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# Methods for non-stigmatic pollination in *Trifolium repens (Papilionaceae)*: seed set with self- and cross-pollinations in vitro

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**Summary.** The stigma and style of *Trifolium repens* L. was successfully by-passed, and viable seed was obtained by placing pollen on a perforation made in either the ovary wall or in the base of the style. The application of pollen to the stump of the style also succeeded in producing viable seed. The presence of the calyx and corolla facilitated seed set in cultured florets. An incision made along the entire length of the ovary to permit pollen entry resulted in necrosis of the floret. Perforation of the ovary did not affect the yield of seeds obtained from stigmatic pollinations. Growth of pollen tubes was observed on wounded tissues and also within the style and cavity of the ovary. A mean yield of 20 embryos was obtained per 100 florets in non-stigmatic pollinations, whereas with stigmatic pollinations in vitro the yield was 148. Non-stigmatic pollinations also gave seeds when *T. repens* was self pollinated, which showed that the gametophytic system of self-incompatibility (SI) could be by-passed. With one genotype, self-pollination via the stigma failed, whereas embryos were obtained via non-stigmatic pollinations.

**Key words:** In vitro culture – Self-compatibility – Pollination – *Trifolium repens*

## Introduction

The processes of pollen germination, growth of pollen tubes, fertilization and seed set can proceed independently of the sporophyte by culturing flowers in vitro. In *Nicotiana* and *Petunia* viable seeds have been obtained in vitro by excising the entire pistil and pollinating the stigma (Dulieu 1966; Rao 1965; Shivanna 1965). An alternative technique of removing the walls of the gynaecium in vitro has also been used extensively, and seeds have been obtained in this way in *Papaver nudicaule* (Olson and Cass 1981), *Petunia hybrida* (Niimi 1970) and in the hybridization of tobacco (Slusarkiewicz-Jarzina and Zenkteler 1983). In these cases whole pistils were cultured, but the ovary wall was excised and pollen was applied to ovules and the placenta (placental pollination).

In some species the entire pistil is not essential, for example in *Papaver somniferum* seeds were obtained by removal of the placenta with ovules attached and by application of pollen to these ovules (Kanta et al. 1962). There is only one report of seed formation arising from the culture of isolated ovules to which pollen was applied in vitro; this was in *Brassica oleracea* (Kameya et al. 1966).

These techniques of non-stigmatic pollination have been useful for by-passing the fertilization barriers that occur in the stigma or style. This has facilitated the production of interspecific (Dhaliwal and King 1978; Stewart 1981) and intergeneric hybrids (Zenkteler 1967; Zenkteler and Melchers 1978). In addition the technique has been used to induce maternal haploids (Hess and Wagner 1974; De Verna and Collins 1984) and to bypass gametophytic or sporophytic systems of self-incompatibility (Rangaswamy and Shivanna 1967; Zenkteler et al. 1987). These and several other applications have been reviewed by Rangaswamy (1977) and Zenkteler (1990).

Success in non-stigmatic pollination in vitro has often been achieved with families in which ovaries contain a large number of ovules and a well-developed placenta: *Solanaceae*, *Caryophylaceae*, *Primulaceae*, *Papaveraceae* (Zenkteler 1990). Attempts at placental pollination have been made with species of the family *Papilionaceae*, such as *Lupinus polyphylus*, *Medicago sativa*, *Pisum sativum* and *Vicia sylvatica*: the ovules withered soon after pollen deposition, and no seeds were set (Zubkova and Sladky 1975; Zenkteler 1980). Few ovules are present in such ovaries, and placentas are poorly developed. In soybean, fertilization by placental pollination has been reported, but the frequency of seed development was not given (Tilton and Russell 1983).

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In *Trifolium* spp. in vitro pollinations could be useful for interspecific hybridization and for by passing self-incompatibility. For example, the hybridization of *T. repens* × *T. ambiguum* is prevented because of the failure of the pollen tubes of *T. ambiguum* to traverse the longer styles of *T. repens* (Richards and Rupert 1980). Similar barriers exist with *T. medium* × *T. repens* (Kazimierska 1978) and *T. pratense* × *T. repens* (Leduc unpublished).

The need for successful techniques of non-stigmatic pollination suited to the *Papilionaceae* led us to develop new methodologies of stylic and ovarian pollination in *Trifolium repens*. In this study we report the first successful non-stigmatic pollination with a species in this family giving rise to viable seeds. Embryo yield after crossing and selfing with stigmatic, stylic and ovarian pollination are also compared.

## Materials and methods

Plants of *Trifolium repens* were raised in pots containing potting compost and maintained in the glasshouse under a 16-h photoperiod. Florets on which the large standard petal was just beginning to open were selected for in vitro pollination. In these florets anthesis would occur within 24 h. Florets with stigmas already bearing pollen grains were discarded.

Sterilization was achieved by immersing excised florets for 20 min in a solution of calcium hypochlorite prepared by mixing 1 part of a filtered solution 7% (w/v) of calcium hypochlorite with 7 parts sterile water. This was followed by three rinses in sterile distilled water.

For all types of pollinations, an incision was first made in the calyx and along the length of the floret through the keel and wing petals that remained attached to the base of the pistil. The stigma, style and upper ovary were probed out through the incision. Pollination was achieved by rubbing the surface of the stigma or the prepared area of the gynaecium with anthers collected on the day of anthesis from other florets. Non-stigmatic pollination included the following methods, and pollen was applied immediately after the incision or perforation was made.

*Pollination on a perforation of the style.* A small perforation was made using the disposable needle-tip of a syringe [Microlance 2 (0.8 × 40 mm) Becton-Dickinson]. The needle was inserted through the epidermis to the stylar canal at the lower fleshy part of the style (Fig. 2). Usually a drop of stylar fluid exuded from this perforation, and pollen grains would stick and hydrate to this fluid.

*Pollination on the cut surface of the style.* The style was cut transversely at the lower fleshy part and removed (Fig. 5). To achieve successful pollination, it was important not to pinch the style when cutting and to ensure the stylar canal was left open.

*Pollination on a perforation of the ovary wall.* A perforation through the ovary wall was made between the two ovules most proximal to the style (Fig. 3). The outline of the ovule was easily distinguishable under the ovary wall, and perforation of an ovule was rare.

*Pollination on a longitudinal incision of the ovary wall.* A longitudinal incision along the full length of the ovary wall was made in the median line and this slightly exposed the ovules.

Florets were cultured by vertically inserting them on agar-solidified (Difco Bacto, 0.8%) B5 medium (Gamborg et al. 1968) under the environmental conditions previously described by Douglas and Connolly (1989). Ten days after pollination, the ovaries were opened and the ovules scored for the presence of an embryo. Most embryos would have reached the horse-shoe stage by then, however globular to torpedo stage embryos could also be found (Fig. 1). The viability of the embryos was ascertained by culturing them on fresh B5 medium to test for germination. Globular or heart-shaped embryos were assumed to be non-viable since they never developed further and would abort. These two types were classified together as "heart-shaped embryos" in the text. All other embryos were recorded as "Torpedo to horse-shoe embryos", and these were transferred onto fresh B5 medium where they germinated. Yield of embryos in both classes was expressed as the number of embryos per 100 cultured florets. Significance between yields was tested by a Student's *t*-test with a probability $P<0.05$.

## Results

Ten days after pollination of the stigmas in cultured florets, the fertilized ovules contained embryos at various stages of development, including horse-shoe shape, torpedo shape and globular to heart shape (Fig. 1). Globular and heart stages were usually reached earlier at 4 or 5 days after pollination. The first two types germinated normally when re-cultured, but heart-staged embryos did not.

Removal of one wall of the ovary to facilitate the deposition of the pollen directly onto ovules resulted in necrosis of the pistil. The effects of removing other organs of the floret on the production of embryos can be compared using entire florets (Table 1). Removal of the corolla and calyx gave significantly fewer embryos. However, removal of these organs did not arrest embryo development since the number of heart-shaped embryos was similar in each treatment. In all subsequent experiments we used florets with attached pedicule, corolla and calyx.

Pollen which was placed on a perforation in the style germinated within an hour. Pollen tubes grew along the epidermis of the style, into the surrounding air (Fig. 2) and also succeeded in penetrating stylar tissue and growing along its length (Fig. 2 inset). Similarly, pollen placed on a perforation of the ovary germinated and the pollen tubes grew into the ovary, around the ovules (Fig. 3) and resulted in fertilization and seed set (Fig. 4). At 10 days after pollination, the calyx pedicule and ovary were green, whereas the stigma, style and corolla had withered (Fig. 4). Pollen also germinated when placed on a stump of the style (Fig. 5) and resulted in fertilization, ovary enlargement and the production of viable seeds (Fig. 6). Pollen which was applied to the bases of non-perforated styles failed to give fertilization and seeds. This showed that the stigma and style could be bypassed only when an incision was made to allow entry of the pollen tubes.

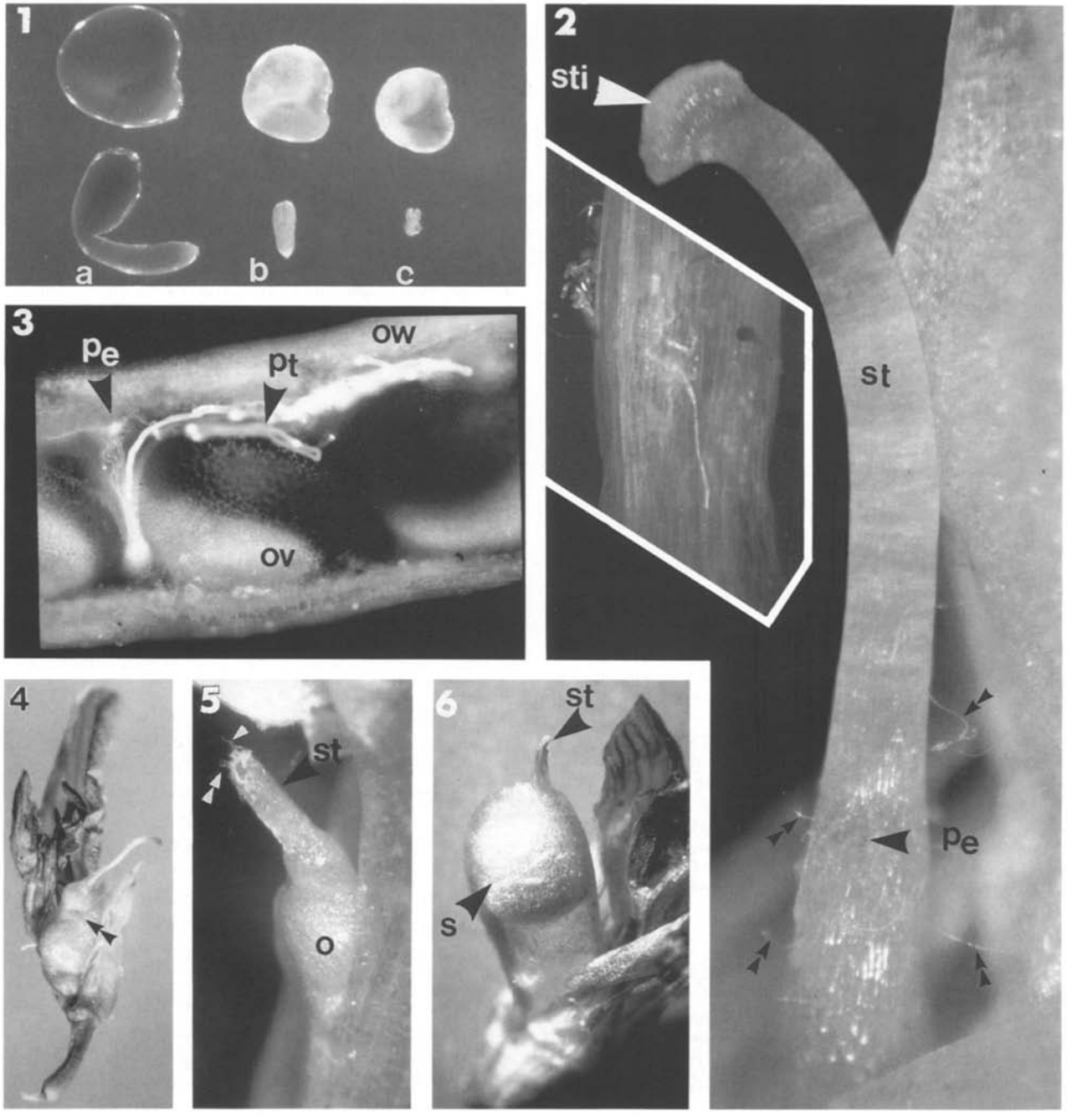


**Fig. 1.** The range of fertilized ovules (*top row*) and their extracted embryos (*bottom row*) at 10 days after in vitro pollination. Embryo stages shown are: horse shoe (*a*) torpedo (*b*) and heart (*c*) ×15

**Fig. 2.** Non-stigmatic pollination via a perforation (*Pe*) of the style (*st*). Note pollen tubes on the surface of the style and surrounding air spaces (*arrows*) and absence of pollen on the stigma (*sti*), ×80. *Inset* shows development of a pollen tube within the style from pollen applied to a perforation; fluorescence micrograph using aniline blue stain. ×160

**Fig. 3.** Non-stigmatic pollination via a perforation (*Pe*) in the ovary wall (*ow*) showing development of pollen tubes (*Pt*) around the ovules (*ov*) and within the ovary. For observation, the non-perforated wall was removed; fluorescence microscopy using aniline blue. ×80

**Fig. 4.** A cultured floret 10 days after pollination via a perforation in the ovary wall showing the perforation point as a necrotic spot (*arrows*) and enlarged fertilized ovule. Note petals and style had withered ×7

**Fig. 5.** Non-stigmatic pollination on the cut surface of the style (*st*) showing pollen tubes (*arrows*) and position of the undeveloped ovule (*o*). ×30

**Fig. 6.** Non-stigmatic pollination on the cut surface of the style at 10 days after pollination. Note drying of the style (*st*) and presence of a developing seed (*s*) from the proximal ovule ×30

**Table 1.** Effect of floral parts (pedicule, calyx and corolla) on yield and development of embryos following stigmatic cross-pollination of *T. repens* in vitro using three genotypes for each type of explant

| Explant cultured | Total no florets | Embryo yield per 100 florets | | |
|---|---|---|---|---|
| | | Stage of development | | |
| | | Heart | Horse shoe and torpedo | Total |
| | | (H) | (HT) | (H+HT) |
| Entire floret | 31 | 48 | 148 | 196 |
| Floret without pedicule | 30 | 30 | 136 | 166 |
| Fluret without calyx and corolla | 29 | 38 | 44* | 82* |

* Significant difference ($P \leq 0.05$) with numbers in the same column

The effects of the four methods of non-stigmatic pollination on yield of embryos can be compared with stigmatic pollinations in Table 2. Pollination via the stigma gave 7 times more embryos than the best methods of non-stigmatic pollination. In addition, pollination of the stigma more frequently gave ovaries that contained more than one fertilized ovule. In such cases, one ovule usually contained a well-developed embryo at the torpedo or horse-shoe stage, while the other contained an immature embryo at the heart stage. The frequency of such heart-stage embryos was 0–31% with a mean of 16% for stigmatic pollinations. In contrast, most of the embryos from non-stigmatic pollinations advanced to the torpedo or horse-shoe stage (Table 2).

Among the non-stigmatic methods of pollination, the perforation methods were superior to excision of the style or incision of the ovary wall, and these were subsequently studied further. The effects of perforating the ovary wall on the subsequent yield of embryos from pollinations via the stigma are given in Table 3. There was no significant difference in the yield of embryos from stigmatic pollination of either entire or perforated ovaries.

There was variability among the genotypes of *T. repens* in their capacity to give seeds by in vitro pollination. Thus, although each of the nine genotypes used for stigmatic pollination in Table 2 gave embryos, the yield of embryos ranged from 81 to 187 per 100 florets. With self-pollination there was also variability and a dramatic reduction in seed yield (Douglas and Connolly 1989; Leduc et al. 1990). To minimize the variability due to genotype and to determine the effects of non-stigmatic methods on self-pollination, we selected four genotypes, and each was either cross- or self-pollinated by alternative methods (Table 4). With cross-pollination the yield

**Table 2.** Effect of site of pollen application on yield and development of embryos following cross-pollinations of *T. repens* in vitro

| Location of pollen applied[a] | No. of geno-types | Total no florets | Embryo yield per 100 florets | | |
|---|---|---|---|---|---|
| | | | Stage of development | | |
| | | | Heart | Horse shoe and torpedo | Total |
| | | | (H) | (HT) | (H+HT) |
| Stigma | 9 | 100 | 16 | 132 | 148 |
| Style perforation | 6 | 51 | 0 | 20 | 20 |
| Ovary perforation | 7 | 102 | 2 | 18 | 20 |
| Style cut | 4 | 22 | 0 | 5 | 5 |
| Ovary cut | 3 | 29 | 0 | 0 | 0 |

[a] Pollen was applied to the stigma (stigma); to a perforation in the base of the style (style perforation); to a perforation in the overy wall (ovary perforation); to the cut surface after excision of the style (style cut); or to the surface of exposed ovules after making a longitudinal incision along the entire length of the overy wall (ovary cut). Details in Materials and methods

**Table 3.** Effects of a perforation in the ovary wall on the embryo yield following stigmatic pollination of *T. repens* in vitro

| Treatment of ovary | No of florets pollinated on stigma | Embryo yield per 100 florets | | |
|---|---|---|---|---|
| | | Stage of development | | |
| | | Heart | Horse shoe and torpedo | Total |
| | | (H) | (HT) | (H+HT) |
| None (control) | 42 | 40 | 86 | 126 |
| Perforated | 55 | 33[NS] | 102[NS] | 135[NS] |

[NS] No significant difference with controls at $P \leq 0.05$

**Table 4.** Embryo yields from cross- and self-pollinations and the effects of three methods of pollen application

| Location of pollen applied[a] | Embryo yield per 100 florets | |
|---|---|---|
| | Cross-pollination | Self-pollination |
| Stigma | 151 (43)[b] | 14 (116) |
| Style perforation | 19 (31) | 4 (79) |
| Ovary perforation | 12 (50) | 5 (64) |

[a] Pollen was applied to the stigma, perforation in the base of the style (style perforation) or to a perforation in the ovary wall (ovary perforation) as described in Materials and methods

[b] Embryo yield expressed as total number of embryos per 100 florets. Number in brackets is the number of florets pollinated per treatment

of embryos obtained by each method was similar to previous results (compare Tables 2 and 4). Thus, cross-pollination via the stigmas gave an 8- to 13-fold increase in the yield of embryos over pollination via perforations. With self-pollinations the yield of embryos obtained using stigmatic pollinations was also superior to selfing via the perforation method. In this case, however, the increase was only three fold (Table 4). Furthermore, in some genotypes non-stigmatic pollination was the only method of obtained self-fertilization and seed. With one of the four genotypes, no embryos were obtained by self-pollination via the stigma (50 florets used). With this same genotype one embryo was obtained via application of pollen to a perforation in the style (36 florets used) and one embryo was obtained via the application of pollen to a perforation in the ovary (30 florets used).

## Discussion

Fertilization and seed set in clover was obtained using non-stigmatic methods of pollination. Limiting the extent of ovary wounding to a small perforation avoided the necrosis of ovary tissue and of the ovules that had been reported in previous attempts with the Leguminosae (Zubkova and Sladky 1975; Zenkteler 1980, Tilton and Russell 1983).

The function of ovary tissues and floral organs in processes of fertilization and embryo differentiation are poorly understood. Previous reports have shown that the removal of floral parts could dramatically impair seed set. Hence, the emasculation of florets prior to stigmatic pollination significantly reduced seed set in soya bean (Walker et al. 1979). In *Brassica* species, seed set was also reduced when the entire ovary wall was removed in order to gain access to the ovules for direct pollination, whereas removal of only a part of the wall gave the best results (Zenkteler et al. 1987). In pea, the biochemical differentiation of developing pod tissues and their contribution to developing ovules has been shown (Srivastava et al. 1980; Carbonell and Garcia-Martinez 1985). In *Trifolium repens*, Richards and Rupert (1980) reported failure of the embryo and endosperm to develop with cultured florets in which the pedicule, calyx and corolla were excised together. The present study has confirmed the necessity to preserve the calyx and corolla to ensure the successful pollination and development of substantial numbers of seeds in vitro. The total yield of embryos was significantly reduced by removal of the corolla and calyx, but there was no increase in the number of heart-staged embryos present (Table 1). Such an increase would represent an arrest in embryo development and might be expected. The overall reduction in embryo yield, therefore, may have resulted from fewer fertilizations and/or an increase in embryo abortion during the early stages of embryogenesis (0–3 days post-pollination).

In comparing the efficiency of the methods of non-stigmatic pollination, those which caused the least injury to the pistil (i.e. pollination via a perforation in the style or ovary) gave the highest yield of embryos. A very small wound, such as a perforation in the ovary wall, had no adverse effect on embryo yield following stigmatic pollination; therefore, this type of wounding was not detrimental to fertilization or embryo development.

Although the stigma and style could be successfully by-passed by using non-stigmatic methods of pollination, we observed that pollination via the stigma was the most efficient method. Thus, stigmatic pollination gave 8–12 times more embryos than non-stigmatic methods in cross-pollinations (Tables 2 and 4). In contrast, stigmatic pollination gave only a three-fold increase in embryos from self-pollinations. Non-stigmatic methods were, therefore, relatively more efficient in self-pollinations and for by-passing the gametophytic system of SI. In addition, no seeds were obtained via stigmatic pollination of one genotype and only non-stigmatic methods gave seed. We assume that in by-passing the stigma and style many of the SI reactions were avoided by pollen but that residual SI reactions were experienced by pollen tubes in the lower style and ovary (Leduc et al. 1990).

The lower overall efficiency of non-stigmatic pollination in giving seed has been noted here and also with other species (Niimi 1970). A variety of causes may contribute to this reduced efficiency. A poor penetration of pollen tubes through wound tissue may be one factor in the present study, despite the excellent germination and growth of pollen tubes on the surfaces of pistil tissues. Alternatively, some pollen tubes may have failed to locate the ovules when pollination was not via the stigma since pistil tissues may have some function in directing pollen tubes toward the ovule or micropyle. A mechanical guidance or a chemotropic gradient may operate in some species (Tilton et al. 1984; Mulcahy and Mulcahy 1985), whereas with others such functions seem to be absent (Kandasamy and Kristen 1987). Non-stigmatic pollination may also result in an abnormal growth or differentiation of the pollen tubes, since normal growth depends on nutrients provided by the pistil tissues through which they grow (Mascarenhas 1975; Heslop-Harrison and Heslop-Harrison 1982). Pollen tubes of *Trifolium* spp. grow in the canal of the style, and their nutrition may have been adversely affected by direct pollination on the ovary or on the lower part of the style (Leduc et al. 1990). The normal pattern of microsporogenesis may have been affected by non-stigmatic pollination. This has been suggested for another binucleated species, *Nicotiana tabacum*, where the number of successful fertilizations was increased when pollen was applied at a distance from the ovules, as opposed to deposition on the surface of the ovules (Balátkova and Tupý 1972). Allowing pollen tubes to grow some distance before reaching the micropyle may facilitate the normal mitosis of the generative cell and maturation of the sperm cells prior to fertilization. Uncoupling the processes which proceed from stigmatic pollination may have also adversely interfered with fertilization. Thus, with barley

and tobacco it has been shown that a synergid cell degenerates soon after stigmatic pollination but before the pollen tube reaches the embryo sac (Cass and Jensen 1970; Mogensen and Suthar 1979). Abnormal interactions between the pollen and pistil with non-stigmatic pollinations may affect this type of response and impair fertilization.

In a previous study with *T. repens* we showed that there was competition among pollen tubes during their process of reaching the ovules (Leduc et al. 1990). Other authors have suggested that through pollen-pistil interaction an active selection process takes place which favours only a few pollen tubes for fertilization (Gawel and Robacker 1986; Shivanna and Shivanna 1985). With non-stigmatic pollination the distance to the micropyle is usually shortened. This would allow for fewer competition/selection processes to take place, thus the presence of pollen tubes of poor quality in the ovary may result in fewer successful fertilizations. This suggestion is supported by results in *Dianthus* where deposition of pollen grains at the base of its long stigma, nearest to the style, resulted in reducing the competition among pollen tubes and a poorer quality of seeds and seedlings (Mulcahy and Mulcahy 1975). However, seed number and seed size were not affected in this case.

Further study of the processes leading to fertilization via non-stigmatic pollination should reveal some causes of its inefficiency relative to stigmatic pollination. In addition, the technique may facilitate genetic manipulation of gametes prior to fertilization or facilitate the production of new interspecific or intergeneric hybrids.

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## References

Balátkova V, Tupý J (1972) Some factors affecting the seed set after in-vitro pollination of excised placentae of *Nicotiana tabacum* L. Biol Plant 14:82–88

Carbonell J, Garcia-Martinez JL (1985) Ribulose-1,5-biphosphate carboxylase and fruit set or degeneration of unpollinated ovaries of *Pisum sativum* L. Planta 164:534–539

Cass DD, Jensen WA (1970) Fertilization in barley. Am J Bot 57:62–70

DeVerna JW, Collins GB (1984) Maternal haploids of *Petunia axillaris* (Lam.) B. S. P. via culture of placenta attached ovules. Theor Appl Genet 69:187–192

Dhaliwal S, King PJ (1978) Direct pollination of *Zea mays* ovules in vitro with *Z. mays*, *Z. mexicana* and *Sorghum bicolor* pollen. Theor Appl Genet 53:43–46

Douglas GC, Connolly V (1989) Self-fertilization and seed set in *Trifolium repens* L. by in situ and in vitro pollination. Theor Appl Genet 77:71–75

Dulieu HL (1966) Pollination of excised ovaries and culture of ovules of *Nicotiana tabacum* L. Phytomorphology 16:69–75

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soyabean root cells. Exp Cell Res 50:151–158

Gawel NJ, Robacker CD (1986) Effect of pollen-style interaction on the pollen growth of *Gossypium hirsutum*. Theor Appl Genet 72:84–87

Heslop-Harrison J, Heslop-Harrison Y (1982) Pollen-stigma interaction in the *Leguminosae*: Constitutents of the stylar fluid and stigma secretion of *Trifolium pratense* L. Ann Bot 49:729–735

Hess D, Wagner G (1974) Induction of haploid parthenogensis in *Mimulus luteus* by in vitro pollination with foreign pollen. Z Pflanzenphysiol 72:466–468

Kameya T, Hinata K, Mizushima U (1966) Fertilization in vitro of excised ovules treated with calcium chloride in *Brassica oleraceae* L. Proc Jpn Acad 42:165–167

Kandasamy MK, Kristen U (1987) Pollen tube growth in the style of *Nicotiana sylvestris* is neither influenced by the ovary nor directed by a gradient. J Plant Physiol 131:495–500

Kanta K, Rangaswamy NS, Maheshwari P (1962) Test-tube fertilization in a flowering plant. Nature 194:1214–1217

Kazimierska EM (1978) Embryological studies of cross compatibility in the genus *Trifolium* L. II. Fertilization, development of embryo and endosperm in crossing *T. repens* L. with *T. medium* L. Genet Pol 19:15–22

Leduc N, Douglas GC, Monnier M, Connolly V (1990) Pollination in vitro; effects on the growth of pollen tubes, seed set and gametophytic self-incompatibility in *Trifolium pratense* L. and *T. repens* L. Theor Appl Genet 80:657–664

Mascarenhas JP (1975) The biochemistry of angiosperm pollen development. Bot Rev 41:259–314

Mogensen LH, Suthar HK (1979) Ultrastructure of the egg apparatus of *Nicotiana tabacum Solanaceae* before and after fertilization. Bot Gaz 140:168–179

Mulcahy BG, Mulcahy DL (1985) Ovarian influence on pollen tube growth as indicated by the semivivo technique. Am J Bot 72:1078–1080

Mulcahy DL, Mulcahy GR (1975) The influence of gametophytic competition on sporophytic quality in *Dianthus chinensis*. Theor Appl Genet 46:277–280

Niimi Y (1970) In vitro fertilization in the self incompatible plant *Petunia hybrida*. J Jpn Soc Hortic Sci 39

Olson AR, Cass DD (1981) Changes in megagametophyte structure in *Papaver nudicaule* L. (*Papaveraceae*) following in vitro placental pollination. Am J Bot 68:1333–1341

Rangaswamy NS (1977) Applications of in vitro pollination and in vitro fertilization. In: Reinert J, Bajaj YPS (eds) Applied and fundamental aspects of plant cell, tissue and organ culture. Springer, Berlin Heidelberg New York, pp 412–425

Rangaswamy NS, Shivanna KR (1967) Induction of gamete compatability and seed formation in axenic cultures of a diploid self-incompatible species of Petunia. Nature 216:937–939

Rao PS (1965) The in vitro fertilization and seed formation in *Nicotiana rustica* L. Phyton (Argenta) 22:165–167

Richards KW, Rupert EA (1980) In vitro fertilization and seed development in *Trifolium*. In Vitro 16:925–931

Shivanna KR (1965) In vitro fertilization and seed formation in *Petunia violacea* Lindl. Phytomorphology 15:183–185

Shivanna M, Shivanna KR (1985) The role of the pistil in screening compatible pollen. Theor Appl Genet 70:684–686

Slusarkiewicz-Jarzina A, Zenkteler M (1983) Development of hybrid plants from ovules of *Nicotiana tabacum* pollinated in vitro with pollen grains of *Nicotiana knightiana*. Experientia 39:1399–1400

Srivastava PS, Varga A, Bruinsma J (1980) Growth in vitro of fertilized ovules of pea, *Pisum sativum* L., with and without pods. Z Pflanzenphysiol 98:347–354

Stewart J McD (1981) In vitro fertilization and embryo rescue. Environ Exp Bot 21:301–315

Tilton VR, Russell SH (1983) In vitro pollination and fertilization of soyabean *Glycine max* (L.) Herr. (*Leguminosae*). In: Mulcahy DL, Ottaviano E (eds) Pollen: biology and implications for plant breeding. Elsevier Sci Publ, Amsterdam, pp 281–286

Tilton VR, Wilcox LW, Palmer RG, Albertsen MC (1984) Stigma, style and obdurator of soyabean, *Glycine max* (L.) Merr. (Leguminosae) and their function in the reproductive process. Am J Bot 71:676–686

Walker AK, Cianzio SR, Bravo JA, Fehr WR (1979) Comparison of emasculation and non-emasculation for hybridization of soyabeans. Crop Sci 19:285–286

Zenkteler M (1967) Test-tube fertilization of ovules of *Melandrium album* Mill. with pollen grains of several species of the *Caryophyllaceae* family. Experientia 23:775–777

Zenkteler M (1980) Intraovarian and in vitro pollination. Int Rev Cytol [Suppl] 11B:137–155

Zenkteler M (1990) In vitro fertilization and wide hybridization in higher plants. Crit Rev Plant Sci 9:267–279

Zenkteler M, Melchers G (1978) In vitro hybridization by sexual methods and by fusion of somatic protoplasts. Theor Appl Genet 52:81–90

Zenkteler M, Maheswaran G, Williams EG (1987) In vitro placental pollination in *Brassica campestris* and *B. napus*. J Plant Physiol 128:245–250

Zubkova M, Sladky Z (1975) The possibility of obtaining seeds following placental pollination in vitro. Biol Plant 17:276–280