PROCEEDINGS

OF THE

26th ANNUAL MEETING

July 29 to August 4, 1990

Mayaguez, Puerto Rico

Published by:

Caribbean Food Crops Society

with the cooperation of the USDA-ARS-TARS

Mayaguez, Puerto Rico

GENETIC ENGINEERING OF ENDOPHYTIC BACTERIA: A NOVEL APPROACH FOR PRODUCING PEST-RESISTANT CORN

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ABSTRACT

Genetic engineering techniques currently permit several approaches to produce crop plants with enhanced resistance to pests. Many groups are directly introducing genes which encode for pest resistance into the plant's genome. Crop Genetics International is developing an endophyte based technology for systemic delivery of biopesticides to corn and other crops.

Defined broadly, an endophyte is a plant dependent microorganism that lives protected within the tissues of its host. Among endophytic bacteria, those associated with plant disease have been the past object of study. However, nonpathogenic bacterial endophytes present an opportunity for systemic delivery of genetically-engineered biopesticides and plant growth regulators. Crop Genetics has chosen a xylem-limited endophytic bacterium, Clavibacter xyli subsp. cynodontis (Cxc) which occurs naturally in bermudagrass and is distributed throughout the southern two-thirds of the U.S. as well as Europe and Asia. Cxc is a fastidious microorganism with precise nutritional and environmental requirements. Cxc survival is brief outside the host plant in plant debris, soil, air or water, and the endophyte is not seed transmitted. Cxc, when modified with recombinant DNA techniques, provides a systemic delivery system for biopesticides within plants.

The first product under development involves Cxc producing the delta-endotoxin of Bacillus thuringiensis toxic to European corn borer (Ostrina nubilalis). Follow-on products will deliver other insecticides, fungicides, and plant growth enhancers.

Crop Genetics has developed an inoculation technology to introduce the bacterium into seeds. The protocol includes imbibition followed by pressure treatment in a buffered solution containing the bacterium. Seeds are removed from the solution, dried, and coated with conventional seed coatings. This inoculation technology does not alter seed vigor or germination and provides a satisfactory shelf life of the product.

INTRODUCTION

The application of recombinant DNA technology to the field of crop protection against insect pests has followed two principal routes. Entomopathogens, particularly the bacterium Bacillus

thuringiensis (Bt), have been the subject of many attempts (some of them successful) to overcome the problems of poor persistence, slow action, and narrow spectrum of activity that, paradoxically, are the principal environmental advantages of microbial insecticides as well as the major factors that have hindered their commercial success. The other principal approach has been to insert genes coding for insecticidal proteins (such as Bt delta-endotoxins and proteinase inhibitors from various plants) directly into the DNA of plant cells, so that the resulting transgenic plant produces these compounds for its own defense.

Another approach that has received attention takes advantage of our increasing awareness of the diversity of relationships between plants and microorganisms, particularly bacteria. For example, root-colonizing bacterial epiphytes such as Pseudomonas fluorescens have been engineered to produce Bt endotoxin. Among endophytic bacteria (those that live within the host plant, rather than upon it), there are some species which are pathogenic to plants and other species which are nonpathogenic. Genetic engineering of nonpathogenic endophytic bacteria presents an opportunity for the systemic delivery of biopesticides within host plant tissues without direct genetic manipulation of the host plant. Such a systemic microbial pesticide should exhibit sustained and protected biopesticidal activity.

InCide Biopesticides

Crop Genetics International (CGI) is currently developing such an endophytic delivery system under the trade name InCide.

CGI's InCide technology utilizes genetically engineered endophytic microbes to produce biological plant protectants. One such microbe is Clavibacter xyli subsp. cynodontis (Cxc), a fastidious, gram positive, coryneform bacterium, that occurs naturally in the xylem of bermudagrass (Cynodon dactylon). The first product involves a genetically-engineered Cxc capable of producing the delta-endotoxin of Bacillus thuringiensis subsp. kurstaki (Cxc/Bt) that is toxic to European corn borer (Ostrinia nubilalis) larvae.

Species of bacteria that are presently classified as belonging to the genus Clavibacter were previously listed as species of the genus Corynebacterium (Davis et al., 1984). Due to the lack of literature references for the subspecies cynodontis Clavibacter xyli, CGI has generated extensive biological and environmental fate data to aid in product development and registration.

Extensive sampling of bermudagrass has shown that Cxc is distributed widely within the geographical range of its natural host. Currently, Cxc has been isolated from bermudagrass in 26 states in the U.S. including important corn producing states such as Nebraska, Iowa, Illinois, and Indiana.

Once introduced into seedling corn by wound or seed inoculation, Cxc rapidly colonizes the xylem of roots, stems, leaves, and husk. Cxc population levels up to 1 x 10^9 bacteria per gram fresh weight tissue is achieved. Since it is xylem limited, Cxc is not present in or transmitted via seed. Host range studies have shown Cxc is capable of colonizing primarily grass species. Recombinant strains of Cxc have patterns of host colonization similar to patterns of the wild type.

Extensive laboratory, greenhouse, and field studies were conducted from 1987 through 1989 to determine the ability of Cxc and/or Cxc/Bt to persist in the environment and disperse beyond the point of introduction. Since Cxc is dependent on live plants for replication, and does not produce spores, persistence in soil, water, and on plant surfaces is brief. In the field, Cxc was undetectable after 2 weeks in soil, 3 weeks in soil-incorporated green residue of colonized corn plants, 5 weeks in buried sections of cornstalks, and 7 weeks in cornstalks standing in the field after harvest. Cxc could not be detected at any time in corn plants grown in inoculated soil, even when the roots were injured deliberately by passing knives through the soil to simulate cultivation damage. Volunteer seedlings originating from colonized parents in the field also were not colonized. Likewise, Cxc was never detected in irrigation run-off from plots of colonized corn. These results indicate that soil, water, or plant debris are unlikely to serve as sources of inoculum for neighboring fields or subsequent crops or weeds in the same field. Dispersal of Cxc from inoculum foci was rarely observed. Mechanical transmission in the field was very limited.

Field studies conducted with a prototype recombinant Cxc (Cxc/Bt) in 1988 again demonstrated poor persistence in soil, incorporated plant material, and cornstalks remaining after harvest. Cxc/Bt populations were undetectable very soon after inoculation of soil in July and August, but persisted somewhat longer when the test was repeated under cooler conditions in October. Cxc/Bt was not detected at all in soil around inoculated plants, even after incorporation of the plant material into the soil. As in 1987, the endophyte was not detected in runoff water, even after colonized plant debris was chopped and incorporated, providing additional evidence that a colonized crop is not likely to provide a source of soil inoculum for subsequent crops.

Field trials in 1988 demonstrated that Cxc/Bt was not naturally dispersed from corn to other corn plants or weeds and that, under normal agricultural conditions, Cxc/Bt was not spread mechanically from corn to other corn plants or weeds. In an attempt to induce artificial transmission, colonized corn plants were repeatedly cut with shears until the shears were wet with sap, and the shears were then used to trim uncolonized weeds; only in this case was any mechanical transmission observed and even then the frequency of transmission was quite low. Cxc/Bt did not

colonize any of the trap plants (corn and bermudagrass) planted around the perimeter of the test sites to monitor for the dispersal of the organism. Field trials conducted in Maryland, Illinois, Minnesota and Nebraska (involving plants from seed inoculated with Cxc and Cxc/Bt).

Genetic Engineering of Cxc

Recombinant DNA techniques have been used to modify wild-type Cxc to produce delta-endotoxin proteins of Bacillus thuringiensis subsp. kurstaki strain HD-73. Different Cxc/Bt recombinant strains contain the intact protoxin gene (coding for the 130 kilodalton protein that is broken down by proteolysis in the alkaline gut of the corn borer to form the activated toxin), gene fusions combining the toxic domain of HD-73 with various marker genes, and the toxic domain itself (coding just for the active endotoxin). Molecular geneticists at CGI construct plasmids that comprise the toxin coding region, regulatory sequences that control transcription of the genetic code to messenger RNA (promoters) and the translation of the message into amino acid sequences (ribosome binding sites), marker genes that confer selectable traits (such as resistance to antibiotics) for detection of transformants, and a DNA sequence (replicon) that is capable of initiating replication of the entire plasmid. The common enteric bacterium Eschericia coli is used as a host for transformation with these cloning vectors for the initial construction of these expression cassettes. Successful cassettes are then cloned into an integration vector which contains a segment of DNA homologous to a segment of the chromosomal DNA of Cxc, which (unlike E. coli), has no plasmids. When the integration vector is inserted into the Cxc cell, crossing-over occurs between the homologous regions of the vector and the host chromosome, resulting in the table insertion of the engineered DNA sequence into the Cxc chromosome. The resulting Cxc/Bt recombinant produces HD-73 toxin proteins that can be identified electrophoretically by reaction with radioactive antibodies against purified HD-73 crystal protein (Western blotting). Because Cxc has no detectable plasmids or prophage (which could mobilize recombinant genes in nature), and because Cxc isolates have proven unable to transmit or exchange integrated DNA sequences with other bacteria, there is minimal risk of genetic exchange of recombinant toxin genes between Cxc and other microorganisms. Cxc/Bt recombinant strains have also been shown to revert spontaneously, losing the engineered gene sequences at a low frequency. Revertants are able to divide more rapidly and hence outcompete recombinants. Consequently the recombinant genes are eventually lost from the Cxc population in the host plant. This phenomenon occurs at a rate slow enough to ensure product performance (i.e. sufficient Cxc/Bt populations) within a growing season, but rapidly enough that toxin genes would not persist in the environment in the unlikely event that Cxc/Bt was transmitted by mechanical means to a noncrop host plant.

Effects of Cxc/Bt on European Corn Borer

Unlike Bt, Cxc/Bt does not sporulate and release crystal toxins into the environment, which are then ingested by caterpillars. Cxc/Bt does not secrete its toxin, and must therefore be digested by the insect in order to release its active ingredient. However, once this is accomplished, the symptoms are similar to those observed in larvae ingesting Bt: feeding slows and eventually stops, and larvae die from starvation or from invasion of the hemocoel by opportunistic microorganisms.

Experiments have demonstrated that Cxc/Bt can prevent or reduce borer damage to inoculated field corn under conditions of artificial infestation in the greenhouse. In four separate trials, test plants were inoculated with a strain of Cxc/Bt approximately 2 weeks after planting (about 6 to 8 leaf stage) by injection approximately 10 cm above the soil line with 7 to 8 log CFU per plant from a suspension of recombinant cells in phosphate buffered saline. Endophyte control plants were inoculated in similar fashion with wild-type Cxc. A sham-inoculated group were injected with sterile buffer only.

Plants confirmed as colonized systemically by Cxc or Cxc/Bt (except for the sham-inoculated group) were infested with neonate ECB larvae 5 or 6 weeks after inoculation. Incidence of colonization was determined by phase contrast microscopy (1000X) of a drop of sap expressed from a leaf taken from each plant. In the first three experiments, each plant was infested with 15 larvae divided equally among 5 holes (6 mm dia.) drilled into the stalk at 5 internodes, a method first used by Chiang (1959) in field experiments with ECB. The holes were sealed with nonabsorbent cotton plugs to prevent escape. Plants were dissected 3 or 4 weeks later to assess tunnelling damage as well as the numbers and condition of surviving insects. In the fourth experiment, plants were infested at or near tasseling with 50 larvae each, distributed among 5 upper leaf axils with a camel hair brush. The first three trials were designed to detect any activity of Cxc/Bt *in planta* against ECB larvae feeding on a more natural substrate than a laboratory diet, and was not a true simulation of a natural infestation. Normally, ECB larvae are physically unable to penetrate the stalk until third instar, about halfway through their larval development. The axil-infestation technique of the fourth trial was used to simulate more realistically a heavy natural infestation by second brood ECB, in which eggs are laid on plants during anthesis and larvae feed on leaf tissues (particularly sheath and collar) and pollen in the axils prior to entering the stalks at third or fourth instar (Showers et al. 1989).

In the first three trials, plants colonized by Cxc/Bt contained, on average, one-third to one-half as many tunnels and one-quarter to one-third as many live insects than control

plants at the end of the 3- to 4-week infestation period (Table 1). Results of the axil-infested trial were less dramatic, possibly due to the opportunity afforded to the larvae to feed and grow on external tissues before tunneling into the stalk (where Cxc/Bt concentrations tend to be 5 to 10 times higher than in the leaves) as third or fourth instars, which may be less sensitive to the effects of the Bt toxin (McGaughey 1978, Dimock, unpublished observations). Nevertheless, even in the fourth trial, Cxc/Bt-colonized plants contained about half as many borer tunnels and live insects per plant as did the controls, and total tunnel damage was also reduced by about 40% (Table 1).

These results demonstrate that sufficient amounts of a microbial insecticide can be delivered by genetically engineered endophytes to reduce the numbers of corn borers and their tunneling damage to inoculated plants. Field tests are planned for 1990 and 1991 to determine how the effects of Cxc/Bt on artificial infestations in the greenhouse are manifest in the field. Under field conditions, activity levels similar to those present in Table 1 should prevent or lessen yield losses due to corn borer infestations, since yield loss is closely related to the number of borers per plant and the tunneling damage they inflict (Showers et al. 1989).

Seed Inoculation Technology

CGI has developed proprietary methods for inoculation of Cxc/Bt into corn seeds for delivery to growers via seed company licensees. Crop Genetics International initiated its seed inoculation program in 1986. Early efforts focused on existing seed delivery systems. Various methods for external seed application were examined, including seed pelleting and coating with Cxc contained in a variety of polymers, oils, and powders. Both needleless injectors and microparticle guns were examined for direct injection into the seed. None of these methods appeared to be commercially useful for producing colonized plants. However, they demonstrated that penetration of the seed embryo was requisite for successful seed inoculation.

The use of a pressure differential to force Cxc-containing suspensions into dry seeds was successful, although this method produced only low percentages of colonized plants and resulted in a precipitous drop in germination and seedling vigor. However, if seeds are subject to a period of imbibition in water prior to pressure inoculation with Cxc, up to 100% of the seeds treated can produce vigorous, endophyte-colonized plants.

The current inoculation protocol calls for imbibition followed by placement of seeds in a pressure vessel containing a suspension of Cxc cells in a buffer solution. Seeds are then removed from the inoculation suspension and dried on a forced air dryer. Conventional seed coatings can then be applied.

Table 1.

X-TEND c.i.p. Rep #1 - Started 5/22/90

Treatment	DAT	Morningglory	Velvetleaf	Cocklebur	Pigweed	barnyardgrass	Yellow Foxtail	Fall Panicum	Johnsongrass
H_2O	4	0.0	0.0	0.0	0.0	0.0	0.0	15.0	0.0
	9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Touchdown	4	0.0	22.5	40.0	20.0	0.0	0.0	5.0	0.0
	9	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
	17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Suspension	4	20.0	22.5	25.0	17.5	0.0	0.0	0.0	0.0
Culture	9	25.0	0.0	27.5	0.0	0.0	0.0	0.0	0.0
	17	32.5	0.0	37.5	0.0	0.0	0.0	0.0	0.0
Plate	4	20.0	12.5	30.0	45.0	0.0	0.0	10.0	0.0
Cultures	9	25.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	17	12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Touchdown	4	40.0	50.0	62.5	75.0	37.5	40.0	50.0	42.5
&									
Suspension	9	60.0	45.0	55.0	97.5	55.0	75.0	25.0	77.5
Cultures	17	40.0	40.0	50.0	100.0	35.0	77.5	42.5	85.0
Touchdown	4	40.0	42.5	52.5	87.5	45.0	45.0	50.0	47.5
& Plate	9	70.0	45.0	47.5	100.0	67.5	82.5	32.5	90.0
Cultures	17	60.0	40.0	80.0	100.0	30.0	85.0	47.5	75.0

DAT = Days After Treatment
#'s are % injury
- Touchdown - .06 lbs/acre (1/8 rate)
- x77 .25% v/v
P. syringae pv. tabaci - 10^8/ml from suspension cultures or plates

Seed treatment with Captan appears to have no adverse impact on bacterial survival or efficacy of inoculation. Storage of inoculated seeds for longer than a year is possible, with only a gradual reduction in bacterial titer and no significant effect on seed germination. Shelf life for crops will vary. One year of shelf life is expected for the first InCide product for corn. Controlled environment and warehouse storage experiments are in progress.

Seed inoculation will be performed by machinery built specifically for that purpose, that will fit into the lines of current seed conditioning plants with little or no need for redesign of existing facilities. CGI is currently working with four seed companies on the development and field testing of InCide technology. These cooperators are DeKalb-Pfizer Genetics (DeKalb, IL), NC+ Hybrids and Hoegemeyer Hybrids of Nebraska, and Rogers Brothers Seed Co. of Idaho.

Crop Genetics has targeted 1993 for the market introduction of its first InCide product.

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