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Infection and mycotoxin production by *Fusarium lactis*, causal agent of internal fruit rot of sweet pepper

by

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Abstract

Internal fruit rot, caused by *Fusarium lactis*, is as an important disease of greenhouse sweet pepper. Fungal growth was studied microscopically during anthesis and fruit development. Hyphae were observed on the stigmatal surface one day after inoculation (DAI), and in the transmitting tissues of the style and inside the ovary at 5 and 6 DAI. Symptomless seeds from infected fruits yielded colonies of *F. lactis* when cultured axenically, and typical disease symptoms were observed when fruits were dissected at 45 DAI. Isolates of *F. lactis* and the related species *F. proliferatum* and *F. verticillioides*, which are also associated with internal fruit rot, produced the mycotoxins beauvericin, moniliformin and fumonisin B_1 in various combinations, both in infected fruits and *in vitro*. These findings suggest that internal fruit rot is initiated through infection of the stigma and style during anthesis, and that mycotoxin contamination of infected fruit could pose a health concern.

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Chapter 1. General Introduction

1.1. Greenhouse Sweet Pepper

1.1.1. History and economic significance

Sweet pepper (*Capsicum annuum* L.) is a New World species that was extensively cultivated by the native populations of Central and South America. After the Spanish conquest of the Americas in the 1500s, sweet pepper was introduced to the Old World, where its cultivation spread throughout Europe and Asia, and the crop became established as an annual in temperate climates. In Canada, however, sweet pepper is produced mainly in greenhouses, as a consequence of its sensitivity to low temperatures. The predominant sweet pepper cultivars grown in Canada produce green, red, yellow and orange fruits.

Sweet pepper is a high-value cash crop, with production centered mainly in British Columbia, Alberta, Saskatchewan, Ontario and Quebec. Canadian production of greenhouse sweet pepper has more than doubled in the last decade, with the cultivated area increasing from 95.6 ha in 2001 to nearly 300 ha in 2007; the value of the crop over this same period increased from $64.5 million to more than $200 million (Table 1, Statistics Canada). Similar trends have been observed specifically within Alberta, as production increased from 559.3 t in 2001 to 1588 t in 2007. Yields of greenhouse sweet pepper range from 22 to 26 kg/m^2, with an average gross revenue of approximately $90/m^2 (Alberta Greenhouse Industry Profile 2007). Thus, even small decreases in yield per unit area can have significant economic consequences for growers.

1.1.2. Biology of sweet pepper

Sweet pepper is a member of the family Solanaceae, which consists of approximately 90 genera and 3000 species. These species exhibit a huge diversity in terms of habit (e.g., trees, herbs, shrubs, vines), preferred habitat (e.g., deserts, rain forests), and morphology of both the flowers (e.g., zygomorphic, tubular, actinomorphic) and the fruits (e.g., berry, capsule, drupe, mericarp) (Knapp et al. 2004). Under greenhouse conditions, sweet peppers usually exhibit an indeterminate growth habit (Government of Alberta 2007), producing flowers and fruit continually over the growing season. The sweet pepper flower has a star-shaped white corolla, usually five stamens (but sometimes up to six or seven) and a single pistil (Fig. 1-1). The pistil is composed of the stigma, style, and ovary. For successful fertilization of the ovules within the ovary, the pollen grains must first land on the stigma, after which they germinate to produce pollen tubes. The pollen tubes penetrate the stigma, grow down the style through the transmitting tissue (Fig. 1-2), and finally enter the ovules inside the ovary.

Sweet pepper flowers are largely self-pollinated. However, cross-pollination has been shown to result in the production of higher quality fruits with greater quantities of seeds (Cruz et al. 2005). Thus, cross-pollination may be important for seed set. In a greenhouse environment, cross-pollination can be performed manually by the grower, or via the activities of insect vectors such as bees. Pests like aphids and thrips may also function as vectors (ENV/JM/MONO, 2006). After successful fertilization, the sweet pepper fruit begins to develop and the seeds are formed. The color of the fruit depends on the specific cultivar grown.

The sweet pepper fruit or mature ovary is of the berry-type. Inside the fruit, there are three chambers (locules), which are divided by septa, and a basal placenta to which multiple seeds (ovules) are attached (Fig. 1-3). The seed micropyle faces the placenta and is imbedded in the placental tissue (Fig. 1-3). Therefore, the pollen tubes need to penetrate the placenta in order to reach the micropyle and ensure successful fertilization. The mature sweet pepper seed is disk-shaped, with the embryo completely enclosed within the endosperm and testa (seed coat) (Fig. 1-4). The germination process consists of three steps, namely: (1) water imbibition by the dry seed, (2) embryo expansion, and (3) radicle protrusion (Finch-Savage and Leubner-Metzger, 2006). To germinate, the embryo must break through two covering layers, the micropylar endosperm and the testa.

Since they can germinate immediately after harvest, sweet pepper seeds were traditionally regarded as non-dormant. However, Randle and Honma (1981) reported some degree of seed dormancy, especially in non-cultivated pepper varieties. Thus, it appears that the dormancy characteristics of sweet pepper are genotype-specific and can vary according to cultivar (Randle and Honma, 1981; Sanchez et al. 1993). It was suggested that pepper seeds should be left in the harvested fruit after harvest, to allow for a short after-ripening period to overcome dormancy and achieve maximum seed germination potential (Randle and Honma, 1981; Sanchez et al. 1993). In general, germination of a non-dormant seed requires water, oxygen and appropriate temperature, and may also be sensitive to other factors such as light or nitrate. Optimal conditions for the germination of

sweet pepper seeds include a temperature of 25 to 26 °C and a relative humidity of 75 to 80% (Government of Alberta 2007).

1.1.3. Diseases, arthropod pests and physiological disorders of greenhouse sweet pepper

Greenhouse sweet pepper is susceptible to numerous diseases and pests, and may also suffer from various physiological disorders. These can have a negative impact on productivity and represent a challenge to crop production. Fungal diseases of greenhouse sweet pepper include damping-off, Pythium crown and root rot, Fusarium stem and fruit rot, gray mold, and less commonly in Canada, powdery mildew.

Damping-off is a seedling disease caused mainly by *Pythium* spp. and *Rhizoctonia solani* Kühn. This disease is less common when seedlings are grown in inert media such as rockwool, compared to when they are grown in soil-based media (Howard et al. 1994). Nowadays, since vegetable seedlings and transplants are generally grown in rockwool, damping-off is not a major concern (Government of Alberta 2007). Pythium crown and root rot is caused by *Pythium* spp., but is not common in greenhouse peppers. It usually occurs in the seedlings as an extension of damping-off, or develops at the time of transplanting as a result of stressful environmental conditions. Thus, maintaining the seedlings under optimal growing conditions is essential for control of this disease (Government of Alberta 2007).

Fusarium stem and fruit rot, caused by *Fusarium solani* (Mart.) Sacc., causes the development of dark brown or black water-soaked lesions around the calyx or on the stems at nodes and wound sites. These lesions eventually spread to the fruit, and under humid conditions, fungal mycelium may proliferate and become visible to the naked eye (Howard et al. 1994). Proper sanitation practices, lower relative humidity, and careful handling of the plants and fruits to avoid injury are important for the prevention of Fusarium stem and fruit rot.

Gray mold, caused by *Botrytis cinerea* Pers.:Fr., is a common disease in greenhouses. Initially, olive-green, sunken and soft lesions develop on the sweet pepper stems and fruit. Eventually, these lesions collapse. On the fruits, injured areas are particularly prone to infection (Howard et al. 1994). A less common disease in Canada is powdery mildew, caused by *Leveillula taurica* (Lév.) G. Arnaud. It was first reported in Ontario in 1999 (Cerkauskas et al. 1999), and later identified as a new threat to greenhouse bell pepper in British Columbia (Cerkauskas and Buonassisi 2003). As is implied by the name, white powdery pustules form on the lower side of the leaves, with a slight chlorosis developing on the upper leaf surface in association with these pustules (Cerkauskas et al. 1999).

Several viral diseases can also affect greenhouse sweet pepper, including the pepper mild mottle virus (PMMV), tobacco mosaic virus (TMV), tomato spotted wilt virus (TSWV), and less commonly in greenhouse sweet peppers, tomato mosaic virus (ToMV). The most significant of these is PMMV, and the leaf symptoms that it causes may be confused with magnesium and manganese

deficiencies. As PMMV progresses, it is associated with the formation of noticeable bumps on the fruit. PMMV can be spread by routine handling of the young plants, especially during transplanting (Portree 1996).

In addition to diseases, a number of arthropod pests can also attack sweet pepper. These consist mainly of aphids, most commonly the green peach aphid *Myzus persicae* (Sulzer), thrips (the western flower thrips, *Franliniella occidentalis* (Pergande), and the onion thrips, *Thrips tabaci* Lindeman), the two-spotted spider mite (*Tetranychus urticae* Koch), and loopers (most commonly the cabbage looper, *Trichoplusia ni* (Hübner)). Some minor pests of greenhouse sweet pepper also include the whitefly (*Trialeurodes vaporariorum* (Westwood)), fungus gnats, lygus bugs (*Lygus* spp.), and earwigs (the European earwig, *Forficula auricularia* L.) (Government of Alberta 2007). In addition to the direct damage that they cause, arthropod pests may serve as vectors of disease and may also cause injuries that increase susceptibility to infection.

Sweet peppers may also suffer from various physiological disorders, which can cause symptoms similar to those associated with disease or pest damage. Important physiological disorders include blossom end rot (associated with environmental stresses such as water or calcium deficiencies), sunscald (associated with exposure to direct sunlight), fruit cracking (associated with sudden changes in the fruit growth rate), fruit splitting (a response to high root pressure), fruit spots (associated with high calcium levels in the fruit, and subsequent formation of calcium oxalate crystals), and misshapen fruit (occurring during periods of poor flower development or poor pollination, which are

generally associated with poor environmental conditions). In order to minimize the impact of these disorders, growers should aim to maintain optimal greenhouse conditions during the production cycle.

1.2. Internal Fruit Rot of Sweet Pepper and Pathogen Biology

1.2.1. Disease and symptoms

Internal fruit rot first emerged as a significant disease of greenhouse sweet pepper in British Columbia in 2001 (Utkhede and Mathur 2003). The disease was subsequently found in Alberta in 2003 (Yang et al. 2005), in Saskatchewan in 2006, and in Ontario in 2007 (Yang et al. 2008). A similar disease was also recently reported in greenhouses in the Flanders region of the Netherlands (Goossens et al. 2008). Disease development is typically associated with the growth of a whitish-grey mycelium on the seeds, placenta, and the inner surface of the pepper fruit wall. Although severe infection may result in the formation of greenish to dark-brown lesions on the outer surface of some fruits, external symptoms are generally rare, resulting in the “internal fruit rot” name of the disease (Utkhede and Mathur 2004).

1.2.2. Causal agent

Fungal isolates recovered from diseased pepper fruit from British Columbia greenhouses were identified as *Fusarium subglutinans* (Wollenweber & Reinking) Nelson, Tousson & Marasas based on morphological characteristics (Utkhede and Mathur 2003, 2004). However, most of the fungal isolates collected from peppers

showing internal fruit rot in Alberta were classified as *Fusarium lactis* Pirotta & Riboni, based on both fungal morphology and nucleotide sequence analysis of the partial elongation factor 1-α, mitochondrial small subunit ribosomal DNA and β-tubulin genes (Yang et al. 2006, 2009). Most *Fusarium* isolates causing internal fruit rot in Saskatchewan and Ontario greenhouses were also classified as *F. lactis*, as was an isolate from the Netherlands (Yang et al. 2006). Moreover, two isolates of *Fusarium* recovered from sweet pepper in British Columbia greenhouses, and originally identified as *F. subglutinans*, were later re-identified as *F. lactis* based on molecular analyses (Yang et al. 2006). Thus, it appears that *F. lactis* is the predominant causal agent of internal fruit rot of greenhouse sweet pepper, at least in Canada.

1.2.3. *Fusarium lactis* and the *Fusarium* genus

The anamorph genus *Fusarium* represents a group of filamentous fungi with teleomorph states in the genera *Gibberella*, *Albonectria* and *Haematonectria* (Leslie and Summerell 2006). Most species of *Fusarium*, however, have *Gibberella* teleomorphs. *Fusarium* spp. are widely distributed in the soil and are often found in association with plants. They may survive as saprophytes, endophytes, or parasites. More than 80 species have been recognized within the genus *Fusarium*, and many plants have at least one *Fusarium*-associated disease (Leslie and Summerell 2006). Diseases caused by *Fusarium* spp. include wilts, root and stem rots, fruit and seed rots, as well as foliar blights associated with the development of necrosis and chlorosis. Important *Fusarium* pathogens include

Fusarium graminearum Schwabe (Goswami and Kistler 2004), cause of *Fusarium* head blight of cereals, *Fusarium oxysporum* Schlechtendahl emend. Snyder & Hansen, the cause of Fusarium wilt in more than 100 plant species (Agrios 2005), and *Fusarium solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen, which causes Fusarium root, stem and fruit rot of non-grain crops. The latter conditions are sometimes also caused by certain *formae speciales* of *F. oxysporum* (Lee and Lee 2002).

Fusarium lactis was first isolated as a contaminant of milk and described by Pirotta and Riboni in 1879. In 1935, Wollenweber and Reinking were the first to report the fungus from figs (*Ficus carica* L.) (reviewed by Leslie and Summerell 2006). Nirenberg and O'Donnell (1998) re-examined cultures of *F. lactis*, confirmed the original description, and re-typified the species (neotype BBA 68590). *Fusarium lactis* is a member of the *Gibberella fujikuroi* (Sawada) Wollenw. species complex (Nirenberg and O'Donnell 1998), and can be morphologically confused with the closely related species *F. proliferatum*, *F. subglutinans* and *F. verticillioides*. Perhaps the most obvious feature that may be used to distinguish *F. lactis* from the other species is that the former produces microconidia in false heads (in which the spores are attached to each other) or in zigzagged chains of short- to medium-length (consisting of < 30 conidia) (Nirenberg and O'Donnell, 1998). In contrast, *F. proliferatum* and *F. verticillioides* usually produce microconidia in longer (> 30 conidia), linear chains. The microconidia of *F. subglutinans* are not produced in chains, but rather only in

false heads. The morphological characteristics of these four species are summarized in Table 2.

1.2.4. Host range

Fusarium lactis has been reported to cause disease on figs (Subbarao and Michailides 1993; Michailides et al. 1996) and fruits of greenhouse sweet pepper (Yang et al. 2006). However, there is limited information on the virulence of this fungus on other hosts. Utkhede and Mathur (2004) inoculated *F. subglutinans* (although this was most likely *F. lactis* [Yang et al. 2006]) isolated from greenhouse sweet pepper on tomato, cucumber and eggplant, but found that the fungus could not induce disease on these hosts. Indeed, the fact that *F. lactis* causes only internal fruit rot, and does not generally decompose the entire fruit, suggests that it is a weak phytopathogen. This may help to explain why this fungus has not been identified on hosts other than fig and greenhouse sweet pepper in the more than 100 years since it was first described.

1.2.5. Pathogen life cycle

As a recently identified pathogen of greenhouse sweet pepper, little is known regarding the life cycle of *F. lactis* on this crop. However, it may be possible to learn about the sequence of events related to pathogen spread and disease development by examining what is known regarding the disease cycle of endosepsis of figs, which is also caused by *F. lactis* (Michailides et al. 1996) and has been more extensively studied. Nirenberg and O'Donnell (1998) noted that

some isolates of *F. lactis* associated with fig endosepsis may have been mis-identified as *F. moniliforme* (Michailides and Morgan 1994, 1998) or as *F. moniliforme* var. *fici* (Subbarao and Michailides 1992).

The fruit of the fig is called a syconium, which consists of numerous fruitlets inside an enlarged, hollow, globose floral receptacle that is open at the apex. The fig plant is gynodioecious and has two forms: the monoecious caprifig (from which the pollen is produced) and the pistillate edible fig. Pollination requires the presence of fig wasps, which develop in the syconia of caprifigs. When mature, the female wasps fly out of the syconia in search of other caprifig syconia in which to lay their eggs. By chance, they may enter the pistillate edible figs, carrying with them the pollen from the caprifigs, thereby serving as pollinators.

During the pollination process, however, the wasps may also introduce fungal pathogens, including *F. lactis*, into the fig syconia. As the wasps enter the syconia through the ostiole at the apex, they must push through the ostiolar scales and consequently lose their wings and most of their antennae (Galil 1977; Michailides and Morgan 1994). As a result, the wasps can never leave the fig syconia, and when they die, the mycelium of *F. lactis* develops on their bodies. When the fig fruit matures, the fungus starts to infect the fruit tissue, thereby causing endosepsis (Michailides and Morgan 1998). Thus, pathogen spread is entirely dependent on the fig wasp and the disease can be considered to be airborne, since dissemination occurs through the activities of flying vectors. Moreover, since the wasps enter the syconia but never leave it, fig endosepsis has no secondary mechanism of spread and can be characterized as a monocyclic disease.

We hypothesize that the life cycle of *F. lactis* on greenhouse sweet pepper is similar to that on fig, particularly since both internal fruit rot of sweet pepper and fig endosepsis are associated with infection of the flowers and development within the fruit. However, unlike the fig, sweet pepper flowers have both male and female reproductive organs and are partially self-pollinated. The dissemination of *F. lactis* on sweet pepper, therefore, may not be as dependent on insect vectors. Moreover, insect vectors would not be trapped within the sweet pepper flowers, and the indeterminate growth habit of this crop would enable continued spread of the pathogen during the entire growing season. As such, unlike fig endosepsis, internal fruit rot of sweet pepper is likely a polycyclic disease. Infested flower and fruit debris could also allow saprophytic growth of *F. lactis* in greenhouses, serving as a source of inoculum for new cycles of infection.

1.2.6. Disease management

Thus far, there are no sweet pepper cultivars with genetic resistance to internal fruit rot (J. Yang, personal communication). Some biological and chemical treatments, however, have been found to significantly reduce the disease rate when applied to sweet pepper flowers (Utkhede and Mathur 2005). These treatments include the microbial fungicides PreStop® (*Gliocladium catenulatum* J.C Gilman & E.V. Abbott strain J1446) and Quadra-137 (*Bacillus subtilis* (Ehrenberg) Cohn), and the chemical fungicides Rovral® (iprodione) and BASF-516 (boscalid and pyraclostrobin).

In addition to chemical and biological control, a number of cultural disease management strategies have been suggested to combat internal fruit rot of sweet pepper. These include: (1) practicing good sanitation (for example, removal of infected or potentially infected plant debris), (2) keeping the relative humidity in the greenhouse at or below 85% (thereby reducing the high moisture conditions favored by many fungi), and (3) avoiding injury of the fruits during harvest (which might facilitate infection by a weak pathogen such as *F. lactis*) (Yang et al. 2008). These strategies are effective for reducing the incidence of many fungal pathogens, and should serve as the foundation for a proactive disease management plan by growers. Specific information on infection of sweet pepper by *F. lactis* should help in the development of additional strategies for controlling internal fruit rot in greenhouses.

1.3. Mycotoxins

1.3.1. *Fusarium* spp. as mycotoxigenic fungi

In addition to the direct yield losses associated with internal fruit rot of sweet pepper, the possibility exists that *F. lactis* could contaminate infected fruit through the production of mycotoxins. Mycotoxins are secondary metabolites of fungal origin that are toxic to humans and/or livestock when consumed in contaminated food and feed (Barkai-Golan and Paster 2008). They are produced by relatively few but universally present fungal genera, including *Aspergillus*, *Penicillium* and *Fusarium*. These fungi can produce fairly large quantities of certain mycotoxins, even in fruits and grains that show few symptoms of infection

(Agrios 2005). Given that internal fruit rot of sweet pepper does not usually cause any external symptoms, it is possible that if infected fruit is not culled before going to market, it could be consumed. Therefore, if *F. lactis* produces mycotoxins in the pepper fruit, then those consuming the infected peppers would be exposed to these toxins. Moreover, mycotoxins can remain in the fruit tissue after the mycelium is removed, and depending on the characteristics of the fruit, may also diffuse into healthy tissues (Restani 2008). Thus, even a mild infection may contaminate the entire pepper fruit.

Several species of the genus *Fusarium* are known to be mycotoxigenic. These fungal strains may produce mycotoxins in infected plants prior to harvest and/or after harvest in stored grains (Logrieco et al. 2002). The major *Fusarium* mycotoxins, including the fumonisins, trichothecenes, and zearalenone, are mainly found in grains and seeds (Dombrink-Kurtzman 2008). These represent the most toxic and frequently detected mycotoxins produced by *Fusarium* species. However, of these, only the fumonisins are produced within the *G. fujikuroi* species complex. Many *Fusarium* spp. within this species complex also produce so-called minor toxins, which consist of moniliformin (MON), beauvericin (BEA), fusaproliferin, fusarins, and other secondary metabolites such as fusaric acid and giberellic acid (Desjardins 2006; Moretti et al. 2007; Nirenberg and O'Donnell 1998). As a member of the *G. fujikuroi* species complex, it is possible that *F. lactis* from greenhouse sweet pepper also produces some of these mycotoxins. Indeed, *F. lactis* isolate BBA 58590, collected from a fig, was found to produce MON (Fotso et al. 2002) and BEA (Moretti et al. 2007). However, no information is available

on mycotoxin production by *F. lactis* isolated from sweet pepper, nor is there information regarding the *in planta* production of mycotoxins by *F. lactis* in sweet pepper. Since the biosynthesis and accumulation of mycotoxins can be influenced by the host species (Jackson and Al-Taher 2008), the issue of mycotoxin production in greenhouse sweet pepper needs to be examined.

1.3.2. Fumonisins

Fumonisins were initially identified from cultures of *F. verticillioides* (Gelderblom et al. 1988). Recently, 53 different fumonisins were described (Bartok et al. 2006), although not all of them occur naturally. Mycotoxins belonging to the fumonisin family can be classified into four main series (A, B, C and P), based on the type of side-chain connected to the fumonisin backbone (Fig. 1-5). In nature, the most abundant fumonisins are the fumonisin B analogs, which are diesters of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane. In the fumonisin B series, FB_1 is predominant (comprising 70 to 80% of total fumonisins), followed by FB_2 (15 to 25%) and FB_3 (3 to 8%) (Bartok et al. 2006). Fumonisin B_1 is also the most toxic metabolite in naturally contaminated food and feed (Krska et al. 2007; Rheeder et al. 2002). In infected cereals, fumonisins are produced mainly by *F. verticillioides* and *F. proliferatum*. As all fumonisins are heat-stable, they can survive normal food processing conditions. Heating aqueous solutions of FB_1 and FB_2 at temperatures of less than 150 °C leads only to small losses, although

steeping contaminants in water or a solution of sodium hydrogen sulphate may significantly reduce fumonisin levels (Arranz et al. 2004).

Fumonisins are associated with various toxicoses. These mycotoxins cause leukoencephalomalacia in equine species, pulmonary edema in pigs, esophageal and hepatic cancer in horses and rats, atherosclerosis in monkeys, immunosuppression in poultry, brain haemorrhages in rabbits, and decreased body mass in broiler chickens and turkey poults (Shephard 1998; Soriano and Dragacci 2004; Zollner and Mayer-Helm 2006). FB_1 is also considered to be a human carcinogen (International Agency for Research on Cancer 2002), and contamination of maize with this mycotoxin was correlated with an increased incidence of human esophageal cancer in the Transkei region of South Africa and Linxian County in China (Marasas 2001). The Scientific Committee for Food of the European Commission (2003) established a provisional maximum tolerable daily intake of 2 μg/kg of body mass for FB_1, FB_2, and FB_3, alone or in combination. The European Commission also established a maximum level of 100 to 500 μg/kg for FB_1 and FB_2 (alone or in combination) in grain-based products (Jestoi et al. 2004a).

1.3.3. Moniliformin

Moniliformin is another low molecular mass mycotoxin, which was first isolated from corn culture that had been inoculated with what was believed to be *F. moniliforme* (Cole et al. 1973; Springer et al. 1974). This culture was later shown to have actually been inoculated with *F. proliferatum*, but the name

"moniliformin" persisted (Munimbazi and Bullermann 1998). This mycotoxin is generally a sodium or potassium salt of 3-hydroxy-3-cyclobutene-1,2-dione (synonym: semisquaric acid) (Fig. 1-6). Pineda-Valdes et al. (2003) investigated the effects of autoclaving, baking, extrusion, frying, and roasting on the stability of MON. They concluded that the thermal stability of MON was similar to or greater than other *Fusarium* mycotoxins, including FB_1. In aqueous environments, MON was most stable at a pH of 4.0 (with no reduction in levels after heating at 100 °C for 60 min) followed by a pH of 7.0 (Pineda-Valdes and Bullerman 2000). However, cooking under alkaline conditions appeared to be an effective method for reducing MON levels in corn (Pineda-Valdes et al. 2002).

Consumption of MON can cause progressive muscular weakness, respiratory distress, cyanosis, histopathological changes in the internal organs (including the kidneys, lungs and pancreas), comas and even death in chickens, ducklings, mice, rats, minks, and sheep (as reviewed by Jestoi 2008). Although toxicological studies are lacking, Bottalico (1998) proposed that MON could be the cause of a fatal heart disease, known as Keshan disease, affecting people in certain regions of China and South Africa.

1.3.4. Beauvericin

Beauvericin was first isolated from an insect pathogen, the fungus *Beauveria bassiana* (Hamill et al. 1969). Since then, it has also been found to be produced by numerous *Fusarium* species, including *F. proliferatum*, *F. avenaceum*, and *F. subglutinans*. This mycotoxin is a cyclic hexadepsipeptide, which contains three

D-α-hydroxyisovaleryl and three N-methyl-phenylalanines residues (Fig. 1-7). Initially, BEA was studied for its insecticidal properties. In a variety of insect bioassays, BEA was found to be highly toxic to blow fly and Colorado potato beetle adults, mosquito larvae, and a Lepidopteran cell line (Desjardins 2006).

Many *in vitro* tests have also been employed to investigate the mechanisms of BEA toxicity. This mycotoxin appears to induce apoptosis (programmed cell death) and DNA fragmentation in mammalian cells, resulting in its toxicity to human hematopoietic, epithelial, and fibroblastoid cells (Logrieco et al. 1997, 1998). It was also shown that BEA can inhibit cholesterol acyltransferase activity, which may be associated with its cardiotoxicity (Jestoi 2008; Jestoi et al. 2004b). However, *in vivo* studies of BEA biological activity in animals are very limited. This mycotoxin was found to have oral and intraperitoneal LD_{50} values of 100 mg/kg body mass and 10 mg/kg body mass, respectively, in mice (Omura et al. 1991). In ducklings, gastric intubation doses of up to 100 mg BEA/kg body mass produced no 7-day median lethal dose response relative to MON (Vesonder et al. 1999). This suggests that BEA is not an acute toxin to ducklings, at least within the context of the bioassay employed (Vesonder et al. 1999). Similarly, no significant BEA toxicity was observed in broiler chickens (Leitgeb et al. 1999; Zollitsch et al. 2003) or turkeys (Leitgeb et al., 2000).

1.3.5. Combined effects of mycotoxins

There is evidence for interactions amongst mycotoxins when they occur together. These combined effects may be synergistic, additive or antagonistic.

For instance, the simultaneous application of FB_1 and α-zearalenol resulted in an enhanced inhibition of porcine cell proliferation in swine whole-blood cultures, relative to when these mycotoxins were administered separately (Luongo et al. 2008). Similarly, the joint application of FB_1 and T-2 toxin resulted in greater decreases in the rate of body mass gain in broiler chicks (Kubena et al. 1997). In contrast, an antagonistic interaction was observed between diacetoxyscirpenol and fusaric acid; together, these mycotoxins had a reduced impact on body mass gain in turkey poults, compared to when they were administered individually (Fairchild et al. 2005). There are few studies that have specifically examined the interactions between FB_1, MON and BEA. No synergistic effect was observed between FB_1 and MON when these were applied together to turkey poults (Bermudez et al. 1997; Li et al. 2000). In broiler chicks, a less than additive effect was detected between FB_1 and MON (Ledoux et al. 2003). Nonetheless, the possibility of synergistic or additive effects between mycotoxins suggests that even if the effects of a single mycotoxin are not significant in terms of human health, the co-occurrence of various mycotoxins or their occurrence with other secondary metabolites may still be a health concern.

1.4. Research Objectives

The relatively recent emergence of internal fruit rot of sweet pepper in Canadian greenhouses means that little is known regarding this disease. Hence, control strategies are based on the limited understanding of the interaction of its causal agent, *F. lactis*, with figs, and on good management practices for fungal

diseases in general. Increased knowledge regarding the life cycle of *F. lactis* on greenhouse sweet pepper could facilitate the development of additional control methods, which could be targeted specifically to this pathogen. An additional area of concern is the possibility of mycotoxin production by *F. lactis* in greenhouse sweet pepper, which seems likely given that an isolate of the fungus collected from figs produced both MON and BEA. If *F. lactis* from sweet pepper also produces mycotoxins, consumption of infected fruits could represent a health risk, particularly since infection often does not result in development of external symptoms (thereby increasing the chances that the fruit would be consumed).

The present research, therefore, had two principal objectives: (1) to evaluate and quantify production of the mycotoxins FB_1, MON and BEA by *F. lactis* and related species, both in culture and in pepper fruits, and (2) to examine the mechanism of sweet pepper infection by this fungus. As a part of objective (2), the possibility of seedborne transmission of *F. lactis* in greenhouse sweet pepper was also examined. The data obtained should help in the development of more efficient disease management strategies, and enable a more accurate assessment of the risk of mycotoxin exposure when consuming infected fruits.

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Table 1-1. Area devoted to cropping of greenhouse sweet pepper, total amount of fruit produced, and value of the crop in Canada and Alberta from 2001 to 2007 (Statistics Canada, 2003-2007).

	Alberta			Canada		
Year	Area (ha)	Production (t)	Value (million $)	Area (ha)	Production (t)	Value (million $)
2001	2.6	559.3	1.9	95.6	20,997.5	64.5
2002	2.9	668.1	2.3	108.1	24,733.4	78.7
2003	3.5	844.1	2.9	139.3	29,864.7	106.5
2004	4.4	1,087.5	4.4	185.2	40,669.1	136.9
2005	5.7	1,274.6	5.2	215.3	51,357.3	166.0
2006	7.9	1,984.5	7.0	266.8	60,571.5	195.8
2007	6.5	1,587.6	6.0	296.4	71,315.4	206.2

Table 1-2. Morphological characters of four similar *Fusarium* species belonging to the *Gibberella fujikuroi* species complex (Leslie and Summerell 2006).

Character		*Fusarium lactis*	*Fusarium proliferutum*	*Fusarium subglutinans*	*Fusarium verticillioides*
Macroconidia	Shape	Slender and straight to slightly curved	Slender and relatively straight	Relatively slender and slightly falcate	Relatively long and slender, slightly falcate or straight
	Apical	Bent	Curved	Curved	Curved and often tapered to a point
	Foot	Notched	Poorly developed	poorly developed	Notched or foot-shaped
	Number of Septa	Usually 3	Usually 3 to 5	Usually 3	3 to 5
Microconidia	Shape	Obovoid, often with a flattened base	Club-shaped with a flattened base	Oval	Oval to club-shaped with a flattened base
	Septa	0-1	0	0	0
	Sporulation	False head and zigzagged chains (short to medium- length)	Moderate length chains and less commonly false heads	False head, in which spores are glued together, no chains	Commonly long chains
Phialide	Shape	Mono- and polyphialide	Mono- and polyphialide	Mono- and polyphialide - the polyphialide may proliferate extensively	Monophialide

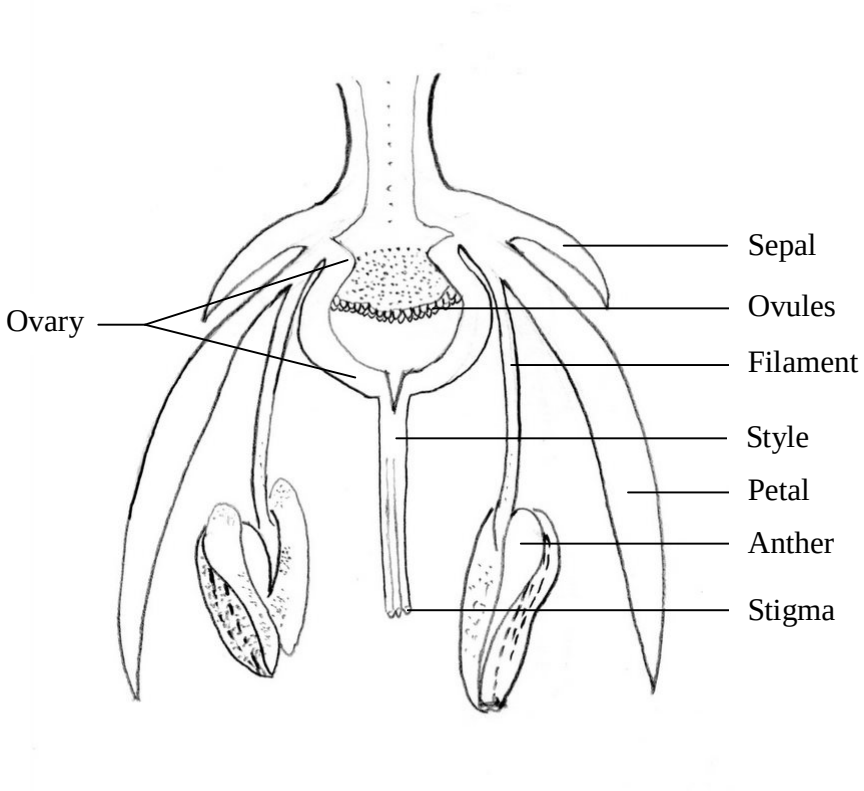


Fig. 1-1. Longitudinal section of a bell pepper flower, showing a complete flower composed of sepals, petals, stamens (including anthers and filaments) and a single pistil (including stigma, style, and ovary).

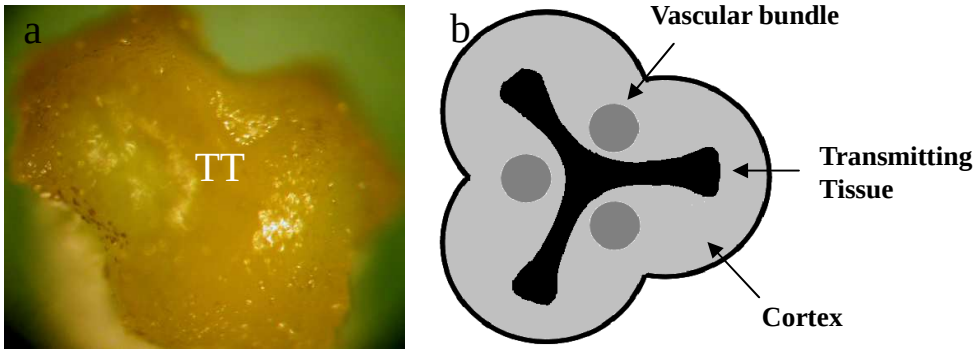


Fig. 1-2. a, Cross-section of a sweet pepper stigma, (magnification ×35), TT= transmitting tissue; **b**, Diagrammatic representation of the cross-section of a stigma (magnification ×35), showing the cortex, three vascular bundles, and the trifurcate transmitting tissue (based on a electron micrograph in Hu and Xu (1985)).

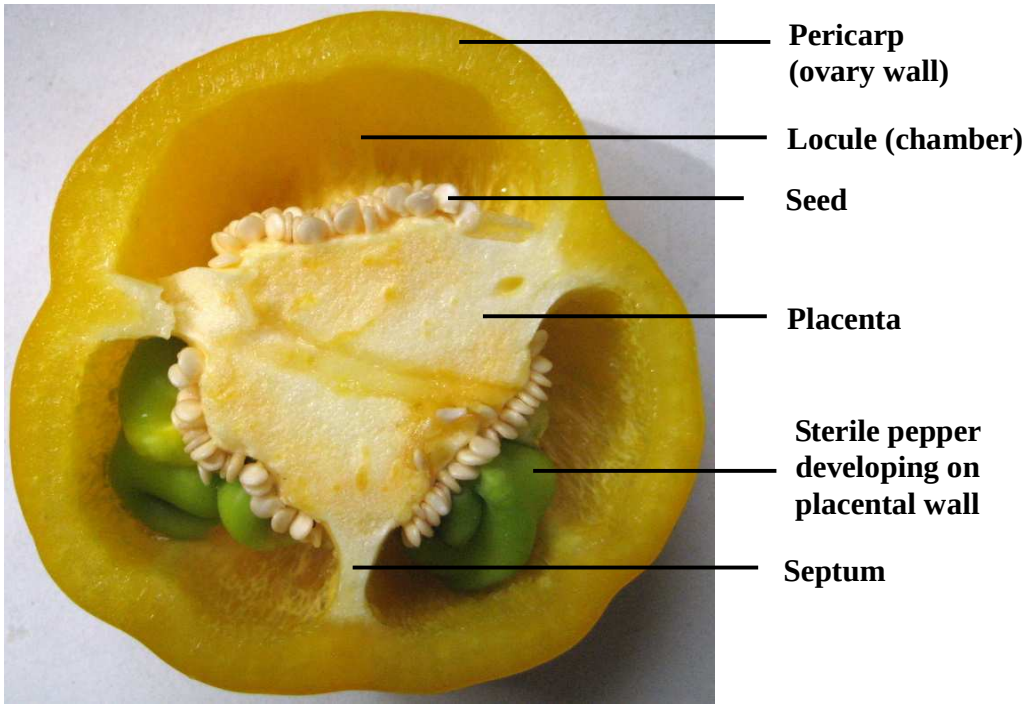


Fig. 1-3. Cross-section of a ripened ovary (fruit) of the sweet pepper.

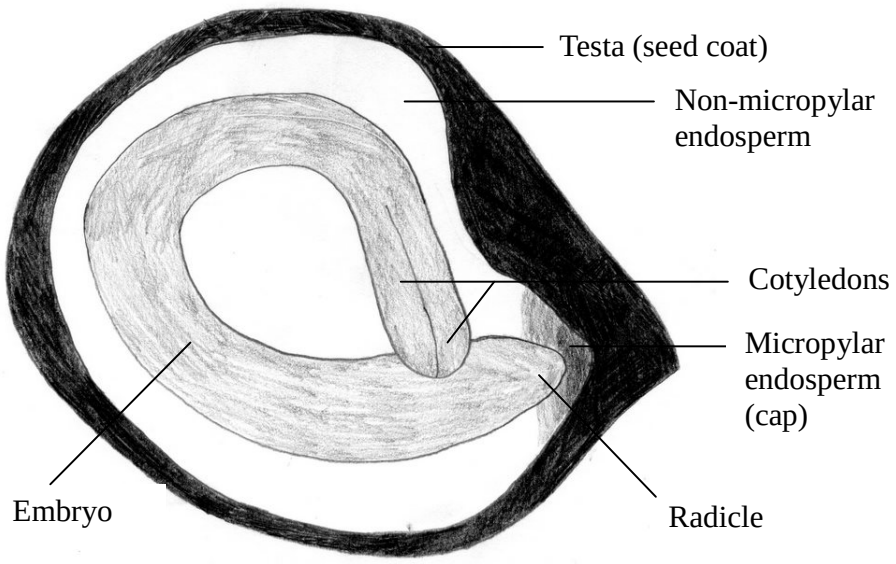
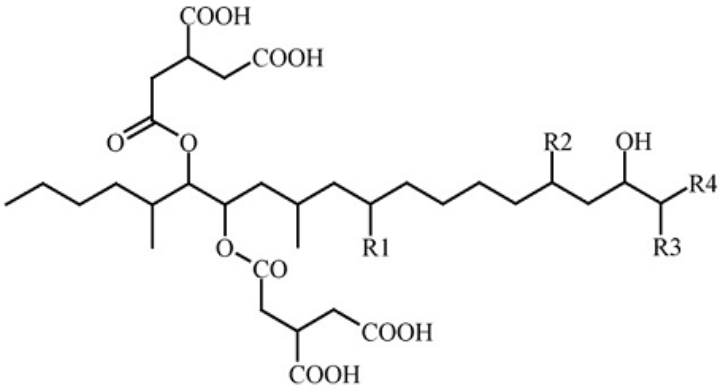


Fig. 1-4. A mature sweet pepper seed (based on Finch-Savage and Leubner-Metzger 2006).



	R_1	R_2	R_3	R_4
FA_1	OH	OH	CH_3CONH	CH_3
FA_2	H	OH	CH_3CONH	CH_3
FB_4	OH	OH	NH_2	CH_3
FB_4	H	OH	NH_2	CH_3
FB_4	OH	H	NH_2	CH_3
FB_4	H	H	NH_2	CH_3
FB_5	OH	H	NH_2	CH_3
FC_1	OH	OH	NH_2	H
FC_3	OH	H	NH_2	H
FC_4	H	H	NH_2	H
FD*	H	H	H	H
FP_1	OH	OH	3-hydroxypyridine	H
FP_2	H	OH	3-hydroxypyridine	H
FP_3	OH	H	3-hydroxypyridine	H

*Hydroxy group between R1 and R3 replaced by hydropen atom.

Fig. 1-5. Summary of mycotoxins belonging to the fumonisin family. The mycotoxins are classified into four main series (A, B, C and P) based on the type of side-chain connected to the fumonisin backbone (modified from Zollner and Mayer-Helm, 2006).

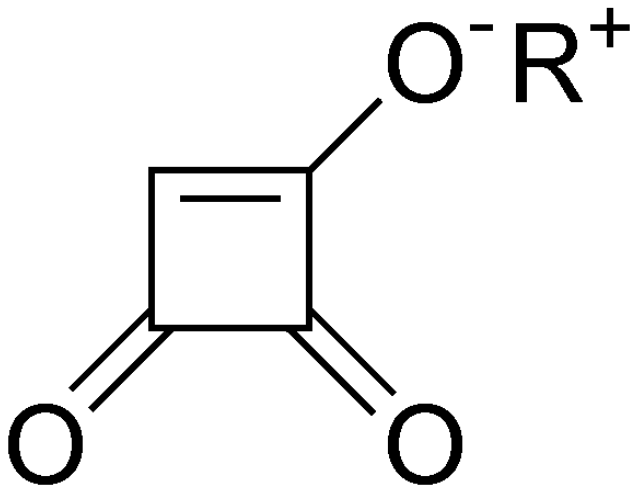


Fig. 1-6. The structure of moniliformin, where R = H, Na or K (image modified from Wikipedia (www.wikipedia.org); accuracy of structure confirmed in Burmeister et al. 1979).

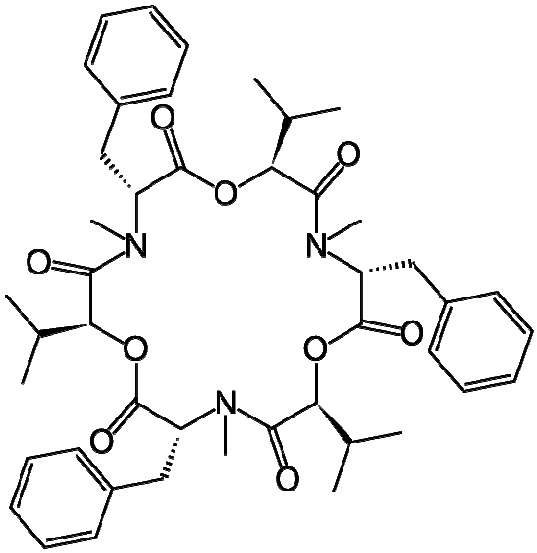


Fig. 1-7. The structure of beauvericin (image from Wikipedia (www.wikipedia.org) and used with permission; accuracy of structure confirmed in Jestoi 2008).

Chapter 2. Histopathology of Internal Fruit Rot of Sweet Pepper Caused by *Fusarium lactis**

2.1. Introduction

Sweet peppers (*Capsicum annuum* L.) are one of the most important vegetable cash crops worldwide, and are generally cultivated in open fields or greenhouses. In Canada, greenhouse sweet peppers are grown mainly in Ontario (over 50% of Canadian production), British Columbia, Quebec, Alberta and Saskatchewan, with production increasing in recent years. In 2007, there were 296.4 hectares of greenhouse pepper grown in Canada with an approximate market value of $206 million CAD (Statistics Canada 2007). Several fungal diseases can cause serious economic losses in greenhouse sweet pepper, including damping-off, Fusarium stem and fruit rot, gray mold and powdery mildew. Fusarium stem and fruit rot of greenhouse pepper, caused by *Fusarium solani* (Mart.) Sacc., was reported in Ontario and British Columbia in 1991, and caused a 5% fruit loss (Howard et al. 1994). However, a severe outbreak of this disease resulted in a 50% yield loss in an Ontario greenhouse in 1990 (Jarvis et al. 1994). Fusarium stem and fruit rot was also reported in Great Britain, with an infection rate of approximately 1% in greenhouse-grown sweet pepper (Fletcher 1994).

During the 2003 growing season, an unusual fruit rot of sweet pepper was observed in a commercial greenhouse in central Alberta (Yang et al. 2004). Unlike the characteristic feature of external fruit rot caused by *F. solani* (Howard et al. 1994), the mature fruit was infected internally by an unknown fungus. Seeds and

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the inner surface of the fruit wall were covered with a white mycelium. Greenish to dark brown lesions formed on the outer surface of some fruits. However, unless they were severely infected, most affected fruits were difficult to cull before delivery to market because external symptoms were not readily visible. Growers tried to grade out the infected fruit based on external observations, but were not successful and a significant amount of the harvested crop had to be destroyed. Consequently, marketable yields were reduced from 24 kg/m^2 to approximately 20 kg/m^2, causing a loss of around \$20/m^2 (M. Mirza, personal communication). Following its initial identification, internal fruit rot has become a major problem in greenhouse sweet pepper in Alberta. The causal agent has been identified as *Fusarium lactis* Pirotta & Riboni, based on fungal morphology and sequence analysis of the elongation factor 1-α, mitochondrial small subunit ribosomal DNA and β-tubulin genes (Yang et al. 2009). A similar internal fruit rot of sweet pepper, caused by *Fusarium subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas, was found in British Columbia in 2002 (Utkhede and Mathur 2003, 2004).

Ngugi and Scherm (2006) classified flower-infecting fungi into three groups, based on the types of infections with which they are associated. These groups include fungi that cause: (1) unspecialized infections, (2) specialized gynoecial entry infections, and (3) specialized systematic infections. One of the gynoecial entry fungi, *Monilinia vaccinii-corymbosi* (Reade) Honey, can infect blueberry flowers through the stigma-style pathway (Ngugi and Scherm 2004). Fungal development in the stylar canal mimics that of the pollen tubes, exhibiting highly

directional growth and adhering to the exudates-lined inner surface of the style (Ngugi and Scherm 2004, 2006). As *F. lactis* grows only on the inside of the pepper fruits, causing few external symptoms, it is possible that this pathogen is also a specialized gynoecial entry, flower-infecting fungus.

Hu and Xu (1985) reported that the mature stylar transmitting tissue of the sweet pepper flower is composed of parenchyma cells with large intercellular spaces, and that the pollen tubes grow through the intercellular spaces of this tissue. They also noted that the transmitting tissue may provide nutrients for pollen tube growth. These characteristics of pepper flowers could also provide a favorable environment for growth of and infection by fungal pathogens. *Fusarium lactis*, which is not very aggressive relative to *F. solani* (Yang et al. 2006), might be able to pass through the intercellular spaces of the transmitting tissue with little damage to the host cells. Ofosu-Anim et al. (2006) reported that pollen tubes reached the ovary within 1.5 days after pollination and entered the ovules within 2 days after pollination. It is thus possible that the fungal hyphae penetrate the ovule shortly after flowering by following the pollen tube pathway. Moreover, the stigmata and styles become browned and withered after anthesis (Ofosu-Anim et al. 2006), and the senescing floral tissues may be easily infected by weak pathogens such as *F. lactis*.

These observations support a previous suggestion that the stigma-style pathway is similar to a natural wound through which pathogens can preferentially enter (Ngugi and Scherm 2006). Utkhede and Mathur (2004) noted that 3% of sweet pepper seedlings germinated from seeds provided by a commercial supplier

were covered with a mycelium similar to that of *F. subglutinans*, another casual agent of internal fruit rot. This also suggests that internal fruit rot pathogens may be seed-transmitted. In the current study, we tested the hypothesis that internal fruit rot of sweet pepper, caused by *F. lactis*, is initiated via infection of the stigma and style at the flowering stage. The objectives of this study were to: (1) examine the mechanism of infection by *F. lactis*, and (2) evaluate the possibility of seedborne transmission of the pathogen in greenhouse sweet pepper. To our knowledge, this is the first comprehensive study of sweet pepper fruit rot caused by *F. lactis*.

2.2. Materials and Methods

2.2.1. Plant material

Seeds of the sweet pepper cv. Sympathy, provided by the Rijk Zwaan Seed Company (De Lier, the Netherlands), were planted in 2.5 cm × 2.5 cm × 2.5 cm rockwool cubes (Westgro, Calgary, AB) soaked in shallow water in plastic trays and maintained in a growth chamber at 25°C/20°C (day/night). When the seedlings were 2 weeks old, they were transplanted to 25 cm diameter pots (at a density of 1 plant per pot) filled with Pro-Mix (Premier Horticulture Inc., Quakerton, PA, USA) growth medium and kept in a greenhouse under the same conditions. Plants were watered with a nutrient solution containing 200 ppm each of nitrogen and calcium, 55 ppm each of phosphorus and magnesium, 318 ppm potassium, 3 ppm iron, 0.5 ppm manganese, 0.12 ppm each of copper and molybdenum, 0.2 ppm zinc and 0.9 ppm boron (Calpas 2001), with a pH of 6.0

and an electrical conductivity of 2.5 $S \cdot cm^{-1}$ in an open hydroponic system in a greenhouse at the Alberta Research Council, Vegreville, AB.

2.2.2. Fungal material and inoculation

Isolate F2004-C of *F. lactis*, which was originally obtained from a sweet pepper fruit from a greenhouse in central Alberta, was grown on carnation leaf agar (CLA, Leslie and Summerell 2006) at 25°C under alternating light/dark conditions (12h/12h) for 2 weeks to induce sporulation. After sporulation, conidial suspensions were prepared by vortexing five culture plugs (each 1 cm in diameter) in 10 mL sterilized distilled water (sdH_2O) in a 20 mL test tube, with 0.001% (v/v) Tween 20 (polyoxyethylene glycol sorbitan monolaurate) included as a surfactant. The conidial suspension was adjusted to a concentration of 0.8×10^6 to 3×10^6 spores/mL by counting both macro- and microconidia on a haemocytometer. Fungal inoculation was performed 24 h after anthesis by placing 10 µL of the conidial suspension on the stigma and anthers, immediately after they were artificially pollinated by gentle brushing. The inoculated flowers were harvested 0.5, 1, 2, 5, 6, or 11 days after inoculation (DAI), and prepared for examination using stereo, fluorescence and scanning electron microscopy as described below. Some of the inoculated pistils were surface-sterilized and cultured on potato dextrose agar (PDA) to confirm infection. Non-inoculated pistils served as controls.

2.2.3. Stereomicroscopy

Pepper flowers were harvested at various DAI and randomly selected ovaries were dissected and examined for evidence of infection with a stereomicroscope at 50-fold magnification. Some of the ovules on which there appeared to be hyphal-like growths were cultured on PDA to confirm infection.

2.2.4. Cryo-sectioning, light and fluorescence microscopy

Freshly harvested stigmata and styles were mounted in a drop of cryomatrix (with 10% polyvinyl alcohol and 4% polyethylene glycol) (Shandon, Thermo Electron Corporation, Waltham, MA, USA) and placed in the cryo-chamber of a cryotome (Shandon, Thermo Electron Corporation, Waltham, MA, USA) that was set to -17 ± 2°C. The frozen samples were then dissected into 9 µm sections and arrayed on microscope slides in the cryo-chamber. After the cryomatrix melted at room temperature, some slides were stained with drops of lactophenol cotton blue and examined by light microscopy. Most sections, however, were stained with several drops of 0.005% aniline blue in 0.15M K_2HPO_4 (Khatypova et al. 2002) and observed with a fluorescence microscope (Standard 14 Fluorescence Microscope, Carl Zeiss, Oberkochen, West Germany) under blue light excitation (480 nm). Images were recorded with a digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) mounted on the microscope and connected to a computer. Software Spot (Diagnostic Instruments Inc.) was used for image processing and analysis.

2.2.5. Scanning electron microscopy

The method of Viret et al. (2004), with some modifications, was used for scanning electron microscopy (SEM). Inoculated stigmata and styles were fixed in 5% glutaraldehyde in 0.03M 1,4-piperazine bis (2-ethanosulfonic acid) (PIPES) buffer (pH 6.8 - 7.0) (Viret et al. 2004) for 2 h under vacuum, rinsed in PIPES buffer and dehydrated in a graded ethanol series (10%, 30%, 50%, 60%, 70%, 80%, 90% and 100%). The samples were then critical-point dried in CO_2, mounted on aluminum stubs, sputter-coated with gold and observed in a scanning electron microscope (Jeol X-Vision JSM6301 FXV, Tokyo, Japan) at 5 to 11 kV.

2.2.6. Cryo-stage SEM

Fresh samples were mounted on stubs with glue, and frozen for 15 min in liquid nitrogen at –210°C in a cryo-stage chamber (Emitech K1250, Ashford, Kent, UK). After a freeze-fracturing step, the samples were placed on a scanning electron microscope (Jeol JSM-6301 FXV) stage for 30 min at -40°C to thaw ice crystals, and were then gold-coated at -178°C in the cryo-stage chamber. The specimens were observed directly by SEM in the cryo-stage.

2.2.7. *In vitro* interaction between germinating pollen grains and fungal conidia

Sweet pepper pollen grains from newly opened flowers were brushed into a 1.5 mL Eppendorf tube and mixed with a 1 mL conidial suspension of *F. lactis* F2004-C. Three sets of freshly excised stigma-style complexes were added to the

suspension and physically crushed with forceps in order to promote pollen grain germination and serve as a source of nutrition. A 150 µL volume of the resulting mixture of pollen grains, *F. lactis* conidia and crushed pepper stigma-style complexes was spread on 2% water agar in 90 × 15 mm Petri dishes, incubated at room temperature for 4, 18 and 24 h, and examined by light microscopy. The experiment was repeated three times. A mixture of pollen grains and crushed stigma-style complexes without conidia was used as a control.

2.3. Results

2.3.1. Pre-penetration

Scanning electron microscopy of inoculated floral tissues revealed hyphal growth on the surface of the stigma (Fig. 2-1a) and along the side of the style (Fig. 2-1b) at 12 h after inoculation. In non-inoculated flowers, pollen grains were visible on the surface of the stigma, but no hyphae were observed on or in the stigma-style complex (data not shown). The hyphae in the inoculated treatments continued to proliferate on the surface of the stigma and were more apparent at 1 DAI (Fig. 2-1c). Spores of *F. lactis* had also germinated and begun to grow on the surface of the anthers at 1 DAI, colonizing many of the pollen grains (Figs. 2-1d and 2-1e). The fungus continued to grow on the anther surface over the next day, so that by 2 DAI extensive hyphal growth could be observed (Fig. 2-1f). Inoculated stigma-style complexes yielded colonies of *F. lactis* when cultured on PDA (data not shown).

2.3.2. Penetration and post-penetration

Hyphae of *F. lactis* could be observed on the stigma throughout the time-course of the study, including at 6 DAI (Figs. 2-2a and 2-2b). Growth within the style, however, was not clearly visible until 5 to 6 DAI after inoculation. Nevertheless, by 6 DAI, penetration and growth of the fungus within the style was evident in the inoculated tissues (Figs. 2-2c, 2-2d and 2-2e). No specialized penetration structures, such as appressoria, were observed at any point in this study. The transmitting tissue in the inoculated stigma-style complexes fluoresced a yellowish-red color, indicative of cell death, by 6 DAI (Figs. 2-3b and 2-3d), while in the non-inoculated stigma-style complexes, the transmitting tissue was generally greener (indicating greater cell viability) (Figs. 2-3a and 2-3c). Pollen tubes, visible as bright green spots or strands in the transmitting tissue of the non-inoculated styles, could also be observed in cross (Fig. 2-3a) and longitudinal tissue sections (Fig. 2-3c), respectively. Close examination confirmed the absence of septa in the pollen tubes (Fig. 2-3e) along with their presence in the fungal germ tubes (Fig. 2-3f). By 11 DAI, the styles in both the inoculated and non-inoculated treatments had turned brown, and by 2 weeks after inoculation, the styles had wilted or fallen off. No hyphal-like structures were observed in or on the non-inoculated stigma-style complexes at any time-point, when these were examined by fluorescence microscopy. However, it was impossible to distinguish fungal hyphae from plant tissues stained with lactophenol cotton blue or acid fuchsin using light microscopy.

2.3.3. Infection of the ovary

The presence of hyphae inside the ovaries of inoculated flowers could also be observed by SEM; at 5 and 6 DAI, the hyphae had colonized the surface of the placenta and the ovules (Figs. 2-4a and 2-4b), and had also penetrated into the placenta (Fig. 2-4c). This hyphal-like material was confirmed to be fungal in origin by culturing of the suspect strands on PDA plates for a 6-day period; *F. lactis* colonies grew from all of the hyphal-like strands and some of the ovules transferred to PDA. In contrast, the ovaries of non-inoculated flowers appeared healthy and no fungal colonies could be recovered when these were cultured on PDA (data not shown).

2.3.4. Infection of the fruit and seeds

Pepper fruits that developed from *F. lactis*-inoculated flowers exhibited no external symptoms at 45 DAI. However, when the fruits were sectioned, fungal mycelium was found growing on the placenta and seeds near the point where the style tissues originally met the ovary (Figs. 2-5a and 2-5b). Some of the infected seeds were grayish-black in color and were covered by mycelium. Examination by SEM on a cryo-stage revealed hyphal growth and sporulation within the fruits and on the seeds (Figs. 2-6a and 2-6b). Furthermore, hyphae could be observed on the inside of the seed coat (Fig. 2-6c) and inside the endosperm (Figs. 2-6d, 2-6e and 2-6f). In some cases, *F. lactis* was also able to colonize the inner wall of the ovary/fruit (data not shown).

Healthy-looking seeds, randomly picked from a healthy-looking but inoculated pepper fruit at 36 DAI, were cultured on PDA directly or after surface sterilization. Five days later, 11% of the surface sterilized seeds and 44% of the non-treated seeds yielded typical colonies of *F. lactis* (Fig. 2-7).

2.3.5. Interaction between germinating pollen grains and fungal conidia

Light microscopy revealed no strong interactions between germinating pollen grains and conidia of *F. lactis* on axenic culture (Figs. 2-8a and 2-8b). In some cases, the young hyphae appeared to colonize the pollen grains, but no fungal proliferation within the pollen tubes was observed (Figs. 2-8c and 2-8d). Colonization of the pollen grains prevented their germination.

2.4. Discussion

Microscopic examination of pepper flowers inoculated with *F. lactis* revealed hyphal growth on the stigma as early as 12 h after inoculation (Fig. 2-1b), and growth within the style (Fig. 2-2) and on the ovary tissues (Fig. 2-4) was evident at 5 and 6 DAI. Fungal colonization of the style likely occurred prior to this time, since it would have presumably preceded infection of the ovary; however, hyphae of *F. lactis* were not readily observed until later, perhaps reflecting the limited proliferation of the mycelium within the style tissue (Fig. 2-2). Nonetheless, the occurrence of mycelial growth on the surface of the stigma and inside the style supports the hypothesis that *F. lactis* infection of sweet pepper fruits is initiated via the infection of the stigma and style during anthesis. The absence of any penetration structures, such as appressoria, is also consistent with

this hypothesis, because it appears that formation of such structures is not required for successful infection. The existence of large intercellular spaces in the transmitting tissue of the style (Hu and Xu 1985) may facilitate hyphal growth within the style, even for a weak pathogen such as *F. lactis*. Senescence of the style appeared to occur later in the current study relative to a previous report (Ofosu-Anim et al. 2006), perhaps as a result of different experimental conditions or cultivars used. Nevertheless, the senescing tissues may have provided a favorable environment for the fungus, and the decreased viability of the transmitting tissue (Fig. 2-3) could indicate selective ingress of *F. lactis* through this tissue. Moreover, at 45 DAI, the fungal hyphae colonized the placenta and seeds at the position close to the original location of the style (Fig. 2-5). Since there is no evidence to suggest that *F. lactis* penetrates directly through the ovary wall from the outside, the stigma-style complex may be the main pathway for pathogen ingress. The strongest support for this mode of entry, however, may come from the microscopic detection of hyphal growth in the stigma-style complex (Fig. 2-2) and on the ovules (Fig. 2-4).

Ngugi and Scherm (2004) found that when the pathogen *M. vaccinii-corymbosi* infected blueberry flowers, it was able to mimic the pollen-pistil interaction by highly directional growth. However, both directed and branched growth patterns were observed in *F. lactis* on/in the stigma and style at the early stages of infection. Thus, although *F. lactis* did not kill the plant tissue immediately and exhibited weak pathogenicity on pepper fruits (Yang et al. 2006), there is no indication that the fungus can mimic the pollen-pistil interaction during

the infection process. Nonetheless, after growing through the stigma and style and entering the ovary chamber, hyphae of *F. lactis* became established on the placenta and ovules (Fig. 2-4). Fungal pathogens may invade the ovule through the micropyle by following the path of the pollen tube or as free-growing mycelium in the locule (Dalbir and Mathur 2004). Since the pepper ovules are anatropous (i.e., the ovule is inverted and straight with the micropyle facing the placenta), and the micropyle of the ovule is fully imbedded in the placenta tissue, the penetration of *F. lactis* into the placenta (Fig. 2-4c) suggests that the pathogen may infect the seed through the micropyle by following the growth path of the pollen tubes.

In some inoculated pepper fruits, a number of the seeds were colonized by fungal mycelium and/or exhibited a strong discoloration (Fig. 2-5), which suggests that *F. lactis* probably utilized nutrients from the ovules/seeds to support its growth and cause internal infection of the fruits. However, symptomless seeds collected from one of the asymptomatic pepper fruits also yielded colonies of *F. lactis* when cultured on PDA (Fig. 2-7), indicating that the fungus can likely be spread via symptomless infection of the seeds to different greenhouses. The possibility of seed transmission was also suggested by Utkhede and Mathur (2004), who found that the fungus *F. subglutinans*, which is also associated with internal fruit rot, could be recovered from commercially purchased seeds. Thus, seed transmission appears to play a role in disease spread, although further work is required to understand how the fungus becomes established in greenhouses and spreads from infected seeds to flowers. It is worth noting that two fungal

isolates from British Columbia, originally identified as *F. subglutinans*, were later reclassified as *F. lactis* based on molecular analyses, and that most *Fusarium* isolates causing internal fruit rot collected from greenhouses in Alberta, British Columbia, Saskatchewan and Ontario were also *F. lactis* (Yang et al. 2006).

In vitro examination revealed that germinating spores of *F. lactis* could colonize the pollen grains, thereby reducing their germination (Fig. 2-8), although proliferation of hyphae within the pollen tubes was not observed. It is possible that the *in vitro* system, consisting of a mixture of pollen grains, spores and crushed pepper stigma-style complexes, did not accurately reflect the *in planta* situation. Additional examination of this issue may be warranted, particularly since colonized pollen grains may be picked up by pollinators and spread amongst flowers in a greenhouse. Indeed, fungal hyphae and intercalary chlamydospores were observed on pollen grains on a bee collected in a pepper greenhouse in Alberta (data not shown).

Based on the observations made in the current study, we propose a model for sweet pepper infection by *F. lactis* that begins with the deposition of conidia on the stigma by insect pollinators or from the air (Yang et al. 2008). After germination and a brief period of growth on the surface, the fungus penetrates the stigma tissue and grows down the style. Hyphae also grow superficially along the outer surface of the style, but rarely penetrate from the outside. Eventually, the fungal hyphae reach the ovary tissue and begin growth on the inner surface of the ovary as well as penetrating the placenta and some of the ovules. This infection through the floral reproductive tissues results in the typical internal fruit rot

symptoms associated with this disease. Moreover, infection of the fruit interior results in the production of infected seeds. Some of these seeds may have no external signs of infection, thus facilitating seed-borne transmission of *F. lactis*. It is likely, therefore, that internal fruit rot will continue to be a problem in greenhouse sweet pepper production. Nonetheless, proper sanitation and careful selection of seed sources may help to reduce the impact of this disease.

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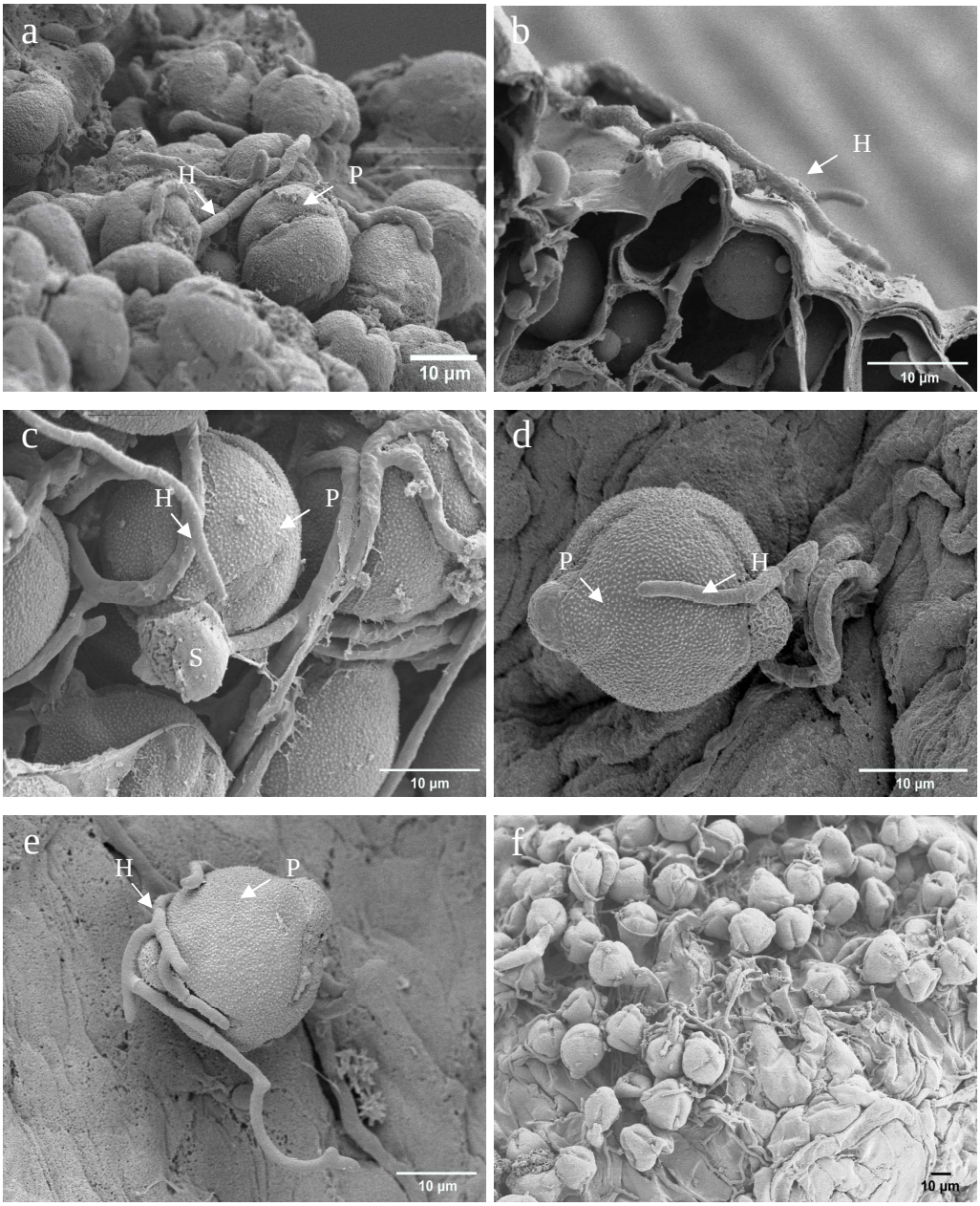

Fig. 2-1. Scanning electron micrographs of sweet pepper stigmata and anthers after pollination and inoculation with *Fusarium lactis*. **a,** Fungal hyphae (H) colonizing pollen grains (P) on the stigma, 0.5 day after inoculation (DAI); **b,** Hyphae growing on the outer surface of the style, 0.5 DAI; **c,** Germinated spore (S) and extensive hyphal (H) growth on the stigma and pollen grains (P), 1 DAI; **d** and **e,** colonization of pollen grains (P) by hyphae (H) on the anther surface, 1 DAI; **f,** extensive hyphal growth on the anther surface 2 DAI.

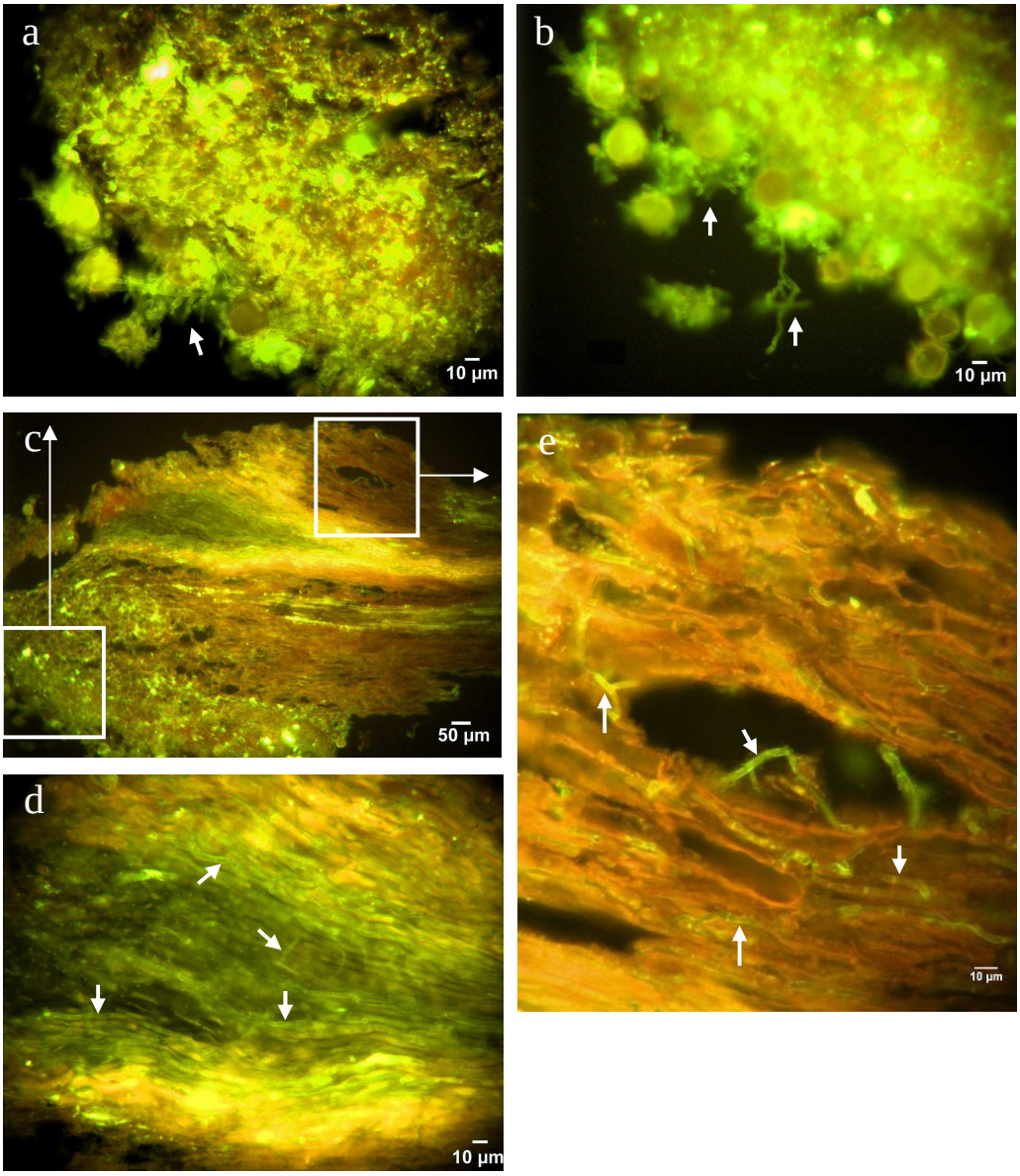

Fig. 2-2. Fluorescence micrographs of the sweet pepper stigma-style complex, 6 days after inoculation (DAI) with *Fusarium lactis*. **a** and **b,** Hyphae (arrows) growing on the stigma; **c,** Longitudinal section of the infected stigma-style complex; **d** and **e**, Hyphae (arrows) growing through the style.

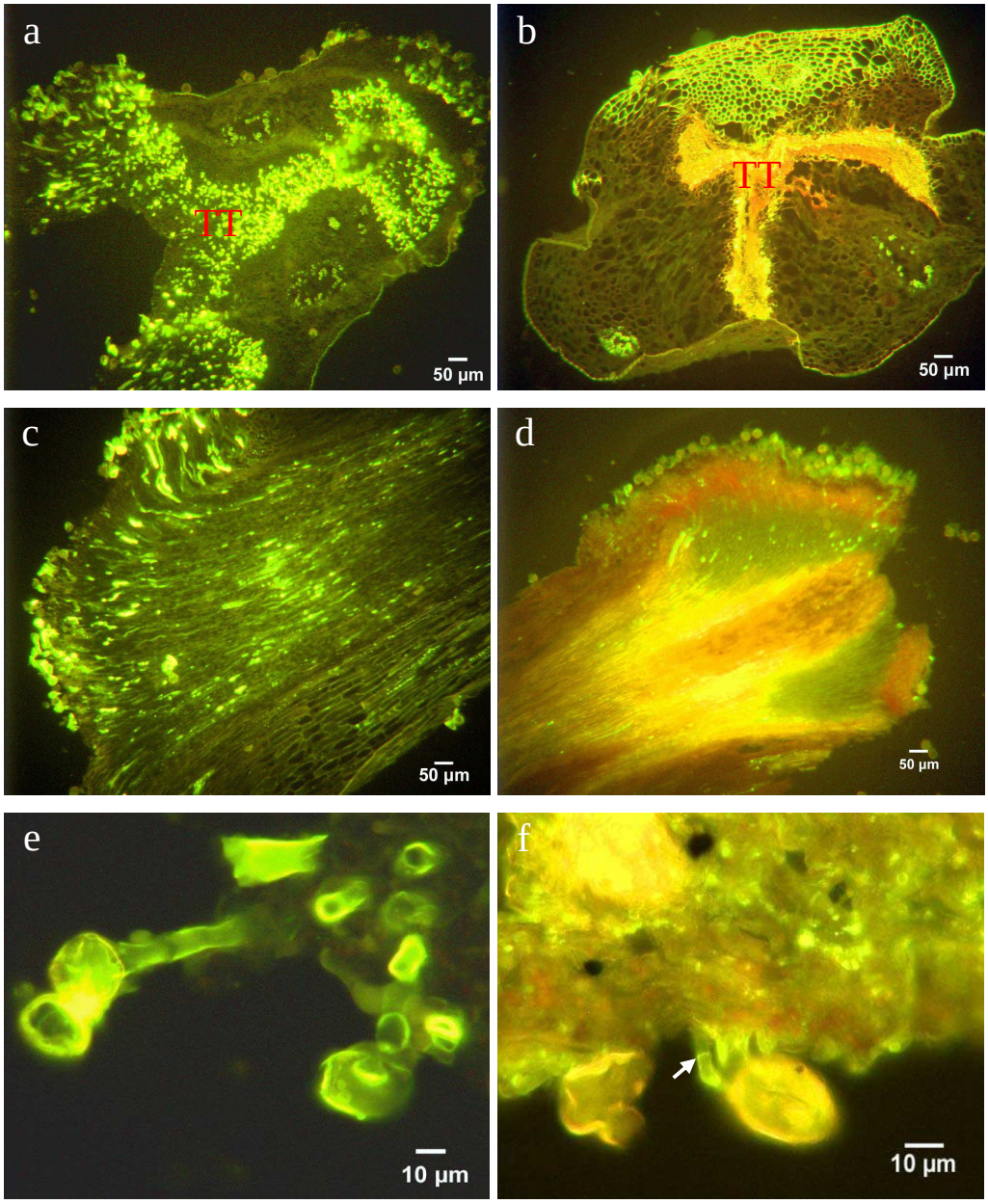

Fig. 2-3. Fluorescence micrographs of the sweet pepper stigma-style complex after pollination and/or inoculation with *Fusarium lactis*. **a,** Cross-section of an uninoculated style, with pollen tubes appearing as vivid green spots in the transmitting tissue (TT); **b,** Cross-section of a style 6 days after inoculation (DAI), with TT fluorescing yellowish-red; **c,** Longitudinal section of an uninoculated style, with pollen tubes appearing as bright green flecks or strands in the TT; **d,** Longitudinal section of an inoculated style 6 DAI, with the TT fluorescing yellowish-red; **e,** A germinating pollen grain on an inoculated stigma; **f,** A germinated microconidium with a septum (arrow) visible in the germ tube. Green staining indicates cell viability, whilst red staining indicates necrosis and yellow intermediate viability.

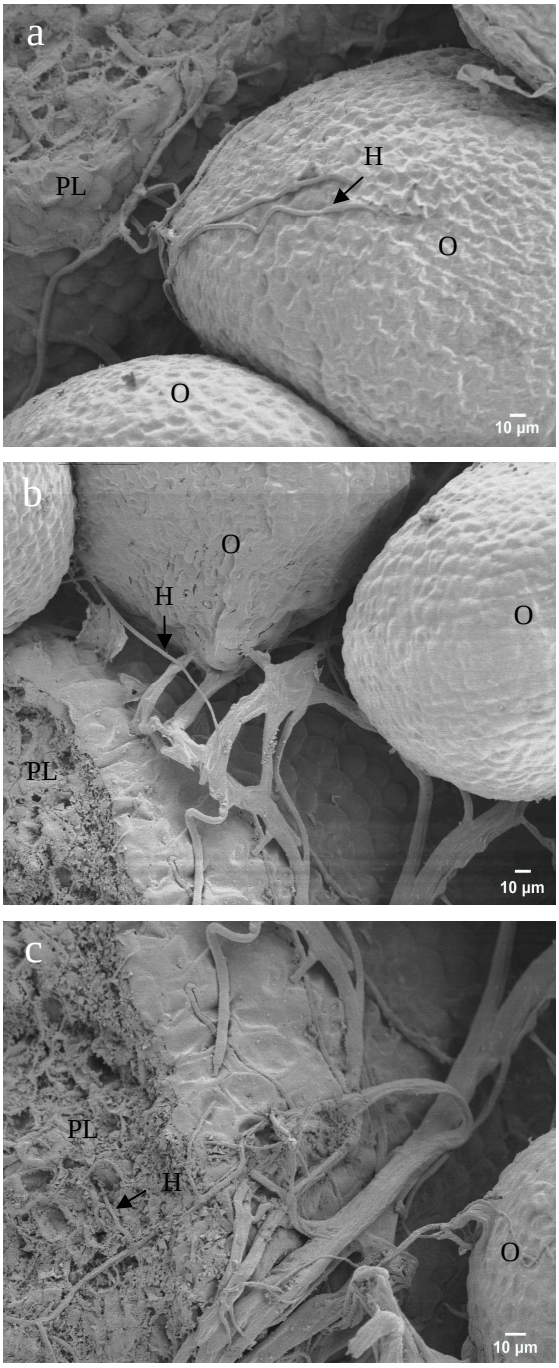

Fig. 2-4. Scanning electron micrographs showing sweet pepper ovules and fungal hyphae 6 days after inoculation (DAI) of flowers with *Fusarium lactis*. **a** and **b**, Growth of hyphae (H) on the ovules (O) inside the ovary, PL = placenta; **c**, Branched hyphae growing into the placenta (PL) at the site beneath the style. Similar results were obtained at 5 DAI (not shown).

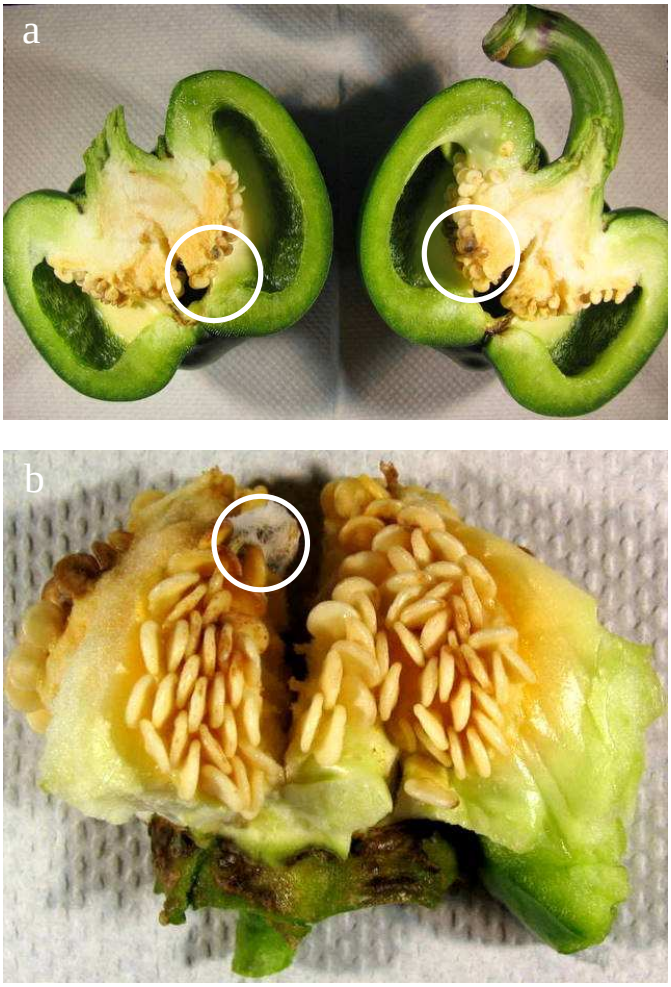

Fig. 2-5. Sweet pepper fruit with symptoms of internal fruit rot caused by *Fusarium lactis*, 45 days after inoculation. **a** and **b,** Proliferation of fungal mycelium (circled in white) on the placenta and seeds near the original style. The fruit exhibited no external symptoms of infection.

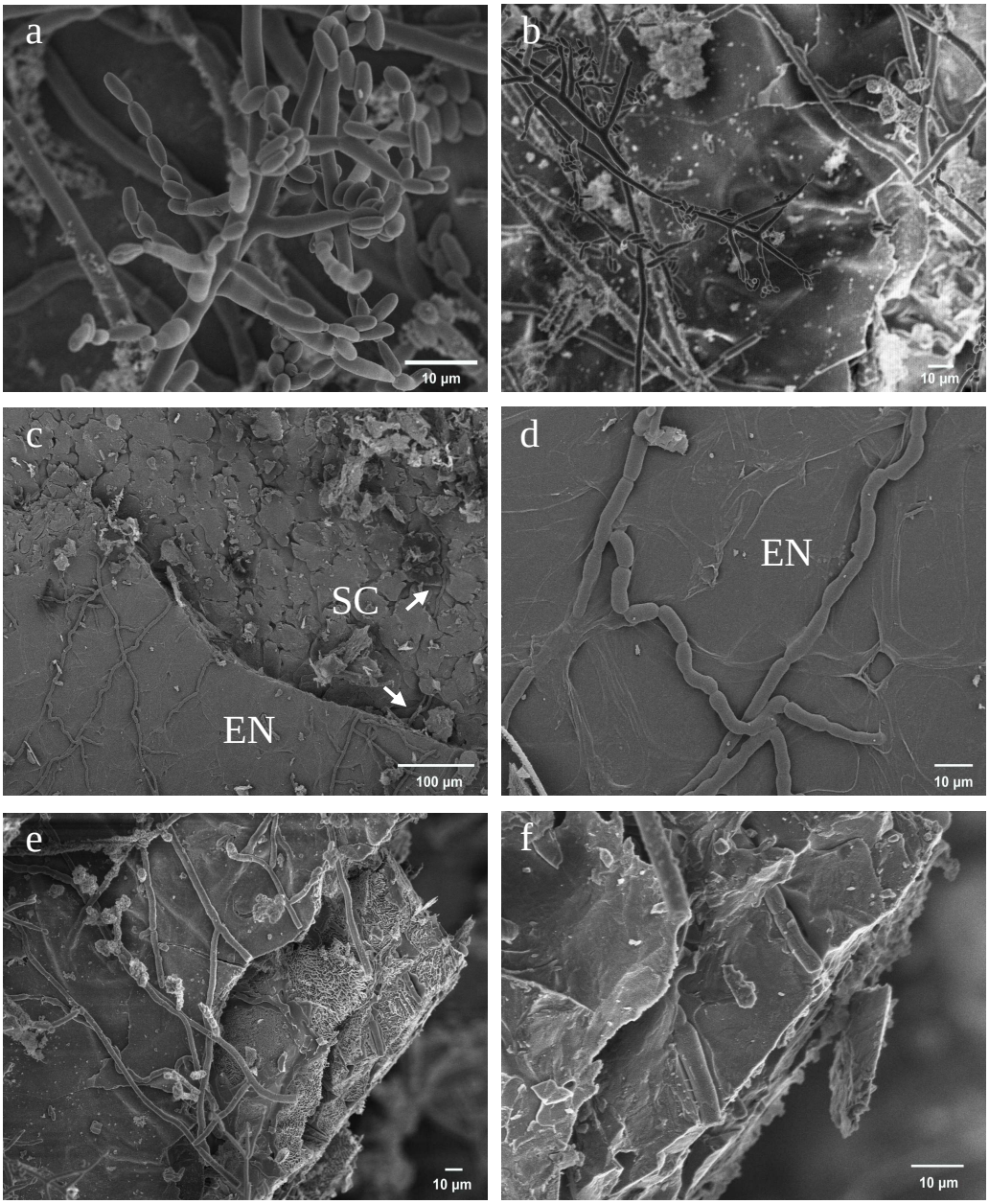

Fig. 2-6. Scanning electron micrographs of *Fusarium lactis*-infected pepper seeds (freeze-fractured) in fruit developed from an inoculated flower 45 days after inoculation. **a** and **b**, Fungal sporulation within an infected fruit; **c**, Hyphal growth on the inner surface of the seed coat (SC) and in the endosperm (EN); **d, e** and **f,** Growth of hyphae inside the endosperm (EN) of an infected seed.

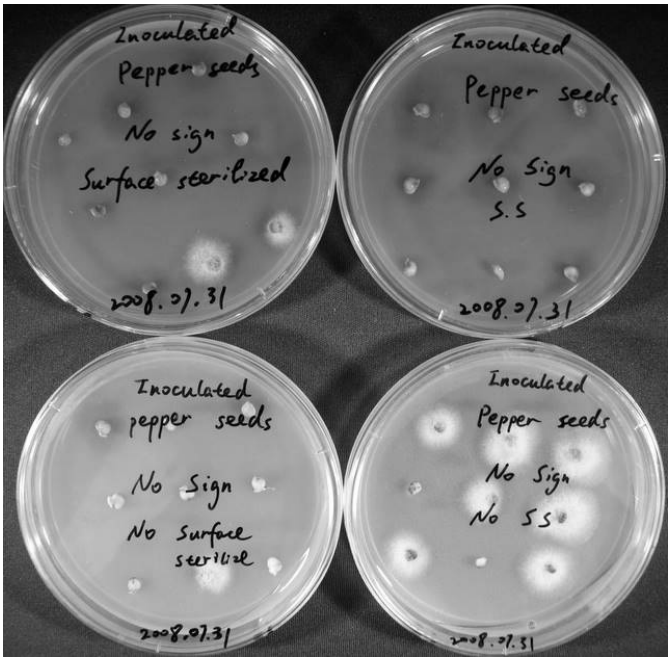

Fig. 2-7. Development of *Fusarium lactis* colonies from symptomless seeds collected from a healthy looking but inoculated pepper fruit, 36 days after inoculation. The seeds were either surface sterilized (top Petri dishes) or not sterilized (bottom dishes) prior to plating on potato dextrose agar for a 5-day period.

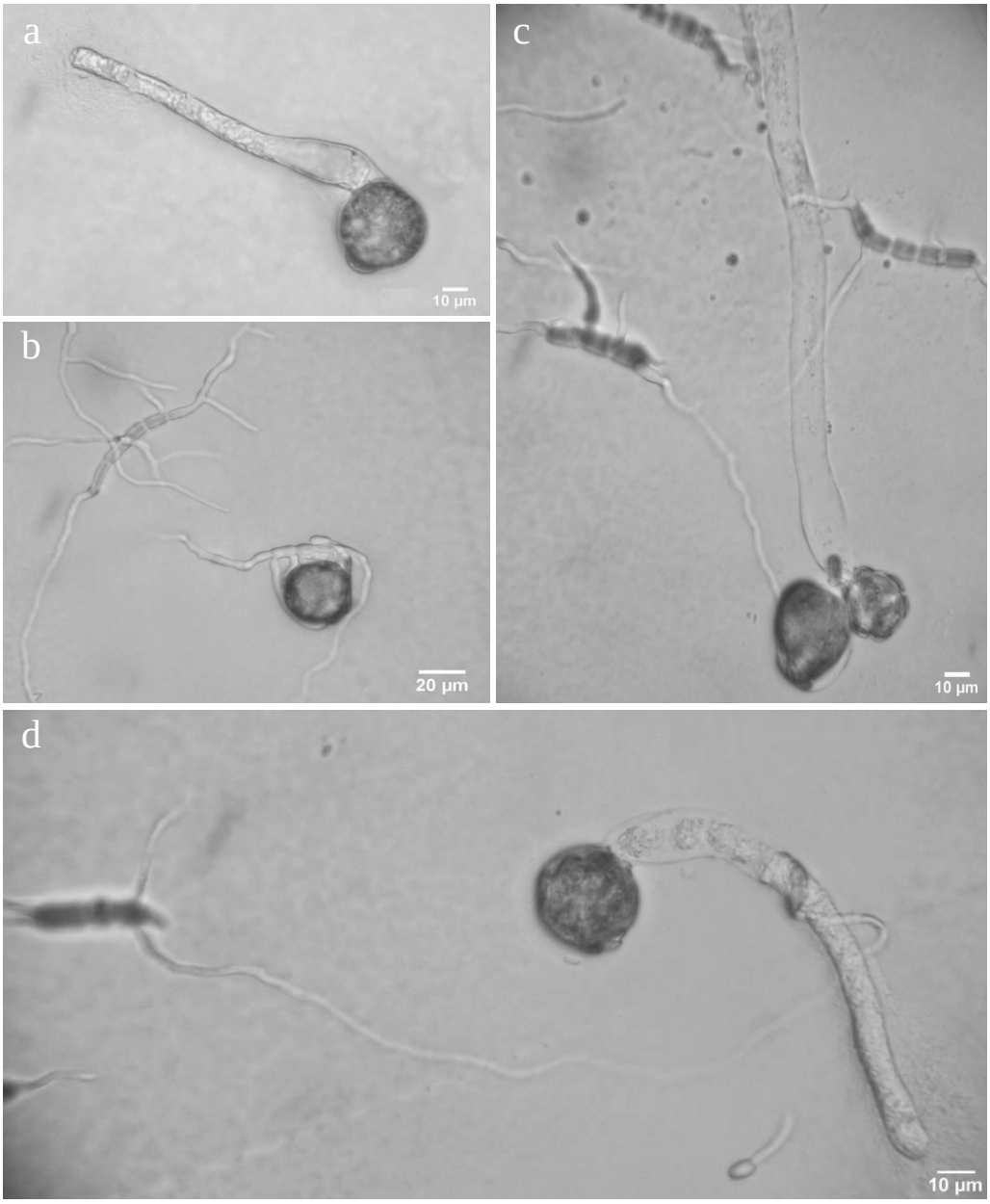

Fig. 2-8. Interaction of germinating pepper pollen grains and macroconidia of *Fusarium lactis* on water agar. **a,** A germinated pollen grain with pollen tube 4 h after plating on agar in the absence of the fungus; **b,** A pollen grain that did not germinate due to colonization by hyphae of *F. lactis*; **c** and **d,** Fungal hyphae did not grow or proliferate within the pollen tubes, even 18 h after plating on the agar.

Chapter 3. Mycotoxin production by isolates of *Fusarium lactis* from greenhouse sweet pepper

3.1. Introduction

Internal fruit rot has emerged as an important disease of sweet pepper (*Capsicum annuum* L.) in Canadian greenhouses (Yang et al. 2009). Typically, disease development is associated with the growth of a whitish-grey mycelium on the seeds, placenta and inner surface of the wall of infected fruits. External symptoms occur only in severe infections, and consist of greenish to dark-brown lesions on the outer surface of some fruits. The principal causal agent of internal fruit rot is *Fusarium lactis* Pirotta & Riboni (Yang et al. 2009), although the closely related fungi *Fusarium proliferatum* (Matsushima) Nirenberg and *Fusarium verticillioides* (Saccardo) Nirenberg have also been implicated in this disease (Utkhede and Mathur 2003; Yang et al. 2009).

As a member of the *Gibberella fujikuroi* (Sawada) Wollenw. species complex (*Fusarium* section *Liseola*), *F. lactis* from greenhouse sweet pepper may produce toxic secondary metabolites or mycotoxins, such as the fumonisins B_1 and B_2 (FB_1 and FB_2), moniliformin (MON), beauvericin (BEA), enniatins, fusaproliferin, fusarins, fusaric acid, and gibberellic acid (Nirenberg and O'Donnell 1998; Moretti et al. 2007). Indeed, an isolate of *F. lactis* from fig (*Ficus carica* L.), on which the fungus causes endosepsis (Michailides et al. 1996), was shown to produce both MON (Fotso et al. 2002) and BEA (Moretti et al. 2007). Since infected pepper fruits show few (if any) external symptoms of disease and may

not be culled before delivery to market, there is a risk that they could be purchased and consumed (Yang et al. In Press). Mycotoxins can persist in infected fruit tissue after removal of the mycelium, and depending on fruit characteristics, may also diffuse into healthy tissues (Restani 2008). Thus, mycotoxin production by *F. lactis* in sweet pepper could represent an important food safety concern, especially given the increased popularity of this fruit as a component in fresh salads, in which it undergoes no additional processing.

The mycotoxin FB_1 is classified as a Group 2B carcinogen (possibly carcinogenic to humans) by the International Agency for Research on Cancer (1993), and a statistically significant correlation was found between exposure to FB_1 by ingestion of moldy corn and the incidence of human esophageal cancer in some regions of South Africa and China (Marasas 2001). The acute and long-term toxicity of MON to humans is not known, although it is highly toxic to many animals, including mice, chickens, minks, and sheep (reviewed in Jestoi 2008). This mycotoxin has also been suggested to be the cause of a fatal heart condition, known as "Keshan disease", which affects people in certain parts of China and South Africa where large amounts of corn are consumed (Bottalico 1998). BEA may induce apoptosis (programmed cell death) and DNA fragmentation, resulting in its toxicity to several human cell lines (Logrieco et al. 1997; 1998). Furthermore, the co-occurrence of FB_1, MON, and BEA with other secondary metabolites may also pose a health concern, since these mycotoxins can act in an additive or synergistic manner to cause increased toxicity (Ledoux et al. 2003).

Despite these risks, there is, to our knowledge, no information available on mycotoxin production by *F. lactis* from sweet pepper, or on the occurrence of FB_1, MON and BEA in infected pepper fruits. Such information is important to properly evaluate the hazards posed by consumption of *F. lactis*-infected sweet pepper fruit. Consequently, the objectives of this study were to: (i) evaluate mycotoxin production by *F. lactis* and closely related fungal species isolated from greenhouse sweet pepper, and (ii) determine whether detectable levels of mycotoxin contamination occur in diseased sweet pepper fruits.

3.2. Materials and Methods

3.2.1. Fungal material

A total of 17 isolates of *F. lactis*, three of *F. proliferatum* and one of *F. verticillioides*, collected from naturally infected pepper fruits in commercial greenhouses in Alberta, British Columbia, Saskatchewan, Ontario and the Netherlands, were included in this study (Table 3-1). The isolates were classified to species based on morphological traits and by sequence analysis of the elongation factor 1-α, mitochondrial small subunit ribosomal DNA, and β-tubulin genes (Yang et al. 2009).

3.2.2. Culture conditions

Fungal isolates were initially grown on Spezieller Nährstoffarmer Agar (SNA, Leslie and Summerell 2006) in 90 mm diameter Petri dishes for a 10 day period, with the cultures kept at room temperature (RT) under 12 h light/12 h dark (Nirenberg

1981), after which they were transferred to rice medium. To prepare the rice medium, 50 g of polished rice were soaked overnight in 22 ml distilled water in a 250 ml flask and autoclaved for 30 min at 121°C (Kostecki et al. 1999). Three 8 mm-diameter agar plugs, bearing fungal mycelium and spores, were excised from the SNA cultures of each isolate and used to inoculate the rice medium. Two flasks were inoculated per isolate, and the flasks were incubated at RT in darkness for 3 weeks. Controls were inoculated with sterile plugs of SNA. After harvest, the duplicate cultures of each isolate were pooled and air dried in a fume hood at RT for a 48 h period. The dried samples were homogenized in a coffee grinder (Fast Touch 203-42, Krups, Toronto, ON, Canada), and the resulting powder was stored in a plastic bag at -20 °C. The controls inoculated with sterile agar were processed in the same manner.

3.2.3. Inoculation of sweet pepper fruits

Mature sweet pepper fruits were inoculated with conidial suspensions of *F. lactis* isolate F2004-C, *F. proliferatum* isolate F2006-AB-5, or *F. verticillioides* isolate F2007-ON-B1-1. Briefly, each pepper fruit was wounded at the inoculation point with a needle and a 1 ml spore suspension (1×10^6 spores/ml) was injected into the wound. A total of four pepper fruits (two red and two yellow), purchased from H & W Produce, Edmonton, Alberta, were inoculated with each *Fusarium* isolate. Peppers inoculated with sterile distilled water served as controls. Each inoculated pepper was incubated in its own sealed plastic bag at RT for 11 days. Following incubation, the fruits were dissected and the fruit

tissue was separated into two groups: (1) heavily diseased tissue exhibiting soft-rot symptoms and the presence of fungal mycelium, and (2) symptomless or lightly diseased tissue, with no obvious mycelial growth but some restricted soft-rot symptoms. The fruit tissues from these two groups were homogenized separately in a blender (700G, Waring Laboratory & Science, Torrington, CT, USA) and processed for mycotoxin detection as described below.

3.2.4. MON analysis

3.2.4.1. Extraction and cleanup

Extraction and preparation of MON for HPLC analysis was based on a protocol provided with a commercially purchased Mycosep^{TM} 240 column (Romer Labs, Inc., Union, MO, USA). Dried and ground rice culture (10 g) was mixed with 50 ml acetonitrile (ACN):water (84:16, v/v) in a 125 ml flask, and incubated on an orbital shaker at 200 rpm for 1 h. This crude extract was filtered under vacuum through Whatman No.1 filter paper, and a 2 ml aliquot of the filtrate was passed through a Mycosep^{TM} 240 MON column (Romer Labs, Inc.). The eluted solution was evaporated to dryness at RT in a speed vacuum (Heto, Birkrod, Denmark) and re-dissolved in 400 µl of methanol (MeOH). The MeOH solution was centrifuged at 16,000 g for 10 min, and the supernatant was collected and stored in a 2 ml HPLC vial at 4°C until injection onto an HPLC. Control cultures inoculated with sterile SNA agar were processed in the same manner. Two sub-samples of dried, ground rice culture were processed for each isolate as well as for the control. Extraction of MON from sweet pepper fruit was

conducted in a similar manner, except that 10 g (fresh mass) of homogenized tissue was extracted in 40 ml ACN:water (84:16, v/v), and the eluate from the Mycosep^{TM} 240 column (Romer Labs, Inc.) was dried under nitrogen and re-dissolved in 500 µl of MeOH.

3.2.4.2. HPLC conditions

MON production was quantified with an HPLC system (Varian, Walnut Creek, CA, USA) consisting of two Prostar 210 pumps, one Prostar 325 detector, and one Prostar 410 auto-sampler. The analytical column utilized was a 150 × 4.6 mm Supelcosil^{TM} (Supelco Inc., Belafonte, PA, USA), 5 µm LC-18 reversed phase column, fitted with a Supelco Pelliguard LC-18 guard column (50 × 4.6 mm). Samples were applied in 20 µl aliquots, with highly concentrated samples diluted 10-fold with MeOH prior to injection. The column was maintained at RT during analysis, and the absorbance of the eluate was monitored at 256 nm. The solvents in the mobile phase consisted of 0.1% tetra-butyl-ammonium hydroxide (TBAH) in water (pH = 4) (A) and ACN (B). Samples were analyzed by means of a linear elution gradient over 48 min at a flow rate of 1 ml/min, starting from 0 to 6% solvent B over 1 min, 6 to 9% solvent B over 25 min, 9% to 100% solvent B over 2 min, an isocratic flow of 100% solvent B for 8 min, and a return to 0% solvent B over 2 min. The final 10 min of the run consisted of an isocratic flow of 100% solvent A. Data were collected using a Galaxie Chromatography Data System version 1.9 (Varian), and MON was quantified based on the UV absorption at 256 nm. The detection limit was 0.05 ppm.

3.2.5. BEA analysis

3.2.5.1. Extraction and cleanup

The method of Kostecki et al. (1999), with slight modifications, was used to extract BEA from rice cultures and infected sweet pepper fruits. Briefly, 8 g ground dry rice culture or 10 g fresh fruit tissue were dissolved in 40 ml of ACN:MeOH:water (16:3:1, v/v/v), and mixed thoroughly by incubation on an orbital shaker at 200 rpm for 1 h. The extracts were then filtered under vacuum through Whatman No.1 filter paper, and a 15 ml aliquot of the filtrate was transferred into a glass test tube and defatted twice with 10 ml of heptane. The bottom layer was dried under nitrogen, re-dissolved in 15 ml of MeOH:water (55:45, v/v), and extracted twice with 10 ml of methylene chloride. The methylene chloride phase was collected and dried again. This residue was re-dissolved in 1.5 or 3 ml of ACN for fruit- or rice culture-derived samples, respectively, and centrifuged at 16,000 g for 10 min. The supernatant (1.4 ml) was transferred into a 2 ml HPLC vial and stored at 4 °C until injection. Two 10 g sub-samples were processed for each isolate as well as for the control.

3.2.5.2. HPLC conditions

The HPLC apparatus and column used for the quantification of BEA were identical to those described above for MON. Twenty μl of each sample were injected into the HPLC system, with highly concentrated samples diluted 10-fold with ACN prior to being re-injected. The column was maintained at RT during the analysis, and the UV absorbance of the eluate was monitored at 205 nm. The solvents used in the mobile phase were water (A) and ACN (B). The samples

were analyzed using an elution gradient at a flow rate of 1 ml/min, starting with an isocratic flow of 70% solvent B for 6 min, followed by 70 to 65% solvent B over 17 min, and finally an isocratic flow of 65% solvent B for 1 min; the column was returned to initial conditions by isocratic flow of 70% solvent B over 2 min. The detection limit for BEA was 0.05 ppm.

3.2.6. FB_1 analysis

3.2.6.1. Extraction and cleanup

A method provided by Romer Labs, Inc., was used for extraction of FB_1. Ground dry rice culture or infected fruit tissue (10 g) was extracted with 40 ml ACN:water (50:50) by incubation on an orbital shaker at 200 rpm for 1 h. The crude extract was filtered under vacuum through Whatman No.1 filter paper and adjusted to pH 6-7 with 0.5 M sodium hydroxide, prior to passing through a MultiSep® 211 Fum column (Romer Labs, Inc.). Briefly, the MultiSep® 211 Fum column was equilibrated with 5 ml of MeOH, followed by 5 ml MeOH:water (3:1, v/v). A 3 ml aliquot of the filtered extract was mixed with 8 ml MeOH:water (3:1, v/v), and the mixture was applied to the column. The column was then washed with 8 ml of MeOH:water (3:1, v/v) followed by 3 ml of MeOH, and the sample was eluted with 10 ml MeOH:acetic acid (99:1, v/v). The eluate was evaporated to dryness under nitrogen, and re-dissolved in 500 µl ACN:water (50:50, v/v). Two 10 g sub-samples were processed for each isolate as well as for the control.

3.2.6.2. HPLC conditions

The HPLC apparatus and column used for quantification of FB_1 were the same as for MON and BEA, above, except that the system was also connected to a Shimadzu RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) at λ_{ex} = 335 nm and λ_{em} = 440 nm. The column was maintained at RT during the analysis. To prepare samples for injection into the system, a 125 µl volume of the fruit or culture extract was mixed with 25 µl ortho-phthaldialdehyde (OPA) reagent (prepared as described by Sedgwick et al. (1991)) and a 50 µl aliquot of this mixture was applied with an auto-injector. Highly concentrated samples were diluted 10- or 100-fold with ACN:water (50:50, v/v) prior to injection. The solvents in the mobile phase consisted of ACN:water:acetic acid (40:60:1, v/v/v) (A) and ACN:acetic acid (99:1, v/v) (B). The sample was analyzed using a linear elution gradient over 18 min at a flow rate of 1 ml/min, with 0 to 35% solvent B over 10 min, 35 to 100% solvent B over 1 min, an isocratic flow of 100% solvent B for 1 min, and a return to initial conditions (100% solvent A) over 3 min. Under these conditions, the detection limit for FB_1 was 0.2 ppm.

3.2.7. Quantification of mycotoxins

Mycotoxins were quantified by means of five-point external calibration curves prepared using commercially purchased standards, with the concentrations calculated based on the area under each curve. The linear range for the MON calibration curve was between 0.1 and 10 ppm (R^2 = 0.9961), whilst for BEA and

FB_1 the linear ranges were between 10 to 200 ppm (R^2 = 0.9975) and 0.5 to 5 ppm (R^2 = 0.9959), respectively.

3.2.8. Recovery of mycotoxins

Rates of each mycotoxin recovery were evaluated by spiking inoculated and non-inoculated rice cultures with commercial mycotoxin standards, as follows: (1) cultures of *F. proliferatum* isolate FGH-15, *F. lactis* F2004-Z, and a non-inoculated control were spiked with 0, 2.5, and 5 ppm MON, (2) cultures of *F. lactis* isolates F2006-SK-2 and SUN SUISI-2, and a non-inoculated control were spiked with 0, 15 and 30 ppm BEA, and (3) cultures of *F. lactis* isolates F2004-C and F2006-SK-3, and a non-inoculated control were spiked with 0, 0.25, and 0.5 ppm FB_1. The spiked samples were processed and analyzed as described above for each mycotoxin, with corresponding non-spiked samples used to monitor basal mycotoxin levels in these cultures. The average recovery rates for MON and BEA in rice culture were 50.8% and 77.1%, respectively. The recovery rate for FB_1 is currently being determined. Data presented in the results were not corrected for the recoveries.

3.2.9. Confirmation of mycotoxin production

Mycotoxins were identified by comparing HPLC retention times with those of each standard; identities were further validated by scanning the UV spectra using a diode array detector (Shimadzu SPD-M10A VP, SpectraLab Scientific Inc., Toronto, ON, Canada). In addition, the presence of MON, BEA and FB_1 in

naturally infected pepper fruits and selected cultures of *F. lactis* was confirmed by liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS-MS). Briefly, for detection of MON, an infected pepper fruit collected in an Alberta greenhouse and rice cultures inoculated with *F. lactis* isolates F2004-C and Fsa-1 were processed as described above, and subjected to LC-MS-MS on a Waters 2695 liquid chromatograph (Waters Corporation, Milford, MA, USA) connected to a MicroMass Quattro Micro triple-quadrupole mass spectrometer (Waters Corporation), with the electrospray ionization probe in the negative mode. For detection of BEA, an infected pepper fruit and a culture of *F. lactis* isolate F-2006-SK-2 were processed as above and subjected to LC-MS (Agilent 1100 LC-MS, Agilent Technologies Inc., Santa Clara, CA, USA), with the electrospray ionization probe in the positive mode, 150 V fragmentor. Finally, the presence of FB_1 was confirmed in a naturally infected pepper fruit and cultures of *F. lactis* isolates F2004-C and F2006-SK-4 by LC-MS-MS, also on an Agilent 1100 liquid chromatograph connected to a MicroMass Quattro Micro triple-quadrupole mass spectrometer. Detection of MON was conducted in the Chemistry Laboratory, Alberta Research Council, Vegreville, Alberta; detection of BEA and FB_1 was conducted in the Mass Spectrometry Laboratory, Department of Chemistry, University of Alberta, Edmonton, Alberta.

3.2.10. Chemicals and standards

All chemicals used in mycotoxin extraction and purification were of HPLC grade. Mycotoxin standards were purchased from Sigma (St. Louis, MO, USA); standards of MON, BEA and FB_1 were prepared in MeOH, ACN and ACN:water (50:50, v/v), respectively. These solutions were stored in a refrigerator at 4°C until needed.

3.2.11 Statistical analysis

Analysis of variance for multiple comparisons (Duncan's multiple range test) was used to compare the mycotoxin concentrations amongst the different treatments. All statistical analyses were conducted using SAS 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

3.3. Results

3.3.1 Mycotoxin production in rice culture

All 21 fungal isolates tested produced BEA in rice culture, at concentrations ranging from 13.28 ppm (*F. lactis* isolate from the Netherlands) up to 1674.60 ppm (*F. lactis* isolate F2004-C from central Alberta) (Table 3-1). Two isolates of *F. lactis* (F2004-C and F2004-Z) and one isolate of *F. proliferatum* (F2006-AB-5), which originated in Alberta, produced more than 1000 ppm BEA. However, the majority of isolates examined produced between 100 ppm and 1000 ppm BEA, whilst the four *F. lactis* isolates from Saskatchewan produced less than 100 ppm BEA, as did the single *F. lactis* isolate from the Netherlands, one *F. lactis* isolate

from southern Alberta (SUN SUISI-2) and two isolates of *F. proliferatum* (F2007-ON-B1-1 and FGH-15) from Ontario and Alberta.

Similarly, 15 *Fusarium* isolates (13 *F. lactis* and 2 *F. proliferatum*) also produced MON in rice culture, at concentrations ranging from 0.23 ppm to 181.85 ppm (Table 1). Amongst the producing isolates, the lowest MON concentrations were observed for *F. lactis* F2004-C, which happened to be the same isolate that produced the highest concentration of BEA. Two isolates of *F. lactis* from Saskatchewan, F2006-SK-1 and F2006-SK4, produced the highest MON concentrations (181.85 ppm and 163.15 ppm, respectively). These latter isolates were amongst the lowest producers of BEA (Table 3-1).

Only four of 17 *F. lactis* isolates tested produced detectable levels of FB_1 in rice culture, at concentrations ranging from below 0.20 ppm to a maximum of 0.28 ppm (for isolate F2004-C) (Table 3-1). In contrast, all three *F. proliferatum* isolates and the *F. verticillioides* isolate produced this mycotoxin, at concentrations that ranged from 6.50 ppm (*F. proliferatum* F2007-ON-A1) to 314.00 ppm (*F. proliferatum* FGH-15). In summary, six isolates (four *F. lactis* and two *F. proliferatum*) produced all three toxins, two isolates (one *F. proliferatum* and one *F. verticillioides*) produced both BEA and FB_1, nine isolates (all *F. lactis*) produced both MON and BEA, and four isolates (all *F. lactis*) produced only BEA.

3.3.2 Mycotoxin production in infected pepper fruits

All three mycotoxins (MON, BEA and FB_1) could be detected in inoculated pepper fruit tissue, whether or not it was heavily diseased (Table 3-2). Nonetheless, mycotoxin concentrations were generally higher in heavily diseased tissue that exhibited soft-rot symptoms and showed visible fungal growth. BEA was detected at concentrations of 13.94 ppm and 19.43 ppm in heavily diseased fruit tissues inoculated with *F. proliferatum* isolate F2006-AB-5 and *F. lactis* isolate F2004-C, respectively. It was also detected at 3.00 ppm in symptomless/lightly diseased tissue inoculated with F2004-C. MON was detected more widely amongst the treatments, and was found in concentrations ranging from 0.03 ppm to 0.27 ppm in lightly and heavily diseased tissues inoculated with *F. proliferatum* F2006-AB-5 and *F. verticillioides* F2007-ON-B1-1, and at 0.07 ppm in heavily diseased tissue inoculated with *F. lactis* F2004-C. The presence of FB_1 was detected in all treatments at concentrations of 0.61 ppm (*F. lactis* F2004-C in symptomless/lightly diseased tissue) to 8.04 ppm (*F. verticillioides* F2007-ON-B1-1 in heavily diseased tissue) (Table 3-2). Interestingly, the concentrations of FB_1 observed in fruit tissue after inoculation with *F. lactis* F2004-C (0.61 ppm – 0.78 ppm) were higher than those observed for the same isolate in rice culture (0.28 ppm) (compare Tables 3-1 and 3-2). In contrast, the quantities of FB_1 produced by *F. proliferatum* F2006-AB-5 and *F. verticillioides* F2007-ON-B1-1 were much higher in rice culture (161.50 ppm and 40.80 ppm, respectively) than in fruit tissue (1.53 ppm – 3.58 ppm and 2.94 ppm – 8.04 ppm, respectively). As expected, FB_1 and MON could not be detected in non-inoculated

control tissue. However, a concentration of 0.18 ppm MON was observed in the control, suggesting that the fruit was naturally contaminated with this mycotoxin.

Mycotoxin contamination in naturally infected sweet pepper fruits and selected cultures of *F. lactis* was confirmed by LC-MS (for BEA) or LC-MS-MS (for MON and FB_1), with identification based on comparison with authentic standards. BEA was detected by the presence of an ion at m/z 784 (Fig. 3-1), corresponding to the quasi-molecular ion $[M + H]^+$. The presence of precursor/product ion pairs at m/z 722.4/334.1 and m/z 722.4/352.1 enabled detection of FB_1 (Fig. 3-2), whilst the presence of a precursor/product ion pair at m/z 97.0/40.5 allowed detection of MON (Fig. 3-3).

3.4. Discussion

In recent years, internal fruit rot has emerged as an important disease of greenhouse sweet pepper in western Canada (Yang et al. 2009). Unfortunately, disease development can be costly to producers, who have in some cases experienced losses of about $20 CAD/$m^2$ (M. Mirza, personal communication). The current study suggests, however, that beyond these economic concerns, consumption of infected fruit could pose a threat to human health. Analysis by LC-MS and LC-MS-MS revealed the presence of BEA, FB_1 and MON in naturally diseased sweet pepper fruits (Figs. 3-1 to 3-3); contamination levels were as high as 19.43, 8.04 and 0.27 ppm, respectively, for each of these mycotoxins, as determined by HPLC with external calibration curves (Table 3-2). Moreover, the possible health risk posed by infected sweet pepper fruit is

exacerbated by the fact that symptomless and lightly diseased fruit tissue was also found to contain detectable levels of MON, BEA and FB_1 (Table 3-2), suggesting that excising obviously infected tissues before the fruit is consumed may not be sufficient to completely eliminate the mycotoxins. Indeed, non-inoculated control tissues from a commercially purchased fruit included in the analysis contained significant levels of MON (Table 3-2); this fruit appeared healthy, but was likely naturally contaminated with this mycotoxin, suggesting some level of fungal infection. Restani (2008) noted that, depending on the characteristics of the particular substrate, mycotoxins may also diffuse into healthy tissues.

The ability to produce BEA appears to be widespread amongst isolates of *F. lactis* (Table 3-1), which is the principal cause of internal fruit rot of greenhouse sweet pepper (Yang et al. 2009). Similarly, most isolates of this fungus also produced MON, although only four isolates produced detectable levels of FB_1. Nonetheless, this study represents the first report of FB_1 production by *F. lactis*, since an isolate of this fungus collected from figs was previously shown to produce MON (Fotso et al. 2002) and BEA (Moretti et al. 2007) but not FB_1. Whilst Moretti et al. (2007) did not specifically test for the occurrence of FB_1, Fotso et al. (2002) did and could not detect this mycotoxin. This discrepancy may be explained by the different origins of the *F. lactis* isolates examined (i.e., sweet pepper versus fig), by intraspecific variability amongst the isolates, or by differences in growth substrate or conditions. *In vitro* mycotoxin production in the current study was evaluated on rice medium, whilst in the earlier report a maize-based medium was used (Fotso et al. 2002).

To determine whether differences in mycotoxin biosynthesis result from true biological differences between the isolates, toxin production by *F. lactis* isolates from fig and sweet pepper would have to be compared under identical conditions. It is interesting to note that FB_1 production by *F. lactis* isolate F2004-C appeared to be greater *in vivo* than *in vitro* (compare Table 3-2 with Table 3-1), highlighting the impact of growth substrate and conditions on mycotoxigenesis. Nevertheless, Leslie et al. (1995) suggested that it may be possible to distinguish species within the *G. fujikuroi* species complex by comparing their production of secondary metabolites. In a similar manner, comparison of mycotoxin production patterns on a suite of media may also serve to distinguish different strains or isolates within the same species.

With respect to the mycotoxins examined in this study, FB_1 represents the greatest health concern, followed by MON and BEA (Desjardins 2006). Although production of FB_1 amongst isolates of *F. lactis* was generally lower than for the other mycotoxins, isolates of *F. proliferatum* and *F. verticilloides* (which are also associated with internal fruit rot of sweet pepper) produced significantly higher quantities of this toxin (Table 3-1). Moreover, additive effects have been reported when MON and FB_1 are found together (Ledoux et al. 2003). Since MON, BEA and FB_1 were all found to be produced individually or in combination by isolates of *F. lactis* (Tables 3-1 and 3-2), infection of fruit tissue by this pathogen does appear to pose a significant health risk, with the occasional occurrence of *F. proliferatum* and *F. verticilloides* serving to intensify this risk. Collectively, these results suggest that development of internal fruit rot of sweet

pepper represents an important food safety concern, and that every effort should be made to cull infected fruit before it makes it to market.

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Table 3-1. Production of moniliformin (MON), beauvericin (BEA) and fumonisin B_1 (FB_1) by isolates of *Fusarium lactis*, *F. proliferatum* and *F. verticillioides* on rice medium

Isolate	Origin	Concentration (ppm)*		
		MON	BEA	FB_1
Fusarium lactis F2004-C	Central Alberta	0.23^{c}	1674.60^{a}	0.28^{e}
F. lactis F2004-T	Central Alberta	ND	684.50^{d,e}	ND
F. lactis F2004-V	Central Alberta	1.10^{c}	807.60^{c,d}	ND
F. lactis F2004-Z	Central Alberta	0.41^{c}	1072.95^{b}	ND
F. lactis P&D-1	Southern Alberta	2.69^{c}	675.80^{d,e}	ND
F. lactis P&D-2	Southern Alberta	ND	553.45^{e,f}	ND
F. lactis SUN SUISI-2	Southern Alberta	ND	18.29^{g}	ND
F. lactis BROXBURN-1	Southern Alberta	9.06^{b,c}	623.00^{d,e,f}	< 0.20^{e}
F. lactis F2007-ON-A3	Ontario	38.50^{b}	469.95^{f}	ND
F. lactis F2007-ON-B2	Ontario	ND	766.80^{c,d}	ND
F. lactis F2007-ON-B4-2	Ontario	0.70^{c}	484.40^{f}	ND
F. lactis Fsa-1	British Columbia	0.66^{c}	778.85^{c,d}	< 0.20^{e}
F. lactis	The Netherlands	2.73^{c}	13.28^{g}	ND
F. lactis F2006-SK-1	Saskatchewan	181.85^{a}	42.37^{g}	ND
F. lactis F2006-SK-2	Saskatchewan	9.60^{b,c}	15.55^{g}	ND
F. lactis F2006-SK-3	Saskatchewan	6.13^{c}	23.92^{g}	ND
F. lactis F2006-SK-4	Saskatchewan	163.15^{a}	35.87^{g}	< 0.20^{e}
F. proliferatum FGH-15	Central Alberta	3.60^{c}	952.20^{b,c}	314.00^{a}
F. proliferatum F2006-AB-5	Central Alberta	ND	1044.20^{b}	161.50^{b}
F. proliferatum F2007-ON-A1	Ontario	10.89^{b,c}	108.85^{g}	6.50^{d}
F. verticillioides F2007-ON-B1-1	Ontario	ND	18.66^{g}	40.80^{c}

*Mean concentration from two replicates; means followed by a common letter in the same column are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test.
ND: Not detected.

Table 3-2. Production of moniliformin (MON), beauvericin (BEA) and fumonisin B1 (FB1) by isolates of *Fusarium lactis*, *F. proliferatum* and *F. verticillioides* on infected fruits of greenhouse sweet pepper

Isolate	Fruit tissue	Concentration (ppm)*		
		MON	BEA	FB_1
F. lactis F2004-C	Symptomless/lightly	ND	3.00^{c}	$0.61^{b,c}$
	Heavily diseased	$0.07^{b,c,d}$	19.43^{a}	$0.78^{b,c}$
F. proliferatum	Symptomless/lightly	$0.14^{b,c}$	ND	$1.79^{b,c}$
	Heavily diseased	$0.03^{c,d}$	13.94^{b}	$1.53^{b,c}$
F. verticillioides	Symptomless/lightly	$0.11^{b,c,d}$	ND	2.92^{b}
	Heavily diseased	0.27^{a}	ND	8.04^{a}
Control	Symptomless	$0.18^{a,b}$	ND	ND

*Mean concentration from two replicates; means followed by a common letter in the same column are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test.
ND: Not detected.

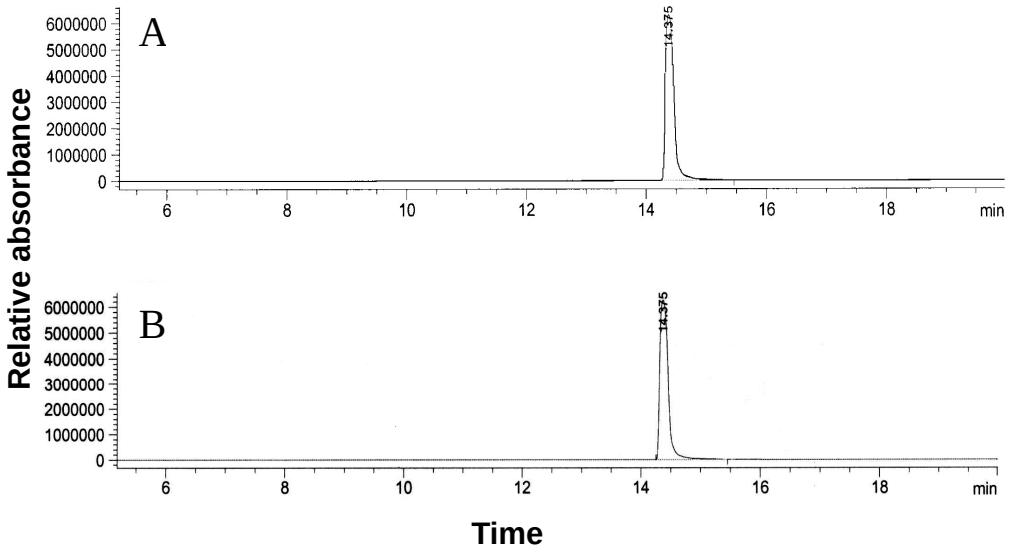

Fig. 3-1. Selected ion monitoring chromatograms for detection of beauvericin from (**A**) a naturally infected sweet pepper fruit exhibiting symptoms of internal fruit rot, and (**B**) a culture of *Fusarium lactis* isolate F2006-SK-2 grown on rice medium. The infected fruit was collected from a greenhouse in central Alberta, Canada. The spectra were obtained by atmospheric pressure ionization (API)-electrospray LC-MS, and chromatograms were recorded at m/z 784 for detection of the mycotoxin. The retention time was 14.38 min.

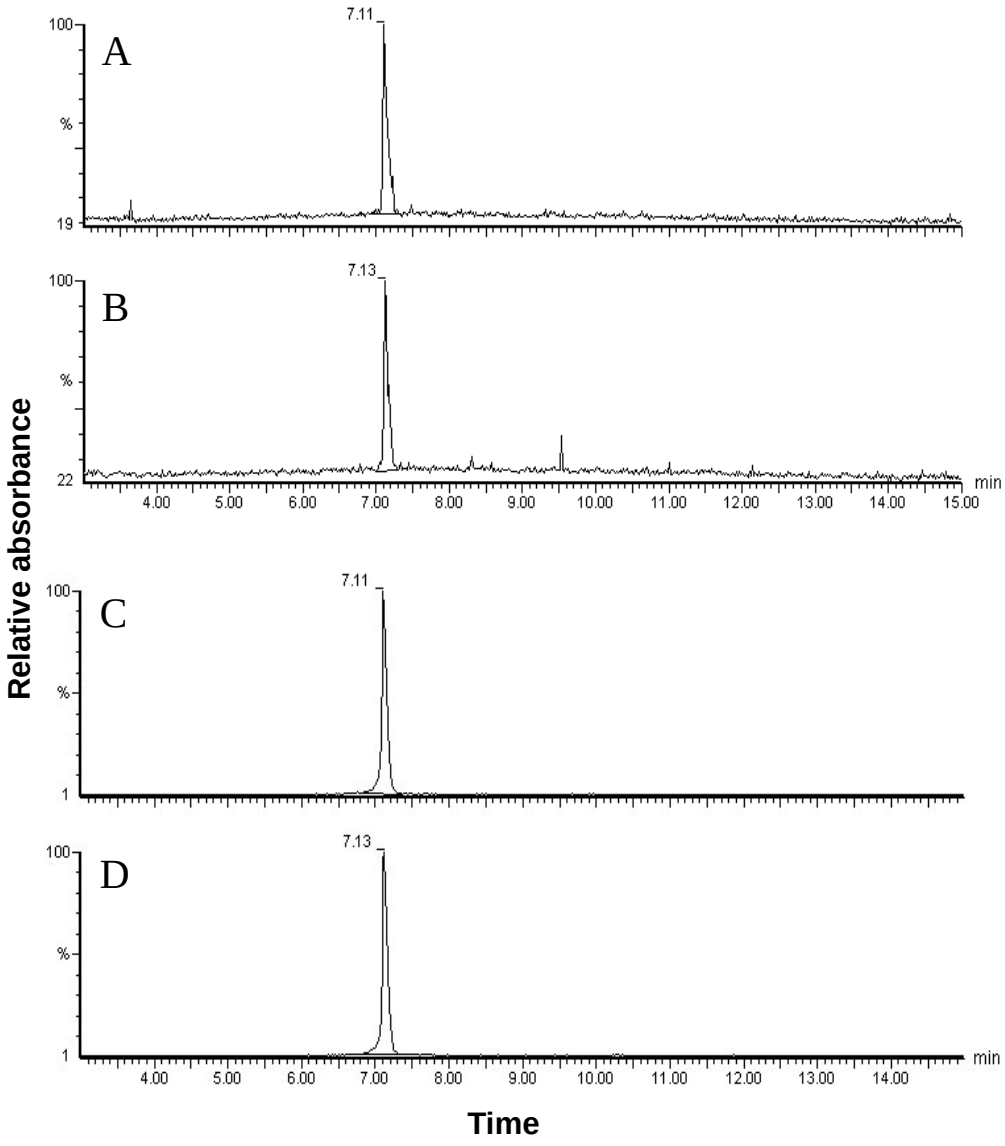

Fig. 3-2. Selected ion monitoring chromatograms for detection of fumonisin B1 from (**A** and **B**) a naturally infected sweet pepper fruit exhibiting symptoms of internal fruit rot, and (**C** and **D**) a culture of *Fusarium lactis* isolate F2004-C grown on rice medium. The infected fruit was collected from a greenhouse in central Alberta, Canada. The spectra were obtained by atmospheric pressure ionization (API)-electrospray LC-MS-MS, and chromatograms were recorded at m/z 722.4 > 334.1 (**A** and **C**) and m/z 722.4 > 352.1 (**B** and **D**) for detection of the mycotoxin. The retention times were 7.11 and 7.13 min, respectively.

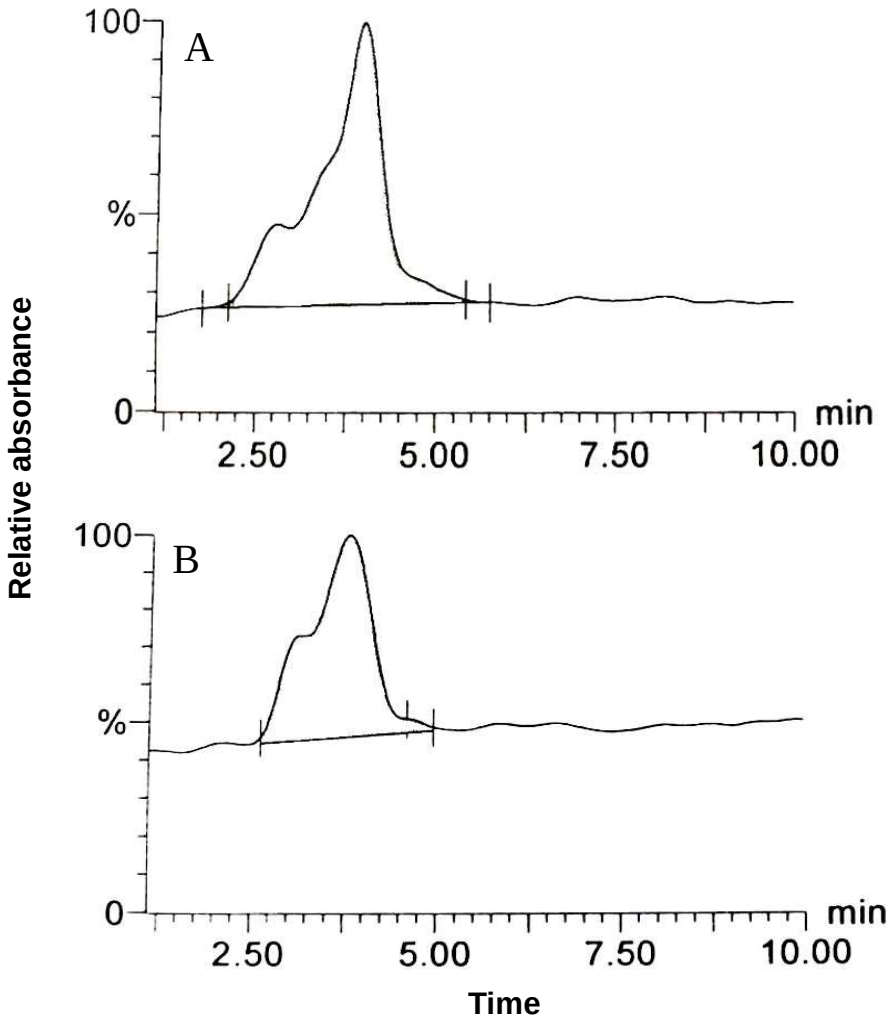

Fig. 3-3. Selected ion monitoring chromatograms for detection of moniliformin from (**A**) a naturally infected sweet pepper fruit exhibiting symptoms of internal fruit rot, and (**B**) a culture of *Fusarium lactis* isolate F2004-C grown on rice medium. The infected fruit was collected from a greenhouse in central Alberta, Canada. The spectra were obtained by atmospheric pressure ionization (API)-electrospray LC-MS-MS, and chromatograms were recorded at m/z 97.0 > 40.5. The retention time was 4.00 min.

Chapter 4. General Discussion

Internal fruit rot, caused primarily by the fungus *Fusarium lactis*, is an emerging disease of greenhouse sweet pepper in Canada (Yang et al. 2009). At the onset of this M.Sc. project, we hypothesized that infection occurs via the flowers, resulting in the development of diseased fruits. This hypothesis was tested by microscopic examination of the infection process. Moreover, as a member of the *Giberella fujikuroi* species complex, *F. lactis* was postulated to be mycotoxigenic, and the production of fumonisin B_1 (FB_1), beauvericin (BEA) and moniliformin (MON) was assessed both in culture and in infected fruits. The work outlined in this thesis, collectively, provides the first comprehensive analysis of the development of internal fruit rot of sweet pepper, and of the risk of exposure to mycotoxins that may be associated with the consumption of infected fruits.

4.1. Infection process

Hyphae of *F. lactis* were found to grow on the surface of the stigma and within the style of inoculated sweet pepper flowers. Growth was eventually also observed within the ovaries (Chapter 2). These observations, combined with the apparent absence of any penetration structures, or of direct penetration by the fungus through the ovary wall from the outside, strongly suggest that the infection of sweet pepper fruits is initiated via infection of the stigma and style at anthesis (Chapter 2). This is consistent with the development of internal symptoms in affected pepper fruits, as well as with the occurrence of seedborne inoculum.

Proper sanitation practices, such as rapidly discarding infected fruits from greenhouses, combined with seed treatments, may therefore be critical in managing and preventing spread of this disease.

During the fertilization process, the pollen tubes follow the transmitting tissue of the sweet pepper style (Hu and Xu 1985). This tissue, which consists of parenchyma cells with large intercellular spaces, may provide nutrients to sustain growth of the pollen tubes (Hu and Xu 1985). It is the availability of these nutrients and the presence of large intercellular spaces that most likely makes this tissue amenable for growth of a weak pathogen such as *F. lactis*. Indeed, it is tempting to speculate that the fungus follows the pollen tubes down the transmitting tissue and into the ovaries. However, although hyphae of *F. lactis* were observed within the style and may have even contributed to accelerated senescence of the transmitting tissue in inoculated flowers (Chapter 2), no evidence was found of fungal growth along the pollen tubes. Moreover, no strong interactions were observed between germinating pollen grains and *F. lactis* conidia on axenic culture. Therefore, based on these results, we may conclude that infection occurs through the stigma and style, but not necessarily along the pollen tubes.

4.2. Mycotoxin production

A *F. lactis* isolate (BBA 58590) collected from fig was previously found to produce MON (Fotso et al. 2002) and BEA (Moretti et al. 2007) in corn and rice culture, respectively. In the current studies with *F. lactis* and closely related

species isolated from greenhouse sweet pepper, production of both of these mycotoxins was also detected (Chapter 3). Furthermore, fungal isolates from sweet pepper also produced the mycotoxin FB_1, which was not detected by Fotso et al. (2002) in *F. lactis* isolated from fig (Moretti et al. [2007] did not test for the occurrence of FB_1).

As discussed by Leslie et al. (1995), differences in the secondary metabolites produced by fungal isolates may help to distinguish species within the *G. fujikuroi* species complex, in combination with morphological studies, *in vitro* fertility tests and DNA analysis. Thus, the detection of FB_1 may serve to differentiate sweet pepper-derived *F. lactis* strains from fig-derived strains, if the latter are confirmed to be non-producers of FB_1. The types and amounts of mycotoxins produced, however, are affected by many factors beyond species or strain, including the specific growth substrate and environmental conditions. Thus, clear criteria (such as growth on the same substrate under identical conditions) would be needed to enable unbiased comparisons of mycotoxin production by different fungal isolates.

Nevertheless, the detection of MON, BEA and FB_1 in cultures of *F. lactis* and in fruit tissue infected by this fungus suggests that internal fruit rot of greenhouse sweet pepper may represent an important food safety concern. The typical absence of external symptoms in this disease means that infected fruit are likely to make it to market, where they may be purchased and consumed. Moreover, sweet pepper is often used as a fresh vegetable ingredient without cooking or additional processing, steps that could be helpful in degrading any mycotoxins present in the

fruit. Even if the sweet peppers are heated prior to consumption, FB_1 and MON have been shown to be heat-stable (Arranz et al. 2004; Pineda-Valdes and Bullerman 2000), and could therefore still represent a health risk.

The safest option to reduce the risk of mycotoxin exposure is to avoid consumption of infected fruits, when and if they are encountered, since Restani (2008) noted that mycotoxins can diffuse from infected into healthy tissues. The extent of this diffusion depends on the characteristics of the particular type of fruit being considered. Mycotoxin diffusion is facilitated by high water content and hindered by the presence of structure-forming polysaccharides. Fortunately, sweet pepper has a consistency similar to that of apple tissue, which was found to reduce diffusion of mycotoxins such as patulin (Restani 2008). Nonetheless, Restani (2008) suggested discarding tissue within a 2 cm radius of moldy areas, even in substrates that are not particularly favorable to mycotoxin diffusion. Since the discarding of infected fruits by growers and consumers is obviously an unfavorable option, every effort should be made to limit the development of internal fruit rot in the greenhouse.

4.3. Future studies

Perhaps the major gap that remains in our understanding of the epidemiology of internal fruit rot of greenhouse sweet pepper relates to its mode of spread between greenhouses. While it is clear that infection of the seeds, especially symptomless infection, may represent an important mechanism for the introduction of *F. lactis* into “clean” greenhouses, it is not clear how the infected

seeds initiate the infection cycle. Spread within the greenhouse is associated with pathogen growth in the floral tissues, presumably after deposition of conidia by pollinators or via air currents (Chapter 2). However, seedborne transmission between greenhouses implies that the germinating seedlings would be infected, with the fungus eventually sporulating to produce the first round of inoculum. It is difficult to envision a weak pathogen such as *F. lactis* growing systemically through infected seedlings, although Utkhede and Mathur (2004) observed that 3% of seedlings which germinated from commercially purchased seeds were covered with a mycelium similar to that of *F. subglutinans*. No information on seedborne infection is available specifically for *F. lactis*. Additional work is therefore required to determine other stages in the sweet pepper lifecycle in which the host is susceptible to infection, and to understand how *F. lactis* first spreads from the infected seeds to cause the typical internal fruit rot symptoms.

Another issue that may merit further study is related to the interaction between *F. lactis* and the germinating pollen grains; on axenic culture, no direct interactions were observed between fungal hyphae and the pollen tubes (Chapter 2). However, this may have been an artefact of the *in vitro* system employed, and additional studies focussed on the *in planta* situation may yield different results. Such information could help conclusively determine whether or not *F. lactis* follows the path of the pollen tubes down the style.

In terms of mycotoxin production, the present study assessed production of FB_1, BEA and MON by *F. lactis* and closely related fungi isolated from greenhouse sweet pepper, but no attempt was made to detect other mycotoxins or

secondary metabolites (such as fusaproliferin and fusaric acid). A complete profile of all mycotoxins produced would be useful not only for fully assessing the health risks posed by infected pepper fruits, but also for possible use as a tool in strain differentiation or other taxonomic applications (Moretti et al. 2007). In this context, it may also be important to re-examine the issue of FB_1 production by *F. lactis* from figs, since this was examined on corn-based medium rather than the rice medium used in the current study. Such work will help to increase understanding of internal fruit rot of sweet pepper and its main cause, *F. lactis*.

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