UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF NEW YORK

ORGANIC SEED GROWERS AND TRADE ASSOCIATION, et al., Plaintiffs, v. MONSANTO COMPANY AND MONSANTO TECHNOLOGY LLC Defendants.	Case No. 1:11-cv-2163-NRB-RLE ECF Case

BRIEF FOR AMICI CURIAE

Farm and Ranch Freedom Alliance, Biodynamic Farming and Gardening Association, Carolina Farm Stewardship Association, Ecological Famers of Ontario, Fair Food Matters, International Organic Inspectors Association, Michigan Land Trustees, Natural Environment Ecological Management, Nebraska Sustainable Agriculture Association, Organic Council of Ontario, Slow Food USA, and Virginia Independent Consumers and Farmers Association

IN SUPPORT OF PLAINTIFFS' OPPOSITION TO DEFENDANTS' MOTION TO DISMISS

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STATEMENT OF INTEREST OF *AMICI CURIAE*

This case presents several issues of first impression, and the outcome will have repercussions for almost every American. While the Plaintiffs are at the most immediate risk of suit for patent infringement by Monsanto, the legal principles involved in this Court's decision will have even broader ramifications. For example, livestock and poultry farmers who feed grain to their animals face issues of GM-contaminated feed. Organic certifiers must make decisions about whether or not, and under what conditions, to require testing for GM contamination as part of the certification process. Food processors, whether they operate on a large-scale or simply bake a few loaves of bread for a local farmers market, use ingredients that may be contaminated with Monsanto's patented products. And, ultimately, almost every American consumer somehow makes use products made from corn, soybeans, canola, sugar beets, or cotton, all of which may implicate the scope and enforceability of Monsanto's patents. The entire food chain is impacted by the spread of Monsanto's patented crops.

Amici organizations include members who feed grain to livestock and poultry, who certify organic production of crops, who use grains or cotton as raw ingredients for other products, and who consume or use products made from these crops. Some of the members of *Amici* are in privity with the affected farmers, either by buying their crops or otherwise conducting business with them such as through organic certification of the crops. All of these individuals have an interest in having the scope and enforceability of Monsanto's patents determined by this Court, both because of the direct issue of their own liability and because of the indirect impact the decision will have on the availability of these crops.

The Farm and Ranch Freedom Alliance (FARFA) advocates for farmers, ranchers, and homesteaders to assure their independence in the production and marketing of their food,

including their right to farm without fear of prosecution for patent infringement. FARFA also advocates for consumers' access to information and resources to obtain healthy foods of their choice, including mandatory labeling of genetically modified (GM) foods.

The Biodynamic Farming and Gardening Association (BDA) is a nonprofit membership association of individuals and organizations in North America who are committed to the transformation of the whole food system, from farm to table, and who draw inspiration from the spiritual-scientific insights of Rudolf Steiner. The BDA's membership includes biodynamic farmers and seed growers who are at risk of being contaminated by genetically modified seed, as well as farmers who feed such crops to their livestock and make other uses of such crops.

Carolina Farm Stewardship Association's (CFSA) mission is to promote local and organic agriculture in the Carolinas by inspiring, educating and organizing farmers and consumers. CFSA is deeply committed to advancing the interests of organic producers in the Carolinas and strenuously opposes any corporate action that would unfairly threaten, hinder, limit, or otherwise impose additional costs on organic agriculture operations. CFSA's membership includes farmers, gardeners, consumers and businesses in North and South Carolina. These members are committed to sustainable agriculture and the development of locally-based, organic food systems.

The Ecological Farmers of Ontario (EFO) develops and provides programs to promote the practice and advancement of ecological agriculture to maintain and enhance the health of the soil, water, crops, livestock and the diversity of the environment. As organic and ecological farmers, committed to growing crops which are free from contamination by genetically modified organisms, its members face the burdens of maintaining buffer zones around corn crops, genetic testing of corn crops and ensuring all equipment from seed cleaning to harvest to storage is free

of any potential GM contamination for both corn and soybeans. With the release of GM alfalfa in the U.S., Ontario's ecological farmers are becoming ever more concerned about the potential genetic contamination of their crops and livestock feed.

Fair Food Matters (FFM) is a Michigan-based nonprofit organization that helps increase awareness of and appreciation for local foods and organic and sustainable production. In doing so, FFM is helping consumers make better choices about what to feed themselves, their families and their communities, and helping those who produce that food to enjoy safe working conditions and a living wage.

The International Organic Inspectors Association (IOIA) is a nonprofit, professional association of organic farm, livestock, and processing inspectors that provides comprehensive organic inspector training worldwide. IOIA promotes consistency and integrity in the certification process, and addresses issues and concerns relevant to organic inspectors, including promoting public confidence in organic agriculture and products. IOIA also serves the Organic Sector by working closely with numerous regional, state, provincial, and international certification agencies, as well as with governmental agencies and IFOAM (International Federation of Organic Agricultural Movements), to develop policies and publications relating to organic inspection and inspector training.

Michigan Land Trustees (MLT) promotes sustainable land management and organic agriculture. MLT helps educate small-scale homesteaders and beginning farmers, while also supporting the development of local and organic-based food systems by providing start up grants to a variety of organizations and projects. Its diverse membership favors the preservation of biodiversity—especially of non-GM crop seeds—as a key element of the social and ecological resilience that is needed to address the challenges of peak oil and climate change.

Natural Environmental and Ecological Management (NEEM) believes that no company should be allowed to irrevocably manipulate the DNA make up of any natural item and commodities level products that make up our basic food supply. NEEM has conducted research that indicates GM crops are unhealthy for humans and damaging to the infrastructure that supports an already fragile industrial food system.

The Nebraska Sustainable Agriculture Society's (NSAS) mission since the 1970's has been to promote agriculture and food systems that build healthy land, people, communities & quality of life, for present and future generations. NSAS has a diverse membership that includes farmers and ranchers, rural and urban consumers, market gardeners, educators, families and restaurateurs.

The Organic Council of Ontario is a membership-based trade organization championing organics in Ontario. A full value-chain organization, OCO has a deep interest in this court case. Many of our members, and the broader organic sector in general, are impacted along the value chain — from seed supplier to producer to processor to retailer — by the inappropriate application of patents for GMOs in food production.

Slow Food USA is a national nonprofit that believes in protecting the diversity of life, including seeds. Slow Food represents a network of both farmers and consumers who are concerned that patents on seed violate several basic truths and who support the rights of farmers to control their own farms.

The Virginia Independent Consumers and Farmers Association's (VICFA) mission is to promote and preserve unregulated direct farmer-to-consumer trade that fosters the availability of locally grown of home-produced food products. VICFA believes Monsanto's patented seeds work contrary to the achievement of its goals.

SUMMARY

Monsanto has a track record of aggressive enforcement of its patent rights. Monsanto has sued or settled with hundreds of farmers, and investigated unknown numbers more. Because of the nature of Monsanto's patented seeds, the individual Plaintiffs and the farmer members of Plaintiff organizations (hereinafter collectively "Plaintiff farmers") cannot avoid infringing on Monsanto's patents unless they entirely abandon growing corn, soybeans, canola, cotton, sugar beets, and, as of this year, alfalfa. While Monsanto tries to downplay the threat of enforcement by pointing to its "commitment" not to sue farmers for "trace" infringement, this provides no enforceable protections for Plaintiffs. Because of the nature of the patented seeds and the realities of farming, it is certain that at least some of the Plaintiff farmers already have more than trace contamination, and the number of such affected farmers will only grow over time. While many of the Plaintiff farmers are certified organic, not all are, so the simple fact that Monsanto has yet to sue a certified organic farmer has no impact on their standing.

Not only does Monsanto's patented technology inevitably lead to infringement through no fault of the Plaintiffs, but, by their design, the majority of Monsanto's patented crops only provide the alleged benefits if a farmer applies herbicides, specifically Roundup®, directly to the crop. Monsanto could easily protect its patent rights by agreeing not to sue for unintentional contamination absent an affirmative action by the farmer to make use of the patented traits. By failing to do so, and instead offering an ambiguous and ultimately meaningless commitment, Monsanto has made it clear that it intends to maintain the threat of patent infringement lawsuits against Plaintiff farmers and those similarly situated.

Plaintiff farmers have, by the simple act of farming corn, soybeans, canola, cotton, sugar beets, or alfalfa crops, undertaken meaningful steps towards infringement. Due to Monsanto's

decision to release patented seeds and market them for widespread planting, it is now impossible for farmers to remain 100% free of genetically modified crops because of the multitude of ways that contamination can occur.

Given the difficulties in minimizing GM contamination, farmers must make numerous decisions about which steps are worthwhile for them and which steps are not. They are not able to make these decisions based on their own and their customers' interests, but must instead make these decisions with the threat of litigation against a giant corporation looming over their heads. The constant threat of a patent infringement suit by Monsanto creates significant, unquantifiable costs for the Plaintiff farmers and similarly situated farmers. Unless this Court allows this case to proceed, the Plaintiff farmers will face the choice of abandoning growing such crops or risking prosecution whenever Monsanto chooses.

ARGUMENT

Monsanto's motion to dismiss notes that its patented technology is self-replicating. *See* Monsanto Br. at p.5. Yet Monsanto wants the court to ignore the real-world repercussions of this fact. Monsanto seeks to reap all of the benefits of a patented seed – particularly the necessity for farmers to buy their products year after year indefinitely, since new seed is needed every single year – but to take no responsibility for the reality that its technology, by its very nature, induces others to infringe.

In *MedImmune*, the Supreme Court recognized that the test for standing to bring suit under the Declaratory Judgment Act involved an analysis of "all the circumstances." *MedImmune, Inc. v. Genentech, Inc.*, 549 U.S. 118, 127, 127 S.Ct. 764, 771 (2007). Following *MedImmune*, the Federal Circuit has held that, to establish an injury in fact traceable to the

patentee, a declaratory judgment plaintiff must allege both (1) an affirmative act by the patentee related to the enforcement of his patent rights, *SanDisk Corp. v. STMicroelecs., Inc.*, 480 F.3d 1372, 1380-81 (Fed. Cir. 2007), and (2) meaningful preparation to conduct potentially infringing activity, *Cat Tech LLC v. TubeMaster, Inc.*, 528 F.3d 871, 880 (Fed. Cir. 2008). As the Federal Circuit recently stated, "no bright-line rule exists for determining whether a declaratory judgment action satisfies Article III's case-or-controversy requirement." *Association for Molecular Pathology v. Myriad Genetics*, No. 2010–1406, 2011 WL 3211513, at *9 (Fed. Cir. 2011) (citing *MedImmune*).

A. Monsanto Has Taken Affirmative Action to Enforce Its Patent Rights.

By patenting a self-replicating product, one virulent in its spread, Monsanto has created a situation in which it can pick and choose targets for enforcement activity. Monsanto has, by its own admission, implemented an aggressive campaign to enforce its patent rights. According to Monsanto's website, in the section addressing lawsuits against farmers for patent infringement, the company has filed 128 lawsuits and settled "almost 700 matters" out of court. *See* E. Freeman, *Settling the Matter - Part 5*, MONSANTO, Nov. 11 2008, http://www.monsanto.com/newsviews/Pages/Settling-the-Matter-Part-5.aspx (last visited Aug. 1, 2011). Monsanto states that only a "rare few choose to seek a resolution in the courts," which would logically imply that it has conducted hundreds, if not thousands, of additional investigations. *See* E. Freeman, *Monsanto Seed Police?*, MONSANTO, Nov. 10 2008, http://www.monsanto.com/newsviews/Pages/Seed-Police-Part-4.aspx (last visited Aug. 1, 2011). Only Monsanto knows how many farmers it has investigated or the harm caused to these farmers.

Monsanto cites *Creative Compounds, LLC v. Starmark Laboratories*, No. 2010–1445, 2011 WL 25195313 (Fed Cir. 2011), in support of its assertion that the court should require

plaintiffs to show action by the patentee directed at the plaintiffs themselves. *See* Monsanto Br. at p.14. But *Creative Compounds* does not stand for such a broad, bright-line rule. Rather, *Creative Compounds* stands for the narrow proposition that one cannot bring a declaratory judgment action to invalidate a patent merely for **economic** gain, such as invalidating a potentially competing patent to reassure one's customers. *See* 2011 WL 25195313 at *11-12.

In contrast, in the case before this Court, there is a clear dispute over legal rights, namely whether Monsanto is legally able to enforce its patent rights against the Plaintiffs. There is an underlying legal cause of action that the declaratory judgment defendant (Monsanto) could bring or threaten to bring, if not for the fact that the declaratory judgment plaintiffs had preempted it. *See Arris Grp., Inc. v. British Telecomm. PLC*, 639 F.3d 1368, 1374-75 (Fed. Cir. 2011).

As discussed in detail below, many Plaintiff farmers undoubtedly have a non-negligible level of GM contamination in their fields because it is simply impossible to remain 100% GM free if one is growing corn, canola, soybeans, cotton, sugar beets, or alfalfa. The exact level of contamination is often unknown because most farmers do not test, possibly in fear of treble damages imposed on "willful" infringers. But, whether they test or not, they remain vulnerable to a patent infringement suit by Monsanto, creating a legal dispute that is properly addressed through the Declaratory Judgment Act.

B. Monsanto's Pledge Not to Sue for "Trace Contamination" is Neither Enforceable Nor Meaningful.

Monsanto seeks to have the court ignore its track record of aggressive enforcement based on an unenforceable "commitment" that Monsanto's policy is to not sue "where trace amounts of our patented seeds or traits are present in [a] farmer's fields as a result of inadvertent means." Monsanto Br. at p.5. The term "trace," however, is ambiguous and unenforceable. Are Plaintiffs and other farmers to assume it means less than 0.9%, the standard in the European Union to

avoid labeling? Two percent? Five percent? Given the realities of farming, as discussed next, it is certain that at least some of the Plaintiff farmers already have contamination that exceeds any of those levels. Moreover, the passage of time and natural biological processes will inevitably lead to higher contamination levels, at which point Monsanto will have created a target-rich environment for its patent enforcement activities.

As a hypothetical, Farmer Smith buys soybean, corn or canola seed from a local seed dealer. Even if the seed is not labeled as GM, there is a very high probability that it is already contaminated to some degree. As another district court found:

> Monsanto's domination of the soybean seed market, combined with the regeneration of the Roundup Ready® trait and the lack of any restriction against the mixing of soybeans harvested from a Roundup Ready® crop from those that are harvested from a crop that was not grown from Roundup Ready® seed, has resulted in the commodity soybeans sold by grain dealers ***necessarily carrying the patented trait*** …

Monsanto Co. v. Bowman, 686 F. Supp. 2d 834, 836 (S.D. Ind. 2009) (emphasis added). *See also* **Attachment E**, L.F. Friesen et al., *Evidence of Contamination of Pedigreed Canola (Brassica napus) Seedlots in Western Canada with Genetically Engineered Herbicide Resistance Traits*, 95 AGRONOMY J. 1342-1347 (2003); **Attachment F**, B.L. Ma et al., *Extent of Cross-Fertilization in Maize by Pollen from Neighboring Transgenic Hybrids*, 44 CROP SCI. 1273-1282 (2004). Assume a relatively low level of contamination of 0.5%. Farmer Smith plants the seed in a 20-acre field without a significant buffer zone because, as discussed below in Section E, the recommended buffer zones are too expensive. His neighbor plants a GM variety of the same crop, and cross-pollination causes an additional 2% of Farmer Smith's field to be contaminated. *Cf.* **Attachment G,** PETER THOMISON, OHIO STATE UNIVERSITY EXTENSION, FACT SHEET, MANAGING "POLLEN DRIFT" TO MINIMIZE CONTAMINATION OF NON-GMO CORN (2004),

available at http://ohioline.osu.edu/agf-fact/0153.html. (noting that research has indicated that a buffer of 660 ft is needed to limit cross-pollination to 1% or less).[1]

Farmer Smith, unaware of the now 2.5% contamination in his field, decides to save seed for next year and hires a local seed cleaner. The seed cleaner does not perfectly clean his machinery in between fields and has some GM grains from a previous field caught in his machinery when he comes to Farmer Smith's farm, adding another 0.5% of contamination. The saved seed now has 3% contamination. If Farmer Smith sells some of the seed, additional contamination can occur due to the transport vehicles and storage facilities. If Farmers Smith plants his saved seed next year, he *starts* with 3% GM contamination, which is then subject to cross-pollination and other vectors of contamination, even though he has never intentionally planted and has always avoided GM seed.

If Farmer Smith tests his seed and finds out that he has 3% contamination, he faces a dilemma. He must choose between planting the contaminated seed (and risking a patent infringement lawsuit by Monsanto, with potentially treble damages for willful infringement since he now knows of the contamination), or disposing of all the seed, a significant loss, and seeking out uncontaminated seed at significant trouble and expense.

The dilemma is inescapable because there is no effective way for a farmer to save seed only from the non-GM portion of his field because the plants intermingle. There are two ways to detect GM contamination. The first is to test a sample of the grain. Such testing will tell the farmer whether or not there is GM contamination, but it will *not* enable the farmer to segregate the GM portion from the non-GM portion because, in order to be a representative sample, the sample must include grains from multiple plants from throughout the field. The second way to

[1] One acre equals 43,650 square feet. A 20 acre-field is 934' x 934', so the recommended buffer zone on two sides would encompass the entire field.

determine GM contamination is to spray Roundup® on the field, killing everything *except* the GM Roundup-Ready® plants and leaving the farmer with no non-GM grain or seed. For farmers wishing to avoid planting GM crops, this second option is entirely counter-productive. Moreover, it only works with crops engineered to be resistant to herbicides, not insecticide-producing GM crops. Thus, Monsanto's threatened enforcement of its patent rights places the burden on the farmer not only to test the seed, but to then either risk a patent infringement suit or bear significant burdens to find non-contaminated seed.

By its very nature, GM technology contaminates non-GM crops. Efforts by the farmers, undertaken at significant expense and burden, can only minimize contamination, not stop it completely. As the contamination compounds, it is reasonable to ask how long it will take for the farmer to have 10% or 20% contamination in his fields, whether he is saving his own seed or buying increasingly contaminated seed because no commercial company can guarantee 100% non-GM seed. At what point does Monsanto contend that this is no longer "trace" contamination, even though it occurred entirely without the farmer's knowledge or intent? Monsanto's pledge not to sue for "trace" contamination is meaningless given the real-world conditions farmers operate under.

By patenting a self-replicating product, one virulent in its spread, Monsanto has created a situation in which infringement is a certainty and occurs at ever-increasing levels. By deliberately keeping their pledge vague, Monsanto perversely expects human error and biological realities to compound the amount of contamination to the point where seed contamination is substantial and near-universal, making infringement unavoidable. A product that, by its very nature, creates inevitable infringement is a case of first impression, and Plaintiffs

have a concrete, tangible, and immediate need to know the scope of their legal rights such that they can plan their activities accordingly.

C. Monsanto's Claim to Have Never Sued a Certified Organic Producer Also Does Not Protect the Plaintiffs

Monsanto expands on its so-called commitment by noting that it has never sued a certified organic producer for GM contamination. But bringing a suit is the final step in a long process that can be used to intimidate and harass farmers, starting with investigations, accusations, harassment, and the threat to file a suit. *Cf. Monsanto v. Scruggs*, 342 F. Supp.2d 602, 605-06 (N.D. Miss. 2004) (Monsanto's investigator placed defendant under video and binocular surveillance, followed defendant and his family members, and questioned defendant's customers). Monsanto can cause significant harm to the Plaintiff farmers without ever filing a lawsuit.

Moreover, certified organic farmers already must answer to their certifiers. While the organic regulations technically require only that certified organic farmers not knowingly plant GM seed, certifiers may spot-check crops and, if GM contamination is found, require the farmer to take steps to reduce contamination. Moreover, certified organic farmers face significant economic losses if they cannot sell their crops as certified organic, or have to pull land out of the program. So they would have a potentially expensive counter-claim against Monsanto for the loss of organic markets from contamination, making it logical that Monsanto has so far hesitated to sue a certified organic producer. *Cf. Johnson v. Paynesville Farmers Union Coop. Oil Co.*, Nos. A10-1596 & A10-2135, 2011 WL 2982473 (Minn. Ct. App., July 25, 2011) (holding that a pesticide applicator was liable to a certified organic producer for damages for trespass connected with the loss of certification). Note that Monsanto has not actually committed that it won't sue certified organic farmers in the future, but merely states that it has not yet done so.

Even if Monsanto were to make an enforceable commitment (which it has not), thousands of farmers would then face the choice of risking infringement suits or becoming certified organic simply to avoid lawsuits. Many farmers around the country are not certified organic, but use organic and sustainable practices. Many other farmers raise conventional crops but have no desire to raise GM crops. Neither category of farmers should be forced to become certified organic, with its attendant expenses and burdens, simply in the hopes of avoiding a patent lawsuit. Monsanto's restraint in the courtroom so far with respect to certified organic farmers does not protect Plaintiff farmers from liability.

D. Monsanto Could Have Offered Enforceable Protections While Still Protecting Its Patent Rights, and Chose Not To.

The ambiguity and narrowness of Monsanto's alleged assurances become even more apparent when considered against the backdrop of the technology at issue. All of Monsanto's GM soybeans and the majority of its other GM crops have been modified to confer resistance to herbicides, specifically Roundup®. *See* U.S. DEPARTMENT OF AGRICULTURE, ECONOMIC RESEARCH SERVICE, ADOPTION OF GENETICALLY ENGINEERED CROPS IN THE U.S., http://ers.usda.gov/data/BiotechCrops/adoption.htm (providing a graph of the adoption of different GM varieties and noting that "soybeans have only HT varieties"). This trait is only useful when the vast majority of the crop is GM. Consider a farmer whose corn field is 50% contaminated with GM Roundup-Ready® corn. If he or she were to spray Roundup® – which is the way Monsanto intends for its technology to be used – then the half of the crop that is **not** GM Roundup-Ready® would be killed along with the weeds.

Amici fully agree with the Plaintiffs that Monsanto's patented technology does far more harm than good. But, just for purposes of this argument over standing, assume that the GM traits confer the benefits Monsanto claims for its products. A farmer gets *none* of those benefits unless

his crop is almost entirely GM. Even when the contamination reaches 30% or 40%, the farmer is unable to make use of it even if he or she is using chemical herbicides. And organic farmers, who do not use Roundup, never receive any benefit at all from Roundup-Ready® crops, even assuming Monsanto's claims were true.

Monsanto is well aware of the uses of its own technology. So why would it limit its alleged assurance not to sue to "trace" contamination when the inadvertent infringer receives absolutely no benefits far past that point? Monsanto could still protect its patent rights by suing only those farmers who make affirmative use of its GM traits, such as by spraying a field with Roundup® and harvesting the resulting crop. Instead, Monsanto has chosen to maintain the threat that it will sue whether or not the farmer intended to use the patented product and whether or not the farmer makes any actual use of the GM traits.

Monsanto's ambiguous, unenforceable pledge does not counter the affirmative steps it has taken in aggressively investigating and harassing farmers for alleged patent infringement.

E. Plaintiff Farmers Have Undertaken Meaningful Preparation to Conduct Potentially Infringing Activity.

Unlike most patented products, patented seeds are not self-limiting. They will, by their very nature, spread even to land where no patented seeds were ever planted. The simple act of farming a wide variety of crops, such as corn, soybeans, canola, cotton, sugar beets, or alfalfa, involves meaningful, if inadvertent, steps towards infringement of Monsanto's patents. In all likelihood, many of Plaintiff farmers have significant levels of GM contamination already in their fields, although they may not have tested for it and definitely do not desire it. While the fault for such GM contamination lies with Monsanto's decision to create and market this self-replicating, uncontrollable technology, the patent doctrine of strict liability means that the farmers still face liability.

Many farmers take steps to avoid contamination because they affirmatively wish to avoid any presence of GM in their crops. Contrary to Monsanto's claims in support of their products, there is a growing body of evidence on the problems caused by both the GM crops themselves and the resulting over-use of the herbicide Roundup®. For example, a recent evaluation of studies on crops genetically modified to produce the insecticide *Bacillus thuringiensis* ("Bt")[2] in India concluded that the studies, which Monsanto claimed supported the safety of its crops, "ignored toxic endpoints" that may have significant implications for human health. Rats fed the GM grain showed damage to the animals' ovaries, livers, and immune systems. *See* **Attachment A**, L. GALLAGHER, BT BRINJAL EVENT EE1: THE SCOPE AND ADEQUACY OF THE GEAC TOXICOLOGICAL RISK ASSESSMENT: REVIEW OF ORAL TOXICITY STUDIES IN RATS (2010) at p.2. Another recent review of the studies on GM crop feeding trials found that the trials used "controversial protocols" and ignored statistically significant results indicating the potential for chronic diseases in the liver and kidney. *See* **Attachment B**, G. Seralini et al., *Genetically Modified Crops Safety Assessments: Present Limits and Possible Improvements*, ENVTL SCI. EUR. 2011, 23:10. The active ingredient of Roundup®, glyphosate, has been found to cause damage to human embryonic and placental cells, and to make plants more susceptible to disease. *See* **Attachment C,** N. Benachour et al., *Time- and Dose-Dependent Effects of Roundup on Human Embryonic and Placental Cells*, ARCH. ENVTL. CONTAM. TOXICOL. 53, 126-133 (2007); **Attachment D**, G.S. Johal and D.M. Huber, *Glyphosate Effects on Diseases of Plants*, 31 EUR. J. AGRONOMY 144-152 (2009).

For these and other reasons unrelated to patent liability, many people are not interested in including Monsanto's products in the food chain. Unfortunately for both farmers and

[2] Strains of the bacteria *Bacillus thuringiensis* ("Bt") produce proteins, known as Bt toxins, that are toxic to certain crop-destroying insects.

consumers, however, avoiding GM contamination is effectively impossible. While Monsanto's brief leaves the impression that avoiding contamination is simple and easy, GM contamination can occur at any stage of the food chain as a result of both natural processes and human intervention: from seed production to crop growing to harvesting to cleaning to storage and transport. To minimize contamination, a farmer must undertake expensive and burdensome measures at every step of production:

1) having the seed tested;
2) implementing buffer zones to avoid cross-pollination;
3) paying for extra time and equipment to ensure that the harvester and cleaner do not contaminate the crop from previous jobs;
4) testing after harvest to check for contamination from events such as seed blowing from a passing truck (a frequent occurrence in an agricultural area);
5) paying to have the truck cleaned prior to hauling non-GM grain to market;
6) paying extra for special storage or storing the grain on the farm after harvest.

Consider the cost of just one of these steps, the buffer zone. According to the Ohio State University Extension, for corn, a buffer zone of 660 feet is required to limit cross-pollination to 1% or less. With a buffer zone of less than 165 feet, the Extension recommends removal of several rows of corn. The actual impact on a small farmer raising 20 acres of corn is significant. Incorporating a 165-foot buffer zone on two sides of a 20-acre field would result in the loss of the use of 35% of that field.[3] The alternative, according to the Extension, is to remove 16 border rows on each side, resulting in losses from the expense of planting that corn, harvesting it

[3] One acre equals 43,650 square feet. A 20 acre-field is 934' x 934'. Incorporating a 165' buffer zone on two sides would reduce the field to 604' x 934', or 564,136 sq. ft or 12.9 acres.

separately and disposing of it. *See* **Attachment G,** PETER THOMISON**,** OHIO STATE UNIVERSITY EXTENSION, FACT SHEET, MANAGING "POLLEN DRIFT" TO MINIMIZE CONTAMINATION OF NON-GMO CORN (2004), available at http://ohioline.osu.edu/agf-fact/0153.html.

And even with these extensive precautions, GM contamination cannot be wholly prevented. For example, gene flow from a GM bentgrass patented by Scotts was observed to have spread as far as 21 kilometers (13 miles) away from the experimental plantings in the direction of prevailing winds. *See* **Attachment H**, L.S. Watrud et al., *Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker*, PROC. NAT'L ACAD. SCI. U.S. 101: 14533-14538 (2004). *See also* U.S. ENVIRONMENTAL PROTECTION AGENCY, WIND MOVES POLLEN WITH ALTERED GENETIC TRAITS BEYOND FIELDS OF EXPERIMENTAL BENTGRASS, http://www.epa.gov/wed/pages/news/04Nov/lead.htm. In Canada, testing of canola seeds from "certified seedlots" revealed GM contamination in all but one seedlot, with approximately 10% of the seedlots showing "very high levels" of contamination, namely greater than 2.0%. *See* **Attachment E,** Friesen et al. at p.9-10. Notably, the seed samples in the Canadian study were taken in 2002, when only 40% of the Canadian canola was estimated to be GM, and the pedigreed crops were required to have extensive isolation distances to try to minimize contamination. *See* Friesen et al. at p.3 & 11. In contrast, 94% of all soybeans, 90% of all cotton, and 88% of all corn planted in the U.S. is currently genetically modified, vastly increasing the probable extent and levels of contamination. *See* U.S. DEPARTMENT OF AGRICULTURE, ECONOMIC RESEARCH SERVICE, ADOPTION OF GENETICALLY ENGINEERED CROPS IN THE U.S., http://ers.usda.gov/data/BiotechCrops/adoption.htm.

The biological reality is that a farmer who raises corn, cotton, soybeans, canola, sugar beets, or alfalfa cannot reliably avoid infringing on Monsanto's patent. The farmer is left with a

choice: risk infringement or refrain from raising these crops at all. That is precisely the type of dilemma that the Declaratory Judgment Act is meant to address. *See MedImmune*, 127 S.Ct. at 773, 549 U.S. at 130 ("The dilemma posed by that coercion – putting the challenger to the choice between abandoning his rights or risking prosecution—is a 'dilemma that it was the very purpose of the Declaratory Judgment Act to ameliorate.'").

CONCLUSION

This problem is of Monsanto's making. By developing a product that is self-replicating, and then marketing it to farmers across the country, Monsanto has ensured that no farmer can entirely avoid infringing. Monsanto has chosen to exploit this problem by an aggressive pattern of enforcement that has left farmers across the country in fear of an enforcement lawsuit even if they have no desire or intent to use the patented seeds. These farmers are placed in the position of abandoning growing valuable crops or investing significant time and effort in protective measures to try to minimize contamination. In the latter case, no matter what measures they take, the farmers still face the threat of a patent infringement lawsuit because of the impossibility of remaining completely GM-free. It is critical for both these farmers and for all the parties involved in the food chain, including the *Amici*, that this Court consider this case and provide a clear declaration of their rights.

Respectfully submitted,

Dated: August 10, 2011

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BT BRINJAL Event EE1

The Scope and Adequacy of the GEAC Toxicological Risk Assessment

Review of Oral Toxicity Studies in Rats

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SUMMARY

This evaluation of Bt brinjal studies is based on requirements for a rigorous evaluation of food safety for the people of India and their health. Departures from Indian and international published standards for the 14-day and 90-day studies are a cause for concern[1].

The current food safety studies for Bt brinjal were not conducted in accordance with published standards, did not accurately summarize results, and ignored toxic endpoints for rats fed Bt brinjal: in particular, rats fed Bt brinjal for 78 out of 90 days (only one dose level) experienced:

- organ and system damage: ovaries at half their normal weight, enlarged spleens with white blood cell counts at 35 to 40 percent higher than normal with elevated eosinophils, indicating immune function changes.
- toxic effects to the liver as demonstrated by elevated bilirubin and elevated plasma acetylcholinesterase.

Major health problems among test animals were ignored in these reports. The single test dose used was lower than recommended by the Indian protocols. Release of Bt brinjal for human consumption cannot be recommended given the current evidence of toxicity to rats in just 90 days and the studies' serious departures from normal scientific standards.

Unanswered concerns regarding the safety assessment of Bt Brinjal

Neurological function, behavioral effects, reproductive performance and biological resilience of test animals were not evaluated in these studies. Further research based on properly conducted and supervised studies is needed to resolve indications that Bt brinjal may have adverse effects on these clinical endpoints.

Dietary equivalence of dried brinjal, dried Bt brinjal and control diets was not addressed. Concentrations of the new insecticide protein Cry1A(c) were not measured in dried brinjal powder. It is important to know how much of this new protein was actually in the dried samples fed to the rats, especially since there is data to suggest that Cry1A(c) is at least partially destroyed in laboratory heating conditions. That omission makes it impossible to compare the test diet with insecticide concentrations expected in cooked human food.

[1] The Indian toxicology standards for 14-day and 90-day feeding trials published by the Department of Biotechnology (DBT) in 1998 and in 2008 fall short of the international standards (OECD 1998 and Codex Alimentarius 2003a-c), allowing a significant loss of scientific rigour. Therefore, although this critique is based on the Indian DBT protocol, meaningful departures and omissions from international standards are noted. It is important to clarify that 14 and 90 day exposures to rodents are insufficient periods of time on which to base food safety decisions for humans.

The use of laboratory animals to test food safety, although widely accepted as a toxicological tool, is only an indication of effects that might be expected from human exposure[2]. It is essential that deviations from standard protocols be evaluated carefully, as these changes can have a profound impact on results. Yet every departure made by INTOX (the laboratory contracted to do the research) from the Indian Department of Biotechnology protocol (1998) has resulted in lower standards being used, with less power to detect changes in rats eating Bt brinjal. These include: skipping important endpoints such as IgE measurement to test for allergenicity, testing only one dose that was lower than human consumption is likely to be, ignorance of toxicological equivalence, lost data, lack of Good Laboratory Practice standards, inadequate observation of animals, a 29% decrease in exposure days in one study (doses were administered 5 days per week instead of 7), etc.

Consequently, the studies submitted by Mahyco are woefully inadequate to determine the safety of Bt brinjal for long-term human consumption.

Table 1. Summary of statistically significant findings in rats fed Bt brinjal in 90 day study with implications for human health

Indicator	What it might indicate	Significant potential adverse effect
Elevated white blood counts from chronic exposure	Inflammation, allergy, tissue injury	√
Higher aspartate aminotransferase in blood from acute exposure	Liver damage	√
Elevated bilirubin in blood		
Altered plasma acetylcholinesterase		√
Smaller ovaries	Reproductive toxicity	√
Enlarged spleens	Chronic infections or blood cancer	√

[2] Significant genetic and phenotypic variation between humans makes it statistically impractical to conduct food safety trials on humans. As a result, the use of animals to test food safety cannot completely resolve all potential health implications because it also introduces uncertainty in risk assessment. In other words, the lingering uncertainties in animal feeding studies require that they be interpreted cautiously and conservatively. Any indication of adverse effect (even if statistically small) should be followed up.

METHODS

The current assessment is of three toxicology studies conducted by commercial toxicology laboratory INTOX PVT LTD on behalf of Maharashtra Seed Company, also known as Mahyco. Study details and raw data have recently been made available to the public through the internet link http://www.envfor.nic.in/divisions/csurv/geac/bt_brinjal.html.

All three studies tested the genetically modified food brinjal (herein referred to as Bt brinjal)[3] containing the insecticide protein Cry1A(c) and other novel genetic components, as it is proposed for sale of seed in India;

- a 14-Day Dose Range Finding Study
- an Acute Oral Toxicity study of Transgenic Bt brinjal containing Cry1A(c) gene in rat (Study No. 218301)
- a 90-day Subchronic Oral Toxicity Study of Transgenic Bt brinjal (Study No. 218304).

These studies are herein referred to as the 'dose-range finding', the '14-day' and the '90-day' studies, respectively.

The dose-range finding study

The first of these published studies is a 14 day dose-range finding study of Bt brinjal in rats. Although this study was submitted by INTOX PVT LTD via Mahyco, it is misleading to do so as it occurs within a report for another study. Only limited information about the study is provided, and then only in summary form on pages 12 and 13 of the 90-day study report, as well as some raw data in Appendix D. This study is disregarded from further consideration for the following reasons:

- Only three animals were tested per dose group, which is insufficient to make any valid conclusions. According to the Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts (DBT 1998), a minimum of ten animals per dose group is necessary. This is considerably less than the OECD standard of ten animals per sex and dose group.
- The problem with using fewer than the recommended number of animals is an increased chance of Type II errors – that is, failing to observe a treatment-related difference when in truth there is one.

In addition to using too few animals to provide confidence in the findings, there were other arbitrary and unjustified methodological practices:

- The rationale for using doses of dried brinjal powder at 500 and 1000 mg/kg was not provided.

[3] Brinjal is also known as eggplant or aubergine

- The study guidelines and laboratory standards for this study were not provided. Statements about following good laboratory practice (GLP) or having GLP certification are also absent. Lack of stated adherence to laboratory standards or protocol puts the quality of the research conducted into question.
- The dates of the study and the names, titles and signatures of the people conducting the study were not provided.

The 14-day and 90-day studies

The 14-day and the 90-day studies are stated to have been conducted according to "Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts"[4] as outlined by the Department of Biotechnology (DBT) in India in 1998, and in compliance with the principles of Good Laboratory Practice as established by the OECD in 1998.

Statements of compliance with Good Laboratory Practices and Quality Assurance (pages 3 and 4 of both study reports) are not signed. This omission does not inspire confidence in the published results.

Inclusion of extra control groups

Only one control group was required according to the Indian protocol (DBT 1998, page 61) and international protocols (OECD 1998, Item 14 page3)(Codex Alimentarius 2003b). INTOX PVT LTD on behalf of Mahyco used three control groups for each single dose test group:

- G I (14 day test) and G1 (90 day test): Controls receiving vegetable oil only (vehicle control)
- G II (14 day test) and G2 (90 day test): Vegetable controls receiving non-transgenic brinjal powder in oil
- G III (14 day test) and G3 (90 day test): Vegetable controls receiving commercially available non-transgenic brinjal powder in oil.

Was the non-transgenic brinjal group included under the assumption that the studies would find no toxicity at the doses used [5000 mg/kg-day in the 14-day study and 1000 mg/kg-day in the 90-day study], and therefore suffice as the 'limit tests' described on pages 54/55 and 62 of the protocol (DBT 1998)? The second and third control groups listed above were not required for the 14-day or the 90-day studies.

[4] Herein referred to as 'the protocol'

The inclusion of extraneous control groups is not scientifically or methodologically valid. Increasing the number of control groups in this manner decreases the chances that true and relevant differences will be consistently observed between the Bt brinjal group and others.

The salient analysis of toxicology results is between equal numbers of individuals from the Bt brinjal group and a single control group. Since it is unnecessary to produce more than one control group and since we have no information to confirm that the commercially-available brinjal did not contain the Cry1A(c) protein, or other agricultural chemicals that may adversely affect the health of animals eating it, commercially available brinjal dose groups (G III and G3) are not considered further in this analysis.

Presence or absence of Cry1A(c) protein in brinjal powder

Were fruit powders received from Mahyco verified for the presence or absence of transgenic material just prior to conducting toxicity tests? The only evidence we have that testing was conducted to confirm the presence of Cry1A(c) protein in Bt brinjal and non-Bt brinjal in these studies is a single page that was produced twice: at the end of the 14-day study report (no page number) and also as Appendix E on page 106 of the 90-day study report. Since there is no date on this page and these two studies were conducted more than one year apart, it is impossible to know which study it was produced for.

Evidence of testing for the Cry1A(c) protein in animal feed is *either* misrepresented in one or both of these reports *or* both studies used the same stored batches of dried brinjal powder. The possibility that transgenic proteins degraded during drying or after storage cannot be ruled out, representing a significant potential loss of potency of the test article. Furthermore, there is no indication of the concentrations of Cry1A(c) protein in dried brinjal powder either before or after several months of storage. In turn, this would be a further loss in representation of laboratory tests at a dose that consumers are likely to be exposed to.

New statistical analyses

Raw data from the published reports were used to calculate statistically significant differences between test groups using a student's t-test for two independent samples with unequal variance using Microsoft Office Excel 2007. The raw data selected were variables noted from visual inspection of the summary tables for each report. This included concentrations of acetylcholinesterase (a neurotransmitter enzyme) from plasma and red blood cells, bilirubin (increases indicate liver complications from infection or chemical exposure), total white blood cells (increased in response to infection) and aspartate aminotransferase (increases are used to diagnose liver or heart damage) in blood. In the 90-day study, organ weights for ovaries (which give an indication of reproductive health), spleen (this organ purifies the blood) and kidneys (which excrete waste products from the body) were also analysed.

Direct statistical comparisons in both studies are made between the main test group (G IV and G4, receiving Bt brinjal powder in peanut oil) and the group receiving peanut oil only (G I and G1).

Comparisons between the Bt brinjal test groups and the control group receiving non-transgenic brinjal in peanut oil (G II and G2) are described in the text and noted in Appendix B of this report.

This report addresses the following questions:

1. Do the two studies meet the stated 1998 protocol standards[5] for India and the OECD standard?
2. Have the studies been accurately summarized to be consistent with the raw data results? Were statistical assumptions valid and adequately described?
3. Would an impartial technical reviewer derive the same conclusion as the laboratory contracted by Mahyco (and accepted by the second Expert Committee or EC II)?

The larger question of whether or not these results are sufficient to draw conclusions of food safety is addressed in the Discussion section of this report.

[5] The DBT protocol was updated in 2008. Since this research was conducted prior to 2008, the 1998 protocol was relevant at the time. Neither of these protocols adhere to international standards.

RESULTS

Table 2 below summarizes the compliance of the 14 day acute toxicity test and the 90 day feeding study with the stated guidelines (DBT 1998).

Table 2. Summary of study characteristics in compliance with protocol guidelines (1998)[6]

Protocol Requirement, Department of Biotechnology 1998	14 day study	90 day study
Sufficient number of animals tested per dose group; 10 for 14 day study and 20 for 90-day study	Yes	Yes
Animals housed singly or in pairs (not a protocol requirement for 90-day study)	Yes	No
Test doses selected according to protocol	Yes	No
Daily (twice daily required for 14-day study) observations of animals to look for signs of toxicity including tremors, convulsions, salivation, diarrhea, lethargy and sleep, dyspnea, coma, nasal bleeding, etc[7].	Undetermined	No
Daily observations of behavioral abnormalities	No	No
Statistical methods described	No	Yes
Statistical methods used	No	No
Statistical results reported	No	No
Significant differences discussed in terms of biological significance and impact on food safety	No	No
Study summary reflects results	No	No

[6] Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts. Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998. Public Printing Service (Delhi) 96 pp.

[7] Updated protocol for 2008 emphasizes the importance of behavioural signs of toxicity not limited to hunched posture, lethargy or persistent recumbancy, labored breathing, any condition interfering with eating or drinking (e.g., difficulty moving), or excessive or prolonged hyperthermia or hypothermia.

The 14-day acute oral toxicity of transgenic Bt brinjal containing Cry1A(c) gene in rat

An acute oral toxicity study (a limit test) was performed on rats fed 5 grams of dried brinjal powder per kg of body weight in peanut oil. Doses were administered over 24 hours and rats were observed for 14 days following dosing.

As shown in Table 2, the 14 day study was conducted with several deviations from the 1998 DBT protocol: the report lacks a description of statistical methods used, study results were not compared using a statistical analysis, and important variations in health endpoint outcomes were not discussed in terms of biological significance. These are critical and unjustifiable omissions by the researchers. Consequently, while the study has been used by Mahyco to provide evidence that Bt brinjal is safe to eat, this conclusion cannot be substantiated.

New Statistical Comparisons for the 14- day study

For the purposes of verifying the conclusions reported in the 14 day study, the following statistical comparisons have been made on endpoints of interest[8] from the following sources in the INTOX report:

- Appendix B1 of INTOX report: Individual animal hematology data
- Appendix B2 of INTOX report: individual animal clinical chemistry data

[8] Endpoints of interest were selected from quick visual inspection of data summary tables. This is not an exhaustive analysis of all raw data from the 14-day report.

Table 3. Results of statistical analysis of raw data from the 14-day study

Toxicological endpoint	Arithmetic mean values for females/males/total	
	Vehicle control group (G I)	Bt brinjal group (G IV)
Total white blood cells ($x10^3$/cmm) females/males/total	8.6/9.0/8.8	7.7/8.2/8.0
Aspartate aminotransferase (IU/L) females/males/total	164.2/154.0/159.1	251.8**/244.8*/248.3**
Plasma acetylcholinesterase (IU/L) females/males/total	641.8/656.2/649.0	534.0/529.3/531.7**
Red blood cell acetylcholinesterase (IU/L) females/males/total	407.6/398.8/403.2	351.9/324.9/338.4
Bilirubin (mg/dl) females/males/total	1.1/0.9/1.0	1.1/1.2*/1.2*

*Statistically significant difference from G IV at $p \leq 0.05$

**Statistically significant difference from G IV at $p \leq 0.01$

Toxicological implications of the results in Table 3 from the 14 day study

Total white blood cell counts were found to be 9 to 12% lower among the rats fed Bt brinjal compared to controls. The toxicological implications of *decreased* white blood cell count following an acute exposure include a possible recent infection or impaired immunological function.

Increases in aspartate aminotransferase (AST) among Bt brinjal-fed rats were 54 to 60% higher than controls. Increased AST indicates damage to the liver or heart. In this case, coupled with elevated bilirubin (another measure of liver dysfunction also noted in this table), damage to the liver from short-term exposure at the dose of 5000 mg/kg-day is indicated.

Plasma acetylcholinesterase was 22% lower among rats fed Bt brinjal than that observed for controls. Significant changes in plasma acetylcholinesterase (a neurotransmitter enzyme) concentrations could be further evidence of liver damage in rats fed Bt brinjal.

Inconsistencies in the 14-day study report

Page 6 of the INTOX report is a summary of the 14-day study. In this summary there are only three study groups mentioned as follows: "…the test article was administered orally to a group of 5 male and 5 female rats as an acute dose at the limit dose of 5000 mg/kg body weight, suspended in peanut oil, as a vehicle. One concurrent control group of 5 male and 5 female rats was similarly gavaged with nontransgenic brinjal powder in peanut oil, while a third group of 5 male and 5 female rats was gavaged with normal powdered rodent diet in peanut oil only, and served as an untreated control." The study summary appears to be at odds with the data reporting results for four study groups, not three.

Page 9 of the report states: The total number of animals tested per sex is 20 (five per dose group using four dose groups) but the table lists only 15 animals per sex. These may only be typographical errors. However, they may also indicate that data for the third test group (G III, not mentioned in the study summary) was added at a later date, as suggested by the study summary.

The results given in Table 3.3 (individual animal fate and pathology findings) of Appendix B (of the INTOX report) for group G III are identical to the results in the next table for Bt brinjal-fed rats among both males and females. This additional suspected formatting/typographical error carries other implications: where are the missing data for these animals and what information is contained in those missing data?

The protocol requires twice daily observation of animals for signs of toxicity since the test article is given in a single acute dose. Tables A1.1 through A1.4 report no clinical abnormalities over 14 days of observation. It is unlikely that clinical observations would not pick up a single abnormality among 40 rats over 14 days, if observations had been conducted by trained researchers.

Statistically significant differences between the Bt brinjal and control groups for toxicological endpoints were not noted or discussed in the INTOX report.

The 90-day Subchronic Oral Toxicity Study of Transgenic Bt brinjal

Animal Husbandry

Caging groups of 5 animals together is considered to be extreme crowding unless unusually large cages were used. Instead, they should have been housed singly or in pairs. Group caging also has the effect of "washing out" individual differences in amounts of food and water consumed over the course of the study. If one animal in five has an abnormal eating or drinking pattern (as was the case among goats fed Bt brinjal) this is unlikely to be observed in a group measurement, even though a health outcome for 20 percent of the population (one in five animals) is of interest. It is noted that this is not strictly a deviation from either the 1998 protocol or the OECD 408 protocol which states that animals may be housed in small groups of the same sex in the 90-day study (OECD 1998).
Were all test group animals placed in the same room to minimize differences in temperature, humidity and air changes that will impact on the overall health of the animals? This needs to be specified but was not.

Were all test group animals obtained from the same source, at the same age, previously unexposed to Bt brinjal and nulliparous at the start of the study? Were animals randomly assigned to dose groups at the start of the study? All of these variables need to be reported as they have the potential to affect health outcomes measured in this study, but none of them were.

Dose groups

The 90-day toxicity study is meant to include 3 doses of Bt brinjal. According to the Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts;

> "The selection of the dose is made on the basis of acute toxicity studies of the test chemical. At least 3 dose levels, one maximum, one minimum and one intermediate are used. Consideration is given that the highest dose may result in toxic effects without causing excessive lethality and the lowest dose may not produce any toxic effects. A group of vehicle controls is also used." –(DBT 1998).

The 90-day study was conducted using a single dose level for which there is no demonstrable toxicology information prior to conducting the study. Without evidence to support the assumption that 1000 mg/kg-day will result in toxic responses in a 90-day study, this particular dose does not make sense scientifically.

One possible outcome of using a dose for which there is no evidence of toxicity would be a false finding of safety because the dose was too small to observe toxic effects in rodents over 90 days. This increases the chance of failing to observe a treatment-related toxic endpoint when in truth there may be one.

Reasons for using the dose of 1000 mg/kg were tacitly given by stating that a dose range finding study had been conducted. Since this test used a total of three animals per test group, and for other reasons listed above, this cannot be considered justification for selecting the 1000 mg/kg-day dose over 90 days.

Moreover, there are justifications for believing that one gram of brinjal per kg body weight is inadequate to determine the health effects of this crop on the Indian people. Brinjal is a crop that is widely consumed in significant amounts in India. The dose used in this study is equivalent to only 40g (about 2 tablespoons) of Bt brinjal/day for a slightly-built woman and 70g/day (about 4 tablespoons) for a reasonably-sized man. Notably, Codex Alimentarius 2003b recommends:

"Information about the known patterns of use and consumption of a food, and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA plant. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage." paragraph 49, Section 1.

This recommendation is emphasized in all guidance for conducting the 90-day subchronic toxicity study, including the 1998 DBT protocol: "expected human exposure may indicate the need for a higher dose level."

Other omissions in the 90-day study

IgE was not measured in this study, even though the report states on page 16 that IgE was analysed. Clinical chemistry data in Appendix B2 report IgE results as <1.00 IU/ml for every observation. Since IgE concentrations vary widely between individual rats (Abadie and Prouvost-Danon 1980) and expected values in rats are greater than 200 IU/ml[9], it is likely that:

- The IgE measurement method used by the researchers using the "Erba Smartlab Random Access Batch Analyser" (page 16 of the report) was not sensitive enough to accurately measure IgE in rats.

- Blood samples were incorrectly stored prior to chemical analysis leading to serious errors in the results.

The lack of IgE data is unfortunate as IgE is especially important as a measure of allergic reactivity. Quantitative evaluation of IgE is required in the protocol on page 62 of DBT 1998 and emphasized in an expert consultation from FAO/WHO (2001). This is a considerable omission and protocol deviation that has not been addressed. Without IgE data, there is a lack of important information about the possible effects of the Cry1A(c) protein on the mammalian gut resulting in possible hypersensitivity/allergic reactions, observed as increased concentrations of IgE compared to controls (Karlsson et al 1979). On the other hand, decreased concentrations of IgE in Bt brinjal rats would be consistent with diseases such as hypogammaglobulinemia, autoimmune diseases, ulcerative colitis, and hepatitis. It is important to know whether the new brinjal may simply act as an irritant that produces allergic responses in the gut, or as an endotoxin conferring damage to the liver with ingestion.

Raw data in Appendix B1 of the INTOX report for differential white blood cells are reported in whole numbers without decimal places, preventing analysis of eosinophil concentrations. The summary table for these results (Table 8 on pages 38-39 of the INTOX report), however, reports concentrations at two decimal places (two more significant digits than the raw data support). Is this is a lack of precision in reporting individual raw data, or is it over-precision in aggregate data? It is impossible to tell if the aggregate data actually reflect the raw data in this case. The overall effect of leaving out these important raw data is to prevent independent analysis of differentiated white blood cell counts in rats fed Bt brinjal.

[9] Background concentrations of Total IgE in Sprague-Dawley rats are 0.6 ug/ml (Abadie and Prouvost-Danon, 1980); this is equivalent to 250 IU/ml. Thus, it is extremely unlikely to measure less than one IU/ml of IgE in any rat, and even more impossible in 80 rats.

New statistical comparisons for the 90-day study

In order to verify the conclusions listed in the 90-day report, statistical comparisons on endpoints of interest[10] have been made using the following sources in the report:

- Appendix A3: Individual animal organ weight absolute values
- Appendix A4: Individual animal organ weight relative values
- Appendix B1: Individual animal hematology
- Appendix B2: Individual animal clinical chemistry

Table 4. Results of statistical analysis of raw data from the 90-day study

Toxicological endpoint	Test group mean values females/males/total	
	Vehicle control group (G1)	Bt brinjal group (G4)
Organ weight – ovaries (g) females only	0.11	0.06**
Organ weight – spleen (g) females/males/total	0.86/1.34/1.10	1.02/1.19/1.11
Organ weight – kidneys (g) females/males/total	1.42/1.34/1.38	1.48/1.19/1.34
Total white blood cells ($x10^3$/cmm) females/males/total	9.3/11.1/10.2	14.0*/12.6/13.3*
Aspartate aminotransferase (AST) females/males/total	134.5/189.5/162.0	151.7/156.5/154.1
Plasma acetylcholinesterase (IU/L) females/males/total	591.6/604.0/597.8	875.0/902.6**/888.8**
RBC acetylcholinesterase (IU/L) females/males/total	299.9/388.3/344.1	265.7/335.6/300.6
Total acetylcholinesterase (IU/L) females/males/total	891.4/992.4/941.9	1140.7/1238.2/1189.4*
Bilirubin (mg/dl) females/males/total	.58/.51/.54	. 81**/.52/.66*

[10] Endpoints of interest were selected from quick visual check of data summary tables. This is not an exhaustive analysis of all raw data from the 90-day report.

*Statistically significant difference from rats fed Bt brinjal at $p \leq 0.05$
**Statistically significant difference from rats fed Bt brinjal at $p \leq 0.01$

According to the DBT 2008 study protocol, toxicological implications of the results must be reported:

"The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth well into adulthood. The study will provide information on the major toxic effects, including possible target organs, and the possibility of cumulative effects." –DBT, 2008

Discussion on the implications of the toxicology results for the 90-day report from INTOX has been left out.

Toxicological implications of the results in Table 4 from the 90-day study

Females who were fed Bt brinjal had smaller ovaries than controls. At just over half the expected size, ovaries of Bt brinjal-fed rats exhibit a consistent[11] and profound reproductive toxicity signal that is statistically significant at $p < 0.0001$[12] even with the small number of animals tested and the relatively short exposure time (90 days). Unfortunately, a 90-day study is not long enough to know what the long term reproductive performance outcome would be for animals fed Bt brinjal. Other research has shown that when mice were fed genetically modified food containing the Bt toxin in a multigenerational study, they had decreased reproductive performance as demonstrated by smaller litter size and lower average litter weight (Velimirov 2008)[13].

Spleen weights among Bt brinjal female rats were 19% higher than the control group (and 26% higher than the vegetable control group – see Appendix B, Table B2). Differences were statistically significant when compared to the vegetable control group (Appendix B, Table B2)[14]. These differences were not noted in the INTOX report.

Significant changes in both the ovarian and spleen weights for the female rats fed Bt brinjal were apparent from summary values listed in Table 6 of the 90-day report. However, page 21 of the report incorrectly summarized the results, saying:

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[11] Vegetable controls also had normal-size ovaries compared to Bt brinjal rats (see Appendix B, Table B2).

[12] Statistical significance remained the same using ovary weights relative to total body weight.

[13] It is worth noting that only one of two reproductive toxicology study protocols was powerful enough to observe this sensitive outcome; the Reproductive Assessment by Continuous Breeding (RACB).

[14] Statistically significant differences are dependent on sample size: doubling the observations of each group G1 and G4 results in statistically significant increases in spleen weights for females fed Bt brinjal.

> "The values of absolute and relative weights of kidneys, liver, adrenals, testes, spleen, brain and ovaries of male/female rats treated with Transgenic Bt brinjal containing Cry 1 A(c) gene, non-transgenic brinjal and nontransgenic brinjal (commercially available) at 1000 mg/kg were found to be comparable to those of the control group rats at termination of the treatment."

Page 22 of the report further incorrectly concludes:

> "No alterations in the absolute and relative organ weights of rats treated at 1000 mg/kg [were found]".

These statements clearly indicate that the authors are not familiar with the principles or procedures for evaluating their own results in the 90-day study. Only the vehicle control group (G1) was required in this study (DBT 1998, page 61 and OECD 1998, Item 14 page3). As shown in Table 4 above, significant measures of organ toxicity (resulting in lower organ weights) to female rats consuming Bt brinjal in the 90-day study were evident. Findings of statistical significance further emphasize the seriousness of these differences.

Rats fed Bt brinjal also displayed elevated white blood cell counts (up to 50 percent higher among females and 33 percent higher overall), compared to controls. This was consistent among vegetable controls (Appendix B, Table B2). In differentiated white blood cell counts, this increase includes a near doubling in the count of eosinophils (a type of white blood cell) among Bt brinjal-fed rats. Eosinophils typically increase in response to allergic disorders, infection or to epidermal inflammations (such as those caused by parasitic infections). Bt brinjal-fed female rats had nearly twice the concentration of eosinophils compared to control groups, consistent with subchronic gut irritation, possibly caused by Cry1A(c) protein in the diet. For reasons discussed above, it is impossible to compare eosinophil concentrations using the raw data presented in the 90-day report.

Bilirubin concentrations are also elevated among female rats, with a 35% increase compared to vegetable controls (Appendix B, Table B2) and a 40% increase compared to vehicle controls. Bilirubin is a measure of liver function. Increased bilirubin in the absence of harmful xenobiotics or infectious hepatitis indicates obstruction of the biliary ducts in the liver. The increases in bilirubin of females in this study are extreme enough to result in statistically significant differences for females and the total groups. It is not clear how the authors missed this result, but the differences are not described or discussed in the 90-day report.

Plasma acetylcholinesterase (a neurotransmitter enzyme) is 20-49% higher among all Bt brinjal-fed rats, both male and female, compared to control groups. Statistical significance for these increases were observed in comparison with the vehicle control group among males and total. The implications for elevated acetylcholinesterase in plasma samples could include hepatotoxicity (Garcia-Ayllon et al 2006), early onset of type II diabetes or neurological impairment (Garcia-Ayllon et al 2010).

When combining these individual signs of toxicity, a more concrete picture emerges:

- Increased white blood cell counts coincide with enlarged spleen weights observed among Bt brinjal-fed rats, further indicating immune responses to toxic exposure.
- Elevated bilirubin concentrations and elevated acetylcholinesterase concentrations are consistent with hepatotoxicity from subchronic exposure in rats fed Bt brinjal.

DISCUSSION

Shortcomings of using these data to approve Bt brinjal for human consumption

Consumers, objective scientists and government representatives need to be aware of the potential health effects of new foods proposed for sale in India so they can take part in the decision about how much uncertainty remains after feeding studies have been conducted and how that affects risk assessment. Although animal feeding studies are limited in their representation of human responses, they form an important basis from which to gauge possible toxic response to new products. Even when these limited short-term feeding trials are conducted correctly using Good Laboratory Practices and following internationally accepted protocols, there will be some exposures that are still untested; chronic (long term) exposure to humans and animals, occupational exposure to people growing Bt brinjal and inhalation exposure to those who cook or process Bt brinjal.

Adverse effects of Bt brinjal exposure may be more easily transmitted by inhalation than by ingestion. As has been shown in a study with Wistar rats, inhalation exposure caused immunomodulation in control rats housed in the same room as those fed a GM Bt rice diet (Kroghsbo et al., 2008). Human reactions to the Bt toxin via inhalation have been observed in occupational settings (Bernstein et al 1999): greenhouse workers exposed to Bt toxin in sprays developed allergic responses and elevated IgE compared to pre-exposure concentrations (Doekes et al 2004).

Multiple control groups and other methods of obscuring toxic response to GM foods

Previous publications from commercial seed producers on the toxicological research of transgenic foods have included multiple control groups (Hammond et al 2006). The use of multiple control groups has the effect of increasing the variation (wider confidence intervals) in the combined controls, which decreases the chance that a difference will be found between the test group and the controls. In some cases, as possibly indicated by group G III in the 14-day test described in this paper, the data are recorded under different circumstances than the animals consuming the test diet. In toxicology research, comparing equal numbers of individuals from two groups that receive different diets while all other variables are kept constant is the established method for investigating health effects related to diet. Establishing dose and response effects requires *at least* three test diet dose groups, as required by DBT 1998 and OECD 2003. Somehow the presentations made by commercial seed producers have allowed the opposite set of comparisons to be made: one or two test doses compared to several control groups.

Extra control groups is only one technique used by commercial operators to attempt to disregard significant differences between animals fed genetically modified foods and those on conventional diets. Other “techniques” that would be expected to disqualify research results from publication (if reviewers and publishers were blinded to the author’s interests) have been discussed previously (Seralini et al 2009). Briefly, these include:

1. A false assertion that males and females must have the same toxicological responses;

2. A false assertion that, if two doses are used, the higher dose must have a greater effect than the lower dose (a so-called dose-response observation);

3. A total omission of any data analysis enabling researchers to write conclusions in the void of data evidence;

4. A total omission of statistical results indicating significant differences in organ weights, haematology and clinical chemistry; and

5. Conclusions that ignore toxicologically significant results.

Other studies confirm toxic responses seen in Bt brinjal

The lack of scientific data on genetically modified food toxicology in the peer-reviewed literature (Domingo 2000 and 2007) indicates a scarcity of independent science in this area (Pryme and Lembke 2003). Specifically, no chronic feeding studies assessing the safety of genetically modified food containing the Cry1A(c) protein in the published, peer-reviewed literature were found at the time of this report.

Previous studies on the immunogenicity of the Cry1A(c) protein have shown that this protoxin is a potent allergen in animal models (Vásquez-Padrón et al 1999, Vásquez-Padrón et al 2000 and Moreno-Fierros et al 2000). Accordingly, this team concluded in 2000:

> "We think that previous to commercialization of food elaborated with self-insecticide transgenic plants it is necessary to perform toxicological tests to demonstrate the safety of Cry1A proteins for the mucosal tissue and for the immunological system of animals."

Although previous research has demonstrated hepatic toxicity in rats fed genetically modified foods (de Vendômois 2009 and Malatesta et al 2002, 2005), this is the first time that a food containing the Cry1A(c) protein has been tested and associated with hepatotoxicity and reproductive disorder.

Reproductive toxicity of Bt brinjal is demonstrated by the reduced ovarian weights resulting from a dose of 1000 mg/kg-day (ie 1 gram/kg body weight each day) using only ten animals, compared to two groups of controls (Appendix table B2). The likely clinical significance of decreased ovarian weights is lower fecundity, although other unintended effects may occur as well. This brings into question the possibility of hormonally-mediated toxicity that has not previously been considered for Bt brinjal but has been observed in other studies on GM foods containing proteins derived from the same *Bacillus thuringiensis* sp *kurstaki* (Bt) bacteria, Cry1Ab (Seralini et al 2007 and Velimirov et al 2008). Brasil et al (2008) found that a Bt soy diet altered ovarian and uterine morphology resulting in fewer follicles (viable eggs) and more corpus luteum (egg sacks without eggs), and a thickened uterus lining, in the second generation of female rats consuming Bt soy diets.

Fares and El-Sayed (1998) found structural damage to the ileum of mice fed Bt-potatoes over a two week period, when examining tissue with electron and light microscopes. The control group of mice fed

conventional potatoes differed significantly, with ileum tissue in its normal state. Elevated white blood cell counts experienced by female rats in the 90-day study are another indication of possible structural damage to tissues involved in processing Bt toxins such as the gut and spleen (Finnamore et al 2008). In the Finnamore study, mice eating Bt corn had increased eosinophil production and granulation, resulting in the production of specific cytokines (IL-6, IL-13, IL12p70 and MIP-1). Furthermore, it was noted that inflammatory and immunologic responses were stronger in weanling (young) mice than in older mice, because of their sensitivity to new allergens.

Compositional analysis of test article and vegetable control

The compositional analysis (reported in section 7.2 of Mahyco 2008) describes Bt brinjal as similar to non-Bt brinjal in content of protein, carbohydrate, oil, calories, ash, nitrogen, crude fibers and moisture content. These analyses were conducted by the seed company (Mahyco) at their own labs, and fall far short of required analytical parameters (Section 7.3 and Checklist 9 of Indian Council of Medical Research 2008, Sections 4.2.6 and 4.2.10 of the European Food Safety Authority 2008, Codex Alimentarius 2003b, sections 44, 45 and 49). Results are not shown in this report so it is impossible to know how large these differences might have been. Was the conventional counterpart of Bt brinjal used for the compositional analyses as recommended by section 44 of Codex Alimentarius (2003b)? The conventional brinjal parent variety was not named in the Mahyco 2008 report or the toxicology study reports. Was the conventional counterpart of Bt brinjal used in the 14-day and 90-day toxicology studies? If specific differences in vitamin, mineral, fatty acid and protein contents of the brinjal and Bt brinjal diets were not known at the time of the studies, there is some uncertainty about nutritional equivalence between test groups, and this may have impacted results.

Brinjal is an exceptional plant with many varieties. It is essential that the non-Bt brinjal comparator would have been the parent (conventional) variety of brinjal (EFSA 2008, Page S9), grown in the same location at the same time as the Bt brinjal to minimize differences in nutrients and solanine content. These important details were not described in the reports reviewed.

In particular, we have no knowledge of whether or not the Cry1A(c) and other protein concentrations in the dried brinjal powder used in this research was representative of actual cooked fresh brinjal at the point of consumption. Storage conditions of this brinjal powder are important, as we are led to believe that both the brinjal and Bt brinjal powder were received by INTOX in a single shipment from Mahyco and fed to rats over a period of years. There is no chain of custody report or acknowledgement of sample receipt, no verification of transgenic material presence and absence upon sample receipt and no documentation of proper labeling or safe storage procedures. It is likely that pesticide concentrations in the non-Bt brinjal and the Bt brinjal were measurable both prior to drying and before feeding to rats, yet we have no data on that either. According to EFSA guidance from 2006,

> "The role of the laboratory animal study is to deliver data from the basic, universal, presumably worst case situation for use in the hazard characterization. In practice, worst case will be decided on a case-by-case basis, but will most often be to test the GM food in its original raw form." -EFSA 2006

On page 105 of Mahyco's 2008 report there are descriptions of the cooking tests used to determine if the Cry1A(c) protein was stable in cooking. There are no data shown on concentrations of the protein before and after cooking trials, but the following statement was made: "Cry1A(c) protein was absent in cooked fruit. This study demonstrated that Cry1A(c) protein is completely degraded in Bt brinjal fruit upon cooking." The sensitivity values of the tests used to detect the protein were not specified, and in fact it was not clear which of these tests was actually used. However, this may indicate that at least some of the protein was lost upon heating. If that is the case, then how much of the protein was actually

in the dried fruit samples? Would this concentration be the same as that in Bt brinjal for cooked human food?

Minimum testing requirements have not been met

Commercial release of this product is not recommended prior to adequate safety testing. The minimum number of toxicity studies as recommended in the DBT 1998 protocol have not been conducted on Bt brinjal. While the OECD standard is superior to the DBT 1998, even meeting the latter would be an improvement.

The toxic effect of Bt brinjal to ovaries in female rats was completely missed by the toxicologists who wrote the report for the 90-day study, Mahyco reviewers who received the report, and the government committee who subsequently reviewed the report. If the statistical analysis had been conducted as indicated in the methods section of the report and the results of the analysis had been included in the summary tables and discussion as required by the regulatory guidance, this would have been impossible to miss.

> "Should there be structural alerts for reproductive/developmental effects or other indications from data available on a GM food and feed, then these tests [multi-generational reproductive toxicity studies] should be considered" – European Food Safety Authority GMO Panel Working Group on Animal Feeding Trials (2008)

Overall, this study fell short of the minimum testing requirements for the following reasons:

- Lack of dietary research to determine a relevant dose of brinjal in human diet, failure to analyse nutritional equivalence of test diets and identify the near isogenic parent line of brinjal, failure to perform quantitative analysis of Cry1A(c) protein before and after drying brinjal powder, failure to perform quantitative analysis of Cry1A(c) protein and pesticides in brinjal at the time of dosing test animals, no indication of proper receipt of test materials, identification or storage of test material by INTOX Lab.
- Failure to follow protocol guildelines relevant at the time the tests were conducted.
- Failure to analyse raw data, summarize results and provide discussion on the toxicological implications of results.
- Under Good Laboratory Practices, the study director is a qualified toxicologist who is responsible for the project from start to finish, and who must sign off on the final report. This did not happen.

CONCLUSIONS

A review of the adequacy of current toxicology studies to address the safety of genetically modified Bt brinjal for commercial release shows that the studies were not conducted according to the published standard, did not accurately summarize results, and ignored toxic endpoints for rats fed Bt brinjal.

For a brief period of time (1998 to 2001) there appeared to be an exemption given to genetically modified foods that showed no signs of toxicity: if a food tested at a dose of 1000 mg/kg-day produced no toxic effects then further testing was not required. According to OECD 1998 (page 3, item 16) and the 1998 DBT protocol quoted here,

> "If a test at one dose of at least 1000 mg/kg body weight (but expected human exposure may indicate the need for a higher dose level) using the procedures described for this study produces no observable toxic effects, then a full study using 3 dose levels may not be necessary."

Although this apparent exemption is no longer part of GM testing protocol (WHO/FAO 2000, Codex Alimentarius 2003a-c, EFSA 2008) the 90-day toxicity study appeared to be conducted at the particular dose of 1000 mg/kg-day with the expectation of finding no evidence of toxicity.

Were the contract laboratory INTOX PVT LTD and the funder Mahyco uncomfortable with results showing evident toxicity among rats fed Bt brinjal at 1000 mg/kg-day? Did the researchers write the conclusions for the 14-day and 90-day studies themselves or did others write conclusions for them? These questions are of interest since the text does not match the data, the researchers did not sign their reports, and the cover page of the 90-day report details a completely new report number (R/2183/SOR-90) from that which may be the original, 05.0002.

Not only has the scrutiny of these data provided insight into the substandard and extremely misleading interpretation of results, but it suggests to the reviewer that urgent changes need to be made to ensure that future studies are properly conducted and interpreted.

In particular, current results from these rat feeding studies indicate that rats eating Bt brinjal experienced organ and system damage: ovaries at half their normal weight, enlarged spleens with white blood cell counts at 35 to 40 percent higher than normal (elevated eosinophils in particular) indicating immune function changes possibly due to allergen response, and toxic effects to the liver as demonstrated by elevated bilirubin along with plasma acetylcholinesterase. Further studies are required to assess the potential outcomes of these indicators of toxicity.

Unanswered concerns regarding the safety assessment of Bt brinjal

Nutritional and toxicological equivalence of dried Bt brinjal samples

Are dried brinjal samples equivalent to cooked brinjal as it is prepared for human consumption, or do dried samples differ in their concentrations of Cry1A(c) and other important proteins, carbohydrate, fat and micronutrients? Would the toxicity profile of Bt brinjal also change as a result of cooking and home processing? Notably, Codex recommends:

"The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps." paragraph 47

Dietary equivalence for brinjal-fed rats, Bt brinjal-fed rats and vehicle control rats was not addressed.

Inhalation exposure to Bt brinjal

Oral ingestion of Bt brinjal does not address the issue of inhalation exposures to people who grow Bt brinjal or live near Bt brinjal crops in the ground. Toxicological responses to proteins that reach the lining of the lungs and nasal cavity, previously found to be of concern for agricultural workers, have not been addressed.

Toxicity testing standards

The main reason for conducting the toxicology studies is to have an objective assessment of whether or not the new food is safe for humans to eat. This needs to be a careful and objective assessment since millions of people with varying nutritional status, age and biological resilience will be exposed in the event of commercial release.

Neither of the 90-day toxicity testing protocols released by the Department of Biotechnology (1998 and 2008) are as methodologically strong as accepted international standards (see Appendix 1). This makes India an "easy target" for developers since the requirements to conduct toxicology studies are less stringent those in the European Union.

The use of laboratory animals to test food safety for humans is already a significant departure from species-specific testing. Deviations and omissions from accepted protocols need to be checked. Yet every departure made by INTOX on behalf of Mahyco has resulted in lower standards with less power to detect changes in rats eating Bt brinjal. These include leaving out important endpoints such as IgE

measurement to test for allergenicity, using only one dose group that is smaller than human consumption is likely to be, ignorance of toxicological equivalence, lost data, lack of Good Laboratory Practices standards, inadequate observations of animals, a 29% decrease in exposure days (doses were administered 5 days per week instead of 7), failure to quantify Cry1A(c) concentrations in dried fruit powder, etc.

The real risk here is that potential health problems attributable to Bt brinjal will be ignored as masses of people eat the very food their government thought was safe. In the long run, it is the people of India who could pay the price for bad science!

Further research studies

The logical next steps for describing the risk profile of Bt brinjal are to:

- Meet international published standards for conducting tests with scientific rigor in independent testing, following Good Laboratory Practice described in Council Directive 2004/10/EC (EC 2004) with quality assurance checks.
- Conduct dietary equivalence tests with quantitative measurement of Cry1A(c) protein before and after processing for administration to test animals according to ILSI 2004 and 2007 and Section 4 of EFSA 2006 to ensure that: nutritional requirements of animals are met equally between dose groups and that concentrations of Cry1A(c) and other proteins at the time of administration to animals are accurately represented..
- Conduct proper exposure assessment prior to laboratory studies so that future doses of Bt brinjal reflect the maximum exposure expected in the human population: 1000 mg/kg-day is not enough for an upper limit.
- Conduct the 90-day sub-chronic feeding study according to OECD guidelines, following Codex Alimentarius (2003 a-c) recommendations. That is, use at least three dose groups with doses given on all 90 days (not 5 days per week), include IgE measurements, perform daily observations on animals and include behavioral tests on individuals, include appropriate statistical analyses comparing the Bt brinjal group with appropriate controls and report results accordingly.
- Complete chronic (2-year) rodent feeding studies and multigenerational studies as suggested by the European Food Safety Authority (2008) to assess reproductive performance, neurological function and behavioral effects.
- Reduced resilience in circumstances of infection or other adverse events needs to be addressed as a potential risk factor for Bt brinjal consumers.

The Bt brinjal EC II Report recommending the commercial approval of Bt brinjal cannot be upheld. Scientifically rigorous safety assessment is needed to dislodge a trust deficit (held by the Indian public) created by the EC II Report. Furthermore, adoption of and adherence to a stronger safety testing protocol in India than the current DBT standard from 2008 is prudent.

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Appendix A: Protocol requirements for 90-day toxicity study by various sources

	DBT 1998[15]	DBT 2008[16]	OECD 1998[17]	FDA Redbook 2003[18]
Maximum number of animals per cage	Not specified	Individually or in groups of no more than 5	Individually or in small groups of the same sex	Individually
Good Laboratory Practices	15 items	Not specified	Not specified	U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations
Number of dose groups	At least 3	One or more	At least 3	At least 3 but ideally 4 or 5
Nutritionally equivalent diets required for each group	No	Yes	No	Yes
Number of animals per dose group	20	20	20	40 (20 if longer-term studies are planned)
Age of animals	Six to eight weeks old	Healthy young adult animals	As soon as possible after weaning, before they are 9	No later than six to eight weeks old

[15] Department of Biotechnology (1998) Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts. Ministry of Science and Technology, Government of India. Public Printing Service (Delhi) 96 pp.

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			weeks old	
Method of administration	Dry powder added to peanut oil and administered by gavage. Oil volume not to exceed 1 ml/100 g body weight	Not specified	Gavage delivery of an aqueous solution/suspension or solution/emulsion in corn oil. Oil volume not to exceed 1 ml/100 g body weight	In the diet, dissolved in drinking water or by gavage. Oil gavage not to exceed 0.4 ml/100g body weight
Control groups required	Vehicle control	Conventional non-GM plant with similar nutritional values	Vehicle control	Vehicle control: Control diet is equivalent in caloric density and contains the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the test groups
Dosing regime	5 days per week	7 days per week	7 days per week	7 days per week
Observation of animals	Daily observations of tremor, convulsion, diarrhoea, lethargy, dyspnea and nasal bleeding	Clinical signs include, but are not limited to: rapid weight loss; diarrhea (if debilitating); progressive dermatitis; rough hair coat; hunched posture; lethargy or persistent recumbency; coughing; labored breathing; nasal discharge; jaundice or anemia; neurological signs; bleeding from any orifice; self-induced trauma; any condition	Clinical observations at least once per day after dosing. Twice daily observations of morbidity and mortality. Opthalmological exams at beginning and end of trial Behavioural tests: sensory reactivity to stimuli of different types (e.g., auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment[19].	Daily or twice daily. Observation of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity including but not limited to changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or

[19] Out-of-the-cage behavioural tests are conducted prior to treatment start and periodically throughout the study

		interfering with eating or drinking (e.g., difficulty moving); or excessive or prolonged hyperthermia or hypothermia		other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern).[20]

[20] Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

Appendix B: New statistical analyses of Bt brinjal-fed rats in 14-day and 90-day feeding trials

Table B1. Results of statistical analysis of raw data from the 14 day study

	Arithmetic mean values for females/males/total		
	Vehicle control group (G I)	**Vegetable control group (G II)**	**Bt brinjal group (G IV)**
Total white blood cells ($x10^3$/cmm) females/males/total	8.6/9.0/8.8	8.7/8.4/8.6	7.7/8.2/8.0
Aspartate aminotransferase (IU/L) females/males/total	164.2**/154.0*/159.1**	165.4*/149.8*/157.6**	251.8/244.8/248.3
Plasma acetylcholinesterase (IU/L) females/males/total	641.8/656.2/649.0**	557.7/621.5/589.6	534.0/529.3/531.7
Red blood cell acetylcholinesterase (IU/L) females/males/total	407.6/398.8/403.2	303.0/369.7/336.4	351.9/324.9/338.4
Bilirubin (mg/dl) females/males/total	1.1/0.9*/1.0*	1.1/1.1/1.1	1.1/1.2/1.2

*Statistically significant difference from G IV at $p \leq 0.05$

**Statistically significant difference from G IV at $p \leq 0.01$

Table B2. Results of statistical analysis of raw data from the 14 day study

	Test group mean values females/males/total		
	Vehicle control group (G1)	**Vegetable control group (G2)**	**Bt brinjal group (G4)**
Organ weight – ovaries (g) females only	0.11**	0.10**	0.06
Organ weight – spleen (g) females/males/total	0.86/1.34/1.10	0.81*/1.20/1.00	1.02/1.19/1.11
Organ weight – kidneys (g) females/males/total	1.42/1.34/1.38	1.49/1.20/1.34	1.48/1.19/1.34
Total white blood cells ($x10^3$/cmm) females/males/total	9.3*/11.1/10.2*	9.3*/10.3/9.8*	14.0/12.6/13.3
Aspartate aminotransferase (AST) females/males/total	134.5/189.5/162.0	152.7/166.0/159.4	151.7/156.5/154.1
Plasma acetylcholinesterase (IU/L) females/males/total	591.6/604.0**/597.8**	731.0/753.2/742.1	875.0/902.6/888.8
RBC acetylcholinesterase (IU/L) females/males/total	299.9/388.3/344.1	332.1/390.1/361.1	265.7/335.6/300.6
Total acetylcholinesterase (IU/L) females/males/total	891.4/992.4/941.9*	1063.1/1143.3/1103.2	1140.7/1238.2/1189.4
Bilirubin (mg/dl) females/males/total	.58**/.51/.54*	.60**/.52/.56**	.81/.52/.66

*Statistically significant difference from rats fed Bt brinjal at $p \leq 0.05$

**Statistically significant difference from rats fed Bt brinjal at $p \leq 0.01$

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Education

2001 PhD in Epidemiology, University of Otago, Wellington, New Zealand

1998 European Educational Programme in Epidemiology, Florence, Italy

MSc, 1991 Environmental Technology, University of Washington, USA

BS, 1985 Human Nutrition and Foods, University of Vermont, USA

Work Experience

2008 - present **: Independent Contracter with the following projects:**

Applied Occupational epidemiology workshop coordinator and lecturer for clinicians studying to gain Occupational Medicine Qualification with the Australasian College of Physicians.

Food safety risk assessment in GM foods, Bt brinjal in India. Funded by GEKKO Foundation and Testbiotech, Germany.

Post-mortem toxicology of antidepressant suicides in New Zealand, collaborative project with the Institute for Environmental Science and Research, Ltd and Otago University.

Project Manager, **Bioremediation of TCDD-contaminated sediment in Whakatane,** Environment Bay of Plenty Regional Council and Te Rununga o Ngati Awa. Phytoremedation pilot project on 35 tonnes of contaminated canal sediment.

Senior Research Advisor, Policy Research and Evaluation Group, Sport and Recreation, Wellington, New Zealand (6 month contract)**.**

Literature review of historical chemical exposure to Maori in New Zealand, Te Atawhai O Te Ao

2008 **Principal Epidemiologist, Research Group at the Office of Australian Compensation Council, Department of Education, Employment and Workplace Relations in Canberra, Australia**

Strategic development of research agenda in consultation with other government agencies (federal and local), contract management, preparation of question-time briefs as needed, presentations to stakeholders.

2004 - 2008 **Environmental Epidemiologist, Institute for Environmental Science and Research, Crown Research Institute, Wellington New Zealand**
Environmental toxicology, forensic science, risk assessment and epidemiology research. Independent and group projects for a variety of government and private clients.

2005, 2006 **6-week Fellowship Appointments with Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Rockville Maryland.**
Work with Risk Assessment Group at CBER to evaluate increase in infectious unit risk to recipients of blood transfusions under current and proposed donor policies.

2001 - 2004 **Senior Research Fellow, Health Services Research Center, Victoria University, Wellington**
Study design and data analysis of multi-center, multi-discipline health services/epidemiological studies. Grant writing, publishing and public presentation of results. Advised graduate students.

1997 - 2001 **Research Fellow, University of Otago Medical School (Wellington)**
Designed studies, obtained external funding, conducted and published epidemiological research relating to occupational and environmental health.

1995 -1996 Environmental Risk Assessment Instructor (CH2MHill International) Kiev, Ukraine

1993 -1995 Risk Assessor (CH2MHill) Corvallis, Oregon, USA

1992 -1993 Risk Assessor (SAIC) Seattle, Washington, USA

1989- 1991 Research Assistant (University of Washington) Seattle, USA

1987- 1989 Environmental Fate Chemist (Springborn Life Sciences) Wareham, Massachusettes

1985- 1986 Dietetic Technician (Concord Hospital) Concord, New Hampshire

Graduate student supervision

2008 to present: PhD Supervisor for Su Mon Kyaw-Myint, National Centre for Epidemiology and Public Health, Australian National University, Canberra, ACT. Identification of Benchmark Doses for Selected Psychosocial Hazards in Relation to Mental Health Symptoms.

2002 – 2003: Master's Thesis statistics advisor for Michelle Ryder-Lewis, Victoria University of Wellington, 2002/2003. Reliability Study of the Sedation-Agitation Scale in an Intensive Care Unit, MA Nursing, completed in 2004.

Peer-reviewed Publications

Submitted/in press:

Heinemann J, Sherman DG, **Gallagher L**, Carman J, Prasad S (2011) Bt Brinjal: A case study in the scope and adequacy of the GEAC to protect India's farming & food security. Delhi, India. 108 pp.

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Conference Publications

Gallagher L, Kliem C and Stallones L. Chemical poisoning as suicide modality in New Zealand. ACT Public Health Forum, Public Health Association of Australia. Canberra, ACT. October 2008.

Gallagher L, Adlam B, Lake R, Dyet K and Donelly T. Prioritization of chemicals for forensic science identification: Designing a model to predict laboratory capability needs. *Society for Risk Analysis Annual Meeting 2006.* Baltimore, MD USA.

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Gallagher L, Panting A, Theis J-C, Williams H, Gandar P. Clinical priority assessment criteria (CPAC) for orthopaedic surgery in New Zealand - what good is it doing us? Presentation to Health Services Research and Policy Conference in Melbourne, Australia, November 2003.

Gallagher L. Designing an MS Database in New Zealand. Presentation to the International Consortium of Databases in Multiple Sclerosis, San Diego, June 2003.

Gallagher L, Crane J, Fitzharris P, Bates M. Occupational risk factors for respiratory symptoms in New Zealand Horse Trainers. (2002) 16th EPICOH Congress on Epidemiology in Occupational Health. La Medicina del Lavoro 93(5):455.

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Panelli R, **Gallagher** L. It's your whole way of life really: Negotiating work, health and gender. Public Health Association of New Zealand National Conference, Dunedin, June 2002.

Successful grant applications

Wellington Medical Research Fund (1998)

New Zealand Lottery Health Board (1999)

The Royal Society ISAT Linkages Fund (1999)

Association for Environmental Epidemiology (2000)

Multiple Sclerosis Society of New Zealand (2002)

New Zealand Ministry for Research, Science and Technology (2003)

Victoria University of Wellington Research Fund (2003)

Health Research Council of New Zealand (2009)

Review Activities

- Reviewer of applications for health research funding with the Health Research Council of New Zealand since 2002
- Reviewer of Scientific Reports for Science Quality and Research Priorities Team, Department for Environment, Food and Rural Affairs. London since 2007.
- Journal submissions as requested

Membership in Professional Societies

Australasian Epidemiological Association since 1996

Member, Wellington Regional Ethics Committee, New Zealand Ministry of Health, April 2002 to November 2004

Society of Risk Assessment, United States

Intergovernmental Consultation in New Zealand

Chemical and microbial threat prioritisation by government agencies in New Zealand: Project to develop capability in forensic testing of chemical and biological threats to New Zealand's health and safety, economic interests and public risk perception. Project involved a group of 5 scientists and nine government agencies to develop methods for ranking hazards using old and new risk assessment frameworks: Cynefin modelling, stochastic methods and elicitation of expert opinion (2006 – 2007).

Sudden Infant Mortality proposed research coordinated with Coroners (Ministry of Justice) (2004), Police, Ministry of Health, and University researchers from three Universities: Auckland, Otago and Massey

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REVIEW Open Access

Genetically modified crops safety assessments: present limits and possible improvements

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Abstract

Purpose: We reviewed 19 studies of mammals fed with commercialized genetically modified soybean and maize which represent, per trait and plant, more than 80% of all environmental genetically modified organisms (GMOs) cultivated on a large scale, after they were modified to tolerate or produce a pesticide. We have also obtained the raw data of 90-day-long rat tests following court actions or official requests. The data obtained include biochemical blood and urine parameters of mammals eating GMOs with numerous organ weights and histopathology findings.

Methods: We have thoroughly reviewed these tests from a statistical and a biological point of view. Some of these tests used controversial protocols which are discussed and statistically significant results that were considered as not being biologically meaningful by regulatory authorities, thus raising the question of their interpretations.

Results: Several convergent data appear to indicate liver and kidney problems as end points of GMO diet effects in the above-mentioned experiments. This was confirmed by our meta-analysis of all the *in vivo* studies published, which revealed that the kidneys were particularly affected, concentrating 43.5% of all disrupted parameters in males, whereas the liver was more specifically disrupted in females (30.8% of all disrupted parameters).

Conclusions: The 90-day-long tests are insufficient to evaluate chronic toxicity, and the signs highlighted in the kidneys and livers could be the onset of chronic diseases. However, no minimal length for the tests is yet obligatory for any of the GMOs cultivated on a large scale, and this is socially unacceptable in terms of consumer health protection. We are suggesting that the studies should be improved and prolonged, as well as being made compulsory, and that the sexual hormones should be assessed too, and moreover, reproductive and multigenerational studies ought to be conducted too.

Background, aim, and scope

Recently, an ongoing debate on international regulation has been taking place on the capacity to predict and avoid adverse effects on health and the environment for new products and novel food/feed (GMOs, chemicals, pesticides, nanoparticles, etc.). The health risk assessments are often, but not always, based on the study of blood analyses of mammals eating these products in subchronic tests, and more rarely in chronic tests. In particular, in the case of GMOs, the number and nature of parameters assessed, the length of the necessary tests, the statistics used and their interpretations are the subject of controversies, especially in the application of Organization of Economic Cooperation and Development (OECD) norms. Confusion is perceived even in regulatory agencies, as in the European Food Safety Authority (EFSA) GMO panel working group and its guidelines. Doubt has arisen on the role and necessity of animal feeding trials in safety and nutritional assessments of GM plants and derived food and feed [1]. Based on the literature data, EFSA first admitted (p. S33) that for other tests than GMOs: "For 70% (57 of 81) of the studies evaluated, all toxicological findings in the 2-year tests were seen in or predicted by the 3-month subchronic tests". Moreover, they also indicated (p. S60) that "to detect effects on reproduction or development [...] testing of the whole food and feed beyond a 90-day rodent feeding study may be needed." We fully agree with these assumptions. This is why we think that in order to protect large populations from unintended effects of novel food or feed, imported or cultivated crops on a large scale, chronic 2-year and reproductive and developmental tests are crucial.

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However, they have never been requested by EFSA for commercial edible crops. We therefore wish to underline that in contrast with the statements of EFSA, all commercialized GMOs have indeed been released without such tests being carried out, and as it was the case recently with maize stacked events without 90-day *in vivo* mammalian tests being conducted. GM stacked events have the cumulated characteristics of first generation of GMOs (herbicide tolerance and insecticide production), which are mostly obtained by hybridization. For instance, Smarstax maize contains two genes for herbicide tolerance and six genes for insecticide production. In fact, this contradictory possibility was already highlighted in the same review by EFSA (p. S60), when substantial equivalence studies and other analyses were performed: "animal feeding trials with rodents [...] adds little if anything [...], and is not recommended." This is why, in this work we will analyze and review deficiencies in GMO safety assessments, not only performed by biotech companies, but also by regulatory agencies.

We will focus on the results of available 90-day feeding trials (or more) with commercialized GMOs, in the light of modern scientific knowledge. We also suggest here an alternative to conventional feeding trials, to understand the biological significance of statistical differences. This approach will make it possible to avoid both false negative and false positive results in order to improve safety assessments of agricultural GMOs before their commercialization for cultivation and food/feed use and imports.

Overview of the safety studies of GMOs performed on mammals

Our experience in scientific committees for the assessment of environmental and health risks of GMOs and in biological, biostatistical research, and medicine, as well as in the research relative to side effects [2-6] allowed us to review and criticize mammalian feeding trials with GMOs and make new proposals. Mammalian feeding trials have been usually but not always performed for regulatory purposes in order to obtain authorizations or commercialization for GM plant-derived foods or feed. They may have been published in the scientific literature afterwards; however, without public access to the raw data.

We have obtained, following court actions or official requests, the raw data of several 28- or 90-day-long safety tests carried out on rats. The thing we did was to thoroughly review the longest tests from both a biostatistical and a biological point of view. Such studies often analyze the biochemical blood and urine parameters of mammals eating GMOs, together with numerous organ weights and histopathology. We have focused our review on commercialized GMOs which have been cultivated in significant amounts throughout the world since 1994 (Table 1). We observe and emphasize that all the events in Table 1 correspond to soybean and maize which constitute 83% of the commercialized GMOs, whilst other GMOs not displayed in the table, but still commercialized, are canola or cotton. However, they are not usually directly consumed [7]. Only Sakamoto's and Malatesta's studies have been more than 90 days long (104 weeks and 240 days with blood analyses in Japanese for the first one). Moreover, such tests are not obligatory yet for all GMOs. No detailed blood analysis is available for Malatesta's study, as it mostly includes histochemistry at the ultrastructural level; moreover, the latter tests have not been used to obtain the commercial release by the firm. However, this work has been performed by researchers independent from the GMO industry; it is an important element to take into account for an objective interpretation of the facts, as pointed out in the case of the risk assessments conducted by regulatory agencies with Bisphenol A. For instance in the latter case, it was observed that none of the industry-funded studies showed adverse effects of Bisphenol A, whereas 90% of government-funded studies showed hazards at various levels and various doses [8]. However, regulatory agencies still continue to refer only to industry-funded studies because they are supposed to follow OECD norms, even if such standards are not always appropriate for the detection of environmental hazards [9]. In this paper, Myers et al. showed that hundreds of laboratory animals and cell culture studies were rejected by regulatory authorities because they did not follow the Good Laboratory Practices (GLP). The Food and Drug Administration and EFSA have based their final decision on two industry-funded studies, claiming that they were superior to the others because they followed GLP. Yet, GLP are based on ancient paradigms. They have serious conceptual and methodological flaws, and do not take into account the latest knowledge in environmental sciences. For example, in the case of Bisphenol A assessment, the animal models used are known to be insensitive to estrogen (CD-1 mouse). Also, assays and protocols in some OECD guidelines are out of date and insensitive. It is obvious that new product assessments should be based on adapted studies using state-of-the-art experiments. The significant gap between scientific knowledge and regulations should be filled also in the case of GMOs [9]. Therefore, some tests presented here show controversial results or statistically significant results that were not considered as biologically significant by EFSA, raising the question of their interpretation.

First of all, the data indicating no biological significance of statistical effects in comparison to controls have been published mostly by companies from 2004 onwards, and at least 10 years after these GMOs were

Table 1 Review of the longest chronic or subchronic toxicity studies in mammals fed with commercialized GM soybean and maize representing more than 80% of edible GMOs (2010)

References	Plant	Pesticide contained	Name of event	Species	Duration	Main observations
[17,38,39,19,15]	Soybean	Roundup herbicide	mCP4 EPSPS	Mouse	240 days	Ultrastructural histochemistry disturbed
[14]	Soybean	Roundup herbicide	mCP4 EPSPS	Rat	91 days	Weight problems
[40]	Soybean	Roundup herbicide	Optimum GAT DP-356Ø43-5	Rat	93 days	Statistical differences[a]
[41]	Soybean	Roundup herbicide	Not precise	Rat	104 weeks	Statistical differences[a]
[42]	Maize	Roundup herbicide	Optimum GAT DP-Ø9814Ø-6	Rat	91 days	Statistical differences[a]
[43,5]	Maize	Roundup herbicide	NK603	Rat	90 days	Controversial results
[44,5]	Maize	mCry1Ab insecticide	MON810	Rat	90 days	Controversial results
[25,2,4,5]	Maize	mCry3Bb1 insecticide	MON863	Rat	90 days	Controversial results
[16]	Maize	mBt insecticide	not indicated	Rat	Multi-generational (F3)	Histopathological, biochemical, organ weights alterations
[45]	Maize	mCry1F insecticide - glufosinate ammonium-based herbicide	DAS-Ø15Ø7-1	Rat	91 days	Statistical differences[a]
[46,47]	Maize	mCry34Ab1, mCry35Ab1 insecticides - glufosinate ammonium-based herbicide	DAS-59122-7	Rat	90 days	Statistical differences[a]
[48]	Maize	mCry1F, mCry34Ab1, mCry35Ab1 insecticides - glufosinate ammonium-based herbicide	DAS-Ø15Ø7-1 × DAS-59122-7	Rat	92 days	Statistical differences[a]

[a]Statistical differences are not biologically meaningful for the authors; however, this can be debated. Oilseed rape and cotton have been excluded because they are not directly edible and not primarily grown for feed. This table includes authorized events for food and feed at least in the European Union and America.

first commercialized round the world. This is a matter of grave concern. Moreover, only three events were tested for more than 90-days in feeding experiments or on more than one generation. This method was not performed by industries which conducted 90-day tests (with blood and organ analyses), but it was in some cases only. However, a 90-day period is considered as insufficient to evaluate chronic toxicity [1,5]. All these commercialized cultivated GMOs have been modified to contain pesticides, either through herbicide tolerance or by producing insecticides, or both, and could therefore be considered as "pesticide plants." Almost all GMOs only encode these two traits despite claims of numerous other traits. For instance, Roundup ready crops have been modified in order to become insensitive to glyphosate. This chemical together with adjuvants in formulations constitutes a potent herbicide. It has been used for many years as a weed killer by blocking aromatic amino acid synthesis by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Most Roundup ready plants have been modified thanks to the insertion of a mutated EPSPS gene coding for a mutated enzyme, which is not inhibited by glyphosate. Therefore, GM plants exposed to glyphosate-based herbicides such as Roundup do not specifically degrade glyphosate. They can even accumulate Roundup residues throughout their life, even if they excrete most of such residues. Glyphosate and its main metabolite AMPA (with its own toxicity) are found in GMOs on a regular and regulatory basis [10,11]. Therefore, such residues are absorbed by people eating most GM plants (as around 80% of these plants are Roundup tolerant). On the other hand, about 20% of the other GMOs do synthesize new insecticide proteins through the insertion of mutated genes derived from *Bacillus thuringiensis* (Bt).

Usually, pesticides are tested over a period of 2 years on a mammal, and this quite often highlights side effects. Additionally, unintended effects of the genetic modification itself cannot be excluded, as direct or indirect consequences of insertional mutagenesis, creating possible unintended metabolic effects. For instance, in the MON810 maize, the insertion of the transgene in the ubiquitine ligase gene caused a complex recombination event, leading to the synthesis of new RNA products encoding unknown proteins [12]. Thus, genetic modifications can induce global changes in the genomic, transcriptomic, proteomic, or metabolomic profiles of the host. The frequency of such events in comparison to classical hybridization is by nature unpredictable. In addition, in a plant producing a Cry1Ab-modified toxin, a metabolomic study [13] revealed that the transgene introduced indirectly 50% changes in osmolytes and branched amino acids.

Review of statistical effects after GMO consumption

Some GMOs (Roundup tolerant and MON863) affect the body weight increase at least in one sex [2,14]. It is a parameter considered as a very good predictor of side

effects in various organs. Several convergent factors appear to indicate liver and kidney problems as end points of GMO diet effects in these experiments [2,5,15,16]. This was confirmed by our meta-analysis of all *in vivo* studies published on this particular topic (Table 2). The kidneys are particularly affected, concentrating 42% of all parameters disrupted in males. However, other organs may be affected too, such as the heart and spleen, or blood cells [5].

Liver parameters

For one of the longest independent tests performed, a GM herbicide-tolerant soybean available on the market was used to feed mice. It caused the development of irregular hepatocyte nuclei, more nuclear pores, numerous small fibrillar centers, and abundant dense fibrillar components, indicating increased metabolic rates [17]. It was hypothesized that the herbicide residues could be responsible for that because this particular GM plant can absorb the chemicals to which it was rendered tolerant. Such chemicals may be involved in the above-mentioned pathological features. This became even clearer when Roundup residues provoked similar features in rat hepatic cells directly *in vitro* [18]. The reversibility observed in some instances for these parameters *in vivo* [19] might be explained by the heterogeneity of the herbicide residues in the feed [20]. Anyway, these are specific parameters of ultrastructural dysfunction, and the relevance is clear. The liver is reacting. The Roundup residues have been also shown to be toxic for human placental, embryonic, and umbilical cord cells [21-23]. This was also the case for hepatic human cell lines in a comparable manner, inducing nuclei and membrane changes, apoptosis and necrosis [24].

The other major GMO trait has to do with the mutated (mBt) insecticidal peptidic toxins produced by transgenes in plants. In this case, some studies with maize confirmed histopathological changes in the liver and the kidneys of rats after GM feed consumption. Such changes consist in congestion, cell nucleus border changes, and severe granular degeneration in the liver [16]. Similarly, in the MON810 studies, a significantly lower albumin/globulin ratio indicated a change in hepatic metabolism of 33% of GM-fed male rats (according to EFSA opinion on MON810 and [5]). Taken together, the results indicate potential adverse effects in hepatic metabolism. The insecticide produced by MON810 could also induce liver reactions, like many other pesticides. Of course, the mCry1Ab and other mBt (mutated Bt toxins derived from native *Bacillus thuringiensis* toxins) in GMOs are proteic toxins; however, these are modified at the level of their amino acid sequence by biotechnologies and introduced by artificial vectors, thus these could be considered as xenobiotics (i.e., a molecule foreign to life). The liver together with the kidneys are the major reactive organs in case of food chronic intoxication.

Kidney parameters

In the NK603 study, statistically significant strong urine ionic disturbances and kidney markers could be explained by renal leakage [5], which is well correlated with the effects of glyphosate-based herbicides (like Roundup) observed on embryonic kidney cells [23]. This does not exclude metabolic effects indirectly due to insertional mutagenesis linked to the plant transformation. Roundup adjuvants even stabilize glyphosate and allow its penetration into cells, which in turn inhibit estrogen synthesis as a side effect, cytochrome P450 aromatase inhibition [21]. This phenomenon changes the androgen/estrogen ratio and may at least, in part, explain differential impacts in both sexes.

Kidney dysfunctions are observed with mBt maize producing mutated insecticides such as in MON863. For instance, we quote the initial EFSA report: "Individual kidney weights of male rats fed with the 33% MON863 diet were statistically significantly lower compared to those of animals on control diets", "small increases in the incidences of focal inflammation and tubular regenerative changes in the kidneys of 33% MON863 males." This was confirmed by the company tests [25] and another counter analysis revealed disrupted biochemical

Table 2 Meta-analysis of statistical differences with appropriate controls in feeding trials

All parameters measured *in vivo* in GMO toxicity studies	Measured by organ (%)/Total (694-698)		Disturbed in each organ (%)/Total disrupted parameters (approximately 9%)	
	Females	Males	Females	Males
Liver	22.9	22.9	**30.8**	26.1
Kidney	23.7	23.7	26.4	**43.5**
Bone marrow	29.5	29.5	29.7	22.8
Total for 3 tissues	76.1	76.1	86.9	92.4

Commercialized soybean and maize GMOs were fed to rats and their blood analyses were obtained. The different parameters are classified according to the tissue [2] to which they are related (e.g., liver, kidney, bone marrow). Of the total parameters measured 76.1% are related to these three organs. The percentages of significantly different parameters to the controls are called "disrupted parameters." There are in total 9% of disrupted parameters and, for instance, 43.5% of these are concentrated in kidneys in males. The bold values are significantly over the parameters measured per organ.

markers typical of kidney filtration or function problems [2]. The first effects were not always but sometimes greater than the ones with non-isogenic maize (called reference lines), which contain different salts, lipids, or sugars. Moreover, both results described are different between males and females; this is quite usual in liver or kidney pesticide reactions. These facts do not exclude that such effects can be considered as treatment-related. Other studies also confirmed effects on kidneys. Tubular degeneration and not statistically significant enlargement in parietal layer of Bowman's capsules were also observed with GM maize fed rats [16].

Last but not least, a total of around 9% of parameters were disrupted in a meta-analysis (Table 2). This is twice as much as what could be obtained by chance only (generally considered as 5%). Surprisingly, 43.5% of significant different parameters were concentrated in male kidneys for all commercialized GMOs, even if only around 25% of the total parameters measured were kidney-related. If the differences had been distributed by chance in the organs, not significantly more than 25% differences would have been found in the kidney. Even if our own counter analysis is removed from the calculation, showing numerous kidney dysfunctions [2], around 32% of disturbances are still noticed in kidneys.

Discussion

Need for chronic tests and other tests

Chronic toxicity tests (both with males and females) and reproductive tests with pregnant females and then with the developing progeny over several generations (none of these steps exist at present) are called as a whole the Toxotest approach (or Risk management test, see "Details on the new suggested Toxotest approach"). This could address the long-term physiological or pathological relevance of the previous observations. The physiological interpretations of 90-day-based effects are otherwise somewhat limited. These studies should be complementary to the present regulations or the Safotest and the sentinel test suggested by EFSA [1]. The Toxotest could provide evidence of carcinogenic, developmental, hormonal, neural, and reproductive potential dysfunctions, as it does for pesticides or drugs. Additionally, it is obvious that the 90-day-long trials on mature animals performed today cannot scientifically replace the sensitivity of developmental tests on neonates. A good example is the gene imprinting by drugs that will be revealed only at maturity; this is an important subject of current research, and many findings have been reported for some chemicals such as bisphenol A [26,27]. Even transgenerational effects occur after epigenetic imprinting by a pesticide [28]. These effects cannot be detected by classical 90-day feeding trials and will be visible after many decades by epidemiology in humans if any, as illustrated in the case of diethylstilbestrol, which induced female genital cancers among other problems in the second generation [29]. The F3 multigenerational study for a GMO (Table 1) was too rarely performed. This is why, because of the number of parameters disrupted in adult mammals within 90 days, the new experiments should be systematically performed to protect the health of billions of people that could consume directly or indirectly these transformed products.

The acute toxicity approach (less than a month of investigations on rodents with high doses) may give effects which are more proportional to the dose, as it might correspond to a rapid poisoning of the animals, generally with force-fed experiments. However, for many pesticide studies in the scientific literature, some long-term side effects of pesticides at environmental doses are described, which are not apparent in short-term experiments [30]. Classical toxicology is quite often based on the concept of revealing linear dose-responses as defined by Paracelsus, which generally fails to evidence U or J curves observed after hormonal sex-specific disruptions. Moreover, the effects of mixtures are also neglected in long-term studies, when supposed active principles of pesticides are not assessed with their adjuvants, which also are present as residues in GMOs. Such pesticides may have the capacity to disrupt the "cell web", i.e., to interfere with a signaling pathway, and this could be unspecific. For instance Roundup is known to disrupt the EPSPS in plants, but is also known to interact with the mammalian ubiquist reductase [21] common and essential to cytochromes P450, a wide class of detoxification enzymes. The so-called Roundup active principle, glyphosate, acts in combination with adjuvants to increase glyphosate-mediated toxicity [21,31], and this may apply to other environmental pollutants [22]. Moreover, all new metabolites in edible Roundup ready GMOs, as acetyl-glyphosate for the new GAT GMOs, have not been assessed for their chronic toxicity [11], and we consider this as a major oversight in the present regulations.

Therefore, as xenobiotic effects are complex, the determination of their toxic effects cannot be determined using a single method, but rather converging pieces of evidence. In GMO risk assessment, the protocols must be optimized to detect side effects, in particular for herbicide-treated GM plants. These cannot be reduced to GM assessment on one side and herbicide residues with any diet on the other side, but unfortunately this has been the case, and this approach has been promoted up to now by regulatory authorities.

In fact, it is impossible, within only 13 weeks, to conclude about the kind of pathology that could be induced by pesticide GMOs and whether it is a major pathology or a minor one. It is therefore necessary to prolong the

tests, as suggested by EFSA, since at least one third of chronic effects visible with chemicals are usually new in comparison to the ones highlighted in subchronic studies [1]. The so-called Toxotests, which are supposed to include the studies of chronic pathologies in particular, should be performed on three mammalian species, with at least one non-rodent, similar to the type of rodents used for pesticides and drugs. However, the chronic feeding tests for GMOs cannot be based on the *no observed adverse effect level*, nor on the *lowest observed adverse effect level* approach, as in classical toxicology. There are several reasons for that. There is not only one chemical, but also several unknown metabolites and components, in Roundup tolerant varieties for instance, and therefore toxicity is enhanced thanks to the fact that they are mixed together. There is also no possibility of increasing the doses of GMOs in an equilibrated diet over an acceptable level. The diets should be rather representative of an equilibrated diet with GMOs like it could be the case in a real population in America. To prolong 90-day subchronic tests with three normal doses of GM in the diet (11%, 22%, 33% for instance) is the solution.

Sex- or dose-specific pathological effects are common

When there is a low or environmental dose impregnation of the feed (with a pesticide GM plant for instance), the chronic effects could be more differentiated according to the sex, the physiological status, the age, or the number of intakes over such and such a period of time in the case of a drug. These parameters (chronic intake, age of exposure, etc.) are more decisive for pathologies like cancers, than the actual quantity of toxin ingested in one intake. This is in part because the liver, kidney, and other cytochrome P450-rich organs are concerned for long-term metabolism and detoxification, and this phenomenon is hormone dependent. It is also due to the process of carcinogenesis or hormone-sensitive programming of cells [32]. The liver for instance is a sex differentiated organ as far as its enzymatic equipment is concerned [4]. An effect in subchronic or chronic tests cannot be disregarded on the rationale that it is not linear to the dose (or dose-related) or not comparable in genders. This would not be scientifically acceptable. However, this reasoning was adopted both by companies and EFSA for several GMOs, as underlined by Doull et al. [33]. Indeed, most xenobiotics or pollutants may have non-linear effects, and/or may have sex- and age-specific impacts.

One of the pivotal requirements for regulators nowadays, in order to interpret a significant difference as biologically relevant, is to observe a linear dose-response. This allows them to deduce a causality. However, this dose-response cannot be studied with only two points, which is nonetheless the case for all major commercial GMOs today, which are given in the diet in 11% and 33% concentrations only, in subchronic tests. This is true overall if no preliminary data has been obtained to choose the given doses, which is the case in regulatory files. As we have already emphasized, most of pathological and endocrine effects in environmental health are not directly proportional to the dose, and they have a differential threshold of sensitivity in both sexes [34]. This is, for instance, the case with carcinogenesis and endocrine disruption.

Improving the knowledge on impacts of modified Bt toxins

One of the interpretations of the side effects observed (Tables 1 and 2) would be that the insecticide toxins in maize lines may have more pleiotropic or specific actions than originally supposed. The toxins could generate particular metabolites, either in the GM plant or in the animals fed with it. The Bt toxins in GMOs are new and modified, truncated, or chimerical in order to change their activities/solubility in comparison to wild Bt. For instance, there is at least a 40% difference between the toxin in Bt176 and its wild counterpart [10]. None of the modified Bt toxins have been authorized separately for food or feed, neither has the wild Bt, and neither have they been tested by themselves on animal or human health to date. Even if some studies were performed, the receptors have not been cloned and the signaling pathways have not been identified as yet, nor required for authorizations, and the metabolism of these proteins in mammals are unknown [35]. Thus, the argument about "safe use history" of the wild Bt protein (not designed for direct consumption, in contrast to several GMOs) cannot, on a sound scientific basis, be used for direct authorizations of the above-cited GM corns, overall without *in vivo* chronic toxicity tests (or Toxotest approach), as it is requested for a pesticide. Some improvements may even be included with regard to pesticide legislation, since these human modified toxins considered as xenobiotics are continuously produced by the plants devoted to consumption.

The proteins usually compared (modified Bt toxins and wild ones) are not identical, and the tests on human cells of Bt proteins are not performed nor are they requested by authorities. Their stability has been assessed *in vitro*, and GM insecticide toxins are never fully digested in vivo [36]. If some consumers suffer from stomach problems or ulcers, the new toxins will possibly act differently; the digestion in children could be affected too; however, these GMOs could be eaten anywhere and all proteins are never fully decomposed in amino acids by the digestive tract.

Details on the new suggested Toxotest approach

The suggested Toxotest would basically include an extension of the existing 90-day tests, but with at least three doses plus controls (0%, 11%, 22%, 33% GMOs for instance; today the equilibrated diets tested contain 0%, 11%, and 33% GMOs in the best regulatory tests). The purpose would be to characterize scientifically the dose-response approach. The latter cannot be taken seriously with only two GM doses. The final goal is the best health protection for the population without really possible clinical trials, in our case for practical and ethical reasons. There is also no epidemiological follow-up for lack of traceability and labeling in GM-producing American countries. In addition, the fact that the Toxotest includes the best possible toxicological approach will also be in favor of the biotechnology economy and the European Community because it is more expensive to address an issue concerning a whole population afterwards, rather than to work with laboratory animals beforehand; it is also more ethical to work on rats and other mammalian experiments, in order to get the relevant information, rather than to give pesticide plants directly to humans on a long-term basis.

As previously underlined, the health effects such as those suggested in Table 2 (if any, are revealed by adapted studies, such as Safotests or Toxotests), could only be due to two possibilities:

Firstly, the side effects may be directly or indirectly due to a pesticide residue and/or its metabolites. The direct effect is about the pesticide effect on the consumer, and the indirect one is about a metabolism disruption that it has provoked within the plant first. This could not be visible by a detailed compositional analysis, such as the one performed to be assessed by a substantial equivalence study. This concept is not a well-defined one (how many cultivations of crops, over how many years, under which climate, and to measure what precise parameters).

Secondly, the pathological signs may be due to the genetic transformation itself, its method provoking either insertional mutagenesis or a new metabolism by genetic interference. This is the reason why separating intended effects (the direct genetic trait consequence itself) from unintended effects (linked to biotechnology, e.g., insertional mutagenesis), such as spiking the control diet with the purified toxin in the Toxotest approach, is clearly inadequate. It could work in the case of a direct action of the toxin in mammals, but conversely one could not conclude, between an insertional mutagenesis and a specific metabolic action in the plant due to the toxin. However, this is more a research question about the mode of genesis of an effect on health, and new research avenues could be, for instance, to compare the GM diet with or without herbicide treatment in long-term tests with the isogenic control diet including herbicide residues added. This is only necessary for the understanding of the potential signs of toxicity and not for a conclusion of the Safotest or the Toxotest, which would rather suggest, if positive, excluding immediately the corresponding GMO from food and feed.

Improvement of statistical analysis

A serious experimental design is based on a proper choice of the groups, with only one question studied per experiment if possible, and balanced sample sizes. In several authorized GMOs, the sample sizes appear inadequate in 90 days: ten animals per group for the measurement of biochemical parameters out of 20, as performed by the major stakeholders, and accepted by EFSA for MON863, MON810, or NK603 for instance. This is too limited a size to ensure that parametric statistical methods used by the company are reliable. Moreover, an important discrepancy between GMO-treated rats (40 measured out of 80) and the total number of animals (400) renders more difficult the evidencing of relevant effects, and confusion factors are brought in at the same time with six different reference diets in addition to the two normal control groups as performed in three commercialized GMOs at least [5,6]. This introduces new uncontrolled sources of variability about the effects of the diets and new unnecessary questions not relevant to the GMO safety. The representation of a standard diet with multiple sources could have been studied with only one control group of the same size than the GMO group, eating a mix of six different regular non-GM diets.

Several questions have been raised by companies and authorities as well as comments on statistically significant effects that would supposedly not be biologically meaningful. A subjective part is introduced at this level because it is necessary to take into account the context and the general and detailed knowledge of toxicology and endocrine disruption, as EFSA underlines. This might be highly expert dependent. This is why, to avoid or prevent any misunderstanding, we suggest, in addition to a new statistical approach based on classical methods, to analyze the 90-day tests, even with control and reference diets called the "SSC method" (according to the initials of the authors in [2]).

Briefly, following the necessity to model and analyze the growth curves, multivariate data analysis and data mining of all parameters can be used to correlate, cluster, and select meaningful variables. This kind of approach is not performed at all today. Thereafter, the detailed comparison between GM-treated and control groups, fed with the near isogenic line (because the real isogenic line does not often exists anymore), will necessarily be followed by the study of specific diet effects,

when there are non-substantially equivalent diets for reference groups. For that purpose, the controls will be first compared using multivariate inference with reference groups, and thereafter, similarly GMO-treated groups with reference groups. The significant differences linked to the GMO and/or the composition of the diet will be classified according to organ and function. The results will appear more clearly than with the simple statistics accepted today by the authorities (that is, comparison of the highest GM dose group with the mean value of all six control groups), and will reveal in addition new information, as it can be demonstrated.

As recommended by EFSA, an appropriate and relevant statistical analysis is crucial. It should follow the following series of steps, allowing the use of several methods depending on the questions raised:

- Obtaining and modeling the growth curves and feed consumption, assessed by non-linear regression, validation, and statistical comparisons in order to test if the curves are significantly different, thus taking into account individual variability. This necessitates the use of time series analysis, selection models, and non-parametric tests, Akaike Information Criteria and related methods. Water consumption should also be an important factor to follow-up and therefore better understand kidney and urine data.
- The study of dose-response predictions using non-linear regression should be the goal, but the only two doses generally used in these tests do not make it possible to evidence linearity as we indicated. Moreover, in the cases where there are not dose-related trends or relationships using the two doses mentioned, the absence of linear dose-response curves cannot be a reason to neglect the effects. For instance, as previously cited, U or J curves may be characteristic of endocrine effects [37], and spiky irregular curves may be detected in carcinogenesis.
- Simultaneous analysis of all observed variables: multivariate data analysis, principal component analysis, correlations analysis, factorial analysis and clustering
- Multivariate comparisons of the different variables: hypothesis testing, multiple ways ANOVA, MANOVA, and others to determinate if the groups differ relative to the different questions: specific GMO effect or diet effect per se. To evidence a detail, when comparing two mean values, SEM should be calculated to determine confidence intervals; however, SD have been used up to now by the company for MON863 and NK603 files for instance.

Apart from empirical curves in some instances, ANOVA and univariate hypothesis testing only the GMO effect, none of the other statistical approaches is currently used nor requested by the authorities.

Human tests and post-market monitoring

For the record, it must be said that very few tests on humans have been carried out up to now. Moreover, epidemiological studies are not feasible in America, since there is no organized traceability of GMOs anywhere on the continent, where, by far, most of edible GMOs are cultivated (97%). As a consequence, a post-market monitoring (PMM) is offered to the population. The Cartagena Biosafety Protocol identifying GMOs at the borders of a country has now been signed by over 150 countries, including the member states of the European Union. PMM may have some value in detecting unexpected adverse effects. It could therefore be considered as a routine need. This approach makes it possible to collect information related to risk management. It can be relied upon as a technique for monitoring adverse events or other health outcomes related to the consumption of GM plant-derived foods, provided that the Toxotest approach, together with the SSC method, should have already been applied. The PMM should be linked with the possibility of detecting allergenicity reactions to GMOs in routine medicine, thanks to the very same routine cutaneous tests that should be developed prior to large-scale commercialization. A screening of serum banks of patients with allergies could be also put forward in order to search for antibodies against the main GMOs and not only their transgenic proteins, since they may induce secondary allergenic metabolites in the plant not visible in the substantial equivalence study.

The traceability of products from animals fed on GMOs is also crucial. The reason for this is because they can develop chronic diseases which are not utterly known today. Such possible diseases could be linked to the hepatorenal toxicity observed in some GMO-related cases (Table 1).

Moreover, labeling animals fed on GMOs is therefore necessary because some pesticide residues linked to GMOs could pass into the food chain and also because nobody would want to eat disabled or physiologically modified animals after long-term GMOs ingestion, even if pesticides residues or DNA fragments are not toxic nor transmitted by themselves.

Conclusion

Transcriptomics, proteomics and other related methods are not ready yet for routine use in the laboratories, and moreover they may be inappropriate for studying toxicity in animals, and could not in any way replace *in vivo* studies with all the physiological and biochemical parameters that are measured with organs weight,

appearance, and histology. By contrast, afterwards, new approaches could well help to explain pathological results or action mechanisms of pesticides present in the GM plants or GM-fed animals, if found.

To obtain the transparency of raw data (including rat blood analyses) for toxicological tests, maintained illegally confidential, is crucial. It has also become crucial to apply objective criteria of interpretation like the criteria described here: sex-specific side effects or non-linear ones. Such data can be put online on the EFSA website with a view to provide a fuller review to the wider scientific community, and in order to better inform the citizen to make biotechnologies more socially acceptable. Since fundamental research is published on a regular basis, it should be the same for this kind of applied research on long-term health effects, as suggested by the CE/2001/18 and the corresponding 1829/2003 regulations.

We can conclude, from the regulatory tests performed today, that it is unacceptable to submit 500 million Europeans and several billions of consumers worldwide to the new pesticide GM-derived foods or feed, this being done without more controls (if any) than the only 3-month-long toxicological tests and using only one mammalian species, especially since there is growing evidence of concern (Tables 1 and 2). This is why we propose to improve the protocol of the 90-day studies to 2-year studies with mature rats, using the Toxotest approach, which should be rendered obligatory, and including sexual hormones assessment too. The reproductive, developmental, and transgenerational studies should also be performed. The new SSC statistical method of analysis is proposed in addition. This should not be optional if the plant is designed to contain a pesticide (as it is the case for more than 99% of cultivated commercialized GMOs), whilst for others, depending on the inserted trait, a case-by-case approach in the method to study toxicity will be necessary.

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Authors' contributions

GES designed and coordinated the review. RM participated in the drafting of the manuscript and final version. EC, SG, JSV and DC helped the writing, compiling the literature, revising in details and proofreading the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Time- and Dose-Dependent Effects of Roundup on Human Embryonic and Placental Cells

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Abstract. Roundup® is the major herbicide used worldwide, in particular on genetically modified plants that have been designed to tolerate it. We have tested the toxicity and endocrine disruption potential of Roundup (Bioforce®) on human embryonic 293 and placental-derived JEG3 cells, but also on normal human placenta and equine testis. The cell lines have proven to be suitable to estimate hormonal activity and toxicity of pollutants. The median lethal dose (LD_{50}) of Roundup with embryonic cells is 0.3% within 1 h in serum-free medium, and it decreases to reach 0.06% (containing among other compounds 1.27 mM glyphosate) after 72 h in the presence of serum. In these conditions, the embryonic cells appear to be 2–4 times more sensitive than the placental ones. In all instances, Roundup (generally used in agriculture at 1–2%, i.e., with 21–42 mM glyphosate) is more efficient than its active ingredient, glyphosate, suggesting a synergistic effect provoked by the adjuvants present in Roundup. We demonstrated that serum-free cultures, even on a short-term basis (1 h), reveal the xenobiotic impacts that are visible 1–2 days later in serum. We also document at lower non-overtly toxic doses, from 0.01% (with 210 μM glyphosate) in 24 h, that Roundup is an aromatase disruptor. The direct inhibition is temperature-dependent and is confirmed in different tissues and species (cell lines from placenta or embryonic kidney, equine testicular, or human fresh placental extracts). Furthermore, glyphosate acts directly as a partial inactivator on microsomal aromatase, independently of its acidity, and in a dose-dependent manner. The cytotoxic, and potentially endocrine-disrupting effects of Roundup are thus amplified with time. Taken together, these data suggest that Roundup exposure may affect human reproduction and fetal development in case of contamination. Chemical mixtures in formulations appear to be underestimated regarding their toxic or hormonal impact.

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Mammals and humans may be exposed to Roundup herbicide residues by agricultural practices (Acquavella *et al*. 2004) or when the residues enter the food chain (Takahashi *et al*. 2001); glyphosate is also found as a contaminant in rivers (Cox 1998).

In our previous work, we have demonstrated that the major herbicide used worldwide, Roundup, was toxic for a human placental cell line at concentrations below that recommended for agricultural use (1–2 %, *i.e.*, with 21–42 mM glyphosate) and had endocrine-disrupting potential on estrogen synthesis at lower nontoxic doses. These cell culture experiments were performed with or without serum only on one cell model and up to 18 or 48 h, respectively (Richard *et al*. 2005). Roundup is believed to be rather specific and less toxic to the ecosystem than other pesticides; transgenic plants tolerant to this compound have even been developed following this argument (Vollenhofer *et al*. 1999, Williams *et al*. 2000). Roundup is in fact a mixture of an isopropylamine salt of glyphosate, quantitatively a minor compound called the active ingredient, and various adjuvants (Cox 1998, Cox 2004) usually considered as surfactants forming an inert part of the composition and a secret of manufacturing. All these adjuvants can be differently used depending on the formulations. Among them are ammonium sulfate, benzisothiazolone, 5-chloro-2-methyl 3(2H)-isothiazolone, FD&C Blue No. 1, glycerine, 3-iodo-2-propynyl butylcarbamate, isobutane, isopropylamine, light aromatic petroleum distillate, methyl p-hydroxybenzoate, methyl pyrrolidinone, pelargonic acid, polyethoxylated tallowamine or alkylamine (POEA), potassium hydroxide, propylene glycol, sodium sulfite, sodium benzoate, sodium salt of o-phenylphenol, and sorbic acid. These products allow for glyphosate penetration through plasmatic membranes, potentialization of its action, increased stability, and potential bioaccumulation. Glyphosate does not appear to have an herbicide action by itself.

A differential effect was noticed in our previous study in favour of Roundup, in contrast to pure glyphosate. The purpose of the present work was to study in more detail the dose-and time-dependent cytotoxicity of both compounds, up to 72 h, comparing the effects on two cell lines from human embryonic kidney and placenta. Moreover, we wanted to examine the combined effects of this chemical mixture Roundup (Bioforce® herein) on a new cellular model. We also

tested the hypothesis that Roundup and glyphosate would inhibit aromatase activity at doses lower that those producing overtly toxic effects. We determined the aromatase disruption potential in 293 cells transfected with aromatase cDNA, and examined the temperature-dependent and direct mechanism of inhibition of aromatase by glyphosate on preparations of fresh human placenta and equine testis, a tissue known to be aromatase-rich (Lemazurier *et al*. 2001).

This was of particular interest since Roundup and/or glyphosate were suggested to disturb human (Savitz *et al*. 1997) and rat pregnancies (Daruich *et al*. 2001, Beuret *et al*. 2005), mouse kidney (Peluso *et al.* 1998), rabbit spermatogenesis (Yousef *et al*. 1995), and other human tissues (Monroy *et al*. 2005).

The cytotoxic and/or genotoxic effects of glyphosate have been reported at several checkpoints of the ecosystem, for instance on fish (Jiraungkoorskul *et al*. 2003), tadpoles and other aquatic species (Pettersson and Ekelund 2006), but also on urchin eggs (Marc *et al*. 2002, 2004, 2005) and human cells (Richard *et al*. 2005, Monroy *et al*. 2005). The endocrine disruption provoked by this compound is less documented. However, it has a very clear target at two crucial steps of steroidogenesis in mammals: at the first rate-limiting level of mitochondrial cholesterol transport (Walsh *et al*. 2000), and at the last irreversible conversion of sexual steroids androgens into estrogens, via a direct action on the aromatase enzyme (Richard *et al*. 2005).

Aromatase is an evolutionarily well conserved cytochrome P450 enzyme. Its superfamily includes numerous proteins able to metabolize xenobiotics (Nelson 1998). Its catalytic action is ensured by the product of the *CYP19* gene (Bulun *et al*. 2003) associated with another moiety, the ubiquitous NADPH-dependent reductase as electron donor. It is considered a limiting factor involved in estrogen synthesis and, thus, in physiologic functions, including female and male gametogenesis (Carreau 2001), reproduction, sex differentiation, and even bone growth. It is also pharmacologically controlled in the treatment of estrogen-dependent cancers (Séralini and Moslemi 2001).

The cytotoxic effect of Roundup on cells, and the direct action of glyphosate on aromatase, could explain some reproduction problems at least in part. Among the two lines, the 293 cells have proven to be very suitable to estimate hormonal activity for xenobiotics after transfection (Kuiper *et al*. 1998). In contrast, JEG3 cells present natural aromatase activity and are also considered a useful model to examine placental toxicity (Letcher *et al*. 1999). These cell lines may be even less sensitive to xenobiotics than primary cultures (L'Azou *et al*. 2005); in this case, the effects measured could well be an indication of human placental toxicity *in vivo*, if sufficient contamination occurs, because the phenomena appear to be amplified with time in cells.

Materials and Methods

Chemicals

N-(Phosphonomethyl) glycine (glyphosate) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The herbicide Roundup used in this work is the formulation available on the market called Roundup Bioforce®, which contains 360 g/L acid glyphosate, equivalent to 480 g/L of isoproplyamine salt of glyphosate, homologation 9800036, Monsanto, Anvers, Belgium. A 2% solution of Roundup (1 or 2% is recommended by the company for agricultural use, *i.e.*, 21–42 mM glyphosate) and an equivalent solution of glyphosate were prepared in Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France). When their effects were compared, the pH of glyphosate solution was adjusted to the pH of the 2% Roundup solution (∼ pH 5.8). Successive dilutions were then obtained with serum-free or serum-containing EMEM. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and all other compounds, unless specified otherwise were obtained from Sigma-Aldrich. MTT was prepared as a 5-mg/mL stock solution in phosphate-buffered saline, filtered through a 0.22-µm filter before use, and diluted to 1 mg/mL in serum-free EMEM.

Cell Lines

The human embryonic kidney 293 cell line (ECACC 85120602) and the human choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308) were provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (mix of penicillin, streptomycin, and fungizone), and 10% fetal calf serum (Biowhittaker, Gagny, France). The JEG3 cell line was suplemented with 1 mM sodium pyruvate. Fifty thousand cells per well were grown at 37°C (5% CO_2, 95% air) during 48 h to 80% confluence in 24-well plates, washed with serum-free EMEM and then exposed to various concentrations of Roundup (0.01, 0.05, 0.1, 0.5, 0.8, 1, 2%), or the equivalent concentrations of glyphosate, in EMEM serum free or not, for various times:1, 24, 48, and 72 h.

MTT Assay

This enzymatic test, based on the cleavage of MTT into a blue-coloured product (formazan) by the mitochondrial enzyme succinate-dehydrogenase (Mossmann 1983), was used to evaluate human cell viability. Cells were washed with serum-free EMEM and incubated with 250 µL MTT per well after each treatment. The plates were incubated for 3 h at 37°C and 250 µL of 0.04 N-hydrochloric acid-containing isopropanol solution were added to each well. The plates were then vigorously shaken in order to solubilize the blue formazan crystals formed. The optical density was measured using a spectrophotometer (Stratagene, Strasbourg, France) at 560 nm for test and 720 nm for reference. The differential effects between glyphosate and Roundup are measured by the surfaces between the curves by the calculation of integrals.

Measurement of Aromatase Activity in Cells

Aromatase activity was evaluated according to the tritiated water release assay (Thompson and Siiteri 1974) with a slight modification as previously described (Dintinger *et al*. 1989). This method is based on the stereo-specific release of 1β-hydrogen from the androstenedione substrate, which forms tritiated water during aromatization. The 293 cells were transfected with the human aromatase cDNA (Auvray *et al*. 1998), exposed to nontoxic concentrations of glyphosate alone or Roundup, and were washed with serum-free EMEM and incubated for 45 min with 200 nM [1β-^{3}H] androstenedione at 37°C (5% CO_2, 95% air).

The reaction was stopped by placing the plates on ice for 5 min and then centrifuging at 2700*g*, at 4°C for 10 min. After adding 0.5 mL of charcoal/dextran T-70 suspension (7%:L5%), the mixture was left at

4°C for 5 min, and then centrifuged similarly. Supernatant fractions were assessed for radioactivity by scintillation counting.

Preparation of Microsomes

Microsomal fractions (containing endoplasmic reticulum) were obtained from full-term placentas of young healthy and nonsmoking women (Centre Hospitalier Régional de Caen, France) and equine testis by differential centrifugations (Moslemi *et al*. 1997). Briefly, tissues were washed with 0.5 M KC1, homogenized in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM Dithiothreiol DTT, and centrifuged at 20,000*g*. The supernatant was then ultracentrifuged at 100,000*g*, and the final pellet was washed twice, dissolved in the same buffer containing 20% glycerol, and stored at −70°C until use. All steps of the preparation were carried out at 4°C.

Measurement of Microsomal Aromatase Activity

Microsomal aromatase activity was evaluated by tritiated water release from radiolabelled substrate [1β-^{3}H] androstenedione as described above. Human placental microsomes (50 μg proteins) were incubated with radiolabelled androstenedione (100 pmol/tube) at 37°C for 15 min, in the presence or absence of various concentrations of Roundup or glyphosate in 1 mL total volume of 50 mM Tris-maleate buffer, pH 7.4. The reaction was started by adding 100 μL of 60 μM H^{+}-NADPH and stopped with 1.5 mL chloroform and then centrifuged at 2700*g* at 4°C for 5 min. After adding 0.5 mL of charcoal/dextran T-70 suspension (7%:1.5%) into the preparation, the centrifugation was repeated for 10 min. Aromatase activity was determined by measuring the radioactivity of 0.5 mL aqueous phase.

Purification of Aromatase Moieties and Measurement of Reductase Activity

Reductase was prepared by chromatographic separation using (ωD-aminohexyl-Sepharose 4B followed by adenosine 2′-5′-diphosphate-agarose, hydrophobic interaction, and affinity columns (Vibet *et al*. 1990), Protein concentration was determined as previously described (Bradford 1976). Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H^{+}-NADPH (Vibet *et al*. 1990) at 550 nm for 2 min at 20°C using a Kontron-Uvikon 860 spectrophotometer. The pH of the preparation was adjusted to 7.4 by adding an appropriate volume of 10 N NaOH. After equilibration, the reaction was started by adding cytochrome C.

Inactivation Study

The inactivation was carried out as previously described (Moslemi and Séralini 1997) by pre-incubation of equine testicular microsomes (200 μg proteins) for different times (0 to 30 min) at 20°C in a 0.5 mL final volume of 50 mM Tris-maleate buffer, pH 7.4, in the presence of saturating concentration of Roundup (11.6%) or in its absence (control). Androstenedione (400 nM) or H^{+}-NADPH (60 μM) were included or not in the preincubation medium. After preincubation, the free Roundup and androstenedione were removed by adding 100 μL of charcoal/dextran T-70 suspension (2%:1%) into the medium. The mixture was then gently mixed and left at 4°C for 15 min; this was followed by a centrifugation at 350*g* at 4°C for 10 min. Residual aromatase activity was then evaluated by incubating 70 μL of the aqueous phase with 200 nM tritiated androstenedione for 15 min at 25°C, in 0.5 mL of 50 mM Tris-maleate buffer, pH 7.4, containing 60 μM H^{+}-NADPH. The efficiency of Roundup adsorption by charcoal/dextran was previously tested without preincubation.

Statistical Analysis

The experiments were repeated at least 3 times in different weeks on 3 independent cultures each time (n = 9). All data were presented as the mean ± standard error (S.E.M.). Statistical differences were determined by a Student t-test using significant levels with $p < 0.01$ (**) and $p < 0.05$ (*).

Results

Cell Viability

We tested the toxicity potential of Roundup on 293 cells derived from a human embryo, at doses (from 0.01 to 2%, *i.e.*, containing 210 μM to 42 mM glyphosate among adjuvants) below that recommended for agricultural use. We tested its effect on cell viability up to 72 h in comparison to glyphosate. We also compared the results of similar exposures on human placental JEG3 cells. The Roundup dilutions and equivalent quantities of glyphosate were adjusted to the same pH, to avoid measuring a specific action of glyphosate acidity.

Roundup always shows the highest time-and dose-dependent cytotoxicity on the 293 cell line in serum-free medium. Its toxic effect is attenuated in the presence of serum (Fig. 1A and B). Fifty percent of embryonic cells degenerate already within 1 h with 0.3% Roundup (LD_{50}) in serum-free medium. Afterwards, the LD_{50} decreases with time in the presence of serum, it reaches only 0.06% Roundup after 72 h. In all instances, Roundup is more efficient than its active ingredient, glyphosate, suggesting an additional effect provoked by the adjuvants. Moreover, the differential effect between Roundup and glyphosate, measured by the surface between the curves, permanently increases with time. This is true except when glyphosate becomes highly toxic alone, and this is only after 48 h on the more sensitive 293 cells in serum-free medium (Fig. 1B). Comparable results are obtained with the slightly less sensitive JEG3 placental cells (Fig. 2A and B). Their relative resistance is visible even with glyphosate alone. The sensitivity of the 293 cells is confirmed essentially for Roundup after 72 h (Fig. 3A and B). The cytotoxic effects of glyphosate and overall Roundup are more important after 72 h with serum in both cell lines, and differential effects between Roundup and glyphosate also become greater than after 1 h in serum-free medium. The cells were not viable in culture after 60 h without serum, but here we show that the short-term serum-free cultures optimize the xenobiotic impacts, which will in any case be visible after longer exposures in the presence of serum. In general, the serum buffers the xenobiotic impacts and the differential effect. It appears to delay the toxicity effect by 1–2 days.

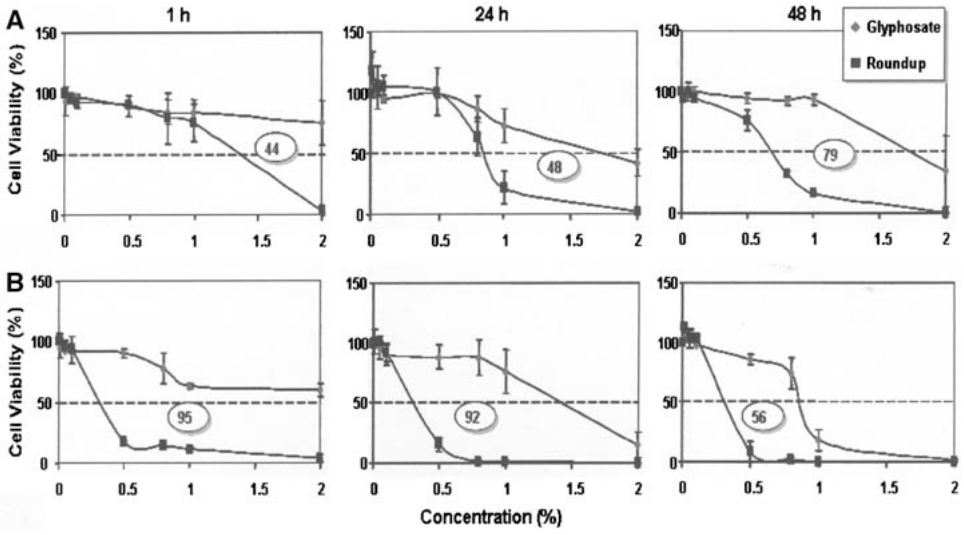

Fig. 1. Effects of Roundup and equivalent quantities of glyphosate on 293 cell viability in serum-containing medium (***A***) or in serum-free medium (***B***) for various times (1, 24, 48 h). This was evaluated by the MTT assay; the results are presented in % comparatively to nontreated cells. Cells were incubated with increasing concentrations of Roundup or equivalent concentrations of glyphosate at the same pH. The LD_{50} is indicated by a dashed line. The differential effects between glyphosate and Roundup are measured by the surfaces between the curves, and indicated within the circles in arbitrary units

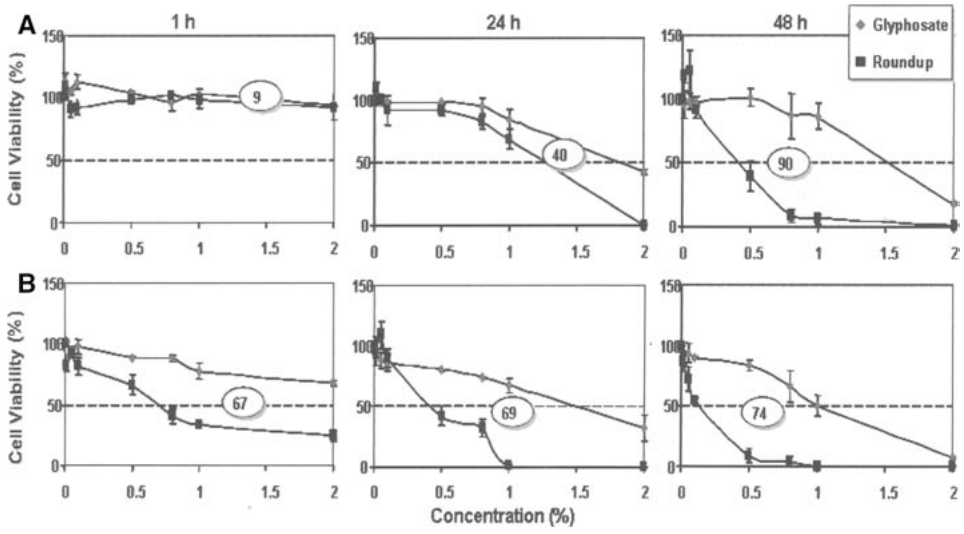

Fig. 2. Effects of Roundup and equivalent quantities of glyphosate on JEG3 cell viability in serum-containing medium (***A***) or in serum-free medium (***B***) for various times (1, 24, 48 h). The other details are indicated in the legend of Figure 1

Aromatase Activity Inhibition

Roundup or glyphosate alone was observed to have another action at lower nontoxic concentrations, namely the inhibited estrogen synthesis. This was demonstrated both after 24 h on the 293 cells transfected with the aromatase cDNA (Fig. 4A), and also after 15 min on fresh human placental cellular extracts (Fig. 4B). The IC_{50} is lowered from 2.4% glyphosate after 15 min in microsomes to 0.8% after 24 h in 293 whole cells. Glyphosate acts directly and independently of the Roundup adjuvants that facilitate its action. Its activity is not solely due to its acidity, as its inhibitory activity is present after neutralization (pH adjusted to Roundup, Fig. 4B). A genomic action is thus not necessary for this endocrine disruption, but is not excluded. In fact, we noticed that the aromatase activity inhibition by glyphosate is 3 times more important at a cellular level after 24 h, than in placental microsomes after 15 min of direct contact. In addition, we also confirmed the specific action of Roundup on another mammalian aromatase. In equine testicular microsomes, the results were very comparable and we noticed and a slight pH effect (Fig. 5). In fact, we documented that Roundup is active in different tissues (cell lines from placenta or embryonic kidney, testicular or placental fresh extracts) and on two species (man and horse).

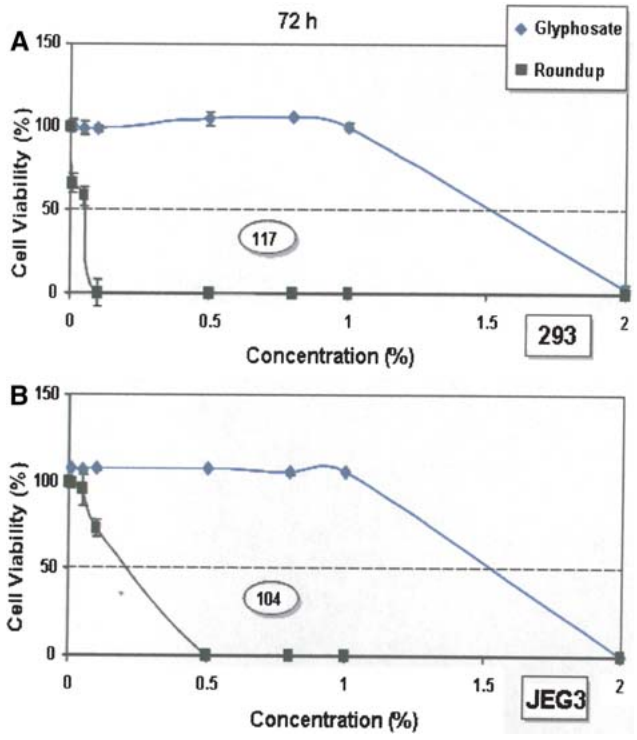

Fig. 3. Effects of Roundup and equivalent quantities of glyphosate on 293 (***A***) and JEG3 (***B***) cell viability in serum-containing medium for 72 h. Without serum, the cells do not survive 72 h. The other details are indicated in the legend of Figure 1

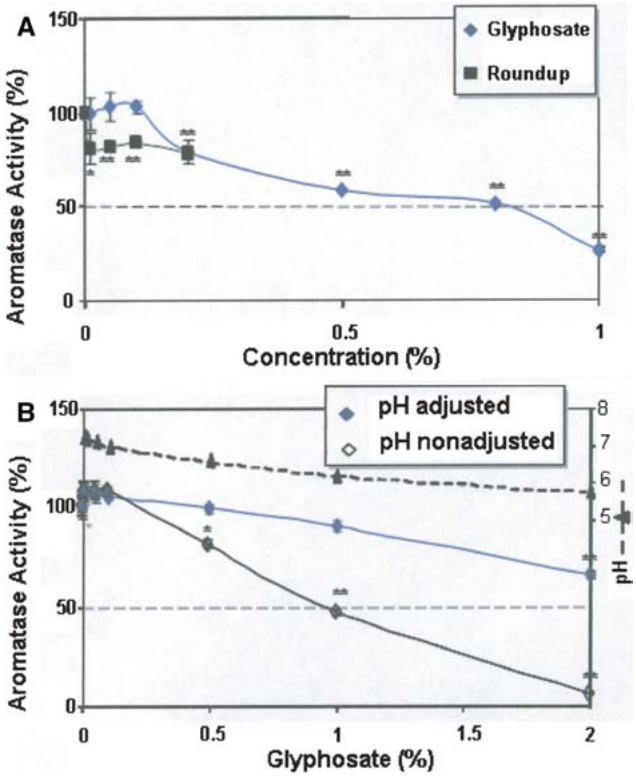

Fig. 4. Effects of glyphosate and equivalent quantities of Roundup on human aromatase activity in 293 cells in serum-free medium after 24 h (***A***) at nontoxic concentrations below 0.2 and 1% for Roundup and glyphosate, respectively. Effects of glyphosate alone on human aromatase activity in placental microsomes after 15 min and at 37°C (***B***) at pH adjusted (to the Roundup pH, - -▲- -) or nonadjusted, decreasing to pH 2.96 at 2%

We further purified the reductase enzyme moiety from the aromatase-rich equine testis, to confirm the level of action of the herbicide on the aromatase complex. A direct temperature-dependent effect was observed on both enzymatic moieties, because not only was the cytochrome P450 aromatase (Fig. 6A) affected, but also the purified reductase (Fig. 6B), to a lesser extent. The inhibition was maximal at body temperature or higher, and it was 3 times higher on the aromatase complex than on reductase alone. We confirmed and clarified this functioning between 25°C and 37°C with human placenta. Aromatase in fresh cellular extracts present a greater sensitivity to Roundup (increasing with temperature) than to glyphosate (Fig. 7A and B). When glyphosate is mixed with the adjuvants, its effect is 2–3 times more important. Moreover, we demonstrate for the first time that the Roundup inhibition is partially irreversible on the aromatase activity (Fig. 8) because in the presence of Roundup during the preincubation, the enzyme is partially inactivated. In contrast, the substrate protects the active site to some extent.

Discussion

In this work, we demonstrated a cytotoxic effect of Roundup for the first time on human embryonic cells, as well as endocrine disruption in this new model at lower nontoxic levels. This major herbicide is used worldwide and composed of glyphosate and a mixture of various adjuvants. The 293 cells were shown to be suitable for the estimation of hormonal activity of xenobiotics after aromatase transfection (Kuiper *et al*. 1998), in particular since they are themselves deprived of steroidogenesis. Our results also confirmed and extended our previous study on the human placental JEG3 cell line (Richard *et al*. 2005). This cell line is considered to be a useful model for examining placental toxicity (Letcher *et al*. 1999). Our studies also revealed that the embryonic cells are more sensitive than the placental ones.

The use of transformed or cancer-derived cell lines allows longer experiments than in primary cultures; moreover, the established cell lines may be less sensitive to xenobiotics than their normal counterparts (L'Azou *et al*. 2005), but still we measure here important impacts of Roundup. In this case, the timing and effects measured may be more important *in vivo* if living tissues are exposed to comparable contamination. Of course, the metabolism in the body will moderate these actions. However, we demonstrate irreversible inhibition and the exposures are also often longer *in vivo.* Thus, our models offer at least a good indication of the potential toxicity of Roundup during agricultural use. We have also worked here with fresh human placenta to determine whether the endocrine disruption by Roundup observed in the cell lines could also be evoked in the microsomal fraction obtained from fresh, normal tissue.

When used in agricultural practice, the formulated concentrated commercial Roundup is diluted on the farm. The farmers

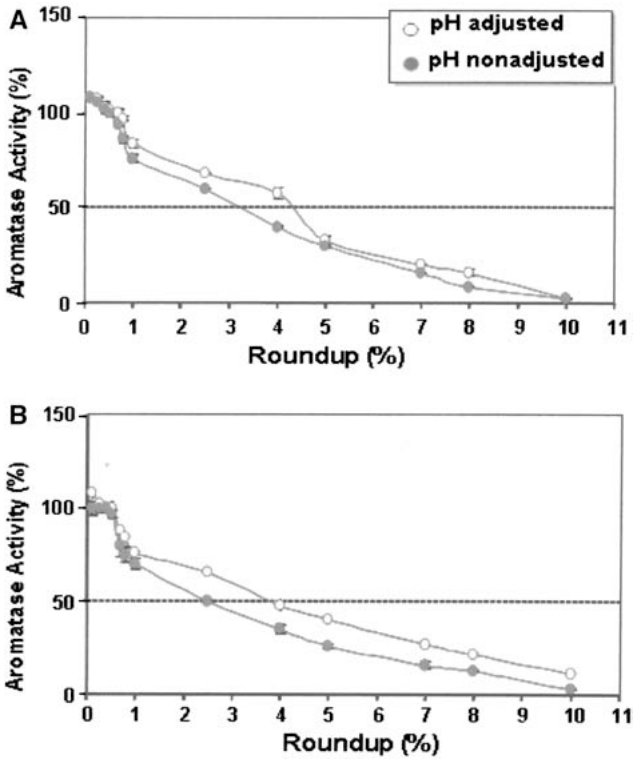

Fig. 5. Effects of Roundup on aromatase activity in human placental microsomes (**A**) and in equine testicular microsomes (**B**). The enzymatic activity was measured with pH nonadjusted decreasing to 4.88 at 10% (●) or pH adjusted to 7.4 (○) at 25°C after 15 min. The IC_{50} is indicated by a dashed line

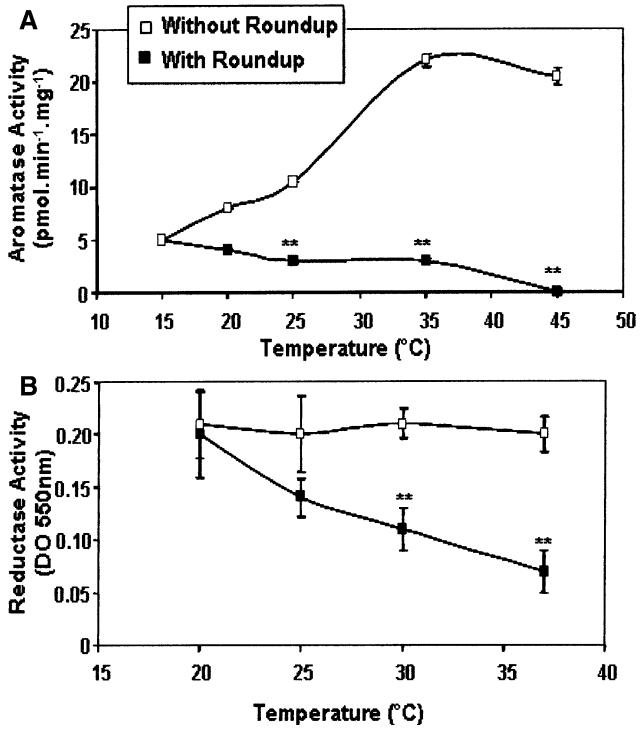

Fig. 6. Temperature influence on aromatase activity (***A***) and on reductase activity (***B***) in equine testicular microsomes or with the purified enzyme moiety, respectively. The enzymatic activity was measured in the absence (□) or in the presence of Roundup at IC_{50} (■)

are then often exposed to concentrated solutions (100%, *i.e.*, 2.13 M glyphosate), and then during spraying to more diluted solutions, up to 1–2%, the latter corresponding to the maximal concentrations used on the cells in this work. Pregnant women with embryonic and placental cells could be exposed during repeated herbicide preparations and generally only a few precautions are applied, since Roundup is believed to be one of the most environmentally friendly pesticides (US EPA 1998, Williams *et al*. 2000).

Our data demonstrated that as little as 0.01% Roundup, within only 24 h, provoked a significant reduction of 19% of estrogen production in transfected 293 cells. Estrogens are known to be necessary for normal fetal development. This Roundup dose became toxic after 72 h of exposure. Serum-binding proteins, including albumin, can buffer the xenobiotic bioavailability (Seibert *et al*. 2002), as we have observed, and our serum-free cultures allowed a shortening of the experiments to mimic longer-term effects, since within 1 h we obtained results comparable to those after 1–2 days in serum.

The endocrine effect was linked to glyphosate, which was directly able to inhibit aromatase in cells, and in the microsomes formed not only by the endoplasmic reticulum out of placental fresh cells but also from equine testis. Glyphosate also inhibited aromatase activity independently of its acidity, and on both enzyme moieties (reductase and cytochrome P450 aromatase). However, the acidity presented very little partial impact in contrast to the formulation Roundup. This interaction was not only demonstrated to be direct with the aromatase active site (Richard *et al*. 2005), it was also found to be temperature-dependent in our work on enzymatic catalytic activity, and all these impacts were promoted by the adjuvants in all instances. It is also suggested that the adjuvants allow a better solubilization of glyphosate and the latter are more active with the increase of the temperature. An indirect pathway on the aromatase gene expression was also observed in JEG3 cells (Richard *et al.* 2005). The action of Roundup disturbing the transcriptional activity of another crucial enzyme has been demonstrated (Marc *et al*. 2005) for the hatching of the sea urchin eggs. In addition, when the cytotoxic effect was noticed in this work, it was due to disruption of the mitochondrial enzyme succinate-dehydrogenase, implicated in a cellular viability process.

Our models are then pertinent to the study of Roundup toxicity. If the agents that it contains bioaccumulate, in case of contamination of a pregnant woman, it is likely that the placenta and embryo will be reached by significant levels of those. Pesticide adjuvants and surfactants, which are present in Roundup, are used in herbicide formulations to favor stability and penetration of the active ingredient into cell membranes (Cox 1998). These adjuvants amplify the cellular effects of herbicides not only in plants but also in animal models (Marc *et al*. 2002, Walsh *et al*. 2000, Nosanchuk *et al*. 2001). Some of them may eventually stick to DNA and bioaccumulate in new and not usually detected forms (Peluso *et al*. 1998). Thus, partial Roundup elimination does not exclude the action of some metabolites at cellular levels, since at least some of the Roundup residues have been

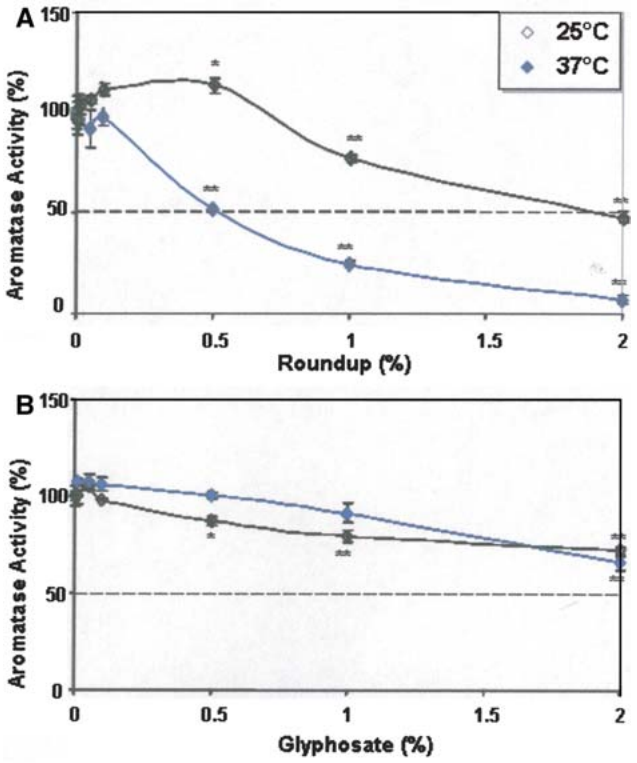

Fig. 7. Comparative effects of Roundup (***A***) and equivalent concentrations of glyphosate (***B***) on human placental aromatase activity in microsomes at 25 and 37°C after 15 mm. The pH was adjusted for glyphosate to the Roundup pH (Fig. 4B). Asteriks (*): $p < 0.05$; (**): $p < 0.01$. Significant effects in comparison to the 100% control

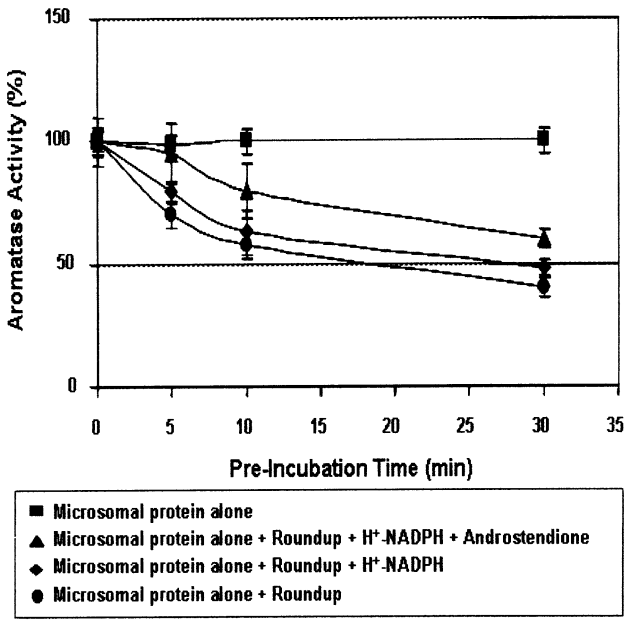

Fig. 8. Partial inactivation of equine aromatase by Roundup. Equine testicular microsomes were pre-incubated in different conditions (see below), when indicated with a saturating concentration of Roundup (11.6%) and 60 µM H^+-NADPH, plus 400 nM androstenedione, in 50 mM Tris-Maleate buffer, pH 7.4 at 20°C for different times. After Roundup removal, aromatase activity was evaluated

demonstrated to be strongly and more permanently bound to mammalian tissues.

This potential bioaccumulation could also induce or explain amplified effects with time. Thus, our results are in favour of the recruitment of other synergistic signalling pathways of action. This is why we have also analyzed the cell viability from 1 to 72 h, which has confirmed a drastic amplification of the cytotoxic effect of Roundup with time. Unfortunately, farmers are exposed often at least for many weeks to this regularly used product, which is also a common contaminant of rivers (Cox 1998). Considering all the data taken together, we cannot conclude like Williams *et al*. (2000) that the effects of the surfactants are antagonistic rather than synergistic.

Finally, we characterized in this work the differential sensitivity for Roundup and glyphosate of human embryonic cells, placental-derived cell lines, and fresh tissue extracts from human placenta and mammalian testis. Moreover, we confirmed the potential endocrine disruption of Roundup in all models on estrogen synthesis. As Roundup was more active than its claimed active ingredient in all instances, the formulation adjuvants probably allow a better cell penetration and stabilization of the product. Chemical mixtures in formulations may thus be underestimated regarding their toxic or hormonal impact (Tichy *et al*. 2002, Lydy *et al*. 2004, Monosson 2005). Most of the tests undertaken in a regulatory context are in fact performed with the active ingredient alone *in vivo* for one or two years (Williams *et al*. 2000). For instance, toxicity was not measured for Roundup treatments during more than 22 days with rats and rabbits. The potency for endocrine modulation was not assessed with the Roundup mixture at all, but only with glyphosate or POEA alone (Williams *et al*. 2000). Consequently, our experiments with Roundup should be also conducted on entire organisms *in vivo*. As emphasized by Brian *et al*. (2005), we can conclude that the failure to account for the combined effects, in particular with adjuvants, will undoubtedly lead to the underestimation of potential hazards, especially at the endocrine disruption level, and hence to erroneous conclusions at a regulatory level regarding the risk that they provoke. Thus, the toxic or hormonal impact of chemical mixtures in formulations appears to be underestimated.

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Glyphosate effects on diseases of plants

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ABSTRACT

Glyphosate, N-(phosphonomethyl)glycine, is the most extensively used herbicide in the history of agriculture. Weed management programs in glyphosate resistant (GR) field crops have provided highly effective weed control, simplified management decisions, and given cleaner harvested products. However, this relatively simple, broad-spectrum, systemic herbicide can have extensive unintended effects on nutrient efficiency and disease severity, thereby threatening its agricultural sustainability. A significant increase in disease severity associated with the wide spread application of the glyphosate herbicide can be the result of direct glyphosate-induced weakening of plant defenses and increased pathogen population and virulence. Indirect effects of glyphosate on disease predisposition result from immobilization of specific micronutrients involved in disease resistance, reduced growth and vigor of the plant from accumulation of glyphosate in meristematic root, shoot, and reproductive tissues, altered physiological efficiency, or modification of the soil microflora affecting the availability of nutrients involved in physiological disease resistance. Strategies to ameliorate the predisposing effects of glyphosate on disease include judicious selection of herbicide application rates, micronutrient amendment, glyphosate detoxification in meristematic tissues and soil, changes in cultural practices to enhance micronutrient availability for plant uptake, and biological amendment with glyphosate-resistant microbes for nitrogen fixation and nutrient availability. Given that recommended doses of glyphosate are often many times higher than needed to control weeds, we believe the most prudent method to reduce the detrimental effects of glyphosate on GR crops will be to use this herbicide in as small a dose as practically needed. Such a frugal approach will not only curtail disease predisposition of GR crops, but will also benefit the grower and the environment.

1. Introduction

Changes in agricultural practices such as crop rotation, crop sequence, tillage, and fertility that affect the soil microflora or nutrient availability generally result in changes in disease expression (Datnoff et al., 2007; Englehard, 1989; Huber and Graham, 1999). This is commonly observed for soilborne diseases where only limited innate resistance is available in commercial cultivars so that cultural controls become important management practices to minimize the impact of these diseases. Threatening to make things worse in this regard is the introduction of herbicide-resistant crops (canola, corn, cotton, soybeans, alfalfa, etc.) that are now grown extensively throughout the world. This new trend in agriculture has increased the usage and intensity of specific herbicides while limiting genetic diversity in the specific crops that have been genetically modified.

Herbicides are known to increase specific plant diseases (Altman and Campbell, 1977; Hornby et al., 1998; Mekwatanakarn and Sivasithamparam, 1987), and several are reported to influence micronutrient availability (Evans et al., 2007; Huber et al., 2004, 2005). Micronutrients are the activators or inhibitors of many critical physiological functions. Thus, a deficiency or change in availability of these regulatory elements can greatly affect plant growth and resistance to diseases and pests (Datnoff et al., 2007). The virulence mechanism of some pathogens such as *Gaeumannomyces*, *Magnaporthe*, *Phymatotrichum*, *Corynespora*, and *Streptomyces* involves Mn oxidation at the infection site to compromise the plant's resistance mechanisms involving the shikimate pathway (Thompson and Huber, 2007). Isolates of these pathogens that cannot oxidize physiologically available Mn^{2+} to the non-available Mn^{4+} are avirulent and not able to cause significant tissue damage (Roseman et al., 1991). Production of the Mn oxidizing enzyme(s) occurs soon after spore germination and during epiphytic growth (Cheng, 2005; Schulze et al., 1995; Thompson et al., 2005). Environmental conditions that reduce the availability of micronutrients for plant uptake also predispose plants to disease (Huber and McCay-Buis, 1993; Huber and Graham, 1999; Thompson and Huber, 2007).

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Table 1
Some diseases increased in glyphosate weed control programs.

Plant	Disease	Pathogen	References
Apple	Canker	*Botryosphaeria dothidea*	Rosenberger and Fargione (2004)
Banana	Panama disease	*Fusarium oxysporum* f. sp. *cubense*	Harper (2007)
Barley	Root rot	*Magnaporthe grisea*	Smiley et al. (1992)
Bean	Anthracnose	*Colletotrichum lindemuthianum*	Johal and Rahe (1984, 1988, 1990)
Bean	Damping off, root rot	*Pythium* spp.	Johal and Rahe (1984)
Bean	Root rot	*Fusarium solani* f. sp. *phaseoli*	Harper (2007)
Bean	Hypocotyl rot	*Phytophthora megasperma*	Keen et al. (1982)
Canola	Crown rot	*Fusarium* spp.	Harper (2007)
Canola	Wilt	*Fusarium oxysporum*	Harper (2007), Large and McLaren (2002)
Citrus	Citrus variegated chlorosis	*Xylella fastidiosa*	Yamada (2006)
Citrus	Crown rot	*Phytophthora* spp.	Yamada (2006)
Cotton	Damping off	*Pythium* spp.	Harper (2007)
Cotton	Bunchy top	Manganese deficiency	Harper (2007)
Cotton	Wilt	*F. oxysporum* f. sp. *vasinfectum*	Harper (2007)
Grape	Black goo	*Phaeomoniella chlamydospora*	Harper (2007)
Melon	Root rot	*Monosporascus cannonbalus*	
Soybeans	Root rot	*Corynespora cassiicola*	Huber et al. (2005)
Soybeans	Target spot	*Corynespora cassiicola*	Huber et al. (2005)
Soybeans	Sudden Death Syndrome	*Fusarium solani* f. sp. *glycines*	Keen et al. (1982)
Soybeans	Root rot	*Phytophthora megasperma*	Keen et al. (1982)
Soybeans	Cyst nematode	*Heterodera glycines*	Geisler et al. (2002), Kremer et al. (2000)
Soybeans	White mold	*Sclerotinia sclerotiorum*	Harper (2007)
Sugar beet	Yellows	*Fusarium oxysporum* f. sp. *betae*	Larson et al. (2006)
Sugar beet	Root rot	*Rhizoctonia solani*	Larson et al. (2006)
Sugarcane	Decline	*Marasmius* spp.	Huber (unpublished)
Tomato	Crown root rot	Fusarium	Bramhall and Higgins (1988)
Tomato	Wilt	*Fusarium oxysporum* f. sp. *pisi*	Harper (2007)
Various	Canker	*Phytophthora* spp.	Harper (2007)
Weeds	Biocontrol	*Myrothecium verrucaria*	Boyette et al. (2006)
Wheat	Bare patch	*Rhizoctonia solani*	Harper (2007)
Wheat	Glume blotch	*Septoria* spp.	Harper (2007)
Wheat	Root rot	*Fusarium* spp.	Fernandez et al. (2005, 2007), Harper (2007)
Wheat	Head scab	*Fusarium graminearum*	Fernandez et al. (2005)
Wheat	Take-all	*Gaeumannomyces graminis*	Hornby et al. (1998)

The herbicide glyphosate, N-(phosphonomethyl)glycine, is a strong systemic metal chelator and was initially patented for that purpose (Bromilow et al., 1993). Its herbicidal action is by chelating with Mn, a cofactor for the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimate pathway, to inhibit this metabolic pathway of plants and many microorganisms (Cerdeira and Duke, 2006; Grossbard and Atkinson, 1985; Jaworski, 1972). Many cations chelate with glyphosate, thus reducing its herbicidal efficacy (Bernards et al., 2005; Hickman et al., 2002). Plants with a compromised shikimate metabolism are predisposed to various plant pathogens (Johal and Rahe, 1988; Rahe et al., 1990), and glyphosate is patented as a synergist for mycoherbicides to enhance the virulence and pathogenicity of organisms used for biological weed control (Boyette et al., 2006; Duke and Cerdeira, 2005). The synergistic activity of glyphosate weed control in predisposing plants to infectious organisms has been observed for many diseases (Table 1), and the extensive use of glyphosate in agriculture is a significant factor in the increased severity or "reemergence" of diseases once considered efficiently managed.

The extensive adoption of Roundup Ready® crops such as soybeans, canola, cotton, and corn has intensified the application of glyphosate in these production systems. The applied glyphosate is readily translocated to roots and released throughout the rhizosphere in root exudates of Roundup Ready® plants as well as glyphosate-sensitive plants (Bromilow et al., 1993; Grossbard and Atkinson, 1985). The toxic microbial effects of glyphosate are cumulative with continued use so that Mn deficiency is now observed in areas that were previously considered Mn sufficient because of reduced populations of Mn-reducing soil organisms (Huber, unpublished). The presence of the glyphosate-resistance gene in corn and soybeans also reduces Mn uptake and physiological efficiency (Dodds et al., 2002a,b,c; Gordon, 2006; Reichenberger, 2007). Along with glyphosate-induced Mn deficiency, there has been a gradual recognition of increased disease severity (Harper, 2007; Larson et al., 2006). A few examples are presented to illustrate this relationship.

2. Some diseases increased by glyphosate

2.1. *Corynespora* root rot of soybean

The damage from Corynespora root rot, previously considered minor, may become economically damaging in Roundup Ready® soybeans since application of glyphosate to Roundup Ready® soybeans greatly increases severity of this disease (Fig. 1). This fungal root rot is more severe when glyphosate is applied to soybeans under weedy conditions even though the weeds may not be hosts for *Corynespora cassiicola*. The weeds serve to translocate and release more glyphosate into the rhizosphere environment to reduce the population of Mn-reducing organisms and increase Mn-oxidizing organisms. This change in soil biology limits manganese availability for plant uptake and active defense reactions, and acts synergistically with *Corynespora* to increase disease (Huber et al., 2005).

2.2. *Take-all of cereal crops*

The most comprehensive understanding of the interaction of micronutrients influenced by glyphosate and disease is with the take-all disease of cereals. Increased take-all of cereals after a preplant "burn-down" use of glyphosate has been recognized for over 15 years (Hornby et al., 1998). Take-all is also increased when glyphosate is applied to Roundup Ready® soybeans the preceding year compared with the use of a non-glyphosate herbicide (Fig. 2). All of the conditions known to affect Mn availability are inversely related to the severity of take-all (and other diseases, Table 2) so that

Fig. 1. Increased severity of Corynespora root rot after glyphosate application to Roundup Ready® soybeans. Non-inoculated control (left), inoculated plants (center), inoculated plants sprayed with glyphosate (right).

Fig. 2. More severe take-all root rot of wheat grown following Roundup Ready® soybeans sprayed with glyphosate (left) than following Roundup Ready® soybeans grown with a non-glyphosate herbicide (right).

Table 2
Some conditions affecting the form of nitrogen, manganese availability, and severity of take-all, rice blast, potato scab, *Phymatotrichum* root rot, and corn stalk rot (after Thompson and Huber, 2007).

Soil factor or cultural practice	Favored N form (NH_4 vs. NO_3)	Manganese availability	Severity of these diseases
Low soil pH	NH_4	Increase	Decrease
Green manures (some)	NH_4	Increase	Decrease
Ammonium fertilizers	NH_4	Increase	Decrease
Irrigation (some)	NH_4	Increase	Decrease
Firm seed bed	NH_4	Increase	Decrease
Nitrification inhibitors	NH_4	Increase	Decrease
Soil fumigation	NH_4	Increase	Decrease
Metal sulfides	NH_4	Increase	Decrease
High soil pH	NO_3	Decrease	Increase
Lime	NO_3	Decrease	Increase
Nitrate fertilizers	NO_3	Decrease	Increase
Manure	NO_3	Decrease	Increase
Low soil moisture	NO_3	Decrease	Increase
Loose seed bed	NO_3	Decrease	Increase

those conditions that increase the availability of Mn for plant uptake generally reduce take-all, and those that reduce Mn availability increase take-all (Huber and McCay-Buis, 1993). Microorganisms proposed for biological control of this disease such as *Bacillus cereus* and *Trichoderma konigii* are all strong Mn reducers that increase Mn availability in the rhizosphere (Huber and McCay-Buis, 1993; McCay-Buis, 1998; Rengel et al., 1996). In contrast, the addition of Mn-oxidizing organisms increases take-all (Crowley and Rengel, 1999; McCay-Buis, 1998; Rengel, 1999; Thompson et al., 1998). *Gaeumannomyces graminis* is a strong Mn oxidizer in soil and as it grows externally along plant roots (Thompson et al., 2000, 2005). Isolates of *Gaeumannomyces* that cannot oxidize Mn are avirulent, and isolates that oxidize Mn only at certain temperatures are virulent only at temperatures where they can oxidize Mn (Roseman et al., 1991).

Species of *Gramineae* such as rye (*Secale cereale* L.) that are efficient in Mn uptake are resistant to take-all compared with the relatively inefficient, highly susceptible wheat (*Triticum aestivum* L.) (Hornby et al., 1998). In contrast, resistance of oats to take-all is associated with glycocyanide root exudates that are toxic to Mn-oxidizing organisms in the rhizosphere. Oats, as a precrop for wheat, provide effective control of take-all in many areas because of the induced shift in soil biological activity that is less favorable for Mn oxidation. The biological activity favoring Mn availability reduces take-all severity for two or more subsequent wheat crops even though there is little change in the pathogen population (Huber and McCay-Buis, 1993). Glyphosate, in contrast to oats, is toxic to Mn-reducing and N-fixing organisms in soil so that the availability of nitrogen and Mn in soil may be markedly compromised (Huber et al., 2004). Low levels of residual glyphosate in soil also reduce root uptake and translocation of Fe, Mn, and Cu (Eker et al., 2006; Ozturk et al., 2008). Increased take-all root, crown, and foot rot of cereals following glyphosate applications (Hornby et al., 1998; Huber and McCay-Buis, 1993) may be the result of reduced resistance from induced Mn deficiency, inhibited root growth from glyphosate accumulation in root tips, modified virulence of the pathogen, or an increase in synergistic Mn-oxidizing organisms in the rhizosphere.

2.3. Diseases caused by *Xylella fastidiosa*

Various diseases caused by *X. fastidiosa* are referred to as "emerging" or "reemerging" diseases as glyphosate weed management

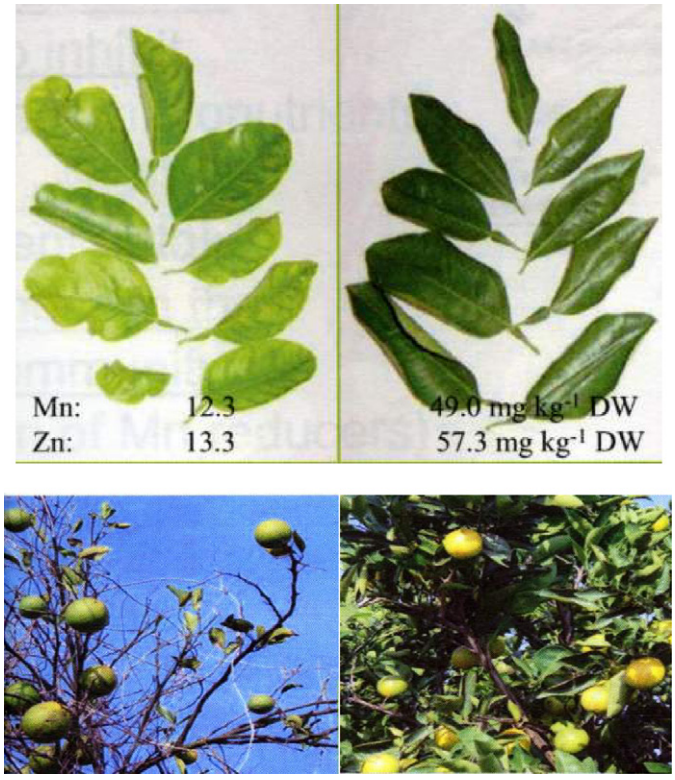

Fig. 3. Expression of citrus variegated chlorosis under glyphosate (left) compared with an alternative mulch (right) weed control program. All trees are infected with the CVC pathogen, *Xylella fastidiosa* (after Yamada, 2006). Left: severe Mn and Zn deficiency, and eventual severe decline in vigor with the glyphosate weed management program. Right: restoration of tissue nutrient levels and productivity of *X. fastidiosa* infected trees under the non-glyphosate mulch system.

programs for their respective crops have intensified. These diseases (Pierce's disease of grapevine, plum scorch, almond scorch, citrus variegated chlorosis, coffee blight, citrus blight, alfalfa dwarf, pecan decline, etc.) are characterized by a loss of vigor, slow decline, micronutrient deficiency, and reduced productivity. The pathogen is an endophytic bacterium that colonizes xylem tissues and restricts nutrient translocation when plants are stressed. Citrus variegated chlorosis (CVC) was first described on oranges in Brazil in 1987 and is also recognized in Puerto Rico. An early symptom of this disease is a variegated chlorosis of foliage (Fig. 3) similar to a deficiency of, and associated with, a drop in tissue levels of Mn and Zn (Li et al., 1996). Normal flushes of new growth are sparse or absent, fruit is small, "skirts" of trees move up, and trees enter a serious decline in growth and productivity. A similar disease referred to as "citrus blight" occurs worldwide and causes the death of several hundred thousand citrus trees annually in the United States (Derrick and Timmer, 2000; Timmer, 2000). Yamada (2006) developed the only known control for CVC, and properly managed trees return to full productivity even though the pathogen may still be present. Control of CVC emphasizes elimination of glyphosate and adoption of an alternative grass mulch weed control program for citrus orchards in Brazil (Yamada and Castro, 2005). This control strategy uses optimally fertilized *Brachiaria* grass grown between the tree rows. The grass is mowed twice a year to provide a 10–15 cm mulch under the citrus trees for weed control and nutrition. Natural mineralization of this mulch inhibits nitrification to provide an ammonium source of nutrition for the citrus trees, Mn and Zn tissue levels are restored to sufficiency levels, and trees in early to mid-decline produce a new flush of growth. Full productivity is restored within a few years. Removing glyphosate from the citrus production system also has significantly reduced the occurrence of *Phytophthora* crown rot.

2.4. *Fusarium* diseases

Various diseases caused by *Fusarium* spp. are increased by glyphosate (Fernandez et al., 2005; Sanogo et al., 2000, 2001). Glyphosate has made crops susceptible to normally non-pathogenic isolates of *Fusarium*, and the population of *Fusarium* increases in soil after glyphosate application (Levesque et al., 1987; Kremer et al., 2000). Glyphosate predisposes tomato to *Fusarium* crown and root rot by inhibiting the plant's structural and defense barriers (Bramhall and Higgins, 1988). Cotton growers in Australia and the Western United States have seen a resurgence of Fusarium wilt since the introduction of Roundup Ready® cotton, and previously high levels of wilt resistance appear to be less effective under glyphosate management programs (Harper, 2007). Glyphosate also breaks resistance to cyst nematodes in soybeans (Geisler et al., 2002). The increased Fusarium yellows and *Rhizoctonia solani* diseases of Roundup Ready® sugar beets prompted Larson et al. (2006) to comment that "precautions need to be taken when certain soilborne diseases are present if weed management for sugar beet is to include post-emergence glyphosate treatments." These authors also reported that the sugar beet variety resistant to *Rhizoctonia* was as susceptible to this pathogen as the susceptible variety after glyphosate application regardless of the time of inoculation.

Fusarium head scab of cereals and other diseases caused by *Fusarium* spp. increase following glyphosate applications (Fernandez et al., 2005; Larson et al., 2006), and previously established "cardinal" conditions (precipitation, flowering, and temperatures above 26 °C) for head scab are modified when glyphosate is applied prior to a susceptible cereal crop (Fernandez et al., 2005, 2007). Glyphosate modifies plant nitrogen metabolism similar to high temperature-induced changes that provide susceptibility to head scab (Huber, unpublished) so that head scab and the mycotoxins produced by the causal fungi are now prevalent in cooler areas where they were rarely observed before the extensive use of glyphosate (Fernandez et al., 2005, 2007). Similar changes in nitrogen and carbohydrate metabolism provide transient resistance of wheat and soybeans to rust after glyphosate application (Anderson and Kolmer, 2005; Feng et al., 2005, 2007).

The Palouse area of Washington, Idaho, and Oregon in the United States has had a long history of pea, lentil, and wheat production on the deep loess soils characteristic of the area; however, pea and lentil yields have been in slow decline as symbiotic nitrogen fixation is reduced and Fusarium diseases increased commensurate with the extensive use of glyphosate for no-till wheat production. Pea and lentil production are now uneconomical in some areas, and production is rapidly moving from the Palouse to Montana where glyphosate usage has been more limited. The loss of legumes in crop rotations in the Palouse area can result in serious degradation of these once highly productive soils with few economical, alternative crops available as replacements. A new Fusarium wilt of canola caused by *F. oxysporum* and *F. avenaceum* has caused severe yield reductions in nutrient poor soils of Alberta and Saskatchewan, Canada since 2000, but has not yet become a problem in the Mn-rich soils in the Red River valley (Lange and McLaren, 2002).

3. Predisposition to disease underlies the herbicidal efficacy of glyphosate

Inhibition of EPSP synthase initially was considered to be the sole target of glyphosate in plants. It was believed that this mode of action would kill plants by starving them of aromatic amino acids through deregulation of the shikimate pathway (Cerdeira

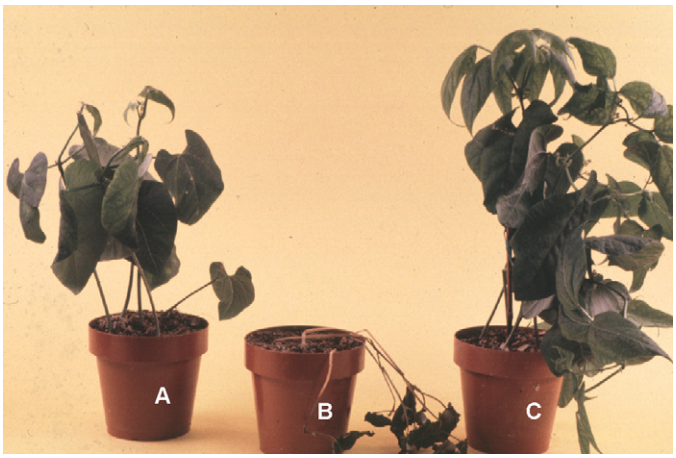

Fig. 4. Fate of glyphosate-treated (10 μg plant^{-1}) bean plants grown in (A) vermiculite and (B) field soil 20 days after glyphosate treatment, and (C) non-glyphosate treated control plants. Glyphosate treated plants in field soil (B) collapsed 10 days after glyphosate treatment from *Pythium* infection.

and Duke, 2006; Grossbard and Atkinson, 1985; Jaworski, 1972). This, however, did not explain some aspects of the death caused by glyphosate. For instance, glyphosate must be translocated to roots to be effective, although growth of the plant stops soon after application of the herbicide. In addition, effects of sublethal doses of this herbicide on perennial plants sometimes appear a year after exposure and persist for two or more years (Rahe et al., 1990). These characteristics of glyphosate-induced injury suggest that the herbicidal action of glyphosate was more than simply the starvation of treated plants of aromatic amino acids as assumed initially (Rahe et al., 1990).

Intrigued by these observations and the possibility that something about the root environment may contribute to the herbicidal action of glyphosate, a systematic research effort was launched in the early 1980s that led to the following findings (Levesque and Rahe, 1992; Rahe et al., 1990):

(1) The herbicidal efficacy of glyphosate is largely due to colonization of roots of affected plants by soil-borne pathogens (Fig. 4) (Johal and Rahe, 1984).
(2) Two pathogens that are most important in this regard are *Pythium*, an oomycete, and *Fusarium*, an ascomycete. Both of these pathogens are ubiquitous in agricultural and other soils.
(3) Plants growing in sterile medium do not die even though their growth is temporarily inhibited by glyphosate.
(4) Amending sterile media with *Pythium* or *Fusarium* restores the ability of glyphosate to kill plants.
(5) Both *Pythium* and *Fusarium* begin to colonize plants within a day or two of glyphosate application to foliar parts of the plant (Fig. 4) (Johal and Rahe, 1984; Levesque et al., 1993).
(6) The amount of glyphosate needed to kill plants in natural soils is much lower than the recommended dose.

These results suggested that glyphosate was somehow compromising the ability of plants to defend against rhizosphere-inhabiting pathogens.

4. Mechanisms of predisposition to disease

Plants rely on multiple components of defense to deter pathogens following infection (Hammond-Kosack and Jones, 2000). Many of these active resistance components are derived from the phenylpropanoid pathway, which acquires almost all of its precursors (notably phenylalanine and chorismate) from the shikimic acid pathway (Hammond-Kosack and Jones, 2000; Dixon et al., 2002). A key inducible defense component associated with the shikimic acid pathway is the production of antimicrobial phytoalexins that accumulate rapidly at the site of infection. Lignification of cell walls at and around the infection site is another shikimate-derived component that functions to fortify cells and ensure isolation of the pathogen at the infection site. Production of salicylic acid (SA) following infection represents another component of inducible defense. SA is synthesized either directly from chorismic acid or indirectly through phenylalanine. Although SA is not antimicrobial per se, it functions to signal and coordinate various defenses following challenge by a pathogen; however, its direct role in plant–pathogen interactions involving root tissue remains unclear. Another defense component that relies on three final products of the shikimic acid pathway – tryptophan, tyrosine and phenylalanine – is the production of a diverse variety of pathogenesis-related (PR) proteins that function to curtail the advance of a pathogen. Many kinds of PR proteins have been identified (Hammond-Kosack and Jones, 2000).

Given the reliance of many plant defenses on the shikimic acid pathway, and the fact that glyphosate blocks this pathway, it is not surprising that this herbicide would render plants more susceptible to pathogens. Keen et al. (1982) were the first to show that by inhibiting the phytoalexin glyceollin, glyphosate was able to compromise resistance of soybeans to *Phytophthora megasperma* f. sp. *glycines*. Using the bean-anthracnose pathosystem, Johal and Rahe (1988, 1990) demonstrated that, while glyphosate did not interfere with the hypersensitive reaction (HR) of incompatible interactions, it suppressed significantly the production of all four of the bean phytoalexins. As a result, the pathogen was able to kill the plant if it escaped the localized HR, a situation that occurred only with glyphosate-treated plants (Fig. 5). The effect of glyphosate on the compatible bean anthracnose interaction was even more dramatic (Johal and Rahe, 1990). Glyphosate almost completely suppressed the production of phytoalexins associated with susceptible lesion containment and permitted the pathogen to invade unimpededly until the entire hypocotyl collapsed (Figs. 6 and 7). As little as 2% of the recommended herbicidal rate of glyphosate was enough to transform normally delimited lesions typical of anthracnose into constantly expanding lesions (Johal and Rahe, 1990).

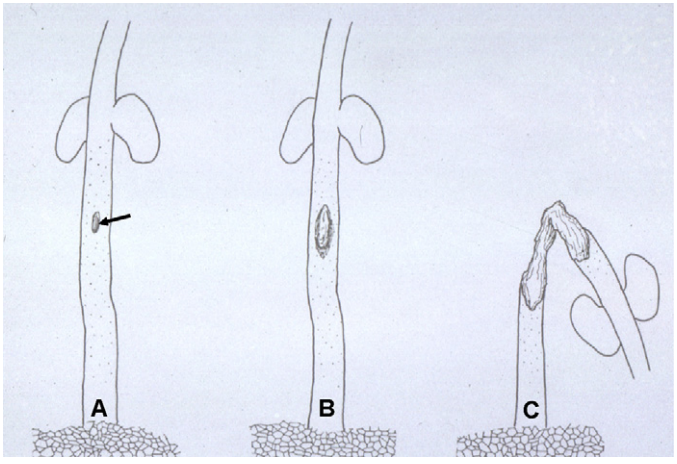

Fig. 5. Diagramatic representation of glyphosate-treated bean seedlings following inoculation with an incompatible race of *Colletotrichum lindemuthianum*. (A) Dots all over the hypocotyl represent hypersensitive reaction (HR) sites (cells) incited by the pathogen on spray inoculation. Arrow indicates the site where a drop of glyphosate (10 μg) was placed. (B) The fungus normally contained inside HR cells sometimes escapes near the glyphosate treatment site and results in a susceptible lesion (arrow). (C) The lesion continues to expand to kill the plant after glyphosate treatment.

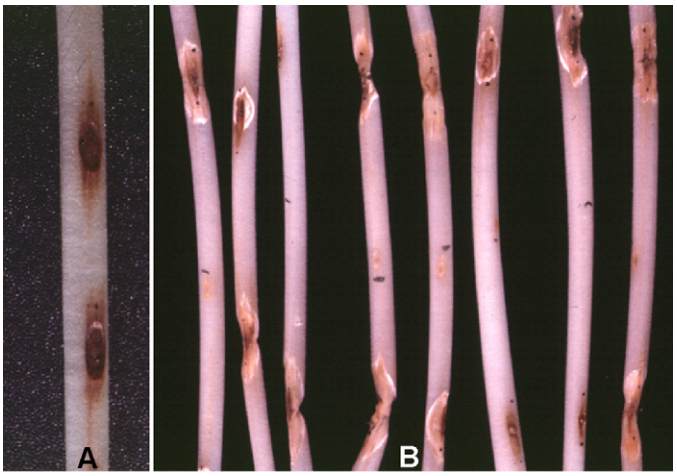

Fig. 6. Anthracnose lesions on bean hypocotyls in the (A) absence of glyphosate and (B) presence of glyphosate. A 10 μg drop of glyphosate (black dot) was placed near the center of the hypocotyls one day after inoculation with *C. lindemuthianum* in (B). Note the loss of lesion delimitation and collapse of tissue seven days after glyphosate treatment.

The defense studies mentioned above were confined largely to diseases of aerial parts of host plants. There are indications that defense components may vary significantly in root tissue that are in intimate and continuous contact with potential pathogens (Hammond-Kosack and Jones, 2000). For instance, roots do not rely on HR-mediated defense to contend with pathogens, although the exact defense components that keep roots pathogen-free are only partially understood. To gain an insight into what contributes to glyphosate-induced susceptibility of French beans (*Phaseolus vulgaris*) to *Pythium*, Liu et al. (1995, 1997) assessed phytoalexins as well as lignification of root tissue in response to glyphosate treatment. By comparing phytoalexins in roots of bean seedlings grown in different media, they concluded that phytoalexins were induced by soil microorganisms. Interestingly, while phytoalexin accumulation was affected only modestly by glyphosate in response to exposure to *Pythium*, lignification (a process requiring Mn) was suppressed significantly. Thus, enhanced colonization by *Pythium* in roots of bean seedlings treated with foliar applied glyphosate occurs as a result of glyphosate interference with lignin-based defense mechanisms (Liu et al., 1997). However, these results also suggest that sustained production of phytoalexins in response to *Pythium* infection is maintained temporarily following glyphosate treatment, whereas lignification is not.

5. Roundup Ready® plants and disease predisposition

Given that the herbicidal activity of glyphosate is mediated largely by its ability to lower plant immunity to pathogens, the status of Roundup Ready® plants with regard to such predisposition following glyphosate treatment becomes a serious consideration. For reasons that were not explained, Cerdeira and Duke (2006) contended that reduced resistance to pathogens in response to glyphosate treatment should not occur in Roundup Ready® plants. This is a misconception that can hold true only if the Roundup Ready® transgene following glyphosate treatment operates and behaves in exactly the same manner as the native EPSP synthase gene does in the absence of glyphosate. Such a scenario is possible only if the Roundup Ready® transgene is completely insensitive to glyphosate and is also as efficacious as the native EPSP synthase gene is in the absence of glyphosate. In addition, the Roundup Ready® gene has to match exactly the transcriptional activity of the native gene in every tissue of the plant and under all conditions, both normal and stressful. This is a tall order of requirements that is unlikely to be fulfilled by the present day Roundup Ready® transgenics, thus making it highly probable that our Roundup Ready® crops are vulnerable to glyphosate toxicity under at least some conditions. One such condition could arise when the level of glyphosate exceeds the ability of the transgenic enzyme to tolerate it, and yet another may develop if the transgene fails to match the transcriptional activity and profile of the native gene under conditions of biotic stress. Both of these scenarios are possible and, if they develop, it is very likely they would enhance the vulnerability of Roundup Ready® plants to fungal diseases following Roundup application.

Glyphosate treatment of transgenic crops to manage weeds can also promote disease damage indirectly by impacting the inoculum potential of pathogens. Shortly after soilborne fungi's causative role was revealed in the herbicidal efficacy of glyphosate (Johal and Rahe, 1984), Levesque et al. (1987) documented a significant, albeit temporary, spike in the level of fungal pathogens in the rhizosphere following glyphosate application to weeds. This prompted the speculation that such a buildup of pathogen load could have ill effects for subsequent crop plants. This, indeed, was found to be the case in barley fields in which significant yield reductions were witnessed if the crop was planted within a few days after glyphosate application (Smiley et al., 1992). Although the latter study was conducted on non Roundup Ready® barley, it is likely that a similar boost in the inoculum potential of pathogens in the rhizosphere (also called 'green bridge') could lead to enhanced root rot problems in Roundup Ready® crops as well.

A prudent way to avoid disease enhancement is to decrease the concentration of glyphosate applied to Roundup Ready® crops. Many studies have documented that the levels of glyphosate necessary to kill or compromise the health of many weeds are several fold lower than the generally recommended application rates (Rahe et al., 1990). An alternative to using insensitive EPSP synthase genes to generate glyphosate-resistant plants might be to use genes that degrade glyphosate. Three such genes which inactivate glyphosate by oxidation (the *Gox* gene), acetylation (the *Gat* gene) or decarboxylation (the *Gdc* gene) have become available in recent years (Cerdeira and Duke, 2006; Dill, 2005). If the problem persists, there is also the possibility of stacking a resistant EPSP synthase gene with a glyphosate metabolism gene as has been done in canola (Dill, 2005). A major disadvantage of this strategy is that it may encourage the application of higher levels of glyphosate than needed. In turn, this would not only impact the environment negatively but also would hasten the evolution of resistant weeds and thereby further threaten sustainability of this herbicide.

6. Strategies to ameliorate glyphosate predisposition to disease

Several strategies may be deployed to reduce glyphosate-induced predisposition to disease. These strategies primarily focus

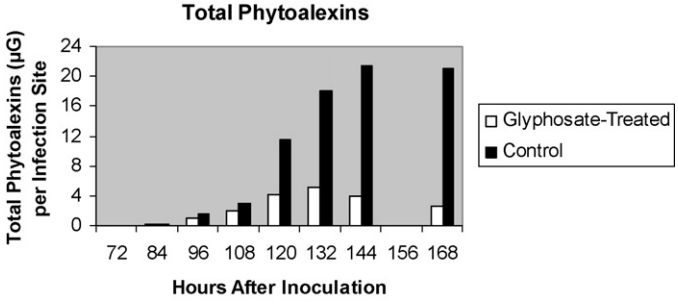

Fig. 7. Glyphosate suppression of phytoalexins in compatible bean anthracnose lesions by 10 μg glyphosate (after Johal and Rahe, 1990).

on four aspects of the glyphosate-disease-environment interaction, i.e.:

(1) minimizing non-target exposure to glyphosate by limiting the rates of glyphosate used,
(2) enhancing micronutrient sufficiency to maintain optimum plant physiological function and resistance,
(3) detoxifying accumulated glyphosate in root tips and other meristematic tissues to restore growth potential, and
(4) moderating glyphosate toxicity to rhizosphere microbes or restoring critical microbial components damaged by glyphosate released in root exudates.

6.1. Minimizing non-target exposure to glyphosate by limiting the rates of glyphosate used

As stated earlier, the rates of glyphosate generally recommended for herbicide use are far in excess of the amount required to kill most weeds. Excess application has occurred primarily as a result of advertising promotions, ease of application, increasing weed resistance, low cost of the product, and apathy towards the extensive non-target environmental effects of glyphosate. Very low levels of residual glyphosate in soil can greatly impede the availability and uptake of Mn, Fe, Cu, and Zn with subsequent translocation to vegetative tissues also impeded (Eker et al., 2006; Ozturk et al., 2008). This limitation in uptake and translocation can greatly impede the "replenishing" of these critical micronutrients and restoration of physiological resistance mechanisms dependent on them after nutrient immobilization in tissues by the applied glyphosate. A more judicious use of glyphosate would appear essential to maintain sustainable crop production efficiency.

6.2. Enhancing micronutrient sufficiency to maintain optimum plant physiological function and resistance

Most micronutrients are readily absorbed after foliar application, a common method of fertilization; however, some micronutrients, such as Mn, are relatively immobile and are not basipitally translocated to roots where soilborne root, hypocotyl, crown and vascular pathogens are established (Marschner, 1995; Thompson and Huber, 2007). Thus, although foliar application of Mn can provide nutrient sufficiency to foliar tissues for this essential element, it would not be effective in detoxifying accumulated glyphosate in root tip meristematic tissues or maintaining physiological resistance dependent on the shikimate pathway in root tissues because it is relatively immobile in the plant and does not move downward in the phloem.

A combination of foliar applied Mn with more mobile elements such as Cu or Zn could be more effective in detoxifying glyphosate in root tissues than Mn alone. Difficulties in meeting plant needs for Mn are further compounded since soil-applied Mn can be readily oxidized by soil organisms to the Mn^{4+} form that is not available for plant uptake (Marschner, 1995; Thompson and Huber, 2007). Reduced physiological efficiency of Roundup Ready® crops (Dodds et al., 2002a,b,c; Gordon, 2006; Zobiole et al., 2009) require higher levels of Mn to achieve nutrient sufficiency and comparable productivity as their non-genetically modified isolines (Reichenberger, 2007). Rates of Mn applied to Roundup Ready® soybeans required for comparable yield with non-RR soybean approached toxicity when applied to the isogenic non-Roundup Ready® soybean (Gordon, 2006; Reichenberger, 2007). The simultaneous application of many nutrients with glyphosate ("tank mixes") results in their immobilization and non-availability for plant physiological functions. Full physiological efficiency from nutrient application may not be achieved unless the micronutrients are applied eight to fifteen days after the glyphosate is applied. This is necessary to prevent chelation and immobilization by residual glyphosate in tissues that renders them physiologically unavailable (Huber et al., 2004; Severson, 2006), although earlier applications may be more effective in detoxifying tissue-bound glyphosate.

6.3. Detoxifying accumulated glyphosate in meristematic tissues

Reduced root growth from the accumulation of glyphosate in root tips results in less contact of the roots with dispersed nutrients in the soil profile and may negate tolerance of plants to soilborne pathogens based on their ability to "outgrow" the damage caused from loss of root tissue. Likewise, glyphosate accumulates in active meristematic tissue in shoots and developing fruits to inhibit growth of these tissues. Calcium, Mg, and micronutrients that chelate with glyphosate can reduce its biological activity and restore some of the potential physiological activity in these tissues. These "detoxifying" elements can come from within the plant or from further uptake from the soil. Thus, it is important to maintain mineral sufficiency in plant tissues and their ready availability in soil for plant uptake. This may be achieved by soil or foliar applied nutrients (Bernards et al., 2005; Huber et al., 2004; Reichenberger, 2007) if other environmental restraints are considered.

6.4. Eliminating glyphosate toxicity to rhizosphere microbes or restoring critical microbial components damaged by glyphosate released in root exudates

Detoxifying glyphosate in root exudates may occur in highly calcareous soils or soils with high levels of soluble metal nutrients through chelation to reduce its impact on soil organisms. Toxicity of glyphosate to Mn-reducing and synergistic nitrogen-fixing organisms in the rhizosphere can have serious consequences for sustainability of legume production. Regular inoculation of legume crops with synergistic nitrogen-fixing organisms may be required in many areas for maximal productivity where extended applications of glyphosate have eliminated them from the soil profile. Development of glyphosate-tolerant nitrogen-fixing and Mn-reducing organisms would be beneficial in many of these situations, and especially for perennial Roundup Ready® legume crops such as alfalfa.

7. Summary

Extended use of glyphosate can significantly increase the severity of various diseases by impacting all four of the interacting components of the "plant disease diamond" comprised of the plant, abiotic and biotic environments, and pathogen (Fig. 8). Reduced growth, impaired defenses, impaired uptake and translocation of nutrients, and altered physiology of plants by glyphosate can affect susceptibility or tolerance to various diseases. Glyphosate chelation of nutrients in the plant and soil can render those nutrients immobile and unavailable for plant use or uptake, while toxicity to essential synergistic and beneficial soil organisms (Purcell, 2001) further reduces availability of nutrients that are critical for a plant's physiological defense to disease. Glyphosate stimulation of fungal growth and enhanced virulence of pathogens such as *Fusarium*, *Gaeumannomyces*, *Phytophthora*, *Pythium*, and *Xylella* can have serious consequences for sustainable production of a wide range of susceptible crops and lead to the functional loss of genetic resistance that is dependent on metabolites through the shikimate pathway (Larson et al., 2006). Nutrient balance is important because each element functions as part of a delicately balanced, interdependent physiological system with the plant's genetics and the environment. Maximal utilization of cultural and management practices that increase the availability of nutrients (Table 2) to negate the deleterious effects of glyphosate should be incorporated

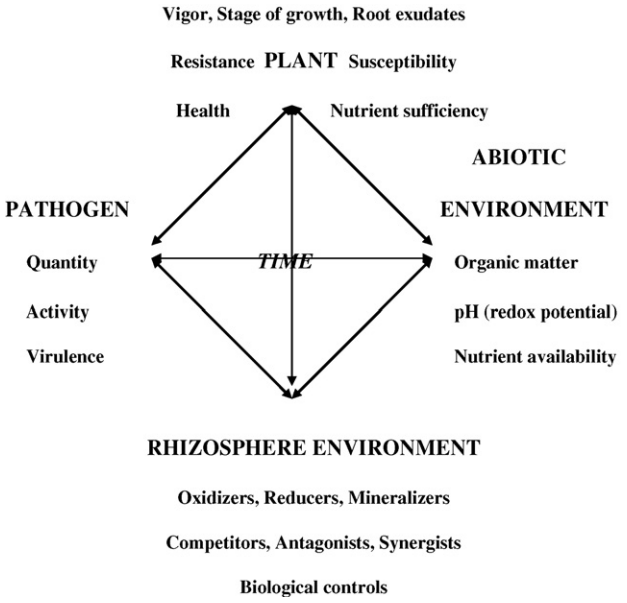

Fig. 8. The four primary interacting factors influencing nutrient availability and disease that are affected by glyphosate.

into crop production programs to facilitate optimal production efficiency and sustainable disease control. It is important to understand the effect of glyphosate on the chemical and biological properties of soils and its overall effects on the agricultural production system to permit its judicious use. Ignoring potential non-target detrimental side effects of any chemical, especially used as heavily as glyphosate, may have dire consequences for agriculture such as rendering soils infertile, crops non-productive, and plants less nutritious (Altman and Campbell, 1977). To do otherwise might well compromise not only agricultural sustainability, but also the health and well-being of animals and humans (Ozturk et al., 2008).

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Evidence of Contamination of Pedigreed Canola (*Brassica napus*) Seedlots in Western Canada with Genetically Engineered Herbicide Resistance Traits

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ABSTRACT

The objective of this study was to survey pedigreed canola seedlots for contaminating herbicide resistance traits, because of complaints from farmers regarding glyphosate [*N*-(phosphonomethyl)glycine] resistant canola volunteers occurring unexpectedly in their fields at densities and in patterns that suggested that pollen mediated gene flow from neighboring fields in previous years was not the source of contamination. Twenty-seven unique, commercial certified canola seedlot samples were collected. Glyphosate resistant seedlot samples were not collected. Canola samples were planted in the field, and when the canola had 2- to 4-true leaves, glyphosate, glufosinate [2-amino-4-(hydroxymethylphosphinyl)butanoic acid], and thifensulfuron [methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate] herbicides were applied. Surviving canola plants were counted. Of the 27 seedlots, 14 had contamination levels above 0.25% and therefore failed the 99.75% cultivar purity guideline for certified canola seed. Three seedlots had glyphosate resistance contamination levels in excess of 2.0%. Unexpected contamination (even at 0.25%) can cause problems for producers that practice direct seeding and depend on glyphosate for non-selective, broad-spectrum weed control. To avoid unexpected problems and costs, it is important that farmers are cognizant of the high probability that pedigreed canola seedlots are cross-contaminated with the various herbicide resistance traits.

Abbreviations: CSGA, Canadian Seed Growers' Association; IR, imidazolinone resistant.

Canola (*Brassica napus* L. and *B. rapa* syn. *B. campestris* L.) is the second most widely grown and the second most valuable crop in western Canada (after wheat) with annual plantings over the past decade of 3.0 to 5.7 million ha (Statistics Canada, 1992-2001). In recent years, over 90% of the canola grown has been *B. napus* cultivars (Canadian Grain Commission, 2002), for reasons that include greater yields and availability of cultivars with novel trait herbicide resistance. There currently are three novel-trait, herbicide resistant *B. napus* types commercially available in western Canada, namely, glyphosate resistant, glufosinate resistant, and imidazolinone resistant (IR). Two of these herbicide resistant types, glyphosate and glufosinate, are transgenic with the genes conferring resistance derived from bacteria (Canadian Food Inspection Agency, 1995a; Canadian Food Inspection Agency, 1995b). The IR trait in canola, which also confers resistance to certain other acetolactate synthase inhibitor herbicides such as thifensulfuron, was derived by *in vitro* microspore mutagenesis and selection (Swanson et al., 1989).

Since its commercial introduction in 1996 (IR canola in 1995), herbicide resistant *B. napus* canola technology has been rapidly and widely adopted by Canadian farmers. In 1998 it was estimated that nearly 60% of a total of 4.9 million ha of canola were planted to herbicide resistant *B. napus* cultivars (Anonymous, 1998). For the year 2000, it was estimated that approximately 1.8 to 2 million ha of glyphosate resistant canola were planted in Canada by 20,000 farmers (40% of the total canola area) (Sharlow, 2002).

The agronomic practice of direct seeding, where the soil is not disturbed in spring prior to planting the crop, has become common in western Canada. This practice is beneficial in terms of minimizing soil erosion and conserving soil moisture, as well as reducing wear on tillage implements and tractors (Lafond et al., 1992). With direct seeding, weeds that have emerged prior to planting must be controlled to minimize subsequent competition and crop yield loss. These weeds, which can be large, established plants if they germinated late in the fall or early in the spring, are normally controlled in one of two ways. Some producers practicing direct

seeding use a planting implement that provides complete disturbance of the soil surface (e.g., discer seeder or large sweep shovels on an air seeder), which kills most annual weeds present at the time of seeding. Other producers spray a broad-spectrum, non-selective, non-residual herbicide (most commonly glyphosate) prior to planting the crop, or after planting but prior to crop emergence, to control emerged weeds. These producers generally plant the crop with an implement that does not cause complete soil disturbance (e.g., narrow openers on an air seeder). The herbicide application prior to crop emergence is often referred to as spring 'burn-off' or 'burn-down'. A direct account of the area treated with glyphosate as a spring burn-off is not publicly available. However, it is estimated that 13, 39, and 27% of the total area prepared for seeding in Manitoba, Saskatchewan, and Alberta, respectively, is seeded following no-till or zero-till practices (a total of 8.1 million ha) (Statistics Canada, 2002). Glyphosate as a spring burn-off treatment would be applied to the majority of this land.

Currently there are no suitable substitutes for glyphosate as a spring burn-off herbicide considering spectrum of activity, efficacy, absence of soil residue, and cost. Consequently, those farmers that expect glyphosate resistant canola volunteers (from previous crops) still use glyphosate as a spring burn-off, but usually add an auxin-type herbicide such as 2,4-D [(2,4-dichlorophenoxy)acetic acid] or MCPA [(4-chloro-2-methylphenoxy)acetic acid] to the spray tank. In addition to extra cost, there are other concerns with tank-mixing glyphosate and an auxin-type herbicide. The auxin-type herbicides have some soil residual activity, and this soil residue can seriously injure certain sensitive broadleaf crops as they emerge such as field pea (*Pisum sativum* L.), field bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* Medic.), chickpea (*Cicer arietinum* L.), and sunflower (*Helianthus annuus* L.) (Saskatchewan Pulse Growers, 2000). Furthermore, volunteer canola plants that emerge early in the spring are generally large, hardy, and robust at the time of spring burn-off, and therefore complete control may be difficult with alternative herbicides such as 2,4-D, MCPA, or thifensulfuron/tribenuron [tribenuron, methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)methylamino]carbonyl]amino]sulfonyl]benzoate].

Thifensulfuron/tribenuron will not control IR canola volunteers, even if these volunteers are small. If there are unexpected glyphosate resistant canola volunteers, due to pollen mediated gene flow from a neighboring field or from a contaminated seed source, these become very obvious five to seven days after application of the spring glyphosate burn-off treatment. Depending on the crop planted (e.g., field bean, lentil, chickpea, sunflower) applying auxin-type herbicides in-crop to the escaping glyphosate resistant canola volunteers may not be an option. Also, glyphosate resistant volunteers escaping the spring burn-off treatment may be relatively large and difficult-to-control by the time alternative herbicides can be applied in-crop. Depending on surviving volunteer canola density and the crop that was sown, the resulting problem may be cosmetic or the competitive growth habit of volunteer canola may actually reduce crop yield and contribute to the glyphosate resistant canola seedbank in the soil.

Currently for Canadian pedigreed canola seed, there are no specific standards regarding the adventitious presence of genetically engineered herbicide resistance traits in seedlots. However, if novel trait herbicide resistance is considered an integral component of a herbicide resistant canola cultivar, then cultivar purity standards would apply. The Association of Official Seed Certifying Agencies allows 0.25% maximum for the presence of other canola cultivars in a certified canola seedlot (Association of Official Seed Certifying Agencies, 1999; Downey and Beckie, 2002). For Breeder and Foundation canola seedlots, the tolerance level for other canola cultivars (genetic purity limit) is 0.05%. Prior to the introduction of novel trait herbicide resistance in canola, there were no definitive genetic markers in canola to easily, quickly, and precisely quantify the levels of genetic impurity in a canola cultivar/seedlot (Downey and Beckie, 2002).

Contamination of pedigreed canola seedlots or commercial crops with herbicide resistance traits can occur in two ways: via either pollen mediated gene flow or whole seed. Whole seed contaminants may be homozygous for the herbicide resistance trait, while contaminants resulting from pollen mediated gene flow will be heterozygous for the resistance trait in the initial progeny generation. Canola seedlings heterozygous for the herbicide

resistance traits (glyphosate, glufosinate, or imidazolinone) can survive and thrive following recommended commercial dosages of these herbicides in the field (Hall et al., 2000; Rieger et al., 2002).

This survey of the purity of pedigreed canola seedlots with respect to herbicide resistance traits was prompted by complaints from several farmers regarding glyphosate resistant canola volunteers occurring unexpectedly in their fields at densities and in patterns that suggested that pollen mediated gene flow from neighboring fields in previous years was not the source of contamination. The authors are aware of only one other study investigating the purity of pedigreed canola seedlots with respect to genetically engineered herbicide resistance traits. Downey and Beckie (2002) tested a total of 70 certified canola seedlots drawn from 14 different conventional, open pollinated *B. napus* cultivars for glyphosate and glufosinate resistance trait contamination. They screened 2,000 seeds from each seedlot per herbicide in a petri dish seed bioassay. They reported that 41 of the 70 seedlot samples had detectable levels of herbicide resistance trait contamination, and that 18 of the 70 samples failed the 99.75% cultivar purity guideline. One of the samples tested had glyphosate resistance trait contamination levels of 6.8%. Downey and Beckie (2002) obtained their seedlot samples directly from pedigreed seed producers, prior to seed treatment and packaging of the seed into bags for commercial sale. Their results may not directly reflect what farmers actually plant in their fields since seed treatment and packaging involves additional handling of the seedlot and opportunities for contamination due to inadvertent seed admixtures.

The objective of this study was not to determine the absolute level of herbicide resistance trait contamination in a given canola cultivar/seedlot, but rather to determine whether pedigreed canola seedlots in western Canada are contaminated with unwanted/unexpected herbicide resistance traits. Samples were drawn from commercially packaged, seed-treated, certified seedlots that were sown by farmers into their fields in May of 2002.

MATERIALS AND METHODS

Thirty-three commercial certified canola (*B. napus*) seedlot samples were collected in the spring of 2002 from local co-operating farmers, representing 27 unique Canadian Seed Grower's Association (CSGA) seedlot numbers (i.e. some seedlots were sampled from different bags purchased by different farmers, but had identical CSGA lot numbers). All canola samples were commercially treated with a combination fungicide/insecticide seed treatment. Eighteen conventional canola samples, eight glufosinate resistant samples, and seven IR samples were collected. Glyphosate resistant samples were not collected because farmers are contractually prohibited from providing seed to third parties for any reason including research and testing. Furthermore, low levels of contamination (5% or less) of glyphosate resistant seedlots by conventional, glufosinate resistant, or IR canola is of minimal agronomic importance, since neither glufosinate nor acetolactate synthase inhibitors normally are used as broad-spectrum spring burn-off treatments. The seedlots were collected without bias or foreknowledge of contamination levels. To minimize inconvenience to the farmer, the collection procedure consisted of opening one bag and scooping out several cups of seed. Bags were not probed in multiple places nor were multiple bags sampled of a specific cultivar held by a given farmer. It was assumed that the seedlot samples would be relatively homogeneous after harvest, handling, and commercial seed treatment. The results presented in Table 1 on seedlots with identical CSGA lot numbers generally support this assumption.

The canola seedlot samples were planted in the field at the University of Manitoba research station at Carman, Manitoba on June 18, 2002 using a small-plot cone seeder with 12 double disk openers spaced 15 cm apart. The plots were located in an area where a canola crop had not been grown for at least eight years. This area was limited in size, which limited the number of replicates that were planted. Strips 2 m wide were left unseeded between each replicate, to function as a check for volunteer canola emergence from the soil seedbank and to separate the various herbicide treatments. The plot area was cultivated just prior to seeding the

canola, and the soil was moist. Individual plot size was 1.8 m x 6 m (11 m^2). Seven replicates were planted with each replicate including all 33 seedlots. Because the canola was not being grown to maturity, seeding rate was higher than that normally used. An average thousand seed weight of 3.5 g was assumed for all samples, and the target seeding rate was 3500 seeds per plot. Canola seedling density prior to herbicide application was determined on July 2 in three of the seven replicates, by counting the number of canola seedlings occurring per 50 cm row length in three adjacent rows in each plot.

Canola seedlings were sprayed on July 3 when they had two to four true leaves. Herbicides were applied using an 'All Terrain Vehicle' mounted sprayer equipped with Teejet 11001 flat fan nozzles (Spraying Systems Co., Wheaton, IL, 60188, U.S.A.) calibrated to deliver 55 L/ha of spray solution at 275 kPa at 8 km/hr. Commercial formulations of the herbicides were applied. Glyphosate was applied at 750 g a.e./ha (445 g a.e./ha is the recommended dosage for glyphosate resistant canola) to three replicates. Glufosinate was applied at 500 g a.i./ha to one replicate. Thifensulfuron was applied at 10 g a.i./ha + 0.2% v/v nonionic surfactant to one replicate. Glyphosate + glufosinate tank mix (at the above dosages) was applied to one replicate, and glyphosate + glufosinate + thifensulfuron tank mix (at the above dosages) was applied to one replicate. In the three-way tank mix, the nonionic surfactant was omitted (the glyphosate and glufosinate commercial formulations include surfactants). Thifensulfuron was used to identify canola seedlings with the IR trait because of the very short persistence of thifensulfuron in soil as compared to an imidazolinone herbicide – herbicide soil residues are a concern on the University of Manitoba Carman research station. Thifensulfuron is registered for commercial use on IR canola in western Canada (Manitoba Agriculture and Food, 2002).

Surviving canola plants in all plots, except those plots treated with thifensulfuron alone, were counted on July 9. Survivors were very obvious and response was very definitive, particularly for plots treated with glyphosate and tank mixtures containing glyphosate (i.e., plants were either dead or alive with no stunted green seedlings). For glufosinate, seedlings exhibiting

obvious crisping of leaf edges were classified as susceptible (i.e., not carrying the glufosinate resistance trait). Plots treated with thifensulfuron were assessed July 15. In the non-IR plots, the majority of canola seedlings treated with thifensulfuron were not dead and desiccated, but were green and very stunted. However, canola plants with the IR trait were bolting at this time and this was the basis for discrimination. For each plot, percentage resistance was calculated by dividing the number of surviving or uninjured canola plants by the total number of seedlings prior to herbicide application and then multiplying by 100.

RESULTS AND DISCUSSION

Canola emerged uniformly and visually grew normally in all plots. A relatively high percentage of the seeds sown successfully established seedlings (approximately 2250/3500 = 64%) (Table 1, refer to overall mean density per plot). Flea beetle (*Phyllotreta* spp.) damage to the canola seedlings was minimal, probably because all seedlots had a seed treatment. There was no emergence of canola outside of the seeded plots indicating no viable canola seed in the soil seedbank.

There was some variability between seedlots in the mean number of seedlings screened per plot (Table 1). This variability may have been due to differences in thousand seed weight between seedlots and cultivars (when calculating the seeding rate, a thousand seed weight of 3.5 g was assumed for all seedlots to facilitate packaging of the seed for the small-plot cone seeder). Furthermore, an occasional seedlot sample had either fertilizer or insecticide impregnated granules mixed with the seed. For example, seedlot 26 had some fertilizer mixed with the seed and this was over-compensated for when packaging the amount of seed per plot (compare seedlings screened to seedlot 25). Regardless, variable seedling density did not compromise either early growth or spray coverage in any of the plots.

Of the 33 seedlots, only one seedlot (seedlot 30) had no detectable herbicide resistance trait contamination based on the numbers of seedlings screened (Table 1). Of the 27 unique

CSGA numbered seedlots, 14 had contamination levels above 0.25% and therefore failed to meet the 99.75% cultivar purity guideline for certified seed. Of the 14 unique seedlots that had contamination levels in excess of 0.25%, nine failed due to glyphosate resistance trait contamination while five failed due to glufosinate resistance trait contamination. One of the unique CSGA numbered seedlots (seedlots 12 and 13) failed to meet the purity guideline because both glyphosate and glufosinate resistance trait contamination exceeded 0.25%. However, double resistant individuals, resistant to both glyphosate and glufosinate, were not detected in seedlots 12 and 13. Three of the unique seedlots had very high levels of glyphosate resistance trait contamination, that is, greater than 2%.

Glufosinate resistance trait contamination (in the non-glufosinate resistant cultivars) occurred at lower levels as compared to glyphosate resistance trait contamination, with no seedlots exceeding 1% glufosinate resistance trait contamination (Table 1). As might be expected, double resistant individuals (glyphosate and glufosinate) were detected only in the glufosinate resistant cultivars. Of the seven unique CSGA numbered glufosinate resistant seedlots, six of the seven had lower levels of double resistant individuals as compared to the levels of glyphosate resistant individuals. These results indicate that some of the glyphosate resistant individuals in the glufosinate resistant seedlots were, in fact, susceptible to glufosinate. This may be a result of whole seed contamination as opposed to pollen mediated gene flow.

The majority of the IR cultivars had undetectable levels of glyphosate resistance trait contamination, while still exhibiting glufosinate resistance trait contamination (Table 1). Since only one company is involved in the breeding and development of IR cultivars in Canada to date, it appears that IR seedlots were screened for glyphosate resistance trait contamination at all stages of pedigreed seed production (and contaminated seedlots were discarded). However, the same vigilance appears to have not been exercised for glufosinate resistance trait contamination. Glufosinate resistance trait contamination does not have the same agronomic implications for farmers practicing direct seeding as does glyphosate resistance trait

contamination. The IR seedlot results indicate that it is possible to produce certified canola seedlots in western Canada with low levels of herbicide resistance trait contamination.

Only six unique CSGA numbered seedlots had detectable levels of IR trait contamination, and contamination levels were 0.05% or less in all instances (Table 1). This may reflect the relative popularity of the various herbicide resistant canola types (refer to the Introduction). Fewer acres would result in fewer opportunities for outcrossing and also reduced whole-seed contamination. No triple resistant individuals (resistant to glyphosate, glufosinate, and thifensulfuron) were detected in this study.

The overall results of this study are comparable to those reported by Downey and Beckie (2002), although somewhat more contamination was identified in the current study [14 out of 27 unique CSGA numbered seedlots failed the 99.75% cultivar purity guideline in the current study as compared to 18 out of 70 seedlot samples which failed in the Downey and Beckie (2002) study].

Given current knowledge of pollen mediated gene flow in *B. napus* (Staniland et al., 2000; Rieger et al., 2002), it is unlikely that pollen flow would cause greater than 0.1% contamination in a single generation of pedigreed seed production. Pedigreed seed crops are grown with mandatory isolation distances from sexually compatible species (Canadian Seed Growers' Association, 2002), which limits pollen mediated gene flow. Therefore, the contamination occurring in certified canola seedlots with contamination levels greater than 0.25% is either the result of inadvertent mechanical mixing of certified seedlots during harvest or handling, or the result of contamination occurring in earlier generations of pedigreed seed production (i.e., Breeder or Foundation seed) that was not tested for or detected (Downey and Beckie, 2002).

The planting of pedigreed canola seedlots that do not exceed the 0.25% contamination guideline for certified seed does not necessarily mean that there will be no agronomic concern the following year with regard to the unexpected presence of herbicide resistance traits in

volunteer canola seedlings. Given some reasonable assumptions regarding canola seeding rates and thousand seed weight (5.5 kg/ha, 4.0 g per thousand seeds), there are approximately 1.4 million seeds planted per hectare. At the 0.25% contamination level of a herbicide resistance trait in a seedlot, there will be 3,500 resistant 'seeds' planted per hectare. If one-half of these seeds result in mature canola plants, which is a typical establishment rate for a commercial canola crop in western Canada, then there will be 1,750 resistant canola plants per hectare. Given a 2,000 kg/ha crop yield and harvest losses of 6% (Gulden et al., 2003), there will be 120 kg/ha of seed remaining in the field. Resistant seeds will be 0.25% of this 120 kg/ha [in the absence of selection and given equal fitness of susceptible and resistant individuals, a resistance trait will remain at the same frequency in a population over time (Jasieniuk et al., 1996)]. Therefore, 300 g of resistant seed will shatter onto the soil per hectare, or 75,000 resistant seeds per hectare. If one-tenth of these seeds successfully establish a seedling the following year, there will be one herbicide resistant volunteer canola plant every 1.3 m^2. If the resistance trait is glyphosate and the farmer practices direct seeding and sprays with glyphosate alone prior to crop emergence, one surviving canola plant every 1.3 m^2 will be a weed problem. Depending on the crop planted, there may not be in-crop herbicide options that will provide satisfactory control of relatively large volunteer canola plants (large because the canola volunteers would have survived the spring glyphosate burn-off applied prior to crop emergence). If the crop planted is not as competitive as cereals (e.g., flax, lentil, or field bean), one volunteer canola plant every 1.3 m^2 may be more than a cosmetic problem and probably will cause crop yield losses. The above scenario applies to pedigreed canola seedlots that meet the cultivar purity guideline of 99.75%. Downey and Beckie (2002) acknowledged this problem and noted that even when the genetic purity standards are met, the sowing of a conventional cultivar will almost certainly result in a significant population of herbicide resistant plants within that field.

Because of the value and popularity of direct seeding to farmers in western Canada and the dependence of direct seeding systems on glyphosate, the adventitious presence of the

glyphosate resistance trait in pedigreed canola seedlots has greater agronomic implications than either the glufosinate or IR traits. Neither glufosinate nor the various herbicides used in IR canola are registered for use as direct seeding, spring burn-off treatments (Manitoba Agriculture and Food, 2002). However, it is possible that IR canola volunteers emerging with a subsequent crop could survive an in-crop herbicide application if an acetolactate synthase inhibitor herbicide is applied alone. In this study, the adventitious presence of the IR trait in canola seedlots was relatively rare.

CONCLUSIONS

The results of this study indicate that the pedigreed canola seed production system in western Canada is cross-contaminated with the various herbicide resistance traits at a high level, and that purchasing and planting a pedigreed conventional canola seedlot does not guarantee the absence of genetically engineered traits. For those producers that grow canola and practice direct seeding, it means that glyphosate no longer is a non-selective, broad-spectrum herbicide that can be used alone as a spring burn-off treatment. Because other herbicides have to be tank-mixed with glyphosate to achieve broad-spectrum vegetation control in the spring burn-off treatment, additional costs will be incurred.

While this survey of pedigreed canola seedlots was not repeated in time or space, we believe that this study has merit despite the lack of repetition. The objectives of this study were not to determine the actual or absolute level of herbicide resistance trait contamination in a given canola cultivar/seedlot, but rather to determine whether pedigreed canola seedlots in western Canada are contaminated with unwanted/unexpected herbicide resistance traits. Of the 27 unique CSGA numbered canola seedlots in this study, 26 had detectable levels of herbicide resistance trait contamination even given the relatively low numbers of individual seedlings screened (as compared to the number of individual seedlings that normally are present in one hectare of a canola crop, for example). Results in all field plots were very clear and easy to assess. Furthermore, the similar results of the Downey and Beckie (2002) canola seedlot study

(refer to the Introduction) confirm our results and indicate that our survey was and is representative of reality. The Downey and Beckie (2002) study is not published in a widely-circulated scientific journal, though. The results of these canola seedlot surveys are extremely important, particularly to those farmers and organizations that are hoping to avoid or minimize the occurrence of GM traits on their land or in their crops.

The pedigreed seed production system can be considered a stringent segregation/identity preservation system complete with mandatory isolation distances, crop rotation restrictions, and inspections (Canadian Seed Growers' Association, 2002). The results of this study indicate that this stringent segregation system does not result in the genetic purity of pedigreed canola seedlots in western Canada. The results of this study can be considered as a model prior to the commercialization of other genetically engineered crops, where segregation/identity preservation systems are being considered. Factors such as the inherent outcrossing rate, seedbank recruitment and longevity, and frequency of the crop in the rotation would influence subsequent contamination levels in conventional pedigreed seed and commercial grain lots. The specific genetically engineered trait also is important in considering the implications of commercializing a genetically engineered crop. For example, the glyphosate resistance trait in plants directly affects the success and economics of a direct seeding crop production system. Furthermore, a successful segregation/identity preservation system requires agreed-upon tolerances for contaminants and enforcement of the standards through frequent testing and discarding of out-of-tolerance seed or grain lots, creating additional costs for the entire production system.

For example, the commercialization of glyphosate resistant wheat in western Canada is currently being contemplated, possibly initially under an identity preservation protocol. Considering that wheat can outcross to nearly the same extent as *B. napus* (Staniland et al., 2000; Hucl and Matus-Cádiz, 2001), has similar seedbank longevity to *B. napus* (Beckie et al., 2001; Légère et al., 2001), and is grown more often in the rotation than canola (Thomas et al.,

1999), it can be predicted that the extent of glyphosate resistance trait contamination in pedigreed conventional wheat seedlots and commercial grain lots will eventually be similar to or greater than the situation currently in canola. The presence of two widely-grown commercial glyphosate resistant crops will cause additional problems and increased costs for those farmers practicing direct seeding in western Canada, whether they choose to seed resistant cultivars or not, and may threaten the viability of this widely adopted and beneficial farming practice (Van Acker and Entz, 2001).

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Table 1. Summary of canola seedling survival percentages for certified seedlots treated with various herbicides. Values based on three replicates (number of seedlings screened per plot and glyphosate treated survivors) are presented as means followed by standard deviation in parentheses.

			Percentage survivors				
Seedlot	Cultivar[†]	Type	Glyphosate (3 reps)[‡]	Glufosinate	Glyphosate + glufosinate	Thifensulfuron	Mean number of seedlings screened per plot
1	A_1	Conv[§]	0.06 (0.08)	0.15	0.00	0.00	2640 (416)
2	A_1	Conv	0.02 (0.04)	0.07	0.00	0.03	3070 (333)
3	B	Conv	0.09 (0.13)	0.04	0.00	0.04	2480 (587)
4	B_1	Conv	0.29 (0.06)	0.04	0.00	0.00	2550 (520)
5	B_1	Conv	0.39 (0.13)	0.04	0.00	0.00	2390 (73)
6	C_1	Conv	0.19 (0.08)	0.10	0.00	0.05	1970 (393)
7	C_1	Conv	0.19 (0.07)	0.21	0.00	0.00	2800 (194)
8	D	Conv	0.24 (0.16)	0.00	0.00	0.00	1920 (173)
9	E	Conv	0.02 (0.03)	0.09	0.00	0.00	2130 (618)
10	F	Conv Hybrid	0.24 (0.11)	0.60	0.00	0.00	1840 (391)
11	G	Conv Hybrid	0.67 (0.10)	0.09	0.00	0.05	2180 (481)
12	H_1	Conv	4.89 (0.69)	0.50	0.00	0.05	2190 (290)
13	H_1	Conv	3.00 (0.64)	0.32	0.00	0.05	1860 (409)
14	I	Conv	0.05 (0.06)	0.31	0.00	0.00	2550 (520)
15	I_1	Conv	0.04 (0.06)	0.43	0.00	0.05	1870 (48)
16	I_1	Conv	0.02 (0.03)	0.19	0.00	0.05	2070 (375)
17	J	Conv HEAR	0.31 (0.08)	0.11	0.00	0.00	1830 (127)

1	18	J	Conv HEAR	0.03 (0.05)	0.20	0.00	0.00	2530 (100)
2	19	K	GluR Hybrid	0.27 (0.07)	NA	0.04	0.00	2340 (182)
3	20	K	GluR Hybrid	0.23 (0.12)	NA	0.28	0.00	2160 (267)
4	21	L	GluR Hybrid	0.08 (0.06)	NA	0.00	0.00	2000 (246)
5	22	L	GluR Hybrid	2.67 (0.18)	NA	1.45	0.00	2270 (431)
6	23	L	GluR Hybrid	0.44 (0.34)	NA	0.20	0.00	1520 (111)
7	24	M	GluR Hybrid	2.13 (0.42)	NA	1.20	0.00	2160 (300)
8	25	M_1	GluR Hybrid	0.32 (0.11)	NA	0.07	0.00	1440 (267)
9	26	M_1	GluR Hybrid	0.38 (0.17)	NA	0.15	0.00	3310 (660)
10	27	N	IR	0.05 (0.05)	0.82	0.00	NA	2190 (581)
11	28	N	IR	0.00 (0.00)	0.25	0.00	NA	2820 (680)
12	29	N	IR	0.00 (0.00)	0.15	0.00	NA	2630 (782)
13	30	O	IR	0.00 (0.00)	0.00	0.00	NA	2370 (227)
14	31	O	IR	0.00 (0.00)	0.12	0.00	NA	1600 (242)
15	32	O	IR	0.00 (0.00)	0.04	0.00	NA	2450 (646)
16	33	O	IR	0.00 (0.00)	0.19	0.00	NA	2100 (529)
17	Mean							2250 (423)

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19 †The subscript '1' following the alphabetic seedlot designation indicates that the two seedlots had identical

20 Canadian Seed Growers' Association (CSGA) Lot numbers. These seedlots were sampled from different

21 25 kg bags purchased by different farmers, but the CSGA Lot number was identical. For the seedlots,

22 CSGA Crop Certificate and Lot numbers are available from the author based on a justifiable request.

23 ‡For glyphosate, the total number of seedlings screened per seedlot is three times the mean number of

24 seedlings screened per plot.

[§]Abbreviations used in table: Conv - conventional, Conv HEAR – conventional high erucic acid rapeseed, GluR – glufosinate resistant, IR – imidazolinone resistant, NA – not assessed.

CROP ECOLOGY, MANAGEMENT & QUALITY

Extent of Cross-Fertilization in Maize by Pollen from Neighboring Transgenic Hybrids

B. L. Ma,* K. D. Subedi, and L. M. Reid

ABSTRACT

There is an increasing concern about the preservation of genetic identity of conventional maize (*Zea mays* L.) and of distance required to segregate non-genetically modified (non-GM) from GM grain production since the introduction of *Bacillus thuringiensis* (Bt) and other transgenic events into commercial hybrids. Field experiments were conducted at three sites in Ottawa, Canada, for 3 yr to determine (i) the extent of cross-fertilization of a maize genotype by foreign pollen of neighboring hybrids and (ii) the practical distance required to isolate conventional maize hybrids from neighboring GM maize fields. At each site, yellow-kernel Bt maize was planted in the center (27 by 27 m) of a field surrounded in all directions by the distance equivalent to 24 or 48 rows (37 m) of white-kernel maize, and a 200-m non-maize crop was maintained in all directions. Phenology and weather conditions were closely monitored during the tasseling and silking period. At maturity, a thorough examination on the cross-fertilization was conducted in the white maize population. Our results showed that the rate of cross-fertilization in maize was dependent upon the distance from the pollen source, wind direction and synchronization of silking and pollen shedding of the two genotypes involved. Up to 82% out-cross was measured in the first row adjacent to the Bt maize. The level of out-cross was <1% beyond the 37th border row (28 m) downwind and the 13th row (10 m) upwind in all site-years. An exponential decline model was fitted well ($P < 0.01$) to the cross-fertilization data as a function of distance from the yellow maize pollen source with R^2 up to 0.64. Our data suggested that it is possible to produce non-GM maize grains by removing the outside rows of non-GM maize plants (about 30 m) neighboring the GM maize field in concern if the acceptance level is set at ≤1% out-cross. The generally recommended 200-m distance between two genotypes (inbreds, populations, hybrids, and wild relatives) appears to be appropriate for Bt or other GM maize, as well.

Maize is a monoecious plant with male (staminate inflorescence) and female (pistillate inflorescence) flowers formed in separate parts of the same plant, leading to a high degree of cross-pollination between plants. It is reported that the cultivated maize plant freely crosses with nearly all members of the genus including several hundred mutants (Burris, 2001). The male inflorescence (tassel) of maize can produce considerably more pollen grains than are required for pollination of a single plant (Schoper et al., 1987). Goss (1968) estimated that as many as 2 to 5 million pollen grains are produced by a typical maize plant. Pollen shed can begin before tassels have completely emerged from the whorl and can continue over a week or longer (Ritchie et al., 1993). Westgate et al. (2003) estimated that individual tassels produced 4.5×10^6 pollen grains and pollen shedding lasted for 5 or 6 d.

Maize pollen grains are one of the heaviest and largest (about 90–100 μm in diameter) among the wind-dispersed pollen grains, thus limiting the distance maize pollen can travel (Raynor et al., 1972; Burris, 2001). Under natural conditions, the majority of pollen grains from a plant are normally assumed to fall within the row space. It is also suspected that a small amount of pollen can be transported over longer distances given favorable wind speeds and appropriate humidity (Kiesselbach, 1949; Garcia et al., 1998). Raynor et al. (1972) recorded only 0.2% pollen deposition per unit area at 60 m from the original source. This is in agreement with the earlier finding of Bateman (1947) that only 1% of the pollen grains at source was found at 27 m. Luna et al. (2001) reported that cross-fertilization in maize could occur at a maximum distance of 200 m from the source. Other biological factors such as pollen density, pollen radius, and sedimentation velocity are also important factors in determining the distance of the pollen drift (Luna et al., 2001). Maize pollen generally remains viable only for 1 to 2 h after dehiscence (Luna et al., 2001). However, depending on the environmental factors, mainly temperature (Goss, 1968; Schoper et al., 1987; Jemison and Vayda, 2001), humidity (Goss, 1968; Barnabas, 1984; Garcia et al., 998; Traore et al., 2000; Jemison and Vayda, 2001) and atmospheric water potential (Luna et al., 2001), it may remain viable for up to 24 h after shed. Cool temperatures and high humidity favor pollen longevity.

The issue of the degree of pollen dispersal and cross-fertilization between maize genotypes has become increasingly important with the recent and continued release of new transgenic maize hybrids. Several transgenic hybrids have been developed with herbicide tolerance or pest resistance, and are now commercially cultivated. One of the most common examples is the maize transformed with a gene from the bacterium *Bacillus thuringiensis* to express the insecticidal 1 epidopteran-active crystalline protein (Cry1Ab) endotoxin for the control of European corn borer [*Ostrinia nubilalis* (Hübner)] (Koziel et al., 1993). Such genes can be naturally transferred to conventional (non-Bt) genotypes in adjacent fields via pollen dispersal. This "loss of control" over the engineered gene is one of the most discussed environmental effects associated with the use of transgenic

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Abbreviations: Bt, *Bacillus thuringiensis;* CHU, crop heat unit; GM, genetically modified.

plants (Scriber, 2001; Saeglitz and Bartsch, 2001). For maize producers, the major issue is that contamination of conventional hybrids by pollen from neighboring transgenic hybrids will restrict the marketing of the grain harvested from the contaminated field. Grain harvested from a contaminated conventional field is essentially declared as transgenic, will not be accepted at all grain elevators and will have to be channeled to specific elevators, processors and even countries who will accept transgenic grain. Thus, there is an urgent need to understand the pollen-mediated gene flow, and the minimum distance required to isolate conventional maize hybrids from neighboring GM maize fields.

Pollen, as the carrier of the male gamete, is an important vector of gene flow from one plant to another. The maintenance of genetic purity in cross-pollinated plants is the most important issue for hybrid and breeder's seed production (Jones and Brooks, 1950; Burris, 2001). Out-crossing in maize varieties is prevented either by time isolation (temporal) or distance isolation (spatial). An isolation distance of 185 to 200 m is recommended between two maize fields for seed production (Luna et al., 2001). Garcia et al. (1998) found complete pollen control at a distance of >184 m. They observed that most of the pollen settled on the soil surface within the source field itself. We, therefore, hypothesized that production of non-GM maize is possible by removing the outside rows of non-GM maize plants adjacent to the GM maize field. For seed production, the recommended distance of 200 m between two genotypes (inbreds, populations, hybrids, and wild relatives) was also appropriate for Bt or other GM maize, as well.

The endosperm of maize kernels can be yellow or white. These colors are easily observable and can be used as markers in genetic studies or tools for evaluating cross-fertilization. It is possible to determine the degree of out-cross between genotypes, by planting a white-kernel hybrid next to a yellow hybrid (Wicks and Mack, 1996). The objective of this study was to determine the extent of cross-fertilization of a maize genotype by foreign pollen from neighboring maize fields. Specifically, by growing white-kernel, non-Bt maize adjacent to yellow kernel Bt maize hybrids sided by side as a model system, we determined the effects of synchronization of the receptive silking period of a maize genotype with the pollination of the other maize hybrids, weather conditions during the flowering period, and distance between the hybrids on the rate of cross-fertilization.

MATERIALS AND METHODS

Site Description

Field experiments were conducted at three sites in Ottawa, Ontario, Canada (45°22′ N, 75°43′ W) for three growing seasons (2000, 2001, and 2002). The three sites were located within 3 km from each other with specific geographic characteristics. Site #1 was a clay loam soil (fine loamy, mixed, mesic Endoaquolls) with a flat surface surrounded by farmland in all directions with at least a 550 m radius. Before 2000, the land was cropped with barley (*Hordeum vulgare* L.). Site #2 was a clay loam (Endoaquolls) soil, gently sloped (about 5%) downward and connected to a meadow field in the west, with a road connecting to a grass field in the north, a manure patch on the east side and farmland on the south. The field was cropped to hay grasses in the past several years. Site #3 was a sandy loam soil (Haplorthods), gently rolled toward east direction, with a grass hilly field in the west, a road in the north, a meadow field in the east and a wheat (*Triticum aestivum* L.)–oat (*Avena sativa* L.) field in the south. All sites were open fields and in all cases, there were no maize crops, fence or block to stop wind flow within at least 200 m in all directions. For all sites, the fields were moldboard plowed in the fall each year.

Field Experiment

At all sites, soil samples (0–30 cm) were taken before planting each year to determine soil nutrient level and general properties to ensure adequate fertilizer applications. Adequate phosphorus (P) and potassium (K) were applied during the land preparation according to the soil test recommendations. Fertilizer urea at 200 kg N ha^{-1} and herbicide [Fieldstar (flumetsulam/clopyralid) 216 g ha^{-1} + Primextra Lite (s-metolachlor/atrazine) 3.3 L ha^{-1}] mixtures were applied and incorporated into the soil before planting at each site in all years. The maize was planted at a density of 73 000 plants ha^{-1} in 76.2-cm row spacing in a north–south row orientation. In each field, the yellow Bt maize was planted in the center (36 rows of 27 by 27 m) while a white maize hybrid was planted in the surroundings to fill a total area of 1 ha (100 by 100 m) for Site #1 and 0.68 ha (82.3 by 82.3 m) for the other two sites (Fig. 1). In this region, the prevailing wind in July and August is generally assumed from the northwest direction. Therefore, the white maize planted in the east and south direction of the yellow Bt maize was assumed to be in the "downwind" direction and designated as downwind, while the white maize in the west and north direction was upwind. Hybrids and planting dates for each site-year are listed in Table 1. In 2000, for Site #1, the hybrids for which the flowering dates of yellow and white maize were synchronized better than for the other sites. In the other years, more synchronised hybrid pairs were chosen. In Sites #2 and #3, the pairs of white and yellow kernel hybrids were planted on the same day as they had similar silking dates. In addition, to provide white maize with an extended period of pollen availability of yellow kernel maize, mixtures of two yellow hybrids with differences up to 60 Crop Heat Units (CHU; Brown and Bootsma, 1993) were used in 2002 in Sites #2 and #3 (Table 1). Phenological progressions of both hybrids at each site were recorded. A plant was considered to be at pollination stage if at least one anther was releasing pollen as checked daily before 1000 h. Similarly, date of silking stage was recorded if at least one silk was emerged from the sheath. Fields were monitored daily from the beginning of tasseling and silking, and times taken to pollen-shed and silking in 50% plants were recorded.

Measurements

At each site, an automated weather station (Wind Sentry, Model 03002-10A; R.M. Young Company, Traverse City, MI) mounted on a 10-m post was set up at the canopy top (3 m above the ground), and hourly wind speed and direction were recorded in a data logger (CR10, Campbell Scientific, Logan, UT) from shortly before tassel emergence to the end of flowering. Daily temperatures and rainfall data were acquired from the Environment Canada Atmospheric and Environment Services station located close to the experimental sites.

At maturity, a systematic sampling of the white maize was conducted to determine the pattern and extent of cross-fertil-

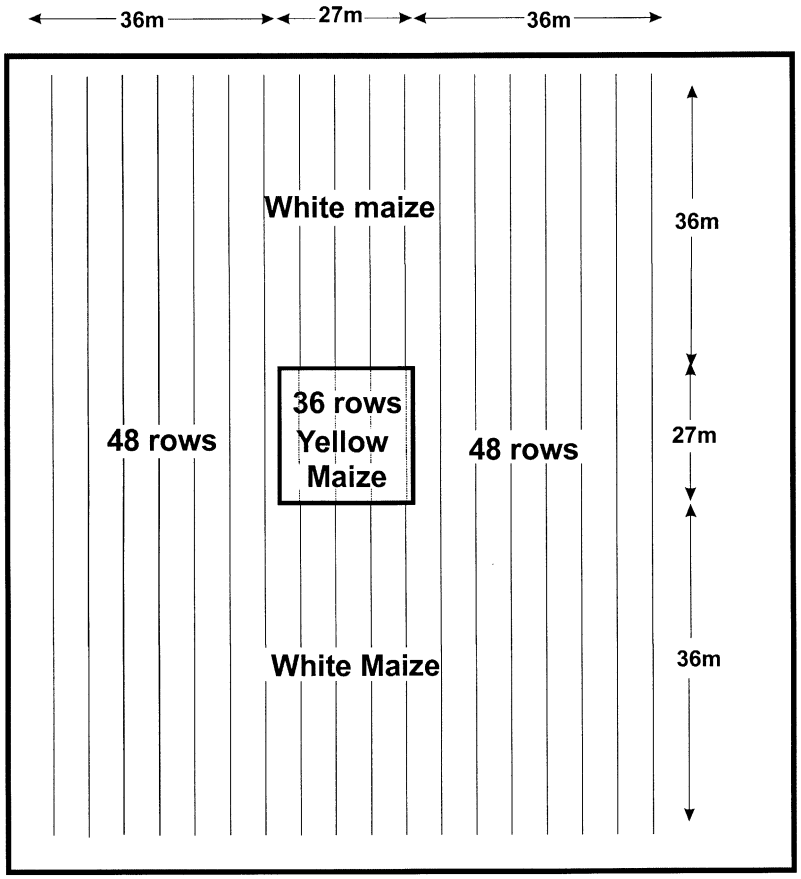

Fig. 1. Outline of the experimental field design showing the allocation of yellow (Bt) and white (non-Bt) hybrids of maize.

ization of white maize by pollen from neighboring yellow Bt hybrid. In Site #1, in both downwind and upwind directions, ears of white maize were sampled from rows No. 1 (the first row of white kernel maize adjacent to the yellow kernel maize), 7, 13, 19, 25, 31, 37, 43, and 48 (37 m) bordering the yellow Bt maize. These rows were chosen on the basis of the fact that the dominant maize header in Ontario is six rows wide, thus contamination after potentially removing 0, 1, 2 . . . 6 header widths could be evaluated. In addition, 36 rows represent three passes of a 12-row maize planter and perhaps the widest isolation buffer maize growers would adopt. In the north and south ends of yellow Bt maize rows, white maize ear samples were taken from rows 1, 7, 13, 19, 25, and 33 (row number was arbitrarily defined, but plant number was counted

Table 1. Maize hybrids; days elapsed since 1 January for planting, 50% pollen shed, and 50% silking; and Crop Heat Units (CHU) accumulated from planting to 50% pollen shed for each of three sites in 2000, 2001, and 2002.

Site	Hybrid	Days after 1 January			CHU to 50% pollen shed
		Planting	50% Pollen shed	50% Silking	
	2000				
Site #1	1) Pioneer 34P93 (White)	124	220	220	1998
	2) Pioneer 38W36 (Yellow, Bt)	136	214–221†	214	1650
Site #2	1) Cargill V414W (White)	138	227	227	1969
	2) Cargill 4521 (Yellow, Bt)	138	224–235	224	1894
Site #3	1) Cargill V414W (White)	126	223	223	2054
	2) Cargill 4521 (Yellow, Bt)	126	215–223	215	1846
	2001				
Site #1	1) Pioneer 34P93 (White)	122	214	213	1950
	2) Pioneer 33V06 (Yellow, Bt)	122	212–221	213	1950
Site #2	1) Cargill V414W (White)	124	211	212	1877
	2) Mycogen 2725 (Yellow, non-Bt)	124	210–220	212	1877
Site #3	1) Cargill V414W (White)	124	208–223‡	214–226	1930–2258
	2) Mycogen 2767 (Yellow, Bt)	124	208–222	213	1883
	2002				
Site #2	1) Cargill V414W (White)	125	221–235	221–235	1947–2316
	2) Mycogen 2767 (Yellow, Bt) and Pioneer 34R07 (Yellow, Bt)	125	221–235	221–235	1947–2316
Site #3	1) Cargill V414W (White)	127	213–229‡	213–229‡	1716–2150
	2) Pioneer 35Y55 and Pioneer 34M95 (Yellow, Bt)	127	219–232‡	219–232‡	1871–2226

† For the yellow kernel maize hybrids, the beginning and ending of pollen shedding period.
‡ Uneven growth (i.e. variability in size and developmental stages of plant due to drought and/or wet conditions).

always starting from the white kernel plant bordering the yellow kernel maize). Sampling scheme was the same in Sites #2 and #3 except that there were only 24 rows of white maize in the assumed upwind directions. In all designated rows, ears from every 10th plant of white maize (i.e., 1st, 11th, 21st, and so on) were collected for a total of 47 to 57 ears per row, marked and stored in onion bags for air-drying before counting the kernels. The relative distance to the yellow maize determined the position of all sampled plants in the field. A plant was considered as 0% cross-fertilization if there were no yellow-colored kernels in the sampled ear as well as in the ears of two adjacent plants (e.g., if the target was an 11th plant, plants 10th, 11th, and 12th were also field-checked to ensure no yellow kernels were present). In the straight south and north sides of the yellow maize rows, every 10th plant of white maize from Rows 1, 7, 13, 19, 25, and 31 was sampled starting from the 1st plant in each sampling row. In this way, a total of about 2800 ears were collected each year except in year 2002 when Site #1 was abandoned due to severe drought and rootworm damage. Within a single ear, the total number of rows, number of kernels per row and the total number of yellow kernels per ear were counted. Percent out-cross was calculated as the number of yellow kernels divided by the total number of kernels (white+yellow) per ear.

The cross-fertilization data with distance to the pollen source of yellow Bt maize were fitted to an exponential equation:

$$Y = Y_0\, e^{-BX} \quad [1]$$

And a modified exponential equation:

$$Y = Y_0\, e^{-BX} + C \quad [2]$$

where Y is the cross-fertilization (%), Y_0 is cross-fertilization extrapolated to $X = 0$, B is a shape coefficient, C is a coefficient that represents the cross-fertilization (%) at the farthest distance, and X is the distance (m) of the sampled ear to the pollen source of the yellow maize.

Means and standard deviations (STD) of cross-fertilization of individual rows were calculated and presented. Synchronization of pollen donor and silking receptor, and patterns of average kernel number, barrenness and percent cross-fertilization were assessed against wind conditions during the flowering period.

RESULTS

Weather Pattern

During the tasseling and silking period, wind speed varied considerably between sites and years (Fig. 2). In 2000, the average daily wind speed during pollination ranged from 1 to 5 km h^{-1} with a maximum of 22 km h^{-1}. In 2001, wind speed ranged from 4 to 10 km h^{-1} with a maximum speed of 24 km h^{-1}. The hourly average wind speeds were slightly higher (5–12 km h^{-1}) in 2002 with a maximum of 26 km h^{-1}. Generally, westerly wind prevailed during the flowering period in all the site-years except in 2000 at Site #2 (Fig. 3), where east wind prevailing. However, non-prevailing wind also came from north, south and east directions.

Precipitation in 2000 was high and generally evenly distributed during the growing season, while the years 2001 and 2002 had encountered an unusual drought. The growing season in 2001 was characterized by precipitation that was far below normal in June, July and August (only 56% of the 30-yr averages), but with excess rainfall in September and October. In 2002, extended

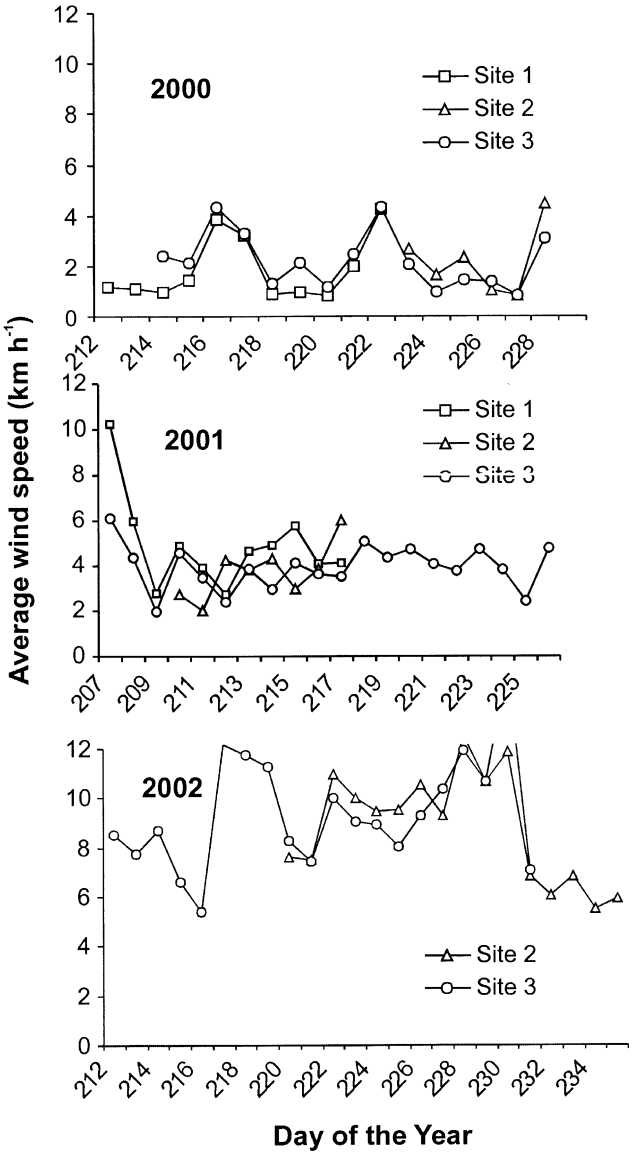

Fig. 2. Mean hourly wind speed (km h^{-1}) during the flowering periods at three sites in 2000, 2001, and 2002.

periods of drought also occurred in July, August and September. Site #1 in 2002 had to be abandoned due to both severe drought and rootworm damage.

Crop Phenology

Pollen shedding and silking dates, time taken to reach the stages and CHU accumulated for both yellow and white kernel maize hybrids are presented in Table 1. The Cargill hybrid 4521Bt (yellow) in Site #2 (year 2000), V414W (white) in Site #3 (year 2001) and both hybrids at Sites #2 and #3 in 2002 had non-uniform plant growth (uneven plant size) and development (phenological stages) resulting in a longer periods to complete their flowering, and probably asynchronous pollination within the population.

Environmental Effects

Seasonal climatic conditions affected the level of cross-fertilization. Overall maximum cross-fertilization occurred

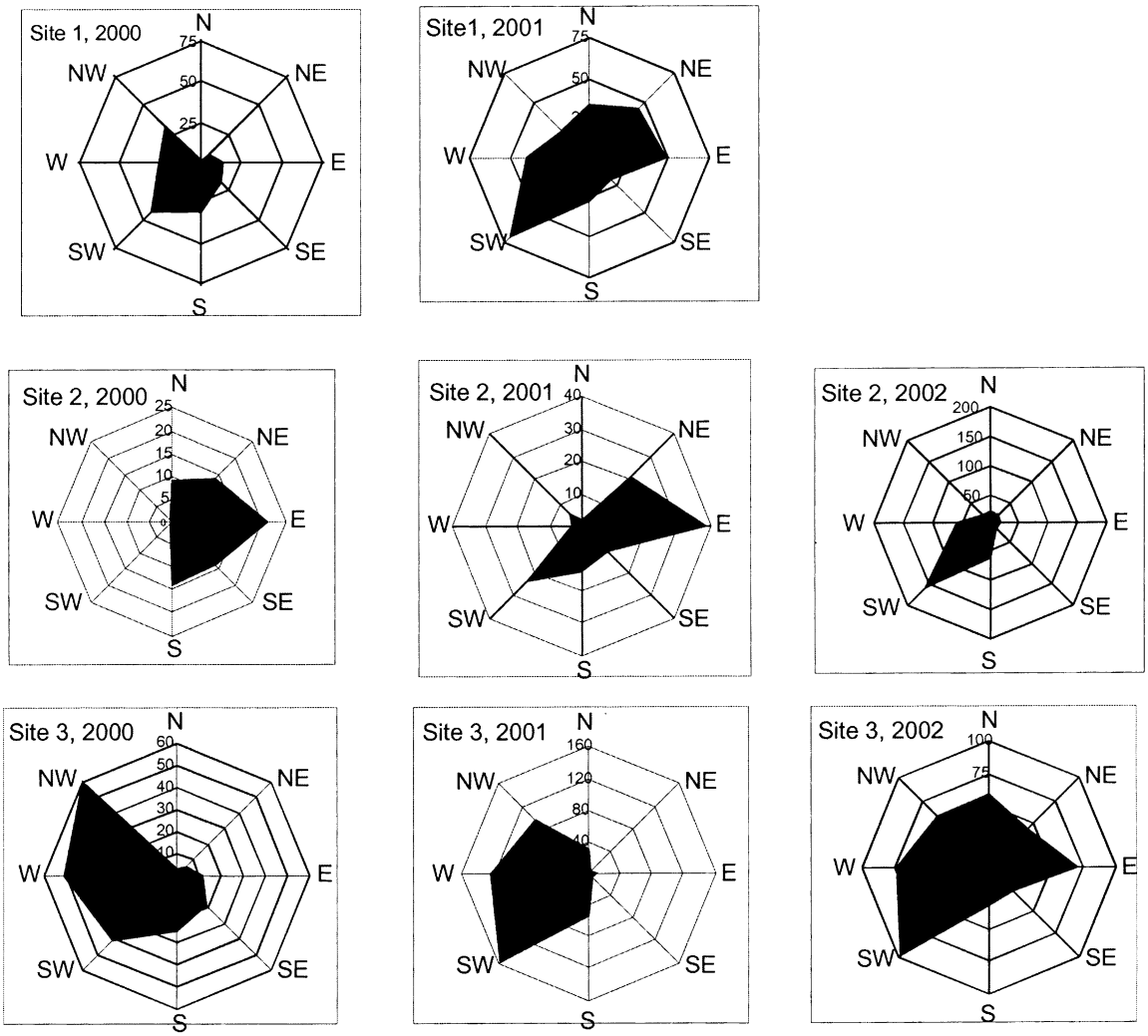

Fig. 3. Frequencies of gust wind directions measured hourly during the flowering periods at three sites in 2000, 2001, and 2002. The scales are hourly occurrence of gust from different directions.

in the first row of the white maize adjacent to the yellow-kernel Bt hybrid. The observed maximum out-cross was over 82% in all years (Table 2). The average level of out-cross in the first adjacent row was greater in 2000 (18.2%) than in 2001 (12.3%) or 2002 (13.3%). A consistent pattern was also observed in subsequent rows (Table 3). However, the effect of wind direction was different among the three years. Although the assumed wind direction was northwesterly, this was not always the case, particularly in Site #2 in 2000 and 2001 (Fig. 3). Non-prevailing wind evidenced in all the site-years (Fig. 3) may have had a large impact on cross-fertilization, which cannot be pinpointed from the current study. In 2000, cross-fertilization in the first adjacent row was on average 27.6% downwind and only 8.7% upwind (Table 3). A similar pattern of out-cross was observed in 2002 (24.9% downwind and only 4.9% upwind). In 2001 this pattern was reversed: downwind had only 10.6% out-crossing compared to 14.0% in upwind direction, indicating the impact of variable wind directions during flowering (Fig. 3). In general, in 2000 and 2002, all sites had greater out-cross in downwind than in upwind directions, but in 2001, Sites #2 and #3 had lower level of out-cross in downwind than upwind while, Site #1 had greater out-cross in downwind than upwind (Table 3). Apparently, instantaneous wind direction changes at the time of silking of the white maize had much larger effect than the overall prevailing wind direction.

The distance that yellow pollen reached was, as expected, much farther downwind than upwind in all years and sites (Tables 2 and 3). Cross-fertilization of some ears in the first adjacent row of white maize was as high as 82% downwind and 73% upwind (Table 2). The unusual drought in 2001 and 2002 resulted in unsynchronized pollen shedding and silking, thus reduced opportunity for cross-fertilization to occur. This led to a large number of barren ears in some rows of Site #1 in 2001 and Site #3 in 2002. Consequently, up to 56% of the ears had 1/3 or more of the tips unfilled in some of the rows sampled.

Site Effect

Cross-fertilization of white maize by yellow kernel Bt hybrid varied in all sites. Irrespective of year and wind direction, the mean percentage of cross-fertilizations recorded in the first adjoining row of white maize was 17% in Site #1, 14.2% in Site #2 and 14.5% in Site #3.

However, the sites did not follow the same pattern over the three years. In 2000, Site #1 had the highest out-crossing (27.4%) in the first adjacent row of white maize followed by Site #2 (14.3%) and Site #3 (12.7%). In 2001, Site #2 had the highest out-crossing (19.1%) followed by Site #3 (10.3%) and Site #1 (6.6%). In 2002, Site #2 had higher cross-fertilizations (20.7%) than Site #3 (9.1%), while Site #1 was abandoned. The lower percentage of cross-fertilization in Site #1 in 2001 was mainly due to uneven plant growth as affected by the drought, which also resulted in more barren plants. The difference between the sites was however, mainly within the 13 rows (10 m) adjacent to the pollen source; thereafter, cross-fertilization of white maize by pollen from

Table 2. Ranges of cross-fertilization (%) in white maize by pollen grains of neighboring yellow Bt maize in different rows and directions in 2000, 2001, and 2002. The values in the parenthesis within the row are distance in meter from the yellow kernel hybrid (source of pollen).

Direction	Site 1†		Site 2			Site 3			Overall mean
	2000	2001	2000	2001	2002	2000	2001	2002	
	%								
				Downwind					
1 (0.76)	14–81	0.0–74	2.0–66	1.7–49.7	0.0–74.9	0.0–50.0	0.0–20.6	0.0–82.2	2.2–62.3
7 (5.32)	0.7–15.7	0.0–6.6	0.0–27.3	1.0–6.0	0.5–11.4	0.0–34.3	0.0–0.3	0.0–2.8	0.3–13.1
13 (9.88)	0.2–5.0	0.0–4.6	0.0–7.6	0.0–0.7	0.0–3.4	0.0–25.4	0.0–3.3	0.0–75.0	0.0–15.6
19 (14.44)	0.2–4.8	0.0–4.2	0.0–1.3	0.0–1.2	0.0–7.1	0.0–13.8	0.0	0.0–3.5	0.0–4.5
25 (19.0)	0.0–1.9	0.0–4.0	0.0–2.8	0.0–0.4	0.0–5.9	0.0–5.2	0.0–0.3	0.0–0.8	0.0–2.7
31 (23.56)	0.0–4.5	0.0–1.7	0.0–0.6	0.0–1.2	0.0–3.3	0.0–2.9	0.0	0.0	0.0–3.1
37 (28.12)	0.2–7.9	0.0–1.1	0.0–11.4	0.0–0.3	0.0–2.9	0.0–11.4	0.0–0.3	0.0–5.1	0.0–3.2
43 (32.76)	0.0–2.3	0.0–2.1	0.0–0.4	0.0	0.0–2.0	0.0–9.8	0.0	0.0	0.0–2.1
48 (36.48)	0.0–1.8	0.0–0.9	0.0–0.2	0.0	0.0–0.5	0.0–1.7	0.0	0.0	0.0–0.6
				Upwind					
1 (0.76)	1.0–22.2	0.0–7.4	0.2–8.1	0.0–55.0	0.6–15.6	0.0–42.3	0.0–72.8	0.0–22.6	0.2–30.7
7 (5.32)	0.0–2.0	0.0–7.3	0.0–0.3	0.0–2.2	0.0–0.8	0.0–12.9	0.0–8.1	0.0–4.7	0.0–4.8
13 (9.88)	0.0–0.2	0.01–0.9	0.0–0.5	0.0–7.5	0.0–0.9	0.0–3.7	0.0–15.7	0.0–0.6	0.0–3.7
19 (14.44)	0.0–0.2	0.0	0.0–0.5	0.0–1.1	0.0–0.3	0.0–6.2	0.0–9.6	0.0–0.3	0.0–2.3
25 (19.0)	0.0–0.2	0.0	N.A.	0.0–3.7 (24)	0.0–1.2 (24)	N.A.	1.0–4.4 (24)	0.0–0.5 (24)	0.2–1.5
31 (23.56)	0.0–0.3	0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0–0.2
37 (28.12)	0.0–0.6	0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0–0.3
43 (32.76)	0.0–1.3 (41)‡	0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0–0.7
48 (36.48)	N.A.	0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0
54 (40.04)	N.A.	0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0

† Site #1 was abandoned in 2002 due to crop damage by extreme drought and corn rootworm beetle.
‡ Numbers in parentheses are rows actually sampled.
N.A., not applicable.

Table 3. Cross-fertilization (mean ± standard deviation) in white maize by pollen of neighboring yellow Bt hybrid in 2000, 2001, and 2002. Rows are limited to the mid section only (Bt range). The exponential and modified exponential decline models for the data: $Y = 27.67e^{-0.4098X}$ or $Y = 28.13e^{-0.464X} + 0.52$, both models with $R^2 = 0.64$, $P < 0.01$ for downwind; $Y = 15.38e^{-0.6468X}$ or $Y = 14.37e^{-0.5139X} + 0.33$ with $R^2 = 0.58$, $P < 0.01$ for upwind directions.

Row (m) Direction	Site 1†		Site 2			Site 3			Overall mean
	2000	2001	2000	2001	2002	2000	2001	2002	
	%								
				Downwind					
1 (0.76)	43.4 ± 18.3	11.7 ± 17.8	26.3 ± 19.2	16.5 ± 13.4	38.2 ± 27.4	13.0 ± 18.2	2.1 ± 15.5	10.8 ± 26.9	19.0
7 (5.32)	7.3 ± 5.8	0.6 ± 1.4	1.5 ± 1.8	1.1 ± 1.7	3.9 ± 3.4	5.8 ± 10.3	0.1 ± 0.06	0.5 ± 0.9	2.6
13 (9.88)	1.4 ± 1.4	0.5 ± 1.0	1.5 ± 1.7	0.1 ± 0.2	1.1 ± 0.9	4.6 ± 7.5	0.3 ± 0.8	6.2 ± 19.8	2.0
19 (14.44)	0.9 ± 1.2	0.2 ± 0.9	0.5 ± 0.4	0.2 ± 0.4	1.0 ± 1.6	1.5 ± 3.5	0.0 ± 0.00	0.4 ± 1.0	0.6
25 (19.0)	0.6 ± 0.6	0.3 ± 0.8	0.3 ± 0.3	0.1 ± 0.1	1.0 ± 1.7	1.1 ± 1.7	0.01 ± 0.01	0.3 ± 0.3	0.4
31 (23.56)	1.2 ± 1.3	0.2 ± 0.5	0.1 ± 0.2	0.1 ± 0.3	0.6 ± 0.8	0.5 ± 1.0	0.00 ± 0.0	0.0 ± 0.0	0.3
37 (28.12)	1.1 ± 1.9	0.2 ± 0.4	0.1 ± 0.1	0.04 ± 0.1	0.6 ± 0.8	2.1 ± 4.2	0.01 ± 0.06	0.4 ± 1.3	0.5
43 (32.76)	0.5 ± 0.6	0.2 ± 0.5	0.1 ± 0.1	0.0 ± 0.0	0.5 ± 0.5	0.8 ± 2.5	0.0 ± 0.0	0.0 ± 0.0	0.3
48 (36.48)	0.4 ± 0.4	0.1 ± 0.2	0.0 ± 0.1	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.4	0.0 ± 10.0	0.0 ± 0.0	0.1
				Upwind					
1 (0.76)	11.3 ± 5.6	1.9 ± 2.5	2.2 ± 1.9	21.7 ± 21.8	2.9 ± 3.4	12.4 ± 14.4	18.4 ± 22.6	6.7 ± 6.2	9.7
7 (5.32)	0.3 ± 0.5	0.4 ± 1.5	0.03 ± 0.1	0.9 ± 0.7	0.2 ± 0.3	3.2 ± 4.0 (8)	1.0 ± 2.3	0.9 ± 1.3	1.3
13 (9.88)	0.04 ± 0.09	0.1 ± 0.2	0.04 ± 0.1	1.2 ± 2.1	0.1 ± 0.3	0.9 ± 1.3 (14)	2.0 ± 3.8	0.1 ± 0.2	0.7
19 (14.44)	0.04 ± 0.04	1.0 ± 0.0	07. ± 0.2	0.3 ± 0.3	0.05 ± .01	0.7 ± 1.6 (20)	1.3 ± 2.3	0.1 ± 0.1	0.3
25 (19.0)	0.01 ± 0.09	0.0 ± 0.0	N.A.§	0.7 ± 1.1 (24)	0.3 ± 0.4 (24)	N.A.	1.2 ± 1.7 (24)	0.1 ± 0.1 (24)	0.4
31 (23.56)	2.0 ± 0.0	0.0 ± 0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0
37 (28.12)	0.2 ± 10.2	0.0 ± 0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.1
43 (32.76)	0.5 ± 0.4 (41)‡	0.0 ± 0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.3
48 (36.48)	–	0.0 ± 0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0
54 (40.04)	–	0.0 ± 0.0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0

† Site #1 was abandoned in 2002 because of crop damage by extreme drought and corn rootworm.
‡ Numbers in parentheses are rows actually sampled.
§ N.A., not applicable.

neighboring yellow Bt maize exponentially declined in all site-years (Table 3).

Distance from the Pollen Source

The level of cross-fertilizations across site-years fluctuated greatly because of the wind directions, but as a rule, the farther away from the yellow Bt pollen source, the lesser was the percent out-cross (Table 3, Fig. 4). Consequently, the first row of white maize adjacent to the yellow Bt hybrid always had the highest cross-fertilizations. The extent of cross-fertilization in the subsequent rows declined exponentially to 0 or near 0% toward the edge of the field. Less than 1% cross-fertilization was found after the 37th border row (28 m) downwind from the prevailing wind direction or the 13th row (10 m) in the upwind direction in all site-years.

In the white maize rows on the straight north and south sides of the yellow Bt maize field, a considerable amount (7–15%) of out-cross was recorded, mainly within the 7.4 m (41st plants) from the pollen source with substantial differences in level of out-cross among site-years (Fig. 4). In the south side of the pollen source, as expected, the level of out-cross was greater in the first plant and then reduced exponentially in the subsequent plants. Surprisingly, an event of about 8% cross-fertilization was recorded in the 181st plant (32.6m) in the south side of Site #1 in 2000. On the north side, as high as 6 to10% out-cross was also recorded at the 31st plants (5.6m), but the out-cross was found to be 0 or almost 0% thereafter (Fig. 4). In all cases, the plant of white maize adjacent to the yellow maize pollen source always had the highest level of cross-fertilization (19–45%). Similar patterns of out-crossing were observed in 2001 and 2002 (Fig. 4), but the extent of out-cross was quite low in 2001.

Rate of cross-fertilization with distance to the pollen source was well represented by both exponential and modified exponential decline functions ($P < 0.01$) with $R^2 = 0.64$ for downwind and 0.58 for upwind (Table 3). Data of cross-fertilization in the north and south sides of yellow Bt maize represented by the Eq. [1] better than Eq. [2] on the basis of the R^2 values (Fig. 4). According to Eq. [1], the estimated zero (or 0.0001%) cross-fertilization would have occurred in the white maize population at about 30 m downwind or 23 m upwind from the pollen source (Table 3). Estimated zero (or 0.0001%) of white maize in the south and north sides of the yellow Bt hybrid had a short distance (11–19 m) from the pollen, suggesting that pollen traveled shorter distances, or cross-fertilization declined more quickly along the same row

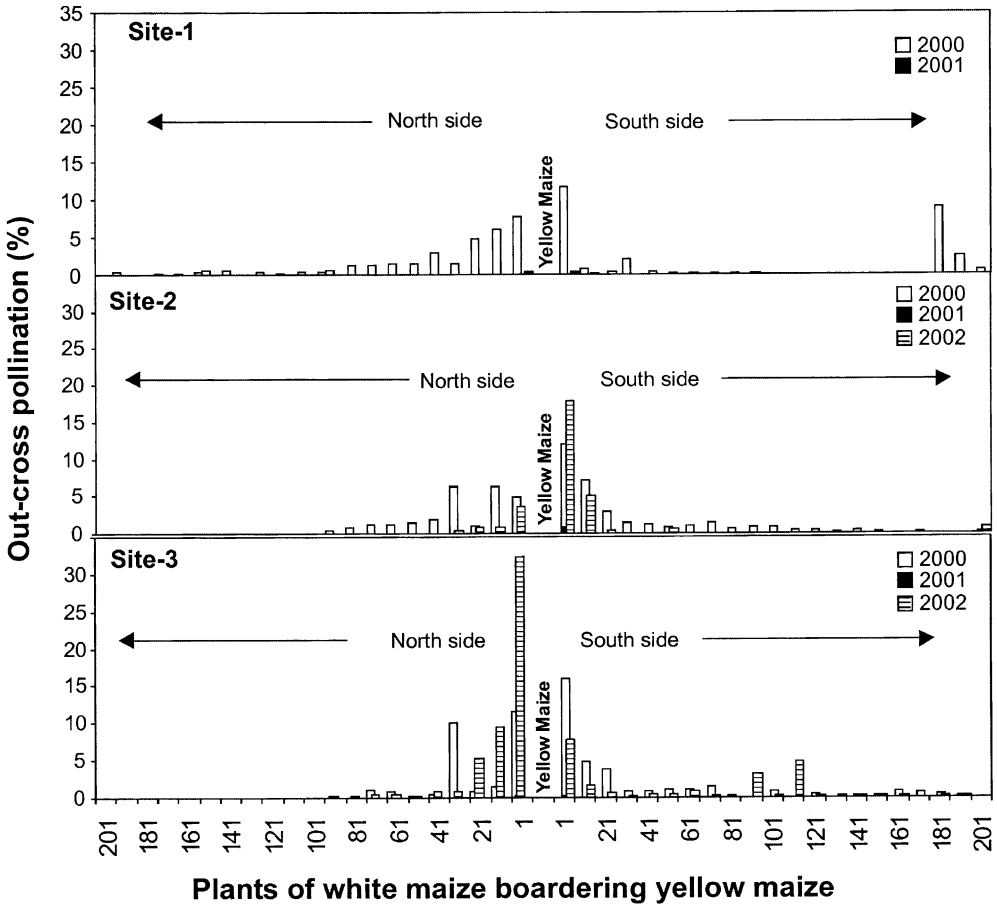

Fig. 4. Extent of cross-fertilization (Y) in the ears of every tenth plant of white maize grown in the north and south sides of the 36 rows of yellow maize in 2000, 2001, and 2002. Each bar represents the average cross-fertilization (%) of 6 white maize plants. The exponential model fits the data. Site #1 north $Y = 14.02e^{-0.63X}$, $R^2 = 0.59$; south $Y = 19.07e^{-0.81X}$, $R^2 = 0.61$. Site #2 north $Y = 18.09e^{-0.97X}$, $R^2 = 0.47$; south $Y = 35.52e^{-1.08X}$, $R^2 = 0.61$. Site #3 north $Y = 26.75e^{-0.90X}$, $R^2 = 0.66$; south $Y = 23.98e^{-1.16X}$, $R^2 = 0.56$, with $P < 0.01$ for all cases.

direction than cross row directions. In general, although the models fit the data, the R^2 values in all cases were not very large (Table 3, Fig. 4), indicating factors other than distance also played important roles in the extent of cross-fertilization.

DISCUSSION

The results of this study demonstrated that the level of cross-fertilization of white maize by pollen from neighboring yellow kernel Bt maize varied with year, site, wind direction, synchrony of flowering, and most importantly with the distance from the yellow pollen source. The majority of the out-crossing was within the adjoining rows. We suspect that if there were no wind at all, almost all pollen would have settled at the source. The limited dispersal of maize pollen away from the source is due to the fact that maize pollen grains are the largest and heaviest among those of wind-pollinated plants (Raynor et al., 1972), and pollen grains in the air have a greater tendency to settle down than to move upward and downward. In general, rate of cross-fertilization in white maize population with distance to the pollen source was well represented by both exponential and modified exponential decline functions.

The extent of pollen transfer and out-cross fertilization also depended upon the synchronization of pollen shedding of the yellow maize with the silking of the white maize, and the amount of pollen available from the yellow maize. Greater level of out-cross was expected in Sites #2 and #3 in 2002 as two yellow hybrids with different maturities were used in each site, and there was a longer period of pollen shed of the yellow kernel maize hybrids (Table 1). However, poor plant growth in size and uneven phenological progression (different silking dates) of the white maize in Site #3 of 2002 (and also 2001) was associated with the extended period of drought, which has caused asynchronous pollination between early and late appearing silks (Table 1), and thus resulted in poor kernel set and a large number of partial or even over 50% of the ear barrenness. Even if wind direction and speed are favorable for pollen dispersal, if silks are not receptive or if the air is too dry, pollen viability will be quickly lost. Therefore, synchronization of pollen dispersal and silking is very crucial in determining the extent of cross-fertilization in maize. In breeding programs, isolation in time is sometimes used to prevent cross-fertilization in seed production fields between materials concerned. For production of non-GM maize, or specialty maize, this method can also be considered.

Hybrid seed production requires close synchrony between receptive silks on the female parent and pollen shed by male parent (Westgate et al., 2003). The variation in the extent of cross-fertilization among site-years indicates that seasonal conditions influenced the level of synchrony and thus out-crossing. In 2001, hot and dry weather conditions in July and August substantially shortened the viability of pollen after shed and maturation of silking may also have been accelerated. When plants are exposed to any stress before anthesis, the time gap between male and female flowers usually lengthens (Cárcova and Otegui, 2001). Drought delayed tassel emergence, silking and grain filling (NeSmith and Ritchie, 1992), and resulted in a water deficit during the tassel and silk emergence period, which can increase the interval from silking to tasseling (Traore et al., 2000) and pollen shed (Herrero and Johnson, 1981). Prolonged drought in 2001 and 2002 seasons may have been associated with two events: (i) drought caused uneven plant growth in size and uneven phenological progress and thus led to unsynchronized flowering and reduced cross-fertilization within and among populations and kernel set and (ii) probably more importantly, drought (low moisture in the atmosphere) reduced pollen longevity. Maize pollen is susceptible to desiccation (Luna et al., 2001) and water loss in pollen grains affects the ability of pollen to germinate in stigma (Barnabas, 1984). Under normal conditions, the gradient in floret development and silk length along the ear at silking determines interval between early and late appearing silk, which results in pollination asynchrony between them (Cárcova et al., 2003). Therefore, some degree of asynchrony should be expected in an experiment involving different hybrids and varying environments. In this study, the impact of the asynchrony on the level of cross-fertilization should have been the same to or smaller for out-cross between the two populations than within a population as percent cross-fertilization was influenced by the synchronization of pollination of donor pollen with the receptive silking across two populations (Table 1) rather than synchronization within a hybrid. We assume that as a cross-pollinated crop, foreign pollen grains are favored for maize receptive silks (out-cross over 50%; Table 2) when pollen grains from both yellow and white kernel maize are available.

The approach of using yellow kernel maize as a marker of cross-fertilization in white maize (Fig. 5) has been proved to be a useful tool. The experimental results clearly showed that majority of the maize pollen grains had settled close to the source itself, and an exponential decrease in pollen dispersal was observed as the distance from the pollen source of the yellow-kernel Bt maize increased. The risk of cross-fertilization of white maize (or other non-GM maize) by pollen from neighboring yellow Bt maize was very low beyond the 37th row (28 m) from the source. However, trace amount of the yellow maize pollen dissemination may have occurred beyond the 48th row (37 m) of the white kernel maize plants; this study was not able to confirm this because of the limited field size. Nonetheless, even if some pollen had reached that distance, the likelihood of significant cross-fertilization would be very low because of the short viability of the pollen grains once shed. Our results are also in agreement with the findings of Bateman (1947), Raynor et al. (1972), Garcia et al. (1998), Luna et al. (2001), Jemison and Vayda (2001), and Halsey et al. (2002) that a sharp decline in pollen dispersal occurs as the distance from the source increases. From a practical point of view, and considering differences in planting dates from neighboring maize fields, and/or different maturity of two hybrids involved, our data suggest that it is possible to produce non-GM maize by removing the

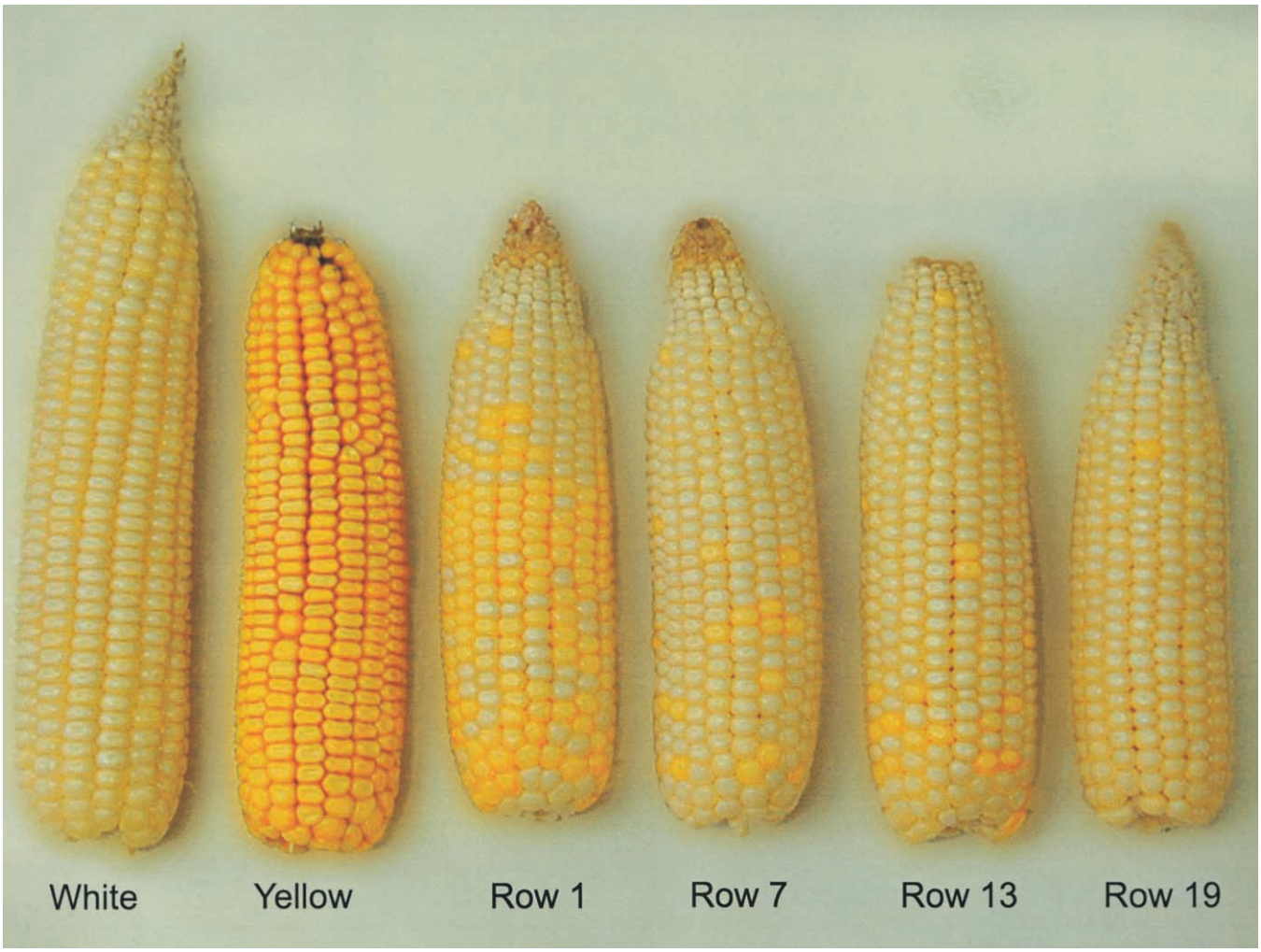

Fig. 5. Example of cross-fertilization of the ears of white maize by foreign pollen of neighboring yellow maize from the first adjoining row up to the 19th row from the yellow maize (source of pollen) as compared with the pure white and yellow maize ears.

outside rows of plants (about 30 m) adjacent to the GM maize field if the acceptance level is set at ≤1% cross-fertilization. Although the chances of cross-fertilization of white maize by pollen from neighboring yellow maize at the distance of 28 m was minimal, this study did not examine the situation in which the Bt and non-Bt maize were separated by a non-maize space. In the study reported here, the white maize rows from 1 to 37 might have also served as a physical barrier for the pollen of yellow maize in addition to compete for viable pollen from the yellow maize. A situation with a non-maize barrier pollen flow could be an area of further study on this topic.

The extent of risk from the escape of transgenes into other maize genotypes or wild relatives and non-target species through pollen dispersal is a matter of great concern. As such, there is a strong likelihood of transferring the GM traits through pollen if the flowering of the source plants and the recipients are synchronized and if they are not adequately isolated in space. It is evident from this study that whatever may be the variability in percentage of out-crossing among the eight site-years, the possibility of distance of pollen grains dispersed over 37 m (48 rows) from the source was very small. Considering the general pattern of pollen dispersal found in this study and findings by others (e.g., Garcia et al., 1998; Luna et al., 2001; Jemison and Vayda, 2001), it could be concluded that control of out-crossing in transgenic maize is possible through an appropriate isolation distance. The generally recommended distance of 200 m for maize breeders to prevent out-cross between two genotypes (inbreds, populations, hybrids, and wild relatives) is appropriate for use with Bt or other GM maize, as well. Other alternatives for the complete control of pollen dissemination may be through isolation in time or using the transgenic plants only as female that are detassel before anthesis (Garcia et al., 1998) in the routine breeding programs.

ACKNOWLEDGMENTS

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Ohio State University Extension Fact Sheet
Horticulture and Crop Sciences
2021 Coffey Road, Columbus, OH 43210
Managing "Pollen Drift" to Minimize Contamination of Non-GMO Corn, AGF-153
AGF-153-04

Peter Thomison
Department of Horticulture and Crop Science

Corn is a cross-pollinating crop in which most pollination results from pollen dispersed by wind and gravity. Pollen drift in corn has received considerable attention in recent years as the result of the development and widespread adoption of new seed technologies containing transgenes or genetically modified organisms (GMOs). Managing pollen drift has always been a major concern in the production of hybrid seed (to ensure genetic purity of inbreds) and specialty corn (to optimize expression of value-added traits, like high oil content). Pollen drift has now become an important consideration in the production of non-GMO corn as an Identity-Preserved (IP) grain crop. Producers of IP non-GMO grain are concerned that pollen drift from GMO hybrids will contaminate, by cross-pollination, nearby non-GMO corn. Farmers growing GMO hybrids approved for export also want to avoid contamination of their crops by GMO corns that have not yet received approval in overseas markets (Nielsen, 2003a).

A significant percentage of U.S. IP corn is earmarked for overseas markets with rigorous GMO restrictions. Japan has set a zero tolerance for seed and food imports containing unapproved GMO material, e.g. StarLink corn (containing the Cry9C Bt transgene); food products containing less than 5% of approved biotech crops like corn and soybeans can be labeled as non-GMO. The European Union (EU) guidelines require that foods, including grains, containing more than 0.9% biotech material be labeled as genetically engineered. Producers of non-GMO corn need to minimize pollen contamination by GMO corn if they are to obtain premiums associated with IP grain contracts. As GMO corn acreage in Ohio increases with the introduction of Bt rootworm corn and wider use of other types of Bt and Roundup Ready corn, the potential for contamination of non-GMO corn is increasing. If growers want to produce non-GMO IP corn successfully, they need to become familiar with some physical and biological characteristics of corn pollen, potential distances that pollen

can travel, and planting practices that reduce the risk of pollen contamination by nearby GMO corn fields.

Characteristics of corn pollen affecting "drift"

Corn pollen is spherical and much larger than the pollen produced by most grasses (Burris, 2002; Gray, 2003). Corn pollen is among the largest particles found in the air. Although it is readily dispersed by wind and gravity, it drifts to the earth quickly (about 1 foot/second) and normally travels relatively short distances compared to the pollen produced by other members of the grass family. Pollen may remain viable from a few hours to several days. Pollen can survive up to nine days when stored in refrigerated conditions. However, under ambient field conditions, pollen is viable for only 1 to 2 hours. High temperatures and low humidity reduce viability. Elevated temperatures have a greater negative impact on pollen viability than humidity, with viability greatly reduced at temperatures above 100 degrees F. At flowering, 60% of pollen fresh weight consists of water; pollen longevity diminishes rapidly if the water content drops below 40%. Corn plants typically shed pollen for 5 to 6 days, whereas a whole field may take 10 to 14 days to complete pollen shed, due to the natural variation in growth and development among plants (Nielsen, 2003b). Peak pollen shed generally occurs 2 to 3 days after 50% of the plants have shed pollen. Individual corn plants produce 4 to 5 million pollen grains. Therefore, even if only a small percentage of the total pollen shed by a field of corn drifts into a neighboring field, there is considerable potential for contamination through cross pollination.

How far can corn pollen travel?

Many studies have been conducted to determine how far pollen will travel ? some have evaluated the density of pollen at varying distances from a corn source, whereas others have measured pollen drift by measuring outcrossing in neighboring corn. This latter approach is probably more meaningful when it comes to assessing the impact of pollen drift from GMO corn fields.

Once released from the anthers into the atmosphere, pollen grains can travel as far as ? mile with a 15 mph wind in a couple of minutes (Nielsen, 2003b). However, most of a corn field's pollen is deposited within a short distance of the field. Past studies have shown that at a distance of 200 feet from a source of pollen, the concentration of pollen averaged only 1% compared with the pollen samples collected about 3 feet from the pollen source (Burris, 2002). The number of outcrosses

is reduced in half at a distance of 12 feet from a pollen source, and at a distance of 40 to 50 feet, the number of outcrosses is reduced by 99%. Other research has indicated that cross-pollination between corn fields could be limited to 1% or less on a whole field basis by a separation distance of 660 ft., and limited to 0.5% or less on a whole field basis by a separation distance of 984 ft. However, cross-pollination could not be limited to 0.1% consistently even with isolation distances of 1640 ft.

Several studies have been performed evaluating the impact of pollen drift from GMO fields on neighboring non-GMO fields. A Colorado study (Byrne et al. 2003) tracked the drift of pollen from blue corn and GMO Roundup Ready corn into adjacent conventional corn. Corn with marker traits (blue kernels or Roundup herbicide tolerance) was planted adjacent to corn without those traits. Cross pollination was greatest at the closest sampling site ? up to 46% outcrossing about 3 ft. from the edge of the test plots containing blue corn. Cross pollination dropped off rapidly with only 0.23% cross pollinated kernels near the blue corn plot at 150 ft. Only 0.75% of the corn showed cross-pollination with the Roundup Ready plot at 150 ft. The farthest distance any cross pollination was detected was 600 ft. These results suggest that 150 ft. may be a reasonable buffer between GMO and non-GMO corn to prevent significant cross pollination due to pollen drifting from one field to another. Planting practices to minimize GMO pollen contamination Isolation and Border Rows

One of the most effective methods for preventing pollen contamination is use of a separation or isolation distance to limit exposure of non-GMO corn fields from pollen of GMO fields. The potential for cross-pollination decreases as the distance between GMO and non-GMO corn fields increases. Several state seed certification agencies that offer IP grain programs for corn programs require that non-GMO IP corn be planted at a distance of at least 660 ft. from any GMO corn. This isolation distance requirement may be modified by removing varying numbers of non-GMO border rows, the number of which is to be determined by the acreage of the non-GMO IP corn field. The border rows ensure that the non-GMO field is "flooded" with non-GMO pollen which will dilute adventitious pollen from a GMO source.

* For corn fields over 20 acres in size, the isolation distance (of 660 ft.) may be modified by post pollination removal of 16 border rows if the actual isolation distance is less than 165 feet

* For corn fields over 20 acres in size, the isolation distance may be modified by post pollination removal of 8 border rows if the isolation distance is between 165 and 660 feet.

These isolation and border row requirements are designed to produce corn grain that is not more than 0.5% contaminated with GMOs.

Planting Dates and Hybrid Maturity

Use of different planting dates and hybrid maturities can also be used to reduce the risk of cross-pollination between fields of GMO and non-GMO corn. For example, planting short season non-GMO corn hybrids followed by full season GMO hybrids later will reduce the chance for pollen from the GMO field to fertilize the early planted, earlier maturity non-GMO hybrid in an adjacent field. However, there are shortcomings with this approach. Differences in maturity between the early and late hybrid may not be large enough to ensure that the flowering periods of each hybrid will not overlap, especially when certain climatic conditions may accelerate or delay flowering. Moreover this strategy will only work if you control the adjacent fields or can closely coordinate your corn planting operations with those of your neighbors.

Prevailing Wind Direction

In Ohio, the importance and consistency of relative wind direction during pollen shed has not been established. However, in states to the west of Ohio, the south and west edges of non-GMO fields are often more vulnerable to pollen drift because the prevailing winds during the summer are from the southwest. Therefore, it may be beneficial to follow recommendations regarding isolation distances and border row on these sides of non-GMO fields.

Other Considerations

Other factors that can negatively impact non-GMO grain purity are volunteer corn plants resulting from no-till or minimum till continuous corn, purity level of the seed planted, planting errors, and drought or flood conditions which stunt border rows and reduce desirable pollen production and flow.

Planting operations to control pollen drift are only part of the process of producing an IP corn grain crop. Other major issues include harvesting, drying and storage, along with thorough record keeping. Seed certification agencies like the Ohio Seed Improvement Association (http://www.ohseed.org/) offer IP programs for grain. These IP programs, which are similar to seed

certification, assist in preserving the genetic identity of a product, and verify specific traits through field inspections, laboratory analysis, and record keeping.

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From The Cover: Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker

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Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with *CP4 EPSPS* as a marker

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Sampling methods and results of a gene flow study are described that will be of interest to plant scientists, evolutionary biologists, ecologists, and stakeholders assessing the environmental safety of transgenic crops. This study documents gene flow on a landscape level from creeping bentgrass (*Agrostis stolonifera* L.), one of the first wind-pollinated, perennial, and highly outcrossing transgenic crops being developed for commercial use. Most of the gene flow occurred within 2 km in the direction of prevailing winds. The maximal gene flow distances observed were 21 km and 14 km in sentinel and resident plants, respectively, that were located in primarily nonagronomic habitats. The selectable marker used in these studies was the *CP4 EPSPS* gene derived from *Agrobacterium* spp. strain CP4 that encodes 5-enol-pyruvylshikimate-3-phosphate synthase and confers resistance to glyphosate herbicide. Evidence for gene flow to 75 of 138 sentinel plants of *A. stolonifera* and to 29 of 69 resident *Agrostis* plants was based on seedling progeny survival after spraying with glyphosate in greenhouse assays and positive TraitChek, PCR, and sequencing results. Additional studies are needed to determine whether introgression will occur and whether it will affect the ecological fitness of progeny or the structure of plant communities in which transgenic progeny may become established.

We developed sampling methods and describe results of a gene flow study that will be of interest to plant scientists, evolutionary biologists, ecologists, and stakeholders assessing the environmental safety of transgenic crops. Creeping bentgrass (*Agrostis stolonifera* L.) is one of the first wind-pollinated, perennial, and highly outcrossing transgenic crops being developed for commercial use. Unlike currently commercialized transgenic crops in the U.S., which have no synchronously flowering relatives in areas of commercial production, the cosmopolitan genus *A. stolonifera* has compatible relatives in a broad variety of habitats. The methods and results of using herbicide resistance as a selectable marker from a genetically modified (GM) crop to measure gene flow will be useful for assessing the potential for GM crops to transfer their novel genes to compatible relatives.

More data are available on gene flow from cultivated crops to other crops than from crops to resident (native, naturalized, or weedy) species (1). Typically, gene flow distances are reported on the scale of meters, much less often on the scale of kilometers. Maximum reported distance for gene flow between radish and wild radish (2) and between cultivated and wild sunflowers is 1,000 m (3); distances of 1,300 m have been reported between cultivated and wild squash (4). In an Australian study, crop-to-crop transfer distance of 3,000 m has been reported from source fields of nonGM herbicide-resistant canola to fields of herbicide-sensitive canola cultivars (5).

In this study, we present evidence that documents multiple instances at numerous locations of long-distance viable pollen movement from multiple source fields of GM creeping bentgrass. We used the *CP4 EPSPS* gene that encodes 5-enol-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* spp. strain CP4 as a selectable marker to track gene movement. This gene confers resistance to glyphosate (*N*-phosphono methyl-glycine), the active ingredient in RoundUp herbicide (Monsanto, St. Louis, MO). Herbicide resistance as a result of expression of the engineered *CP4 EPSPS* gene was observed in seedling progeny of sentinel *A. stolonifera* and resident *Agrostis* spp. located at distances up to 21 km and 14 km, respectively, from the crop fields. Eight source fields totaling ≈162 hectares (ha) were located on an irrigated plateau above the Deschutes River in central Oregon. The fields were contained within a 4,453-ha GM bentgrass control district (http://arcweb.sos.state.or.us/rules/OARS_600/OAR_603/603_052.html; ref. 6) located ≈144 km east of commercial nonGM bentgrass seed production areas in Oregon's Willamette Valley. When the source fields of GM creeping bentgrass flowered for the first time during the summer of 2003, they presented a unique opportunity to use the *CP4 EPSPS* gene as a marker to quantify viable GM pollen movement and potential gene flow to compatible resident and sentinel plants located in areas beyond the crop source fields. Results presented here use multiple lines of evidence based on assays of seedlings germinated from seed harvested from sentinel and resident plants. These assays include tests in greenhouse settings for survival after spraying with RoundUp and tests for presence and expression of the *CP4 EPSPS* marker.

A. stolonifera is a cool season, wind-pollinated perennial grass used on golf courses around the world (7). It also is of interest as a forage crop (8), for phytoremediation of heavy metals in soils (9), and for water quality improvement by biofiltration (10). The taxonomically uncertain genus *Agrostis* is estimated to include >200 species worldwide (11, 12). In North America, 26 species of *Agrostis* are considered native, including 14 species found in Oregon (http://plants.usda.gov). *Agrostis* is found in riparian habitats, agronomic and urban settings, mountain meadows and woodlands, coastal sand dunes, fresh and salt water marshes, ditches, pastures, grasslands, and roadsides (13, 14). The small seeds of *A. stolonifera* (up to 6×10^6 per pound) are readily dispersed by wind, water, and animals (13, 15). Introduced and widespread in the U.S., *A. stolonifera* is sometimes considered an economic weed, e.g., as a volunteer in grass seed or other agronomic production fields and as a colonizer of nonagricultural habitats; it has been reported as weedy in Japan, Australia, New Zealand, Chile, Germany, Denmark, the United Kingdom, and Canada (16).

A. stolonifera is generally considered to be an obligate outcrosser (17); however, self-fertility also has been reported (18).

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Abbreviations: GM, genetically modified; ha, hectare.

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The species is most typically an allotetraploid (19, 20) and has cytotypes of higher ploidy (21). Naturally forming interspecific F_1 hybrids generally are low in fertility or sterile; in favorable habitats, some hybrids (e.g., F_1 hybrids of *A. stolonifera* and *Agrostis capillaris* L.) have been reported to out-compete both parents (22). There are few clear examples of F_2 hybrids (23) or of backcrosses of F_1 hybrids to a parental species (18). Although native or naturalized hybrids may be sterile, they can constitute a significant component of plant communities because of vegetative spread by means of stolons (24).

Field studies of hybridization between *A. stolonifera* and other species of *Agrostis* or between *A. stolonifera* and closely related *Polypogon* spp. (18, 25, 26) have produced similar findings on outcrossing ability. In a field study that included several hundred plants as sources of pollen from bentgrass engineered to be resistant to glufosinate herbicide, a gene flow distance of 298 m was reported (25). Natural hybrids of *A. stolonifera* have been reported with six other native species.: *Agrostis canina* L., *A. capillaris* L., *Agrostis castellana* Boissier and Reuter, *Agrostis gigantea* Roth, *Agrostis mertensii* Trinius, and *Agrostis vinealis* Schreber (www.essentialbiosafety.info/docroot/articles/02-281-009.pdf). A computer model (27) found that pollen dispersal and gene introgression would be limited at some sites and extensive at others, depending on local wind conditions.

Materials and Methods

Sampling Design. A sampling grid (Fig. 1) was designed to determine the extent of viable GM pollen flow based on the temporary deployment of 178 compatible *A. stolonifera* sentinel plants and the monitoring of naturally occurring compatible resident plants. Critical assumptions in the sampling design included a maximal pollen viability of up to 3 h (28) and prevailing winds of 10 km/h from the north and northwest (data are from the Pacific Northwest Cooperative Agricultural Weather Network weather data archive, see www.usbr.gov/pn/agrimet/) during the expected period and hours of anthesis (e.g., mid-June to early July, from 11 a.m. to 2 p.m.) of the source GM creeping bentgrass crop fields. Thirty locations with resident plants of *A. stolonifera,* 39 locations with resident plants of *A. gigantea*, and 10 locations with resident plants of *Polypogon monspeliensis* (L.) Desfontaines also were included in the study. Plants of *A. stolonifera* (experimental population no. 1 CRBP, Seed Research of Oregon, Corvallis, OR) cultivated in a field in the Willamette Valley of western Oregon were transplanted to 23-cm diameter pots and used as sentinel plants. Before their transport to central Oregon, each of the sentinel plants tested negative for *CP4 EPSPS* by the TraitChek immunological lateral flow test strip method (Strategic Diagnostics, Newark, DE). Each of the 69 resident *Agrostis* plants and the 10 *P. monspeliensis* resident plants were tested by using the TraitChek method to ensure that they were negative for the *CP4 EPSPS* protein that confers resistance to glyphosate. In mid-June, sentinel plants were deployed to field positions at times of day when anthesis from the source fields was considered unlikely (i.e., before 8 a.m. and after 6 p.m.). Additional steps taken to minimize incidental pollination of sentinel plants included bagging each plant during transit and distribution of the plants by geographic sector. Within sectors, the first plants that were put in place were those at the greatest distance from the perimeter of the control district; the last plants that were placed within a sector were those closest to the control district perimeter. In mid-July, after anthesis in the source fields had ended, panicles were bagged in the field. Bagged sentinel plants with bagged panicles and bagged panicles from resident populations were collected several weeks later. These measures allowed for *in situ* seed fill and for temporal separation with seed harvesting activities on the GM bentgrass fields. An additional precaution taken to prevent dissemination of any potentially transgenic F_1 seedling progeny from the field collections was the use of sealed boxes to transport the doubly bagged sentinel plants and the bagged resident plant panicles during their transport to greenhouses.

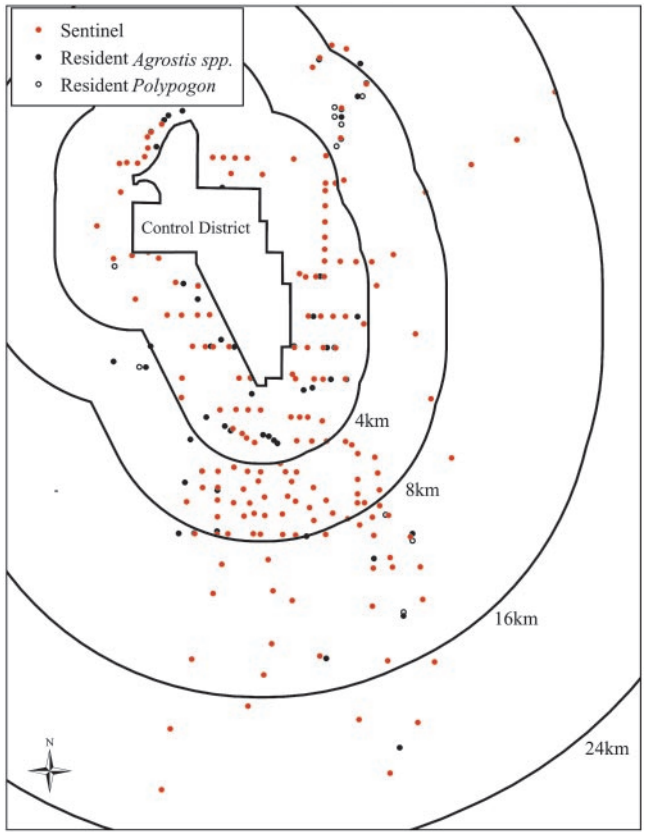

Fig. 1. Sampling design to determine gene flow from source fields within the control district to potentially compatible plants outside the control district. A total of 178 sentinel *A. stolonifera* plants (red circles) were placed outside the control district (6) near accessible public roads spaced 1.6 km apart in the north–south direction and 0.8 km apart in the east–west direction. Given a prevailing wind of 10 km/h from the north or northwest, 76 sentinel plants were located downwind from the control district in a 9.6-km-wide by 3.2-km-deep grid with ≈0.8-km spacing. Remaining sentinel plants were placed at 1.6-km intervals for the next 4.8 km and 3.2-km intervals for the next 6–10 km out to a distance of 16–21 km along six transects corresponding to major highways. In addition to the sentinel plants, 69 compatible resident *Agrostis* plants (black circles) of *A. stolonifera* and *A. gigantea*, plus 10 *P. monspeliensis* (open circles) located primarily along waterways and in moist soils, were included in the study.

Greenhouse Assays. Seeds harvested from sentinel and resident plants were chilled at 5°C for 7–10 days in moist sand and grown in trays of a peat-based potting medium (Seedling Mix no. 1, OBC Northwest, Canby, OR) in the greenhouse until the two-leaf stage and then sprayed with the field rate (2.3 liters/ha) of RoundUp herbicide by using a track sprayer (model RC-500-100-EP, Mandel, Guelph, ON, Canada). Seedlings that survived the initial spraying with the field rate of RoundUp or emerged after the spray event were subjected to spraying with herbicide at twice the field rate (4.6 liters/ha) ≈2 weeks later. Survivors of the second cycle of herbicide spraying identified as presumptive positives were confirmed by means of the TraitChek test.

Molecular Characterization. DNeasy Plant Mini kits (Qiagen, Valencia, CA) were used to extract genomic DNA from leaves of seedling progeny derived from 130 sentinel and 45 resident plants that were both herbicide resistant and TraitChek-positive for *CP4 EPSPS*. Primers for amplification and sequencing of a

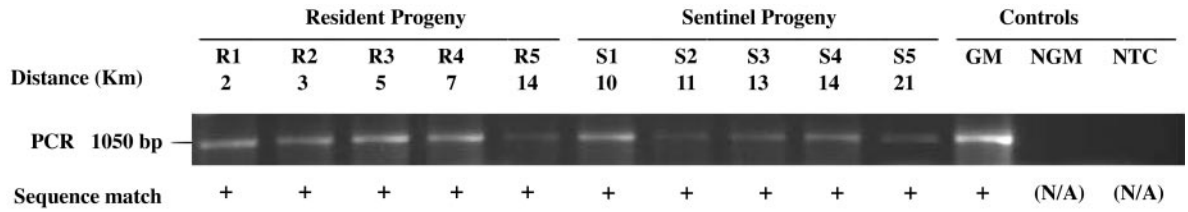

Fig. 2. Molecular confirmation of the presence of the engineered *CP4 EPSPS* herbicide-resistance gene. The presence of the *CP4 EPSPS* gene as verified in a subsample of TraitChek-positive progeny from resident (R1–R5) and sentinel (S1–S5) plants located at various distances from the control district perimeter. All PCR products had the same size and DNA sequence as that amplified from the GM-positive control (*A. stolonifera*, designated event ASR368). BLASTN searches (29) revealed that the DNA sequences also matched GenBank accessions AF464188.1, *Glycine max CP4 EPSPS* (score = 1,271, $E = 0.0$), and AY125353.1, a synthetic *CP4 EPSPS* construct. Negative controls included DNA from nonGM (NGM) *A. stolonifera*, variant Penncross, and a nontemplate control (NTC). +, positive sequence matches; N/A, not applicable.

1,050-bp segment of the *A. stolonifera CP4 EPSPS* coding region were designed with PrimerSelect (DNASTAR, Madison, WI) based on *Glycine max* (L.) Merr. *CP4 EPSPS* (GenBank accession no. AF464188.1). Amplifications with P217F (5′-ACTATGGGCCTCGTCGGGGTCTA-3′) and P218R (5′-GGCAGCCTTCGTATCGGAGAG-3′) were conducted for 40 cycles of 94°C for 30 sec, 64°C for 30 sec, and 72°C for 90 sec. PCR products were purified with QIAquick Gel Extraction kits (Qiagen). Cycle-sequencing reactions used BigDye v3.1 chemistry and the standard thermal profile suggested by the manufacturer (Applied Biosystems). Labeled fragments were purified with CleanSeq kits (AgenCourt Bioscience, Beverly, MA). Sequence data were collected on a Prism 3100 Genetic Analyzer (Applied Biosystems). Sequences were then compared with matching GenBank accessions by using BLASTN searches (29).

Statistical Analyses. The percentage of positive seedling progeny was calculated as the number of seedlings that survived two sprays with RoundUp and had positive TraitChek tests for the *CP4 EPSPS* gene divided by the total estimated number of seedlings germinated in the greenhouse. Maximum likelihood estimation was used to fit the two-parameter gamma distribution (30), $f(x) = x^{(\alpha - 1)} \exp(-x/\beta)/(\Gamma(\alpha)\beta^{\alpha})$, where α and β are the model parameters and $\Gamma(\cdot)$ is a complete gamma function, to the observed distances from the control district perimeter at which positive seedling progeny were found. The adequacy of the gamma distribution was tested by using the one-sample Kolmogorov–Smirnov test (31, 32). The two-sample Kolmogorov–Smirnov test (33) was used to compare the probability distributions of the positive seedling progeny of sentinel and resident plants. Nonparametric kernel smoothing (34) was applied to percent positive seedling progeny to generate spatial maps of gene flow transfer for sentinel and resident plants separately. The estimation and hypothesis testing of the gamma distributions were performed by using S-PLUS v6.01 (Mathsoft, Cambridge, MA). Kernel smoothing and spatial maps were undertaken by using ARCMAP v8.3 and the ARCGIS SPATIAL ANALYST 8.3 (Environmental Systems Research Institute, Redlands, CA).

Results

Gene Flow to Sentinel and Resident Agrostis. Molecular analyses by PCR (see Fig. 2) and by sequencing (sequence data not shown) confirmed the presence of the *CP4 EPSPS* marker in seedling progeny that had survived two cycles of spraying with RoundUp herbicide. The sequence matched that of GenBank accession AF464188.1 for a *CP4 EPSPS* construct in glyphosate-resistant soybean (*G. max*). The highest relative frequencies of pollen-mediated gene flow to *A. stolonifera* sentinel and *A. stolonifera* and *A. gigantea* resident plants were observed within 2 km of the control district perimeter. Maximal distances at which gene flow was observed in sentinel and resident *A. stolonifera* and resident *A. gigantea* plants were ≈21 km, 8 km, and 14 km, respectively. Viable pollen dissemination distances for sentinel plants may be biased low because this distance of 21 km represented the limit of the sampling design (Fig. 1). An additional source of bias is that distances from source fields to the control district perimeter were unknown. Based on the one-sample Kolmogorov–Smirnov goodness-of-fit test, the empirical distribution of minimum distances of the 75 positive sentinel *A. stolonifera* plant locations (Fig. 3*A*) was adequately described by a gamma distribution with $\alpha = 0.93$ and $\beta = 4.1$ ($P = 0.67$); that of 16 positive resident *A. stolonifera* plant locations (Fig. 3*B*) was adequately described by a gamma distribution with $\alpha = 0.74$ and $\beta = 4.0$ ($P = 0.77$); and that of 13 positive resident *A. gigantea* plant locations (Fig. 3*C*) was adequately described by a gamma distribution with $\alpha = 0.74$

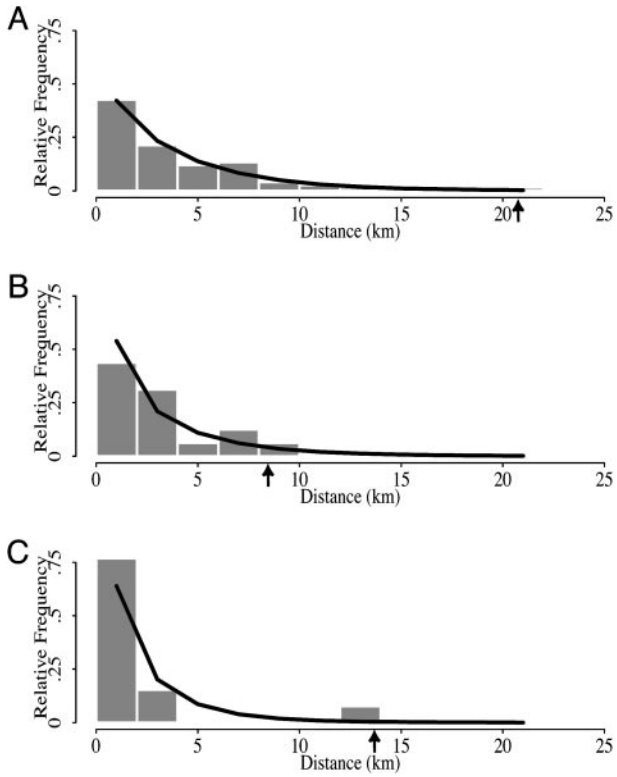

Fig. 3. Skewed distribution of GM bentgrass pollen-mediated gene flow to sentinel and resident plants in 2003. Based on the presence and expression of the *CP4 EPSPS* gene for herbicide resistance, relative frequencies of gene flow among sentinel and resident plant seedling progeny were highest within the first 2 km from the perimeter of the control district and decreased with distance. Arrows depict maximal gene flow distances that were observed. *A*, *B*, and *C* represent locations of sentinel *A. stolonifera*, resident *A. stolonifera*, and resident *A. gigantea* plants, respectively.

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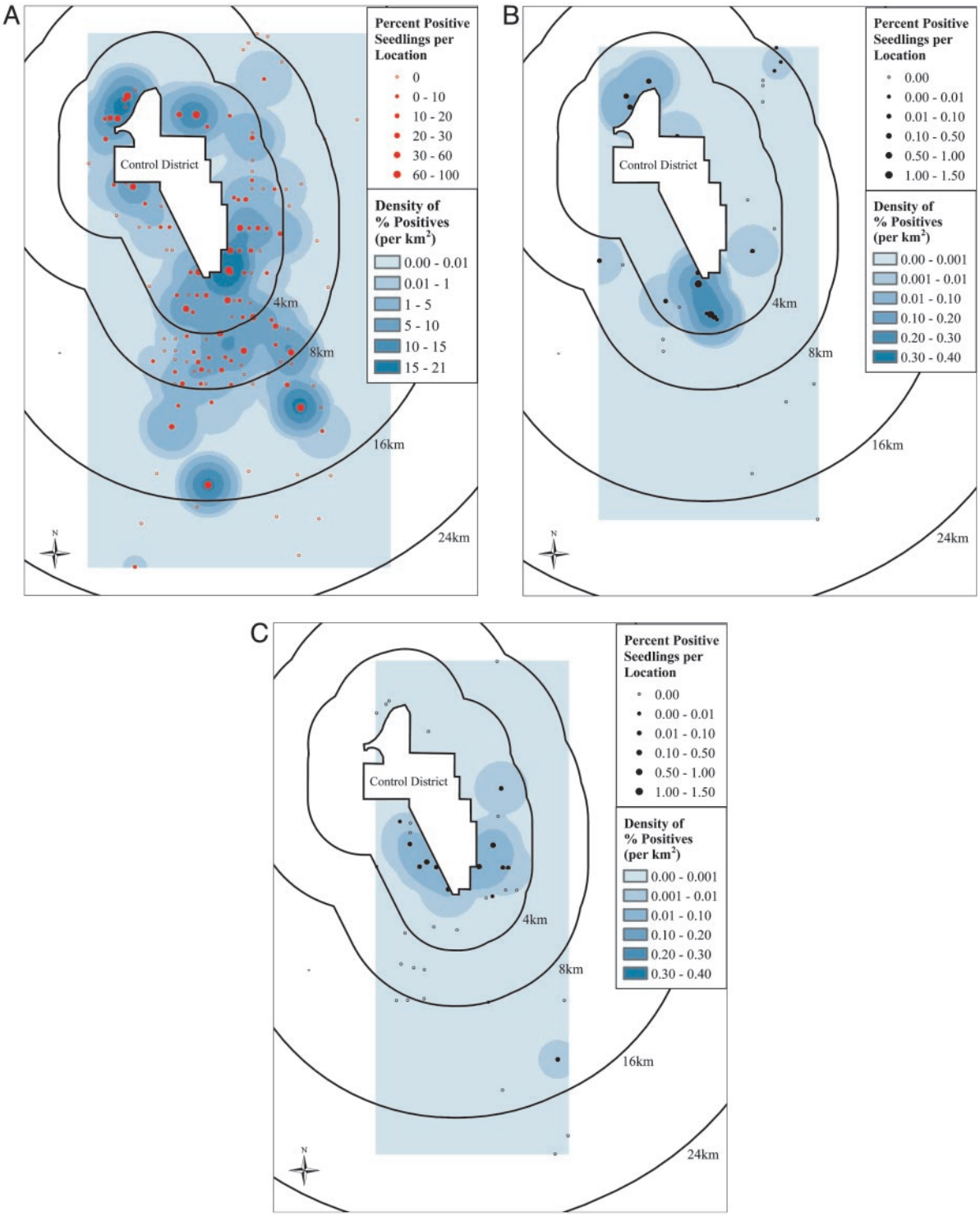

Fig. 4. Prevalence of gene flow based on percent positive seedling progeny of sentinel and resident plants at various distances from the control district perimeter. Kernel smoothing (34) was applied to percent positive seedling progeny (filled circles) of sentinel *A. stolonifera* plants (*A*), resident *A. stolonifera* plants (*B*), and resident *A. gigantea* plants (*C*) to generate spatial maps of the density of percentage positives. Open circles indicate locations where no positive seedling progeny were found. The highest densities of percent positive seedling progeny of sentinel and resident *A. stolonifera* plants occurred southeast and/or due south of the perimeter of the control district, in the direction of the prevailing winds.

and $\beta = 2.8$ ($P = 0.42$). The mean ($\alpha\beta$) and variance ($\alpha\beta^2$) of a gamma distribution decrease monotonically with respect to α and β. Consequently, higher α and β values indicate density distributions of viable pollen that hybridized with sentinel or resident plants that were spread farther from source fields. The gamma distributions for sentinel and resident *A. stolonifera* locations were not significantly different at the 0.05 level based on the two-sample Kolmogorov–Smirnov test ($P = 0.63$) but were significantly different from that for resident *A. gigantea* locations ($P = 0.031$ and 0.047, respectively). The mean distance

Table 1. Prevalence and incidence of *CP4 EPSPS*-positive plants and seedling progeny

Species	Plants with positive seedling progeny,* %	No. tested†	No. positive seedling progeny (%)
Sentinel *A. stolonifera*	54 (75/138)	32,000	625 (2.00)
Resident *A. stolonifera*	53 (16/30)	565,000	157 (0.03)
Resident *A. gigantea*	33 (13/39)	397,000	159 (0.04)
Resident *P. monspeliensis*	0 (0/10)	190,000	0 (0.00)

*Values in parentheses represent the ratio of plants with positive seedling progeny to the total number of plants.
†Number of seedling progeny tested in greenhouse.

from the perimeter of the control district ranged from 2.1 km for resident *A. gigantea* plant locations to 3.8 km for sentinel plant locations.

Spatial Patterns of Gene Flow. For both sentinel and resident *A. stolonifera* plants, the greatest spatial density of percent positive seedlings was found southeast and south of the control district in the direction of prevailing winds (Fig. 4 *A* and *B*). Positive seedlings derived from resident *A. gigantea* were found primarily east and west of the southern portion of the control district (Fig. 4*C*). In addition, some *CP4 EPSPS*-positive *A. stolonifera* seedling progeny were obtained from seeds harvested from plants near and below the northwest section of the control district perimeter (Fig. 4 *A* and *B*). This finding may be due to localized temperature gradients and wind conditions near the rim of the Deschutes River canyon, which brought viable pollen down to the canyon floor. In comparison, the percentage of positive sentinel plants was about an order of magnitude higher than that for resident plants.

Resident *Agrostis* typically were found in moist soils, e.g., riparian areas and along irrigation or drainage ditches. Most of the *A. stolonifera* resident plants with positive seedling progeny were located in sagebrush steppe or other nonagronomic land use areas (50% and 25%, respectively), whereas the majority (78%) of positive *A. gigantea* plants were located in agronomic production areas. Forty of 178 sentinel plants were lost to various causes, e.g., transplant shock and grazing. As shown in Table 1, hundreds of *CP4 EPSPS*-positive seedling progeny were found among *A. stolonifera* sentinel and resident plants and *A. gigantea* residents.

Discussion

Our multiple lines of evidence from greenhouse and laboratory tests document movement of viable GM creeping bentgrass pollen on a landscape level that encompassed ≈310 km^2. The gene flow evidence presented here contrasts quantitatively with previous studies with *A. stolonifera* (18, 25, 26) with significantly higher numbers of occurrences and maximally observed linear distances. The higher number of observed occurrences may reflect greater total acreage of source fields in this study (162 ha) as compared with much smaller experimental field plots of previously reported studies with *Agrostis* in which only several hundreds of plants served as pollen donors. The long period of flowering (estimated at 4–5 weeks rather than a more typical flowering period of 2–3 weeks for creeping bentgrass in the Willamette Valley), may have been due to asynchronous flowering of GM crop source fields. Potential causes of floral asynchrony include differences in cultivars, soil characteristics, and microclimates among source fields. The long gene flow distances we observed may, in part, reflect our sampling design, which purposefully looked at a range of distances in directions guided by historic information on prevailing winds (www.usbr.gov/pn/agrimet) as well as a 3-h window of assumed pollen viability (28). Our landscape level sampling design was distinct from "wagon-wheel" designs typically used for gene flow determinations in agronomic settings; i.e., with regard to its geographic scale of several hundreds of kilometers-squared rather than linear meters, in the broad variety of nonagronomic as well as agronomic habitats that it encompassed, and in the use of both sentinel and resident plants.

Lower frequencies of gene flow observed in resident *Agrostis* as compared with sentinel plants are likely primarily due to initiation of flowering of resident plants 2–3 weeks later than crop source fields. Pollen competition, i.e., pollen loads in the vicinity of patches of resident plants were higher than around individual sentinel plants, may also have reduced the relative availability of stigma sites and GM pollen in resident plants. Diverse factors (35) may have resulted in our lack of observations of gene flow to *P. monspeliensis* resident plants; two reasons we consider most likely are flowering of *P. monspeliensis* residents 2–3 weeks later than the bentgrass fields and their generally upwind locations.

Our results clearly document pollen movement and gene flow from large source populations of GM creeping bentgrass into much smaller numbers of resident *Agrostis* plants and individual sentinel plants of *A. stolonifera*. Conceivably, gene flow to resident plants from small-scale field trials of GM creeping bentgrass initiated within the control district before 2003 (www.agcomm.ads.orst.edu/agcomwebfile/edmat/html/sr/sr1046.9htm; ref. 36), e.g., by wind-dispersed pollen or seeds, may have contributed to the observations we report here. However, all tests done to date on leaf and panicle tissue samples of resident plants that produced *CP4 EPSPS*-positive seedling progeny in our greenhouse assays have proven negative for the marker. Efforts will continue over the next few years to identify potential establishment and recruitment of resident *Agrostis* that express the *CP4 EPSPS* marker. More detailed molecular analyses of positive seedlings and of maternal or paternal crop or resident plant parents are planned to distinguish hybridization events between GM crop and resident plants from GM crop seed dispersal. Multiyear sampling to monitor potential introgression of the *CP4 EPSPS* marker into resident populations and for potential effects on plant community structure and the ecological fitness of progeny also is planned.

In competitor–stress tolerator–ruderal characterization of plant functional types (13, 37), *A. stolonifera* is considered to have both competitive and ruderal features; thus, its invasive root and stolon growth can contribute to weediness, and new plants can be established either by seeds or by dispersal of stolon pieces (13, 15, 38, 39). The particular engineered trait for herbicide resistance (*CP4 EPSPS*) that we used as a selectable marker would not be anticipated *per se* to confer a selective advantage in the absence of herbicide selective pressure. However, in areas where weed control or restoration efforts are being practiced, hybrid *Agrostis* progeny resistant to glyphosate herbicide might be expected to have a selective advantage. Further studies should continue over the next few years within resident plant populations to monitor for introgression, spread, or extinction of the engineered *CP4 EPSPS* gene, and for potential effects on ecological fitness of progeny and plant community structure in various, largely nonagronomic habitats.

Biological confinement strategies (e.g., male sterility, gene insertion into organelles or into targeted chromosomes or chromosome sites) are of interest to try to restrict gene flow; however, recent reports (40, 41) suggest that gene leakiness may make fully effective, long-term containment of transgenes unlikely. Studies, such as the one reported here, that use both

sentinel- and resident-compatible plants in an appropriately large sampling design that includes nonagronomic and agronomic habitats may be useful to quantify potential rates of gene exchange between GM or conventional crops and nonagricultural resident plants when conducting assessments of ecological risks (35) and evaluating potential mitigation technologies (41). Similar approaches could be used to develop sampling designs to test for potential long-distance wind dispersal of GM seeds. Our methods and findings contribute significantly to the ongoing discussion about potential risks of gene flow from GM crops and thus are anticipated to be of interest to plant scientists, evolutionary biologists, ecologists, policy makers, and regulators.

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