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Interspecific Hybridisation Prospects in the Genus *Trifolium*

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Abstract: Study of pre- and post-fertilisation barriers of crossability after interspecific hybridisation of *Trifolium pratense* and wild species *T. alpestre*, *T. medium* and *T. sarosiense* is presented in this work. Growth of pollen tubes was arrested after interspecific crosses to a lesser extent. The decisive meaning for hybrid obtaining had post-fertilisation barriers traced by two clearing treatment of immature seeds. The most promising genotypes for interspecific crosses was *T. pratense* (4×) as a female and *T. medium* as a male. From the cross of *T. pratense* cv. Tatra × *T. medium*, 14 embryos at the early torpedo stage were cultivated *in vitro* and 12 F_1 hybrid plants were produced. Cytological studies by flow cytometric analysis of BC_2 progeny revealed 30 somatic chromosomes in 443 plants, 42 and 44 chromosomes in 6 plants.

Keywords: *Trifolium;* interspecific hybridisation; barriers of crossability; embryo culture

In the research directed to increasing tolerance to diseases and unfavourable environmental effects and persistence in promising *Trifolium pratense* L. 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) are the main features of this procedure. The overcoming of these barriers helps to solve various *in vitro* methods such as embryo culture, protoplast fusion and *in vitro* pollination (Taylor & Quesenberry 1996). An alternative procedure, currently, is gene introduction by *Agrobacterium tumefaciens*.

Interspecific hybridisation within the genus *Trifolium* has thus far been based exclusively on the culture of immature zygotic embryos, which has facilitated to overcome incompatibility. *T. pratense* was successfully crossed with 5 species: *T. sarosiense* Hazsl. (Phillips *et al*. 1982), *T. medium* L. (Vogt & Schweiger 1983; Merker 1984; Sawai *et al*. 1990), *T. alpestre* L. (Merker 1988), *T. ambiguum* M. Bieb. (Vogt & Schweiger 1983) and *T. diffusum* Ehrh. (Schwer & Cleveland 1972). On the other hand, the successful genetic transformation and transmission of genes introduced through sexual generation has been quite sporadic in *T. pratense* (Quesenberry *et al*. 1996). From this point of view, various *in vitro* methods seem to be still prospective and promising for the future.

The main objective of the presented work was to study pre- and post-fertilisation barriers to compatibility after interspecific hybridisation of *T. pratense* and the wild species *T. alpestre*, *T. medium* and *T. sarosiense*. As a part of the work, the obtaining of *T. pratense* and *T. medium* hybrids by *in vitro* embryo rescue and their cytological evaluation was carried out.

MATERIALS AND METHODS

Plant material and crossing techniques: The experiments involved 4 clover species: 5 diploid ($2n = 2x = 14$) and 14 tetraploid *T. pratense* ($2n = 4x = 28$), wild species were substituted by *T. medium* ($2n = 56$), *T. sarosiense* ($2n = 48$) and *T. alpestre* ($2n = 16$). Intraspecific and interspecific crosses were performed. Flowers of the female plants were manually emasculated and hand pollinated in all possible combinations including reciprocal combinations.

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 40 min. After washing for 24 h in running tap water, the flowers were stained with 0.1% aniline blue in 0.7% K_3PO_4.

Post-fertilisation barriers: Post-fertilisation barriers (endosperm and embryo development) were traced by two clearing treatments after Mayer *et al*. (1993) and Hoshino *et al*. (2000) with modifications. Ovaries or immature seeds were dissected from immature pods in different stages of development (1, 3, 4, 7, and 8 days after pollination [DAP]), fixed in FAA mixture (formaldehyde, acetic acid, ethanol [5:5:90, v/v/v]) for 4 h at room temperature. Seeds were cleared in chloral hydrate (Mayer *et al*. 1993) overnight. For the other clearing procedure, after Hoshino *et al*. (2000), the following modifications were performed: fixation for 4 h, the last step of clearing in benzyl benzoate and dibutyl phtalate (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.

Hybrid embryo rescue and hybrid plants evaluation: Hybrid embryos from *T. pratense* cv. Tatra × *T. medium* were cultivated *in vitro* on L2 cultivation medium (Phillips & Collins 1979) with 10% sucrose to produce intact plants. The progeny obtained by immature embryo rescue was tested by flow cytometric analysis.

RESULTS AND DISCUSSION

Growth of pollen tubes was arrested after interspecific crosses to a lesser extent, namely in the combinations *T. pratense* (2*x*) × *T. alpestre*, *T. medium* and *T. sarosiense* × *T. pratense* (2*x*). In *T. sarosiense* × *T. pratense* (4*x*), successful growth depended on the particular combination of genotypes used. In most cases, the pollen tubes grew up to the base of a style and successful fertilisation could be expected.

The use of the procedure of immature seed clearing was convenient for embryos up to the torpedo stage, which was sufficient for the evaluation of hybrid embryo development *in situ*. Defects of the embryo sac were observed after interspecific crosses in various stages of embryogenesis and in various cross combinations; this have decisive meaning for hybrid embryo viability. The hybrid endosperm remained at the nuclear stage and was disintegrated. Importance of post-fertilisation barriers in *Trifolium* after interspecific hybridisation is of common acceptance (Taylor *et al*. 1980).

In our work, the most promising genotypes for interspecific crosses was *T. pratense* (4*x*) as a female and *T. medium* as a male. The optimal period of *in situ* embryo development and for *in vitro* embryo culture was on the 9th DAP; the embryo was at the early torpedo stage (Figure 1).

From the cross of *T. pratense* cv. Tatra × *T. medium*, out of several hundred pollinated flowers, 14 embryos at the early torpedo stage were found which were suitable for dissection. The embryos were cultivated *in vitro* to produce intact plants. 12 F_1 hybrid plants were produced and trans-

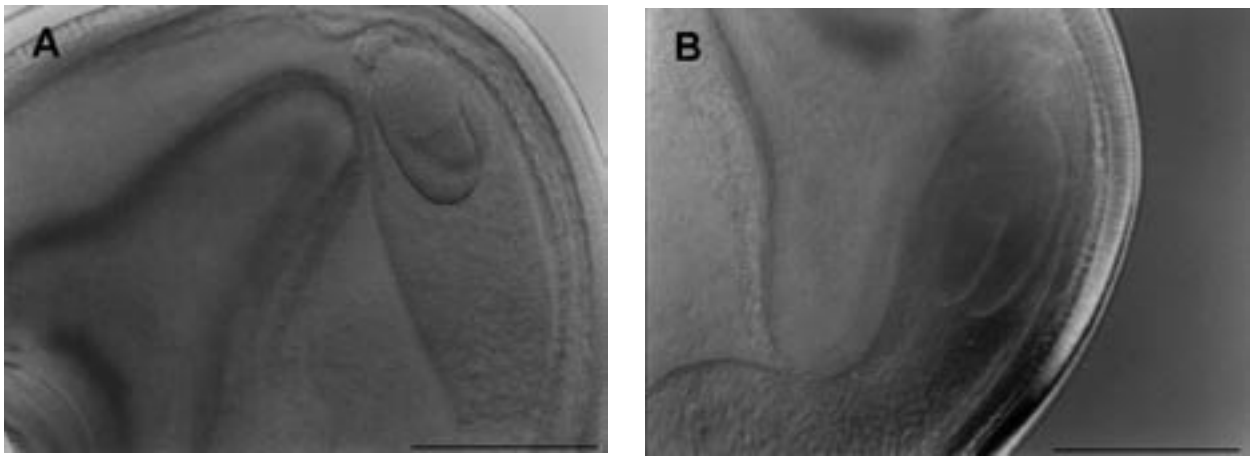

Figure 1. Hybrid embryo of *T. pratense* (4*x*) × *T. medium* at the early torpedo stage on the 7th DAP (A), in comparison with the embryo of *T. pratense* at the torpedo stage on the 8th DAP (B)

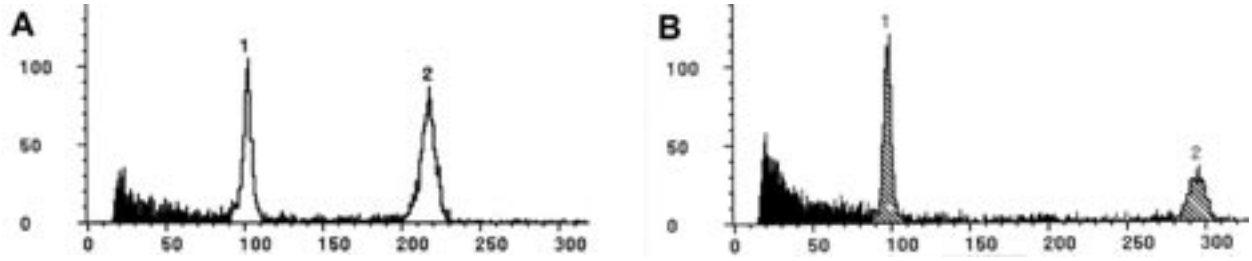

Figure 2. Histograms of DNA content in two hybrid plants corresponding to 30 somatic chromosomes (A); and 42 somatic chromosomes (B). *T. pratense* (2*x*) was used as a control plant (1)

ferred into nonsterile conditions. Hybrid plants were subjected to one generation of intercross and two generations of backcross with *T. pratense* cv. Amos. Cytological studies by flow cytometric analysis of this BC_2 progeny revealed 30 somatic chromosomes (Figure 2A) in 443 plants. Furthermore, 42 (Figure 2B) and 44 somatic chromosomes were detected in 6 plants and 45 chromosomes in 1 plant.

Hybrid plants showed an intermediate character between the two parent species. Morphologically the hybrids resembled *T. pratense*; leaf morphology was widely varied. As for economic traits introduction from *T. medium*, rhizomatous habits of plants and resistance to diseases will be evaluated and published in a future study.

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