

## Simulation and Assessment of Possible Environmental Risks Associated with Release of Genetically Modified Peas (*Pisum sativum* L.) into Environment in Central Europe

RADMILA DOSTÁLOVÁ<sup>1</sup>, MAREK SEIDENGLANZ<sup>2</sup> and MIROSLAV GRIGA<sup>3</sup>

<sup>1</sup>Department of Grain Legumes, <sup>2</sup>Plant Protection Department, <sup>3</sup>Plant Biotechnology Department, AGRITEC, Ltd., Šumperk, Czech Republic

**Abstract:** Environmental risks connected with the release of genetically modified peas into the environment were studied in simulated field experiments. They included (1) an assessment of pollen transfer and the rate of natural outcrossing between commercial peas (*Pisum sativum*), (2) the incidence and behaviour of insects visiting pea flowers and their role as potential pollinators, and (3) the inventory of other insect taxa in pea crop (not related directly to pea reproduction organs). Field trials were established (2001–2003) with two non-GM pea cultivars differing in flower colour, seed coat colour and whole plant habit that were grown in close proximity. Cv. Zekon with recessive traits served as a trap variety, cv. Arvika with dominant traits as a pollen donor. The seeds of the trap variety were completely harvested and sown each successive year (2002, 2003) to monitor the incidence of dominant traits in F<sub>1</sub> generation. In the case of outcrossing, dominant traits would occur already in F<sub>1</sub> generation. However, the occurrence of plants with dominant traits in the progeny of the trap variety was not recorded in any case during the whole experimentation period (ca 40 thousand F<sub>1</sub> plants screened each year). Based on the obtained data we assume that the probability of outcrossing in recent commercial peas is extremely low (zero in our experiments). Among insect species visiting regularly pea flower buds/flowers (pests, pollinators) the most frequent were pea weevil (*Bruchus pisorum*), pea aphid (*Acyrtosiphon pisum*), pea thrips (*Kakothrips robustus*), honey-bee (*Apis mellifera*), bumble-bees (*Bombus* sp.). *Bruchus pisorum* is a possible candidate for pollen transfer in unopened pea flowers. A list of insect taxa occurring in pea fields was created on the basis of the four-year monitoring (2001–2004).

**Keywords:** pea; *Pisum sativum*; transgenic plants; insect pollinators; outcrossing; environmental risks

As compared to soybean, which represents a model species for genetic transformation within grain legume crops and whose herbicide-tolerant and insect-resistant cultivars have recently been grown widely in the USA and Argentina (CHRISTOU 1997; GRIGA *et al.* 2001; JAMES 2003), the progress of pea transformation and transgenic line commercialisation is rather limited. The first reports on a transient expression of foreign DNA in pea tissue cultures (without plant regeneration) were published at the end of the eighties of the last century, the principal breakthrough was done in 1990,

when the first fertile transgenic pea plants were obtained via *Agrobacterium*-mediated transformation (PUONTI-KAERLAS *et al.* 1990). Later the same authors confirmed the stable transfer of transgenes to two following seed generations (PUONTI-KAERLAS *et al.* 1992). During the nineties – besides the optimisation of pea transformation protocols – the first successful modifications of pea with “useful” genes (herbicide-tolerance, insect-resistance, virus-resistance) were reported (SCHROEDER *et al.* 1993, 1995; SHADE *et al.* 1994; GRANT *et al.* 1995; BEAN *et al.* 1997; JONES *et al.* 1998). Recently, this research

resulted in a successful proof of pea insect and virus resistance in the field conditions (MORTON *et al.* 2000; TIMMERMAN-VAUGHAN *et al.* 2001). Although the field testing of GM peas has been carried out at least since the end of the nineties of the last century (Australia, New Zealand, Canada), there is a lack of studies dealing with environmental risks connected with GM pea growing (POLOWICK *et al.* 2002). Hypothetically, the main risk may consist in unintended pollen transfer from GM peas to wild relatives (taxonomically related species) or commercially grown peas resulting in cross-pollination and uncontrolled transgene spreading. From this aspect, critical factors are (1) pea flower biology, i.e. mechanisms of pollination/fertilisation, (2) factors improving the probability of successful cross-pollination – here, the insects visiting pea flowers (LOENNIG 1985; CLEMENT *et al.* 1988; CLEMENT 1992), (3) sexual (reproductive) compatibility/incompatibility of pea with its wild relatives (CONNER *et al.* 2003). Finally, a special attention should be paid to other organisms, mainly insects living in pea fields which may potentially be affected by expressed transgenes.

The pea is a self-fertilising, cleistogamous crop – the pollination is completed in closed flower buds (flowers open 24 h after pollination – COOPER (1938)), thus the cross-pollination mediated by honey- or pollen-collecting insects is very low (less than 1% – GRITTON (1980)). Allogamy sometimes occurred under subtropical or tropical conditions (GOVOROV 1928; HARLAND 1948). In Central Europe, there are not any naturally occurring wild *Pisum* species/forms crossable with cultivated pea (*Pisum sativum* L.). These primitive and wild peas (*P. fulvum*, *P. elatius*, *P. humile*) occur in the Near East, Ethiopia and Central Asia in the centres of the genus *Pisum* evolution and domestication. All wild *Pisum* species/forms have a diploid chromosome number  $2n = 14$  and they are crossable – at least unidirectionally – with cultivated pea – the  $F_1$  progeny is fertile or semi-sterile (BEN-ZE'EV & ZOHARY 1973; SMARTT 1979; GOLUBEV & YANKOV 1982; GANTOTTI & KARTHA 1986; ERRICO *et al.* 1991, 1996; CONICELLA & ERRICO 1993; DAVIES 1993; ELVIRA-RECUENCO & TAYLOR 1998). However, the majority of reported hybrids were obtained after artificial (manual) hybridisation, sometimes with the use of another wild *Pisum* form as a bridging cross (WROTH 1998). The wild forms do not usually survive in climatic conditions of Central or Western Europe in the case of occasional introgression. In contrast, there

is an evidence in the literature on a certain extent of natural cross-pollination within cultivated peas (GOVOROV 1928; HARLAND 1948; OLIVEIRA 1963; LOENNIG 1983, 1984, 1985; GRITTON 1980; GIORDANO *et al.* 1991; Tyller (SELGEN Ltd. Prague, Breeding Stn. Chlumec nad Cidlinou, CR) – unpublished breeder's observations; Liška (SELGEN Ltd. Prague, Breeding Stn. Lužany, CR) – personal communications). The cultivated peas do not cross naturally with other cultivated (genera *Phaseolus*, *Vicia*, *Glycine*, *Lathyrus*) or wild European species of the family *Fabaceae*. In an effort to expand the gene pool available for use in *Pisum*, GRITTON (1969) tried to cross some of the genera of the tribe *Vicieae* with *Pisum*. The intergeneric crosses involved *Cicer arietinum*, *Lathyrus aphaca*, *L. sativus*, *L. cicera*, *L. ochrus*, *L. inconspicuus*, *L. cholranthus*, *L. hirsutus*, *L. annus*, *L. clymenum*, *L. szowitzii*, *L. articulatus*, *Vicia angustifolia*, *V. ervilia*, *V. sativa*, *V. faba*, *Lens culinaris*. All pollinations included *Pisum* as the female component and in some cases reciprocal pollinations were made. The only pollination which resulted in any observable carpel or ovule development was *P. sativum* × *V. faba*. Later experiments led only to a few cell divisions and embryo abortion at a very early stage (GRITTON & WIERZBICKA 1975). Promising results of ZENKTELER (1980) with *in vitro* pollination of *Pisum sativum* and *Lathyrus odoratus* were not followed by hybridisation attempts. Recently, heterokaryons or hybrids between *Pisum* and *Lathyrus* were reported as products of intergeneric protoplast fusion (DURIEU & OCHATT 2000) or manual cross-pollination (OCHATT *et al.* 2004).

Recently, in AGRITEC we have produced several tens of transgenic pea lines ( $T_1$  to  $T_3$  generations) modified by model constructs (containing reporter gene *gus-int* and selection marker genes *nptII* or *bar*), which are tested in laboratory and greenhouse conditions (ŠVÁBOVÁ *et al.* 2002, 2004, 2005). The pea lines with resistance to virus diseases (PSbMV, PEMV) are under development. As we plan to evaluate the above-mentioned GMOs in field conditions (i.e. release into the environment according to Czech Directive 78/2004), we started research simulating potential environmental risks of GM peas in the natural conditions of the Czech Republic (DOSTÁLOVÁ *et al.* 2004; RAKOUSKÝ *et al.* 2004). The aim of research was (1) to estimate the extent of cross-pollination among commercial peas, (2) to assess the potential role of insect pollinators in pea pollen transfer, and (3) to monitor the spectrum of other organisms, mainly insects

occurring in pea fields which may potentially be affected by expressed transgenes.

## MATERIAL AND METHODS

**Assessment of cross-pollination among commercial peas in field conditions.** To investigate possible cross-pollination within commercial pea cultivars, field experiments were carried out in 2001–2003 at Šumperk location, Czech Republic, with two non-GM pea cultivars. Easily distinguishable dominant and recessive traits were used for easy and quick discrimination of hybrids.

Cv. Zekon – *Pisum sativum* ssp. *sativum* L.; dry-seeded, white-flowering (anthocyan inhibition, recessive gene *a*), colourless seed coat (recessive gene *pallens pal*), green cotyledons (recessive gene *i*), reduced foliage (semi-leafless type, recessive gene *afila af*). Cv. Zekon was used as a trap variety.

Cv. Arvika – *Pisum sativum* ssp. *arvense* L.; fodder, violet-flowering (with anthocyan, dominant gene *A*), coloured seed coat with violet spots (dominant gene *violaceopunctata F*), yellow cotyledons (dominant gene *I*), standard foliage (dominant gene *Af*). Cv. Arvika was used as a pollen donor.

Flowering periods of both cultivars overlap thus the prerequisite for hypothetical pollen transfer is fulfilled. The experimental design is illustrated in Figure 1A. Two 5 m<sup>2</sup> (1.25 × 4 m, 80 seeds in a row, 6 rows per plot, 20 cm row distance, 60 cm plot distance) plots of the trap cv. Zekon were surrounded by ten 5 m<sup>2</sup> plots of the pollen donor cv. Arvika and *vice versa*. Standard fertilisation and integrated plant protection was applied during vegetation. The harvest of seeds of both cultivars was carried out separately. All harvested seeds of cv. Zekon were sown each successive year (square ca 430 m<sup>2</sup>; Figure 1B) and the occurrence of F1 hybrids expressing dominant genes was recorded (40 thousand F1 plants evaluated yearly). In the case of cross-pollination, dominant traits should be expressed already in F<sub>1</sub> generation.

**Monitoring of insects visiting and damaging pea flower buds/flowers.** During the vegetation, the incidence of potential insect pollinators was monitored by four methodological approaches: beating down, sweeping, glue strips and direct counts of individuals per certain unit (e.g. one plant; one inflorescence, one flower, etc.). Insect monitoring was carried out both in the above-men-

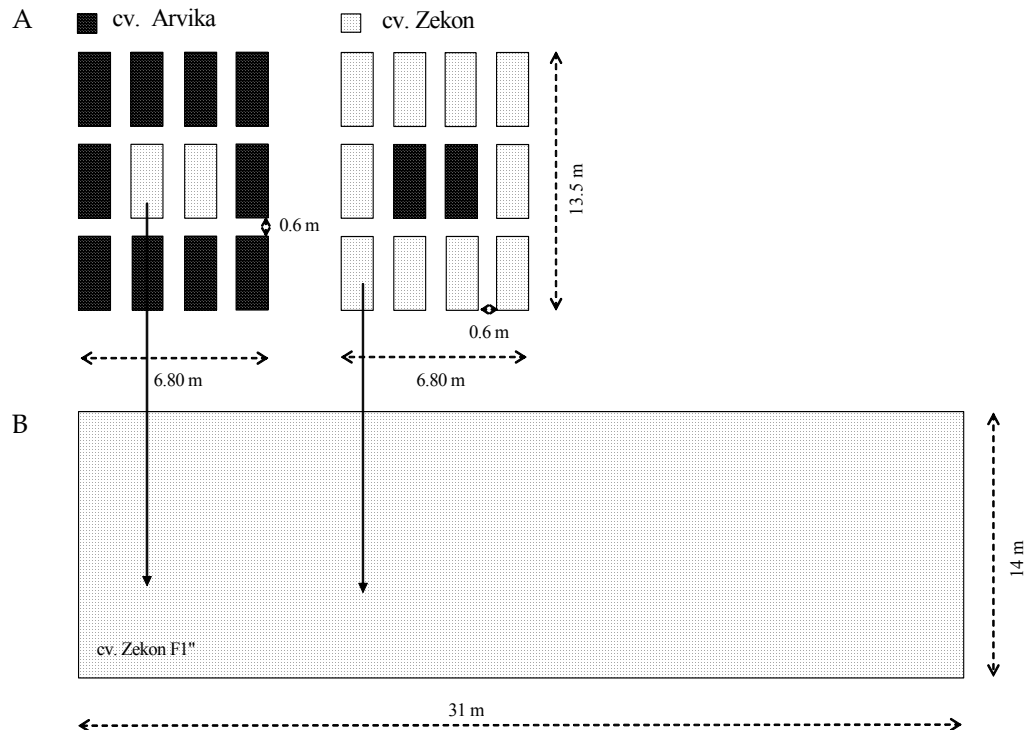


Figure 1. Experimental design of field experiments monitoring pea pollen dispersal from donor cv. Arvika with dominant marker genes to trap cv. Zekon with recessive marker genes. (A) "First" year (2001, 2002) experiments with parental varieties. (B) "Second" year (2002, 2003) experiments with F1 plants of cv. Zekon, potentially containing hybrid plants (For details see Material and Methods)

tioned trials and in neighbouring pea fields of the same location. An emphasis was laid on pea pests damaging pea reproductive structures (*Bruchus pisorum*, Coleoptera, Chrysomelidae; *Acyrtosiphon pisum*, Homoptera, Aphididae; *Kakothrips robustus*, Thysanoptera, Thripidae).

#### **Inventory of other insects occurring in pea crop.**

The occurrence of the insect taxa was monitored in 2001–2004: firstly, insect pests infesting various organs of the pea plant, and secondly, insect species occurring regularly and abundantly in the crop, but without direct trophic relations to pea. The used methods of monitoring were sweeping, direct counting of individuals per certain units (e.g. one plant) and recording of injury symptoms (as a consequence of the presence of some phytophagous species) on the various parts of the pea plant.

Besides the above-described group of insects, the carabids (and other ground beetles) were monitored both in the above-mentioned trials and in neighbouring pea fields of the same location. Insects were caught in pitfall traps, plastic pots 75 mm in diameter and 8 cm in height. The pots were buried into the soil, with the rim at the soil surface. Half of the traps were equipped with safety screens as

protection against rain and sunshine. A few clods of soil on the bottom of the traps provided a shelter for trapped individuals. The traps were emptied at five-day or seven-day intervals. 4–8 traps were used for monitoring during the vegetation of pea every year.

## **RESULTS**

### **Assessment of cross-pollination among commercial peas in field conditions**

The analysis of potential pea hybrids was carried out in 40 thousand F1 pea plants of cv. Zecon (as a female component) in 2002 and 2003. Despite of the incidence of several insect species visiting pea reproductive organs (see the text below), no plants expressing dominant genes of the studied traits were recorded within the F<sub>1</sub> population either in 2002 or 2003. The meteorological data 2001–2004 are shown in Figure 2, the flowering periods of both studied cultivars in Table 1. The existence of many complicated relationships between the development of some meteorological elements and life processes (e.g. longevity of hibernation,

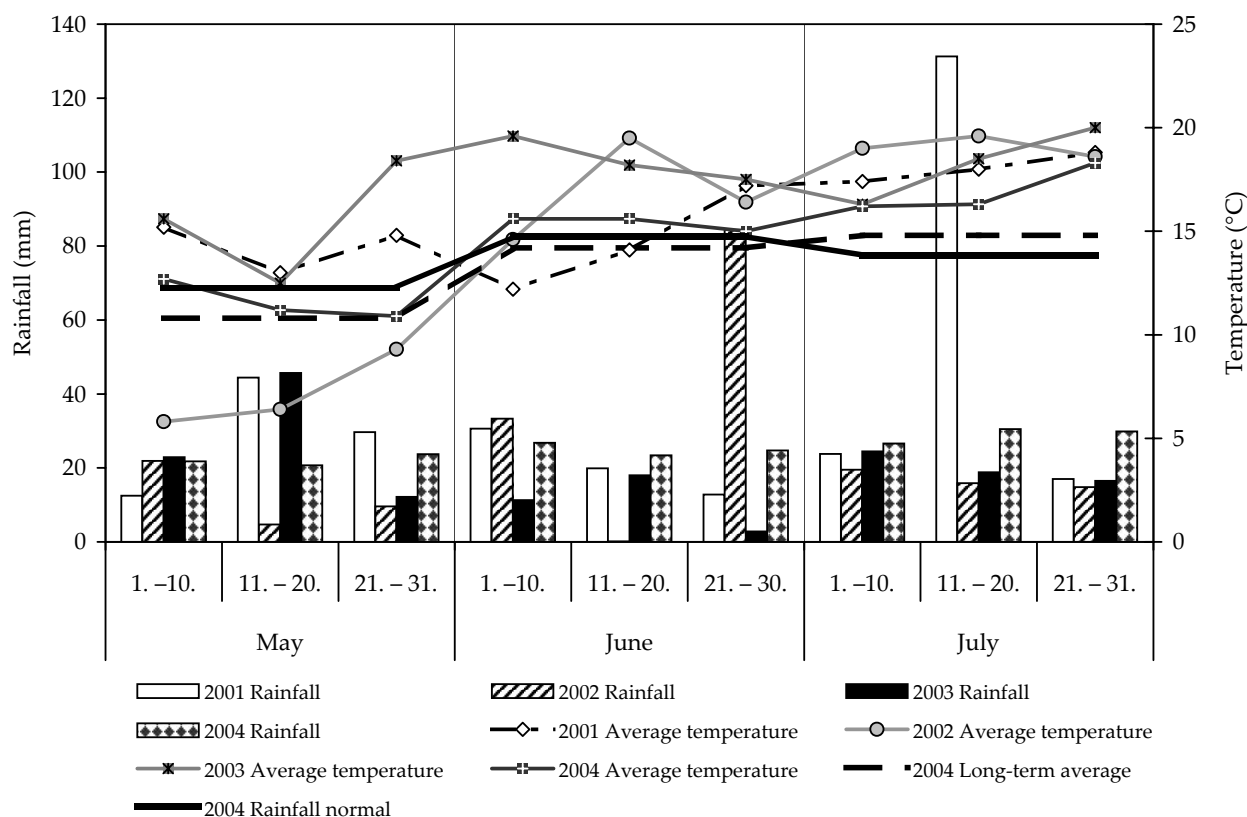


Figure 2. Rainfall sums and temperatures in the period May–July (2001–2004). The values represent the means of decades in particular months, long-term average temperatures and long-term rainfall normal

Table 1. The flowering periods of both pea cultivars included in field experiments

Experiment*	Year of observation	Cultivar	Date of sowing	Start of flowering	End of flowering
A	2001	Zekon	28.4.	25.6.	5.7.
		Arvika	28.4.	30.6.	11.7.
	2002	Zekon	4.4.	6.6.	19.6.
		Arvika	4.4.	11.6.	25.6.
	2002	Zekon	11.4.	13.6.	26.6.
		Arvika	11.4.	17.6.	29.6.
B	2002	Zekon	4.4.	7.6.	19.6.
	2003	Zekon	23.4.	13.6.	23.6.

\*For the design of experiments (A, B) see Figure 1

reproductive behaviour, activity in general, the ability for hunting for forage/prey, etc.) of many species and other taxa of insects is satisfactorily validated. A possibility of recording *Bruchus pisorum* imagoes and for instance many members of *Homoptera* on pea flowers is much higher during a warm sunny afternoon than in the course of a cold and cloudy day. The rate of reproduction of the pea aphid (*Acyrtosiphon pisum*) colonies is in close correlation with the pattern of mean daily temperatures (1 new generation per 10 days when mean daily temperatures reach 20°C).

#### Monitoring of insects visiting and damaging pea flower buds/flowers

The abundance of adult pea weevils (*Bruchus pisorum*, *Coleoptera*, *Chrysomelidae*) was very low in the pea trials during 2002 and 2003 although the beetles were recorded in the crop from the beginning of flowering (or already from the stage when the first flower buds have visible white petals) (Table 2). The pea weevil abundance was higher in the pea trials in 2004. The first individuals were monitored at the beginning of flowering (the first buds in the lowest layer were opened or almost opened) (Table 2). The first orange-yellow eggs occurred on small pods (25–30 mm) and rarely on the calyces of pods approximately 10 days after the crop infestation with imagoes. The period during which new eggs were still found on plants in the experimental locality was about 4 weeks long. In that period adult beetles changed places of feeding during the trial (several varieties). They were observed on opened flowers and also on closed flower buds. The beetles made small linear holes

into the base part (calyx) of flowers. But this was observed very sporadically and on opened flowers only.

The abundance of the pea aphid (*Acyrtosiphon pisum*, *Homoptera*, *Aphididae*) had a quite stable pattern in 2002–2004. The difference in the time of recording the first females on pea plants was not more than 14 days in particular years (Table 2). The invasion of viviparous winged females to the pea crop was not of mass character but it was a slow and quite continuous process. The rate of reproduction of individuals present on pea plants was of crucial importance for the resulting abundance of the aphid in the crop. The rates of population growth were only slightly different in the course of the compared seasons (Table 2). The beginning of the fast growth of aphid population (the only separated colonies) coincided with the onset of flowering in 2002–2004. The aphid colonies are localised especially in the area of the growth apex where the flower buds at various stages of development are placed. The aphids suck from the tissues of the buds, but it does not seem to be probable, they are able to penetrate toward generative organs inside the flower buds. The aphids were not found inside the flowers until they opened. The movement of an individual seems to be minimal during its whole development, which concerns the possibility of pollen transfer from plant to plant. Additionally, the winged females were not observed inside the flowers at all.

Differences in the occurrence of *Thysanoptera* in the pea crop in 2002–2004 are documented in Table 2. *Kakothrips robustus* was a clearly predominating species. Larvae and imagoes were present together at the time shortly before the first flow-

Table 2. The list of insect taxa visiting and damaging pea flower buds/flowers at locations Šumperk-Vikýřovice and Šumperk-Temenice in 2002–2004

Species	Family	Order	Year of monitoring	Level of infestation
<i>Bruchus pisorum</i>	<i>Chrysomelidae</i>	<i>Coleoptera</i>	2002	1 beetle per 25 sweeps; less than 1% of seeds infested by larvae of the pest
			2003	maximally 8 beetles per 25 sweeps; less than 1% of seeds infested by larvae of the pest
			2004	20 beetles per 25 sweeps (sunny days); 10% of seeds infested by larvae of the pest
<i>Acyrtosiphon pisum</i>	<i>Aphididae</i>	<i>Homoptera</i>	2002	first winged viviparous females recorded at the end of May the level of 3–5 individuals per plant reached on 28. 5. 2002
			2003	first winged viviparous females recorded at the beginning of June the level of 3–5 individuals per plant reached on 5. 6. 2003
			2004	first winged viviparous females recorded at the beginning of June the level of 3–5 individuals per plant reached on 8.–12. 6. 2004
<i>Kakothrips robustus</i> and other <i>Thysanoptera</i>	<i>Thripidae</i>	<i>Thysanoptera</i>	2002	below 20% of plants infested with the pest during the stage of flowering
			2003	40% of plants infested with the pest during the stage of flowering (1–8 individuals per generative part of an infested plant)
			2004	40–60% of plants infested with the pest during the stage of flowering
<i>Apis mellifera</i> <i>Bombus</i> sp.	<i>Apidae</i>	<i>Hymenoptera</i>	2002–2004	frequent visitors of fully opened flowers of pea
<i>Melitta leporina</i>	<i>Melitidae</i>	<i>Hymenoptera</i>	2002–2004	frequent visitor of fully opened flowers of pea
<i>Andrena</i> sp. <i>Panurgus</i> sp.	<i>Andrenidae</i>	<i>Hymenoptera</i>	2002–2004	frequent visitors of fully opened flowers of pea

ers opened. They were localised along the growth apex – especially in various folds of flower buds and stipule bases. They were also found in open flowers. In 2004, winged imagoes were found to change the host plants. But they are not probably capable of getting into flower buds before their opening.

During the flowering period the pea crop was visited abundantly by several members of the taxon *Apoidea* (in each year of monitoring). The most abundant were *Apis mellifera* (*Apidae*, *Hymenoptera*) and *Andrena* sp. (*Andrenidae*, *Hymenoptera*). The other members of the taxon *Apoidea* recorded in pea are listed in Table 2. However, it is obvious

this group of insects is entirely attracted by fully opened flowers.

#### Inventory of other insects occurring in pea crop

In all years of monitoring (2001–2004), practically from the beginning of emergence of pea plants it was possible to observe the first indications (U-shaped notches on leaves and stipules) of the presence of *Sitona* beetles (*Curculionidae*; *Coleoptera*). *Sitona lineatus* was a dominant species of this genus. Overwintered imagoes (1<sup>st</sup> generation) frequently cause a significant reduction of the assimilation area of pea leaves and stipules. From the begin-

Table 3. The list of insect taxa which regularly occurred in the pea crop at locations Šumperk-Vikýřovice and Šumperk-Temenice in 2001–2004

Species	Family	Order	Relation of the time of occurrence to the growth stage of the plant	Relation of the occurrence to a certain place on the plant
<i>Lygus rugulipennis</i> <i>Lygus pratensis</i> <i>Calocoris norvegicus</i> <i>Adelphocoris seticornis</i>	<i>Miridae</i>	<i>Heteroptera</i>	during the second half of flowering to the beginning of ripening	especially upper parts of plants
<i>Carpocoris fuscispinus</i>	<i>Pentatomidae</i>	<i>Heteroptera</i>	from flowering to harvest	especially upper parts of plants
<i>Coreus marginatus</i>	<i>Coreidae</i>	<i>Heteroptera</i>	from flowering to harvest	especially upper parts of plants
<i>Empoasca</i> sp.	<i>Cicadellidae</i>	<i>Auchenorrhyncha</i>	ripening, to harvest	above the ground and on weeds
<i>Psammotettix</i> sp.	<i>Cicadellidae</i>	<i>Auchenorrhyncha</i>	ripening, to harvest	above the ground and on weeds
<i>Apis mellifera</i> <i>Bombus</i> sp.	<i>Apidae</i>	<i>Hymenoptera</i>	in the course of flowering	open flowers
<i>Melitta leporina</i>	<i>Melittidae</i>	<i>Hymenoptera</i>	at the end of flowering	open flowers
<i>Andrena</i> sp. <i>Panurgus</i> sp.	<i>Andrenidae</i>	<i>Hymenoptera</i>	during flowering	open flowers
<i>Aphidius ervi</i>	<i>Braconidae</i>	<i>Hymenoptera</i>	after the infestation of pea with <i>A. pisum</i>	parasitoid of <i>A. pisum</i>
<i>Triaspis</i> sp.	<i>Braconidae</i>	<i>Hymenoptera</i>	frequent in seeds infested with <i>B. pisorum</i>	parasitoid of <i>B. pisorum</i>
<i>Aphis fabae</i>	<i>Aphididae</i>	<i>Homoptera</i>	during flowering	especially on tendrils and on the weed ( <i>Chenopodium album</i> )
<i>Scaeva pyrastris</i> <i>Epistrophe balteata</i>	<i>Syrphidae</i>	<i>Diptera</i>	during flowering	larvae – in colonies of <i>A. pisum</i> ; imagoes – on flowers
More than 10 various species of <i>Diptera</i>	–	<i>Diptera</i>	during the whole vegetation	upper parts of pea plants
Complex of species <i>Chrysopa carnea</i>	<i>Chrysopidae</i>	<i>Neuroptera</i>	during flowering	nearby to <i>A. pisum</i> colonies
<i>Coccinella septempunctata</i> <i>Adonia variegata</i>	<i>Coccinellidae</i>	<i>Coleoptera</i>	during flowering	larvae and imagoes – nearby (or inside) <i>A. pisum</i> colonies
<i>Melanchra pisi</i> <i>Melanchra persicariae</i> <i>Mamestra brassicae</i> <i>Autographa gamma</i>	<i>Noctuidae</i>	<i>Lepidoptera</i>	during the whole vegetation	without exact relation
<i>Aglais urticae</i>	<i>Nymphalidae</i>	<i>Lepidoptera</i>	at the end of flowering	without exact relation
<i>Cantharis fusca</i> <i>C. lateralis</i>	<i>Cantharidae</i>	<i>Coleoptera</i>	at the end of flowering	without exact relation
<i>Forficula auricularia</i>	<i>Forficulidae</i>	<i>Dermaptera</i>	at the end of vegetation, before and in the course of harvesting	without exact relation

Table 3 to be continued

Species	Family	Order	Relation of the time of occurrence to the growth stage of the plant	Relation of the occurrence to a certain place on the plant
<i>Amara</i> spp.				
<i>Anchonemus dorsalis</i>				
<i>Bembidion lampros</i>				
<i>Calathus fuscipes</i>				
<i>Carabus granulatus</i>	<i>Carabidae</i>	<i>Coleoptera</i>	during the whole vegetation	pitfall traps
<i>Carabus scheidleri</i>				
<i>Harpalus affinis</i>				
<i>Poecilus cupreus</i>				
<i>Pseudoophonus rufipes</i>				
<i>Pterostichus melanarius</i>				
<i>Trechus quadristriatus</i>				
More than five species of <i>Staphylinidae</i>	<i>Staphylinidae</i>	<i>Coleoptera</i>	during the whole vegetation	pitfall traps
Several species of <i>Collembola</i>	–	<i>Collembola</i>	during the whole vegetation	pitfall traps
Several other species of <i>Thysanoptera</i>	–	<i>Thysanoptera</i>	during flowering	on leaves, stipules and generative organs

ning of June infested bacterial nodules with *Sitona* larvae were observable on the roots (the first finds of larvae on the roots usually coincided with the beginning of flowering). A majority of the infested root nodules was abandoned at the end of July. Imagoes of the 2<sup>nd</sup> generation were recorded in the crop from the second decade of July.

In the course of 2002–2004, the monitoring of *Cydia nigricana* (*Tortricidae*; *Lepidoptera*) flight activity was carried out in sex-pheromone traps. A high occurrence of males was recorded only in 2002. It was possible to identify two peaks of flight activity during the summer of 2002: 17. 6. 2002 (51.3 males per 2 traps and 1 day of flight activity) and 11. 7. 2002 (20.3 males per 2 traps and 1 day of flight activity). The percentage of damaged seeds from untreated plots was in the range of 30–40%. The occurrence of moths in the pea trials conducted in 2003 and 2004 was very low. The flight activity of *Cydia nigricana* has quite a variable pattern, which was evident from a comparison of the records of infestations from particular years and localities.

*Liriomyza* larvae (*Agromyzidae*; *Diptera*) were found inside leaves and stipules of some plants during all years of monitoring (2001–2004). Not more than 10–15% of plants were infested with the pest. The most infested were the leaves from the two bottom layers. Prevalent species were *Liriomyza pisiivora* and *L. strigata*.

A list of insect taxa (other than those visiting targetly pea reproduction organs, some less important insect pests) recorded regularly in the pea crop is given in Table 3.

## DISCUSSION

The cross-pollination of cultivated peas with wild peas in Central Europe is limited due to the absence of natural occurrence of the last mentioned taxa in this region. Natural intergeneric hybrids of *Pisum* with other genera of cultivated grain legumes and wild relatives from the tribe *Vicieae* were not reported (SMARTT 1979), and even experimental intergeneric hybridisation is extremely difficult and it has been only partially successful until now (GRITTON 1969; GRITTON & WIERZBICKA 1975; DURIEU & OCHATT 2000; OCHATT *et al.* 2004). Due to the specific reproductive biology of pea (cleistogamy), it makes a little sense to study pollen transfer from peas to wild leguminous relatives and *vice versa*. Thus, the environmental risks resulting from GM-pea growing and vertical transgene flow via pollen dispersal should be restricted to commercial pea germplasm. Literature data show that a certain degree of natural outcrossing within cultivated peas may be detected (GOVOROV 1928; HARLAND 1948; OLIVEIRA 1963; GRITTON 1980; LOENNIG 1983, 1984, 1985). It seems that the reliability of experi-



mentally obtained data may be affected by several factors – (1) selection of suitable parent genotypes (overlapping flowering periods; better or worse affinity for cross-pollination due to a complex of physiological/biochemical characteristics affecting the pollen-stigma affinity of included genotypes; competition in own versus foreign pollen germination and growth, etc.; easy/difficult scorability of genetic markers demonstrating the hybrid state), (2) spatial distance between the pollen donor and recipient plants, (3) climatic conditions, particularly during the flowering period, (4) incidence of possible insect pollinators.

OLIVEIRA (1963) found 0.98 to 2.19% cross-pollination in peas grown in the south of Brazil. The marker genes *I* (green cotyledons) and *r* (wrinkled seed cotyledons) were strongly affected by the environmental conditions at the harvest time and it was difficult to precisely identify the seed colour (due to bleaching) and seed shape. GIORDANO *et al.* (1991) chose less environmentally affected marker genes (*af* – *afila*, *st* – reduced stipules; cvs. Mikado and Majestic – *AfAfStSt*, cv. Filby – *afafstst*) in the field experiments (Brazil) arranged as alternated rows or concentric circles. In the circular design, the rate of cross-pollination decreased with the distance of spatial isolation: 0.24% at 1 m, 0.14% at 2 m, 0.12% at 3 m. In alternated row planting with a row spacing of 1 m the cross-pollination increased to 0.80%. The only data on transgene flow from field-grown GM-peas were published by POLOWICK *et al.* (2002). The trap cvs. Montana, Carneval and Tipu exhibited the recessive traits semi-leafless (*af*) and absence of *gusA* gene, GM cv. Greenfeast had a dominant normal leaf form (*Af*) and was homozygous for the *gusA* gene. During the experimentation, a high frequency of normal leaf forms not associated with GUS activity was recorded (e.g. 10.4% in cv. Tipu) – later it was proved that commercial seed lots of cv. Tipu displayed a significant heterogeneity (both normal and semi-leafless plants included). Thus, the use of the dominant normal leaf form was considered as unreliable as a sole indicator of outcrossing. If both dominant traits were strictly taken into account, the mean frequency of outcrossing over a two-year trial was 0.07% (Montana 0.11%, Tipu 0.09%, Carneval 0%). Our results (i.e. 0% outcrossing) are more similar to those of POLOWICK *et al.* (2002) as compared to those of OLIVEIRA (1963) and GIORDANO *et al.* (1991). Despite of the selection of different pea varieties (and marker genes) for ex-

periments and different designs of field trials, an important role may be played by significantly different geographic and climatic conditions (Canada and Czech Republic – temperate zone, ca 50 to 55°C latitude versus Brazil – tropical to subtropical zone, ca 0 to 30° latitude) connected with different spectra of potential insect pollinators (see below). GOVOROV (1928) and HARLAND (1948) also found a higher tendency to cross-fertilisation in peas under subtropical (Afghanistan) and tropical conditions (Peru). LOENNIG (1983) reported the frequency of cross-fertilisation 1.20–2.39% with the use of two fasciated pea lines as the trap plants – the fact that fasciated plants tend to have more flowers open at a time than normal type plants is probably a reason for higher cross-pollination (by bumble-bees) as compared to normal pea plants. The comparison of cross-fertilisation in two locations differing in the incidence of insect pollinators did not result in a dramatically higher cross-pollination rate in the insect-rich location (1.92% versus 1.20%) (LOENNIG 1984). *Bombus* species were likely the candidates for pollen transfer (LOENNIG 1983, 1984, 1985).

According to the Czech pea breeders (TYLLER; LIŠKA – personal communications), extremely high temperatures during the flowering period may cause an outcrossing as a result of precocious flower opening, i.e. still before the self-pollination is fully accomplished. In addition, some pea cultivars tended to be more sensitive to outcrossing than others – e.g. German white-flowering fodder pea cv. Edit exhibited a higher rate of outcrossing, especially when grown in the proximity of violet-flowering fodder pea. Another factor which may increase the outcrossing rate would be a pesticide treatment immediately before flowering and in the course of flowering period.

Recently, BOGDANOVA and BERDNIKOV (2000) tried to explain (in a greenhouse experiment) a possible mechanism of pea outcrossing with the use of 218 pea accessions from different parts of the world (Afghanistan, Caucasus, Balkans, Asia Minor, Ethiopia, Dnieper Basin, North Russia, Pamirs). Immediately after the flower opened, its keel was cut off and pollen from the tester pea line was applied to the stigma (already covered by its own pollen). Surprisingly high rates of outcrossing (4.4 to 28.6%) were attributed to a possibility that some ovules remained unfertilized until after the pea flower opened.

*Hymenopterous* insects (bees, bumble-bees) usually visit pea flowers mostly opened and thus already

pollinated. The cross-pollination may be realised in this way only exceptionally via better affinity of foreign pollen to the host stigma. In contrast, cross-pollination may be caused by small pests or other insect species which visit undeveloped flowers – aphids (*Acyrtosiphon pisum*, Homoptera, Aphididae), thrips (*Kakothrips robustus*, Thysanoptera, Thripidae), and especially by the pea weevil (*Bruchus pisorum*, Coleoptera, Chrysomelidae). Sexually immature females of the beetle probably must feed on pollen before they can lay eggs (PAJNÍ 1981; PESHO & VAN HOUTEN 1982; CLEMENT 1992). However, the adults of *B. pisorum* also feed (and thus injure) on other parts of the pea flower, notably petals (ANNIS & O'KEEFFE 1984) and female organs (gynaecium) (CLEMENT 1992) and they also suck nectar (PAJNÍ 1987; CLEMENT *et al.* 1988). Both in the field and in laboratory conditions, *B. pisorum* individuals were observed to perforate small holes in the base of the flower calyx (1 to 2 mm in length) and to feed accumulating nectar. Thus, there is a possibility of carrying the pollen on the insect body from pea flower to pea flower. Pea weevils appear in pea fields from the beginning of the flowering period. It means that beetles come into contact with already pollinated/fertilised flowers, and also with flower buds containing ripe pollen and stigmas ready to pollination. Based on studies of mutual relations between the pea host and phytophagous insects, namely pea weevil (HORNE & BAILEY 1991), there exists at least a theoretical possibility of cross-pollination. The role of possible insect pollinators in natural outcrossing of peas needs further research.

The reported inventory of organisms occurring in pea fields may serve as a basis for the risk assessment procedure when GM-pea is released into the environment in climatic conditions of Central Europe.

## CONCLUSION

The results of our field experiments confirmed the working hypothesis as well as some literature data that natural outcrossing in cultural peas was extremely low (zero in our experiments) in climatic conditions of the temperate zone. The factors which may influence the natural outcrossing rate hypothetically positively include the pea genotype, increased temperature during the flowering period, insect pollinators visiting unopened pea flowers and pesticide treatments just before/during the flowering period.

Thus from the aspect of pollen transfer GM-pea represents a negligible or nearly zero environmental risk in climatic conditions of Central Europe and relatively short isolation distances may enable the non-problematic coexistence of GM and non-GM pea cultivation (conventional versus ecological farming). This situation would change if genetic modifications dramatically changed flower biology and mechanisms of pollination/fertilisation in pea (morphology of reproductive structures, increased extent of allogamy).

## References

- ANNIS B.A., O'KEEFFE L.E. (1984): Effect of pollen source on oogenesis in the pea weevil, *Bruchus pisorum* L. (Coleoptera: Bruchidae). *Protection Ecology*, **6**: 257–266.
- BEAN S.J., GOODING P.S., MULLINEAUX P.M., DAVIES D.R. (1997): A simple system for pea transformation. *Plant Cell Reports*, **16**: 513–519.
- BEN-ZE'EV N., ZOHARY S. (1973): Species relationships in the genus *Pisum* L. *Israel Journal of Botany*, **22**: 73–91.
- BOGDANOVA V.S., BERDNIKOV V.A. (2000): A study of potential ability for cross-pollination in pea originating from different parts of the world. *Pisum Genetics*, **32**: 16–17.
- CHRISTOU P. (1997): Biotechnology applied to grain legumes. *Field Crops Research*, **53**: 83–97.
- CLEMENT S.L. (1992): On the function of pea flower feeding by *Bruchus pisorum*. *Entomologia Experimentalis et Applicata*, **63**: 115–121.
- CLEMENT S.L., LATHROP L.A., MUEHLBAUER F.J. (1988): More on the pea's nectaries and insect visitors. *The Pisum Newsletter*, **20**: 3–4.
- CONNER A.J., GLARE T.R., NAP J.-P. (2003): The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. *The Plant Journal*, **33**: 19–46.
- CONICELLA C., ERRICO A. (1993): Embryology of ovule abortion in reciprocal crosses between diploids and tetraploids in *Pisum sativum* and *P. fulvum*. *Journal of Genetics and Breeding*, **47**: 157–162.
- COOPER D.C. (1938): Embryology of *Pisum sativum*. *Botanical Gazette*, **100**: 123–132.
- DAVIES D.R. (1993): The pea crop. In: CASEY R., DAVIES D.R. (eds): *Peas: Genetics, Molecular Biology and Biotechnology*. CAB International, Wallingford, 1–12.
- DOSTÁLOVÁ R., SEIDENGLANZ M., GRIGA M. (2004): Monitoring of environmental risks of genetically modified pea (*Pisum sativum* L.) in CR: model solution. In:

- OVESNÁ J., KUČERA L. (eds): Problems of biological safety, GMOs and international involvement of the Czech Republic. VÚRV Praha-Ruzyně, 25–34. (in Czech)
- DURIEU P., OCHATT S.J. (2000): Efficient intergeneric protoplast fusion between pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.). *Journal of Experimental Botany*, **51**: 1237–1242.
- ELVIRA-RECUENCO M., TAYLOR J.D. (1998): Inheritance of potential race non-specific resistance derived from *Pisum abyssinicum* for the control of pea bacterial blight (*Pseudomonas syringae* pv. *pisi*). In: Proceedings 3<sup>rd</sup> European Conference on Grain Legumes – Opportunities for High Quality, Healthy and Added-Value Crops to Meet European Demands. Valladolid, Spain, 234.
- ERRICO A., CONICELLA C., VENORA G. (1991): Karyotype studies on *Pisum fulvum* and *Pisum sativum* using a chromosome image analysis system. *Genome*, **34**: 105–108.
- ERRICO A., CONICELLA C., DE MARTINO T., ERCOLANO R., MONTI L.M. (1996): Chromosome reconstructions in *Pisum sativum* through interspecific hybridization with *P. fulvum*. *Journal of Genetics and Breeding*, **50**: 309–313.
- GANTOTTI B.V., KARTHA K.K. (1986): Pea. In: EVANS D.A., SHARP W.R., AMMIRATO, P.V. (eds): *Handbook of Plant Cell Culture*, Vol. 4, Techniques and Applications. Macmillan Publ. Comp., New York, 370–418.
- GIORDANO L.B., MARQUES M.R.C., MELO P.E. (1991): Estimates of natural outcrossing in peas in Brasilia – DF. *Horticultura Brasileira*, **9**: 82–83 (in Portuguese).
- GOLUBEV A.A., YANKOV I.I. (1982): Prospects for wide hybridization in pea as related to the problem of sources of resistance to diseases. *Trudy po Prikladnoj Botanike, Genetike i Selekcii*, **71**: 75–78 (in Russian).
- GOVOROV L.I. (1928): The peas of Afghanistan. *Trudy po Prikladnoj Botanike, Genetike i Selekcii*, **19**: 517–522 (in Russian).
- GRANT J.E., COOPER P.A., MCARA A.E., FREW T.J. (1995): Transformation of peas (*Pisum sativum* L.) using immature cotyledons. *Plant Cell Reports*, **15**: 254–258.
- GRIGA M., KOSTURKOVA G., KUCHUK N., ILIEVA-STOILEVA M. (2001): Biotechnology. In: HEDLEY C. (ed.): *Carbohydrates in Grain Legume Seeds: Improving Nutritional Quality and Agronomic Characteristics*. CAB International, Wallingford Oxon, U.K., 145–207.
- GRITTON E.T. (1969): Crosses between *Pisum* and closely related genera. *The Pisum Newsletter*, **1**: 7.
- GRITTON E.T. (1980): Field pea. In: FEHR W.R., HADLEY H.H. (eds): *Hybridization of Crop Plants*. American Society of Agronomy, Madison, Wisconsin, 347–356.
- GRITTON E.T., WIERZBICKA B. (1975): An embryological study of a *Pisum sativum* × *Vicia faba* cross. *Euphytica*, **24**: 277–284.
- HARLAND S.C. (1948): Inheritance of immunity to mildew in Peruvian forms of *Pisum sativum*. *Heredity*, **2**: 263–269.
- HORNE J., BAILEY P. (1991): *Bruchus pisorum* L. (Coleoptera; Bruchidae) control by a knockdown pyrethroid in field peas. *Crop Protection*, **10**: 53–56.
- JAMES C. (2003): Preview: Global status of commercialized transgenic crops: 2003. ISAAA Briefs No. 30. ISAAA, Ithaca, N.Y.
- JONES A.L., JOHANSEN I.E., BEAN S.J., BACH I., MAULE A.J. (1998): Specificity of resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (*Nib*) gene. *Journal of General Virology*, **79**: 3129–3137.
- LOENNIG W.E. (1983): Cross-fertilization in peas. *The Pisum Newsletter*, **15**: 40.
- LOENNIG W.E. (1984): Cross-fertilization in peas under different ecological conditions. *The Pisum Newsletter*, **16**: 38–40.
- LOENNIG W.E. (1985): The peas' nectaries and insect visitors. *The Pisum Newsletter*, **17**: 47–49.
- MORTON R.L., SCHROEDER H.E., BATEMAN K.S., CHRISPEELS M.J., ARMSTRONG E., HIGGINS T.J.V. (2000): Bean  $\alpha$ -amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. *Proceedings of the National Academy of Sciences of the USA*, **97**: 3820–3825.
- OCHATT S.J., BENABDELMOUNA A., MARGET P., AUBERT G., MOUSSY F., PONTÉCAILLE C., JACAS L. (2004): Overcoming hybridization barriers between pea and some of its wild relatives. *Euphytica*, **137**: 353–359.
- OLIVEIRA H.A. DE (1963): Occurrence of allogamy in pea. *Revista de Olericultura*, **3**: 83–90 (in Portuguese).
- PAJANI H.R. (1981): Trophic relations and ecological status of the adults of *Bruchus pisorum* L. and allied field species of *Bruchidae* (Coleoptera). In: LABEYRIE V. (ed.): *The Ecology of Bruchidae Attacking Legumes (Pulses)*. Junk, Dordrecht, the Netherlands, 125–129.
- PAJANI H.R. (1987): Ecological status of host range and polymorphism in *Bruchidae*. In: DONAHAYE E., NAVARRO S. (eds): *Proceedings 4<sup>th</sup> International Working Conference on Stored Product Protection*. Tel Aviv, Agricultural Research Organization, Bet Dagan, Israel, 506–516.
- PESHO G.R., VAN HOUTEN R.J. (1982): Pollen and sexual maturation in the pea weevil (Coleoptera; Bruchidae). *Annals of the Entomological Society of America*, **75**: 439–443.

- POLOWICK P.L., VANDENBERG A., MAHON J.D. (2002): Field assessment of outcrossing from transgenic pea (*Pisum sativum* L.) plants. *Transgenic Research*, **11**: 515–519.
- PUONTI-KAERLAS J., ERIKSSON T., ENGSTRÖM P. (1990): Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. *Theoretical and Applied Genetics*, **80**: 246–252.
- PUONTI-KAERLAS J., ERIKSSON T., ENGSTRÖM P. (1992): Inheritance of a bacterial hygromycin phosphotransferase gene in the progeny of primary transgenic pea plants. *Theoretical and Applied Genetics*, **84**: 443–450.
- RAKOUSKÝ S., ONDŘEJ M., SEHNAL F., HABUŠTOVÁ O., HUSSEIN H.M., OVESNÁ J., KUČERA L., KOCOUREK F., ŘÍHA K., DOSTÁLOVÁ R., SEIDENGLANZ M., TEJKLOVÁ E., GRIGA M. (2004): Transgenic plant products and their introduction into the environment and crop protection systems, a risk assessment. In: NAP J.-P., ATANASSOV A., STIEKEMA W.J. (eds): *Genomics for Biosafety in Plant Biotechnology*. IOS Press, Amsterdam, 173–184.
- SCHROEDER H., SCHOTZ A., WARDLEY-RICHARDSON T., SPENCER D., HIGGINS T. (1993): Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). *Plant Physiology*, **101**: 751–757.
- SCHROEDER H.E., GOLLASCH S., MOORE A., TABE L.M., CRAIG S., HARDIE D.C., CHRISPEELS M.J., SPENCER D., HIGGINS T.J.V. (1995): Bean alpha-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum*). *Plant Physiology*, **107**: 1233–1239.
- SHADE R.E., SCHROEDER H.E., PUEYO J.J., TABE L.M., MURDOCK L.L., HIGGINS T.J.V., CHRISPEELS M.J. (1994): Transgenic pea seeds expressing the  $\alpha$ -amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio/Technology*, **12**: 793–796.
- SMARTT J. (1979): Interspecific hybridization in the grain legumes – a review. *Economic Botany*, **33**: 329–337.
- ŠVÁBOVÁ L., ONDŘEJ V., GRIGA M. (2002): Genetic modification of pea (*Pisum sativum* L.) with the use of multiple shoot culture and *in vivo* regeneration system. In: GRIGA M., ONDŘEJ V., HORÁČEK J. (eds): *Utilization of molecular markers in plant biology, plant breeding and plant genetic resources conservation*. AGRITEC, Šumperk, 207–214 (in Czech).
- ŠVÁBOVÁ L., ONDŘEJ V., HORÁČEK J., GRIGA M. (2004): *Agrobacterium*-mediated pea transformation by *in vitro* and *in vivo* approaches. In: *Proceedings 5<sup>th</sup> European Conference on Grain Legumes – Legumes for the Benefit of Agriculture, Nutrition and the Environment: Their Genomics, Their Products, and Their Improvement*. Dijon, France, 198.
- ŠVÁBOVÁ L., SMÝKAL P., GRIGA M., ONDŘEJ V. (2005): *Agrobacterium*-mediated transformation of *Pisum sativum* *in vitro* and *in vivo*. *Biologia Plantarum*, **49**: 361–370.
- TIMMERMAN-VAUGHAN G.M., PITHER-JOYCE M.D., COOPER P.A., RUSSEL A.C., GRANT J.E. (2001): Partial resistance of transgenic peas to alfalfa mosaic virus under greenhouse and field conditions. *Crop Science*, **41**: 846–853.
- WROTH J.M. (1998): Possible role for wild genotypes of *Pisum* spp. to enhance ascochyta blight resistance in pea. *Australian Journal of Experimental Agriculture*, **38**: 469–479.
- ZENKTELER M. (1980): Intraovarian and *in vitro* pollination. *International Review of Cytology*, Suppl. **11B**: 137–156.

Received for publication February 2, 2005

Accepted after corrections June 13, 2005

## Souhrn

DOSTÁLOVÁ R., SEIDENGLANZ M., GRIGA M. (2005): **Simulace a hodnocení možných environmentálních rizik spojených s uvolňováním geneticky modifikovaného hrachu (*Pisum sativum* L.) do prostředí v podmínkách střední Evropy.** *Czech J. Genet. Plant Breed.*, **41**: 51–63.

Environmentální rizika spojená s uvolňováním geneticky modifikovaného hrachu do životního prostředí byla studována v simulovaných polních experimentech. Pokusy zahrnovaly (1) odhad možností přenosu pylu a rozsah přirozeného cizosprašení mezi komerčními odrůdami hrachu (*Pisum sativum*), (2) výskyt a chování hmyzu navštěvujícího květy hrachu a jejich roli jako potenciálních opylovačů, (3) inventarizaci dalších taxonů hmyzu v porostech hrachu (bez přímého vztahu k reprodukčním orgánům hrachu). V letech 2001–2003 byly založeny polní pokusy se dvěma odrůdami hrachu lišícími se barvou květu, barvou osemení a celkovým habitem rostlin, které byly umístěny v těsné blízkosti. Odrůda Zekon s recesivními znaky sloužila jako recipient pylu, odrůda Arvika s dominantními

znaky jako donor pylu. Semena recipientní odrůdy byla kompletně sklizena a v každém následujícím roce (2002, 2003) vyseta za účelem monitorování dominantních znaků v  $F_1$  generaci. V případě cizosprášení by se již v  $F_1$  generaci objevily dominantní znaky. Výskyt rostlin s dominantními znaky v potomstvu recipientní odrůdy však nebyl zaznamenán ani v jednom roce zkoušení (každoročně bylo hodnoceno asi 40 tisíc  $F_1$  rostlin). Na základě získaných dat můžeme konstatovat, že pravděpodobnost cizosprášení u současných komerčních odrůd hrachu je extrémně nízká (v našich experimentech nulová). Mezi druhy hmyzu pravidelně navštěvující květní poupata/květy (škůdci, opylovači) se nejčastěji vyskytovali zrnokaz hrachový (*Bruchus pisorum*), kyjatka hrachová (*Acyrtosiphon pisi*), třásněnka hrachová (*Kakothrips robustus*), včela medonosná (*Apis mellifera*) a čmeláci (*Bombus* sp.). Možným kandidátem na přenos pylu v uzavřených květech hrachu je *Bruchus pisorum*. Na základě čtyřletého monitoringu (2001–2004) byl zpracován seznam hmyzích taxonů vyskytujících se v porostech hrachu.

**Klíčová slova:** hrách; *Pisum sativum*; transgenní rostliny; hmyzí opylovači; cizosprášení; environmentální rizika

---

Corresponding author:

RNDr. MIROSLAV GRIGA, CSc., AGRITEC, Plant Research, s. r. o., oddělení biotechnologií, Zemědělská 16,  
787 01 Šumperk-Temenice, Česká republika  
tel.: + 420 583 382 126, fax: + 420 583 382 999, e-mail: griga@agritec.cz

---