PCR-based identification of *Fusarium* spp. and impact of wound healing time on dry rot infection

Thomas Assefa Jima

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Swedish University of Agricultural Sciences (SLU)

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Thomas Assefa Jima

Supervisor:

Prof. Dan Funck Jensen
Swedish University of Agricultural Sciences
Department of Forest Mycology and Plant Pathology

Co-supervisors:

Dr. Nicklas Samils
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences

Assistant Prof. Magnus Karlsson
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences

Examiner:
Prof. Jonathan Yuen
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences

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Abstract

Dry rot is an important fungal storage and field rot found on potatoes world wide, which is caused by various *Fusarium* spp. Out of the thirteen *Fusarium* species reported, *F. sambucinum* (*Gibberella pulicaris*) and *F. coeruleum* (*F. solani* var. *coeruleum*) are more frequently associated with dry rot of tubers. Quick and reliable identification of dry rot pathogens is important in the diagnosis, phylogenetics and suggesting the right management practices and storage possibilities for processors and growers. Modern molecular-based techniques play a major role in quick, reliable and accurate identification of pathogens. In this study, the process of amplifying the ITS and LSU region of the rDNA from *F. sambucinum* and *F. coeruleum* using the universal primers was laborious and time consuming. The universal primer pairs ITS1 x ITS2, ITS1-F x ITS 2, ITS 1 x LR 1, ITS 1-F x LR 1 were able to amplify *F. sambucinum* DNA samples but failed to get a product using primer pair ITS 1 x ITS 4. The *F. sambucinum* primer pair S3F x S3R has failed to discriminate the DNA samples from *F.coeruleum* and *T. viride*. Therefore, these regions of rDNA were not reliable for effective diagnosis of *Fusarium* strains that cause dry rot. However, it helped to investigate the polymorphism between *Fusarium* spp. used to identify a specific ITS region.

The impact of wound healing on dry rot infection was also investigated by this work. A decline in relative mean rot volume of laboratory infected tubers with *F. avenaceum* and *F. coeruleum* spores was observed after 4-6 days of wound healing time. The highest mean rot recorded for both *F. coeruleum* and *F. avenaceum* infected tubers was at 0 and 2 days wound healing time, which might be due to the combined effect of bacteria and fungal infection. Furthermore, the *F. avenaceum* strain used in this study was more aggressive with the highest rot symptoms compared to the *F. coeruleum* strain.

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I. Introduction

Potato, *Solanum tuberosum* L, is among the top five crops growing world wide following cereals wheat, rice, corn and barley due to its high carbohydrate content and adaptability. It is a co ld temperate crop of altitudes approximately 2000 m or more in the tropics. Global annual production of potato in 2010 was about 324.3 metric tonnes (FAOSTAT). It is an important source of calorie, protein and fat for humans, industrial raw material for starch and alcohol production and also feed for animals. From the overall annual production a considerable amount is also used as seed potato. By the year 2007 in northern Europe around 9.4, 8.7 and 4 % of the total potato produced was used for industrial processing, seed potato and animal feed, respectively (FAOSTAT).

Both field and postharvest diseases are prominent potato production determining factors. As it is listed in table 1 the pathogens include oomycetes, fungi and bacterial rots that can cause multiple infections at different stages of potato production, handling and storage (Atallah and Stevenson 2006) (Table 1). Among the most common fungal pathogens that cause tuber rot are: *Phytophtora infestans* (late blight), *P. erythroseptica* (pink rot), *Pythium ultimum* var. ultimum (leak), *Fusarium sambucinum* and/or *F. coeruleum* (dry rot). Also bacterial soft rot (*Erwinia carotovora* sp.*carotovora* and *E. carotovora* sp. *atroseptica*) cause serious symptoms (Atallah and Stevenson 2006).

Table 1. Important diseases of Potato (Atallah and Stevenson 2006).

Disease name	**pathogens**
Late blight	*Phytophtora infestans* Stem
canker and black scurf	*Rhizoctonia solani*
Common scab	*Streptomyces scabiei*
Powdery scab	*Spongospora subterranean*
White mold	*Sclerotinia sclerotiorum*
Pink rot	*Phytophthora erythroseptica*
Pythium leak	*Pythium spp.*
Silver scurf	*Helminthosporium solani*
Verticillium wilt	*Verticillium dahliae*
Fusarium dry rot	*Fusarium sambucinum*
Soft rot	*Erwinia carotovora* subsp.carotovora
Soft rot and balckleg	*E. carotovora* subsp. Atroseptica
Ring rot	*Clavibacter michiganensis* subsp.*sepedonicus*

Potatoes can be stored as piles or in pallets for a period up to one year using modern storage facilities. The storage facilities provide optimum humidity and air ventilation that can favor occurrence of several diseases (Atallah and Stevenson 2006). These postharvest diseases were reported to cause a substantial amount of yield loss (Rowe 1993). This paper deals specifically with dry rot of potato caused by *Fusarium* spp. It is an economically important disease that affects almost all commonly grown potato cultivars (Leech and Webb 1981).

Harvesting, Curing and Cold storage of potato tubers

Harvesting is an important stage in potato production that can determine the quality of tubers. A considerable amount of damage occurs during digging, loading and transportation of tubers. The use of proper agronomic practices and harvesting procedures can alleviate these damage problems. For example, avoiding of excess nitrogen and water and maintaining good soil

aeration can reduce the damages on tubers (Schweers and Voss 1973). After harvesting is completed potato tubers are stored with a temperature of around 12^{o}C for a couple of weeks to initiate wound healing (Kushalappa et al. 2002). Temperature is the critical factor for the wound closure process and varies with the range of 2.5 to 20^{o}C as per the storage condition and potato cultivar (Lui and Kushalappa 2002). More often potato tubers can be culled for significant physical damages and diseased severity (Kushalappa et al. 2002). For long time storage facilities are supplied with constant temperature and high relative humidity with good ventilation to reduce tuber dehydration and weight loss (Atallah and Stevenson 2006). Storage temperature varies depending on the potato tuber use; for processing (Chipping, fries …etc) it is maintained at 7 to 13^{o}C , 8 to 10^{o}C for table potatoes and 4 to 5^{o}C for seed potatoes (Atallah and Stevenson 2006; Lui and Kushalappa 2002). However, in most cases the storage conditions favor disease inoculum, pathogen distribution and new and rapid tuber infections (Atallah and Stevenson 2006).

Dry Rot of Potato

Dry rot is an important fungal storage and field rot found on potatoes world wide, which is caused by various *Fusarium* spp. (Leech and Webb, 1981; Hanson et al. 1996). Significant yield losses of 6% -25% is recorded in the field and severe dry rot infections of up to 60% occur in storage conditions (Chelkowski 1989; Carnegie et al., 1990; Stevenson et al., 2001). Around thirteen species of *Fusarium* are reported to cause dry rot (Cullen et al. 2005) like *F. oxysporum* , *F. avenaceum*, *F. culmorum* and *F.equiseti* strains even though most infections are associated with *F. sambucinum* and/ or *F. coeruleum* (syn. *F. solani* var. *coeruleum*) (Al-Mughrabi 2010).

F. sambucinum is the most pathogenic species in North America and parts of Europe (Hide et al. 1992).

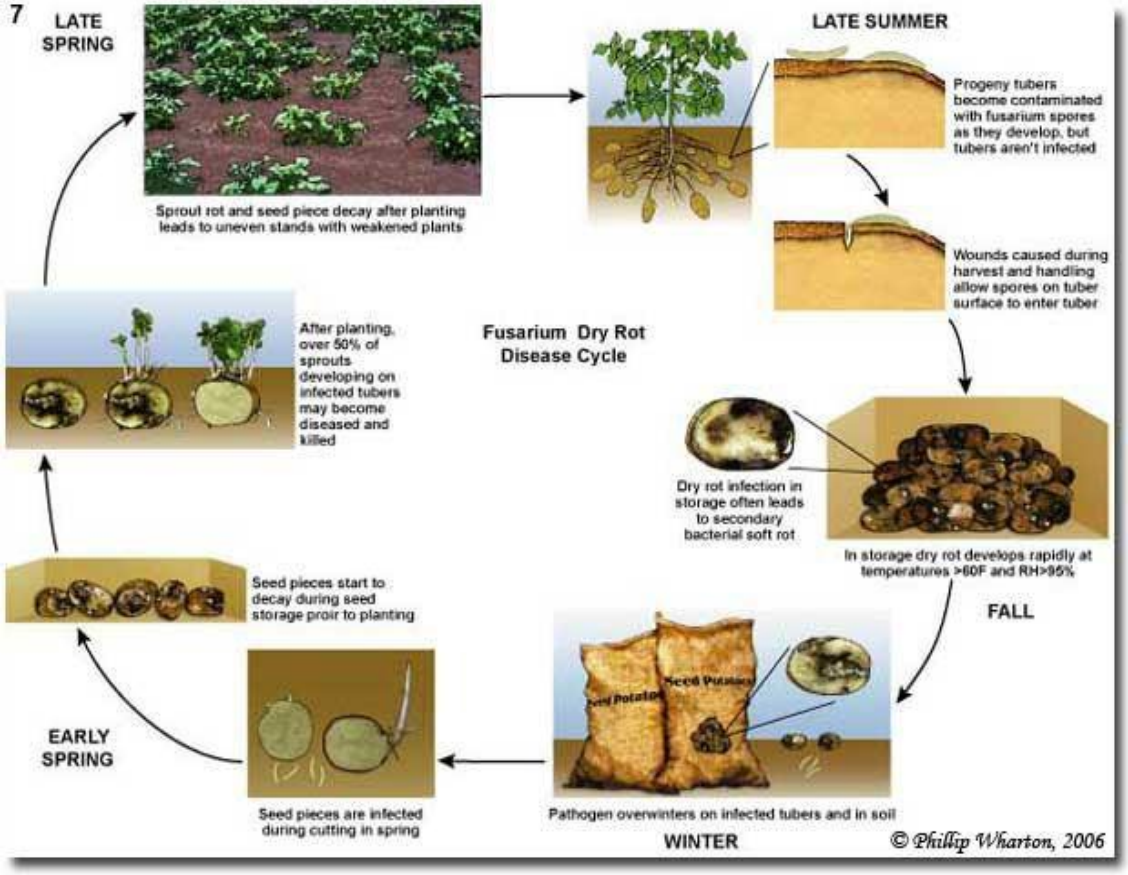
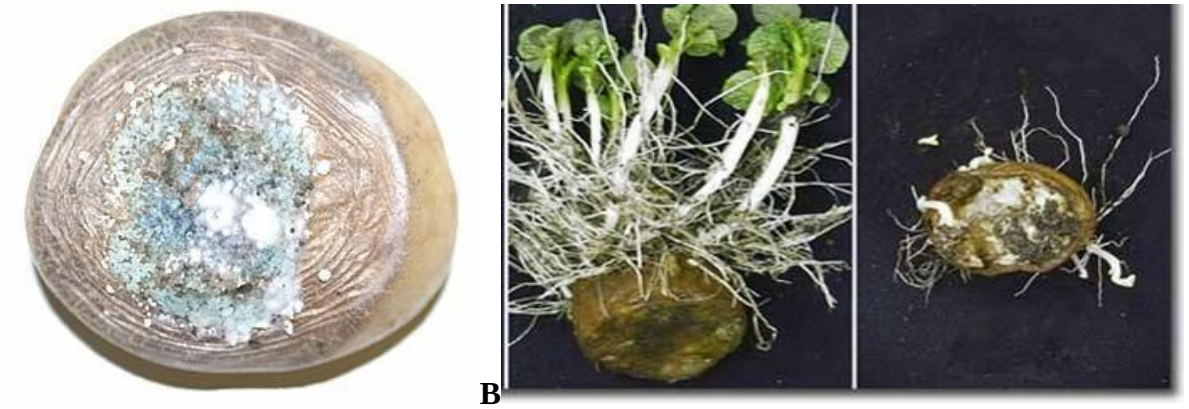


Figure 1. Disease cycle of dry rot of potato (Wharton et al. 2006)

The spores overwinter in soils where potatoes are cultivated or on decaying plant debris and can persist for many years (Al-Mughrabi 2010). It is depicted in the disease cycle above that inoculum sources for infections are mainly tubers with decayed periderm, seed tubers with spores or spore contaminated soil on the surfaces (Al- Mughrabi 2010). Infected mother tubers that are devoid of roots are preferred for the sporulation of *F. sambucinum*, unlike *F. coeruleum* spores, which sporulate from the outside of rooted mother tubers (Adams and Lapwood 1993). Entry in to the host is mainly through mechanical injury at and after harvest (Dean, 1994). In fall

and winter the disease appears on tubers during storage and after planting as a seed (Leach and Nielsen 1975) (Figure 1). In storage, small brown lesions develop at the wound area after 3-4 weeks and gradually enlarge (Figure 2) (Boyd 1952). The rotting tissue shrinks slowly and allows the periderm to collapse and often contains mycelia and sometimes concentric rings of dead dry tissues appear (Al-Mughrabi 2010).



A **B**

Figure 2. A) Dry rot symptom caused by *F. coeruleum* (stackyard.com) B) Dry rot infected seed potato (right) and fungicide treated seed potato against dry rot (left) (Wharton et al. 2006).

During humid conditions soft rotting bacteria colonize through the lesions and further facilitate the decay of tubers (Boyd 1952). In the field, decaying tissues may attract insects that can vector soft rotting bacteria like *Erwinia* species (Mckef 1954). Both the yield, market value and seed quality of potato tubers is affected by *Fusarium* dry rot. In addition, reports have shown the production of mycotoxins like trichothecene by these *Fusarium* fungi is toxic to humans and animals (Senter et al. 1991; Sveeney and Dobson 1999).

Fusarium sambucinum and *F. coeruleum* Biology, Identification, Pathogencity and Resistance.

Fusarium sambucinum (teleomorph: *Gibberella pulicaris*) and *F. coeruleum* (*F. solani* var. *coeruleum*) are more frequently associated with dry rot of tubers but *F. coeruleum* is considered to be a more aggressive pathogen in most parts of Europe (Lenc et al. 2008) including Sweden (Olofsson 1976). Infections of tubers occur at optimum temperature of 10-20^{o}C and high relative humidity. The morphological identification of cultures grown on potato-dextrose agar at an ambient temperature of 20-25^{o}C describes *F. sambucinum* as having more vigorous growth of thin, white mycelia with pink to salmon spores. However, *F. coeruleum* has a delayed growth of white mycelia with a purple pigment and sparse sporulation. Initial hyphal growth is intercellular and later fully colonizes the dead cells with more mycelium concentrated in the center of the lesion. In resistant tissues lesion enlargement is confined to the infection spot, which might be due to the suberin deposition in the host cells.

Wound Healing and Periderm Formation

A wound can be defined as an external or internal damage on the outer protective layer of a plant that causes the breakdown of cells in specific tissue (Davies 1987; Hooker 1981). This can be inflicted by practices of man, weather conditions, animals and insects and also by the physiological development of the plant (Bostock and Stermer 1989). Wounds can facilitate the entrance of plant pathogens that can cause serious diseases. Hence, wound healing or wound closure in plants hinders the penetration of pathogens through the wound-induced responses. Many studies have been conducted on wound anatomy and indicated that it is a progressive process which results in a barrier zone and wound periderm formation (Beckman 1980; Biggs 1984). It involves formation of lignin, suberin, waxes or wound gums in the immediate cells next

to the wounded surface. Wound repair in potato tuber is most advanced and involves the autolysis and death of cells surrounding the wound surface, formation of impermeable layers through the redifferentiation of parenchyma cells, creation of lignosuberized region, and creation of a suberized wound periderm in the lignosuberized area (Bostock and Stermer 1989). Wound periderm is comprised of meristematic cells or the phellogen layer, parenchyma cells constructed inwards from the meristematic cells or phelloderm and a suberin region to the out side of the phellogen called phellem (Bostock and Stermer 1989). The maturation of phellem cells is followed by high suberin deposition and infusion with tannins, which later exhibit the corky look of the periderm layer (Bostock and Stermer 1989). From the context of plant-pathogen interaction the formation of cork barriers is involved in physically limiting the pathogen outside the plant surface (Bostock and Stermer 1989; Wood 1967). However, further studies imply that formation of lignin and suberin components of periderm may actively be involved in the defense of pathogens (Vance et al. 1980).

Chemical Responses in the Wound Healing Process

Suberization of wounds involves initiation of phenylalanine ammonialyase (PAL) and biosynthesis of phenylpropanoid (Bernards 2002; Lulai 2007). Suberin is a complex heteropolymer with a phenolic (aromatic or lignin-like) component imbedded to the cell wall and the hydrophobic (aliphatic, lipid) domain attached to the earlier phenolic component (Kolattukudy 1987). The poly(aliphatic) and poly(phenolic) domains have a characteristic chemical composition and appear as separate components of the suberin (Bernards and Lewis 1998). An alkylaryl ether bond were assumed to be active between the poly(aliphatic) and

poly(phenolic) domain of suberin macromolecule (Bernards and Lewis 1992; Gil et al. 1997; Lopes et al. 2000).

Cottle and Kolattukudy (1982) suggested that the phenolic domain is mainly constituted by ρ-coumaryl and coniferyl alcohol moieties. However, Borg-Olivier and Monties (1989) indicated that only guaiacyl-and syringyl moieties were observed from suberized potato wounds. In other cases evidences showed the absence of lignin like substructure linkage by NMR method using the ^{1}H and ^{13}C (Zimmermann et al. 1985; Garbow et al. 1989). Bernards and Lewis (1992) rather suggested phenolic acids and/or esters through caroboxylic acid and ester signals obtained using the ^{13}C NMR spectra. Suberized tissues also contain phenolic compounds like amides, hydroxycinnamic acids or monolignols and tyrosine and tyramine bounded in the cell wall (Riley and Kolattukudy 1975; Clarke 1982; Borg-Olivier and Monties 1993). Bernard et al. (1995) further strengthened that covalently bonded hydroxycinnamic acids were a principal part of subrine poly(phenol) moiety. Generally, irrespective of what is expected from a lignified tissue it was showed that suberized tissues are mainly comprised of hydroxycinnamic acid and a minority of monolignols (Bernards 2002). This implied that there is a significant difference in the amount of phenolic monomer profiles for suberized and lignified tissues and also determine the physiological and cellular origin variations among them (Bernards 2002). Suberized root meristem initials are different from lignified cells of the stele (Malamy and Benfey 1977).

The aliphatic moiety of suberin is principally arranged from a sequence of aliphatic blocks that constitute 40-50% of the suberized cell walls (Marques et al. 1994; Graca and Pereira 1997). Among the aliphatic components ω-hydroxyalkanoic acids, midchain epoxide and di and tri-

hydroxy-substituted octadecanoates,α,ω-dioic acids, glycerols, and 1-alkanols constitute a major portion of the monomers (Holloway 1983; Graca and Pereira 1997). Adamovics et al. (1977) detected accumulation of long chain alkanols of ferulates in suberin deposited periderm. The biosynthesis poly(aliphatic) domain depicted both short chain (C-18)-oxidized fatty acids and very long chain 1-alkanols, fatty acids, ω-hydroxy fatty acids and α,ω-dioic acids involved as a monomer (Bernards 2002).

Therefore, the structure of suberin as demonstrated in figure 3 has distinct arrangement of the poly(aliphatic) and poly(phenolic) moieties in which the later is attached directly to the primary cell wall(Rittinger et al. 1987 ; Thomson et al. 1995). These two domains are covalently joined (Stark and Garbow 1992; Lopes et al. 2000) and esters of acyl-glyceryl and hydroxycinnamoyl-glyceryl were also detected (Graca and Pereira 1999) (Figure 3). The poly(phenolic) moiety is distinct in the precursor composition with lignin but its subcellular position and build up of macromolecules are similar (Bernards 2002). Moreover, glycerol serves as a structural linkage between successive aliphatics and the aliphatic and aromatic domains (Schmutz et al. 1994; Graca and Pereira 2000) (Figure 3).

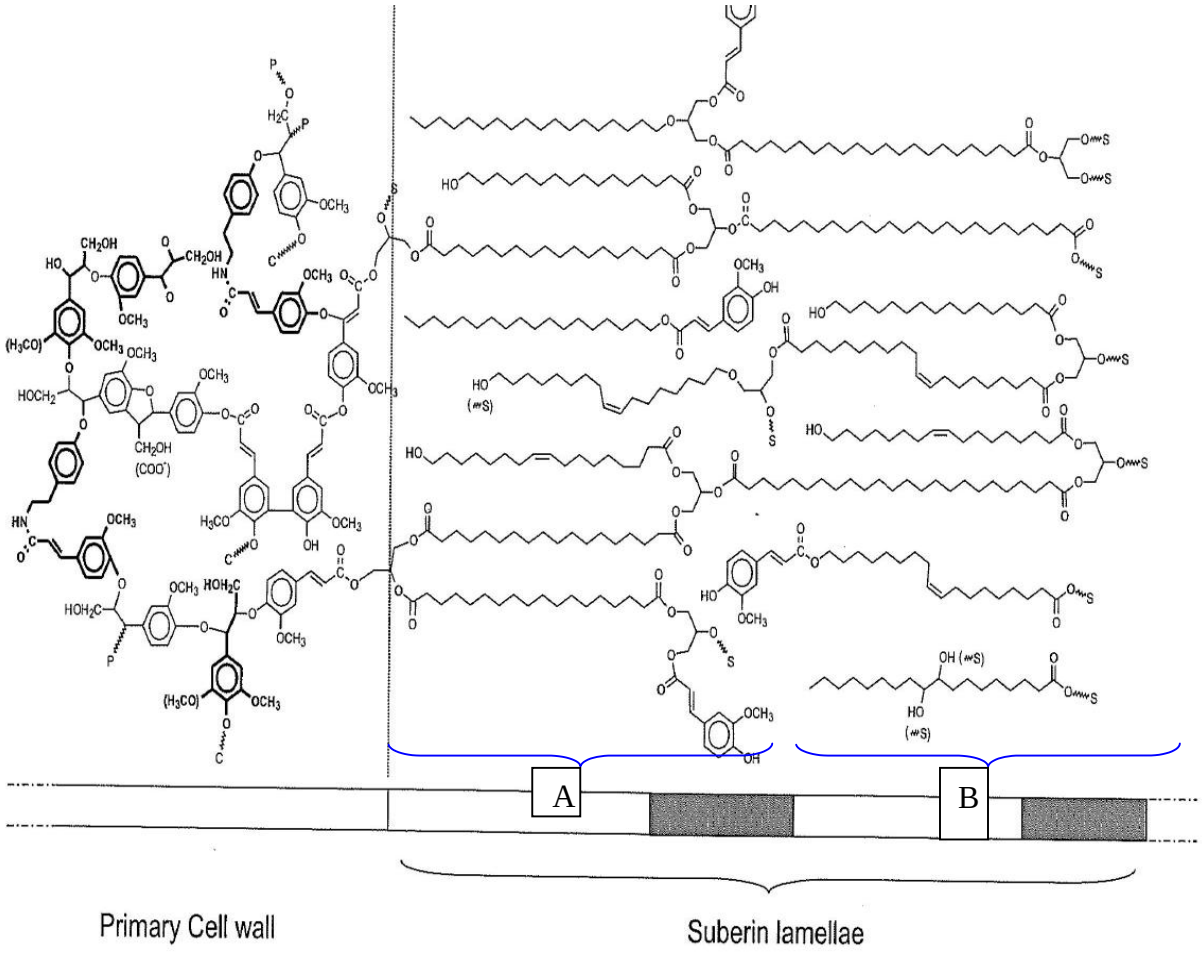


Figure 3. Ideal drawing describing part of a potato suberin with the poly(phenolic) (A) moiety and situated in the primary cell wall and liked to the poly(aliphatic) (B) domain with covalent bond and also to the carbohydrate unit (Bernards 2002).

Wound healing is an independent process from the normal development of the plant and initiated in action with a specific wounded tissues (Borchert and McChesney 1973).

This process might utilize a temporary program from the preexisting control system or in some cases the development of new once and it will disappear after the full healing of the wound (Borchert and McChesney 1973). The specific origin of cell walls that are to be suberized might

determine the chemical and physiological roles they have (Bernards 2002). Below ground plant tissue parts, which undergo suberin deposition, are mainly from the root meristem initials like the epidermis, exodermis, tuber and root phellem (Esau 1965). Wound induced initiation of metabolic activities has been preceded by transcription of genetic information and protein synthesis (Borchert and McChesney 1973). These activities include respiration (Click and Hackett 1963), phenylalanine-ammonia-lyase (Zucker 1965), fatty acid synthetase (Willemot and Stumpf 1967) and cell division and differentiation to suberize the cell wall surrounding the wound (Borchert and McChesney 1973). During the suberin deposition process the cell division and differentiation occur in a specific spatial pattern that is influenced by the location of the wound (Borchert and McChesney 1973).

Wound healing process can also reduce the rate of water loss from the tubers by the long chain waxy layer in the suberin structure (Kolattukudy 1987; Lulai and Orr 1994; Soliday et al. 1979). Lulai and Orr (1995) indicated that the action of soluble waxes to halt the water vapor loss comes prior to the deposition of suberin. The loss of turgor is crucial in keeping the cells around the wound alive for the coming suberin deposition (Lulai et al. 2008). The reduction in the water vapor loss has been initiated during the first 24 h time following wounding, before the deposition of any poly(phenolic) layer and days before the development of the alphatic moiety (Lulai and Orr 1995). This is supported by the idea that epidermal tissues that can be suberized are devoid of cuticle (Esau 1965; Matzke and Riederer 1991). Little is known about the role of hormones in regulating suberization and reduction in water vapour loss (Lulai et al. 2008). However, Lulai et al. (2008) noticed the trend of abscisic acid (ABA) content in healing tuber discs decreased after wounding, attain the minimum by 24 h and continued to increase from 3rd to 7th day. It was

stressed in the same paper that inhibition of endogenous ABA reduced the PAL activity and successively the deposition of the poly(phenolic) and poly(aliphatic) moieties (Lulai et al. 2008). Moreover, wax accumulation is also influenced by endogenous ABA, which in turn regulates dehydration of tissues (Lulai et al. 2008).

The presence of wounds on plant tissues induces the production of reactive oxygen species like superoxide, hydrogen peroxide and hydroxyl radicals, which protect the exposed tissues (Hammond-Kosack and Jones 1996; Kumar and Knowles 2003). Razem and Bernards (2003) claimed the role of reactive oxygen species (ROS) in the oxidative coupling of phenolic monomers during suberin deposition in wounded potato tubers. In suberized potato tubers a peroxide anionic isoform is suggested in the radical coupling of the phenolic monomers (Bernards et al. 2000). This was supported by biochemical analysis that identified anionic peroxidase in suberized wounds of potato (Bernards et al. 2000). Moreover, this anionic peroxidase also reacts with feruloyl-substituted substrates that are typical for healing wounds of potato tuber (Bernards et al. 2000). Bradley et al. (1992) also reported anti-microbial effect of ROS and facilitating the reaction in cross-linking cell wall proteins. It also serves as a signaling molecule for up-regulated defense genes (Levine et al. 1994; Tenhaken et al. 1995). In wounded potato tubers the oxidative burst and production of superoxide radicals is related to the plasma membrane-bound NADPH oxidase (NOX) (Park et al.1998; Sagi et al. 2004) that is produced at the time of wounding.

However, Lulai and Corsini (1998) indicted that inhibition of suberin deposition aggravated infection by pathogens. Resistance to bacterial infection is detected after the formation of the

first poly(phenolic) domain (Lulai and Corsini 1998). However, with the continued deposition of the first layer of the poly(aliphatic) moiety within 5-7 days fungal advancement was also stopped. In resistant tuber tissues with suberin deposition, steroid glycoalkaloids α-chaconine and α-solanine are produced to inhibit spore germination (Zeng 1993). Reports also showed production of sesquiterpenoid phytoalexins lubimin and rishitin in *Fusarium* infected tubers (Desjardins et al. 1989; Desjardins and Gardner 1991). Many other defense related genes encoding for enzymes like hydrolases, peroxidase, HMGCoA reductase and lypoxygenase were also upregulated in tubers infected with *Fusarium* dry rot fungi (D'Ippolito et al 2010). However, the susceptible cultivars fail to establish a uniform suberin deposition on the wounded potato tubers (Ray and Hammerschmidt 1998). Extended tuber storage period of over 30 months can depreciate the wound healing process, resistance to pathogens and production of superoxide (Kumar and Knowles 2003).

Resistance of Potato Tubers to Dry Rot

Tuber tolerance to *Fusarium* dry rot is higher during harvest but continues to decline in storage and even become more susceptible at planting (Kumar and Knowles 2003). Inducing wound healing and wound periderm can prevent infection (Vance et al. 1980). After harvest, storage temperature of around 12 to 15^{o}C at optimum air circulation and humidity, wound periderm can be formed within couple of weeks (Kushalappa et al. 2002). However, at a lower temperature the wound periderm formation can be extended and wound healing is not complete (Leach and Nielsen 1975).

Like other potato diseases the resistance to dry rot is considered as moderate and it varies among potato cultivars (Secor and Gudmestad 1999; Bukhart et al. 2007). Independent genetic factors are involved in controlling resistance against major *Fusarium* species (Wastie et al. 1989; Huaman et al. 1989). The *Fusarium* spp. used for inoculation also determines the strength of resistance and hence makes the breeding process difficult (Lynch et al. 2003). Some *Fusarium* species like *F. sambucinum* have also the capacity to overcome host resistance through sesquiterpenoid detoxification of phytoalexins and steroid glycoalkaloids (Desjardins and Gardner 1991; Weltring et al. 1997). In another study potato cultivars infected with the most widespread potato virus X (PVX) are observed to show better resistance to dry rot diseases (Jones and Mullen 1974).

Detection and Identification of Dry Rot

Identification and quantification of pathogens can help in diagnosis, phylogenetics and suggesting the right management practices and storage possibilities for processors and growers. In addition to symptom analysis, culturing and microscopy modern molecular-based techniques play a major role in quick, reliable and accurate identification of fungal pathogens (O'Donnell et al 1998). The available tools can be utilized in detecting the pathogen load for further decision making whether to process or store for extended use (Atallah and Stevenson 2006). Currently, the conventional polymerase chain reaction (PCR) technique which can make use of the internally transcribed spacer region (ITS) of the ribosomal DNA (rDNA) is utilized for limited application (Atallah and Stevenson 2006). However, higher sensitivity and target quantification can be achieved by using the real-time quantitative PCR and (Q-PCR) (Winton et al. 2002; Wen et al. 2005). The use of PCR-restriction fragment polymorphism assays can also facilitate routine

detection and identification by avoiding sequencing time and cost (Nitschke et al. 2009). This ITS region is comprised of two non-coding regions situated between 5.8S and a larger subunit rRNA genes in the rDNA (Gardes and Bruns 1993). The fungal ITS region is preferred for molecular identification due to its size that extends between 280 to 600 bp and can be also amplified easily by universal primers that compliment with the rRNA genes (White et al. 1990). The other merits of this region is the capacity to be amplified from a small, diluted or highly degraded DNA samples and its variability to morphologically specific fungal species (Gardes et al.1991; Gardes and Bruns 1993). However, many other reports suggest that intraspecific variation in the ITS region is considered low (Anderson and Stasovski 1992; Baura et al. 1992; Chen et al 1992). Limitations in comparing sample quantifications might be caused due to variations in rDNA with age and stage of the fungus (Paris and Lamattina 2002), and other non-orthologous copies of ITS region from the same species and among strains (Ko and Jung 2002).

The other sets of genes that have significant importance in the identification of fungi are the translation elongation factor 1-α (TEF) genes (Geiser 2003). These protein coding genes can offer more accurate and descriptive classification of *Fusarium* at species level (Geiser et al. 2004). Moreover, the universal primers that have been obtained from this region are applicable for the entire genus (Geiser et al. 2004). It is more appropriate for phylogenetic and taxonomic studies than for routine use (Geiser et al. 2004). Despite all the merits, this single-locus based identification method is more time consuming and laborious (Geiser et al. 2004).

Management Practices

Postharvest management of *Fusarium* dry rot for seed potato production has been done by treating tubers with fungicides such as thiabendazole (Al- Mughrabi 2010). However, resistance against this bendimidazole fungicide has been reported from *F. sambucinum* (Tivoli et al. 1986; Desjardins et al. 1993; Hanson et al. 1996), and *F. coeruleum* (Hanson et al. 1996). Other compounds like imazalil and mixtures of thiabendazol have been reported to be promising against dry rot (Carnegie et al. 1990; Carnegie et al. 1998). In general no other alternative control measures have been successful in managing *Fusarium* dry rot (Al-Mugharbi 2010). Biological control of postharvest diseases has been recommended to be effective and feasible (Janisiewez 1988; Wisniewski and Wilson, 1992). Whole-tube assay, laboratory and commercial storage tests showed that various gram-negative bacteria and strains from the *Pseudomonas*, *Enterobacter* and *Pantoea* genera were reported to suppress the growth of dry rot (Schisler and Slininger, 1994; Schisler and Slininger 1997; Schisler and Slininger 2000). Moreover, field trials on the use of antagonists *Pseudomonas fluorescens* and *Enterobacter cloacae* against *F. sambucinum* revealed average disease reduction of 35% and 26.5% respectively (Al-Mughrabi 2010). In general, integrated *Fusarium* dry rot control and management encompass selecting cultivars with better resistance, harvesting tubers for storage and seed from dead vines, reducing mechanical damage during harvesting and storage. Moreover, creating conducive environment for wound healing during storage, pre-warming of cold stored seed tubers at a temperature of 20-25^{0}C before preparing for planting, immediate planting of cuttings and proper hygiene to avoid contamination is recommended (Leach and Nielsen 1975). Application of fungicides both in storage and after planting of seed tubers can control dry rot disease (Leach and Nielsen 1975).

II. Objectives

General Objectives

The main aim of this study is an experimental work aiming at the identification of *Fusarium* species (*F. sambucinum* and *F. coeruleum*) by sequencing internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and design functional PCR-primers within this site.

Specific Objectives

Testing wound healing in potato tubers at different healing time with optimum temperature of 12^{o}C and 95% relative humidity.

Quantify the *F. sambucinum* and *F. coeruleum* infection in tubers at different time by measuring wound size.

Research Questions

Does the age of the wound affect the success of infection?

How does the duration of wound healing process at 12^{o}C affect infection and resistance in tubers?

How do the different species of *Fusarium* succeed in infection when compared?

III. Materials and Methods

Culture Preparation

Potato Dextrose Agar (PDA) solution was prepared with 36 g PDA dissolved in a liter of deionized water and autoclaved. Petri dishes were filled with PDA solution and allowed to cool down in a laminar flow hood. Isolates of *F. sambucinum* and *F. coeruleum* which were obtained from Norway were transferred to the PDA plates as subcultures by transferring about 0.4 cm^2 of mycelium and left to grow for 14 days at 25^oC.

For DNA extraction, 300 ml liquid culture media containing Potato Dextrose Broth (PDB) at a concentration of 24 g per liter of deionized water were prepared. Approximately 50 ml of the solution were poured in to six mini flasks, covered with aluminum foil and autoclaved.

DNA Extraction

A 200 ml liquid culture media containing Potato Dextrose Broth (PDB) with 24 g/lt of PDB and deionized water were prepared in two flasks. The top of each flask was covered with aluminum foil, autoclaved and was allowed to cool down in a hood for about 40 minutes. Mycelia from PDA plates of *F. sambucinum* and *F. coeruleum* were gradually scrapped from the agar using sterilized scalpels. Then each flask was inoculated by adding the mycelia from *F. sambucinum* and *F. coeruleum* respectively. The flasks were incubated in a regulated room at 25^oC for about 10 days. The mycelia were harvested and kept in a -20^oC freezer overnight and used for the DNA extraction. The DNA extraction was performed using a 3% cetyl trimethylammonium bromide (CTAB), phenol:chloroform:isoamyl alcohol , chloroform, ispropanol and 70% ethanol. A piece of mycelium was placed in a 1.5 ml eppendorf tube and grinded by a pestle. The grinded

mycelium was mixed with 600 µl of CTAB and kept on a heat block at 65^{o}C for 1.5 hrs. Then phenol:chloroform:isoamyl alcohol was added in equal volume to the earlier added CTAB and centrifuged for 10 min at 6000 rpm. The supernatant was transferred to a new eppendorf tube and the same volume of phenol:chloroform:isoamyl alcohol added and centrifuged it again for 10 min at 6000 rpm. The supernatant was transferred to a new eppendorf tube and chlorophorm was added to the same volume as the CTAB mixed in the earlier steps and further centrifuged for 10 min at 6000 rpm. The supernatant was transferred to a new eppendorf tube and 600 µl of ice cold isopropanol was added and settled overnight at -20^{o}C. Following morning the precipitated DNA was centrifuged for 30 min at 6000 rpm and the supernatant was discarded. The pellet was then washed with 1 ml 70% ethanol and centrifuged for 10 min with 6000 rpm and this step was repeated three times. Finally, all the ethanol was removed, the pellet was allowed to dry briefly and dissolved in 50 µl water and the DNA concentration and quality was measured on a Nanodrop.

Amplification of the ITS region from *F. sambucinum* , *F.coeruleum* and a strain of *Trichoderma viride* as reference strain was done according to the following protocol .The PCR cocktail of 25 µl each with 16 µl H_2O, 2.5 µl 10x buffer, 2.5 µl Dntp (2 mM),1 µl primers (10 mM), 0.75 µl $MgCl_2$ (50 mM) , 0.25 µl Taq polymerase (5 µ/ µl) and 1µl (8-15ng/ µl) DNA was amplified with the program on the thermocycler as 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s, 72^{o}C for 1 min and a final extension at 72^{o}C for 7 min. For this activity six primers that are used for the amplification of fungal ITS region were used including ITS 1, ITS1-F, ITS 2, ITS 3,ITS 4, and LR 1 (White et al. 1990, Gardes and Bruns 1993). A total of six

forward and reverse primer combinations were considered as described in the table below (Table 2). The PCR product was verified using gelelectrophoresis with 1% agrose gel, SB buffer and 1µl greenbuffer for staining. The gel was observed under ultraviolet light and photos were taken using the program Quantity one®.

Table 2. Primers used for the amplification of fungal ITS region.

Primer name	**Sequence (5'--> 3')**	**Reference**
ITS 1	TCCGTAGGTGAACCTGCGG	White et al. 1990
ITS 2	GCTGCGTTCTTCATCGATGC	White et al. 1990
ITS 3	GCATCGATGAAGAACGCAGC	White et al. 1990
ITS 4	TCCTCCGCTTATTGATATGC	White et al. 1990
ITS 1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns, 1993
LR 1	GGTTGGTTTCTTTTCCT	

Primer Design

The PCR amplification products obtained by using ITS 1-F x LR 1 and ITS 1 x LR 1 on *F. sambucinum, F. coeruleum* and positive control were selected for sequencing. Sequencing was performed by Macrogen Incorporation, Amsterdam, the Netherlands. These sequences were further analyzed using the Lasergene 9®, SeqMan Pro® software and aligned using ClustalW. Basic local Alignment Search (BLAST) was also conducted to confirm the species identity of the sequences. Primers were designed using Primer3® and Primer select® in Lasergene 9 software package and the results were thoroughly crosschecked for similarity. A total of eight and six

primers were selected from *F. coeruleum* and *F. sambucinum*, respectively (Table 3). Based on these sequences the primers were synthesized by Biomers.net®, Stuttgart, Germany.

Table 3. List of primers designed from the sequences of *F. sambucinum* and *F. coeruleum* ITS region using primer set ITS1 x LR1.

Primer name	primer sequence (5'--->3')	Sequence origin
C1F	TCAAGCTCTGCTTGGTGTTG	*F. coeruleum*
C1R	TTGACCTCGGATCAGGTAGG	*F. coeruleum*
C2R	ACTCGCCTCAAAACAATTGG	*F. coeruleum*
C3R1	ATTTCGGAGCGCAGTACATC	*F. coeruleum*
C4F	ACAAGGTTTCCGTAGGTGA	*F. coeruleum*
C4R	AGACTCGCCTCAAAACAATT	*F. coeruleum*
C5F	GCTTTGCCTGCTACTATCTCTTAC	*F. coeruleum*
C5R	TCAATAAGCGGAGGAAAAG	*F. coeruleum*
S1F	ATCTCTTGGTTCTGGCATCG	*F. sambucinum*
S1R	AAATACATTGGCGGTCTTGC	*F. sambucinum*
S2R	CGAAATACATTGGCGGTCTT	*F. sambucinum*
S3F	GGAGGGATCATTACCGAGTTTACA	*F. sambucinum*
S3R	TAAAACCCCAACTTGTGAATGTGA	*F. sambucinum*

To determine the optimal annealing temperature for each primer pairs PCR with temperature gradients were run from 48 to 58^{o}C with 2^{o}C intervals using the PCR cocktail protocol described above. The efficiency of both primer sets, developed from the sequences obtained from the ITS-region of *F. sambucinum and F. coeruleum* were also tested against the *F. sambucinum*, *F. coeruleum* and *T. viride* DNA samples, using the same PCR cocktail described above. The same PCR condition mentioned above were also applied except for the annealing temperature of 56

and 54 ^{o}C for the primers designed from the ITS regions of *F. sambucinum* and *F. coeruleum*, respectively.

The primer combinations were also tested with a gradient of DNA concentration (10, 10^2, 10^3,10^4 and highest concentration) of *F. sambucinum* and *F. coeruleum* and *T. viride*. The gel was visualized under ultraviolet light and photos were taken. In order to check the effect of unknown contaminants samples were also run with 50 µl of PCR mixture with doubled $MgCl_2$ concentration.

Testing *Fusarium* spp infected potato tubers in the laboratory.

Dry rot susceptible potato variety Melody was selected and 10 potato tubers for each *Fusarium* species were damaged using a nail tip with 1 cm deep, 50 mm diameter wound size and inoculated with mycelia of *F. sambucinum, F. coeruleum* and *F. avenacium*, respectively. The tubers were stored at room temperature of 20 $\pm$ 2°C in separate plastic boxes with sufficient relative humidity for about 8 weeks. Samples of mycelia from the periderm and cross-section of the rotten potatoes were taken. The DNA was extracted using the procedure described above and amplified using PCR with the S3F x S3R , as well as the ITS1F x LR1 primers.

The primer pair S3F x S3R designed to amplify *F. sambucium* was the most consistent primer pair during early testing and was therefore tested using DNA samples extracted from *F. cerealis, F. culmorum, F. avaenaceum, F. coeruleum, F. sambucinum* and *T. viride*.

Bioassay Test in Phytotrone

The effect of wound healing time on the infection level of dry rot disease was tested parallel with the molecular work. The susceptible variety named Melody which was harvested in 2011 cropping season from the Uppsala län area was used. Ten potato tubers per treatment were carefully semi-sterilized using 70% ethanol and damaged using a sterilized nail tip with depth of about 1cm and 50 mm diameter wound size. The wounds were made in two days interval from day 0 to 14 (0, 2, 4, 6, 8, 10, 12, 14 days post *Fusarium* infection) and tubers were stored at an average wound healing temperature of 11°C with high relative humidity (72%) in darkness. The potatoes were then inoculated using 40 µl spore suspension (50 spores /µl) of *F. avenaceum* and *F. coeruleum* per wound. The inoculated potato tubers were further stored at a temperature of 2°C and 80% RH for about 4 weeks and later taken to 21°C with higher RH (110%) for another 2 weeks to mimic potential fluctuations during storage conditions. Then data was taken on the presence of rot or not (rot = 1 and non-rot= 0) and the infection was further quantified by measuring the lesion size. The potato tuber was cross-sectioned into equal parts starting from the wound point and the diameter and depth of the lesion was measured and recorded. A relative volume of the rot was calculated using the formula for the volume of a cone ($\pi r^2 h/3$). The data was analyzed using Excel® sheet and were subjected to analysis of variance (ANOVA). Then the means were compared using F-test and statistical significance were checked at 5% significance level ($\alpha = 0.05$) (Figure 9)

IV. Results

Amplification of the ITS region using universal primers

The universal primer pairs tested against *F. sambucinum*, F.coeruleum and *T. viridae* DNA samples had different PCR products (Table 4).The amplification of the *F. sambucinum*, *F. coeruleum* and *T. viridae* DNA samples using the primer pairs ITS 1 x ITS 2 and ITS 1-F x ITS 2 yielded a product around 280 bp for *F. sambucinum* and the reference strain (Figure 4).

Table 4. PCR amplification of DNA samples from *F. coeruleum*, *F.sambucinum* and *T. viride* using different universal primer sets.

Primer pairs tested	presence of PCR product (+ or -)			approximate band size (bp)
	F. coeruleum.	*F.sambucinum*	*T. viride* (+ve control)	
ITS 1 x ITS 4	-	-	+	520
ITS1-F x ITS 4	-	-	+	520
ITS 1-F x ITS 2	-	+	+	280
ITS 3 x ITS 4	-	-	+	280
ITS 1 x ITS 2	-	+	+	280
ITS 1 x LR 1	-	+	+	530
ITS1-F x LR 1	+ (faint)	+	+	530
ITS 3 x LR 1	-	+	+	280

However, the primer pairs ITS 3 x ITS 4 yielded a product of 280 bp only for *T. viridae* DNA sample (Figure 4). The *F. coeruleum* DNA samples were not amplified by the three primer pairs tested. The second set of test was conducted by replacing the reverse primer ITS 2 in three primer pairs used above to LR 1, which binds to another site in the large subunit. Again *F. sambucinum* and *T. virdae* DNA samples were amplified by primers ITS 1 x LR 1 and ITS 1-F x

LR 1and resulted in a product that is around 530 bp (Figure 5). The third primer pair ITS 3 x LR 1 also yielded a product for *F. sambucinum* and *T. virdae* but shorter in size, 280 bp (Figure 5). Contrary to what was observed in the above primer pairs the ITS 1-F x LR 1 yielded a faint product of 530 bp for the *F. coeruleum* DNA samples. Later, a nested PCR was performed on this product and the results had improved but the band on the gel was still faint compared to the other results.

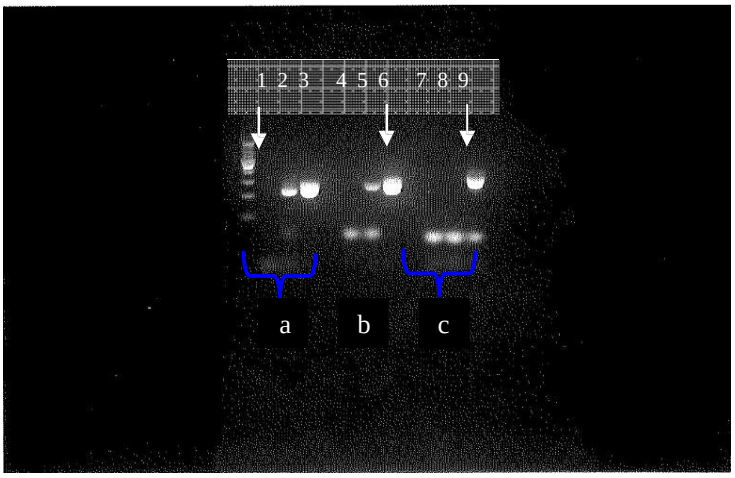


Figure 4. PCR products of *F. coeruleum* (wells 1, 4, 7), *F. sambucinum* (2, 5, 8) & *T. viride* (3, 6, 9) DNA samples tested using the primer pairs ITS 1 x ITS 2 (a), ITS 1F x ITS 2 (b) & ITS 3 x ITS 4 (c).

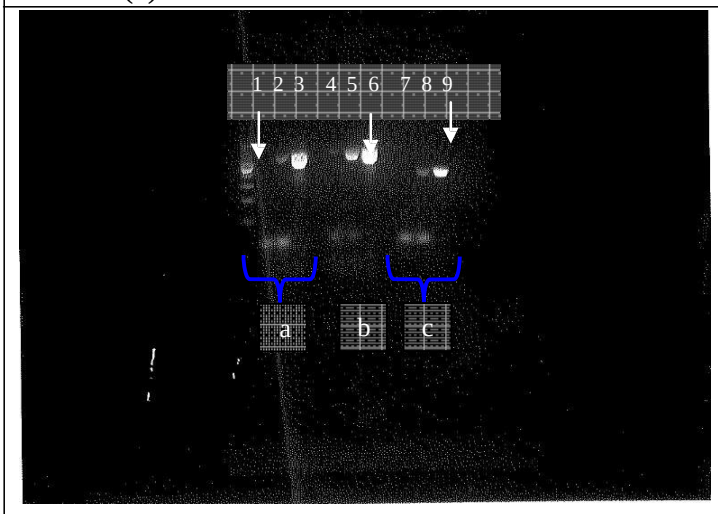


Figure 5. PCR amplification of *F. coeruleum* (wells 1, 4, 7), *F. sambucinum* (2, 5, 8) & *T. viride* (3, 6, 9) DNA samples tested using the primer pairs ITS 1 x LR 1(a), ITS 1-F x LR 1(b) & ITS 3 x LR 1 (c).

In addition, DNA samples taken from the periderm (P) and cross section (CS) of potato tubers infected with *F. sambucium, F. avenaceum* and *F. coeruleum* were amplified using ITS 1 x LR 1 and ITS 1-F x LR 1 primer pairs. A clear band was noticed for *T. viride* only using the ITS 1 x LR 1 primer pair (Figure 6). However, the ITS 1-F x LR 1 primer pair successfully amplified all the samples taken from the rotten potato tubers and the reference *T. viride* (Figure 6).

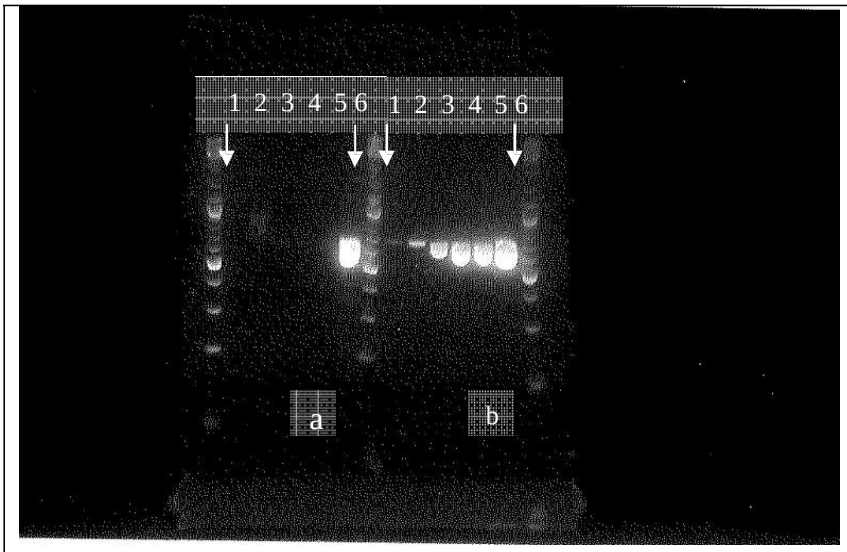


Figure 6. PCR amplification of DNA samples of *F.coeruleum* (wells 1-P, 2 -CS), *F. avenaceum* (wells 3-P, 4-CS), *F. sambucinum* (well 5) and *T. viride* (well 6) taken from periderm(P) and cross section (CS) of rotten tubers using primer pairs ITS 1 x LR 1 (a) and ITS 1-F x LR 1 (b), respectively.

Primer Designs

The primer pairs in table 5 amplified parts of the ITS region of *F. sambucinum* and *F. coeruleum*. Assigning primer pairs were conducted by evaluating the minimum self-compatibility and formation of secondary structures that can occur through intermolecular and intramolecular interactions. In the case of *F. coeruleum*, some primers were cross-paired with those that are designed from *F. sambucinum* (S1F x C2R and S1F x C3R). Moreover, primer

pairs that amplifed *F. sambucinum* and *F. coeruleum* DNA with annealing temperature of 56 and 54^{o}C respectively were considered.

Table 5. PCR amplification of DNA samples from *F. coeruleum, F.sambucinum* and *T. viride* using primer sets designed from the sequences of *F.sambucinum* and *F. coeruleum* ITS regions.

Primer pairs tested	presence of PCR product (+ or -)			approximate band size (bp)
	F. coeruleum.	*F.sambucinum*	*T. viride* (+ve control)	
C1F x C1R	-	-	-	
S1F x C2R	-	-	-	
S1F x C3R	-	-	-	
C4F x C4R	-	-	-	
C5F x C5R	-	-	-	
S1F x S1R	-	-	+	230
S1F x S2R	+	+	+	230
S3F x S3R	+	+	+	491

The primers were also tested with a dilution series (1, 10, 10^2, 10^3,10^4) of the sample DNA from *F. sambucinum,*and *F. coeruleum*. The reference strain (*T. viride*) used as a positive control. The primer pair S1F x S1R did not amplify any product except the reference. The second set of primers (S1F x S2R) from *F. sambucinum* had a strong result with the lowest dilution rate (10x) of *F. sambucinum* samples. However, 10^3x diluted *F. coeruleum* sample resulted in a clear and visible product compared to the rest of the treatments. Moreover, the same procedure were run for the *F. coeruleum* selective primer pairs; S1F x C2R, S1F x C3R, C4F x C4R and C5F x C5R that did not amplify any product, including *T. viride*.

Primer Specificity

The primer pairs designed from the *F. sambucinum* sequence, S1F x S2R and S3F x S3R specificity were tested with the PCR cocktail used above but in a total volume of 50 µl and doubled $MgCl_2$ concentration. Both *F. sambucium* and *F. coeruleum* DNA samples in dilution series were used. Only the S3F x S3R primer pair tested with the highest concentration of *F. sambucinum, F. coeruleum* DNA and the *T. viride* reference strain yielded a product of approximately 491 bp. However, the S1F x S2R primers amplified a product of around 230 bp only for the *T. viride* DNA samples with highest DNA concentration (Figure 7).

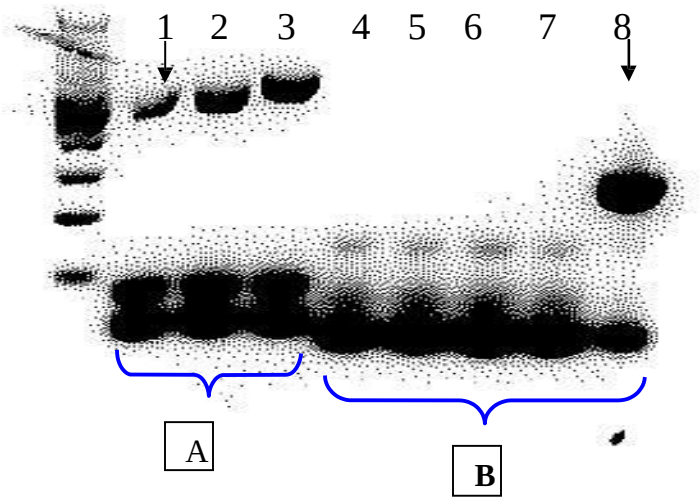
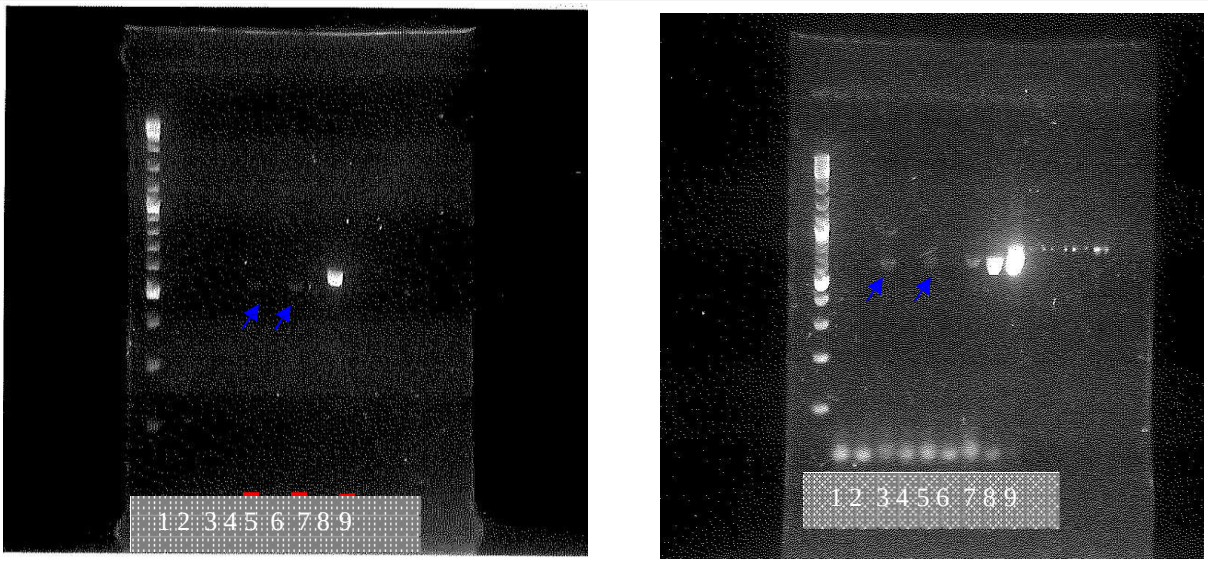


Figure 7. PCR amplification by primer pair: A) S3F x S3R with DNA samples of *F. coeruleum* (well 1), *F. sambucinum* (2) & *T. virdea* (3) at highest concentration, respectively.
B) S1F x S2R with DNA sample of *F. coeruleum* at concentrations 10^3x (well 4) and 10^4x (well 5), *F. sambucinum* at concentrations 10x (well 6), 10^2x (wells 7) and *T. virdea* at*:* highest conc. (well 8), respectively

The DNA samples obtained from infected potato tubers using *F. sambucium, F. coeruleum, F. avenaceum* and *T. viride* amplified using the primer pair S3F x S3R resulted in a product of the desired size (491 bp) only for the *F. sambucium* and *T. viride*. Moreover, the specificity of this primer pair was tested against *F. cerealis, F. culmorum, F. avaenaceum, F. coeruleum, F.*

sambucinum and *T. viride* DNA samples. The result revealed non-specific amplification of DNA samples with the band size of around 491 bp for *F. avenaceum* , *F. sambucium* and *T. viride*. The bands for the *F. avenaceum* and *F. sambucium* were faint and might be due to the DNA quality (Figure 8).



A **B**

Figure 8. PCR products by using primer sets S3F x S3R (A) and ITS 1-F x LR 1 (B) with DNA samples of *F. cerealis* (wells 2,3), *F. culmorum* (1, 4), *F. avaenaceum* (5,6), *F.coeruleum* (8), *F. sambucinum* (7) and *T. viride* (9).

Bioassy Test in Phytotron

The mean relative rot incidence on potato tubers assessed after storing at wound healing temperature (12°C), four weeks at -4°C and two weeks at 21± 2°C revealed that tubers infected with *F. coeruleum* and *F. avenaceum* immediately after damaging (without wound healing) had the highest rot volume of about 2.3 and 8.1 mL, respectively (Figure 9). The relative rot size for *F. avenaceum* remained high at 4.7 and 3.7 mL for potato tubers that are stored at wound healing temperature for 2 and 4 days respectively (Figure 9).

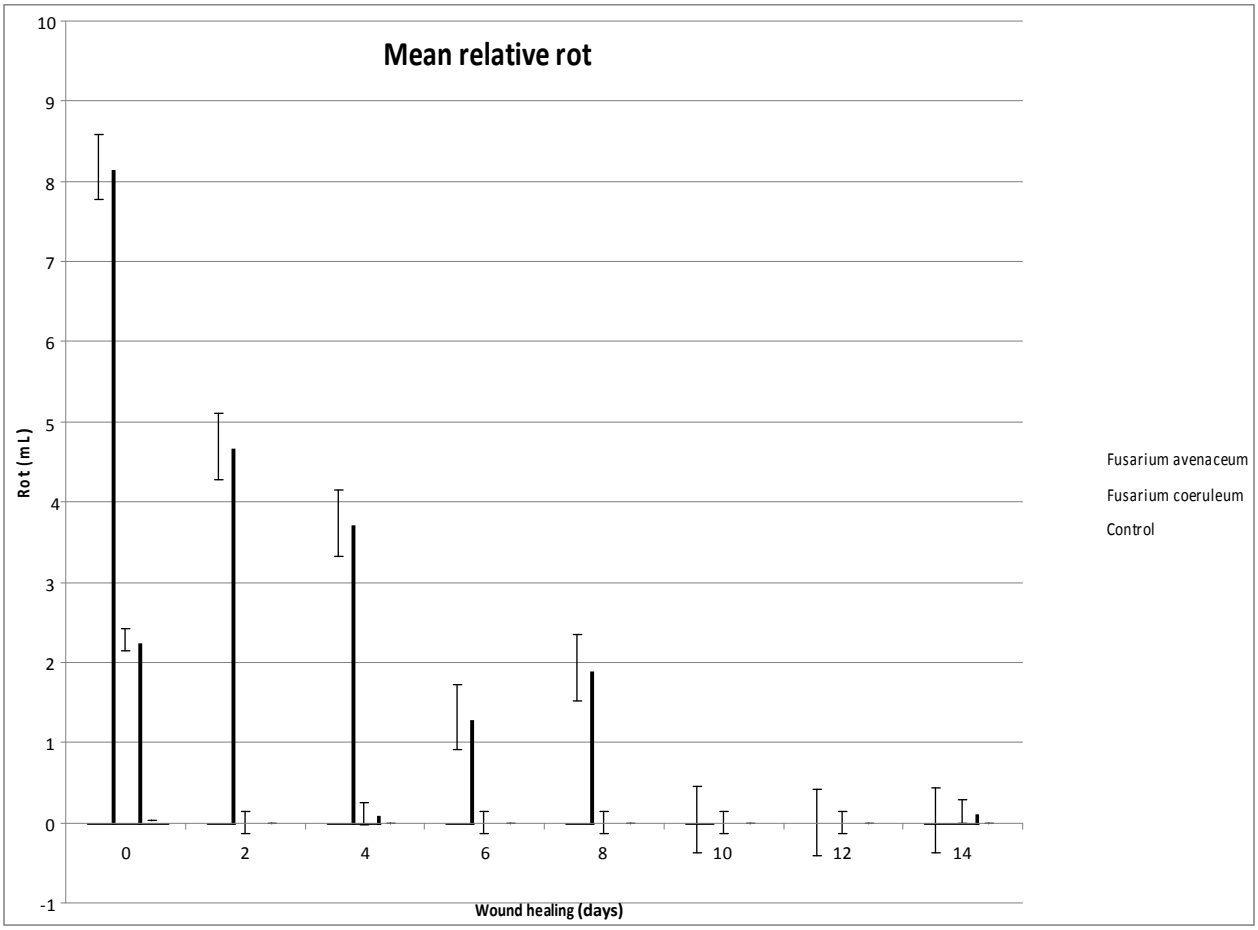
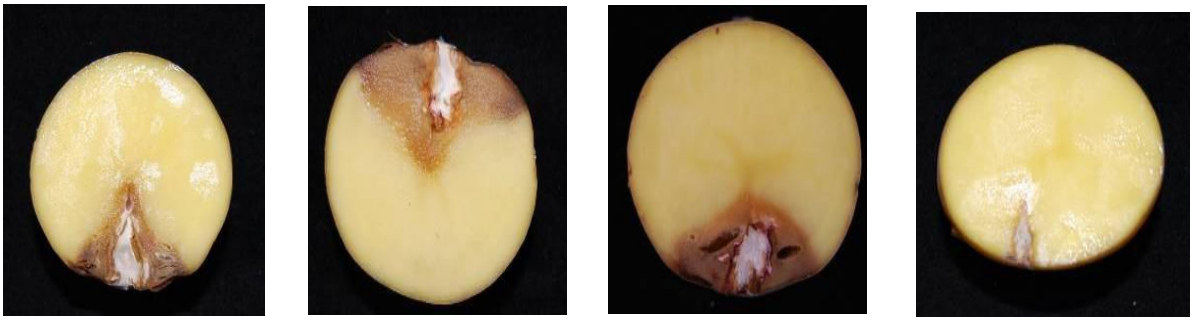


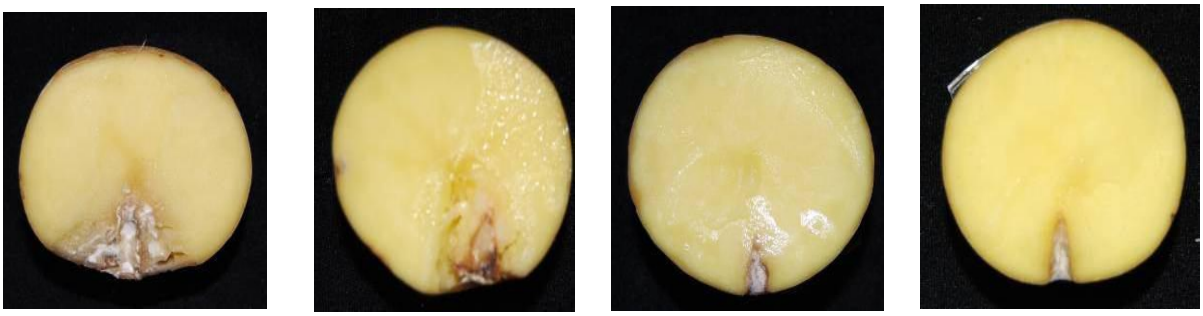
Figure 9. The mean relative rot volume of *F. avenaceum* and *F. coeruleum* laboratory infected tubers ± SE.

However, for the *F. coeruleum* infected tubers the mean rot volume has reduced significantly for tubers treated for 6 days or more in wound healing temperature. In the case of *F. avenaceum* the rot has completely avoided after keeping the potato tubers in wound healing temperature for a week (Figure 10). Moreover, this quantification of rotten tissues of laboratory infected potato tubers implied that *F. avenaceum* species had the highest mean rot size and frequency which was significant ($p=0.018$) compared to *F. coeruleum*.



A. 0 days B. 2 days C. 4 days D. 6 days

I. *F. avenaceum* infected tubers.



A. 0 days B. 2 days C. 3 days D. 6 days

(Picture: Thomas and Nicklas 2012)

II. *F.coeruleum* infected tubers.

Figure 10. Dry rot symptom of laboratory infected tubers with *F. avenaceum* (I) and *F. coeruleum* (II) spores and stored at different days of wound healing.

V. Discussion

In this study, the strains of *F. coeruleum* and *F. avenaceum* were identified as being more aggressive pathogens compared to the tested *F. sambucinum* strain. It was also indicated in the work of Al-Mughrabi (2010) that *F. coeruleum* was more infectious compared to *F. sambucinum*. However, this specific study was not comprehensive enough to generalize *F. avenaceum* as more aggressive than *F.coeruleum* due to the limitation of using one strain per species for the experiment which emphasized a specific characteristic for the strains than the species as a whole. However, according to Hide et al. (1992) *F. sambucinum* is the most associated pathogen species to potato tubers in North America and parts of Europe.

Phenotypic classification is often complicated expertise, time consuming, and misleading due to the various characters involved (O'Donnell 2000). Fungal diagnostics using molecular techniques can be used to confirm cultural and morphological characterization and classification (Lenc et al. 2008). It is based on the nucleotide sequencing where variation in the DNA sequences is used to distinguish between species, or even between individuals (O'Donnell et al.
1998). According to Geiser (2003) the markers used for molecular fungal characterization are intron-rich parts of the protein coding genes. However, the ITS region is more frequently used and evolve at a slower rate in species compared to the other regions (O'Donnell, 2000). Identification of *Fusarium* spp. using PCR amplification of ITS region of the rDNA using the right primer pairs is quick, accurate and reliable. Duggal et al. (1997) stated that the ITS region shows polymorphisms between and within *Fusarium* spp. This is supported by White et al. (1990) and O'Donnell (1992) which emphasizes the feasibility of using the ITS region as a genetic marker to amplify *Fusarium* spp. It was also stated in these works that the rDNA region of *F. sambucinum* is highly conserved. Nevertheless, the presence of non-orthologous copies of

the ITS 2 is observed in *Fusarium* genus, which may result in wrong identification (O' Donnell and Cigelnik, 1997; O'Donnell et al. 1998). Moreover, very small sequence divergence was observed in the ITS region that can discriminate species from the same clan (Turner et al. 1998) Hence, recent studies suggest the translation elongation factor 1-α (TEF) gene is more applicable for characterization of *Fusarium* at species level (O'Donnell et al. 1998 ; Seifert and Levesque 2004). It is also argued that the gene has not been shown to have non-orthologous copies and universal primers are available for the genus (Geiser et al. 2004). It is also implied that comparable or even lesser time is required to clone and sequence TEF-1α than to use specific PCR primers (Nitschke et al. 2009).

The process of amplifying the ITS region from *F. sambucinum* and *F. coeruleum* using the universal primers was laborious and time consuming. It was also characterized by the presence of unknown contaminants that negatively affected the DNA extraction and PCR reactions. Incorporation of higher $MgCl_2$ in the PCR protocols has elevated this problem and resulted in more precise outcomes. Hence, developing primer pairs with species-specific resolution is time saving, easy to apply and reliable. This is in line with the work of Lenc et al. (2008) which emphasize the use of species-specific primers for PCR amplification of *F. sambucinum* .The universal primer pairs ITS1 x ITS2, ITS1-F x ITS 2, ITS 1 x LR 1, ITS 1-F x LR 1 were able to amplify *F. sambucinum* DNA samples. In contrary to this study which failed to get a product using primer pair (ITS 1 x ITS 4), O'Donnell (1992) has reported that primer pair ITS 4 x ITS 5 has amplified PCR product of the required size from *F. sambucinum* samples. Moreover, his work identified that *F. sambucium* is not a single species and has three divergent ITS types fixed in the nuclear rDNA (O'Donnell 1992).

The *F. sambucinum* primer pair S3F x S3R has failed to discriminate the DNA samples from *F.coeruleum* and *T. viride*. This might be supported by the result from Solexa® data analysis of the ITS region of the *F. sambucinum* and *F. coeruleum* strains used in this study, that showed only about 7 bp difference in the sequence (Karlsson and Samils, unpublished). *F. sambucium* was detected using the primer pairs S3F x S3R in DNA samples from tubers infected in the laboratory. As opposed to our result, Mishra et al. (2003) designed *Fusarium* species-specific PCR primers from the nrDNA ITS region that effectively differentiate five *Fusarium* species. The *F. sambucinum* selective primer pairs (FSF-1 x FSR-1) amplified the expected product size of about 315 bp for the specific *F. sambucinum* isolates (Mishra et al. 2003).

Therefore, the ITS region of rDNA can be used for effective diagnosis of many fungal genera but for *Fusarium*, the translation elongation factor 1-α (TEF) has proven to be more reliable, especially when aiming for resolution between species. However, PCR amplification of DNA samples from *Fusarium* spp. using the universal primer pairs designed from the ITS region of rDNA can also offer useful and reliable information. The method helped to investigate the polymorphism between *Fusarium* spp. used to identify a specific ITS region. This ITS region which has a size of 280 to 600 bp can also be easily amplified by universal primers that can compliment with the rRNA genes (White et al. 1990). Moreover, the high copy number of rRNA genes from the ITS region makes it easy to amplify from a small, diluted or highly degraded DNA samples (Gardes et al. 1991; Gardes and Bruns 1991). Hence, the PCR protocols specified in this paper can be used as a reference for proper amplification of the required product size and quality.

Effect of wound healing on dry rot disease development

Formation of wound periderm in damaged plant tissues can restrict pathogen progress to a specific site. It is also noticed from this study that wound healing time of about one week can decrease infection by *F. coeruleum* and *F. avenaceum* on potato tubers and delimit the lesions caused by these pathogens. Many studies imply that the wound healing process involves the development of impermeable layers that protect the damaged tissues from infection and avoids the loss of moisture (Vogt et al. 1983; Lulai and Corsini 1998; Schreiber et al. 2005). Lulai and Corsini (1998) reported that resistance to fungal infection occurred after the deposition of the first polyaliphatic component of the suberin layer with in 5-7 days of the wound healing process. This finding is also in line with our study where we observed the decline in relative mean rot volume of laboratory- infected tubers with *F. avenaceum* and *F. coeruleum* spores after 4-6 days of wound healing time. The highest mean rot recorded for both *F. coeruleum* and *F. avenaceum* infected tubers was at 0 and 2 days wound healing time, which might be due to the combined effect of bacteria and fungal infection. This complies with the work of Lulai and Corsini (1998), which emphasize the role of suberin polyphenolic component accumulated in 2-3 days of the wound healing to halt bacterial infections.

Furthermore, the *F. avenaceum* strain used in this study was more aggressive with the highest rot symptoms compared to the *F. coeruleum* strain. From a study done in Scotland, *F. avenaceum* was reported to cause the highest incidence of postharvest rotting (Choiseul et al. 2007; Cullen et al 2005). Peters et al. (2008) implied *F. coeruleum* as a more commonly isolated species when compared to *F. avenaceum* and *F. sambucinum*, but larger tuber rots were observed in most cultivars infected with *F. sambucinum* at 10°C.

To conclude, dry rot disease caused by *Fusarium* species is an economically important field and postharvest disease through out the world. The species *F. coeruleum*, *F. avenaceum* and *F. sambucinum* are most commonly associated with dry rot in Europe and US. The quick and reliable identification of these species can be performed using available universal primers or by designing desired primers from the ITS region of rDNA, or other sites in the DNA. Allowing wounded potato tubers to heal at 12°C for about 6-8 days can reduce the dry rot symptoms of tubers exposed to *F. avenaceum* and *F. coeruleum*. This study was also concluded that the investigated strain of *F. avenaceum* was aggressive and caused more severe tuber rots compared to the other tested species. Finally, further field surveys and quantification of the disease using QPCR may be applied as a tool to study the disease incidence. Integrating the application of wound healing with biological control methods can be also considered for effective management of dry rot disease and should receive further attention.

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VI. References

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