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Identification of barriers to interspecific crosses in the genus *Trifolium*

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Summary

A study of pre- and post-fertilisation barriers after interspecific crosses of diploid and tetraploid *Trifolium pratense* L. and wild species *T. alpestre* L., *T. medium* L. and *T. sarosiense* Hazsl. was aimed at finding of a promising cross combination for obtaining hybrids. The growth of pollen tubes was arrested in interspecific crosses mainly when *T. pratense* was at a diploid level. To investigate the post-fertilisation barriers in detail, the hybrid embryo viability was traced by two clearing treatments of immature seeds: (1) using chloral hydrate (which proved to be most appropriate); and (2) a mixture of benzyl benzoate and dibutly phthalate. In interspecific combinations *T. pratense* (4×) × either *T. alpestre* or *T. sarosiense*, enlargement of immature seeds occurred, but no hybrid embryo was traced. Of the wild species used as a male parent for crosses, *T. medium* was the only exception from the point of view of fertilisation. Globular, heart and the early torpedo stages of hybrid embryos were observed 7 days after pollination (DAP) but only when *T. pratense* was at a tetraploid level. When *T. pratense* (2×, 4×) was used as a male parent for interspecific crosses with *T. alpestre*, *T. medium* and *T. sarosiense*, strong defects in various stages of embryogenesis were observed, particularly wrinkled and narrowing embryo sacs caused by an expansion of endothelial cells. We conclude with the following finding: (1) to make crosses only in one direction with *T. pratense* as a female parent and *T. medium* as a male; (2) to use tetraploid plants of *T. pratense*; (3) and to excise hybrid embryos at an early torpedo stage, about 7 DAP.

Introduction

Genus *Trifolium* occupies an important place within the *Fabaceae* family and includes economically valuable species cultivated extensively throughout the world (*T. pratense* L. and *T. repens* L.). Economically valuable characteristics improved in breeding programmes are yield, protein content, resistance to the various biotic and abiotic stress conditions and persistence. In the research directed to increasing tolerance to diseases, pests and unfavourable environmental effects and persistence in promising *Trifolium* varieties, attention has been concentrated on the utilisation of genetic resources of wild species through interspecific hybridisation. Various types of barriers of crossability (Hughes, 1986), which make conventional crossing procedures and obtaining viable hybrid seeds entirely unsuccessful, are the main features of this procedure. To overcome these barriers, various *in vitro* methods are necessary such as embryo culture, protoplast fusion and *in vitro* pollination (Taylor & Quesenberry, 1996). An alternative procedure, currently, is incorporation of foreign genes by genetic transformation by means of *Agrobacterium tumefaciens*. Most transformation methods require regeneration of transformed cells into whole plants from cell suspension, protoplasts or callus tissue culture, but the frequency of regeneration is very low, particularly in red clover. In *T. pratense*, the successful transformation and transmission of introduced genes through a sexual generation has been quite sporadic (Quesenberry et al., 1996). In comparison with *T. pratense*, the results obtained in *T. repens* are quite promising (Pittock et al., 1997; Scott et al., 1998;

Sharma et al., 1998; Christiansen et al., 2000; Lee et al., 2001).

For the above-mentioned reasons, it is worth continuing interspecific hybridisation with *T. pratense* and improving procedure of embryo rescue. Post-fertilisation barriers in *Trifolium* are of greater importance (Kazimierska, 1978; Taylor et al., 1980). The study of this type of barriers requires the knowledge of embryogenesis. Mackiewicz (1965) and more recently Algan and Bakar (1996) have studied the ultrastructure of embryo development and endosperm formation in *T. pratense* in detail by light and electron microscopes. Kazimierski et al. (1972), Kazimierska (1978) and Sawai and Ueda (1987), dealt with the histological observations of the process of embryogenesis after interspecific hybridisation of *T. pratense* and any other species. These studies were helpful for understanding the post-fertilisation barriers in *Trifolium*. Inclusion of diploid and also tetraploid genotypes of *T. pratense* into interspecific crosses and their comparison from the viewpoint of probability of fertilisation remains to be investigated. In order to perform this examination, the conventional procedure of embryo embedding in paraffin and sectioning is very time consuming. More expeditious and convenient procedure could be whole-mount clearing treatment of immature seeds leaving the cell walls of tissues intact. This procedure has been routinely used in *Arabidopsis thaliana* L. (Heynh.) for the detailed study of embryo and endosperm development (Mayer et al., 1993; Aida et al., 1997; Herr, 1999). Clearing procedure has been applied in other plant species, for example in *Glycine max* and *Phaseolus aureus* (George et al., 1979), *Planera aquatica* and *Cassia occidentalis* (Herr, 1982), *Solanum* species (Stelly & Peloquin, 1983), *Vicia faba* (Ramsay & Pickersgill, 1986), *Linum usitatissimum* (Huyghe, 1987), *Dianthus* species (Hoshino et al., 2000), to the study of ovule and megagametophyte development, microsporogenesis and embryological investigation (Herr, 1982).

In *T. pratense*, study of pre-fertilisation and post-fertilisation barriers after interspecific hybridisation resulted in determination of promising cross combinations concerning parental species. Influence of different ploidy level on the probability of fertilisation was not performed in large extent, though this could bring some success. Therefore, the main objective of the presented work was to study pre- and post-fertilisation barriers to compatibility after interspecific crosses of diploid and tetraploid *T. pratense* and wild species *T. alpestre* L., *T. medium* L. and *T. sarosiense* Hazsl. The analysis of post-fertilisation barriers was aimed at *in situ* embryo development search by means of clearing treatment in various *Trifolium* species and in hybrid combinations. The investigation was focused on the determination of the most promising genotypes for the interspecific crosses, the examination of maximum level of hybrid embryo development and so optimal period for *in vitro* embryo cultivation.

Material and methods

Materials and growth conditions

The experiments involved 4 clover species; diploid and tetraploid *T. pratense*, wild species *T. medium* ($2n = 56$), *T. sarosiense* ($2n = 48$) and *T. alpestre* ($2n = 16$). All plants of *T. pratense* used in this study were obtained from Plant Breeding Station of Hladké Životice; namely 5 diploid ($2n = 2x = 14$) and 14 tetraploid ($2n = 4x = 28$) experimental breeding materials, altogether 29 diploid and 136 tetraploid plants.

Plants were grown to maturity and induced to flower in a controlled environment growth chamber, at 20–22°C and irradiation of 70 μmol m^{-2} s^{-1}, under 16 h light/8 h dark cycles.

Crossing technique

Intraspecific and interspecific crosses were performed. Flowers of the female plants were manually emasculated and hand pollinated in all possible combinations including reciprocal combinations. Flowers were collected at various intervals after pollination. Ovaries were examined for viable ovules.

Pre-fertilisation barriers

The callose staining method in the growing pollen tubes by aniline blue was used. After intra- and interspecific crosses, flowers were collected in intervals of 1 h, up to 72 h, after pollination. Flower maceration was performed in 1N NaOH on a water bath (60°C) for 20–40 min. After washing for 24 h in running tap water, the flowers were stained with aniline blue. The staining solution was prepared from 7 g K_3PO_4 dissolved in 1 lit of distilled water and 1 g aniline blue. The same solution was used for long term sample storage under 10°C. Microscopic samples were prepared by pistil excision, mounting in glycerol on a glass slide and gentle pressure on a cover slide. Fluorescent signals were evaluated in Olympus BX-60 microscope with excitation filter 400–455 nm and emission filter 475 nm.

Post-fertilisation barriers

Post-fertilisation barriers (endosperm and embryo development) were traced by two clearing treatments by means of chloral hydrate (Mayer et al., 1993) and a mixture of benzyl benzoate and dibutyl phthalate (Hoshino et al., 2000), both with modifications.

Ovaries or immature seeds were dissected from immature pods in different stages of development (1, 3, 4, 7, 8 and 9 days after pollination [DAP]). Immature seeds were transferred to Eppendorf tubes and fixed in FAA mixture (formaldehyde, acetic acid, ethanol [5:5:90, v/v/v]) for 4 h at room temperature. After washing 3 times in 96%, 70%, 30% ethanol and finally in distilled water, seeds were cleared in chloral hydrate (Mayer et al., 1993) overnight at room temperature or at 4°C.

For the other clearing, after Hoshino et al. (2000), the following modifications were performed: fixation for 4 h, the last step of clearing in benzyl benzoate and dibutyl phthalate (1:1, v/v) for 24 h.

A differential interference contrast (DIC) Olympus BX-60 microscope was used after clearing treatment. Photographs were taken using Olympus camera and Lucia 4.21 software.

Results

Pre-fertilisation barriers

40 min long maceration in the water bath showed to be sufficient in comparison with 20 min interval. In diploid plants of *T. pratense* the pollen tubes reached the base of the style 17 h after pollination, whereas in tetraploid plants of *T. pratense*, wild species *T. alpestre*, *T. medium* and *T. sarosiense* the pollen tubes entered the ovary 20 h after pollination. After interspecific crosses, the growth of pollen tubes was slower; they entered the ovary 30 h after pollination. The growth of pollen tubes after interspecific crosses was completely arrested in the combination *T. pratense* (2*x*) × *T. medium* including its reciprocal cross, further in *T. pratense* (2*x*) × *T. alpestre* and *T. sarosiense* × *T. pratense* (2*x*). Fully successful pollen tubes growth was observed in *T. pratense* (4*x*) × *T. medium*, *T. pratense* (4*x*) × *T. alpestre* including their reciprocal crosses. Moreover, crosses successful from this viewpoint also were *T. alpestre* × *T. pratense* (2*x*) and *T. pratense* (both 2*x* and 4*x*) × *T. sarosiense*. In *T. sarosiense* × *T. pratense* (4*x*) successful growth depended on the used combination of genotypes. Within the used *T. pratense* (4*x*) plants, some appear to be successful, others not. These results are summarised in Table 1.

Table 1. The results of the study of pre-fertilisation barriers of crossability between *Trifolim pratense* and wild species *T. medium*, *T. alpestre* and *T. sarosiense*

Parental combination	No. of crosses	No. of genotypes	No. of pollen tubes[a]
T. pratense (2x) × *T. medium*	60	2	0
Reciprocal	60	2	0
T. pratense (4x) × *T. medium*	160	14	160
Reciprocal	160	4	160
T. pratense (2x) × *T. alpestre*	40	2	0
Reciprocal	40	2	40
T. pratense (4x) × *T. alpestre*	130	13	130
Reciprocal	130	2	130
T. pratense (2x) × *T. sarosiense*	30	3	30
Reciprocal	30	2	0
T. pratense (4x) × *T. sarosiense*	70	7	70
Reciprocal	70	2	50

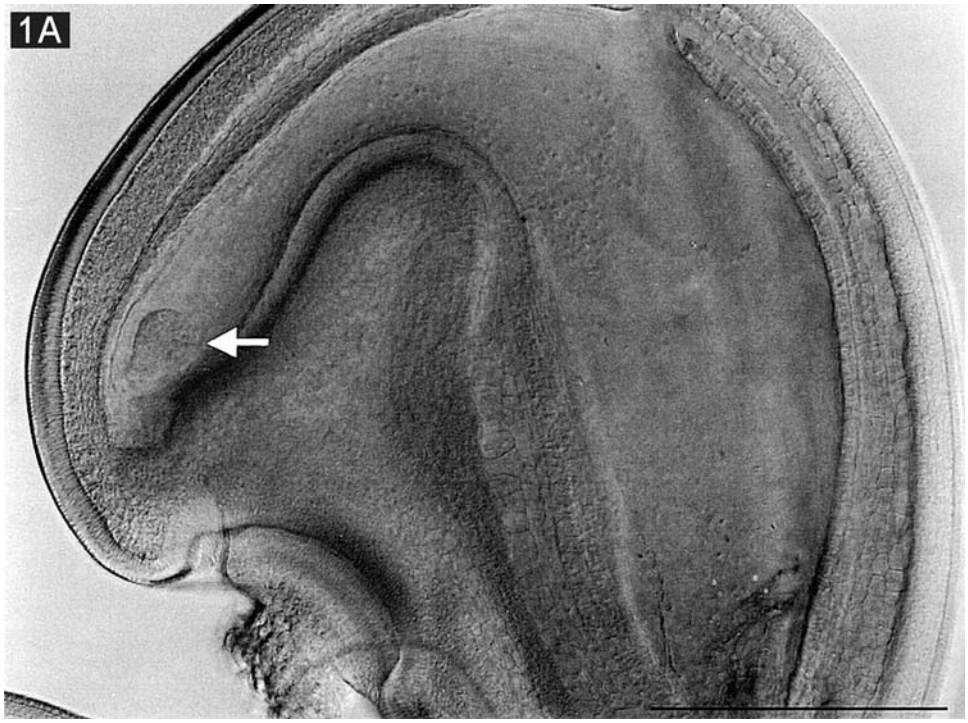
[a]Number of ovaries with observed pollen tubes.

Post-fertilisation barriers

Intraspecific pollinations were used for optimisation of clearing method and reliable methodology was accomplished. The optimal length of fixation for both procedures was 4 h; a shorter period had negative influence on the subsequent clearing. Total period of immature seed clearing was decisive for sufficient clearing of embryo proper. Based on our experiments, the optimal length of action of chloral hydrate was 20 h, and for BBD was 24 h.

The following embryo stages were observed in reviewed time intervals after intraspecific pollination *T. pratense* 2*x* and 4*x*: 24 h – zygote at the micropylar end, 2 DAP – dividing of zygote, 3–4 DAP – globular stage (Figure 1A), 5–6 DAP – heart stage (Figure 1B), 8 DAP – torpedo stage (Figure 1C). In wild species *T. alpestre*, *T. medium* and *T. sarosiense* the same time course in relation to individual embryo stages was observed.

In interspecific combinations *T. pratense* (4*x*) × *T. alpestre* and *T. pratense* (4*x*) × *T. sarosiense*, enlargement of immature ovules occurred, but no hybrid embryo was traced. Normal shape of embryo sacs was observed. Similar results were found in *T. pratense* (2*x*) × *T. alpestre* and *T. pratense* (2*x*) × *T. sarosiense* crosses.



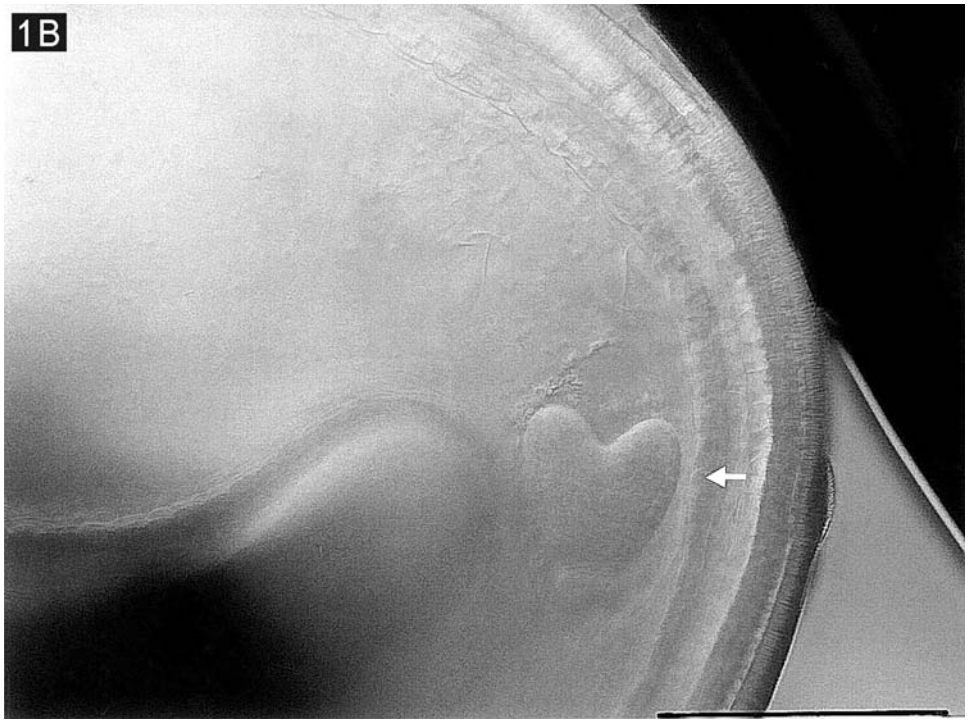


Figure 1. The embryos of *T. pratense* after intraspecific pollination: globular stage on the 4th DAP with nuclear endosperm (A), heart stage on the 6th DAP with cellular endosperm (B), torpedo stage on the 8th DAP with cellular endosperm (C). Bar = 50 μm.

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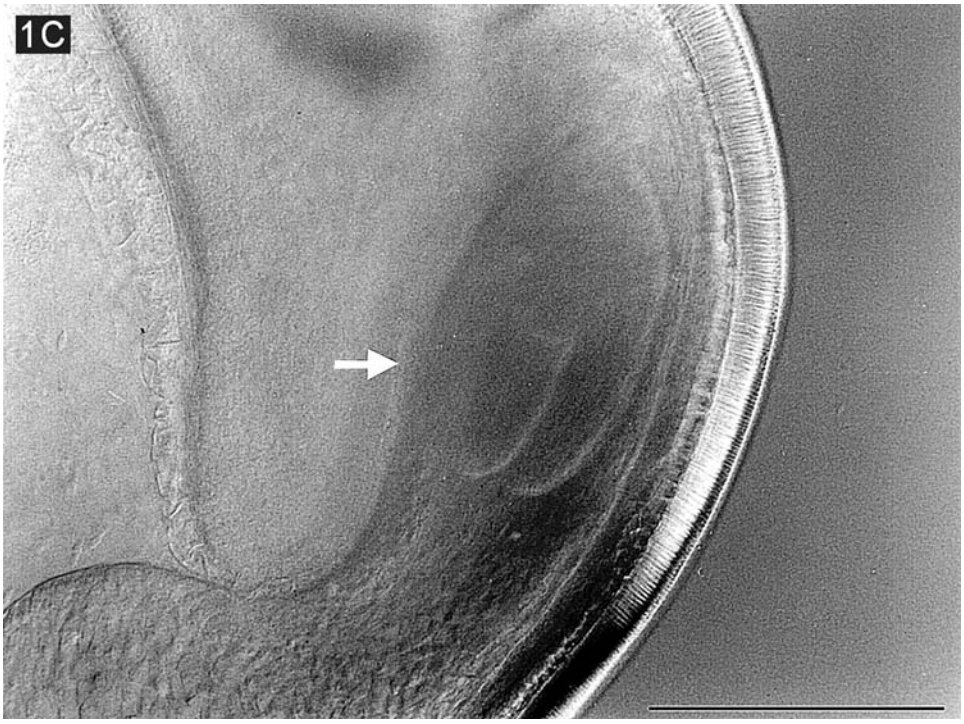

Figure 1. (*Continued*)

In *T. pratense* (4*x*) × *T. medium*, globular stage of hybrid embryo (Figure 2A), heart embryo (Figure 2B) and early torpedo embryo were observed 7 DAP. Diploid genotypes of *T. pratense* used as a female parent for crosses with *T. medium* were unsuccessful from the point of view of hybrid embryo development.

When *T. pratense* (2*x*, 4*x*) was used as a male parent for interspecific crosses with *T. alpestre*, *T. medium* and *T. sarosiense*, defects in various stages of embryogenesis were observed (Figure 3). These defects were crucial for lack of hybrid embryo viability. As for defects, the wrinkled and narrowing embryo sacs were traced very frequently (Figure 4). These results are summarised in Table 2.

Discussion

Hybridisation between species of the genus *Trifolium* under natural conditions is considered very improbable, even in closely related species (Hendrych, 1990). Nevertheless, hybrids are of great demand as they could recombine economically desirable genes. From the 1970s to the 1990s, the method of embryo culture seemed to be a possible way of overcoming interspecific, post-fertilisation, barriers in *Trifolium*, even if in very sporadic cases. It has facilitated to overcome incompatibility caused by abnormal endosperm development and insufficient embryo nutrition resulting in

Table 2. The results of the study of post-fertilisation barriers between *Trifolium pratense* and wild species *T. medium*, *T. alpestre* and *T. sarosiense*

	No. of		
Parental combination	crosses	globular embryos[a]	heart embryos[b]
T. pratense (4x) × *T. medium*	160	3	1
Reciprocal	240	0	0
T. alpestre × *T. pratense* (2x)	20	0	0
T. pratense (4x) × *T. alpestre*	110	0	0
Reciprocal	130	0	0
T. pratense (2x) × *T. sarosiense*	20	0	0
T. pratense (4x) × *T. sarosiense*	190	0	0
Reciprocal	230	0	0

[a,b] Embryos observed after clearing treatments.

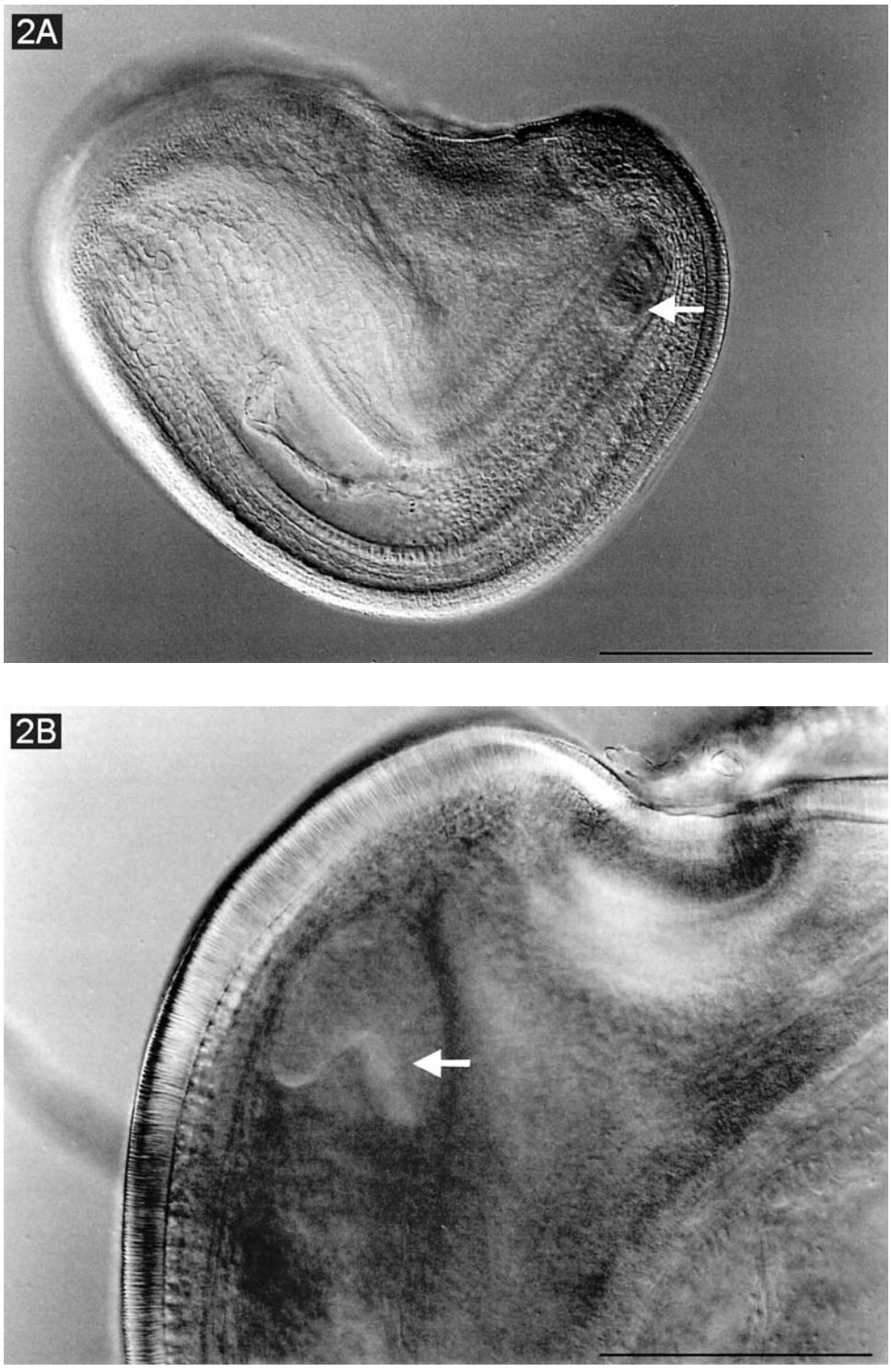

Figure 2. The hybrid embryos of *T. pratense* (4x) × *T. medium*: globular stage on the 7th DAP (A), heart stage on the 7th DAP (B). Bar = 50 μm.

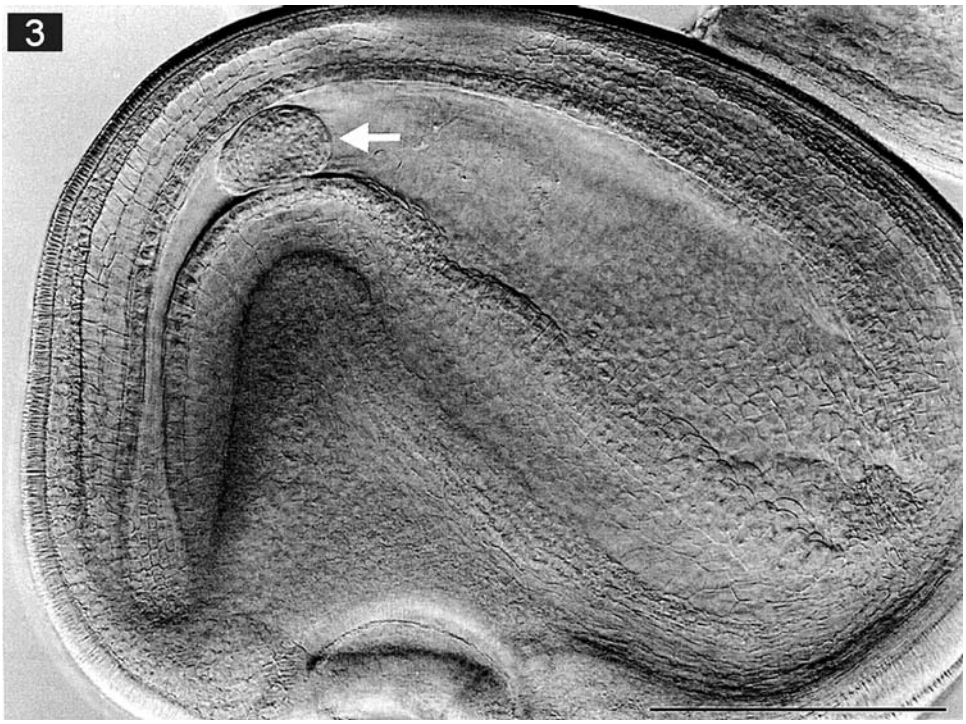

Figure 3. The globular embryo disintegration after the interspecific cross *T. sarosiense* × *T. pratense* (4x) on the 7th DAP. Bar = 50 μm.

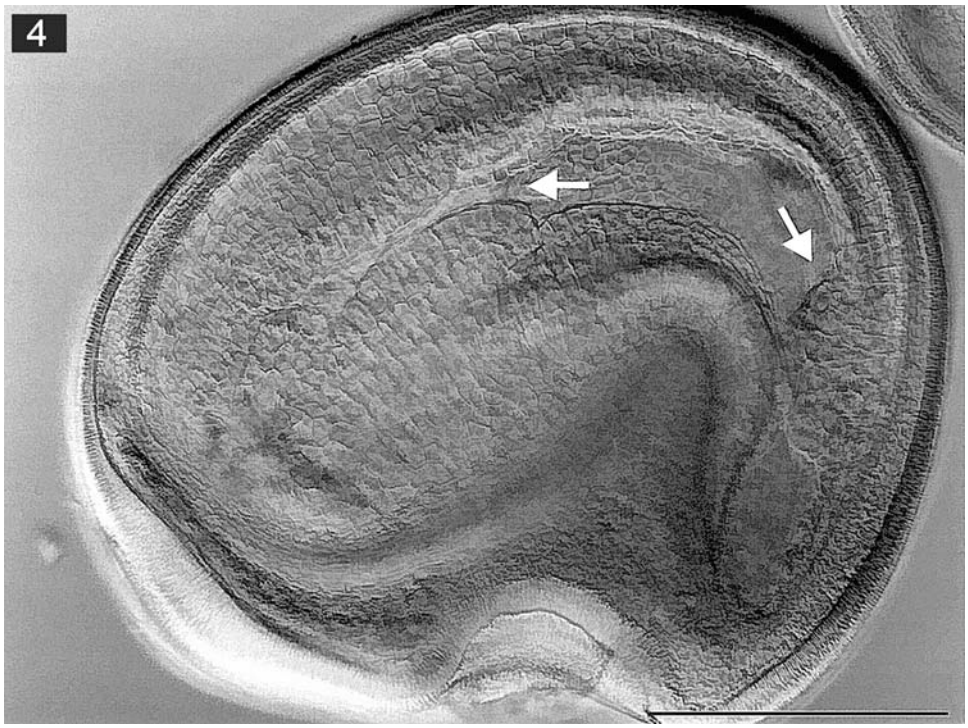

Figure 4. The expansion of endothelial cells of embryo sac after the interspecific cross: *T. alpestre* × *T. pratense* (4x) on the 7th DAP. Bar = 50 μm.

abortion (Kazimierska, 1980). Efforts for interspecific hybridisation in *T. pratense* persist up to now. So far, *T. pratense* was successfully crossed with 5 species: *T. sarosiense* Hazsl. (Collins et al., 1981; Phillips et al., 1982), *T. medium* L. (Collins et al., 1981; Vogt & Schweiger, 1983; Merker, 1984; Sawai et al., 1990; Nedbalkova et al., 1995), *T. alpestre* L. (Collins et al., 1981; Merker, 1988; Phillips et al., 1992), *T. ambiguum* M.Bieb. (Vogt & Schweiger, 1983) and *T. diffusum* Ehrh. (Schwer & Cleveland, 1972).

Our experience with pre-fertilisation and post-fertilisation barrier examinations shows that selection of promising material is of crucial importance. Study of pre-fertilisation barriers of crosses revealed that red clover plants as a tetraploid level were more effective for fertilisation in comparison with diploid ones. Strong pre-fertilisation barriers occurred after the cross of diploid *T. pratense* with *T. medium* in both parental combinations, *T. alpestre* used as male parent and *T. sarosiense* used as female parent. Fertilisation after the cross of tetraploid *T. pratense* was fully effective in all three cross combinations and both parental combinations. The explanation is apparent mainly for the crosses with *T. medium* and *T. sarosiense*. Generally considered, the both species are polyploids, therefore, higher imbalance in chromosome numbers might be expected after the cross with diploid *T. pratense* in comparison with tetraploid. This chromosome imbalance occurs between growing pollen tubes and the surrounding tissues whereas the usual situation requires a chromosome ratio 2 (pistil) : 1 (pollen). The reason of unsuccessful growth of haploid pollen tubes of *T. alpestre* through diploid tissue of pistils of *T. pratense*, and, on the contrary successful growth in case of reciprocal crosses is difficult to explain unambiguously. These species have different basic chromosome numbers 7 and 8 and differential genomes.

For searching of *in situ* embryo development in various *Trifolium* species and their hybrids, the successful optimisation of a method of clearing was crucial. Clearing of immature seeds and inner embryos up to torpedo stage was carried out after modifications of known procedures (Mayer et al., 1993; Hoshino et al., 2000). This approach constituted meaningful acceleration in our examination in comparison with embryo embedding in paraffin and its sectioning. Our experience obtained from this investigation showed the clearing treatment with chloral hydrate superior to the mixture of benzyl benzoate and dibutyl phthalate. All figures presented here (Figure 1 up Figure 4) were taken after chloral hydrate treatments.

In situ hybrid embryo development has been reported in *T. medium* × (4*x*) *T. pratense* (Sawai and Ueda, 1987). They histologically examined hybrid embryos of *T. medium* × (4*x*) *T. pratense* that reached up to early heart stages and then became vacuolated and disintegrated. Phillips et al. (1982) observed torpedo shaped embryos after *T. pratense* × *T. sarosiense* cross.

In our observation, defects in embryo sacs, mainly when wild species was used as a female parent, were revealed. In *T. pratense* (4*x*) × *T. alpestre*, enlarging of ovules without embryo development was observed. It is believed fertilisation probably occurred but embryo dies in early stage of its development. In the reciprocal combination of cross, the buckling of embryo sac by the hyperplastic growth of endothelium was observed 7 DAP. It seems this feature is of a common trait and results from the strong barriers of crossability. Bharathi et al. (1982) observed the same phenomenon in *Arachis hagenbeckii* and *A. glabrata*. Merker (1988) succeeded in overcoming the cytological barriers of crossability of *T. pratense* and *T. alpestre* by tetraploidy induction in somatic chromosomes of *T. alpestre*.

From wild species used for interspecific crosses, only *T. medium* gave successful results as concerned obtaining of the viable hybrid embryos. Globular stage of hybrid embryo was observed 7 DAP. In *T. pratense* this stage was observed 3 to 4 DAP. The possible explanation of these inconsistent results is either by somewhat delayed course of hybrid embryo development or embryo remained in heart stage and further development did not occur. The maximum degree of *T. pratense* (4*x*) × *T. medium* hybrid embryo development was determined as early torpedo stage. In this stage embryos are capable of autotrophic nutrition, *in vitro* cultivation and completion of their development up to intact plants.

T. medium belongs to the tertiary gene pool of *T. pratense* relatives (Morris & Greene, 2001) along with *T. alpestre*. Plant breeders use the secondary gene pool if they cannot find desirable alleles in primary gene pool (cultivars, landraces, wild/naturalised, botanical varieties) and they turn to the tertiary gene pool only after exhausting the secondary pool (Fehr, 1987). *T. sarosiense* is in secondary gene pool of *T. medium* relatives. Thus *T. sarosiense* is generally considered as a nearer relative to *T. pratense* in comparison with *T. medium*. The following consideration is obvious, namely that also *T. sarosiense* should be perspective for interspecific hybridisation with *T. pratense* even if our study did not confirm this assumption. Previously, other examination performed by Phillips et al. (1982)

confirmed successful hybridisation *T. pratense* with *T. sarosiense*.

From our work, it can be concluded the following findings; to make crosses only in one direction with *T. pratense* as a female parent and *T. medium* as a male, to use the tetraploid plants of *T. pratense*, and to carry out hybrid embryos excision at early torpedo stage at time interval of about 7 DAP.

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