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COULD BT-ENGINEERED PEST RESISTANT TREES BE HAZARDOUS TO AQUATIC INVERTEBRATES?

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Abstract.--Bacillus thuringiensis, commonly known as Bt, is a naturally occurring bacterium that has been used as a commercial pesticide for over 65 years. Its pesticidal properties are derived from alkali-soluble crystal proteins produced in its dormant spores. A traditional formulation of Bt var. *kurstaki* (Btk) containing dried bacteria and spores is sprayed on forests to control defoliation by Lepidopteran and Coleopteran larvae but persistence of the traditional formulation is limited. Toxicity testing of Btk products has shown minimal activity against non-target organisms, including aquatic invertebrates, but questions are raised about the validity of the tests. In recent years, many Bt genes coding for toxins have been identified, cloned, and inserted into the chromosomes of trees, possibly increasing Bt's toxicity spectrum and certainly increasing pesticidal persistence. Results of toxicity testing for traditional Bt formulations are being applied to genetically engineered trees. A literature review and consultation with experts suggest that they should not be. Aquatic ecosystems, including fish populations, could be altered by exposure to shed leaves of transgenic trees. Toxicity of leaves from bioengineered trees to aquatic invertebrates and other non-target organisms should be determined before large scale commercialization is approved.

Bacillus thuringiensis (Bt) is a bacterium that is used to kill pest insects in forested, agricultural, and urban areas. The organism was first isolated from diseased silkworm larvae in Japan in 1901 (Swadener 1994) and named *Bacillus thuringiensis* in 1911. Bt was first used commercially in France in 1938 and in the United States in 1958 against Lepidopteran larvae (Melin and Cozzi 1990). Perhaps as many as 50,000 isolates representing several thousand unique strains have been identified (Peferoen 1997).

When conditions for bacterial growth are suboptimal, Bt organisms form dormant spores containing crystalline proteins that are toxic to certain insects when ingested. By 1989, commercial formulations containing dried spores and toxin crystals had garnered 90-95% of the biopesticide market (Swadener 1994). The toxin crystals are only soluble in alkaline solutions and gut alkalinity of the target insect is a component of the insecticide's specificity. If the crystal dissolves in the insect gut, digestive enzymes break the protein into protoxins that are activated by a protease to form toxic compounds called δ-endotoxins that bind to specific receptors in the midgut (Adang 1991). After binding, ATP levels decrease and 78% of the potassium ion transport is inhibited within 10 minutes. Midgut cells then imbibe water, burst, and form lesions (Reichelderfer 1991). The insect larva ceases

feeding and eventually starves to death (Swadener 1994).

The advent of transgenic plants in 1982 (Fraley et al. 1988) introduced a new method of utilizing the insecticidal properties of Bt, overcoming the drawbacks associated with commercial Bt products. Traditional Bt insecticides are applied to foliage by dusting or spraying aqueous suspensions to control Coleopteran and Lepidopteran insects. The toxin is photo labile, limiting its persistence (Kleiner et al 1995; Ellis and Raffa 1997). In addition, traditional Bt formulations must be applied to coincide with the susceptible life stage of the pest insect (Lewis et al. 1974; Andreadis et al. 1983; Kleiner et al. 1995). The first Bt crystal protein (Bt Cry protein) encoding genes were cloned in 1985 (Adang et al. 1985; Schnepf et al. 1985; Peferoen 1997) and incorporated into plants in 1987 (Adang et al. 1987; Barton et al. 1987; Vaeck et al. 1987). McCown et al. (1991) reported successful incorporation of Bt genes in poplar trees (*Populus sp.*) in 1991 and Kleiner et al. (1995) confirmed that new leaves were toxic to target organisms after winter dormancy. Several other tree species have been engineered to include Bt encoding genes since 1991 (Peña and Seguin 2001). Of the 54 known toxin-producing genes, 24 are known to be toxic to Lepidoptera. 15 to Coleoptera (two to both), five to Diptera, and six to Nematoda (Peferoen 1997). Six genes produce toxins with unknown insect toxicology.

Reports of possible toxicity to non-target organisms (NTO's) from commercial Bt formulations suggest possible hazards to aquatic insects from transgenic trees. Eidt (1985) reported a sharp decrease in survival of stoneflies (Plecoptera) in all treatment doses of Bt var. *kurstaki* (Btk) after 12 d but concluded that the insecticide was not responsible for the mortalities because there was no significant dose-effect and because 12 d was longer than the expected response interval of 3-4 d (Van Frankenhuyzen 1990). Kreutzweiser et al. (1992) reported 30% mortality of the Plecoptera species *Taeniopteryx nivalis*, one of twelve non-target species exposed to the high dose treatment of 600 IU (international units)/ml of Btk. They concluded, however, that hazard to non-targets was minimal

because concentrations that high would never occur in a normal application. Similarly, unexpected mortality of a Heptageniid mayfly from a high dose of Bt var. *israelensis* (Bti) was reported by Wipfli and Merritt (1994), but again, its significance was minimized because of the extraordinarily high dosage. If Bt transgenic trees are planted near streams or lakes and the leaves are toxic to aquatic invertebrates, fish communities would be negatively affected.

Sub lethal effects of Bt engineered crops and traditional Bt formulations on NTO's have also been reported. In studies of the toxicity of Bt engineered corn pollen to monarch butterflies, Sears et al. (2001) reported that "...growth inhibition (and mortality) would likely occur within or near corn fields..." when monarch instars consumed milkweed leaves containing pollen from Bt engineered corn. Eidt (1985), experimenting with a traditional formulation of Btk, reported no emergence of a Tanytarsid mayfly at a dosage of 430 IU/ml, a dose higher than normally applied to control insects. Richardson and Perrin (1994) reported that Btk-treated leaf packs with adsorbed spores had significantly less reduction in mass than controls in feeding experiments, and suggested that the insects avoided consuming the treated leaves. They also reported marginally elevated drift rates of a Baetid mayfly. Pistrang and Burger (1984) reported a 14 fold increase in drift of *Epeorus fragilis* and a 29 fold increase in drift of *Baetis brunneicolor*, McDonnough (both species are Ephemeropterans) following a Bti exposure of 10 ppm, a dose typically used for black fly (Diptera sp.) control. Wipfli and Merritt (1994) reported increased drift rates of Perlid stoneflies from high test doses (100 ppm) of Bti, an insecticide reportedly only toxic to Dipterans. Jackson et al. (1994) reported dramatic reductions in densities and increases in drift of the aquatic moth *Petrophila* sp, after application of Bti to control black flies. By compromising the integrity of the gut lining, sub lethal effects of Bt could disrupt aquatic food webs by leaving immature insects susceptible to secondary infections, reducing growth rates, vigor, and fitness (Loszy et al. 2002). Sub lethal effects could even exceed the effects of direct mortality.

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Although terrestrial Lepidopterans and Coleopterans are the most likely target pests, it should not be assumed, based on results from past studies using traditional Bt formulations, that leaves containing engineered toxins are not toxic to aquatic instars of NTO's. In many reviewed studies, when mortalities of aquatic NTO's were observed, the null hypothesis was not rejected because of conflicting evidence, or the biological significance was minimized because the dosages greatly exceeded those in a normal insecticide applications. In reviewed literature, consumption of the toxin was assumed but never verified. Many of the insects studied were grazers and shredders that may not have ingested Bt spores and crystals that were simply introduced into their aquatic environment. Toxin specificity due to its alkali-solubility is often cited to dismiss concern, however, the solubility of transgenic product is not necessarily the same as the native protein (B. Oppert, U. S. Department of Agriculture, personal communication.). Traditional Bt formulations contain pro-toxins that are converted to toxic δ-endotoxins in the insect gut. Genetic engineers can truncate and activate Bt genes that are inserted into tree genomes so that they directly code for the δ-endotoxins, which could increase the toxicity spectrum of some protein families (D. Andow, University of Minnesota, personal communication). Finally, the U. S. Environmental Protection Agency (USEPA) approval of Bt products as safe for aquatic organisms has been based on toxicology tests utilizing the water flea *Daphnia magna* as the test organism (USEPA 2001). Daphnia may not be a good surrogate species for aquatic insects because of Bt's specificity in traditional formulations and because reasonable doubts exist that the toxin can be eaten by the organism as they are exposed to it (Greenpeace International 2000).

Conclusions about the low risk of genetically engineered food crops to NTO's should not be applied to transgenic trees. Referring to previously registered food crops, the USEPA has concluded, "In general, the reviewed publications, recent research data, and information submitted as a result of the data call-in (DCI) provide a weight of evidence assessment indicating no unreasonable adverse effects of Bt Cry proteins expressed in plants to non-target wildlife or beneficial invertebrates, ..."[from USEPA 2001]. This conclusion was based on the known toxicity spectrum of the pesticide, bioassays using *Daphnia magna* as a test organism, and on the assumption that exposure to aquatic invertebrates is minimal, a justifiable argument for food crops but not for trees. In small headwater streams, leaf litter and their decay provide most of the stream's energy (Murphy and Meehan 1991).

Tree species of the genus *Populus* (poplars), a ubiquitous group, "...have emerged as a model organism for forest biotechnology, and genetic modification..." [from Strauss et al. 2001]. Transgenic poplars engineered for pest control are not yet widely distributed in the United States; however, their distribution is expected to increase. To date, transgenic poplars have only been planted in isolated test plots. On 13 April, 2005, no applications for field tests were listed as currently in effect on the Information Systems for Biotechnology web site (ISB 2005). However, demand for wood products is growing (Strauss et al. 2001), and intensified forest management and tree plantations will likely increase to meet the need. If Bt engineered trees are planted in riparian areas, leaves containing viable Bt toxins could be consumed by aquatic invertebrates with unpredictable consequences.

Published literature suggests that environmental risks are of concern to scientists developing the bioengineered trees (Raffa et al. 1997; James et al. 1998; Strauss 1999) and many academic scientists entered the field because of its potential environmental benefits. Discussion of risk assessment is well represented in the literature (Raffa et al. 1997; Mullin and Bertrand 1998; Pascher and Gollmann 1999) but despite numerous personal contacts, including experts at Oregon State University, Animal and Plant Health Inspection Service, USEPA, USDA, and several other universities, I was unable to find any peer reviewed studies or any scientists working to directly assess the risks of genetically modified trees to aquatic invertebrates. Many questions must be answered before commercialization of Bt engineered trees to insure the safety of aquatic ecosystems. The first question that should be answered: Are the engi-

neered toxins persistent in the environment? Steve Strauss, a co-developer of Bt engineered poplar, hypothesized that enzymes degrade the toxins prior to leaf fall (Oregon State University, personal communication). If true, then the Bt toxins are not a threat to aquatic invertebrates and no further toxicological work is necessary. However, Hay et al. (2002) found that fragments of recombinant plant marker genes were detectable in decaying terrestrially deposited leaves for up to four months. Leaves that are deposited in the water can take up to a year to decay (Gregory 1992). Do the toxins degrade more quickly than the leaves themselves as hypothesized by Dr. Zigfridas Vaitusis (USEPA, personal communication)? If so, then it must be determined if the decay is "quick enough" to avoid consumption of harmful quantities of toxin by aquatic invertebrates. How does the altered solubility of engineered proteins affect toxin specificity? If the toxins persist, how does the continuous exposure to NTO's alter their toxicology? What are the long-term consequences of sublethal effects? How extensive are food web effects? Does pollen from engineered trees pass toxin-producing genes on to hybrids that would eventually replace mature trees in non-engineered riparian buffers? What are the toxicity spectra of truncated and activated proteins?

Advocates and critics agree that more assessments of environmental risk are needed. Funding of risk assessment studies has been small compared to the millions spent on tree development (Strauss et al. 2000; Kaiser 2002). Furthermore, past scientific enquiry that seems to be the basis for safety assurances has focused on traditional Bt formulations, and these results may not apply to genetically modified constructs. Genetically modified trees are a new vector requiring new enquiry.

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