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Full Length Research Paper

Pollen characteristics and *in vitro* pollen germination of *Cedrus libani* A. Rich.

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This study aims to determine the germination characteristics, pollen tube developments, effects of germination media and temperature and incubation durations of the pollens obtained from the four clones (11342, 11344, 11345 and 11351) of *Cedrus libani* A. Rich. (Lebanon Cedrus) obtained from clonal seed orchard (with national registration no: Eskisehir 117) between 2004 and 2006 as well as of those taken from clone no: 11351 in 2004. They were stored at 3°C for 13 months till they were investigated experimentally. MS medium was preferred for pollen germination for its relative superiority. Three-day incubation period at 33°C temperature, in dark was applied along with the MS medium. The highest germination rate in MS medium was achieved in clone no.11342 with 84.77% among the pollen samples of 2005. On the other hand the germination rate of the pollens taken from clone no: 11351 was determined as 49.95%.

Key words: *Cedrus libani*, pollen germination, pollen tube.

INTRODUCTION

Cedrus genus has four species spread out worldwide. Among these species, *Cedrus libani* A. Rich. (Lebanon Cedar) is a favored one due to its scent, color of wood and strength. *C. libani* A. Rich is mainly distributed across Taurus Mountains with the exceptions of some forests in the north of Lebanon and in Syria (Isık, 1994). The species is spread out in the South of Turkey, the Mediterranean Region, particularly in Taurus Mountains and holds a remarkable status in forestry and reforestation practices in Turkey (Atalay, 1987). *C. libani* was declared as a “species of conservation concern” by the Food and Agriculture Organization (FAO) within its spread area (Isik and Yildirim, 1990; FAO, 1997, 2004).

Since the pollen is among the main source for hybridization breeding, it is crucial to have information that associated with germination characteristics, lifetime and storage requirements. There are studies on the storage and *in vitro* germination of gymnosperm pollens such as *Cedrus atlantica* (Endl.) Monetti ex Carriere, *Pseudotsuga menziesii* (Mirbe) Franco, *Picea abies* L. Karst., *Pinus nigra* Arnold., *Pinus pinea* L., *Pinus strobus* L., *Pinus sylvestris* L., *Pinus uncinata* Mill. Ex Mirp., *Pinus monticala* Dougl. Ex D. Don, *Picea sitchensis* (Bong.) Carr., *Larix occidentalis* Nutt., *P. menziesi* (Mirbe) Franco, *P. menziesii* (Mirbe) Franco and *Pinus radiata* (D. Don) (Copes et al., 1991; Lanteri et al., 1993; Dumont-Beboux et al., 1998; Fernando et al., 1998; Siregar and Sweet, 2000; Krouchi et al., 2004). The purpose of the study is to determine *in vitro* germination conditions of *C. libani* A. Rich. (Taurus Cedar) pollens as the main sources of hybridization breeding and to determine the characteristics of those germinated.

MATERIALS AND METHODS

Plant material

The pollens of *C. libani* A. Rich used in this study were obtained from Afyon-Cay Cedar clonal seed orchard (national reg. no: 117), established in 1990, by the Department of Forest Tree Seeds and Tree Breeding Research of the Ministry of Environment and Fores-

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Figure 1. Cedar clonal seed orchard with national reg. no: 117 (A). Drying male flowers (B).

try (Figure 1a). The research materials were taken from the four clones of the clonal seed orchard (national reg. no's: 11342, 11344, 11345, 11351) in 2005. In addition, samples were taken from the clone no: 11351 in 2004 and stored at 3°C with 12% humidity for 13 months.

The male flowers were handpicked by the end of September between 09.00 and 10.30 A.M. in good weather conditions (20 - 21°C) (Figure 1b). They were subsequently taken to laboratory and left to dry in carton boxes inside covered with filter paper at room temperature (24 - 25°C) and 35 - 45% relative humidity (Weber and Painter, 1996). The papers in the carton boxes were changed frequently during the drying process. Following the drying process of five-nine days, the male flowers started to blossom and spread out pollens. At this phase, sieving process was carried out in 80 and 100 µm sized telescopic sieves. Just 5 g of the sieved pollens was weighed and their initial humidity (the humidity during the spread of pollens) was measured in Sartarius L310D device. The samples from 2004 were placed into sterile tubes (cotton-slica gel-cotton-pollen- cotton-silica gel-cotton) and left for storage at 3°C.

In vitro pollen germination

Then the pollens were placed in-between the folded sterile papers in the cabin were first kept for five minutes in a petri dish with 5% sodium hypochlorite and subsequently washed in sterile water three

Table 1. Three-day germination rates of *Cedrus libani* A. Rich pollens (2004 and 2005) in MS medium, at 33°C.

Clone no.	Initial humidity (%)	Germination (%)		
		Day one	Day two	Day three
11342	29.60	1.36	72.46	84.77±2.2
11344	14.71	2.19	79.17	81.23±1.8
11345	23.53	9.20	61.04	57.69±0.4
11351 - 2005	14.51	12.83	42.08	40.52±1.7
11351 - 2004 (+3°C)	12.43	10.92	50.88	49.95±

times.

Three different media (A hormone-free MS medium (pH 5, 8 and 8% agar) (Murashige and Skoog, 1962), a medium composed of Brewbaker and Kwack mineral salts (Weber and Painter, 1996) and a medium composed of only agar and sucrose (Krouchi et al., 2004) were used for the germination of the collected pollens.

The sterilized pollens were distributed via sterile brushes onto the germination medium in glass petri dishes of 6 cm diameter. Ambient temperatures of 33°C and 24-25°C were tested in the preliminary experiments. The *in vitro* germination at 33°C, which produced more superior results, was observed for three days. Incubation was performed at 33°C with constant temperature, in dark. The preparations were examined with Leica DM LB2 Light Microscope and counted. They were photographed with Leica DFC320 camera. The counts were based on the principle of pollen tube production among the germinated pollens up to stem (Lanteri et al., 1993). Protein and starch grains of the germinated pollens were stained with Lugol's solution, while the nuclei were stained with hematoxylen (Yakar-Olgun, 1958; Algan, 1981).

In each experiment, 300-350 pollens were seewed and experiments were repeated three times.. Standard errors were calculated for the data on germination and tube lengths (in each of the three rounds, measurements were made in 50 pollens).

RESULTS AND DISCUSSION

The purpose of this study is to determine the viability of the *C. libani* A. Rich. pollens sampled in 2004 and 2005, which is a species naturally spread out in Turkey having significance in terms of economical value and aforestation practices.

The humidity measurements of the pollens were recorded as 29.60% for the clone 11342, 23.53% for the clone 11345, 14.71% for the clone 11344 and 14.51% for the clone 11351. Among the pollens of *P. menziesii* (Mirbe) Franco male flowers, which were collected in fourth phase of the development and extracted after a 48 h drying process, the humidity was below 10% (Webber and Painter, 1996). Researchers stated that humidity rate of *P. menziesii* pollens was lower than the humidity of the pollens of our clones. This is particularly important for extending pollen viability.

Three different media were used in our preliminary studies as pollen germination media. In accordance with the findings of preliminary experiments, Brewbaker and Kwack germination medium resulted in considerably poor germination accompanied with contaminations in the subsequent days. The experiments evaluating the temperature have shown that the ambient temperature of 33°C increased the speed of germination; therefore the study was carried on under these conditions. Furthermore, the pollens of clone no: 11342 germinated in 2% agar + 5% sucrose medium yielded a germination rate of 76.30% on the third day. Since the highest germination rate was achieved in MS medium, the experiments were continued in this medium (Table 1).

Dumont-Beboux et al. (1998) sterilized male flowers in 70% alcohol and subsequently with 1% sodium hypochloride for 30 s to eliminate the contamination. Although they achieved successful results, the most successful result in our experiments in terms of pollen surface sterilization was obtained by using higher amount of, that is, 5%, sodium hypochloride.

Since the fluid germination medium of Brewbaker and Kwack as well as the solid 2% agar + 5% sucrose mixture did not yield successful outputs, experiments were carried out in MS medium. MS germination medium was used in this study for pollen viability tests. Different researchers have utilized diverse media for this aim. For the germination of *P. menziesii* (Mirbe) Franco pollens, MSBK germination medium was utilized which was formed by the modification of the mineral salts of Brewbaker and Kwack into MS germination medium (Fernando et al., 1998). Experiments were performed by hanging-drop method and various mixtures of sugar and water to examine the germination of *P. sylvestris* L. pollens, which is a naturally spread pine species in Turkey. The germination rates achieved were 91% in distilled water, 91% in 5% sweetened water, 92% in 10% sucrose and 89% in 30% sweetened water (Boydak, 1977).

Among the pollens taken in 2005 and put onto MS medium, the highest and lowest germination rates by the third day were recorded respectively in clone no: 11342 (84.77%) and clone no: 11351 (40.52%). The rest of the germination rates were 81.23% for clone no: 11344 and 57.69% for clone no: 11345. The clones with high germination rates on the first day (11345-11351) resulted in relatively lower germination rates by the third day whereas the clones with low germination rates on the first day (11342-11344) resulted in relatively higher germination rates by the third day (Table 1). The germination rate of 2004 samples of clone no: 11351 that were preserved at 3°C was 49.95%. On the other hand the

Table 2. The statistical values on the pollen tube lengths of 2004 and 2005 samples of clone no: 11351 by the third day.

Year	n	Average (µm)	Std. deviation	Std. error	Minimum	Maximum
2004	50	544.56	56.98	8.06	439.00	668.33
2005	50	434.31	72.04	10.19	228.67	583.00
Total	100	489.43	85.12	8.52	228.67	668.33

n: 50 pollen tube lengths.

germination rate yielded by 2005 samples that were immediately planted was 40.52% (Table 1). These results point out the existence of differences among clones with respect to pollen viability. While the highest germination rate (84.77%) was achieved in clone no: 11342 by the third day, the germination rate was higher in *P. radiata.* The average germination rate of *P. radiata* (D. Don) at the end of 48 h incubation in 2% sucrose + 1% agar was identified as 93% along with significant clone-wise differences (Siregar and Sweet, 2000).

It is known that there is a close relationship between pollen viability and seed storage durations (Sehirali and Ozgen, 1987). The germination rates and resistance of seeds are low in the years of poor seed yield. For example, while the average germination rate of *Picea orientalis* (L.) Link seeds in the years of rich seed yield was 89.1%, the same rate has decreased to 30.8% in the years of poor seed yield (Atay et al., 1970). The germination rate of 2005 samples of clone no: 11351 (40.52%) is lower than that of the pollens preserved at +3°C for 13 months (49.95%) (Table 1). Furthermore, after a three-day germination in the samples of the same clone, it was determined that the tube length of 2004 samples was 120 µm higher than that of 2005 samples (Table 2). These two findings reveal that 2005 was a year of poor seed yield in terms of cedar. In accordance with the findings of another project carried out in the same clonal seed orchard, while plenty of female and male flowers existed in 2004, there were almost none in 2005. In the tree of clone no: 11351, there were 1047 and 272 male flowers on average, in 2004 and 2005, respectively (Anonymous, 2006). Moreover, another noteworthy remark was the high amount of contamination in 2005 samples contrary to those of 2004 among which contamination was either very low or non-existent. The high prevalence of contamination among 2005 samples is possibly due to either the poor seed yield or the heavy rain in the previous day of collection of male flowers.

Along with the start of germination, the exine layer thinned, the bulb has started to appear due to the swelling and ruptures in the germinal zone and the nutrients (proteins, starch grains) left the pollen. It was identified that among the pollens with high germination rates on the first day of incubation, the length of the pollen tubes were up to two times that of pollen stem on the first day of germination, up to five-six times that of pollen stem on the second day of germination and up to seven-eight times that of pollen stem on the third day of germination (Figure 2).

The measurements indicated that among 2005 samples of clone no: 11351, the average tube lengths were respectively 274 and 434 µm by the second and third days, while a tube length of 545 µm was reached by the third day among 2004 samples. 2005 values of tube lengths were lower than those of 2004 by the third day (Table 2).

The germinated pollens of clone no: 11351 were stained with lugol and hematoxylen. In the pollen tubes, there were starch grains in dark purple stained with lugol (Figure 3a). Concurrently, two to four nuclei in violet-blue colour were identified in hematoxylen staining. It is estimated that the first and second nuclei in Figure 3b are sperm nuclei and the remainder two are the tube and stalk cells (Von Denffer et al., 1971; Owens, 1993).

During the observations of germination, a few cases of abnormal tube growth were determined such as; tube growth of more than one (poly-tube growth) (Figure 4a), swelling at the tip of the tube (Figure 4b) and tube growth at the opposite poles of the germinal zone (Figure 4c).

Pollen tube length is a significant determinant of germination rate (Dillion and Zobel, 1957; Stairs and Troendle, 1967). Pollen tube growth is faster in angiosperms compared to that of gymnosperms. In a study on *Trifolium pratense* L. pollens comprising a 12 h observation period, the pollen tube length of 177.6 µm in the first hour was noted to have reached to 732 µm by the end of the 12th hour (Buyukkartal, 2003). In the species of *C. atlantica* (Endl.) Monetti ex Carriere, the pollen tube lengths ranged between 194 and 559 µm by the third day of germination whereas the values for *P. menziesii* (Mirbe) Franco pollens were between 90 and 1170 µm by the seventh day of germination (Fernando et al., 1998; Krouchi et al., 2004). As indicated by the measurements in this study, the average tube length of 2005 samples of clone no: 11351 by the third day was 434 µm, whilst the same sample preserved at +3°C for one year yielded an average tube length of 545 µm by the third day (Table 2). On the basis of these data, *C. libani* A. Rich. pollens proved to adapt cold storage conditions. Hence, pollens of different clones might be stored at +3°C during breeding studies. Further experiments on storage durations are planned in the forthcoming studies.

The determination of pollen germination criteria is of considerable importance for the investigation of the impacts

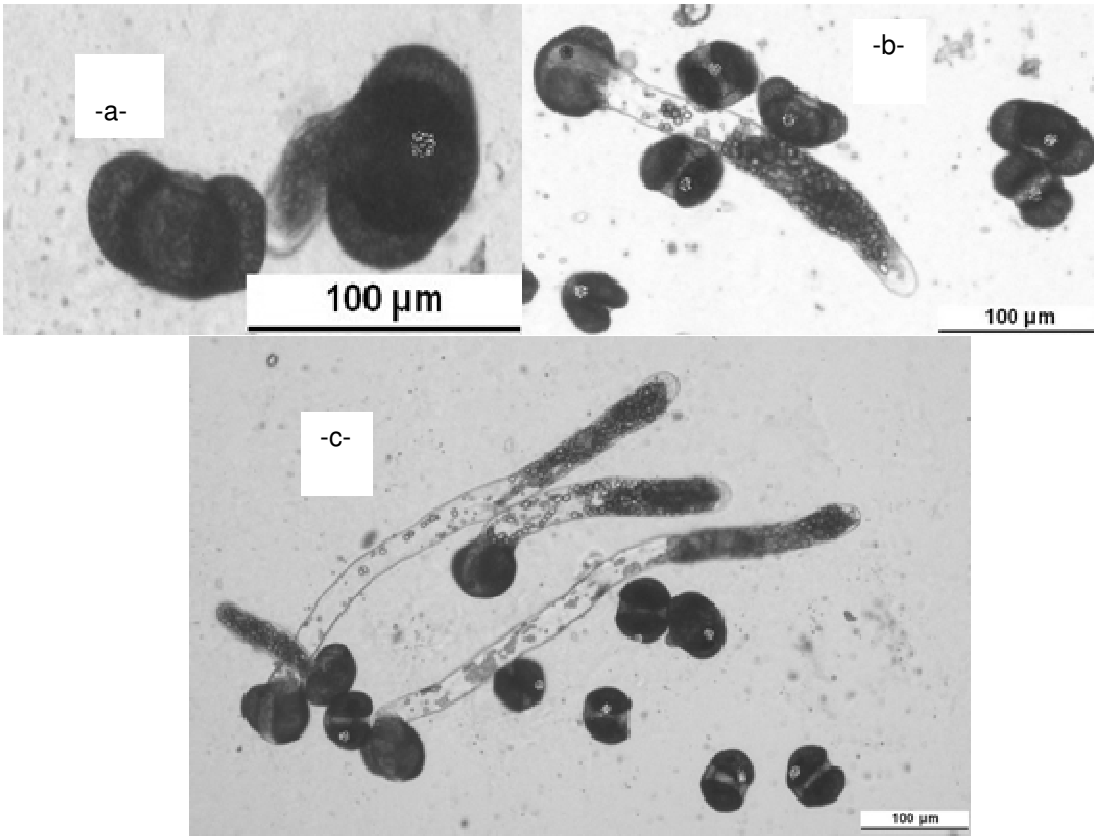

Figure 2. The germination of 2005 samples of clone no: 11351: a) Day one; b) Day two; and c) Day three (Bars: 100 µm).

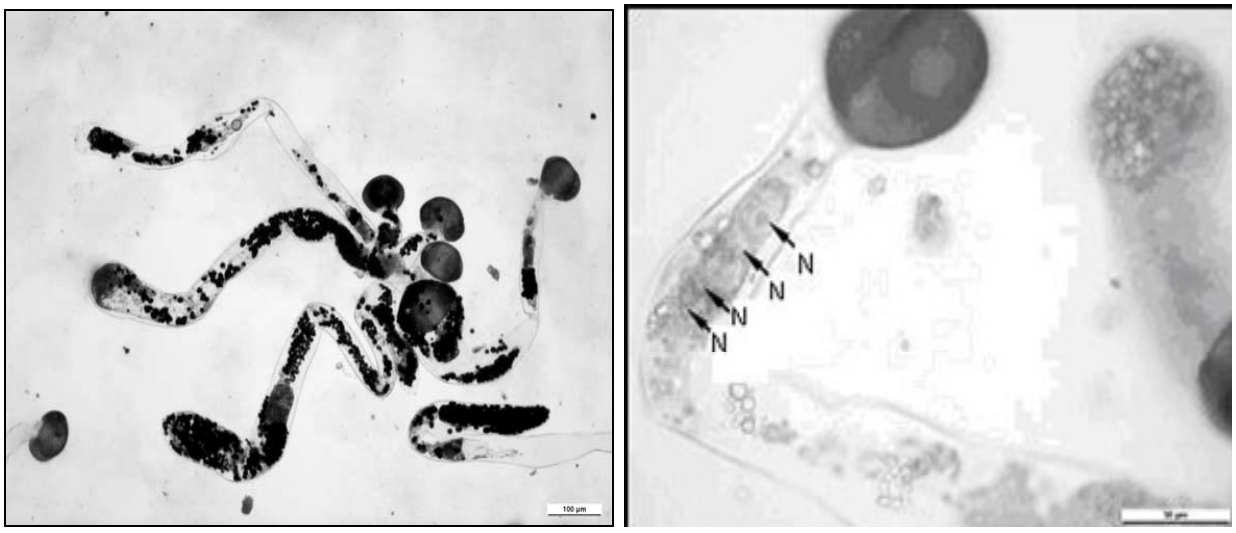

Figure 2. a) Starch grains in germinated pollens (Bar: 100 µm); b) Numerous nuclei in the pollen tube (Bar: 50 µm, arrows: nuclei).

of yield-enhancing hormonal practices in breeding facilities (seed orchards, clone parks etc.) and environmental pollution (radiation etc.) on pollen biology. The findings have shown that *C. libani* A. Rich. pollens and stored pollens can germinate on MS medium. This study on *in vitro* germination and storage practices of *C. libani* pollens constitutes the baseline data for further clarifycation of hybridization breeding.

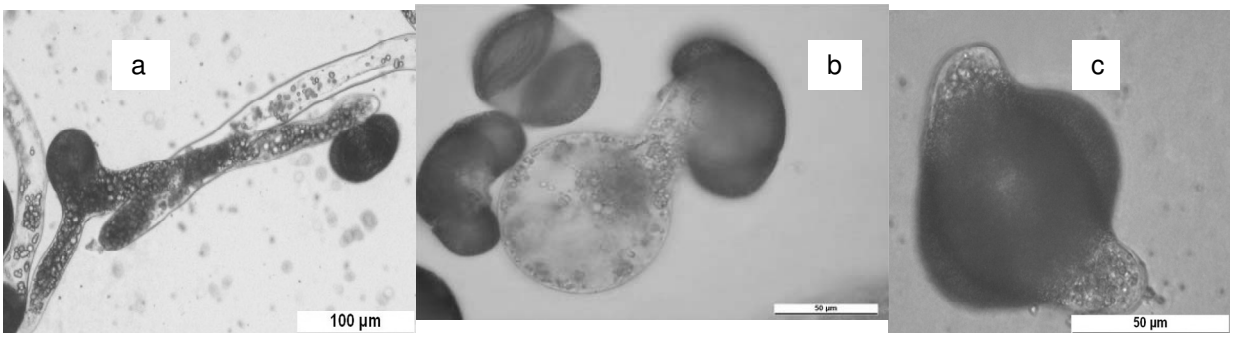

Figure 3. Abnormal tube growths: a) Poly-tube growths (Bar: 100 µm); b) Swelling at the tip of the tube r: 50 µm); c) Tube growth at the opposite poles (Bar: 50 µm).

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