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Hybridological Relationships between *Pinus nigra* Arn., *Pinus thunbergii* Parl. and *Pinus tabulaeformis* Carrière

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Abstract

Artificial hybridization of *Pinus nigra* ARN., *P. thunbergii* PARL. and *P. tabulaeformis* CARRIÈRE resulted in a high degree of crossability between the species *P. nigra* x *P. thunbergii* and *P. nigra* x *P. tabulaeformis* approaching by its efficiency the variants from controlled cross-pollination and open pollination of *P. nigra*. The inbreeding effect of selfing in *P. nigra* has already been evident at the seed level as well as the heterotic growth of one-year old seedlings of both interspecific crosses. Cytologically the high genetic affinity of the species was illustrated by the common developmental changes which take place in pollinated ovules of *P. nigra* during the first vegetative period. The hybrid nature of the interspecific crosses was confirmed by the isoenzyme markers, partially also by the cross-sections of needles in *P. nigra* x *P. thunbergii* combination.

Key words: *Pinus nigra*, *P. thunbergii*, *P. tabulaeformis*, crossability, isoenzymes, needles.

Introduction

The relative abundance of both the natural and artificial hybrids is regarded to be a noteworthy feature of the genus *Pinus* (JOHNSON, 1952). In particular it concerns the group of white pines (subgenus *Strobus*) which manifest a high degree of interspecific compatibility with a total absence or with only very weak hybridological barriers involved (CRITCHFIELD, 1975; KRIEBEL, 1967). On the contrary, the group of hard pines (subgenus *Pinus*) differs profoundly in this respect displaying conspicuously expressed specific combining abilities of the species which only partially correlate with their taxonomic positions and geographic distribution. Also, the genetic barriers between species of the subgenus are considered to be more highly developed than in most genera of woody plants. In our opinion, it is preferably this group of pines to which MIROV'S (1967) conclusion of the irregularity of hybridological relationships within the genus *Pinus* can be applied.

As an experimental evidence supporting the validity of this assumption, the results of hybridization experiments are presented in which the European black pine (*Pinus nigra* ARN.), the Japanese black pine (*Pinus thunbergii* PARL.) and the Chinese representative of *Pinus tabulaeformis* CARRIÈRE were involved. All the three species belong taxonomically into the group of Lariciones of the subsection Pinaster according to classification by SHAW (1914) based on morphological characters and wood anatomy or to the section Eupitys according to PILGER's (1926) taxonomic account which regards the number and anatomy of needles. On the background of an extraordinary reproductive behaviour of the hard pines, the established compatible hybridological relationships between these species is remarkable mainly from the standpoint of their geographic isolation.

Materials and Methods

Artificial pollination

Four individuals of the European black pine (*Pinus nigra* ARN.) growing at the artificial stand of the species near Nitra were chosen as maternal trees while the two trees of each of Japanese black pine (*Pinus thunbergii* PARL.) and the Chinese pine (*Pinus tabulaeformis* CARRIÈRE) introduced to arboretum Mlynany served in experiment as the paternal species. A classical technique of controlled pollination was applied using the paper bags for isolation of the female strobili and pollinator of own construction for delivering the pollen to receptive macrostrobili.

Except of the interspecific combinations of *P. nigra* x *P. thunbergii* and *P. nigra* x *P. tabulaeformis*, the intra-

specific variants involving self-pollination, controlled cross-pollination and open pollination of the maternal trees were tested as well. Throughout the entire experiment only freshly collected pollen was used. The quality of extracted mature seeds was tested by X-ray analysis (SIMAK, 1974) in accord with the Czechoslovak standard no. 48 1211 (number of filled seeds per 400 seeds analyzed). The degree of crossability was calculated according to the formulae

$$\frac{\text{per cent of filled seeds in interspecific combination}}{\text{per cent of filled seeds in a controlled outcrossing}} \times 100$$

The growth potentials of the offspring was evaluated with regard to the height growth of one-year old seedlings obtained in the nursery.

Cytological study of the ovules and needles

Cytological analysis of pollinated ovules involved the interspecific combinations of *P. nigra* x *P. thunbergii* and *P. nigra* x *P. tabulaeformis* as well as the variant from controlled intraspecific crossing of *P. nigra* individuals. The ovules were excised from the strobili two weeks after pollination and in October of the first vegetative period and processed immediately for the block preparations. Glutaraldehyde was used as a fixative, osmium oxide as a postfixative, acetone as a dehydrating agent, and durcupan ACM (FLUKA) as an imbedding medium, respectively. Semithin sections of the ovules were stained first in 1% (w/v) toluidine blue and subsequently in 0.1% (w/v) basic fuchsin (LUX, 1981).

The anatomical structure of needles has been analysed only in the interspecific hybrid of *P. nigra* x *P. thunbergii* and in the corresponding parental species using the nee-

Table 1. — Summary of the results obtained by the artificial hybridization of the species *Pinus nigra* ARN., *Pinus thunbergii* PARL. and *Pinus tabulaeformis* CARRIÈRE.

type of pollination	number		total number of seeds obtained	sound seeds		degree of crossability (%)
	pollinated female strobili	collected mature cones		number	per cent	
<i>P. nigra</i> 1 - selfing	7	5	31	23	74,19	
<i>P. nigra</i> 1 - open pollination	-	8	36	29	80,55	
<i>P. nigra</i> 1 x <i>P. nigra</i> 3	15	12	219	193	88,12	
<i>P. nigra</i> 1 x <i>P. thunbergii</i>	11	4	20	13	65,00	73,76
<i>P. nigra</i> 2 - selfing	12	4	91	12	13,18	
<i>P. nigra</i> 2 - open pollination	-	21	363	230	63,36	
<i>P. nigra</i> 2 x <i>P. nigra</i> 5	11	5	146	110	75,34	
<i>P. nigra</i> 2 x <i>P. thunbergii</i>	8	5	70	35	50,00	66,36
<i>P. nigra</i> 3 - selfing	7	5	44	7	15,90	
<i>P. nigra</i> 3 - open pollination	-	4	83	64	77,10	
<i>P. nigra</i> 3 x <i>P. nigra</i> 4	16	10	72	63	87,50	
<i>P. nigra</i> 3 x <i>P. thunbergii</i>	28	20	352	296	84,09	96,10
<i>P. nigra</i> 4 - selfing	19	7	186	11	5,91	
<i>P. nigra</i> 4 - open pollination	-	16	439	317	72,20	
<i>P. nigra</i> 4 x <i>P. thunbergii</i>	32	26	133	59	44,36	70,04
<i>P. nigra</i> 4 x <i>P. tabulaeformis</i>	12	4	117	61	52,13	82,31



Figure 1. — Cytological structure of the ovules of *Pinus nigra* ARN. revealed two weeks after pollination with the pollen grains of *P. thunbergii* (a; 400x) and at the end of the first vegetative period in the intraspecific cross of *P. nigra* x *P. nigra* (b; 220x) and interspecific combinations of *P. nigra* x *P. tabulaeformis* (c; 230x) and *P. nigra* x *P. thunbergii* (d; 320x) P-pollen grain, Pt-pollen tube, N-nucellus, I-integument.

dles of four year old seedlings which have been obtained by us earlier (KORMUTAK and LANAKOVA, 1988). The needles were collected at the end of vegetative period and

processed according to the procedure described by TANG SHUI-LIU (1971). To avoid the variation within a needle, only 5 mm segments excised from the middle parts of one-

year old needles were used.

Karyological study of the hybrids

Karyological characteristics of the interspecific hybrids of *P. nigra* x *P. thunbergii* and *P. nigra* x *P. tabulaeformis* were derived from the root-tip meristems following pre-treatment of germinating seeds in 0.2% (w/v) colchicine for the period of 12 hours and 25 °C. After fixation in Carnoy I solution, the root tips were washed thoroughly under tap water, hydrolyzed in 1 N HCl for 8 minutes (60 °C) and stained in Feulgen solution. The squash pre- parates made in 45% (v/v) acetic acid were examined microscopically using immersion oil.

Isoenzyme analysis

Mature seeds of *P. nigra* x *P. thunbergii* and *P. nigra* x *P. tabulaeformis* crossings were moistured for three days to stimulate the metabolic activity and subsequently used for the isozyme analysis. The excised megagametophyte and the corresponding embryos were separately homo- genized in 3 drops of 100 mM Tris-borate-EDTA buffer, pH 7.5 and used immediately as the crude extracts for electrophoretic separation of the enzymes in 12.5% starch gel. All separations were performed in Ashton buffer, pH 8.1 (LUNDKVIST, 1978).

Six seeds of each combination derived from a given tree were analyzed representing the genotypic constitu- tion of both the maternal tree and the corresponding hybrid combination. Owing to the lack of seeds in *P. thunbergii* and *P. tabulaeformis*, the attempt has been made to characterize the genetic constitutions of the pa- ternal trees by using their vegetative buds. However, except for the fluorescent esterase, no activity of gluta- mate-oxaloacetate-transaminase and alcohol dehydro- genase was detected in the material collected at the end of vegetative period.

The isozymes of glutamate-oxaloacetate-transaminase (GOT; E. C. 2.6.1.1.), alcohol dehydrogenase (ADH; E.C. 1. 1.1.1.) and fluorescent esterase (F EST; E.C.3.1.1.1.) were visualized according to the methods described by RUDIN and RASMUSON (1973).

Results

The hybridological relationships between the European species of *P. nigra* and the Asiatic representatives of *P. thunbergii* and *P. tabulaeformis* seem to be very close. As it follows from table 1, the degree of crossability cal- culated for the interspecific combination of *P. nigra* x *P. thunbergii* ranged within the limits of 66.36% to 96.10%,



Figure 3. — Allozyme patterns of ADH in megagametophytes of *Pinus nigra* no. 4 (B) and its hybrid combinations of *P. nigra* x *P. tabulaeformis* (A) and *P. nigra* x *P. thunbergii* (C).

averaging at 76.56% in the four maternal individuals. The corresponding parameter of *P. nigra* x *P. tabulaeformis* combination obtained within the maternal tree no. 4 has also reached the level of 82.31%.

According to the efficiency of hybridization, measured by the proportion of sound seeds, both the above crossings occupy intermediate positions between the variants from controlled intraspecific pollination and open pollination of the maternal trees. The only exception was in this respect the maternal tree no. 3 with the hybrid progeny of *P. nigra* x *P. thunbergii* surpassing by its quality (84.09% of filled seeds) the offspring from open pollination (77.1% of filled seeds).

The selfing has distinctly deviated from the remaining pollination types by exerting the profound inbreeding effect at the seed level. Of the four maternal trees pollinated, the conspicuous reduction in number of sound seeds had occurred in the progenies of three individuals (no. 2,3,4) with the proportions of fully developed seeds ranging between 5.91% and 15.90%. The maternal tree no. 1 has however been remarkable by incomparably higher quality of its progeny from selfing. The latter contained as high as 74.19% of sound seeds approaching in this respect the variants from both the intraspecific crossings of trees no. 1 and 3 and open pollination of the maternal tree.

At the cytological level a high degree of genetic affinity between all the three species of pines has been illustrated utilizing the mode of mutual interactions of the pollen grains of *P. thunbergii* and *P. tabulaeformis* with the nucellar tissue of *P. nigra* ovules. Figure 1 refers to the situation as revealed two weeks after pollination when the pollen grains of *P. thunbergii* started to germinate at the top of the nucellus (Fig. 1a). At the end of the first vegetative period the pollen tubes have already penetrated the apical layer of the ovules providing thus a stimulus

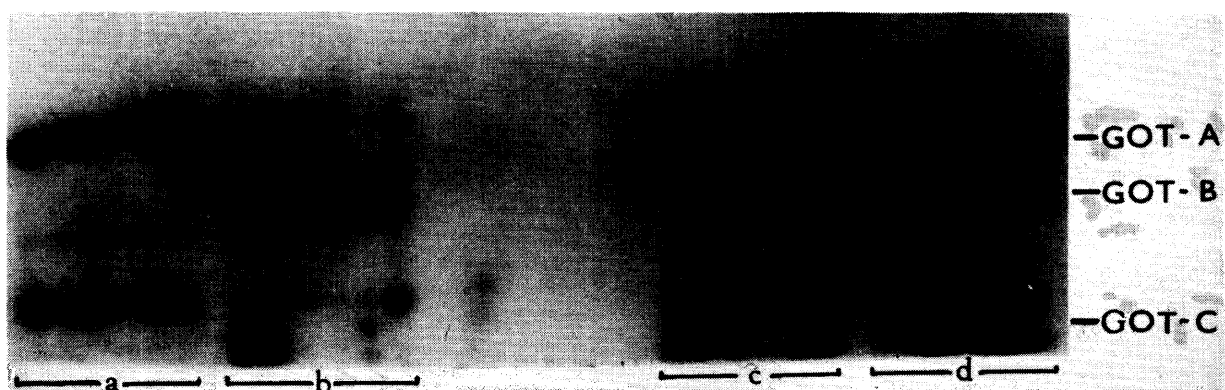


Figure 2. — Isozyme patterns of GOT found in megagametophytes of the maternal trees of *Pinus nigra* no. 1 (a) and no. 3 (d) and in the hybrid embryos of *P. nigra* no. 1 x *P. thunbergii* (b) and *P. nigra* no. 3 x *P. thunbergii* (c).

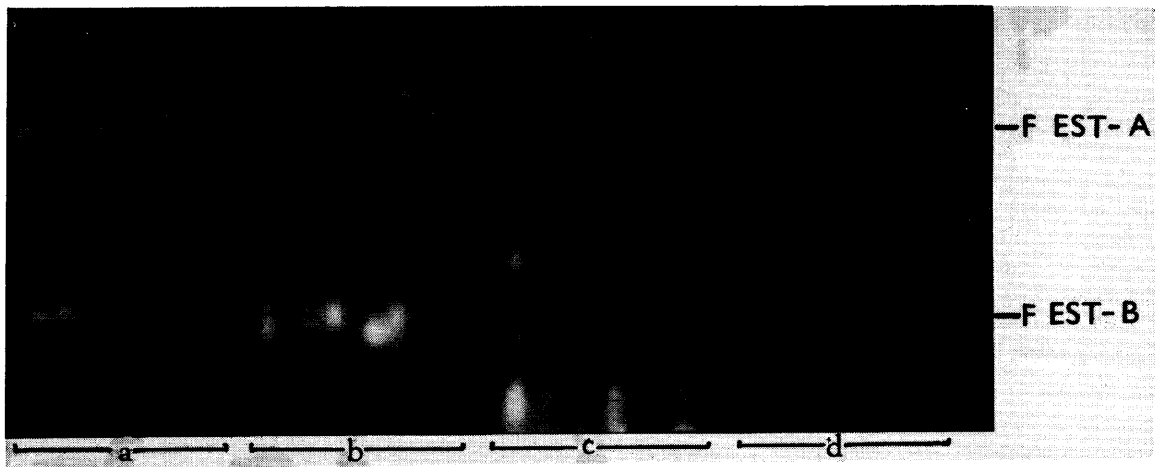


Figure 4. — Isozyme patterns of F EST found in megagametophytes of the maternal tree of *Pinus nigra* no. 4 (c), in vegetative buds of *Pinus tabulaeformis* (a) as well as in the hybrid embryos of *P. nigra* x *P. tabulaeformis* (b) and *P. nigra* x *P. thunbergii* (d).

indispensable at this period for the ovules to overwinter as well as for the entire strobili to develop into mature cones. It seems that intensity of the lytical activity of the pollen tubes correlates to some degree with the established extent of species crossability being higher in the intraspecific crossing of *P. nigra* (Fig. 1b) than in the ovules of *P. nigra* x *P. tabulaeformis* combination (Fig. 1c). However, the most expressive was in this respect the interspecific crossing of *P. nigra* x *P. thunbergii* with the pollen grains developing into polysiphonic tubes which form a dense haustorium within the nucellus (Fig. 1d).

Biochemically, it was possible to confirm the hybrid nature of both the above interspecific crosses using the enzyme systems of GOT, ADH and F EST of seeds. Except of one allele revealed in all the three loci of GOT in the female gametophyte of the maternal tree no. 1, the hybrid embryos of *P. nigra* x *P. thunbergii* have for example been enriched of some additional alleles in their GOT-B and GOT-C loci (Fig. 2b). The same is true of the maternal tree no. 3 which in the hybrid combination with *P. thunbergii* possessed the hybrid enzymes in GOT-C locus not revealed in the female gametophyte (Fig. 2c). However, owing to the difficulty with visualization of the enzyme activity in vegetative buds of *P. thunbergii* (the middle part of an electrophoregram) it was not possible to involve into comparison both the parental species.

Alcohol dehydrogenase system consisting of three alleles in the female gametophyte tissue of the maternal tree no.4 (Fig. 3b) was found to contain a new allele in the hybrid combinations of *P. nigra* x *P. tabulaeformis* (Fig. 3a) and *P. nigra* x *P. thunbergii* (Fig. 3c) which can be ascribed to the paternal species.

In case of F EST both its loci represent in *P. nigra* 4 x *P. tabulaeformis* crossing (Fig. 4b) a combination of the electrophoretic variants which are manifested by the parental species at the haploid level (Fig. 4a, c). Because of a low activity of the enzyme in the dormant buds of *P. thunbergii*, the hybrid nature of the F EST-A locus in *P. nigra* x *P. thunbergii* combination (Fig. 4d) is apparent only in relation to the maternal tree of *P. nigra* (Fig. 4c).

As far as the height growth of the hybrids is concerned, we can only state that 1-year old seedlings of both interspecific combinations reached on average higher values of the kind than the offspring from a controlled intraspecific crossing of *P. nigra*. By its mean height of 2.61 cm the hybrid combination of *P. nigra* x *P. thunbergii* has significantly differed not only from a control averaging at 1.82 cm but also from the hybrid seedlings of *P. nigra* x *P. tabulaeformis* which being tall of 2.01 cm occupied intermediate position between both the above variants (Fig. 5). Though very preliminary, these data indicate a heterotic nature of the hybrids.

Karyological characteristics of the hybrids conform the classical formulae of Sax and Sax (1933) referring to the chromosome structure of pines. There can accordingly be distinguished eighteen isobrachial chromosomes of a long size, two chromosomes of the same morphology but of intermediate size and four short chromosomes of heterobrachial type, respectively, within a diploid chromosome set of *P. nigra* x *P. thunbergii* (Fig. 6a). Essentially similar karyotype is typical also for the *P. nigra* x *P. tabulaeformis* combination with the difference that in two of the long isobrachial chromosomes of the hybrid the secondary constrictions have been recognized. Also, it seems that instead of two chromosomes of an intermediate size with a median position of their centromeres in *P. nigra* x *P. thunbergii*, the hybrid combination of *P. nigra* x *P. tabulaeformis* contains four chromosomes of the kind (Fig. 6b).

According to the needle anatomy, there exists a high degree of resemblance between the interspecific hybrid

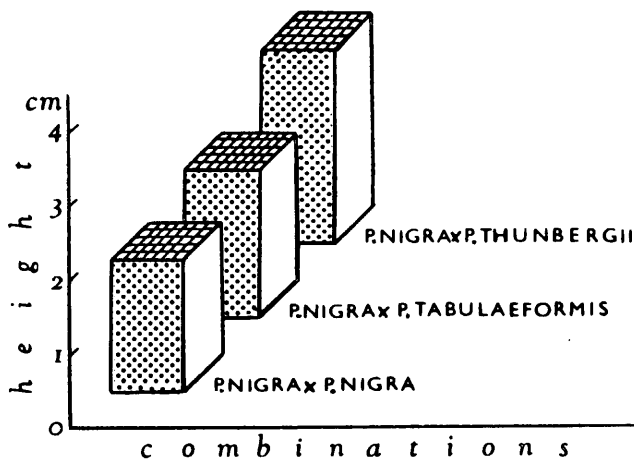


Figure 5. — Height growth parameters of one-year old seedlings.

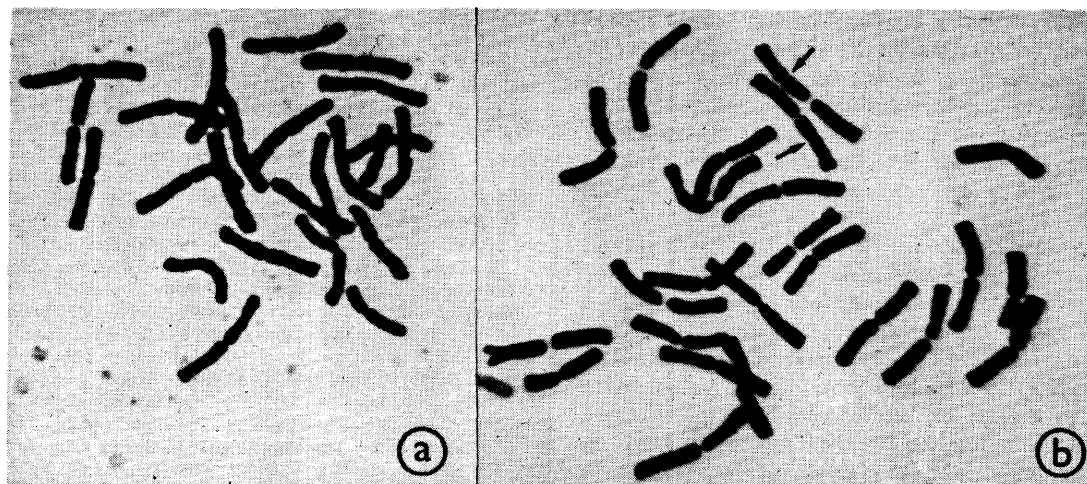


Figure 6. — Diploid chromosome sets in the interspecific hybrids of *P. nigra* x *P. thunbergii* (a) and *P. nigra* x *P. tabulaeformis* (b); 750x, arrows indicate secondary constrictions.

of *P. nigra* x *P. thunbergii* and its paternal species of *P. thunbergii* although some features of *P. nigra* are shared by the hybrid needles as well. The principal resin ducts so distinct in *P. nigra* (Fig. 7a) by their size and median position between the endodermis and the outer angles of the leaf are in the hybrid combination *P. nigra* x *P. thunbergii* (Fig. 7b) much smaller and are of marginal type resembling thus *P. thunbergii* (Fig. 7c). The presence of additional ducts around the periphery of needles is another feature commonly occurring in the hybrid and its paternal parent as well as their mesophyll tissue consisting of two layers of elongate cells instead of three layered mesophyll of *P. nigra*. Elongate shape follow also the cells of transfusion tissue in both *P. nigra* x *P. thunbergii* and *P. thunbergii* as compared with the oval cells surrounding the double vascular bundle of *P. nigra*.

Discussion

Within context of postulated irregularity of hybridological pattern in the genus *Pinus*, the interspecific combination of *P. nigra* x *P. thunbergii* is given as an example of surprising reproductive behaviour of pines isolated from each other for millions of years and separated by thousands of miles (Sax and Sax, 1933; Mirov, 1967). Obtained at the Morris Arboretum near Philadelphia the hybrid was shown to be slightly larger at the seedling stage than the intraspecific cross of *P. nigra* but 15 years later this difference had already reached the level of 10% to 15%. Also, survival of the hybrid was of 40% better than a control what makes it promising for many parts of the Northeast of USA (Garrett, 1979). Being taller than *P. nigra*, the hybrid seedlings of *P. nigra* x *P. tabulaeformis* have also been mentioned in this connection. None of them has however been outplanted to follow the maintenance of this property by the hybrid during its ontogeny. In the light of presented results of hybridological experiments all the above data serve as an indisputable evidence of the compatible relationships between *P. nigra*, *P. thunbergii* and *P. tabulaeformis* what coincides also with the modified systematics of the group *Lariciones* proposed by Duffield (1952) on the basis of crossability data.

Cytologically it is illustrated by a high affinity of the pollen grains of both the paternal species towards the nucellar tissue of *P. nigra* ovules which is of key im-

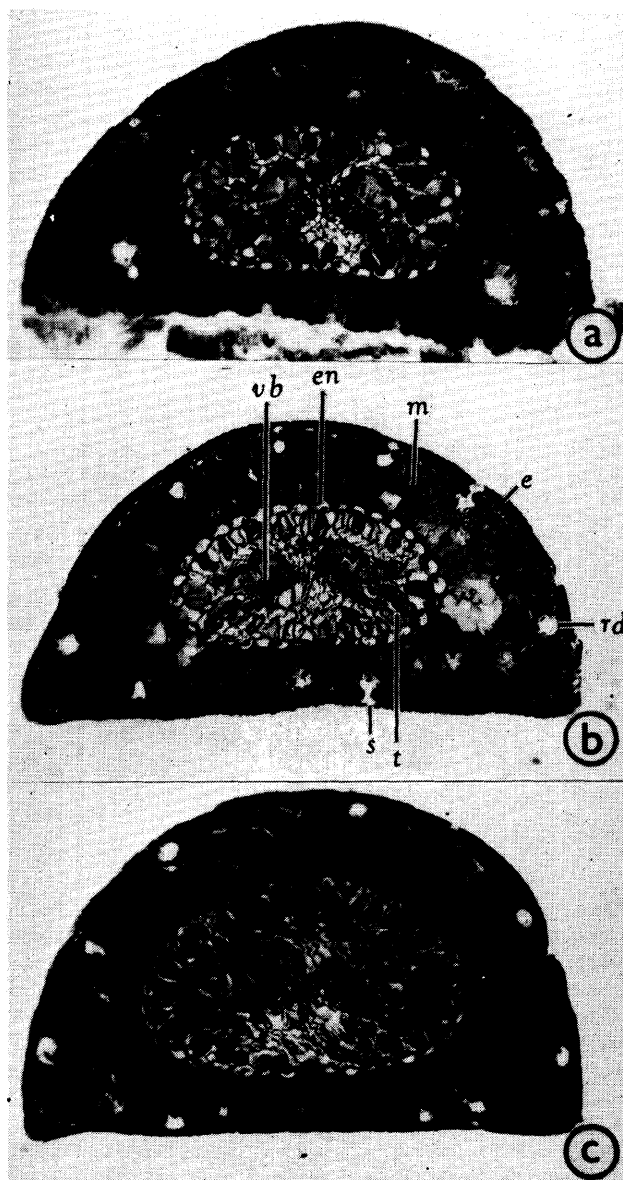


Figure 7. — Needle anatomy in *Pinus nigra* (a), *P. nigra* x *P. thunbergii* (b) and *Pinus thunbergii* (c); vb-vascular bundle, en-endodermis, e-epidermis, m-mesophyll, rd-resin ducts, t-transfusion tissue, s-stomata.

portance for survival of the ovules during winter period and subsequent fertilization of the egg cell in the second developmental season. It is evident therefore that this feature of pollinated ovules reflects very sensitively the hybridological status of the species under investigation and that it may be utilized effectively in the evolutionary study of the hard pines as recommended earlier by Mc WILLIAM (1959).

A close relationship between *P. nigra* and *P. thunbergii* at the biochemical level has been demonstrated in our comparative study concerning the serological properties of the pollen grains (KORMUTAK and LANAKOVA, (1988) and the restriction profiles of the chloroplast DNA (KORMUTAK et al., in press). All the findings obtained so far correlate positively with the established compatibility of *P. nigra* x *P. thunbergii* combination. PRUS-GLOWACKI et al. (1985) in their serotaxonomical investigation of pines revealed the presence of seed antigens common for both the above species. The authors share opinion that except for the continuing speciation of pines and considerable intra-specific variability, it is irregular hybridization which complicates the systematics of the genus *Pinus*. The last mentioned aspect is fully applicable to the group *Laricines* as well. Of no less strange than compatible hybridological status of geographically distinct species of *P. nigra* x *P. thunbergii* and *P. nigra* x *P. tabulaeformis* is also a strong reproductive isolation of the species combinations of *P. mugo* x *P. nigra* (KORMUTAK and LANAKOVA, 1988), *P. nigra* x *P. sylvestris* and reciprocal (VIDAKOVIČ and JURKOVIČ-BEVILACQUA, 1970; VIDAKOVIČ and BORZAN, 1973) as well as a negligible crossability between *P. sylvestris* and *P. mugo* (DOBRINOV and JAGDZIDIS, 1971; NEET-SARQUEDA et al., 1988) the evolution of all of which was to a large degree bound to common territory of Europe.

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Clonal Variation in Heat Tolerance and Heat Shock Protein Expression in Black Spruce

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Summary

One-year-old *Picea mariana* rooted cuttings representing 9 families and a total of 24 clones were used to assess variability in heat tolerance and, for 2 clones, differences in heat shock protein (HSP) synthesis. Different clones of the same family exposed to a temperature of 47 °C for 30 minutes varied widely in damage, indicating a large component of single tree variability in the factors contributing

to heat tolerance. The predominance of clones from family 347 possessing above-average heat tolerance suggested that tolerance is, in part, also heritable within families. Clone 86–109 was both more heat tolerant and possessed higher constitutive levels of HSP compared to clone 347–36. Levels of HSP synthesis offer promise as a means of molecular selection for tree breeding and clonal propagation. Rapid screening for heat tolerance of large numbers of individuals is possible by means of direct hot water immersion of shoots.

Key words: *Picea*, stress tolerance, rooted cutting, heat shock.

Introduction

For black spruce (*Picea mariana* MILL. B. S. P.), a boreal temperate zone species with a vast range spanning the

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