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In vitro Pollen Performance of *Pinus densiflora* and *P. rigida*: Temperature and Medium Nutrient Effects

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ABSTRACT: Effects of environmental factors on *in vitro* pine pollen performance were investigated. *Pinus densiflora* and *P. rigida* pollen grains collected at Mt. Kwanak, Korea were used. Three environmental factors, such as pollen storage temperature, pollen culture temperature and nutrient condition in medium, were tested. To determine the storage temperature effects on pollen viability, pine pollen was stored at -70°C, -12°C, 4°C and 22°C. Pollen viability was substantially extended at the storage temperatures of -12°C and 4°C for more than 300 days. To elucidate the culture temperature effects on pine pollen germination and tube growth, pollen grains were cultured at the temperatures from 5°C to 40°C at 5°C intervals. The germination rate and tube growth were highest at 25°C and decreased above 30°C. To investigate boron and sucrose effects on pollen tube growth, the pollen was cultured at different sucrose and boric acid concentrations. Germination rate was optimal in germination medium containing 3 or 5% sucrose with 0.01% boric acid. These results indicate that the pine pollen can be stored for considerable length of time without noticeable loss of viability at storage temperature below or near 0°C. Optimal germination medium conditions were established for pine pollen. Therefore, pine pollen can be used for many biological and environmental monitoring researches.

Key words : Germination medium, *Pinus densiflora, P. rigida*, Pollen viability, Temperature

INTRODUCTION

In nature, pollen germination occurs on the stigma where a pollen tube from germinated pollen grain penetrates style and grows towards ovary. In extreme cases, depending on the species involved, the tube length can reach several thousand times of pollen diameter (Stanley and Linskens 1974, Shivanna and Johri 1985). Pollen can be easily collected and, in some species, pollen can be stored for a considerable length of time without loss of viability (Shivanna and Johri 1985).

Generally it is known that pollen performance is affected by several environmental factors, such as temperature, moisture, nutrients, ozone, H_2S, air pollutants and acidic precipitation (Shivanna and Johri 1985, Lankinen 2001). Also other factors influencing pollen tube growth include plant species, collection time, collection method and storage condition. Temperature effects on pollen germination are well documented in several species. Temperature during pollen development has been found to influence the chemical composition of pollen, pollen viability, pollen tube growth rate and the synthesis of heat shock proteins in pollen grains. Both development and germination temperature are important for pollen performance (Hormaza and Herrero 1996, Delph *et al.* 1997, Lankinen 2001). Many chemical and physical factors are now known to influence pollen germination *in vitro*. Some chemicals stimulating germination, *e.g.* boron, calcium and magnesium, are first noted as similar or identical to factors found in a style tissue or stigmatic fluid in which the pollen naturally germinates. Requirements for pollen germination vary appreciably from species to species. Pollen germinations usually need water, carbohydrate sources, boron and calcium. When pollen grains are placed in solution containing a high boron concentration, pollen germination can be increased (Stanley and Linskens 1974). But few studies are reported about environmental factors affecting pine pollen performance *in vitro* and pine pollination ecology (Shivanna and Johri 1985, Delph *et al.* 1997, Kang 1999).

This study was undertaken to understand the effects of temperature and medium nutrient on the performance of pine pollen *in vitro*. Three environmental factors, such as pollen storage temperature, pollen culture temperature and nutrient condition were tested. *Pinus densiflora* and *P. rigida* pollen grains collected at Mt. Kwanak, Korea were used. We select these two pine species since *P. densiflora* is a naturally grown pine species and *P. rigida* is an introduced pine in Korea for re-vegetation several decades ago. Specific objectives of this study include the following: 1) to determine whether pollen storage temperature affects on pine

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pollen performance; 2) to examine the culture temperature effects on pollen tube growth; and 3) to find an optimal nutrient medium for *in vitro* pine pollen study.

MATERIALS AND METHODS

Collection, processing and storage of pollen

Male cones of *Pinus densiflora* Siebold et Zucc. and *Pinus rigida* Miller were collected in May 1999 from trees located at Mt. Kwanak (37° 27′ N, 126° 58′ W) in Seoul, Korea. Collected male cones were air-dried in the shadow at 23℃ for one week. After drying, pollen was shaken off, screened and placed in glass vials. And the vials were then sealed and stored in a refrigerator at 4℃ until used. Petri dishes containing germination medium were used for pollen culture. Standard liquid germination medium contained 5% sucrose and 0.01% boric acid in double distilled water at adjusted pH of 6.7 (Stanley and Linsken 1974). Pollen was dusted over the surface of germination medium for pollen growth. Germination rate and pollen tube length were recorded after 48 hours of incubation under an Olympus BX50 microscope (100×). Pollen germination tubes were determined by scoring 250 pollen grains after staining with methylene blue (0.5% methylene blue in DDW). Three replicates were used for each treatment and every experiment was repeated twice.

Storage temperature effect on pollen viability

To determine whether pollen storage temperature affects pollen viability, air-dried pollen was placed in 10 mL vial and stored at -70℃, -12℃, 4℃ and 22℃, respectively. Pollen viability was determined at an interval of 10 days by scoring 250 pollen grains each of 4 temperature regimes after staining with 0.5% methylene blue.

Relation between temperature and pollen growth

Pollen grains of *P. densiflora* and *P. rigida* were cultured at the temperatures from 5℃ to 40℃ at the rate of 5℃, respectively for 48 hours. After 48 hours, the pollen grains were stained with 0.5% methylene blue and viewed at ×100 under an Olympus BX50 microscope. Two hundreds and fifty grains selected at random were scored for germination and pollen tube growth were measured.

Effect of sucrose and boric acid concentrations on pollen growth

To investigate requirements for pollen growth on germination medium, we made three kinds of germination media with different concentrations, sucrose only, boric acid only, sucrose with 0.01% boric acid. We made media containing 0, 1, 3, 5, 10% sucrose in DDW, 0.001, 0.005, 0.01, 0.05, 0.1% boric acid in DDW and 0, 1, 3, 5, 10% sucrose with 0.01% boric acid in DDW. And the samples were incubated at 25℃ for 48 hours in a growth chamber before observation.

RESULTS

Storage temperature effects on pollen viability

Pollen viability of *P. densiflora* significantly decreased as the duration of the pollen storage days increased which was stored at room temperature after 30 days of storage and noticeably decreased at -70℃ after 150 days. Pollen grains stored at -12℃ and 4℃ maintained viability showing the germination rate over 80% (Fig. 1A). Pollen of *P. densiflora* which were stored at -12℃ and 4℃ observed 86.0±7.9% and 79.6±2.6% in germination rate, 108.0±5.4 ㎛ and 85.4±6.2 ㎛ in pollen tube length after 150 days, also 82.8±5.6% and 61.2±4.1% in germination rate and 102.8±6.9 and 50.0±5.3 ㎛ in pollen tube length after 300 days, respectively (Fig. 1A and 1C).

Germination rates of *P. rigida* stored at a room temperature were greatly decreased after 30 days as shown in *P. densiflora* (Fig. 1B and 1D). But, it showed that viability can be substantially extended at temperatures of -70℃, -12℃ and 4℃. Pollen of *P. rigida* which were stored at -12℃ and 4℃ showed 88.8±1.1% and 79.2±4.1% in germination rate, 116.0±5.6 ㎛ and 105.6±7.1 ㎛ in pollen tube length after 150 days, also 80.8±6.3% and 65.2±4.8% in germination rate and 91.0±6.1 ㎛ and 67.8±6.2 ㎛ in pollen tube length after 300 days, respectively. Viability can be extended beyond 300 days when stored at -70℃, -12℃ or 4 ℃.

Pollen culture temperature effects on pollen growth

Pollen of *P. densiflora* and *P. rigida* that were cultured at 5℃ for 48 hours showed 0% in germination rate and any pollen tubes were not grown. As the temperature increased, the pollen of both *P. densiflora* (Fig. 2A) and *P. rigida* (Fig. 2B) showed higher germination rate and pollen tube length. At 25℃, the germination rate of *P. densiflora* pollen was 85.2±4.6% and that of *P. rigida* pollen was 83.2±3.0%. In pollen tube growth, the tube length was 101.6±6.0 ㎛ in *P. densiflora* and 124.6±6.9 ㎛ in *P. rigida*. It showed that the germination rate and pollen tube growth were maximum at 25℃ among the temperature regimes. Over 25℃, the pollen of *P. densiflora* and *P. rigida* were decreased in germination rate and pollen tube length. The germination rates of *P. densiflora* and *P. rigida* pollen were 13.6±3.8% and 12.0±4.0% at 40℃. Also there were 2.4±0.7 ㎛ (*P. densiflora*) and 2.2±0.6 ㎛ (*P. rigida*) in pollen tube length.

Effects of sucrose and boric acid concentrations on *in vitro* pollen growth

Germination media contained 3% and 5% sucrose showed 62.4±7.8% and 56.8±7.6% of germination rate in *P. rigida*

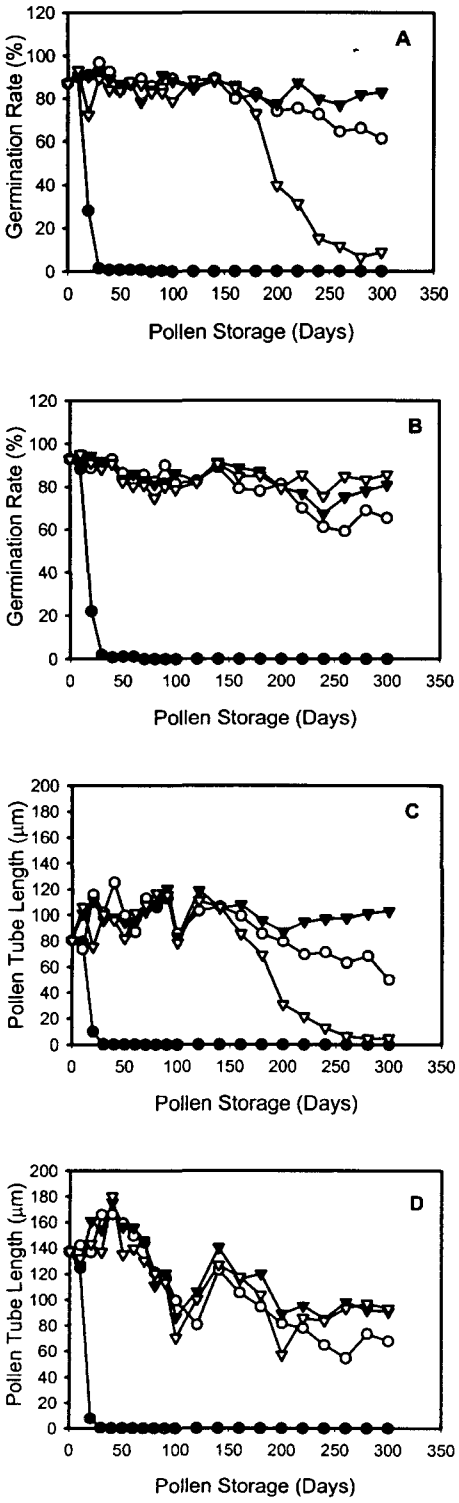

Fig. 1. *In vitro* pollen germination rate of *Pinus densiflora* (A), *P. rigida* (B) and pollen tube length of *P. densiflora* (C), *P. rigida* (D) pollen stored at -70℃, -12℃, 4℃ and 22℃, respectively (● stored at 22℃, ○ stored at 4℃, ▼ stored at -12℃ ▽ stored at -70℃).

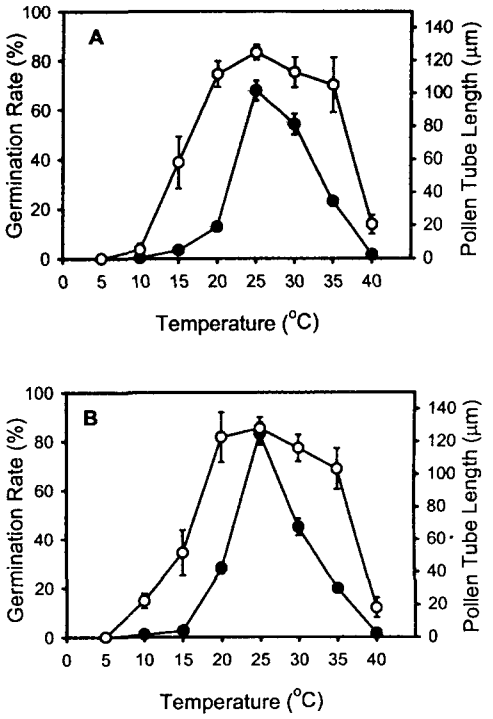

Fig. 2. Culture temperature effect on *in vitro* pollen growth of *P. densiflora* (A) and *P. rigida* (B) (○ germination rate, ● tube length).

pollen (Fig. 3A). Pollen growth in several sucrose concentrations with 0.01% boric acid showed similar performance from 1% to 10% in sucrose concentrations (Fig. 3b). In addition, germination media contained several boric acid concentrations showed that germination rate and tube length were greatest in 0.01% boric acid in DDW (Fig. 3C).

DISCUSSION

Storage temperature and pollen viability

Our results showed that storage temperature considerably affected on pollen performance *in vitro*. Generally lower temperatures supported longer pollen viability in two pine species except the *P. densiflora* pollen stored at -70℃ after 150 days. *Pinus densiflora* and *P. rigida* pollen did not show difference in pollen viability after 300 days of storage. But *Pinus rigida* pollen tube growth was generally better than *P. densiflora*. Pollen viability is one measure of male fertility. Viability tests are often conducted in breeding experiments in agriculture and to monitor the condition of stored pollen (Heslop-Harrison *et al.* 1984, Iwanami 1972). There are direct and indirect measures for pollen viability. Indirectly pollen germination can be scored *in vitro*. Indirect methods rely on the correlation between ability to fertilize an ovule and some physiological and physical characteristics that can be

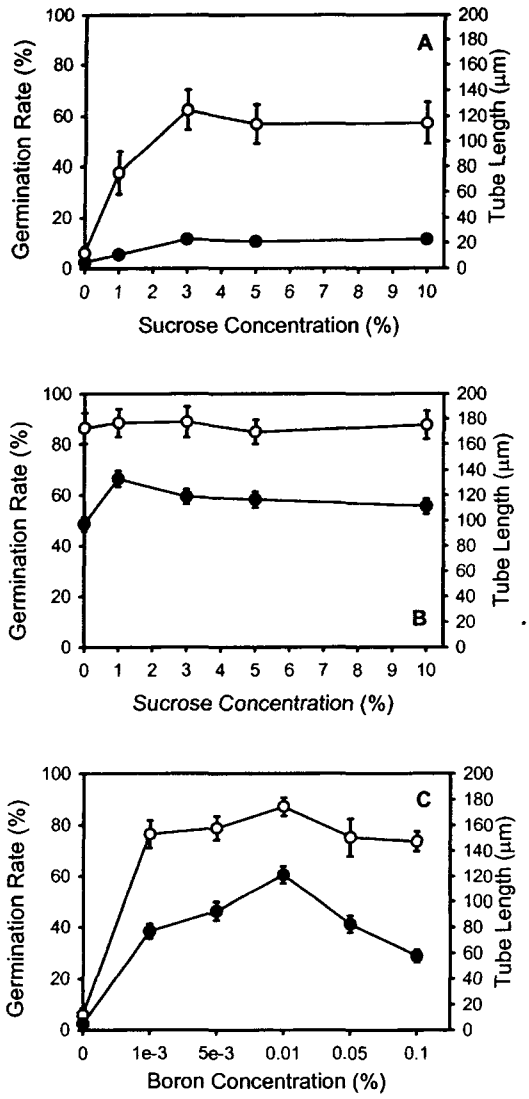

Fig. 3. *In vitro* pollen growth of *Pinus rigida* in several sucrose concentrations (A), in sucrose medium with 0.01% boric acid (B), and in several boric acid concentrations (C) (○ germination rate, ● tube length).

determined more rapidly *in vitro*.

In general, pollen longevity is affected by storage temperature and humidity (Shivanna and Johri 1985, Shivanna *et al.* 1991). So if pollen is not used in experiment immediately, pollen has be to dried to retain maximum viability. In addition, pollens collected at different times of the day (Stanley and Linskens 1974) or at different stages of floral development (Leduc *et al.* 1990) may differ in viability. Tree pollen from several species showed the same pattern. Pollen viability of *P. densiflora* and *P. rigida* significantly was decreased as the duration of the pollen storage days increased at room temperature after 30 days. The primary reason for decreased pollen viability is probably related to reduced enzyme activities in pollen. The mechanism by which the pollen retains its viability during the storage is related to the intercellular rates of respiration, *e.g.* the conversion of sugars to organic acids (Stanley and Linskens 1974). Reduction of germination capacity under certain storage conditions can, therefore, be interpreted as an inactivation of enzymes and metabolic substrates essential for germination. Furthermore, an accumulation of secondary metabolic products, such as organic acids during storage, may inhibit subsequent pollen growth (Stanley and Linskens 1974). Smith-Huerta and Vasek (1984) found that *Clarkia* (Onagraceae) pollen viability decreased with time at room temperature both on the plant or in the lab, but the pollen stored at 5°C retained its ability to fertilize ovules for longer periods. In this study *P. densiflora* and *P. rigida* pollen stored at -12 °C and 4°C, remains viability for at least 300 days. *P. densiflora* pollen stored at -70°C gradually decreased in viability after 150 days. This result shows that *P. densiflora* pollen is a bit more vulnerable when compared to *P. rigida* pollen which can survive longer in storage at -70°C. It is known that at the extremely low temperatures, from -180 to -271°C, obtained by liquid gases, it may be assumed that cytoplasmic activity is reduced to nearly zero (Stanley and Linsken 1974). Our results indicate that the pollen of *P. densiflora* and *P. rigida* can be easily collected and can be stored for a considerable length of time without loss of viability. Therefore, pine pollen may be used as a good material for biological and environmental monitoring researches.

Culture temperature and pollen growth

The optimal culture temperature condition was 20-30°C for pine pollen growth. Pollen germination and tube elongation were inhibited or suppressed in condition of over 30°C or less than 15 °C. It might be assumed that the optimal temperature of pine pollen is consistent with the air temperature during early or middle of May when the pollen grains are dispersed from male cones. However, pollen of *Camellia*, which flowers in winter, can germinate at even below 5°C (Richards 1986; Shivanna *et al.* 1991; Iwanami 1988). Factors influencing *in vitro* growth include the species of pollen, time of collection, season of the year, method of collection and storage condition.

Germination medium and pollen growth

In this study, pollen growth was examined based on the effect of sucrose and boric acid concentrations in medium. *In vitro* pollen germination can take place (1) in water (some gymnosperms), (2) in a sucrose solution, or (3) in a sucrose solution on agar or gelatin as a medium (Stanley and Linskens 1974). Germination media with sucrose were made at different concentration such as 1, 3, 5, 10% . The optimal sucrose solution can be determined by germination trials at different sucrose concentrations (*e.g.*, 1, 2.5, 5, 10, 20, 30%; R. Peakall, personal communication). If the concentration is too low, germination is poor

and pollen tubes are short. At extremely high or low concentration, pollen will burst or shrink. In this study, the highest growth was observed at sucrose concentration of 5% in *P. densiflora* (82.4±4.1 in germination rate) and 3% *P. rigida* (62.4±7.8 in germination rate). This result coincided with the observation that stored pollen requires higher concentration of sugar for normal germination than fresh pollen (Vasil 1964). The increased sucrose concentration required to obtain optimal germination has also been attributed to a decrease in pollen permeability. Sucrose solutions range from 2% to 40% depending on the optimum for the species were established empirically. A 20% solution has been used successfully with *Brassica* (Brassicaceae) pollen, whereas a 2.5% solution was better for *Microtis* (Orchidaceae) pollen (Peakall and Beattie 1989). Our results indicated that the pollen growth cultured in the medium with sucrose and boric acid was much longer than cultured in those with sucrose only. Boric acid levels in pollen were influenced by the amount of boron available to the plant during development (Vasil 1964). He reported that developing floral organs were placed in solution containing a high boron concentration the resulting pollen germinated *in vitro* better than pollen having developed with little or no pollen could be increased by externally added sources. In nature, water, sugar, and amino acids are supplied by the style to nourish the growing pollen tube. For many species, boron and calcium are also required for pollen tube growth. Boron, provided by stigmas and styles of these species facilitates sugar uptake and has a role in pectin production in the pollen tube (Richards 1986). *In vitro* pollen tube growth is generally slower than in vivo and tubes do not get as long. In conclusion, *in vitro* pine pollen germination was affected by storage conditions, culture temperature as well as sucrose and boric acid concentrations in the culture medium.

LITERATURE CITED

Delph, L. F., M. H. Johannsson and A. G. Stephenson. 1997. How environmental factors affect pollen performance: ecological and evolutionary perspectives. Ecology 78: 1632-1639.

Heslop-Harrison, J., Y. Heslop-Harrison and K. R. Shivanna. 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. Theor. Appl. Genet. 67: 367-375.

Hormaza, J. I. and M. Herrero. 1996. Dynamics of pollen tube growth under different competition regimes. Sexual Plant Reprod. 9: 153-160.

Iwanami, Y., T. Sasakuma, and Y. Yamada. 1988. Pollen: Illustrations and scanning electronmicrographs. Kodansha, Tokyo.

Kang, Hyesoon. 1999. Variations in the seed production of *Pinus densiflora* trees. Korean J. Biol. Sci. 3: 29-39.

Lankinen, A. 2001. *In vitro* pollen competitive ability in *Viola tricolor* : temperature and pollen donor effects. Oecologia 128: 492-498.

Leduc, N., M. Monnier and G. C. Douglas. 1990. Germination of trinucleated pollen: Formulation of a new medium for *Capsella bursa-pastoris*. Sex. Plant Reprod. 3: 228-235.

Peakall, R. and A. J. Beattie. 1989. Pollination of the orchid *Microtis parviflora* R. Br. by flightless worker ants. Funct. Ecol. 3: 515-522.

Richards, A. J. 1986. Plant Breeding Systems. George Allen and Unwin, London.

Shivanna, K. R. and B. M. Johri. 1985. The Angiosperm Pollen. John Wiley & Sons, New York. pp. 154-177.

Shivanna, K. R., H. F. Linskens and M. Cresti. 1991. Responses of tobacco pollen to high humidity and heat stress: Viability and germinability *in vitro* and *in vivo*. Sex. Plant Reprod. 4: 104-109.

Smith-Huerta, N. L. and F. C. Vasek. 1984. Pollen longevity and stigma pre-emption in Clarkia. Am. J. Bot. 71: 1183-1191.

Stanley, R. G. and H. F. Linskens 1974. Pollen: Biology, Biochemistry and Management. Springer Verlag, New York. pp. 124-128.

Vasil, I. K. 1964. Effect of boron on pollen germination and pollen tube growth. *In* H. F. Linskens (ed.), Pollen Physiology and Fertilization. North-Holland, Amersterdam. pp. 107-119.

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