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## Preliminary results on studies of *in vivo* and *in vitro* sexual reproduction of *Salix viminalis* L.

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**Abstract:** *In vivo* and *in vitro* self-pollination of whole pistils of some clones of *Salix viminalis* enabled to obtain mature seeds containing cotyledonary embryos which after the transfer to MS medium developed into wholly formed seedlings. Pollination *in vitro* of placenta led to abundant pollen germination and formation of tubes which occasionally they were entering the ovules through micropyle. Fertilized ovules normally developed into germinable seeds.

Distant pollination of stigmas *in vivo* and *in vitro* with pollen grains of *Populus tremula*, *P. tomentosa*, *P. lasiocarpa* showed the ability of pollen to germinate and to form tubes several hours after pollination. Some tubes penetrated the styles but did not enter into the placenta. When placenta were directly pollinated than pollen germinated abundantly and occasionally pollen tubes were found entering the micropyle. Embryological analysis of those ovules performed 3–5 days after pollination demonstrated the presence of globular embryos with several endosperm nuclei.

The technique of *in vitro* placental pollination works well for *Salix viminalis* and it could probably be applied to other *Salix* species.

**Additional key words:** willow, poplar, *in vivo*, *in vitro* stigma and placental pollination, embryo development

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### Introduction

The genus *Salix* belongs to family *Salicaceae* and it contains from 300–500 species (Boratyński and Boratyńska 1990) characterized as dioecious, however the sporadic occurrence of androgyny and polygamy occurs among some lowland species (Faliński 1998). Among *Salix* species valuable hybrids, both natural and artificial are known (Giertych 1990). There are also some selected clones for special pur-

poses particularly for timber or biomass production (Weih 2001; Weih and Nordh 2002; Heinsoo et al. 2002). Research concerning the productivity and economic potential of *Salix* has centered on *S. viminalis* as a fast growing tree species (Dubas 2001; Szczukowski et al. 2001). Informations are now being collected and checked in relation to biology of this species, including among others genetic parameters of growth characters as well as generative and vegetative reproduction (Rönnerberg-Wastjung and Gullberg 1994). Swed-

ish breeding programmes mainly focused on interspecific crossing and building up a set of inbred lines for two selected species, *S. viminalis* and *S. dasyclados* (Gullberg 1993).

The somatic chromosome number in *Salix viminalis* is 38, however in different other species natural diploids, triploids, tetraploids, heksaploids and octoploids have been described (Giertych 1990). Investigations carried out on crosses between clones of *Salix viminalis* originating from various aeries showed that hybridization among plants occurs easily (Rönnerberg-Wastjung 1994; Rönnerberg-Wastjung and Thorsen 1988). The value of the hybrids in breeding programmes may be most valuable, particularly for the establishing of plantation of selected clones for biomass production.

Most *S. viminalis* plants are a sexually propagated clones derived from hybrids. In contrast to well documented techniques concerning vegetative propagation of *Salix* species, little attention has been given to the process of sexual reproduction. The acquisition of new, detailed knowledge of *in vivo* and *in vitro* sexual reproduction has both fundamental and applied value, especially since the scanty study on the *Salix* genus is almost 100 years old. Chamberlain (1897) gave an account of the life history of *Salix* in which the development of the male and female gametophytes was described. According to the author's statement the embryo sac was tetrasporic. Maheshwari and Roy (1951) were unable to confirm Chamberlain's findings as their investigations of several *Salix* species demonstrated that the embryo sac was of monosporic, eight-nucleates type.

Sexual reproduction in angiosperms is initiated when pollen grains transferred from anther to the stigma. Well documented information on the structure of embryo sac, fertilization and embryo development in *S. viminalis* creates a growing interest in the use of *in vivo* and *in vitro* fertilization procedure to obtain new interspecific and intergeneric hybrids. The major objectives of the present studies were as follows: 1) to analyze the embryo and endosperm development after *in vivo* and *in vitro* self-crosses; 2) to reveal if application of the *in vitro* pollination technique would enable to cross *S. viminalis* with various *Populus* species.

## Material and methods

### *In vivo* self-pollination of female flowers

*Salix viminalis* is a tall shrub, it grows to over 7 m, and it has girth of 20–30 cm at ground level. Plants representing several 3-years old clones (female 2, 4, 10, 38, 53; male 75, 72, 37, 71) of *Salix viminalis* grown under the field conditions in the Institute of Plant Genetics, Polish Academy of Sciences Poznań, were used for the experiments. Under the climate of

Poznań, *Salix viminalis* flowers during the end of February and the beginning of April. Stem sections of a length of 60 cm with closed catkins were cut off and placed in jars of water standing on the windows' still in laboratory rooms at the temperature of  $\pm 20^{\circ}\text{C}$ . Stems with female catkins were bagged. Several days later anthers begin to dehisce usually at the same time as stigmatic papilae of the female catkins were present all over the radiating arms of the stigmas. Pollen grains were removed from the inflorescences of the male catkins and using a fine paintbrush were transferred on the stigmas of female catkins. Similar experiments were done in the field. Here female catkins were bagged before anther dehiscence. Few days later when stigmas were mature pollen grains were shed all over the female catkins and again bagged. Plants were maintained for the next 3–4 weeks until seed set. Pollinated pistils were fixed in formalin-acetic-alcohol at intervals of 2 days until the 3-th week. Whole ovaries or single enlarged ovules were embedded in paraplast, cut on a microtome to 10–12  $\mu\text{m}$  sections and stained with Heidenhein's iron hematoxyline and fast green or with crystal violet counterstained with orange G.

### *In vitro* self- and cross-pollination of pistils and placentae

Whole female catkins at the stage of fully developed stigmatic papilae were sterilized for 6–10 minutes in chlorine water and later three times thoroughly washed with sterile water. Single pistils were excised from catkins and their stigmas wiped with sterile filter paper. Pollen grains were applied directly on the stigmas and the pistils thus pollinated were placed in the test tubes containing macro- and microelements of MS medium (Murashige and Skoog 1962) with 3% sucrose. In the case of placenta pollination stigmas along with the styles were cut off and pollen were deposited on the cut surface of the opened ovary. At 24 and 72 hours after pollination some of the styles and ovules were removed, stained in aniline blue and examined for pollen tube growth using epifluorescence microscopy. Pollen grains of *S. viminalis* used for *in vivo* and *in vitro* pollination were collected from freshly dehiscent anthers of the shrubs growing in the field or from the cut stems kept in laboratory.

Pollen grains of *Populus* species were collected from male catkins of the trees growing in Botanical Garden. In total 96 pistils and 160 placentae of clones 2, 4, 10, 38, 53 were pollinated with pollen grains of clones 75, 72, 37, 71. Moreover 65 pistils and 210 placentae of the same clones were pollinated with pollen grains of *Populus tremula*, *P. tomentosa* and *P. lasiocarpa*. Fully developed cotyledonary embryos were aseptically isolated from ovules and cultured on MS medium lacking growth regulators. The pH of the medium was adjusted

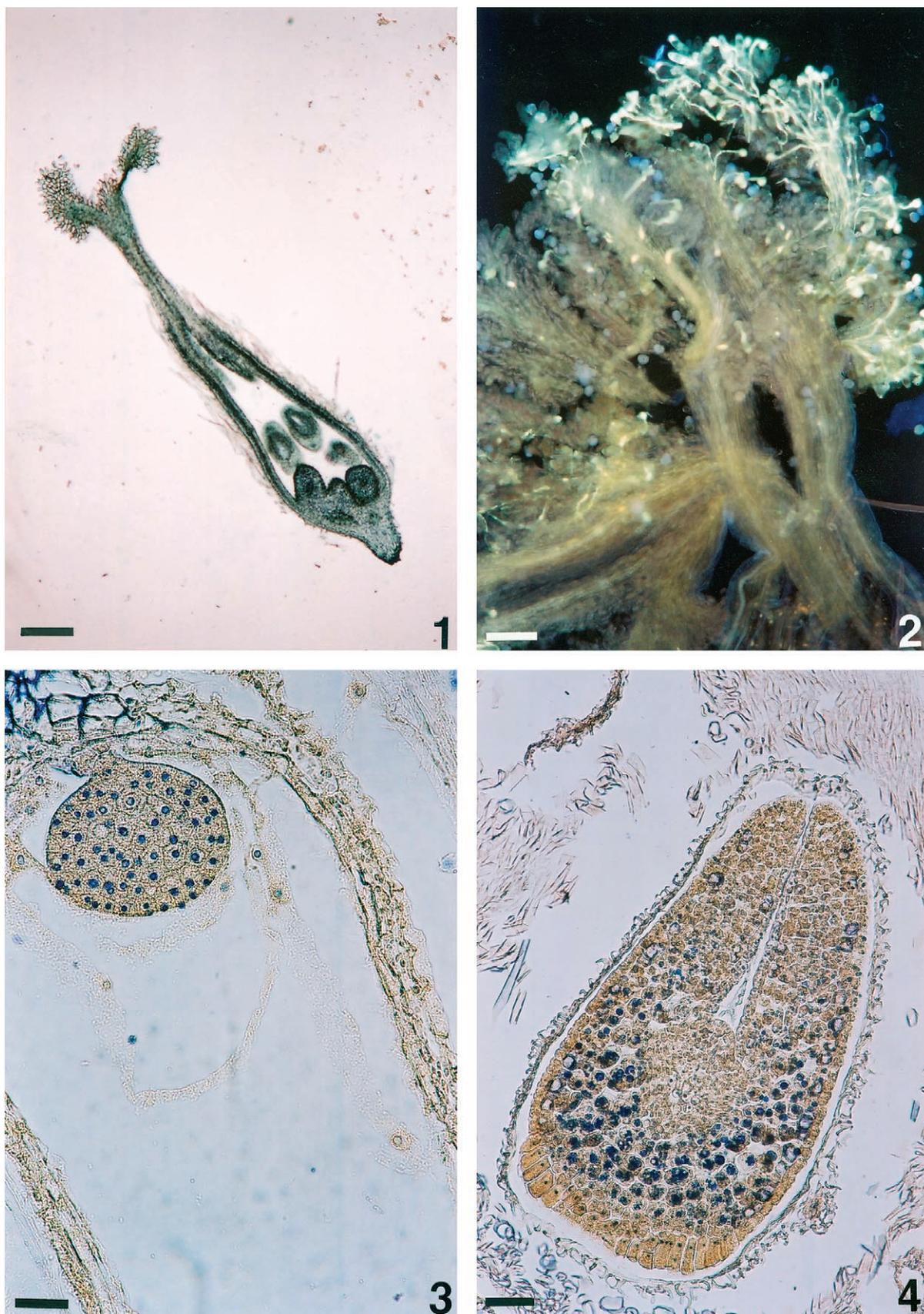


Fig. 1. A longitudinal (L.s.) section of a pistil of *Salix viminalis* (S.v) with 2 ovules (the remaining ones on other sections); bar=100  $\mu\text{m}$

Fig. 2. Germinating pollen grains on stigmas of S.v. 24 hours after *in vivo* pollination of pistil; bar=50  $\mu\text{m}$

Fig. 3. 5 day-old globular embryo of S.v from *in vivo* self-pollinated pistil; bar=50  $\mu\text{m}$

Fig. 4. L.s. of an ovule with a well developed embryo 20 days after self pollination of pistil; bar=50  $\mu\text{m}$

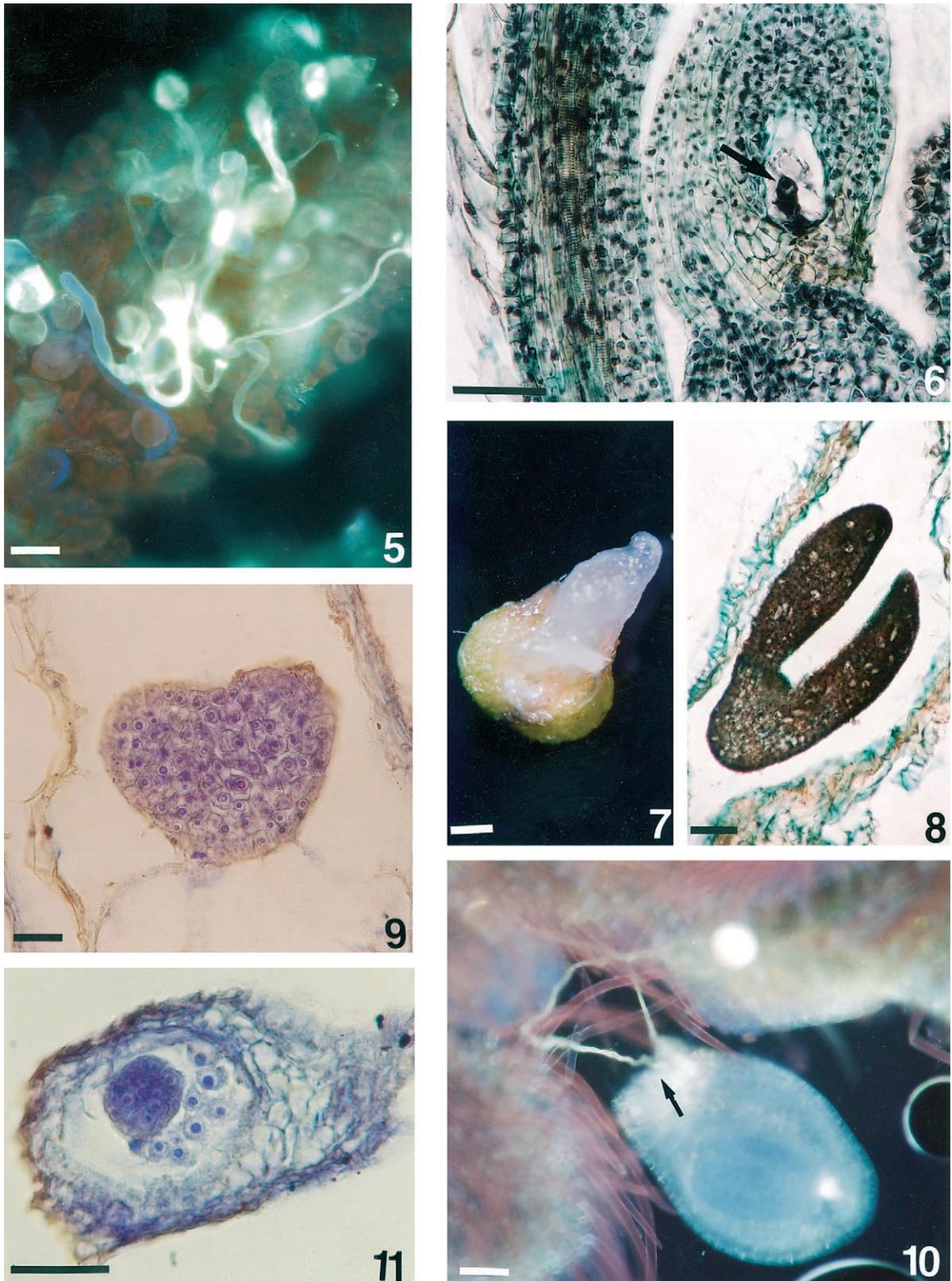


Fig. 5. Germinating pollen grains on placenta of *S.v.* 20 hours after *in vitro* pollination; bar=50  $\mu$ m

Fig. 6. Remnants of pollen tube inside an embryo sac of *S.v.* after *in vitro* self-pollination of pistil (arrow); 50  $\mu$ m

Fig. 7. Enlarged ovule of *S.v.* 5 days after placental self-pollination; bar=0,5 mm

Fig. 8. 21 day-old embryo of *S.v.* inside an ovule after self pollination of placenta; bar=50  $\mu$ m

Fig. 9. L.s. of an ovule with heart-shape embryo 7 days after self-pollination of placenta; bar=50  $\mu$ m

Fig. 10. Pollen tubes of *Populus tremula* inside the micropyle of *S.v.* ovule, 24 hours after placental pollination (arrow); bar=50  $\mu$ m

Fig. 11. 5 day-old embryo and endosperm cells developed after placental pollination of *S.v.* with *P. lasiocarpa*; bar=50  $\mu$ m

to 5.8 before autoclaving. The cultures of pollinated *in vitro* pistils and placentae were incubated at  $23^{\circ}\text{C}\pm 2^{\circ}\text{C}$  under the cool white fluorescence light.

## Results

### *In vivo* self-pollination

In the unilocular ovary of *Salix viminalis* 7–9 anatropous ovules are attached to the placenta by a pedestal like funiculus (Fig. 1). The mature gametophyte is composed of an egg apparatus consisting of two synergids and an egg resides in the micropylar region. Two polar nuclei are located in the central cell which is occupied by a large vacuole. Antipodals in the mature gametophyte are not distinguished.

The stigmas of the freshly opened flowers were pollinated with an abundant number of pollen grains. After pollen have germinated (Fig. 2) (24 hours after pollination fully formed tubes were observed), the papillae begin to collapse and later on stigmas started drying. Unpollinated female flowers remained healthy for about a week, but later turned brown and dried up. The pollen tubes grew rapidly after selfing and 48 hours following pollination in some ovules few-celled proembryos and free endosperm nuclei were observed. Although stages in double fertilization were not seen by us, this may be assumed to take place normally, for the development of the embryo and endosperm followed the normal course. The embryo attained a globular shape (Fig. 3) within 5 days, heart-shape within 12 days and in a 20-day-old ovules well differentiated embryos showing immense cotyledons with a short axis were present (Fig. 4). The endosperm remained free nuclear for the first days, after which cell walls were formed. In a 20-day-old ovules there were few vacuolated endosperm cells and in the 28-day-old seeds only residues of endosperm could have been distinguished. The number of seeds per fruit (ovary) varied. In catkins pollinated in field usually 5–7 seeds were present in mostly all fruits, instead in those pollinated in laboratory in that less seeds (1–3) were demonstrated. Isolated embryos (Fig. 4) from 28-old seeds transferred into the medium developed into healthy seedlings. After 4–5 weeks of culture the plantlets with well formed roots were transferred into soil.

### *In vitro* self-pollination

Sterile conditions did not hinder germination and pollen tube growth on the stigmas and placentae. Pollen germinated abundantly on stigmas and some grew alongside the styles attaining ovules in 18–20 hours. The remnants of pollen tubes inside the micropyle region of embryo sacs were noticed (Fig. 6). On directly pollinated placentae pollen also germinated and their tubes grew around the ovules (Fig. 5). Within 5–7

days an enlargement of some of the ovules could be observed (Fig. 7). Histological analysis of the enlarged ovules 5 days following pollination revealed the presence of globular embryos and 2 to 3 days later the heart-shape ones with few vacuolated endosperm cells (Fig. 9). Pollination *in vitro* of whole pistils resulted in the development of 1–3 seeds per one ovary. Much less seeds were obtained after placental pollination as usually only 1, very seldom 2 seeds were found in some drying ovaries. Cotyledonary embryos (Fig. 8) obtained from *in vitro* pollinated stigmas or placentae were fully viable and their behavior was identical with that of normal ones obtained *in vivo*. In results of stigma pollination 5–10% of ovules grew to maturity as placental pollination resulted in only 1–2% maturing ovules containing cotyledonary embryos.

### *In vivo* and *in vitro* cross-pollination

One of the purposes of the work presented here was to look whether pollen grains of *Populus* species would germinate when placed on the stigmas and placentae of *Salix viminalis* and if so, what will happen later when tubes reach the ovules. We wished to know whether barriers to pollen penetration exists within megagametophytes. Furthermore, we were interested if an hybrid embryo will develop after pollen tube entrance into the embryo sac.

There is no information in the available literature on crossing *Salix* with *Populus*. This study gives only preliminary results on experiments carried out during flowering both groups of plants in March – beginning April 2003. The up to date results are as follows: 1) pollen grains of *Populus tremula*, *P. tomentosa* and *P. lasiocarpa* germinated *in vivo* on stigmas of *Salix viminalis*, some tubes penetrated the styles but did not enter into the placentae; 2) stigmas pollinated *in vitro* resulted in abundant pollen germination and penetration of some tubes through the style, however the entrance into the ovary were never noticed; 3) when styles were cut off pollen germinated on the placentae, few pollen tubes did run into the direction of ovules where sporadically they were entering the micropyle (Fig. 10). The process of fertilization was not found. Globular embryos at various developmental stages and several endosperm nuclei were observed between 4–5 days after pollination (Fig. 11). We failed to obtain embryos at later stages of development as in those crosses a contamination appeared early, usually on the 2–3 day after transfer pollen on the placenta. Probably the procedure currently employed in our laboratory for application unsterile pollen grains of *Populus* species was not suitable. Irrespectively of the donor plants pollen grains of *Populus* species germinated on stigmas and placentae, however sporadic globular embryos were obtained only after placental pollination.

## Discussion

The simplest technique available to effect fertilization *in vitro* is to culture whole pistils or in some cases placentae and apply pollen to the stigmas and ovules when they are mature for fertilization. Using this method successful self- and cross-pollination and fertilization resulted in certain species of angiosperms, mainly among species representing *Solanaceae*, *Caryophyllaceae*, *Brassicaceae* and *Liliaceae* families (Zenkteler 1999). However, no data are available on applying this technique on distant crosses in species representing woody plants. It is interesting to note that when self-pollinating *in vitro* mature seeds with viable embryos developed. From those embryos after transfer on macro- and micro- MS medium fully formed fast growing plants were obtained. Placental pollination may prove useful to geneticists when failure to secure desired hybrids is due to the lack of pollen tube growth and fertilization. It ensue from our experiments that *Salix viminalis* may be a convenient species for *in vitro* placental pollination as pollen tubes elongate toward ovules and penetrate them.

Recent research also demonstrate that stigmas *in vivo* and *in vitro* enable germination of *Populus* pollen. The incompatibility mechanism exists later acting in the style.

Much more interesting is our finding that pollen grains of *Populus* species after transfer on placentae of *Salix viminalis* germinated and produced tubes of which some entered the ovules. Our results indicate that those placental pollination may bypass stylar barrier preventing hybridization. Unfortunately, we were unable to observe male gametes penetrating female gametophytes what would be a direct proof that the developed globular embryos were a hybrid origin. When new experimental material will be applicable in the next season, attempts will be made to increase the frequency of pollen tube penetration, and examine the fate of pollen tube during penetration. It has also to be work out a suitable procedure enabling to obtain pollen without microbial contamination.

Our findings on *in vitro* pollination in *Salix viminalis* show that in conjunction with advances in biotechnology and genetic engineering will have a great impact on generative reproduction of this species in the future. In particular, finding proper pollinators representing precise genes, will become powerful breeding tools on a practical basis.

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