Andi Wijaya

Towards Interspecific Hybridization in *Vicia faba* L.

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Towards Interspecific Hybridization in *Vicia faba* L.

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by

Andi Wijaya

born in Pangkal Pinang, South Sumatra,

Indonesia

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1 Introduction

Production and distribution of faba bean

Faba bean (*Vicia faba* L.), also referred to as broad bean, horse bean or field bean, has been cultivated in 2002 on nearly 2.4 x 10^6 ha world wide (FAO 2002). In developing countries faba bean is used mainly for human consumption. Feeding value of faba bean is high, and is considered in some areas to be superior to that of field peas or other legumes. *V. faba* is known to have been cultivated from the early Neolithic, from India to the Western Mediterranean countries (Cubero 1973, Cubero 1974). It is generally accepted that the geographic origin of *V. faba* was the Near East (Duc 1997). Ladizinsky (1975) argued that assuming the Middle East as place of origin of the broad bean is inconsistent with archaeological evidence. He suggested Central Asia to be the center of origin of *V. faba*.

The *V. faba* species is divided into two sub-species: ssp. *paucijuga* and ssp. *eu-faba*. The latter subspecies comprises three botanical types differing in seed size. Small seeded types with 1000-seed weight less than about 500 g (*V. faba minor*) are found in the Ethiopian area and have been favored by North European agriculture. Medium seeded types (*V. faba equina*) have developed throughout Middle East and North Africa with large seeded types with 1000-seed more than 1000 g (*V. faba major*) concentration in Egypt (Duc 1997).

With a world production of 3.7 million tons according to the 2002 FAO year book, *V. faba* ranks among the most important grain legumes. China contributes 41% of the faba bean world wide production, whereas Europe contributes 15%. However, the total harvested area of faba bean decreased in China and Europe over the past 20 years (Figure 1). Yield instability (Figure 2) and low prices are the main reasons for the decreasing harvest area.

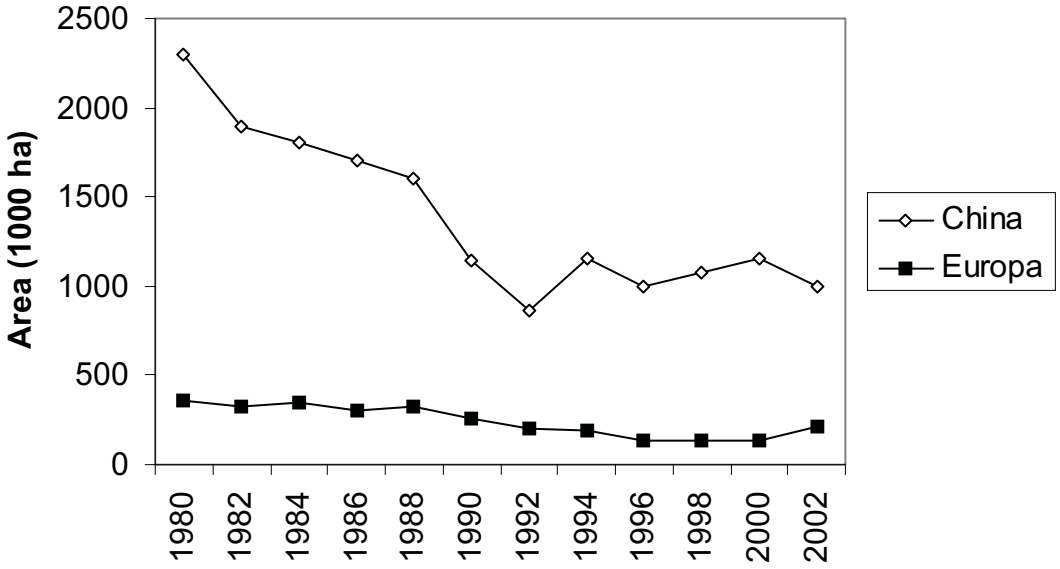

Figure 1. Total harvest area of faba bean in the period 1980-2002 (FAO 2002).

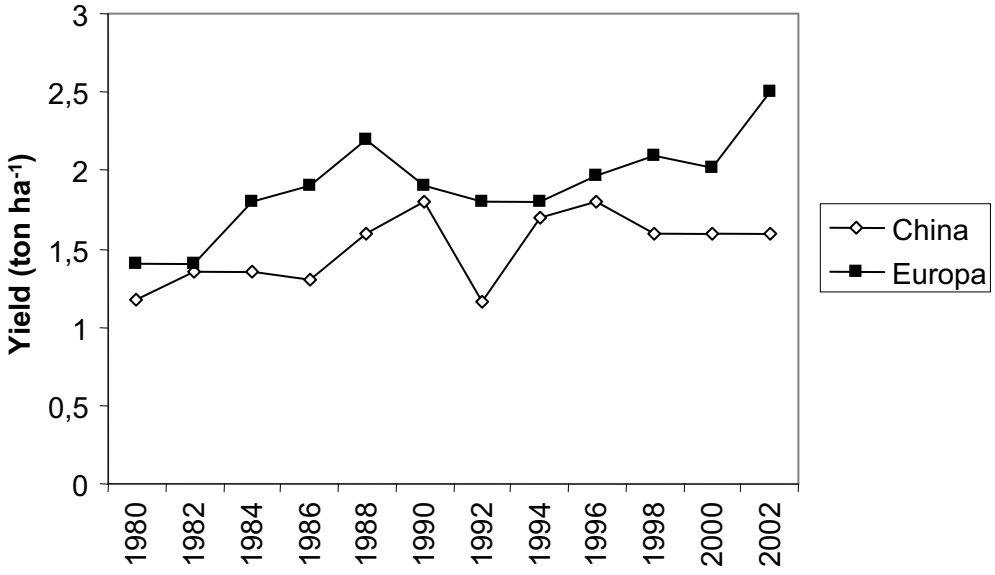

Figure 2. Yield of faba bean in the period 1980 – 2002 (FAO 2002).

In Europe, since 2001, interest in faba bean has been reinforced due to the organic farming demand, interest in poultry feed and demand for food safety and

as replacement for pea in soil infected by *Aphanomyces* (Duc and Marget 2002). Bond et al. (1985) suggested that its low fertilizer and pesticide requirement makes this crop environmentally acceptable in sustainable agriculture.

Breeding

The creation of the International Center for Agriculture in Dry Areas (ICARDA) in 1977 was also a strong support to faba bean breeding research. ICARDA has collected and maintains more than 9000 accessions. It is the largest world collection and is used by many breeding programs. Other collections are maintained at the Institute of Genetics and Crop Plants Research at Gatersleben in Germany, Consiglio Nazionale delle Richerche (CNR)-Bari in Italy, Escuela Técnica Superior de Ingenieros Agronomos de Montes (ETSIA)-Cordoba in Spain, Institut National de la Recherche Agronomique-France (INRA)-Dijon and Rennes in France (Ward and Chapman 1986, Duc 1997).

Nowadays, the most important breeding goals in faba bean are breeding for disease resistance, improved yield, improved seed protein quality, improved drought and frost tolerance (winter beans). Difficulties in pollination control, the limited gene pool and the fact that faba beans have for a long time been a crop with minor breeding input has led to a slow progress in varietal improvement (Bond 1987). Today, breeding programmes of *V. faba* could be supplemented with recombinant DNA technology with the purpose of introducing genes conferring fungal resistance or the improvement of nutritional quality of seed proteins. Böttinger et al. (2001) reported the successful production of stable transformed lines of faba bean by using an *Agrobacterium tumefaciens*-mediated gene transfer

system. However, they argued that this method is extremly time consuming and of relatively low efficiency.

Interspecific hybridization in *Vicia faba*

Bond et al. (1994) argued that the sensitivity to biotic and abiotic stress causes the yield instability of *V. faba.* In view of insufficient genetic variability for these traits, chances of conventional breeding approaches to improve this crop for these traits are limited. Treopoulos et al. (2003) warned that there is a continuous genetic erosion due to the fact that bean breeding is based only on populations within the species. Some other *Vicia* species (vetches) are expressing winter hardiness, disease and drought tolerance to a higher degree than *V. faba* (Bond et al. 1994 and Cubero 1982). Therefore, interspecific hybridization with species closely related to *V. faba* could be applied to widen the existing genetic variability in this crop. However, many attempts to obtain interspecific hybrids between faba bean and other related species by the sexual and somatic system have been unsuccessful (Link et al.1995, Tegeder 1996, Zenkteler et al. 1998).

Appropriate selection of the vetch species, among genotypes within species and between species, to be used in interspecific hybridization can be crucial for the success. The phylogenetic relationship among the vetch species and *V. faba* are one criterion to select a vetch as hybrid partner for faba bean. Even more decisive than the phylogenetic relationship, interspecific pollen tube growth and ovule and pod development are regarded as important parameters for selecting hybrid partners. The phylogenetic relationship in some *Vicia* species is shown in Figure 1.

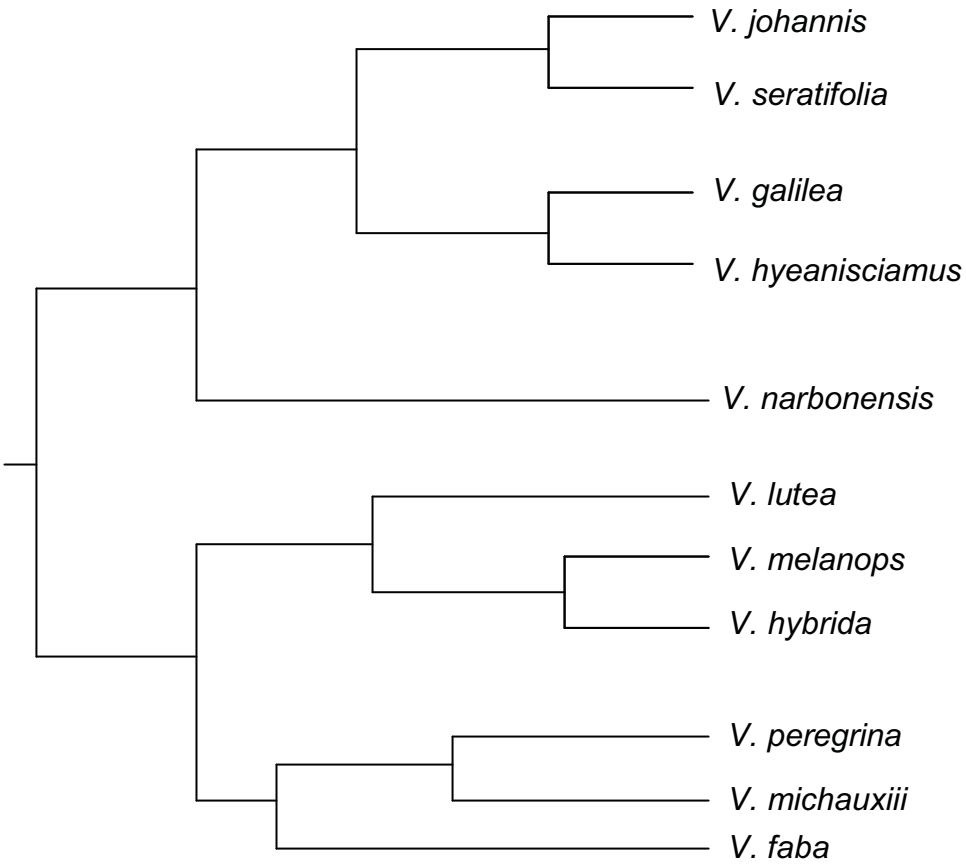

Figure 1. Phylogenetic relationship of *Vicia* species based on RFLP and PCR data, the figure is modified from Van de Ven et al. (1993).

Some researchers concluded that postzygotic barriers prevent development of embryos which were derived from interspecific crosses between *V. faba* and its related species. Indeed, they observed that the ovules were fertilized in at least some of the interspecific combinations among *V. faba* and its related species (Ramsay et al. 1984, Ramsay and Pickersgill 1986, Roupakias 1986 and Zenkteler et al. 1998). Van Tuyl and De Jeu (1997) suggested that application of phytohormones to flowers in combination with embryo rescue technique following wide crosses can overcome postzygotic barriers in a range of crops. However, at

present no information about the applicability of this system to *Vicia* species has been reported.

2 Objectives

The objectives of this study were:

1) to optimize the *in vitro* culture conditions using rescued embryos from different faba bean genotypes as a prerequisite for successful interspecific sexual hybridization in *V. faba*,

2) to study the effects of phytohormone treatment of flowers with the aim to postpone premature pod abscission following interspecific pollination in *V. faba*, and

3) to perform a large scale interspecific pollination experiment using previously identified highly responding *V. faba* genotypes and related *Vicia* species with the aim to produce interspecific *Vicia* hybrids.

3 Plant material

The study was based on nine genotypes of *V. faba* and 11 related *Vicia* species obtained from different gene banks and institutes (Table 1).

Table 1. *V. faba* genotypes and related species used in this study.

Species, genotypes	Abbreviation	Gene bank code/ Seed donor	Seed source
***V. faba* genotypes**			
V. faba minor Mythos	Myth	Collection Göttingen	BPC 2000
V. faba minor Hedin	Hedi	Collection Göttingen	BPC 2000
V. faba equina F2(Hedin x Pietranera)	F2	Collection Göttingen	BPC 2000
V. faba paucijuga vf 78	P78	CNR	GH 98/99
V. faba paucijuga vf 163	P163	CNR	GH 98/99
V. faba paucijuga vf 172	P172	CNR	GH 98/99
V. faba major Hangdown	Hang	Collection Göttingen	BPC 2000
V. faba major Peru	Peru	IPK	IPK 2000
V. faba major Pietranera	Piet	Collection Göttingen	BPC 2000
Vetch species			
Vicia bithynica	*V. bith*	VIR-VB-34427	GH 98/99
Vicia galeata	*V. gale*	USDA-VG-PI.602380	USDA 98
Vicia galilea	*V. gali*	IPK-VG-NAR44/80	GH 98/99
Vicia hybrida	*V. hybr*	VIC 309/96	IPK 98
Vicia johannis	*V. joha*	USDA-VH-W6.17061	GH 98/99
Vicia lutea	*V. lute*	VIR-VL-34863	GH 98/99
Vicia melanops	*V. mela*	IPK-VM-VIC474/95	GH 98/99
Vicia narbonensis	*V. narb*	BAZ-VN-45614	GH 98/99
Vicia narbonensis	*V. narb*	VIR-VN-35391	GH 98/99
Vicia michauxii	*V. mich*	IPK_VM_VIC47/95	IPK 1999
Vicia peregrina	*V. pere*	IPK-VP-VIC747/78	IPK 98
Vicia serratifolia	*V. serr*	IPK-VS-NAR142/83	IPK 98

BAZ = Bundesanstalt für Züchtungsforschung Genbank, Braunschweig

CNR = Nazionale delle Richerche (CNR)-Bari

IPK = Institut für Pflanzengenetik und Kulturpflanzenforschung Genbank, Gatersleben

USDA = United States Department of Agriculture, Washington

VIR = Vavilov Research Institute of Plant Industry, Petersburg

GH = Green house, Institute of Agronomy and Plant Breeding, Göttingen

BPC = Bee-proof cages, Institute of Agronomy and Plant Breeding, Göttingen

4 Results and Discussion

Optimization of embryo rescue technique in *Vicia faba* (Manuscript I)

Hybrids between *V. faba* and its related species 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. However, until now studies on embryo rescue techniques in *V. faba* are rare. The available techniques allowed a maximum of 3.3 % germinated embryos derived from 11-days-old embryos obtained after self-fertilization (tripping) in *V. faba*. The plantlet regeneration from embryos rescued earlier than 11 days after pollination has in no case been successful (Lazaridou et al. 1993).

Within the present study the embryo rescue technique in *V. faba* has been optimized. Four different basal media (KM, B5, MS, NLN) were tested in liquid and in solid form with *V. faba* cv. Mythos. The results showed that in solid medium percentage of surviving embryos and percentage of plant regeneration were higher than in liquid medium. Selva et al. (1989) argued that considerable difficulties exist in *in vitro* culture of *V. faba*, because explanted tissue and callus cells tend to produce high levels of phenolic compounds, resulting in subsequent death of the tissue. Once these phenolics compounds are produced, they probably spread faster in liquid media than in solid medium, causing toxic effects to other ovules.

The use of the NLN-medium in the solid form resulted in the highest percentage of surviving embryos (52%) and subsequent plant regeneration (29%) following the *in vitro* culture of ovules dissected eight to 10 days after self-pollination in *V. faba* cv. Mythos. NLN-medium is low in nitrate and high in organic nitrogen. Both factors

are believed to play a significant role in embryo culture of faba bean. A similar phenomenon has been found by Pellegrineschi et al. (1997) in cowpea embryo rescue. They found that an increase of organic nitrogen in the culture medium had positive effects on the final percentage of plant regeneration.

The addition of gibberellic acid and indole-3-butyric acid (GA_3+IBA) to the medium had negative effects on the embryo development in nine different *V. faba* genotypes. If the auxin was substituted by a cytokinin (GA_3+BAP), the percentage of surviving ovules, embryos and plant formation increased. Kramer (2002) found that the number of surviving calli, their size and visual performance are better in media without auxin compared to cultures in media with auxins. It has been found that *V. faba* contains a relatively high concentration of endogenous auxin (Manabe et al. 1999). Hofinger and Böttger (1979) observed that immature *V. faba* seeds had a high amount of 4-chloroindolylacetic acid (4-Cl-IAA). The 4-Cl-IAA is a potent auxin, generally showing a higher activity than IAA. The nine different genotypes varied significantly in the percentage of plant formation. This indicates that a screening of a larger germplasm collection could lead to the identification of genotypes with an even higher *in vitro* culture response.

Effects of gibberellic acid and naphthyl acetic acid treatments of flowers on pod and ovule development in *Vicia faba* (Manuscript II)

Application of phytohormones, such as auxins, cytokinins and gibberellins to the pedicel or the ovary at the time of, or soon after pollination may improve pod and ovule development after interspecific pollination (Van Tuyl and De Jeu 1997). Application of phytohormones to delay pod abscissions shows positive effects on

the development of young pods derived from interspecific pollination of *Arachis hypogea* and *A. monticola* with *A. glabrata* (Sastri and Moss 1982) and from interspecific crosses between *Cicer arietinum* and *C. pinnatifidum* (Mallikarjuna 1999). The present study was performed to determine the influence of GA_3 and NAA treatments of flowers on subsequent young pod and ovule development. Application of phytohormones to flowers may help to overcome early pod abscission in *Vicia* interspecific hybridization experiments.

The GA_3 treatment of unfertilized flowers led to a young pod fresh weight, pod length and mean ovule size per pod similar to those of fertilized flowers as determined 14 days after pollination. The NAA treatment alone had a negative effect on the pod fresh weight, pod dry matter, pod length and mean ovule size per pod. The results showed that GA_3 has amore important role than NAA for pod and ovule development. One week after pollination, the percentage of produced pods as obtained following phytohormone treatments of unfertilized flowers was not significantly different from that of fertilized flowers. After two weeks the number of produced pods derived from unfertilized-untreated flowers significantly decreased due to the abscission of young pods compared to fertilized flowers and unfertilized-treated flowers. The application of GA_3 in combination with NAA gave better results compared to application of GA_3 alone, however, differences were mostly not significant. This study demonstrated that the GA_3 and GA_3+NAA treatments postpone pod abscission in unfertilized flowers. Perhaps, the same treatments could have a similar positive effect on pod and ovule development following interspecific *Vicia* pollinations and allow interspecific embryos to grow until their size and stage is adequate for successful application of the embryo rescue technique.

Interspecific hybridization in *Vicia faba* (Manuscript III)

Many attempts to obtain interspecific hybrids by crossing *V. faba* with its related species have been unsuccessful. Cubero (1982) mentioned that already Mettin (1962) undertook efforts to cross those species but failed to obtain hybrids. After that, much more work has been devoted to the same problem. However, all attempts have failed (Roupakias 1986, Ramsay and Pickersgill 1986, Roupakias and Tai 1986, Yamamoto 1986, Zenkteler et al. 1998). Their studies indicated that postzygotic barriers are the main cause for the lack of hybrid seed formation between faba bean and other *Vicia* species, as visible through early hybrid seed abortion.

Application of the optimized embryo rescue technique in combination with the phytohormone treatment of flowers after interspecific pollination of selected parents could enable the regeneration of interspecific hybrids between *V. faba* and its related species. By considering phenotypic similarity, genetic relationship and relative DNA content, six out of eleven vetch species were chosen for the further experiments. The selected vetch species were reciprocally pollinated with a set of nine highly diverse *V. faba* genotypes. Pollen tube growth observation and percentage of produced pods were recorded and used as criteria for the selection of three superior vetch species and three *V. faba* genotypes.

Based on the pollen tube observation and the percentage of produced pods. *V. faba* F2 (Hedin x Pietranera), *V. faba paucijuga* vf 172 and *V. faba* Peru (with seven chromosomes) as well as the vetch species *V. galilea*, *V. narbonensis* and *V. peregrina* were chosen as parents for a large scale interspecific pollination

experiment. The selected *V. faba* genotypes and vetch species are showed in Figure 3.

The results showed that the combination of *V. faba* F2 (Hedin x Pietranera) x *V. galilea* produced the highest number of pods and showed a better pod and ovule development compared to the other interspecific pollinations, followed by the combination *V. faba paucijuga* vf 172 x *V. galilea.* However, even these very intensive efforts to regenerate interspecific hybrids proved unsuccessful. A more detailed and intensive project study is needed to ultimately overcome postzygotic barriers hindering interspecific hybrid generation in *V. faba*.

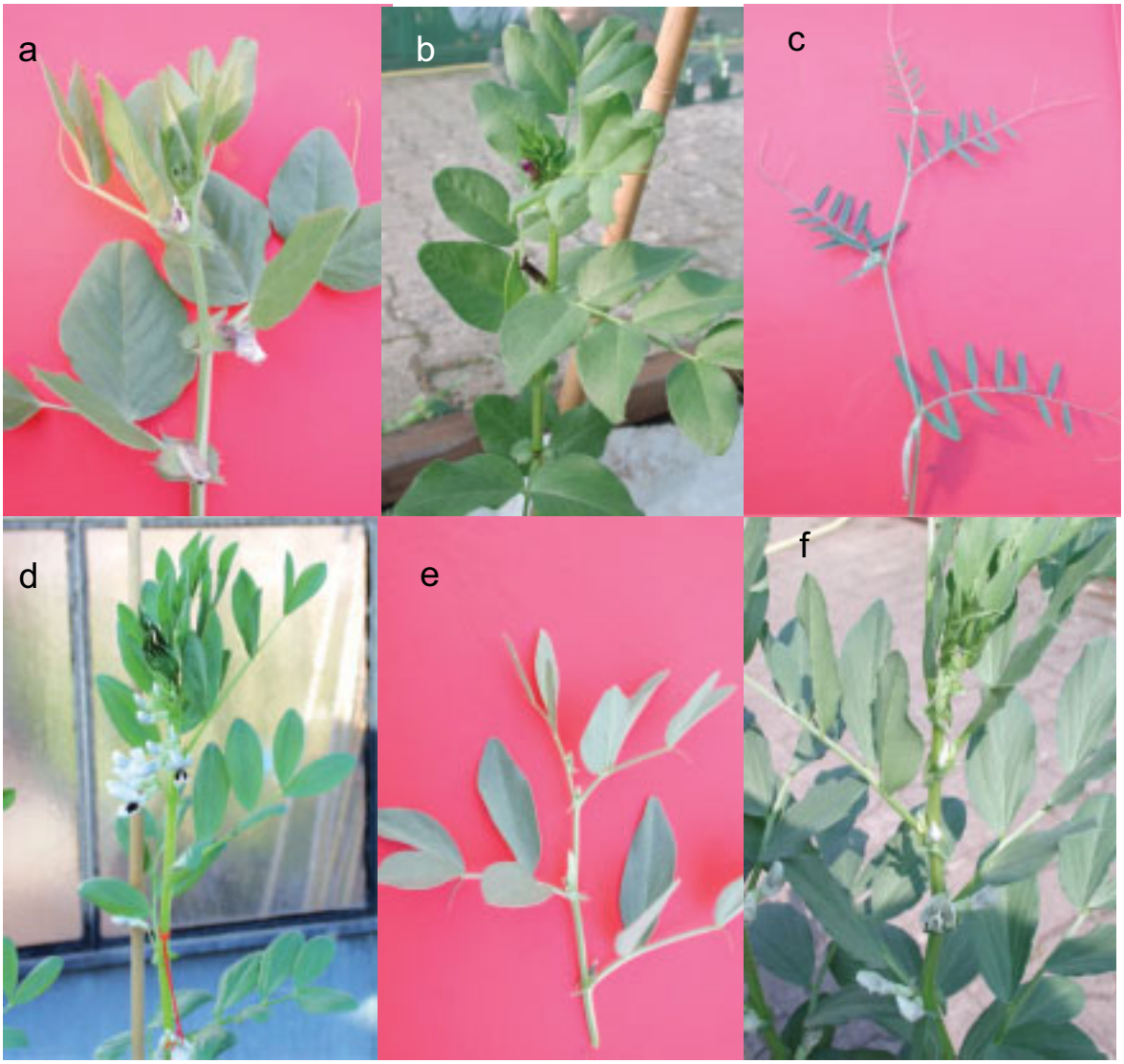

Figure 3. The selected *Vicia* species and *V. faba* genotypes, *V. galilea* (a), *V. narbonensis* (b), *V. peregrina* (c), *V. faba* F2 (Hedin x Pietranera) (d), *V. faba paucijuga* vf 172 (e) and *V. faba* Peru (f).

Summary

In the present work, the embryo rescue technique was optimized (I) and the effect of phytohormone treatments of flowers on pod and ovule development was studied to overcome postzygotic barriers (II) which hinder development embryos obtained after interspecific hybridization in *Vicia* species. The results of these studies were applied to a large scale interspecific pollination experiment using selected superior performing *V. faba* genotypes and vetch species (III).

(I) To optimize the embryo rescue technique four different basal media (KM, B5, MS, NLN) were tested in liquid and in solid form with *V. faba* cv. Mythos. The use of the NLN-medium in the solid form resulted in the highest percentage of surviving embryos and subsequent plant regeneration. The addition of phytohormones (BAP+GA_3 and IBA+GA_3) to the NLN-solid medium was tested with nine *V. faba* genotypes. In general this resulted in a lower percentage of plant formation from rescued embryos. The use of highly responsive *V. faba* genotypes in combination with the NLN-solid medium provides an improved basis for interspecific hybridization in *V. faba*.

(II) The effects of three phytohormone treatments (NAA 10 mgl^{-1}, GA_3 75 mgl^{-1} and GA_3 75 mgl^{-1}+NAA 10 mgl^{-1}) of unfertilized flowers on pod and ovule development in comparison to untreated-unfertilized and self-fertilized flowers were studied. The GA_3 treatment of unfertilized flowers led to a young pod fresh weight, pod length and mean ovule size per pod similar to those of fertilized flowers. The NAA treatment alone had a negative effect on the pod fresh weight, pod dry matter, pod length and mean ovule size per pod. The application of GA_3 in combination with NAA led to the development of significantly longer pods compared to the self-

fertilized flowers and all other treatments. This study demonstrated that either GA_3 or GA_3+NAA treatment postpones early pod abscission of unfertilized flowers. It is concluded that this treatment could also postpone premature pod abscission in *V. faba* interspecific hybridization experiments.

(III) A study was undertaken to obtain interspecific hybrids from crosses between nine highly diverse *V. faba* genotypes and several vetch species. From eleven vetch species examined, six were chosen based on their phenotypic similarity, phylogenetic relationship to *V. faba* and on their relative DNA content. Pollen tube growth observation and percentage of produced pods were recorded and used as criteria for the further selection of three superior vetch species and three *V. faba* genotypes. Based on these criteria *V. faba* F2 (Hedin x Pietranera), *V. faba paucijuga* vf 172 and *V. faba* Peru (with seven chromosomes) as well as the vetch species *V. galilea*, *V. narbonensis* and *V. peregrina* were chosen as parents for a large scale interspecific pollination experiments. The results showed that the combination of *V. faba* F2 (Hedin x Pietranera) x *V. galilea* produced the highest number of young pods and showed the most promising pod and ovule development, followed by the combination *V. faba paucijuga* vf 172 x *V. galilea.* However, even very intensive efforts to regenerate interspecific hybrids proved unsuccessful. A more detailed and longer lasting sequence of study is needed to ultimately overcome the postzygotic barriers hindering development of interspecific hybrids of *V. faba*.

Zusammenfassung

In der vorliegenden Arbeit wurde die Embryo Rescue Technik optimiert (I). Postzygotische Hindernisse (II) beeinträchtigen die Entwicklung der Embryonen nach interspezifischer Hybridisierung von Vicia Arten. Um die Hindernisse zu überwinden wurde der Einfluß phytohormoneller Behandlungen der Pflanzen auf die Hülsen- und Samenanlagenentwicklung untersucht. Die Ergebnisse dieser Untersuchungen wurden als Grundlage für ein breit angelegtes interspezifisches Kreuzungsprogramm herangezogen bei dem ausgewählte überlegene *V.faba* Genotypen sowie verschiedene Wickenarten (III) eingesetzt wurden.

(I) Vier unterschiedliche Grundmedien (KM, B5, MS, NLN) wurden in flüssiger und fester Form an *V.faba* cv. Mythos getestet. Das NLN-Medium in fester Form zeigte die höchste prozentuale Anzahl überlebender Embryos und daraus folgend die höchste Anzahl regenerierter Pflanzen. Die Beigabe von Phytohormonen (BAP+GA_3 und IBA+GA_2) zu dem festen NLN-Medium wurde an neun verschiedenen *V.faba* Genotypen untersucht. Im Allgemeinen führte dies zu einem geringeren Erfolg des Embryorescues. Der Einsatz von *V.faba* Genotypen mit gutem Regenerationsvermögen in Kombination mit dem NLN-Festmedium stellt eine verbesserte Basis für die interspezifische Hybridisierung dar.

(II) Die Effekte drei verschiedener Phytohormonbehandlungen (NAA 10 mgl^{-1}, GA_3 75 mgl^{-1} und GA_3 75 mgl^{-1}+NAA 10 mgl^{-1}) auf die Hülsen- und Samenanlagenentwicklung unbefruchteter Pflanzen wurden untersucht. Hierbei wurden Vergleiche mit unbehandelten selbstbefruchteten und unbefruchteten Pflanzen durchgeführt. Die GA_3-Behandlung der unbefruchteten Pflanzen führte zu höheren Hülsen Frischmassegewichten, Hülsenlängen und zu einer größeren

durchschnittlichen Samenanlage pro Hülse, vergleichbar mit denen der befruchteten Pflanzen. Die NAA-Behandlung zeigte einen negativen Einfluß auf die Hülsen Frischmasse, Hülsen Trockengewicht, Hülsenlänge sowie die durchschnittliche Größe der Samenanlage. Die Applikation von GA_3 in Kombination mit einer NAA-Behandlung erzielte bessere Ergebnisse als die alleinige Applikation von GA_3. Die hier festgestellten Unterschiede waren statistisch nicht signifikant. Die Untersuchungen zeigten, dass die Behandlung der unbefruchteten Pflanzen mit GA_3 und GA_3+NAA eine verzögerte Abtrennung der Hülsen zur Folge hatten. Diese Eigenschaft kann für die interspezifische Hybridisierung von *V.faba* genutzt werden, um die frühzeitige Abtrennung der Hülsen zu vermeiden.

(III) Es wurden interspezifische Hybriden aus Kreuzungen neun diverser *V.faba* Genotypen und elf Wickenarten hergestellt. Sechs der untersuchten Wickenarten wurden aufgrund ihrer phenotypischen Ähnlichkeit, der genetischen Verwandtschaft zu *V.faba* und der ähnlichen DNA Mengen zu *V.faba,* drei weitere Wickenarten und drei *V.faba* Genotypen wurden anhand des Pollenschlauchwachstums und der prozentualen Hülsenproduktion ausgewählt. Ebenfalls auf diesen Merkmalen basierend wurden *V.faba* F2 (Hedin x Pietranera), *V.faba paucijuga* vf 172 und *V.faba* Peru (sieben Chromosomen) und die Wickenarten *V.galilea, V.narbonensis* und *V.peregrina* als Elternpflanzen für ein breit angelegtes interspezifisches Kreuzungsprogramm ausgewählt. Die Ergebnisse zeigten, dass die Kreuzung von *V.faba* F2 (Hedin x Pietranera) x *V.galilea* die Nachkommen mit der höchsten Hülsenanzahl und der besten Hülsen- und Samenanlagenentwicklung hervorbrachte. Auch die Kreuzung von *V.faba paucijuga* vf 172 x *V.galilea* zeigte gute Ergebnisse bezüglich der oben genannten

Merkmale. Schließlich blieb, trotz intensiver Aufwendungen, die Herstellung interspezifischer Hybriden ohne Erfolg. Um die postzygotischen Hindernisse bei der interspezifischen Hybridisierung von *V.faba* zu überwinden bedarf es weiterer, detailierterer Projekte.

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Appendix

Manuscripts I – III

The present thesis is based on the following manuscript, which are referred to by their Roman numbers.

I. Optimization of embryo rescue technique in *Vicia faba* (in preparation for Plant Cell, Tissue and Organ Culture Journal)

II. Effects of gibberellic acid and naphthyl acetic acid treatment of Flowers on pod and ovule development in *Vicia faba* (in preparation for a Plant Growth Regulation)

III. Interspecific hybridization in *Vicia faba* (in preparation for the 5th European Conference on Grain Legumes, Dijon 2004)

Optimization of embryo rescue technique in *Vicia faba**

Abstract

Interspecific hybridization between faba bean (*Vicia faba* L.) and related *Vicia* species could be an important tool for the improvement of the faba bean crop. Postzygotic barriers are the main cause for an early abortion of hybrid seeds obtained following interspecific sexual hybridization in *V. faba*. The application of the embryo rescue technique may help to overcome this problem. This study was conducted to optimize the embryo rescue technique in *V. faba*. Four different basal media (KM, B5, MS, NLN) were tested in liquid and in solid form with *V. faba* cv. Mythos. The use of the NLN-medium in the solid form resulted in the highest percentage of surviving embryos (52%) and subsequent plant regeneration (29%) following the *in vitro* culture of ovules dissected eight to 10 days after self-pollination. The addition of phytohormones (0.5 mgl^{-1} BAP+0.5 mgl^{-1} GA_3 and 0.5 mgl^{-1} IBA+0.5 mgl^{-1} GA_3) to the NLN-solid medium was tested with nine *V. faba* genotypes. In general this resulted in a lower percentage of plant formation from rescued embryos. The use of highly responsive *V. faba* genotypes in combination with the NLN-solid medium provides an improved basis for interspecific hybridization in *V. faba*.

Key words: Ovule culture, embryo rescue, *Vicia faba*

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*This chapter is in preparation as manuscript to be submitted to the Journal Plant Cell, Tissue and Organ Culture for publication

Introduction

Faba bean (*V. faba*) is a traditional crop in Europe used with very small (200g 1000-seed-weight) to medium size seeds as field bean (*V. faba* spp*. paucijuga, minor* and *equina*) for animal feeding and with large seeds (more than 1000g 1000-seed-weight) as broad beans (*V. faba major*) for human consumption. In *V. faba*, it is generally accepted that this species is divided into two sub-species: ssp. *paucijuga* and ssp. *eu-faba* (Abdalla, 1977). Moreno (1979) argued that the primitive wild form of *V. faba* may have been close to the present day *paucijuga* types. Botanically, *V. faba (eu-faba)* has been divided on the basis of seed size into varieties major, equina and minor (Witcombe, 1981).

Faba bean yield is rather unstable due to diseases and abiotic stresses. According to Ahmed et al. (2000), Bond et al. (1994) and Cubero (1982) some other *Vicia* species express winter hardiness, disease and drought tolerance to a higher degree than *V. faba*. Therefore, interspecific hybridization between faba bean and its related species could be an important tool for the improvement of the faba bean crop. However, until now no report on the successful hybridization between faba bean and other *Vicia* species has been published. Several studies indicate that postzygotic barriers are the main cause for the lack of hybrid embryo formation between faba bean and other *Vicia* species, as visible through early hybrid embryo abortion (Ramsay et al., 1984, Ramsay and Pickersgill, 1986, Roupakias, 1986, Zenkteler et al. ,1998).

One technique to overcome early seed abortion is embryo rescue (Zenkteler, 1990). Immature embryos call for a far more specific medium composition than required for mature embryos (Pierik, 1997). Lazaridou et al. (1993) tested five

different liquid media to evaluate the *in vitro* response of *V. faba* ovule cultures. The results showed that a modified Gamborg medium (B5) gave the best and most consistent results. Meija-Jimenez et al. (1994) successfully produced hybrids between common bean (*Phaseolus vulgaris*) and tepary bean (*Phaseolus acutifolius*) using the embryo culture technique based on a modified Murashige and Skoog medium (MS, Murashige and Skoog, 1962). Lichter (1981) developed the NLN-medium for anther and isolated microspore cultures of *Brassica napus* (Möllers et al., 1994). This medium is similar in its composition to other media, e.g. the N6-medium, used for anther and microspore culture in cereals. Embryogenic microspores are to some extent similar to fertilized egg cells, both follow an embryogenic programme. Therefore, a medium like NLN that supports microspore regeneration should be a good candidate medium to promote sexual embryo regeneration.

The effect of hormones on growth and morphogenesis of embryos *in vivo* has been studied extensively, and it can be concluded in general that a low concentration of auxins promote embryo growth, whereas a high concentration is inhibitory. Gibberellins (GA_3) and cytokinins (CK) are found in relatively high concentrations in the liquid endosperm during early seed development (Rock and Quatrano 1995). It has been suggested that CK activity at this stage is responsible for enhancing seed size by increasing cell number, resulting in a larger storage capacity. A number of *in vitro* studies suggested that exogenous GA_3 can substitute for endogenous GA_3 in supporting embryo growth in culture (Garcia-Martinez et al., 1991). Pellegrineschi et al. (1997) studied the effect of phytohormone factors on embryo development *in vitro* from 18-days-old cowpea embryos. They found that a treatment with a low CK level is required for the

continued growth of the embryos. With younger, 10-days-old embryos a higher CK level was required. Mallikarjuna (1999) obtained a maximum number of regenerated plants from 8-days-old interspecific embryos of chickpea when they were cultured on a medium with a low auxin (0.25 mgl^{-1} IAA/ indoleacetic acid) and a high cytokinin concentration (1.0 mgl^{-1} zeatin).

Until now, studies on embryo rescue techniques in *V. faba* are rare. The available techniques allowed a maximum of 3.3 % (only one embryo) germinated embryos derived from 11-days-old embryos obtained after self-pollination. The plantlet regeneration from embryos younger than 11 days has in no case been successful (Lazaridou et al. 1993). Roupakias (1986) reported that interspecific hybrid embryos aborted nine days after pollination in the cross *V. faba* x *V. narbonensis,* indicating that younger embryos need to be rescued for successful interspecific hybridization. In interspecific crosses of *V. faba* with other vetches, *V. faba* seems to be better responding as female parent compared to other *Vicia* species. Zenkteler et al. (1998) observed 3.02% globular embryos derived from crosses between *V. faba* x *V. narbonensis*, whereas the reciprocal cross produced only 1.5% globular embryos. Similar results were found by Ramsay et al. (1984) who did interspecific hybridization between *V. faba* and different vetches (*V. galilea*, *V. johannis*, *V. narbonensis* and *V. bithynica*). Hence, it is important to improve the embryo rescue technique for young embryos in faba bean to ultimately allow the technique to be applied for interspecific hybridization of faba bean with other *Vicia* species.

The present study was undertaken to improve the embryo rescue technique in faba bean. To achieve this, different basal media were tested in liquid and in

solidified form and the effect of phytohormone treatments on the number of rescued embryos of different faba bean genotypes following self-pollination was studied.

Materials and Methods

Two experiments were carried out. In the first experiment, the effects of different basal media on the development of *V. faba* cv. Mythos embryos dissected eight to ten days after self pollination was studied. In the second experiment the effect of different phythohormone treatments and of different *V. faba* genotypes on the survival of isolated embryos obtained after selfing and their plant formation were studied. Nine highly diverse *V. faba* genotypes were used in the second experiment as shown in Table 1. The *V. faba paucijuga* genotypes were obtained from the Instituto del Germoplasma del CNR – Bari, Italy. The *V. faba* Peru (which has seven chromosomes) was obtained from the gene bank of Plant Genetics and Crops Plant Research Institute, Gatersleben, Germany. The other genotypes were obtained from the Agronomy and Plant Breeding Institute, Göttingen, Germany.

Table 1. *V. faba* genotypes used in the second experiment.

No	*V. faba* genotype	Seed type	Thousand seed weight (g)
1	F1(Hedin x Pietra.)	*Equina*	800
2	Hangdown	*Major*	1000
3	Hedin	*Minor*	300
4	Mythos	*Minor*	400
5	*paucijuga* vf 78	*Paucijuga*	300
6	*paucijuga* vf 163	*Paucijuga*	300
7	*paucijuga* vf 172	*Paucijuga*	200
8	Peru	*Major*	1000
9	Pietranera	*Major*	2000

Green house conditions

The experiments were carried out in winter 2001. The plants were grown in the green house at 16 hours light by using additional 400 Watt Sodium-steam lamp. Temperature was set at 20^{o}C at day and 15^{o}C at night. They were cultivated in 18 x 18 cm^{2} pots containing compost mixture and sand (4 : 1). The flowers were manually tripped (Link 1990) on the first day of anther dehiscence. The flowers were tripped as early as possible after the opening of flowers. According to our experience and to those of Schimd (1976) anther dehiscence is started at the time of flower opening. This day was defined as the day of self pollination. Pods were collected at eight to ten days after self pollination. Pod length and ovule size were recorded at the day of collection. Four to five flowers per inflorescence were manually tripped at opening of the flowers. Only 3 young produced pods per inflorescence were used and 12 to 16 flowers were used per plant.

***In vitro* culture experiments**

In the first experiment, several basal media were used which included the modified B5 medium used by Newell and Hymowitz (1982), the modified MS medium used by Meija-Jimenez et al. (1994), the Kao-Michayluk (KM) medium used by Tegeder et al. (1995) and the NLN medium derived from the medium described by Lichter (1981). Media were prepared following the published recipies in the solid (S) and liquid (L) form, except the KM-medium which was only used in liquid form. For the solid form, the media were solidified by adding 0.3% Gelrite (Duchefa Biochemie Haarlem, The Netherlands) before autoclaving, except the NLN medium which vitamin and micro elements were filtered sterilization. The pods obtained eight to 10 days after selfing were surface disinfected for 15 minutes in 3% calcium hypochlorite $Ca(OCl)_2$ containing few drops of Tween-20 (Merck-Schuchard

Hohenburn, Germany). The pods were then rinsed three times in sterile distilled water. The pods were carefully opened under a binocular and the ovules were excised. Ten ovules were cultured together in plastic petri dishes (diameter 55 mm). Four weeks after start of ovule culture, the embryos were excised from ovules and cultured in fresh medium. *In vitro* cultures were kept in a growth chamber at a 16-h photoperiod and at 23^0C day and 21^0C night temperature.

In the second experiment, the phytohormone solutions were filter sterilized and added to the medium after autoclaving. The *in vitro* culture conditions and ovule preparations were as described for the first experiment.

Experimental design

In the first experiment, seven different basal media were tested in a completely randomized block design with six replications. One replication consisted of 350 ovules (35 petri dishes, 5 petri dishes per medium). The replications were done in different weeks, which represented the blocking factor. The observed traits in this experiment were: the percentage of surviving ovules, the percentage of surviving embryos and the percentage of plant formation. The percentage of surviving ovules was counted per petri dish 3 weeks after start of ovule culture (percentage of number of surviving ovules from the total number of cultivated ovules). The percentage of surviving embryos was counted per petri dish 4 weeks after start of ovule culture. It was the percentage of surviving embryos from the total number of cultivated ovules. The percentage of plant formation was counted per petri dish 5 weeks after start of ovule culture, it was the percentage of plant formation from the total number of cultivated ovules. An analysis of variance (ANOVA) using Plabstat

(Utz 1997) was applied to the data. The mean values of the treatments were compared using Fischer's Least Significant Difference Test (LSD test).

In the second experiment, nine genotypes (see Table 1) and three phytohormone treatments were tested in a completely randomized block design with five replications. Each replication in the experiment consisted of 270 ovules (27 petri dishes, 3 petri dishes per one combination of genotype and phytohormone treatment). The best medium from the first experiment was used. The tested phytohormone treatments were:

1. Control (NLN solid medium without phythormone treatment).
2. NLN medium with 0.5 mgl^{-1} gibberellic acid (GA_3) + 0.5 mgl^{-1} benzylaminopurine (BAP).
3. NLN medium with 0.5 mgl^{-1} gibberellic acid (GA_3) + 0.5 mgl^{-1} indole-3-butyric acid (IBA).

Culture conditions, trait recording and statistical analysis of the data were as described for the first experiment.

Results

Eight to ten days after selfing, the immature pods of *V. faba* cv. Mythos were 2.51 ± 0.21 cm in length and contained ovules of 2.02 ± 0.38 mm in size. After three weeks of *in vitro* culture of these excised ovules, the media showed significantly different effects on the percentage of surviving ovules (Figure 1).

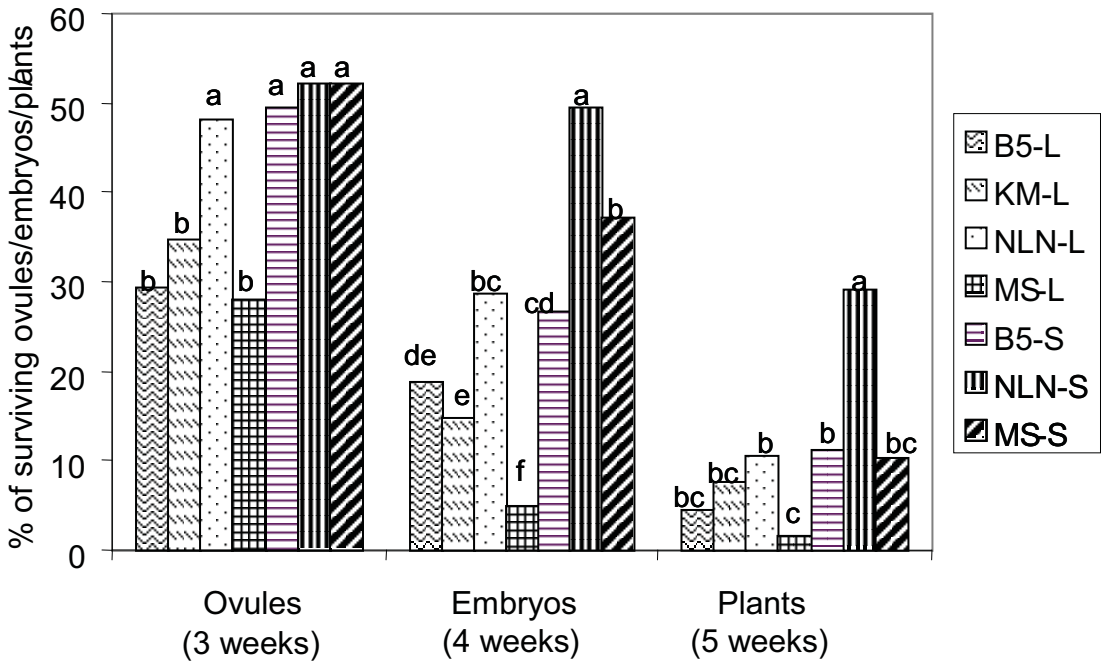

Figure 1. Percentage of surviving ovules (three weeks after ovule culture), surviving embryos (four weeks after ovule culture) and percentage of plant formation (five weeks after ovule culture) of *V. faba* cv. Mythos ovules dissected eight to 10 days after self pollination, and cultured in different basal media. Values with different letters within a time period are significantly different according to Fisher's LSD test (α = 0.05).

Figure 2. Plantlet obtained from an embryo dissected nine days after self-pollination.

The percentage of plant formation was significantly ($p = 0.01$) higher in the solid media (mean value of 16.87 %) than in the liquid media (mean value of 6.05%). The use of the NLN-medium in the solid form resulted in the highest percentage of surviving embryos (52%) and subsequent plant regeneration (29%, Figure 1). In liquid medium, browning of the embryos occurred more often and to a higher extent than in solid medium (data not given). The plantlets which developed five weeks after start of ovule culture had a well developed shoot and root system (Figure 2).

Since the NLN-solid medium was found to be superior to the other media, it was used as the only basal medium in the second experiment. It can be discerned from Table 2 that there were highly significant differences in percentage of surviving ovules, surviving embryos and plant formation after three, four and five weeks of ovule culture, respectively, between the genotypes, phytohormone treatments and due to their interactions. The GA_3+IBA phytohormone treatment had a negative effect on embryo development of virtually all genotypes (see Table 3, 4 and 5). The GA_3+BAP treatment positively effected the percentage of surviving ovules and embryos of genotypes *Paucijuga* vf 163, vf 172 and of cv. Pietranera. In general, the phytohormone treatments (GA_3+BAP and GA_3+IBA) induced a lower number of plant formation for the genotypes compared to the control (without phytohormone). As an exception, *V. faba* Pietranera, when treated with GA_3+BAP had a higher percentage of plant formation than its control.

Table 2. Mean squares and F-test of percentage of surviving ovules, surviving embryos and plant formation (second experiment).

Source of variation	DF	Parameters					
		Percentage of surviving					
		Ovules after 3 weeks		Embryos after 4 weeks		Plantlets after 5 weeks	
		MS	F	MS	F	MS	F
Genotype	8	5236.30	37.22**	2465.00	25.35**	654.63	21.85**
Phytohormone	2	4298.52	30.56**	4275.56	43.98**	982.96	32.82**
Replication	4	79.63	0.57	56.67	0.58	38.15	1.27
Genotype x Phytohormone	16	507.69	3.61**	705.56	7.26**	124.63	4.16**
Error	64	126.30		97.22		29.95	

** : significant at p = 0.01

DF: degrees of freedom

Table 3. Effect of genotypes and phytohormone treatments on percentage of surviving ovules after three weeks of ovule culture (second experiment).

V. faba genotype	Phytohormone treatment			Genotype mean
	Control	GA_3+BAP	GA_3+IBA	
F1 (Hedin x Pietra.)	52	48	38	46.00
Hangdown	80	54	42	58.67
Hedin	46	44	28	39.33
Mythos	74	66	58	66.00
Paucijuga vf 78	80	66	60	68.67
Paucijuga vf 163	8	32	8	16.00
Paucijuga vf 172	48	70	30	49.33
Peru	28	20	8	18.67
Pietranera	32	60	30	40.67
Phytohormone mean	49.78	51.11	33.56	44.81

LSD 0.05 for mean of genotypes = 8.65

LSD 0.05 for mean of phytohormone treatments = 5.00

LSD 0.05 for individual combinations of genotype x phytohormone = 14.99

Table 4. Effect of genotypes and phytohormone treatments on percentage of surviving embryos after four weeks of ovule culture (second experiment).

V. faba genotype	Phytohormone treatment			Genotype mean
	Control	GA_3+BAP	GA_3+IBA	
F1 (Hedin x Pietra.)	32	28	18	26.00
Hangdown	60	34	22	38.67
Hedin	36	34	18	29.00
Mythos	32	24	16	24.00
Paucijuga vf 78	60	38	44	47.33
Paucijuga vf 163	0	30	0	10.00
Paucijuga vf 172	38	66	20	41.33
Peru	16	16	0	10.67
Pietranera	20	52	20	30.67
Phytohormone mean	32.67	35.78	17.56	28.67

LSD 0.05 for mean of genotypes = 7.19

LSD 0.05 for mean of phytohormone treatments = 4.15

LSD 0.05 for individual combinations of genotype x phytohormone = 12.46

Table 5. Effect of genotypes and phytohormone treatments on percentage of plant formation after five weeks of ovule culture.

V. faba genotype	Phytohormone treatments			Genotype mean
	Control	GA_3+BAP	GA_3+IBA	
F1 (Hedin x Pietra.)	16	14	0	10
Hangdown	24	8	12	14.67
Hedin	0	0	0	0
Mythos	20	16	6	14
Paucijuga vf 78	30	16	14	20
Paucijuga vf 163	0	0	0	0
Paucijuga vf 172	20	10	8	12.67
Peru	0	0	0	0
Pietranera	8	16	0	8
Phytohormone mean	14.67	9.56	5.33	9.58

LSD 0.05 for mean of genotypes = 3.99

LSD 0.05 for mean of phytohormone = 2.30

LSD 0.05 for individual combinations of genotype x phytohormone = 6.91

The results show significant higher percentage of plant formation for *V. faba* cv. Mythos, Paucijuga vf 78, Hangdown and Paucijuga vf 172 compared to the other cultivars. Three genotypes failed to produce plants, namely *V. faba* Hedin, *V. faba* Paucijuga vf 163 and *V. faba* Peru.

Discussion

Embryo stage is a crucial factor for embryo rescue. To our knowledge there is no publication related to *V. faba* which reports on the successful embryo rescue in faba bean if the embryos were rescued earlier than 11 days after pollination. In the present experiments, the use of the embryo rescue method with a solid medium and two phases of culture (the first is ovule culture followed by embryo culture)

allowed to germinate embryos dissected from eight to 10 days old pods of *V. faba.* According to Borisjuk et al. (1995) six to nine days old embryos of *V. faba* var. *minor* cv. Fribo are in the globular stage and nine to 11 days old are in the early heart stage. The ovules of these stages were reported to be 1.3 – 3.0 mm in size. In our work, the ovule size was observed to be 2.02 ± 0.38 mm, indicating a similar embryo size. Pierik (1997) argued that generally very small undifferentiated embryos are virtually unable to grow *in vitro*. Lazaridou et al. (1993) concluded that faba bean embryos younger than 11 days old do not germinate. A very well optimized culture medium is needed to regenerate embryos derived from earlier stages of embryos (Pellegrineschi et al., 1997).

The results of the present experiments also show that the percentage of surviving embryos and plant formation was higher in solid medium than in liquid medium. Use of solid medium confirmed from earlier results in faba bean protoplast regeneration: the faba bean protoplasts became brown when cultured in liquid media, the cells did not divide and finally burst. Cell death rapidly ensued and was probably caused by phenolic oxidation (Wijaya 2000). Analogous to this, it can be assumed that in the present experiments browning ovules exuded phenolics into the media and that these phenolics probably spread faster in liquid media than in solid medium, causing toxic effects to the other ovules.

The results of the first experiment showed that the NLN-solid medium produced the highest percentage of surviving ovules and of plants followed by the MS-solid medium. In contrast to the other media, NLN-medium is low in nitrate and high in organic nitrogen. Both factors are believed to play a significant role in embryo culture of faba bean: leguminous species receive most of the plant nitrogen

essentially as ammonium via the *Rhizobium* symbiosis (Nutman 1965). Legumes have different nitrogen requirements compared to non-legumes. A similar phenomenon has been found by Pellegrineschi et al. (1997) in cowpea embryo rescue: they concluded that an increase of organic nitrogen in the culture medium had positive effects on the final percentage of plant regeneration. Further improvement of the embryo rescue results may be obtained by optimizing the organic nitrogen content and composition of the NLN-medium.

The addition of exogenous gibberellic acid and indole-3-butyric acid (GA_3+IBA) to the media had negative effects on the embryo development. If auxin is substituted with cytokinin (GA_3+BAP), the percentage of surviving ovules, embryos and plant formation increased. Kramer (2002) found that auxins are not really important for callus induction in *V. faba.* Her experiments showed that the number of surviving calli, the size and visual performance are better in media without auxin compared to cultures in media with auxin. It is expected that *V. faba* contains a relatively high concentration of endogenous auxin (Manabe et al., 1999).

The faba bean genotypes that were tested in the present experiment belong to three taxonomical groups, however, concerning the *in vitro* response no difference between the three groups was found. The nine different genotypes varied significantly in the percentage of plant formation. This indicates that a screening of a larger germplasm collection could lead to the identification of genotypes with an even higher *in vitro* culture response.

However, this study was carried out in winter season. In further study (see manuscript II), it was observed that pod and ovule development of *V. faba* was different in winter and in summer. Pod and ovule development in summer was

faster than in winter. Therefore, a further study is needed for the optimisation of embryo rescue technique in *V. faba* in summer condition.

In conclusion, the results show that using the NLN-solid medium eight to 10 days old *V. faba* embryos obtained from a number of *V. faba* genotypes can be regenerated *in vitro* to plantlets. The use of these genotypes in combination with the NLN-solid medium provides an improved basis for future interspecific hybridization experiments in *V. faba*.

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Effects of gibberellic acid and naphthyl acetic acid treatments of flowers on subsequent pod and ovule development in *Vicia faba**

Abstract

Premature pod abscission is still a major bottle neck in interspecific hybridization of *Vicia faba* L. This study was conducted to postpone such premature pod abscission. The effects of three phytohormone treatments (NAA 10 mgl^{-1}, GA_3 75 mgl^{-1} and GA_3 75 mgl^{-1}+NAA 10 mgl^{-1}) of unfertilized flowers on young pod and ovule development in comparison to untreated-unfertilized and self-fertilized flowers were studied. All treatments had a positive effect on the percentage of produced pods, pod weight and ovule size per pod when compared to unfertilized flowers. However, with respect to these traits, only the GA_3 and GA_3+NAA treatment gave similar results as found for self-fertilized flowers. The application of GA_3 in combination with NAA led to the development of significantly longer pods compared to the self-fertilized flowers and all other treatments. This study demonstrated that either GA_3 or GA_3+NAA treatment postpones early pod abscission of unfertilized flowers. It is concluded that this treatment could possibly postpone premature pod abscission in *V. faba* interspecific hybridization experiments.

Key words: gibberellic acid, naphthyl acetic acid, flower, ovule and pod development, *Vicia faba*

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Introduction

Interspecific hybridization of *Vicia faba* L. with closely related species is still an unsolved task. Many attempts to obtain interspecific hybrids by crossing *V. faba* with one of the other *Vicia* species have been unsuccessful (Pickersgill 1993 and Duc 1997). Several previous studies reported that pollen tubes are capable of reaching and fertilizing the ovules in some of the interspecific combinations, but that postzygotic barriers prevent development of hybrid embryos (Ramsay et al. 1984, Ramsay and Pickersgill 1986, Roupakias 1986 and Zenkteler et al. 1998). In previous work with 54 interspecific crossing combinations between nine different faba bean genotypes and six other *Vicia* species, (Manuscript III) it was found that in some combinations pollen tubes were capable of reaching the ovules. However, application of ovule culture and embryo rescue of eight to ten days old pods, which were obtained after interspecific pollination, was not successful. Roupakias (1986) studied pod growth following pollination of *V. faba* with pollen of *V. narbonensis*. He found that at day nine or 10 after pollination the growth rate of the interspecific embryos decreased and that by day 13 pod growth had almost stopped.

Phytohormone treatments are commonly applied after pollination in interspecific and intergeneric wide crosses to postpone embryo abortion. Amrani et al. (1993) obtained haploid wheat plants following pollination of tetraploid and hexaploid wheat with maize pollen. To postpone embryo abortion, pollinated flowers were treated with 10 mgl^{-1} 2,4-D. Sastri and Moss (1982) found that when phytohormones were applied after interspecific pollination of *Arachis hypogea* and *A. monticola* with *A. glabrata*, the fertilized ovules survived longer than untreated ovules, enabling some embryos to reach a size at which they could be cultured successfully *in vitro*. They also found that the use of 10 mgl^{-1} of naphthyl acetic

acid (NAA) was more effective than other concentrations of NAA or other hormones like indole-3-acetic acid **(**IAA) and Kinetin (Kn). Mallikarjuna (1999) reported that application of gibberellic acid (GA_3, 75 mgl^{-1}) was a crucial precondition to obtain pods and interspecific hybrids between *Cicer arietinum* and *C. pinnatifidum.*

In pea (*Pisum sativum*), normal pod growth requires the presence of seeds. The developing seeds contain a high level of GA_3 (Maki et al. 1986). Seeds as a precondition for pod development can be substituted by an application of GA_3 (Euwens and Schwabe 1975). It has been assumed that the GA_3 synthesized by seeds is transported to the pod wall and regulates pod growth (Sponsel 1982). But Ozga et al. (1992) proposed an alternative hypothesis, that seeds may promote pod growth by maintaining GA_3 biosynthesis in the pod wall.

Time series experiments showed that GA_3 stimulated both elongation and inflation growth in unfertilized pea ovules, while the auxin 2,4-D only stimulated pod elongation of unfertilized ovules (Garcia-Martinez and Carbonell 1980). Ozga and Reinecke (1999) reported that unfertilized pods of pea responded differently to exogenous GA_3 and to the auxin 4-chloroindole-3-acetic acid (4-Cl-IAA). However, GA_3 was more active in stimulating pod growth than 4-Cl-IAA. They have also observed that there was a positive synergistic effect of simultaneous application of auxin and GA_3 on unfertilized pod growth.

Phytohormone treatments of flowers could postpone premature pod abscission and hence may help to overcome early embryo abortions in *Vicia* interspecific hybridization experiments. The present study was performed to determine the

effects of GA_3 and NAA treatments of flowers on subsequent pod and ovule development.

Materials and Methods

Plant material

Faba bean plants were cultivated in 18 x 18 cm^2 pots containing compost mixture and sand (4 : 1). During winter the plants were grown in a green house providing 16 hours light by using additional 400 Watt Sodium-steam lamps. Temperature was set at 20^oC during the day and 15^oC at night. In summer, the plants were cultivated in bee-proof isolation cages under natural temperature and day light. F2-plants derived from the cross *V. faba* Hedin x *V. faba* Pietranera were used in this study. In an earlier study on interspecific hybridization in *Vicia faba,* these plants showed a relatively good performance as female parents (Manuscript III).

Method

A randomized complete block design with three replications (experiments) was used in each of three seasons: winter 2001, summer 2002 and winter 2002. Thirty flower buds were used for each treatment in one season. Only one flower bud was used per inflorescence. Allocation of treatments on inflorescences and plants was randomized. Up to three treatments were placed on one plant. Eight to ten plants were used for one treatment in one season. Flower buds were manually emasculated one day before anther dehiscence (see below). Phytohormone treatments were applied 24 hours after emasculation, using a micro pipette. Five µl phytohormone solution was injected into flowers into the space between stigma and corolla. To reduce any cohesion effect, 0.1 % aqueous Tween 80 (Merck-

Schuchard Hohenburn, Germany) were added to each solution. Percentage of produced pods, pod fresh weight, pod dry matter, pod length, mean ovule size per pod and number of ovules per pod were determined. The parameters were measured 14 days after phytohormone treatment. An analysis of variance (ANOVA) using Plabstat (Utz 1997) was applied to the data. The mean values of the treatments were compared using Fischer's Least Significant Difference Test (LSD test).

The following treatments were tested:

1) Control I, emasculation, no phytohormone treatment (injection of water added with 0.1% Tween 80), no pollination (unfertilized flower).
2) Control II, emasculation, no phytohormone treatment (injection of water added with 0.1% Tween 80), with manual self pollination (fertilized flower).
3) GA_3, emasculation, phytohormone treatment (injection of water with 0.1% Tween 80 and GA_3 75 mgl^{-1}), no pollination (unfertilized flower).
4) NAA, emasculation, phytohormone treatment (injection of water with 0.1% Tween 80 and NAA 10 mgl^{-1}), no pollination (unfertilized flower).
5) GA_3+NAA, emasculation, phytohormone treatment (injection of water with 0.1% Tween 80, GA_3 75 mgl^{-1} and NAA 10 mgl^{-1}), no pollination (unfertilized flower).

Results

In the present experiment, the effects of GA_3 and NAA treatments of the unfertilized *V. faba* flowers on the young pod and ovule development were evaluated and compared to that of fertilized flowers. The results of the analysis of variance showed that the effects of the phytohormone treatments and seasons were highly significant (*p = 0.01,* Table 1). Their interactions (phytohormone treatment x season) were also significant (*p = 0.05*) for some of the observed parameters.

Table 1. Means squares (MS) and F-test of percentage of produced pods, pod fresh weight, pod dry matter, pod length, mean ovule size per pod and mean ovule number per pod of in 14 days old pods.

Parameters/ DF	Phytohormone treatment(T)		Season (S)		TxS	
	MS	F	MS	F	MS	F
DF	4		2		6	
Percentage of produced pods	800	5.72**	4792	34.25**	489	4.82*
Pod fresh weight	0.44	13.85**	0.68	21.43**	0.068	2.13^{+}
Dry matter pod	3598	6.87**	22980	43.86**	1386	2.65*
Pod length	3.90	11.57**	8.80	26.15**	0.98	2.91*
Mean ovule size per pod	0.67	9.35**	2.39	33.37**	0.13	1.79
Mean ovule number per pod	0.065	0.82	0.002	0.03	0.13	1.56

**, *, + : Statistically significant difference at p = 0.01, p = 0.05, p = 0.10; F-test in ANOVA

DF = degrees of freedom

One week after pollination, the percentage of produced pods were similar for the unfertilized-untreated flowers and the others treatments (data not shown). However, after two weeks, the percentage of produced pods derived from unfertilized-untreated flowers decreased due to the abscission of young pods. The phytohormone treatments of unfertilized flowers led to an increase of the percentage of produced pods (Figure 1) as determined two weeks after pollinations. The phytohormone treatments (GA_3, GA_3+NAA and NAA) induced a significantly higher percentage of produced pods (mean 73%) compared to the untreated-unfertilized flowers (Control I, 54%). As to the percentage of produced pods, the phytohormone treatments gave an effect similar to the results of the fertilized flowers (Control I, 76%). The percentage of produced pods was higher in summer than in winter.

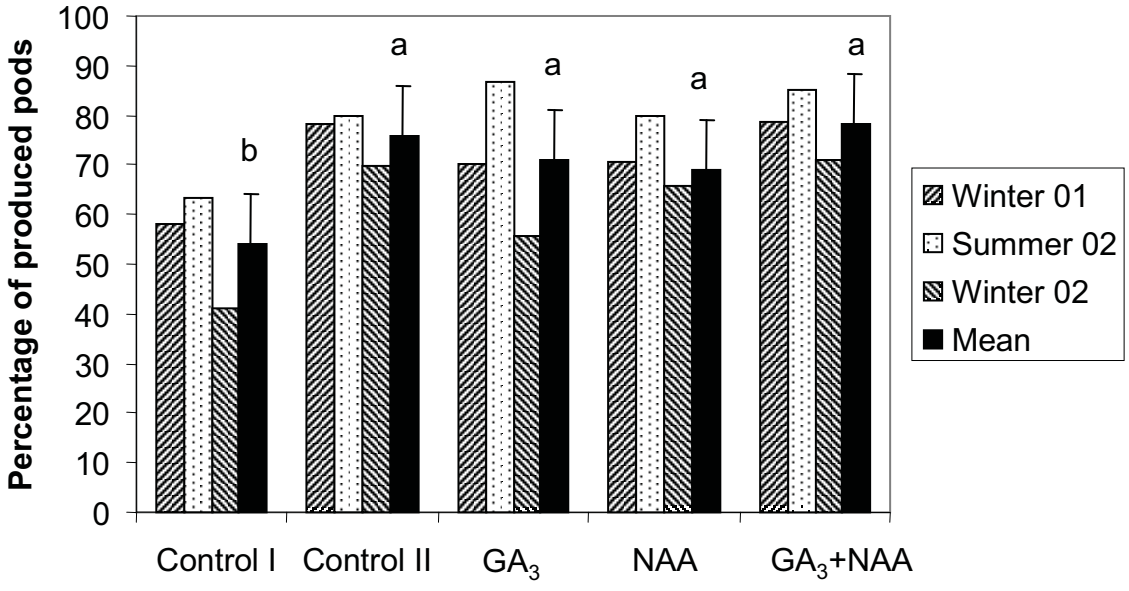

Figure 1. The effect of phytohormone treatments of unfertilized flowers on percentage of produced pods in three seasons as determined two weeks after pollination.

The GA_3+NAA treatment of unfertilized flowers led to a significantly longer pod length (4.24 cm) compared to the other treatments (Figure 2). On the other hand, Control I and NAA produced significantly smaller pods compared to the Control II, GA_3 and GA_3+NAA treatments.

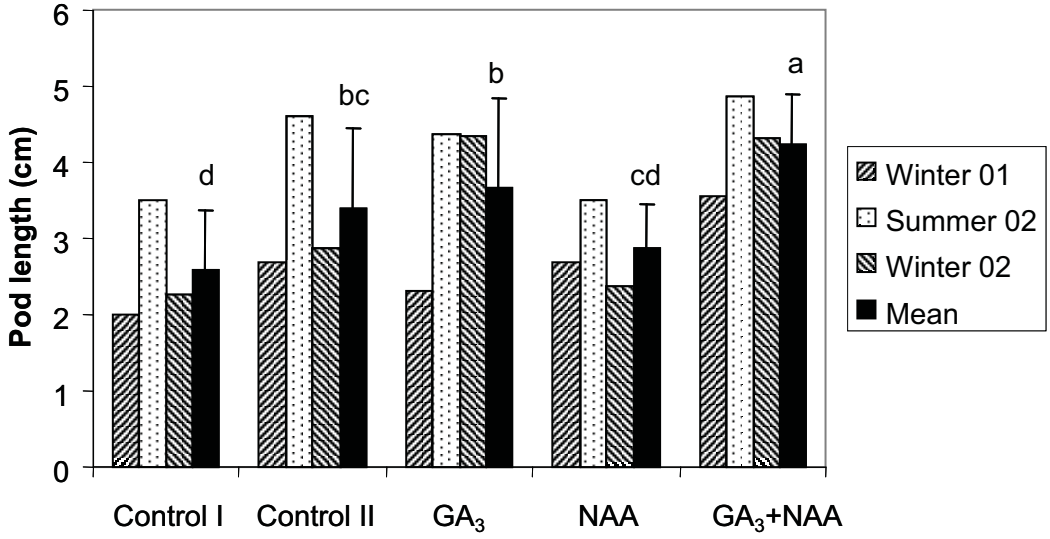

Figure 2. The effect of phytohormone treatments of unfertilized flowers on pod length in three seasons as determined two weeks after pollination

Although the GA_3+NAA treatment produced significantly longer pods, this treatment did not result in an increased pod weight compared to the Control II and GA_3 treatment (Figure 3). However, the Control II, GA_3 and GA_3+NAA treatments produced significantly longer and heavier pods than the untreated-unfertilized and the NAA treated flowers (see Figures 2,3 and 4).

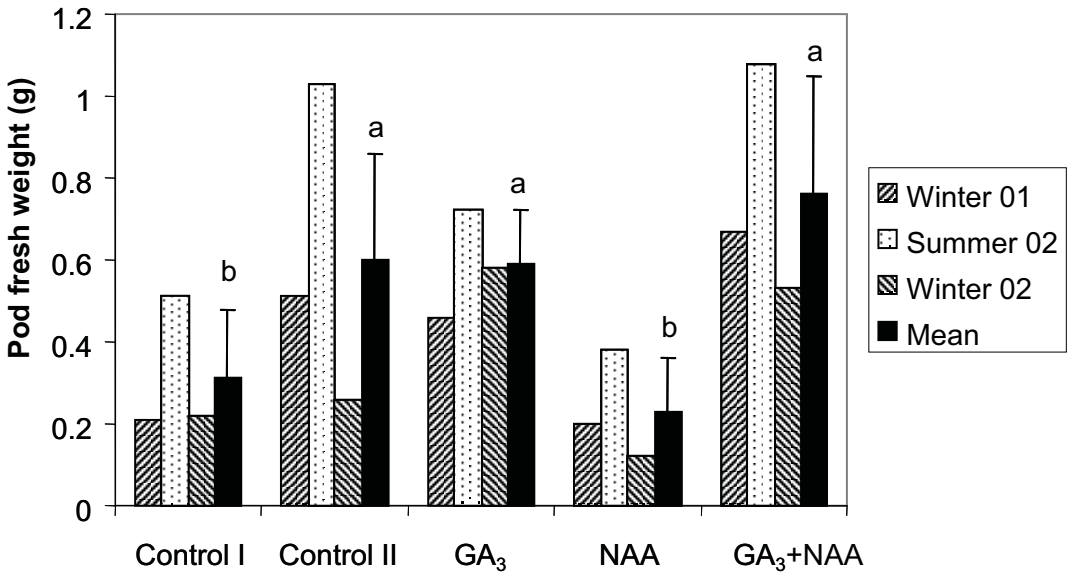

Figure 3. The effect of phytohormone treatments of unfertilized flowers on pod weight in three seasons as determined two weeks after pollination

The fertilized flowers (control II) produced significantly bigger ovules compared to control I and the NAA treatment (Figure 5). Taken together, the results showed that the NAA treatment had a negative effect on the pod fresh weight, pod length and mean ovule size per pod. The GA_3 treatment was in general less effective on the pod fresh weight than the GA_3+NAA treatment. The results also indicate that in summer the pods and ovules were bigger than in winter.

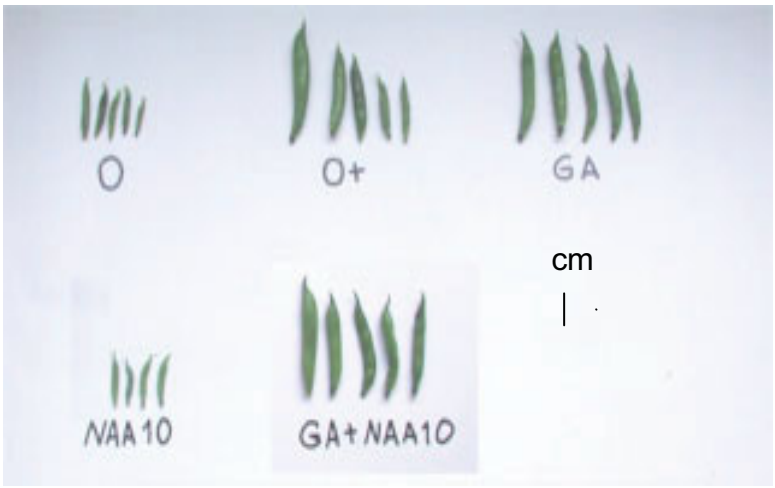

Figure 4. Pod performance 14 days after phytohormone treatments of unfertilized flowers of *V. faba* F2 (Hedin x Pietranera). 0 is control I (unfertilized pods), 0+ is control II (fertilized pods)

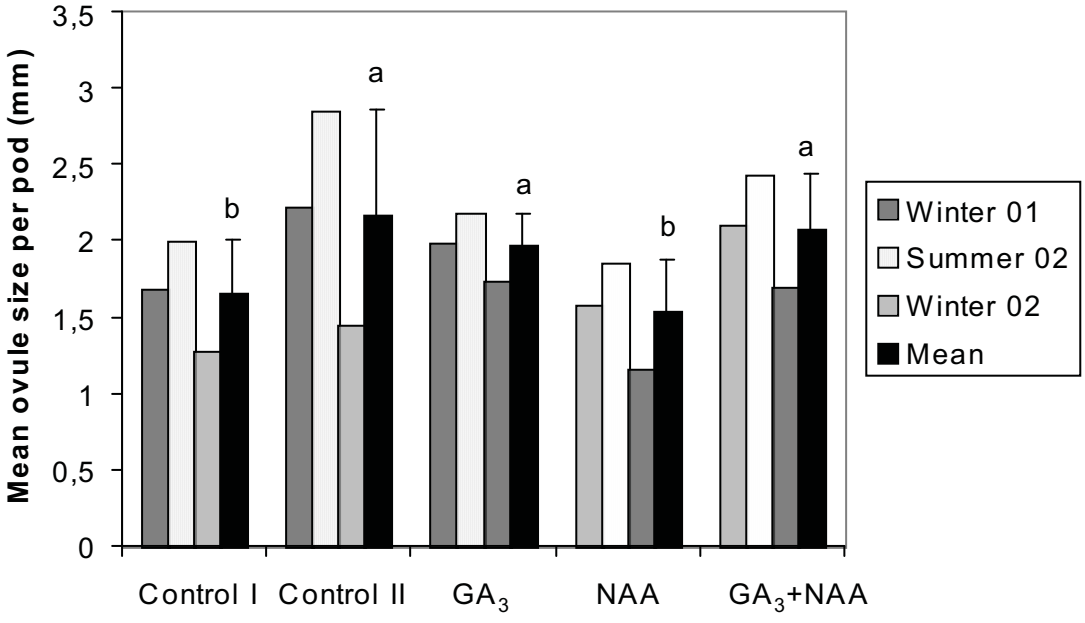

Figure 5. The effect of phytohormone treatments of unfertilized flowers on mean ovule size per pod in three seasons as determined two weeks after pollination

Discussion

In our work we tried to substitute the effects of a successful fertilization on early pod development by exogenous treatments of unfertilized flowers with NAA and GA_3. The F-test of the ANOVA showed significant effects for the phytohormone treatments, seasons and their interactions (phytohormone treatment x season, TxS, Table 1). For the seasons this was expected because of the extremely different conditions in summer (isolation cage) and in winter (green house). The TxS interactions were significant for three of the six observed parameters, indicating that the effect of phytohormone treatments markedly depends on the environmental conditions. An explanation for this could be that the effect of phytohormone treatments on flowers may depend on sink activity (Brenner et al. 1985). The sink activity is limited by the source potential, which in summer probably is higher than in winter due to generally better growth conditions. Dantuma and Thompson (1983) also gave examples of apparently good growing conditions leading to heigher levels of total dry mater.

The percentage of produced pods as obtained two weeks after phytohormone treatments of unfertilized flowers was not significantly different from that of fertilized flowers. It was observed that one week after pollination the unfertilized-untreated flowers also produced a similar number of pods to fertilized flowers. After two weeks, the number of produced pods derived from unfertilized-untreated flowers was significantly lower due to the abscission of young pods compared to fertilized flowers and unfertilized-treated flowers. In this study, one half of the unfertilized-untreated flowers produced pods, a phenomenon was observed ealier

by Chapman et al. (1979). They found that numerous parthenocarpic pods were formed when all flowers of *V. faba* were emasculated-unpollinated and the apex were removed, the aim of this treatment to reduce a competition within plant to assimilate sinks.

The GA_3 and the GA_3+NAA treatments led to pod and ovule performances similar to those obtained from fertilized flowers. A similar phenomenon was found in pea by Sponsel (1982). He observed that the application of GA_3 could replace the presence of seeds which were a requirement for pod development. In the present study the fertilized flowers produced slightly bigger ovules than those obtained following GA_3 and GA_3+NAA treatments (Figure 5). This result is consistent with the report of Ozga et al. (1992) who reported that normal fertilized ovules of peas were bigger in size than the ovules without embryos obtained from pods which were treated with GA_3.

The results of the present work show that GA_3 is more important than NAA for pod and ovule development. However, the application of GA_3 in combination with NAA gave better results compared to application of GA_3 alone. Meija-Jimenez et al. (1994) argued that besides GA_3, an auxin at a low concentration is needed to promote embryo growth. The results of Euwens and Schwabe's (1975) experiment supported this argument: their GA_3+NAA treatment re-established the normal development of pea pods after mechanically killing developing embryos.

This study demonstrates that the GA_3+NAA treatment postpones pod abscission unfertilized flowers. It can be anticipated that this same treatment may have a similar positive effect on pod and ovule development obtained following

interspecific pollinations. Perhaps, this treatment allows interspecific embryos to grow until their size and developmental stage is adequate for successful embryo rescue.

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Interspecific hybridization in *Vicia faba**

Abstract

All attempts to obtain interspecific hybrids between faba bean and related *Vicia* species (vetch) have so far been unsuccessful. It was concluded that postzygotic barriers prevent the development of interspecific hybrid embryos. Selection of appropriate parents, phytohormone treatments of flowers and the application of the embryo rescue technique may help to overcome postzygotic barriers. A corresponding study was undertaken to obtain interspecific hybrids from crosses between nine highly diverse *V. faba* genotypes and several vetch species. From eleven vetch species examined, six were chosen based on their phenotypic similarity, genetic relationship and relative DNA content when compared with *V. faba.* Pollen tube growth and percentage of produced pods were recorded in these interspecific crosses and used as criteria for the further selection of three superior vetch species and three *V. faba* genotypes. Based on these criteria *V. faba* F2 (Hedin x Pietranera), *V. faba paucijuga* vf 172 and *V. faba* Peru (with seven chromosomes) as well as the vetch species *V. galilea*, *V. narbonensis* and *V. peregrina* were chosen as parents for a large scale interspecific pollination experiment. The results showed that the combination of *V. faba* F2 (Hedin x Pietranera) x *V. galilea* produced the highest number of pods and showed a better pod and ovule development compared to the other interspecific pollinations, followed by the combination *V. faba paucijuga* vf 172 x *V. galilea.* Together, N = 3978 flowers were interspecifically pollinated. However, even these very intensive efforts to regenerate interspecific hybrids proved unsuccessful. A more detailed and intensive study is needed to ultimately overcome postzygotic barriers hindering interspecific hybrid generation in *V. faba*.

Key words: Insterspecific pollination, *Vicia faba*, vetches

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1 Introduction

Vicia faba L. is a crop that is capable of high yields but which is sensitive to stress. Therefore, breeding for resistance to abiotic and biotic stress is important (Bond et al. 1994). Due to a low genetic variability for most of these traits within the *V. faba* species, the probabilities of markedly improving these traits by conventional breeding are rather low. Interspecific hybridization opens a new perspective to improve these traits by introducing genes from other *Vicia* species (in the following collectively termed vetches). Cubero (1982) reported that some vetches are disease and drought resistant and well adapted to soils of low fertility. Ahmed et al. (2000) found that many germplasm accessions of narbon (*V. narbonensis*) and common vetch (*V. sativa*) were highly resistant to downy mildew.

Interspecific hybridization attemps with *V. faba* were mainly performed using *V. narbonensis* (Metin 1962, Roupakias 1986, Roupakias and Tai 1986, Lazaridou et al. 1989 and Zenkteler et al. 1998). However, Ramsay et al. (1984) crossed four botanical types of *V. faba* (*paucijuga*, *minor*, *equina* and *major*) with *V. bithynica, V. galilea,* and *V. johannis.* Ramsay and Pickersgill (1986) also crossed faba bean with *V. melanops, V. lutea* and *V. johannis*. But so far all attempts have not resulted in any regeneration of interspecific hybrids.

Appropriate selection of the species and the genotypes to be used in interspecific hybridization can be crucial for the success. The phylogenetic relationships among the species in the subgenus *Vicia* are one criterium to select a vetch species as a hybrid partner for faba bean. Van de Ven et al. (1993) examined the phylogenetic relationships between *Vicia* species using restriction fragment length polymorphism (RFLP) and Polymerase Chain Reaction (PCR) methods. They suggested that *V. faba* is more closely related to *V. peregrina* and *V. michauxii*

followed by *V. lutea*, *V. hybrida* and *V. melanops* than to *V. narbonensis*. Potokina et al. (1999) evaluated the phylogeny of the *Vicia* subgenus *Vicia* (Fabaceae) based on Random Amplification of Polymorphic DNA (RAPD) analyses of total genomic DNA and on PCR-RFLP analyses of chloroplast genes. They came to similar results as Van de Ven *et al*. (1993). Furthermore, they found that *V. bithynica* is more closely related to the faba bean than *V. narbonensis*. However, Maxted (1992) argued that *V. faba* was more isolated from its related species than these species from each other.

In *V. faba*, it is generally accepted that this species is divided into two sub-species: ssp. *paucijuga* and ssp. *eu-faba* (Abdalla 1977). Moreno (1979) argued that the primitive wild form of *V. faba* may have been close to the present day *paucijuga* types. Botanically, the sub-species *eu-faba* has been divided on the basis of seed size into sub-goups *major*, *equina* and *minor* (Witcombe 1981).

Schubert and Rieger (1991) reported on chromosomal and morphological mutants of faba bean. They found that some genotypes have seven chromosomes instead of the normal chromosome number six. One of these genotypes is *V. faba* cv. Peru, in which the longest chromosome is broken into a short and a long arm chromosome. Vetches generally have seven chromosomes. It is estimated that the interspecific cross between any *V. faba* genotype which has this karyotype and any vetch will have higher possibility for chromosome pairing than the crosses with a normal karyotype.

Cubero (1982) argued that a big difference of DNA content per genome and per chromosome between *V. faba* and vetches causes a collapse of interspecific

embryos, which means that its DNA reproductive cycle must be different. 'Contradictory orders' could thus simply be the lack of co-adaptation of two different DNA sets.

Even more decisive than the phylogenetic relationships, interspecific pollen tube growth and ovule and pod development are regarded as important parameters for selecting hybrid partners for interspecific crossses. Ramsay and Pickersgill (1986) pollinated *V. faba* with pollen of *V. bithynica, V. galilea, V. narbonensis* and *V. johannis*. They found that pollen tubes were able to germinate and penetrate the faba bean stigma, but in pollinations of *V. bithynica, V. galilea* and *V. johannis* as female with *V. faba* as male, pollen tubes became arrested in the styles which is the normal site of self-incompatibility in the *leguminosae*. Zenkteler et al. (1998) studied the reciprocal crosses between *V. faba* and *V. narbonensis*. They found that only few pollen tubes grew straight through the style and into the micropyles.

In an earlier work we have developed an embryo rescue method which allows to regenerate embryos dissected from 8 to 10 days old self pollinated pods of *V. faba* (Manuscript I). Furthermore, we have found that treatment of non-pollinated *V. faba* flowers with GA_3 (gibberellic acid) + NAA (naphthyl acetic acid) led to the development of seedless pods similar in size to those of normal self-fertilized pods (Manuscript II). These results indicated that a phytohormone treatment of flowers could postpone premature pod abscission in interspecific hybridization of *V. faba.*

The aim of the present work was to obtain interspecific hybrids from crosses between *V. faba* and related vetch species. This goal was intended to be achieved through (1) selecting superior *V. faba* genotypes and vetch species (one genotype per species) based on observations of pollen tube growth and pod production, and

(2) evaluation of pod and ovule development from selected interspecific cross and regeneration of hybrid plants following embryo rescue.

2 Materials and Methods

Two experiments were carried out. The aim of the first experiment (Experiment 1, selection of parents) was to identify promising interspecific parental combinations. Eleven vetch species related to *V. faba* (Table 1) were studied. From these, six vetches were chosen based on phenotypical similarity, genetic relationship and DNA content for the further experiments. The six vetches were reciprocally crossed with nine *V. faba* genotypes (Table 1). Pollen tube growth and percentage of produced pods were observed in these crosses. From Experiment 1 three *V. faba* genotypes and three vetches were selected for the second experiment (Experiment 2), study of interspecific pollinations of selected parents. These were reciprocally crossed, self-pollination and non-pollination of emasculated flowers were used as controls. The phytohormone flower treatment and the embryo rescue technique were applied as described in Manuscript I and II.

Plant cultivation

The experiments were conducted in Göttingen, from winter 2000 to winter 2002. The plants were cultivated in 18 x 18 cm^2 pots containing a mixture of compost and sand (4:1). During winter the plants were grown in a green house providing 16 hours day light by using additional 400 Watt Sodium-steam lamp. Temperature was set at 20^oC at day and 15^oC at night. In summer 2001, the plants were cultivated in bee-proof cages under natural temperature and day light. Insecticides and fungicides were applied whenever necessary.

2.1 Experiment 1 (selection of parents)

2.1.1 Plant materials

The vetch and *V. faba* genotypes used in Experiment 1 are shown in Table 1.

Table 1. Species and genotypes used in Experiment 1 (selection of parents)

Species	Abbreviation	Gene bank code/ Seed donor	Seed source
***V. faba* genotypes**			
V. faba minor Mythos	Myth	Collection Göttingen	BPC 2000
V. faba minor Hedin	Hedi	Collection Göttingen	BPC 2000
V. faba equina F2(Hedin x Pietranera)	F2	Collection Göttingen	BPC 2000
V. faba paucijuga vf 78	P78	CNR	GH 98/99
V. faba paucijuga vf 163	P163	CNR	GH 98/99
V. faba paucijuga vf 172	P172	CNR	GH 98/99
V. faba major Hangdown	Hang	Collection Göttingen	BPC 2000
V. faba major Peru	Peru	IPK	IPK 2000
V. faba major Pietranera	Piet	Collection Göttingen	BPC 2000
Vetch species			
*Vicia bithynica**	*V. bith*	VIR-VB-34427	GH 98/99
Vicia galeata	*V. gale*	USDA-VG-PI.602380	USDA 98
*Vicia galilea**	*V. gali*	IPK-VG-NAR44/80	GH 98/99
Vicia hybrida	*V. hybr*	VIC 309/96	IPK 1998
Vicia johannis	*V. joha*	USDA-VH-W6.17061	GH 98/99
Vicia lutea	*V. lute*	VIR-VL-34863	GH 98/99
*Vicia melanops**	*V. mela*	IPK-VM-VIC474/95	GH 98/99
*Vicia narbonensis**	*V. narb*	BAZ-VN-45614	GH 98/99
Vicia narbonensis	*V. narb*	VIR-VN-35391	GH 98/99
*Vicia michauxii**	*V. mich*	IPK_VM_VIC47/95	IPK 1999
*Vicia peregrina**	*V. pere*	IPK-VP-VIC747/78	IPK 1998
Vicia serratifolia	*V. serr*	IPK-VS-NAR142/83	IPK 1998

*These six vetches were later on chosen for experiment 2 based on phenotypical similarity and genetic relationship to *V. faba* and appropriate DNA content.

Table 1. continued

BAZ = Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Genbank, Braunschweig

CNR = Nazionale delle Richerche (CNR), Bari

IPK = Institut für Pflanzengenetik und Kulturpflanzenforschung, Genbank, Gatersleben

USDA = United States Department of Agriculture, Washington

VIR = Vavilov Research Institute of Plant Industry, Petersburg

GH = Green house at Göttingen

BPC = Bee-proof cage at Göttingen

2.1.2 Phenotypic similarity

One criterion to select the vetches for interspecific hybridization experiments with *V. faba* was their phenotypic similarity with *V. faba*. There were four characters which were evaluated. These were general habitus, flower, leaf and pod morphology. *V. faba* cv. Mythos was used as a standard to denote *V. faba*. Ten plants in each species were observed for phenotypic similarity. The plants were cultivated in the green house winter 2000/2001.

2.1.3 DNA measurement

A further criterion was DNA content. Three young fully developed leaves from three plants per species were mixed for analysis. Measurement of each sample was repeated three times. Relative nuclear DNA content was estimated by using a Flow Cytometer (Ploidy Analyser, Partec GmbH, Germany). For nuclei extraction about 0.5 cm^2 leaf area (mixed from three leaves) was chopped with a new razor blade in a Petri dish continuing a few drops of Partec buffer DAPI (4,6-diamidino-2-phenylindole). After adding another 2 ml DAPI buffer, the suspension with the nuclei was filtered through a 50μm pore size nylon filter. Relative fluorescence

intensity of the nuclei was analyzed using Partec DPAC software. The DNA content relative to *V. faba* cv. Mythos were calculated from sample peak means.

2.1.4 Observation of pollen tube growth

Pollen tube growth in interspecific pollinations was studied. The method for pollen tube observation was modified from Martin (1959) and Kho and Baer (1968). The flower buds were collected 24 hours after pollination. The petals and corollas were removed. The pistils were fixed in a solution containing 70% ethanol : 100% acetic acid : 35% formaldehyde (90 : 5 : 5) for at least 24 hours. After washing out the solution with tap water, the pistils were incubated 6 hours in 1 N NaOH (maceration) at 20 ^{o}C and stained with in 0.1 % aniline blue in 0.1 M K_3PO_4 for 24 hours in darkness. The pistils were carefully squashed and then observed under a fluorescence microscope (microscope-camera MC 80 Zeiss) using ultraviolet light (excitation filter FT 580 and LP 590 from Zeiss). Three to five flower buds per combination were observed and the existence of the pollen tubes in styles was recorded.

2.2 Experiment 2 (Study of interspecific pollinations of selected parents)

2.2.1 Plant materials

Based on the pollen tube observation and the percentage of produced pods (Experiment 1), *V. faba* F2 (Hedin x Pietranera), *V. faba paucijuga* vf 172 and *V. faba* cv. Peru (with seven chromosomes) as well as the vetches *V. galilea*, *V. narbonensis* and *V. peregrina* were chosen as parents for the experiment 2. *V. bithynica* as pollinator showed high scores for pollen tube growth and percentage of produced pods, but this species showed a very low performance when used in

winter season (results were not shown). Therefore, this species was not chosen for Experiment 2.

2.2.2 Emasculation, pollination, phytohormone application and embryo rescue

All flowers used were manually emasculated one day before anther dehiscence. The flowers were pollinated directly after emasculation. The phytohormone treatment (GA 75 mgl^{-1} + NAA 10 mgl^{-1}) was applied 24 hours after emasculation and pollination. Using a micro-pipette, five μl phytohormone solution were injected in the flowers into the space between stigma and corolla. To reduce any cohesion effect, 0.1 % Tween 80 (Merck-Schuchard Hohenburn, Germany) were added to the solutions (Manuscript II). Eight to ten days old pods were collected and ovules were prepared and cultured in NLN medium according to Lichter (1981) as described earlier (Manuscript I).

2.2.3 Statistical analysis

In the second experiment, eighteen interspecific pollination combinations and 12 control treatments (self-pollination and non-pollination, Table 2) of selected *V. faba* genotypes (N = 3) and vetches (N = 3) were conducted in a completely randomized design with two replications. The first replication was in winter (green house) and the second replication was in summer (bee-proof cages). In the following, the pollination combinations and the control treatments are collectively termed ‘treatment’. One treatment consisted of 60 to 420 treated flowers. Eight to ten days after pollination the young pods were collected. The percentage of produced pods was calculated, pod length, pod weight, ovule size and ovule number were recorded. An analysis of variance (ANOVA) using Plabstat (Utz

1997) was applied to the data means of flowers per treatment and per replicate were the most of analysis. The mean values of the treatments were compared using Fischer's Least Significant Difference Test (LSD test).

Table 2. The treatments used for studying interspecific pollinations of selected parents (Experiment 2).

No	Treatments (♀x ♂)	Pollination type	Number of emasculated and treated flowers
1	F2 x *V. gali*	Interspecific	420
2	F2 x *V. pere*	Interspecific	210
3	F2 x *V. narbo*	Interspecific	100
4	F2 x F2	Self	130
5	F2 x 0	Non	112
6	P172 x *V. gali*	Interspecific	358
7	P172 x *V. pere*	Interspecific	190
8	P172 x *V. narbo*	Interspecific	100
9	P172 x P172	Self	110
10	P172 x 0	Non	100
11	Peru x *V. gali*	Interspecific	243
12	Peru x *V. pere*	Interspecific	220
13	Peru x *V. narbo*	Interspecific	130
14	Peru x Peru	Self	110
15	Peru x 0	Non	110
16	*V. gali* x F2	Interspecific	392
17	*V. gali* x P172	Interspecific	390
18	*V. gali* x Peru	Interspecific	245
19	*V. gali* x *V. gali*	Self	110
20	*V. gali* x 0	Non	110
21	*V. pere* x F2	Interspecific	220
22	*V. pere* x P172	Interspecific	190
23	*V. pere* x Peru	Interspecific	220
24	*V. pere* x *V. pere*	Self	60
25	*V. pere* x 0	Non	60
26	*V. narbo* x F2	Interspecific	110
27	*V. narbo* x P172	Interspecific	130
28	*V. narbo* x Peru	Interspecific	110
29	*V. narbo* x *V. narbo*	Self	60
30	*V. narbo* x 0	Non	60

3 Results

3.1 Experiment 1 (Selection of parents)

The DNA content, genetic relationship and phenotypic similarity to *V. faba* of 11 vetch species are summarized in Table 3.

Table 3. DNA content, genetic relationship and phenotypic similarity of different vetch species to *V. faba*.

Species	DNA content relative to *V. faba*	Genetic* relationship	Phenotypic** similarity
V. faba cv. Mythos	1.00	++++	++++
V. bith	0. 53	++	++
V. gale	0. 45	n.d.	+
V. gali	0.40	+	+++
V. hybr	0.41	++	+
V. joha	0.26	+	+
V. lute	0.11	++	++
V. mela	0.54	+++	+
V. michi	0.47	+++	+
V. narb	0.40	+	+++
V. pere	0.44	+++	+
V. serr	0.42	+	++

* According to Van de Ven et al. (1993) and Potokina et al. (1999) (+ - ++++ increasing genetic relatedness).

**General similarity between the species, based on habitus, leaf and pod morphologhy (+ = low similarity, ++++ = high similarity).

n.d. = not determined

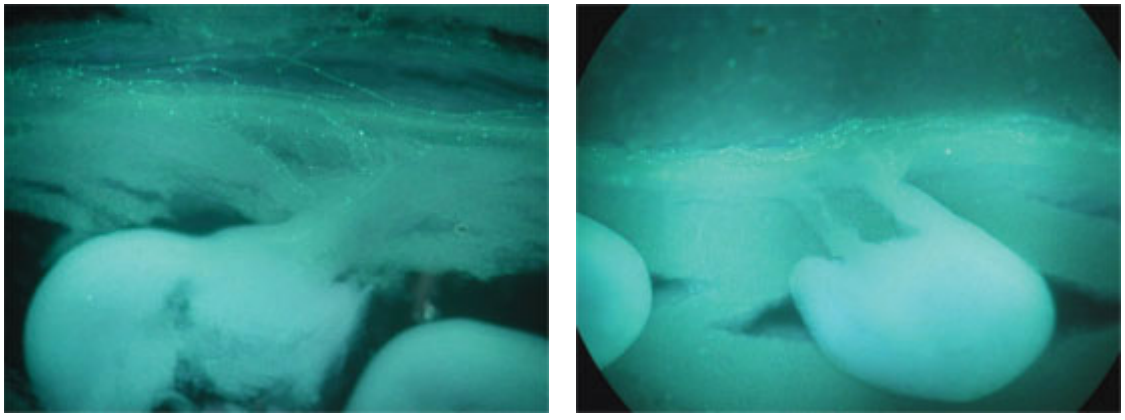
According to Van de Ven et al. (1993) and Potokina et al. (1999), *V. melanops, V. michauxii* and *V. peregrina* are closely related to *V. faba* followed by *V. bithynica*, *V. hybrida* and *V. lutea*. The narbon vetch *V. narbonensis* and *V. galilea* are

genetically not closely related to *V. faba.* However according to our observations these species are phenotypically very similar to *V. faba* (Table 3).

In the present experiment, a flow cytometer was used to estimate the relative DNA content. All vetch species had a DNA content much lower than *V. faba* ranging from 0.53 to 0.11 when expressed as relative DNA content. Considering the DNA content, genetic relationship and phenotypic similarity, *V. melanops, V. michauxii, V. peregrina, V. narbonensis*, *V. galilea* and *V. bithynica* were chosen as vetch parents for the interspecific crossing experiments with *V. faba*.

The most promising combinations

Fluorescence microscopy proved very suitable for the detection of aniline blue stained pollen tube growth in the style of interspecific reciprocal cross combinations between nine faba bean genotypes and six different vetch species. The pollen tube tissues could be clearly distinguished from the surrounding tissues as shown in Figure 1.



A B

Figure 1. Pollen tube growth following intraspecific pollination in *V. faba* F2 (Hedin x Pietranera) (A), pollen tube growth of *V. galilea* in the style of *V. faba* F2 (Hedin x Pietranera) (B).

Many pollen tubes were observed in all *V. faba* styles if the flowers were pollinated with *V. faba* cv. Mythos (intraspecific, Table 4). In interspecific pollinations, there were less pollen tubes and in some combinations no pollen tubes could be observed.

Table 4. Detection of pollen tubes in styles derived from interspecific reciprocal pollinations

♀ \ ♂	*V. mela*	*V. mich*	*V. pere*	*V. narb*	*V. gali*	*V. bith*	Total *)	*V. faba* Mythos
F2	+ / -	- / -	+ / +	+ / +	+ / +	+ / +	5/4	+ / +
Hang	- / -	- / -	- / -	- / -	+ / -	+ / -	2/0	+ / +
Hedi	+ / -	- / -	- / -	+ / +	+ / +	+ / +	4/3	+ / +
Myth	- / -	- / -	- / -	- / -	- / +	- / +	0/2	+ / +
P78	- / -	- / -	- / -	- / +	- / +	- / -	0/2	+ / +
P163	- / -	- / -	- / -	- / +	- / +	- / +	0/3	+ / +
P172	- / -	- / -	+ / -	+ / +	+ / +	- / +	3/3	+ / +
Peru	- / -	- / -	+ / -	+ / +	- / +	- / -	2/1	+ / +
Piet	+ /-	- / -	+ / -	- / -	+ / +	- / -	3/1	+ / +
Total*)	3/0	0/0	4/1	4/6	5/8	3/6		+ / +

*) : Total of pollen tubes which existed in styles

\- : No pollen tubes that were visible in styles

\+ : More than one pollen tube was visible in styles

/ : Faba bean as female parent / faba bean as male parent

The results of percentage of produced pods parameter showed that most of the faba bean cultivars produced young pods without pollination, except for *V. faba* Hangdown, Mythos and *paucijuga* vf 78 (Table 5).

Table 5. Percentage of pods with ovules obtained from interspecific reciprocal pollinations between *V. faba* and vetches

♀ \ ♂	*V. bith*	*V. gali*	*V. mela*	*V. mich*	*V. narb*	*V. pere*	Mean	*V. faba* Mythos	MS*
F2	30/50	30/70	40/0	0/0	40/20	30/0	28/23	40	20
Hang	0/60	0/0	0/0	0/0	0/0	0/0	0/10	20	0
Hedi	30/80	30/30	30/0	0/0	30/50	20/0	23/27	80	30
Myth	0/60	0/50	0/0	0/0	0/0	0/30	0/23	80	0
P78	0/0	0/30	0/0	0/0	0/30	0/0	0/10	30	0
P 163	0/0	0/30	20/0	0/0	20/30	20/0	7/10	30	10
P 172	0/40	20/50	50/0	0/0	50/0	50/0	28/15	70	20
Peru	80/30	30/70	0/0	0/0	0/50	30/0	23/25	80	30
Piet	50/0	0/70	30/0	0/0	30/10	30/0	23/13	70	20
Mean	21/36	12/44	19/0	0/0	19/21	20/0	15/17	56	

* Emasculated flowers without pollination, numbers of pods (all with very small ovules)

/ : Faba bean as female parent / faba bean as male parent

The observation of pollen tube growth and percentage of produced pods showed that *V. faba* genotypes and vetches had a similar potency as female parents and male parents. In *V. faba*, *V. faba* F2 (Hedin x Pietranera) had the highest score of pollen tube growth and percentage of produced pods followed by *V. faba* Peru and *V. faba paucijuga* vf 172. For the vetches, *V. galilea* had the highest score for pollen tube growth and percentage of produced pods followed by *V. bithynica, V. narbonensis* and *V. peregrina.*

3.2 Experiment 2 (Study of Interspecific pollination of selected parents)

The results from the analysis of variance and the F-test showed the treatments (interspecific cross combination) as a highly significant source of variation ($p = 0.01$). The analyses of variance for percentage of produced pods, pod length, pod weight, ovule size and ovule number of selected parents are shown in Table 6.

The non-pollinated flowers of faba bean genotypes produced more pods compared to the vetches (Figure 2). The pollination of *V. faba paucijuga* vf 172 with *V. galilea* produced a similar number of pods as the self pollinated flowers, somewhat less in the pollination of *V. faba* F2 (Hedin x Pietranera) with *V. galilea*. When the vetch species were used as female parents, the percentage of produced pods clearly decreased compared to self pollinated flowers, data given in the Figure 2. However this phenomenon was not found in *V. faba* Peru. The self pollinated flowers of *V. faba* Peru produced less pods (36 %) compared to the other two faba beans used in this experiment (Figure 2). Generally the crossing attempts in summer were more successful than in winter (results are not presented).

Table 6. Mean square and F-test from the analyses of variance of percentage of produced pods, pod length, pod weight, ovule size and ovule number .

Source of variation	DF	Percentage of produced pods		Pod length		Pod weight		Ovule size		Ovule number	
		MS	F	MS	F	MS	F	MS	F	MS	F
Treatment	29	896.41	3.4**	3.00	14.21**	0.04	4.07**	0.46	9.20**	1.86	4.84**
Replication	1	16677.37	64.12**	0.01	0.04	0.09	9.62**	0.58	11.63**	2.75	7.15*
Treatment x Replication	21	260.08		0.21		0.01		0.05		0.39	

**, * : Statistically significant difference at P=0.01, P=0.05

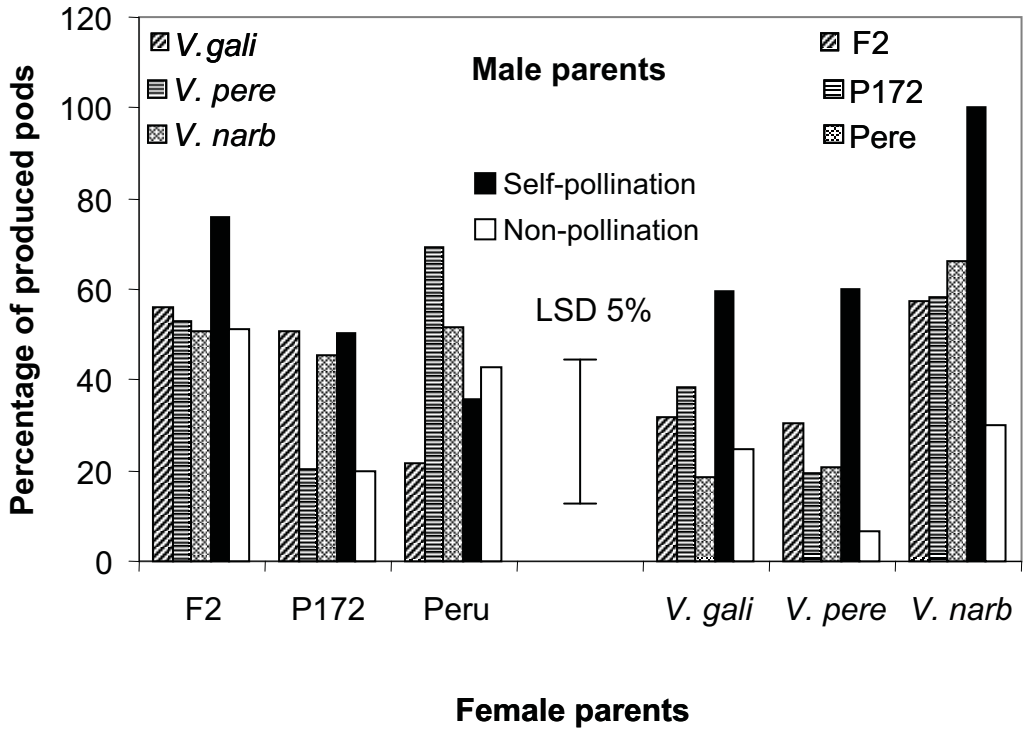


Figure 2. Mean percentage of produced pods derived from interspecific pollination, self pollination and from non-pollinated flowers of *V. faba* and vetches.

In general, the results showed that the pod and ovule performances depended largely on the female parents (Figure 3, 4 and 5). The LSD test showed that there are barely any statistically significant differences among the treatments for pod and ovule performance values within a given female parent. Pollinated flowers produced longer and heavier pods compared to non-pollinated flowers (Figure 3 and 4). As an exception, the non-pollinated flowers of *V. faba* Peru produced a longer and heavier pod after interspecific pollination than after self pollination.

Faba bean genotypes produced shorter pods compared to vetches (Figure 3). Some interspecific pollinations (*V. faba paucijuga* vf 172 x *V. galilea*, *V. faba paucijuga* vf 172 x *V. narbonensis* and *V. faba paucijuga* vf 172 x *V. galilea*) had a similar performance in pod length as the self pollinated flowers. If the vetches were

used as female parents, the non-pollinated flowers produced a similar pod length as the pods derived from the self pollinated flowers or interspecific pollinated flowers. In contrast to this, pod weights showed that pods derived from non-pollinated flowers were clearly lighter than pods derived from self pollinated and interspecific pollinated flowers, especially, in *V. peregrina* and *V. narbonensis* (Figure 4) .

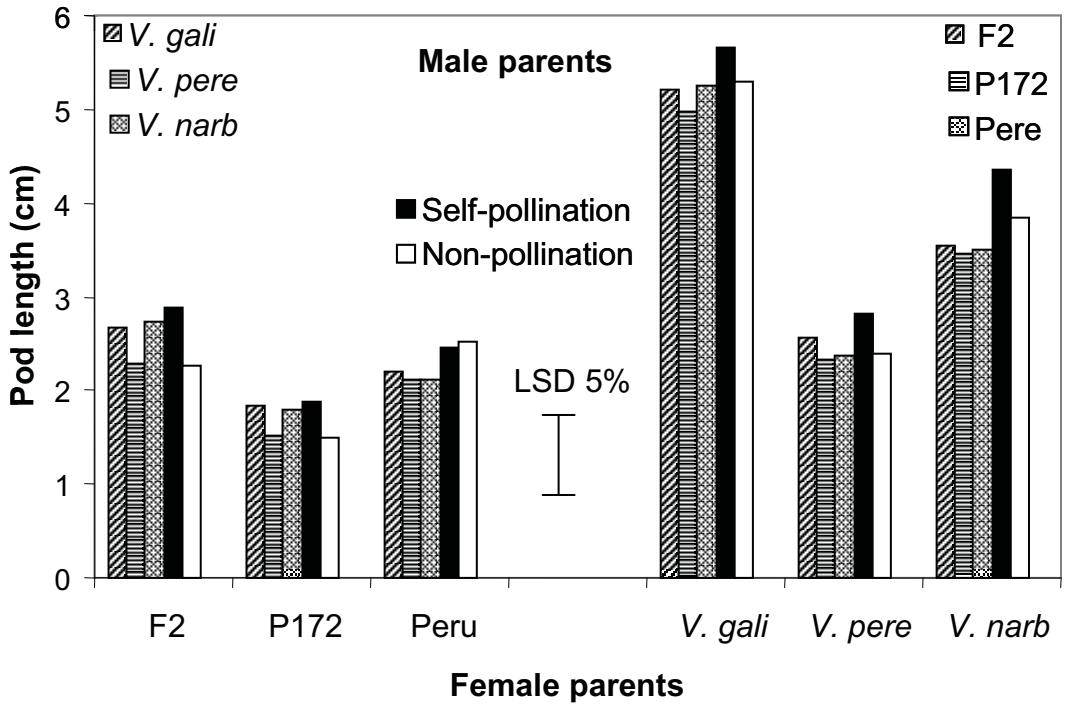


Figure 3**.** Mean pod length of the pods derived from interspecific pollination, self pollination and from non-pollinated flowers of *V. faba* and vetches.

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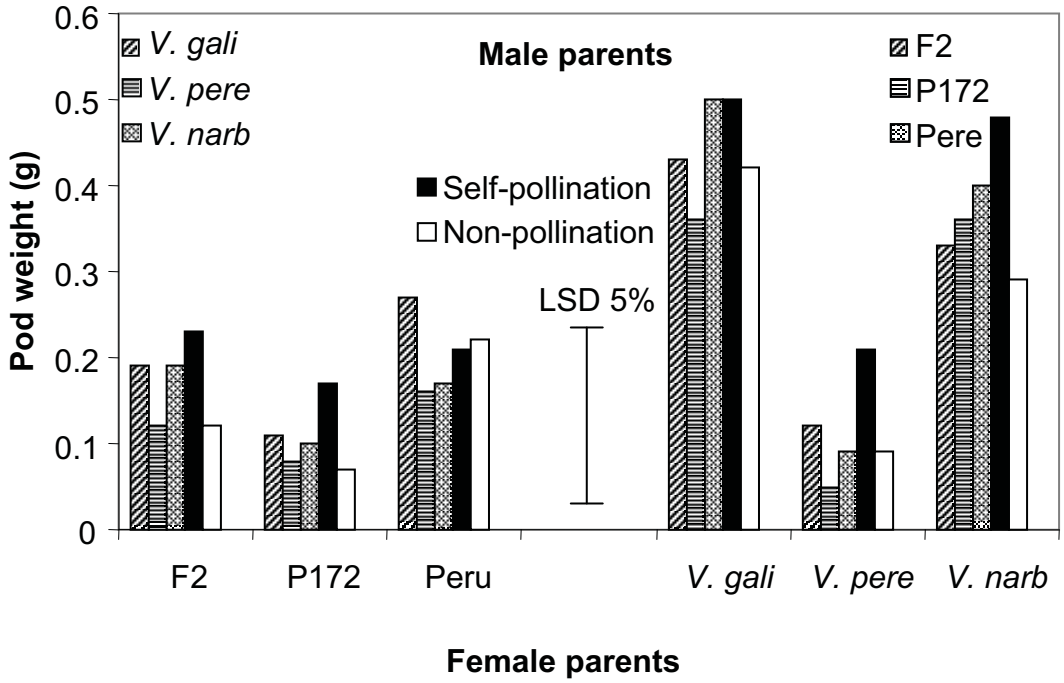

Figure 4. Mean pod weight of the pods derived from interspecific pollination, self pollination and from non-pollinated flowers of *V. faba* and vetches.

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The ovule size results showed that the self pollinated flowers produced bigger ovules than interspecific pollinations and non-pollinated flowers (Figure 5.) Moreover, flowers of faba bean genotypes pollinated with *V. galilea* produced bigger ovules compared to the interspecific pollination with other vetches. Especially the pollination of *V. faba* F2 (Hedin x Pietranera) with *V. galilea* pollen and also its reciprocal direction showed a higher ovule size compared to the other interspecific pollinations. The non-pollinated flowers produced smaller ovules compared to self pollination and interspecific pollinations (Figure 6). In contrast to this, the number of ovules did not show marked differences among treatments within a given female parent even if pods derived from non-pollinated flowers and self pollinated flowers were compared (Figure 7).

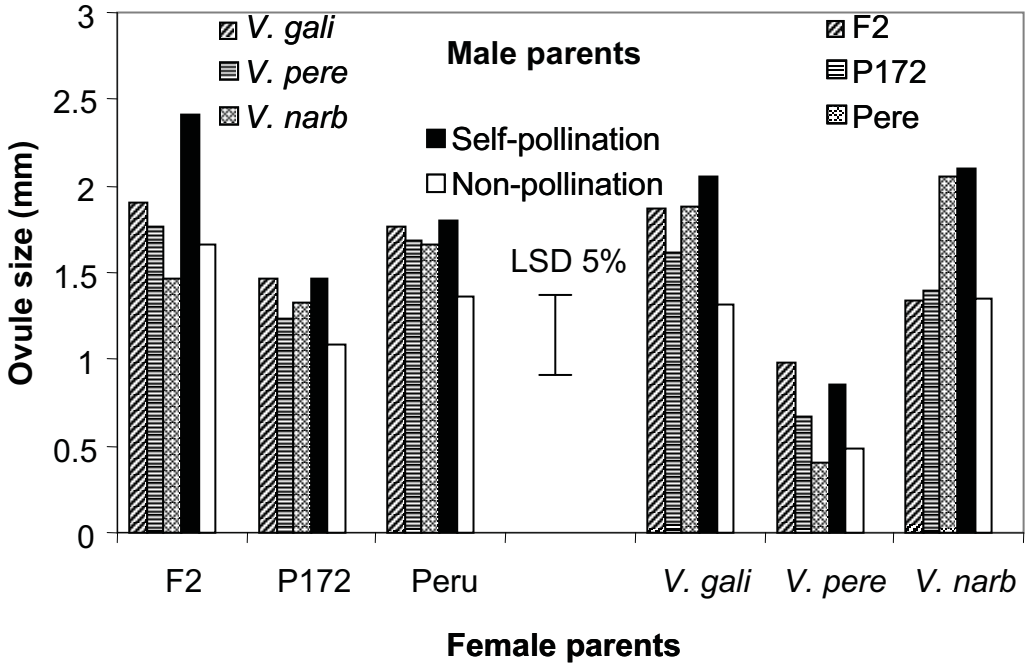

Figure 5**.** Mean ovule size derived from interspecific pollination, self pollination and from non-pollinated flowers of *V. faba* and vetches.

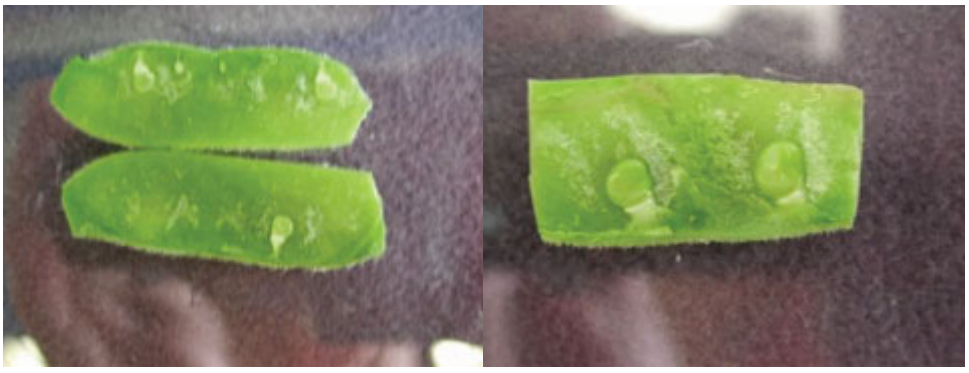

Figure 6. Ovules of *V. narbonensis* without pollination (left) and pollinated with *V. faba paucijuga* vf 172.

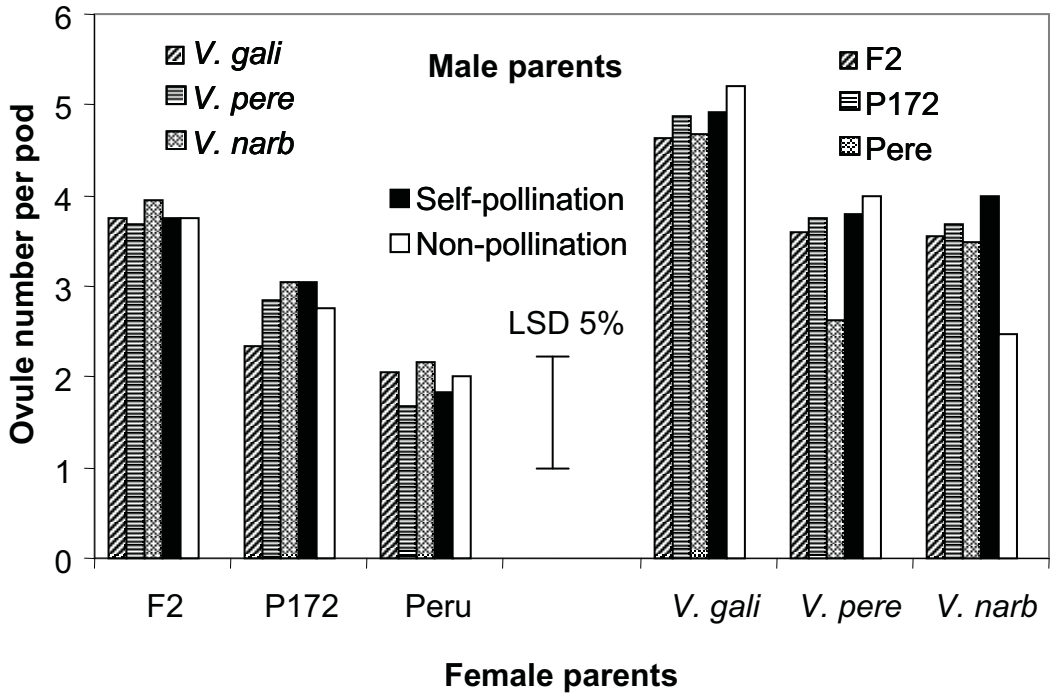

Figure 7. Number of ovules per pod of 8 to 10 days old pods derived from interspecific pollination, self pollination and from non-pollinated flowers of *V. faba* and vetches.

4 Discussion

4.1 Experiment 1 (Selection of parents)

Vicia narbonensis is considered by many authors to be phenotypically the closest wild relative of faba bean (Hanelt et al. 1972, Maxted 1992, Schäfer 1973). Other authors (Zohary and Hopf 1973) tentatively suggest that another member of the complex, *V. galilea,* may be the faba bean ancestor because it is phenotypically also very similar to *V. faba.* Our observations on the phenotypical characters corroborated these finding. The *V. narbonensis* complex species (*V. narbonensis, V. galilea*, and *V. serratifolia*) had a convincingly higher phenotypical similarity to *V. faba* than the other vetch species.

As has been reported by Cubero (1982), the vetches related to *V. faba* contain about half as much DNA in the nucleus compared to the faba bean. Similar results have been found in the present experiment by using a flow cytometer to estimate the DNA content. Cubero (1982) also proposed that the big difference in DNA content may cause a different mean cell doubling time between *V. faba* and its related species. Foster and Dale (1983) observed the abnormalities in early seed development producing from crosses between *Hordeum vulgare* and *H. bulbosum.* They argued that the abnormalities were caused by difference of the mean cell doubling time of parental embryos. Such difference could prevent synchrony in the parental genome and give rise to the mitotic aberrant found. Therefore, it could be interesting to cross faba bean with vetches which have a relatively high DNA content.

The most promosing parental combinations

The walls of the pollen tubes contain callose, which is lacking in the surrounding style tissue. Thus, the pollen tube growth in the style can be clearly followed by use of fluorescence microscopy. It is this callose that takes up aniline selectively and consequently fluorescences when illuminated by blue or ultraviolet light (Kho and Baer 1968). Under a fluorescence microscope the callose exhibits a bright yellow-green fluorescence, contrasting markedly with the dark background. Due to the reliability of this method, pollen tube growth observations have been used in many crops to check their cross compatibility (Akhond et al. 2000, Busmann-Loock 1990, Hamzah et al. 2002, Vervaeke et al. 2002).

Ample pollen tube growth was observed in all *V. faba* stigmas if the flowers were pollinated with *V. faba* cv. Mythos (intraspecific). Hence, there is no intraspecific incompatibility between *V. faba* cv. Mythos and other *V. faba* genotypes. A higher abundance of pollen tubes grew into the style in intraspecific pollinations compared to interspecific pollinations (see Figure 1). The *V. faba* and vetches showed a similar score for pollen tube observation in styles (Table 3). Similar results have been found for the percentage of produced pods (Table 4). The *V. faba* genotypes and vetches produced a similar mean of percentage produced pods. In contrast to these results, other authors reported that the percentage of pod set in *V. faba* x *V. narbonensis* crosses was higher than in reciprocal pollinations (Roupakias 1986, Roupakias and Tai 1986; Lazaridou et al. 1989).

4.2 Experiment 2 (Study of interspecific pollination of selected parents)

In general, this study showed that the pod and ovule performances are more influenced by the female parents than by the male parents. The strong maternal influence on final pod and ovule size was already observed by Davies (1975) in pea. In *V. faba* genotypes, the unpollinated flowers produced more pods compared to the unpollinated flowers of vetches (Figure 2). The *V. faba* genotypes produced small pods even if emasculated flowers were not pollinated. The similar phenomena was observed by Chapman et al. (1979). They observed numerous parthenocarpic pods from emasculated-unpollinated flowers of *V. faba* and treated with an apex removed. This treatment minimised competition within the plant by removing all the normal assimilate sinks. The results on percentage of produced pods in Experiment 2 showed that *V. faba* genotypes were more promising as female parents than the vetch species. The results of Experiment 2 were different

from the results of pollen tube observation and percentage of produced pods test of Experiment 1 (see 4.1). These differences between the two experiments can be explained by the fact that *V. faba* genotypes which had a low performance for pollen tube growth and percentage of produced pods in Experiment 1 were not included in Experiment 2. Similar results were reported by Ramsay and Pickersgill (1986) who pollinated *V. faba* with pollen of other *Vicia* species (*V. bithynica, V. galilea, V. narbonensis* and *V. johannis*). They found that pollen tubes were able to germinate and penetrate the stigma, but in pollinations of *V. bithynica, V. galilea* and *V. johannis* with *V. faba*, pollen tubes became arrested in these styles.

Eight to ten days after pollination, the *V. faba* genotypes produced smaller and lighter pods compared to vetches (Figure 3 and 4). The difference of pod size is discussed in connection to cell cycle time. The cell cycle time is defined as the time needed for one cell to divide into two cells. Roupakias (1986) reported that *V. narbonensis* was at least four days faster in cell cycle time of endosperm and embryo development than *V. faba*. Therefore, the *V. faba* genotypes had smaller and lighter pods than the vetch species at the same period after pollination.

Both, self pollination and interspecific pollination produced bigger pods and ovules than non-pollinated pod (Figure 7). This fact demonstrated that pollen of other species stimulated the pod and ovule development. The growth of pollen tubes into ovules were observed in our Experiment 1. Some researchers have observed the double fertilization in interspecific *Vicia* crosses, but thereafter the fertilized cells did not grow (Roupakias 1986, Lazaridou et al. 1989, Ramsay et al. 1984 and Zenkteler et al. 1998). It is assumed that the fertilized cells initiated the synthesis of some important phytohormones for pod development e.g. gibberellic

acid (GA_3) as well as auxin. Garcia-Martinez and Carbonell (1980) argued that plant hormones such as auxin and gibberellin originating from pollen and/or the pollination event play an important role in signalling processes required for further ovary development.

A consideration of the mean values of all parameters showed that the combination of *V. faba* F2 (Hedin x Pietranera) x *V. galilea* produced the highest percentage of produced pods and a superior pod and ovule development followed by *V. faba paucijuga* vf 172 x *V. galilea.* However, intensive efforts in these combinations as well as in others to regenerate interspecific hybrids following phytohormone treatments of flowers and in vitro culture of ovules proved unsuccessful.

From our work, it could be estimated that prezygotic barriers are not the main problem in interspecific hybridizations between *V. faba* and these vetches. In some interspecific combinations, we have observed that pollen tubes could reach the ovules. This was also reported by other researchers (Ramsay and Pickersgill 1986, Roupakias 1986, Zenkteler et al. 1998, Roupkias and Tai 1986). However, after the fertilization of ovules obviously postzygotic barriers prevent further embryo development. Pickersgill et al. (1983) reported that the endosperm is a major site at which interspecific hybridization may be blocked. Their observation of interspecific hybridization of *V. faba* showed that the endosperm was abnormal from the time of its first nuclear division. Cubero (1982) reported that intergeneric endosperm cells show many chromosomal abnormalities and formed scattered masses of densely stained nuclei.

It is concluded from this present study that a more detailed study is needed for understanding and overcoming existing postzygotic barriers. The efforts of any subsequent study should be concentrated on the promising results obtained with the pollinations of *V. faba* F2 (Hedin x Pietranera) x *V. galilea* and *V. faba paucijuga* vf 172 x *V. galilea.*

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Curriculum Vitae

Personal Identification

Name:	Andi Wijaya
Nationality:	Indonesian
Date of birth:	3 October 1965
Place of birth:	Pangkal Pinang, Indonesia
Marital Status	Married since 1991 and has three daughters
Permanent address:	Jalan Enim Blok O No: 3 Palembang, South Sumatra Indonesia
Email:	Andiswijaya@yahoo.com

Educational background

1971-1977	Attended and finished Primary School, Palembang
1977-1981	Attended and finished Junior High School, Palembang
1981-1985	Attended and finished Senior High School, Palembang
1985-1990	Studied at the Faculty of Agriculture, University of Sriwijaya, Palembang-Indonesia, (Degree: Ir. Equal to BSc.)
1998-2000	Post-Graduate program on integrated aspects of tropical agriculture and forestry science, Georg-August University, Göttingen, Germany (Degree: Master of Science: MSc.)
2000- Now	Ph.D. student at institute of Plant Production and Plant Breeding University of Göttingen, Germany