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Storage of Pollen of Norway Spruce and Different Pine Species

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Summary

The effect of 2 temperatures on pollen storage ability of the following conifers was investigated: *Picea abies* L., *Pinus nigra* ARNOLD, *Pinus pinea* L., *Pinus strobus* L., *Pinus sylvestris* L. and *Pinus uncinata* MIRB. Pollen was stored at —18° C and —196° C for 24 months and was assayed for viability every 2 months by means of *in vitro* germination tests, which are one of the most convenient methods for evaluating pollen reactivity to storage, although they may not necessarily provide the best indication of potential fertility.

The variability in germination response to the 2 storage temperatures was dependant on the species. At the end of the considered period, only the pollen germinability of *Picea abies* and *P. nigra*, in both storage conditions, did not show any decrease, while in the other species a decrease in pollen germination percentage was observed in freezer as well as in liquid nitrogen storage. The temperature of —196° C proved clearly to preserve the pollen better only in *P. uncinata*. In three of the species considered: *Picea abies*, *P. sylvestris* and *P. uncinata*, an increase in germination during the first months of storage at —196° C was observed.

Key words: Conifers, pollen storage.

Introduction

The preservation of viable pollen is very important for plant breeding. Successful pollen storage in the short term allows the hybridization of species which flower at different times or of populations which are separated geographically. The preservation of viable pollen for several years is one of the methods used for long term storage of plant germplasm. Pollen preservation for germplasm conservation is convenient, economical and space-saving. This is particularly evident for woody plants, which take several years to flower from the seedling stage.

The preservation of pollen viability presents problems which are quite similar to the problems with seeds. Optimal storage environments for pollen differ according to species, but common factors of importance are moisture content and storage temperature. Other factors, such as the composition and pressure of the gas phase around the pollen are known to affect longevity, but are rarely manipulated for optimum storage, except in storage under vacuum (TOWILL, 1985).

Unlike angiosperm pollen, whose reduction of moisture content below a certain level (20% to 30%) is usually fatal, in conifers the longevity of stored pollen increases with the decrease of its moisture content. High moisture content allows greater metabolic activity and also promotes the destructive activities of fungal and bacterial contaminants (MATTHEWS and KRAUS, 1981). For most conifers species pollen is best stored in a range of 10% to 20% moisture content and its viability is retained longer when a minimum of humidity fluctuation during the storage period is insured (HARRINGTON, 1970). Preservation at deep freeze temperatures (—18° C to —20° C) usually gives satisfactory results and is a common and practical way of storing pollen from one season to another, while pollen of several crop plants was successfully maintained in a viable state for prolonged periods in liquid nitrogen (FARMER and BARNET, 1974; NATH and ANDERSON, 1975; BARNABAS and RAJKI, 1976; TOWILL, 1981; TISSERAT *et al.*, 1983; COPES, 1985, 1987; GANESHAN, 1986). Nevertheless, the ideal storage conditions for short and long-term preservation of the pollen of many coniferous trees are not well-known and available data are approximate and incomplete.

Most data found in literature are descriptive and report survival under a given set of conditions after a certain period of time. They do not consider variation in viability during storage and usually the final test is field fertility (CRAWFORD, 1937; DUFFIELD and CALLAHAM, 1959; CALLAHAM and STENHOFF, 1966). Furthermore, investigations involved only some species and it is difficult to extrapolate whether all pollen from a genus or family behaves similarly in storage or not.

After a period of storage the viability of pollen can be measured *in vitro* by means of indirect or direct tests. Among the former the most frequently used are specific vital stainings, which assess the pollen contents with varying degrees of specificity for different constituents; the enzyme tests, which establish the activity of various enzymes of the vegetative cells; and the fluorochromatic procedure, which is an indirect measure of the integrity of the plasma membrane (HESLOP-HARRISON and HESLOP-HARRISON, 1970; HESLOP-HARRISON *et al.*, 1984). The plasmalemma integrity, which proved to be a reliable assay for viability, can also be evaluated by means of conductivity measurements of the leachate released by pollen in distilled water (CHING and CHING, 1976). BINDER and BALLANTYNE (1975) suggested that also the respiration rate can be correlated to viability in Douglas-fir pollen.

To the contrary, the direct *in vitro* germination tests provide a direct assessment of viability. Therefore, as long as there is a suitable medium for obtaining consistent pollen germination and pollen tube growth for the species in study, said tests are convenient laboratory methods for evaluating pollen reactivity to storage (TOWILL, 1985). In spite of this, it must be pointed out that *in vitro* germination tests do not necessarily predict the precise yield of sound seeds that will result from the use of a pollen batch; in fact stored pollen of some species having an excellent pollen tube elongation in cultures, will not set seeds, while stored pollen of other species may show no germination in cultures, but will set fruit (HARRINGTON, 1970). However, by optimizing media constituents and using prehydration techniques, the correlation between *in vitro* germination percentage and pollen ability to set seeds may improve substantially (WEBBER, pers. comm.).

In a previous paper the authors described the best procedure for *in vitro* pollen germination of the species studied in this experiment (LANTERI and BELLETTI, 1987). For each species three germination techniques were assayed: water vapour test, water drop test and test on agar (1%). In the last 2 tests, the medium was supplied with sucrose (5%, 10%, 15%, 20%) and boron. The latter was always added as boric acid solution at a concentration of 0.01% boron anion. These nine germination tests were performed at 4 temperatures (15° C, 20° C, 25° C, 30° C) and therefore 36 conditions of germination were compared. The germination conditions which allowed for the highest value of germination percentage on fresh pollen in each species are reported in *table 1*.

The present investigation was undertaken to determine the pollen longevity of various conifers at two different storage temperatures: —18° C (freezer) and —196° C (liquid nitrogen). Differences in the storability of pollen at —20°C among the different species of *Pinus* were observed previously by CALLAHAM and STENHOFF (1966).

Table 1. — Best pollen germination conditions for the species in study. Where 2 or more conditions are reported, the conditions not utilized in this study are reported between brackets. In the drop test, pollen was sprinkled on a drop of water placed on a glass slide. In the vapour test pollen was sprinkled on a dry glass slide. In both cases slides were placed in a high humidity room obtained by lining a 110 mm petri dish with filter paper saturated with water.

SPECIES	MEDIUM	SUCROSE CONCENTRATION (%)	TEMPERATURE (°C)
Picea abies	Agar 1%	10 (15)	25 (30)
Pinus nigra	H_2O drop	10	25
Pinus pinea	H_2O vapour	0	25
	(Agar 1%)	(5)	
Pinus strobus	Agar 1%	(5) 10	30
Pinus sylvestris	H_2O vapour	0	(20) 25 (30)
	(Agar 1%)	(5)	
Pinus uncinata	H_2O drop	10 (15)	25

Materials and Methods

Pollen collection and management

Ripe microsporangiate strobili of the following species: *Picea abies* L., *Pinus nigra* ARNOLD, *Pinus pinea* L., *Pinus strobus* L., *Pinus sylvestris* L. and *Pinus uncinata* MIRB. were collected from at least eight trees per species, about 30 years old.

Microstrobili were gathered, just before shedding time, in the early morning, during the spring of 1986, and transferred to the laboratory in plastic bags, without separating those belonging to the different trees of the same species. After a few hours they were placed in uncovered petri dishes inside a growth chamber at 21° C and 30% relative humidity for about 3 hours to facilitate dehiscence. After shaking the catkins and sieving the pollen, the latter was spread in a thin layer on paper sheets in a room free of air turbulence, overnight; the room temperature was approximatively 25° C.

Pollen storage

Before storage, approximatively 20 g of pollen of each species was placed in a thin layer inside a fridge at +6° C, in a dessicator over silica gel. This step was carried out to further dehydrate the material. After 12 hours the pollen moisture content of each species ranged from 9.5% to 10.1% (*Table 2*), values obtained by calculating the difference in weight between air-dried and oven-dried pollen divided by the oven-dried weight. Oven-dried weight was obtained from about 1 g of pollen dried at 80° C for two days. Before storage, pollen of each species was subdivided into 2 samples and transferred to 2 sets of plastic, screw-cap vials of 2 ml, half full. One set of vials was stored at —18° C (freezer) and one at —196° C (liquid nitrogen) for 24 months. Cryopreservation was accomplished in a TAYLOR-WHARTON cryobiological system by direct immersion of the vials in liquid nitrogen. Based

Table 2. — Moisture content (% on a dry weight basis) of the pollen before storage.

SPECIES	
Picea abies	10.1
Pinus nigra	9.6
Pinus pinea	9.8
Pinus strobus	9.5
Pinus sylvestris	9.5
Pinus uncinata	9.8

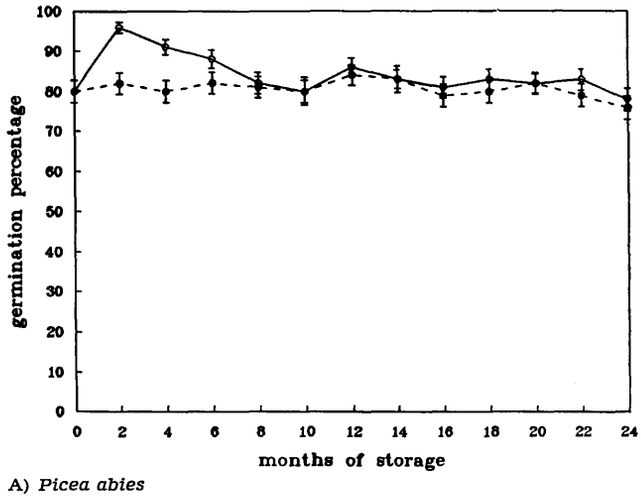
Table 3. — Germination percentage *in vitro* of fresh pollen and pollen tested after 24 months of storage at —18° C and —196° C. Mean values for each species followed by identical letters are in the same range according to the LSD test ($p < 0.01$).

SPECIES	FRESH POLLEN	POLLEN STORED -18°C	POLLEN STORED -196°C
Picea abies	80.7 a	76.2 a	78.2 a
Pinus nigra	74.7 a	68.0 a	65.8 a
Pinus pinea	93.0 a	44.0 b	48.3 b
Pinus strobus	96.2 a	76.2 b	82.4 b
Pinus sylvestris	70.0 a	50.1 b	54.3 b
Pinus uncinata	84.7 a	46.3 c	61.3 b

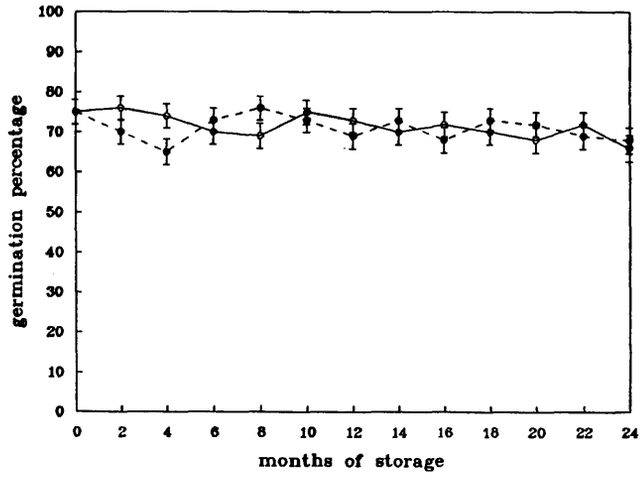
on the previous use of a pre-cooling period by several researchers (Layne and Hagerdorn, 1963; Ichikawa and Shidei, 1972a and b; Ganeshan, 1986; Bowes, 1990) the pollen was pre-cooled at —18° C for 3 hours before dipping it into liquid nitrogen.

Germination techniques

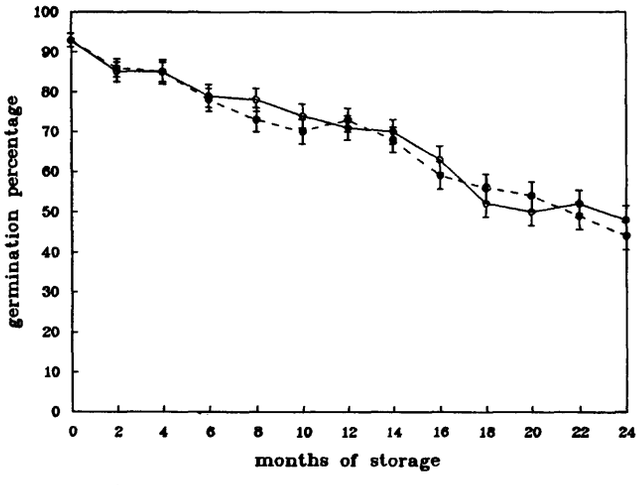
Pollen was assayed for viability before storage and every two months during storage using *in vitro* germination. The germination values for fresh pollen were completed on the same pollen used for the storage trial; before germination testing, fresh pollen was first dehydrated and then rehydrated right away. One vial per testing time per species was utilised. As previously mentioned, the *in vitro* tests utilised were those found to be the best ones for each species (Lantari and Belletti, 1987) and are listed in *table 1*. Prior to incubation in the germination medium, the amount of pollen contained in a vial was spread in a 60 mm diameter plastic petri dish, which was placed, for 20 hours at room temperature, in a 150 mm dish lined with filter paper saturated with water. Under these conditions, the moisture content approximated that of fresh pollen, ranging from 30% to 35%. Germination tests were carried out in two replicas for each sample. Before this step, pollen stored at —196° C was kept in a freezer at —18° C for 2 hours.



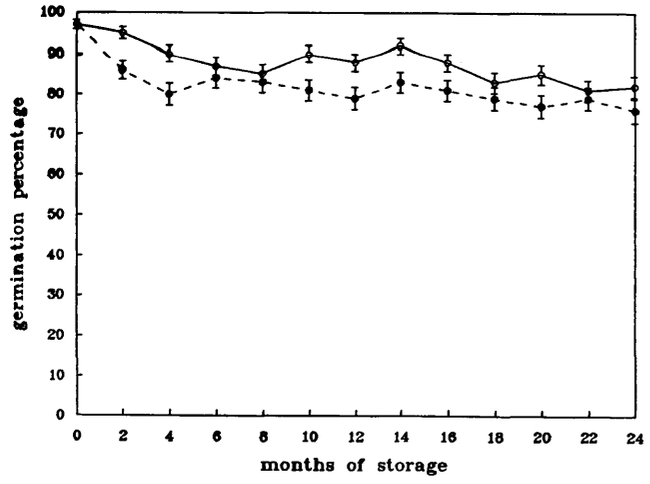
A) *Picea abies*



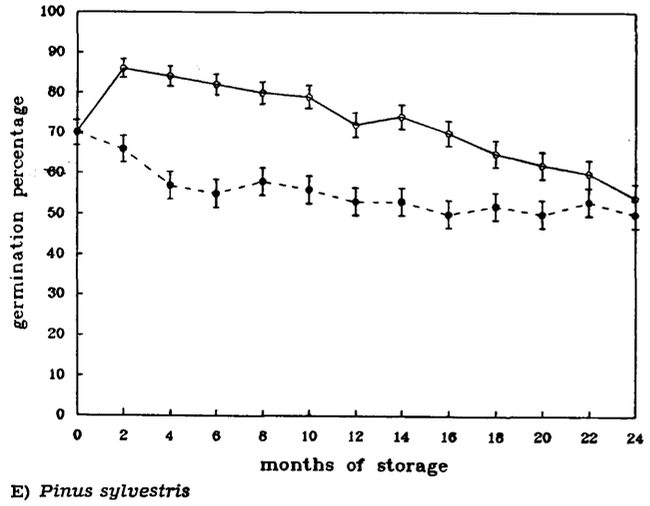
B) *Pinus nigra*



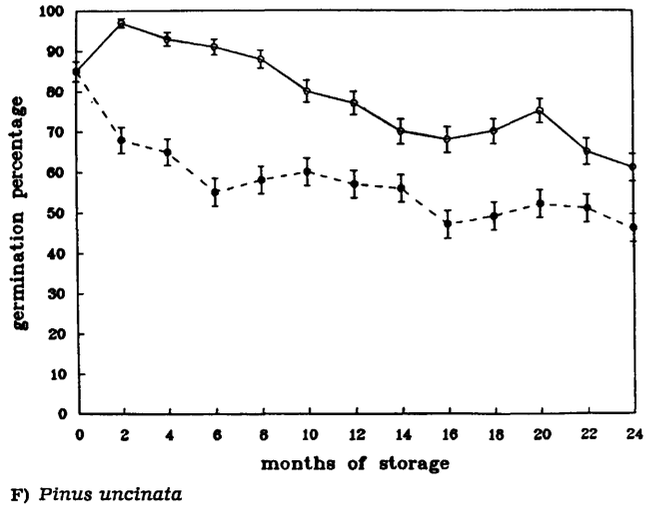
C) *Pinus pinea*



D) *Pinus strobus*



E) *Pinus sylvestris*



F) *Pinus uncinata*

Figure 1. — Germination data of pollen stored for 24 months at —18° C (solid line) and —196° C (dashed line). 95% confidence limits (I) are reported.

Data collection

Germination counts were taken after 24 hours. A longer period was not adopted because of the overlap of pollen tubes and bacterial and fungi contamination, which made the tallying of germination very difficult. Five to ten optic fields for each replica were randomly selected under the microscope and the number of germinated and ungerminated grains was recorded. At least 500 pollen grains were recorded for each replica. The pollen was considered germinated when at least one recognisable pollen tube was present and when the tube's length exceeded the maximum width of the grain. No attempt was made to measure pollen tube length.

Statistical analysis

The data are presented in terms of germination percentage (GP). Confidence limits were calculated using standard error of binomial distribution, according to SNEDECOR and COCHRAN (1967). Probit analysis of data was performed by maximum likelihood estimates (FINNEY, 1962) in order to assess the effect of storage time on pollen viability. A linearity test on regression lines was performed and, when possible, the regression lines referring to the 2 storage conditions in each species were compared by means of a parallelism test. For each species, GP values at the beginning and at the end of the storage period were also compared, after inverse sine transformation, by means of LSD (Least Significant Difference).

Results

Table 3 shows, for each species, the GP of the fresh pollen and the pollen preserved in freezer and in liquid nitrogen for 24 months, while *Figure 1A* to *F* shows the variation in GP during the same period.

The reaction of the pollen to storage was variable with respect to the species tested.

As regards *Picea abies*, probit analysis showed that the viability of pollen stored in freezer did not decrease during the 24 months of storage. Since a deviation from the linearity, due to an increase in pollen germination during the first months of storage at —196° C was observed, a linear regression did not match the data of pollen stored in this condition (*Table 4*). However, starting from the eighth

Table 4. — Regression lines of germination percentage of pollen stored in freezer (—18° C) and in liquid nitrogen (—196° C) for 24 months. Equations not given refer to significant deviation from linearity.

SPECIES	FREEZER	LIQUID NITROGEN
Picea abies	y = 5.8 - 0.01x (n.s.)	-
Pinus nigra	y = 5.7 - 0.05x (n.s.)	y = 5.7 - 0.05x (n.s.)
Pinus pinea	y = 6.7 - 0.5 x (**)	y = 6.7 - 0.5 x (**)
Pinus strobus	y = 6.1 - 0.1 x (**)	y = 6.6 - 0.2 x (**)
Pinus sylvestris	y = 5.4 - 0.1 x (**)	-
Pinus uncinata	y = 5.7 - 0.2 x (**)	-

y = probit of germination percentage
x = natural log. of months of storage
Between brackets is reported the significance of the slope:
n. s. = not significant
** = significant for $p < 0.01$

month of conservation, the confidence limits of PG data of the two storage conditions partially overlapped (*Figure 1A*).

In *P. nigra* statistical analysis showed no loss in viability in pollen stored in freezer, nor in that stored in liquid nitrogen (*Table 3*) and therefore the 2 regression lines proved to be identical (*Table 4* and *Figure 1B*).

A significant decrease in pollen viability was observed in both storage conditions in *P. pinea* (*Table 3* and *Figure 1C*). The regression lines were identical, as their slopes did not differ significantly at the Student's T-test ($p < 0.05$) (*Table 4*).

In *P. strobus* loss in pollen viability was observed in both storage conditions (*Table 3*). Although in some cases the confidence limits of GP values scored in the two storage conditions overlapped, the slopes of the two regression lines were statistically different ($p < 0.01$ at the Student's T-test) (*Table 4* and *Figure 1D*).

In *P. sylvestris* and *P. uncinata* data of pollen stored in liquid nitrogen showed deviation from linearity, due to the initial germination increase (*Figures 1E* and *1F*); therefore a linear regression analysis could be performed only for data referring to freezer conservation (*Table* 4). In this storage condition, in both species, a statistically significant decrease in viability was observed (*Table 3*). A significant difference between GP values at the beginning and at the end of the storage period in liquid nitrogen was also found. In *P. sylvestris,* at the 24th month of storage, the difference in GP of the two storage conditions did not differ significantly.

Discussion

As expected, in most of the species in study, the viability of stored pollen decreased with the increased duration of storage.

Only the pollen of *Picea abies* and *P. nigra* did not undergo a decrease in viability after 24 months of storage, while the GP of the pollen of the other species decreased to various levels. The maximum decrease was observed in *P. pinea*, where GP in both storage conditions was reduced to about one half. In this regard, it is important to emphasize that the germination tests of *P. pinea* and *P. sylvestris* pollen were carried out in H_2O vapour and that of *P. uncinata* in an H_2O drop; the latter supplied only 5% of sucrose in the media. These tests were utilised because they proved to be the best ones when applied on fresh pollen, first dehydrated and then rehydrated right away (Lanteri and Belletti, 1987). Nevertheless, it was shown that stored pollen may require a higher concentration of sugar for germination, probably due to a decrease in the pollen wall permeability (Stanley and Linskens, 1974; Polito and Luza, 1988) and this could have influenced the results obtained in these species.

Germination response of pollen stored at the 2 temperatures also varied. Among the species where a statistically significant decrease in viability was observed, only in *P. pinea* did statistical analysis not prove the advantage of liquid nitrogen storage compared to that of —18° C. It is possible that, in this species, the relatively short period of storage did not permit the presumed advantage of preservation in liquid nitrogen to be proven.

In *Picea abies, P. sylvestris* and *P. uncinata* an increase in pollen germination during the first months of storage at —196°C was observed. The same phenomenon was already observed by Ichikawa and Shidei (1972c) and Stanley and Linskens (1974). Nevertheless, the latter demonstrated no effect of the increased GP on the ability to set seeds and suggested that the increase of GP could be due to the freezing process which causes the release of some necessary nutrients into the medium. The increase in germination does not seem attributable to after-ripening processes occurring after shedding since it was observed only in pollen stored in liquid nitrogen.

The results of *in vitro* germination tests show that pollen of *Picea abies* and some species of *Pinus*, after storage at low temperatures, is alive. Therefore, low temperatures are recommended for good preservation. In fact, it was previously shown that the pollen of *P. nigra,* stored at +5°C, lost 80% of its viability after one year, while the pollen of *P. strobus* decreased in GP from 93% to 20% after about 13 months of storage at +18° C (Stanley and Linskens, 1974).

An important step to take before storage is to reduce the moisture content of the pollen by at least 10% prior to low temperature exposure. The authors proved that pollen put into storage, without being previously dessicated, lost about 50% of its viability after one month, probably as a consequence of the ice crystallisation of its water (Lanteri and Belletti, 1987). Below this moisture content it is possible, but not necessary, to pre-cool the material before dipping it into liquid nitrogen; however, as a precaution, pre-cooling was adopted in this experiment. Copes (1985; 1987), in fact, observed fertility of Douglas-fir pollen, dehydrated at 6.5% moisture content, after up to 3 years of cryogenic storage, carried out by placing it directly into liquid nitrogen without pre-chilling.

It can be concluded that, in general, storage at —18° C can be useful in hybridisation programmes, where the pollen donor plant sheds pollen before the female parent is receptive and also where the pollen of the male parent must be stored from one season to another. On the other hand, the more elaborate procedure of storage in liquid nitrogen would be advantageous for the preservation of male germplasm in gene banks.

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Genetic Analysis of Isoenzyme Variation in Mediterranean Cypress (Cupressus sempervirens L.)

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Abstract

Our inability to separate the haploid megagametophytic tissue from the diploid, maternal perisperm in the seeds of Mediterranean cypress was the reason for using single tree progenies from open pollination for the genetic analysis of isoenzyme phenotypes. Isoenzyme patterns from seed perisperms (maternal tissue) and seed embryos (progeny tissue) were compared and tested qualitatively and quantitatively for the mode of inheritance. In total, 8 variable proposed gene loci coding for 7 enzyme systems could be identified. The data of genetic analysis supported the intuitive genetic interpretation of the isoenzyme patterns.

Key words: *Cupressus sempervirens*, perisperm, enzyme gene loci, segregation analysis.

1. Introduction

Previous studies dealing with enzyme systems in Mediterranean cypress were limited to the application of isoenzyme phenotypes (zymograms) to distinguish cypress clones (Raddi et al., 1990). Their inability to isolate the haploid endosperm from other seed tissues was the major reason for not performing haploid segregation analysis. The endosperm is connected with the perisperm, a diploid tissue which surrounds the endosperm and originates from the parent tree (Raddi et al., 1990). However, the isoenzyme analysis of this tissue combination gives a clear zymogram that differs from the embryonic one and is identical to the parent tree zymogram. The genetic analysis described in this paper is simultaneously a test for the use of perisperm to obtain parent tree isoenzyme genotypes. This paper presents the results of the genetic analysis of 7 polymorphic enzyme systems in order to obtain biochemical genetic markers for population genetic studies. Due to lack of controlled cross progenies, the genetic analysis is based on single tree progenies derived from open pollination (Gillet and Hattemer, 1989).

2. Material and Methods

2.1 Material

The material used consisted of perisperm and embryo of single seeds. In most conifers, seeds consist of 2 tissue types: Seed endoperm (haploid megagametophytic tissue) and seed embryo (diploid progeny tissue). Seed perisperm is a tissue that surrounds the endosperm and in most conifers takes the form of a dry brown layer lacking enzyme activity. Thus, the analysis of the seed material after removing the embryo gives the zymograms of the haploid female gamete.

In our first experiments with cypress seeds, the zymograms obtained from the analysis of seed material after removing the embryo appear to present diploid patterns. These diploid zymograms were usually different from the embryonic ones. For each tree, the non-embryonic seed zymograms for all seeds were always identical, yet they differed from tree to tree. The nonembryonic and embryonic zymograms of each seed always had at least one isoenzyme variant in common. These results clearly suggest that the isoenzyme patterns obtained originate from the seed perisperm. Raddi et al. (1990) also reported that the perisperm is strongly connected with the seed endosperm, this being the major problem of obtaining isoenzyme patterns from haploid megagametophytic tissue. The perisperm was identified as diploid tissue, genetically identical to the maternal tree. It seems that in the case