EMBRYOLOGICAL STUDIES OF RECIPROCAL CROSSES BETWEEN *VICIA FABA* AND *VICIA NARBONENSIS*

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ABSTRACT

A study was undertaken to assess the reciprocal crossability between *Vicia faba* and *Vicia narbonensis*. Flower buds or only ovaries of several varieties and genotypes were cross-pollinated in vivo (green house and field) and in vitro. Only few pollen tubes passed the style and entered into the ovary. On the whole number of 5320 cross pollinated in vivo and in vitro flowers and ovaries of *Vicia narbonensis* only 78 globular hybrid embryos were observed. After cross pollination in vivo of 3860 flower buds and ovaries of *Vicia faba* globular embryos developed in 124 ovules. The highest number of globular embryos were obtained when the *Vicia faba* line 1/33 was pollinated with *Vicia narbonensis* lines P₃, P₅, 150, SE.

Embryogenesis proceeded till the 6-10 day after pollination, however, karyological disturbances in the cells of embryos and endosperm were often noticed at earlier stages.

In vitro pollen grains of *Vicia faba* germinated on stigmas and ovaries of *Vicia narbonensis*, a significant increase in the growth of pollen tubes was noticed after ovary pollination. The technique of in vitro pollination was not suitable for *Vicia faba* as the inoculated explants died shortly after transferring onto the medium. The results indicate that finding a more suitable genotype for crossing may give a chance to obtain higher number of embryos (example line 1/33) – thus sufficient number for culturing them on media.

KEY WORDS: *Vicia faba*, *Vicia narbonensis*, interspecific in vivo and in vitro pollination, embryo abortion, endosperm disintegration.

INTRODUCTION

Vicia faba is an important annual leguminous crop widely grown in European countries however, its yield is often unstable due to disease susceptibility and higher abortion rates. According to Cubero (1982) *Vicia narbonensis* is a wild relative of *Vicia faba* and is disease and drought resistant and well adapted to soils of low fertility. Many attempts to obtain interspecific hybrids by crossing *Vicia faba* with *Vicia narbonensis* have been unsuccessful. Cubero (1982) is mentioning that already in 1974 Van Cruchten as well as Jamanoto and Rousselle undertook efforts to cross those species but failed to obtain hybrids. During the last 10 years much work has been devoted to the same problem by Roupakias (1986), Roupakias and Tai (1986), Lazaridou et al. (1989), Lazaridou and Roupakias (1993), Lazaridou et al. (1993) and Lazaridou and Roupakias (1995). The main results of those investigations can be summarized as follows:

1) when selfing pods and ovules develop faster in *Vicia narbonensis* than in *Vicia faba* and also the initial development of endosperm and embryo is faster in *Vicia narbonensis* than in *Vicia faba*;

- 2) double fertilization occurs in both reciprocal crosses and the percentage of pod set in *Vicia faba* x *Vicia narbonensis* crosses ranges between 9-59% while in reciprocals from 12% up to 30%;
- 3) in the cross *Vicia narbonensis* x *Vicia faba* the ovules stop growing 9 days after pollination (embryos abort after 5 days), while in the reciprocal cross they stop growing 15 days after pollination (embryos abort after 9 days);
- 4) the percentage of successful fertilization is associated with the parental genotypes;
- 5) *Vicia narbonensis* selfed embryos younger than 5 days and *Vicia faba* selfed embryos younger than 9 days after pollination are too small for excision. However, in-ovulo culture enables to induce the growth of embryos when ovules of *Vicia narbonensis* are inoculated 6-8 days and *Vicia faba* 14 days after pollination.

So far, hybrids between *Vicia faba* and *Vicia narbonensis* have not been obtained by using conventional techniques and, therefore it is presumed that hybrids will not develop unless an embryo rescue technique becomes available. The aim of the study presented here was to determine the best technique

Fig. 1. Germinating in vivo pollen grains of *Vicia faba* (Albatros) on the stigma of *Vicia narbonensis* (SE). Bar x 200 mm

Fig. 2. Germinating in vivo pollen grains of *Vicia faba* (Boss) on the stigma of *Vicia narbonensis* (P₃), stigma moistured with 40% of sucrose solution. Bar x 200 mm

Fig. 3. Pollen tubes of *Vicia faba* (1/33) inside the style of *Vicia narbonensis* (SE); 24 hours after in vivo stigma pollination, stigma moistured with 30 % of sucrose solution. Bar x 200 mm

Fig. 4. Germinating in vitro pollen grains of *Vicia faba* (1/33) on stigma of *Vicia narbonensis* (SE). Bar 200 mm

Fig. 5. Enlarged ovaries of *Vicia narbonensis* (150) 4 days after in vitro pollination with pollen grains of *Vicia faba* (1/33), 2x.

Fig. 6. Pollen tubes of *Vicia faba* (1/33) inside the ovary of *Vicia narbonensis* (SE); 24 hours after in vitro direct ovary pollination. Bar x 200 mm

and environmental conditions to overcome pre- and post-zygotic barriers of incompatibility in reciprocal crosses between *Vicia narbonensis* and *Vicia faba*.

MATERIAL AND METHODS

The following lines and varieties of *Vicia narbonensis* and *Vicia faba* were used in the experiments:

- 1) *Vicia narbonensis*: P₃, P₅ (protoplast derived lines from a line kindly provided by Hoechst Company, Frankfurt; Tegeder et al., 1996), SE (somatic embryo derived line from the line of the same origin; Pickardt et al., 1989), 148, 150 and 151 (ICARDA, Aleppo).
- 2) *Vicia faba*: The varieties Albatros, Carola, Mythos, Boss, Troy, Piccolo (Norddeutsche Pflanzenzucht, Hohenlieth), Kristall (Lechow Petkus, Bergen), Optica (Zwaan, The Netherlands), Geo (Pflanzenzucht Oberlimpurg, Schwäbisch Hall) and the lines 1/33 and 14/338 (Genbank of the Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben).

Plants were grown in a green house in the Institut für Angewandte Genetik, Freie Universität Berlin and in the field belonging to the Botanical Garden, Poznań, Poland. The plants grown in green house were watered by an automatic sand-bed watering system. All the experiments were carried out during Spring-Autumn 1995 and 1996. Flower buds were emasculated one or two days before anthesis and stigmas or ovaries (stigma cut off) were pollinated on the same or on the next day with pollen grains from the newly opened flowers of *Vicia faba* and from the still closed flower buds of *Vicia narbonensis*. Pollination was performed by using only one donor line or variety, a mixture of pollen from various genotypes was never used. Emasculated and pollinated flowers of plants growing in the field were covered with isolators. As pollen grains usually germinated poorly, in some combinations of crosses a drop of a sucrose solution at concentrations of 30 or 40% was put onto the stigma or on the upper part of the ovaries (when the stigmas were cut off) just before pollination. 4900 flowers and ovaries of *Vicia narbonensis* and 3860 flowers and ovaries of *Vicia faba* were in vivo cross pollinated.

Irrespectively of the experiments carried out in vivo additional investigations were performed on pistils and ovaries cultured in vitro. Closed flower buds just before anthesis were used as the experimental material. Two following procedures were applied: 1) anthers were cut off from the buds and immediately the stigmas were covered with pollen grains from the donor plant. The whole bud with the calyx lobes, corolla and part of the pedicle were transferred onto the medium;

2) the style was cut off and pollen grains were put on the upper part of the ovary. Ovary with the calyx and a part of the corolla and pedicle were transferred onto the medium. In some cases stigmas and the opened ovaries were covered with a drop of sucrose solution. The pollinated buds and ovaries were cultured on Gamborg (1968) or MS (1962) medium. All together 240 pistils and 180 ovaries of *Vicia narbonensis* and 130 pistils and 110 ovaries of *Vicia faba* were in vitro cross pollinated (Table 1).

Young pods were collected at intervals from 1-20 days. Whole ovaries or only the enlarged ovules were fixed in Carnoy's solution for 24 or 48 hours and stored in 70% alcohol. Then they were processed by the alcohol-xylol paraplast embedding method and sectioned 10-12 mm in thickness with a rotary microtome. Slides were stained with Heidenhein's hematoxyline and fast green or cristal violet and orange G. For culturing ovules with globular embryos several media were used including the modified Murashige and Skoog medium of Mok et al. (1978), modified B₅ used by Newell and Hymowitz (1982) and Gamborg (1968) medium. The medium described by Mok et al. was enriched by supplementing thidiazuron at the concentrations of 0.5 or 1.0 mg/l.

RESULTS

In vivo interspecific pollination of *Vicia narbonensis* with *Vicia faba*

Irrespectively of the donor plants pollen grains germinated with various intensities and usually short pollen tubes were noticed on the stigmas (Fig. 1). The abnormalities in pollen tube growth included non-directional growth in the style and an arrest of growth in the style. For all crosses, the number of pollen tubes in the style was found to be much lower than the number of germinated pollen grains observed on the stigmas. During the hot summer days conditions were not favourable for pollen germination however they germinated abundantly when stigmas were moistened with a drop of sucrose solution at concentrations of 30 or 40% (Fig. 2). Pollen tubes did not follow any particular direction, only few of them could have been discerned alongside the style (Fig. 3). When styles were cut off pollen germinated on the upper part of the ovaries but only few of them did run into the direction of ovules. Histological sections of ovules showed that embryo sacs at the time of pollination was mature. The egg apparatus was situated at the micropylar region of the ovule and the central cell occupied the wider part near the chalaza but antipods were yet not present. The process of fertilization was never found, only the remnants of pollen tubes were observed in the micropylar region of some embryo sacs (Fig. 7). In the dividing cells of the globular embryos the number of chromosomes was $2n=13$.

TABLE 1. In vivo reciprocal pollination of *Vicia narbonensis* and *Vicia faba*

Number of in vivo and in vitro pollinated flowers of <i>Vicia narbonensis</i>	Number of in vivo and in vitro pollinated flowers of <i>Vicia faba</i>	Number of analysed ovules of <i>Vicia narbonensis</i> (1)	Number of analysed ovules of <i>Vicia faba</i> (1)	Number of globular embryos in cross <i>Vicia narbonensis</i> x <i>Vicia faba</i>	Number of globular embryos in cross <i>Vicia faba</i> x <i>Vicia narbonensis</i>
5320	4100	2160 ^x 1800 ^x 360 ⁺	250 160 ^x 90 ⁺	78	124

(1) — analysis was performed only on the enlarged ovules

^x — under the binocular by using needles

⁺ — embryological permanent slides

Fig. 7. Remnants of pollen tube (arrow) of *Vicia faba* (Albatros) inside the micropyle region of embryo sac of *Vicia narbonensis* (P₃); 48 hours after in vivo pollination. Bar x 100 mm

Fig. 8. Globular embryo of *Vicia narbonensis* (P₃) x *Vicia faba* (Boss), endosperm absent; 9 days after in vivo pollination. Bar x 50 mm

Fig. 9. Globular embryo of *Vicia narbonensis* (SE) x *Vicia faba* (1/33), little endosperm inside the embryo sac; 10 days after in vivo pollination. Bar x 50 mm

Fig. 10. Completely degenerated proembryo of *Vicia narbonensis* (SE) x *Vicia faba* (1/33); 14 days after in vivo pollination. Bar x 50 mm

Fig. 11. Masses of degenerating endosperm nuclei in embryo sac of *Vicia faba* (1/33) x *Vicia narbonensis* (SE); 16 days after in vivo pollination. Bar x 100 mm

Fig. 12. Globular embryo of *Vicia faba* (Albatros) x *Vicia narbonensis* (SE) inside an embryo sac, endosperm absent; 8 days after in vivo pollination. Bar x 20 mm

Fig. 13. Globular embryo of *Vicia faba* (1/33) x *Vicia narbonensis* (SE), some nuclei are still dividing; 10 days after in vivo pollination. Bar x 20 mm

Fig. 14. Heart stage embryo of *Vicia faba* (1/33) x *Vicia narbonensis* (SE); 18 days after in vivo pollination. Bar x 100 mm

Those observations were based on 360 ovaries sectioned longitudinally and stained with hematoxyline or cristal violet 2-12 days following pollination.

On the whole number of 2160 enlarged ovules analysed between 2-20 days after pollination globular embryos at various developmental stages were found only in 78 ovules (Table 1).

In some ovules the globular embryos developed together with a poor endosperm (Fig. 9), but in others endosperm was not present (Fig. 8). Whereas in *Vicia narbonensis* selfed endosperm nuclei divided normally and endosperm developed throughout into the ovule cavity and around the embryo. After cross pollination endosperm often formed scattered masses of densely stained nuclei, only sometimes those masses were in close proximity to the hybrid embryo. In all populations studied, the globular embryo with suspensor was located in the centre of the embryo sac. The elongated highly vacuolated suspensor cells were packed with nuclei of various size and shape and with various number of nucleoli.

Most globular hybrid embryos failed to persist beyond the 10-th day (Fig. 10). Those embryos did not develop normally when compared with the selfed ones. Five to six days after pollination globular embryos, in some cases surrounded by few degenerating endosperm nuclei, were detected in the embryo sac cavity. During the next 4 days the embryos were still globular with strongly vacuolated cells. The enlargement of pods as a consequence of cross pollination was often not an indication of the presence of an embryo. After 3-4 weeks after pollination a high percentage of pods, particularly in lines 150 and 151 of *Vicia narbonensis* were green and had increased in size, although they did not contain seeds. Many ovules considerably increased in length and width but did not contain embryos. Histological analysis of those ovules revealed the presence of only well developed nucellus tissue. In some younger stages it was possible to discern a narrow curved cavity being the only remnant of the earlier degenerated female gametophyte. On the basis of pod's development the results of interspecific pollination *in vivo* were divided into the following groups:

1. pistils did not grow and flowers dropped off the plants several days after pollination;
2. pistils grew significantly and reached the length of a control plant. Enlarged ovules contained nucellus tissue only;
3. pistils and pods enlarged till the 8-10th day after pollination. Pods were thin and contained ovules at various size. Inside some enlarged ovules globular embryos were present.

In vivo interspecific pollination of Vicia faba with Vicia narbonensis

The seed yield in the populations of *Vicia faba* after selfing was considerably low and it differed in accordance to the climatic conditions. We presume that pollination was one major barrier to fertilization of the ovules, since no obstacles were evident in the styles to prevent penetration. Often after selfing pollen did not germinate on the stigmas or on the ovaries when the styles were cut off. The other barrier to fertilization might have been attributed to the absence of embryo sacs in ovules as histological analysis of ovaries revealed that some ovules were filled up only with the nucellar cells. Neither the position of the ovule within the ovary nor the position of the raceme on the stem had any effect on the development of seeds. It is worth to notice that irrespectively of the variety an abundant number of flowers dropped off the plants not only after cross pollination but also after selfing.

The procedure of pollination of flowers of *Vicia faba* with pollen grains of *Vicia narbonensis* was similar to that which was applied in reciprocal crosses. However, in the case of *Vicia faba* more efforts were devoted on pollinating stigmas, yet much less ovaries, as we found that shortly after ovarian pollination they shriveled and dried soon. The addition of sucrose was not much helpful.

Several hours after cross pollinating stigmas of *Vicia faba* pollen tubes were growing in various directions, they were short and only some longer ones were observed alongside the styles. The intensity of pollen germination was not dependant of the donor population. The presence of sucrose solution on the stigmas enhanced pollen germination but without an evident further effect on their growth alongside the style. During the investigations carried out in 1995 we found that much more globular embryos have developed in the line 1/33 of *Vicia faba* after crossing with one of the lines SE, 148 and P₃. Due to this in 1996 this line constituted our main experimental object. On the whole number of 1500 cross pollinated flowers of line 1/33 68 globular embryos at different developmental stages were found in the enlarged ovules (Fig. 13). In some ovules globular embryos aborted very shortly after pollination, and in some others only masses of degenerating endosperm were observed (Fig. 11). It is worth to notice that in 4 ovules embryos developed till the heart stage (Fig. 14). After cross pollinating 2600 flowers of the remaining varieties hybrid globular embryos were observed only in 56 of the enlarged ovules (Fig. 12).

In vitro cross pollination and in-ovule culture

The aim of *in vitro* technique has been mainly devised to overcome the pre- and post-zygotic barriers for seed set. Pollen grains of *Vicia faba* germinated abundantly on stigmas and ovaries of *Vicia narbonensis* (Figs 4-6). In the presence of sucrose solution much more pollen germinated on the ovaries and many pollen tubes were entering the ovary. On the whole number of 240 flower buds and 180 ovaries pollinated *in vitro* globular embryos were found in 6 ovules after ovarian pollination and in 2 ovules after stigmatic pollination. Cross pollination *in vitro* of buds and ovaries of *Vicia faba* were unsuccessful mainly due to the phenomenon that the explants were very sensitive to the applied sterilization agents, particularly to the ethanol. After ethanol treatment the whole explants became dark shortly after transferring onto the media. Dilluted chlorine water was applied only for a short time as it also "killed" the material. When the explants were disinfected too short than an abundant infection appeared already on the third day after inoculation.

To prevent the degeneration of globular hybrid embryos obtained *in vivo* 42 enlarged ovules, mainly those ones of line 1/33 were excised 6 to 10 days after pollination for embryo rescue. On the second week following the transfer embryos together with the surrounding nucellus started to degenerate and during the next few days they shriveled and turned brown. No indication of embryo development was found in ovules cultured on the applied media.

DISCUSSION

Our studies on interspecific cross pollination between *Vicia faba* and *Vicia narbonensis* did not allow to determine the reason of the low development of globular embryos. Pollen tubes did not penetrate frequently the ovules and only few of

the pollen tubes grew straight to the ovary in to the micropyles. In spite of microscopical analysis of an abundant number of ovules it was not possible to find an exact moment of the process of double fertilization and the division of the hybrid zygote. A diploid number of chromosomes as well as karyological disturbances present in the cells of globular embryos and in the endosperm nuclei may be of an indirect proof that double fertilization took place. It is surprising that in the recent literature concerning interspecific hybridization between *Vicia faba* and *Vicia narbonensis* there is no convincing information, based on the cytoembryological preparations, that double fertilization did occur (Roupakias and Tai, 1986; Roupakias, 1986; Lazaridou and Roupakias, 1993). Fertilization depends on the regular development and function on the male and female gametophytes. Pollen grains in both species of *Vicia* were normally developed and in high percentage fertile. Our observations on female gametophytes in *Vicia narbonensis* have shown that in most of the analysing ovules embryo sacs were fully developed – thus there was no female sterility. However, some ovules of *Vicia faba* were sterile, as embryo sacs were not discerned and only the nucellus constituted the main tissue. Irrespectively of the combinations of crosses pollen grains started to germinate on stigmas and ovaries, in some cases even abundantly, particularly in the presence of the sucrose solution. An incompatibility reaction came to force shortly after pollen germination as probably the stylar tissues did not provide suitable environment for pollen tubes growth and only sporadically pollen tubes grew through the style into the direction of the ovary. Interaction between the male and female gametophytes can depend on the suitability of genomes of both partners and receptioness of ovules to fertilization. It is probable that both factors are not active during interspecific crosses in both species of *Vicia*. Large genetical differences between those two species contribute to the failure in hybrid development. Prezygotic barriers of incompatibility are mainly responsible for the low number of pollen tubes penetrating into the ovules. However, much more difficult to overcome are the post-zygotic barriers of incompatibility. Post-zygotic barriers, involving an unbalanced genome constitution in hybrid embryo and endosperm are the main causes of failure of hybrid seed development. Collapsing of young globular embryos, abnormalities in endosperm development or even the lack of endosperm, were the main obstacles in culturing in vitro embryos. Lazaridou et al. (1993) has shown that in vitro technique can be successfully applied for *Vicia narbonensis* selfed embryos 6-8 days after pollination and for *Vicia faba* 10-14 days after pollination. Therefore, the lack of a suitable cell cycle time of the hybrid endosperm and embryo may also bring about the karyological disturbance in globular embryos shortly after fertilization. Thus our efforts in culturing globular hybrid embryos have been unsuccessful.

It is concluded from the present investigations that a more detailed study is needed for finding new genotypes for inter-

specific crossing as the promising results obtained with genotype 1/33 as a mother plant enhances to concentrate efforts in this direction.

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BADANIA EMBRIOLOGICZNE
NAD OBUSTRONNYM KRZYŻOWANIEM *VICIA FABA* I *VICIA NARBONENSIS*

STRESZCZENIE

W celu uzyskania roślin mieszańcowych przeprowadzono obustronne krzyżówki między różnymi genotypami i liniami *Vicia faba* i *Vicia narbonensis*. Zarówno w warunkach in vivo jak i in vitro stwierdzono obfite kiełkowanie ziarn pyłku na znamionach i zalążkach, jednak wrastanie łagiewek, poprzez szyjkę słupka w kierunku zalążni, następowało tylko sporadycznie. W wyniku oddalonego zapylania in vivo i in vitro 5320 kwiatów i zalążni *Vicia narbonensis* otrzymano 78 zarodków w stadium kulistym. Natomiast w rezultacie zapylania in vivo 3860 kwiatów i zalążni *Vicia faba* zarodki kuliste obserwowano w 124 zalążkach. Najwięcej zarodków w stadium kulistym (68) i w stadium serca (4) otrzymano po zapyleniu linii 1/33 *Vicia faba* pyłkiem linii P₃, P₅, 150 i SE *Vicia narbonensis*. Proces embriogenezy przebiegał z zakłóceniami i zarodki zamierały po ok. 10 dniach od zapylenia. Podziały jąder bielma przebiegały również z zakłóceniami, a w woreczkach zalążkowych szczególnie często obserwowano silnie zagęszczoną cytoplazmę wraz z resztkami jąder i jąderek. W niektórych woreczkach zalążkowych pomimo obecności kulistych prazarodków bielmo się w ogóle nie rozwijało. Kultury całych zalążków zawierających zarodki zamierały wkrótce po przeniesieniu zalążków na pożywkę. Ponieważ w wyniku oddalonego zapylania linii 1/33 *Vicia faba* rozwijało się stosunkowo wiele zarodków kulistych, wydaje się być celowym dalsze kontynuowanie badań nad tą linią oraz na materiale poszerzonym o nowe genotypy *Vicia faba* i *Vicia narbonensis*.

SŁOWA KLUCZOWE: *Vicia faba*, *Vicia narbonensis*, międzygatunkowe zapylanie in vivo i in vitro, degeneracja zarodka, rozpad bielma.