>x</sup> (days)
GA <sub>3</sub>	Kinetin			
0	0	16.3d <sup>w</sup>	74.2a	-
	10	51.8b	52.9ab	8.7c
	20	32.0b-d	52.2ab	-
100	0	44.8bc	57.2ab	20.0c
	10	26.0cd	59.4ab	-
	20	49.5b	53.0ab	17.5c
200	0	70.5ab	54.4ab	18.5c
	10	72.0ab	46.6ab	11.3c
	20	25.0cd	33.0bc	-
500	0	45.8b	15.8c	39.0b
	10	84.5a	54.8ab	9.8c
	20	44.8bc	46.5ab	53.0a
Significance				
GA <sub>3</sub>		*	*	*
Kinetin		***	ns	***
GA <sub>3</sub> × Kinetin		***	*	*

<sup>z</sup>Percent germination.

<sup>y</sup>Germination energy: (Number of germinated seeds at day 7/number of total germinated seeds)×100.

<sup>x</sup>Days required for 50% seed germination.

<sup>w</sup>Mean separation within columns by Duncan's multiple range test,  $p < 0.05$ .

ns,\*,\*\*\* non-significant or significant at 5 or 0.1% level, respectively.

### GA<sub>3</sub> and kinetin treatment promoting seed germination

The 200 mg·L<sup>-1</sup> GA<sub>3</sub> produced the best result of 70.5% germination rate, a 54.2% improvement over the control (Table 1). The 10 mg/L kinetin produced the final germination rate of 51.8%, an 35.5% improvement over the control, but failed to match that of GA<sub>3</sub>.

As mixture, 500 mg/L GA<sub>3</sub> and 10 mg/L kinetin significantly improved the germination rate to 84.5%. In general, greater the concentration of GA<sub>3</sub>, to produce better germination rate. With kinetin more dilute the solution, better the germination rate.

In terms of germination energy, the control rate of 74.2% could not be matched by any treatment. A highly concentrated (500 mg/L) solution of GA<sub>3</sub> caused the germination energy to

drop to 15.8% and in general the increase in concentration caused a noteworthy decrease in germination energy. T<sub>50</sub> was longest in 500 mg/L GA<sub>3</sub> and 20 mg/L kinetin mixture (53.0 days) and in 500 mg/L GA<sub>3</sub> (39.0 days). The high concentrations of GA<sub>3</sub> cause a significant increase in T<sub>50</sub>. T<sub>50</sub> in other treatments ranged from 8.7 to 20.0 days and there was no statistically significant difference.

## Discussion

There are two types of Ranunculaceae embryos; the smaller and elliptical rudimentary type and the more straight linear type (Geneve, 2003; Nomizu *et al.*, 2004). Both types epitomize the immature embryo. Lee *et al.* (2012b) reported that, wild *Adonis amurensis*, *Aquilegia buergeriana* var. *oxysepala* and *Ranunculus crucilobus* are rudimentary type while, *Pulsatilla tongkangensis* is linear type. They are both underdeveloped embryos. The genus *Thalictrum* studied in this paper has an unclear boundary, immature, and elongated linear embryo and is very similar morphologically to compare other Ranunculaceae. Consequently, the embryo being analyzed deemed the immature and linear type.

In categorizing dormancy, the immature embryo seeds can be divided into morphological dormancy, embryonic immaturity and morphophysiological dormancy caused by physiological conditions such as embryo growth retardant. In addition, these types of dormant seeds can be broken out of dormancy and force embryonic growth by wet chilly or wet high temperature treatments (Baskin and Baskin, 2004).

Seeds of oleaster were broken out of physiological dormancy by storing at low temperature for 90 days - upon which coumarin, a germination catalyst started to be created (Hamilton and Carpenter, 1975). Seeds of *Pinus taeda* saw the concentration of germination catalysts increase over the course of 42 days at low temperature. The concentration of ABA, a retardant, decreased to almost zero (Paul *et al.*, 1973). Considering also the result of this research, dormancy breaking and embryo growth that follows wet-chilling treatment was thought to be the grade of germination retardant and catalyst within the seed.

Morphophysiological dormancy (MPD) demonstrates traits of both morphological dormancy (MD) and physiological

dormancy (PD). This dormancy type requires PD to be broken before or after the underdeveloped embryo develop and the embryo to growth to certain size before radicle emerges. Low or high temperature induced embryonic development and whether dormancy is broken by GA<sub>3</sub> can be used to further (sub) categorization (Baskin and Baskin, 2004). Lee et al. (2012a; 2015) used the categorization criteria to conclude that seed of *T. rochebrunianum* var. *grandisepalum* is a non-deep simple type because its seeds rapidly grew at 25/15°C after a 4-8 weeks of low temperature treatment at 1 or 5°C, that is embryo seemed to grow only in high temperature.

However, we have observed a continual growth at low temperature and subjected the seeds to at least 10 weeks of low temperature to induce germination, embryo development and dormancy breaking. We conjecture that seed of *T. rochebrunianum* var. *grandisepalum* may actually be the type non-deep complex or intermediate complex-subtypes of MPD where embryo develops in low temperature.

Germination rate of seeds that undergo MPD can be improved by GAs (Hidayati et al., 2000). *Sambucus sieboldiana* var. *pendula* seeds, of which's dormancy types are reported to be non-deep complex and intermediate complex, dormancy breaking was significantly bettered by a 100 mg/L GA<sub>3</sub> at 5°C (Kim et al., 2013) and produced a similar result with one presented in this paper.

The results suggest that submerging them in a mixture of 500 mg/L GA<sub>3</sub> and 10 mg/L kinetin can significantly better the seeds with development enough embryos of *T. rochebrunianum* var. *grandisepalum*. T<sub>50</sub> is relatively short at 9.8 days so germination may be achieved in a short period of time and seedling produced with consistency. Furthermore, an analysis of correlation between germination rate and concentrations of PGRs is statistically significant within 5% of GA<sub>3</sub> alone, and 0.1% of kinetin and GA<sub>3</sub> in mixture (Table 1). It is thought that seed propagation of *T. rochebrunianum* var. *grandisepalum* can be facilitated by 10 weeks of wet-chilling treatment, submerging the seeds in a mixture of 500 mg/L GA<sub>3</sub> and 10 mg/L kinetin for 24 hours then sowing them in dark condition at 25°C. Thus the effect of plant growth hormones coupled with chilling temperature on seed breaking dormancy provide a subsequent growth of seedlings for successful plantation.

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