# Chapter 9

# Endophytic Bacteria for the Delivery of Agrochemicals to Plants

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A novel delivery system (InCide) utilizing naturally occuring plant endophytic bacteria is being developed for the systemic delivery of agrichemicals in-planta. Host crops are systemically colonized by the endophyte, thus providing an environmentally safe vehicle with which to deliver potent, targeted, biologically-derived agrichemicals to the plants. InCide products, including those having insecticide, fungicide, bactericide, viricide, nematicide and plant growth enhancer activity are designed to be applied as a seed, seedling or young plant treatment only once during the life of a plant. They exploit the biological characteristics of a natural endophytic microbe by systemically colonizing the xylem and achieving sustained levels in their host plants. Because of their in-planta growth, effects on non-target organisms and the environment are minimized and significant advantages are afforded over current externally applied agrichemicals. The first InCide product is an endophyte of corn that has been genetically modified by insertion of a gene from Bacillus thuringiensis encoding for the production of a highly specific insecticidal protein (delta-endotoxin) active against the European Corn Borer. Refinement and scale-up of techniques for inoculation of this product into corn seeds will permit rapid commercialization of this new delivery system.

An examination of plant-associated microbes will illustrate the diversity of relationships existing between plants and "internally dwelling" plant-neutral or beneficial microbes. Just how some of these relationships evolved is not fully understood, but there are a variety of stable, mutualistic or symbiotic relationships between

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higher plants and microbial endophytes. Endophytes are microbes which live within plants. Maintenance of all plant-endophyte relationships require that although the plant may recognize the invader, it does not trigger any overt pathogenic response to the microbe or that the microbe successfully avoids the plant's defense reactions and does not cause disease. Numerous plant-endophyte interactions have been identified over the past century. To date, only the Rhizobium species, not even true endophytes but mild plant pathogens of the legume family, have been commercialized for their agricultural potential as symbionts.

Naturally Occurring Endophytes

The nodulation of legumes like soybean, clover and alfalfa by the gram-positive Rhizobium & Bradyrhizobium is by far the most thoroughly characterized plant-microbe relationship and the only one which has been exploited commercially. Rhizobium species in symbiotic association with plants are responsible for the conversion of as much as $2 \times 10^7$ tons of atmospheric $N_2$ per year to ammonium (1). This "fixed" nitrogen is then utilized by plants for the synthesis of amino acids and protein. In Rhizobium symbioses, the bacteria infect plant roots and are then contained within specialized structures called root nodules. A highly integrated expression of genes from both the plant and the bacteria lead to the formation of a morphologically distinct nodule, with its own meristematic regions and vascular innervation. A molecule very similar to the hemoglobin found in blood is synthesized to protect the oxygen-sensitive enzyme, nitrogenase, and the bacteria actually alter their morphology once established in a developing nodule. Without the protective environment and carbon supply afforded the bacteria by the plant, the bacteria would not perform the energy-intensive process of nitrogen reduction. Without the bacteria's significant nitrogen input, the plants would not, in many cases, be able to prosper in an otherwise nitrogen poor environment. This natural plant-bacterial symbiosis can therefore save farmers growing many leguminous crops from the considerable expense associated with fertilizer nitrogen input and may permit certain species to thrive in areas which they might otherwise not occupy.

Certain non-legumes also form root nodules which are responsible for nitrogen fixation. The microbe found in all non-legume root nodules (actinorhizal plants) studied to date is the actinomycete, Frankia. Most actinorhizal symbyotic associations are found in woody plants in the temperate or cold zones of the Northern Hemisphere. Over 178 species in 20 different genera have been documented to contain actinorhizal symbionts (2). There are two major morphological types of actinorhizal nodules: The Alnus-type or coralloid nodules are short, stubby, dichotomously branched growths on lateral roots. Myrica-type or lobed nodules are thin, and produce negatively geotropic rootlets at their apex (3).

Stem nodules, too, are formed on certain members of the Leguminosae family by Rhizobium bacteria. These nitrogen-fixing symbioses have been documented in three genera of predominantly tropical or hydrophytic plants --- Aeschynomene, Sesbania and Neptunia, which are represented by about 200, 70 and 15 species respectively (4). Nitrogen fixation by the endophyte in stem nodules and the protection of the oxygen-sensitive microbial enzyme seems to be accomplished via a similar strategem of leghemoglobin and nodule morphology to that adopted by the legume root nodules.

Leaf nodules occur predominantly in the families Rubiaceae and Myrsinaceae which contain over 400 nodule-forming species (5). These families of predominantly sub-tropical shrubs are nodulated by a gram negative, pleiomorphic bacterium in an association which in at least one species (Psychotria bacteriophila) has been shown to be obligate (6). In addition, one family of monocots, Dioscoreaceae has been identified as having a species with leaf nodules colonized by endophytic bacteria (7). Although the nature of these symbioses are not fully understood, it is hypothesized that the bacteria have a growth promoting effect.

There is at least one well-characterized association of a heterocystous, nitrogen-fixing blue green alga (cyanobacterium) with a plant. The cyanobacterium Anabaena sp. develops in a mutualistic relationship with the water fern (Azolla sp.). Anabaena is a filamentous, photosynthetic cyanobacterium containing heterocysts at intervals along a chain of vegetative cells. These heterocysts contain the requisite enzymes for di-nitrogen fixation. Azolla provides "safe harbour" for the $N_2$-fixing symbiont within cavities or nodules on the undersides of its leaves.

Another important and extremely widespread group of endophytes is the mycorrhyzal fungi. These fungi ramify throughout the root cells of plants and are hypothesized to function by aiding in the mobilization of relatively insoluble soil nutrients such as phosphate and zinc to the plant. Ascribing a generalized function to these endophytes is the object of considerable controversy. Endo-mycorrhyzae penetrate and ramify throughout certain root cells. Ecto-mycorrhyzae are those whose hyphae do not penetrate the root cells, but either envelop the roots and/or enter the space between root cells, usually outside the endodermis. The majority of higher plant taxa form associations with mycorrhyzal endophytes.

The Acremonium-type fungi are endophytes of certain types of grasses. They have been responsible for significant outbreaks of livestock toxicity in forage grasses, however, they are also responsible for conferring beneficial qualities to certain of their hosts. Enhanced performance, enhanced insect resistance and improved persistance of Lolium and Festuca species have been documented in turfgrass stands colonized by species of Acremonium (8).

And finally, there are the xylem-inhabiting bacteria. These bacteria are not ubiquitous, but certain species can reliably be recovered from their host plant species in a pattern which suggests that these bacteria may be part of the normal microflora of those hosts. Bacteria from 13 different genera were isolated from the xylem of healthy Citrus trees (9) which were colonized at levels ofup to 2 x $10^4$ CFU/g. The presence in Citrus of large numbers of diverse endophytic bacteria has been confirmed in subsequent studies (Zablotowicz, R.M., Allelix, personal communication),although this phenomenon appears to be the exception and not the rule. Xylem inhabiting endophytic bacteria may be responsible for the frequently observed inability of plant tissue culturists to "disinfest" cultures of rigorously surface-sterilized explant material. The failure of certain species to grow in-vitro could in fact be related to a stimulatory effect of an endophytic microbe which cannot be reproduced in the culture of isolated, bacteria-free explants. It is these xylem-inhabiting endophytes which we are exploiting.

## Crop Protection

Today's crop protection market was created and is presently dominated by the chemical industry. Biologicals account for only $100 million of this $14 billion market. Although synthetic chemicals presently dominate the market, the safety of these chemicals has been called into question. Biological pesticides are known to be safe but have lacked the efficacy of synthetic chemicals. The safety issue of chemicals and the efficacy problem of biological pesticides are consequences of their external application. Currently utilized methods for application of either chemicals or biologicals (e.g. foliar sprays, soil applications or seed treatments), all involve placing the treatment outside the plant even if the treatments themselves have systemic activity.

Without conventional pest control, food production in the U.S. alone could drop by as much as one-third. Despite the presence of chemical pesticides, more than one-third of the world's potential crop yield is still lost to fungal diseases and insect pests. Some chemical products leave toxic residues on the crop, leach into groundwater and are toxic to farm workers. While chemicals are effective against a broad spectrum of plant pests, they can destroy natural pest predators and other beneficial organisms. For many pesticides and for externally delivered biologicals, multiple applications are required because the products are diluted by rain, dissipated by wind and degraded by sunlight and microorganisms. The effectiveness of chemicals may decline over time due to the development of resistance by the target pests. There is much less risk of target pests developing resistance to endophyte-delivered agrichemicals because of the manner in which they are exposed and the vastly smaller quantities of active ingredient which thus need to be present in the crop. Finally, government regulations protecting consumers, workers and the environment are increasingly restricting the use of presently-registered chemical products and are making the introduction of new chemical products more difficult. The regulatory ground-rules for the introduction of biologicals are currently being written as numerous groups are in the process of attempting to field-test and register such products.

Endophytic Bacteria, A New Delivery System

InCide biopesticide technology is a system of crop protection in which biology is substituted for chemistry: Incide products are microorganisms genetically engineered to be both environmentally safe and efficacious. InCide products are designed to function internally in a plant's vascular system. They involve the use of naturally-occuring endophytes for the production and delivery of crop protectants and growth enhancers. Methods have been developed to screen, identify, recover and characterize endophytic microorganisms. CGI has conducted an extensive search for and analysis of endophytes capable of colonizing the major crops and now has a large collection of endophytes. Microorganisms are selected for the Company's collection based on ability to live inside, and inability to live outside, the target crops. CGI has indentified endophytes capable of colonizing corn, cotton, soybeans, wheat and rice as well as other major crops. InCide products are being developed to solve many of the problems associated with externally applied chemicals and biologicals. There are numerous economic, environmental and technological advantages to be realized by using endophytic bacteria for the delivery of agrichemicals to plants. Some of the advantages of this technology over conventional delivery of pesticides includes:

Economic Advantages.

- Single Application. Seed inoculation or inoculation of the juvenile plants with an appropriate endophyte results in colonization of that plant. The endophytes live inside the plant and are thus protected from the external environment.
  Externally applied chemicals and biologicals are unprotected and often require multiple applications.

- Minute Dosage. Seed application of endophytes can be accomplished with only milligram quantities of bacteria per acre. After application, the endophytes multiply inside each plant so that the final manufacturing step occurs after the point of sale.
  Conventional externally applied chemicals are generally applied in pounds per acre.

- Sustained Potency. Endophytes can thrive and produce the desired agrichemical for the duration of the plant's life.
  Externally applied products are adversely affected or rendered ineffective by the environment and by subsequent plant growth.

Environmental Advantages.

- Contained Activity. Endophytes survive and function only within the plant which they protect or enhance. Externally applied products are dispersed widely each time they are applied.

- Plant Dependency. Endophytes, by nature, do not surviveoutside the plant and therefore do not multiply or spread in the environment or remain active after harvest. Externally applied microorganisms must survive in the environment to be effective.

- No Toxic Residues. Endophyte products can be designed to be environmentally safe and degradable. Most externally applied pesticides must resist degradation to be effective.

Technological Advantages. Certain endophyte products may have the following technological advantages over genetically improved plants:

- Rapid Development. Changing the genetics of microorganisms is a more rapid and simpler process than changing the genetics of plants.

- Early Commercialization. Development of endophyte-based products does not require multi-year plant breeding programs.

- Wide Applicability. Endophyte-based products can be designed to function in a wide range of commercially useful varieties of the targeted crop.

- Yield. Endophyte delivery has minimal impact on host plant physiology (e.g. little or no effect on yield, vigour or quality). In many instances, changing the genetics of plants can reduce yield substantially.

- Repeat Sales. Many endophytes are not seed transmitted and farmers will need to purchase products based on these organisms each growing season. New plant varieties normally have repeat sales opportunities only when the purchased seed is a hybrid.

Progress is underway to develop the InCide delivery system for the delivery of insecticides, fungicides, nematicides, viricides and bactericides to protect corn, cotton, soybeans, rice and wheat, as well as other vegetable, forestry, and horticultural crops. We are developing a family of genetically engineered biopesticides using endophytes that colonize the major crops and genes that protect against major plant pests.

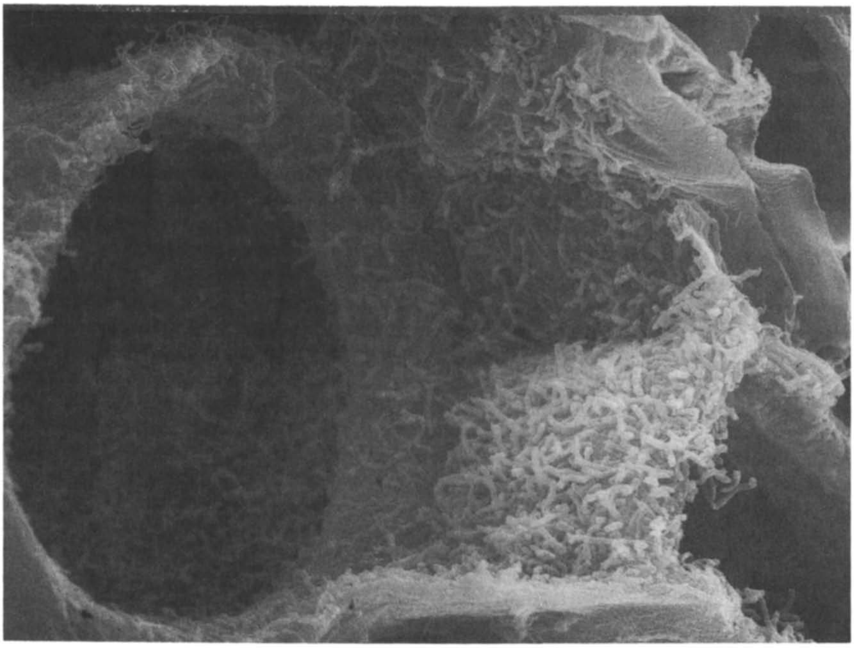

Figure 1. Lumen of xylem aerenchyma of Zea mays var. FR632 colonized with endophytic bacterium Clavibacter xylii subsp. cynodontis. Freeze-fracture surface reveals annular rings on left of plate. Magnification = 4250 X

The first InCide product under development is an insecticide for the United States and French corn market. We are conducting extensive field and greenhouse trials with our best corn endophyte. These tests are designed to accumulate data for product registration in the United States and France. We are seeking regulatory approval to field test our first recombinant corn insecticide product in the United States and France in the spring of 1988. This product uses the endophyte, Clavibacter xyli subsp. cynodontis, a Coryneform bacterium which was originally isolated from a non-crop plant and can be reproducibly introduced into corn (Fig. 1). This bacterium has been transformed by inserting a gene encoding for production of an insecticidal protein from the bacterium Bacillus thuringiensis (B.t.). B.t. has experienced decades of safe use as an insecticidal product. B.t. was first sold in France in 1939 and has been a leading biological insecticide in the U.S. since registration in 1961. There are more than one thousand B.t. isolates, each with one or more genes having a specific spectrum of insecticidal activity. Some B.t. genes are active against certain lepidoptera (caterpillars) and others are toxic to certain coleoptera (beetles). For its initial products, the Company purchased the rights to a B.t. gene effective against the caterpillar stage of European Corn Borer. Insect feeding trials have shown that the toxin produced by the B.t. gene is lethal to the European Corn Borer.

The wild-type endophyte rapidly colonizes the xylem of inoculated corn plants and achieves average levels of up to 1 x $10^8$ CFU/g fresh weight of tissue. It systemically colonizes the roots, stem, leaves and husks of inoculated plants and can be detected within a week of inoculation but it does not transmit via the seed of colonized plants.

Endophytes can be found throughout the plant kingdom. Natural endophytes exist which provide manifold benefits to the plants with which they are associated. We are using the tools of biotechnology to add specific beneficial qualities to carefully chosen endophytes. By selective enhancement, endophytes can be engineered to help solve some of agriculture's most pressing problems using a biological system which has been around for millions of years.

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