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Swedish University of  
Agricultural Sciences

**Postharvest Biological control of Fusarium dry-rot disease in potato tubers  
using *Clonostachys rosea* strain IK726.**

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## Abstract

The common saprophytic fungus *Clonostachys rosea* (*Gliocladium rosea*) has been reported for its biological control capacity against plant pathogenic fungi. Postharvest application of *C. rosea* strain IK726 and the mechanism of action against potato dry rot disease are not properly investigated. In this piece of study the biological control potential of strain IK726 against *F. avenaceum* and *F. coeruleum* was investigated considering the postharvest processing and handling procedures used in the potato industry. Moreover, its effect on the rot development, wound colonization potential and interactions with the *Fusarium spp.* were studied in potato tubers and culture media. In phytotron bioassay the mean number of rot incidence has reduced significantly ( $p= 0.018$ ) to 16.25% and 20% in tubers treated with *C. rosea* strain IK726 and artificially infected with *F. avenaceum* and *F. coeruleum*, respectively. It was about 45% reductions in the mean number of rot compared to the non-treated ones. *C. rosea* strain IK726 had also survived the fluctuation in temperature from 12, 4 and 22 °C overtime and managed to give significant control. Dual culture tests showed lack of clear inhibition zone and the mycelia of *C. rosea* had grown into and covering the *Fusarium* mycelia and gradually suppressed its growth. The microscopy study revealed a mycoparasitic-like interaction in which direct contact and growth of strain IK726 on and along the hyphae of *F. avenaceum* and *F. coeruleum*. Finally, time-lapse spore interaction tests implied delay in *F. coeruleum* spore germination to 8 to 10 hours and the germ tube growth was also affected in the presence of *C. rosea* strain IK726. Therefore, *C. rosea* strain IK726 has the potential to control dry-rot disease in potato tubers in combination with postharvest handling practices and storage conditions. Moreover, effective colonization of tuber wounds, antibiosis and mycoparasitic-like action could possibly be the mode of action against the *Fusarium spp.*

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## **Introduction**

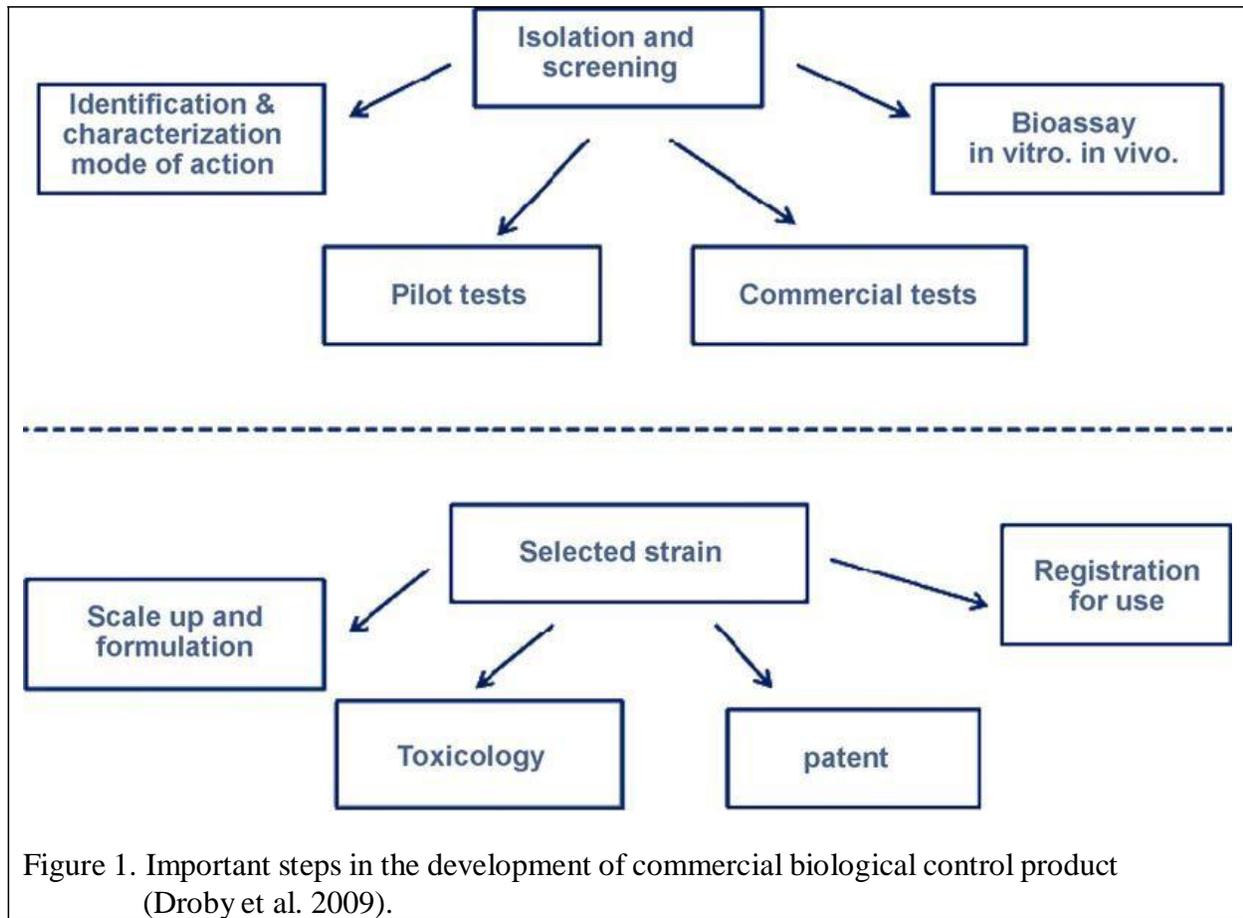
### **Postharvest application of Biological Control**

The economic significance of postharvest loss of fruits and vegetables due to rot by pathogens is immense (Sharma et al. 2009). In the developed world these postharvest losses that occur during transportation and storage account up to 25% of the total harvest (Dorby 2006; Singh and Sharma 2007). However, in countries where these facilities are inadequate the losses might even be more severe (El-Ghaouth et al. 2004; Zhu 2006).

The postharvest disease management of food crops has earlier been restricted to the use of chemical pesticides, storage facilities, heat and UV irradiation (Eckert 1967). Easy application and relatively cheaper cost have made postharvest applications of agrochemicals more preferred. However, the high health risks posed and awareness created by the public have been the limiting factors for type and number of pesticides allowed for postharvest use (Mari et al. 2007; Sharma 2009). More research trials also indicated increased resistance against postharvest chemicals (Delp 1980). This growing sentiment against the use of chemicals on harvested products and limited application of other alternative measures have been the driving factors for looking noble and effective means (Sharma et al. 2009). At this point, biological control of postharvest pathogens came in to the picture (Wilson and Pusey 1985).

In general, the development of a biological product for commercialization is time consuming, expensive and determined by various factors (Blachinsky et al. 2007; Droby et al. 2009) (Figure

1). Hence, limited progress has been made in an attempt to secure safe, effective and economically feasible biological products (Droby et al. 2009).



Recently the application of microbial antagonists like bacteria, yeasts and fungi to control postharvest decay is gaining attention (Wisniewski and Wilson 1992, Sharma et al. 2009). The capacity to determine and modify environmental conditions in storage facilities, efficient spot specific application of biocontrol products and the cost benefit in using various control protocols in stored food than field application could be the merits of postharvest biocontrol (Wilson and Pusey 1985; Janisiewicz and Korsten 2002). This has been substantiated by various reports made on the efficacy of biocontrol agents on postharvest pathogens (Wilson and Wisniewski 1994) For example, gray mold disease on strawberries were considerably checked both before and after

harvest by the application of *Trichoderma* spp. (Tronsmo and Dennis 1983) and *C. rosea* isolate Pg 88-710 ( Sutton et al., 1997; Sutton and Peng 1993). In potato tubers, yeast strains were reported to effectively control dry rot incidence caused by *F. sambucinum* (Schisler et al. 1995). Among the reported antagonists that have been effective under laboratory conditions only few products were commercialized (Table 1).

Table 1. Biological control products available in the market for postharvest diseases (Sharma et al. 2009)

Products	Microbial agent	Fruits/vegetables	Target disease (s)
AQ-10 bio-fungicide	<i>Ampelomyces quisqualis</i> Cesati ex Schlechtendahl	Apples, grapes, strawberries, tomatoes and cucurbits	Powdery mildew
Aspire	<i>Candida oleophila</i> strain 1-182	Apple, pear, citrus	Blue, gray and gree-mold
Biosave 10LP, 110	<i>Pseudomonas syringae</i> (strain 10LP, 110)	Apple, pear, citrus, cherries, potatoes	Blue and gray mold, mucor, and sour rot
Blight Ban A 506	<i>P.fluorescence</i> A 506	Apple, pear, strawberries, potatoes	Fire blight, soft rots
Contans WG, Intercept WG	<i>Coniothyrium minitans</i>	Onion	Basal, neck rots
Messenger	<i>Erwinia amylovora</i> (Burrill)	Vegetables	Fire blight
Rhio-plus	<i>Bacillus subtilis</i> FZB 24	Potatoes , other vegetables	Powdery mildew, root rots
Serenade	<i>Bacillus subtilis</i>	Apple, pear, grapes and Vegetables	Powdery mildew, late blight, brown rot, fire blight

The modes of action of microbial antagonists have been the point of discussion in several research works (Korsten et al. 1997; El-Ghaouth et al. 2004; Droby et al. 2009; Sharma et al. 2009). However, the mechanism of influence applied by the biological control agents on the pathogens has not yet completely understood (Droby et al. 2009; Sharma et al. 2009). Among the suggested ways of action by antagonists, competition for nutrient and space, production of antibiotics, direct parasitism and induced resistance have been frequently stressed (Wilson et al. 1993; Janisiewicz et al. 2000; El-Ghaouth et al. 2004; Sharma et al. 2009). In general,

understanding these mechanisms can help to study the existing biological control agents for better performances. Moreover, it will assist in selecting noble and more effective products for future (Wisniewski and Wilson 1992; Sharma et al. 2009).

### **Dry rot disease**

Dry rot is a storage and in field potato tuber disease widely recognized for its economic importance (Leach and Webb, 1981; Hanson et al. 1996; Lenc et al. 2008). It is a fungal disease caused by different *Fusarium* spp. and the most frequently associated ones include *F. coeruleum* (*F. solani*), *F. sambucinum* (*F. sulphureum*), *F. avenaceum*, *F. culmorum* and *F. equiseti* (Cullen et al. 2005; Lenc et al. 2008; Peters et al. 2008). In most dry rot cases more than one *Fusarium* spp. are responsible although reports of a single species infection is rarely noticed (Latus-Zietkiewicz 1993; Lenc et al. 2008). Primary infection of tubers is through fresh wounds caused mainly during harvesting, sorting and transportation (O'Brien and Leach 1983; Ray and Hammerschmidt 1998; Peters et al. 2008). The spores overwinter from the previous seasons in the soil and remains of decaying tubers can serve as the inoculum source (Al-Mughrabi 2010). In other cases the seed tubers and contaminated soils covering the seed tubers can be also the source of spores. During the preparation seed pieces may be infected with spores and start to decay prior to planting in storage and after planting (Wharton et al. 2006). In the field the disease progresses and more than half of the sprouts from the infected planting material will rot resulting in sparse crop stand (Wharton et al. 2006). This type of severe incidences can result up to 25% yield loss (Chelkowski 1989). However, the postharvest infection of tubers with this disease might extend up to 60% (Theron 1991).

The characteristic dry rot symptom of small brown to dark lesion appears 3 to 4 weeks after the infection occurs in the wounded area (Boyd 1972; Lui and Kushalappa 2002). Later, the rotting of tissues from inside force the periderm to wrinkle and collapse (Al-Mughrabi 2010). The presence of moisture and lack of enough air circulation allow secondary microorganisms like bacteria to establish easily and worsen the rotting of tubers (Burton 1989). In addition to the reduced quality and marketability of tubers, *Fusarium* spp. produce secondary metabolites like mycotoxins and trichothecene which pose a health hazard to humans and animals (Leach and Webb 1981; Latus-Zietkiewicz 1993; Senter et al. 1991; Sweeney and Dobson 1999).

The distribution in the pathogenicity of *Fusarium* spp. differs from region to region (Lenc et al. 2008). Olofsson (1976) indicated that *F. coeruleum* is mainly responsible for dry rot of potato tubers in Sweden as same in Germany (Lenc et al. 2008) and Finland (Seppänen 1981). In UK up to 52 % of the dry rot incidence was associated with *F. coeruleum* in the survey between 2000 to 2002 even though *F. sambucinum* was the most aggressive one (Petters et al. 2008).

### **Dry rot disease management.**

The management of *Fusarium* dry rot mostly concentrates on preventing wounded potato tubers from infection (O'Brien and Leach 1983). Curing of mechanically wounded potato tubers during harvesting, transport and storage is important to avoid entry of disease causing pathogens by inducing wound response. The wound responses include reduction of moisture loss from wounded tissues (Soliday et al. 1979; Lulai and Orr 1994), covering of exposed tissues by reactive oxygen species like superoxide and hydrogen (Kumar and Knowles 2003) and production of compounds like steroid glycoalkaloids,  $\alpha$ -chaconine and  $\alpha$ -solanine that hinders

spore germination (Zeng 1993). Suberization and formation of wound periderm can be achieved in few weeks at a storage temperature of 12 to 15°C, optimum humidity and air circulation (Kushalappa et al. 2002). Detail studies by Lulai and Corsini (1998) claimed that wound curing of 5 to 7 days can halt fungal growth in potato tubers.

### **Chemical control**

Postharvest treatment of seed potato using fungicide thiabendazole (TBZ) might be used for partial control of dry rot (Cayley and Hide 1980; Peters et al. 2008). However, frequent reports have been made on the resistance of *Fusarium* strains to this chemical (Hide et al. 1992; Desjardins et al. 1993; Hanson et al. 1996; Satyaprasad et al. 1997). The extent of frequency and severity of the disease forced growers to look for more efficient new molecules and formulations with mixture of active ingredients (Carnegie et al. 1998). In other attempts the efficacy of fungicides were elevated by combing it with proper storage conditions (Lui and Kushalappa 2002). In general, the risk of disease resistance together with increased public awareness towards the health and environmental risks of agrochemicals became the driving force to search noble and better performing control strategies.

### **Resistant cultivars and disease tolerance**

The resistance of potato tubers to dry rot is limited and difficult to determine (Secor and Gudmestad 1999; Burkhart et al. 2007; Petters et al. 2008). The cultivars available in the market give variable and unreliable degree of resistance to the disease (Petters et al. 2008). This might be due to the viability in the distribution and aggressiveness of dry rot causing *Fusarium* strains

(Corsini and Pavek 1986; Lees et al 1998). In resistant cultivars suberin deposition, distribution and chemical responses of the wound healing process determine the effectiveness (O'Brien and Leach 1983; Corsini and Pavek 1980).

### **Cultural practices**

The implementation of practices that can reduce the infection and dispersal of dry rot in storage is important in order to avoid epidemics (Kushalappa et al. 2002). These include, checking quality and sorting of diseased potato tubers, minimizing bruises and damages of tubers, allowing damaged potatoes to cure before long term storage, minimizing moisture availability during wound healing time and proper monitoring of storage conditions (Sommer 1982; Lui and Kushalappa 2002; Kushalappa et al. 2002). In field condition, the warming of seed tubers prior to planting, careful preparation of cuttings to avoid infection and quick planting of prepared cuttings are also recommended (Leach and Nielsen 1975).

### **Storage**

Moreover, storing potato tubers at 7 °C can limit the development of dry rot (Burton 1989). This might be more effective if the potatoes are subjected to wound curing for few weeks with a temperature of 12 to 15 °C (Kushalappa et al. 2002). The capacity to operate this storage conditions might also be considered as an advantage to integrate it with other control strategies like biological control (Wilson and Pusey 1985). In this way it might be possible to create an environment in which the biological control agent could be supported better without affecting proper storage of the product (Wilson and Pusey 1985). However, the high relative humidity and

ventilation facilities in the ware house required for potato storage can favor disease progress (Attallah and Stevenson 2006). The piling of tubers during storage which is usually practiced by the industry might also aggravate the dissemination of inoculum to adjacent tubers (Attallah and Stevenson 2006).

### **Biological control of dry rot disease in potato**

The screening of potential biological control agents against dry rot disease has been considered important due to the limited number of pesticides allowed for post harvest application. Moreover, the disease development necessarily requires wounds on the tuber and infection can occur before the wound healing process which usually takes about 4 to 6 days. Hence, protecting the wounds by applying the antagonist before the introduction of the disease causing agent can be a crucial step for successful control of dry rot (Hooker 1981; Schisler et al. 1998). Soil dwelling bacteria like *Enterobacter*, *Pantoea*, *Pseudomonas* and *Bacillus* have been promising in controlling *F. sambucinum* (Schisler and Shninger 1994; Sadfi et al. 2002). In laboratory studies some bacterial isolates have shown to be antagonists against dry rot disease (Schisler and Shninger 1994; Kiewnick and Jacobsen 1997). At wound healing and marketing temperature the bacterial isolates *Serratia grimesii* 4-9 and *S. plymuthica* 5-6 had effective control of *F. sambucinum* both on artificial medium, tuber slices and whole potato tubers (Gould et al. 2008). In addition, there happened to be no deterioration in the tuber quality due to the addition of these biological control agents (Gould et al. 2008). Another trial using the *P. fluorescens* isolate P22:Y:05 as antagonist to *F. sambucinum* offered a considerable management as the standard fungicide TBZ (Schisler et al. 2000). The antagonist bacteria *Enterobacter cloacae* S11:T:07 were proven to suppress dry rot disease of potato by producing different anti-fungal metabolites

like phenylacetic acid, indole-3-acetic acid and tyrosol (Slininger et al. 2004). Moreover, yeasts and arbuscular mycorrhizae fungi were also detected as potential biological control agents against dry rot of potato tubers (Schisler et al. 1995; Niemira et al. 1996).

### **Biological control using *C. rosea***

*Clonostachys rosea*, Schroers, Samueles, Serfet and Gams (Syn. *Gliocladium roseum*; teleomorph: *Bionectria ochrdeuca*) is a world wide common saprophytic fungus in the family Bionectriaceae (Schroers et al. 1999). It is abundantly available in temperate and tropical arid weather even though reports were made from the extreme subarctic and desert conditions. *C. rosea* has a characteristic mode of life as antagonist against other plant pathogenic fungi, nematodes and insects and also endophytic in different plant parts (Toledo et al. 2006; Sutton et al. 1997; Knudsen et al. 1995; Stewart and Harrison 1989, Jensen 2002). The mechanism of actions for this antagonistic feature are not properly studied but mycoparasitism, competition for substrate and available nutrients, enzymatic activity, antibiosis and induced resistance can be stated to play part (Sutton et al. 1997). *C. rosea* has been reported to be competent against both rhizosphere and phyllosphere pathogenic fungi (Chatterton et al. 2008; Sutton et al. 1997; Li et al. 2004; Luongo et al. 2005). Moreover, it has been observed to have ecological versatility and can effectively colonize living and dead plant material (Schroers et al. 1999).

### **Mycoparasitism**

The mycoparasitic interaction of *C. rosea* has not been thoroughly studied but its potential to secrete cell wall degrading enzymes can be an important factor. The distraction of pathogenic

fungi *S. sclerotiorum* and *Fusarium* spp hyphae were identified without the penetration by antagonist mycelia (Huang 1978). However, Xue (2003) has noticed the growth of lateral hyphal branches of *C. rosea* strain ACM941 which made a direct contact with pathogen hyphae. In another study by Barnett & Lilly (1962), the entwining by *C. rosea* hyphae has resulted in gradual penetration and disintegration of mycelia. The hyphae of *C. rosea* which was devoid of appressoria had the capacity to penetrate the conidia and germ tubes of *Botrytis cinerea* (Li et al. 2002). The same study argued that the infection strategy of *C. rosea* deployed a physical pressure to break the cell wall of *B. cineria* hyphae.

### **Enzymatic activity**

Enzymes like chitinases, glucanases and other proteases were identified from *C. rosea* interaction with other pathogenic fungi and nematode hosts (Zhao et al. 2005; Li et al. 2006; Gan et al. 2007; Chatterton & Punja 2009). The antagonistic potential of *C. rosea* against the tobacco root rot pathogen *Rhizoctonia solani* might have been associated with the chitinase CrChi1 (Gan et al. 2007). Chitinase and  $\beta$ -1-3 glucanase were also detected from dual culture plates of *C. rosea* and *Fusarium* spp. These enzymes were later related with hydrolysis and distraction of fungi like *Fusarium* spp. which have chitin and  $\beta$ -1-3 glucan as a predominant component of the cell wall (Chatterton & Punja 2009). In another separate experiment inhibition of *Pythium ultimum* by *C. rosea* were attributed to enzymes glucanases or carboxymethyl cellulase (Mamarabadi et al. 2008). Moreover, further studies from the antagonist fungus *Trichoderma* spp. stressed the secretion of a 42 kDa extracellular chitinase with biological control mode of action against many phytopathogenic fungi (Baek et al. 1999; Kim et al. 2002; Ramot et al. 2004; Seidl et al. 2005). In a related study, *Trichoderma* spp. chitinase *ech42* were triggered by

fungal cell wall material, colloidal chitin and shortage of carbon (Garcia et al. 1994, Margolles-Clark et al. 1996). Hence, both the physical interaction and chemical constituents of *C. rosea* cell wall might be the reasons for the mycoparasitic property (Viccini et al. 2009).

### **Competition for substrate and nutrients**

The antagonistic behavior of *C. rosea* can be also associated with the competition for nutrient and substrate in the rhizosphere (Sutton et al. 1997). *C. rosea* strain ACM941 has shown a vigorous mycelia establishment to undermine other pythopathogenic colonies Xue (2003). In controlled trials on rose plants, *C. rosea* was effective in competing for niches, available resources and suppress sporulation of *B. cinerea* (Sutton et al. 1997; Morandi et al. 2000; Morandi et al. 2001). Another related study also indicated that strawberry gray mold incidence were checked by efficient colonization of strawberry leaves by *C. rosea* strain Pg 88-710 (Cota et al. 2008).

### **Antibiosis**

The possible production of diffusible compounds might play a role in the antifungal property by *C. rosea*. According to Xue et al. (2009), dual-culture tests using *C. rosea* strain ACM941 and *Gibberella zia* depicted the formation of a clear inhibition zone and lack of spore germination which may be due to antibiosis.

## **Induced resistance**

Besides the pathogen suppression effect *C. rosea* has been reported to enhance plant growth through the effect on plant hormones, signaling factors (Lahoz et al. 2004) and nutrient utilization (Sutton et al. 2008). It was demonstrated that the endophyte *C. rosea* have direct impact on the establishment of roots and shoots, physiology of leaves, flowers and fruits and ultimately on the productivity of plants (Sutton et al. 2008).

Therefore, this project aims at studying the postharvest application of fungal biological control agent *C. rosea* strain IK726 against the pathogenic *Fusarium* spp. that cause dry rot disease in potato tubers. The study will test procedures in screening potential biological control agents for postharvest application by mimicking the storage conditions and handling processes that is widely practiced in the sector. It also investigates the *C. rosea* strain IK726 – *F. avenaceum* and *F. coeruleum* interactions both on potato tubers and culture media with emphasis on biological control.

## Objectives

### Major Objective

To investigate the potential application of biological control agent *C. rosea* strain IK726 for the control of dry rot caused by *F.avenaceum* and *F.coeruleum* using a standard infection system.

### Specific Objectives

To test the postharvest application system of biological control agent *C. rosea* strain IK726 against the infection by *Fusarium* spp. that causes dry rot in potato tubers.

Evaluating the effect of biological control agent *C. rosea* strain IK726 on the rot development caused by *F.coeruleum* and *F.avenaceum*.

Studying the wound colonization potential of *C. rosea* strain IK726 against pathogens *F.coeruleum* and *F.avenaceum* using microscopy.

To study the interactions between *C. rosea* strain IK726 with *F. coeruleum* and *F.avenaceum* both on potato tubers and culture media.

### Study Questions

- Does the infection system developed for the *Fusarium* spp. effectively work?
- Does the protocol of applying the biological control agent *C. rosea* strain IK726 in postharvest tuber rot control work? Can the storage conditions support *C. rosea* strain

IK726 to establish on the potato tubers? Is *C. rosea* strain IK726 pathogenic to potato during storage conditions?

- Is there a significant reduction in the dry rot disease development after the treatment with *C. rosea* strain IK726?
- Are there any hyphal interaction between *C. rosea* strain IK726 and *F. coeruleum* and *F. avenaceum*.
- Is there any change in the morphology and physiology of the hyphae while interacting?
- What can be the possible mechanisms used by the antagonist to reduce the growth of the *Fusarium* spp.?

## Materials and Methods

### Bioassay in Phytotron

The bioassay test was conducted June to August, 2012 in Phytotron at the BioCentrum, SLU. Potato tubers artificially inoculated with dry rot causing pathogens *F. coeruleum* and *F. avenaceum* alone and in combination with the biological control agent *C. rosea* strain IK726 were considered as treatments. These include tubers treated with *C. rosea* strain IK726 and inoculated with *F.coeruleum*, treated with *C. rosea* strain IK726 and inoculated with *F.avenaceum*, tubers inoculated only with *F.avenaceum* and *F.coeruleum*, and non-inoculated tubers treated with *C. rosea* strain IK726. Tubers treated only with deionized water were considered to see the natural infection in the absence of artificial inoculum source (Table 2). The treatments were replicated in 80 potato tubers except the *C. rosea* strain IK726 and water treated with 30 and 50 tubers, respectively.

Table 2. List of treatments used for bioassay in phytotron.

Treatments	Remarks
<i>C. rosea</i> strain IK726 + <i>F.coeruleum</i>	
<i>C. rosea</i> strain IK726 + <i>F.avenaceum</i>	
Infected only with <i>F.avenaceum</i>	control
Infected only with <i>F.coeruleum</i>	control
Treated with <i>C. rosea</i> strain IK726	no artificial infection
Treated with deionized water	no artificial infection

Potato variety Melody which was harvested in the 2011 cropping season was purchased from growers in the Uppsala area. The tubers were packed in paper bags and stored in 4°C rooms.

Healthy and undamaged tubers were selected and carefully washed with clean water. The paper towel dried tubers were semi-sterilized using 70% Ethanol and damaged using a flame sterilized nail tip with 1 cm depth and 0.5 cm diameter.

### **Culture Preparations**

Culture plates were prepared from Potato Dextrose Agar (PDA) solutions with 36g PDA dissolved in a liter of deionized water. The PDA plates were sub-cultured by transferring 0.4 cm<sup>2</sup> mycelium of *F. coeruleum* and *F. avenaceum* isolates obtained from Norway and *C. rosea* strain IK726 from the department at SLU. The cultures were allowed to grow for 14 days at 25 °C in the dark room. The well grown mycelia were allowed to sporulate by further keeping the plates for 6 days close to a light source.

### **Spore solution preparation**

The glass funnels, glass wool, glass rods, and deionized water were autoclaved. The culture plates with sporulating mycelia were flooded with autoclaved deionized water using pipettes and gradually stirred with a glass rod. The spore solution was poured through a funnel covered with the glass wool and the filtrate was collected in a falcon tube. After diluting the filtrate with deionized water the number of spores in the solution was counted using a hemocytometer. A standard working spore solution of 100 spores/ µl were prepared to inoculate the potato tubers.



Figure 2. Spore preparation and treating of artificially damaged potato tubers with *C. rosea* strain IK726 prior to inoculation with *F. avenaceum* and *F. coeruleum* spores.

### **Tuber inoculation**

A standard infection system developed for *Fusarium* spp. on potato tubers were used (Jima 2012). However, for biological control application the wounds were first inoculated with 40 $\mu$ l spore solution of *C. rosea* strain IK726 prior to inoculation with the pathogens. Subsequently, they were inoculated with 40 $\mu$ l of spore solution of *F. avenaceum* and *F. coeruleum* (Figure 2).

### **Phytotron arrangement**

The test was carried out in two separate chambers that were used to accommodate the different treatments. Those boxes with tubers which were treated with *C. rosea* strain IK726 were kept separately to avoid spore contamination. This bioassay test was done according to the procedures

that the industry uses in postharvest handling of potato tubers. The experiment started by storing the tubers at wound healing temperature of 12°C and high relative humidity (> 95%) for 14 days that facilitate the curing of wounds which occur in the harvesting and transportation process. Then the temperature was lowered to 4°C and high relative humidity (> 95%) for 35 days which was considered as long term storage. Later, the temperature was raised to 22 °C for 14 days with the same high relative humidity which mimics the temperature before processing or table consumption.

### **Scoring of the data**

The scoring on the effect of the antagonist on the dry rot disease development was done through visual observation. The presences or absence of the characteristic brown rot in the wounded area was recorded as (rot = 1 and non-rot= 0). The rotten tubers were further dissected approximately into equal parts taking the centre of the wound as a reference. Then the diameter and depth of the rot were measured and recorded. The data was subjected to analysis using Excel<sup>®</sup> sheet and later analysis of variance (ANOVA) was done using Minitab<sup>®</sup>. Finally, the means were compared using F-test and statistical significance are checked at 5% significance level ( $\alpha = 0.05$ ).

### **Microscopy**

The fungal hyphae growth within the potato tuber cells were diagnosed using Leica<sup>®</sup> light microscopy. Samples for microtome were randomly taken from potato tubers inoculated with *Fusarium* pathogens and/or treated with *C. rosea* strain IK726. The Leica<sup>®</sup> constant cryostat microtome was used to prepare slices of thickness 30µm from each sample and mounted on

microscope slides. The specimens were stained using aniline blue and observed under the microscope. Wet mounted slides were also made for comparison. Pictures were taken using Leica<sup>®</sup> DFC 420 C camera on the microscope with bright field contrast (BF) in transmitted light axis.

### **Dual culture assay**

The fungal interactions between *C. rosea* strain IK726 and *F. avenaceum* and *F. coeruleum* were done using two methodologies by Jones and Deacon (1995) and Elad (1983). In the first method autoclaved coverslips of size 18 x 18 mm were placed on the center of Potato Dextrose Agar (39 g/l) culture plates. The plates were inoculated in each side approximately 3 - 5 cm away from the coverslips with mycelium plugs (5 mm) from one week old culture plates of *C. rosea* IK726 Green Fluorescent protein (GFP) mutant and *F. avenaceum* or *F. coeruleum* obtained from the department. The culture plates were allowed to grow in dark growth room at  $25 \pm 2$  °C. The plates were visually observed for any possible phenomena of interest starting from 24 hrs after inoculation. The observations under the Leica<sup>®</sup> microscope were done both in Leica<sup>®</sup> DFC 420 C normal and Leica<sup>®</sup> DFC 360 FX fluorescence camera. It was started on the 4<sup>th</sup> day when the respective hyphae gradually approaching each other. The microscope observation continued up to two week time and pictures were taken whenever necessary using the Leica<sup>®</sup> V4 and AF software.

The methodology described by Elad et al. (1983) was also followed in parallel with the above protocol. Cellophane membrane were spread on top water agar (20 g/l) plates. The plates were inoculated in the opposite sides with mycelium plugs (5 mm) of *F. avenaceum* or *F. coeruleum*

confronted with *C. rosea* strain IK726 GFP mutant plugs. The cultures were allowed to grow in dark growth room at  $25 \pm 2$  °C. All possible interactions were visually observed starting from 24 hrs after inoculation. Later when the hyphae grew towards each other, piece of the cellophane membrane were taken to prepare slides for microscopy. The Lecia<sup>®</sup> DFC 420 C normal and Lecia<sup>®</sup> DFC 360 FX fluorescence camera attached to the light microscope were used to take pictures.

### **Spore interaction study**

The interaction of spores between *C. rosea* strain IK726 and *F. avenaceum* and *F. coeruleum* were studied using time-lapse Lieca<sup>®</sup> microscopy. A standard working solution 200 spores/ $\mu$ l from *C. rosea* strain IK726 and *F. avenaceum* and *F. coeruleum* were prepared. The slides for microscopy were made by pipetting 10  $\mu$ l liquid PDA agar (39 g/lt), spore solution from *C. rosea* strain IK726 and the *Fusarium* spp. which was thoroughly mixed using the pipette tip before the agar solidify and covered with autoclaved coverslips. The interactions were then studied using Lecia<sup>®</sup> DFC 360 FX fluorescence camera microscopy with bright field contrast (BF) in transmitted light axis and pictures were taken in an hour interval for 5 consecutive days.

## Results

### Bioassay in Phytotron

The dry rot incidence in artificially infected tubers with two different *Fusarium* strains were compared with tubers treated with antagonist *C. rosea* strain IK726 and inoculated with *Fusarium* spores. The highest mean rot of 35 and 31.25% was observed in tubers inoculated only with *F. avenaceum* and *F. coeruleum*, respectively (Figure 1). The rot incidence had minimized significantly to 16.25% in tubers infected with *F. avenaceum* and treated with *C. rosea* strain IK726. The same trend in rot reduction was noticed in *F. coeruleum* infected and *C. rosea* strain IK726 treated potato tubers to 20% (Figure 3).

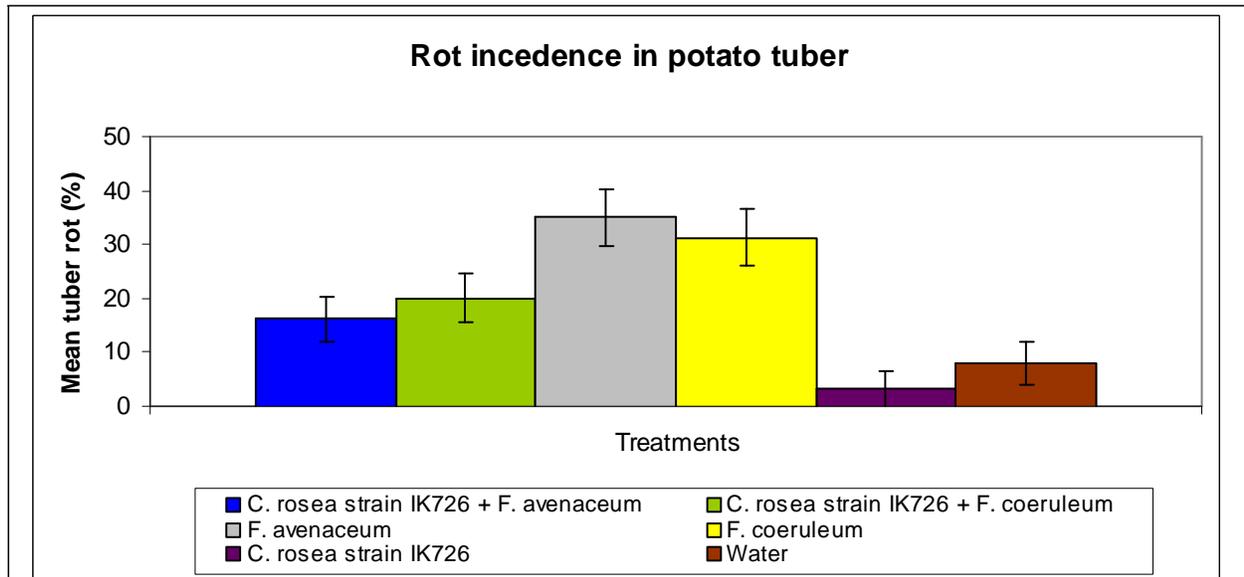
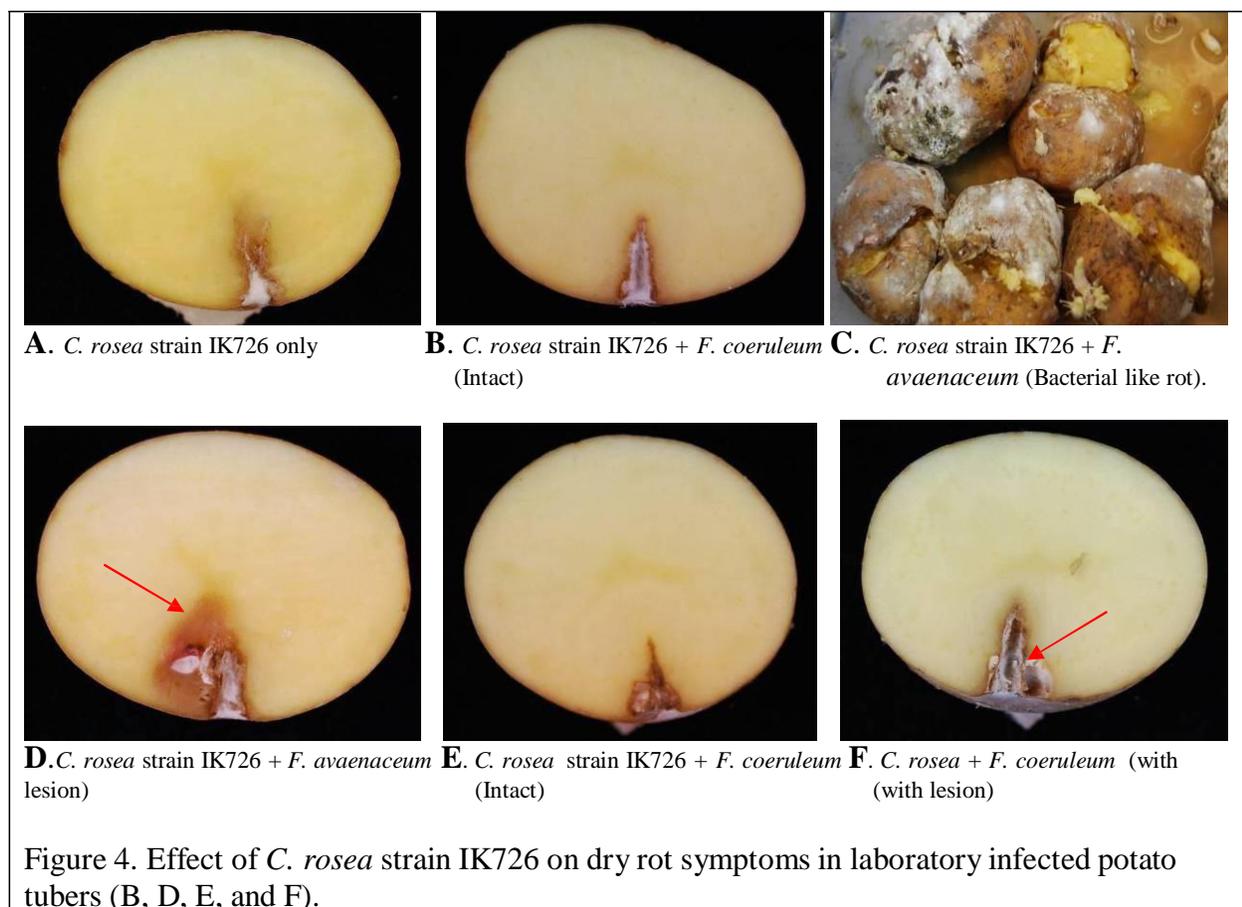


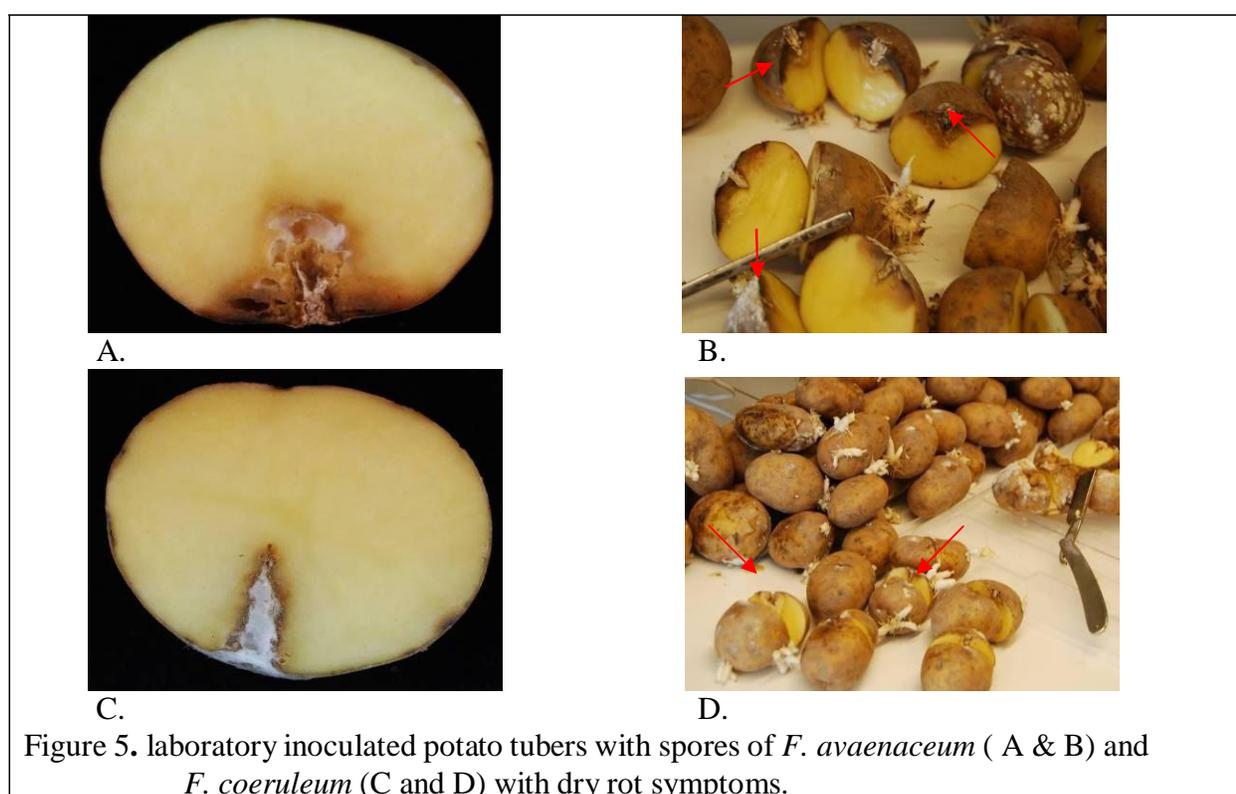
Figure 3. Influence of *C. rosea* strain IK726 on the mean number of *Fusarium* dry rot in artificially damaged potato tuber  $\pm$  SE.

The reduced mean rot due to the application of antagonist *C. rosea* strain IK726 on *F. avenaceum* and *F. coeruleum* infected tubers was significant ( $p=0.018$ ) compared to the non-treated tubers.

The visual observation of *C. rosea* strain IK726 treated tubers before it was inoculated with the respective pathogens *F. avenaceum* and *F. coeruleum* had intact tissues around the wounded area that were covered with mycelia of the antagonist (Figure 4). In most of the healthy tubers there was no characteristic lesion of dry rot disease symptom developed from the point of inoculation (Figure 4 B and E). However, from the few rot cases witnessed in the *C. rosea* strain IK726 treated tubers most of the symptoms, 76.9 and 50% (for *F. avenaceum* and *F. coeruleum* infected tubers, respectively) were like soft rot of bacteria (Figure 4 C). The rest were categorized as typical to Fusarium dry rot symptoms (Figure 4 D).



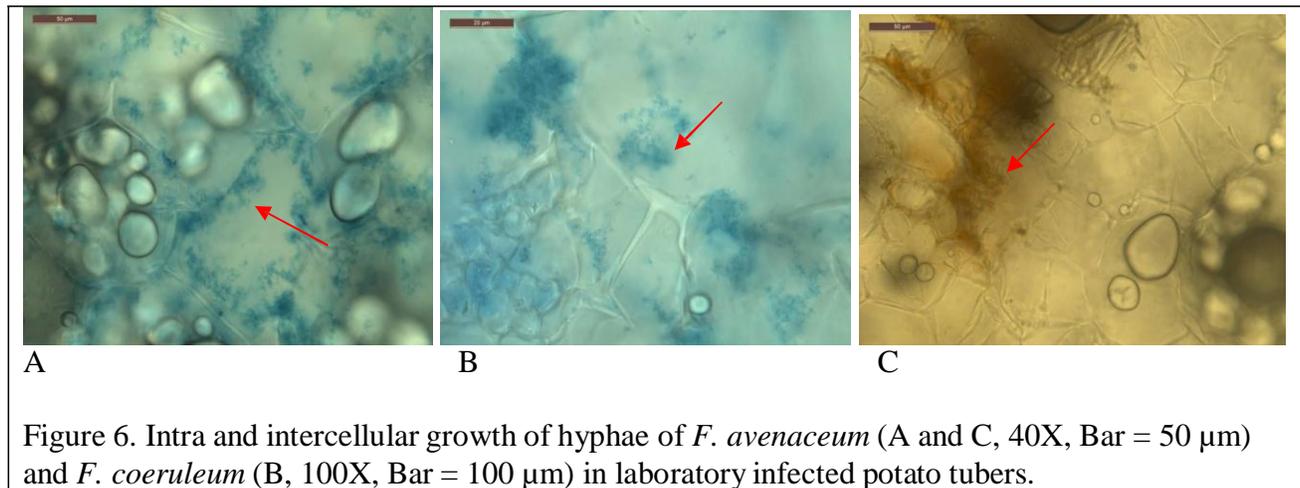
Potato tubers that were infected with the *F.avenaceum* and *F. coeruleum* spores only had sustained the infection with lesions originated from the point of damage and growing wider in to the inner tissues . The lesions were brown to dark brown with dried periderm which looks intact and covered with mycelia in certain cases that are characteristic to Fusarium dry rot disease (Figure 5). In these tubers around 93 % of the rot signs were associated with Fusarium infection and the rest 7% had bacterial like soft rot.



### Microscopy assay

The microscopy pictures taken from the samples that were artificially infected only with *F. avenaceum* and *F. coeruleum* spores had hyphae grown both intracellular and intercellular (Figure 6). It was also observed that the hyphal growth was mostly intercellular in intact potato

tissues. However, the trend had changed to intracellular growth as the tissues got rotten (Figure 6). Moreover, in these samples the hyphal development had progressed deeper from the point of inoculation into the inner cells and were effectively colonized (Figure 6).



In the samples taken from tubers that were inoculated with *C. rosea* strain IK726 in the absence of the pathogen spores; the pictures had revealed hyphal growth only on the outer cells (Figure 7A). The growth was intercellular and did not progress deeper in to the non-inoculated cells. The same phenomenon was noticed in the tissues treated with *C. rosea* strain IK726 and inoculated with *F. avenaceum* or *F. coeruleum*. The hyphae development was limited only in the outer cells at the point of inoculation (Figure 7 B and C). The cells beneath were intact and healthy with no hyphae growth.

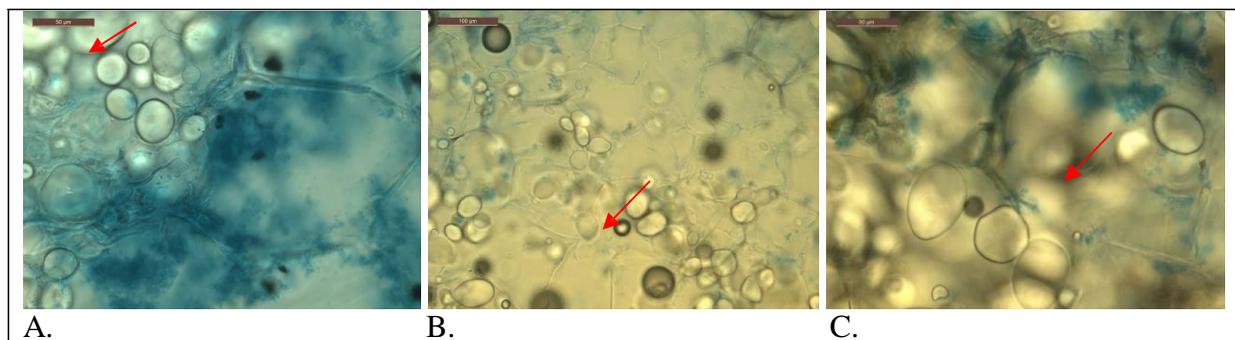


Figure 7. Limited hyphae growth only on the outer potato tuber cells that are treated with *C. rosea* strain IK726 spores only (A, 40X, Bar = 50 µm), and treated prior to laboratory infection with *F. avenaceum* (B, 40X, Bar = 50 µm) and *F. coeruleum* (C, 100X, Bar = 100 µm)

### Dual culture assay

In both dual cultures of *C. rosea* strain IK726 with *F. coeruleum* and *F. avenaceum* the mycelia grew heading towards each other without the formation of a clear inhibition zone. After 3 - 4 days the mycelia started to contact, and coverslips and/or piece of cellophane membrane containing the possible area of interaction were taken to prepare slides for microscopy. It was noticed that there were no clear interactions in the first few days after the hyphae approached each other (Figure 8). Rather the *C. rosea* strain IK726 hyphae grew deeper in to the *F. avenaceum* and *F. coeruleum* mycelia. Later as the cultures were ageing a clear interaction zone was observed and the *C. rosea* strain IK726 mycelia established well in the interaction zone (Figure 8).

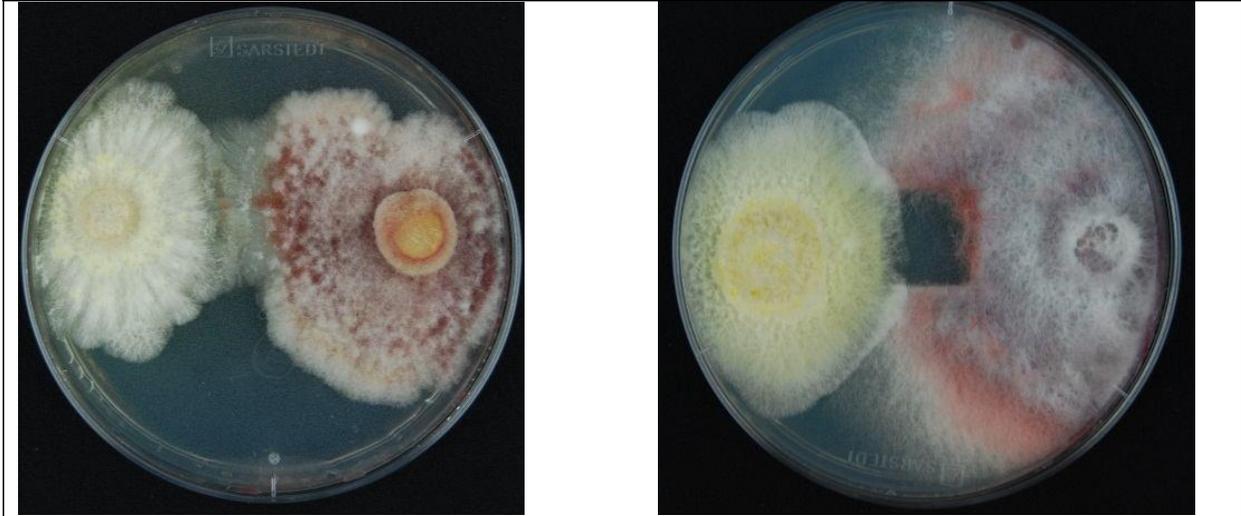


Figure 8. Mycelia of *C. rosea* strain IK726 growing deeper and on top of *F. avenaceum* or *F. coeruleum* mycelia.

The *C. rosea* strain IK726 hyphae grew attached on top and along *F. avenaceum* and *F. coeruleum* (Figure 9). There was a typical one to one allocation of *C. rosea* strain IK726 hyphae growing on top of the *F. avenaceum* and *F. coeruleum* hyphae.

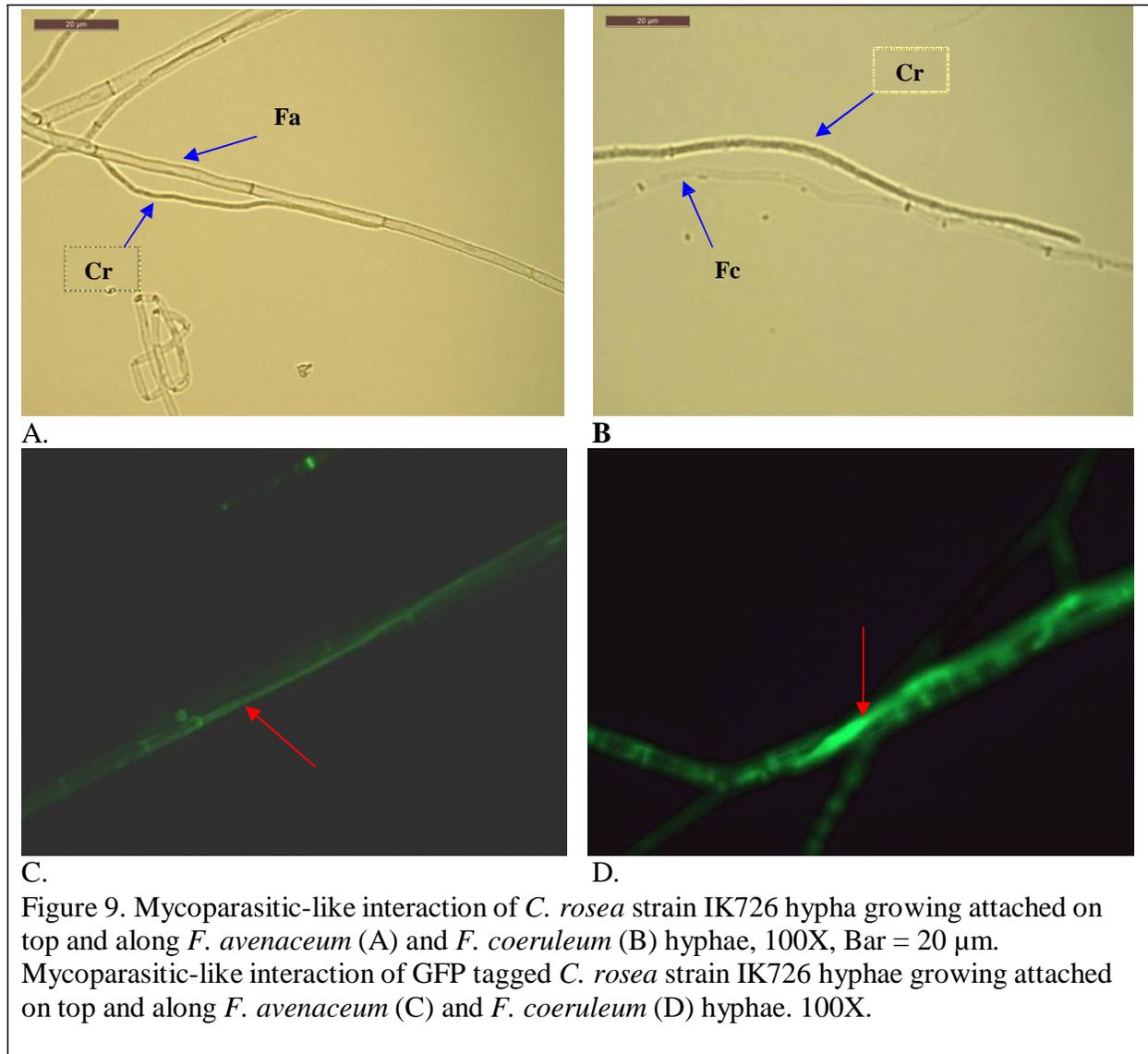
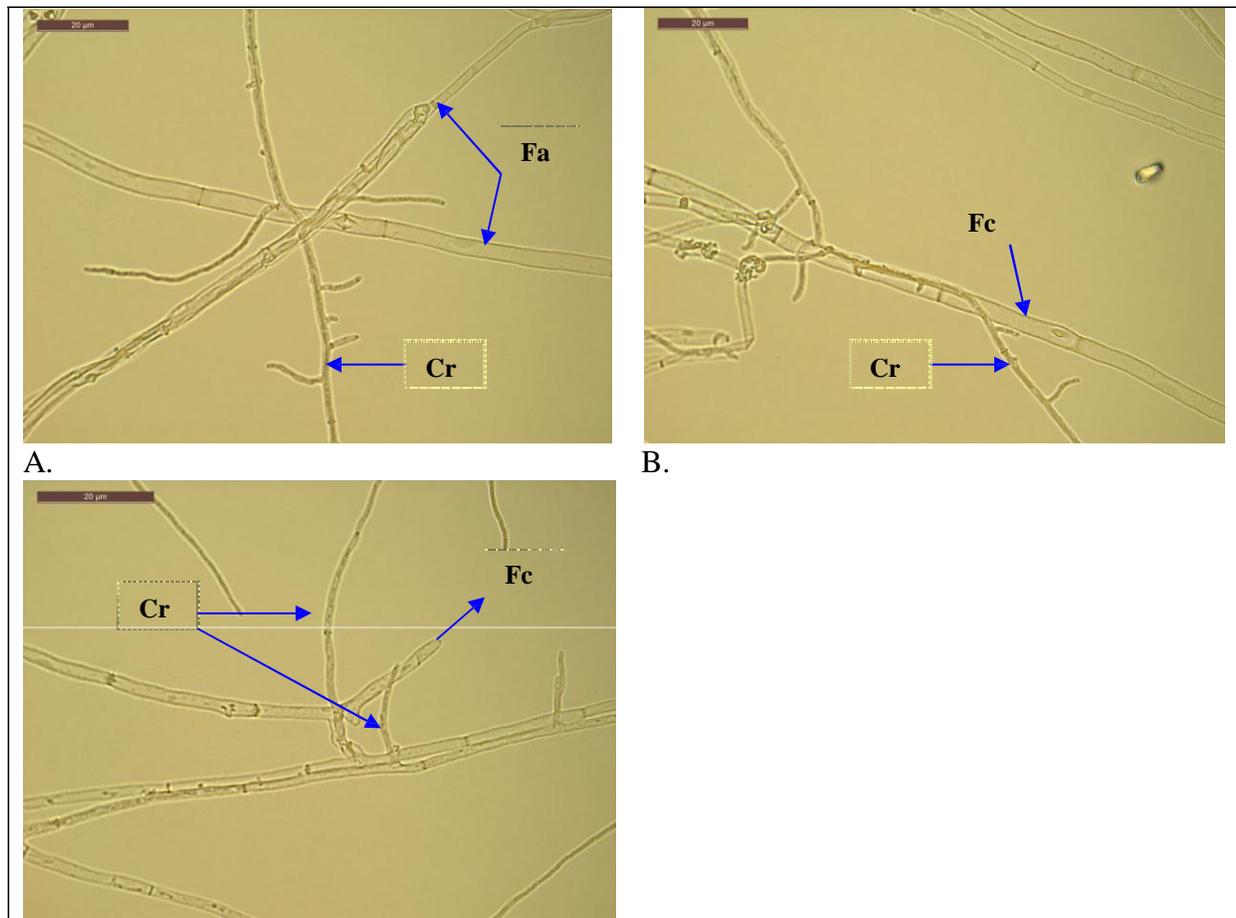


Figure 9. Mycoparasitic-like interaction of *C. rosea* strain IK726 hypha growing attached on top and along *F. avenaceum* (A) and *F. coeruleum* (B) hyphae, 100X, Bar = 20 µm. Mycoparasitic-like interaction of GFP tagged *C. rosea* strain IK726 hyphae growing attached on top and along *F. avenaceum* (C) and *F. coeruleum* (D) hyphae. 100X.

Moreover, *C. rosea* strain IK726 hyphae had frequent branches laterally when it encountered the *F. avenaceum* and *F. coeruleum* hyphae (Figure 10). It was also characterized by active cytoplasmic streaming. The *F. avenaceum* and *F. coeruleum* hyphae also had short and irregular branches in the presence of *C. rosea* strain IK726 hyphae (Figure 10). They were also vacuolated and had no cytoplasmic action in the presence of *C. rosea* strain IK726 attached on the hyphae of *F. avenaceum* and *F. coeruleum* hyphae (Figure 10).



A.

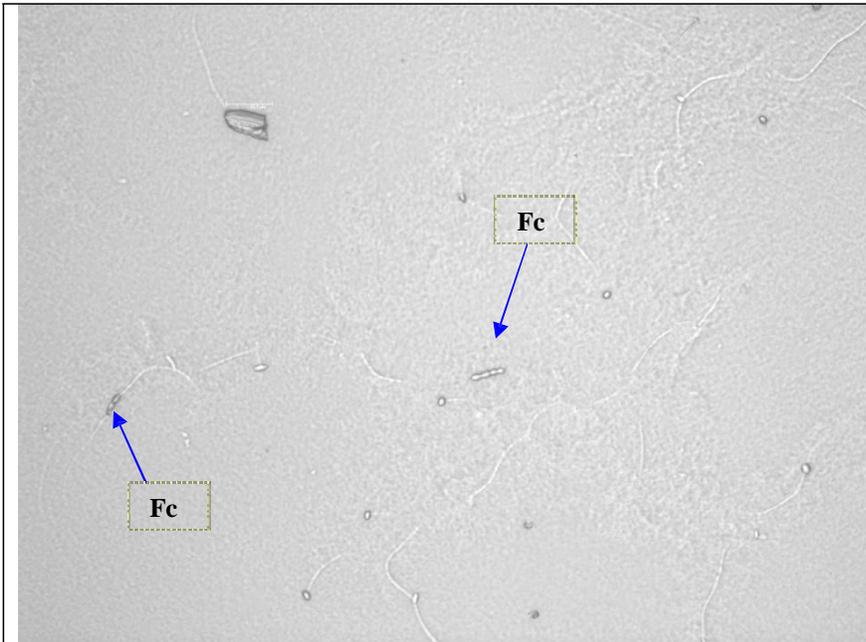
B.

C.

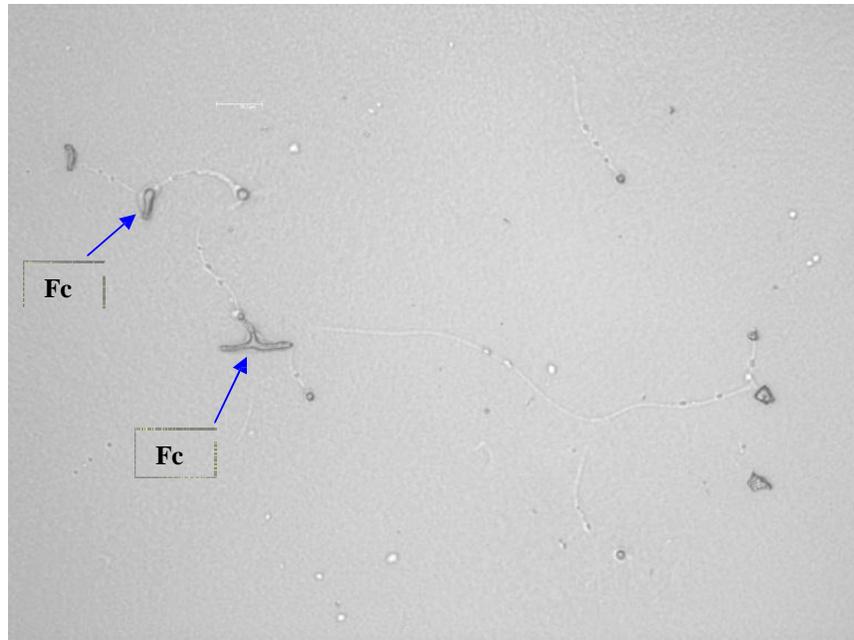
Figure 10. Frequent lateral branching of *F. avenaceum* (A) and *F. coeruleum* (B) hyphae in the presence of *C. rosea* strain IK726. *Fusarium* spp. with short and irregular branches when encountered with *C. rosea* strain IK726 (C). 100X. Bar = 20 µm.

### Spore interaction study

After the dual inoculation of conidial solution of *F. coeruleum* with *C. rosea* strain IK726 spore solutions, most of the conidia did not germinate within 8 to 10 hour time (Figure 11). The germ-tubes were characterized by the formation of short, septated and granular tips with no tactile cytoplasmic streaming. The non-germinated conidia also had no cytoplasmic streaming (Figure 12).



A.



B.

Figure 11. A- Spore interaction between *C. rosea* strain IK726 and *F. coeruleum* 9 hour after dual inoculation, 20X.

B – Hyphae or germ-tube interaction between *C. rosea* strain IK726 and *F. coeruleum* 24 hours after dual inoculation, 20X.

However, spores of *C. rosea* strain IK726 had mass germination in 6 to 8 hours after inoculation with one or two active germ-tubes which are gradually tilted to the plane of *F. coeruleum* conidia (Figure 11). There were active cytoplasmic streaming and movement of nuclei with elongation and formation of hyphal branches. In most cases the germ-tubes or hyphae of *C. rosea* strain IK726 had made visible contact with the germinating conidia of *F. coeruleum* (Figure 11).

## Discussion

The high expectancy to develop effective biological control agents for postharvest application has emanated from the advantage of exploring the physical environment in storage rooms, targeted application of products and the economic value of the produce (Droby et al. 2009). The importance of a viable system for postharvest biological control application which is compatible with the processing and handling procedure in storage facilities has a significant importance. Only few studies have been demonstrated application of biological control agents in commercial storage environments (Schisler et al. 2000). This study is unique in that *C. rosea* strain IK726 was tested against the two *Fusarium* spp. that cause dry rot by mimicking the processing and handling procedures used in the potato industry. In addition, attempts were made to investigate the wound colonization potential of *C. rosea* strain IK726 with reference to biological control in potato tubers. It also studied spore, hyphae and mycelia interactions related to biological control mechanisms. These different studies can help to suggest the possible interactions that the biological control agent had to check the pathogen development.

According to the phytotron bioassay more than 45 % reduction in the mean number of rot incidence were witnessed in potato tubers that were treated with *C. rosea* strain IK726 compared to non-treated ones. *C. rosea* strain IK726 had also survived the fluctuation in temperature from 12, 4 and 22 °C over time and managed to give a significant control of *F. avenaceum* and *F. coeruleum*. In previous studies the same strain had proven to be effective against *F. culmorum* and withstand temperature variations (Jensen et al 2000). The antagonist mycelia have effectively colonized the damaged area suppressing both *F. avenaceum* and *F. coeruleum*. This might be due to the high efficiency of *C. rosea* strain IK726 mycelia to compete for space and

available resources. The high capacity of *C. rosea* to colonize senescent leaves efficiently compared to pathogens is directly associated to its efficacy (Morandi et al. 2001; Morandi et al. 2003). Moreover, the 4 °C cold storage temperature which inhibits the growth of *Fusarium* spp. can give advantage to *C. rosea* IK726 to establish better in the wounded area. This capacity to tolerate and grow in cold storage temperature can be an important merit for *C. rosea* IK726 to be used together with other postharvest handling practices. *Fusarium* dry rot incidence has effectively reduced by combining practices of tuber wound healing at 12 °C (Kushalappa et al. 2002) and keeping in storage facilities with temperature below 7 °C (Burton 1989).

In dual culture tests both mycelia and hyphal interactions between *C. rosea* strain IK726 and the *Fusarium* spp. were studied with respect to biological control. The result indicated that mycelia of the *C. rosea* strain IK726 and the *Fusarium* spp. have grown towards each other and eventually merged after 3 to 4 days. The lack of a clear inhibition zone might indicate that there was no antifungal metabolite released by the *C. rosea* strain IK726 with an action to inhibit the growth of *F. avenaceum* and *F. coeruleum*. It was also observed that the mycelia of *C. rosea* strain IK726 have grown progressively into and covering the *Fusarium* mycelia which gradually suppressed its growth. In contrary to this study, antibiosis was reported to be the primary mode of action by *C. rosea* strain ACM941 against *Gibberella zeae* (*F. graminearum*) *in vitro*, greenhouse and field condition (Xue et al. 2009). Moreover, the microscopy study done after the formation of an interaction zone revealed a direct contact and growth of *C. rosea* strain IK726 on and along the hyphae of *F. avenaceum* and *F. coeruleum*. This opposes the idea that suppression of *Fusarium* spp. by *C. rosea* is mainly due to effective colonization but it can also be by mycoparasitic interaction. It is supported by the work of Xue (2003), which stated the

direct contact by *C. rosea* strain ACM941 lateral branches with the pathogen mycelia. Li et al. (2002) also indicated the lack of appressoria by *C. rosea* rather deployed physical pressure to break the cell-wall of *B. cinerea*. Moreover, it was stressed that degradation of *S. sclerotiorum* and *Fusarium* spp. hyphae in the absence of mechanical penetration by *C. rosea* (Huang 1978). However, as stated by Barnett and Lilly (1962) there was no coiling of *C. rosea* strain IK726 hyphae on *F. avenaceum* and *F. coeruleum* hyphae to penetrate and disintegrate the host mycelia. This mycoparasitic-like interaction by *C. rosea* strain IK726 might also help to suppose that enzymes like chitinases and glucanases that can degrade the host cell-wall can be involved. It is in line with the work of Viccini et al. (1997) that physical contact and release of chemical substances by *C. rosea* were attributed to the mycoparasitic behavior. Gan et al. (2007) also strengthened the idea that mycoparasitic potential of *C. rosea* has been related to the secretion of enzymes like chitinases CrChil (Gan et al. 2007). Other related studies also depicted  $\beta$ -1-3 glucanase together with chitinase involved in the degradation of *Fusarium* mycelia wall (Mamarabadi et al. 2008; Chatterton & Punja 2009).

In addition, frequent lateral branching of *C. rosea* strain IK726 hyphae were witnessed when it was in contact with the *F. avenaceum* and *F. coeruleum* hyphae. This can be a strategy to out-dominate the competition by the pathogen in terms of attaching on the host, and compete for space and/or nutrient. Nonetheless, *F. avenaceum* and *F. coeruleum* had irregular branching with short segments ( $\leq 40 \mu\text{m}$ ). It seems a strategy to have many but short and unsightly hyphae to avoid the attachment by *C. rosea* strain IK726. Moreover, it can save energy that can be better utilized to survive longer. There was no difference noticed in the outcome of mycelia and/or hyphal interaction due to the change in the nutrient media.

Finally, the spore interaction study implied delay in *F. coeruleum* spore germination from the expected 2 to 4 hours of incubation on glass surface (Wagacha et al. 2012). In addition, the germ tube growth was also affected in the presence of *C. rosea* strain Ik726 spores. This might be due to antibiosis effect of *C. rosea* strain IK726 germinating spores of *C. rosea* strain IK726. In other *in vitro* test the release of diffusible substances that have antifungal capacity against other *Fusarium* spp. were suggested (Xue et al. 2009). It is also claimed that the biocontrol efficiency of *C. rosea* is attributed to diverse mode of action (Gan et al. 2007).

To conclude, *C. rosea* strain IK726 has the potential to control *F. avenaceum* and *F. coeruleum* that cause dry-rot disease in potato tubers in combination with postharvest handling practices and storage conditions. Moreover, effective colonization of tuber wounds, antibiosis and mycoparasitic-like action could possibly be the mode of action against the *Fusarium* spp. The mycoparasitic-like interaction could be the predominant mechanism by *C. rosea* strain Ik726 against *F. avenaceum* and *F. coeruleum* on growth media. Further investigations should be done to fully understand the mycoparasitic interaction and identification of antifungal substances that could be released by the IK726 strain. It is also important to have detail transcriptome study that might help to better understand the interaction.

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