Regular Article
pISSN: 2288-9744, eISSN: 2288-9752
Journal of Forest and Environmental Science
Vol. 37, No. 4, pp. 323-330, December, 2021
https://doi.org/10.7747/JFES.2021.37.4.323

JFES
Journal of Forest and Environmental Science

Propagation by In Vitro Zygotic Embryos Cultures of the *Quercus myrsinifolia*

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Abstract

Zygotic embryo culture was performed to propagate evergreen oak, *Quercus myrsinifolia*, which has recalcitrant seeds and is difficult to propagate by cuttings. Zygotic embryos appeared in WPM medium after 14 days, and after 56 days, they developed into complete plants with cotyledons and roots. The medium suitable for zygotic embryo culture was 1/4 WPM medium, showing a shoot growth of 2.43 cm and root growth of 8.7 cm after 8 weeks of culture. As a result of investigating the effect of GA_3 on the growth of plants germinated from zygotic embryos through GA_3 treatment, the best growth was shown in 0.5 mg/l GA_3 treatment. The in vitro rooting and growth of IBA-treated zygotic embryo-derived plants were good in the 0.5 mg/l IBA treatment and rooting and shoot growth were not observed at higher concentrations. And the callus induction rate also increased as the concentration of IBA increased. Plants grown in vitro were transferred to a plastic pot containing artificial soil and acclimatized in a greenhouse for about 4 weeks, resulting in more than 90% survival. As a result of this study, the zygotic embryo culture method was confirmed to be effective for mass propagation of *Q. myrsinifolia*. The results of this study are expected to contribute significantly to the mass propagation of elite *Q. myrsinifolia*.

Key Words: zygotic embryo culture, *Quercus myrsinifolia*, germination in vitro rooting, acclimatization

Introduction

Due to climate change, vegetation is changing rapidly. In the case of Korea, the northward shift of warm temperate tree species is visible (Son et al. 2011). Therefore, it is necessary to lay the foundation for an industry that can use forest resources such as wood and medicine through the preservation of the native habitats of temperate forests and long-term cultivation of promising resource-converted species among warm temperate evergreen broadleaf species (Lewis et al. 2006; Metz et al. 2007).

The demand for seedlings for warm temperate evergreen broadleaf species is increasing for restoration of temperate forests and new reforestation due to climate warming, for landscape planting around cities, road trees, park trees, and garden trees, and the planting area is also expanding (Park et al. 2010; Song et al. 2015).

Evergreen *Quercus* trees are promising tree species for preparing climate change due to their tolerant heat and drought-heat dual stress (Park et al. 2019). In the Korean

Received: August 2, 2021. Revised: October 27, 2021. Accepted: October 27, 2021.

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peninsula, there are 6 species of evergreen *Quercus* species: *Q. acuta*, *Q. gilva*, *Q. glauca*, *Q. myrsinaefolia*, *Q. phillyraeoides* and *Q. salicina* (Korea Forest Service 2018). These typical evergreen tree species of the warm-temperature zone, except *Q. phillyraeoides*, are naturally grown in the Korea peninsula's south shore or southern islands.

The high survival rate of evergreen oaks when transplanted is considered a significant problem (Park 2007). In addition, there is a problem that the cultivation area is limited because of its weak cold resistance. Also, although evergreen oaks can be cut or planted, they are not used as a propagation method due to poor performance. Therefore, finding out other propagation methods other than actual propagation by seed sowing for evergreen *Quercus* is necessary.

Quercus species have recalcitrant seeds, so storage of the seeds is difficult, and propagation is difficult (Bonner 1996). Usually, seeds are sown in the year of collection or stored in an open-air store or a 5-10°C refrigeration facility and sown in April of the following year, and long-term storage is not carried out. In general, when *Quercus* species seeds are harvested and stored in a dry state, their vitality is lost within 1 to 2 months, and even in a 5-10°C refrigeration facility, the vitality gradually decreases after 6 months or more, and the germination rate in the forge after 1 year. Since this is significantly lowered, sowing is usually carried out within 6 months, and long-term storage is not generally performed. This series of operations are cumbersome and inefficient. Therefore, it is necessary to find out an efficient live propagation method.

Zygotic embryo culture plays an essential role in the rapid in vitro propagation of an endangered and valuable forest tree species to overcome physical and biotic stress (Stanys et al. 2007). Also, embryo culture can help overcome embryo non-viability, seed dormancy and related problems. However, there is no study on the proliferation of evergreen *Quercus* by zygotic embryo culture. This study was conducted to investigate various optimal factors affecting the zygotic embryo culture of *Quercus myrsinaefolia*.

Materials and Methods

Plant materials and in vitro germination

The seeds used in this study were selected from Gyeongsang National University and Gajwa Experimental Forest for 3 years, from September 2017 to November 2019. At the time of collection, the damaged seeds were visually selected and dried at room temperature for a week. Until germination, those were stored in a low-temperature refrigerator at 4°C.

Disinfection of seeds was performed by placing the seeds in a culture bottle, washing the seeds with the surfactant, and then sonicating them at 50°C for 30 minutes. Then, the seeds were sterilized three times in 70% ethanol for 1 minute and 3% NaClO for 2 minutes on a clean bench. The surface-sterilized seeds were left in distilled water for 10 minutes to remove phenol substances, washed several times with sterile water, and dried for 40 minutes.

The extracted zygotic embryos were transferred to MS and WPM media adjusted for each salt concentration. *Q. Myrsinifolia* germinated seedlings that were not normal were found when zygote embryos were cultured. Abnormal germinated seedlings developed with one cotyledon instead of two cotyledons and had only cotyledons and did not develop into roots. In this study, the germination rate and frequency of abnormal seedlings according to the type of medium after 8 weeks of sowing were investigated. At this time, the culture conditions were cultured at a temperature of 3,000 lux light 25°C (16 hours light, and 8 hours dark). Subculture was performed in the same medium every 4 weeks. It was transferred to a culture medium and cultured for 16 weeks to establish an in vitro plant.

The effect of GA_3 treatment on the growth of germinated plants from *Q. myrsinifolia* embryos was investigated. GA_3 treatment was added at a concentration of 0.1, 0.25, 0.5, 0.75, 1.0, 2.0 mg/l to 1/4 WPM medium. After culturing for 8 weeks, the growth amount of shoot and roots was measured, and the the total length of both the shoot and the root is represented. The culture conditions were the same when irradiating the above culture medium.

Shoot elongation of germinated plants

Plants germinated from zygote embryos were investigated for optimal conditions for growth, such as culture medium and growth regulators. MS (Murashige and Skoog 1962) and WPM (Lloyd and McCown 1981) were used as the medium for the optimal growth of germinated seedlings. These media were adjusted to 1/4, 1/2, and origi-

nal concentrations of the original media composition. 3% (w/v) of sucrose as a carbon source was added to the medium, 0.4% (w/v) of gelite was used as a gelling agent, and the pH was adjusted to 5.7. Plant growth according to each medium was investigated 4 weeks after culture in a petri dish (87×15 mm), and the length of the shoot, the number of shoots, length of the roots, and the number of roots were measured.

In vitro rooting and propagation

The difference in growth according to the concentration treatment of the GA_3 solution from embryos from immature zygotic embryos was examined. Zygotic embryos were transplanted in 1/4 WPM medium to which GA_3 was added at concentrations 0.1, 0.25, 0.5, 0.75, 1.0, 2.0 mg/L, respectively. For each treatment, 12 immature zygotic embryos per petri dish were transplanted, and This experiment was repeated 3 times per treatment. The germination of the direct embryo was visually examined based on the growth of 2 mm or more on the surface of immature embryos among the immature embryos after culture for 8 weeks. In addition, IBA was used for concentration in the 1/4 WPM medium 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg/L for in vitro rooting. Therefore, shoot growth, leaf number, rooting rate, root length, and callus induction rate were analyzed as root growth indicators.

The in vitro growth of zygote embryo-derived plants according to the culture period was investigated. The survey items were indicated by measuring stem and root length, rootlet length, etc., by week.

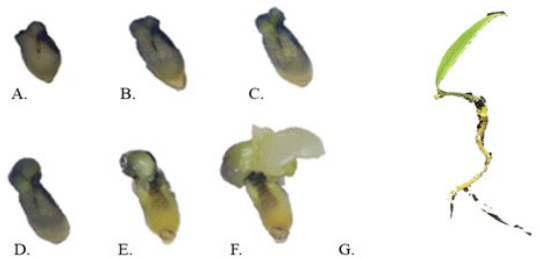

Fig. 1. Germination pattern according to the culture period of zygote embryos in *Q myrsinifolia*. (A). Dissorciated zygotic embryo from endosperm, (B). 7 Days after culture, (C). 14 Days after culture, (D). 21 Days after culture, (E). 28 Days after culture, (F). 48 Days after culture, and (G). Complete plant formation after 56 days of culture.

Acclimatization of in vitro plants

The in vitro plant was taken out of the culture bottle, the medium attached to the root was removed, washed with running water, and then planted in artificial soil. Plants were planted in artificial soil mixed with peat moss: perlite: vermiculite 6:0:0, 4:1:1, 2:2:2, 1:1:4, 0:0:6 (v/v), acclimated in the greenhouse. Acclimatization kept the relative humidity over 90% for one week and gradually lowered the humidity. Acclimatization was planted in a plastic pot with a diameter of 10 cm and covered with vinyl to not dry in the growth chamber, and sprayed from time to time to maintain humid conditions. The experiment was repeated three times to see the effect on artificial soil, and the results were analyzed 8 weeks after acclimatization.

Statistical analysis

To verify the significance of the measured values for each treatment, ANOVA was performed using SPSS version 25, and Duncan's multiple range test (DMRT) was conducted to analyze differences between treatments.

Results and Discussion

Germination of in vitro zygotic embryos

In order to investigate the efficient propagation method of *Q. myrsinifolia* trees, zygotic embryos were cultured. Surface-sterilized zygote embryos began to swell after 7 days of culture in WPM medium (Fig. 1A), and cotyle-

Table 1. Germination rate and frequency of abnormal plant of *Q. myrsinifolia* trees zygotic embryos according to the culture medium

Culture medium	Germination (%)	Frequency of abnormal plant (%)
1/4 MS*	43.97 ± 7.17^{d}*	10.00 ± 1.43^{NS}
1/2 MS	56.03 ± 4.32^{c}	12.06 ± 1.09
MS	46.99 ± 4.29^{d}	13.01 ± 4.29
1/4 WPM	80.00 ± 2.86^{a}	11.95 ± 0.83
1/2 WPM	66.98 ± 4.29^{b}	9.84 ± 2.75
WPM	56.51 ± 1.98^{d}	13.02 ± 1.46

*The data are presented as means±SD of three times of independent test. Means with different letters are significantly different at a ≤ 0.05 by Duncan's multiple range test.
NS, no significance.

dons began to develop after 14 days of culture (Fig. 1B). After 21 days of culture, cotyledons were further developed, and the root radicle tissue changes were observed (Fig. 1D). After 28 days of incubation, the cotyledons developed more and turned light green, and the root radicle tissue was observed (Fig. 1E). After 56 days of incubation, a complete plant with bud and root was formed (Fig. 1G).

The germination rate differed according to the type of medium (Table 1). Among the culture media, the germination rate was higher in the WPM medium than in the MS medium. The best medium for germination was 1/4 WPM medium, and the medium with the lowest germination rate was 1/4 MS medium.

In addition, it was also observed that the plant germinated from the zygotic embryo developed in an abnormal form (Table 1). There was no significant difference in the frequency of abnormal plants depending on the type of medium. Abnormal plants appeared at a frequency of approximately 10%. These abnormal plants did not develop into normal plants.

The germination rate of the zygotic embryo showed a difference according to the culture medium (Table 1). The germination rate of the zygotic embryo was higher in WPM medium than in MS medium. In germinated seedlings, a zygotic embryo with abnormal plant development was also observed. The proportion of abnormally germinated seedlings was about 10%. The treatments with the highest germination rate were 1/4 WPM medium.

The culture medium has a significant effect on plant seed germination. The choice of culture medium had a significant impact on germination, probably because of differences in the balance and supply of organic and inorganic nutrients (Zeiger et al. 1985; Van Waes and Debergh 1986). In particular, the nitrogen component in the medium is known to affect germination. Dohling et al. (2008) also reported the importance of NH^{4+} and NO^{3-} ions (individual or combination) in vitro culture of orchid seeds.

The zygotic embryo germination of *Q. myrsinifolia* seeds was achieved in a medium containing a small amount of inorganic salt. MS medium is widely used for plant culture due to its high salt concentration, such as nitrogen and potassium. In contrast, WPM medium is used for culturing woody species showing sensitivity to salt (Koene et al. 2019). WPM medium has a similar composition to MS medium in ammonium/nitrate ratio and potassium concentration, but with lower NH^{4+} and NO^{3-} concentrations.

WPM and MS medium with half-strength macronutrients enhanced shoot induction and growth in *Q. rubra* and other *Quercus* species (Ostrolucka and Bezo 1994). Chalupa (1984) reported WPM was better for shoot growth and development than MS or Gresshoff and Doy (GD) medium for *Q. rubra*.

Shoot elongation of germinated plants

The culture medium suitable for the growth of plants germinated from *Q. myrsinifolia* embryos was found. As a result, the growth of plants showed a lot of difference in growth according to the type and concentration of the culture medium (Table 2). Germinated seedlings of *Q. myrsinifolia* showed a difference in medium concentration, and the lower the medium concentration, the better the shoot growth. Also, WPM medium was better for shoot growth

Table 2. Germination rate and frequency of abnormal plant of *Q. myrsinifolia* trees zygotic embryos according to the culture medium

Culture medium	Shoot growth (cm)	Shoot number	Root length (cm)	Root number
1/4 MS*	0.53 ± 0.26^{b}	1 ± 0.33^{NS}	5 ± 0.61^{bc}	1 ± 0.33^{NS}
1/2 MS	1.27 ± 0.54^{ab}	1 ± 0.33	7.1 ± 0.83^{a}	2 ± 0.67
MS	0.83 ± 0.33^{b}	1 ± 0.00	4.2 ± 1.27^{c}	1 ± 0.33
1/4 WPM	2.43 ± 0.35^{a}	1 ± 0.00	8.7 ± 1.68^{a}	3 ± 1.67
1/2 WPM	1.67 ± 0.52^{ab}	2 ± 0.67	6.96 ± 0.9^{ab}	1 ± 0.33
WPM	0.7 ± 0.4^{b}	1 ± 0.33	5.06 ± 0.91^{bc}	1 ± 0.88

*The data are presented as means±SD of three times of independent test. Means with different letters are significantly different at $a\leq0.05$ by Duncan's multiple range test.
NS, no significance.

than MS medium. As a result of finding the optimal growth medium conditions for in vitro culture, the best average medium for growth in shoots was 1/4 WPM, and the growth was 2.43 cm for 4 weeks. It grew to 1.67 cm in 1/2 WPM, 1.27 cm in 1/2 MS, 0.83 cm in original MS medium concentration, 0.7 cm in WPM, and 0.53 cm in 1/4 MS. The shoot growth of *Q. myrsinifolia* did not grow well at the original concentrations of MS and WPM.

The number of stems of *Q. myrsinifolia* did not show a significant difference depending on the culture medium. In the germinated plant cultured for 4 weeks, 2 stems were generated at 1/2 WPM, and 1 plant was developed in other treatments.

Root growth also showed a difference according to the culture medium. The treatment with the best root growth was 1/4 WPM, and after 4 weeks, it grew 8.7 cm. Root growth was achieved at 7.1 cm at 1/2 MS, 6.96 cm at 1/2 WPM, and 5.06 cm at WPM. The root growth of germinated seedlings also showed better growth as the salt concentration of the medium was lower.

Germinated seedlings were found to be greatly affected by the type of medium and salt concentration. During the 4-week incubation period, the best medium for root development was 1/4 WPM, and 3 or more roots were developed. Among MS medium, more than 2 roots were developed in 1/2 MS medium, and about 1 root was generated in the rest.

Overall, the best medium with good rooting and shoot growth was 1/4 WPM medium and an MS; the shoot growth was relatively good in 1/2 MS medium with salt concentration reduced.

The culture medium is also involved in the germination rate and growth after germination (Koene et al. 2019). Vudala and Ribas (2017) reported that the germination rate was also high in WPM medium, and the seedlings grew faster with development. In the case of *Hadrolaelia grandis* (Orchidaceae), MS medium was effective for early seed germination, but it was later reported that the mortality rate was high due to high concentrations of ammonium and nitrate (Vudala and Ribas, 2017).

The effect of GA_3 treatment on the growth of germinated plants from *Q. myrsinifolia* embryos was investigated. (Fig. 2). Root growth started earlier than that of no treatment at 1 week, and at 0.5 mg/l GA_3, the growth was excellent, and the root growth was high. The 0.5 mg/l GA_3 treatment showed double the growth rate compared to the 1.0, 0.25, 0.75 mg/l GA_3 treatment, and the 0.1 and 2.0 mg/l GA_3 treatments showed 3 times less growth than the 0.5 mg/l treatment. When cultured for 8 weeks, the lowest growth rate was 0.75 mg/l GA_3 treatment.

Gibberellins (GAs) are essential plant regulators for several plant development processes, including seed germination, shoot elongation, leaf expansion, pollen maturation, and induction of flowering (Urbanova and Leubner-Metzger 2016). The effect of GA_3 treatment depends on the plant species, the concentration used, and the treatment time. Treatment with various concentrations of GA_3 (20-400 mg/L) reported improved *Cyclamen percicum* seed germination and found significant differences between cultivars (Hanyan et al. 2006). Treatment of GA_3 at a 25 to 100 mg/L concentration on the leaf surface effectively increased petiole length and flower development (Cornea-Cipcigan et al. 2020). Additional application with GA_3 (5-100 mg/L) increased shoots of salt-stressed rice (Wen et al. 2010). Treatment with GA_3 at 50, 100 and 200 ppm increased the plant height, number of shoots, and number of flowers per cv. Pusa Narangi Gainda (Kumar et al. 2010).

In vitro rooting of propagated seedling

The effect of IBA treatment concentration on rooting and root growth of zygotic embryo-derived plants was investigated (Table 3). As a result, the rooting and root growth of plants were different according to the concentration of IBA. In addition, IBA treatment formed callus at

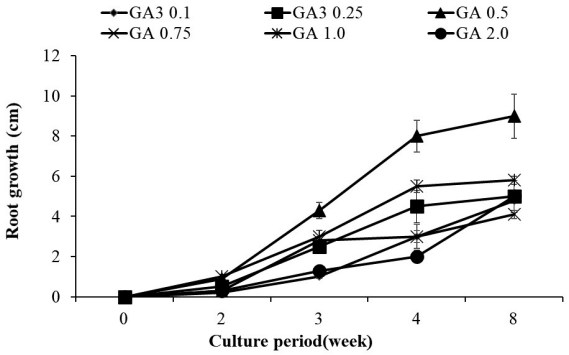

Fig. 2. Effect on shoot and root growth of plants germinated from *Q. myrsinifolia* zygote embryo. The data are presented as means±SD of three times of independent test.

Table 3. Effect of IBA treatment on shoot and root growth of plants derived from *Q. myrsinifolia* zygotic embryos

IBA Conc. (mg/L)	Shoot growth (cm)	Leaf number	Rooting (%)	Root length (cm)	Callus frequency (%)
0	$3.21\pm0.53^{NS*}$	2.42 ± 0.20^{NS}	5	1.00 ± 0.48^{b}	52
0.1	3.59 ± 0.49	2.63 ± 0.26	3	1.10 ± 0.35^{b}	37
0.2	3.56 ± 0.54	2.17 ± 0.4	9	1.21 ± 0.33^{b}	42
0.5	3.47 ± 0.27	2.00 ± 0.29	20	3.00 ± 0.33^{a}	83
1.0	3.67 ± 0.39	2.90 ± 0.28	-	0.00 ± 0.00^{c}	90

*The data are presented as means±SD of three times of independent test. Means with different letters are significantly different at a≤0.05 by Duncan's multiple range test.
NS, no significant.

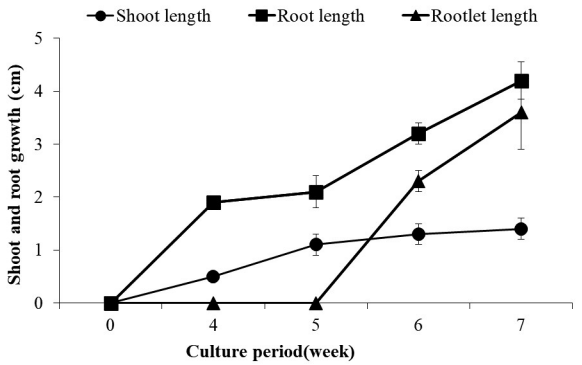

Fig. 3. In vitro growth of germinated seedlings of *Q. myrsinifolia* according to the culture period. The data are presented as means±SD of three times of independent test.

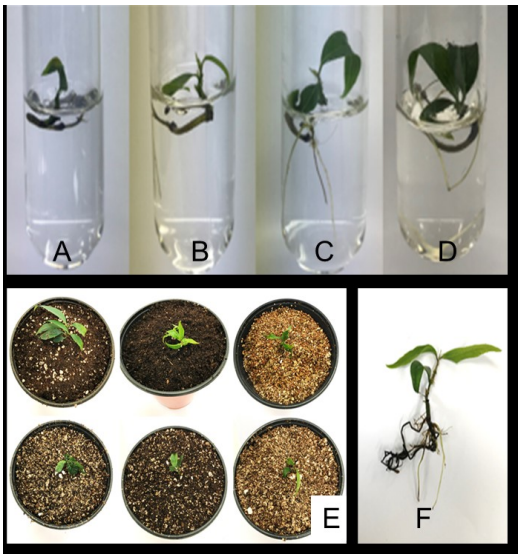

Fig. 4. In vitro rooted plants (A-D) and acclimatized plants (F) and well-established plant of *Q. myrsinifolia* zygotic embryo cultures.

the base of shoots at all concentrations, which had a significant effect on stem growth and root growth.

The in vitro rooting rate was as low as less than 20%. The IBA treatment with the highest rooting rate was 0.5 mg/l, followed by 0.2 mg/L. The length of roots also differed according to the concentration of IBA treatment. The best treatment for root length was 0.5 mg/L, and the other treatments had similar root length growth.

The growth of shoots also showed a similar tendency to root development and root growth. The best IBA-treatment for stem growth were 0.1 and 0.2 mg/l, but there was no significant difference between the treatments. The number of leaves was the best at 1.0 mg/l, but there was no significant difference between treatments.

IBA is considered a very important growth hormone for the rooting of in vitro cultured shoots (Frick and Strader 2018). It has been reported that the concentration and exposure time of IBA treatment significantly affect the in vitro rooting of *Quercus* sp. plants. Sanches et al. (1996) reported that the best rooting efficiency was achieved by culturing 25 mg/L IBA in *Quercus* robur in vitro culture for 24 hours and then transferring to an auxin-free medium. Vidal et al. (2003) also reported that rooting is easy when IBA is treated in the lower part of the in vitro cultured *Quercus robur* and *Q. rubra* plants in culture.

In vitro growth according to the cultivation period

The in vitro growth of zygotic embryo-derived plants according to the culture period was investigated (Fig. 3). The shoot started after 2 weeks of culture and grew at a relatively fast rate until 5 weeks of culture, and then gradually grew after that. Root growth started after 2 weeks of culture and grew rapidly until 4 weeks of culture, and then showed

moderate growth until 5 weeks, and then rapidly grew again. Rootlet appeared from the 5th week of culture, and then it was found that it grew very rapidly.

Acclimatization of in vitro plants

Plants cultured in vitro were removed from the culture bottle, the medium was removed, washed, and then transferred to a plastic pot containing 4 types of sterilized artificial soil and acclimatized in a greenhouse for about 4 weeks. As a result, more than 90% of acclimatized seedlings were obtained (Fig. 4). Plants acclimatized in artificial soil produced new leaves and roots after 3 weeks and grew normally.

Evergreen oaks are recalcitrant seeds, so they are difficult to propagate. Propagation is complicated due to difficulty cutting. Therefore, it is necessary to find an efficient multiplication method. In this study, the proliferation method through zygotic embryo culture was investigated. Although plants were successfully obtained through in vitro cultivation, it seems that the appearance of abnormal plants and difficulties in vitro rooting due to the formation of callus during in vitro rooting should be resolved through future research. However, this study is expected to contribute to the proliferation of evergreen oaks, growing in importance due to climate change.

Acknowledgements

This study was carried out with the support of "Forest BioResource Collection, Conservation and Characteristic Evaluation" of the National Forest Seed and Variety Center.

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