Regular Article
ISSN 2287-2396
Journal of Forest and Environmental Science
Volume 29, No. 2, pp. 173-180, May 2013
http://dx.doi.org/10.7747/JFS.2013.29.2.173

Germination and Seedling Induction of *Viscum album* var. *coloratum* (Kom.) Ohwi after Artificial Inoculation on the Branch of Host Plants

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Abstract

Berries of Korean mistletoe (*Viscum album* var. *coloratum* [Kom.] Ohwi) contained one seed, which have, in general, one or two embryos but very rarely three embryos. Mucilaginous substances in berries may help them adhere to the branches of host trees. It was observed that seeds need more than one and half years to develop into normal and healthy seedlings from the time of inoculation. Many factors such as adhesion of berry, thickness of host branch, orientation of haustorial root, etc. influenced the successful development of mistletoe plants. Through the application of six-year observation results on the germination of seeds and growth of seedlings, about 80% of germination rate for mistletoe seeds and 61% of survival ratio for germinated seeds, which is more than 23 times higher in natural conditions, were obtained after inoculation of seeds on the one-year-old branches of *Malus pumila* var. *dulcissima* and *Quercus mongolica* trees. The technological aspects of the success can be applied to other host plants and provide a critical clue to an artificial propagation system, for this medicinally valuable genus. This is the first successful report on artificial inoculation and plant development of Korean mistletoe.

Key Words: *Viscum album* var. *coloratum*, haustorium, cultivation, viscosin, infection

Introduction

Domestic mistletoe (*Viscum album* var. *coloratum* [Kom.] Ohwi) grows on the top of Mongolian oak [*Quercus mongolica*] where light is well received. Is an evergreen perennial parasitic plant found mostly on Chinese cork oak [*Quercus variabilis*], chestnut, stone pear, pear, and a few are also found on elm and birch. However, it is not a complete parasitic plant that relies entirely on moisture and nutrients in the host plant, but a semi-parasitic plant that has photosynthetic ability by itself (Visser 1981).

Mistletoes native to Korea are also distributed in Japan, Taiwan, China, Europe and Africa. The [Korean] mistletoe plants are in 4 genera and 5 species: Camellia mistletoe (*Korthalsella japonica* [Thunb.] Engl.), tailed mistletoe (*Loranthus tanakae* Franch. & Sav.), oak mistletoe (*Taxillus yadoriki* [Siebold ex Maxim.] Danser), red mistletoe *Viscum album* f. *rubroauranticum* [Makino] Ohwi), and mistletoe (*Viscum album* var. *coloratum* [Kom.] Ohwi) (KNA 2007).

Studies on mistletoe in and outside of Korea (Bloksma et al. 1979; Franz et al. 1981; Khwaja et al. 1986; Ribéreau-Gayon et al. 1986; Doser et al. 1989; Hajto et al. 1990; Kuttan and Kuttan 1992; Bocci 1993; Schink 1997; Yoon 1997; Choung 1999; Park 1999; Jang and Na 2000; Lee 2002; Lee 2003; Choi et al. 2004; Jeon 2004; Jung 2004; Seong 2004) are mostly about chemical components and their effects. The main components related to anti-cancer are lectin and viscotoxin. In European mistletoe (*Viscum album* L.), the components extracted from lectin and viscotoxin are for cancers. It has been developed and used as a therapeutic injection, but currently in Korea there are no injections developed using Korean mistletoe, therefore, injections are imported from Europe.

Most native mistletoe reported by some researchers are distributed in the mountains at altitudes high above sea level, and the mistletoe is located in the host tree more than 7-10 m high (Seong 2000; Park et al. 2003). Because of the risk of having to climb the tree for harvesting, the collector often cuts it down and this causes problems such as habitat and ecosystem destruction.

Although few attempts have been made to establish mistletoe through artificial cultivation techniques (Seong 2000; Lee 2010), the mistletoe root (haustorium), plays a decisive role in rooting the mistletoe to the host and is thus critical to its life history. Importantly, the methodology for artificial cultivation of mistletoe has not been achieved to date.

This study investigates and analyzes a series of host inoculation processes, including the observation on mistletoe seed roots, and pays primary attention to fixation of the parasitic to the host. This is a report of successful artificial inoculation of seedlings onto host plants, and it is thought that this artificial mistletoe methodology can be applied to various types of host plants.

Materials and methods

Morphological characteristics of seeds

In 2009, mistletoe seeds from Hongcheon, Yanggu, Hwacheon, Inje, Wonju, and Jeongseon in Gangwon-do were used. Most of the mistletoe host trees are Mongolian oak, and fewer host trees such as oak, chestnut, birch, elm, and pear trees have been observed. One hundred mature fruits were selected to measure their length and width; the length and width of embryos from these seeds were also measured.

Seed maturity, germination characteristics and development of young plants

From 2005 to 2010, the development and maturity of seeds were observed by examining the flowers and fruits of mistletoe growing in native areas. After transplanting into a pot a 3-year old Mongolian oak tree in 2007, mistletoe seeds were inoculated on 2-3 February 2008. The germination, growth and development and of young mistletoe plants were investigated for 4 years. A total of 160 seeds were attached to the branches using gauze and tape.

Artificial cultivation to increase survival rate

The host plants were selected from 5 apple trees on the grounds of Gangwon University located in Sinbuk-myeon, Chuncheon-si, Gangwon-do, and 4 new Mongolian oak trees on the university campus. The apple tree is estimated to be over 20 years old, and the Mongolian oak 5-10 years old.

The mistletoe seeds used in the experiment were collected from Hongcheon, Yanggu, Hwacheon, Inje, Wonju, and Jeongseon in Gangwon-do from January to February 2009, and mature and intact fruits were selected and stored in the refrigerator (4-10˚C). At this time, in order to maintain the moisture, the seeds were stored in zip-lock bags that are used for food storage, and the mucilaginous flesh was not removed.

Inoculation of mistletoe seeds was carried out by using a special location fixing method (Yi and Kim 2011). On the 16th and 17th of March, seeds were fixed onto one year-old branches of the host tree. When inoculated onto the branches of host plants in the presence of pulp, it is very difficult to position the roots due to the spherical nature of the fruit and the sticky nature of the popped pulp. Seeds were inoculated only on the first-year shoots of selected host plants.

Table 1. Characteristics of fruit and embryo of Korean mistletoe

Fruit (mm)		One embryo in seed (mm)		Two embryos in seed (mm)	
Length	Width	Length	Width	Length	Width
8.74±0.55	8.38±0.51	5.73±0.39	3.94±0.20	5.68±0.28	4.46±0.24

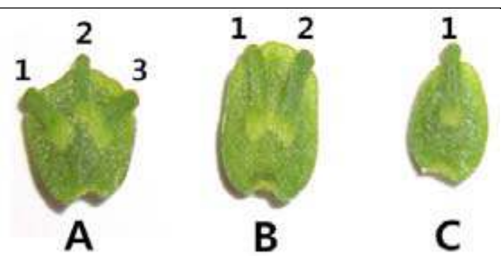

Fig. 1. Number of embryos of Korean mistletoe seed (A: Three, B: Two and C: One).

Results

Morphological characteristics of seeds

The mistletoe fruit (including the flesh) was 8.74 mm long and 8.38 mm wide, nearly spherical, and the size was measured by removing the flesh and separating the one or two embryos from the seeds (Table 1). In the case of having one embryo, the length of the embryo was 5.73±0.39 mm, which tended to be greater than 5.68±0.28 mm when there were two embryos, but the width of the seed was 4.46±0.24 mm. It was wider than 3.94±0.20 mm in that case. It was very rare to have three embryos, and only the shape is shown in Fig. 1.

It is common for a seed to contain one embryo. However, a mistletoe seed has 1, 2 or 3 embryos, but most of them have 1 or 2

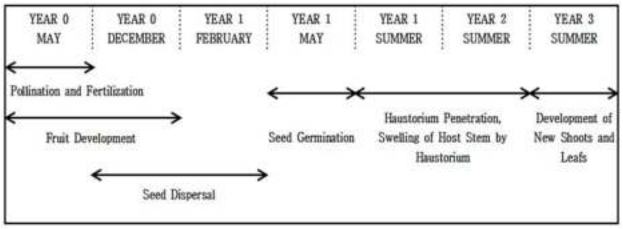

Fig. 2. Development of Korean mistletoe seedling on host plants.

embryo. Of the 100 seeds surveyed in this study, 36% were found to have 1 embryo,62% had 2 embryos, and 2% had 3 embryos, which may be very different depending on the number of seeds collected. The bottom of the mistletoe seed takes root when it is attached to the branch of the host plant, which is called the haustorium, and is the organ corresponding to the root of the ordinary plant.

Fig. 1, A, B, and C are the mistletoe seeds. The part of the embryo that comes out of the seed, that grows and touches the bark of the host plant, creates a root that can absorb nutrients from the stem of the host plant. The part of the embryo inside the seed is the place where the embryonic leaves of the mistletoe [cotyledons] absorb the nutrients necessary for the growth and the implantation of the roots into the bark of the host plant, so as to be able to survive.

General developmental process of young plants

The mistletoe is a male and female shrub, and both male and female flowers are divided into four parts. In May, the mistletoe male flowers open, and the female flowers secrete a moist viscous substance as the petals open. When moisture is formed, the mucous substance disappears and the fruit begins to develop.

The difference between the germination of seeds and the growth process of seedlings can provide important data for taxonomic classification in *Viscum*. See Figure 2.

The chronological processes of Korean mistletoe plants are as follows.

-Most of the pollination is completed in May, and it seems that fertilization will occur afterwards.

-Seeds develop from May to December.

-Seeds are dispersed in Jan-Feb in the following year. When the fruit wall [pericarp] is opened by any physical action, seeds are attached to the host tree by the mucilaginous material of the pulp (Peter et al. 1986).

-Among the mistletoe seeds that have fallen in May of the same year, the seeds that reach host plants site can start to germinate.

-Haustorial roots grow in the first year and penetrate through the bark of the host plant branch. From the end of the first year to the second year, a very small cotyledon of 1-2 mm is observed toward the green haustorium.

-In the second year, the cotyledon observed in the first year develops and grows like a young leaf in the shape of an ellipse, and between two cotyledons, a young shoot develops and begins to grow. The shoot usually develops two degrees, and the branch of the host tree is more dilated.

-In the third year, the haustorium seems to have completely penetrated the stem of the host plant, and the diameter of the branch of the inoculation site is observed 1.5-2.0 times larger than the other parts. A new leaf occurs and grows, and a young stem develops and grows between the two leaves (Fig. 3).

Fig. 3 is a picture taken by observing the same plant on the same site for 4 years after inoculating mistletoe seeds. (A) Mistletoe seed with endosperm and the root (haustorium) of the embryo growing and using (degenerating) it as the nutrient necessary to adhere to the host plant. (B). However, there are cotyledons and epicotyl on the side (B) toward the haustorium. From the year of inoculation, the roots of mistletoe on the stem of the host plant were observed to be enlarged, and a large portion (C) was observed. In the beginning of the second year after inoculation, a complete mistletoe individual (D) appears, but the young shoot of long oval leaves is seen developing between cotyledons. At the beginning of the 3rd year, an individual (E) with 2 branches was observed, with young buds between the small leaves of the last node.

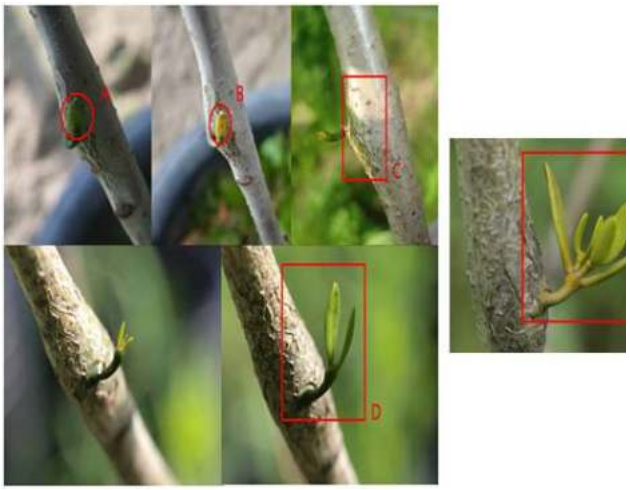

Fig. 3. Growing process of Korean mistletoe seedlings.

Developmental characteristics and rooting of parasitic seedlings

In the mistletoe seed inoculation experiment on the apple and the red oak trees, the direction of growth of the seeds with 1-2 embryos was examined after inoculation. A total of 100 seeds were inoculated. Here, 160 embryos developed, and as a result of observing the growth, only 15 (9.4%) of the haustorial roots attached to the bark (Table 2).

Table 2. Successful adhesions of Korean mistletoe embryos

Number of seeds	Number of embryos	Number of embryos grown to bark
100	160	15

The haustorium does not grow by recognizing the bark of the host plant, and is not informed of the direction of the host, that is, the direction of orientation is not constant. This developmental characteristics of the mistletoe haustorium seem to be one of the reasons for the low attachment rate in natural or artificial inoculations (Table 2).

The sticky pulp of mistletoe helps the seeds adhere to the branches of plants when the pericarp is damaged (Peter et al. 1986). As the mucilage dries after attachment, the seeds stick to the stem surface of the host plant and are fixed to enable adhesion. In the natural state, the factor in which the mistletoe seed has a low rate of host attachment is a subject to be examined from various angles.

Artificial cultivation experiment to increase the survival rate

According to the results of studies on the cultivation of mistletoe, the artificial seeding rate of mistletoe was very low (Seong 2000; Lee 2010). When the seeds were inoculated into host plants, artificial control experiments were conducted using the results obtained from the experiment where it was determined that the inoculation position was a very important factor for increasing the adhesion rate.

One-year-old branches of host plants are selected for cultivation

The mistletoe seedling grows and attaches to the branches of the host plant. If bark is present it is very difficult for the seedling to penetrate. In this experiment, only 1-year-old host branches that grew in the previous year were used as the test subjects. Although the survival rate was lower than 3%, the experimental results (Seong 2000) show that the survival rate is higher in a 1-2 year-old stem than for a stem with thick bark over 3 years old. Because the bark of a 1-year-old host plant branch is very thin, it is thought that it has the proper conditions for the mistletoe to penetrate the stem. In addition, when comparing 16-22-year-old Mongolian oak growing in the upper forest layer, it was confirmed that the difference in age of the host branch and mistletoe was under 4 years. As a result of examining the branches with 1-year-old mistletoe, the host was 1-3 years old (Table 3).

Table 3. Age relationship between mistletoe and host plant

Individual trees of overstory	1	2	3	4	5
Host plant (years)	18	21	22	19	24
Mistletoe (years)	16	17	18	18	22
Individual trees of understory	1	2	3	4	5
Host plant (years)	2	3	1	1	2
Mistletoe (years)	1	1	1	1	1

Fixing the position of the seedling so that it touches the bark of the host plant according to the growth direction of the seedling

Since the mistletoe seedling does not grow by recognizing the bark of the host plant, in order to increase the adhesion rate, the seedling must be artificially adjusted to contact the bark of the host plant (Yi and Kim 2011) (Tables 4, 5, Fig. 4).

Fig. 4 an 5 are for a 3 year-old mistletoe that grows after fully adhering to the branch of its major hosts the apple and oak trees. In this experiment, the apple tree was inoculated with 10-17 seeds per tree, and the Mongolian oak was inoculated with 10-12 seeds. Germination began 2-3 weeks after inoculation, and the germination rates of 78.5% and 79.6%, respectively, were observed in apple and Mongolian oak trees (Tables 4 and 5).

Table 4. Germination rate of seeds and survival rate of seedlings of Korean mistletoes inoculated on *Malus pumila* var. *dulcissima*

Content	Control	Individuals tested					
		1	2	3	4	5	Mean
Number of seeds tried	50	12	16	10	17	16	-
Number of seeds germinated	38	10	13	7	12	14	-
Germination rate (%)*	76	83.3	81.3	70	70.6	87.5	78.5
Number of plants survived	1	9	8	4	9	6	-
Survival rate (%)**	2.6	90	61.5	57.1	75	42.9	65.3

*Germination rate (%)=(Number of seeds germinated/Number of seeds tried)x100; **Survival rate (%) = (Number of seedlings survived/Number of germinated seeds) x100.

Table 5. Germination rate of seeds and survival rate of seedlings of Korean mistletoes inoculated on *Quercus mongolica*

Content	Control	Individuals tested					
		1	2	3	4	5	Mean
Number of seeds tried	50	11	12	10	12	16	-
Number of seeds germinated	38	9	10	7	10	14	-
Germination rate (%)*	76	81.8	83.3	70	83.3	87.5	79.6
Number of plants survived	1	5	6	4	7	6	-
Survival rate (%)**	2.6	55.6	60	57.1	70	42.9	60.7

*Germination rate (%)=(Number of seeds germinated/Number of seeds tried)x100; **Survival rate (%) = (Number of seedlings survived/Number of germinated seeds) x100.

Kim et al. (2008) got higher germination rate than experiments under environmental conditions and growth control agents. Among the young plants that germinated and were planted, the survival rates of 65.3% and 60.7%, respectively, on the apple tree and the Mongolian oak tree at the third year of inoculation are shown (Tables 4 and 5). This is much higher than the result of Seong (2000), who showed that the pine, cedar, cottonwood (*Populus tomentiglandulosa*), and *Cornus* trees have mistletoe germination less than 2%, and the mulberry, hawthorn, quince, oak, and apricot trees have germination less than 2%.

Kim et al. (2008) investigated germination rates by treating light, temperature, and growth regulators, and showed germination rates of 35% in light culture treatment, 40% in 25˚C treatment, .8% in indole acetic acid treatment, and 48.8% in ethylene treatment. In this study, a germination rate of about 80% was obtained even in the natural state without artificial treatment (light, temperature, growth regulators, etc.). In the experiment of Kim et al. (2008), it seems that the germination rate of mistletoe seeds was inhibited by the artificial treatment. In order to increase the germination rate in the ethylene chamber by artificial treatment, it is necessary to do further experiment with various conditions.

Fig. 4. Seedlings of Korean mistletoe on *Malus pumila* var. *dulcissima* (1 and 2) and on *Quercus mongolica* (3 and 4).

Review

Most research on mistletoe in domestic and foreign countries is on the use of [chemical compondents]; research on reproduction and cultivation is very insufficient. Prior to this commercial efforts, such as various component analysis and product development, centered on use. Therefore, it is urgent to secure raw materials, and to accomplish this, research on reproduction and cultivation should be done. The scarcity of the mistletoe and the difficulty in collecting seeds are two of the reasons for making such work difficult. Mistletoe seeds are spherical as shown in Table 1 and are berries such as grapes and tomatoes. The mesocarp area of the flesh is made of mucilage [viscin], which is a clue that plants of the Loranthaceae can parasitize trees (Visser 1981).

The polyembryony phenomenon in which 2-3 embryos are observed is mainly observed in the seeds of conifers and is very rare in hardwoods (Singh and Johri 1972). It is very rare in this study, but the discovery of three embryonic seeds is likely to be an interesting anatomical and genetic research subject for the process of development of this species. This is because multiple embryos may be resulting from apomixis, but they may also be caused by the development of two or three embryos from the same zygote or from embryos derived from nucellar cells. According to the former, each embryo is genetically identical, but not in the latter (Hartmann et al. 1990).

In the natural state, the seeding rate of the mistletoe is very low, thus long-term research and analyses of results from various different places are required:

1) When the seeds mature, most of them fall to the ground and some of them fall and stick to the branches of other trees around them. Seeds that have fallen to the ground degenerate.

2) Even if the mistletoe seeds are attached to the body of a bird or the like and are moved to a place for growth, the probability that the seeds adhere to branches of other trees is very low.

3) In order to be successful, it is advantageous that the seeds fall on the upper part of the host tree branches that are less than 3 years old. The bark may have different characteristics for each species, but branches of 4 years or older seem to be difficult for the mistletoe seedling to penetrate due to the bark thickness.

4) Even if it is attached to a branch, the mistletoe seeds must grow in the direction of the branch bark, and if it grows in the air (opposite the branch), it will soon die.

5) Most seeds do not adhere to the branches of host plants that have favorable conditions. Even when attached to the stems of host plants that are suitable, the seeds attached to branches can fall because of animal movement and rain. The mucilaginous substance becomes hard when it dries and adheres to the host plant, but when water is supplied, it changes back to mucilage and can cause loss due to detachment from the bark.

Due to the above factors, mistletoe seeds are considered to have a very low rate of adhesion in the natural state. Based on this, artificial cultivation experiments were conducted on apple trees (*Malus pumila* var. *dulcissima*) and Mongolian oak (*Quercus mongolica*) trees. It is recognized that it is economically valuable because it has been used as a natural anti-cancer agent (injection agent) extracted from mistletoes in cultivation. So far in Korea, various species have been tested as host plants, but apple trees have not been used as host stocks and there have been no successful cases of inoculation. The reason for the selection of Mongolian oak trees is that most of the mistletoe native to the Gangwon-do region were determined to be on these trees which provide the most suitable conditions for the growth of the mistletoe. The choice of host plants for large-scale projects is very important, and it is necessary to examine a variety of woody plants, and further research and discussion are required in the future. In particular, it is considered that the experiment in the growth chamber can obtain a large number of seedlings at a time rather than the germination rate, and further study is needed to increase the germination rate to facilitate management. In the future, more effective results can be obtained if appropriate host species are used, which was the reason for selecting the apple tree used in this study.

Conclusions

Until recently, most of the work on mistletoes were studies on physiological activity and anti-cancer effects, and several attempts were made to artificially cultivate mistletoe, but they did not show practical cultivation techniques due to their very low adhesion rate. This study revealed that the development of the mistletoe seedling (or root haustorium) plays the most important role in host establishment and this applies to artificial cultivation. The first was to inoculate a thin bark of about 1 year old, and the second was to artificially adjust the seedling to develop toward the host plant; a specially developed positioning device was very effective for this adjustment. When the mistletoe seeds were inoculated onto apple and oak trees and the seeds artificially manipulated, they showed a high germination rate of 80% compared to 3% in the natural state and 60% survived. In the year of inoculation, parasitic roots with young cotyledons that appeared to have degenerated were observed as whole plants, and in the second year, the mistletoe plants were formed. These results are the first successful reports to show that mistletoes can be artificially propagated on various host plants.

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