ONE BIOTECH COMPANY'S SOLUTIONS TO TRADITIONAL AGRICHEMICAL PROBLEMS

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Summary

Research at Crop Genetics International (CGI) is focused on the creation of biological crop protection products that are effective, cost competitive and compatible with the environment. The company's InStar™ division is developing manufacturing processes for viral insecticides. Naturally occurring insect viruses exhibit specificity against the intended pests and pose no human or environmental hazards. Initial efforts are concentrated on in vivo production systems for viruses targeting lepidopterous pests of various row and orchard crops. Field tests in 1991 demonstrated that the company's beet armyworm virus, Spod-X™, performs as well as or better than Bt-based insecticides, and has a field half-life of 7 to 11 days. The company's InCide® technology is designed to use genetically engineered plant inoculants to protect corn, rice and other major row crops from insects and fungi. The InCide® delivery systems that are being developed are proprietary plant "vaccine" systems in which seeds are inoculated with genetically engineered microorganisms. These microorganisms, which live and multiply within the growing plant's vascular system, are engineered to produce biopesticidal compounds that protect the plant against targeted insects or fungal diseases. Functioning within the plant, the InCide® delivery system is not degraded or dispersed by rain, sunlight, wind and other forces that limit the effectiveness of externally applied insecticides and fungicides. CGI's X-tend™ group is focused on developing weed control systems that combine biological and synthetic herbicidal agents. The broad spectrum systems being developed combine chemical herbicides, in amounts substantially lower than are currently applied, with selected naturally occurring bacterial plant pathogens. Applied as a mixture, the low doses of chemical herbicides stress the weeds, allowing the bacterial pathogens to kill the stressed weeds. The company's Kleentek® division has developed "disease-free" sugarcane seed products using advanced cell culture technologies. Traditionally, sugarcane growers used their own seedcane to replant their crops. However, these stalks or seedcane pass viral, fungal and bacterial diseases directly to the next season's crop. The use of certified Kleentek® seedcane for planting stock has permitted dramatic yield enhancements.

1. Introduction

Although there is certainly a market for genetically engineered plants, CGI has specifically chosen to pursue microbial solutions to agricultural problems. Microbial based systems or products seem to be more adaptable and manipulable over the short and perhaps the long term. We have focused on the creation of unique biopesticides that are effective for food production, cost competitive with synthetic chemicals and compatible with the environment. We are developing four technologies for so doing -- (1) a low-cost manufacturing processes for insecticidal virus products; (2) genetically engineered plant "vaccines" to protect corn, rice and other crops from insects and fungi; (3) environmentally sound weed control systems that combine biological and synthetic herbicidal agents; and (4) the production of disease-free sugarcane "seed" (which has been a business for the company for a number of years).

2. Insecticidal Viral Products

The worldwide insecticide market of an estimated $7.6 billion is dominated by well-established international producers of synthetic chemicals. However, the traditional effectiveness and cost advantages of synthetic insecticides on major crops, such as vegetables, cotton, and fruits, are being diminished by environmental, regulatory and economic forces. One alternative to chemicals is insecticidal agents produced by biological organisms. While bioinsecticides are considered environmentally safer than synthetics, they currently account for less than two percent of worldwide insecticide sales.

Insect viruses of the genus *Baculovirus* are biological insecticides that are environmentally friendly alternatives to synthetic compounds. Of the more than 600 baculoviruses known, the majority are extremely host-specific. These viruses generally replicate in only one or a very narrow range of species (predominately Lepidoptera), and pose no known threat to crops, beneficial insects, wildlife, or humans (Groner, 1986). They are easily formulated and applied using conventional methods, and can be used alone or in combinations with other insecticides.

The InStar™ Division of CGI, in an alliance agreement with E. I. du Pont de Nemours & Co., is pursuing a strategy of becoming a producer of high quality virus products for pest control. CGI will focus on registration and development of production technology, while DuPont will provide formulation, field development, and marketing expertise. In 1991, CGI submitted three registration packages to the EPA: (1) Spod-X™, the nuclear polyhedrosis virus (NPV) of the beet armyworm (*Spodoptera exigua*); (2) Gusano™, the alfalfa looper (*Autographa californica*) NPV and (3) Cyd-X™, the granulosis virus (GV) of the apple codling moth (*Cydia pomonella*). Spod-X and Gusano target pests of vegetables, cotton, ornamentals, and other row crops; the codling moth GV is intended for use in apple and pear orchards.

Because viruses reproduce exclusively in living cells, there are only two production methods available: (1) the viruses can be produced in living insect hosts (in vivo); or (2) they can be produced in cell cultures in which the virus replicates in fermentation vats of growing insect cells (in vitro). CGI is focusing its development efforts on in vivo production systems because it believes this methodology provides the best opportunity for a cost effective way to produce large amounts of high quality product. The company is developing technologies and techniques to raise large numbers of healthy insects in aseptic conditions. The insects are then dosed with controlled quantities of the intended virus and maintained in carefully controlled environmental chambers. After harvesting the virus-killed cadavers, a separation and filtration system is used to isolate virus from the insect bodies. This process results in a technical grade product ready for formulation and packaging.

In 1991, a technical grade, lyophilized preparation of Spod-X was field tested in small plots and compared with *Bacillus thuringiensis* (Bt) for control of the beet armyworm (BAW) on tomatoes, peppers and garbanzo beans in the Central Valley of California and on tomatoes in the Guasave Valley of Sinaloa, Mexico (Kolodny-Hirsch et al., 1992). Weekly application at dose rates of 2.5×10^{11} and 12.5×10^{11} OB/ha [an OB or occlusion body is a proteinaceous matrix containing multiple virions] gave season-long control of BAW and provided significant reductions in plant damage compared with untreated control plots. On pepper and garbanzo bean, respectively, the level of insect reduction and foliar protection was significantly greater in plots treated with 12.5 x 10^{11} OB/ha of Spod-X than in plots treated with Bt. However, the degree of control achieved at the low rate of the virus was statistically similar to the high rate and to Bt on all crops tested. Bioassay of chrysanthemum leaves sprayed with Spod-X killed >98% of BAW immediately after application and retained ca. 50% of its original pathogenicity for 7-11 days. Two candidate ultraviolet light protectants, Orzan LS and skim milk, improved virus persistence under field conditions. The results of these tests confirm the efficacy of Spod-X, and provide encouragement for the continued development of other viral insecticides.

3. Endophytes for Crop Protection

Although numerous plant-microbe interactions have been identified over the past century, only a few species of microorganisms (e.g. *Rhizobium*) have been used commercially for their agricultural potential as symbionts. Genetic engineering of nonpathogenic endophytic bacteria presents an opportunity for the systemic delivery of biopesticides (fungicides and insecticides) within host plant tissues without direct genetic manipulation of the host plant. The advantages of such a strategy include sustained and protected activity of the biopesticidal compound.

Such products can be delivered as single application seed or mechanical inoculation treatments. This approach exploits the biological characteristics of a natural endophytic microbe, which can systemically colonize the xylem, and persist in host plants. Because of the containment of the biocontrol agent within the plant's vascular system and its inability to survive when the host dies, effects on nontarget organisms and the environment are minimal. Thus, the use of endophytes should have significant economic, environmental, and technological advantages over the use of current externally applied agrichemicals.

CGI scientists have conducted an extensive search for endophytes capable of colonizing the major crop species and have developed a collection of putative endophytes. Microorganisms were selected for this collection based on their ability to live inside and inability to survive outside the target crops. One such microbe is *Clavibacter xyli* subsp. *cynodontis* (Cxc), a fastidious, Gram-positive, coryneform bacterium. CGI has used Cxc as the base of a new class of biopesticides named InCide®.

Once introduced into corn seedlings by wound inoculation of the stems of young plants, or by seed inoculation, Cxc colonizes the xylem of roots, stems, leaves, and husks. Cxc populations of over 10^9 bacteria per gram of fresh stem tissue can be achieved within 2-4 weeks of planting inoculated seed. Cxc has been inoculated into over 100 commercial hybrids, all of which were colonized, generally in the range of 10^5-10^9 CFU/g fresh tissue. Since it is a vascular-limited endophyte, Cxc is not present in the seed produced by colonized plants. Recombinant strains of Cxc that are potential InCide candidates show host colonization patterns very similar to the wild-type organism.

The first InCide product involves a genetically engineered Cxc capable of producing the delta endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 (Cxc/Bt), a protein that is toxic to the larvae of many species of Lepidoptera (caterpillars). This product can be inoculated into corn to control the European corn borer (ECB) *Ostrinia nubilalis*. The extreme specificity of Bt toxins accounts for their record as an environmentally safe insecticide, with little or no toxicity toward vertebrates or beneficial insects. Since there had been little published information on Cxc, CGI has generated extensive data (Experimental Use Permit applications submitted to the U.S. EPA in 1987-1990) on the biology, ecology and environmental fate of this organism in order to aid in product development and registration.

Recombinant DNA techniques were used to modify wild-type Cxc to produce delta-endotoxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* strain HD-73. The Cxc/Bt recombinant strains contain either: 1) the intact cryIA(c) protoxin gene of HD73 (coding for the 133 kDa protoxin that is broken down via proteolysis in the alkaline gut of the corn borer to form the activated toxin); 2) gene fusions combining the toxic domain of HD-73 with various marker genes; or 3) truncated genes coding for the active toxic domain itself. Molecular geneticists at CGI have constructed plasmids that include: 1) the Bt coding region; 2) regulatory sequences that control transcription of the genetic code to messenger RNA (promoters); and 3) marker genes that confer selectable traits (such as resistance to antibiotics) for detection of transformants. The common enteric bacterium *Escherichia coli* is used as a host for transformation with the cloning vectors for the initial construction of these expression cassettes. Successful cassettes are then cloned into proprietary integration vectors containing a segment of DNA homologous to a segment of the chromosomal DNA of Cxc. When the integration vector is transformed into Cxc, crossing-over occurs between the homologous regions of the vector and the host chromosome, resulting in the stable insertion of the Bt expression cassette into the Cxc chromosome. The resulting Cxc/Bt recombinant produces HD-73 toxin proteins that can be identified by Western blotting (Burnette, 1981) using polyclonal antisera raised against crystal protein purified from sporulated HD-73 cultures.

Since 1986, CGI has developed proprietary methods for inoculation of Cxc/Bt into corn seeds. The current inoculation protocol involves imbibition followed by application of a pressure differential in a vessel containing a buffered suspension of Cxc cells. Seeds are then removed from the inoculation suspension and dried on a forced air dryer after which conventional seed treatments can be applied. 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. Although shelf life for crops is expected to vary, a shelf life of one season is expected for the first commercial corn seed product.

Development of an effective seed inoculation technology made large-scale field trials possible in 1989. These trials were conducted in Maryland, Illinois, Minnesota and Nebraska using plants from seed inoculated with Cxc and Cxc/Bt. Field tests in 1989-1991 (Fahey et al., 1991; Kostka, 1991; Turner et al., 1991) were conducted using both artificial and natural infestation and multiple corn hybrids. Inoculation with Cxc/Bt caused significant overall reduction in borer survival and tunnel damage, although the magnitude and consistency of this effect varied between hybrids. Current efforts are aimed at development of more potent strains of Cxc/Bt (producing more of the Bt toxin), elucidation of the relationship between Cxc/Bt population and Bt expression levels in plants and activity against ECB, and selection of corn hybrids that interact most favorably with Cxc/Bt colonization to produce the desired insect suppression and yield benefit.

Industrial scale seed inoculation will be performed with custom-built machinery designed to be incorporated 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 Plant Genetics (DeKalb, IL), NC+ Hybrids and Hoegemeyer Hybrids (Nebraska), and Rogers Brothers Seed Co. (Idaho).

4. Enhancing the Effect of Herbicides with Biologicals

The world market for synthetic chemical herbicides is an estimated $11.6 billion annually. This market reflects the single largest sector of pesticide products utilized world-wide. The growing concern over the continued use of synthetic herbicidal agents in agricultural and industrial applications has prompted efforts to identify biological methods to control major weeds. Although biological herbicides are environmentally attractive, these products have only seen limited commercialization because of (1) slow and/or insufficient activity, (2) limited weed control spectrum, (3) inconsistent activity and (4) high cost of production.

CGI is developing an enhanced weed control technology, X-tend™, in which bacteria are applied in combination with reduced rates of synthetic herbicides. The bacteria utilized in this system represent a range of well-characterized genera and species, as well as, several unidentified plant-associated strains. When applied as a tank mix with reduced rates of a synthetic herbicide, the resulting herbicidal activity is equivalent to or greater than that with the full labelled rate of the herbicide applied alone. In addition, the microbe/herbicide combination may expand the spectrum of weeds controlled. The synergy from sublethal levels of synthetic herbicides with bacteria that kill stressed plants provides an attractive, environmentally acceptable means of weed control. A critical feature of this system is that the microbial/herbicide mixture is prepared as a tank mix and is applied using standard application equipment.

Greenhouse screens conducted during 1990 and 1991 identified bacteria that synergized the herbicidal activity of synthetic herbicidal agents. These assays established a spectrum of activity, as well as compatibility and synergy with major synthetic herbicides. The herbicidal compounds when applied alone produced low levels of activity, while the microbe preparations did not cause any observable symptoms on treated plants. Under greenhouse conditions, bacterial preparations could synergize reduced levels of a synthetic herbicide (Christy et al., 1992). For example, a microbial preparation applied with 68 g/ha sulfosate yielded greater control than did the herbicide alone. Moderate to good control was observed with the broadleaf weeds morning glory and pigweed. Injury on narrowleaf weeds was twice that observed on plants treated with the herbicide alone.

Field studies conducted during 1991 evaluated six bacterial preparations in 21 tests conducted in eight states. Up to five herbicides were tested at each site (sulfosate, glufosinate, sethoxydim, fluazifop, nicosulfuron). The results of these trials confirmed that combining low rates of synthetic herbicides with a bacterial preparation increased weed control in comparison to treatments that only received the herbicide (Christy et al., 1992). Synergy between the herbicide and the bacterial preparations was observed when 1/4 to 1/5 the field rate of a herbicide was used in the combination. As the herbicide rate approached the recommended labelled rate, less synergy with the bioherbicide was observed. Although synergy was observed, the levels of activity were not sufficiently consistent across a range of environmental conditions.

Bacterial preparations with improved activity have been identified in greenhouse screens and are being evaluated in the field during 1992. Fermentation conditions are being optimized, and surfactants and novel adjuvants screened. Combinations of bacterial preparations, herbicides and adjuvants are being formulated for both greenhouse and field tests.

5. Kleentek® In-Vitro Sugarcane

Sugarcane is a vegetatively (nonsexually) propagated, perennial crop that is highly susceptible to disease. Seedcane, the stalks that are cut up and replanted, transmit viral, fungal or bacterial diseases directly to the subsequent crop. Since these diseases build up over two or three growing seasons, cane farmers in Louisiana must typically replant their fields. Growers typically use their own cane, thus propagating any diseases that were present in that field at the time of planting. About 80,000 acres are replanted every year, requiring 10,000 acres for the production of seedcane.

CGI has developed "disease-free" Kleentek seedcane in order to break the cycle of disease. Common, commercial varieties of sugarcane are used and the transmission of disease is eliminated by using specialized agronomic practices followed by cell culture techniques. Seedcane produced from these tissue culture-derived plants are thus disease-free at the time of planting. By planting Kleentek cane, growers achieve as much as 40% higher yields during the first year and average 15% more sugar per acre by planting Kleentek products. In addition to getting more sugar from their cane, farmers can send to the mill the 10-15% of their crop that normally is used as replant material in order to generate additional revenue for the grower and the mill. Diseases are eventually spread by wind, insects and farming equipment into fields planted with Kleentek seedcane. These diseases build up over time until yields decline and replanting becomes necessary. Repeat sales are therefore generated, but only after allowing the farmer to realize considerable additional profit.

CGI maintains a disease-free collection of the leading Louisiana sugarcane varieties at one of its Maryland facilities. Random sampling of this collection for laboratory analysis and rigorous visual inspection permits verification that the collection remains free of known diseases. Cell culture techniques have been refined by CGI such that large quantities of plantlets can be propagated. Trueness-to-type is not an issue since there is no appreciable somaclonal variation among regenerants. Thousands of disease-free plantlets can thus be quickly and economically produced from a single stalk of sugarcane.

After initial production, plantlets may be stored for up to 1½ years using long-term storage technology developed at CGI. This ability to store plantlets for months eliminates fluctuations in the labor required for the tissue culture process --- an issue of economics that plagues those involved with in vitro production of other crops. Plantlets are then shipped in sealed containers to Louisiana greenhouses, conditioned for 2 months and planted on isolated seedfarms where the risk of infection from diseased sugarcane is low. Plant pathologists and plant protection personnel regularly inspect Kleentek seed farms to monitor quality and control diseases. CGI uses proprietary equipment to harvest and distribute its seedcane to growers. The equipment strips leaves from the seedcane and harvests the seedcane in bundles to facilitate delivery. This proprietary equipment causes less damage to seedcane than conventional industry practices.

During the fall planting season, the Kleentek Division delivers 15,000 tons of product within hours of harvesting. Today more than half of Louisiana sugarcane farmers buy Kleentek seedcane, adding $10 million annually to the state's economy.

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References

Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203.

Christy, A.L., Herbst, K.A., Kostka, S.J., Mullen, J.P., and Carlson, P.S., 1992. Synergizing chemical herbicides with microbes. ACS Symposium Series. (In press).

Fahey, J.W., Dimock, M.B., Tomasino, S.F., Taylor, J.M., and Carlson, P.S., 1991. Genetically engineered endophytes as biocontrol agents: A case study from industry. In: Andrews, J.H., and Hirano, H.S. (eds.), Microbial Ecology of Leaves. Springer-Verlag, New York:401-411.

Groner, A., 1986. Specificity and safety of baculoviruses. In: Granados, R.R. and Federici, B.A. (eds.), The Biology of Baculoviruses, vol. I. CRC Press, Boca Raton:177-202.

Kolodny-Hirsch, D. M., Warkentin, D. L., Alvarado-Rodriguez, B. and Kirkland, R., 1992. *Spodoptera exigua* nuclear polyhedrosis virus as a candidate viral insecticide for the beet army worm. Submitted to J. Econ. Entomol.

Kostka, S.J., 1991. The design and execution of successive field releases of genetically-engineered microorganisms. In: MacKenzie, D.R., and Henry, S.C., (eds.), The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms. Agricultural Research Institute, Bethesda, MD:167-176.

Turner, J.T., Lampel, J.S., Stearman, R.S., Sundin, G.W., Gunyuzlu, P., and Anderson, J.J., 1991. Stability of the δ-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. *cynodontis*. Appl. Environ. Microbiol. 57:3522-3528.

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