6th Pacific Rim Conference on the Biotechnology of Bacillus thuringiensis *and its Environmental Impact,* Victoria BC, 2005
Côté, J.-C., Otvos, I.S., Schwartz, J.-L. and Vincent, C. (eds)

Bt Crop Straw is an Effective Source of Active and Stable Cry1Ac Toxin for Spray Bio-Formulations

Mohsin Abbas Zaidi[1], Xiongying Cheng[1], and Illimar Altosaar[1*]

[1]Agricultural Biotechnology Laboratories, Biochemistry, Microbiology and Immunology Dept., Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada, KIH 8M5.

Keywords: *Bacillus thuringiensis*, Cry1Ac, transgenic rice, *Pieris rapae, ex situ* application, Chinese cabbage, *Brassica juncea*.

Accumulated Cry1Ac protein in cereal leaves was applied to non-transgenic plants to test if such ex situ application could successfully control insect pests. A homogenized aqueous suspension of transgenic rice (*Oryza sativa*) leaves containing Cry1Ac was spread over the leaves of Chinese cabbage (*Brassica juncea*) to determine its toxicity against *Pieris rapae larvae*. A mortality of 78 to 96% was observed for different instar larvae. Furthermore, Cry1Ac-treated larvae had 22% and 39% lower adult emergence than their controls. The in-leaf particle *Bt* toxin treatment reduced the egg production of surviving moths to 92 eggs per female versus 211.4 eggs per female in the control. The viability of these eggs was also effected, 7.1% versus 50.2% and 42.8% in the controls.

Introduction

Bt sprays for crop protection rely on fermentation for the production of bacterial crystals (containing the toxins) and spores. However, microbial production technology is expensive, tedious and the quality control is demanding, given the needs to avoid production of the closely related *B. cereus* and *B. anthracis*, which infect vertebrates. Nowhere is the need for cheaper production methods desired more than in developing countries (4). Given that at least 17 countries around the world are now using *Bt*-modified crops, an overlooked source for *Bt* toxin proteins is in crop residues, such as transgenic *Bt* cotton and *Bt* maize. The development of cost-effective protocols for the mass production of *Bt* can be significantly advanced by the utilization of plant residues containing Cry proteins. Such an alternative source of these toxins, e.g. Cry1Ab or Cry1Ac, can provide an *ex situ* protection of another plant against the same or other target insects (7). To test this *ex situ* strategy, white butterfly larvae, *Pieris rapae* (L.) were fed cabbage leaves treated with Cry1Ac-modified rice leaves. Furthermore, stability of Cry1Ac in *Bt* rice leaves was also analyzed for its long term storage and availability.

Materials and methods

Transgenic Cry1Ac-rice [*Oryza sativa* (Poales: Poaceae)] plants used in this study were produced and grown as previously described (3). Leaf tissues of these plants at ripening stage were homogenized in distilled water using 2 mL/g plant tissue and applied onto the surface of detached leaves of fresh non-transgenic Chinese cabbage [*Brassica juncea* (Brassicales: Brassicaceae)] (1mL per 4g of leaf tissue). Eggs and 1st to 3rd instar larvae of *Pieris rapae* (Lepidoptera: Pieridae) were collected from local farms at Zhejiang University, China. Eggs were hatched in a lab setting to obtain neonates. In no-choice feeding assays, 10 to 15 (neonate or 1st to 3rd instar) larvae of *P. rapae* were fed 2 g of the *Bt*-treated cabbage leaves per Petri plate for 2 days. The larvae were subsequently transferred to fresh untreated leaves until mortality or pupation occurred. The larvae were reared in Petri dishes in entomological incubator (ZAU Industries, China) at conditions described previously by Wittstock et al. (6). Distilled water and homogenized leaf tissues from non-transgenic rice plants were applied on cabbage leaves as negative controls with 10 to 15 larvae per Petri plate. The experiment was replicated three times (as per conformity to Table 1). The emerging female adults were allowed to mate with field collected males to establish homogeneity within the experimental population with respect to lab versus field environmental effects. The eggs were allowed to hatch in an entomological incubator at the conditions described previously by Wittstock et al. (6). The number of emerging moths, the number of eggs per female, and hatching rates were recorded for the F_1 generation from the treated and control larvae. To determine Cry1Ac stability in rice, detached leaf samples were stored at

* Corresponding author. Mailing Address. Agricultural Biotechnology Laboratories, Biochemistry, Microbiology & Immunology Dept., Faculty of Medicine, University of Ottawa, 451 Smyth Rd. Ottawa, Ontario, Canada, KIH 8M5.Tel +1 613 562 5459, Fax +1 613 562 5440. Email: altosaar@uottawa.ca

4°C or 37°C for up to 150 days and *Bt* protein levels were determined at monthly intervals using ELISA blots and quantified using Sigmagel™.

Results and discussion

Mortality of field collected larvae exposed to the *Bt* rice homgenate-treated cabbage was high, 78% (Table 1) on day 7 of the bioassay. Some of the larvae that survived after feeding for 2 days on *Bt* rice-treated cabbage leaves were able to pupate when transferred to field-grown cabbage plants. Moth emergence was 39% and 22% lower in the *Bt* treatment compared to those in the water treated and non-transgenic rice control treatments, respectively (Table 2). Moths from the *Bt* toxin-treated larvae produced an average of only 92 eggs per female, while non-transgenic rice tissue-treated and water-treated female moths laid an average of 211.4 and 462 eggs per female, respectively. Moreover, the egg hatching rate of the surviving *Bt* toxin-treated larvae was much lower (7.1%) than in the controls (50.2% and 42.8%, respectively). Clearly, feeding on Cry1Ac-rice-treated cabbage leaves for 2 days resulted in high larval mortality and had serious deleterious physiological effects on *P. rapae*. These effects strongly suggest that field application with homogenates from transgenic *Bt* rice leaf tissue would contribute to an overall reduction of the insect population. Mortality of laboratory-reared neonate larvae was higher than that of older larvae, nearly 100%, and none survived to reach pupation stage after the feeding period (Table 3).

To determine toxin stability, the Cry1Ac contents of leaf tissues harvested from mature transgenic rice were determined after 1 to 5 months storage at 4°C and 37°C. ELISA assays indicated a 20% decline in Cry1Ac contents at 4°C and 15% at 37°C. The concentration of Cry1Ac in transgenic tissue declined slowly when stored under dry conditions, offering large-scale industrial use, particularly in developing countries. Much of the decline took place within the first 30 days of storage period. After that no significant decline was observed (Fig. 1). It appears that the level of water content in rice biomass is very important for Cry1Ac stability. High water contents during the initial period of 30 days may facilitate some enzymatic degradation of the toxin protein. Cry1Ac concentrations decreased to a non-significant level as the water contents decreased from month 2 through month 5. This stability implies that before the rice biomass is directly applied as a dry powder or before plant-derived *Bt* proteins are prepared for spray applications, they can be stored under normal warehouse conditions for up to 5 months. Just as commercial formulations of *Bt* subsp. *kurstaki* (*Btk*) are being widely used against lepidopteran forest defoliators, their spray application prescriptions are still being optimized (1, 2). Furthermore, current research into conventional *Bt* sprays involves novel adducts, adjuvants, safeners and other 'stickers and spreaders' like petroleum spray oil (5). Because rice or maize leaf and stem cellular structure may yield a favorable particle size distribution upon grinding, *ex situ* solutions of *Bt* straw may have unique pesticide delivery attributes. For example, rice and maize cell wall lignins, leaf waxes and pigment contents, even in dead cells, may serve as UV protectants. Also epidermal cell trichomes and leaf hairs, maintained in *Bt*-straw particles, may play very beneficial roles in spray modalities. Consequently, we anticipate similar foliage protection to be achieved with *ex situ* solutions compared to conventional sprays. Cry protein concentrations equivalent to conventional spray doses [billion international units per hectare (BIU/ha)] should now be made operational.

References

1. **Brar S. K., M. Verma, R. D. Tyaci, and J. R. Valero.** 2006. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. Process Biochem. **41:** 323-342.
2. **Chattopadhyay, A., N. B. Bhatnagar, and R. Bhatnagar.** 2004. Bacterial insecticidal toxins. Crit. Rev. Microbiol. **30:** 33-54.
3. **Cheng, X., R. Sardana, H. Kaplan, and I. Altosaar.** 1998. *Agrobacterium*-transformed rice plants expressing synthetic *cry1A(b)* and *cry1A(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. **95:** 2767-2772.
4. **Devi, P. S. V., T. Ravinder, and C. Jaidev.** 2005. Barley-based medium for the cost-effective production of *Bacillus thuringiensis.* World J. Microbiol. Biotechnol. **21:** 173-178.
5. **Mensah, R. K., W. Liang, D. Gibb, R. Coates, and D. Johnson.** 2005. Improving the efficacy of nuclear polyhedrosis virus and *Bacillus thuringiensis* against *Helicoverpa* spp. with ultra-violet light protected petroleum spray oils on cotton crops in Australia. Int. J. Pest Manag. **51:** 101-109.
6. **Wittstock, U., N. Agerbirk, E. J. Stauber, C. E. Olsen, M. Hippler, T. Mitchell-Olds, J. Gershenzon, and H. Vogel.** 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proc. Natl. Acad. Sci. USA **101:** 4859-4864.
7. **Zaidi, M. A., X. Cheng, H. Xu, and I. Altosaar.** 2006. Ex situ application of foliar-produced lepidoptericides from transgenic rice to control *Pieris rapae* and Cry1Ab stability in vivo. Crop Prot. **25:** 748-752.

TABLE 1. Mortality of field-collected *P. rapae* (1st to 3rd instar) larvae after 2-day feeding on cabbage leaves treated with Cry1Ac transgenic rice leaf homogenate.

Treatment	Toxin	Total no. of larvae in all three replicates	Mortality (%)				
			Days after feeding				
			3	4	5	6	7
Water (control)	None	60	0.0	3.3	3.3	8.3	10.0[a]
Non-transgenic rice	None	84	4.3	8.6	14.3	14.3	14.3[a]
Transgenic rice	Cry1Ac	54	21.7	34.7	57.2	73.9	77.9[b]

[a, b] Values with different superscripts differ significantly ($p < 0.05$).

TABLE 2. Development and rearing of *P. rapae* larvae after 2-day feeding on cabbage leaves treated with Cry1Ac transgenic rice leaf homogenate, until pupation.

Treatment	Toxin	No. of pupae examined	Moths emerged		No. of eggs laid per female	No. of eggs hatched
			No.	%		
Water	None	25	24	96.0a	412.3 ± 56.7[a]	42.8[a]
Non-transgenic rice	None	28	22	78.6b	211.4 ± 33.6[b]	50.2[b]
Transgenic rice	Cry1Ac	14	8	57.1c	92.0 ± 9.9[c]	7.1[c]

[a, b, c] Values with different superscripts differ significantly ($p < 0.05$).

TABLE 3. Mortality of laboratory-hatched *P. rapae* neonate larvae after 2-day feeding on cabbage leaves treated with Cry1Ac transgenic rice leaf homogenate.

Treatment	Toxin	Total no. of larvae in all three replicates	Mortality (%)				
			Days after feeding				
			3	4	5	6	7
Water	None	81	0.0	3.3	3.3	8.3	15.0[a]
Non-transgenic rice	None	74	8.3	10.6	11.3	24.3	24.3[b]
Transgenic rice	Cry1Ac	104	31.7	54.7	77.2	95.9	95.9[c]

[a, b, c] Values with different superscripts are significantly different ($p < 0.05$).

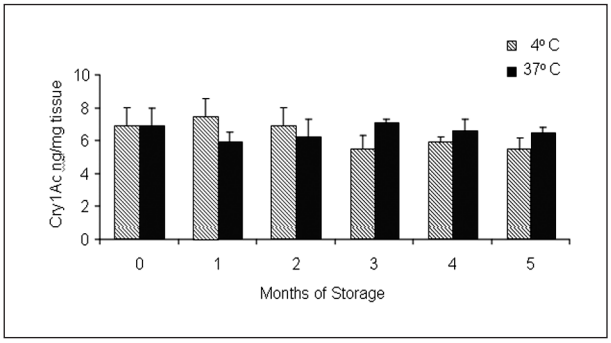

FIG. 1. Cry1Ac levels in detached leaf tissues of transgenic rice lines after storage at 4ºC and at 37ºC. Cry1Ac from the stored leaf tissue was extracted and immunologically quantified by ELISA using anti-Cry1Ac antibody. Vertical bars represent standard errors.