Environ. Biosafety Res. 2 (2003) 117–132

DOI: 10.1051/ebr:2003007

Selection of relevant non-target herbivores for monitoring the environmental effects of *Bt* maize pollen

Gregor SCHMITZ[1],*, Detlef BARTSCH[2], and Peter PRETSCHER[3]

[1] Botanical Garden, University of Konstanz, Universitätsstr. 10, 78457 Konstanz, Germany
[2] Robert Koch Institute, center for Gene Technology, Postfach 650280, 13302 Berlin, Germany
[3] Federal Agency for Nature Conservation (BfN), Konstantinstr. 110, 53179 Bonn, Germany

Genes of *Bacillus thuringiensis* var. *kurstaki* (Berliner) that encode lepidopteran-specific toxins were engineered into maize for protection against the European Corn Borer, *Ostrinia nubilalis* (Hbn.). Recent data suggest that Lepidoptera may be negatively affected, if maize pollen contains high amounts of *Bt* toxin and is diposited on host plants near maize fields. Monitoring the environmental effects of commercial *Bt* maize fields requires effective use of limited financial and logistical resources. The aim of this study was to develop and apply tools for selecting relevant herbivore species for the field monitoring of environmental *Bt* toxin effects via pollen deposition. We first present a theoretical selection tree based on "risk index of *Bt* pollen for herbivores" (I_{Btp}). Our index consists of five classes from zero (not relevant) to four (highly relevant) derived from data on potential temporal and spatial coincidence of pollen exposure (A), feeding mode (B), susceptibility to lepidopteran-specific *Bt* toxins (C) and hazard to rare and/or endangered species ("Red List") (D). We then screened the Macrolepidoptera database LEPIDAT to identify relevant species in Germany. Finally, we also applied the index to species found in a local biocoenotic field study (Bonn, Western Rhineland, Germany). Approximately 7% of the German Macrolepidoptera species mainly occur in farmland areas and were selected as being potentially affected by *Bt* pollen exposure. Of these species, 14% (= 1% of total) were found to be potentially exposed on a regional scale. The combination of I_{Btp} and database screening enables us to pre-select species for monitoring purposes.

Keywords: monitoring / transgenic insect resistant maize / *Bt* maize / biosafety / pollen exposure / non-target effects / Lepidoptera conservation

INTRODUCTION

Over the last decade, genes of *Bacillus thuringiensis* var. *kurstaki* (Berliner) ("*Bt*") that encode lepidopteran-specific toxins (cry1A(b), cry1A(c), cry9) were engineered into maize for protection against the European Corn Borer (ECB, *Ostrinia nubilalis* (Hbn.)). Arguments in favor of the introduction of *Bt* maize claim that the ECB and other harmful Lepidoptera can be controlled effectively, selectively and in an environmentally friendly manner. However, questions have been raised on the environmental risks of these transgenic plants (NAS 2002). For instance, laboratory studies have shown evidence of potentially adverse effects on non-target organisms: Hilbeck et al. (1998a; 1998b) raised concerns about lacewings negatively affected by lepidopteran prey previously fed with *Bt* maize; Losey et al. (1999) published that the deposition of *Bt* maize pollen on host plants can reduce the vitality of phytophagous insects (Monarch caterpillars, *Danaus plexippus*) feeding on them. Due to the potential relevance of this interaction, this study led to an extensive discussion on the environmental risks of genetically modified organisms (Kleiner, 1999) and intensified research on the effect of *Bt* maize pollen deposition on the Monarch Butterfly (cf. Monarch Butterfly Research

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* Corresponding author:
Tel.: 00497531/883597; fax: 00497531/884856; e-mail: Gregor.Schmitz@uni-konstanz.de

Symposium, Chicago, Nov. 1999) and other non-target butterflies (Felke et al., 2002; Villiger, 1999; Wraight et al., 2000). Up to now, medium and large scale field studies on this and other ecological pathways do not confirm the negative effects of *Bt* maize found in the lab (Bourguet et al., 2002; Hellmich et al., 2001; Lozzia et al., 1998; Lozzia, 1999; Oberhauser et al., 2001; Pleasants et al., 2001; Sears et al., 2001; Stanley-Horn et al., 2001; Wraight et al., 2000; Zangerl et al., 2001).

However, considering the high diversity of herbivores, many species of which inhabit agricultural landscapes, and the high complexity of interactions even in agricultural biocoenoses, more biosafety research and monitoring on the effect of *Bt* pollen deposition is indicated. The adequate protection of herbivores, particularly Lepidoptera, in the agricultural landscape is important for general environmental protection efforts (Declaration of Rio: New et al., 1995; integrative concept of nature conservation: Plachter, 1991). In addition, integrated pest management strategies rely on sufficient non-target-species that serve as alternative hosts for parasitoids of economic relevance (Franz and Krieg, 1982).

The concept of the ecological risk assessment and risk management of GMOs, as it is defined in the new EU Directive 2001/18, relies on pre-commercialization biosafety research and post-commercialization monitoring, which can be “case specific” or general (“general surveillance”). Because laboratory and field biosafety studies can only target a limited number of species and parameters (Wagner et al., 1996), broad and long-term monitoring programs are indicated to detect potential side effects (*e.g.* population decline based on lethal and sub-lethal effects, biometrical changes, changes in species composition). The US National Academy of Science recommends that “general ecological monitoring” (cf. general surveillance above) should be used to assess unanticipated or long-term, incremental environmental impacts of transgenic plants (NAS 2002). This is even more important since *Bt* maize is already commercialized in the United States, Canada, Argentina, and South Africa. In Europe, only one cultivar based on the *Bt*176 event has passed legal-variety approval in Spain, where 22 000 ha have been planted yearly since 1998 (Brookes, 2002). Thus far, the only plans for monitoring *Bt* maize are those established by the US Environmental Protection Agency (2001) to determine resistance development in the European Corn Borer.

The problem of monitoring the adverse effects of pollen deposition is that potential non-target species have to be selected from a high number of species in the field. However, there are still no tools or methods of standardization for the selection of species relevant for monitoring. As a first step in establishing monitoring plans, we develop and apply a tool for pre-selecting relevant herbivore species that is based on risk assessment principles.

The study consists of the following steps:

(1) Establishment of a risk index for *Bt* pollen (decision tree). This index includes four general factors: (a) pollen exposure; (b) relative amount of pollen ingestion partly mediated by the feeding mode; (c) physiological susceptibility of herbivores to the *Bt* toxin; and (d) significance of the species in terms of its degree of endangerment.

(2) Screening of a national database of Macrolepidoptera (LEPIDAT) maintained by the German Federal Environmental Protection Agency for a larger-scale evaluation of relevant species.

(3) Selection of relevant species on a regional level (from a field study near Bonn, Germany).

RESULTS

Risk index

A decision tree (Fig. 1) was primarily developed exclusively for herbivorous species that accidentally ingest pollen while feeding on the host plant's tissue. Consequently, it does not give consideration to the *Thysanoptera, Psocoptera* or *Heteroptera* (*e.g. Orius*), for example, which explore pollen depositions on plant surfaces as a major resource.

The decision tree comprises five decision steps, each of which leads either to a relevance level or to the next decision step. While steps 1 to 3 correspond to the probability of pollen consumption from large to small spatial scales, step 4 takes the specific susceptibility into account and step 5 the risk status of extinction or endangerment in Germany based on the Red List ranking. Due to the fact that steps 1 and 2 are of similar importance for excluding irrelevant species, they both lead to the “non-relevant” level.

The decision steps within the risk index were generated as follows:

Step 1: If the herbivorous stages of the species occur during the time of pollen shed (in early July until mid-October) close to potential maize fields (*i.e.* in herbaceous vegetation within a distance of 10 m from a maize field at altitudes of less than 800 m above mean sea level).

Step 2: If the herbivorous stages may ingest pollen due to their ectophytic feeding mode and their chewing mouth parts.

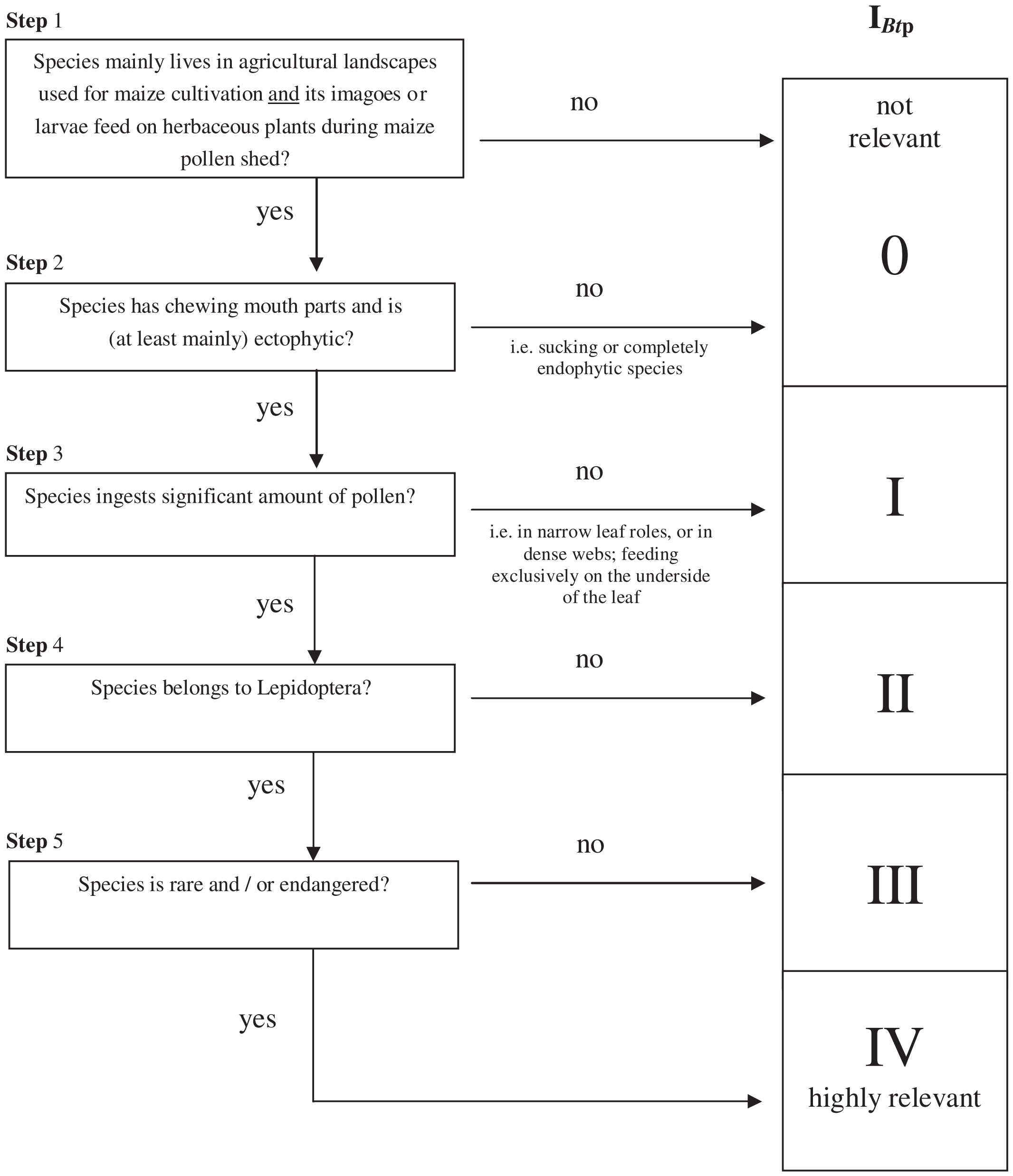


Figure 1. Decision tree for assessing the relevance of herbivores on the adjusted vegetation for investigations on the effect of *Bt* maize pollen (for more detailed specifications see text).

Step 3: If the herbivores ingest larger amounts of pollen because they do not live in leaf rolls or feed exclusively on the underside of leaves.
Step 4: If the herbivore belongs to the Lepidoptera, as the *Bt* toxin is primarily specific to this order.
Step 5: If the species is naturally rare and/or endangered (synonymous to ranking on the German Red List).

Screening of the national lepidopteran database LEPIDAT

Ninety-six species of “Macrolepidoptera” typically occur in the German agricultural landscape and may get into contact with (*Bt*-) maize pollen due to their phenology and habitat preference (step 1–4, Fig. 1; Tab. 1).

Table 1. Macrolepidoptera potentially affected by *Bt* maize pollen deposition based on an analysis of the LEPIDAT database.

	Description of the larval habitat (cf. step 1 in Fig. 1)										
	Larval time	Lucerne, clover, sainfoin	Cereal crop	Row crop	Field edge	Habitat code (see Tab. 3)				RL	I_{Btp}
ZYGAENIDAE											
Zygaena ephialtes (L.)	b9-e6				++	2.6	2.17	2.20		3	IV
Zygaena loti (Denis and Schiff.)	b8-e5				++	2.8				3	IV
Zygaena viciae (Denis and Schiff.)	b8-e5	+			+	2.17	2.8			V	IV
SPHINGIDAE											
Acherontia atropos (L.)	b7-e9			++		1.1.3					III
Agrius convolvuli (L.)	b7-e9			+++	++	1.	2.13	2.14	2.15		III
Marcoglossum stellatarum (L.)	b6-b10				+	2.20					III
HESPERIIDAE											
Carcharodus alceae (Esper)	b6-e4				+++	2.13	2.8	2.19		3	IV
Thymelicus acteon (Rottemb.)	b9-e6				+	2.14				3	IV
Thymelicus lineola (Ochsenh.)	b9-e6				+	2.14					III
PIERIDAE											
Colias croceus (Geoff.)	b8-e9	+++			++	1.1.4					III
Colias hyale (L.)	b9-e4	+++			++	1.1.4	2.13				III
Pieris brassicae (L.)	b6-e9	++		++		1.1.2					III
Pieris napi (L.)	b8-e9	+++		++	+	1.1.4	1.2.1	2.2			III
Pieris rapae (L.)	b9-e10	++		++	++	1.1.4	2.	2.19			III
Pontia daplidice (L.)	b8-e9				++	2.2	1.2.1	2.8			III
LYCAENIDAE											
Lycaena phlaeas (L.)	b6-e4				+	2.5	2.11				III
Plebeius argyrognomon (Bergstr.)	b6-e7				+	2.8	2.13	2.19	2.20	3	IV
Polyommatus icarus (Rottemb.)	b9-e7				+	2.8	2.19	2.20			III
Polyommatus thersites (Cantener)	b9-e5	+++				1.1.5				3	IV
NYMPHALIDAE											
Aglais urticae (L.)	b7-e8				+++	2.12	2.16				III
Vanessa atalanta (L.)	b8-e9				+++	2.16					III
Vanessa cardui (L.)	b6-e9				+++	1.1.4	2.12	2.8	2.17		III
Cupido osiris (Meigen)	m5-e4	++				1.1.5				0	IV

Table 1. Continued.

	Description of the larval habitat (cf. step 1 in Fig. 1)										
	Larval time	Lucerne, clover, sainfoin	Cereal crop	Row crop	Field edge	Habitat code (see Tab. 3)				RL	I_{Btp}
Issoria lathonia (L.)	b6-e4			++	+++	1.	1.4	1.2	2.		III
HEPIALIDAE											
Korscheltellus lupulina (L.)	b7-e4				++	2.14	1.1.5	2.			III
GEOMETRIDAE											
Chiasmia clathrata (L.)	b8-e9	++	++	++	+	1.1.4	1.	2.			III
Eupithecia absinthiata (Clerck)	b8-e10				++	2.10	2.9				III
Eupithecia icterata (De Villers)	b9-m10				++	2.10	2.				III
Eupithecia innotata (Hufn.)	m9-m10				+++	2.10	2.9	2.16			III
Eupithecia linariata (Denis and Schiff.)	b7-e8 / b9-e10				+++	2.7	2.8	2.19			III
Eupithecia simpliciata (Haworth)	b8-e9			+++		1.2	1.2.2	2.3			III
Eupithecia sinuosaria (Eversmann)	e7-e8				+++	1.2.2	2.3				III
Eupithecia succenturiata (L.)	m7-e10				+++	2.10	2.				III
Eupithecia tripunctaria (Herr.-Schäffer)	b6-e9				++	2.7					III
Eupithecia trisignaria (Herr.-Schäffer)	e7-e10				++	2.15	2.7				III
Idaea rufaria (Hbn.)	b9-e6			++	+	1.2	2.5			3	IV
Idaea subsericeata (Haworth)	b7-e5				+	1.2	2.13			3	IV
Larentia clavaria (Haworth)	b5-e7				+++	2.13	2.8	2.1		3	IV
Lithostege farinata (Hufn.)	b6-e7		+++	+++	++	1.	2.	2.2	1.2.1	2	IV
Lithostege griseata (Denis and Schiff.)	b6-e7				+++	2.2	2.			2	IV
Lythria purpuraria (L.)	b8-e10		+	++	++	1.	2.13	2.4		2	IV
Pelurga comitata (L.)	b8-e9			+	+++	1.2.2	2.3	2.10	2.8		III
Perizoma alchemillata (L.)	b7-e9			++	+++	1.4	2.	2.16			III
Perizoma bifaciata (Haworth)	b8-e9				++	2.13				3	IV
Perizoma flavofasciata (Thunberg)	b8-e9				+++	2.15					III
Phyllophila obliterata (Rambur)	b7-b8				+++	2.10					III
Rhodostrophia vibicaria (Clerck)	b8-e5	+				1.1.5					III
Scopula subpunctaria (Herr.-Schäffer)	b8-e5				++	2.8	2.9	2.19		3	IV
Xanthorhoe fluctuata (L.)	b8-e10			+	++	1.	2.	2.21			III

Table 1. Continued.

	Description of the larval habitat (cf. step 1 in Fig. 1)										
	Larval time	Lucerne, clover, sainfoin	Cereal crop	Row crop	Field edge	Habitat code (see Tab. 3)				RL	I_{Btp}
NOCTUIDAE											
Acontia lucida (Hufn.)	b8-e9			++	+++	1..2.	2.1	2.2	2.13	0	IV
Actinotia radiosa (Esper)	e5-e9				++	2.7	2.16	2.19		1	IV
Aedia funesta (Esper)	m7-m5				+	2.15					III
Agrotis cinerea (Denis and Schiff.)	b6-b5				+	2.4				3	IV
Agrotis crassa (Hbn.)	e9-e5		+			1.3				V	IV
Agrotis exclamationis (L.)	b8-e5		++	+++	++	1.1	.				III
Agrotis ipsilon (Hufn.)	b5-e9			++	++	1.1	.				III
Agrotis segetum (Denis and Schiff.)	b7-e4		+++	+++	++	1.1	.				III
Apamea anceps (Denis and Schiff.)	b8-b5		++		+	1.1.1	2.				III
Apamea sordens (Hufn.)	b8-e4		++		++	1.1.1	2.	2.14			III
Autographa gamma (L.)	b5-e10	+++	+++	+++	++	1.1	2.				III
Autographa pulchrina (Haworth)	e8-e5				+	2.15	2.6	2.7			III
Chersotis margaritacea (De Villers)	b9-e4				+	2.17	2.8	2.20		3	IV
Chloantha hyperici (Denis and Schiff.)	b6-e10				+++	2.7	2.10	2.8	2.19		III
Cucullia absinthii (L.)	b7-e9				+++	2.14	2.10	2.8	2.19	V	IV
Cucullia artemisiae (Hufn.)	m7-b10				+++	2.16	2.14	2.8	2.16	V	IV
Cucullia chamomillae (Denis and Schiff.)	e5-e8		+++		++	1.3	1.4	2.13	2.2	V	IV
Cucullia fraudatrix (Eversmann)	b8-e9				+++	2.10	2.8	2.	2.16	V	IV
Cucullia lactucae (Denis and Schiff.)	b6-e9			++	++	2.1	2.7			V	IV
Cucullia tanaceti (Denis and Schiff.)	b7-e9				+++	2.9	2.10	2.8	2.16	V	IV
Discestra trifolii (Hufn.)	b8-e10			+++	++	1.2	2.				III
Emmelia trabaelis (Scop.)	b8-e10		+	++	+++	2.	2.8	2.14		V	IV
Emmelia ochroleuca (Denis and Schiff.)	b5-e7				+++	2.14	2.2	2.5	2.7	3	IV
Euchalcia consona (F.)	e7-m8		+++	++	++	1.	1.3	1.4	2.	1	IV
Euclidia glyphica (L.)	e5-e10	+				1.1.4	2.19				III
Euxoa nigricans (L.)	b9-e6				++	1.1.1	2.5	2.9	2.10		III
Euxoa tritici (L.)	b9-e5		++	++	+	1.1.1	1.1.3	2.14			III
Heliothis ononis (Denis and Schiff.)	b8-e9				++	2.8	2.19			1	IV
Heliothis peltigera (Denis and Schiff.)	b7-e8	++			++	1.1.4	2.				III

Table 1. Continued.

	Description of the larval habitat (cf. step 1 in Fig. 1)									
	Larval time	Lucerne, clover, sainfoin	Cereal crop	Row crop	Field edge	Habitat code (see Tab. 3)			RL	I_{Btp}
Lacanobia oleracea (L.)	b9-m10			+++	++	1.2.2	1.1	2.		III
Lacanobia suasa (Denis and Schiff.)	b8-m10			+	++	1.1	2.	2.19		III
Lacanobia thalassina (Hufn.)	b7-e9	+				1.1.4				III
Mamestra brassicae (L.)	b7-e10			+++	++	1.1.2	2.			III
Melanchra pisi (L.)	b7-m10			++		1.1				III
Orthosia gracilis (Denis and Schiff.)	b5-e7	+				1.1.4	2.10			III
Paradrina clavipalpis (Scop.)	b7-e8		++		+++	1.1.1	2.			III
Periphanes delphinii (L.)	b7-e8		+++		+	1.3	2.8	2.	0	IV
Pseudeustrotia candidula (Denis and Schiff.)	b8-e9				+	2.13	2.11		2	IV
Rhyacia lucipeta (Denis and Schiff.)	b9-e5				++	2.8	2.19		2	IV
Schinia cardui (Hbn.)	b8 b9				++	2.8	2.7		0	IV
Shargacucullia lychnitis (Rambur)	e6-e9				+++	2.18				III
Shargacucullia scrophulariae (Denis and Schiff.)	b6-e9				++	2.18				III
Shargacucullia verbasci (L.)	m5-e7				+++	2.18	2.8			III
Sideridis albicolon (Hbn.)	b7-e9				+++	2.16	2.8	1.2	3	IV
Xylena exsoleta (L.)	b5-e7			++	++	2.	1.	1.22	V	IV
ARCTIIDAE										
Euplagia quadripunctaria (Poda)	b9-e6				++	2.8	2.19	2.20	V	IV
Spilosoma lutea (Hufn.)	b7-e9				++	1.1	2.	2.13		III

The list includes herbivorous species (a) typically inhabiting herbaceous vegetation in agricultural landscapes (see habitat description) potentially culturable for maize and (b) which feed on plants between the beginning of July and mid-October, *i.e.* time of maize pollen shed in Central Europe (cf. step 1 in Fig. 1). The species could ingest deposed pollen, since the larvae have chewing mouthparts and live mainly ectophytically (cf. step 2 in Fig. 1). In addition the larvae of the selected species do not feed hidden in webs, leaf roll or exclusively on the leaf underside (cf. step 3 in Fig. 1).

Abbreviations: RL = status of endangerment according to the German Red List (Pretscher 1998); 0 = extinct in the wild; 1 = critically endangered; 2 = endangered; 3 = vulnerable; V = lower risk – nearly threatened. Larval occurrence: b = begin; m = mid; e = end of a month (*e.g.* b9-e6 = beginning of September to the end of June). Plant community code see Table 3: + = low; ++ = moderate; +++ = high significance of the habitat.

Thirty-eight of these are rare or endangered (= 5.3% of the Red List species, Pretscher, 1998). 6.6% of the 1450 Macrolepidoptera species in the German fauna (Pretscher, 1998) is listed here. Of the families, the Pieridae are represented by a relatively high number of species (6 of the 17 German species, Gaedike and Heinicke, 1999).

Selection of locally relevant species

A total of 26 herbivore species found in our studies (Schmitz and Bartsch, 2001) are assigned to the levels of relevance (Tab. 2). Arthropods that exclusively suck on plants (Acari, Homoptera and most Heteroptera) are not relevant for further studies of the effect of pollen

Table 2. Herbivores found on *Chenopodium album* (Ch), *Solanum nigrum* (So), *Echinochloa crus-galli* (Ec), *Amaranthus* cf. *retroflexus* (Am), *Calystegia sepium* (Ca) 1999 in maize fields near Bonn and Aachen (numbers under the species abbreviations indicate the numbers of sites investigated).

					Ch	So	Ec	Am	Ca	Si^2	add.^{1}	
		Taxon	**FT**	**Ph**	**14**	**9**	**9**	**1**	**1**	**4**		**I$_{Btp}$**
Hym.	Tenthred.	*Dolerus* sp.	F	M							Ea	II
		Athalia sp.	F	O						+++		II
Col.	Chrysom.	Halticinae	F	?	++							II
		Epithrix pubescens (Koch)	F	M		+++						II
		Gastrophysa polygoni (L.)	F	O	+							II
		Gastrophysa viridula (L.)	F	O							Ro	II
	Curcul.	*Rhinoncus gramineus* (Herbst)	F								Pat	II
Lep.	Lyonetiidae	*Bedellia somnulentella* (Zeller)	F, M	M					++			0
	Pteroph.	*Emmelina monodactyla* (L.)	F	M					++			III
	Plutellidae	*Plutella xylostella* (L.)	F	O						+++		III
	Oecoph.	*Schiffermuelleria schaefferella* (L.)	F, M	M	++							0
		Dinisia stipella (L.)	F, M	M	++							0
	Pieridae	*Pieris rapae* (L.)	F	O						+++		III
		Pieris napi (L.)	F	O						++		III
	Noctuidae	*Diachrysia* cf. *chrysitis* (L.)[3]	F	P	+				+			0
		Autographa gamma (L.)	F	P				+	+			III
		Macdunnoughia confusa (Steph.) [3]	F	P	+							0
		Lacanobia oleracea (L.)	F	P	++	+		+++	++	+		III
		Phlogophora meticulosa (L.) [3]	F	P	+							0
		Discestra trifolii (Hfn.)	F	P	++			+++	+		Ap, Pp	III
		Xestia c-nigrum (L.) [3]	F	P	+					+		0
		Trachea atriplicis (L.) [3]	F	P	+							0
		Noctua pronuba (L.) [3]	F	P	++							0
	Geomet.	*Eupithecia* sp.	F	P?	+						Ro	III
Dipt.	Agrom.	*Liriomyza solani* Meig.	F, M	M		++						0
	Anthom.	*Pegomyia hyosycami* Pz.	F, M	P	+	+						0

[1] Additional plant species (add.): *Atriplex patula* (Ap), *Polygonum persicaria* (Pp), *Polygonum amphibium* var. *terrestris* (Pat), *Rumex obtusifolius* (Ro), *Equisetum arvense* (Ea). FT = Feeding type (F = External feeder, M = Miner); Ph = degree of host plant specificity (P = polyphagous, O = oligophagous, M = monophagous). Frequency from "+" (rare) to "+++" (very frequent). [2] *Sinapis alba* (Si) was investigated as a representative of the Brassicaceae on an experimental site near Bonn in 2000. [3] Macrolepidoptera species inhabiting a vast variety of open biotopes and which are thus not listed in Table 1.

deposition and are thus excluded from the list. Lepidopteran species that feed ectophytically on the plant belong to relevance level III, since these are not rare or endangered.

Of the Macrolepidoptera species listed in Table 1, six species (= 6.25%) were found in this local study. The relevance levels of six additional Marcolepidoptera species are categorized in I$_{Btp}$ "0", since they also occur in a wide range of open, non-agricultural habitats and are therefore not listed in Table 1. Their populations are unlikely to be affected by changes in agricultural practices.

DISCUSSION

General aspects and decision tree

Since risk is defined as a function of both *exposure* and *effect* (hazard), it is scientific consensus that risk assessment must target both the exposure (*e.g.* frequency of *Bt* transgenes and their expression products), and the specific effects associated with the new technology (Bartsch and Schuphan, 2002). Risk-related monitoring concepts are rare for the environmental effects of genetically modified plants. Only gene flow and hybridization to wild relatives have already been put into practice (Ammann et al., 1996). Our risk index is the first attempt to formulate and rank criteria, and thus to standardize the selection of species potentially affected by *Bt* maize pollen. The selection procedure integrates essential factors of risk assessment: exposure as the likelihood of pollen ingestion (steps 1 to 3), the effect as the potential susceptibility (step 4) and relative threat of extinction for populations that are rare and/or endangered (step 5).

However, the decision tree, especially the Lepidoptera listed, do not directly indicate the risk of *Bt* maize cultivation at all, but they can be used to select species for more detailed lab and field studies. In this context, a definite distinction should be made between a relatively small group of species that primarily live on weeds in fields and field edges in agricultural landscapes where maize can be cultivated (cf. Tab. 1), and the vast group of species that theoretically could be affected by *Bt* maize pollen deposition (some species in Tab. 2).

Pollen exposure

While the absolute amounts of pollen deposition in relation to the distance from field margins varies in the different studies, an exponential decrease in the first few meters was in every case reported (Pleasants et al., 2001). Assuming that species are unlikely to be affected at distances of more than 10 m from maize field margins, the number of species subject to monitoring is quite limited. This short distance is the reason for the focus on species typically inhabiting fields and field edges. However, more species can potentially be affected in diverse agricultural landscapes characterized by small fields, high crop diversity, and integrated non-agricultural areas. These landscapes can be extensively found in hilly regions of Central Europe.

The amount of pollen ingestion depends on the feeding mode, which can vary significantly within the life span of the herbivorous stage. For example, the Agrotinae (Noctuidae) feed externally on leaves as young larvae, but live hidden at the roots and the stem base as mature larvae. In contrast, the pierid butterflies first live hidden in leaf mines, but later exposed on the leaves. Species that mainly feed endophytically would only ingest pollen by boring into a new feeding place (*Hadena* spp. in capsules of *Silene* and relatives). In accordance with the fact that, unlike the “Microlepidoptera”, virtually none of the “Macrolepidoptera” live exclusively in narrow leaf rolls, dense webs or on leaf undersides, our list contains no species belonging to relevance level II (Fig. 1).

On a micro-spatial scale, the characteristics of both the plant architecture and the surface probably influence the amount and duration of pollen load. While pollen would accumulate on rough, hairy or glandulous leaves (*e.g.* species of Boraginaceae, Urticaceae, Lamiaceae), smooth and waxy leaves (*e.g. Brassica* spp.) would minimize pollen deposition due to self-cleaning effects. Pollen can also accumulate on leaves covered with a layer of honeydew. Although these aspects are important criteria, they are not integrated into the decision tree due to the lack of unequivocal data.

Toxin-specific effects of the pollen

Bt proteins may cause sub-lethal effects, which are of course difficult to detect in field studies, mainly due to the multifactorial nature of ecosystems. Thus, there is not enough data at present on the food web and intergenerational effects of prolonged sub-lethal exposure to *Bt* proteins. Most of the susceptibility data known to date have been collected with *Bt* formulations containing the inactive protoxin or crystalline forms of several different *Bt* protein types. Many *Bt* sprays also contain the spores of bacilli known to interact with the *Bt* proteins (Fearing et al., 1996). In addition, still too little is known about the effects and modes of action of the various *Bt* proteins. Gilliland et al. (2002) reported a complex mode of action of *Bt* toxin Cry1, which was inconsistent for the species-instar-toxin combination used in their studies. While the usual applications of *Bt kurstaki* (especially as sprays) cause comparable mortalities in lepidopteran species, it should be considered that the toxic effect varies significantly between species in low dose application (Glare and O'Callaghan, 2000). Studies of chemical pesticide effects show that polyphagous species are generally less susceptible to insecticides due to their pronounced detoxification ability (*e.g.* Gordon, 1961). Up to now, there is no evidence that comparable reactions can be assumed for *Bt* toxins (Burges, pers. comm., 2001).

On the intra-specific level, variation of toxic effects is reported. For instance, young lepidopteran larvae are generally more susceptible than older ones (Glare and O'Callaghan, 2000; Felke et al., 2002). Therefore, it is more relevant when young larvae of a given species hatch from eggs during the main time of pollen shed (Germany: July/August).

It is difficult to assess the effect of a given *Bt* maize variety/transformation event on a specific herbivore species due to the presence of various influencing factors. For instance, the specific amount of *Bt* toxin in the pollen differs significantly between the different strains of *Bt* maize. The cry1A(b) protein content [$\mu g.g^{-1}$ fresh weight] varies from 0.09 (Mon 810) to 7.1 (*Bt* 176) (Sears et al., 2001). No data are available on the change in *Bt* toxin concentration during the ageing process and by the influence of UV-light. Up to now, adverse effects have only been found with the high expressing pollen of *Bt* 176 (Zangerl et al., 2001), but currently available exposure data confirm that the risks of *Bt* 176 pollen to the Monarch Butterfly are likely to be minimal. In other transgenic events with low *Bt* toxin concentration in pollen (Mon 810) it seems much more unlikely that direct lethal effects will be detected on non-target herbivores (Hellmich et al., 2001).

Monitoring of *Bt* maize in the field should also focus on unanticipated effects (see EU Directive 2001/18). Hypothetically even small amounts of *Bt* toxin may alter the physiological and ethological features of the herbivores (Theiling and Croft, 1988: pesticides; Schuler et al., 1999: *Bt* oilseed rape; Salama and Sharaby, 1988). For instance, a prolonged larval stage, or a change in defense or escape behavior, could lead to a less effective avoidance of attack by parasitoids and predators (Schuler et al., 2001). In addition, sub-lethal effects may also include a reduced number of matings, eggs and successful hibernations. Even slight reductions in the abundance of non-target herbivores could be severe for locally distributed and rare species.

Abundance of species in view of I_{Btp}

Of course, the frequency and threat to a given species vary significantly between the different agricultural areas of a country. For the Monarch Butterfly, the probability of a *Bt* pollen effect was found to be low based on the temporal and spatial overlap of larvae and maize pollen (Oberhauser et al., 2001; Sears et al., 2001). However, in order to select indicator species in a solid and thus practicable manner, further differentiation is necessary: the aspect of practicability (*e.g.* availability, handling, rearing methods) should be taken into account when selecting species for monitoring programs or biosafety research from a list of pre-selected species. In addition, the strict application of the Red List status may sometimes be insufficient. First, the Red List does not exist for each Lepidoptera family (especially Microlepidoptera). Second, other value judgments than rareness or endangerment may be more appropriate for species selection, *e.g.* "symbolic" or "heritage" characters.

Macrolepidoptera list

In terms of susceptibility, the specificity of *Bt* for Lepidoptera is well documented and accepted. Therefore, the detailed knowledge of the "Macrolepidoptera" made it seems practicable to apply the index criteria – as a first step – to this herbivore group and on the base of LEPIDAT. In order to focus on species that are significantly affected by *Bt* maize pollen, species inhabiting meadows, pastures, hedges or natural biotopes are excluded. Since species inhabiting an extremely wide range of biotopes in non-forest rural areas would hardly be endangered by *Bt* pollen, they are also excluded from the list, even though they can colonize agricultural biotopes in high densities.

A simple listing of diurnal butterflies whose larvae live during pollen shed in the altitudes where maize could be cultivated appears to be inadequate. For instance, many of the species listed by Villiger (1999) do not live in the close vicinity of maize fields and would therefore hardly ingest corn pollen in risky amounts. It should be considered that, like other herbivores, Lepidoptera species might differ in their habitat choice from region to region. Species that rarely occur in or at the margin of fields in the northern lowlands of Central Europe may be abundant in the southern hill regions. In addition, it should be taken into account that regional differences in host plant preference may alter the susceptibility to *Bt* toxins (Glare and O'Callaghan, 2000). As a result, the selection of species as candidates for biosafety research, particularly in long-term monitoring programs, requires a detailed evaluation of their distribution and biology. In addition, regional monitoring should include regional cropping patterns and practices.

Combination of local data and the LEPIDAT list

Assuming that species are very unlikely to be affected at distances of more than 10 m from maize field margins, the number of Lepidopteran species potentially affected is quite small on a local scale. Our study demonstrated that corresponding habitats are clearly dominated by ubiquitous species. Differences in abundances and species combinations between various local sites are likely due to geographic (*e.g.* habitat fragmentation) and (micro-) climatic parameters. As a consequence, regional monitoring plans should take into account the regional differences in species compositions and a countrywide monitoring plan should include various representative ecological regions.

Outlook

In view of the great diversity of herbivore species in Central Europe (10 600 species, Klausnitzer, 1977), it is necessary to select relevant non-target herbivores for the purpose of effective monitoring. Biosafety research and monitoring should not be restricted to the Macrolepidoptera. Although many "Microlepidoptera" live endophytically in leaf rolls or on leaf undersides, this group should be considered. Their host-specificity and their low mobility make them effective profound indicator species. Finally, the harmless effect of *Bt* maize pollen on larvae of the external feeders Tenthredinidae and Chrysomelidae has not been verified yet (Glare and O'Callaghan, 2000).

Tritrophic relationships should be observed more intensively. Indirect effects mediated by sub-lethal doses must certainly be included in risk research programs (Frederick and Pilsucki, 1991). While sub-lethal doses could reduce the attraction of a herbivore as a host or prey for antagonists in some cases, due to decreased larval weights (Schuler et al., 1999), the prolonged larval phase and decreases in the effectiveness of defense (*e.g.* enemy escape) by behavior and the immune response may lead to increased predation and parasitization.

Due to the potentially toxic effect of *Bt* maize pollen, the new generation of *Bt* maize contains low amounts of *Bt* toxin in the pollen (*e.g. Bt*11). However, it is important to be aware of the toxin concentration in the pollen of *Bt-tenebrionis* maize currently being engineered to combat the Western corn rootworm, *Diabrotica virgifera*, a chrysomelid beetle invading maize cultivation areas in Europe. Potentially affected insects would probably be beetles, meaning that the selection procedure would require a modified decision tree.

Insects searching for pollen in the male flowers and in deposits on the plant surface (Psocoptera, Thysanoptera, Syrphidae, some Heteroptera) are excluded from the selection process developed in this paper. Of course, these true pollen feeders are generally worth investigating within the risk assessment of *Bt* maize.

A combination of case-specific monitoring and general environmental surveillance will help us to prove the risk assumptions made prior to commercialization of *Bt* maize. Intelligent monitoring should use causal analytic approaches based on the comparison of transgenic and isogenic plants. However, as in other non-experimental field studies, researchers will be confronted with the difficulty of identifying a single effect. This is due, for instance, to the high complexity of the ecosystem, "natural" fluctuations and regional differences in the biocoenoses. Finally, any measurable effect of *Bt* transgenes on non-target herbivores must be evaluated to determine whether the effect is undesirable or acceptable.

METHODS

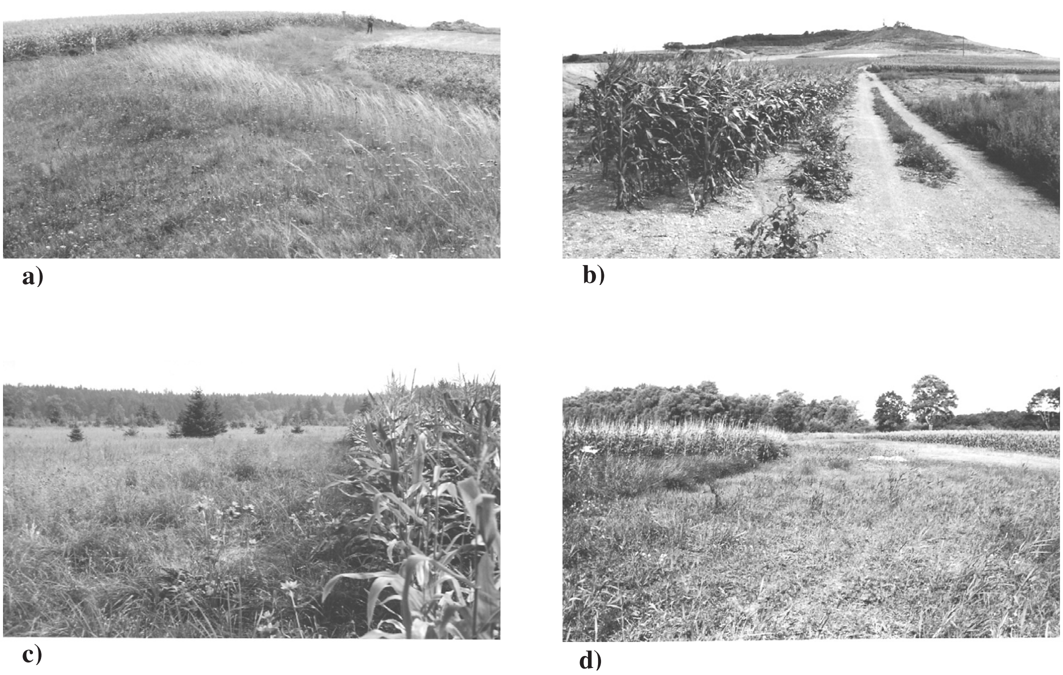
Developing of a "risk index of *Bt* pollen for herbivores" (I_{Btp})

In order to develop the risk index, we first analyzed exposure, and then the parameters that potentially mediate specific effects. These aspects include the occurrence of the herbivorous stages and the pollen at the same time and place, as well as the susceptibility and frequency/endangerment of the species.

Exposure to *Bt* maize pollen

Large spatial scale

The former expansion of maize areas even on poor soils was made possible in Germany by amelioration and nitrogen fertilization. Today maize grows in a wide variety of low and hilly landscapes. An extensive assessment conducted by Pretscher in the 1980s showed that 22 to 29% of nature conservation areas in Germany are immediately surrounded by agricultural areas (Haarmann and Pretscher, 1993). If located at altitudes of less than 800 m above mean sea level (Villiger, 1999), these areas are potential useful for maize. It is thus probable that, after commercialization, *Bt* maize will be cultivated in close proximity to biotopes that are important for species conservation. Figure 2 presents four examples of close spatial contact of (conventional) maize fields to areas important for butterfly conservation.



a) b) c) d)

Figure 2. Examples of the close spatial contact of (conventional) maize fields to areas important for butterfly conservation, including records of endangered Macrolepidoptera species (*i.e.* on the German Red List or of the Red List of at least one state, Pretscher, 1998), which larvae occur within the time of maize pollen shed:
(a) Dry meadow with *Stipa* on a plaster hill near Külsheim (Bad Windsheim, northern Bavaria): *Boloria dia* L., *Melitaea aurelia* Nickerl, *Melitaea didyma* Esper (Nymphalidae), *Papilio machaon* L. (Papilionidae), *Cupido minimus* Fuesslin, *Polyommatus bellargus* Rottembg. (Lycaenidae).
(b) Former pumice mining region and nature conservation area "Korretsberg" (near Kruft, Mayen-Koblenz district, Rhineland-Palatinate) with thermophilous ruderal vegetation and dry bush and forest margins on pumice tuff: *Antonechloris smaragdaria* F. (Geometridae), *Euplagia quadripunctaria* Poda, *Thyria jacobaeae* L. (Arctiidae), *Hamearis lucina* L. (Lycaenidae: Riodinae), *Hyles euphorbiae* L. (Sphingidae), *Lycaena alciphron* Rottembg (Lycaenidae), *Nymphalis polychloros* L. (Nymphalidae), *Pyronia tithonus* L. (Nymphalidae: Satyrinae), *Zygaena transalpina* Esper, *Zygaena ephialtes* L. (Zygaenidae).
(c) Calcareous low moor with reed and sedge meadows in the nature conservation area "Görbelmoos" (near Gilching, Starnberg district, Bavaria): *Boloria eunomia* Esper, *Brenthis ino* Rottembg., *Euphydryas aurinia* Rottembg. (Nymphalidae), *Coenonympha hero* L., *Coenonympha tullia* F.O. Müller, *Minois dryas* Scop. (Nymphalidae: Satyrinae).
(d) Inland salt marsh near Schreyahn (Lüchow-Dannenberg district, Lower Saxony) with reed and halophyte vegetation: *Acronicta cuspis* Hbn., *Archanara dissoluta* Treitschke, *Chilodes maritima* Tauscher, *Eremobia ochroleuca* Denis and Schiff., (Noctuidae), *Pelosia obtusa* Herr.-Sch., *Coscinia cribraria* L. (Arctiidae).

Local spatial scale

Studies on the spread of maize pollen (non-transgenic and transgenic) have shown that the number of pollen grains decreases rapidly and exponentially with the distance from the field due to the high specific weight of the pollen. According to Sears and Stanley-Horn (2000), 90% of pollen is deposited in the first five meters from the field margin. However, distance is not the only factor affecting pollen dispersal. Larger fields may result in poller dispersal over a wider area. In addition, wind characteristics and weather conditions also have a significant impact. As a simplified assumption, we suggest a zone of less than 10 m from the field margin in which the impact of pollen deposition could be significant. We excluded species that are aquatic or live exclusively in tree canopies, since they hardly come into contact with maize pollen.

Temporal overlapping

While the pollen production of individual maize plants/ fields is restricted to about two weeks, the time of pollen emission varies widely from year to year. In Germany, pollen exposure can occur from early July to mid-October, although maize pollen rarely sheds after

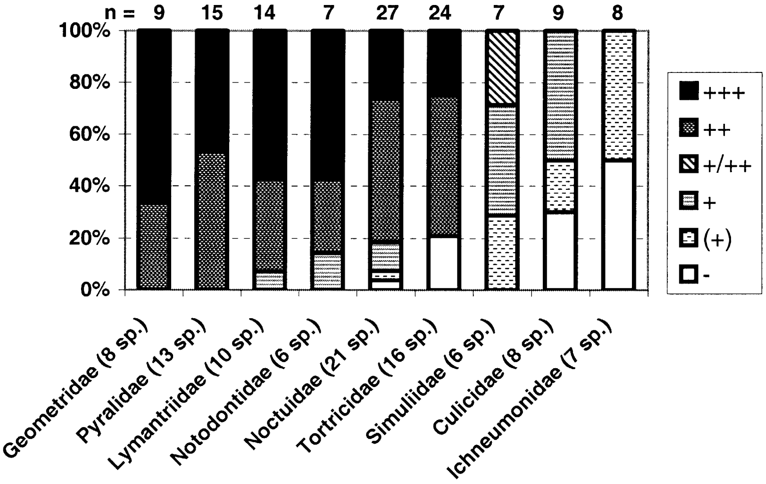


Figure 3. Susceptibility of larvae (and imagoes in the case of Ichneumonidae) of different insect families to toxin spray of *Bt* (raw data taken from Krieg and Langenbruch, 1981). The degrees of susceptibility range from highly susceptible (= "+++") to not susceptible ("-"). The number of individual results (= n) and the number of species ("sp.") is noted. Only families with N > 5 are included.

mid-September. Pollen can be preserved on the plant surfaces for a time, or blown onto weedy plants from deposits on maize leaves. Therefore the critical time of pollen exposure was defined to last until mid-October. Insect species are potentially affected whose herbivorous stages exist within this window of time. Unfortunately, no data are available on the age and toxicological significance of primary and secondary deposits.

Ingestion of pollen and susceptibility to Bt toxin and pollen

Any effect of *Bt* in pollen essentially depends on the degree of pollen ingestion (mediated by feeding mode and behavior) and the physiological susceptibility to *Bt* toxins. The ingestion of pollen basically depends on the occurrence of chewing mouthparts (true herbivorous plant-suckers do not eat pollen at all). In addition, exclusively endophagous herbivores may never ingest pollen from the plant surface. Finally, the degree of pollen ingestion depends on the amount of pollen on the feeding site of the herbivores on the plant. For instance, pollen deposition is very low on basal leafs, leaf undersides and in leaf rolls, but probably high on the top of the leaf blade and especially at the leaf base.

The physiological susceptibility of *Bt* to certain organisms has been studied intensively, although the direct molecular mechanism is not fully understood (Hua et al., 2001; Guihard et al., 2001). We use the term 'susceptibility' for any direct or indirect, short-term or long-term, lethal or sub-lethal response of a given organism to *Bt*. According to the existing database of the direct effects of conventional *Bt* toxins (review: Glare and O'Callaghan, 2000), a more or less safe prediction can be applied to the relative susceptibility of many species. On the basis of the review by Krieg and Langenbruch (1981) (data included in Glare and O'Callaghan, 2000), we attempted to summarize and visualize the results of various studies on the susceptibility of insects to *Bt kurstaki* spray (Fig. 3). Among those groups for which sufficient data were available (*i.e.* N > 7 species), the Geometridae appeared to be the most susceptible family. In contrast, the Noctuidae are relatively resistant to *Bt* spray. Some Diptera even seemed to be affected by *Bt* spray, albeit on quite a low level. Overall, the literature confirms the lepidopteran-specific toxicity of *Bt kurstaki* toxins.

Relevance to rare and endangered species

The negative effects of *Bt* maize pollen on herbivore populations are more critical in rare and endangered species. Thus, species listed in the "Red List of Endangered Species" (Pretscher, 1998) certainly have greater relevance for monitoring studies than ubiquitous species. As a simplification, we do not differentiate between the different categories of endangerment.

The Macrolepidoptera Database LEPIDAT

LEPIDAT is maintained in the German Federal Environmental Protection Agency and represents the first part of the planned fauna database FAUNADAT. The database refers to Germany and includes approximately 1450 "Macrolepidoptera", a polyphyletic group defined here in accordance with Pretscher (1998), (*e.g.* including

Table 3. Plant communities (as habitat for Lepidoptera larvae) that occur in or near maize fields.

1. Weed communities of row and cereal crop

1.1 Crop fields, mainly of row crop
1.1.1 Row crop fields (excl. maize)
1.1.2 Row crop fields (excl. potato)
1.1.3 Potato fields
1.1.4 Clover and lucerne fields
1.1.5 Sainfoin fields
1.2 Annual weed communities of ruderal sites or row crop fields (Sisymbrion[1] and Chenopodion[1])
1.2.1 *Sisymbrium officinale* community
1.2.2 *Atriplex-Chenopodium* community
1.3 Communities of annual weeds in cereal crop of calcareous or base rich soils (Secalietalia[1])
1.4 Communities of annual weeds in cereal crop of acidic or neutral silicate soils (Aperetalia[1])

2. Ruderal vegetation

2.1 Nitrophytic Sisymbrion[1] communities of dry to moist soils
2.2 Sisymbrion[1] communities dominated by *Sismbrium* species or *Descurainia sophia* at dry sites
2.3 Highly nitrophytic *Chenopodium* communities
2.4 Annual vegetation with short species on dry ruderal sites
2.5 Annual vegetation with tall species on dry ruderal sites
2.6 Weedy tall herb communities without umbellifers
2.7 Ruderal vegetation characterized by high umbellifers (*Conium*, *Heracleum*, *Pastinaca, Daucus*)
2.8 Thermophile tall herb communities (Onopordion[1])
2.9 *Artemisia campestris* stands
2.10 *Tanacetum vulgare-Artemisia vulgaris* community
2.11 *Rumex* stands
2.12 *Urtica dioica* stands of dry sites
2.13 Weed and grass communities in nutrient rich places of farmland (Plantaginetalia[1]/Sisymbrion[1])
2.14 *Agropyron repens* communities in dry places (Agropyretea[1])
2.15 Perennial weed communities in moist semi-shaded places with *Aegopodium* or *Calystegia*
2.16 Perennial weed communities in moist ruderal places with *Ballota*, *Artemisia*, *Urtica* or *Conium*
2.17 Open patches in dry meadows with thistle stands
2.18 Sites of soil wounding with occurrence of *Verbascum* species
2.19 Vegetation dominated by *Echium vulgare* and *Melilotus* spp.
2.20 *Origanum vulgare* stands on field edges
2.21 Nitrophytic vegetation of moist semi-shaded forest edges dominated by Umbellifers

[1] Habitat codes: terms of plant communities ending with "-etea", "-etalia" and "-ion" correspond to the syntaxonomy of Oberdorfer (1983).

Psychidae, Zygaenidae, Sesiidae). LEPIDAT contains species-specific information intended to answer questions on butterfly ecology, distribution and conservation. Among other things, the EDP-based tool contains data on the life cycle/phenology, ecological demands on the environment (habitat), trophic requirements (host plants), behavior, species composition in biotopes, historic and current distribution, causes and status of endangerment (Red List categories) (Pretscher and Kleifges, 2000). It was already an important resource for drawing up the Red

List of Macrolepidoptera in Germany (Pretscher, 1998) and is used here to select species that may be affected by *Bt* maize due to their co-occurrence in time (larval time) and space during maize pollen shed.

For each Lepidoptera species in LEPIDAT, the habitat range is described by a list of vegetation units of decreasing habitat significance. The habitat catalogue is based on vegetation units defined by Oberdorfer (1983; 1994), descriptions in Friese et al. (1973) and categories exclusively developed for the database. The habitats of each species are arranged in an ordinal scale from 1 (most significant habitat) to 9 (least significant habitat). These ranks can be used to select characteristic lepidopteran elements from biocoenoses near maize fields. In order to select species that primarily occur in agricultural biotopes, only those species with a specific preference to agricultural vegetation units were listed (see Tab. 3 for vegetation types selected as being characteristic for agricultural biotopes).

Collecting biocoenotic data on a local level (Western Rhineland, Germany)

The herbivorous arthropod species were screened in maize fields in and at the border of a given ECB infestation area in northwestern Germany in 1999. The arthropods were collected manually, mainly by shaking associated weeds from late July to late September (the end of the maize flowering season) in 18 maize fields 25 km around Bonn and in 2 maize fields west of Aachen. In addition, the caterpillars from a weed strip within a maize field near Rheinbach (15 km west of Bonn) were inventoried in 2000 (Schmitz and Bartsch, 2001).

ACKNOWLEDGEMENTS:

We thank H.D. Burges (West Sussex) for his helpful comments on interpreting the susceptibility levels of various insect families. We also thank two anonymous reviewers and D.K. Letourneau for critical comments.

Received September 29, 2001; accepted February 12, 2003.

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