CHAPTER II

LITERATURE REVIEWS

1. Endophytic Fungi

1.1 Definition of Endophyte

The terms endophyte and endophytic were adopted by scientists investigating asymtomatic fungal infection of grasses caused by species of Clavicipitaceae and those investigating other microbes. Basically, the mainly clavicipitaceous systemic grass endophytes, reportedly living in a mutualistic symbiosis with their hosts, and the endophytes of trees, shrubs, and herbs (including the non-clavicipitaceous grass endophytes as well) can be distinguished. Application of the same terms to different systems has contributed to some confusion and controversy (11). Contemporary application of the terms is not always consistent or accepted among all workers. Recent observations on pathogens living latently within the tissues of their host have shown that the definition of endophyte symbiosis as an almost exclusively mutualistic one encompasses probably only a limited number of cases. Therefore definition of endophyte has been expanded to include all those organisms that, during a more or less long period of their life, colonize symptomlessly the living internal tissues of their hosts (11).

1.2 Distribution of Endophytic Fungi

Fungal endophytes live internally, either intercellularly or intracellularly, and asymtopmatically (i.e. without causing overt signs of tissue damage) within plant tissues. Endophytes usually occur in aboveground plant tissues, but also occasionally in roots, and are distinguished from mycorrhizal by lacking external hyphae or mantles (12). They have long been known in grasses, and more recently, their occurrence in a variety of woody plants has also been reported. They have been found in virtually any host plant investigated, usually by rigorous surface sterilization of leaf or stem samples and subsequent incubation of the samples in nutrient media. With appropriate clearing and staining techniques endophytic hyphae can often be

demonstrated visually within plant tissue. Endophytic infection frequencies can vary from rare to 100% depending on the size of the sample and its provenance. Carroll and Petrini have shown that climate and density of vegetation probably serve as important determinants of infection frequency (13).

Grass endophytes, such as *Neotyphodium*, are generally thought to form systemic infections throughout the host, although mycelial biomass may be unevenly distributed within plants. However, few systemic endophyte-grass associations have been studied intensively for within-plant distribution. In *Neotyphodium*-infected Arizona fescue plants, for example, infection of individual tillers is variable, ranging from 10% to 100%. Even in the intensively studied, *Neotyphodium*-infected cultivars of tall fescue and perennial ryegrass, uninfected seeds are often produced by infected plants, which suggests less than complete systemic infections within plants (14).

Infections by endophytes of woody plants are usually highly localized within leaves, petioles, bark, or stems. However, under certain conditions, such as when leaves age or senesce, localized infections can become more widespread, although the term endophyte may no longer apply because the infections become external. Nevertheless, Petrini *et al* caution that far too little is known about endophytes of woody plants to assume that they all nonsystemic. Likewise, little is known about nonsystemic endophytic infections in grasses because virtually all studies have focused on systemic endophytes. However, a recent study indicates that in addition to systemic infections, endophytes that form localized infections in grasses may be as diverse and abundant as those found in woody plants (12).

Research on species composition of endophyte communities of several hosts has shown that generally a large number of fungal taxa can be recovered from the tissues of a single host species after surface sterilization (15). Endophytic communities of trees of the same species growing at the same location are generally similar, but marked differences in species richness and distribution of selected fungal species can be detected between young and old individuals of the same host. Higher colonization rates by endophytic fungi can be observed for samples from homogeneous stands with closed canopy. There may be a correlation between elevation above sea level and/or humidity and moisture and infection by a given endophyte. In general, a correlation between increase in species richness and/or frequency of colonization and the age of the tissues has been demonstrated. Usually, distinct endophyte communities characterize leaf samples of different age classes.

Changes in diversity of the endophytic associations are mostly correlated with the differential distribution of some species in young versus old leaves. There is a tendency for a marked increase of overall colonization rates and changes in species diversity with increasing height of the canopy; no significant differences, on the other hand, have been found for leaf and twig samples taken from different compass directions on the same tree (16).

Studies on seasonal changes of the endophyte populations and communities have yielded partially contrasting results. In 1984, Widler and Muller described a seasonal pattern in the occurrence of some endophytes of *Arctostaphylos uva-ursi* (L.) Sprengle, but the species undergoing seasonal variation were never host-specific, frequently observed taxa. In 1987, Sieber and Hugentobler detected no distinct seasonal patterns in the species composition of endophyte communities in beech leaves; they have reported, however, marked seasonal variations in colonization rates by *Apiogomonia errabunda*, *Diaporthe eres* Nitschke, and *Bisporella* sp., the three most frequent endophytes (16).

1.3 Fungal Endophytes and their Secondary Metabolites

Bioactive compounds from endophytic fungi have been reported increasingly. Endophytic fungi isolated from healthy black spruce needles such as *Cryptocline abretina*, *Aureobasidium pullulans*, and *Hormonema dematioides*, produce secondary metabolites with insecticidal activity (17). Paclitaxel (Taxol®), anticancer agent from Pacific yew bark (*Taxus brevifolia*), is a secondary metabolite of *Taxomyces andreanae* isolated from Pacific yew and *Pestalotiopsis microsopra* isolated from *Taxus wallachiana* (18,19). *Acremonium* sp. occurs as an endophyte in European yew (*Taxus baccata*). It produces a series of peptide antifungal-anticancer agents known as the leucinostatins (20). Two new compounds, 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran and 5 – hydroxy – 2 - (1' – hydroxy - 5' – methyl - 4' - hexenyl) benzofuran, show toxicity to spruce budworm (*Christoneura fumifercina* Clem.) cells, and 5 – hydroxy – 2 - (1' – oxo - 5' –methyl - 4' - hexenyl) benzofuran is also toxic to the larvae (21). Endophytes which produce secondary metabolites toxic to animals confer a selective advantage on their plant hosts by preventing grazing or insect attack. Members of the Hypocreales related to the ergot fungus are found in some grasses where they produce alkaloids that deter insect attack. The

weed known as darnel is the grass *Lolium temulentum* infected by a toxin-producing fungal endophyte which prevents animals feeding on it (7). A unique lipopeptide antimycotic, termed cryptocandin, is described from, an endophytic fungus, *Cryptosporiopsis* cf. q*uercina*. Cryptocandin exhibits activities against isolates of *Candida albicans*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. Cryptocandin is also active against a number of plant-pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea* (22). CR377, a new pentaketide antifungal agent, is isolated from an endophytic fungus, *Fusarium* sp. CR377, collected in the Guanacasted Conservation Area of Costa Rica. CR 377 shows potent activity against *Candida albicans* (23). A new antimicrobial metabolite, named colletotric acid, is isolated from *Colletotrichum glocosporioides*, an endophytic fungus colonized inside the stem of *Artemisia mongolica*. Colletotric acid inhibits the growth of *Bacillus subtilis, Staphylococcus aureus, and Sarcina lutea* (24). Endophytics inside *Artemisia annua* exhibit antifungal activity against crop-threatening fungi *Gaeumannomyces graminis* var *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum*, *Fusarium graminearum*, *Gerlachia nivalis*, and *Phytophthora capsici* (25). *Sporormia minima, Trichothecium* sp. and an unidentified dimorphic fungus of *Taxus wallachiana* from Himalayan yew in Nepal, were shown to produce paclitaxel too. Each of these fungi represents a new report as a paclitaxel producer (26).

2. Genetic Basis of Antibiotic Resistance

The history of drug resistance has been closely paralleled to the history of chemotherapy. Antibiotic resistance may develop in microbes within the population. Antibiotics do not create resistant cells or cause mutations that produce resistant organisms. They do, however, selectively favor the survival and proliferation of drug-resistant strains, which otherwise are only a small subpopulation within the vast majority of sensitive cells. Antimicrobial resistance is acquired either by mutation in the pathogen's chromosome or by direct transfer of R-factor plasmids from antibiotic-resistant strains to sensitive recipients (27).

Microbial populations, particularly large inocula, often contain a few resistant organisms before the initiation of therapy. In some cases, the initial population is comprised solely of drug-sensitive cells, but one or a few organisms subsequently become resistant and are selected out during therapy. The chromosomal type of

resistance that arises because of mutation may change antibiotic sensitivity greatly or moderately, depending upon the location, type, and biological consequence of the mutation (28). Random mutations in chromosomal genes may produce a few cells that are resistant to antibiotics. These mutants become significant only when prolonged exposure to the drug favors their survival over that of sensitive cells. In bacteria, genetic information for resistance may also be carried by plasmids, which are readily transferred to susceptible cells by conjugation or transformation. The normal flora often acquires such transmittable resistance. The overuse of antibiotics favors these plasmid-carrying strains, establishing a reservoir of R factors in the normal flora. Since plasmids can be transferred among different species, drug-sensitive pathogenic bacteria may acquire R factors from normal flora bacteria, and a patient's disease suddenly becomes untreatable by antibiotics that would have been effective prior to the plasmid transfer. Therefore, prolonged exposure to even a single antibiotic may favor the proliferation of bacteria resistant to several drugs (29).

3. Biochemical Mechanisms of Antibiotic Resistance

Some microorganisms are naturally resistant to certain antibiotics because they lack the target that the antibiotic affects or because the drug cannot reach its site of action. Fungi, protozoa, and viruses, for example, contain no peptidoglycan and are naturally resistant to penicillin and other inhibitors of bacterial cell wall synthesis. Most antibiotic resistance in microorganisms fits one of several general mechanisms, i.e. decreased drug uptake or increased efflux of the drug, enzymatic inactivation of drug, decreased conversion of a drug to the active growth inhibitory compound, increased concentration of a metabolite antagonizing the drug action, altered amount of drug receptor, and decreased affinity of receptor for the drug (29,30).

3.1 Decreased Drug Uptake or Increased Efflux of the Drug

Most chemotherapeutic agents must be able to penetrate the cell wall and plasma membrane to achieve effective concentrations at an internal target site. A modification in the plasma membrane may reduce its permeability to the drug, thereby increasing the microbe's resistance. Altered membrane permeability, however, does not confer resistance against penicillin and cephalosporins, as these antibiotics block extracellular assembly of peptidoglycan. This mechanism is the principal mechanism of tetracycline resistance in bacteria. In recent years active

efflux systems have been responsible for resistance of mammalian cells to a variety of structurally unrelated antibiotics and toxic compounds. This mechanism is being recognized more frequently in a variety of bacteria (29,30).

3.2 Enzymatic Inactivation of Drug

Many microorganisms produce extracellular enzymes that destroy an antibiotic's activity. Penicillinases, for example, are produced by many bacterial species, including *Staphylococcus, Neisseria, Pseudomonas, Proteus, Mycobacteria, Yersinia, Salmonella,* and *Shigella.* Other bacterial enzymes chemically modify antibiotics to forms that are poorly absorbed by the microbe. This mechanism is the principal mechanism of resistance to penicillins, aminoglycosides, and chloramphenicol (29,30).

3.3 Decreased Conversion of a Drug to the Active Growth Inhibitory Compound

Flucytosine, an antifungal drug, must be converted in the organism to fluorouracil, which is further metabolized to the active from of the drug. Fungi become resistant to flucytosine by losing the activity of enzymes along the activation pathway (29).

3.4 Increased Concentration of a Metabolite Antagonizing the Drug Action.

Some bacteria acquire resistance to antimetabolites by bypassing the metabolic step inhibited by the drug. For example, bacteria that acquire the ability to absorb folic acid no longer depend on the biosynthetic step blocked by sulfa drugs. The drug continues to interfere with the reaction, but the formerly sensitive cell is unaffected because it has an alternative source of this essential coenzyme (30).

3.5 Altered Amount of Drug Receptor

Microorganisms commonly acquire resistance when a structure or enzyme that is normally impaired by an antibiotic is modified and is no longer recognized by the drug. Bacteria resistant to streptomycin, for example, produce modified ribosomes to which the antibiotic cannot bind. Protein synthesis continues unimpaired in these bacteria even in high concentrations of streptomycin (29,30).

3.6 Decreased Affinity of Receptor for the Drug

This mechanism has been defined in bacteria resistant to sulfonamides, trimethoprim, streptomycin, erythromycin, rifampin, and several other antibiotics (30).