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Original Research Article

Effect of Storage Conditions and Scarification on *in vitro* Seed Germination in *Lorathus tanakae* Hosok

Amal Kumar Ghimeray[1], Hyun Woo Lee[1], Bo-Duk Lee[1], Pankaja Sharma[1], Ie Sung Shim[2] and Cheol Ho Park[1]*

[1]Department of Bio-health Technology, Kangwon National University, Chuncheon 200-701, Korea.
[2]Departments of Horticulture, Seoul City University, Seoul 130-743, Korea

Abstract - *Loranthus tanakae* (Franch. & Sav.) is an endangered species of mistletoe, distributed in Korean peninsula. The objective of our research is to determine the effect of storage duration and conditions [air flow (AF) and air tight (AT)] at different temperatures for survivability and germination of mistletoe seeds, and also to monitor the effect of seed scarification on germination *in vitro*. The result revealed that the seeds stored in natural conditions (no stratification) showed highest survival rate of 100% and retained up to 93.3% even after two months of storage in natural conditions and showed higher germination percentage (90%) compare to after ripened seeds. However, the seed stored at 0°C decreased the germination percentage (ranged from 63 to 73%). Therefore, it can be confirmed that mistletoe does not need after ripened treatment to promote germination. Our research also showed that the storage of *L. tanaka* seeds in freezing temperatures of -20°C and in room temperature for long time either in AT or AF conditions caused the loss of survival and germination rate. On the other hand, the chemical scarification (0.01N HCl incubation for 12 hrs. at 38°C) method was proven more effective to enhance germination percentage of *L. tanakae*. Regarding the temperature regime, 22°C showed early germination of mistletoe seeds *in vitro*.

Key words - Endangered species, Stratification, Scarification, *Loranthus tanakae*, Germination

Introduction

Loranthus tanakae (Franch. & Sav.) Hosok. a species of mistletoe, synonymously called *Hyphear tanakae* Hosokawa, is a semi-parasitic, deciduous shrub of Loranthaceae family that grows on the branches or twigs of trees and is distributed in Korean peninsula and China (Huaxing *et al.,* 2003). The plant is considered as an endangered species and grows in different host plants like *Quercus, Prunus, Morus* and also in *Poppulus* species (Kim *et al.,* 2013; Lee 2010b). It develops a special structure called haustorium which penetrates into the phloem tissue of the host plan to absorb water and nutrients (Kim *et al*., 2013; Calvin and Wilson 1998; Lee *et al.,* 2009). It is considered as a medicinal plant as it is known to produce variety of bioactive compounds (quercetin glycosides, kaempferol glycosides, etc) which have significant anti-tumor properties (Kim *et al.,* 2004).

*Corresponding author. E-mail : chpark@kangwon.ac.kr

In Korea, the leaves, twigs and stem of mistletoe are used traditionally to make herbal medicine. A decoction of leaves and stem consumed traditionally is believed to cure number of ailments, including immune stimulation. However, due to over exploitation, the plant became endangered. Therefore, this research aims to restore its population and multiplication of plant by *ex-situ* method. For that, here we studied the survivability and germination of mistletoe seeds after harvest, and the effect of storage duration and conditions (air flow and air tight) at different temperatures were determined. We also examined the effect of seed scarification on mistletoe seed germination *in vitro.*

Materials and methods

Sample collection

Mature mistletoe (*Loranthus tanakae*) plants with yellow fruits in a dark brown stem, grown in the oak trees in natural habitat were harvested in 2nd week of Nov. 2013 from Yuljeon-ri,

Hongcheon-gun, Gangwon-do (37.74N, 128.32E), Korea.

Storage conditions of mistletoe seeds at different temperatures

After harvesting, mistletoe fruits were separated from the stem which was considered as non-after-ripened seeds and subjected to germination test (total 30 seeds in 3 replications). Remaining fruits were stored at different temperatures (-20, 0, +4 and room temperature (22 ± 2°C) in air tight (AT) or air flow (AF) conditions for one and two months duration (considered as after-ripened). Other fruits were preserved in a natural environment for two months to compare the germination pattern with stratified seeds.

Chemical and Mechanical scarification of seed

In chemical scarification method, 30 seeds (without pulp) in three replications were scarified by immerging in 0.01N HCl of appropriate volume and incubated at a constant temperature of 38°C for 12 hrs. In mechanical scarification, seeds were placed on sterilized river sand moistened with distilled water in a plastic container and rotated gently for 2 hrs. After scarification, the seeds were incubated at 38°C for 12 hrs. The unscarified seeds were taken as a control. After the completion of incubation time, the scarified and unscarified seeds were subjected to germination test after sterilization.

Sterilization of mistletoe seeds and germination test

The pulp of fruits (non-after ripened or after ripened) were removed manually by rubbing them against paper towel. Next, the seeds were washed in running water and dried in paper towel. To avoid contamination from microorganisms, 30 seeds were washed by immersing in 50 ml of sodium hypochlorite solution (1.5%) for 3 min and were shaken vigorously. They were then washed with six changes of distilled water. Another 30 seeds were washed only with distilled water for six changes which served as control. After draining out the liquid of the final wash, three replications of 30 seeds were placed on absorbent paper that had been moistened with distilled water in Petri dishes (9-cm). The petri dishes were then placed at two different temperature (15°C and 22°C) regimes in germinator under 16/8 hrs photoperiod with 80% relative humidity. During the germination period, petri dishes were watered with distilled water as per need to ensure adequate moisture for seed germination. The seeds in the dishes were maintained for germination with close observation. After 4 weeks, the germination percentage of each treatment was calculated from the average of 3 replications' percentage. The survivability of the seeds was also evaluated every week by counting the remaining individuals whose seed coat color were not changed from green to brown.

Statistical analysis

The data on germination (%) and survival rate (%) were analysed using ANOVA. The treatment means were tested by the Tukey tests at the 5% level of significance.

Results

Survival rate of seeds during *in vitro* germination process

The temperatures at which the seeds were stored had a marked influence on the survivability of mistletoe. According to the data observed after 28 days, the freshly harvested seeds when allowed to germinate in Petri dish showed the highest survival rate up to 100% (one month) and retained up to 93.3% even after two months of storage in natural conditions (Table 1 and 2). The survival rates of seeds stored for one and two months were not significantly different with the seeds stored at 0°C either in AT or AF conditions, where the survival rate ranged from 80 to 90% at 15 and 22°C germinator.

The seeds stored at 4°C also showed higher rate of survivability (observed in a germinator at 15°C) with 93.3% (one month stored) and 80% (two months stored) in AF conditions. In the same conditions, the survivability rates were 83.3 and 87% respectively for the one and two months stored seeds at 22°C germinator. In the AT condition, 80 (one month stored) and 73.3% (two months stored) survivability was observed at 15°C germinator and 83.3 (one month stored) and 76.7% (two month stored) survivability were observed at 22°C germinator. The one month stored seeds at room temperature in AF condition showed 50 and 73.3% survivability when tested in a germinator at 15 and 22°C respectively. However, 0% percentage survivability was observed in the seeds stored for one month in AT condition and also in the seeds stored for two months in both AF and AT conditions. The after-ripened seeds stored at -20°C for a month in AF

Table 1. Survival rate of 1 month stored mistletoe seeds in different temperature and conditions as determined by germination test at 15 and 22˚C

			Number of seed survived (% in parentheses)				
Conditions	Temp. used for germination (˚C)	No. of seeds used in 3 replications	7 days	14 days	21 days	28 days	Total % of survival after 28 days
Non-after ripened seeds	15	30	30(100)	30(100)	30(100)	30(100)	100^{a}
	22	30	30(100)	30(100)	30(100)	30(100)	100^{a}
-20˚C AF	15	30	4.3(14.3)	3((10)	3(10)	3(10)	10.0^{f}
	22	30	11(36.7)	3(10)	1(3.3)	1(3.3)	3.3^{gh}
-20˚C AT	15	30	3.7(12.3)	3.7(12.3)	2.7(9)	1.7(5.7)	5.6^{fg}
	22	30	7(23.3)	4(13.3)	3(10)	1(3.3)	3.3^{gh}
0˚C AF	15	30	29.1(97)	27(90)	27(90)	27(90)	90.0^{b}
	22	30	30(100)	28(93.3)	27(90)	26(86.7)	87.0^{bc}
0˚C AT	15	30	30(100)	27(90)	25(83.3)	25(83.3)	83.3^{c}
	22	30	30(100)	28(93.3)	27(90)	27(90)	90.0^{b}
+4˚C AF	15	30	30(100)	30(100)	28(93.3)	28(93.3)	93.3^{ab}
	22	30	28(93.3)	27(90)	26(86.7)	25(83.3)	83.3^{c}
+4˚C AT	15	30	26(86.7)	25(83.3)	25(83.3)	24(80)	80.0^{c}
	22	30	26(86.7)	26(86.7)	26(86.7)	25(80)	83.3^{c}
22±2°C AF	15	30	24(80)	15(50)	15(50)	15(50)	50.0^{e}
	22	30	24(80)	23(76.7)	23(76.7)	22(73.3)	73.3^{d}
22±2°C AT	15	30	2(6.7)	0(0)	0(0)	0(0)	0.0^{h}
	22	30	3(10)	0(0)	0(0)	0(0)	0.0^{h}

Small letters in superscripts represent significant differences at the level of 5% according to Tukey Test.

Table 2. Survival rate of 2 months stored mistletoe seeds in different temperature and conditions as determined by germination test at 15 and 22˚C

			Number of seed survived (% in parentheses)				
Conditions	Temp. used for germination (˚C)	No. of seeds used in 3 replications	7 days	14 days	21 days	28 days	Total % of survival after 28 days
Non-after ripened seeds	15	30	30(100)	28(93.3)	28(93.3)	28(93.3)	93.3^{a}
	22	30	30(100)	29(96.7)	28(93.3)	28(93.3)	93.3^{a}
-20˚C AF	15	30	7(23.3)	2(6.7)	1(3.3)	0(0)	0.0^{e}
	22	30	22(73.3)	12(40)	4(13.3)	2(6.7)	6.7^{d}
-20˚C AT	15	30	10(33.3)	2(6.7)	0(0)	0(0)	0.0^{e}
	22	30	19(63.3)	7(23.3)	2(6.7)	0(0)	0.0^{e}
0˚C AF	15	30	30(100)	28(93.3)	25(83.3)	25(83.3)	83.3^{b}
	22	30	30(100)	30(100)	28(93.3)	27(90)	90.0^{a}
0˚C AT	15	30	28(93.3)	25(83.3)	25(83.3)	25(83.3)	83.3^{b}
	22	30	30(100)	28(93.3)	26(86.7)	24(80)	80.0^{bc}
+4˚C AF	15	30	28(93.3)	28(93.3)	26(86.7)	24(80)	80.0^{bc}
	22	30	30(100)	29(96.7)	27(90)	26(86.7)	87.0^{ab}
+4˚C AT	15	30	25(83.3)	25(83.3)	24(80)	22(73.3)	73.3^{c}
	22	30	25(83.3)	25(83.3)	25(83.3)	23(76.7)	76.7^{c}
22±2°C AF	15	30	0(0)	0(0)	0(0)	0(0)	0.0^{e}
	22	30	0(0)	0(0)	0(0)	0(0)	0.0^{e}
22±2°C AT	15	30	0(0)	0(0)	0(0)	0(0)	0.0^{e}
	22	30	0(0)	0(0)	0(0)	0(0)	0.0^{e}

Small letters in superscripts represent significant differences at the level of 5% according to Tukey Test.

condition showed 10 and 3.3% survival rate at 15 and 22°C germinator respectively. In same condition after two months, nearly 6.7% seeds retain their survival capacity. At the same temperatures in AT condition, the survival rates were further decreased to 5.6 and 3.3% at 15 and 22°C germinator respectively. Furthermore, the survival rate was decreased to 0% in the two months stored seeds in AT conditions.

Effect of storage temperatures on germination rate

The germination tests were performed at two constant temperature regimes of 15 and 22°C and the results are shown in Table 3 and 4. When the total germination of seeds was counted after 28 days, the freshly harvested (non-after-ripened) seeds showed 67 and 70% germination at 15 and 22°C respectively. The mistletoe seeds kept for 2 months in natural condition (as described in method) without any treatment and allowed to germinate at both temperature regimes of 15 and 22°C showed higher percentage of germination (90%). However, the seeds stratified at 0°C decreased the germination by 20%. The germination rate of one and two months stored seed at 0°C in AF and AT conditions were not significantly different, where the germination ranged from 63 to 73% in the germinator at 15°C and 22°C.

The seeds stored at 4°C for one and two months in AF condition did not show any significant difference in germination, where the percentage ranged from 73 to 77 % at 15°C germinator (Table 3 & 4). However in AT condition, the germination percentage of the seeds stored for two months was reduced to 43.3% from 57% of one month stored seeds. Similarly, one and two months stored seeds at 4°C also showed a low germination percentage (47 to 50%) in 22°C germinator with no significant difference between the seeds stored in AF and AT conditions. Previously, Lee *et al*. (2010a) also conducted an *in vitro* germination test of mistletoe seeds on 4°C stored seeds in different growth media (without viscin) where they found 60-69% of germination in freshly harvested seed, later the germination percent was reduced with increase in storage duration.

The seeds stored for one month in room temperature (22 ± 2°C) in AF condition showed 70 and 83.3% of germination at 15 and 22°C germinator respectively within two weeks. However, it was reduced to 0% germination for the seeds stored for one month in AT condition and also for the seeds stored for two months in both AF and AT conditions. The stratified after-ripened seeds stored for one month at -20°C in AF condition showed 10 and 3% germination at 15 and 22°C germinator respectively. However, the germination was decreased to 0% with an increase in storage duration for 2 months in AT or AF condition. In another study by Scharpf (1970) in dwarf mistletoe (*Arceuthobium abietium* and *A. occidentale)* found that the temperature affected the viability and germination most noticeably. Our results are in agreement with the findings of Scharpf (1970), in which the dwarf mistletoe seeds kept for two months for after-ripened showed significantly lower percentage of germination than did fresh seeds allowed to germinate immediately.

Germination pattern

The non-after-ripened seeds, after ripened seeds (at -20 and 0°C) and scarified seeds showed significantly different germination pattern when subjected to germinator at two constant temperature regimes of 15 and 22°C (Table 3 and 4). The freshly harvested (non-after ripened) seeds started germination within 9 days after placing the seeds in germinator at 22°C and reached maximum 43.3% after two weeks, However, the germination started after 11 days at 15°C germinator and showed maximum germination of 30% after three weeks. On the other hand, the mistletoe seeds which were kept for 2 months in natural condition showed higher percentage of germination (60 and 83.3% at 15 and 22°C respectively) within two weeks.

The 0°C stratified seeds kept for a month in AF and AT conditions showed maximum germination of 46.7 & 36.7 % respectively within three weeks at 15°C germinator. However, at 22°C germinator, the rate was 40 & 33.3% in AF and AT conditions respectively within two weeks. Similar trends of germination were observed for the 2 months stratified seeds at 0°C where the seeds were germinated maximum within 2 to 3 weeks in the range of 30 to 53.3%.

With 4°C stratification, the one month stored seeds showed early commencement (after 5 days) of germination which was in the range of 10 to 23.3% within a week. The maximum germination rate was recorded in a germinator at 15°C within 2 weeks with 53.3% in AF conditions, while germination at the 22°C germinator was found maximum 30% in AT after 2

Table 3. Germination rate of 1 month stored mistletoe seeds in different temperature and conditions as determined by germination test at 15 and 22˚C

			Number of germinations (% in parentheses)					
Conditions	Temp. used for germination (˚C)	No. of seeds used in 3 replications	7 days	14 days	21 days	28 days	Total germinated seeds after 28 days	% of germination after 28 days
Non-after ripened	15	30	0(0)	7(23.3)	9(30.0)	4(13.3)	20	67.0^{cd}
	22	30	0(0)	13(43.3)	4(13.3)	4(13.3)	21	70.0^{c}
-20˚C AF	15	30	0(0)	3(10)	0(0)	0(0)	3	10.0^{g}
	22	30	0(0)	0(0)	0(0)	1(3.3)	1	3.3^{h}
-20˚C AT	15	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{i}
	22	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{i}
0˚C AF	15	30	0(0)	7(23.3)	14(46.7)	0(0)	21	70.0^{c}
	22	30	0(0)	12(40)	8(26.7)	1(3.3)	21	70.0^{c}
0˚C AT	15	30	0(0)	7(23.3)	11(36.7)	2(6.7)	20	67.0^{cd}
	22	30	0(0)	10(33.3)	8(26.7)	2(6.7)	20	67.0^{cd}
+4˚C AF	15	30	4(13.3)	16(53.3)	2(6.7)	1(3.3)	23	77.0^{b}
	22	30	7(23.3)	7(23.3)	2(6.7)	2(6.7)	18	57.0^{e}
+4˚C AT	15	30	3(10)	16(53.3)	1(3.3)	0(0)	20	70.0^{c}
	22	30	6(20)	9(30)	0(0)	0(0)	15	50.0^{f}
22±2°C AF	15	30	17(56.7)	4(13.3)	0(0)	0(0)	21	70.0^{c}
	22	30	22(73.3)	3(10)	0(0)	0(0)	25	83.3^{a}
22±2°C AT	15	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{i}
	22	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{i}

Small letters in superscripts represent significant differences at the level of 5% according to Tukey Test.

Table 4. Germination rate of 2 month stored mistletoe seeds in different temperature and conditions as determined by germination test at 15 and 22˚C

			Number of germinations (% in parentheses)					
Conditions	Temp. used for germination (˚C)	No. of seeds used in 3 replications	7 days	14 days	21 days	28 days	Total germinated seeds after 28 days	% of germination after 28 days
Non-after ripened	15	30	0(0)	18(60)	9(30)	0(0)	27	90.0^{a}
	22	30	0(0)	25(83.3)	2(6.7)	0(0)	27	90.0^{a}
-20˚C AF	15	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}
	22	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}
-20˚C AT	15	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}
	22	30	0(0)	0(0)	0(0)	1(3.3)	0	0.0^{f}
0˚C AF	15	30	0(0)	9(30)	11(36.7)	2(6.7)	22	73.3^{b}
	22	30	0(0)	16(53.3)	4(13.3)	0(0)	21	70.0^{b}
0˚C AT	15	30	0(0)	10(33.3)	9(30)	0(0)	19	63.3^{c}
	22	30	0(0)	13(43.3)	6(20)	0(0)	19	63.3^{c}
+4˚C AF	15	30	2(6.7)	10(33.3)	8(26.7)	3(10)	22	73.3^{b}
	22	30	9(30)	6(20)	1(3.3)	0(0)	16	53.3^{d}
+4˚C AT	15	30	2(6.7)	10(33.3)	1(3.3)	0(0)	13	43.3^{e}
	22	30	9(30)	5(16.7)	0(0)	0(0)	14	47.0^{de}
22±2°C AF	15	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}
	22	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}
22±2°C AT	15	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}
	22	30	0(0)	0(0)	0(0)	0(0)	0	0.0^{f}

Small letters in superscripts represent significant differences at the level of 5% according to Tukey Test.

weeks. Similar trends of germination were also observed in two months stored seeds where the maximum germination was

33.3% in AF within two weeks. However, in AT conditions at 4°C, 30% germination was observed within first week. The germination patterns of seeds stored at room temperature were varied. The maximum germination was found within first week in the seeds stored for one month with 73.3% and 56.7% in the germinator at 22 & 15°C respectively in AF conditions.

Effect of chemical and mechanical scarification on seed germination

Scarification (chemical, mechanical or thermal method) has been used to break seed dormancy. It increases seed coat permeability to allow water or gases exchange and encourage germination (Crocker and Barton 1953). According to our data (Table 5 A & B), chemical scarification of the seeds showed enhanced germination rate (93%) as compared to the non-scarified seed, but the mechanical methods (sand scarification followed by 12 hrs incubation at 38°C) failed to stimulate germination (63.3%). The scarified and non-scarified seeds showed similar pattern of germination, where the maximum rate was observed within two weeks in the range of 40 to 43.3%. The sand scarification seeds showed lower survival rate with 70% compared to 100% in non-scarified seeds. In previous report, Wicker (1962) showed that chemicals like hydrogen peroxide can stimulate germination of *Arceuthobium* spp. However, Scharpf (1970) failed to stimulate germination by using sulphuric acid in *A. occidental* (type of mistletoe).

Discussion

Our study showed that the temperature played an important role on the germination of mistletoe. The stratified seeds at different temperatures [-20, 0, 4°C and room temperature (22 ± 2°C)] and conditions (AF & AT) showed different germination pattern when allowed to grow in two constant temperature regimes of 15 and 22°C. In this experiment, we followed the international seed testing rules (ISTA, 1999) according to which the seed germination was defined as the appearance of a radicle at least of 2 ㎜ long, therefore, in our experiment, radicle appearance of upto 2 ㎜ long was monitored every week for 4 weeks and considered as germinated seeds.

According to the data, seeds kept for two months in natural condition showed significantly higher percentage (above 90%) of germination than that of freshly harvested seeds (average 70%). This lower percentage of germination in freshly harvested seeds could be due to lack of full maturation of some seeds during harvesting time.

In our research, the mistletoe seeds stored at freezing

Table 5. Survivability (A) and Germination rate (B) of mistletoe seeds after chemical and mechanical scarification

(A)		Number of seed survived (% in parentheses)				
Conditions	No. of seeds used in 3 replications	7 days	14 days	21 days	28 days	Total % of survival rate after 28 days
Control (no treatment)	30	30(100)	30(100)	30(100)	30(100)	100[a]
HCl (0.01N 12 hrs 38 ˚C)	30	30(100)	28(93.3)	28(93.3)	28(93.3)	93[b]
Sand Scarification (12hrs 38 ˚C)	30	27(90)	23(76.7)	21(70)	21(70)	70[c]
(B)		Number of germinations (% in parentheses)				
Conditions	No. of seeds used in 3 replications	7 days	14 days	21 days	28 days	Total germinated seeds after 28 days / % of germination after 28 days
Control (no treatment)	30	0(0)	13(43.3)	4(13.3)	4(13.3)	21 / 70[b]
HCl (0.01N 12 hrs 38 ˚C)	30	0(0)	13(43.3)	9(30)	5(16.7)	28 / 93.3[a]
Sand Scarification (12hrs 38 ˚C)	30	0(0)	12(40)	3(10)	3(10)	18 / 63.3[c]

temperature (0°C) showed similar results as that of non-after-ripened seeds. However, the seeds stored at -20°C showed lower survival and germination percentages that could be due to intracellular freezing injury, where the seed tissues might get injured by causing membrane destruction, dehydration, lipid phase separation (Burke *et al*., 1976). During our research it was observed that the prolonged storage of seeds at room temperature was contaminated by fungal spores, which also cause the reduction in germination rate. As it was reported that the fungal spores cause seed discoloration, death of ovules and embryos resulting in the decline in survival and germination rate of seeds (Christensen, 1957). The sand scarified seeds showed lower survival rate with 70% compared to 100% in non-scarified seeds. In other study by Scharpf (1970) also found that the seeds stratified in sand lost viability rapidly because of deterioration by mold fungi.

The scarified seeds showed significantly higher rate of germination than that of the non-scarified seeds, which could be due to the removal of some chemicals (viscin) of the seeds that prevent germination during hydrochloric acid (0.01N HCl) scarification. In previous studies, Crocker and Barton (1953); and Beckman and Roth (1968) also found that fleshy coating of some seeds or viscin layer of mistletoe seeds could prevent germination.

In conclusion, higher percentage (90%) of germination and survivability (100%) of two months stored seeds in natural condition confirmed that mistletoe does not need after ripened treatment in freezing temperature to promote germination. In a previous research, Scharpf and Parmeter (1962) also found that seeds of *A. occidentale* (kind of mistletoe) do not need freeze treatment to support germination. According to this research, if *L. tanakae* seeds need to preserve, 0 to 4°C is preferred for short (less than one month) duration in AF conditions where the survivability and germination rate ranged between 83.3 to 90% and 67 to 77% respectively. Our research also showed that the storage of *L. tanaka* seeds in freezing temperatures at -20°C and in room temperature either in AT or AF conditions caused the loss of survival and germination rate. On the other hand, the chemical scarification (0.01N HCl incubation for 12 hrs) method was proven more effective to enhance germination percentage of *L. tanakae*. Regarding the temperature regime, 22°C showed early germination of mistletoe seeds *in vitro*.

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