LITERATURE REVIEW

This review of literature is organized into eight topics relating to the study of water deficit on peanut genotypes with *Aspergillus flavus* infection on peanut pods. These topics are: Peanut or groundnut, Minirhizotron, WinRhizo, Root measurement system, Quantitative Analysis of Color System, Drought resistance and tolerance, Green fluorescent protein *Aspergillus flavus* and Root exudates.

The focus of the study was on the effect of water deficit on peanut root systems, root exudate or leachates and sloughed dead cells from roots and pods that might promote growth of *A. flavus. A. flavus* infection on peanut plant was used for screening method of aflatoxin resistance of peanut genotypes. The details of peanut plant are presented in the first topic. Detailed study of root systems requires modern methodology for observing roots. Thus, a review of minirhizotron and WinRhizo as modern tools for studying root systems and associated problems regarding the use of both tools are also presented. The sixth topic, drought resistance and tolerance, provides a background for the whole study on water deficit effects. Seventh topic describes growth of *A. flavus* and infection of flower, ovary, peg, and pod, which provides a background for the study of *A. flavus* survival under natural soil and plant conditions with water deficit. The last review topic describes root exudate or leachates, which provide substrates for *A. flavus* growth in soils. The most important substrates for *A. flavus* are likely to be dead leaves, flower, dead roots and some dead

bacteria. A more specific review of literature is presented at the introduction of each chapter in this study.

Peanut or Groundnut

Peanut or groundnut (*Arachis hypogaea* L.) is Plantarum species, in the family Leguminosae (2n= 4x= 40). Peanut originated in the area of southern Bolivia and north-western Argentina of South America. Peanut is now grown in many tropical, subtropical and temperate countries between 40 °N and 40 °S latitude, especially in Africa, Asia, North and South America. In Asia, peanut is a major crop in India, China, Indonesia, Burma, Thailand and Vietnam. Most production in each country is consumed locally, only Vietnam and Thailand export significant amounts of peanut.

Peanut plants grow and develop adequately on clay soil. For optimum growth, pH should be in the range 5.5-6.5. Spanish type is more tolerant to acid conditions (to pH 4.5) and some cultivars grow well in alkaline soil to pH 8.5.

Peanut can flower over a long period (20-60 days) depending on plant water status, irradiance, temperature and photoperiod. After fertilization, a stalk-like structure called " peg" or gynophore elongates below the ovary with and intercalry meristem. The peg grows down towards the soil, carrying the ovary at its tip, which becomes hardened into a protective cap as the peg enters the soil.

The peanut seed has two large cotyledons, an epicotyl with leaf and bud primodia, a hypocotyl and the primary root. Per 100 g edible portion, peanut contains roughly: 5.4 g water, 30.4 g protein, 47.7 g fat, 11.7 g carbohydrates, 2.5 g fibre and 23 g ash. The energy content averages 2,457 KJ/ 100 g. Peanut seeds are high in oil and protein and a good source of vitamins B and E.

Peanut pods harvested 110 days after seedling emergence in the field showed a higher germination percentage of seeds and vigorous seedling growth (Dey *et al*., 1999) and increased percent pod loss would be expected when digging was delayed (Shushu and Cutter, 1990). Slow drying of peanut pods helps to avoid physical damage, and reduces loss of vigor and viability (Dey *et al*., 1999).

Peanut is particularly susceptible to infestation by molds that produce the poisonous substrate aflatoxin. Reductions of aflatoxins in peanut are resistant cultivars, thick shells, waxy testa, improved farm management techniques and postharvest involving drying and storage (Pitt *et al*., 1991).

Minirhizotrons

Minirhizotrons are tools for non-destructive observation of roots in soil. They allow observation at different depths and locations throughout the crop growth duration. Minirhizotron are particularly appropriate for the study of temporal and spatial root responses to water deficit.

Minirhizotron are clear plastic tubes, which are usually made of acrylic or cellulose acetate butyrate. Tubes are inserted into holes bored in the soil and allow periodic observations of roots through the use of a digital camera. Horizontal minirhizotron tube orientation avoids overestimation of roots at the soil-tube interface as compared with actual roots in nearby soil. Thus, horizontal installation of minirhizotron tubes is preferred over other tube orientations. Horizontal tube orientation can easily be done in a greenhouse experiment set up through the use of large containers for growing plants.

The soil-tube interface may affect root growth and density if light penetrates through the minirhizotron tube (Klepper and Kaspar, 1994; Box, 1996). The end of tubes must be well sealed to prevent light from entering the tube, and inspection for light leaks is important.

New models of minirhizotron cameras (Bartz Technology Corp., Santa Barbara, CA) allow capture and storage of root images directly into a laptop computer. Root measurement can also be done from digital images using imaging software (Smucker *et al*., 1987). Digital image measurements using software packages may provide a faster and more accurate root measurements than the soil core method.

Estimation of root length density assumes that minirhizotron cameras view roots to 1 to 3 mm depth into the soil, depending on soil texture (Upchurch, 1987). Soil volume is estimated by multiplying the surface area observed by the minirhizotron camera by the assumed depth of view into the soil. Root length density is calculated from measured root length divided by the soil volume.

Root Analysis Software

WinRhizo

Several software packages have been used in analyzing digital root images in previous research, for example, GUNSCAN program (Kirchhof, 1992), Khoros software (Andren *et al*., 1996), and ROOTS (Goins and Russelle, 1996; Majdi and Nylund, 1996). These packages work best for clean root images, where roots were washed from soil cores, cleaned, dyed, and scanned against a white background producing sharp contrast between roots and soil. However, use of imaging software to

measure root dimension from minirhizotron images if difficult because there is hazy contrast between roots and soil background. There is a large background image noise, which translates to errors with automated image measurements.

WinRhizo is an image analysis system specifically designed for measurement of cleaned, stained roots. It can measure root morphology (length, area, volume…), and analyze root topology, architecture and color. It is comprised of a computer program and image acquisition component that are adapted. The WinRhizo *Basic*, *Reg* or *Pro* program does automatic root morphology analysis and more. It runs on standard desktop or portable computers.

- WinRhizo displays the analysis over the image. The color used to draw the root skeleton indicates into which diameter class the part of the root has been classified. The same color is used for drawing the root distribution graphic above the image.
- Root distribution graphic displays the root length, area, volume or number of tips as a function of root diameter or color. The number and the width of the classes are user-definable and can be change at any time.
- Measurement data of the sample under analysis is summarized on screen and is available in detail on data files.

Root position: Simply place the roots directly on the scanner glass or the Regent's waterproof trays. Root positioning is easy and fast with Regent's positioning system for optical scanners. Root can overlap and do not need to be randomly distributed.

Acquire the image: WinRhizo controls the scanner (or a digital camera) directly. It is TWAIN compatible, meaning that it can get images from many scanners or cameras. It can also analyze images stored in TIFF or JPEG files.

Analyze the roots: The analysis is complete and roots found by WinRhizo are identified by coloured lines in the image. The colours used for drawing the roots are coded according to their diameter.

Root length and diameter are measured with REGENT's unique method and with an indirect statistical method. With REGENT's method, measurements are made continuously at each point along the root. Root overlap, forks and tips are taken into account to provide accurate measurements of length and area. Image edition is also available to override decisions made by the system.

Save the measurement data: WinRhizo knows when data are ready to be saved and does this automatically. Data files are in ASCII (text) format easily readable by spreadsheet style programs like Microsoft Excel. Images can also be saved in files for later validations, analyzes or for visualization in other programs (www.regent.qc.ca/products/rhizo/Rhizo.html).

Root Measurement System (RMS)

Root Measurement System (RMS, Copyright, The University of Georgia) Version 2.5 was written in Visual Basic for MS Window 95 or higher to measure length and diameter of roots from digital images. Version 2.5 of RMS accepts only images of 640 by 480 pixels in JPEG format. RMS recorded number of roots in an image and calculates total root volume, total root surface area, and root length density.

For minirhizotron images collected in a filed study, an operator could analyze from 17 to 38 images hr^{-1} depending on number and length of roots in the images. With its speed, accuracy and versatility, RMS offers the possibility to analyze sufficient number of minirhizotron images to allow detection of treatment effects even under field conditions with large variability.

Quantitative Analysis of Color System (QuaCos)

QuaCos, a program written in Visual Basic, analyzes red, green, and blue values of pixels in a digital images, producing a spreadsheet of values for the colors that user selects. Users may also choose the resolution of analysis, from individual pixels to 500 × 500 pixel squares. QuaCos stores color values in a text file (ASCII), which may be read with eith a word processor or a spreadsheet program. Color intensity was scored on a 0 to 255 scale.

QuaCos operates under Windows 98 or higher operating systems and requires approximately 10 MB available on a hard drive.

Peanut drought resistance and tolerance

Water availability is a critical problem for most agricultural production. Water has become a limiting factor in world agriculture, and most crops are sensitive to even mild dehydration stress. Drought is an insidious hazard of nature, and it is the major factor responsible for low productivity under low-input conditions. Under a commercial system, water may be also a limiting factor. Thus, cultivars which are efficient in water utilization are required. Drought tolerance and resistance is part of the solution to low and erratic water availability.

Drought resistance. Drought resistance mechanisms include avoidance, tolerance, escape and recovery (Kramer, 1980) and integrated traits assisting crop performance (Subbarao *et al*., 1995). Drought tolerance is the ability of plant to endure low tissue water potential (Turner, 1979). Drought avoidance occurs when plants are able to maintain a water status within reasonable limits for normal metabolic functioning with limited water supply (Subbarao *et al*., 1995). This drought

avoidance is related through an improved good root system which is used for water absorption. There is some evidence that such tolerance may increase if the stress develops slowly. Leaf rolling and stomatal closure are also avoidance mechanism which restricts water loss by transpiration.

Deficient water at planting may hinder germination, lead to low plant populations and reduction of final yield that varies at stages of crop development. An initial plant response to water deficit is reduced opening of stomata which restricts water loss by transpiration. Leaf rolling may reduce transpiration mechanism in plant. The plant tissue elasticity as its affects the maintenance of turgor is more complex and involves the relationship of turgor to cell volume. Small cells are more elastic than large cells, hence tissue made up of small cells are more tolerant of drought stress.

Other drought tolerance mechanisms exhibited by plant include; efficient water transport system through greater lateral root biomass, modifications in root structure such as accelerated secondary thickening of lateral roots (Wan *et al*., 1996), high concentration of water-soluble carbohydrates in stem, slower shoot growth rate (Volarie *et al*., 1995) deep root systems, large root density and greater extraction of water from deeper soil profile (Senthong and Pandey, 1989; Senthong and Pandey, 1998; Boonpradub, 2000). Root length in deeper soil layers is important because deep root enables plant to resist water deficit, but the root system must be well established before the stress occurs to provide this resistance (O'Toole and Bland, 1987). Root systems are often overlooked in breeding programs because of difficulty in assigning root growth as compared to the shoots and other visible parts of the plant.

High root to shoot ratio has been associated with drought tolerance (Fernandez *et al*., 1996). Deeply-rooted plants can continue to absorb water until the drought

becomes severe and reaches deep into the soil. As roots grow, the root-to-shoot ratio changes. Increased root growth may result in less shoot growth, or the increased length and density of roots may change the ratio.

Aspergillus flavus Infection and Aflatoxin Accumulation

Aspergillus flavus is a soil-borne fungus that colonizes many substrates. This fungus is notorious for producing aflatoxin which is toxic to human and animal (CAST, 1976), thus many countries regulate the quantity of aflatoxin allowed in food and feeds (Park *et al*., 1988). Both *A. flavus* and aflatoxin can be detected from soil, peanut plants and stored products (seed, ground peanut, peanut meal and peanut oil). Many factors may affect the growth of fungus and toxin production, for instance, relative humidity, seed moisture, O_2: CO_2 ratio, characteristic of pod and seed, initial level of mold concentration and genetic property of peanut (Sander, 1986). It has been known that the best way to cope with this problem is to prevent contamination of *A. flavus* in the field and then sanitize peanut products during storage or handling. The best chemical to inhibit growth of *A. flavus* on peanut seeds was benomyl 50% (Research Report, 1990).

Accumulation of aflatoxin was neither dependent on moisture content nor colonization but on the nature of seed parts. Cotyledons and embryos are the two major parts that contain the toxin of 275.5 and 274.0 ppb, respectively (Jatumanussiri and Sommartya, 1988). Jatumanussiri and Sommartya (1988) also reported that growing peanut in infected soil would be a more appropriate screening technique for *A. flavus* resistance. Waranyuwat and Bhumibhamon (1989) reported that peanut seeds infection by *A. flavus* for 7 peanut lines was only 2.5% under laboratory

screening. When duration of seed storage was considered, infection percentage increased with increased storage time. Even resistant lines were infected, but at slower rates than the susceptible lines. Old seeds had more aflatoxin B_1 than new seeds.

Yingthongchai (1994) reported that both flowering and pod maturing stages were the most critical growth stage for *A. flavus* infection in peanut, but the degree of infection at both growth stages varied among resistant cultivars. He also reported that less than 30% of seeds infected by *A. flavus* was observed among the resistant cultivars and CMU1 collection, which showed the most resistance to *A. flavus* infection of seeds 0.65-5.00%. Manzo and Misari (1989) and Pitt (1989) reported that *A. flavus* can infect peanut plants during the flowering and pod filling stages. At the flowering stage, *A. flavus* can infect 7% as compared to the peg initiation stage, which infects only 0.3-1.5%.

Suriyong (1997) reported that thickness of seed coat of peanut has no correlation with the resistance to *A. flavus*. Resistant genotypes have an unchanged seed coat structure. In contrast, susceptible genotypes show a breakdown in seed coat structure when infected with *A. flavus*. This phenomenon indicated that the seed coat of resistant genotypes might contain some chemical or tissues that block the pathway and inhibit the spread of the fungus. Pettit *et al*. (1989) reported that seeds of Florunner cultivar peanut produced phytoalexins that could inhibit aflatoxin production.

***Aspergillus flavus* description.** *A. flavus* belongs to the fungi, organisms that are devoid of chlorophyll and thus unable to utilize the carbon dioxide of the air for the production of carbohydrates through photosynthesis. *A. flavus* is dependent on previously-elaborated compound for their supply of carbon (Hawker, 1950) and lives

on substrate materials including those from plant organism such as peanut, corn, cotton, soybean. *A. flavus* is an imperfect fungus with no known sexual stage in its life cycle, usually reproducing by conidia.

Outstanding characters of the *A. flavus* group are: i) Conidial heads globose to radiate or columnar; very light yellow- green, deep yellow green, olive-brown, or brown; ii) Conidiophores colorless, usually roughened but varying from smooth or nearly so to coarsely roughened; iii) Vesicles globose or subglobose at maturity in species with large heads, remaining clavate or flash shaped in species with small heads; fertile over most of their surface; iv) Sterigma uniseriate or biseriate with both conditions commonly seen in the same strain or on a single vesicle; v) Conidia in most species globose or subglobose when mature with roughening conspicuous or almost absent and often showing considerable intra strain variability in size; Sclerotia dark red brown to purple brown or black at maturity; globose; subglobose or vertically elongate (Raper and Fennel, 1973; Thom and Raper, 1945).

***Aspergillus flavus* life cycle**. The *A. flavus* life cycle starts from the spore and ends with spore formation in conidial structures. Life cycle duration is largely dependent on the kind of substrate present and conditions in the growing environment.

A. flavus spores are non motile. The spore is able to withstand a period of unfavorable conditions, which would be fatal to the vegetative parts of the fungus. *A. flavus* spores do not have any special method of spore discharge and depend on physical or biotic agents in nature for getting the spores away from the conidia fruiting body, or usually germinate within the conidia.

A spore germinates when it falls on a suitable moisture substrate with other conditions favorable. The general requirements for germination of spores are: suitable temperature, adequate moisture supply, adequate oxygen supply, suitable pH, and viable spores. Only a small proportion of fungal spores reaches a favorable substrate with favorable conditions for germination (Hawker, 1950). Spore bulges then elongate to form slender threads or germ tubes. Germinated spores become vegetative and form a thin but close textured basal mycelium.

Abundant conidial structures, forming conidiophores are then produced directly from the substrate mycelium. Conidiophores, heavy walled and coarsely roughened, are usually less than 1 mm in length, having stalk diameters immediately below the vesicles ranging from 10 to 20 μm. Conidiophores are upright, simple, terminating, globose or clavate swelling, with the phialides at the apex radiating from the entire surface (Barnett, 1960). Young conidial heads have yellow shades near strontium yellow or yellowish citrine. This transforms to dark yellow green shades and finally become deep grape green in age. Conidial heads radiate, splitting into several poorly defined columns less than 600 μm in diameter. Spores are borne in the conidial head. Spores ripen and mature ready to start the next cycle (Raper and Fennel, 1973).

Green Fluorescent Protein (GFP) *Aspergillus flavus*

A gene for a natural fluorescent protein from a jellyfish (*Aequorea victoria*) was tested for its ability to differentiate genetically-modified *A. flavus* from wild type fungus. This gene for green fluorescent protein (GFP) allowed detection of genetically-modified fungus. Detection of infection with the modified *A. flavus* could be performed without expensive equipment, faster and easier detection of plants. No microscope or light filters were required. Any black light was sufficient for detection of the GFP gene (www.nal.usda.gov).

Root Exudates and Apparent *Aspergillus flavus* Resistance

Among the carbon sources utilized by fungi are hexose, pentose, organic acids, disaccharide, starch, pectin, cellulose, fats and lignin, which is particularly resistant to bacteria degradation. Reduced nitrogen frequently comes from ammonium or nitrate, but protein, nucleic acids or other organic nitrogenous complex serves as well. Some species are nutritionally dependent, requiring B vitamins, amino acids or other growth factors for active proliferation, but many develop fully in media containing only a sugar and inorganic salts.

Plant and root systems are associated not only with an inanimate environment composed of organic and inorganic substances but also with a vast population of metabolically-active microorganisms. Because the crop plays a greater role than the soil, the nature of the plant's excretions and the chemical constituents of its tissues probably determine to a large extent the microbiological composition of the environment.

The vast number of viable cells so close to the root indicated that the plant is excreting and sloughing off large quantities of organic substances. The mechanism of greater importance, excretion or sloughing, has yet to be ascertained. Regardless of the precise explanation, however, products ultimately encountered by microorganisms vary from plant to plant. Some substances released by plants have been characterized, but the list is far from complete. Excreted compounds include amino acids, simple sugar, and nucleic acid derivatives. These substances are largely true excretions, not the result of sloughing or decomposition, since the compounds are isolated from aseptically-grown plants in the early phases of development. The most important plant contribution to the rhizosphere flora is the provision of excretion products and sloughed-off tissue to serve as sources of energy, carbon, nitrogen or growth factors.

Exudation of organic compounds from roots plays an important role in the ecology of the rhizosphere and also may be important in the colonization of underground peanut fruits by toxin- producing soil fungi such as *Aspergillus flavus* Link. ex. Fries. Griffin (1972) stated that there is an increase in populations of fungi, actinomycetes and bacteria in the geocarposphere, which is the soil adjacent to developing peanut pods, and that clamydospore of *Fusarium spp*. germinated readily in geocarposphere soil soon after peanut peg (gynophore) entered the soil. In contrast, appreciable germination of *A. flavus* conidia occurred in peanut geocarposphere soil only following mechanical injury of peanut pods, and the colonization of peanut pods by *A. flavus* is also favored by injury (Ashworth *et al*., 1965; Schroeder and Ashworth, 1965).

Several researchers have shown that injury increases exudation from roots. Sites of lateral root and adventitious root development have shown large amounts of

exudation (McDougall, 1968; McDougall and Rovira, 1970). McDougall and Rovira (1970) state that the lateral root zone was the major region of exudation but this was exudation mainly from the apices of the emerging lateral roots and not from points of rupture nor injury to the emerging root tips. Hale and Griffin (1976) reported that injury of the peanut pod surface resulted in a 10-20 fold increase in sugar exudation and an increase in the amount of amino-N exuded. Of sugars, exuded by peanut pods, glucose was the most stimulatory for *A. flavus* conidial germination (Pass and Griffin, 1972). Glucose plus amino-N supported the highest percentage of germination. Increasing the concentration of glucose, other single sugars or amino acids in axenic culture or of glucose plus peptone in soil increased *A. flavus* conidial germination (Griffin, 1969; Pass and Griffin, 1972).

Shay and Hale (1973) examined the effects of calcium levels on exudation of sugars and sugar derivatives from peanut root grown under axenic conditions and found that four times more sugar was exuded at 10 mg than at 50 mg of Ca^{2+} per liter. Ion influx measurements indicated that low levels of Ca^{2+} increased the root-cell-membrane permeability, resulting in a quantitative increase in exudation of sugar from peanut roots. A system of axenic culture is essential for the most accurate evaluation of the nature of exudates, a combination of exudates and sloughed matter actually provides the influencing principle affecting microbial populations and their many interactions.