Table 1. Toxicity of different combinations of Cry1Ac/Cry1F toxin mixtures to *H. armigera*

Ratio (Cry1Ac : Cry1F)	Observed EC^{*}_{50}	Slope ± SE	Expected EC^{**}_{50}	Expected/observed
1 : 0	8.0	0.86 ± 0.01	–	–
0 : 1	870.0	0.49 ± 0.03	–	–
2 : 1	0.5	1.15 ± 0.04	11.9	23.8
1 : 1	0.6	0.89 ± 0.02	15.8	26.3
1 : 2	1.9	1.15 ± 0.04	23.6	12.3

EC^{*}_{50}: Concentration of Cry toxins causing 50% larval growth reduction.

EC^{**}_{50}: Calculated by the formula used by Tabashnik[4].

sorghum, etc.[5]. Here we report synergism between two Bt δ-endotoxins in relation to their toxicity to *H. armigera*.

The genes (*cry1Ac, cry1Ab, cry1F* and *cry2Aa*) coding for Bt δ-endotoxins were overexpressed in *E. coli* strain JM103 using the expression vectors (from Donald Dean, Ohio State University, USA). The protoxins were purified and solubilized as described by Lee *et al.*[6]. The solubilized protoxin was digested with trypsin in a trypsin : protoxin ratio of 1 : 25 (by mass) for 2 h at 37°C. Activated toxins were dialysed against 50 mM sodium carbonate buffer, pH 9.5. The purity of the protoxin and activated toxin was examined on 10% SDS-PAGE[7]. The toxins at different concentrations were spread on semi-artificial diet in 24-well Costar plates and one larva (I instar) per well was released[5]. Larval mortality and growth inhibition were recorded after 6 days. The data were subjected to probit analysis[8]. Synergism between the toxins was calculated according to the equation of Tabashnik[9].

$$LD_{50(m)} = \left[\frac{r_a}{LD_{50(a)}} + \frac{r_b}{LD_{50(b)}}\right]^{-1},$$

where $LD_{50(m)}$ represents the expected LD_{50} of the mixture, $LD_{50(a)}$ and $LD_{50(b)}$ represent the LD_{50} for toxin a and b respectively, and r_a and r_b represent the relative proportion of toxins a and b in the mixture respectively.

We have previously observed that four toxins, viz. Cry1Ac, Cry1Aa, Cry1Ab and Cry2Aa were highly effective against *H. armigera* larvae and toxins such as Cry1F caused only growth inhibition[5]. Three combinations of toxins (Cry1Ac + Cry1Ab, Cry1Ac + Cry2Aa and Cry1Ac + Cry1F) were tested for their efficacy in the present study. No significant alteration in the toxicity was observed when the combinations of Cry1Ac + Cry1Ab and Cry1Ac + Cry2Aa were used (data not shown). On the other hand, Cry1Ac and Cry1F exhibited an interesting interactive effect (Table 1). Cry1Ac toxin is about 100 times more toxic than Cry1F toxin towards *H. armigera*. Mixture of Cry1Ac and Cry1F toxin (1 : 1) showed a synergistic effect in that the EC_{50} of Cry1Ac toxin was lowered 13 times due to the presence of Cry1F. The observed toxicity of the Cry1Ac + Cry1F mixture was about 26 times higher than the expected toxicity, strongly suggesting a synergism. Although we have no definitive explanation for the synergistic effect of the Cry1Ac and Cry1F mixture, two possible mechanisms can be speculated. The mechanism of Bt δ-endotoxin action involves binding of the toxins to the receptors, insertion into the membrane and pore formation[1]. It has been suggested that the toxin might oligomerize before or after binding to the receptors[10]. It is possible that a hetero-oligomer comprising Cry1Ac and Cry1F has better insertional ability than the Cry1Ac homo-oligomer complex, either during or subsequent to receptor binding. Another possibility is that the toxins Cry1Ac and Cry1F bind to different receptors in the midgut epithelium of the larvae and each individual pore made by different toxins act together and show higher toxicity than the individual pores. Receptor binding analysis and voltage clamp experiments can resolve the mode of synergism.

In conclusion, we suggest that the toxins Cry1Ac and Cry1F can be expressed together in transgenic crop plants for effective control of *H. armigera* and also as a durable resistance management strategy.

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Plant regeneration from mature leaves and roots of *Eryngium foetidum* L., a food flavouring agent

Eryngium is a South American genus of family Apiaceae, with approximately 230 species, of which *Eryngium foetidum*, one of the rare species, is restricted to certain parts of India. It is an aromatic plant, commonly called spiny coriander, and as important as *Coriandrum sativum*. It is strong smelling and contains aromatic essential oil comprised of about forty compounds[1,2]. The leaves are used as a condiment for culinary purposes in north-east India[3]. Its aromatic oil is indispen-

sable to perfumery, flavour and pharmaceutical industries. The leaves possess antiinflammatory and analgesic properties as well[4]. Chemical analysis of root and leaf oil has been the subject of investigation for many years[1,2]. Recent developments in tissue culture techniques such as cell culture and organ cultures offer new avenues in the improvement of many plant species towards the production of essential oil in both quality and quantity. In plantlets derived from *in vitro* cultures, the oil composition could be controlled by changing the composition of the culture medium, and organized or differentiated cultures often accumulate high levels of secondary metabolites[5]. There is an increasing demand for it in Europe and north America[6]. We report here for the first time the plant regeneration from callus cultures of mature leaves, and direct organogenesis from roots.

Eryngium foetidum L. plants were collected from Andaman and Nicobar islands and were grown in the garden at Entomology Research Institute, Loyola College, Chennai. Mature leaves and roots were washed in running tap water for one hour, surface sterilized with 70% alcohol for 30 sec and 0.1% $HgCl_2$ (w/v) for 5 min and given six washes with sterile distilled water. The disinfected leaves and roots were incised using a sterile knife and then placed on the culture slants of LS medium[7] containing varying concentrations of NAA, 2,4-D and BAP (0.5–2.0 mg/l each) for callus induction. The leaves were placed with their abaxial surface in contact with the medium.

For plant regeneration, four-week-old calli derived from leaves and roots were cultured on MS medium[8] supplemented with varying concentrations of cytokinins (BAP and kinetin) (0.5–2.0 mg/l each) plus auxins (IAA and NAA) (0.01–1 mg/l each). The differentiated shoots were subcultured in the same medium for multiplication. The regenerated shoots were transferred to MS medium containing different concentrations of IBA, IAA, NAA singly (0.01–1 mg/l), and in combination with GA_3 (0.5–2.0 mg/l) (filter sterilized) for root induction and elongation, respectively. The pH of the media was adjusted to 5.7 and gelled with 0.7% agar before autoclaving at 15 lb for 15 min. The cultures were incubated at 27 ± 2°C and given a light and dark cycle of 16/8 h.

The fully developed plants were transferred to paper cups containing sterile vermiculite, covered with polythene bags, and kept in growth chamber for two weeks. The plants were irrigated initially with half-strength MS basal medium for seven days and subsequently with tap water. Polythene bags were removed from the cups at the end of third week and plants were transferred to garden soil after four weeks.

In evaluating the influence of various plant growth regulators in the formation of callus from root and leaf explants on LS medium, it was observed that callus was initiated from leaves on LS medium fortified with 0.5 mg/l of 2,4-D, 1 mg/l of NAA, and 2 mg/l of BAP after ten days. A small amount of callus developed from root explants at 0.5 mg/l 2,4-D. While in root explants grown in a medium with either NAA or BAP alone sometimes no callus formation was observed (Table 1), when the medium contained these hormones in combination, a meagre amount of callus developed. A high frequency of callus induction was noted in explants from leaf tissues (Figure 1 *a*) compared with explants from root tissue. While the presence of 0.5 mg/l 2,4-D and 1 mg/l NAA resulted in the development of friable, brown and shiny callus from leaf tissue, in the presence of BAP at 2 mg/l compact, brown and shiny callus developed. The growth rate and amount of the leaf callus were higher in the presence of BAP than with other hormones, and the callus growth rate was significantly correlated to the types of hormones used in the following sequence: BAP > 2,4-D > NAA. Among the hormones used, 2 mg/l BAP was found to be the optimal concentration required for callus induction from the leaves. The callus derived from the leaves in the presence of NAA and 2,4-D, often resulted in differentiation of roots in the same medium itself after five weeks. Hence calli formed on the medium with BAP at 2 mg/l were subcultured in the same medium once in four weeks for maintenance and regeneration. Generally there is a requirement for high exogenous concentrations of auxin to promote intensive callus induction[9]. However, our observations showed that in *Eryngium foetidum* BAP alone was necessary for obtaining organogenic callus. The observed brown colour of the callus might be due to the exudation of aromatic oils and some other secondary metabolites which could not leach out into the medium, and were not inhibitory to differentiation of shoots and roots.

Fifteen to twenty days after culturing of leaves in the regeneration medium, small globular protuberances developed at 0.1 mg/l IAA and 1 mg/l BAP (Figure 1 *a*) and later shoots originated from these protuberances (Figure 1 *b*). The regenerated shoots were divided into 2–3 clumps and cultured in the same medium for further proliferation to increase the number of shoots. These clumps, in turn, proliferated into multiple shoots. For continuous production of these shoots, the clumps were subcultured every 4–6 weeks. However, the vigour of the shoot

Table 1. Influence of plant growth regulators in callus formation of leaf and root explants on Linsmair and Skoog medium

Hormones	Concentration (mg/l)	Leaf explants		Root explants	
		Quantity	Nature	Quantity	Nature
2,4-D	0	–	–	–	–
	0.5	+++		++	B
	1.0	++	BFS	+	B
	2.0	+		A. roots	–
NAA	0	–		–	–
	0.5	++		A. roots	–
	1.0	+++	BFS	A. roots	–
	2.0	+		A. roots	–
BAP	0	–		–	–
	0.5	++		–	–
	1.0	+++	BCS	–	–
	2.0	++++		–	A. shoots

B, Brown; F, Friable; C, Compact; S, Shiny; A, Adventitious; –, No callus; +, Meagre amount of callus; ++, Small amount of callus; +++, Large amount of callus; ++++, Profuse callus.

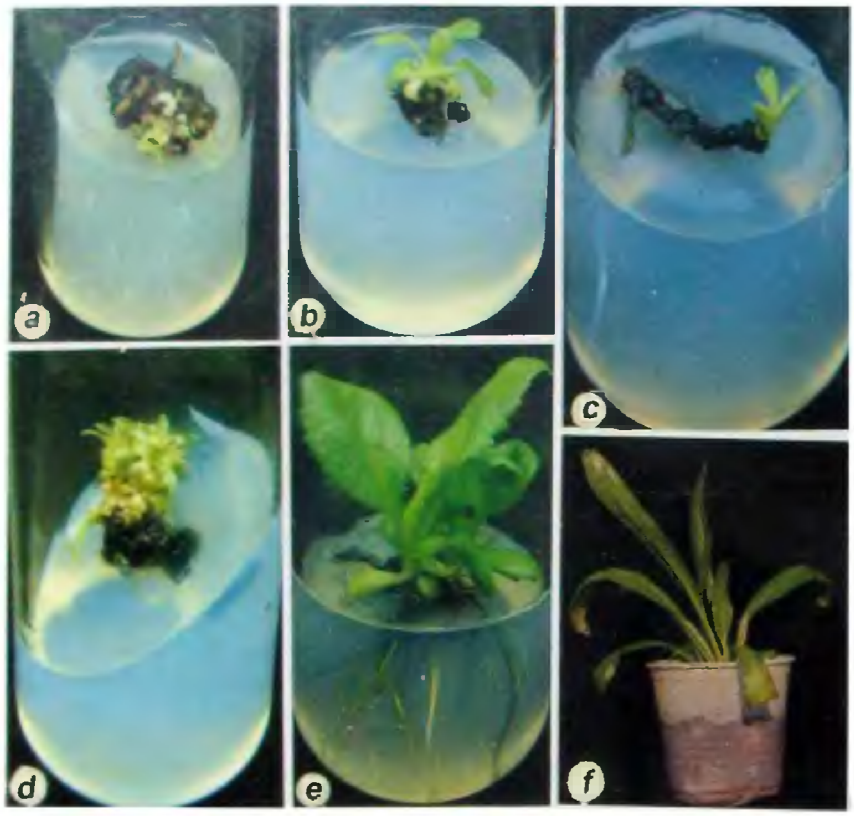

Figure 1. *a*, Callus obtained from leaves showing protuberances. *b*, Regenerated shoots obtained from leaf callus. *c*, Root explant showing direct differentiation of shoots. *d*, Root showing aberrant leaf morphology. *e*, Regenerated shoots showing multiplication and rooting. *f*, Regenerated plants in *in vivo* conditions.

Table 2. Influence of IAA and BAP on plant regeneration from leaf callus of *Eryngium foetidum* L. on MS medium

IAA (mg/l)	BAP (mg/l)	Average number of regenerated shoots from the callus	Average number of shoots that multiplied after subculture
0.01	0.5	–	–
0.1	1.0	6.6 ± 1.3	11.2 ± 1.20
0.5	1.5	4.0 ± 1.0	6.32 ± 1.00
1.0	2.0	–	–

multiplication declined after 3–4 subcultures.

In callus obtained from the root explants, no regeneration was observed at all; but the root explants differentiated directly into shoots at 2 mg/l BAP from the distal end of the roots after five weeks (Figure 1 *c*; Table 1). Some of them showed aberrant morphology of leaves when the explants were placed vertically on the medium (Figure 1 *d*). The aberrant leaves showed arrow-shaped and needle-like structures and were smaller compared to the other normal leaves. Thus, the orientation of the explants in the medium might have significance and might affect the morphology of the plant by giving them a disturbed hormone physiology. This observation coincides with the findings of Dillen *et al.*[10] in *Phaseolus acutifolius*. Further, selection of somaclones derived from tissue culture may serve as an alternative tool for improving plants for higher yield of essential oil, and perhaps for other desired qualities as well.

Root induction and elongation occurred when the shoots were transferred to MS medium with 0.1 mg/l of IBA and 1 mg/l of GA_3 (Figure 1 *e*). Rooting was initiated within seven days and profuse root system was formed without intervening callus development. Initially the roots were white but subsequently turned brown, which may be indicative of biosynthetic activity of oils in roots. IBA was found to be better suited for root induction than other auxins. Further, the addition of IBA and GA_3 in combination on MS medium was ideal for simultaneous occurrence of rooting and elongation. The plantlets were healthier in this medium due to the effect of GA_3. In the present investigation the best shoot regeneration response (90%) was observed in cultures initiated from leaf callus, while the percentage of direct differentiation of shoots from root explants was lower (45%). Further, the number of shoots obtained from leaf callus was also higher (11–12) (Table 2) than the number from roots (3–5). The plantlets showed 85–90% survival under field conditions (Figure 1 *f*). This simple procedure offers a potential for generating sufficient material through mass multiplication in commercial planting.

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