Apidologie 34 (2003) 139–145

DOI: 10.1051/apido:2003003

Original article

Environmental risk assessment of transgene products using honey bee (*Apis mellifera*) larvae

Henrik F. BRØDSGAARD*, Camilla J. BRØDSGAARD, Henrik HANSEN, Gábor L. LÖVEI

Department of Crop Protection, Danish Institute of Agricultural Sciences, Research Centre Flakkebjerg, 4200 Slagelse, Denmark

(Received 3 January 2002; revised 25 June 2002; accepted 30 August 2002)

Abstract – An environmental concern regarding the cultivation of transgenic crop plants is their effect on non-target organisms. Honey bees are obvious non-target arthropods to be included in a risk assessment procedure but due to their complex social behaviour, testing transgene products on individual bees is not possible in bee colonies. We employed a laboratory larval rearing technique to test the impacts of such transgene products on honey bees. A serine proteinase inhibitor (Kunitz Soybean Trypsin Inhibitor, SBTI), that is a source of insect resistance in transgenic plants, was used as a model insecticidal protein on honey bee larvae reared individually in the laboratory. The addition of 1.0% SBTI (w:w of total protein) to the larval diet created significant additional larval mortality, slowed juvenile development and significantly decreased adult body mass. Our results suggest that the larval rearing technique can be used to monitor direct side-effects of transgene products on individual honey bee larvae.

***Apis mellifera* / risk assessment / larval rearing / transgenic plants / proteinase inhibitor**

1. INTRODUCTION

Gene technologies may prove to be a powerful tool for generating new plant cultivars that possess improved traits in relation to crop production (Jouanin et al., 1998; Schuler et al., 1998, 1999; Hilder and Boulter, 1999). However, before releasing genetically modified (GM) cultivars, a thorough environmental risk assessment is required (Wolfenbarger and Phifer, 2000). Widely accepted risk assessment protocols do not exist but a general requirement is to test that the GM plants to be released do not cause adverse effects to the environment. Organisms to consider should include non-target arthropods (EU, 1990; USDA/APHIS, 1991). Honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae) are obvious non-target arthropods to be included in a risk assessment analysis; honey bees are of huge economic importance as the most important insect pollinators of wild and cultivated plants (Free, 1993). Honey bees are also important in the public perception of biodiversity and a healthy environment and are considered to be under human-induced environmental risk (Buchmann and Nabham, 1996). Adult bees, by feeding on nectar, pollen (also of wind-pollinated crops (McGregor, 1976; Free, 1993)) or by collecting resin are potentially exposed to compounds expressed by GM plants. Bee larvae are fed by nurse bee secretions and pollen collected by adults, and thus they can also potentially be affected by

* Correspondence and reprints
E-mail: hfb@bigfoot.com

such compounds. Published methods of testing non-target effects of GM plants (Jepson et al., 1994) or pesticides (EPPO, 1999) on bees have so far been restricted to adults. Effects on larval development cannot be tested using these methods. The larvae could be more sensitive than adults because they need a complex diet to complete their development and because they are in a no-choice situation when feeding in the brood cells. Investigation of the impact of larval food on individual larval development is not possible in bee colonies because the larvae are tended by nurse bees. Nurse bees will detect and remove diseased larvae several days before symptoms are visible to the human eye (Brødsgaard et al., 2000). To follow possible influence of food composition at the individual level, it is, therefore, necessary to rear the larvae in vitro without the interference of nurse bees (Brødsgaard et al., 1998). Here we report on the application of such an in vitro rearing system using a plant-based proteinase inhibitor (PI) as a model GM product.

Genes coding for plant derived PIs can contribute to pest resistance in host plants (Ryan, 1990). Though the full physiological effects of PIs on insects remain unknown (Schuler et al., 1998), PIs have been reported to inhibit growth and development and to reduce adult longevity of a range of insect juveniles (Broadway and Duffy, 1986; Orr et al., 1994; Burgess et al., 1996). Reduced adult longevity (Malone et al., 1995, 1998; Girard et al., 1998; Pham-Delègue et al., 2000) and behavioural disturbances (Girard et al., 1998; Pham-Delègue et al., 2000) also have been reported for honey bees. Acute toxicity to PIs is seldom seen in insects (Schuler et al., 1998) and not in honey bees (Belzunces et al., 1994). By 1998, at least 14 different PI genes had successfully been introduced into crop plants (Schuler et al., 1998). One of these, the Kunitz Soybean Trypsin Inhibitor (SBTI), a serine proteinase inhibitor, is reported to have been engineered into potato, tobacco (Marchetti et al., 1994), and rice (Lee et al., 1999). Honey bees have serine proteinases as digestive enzymes (Moritz and Crailsheim, 1987). SBTI may therefore have an impact on honey bee protein digestion and, thus, especially on larval development.

2. MATERIALS AND METHODS

The test larvae originated from colonies with sister queens, which were descendants of a line bred on the Danish island Drejø. Average cubital index and genetic analysis showed that this line was *Apis mellifera ligustica* Spinola (Svendsen et al., 1992; Itenov and Skjøth, 1993). The queens were mated on an isolated island with a known line of drones. The larvae were reared using the method by Brødsgaard et al. (1998) with the modifications that the larvae were reared in sterile tissue culture multi-wells (∅ 16.2 mm) (Orange Scientific #2030300) and grafted daily to new wells with fresh food. Handling was hereby reduced to once daily with no additional feeding (Peng et al., 1992). We repeated the experiment four times with a total of between 188 and 312 larvae per experimental group.

Expression levels of transgenes in GM plants vary according to plant tissue, plant species (Schuler et al., 1998), and the promoter used (Malone and Pham-Delègue, 2001). To our knowledge, there are no studies suggesting that GM products can be passed on to the larvae from the nurse bees in the hypopharyngeal excretions. However, as proteinase inhibitors are known to be extremely stable (as with pancreatic trypsin inhibitors found in mammals), is possible that PIs will be passed to the larvae with the larval jelly. However, if GM products only reach the larval food through modified pollen, the average amount of GM product in larval food in bee colonies would be between 0.1% and 0.2% if the pollen express 1% PI of total protein (calculation based on Wille et al., 1985 and Malone et al., 2002). Therefore, we chose 0.1% and 1.0% SBTI of total soluble protein as realistic low and high expression levels, respectively (Jouanin et al., 1998), and investigated the juvenile development, mortality, and adult body mass with larval diets containing 0.1% or 1.0% (w:w) SBTI of total protein. A control group was fed with a larval diet containing 1.0% (w:w) of the neutral protein Bovine Serum Albumin (BSA). Pure SBTI and BSA were obtained from Sigma (St. Louis, USA) and mixed into the standard food (Rembold and Lackner, 1981) in concentrations of 0.1% and 1.0% (w:w) of total protein content in the food.

The larvae and pupae were monitored once daily until adult emergence. Larval and pupal stage as well as survival were noted. Larval development time was calculated at the LS stage (Rembolt and Kremer, 1980) (the larvae stop feeding and defecate in this stage to begin pupation) and at adult emergence. The newly emerged adults were weighed to investigate differences in body mass. Differences in development time and adult body mass among treatments were tested using pair-wise

Table I. Development time from egg to LS stage (larval stages L_1 to LS are feeding) and egg to adult, adult body mass, and juvenile mortality of *Apis mellifera ligustica* reared in vitro on standard diets (Rembolt and Lackner, 1981) containing bovine serum albumin (BSA) or soybean trypsin inhibitor (SBTI).

Diet	Development time from egg to		Adult body mass	Juvenile mortality
	LS stage	adult	(g[#] ± SE (n)[¤])	(%~± SE (N)[≈])
	(days[#] ± SE (n)[¤])	(days[#] ± SE (n)[¤])		
Standard + 1.0%BSA	9.62a ± 0.06 (188)	21.05a ± 0.16 (99)	0.138a ± 0.003 (99)	48.49a ± 6.83 (4)
Standard + 0.1%SBTI	9.79a ± 0.03 (312)	20.73a ± 0.17 (121)	0.139a ± 0.003 (120)	59.05ab ± 10.79 (4)
Standard + 1.0%SBTI	10.29b ± 0.03 (258)	22.06b ± 0.13 (74)	0.109b ± 0.002 (74)	74.04b ± 3.80 (4)

[#] Means not followed by the same letter are significantly different (pair-wise t-tests, $P < 0.0001$).
[¤] Number of individuals.
~ Means not followed by the same letter are significantly different (pair-wise t-tests on arcsine transformed data; $P < 0.05$).
≈ Number of repetitions.

t-tests. Differences in per cent juvenile mortality were tested using pair-wise t-tests on arcsine transformed data.

3. RESULTS

The addition of BSA to the larval diet did not influence juvenile development time or mortality, or adult body mass at emergence (Brødsgaard et al., 1998, and unpublished results). However, the addition of SBTI generated several effects. With respect to the control (normal diet + 1.0% BSA), there was an additional juvenile mortality of 11.7% when the diet contained 0.1% SBTI, but this was not significant. The addition of 1.0% SBTI, however, generated significant additional juvenile mortality (Tab. I).

Juvenile development was slower in the 1.0% SBTI treatment for both the feeding (egg to LS stage) and the non-feeding stages (Tab. I). With respect to the control, development from egg to LS stage was about 0.6 days slower, and the total development 1 day slower. Both these differences were significant (Tab. I). Development times were not significantly different in the 0.1% SBTI-containing diet for either response variable.

There were similar trends found in adult body mass. Adults that emerged after being fed diet containing 0.1% SBTI treatment as larvae had the same body mass as control bees (Tab. I). However, surviving adults in the 1.0% SBTI treatment at emergence, weighed about 21% less than bees emerging from the control treatment. This difference was significant (Tab. I).

4. DISCUSSION

The present study suggests that the development of honey bee larvae is influenced by the serine proteinase inhibitor SBTI and that the larvae respond to the SBTI with a dose-dependent relationship. This is not unexpected because adult honey bees and possibly larvae have serine proteinases as digestive enzymes. Also SBTI does not act as a toxin; rather it affects the molecule-to-molecule binding action. The dose-dependent relationship is illustrated by the fact that the larvae were able to compensate for the digestive inhibition of SBTI at 0.1%, but at 1.0% significant effects on the juvenile development and mortality were evident (Tab. I). A dose-response relationship of SBTI has also been reported for adult longevity of both honey bees (Burgess et al., 1996; Pham-Delègue, 2000) and bumble bees (Malone et al., 2000).

The larvae that ingest 1% of SBTI but survive to adulthood will have a smaller body mass than larvae that receive 0.1% or less SBTI in the food (Tab. I). Such a reduction of mass gain due to PIs in the food was also reported for other herbivorous insects (Broadway and Duffy, 1986; Burgess et al., 1991). These smaller surviving adult bees will

probably have reduced performance as adults, regardless of their food intake during adult stage. This is often seen in Hymenoptera and within several species of bumble bees where some workers receive less food than their sisters in the brood period and therefore become smaller adults with a poorer performance than their larger sisters (Free and Butler, 1959).

Pollen is the major source of protein to honey bees. Adult worker honey bees eat more pollen as nurse bees, with a peak in pollen intake at day nine after emerging. The pollen intake and, thus, the amount and type of digested protein, is correlated with the developmental status of the hypopharyngeal glands (Standifer et al., 1970; Crailsheim et al., 1992). The secretions of these glands are important components of the larval food. It is therefore likely that nurse bees that ingest PIs will be poorer producers of larval food in terms of both quantity and quality and, thus, sub-optimal tenders of larvae as nurse bees (Malone and Burgess, 2000). Furthermore, the longevity and learning ability of adult bees is reduced if they are influenced by a SBTI-containing pollen or nectar source (Malone et al., 1995, 1998; Burgess et al., 1996; Pham-Delègue, 2000) which influences their performance as forager bees. A crop expressing SBTI at a 1.0% concentration in pollen or nectar could have a direct impact on honey bee larvae through digestive inhibition, resulting in increased development time, increased juvenile mortality, and surviving individuals being smaller adults. It also could have an indirect impact on nurse bees which in turn may influence their provisioning ability.

Expression levels of SBTI up to 2.5% of total protein in transgenic plants have been reported (Lee et al., 1999). However, little is known about SBTI expression levels in pollen or nectar. Since expression levels of GM products are dependent on a wide range of variables, estimation of actual expression levels must be done on a case-by-case basis.

Honey bees are important non-target organisms in relation to GM plants and the larval instars of the honey bee will probably be the most sensitive stages to the GM products. The in vitro rearing technique presented here makes it possible to monitor individual larval development without interference by nurse bees. Therefore, we suggest that a bio-assay using the in vitro larval rearing technique should be included in environmental risk assessment procedures before releasing transgenic plants for field planting. Furthermore, such a system can be used by plant breeders as a pre-plant test evaluation method, using artificial diets containing the concentration of the gene product at a level which approximates what bee larvae may be exposed to when the adults of the colony forage on those transgenic plants.

ACKNOWLEDGEMENTS

We thank Lene H. Andersen, P. Asger Marthin and Ulla Sandberg for technical assistance and two anonymous reviewers for comments on the manuscript.

Résumé – Utilisation des larves d'abeilles (*Apis mellifera*) pour évaluer les risques environnementaux des produits transgéniques. Avant d'implanter dans la nature des cultivars génétiquement modifiés (GM), il est nécessaire de faire une évaluation approfondie des risques pour l'environnement. Les abeilles domestiques (*Apis mellifera* L.) sont des arthropodes non cibles qui doivent être inclus dans une procédure d'évaluation des risques mais, en raison de leur comportement social complexe, il n'est pas possible de faire des tests individuels sur les abeilles dans les colonies. Nous avons utilisé une technique d'élevage des larves en laboratoire pour tester l'impact des produits transgéniques sur les abeilles. Un inhibiteur de protéinase à sérine (inhibiteur de trypsine soja de Kunitz, SBTI), qui est une source de résistance des insectes aux plantes transgéniques, a été utilisé comme modèle pour une protéine insecticide sur des larves d'abeilles élevées individuellement en laboratoire.
Nous avons ajouté 0,1 % et 1 % de SBTI de la protéine soluble totale au régime des larves, ce qui correspond à des quantités réalistes si des cultures transgéniques exprimant un niveau de SBTI respectivement faible ou fort constituent la seule source de nourriture des abeilles. Un groupe témoin a été nourri avec un régime contenant 1,0 % de la protéine neutre albumine de sérum bovin (BSA). La durée du développement larvaire a été calculée au stade LS (la larve arrête de s'alimenter et de déféquer et commence à se nymphoser) et à l'émergence de l'adulte. Les adultes qui venaient de naître ont été pesés pour rechercher d'éventuels différence dans la masse corporelle.
L'addition de BSA au régime larvaire n'a pas influencé la durée du développement juvénile, ni la mortalité, ni la masse corporelle des adultes à la naissance. Mais l'addition de SBTI a provoqué une mortalité juvénile supplémentaire de 11,7 %

lorsque le régime contenait 0,1 % de SBTI (non significatif). L'addition de 1,0 % de SBTI a par contre provoqué un supplément significatif de mortalité juvénile (Tab. I). Comparé au témoin, le développement de l'œuf au stade LS dans le traitement à 1,0 % de SBTI a été plus lent de 0,6 jours et le développement total de une journée (les deux différences sont significatives, voir Tab. I). Avec le régime à 0,1 % de SBTI les durées de développement n'ont pas été significativement différentes. Les larves nourries avec le régime à 0,1 % de SBTI ont donné naissance à des adultes dont le poids était le même que celui des abeilles témoins (Tab. I). Par contre, les adultes issus des larves ayant reçu le régime à 1,0 % de SBTI pesaient à l'émergence environ 21 % de moins que ceux issus du traitement témoin (différence significative, Tab. I).

Notre étude suggère que le développement des larves d'abeilles est influencé par l'inhibiteur de protéase à sérine SBTI et que les larves réagissent au SBTI en fonction de la dose. Les larves qui ingèrent 1 % de SBTI mais survivent jusqu'à l'âge adulte ont une masse corporelle plus faible que les larves élevées avec de la nourriture renfermant 0,1 % ou moins de SBTI (Tab. I). Ces abeilles adultes plus petites auront certainement des performances d'adultes moindres, quelle que soit la nourriture qu'elles ingéreront à l'âge adulte. Les stades larvaires de l'Abeille sont probablement les stades les plus sensibles aux produits GM. La technique d'élevage in vitro présentée ici permet de suivre le développement larvaire individuel sans que les nourrices n'interfèrent. Nous suggérons donc qu'un test biologique utilisant cette technique soit inclus dans les procédures d'évaluation des risques environnementaux avant d'installer des plantes transgéniques dans les champs.

***Apis mellifera* / évaluation des risques / plante transgénique / élevage larvaire / inhibiteur de protéase**

Zusammenfassung – Risikoabschätzung von transgenen Produkten für die Umwelt mit Bienenlarven (*Apis mellifera*). Bevor genetisch veränderte Kulturpflanzen (GM) freigesetzt werden, ist eine sorgfältige Risikoabschätzung für die Umwelt erforderlich. Honigbienen gehören sicher zu den schonungsbedürftigen Gliedertieren, die beim Prozess der Risikoabschätzung berücksichtigt werden müssen, die aber auf Grund ihres komplexen Sozialverhaltens nicht im Volk getestet werden können. Wir verwendeten eine Technik zur Aufzucht von Larven im Labor, um die Wirkung von solchen transgenen Produkten auf die Honigbienen zu testen. Ein Hemmer der Serinproteinase (Kunitz Soybean Trypsin Inhibitor, SBTI), der eine Ursache für die Insektenresistenz von transgenen Pflanzen ist, wurde als Modell für ein insektenschädliches Protein genutzt und individuell bei im Labor gezogenen Larven der Honigbienen verwendet.

Wir fügten 0,1 % und 1,0 % SBTI zur Gesamtmenge der löslichen Proteine der Larvendiät hinzu: dies entspricht einer realistischen Größe, wenn transgene Trachtpflanzen mit niedriger oder hoher Expression von SBTI die einzige Nahrungsquelle der Bienen sind. Wir überprüften die Dauer der Juvenilentwicklung, die Sterblichkeit und das Körpergewicht der adulten Tiere nach dieser Diät. Die Kontrollgruppe wurde mit einer Diät mit 1,0 % des neutralen Proteins Rinder Serum Albumin (BSA) gefüttert. Die larvale Entwicklungszeit wurde vom LS Stadium (die Larve hört auf zu fressen, setzt Kot ab und beginnt mit der Verpuppung) und dem Schlupf gemessen. Die frisch geschlüpften Bienen wurden zur Bestimmung der Gewichtsunterschiede gewogen.

Die Zugabe von SBTI bewirkte eine zusätzliche juvenile Sterblichkeit, im Fall von 0,1 % SBTI betrug sie 11,7 %, war aber nicht statistisch signifikant. Bei der Konzentration von 1,0 % SBTI war die höhere Sterblichkeit jedoch signifikant (Tab. I). Im Vergleich zur Kontrolle war die Entwicklung vom Ei bis zum LS Stadium bei der 1 % SBTI Diät etwa 0,6 Tage langsamer und die Gesamtentwicklung 1 Tag langsamer (beide Werte sind signifikant, Tab. I). Bei der 0,1 % SBTI Diät waren die Unterschiede in den Entwicklungszeiten in beiden Fällen nicht signifikant. Die adulten Tiere hatten nach der 0,1 % SBTI Diät dasselbe Körpergewicht wie die Kontrollbienen (Tab. I). Die bei der 1,0 % SBTI Behandlung überlebenden adulten Bienen dagegen hatten ein Schlupfgewicht, das etwa 21 % niedriger war als das der Kontrolle. Dieser Unterschied war signifikant. (Tab. I).

Diese Untersuchung legt nahe, dass der Serin Proteinase Hemmer SBTI die Entwicklung der Brut der Honigbienen beeinflusst, und dass die Reaktion der Larven auf SBTI Dosis abhängig ist. Die Larven, die 1 % SBTI aufnehmen und bis zum Adultstadium überleben, haben ein geringeres Körpergewicht als Larven, die mit 0,1 % oder weniger SBTI im Futter gezogen wurden (Tab. I). Diese kleineren überlebenden adulten Bienen werden wahrscheinlich auch nur eine geringere Leistung zeigen, unabhängig von ihrer Futteraufnahme während der adulten Phase.

Die Larven der Honigbienen sind die Stadien, die wahrscheinlich am empfindlichsten auf GM Produkte reagieren. Die in vitro Aufzuchtstechnik, die hier beschrieben ist, ermöglicht ein Monitoring der Entwicklung von individuellen Larven ohne eine Beeinflussung durch Ammenbienen. Deshalb empfehlen wir, dass ein Biotest mit in vitro aufgezogenen Larven in die Prozedur der Umwelt – Risiko - Abschätzung aufgenommen werden sollte, bevor die Pflanzen auf den Feldern ausgebracht werden dürfen.

***Apis mellifera* / Risikoabschätzung / Aufzucht von Larven / Transgene Pflanzen / Proteinase Inhibitoren**

REFERENCES

Belzunces L.P., Lenfant C., Pasquale S.D., Colin M.E. (1994) In vivo and in vitro effects of wheat germ agglutinin and Bowman-Birk soybean trypsin inhibitor, two potential transgene products, on midgut esterase and protease activities from *Apis mellifera*, Comp. Biochem. Physiol. 109 B, 63–69.

Broadway R.M., Duffy S.S. (1986) Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of *larval Heliothis ze*a and *Spodoptera exigua*, J. Insect Physiol. 32, 827–833.

Brødsgaard C.J., Hansen H., Ritter W. (2000) Progress of *Paenibacillus larvae larvae* infection in individually inoculated honey bee larvae reared singly in vitro, in micro colonies, or in full-size colonies, J. Apic. Res. 39, 19–27.

Brødsgaard C.J., Ritter W., Hansen H. (1998) Response of in vitro reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores, Apidololologie 29, 569–578.

Buchmann L.S., Nabham G.P. (1996) The forgotten pollinators, Island Press, Washington, USA.

Burgess E.P.J., Malone L.A., Christeller J.T. (1996) Effects of two proteinase inhibitors on the digestive enzymes and survival of honey bees (*Apis mellifera*), J. Insect Physiol. 42, 823–828.

Burgess E.P.J., Stevens P.S., Keen G.K., Laing W.A., Christeller J.T. (1991) Effects of protease inhibitors and dietary protein level on the black field cricket *Teleogryllus commodus*, Entomol. Exp. Appl. 61, 123–130.

Crailsheim K., Schneider L.H.W., Hrassnigg N., Buhlmann G., Brosch U., Gmeinbauer R., Schoffmann B. (1992) Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): Dependence on individual age and function, J. Insect Physiol. 38, 409–419.

European and Mediterranean Plant Protection Organization (EPPO) (1999) Side effects on honeybees, EPPO Standards, Guideline for the efficacy evaluation of plant products, 1, 161–164.

EU (1990) Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms, Official Journal L 117, 08/05/1990, 15-27, also available on line at: http://biosafety.ihe.be/GB/Dir.Eur.GB/Del.Rel./90.220/TC.html (consulted 3 Dec. 2002).

Free J.B. (1993) Insect pollination of crops, Academic Press, London, Great Britain.

Free J.B., Butler C.G. (1959) Bumblebees, Collins, London, Great Britain.

Girard C., Picard-Nizou A.L., Grallien E., Zaccomer B., Jouanin L., Pham-Delègue M.H. (1998) Effects of proteinase inhibitor ingestion on survival, learning abilities and digestive proteinase of the honeybee, Transgenic Res. 7, 239–246.

Hilder A.H., Boulter D. (1999) Genetic engineering of crop plants for insect resistance – a critical review, Crop Prot. 18, 177–191.

Itenov K., Schøth F. (1993) Uniformity within race and genetic constitution of a population of the brown honeybee on the island of Læsø, SP-rapport 4, 35 p.

Jepson P.C., Croft B.C., Pratt G.E. (1994) Test systems to determine the ecological risks posed by toxin release from *Bacillus thuringiensis* genes in crop plants, Mol. Ecol. 3, 81–89.

Jouanin L., Bonadé-Bottin M., Girard C., Morrot G., Giband M. (1998) Transgenic plants for insect resistance, Plant Sci. 131, 1–11.

Lee S.I., Lee S.H., Koo J.C., Chun H.J., Lim C.O., Mun J.H., Song Y.H., Cho M.J. (1999) Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgene rice, Mol. Breed. 5, 1–9.

Malone L.A., Burgess E.P.J. (2000) Interference of proteinase inhibitors on non-target organisms, in: Michaud D. (Ed.), Recombinant Protease inhibitors in plants, Landes Bioscience, Georgetown, TX., USA, pp. 89–106.

Malone L.A., Pham-Delègue M.H. (2001) Effects of transgene products on honey bees (*Apis mellifera*) and bumble bees (*Bombus* sp.), Apidologie 32, 287–304.

Malone L.A., Giacon H.A., Burgess E.P.J., Maxwell J.Z., Christeller J.T., Laing W.A. (1995) Toxicity of trypsin endopeptidase inhibitors to honey bees (Hymenoptera: Apidae), J. Econ. Entomol. 88, 46–50.

Malone L.A., Burgess E.P.J., Christeller J.T., Gatehouse H.S. (1998) In vivo responses of honey bee midgut proteinases to two proteinase inhibitors from potato, J. Insect Physiol. 44, 141–147.

Malone L.A., Burgess E.P.J., Stefanovic D., Gatehouse H.S. (2000) Effects of four proteinase inhibitors on the survival of worker bumblebees, *Bombus terrestis* L., Apidologie 31, 25–38.

Malone L.A., Tregidga E.L., Todd J.H., Burgess E.P.J., Philip B.A., Markwick N.P., Poulton J., Christeller J.T., Lester M.T., Gatehouse H.S. (2002) Effects of ingestion of a biotin-binding protein on adult and larval honey bees, Apidologie, 33, 447–458.

Marchetti S., Giordana A., Oliviere A.M., Fogher C., Delledonne M. (1994) Genetic engineering for insect resistance in potato: introduction of foreign proteinase-inhibitor genes, Potato Res. 37, 450–451.

McGregor S.E. (1976) Insect pollination of cultivated plants, Agriculture Handbook No. 496, Agric. Res. Serv. USDA.

Moritz B., Crailsheim K. (1987) Physiology of protein digestion in the mid gut of the honey bee, J. Insect Physiol. 33, 923–931.

Orr G.L., Strickland J.A., Walsh T.A. (1994) Inhibition of *Diabrotica* larval growth by a

multicystatin from potato tubers, J. Insect Physiol. 40, 893–900.

Peng Y.-S.C., Mussen E., Fong A., Montague M.A., Tyler T. (1992) Effects of chlortetracycline of honey bee worker larvae reared in vitro, J. Invertebr. Pathol. 60, 127–133.

Pham-Delègue M.H., Girard C., Le Métayer M., Picard-Nizou A.L., Hennequet C., Pons O., Jouanin L. (2000) Long-term effects of soybean proteinase inhibitors on digestive enzymes, survival and learning abilities of honeybees, Entomol. Exp. Appl. 95, 21–29.

Rembold H., Kremer J.-P. (1980) Characterization of postembryonic developmental stages of the female castes of the honey bee, *Apis mellifera* L., Apidologie 11, 29–38.

Rembold H., Lackner B. (1981) Rearing of honeybee larvae in vitro: effect of yeast extract on queen differentiation, J. Apic. Res. 20, 165–171.

Ryan C.A. (1990) Proteinase inhibitors in plants: genes for improving defences against insect and pathogens, Annu. Rev. Phytopathol. 28, 939–943.

Schuler T.H., Poppy G.M., Kerry B.R., Denholm I. (1998) Insect-resistant transgenic plants, Trends Biotechnol. 16, 168–175.

Schuler T.H., Poppy G.M., Kerry B.R., Denholm I. (1999) Potential side effects of insect-resistant transgenic plants on arthropod natural enemies, Trends Biotechnol. 17, 210–216.

Svendsen O., Bertelsen I., Meyer I.-L. (1992) Identification of honeybee subspecies (*Apis mellifera mellifera* versus *Apis mellifera ligustica*) by cubital index on the island of Læsø, Denmark, Tidsskr. Planteavl 96, 319–324.

Standifer L.N., MacDonald R.H., Levin M.D. (1970) Influence of the quality of protein in pollens and a pollen substitute on the development of the hypopharyngeal glands of honey bees, Ann. Entomol. Soc. Am. 63, 909–910.

USDA/APHIS (1991) User's guide for introducing genetically engineered plants and microorganisms, United States Department of Agriculture, Animal and Plant Health Inspection Service, Tech. Bull., 1783.

Wille H., Imdorf A., Bühlmann G., Kilchenmann V., Wille M. (1985) Beziehung zwischen Polleneintrag, Brutaufzucht und mittlerer Lebenserwartung der Arbeiderinnen in Bienenvölkern (*Apis mellifera*), Mitt. Schweiz. Entomol. Ges. 58, 205–214.

Wolfenbarger L.L., Phifer P.R. (2000) The ecological risks and benefits of genetically engineered plants, Science 290, 2088–2093.