2 Pathogenesis

Pathogenesis refers to the origin, development and resultant effects of a disease, from the initial appearance of disease all the way to its end stages. The pathogenesis can also describe the origin and development of the disease and whether it is acute, chronic or recurrent. The study of pathogenesis is important to diagnose and manage diseases. The term 'pathogenesis' comes from the Greek words for 'disease' and 'beginning.' The origins of disease are the first step in pathogenesis. Studying pathogenesis can also provide insight into the ways in which diseases spread and potentially contribute to the development of a program which is designed to slow the spread of disease.

2.1 DISEASE DEVELOPMENT

Disease development in brief involves a number of distinct events including the dissemination of the pathogen, attraction of the pathogen to the host, prepenetration, penetration of the pathogen into the host, invasion and spread of the pathogen, reproduction of the pathogen and the survival of the pathogen.

2.1.1 Chain of Infection

For infection and disease to occur in an individual, a process involving six related components must occur. This process has been referred to as the 'chain of infection.' The six steps or 'links' in the chain include an etiologic agent, reservoir, portal of exit, mode of transmission, portal of entry and a susceptible host. This module helps in understanding infectious disease transmission. To stop the spread of disease, one or more of these links must be broken. The infection process extends from the germination or multiplication of an infective propagule within or on the surface of a potential host to the time the pathogen establishes some form of parasitic relationship with its host. The nature of the host-parasite interaction established is determined by the genotypes of the host and the pathogen and is influenced by the environment.

2.1.2 Etiologic Agents

Several categories of biological agents can cause infectious diseases. Each has its own particular characteristics. Major types of agents are fungi, bacteria, viruses, viroids, nematodes and phanerogamic plant parasites.

through the feeding organ; stylet or ectoparasites may just stay outside the tissue and absorb cell contents by the stylet.

Germination is essentially the change from low metabolic rate to a high metabolic rate and involves a change from near dormancy to intense activity; for this an energy source is needed such as a carbohydrate or fat reserve in the propagule. Fungal invasion is chiefly by germ tubes or structures derived from them. In some fungi like *Rhizoctonia solani* and *Armillariella mellea*, the hypha act in a concerted way to achieve the penetration. In *Rhizoctonia solani*, the fungus on coming in contact with root surface, first forms infection cushions and appressoria and from these multiple infections takes place by means of infection pegs. In *Armillariella mellea*, the hyphae form the rhizomorphs and only these can cause infection.

The survival of the propagules in the external environment is sensitive to interactions between the pathogen and host and the biological, physical and chemical components of the surrounding environment (David Guest and John Brown, 1997). To become infected, a plant must first be inoculated. Inoculation is the process whereby spores or other infective propagules of a parasite come into contact with potential hosts. Plant surfaces differ between species and cultivars in their chemical, physical and biological characteristics. Root surfaces are different from leaf surfaces. For example, the type of wax that forms the cuticle, the thickness of the cuticle, the surface topography, size, shape and number of leaf hairs and stomata are all species-specific characteristics of leaves. Leaf surfaces are subject to extremes of temperature, ultraviolet radiation, and moisture. Roots are buffered against these environmental extremes by the soil environment. However, root pathogens face intense biological competition for nutrients.

Preentry or prepenetration phase includes inoculation, spore germination, germ tube growth and formation of infection while entry or penetration phase involves the actual entry and colonization or postpenetration phase includes colonization, first symptoms of the disease and the production of spores and their dispersal. The initial contact between infective propagules of a parasite and a potential host plant is termed 'inoculation.' Pathogens use a variety of stimuli to identify a suitable entry point. Several fungi use topographical cues on the plant surface to guide them towards a likely stomatal site. Once the hypha reaches a stoma, volatile compounds escaping from the pore appear to provide a signal for the formation of a specialized penetration structure, the 'appressorium.' Sugars, amino acids, and minerals secreted by plants at the leaf surface can nonspecifically trigger spore germination or provide nutrition for the pathogen. Some pathogenic spores will not germinate in the absence of these substances. Pathogen development is influenced by temperature, moisture, light, aeration, nutrient availability and pH. The conditions necessary for survival and successful infection differ between pathogens.

Predation by soil fauna and hyperparasitism of fungal pathogens, such as that of *Phytophthora cinnamomi* by species of *Gliocladium* and *Trichoderma*, also affect prepenetration growth (David Guest and John Brown, 1997). These interactions have sometimes been exploited in biological control programs. Surface topography is used by some pathogens

to locate suitable infection sites. Germ tubes of the wheat stem rust fungus (*Puccinia graminis tritici*) and the wheat powdery mildew pathogen (*Blumeria graminis*) grow at right angles to parallel ridges on leaf surfaces. Because of the staggered arrangement of stomata along wheat leaf veins, this increases the chances of germ tubes finding stomata. Once a germ tube reaches a stoma, yet unidentified volatile compounds escaping from the stomatal pore initiate the differentiation of a specialized penetration structure, the appressorium. Hyphae of *Rhizoctonia solani* grow along the valleys above epidermal cell junctions where they penetrate after forming infection cushions. Conidia of the banana anthracnose pathogen, *Colletotrichum musae* settle in these valleys and germinate to form appressoria.

Valleys in the cuticle surface connect to ectodesmata which are pits in the outer wall of underlying epidermal cells where the plasma membrane is exposed. Plant metabolites leak from ectodesmata and accumulate in valleys on the leaf surface, providing nutrients for pathogens and saprophytes. Physical stimuli like scratches on glass, aluminum, gold or polythene surfaces also initiate appressorium formation in some pathogens (e.g., cereal rusts), presumably by imitating the surface topography of host surfaces.

Very few microorganisms that come into contact with a plant surface develop a successful parasitic relationship. Either their nutrient requirements are not satisfied, or their growth and development is inhibited. Sugars, amino acids, and minerals secreted onto the leaf surface (the phylloplane) often stimulate the germination and growth of pathogens. Leaf and petal surfaces contain secretions that nonspecifically stimulate the germination and attract the directional movement or growth of leaf pathogens, including *Botrytis cinerea.* Motile cells of the vascular wilt pathogen *Ralstonia solanacearum* are attracted to stomata by chemical gradients and subsequently enter the host through stomatal apertures. Germinating seeds produce a similar mix of stimulatory plant exudates which influence infection by pathogens such as *Pythium ultimum.* The rhizosphere (the zone of soil adjacent to and influenced by roots) also contains exudates. The concentration of exudates is the highest adjacent to the root surface, and the gradient of plant diffusates away from the root directs the movement of zoospores, bacteria and some nematodes towards the root surface. The rhizosphere supports a higher population of saprophytic and sometimes antagonistic microorganisms than the rest of the soil. These microorganisms may affect pathogen development.

Numerous fungi, oomycetes and parasitic plants produce feeding structures, known as 'haustoria.' They are formed within living cells of the host organism, assumed to play a major role in the absorption of plant metabolites and thus infection. Haustoria of rust fungi can be purified biochemically. Several genes that are expressed in haustoria have been identified. An amino acid transporter protein of the rust fungus, *Uromyces fabae* on broad bean is specifically localized in haustoria, which confirms a role in nutrient assimilation. Signal transduction pathways allow the pathogen to recognize and respond to the plant surface/plant tissue environment. Proteins involved in these signal transduction pathways can then be predicted to be essential for pathogenicity. These signal transduction pathways are often conserved between pathogenic and nonpathogenic organisms.

Puccinia striiformis is an obligate biotrophic fungus, which during infection forms an intimate relationship with the host plant. This includes the formation of highly specialized structures, haustoria, which are formed between the cell wall and plasma membrane of host cells in leaves (Chris Khadgi Sørensen, 2012).

Haustoria play an important role in the acquisition of water and nutrient from the host and are further involved in signaling processes, including suppression and induction of plant defense. Due to its biotrophic nature P. *striiformis* cannot be grown in culture, and infection structures can thus only be studied in leaf tissue. In this study, a classical staining technique was used in combination with advanced 2-photon microscopy to study haustoria in fixed-whole leaves. This allowed 3D reconstruction of fungal structures and revealed several characteristics of haustoria not previously reported. The haustorial body gradually changed from small and spherical to highly irregular and apically branched, taking up significant space in the host cell. The haustorial neck area, which is thought to play an important role in the formation of haustoria, showed significant changes during haustoria development. These results indicate that haustorium formation is a highly dynamic process, which should be considered in future studies on the function and constitution of haustoria of *P. striiformis*.

Pathogen development is influenced by temperature, moisture, light, aeration, nutrient availability and pH. Each species of the pathogen and each strain of many pathogenic species has its own distinct requirements for parasitic development. The spores of most parasitic fungi germinate only in the presence of free moisture. However, conidia of some powdery mildew fungi, like many saprophytic molds, germinate at a relative humidity as low as 70%. Most plant pathogens develop rapidly at temperatures between 15 and 25°C, although some species prefer colder or warmer temperatures. These temperatures may not always coincide with the optimum temperature for disease severity, as this is a product of the effects on the host-pathogen interactions.

The movement of zoospores of *Phytophthora cinnamomi* in the rhizosphere of potential host roots illustrates the interacting environmental factors that determine pathogen development. Zoospores are aerobic and swim towards the air-water interface where oxygen concentrations are highest. They also swim towards higher sugar concentrations and respond to electrical charges on the root surface. Wound pathogens such as *Pythium ultimum* have a positively charged anterior flagellum that is attracted to negatively charged surfaces around root hairs and wound sites. In contrast zoospores of most species of *Phytophthora* have a negatively charged anterior flagellum and are attracted to the positively charged surface at the zone of root elongation. At each stage of the journey, the zoospores are susceptible to antagonistic and parasitic microbes or inhibitory plant exudates and also to temperature and moisture changes.

2.2.1 Entry

Pathogens exploit every possible pathway to enter their host, although individual species of pathogen tend to have a preferred method. Fungal pathogens often use direct penetration of the plant surface to enter the host. This requires adhesion to the plant

share many common features. These include dispersal of an infectious particle, host adhesion, recognition, penetration, invasive growth, and lesion development (Shaowu Meng *et al.*, 2009). Previously, many of these common processes did not have corresponding Gene Ontology (GO) terms. For example, no GO terms existed to describe processes related to the appressorium, an important structure for infection by many fungi and oomycetes. In this chapter, we have identified common features of the pathogenic processes of fungi and oomycetes and created a pathogenesis model using 256 newly developed and 38 extant GO terms, with an emphasis on the appressorium and signal transduction. This set of standardized GO terms provides a solid base to further compare and contrast the molecular underpinnings of fungal and oomycete pathogenesis.

Sixty-four new GO terms were developed to describe the biological process of penetration into the host, which were formed into two groups (Shaowu Meng *et al.*, 2009). The first group included 43 new GO terms related to infection structures established on the outside of the host tissue, such as appressoria, hyphopodia, infection cushions and haustorium mother cells. The second group had 21 new terms related to specialized structures that directly pierce the surface of the host, for example, penetration pegs, penetration hyphae and haustorium necks. Two hundred fifty-six new GO terms were developed to annotate genes or gene products involved in common pathogenic processes in fungi and oomycetes, including spore dispersal, host adhesion, recognition, penetration, and invasive growth. These new GO terms provide the opportunity to apply a standard set of terms to annotate gene products of fungi, oomycetes, and their associated hosts, as well as those of other plant-associated pathogens and their hosts. The ability to compare and contrast these annotations for widely different plant-associated microbes and their hosts, using a standardized vocabulary, will greatly facilitate the identification of unique and conserved features of pathogenesis across different kingdoms. In addition, such comparisons should provide insight into the evolution of pathogenic processes.

Successful pathogenesis requires a number of coordinated processes whose genetic bases remain to be fully characterized. Schreiber *et al*. (2012) utilized a high-throughput, liquid media-based assay to screen transposon disruptants of *Pseudomonas syringae* pv. *maculicola* ES4326 to identify genes required for virulence in *Arabidopsis*. Many genes identified through this screen were involved in processes such as type III secretion, periplasmic glucan biosynthesis, flagellar motility, and amino acid biosynthesis. A small set of genes did not fall into any of these functional groups, and their disruption resulted in context-specific effects on in planta bacterial growth.

2.2.3 Direct Penetration

Probably the most common mode of entry by fungal pathogens is by direct penetration of the plant surface (Fig. 2.2). Plant surfaces are complex and robust and resistant to penetration by the vast majority of microorganisms. During penetration, pathogen genes are expressed in a coordinated sequence that results in adhesion, followed by the application of physical pressure to the plant surface and the enzymic degradation of the

cuticle and different layers of cell walls. Physical and chemical signals switch pathogen genes on and off precisely, so that cutinase, followed by cellulase, then pectinase and protease digest the host cuticle, cell wall, and middle lamella in the order they are confronted.

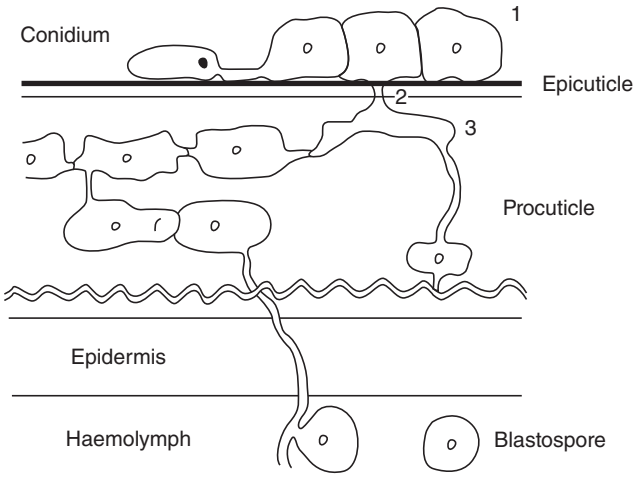


Fig. 2.2 Penetration of host cuticle by a Deuteromycetes entomopathogens 1 = appressorial complex, 2 = penetration peg, 3 = penetration plate

Enzymic degradation of the cuticle by fungal enzymes is necessary for adhesion and penetration by some pathogens. Inhibition of cutinase activity using enzyme inhibitors or cutinase-specific polyclonal antibodies reduces infection of unwounded pea stems by *Fusarium solani* and of unwounded pawpaw fruits by *Cotletotrichum gloeosporiotdes*. These inhibitors do not affect penetration if the cuticle of the inoculated pea stem or pawpaw fruit is first mechanically damaged (David Guest and John Brown, 1997).The pathogenicity of nonpathogenic cutinase-deficient mutants of *C. gtoeosporioides* is restored in the presence of added cutinase. *Mycosphaerella* sp. normally only pathogenic to wounded pawpaw fruit became able to enter unwounded fruit when purified cutinase from *C. gloeosporioides* was added to the inoculum. Pathogenicity was also restored when the fungus was transformed with a cutinase gene from *Nectria haematococca*. These findings provide solid evidence, although not conclusive proof, that cutinase activity is required for pathogenesis.

Root pathogens face a surface composed of suberized tissue, the periderm. Suberin is extremely resistant to enzymic degradation and to physical pressure. However, the take-all pathogen of cereals and the root-rot fungus of many trees, *Arrnitlaria mellea*, penetrate root surfaces using specialized runner hyphae or rhizomorphs, respectively, that apply intense pressure to the infection point. Secondary cell walls are degraded by only a few wood rotting fungi, forcing most pathogens to grow in between cells, or within their lumens, as happens with vascular pathogens.

2.2.3.1 Breakdown of physical barriers

Viruses have no physical force or enzyme system of their own to overcome structural or chemical barriers of the host and, therefore, come in contact with the host protoplasm only through wounds. Bacteria are mostly weak parasites and cannot employ force to effect penetration. Fungi and nematodes are the only groups of plant pathogens that employ force for direct penetration of the host. Fungi penetrate host plants directly through a fine hypha produced directly by the spore or mycelium or through a penetration peg produced by an appressorium. These structures exert pressure on the surface which results in stretching of the epidermis which becomes thin. Then the infection peg punctures it and affects its entry.

2.2.3.2 Breakdown of chemical barriers

The host is provided with defense mechanisms against invasion which include the presence of cuticular layer on the epidermis, lack of suitable nutrients for the pathogen in the host cells, presence of inhibitory or toxic substances in the host cells and exudation of substances toxic to pathogen or stimulatory to antagonists of the pathogen [*Example*: The glands in leaf hairs of Bengal gram contain maleic acid which is antifungal and provide resistance to infection by the rust fungus (*Uromyces ciceris arietini*)]. Similarly, protocatechuic acid and catechol in the red scales of onion provide resistance to onion smudge pathogen, *Colletotrichum circinans*. To overcome these physical and chemical barriers, the fungi produce various enzymes, toxins organic acids and growth regulators.

2.2.4 Indirect Penetration–Through Natural Openings

Plants have many natural openings that facilitate gas exchange between plant tissues and their environments. These openings are also used by pathogens as entry points. Major among them are stomata, hydathodes, lenticels, and nectaries.

2.2.4.1 Stomata

Stomata are microscopic pores formed by pairs of guard cells in the epidermis of terrestrial plants and are essential for gas exchange with the environment and controlling water loss. Accordingly, plants regulate stomatal aperture in response to environmental conditions, such as relative humidity, CO_2 concentration, and light intensity. Stomatal openings are also a major route of pathogen entry into the plant and plants have evolved mechanisms to regulate stomatal aperture as an immune response to bacterial invasion. Stomata represent a major route of pathogen invasion, and recent studies have begun to shed light on the signal transduction cascades underlying bacterial regulation of stomatal closure and opening. It was suggested that stomatal closure was a functional output of both PAMP-triggered and effector-triggered immunity. Because stomata respond to both abiotic and biotic signals, pathogens might exploit abiotic environmental conditions, such as high humidity and/or produce virulence factors to actively suppress stomatal closure as part of their infection strategy (Weiqing Zeng *et al.*, 2011). In the future, it would be

discriminate between open and closed stomata, this may be driven by a chemotactic process that could involve sensing of nutrients derived from the leaf interior and subsequent directed bacterial movement that is propelled by the flagellin-based motility apparatus. Directed motility by chemotaxis is required for virulence and competitive fitness of *Ralstonia solanacearum*, which invades host plants via their roots. *R. solanacearum* is attracted by diverse amino acids and organic acids present in exudates from roots of their host plants; mutants lacking core proteins that regulate chemotaxis exhibit reduced virulence despite retaining normal motility.

Stomata-based immunity might also have relevance for other classes of pathogens, such as parasitic fungi. Epiphytic hyphae of the basidiomycete rust fungus move directionally toward stomata to enter the leaf interior. Also, chitosan, a β-1,4-linked glucosamine derived from fungal cell walls, induces stomatal closure. Unlike bacteria, however, many parasitic fungi including rusts must penetrate the plant cell wall to accommodate specialized feeding structures in plant cells for nutrient uptake. Recent evidence suggests that fungal entry into plant cells is restricted by two secretory pathways that likely deliver defensive compounds into the extracellular space upon PAMP recognition. Ascomycete powdery mildew fungi appear to engage a plasma membrane protein of the host to manipulate these secretory pathways for entry into plant cells.

Foliar diseases have been reported to decrease crop water-use efficiency (WUE) substantially, yet the effects of plant pathogens are seldom considered when methods to improve WUE are debated. Michael K. Grimmer *et al.* (2012) reviewed the effects of foliar pathogens on plant water relations and the consequences for WUE. The effects reported varied between host and pathogen species and between host genotypes. Some general patterns emerged, however. Higher fungi and oomycetes cause physical disruption to the cuticle and stomata and also cause impairment of stomatal closing in the dark. Higher fungi and viruses are associated with impairment of stomatal opening in the light. A number of toxins produced by bacteria and higher fungi have been identified that impair stomatal function. Deleterious effects are not limited to compatible plant–pathogen interactions. Resistant and nonhost interactions have been shown to result in stomatal impairment in light and dark conditions. Mitigation of these effects through a selection of favorable resistance responses could be an important breeding target in the future. The challenges for researchers are to understand how the effects reported from work under controlled conditions translate to crops in the field, and to elucidate underlying mechanisms.

The hypothesis that stomatal wax plugs in *Agathis robusta* (Araucariaceae) protect leaves against fungal invasion by preventing hyphae entering the stomatal pore was tested by Mansour A. Mohammadian *et al.* (2009). Leaves with intact wax plugs and leaves from which wax plugs had been experimentally removed were inoculated with either *Botrytis cinerea* or *Alternaria solani*. Interactions of fungal hyphae with stomata were subsequently analyzed by scanning electron microscopy. Wax plugs blocked the penetration of fungal hyphae into stomata of *A. robusta* in 100% of encounters. In contrast, hyphae readily penetrated stomata of leaves from which wax plugs had been removed or where the wax plugs were damaged. On rare occasions, hyphae were also observed to penetrate directly through the cuticle. Florin rings around the external surface of

the leaf surface, a characteristic sign of the disease and a source of secondary inoculum. Xoc, by contrast, penetrates the leaf mainly through stomata multiplies in the substomatal cavity and then colonizes the intercellular spaces of the parenchyma. Like Xoo, Xoc may also gain access through wounds, but it remains restricted to the apoplast of the mesophyll tissue and does not invade the xylem. Xoc also exudes from natural openings in the leaf in chains or strands, or under moist conditions as small beads of ooze. Yellow exudate on the leaf surface is a typical sign of bacterial leaf streak, and as is the case for Xoo, it may fall into irrigation water or be dispersed by wind, rain, insects or other means, and contribute to the spread of the disease.

2.2.4.3 Lenticels

Lenticels are raised pores that facilitate gas exchange across the bark of woody plant tissues. Although they adequately exclude most microorganisms, pathogens such as *Spongospora subterranea* (powdery scab of potatoes), Penicillium *expansum* (blue mold of apples) and *Monilinia fructicola* (brown rot of stone fruits), enter through lenticels. A lenticel is an opening that allows gasses to be exchanged between air and the inner tissues of a plant. They can be commonly found in bark and on the outside of fruits. Some are more noticeable than others. Ectodesmata, styles and nectaries also provide potential entry points for specialized pathogens. Surprisingly, pathogens appear unable to follow the route taken by pollen tubes through the stigma and style to invade the ovaries. Bacteria can enter, and exit, from stomata along the sides of the style. The alternative pathway for pathogen entry is via a preexisting opening in the plant surface. This can be a natural opening or a wound. Pathogenic bacteria and nematodes often enter through stomatal pores when there is a film of moisture on the leaf surface.

Blackleg is caused by the bacterium *Erwinia carotovora* subsp. *atroseptica*. The blackleg organism may also infect lenticels, causing them to be slightly sunken and brownish to black in color. The infected lenticels can be up to 1/4 inch in diameter. The tissues under the infected lenticels are brownish and usually dry. This extends less than 1/8 inch into the tuber flesh. *Pseudomonas syringae* pv. *actinidiae* (Psa) is a bacterium whose virulent form (Psa-V) causes severe infections of kiwifruit, particularly *Actinidia chinensis* 'Hort16A.' Lenticels on kiwifruit canes function as pores, allowing gaseous exchange. The lenticellular structure penetrates through the periderm, potentially allowing bacterial entry and subsequent cortex infection (Larsen *et al.*, 2012). Bacteria have been observed inside and directly below lenticels from ca 3-year-old woody tissue from the field. To investigate this pathway of bacterial infection further, lenticels from three wood ages were inoculated with a strain of Psa-V at 109 cfu/ml. Brown staining was observed on lenticels three days after inoculation on the youngest wood (less than one-year-old). Lenticels were sectioned two and four weeks after inoculation, and isolations were conducted from sterilized tissue after four weeks. Psa was visible inside lenticels of the youngest wood two weeks postinoculation and was also isolated from the youngest tissue after four weeks. Bacteria were not observed in lenticels of older wood and Psa was not isolated.

caused by farm operations, hail storms, or insects will help in the entry of different plant pathogens into the host cells. Organisms which cause storage diseases and ripe rots will enter through the wounds caused by farm operations. (*Example: Rhizopus, Gloeosporium, Aspergillus, Penicilium, Colletotrichum, Diplodia,* etc.). Weak parasites enter through the wounds caused by hail storms and freezing (*Example: Macrophomina phaseolina*). Pathogen causing brown rot of fruits (*Sclerotinia fructicola)* enters through the wounds caused by insect punctures. Similarly, the causal organism of Dutch elm disease (*Ceratostomella ulmi)* enters through the wounds caused by elm bark beetle.

Penicillium digitatum (blue mold of citrus) only infects fruit wounded during harvest or postharvest handling. Postharvest handling damage allows the entry of many fruit and vegetable rot pathogens, including *Rhizopus nigricans* and *Sclerotium rolfsii.* Wounds also occur naturally. Wounds caused by farm operations, hail storms, or insect punctures, etc., will help in the entry of different plant pathogens into the host cells. Organisms that cause storage diseases and ripe rots will enter through the wounds caused by farm operations (*Example*: *Rhizopus, Gloeosporium, Aspergillus, Penicilium, Colletotrichum, Diplodia*, etc.). The points of lateral root emergence enable the fungus *Chalara elegans* to enter tobacco roots. Leaf scars provide similar infection sites for the apple canker pathogen, *Nectria gattigena*. Damage also results from wind, hail, frost or extreme heat or from the effects of insects, herbivores or humans. Most plant viruses enter through wounds, such as those created by their insect vectors.

The mechanical wounding of plant leaves, which is one of the first steps in pathogen infection and herbivore attack, activates signal transduction pathways and airborne signals to fend-off harmful organisms. The mechanisms by which these signals promote plant immunity remain elusive (Yuri L. Dorokhov *et al.*, 2012). It was demonstrated that plant-leaf wounding results in the synthesis of a cell wall enzyme, pectin methylesterase (PME), causing the plant to release methanol into the air. Gaseous methanol or vapors from wounded PME-transgenic plants induced resistance to the bacterial pathogen, *Ralstonia solanacearum* in the leaves of nonwounded neighboring 'receiver' plants. To investigate the mechanism underlying this phenomenon, the methanol inducible genes (MIGs) were identified in *Nicotiana benthamiana*, most of which fell into the category of defense genes. Genes were selected and isolated, viz., *noncell-autonomous pathway protein (*NCAPP*), β-1,3-glucanase (*BG*),* and the previously unidentified MIG-21. It was later, demonstrated that BG, MIG-21, and NCAPP could enhance cell-to-cell communication and *Tobacco mosaic virus (*TMV) RNA accumulation. Moreover, gaseous methanol or vapors from wounded plants increased TMV reproduction in 'receivers.' Thus, methanol emitted by a wounded plant enhances antibacterial resistance as well as cell-to-cell communication that facilitate virus spreading in neighboring plants.

2.2.5 Colonization

A successful infection requires the establishment of a parasitic relationship between the pathogen and the host, once the host has gained entry to the plant (David Guest and John Brown, 1997). There are two broad categories of pathogens are biotrophs (those that establish an infection in living tissue) and necrotrophs (those that kill cells before

specialized biotrophic relationships with their hosts. Intercellular hyphae of downy mildew colonize host mesophyll cells and form haustoria. The mildew sporulates and the infected cells eventually die, although necrosis is delayed and contained, compared to that caused by necrotrophic pathogens. Rust fungi can also delay senescence in infected cells while they sporulate. Vascular infections usually cause wilting and discoloration as a result of the physical blockage of infected xylem vessels. True vascular wilt pathogens colonize the vascular tissue exclusively, although other pathogens can cause the same symptoms if they infect the vascular system as well as other tissues. There are a few pathogens that manage to achieve systemic infection of their host. For example, many viruses can spread to most parts of the plant, although not necessarily all tissues. Some downy mildews can also systemically infect their host by invading the vascular tissue and growing throughout the host, causing deformation, rather than necrosis. Finally, there are some pathogens that complete their entire life cycle within the cells of their host, and may spread from cell to cell during cytokinesis. These are endobiotic infections.

The characteristics of idealized biotrophic and necrotrophic relationships are summarized in Table 2.1 (David Guest and John Brown, 1997). In reality, this distinction is not always clear. Every host-pathogen combination is unique, and few archetypal necrotrophs or biotrophs exist. Symbiotic fungi, such as mycorrhizal fungi and endophytes and symbiotic bacteria such as *Rhizobium* spp., represent highly coevolved examples of biotrophic parasitism. Most interactions are hemibiotrophic, initially biotrophic, but at later stages of parasitism, necrotrophic. Hemibiotrophs utilize parasitic strategies of both biotrophs and necrotrophs. *Phytophthora palmivora*, *Fulvia fulva,* and *Colletotrichum lindemuthianum* at first colonize and parasitize living cells. Nevertheless, these cells eventually collapse and die, triggering necrotrophic growth that often coincides with sporulation.

Infection of plants by pathogenic bacteria can generally be considered in terms of three interrelated phases, viz., population build-up, competition and migration of bacteria at the plant surface, bacterial entry into plant tissue and the migration of bacteria within the plant to and from regions of multiplication.

2.2.5.1 Population build-up

The presence of epiphytic pathogens on host plants does not imply that disease will necessarily develop, and many cases have been reported where quite high levels of pathogenic bacteria were present on symptomless foliage. This has been noted, for example, for *Pseudomonas syringae* pathovars on red maple and snap beans and for *Erwinia amylovora* on apple and pear blossom.

In other situations, the presence of epiphytic bacteria does lead to disease development. The presence of *Pseudomonas syringae* pv. *morsprunorum* as an epiphyte on cherry foliage leads to canker formation. The relationship between epiphytic occurrence and disease development has subsequently been investigated for a wide range of bacterial pathogens by monitoring naturally occurring populations and carrying out experimental inoculations of plant surfaces. These studies have shown that plant infection and disease development

2.2.5.3 Molecular genetics: identification and investigation of bacterial genes

Bacterial genes, occurring on either chromosomal or plasmid DNA, are involved in the determination of a wide range of phenotypic characteristics. In recent years, new techniques of molecular biology have been particularly successful in the genetic analysis of plant pathogenic bacteria and have been described in detail in a number of recent texts. The major objectives of molecular genetics are identification and isolation (cloning) of specific genes with defined functions.

In natural environments, where a particular host species occurs within mixed vegetation, the development, and spread of disease is probably limited to some extent by the separation of individual plants within the area. This constraint does not apply in the crop situation, where localized infection and progression of disease within the homogeneous plant population can occur rapidly. In this artificial situation, where the natural balance between pathogen and host does not apply, special control measures often have to be adopted if the large-scale occurrence of disease and consequent major crop loss are to be avoided. These measures fall into four main categories: chemical control, biological control, breeding of resistant cultivars and sanitary procedures.

2.2.5.4 Disease physiology

While necrotrophs have little effect on plant physiology since they kill host cells before colonizing them, biotrophic pathogens become incorporated into and subtly modify various aspects of host physiology, such as respiration, photosynthesis, translocation, transpiration and growth and development. The respiration rate of plants invariably increases following infection by fungi, bacteria or viruses. The higher rate of glucose catabolism causes a measurable increase in the temperature of infected leaves. An early step in the plant's response to infection is an oxidative burst, which is manifested as a rapid increase in oxygen consumption, and the release of reactive oxygen species, such as hydrogen peroxide (H_2O_2) and the superoxide anion (O^{2-}). The oxidative burst is involved in a range of disease resistance and wound repair mechanisms.

2.2.5.5 Link to rapid active defense

In resistant plants, the increase in respiration and glucose catabolism is used to produce defense-related metabolites via the pentose phosphate pathway. In susceptible plants, the extra energy produced is used by the growing pathogen. Pathogens also affect photosynthesis, both directly and indirectly. Pathogens that cause defoliation rob the plant of photosynthetic tissue, while necrotrophs decrease the photosynthetic rate by damaging chloroplasts and killing cells. Biotrophs affect photosynthesis in varying degrees, depending on the severity of the infection. A biotrophic infection site becomes a strong metabolic sink, changing the pattern of nutrient translocation within the plant and causing a net influx of nutrients into infected leaves to satisfy the demands of the pathogen. The depletion, diversion and retention of photosynthetic products by the pathogen stunts plant growth and further reduced the plant's photosynthetic efficiency. In addition, pathogens affect water relations in the plants they infect. Biotrophs have

little effect on transpiration rate until sporulation ruptures the cuticle, at which point the plant wilts rapidly. Pathogens that infect the roots directly affect the plant's ability to absorb water by killing the root system, thus producing secondary symptoms such as wilting and defoliation. Pathogens of the vascular system similarly affect water movement by blocking xylem vessels. Growth and development, in general, are affected by pathogen infection, as a result of the changes in source-sink patterns in the plant. Many pathogens disturb the hormone balance in plants by either releasing plant hormones themselves, or by triggering an increase or a decrease in synthesis or degradation of hormones in the plant. This can cause a variety of symptoms, such as the formation of adventitious roots, gall development, and epinasty (the down-turning of petioles).

2.2.6 Growth and Development

Because pathogens affect the source-sink patterns in plants, normal growth and development are affected. In addition to the direct damage to plant tissue and nutrient deprivation, many pathogens disturb the hormone balance in plants. Plant growth and development depends on subtle shifts in the balance between auxin, gibberellin, cytokinin, ethylene and abscisic acid. Pathogens may disturb the hormone balance by releasing plant hormones or by inducing increased or decreased synthesis or degradation of plant hormones. Gibberellic acid, the first plant hormone described, was discovered during a study of bakanae, or 'foolish seedling' disease of rice. Infected seedlings are unusually tall, and flowering is suppressed. Following the discovery in the 1930s that cell-free culture filtrates of the pathogen, *Gibberella fujikuroi,* cause the same symptoms, the active chemical was identified as gibberellic acid. Gibberellic acid was subsequently shown to be produced by plants, where it naturally functions as a plant hormone. Epinasty, the down-turning of petioles, is a symptom typical of many systemic bacterial infections such as bacterial wilt caused by *Ralstonia solanacearum.* The fact that the addition of ethylene can also induce this symptom suggests that this plant stress hormone is involved in symptom development. Green islands formed around rust pustules result from sustained cytokinin levels in infected cells. Cytokinin generates a metabolic sink and maintains cell viability in parasitized cells while nonparasitized cells surrounding the pustule senesce and die prematurely.

Hormonal disturbances are also involved in the appearance of witches' broom and broomsticking symptoms caused by phytoplasmas (e.g., big bud diseases) and some fungi, such as *Crinipellis perniciosa* (witches' broom of cocoa), and *Oncobasidium theobromae* (vascular-streak dieback of cocoa). Vascular wilt infections upset auxin and ethylene balances in plants, inducing epinasty and the formation of adventitious roots on the lower stem. Galls and club-roots caused by fungi, nematodes, insects, and bacteria develop because of excessive cell growth (hypertrophy) and division (hyperplasia). Perhaps the best-studied example of symptoms associated with hormonal disturbance is crown gall, a disease of a wide range of plants caused by the bacterium *Agrobacterium tumefactens*. Genes from the pathogen, after incorporation into the plant genome, condition the overproduction of hormones that induce cell division and gall formation. Giant cells formed in roots infected by the root knot nematode, *Meloidogyne* species result from the breakdown of cell walls as well as excessive cell growth.

2.2.7 Exit of Pathogen

After invasion and colonization of the host, the pathogens come out of the host to maintain the continuity of the infection chain or disease cycle and escape death due to overcrowding. Once the pathogens exit from the host, they survive and are disseminated to other hosts and continue the infection cycle. Viruses can exist only with the living protoplasm and hence disseminated through their animate vectors like insects, fungi, and nematodes. The bacteria ooze out in the form of slime on the host surface from where they can be disseminated through water and insects. However, the fungi have the most elaborate system of exit, grow out on the host surface and produce repeating spores (secondary inoculum), usually asexually, under favorable conditions. The spores thus formed are disseminated through the wind, water, soil, seed, and vegetative propagating material and agricultural implements. Phytonematodes, however, come out of dead tissues, either infest another healthy tissue/plant part or survive in the soil.

Dothideomycetes is the largest and most ecologically diverse class of fungi that includes many plant pathogens with high economic impact. Currently 18 genome sequences of *Dothideomycetes* are available, 14 of which are newly described allowing unprecedented resolution in comparative analyses (Ohm *et al.*, 2012). These 18 organisms have diverse lifestyles and strategies of plant pathogenesis. Three feed on dead organic matter only, six are necrotrophs (killing the host plant cells), one is a biotroph (forming an association with and thus feeding on the living cells of the host plant cells), and 8 are hemibiotrophs (having an initial biotrophic stage, and killing the host plant at a later stage). These various lifestyles are also reflected in the gene sets present in each group. For example, sets of genes involved in carbohydrate degradation and secondary metabolism are expanded in necrotrophs. Many genes involved in pathogenesis are located near repetitive sequences, which are believed to speed up their evolution. Blocks of genes with conserved gene order were identified. In addition to this we deduce that the mechanism for mesosynteny, a type of genome evolution particular to *Dothideomycetes*, is by intrachromosomal inversions.

The genome and transcriptome analyses of *Colletotrichum higginsianum* infecting *Arabidopsis thaliana* and *Colletotrichum graminicola* infecting maize was reported by Richaard J O'Connell *et al.* (2012). Comparative genomics showed that both fungi have large sets of pathogenicity-related genes, but families of genes encoding secreted effectors, pectin-degrading enzymes, secondary metabolism enzymes, transporters, and peptidases are expanded in *C. higginsianum*. Genome-wide expression profiling revealed that these genes are transcribed in successive waves that are linked to pathogenic transitions: effectors and secondary metabolism enzymes are induced before penetration and during biotrophy, whereas most hydrolases and transporters are upregulated later, at the switch to necrotrophy. These findings showed that preinvasion perception of plant-derived signals substantially reprograms fungal gene expression and indicate previously unknown functions for particular fungal cell types.

Bacteria have many export and secretion systems that translocate cargo into and across biological membranes. Seven secretion systems contribute to pathogenicity by translocating proteinaceous cargos that can be released into the extracellular milieu or directly into

recipient cells. Chang *et al.* (2014) described these secretion systems and how their complexities and functions reflect differences in the destinations, states, functions, and sizes of the translocated cargos as well as the architecture of the bacterial cell envelope. They examined the secretion systems from the perspective of pathogenic bacteria that proliferate in plant tissues and highlight examples of translocated proteins that contribute to the infection and disease of plant hosts.

2.2.8 Major Steps in Pathogenesis

Fungi must find the host and the appropriate entry site, develop specialized infection structures, overcome preformed and induced host defenses, take up nutrients, grow and colonize host tissue.

2.2.8.1 Finding the host

It may be either passive vs. active and involves steps like wait in favorable site for the host; dispersal by animal vectors and use of dispersal stages like spores, zoospores, etc.

2.2.8.1.1 Chemotaxis of Phytophthora zoospores

Tactic response allows zoospores to reach infection sites in roots of host plants, positive chemotaxis towards the plant, compounds (*Example: P. sojae* to isoflavones), electrotactic responses also noted (weak electric fields generated by roots). *Phytophthora* and *Pythium* zoospores swim towards amino acids and sugars. *Phytophthora palmivora* marked is attracted to the root tip (electrotaxis) while *Pythium aphanidermatum* is attracted to wounds (Sophien Kamoun, 2010).

2.2.8.2 Developmental processes and infection

Morphological changes are essential to penetration and adaptation to plant issue. Many fungal/oomycete pathogens develop highly specialized infection structures in response to signals such as contact with the plant surface and plant tissue environment. Most important structures are appressoria and haustoria. Not all fungal plant pathogens produce specialized infections structures. Forcible entry is seen in rice blast fungus. A spore from *Magnaporthe grisea* has germinated on the surface of a rice leaf and formed a dome-shaped appressorium. The appressorium has to breach the thin but tough rice leaf cuticle to invade the leaf and cause disease (Talbot, 1999).

2.2.8.3 Penetration of plant epidermis

Infection process by appressoria can involve enzymatic action; external matrix around appressoria contains cutinase, cellulases, and other enzymes to help soften the cuticle, thereby aiding adhesion and penetration. However, some fungi can physically force their way through plant cuticles. Fungi such as *Colletotrichum* and *Magnaporthe* produce appressoria with tough melanin-pigmented cell walls. Appressoria generate pressures between 6-8 Mega Pa (30-40x pressure of a car tire), produce hydrostatic turgor by

accumulating molar concentrations of glycerol, and can puncture artificial plastic membranes. Melanin mutants are nonpathogenic and cannot accumulate turgor pressure.

2.3 INFECTION BY BACTERIA

Bacteria can enter through cuts our other areas of damage that occur in the leaves or stems due to wind bending the plant, objects hitting the plant, aphids sucking the plant sap or animals grazing on it. The symptoms then depend on which internal tissues and structures where the infection takes a hold. Although thousands of species of bacteria can cause disease in animals and humans, a much smaller number, probably about one hundred, are able to infect plants and damage them. Bacterial plant diseases are generally due to bacilli, bacteria that are shaped by rods, some of which are Gram-negative bacteria and some of which are Gram-positive. The majority of plant diseases due to bacterial infection occur in parts of the world that have a tropical weather pattern.

Once a species of pathogenic bacteria gains entry to a plant it causes one of four main problems that leads to a less than a healthy plant. Some bacteria produce enzymes that break down the cell walls of plants anywhere in the plant. They do this to break open to the cell to gain access to the nutrients inside, but the plant cells affected die quickly, causing parts of the plant to start rotting. This is why plant diseases that develop due to cell wall degrading enzymes are generally known as a type of 'rot.' Some bacteria produce toxins that are damaging to plant tissues generally, usually causing early death of the plant. Others produce large amounts of polysaccharide sugars that have long chains and are very sticky. As these travel in the water carrying vessels, the xylem, they block the narrow channels, preventing water getting from the plant roots up to the shoots and leaves, again causing rapid death of the plant. Finally, some bacteria produce proteins that mimic plant hormones. These lead to overgrowth of plant tissue and tumors form. These grow rapidly, taking up valuable nutrients and energy resources that the plant would otherwise use for its own tissue growth, and it becomes weakened and susceptible to attack by other pathogens such as fungi.

Infection of plants by pathogenic bacteria can generally be considered in terms of three interrelated phases, viz., population build-up, competition and migration of bacteria at the plant surface; bacterial entry into plant tissue and migration of bacteria within the plant to and from regions of multiplication (David C Sigee, 2009).

2.3.1 Build-up and Activity of Epiphytic Populations

The presence of epiphytic pathogens on host plants does not imply that disease will necessarily develop, and many cases have been reported where quite high levels of pathogenic bacteria were present on symptomless foliage. This has been noted, for example, for *Pseudomonas syringae* pathovars on red maple and snap beans and for *Erwinia amylovora* on apple and pear blossom. In other situations, the presence of epiphytic bacteria does lead to disease development. The relationship between epiphytic occurrence and disease development has subsequently been investigated for a wide range of bacterial pathogens by monitoring naturally occurring populations and carrying out experimental

spread of bacteria to surrounding tissues. This reaction was induced by a range of bacteria comprising various pathovars of *Pseudomonas syringae*. The disease reaction involving a delayed host cell response with the spread of bacteria to other parts of the plant was induced by *Pseudomonas syringae* pv. *tabaci* and resulted in wildfire disease and, finally, no reaction was observed after infiltration of the saprophytic bacterium *Pseudomonas fluorescens*.

Although compatible phytopathogenic bacteria share a common ability to spread and multiply within the host plant, the manner in which they do this and the effect they have on the host plant (disease) vary considerably. This chapter considers general aspects of disease induction, different types of disease that are caused by plant pathogenic bacteria and the range of bacterial characteristics that are important in disease development.

2.3.3 Induction of Bacterial Disease

The ability of plant pathogenic bacteria to cause disease in a particular host plant depends on many features, including environmental aspects, plant physiology and development, and the expression of pathogenicity and virulence factors by the bacterial cells.

2.3.4 Environmental and Physiological Factors Affecting Disease Development

Environmental factors are important in the development of plant disease for their direct effects on infection) and for their indirect effects in determining the physiological status of the plant. The various aspects of the plant that affect disease development have been well discussed and include nutritional status, photoperiodic conditioning, and stage of maturity and development. Levels of macronutrients have been shown to be important in plant susceptibility to *Erwinia stewartii*, where elevated levels of N and P increased susceptibility, and high levels of Ca and K increased resistance.

The ability of plant pathogenic bacteria to survive and multiply outside and inside plants, and to cause disease, is determined to a large extent by their genetic constitution. The genetic analysis of plant pathogenic bacteria currently involves the application of molecular techniques for the identification and investigation of bacterial genes that are important in all of these aspects, and will be considered first. Following sections discuss the role of specific genes and gene systems in the activity of plant pathogenic bacteria in relation to the determination of compatibility and incompatibility, disease virulence, and nonpathogenic characteristics. The final part of this chapter deals with the occurrence and role of plasmids in these bacterial cells.

2.3.5 Molecular Genetics – Identification and Investigation of Bacterial Genes

Bacterial genes, occurring on either chromosomal or plasmid DNA, are involved in the determination of a wide range of phenotypic characteristics. In recent years, new techniques of molecular biology have been particularly successful in the genetic analysis of plant pathogenic bacteria and have been described in detail in a number of recent texts. The major objectives of molecular genetics include identification and isolation (cloning) of specific genes with defined functions.

In natural environments, where a particular host species occurs within mixed vegetation, the development, and spread of disease is probably limited to some extent by the separation of individual plants within the area. This constraint does not apply in the crop situation, where localized infection and progression of disease within the homogeneous plant population can occur rapidly. In this artificial situation, where the natural balance between pathogen and host does not apply, special control measures often have to be adopted if the large-scale occurrence of disease and consequent major crop loss are to be avoided. These measures fall into four main categories: chemical control, biological control, breeding of resistant cultivars and sanitary procedures.

To exploit plant nutrients, phytopathogenic bacteria have evolved sophisticated infection strategies (Roberto Buonaurio, 2008). A number of biotrophic Gram-negative bacteria are able to cause diseases in plants through a type III secretion system, composed of a protruding surface appendage, known as the hrp pilus, through which the bacterium injects effector proteins into the host cells to manipulate plant cells, in particular, to suppress plant defenses. A unique infection strategy in plant-bacterial interactions is adopted by *Agrobacterium tumefaciens*, which genetically transforms its host by transferring T-DNA from its tumor-inducing plasmid to the chromosome of a plant cell. This transfer is mediated by the pilus T, belonging to the type IV secretion system. Other important virulence factors of phytopathogenic bacteria are phytotoxins, extracellular polysaccharides, phytohormones and plant-cell-wall-degrading enzymes. These latter factors are fundamental to the pathogenesis of necrotrophic bacteria. Plants defend themselves from bacterial attacks through a multilayered system of passive and active defense mechanisms, which can interfere with entry of bacteria into the plant tissue and restrict bacterial growth when the ingress has been gained. A number of hypotheses have been advanced to explain the restriction of the bacterial growth observed during the hypersensitive reaction and systemic acquired resistance development.

Except for a few rare cases, phytopathogenic bacteria provoke diseases in plants by penetrating into host tissues. Penetration occurs through natural openings, such as stomata, hydathodes, lenticels, nectarthodes, stigma, etc., or through wounds (Scurtleff and Averre, 1997). Bacteria colonize the apoplast that is the intercellular spaces or xylem vessels, causing parenchymatous and vascular or parenchymatous-vascular diseases, respectively. Besides the endophytic habitat, some bacterial species also have the capacity to survive as epiphytes on plant surfaces (phylloplane, rhizoplane, carpoplane, etc.). From an epidemiological point of view, this poses a particular danger because they are quick to infect plants in favorable conditions. Once inside plant tissues, bacteria may implement two main attack strategies to exploit the host plant nutrients: biotrophy, in which the plant cells are kept alive as long as possible, and bacteria extract nutrients from live cells, and necrotrophy, in which bacteria kill plant cells and extract nutrients from dead cells. Interactions of bacteria with plants can be either incompatible or compatible. Incompatible interactions occur when the bacterium encounters a nonhost plant (nonhost resistance) or a resistant host plant (cultivar-specific resistance) and they are frequently associated with a hypersensitive response, i.e., a rapid, programmed death of plant cells that occurs at the site of infection. Compatible interactions occur when the bacterium infects susceptible host plants causing disease symptoms.

A bacterium could be pathogenic yet have varying degrees of virulence. Pathogenicity and/or virulence of Gram-negative plant pathogenic bacteria are strictly dependent on the presence of secretion apparatuses in their cells, through which they secrete proteins or nucleoproteins involved in their virulence in the apoplast or inject into the host cell. To date, five secretion systems, numbered from I to V have been described in both animal and plant Gram-negative pathogenic bacteria. In addition, it is now emerging that the expression of virulence factors is under the control of quorum sensing, a communication mechanism by which bacteria regulate the expression of certain genes in response to their population density.

The pathogenicity of a number of biotrophic Gram-negative bacteria in the genera *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia,* and *Pantoea* is mainly due to their ability to produce a type III secretion system (T3SS), also called injectisome (Desvaux *et al.*, 2004), by which the bacterium injects proteins involved in its virulence into plant cells (T3SS effectors).

It is worth remembering that if the effector leads to the development of disease symptoms in the plant it is called a virulence protein, while if it triggers a defense reaction that leads to the HR, it is referred to as an avirulence protein. The T3SS is encoded by hrp (HR and pathogenicity) and hrc (HR and conserved) genes, whose mutations eliminate bacterial pathogenicity in susceptible host plants and the ability to elicit HR in nonhost or cultivar-specific resistant plants.

2.3.6 Infection Process *in Agrobacterium tumefaciens*

The particular infection modality of *Agrobacterium tumefaciens*, the causal agent of crown gall in many woody and herbaceous dicotyledonous plants, is worthy of separate discussion. This Gram-negative soil bacterium is able to genetically transform healthy host cells in tumoral cells by inserting a part of its DNA (transferred DNA; T-DNA), contained on the tumor-inducing (Ti) plasmid, into the plant genome (Sheng and Citovsky, 1996). T-DNA carries genes involved in the synthesis of plant growth hormones (auxins and cytokinins) and synthesis and secretion of amino compounds called opines, tumor-specific compounds that are exclusively assimilated by the pathogen as major carbon and nitrogen sources. The actively growing tumoral cells for the high plant growth hormone levels and the opine they produce render the tumor a favorable biological niche for *A. tumefaciens* growth.

At least three genetic components are required for tumorigenesis: (i) T-DNA; (ii) the virulence (*vir*) region located on the Ti plasmid, which genes respond to specific plant-released signals, to generate a copy of the T-DNA and mediate its transfer into the host cell; and (iii) a suite of chromosomal virulence (*chv*) genes, involved in bacterial chemotaxis toward-and attachment to the wounded plant cell wall (Sheng and Citovsky, 1996). In natural infections, *A. tumefaciens* cells present in the soil, reach wounds (the main infection sites) of roots and the subterranean part of the stem by the means of flagella, attracted by the compounds (e.g., sugars) released from plant wounds. The importance of motility in the infection process is illustrated by the fact that mutations in genes encoding flagellin abolish motility and reduce tumorigenesis (Chesnokova *et al.*, 1997; Hawes and Smith, 1989).

symptoms that they cause. Induced or repressed genes belong to a broad range of cellular processes, such as hormonal regulation, cell cycle control and endogenous transport of macromolecules, among others. In addition, recent evidence indicates the existence of an interplay between plant development and antiviral defense processes, and that interference among the common points of their signaling pathways can trigger pathological manifestations.

More than one thousand viruses are currently known to be potentially capable of infecting plants. Despite a large number of possible combinations, the development of the disease is an exception rather than a common outcome and thus, in most cases, plants are capable of counteracting the harmful effects of viruses. This resistance is owed to the absence of essential host susceptibility factors (passive resistance) or to the existence of several defense layers that the virus has to overcome. First, the virus needs to overcome a series of preexisting physical and chemical barriers in plants. If a pathogenic virus succeeds in overcoming this first line of defense, it would have to face the nonspecific defensive reactions with which the plant responds to some molecular patterns that are common to different pathogens (Jones and Dangl, 2006). If a virus has evolved to acquire virulence factors to counteract this basal defense, it is in a position to be able to trigger infection. In many cases, however, plants are able to recognize these virulence factors and create a new, more specific resistance layer that is only induced when faced with viruses expressing this virulence factor. A virus can cause productive infection only in those plants that have not developed specific defensive responses to its virulence factors.

A virus not only needs to escape the defenses that plants erect but must also tackle different processes to complete its productive cycle (Maule *et al.*, 2002). The initiation of this cycle depends on the nature of the genetic material of the virus. Positive-polarity RNA viruses are the most abundant in the plant kingdom. For these viruses, genomic RNA must be uncoated and translated after viral particles have entered the plant cell, and both processes are highly coordinated. There also seems to be some kind of coupling between the synthesis of viral proteins and the assembly of some of these proteins with genomic RNA and host factors to form replication complexes. The next stage of the virus cycle entails its movement to neighboring cells and its dissemination throughout the plant. Interactions of viral and cellular factors may not only contribute to facilitate these viral infection steps and help to establish optimum infection susceptibility conditions but may also indirectly affect host physiological processes. Although many viral infections progress efficiently without symptom development, induction of plant defense mechanisms, their suppression by counteracting viral strategies and the cooption of host factors required for virus replication, and movement can confer a pathological character upon the viral infection. There is experimental evidence of the individual contribution of these elements in different viral infections; nonetheless, a model that includes them in the specific development of a particular pathology is lacking (Culver and Padmanabhan, 2007). This review does not intend to explain how different viral plant diseases develop but to describe some specific examples of viral and plant factors that contribute to viral pathogenesis.

Since the possible interference of viruses with cellular transport processes is a potentially effective form of altering plant physiology, it is intuitive to believe that the translocation of viruses among cells throughout the plant body strongly influences the pathogenesis process. Virus multiplication and movement are necessary for the symptoms of the disease to develop. Thus, the rate and extent to which these processes occur can be primary determinants of symptom development. The infective viral cycle in a susceptible host mostly begins through epidermal cells, or through roots, as a result of either mechanical damage or being assisted by biological vectors (e.g., insects, nematodes, fungi, etc.). Once the first viral genome replication cycles have been completed, the progeny viruses must be capable of translocating from one cell to another until they reach the vascular system, through which the viruses could invade the distal plant parts. The first of these phases is known as local or cell-to-cell movement, and the second is called systemic or vascular virus movement (Waigmann *et al.*, 2004). In both types of movement, but especially in the first one, the involvement of the virus-encoded movement proteins (MP) is essential (Fernadez-Calvino *et al.*, 2011). MPs can act by forming ribonucleoprotein complexes with the viral genome or tubular structures that hold virions to allow them to cross plasmodesmata (Lucas, 2006). One of the most common ways to restrict the invasion of a given virus is to block its cell-to-cell and long-distance movements. Thus, alterations in the viral movement function have a direct effect on the symptomatology. Most of the data that correlate this function with pathogenesis originate from studies conducted with natural or artificial mutants of the corresponding MPs, or with pseudorecombinant viruses.

Viruses need living tissue for their multiplication and thus do not normally cause the death of the host, although there are exceptions. A large body of evidence has recently shown that to accomplish their life cycle; plant viruses need to confront plant defense mechanisms and to hijack the functions of different host factors. As a consequence, viral components must interact and/or interfere with host components that, in turn, in some instances would cause an alteration in the plant physiology resulting in the development of symptoms. Indeed, recent discoveries have evidenced that plant development is affected by plant–virus interactions, which interfere with a broad range of cellular processes, such as hormonal regulation, cell cycle control and endogenous transport of macromolecules, among others. One important landmark along this line of experimentation has been the demonstration of an interplay between plant development and antiviral defense processes, and that the interference among the common points of their signaling pathways can trigger pathological manifestations (Chapman *et al.*, 2004; Gomez *et al.*, 2009; Kassachau *et al.*, 2003).

2.4.1 Infection Cycle

2.4.1.1 Entry

The entry process includes attachment of virions to a cell surface and a subsequent step for the virus to enter the cell or for viruses to be directly deposited into the cytoplasms in the case of plant viruses. Various ways viruses can enter into host plant cells which are briefed below.

infection cycle of viruses. Thus, several genes may be encoded on a single viral RNA. However, eukaryotic mRNA is monocistronic in nature, i.e., only the first open reading frame is translated. To circumvent this restriction, viruses utilize various strategies to package and express their genomes. These include genome segmentation: Viral genome is broken into several pieces. Each piece of the genome contains one or a few open reading frames (ORFs) and effectively becomes a monocistronic mRNA; terminator read-through and ribosomal frameshifting that allows translation of two consecutive genes to a larger fusion protein and proteolytic processing (polyprotein cleavage). An ORF encoding several genes is expressed as a large polyprotein, which is then cleaved into smaller, functional proteins by an array of proteases encoded by viruses.

Subgenomic RNA (mRNA) synthesis: Some viral genes located internally in the viral RNA are not accessible to the host translation machinery. Thus, they need to be transcribed from the genomic RNA into subgenomic RNAs (mRNA) for gene expression.

2.4.2.2.2 Transcription before translation

For dsRNA viruses, (–)-strand RNA viruses, and DNA viruses, the first step in the gene expression is transcription. mRNAs encoding virus-specific proteins are first made and then serve as templates for translation. Dictated by the nature of viral genomes, these viral genomes cannot serve as mRNA directly. Therefore, an intermediate process (transcription) is taken before viral genes can be expressed. Transcription of most DNA viruses is accomplished entirely by host DNA-dependent RNA polymerase. However, all ds- or (-) RNA viruses must carry the necessary enzymes within the virus particles. These enzymes transcribe mRNA from the viral ds- or (–) RNA.

2.4.2.3 Replication

Replication of viruses is as diverse as viruses themselves. Viral replication varies depending on the type of viral genomes.

2.4.2.4 dsDNA viruses that replicate through dsDNA

None of plant viruses are true dsDNA viruses in this sense. However, this group contains numerous viruses that infect various organisms. Replication of the viruses is in all cases by the semiconservative method favored by cellular genomes. Among the viruses of Eukaryotes, replication mainly occurs in the nucleus, using cellular enzymes such as polymerases, methylases, etc.

2.4.2.5 ssDNA viruses that replicate through ds DNA intermediates:

Two plant virus groups, geminiviruses and banana bunchy top virus contain circular, single-stranded (ss) DNA. Replication of all of the viruses requires formation of a 'replicative form' (RF) double-stranded DNA intermediate: this is formed soon after infection, almost certainly by the host cell DNA polymerases engaging in 'repair' of the ssDNA. In the case of circular genomes, these get converted into covalently closed circular double-stranded (cccds) (plasmid-like)-DNA in the nucleus and become associated with

second strand and creates a gap. There are one or two missing nucleotides at the gap. Again the RNase H activity of the reverse transcriptase digests away RNA in the DNA: RNA hybrid. However, two G-rich polypurine RNA fragments are undigested around nucleotide 1600 and 4200. These two RNA fragments serve as a primer for second strand DNA synthesis of the first and second strands. During the synthesis of strand, there is template switch event since the second strand overlaps with the gap in the strand.

2.4.2.8 Assembly

When both viral capsid proteins and viral genomic nucleic acids are produced in large quantities, the proteins encapsidate viral genomes within a protective shell (virions) by a self-polymerization process. The virion assembly process can be reproduced in test tubes given purified viral capsid proteins and viral nucleic acids.

2.4.3 Cell-to-cell Movement

To infect the entire host plants, viruses will have to move from initially infected cells to neighboring cells (cell-to-cell movement) and to spread from inoculated leaves to other leaves through plant vascular systems (long distance translocation). These two processes involve different mechanisms. Viral proteins are actively involved**.**

Plant cells are surrounded by thick layers of cell walls. Each cell, however, is interconnected by plasmodesmata. Plasmodesmata are membrane-lined cylindrical pores across the cell wall. The pore size of the plasmodesmata is usually very small, in the range of less than one nanometer (nm), much bigger than virus particles. To move out of the initially infected cells, viruses need to overcome the plasmodesmata barrier. Recent experiments have shown all viruses encode a special protein call movement protein that is able to increase the pore size of the plasmadosmata and target the viral RNA-protein complex into the plasmodesmata.

2.4.3.1 Long distance translocation

Long distance translocation is the spread of viruses from an inoculated leaf to other parts of the plant through the vascular system. The movement of the virus appears through the phloem of the vascular system. As a result, viruses flow with the carbohydrate materials in the plant. Systemic symptoms of a viral infection often occur in young growing areas such as young leaves at a plant tip or young roots. In some viruses, long distance translocation requires functional capsid proteins. But this is not the case in some other viruses. Capsid proteins may be required for virion formation. Viral nucleic acids in virions may be better protected from the adverse environment in the phloem tissues.

2.5 INFECTION PROCESS IN PHYTONEMATODES

Nematode parasitism of plants is a complex and dynamic interaction and may involve sensory abilities, host-finding behavior, recognition phenomenon, cytopathology, hatching stimuli, attraction to the host, penetration, feeding site formation and host response

that particular nematode species aggregate around particular zones of the root (*Example*: *Meloidogyne* and *Paratrichodorus* congregate and penetrate about the meristematic region behind the root cap). Juveniles also enter where secondary roots emerge and at surface cracks and lesions of enlarged roots. The nematodes may be responding to kairomones or chemical cues that induce a favorable behavioral and physiological response in the receiver. Perhaps after being directed to within several centimeters of the root by heat or CO_2 gradients, nematodes respond to another cue, the kairomone, which triggers their next behavioral response. This stage would initiate the recognition of the host by the nematode.

The intrigues and complexities involved in the host recognition by the plant parasitic nematodes in most cases remain unresolved. However, two theories were proposed for this process, viz., the orientation of nematodes to plants – the attraction of plant parasitic nematodes by the plant root exudates – and random movement of nematodes, trapped in free water in root vicinity.

2.5.3 Feeding Site Establishment

The host nematode interaction is a complex development system with genes from both sources interacting and failure of the association may result at any stage, from the initial parasitism of the first cell to the maturation of the adult nematode. The failure of the compatible response could result from the synthesis of metabolic inhibitors, the death of feeding site or the failure of host tissue to keep pace with nutritive demands of the nematode and all of these are, in one way or another, resistant responses. The multimechanismic nature of compatibility-incompatibility is evident in the differential development of *Heterodera glycines* on soybean, kidney bean, and other plants. The rates of development of juvenile development remained same on these hosts, but young females on kidney bean were often small and poorly developed and produced fewer eggs than on other hosts. Responses to compatible sedentary endoparasites include the development of the coenocytic cells and syncytia. The susceptible response may begin by recognition of the parasite by the plant, followed by a rapid accommodation of the parasite through genome-modifying sequences utilizing energy or the susceptible response may begin with a passive failure of the host to recognize the pathogen and respond to limit its development. Various models provide scenarios for recognition of self, facilitating the development of the pathogen or nonself, leading to restriction of the pathogen.

2.5.4 Host Penetration

Initial disturbance to the plant is caused when the nematode comes to the root surface. Most plant parasitic nematodes are known to produce enzymes like cellulase and B-1,4-endoglucanases to soften the cell wall before penetrating the plant cell. Pectinases, known to degrade pectin in middle lamellae, are also produced by *Pratylenchus zeae*, *Heterodera trifoli*, *Meloidogyne* spp., *Radopholus similis* and *Ditylenchus dipsaci.* Before penetration, nematode presses its lips to the plant root surface and thrusts backward and forward by using the stylet. By doing this process, if gets compatible response nematode penetrate

just a passive substrate on which the nematodes' secretions act, instead, nematodes may have developed systems for directing the host's metabolism or its initiating physiological processes that achieve the same objective without the direct injection of an enzyme. *Ditylenchus dipsaci* is normally associated with host cell separation, which produces a large amount of pectinase. Different symptoms produced in different host plants by populations of this nematode may be caused by different pectolytic enzymes associated with these populations. The granular exudation, which adult female of *Meloidogyne javanica* injects from the dorsal esophageal gland into host cells, may contain material capable of synthesizing enzymes only when placed in the cytoplasm of the host. Plant parasitic tylenchid nematodes are well adapted for parasitism. In addition to a protrusible stylet, these nematodes have three large and complex esophageal secretary glands, one dorsal and two-subventral (Ravichandra, 2008). These secretions are spherical membrane bound secretary granules (dense core vesicles) formed in both dorsal and subventral esophageal glands.

2.6.1 Nature of Secretions

Elucidating the chemical nature of nematode secretions and characterizing their biological activity remains one of the most intriguing and challenging areas of future research in Nematology. Analysis of secretion composition showed the presence of proteins, glycoproteins, amino acids like lysine, histidine and arginine, carbohydrates and cytokinins like compounds and enzymes like cellulose, proteinase, and B-1,4-endoglucanases. These secretions play an important role in penetration, induction, and maintenance of feeding sites and digestion process of plant-nematode interaction.

Therefore, types of tissues damaged by the nematodes are important and in general, disturbance of cells in the stele or the region of the root tip are likely to have the most influence on the plant nematodes injury to plants may be a mechanical, i.e., penetration and movement through plant tissues. It may include the following.

(a) Cellular changes: Death of cells (necrosis), changes in growth of cells.
(b) Physiological changes in host: Interruption in uptake and flow of water and nutrients from roots, interruption in the flow of food from leaves to roots.
(c) Create openings for entry of other microorganisms.
(d) Interaction with other disease-producing agents.
(e) Transmission of other disease-producing agents.
(f) Increase susceptibility to environmental stress.

2.7 ROLE OF ENZYMES, TOXINS, GROWTH REGULATORS AND POLYSACCHARIDES IN PATHOGENESIS

The term pathogenesis means step-by-step development of a disease and the chain of events leading to that disease due to a series of changes in the structure and/or function of a cell/tissue/organ being caused by a microbial, chemical or physical agent. The pathogenesis of a disease is the mechanism by which an etiological factor causes the

Pectinases and pectolytic enzymes are pectin methyl esterases (PMEs), polygalacturonases (PGs) and pectin lyases (PLs).

2.7.1.3.1 Pectin methyl esterases

Pectin methylesterases catalyze the demethylesterification of cell wall polygalacturonans. In dicot plants, these ubiquitous cell wall enzymes are involved in important developmental processes including cellular adhesion and stem elongation. It breaks ester bonds and removes methyl groups from pectin leading to the formation of pectic acid and methanol (CH3OH). Cell-wall pectin methyl esterification can influence plant resistance because highly methyl-esterified pectin can be less susceptible to the hydrolysis by pectic enzymes such as fungal endopolygalacturonases (PG). Pectin is secreted into the cell wall in a highly methylesterified form and, here, is de-methyl esterified by pectin methyl esterase (PME). The activity of PME is controlled by specific protein inhibitors called PMEI; consequently, an increased inhibition of PME by PMEI might modify the pectin methyl esterification. To test the possibility of improving wheat resistance by modifying the methyl esterification of pectin cell wall, durum wheat transgenic lines were produced expressing the PMEI from *Actinidia chinensis* (AcPMEI). The expression of AcPMEI endows wheat with a reduced endogenous PME activity, and transgenic lines expressing a high level of the inhibitor showed a significant increase in the degree of methyl esterification. These lines showed a significant reduction of disease symptoms caused by the fungal pathogens *Bipolaris sorokiniana* or *Fusarium graminearum*. This increased resistance was related to the impaired ability of these fungal pathogens to grow on methyl-esterified pectin and to a reduced activity of the fungal PG to hydrolyze methyl-esterified pectin. In addition to their importance for wheat improvement, these results highlight the primary role of pectin despite its low content in the wheat cell wall.

2.7.1.3.2 Polygalacturonases

Split pectin chain by adding a molecule of water and breaks the linkage between two galacturonan units. These enzymes catalyze reactions that break α-1,4-glycosidic bonds. Polygalacturonases (PGs) are produced by fungal pathogens during early plant infection and are believed to be important pathogenicity factors (D'ovido *et al.*, 2004). Polygalacturonase-inhibiting proteins (PGIPs) are plant defense proteins which reduce the hydrolytic activity of endoPGs and favor the accumulation of long-chain oligogalacturonides (OGs) which are elicitors of a variety of defense responses. PGIPs belong to the superfamily of leucine reach repeat (LRR) proteins which also include the products of several plant resistance genes. A number of evidence demonstrate that PGIPs efficiently inhibit fungal invasion.

Polygalacturonases otherwise referred to as pectic hydrolyases are pectic enzymes that hydrolyse pectic substances into their monomeric units. They are known to be responsible for the hydrolytic cleavage of the B-1,4-glycosidic of the galacturonan, a moiety of pectic substances (Arotupin Daniel Juwon *et al.*, 2012). However, extracellular polygalacturonases catalyze the hydrolytic cleavage of terminal X-1,4-glycosidic bonds (pectic acid) releasing galacturonic acid as the main product. Polygalacturonases

appeared to be the most frequently encountered pectic enzymes. They are formed in the majority of plant tissues particularly in ripening fruits. Also many plant-pathogenic and saprophytic microorganisms produced polygalacturonases. Most fungi that exhibited pectolytic activity produce polygalacturonases either as sole pectic enzymes as observed in *Sacchromyces fragilis* (Phaff, 1966) or in association with either or both pectinmethylesterase and pectin lyase. The critical role of these enzymes in the degradation of the host middle lamella and cell walls, leading to plant tissue maceration and cellular death had been documented. The infection of cotton seedlings by *Rhizoctonia solani*, soft rot yam and sweet potato, brown rot of apple by *Monilinia* species and deterioration of tomato by *Botryoplodia theobromae* Pat have been reported to produce polygalacturonases and other cell wall degrading enzymes. The culture filtrates of *Collectotrichum lagnarum* exhibited polygalacturonase activity.

2.7.1.3.3 Pectin lyases

A pectin lyase also known as pectolyase is a naturally occurring pectinase, a type of enzyme that degrades pectin. Pectin lyases are the only known pectinases capable of degrading highly esterified pectins (like those found in fruits) into small molecules via β-elimination mechanism without producing methanol, which is toxic, in contrast to the combination of PG and PE, which are normally found in commercial products. In addition, the presence of undesirable enzymatic activity in commercial pectinases may be detrimental to aroma because they are responsible for producing unpleasant volatile off flavor.

Pectin lyase acts on the pectic substances that occur as structural polysaccharides in the middle lamella and primary cell walls of higher plants. This enzyme has potential applications in food, paper and textile industries. Since new applications of this enzyme are emerging, there is a scientific need to explore the important aspects of the enzyme specifically the catalytic efficiency and possible sources. Split pectin chain by removing a molecule of water from the linkage, thereby breaking it and releasing products with unsaturated double bonds. These pectin enzymes can be exopectinases (break only terminal linkage) or endopectinases (break pectin chain to random sites). Pectin degradation results in liquefaction of the pectic substances and weakening of cell walls, leading to tissue maceration (*Example*: Soft rot bacterium, *Erwinia caratovora* subsp. caratovora and other fungi like *Botrytis cinerea, Sclerotium rolfsii*, etc.).

Pectinases have multiple biological functions. Pectate lyases can act as extracellular virulence agents and their role in the release of cell wall oligogalacturonides is important for activation of plant defense mechanisms (Jun Cao, 2012). Furthermore, pectate lyases may be important for fruit ripening and softening, as well as plant growth and development. Similarly, polygalacturonases contribute to pectin disassembly during many stages of plant development, such as those that require cell separation. Polygalacturonase genes also have roles in pollen maturation and pollen tube growth, as well as intine and exine formation. In addition, like pectate lyase and polygalacturonase, pectin lyase can enhance the reconstituted expansin-induced extension of the apical (elongating) segments of cucumber hypocotyls. Most pectin lyases are produced by microorganisms (such as *Aspergillus*, *Penicillium*, and *Fusarium*). In these microorganisms,

expression of pectin lyase gene is generally induced by medium pH, carbon sources, and pectin, and is generally repressed by glucose.

The early steps in the interaction between a pathogen and a host are confined to the phase when the pathogen gains entry into the host tissues, into the cells or between the cells by dissolving the cell walls. At this stage, the ability of the pathogen to degrade the cell wall constituents such as cellulose, pectin, hemicelluloses and protein by secreting the appropriate hydrolytic enzymes seems important (Subramanian, 1969). Although evidence for such enzymatic breakdown of cell walls has been obtained in some cases such as soft rot, damping-off of seedlings, foot-rot of cereals, the role of these extracellular enzymes of the pathogen in other diseases is less clear.

The other facet of this problem, namely, the reaction of host tissues to the action of these pathogen factors is also important in the development of the disease. Several enzymes are either stimulated or inhibited in and around the infection loci. For example, oxidases such as polyphenolase and peroxidase show increased activity in several host-pathogen interactions studied. Increased activity of these enzymes could have been owing to their activation consequent to the release from 'inactive' combinations with other cellular components, or it could mean a synthesis of these enzymes *de novo* in response to the activity of the pathogen. Be that as it may, the excessive activity of these enzymes would bring about the death of cells and tissues if the stimulation is violent enough as encountered in 'hypersensitive' reaction. Perhaps, plants can afford to lose the diseased portions in this battle without any serious repercussion on the rest of the portions.

2.7.1.3.4 Pectic enzymes

Plant cell walls are primarily polysaccharide in composition. A simple but major pathogenic mechanism in plants involves degradation of the cell wall by a battery of polysaccharidases secreted by pathogens. Most of the degradative enzymes are glycoside hydrolases, which degrade the cellulose and pectate matrices by the addition of water to break the glycosidic bonds. The pectate network is also degraded by polysaccharide lyases, which cleave the glycosidic bonds via a β-elimination mechanism (Steven R. Herron *et al.*, 2000). To better understand the latter virulence mechanism, research has been carried out on pectate lyase C, a pectolytic enzyme secreted by the pathogenic bacterium *Erwinia chrysanthemi*. The story of pectate lyase C illustrates how structural techniques have contributed to a detailed understanding of polysaccharide recognition and the lyase cleavage mechanism. In the process, a novel protein structural fold and a unique catalytic role for an arginine have been discovered. The structural results have also provided the first atomic description of a pectate fragment, which differs considerably from the popular view in conformation as well as the mode of interactions with Ca^{2+} ions. Finally, the growing structural database of pectolytic enzymes is enabling researchers to elucidate subtle structural differences that are responsible for the specific recognition of a unique oligosaccharide sequence from a heterogeneous mix in the plant cell wall. Such knowledge will ultimately lead to a better understanding of the characteristics that render the host susceptible to attack by a particular pathogen.

years of crystallography (Yoder *et al.*, 1993). *PelC* folds into a large right-handed coil termed the parallel β helix. There are eight coils in the helix, and each is comprised of three β strands, connected by three turns with unique features (Fig. 2.3). When the coils are stacked, the structure has the appearance of three parallel β sheets, stabilized by an extensive network of interstrand hydrogen bonds. Another notable feature of the *PelC* fold is the internal organization of the amino acids that form the core of the parallel β helix. All of the amino acids, which are oriented toward the interior, are regularly aligned with amino acids from neighboring coils, giving rise to long ladders of hydrophobic, aromatic, or polar amino acids. In contrast, the exterior amino acids are randomly oriented and comprise loops, of varying length and composition, which protrude from the central core. From the perspective of an effective plant virulence factor, the most important feature of the parallel β helix fold is the stability that it confers upon an enzyme that must function in the hostile extracellular environment.

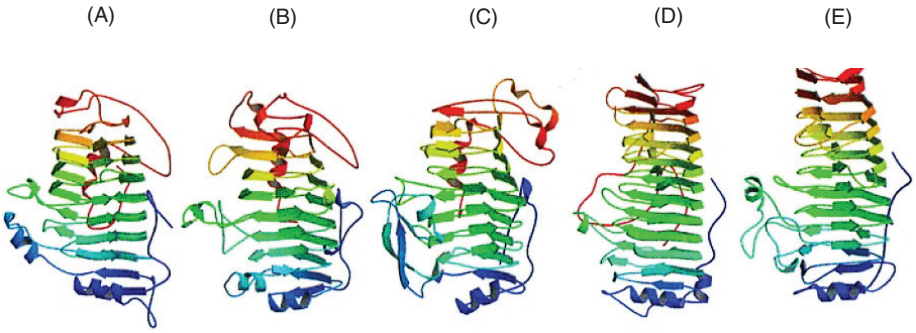


Fig. 2.3 Five examples of plant cell wall degradative enzymes that fold into a parallel β helix motif. The predominant secondary structural features of the proteins are illustrated as β strands, and the coils represent β helices. (A) *E. chrysanthemi* pectate lyase C; (B) *E. chrysanthemi* pectate lyase E; (C) *A. niger* pectin lyase B; (*D*) *E. carotovora* polygalacturonase; and (*E*) *A. aculeatus* rhamnogalacturonase A

2.7.1.3.7 Significance of multiple isozymes for pathogenesis

One enigma, yet to be resolved in the pectate lyase field, is the function of multiple, independently regulated pectate lyase isozymes and the role, if any, in pathogenesis (Steven R. Herron *et al.*, 2000). The composite results of the structural studies suggest that the answer may lie in the heterogeneous nature of the substrate. The pectate component is composed of repeating units of negatively charged galacturonic acid. The uronic acid group on C-6 is frequently esterified with a methyl group. Methylation neutralizes the negative charge of an individual Gal*p*A unit and, consequently, alters the surface charge of the pectate polymer. The percentage and the positional sequence of methylated Gal*p*A units (mGal*p*A) vary during the life cycle of the plant and from one type of plant to another. Thus, a pathogen that secretes a battery of pectic enzymes, each of which uses a similar catalytic mechanism, but recognizes a different sequence of methylated and nonmethylated oligogalacturonate units, would be expected to have a broader host range.

enzymes equal to those produced by soft-rotting *Erwinia* species. Induction of polygalacturonase and pectate lyase in *A. nidulans* required substrate and was completely repressed by glucose. Surprisingly, inoculation of excised plant tissues with *A. nidulans* conidia lead to the formation of necrotic, water-soaked lesions within which the organism sporulates. Thus, *A. nidulans* has phytopathogenic potential. The release of glucose and other sugars from wounded tissues may repress pectolytic enzyme production and limit disease development. They tested creA204, a mutation that relieves glucose repression of some *A. nidulans* carbon utilization enzymes, for its effect on the production of pectolytic enzymes. The creA204 failed to relieve catabolite repression of polygalacturonase or pectate lyase and had no effect on disease severity.

Cutinases, cellulases, pectinases and lignases are often secreted by the pathogenic organism. Fungi, nematodes, and bacteria are all known to produce one or more of the above enzymes in specific pathogen-host combinations. Viruses and viroids are generally not considered to secrete enzymes, although some viruses may encapsidate an enzyme in their particle. Pathogenic organisms either continually secrete enzymes or upon contact with the host plant.

In fungal plant pathogenesis, enzymes play a crucial role, and they are involved in the external and internal interactions (Ales Lebeda *et al.*, 1999). To restrict the development of fungal pathogens, the plants formed many defense mechanisms. They built mechanical barriers from lignin, suberin, and callose and produced a lot of antimicrobial compounds with low molecular weight like phenols, chinons, alkaloids and others. Enhanced production of some enzymes and their activity increasing is one of the most important processes in plant defense. These enzymes frequently occur in many isoforms and are involved in the synthesis of defense substances or have a direct antimicrobial activity.

The first surface an organism comes into contact with is cuticle and the cell wall of the plant. The cuticle is comprised of a complex wax, cutin, which impregnates the cellulose wall. The cell wall is comprised of cellulose, which makes up the structural framework of the wall, along with the matrix molecules hemicellulose, glycoproteins, pectin, and lignin. Thus, penetration into living parenchymatous tissues and degradation of the middle lamella is due to the action of one or more enzymes which degrade these chemical substances. Cutinases degrade the cutin on the cuticle layer presoftening the tissue for mechanical penetration or as a first step in tissue degradation. Studies have shown that several fungi and at least one bacterial species produce cutinases. Further, evidence indicates that cutinases are continually produced, albeit in low concentrations, with degradation products often inducing even higher levels of cutinase secretion.

Studies have shown that in some organisms, cutinase production may be linked to virulence. Pectic substances comprise the middle lamella and also form an amorphous gel between the cellulose microfibrils in the primary cell wall. Pectin degrading substances often termed pectinases or pectolytic enzymes include pectin methylesterases (PME), Polygalacturonases (PG) and pectin lyases or transeliminases. Pectin methylesterases remove small groups such as methyl groups (CH3) often altering solubility and thus affecting the rate of chain splitting by polygalacturonase and pectin lyase. Polygalacturonases split chains by adding a molecule of water while pectin lyases split chains by removing a water molecule from the linkage. Pectin degrading enzymes are

involved in a wide range of plant diseases particularly in the soft rot diseases (*Example*: *Erwinia carotovora, Sclerotinia sclerotiorum* and *Rhizoctonia solani*). Organisms such as these, as well as, elaborate pectic enzymes lead to tissue maceration. In fact, these enzymes are sometimes referred to as macerating enzymes.

2.7.1.4 Cellulases

Cellulose is the major framework molecule of the plant cell wall existing as microfibrils with matrix molecules (glycoproteins, hemicelluloses, pectins, lignins) filling the spaces between the microfibrils and cellulose chains. Cellulases have been shown to be produced by many pathogenic fungi, bacteria, and nematodes. Cellulolytic enzymes play a role in softening and disintegration of cell walls. No doubt cellulolytic enzymes are involved in the invasion and spread of the pathogen, but also are instrumental in the collapse of cells and tissues. Indirectly cellulolytic enzymes participate indirectly in disease development by releasing soluble sugars that may be used as nutrients by pathogens and also may be involved in the release of materials in the vascular system interfering with transport or translocation of water. Cellulose is degraded by cellulases. Cellulase one (C1) attacks native cellulose by cleaving cross-linkages between chains. A second cellulase (C2) also attacks native cellulose and breaks into shorter chains. These shorter chains are then attacked by Cx enzyme, which degrade them into disaccharide, cellobiose. Finally, cellobiose is degraded by the enzyme, β-glucosidase into glucose. Cellulase degrading enzymes play a role in softening and degradation of cell wall material and facilitate easy penetration and spread of the pathogen in the host. (*Example*: Basidiomycetes fungi).

The ubiquitous oomycete *Pythium oligandrum* is a potential biocontrol agent for use against a wide range of pathogenic fungi and an inducer of plant disease resistance. The ability of *P. oligandrum* to compete with root pathogens for saprophytic colonization of substrates may be critical for pathogen increase in the soil, but other mechanisms, including antibiosis and enzyme production, also may play a role in the antagonistic process. Karine Picard *et al.* (2000) used transmission electron microscopy and gold cytochemistry to analyze the intercellular interaction between *P. oligandrum* and *Phytophthora parasitica*. The growth of *P. oligandrum* towards *Phytophthora* cells correlated with changes in the host, including retraction of the plasma membrane and cytoplasmic disorganization. These changes were associated with the deposition onto the inner host cell surface of a cellulose-enriched material. *P. oligandrum* hyphae could penetrate the thickened host cell wall and the cellulose-enriched material, suggesting that large amounts of cellulolytic enzymes were produced. Labeling of cellulose with gold-complexed exoglucanase showed that the integrity of the cellulose was greatly affected both along the channel of fungal penetration and also at a distance from it. They measured the cellulolytic activity of *P. oligandrum* in substrate-free liquid medium. The enzymes present were almost as effective as those from *Trichoderma viride* in degrading both carboxymethyl cellulose and *Phytophthora* wall-bound cellulose. *P. oligandrum* and its cellulolytic enzymes may be useful for biological control of oomycete pathogens, including *Phytophthora* and *Pythium* spp., which are frequently encountered in field and greenhouse production.

Uredospores of the obligatory biotrophic broad bean rust fungus *Uromyces viciae-fabae* form infection structures on artificial membranes providing a thigmotropic signal (Heiler *et al.*, 1993). In nature, these are essential for invasion of the host plant through the stomata. This experimental system was used to analyze the production of cellulolytic enzymes during the differentiation of rust infection structures. Low cellulase activity was detected in dormant spores and germ tubes. Enzyme activity increased during appressorium development and reached a maximum when infection hyphae and haustorial mother cells were formed. Seven cellulolytic enzymes were separated by chromatofocusing on DEAE = Si500 and PBE 94 exchangers. At least two cellulases, characterized by isoelectric points of 7.1 and 7.3, were identified as endocellulases. The neutral enzyme forms increased from 3.3% of the total activity at appressorium formation to 36.6% when infection hyphae were formed (18 h p.i.), and to 45.4% in haustorial mother cells (24 h p.i.). Cellulase activity of the 'rust' fungus is neither substrate-inducible nor catabolite repressible. The regulation of these enzymes appears to be strictly controlled by the differentiation of infection structures and is, therefore, distinct from cellulases of necrotrophs and saprophytes investigated thus far.

Cellulases are produced by rust fungi. The expression of cellulase activity by *U. viciae-fabae* is controlled by mechanisms active during differentiation of infection structures (Deising & Mendgen, 1992). Substrate induction and catabolite repression are not involved in the regulation of cellulase activity. These findings are in contrast to results obtained from other fungi and support the concept that polymer-degrading enzymes in obligate biotrophs and necrotrophs or saprophytic fungi are regulated by essentially different mechanisms.

In a study on cellulolytic and pectolytic activity of *Cylindrocarpon destructans*, Dahm and Strzelczyk (1987), found that only one of the twenty isolates studied exhibited cellulolytic activity. The total activity of this isolate was similar in media with CMC and powdered cellulose. The specific activity was, however, two times higher with powdered cellulose. All isolates identified as pathogenic to fir and to pine produced pectolytic enzymes. Not all of them, however, exhibited exo- and endo-PMG activity. In general, an increase of total activity of exo-PMG was accompanied by an increase in the specific, activity. Of the nonpathogenic isolates, only one did not show pectolytic activity. There exist no significant differences in pectolytic activity between the isolates pathogenic and nonpathogenic to fir and pine. Also, the isolates belonging to both groups were not cellulolytic except one nonpathogenic.

2.7.1.5 Hemicellulases

Hemicelluloses are complex polysaccharide polymers that link the ends of pectic compounds to cellulose microfibrils. Since hemicelluloses are such a diverse group of polymers such as xyloglucans, glucomannans, glactomannans, arabinoglucans, etc., several hemicellulases have been identified in many plant pathogenic fungi. The mechanism by which they participate in cell wall breakdown is not clear, nor is it known how they contribute to pathogenesis. These are the major constituents of the primary cell wall and also seen in middle lamella and secondary cell wall. The hemicellulose

polymers include primarily xyloglucan but also glucomannans, galactomannans, arabinogalactans, etc. Hemicelluloses link the ends of pectic polysaccharides and various points of the cellulose microfibrils. Hemicellulases degrade hemicelluloses and depending on the monomer released from the polymer on which they act; they are termed xylanase, galactanase, glucanase, arabinase, mannose, and so on (*Example: Sclerotinia sclerotiorum*, *Sclerotinia fructigena*).

2.7.1.6 Cell wall proteins

Cell wall proteins are similar to other proteins, except that they are rich in amino acid, hydroxyproline. Five classes of structural proteins are found in cell walls: extensins, proline-rich proteins (PRPs), glycine-rich proteins (GRPs), Solanaceous lectins and arabinogalactan proteins (AGPs). Proteins are degraded by means of enzymes, proteases or proteinases or peptidases. Cell wall proteins are essential constituents of plant cell walls involved in modifications of cell wall components, wall structure, signaling, and interactions with plasma membrane proteins at the cell surface. The nature of cell wall proteins is as varied as the many functions of plant cell walls. With the exception of glycine-rich proteins, all are glycosylated and contain hydroxyproline (Hyp) (Cassab, 1998). Again excepting glycine-rich proteins, they also contain highly repetitive sequences that can be shared between them. The majority of cell wall proteins is cross-linked into the wall and probably has structural functions, although they may also participate in morphogenesis. On the other hand, arabinogalactan proteins are readily soluble and possibly play a major role in cell-cell interactions during development. The interactions of these proteins between themselves and with other wall components are still unknown, as is how wall components are assembled. The possible functions of cell wall proteins are suggested based on repetitive sequence, localization in the plant body, and the general morphogenetic pattern in plants.

The discovery and development of novel plant cell wall degrading enzymes is a key step towards more efficient depolymerization of polysaccharides to fermentable sugars for the production of liquid transportation biofuels and other bioproducts (Brian C. King *et al.*, 2014). The industrial fungus *Trichoderma reesei* is known to be highly cellulolytic and is a major industrial microbial source for commercial cellulases, xylanases and other cell wall degrading enzymes. However, enzyme-prospecting research continues to identify opportunities to enhance the activity of *T. reesei* enzyme preparations by supplementing with enzymatic diversity from other microbes. Genomic analysis of lignocellulose-degrading fungi shows that a single species can have the genetic capacity to produce many different enzymes with similar functional designations (cellulase, xylanase, and others). For example, the genome of the phytopathogen *Magnaporthe grisea* is predicted to encode at least 30 enzymes in six GH families for the degradation of cellulose and 44 enzymes in 11 families for the degradation of hemicelluloses.

Most plant-associated microbes (both pathogenic and saprophytic) that break down plant cell walls have the genetic capacity to produce enzymes for the degradation of the major structural polysaccharides found in the cell wall, namely cellulose, xylan and pectin (Brian C. King *et al.*, 2014). In particular, plant pathogens have intimate relationships

Lipolytic enzymes, called lipases (phospholipases, glycolipases) hydrolyze lipids and release fatty acids.

2.7.1.9 Starch

Starch is the main reserve polysaccharide found in plant cells. It is a glucose polymer and exists in two forms: amylose, a linear molecule, and amylopectin, a highly branched molecule. Starch is degraded by the enzyme amylases.

2.7.1.10 Proteinaceous enzymes

In the course of evolution, plants have elaborated protective mechanisms that allow them to successfully resist different kinds of unfavorable conditions including insects and phytopathogenic microorganisms. The most important components of all protective mechanisms are proteinaceous compounds. These include enzymes such as β-1,3-glucanases and chitinases, inhibitors of proteases and α-amylases, lectins, and also other proteins and peptides which have antimicrobial activity (Valueva and Mosolov, 2004).

Many phytopathogenic microorganisms produce active extracellular proteinases that along with other enzymes play an important role in pathogenesis, e.g., polygalacturonases, pectolyases, and xylanases. It was demonstrated that the *Colletotrichum lindemithianum*, when grown on plant cell walls or on artificial nutrient medium, secretes an active protease of 25 kD molecular weight and pH optimum at 8.6. This was the first extracellular proteinase of a plant pathogen obtained in its pure form. In recent years, many extracellular proteases produced by phytopathogenic microorganisms have been isolated and characterized to some extent. Among these serine proteinases prevail, but there are enzymes belonging to other mechanistic classes. All known serine proteinases of phytopathogens can be divided into trypsin-like and subtilisin-like enzymes. The first group contains proteinases that are produced by *Cochliobolus carbonum*, *Verticillium dahliae*, *Stagonospora* (*Septoria*) *nodorum* and *Phytophtora infestans*. Subtilisin-like enzymes are secreted by *C. carbonum*, *P. infestans*, *Acremonium typhium*, *Magnaporthe poae*, *Trichoderma harzianum* and *Fusarium oxysporum*.

Among extracellular proteinases of phytopathogens, the aspartic proteinases are fairly widespread. These include the enzymes produced by *Botrytis cinerea* (Valueva and Mosolov, 2004) *Cryphonectria parasitica* (endothiapepsin) and *Glomerella cingulata*. Cysteine proteinase is secreted by the fungus *Pyrenopeziza brassicae*. Metalloproteinases include a family of Zn-dependent bacterial enzymes belonging to the genus *Erwinia*. One of these proteinases extracted from *Erwinia carotovora* subsp. carotovora is similar in its properties to thermolysin from *Bacillus thermoproteolyticus*. Extracellular proteinases apparently play an active role in the process of pathogenesis. For instance, it has been revealed that in *P. brassicae* nonpathogenic mutants are unable to produce extracellular cysteine proteinase. Recovery of pathogeneses in these mutants was accompanied by the recovery of their ability to produce the proteinase. An important role in disease progression is also played by aspartic proteinase of the fungus *B. cinerea*, which is a wide profile pathogen.

Proteinases found in pathogens can also play an active role in the degradation of other proteins involved in plant protection, for instance, such enzymes as chitinase and β-1,3-glucanase (Alexander *et al.*, 1994). Proteinases of phytopathogenic microorganisms can also perform other specific functions. For instance, in the bacterium *E. chrysanthemi* extracellular metalloproteinase catalyzed the transformation of pectate lyase into the mature form of this enzyme, which is crucial for plant tissue maceration. It has been assumed that certain peptides released by the action of extracellular proteinases of phytopathogenic microorganisms can act as elicitors, activating plant protection reactions. Proteinase inhibitors in plants are able to suppress the enzymatic activity of phytopathogenic microorganisms.

2.8 ROLE OF TOXINS IN PATHOGENESIS

Toxins are compounds that are produced by the pathogens and cause part or all of the symptoms of a disease. Genetic and biochemical studies revealed that at least in part of the plant-pathogen interactions toxins are the determinants of specificity. In such cases, resistance or susceptibility to the fungus correlates with insensitivity or sensitivity to the toxin (Slavov, 2005). Microbial toxins have been the objects of extensive studies as possible pathogenicity or virulence factors for the producer pathogens. Toxins are considered to be the special weapons of the plant pathogens to evade or overcome the inherent resistance strategies of host plants (Kimura *et al.*, 2001). Plants themselves have a broad spectrum of defense barriers to protect themselves from invading organisms. Pathosystems are very diverse, and there is neither a single model of plant-pathogen interactions nor a sample and common resistant mechanism. In some plant diseases, phytotoxins may play a critical role in the development of the disease symptoms.

Microbial toxins are metabolites produced by plant pathogens, which play a role in host-pathogen interactions and disease expression. They are low molecular weight substances produced by some pathogens which are capable of reproducing symptoms similar to that found in natural infections in plants (Bilgram and Dube, 1976). Several plant pathogens often damage their host (plants) tissues by producing toxic metabolites, which induced various symptoms such as necrosis, chlorosis, wilting, water soaking and eventually the death of plants. These toxic metabolites also known is one of the weapons used by pathogen inducing disease condition in susceptible host plants. Many pathogens are known to produce toxins both *in vitro* and *in vivo,* and these toxins have been implicated in the symptom development on the host tissues (Amusa, 2006).

Several phytotoxic metabolites have been found associated with pathogens including bacteria and fungi, which, causes symptoms similar to those caused by the pathogen. Such toxic metabolites include pinolidoxin from *Ascochyta pinodes*, deoxyradicin and maculosin from *Alternaria helianthi* and *Alternaria alternata* (Stierle *et al.*, 1988). Identified metabolites from other pathogens include piricularin from *Piricularia oryzea*, victorin from *Cochliobolus vitoriae,* phaseolotoxin from *Pseudomonas syringae* pv. *phaseolicola*, a toxin from *Periconia circinata,* saccharitoxin from *Helmithosporium sacchari*, cercosporin from *Cercospora* spp. Phytotoxic metabolites of most of these pathogens have been reported to play a significant role in pathogenesis (Amusa, 2006).

Several characteristics have been used for the classification of toxins that affect plants. Such features include their chemistry. Based on this, some phytotoxins are regarded as low molecular weight peptides, others have terpenoid structures, and still others contain carbohydrates (Amusa, 1991). However, few other structures are known for toxins that play an unquestionable role in plant disease. Another form of classification is based on the producing organism. This is, however, of no predictive value since more than one type of phytotoxins can be produced by one organism. Phytotoxin classification has also been based on biological activities such as enzyme inhibitors, antimetabolites, membrane-affecting compounds (Amusa, 2006). However, the widely accepted classification is that based on toxic selectivity to plant genotypes (host selective or nonhost selective) and on the general role in disease development.

Toxins have been implicated in plant disease as far back as deBary who advanced a theory of plant disease often termed the 'toxin theory.' A primary tenant of the toxin theory is that a toxin elaborated by a pathogen may produce all of the symptoms of the disease. As more information was developed the theory was largely discarded. As we will see a little later in this discussion, the discovery of the toxin victorin, a host-specific toxin, revived interest in the toxin theory of plant disease. Toxins may act directly on living host cells, damaging or even killing the host. Some toxins are active on a wide range of plant species (nonhost-specific) or in some cases, as with the toxin victorin (host-specific).

A toxin can be defined as a microbial metabolite excreted (exotoxin) or released by lysed cells (endotoxin) which in very low concentration is directly toxic to the cells of the suscept (host). The term toxin is used for a product of the pathogen, its host, or pathogen-host interaction which even at very low concentration directly acts on living host protoplasm to influence disease development or symptom expression. Toxins are different from enzymes in that they do not attack structural integrity of host tissues but affect the metabolism of the host because the toxins will act on protoplast of the cell.

2.8.1 Toxin Hypothesis

It states that a toxin should produce all symptoms characteristic of the disease; correlation of toxin sensitivity with susceptibility to pathogen and toxin production by the pathogen will be directly related to its ability to cause disease. Except, victorin, the toxic metabolite of *Cochliobolus victoriae*, the vast majority of toxins associated with plant diseases fail to exhibit all the above characters (Luke and Wheeler, 1955).

2.8.2 Classification of Toxins

According to the source of origin, toxins are divided into three broad classes, namely, pathotoxins, vivotoxins, and phytotoxins **(**Wheeler and Luke, 1963).

2.8.2.1 Pathotoxins

These are the toxins which play a major role in disease production and produce all or most of the symptoms characteristic of the disease in susceptible plants. Most of these

toxins are produced by pathogens during pathogenesis. A pathotoxin is a chemical of biological origin, other than an enzyme and plays an important causal role in a plant disease. Most pathotoxins are produced by plant pathogenic fungi or bacteria, but some are produced by higher plants, and one has been reported to be the product of an interaction between a plant and a bacterial pathogen. Some pathogen-produced pathotoxins are highly selective in that they cause severe damage and typical disease symptoms only on plants susceptible to the pathogens that produce them. Others are nonselective and are equally toxic to plants susceptible or resistant to the pathogen involved. A few pathotoxins are species-selective and are damaging to many but not all plant species. In these instances, some plants resistant to the pathogen are sensitive to its toxic product [(*Example*: Victorin: *Cochliobolus victoriae (Helminthosporium victoriae*)], the causal agent of Victoria blight of oats. This is a host-specific toxin. It fully meets the strict requirements of a pathotoxin.

2.8.2.1.1 Victorin

The fungus, *Cochliobolus victoriae (Helminthosporium victoriae*), secretes a toxin known as victorin that is toxic to susceptible oat leaves at very low concentrations. Victorin is an unusual, halogen containing cyclic pentapeptide (Fig. 2.4). Toxin synthesis is inherited at a single locus TOX3, and it would seem reasonable to hypothesize that it is synthesized in a manner analogous to other cyclic peptide toxins, that is, by a nonribosomal (cyclic) peptide synthetase (Jonathan E. Markham and Jacques Hille, 2001). Enzymes must also be present to synthesize the novel amino acids required by such a peptide synthetase, but unfortunately to date no reports on the identification of any genes necessary for victorin synthesis are known.

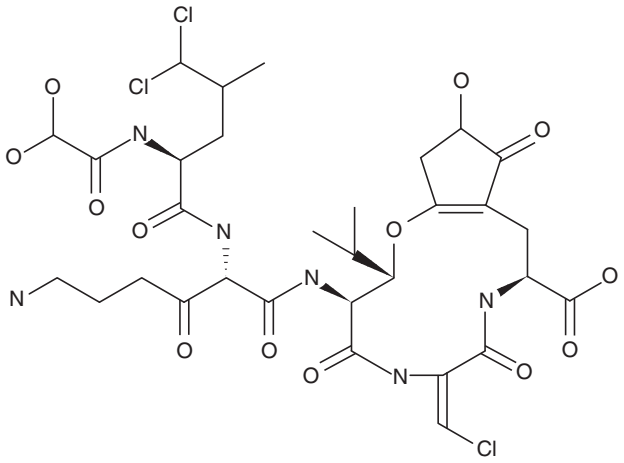


Fig. 2.4 Victorin

Victorin is required for *C. victoriae* to infect its host successfully. Fungal strains that do not produce victorin are not pathogenic. Victorin also reproduces the disease symptoms in the absence of the fungus. Oat sensitivity to victorin is dominant and is

determined by a single plant gene, designated *Vb*. Therefore, the study of susceptibility and also host range is simplified to the study of the interaction of a single fungal metabolite, victorin, with the product of a single dominant oat gene *Vb*. The structure of the most prevalent form of victorin has been identified as a cyclized pentapeptide of 814 D. A biologically active, I^{125}-victorin derivative was produced and used to search for oat victorin binding proteins. Several binding proteins were found, one of which binds victorin in leaf slices from susceptible but not resistant cultivars. The gene encoding this protein was cloned and identified as the P protein component of the glycine decarboxylase complex (GDC), an important enzyme complex in the photorespiratory cycle. Picomolar victorin concentrations inhibit GDC in leaf slices, and micromolar concentrations inhibit GDC activity *in vitro*.

The host-selective toxin victorin is produced by *Cochliobolus victoriae*, the causal agent of victoria blight of oats. Victorin has been shown to bind to the P-protein of the glycine decarboxylase complex (GDC) in mitochondria and induces defense-related responses such as phytoalexin synthesis, extracellular alkalization, and programmed cell death. However, evidence demonstrating that the GDC plays a critical role in the onset of cell death is still lacking, and the role of defense-like responses in the pathogenicity has yet to be elucidated. Cytofluorimetric analyses, using the fluorescein (VicFluor) or bovine serum albumin–fluorescein derivative of victorin (VicBSA), demonstrated that victorin-induced cell death occurs before these conjugates traverse the plasma membrane (Yasuomi Tada *et al.*, 2005). As with native victorin, VicBSA clearly elicited apoptosis-like cell death, production of phytoalexin, extracellular alkalization, and generation of nitric oxide and reactive oxygen intermediates. These results suggested that the initial recognition of victorin takes place on the cell surface, not in mitochondria and leads to the activation of a battery of victorin-induced responses. Pharmacological studies showed that extracellular alkalization is the essential regulator for both victorin- and VicBSA-induced cellular responses. A model was proposed where victorin might kill the host cell by activating an HR-like response, independent of the binding to the GDC, through ion fluxes across the plasma membrane.

Previously, victorin was shown to be bound specifically to two proteins of the mitochondrial glycine decarboxylase complex, at least one of which binds victorin only in toxin-sensitive genotypes *in vivo* (Duroy A. Navarre and Thomas J. Wolpert, 1999). This enzyme complex was involved in the photorespiratory cycle and was inhibited by victorin, with an effective concentration for 50% inhibition of 81 pM. The photorespiratory cycle began with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and victorin was found to induce a specific proteolytic cleavage of the Rubisco large subunit (LSU). Leaf slices incubated with victorin for 4 hours in the dark accumulated a form of the LSU that is cleaved after the 14th amino acid. This proteolytic cleavage was prevented by the protease inhibitors E-64 and calpeptin. Another primary symptom of victorin treatment is chlorophyll loss, which along with the specific LSU cleavage was suggestive of a victorin-induced, senescence-like response. DNA from victorin-treated leaf slices showed a pronounced laddering effect, which is typical of apoptosis. Calcium appeared to play a role in mediating the plant response to victorin because $LaCl_3$ gave

near-complete protection against victorin, preventing both leaf symptoms and LSU cleavage. The ethylene inhibitors amino-oxyacetic acid and silver thiosulfate also gave significant protection against victorin-induced leaf symptoms and prevented LSU cleavage. The symptoms resulting from victorin treatment suggested that victorin causes premature senescence of leaves.

2.8.2.1.2 Other examples

(1) *Host selective/specific:* T-toxin (*Helminthosporium maydis* race T); HC-toxin (*Helminthosporium carbonum);* HS-toxin (*Helminthosporium sacchari*); Phyto-alternarin (*Alternaria kikuchiana);* PC-toxin (*Periconia circinata*).

(2) *Nonselective/Nonspecific:* Tentoxin (*Alternaria tenuis);* Tabtoxin or wildfire toxin (*Pseudomonas tabaci); Phaseolotoxin* (*Pseudomonas syringae* pv. *Phaseolicola)*. Table 2.2 gives the major differences between host-specific and nonhost-specific toxins.

Table 2.2 Differences between Host-Specific and Nonhost-Specific Toxins

Host specific	*Nonhost specific*
1. Selectively toxic only to susceptible host of the pathogen	1. No host specificity and can also affect the physiology of those plants that are normally not infected by the pathogen
2. Primary determinants of disease	2. Secondary determinants of disease
3. Produce all the essential symptoms of the disease	3. Produce few or none of the symptoms of the disease
4. *Example:* Victorin, T-toxin	4. *Example:* Tentoxin, Tabtoxin

(3) *Produced by plant or plant X pathogen interaction:* Amylovorin: *Erwinia amylovora* (Fire blight of apple and pears)

2.8.2.2 Phytotoxins

These are the substances produced in the host plant due to host-pathogen interactions for which a causal role in disease is merely suspected rather than established. These are the products of parasites which induce few or none of the symptoms caused by the living pathogen. They are nonspecific, and there is no relationship between toxin production and pathogenicity of the disease-causing agent (*Example*: Alternaric acid–*Alternaria solani*).

The ability of a pathogen to infect and invade a compatible host may be facilitated by the production of toxins that induce cell death in the proximity of the invading organism (Dangl and Jones, 2001). These toxins were also reported to play important roles in inhibiting the physiological processes in cells surrounding the point of infection, enabling the spread of the disease (Staskawicz *et al*., 2001). Gaumann (1950) has earlier suggested that some pathogens would be unsuccessful if the toxin did not kill the cells in advance of the fungus and permitted it to establish itself continually on dead or dying cells and produce more toxins. While Baker *et al*. (1997) reported that the virulence of an organism is sometimes enhanced by its ability to produce phytotoxins that kill cells

in the tissue surrounding the point of infection. In some plant diseases, especially with yam anthracnose, toxins often produced a more rapid and extensive invasion by the pathogen than would be in the case in the absence of toxins (Amusa, 1991). Amusa *et al.* (1993) reported the extraction of phytotoxic metabolites from *Colletotrichum gleoesporioides* infected yam leaves. The extracted phytotoxic substance-induced necrotic lesion was similar to the symptoms induced by the pathogens on healthy yam leaves. Phytotoxins often act as the initiation factor for successful pathogenesis. Spores of some fungal pathogen have been associated with phytotoxin production, which probably kill cells of susceptible host paving the way for the penetration of the germ tube.

Most of the phytotoxic metabolites acts by modifying the metabolism of the host plants while some are toxic to the plant tissues once accumulated and poison the plant tissues. A phytotoxin secreted by *Psuedomonas syringae* pv. *tabaci*, the pathogen inducing wildfire disease of tobacco, drastically modifies the amino acid metabolism of the plant with the eventual accumulation of ammonia in tobacco leaves, which causes extensive blighting. Interestingly, the pathogens that synthesize the phytotoxin remain unaffected by the toxin (Balasubramanian, 2003). The development of an effective phytotoxin for use in plant disease control will require a comprehensive understanding of the pathogen(s) involved including its virulence and the biology of the target host plant.

Phytotoxins often act as the initiation factor for successful pathogenesis. Spores of some fungal pathogen have been associated with phytotoxin production, which probably kill cells of susceptible host paving the way for the penetration of the germ tube (Amusa, 2006). All known host specific toxins can be detected from the spore germinating fluids of each virulent pathogen but not from those of the avirulent ones. Thus, specificity found to be characteristically associated with host-specific toxin suggests the early participation of toxin at the site of initial contact with inoculated and host surface. Several phytotoxins are now known, beyond a reasonable doubt, to be the determinant factor in pathogenesis, and some can even act as reliable surrogates for the pathogen that produce them. The partially purified metabolites of *Colletotrichum* spp. induced necrotic lesion of varying sizes on leaves and stems of susceptible hosts. While the phytotoxic metabolites of *Colletotrichum graminicola*, *C. truncatum,* and *C. lindemutianum* inhibited seed germination in respective host crops.

The applications of phytotoxins and toxic containing culture filtrates of plant pathogens combined with tissue culture techniques successfully resulted in obtaining resistant lines in some crops as banana, carnation, wheat, tobacco, and grapevine. This approach is an important complement to classical breeding methods. Although many resistant breeding lines were obtained by *in vitro* selection, there are not many cultivars in agricultural production whose resistance is based on these techniques. The interest of utilizing the *in vitro* methods for improving resistance to plant pathogens remains. They could be considered as methods to contribute to classical selection methods and in combination with them to accelerate obtaining of disease resistant plants since *in vitro* breeding doesn't meet the antipathy of public. Very precise preliminary bioassays and inoculation experiments must be conducted before choosing the successful combination plant pathogen – selective agent – host plant selection level (Slavov, 2005).

of protozoa produce photoactivated extended quinines that are involved in photomovement responses (Giese, 1981).

Table 2.3 Photoactivated Perylenequinones Produced by Fungi

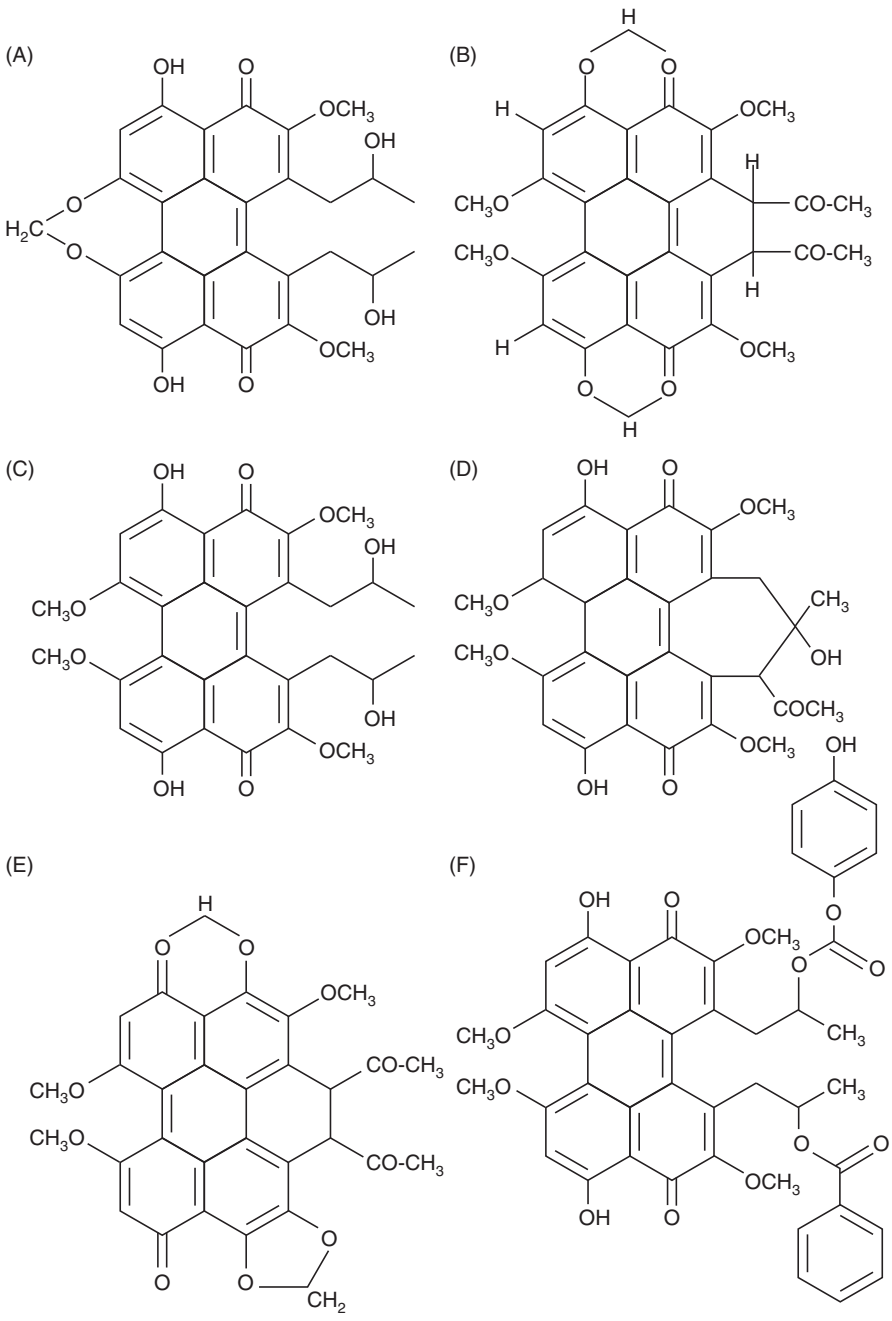
Perylenequinone references	*Fungal species*
Alteichin	*Alternaria alternata*
Alterlosins	*Alternaria eichorniae*
Altertoxins	*Stemphylium botryosum*
Cercosporin	*Cercospora* spp.
Isocercosporin	*Scolecotrichum graminis*
Acetylisocercosporin	*Stagnospora convolvuli*
Calphostin C	*Cladosporium cucumerinum*
Cladochrome	*Cladosporium cladosporioides*
Ent-isophleichrome	*Cladosporium herbarum*
Phleichrome	*Cladosporium phlei*
Elsinochromes	*Elsinoe* spp.
	Stagnospora convolvuli
Hypocrellins	*Hypocrella bambusae*
Isohypocrellin	*Shiraia bambusicola*
	Graphis hematites
Hypomycin A	*Hypomyces* spp.
Shiraiachromes	*Shiraia bambusicola*
Stemphyltoxins	*Stemphylium botryosum*
Other perylenequinones	*Bulgaria inquinans*

2.8.2.5.1 Cercosporin

Cercosporin is one of the most widely studied toxins. Several photoactivated perylenequinones are produced by a number of major fungal plant pathogens, including species of *Alternaria*, *Cercospora*, *Cladosporium*, *Elsinoe*, and *Hypocrella*, among others. The toxins produced by these pathogens are red in color and share a similar structure. Of the perylenequinone toxins, the best studied are the hypocrellins and cercosporin. The hypocrellins have been studied, not for their role in plant disease, but rather due to their pharmaceutical potential as anticancer agents through photodynamic tumor therapy. Cercosporin, by contrast, has been shown to play an important role in *Cercospora* diseases of diverse hosts. Cercosporin is a red pigment, and toxin-deficient mutants can be visually identified on agar plates.

Cercosporin and the other perylenequinone toxins are photoactivated and lack toxicity in the dark. In the light, these compounds absorb light energy and are converted to an energetically activated triplet state. The triplet molecule then reacts with oxygen and results in the generation of toxic, activated oxygen species such as singlet oxygen ($^1O_2$)

and superoxide (O_2^-) (Fig. 2.5). This property classifies cercosporin as a photosensitizer, a term that dates to the early 20th century and was used to describe compounds that



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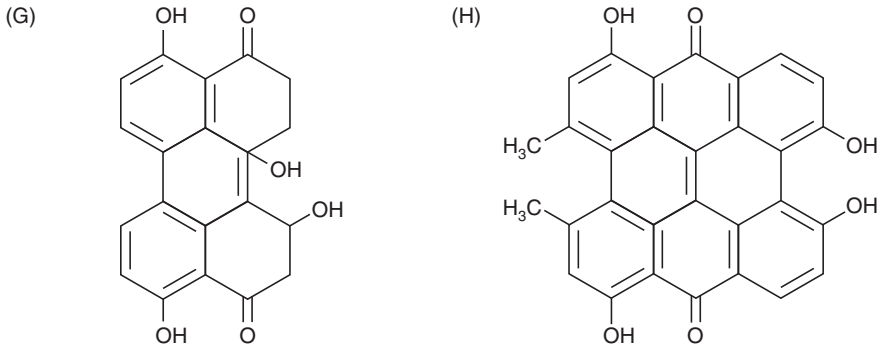

Fig. 2.5 Structure of photoactivated perylenequinones produced by fungi (A-G) and H (Plants)- A(Cercosporin), B (Elsinochrome), C (Phleichrome), D (Hypocrellin), E (Hypomycin), F (Calphostin), G (Altertoxin), H (Hypericin from a herb, *Hypericum perforatum*)

'sensitized' cells to visible wavelengths of light. Photosensitizers are structurally diverse and include common dyes such as methylene blue and acridine orange as well as natural products such as porphyrins, flavins, chlorophyll, and extended quinones. Toxicity of photosensitizers is due to oxidative damage to lipids, proteins, and nucleic acids, with the cellular target dependent on where the photosensitizer molecule localizes in cells (Valenzeno and Pooler, 1987). Cercosporin is a membrane sensitizer and a potent producer of singlet oxygen. Exposure of plant cells and tissues to cercosporin results in peroxidation of the membrane lipids, leading to membrane breakdown and death of the cells. It is hypothesized that membrane damage allows for leakage of nutrients into the leaf intercellular spaces, allowing for fungal growth and sporulation (Daub and Chung, 2007).

Production of cercosporin is mediated by multiple signal transduction pathways. Studies using pharmacological inhibitors implicated the involvement of calcium and calmodulin signaling in cercosporin production (Chung, 2003.). A study also revealed that cercosporin production was associated with a gene encoding an MAP kinase kinase kinase in *C. zeae-maydis* (Shim and Dunkle, 2003). In addition, cercosporin biosynthesis is regulated by another transcription factor, *CRG1*. The *CRG1* gene was originally identified as a gene involved in cercosporin resistance, but disruption mutants also showed a reduction in production. Cercosporin plays an important role in *Cercospora* pathogenicity and lesion formation in several crop species. Thus, any means of reducing cercosporin toxicity promises to have an important application for managing Cercospora leaf spot diseases in the field. In addition, cercosporin is very toxic due to the production of reactive oxygen species. Breeding for Cercospora disease resistance has been difficult since cercosporin has general toxicity to cells. Through our studies, we found that biosynthesis and regulation of cercosporin is a complicated process (Daub. and Chung, 2007). Also, the mechanisms by which the *Cercospora* fungi operate to protect themselves against cercosporin toxicity likely involve multiple factors.

2.8.2.5.2 Mode of action of perylenequinone toxins

The fungal perylenequinone toxins fit the classification of a group of compounds known as 'photosensitizers,' which signifies the ability of these compounds to 'sensitize' living cells to visible wavelengths of light. Beyond the perylenequinones and synthetic dyes, there are many classes of compounds that have photosensitizing activity. These include numerous compounds isolated from plants such as chlorophylls, coumarins, thiophenes, and acetylenes, compounds important to human health such as riboflavin and porphyrins, and other commonly used dyes such as methylene blue, rose bengal, and acridine orange (Spikes 1989). Due to their toxicity, photosensitizers are being investigated for possible utility as herbicides and insecticides, as antiviral agents, and in photodynamic tumor therapy (Hudson and Towers 1991).

Photosensitizers are colored and absorb visible wavelengths of light. The absorbed light energy causes the photosensitizer to be converted to the energetically activated and long-lived 'triplet state.' Triplet state photosensitizers may react through radical or energy transfer reactions. In the so-called 'type I reaction' the triplet sensitizer reacts through a reducing substrate, leading to the formation of a reduced photosensitizer (Margaret E. Daub and Kuang-Ren Chung, 2009). The reduced photosensitizer in turn may react with cellular macromolecules such as lipids, proteins, or nucleic acids, causing oxidative damage and leading to the production of lipid peroxides and other free radical compounds. The reduced photosensitizer may also react with oxygen, leading to the production of reduced and free radical forms of oxygen including superoxide (O_2^-), hydrogen peroxide (H_2O_2), or the hydroxyl radical (OH).

Photosensitizers may also react directly with oxygen via energy transfer, a reaction known as a 'type II reaction.' These reactions result in the production of the highly toxic singlet oxygen ($^1O_2$). In cells, the type of damage resulting from photosensitization is due to where the photosensitizer molecule localizes (Ito 1981). Lipid-soluble photosensitizers, for example, generally cause membrane damage whereas those that localize in nuclei damage DNA Photoactivated perylenequinone toxins play an important role in diseases caused by *Cercospora* species and likely also by a diversity of other fungal pathogens that synthesize these interesting compounds. Significant advances have been made in the understanding of the mode of action of these toxins, their biosynthesis and regulation, and possible mechanisms involved in resistance to their toxic effects. Although efforts thus far to engineer resistance in crop plants to cercosporin and *Cercospora* pathogens have not yet been successful, the strategy of targeting cercosporin in genetic engineering efforts remains a promising strategy for developing crop species resistant to these damaging pathogens (Margaret E. Daub and Kuang-Ren Chung, 2009).

2.8.2.6 Toxin classification based on their specificity

The idea that toxins play causal roles in plant diseases is attractive and dates back to the time of deBary. When one reduces the toxin theory to its most elementary form, it can be stated that all of the symptoms of a given disease result from the direct action of a

Table 2.4 Fungi Producing Host-Selective Toxins

Organism	*Toxin*	*Structure*
Cochliobolus carbonum	HC-toxin	Cyclic tetrapeptide
C. heterostrophus	T-toxin	Polyketide
C. victoriae	Victorin	Cyclic pentapeptide
Alternaria alternata f. sp. *lycopersici*	AAL-toxin	Aminopentol ester
A.alternata f. sp. *kikuchiana*	AK-toxin	Epoxy-decatrienoic acid backbone
A. alternata f. sp. *fragariae*	AF-toxin	Epoxy-decatrienoic acid backbone
A.alternata f. sp. *citri tangerine*	ACT-toxin	Epoxy-decatrienoic acid backbone
A. alternata f. sp. *mali*	AM-toxin	Four members cyclic depsipeptide

Fungi can fail to be pathogenic because they induce defenses. Induced resistance involves detection of the fungus and the mounting of an increased localized and systemic defense against fungal invasion. This involves the biosynthesis of pathogenesis-related proteins and phytoalexins. The hypersensitive response implicates cell death as a mechanism of resistance, but some necrophytic or facultative saprophytic fungi utilize cell death to their advantage. By secreting toxins which are able to kill cells of susceptible hosts, these fungi are able to infect, colonize and feed off their host and in the meantime complete their own life cycle. In this case, cell death is associated with susceptibility, and resistance is mediated by insensitivity to the effects of the toxin. In most cases, application of purified toxin is sufficient to cause cell death in susceptible plants. These toxins are an absolute requirement for successful infection of susceptible hosts and are therefore compatibility factors (Briggs and Johal, 1994).

Host-selective toxins are known determinants of compatibility in plant–fungus interactions and provide a powerful model for understanding the specificity of these associations. The identification of genes required for toxin biosynthesis has shown that the genes are unique to the toxin-producing species and cluster in complex loci. These loci may have been acquired by horizontal gene transfer (Jonathan E. Markham and Jacques Hille, 2001). Many, if not all, host-selective toxins act by disrupting biochemical processes and in several cases the resulting cell death has the characteristics of programmed cell death. This ability to make dead tissue from living has enabled these facultative saprophytic fungi to become plant pathogens.

By killing the plant cells with a secreted, soluble toxin, the fungi are able to circumvent the innate defenses of living plant cells (Van Loon, L.C., 1997). In all cases examined so far, a single plant gene has conferred a stable form of resistance (or susceptibility) to this type of infection strategy, but several genes are associated with toxin production in the fungus. Thus the gene-for-gene interaction model does not apply for this type of plant–fungus interaction. The toxins and host sensitivity to them are a major determinant of the fungus' host range, and so they are commonly referred to as host-selective toxins (Pringle and Scheffer, 1964). The genera *Alternaria* and Cochliobolus contain species that produce a variety of host-selective toxins and, as a result, have different host ranges. These species provide a unique model for understanding host-selectivity and the

strategies deployed by pathogens to allow infection. Both genera also contain saprophytic species or usually isolates of the same species, which have no known specific plant host.

Cochliobolus and *Alternaria* contain several pathogens which show enhanced pathogenicity towards their hosts. The enhanced pathogenicity in these species is linked to toxin production because nontoxin producing strains can be just as pathogenic as toxin producing strains if they are inoculated on toxin-sensitive host varieties in the presence of the host-selective toxin (Comstock and Scheffer, 1973). Toxins are usually a variety of acyl or ester derivatives of common structures with varying degrees of toxicity. As it is not always clear if a single molecular species is being used and because there is no consensus in the literature on the use of plural vs. singular names, we refer to all toxins in this review in the singular. While toxins are required for pathogenicity; they are not necessarily the sole determinants of pathogenicity. Extracellular proteins, enzymes, and nonhost selective toxins may all play a role in overall pathogenicity (Tonukari *et al.*, 2000). It is vital for our understanding of these diseases to know what genetic changes have created the enhanced pathogenicity. The *Cochliobolus* species are common pathogens of monocotyledons, which include important crop species such as rice, maize and oat (Agrios, 1997). *Cochliobolus* (anamorph *Bipolaris*) species are true fungi of the *Ascomycota.* Most isolates of *Cochliobolus* are non- to only weakly-pathogenic, but strains with increased pathogenicity due to the production of host-selective toxins have emerged.

The genera *Alternaria* and *Cochliobolus contain* species that produce a variety of host-selective toxins and, as a result, have different host ranges [(Table 2.4) (Jonathan E. Markham and Jacques Hille, 2001)]. These species provide a unique model for understanding host selectivity and the strategies deployed by pathogens to allow infection.

2.8.2.8 Toxins promoting pathogenicity of Cochliobolus and Alternaria species

These genera contain several pathogens which show enhanced pathogenicity towards their hosts. The enhanced pathogenicity in these species is linked to toxin production because non-toxin-producing strains can be just as pathogenic as toxin producing strains if they are inoculated on toxin-sensitive host varieties in the presence of the host-selective toxin (Comstock and Scheffer, 1973). Toxins are usually a variety of acyl or ester derivatives of common structures with varying degrees of toxicity. While toxins are required for pathogenicity; they are not necessarily the sole determinants of pathogenicity. Extracellular proteins, enzymes, and nonhost selective toxins may all play a role in overall pathogenicity (Annis and Goodwin, 1997). Recent progress in the isolation of genes involved in toxin biosynthesis has shown that the arrangement of toxin biosynthesis genes follows a similar pattern in this diverse range of fungi and suggests a common mechanism by which toxin producing strains may have arisen.

2.8.2.8.1 HC-toxin

HC-toxin is a cyclic tetrapeptide of structure cyclo (D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid (Fig. 2.6). It is a determinant of

specificity and virulence in the interaction between the producing fungus, *Cochliobolus carbonum*, and its host, maize. HC-toxin qualifies as one of the few microbial secondary metabolites whose ecological function in nature is understood. Reaction to *C. carbonum* and HC-toxin is controlled in maize by the Hm1 and Hm2 loci. These loci encode HC-toxin reductase, which detoxifies HC-toxin by reducing the 8-carbonyl group of Aeo. HC-toxin is an inhibitor of histone deacetylases (HDACs) in many organisms, including plants, insects, and mammals (Walton, 2006), HC-toxin appears to induce the degradation of defense gene transcripts in maize. Another leaf spot disease of maize is caused by the pathogen *C. carbonum.* Races 2 and 3 are weakly virulent, producing small chlorotic or necrotic flecks on leaves, but race 1 is extremely pathogenic on susceptible varieties causing large necrotic lesions on leaves (Pitkin *et al.*, 2000). The increased pathogenicity of race 1 is due to the production of HC-toxin, a cyclic tetrapeptide containing D-amino acids (Kawai *et al.*, 1983). It was the first host-selective toxin to be identified and consequently provides a model for studies concerning other host-selective toxins.

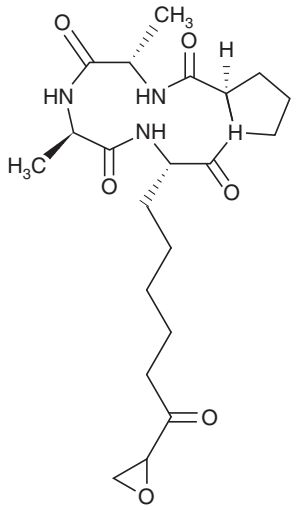

Fig. 2.6 HC-toxin

The genes for HC-toxin biosynthesis have been mapped to a single locus, *TOX2*, which, depending upon the isolate is located on either a 3.5 Mbp or 2.2 Mbp chromosomes. The locus covers at least 540 kbp and is thought to contain all the genes required for toxin biosynthesis. The central gene for toxin biosynthesis encodes the enzyme HC-toxin synthetase (HTS1), a 570 kDa nonribosomal peptide synthase. Other genes, *TOXCTOXD, TOXF*, and *TOXG*, encode enzymes which may synthesize the substrates for HC-toxin synthetase (Ahn and Walton, 1997). *TOXA* is closely linked to *HTS1* and encodes a putative HC-toxin efflux carrier (Pitkin *et al.*, 1996). All these genes, except *HTS1*, are under the transcriptional influence of *TOXE.*

Some effects of HC-toxin on host plant tissues could be construed as beneficial. Many of these effects are directly opposed to those induced by other host selective toxins.

Instead of inducing a general leakage of electrolytes, HC-toxin increases uptake of organic and inorganic solutes by susceptible maize roots and stimulates uptake, accumulation, and reduction of nitrate. In contrast to the effects of most *HST*s on cell membranes, HC-toxin induces membrane hyperpolarization of susceptible maize cells rather than depolarization. Further, instead of killing protoplasts, HC-toxin increases the long-term viability of leaf protoplasts a few days beyond that of protoplasts incubated in the absence of HC-toxin. Most of the effects of HC-toxin also are observed with tissues of the resistant genotype but only at toxin concentrations 100- to 200-fold higher than those that affect the susceptible genotype (Thomas J. Wolpert *et al.*, 2002).

2.8.2.8.2 T-toxin

T-cytoplasmic male sterile (T-cms) maize is sensitive to T-toxin, a polyketide synthesized by the fungus *Cochliobolus heterostrophus*, which is responsible for Southern Corn Leaf Blight disease (Wise *et al.*, 1999). T-Toxin is a trichothecene that, unlike the other trichothecenes that inhibit protein synthesis, also causes plant plasma membrane leakage of electrolytes at low concentrations.

The genes for T-toxin biosynthesis are located at the locus TOX1, which has become separated by a chromosome translocation into two loci, TOX1A found on chromosome 12;6 and TOX1B on chromosome 6:12. The loci are inseparably linked to a four-armed linkage group which causes the TOX1 locus to inherit as a single locus. Karyotype comparisons between race T and non-T-toxin producing race show 1.2 Mbp more DNA in race T, which maps to the TOX1A and TOX1B loci (Jonathan E. Markham and Jacques Hille, 2001). Two genes have been identified which are necessary for T-toxin biosynthesis, a polyketide synthase (*PKS1*) that is a multifunctional enzyme similar to fatty acid synthases and *DEC1* which is thought to act as a decarboxylase to remove one carbon as CO_2 from the even-numbered carbon chain to produce the odd numbered final T-toxin (Yoder, 1998). T-toxin, the HST produced by *C. heterostrophus* (*Helminthosporium maydis* = *Bipolaris maydis*) race T and *Mycosphaerella zeae-maydis* (*Phyllosticta maydis*), is certainly the best-characterized HST in regard to its site and mode of action (Lindgren, 1997). Both organisms produce a group of structurally related polyketides that are selectively toxic to maize. Toxin-producing isolates are considerably more virulent than nonproducing isolates, and mutations in structural proteins required for toxin production clearly reduce virulence (Thomas J. Wolpert *et al.*, 2002). T-2 is a trichothecene mycotoxin (Fig. 2.7). It is a naturally occurring mold by-product of *Fusarium* spp. the fungus which is toxic to humans and animals.

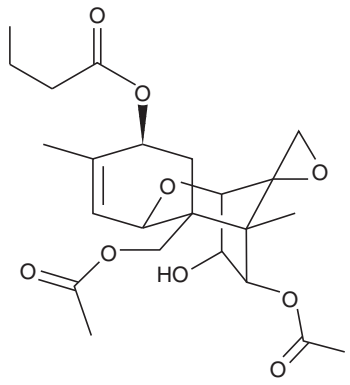

Fig. 2.7 T-2 mycotoxin

The toxin appears to have two modes of action in T mitochondria, viz., as an uncoupler and as an inhibitor of electron transport at Complex I. For example, NADH- or succinate-dependent respiration

(O_2 uptake) in isolated T mitochondria is stimulated by HmT toxin and not coupled to ATP production. The toxin may act at multiple sites on the inner mitochondrial membrane or that the toxin increased membrane permeability in T mitochondria (Marcia J. Holden and Heven Sze, 1984). T-toxin increases membrane permeability specifically in T-mitochondria. Ca^{2+} uptake into T mitochondria driven by either malate succinate, or ATP generated a Ca^{2+} gradient that was dissipated by either the Ca^{2+} ionophore (A23 187) or HmT toxin.

2.8.2.8.3 Victorin

The fungus *Cochliobolus victoriae* causes Victoria blight disease in oats. The fungus secretes a toxin known as victorin that is toxic to susceptible oat leaves at very low concentrations (Wolpert *et al.*, 1995). Victorin is an unusual halogen-containing cyclic pentapeptide. Victorin-C is a heterodetic cyclic peptide (Fig. 2.8). Toxin synthesis is inherited at a single locus TOX_3, and it would seem reasonable to hypothesize that it is synthesized in a manner analogous to other cyclic peptide toxins, that is, by a nonribosomal (cyclic) peptide synthetase. Enzymes must also be present to synthesize the novel amino acids required by such a peptide synthetase, but unfortunately to date no reports on the identification of any genes necessary for victorin synthesis are known. Victorin appears to induce many of the plant responses classically associated with avirulence elicitors such as callose deposition, the respiratory burst, lipid peroxidation, ethylene evolution, extracellular alkalinization phytoalexin synthesis and KC efflux. Thus, genetic and physiological studies support the perception that victorin functions as an elicitor to induce components of a resistance response similar to those induced by avirulence factors. Victorin causes premature senescence of leaves.

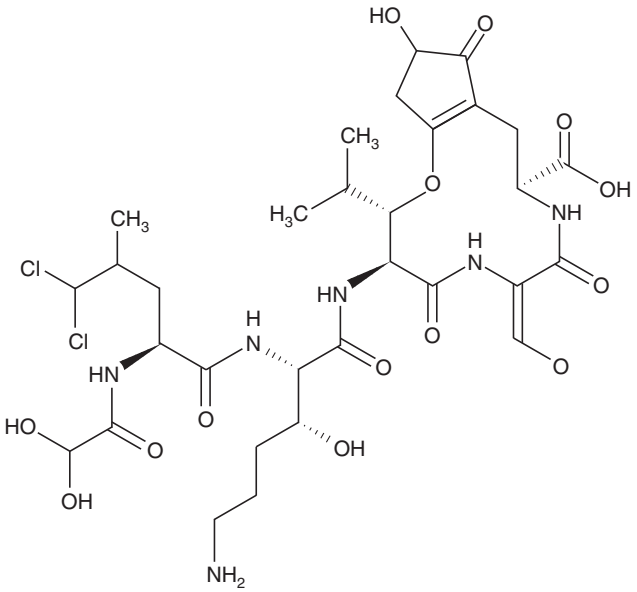

Fig. 2.8 Victorin-C

Evidence is accumulating that the hypersensitive cell death response often associated with induced resistance in gene-for-gene interactions is a type of programmed cell death (PCD) (Thomas J. Wolpert *et al.*, 2002). PCD is a genetically controlled, organized form of cellular suicide that normally functions to eliminate unnecessary or aged cells during morphogenesis (i.e., by cell selection) or cellular maturation. It is a 'normal' form of cell death essential to cellular homeostasis in multicellular organisms. Thus, PCD is distinct from necrotic cell death, which typically arises as a result of severe cellular damage. Necrosis does not entail the participation of the cell in its own destruction, as does PCD, but rather arises from a loss of function. The distinction between PCD and necrosis may be envisioned as the difference between 'organized disassembly' and 'death from damage.' Victorin induces a form of PCD that shares many of the biochemical and morphological characteristics of apoptosis, including DNA laddering, heterochromatin condensation, cell shrinkage and protease activation. The signaling events leading to the host response to victorin also share characteristics with signaling events in resistance, including changes in calcium homeostasis and the participation of kinases and ethylene (Ishihara *et al.*, 1996). Further, victorin contributes to mitochondrial dysfunction (Curtis and Wolpert, 2002). This finding is noteworthy because, as mentioned above, mitochondrial dysfunction is commonly associated with the induction of PCD and because voctorin had previously been shown to bind to members of the mitochondrial enzyme complex, glycine decarboxylase (GDC) (Navarre and Wolpert, 1995).

2.8.2.8.4 Alternaria species

The fungal genus *Alternaria* contains at least ten host-selective toxin-producing plant pathogens which are all pathogens of dicotyledons (Jonathan E. Markham and Jacques Hille, 2001). The largest group of pathogens is proposed to be all pathotypic variants of the species *Alternaria alternata*, a very common, imperfect fungus (Nishimura and Kohmoto, 1983). Molecular phylogenetics by analysis of DNA–DNA reassociation kinetics, rDNA regions and internal transcribed spacer regions from different *Alternaria alternata* pathotypes support this proposal.

2.8.2.8.5 AAL-toxin

The fungus *Alternaria alternate* f. sp. *lycopersici* causes *Alternaria* stem canker of susceptible tomatoes. The fungus produces an aminopentol ester toxin which is very similar in structure to fumonisin, a toxin first identified in *Fusarium moniliforme*. The consumption of food contaminated by fumonisin is implicated in the development of several human and animal diseases. Identification of the genes involved in AAL-toxin (Figure 2.9) biosynthesis is not possible by traditional genetic methods because *A. alternata* has no known sexual cycle. By using REMI however, it has been possible to create AAL-toxin deficient mutants (Jonathan E. Markham and Jacques Hille, 2001). These mutants are not pathogenic, confirming the requirement of the toxin for pathogenesis. The number of mutants recovered is 1 per 100 screened, which suggests that there may be many genes involved, or that there is instability in the locus during the REMI transformation procedure. Isolation of the sequences adjacent to the insert has not been reported in *A. alternata* f. sp. *lycopersici*, but this technique has been successful for other *A. alternata* pathotypes.

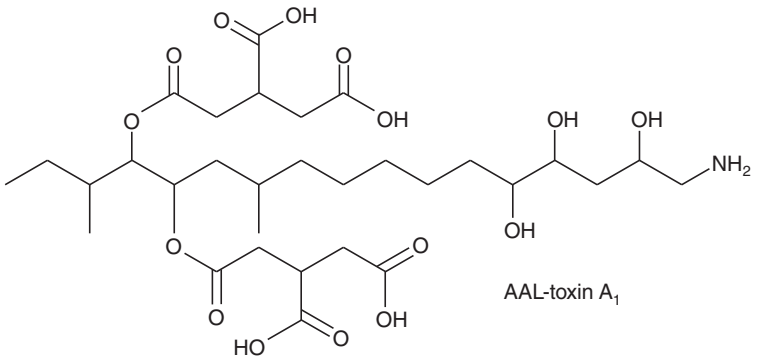

Fig. 2.9 AAL toxin

The AAL-toxins induce an apoptotic-like response in toxin-sensitive tomato, as demonstrated by TUNEL-positive cells, DNA laddering, and the formation of apoptotic-like bodies (Wang *et al*., 1996). This PCD response, as in the HR, appears to involve calcium and ethylene and therefore, exhibits similarities to an induced resistance response. The AAl-toxins are part of a larger group of compounds structurally related to sphingosine and sphinganine and are referred to as sphinganine analog mycotoxins, or SAMs. Other SAMs, known as fumonisins are produced by the unrelated fungus *Fusarium verticillioides* (syn. *F. moniliforme*) and other *Fusarium* species Fumonisin B1 (and other fumonisins) is as selectively toxic to the AAL-toxin sensitive tomato genotypes as the AAL-toxins. Both fumonisin B1 and TA inhibit ceramide synthase in rat hepatocytes and microsomal preparations from green tomato fruit, with a concomitant alteration in the concentration of sphingoid bases (Thomas J. Wolpert *et al*., 2002).

2.8.2.8.6 AM-toxin

Alternaria blotch is a worldwide disease of susceptible apple caused by *A. alternata* f. sp. *mali* which is known to produce AM-toxin, a four-member cyclic depsipeptide (Figure 2.10). Cloning of the genes involved in toxin biosynthesis is underway with the isolation of a gene for AM-toxin synthetase (AMT), a potential cyclic peptide synthetase (Johnson *et al*., 2000). A new assay was developed to measure the necrotic activity of AM-toxin analogs using cultured leaves from meristem cells (Miyashita *et al*., 2001). This method was not only more sensitive to AM-toxin I but also more reliable than the previous one that used tree leaves due to the homogeneous nature of cultured leaves and to the method of application of toxins. A structure-activity relationship of AM-toxin analogs synthesized was developed. Most residues and the macrocyclic ring structure were strictly recognized by AM-toxin putative receptor, whereas the L-Ala binding subsite of the receptor allowed for side chain structures with various stereoelectronic properties. One of the most homologous genes of AM-toxin synthetase is HC-toxin synthetase from *C. carbonum*. Similarly, as for HC-toxin synthetase, there is also evidence for more than one copy of AMT in the *A. alternata* f. sp. *mali* genome located together on one chromosome. Such an arrangement of genes is highly similar to the characterized TOX2 locus for HC-toxin biosynthesis. It will be interesting to see whether the small chromosome of *A. alternata*

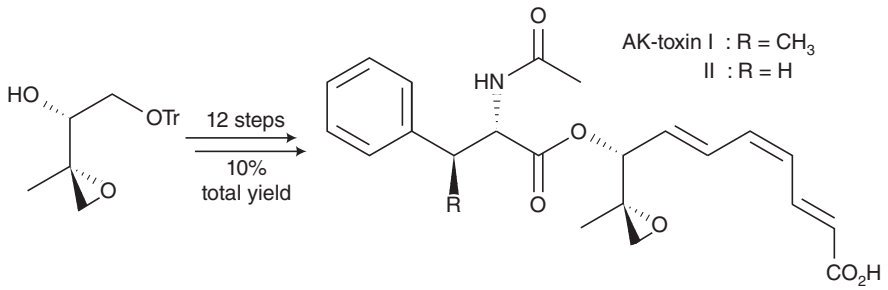

Fig. 2.11 AK-toxin

The filamentous fungus *Alternaria alternata* includes seven pathogenic variants (pathotypes) which produce different host-selective toxins and cause diseases on different plants (Ai Imazaki *et al*., 2010). The Japanese pear pathotype produces the host-selective AK-toxin, an epoxy-decatrienoic acid ester, and causes a black spot of Japanese pear. It was shown that genes, *AKT1*, *AKT2*, *AKT3*, and *AKTR* were involved in AK-toxin biosynthesis. *AKT1*, *AKT2*, and *AKT3* encode enzyme proteins with peroxisomal targeting signal type 1 (PTS1)-like tripeptides, SKI, SKL, and PKL, respectively, at the C-terminal ends. The peroxisome localization of *Akt1, Akt2*, and *Akt3* was verified by using strains expressing the N-terminal green fluorescent protein (GFP)-tagged versions of the proteins. To assess the role of peroxisome function in AK-toxin production, Aa*PEX6 was isolated* which encodes a peroxin protein essential for peroxisome biogenesis, from the Japanese pear pathotype and made Aa*PEX6* disruption-containing transformants from a GFP-*Akt1*-expressing strain. The ΔAa*PEX6* mutant strains did not grow on fatty acid media because of a defect in fatty acid β oxidation. The import of GFP-Akt1 into peroxisomes was impaired in the ΔAa*PEX6* mutant strains. These strains completely lost AK-toxin production and pathogenicity on susceptible pear leaves. This proved that peroxisomes were essential for AK-toxin biosynthesis. The ΔAa*PEX6* mutant strains showed a marked reduction in the ability to cause lesions on leaves of a resistant pear cultivar with defense responses compromised by heat shock. It was suggested that peroxisome function was also required for plant invasion and tissue colonization in *A. alternata*. Mutation of Aa*PEX6* caused a marked reduction of conidiation.

Restriction enzyme-mediated integration (REMI) mutagenesis was used to tag genes required for toxin biosynthesis (Tanaka *et al*., 1999). Protoplasts of a wild-type strain were treated with a linearized plasmid along with the restriction enzyme used to linearize the plasmid. Of 984 REMI transformants recovered, three produced no detectable AK-toxin and lost pathogenicity on pear leaves. Genomic DNA flanking the integrated plasmid was recovered from one of the mutants. With the recovered DNA used as a probe, a cosmid clone of the wild-type strain was isolated. Structural and functional analyses of an 8.0-kb region corresponding to the tagged site indicated the presence of two genes. One, designated *AKT1*, encodes a member of the class of carboxyl-activating enzymes. The other, *AKT2*, encodes a protein of unknown function. The essential roles of these two genes in both AK-toxin production and pathogenicity were confirmed by transformation-mediated gene disruption experiments. DNA gel blot analysis detected

AKT1 and *AKT2* homologs not only in the Japanese pear pathotype strains but also in strains from the tangerine and strawberry pathotypes. The host-specific toxins of these two pathotypes are similar in structure to AK-toxin. Homologs were not detected in other pathotypes or nonpathogenic strains of *A. alternata*, suggesting acquisition of *AKT1* and *AKT2* by horizontal transfer.

2.8.2.8.8 Other alternaria alternata pathotype toxins

A. alternata f. sp. *fragariae*, which infects strawberry and *A. alternate* f. sp. *citri,* pathotype tangerine, produce toxins with epoxydecatrienoic ester backbones, designated AF- and ACT-toxin, respectively (Otani *et al.*, 1995). This structure is also present in AK-toxin; indeed certain forms of AF- and ACT-toxin are also toxic to AK-toxin susceptible cultivars of Japanese pear. Cross pathotype hybridization studies and PCR cloning have revealed that the strawberry and tangerine pathotypes contain homologs of the genes implicated in AK-toxin biosynthesis (Masunaka *et al.*, 2000).

2.8.3 Mycotoxins in Plant Pathogenesis

Mycotoxins are defined as low molecular weight fungal metabolites that are toxic to vertebrates. Mycotoxins can have dramatic adverse effects on the health of farm animals and humans that eat contaminated agricultural products. Mycotoxicology has not been a traditional field of plant pathological research. Mycotoxin research has historically been performed by natural product chemists, mycologists, animal toxicologists, and human disease epidemiologists. The apparent lack of specificity of mycotoxins has hindered the acceptance of a role for mycotoxins in plant pathogenesis. In addition, mycotoxin contamination was perceived to be a postharvest problem of stored grain. But it is now well established that many mycotoxin-producing fungal species cause plant disease under field conditions. It thus becomes logical to ask whether mycotoxins themselves play a role in plant pathogenesis in addition to their role in animal diseases. A wide variety of fungal metabolites are both mycotoxic and phytotoxic (Table 2.5). The identification of toxins as probable virulence factors by fungal genetic analysis should be confirmed by plant genetic analysis. If the production of a toxin increases pathogen virulence then increased host resistance to the toxin should increase host resistance to the disease (Anne E. Desjardins and Thomas M. Hohn, 1997).

Table 2.5 Major Mycotoxins

Fungus	*Substrate*	*Mycotoxin*
Aspergillus flavus, *A. parasiticus*	Maize, groundnut, cotton	Aflatoxin
A. ochraceus, *Penicillium viridictum*	Wheat, barley, maize	Ochratoxin
Fusarium moniliforme, *F. culmorum*, *F. avenaceum*, and *F. nivale*	Wheat, barley, maize	Fumonisins
Fusarium moniliforme, *F. equiseti*, *F. culmorum*, *F. solani*, *F. avenaceum*, *F. roseum*, and *F. nivale*	Wheat, barley, maize, oats, rye.	Trichothecene

Contd...

Table 2.6 Physical and Chemical Properties of Selected Ergot Alkaloids

	Molecular formula	*Molecular weight*	*Melting point (°C)*
Ergotamine	$C_{33}H_{35}O_5N_5$	581.6	212–214 (dec)
Ergocornine	$C_{31}H_{39}O_5N_5$	561.7	182–184 (dec)
Ergocristine	$C_{35}H_{39}O_5N_5$	609.7	165–170 (dec)
Ergocryptine	$C_{32}H_{41}O_5N_5$	575.7	173 (dec)
Agroclavine	$C_{16}H_{18}N_2$	238.2	210–212 (dec)
Ergometrine	$C_{19}H_{23}O_2N_3$	325.4	175–180 (dec)

*dec = decomposition

The ergot sclerotium contains high concentrations (up to 2% of dry mass) of the alkaloid ergotamine (Fig. 2.12), a complex molecule consisting of a tripeptide-derived cyclol-lactam ring connected via an amide linkage to a lysergic acid (ergoline) moiety, and other alkaloids of the ergoline group that are biosynthesized by the fungus. Ergot alkaloids have a wide range of biological activities including effects on circulation and neurotransmission Ergot alkaloids can be classified into two classes, viz., derivatives of 6,8-dimethylergoline and lysergic acid derivatives (Eadie, 2003).

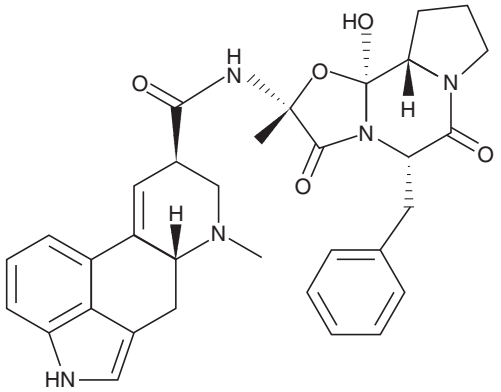

Fig. 2.12 Ergotamine

Biosynthesis of the ergopeptines is catalyzed by a nonribosomal peptide synthetase that employs D-lysergic acid, D-proline, and two additional amino acids as substrates. For ergotamine, these unspecified amino acids are L-alanine and L-phenylalanine. Genes encoding specific peptide synthetases involved in ergopeptine biosynthesis have not been identified in *C. purpurea.* However, portions of genes for putative peptide synthetases have been amplified by polymerase chain reaction from *C. purpurea* and the closely related species *Acremonium coenophialum* (Panaccione 1996). Outbreaks of ergotism occur in animals that eat grain contaminated with *C. purpurea* and other *Claviceps* spp. Similar mycotoxicoses occur in livestock that graze on pastures of certain fescue and ryegrass species that are infected with various *Acremonium* endophytes. These endophytic fungi

appear to enhance growth, disease resistance, and drought tolerance of their grass hosts, but also contaminate them with ergot alkaloids that produce gangrene, convulsions, and other neurological disorders in animals that graze infected pastures.

2.8.3.2 Aflatoxins

With Turkey X disease and the discovery of aflatoxins, the modern era of mycotoxicology began in England in 1960. Toxicity of animal feeds containing contaminated peanut meal led to the deaths of more than 100,000 turkeys, and of other farm animals, by acute liver necrosis. Scientists in England quickly identified the toxin-producing organism as *Aspergillus flavus* and the toxic agents as a group of related bisfuranocoumarins that were named aflatoxins B1, B2, G1, G2, etc. [(Fig. 2.13) (Reddy and Waliyar, 2012)]. Subsequent studies have shown that aflatoxins are potent liver toxins and liver carcinogens in a wide variety of animals, causing hepatocellular carcinomas in some species at dietary levels below 1.0 μg per kg of feed. Human exposure to aflatoxins can result from consumption of contaminated peanuts, corn, and other agricultural commodities, but also from consumption of meat, milk, and eggs from animals that have consumed contaminated feeds. The occurrence of aflatoxins in milk is of particular concern worldwide (Marasas and Nelson, 1987).

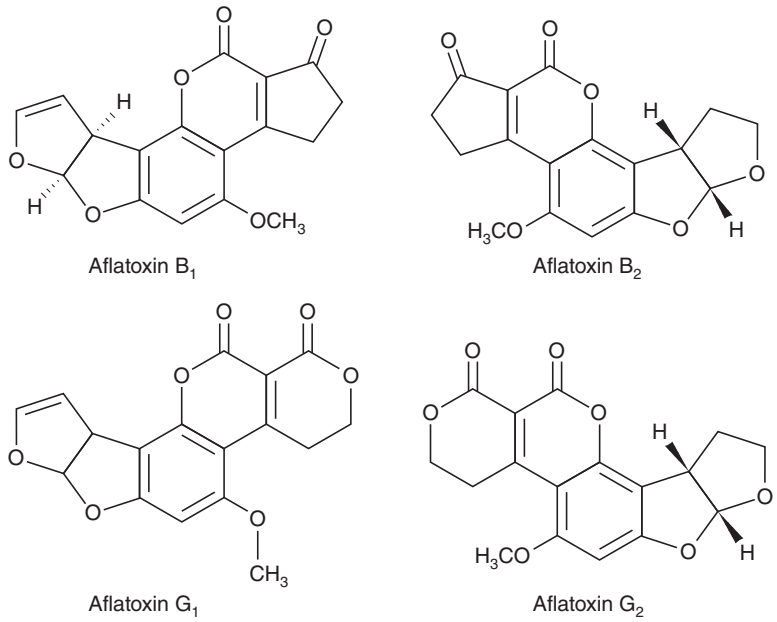

Fig. 2.13 Structures of the major aflatoxins

The various food products contaminated with aflatoxins include cereals like maize, sorghum, pearl millet, rice and wheat, oilseeds such as groundnut, soybean, sunflower and cotton, spices like chillies, black pepper, coriander, turmeric and ginger; tree nuts such as almonds, pistachio, walnuts and coconut, and milk and milk products.

Groundnuts are often contaminated with aflatoxins B_1 and B_2, less often with aflatoxins B_1, B_2, G_1, and G_2, so it is important to have analytical values that represent the total aflatoxin content.

Aspergillus fumigatus is known to produce various immunosuppressive mycotoxins including gliotoxin (KameI and Watanabe, 2005). However, none of these mycotoxins has been confirmed as being directly related to the pathogenesis of aspergilli. Recent studies have made substantial progress in the determination of mycotoxins as virulence factors. Gliotoxin was found to be produced much faster than previously believed under certain culture conditions, such as high oxygen content, which is close to the environment in the host. Gliotoxin was also found to be detectable in the sera of aspergillosis mice and aspergillosis patients. Based on these findings, it is evident that gliotoxin is produced in the infected organs of patients with aspergillosis at a significant level. In addition to these known mycotoxins, *A. fumigatus* produces many mycotoxins apparently different from known toxins. From the aspect of gene analysis, the deletion of laeA was found to block the expression of metabolic gene clusters such as sterigmatocystin, and the gene is also expected to be related to the production of gliotoxin.

Aflatoxins are produced by *A. flavus* and *A. parasiticus,* whereas a wide range of *Aspergillus* spp. produce the aflatoxin precursor sterigmatocystin, which also is an animal toxin and carcinogen (Table 2.7). The aflatoxin/sterigmatocystin pathways of *Aspergillus* spp. are perhaps the most thoroughly studied fungal polyketide pathways. The first step in the biosynthesis of sterigmatocystin/aflatoxin is catalyzed by a type I polyketide synthase (Chang *et al.,* 1995). In contrast to most polyketide synthases, which utilize acetate as a starter unit, the starter unit for the aflatoxin/sterigmatocystin enzyme is hexanoate (Brobst and Townsend, 1994). The synthase reaction product and first stable intermediate in the pathway is norsolorinic acid, which undergoes a complex series of modifications to yield sterigmatocystin and finally, aflatoxin. Studies of the sterigomatocystin pathway in *A. nidulans* have shown that the gene encoding the polyketide synthase (*pksST*) is part of a gene cluster containing at least 25 pathway-related genes (Brown *et al.,* 1996). This gene cluster occupies a 60-kb region and contains genes for regulatory factors in addition to all of the required pathway enzymes. The genes for the aflatoxin pathways in *A. flavus* and *A. parasiticus* are similarly organized and in most cases appear to contain closely related homologs of the sterigmatocystin pathway genes (Anne E. Desjardins and Thomas M. Hohn, 1997).

Both *A. flavus* and *A. parasiticus* are pathogenic on a variety of plant species, although *A. flavus* predominates on most hosts except peanuts. Aflatoxin production is widespread in both species; field strains of *A. parasiticus*, in particular, rarely lose the ability to produce aflatoxins (Payne, 1983). Aflatoxins, their metabolites, the aflatoxin-8,9-epoxide and the generated ROS causes deleterious effects on the various body organs and body systems including the development of cancers especially the liver cancer mainly due to AFB_1 exposure. Aflatoxins are also responsible for the suppression of both the humoral and cell-mediated immunity and thus making individuals susceptible to infectious diseases. Aflatoxins also responsible for the malabsorption of various nutrients thus leading to nutritional deficiencies, impaired immune function, malnutrition and stunted growth and hence the development of kwashiorkor and marasmus in infants. Aflatoxins

2.8.3.3 Trichothecenes

For more than 100 years, both acute and chronic mycotoxicoses in farm animals and in humans have been associated with consumption of wheat, rye, barley, oats, rice, and maize contaminated with *Fusarium* spp. that produce trichothecene toxins. A number of *Fusarium* spp. produce trichothecenes, viz., *F. acuminatum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. graminearum* (*G. zeae*), *F. lateritium*, *F. poae*, *F. sambucinum* (*G. pulicaris*), *F. solani*, and *F. sporotrichioides* (Marasas *et al.,* 1984; El-Banna *et al.,* 1984; and Clark *et al.,* 1995). Trichothecene-producing *Fusarium* spp. are destructive pathogens and attack a wide range of plant species. The acute phytotoxicity of trichothecenes and their occurrence in plant tissues also suggest that these mycotoxins play a role in the pathogenesis of *Fusarium* on plants.

The trichothecenes (Fig. 2.14) constitute a family of more than sixty sesquiterpenoid metabolites produced by a number of fungal genera, including *Fusarium, Myrothecium*, *Phomopsis, Stachybotrys*, *Trichoderma*, *Trichothecium*, and others. The term trichothecene is derived from trichothecin, which was the one of the first members of the family identified. All trichothecenes contain a common 12,13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions.

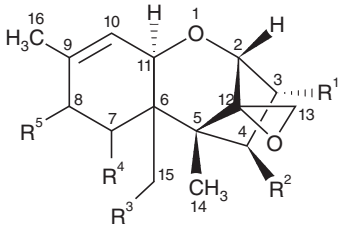

Fig. 2.14 Trichothecenes

Experiments with chemically pure trichothecenes at low dosage levels have reproduced many of the features observed in moldy-grain toxicoses in animals, including anemia, immunosuppression, hemorrhage, emesis, and feed refusal. Historical and epidemiological data from human populations indicate an association between certain disease epidemics and consumption of grain infected with *Fusarium* spp. that produce trichothecenes. In particular, outbreaks of a fatal disease known as alimentary toxic aleukia, which has occurred in Russia since the nineteenth century, have been associated with consumption of overwintered grains contaminated with *Fusarium* spp. that produce the trichothecene T-2 toxin (Anne E. Desjardins and Thomas M. Hohn, 1997). In Japan, outbreaks of a similar disease called akakabibyo or red mold disease have been associated with grain infected with *Fusarium* spp. that produce the trichothecene deoxynivalenol and related compounds.

There is more direct evidence that trichothecenes were responsible for recent human disease outbreaks in India and Japan, where trichothecenes were detected in the toxic grain samples themselves (Beardall and Miller, 1994). In addition, symptoms produced by the trichothecene diacetoxyscirpenol in clinical trials conducted with terminally ill

cancer patients were similar to reported symptoms of alimentary toxic aleukia and akakabibyo (Anonymous, 1983). Trichothecenes constitute a large family of sesquiterpene epoxides that inhibit eukaryotic protein synthesis. The biosynthesis of trichothecenes by *Fusarium* spp. proceeds from the hydrocarbon trichodiene through a complex series of steps to trichothecenes such as diacetoxyscirpenol, deoxynivalenol, and T-2 toxin.

2.8.3.4 Fumonisins

Fumonisins are amino polyalcohols and are structurally similar to the long-chain base backbones of sphingolipids. Fumonisins inhibit the activity of sphingosine N-acetyltransferase, which leads to the accumulation of toxic sphingoid bases (Fig. 2.15). The toxicity of maize contaminated by *F. moniliforme* has been well documented for more than 100 years. A disease of farm animals known as moldy corn poisoning or blind staggers was first described in the USA in 1850. The most dramatic manifestation of moldy corn disease is leucoencephalomalacia, a fatal brain disease of horses, donkeys, mules and rabbits. In 1988, a South African research group reported the isolation of fumonisin B1 from cultures of *F. moniliforme* (sexual stage, *G. fujikuroi* mating population A) (Anne E. Desjardins and Thomas M. Hohn, 1997). The structural similarity of fumonisins to the long chain sphingolipid bases suggests that fumonisin biosynthesis may be similar to sphingolipid biosynthesis. The latter begins with the condensation, catalyzed by serine palmitoyltransferase, of an amino acid with a fatty acyl-CoA. If fumonisin B1 is synthesized in a similar manner, then alanine would replace serine and an 18-carbon fatty acyl-CoA would replace palmitoyl-CoA. Isotope-feeding studies determined that alanine is a biosynthetic precursor of fumonisin B1 and that the polyalcohol moiety is derived from acetate (Blackwell *et al.*, 1996).

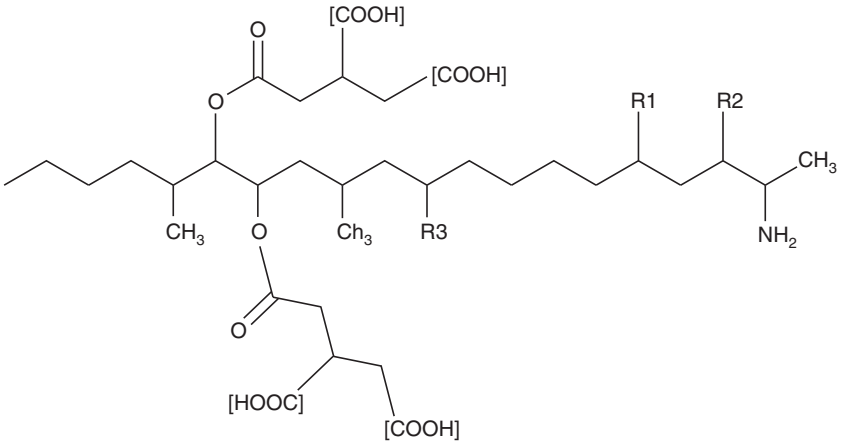

Fig. 2.15 Structural formula of fumonisin B_1-B_4: Fumonisin B1: R1= OH; R2= OH; R3= OH; Fumonisin B2: R1= H; R2= OH; R3= OH; Fumonisin B3: R1= OH; R2= OH; R3= H; Fumonisin B4: R1= H; R2= OH; R3= H

Fumonisins are produced by several members of the *G. fujikuroi* species complex, including serious pathogens of maize, sorghum, millet, and rice, but the most consistent and important producer of fumonisins is *G. fujikuroi* mating population A [*Example*: *Fusarium verticillioides* (formerly *Fusarium moniliforme* = *Gibberella fujikuroi*), *Fusarium proliferatum,* and *Fusarium nygamai,* as well as *Alternaria alternata* f. sp. *Lycopersici].*

The high frequency (>95%) of fumonisin production among strains of *G. fujikuroi* mating population A from maize and the high frequency of fumonisin contamination in maize raise the possibility that fumonisins play a role in the pathogenesis of maize. Further indirect evidence is provided by the structural similarity of fumonisins to AAL-toxin, which plays a role in the pathogenesis of *Alternaria alternata* f. sp. *lycopersici* on certain genotypes of tomato (Winter *et al.,* 1996). In addition, pure fumonisins at low concentrations have been shown to cause necrosis and other symptoms in maize seedlings, tomato seedlings, and other plants (Lamprecht *et al.,* 1994). Fumonisins are acutely toxic to the liver and kidney of a wide range of experimental animals. Consumption of feed contaminated with fumonisins or an intravenous injection of pure fumonisin B1 can produce a fatal lung edema in pigs. Although the role of fumonisins in some moldy corn diseases of livestock has now been well established, their role in human diseases and, most particularly, their carcinogenic potential in humans are much more difficult to determine.

2.8.4 Other Mycotoxins

A number of other mycotoxins warrant closer scrutiny with respect to their role in plant pathogenesis. These include a diverse array of metabolites produced by *Fusarium*, *Aspergillus,* and *Penicillium* spp. (Anne E. Desjardins and Thomas M. Hohn, 1997).

2.8.4.1 Fusarium mycotoxins

Mycotoxins produced by *Fusarium* spp. are of two general types, the nonestrogenic trichothecenes, including DON, nivalenol, T-2 toxin, and diacetoxyscripenol, and the mycoestrogens, including Zearalenone (ZEN) and zearalenol.

Zearalenone and zearalenol are both estrogenic resorcylic acid lactone compounds produced by the fungi *Fusarium* spp. (Diekman and Green, 1992). Zearalenone {(6-[10-hydroxy-6-oxo-*trans*1-undecenyl]-B-resorcyclic acid lactone) (Fig. 2.16)}, a secondary metabolite from *Fusarium graminearum* (teleomorph *Gibberella zeae*) was given the trivial name zearalenone as a combination of *G. zeae*, resorcylic acid lactone, -ene (for the presence of the C-1′ to C-2 double bond), and -one, for the C-6′ ketone. Zearalenone is better classified as a nonsteroidal estrogen or mycoestrogen. Sometimes it is called a phytoestrogen. Zearalenones are the strongly estrogenic polyketides produced by *F. graminearum* and related species, viz., *F. culmorum*, *F. equiseti* and *F. crookwellense*.

Consumption of feeds contaminated with zearalenones causes severe reproductive and fertility problems in animals (Marasas *et al.*, 1984). The enniatins and beauvericins constitute a family of cyclic depsipeptides that are produced by many *Fusarium* spp. and demonstrate toxicity to both vertebrates and plants. Despite their structural

dissimilarity to the steriodal estrogens, ZEN and several of its derivatives possess estrogenic activity. ZEN undergoes a folding such that hydroxyl or potential hydroxyl groups become appropriately orientated to facilitate binding to tissue receptors that normally bind estrogens. Similar binding affinities for ZEN have been determined for the estrogen receptor in sheep and calf uterus (Diekman and Green, 1992).

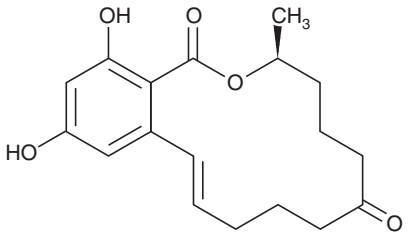

Fig. 2.16 Zearalenone

2.8.4.2 Moniliformin

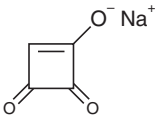

Fig. 2.17 Moniliformin

This is an unusual cyclobutane derivative with phytotoxicity and mycotoxicity, especially to avian species and has been reported to inhibit mitochondrial oxidative enzymes (Leslie *et al.*, 1996). Strains of *G. fujikuroi* associated with the Bakanae disease of rice produce particularly high levels of moniliformin [(Marasas *et al.*, 1986) (Fig. 2.17)]. Moniliformin is formed in many cereal types by a number of *Fusarium* species that include *F. moniliforme*, *F. avenaceum*, *F. subglutinans*, *F. proliferatum*, *F. fujikuroi* and others. Four new moniliformin-producing species of *Fusarium* were reported by Rabie *et al.* (1982), viz., *F. acuminatum, F. concolor, F. equiseti,* and *F. semitectum*. Isolates of *F. acuminatum and F. concolor* produced large amounts of moniliformin (3.4 and 9.5 g/kg, respectively), whereas isolates of the other three species yielded less than 30 mg/kg. Moniliformin is mainly cardiotoxic and causes ventricular hypertrophy. Moniliformin actually causes competitive inhibition of the activity of pyruvate dehydrogenase enzyme complex of respiratory reaction, which prevents pyruvic acid, a product of glycolysis, to convert to acetyl CoA. Moniliformin has been found in nature in cereal grains together with other toxins such as deoxynivalenol (DON), zearalenone (ZEA) and fusarin C. Moniliformin is produced by approximately 50% of the isolates tested, and its presence might mask unknown toxins. This study indicates that moniliformin found in the culture at a concentration of 1,000 ppm (1,000 μg/g) or higher and when incorporated into a rat diet, most likely accounts for the toxicity observed (Hamed K. Abbas *et al.*, 1990).

2.8.4.3 Patulin, Ochratoxins, and Citrinin

These mycotoxins are produced by *Aspergillus, Penicillium,* and *Byssochlamys.* Patulin is a secondary metabolite synthesized by a number of anamorphic fungal genera. Patulin is a cyclic tetraketide with phytotoxic activity [(McKinley and Carlton 1991) (Fig. 2.18)]. It was originally considered an antibiotic but now recognized as a mycotoxin displaying

and soy sauce (*Aspergillus oryzae*). Citrinin is associated with yellow rice disease in Japan and acts as a nephrotoxin in all animal species tested. Although it is associated with many human foods (wheat, rice, corn, barley, oats, rye, and food colored with Monascus pigment), its full significance for human health is unknown. Citrinin can also act synergistically with Ochratoxin-A to depress RNA synthesis in murine kidneys.

Citrinin (Fig. 2.20) was first isolated from *Penicillium citrinum* prior to World War II; subsequently, it was identified in over a dozen species of *Penicillium* and several species of *Aspergillus* (e.g., *Aspergillus terreus* and *Aspergillus niveus*), including certain strains of *Penicillium camemberti* (used to produce cheese) and *Aspergillus oryzae* (used to produce sake, miso, and soy sauce). More recently, citrinin has also been isolated from *Monascus ruber* and *Monascus purpureus,* industrial species used to produce red pigments. Citrinin has been associated with yellow rice disease in Japan. It has also been implicated as a contributor to porcine nephropathy. Citrinin acts as a nephrotoxin in all animal species tested, but its acute toxicity varies in different species. The 50% lethal dose for ducks is 57 mg/kg; for chickens it is 95 mg/kg, and for rabbits it is 134 mg/kg. Citrinin can act synergistically with ochratoxin A to depress RNA synthesis in murine kidneys. Wheat, oats, rye, corn, barley, and rice have all been reported to contain citrinin. With immunoassays, citrinin was detected in certain vegetarian foods colored with *Monascus* pigments. Citrinin has also been found in naturally fermented sausages from Italy.

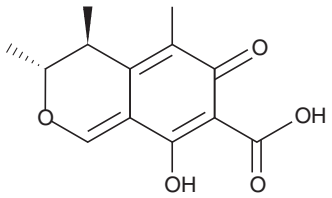

Fig. 2.20 Citrinin

Paenibacillus polymyxa, a Gram-positive low-G+C spore-forming soil bacterium, belongs to the plant growth-promoting rhizobacteria. The swarming motility of *P. polymyxa* strain E681 was greatly induced by a secondary metabolite, citrinin, produced by *Penicillium citrinum* KCTC6549 in a dose-dependent manner at concentrations of 2.5–15.0 μg mL^{-1} on tryptic soy agar plates containing 1.0% (w/v) agar (Soo-Young Park *et al*., 2008). Flagellum staining showed that citrinin activated the production of flagella by *P. polymyxa*. This result was supported by reverse transcriptase-PCR analysis of gene expression, which showed increased transcriptional levels of *sigD* and *hag* homologs of *P. polymyxa* E681 in the presence of citrinin. The results presented here show that a mycotoxin, citrinin, has a newly identified function of inducing bacterial motility by transcriptional activation of related genes. This finding contributes to our understanding of the interactions between bacteria and fungal strains in nature.

2.8.4.4 Nonspecific/Nonselective toxins

These are the metabolic products of the pathogen, but do not have host specificity and affect the protoplasm of many unrelated plant species that are normally not infected by

the pathogen. [*Example*: Ten-toxin, Tab-toxin, Fusaric acid, Piricularin, Lycomarasmin and Alternaric acid].

2.8.5 Effect of Toxins on Host Tissues

2.8.5.1 Changes in cell permeability

Toxins kill plant cells by altering the permeability of plasma membrane, thus permitting loss of water and electrolytes and also the unrestricted entry of substances including toxins. The cellular transport system, especially, H^+/K^+ exchange at the cell membrane is affected.

2.8.5.2 Disruption of normal metabolic processes

The major processes include increase in respiration due to disturbed salt balance, malfunctioning of enzyme system (Example: Pyricularin inhibits polyphenol oxidase) and uncoupling of oxidative phosphorylation).

2.8.5.3 Interfere with the growth regulatory system of host plant

Example: Restricted development of roots induced by *Fusarium moniliforme*.

2.8.5.3.1 Tentoxin

Tentoxin is a natural cyclic tetrapeptide (Fig. 2.21) produced by phytopathogenic *Alternaria alternata*. It selectively induces chlorosis in several germinating seedling plants. Therefore, tentoxin may be used as a potential natural herbicide. Tentoxin was first isolated from *Alternaria alternata* (syn. *tenuis*) and characterized by George Templeton *et al.* in 1967. Tentoxin has also been used in recent research to eliminate the polyphenol oxidase activity from seedlings of higher plants (Duke and Vaughn, 1982.). At a concentration of 2 ppm, the toxin is effective. Acid hydrolysis of the toxin produces leucine, glycine, and N-methylalanine. It causes a reduction in chlorophyll content, inhibits cyclic photophosphorylation and stomatal closure in sensitive plants.

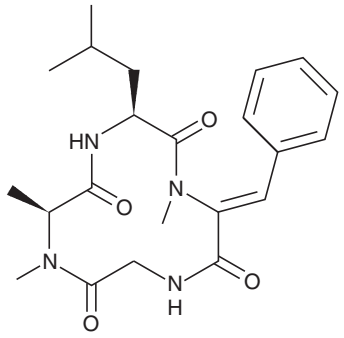

Fig. 2.21 Tentoxin

The disease induced by this pathogen is primarily a seedling disease in a wide range of plant species. Seedling death results when greater than one-third of the leaf area become chlorotic, and reduce vigor with less than that amount of leaf chlorosis. The toxin is a cyclic tripeptide that binds to and inactivates a chloroplast-coupling factor protein involved in energy transfer and also the inhibition of light-dependent phosphorylation of ADP to form ATP.

Tentoxin inactivated photophosphorylation and coupling factor 1 (CF_1) ATPase in lettuce, a sensitive species (John A. Steele *et al.*, 1976). This effect was due to binding of tentoxin with CF_1 at a single site (affinity constant 1.3 to 20 × 10^7 M^{-1}). Neither AMP nor adenyl-5′-yl imidodiphosphate appeared to bind to this site. In radish, an insensitive species, 20 times more tentoxin was required for 50% inhibition of photophosphorylation. In this species CF_1ATPase was unaffected by tentoxin, and its CF_1 bound tentoxin only weakly (affinity constant less than 1 × 10^4 M^{-1}). The sensitivity of photophosphorylation to tentoxin was correlated with chlorosis sensitivity in six other species examined.

Tentoxin-treated mung bean plants were shown to lack chloroplast polyphenol oxidase (PPO) by enzymatic, electrophoretic and cytochemical analysis. Incorporation of PPO (a protein coded by nuclear DNA into the plastid may occur via concentration of the protein into inner envelope-derived vesicles (Kevin C. Vaughn and Stephen O. Duke., 1981). PPO integration into the plastid is apparently blocked by a tentoxin treatment although fraction I protein (and hence the proteins for chloroplast ribosome production) is not affected by this fungal toxin. Both apical and etiolated plastids from teotoxin-treated plants lack PPO. Thus, it is unlikely that the primary effect of tentoxin is due to the binding of the chloroplast coupling factor, as previously supposed.

Holland *et al.* (1997) clarified the mechanism of tentoxin-induced chlorosis in *Nicotiana* spp. seedlings, who observed that chlorosis did not correlate with the inhibition of chloroplast ATP synthesis *in vivo,* since it occurs at tentoxin concentrations far higher than that required for the inhibition of photophosphorylation measured in the same seedlings. However, tentoxin-induced chlorosis did correlate with *in vivo* overenergization of thylakoids. They demonstrated that tentoxin induced overenergization in intact plants and isolated thylakoids, probably via multiple interactions with ATP synthase. Furthermore, gramicidin D, a protonophore that relieves overenergization, also relieved chlorosis. Two lines of evidence suggested that reactive oxygen species may be involved in the process of chlorosis: ascorbate, a quencher of oxygen radicals, significantly protects against chlorosis, whereas transgenic *Nicotiana* spp. mutants overexpressing chloroplast superoxide dismutase were partially resistant to tentoxin-induced chlorosis. It was proposed that chlorosis in developing seedlings resulted from overenergization of thylakoids, which lead to the generation of oxygen radicals.

Tentoxin and to a lesser extent, dihydrotentoxin (both at 10 mmol m^{-3}) reduced stomatal opening in epidermal strips of *Commelina communis* in the light but not in darkness (Dahse *et al.*, 1990). This effect was significantly greater in the normal air than in CO_2-free-air. Fusicoccin overcame the tentoxin effect. However, tentoxin did not inhibit stomatal opening in the light in epidermal strips of *Paphiopedilum harrisianum*, a species which lacks guard cell chloroplasts. It is concluded that tentoxin exerts its action on

stomata not by an ionophorous effect in the plasmalemma of guard cells but by the inhibition of photophosphorylation in their chloroplasts. The effects of DCMU and tentoxin on guard cells are discussed in terms of their effects on chloroplasts and the extent to which energy is supplied from this organelle during the stomatal opening in the light. The results indicate that neither photophosphorylation nor noncyclic electron transport in guard cell chloroplasts is essential for stomatal opening.

2.8.5.3.2 Tabtoxin

Tabtoxin is strongly antimicrobial and functions by inhibiting glutamine synthetase and ornithine carbamoyltransferase, respectively. Genetic analysis has revealed the mechanisms responsible for toxin biosynthesis. Tabtoxin is derived from the lysine biosynthetic pathway, whereas syringomycin, syringopeptin, and phaseolotoxin biosynthesis require peptide synthetases. Activation of phytotoxin synthesis is controlled by diverse environmental factors including plant signal molecules and temperature.

Tabtoxin is a monocyclic β-lactam produced by *P. syringae* pv. *tabaci*, *coronafaciens*, and *garcae* [(Mitchell, 1991) (Fig. 2.22)]. The dipeptide toxin contains tabtoxinine-β-lactam (TβL) linked by a peptide bond to threonine. Although tabtoxin is the primary intracellular metabolite produced, the chlorosis-inducing activity occurs only after hydrolysis of the peptide bond by aminopeptidases of plant or bacterial origin. Cleavage of the peptide bond in tabtoxin releases TβL, the toxic moiety (Levi and Durbin 1986).

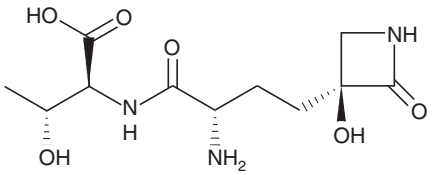

Fig. 2.22 Tabtoxin

TβL irreversibly inhibits glutamine synthetase. The inhibition of glutamine synthetase has at least two major effects; first, the enzyme is presumably the major route for glutamine synthesis in plants, and second, the enzyme is the only way to efficiently detoxify ammonia. A variety of harmful effects has been attributed to ammonia in plants, including disruption of the thylakoid membrane of the chloroplast and uncoupling of photophosphorylation. Protection of the bacteria from the toxin has been associated with the adenylation of glutamine synthetase, which renders the target enzyme less susceptible to inactivation by TβL (Knight *et al.*, 1986). A second potential detoxification mechanism involves the production of β-lactamases which hydrolyze the β-lactam ring of TβL to liberate the nontoxic metabolite, tabtoxinine.

Pseudomonas syringae pv. *coronafaciens*, a pathogen of oats, was mutagenized with Tn5 to generate mutants defective in tabtoxin production (Barta *et al.*, 1992). From a screen of 3,400 kanamycin-resistant transconjugants, seven independent mutants that do not produce tabtoxin (Tox-) were isolated. Although the Tn5 insertions within these seven mutants were linked, they were not located in the previously described tabtoxin

Fusaric acid is a mycotoxin with low to moderate toxicity, which is of concern since it might be synergistic with other co-occurring mycotoxins. Fusaric acid is widespread on corn and corn-based food and feeds and is frequently found in grain, where *Fusarium* spp. are also isolated. Fusaric acid (5-butylpicolinic acid) was first discovered during the laboratory culture of *Fusarium heterosporum* Nees by Yabuta *et al*. (Yan *et al*., 1937). This compound was one of the first fungal metabolites implicated in the pathogenesis of tomato wilt symptoms caused by *F. oxysporum* f. sp. *lycopersici* Schlecht. Emend. Snyd & Hans (Bacon *et al*., 1996). In addition to the suggested role in plant pathogenesis, fusaric acid is potentially toxic to animals. Fusaric acid is mildly toxic to mice, and it has several important. *Fusarium moniliforme* Sheld (sexual stage *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura) of the section *Liseola* is associated with corn as a nonobligate and usually symptomless endophyte. The endophytic association of corn with this fungus is a cause for concern since several mycotoxins may be produced during its symptomless endophytic colonization. *F. moniliforme* produces fumonisins, fusarins, moniliformin, and beauvericin.

Fusaric acid is known to enhance the toxicity of other mycotoxins in terms of both animal and plant toxicities. This result could be due to the presence of numerous *Fusarium* spp. and strains within the contaminated feed samples or to the fact that with multiple mycotoxins being produced any analysis that focuses on only a single toxin is unlikely to show a strong correlation between toxicity and the amount of any single mycotoxin. Reports of multiple mycotoxins being present in some toxic samples are not uncommon, and work of this type should be encouraged, especially in light of the widespread ability of strains of numerous *Fusarium* species to synthesize fusaric acid (Bacon *et al*., 1996).

Fusaric acid (5-butylpicolinic acid) was first discovered during the laboratory culture of *Fusarium heterosporum* and was one of the first fungal metabolites implicated in the pathogenesis of wilt symptoms of plants especially under adverse conditions (Bacon Charles *et al*., 2006). In addition to a primary role in plant pathogenesis, fusaric acid is mildly toxic to mice and has other pharmacological properties. During tests for control of the fungus *F. verticillioides* and reduction of the mycotoxin fumonisin B1, it was determined that fusaric acid was produced in planta and appears to control the growth of the biocontrol endophytic bacterium, *Bacillus mojavensis*, without any apparent symptoms of a disease. Since fusaric acid is considered a wilt toxin, we examined it in planta production and role in the wilt of field maize. Using plants infected with fusaric acid producing and nonproducing strains of *F. verticillioides*, they isolated, identified, and measured fusaric acid in roots of seedlings grown with and without drought stress. It was determined that fusaric acid was produced in planta at the same concentrations regardless of drought stress, and there were no symptoms of wilt disease in the one field maize cultivar tested. Perhaps its major importance was like an antibiotic against *B. mojavensis*, other biocontrol species and endophytic competing bacteria that co-occur with *Fusarium* species and in the soil, suggesting that fusaric acid does not function solely as a wilt toxin in maize.

Suppression of soil-borne diseases by biocontrol agents involves complex interactions among biocontrol agents and the pathogen and between these microorganisms and the plant (Landa *et al*., 2002). They studied the diversity among strains of fluorescent

Pseudomonas spp., *Bacillus* spp., and *Paenibacillus* sp. for their sensitivity to fusaric acid (FAc) and phytoanticipins from different host plants, the diversity of pathogenic and nonpathogenic *Fusarium oxysporum* isolates for their sensitivity to phytoanticipins, and the influence of FAc on the production of pyoverdine by fluorescent *Pseudomonas* spp. tolerant to this compound. There was a great diversity in the response of the bacterial strains to FAc; however, as a group, *Bacillus* spp. and Paenibacillus macerans were much more sensitive to FAc than *Pseudomonas* spp. FAc also affected the production of pyoverdine by FAc-tolerant *Pseudomonas* spp. strains. Phytoanticipins differed in their effects on microbial growth, and sensitivity to a phytoanticipin varied among bacterial and fungal strains. Biochanin A did not affect the growth of bacteria, but coumarin inhibited the growth of *Pseudomonas* spp. strains and had no effect on *Bacillus circulans* and *P. macerans.* Conversely, tomatine inhibited the growth of *B. circulans* and *P. macerans*. Biochanin A and tomatine inhibited the growth of three pathogenic isolates of *F. oxysporum* but increased the growth of three nonpathogenic *F. oxysporum* isolates. Coumarin inhibited the growth of all pathogenic and nonpathogenic *F. oxysporum* isolates. These results indicated that complex interactions can occur among plants, pathogens and biological control agents in the rhizosphere and on the root surface. Also, these results helped to explain the low efficacy of some combinations of biocontrol agents, as well as the inconsistency in achieving disease suppression under field conditions.

2.8.5.3.4 Piricularin

Piricularin, one of the toxic substances from *Pyricularia oryzae,* produces brown necrotic spots and inhibits seedling growth (Tamari and Kaji, 1959). It exists in two froms, pyricularin and α-picolinic acid (Fig. 2.24). At low concentrations, it inhibits the germination of the conidia of *P. oryzae*, which produces a piricularin binding protein, a copper oxidase that binds [iricularin and destroys its fungi-toxicity but not its phytotoxicity. This toxin induces an increase in the concentrations of polyphenols and oxidases and its toxicity is counteracted by Chlorogenic acid, one of the principal polyphenols of the rice plant (Mehrothra, 1981). A novel phytotoxin, tenuazonic acid, has also been reported in rice plants. Piricularin increases respiration and growth at low doses but inhibits at higher doses. Aspargine was the best nitrogen source for the *in vitro* production of toxins by the pathogen followed by urea, ammonium sulphate and sodium nitrate.

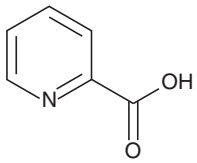

Fig. 2.24 α-picolinic acid

Piricularin plays an important role in the blast-fungal infection together with picolinic acid suppressing the resistant reaction, i.e., the hypersensitive reaction, of the host tissue to the infection (Kinjiro TAMARI *et al.*, 1966). To determine whether or not piricularin takes part in the blast fungal infection, they investigated the effect of a piricularin-

these wilt toxins are only produced in still cultures. The minimum dose of this toxin needed to induce injury to plants like tomato shoot is 150 mg/kg fresh weight. The structure of lycomarasmin (Figure 2.25) indicates that at least some parts of the molecule could well be derived from simple amino acids or their deaminated keto analogs. A subdivision of the molecule into its apparent structural units gives rise to a series of well-known metabolic intermediates all of which could conceivably serve as immediate biogenetic precursors. Thus, acid hydrolysis of lycomarasmin gives rise to aspartic acid, pyruvic acid, and glycine (I), some or all of which might serve as building units.

This toxin yields glycine and aspartic acid on hydrolysis and has been shown to be a derivative of asparagine. Thus, the third N atom, which had previously not been assigned position, was located. Because of the reactions which lycomarasmin underwent, a structural formula for it was proposed which contained the new amino acid, alpha-hydroxyalanine. This was attached to a common N atom (i.e., the amino group) to the amino group of glycylasparagine. A synthetic product was isolated from the reaction of ethyl-alpha-acetoxy-alpha-bromopropionate and the methyl ester of glycylasparagine, which had properties in common with lycomarasmin, including quantitatively the same biological activity. Some reasons were advanced for regarding lycomarasmin as a much-simplified model of proteins.

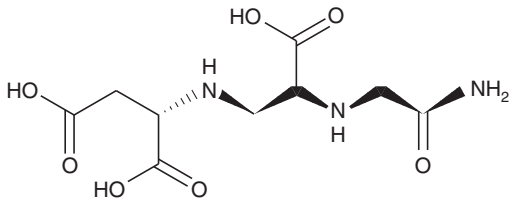

Fig. 2.25 Lycomarasmin

A number of new derivatives of aspartic acid and asparagine have been prepared to examine the effects of various structural changes on the activity of lycomarasmin, which is a peptide derived from asparagine, glycine, and probably a-hydroxyalanine (Woolley, 1948). It has been shown that quantitative change in the constitution of Richard's medium causes a corresponding change in the quantity of toxins produced by the various strains of *F. lycopersici*. Thus, the virulent strain, producing lesser amounts of toxins (used in the widest sense of the word) in Richard's medium, produces more than the avirulent strain when the quantities of carbon and nitrogen are varied in the medium. With glycine as the exclusive carbon source in the medium, *F. lycopersici* produces all the three known toxins (vasinfuscarin, fusaric acid and lycomarasmin).

Thus, glycylserylaspartic acid, glycyl-P-aspartylserine, acetyllactylglycylasparagine, cY, a-diacetaminopro-pionylglycylasparagine, the corresponding aspartic acid compound, 01-hydroxy-cu-acetaminopropionylglycylaspartic acid, and pyruvylglycylaspartic acid were synthesized. These, along with known compounds such as glycylaspartic acid, glycylasparagine, aspartic acid, asparagine, glycine, serine, pyruvylglycine, serylglycylaspartic acid, serylglycylglutamic acid, and glutathione, were compared with lycomarasmin for the ability to cause excised tomato leaves to wilt and curl. Several of

the peptides were somewhat less active in this respect than was lycomarasmin, but one of them, ar-hydroxy-cr-acetaminopropionylglycylaspartic acid, was about equal to the natural toxin in activity. Some produced no detectable effect. A comparison was made of the relative effect on the activity of changing the position of an amino acid in a peptide with that of altering the nature of the amino acid.

2.8.5.3.6 Alternaric acid

Alternaric acid, a metabolic product of some strains of *Alternaria solani*, is highly phytotoxic. Alternaric acid is an antifungal and natural product, which bears a substituted glycolic acid in the functionally and stereochemically dense core of the molecule (Fig. 2.26). It is optically inactive, unsaturated dibasic acid and it inhibits the growth of the fungal germ tubes without actually preventing germination.

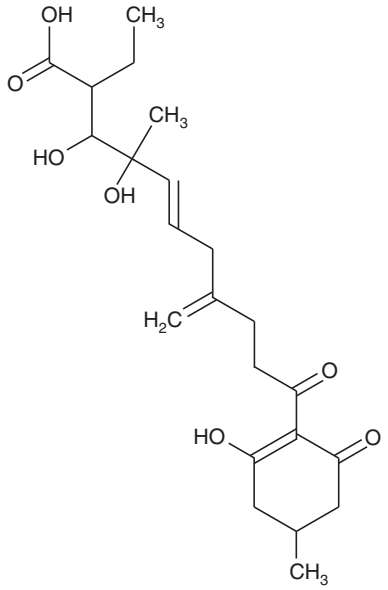

Fig. 2.26 Alternaric acid

In 1960, the planar structure of alternaric acid (Bartels-Keith, 1960), a phytotoxin isolated from *Alternaria solani,* causal fungus in the early blight disease of potato and tomato, was determined by using classical chemical methods. However, the total stereostructure of 1 remained to be elucidated. *A. solani* also produces several secondary metabolites, and some of them were isolated in our laboratory. After that, alternaric acid was shown to contribute to the disease development of the host by *A. solani* in a manner similar to the mode of action of the group of compounds classified as host-specific toxins, although all of the requirements as a primary disease determinant were not fulfilled.

If introduced into the vascular system of cut shoots of tomato or potato, this toxin travels upwards and causes necrotic lesions of the stem, petioles, and leaf blades, very

similar in appearance to the lesions appearing in some phases of natural attack by *A. solani.* Alternaric acid also produces similar lesions on plants outside the host range of *A. solani.* Associated with the production of visible lesions is a disturbance of the water-balance of shoots. Transpiration is increased and, in spite of a simultaneous but smaller increase in water uptake; this eventually leads to complete desiccation of the shoots (Brian *et al.*, 2008). A strain of *A. solani* known to produce alternaric acid in synthetic culture media produces a substance with similar biological properties when inoculated into tomato fruits. Since other workers have shown that some of the symptoms of *A. solani* attack are toxigenically induced it is tempting to suggest that alternaric acid is the toxin concerned. On the other hand, there is no correlation between virulence of strains of *A. solani* and their ability to produce alternaric acid in synthetic culture media or tomato fruits. Some highly virulent strains produce little or no alternaric acid under any conditions tested. There is some evidence that other toxins besides alternaric acid are produced by *A. solani*, not necessarily in the same proportions by all strains.

2.8.5.3.7 Phaseolotoxin

This is the toxin involved in one of the bacterial bean blights called 'halo blight.' Symptoms of the disease incited by the bacterium can be produced by the toxin alone. Within cells, the toxin is enzymatically cleaved releasing phosphosulfinylornithine which is the toxic moiety. Cellular effects are a result of the inactivation of the enzyme ornithine carbamoyltransferase.

Phaseolotoxin is a modified tripeptide [Nδ-(N'-sulfodiaminophosphinyl)-ornithyl-alanyl-homoarginine] (Fig. 2.27) produced by certain strains of *Pseudomonas syringae* pv. *phaseolicola*, *Pseudomonas syringae* pv. *actinidiae* and strain *Pseudomonas syringae* pv. *syringae* CFBP 3388 (Bender *et al.*, 1999) Phaseolotoxin is a reversible inhibitor of the enzyme ornithine carbamoyltransferase, which catalyzes the formation of citrulline from ornithine and carbamoylphosphate in the arginine biosynthetic pathway. Phaseolotoxin is an effective inhibitor of OCTase activity from plant, mammalian, and bacterial sources and causes a phenotypic requirement for arginine. Additionally, phaseolotoxin inhibits the enzyme ornithine decarboxylase (EC 4.1.1.17), which is involved in the biosynthesis of

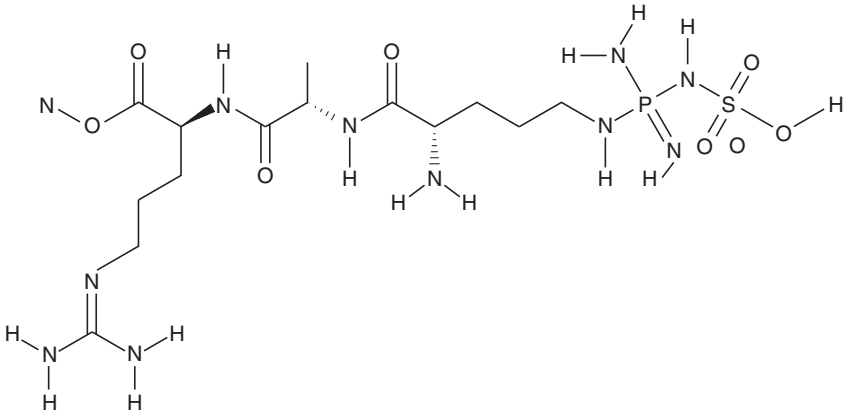

Fig. 2.27 Phaseolotoxin

polyamines. Phaseolotoxin is an effective inhibitor of OCTase activity from plant, mammalian, and bacterial sources and causes a phenotypic requirement for arginine.

Tourte and Manceau (1995) isolated a bacterial strain, CFBP 3388 from Vetch (*Vicia sativa* L.) which was identified as *P. s.* pv. *syringae* on the basis of nutritional and biochemical patterns which were obtained with classical tests and the Biolog™ system. It caused necrotic symptoms typical of *P. s.* pv. *syringae* on bean leaves and pods after artificial inoculation. However, the isolate caused a citrulline-reversible inhibition of *E. coli* in phaseolotoxin bioassay. Furthermore, with CFBP 3388 DNA as template a 1900 bp DNA fragment, specific for the phaseolotoxin DNA cluster of *P. s.* pv. *phaseolicola* was amplified by PCR. This is the first demonstration that an isolate of *P. syringae* that is not pv. *phaseolicola* can produce phaseolotoxin.

2.8.6 Overall Role of Pathogen Toxins in Plant Pathogenesis

The interaction between plant pathogens and their hosts is extremely complex. There are many factors affecting the plant disease development, plant, and pathogen physiology, metabolism of the host plant, physical environment and the way it impacts the plant growth and development, biochemistry and genetics of the pathogen-plant interactions (Slavov, 2005). An area of special interest in the specificity of these interactions is the role of pathogen toxins in the disease development. Toxins are compounds that are produced by the pathogens and cause part or all of the symptoms of a disease. They are of various chemical types and include peptides, glycoproteins, polysaccharides, organic acids, fatty acids and derivatives, polyketides and terpenoids (Turner, 1984). The best-known mycotoxins are coming from the fungi of genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Claviceps*, *Stachybotrys*, *Myrothecium*, *Phoma,* and *Diplodia*. There are also toxin-producers among bacteria, like those from genera *Xanthomonas*, *Pseudomonas,* and *Clavibacter (*Švàbova and Lebeda, 2005). There are large differences between the toxin-producing pathogens and mode of plant-pathogen interactions. The role of a toxin as a disease determinant is proved by the occurrence of the toxin in an infected plant and the ability of the toxin alone to elicit at least part of the symptoms of the disease (Hamid and Strange, 2000).

The major visible symptoms caused by the toxins are chlorosis, necrosis, and wilting. Some of the toxins have general phytotoxic properties and are active toward a broad range of plant species. Those are nonhost-specific toxins. They contribute to the virulence or symptom development in the disease in which they occur, but are not primary determinants of host range. Many non-host-specific toxins as brefeldin A, tenuasonic acid, tentoxin, and zinniol are produced from several *Alternaria* species. They exert their phytotoxic activities through different modes (Thomma, 2003). Other toxins are host-specific ones and affect only certain plant varieties or genotypes (Osbourn, 2001). The host-specific toxins (HSTs) are playing a role in determining the host range of specificity of plant pathogens and can act as an agent of virulence of those pathogens. HSTs appear to be specific for individual plant species, and in some cases their effects are mediated by gene-for-gene interactions. Most of the HSTs exist as families of compounds, each number of which are produced in different amount and have a different potency. They

are acting in very low concentrations ranging from 10 μM to 1 μM. HSTs can be very diverse by chemical structure even when they are produced by pathogens belonging to one genus, as it is in species *Alternaria alternata*.

Different pathotypes of this plant pathogen produce different toxins known as AK-, AF-, ACR-, ACT-, ACTG-, AM-, and AAL-toxins, which differ in their structure and host range (Otani *et al.*, 1995). Despite the differences in chemical structure of those toxins, *Alternaria* pathotypes cause similar infection patterns. Species of genus *Cochiobolus* produce victorin, T-toxin, HC-toxin or HS toxin, which differ in the chemical structure. Some fungi that are not closely related can produce structurally very similar toxins as it is in the case of T-toxin of *Cochiobolus heterostrophus* race T and PM-toxin from *Phyllosticta maydis*. One pathogen, tangerine type of *Alternaria alternata* is known to produce more than one class of HSTs – ACT-, and ACTG-toxins (Kohmoto *et al.*, 1993). The role of toxins in infection and colonization of the host plant by the pathogen is of critical importance. It was proved in experiments with a nontoxic mutant of the pathogens as it was in the case of *Pseudomonas syringae* pv. *tabaci* (Turner and Taha, 1984.). The toxigenicity is partially or fully determinant of pathogenicity, for example of victorin and *Helmintosporium victoriae* (Scheffer, 1976.). This toxin determines the host range and parasitic ability of the pathogen.

2.8.6.1 Toxins produced by plant or plant X pathogen interaction–Amylovorin

Amylovorin is a high-molecular-weight polysaccharide, characterized as a host-specific toxin associated with *Erwinia amvlovora*-infected tissues. The common characteristic of infection of rosaceous plants by *Erwinia amylovora*, the fire blight pathogen, is the appearance of wilt, necrosis, and the production of copious amount of exudates, or 'ooze.' In the 1930s, experiments established that the ooze was bacterial in origin and responsible for the induction of wilt symptoms in pear shoot cuttings (Dwayne *et al.*, 2013). A majority of the field samples of *E. amylovora* examined by Billing were encapsulated by the ooze, with less than 1% having no capsule. The ooze was named polysaccharide amylovorin, to reflect its toxic effect on plant tissues. Amylovorin was subsequently renamed amylovoran to be consistent with polysaccharide nomenclature. In due course, the linkages among *E. amylovora*'s capsule, slime, polysaccharides, ooze, and pathogenicity were made. The growth of the bacterium on sucrose-, glucose-, or sorbitol-enriched media results in the production of excess quantities of two exopolysaccharides (EPSs), one acidic and one neutral. The acidic EPS, amylovoran, may form a capsule, slime, and/or float free in the liquid medium. Amylovoran is a heterogeneous polymer consisting of repeating units of one glucuronic acid and four galactose residues. The *ams* region of the genome controls the production of the EPS, with *rcsA* and *rcsB* genes being required for synthesis. Mutation in *rcsA* or *rcsB* results in reduced amylovoran production. The neutral EPS, levan, was synthesized in the presence of sucrose by the enzyme levansucrase, which cleaves the sugar and polymerizes fructose into a polyfructan (β-2,6-D-fructofuranan). Levan synthesis took place extracellularly through the action of the enzyme encoded by the *lsc* gene.

in the regulation of plant immune responses. In addition, other plant hormones, such as auxins, abscisic acid, cytokinins, gibberellins, and brassinosteroids, that have been thoroughly described to regulate plant development and growth, have recently emerged as key regulators of plant immunity (Nicolas Denance *et al.*, 2013). Plant hormones interact in complex networks to balance the response to developmental and environmental cues and thus limiting defense-associated fitness costs. The molecular mechanisms that govern these hormonal networks are largely unknown. Moreover, hormone signaling pathways are targeted by pathogens to disturb and evade plant defense responses.

The resistance response is regulated by phytohormones, which are small molecules which synergistically and/or antagonistically work in a complex network to regulate many aspects of plant growth, development, reproduction, and response to environmental cues (Jaillais and Chory, 2010.). Recent progress has been made in understanding the complex hormone network that governs plant immunity, giving rise to a database containing information on the hormone-regulated genes (e.g., in *Arabidopsis thaliana*) and the phenotypic description of hormone-related mutants. In parallel, it has been found that pathogens have developed sophisticated molecular mechanisms to deregulate the biosynthesis of hormones and/or to interfere with hormonal signaling pathways, thus, facilitating the overcoming of plant defense mechanisms. The essential roles of salicylic acid and ethylene/jasmonic acid mediated signaling pathways in resistance to pathogens are well described.

Hormones such as auxins and abscisic acid (ABA), originally described by their function in the regulation of plant growth processes and the response to abiotic stresses, have recently emerged as crucial players in plant–pathogen interactions. All the phytohormone pathways are linked to each other in a huge, complex and still obscure network. For example, ET, ABA, auxin, gibberellins, and cytokinins pathways are considered as hormone modulators of the SA–JA signaling backbone (Jaillais and Chory, 2010.).

Growth regulators are of two types, growth promoting substances and growth inhibiting substances. Auxins, gibberellins, and cytokinins are growth promoting substances, whereas, ethylene and abscisic acid are major growth inhibiting substances. The imbalance in growth promoting and growth inhibiting substances causes hypertrophy (excessive increase in cell size) and atrophy (decrease in cell size). Symptoms may appear as tumors, galls, knots, witches broom, stunting, excessive root branching, defoliation and suppression of bud growth. There is an increasing evidence that both the pathogen and the host have the capacity to synthesize various PGRs. Alterations in the levels of PGRs and disease susceptibility or resistance reaction are associated with plant-pathogen interaction (Singh *et al.*, 1997). Some investigations indicated that naphthalene acetic acid is a potential antifungal agent (Michniewicz and Rozej, 1988). Auxin strongly inhibited mycelial growth, sporulation, and spore germination of *Fusarium culmorum in vitro* NAA, indole acetic acid (IAA), 2,4-diphenol acetic acid (2,4,D) and abscisic acid (ABA) were exogenously applied to control *Alternaria solani* caused early blight of potato. Auxins such as IAA, naphthalene acetic acid ethyl ester and N-metatotyl phthalamic acid reduced *Botrytis* blight of cut rose flowers (Elad, 1995).

2.9.1 Growth Promoting Substances

2.9.1.1 Auxins

Auxins are a group of molecules including IAA (indole-3-acetic acid) that regulate many aspects of plant development, such as apical dominance, root gravitropism, root hair, lateral root, leaf, and flower formation, and plant vasculature development (Jaillais and Chory, 2010). It is continuously produced in young meristematic tissue and moves rapidly to older tissues. If auxin concentration is more, its concentration is reduced by the enzyme, IAA oxidase. Auxins are the major growth regulator in plants and are defined as growth regulators whose major mode of action is cell elongation; they resemble indole-3-acetic acid in their activity. The naturally occurring auxin is indoleacetic acid [(IAA) (Fig. 2.28)].

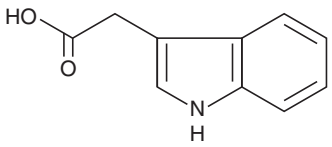

Fig. 2.28 Auxin (IAA)

Increased levels of auxin occur in plants infected with fungi, bacteria, viruses, mollicutes and nematodes. Increased IAA resulted in hypertrophy and decreased IAA results in atrophy. Increased IAA may be due to inhibition of IAA oxidase (*Example: Ralstonia solanacearum*, the causal agent of wilt of Solanaceous plants, induces a 100 fold increase in IAA level in diseased plants). Increased plasticity of cell walls as a result of high IAA levels renders the pectin, cellulose and protein components of the cell wall more accessible to pathogen degradation. The increase in IAA levels may also inhibit lignifications of tissues. Increased IAA levels have been reported in plants infected with the following pathogens: *Phytophthora infestans* (late blight of potato), *Ustilago maydis* (maize smut), *Plasmodiophora brassicae* (clubroot of crucifers), *Sclerospora graminicola* (downy mildew of sorghum), *Agrobacterium tumefaciens* (crown gall of apple), and *Meloidogyne* (root knot nematode).

Both direct and indirect effects of auxins on the regulation of pathogen resistance responses in plants have been described. Indirect effects may be caused by auxins regulation of development-associated processes, such as cell wall architecture, root morphology, and stomata pattern. For example, treatment of rice with IAA impaired the resistance to *Xanthomonas oryzae* pv. *Oryzae* probably as a consequence of the activation of the biosynthesis of cell wall-associated expansins that lead to cell wall loosening, which facilitates pathogen growth.

Many pathogenic microbes and plant growth promoting rhizobacteria have evolved complete pathways for auxin biosynthesis with tryptophan as the main precursor. Auxin-producing phytopathogenic bacteria are mostly, but not exclusively, gall-inducing microbes. They include, for instance, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Erwinia chrysanthemi*, *Erwinia herbicola*, *Pseudomonas fluorescens*, *P. putida*, *Pseudomonas savastanoi*, *P. syringae, R. solanacearum* and *Rhodococcus fascians*. In *R. solanacearum*, auxin

biosynthesis is governed by HrpG, a major regulator of bacterial virulence and response to metabolic signals. In *Agrobacterium tumefaciens*, two genes required for conversion of tryptophan to auxin are localized in the T-DNA region of the Ti plasmid injected into plant cells. Auxin biosynthesis is necessary for tumor gall formation and pathogenicity of *Agrobacterium.* Auxins negatively regulate the expression of genes necessary for the transfer of *Agrobacterium* T-DNA in plants and also inhibit the growth of several bacterial species *in vitro* (Nicolas Denance *et al*., 2013). Auxin biosynthesis in fungal pathogens seems to be limited to a few species. In *Ustilago maydis*, *U. esculenta*, and *U. scitaminea* auxin is produced. Additionally, other fungi have enzymatic tools to produce auxins, such as *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, *Colletotrichu macutatum*, and *F. proliferatum.*

Phytonematodes have developed the capacity to sense and respond to chemical signals of host origin, and the ability to orientate towards plant roots enhances the nematode's chance of survival. Root exudates contain a range of compounds which mediate belowground interactions with pathogenic and beneficial soil organisms. Chemical components of root exudates may deter one organism while attracting another and these compounds alter nematode behavior and can either attract nematodes to the roots or result in repellence, motility inhibition or even death (Curtis, 2008). *In vitro* plant signals present in root exudates, triggered a rapid alteration of the surface cuticle of *Meloidogyne incognita* and the same changes were also induced by indole-acetic acid (IAA). IAA bound to the chemosensory organs of *M. incognita*, and it was opined that IAA might act as a signal that orientates the nematode on the root surface in the rhizosphere and/or inside the root tissue and thereby promoted nematode infection.

Auxins can negatively impact plant defense by interfering with other hormone signaling pathways or with PTI. The bacterial PAMP flg22, a peptide from flagellin protein (Pel and Pieterse, 2013) induces an *Arabidopsis* microRNA (miR393), which negatively regulates the mRNA levels of auxins receptors TIR1 (transport inhibitor response 1), AFB2 (auxin signaling F-box 2), and AFB3. Thus, the flg22-triggered suppression of auxin signaling leads to increased resistance to the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) and also to the oomycete *Hyaloperonospora arabidopsidis.* The flg22-induced resistance to these biotrophic pathogens was explained by the observed induction of the SA signaling pathway. Supporting this hypothesis, it was found independently that treatment of *Arabidopsis* leaves with flg22 induces salicylic acid accumulation (Jaillais and Chory, 2010).

The conjugated auxin–aspartic acid (IAA–Asp) has been recently reported to play a key role in regulating resistance to the necrotrophic fungus *Botrytis cinerea* and *Pst*DC3000. In *Arabidopsis*, tomato, and *Nicotiana benthamiana* infected with these pathogens there is an enhanced expression of *GH3.2* and *GH3.4* genes, which encode two enzymes required for conjugation of auxins with Asp. Thus, upon pathogen infection, accumulation of IAA–Asp takes place, promoting the development of disease symptoms in infected plants (González-Lamothe *et al*., 2012). The negative effects of auxins on the activation of plant resistance are further supported by the observed enhanced susceptibility of auxin-treated rice to *X. oryzae* and of auxin-treated *Arabidopsis* to *Pst*DC3000 and *Fusarium culmorum*. Disruption of auxin signaling in *Arabidopsis* mutants, such as *axr1*, *axr2* and *axr3*, leads

was a significant effect of GA in reducing infection in Nipponbare against *H. oryzae* but the experiment was not repeated to confirm this result. A significantly higher number of *H. oryzae* per plant was found in GA-deficient mutant waitoC compared to WT and a lower number in GA-insensitive mutant gid1-3 plants. Experiments with *M. graminicola* showed that galling was significantly and reproducibly lower in mutants, whether it was GA-insensitive or GA-deficient than in wild type (Taichung 65). There were even significantly lower galls in these GA-deficient mutants after GA treatment than in nontreated mutant plants. There was no difference in the expression of GA signaling gene *Osgid1-3* after *M. graminicola* infection in Taichung 65, neither in root tip nor leaf tissue. The expression of GA signaling gene *Osgid1-7* was significantly higher only in the whole root at 2 dpi but not in leaves or root tips. Even this gene was not differentially expressed in root and leaf tissues at a later stage (6 dpi) of infection. The nematode inhibited biosynthesis of *OsGA20ox* in leaves at 2 dpi and in both of leaves and roots at 6 dpi. The GA response gene *AK073385* was also less expressed in root tip at 6 dpi. These might help nematodes to continue their infection in Taichung 65.

Gibberellins play a vital role in plant growth and development. However only 12 fungi, associated with plants and/or soil have been reported as GA producers (Muhammad Hamayun *et al.*, 2010). Endophytic strains of the *Cladosporium sphaerospermum* and *Penicillium citrinum* have been reported as GA producers. Genus *Cladosporium* includes several plant-associated saprophytic and some plant pathogenic species. Some species are widely distributed dematiaceous molds, and some are predominant in tropical and subtropical regions. Some *Cladosporium* species such as *C. fulvum* are pathogenic to plants, causing scab diseases as well as leaf spots and blights. *Cladosporium* sp. MH-6 is a novel GA-producing fungus that secretes comparable amounts of GA to *F. fujikuroi*. Therefore, this isolate might have the potential for use as a biofertilizer with minimal environmental risks. There are two classes of gibberellins, the 19-carbon gibberellins, and the 20-carbon gibberellins. The 19-carbon gibberellins, formed from 20-carbon gibberellins, are the biologically active forms. Gibberellins also vary according to the position and number of hydroxyl groups linked to the carbon atoms of the *ent*-gibberellane skeleton (Fig. 2.29). Hydroxylation has a profound influence on biological activity.

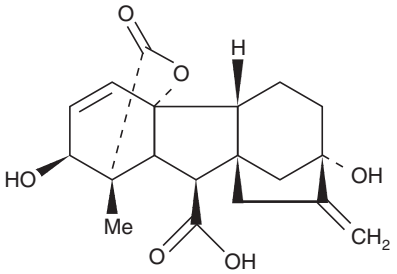

Fig. 2.29 Gibberellic acid

The causative agent *Gibberella fujikuroi* contains several different types of gibberellin (abbreviated to GA, after gibberellic acid, the first form discovered), some of which also exist in plants. Plants possess many other unique gibberellins, and collectively there are

well over 100 identified compounds. This intimidating complexity can be reduced to a comprehensible level by realizing that each species contains only about 25 of these gibberellins and that most gibberellins are biosynthetic intermediates or inactive end-products, and not active in their own right. There are many steps and enzymes involved in building up the 19- and 20-carbon gibberellin molecules from five-carbon mevalonic acid. Several parallel pathways exist differing only in the number of hydroxyl (–OH) groups. Hydroxyl groups are the key to gibberellin functions: some positions (3b) are generally essential for activity, whereas others (2b) completely abolish it. Inactivation by conjugation to glucose also occurs. Gibberellin synthesis takes place mainly in developing leaves and stems, in developing seeds and during germination. Gibberellins function in dormancy release and germination, as well as in growth promotion (e.g., stem elongation, fruit tissue expansion).

2.9.1.3 Cytokinins

Cytokinins are plant-specific chemical messengers (hormones) that play a central role in the regulation of the plant cell cycle and numerous developmental processes (Schmülling, 2004). The first cytokinin discovered was an adenine (aminopurine) derivative named kinetin. Cytokinins are present in all plant tissues. They are abundant in the root tip, the shoot apex, and immature seeds. Their endogenous concentration is in the low nM range. Cytokinins may also act on the cell that produced them (autocrine signaling). Cytokinins are necessary for cell growth and differentiation. It inhibits the breakdown of proteins and amino acids and thereby inhibits senescence, and they have the capacity to direct the flow of amino acids and other nutrients towards high cytokinin concentration. Cytokinin activity increases in club root, in crown galls and in rust infected bean leaves (*Example*: Green islands are formed around infection in bean (*Phaseolus vulgaris)* leaves infected by *Uromyces phaseoli*).

In many ways, cytokinins are opposites of auxins, being synthesized in roots but with most dramatic effects on shoot development. However, shoot tissues can also produce cytokinins as can developing seeds. A classic example of the latter is coconut milk, the copious liquid endosperm from coconut seed, which is still a popular cytokinin source in plant tissue culture media. Cytokinins were originally named for their ability to promote cell division, but they also function in the initiation of new shoot structures, dormancy release and retardation of senescence. Cytokinins are derivatives of adenine, one of the purine bases found in all DNA and RNA. Indeed, cytokinins were originally thought to be products of transfer RNA (tRNA) breakdown. Cytokinins, such as zeatin and isopentenyl adenosine (IPA) have been isolated from plants.

Cytokinins have emerged as a major factor in plant-microbe interactions during nodule organogenesis and pathogenesis. Microbe-originated cytokinins confer abnormal hypersensitivity of cytokinins to plants, augmenting the sink activity of infected regions. However, recent findings have shed light on a distinct role of cytokinins in plant immune responses. Plant-borne cytokinins systemically induce resistance against pathogen infection. This resistance is orchestrated by endogenous cytokinin and salicylic acid signaling. Cytokinins are also produced by cyanobacteria, some plant pathogenic bacteria (e.g., *Agrobacterium tumefaciens, Pseudomonas savastanoi, Rhodococcus fascians*) and the slime

mold *Dictyostelium discoideum*. Naturally occurring cytokinins are adenine derivatives with a side chain at the N6-position (Fig. 2.30). The structure and conformation of the N6-attached side chain can markedly influence the biological activity of the cytokinin (Schmülling, 2004). The cytokinin zeatin was named after the genus of corn, *Zea,* in which it was discovered.

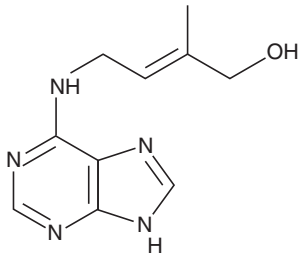

Fig. 2.30 Cytokinin zeatin

Depending on the structure of the N6-substituent, cytokinins are classified as isoprenoid or aromatic cytokinins. The biological activities of both classes are qualitatively similar, but they may differ quantitatively in different processes. Isoprenoid cytokinins are the most abundant class.

Cytokinins are produced by several plant pathogenic bacteria and play a role in pathogenicity. One such pathogen is *Agrobacterium tumefaciens*, the causative agent of the crown gall disease. During the infection process, *A. tumefaciens* transfers a small stretch of DNA, the T-DNA, to the host plant, where it becomes integrated into the nuclear genome. The T-DNA harbors an *IPT* gene, which is expressed in the host cell and causes cytokinin overproduction. This leads, together with enhanced auxin content, to tumorous cell proliferation. Other cytokinin-synthesizing pathogens are *Pseudomonas syringae*, which induces gall formation and *Rhodococcus fascians*, which causes fasciation and a growth abnormality called witches´ broom disease. The root-nodule forming and nitrogen-fixing plant symbiont, *Rhizobium* species is also known to produce cytokinin.

It is known that plants utilize hormone signal molecules to regulate plant development and respond to the environment. Historically, direct evaluation of the role of hormones in various plant signaling pathways was hampered by extremely low levels of hormones present in any tissue. New technologies based on the discovery that certain plant proteins can be used as markers for the presence of the group of plant hormones known as cytokinins are allowing the investigation of the role of cytokinins in plant development and responses to the environment at the molecular and plant physiology levels (Prasad *et al.*, 2004). An attempt was made to identify and correlate the presence of endogenous cytokinins in different regions of developing roots and roots exposed to symbiotic nodulating bacteria or pathogenic root knot nematodes. As assayed by protein expression for cytokinin responsiveness, the hormones appeared to regulate spatially and temporally in the formation of lateral roots. A role for cytokinins was implicated in the formation of nitrogen-fixing nodules, and it was found that a cytokinin response was present subsequent to nematode root penetration and migration. In addition, experiments with

Ethylene exerts a variety of effects on plants, viz., chlorosis, leaf abscission, epinasty, stimulation of adventitious roots, fruit ripening and increased permeability of cell membranes. Ethylene production in infected tissues can be dramatically induced. This induction is largely dependent on activation of the ethylene biosynthetic pathway in plant tissues. Genes encoding several key enzymes involved in the ethylene biosynthesis are highly activated at the transcriptional level. It has not been shown that ethylene is produced directly by plant pathogenic fungi and bacteria.

The role of ethylene in the hormonal regulation of plant development has been well established (Leendert C. van Loon *et al.*, 2006). In addition, it has been implicated in biotic stress, both as a virulence factor of fungal and bacterial pathogens and as a signaling compound in disease resistance. This apparent discrepancy has stimulated research on the effects of various types of pathogens on mutant and transgenic plants that are impaired in ethylene production or perception. It has become clear that ethylene differentially affects resistance against pathogens with different lifestyles and plays an important role in mediating different types of induced resistance. It is a gaseous plant hormone, which plays a role in plant development, defense, and climacteric fruit ripening. Both genetic and biochemical evidence suggest that the response of plants to ethylene is mediated by a specific ethylene receptor.

Ethylene has been regarded as a signal in the plant for wounding and senesces responses. Recent studies show that ethylene together with another signal component jasmonic acid may play an essential role in plant defense responses of several pathosystems and induces ethylene production in infected tissues. Ethylene induces expression of certain types of *PR*-genes and some signal components involved in defense signaling. Ethylene insensitive mutants block several *PR*-gene-expression, and ethylene-insensitive mutants can be beneficial against some pathogens but deleterious to resistance against other pathogens in a specific a gene-for-gene manner. Other chemical weapons include polysaccharides, plant defense suppressors, transporters, etc. Plant viruses and viroids are not known to produce any substances themselves, but they adapt, induce and manipulate the host metabolism to replicate themselves.

Example: Ethylene is involved in the premature ripening of fingers in banana infected by *Pseudomonas solanacearum*, the causal agent of moko disease of banana. Ethylene was also detected in leaf epinasty symptom of the vascular wilt syndrome [*Example: Fusarium oxysporum* f. sp. *lycopersici* (wilt in tomato)].

Enhanced ethylene production is an early, active response of plants to the perception of pathogen attack and is associated with the induction of defense reactions (Tzeng and DeVay, 1985). It is generally assumed that ethylene production during stress contributes to stress alleviation, but several plant pathogenic fungi and bacteria are capable of producing ethylene as a virulence factor, which improves their ability to colonize plant tissues. For instance, the ability of the bacterial leaf pathogen *Pseudomonas syringae* pv. *glycinea* to proliferate in the leaves of its host plant soybean is impaired in mutants that lack the capacity to produce ethylene, which indicate that ethylene produced during infection promotes disease rather than alleviates it. Indeed, ethylene is responsible for the epinasty and defoliation caused by the soil-borne fungus *Verticillium dahliae* in cotton and for the stunting and chlorosis of cucumber infected by cucumber mosaic

further delineating the involvement of ethylene in plant defense responses and the elucidation of the underlying mechanisms.

The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play crucial roles in the signaling network that regulates induced defense responses to biotic stresses (Antonio Leon-Reyes *et al.*, 2009). Antagonism between SA and JA operates as a mechanism to fine-tune defenses that are activated in response to multiple attackers. In *Arabidopsis thaliana*, 'Nonexpressor of Pathogenesis-Related Genes1' (*NPR1*) was demonstrated to be required for SA-mediated suppression of JA-dependent defenses. Because ET is known to enhance *SA/NPR1*-dependent defense responses, the role of ET was investigated in the SA-JA signal interaction. Pharmacological experiments with gaseous ET and the ET precursor 1-aminocyclopropane-1-carboxylic acid showed that ET potentiated *SA/NPR1*-dependent *Pathogenesis-Related1* transcription, while it rendered the antagonistic effect of SA on methyl jasmonate-induced *PDF1.2* and *VSP2* expression *NPR1* independent. This overriding effect of ET on *NPR1* function in SA-JA crosstalk was absent in the *npr1-1/ein2-1* double mutant, demonstrating that it is mediated via ET signaling. Abiotic and biotic induction of the ET response similarly abolished the *NPR1* dependency of the SA-JA signal interaction. Furthermore, JA-dependent resistance against biotic attackers was antagonized by SA in an *NPR1*-dependent fashion only when the plant-attacker combination did not result in the production of high levels of endogenous ET. Hence, the interaction between ET and *NPR1* plays an important modulating role in the fine tuning of the defense signaling network that is activated by pathogen and insect attack. A model was suggested in which ET modulates the *NPR1* dependency of SA-JA antagonism, possibly to compensate for enhanced allocation of *NPR1* to function in SA-dependent activation of *PR* genes.

Ethylene evolution occurs concomitantly with the progression of disease symptoms in response to many virulent pathogen infections in plants. A tomato mutant impaired in ethylene perception,'never ripe', exhibited a significant reduction in disease symptoms in comparison to the wild type after inoculations of both genotypes with virulent bacterial (*Xanthomonas campestris* pv. v*esicatoria* and *Pseudomonas syringae* pv. *tomato*) and fungal (*Fusarium oxysporum, F.* sp*lycopersici*) pathogens (Steven T. Lunda *et al.*, 1998). Bacterial spot disease symptoms were also reduced in tomato genotypes impaired in ethylene synthesis (1-aminocyclopropane-1-carboxylic acid deaminase) and perception (14893), thereby corroborating a reducing effect for ethylene insensitivity on foliar disease development. The reduction in foliar disease symptoms in 'n*ever ripe*' plants was a specific effect of ethylene insensitivity and was not due to reductions in bacterial populations or decreased ethylene synthesis. *PR-1B1* mRNA accumulation in response to *X. c. vesicatoria* infection was not affected by ethylene insensitivity, indicating that ethylene is not required for defense gene induction. Broad tolerance of diverse vegetative diseases may be achieved via engineering of ethylene insensitivity in tomato.

2.9.2.2 Abscisic acid

It exerts dormancy in seeds, closure of stomata, inhibition of seed germination and growth and stimulated germination of fungal spores. It is one of the factors involved in stunting

of plants. Abscisic acid is a single compound unlike the auxins, gibberellins, and cytokinins. It was called 'abscisin II' originally because it was thought to play a major role in abscission of fruits. At about the same time, another group was calling it 'dormin' because they thought it had a major role in bud dormancy. The name abscisic acid (ABA) was coined by a compromise between the two groups. Though ABA generally is thought to play mostly inhibitory roles, it has many promoting functions as well.

Abscisic acid (ABA) is an isoprenoid compound that regulates developmental processes, such as seed development, desiccation, and dormancy (Nicolas Denance *et al*., 2013). In addition, the function of ABA as a regulator of abiotic stress has been thoroughly described. ABA has also emerged as a complex modulator of plant defense responses (Sánchez-Vallet *et al*., 2012). ABA can function as a positive or a negative regulator of plant defense depending on the plant–pathogen interaction analyzed. ABA-impaired (biosynthesis or signaling) mutants in tomato (*sitiens*) and *Arabidopsis* (*abi1-1*, *abi2-1*, *aba1-6*, *aba2-12*, *aao3-2*, and *pyr1pyl1pyl2pyl4*) were shown to overexpress defensive-signaling pathways, leading to enhanced resistance to different pathogens such as *B. cinerea*, *P. syringae*, *F. oxysporum*, *Plectosphaerella cucumerina*, and *Hyaloperonospora parasitica.* Negative interactions of ABA with the major hormones involved in plant defense response (SA, JA, and ET) have been described by means of exogenous hormone treatments. ABA plays a direct role in regulating R (resistance) protein activity. ABA and exposition of plants to high temperature both reduce the nuclear accumulation of SNC1 (suppressor of *npr1-1*, constitutive1) and RPS4 (resistant to *Pseudomonas syringae* 4) compromising disease resistance to *P. syringae* (Mang *et al*., 2012). Abscisic acid (Fig. 2.32) can also positively regulate the resistance to some pathogens, such as *Alternaria brassicicola*, *R. solanacearum*, and *Pythium irregulare*, as ABA-deficient and -insensitive mutants (*abi1-1*, *abi2-1*, *abi4-1*, *aba1-6*, *aba2-12*, *aao3-2*, and *npq2-1*) were found to be more susceptible than wild-type plants to these pathogens.

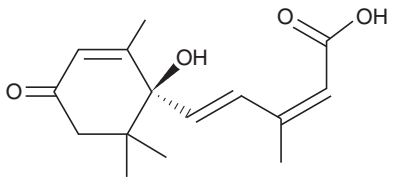

Fig. 2.32 Abscisic acid

Several fungal species produce ABA, including *B. cinerea*, *Rhizoctonia solani*, *Ceratocystis fimbriata*, and *Rhizopus nigricans.* Unlike plants, fungi such as *B. cinerea* and *Cercospora* sp. use the mevalonate pathway to produce ABA. It is a key hormone in *Arabidopsis* response to *R. solanacearum* infection, as 40% of the genes up-regulated during the development of wilting symptoms were related to ABA, including those encoding proteins for ABA biosynthesis [i.e., 9-*cis*-epoxycarotenoid dioxygenase3 (NCED3)] or signaling [i.e., ABA-insensitive1 (ABI1) and ABI5. More recently, it has been shown that preinoculation of *Arabidopsis* with an avirulent strain of *R. solanacearum* activates plant resistance to virulent isolates of this bacterium, and this resistance was correlated with

pathogenic bacterium is responsible for soft rot disease in many plant species, causing maceration symptoms mainly due to the production and secretion of pectinolytic enzymes. On wild-type (WT) tomato cv Moneymaker, *E. chrysanthemi* leaf inoculation resulted in maceration both within and beyond the infiltrated zone of the leaf, but sitiens showed a very low occurrence of tissue maceration, which never extended the infiltrated zone. A single ABA treatment prior to infection eliminated the effect of pathogen restriction in sitiens while repeated ABA spraying during plant development rendered both WT and sitiens very susceptible. Quantification of *E. chrysanthemi* populations inside the leaf did not reveal differences in bacterial growth between sitiens and WT. Sitiens was not more resistant to pectinolytic cell-wall degradation, but upon infection it showed a faster and stronger activation of defense responses than WT, such as hydrogen peroxide accumulation, peroxidase activation, and cell-wall fortifications. Moreover, the rapid activation of sitiens peroxidases was also observed after application of bacteria-free culture filtrate containing *E. chrysanthemi* cell-wall-degrading enzymes and was absent during infection with an out *E. chrysanthemi* mutant impaired in the secretion of these extracellular enzymes.

Studies involving plant-nematode interactions provide an opportunity to unravel plant defense signaling in root tissues. Nahar *et al.* (2012) characterized the roles of salicylate (SA), jasmonate (JA), ethylene (ET) and abscisic acid (ABA) in plant defense against the rice root nematode, *Hirschmanniella oryzae* in the monocot model plant rice (*Oryza sativa*). Experiments with exogenous hormone applications, biosynthesis inhibition, and mutant/transgenic lines were executed to test the effect on *H. oryzae* parasitism in rice roots. It was observed that an intact ET, JA, and SA biosynthesis pathway was a prerequisite for defense against *H. oryzae*. By contrast, exogenous ABA treatment drastically compromised the rice defense towards this nematode. Gene expression analyses using quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrated that the disease-inducing effect of ABA was likely to be the result of an antagonistic interaction between this hormone and the SA/JA/ET-dependent basal defense system. Collectively, in rice defense against *H. oryzae*, at least three pathways, namely SA, JA and ET, are important, while ABA plays a negative role in the defense. It was further suggested that the balance of ABA and SA/JA/ET signaling was an important determinant of the outcome of the rice-*H. oryzae* interaction.

The *in vitro* effects of abscisic acid (ABA) and nitric oxide (NO) on the nematode-trapping fungus *Drechslerella stenobrocha* AS6.1 were examined (Ling-Ling XU *et al.*, 2010). The average number of traps (constricting rings) per colony and the percentage of nematodes (*Caenorhabditis elegans*) trapped were greatly increased by the addition of ABA but greatly suppressed by addition of sodium nitroprusside (SNP, an NO donor) to corn meal agar. The suppressive effect of SNP was not negated by the addition of an NO synthase competitive inhibitor (l-naphthylacetic acid, L-NNA) or an NO-specific scavenger [2-(4-carboxyphenyl)-4,4, 5,5- tetramethylimidazoline-1-oxyl-3-oxide, cPTIO]. When added without SNP however, L-NNA and cPTIO caused moderate increases in trap number and trapping. The results indicated that the trap formation and nematode-trapping ability of *D. stenobrocha* were enhanced by ABA but decreased by exogenous NO.

2.9.3 Other Plant Growth Regulators

Various other plant growth regulators include Jasmonic acid, Salicylic acid, Polyamines, Brassinolides, Nitrobenzene, Seaweed products, etc., among which, few are very important.

2.9.3.1 Jasmonic acid

Jasmonic acid (JA) is a fatty-acid-derived plant hormone that is similar in overall structure, to physiologically active small molecules from animals called prostaglandins. In plants, jasmonic acid is firmly associated with pathogen defense pathways. The physical stimuli of certain insects can trigger the synthesis of JA, which then functions to increase expression of genes involved in defending the plant. Microbial and viral pathogens can also trigger JA synthesis. The study of JA-mediated events in the plant cell is of interest to plant pathologists who wish to engineer transgenic plants that are disease-resistant.

Jasmonic acid and salicylic acid are essential compounds in the pathogen- and wound-signaling pathways accompanying induced expression of acidic and basic pathogenesis-related (PR) protein genes, respectively (Tomoya Niki *et al.*, 1998). However, on the effect of exogenously supplied SA and JA in the induction of *PR* gene expression, conflicting results have been obtained using various plant materials at different developmental stages. There is no clear evidence on these effects in the presence of both signals at the same time. The effect of SA on the wound- and JA-induced basic *PR* gene expression was analyzed and that of JA on SA-induced acidic Pi gene expression in mature tobacco leaves. Wound-induced accumulation of transcripts for all four basic *PR* genes tested was enhanced in the presence of MeJA, and inhibited in the presence of SA. On the other hand, expression of all three acidic *PR* genes tested was induced by SA and was inhibited by MeJA. Using antibodies raised against acidic PR-1 and PR-2 proteins, these effects were confirmed at the protein level. These results indicated that JA works as an inducer of basic *PR* genes, and also as an inhibitor of acidic *PR* genes, while SA does the opposite.

The jasmonates are key signal compounds in the elicitation process leading to *de novo* transcription and translation and, ultimately, to the biosynthesis of secondary metabolites in plant cell cultures. To deter pathogenic microorganisms and herbivores, plants have developed an inducible chemical defense system. It is known that the induced synthesis of low molecular weight compounds can be provoked by exposing cultured cells to fungal cell wall fragments. Heidrun Gundlach *et al.* (1992) demonstrated that endogenous jasmonic acid and its methyl ester accumulate rapidly and transiently after treatment of plant cell suspension cultures of *Rauvolfia canescens* and *Eschscholtia caisfornica* with a yeast elicitor. Thirty-six plant species tested in cell suspension culture could be elicited with respect to the accumulation of secondary metabolites by exogenously supplied methyl jasmonate. The addition of methyl jasmonate initiates *de novo* transcription of genes, such as phenylalanine ammonia lyase, that are known to be involved in the chemical defense mechanisms of plants. These data demonstrated the integral role of jasmonic acid (Fig. 2.33) and its derivatives in the intracellular signal

cascade that begins with the interaction of an elicitor molecule with the plant cell surface and results, ultimately, in the accumulation of secondary compounds.

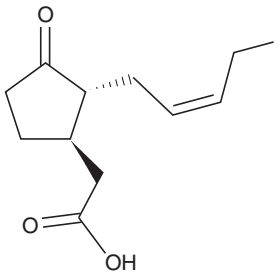

Fig. 2.33 Jasmonic acid

Jasmonic acid is a plant hormone that plays an important role in a wide variety of plant physiological processes. The plant pathogenic fungus, *Lasiodiplodia theobromae* also produces JA. However, its biosynthesis in this fungus has yet to be explored (Tsukada *et al*., 2010). Administration of NaOAc into *L. theobromae* established that JA in this fungus originates from a fatty acid synthetic pathway. The methyl ester of 12-oxo-phytodienoic acid (OPDA) was detected in the culture extracts of *L. theobromae* by GC-MS analysis. This finding indicated the presence of OPDA, a known intermediate of JA biosynthesis in plants, in *L. theobromae*. (2)H NMR spectroscopic data of JA produced by *L. theobromae* with the incorporation of linolenic acid showed that five deuterium atoms remained intact. In plants, this is speculated to arise from JA being produced by the octadecanoid pathway. However, the observed stereoselectivity of the cyclopentenone olefin reduction in *L. theobromae* was opposite to that observed in plants. These data suggested that JA biosynthesis in *L. theobromae* was similar to that in plants but differed in the facial selectivity of the enone reduction.

Responses of resistant (*Mi-1/Mi-1*) and susceptible (*mi-1/mi-1*) tomato to root-knot nematodes infection were monitored using cDNA microarrays and the roles of salicylic acid (SA) and jasmonic acid (JA) defense signaling were evaluated in these interactions (Kishor K. Bhattarai *et al*., 2008). Array analysis was used to compare transcript profiles in incompatible and compatible interactions of tomato roots 24 hr after nematode infestation. The *jai1* and *def1* tomato mutant, altered in JA signaling, and transgenic tomato line *NahG*, altered in SA signaling, in the presence or absence of the nematode resistance gene *Mi-1*, were evaluated. The array analysis identified 1,497 and 750 genes differentially regulated in the incompatible and compatible interactions, respectively. Of the differentially regulated genes, 37% were specific to the incompatible interactions. *NahG* affected neither *Mi-1* resistance nor basal defenses to nematodes. However, *jai1* reduced tomato susceptibility to nematode while not affecting *Mi-1*resistance. In contrast, the *def1* mutant did not affect nematode susceptibility. These results indicated that JA-dependent signaling did not play a role in the *Mi-1*-mediated defense. However, an intact JA signaling pathway was required for tomato susceptibility to nematodes. In addition, low levels of SA might be sufficient for basal and *Mi-1*resistance to nematodes.

2.9.3.2 Salicylic acid

Salicylic acid (SA) is a phenolic phytohormone and is found in plants with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA also induces specific changes in leaf anatomy and chloroplast structure. SA is involved in endogenous signaling, mediating in plant defense against pathogens. It plays a role in the resistance to pathogens by inducing the production of pathogenesis-related proteins. It is involved in the systemic acquired resistance in which a pathogenic attack on one part of the plant induces resistance in other parts. The signal can also move to nearby plants by salicylic acid being converted to the volatile ester, methyl salicylate.

The function of SA in activating resistance against pathogens has been thoroughly described. In *Arabidopsis*, SA is synthesized from chorismate (a precursor of tryptophan and, consequently, of auxins) *via* two pathways, either through phenylalanine or isochorismate. This second pathway, in which SID2/ICS1 (salicylic acid induction-deficient 2/isochorismate synthase 1 is involved, is activated upon pathogen infection, such as *Erysiphe* or *P. syringae*, and after plant recognition of pathogen effectors or PAMPs. Deficiency of SA biosynthesis in *sid2-1* mutant leads to reduced resistance response in *Arabidopsis* plants. SA (Fig. 2.34) is a regulator of plant resistance to biotrophic and hemibiotrophic pathogens, such as *Hyaloperonospora arabidopsidis* and *P. syringae*, and it also regulates systemic acquired resistance, a well-studied type of induced resistance. In addition, SA is a central regulator of immunity. It interacts with other signaling pathways (e.g., ET and JA pathways), as a strategy to induce the proper resistance responses and to reduce the associated fitness costs (Nicolas Denance *et al.*, 2013).

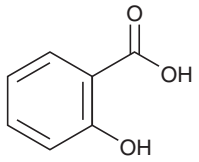

Fig. 2.34 Salicylic acid

Significant insight has been gained in the past year into the roles of salicylic acid in plant-pathogen interactions. The ability to accumulate SA has been shown to be essential for systemic acquired resistance in tobacco plants (Vernooji *et al.*, 1994). It has been shown that SA is apparently not a systemic, vascular-mobile signal, but rather is required for signal transduction at the local level. Its mode of action may include inhibition of catalase activity, leading to increased levels of hydrogen peroxide. After a hypersensitive response to invading pathogens, plants show an elevated accumulation of SA-induced expression of plant defense genes and systemic acquired resistance (SAR) to further infection by a broad range of pathogens (Marianne *et al.*, 2000). There is compelling evidence that SA plays a crucial role in triggering SAR. Tobacco was transformed with two bacterial genes coding for enzymes that convert chorismate into SA by a two-step process. When the two enzymes were targeted to the chloroplasts, the transgenic (CSA,

elevated. It was concluded that SA acted via NPR1 to inhibit nematode parasitism which, in turn, was negatively regulated by SNI1. An inverse correlation between root basal PR-1 expression and plant susceptibility to *H. schachtii* was noticed, and it was suggested that successful cyst nematode parasitism might involve a local suppression of SA signaling in roots.

2.9.3.3 Polyamines

Polyamines are small polycationic molecules found ubiquitously in all organisms and function in a wide variety of biological processes. In the past decade, molecular and genetic studies using mutants and transgenic plants with an altered activity of enzymes involved in polyamine biosynthesis have contributed much to a better understanding of the biological functions of polyamines in plants. A polyamine is an organic compound having two or more primary amino groups–NH2. This class of compounds includes several synthetic substances that are important feedstocks for the chemical industry. It also includes many substances that play important roles in both eukaryotic and prokaryotic cells, such as putrescine, cadaverine, spermidine and spermine (Figures 2.35–2.38).

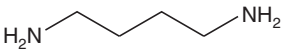

Fig. 2.35 Putrescine

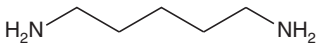

Fig. 2.36 Cadaverine

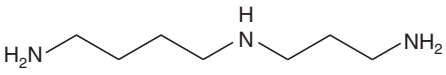

Fig. 2.37 Spermidine

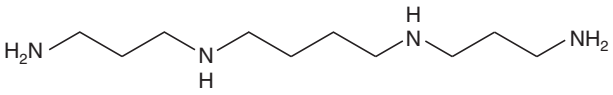

Fig. 2.38 Spermine

Ubiquitous in nature, polyamines are a group of aliphatic amines, cationic at neutral pH, that are essential for cell growth and viability. Because of their positive charge, polyamines are able to bind by electrostatic linkages to many cellular macromolecules, including DNA, RNA, and proteins. Spermidine is essential for the survival of *Arabidopsis embryos*. One of the reasons may lie in the fact that spermidine serves as a substrate for the lysine → hypusine posttranslational modification of the eukaryotic translation initiation factor 5A, which is essential in all eukaryotic cells (Taku Takahashi and JunIchi Kakehi, 2010). Spermine is not essential but plays a role in stress responses, probably through the modulation of cation channel activities, and as a source of hydrogen peroxide during pathogen infection. Thermospermine, an isomer of spermine, is involved in stem elongation, possibly by acting on the regulation of upstream open reading frame-mediated translation.

The diamine putrescine, the triamine spermidine, and the tetraamine spermine are ubiquitous in plant cells while other polyamines are of more limited occurrence. Their chemistry and pathways of biosynthesis and metabolism are well characterized (Galston and Sawhney, 1990). They occur in the free form as cations, but are often conjugated to small molecules like phenolic acids and also to various macromolecules. Their titer varies

from approximately micromolar to more than millimolar and depends greatly on environmental conditions, especially stress. In cereals, the activity of one of the major polyamine biosynthetic enzymes, arginine decarboxylase, is rapidly and dramatically increased by almost every studied external stress, leading to 50-fold or greater increases in putrescine titer within a few hours. The physiological significance of this increase is not yet clear, although most recent work suggests an adaptive, protective role. Polyamines produced through the action of ornithine decarboxylase, by contrast, seem essential for DNA replication and cell division. The application of exogenous polyamines produces effects on patterns of senescence and morphogenesis, suggesting but not proving a regulatory role for polyamines in these processes. The evidence for such a regulatory role is growing.

Polyamines are unique as they are effective (and are applied) in relatively high concentrations. Typical concentrations range from 5 to 500 mg/L. Polyamines such as putrescine, spermidine or spermine influence flowering and promote plant regeneration. Transgenic tomato (*Solanum lycopersicum*) lines overexpressing yeast spermidine synthase (*ySpdSyn*), an enzyme involved in polyamine (PA) biosynthesis, were developed (Savithri Nambeesan *et al.*, 2012). These transgenic lines accumulated higher levels of spermidine (Spd) than the wild-type plants and were examined for responses to the fungal necrotrophs *Botrytis cinerea* and *Alternaria solani*, bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000, and larvae of the chewing insect tobacco hornworm (*Manduca sexta*). The Spd-accumulating transgenic tomato lines were more susceptible to *B. cinerea* than the wild-type plants; however, responses to *A. solani*, *P. syringae*, or *M. sexta* were similar to the wild-type plants. Exogenous application of ethylene precursors, *S*-adenosyl-Met, and 1-aminocyclopropane-1-carboxylic acid or PA biosynthesis inhibitors reversed the response of the transgenic plants to *B. cinerea*. The increased susceptibility of the *ySpdSyn* transgenic tomato to *B. cinerea* was associated with down-regulation of gene transcripts involved in ethylene biosynthesis and signaling. These data suggested that PA-mediated susceptibility to *B. cinerea* was linked to interference with the functions of ethylene in plant defense.

Polyamine (PA) biosynthesis inhibitors, difluoromethylornithine (DFMO), difluoromethylarginine (DFMA), methylglyoxal bis-(guanylhydrazone) (MGBG) and bis-(cyclohexylammonium) sulphate (BCHA) have been tested for their effects on colony diameters at different intervals after inoculation of four plant pathogenic fungi (*Helminthosporium oryzae, Curvularia lunata, Pythium aphanidermatum and Colletotrichum capsici*) (Rajam and Rajam, 1996). All these inhibitors, except DFMA, had strongly retarded the growth of four fungi in a dose- and species-dependent fashion, and *H. oryzae* and *C. lunata* were found to be most sensitive to the effects of PA inhibitors. *P. aphanidermatum* and *C. capsici* were relatively insensitive and required rather high concentrations of inhibitors to get greater inhibition of mycelial growth, except DFMA, which had a stimulatory effect on the growth of these two fungi. However, DFMA had greatly suppressed the growth of *H. oryzae* and *C. lunata*. The effect was generally more pronounced with MGBG than with DFMO and BCHA, and 1 mM Put completely prevented the inhibitory effects of 1 and 5 mM DFMO. Analysis of free and conjugated PAs in two sensitive fungi (*H. oryzae* and *C. lunata*) revealed that Put was present in

highest concentrations followed by Spd and Spm, and their levels were greatly reduced by DFMO application, and such inhibitions were totally reversed by exogenously supplied Put. In fact, PA titers were considerably increased by 1 mM Put alone and in combination with 1 mM DFMO. These results suggest that PA inhibitors, particularly DFMO and MGBG may be useful as target-specific fungicides in plants.

The levels of polyamines in leaves of *Gynura aurantiaca* DC and tomato, *Lycoperskon esculentum* cv Rutgers, infected with citrus exocortis viroid (CEVd) or treated with silver nitrate or ethephon (2-chloroethylphosphonic acid) were measured by HPLC in relation to the development of symptoms (Jose M. Belles *et al.*, 1991). Previously it had been demonstrated that treatment of *G. aurantiaca* plants with silver nitrate or ethephon closely mimicked the effects of viroid infection in the plants. In the studies reported here, a marked decrease in putrescine level was observed in plants infected by CEVd or treated with silver ions or ethephon. There was no significant change in either spermidine or spermine levels. Treatment of *G. aurantiaca* plants with specific inhibitors of ethylene biosynthesis (aminoethoxyvinylglycine, Co^{2+}) or ethylene action (norbomadiene) prevented the decrease of putrescine associated with silver nitrate treatment and had no effect on spermidine or spermine levels. The development of viroid-like symptoms, the production of associated pathogenesis-related proteins, and the rise in protease activity induced by silver nitrate, were all suppressed by exogenous application of putrescine.

Cyst nematodes are sedentary endo-phytoparasites that cause dramatic cellular changes in the plant root to form feeding cells, so-called syncytia. 10A06 is a cyst nematode secretory protein that is most likely secreted as an effector into the developing syncytia during early plant parasitism (Tarek Hewezi *et al.*, 2010). A homolog of the uncharacterized soybean cyst nematode (*Heterodera glycines*), the *10A06* gene, was cloned from the sugar beet cyst nematode (*H. schachtii*), which is able to infect *Arabidopsis* (*Arabidopsis thaliana*). Constitutive expression of *10A06* in *Arabidopsis* affected plant morphology and increased susceptibility to *H. schachtii* as well as to other plant pathogens. Using yeast two-hybrid assays, Spermidine Synthase2 (SPDS2), a key enzyme involved in polyamine biosynthesis, was identified, as a specific 10A06 interactor. In support of this protein-protein interaction, transgenic plants expressing *10A06* exhibited elevated SPDS2 mRNA abundance, significantly higher spermidine content, and increased polyamine oxidase (PAO) activity. Furthermore, the SPDS2 promoter was strongly activated in the nematode-induced syncytia, and transgenic plants overexpressing *SPDS2* showed enhanced plant susceptibility to *H. schachtii*. In addition, in planta expression of *10A06* or SPDS2 increased mRNA abundance of a set of antioxidant genes upon nematode infection. These data lend strong support to a model in which the cyst nematode effector 10A06 exerts its function through the interaction with SPDS2, thereby increasing spermidine content and subsequently PAO activity. Increasing PAO activity results in stimulating the induction of the cellular antioxidant machinery in syncytia. An apparent disruption of salicylic acid defense signaling as a function of 10A06 was observed. Increased antioxidant protection and interruption of salicylic acid signaling were regarded as key aspects of 10A06 function in addition to other physiological and

morphological changes caused by altered polyamines, which are potent plant signaling molecules.

2.10 ROLE OF POLYSACCHARIDES IN PATHOGENESIS

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic bonds. They range in structure from linear to highly branched (Fig. 2.39). Examples include storage polysaccharides such as starch and glycogen and structural polysaccharides such as cellulose and chitin. Nutrition polysaccharides are common sources of energy. Many organisms can easily break down starches into glucose; however, most organisms cannot metabolize cellulose or other polysaccharides like chitin and arabinoxylans. These carbohydrate types can be metabolized by some bacteria and protists. Polysaccharides have a general formula of $C_x(H_2O)_y$ where x is usually a large number between 200 and 2500. Considering that the repeating units in the polymer backbone are often six-carbon monosaccharides, the general formula can also be represented as $(C_6H_{10}O_5)_n$ where $40 \leq n \leq 3000$.

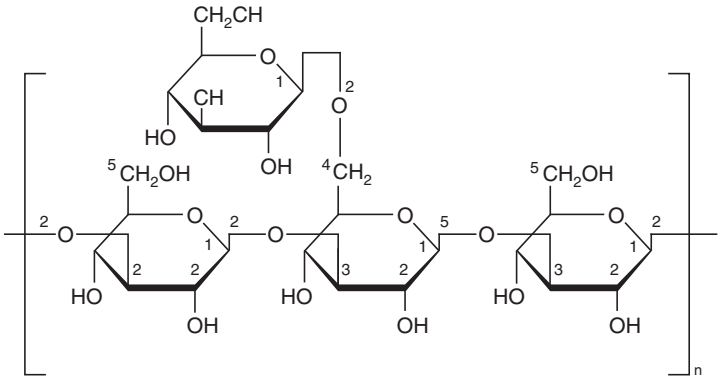

Fig. 2.39 Polysaccharides

Plant biomass is the most abundant organic matter on earth and the major substrate for the majority of fungal species. It consists mainly of polysaccharides, but also contains proteins and the aromatic polymer lignin. Plant polysaccharides can be divided into plant cell wall polysaccharides (cellulose, hemicelluloses, pectin) and storage polysaccharides (e.g., starch, insulin, gums). They consist of many different monomeric components that are attached to each other by a variety of linkages. Fungi, bacteria and nematodes release varying amounts of mucilaginous substances that coat their bodies and provide an interface between the outer surface of the microorganism and its environment. The role of slimy polysaccharides is of utmost importance in wilt diseases. In the vascular wilts, large polysaccharide molecules released by the pathogen in the xylem causes mechanical blockage of vascular bundles and initiate wilting as in *Ralstonia solanacearum*. The high degree of structural complexity of plant cell wall polysaccharides has led to suggestions that some components might function as latent signal molecules

EPS and LPS were extracted from bacterial cultures, applied to potato roots, and tested for activity as an inducer of plant resistance to the plant-parasitic nematode. Whereas EPS did not affect *G. pallida infection*, LPS reduced nematode infection significantly in concentrations as low as 1 and 0.1 mg mL^{-1}. Split-root experiments, guaranteeing a spatial separation of inducing agent and challenging pathogen, showed that soil treatments of one-half of the root system with LPS resulted in a highly significant (up to 37%) systemic induced reduction of *G. pallida* infection of potato roots in the other half. The results clearly showed that LPS of *R. etli* G12 act as the inducing agent of systemic resistance in potato roots.