Chapter 5

BIOLOGICAL AND BIOTECHNOLOGICAL CONTROL ADVANCES IN *ARACHIS* DISEASES

JOHN L. SHERWOOD, MARVIN K. BEUTE, DON W. DICKSON, VERNON J. ELLIOTT, RICHARD S. NELSON, CHARLES H. OPPERMAN, and BARBARA B. SHEW

INTRODUCTION

Disease is managed or controlled by interrupting the disease triangle, the interaction of the host, pathogen and environment, that results in disease. Cultural methods, chemicals or resistant cultivars traditionally have been used to manage diseases, but in some cases these methods have not been successful. As knowledge accumulates about peanut (*Arachis hypogaea* L.), its pathogens, and the environment in which it grows, the application of biological and biotechnological approaches can be explored, expanded, and applied to manage peanut diseases.

FUNGAL PLANT PATHOGENS

Biological Control of Foliar Fungal Pathogens

Advantages of Biologically Based Disease Control. Control of diseases by biological agents should have minimal environmental impact and be compatible with sustainable agriculture. In addition, it has been proposed that biological control agents are less expensive, take less time to develop, and are less expensive to market than chemical control agents (Carlton, 1990; Nelson, 1991). However, regulatory issues relating to the release of biological control agents, particularly genetically engineered microorganisms, may present an obstacle for the successful adoption and commercialization of biological control agents (Cook, 1993).

A Biological Perspective of Biological Control. A considerable number of microbes antagonistic to peanut pathogens have been discovered. However, due to the variability in efficacy under field conditions, only a few biological control agents have been marketed. Lack of success may be a result of following a "chemical model" in development of biological control agents in which candidate agents are screened for activity *in vitro*, followed by controlled environment and greenhouse testing. Promising candidates proceed to small scale field trials where information on efficacy against pathogens, phytotoxicity to plants, and formulation are collected. This model works well with properly formulated chemical agents because they are not influenced much by the complex biological and physical environment at

the plant surface and only have to persist as a deposit or in plant tissue to be effective. By contrast, biological agents must colonize the habitat, compete with resident microflora, and actively metabolize to control disease. Information on microbial ecology, epidemiology, plant ecology, and plant molecular biology is required to develop successful biological controls. For routine development of biological controls a process that evaluates the parameters controlling efficacy of biocontrol agents must be developed.

Basic Mechanisms of Biological Control. Biological control has been formally defined as "the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man" (Cook and Baker, 1983). Biological control can be achieved through antagonism (including competition, antibiosis, and parasitism and predation) and by inducing plant defense reactions. Induced resistance or plant immunization has not been reported in the genus *Arachis*. Host resistance, although considered to be a form of biological control, is not covered here. Because the "chemical model" has been followed in the development of biological controls, reports of antibiosis have been considerable. Antibiosis is the inhibition or destruction of one organism by the metabolic products of another organism. Products may be antibiotics, toxins, enzymes, or compounds like ammonia and hydrogen cyanide; some antagonists can exhibit several mechanisms (Chet, 1987).

Competition. Microorganisms occur in mixed communities with limited resources. Microorganisms have mechanisms to increase the efficiency of nutrient uptake and utilization, and competition can be an effective form of biological control when the proper selection of antagonist is added to the soil. For example, competitiveness for iron can be exploited for inhibiting pathogens. Although iron is abundant in the soil, the free ferric ion (Fe^{3+}) needed by microorganisms can be limiting in many soils. Bacteria have siderophores, high iron affinity molecules that are excreted into the aqueous environment around the cell, and outer membrane transport proteins which bind the siderophore-Fe^{3+} complex and transport the Fe^{3+} inside the cell (Neilands, 1981). Fusarium wilt of cucumbers (*Cucumis anguria* L.), flax (*Linum usitatissimum* L.), and radish (*Raphanus sativus* L.) was reduced by the addition of a siderophore-producing strain of *Pseudomonas putida* to soil (Scher and Baker, 1982), but efficacy was high only when Fe^{3+} was limiting in the system. *In situ* examination of Fe^{3+} regulated siderophore biosynthesis indicated that Fe^{3+} is only slightly limiting on leaf and root surfaces (Lindow and Loper, 1990; Lindow, 1991). A detailed discussion on the role of siderophores in biological control has been published (Leong, 1986).

The competition for other nutrients such as carbon and nitrogen is less characterized. At the plant surface there are resident microbes which can protect the plant from pathogens (Andrews, 1981, 1992; Dik, 1991). The protection due to competition is seldom noticeable in the field, but can be experimentally demonstrated. Benomyl application on rye (*Secale cereale* L.) foliage decreased saprophytes by tenfold (Fokkema *et al.*, 1975) which resulted in infection by a benomyl resistant strain of *Cochliobolus sativus* (Ito and Kuribayashi) Drechs. ex Dastur and an increase in disease.

The epiphytic microflora of peanut leaves is quite complex. Early to midseason bacterial communities consist of species of *Clavibacter, Curtobacterium, Erwinia, Aureobacterium, Methylobacterium, Arthrobacterium, Xanthomonas, Pseudomonas,* and *Flavimonas.* These genera, listed in order of frequency, account for 70% of the population. An additional 24 genera account for the rest of the population (V.J. Elliott, unpubl. data, 1993). Yeast and filamentous fungi are also present but have not been characterized. Little is known about the influence of nutrients on infection of peanut by foliar pathogens so it is difficult to predict how epiphytic microflora might affect infection.

Antibiotics. Antibiotics are metabolic compounds secreted by microorganisms which are inhibitory to other microorganisms. There is no clear distinction between antibiotics and other toxic metabolites, but the term antibiotic generally is used for metabolites that are inhibitory at low concentrations. Antibiotics produced by microorganisms in culture may be detected by a zone of inhibition of microbial growth around the antibiotic producing colony. Antibiotics have received attention in biological control research because such mechanisms are readily detected in the chemical model of research. The role of antibiotics in biological control has been reviewed (Fravel, 1988).

Antibiotic producing bacteria are effective against foliar diseases (Baker *et al.*, 1985; Thomashow and Weller, 1988; Kempf and Wolf, 1989; Rytter *et al.*, 1989). A bacterial suspension or culture filtrate of *Erwinia herbicola* inhibited subsequent establishment of *Puccinia recondita* f. sp. *tritici* on wheat. *Erwinia herbicola* produces herbicolin A which inhibits uredospore germination (Kempf and Wolf, 1989). Efficacy under field conditions was not evaluated. *Bacillus thuringiensis* or *Pseudomonas cepacia*, bacteria both strongly antagonistic to *Cercospora arachidicola* Hori *in vitro*, were evaluated on peanut in the field (Knudsen and Spurr, 1987). *Pseudomonas cepacia* strain Pc742 produces an antibiotic tentatively identified as pyrrolnitrin (Janisiewicz and Roitman, 1988). The antagonistic mechanism of *B. thuringiensis* strain HD-1 is unknown, but *B. thuringiensis* is closely related to *B. cereus,* that produces antifungal antibiotics. Disease control from applications of bacterial antagonists at 14-day intervals, although statistically better than unsprayed controls, was considered unacceptable in comparison to standard fungicide treatment. Even applications at 7-day intervals were not effective (V.J. Elliott and H. Spurr, unpubl. data, 1989). Either poor survival of the biological control agents on foliage or limited nutrients present on the leaf surface to support antibiotic biosynthesis may have contributed to the lack of efficacy.

Some antagonists produce enzymes that act on the pathogen or catalyze the production of toxic products from substrates in the environment. Kokalis-Burelle *et al.* (1991) found that addition of chitin amendments to peanut foliage resulted in an increase of chitinolytic bacteria from 1 to 40% of the total population. Application of a chitinolytic strain of *B. cereus* to peanut foliage with chitin reduced infection by *C. arachidicola* by 60%. Fungi such as *Trichoderma* spp. also produce the enzymes chitinase and β-1,3-glucanase,

which degrade the chitin and β-glucans of fungal cell walls (Elad *et al.*, 1982a). These enzymes are important in the mycoparasitic ability of *Trichoderma* spp. (Chet, 1987).

Indirect enzymatic action also can function in antibiosis. The bacterial antagonist *Enterobacter cloacae* produces enough ammonia to be toxic to soilborne pathogens like *Pythium ultimum* (Howell *et al.*, 1988). Ammonia is thought to be a byproduct of the deamination of amino acids metabolized as a carbon source when sugars are not available. Ammonia production by *E. cloacae* is inhibited by the sugars D-glucose, D-galactose, sucrose, and β-methyl-D-glucoside. This may explain the failure of biological controls on seeds that produce large amounts of sugar during germination (Nelson *et al.*, 1986). Enzymatic mechanisms also have been identified in certain fungal antagonists. *Talaromyces flavus* produces glucose oxidase, which catalyzes the conversion of glucose into gluconic acid and hydrogen peroxide. Hydrogen peroxide can accumulate and kill microsclerotia of *Verticillium dahliae* Kleb. (Fravel *et al.*, 1987; Kim *et al.*, 1988).

Parasitism and Predation. Mycoparasitism is the parasitism of one fungus by another and has been extensively reviewed (Kranz, 1981; Sundheim, 1986; Whipps *et al.*, 1988; Manocha, 1991). Numerous mycoparasites of peanut pathogens have been reported (Table 1) and the use of mycoparasites for biocontrol has been investigated (Mitchell *et al.*, 1986; Ghewande, 1990; Subrahmanyam *et al.*, 1990). In laboratory tests, detached leaves from peanut previously inoculated with *Verticillium lecanii* had fewer infections when subsequently inoculated with *Cercosporidium personatum* (Berk. and Curt.) Deighton and *Puccinia arachidis* Speg. (Subrahmanyam *et al.*, 1990). In another study, culture filtrates of *V. lecanii* and *Penicillium islandicum* were the most effective of several mycoparasites when evaluated in the laboratory and field for biological control of *P. arachidis* (Ghewande, 1990).

Table 1. Mycoparasites reported on foliar pathogens of *Arachis hypogaea*.

Mycoparasite	Pathogen	Reference
Acremonium persicinum	*Puccinia arachidis*	Ghewande, 1990
Acremonium salmoneum	*Puccinia arachidis*	Sharma and Agarwal, 1988
Darluca filum	*Puccinia arachidis*	Subrahmanyam & McDonald,1987
Dicyma pulvinata	*Cercosporidium personatum*	Shokes & Taber, 1983; Mitchell *et al.*, 1986
Eudarluca caricis	*Puccinia arachidis*	Ghewande, 1990
Hyalodendron sp.	*Puccinia arachidis*	Reddy, 1989
Penicillium islandicum	*Puccinia arachidis*	Ghewande, 1990
Trichoderma harzianum	*Puccinia arachidis*	Govindasamy & Balasubramanian, 1989
Tuberculina costaricana	*Puccinia arachidis*	Ghewande, 1990
Verticillium lecanii	*Puccinia arachidis*	Ghewande, 1990; Subrahmanyam *et al.*, 1990
Verticillium lecanii	*Cercospora arachidicola*	Subrahmanyam *et al.*, 1990
Verticillium lecanii	*Cercosporidium personatum*	Subrahmanyam *et al.*, 1990

Using culture filtrates might circumvent the problems of establishing the biological control agent on the plant.

Dicyma pulvinata was shown to colonize up to 87% of *C. personatum* lesions under field conditions, but the amount of disease control was not reported (Mitchell *et al.*, 1987). Although this mycoparasite was not effective as a protectant, it might reduce sporulation. Isolates of *D. pulvinata* varied in their ability to colonize lesions, indicating that improvement of control by strain selection may be feasible (Mitchell *et al.*, 1986).

Whipps *et al.* (1988) attributed the lack of commercialization of mycoparasites to a poor understanding of their behavior and an empirical approach to biological control. Parasites and predators are important in regulating many insect pests, but the effects of mycoparasites on pathogen population are not understood.

Although the terms predation and parasitism often are used interchangeably, predation on plant pathogenic microorganisms seems to be rarer than parasitism. Mycophagous amoebae present in soil prey on chlamydospores and hyphae of *Thielaviopsis basicola* and conidia of *Cochliobolus sativus* (Anderson and Patrick, 1978). It is likely that such soil inhabiting predators also prey on peanut pathogens, but the effect on disease is not documented. A mycophagous thrips, *Euphysothrips minozzii* Bagnall, has been observed feeding on pustules of *P. arachidis* (Shanmugam *et al.*, 1975)

Induced Resistance. Induced resistance or plant immunization, a resistance resulting from a challenge by a potential pathogen, has been associated with structural and chemical changes in the plant. Other stimuli that induce changes include chemical compounds derived from the pathogen, nonbiological chemicals, and physical treatments (Kuc, 1987). Localized effects include the accumulation of phytoalexins, lignin and hydroxyproline rich proteins. Systemic effects include the production of chitinase, glucanase, protease inhibitors, and peroxidases (Sequiera, 1987). These structural and chemical changes make the plant resistant to a wide range of pathogens and abiotic stresses (Tuzun and Kuc, 1991). Induced resistance is a widespread phenomenon and has been reported in several legume species of *Glycine*, *Pisum*, *Vigna*, and *Phaseolus*, but not in *Arachis* (Tuzun and Kuc, 1991). Although peanut does have typical plant defense mechanisms such as the production of phytoalexins (Strange *et al.*, 1988), it is uncertain whether peanut has been examined for induced resistance responses.

Potential for Biological Controls of Foliar Peanut Diseases

The availability of effective and affordable fungicides in the U.S. has reduced the impetus for generating the information base needed to develop biological control agents. Two major foliar diseases of peanut illustrate the information needed for developing biological controls.

***Early Leaf Spot* (Cercospora arachidicola) *and Late Leaf Spot* (Cercosporidium personatum).** Early and late leaf spot are generally considered difficult diseases to control because a considerable amount of new, unprotected, foliage can develop between fungicide applications when

the canopy is rapidly growing (Elliott and Spurr, 1993). A fungicide must be effective at suppressing inoculum on the older part of the canopy or the new foliage will be infected. A biological control agent that colonizes leaves as they emerge would be analogous to a fungicide sprayed on a daily basis. Hence, biological control agents could prove highly effective if they could rapidly colonize new leaves.

Although current efforts for the biological control of early and late leaf spots are directed at protecting the foliage, controlling the overwintering inoculum also might be feasible. Dormant stromata in leaf spot lesions are the most likely mode of survival in the soil (Nuesry, 1981; Porter *et al.*, 1982) and crop rotation is recommended to reduce initial inoculum (Hemingway, 1954; Kucharek, 1975; Porter *et al.*, 1982). Hemingway (1954) reported that longer rotations were required when the biological activity of the soil was low. However, reduction of inoculum may be accomplished with biological controls. The biological and physical factors causing the destruction of inoculum are not well understood. Termites were reportedly responsible for destruction of infected peanut leaf litter in soils in Tanganyika (Hemingway, 1954). The role of other soil organisms is not known. Mycoparasites which could destroy overwintering inoculum in crop debris in the soil might prove to be effective biological controls. *Dicyma pulvinata* was reported to colonize *Cercosporidium* lesions on leaflets on the soil surface (Mitchell *et al.*, 1987). More epidemiological studies on fate of overwintering inoculum and initiation of seasonal epidemic are necessary.

Summary. Several antagonists have been evaluated against leaf spots and rust (Knudsen and Spurr, 1987; Mitchell *et al.*, 1987; Ghewande, 1990; Kokalis-Burelle *et al.*, 1991), and early leaf spot was reduced with chitin amendments and chitinolytic bacteria (Kokalis-Burelle *et al.*, 1991). However, disease control in the field with biological control agents has generally been poor. For foliar biological control agents to be realized, research in an ecological framework should be conducted rather than following a chemical model of development. About $100 million is spent annually in the U.S. for fungicide based disease control in peanut, yet relatively few dollars have been spent on gaining information on the epidemiology, population genetics, peanut microflora, or host-pathogen interaction of peanut diseases. Biological control could make considerable gains if investment in basic research on peanut diseases were made.

Biological Control of Soil-borne Fungal Pathogens

Biocontrol has been studied extensively for several soilborne pathogens of peanut, including *Sclerotium rolfsii* Sacc., *Sclerotinia minor* Jagger, and *Rhizoctonia solani* Kuhn. Biological control of these pathogens has been demonstrated in specialized production systems, but there have been few reports of successful biocontrol of peanut diseases caused by soilborne pathogens. In horticultural and greenhouse crops, biocontrol agents may be applied after fumigation, as dips or with high rates of organic amendments. These practices are not profitable on peanut. In addition, the unique fruiting habit of peanut reduces options that couple biological with cultural control.

Peanut requires protection against soil-borne pathogens throughout the growing season. The crop is in the field for a minimum of 90 to more than 150 days, and reproductive growth may extend over many weeks. Agents that control diseases caused by soil-borne pathogens must either be effective in reducing initial inoculum, or be active and persistent in suppressing disease development. Most biological control agents lack these properties.

Biocontrol in peanut, as in most crops, has focused on a few genera of antagonists, such as *Trichoderma* and *Gliocladium*. These antagonists are abundant in soil, easy to isolate and culture, and tolerate a wide range of temperatures and pH. Unfortunately, *Trichoderma* spp. are poor competitive saprophytes because they require a food base to ensure high levels of activity (Papavisis, 1985). *Trichoderma* spp. also have low rhizosphere competence, i.e. colonization is limited to the root surface and the soil influenced by the root. Recent emphasis on antagonists such as *Sporidesmium* and *Bacillus* has attempted to address these problems. Results outlined below with individual soil-borne pathogens of peanut illustrate some successes and challenges in biological control of the diseases they cause.

Sclerotium rolfsii. *Trichoderma* spp. parasitize sclerotia and mycelium of *S. rolfsii in vitro* and *in vivo* (Papavisas, 1985). Increased incidence and severity of southern blight epidemics on peanut, after application of fungicides for leaf spot control, was attributed to the inhibition of *Trichoderma viride* by benomyl (Backman *et al.*, 1975). Leaf spot sprays suppressed natural biocontrol of southern blight by inhibiting *Trichoderma*. *Sclerotium rolfsii*, which is relatively insensitive to benomyl, grew profusely under these conditions.

In early studies on various crops, colonized amendments applied to control *S. rolfsii* were used at rates too high to be of practical use in peanut culture (e.g., Wells *et al.*, 1972). An application method for peanut was later developed by Backman and Rodriguez-Kabana (1975) by formulating *Trichoderma harzianum* in diatomaceous earth impregnated with molasses. The dried formulation was stored at 5 C until application at 70 and 100 days after planting at a rate of 140 kg/ha and southern blight was controlled as well as with PCNB (pentachloronitrobenzene).

Trichoderma spp. have been cultured in commercial fermenters on inexpensive liquid media such as molasses plus yeast (Papavisas *et al.*, 1984). These cultures can be dried, ground, and formulated with Pyrax (anhydrous aluminum silicate), or pelleted with alginate and bran (Fravel *et al.*, 1985). When these formulations were compared in greenhouse tests, only *T. harzianum* applied as young mycelial cultures on moist bran controlled *S. rolfsii* on bean (*Phaseolus vulgaris* L.) (Papavisas and Lewis, 1989).

Timing of application for maximizing biological control has not received much attention. Csinos *et al.* (1983) applied four different formulations of *T. harzianum* to peanut at pegging, or on demand (disease initiation) and were unable to control southern blight or increase yield. They suggested that the treatment failed because the antagonist was active only until the added food base was exhausted (3 to 8 days), whereas *S. rolfsii* was active for several additional weeks.

Although *Trichoderma* spp. have received the most attention for activity against *S. rolfsii*, other fungi have been tested. *Talaromyces flavus* suppressed *S. rolfsii* on bean in greenhouse tests (Madi *et al.*, 1991) and has been formulated for field use (Fravel *et al.*, 1985). *Gliocladium virens* was more effective than *T. harzianum* in suppressing damping-off of bean caused by *S. rolfsii* in greenhouse tests (Papavisas and Lewis, 1989), but only against isolates that produced tan sclerotia. Isolates producing more melanized sclerotia were not controlled. *Gliocladium virens* (formulated in alginate and wheat bran pellets) reduced survival of sclerotia in tomato fields by 56 to 100% after 6 weeks, but control of southern blight of tomato (*Lycopersicon esculentum* Mill.) was effective only at low levels of disease (Ristaino *et al.*, 1991). More thorough experimentation is needed to determine efficacy of these organisms against *S. rolfsii* and optimal methods for their use on peanut.

Chemical control of southern blight on peanut is difficult because disease outbreaks are unpredictable, resulting in poorly timed or unnecessary fungicide applications. Even if applications are timely, the fungicide may not reach the fungus. Similar problems have impeded progress in biocontrol of southern blight. Several investigators have attempted to address these problems in other crops by integrating biological with other control methods.

Solarization, the solar heating of soil under plastic mulch, reduces inoculum density of *S. rolfsii* in soil, and has been used with antagonistic fungi to control southern blight of tomato (Ristaino *et al.*, 1991), bean, and potato (*Solanum tuberosum* L.) (Elad *et al.*, 1980). Even if elevated soil temperatures are too low to kill the fungus, sclerotia are weakened and become vulnerable to attack by native or added antagonists (Henis and Papavisas, 1983; Lifshitz *et al.*, 1983; Ristaino *et al.*, 1991). Solarization may have limited use for long season peanut grown in temperate regions because effective treatment requires several weeks of warm, sunny days during noncropping periods (Mihail and Alcorn, 1984; Ristaino *et al.*, 1991). Applying and maintaining covering tarps for solarization is too expensive for normal peanut culture, but solarization coupled with biocontrol might be useful in organic production of short season peanut in the U.S.

Fumigation with methyl bromide kills natural antagonists of *S. rolfsii*, and surviving or introduced sclerotia may initiate more disease than in untreated plots (Elad *et al.*, 1982b). Nevertheless, biocontrols can be effective following fumigation because antagonists readily attack sclerotia weakened by such treatment (Elad *et al.*, 1982b).

The increasing use of metam sodium on peanut offers encouraging opportunities for integrated control of southern blight. Sublethal doses of metham sodium weaken sclerotia of *S. rolfsii*, which then can be attacked and killed by biocontrol agents (Henis and Papavisas, 1983). Unfortunately, when *Trichoderma* spp. were reintroduced into metam sodium-treated soil as conidia they did not proliferate (Lewis and Papavisas, 1984). Recolonization might be aided by introducing mycelia of *Trichoderma* spp. in a food base (Papavisas, 1985).

Sclerotinia minor. Biocontrol of *S. minor* on peanut has received little

attention, but much effort has been directed towards biocontrol of *Sclerotinia* spp. on other crops particularly with *Sporidesmium sclerotivorum* which parasitizes sclerotia of *Sclerotinia* spp. (Adams and Ayres, 1981; Adams, 1990). *Sporidesmium sclerotivorum* was isolated from 34 of 73 field soils collected throughout the U.S. and it suppressed *Sclerotinia* (Ayres and Adams, 1979). Unlike most other fungi studied for biocontrol, *S. sclerotivorum* is a hemibiotroph (Whipps, 1991); it is difficult to culture and lives in close association with its host. Macroconidia in soil germinate only in the presence of host sclerotia, which may be attacked from a distance of more than 2 cm. Likewise, hyphae of *S. sclerotivorum* may grow through soil from colonized sclerotia and establish secondary infection cycles (Adams, 1990).

Methods have been developed for culture and field application of *S. sclerotivorum* as a biocontrol agent (Ayres and Adams, 1983). The fungus can be grown in a vermiculite medium on a commercial scale, suspended in water, and sprayed on the crop. When applied at rates ranging from 0.2 to 20 kg/ha, the highest rate was the most effective in controlling *S. minor* on lettuce (*Lactuca sativa* L.) (Adams and Fravel, 1990). The spray was applied to the diseased mature crop, which was disked immediately. In the subsequent lettuce crop, disease incidence was 54% in treated plots and 89% in untreated plots. Inoculum density of *S. minor* also was significantly reduced. Postharvest application of biocontrols might be used to reduce inoculum densities of *S. minor* or *S. rolfsii*. Unfortunately, inoculum of both pathogens is very efficient; severe epidemics of Sclerotinia blight on peanut can develop at inoculum densities of one or two sclerotia/100 g soil (Porter and Steele, 1983).

Application of *S. sclerotivorum* at the end of the season has not been tested on peanut. In addition to reducing inoculum in later crops, this method might be used to avoid unfavorable interactions among *S. minor*, *S. sclerotivorum*, and peanut fungicides. Chlorothalonil, which is used for peanut leaf spot control, enhances Sclerotinia epidemics under some conditions (Porter, 1980), and stimulates toxin and enzyme production by *Sclerotinia* (Hau and Beute, 1983). Chlorothalonil also is toxic to the antagonist *S. sclerotivorum*, as is PCNB used for control of southern blight (Adams and Wong, 1991)

Sporidesmium sclerotivorum has received limited testing on peanut. A formulation applied at planting and broadcast four times during the season at 37.4 L/ha reduced Sclerotinia blight by 47%. In comparison, the fungicide Rovral reduced disease by 81% (Phipps and Porter, 1990).

Coniothyrium minitans is an antagonist of *Sclerotinia* spp. (Turner and Tribe, 1976; Huang, 1980), but field tests in several locations and diverse crops have met with limited success (Adams, 1990). Several other fungi including *T. harzianum*, *Talaromyces flavus* (McLaren *et al.*, 1983), and *G. virens* (Tu, 1980) are antagonists of *Sclerotinia* spp. (Adams, 1990) and might suppress Sclerotinia blight. The incidence of Sclerotinia wilt in sunflower (*Helianthus* sp.) was reduced from 81% in control to 27% in treated plots with *T. flavus* (McLaren *et al.*, 1983).

Biocontrol of Sclerotinia blight in conjunction with other physical or

chemical methods has not been extensively studied. In Texas, significantly fewer sclerotia of *S. minor* were found than in untreated soils in which peanut had been grown (Woodard and Simpson, 1991). Antagonists (a *Trichoderma* spp. or a *Gliocladium* spp.) used in this test did not reduce inoculum densities more than solarization alone.

Cylindrocladium crotalariae *(Loos) Bell and Sobers.* Research on biocontrol of Cylindrocladium black rot (CBR) has focused on attempts to identify, characterize, and create soils suppressive to CBR. Peanut soils have not been surveyed extensively for suppressiveness, but several lines of evidence suggest that biological suppression of CBR exists. First, the amount of CBR that developed from similar inoculum densities of *C. crotalariae* differed in two field soils tested in a uniform greenhouse environment even though the soils had similar physical properties (Barron and Phipps, 1983).

Peanut grown after soybean [*Glycine max* (L.) Merr.] in rotation experiments consistently had less disease than peanut grown after peanut at similar pathogen inoculum densities (Black and Beute, 1984; Sidebottom and Beute, 1989). Disease suppression in soybean rotations was attributed to higher soil nitrogen levels (Pataky *et al.*, 1984), decreased microsclerotial size and efficiency (Black and Beute, 1984), and changes in soil microbe populations (Black and Beute, 1985).

Fumigation appeared to release soybean-induced suppressiveness to CBR in some soils, but not others (Black and Beute, 1985; Sidebottom, 1988). The total population of bacteria and actinomycetes also were greater in bulk soils from soybean rotations and were statistically associated with lower root rot ratings. However, root rot ratings were not affected when actinomycetes isolated from soybean soils were introduced into steamed or field soils.

Three or more years of continuous peanut induced suppression of CBR (Black and Beute, 1984; Sidebottom and Beute, 1989). Monoculture induced decline has been observed in several pathosystems, and multiple mechanisms have been proposed (Schippers and Gams, 1979). None of these mechanisms has been explored in the peanut-CBR pathosystem.

Biocontrol by addition of specific antagonists to *C. crotalariae* has received limited attention. Several organisms antagonistic to *C. crotalariae in vitro* did not suppress disease in field microplots (P. M. Phipps, pers. commun., 1993). *Trichoderma* spp. (Sidebottom, 1988) and *Gliocladium* spp. have been observed in association with *C. crotalariae*, but they have not suppressed CBR in greenhouse tests; formulated biological control agents have not controlled CBR in field trials (P. M. Phipps, pers. commun., 1993).

Certain cruciferous cover crops could be used to suppress population densities of *C. crotalariae* because these plants release isothiocyanate compounds as they decompose in soil (Lewis and Papavisas, 1971). Microsclerotia of *C. crotalariae* are sensitive to methyl isothiocyanate and this sensitivity is exploited in chemical control of CBR with metham sodium (Phipps, 1990). Soil amendments of rapeseed (*Brassica napus* L.) meal reduced inoculum densities of microsclerotia in peanut soils in five of six

greenhouse tests (Adamsen *et al.*, 1992). Use of cruciferous cover crops or residues for suppression of CBR need further investigation.

Rhizoctonia solani. Natural suppressive soils to *R. solani* was associated with *T. hamatum* and low pH, which favors growth of *Trichoderma* spp. (Chet and Baker, 1980, 1981). Biocontrol of *R. solani* on many hosts has been attempted with *Trichoderma* spp. which induces suppressiveness to *R. solani*. Because *R. solani* readily colonizes organic residues in soil, when biocontrol agents were applied in a food base carrier disease was enhanced in the carrier-only checks. To overcome the stimulatory effects of any added food base, biocontrol agents used as biopesticides must be antagonistic, have competitive saprophytic ability, or be efficacious without an added food base.

Multiple isolates of *T. harzianum, T. hamatum, T. viride*, and *G. virens* have been formulated in alginate pellets for control of *R. solani*. Eight of 11 isolates tested were effective in decreasing survival and colonization in soil, but only one isolate of *T. hamatum* and one isolate of *G. virens* prevented damping-off of cotton (*Gossypium hirsutum* L.) in the greenhouse.

Field applications of *Trichoderma* spp. for biocontrol of *R. solani* have given mixed results. An isolate of *Trichoderma* was as effective as deep plowing in controlling fruit rot of cucumber (Lewis and Papavisas, 1980), but deep plowing was the only effective treatment in controlling root rot of bean (Lewis *et al.*, 1983). Deep plowing suppresses disease by inhibiting colonization of crop residues by *R. solani*, thereby decreasing inoculum potential.

As with *S. rolfsii* and *S. minor*, agents for biocontrol of *R. solani* have been combined with physical and chemical treatments. Application of *T. harzianum* combined with solarization or fumigation with methyl bromide gave control on potato superior to that obtained with single treatments (Elad *et al.*, 1980).

Inconsistent results in biocontrol of *R. solani* could be related to differences in the diseases and isolates targeted. The anastomosis group (AG) of a *R. solani* isolate typically influences the hosts attacked, the symptoms that develop (Ogoshi, 1987) and possibly the success of biological control. In peanut, *R. solani* attacks seed, seedlings, roots, limbs, crowns, pegs, and pods, and sometimes occurs in disease complexes. Isolates of AG-1, AG-2, and AG-4 have been reported from peanut, with AG-4 apparently predominating (Bell and Sumner, 1984). Biocontrol of damping-off and root rot of peanut caused by an AG-4 has been attempted with *T. harzianum* and two isolates of *G. virens*. The antagonist was grown on a lignite and stillage carrier and applied in furrow at 9 g/m of row. A gliotoxin-minus isolate of *G. virens* significantly suppressed damping-off and disease development and significantly increased root and shoot growth in comparison to *R. solani*-infested controls. Disease and growth suppression occurred in the *Gliocladium* treatment, but the carrier alone had greater amounts of disease (Jones *et al.*, 1984). The AGs of *R. solani* isolates used in future work should be specified to allow for comparison of results.

Control of *R. solani* with binucleate *Rhizoctonia*-like (BNR) fungi or nonpathogenic isolates of *R. solani* has been tried (Burpee and Goulty, 1984; Cardoso and Echandi, 1987b). These fungi were cultured on a medium such

as oat grains and applied in-furrow with a cover crop or at planting (Cardoso and Echandi, 1987a). Cultures of BNR fungi can be stored several weeks, with some isolates withstanding a broader range of environments than others (Escande and Echandi, 1991). The antagonism may involve competition for nutrients or induced host resistance (Cardoso and Echandi, 1987b). Many isolates of BNR fungi have been screened for antagonism to *R. solani* and suppression of disease (Cubeta, 1991), and some BNR isolates controlled root rot caused by AG-4 isolates on bean where *T. harzianum* was not effective (Cardoso and Echandi, 1987a). Preliminary trials with commercially developed BNRs for control of *R. solani* on peanut have not been encouraging (Phipps and Hagedorn, 1991).

Study of naturally occurring suppression of *R. solani* may provide improved strategies for biocontrol of the diseases it causes. Monoculture-induced declines of *R. solani* have been associated with the presence of *Bacillus* spp. and other gram negative bacteria (Hyakumachi *et al.*, 1990). Suppression of *R. solani* was related to a combination of chemical, physical, and biological factors in Hawaiian soils (Ko and Ho, 1983; Kobayashi and Ko, 1985). Low pH and high concentrations of Al ions apparently are the primary causes of suppression in these soils. Soil microorganisms can enhance suppressiveness by tying up nutrients that would otherwise help overcome Al toxicity to *Rhizoctonia* (Kobayashi and Ko, 1985).

Seed Treatments. *Bacillus subtillus* is a spore-forming bacterium that has activity as a biocontrol agent. Endospores are long-lived and more resistant to heat and desiccation than fungal mycelium or propagules. The bacterium can be grown in commercial fermenters, formulated in a dry powder, and applied to seed along with conventional fungicide treatments. Once treated seed are planted, the bacterium readily increases and can be redistributed along the root system, although movement is passive and little colonization of secondary roots is seen in peanut (Turner and Backman, 1991). *Bacillus subtillus* is being marketed commercially by Gustafson (Dallas, TX) as a biological seed treatment (Kodiak) for peanut and other crops. Seed can be treated before sale to the farmer or the farmer can apply the treatment at planting. Seed treatment with the commercial formulation of *B. subtillus* was associated with yield increases in the southern U.S., but not in North Carolina or Virginia (D. S. Kenney, pers. commun., 1993). When growing conditions were suboptimal, such as after early planting, in poor rotations, or during droughts, root growth and yields were greater with *Bacillus* plus fungicide seed treatment than with fungicides alone.

Although *B. subtillus* is antagonistic to plant pathogenic fungi *in vitro*, yield responses seen *in vivo* were not correlated with disease level (Turner and Backman, 1991). Treatment with *B. subtillus* did not affect root disease, seedling emergence, incidence of seedling diseases, or root senescence in field tests. *Bacillus subtillus* may facilitate vigorous root growth and increased nutrient uptake by inhibiting deleterious rhizobacteria or by producing plant growth regulators (Turner and Backman, 1991).

Summary. Successful biocontrol of soil-borne pathogens requires better understanding of microbial ecology and better approaches to manipulating

soil ecosystems. The microbial composition of peanut rhizospheres and geocarpospheres in field soils is just beginning to be quantified (Kloepper and Bowen, 1991; Kloepper *et al.*, 1992). Methods that emphasize long-term reductions of disease through manipulation of microbial communities, inoculum reduction, and pathogen suppression may have more utility than approaches that address immediate disease outbreaks.

Potential Use of Molecular Biology for Modification of Organisms for Biological Control

Many exciting projects will be undertaken upon the development of routine genetic transformation systems for peanut, although incorporating resistance to necrotrophic pathogens such as *S. rolfsii, S. minor*, and *C. arachidicola* by transformation might be difficult. However, genetic manipulation of microbes in the plant environment could be an alternative or adjunct to producing transgenic plants for disease control.

Genetic modification of microorganisms has great potential for enhancing the efficacy of biological control of peanut diseases. Realization of this potential depends on our ability to develop transformation techniques, understand microbial ecology, and confront obstacles to commercialization. The ability to routinely and predictably obtain useful isolates of biocontrol organisms is a primary requirement for developing new technologies. Several useful isolates have been obtained from mutagenesis, but molecular techniques allow more precise control of genetic modification and introduction of genes from a wider range of organisms.

Bacteria, especially *Escherichia coli,* and fungi, such as *Saccromyces cerevisiae, Aspergillus nidulans,* and *Neurospora crassa* (Fincham, 1989), are routinely transformed, but these model organisms usually do not occur on peanut leaves, roots, or pods, or in peanut soils. Adaptability of these nonnative microorganisms to peanut niches may be limited; in addition, application of genetically engineered microorganisms to nonnative niches could face considerable regulatory resistance.

However, *E. coli* or *S. cerevisiae* could be transformed to produce large quantities of the antibiotics, enzymes, or toxins produced by biocontrol organisms, and these products could be applied as conventional pesticides. Although this approach overcomes difficulties of working with microorganisms in field situations, it also negates the desirable properties of biocontrol organisms, such as the ability to increase through colonization. Natural products that are toxic might not meet regulatory requirements for application to crops as pesticides. On the other hand, biocontrol organisms are thought to be safe, because presumably they release only small amounts of biologically active compounds, and only at the site and time of pathogen contact.

Species of bacteria that may have utility in biocontrol and have been transformed, include members of the genera *Pseudomonas, Xanthomonas, Bacillus* (Mills, 1985), and *Enterobacter* (Fravel *et al.*, 1990). Phylloplane- or soil-inhabiting fungi that have been transformed include *Trichoderma reesei* (Gruber *et al.*, 1990; Peterbauer *et al.*, 1992), *T. harzianum* (Pe'er *et al.*, 1991), *T. viride* (Sivan *et al.*, 1992), *G. virens* (Ossanna and Mischke, 1990), *Cercospora kikuchii* (Upchurch *et al.*, 1991), and *Aspergillus flavus*

(Woloshuck *et al.*, 1989).

Several approaches to create "designer" biocontrol agents are possible through genetic transformation. First, transformation could be used to improve or enhance antagonism, competitiveness, colonization, or survival of organisms currently used for biological control. For example, a gene in *T. reesei* confers both benomyl resistance and cellulase production (Peterbauer *et al.*, 1992). Pesticide resistance allows application of the biocontrol agent and other pesticides together. Elevated cellulase production is a further advantage because it is associated with rhizosphere competence as an enhanced ability to colonize the root-soil interface (Ahmad and Baker, 1987a,b). This approach is appealing because some biocontrol organisms have been studied extensively and much is known about their culture and application. Genes coding for the most toxic forms of an antibiotic or most active enzymes also could be cloned into these organisms. In addition, genes that enhance survival, competitiveness, or adaptability to plant and agricultural niches could be used to improve performance of conventional biocontrol organisms. Another approach could be modification of organisms that readily colonize and survive on peanut plant surfaces and agricultural soils, but are not considered biocontrol agents, to produce toxins or enzymes antagonistic to plant pathogens. However, the characters that contribute to effective biocontrol, colonization of plant surfaces, and survival still need to be determined. Probably many of these characters are controlled by several genes. Handelsman and Parke (1989) outlined the experimental procedures and standards of proof that molecular biologists could use to elucidate mechanisms of biological control of soilborne plant pathogens.

The classic division of biocontrol mechanisms into antibiosis, competition, parasitism, and induced resistance was discussed earlier in the chapter, but the importance of the various mechanisms in a particular biocontrol scheme often is a matter of conjecture. For example, production of antibiotics may be associated with a "cost" to the organism of decreased vigor or survival ability (Fravel, 1988) as vital metabolites are channeled into antibiotic synthesis. Thus, transformation to enhance antibiotic production may not enhance biocontrol.

Ironically, biotechnology may present the best opportunity to answer questions about mechanisms of biological control. Many biocontrol organisms produce antibiotics and other toxins *in vitro*, but these substances generally are difficult to detect *in vivo*. The subject of antibiotics and toxic metabolites has been controversial in the biocontrol literature (Fravel, 1988), and the creation of well defined mutants for antibiotic production may resolve this issue. In one study, transposon mutagenesis was used to generate antibiotic minus mutants of *Pseudomonas flourescens*, a bacterium associated with take-all decline. Although these mutants retained some ability to suppress disease, they were less effective than the wild type. Full suppressiveness could be restored by complementation for antibiotic production. In this example, antibiotic production was a component of biocontrol by the antagonist, but apparently other mechanisms of disease suppression (including Fe^{3+} iron competition) also were important (Thomashow and Weller, 1988, 1990).

If antibiotics are important determinants of antagonism, biocontrol organisms could be transformed for antibiotic production. Unfortunately, antibiotics are secondary metabolites that are produced in multistep pathways, and several clusters of genes (which may even be located on different chromosomes) can be required for the synthesis of an antibiotic (Martin and Larias, 1989). Therefore, successful transformation for production of a given antibiotic could be quite difficult.

Genetic transformation could be used to investigate the importance of other toxic compounds in biocontrol. Metabolites such as sulfur compounds (Lewis and Papavisas, 1971), ammonia (Schippers *et al.*, 1982), and hydrogen peroxide (Fravel, 1988) are thought to be involved in biological control and other forms of microbial antagonism. The ability to produce a toxic metabolite, or to tolerate the presence of such compounds, might make a biocontrol agent more effective or competitive. Transformation for these characters should be possible.

Antibiosis between biocontrol agents and plant pathogens includes parasitism and predation. Most of the best known biocontrol agents, including species of *Trichoderma, Gliocladium, Talaromyces,* and *Sporidesmium*, are parasites of plant pathogens and produce the structures and enzymes (such as β-1,3-glucanase and chitinases) necessary for their exploitation. Genes that code for the production of chitinase have been isolated, cloned, and are being used to transform plants, such as tobacco (*Nicotiana tabacum* L.), to increase disease resistance (Moffat, 1992).

Chitinase genes might be used to transform adapted microorganisms for biocontrol. Regulation of chitinase gene expression in biocontrol organisms also could be altered. Although many biocontrol organisms produce enzymes that break down fungal cell walls, constitutive production is usually low; enzyme production is induced by the substrate (Vantilburg and Thomas, 1993). This need for enzyme induction may account for some instances of poor biocontrol by known parasites. Biocontrol might be improved by using genetic transformation to insert an efficient promoter [such as the 35S promoter of cauliflower mosaic virus (CaMV)] before the genes coding for enzyme biosynthesis (Carr and Klessig, 1989). Increasing the number of copies of these or other desirable genes might also be accomplished through genetic engineering (Pe'er *et al.*, 1991).

Microbial competition for nutrients or infection sites is thought to be an important mechanism of biocontrol in some pathosystems, but competition is extremely difficult to demonstrate conclusively, even with molecular techniques (Handelsman and Parke, 1989). Transposon mutagenesis and cloning have been used to elucidate the role of iron competition by siderophore producers in some pathosystems (Handelsman and Parke, 1989). Little is known about the iron requirements of peanut root pathogens, but Fe^{3+} is not likely to be limiting in the acidic soils where peanut typically is grown (Cox *et al.*, 1982). Availability of Ca or the form of Al ions in these acid soils may influence plant development, microbial competition (Shamsuddin *et al.*, 1992), and development of diseases such as pod rots (Csinos and Gaines, 1986). Cellular uptake of Ca and possibly Al ions is mediated by a

conserved protein, calmodulin. Molecular techniques are being used to determine the role of calmodulin and these ions in pathogen suppression (Hood *et al.*, 1993).

Some microorganisms, possibly including *B. subtilis* on peanut, may control disease development through stimulation of host growth rather than pathogen suppression (Turner and Backman, 1991). These agents produce plant growth regulators such as indole-3-acetic acid. Genes coding for indole-3-acetic acid biosynthesis have been isolated (Kerr, 1987) and could be used to transform biocontrol agents having other desirable properties.

The microbial composition of the phylloplane and rhizosphere also needs to be characterized. Until recently, our ability to detect possible biocontrol agents has been limited by methods used to isolate and identify organisms from peanut surfaces and soils. The organisms that are active at the time of assay, and are easily cultured on standard selective media, are the most likely to be isolated. The polymerase chain reaction (PCR) and random amplified polymorphic DNA (RAPD) techniques have great potential for characterizing microbes which could lead to more effective biocontrol. For example, molecular markers could be used to track the fate of different isolates of biocontrol agents on peanut, allowing selection of the most effective one. *Agrobacterium tumefaciens* and *Frankia* spp. have been detected and enumerated in soil with PCR methods (Picard *et al.*, 1992).

In addition to population identification and enumeration, molecular biology provides techniques for studying plant-microbe and microbe-pathogen interactions. Use of reporter genes such as *lac* for production of β-galactosidase allows researchers to determine when microbial genes of interest are being expressed in soil (Howie and Suslow 1987) and transformation of microorganisms with a luciferase (*lux*) gene allows the distribution of the organisms on roots or leaves to be seen (Fravel *et al.*, 1990).

Perhaps the biggest hindrance to successful commercialization of biocontrol has been the inconsistent performance of biocontrol agents. The use of molecular markers to precisely identify isolates that perform well in agricultural settings may reduce this inconsistency.

Experimental or commercial release of microorganisms, genetically modified as proposed, would be difficult under current regulatory guidelines. Development and release of transgenic plants remains controversial, but research and development has continued for commercial purposes (Kessler *et al.*, 1992).

Progress towards release of genetically altered microorganisms has proceeded more slowly. Unlike plants, we cannot expect microorganisms to always remain in the field where they were applied. Microorganisms also have reproductive capacities several orders of magnitude greater than most plants. Many of the proposed modifications of biocontrol organisms involve introduction of foreign genes. However, the least controversial approaches to genetic modification are the deletion, inversion, or amplification of existing genes. Resistance to further introductions of transgenic organisms should lessen as the public and regulatory agencies become increasingly familiar with genetic engineering, and a history of safe introductions develops.

Conversely, unforeseen "genetic accidents" could doom hopes for commercialization of recombinant technologies. Thoughtful consideration and responsible action by scientists and commercial interests is needed to assure that the public experience with molecular biology is positive and the potential benefits are realized.

NEMATODES

Plant parasitic nematodes are estimated to cause annual monetary losses on peanut of more than $1 billion a year (Sasser and Freckman, 1987). These losses would be even greater were it not for the use of nematicides, crop rotation, and other cultural practices. Peanut root-knot nematode, *Meloidogyne arenaria* (Neal) Chitwood race 1, or the northern root-knot nematode, *M. hapla* Chitwood, cause the majority of damage. Other nematode species affecting peanut include the lesion nematode, *Pratylenchus brachyurus* (Godfrey) Filipjev and Schuurman-Stekhoven, the sting nematode, *Belonolaimus longicaudatus* Rau, and the ring nematode, *Macroposthonia ornata* (Raski) de Grisse and Loof (Minton and Baujard, 1990). A few other nematodes cause diseases that are localized problems.

Meloidogyne arenaria is the most important soilborne pest of peanut in most regions of the world. Initial population densities as low as one or two juveniles of *M. arenaria*/100 cm^3 in a deep sandy soil result in economic yield suppression of peanut (Rodriguez-Kabana *et al.*, 1982; McSorley *et al.*, 1992). Because population densities of this species increase rapidly near the end of the relatively long peanut growing season (Dickson and Hewlett, 1989), peanut quality and yield is affected because of heavy galling of pegs, pods, and roots (Rodriguez-Kabana *et al.*, 1986), and the interaction of the nematode diseased tissue with certain soilborne fungi (Garcia and Mitchell, 1975). Effective management of this nematode can result in up to 20-fold higher yield differences compared to nontreated control plot yields (Dickson and Hewlett, 1989).

Root-knot nematodes are sedentary endoparasites and the permanent feeding sites established within plant roots is a complex and intimate parasitic relationship. The specialized feeding sites that form in the plant, called giant cells, act as sinks for the nematodes to obtain nutrients (Hussey, 1985). The five to seven giant cells that form in the root vascular tissue undergo rapid nuclear division, resulting in enlarged lobed nuclei, and the cells become metabolically active (Huang, 1985). The giant cells have an elaborate network of membranous cell wall in-growths that give these cells features of transfer cells (Jones, 1981). During establishment of the giant cells the root-knot nematode completes its development; the ability to move is lost and the eel-worm shaped juvenile develops into a pear-shaped female.

Lesion nematodes are migratory endoparasites. They do not establish specialized feeding sites within the root. The juveniles and adults migrate through the cortical tissue and feed on cells within this portion of the root. They can also feed on pegs and pods. The damage caused by lesion nematodes may be accompanied by secondary infections from other soilborne

pathogens. Early infection of pegs often cause pod development to abort. The sting and ring nematodes are ectoparasitic forms that feed deep within plant root cells by inserting their long stylets, but the body of the nematode does not penetrate the root tissue, or at least not completely as in the case of root-knot or lesion nematodes.

In the U.S., plant parasitic nematodes traditionally have been managed with nematicides (Rodriguez-Kabana *et al.*, 1985; Dunn, 1993), crop rotation (Norden *et al.*, 1977; Rodriguez-Kabana *et al.*, 1991) or both. Prior to the suspension of registration by the U.S. EPA, dibromochloropropane (DBCP) (until 1977-78) and ethylene dibromide (EDB) (until 1985-86), were effectively and widely used, particularly in the southeastern U.S., for nematode control on peanut. Nematode control practices in the U.S. have since dramatically changed. Nematicides are the primary control for nematodes where crop rotation is impossible or not practical. The fumigant 1,3-dichloropropene; or the nonfumigants aldicarb, ethoprop, fenamiphos, and oxamyl; or the multi-purpose chemical metham sodium are the only chemicals for managing nematode diseases on peanut (Dickson, 1985; Dunn, 1993). Currently, no genetic resistance or tolerance to the peanut root-knot nematode is known in any commercially cultivated peanut (Noe *et al.*, 1992). Methods for managing nematode diseases by biological controls and application of molecular biology are being explored since genetic resistance to nematodes is restricted to only a few unadapted peanut lines and several *Arachis* spp.; thus, traditional methods in plant breeding will require 5 to 10 years to produce a viable cultivar.

Use of Antagonistic Organisms to Control Nematodes

Interest in using biological antagonists to control plant parasitic nematodes was stimulated with the discovery of predatory nematodes (Cobb, 1920) and nematode trapping fungi (Linford and Yap, 1939). Shortly thereafter, a bacterium, first described as *Duboscqia penetrans* (Thorne, 1940), but later renamed *Pasteuria penetrans* (Sayre and Starr, 1985) was reported to be parasitizing nematodes. Today many microorganisms are recognized as antagonists of plant parasitic nematodes (Kerry, 1987; Poinar and Jansson, 1988; Stirling, 1991a,b).

Bacterial Parasites*—Pasteuria *spp. Of the numerous microorganisms that are nematode antagonists, *Pasteuria* spp. have the greatest potential for biological control of the important nematode genera that cause diseases of peanut. Isolates of this obligate parasite of nematodes specific to the peanut root-knot nematode have been recovered from peanut fields in the southeastern U.S. (Minton and Sayre, 1989; Dickson *et al.*, 1991). *Pasteuria* produces nonmotile endospores resistant to desiccation, and they readily attach to the nematode cuticle on contact. A single nematode may have a few to several hundred endospores attached to its cuticle (Fig. 1). The endospores germinate after attachment, produce an infection peg that penetrates the nematode's cuticle and then enters the nematode body. Once inside, the infection peg develops into a vegetative, spherical colony consisting of a dichotomously branched, septate mycelium (Fig. 2) (Sayre and Starr, 1988;

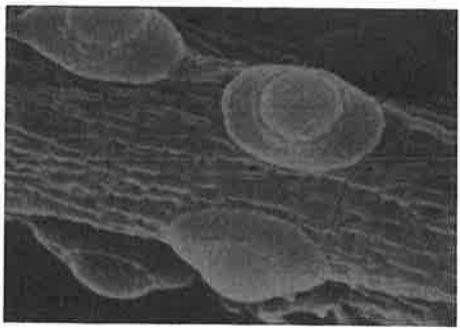

Fig. 1. Scanning electron photomicrograph of endospores from *Pasteuria penetrans* attached to the cuticle of a root-knot nematode juvenile (photograph courtesy of Dr. Khuong B. Nguyen, Univ. of Florida).

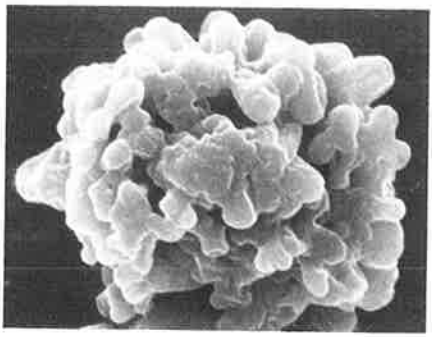

Fig. 2. Scanning electron photomicrograph of the irregular shaped, dichotomously branched early developmental vegetative stage of a mycelial colony of *Pasteuria penetrans*.

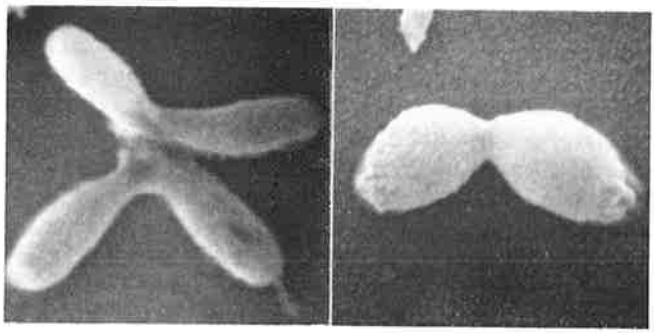

Fig. 3. Scanning electron photomicrograph of doublet and quartet sporangial development of *Pasteuria penetrans*.

Hatz and Dickson, 1992). This mycelium produces many microcolonies, which break up to produce doublets and quartets of club-shaped sporangia (Fig. 3). These sporangia develop into mature endospores that eventually fill the nematode body. Nematodes parasitized by *Pasteuria* spp. remain alive and continue development. In most cases, parasitized nematodes reach the adult stage, but egg laying is greatly reduced or completely blocked (Mankau, 1980; Sayre, 1980). A single parasitized *M. arenaria* female may contain more than 2 million endospores (Sayre and Wergin, 1977; Stirling, 1981) and endospore-filled males of *M. arenaria* also have been observed (Hatz and Dickson, 1992).

The life cycle of *Pasteuria* spp. appears to be in synchrony with that of their nematode hosts and about 30 to 35 days are required for *Pasteuria* to complete its life cycle on *M. arenaria* at 35 C (Hatz and Dickson, 1992). The bacterium developed more quickly within its host at 30 and 35 C than at 25 C or below, whereas the rate of endospore attachment on *M. arenaria* second-stage juveniles (J2) was the highest at 30 C (Hatz and Dickson, 1992). It is thought that endospores are released into the soil pore spaces once the life cycle is completed and the parasitized nematode body disintegrates. Little is known about dispersal or survival of endospores once they enter the soil environment.

Species of* Pasteuria *spp. and Their Diversity. *Pasteuria* spp. are found on many species of nematodes worldwide (Sayre and Starr, 1988), but only the host range and morphology of three species—*P. penetrans*, *P. thornei*, and *P. nishizawae*—specific to root-knot, lesion, and cyst nematodes, respectively, have been described (Sayre and Starr, 1985; Starr and Sayre, 1988; Sayre *et al.*, 1991). Isolates of *Pasteuria* that parasitize sting and ring nematodes are also known. Pathogenicity on and development of *Pasturia* spp. in nematodes has not been well examined, but differences in endospore sizes and shapes, and attachment and reproduction in different nematode species indicate considerable diversity among isolates (Sayre and Starr, 1988; Oostendorp *et al.*, 1990).

Population Development of* Pasteuria penetrans *in the Field. *Pasteuria penetrans* has been effective in several short duration tests (Mankau, 1975; Stirling, 1984; Brown *et al.*, 1985; Dube and Smart, 1987). One isolate of *P. penetrans*, specific for *M. arenaria* on peanut, has been studied in field microplots over a 7-year period to determine the effect of different winter cropping systems [rye, vetch (*Vicia* sp.), wheat (*Triticum aestivum* L.), bare fallow] on population changes of the control agent (D.W. Dickson, unpubl. data, 1994).

Microplots were infested with *M. arenaria* alone or with *P. penetrans*. No effect of *Pasteuria* was observed during the first year (Oostendorp *et al.*, 1991). In the second year, the penetration of second stage juveniles (J2) into roots of peanut was reduced 38 days after planting in *P. penetrans*-infested plots compared with plots of *M. arenaria* alone. The number of endospores attached per J2 was affected by the cropping sequence. Endospore attachment increased continuously during the first 3 years of the experiment. After 3 years the population density of J2 dropped in plots with *P. penetrans* compared to plots with *M. arenaria* alone. Endospore attachment to J2 was

greater in rye and vetch plots than in fallowed plots.

After three growing seasons, yield was higher in *P. penetrans*-infested plots than in plots with *M. arenaria* alone. Significant yield differences were also observed in the fourth through sixth peanut crops grown in *P. penetrans*-infested plots.

Peanut grown in deep (>45 cm) sandy soil is an extreme test situation for determining the efficacy of *P. penetrans* because root-knot nematode is severe under this condition (Dickson and Hewlett, 1989). Technical difficulties in growing *P. penetrans* mandated that only low amounts of endospores could initially be added to the microplots. If more endospores could initially have been added, *M. arenaria* population densities might have decreased sooner.

Exploitation of Naturally Suppressive Soils. Suppressive soils, soils in which disease development is suppressed even though the pathogen is introduced in the presence of a susceptible host (Huber and Schneider, 1982), have been studied only during the past 2 decades for control of nematodes. Most work has been with soil with fungi antagonistic to nematodes (Gair *et al.*, 1969; Stirling and Mankau, 1979; Jaffee and Zehr, 1982; Kerry, 1982).

Only a few suppressive soil sites infested with *P. penetrans* and root-knot nematodes have been studied (Mankau, 1980; Stirling and White, 1982; Bird and Brisbane, 1988; Minton and Sayre, 1989; Dickson *et al.*, 1991). A field near Williston, FL had been used for peanut nematode research for several years because of the heavy infestation of *M. arenaria*. However, over a period of 4 to 5 years (1982-87) peanut yield increased and root-knot nematode galling on pegs and pods greatly decreased (Dickson *et al.*, 1991). A similar situation was reported in peanut fields in southern Georgia (Minton and Sayre, 1989).

To examine whether the suppressiveness of the soil at the Williston, FL site was due to a biological agent, soil samples were collected and then autoclaved, air dried for 2 weeks, drenched with formalin, or stored at 10 C for 2 weeks. Suppressiveness of each soil was assessed in pots with tomato inoculated with none or 1000 J2 of *M. arenaria*. Fewer galls and egg masses developed on plants in air dried and stored soil (untreated) from two soil depths, 0 to 15 and 15 to 30 cm, compared with autoclaved and formalin-treated soil. Formalin-treated soil had fewer galls and egg masses compared with autoclaved soil. Soil from the 0 to 15-cm depth was more suppressive than soil from the 15- to 30-cm depth. Egg mass counts from the different treatments were not different, but galling was less in soil collected at 0 to 15 cm deep when air dried or stored compared with autoclaved or formalin-treated soil.

The density of *P. penetrans* endospores in the air-dried soil was determined by adding 1000 J2 per sample. After 3 days, 82 to 100% of the J2 had endospores attached, indicating *P. penetrans* contributes to suppression of the population density of *M. arenaria*. However, other soilborne fungi parasitic on *M. arenaria* were isolated also. The two most common fungi isolated from the females and egg masses were *Paecilomyces lilacinus* and a

Penicillium sp., but *Verticillium chlamydosporium*, *Neocosmospora* sp., and *Aspergillus* sp. also were found.

Summary. Low numbers of endospores of *P. penetrans* added to soil increased over time to densities effective for control of peanut root-knot nematode. This demonstrates that *P. penetrans* can be important in nematode management in peanut. One drawback for the development of *Pasteuria* spp. as a control is the lack of a method to produce endospores for release. As obligate parasites of plant parasitic nematodes, *Pasteuria* spp. are fastidious in their requirements for growth and reproduction.

Methods have been developed to produce *P. penetrans* endospores in *Meloidogyne* females parasitizing plant roots (Stirling and Wachtel, 1980; Stirling, 1984; Sharma and Stirling, 1991). These roots are washed, dried, and ground to a powder. The resulting product can be applied to soil infested with *Meloidogyne* spp. Maintaining such a production system and the variable yield of endospores make large scale use of this approach impractical. When *P. nishizawae* was maintained *in vitro* for up to six transfers over an 8-month period, all stages of the life cycle were observed (Reise *et al.*, 1991). These results indicate that *Pasteuria* spp. may be grown *in vitro*.

Even if large-scale production of this parasite proves to be impossible or too costly, we may be able to capitalize on natural infestations of *Pasteuria* by crop management to maintain or enhance suppression of nematodes. To do this, factors that affect changes in population densities of the bacterium and nematode need to be determined.

Although stringent host specificity is an advantage for using *Pasteuria* as a biocontrol, it may impede commercial development. Isolates are being identified that have broader host ranges and appear to be more virulent. As more is learned about *Pasteuria* spp., it may be possible to alter the specificity of isolates by chemical, mechanical, or genetic manipulations.

Because *Pasteuria* spp. cannot be cultured, techniques currently are not available to determine the purity of isolates. Information on genetic makeup and stability is lacking. The taxonomic status of the genus is far from being resolved, especially for the economically important isolates that attach to root-knot nematodes. Currently, morphological differences that may not be reliable are used in taxonomy. Methods for evaluating similarities and differences in the DNA of different isolates may provide useful information and result in development of molecular probes for identification and detection of *Pasteuria* spp. Such techniques would allow monitoring the genetic stability of isolates and determining the dispersal and population changes when the bacterium is added to soil. Unfortunately, at this time each bacterial isolate must go through time consuming and costly evaluations, and culture methodologies must be developed and optimized before isolates can be released for control of specific nematodes. Thus, some of the same cost benefit constraints that operate with pesticide development for specialized pest targets may apply to the commercialization of isolates of *Pasteuria*.

Potential Use of Molecular Biology for Modification of Organisms/Host for Biological Control of Nematodes

Genetic resistance to root-knot nematodes is the most desired means to

manage these pathogens. One common mechanism that protects plants against invading nematodes is the hypersensitive response (Kiraly, 1980). The hypersensitive response is a rapid localized necrosis of cells at the site of giant cell initiation, limiting the parasitic ability of the nematode. Little is understood of the molecular and cellular mechanisms involved in plant resistance to nematodes. It is known that many plant genes in both resistant and susceptible hosts are either induced or altered in their expression during nematode infection. For example, genes encoding proteins such as chitinases, glucanases, and other enzymes may be induced (Bowles, 1990). In addition, the expression of other genes, such as those encoding structural proteins or constitutive enzymes may be enhanced to accommodate the increased cellular metabolism occurring during nematode infection.

Nematicidal Transgenic Plants. Crop plants resistant to particular pests have been produced through molecular biology. One approach used is to engineer into a plant foreign gene sequences that have deleterious effects on the target pest. This strategy has been used to produce transgenic plants that express the insect toxin gene (BT toxin) from the bacterium *B. thuringiensis* (Barton *et al.*, 1987). When ingested, BT toxin causes insect gut paralysis. Thus, insects feeding upon plants expressing this gene are poisoned. Presently, there are no examples of genetically engineered crops with nematode resistance.

Many protein toxins known to be active against vertebrates, including neuroactive peptides and some snake venoms, also act upon nematodes (C.H. Opperman, unpubl. data, 1993). However, these compounds are undesirable due to the broad spectrum of activity they exhibit. Several invertebrate specific toxins do exist and can be tested against nematodes. For example, one such compound is the TxP-I toxin from female mites of the species *Pyemotes tritici*. This venom is extremely potent against a wide range of insect species, but does not affect mice (Tomalski and Miller, 1991). The toxin causes immediate muscle paralysis in insects upon exposure to low dosages. The gene encoding TxP-I has been isolated and characterized, and the cDNA contains an open reading frame encoding a polypeptide of 291 amino acids (Mr 33K). Analogous moieties may exist in natural enemies of nematodes. The fungal endoparasite of *Nematoctonus* spp. secretes a nematoxic substance during spore germination (Stirling, 1991a,b). This substance causes rapid paralysis and death of the nematode. The predatory nematode *Seinura* injects a toxin into its nematode prey, causing immobilization within seconds (Stirling, 1991a,b). Although the nature of these molecules is unknown, it may be possible to use them in designing a nematicidal plant. If the toxin is a peptide, it would be possible to engineer it into plants to function in a manner similar to BT-transformed plants. Other fungal and bacterial antagonists are known to produce substances toxic to nematodes, but little information is available on the composition of these compounds.

A different approach to designing nematicidal plants is to express a nematode gene in a plant that is detrimental to the nematode. One possible molecule is collagenase. The major structural component of nematodes is collagen, a polymer unique to animal species, and is found in the cuticle,

stylet, and lining of the esophagus and intestine (Bird and Bird, 1991). During nematode molting, the old cuticle is partially to completely digested by the nematode, and presumably nematode collagenases are utilized in this activity. It may be possible to isolate genes encoding nematode collagenases and use these to design transgenic plants. This enzyme should have no effects on plants, which do not contain collagen. The nematode which feeds upon cells containing collagenase would suffer disruption of the major structural features of its anatomy, terminating development.

One of the theoretical advantages to these strategies is that any nematode which feeds for a prolonged period upon cells containing the toxic moieties is doomed. Possible disadvantages to this approach are that the constitutive expression of any "toxin" gene may place the pest population under strong selective pressure for resistance, the peptide toxins useful in this approach are narrow in their toxic spectra (as is the case with the BT toxins); the host plant suffers a negative energy balance when there is no pest pressure; and that the global constitutive expression of toxin genes guarantees that nontarget species, including humans, will be exposed to the protein products.

Genetically Engineered Host Resistance. Tissue specific regulation of the foreign gene(s) or pest induced expression might overcome some of the disadvantages described for expressing novel pest resistance genes. Molecular studies of organ specific expression have focused upon tissues derived from the shoot meristem, the organ-specific and light regulation of genes expressed in leaves, genes expressed during flower development, and genes encoding numerous seed storage proteins (Benfey *et al.*, 1989). Few root specific genes have been identified (Benfey and Chua, 1989).

Numerous genes induced during pathogen (primarily viral) infection have been cloned. Proteins encoded by these genes (pathogen related proteins) include enzymes of known (e.g., chitinases) and unknown functions (Metraux *et al.*, 1989; Bowles, 1990). The promoters of these organ specific and pathogen related genes have been analyzed by mutational analysis. Models of gene regulation propose that *cis*-acting sequences regulating gene expression in higher plants are often composites of several regions, acting as transcriptional enhancers or silencers. These sequences interact with various nuclear DNA-binding proteins, and perhaps one another, resulting in regulated transcription (Benfey *et al.*, 1989).

The availability of promoters that direct expression of foreign genes in tissues targeted in root-knot nematode infection (giant cells in the developing vascular cylinder) permits the development of novel strategies for nematode control. Because root-knot nematode affects gene expression patterns, it will be possible to isolate and characterize the promoters that control these changes. Hence, nematode induction could be uncoupled from control of normal gene expression which would permit designing a pathogen-specific, transgenic-resistant plant. Studies on induction of defense related genes from other plant species may also provide insight into pathogen induction and specificity.

Tissue specific expression, constitutive expression, and nematode-inducible expression of a protein toxic to nematodes are three potential approaches for developing nematode-resistant plants. The most durable and elegant of

these approaches would be a nematode-induced expression of a protein toxic to the giant cells, i.e., nematode induced hypersensitivity. For this approach to succeed, the peptide toxin must remain confined to the cells expressing the toxin and should not represent a human health hazard. DNases, RNases, or proteases could be used to disrupt cellular functions necessary for initiation of root-knot nematode feeding sites. This strategy has been used to produce transgenic tobacco plants exhibiting male sterility (Maraini *et al.*, 1990). In this study, expression of a gene encoding an RNase (*Barnase*) was directed by a tapetal specific promoter. Expression of the RNase gene in the tapetal cells resulted in cell death, and consequently male sterility. Histochemical analysis of the transgenic plants showed that the surrounding cells were unaffected. Using similar strategies for root-knot nematode resistance, provided that sufficiently restrictive promoter sequences were available, would overcome the disadvantages of using a toxic peptide directed against the pathogen.

Unfortunately, this strategy is not applicable to the migratory endoparasitic and ectoparasitic nematodes. Their less intimate relationships with the host suggest that transgenic control for these nematode species will have to rely upon the use of toxins. Progress in understanding the relationship of these nematode species to the host plant may suggest novel strategies that do not rely on toxin molecules for nematode management.

Future Prospects. Once a routine and consistent transformation system for peanut is developed, it will be possible to design and test strategies for transgenic resistance. Several other factors also compound the nematode problem on peanut. Because much of the damage caused by nematodes is to the developing pegs and pods, gene expression only in roots may not provide sufficient protection to avoid economic losses. Constitutive promoters, such as the CaMV 35S promoter may have to be employed to drive the transgene. Alternatively, root specific and pod specific promoters could be chained to provide specificity, but either of these expression strategies will result in the transgene product being present in the seed. The life histories and feeding habits of the lesion, sting, and ring nematodes are not compatible with an induced hypersensitivity approach. Therefore, a toxin based approach would provide the broadest possible protection from nematodes, but potentially would be the least durable. The lack of currently identified nematode specific toxins further complicates this possibility.

The unique nature of the peanut growth habit and nematode parasitism will make development of transgenic nematode resistance both challenging and difficult. Root-knot nematode inducible promoters offer a viable and exciting option, but other nematode species must be considered also. The problem of pod protection might be negated by strong early season control through root specific gene expression, but pod protection also should be pursued in any transgenic resistance strategy. If transgenic nematode resistant peanut are to be a reality in the future, the foundation must be built by identification of useful promoter and gene sequences.

VIRUSES

The use of developing technologies for detection or control of viruses

perhaps has not been applied as rapidly to peanut as to other crops because of the difficulties in isolating and characterizing some of the viruses that infect peanut and the recalcitrant nature of peanut to transformation (below and Chapter 3 by Knauft and Ozias-Akins). A number of virus diseases limit peanut production (Porter *et al.*, 1982, 1984; Sreenivasulu *et al.*, 1991; Sherwood and Melouk, 1994). Some of these virus diseases, such as groundnut rosette, are found only in a limited geographical area, and others, such as peanut mottle (PMV), have been found in most areas where peanut is grown. In addition to proven methods such as breeding for resistance to ameliorate the effects of virus diseases, molecular techniques provide another avenue for investigating strategies and tactics for management and control of virus diseases.

Current Use of Molecular Biology on Viruses of Peanut

Enhanced Methods of Detection. A number of techniques are widely used for the detection of plant viruses. These include inoculation to susceptible hosts, examination for viral inclusion bodies by light or electron microscopy, examination for virus particles in leaf dips or thin sections of tissue, and assaying for viral components (e.g., viral structural and nonstructural proteins, viral nucleic acid, double-stranded RNA [ds-RNA]) (Matthews, 1991). Although the biological activity of a virus is the ultimate assay for its presence, molecular techniques have been and will continue to be used to facilitate the detection of viruses of peanut.

The purpose of this section is to provide examples, not an exhaustive review, of currently developed techniques for detection of viruses of peanut. Wherever there is a virus of importance, someone most likely is trying to apply the most current technology to its detection.

Serology has long been used for detection of viruses in peanut (Sreenivasulu *et al.*, 1991). Polyclonal antibodies can be used in a variety of serological assays (Hampton *et al.*, 1990) and the enzyme linked immunosorbent assay (ELISA) is commonly used for detection of viruses or determining taxonomic relationships (Matthews, 1991).

Monoclonal antibodies (MABs), which are produced in cell culture as a result of fusing antibody-producing cells with cells that grow in culture (Halk and DeBoer, 1985), have provided an additional tool for serological diagnosis. Polyclonal antibodies react to a number of different epitopes or sites on the antigen, but a MAB reacts to a single epitope on a protein. There are advantages and disadvantages to this specificity. Polyclonal antiserum produced to peanut stripe virus (PStV) also reacts with blackeye cowpea mosaic virus (BICMV) (Demski *et al.*, 1988; Culver *et al.*, 1989), but a MAB only reacted with PStV (Culver *et al.*, 1989), indicating PStV has at least one epitope distinct from BICMV. Although a number of isolates of PMV have been characterized based on their biological properties (Paguio and Kuhn, 1973), several MABs made to PMV did not show serological differences among these isolates (Sherwood *et al.*, 1987). Viruses in the same taxonomic group can share epitopes. Thus, Rajeshwari *et al.* (1987) were able to use a MAB to potato leafroll virus (PLRV) to detect groundnut rosette assistor virus (GRAV). Both viruses are luteoviruses.

Serology has been used also for detection of viruses that are transmitted in seed. Because peanut seed is large, a portion from the nongerm end of the seed can be removed for assay without significantly affecting germination. This has been done for detection of PStV using either polyclonal antiserum or a MAB (Demski and Lovell, 1985; Demski and Warwick, 1986; Culver and Sherwood, 1988). Although either assay worked well with individual seed, the limit of detection using either polyclonal antibody or a MAB for groups of seed was about one infected seed piece with 32 uninfected seed pieces. This is not adequate for screening large seed lots.

The need for greater sensitivity in virus detection might be addressed with nucleic acid hybridization, isolation of ds-RNA, or the PCR. Nucleic acid hybridization detects the viral nucleic acid directly by binding a labeled probe to target nucleic acid in the sample, and the PCR results in increased copy number of the target nucleic acid so it can be visualized on a gel after electrophoresis. Both of these techniques have had limited use with viruses of peanut because of the limited work done to date on sequence analysis of many of the viruses that infect peanut. Bijaisoradat and Kuhn (1988) used complementary (c) DNA probes produced by random priming to enhance detection of PMV and PStV in seed. One part infected seed mixed with 99 parts healthy seed could be detected. Since that time, clones of PStV and PMV have been produced (see below) which might be used to develop probes that could provide greater sensitivity. A similar approach, except using cDNA from cloned portions of the tomato spotted wilt virus (TSWV) genome, was shown useful for detection of TSWV (Sreenivasulu *et al.*, 1991).

The detection of ds-RNA associated with virus infection can be a useful tool for detection of some viruses (Valverde *et al.*, 1990). The ds-RNA detected may be a ds molecule representing both the (+) and (-) sense of the viral genome or subgenomic nucleic acid produced during viral replication, or the ds-RNA can be induced as a result of virus infection. The latter was the case for the ds-RNA specifically associated with groundnut rosette disease (Breyel *et al.*, 1988). Analysis of plants for ds-RNA must include adequate controls because some plant species contain naturally occurring ds-RNA.

As nucleic acid sequences of viruses that infect peanut are obtained, the PCR will have greater utility for detection of viruses. The PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated (Saiki, 1990). Two oligonucleotides primers that flank the region of the DNA to be amplified are used, and generally are synthesized based on sequence analysis of the virus of interest. Because most plant viruses that infect peanut have RNA as the genome, a cDNA is first made using the viral RNA as template and one of the primers to be used in the PCR to prime the synthesis of the cDNA. The cDNA produced is then used in the PCR reaction with the two primers. The rapid and extensive amplification of the target region results in the production of enough DNA in the PCR to be seen on a gel. Thus, no radioactive materials or serological materials are needed. In addition, sample preparation is relatively rapid, primers can be ordered and quickly obtained commercially, and the reagents needed for cDNA synthesis and the PCR are generally commercially available

in comprehensive kits.

The unavailability of virus sequences has limited the application of the PCR for detection of peanut viruses. The PCR has been used for the detection of TSWV (de Haan, 1991), but not necessarily in peanut and for detection of PStV and PMV in peanut (Sherwood *et al.*, 1992; J.L. Sherwood, unpubl. data, 1992). The sensitivity of the PCR using primers in the coat protein region of PStV was found to be 6 pg of virus. Thus, the sensitivity of the PCR may provide a method for determining the extent of virus infection in much larger samples of seed than can be confidently examined with serology or nucleic acid hybridization. The extreme sensitivity of the PCR may not be entirely beneficial. Theoretically, the PCR can detect a single copy of a piece of nucleic acid; even pieces of nucleic acid from broken uninfectious virus particles might be detected. Thus, biological assays for infectivity should be coupled with the PCR as virus detection methods are explored.

Cloning of Virus Genes. Effective utilization of the PCR and nucleic acid hybridization for detection of viruses in peanut is dependent on progress made in the cloning and subsequent sequencing of viral genomes. Of the 23 virus diseases that naturally occur on peanut, only groundnut rosette, peanut clump, peanut mottle, peanut stripe, and tomato spotted wilt (or bud necrosis) are considered to cause economic loss (Sreenivasulu *et al.*, 1991). TSWV and bud necrosis virus are caused by tospoviruses, which have been found to have serological differences and the diseases may be considered to be caused by different, but related, viruses (Reddy *et al.*, 1992). Of the viruses that cause these diseases, TSWV (de Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992) and PStV (Cassidy *et al.*, 1993; B. G. Cassidy, pers. commun., 1993) have been completely cloned and sequenced.

Portions of the genome, primarily the coat protein regions, of two isolates of PMV [ATCC #PV-413 and a PMV-OK isolate from Oklahoma (Sherwood *et al.*, 1987)] have been cloned and sequenced (Gunasinghe *et al.*, 1992). However, another PMV isolate (ATCC #PV-206) may differ in the coat protein region from PV-413 and PMV-OK since primers used for cloning of the latter were not successful in cloning PV-206. Kittipakorn *et al.* (1993) found that five isolates of potyvirus from peanut in Thailand were similar to PStV based on protein profiles in high performance liquid chromatography of tryptic peptide digests of virion coat protein and that an isolate of PMV was quite different from PStV. Thus, there is considerable need to elucidate the relationships among potyviruses that infect peanut. The RNA 2 of peanut clump virus (PCV) has been cloned and sequenced (Manohar *et al.*, 1993).

Since there is conservation in many of the open reading frames of viruses in the same taxonomic group, it is possible to use the sequence obtained from one virus in a group to facilitate cloning for sequencing or for application of the PCR for detection for a related virus. For example, Langeveld *et al.* (1991) used a series of primers for detection of several potyviruses that infect bulb plants. Unfortunately, the primers described did not detect the potyviruses PMV or PStV (J.L. Sherwood, unpubl. data, 1992). However, a consensus sequence of several potyviruses was used to design primers for cloning PStV (B. G. Cassidy, pers. commun., 1992), indicating the utility of

using sequence of related viruses. Thus, a similar approach could be useful for obtaining sequence information on the viruses that cause groundnut rosette or the virus that causes Indian peanut clump. This is particularly true with the luteovirus (groundnut rosette assistor virus) involved in groundnut rosette and the furovirus that causes Indian peanut clump. Some luteoviruses have been cloned, sequenced, and extensively studied. Luteoviruses that have been completely or partially sequenced include barley yellow dwarf virus (Miller *et al.*, 1988) and potato leafroll virus (Mayo *et al.*, 1989). The sequencing of PCV should be useful for designing primers for detection of Indian peanut clump virus. Extensive collaboration between individuals with varied interests is required to expediently use the information obtained by molecular biology about viruses that infect peanut.

Transformation and Regeneration of Peanut as Related to Virus Protection

A widely adaptable transformation procedure for peanut must be developed to reap full benefit from the cloning of peanut virus genes (see Chapter 3 by Knauft and Ozias-Akins). Methods for transformation of other legume species have direct relevance to peanut transformation research. This section focuses on which gene(s) might offer the best protection against viruses or other pathogens once a routine peanut transformation system is obtained.

Soybean Transformation. Of the many approaches used to transform and regenerate soybean (reviewed in Christou *et al.*, 1993), three have resulted in transgenic soybean plants. Hinchee *et al.* (1988) used *Agrobacterium* to inoculate cotyledon explants and observed shoots forming at the base of the explant. The transformation frequency (number of transformed plants/100 explants) was about 0.4 to 2%, but the cultivars that were transformed are not grown commercially in the U.S. Parrott *et al.* (1989) obtained R_0 generation plants from somatic embryos derived from immature cotyledons after co-cultivation with *Agrobacterium*. The R_1 generation plants were untransformed leading the authors to speculate that the primary transformants were chimeras. McCabe *et al.* (1988) and Christou *et al.* (1990) optimized a system to introduce DNA to embryonic axes via electric discharge particle acceleration that appears to be less cultivar dependent. Christou *et al.* (1989) transformed the cv. Williams, which has been widely grown in the U.S. The R_0 plants often were chimeras of transformed and nontransformed tissue. The transformation frequency for the cultivar Mandarin by this approach was approximately 0.8% (McCabe *et al.*, 1988). In later work, 22 germ-line transformation (i.e., trait passed to R_1 progeny) events were determined for 899 regenerating shoots expressing β-glucuronidase (GUS) activity in their stem segments (Christou and McCabe, 1992). However, if stem segments which expressed GUS activity from the epidermis through the cortex to the pith were identified, 38% of the shoots had germ-line transformation events. The number of plants to maintain in culture was decreased further by determining that most plants that had GUS activity in the pith, or pith and cortex, of petiole and midrib sections from the second or third trifoliates had germ-line transformation events. This continuous screening greatly decreases the number of explants maintained

through the rooting stage. However, the procedure required much labor initially because only 12 to 15% of all shoots had GUS activity for an overall transformation and recovery frequency of 1.2 to 1.5%, if one assumes four shoots were obtained per shoot axis treated (data derived from Christou and McCabe, 1992). The transformation frequency is only about 0.3 to 0.4% if the total number of initial explants is considered. Researchers who have only one or two constructs could certainly utilize such a system, but large-scale deletion analyses of promoters or structural genes would require much space and effort.

Bean Transformation. Bean has been transformed using electric-discharge particle acceleration (Russell *et al.*, 1993). The transformation frequency was 0.06% and the inserted gene has been expressed through five generations, but the procedure can be cultivar dependent. Bean explants (cotyledons, leaf discs, and hypocotyls) have also been transformed using *Agrobacterium*, but no plants have been regenerated (McClean *et al.*, 1991; Franklin *et al.*, 1993b). Southern blot border analyses indicated stable integration (Franklin *et al.*, 1993b).

Pea Transformation. Pea (*Pisum sativum* L.) has been transformed using *A. tumefaciens* and regenerated (De Kathen and Jacobsen, 1990; Puonti-Kaerlas *et al.*, 1990, 1992; Davies *et al.*, 1993; Schroeder *et al.*, 1993), but each protocol has a caveat which should be considered before attempting transformation. Puonti-Kaerlas *et al.* (1990, 1992) determined an optimal culture sequence utilizing hygromycin that was best for cv. Stivo. The plants expressed the transgene through the R_2 generation, but all plants were tetraploid. The chromosome doubling could be due to the 9 months on regeneration media in the presence of growth regulators. De Kathen and Jacobsen (1990) obtained transgenic shoots from nodal explants with efficiencies of 1 to 5% depending on whether selection was initiated immediately after co-cultivation (higher percentages) or at the time of rooting (lower percentages). Shoots were obtained by selection with hygromycin or kanamycin, but R_1 seed analysis has not been published. Davies *et al.* (1993) obtained transgenic plants from cotyledonary lateral buds inoculated with *Agrobacterium* using glass needles drawn in an electrode puller. The transformation frequency was approximately 1.3%. The time from inoculation to transfer of rooted plants was 4 months. Kanamycin was used for selection, but escapes occurred. However, this procedure is promising since the time to obtain plants is short and the procedure appears to be cultivar independent. Southern blot analysis and GUS activity of R_1 plants indicated the genes are stably integrated. Schroeder *et al.* (1993) obtained R_0 and R_1 generation transgenic plants using explants from longitudinal sections minus the radicle of embryonic axes of immature seeds. The explant source is not as easily available as in the other protocols, but a transformation frequency of 2% was obtained with the cvs. Greenfeast and Rondo. For maximum transformation efficiencies the embryonic axes had to be harvested at 2 to 5 days after the time of maximum seed fresh weight, *A. tumefaciens* strain AGL1 [a derivative of EHA101 (Hood *et al.*, 1986; Lazo *et al.*, 1991)] and not LBA4404 should be used, and growth regulators needed to be present during co-cultivation.

Peanut Transformation. Peanut is a host to *Agrobacterium rhizogenes* (Riker, Banfield, Wright, Keitt and Sagan) Conn and *A. tumefaciens* (Mugnier 1988; Dong *et al.*, 1990), and DNA integration into the genome of *A. hypogaea* after incubation with *A. tumefaciens* strain A281 was reported (Lacorte *et al.*, 1991). The susceptibility of *A. hypogaea* to *A. tumefaciens* was both cultivar- and strain-specific. Franklin *et al.* (1993a) transformed callus from hypocotyl explants (*A. hypogaea* cv. Okrun) using disarmed *Agrobacterium* strains EHA101, LBA4404, and ASE1, and obtained callus lines with single integration sites. In addition to *A. tumefaciens*-mediated transformation, microprojectile bombardment of peanut leaflets from mature zygotic embryos has resulted in stably transformed callus (Clemente *et al.*, 1992). Recently, two groups have reported the production of transgenic peanut plants. Ozias-Akins *et al.* (1993) produced transgenic cell lines through microprojectile bombardment of embryogenic tissue culture. One hundred plants were regenerated from these lines and one plant was shown to contain foreign DNA by Southern hybridization analysis. Data for R_1 generation seed was not reported. Brar *et al.* (1994) produced transgenic plants from shoot meristems of mature embryonic axes which were bombarded with DNA via electric discharge particle acceleration. The embryos were from the widely cultivated peanut cultivars, Florunner and Florigiant. At least two transgenic R_0 plants out of 226 embryonic axes bombarded produced progeny that were shown to be transformed by Northern and Southern hybridization analyses. Plants through the R_2 generation were resistant to a wide spectrum herbicide due to expression of a transgene.

Summary. Both DNA transfer by an electric discharge particle acceleration or *Agrobacterium* can be used to transform plants. Although the electric discharge particle acceleration protocol is labor-intensive, it functions consistently across genera. The *Agrobacterium*-mediated transformation of pea by injection of cotyledonary lateral buds or treatment of embryonic axes of immature seeds also may have promise for peanut transformation. The transformation frequencies were greater than 1% when using the cotyledonary lateral bud and could allow production of an adequate number of independent transformants for promoter deletion analyses.

Transgenes for Virus Resistance Genes in Plants

In the past 7 years at least nine strategies for protecting plants against viruses have been identified, and some of these are being tested. Several review articles have discussed these strategies and what the protection afforded by transgenic plants might tell us about the mechanism of virus replication (Hanley-Bowdoin and Hemenway, 1992; Register and Nelson, 1992; Wilson, 1993).

One of the original strategies to produce transgenic plants resistant to virus infection involved the constitutive expression of the coat protein (CP) of a virus in plant cells (reviewed in Beachy *et al.*, 1990). Coat proteins from at least 20 viruses from 13 virus groups have been expressed in plants and each provided at least partial protection against virus challenge by the homologous virus. Included in this group are CPs from viruses that infect

both monocots and dicots in transgenic monocot and dicot plants (reviewed in Hanley-Bowdoin and Hemenway, 1992; see also Hayakawa *et al.*, 1992; Brault *et al.*, 1993; Fang and Grumet, 1993). Although the CP has been shown to be an effector in CP-mediated protection (CPMP) (Van Dun *et al.*, 1988; Powell *et al.*, 1990; Tumer *et al.*, 1991), it is now becoming clear that resistance may be achieved by expression of the CP transcript alone in certain instances. Where the CP has been shown to be effective, the protection is manifest as a delay or absence of symptom appearance with a decrease or absence of virus replication. Protection can be overcome with increasing virus inoculum concentration and is greatest against the homologous virus but with some horizontal protection against viruses from different groups (reviewed in Hanley-Bowdoin and Hemenway, 1992; Register and Nelson, 1992). It is unclear whether there is a positive correlation between the level of CP accumulation in the transgenic plants and resistance to the homologous virus. Field tests have shown the efficacy of this approach (Gonsalves *et al.*, 1992; Jongedijk *et al.*, 1992; Sanders *et al.*, 1992; and references within these articles), but concerns about the risk assessment of this approach remain. de Zoeten (1991) indicated the potential for transcapsidation of challenge virus RNA or DNA by the CP encoded by the transgene. Since viruses are often vectored by insects and the CP is a determinant in this process, a new host range might result if a challenge virus successfully infects a CP-expressing plant. This has been shown to occur in transgenic plants (Farinelli *et al.*, 1992), but probably transcapsidation occurs frequently during natural dual infections (Creamer and Falk, 1990). Thus, considering the time frame during which natural transcapsidation has occurred it seems unlikely that transgenic plants will alter the host ranges of challenge viruses. Nevertheless, the possibility of using modified CPs to prevent insect transmission, but still afford protection, needs to be examined. A second concern is the safety of expressing drug resistance genes in plants. The consensus in the scientific literature on expression of kanamycin resistance is that government clearance of this trait is acceptable (Flavell *et al.*, 1992; Nap *et al.*, 1992).

Plants transformed with sequences derived from viral genes involved in virus replication (i.e., viral replicases) protect these plants against virus challenge (reviewed by Carr and Zaitlin, 1993). The protection exhibited by some of these plant lines is near immunity. The protein derived from the transgene appears necessary for protection, possibly indicating competition for host or viral factors (see Carr and Zaitlin, 1993).

A third approach to obtaining virus resistant plants is to express sense (i.e., transcriptional competent transgenes minus a start codon which would produce greatly truncated protein products) or antisense transcripts of viral RNA (reviewed by Hanley-Bowdoin and Hemenway, 1992; Register and Nelson, 1992; Wilson, 1993; see also Nelson *et al.* 1993). Expression of nontranslatable sense transcripts of CP genes or transcripts allowing extremely truncated CPs to theoretically be produced in transgenic plants have resulted in resistances to virus challenge equal to or greater than that observed for CP-expressing plants (Lindbo and Dougherty, 1992a,b; van der Vlugt *et al.*, 1992). The importance of translation from the downstream start

codons or from the truncated open reading frames in these sense constructs for protection to occur remains to be determined. Expression of antisense transcripts of the CP or nucleocapsid protein genes also have resulted in protected plants ranging from partial protection (less than that observed for CP-expressing plants) to near immunity (see review by Wilson, 1993). de Zoeten (1991) described a concern that recombination between transgene transcripts and a challenge virus genome could result in a new virus. Recombination occurs between viruses (see reviews by Lai, 1992; Bujarski *et al.*, 1994); and, as in the case of transcapsidation, the question is if the transgenic plants afford greater potential for recombination compared with natural infections. Considering that dual virus infections of plants occur in nature and that ample opportunity has existed for such recombinations, it seems remote that this is an undue risk. In addition, the expression of transcript or CP in plants that are natural hosts for that virus diminishes the possibility that the transgene product will encounter a novel virus. Although the consequences of constant exposure of a challenge virus to a transgene product cannot be dismissed, present constructs of "protection" transgenes should be advanced and used while new "disarmed" constructs are developed.

Although expression of only transcripts of viral sequences would allay the fears associated with protection mediated by CP, replicase and other foreign protein expression, it would be incorrect to consider protein expression systems expendable since the mechanism of protection mediated by protein versus transcript most likely differs. Yamaya *et al.* (1988) expressed the entire genome of a mild strain of TMV in tobacco and found that strong protection was exhibited against both TMV and TMV-RNA challenge, whereas only moderate protection was exhibited against TMV-RNA with CPMP (Nelson *et al.*, 1987). This protection more closely mimics natural cross protection (see reviews by Gonsalves and Garnsey, 1989; Urban *et al.*, 1990) and replicase-mediated protection (see review by Carr and Zaitlin, 1993), and indicates that viral transcripts or the replicase play a role in protection independent of the CP. Thus, as elite plant lines having multiple resistance mechanisms have prevented simple mutations of the virus from overcoming disease protection, transgenic plants expressing the CP, replicase, and transcripts might provide durable resistance.

Other directions being taken to produce virus resistant transgenic plants include the expression of satellite RNA or defective interfering particle sequences, antibodies against viral proteins, ribozymes against viral sequences, or suicide genes that express their lethal trait only in the presence of a challenge virus (reviewed by Wilson, 1993). Protection against cucumber mosaic virus and tobacco ringspot virus by expression of their respective satellite RNAs in transgenic plants has been demonstrated (Gerlach *et al.*, 1987; Harrison *et al.*, 1987). Recently, this protection has been combined with CPMP resulting in greater protection than that conferred by expression of the satellite RNA or the CP alone (Yie *et al.*, 1992). Use of satellite-mediated resistance in the field should be carefully considered because a satellite may ameliorate symptoms in one host, but intensify symptoms in

another, and a single mutation in the satellite sequence may lead to a virus-satellite combination producing severe symptoms on the host plant. Defective interfering particles have now been shown to give protection in transgenic plants (Kollar *et al.*, 1993) and present an alternative to satellites. However, defective interfering particles are similar to satellite RNAs in that they can intensify symptoms caused by some helper viruses (Li *et al.*, 1989).

With the first demonstration of the production of functional antibodies in plants (see review by Hiatt and Mostov, 1993) and the subsequent demonstration that expression of antibody *in planta* will protect against virus infection (Taviadoraki *et al.*, 1993), the expression of antibodies to plant viruses in plants provides another avenue for conferring virus resistance to plants. It is important to verify the functionality of the polypeptides which comprise the holoprotein since hybridomas can have unstable chromosomes (Harlow and Lane, 1988), and although the myeloma cell line produces no immunoglobulin protein (Harlow and Lane, 1988), they may produce transcript (M. Hein, pers. commun., 1993). In addition, it will be critical to express the antibodies in subcellular locations where they have contact with the antigen (i.e., the virus). Since most viruses replicate and accumulate in the cytosol, and antibodies are normally assembled in the endoplasmic reticulum and exported to the extracellular space, attempts are being made to produce altered antibodies (i.e., single chain antibodies containing both antigen binding domains) that are stable and have binding activity in the cytosol. In addition, the target of the antibody should be carefully considered with those viral proteins having the lowest accumulation during infection being a logical choice.

The use of RNA molecules that catalyze the cleavage of RNA substrates (i.e., ribozymes) in *trans* has also been investigated (see review by Symons, 1991; Edington *et al.*, 1993). Successful cleavage of plant viral RNA substrates has been demonstrated only *in vitro* (Lamb and Hay, 1990; Edington and Nelson, 1992; Xue *et al.*, 1992; van der Vlugt *et al.*, 1993). Although cotransfection experiments gave anomalous results on the efficacy of this approach (Edington *et al.*, 1993), inhibition of human immunodeficiency virus type 1 protein accumulation was demonstrated when HIV-1 proviral DNA and either a plasmid containing a ribozyme targeted against the RNA leader sequence of the virus or *in vitro* transcribed catalytic RNA targeted against the 5'-leader-*gag* region were cotransfected into HeLa or human SW480 cells, respectively (Ojwang *et al.*, 1992; Homann *et al.*, 1993). An additional consideration must be that inhibition by an antisense sequence may have a greater effect than that caused by a ribozyme (Lo *et al.*, 1992).

The use of suicide genes to prevent virus infection is also in the formative stages of research (see review by Wilson, 1993). As an example, attempts have been made to stably transform tobacco plants with an enzymatically active subunit A of diphtheria toxin (Czako and An, 1991). These researchers found that they could not obtain any transgenic plants expressing this protein when the gene was driven by a 35S promoter; an expected result since even one molecule of the A subunit could be lethal to a mammalian cell (Yamazumi *et al.*, 1978). Thus, use of suicide genes will require expression behind

promoters that are active only in the presence of pathogen and even then will pose risks during the time of expression.

The production of virus resistant plants via plant transformation is no longer a novelty to be discussed as an appendage to other breeding procedures. Once peanut transformation is routine, expression of genes involved in virus replication should result in plants that are resistant to virus challenge. Risk assessment is required for all these approaches, but it should be considered in relation to the scientific evidence and not to unsubstantiated worries. Protection of plants through expression of satellites, defective interfering particles, or toxic gene products should proceed in hand with evaluation of the associated risks. Use of ribozymes in transgenic plants has little risk but requires further demonstration of its successful use *in vivo*.

PROSPECTS FOR USE OF NEW APPROACHES FOR CONTROL OF DISEASES OF PEANUT

The subject area of this chapter is considerable since there are a variety of tactics that can be used to interrupt the disease triangle to mitigate the effects of disease. However, there are two areas where information is needed if new control tactics are to be implemented. First, a better understanding of the interactions that occur between organisms on the aerial and subterranean portions of peanut is needed to facilitate biological control. A better understanding of the agroecosystem is needed to progress beyond the conventional chemical approach to developing biological controls. Second, the application of molecular biology to peanut disease control needs the development of an exploitable method for the transformation and regeneration of peanut. Once this has been accomplished, the experiments to do with peanut are reasonably apparent based on successes that has been achieved with other crops. Development of a suitable transformation method for widespread use will likely come in the near future; but without it some experiments outlined in this chapter will remain the subject of speculative chapters, such as this one, rather than the subject of refereed journal articles.

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