PATHOLOGICAL STUDIES OF FUSARIUM OXYSPORUM (SCHLECHT.) F.SP. TULIPAE APT. IN TULIP (TULIPA GESNERIANA L.) BULBS AFTER HARVEST

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PATHOLOGICAL STUDIES OF FUSARIUM OXYSPORUM (SCHLECHT.) F.SP. TULIPAE APT. IN TULIP (TULIPA GESNERIANA L.) BULBS AFTER HARVEST

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We studied the infection process and ethylene production by *Fusarium oxysporum* (Schlect.) f.sp. *tulipae* Apt. (F.o.t.) in tulip bulbs after harvest.

This dissertation aimed: 1) to develop screening assays to study the infection and ethylene evolution of F.o.t. in tulip bulbs, 2) to determine the degree of resistance to *Fusarium* between several cutlivars, and members of lineages of cultivar sports, and 3) to generate a profile of metabolites of two cultivars (one susceptible, and one resistant) involved in the ethylene biosynthesis pathway of F.o.t., and compounds involved in confering resistance to this pathogen.

Chapter 2 presents a literature review.

Chapter 3 describes a multi-step procedure to isolate, characterize and identify *Fusarium* strains allegedly causing *Fusarium* rot in tulip bulbs.

Chapter 4 evaluates various inoculation methods on the time course of fresh weight loss and ethylene production by F.o.t. Moist incubation conditions free of condensation led to healthy fungal development. Change in fresh weight can be used as a predictor of ethylene production by the fungus.

Chapter 5 explores the correlation between ethylene evolution, visual infection rating, and FW loss of 38 cultivars and 2 species when inoculated with F.o.t. A Disease Severity Index (DSI) was developed to determine the degree of resistance to F.o.t. Cultivars and species ranged from resistant to susceptible. Time lapse videos show the infection process by F.o.t. in a susceptible cultivar and fungal growth suppression in a resistant cultivar.

Chapter 6 presents the ethylene production *in-vitro* by F.o.t. in organs explants, and ethylene production in organs from whole inoculated bulbs. Results in organs from whole inoculated bulbs were similar to the *in-vitro* assay, however, biological contamination created experimental noise. Amino acid content and tulipaline-A in crude extracts from organs of two cultivars before inoculation did not correspond to ethylene production and fungal biomass of F.o.t. We postulate that tulipaline-A is a phytoanticipin in tulip bulbs, which in resistant cultivars may increase to fungitoxic levels under pathogen attack as a result of tuliposide breakdown by enzyme activity or chemical depolymerization by pH change in the tissue.

BIOGRAPHICAL SKETCH

Gerardo Suazo obtained his B.S. at the Univesidad Autonoma del Estado de Morelos (UAEM) in Mexico. During that time he participated as a student in an international rural development exchange between Mexico and Canada. After graduation he worked as a landscape manager before becoming the academic supervisor of the exchange in which he previously participated. His involvement in research started at the Colegio de Postgraduados in Mexico where he worked as an adjunct researcher. He studied his M.S. in International Horticulture in the UK and the Netherlands where he conducted a study for an international consultancy company on marketing strategies to increase the US market share of cut flowers produced in Mexico. Upon conferral of the M.S. degree from Essex University, he worked for a short period as a marketing manager for a local company in Mexico. He pursued his Ph. D. in horticulture at Cornell University under the guidance of Dr. William B. Miller where he obtained solid research foundations in post harvest physiology, plant pathology, and physiology of tulip bulbs.

Gerardo has always been interested to work internationally in the horticulture industry. At the time of the publication of this dissertation he was completing a one-year research and development internship at Conrad Fafard (a Syngenta Group company) where he worked with global innovation projects that involved multidisciplinary teams. He plans to enroll in an MBA program in the near future.

Dedico esta disertación a:

Mi esposa Ana María por su amor incondicional, por animarme y apoyarme en tiempos buenos y difíciles.

Matias: has traido luz a nuestras vidas. Tus risas son el resplandor que me impulsa a no darme por vencido.

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EPILOGUE

My motivation to pursue a Ph. D. was to generate knowledge in a relevant area of the floriculture industry. When I started the Ph. D. program I learned through my major advisor, Dr. William Miller, that the Dutch tulip industry was loosing an estimate of 20 million dollars every year due to the infection of *Fusarium oxysporum* f. sp. *tulipae.* Since this project fulfilled my objectives I did not hesitate to work on it.

There were three major challenges that I had to face during my studies. The first was generating the proper conditions that would enable successful establishment and infection of the fungus in the bulbs. Each experiment required at least four weeks, and the time when we obtained the bulbs left four months available for optimal experimentation. After two seasons I learned how to conduct simultaneous and staggered experiments in order to take advantage of this short period of time. The second was overcoming the anxiety to learn and use analytical instruments. Fortunately I met people at Cornell who were always available to help. The third was deciding to stop experimenting and write this dissertation. It was then when I learned from Dr. Gary Bergstrom that the beauty of science is that there will always be more questions to answer, and that one man's life is not enough to address all of them.

Although the information contained in this document does not conclusively solve the *Fusarium* infection problem, it does provide robust guidelines for a grower or an exporter how to screen cultivars to define *Fusarium* resistance, and determine ethylene production values to calculate proper ventilation rates. Scientists may select cultivars listed here to further explore biochemical and molecular defense mechanisms of tulips when challenged with *Fusarium*. It will be important for future studies to characterize changes in tuliposides and tulipalines during pathogen attack in order to develop genetic markers that could be used to select resistant lines during the early breeding phases and shorten the time that it takes to

bring a cultivar to market. This could eventually lead to the development of kits (eg. ELISA tests) that can be used in the field.

As a side project from my studies, a collaboration project was initiated between the Flower Bulb Research Program at Cornell, and the post harvest lab at Washington State Universtity (Dr. John Fellman, and Dr. Scott Mattinson) in order to identify Organic Volatile Compounds specific to *Fusarium oxysporum* f. sp. *tulipae*. Preliminary results identified molecules that could be used to detect early infections. The applications of this research could translate into technologies (such as an electronic nose) that could separate infected and healthy bulbs in conveyor lines after harvest. This would mean reduced energy and costs for ventilation during transport and storage, smaller carbon foorprint, and fewer losses at bulb forcing in greenhouse.

In tough economic times funding for horticultural research is becoming slim, extension services are disappearing, and fewer students are getting involved in horticultural programs. It is important for growers and their organizations to partner with key research institutions in order to support each other. The money and time invested in targeted research topics can, in the short to middle term, provide solutions with a higher return on investment for both sides. The challenge is how to multiply successful models like the Anthos-Cornell Flower Bulb Research Program, across the world.

CHAPTER ONE: INTRODUCTION

The tulip bulb industry faces economic losses due to *Fusarium oxysporum* (Schlecht.) f.sp. *tulipae* Apt. (F.o.t.) which infects bulbs in the field and carries over after harvest. Upon infection the fungus causes bulb rot and synthesizes high amounts of ethylene.

As the fungus colonizes the tissue it makes the bulb unsuitable for flower production and causes indirect losses when healthy bulbs exposed to this gas (generally during storage after harvest) suffer from several physiological disorders (De Hertogh et al. 1980), which may not be visible until greenhouse forcing (De Munk 1973).

Tulip growers and exporters have noticed that over the last two decades F.o.t. infections have increased. Climate change (hot summer and warm fall) together with mechanization practices (which cause mechanical damage during and after harvest) have likely aggravated the problem. Another allegedly increasing problem is the development of highly virulent *Fusarium* strains which are capable of infecting cultivars that were formerly considered resistant to F.o.t. (Miller 2009).

Although this pathosystem has been studied intermittently over the last 60 years, there is limited information about the morphological (Saniewska et al. 2004), biochemical (Saniewska et al. 2005), and molecular interactions that occur between the host and pathogen. Moreover, there are few practical tools that growers can use to screen and select cultivars for *Fusarium* resistance.

This work provides methods that can be used under both lab and field conditions to conduct inoculations, assess fungal virulence, study fungal growth and ethylene production either in whole bulbs or in bulb organs, and carry out metabolic studies to determine chemicals involved in the ethylene biosynthesis pathway of *Fusarium*, as well as compounds in tulips that confer resistance to this pathogen.

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CHAPTER TWO: LITERATURE REVIEW

Overview of the tulip industry

Tulip cultivars mainly belonging to *Tulipa gesneriana* L. are used for ornamental purposes as cut flowers, pot plants, or for landscaping. *Tulipa gesneriana* is the most important ornamental bulb crop world-wide (Flower Council of Holland 2006) ranking in 2004 as the third most important cut flower. In 2000 tulips accounted for 39% of the total world acreage of ornamental bulbs (De Hertogh and Le Nard 2003). In the same year in the Netherlands, the leading tulip bulb producer, there were about 10,000 hectares grown from which 1 billion flowers and 2 billion export bulbs were produced (Straathof and Inggamer 1992).

The three most important cut flowers in the USA are lilies, roses and tulips. Tulips had a wholesale value of $57 million in 2010 (Figure 2. 1). The production doubled between 2000 and 2010, with California, Washington, Minnesota, Oregon, and recently New Jersey as the most important producing states (USDA 2001-2011).

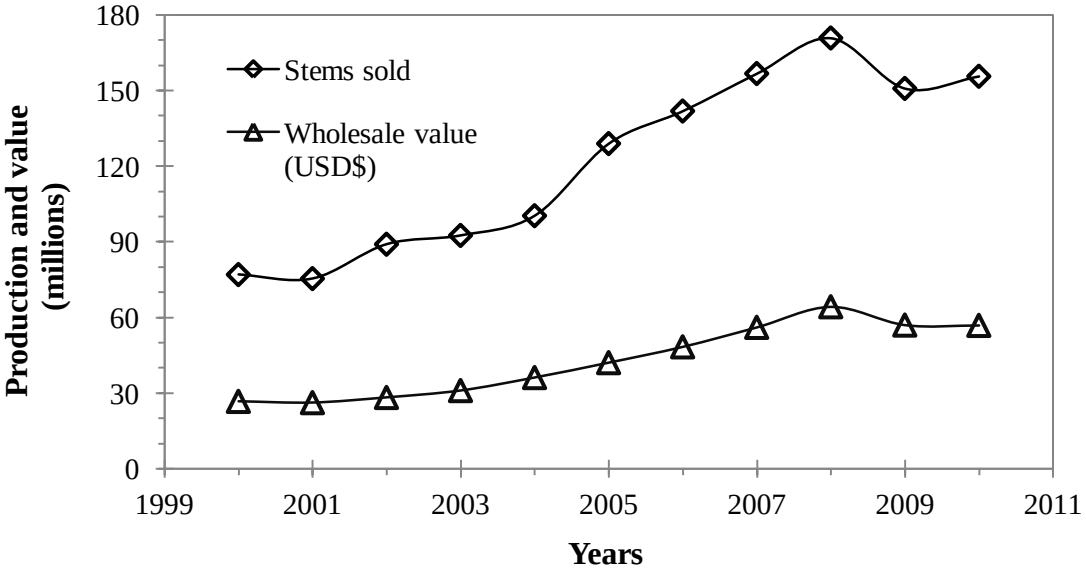

Figure 2. 1. Production and wholesale value of cut tulips in the USA from 2000 to 2010.

Production of tulips

Tulip bulbs are planted in the fall, they overwinter, grow and flower in the spring, and finally they are harvested in July of the following year (Bergman and Beijersbergen 1968).

In The Netherlands after harvest tulip bulbs go through processes in which they are cleaned and the outer small bulbs are removed. After this, bulbs are sorted for size by machines. These processes likely cause mechanical injuries to the bulbs and are thought to help spread *Fusarium* (Miller et al. 2005; Miller 2009, Personal communication).

Most tulip bulbs produced in the Netherlands that are exported to the USA are transported in temperature-controlled shipping containers, and this period takes from two to four weeks (De Hertogh et al. 1980). After arrival, they are transported by truck to their final destination.

De Hertogh and Le Nard (2003) mention that once tulips are cleaned and sorted they are stored at 23 to 25°C for 3 to 4 weeks and then temperatures are slowly decreased to 17°C; this temperature is maintained until differentiation of the flower to G stage is reached (conversion of vegetative meristem into flower primordia). Subsequently bulbs are cooled for 12 to 15 (or more weeks) depending on the cultivar to meet their cold requirement. In commercial operations, two types of precooling regimes are practiced: *standard* and *special precooling*. In *standard* bulbs are planted and maintained at low temperatures (5°C or less for ca. 16 weeks) to promote rooting and scape growth; flowering occurs a few weeks after transfer to the greenhouse. In *special precooling*, bulbs are dry stored (at least 12 weeks) at 2-5°C; upon completion of the cold period, then they are planted and moved immediately into the greenhouse at 12-15°C for forcing (Kawa et al. 1993). The quality of the flowers is correlated with the length of the temperature treatment (Kanneworff and van der Plas 1990). The primary purposes of low temperatures in the storage of tulip bulbs are to give dormancy remission, and to avoid ethylene damage because this gas is potentially dangerous in tulip bulbs at temperatures above 13°C.

Physiological responses of tulips to ethylene, and other chemical factors

Once harvested, tulip bulbs may be accidentally exposed to ethylene from combustion fumes, or by bulbs infected with *Fusarium* which is the main source of ethylene contamination in the storage atmosphere (Kamerbeek and De Munk 1976).

Ethylene contamination can cause many physiological or cultural disorders in tulips such as increased respiration (Kamerbeek and De Munk 1976), fresh weight loss (De Munk et al. 1992), bud necrosis (De Munk 1971; De Munk and Beijer 1971; De Munk 1972, 1973a), flower malformation and abortion (flower blasting), delayed growth of roots (De Munk 1973b), and gummosis -seen as polysaccharide secretion (Kamerbeek et al. 1971). Treatment with ethephon, which generates ethylene, has similar effects (Kawa et al. 1993).

Physiological alterations caused by ethylene are dependent on several factors such as timing of exposure, concentration, temperature, and cultivar. When tulip bulbs were exposed for 24 h to 1 ppm ethylene 2 weeks after harvest they produced gummosis, however, this disorder was not observed 4 months later in bulbs exposed even to 1000 ppm. In some cultivars, however, when the flower inside the bulb is formed, small ethylene concentrations (0.5 ppm for 7 days) can cause 100% flower blasting and sensitivity increases with storage time. This suggests that the physiological disorders caused by ethylene are developmentally controlled (De Munk and Beijer 1971; Kamerbeek et al. 1971; De Munk 1972, 1973b).

The most important determinants for ethylene damage are the combination of temperature, concentration and duration of exposure. As temperature increases, less ethylene is needed to cause damage. De Munk (1973b) showed that bubs treated with 100 ppm of ethylene at 13°C did not show flower blasting, but at 20°C even one ppm was enough to cause damage. This effect is thought to be caused by alterations in internal source: sink relationships since ethylene-induced flower blasting is reversible by injecting gibberellins and kinetins into the buds (De Munk 1975).

Ethylene exposure of tulip bulbs has two types of effect: *immediate* and *delayed*. *Immediate effects* such as gummosis and some bud injury can be detected a short time after

exposure. Bulbs exposed continuously to ethylene at forcing show inhibition of shoot and root growth, however, if ethylene exposure stops, elongation growth resumes (De Munk and de Rooy 1971). *Delayed effects* such as flower abortion (also called flower blasting), cannot be detected immediately, but until bulbs are grown in the greenhouse.

Ethylene in the soil (i.e. when *Fusarium*-infected bulbs are present) has negative effects in the greenhouse plants by inhibiting growth of leaf, stem and root, and causing flower blasting up to 25 cm from *Fusarium*-infected bulbs forced in sand. The diffusion of ethylene is dependent on the structure and air porosity of the soil (De Munk and de Rooy 1971).

Ventilation

Before cooling starts, ventilation is needed during storage and transport to prevent decrease of O_2 and accumulation of CO_2, ethylene and water vapor (De Munk and Duineveld 1986).

Bulbs need a constant temperature (17°C) and high ventilation regimes to remove ethylene from the atmosphere during transportation. As discussed above, tulip bulbs exposed to ethylene can suffer serious physiological disorders that affect the flower and plant quality and for that reason the industry has near zero-tolerance for ethylene during transport and storage. *Fusarium* infected bulbs are a big source of ethylene, and continuous air exchange rates of 150 m^{-3} h^{-1} per m^{-3} of bulbs are needed to remove ethylene and prevent physiological injuries (De Hertogh and Le Nard 2003; Miller et al. 2005). The tulip industry utilizes high ventilation rates in order to maintain ethylene concentration within safe limits (below 0.1 ppm). However this may incur unnecessary energy costs since high ventilation is used even when the danger of ethylene is low.

Ethylene production by tulip bulbs

Kanneworff and van der Plas (1990) studied ethylene production of intact tulip bulbs at different storage times and temperatures. Their results show that healthy bulbs produce very low amounts (1-35 nl C_2H_4 h^{-1} kg fw^{-1}) and the production increases as the storage period (either at 20°C or at 5°C) progresses. Bulbs stored at 17°C produce almost the same amounts of ethylene as those stored at 5°C until late November. After that date ethylene production by bulbs at 17°C was higher than those at 5°C.

Wegrzynowicz and Saniewski (1992) found that ethylene production in the bulbs takes place mainly in the pistil and the first leaf (500-20,000 nl kg fw^{-1} h^{-1}), while the outer scale did not produce detectable levels of ethylene. The maximum ethylene production was 20,000 nl kg fw^{-1} h^{-1} and took place in the first leaf before cooling. They reported low levels of 1-aminocyclopropane-1-carboxylic acid (ACC) versus high levels of ACC oxidase (ACO); both fluctuated during storage and at planting. Leaves produced relatively high amounts of ethylene before bulb cooling and in early stages of shoot growth after planting (20,000 and 10,000 nl kg fw^{-1} h^{-1}, respectively). Treatment of pistil and stigma with auxins did not induce ethylene production, but wounding (slicing) of the pistil induced it greatly. They concluded that ACC biosynthesis is the limiting factor of ethylene production in these organs.

Effect of wounding

Kawa (1993) wounded cooled tulip bulbs and found that wounding increased ethylene production within 2-4 days, but did not cause flower abortion. Wounded bulbs flowered 2 to 8 days earlier than controls but after 12 weeks of cold storage there were no differences in flowering. It was suggested that scale wounding acts as a partial substitute for cold treatment. The level of ethylene produced by uncut bulbs during the 5 days after cutting was very low (40-210 nl kg fw^{-1} h^{-1}) and after wounding the bulbs produced 1,500 nl kg fw^{-1} h^{-1}. It was also reported that wound ethylene is cultivar-dependent. These results suggest that ethylene

exposure at relatively low concentrations and a short duration had a favorable effect on tulip growth and flowering.

Gummosis

Kamerbeek (1971) found that gummosis on tulip bulbs is caused by ethylene exposure (as low as 0.1 ppm for 1 day at 20°C) and can be promoted by rough handling or mechanical damage in the period just after lifting. The same disorder can be induced locally in bulbs and aerial parts of the plant by treating them with 5% ethephon in lanolin paste, and with methyl jasmonate (De Munk and Saniewski 1989; Saniewski et al. 1998).

Gummosis is visible inside or outside the bulb with the formation of gum in certain layers below the epidermis two days after the exposure. If the gum mass increases too much, blisters are formed, which can burst and gum is then extruded. The chances of ethylene-induced gummosis decrease with time after harvest. Bulbs are more sensitive just after lifting; after 4 weeks of storage the response to ethylene decreases, and at 4 months from harvest it disappears (Kamerbeek et al. 1971). The occurrence and severity of gummosis are dependent on the age of the bulb, the concentration of ethylene, and temperature -higher temperatures increase gummosis (Kamerbeek 1975).

It has been suggested that in tulip bulbs gummosis is formed from the carbohydrate metabolism of fructosans and components of the middle lamellae. Gums are a complex of different substances but the most important constituents are polysaccharides (xylose, arabinose, and traces of glucose, manose, and uronic acid) (De Munk and Saniewski 1989).

Although gums have a function in limiting the spread of fungal and bacterial pathogens by isolating the infected tissues, tulip gums added to different growing media (PDA, CzDA, and MEA) in presence of *Fusarium oxysporum* f.sp. *tulipae* (F.o.t.) have a great stimulatory effect on mycelium growth and sporulation (Saniewska 2001). It has not been investigated what the effects of gum induced bulbs have in infection and ethylene production by *F. oxysporum* f.sp. *tulipae*.

Fusarium oxysporum f.sp. tulipae

Fusarium oxysporum is an important pathogen of ornamental bulbous plants. Several *formae speciales* can attack specific or multiple hosts. For flower bulbs the most important *formae speciales* are: *narcissi*, *lili*, and *tulipae*. These pathogens usually cause dry rots in the neck, scales and basal plate (Schenk and Bergman 1969; Linfield 1990; Löffler and Mouris 1992).

Löffler (1992) observed that lily can be infected by *F. oxysporum* isolates from several bulb-infecting *formae speciales* and reported that *Fusarium oxysporum* f.sp. *tulipae* can be heterogenic for pathogenicity towards different *Lilium* cultivars, however, little is known about the pathogenicity of other *F. oxysporum formae speciales* on tulip bulbs.

Fusarium oxysporum f.sp. *tulipae* is a necrotrophic (it kills the host tissue to obtain nutrients) soil-borne fungus. A characteristic of this fungus is its ability to produce high amounts of ethylene when growing on tulip bulb tissue (5,100 times more compared to other *formae speciales*) that may be related with its pathogenicity (Swart and Kamerbeek 1976). Upon infection *Fusarium* causes a dry rot of the fleshy bulb scales, and the symptoms become evident during storage after lifting. Conditions leading to infection of tulip bulbs are insufficient disinfection, heavy soil contamination or latent infections inside or close to the basal plate (Bergman and Beijersbergen 1968; Bergman and Bakker-van der Voort 1980).

The infection is caused either by direct interaction of the fungus with the bulb, or by contact of the fungus with wounds caused mechanically by mites, and both agricultural and postharvest practices.

Bergman and Bakker-van der Voort (1980) made inoculations of *F. oxysporum* f.sp. *tulipae* in some varieties and based on the result they were divided into very susceptible (Paul Richter), susceptible (Lustige White), fairly susceptible (Rose Copland), resistant (Black Parrot), and fairly resistant (Aristocrat).

Etiology aspects

F. oxysporum f.sp. *tulipae* Apt. does not behave as a vascular parasite in tulips. In natural conditions the fungus has two sites of infection: in the root tips and in the scales. At the root tips the hyphae grow in the parenchymatous tissue; when the fungus has overgrown in the root tips it then colonizes the vascular bundles in the basal plate. The hyphae grow abundantly in the intercellular spaces around the root bases where they probably secrete pectolytic enzymes that disrupt the middle lamellae. Finally the fungus invades the cell lumina through secretion of cell wall-degrading enzymes (and possibly fusaric acid) which are responsible for the rot syndrome. By killing the host cells in advance of the hyphae defense responses are defeated and cortical rot is facilitated (Schenk and Bergman 1969).

Fusaric acid, a plant toxin secreted by *Fusarium*, is thought to accumulate *in-vivo* to toxic concentrations in *Lilium*. Growth of tulip embryo, shoot and callus was inhibited *in-vitro* with 0.1-0.5 mmol L^{-1}. The degree of growth inhibition differs among cultivars and the results of the *in-vitro* assays did not reflect those of field trials (Baayen 1992; Podwyszynska et al. 1998; Podwyszynska et al. 2001)

Within the first 2 weeks of forcing tulips in the greenhouse, fungal growth causes retardation and yellowing of the leaves and plants grown at 16°C from pre-cooled bulbs at 5°C are usually killed by obstruction of the xylem vessels; the phloem cells die and show degraded cell walls. On the other hand plants grown at 12°C can still produce a marketable flower, although some damage in roots and bulbs is seen (Schenk and Bergman 1969).

In most bulbs penetration by *Fusarium* starts at rupture sites where roots emerge from the basal plate; in undamaged roots it enters through the root cap cells or via the anticlinal walls of the epidermal cells in the zone of elongation; in bulb scales it is thought that infection takes place through the stomata when latent infections are present (Baayen 1992).

Fusarium infected bulbs

Different studies have shown that *Fusarium oxysporum* f. sp. *tulipae* infects tulip bulbs (Schenk and Bergman 1969) and it is responsible for producing ethylene in presence of oxygen (Hottiger and Boller 1991). Bulbs infected with *Fusarium* produce considerable quantities of ethylene, enough to cause gummosis, flower blasting and flower abortion if diseased and healthy bulbs are stored in the same storage room (Kamerbeek 1975).

De Munk and de Rooy (1971) suggested that the *Fusarium* ethylene producing system is exhausted after about a month, when the bulbs are totally rotted. They also observed that when *Fusarium* infected tulip bulbs were planted in the ground, ethylene was detectable 25 cm from the diseased bulbs and within this distance all flowers became blasted.

Miller and co-workers (2005) inoculated live and heat-killed bulbs with *Fusarium* isolated from tulip bulbs and measured the ethylene production on 36 cultivars. They divided the cultivars in different categories (high, medium and low producers) based on the ethylene production resulting from the infection. They also found that ethylene levels of heat killed bulbs at day 15 of cultivars 'Friso' and 'Prominence' were three times greater than in live bulbs, while live bulbs of 'Furand' produced 30% more ethylene than the heat-killed bulbs.

De Munk (1972) stated that the level of ethylene in storage rooms under conditions of poor ventilation can rise to such a degree that the concentrations are capable of causing open buds and cause bud necrosis if *Fusarium*-infected bulbs and bulb mites (*Rhizoglyphus echinopus* and *Tyrophagus putrescentiae*) are present.

These circumstances have lead to essentially zero ethylene tolerance, which has created the need of some high-cost practices such as continuous ventilation, and collection and discarding of *Fusarium*-diseased tulip bulbs before planting in the greenhouse.

When some plants are infected by pathogens they can produce ethylene as an early resistance response leading to activation of plant defense pathways (Chagué et al. 2006). It is not known if tulip bulbs produce ethylene upon challenge with non-pathogenic strains of

fungus, bacteria, or metabolites of *Fusarium oxysporum* f.sp *tulipae*. It should be reiterated that in the tulip-*Fusarium* system the fungus is the main, if not the only, source of ethylene.

Bulb mites and Fusarium oxysporum f.sp. tulipae

Czajkowska (2002) showed that bulb mites feeding on fungal mycelium are a significant vector to spread *Fusarium oxysporum* f.sp. *tulipae* to healthy plant material. F.o.t. was a better diet for *Rhyzoglyphus echinopus, Tyrophagus putrescentiae* and *T. neiswandery* than *Fusarium oxysporum* f.sp. *lilii*. In preliminary experiments, we observed high infestations of tulip mites (genus not identified) on bulbs after being inoculated with *F. oxysporum* and this may have caused cross contamination with *Fusarium* between treatments and some of the controls.

Role of tuliposides and tulipaline A in Fusarium infection

Tulipalines (α-methylene butyrolactone) and their precursors, the tuliposides, occur in various members of the Liliflorae (*Erythronium americanum, Tulipa sylvestris, and T. turkestanica)* and Alstromeriaceae. Additionally there are other glycosides (picrocrocin and raninculin) known from crocus and ranunculus, respectively. Although there are several types of tulipalines, the best characterized is tulipaline A (α-methylene-γ-butyrolactone) (Christensen 1999).

Tulipaline A can be present in the outer-most layer of the tulip bulb (also called white skin) as both a preformed lactone or as its precursor, tuliposide A. The tuliposide precursor is a glucose ester of γ –hydroxy-α-methylene butyric acid, and is not toxic to *F. oxysporum* at pH 5.5, but it produces tulipaline spontaneously above pH 6.0 or after heating. Although the highest concentrations of tulipaline A is found in the white skin, its precursor, tuliposide A is also found in lower quantities in extracts from tulip roots and from the lowest, subsoil part of the tulip stem.

Tulipaline A has fungitoxic activity *in-vitro* against some strains of *F. oxysporum* f.sp *tulipae* at 100-300 ppm, and causes fungistasis above 72 ppm under *in-vitro* conditions (Bergman and Beijersbergen 1968, 1971; Beijersbergen and Lemmers 1972; Bergman and Bakker-van der Voort 1980; Baayen 1992). The concentration of this lactone flucuates across developmental stages of the organs and thre is evicence that it may behave as a phytoanticipin (see definition in VanEtten et al. 1994) in some cultivars. In three out of four cultivars, the bulb tulipaline level was above toxic levels to *Fusarium*: 'Madame LeFeber' had <3 ug g^{-1} FW, while 'Apeldoorn', 'Lustige Witgwe', and 'Gander' had >130 ug g^{-1} FW (van Rossum et al. 1998). In the same study it was observed that the content of this compound in tulip bulb explants *in-vitro* increased ca. 70 fold within five days of dissection.

Wounding is frequent in nature and during cultivation and postharvest handling. It has been observed that tulipaline only confers resistance to the bulb when it is intact because superficial wounding of the fleshy scale always leads to heavy infections. If the bulb is wounded, tulipaline does not inhibit fungal colonization (Bergman and Beijersbergen 1968).

Observations made in the Netherlands show that *Fusarium oxysporum* is able to penetrate into the tulip bulb almost exclusively in a short window period in late June to early July. It has been reported that tulipaline A diminishes rapidly during the last weeks before the skin turns brown, and it could not be found when the skin is completely brown. Tulip bulbs inoculated a few weeks before harvest (2nd week of June), and the first weeks after harvest (typically during the last week of June to 2nd week of July) showed 60% vs. 100% infection respectively; this principle is used by growers to harvest early and reduce the disease occurrence. Although climatic conditions, specially soil temperature and hot summers greatly influence disease incidence, the factor responsible for the infection before harvest is a reduction of the enzymatic activity that liberates tulipaline A in the white skin rather than the potential concentration of tulipaline in the tissue (Bergman and Beijersbergen 1968). Interestingly, it was observed that when bulbs were in the soil and the skin was still white the tulipaline concentration was less than 200 ppm in freshly harvested bulbs (anytime between

May and the end of June), however, tulipaline in the white skin increased to 2300 ppm when bulbs were stored for four days after harvest (Bergman and Beijersbergen 1971). No reports have investigated the concentrations of tulipaline in bulbs throughout storage.

F. oxysporum may overcome the tulipaline barrier by absorbing and metabolizing it in the hyphae, or by inhibiting the synthesis of tuliposide A. Beijersbergen and Laroo-Lemmers (1975) noticed that when tulipaline was added to liquid media with already growing *F. oxysporum* mycelium, the growth of the fungus was temporarily inhibited. The concentration of tulipaline dropped to a level that allowed resumption of fungal growth, suggesting that the fungus could absorb or metabolize tulipaline. In the second case, synthesis of tuliposide A is inhibited completely when ≥ 2 ppm ethylene is present in the air surrounding the bulbs. If the concentration of the precursor in the tissue is already high, as is the case for the white skin, ethylene does not influence that concentration (Beijersbergen and Bergman 1973).

Ethylene: a gaseous plant hormone

Ethylene is one of the simplest organic molecules (C_2H_4, molecular weight 28) and it exists in the gaseous state under normal physiological conditions. It is produced in all the tissues of plants and functions as a plant hormone. Ethylene plays an important regulatory role in the physiology of plants from germination to senescence and it can cause responses at concentrations well below 1 $\mu l\ l^{-1}$ (Arshad and Frankenberger 1989; Mathooko 1996).

Ethylene has significant commercial application in horticulture from nursery production to the postharvest handling of produce. It is used in the production and at the postharvest stage of fruits, flowers and vegetables. Depending on the physiological stage, nature of the tissue and species, its role can be beneficial (i.e. triggering defense mechanisms, wound responses) or detrimental (i.e. causing triple response of seedlings, or inhibition of root growth in cuttings) (Mathooko 1996). In some ornamental crops (i.e. bromeliads) it is used to trigger flowering, and to produce compact pot plants (i.e. hyacinth). In climacteric fruits like

bananas it is used to hasten ripening. In ethylene-sensitive cut flowers (such as carnations) ethylene causes rapid senesce, shortening shelf life.

Ethylene plays a critical function in the pathogenesis of plant pathogens, activating defense mechanisms (through cross talking with other plant hormones) of higher plants vs. pathogens, and in wounding responses.

The ethylene biosynthesis pathway in higher plants

The ethylene biosynthesis pathway in plants is differentially regulated by external and internal factors. Until recent times molecular biologists and biochemists pointed out the key elements of the biosynthesis pathway by using ethylene biosynthesis inhibitors or mutant plants altered in the biosynthesis or perception of ethylene. It is now known that the starting component in the ethylene biosynthesis is methionine, which through a series of enzymatic reactions is transformed into ethylene.

Ethylene biosynthesis occurs through a rather simple metabolic pathway by conversion of methionine, derived from the Yang cycle, to ethylene through the following sequence: L-methionine → S-adenosylmethionine (SAM) → 1-aminocyclopropane-carboxylic acid (ACC) → C_2H_4. Ethylene is formed from carbons C3 and C4 from methionine; the enzymatic reaction is oxygen-dependent and it produces carbon dioxide (Yang and Hoffman 1984; Mathooko 1996).

Ethylene biosynthesis starts with the conversion of methionine to SAM (also called AdoMet) by the enzyme Met Adenosyltransferase. This step is sensitive to aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor; this process utilizes the largely tissue-constitutive enzyme ACC synthase (ACS) that converts SAM to ACC and 5'-methylthioadenosine (MTA), which is recycled to L-methionine through the Yang cycle. This allows for levels of L-methionine to remain relatively unchanged even during high rates of ethylene production. It has been suggested that this is the most significant step in the ethylene biosynthesis pathway, since the ACC synthase enzyme is very unstable and has been shown

to: (a) be rate limiting and (b) to rise proportionally to ethylene levels within the tissues of some plants. The gene for this enzyme is part of a multigene family, and considerable evidence indicates that the transcription of different forms are induced under different environmental or physiological conditions. The final step is the conversion of ACC to ethylene by ACC oxidase (ACO), which is present in most tissues at very low levels (Yang and Hoffman 1984; Mathooko 1996; Capitani et al. 1999; Woeste et al. 1999).

Both ACC synthase, and ACC oxidase [formerly known as the ethylene-forming enzyme (EFE)] are developmentally regulated and are expressed in response to diverse inducers such as ethylene, auxin, wounding, temperature and metal ions such as Cd^{2+} and Li^{+}. In wound-induced ethylene these enzymes are regulated by ethylene: ACS is negatively regulated in presence of ethylene while ACO is positively regulated (Mathooko 1996).

Ethylene biosynthesis by microorganisms

Ethylene biosynthesis is not only limited to plants. It has been shown that several microorganisms are also able to synthesize it (Fukuda et al. 1993; Akhtar et al. 2005). Pathogenic fungi (i.e. *Fusarium* and *Penicillium*) and bacteria (i.e *Pseudomonas* and *Ralstonia*) also produce different ethylene levels during the infection process (Jacobsen and Wang 1968; Weingart et al. 1999; Akhtar et al. 2005).

It is known that the slime mold *Dictyostelium mucoroides* and the fungus *Penicillium citrinu* are the only microorganisms to have an ethylene biosynthetic pathway that uses ACC as intermediate, like plants do (Chagué et al. 2002). There are two additional ethylene biosynthetic pathways in microorganisms: the first utilizes 2-oxoglutarate as precursor and releases ethylene using L-glutamic acid as substrate by an Ethylene Forming Enzyme (EFE) and requires additional amino acids (arginine, or histidine) and ferrous ions as cofactors. The EFE in micro organisms (most likely a different enzyme than ACC-oxidase in plants) is a member of the super family of Fe^{2+}/ascorbate oxidases and is encoded by the *efe* conserved gene among several indigenous plasmids of *P. syringae*. The difference between the ethylene

forming *Pseudomon*as from non ethylene producers is a difference in substitution of two histidine resides that are essential for catalytic activity and iron-binding (Weingart et al. 1999).

The second microbial ethylene pathway is the α-keto-γmethylthiobutyric acid (KMBA) pathway which uses L-methionine as susbtrate. This pathway has been found in a range of bacteria, *Saccharomyces cerevisiae,* among other higher fungi. Reports show that *Botrytis cinerea* releases KMBA into the growing medium and it is oxidized into ethylene in the presence of light by a non-enzymatic reaction, or by adding peroxidase to dark-grown cultures (Chagué et al. 2006). In a different case, *Penicillium digitatum* showed a differential ethylene biosynthetic pathway: when the fungus was grown statically it used glutamate as substrate for ethylene, and it used methionine when grown on a shaker (Chalutz and Lieberman 1977).

Weingart et al., (1999) suggested that the majority of microbes that synthesize ethylene via the KMBA pathway do so at low rates. In contrast, the higher ethylene producers *Penicillium digitatum* (Fukuda et al. 1989), *Fusarium oxysporum* f. sp. *tulipae* (Hottiger and Boller 1991), *Pseudomonas syringae* pv. *pisi* (Weingart et al. 1999) utilize the 2-oxoglutarate-dependent pathway.

The ethylene production by fungi is affected by the type of ethylene pathway employed by each fungus species or *formae specialis* (Fukuda et al. 1993), the composition of the growing medium such as pH and type of substrates, oxygen availability, temperature and exposition to light (Chalutz and Lieberman 1977; Arshad and Frankenberger 1989; Chagué et al. 2006).

It is not completely clear why pathogens need to synthesize ethylene during infection, but it has been shown that ethylene acts as an elicitor by inducing expression of Pathogenicity-Related (PR) genes. *Arabidopsis* plants infected by *Botrytis cinerea* express an ethylene-mediated mechanism which induce a plant defensin gene (PDF1.2), a chitinase gene

(PR-3) and an acidic hevein-like gene (PR-4) that confer resistance against *B.cinerea*, but those mechanisms are not effective against all pathogens (Thomma et al. 1999).

There have been numerous publications that have characterized the ethylene production in-vitro by several fungi, but the most extensive fungus studied *in-vitro* and *in-vivo* is *Botrytis cinerea* (Chagué et al. 2002; Cristescu et al. 2002).

Several workers have studied the defense response mechanisms of *Arabidopsis* (Govrin and Levine 2000; Dıaz et al. 2002; Govrin and Levine 2002; Chagué et al. 2006; Govrin et al. 2006) and the involvement of ethylene in the infection process of *B. cinerea* in *Arabidopsis* (Thomma et al. 1999; Govrin et al. 2006).

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CHAPTER THREE: ISOLATION AND IDENTIFICATION OF FUSARIUM STRAINS FROM INFECTED TULIP BULBS

SUMMARY

A multi step procedure was conducted to isolate, characterize and identify *Fusarium* strains allegedly causing *Fusarium* rot and ethylene production in tulip bulbs. Forty fungal strains were isolated and when grown in PDA the pigmentation of the mycelium was either purple (75%) or white-cream (25%). Morphological observations made in our lab and corroborated by the Fusarium Research Center at Penn State University indicated that the purple isolates were *F. oxysporum*, while the white-cream isolate was classified as *F. solani*. Isolation of genetic marker translation elongation factor 1-α (TEF1) and comparison of strains on a phylogenetic tree further identified the purple isolates as *F. oxysporum* f. sp. *tulipae*, and the white isolate as *F. solani*. The forma specialis *tulipae*, was tested for ethylene production by inoculating the strains onto five flower bulb species, and large quantities of ethylene (ca. 0.6 ul g^{-1} FW h^{-1}) were seen only on tulip bulbs. *F. solani* produced only trace amounts of ethylene. Strain Dy5 of *Fusarium oxysporum* f. sp. *tulipae* produced the highest amounts of ethylene in both 'Friso' and 'Calgary' tulip bulbs, however, the ethylene data were highly variable, possibly due to non-optimal incubation conditions (i.e. low relative humidity).

The procedure we followed allowed us to conclusively identify various strains of *Fusarium*. Preliminary data were obtained to conduct further experiments with various isolates of *F. oxysporum* f. sp. *tulipae* Apt.

INTRODUCTION

Fusarium oxysporum is a widely spread pathogenic fungus of plants, as well as immunocompromised animals and humans. This fungus has one or several clonal lines known as formae speciales (f. sp.) that may have co-evolved to infect and cause wilts in one or a few plant hosts (Gordon 1997; 2003; Ortoneda et al. 2004; Michielse and Rep 2009).

Fusarium oxysporum Schlecht. f. sp. *tulipae* Apt. (F.o.t) is a soil-borne fungus that infects tulip bulbs at the end of the growing season (Bergman 1965) and it is characterized by producing up to 5,000 times more ethylene than other *Fusarium* species and formae speciales (Swart and Kamerbeek 1976). This fungus causes direct economic losses infecting the crop in the field, and latent infections become important sources of ethylene during storage or forcing in the greenhouse (Bergman 1965; Schenk and Bergman 1969; Bergman and Bakker-van der Voort 1979). An indirect economic impact is caused by the use of high ventilation rates in storage rooms to avoid physiological disorders to healthy bulbs due to ethylene exposure (Kamerbeek and De Munk 1976; De Hertogh and Le Nard 1993; De Wild et al. 2002a; De Wild et al. 2002b).

The study of F.o.t. infection and its effects on tulip bulbs has been documented by several researchers who have utilized diverse sources of inoculum (including some with non-purified cultures) to conduct their experimental work (Schenk and Bergman 1969; De Munk and Beijer 1971; Kamerbeek et al. 1971; De Munk 1973; Kamerbeek 1975; Swart and Kamerbeek 1975; van Eijk et al. 1978; Bergman and Bakker-van der Voort 1979; Baayen and Rijkenberg 1999; Saniewska et al. 2004; Miller et al. 2005). The first step in conducting reliable pathogenic experiments with this organism is to obtain a pure culture and properly identify it. Before the advent of molecular biology, identification of *Fusarium* species was done mainly by describing disease symptoms of the host, fungal morphology (i.e. micro and macro conidia), comparing growth rates and geographical distribution (Booth 1971; Toussoun and Nelson 1976). Currently, together with the above diagnostic procedures, gene markers

such as the translation elongation factor 1-α (TEF1) are being used to complement the correct identification of *Fusarium* species (Summerell et al. 2003; Geiser et al. 2004). An extra step after identification of the *Fusarium* species of interest is to fulfill Koch's postulates, which are a series of steps to determine whether a suspected organism is the causal agent of disease (Agrios 2005).

This work describes the procedure that was conducted to isolate, purify and identify *Fusarium oxysporum* f. sp. *tulipae* prior to more in depth experiments on ethylene production in tulip bulbs. Two experiments were conducted to validate the ethylene production of the fungus in tulip bulbs, and to determine if F.ot. could synthesize ethylene in other flower bulb species, and to confirm its formae specialis.

MATERIALS AND METHODS

Isolation of Fusarium strains

Fusarium infected bulbs of four tulip cultivars were obtained from commercial sources. Tulip cultivars were: Monsella (Mo), Leen van der Mark (Lvd), Gabriella (Ga), and Dynasty (Dy). *Fusarium* strains were isolated by excising 1 cm^2 of visibly infected tissue which was then surface sterilized for 1 minute in 10% commercial bleach solution and rinsed in sterile distilled water. Three tissue sections were transferred onto *Fusarium*-selective media (Schmale III et al. 2007). Five days after plating, fungal tips growing out of from each of the two infected tissues were transferred to ¼ PDA and grown for 4 days. Conidia were suspended in sterile distilled water, spread onto 2% water agar, and a single macro conidium per bulb tissue was re-transferred to ¼ PDA where each grew for 6 days. A total of 40 isolates were obtained and stored (as stock conidia) at -80°C in 15% glycerol until used.

Morphological observations

A frozen aliquot of each one of the stock conidial suspensions was plated onto ¼ PDA and grown for 4 days. Microscopic morphological observations were made to further characterize the fungal strains. Samples of the strains were submitted to the *Fusarium* research center at Penn State University for further validation.

Genetic characterization

Four putative *Fusarium* strains (Dy1, Dy5, Ga2, Mo1) were randomly chosen among the isolated strains for genetic identification. Three of the isolates were purple (Ga2, Mo1, Dy5) and one was white (Dy1). Stock cultures were grown for four days in Petri dishes with 1/4 strength PDA agar (w/v) (BD, Franklin Lakes, NJ). The mycelium and spores were scraped off the surface of the agar, transferred to 50 ml centrifuge tubes containing 30 ml of ½ PD broth and incubated in a rotary shaker at 150 rpm for five days. After this period, the liquid was decanted, the mycelium slightly centrifuged, rinsed with sterile distilled water, and centrifuged again. After lyophilizing the mycelium, gDNA was extracted using Qiagen's DNeasy Plant Minikit (Qiagen, Chatsworth, CA) following the manufacturer's directions. The translation elongation factor 1-α (TEF1) gene was isolated and amplified using primers previously described (Geiser et al. 2004) with the following sequences:

ef1 (forward primer): 5'-ATG GGT AAG GAA GAC AAG AC-3'

e2AG (reverse primer): 5'-GGA AGT AAC AGT GAT CAT GTT-3'

The TEF1 sequences of the isolates were blasted in GenBank®, the matching accessions were recorded and a phylogenetic tree was built.

Inoculum density and ethylene evolution

Five putative *Fusarium* strains (Dy1, Dy5, Ga2, Lvd1, Mo1) were grown for five days in 250 ml Erlenmeyer flasks with 100 ml 3.5% (w/v) Czapek dox broth (BD, Sparks, MD) and held in constant agitation (220 rpm) at 25 °C. After incubation, the liquid culture

consisted mainly of micro conidia. Inoculum suspensions were made by straining the liquid culture through four layers of sterile cheese cloth, then the liquid was centrifuged at 3,000 g for five minutes and the supernatant was decanted and replaced with distilled water with 0.1 % (v/v) Tween 20. After repeating the centrifugation step twice, the conidia were counted and three suspensions made with $3x10^4$, $3x10^5$, and $3x10^6$ conidia ml^{-1}.

The brown tunic of each bulb was removed, and 1 cm of the bulb tip was excised. A sterile toothpick was used to make three wounds (1 cm deep) around the transition zone between the root collar and the scales of the bulb. Each wound was inoculated by injecting 150 ul of conidial suspension, or 0.1% Tween 20 in water as control. One bulb was placed inside a 1 US pint glass jar and 15 jars were placed inside a 32 l capped plastic box containing 1 l deionized water, and covered with a perforated lid (9 holes, 5/8 diam.). Boxes were kept in darkness inside a growth chamber at 21 °C. Ethylene was analyzed after 14, 21, 28, 35, and 41 days.

Host specificity

Fusarium strain Dy5 was inoculated on five species of flower bulbs: 1) *Crocus* 'Remembrance', 2) *Hyacinth* 'Pink Pearl', 3) *Muscari armeniacum*, 4) *Narcissus* 'Carlton', 5) *Narcissus* 'Primeur', 6) *Tulipa gesneriana* 'Leen van der Mark'
and ethylene production recorded after 26 days. Bulbs of *Crocus*, *Tulipa*, and *Muscari* were wounded once and inoculated with $5x10^5$ conidia suspended in 150 ul of water. Since bulbs of *Hyacinth* and *Narcissus* were two to three times heavier than tulip bulbs, inoculations were made in three sites (each site was inoculated with 150 ul of conidial suspension, two on the scales, and one in the root collar).

Ethylene analysis

The jar atmosphere was flushed for 30 seconds using a fan, then jars were sealed with lids containing a rubber septum, and a one ml sample was collected with a hypodermic

syringe after one hour of headspace accumulation. Samples were injected onto a gas chromatograph (Model 310, SRI instruments, Torrance, CA) equipped with an alumina column (90 cm long, 80/100 mesh, 180°C oven temperature) and a FID detector (200°C).

RESULTS

Fusarium infection symptoms and bulb mites

The tulip bulbs that were used to isolate the fungal strains showed typical symptoms of infection by *Fusarium*. Healthy bulbs were ivory-colored with occasional mechanical damage (Illustration 3. 1. A), however, bulbs infected with *Fusarium* had a dry-rot spreading from the scales (Illustration 3. 1. B and C) or from the base plate (Illustration 3. 1. D and E) with Penicillum occasionally growing on necrosed tissue (Illustration 3. 1. E2). The color of *Fusarium* rot ranged from light to dark brown, and gum blisters were irregularly observed in or around infected tissue. White and dry mycelium was almost absent on the outside of the bulb, but it was more frequently found between the bulb scales (Illustration 3. 1. D3 and D4). Infected bulbs had a peculiar smell, referred to in The Netherlands as sour ('zuur').

Healthy dissected bulbs had firm ivory-colored scales and yellow flower buds. On the other hand the scales of infected bulbs had a soft-crumbling texture and showed a darker ivory or brown color while the flower buds were generally withered (Illustration 3. 1. rows 3 and 4). As in the case of *Fusarium* rot on the outer scales, *Penicillium* was often seen growing on infected tissue of the inner organs (Illustration 3. 1. D3).

Fusarium often protruded from outer tulip scale or base plate as white to brown sporodochia where tulip mites (genus and species not identified) were regularly found feeding on fungal tissue (Illustration 3. 1 D2, and Illustration 3. 2 A and B).

Bulb mite populations were observed in the inoculation density experiment starting at 14 DPI, and invasive proportions were seen after 21 DPI. Mites fed on *Fusarium* of

inoculated bulbs, migrated from inoculated to non-inoculated treatments, and were found on the outside of the incubation vessels.

Characteristics of isolated Fusarium strains

From the 40 isolates that were obtained (ten from each cultivar) the mycelium showed either purple or white pigmentation (Illustration 3. 3). Table 3. 1 shows that there was an overall 3:1 ratio of purple to white isolates. Based on color similarities with a known strain used in preliminary experiments, isolates showing purple coloration were preliminarily classified as F. o. t.

Table 3. 1. Tulip cultivars and color of *Fusarium* strains isolated from each.

Tulip cultivar	**No. Isolates**	**Purple**	**White**
Leen van der Mark	10	9	1
Gabriella	10	8	2
Monsella	10	7	3
Dynasty	10	6	4
Total	40	30	10

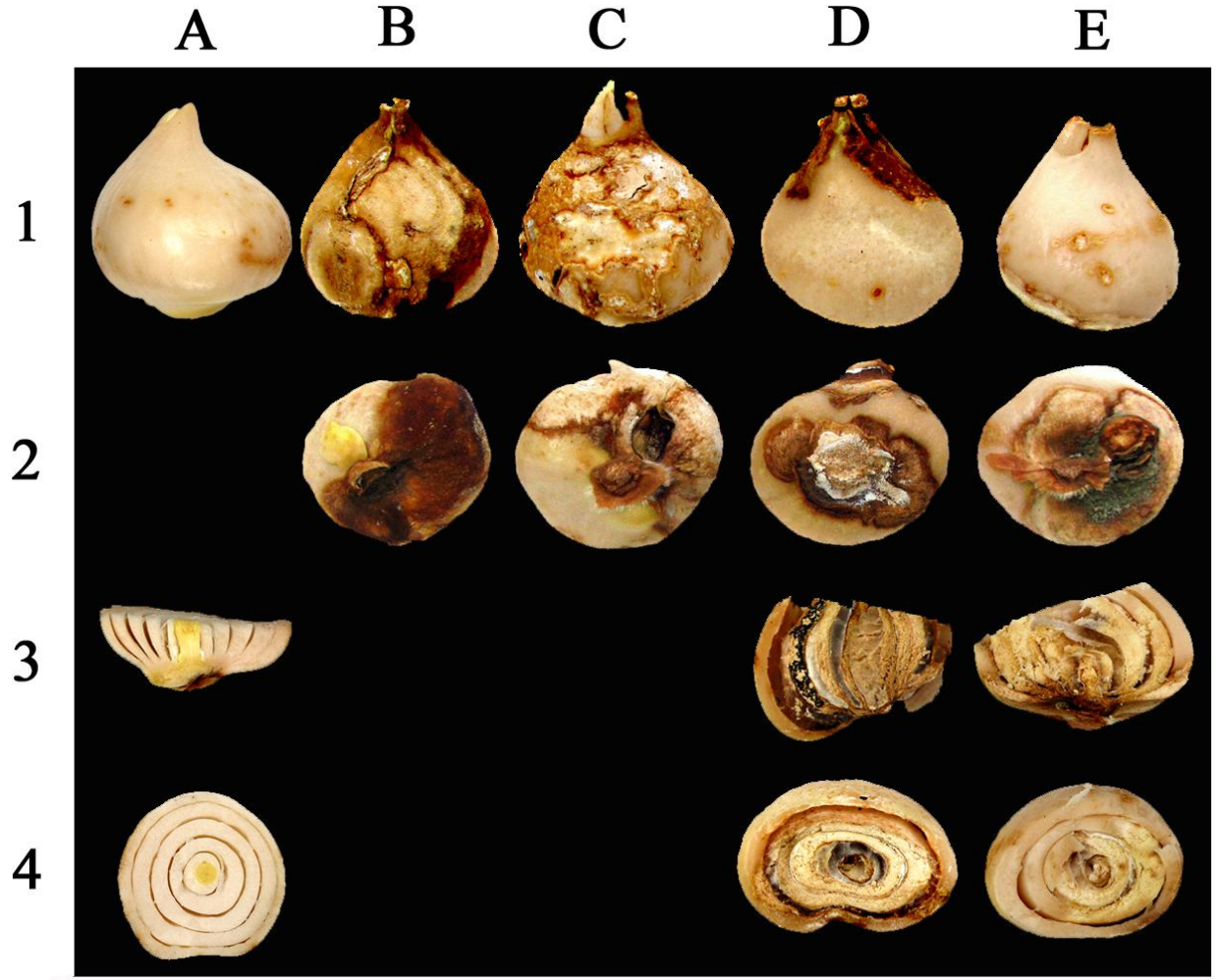
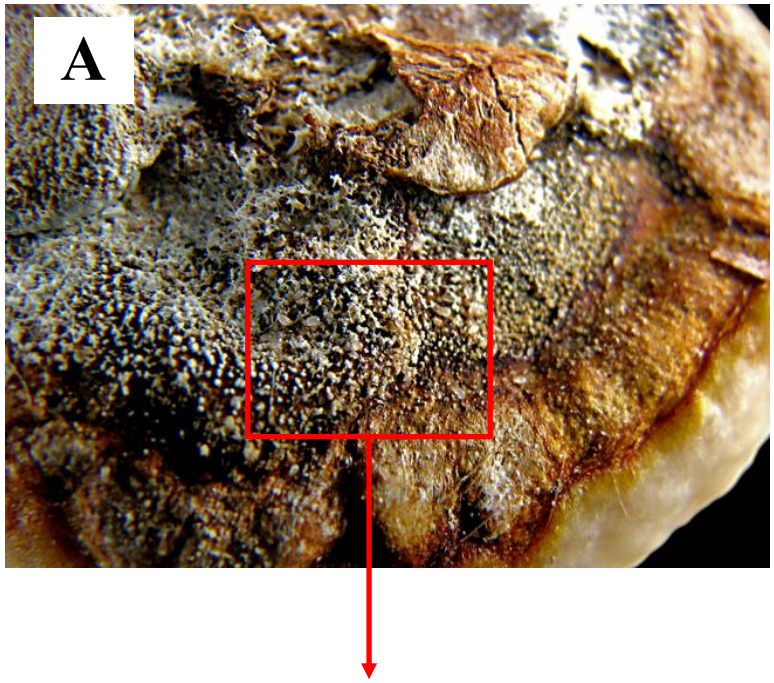


Illustration 3. 1 Whole and dissected tulip bulbs showing different degrees of *Fusarium* infection. Each column shows the same bulb. Rows present the bulb in different planes: 1) Anterior view of intact bulb, 2) Basal view of intact bulb, 3) Longitudinal section with distal end removed, 4) Transverse section with distal end removed. Column A shows a healthy bulb, all other columns depict *Fusarium* infected bulbs.



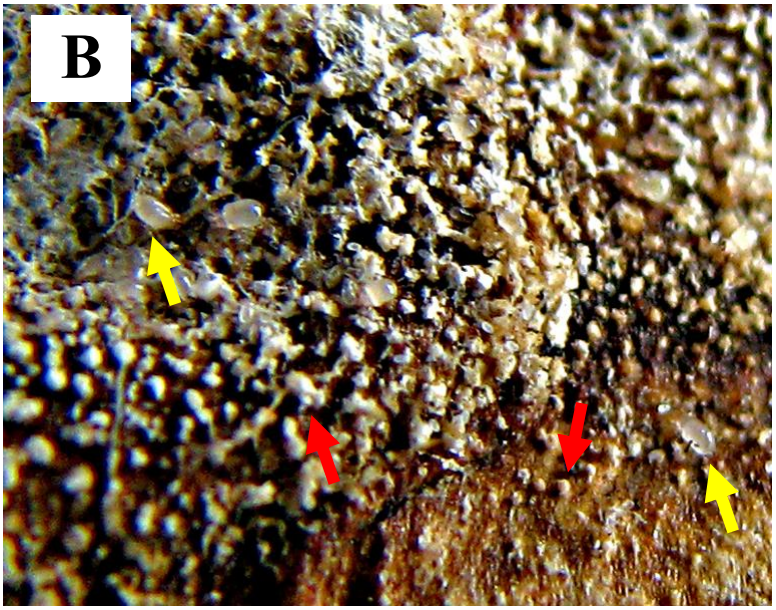


Illustration 3. 2 . A) *Fusarium* growing on root collar of tulip bulb. B) Insert shows *Fusarium* sporodochia (red arrows) and bulb mites (yellow arrows) feeding on fungal structures.

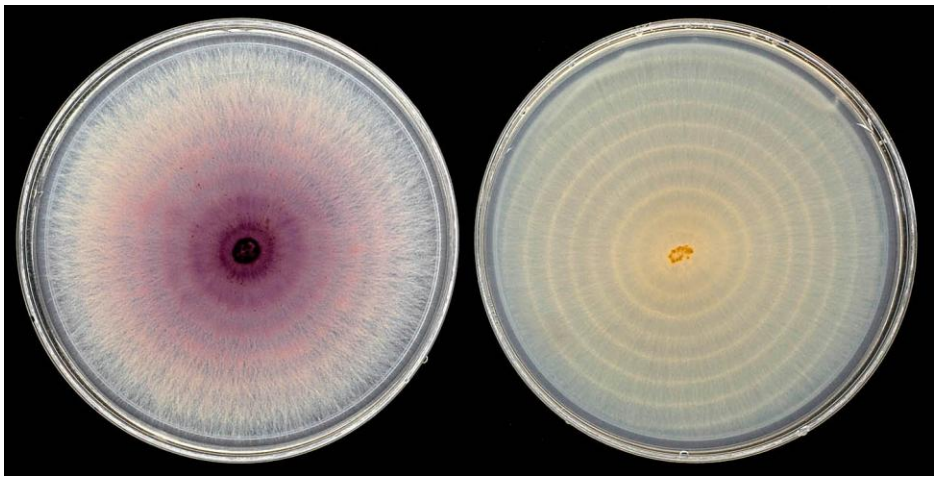


Illustration 3. 3 Pigmentation of *Fusarium isolates* growing on PDA. *Fusarium oxysporum* f. sp. *tulipae* (left), and *Fusarium solani* (right).

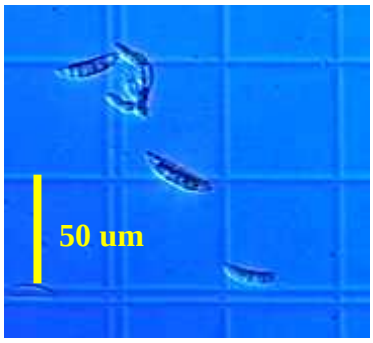


Illustration 3. 4 Macroconidia of Fungal strain visually identified as *Fusarium oxysporum*.

Morphological identification

Microscopic observations of fungal structures (microconidia, macroconidia, and phialides), were recorded (Table 3. 2) and compared with graphical and taxonomic guides (Booth 1971; Toussoun and Nelson 1976; Booth 1977).

The characteristics observed in the purple isolates were typical of *F. oxysporum* (Illustration 3. 4), while the white-cream isolates were typical of *F. solani*. The *Fusarium* research center at Penn State University corroborated the purple isolates as *F. oxysporum* and the white-cream cultures as *F. solani*.

The TEF1 sequences of white and purple *Fusarium* isolates were blasted in GenBank® to identify matching nucleotide candidates. Comparison of the nucleotide sequences on a phylogenetic tree showed that the purple isolates matched accession AF246891 corresponding to *Fusarium oxysporum* f. sp. *tulipae* strain NRRL28974 and were clustered in the same clade. The sequence of the white isolate matched accession DQ247709 belonging to *Fusarium solani* strain FRC S1607.

Table 3. 2. Morphological observations of the two pigmented *Fusarium* isolates.

Colony color	**Microconidia**	**Macroconidia**	**Phialides**
Purple	Not septated	Thin curve-shaped (approx. 25-40 um long), 4-5 septate	Short
White-cream	Not or single septated. More abundant than purple isolates.	Wider diameter than purple isolate with round ends, curve-shaped, (approx. 25-40 um long), 6 septate	Longer than purple isolate

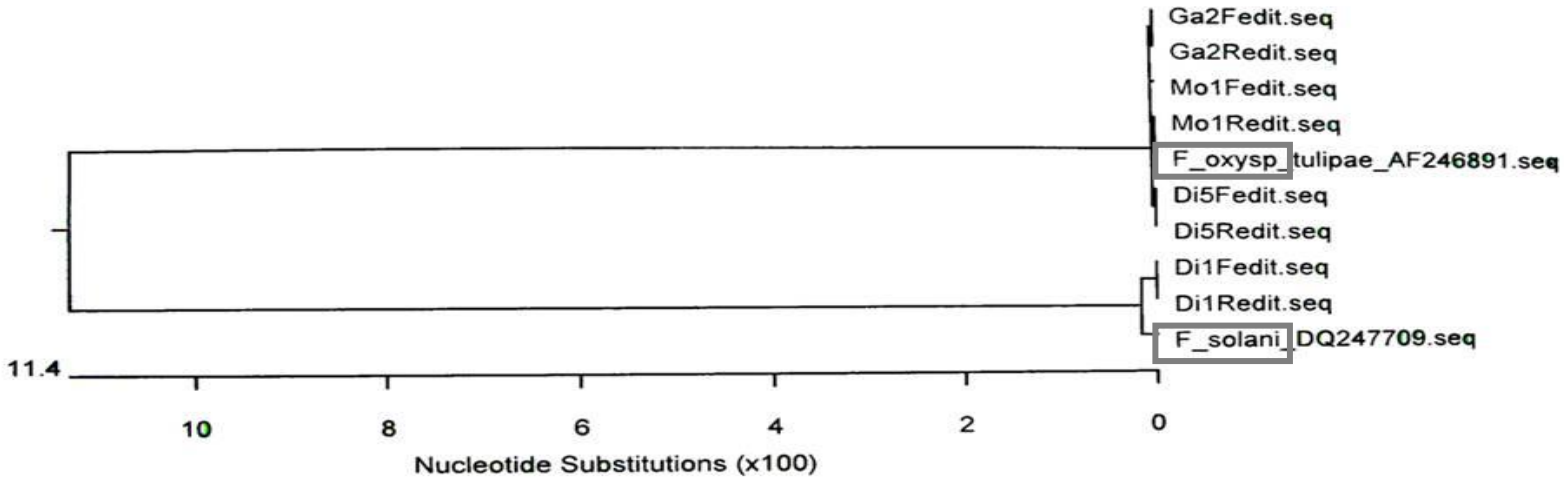


Figure 3. 1. Phylogenetic tree grouping *Fusarium* isolates into two branches belonging to *F. oxysporum* f.sp *tulipae* (upper branch) and *F. solani* (lower branch).

Genetic identification

Inoculation density and ethylene evolution

In 'Friso', ethylene production generally increased with higher inoculation densities (Figure 3. 2). The lowest ethylene levels were observed in Dy1 (*F. solani*) at the three inoculation concentrations, followed by Lv1. Ethylene production by strain Ga2 did not change as inoculation density increased. Strain Mo1 produced much more ethylene at the highest inoculation density. Dy5 produced high amounts of ethylene at the two highest inoculation densities. Ethylene production by Dy5 at $4.5x10^5$ microconidia $bulb^{-1}$ was 105 times higher than Dy1 (*F. solani*) which produced only traces of ethylene.

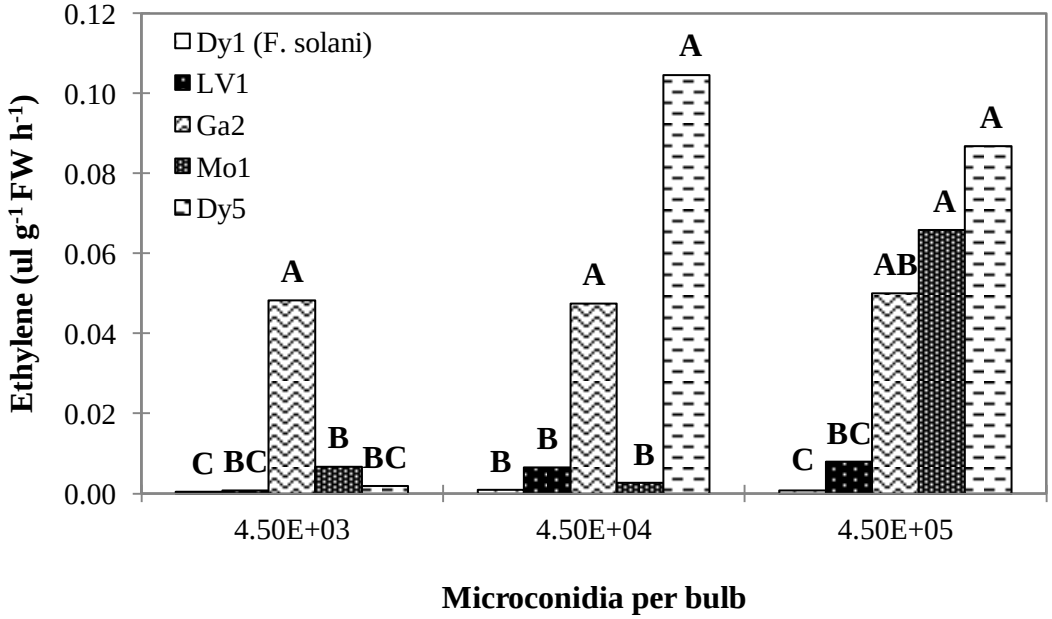


Figure 3. 2. Ethylene production by five *Fusarium* strains in tulip 'Friso'.

The data obtained with 'Friso' under the described experimental conditions was highly variable and Tukey's test did not detect significant differences between inoculation densities and DPI (Figure 3. 3), however, an increasing trend in ethylene production was observed with higher inoculum densities.

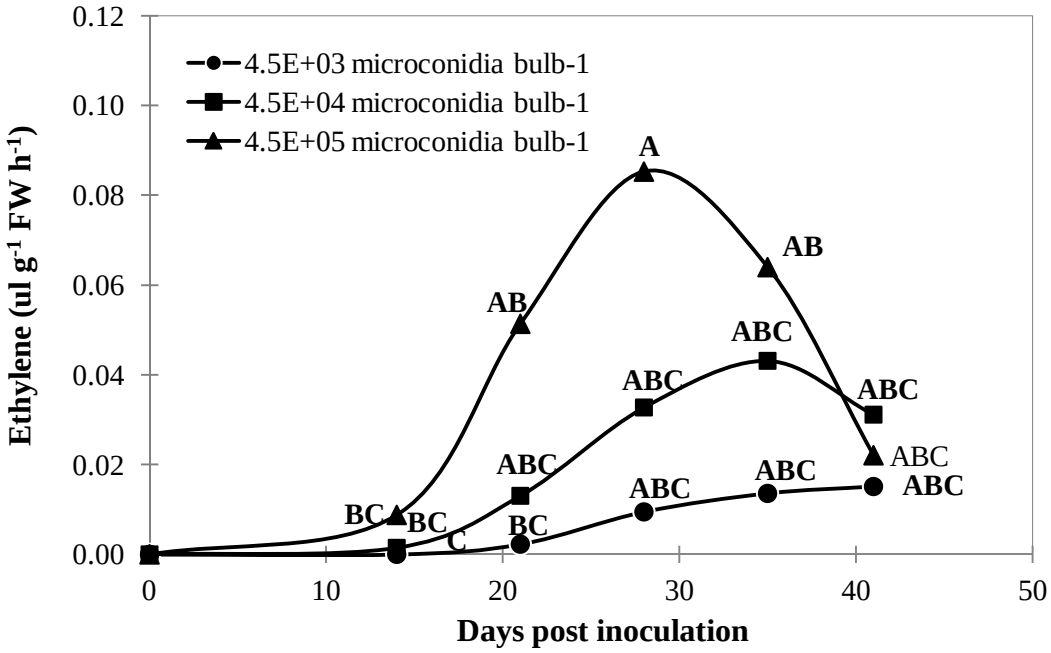


Figure 3. 3. Ethylene production by *Fusarium* at different inoculation densities on tulip 'Friso'.

No significant inoculation density or strain x DPI interaction was detected among F.o.t. strains in 'Calgary' bulbs. Ethylene production by Dy1 was the lowest among all the tested strains, and it was fifteen fold lower than isolate Dy5 (Figure 3. 4). Among the F.o.t. isolates, only Lv1 and Dy5 were statistically different. Overall ethylene production rates were significantly lower than in 'Friso'.

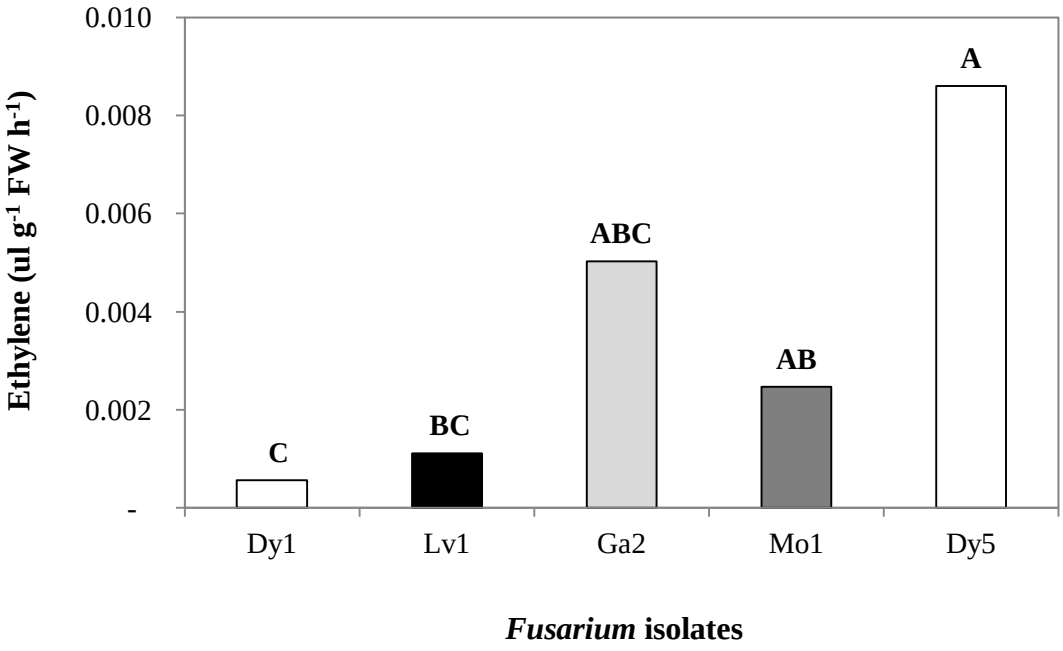


Figure 3. 4. Ethylene production by four F.o.t. strains and *F. solani* (Dy1) when inoculated in 'Calgary'.

Host specificity

Strain Dy5 was inoculated onto six cultivars of five flower bulb genera to test its ability to produce ethylene. At day 26 only tulip (cultivar 'Leen van der Mark') supported significant ethylene production which was 50 and 700 fold higher from *Crocus*, Hyacynth, *Muscari*, or *Narcissus* (Figure 3. 5).

Fungal samples of tulip 'Leen van der Mark' were taken and observed under the microscope. The characteristics of fungal colonies were identical to those previously recorded (Table 3. 2).

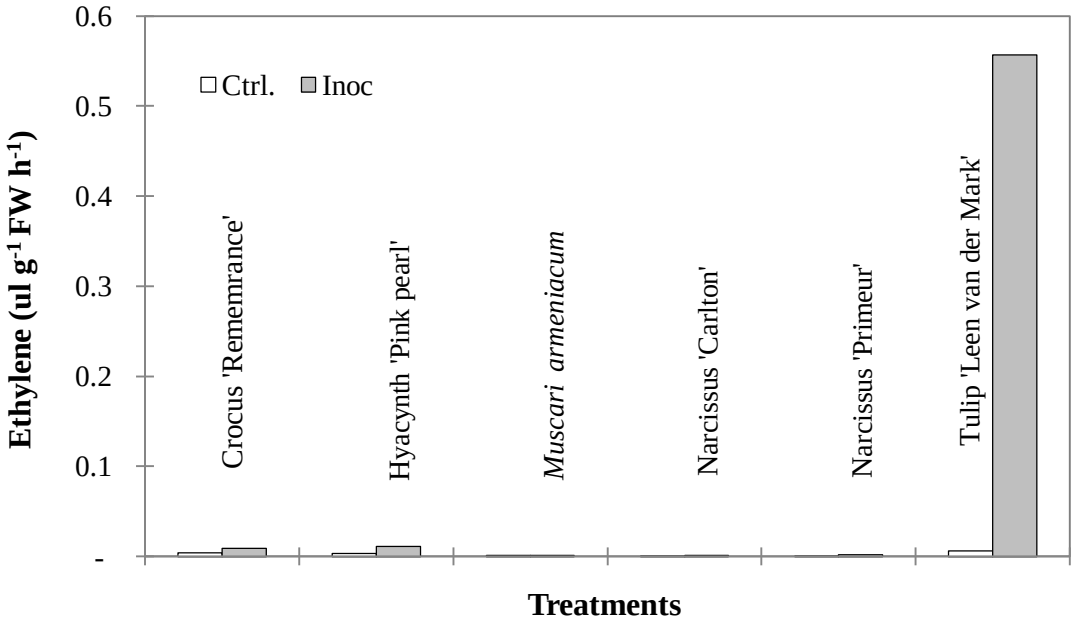


Figure 3. 5. Ethylene production by *Fusarium oxysporum* f. sp. *tulipae* strain Dy5 on six cultivars of five flower bulb genera. Bars are constructed with n=8 for inoculated treatments, and n=4 for control treatments. Data shown are results from two experiments.

DISCUSSION

Fusarium rot in tulip

Symptoms of *Fusarium* infected bulbs obtained from commercial sources presented the characteristic sour smell previously reported (Bergman 1965) and showed dry rotted tissue on the outside of the bulb with white-brown sporodochia. *Fusarium* infection in tulip bulbs is commonly known as basal rot because it is thought that the fungus primarily invades this part of the bulb, however, Bergman (1965) observed that *Fusarium* infection in field grown bulbs was more prevalent on the bulb scales than on the base plate. As seen in Illustration 3. 1, *Fusarium* infection in commercially available tulip bulbs may appear on the outside of the bulb as spreading from the scales, or from the base plate. It was frequently observed that the extent of the rot on the outside of the bulb does not reflect the extent of the fungus in the

internal bulb scales (Illustration 3. 1D and E), however, no data were collected to quantify this statement.

Tulip mites

Tulip mites were often observed on the outside of the bulb feeding on fungal colonies. The mites were not identified but may belong to *Rhizoglyphus echinopus* or *Tyrophagus putrescentiae* (De Munk 1972; Czajkowska and Conijn 1992). It is not known whether these mites can serve as *Fusarium* dispersing vectors by carrying conidia from infected to healthy bulbs, however, we observed an explosive increase in mite population in our experiments after 21 days. Future work should consider treating tulip bulbs against mites to reduce potential cross contamination.

Fusarium isolation and identification

Several steps were performed to correctly identify *Fusarium* strains colonizing infected bulbs (Summerell et al. 2003). When grown on PDA, one quarter of the forty isolated strains showed a white-cream color and the rest were purple. Colony pigmentation in *Fusarium* changes across growing conditions and fungal strains, and is not a reliable parameter for *Fusarium* identification (Booth 1971; Toussoun and Nelson 1976). Known F.o.t. strains used in our preliminary experiments showed purple pigmentation when grown on PDA, and a similar color was observed on strains used by Saniewska (2008). Further identification steps such as fungal morphological observations (Illustration 3. 4), and comparison of the TEF1 gene (Geiser et al. 2004) from isolates on a phylogenetic tree (Figure 3. 1) confirmed that the white-cream isolate was *F. solani*, and the purple isolates *F. oxysporum* f. sp. *tulipae*.

The forma specialis *tulipae* (strain Dy5) was further confirmed by analyzing ethylene production by the fungus in five flower bulb species. A distinctive feature of F.o.t. grown *in-vitro* is its ability to produce high amounts of ethylene compared to other *Fusarium* species

and formae specialis. Ethylene produced by F.o.t. on tulip bulbs was 50 to 700 higher than the other four inoculated species of flower bulbs (Figure 3. 5). While *F. solani* produced only traces of ethylene, the values detected on F.o.t. isolates were several times higher, and these findings are in agreement with Swart (1976) under *in-vitro* conditions.

Fusarium solani may appear in infected tissue of tulip bulbs as an opportunistic or saprophytic organism, as observed with *Penicillium* colonies (Illustration 3. 1 E2). *F. solani* has been associated in plant wounds with *Pythium*, *Phytophthora*, *Rhizoctonia*, and other Fusaria (Booth 1971). The role of this organism as a pathogen of tulip bulbs is beyond the scope of this study; we only inoculated this strain in tulip bulbs to rule out its contribution in the ethylene originating from *Fusarium* infected tissue. No visual fungal development or ethylene production of this isolate was found on the tissue of inoculated tulip bulbs (data not shown).

Identification of F.o.t. as the causal agent of *Fusarium* rot in tulip bulbs was confirmed by proving Koch's postulates. The criteria to fulfill the postulates indicate that the causal agent must: 1) be present in the diseased organism, 2) the organism must be isolated and grown in pure culture, 3) when inoculated in a susceptible host the suspected agent must induce the disease symptoms, 4) the causal agent must be re-isolated from the inoculated and infected host (Agrios 2005). All these criteria were tested and completed in this work.

The growing conditions (PDA) that were used to mass produce the purified *Fusarium* strains are not optimal. Summerell (2003) indicates that PDA can be used for morphological observations and colony pigmentation, but not as growing medium to obtain pure cultures for storage as stock. For such purposes carnation leaf agar (CLA) should be used instead. Therefore as work in this dissertation progressed protocols related to fungal growth and handling were improved.

Fusarium strains and inoculum density

Ethylene production by five *Fusarium* strains was assessed in tulip cultivars 'Friso' and 'Calgary' inoculated at three different inoculum concentrations. Results indicated that F.o.t. produced several times more ethylene when colonizing 'Friso' than 'Calgary'.

In 'Friso', isolate Ga2 produced the same amount of ethylene across the three inoculation densities, while isolate Dy5 showed highest ethylene production at $4.5x10^4$ and $4.5x10^5$ conidia $bulb^{-1}$. Generally, ethylene production among isolates was highest when inoculated at $4.5x10^5$ conidia $bulb^{-1}$, after 28 days of inoculation (Figure 3. 3).

Ethylene levels among *Fusarium* strains in 'Calgary' were several times lower than in 'Friso'. Isolate Dy5, was the highest producer but values recorded in 'Calgary' were up to 12 times lower than those in 'Friso'.

Overall, *F. oxysporum* f. sp. tulipae, strain Dy5 produced the highest level of ethylene in both cultivars, while *F. solani* (Dy1) produced traces of ethylene.

The data obtained from the *Fusarium* strains and inoculation density experiment was highly variable which is evident in Figure 3. 3 and Figure 3. 4. The data variability phenomenon has been previously described (Bergman 1975) and when incubated at high humidity the fungus grows vigorously (Saniewska et al. 2004). It is suspected that the relative humidity conditions of the experimental setup were not optimal since water in the incubation boxes had completely evaporated (and was not refilled) at 14 days. Experiments to optimize infection and reduce variability in ethylene production are discussed in Chapter 4.

CONCLUSION

Proper identification of *Fusarium* strains requires conducting a series of steps progressing from disease observations, fungal characteristics, and finally molecular confirmation. Morphological observations have been historically used to identify pathogenic *Fusarium* species, however, modern genetic marker technologies allow preliminary identification of *Fusarium* strains in a short period of time. In order to complete the pathogenic role of a given *Fusarium* strain, it is required to fulfill Koch's postulates.

Although the characteristics of a fungal colony should not be used as a sole indicator of a given *Fusarium* species, F.o.t. grown in PDA shows a particular purple pigmentation. Preliminary experiments ruled out the role of *F. solani* as a potential tulip pathogen and ethylene producer.

It was observed that *F. oxysporum* f. sp. *tulipae* is non-pathogenic and did not produce significant amounts of ethylene on bulb species (*Hyacinthus*, *Muscari*, *Narcissus*, and *Crocus*) other than tulip, confirming its formae specialis. Strains identified in this chapter, notably strain Dy5, were used in experiments described in the later chapters of this dissertation.

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CHAPTER FOUR: EFFECT OF INOCULATION METHODS AND INCUBATION CONDITIONS ON ETHYLENE EVOLUTION BY FUSARIUM OXYSPORUM SCHECHT. F.SP. TULIPAE APT. IN TULIP (TULIPA GESNERIANA L.) BULBS

ABSTRACT

We studied ethylene evolution and fresh weight loss in tulip bulbs inoculated with microconidia of *Fusarium oxysporum* Schecht f.sp. *tulipae* Apt. (F.o.t.) using a combination of inoculation and incubation treatments. Over the course of 33 days, ethylene production in inoculated bulbs had a sigmoidal curve with the following features: 1) a lag phase between 0 to 12 days post inoculation (DPI) 2) an exponential phase between 12 and 26 DPI, and 3) a deceleration phase between 26 and 33 DPI. Wounded but not inoculated bulbs produced 550 times less ethylene than inoculated bulbs.

Compared with other conditions, inoculated bulbs incubated in open jars within a 70-80% relative humidity (R.H.) atmosphere, had a dry-looking rot on the tissue, produced the highest ethylene values, and caused the largest change in fresh weight (25%). In contrast, when incubated in saturated R.H. (capped jar) the fungus initially protruded out of the tissue as a white downy mycelium, but after 19 days, this environment caused condensation and had deleterious consequences on fungal growth that eventually reduced ethylene production, and less change in fresh weight. No differences in fresh weight were observed between inoculated and control bulbs incubated in capped jars. We report for the first time the correlation between weight loss and ethylene production as an indicator of pathogenesis in the *Fusarium*-tulip system.

Priming the wounds with sterile distilled water prior to inoculation did not have any effect on amount or speed of ethylene produced. Regardless of the inoculation site (base plate or scales). It takes approximately 33 days for the fungus to show maximum ethylene

production levels. Incubation conditions with continuous air exchange free of condensation are necessary to provide conditions for healthy fungal development, continuous ethylene production, and to avoid artifactsThe procedures described in this chapter can be used to screen a large number of samples making individualized and repeated observations with minimum disturbance of the specimens.

INTRODUCTION

The tulip bulb industry can sustain large losses due to the direct and indirect effects of *Fusarium oxysporum* Schlecht f.sp. *tulipae* Apt. (F.o.t.). This fungus can cause extensive damage of some cultivars in the field. During the infection process, F.o.t. produces ethylene, to which tulip bulbs are remarkably susceptible. In storage, bulbs exposed to ethylene can immediately suffer detectable physiological disorders such as gummosis (when exposure occurs early after harvest), and increased respiration and changes in fresh weight throughout storage (Kamerbeek et al. 1971; De Munk et al. 1992; De Wild et al. 2002). However, ethylene also causes "hidden and permanent" injuries such as flower abortion, poor rooting, and shortened stem growth, which become visible during greenhouse forcing the following spring (Schenk and Bergman 1969; De Munk 1973).

The phenomena of ethylene production by *Fusarium* when infecting tulips has been well studied, and various authors have reported different inoculation procedures. Some of these methods include bulb contact with inoculated soil (van Eijk et al. 1978); inoculating with agar plugs colonized with *Fusarium* (Goodenough and Price 1973; Saniewska et al. 2004); or dipping wounded bulbs in a liquid suspension of fungal colonies grown in agar (Miller et al. 2005).

Previous reports state that after inoculation it is necessary to maintain high relative humidity to obtain visible infection symptoms (Gabor; Bergman 1975; Bergman and Bakker-van der Voort 1979). While successful colonization of the bulbs has been achieved with these

methods, to our knowledge, there are no reports on the feasibility of using conidia for inoculation, nor have environmental factors during colonization been investigated.

The objectives of this study were to: 1) explore the potential of using a standardized number of *Fusarium* conidia to inoculate tulip bulbs, 2) investigate how inoculation and incubation conditions affect ethylene production by F.o.t. and the change in fresh weight of tulip bulbs; 3) determine if fresh weight loss and ethylene production are related, with the hypothesis that fresh weight loss is a quantitative predictor of ethylene production in inoculated treatments.

MATERIALS AND METHODS

Plant material

Tulip bulbs (*Tulipa gesneriana* L.) 'Leen van der Mark' 11/12 cm in diameter grown in Chile were obtained from commercial sources in the USA and held at 17°C until use.

Treatments and experimental design

A completely randomized experiment was conducted with four factors: 1) wound priming before inoculation (water added or not), 2) inoculation site (base plate or scales), inoculation (inoculation or mock inoculated), and incubation jar cover (capped or open) for a total of sixteen treatments (Table 4. 1). One bulb was an experimental unit and each inoculation treatment had twelve replicates, while the control non-inoculated treatments had six replicates. To avoid a seasonal effect, individual experiments were started one week apart (June 5 and June 12) for a total of 288 bulbs in both experiments.

Inoculum preparation

Microconidia of strain Dy5 of *Fusarium oxysporum* f.sp. tulipae was grown and suspended in 0.1% Tween 20 at 3.3 x 10^6 conidia ml^{-1} as previously described (Chapter 3).

Table 4. 1. Treatments applied to tulip bulbs. Numbers in inoculation rows refer to the number of bulbs (replicates) in each treatment.

Priming	Wet priming				Dry priming			
Inoculation site	Scales		Base Plate		Scales		Base Plate	
Jar Capping	Open	Capped	Open	Capped	Open	Capped	Open	Capped
Non-inoculated	6	6	6	6	6	6	6	6
Inoculated	12	12	12	12	12	12	12	12

Bulb wounding

After removing the brown tunic, bulbs were surface decontaminated by 10 minute immersion in 10% (v/v) commercial bleach solution and 0.02% Tween 20, followed by two rinses in sterile deionized water. Bulbs were wounded with a brass core borer (8 mm diameter). For scale inoculation the wound was made half way down on the round side of the bulb on its vertical axis (Illustration 4. 1 B), or for base plate inoculation in the transition zone between the root collar and the scales (Illustration 4. 1 C). The wound was approximately 1.5 cm deep and went through all the layers of the bulb until reaching and wounding the flower bud (Illustration 4. 1 D).

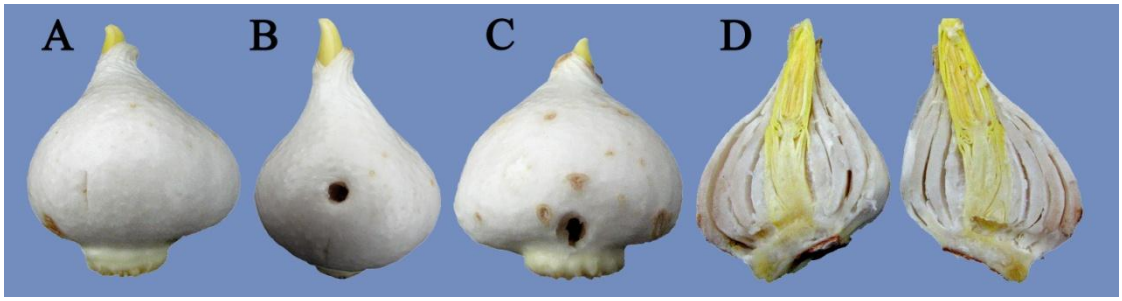

Illustration 4. 1. Tulip bulbs showing wounding sites. A) Intact bulb, B) Wounding on the scales, C) Wounding on the base plate, D) Longitudinal section showing the depth of the wound made on the base plate.

Wound priming

Wet priming of the wound consisted of adding 150 ul sterile distilled water to the wound 30 minutes prior to inoculation; for dry priming treatments, no water was added.

Inoculation and placement in jars

Bulbs were inoculated by depositing 150 ul of conidia suspension throughout the wound with a pipetter; sterile distilled water was used as mock (control) inoculation. One bulb was placed inside a one U.S. pint (nominal 473 ml), wide-mouth glass jar which was either open ("open" treatment), or capped with a piece of "Press and Seal®" plastic film (Glad-The

Clorox Company, Oakland, CA) -"capped treatment"-. One puncture (0.5 cm diameter) was made in the center of the plastic film using a one ml plastic pipette tip to allow aeration; each week two more openings were made on the plastic film until completing seven holes.

Incubation

Twelve jars were placed in a greenhouse pot-carrying tray (CTR415, Dillen Products, Middlefield, OH) with 4" diameter cells. Each tray was kept in a 32 quart capped plastic box (PN 1756, Sterilite Corp, Townsend MA) containing with 5 liters of deinonized water. The box lid had 24 holes (3/16" diameter) for aeration. Boxes were placed in darkness (except during manipulation) in a growth chamber at constant 21°C with continuous air exchange. Relative humidity inside the incubation box was recorded with HOBO® data logers (Onset Computer Corp., Bourne, MA) and fluctuated between 80 and 95%.

Ethylene analysis

Ethylene was measured 12, 19, 26, and 33 days post inoculation. The jar atmosphere was flushed for 30 seconds with humidity-saturated and filter-sterilized air (PTFE filter, 0.2 um pore, PN 4251, Acro®50, Pall Corporation, East Hills, NY) at 4.6 L min^{-1}. Jars were sealed for 30 minutes and 1ml headspace was collected with a hypodermic syringe.

Ethylene was detected and analyzed with a gas chromatograph (Model 310, SRI instruments, Torrance, CA) equipped with an alumina column (90 cm long, 80/100 mesh, 180°C oven temperature) and a FID detector (200°C) with hydrogen at 20 PSI, and helium at 15 PSI as carrier gas. The ethylene peak eluted in approximately 20 seconds.

Fresh weight change and ethylene production rate calculation

The weight of the incubation vessel (with or without the plastic film) was recorded (W_a), then the wounded bulb (before inoculation treatment) was placed inside the jar and the weight was recorded again (W_b). The 150 mg of the inoculation (or control) suspension was

not factored into the calculations since water readily evaporates a few hours after being added. The difference in weight (W_b-W_a) was the fresh weight of the bulb (W_1). The weight of the bulb and incubation vessel were subsequently recorded (W_2, W_3,…,W_n) on the same the day of the ethylene measurements, and the percent change in fresh weight was calculated accordingly (W_1-W_n/W_1)*100

The fresh weight of each bulb at day zero was used as a constant to calculate the ethylene production per gram (ul g^{-1} FW h^{-1}) at each sampling date with the following formula:

$$\text{ul ethylene } g^{-1}\text{ FW } h^{-1} = \left[\frac{\frac{S_{(C2H4)}}{W_1}}{1000} \right] \bullet V_1 \bullet T$$

Where:

- $S_{(C2H4)}$ is the ethylene reading of the gas sample (nl·ml^{-1} of sample) at any given time
- W_1 is the weight (grams) at day zero of the bulb in the container
- 1000 is the factor to convert nl·ml^{-1} to ul·ml^{-1}
- V_1 is the volume of the container in ml
- T is the time (in hours) of headspace accumulation

Statistical analysis

Data were analyzed with a mixed effects model using the statistical package JMP (Version 8.0, SAS institute, Inc. Cary, NC) to generate ANOVA tables. Pair wise comparisons were calculated using (where appropriate) Student's-T, or Tukey's HSD test at p = 0.05 level.

RESULTS

Bulb weight

The bulbs used in the experiment were randomly selected from a large bulb consignment. After wounding, the weight of each bulb was recorded. The median weight was 26.65 g, and the mean of the distribution was 27.74 g (±S. D. 5.9). The smallest bulb weighed 17 grams, while the largest weighed 47.4 g.

Ethylene production

Throughout the experiment, control bulbs did not show substantial ethylene increase compared to the inoculated treatments. By day 26 of the study, ethylene in control treatments was 560 times lower than inoculated bulbs (Figure 4. 2). Since the ethylene production between inoculated and control treatments was evidently dissimilar, comparisons between inoculated and control treatments were done only in the following "Inoculation" section. Ethylene data analysis in consecutive sections excludes non-inoculated treatments and only explores differences in ethylene production between treatments when inoculated with F.o.t.

Inoculation

Analysis of variance of ethylene showed a highly significant interaction (p=<0.0001) between inoculation treatments and days post inoculation –DPI- (Table 4. 2).

Table 4. 2 ANOVA of ethylene production in inoculated and control treatments.

Source	Nparm	DF	DFDen	F Ratio	Prob > F *
Inoc.	1	1	280.7	5601.38	<0.0001
DPI	4	4	1044	2373.11	<0.0001
Inoc.*DPI	4	4	1044	1273.87	<0.0001

[1]* Significant P-values (<0.05%) according to Tukey's Honestly Significant Difference test.

At day zero, two hours after bulbs were wounded and challenged either with water or F.o.t. no traces of ethylene were found (lowest detection limit 0.02 ul L^{-1}). Non-inoculated treatments showed a 3.5 fold linear increase in ethylene production (from 0.0006 to 0.0021 ul g^{-1} FW h^{-1}) between 12 and 33 DPI (Figure 4. 1). In contrast, ethylene evolution in *Fusarium*-inoculated bulbs was much higher (Figure 4. 2) and exhibited a sigmoid shape with three distinctive phases: 1) a lag phase between 0 to 12 DPI, 2) an exponential phase between 12 and 26 DPI, and 3) a deceleration phase occurring between 26 and 33 DPI when ethylene biosynthesis decreased and maximum values were observed.

Analysis of inoculated treatments

Analysis of variance (Table 4. 3) of ethylene production (including only data from inoculated treatments) detected three highly significant (p=<0.0001) two-way interactions indicated as follows: 1) wound priming x DPI, 2) Inoculation site x DPI, 3) Jar capping x DPI.

Table 4. 3 ANOVA of ethylene production of inoculated treatments only.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Wound priming	1	1	182.8	9.07	0.0030 *
Inoc. site	1	1	182.8	25.79	<0.0001*
Jar capping	1	1	182.9	1.50	0.2230
DPI	4	4	729.7	2,169.3	<0.0001*
Wound priming*DPI	4	4	729.6	3.45	0.0084*
Inoc. site*DPI	4	4	729.6	12.61	<0.0001*
Jar capping*DPI	4	4	729.7	19.68	<0.0001*

* Highly significant p-values according to Tukey's Honestly Significant Difference test.

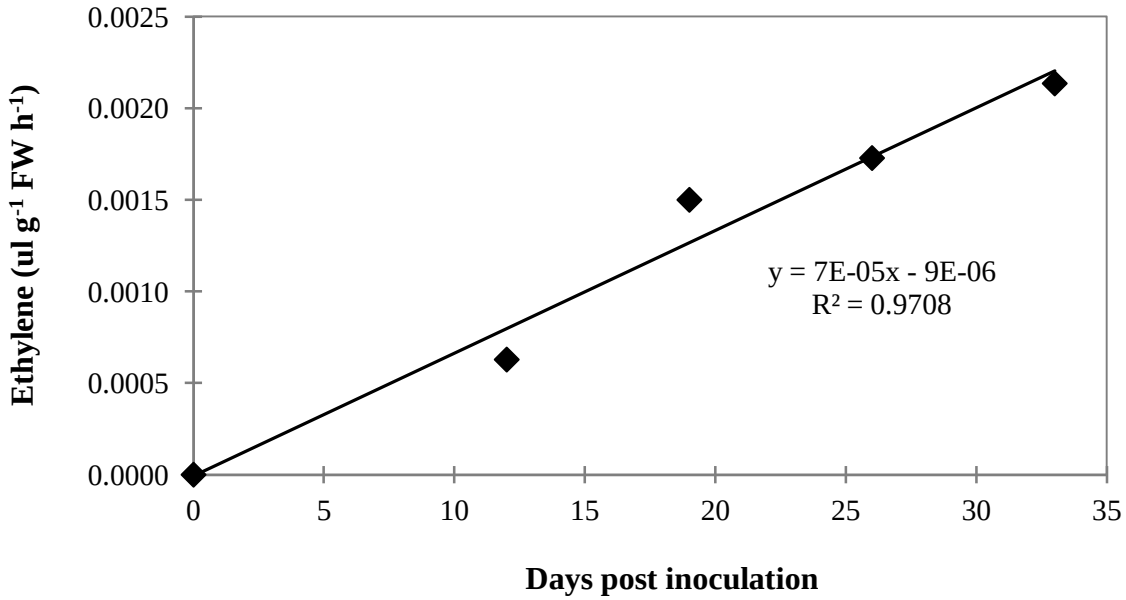

Figure 4. 1 Ethylene production in non-inoculated bulbs. Values merge all inoculation site, wound priming, and jar capping treatments with n=96 at each data point.

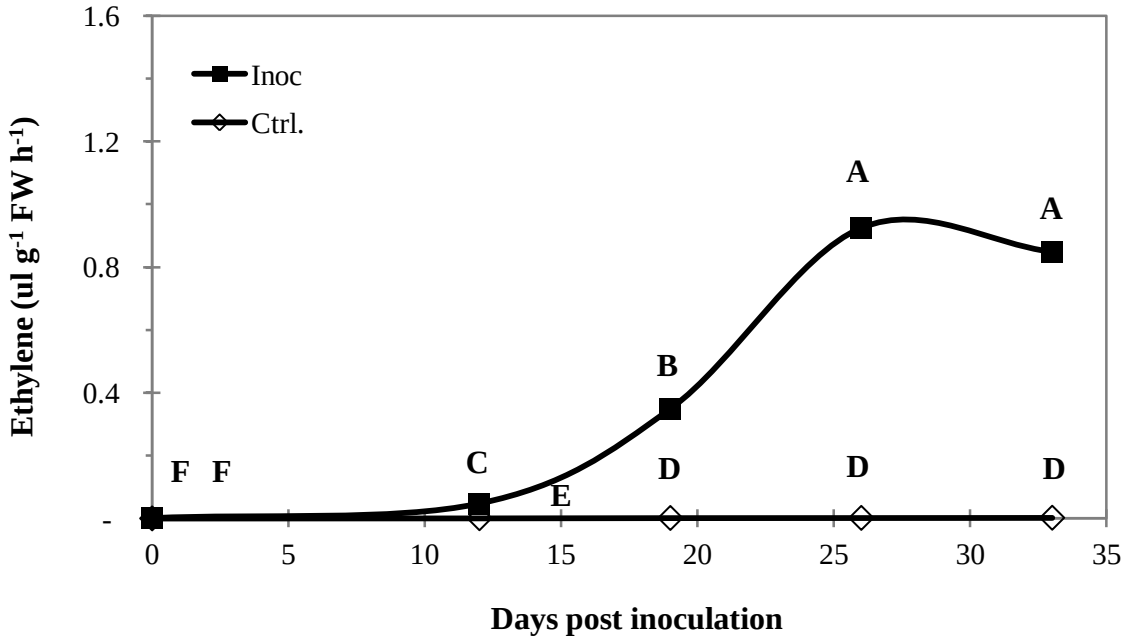

Figure 4. 2 Ethylene production patterns in control (non-inoculated) and inoculated bulbs. Values merge all inoculation site, wound priming, and jar capping treatments. Data points not connected by the same letter are significantly different according to Tukey's Honest Significance Difference test ($p<0.0001$)with n=192 for inoculated, and n=96 for control non-inoculated treatments.

Wound priming

Before inoculation, bulb wounds were made and were either wet or dry primed. Wet priming consisted of adding 150 ul water, while no water was added to dry primed wounds. Wound priming interacted only with DPI ($p=<0.0001$) to affect ethylene production. As observed in Table 4. 3, significant differences between treatments were detected at 12 DPI when ethylene production in dry primed bulbs was 1.5 times higher than the wet primed treatments (0.04 vs. 0.05 ul g^{-1} FW h^{-1}). At 19 DPI, ethylene production of dry primed bulbs was 40% higher than wet primed bulbs (0.49 and 0.28 ul g^{-1} FW h^{-1}). Ethylene production slowed down in both treatments between 26 and 33 DPI. While no significant differences were found between wet and dry primed treatments at 26 and 33 DPI, dry primed bulbs tended to produce approximately 30% more ethylene than the wet-primed bulbs.

Inoculation site

Between 12 and 19 days ethylene production was significantly higher in bulbs inoculated in the scales. While bulbs inoculated in the base plate had 35% less ethylene production at day 33, this difference was not statistically significant (Figure 4. 4). A pattern similar to the priming results was observed in the inoculation site treatments where a higher trend was noticeable in scale-inoculated bulbs than those challenged in the base plate.

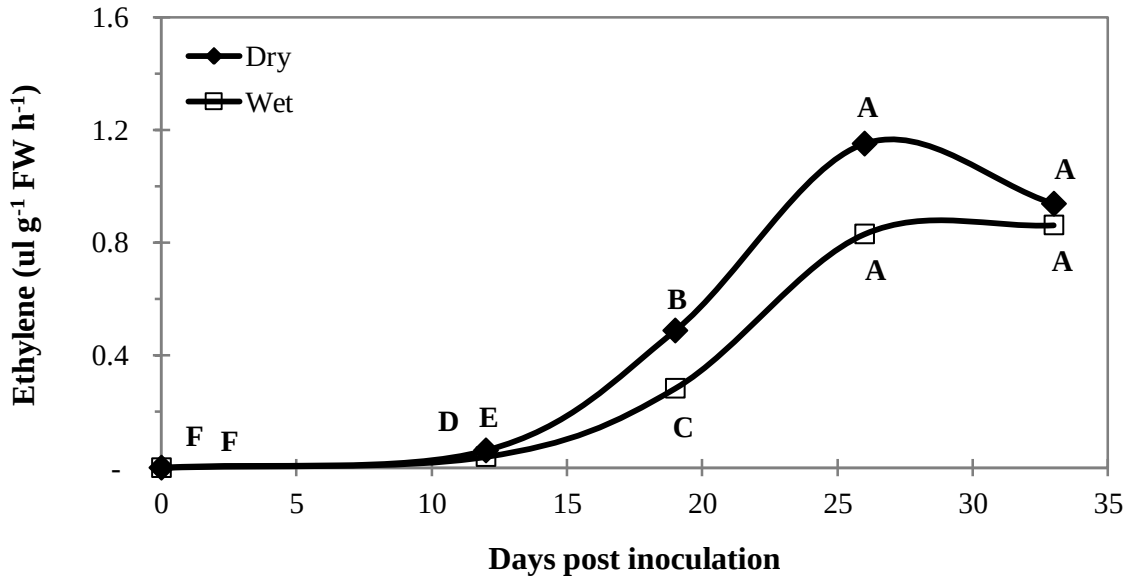

Figure 4. 3. Effect of wound priming before inoculation on ethylene production in bulbs infected with F.o.t. Values merge all inoculation site, and jar capping treatments. Data points not connected by the same letter are significantly different according to Tukey's Honest Significance Difference test (p=0.0084) and n=96 for each priming treatment.

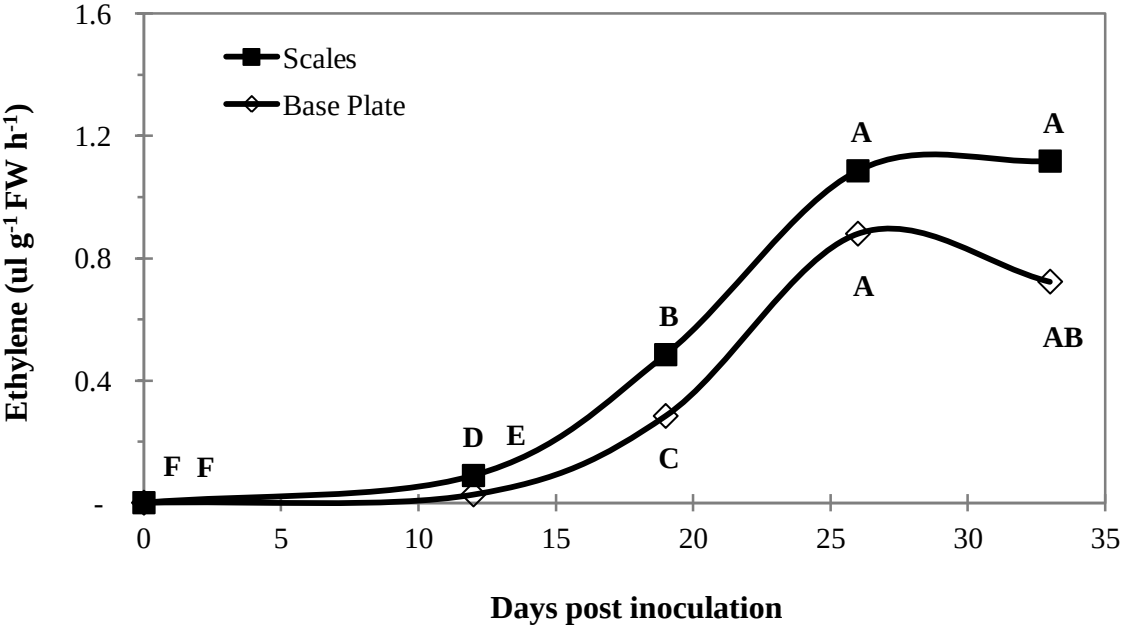

Figure 4. 4. Influence of inoculation site on ethylene production by F.o.t. Values merge all wound priming, and jar capping treatments. Data points not connected by the same letter are significantly different according to Tukey's Honest Significance Difference test (p<0.0001) and n=96 for each treatment.

Jar capping

Post inoculation conditions during incubation (bulbs held in open or capped jars) significantly influenced the amount of ethylene synthesized by F.o.t. (Figure 4. 5). Ethylene production by infected bulbs incubated in both open and capped jars increased linearly between 12 and 26 DPI whereas bulbs held in open jars showed an increasing rate of ethylene production. Between days 12 and 26, capped bulbs showed a 104% daily increment rate (from 0.056 to 0.87 ul g^{-1} FW h^{-1} respectively), while in open jars this value was 175% (from 0.043 to 1.1 ul g^{-1} FW h^{-1}).

In terms of maximal ethylene production, bulbs incubated in open jars produced 70% more ethylene (1.48 ul g^{-1} FW h^{-1} at 33 DPI) than those kept capped (0.87 ul g^{-1} FW h^{-1} at 26 DPI).

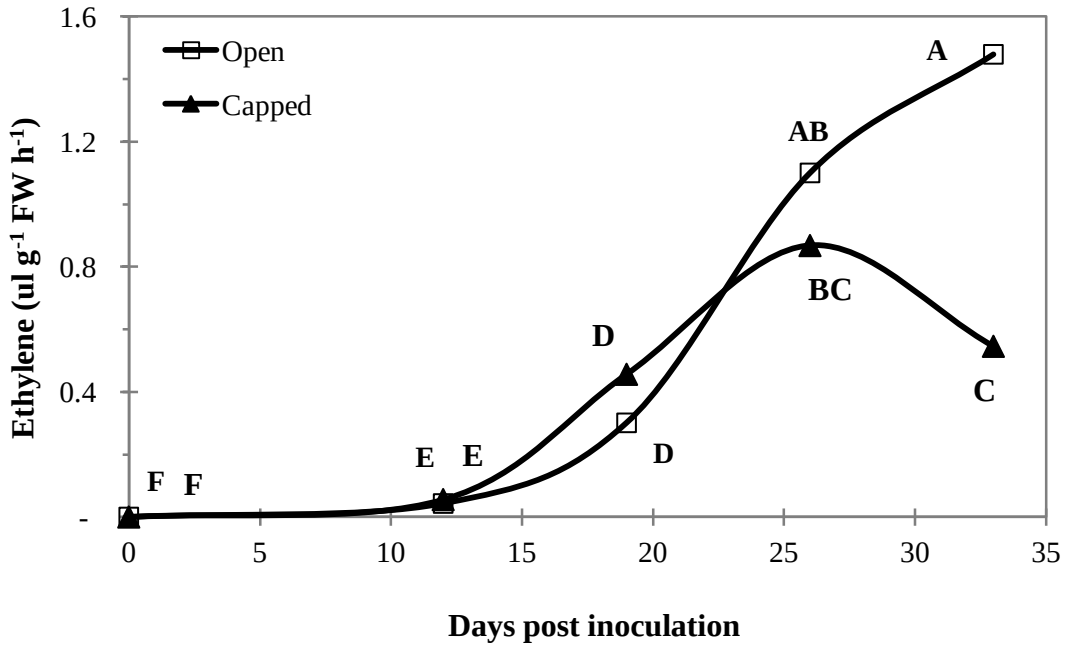

Figure 4. 5. Ethylene production in inoculated bulbs incubated in open or capped jars. Values merge all wound priming, and inoculation site treatments. Data points not connected by the same letter are significantly different according to Tukey's Honest Significance Difference test ($p<0.0001$) and n=96 for each treatment.

Effect of initial bulb weight on ethylene evolution

Since only bulbs incubated in open jars had an increasing trend in ethylene production after 26 days, data from capped treatments was excluded to further investigate the effect of bulb weight on ethylene production on a per gram, and whole bulb basis. Bulbs weights were separated in three categories: 1) small bulbs with < 26g, 2) medium bulbs weighing between 26-32 g, and 3) large bulbs weighing > 32 g.

When ethylene production was expressed on a per gram basis (ul g^{-1} FW h^{-1}) no differences were observed between bulb size groups (Figure 4. 6). However, the rate of increase in ethylene production was less with larger bulbs. A general trend in ethylene production was observed by 33 DPI: bulbs weighing less than 26 g leveled off while heavier bulbs showed rising ethylene production.

On a per bulb basis, no differences were detected in ethylene production in any of the three groups (Figure 4. 7). Similar to the observed ethylene production on a per gram basis, the < 26 g group showed maximal ethylene production by 26 DPI (32 ul $bulb^{-1}$), while at 33 DPI medium and large bulbs continued to increase ethylene production.

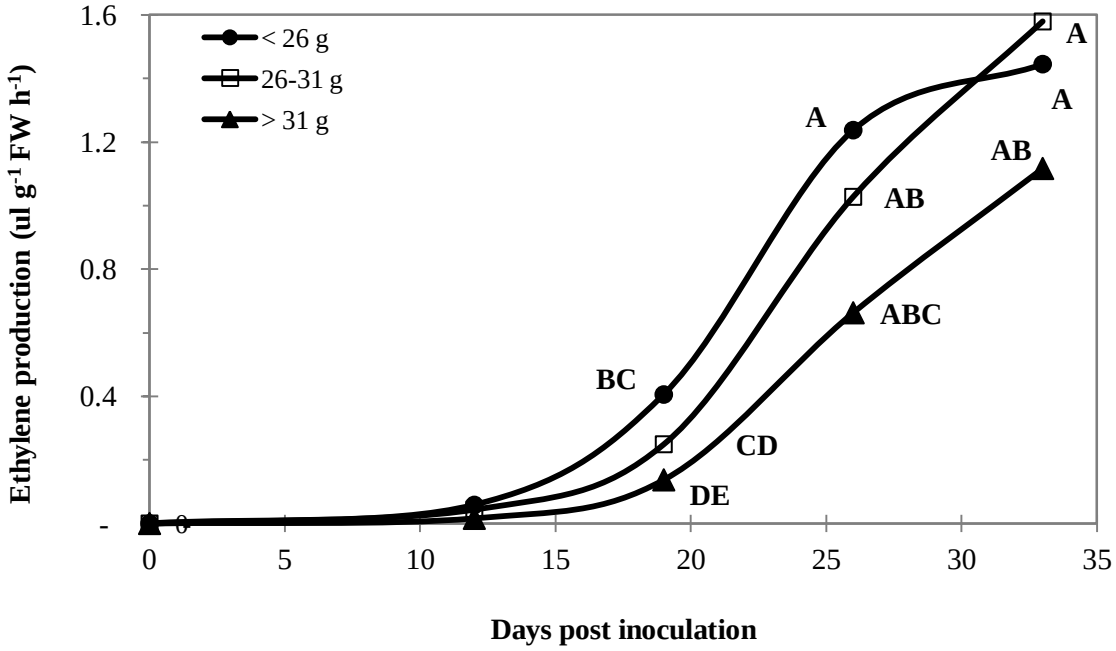

Figure 4. 6. Influence of initial tulip bulb weight on ethylene evolution by F.o.t. in bulbs incubated in open jars. Values (n=96) merge all wound priming, and inoculation site treatments.

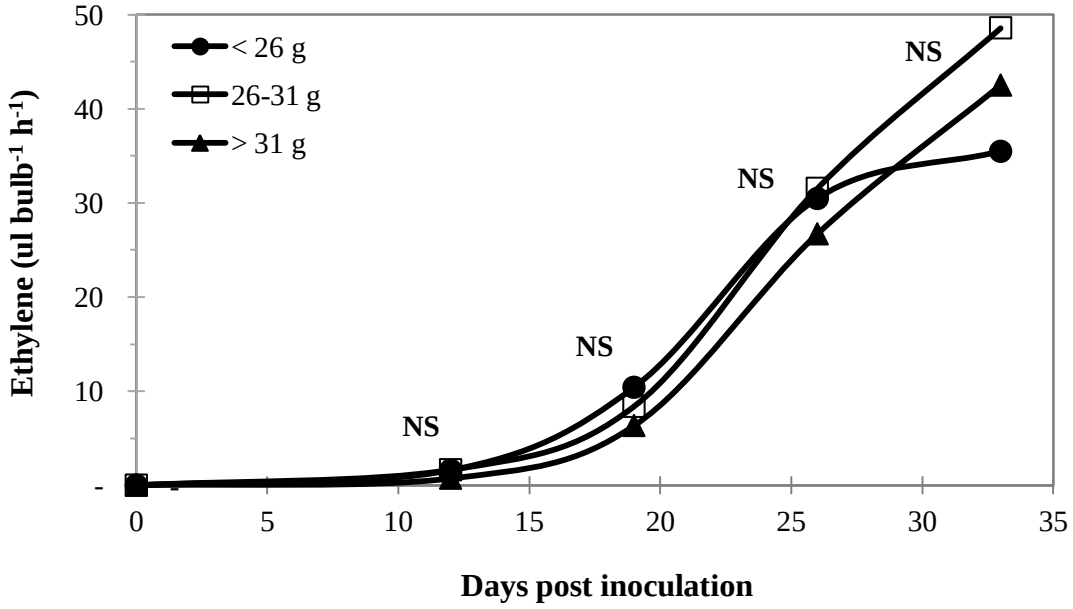

Figure 4. 7. Ethylene production by per bulb of different weight groups. n=96, NS indicates no significant differences at p=0.05 according to Tukey's HSD test.

Fresh weight loss

The percentage of fresh weight loss was significantly influenced by inoculation and incubation treatments. During the course of the experiment, fresh weight loss was almost linear in most treatments (Figure 4. 8) but in the inoculated and open treatments the weight loss trend was exponential. Among treatments the highest differences in fresh weight loss occurred at 33 DPI. At the end of the experiment the non-inoculated and capped treatment had the least fresh weight loss (5%), while inoculated bulbs held in open jars had a 26% decrease in fresh weight.

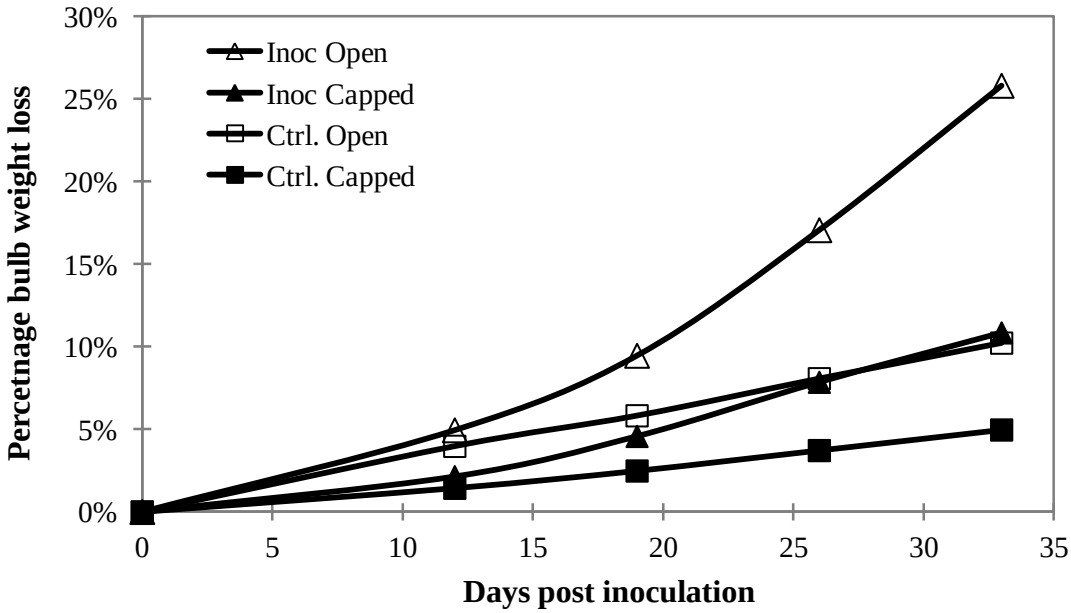

Figure 4. 8. Change in fresh weight due to inoculation, and capping during incubation.

Influence of initial bulb weight on fresh weight loss

The percentage of fresh weight loss in inoculated bulbs held in open jars was dependent on the initial weight of the bulb (Figure 4. 9). The percentage of fresh weight loss did not interact with days, however, it was observed that medium and large bulbs (> 26 g) lost less weight during the course of the experiment than small bulbs (< 26 g).

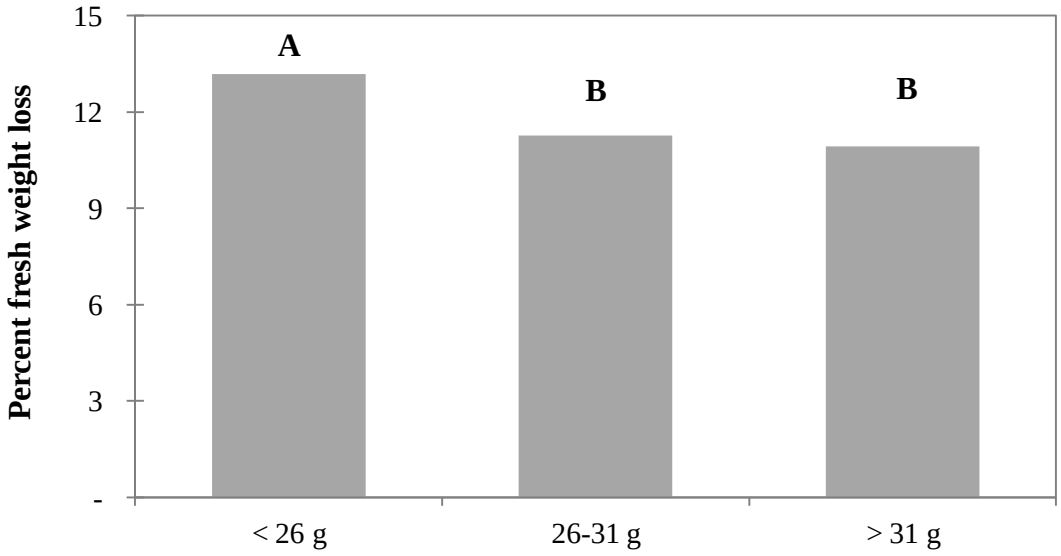

Figure 4. 9. Percentage of fresh weight loss between bulbs of various weights infected with F.o.t. and incubated in open jars (Bars represent average of FW loss recorded during the experiment).

Ethylene and fresh weight loss

Ethylene production by bulbs incubated in open jars was positively correlated with change in fresh weight (Figure 4. 10). Ethylene regression values of inoculated treatments versus percentage fresh weight loss (r^2=0.67) showed an exponential increase in ethylene up to 25% FW loss. In control treatments ethylene values were negligible relative to FW loss.

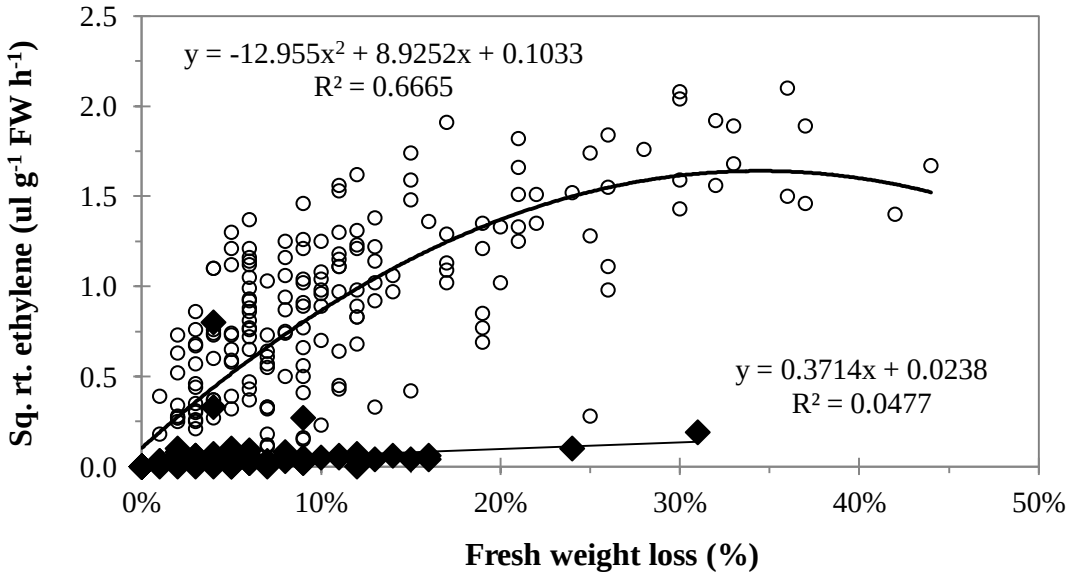

Figure 4. 10. Ethylene production as a function of change in fresh weight in inoculated or control treatments incubated in open jars.

Fungal development

Five days after inoculation, F.o.t. protruded from the wounds as a white and light pink downy mycelium. No changes were observed in wounds of control bulbs. During the first 19 days visible downy mycelium growth was more vigorous in capped jars, while colonization of the fungus in open jars started as a sunken ivory color rot extending from the wound. As rot increased in size mycelium grew on the tissue, however, the appearance of the mycelium was not as dense in open jars as in capped jars.

On day 19 the inoculated and capped treatments started showing condensation on the jar walls, the healthy parts of the bulb, and sometimes covering the fungal mycelium. On day 26, bulbs on which condensation had formed, started turning reddish, and a foul smell was perceived. Finally by day 33, the fungal mycelium looked brown, water-soaked, and a strong foul smell was prevalent in the majority of capped and inoculated treatments. Control-capped bulbs often showed *Penicillium* contamination, mainly around the wounds and spots with dead tissue (i.e. mechanically damaged).

DISCUSSION

Use of conidia as material for inoculation and bulb weight

Past researchers have utilized various inoculum sources and inoculation methods such as mycelium plugs, adding inoculum to the soil, or dipping bulbs in liquid fungal suspensions (van Eijk et al. 1978; Saniewska et al. 2004; Miller et al. 2005). Here we present a method to challenge simultaneously all the organs of a tulip bulb using F.o.t. conidia. This experimental setup allows challenging tulip bulbs using a known amount of conidia, inducing fungal colonization of healthy tissue, and permits repeated data collection on the same subject (i.e. fresh weight change and ethylene evolution) with minimal manipulation and disturbance of the fungus and bulb.

In this study, the amount of inoculated conidia was the same for all bulbs, regardless of their initial weight. The distribution of the bulb weights observed in the experiment was skewed to the right with weights ranging from 17 to 47.4 grams. Although ethylene production (expressed on either FW or per bulb basis) was similar across bulbs of different weights (Figure 4. 6 and 4.7) choosing bulbs within a defined weight range would reduce the variability of ethylene production. The time required to complete this experiment was 33 days, however, we speculate that shorter experiments could be conducted if two or more sites were inoculated simultaneously (for example, in the scales and base plate).

Ethylene evolution

Ethylene evolution in F.o.t. inoculated bulbs was more than two orders of magnitude greater than non inoculated bulbs, which is in agreement with previous articles reporting that ethylene production levels by F.o.t. is several fold higher compared with other *Fusarium* strains, or ethylene from bulb metabolism (De Munk 1972; Swart and Kamerbeek 1976, 1977; Hottiger and Boller 1991; Kanneworff and Van der Plas 1994; Miller et al. 2005).

Miller (2005) measured ethylene in control and *Fusarium*-inoculated tulip tissue that had been previously heat killed (thus removing any metabolic activity). Ethylene produced by F.o.t. in heat-killed tissue was several times higher than the non-inoculated tissue (alive or heat-killed), providing evidence that ethylene production in tulip bulbs infected by *Fusarium* proceeds from fungal metabolic activity.

The pattern of ethylene evolution during this time course study is comparable to other reports of F.o.t. and *Botrytis cinerea* grown *in-vitro* (Swart and Kamerbeek 1977; Fukuda et al. 1986; Chagué et al. 2002; Cristescu et al. 2002), and in *in-vivo* (Chagué et al. 2006). Infected tulip bulbs had a lag phase of ethylene production of 12 days, an exponential phase from 12 to 26 DPI, and finally a deceleration phase between days 26 and 33.

In-vitro studies of F.o.t. (Swart and Kamerbeek 1977) show that during the lag phase low ethylene values were observed, fungal biomass increased exponentially, and at the same time the presence of oxygen was not required. It is thought that as active growth occurs the fungus may "accumulate" precursors for ethylene biosynthesis.

In-vitro, the exponential phase of ethylene production starts after active fungal growth stops, and during this period the fungus requires oxygen to sustain ethylene biosynthesis. In aerated liquid cultures, F.o.t. shows a narrow peak of ethylene production 6-8 days after inoculation (Swart and Kamerbeek 1977). In contrast, ethylene production by *Fusarium* infecting live tulip bulbs incubated open jars is much slower than the *in-vitro* system, showing an exponential phase up to 33 DPI (Figure 4. 5). In capped jar treatments ethylene production showed a deceleration trend after 26 DPI, which is a typical response of microorganisms due to lower nutrient availability (Chagué et al. 2002) and the accumulation of by products. Decreasing ethylene emissions were observed in tomatoes infected with *B. cinerea* as disease symptoms reached their peak (Cristescu et al. 2002). Unlike F.o.t., ethylene production in *Botrytis cinerea* coincides with hyphal growth, while the amount produced by the fungus is related to the amount of inoculum (Cristescu et al. 2002). Although no respiration data were recorded, the deceleration phase might have been a response of nutrient depletion in colonized

tissues and senescing fungus, but not a direct consequence of low availability of atmospheric oxygen in the system.

At the end of the experiment, ethylene production values in non-inoculated bulbs were 560 times lower than in inoculated bulbs. In a previous report ethylene emissions were not detected in healthy and intact bulbs of cv. 'White Sail' (De Munk 1972). Wound ethylene in non-inoculated tulip bulbs remained close to zero during the first 24 hours after injury, it became more visible after 48 hours, leveled off between 3 to 4 days, and the amount produced (1.5-3.0 nl g^{-1} FW h^{-1}) was cultivar dependent (Kawa et al. 1993). The values that we observed in wounded non-inoculated bulbs were similar to Kawa *et al.* (1993) and reached 2 nl g^{-1} FW h^{-1} at 33 days (Figure 4. 1).

In healthy tulip bulbs, metabolic ethylene increases during storage. For example, with 'Apeldoorn' no ethylene was recorded in September, but there was a gradual rise over time reaching 30 nl g^{-1} FW h^{-1} in March during 17 °C storage (Kanneworff and Van der Plas 1994). In the same experiment, 24 hours after detachment, healthy tulip anthers produced 20 times more ethylene (0.014 nl g^{-1} anther FW h^{-1}) than at four hours after abscission, showing that injured anthers may contribute up to 30% of the total wound ethylene in 'Apeldoorn' tulips. Bulbs used in our experiment were held at 17C for several months after harvest and wounds were made across all the organs of the bulb. Dissection of control (wounded, non-inoculated) bulbs at the end of the experiment showed that the entire flower bud had decayed, and there was presence of *Penicillium sp.* growth on the wounds (data not presented).

Ethylene production observed in the non-inoculated bulbs could be due to the combination of metabolic activity (bulb aging), wounding which may have induced the anthers to progressively synthesize increasing amount of ethylene, and microbiological contamination (i. e. bacteria, *Penicillium spp*.). The data recorded probably indicates "normal" levels of ethylene production in stored tulip bulbs.

Priming

Although treatments were not statistically different, throughout the experiment wet-primed bulbs showed a lower trend in ethylene production than the dry-primed treatments. In any case, the addition of water to wounds is not required for successful colonization of the fungus in the wounded tissue.

Inoculation site

Even though F.o.t. causes a characteristic basal rot, similar ethylene production was seen from inoculations to either scales or base plate (Figure 4. 4). At 26 days, scale inoculation caused 20% higher ethylene production than base plate inoculation and by 33 DPI it was almost 50% higher, but the differences were not statistically different.

Incubation conditions

The most dramatic effect on ethylene production was observed between incubation conditions (open or capped jars) in which the bulbs were maintained during the experiment.

By the end of the experiment, bulbs held in open jars (Figure 4. 5) produced twice as much ethylene as bulbs incubated in capped jars. The lower and premature decline in ethylene yield of capped treatments coincided with observed condensation inside the incubation jars and on the fungal mycelium. Compared to bulbs kept in open jars (but still in humid chambers), excess humidity in capped jars caused early death of the fungus which was evident by the mycelium turning brown, having a water-soaked appearance, and emitting a putrid smell. Slight temperature decrease near the dew point causes water to condense; this phenomenon became increasingly frequent three weeks after inoculation, and was concurrent with the exponential phase of ethylene production. As noted before, ethylene production in F.o.t. is an oxygen-dependent process that increases as fungal growth slows and the combination of these factors is a sign of fungal senescence. In *P. digitatum* the senescence stage is characterized by higher respiration than during active growth (Spalding and

Lieberman 1965). Condensation in capped treatments could have been a result of saturated atmospheres due to high respiration rates of the fungus, the uncontrolled water loss by the infected bulb tissue, or small fluctuations in the growth chamber temperature.

A decline in ethylene production in *P. digitatum* was observed when fungal mats grown in stationary liquid conditions were accidentally submerged (Spalding and Lieberman 1965), while in F.o.t. grown *in-vitro,* ethylene production dropped rapidly as shaking was suppressed, then resumed when shaking was restarted (Swart and Kamerbeek 1977). These reports suggest that oxygen dissolved in the liquid medium is rapidly consumed by the actively ethylene-producing fungus and that the rate of oxygen diffusion through water without agitation is not sufficient to sustain continuous ethylene production. We may speculate that as condensation formed inside the incubation vessel, on the surface of the mycelium, and possibly between the tulip layers, the oxygen available to the fungus was reduced, ethylene production dropped, and a combination of these factors may have eventually lead to death of the *Fusarium*.

Three weeks after inoculation bulbs incubated in open jars showed *Fusarium* growth on the surface of the tulip tissue, but unlike the mycelium observed in capped treatments, the mycelium was visually less dense and vigorous ethylene biosynthesis was sustained.

The degree of infection of F.o.t. on tulip bulbs has been assessed comparing fresh weight change between non-inoculated and inoculated bulbs (Goodenough and Price 1973). The largest change in fresh weight (Figure 4. 8) was observed in bulbs incubated in open jars regardless of the inoculation treatment. Bulbs held in capped jars lost one-half (non-inoculated) to one and a half times (inoculated) less weight than their corresponding open treatments. Lower values in fresh weight change of capped jars are mainly due to water condensation in the jars, which may result from water loss of the bulb tissue, high fungal respiration, or a combination of both concluding in lower ethylene values in the same inoculated treatment (Figure 4. 5).

In inoculated bulbs the correlation of ethylene production by fresh weight loss (Figure 4. 10) was positive (r^2 0.67) while no relationship was observed in control bulbs (r^2=0.05). The plot utilizes percent fresh weigh loss as the independent variable because previous authors (Goodenough and Price 1973) measured pathogenicity as a function of fresh weight loss. These observations confirm that *Fusarium* infection is correlated with both changes in fresh weight and ethylene production. Given that all treatments were randomly assigned to each box, we suspect that control bulbs were inherently exposed to ethylene from inoculated bulbs (no data was collected from the box atmosphere), which in combination with wound stress may have contributed to higher respiration rates and fresh weight loss (Kanneworff and Van der Plas 1994; De Wild et al. 2002) than if intact bulbs and non-ethylene exposed bulbs were used.

This is the first report correlating change in fresh weight and ethylene production in *Fusarium* infected bulbs, however, further studies testing several tulip cultivars are needed to completely prove and establish this finding.

Our findings demonstrate that it is essential to consider incubation conditions when reporting changes in fresh weight and ethylene evolution in the tulip-*Fusarium* system. Jarecka and Saniewska (2008) incubated *Fusarium*-infected bulbs in tightly sealed jars for 8, 16, and 28 days and measured ethylene evolution at each time point. Ethylene evolution in their work showed maximal values at 16 DPI and decreasing production at 28 DPI; the shape of the ethylene time course resembles the results of our capped treatments, and probably indicates that they did not obtain maximal potential ethylene production values.

CONCLUSIONS

It has been shown that tulip bulb inoculation with F.o.t. microconidia produces successful and consistent ethylene production. Adding water to the wound prior to inoculation has no effect on the amount or speed of ethylene produced. Regardless of the inoculation site (base plate or scales) it takes approximately 33 days for the fungus to show maximum ethylene production levels. Incubation conditions with continuous air exchange free of condensation are necessary to provide conditions for healthy fungal development, continuous ethylene production, and to avoid artifacts.

Change in fresh weight in inoculated tulip bulbs can be used as a predictor of ethylene production as long as there is continuous air supply, and no condensation is present in the incubation setup.

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CHAPTER FIVE: SCREENING FOR RESISTANCE AND ETHYLENE PRODUCTION BY FUSARIUM OXYSPORUM F.SP. TULIPAE IN TULIP BULBS OF 18 CULTIVAR SPORTS, 20 CULTIVARS, AND TWO SPECIES

SUMMARY

Tulip cultivars showed a wide range of patterns and maximal values of ethylene production when inoculated with *Fusarium oxysporum* f. sp. *tulipae*. No significant differences in ethylene production were detected between members of mutant (sport) lineages. There were, however, significant differences between lineages. Cluster analysis was used to group the 40 cultivars and 2 species into five categories based on the amount of ethylene produced upon infection. *Fusarium* produced 46 times more ethylene in *T. turkestanica* (the highest recorded value, 2.75 ul g^{-1} FW h^{-1}) than in 'Bright Parrot' (the lowest at 0.06 ul g^{-1} FW h^{-1}).

Ratings of visual external *Fusarium* infection on the bulb had low correlation with ethylene production ($r^2 = 0.32$). A Disease Severity Index (DSI) was developed to determine the degree of resistance to *Fusarium* under laboratory conditions. DSI results were useful to rank 20 cultivars and two species into four groups ranging from resistant to susceptible. Results from this assay can be obtained in 28 days.

Fusarium infection and defense responses of susceptible and resistant cultivars were recorded in two time lapse movies. While the fungus grew extensively on 'Ad Rem', the infection was contained five days post inoculation around the site of infection in 'Strong Gold' indicating a possible hypersensitive response.

INTRODUCTION

Fusarium oxysporum f. sp. *tulipae* (F.o.t.) infects tulip bulbs mainly in the soil before harvest (Bergman 1965), however increasing mechanization and standard handling practices at and after harvest (wetting, peeling, and sorting of the bulbs) can aggravate infection (Miller 2009). Latent *Fusarium* infections can lead to fusariosis during storage resulting in high amounts of ethylene, which may induce detrimental physiological disorders (Kamerbeek and De Munk 1976; Bergman and Bakker-van der Voort 1979; De Hertogh et al. 1980).

Many plants are known for "sporting", when an individual member of a clone spontaneously mutates to yield a different phenotype (e.g. flower color, flower shape, or other horticultural attribute). Sporting is important in tulips as the sports allow a greater color range within a given cultivar family. It is generally accepted that tulip sports have identical attributes as the parents (i.e.plant stature, leaf color, forcing characteristics, etc.). It is unknown whether sports would behave similarly following F.o.t. inoculation. The hypothesis is that members of a sport family support simmilar levels of ethylene production after F.o.t. infection.

Previous studies have shown the potential of F.o.t. to produce ethylene upon infection in tulip bulbs (Kamerbeek 1975; Miller et al. 2005) and methods to determine their resistance to F.o.t. (van Eijk et al. 1979), however, the correlation between ethylene production and cultivar resistance has not been studied in detail.

A quick and repeatable method to determine ethylene production rates and resistance to the fungus in a short period of time would be a useful tool for growers, exporters and breeders to make decisions on selecting cultivars for planting, designing appropriate ventilation rates during storage and transport, to define parent lines for breeding and screen progeny to select for low ethylene producing cultivars.

The objectives of this research were 1) to determine the pattern of ethylene production by F.o.t. in vegetative sports of six independent tulip lineages, 2) to determine ethylene

production by F.o.t. in 40 tulip cultivars and two tulip species, 3) to develop a model to quantify resistance of tulip bulbs to infection by F.o.t.

MATERIALS AND METHODS

Plant material

Bulbs of forty-two tulip (*Tulipa gesneriana* L.) cultivars (mostly size 12+ cm) were obtained from commercial sources in the Netherlands. Bulbs were harvested in July 2008 and held at 17°C until November. Two experiments were conducted in the following fashion.

Experiment one- Tulip lineages [1]

Eighteen tulip cultivars from six known lineages with sports (vegetative mutants) spanning up to four mutant generations were selected (Table 5. 1) and inoculated with F.o.t. to record ethylene evolution over 28 days, and fungal infection at the end of the experiment.

Experiment two- Twenty two assorted tulip cultivars

Twenty two economically important tulip cultivars (Table 5. 2) were selected and tested similarly as in experiment one.

Experimental design

Each of the two experiments were performed as a randomized incomplete block design. Each cultivar consisted of eight replicates with one bulb as an experimental unit. Experiment one consisted of 144 bulbs, while 192 bulbs were used for experiment two. Bulbs were inoculated on November 3, 2009 and a repeat of each experiment was inoculated one day later to minimize seasonal effect. Thus, a total of 672 bulbs were used.

[1] The terms lineage, family, and group are used interchangeably throughout this document.

No controls (non-inoculated) bulbs were tested in this experiment since it is known from previous experiments (Chapter 4) that metabolic ethylene from wounded and non-infected bulbs is essentially zero.

Table 5. 1. Tulip cultivar lineages[+] used in experiment one.

Mother cultivar	Daughter cultivars	Granddaughter cultivars	Great granddaughter cultivar
Wirosa (WS)	Top Lips (TL) Pink Star (PS)		
Couleur Cardinal (CC)	Rococo (RC) Prinses Irene (PI)	Prinses Margriet (PM) Hermitage (HR)	
Apeldoorn (AP)*	Golden Apeldoorn (GA)	- - - -	Banjaluka (BJ)
Yellow Present (YP)	Red Present (RP)		
Ad Rem (AR)	Ad Rem's Beauty (AB)		
Leen van der Mark (LV)	Dow Jones (DJ) Markant (MK)		

[+] The lineages of these cultivars were confirmed by consulting the database http://www.kavb.nl/ of the Koninklijke Algemeene Vereeniging voor Bloembollencultuur (The Royal General Bulb Growers' Association), then selecting "Geregistreerde cultivars".
* The Apeldoorn series allowed examination of three members with up to four generational descendants (mother, daughter and great granddaughter) without the granddaughter.

Table 5. 2. Tulip cultivars used in experiment two.

1. Bright Parrot (BP)	2. Strong Gold (SG)	3. Parade (PD)	4. *T. tarda* (TT)
5. Negrita (NG)	6. Judith Leyster (JL)	7. Flaming Parrot (FP)	8. Cummins (CM)
9. Pink Impression (PK)	10. Spryng (SY)	11. Calgary (CY)	12. World's Favourite (WF)
13. Ile de France (IF)	14. Blue Ribbon (BR)	15. Yellow Flight (YF)	16. Passionale (PL)
17. Purple Flag (PF)	18. Yokohama (YK)	19. Kikomachi (KK)	20. Christmas Dream (CD)
21. Mondial (MD)	22. Oscar (OR)	23. *T. turkestanica* (TK)	24. Friso (FS)

Inoculation

A conidial suspension was prepared as in Chapter 4, with the following modification. The conidial suspension was counted and thoroughly mixed with sterile pre-hydrated (1:262.5 w/v) 80 mesh sodium polyacrylate (Waterlock® B204, Grain Processing Corporation, Muscatine, IA.) which formed a slurry with a final concentration of $3.3X10^6$ conidia ml^{-1}. After removing the tunic, surface sterilizing, and wounding the bulbs (Chapter 4), 150 ul of the conidia suspension slurry was evenly deposited throughout the wound with a pipetter. One bulb was placed inside a 1 pint wide-mouth mason glass jar which had a small kimwipe® (Kimberly Clark, Dallas, TX) tissue moistened with 2 ml sterile distilled water. Jars were sealed with a piece of Press and Seal® plastic film (Glad-The Clorox Company, Oakland, CA). Three holes were made in the plastic film using a 1 ml plastic pipette tip to allow aeration; after one week the kimwipe was removed, and two more holes were made on the plastic film each week until completing 9 holes.

Twelve jars were placed in a greenhouse pot-carrying tray (CTR415, Dillen Products, Middlefield, OH) with 4" diameter cells. Each tray was kept in a 32 quart capped plastic box (PN 1756, Sterilite Corp, Townsend, MA,) filled with 5 liters of reverse osmosis water. The box lid had 25 holes (3/16"of diameter) to facilitate aeration. Boxes were placed in darkness (except during manipulation) in a growth chamber at constant 21°C with continuous air exchange.

Ethylene analysis

Ethylene was measured every week following the procedure mentioned in Chapter 4.

Fusarium bulb coverage

Colonization of *Fusarium* on the outer scale of each bulb was rated (Illustration 5. 1) and recorded at the end of the experiment. The cover rating scale used was: 1) healthy tissue - no visible or minimal *Fusarium* growth around the wound, 2) *Fusarium* growing around the

wound and colonizing less than 50% of the base plate, 3) fungus growing on 50-100% of the base plate, 4) base plate fully colonized and less than 50% on the scales, 5) bulb completely colonized.

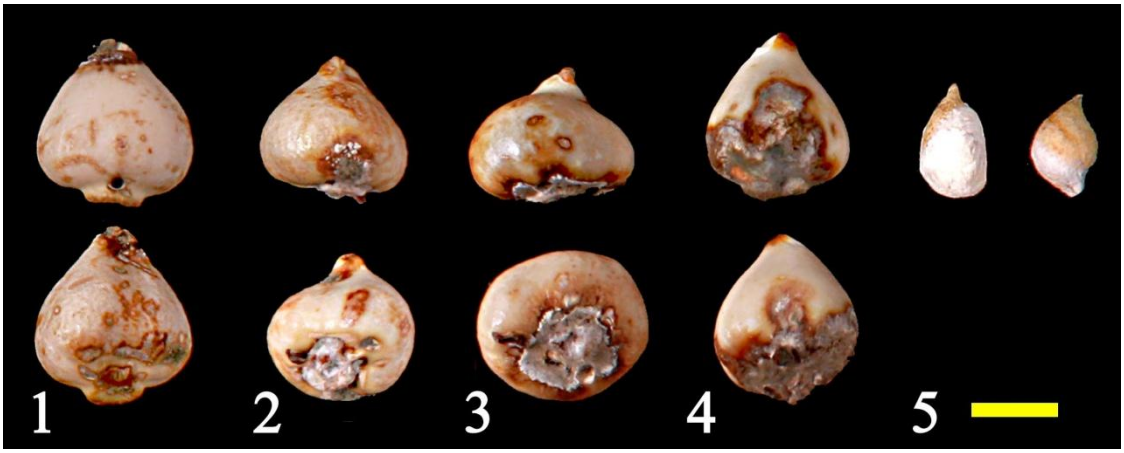

Illustration 5. 1 *Fusarium* cover rating on bulbs. Columns show the same bulb. Top row shows lateral view of the bulb on the round side; bottom row shows the base plate of the bulb. Columns depict: 1) Healthy bulb (BP), 2) Fungus on 50% base plate (CY), 3) *Fusarium* on less than 50% base plate (YP), 4) 50-100% base plate and less than 50% scales (PD), 5) Bulb completely decayed (TK). For tulip name legends (in parenthesis) see table 5.1 and 5.2.

Disease Severity Index

Disease severity index (DSI) for experiment two was constructed by multiplying the raw data of mycelial cover area by percent FW loss at day 28 post inoculation. The index was subject to log transformation and analyzed with analysis of variance (described in the statistical analysis section). Predicted values were back-transformed to obtain the DSI for each cultivar.

Video of Fusarium infection and symptoms development

The time course of *Fusarium* infection was recorded in two time-lapse movies. Tulip bulbs were placed inside an aquarium with 3 gallons of water and paper towels to increase evaporation. Bulbs were arrayed on a black board inside the aquarium which was covered with a glass, allowing adequate ventilation to maintain R. H. at approx. 90%. Images were recorded every 30 minutes for 31 days with a 12.2 megapixel digital camera (model Rebel XSI, Canon USA, Lake Success, NY) controlled with the remote shooting tool of the EOS utility software (same manufacturer) installed on a personal computer. Individual pictures were edited in Photoshop CS5 (Adobe Systems Inc., San Jose, CA) and compiled into time lapse movies with QuickTime (Apple Inc., Cupertino, Ca).

Statistical analysis

The maximum ethylene value from each replicate was log-transformed and analyzed using the statistical package JMP (Version 8.0, SAS institute, Inc. Cary, NC). Pair-wise comparisons between cultivars were made using Tukey's HSD test at the 5% significance level. Cultivars within one lineage were compared using t-test or Tukey's HSD test when appropriate.

The ordinal *Fusarium* cover rating data were analyzed with the ordinal logistic tool of the fit model platform in JMP to obtain the most likely cover rating per cultivar (saving the probability formula); the expected ordinal cover values were used to make correlation plots. Clusters were determined building a dendrogram with the Ward method of the Cluster platform.

RESULTS

In both experiments, ethylene production had a lag phase of 7 to 14 days depending on the cultivar. The time to reach maximum ethylene production across replicates of the same cultivar was not fully synchronized. In order to reduce data variability due to the day-to-day effect, the highest recorded value of each experimental unit (typically 21 or 28 DPI) was used for mean separation between cultivars.

Experiment one: tulip lineages

Ethylene production

The pattern of ethylene evolution, and maximal ethylene values were similar between cultivars of the same lineage (Figure 5. 1). The lag phase in most families was 7 days, except for the Apeldoorn lineage, which had a lag phase of 14 DPI. Highest ethylene values were observed between 21 and 28 DPI.

Ethylene evolution in the 'Wirosa' group (maximum ethylene production of 0.31-0.4 ul g^{-1} FW h^{-1}) showed a flat production pattern. Maximum ethylene production in the five cultivars of the 'Couleur Cardinal' family ranged from 0.41 to 0.64 ul g^{-1} FW h^{-1} and showed a rising trend until day 28. The 'Apeldoorn' group had a lag phase of 14 DPI and showed maximal values (0.58 to 0.87 ul g^{-1} FW h^{-1}) at 28 DPI; in 'Yellow Present' and 'Red Present' ethylene production peaked at 21 DPI (0.93 ul g^{-1} FW h^{-1}); 'Ad Rem' and 'Ad Rem's Beauty' behaved similarly until 21 DPI, however, 'Ad Rem's Beauty' kept increasing until 28 DPI with almost identical maximal values (0.97-1.05 ul g^{-1} FW h^{-1}); the 'Leen van der Mark' group showed highest values at 21 DPI (1.72 to 2.46 ul g^{-1} FW h^{-1}).

No significant differences ($p<0.0001$) were observed between cultivars of the same lineage, and the generational distance between cultivars of the same family did not have an influence on maximal ethylene production (Figure 5. 2). There were, however, significant

differences ($p < 0.001$) between tulip lineages (Table 5. 3). The difference in ethylene production between the lowest and highest ethylene values observed between lineages ('Wirosa' and 'Leen van der Mark') ranged around 8 fold.

Fusarium coverage on bulb

At the end of the experiment the bulb surface area covered by the fungus was similar among members of the same lineage (Illustration 5. 2). The *Fusarium* cover rating on bulbs of cultivars of the same family was similar except in the 'Yellow Present' family. Cultivars in the 'Wirosa' and 'Couleur Cardinal' lineages showed the lowest scores. The recorded cover rating by lineage is as follows: 'Wirosa' (2), 'Couleur Cardinal' (3), 'Ad Rem' (4), 'Apeldoorn' (4), and 'Leen van der Mark' (4). The cover in 'Red Present' was 4, and 3 for 'Yellow Present'.

Correlation between Fusarium infected area and maximal ethylene production

The linear correlation (r^2) between cover rating and maximal ethylene production was 0.29 (Figure 5. 3). However, the correlation within family groups was higher: 'Wirosa' group 0.89; 'Couleur Cardinal' group 0.68; 'Yellow Present' group 1.0; 'Ad Rem' group 1.0; 'Apeldoorn' group 0.76; and 'Leen van der Mark' group 0.97.

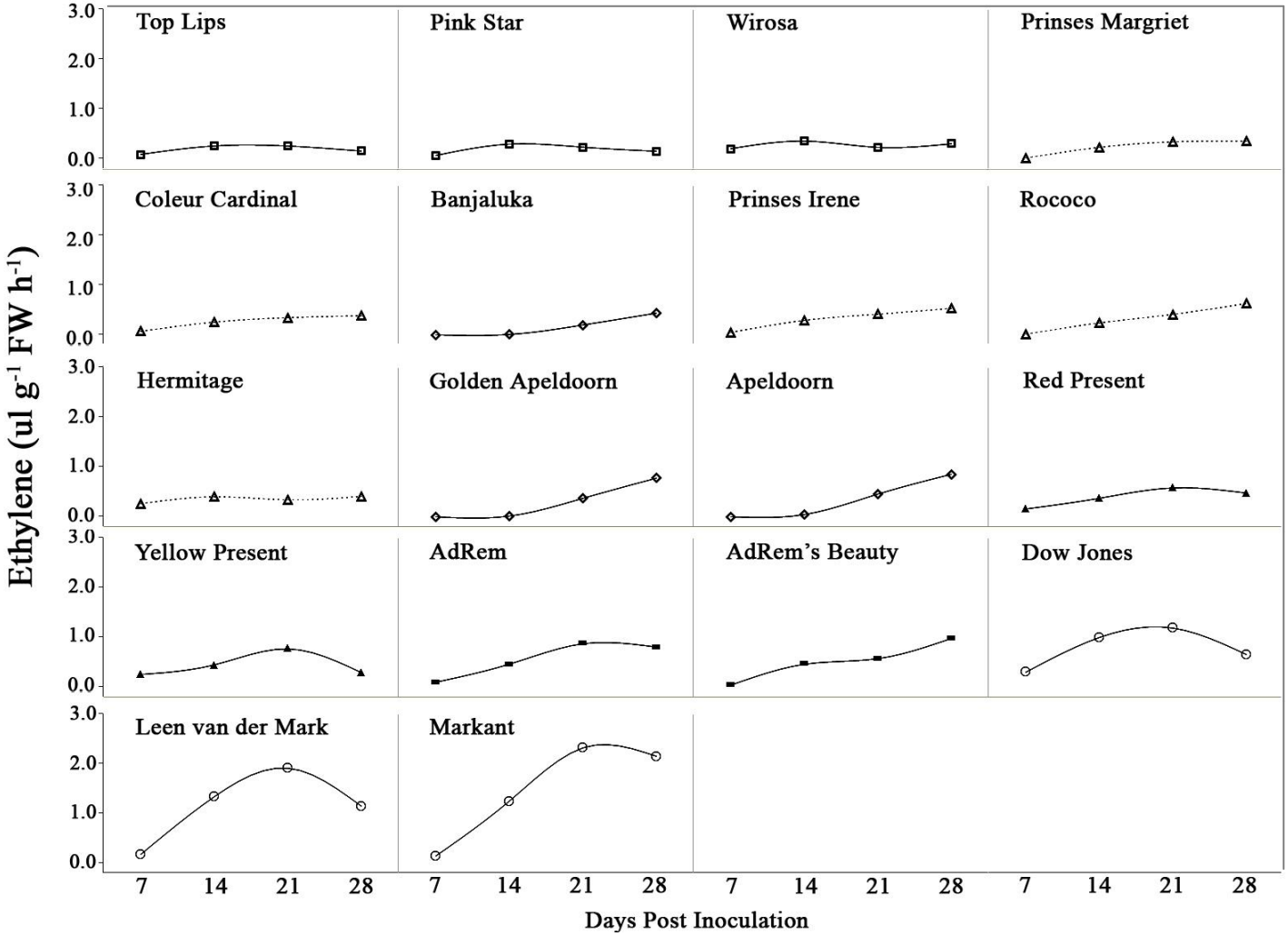

Figure 5. 1. Time course of ethylene production by F.o.t. in cultivars of six tulip lineages. Cultivars with same symbols belong to one lineage. Data points are predicted values with n=16.

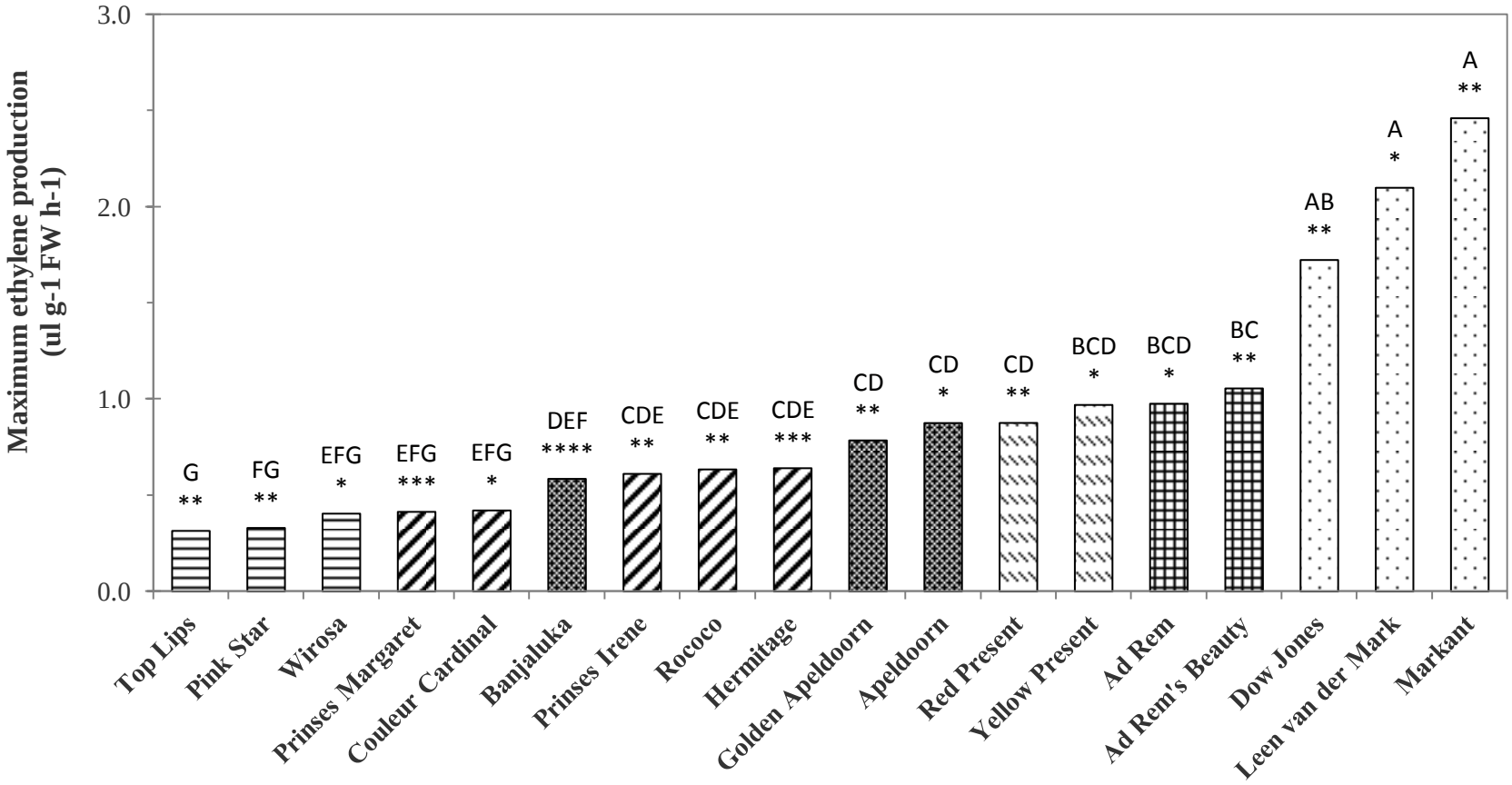

Figure 5. 2 Maximal ethylene values by cultivar. Bars with the same pattern belong to an individual lineage. Cultivars not connected by the same letter are significantly different at p=0.05 usign Tukey's HSD with n=16. Asterisks indicate the number of successive generations of each lineage where *=Mother, **Daughter, ***=Granddaughter, ****=Great granddaughter.

Table 5. 3. Maximal ethylene production by tulip lineage. Values not connected by the same letter are significantly different ($p < 0.0001$) according to Tukey's HSD test.

Lineage	Maximum ethylene (ul g^{-1} FW h^{-1})
Wirosa	0.35 D
Couleur Cardinal	0.53 C
Apeldoorn	0.74 B
Yellow Present	0.92 B
Ad Rem	1.01 B
Leen van der Mark	2.07 A

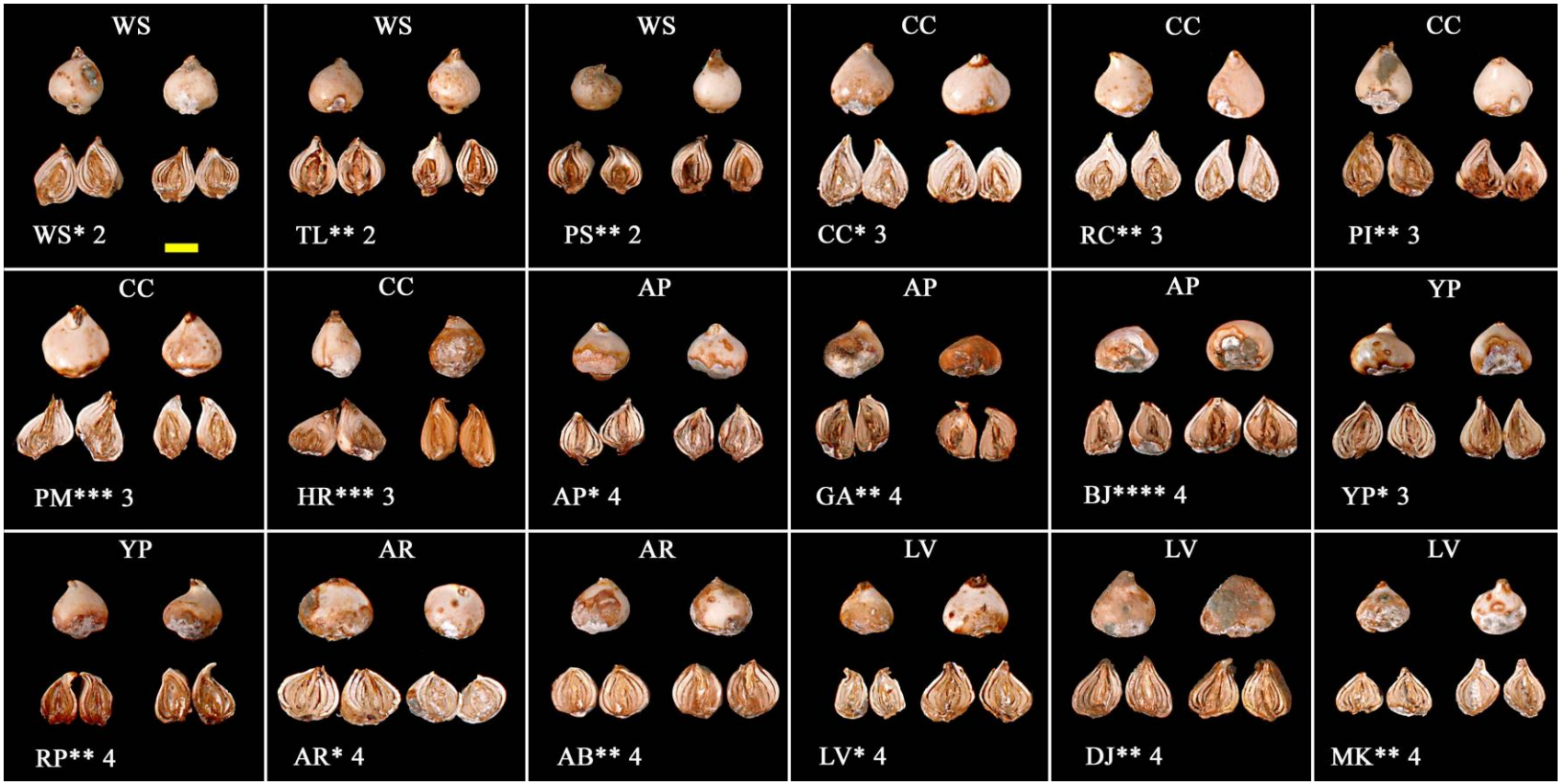

Illustration 5. 2. *Fusarium* cover on tulip bulbs 28 days post inoculation. Letters at the center top of each frame are abbreviations of tulip lineage. Legends at the bottom left corner of each frame depict: cultivar name,(Table 5. 1, page 79); asterisks indicate the number of successive generations of each lineage, and numbers depict the cover rating. Yellow scale bar corresponds to 2.5 cm.

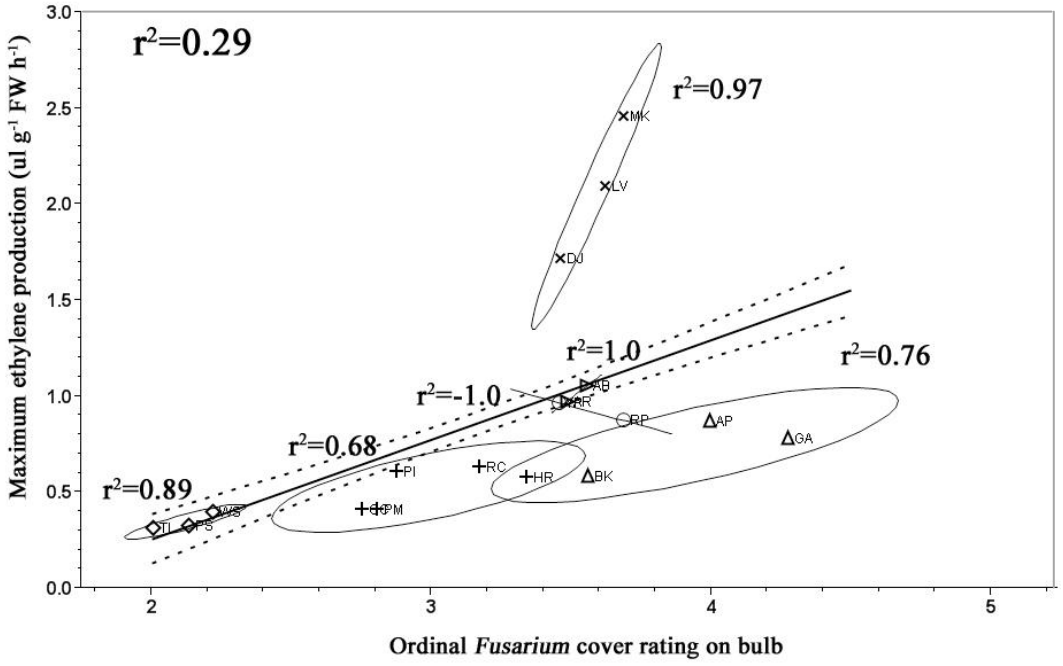

Figure 5. 3. Correlation between *Fusarium* cover rating and maximal ethylene production. Diagonal line represents fitted model; dashed lines show 95% confidence interval of the linear model fit; tulips of the same lineage are grouped with symbols of the same type.

Experiment two: Cultivars and tulip species

Ethylene production

The lag period for ethylene production was seven days for almost all cultivars (Figure 5. 4). At 7 DPI, ethylene production in *T. turkestanica* was about 0.5 ul g^{-1} FW h^{-1}, suggesting that the lag period of this species was shorter than for all other cultivars in the two experiments

The pattern of ethylene evolution in 22 cultivars and 2 tulip species (Figure 5. 4) resembled those observed in the tulip lineage experiment. Ethylene production in four cultivars ('Bright Parrot', 'Strong Gold', 'Negrita', 'Flaming Parrot') and *T. tarda* remained relatively low (<0.3 ul g^{-1} FW h^{-1}) and was almost flat throughout the course of the experiment. Nine cultivars showed ethylene values that continued to increase until the end of the experiment ('Parade', 'Judith Leyster', 'Pink Impression', 'Spryng', 'Ile de France', 'Yellow Flight', 'Passionale', 'Purple Flag', 'Yokohama'); while eleven cultivars reached maximum ethylene values between 14 and 28 days ('Flaming Parrot', 'Cummins', 'Calgary', 'World's Favourite', 'Blue Ribbon'. 'Kikomachi', 'Christmas Dream', 'Mondial', 'Oscar', 'Friso', and *T. turkestanica*).

Maximal ethylene production was highly influenced by tulip cultivar or species, *Fusarium* cover on the bulb, and the % FW loss (Table 5. 4).

Table 5. 4. Analysis of variance of maximal ethylene production.

Source	**Nparm**	**DF**	**DFDen**	**F Ratio**	**Prob > F**
Cultivar	23	23	178.4	12.2201	<.0001*
Fusarium cover	4	4	349.2	19.0480	<.0001*
% FW Loss	1	1	302.3	12.2744	0.0005*

The lowest maximum ethylene value (Figure 5. 5) was observed in 'Bright Parrot' (0.06 ul g^{-1} FW h^{-1}), while the highest was *T. turkestanica* (2.75 ul g^{-1} FW h^{-1}). Fifty percent of the cultivars produced < 0.5 ul g^{-1} FW h^{-1}, 29% produced between 0.5 and 1.0 ul g^{-1} FW h^{-1}, and 21% produced more than 1.0 ul g^{-1} FW h^{-1}. *T. turkestanica* produced 80% more ethylene than 'Friso' (the highest of all cultivars), while the difference with 'Bright Parrot' (the lowest ethylene-sustaining cultivar) was 46 fold.

Fusarium coverage on bulb

At the completion of the experiment, three cultivars ('Bright Parrot', 'Strong Gold', and 'Negrita') did not show any external symptoms of *Fusarium* infection (Table 5. 8). The cross section of these cultivar bulbs showed, however, that the flower bud was decayed, but the scales remained non-infected. Four cultivars ('Flaming Parrot', 'Judith Leyster', 'Cummins', and 'Calgary') rated 2; five cultivars rated 3 ('Blue Ribbon', 'Ile de France', 'Christmas Dream', 'Mondial', and 'Friso"); Ten cultivars rated 4 ('Parade', 'Pink Impresion', 'World's Favourite', 'Spryng', 'Yellow Flight', 'Kikomachi', 'Passionale', 'Purple Flag', 'Yokohama', and 'Oscar'); and the two tulip species rated 5 (*T. tarda*, and *T. turkestanica*).

Interestingly, 'Parade' and *T. tarda* are low in *Fusarium*-ethylene production, but had coverage ratings of 4 and 5, respectively. 'Parade' showed extensive areas with brown edges on the outer scale with mycelium growing on them; approximately 75% of the internal organs showed necrosed tissue. On the other hand, the outer scale of *T. tarda* was mostly brown with marginal growth of *Fusarium* mycelium on it; the interior organs of the bulb were completely necrosed or dry-looking.

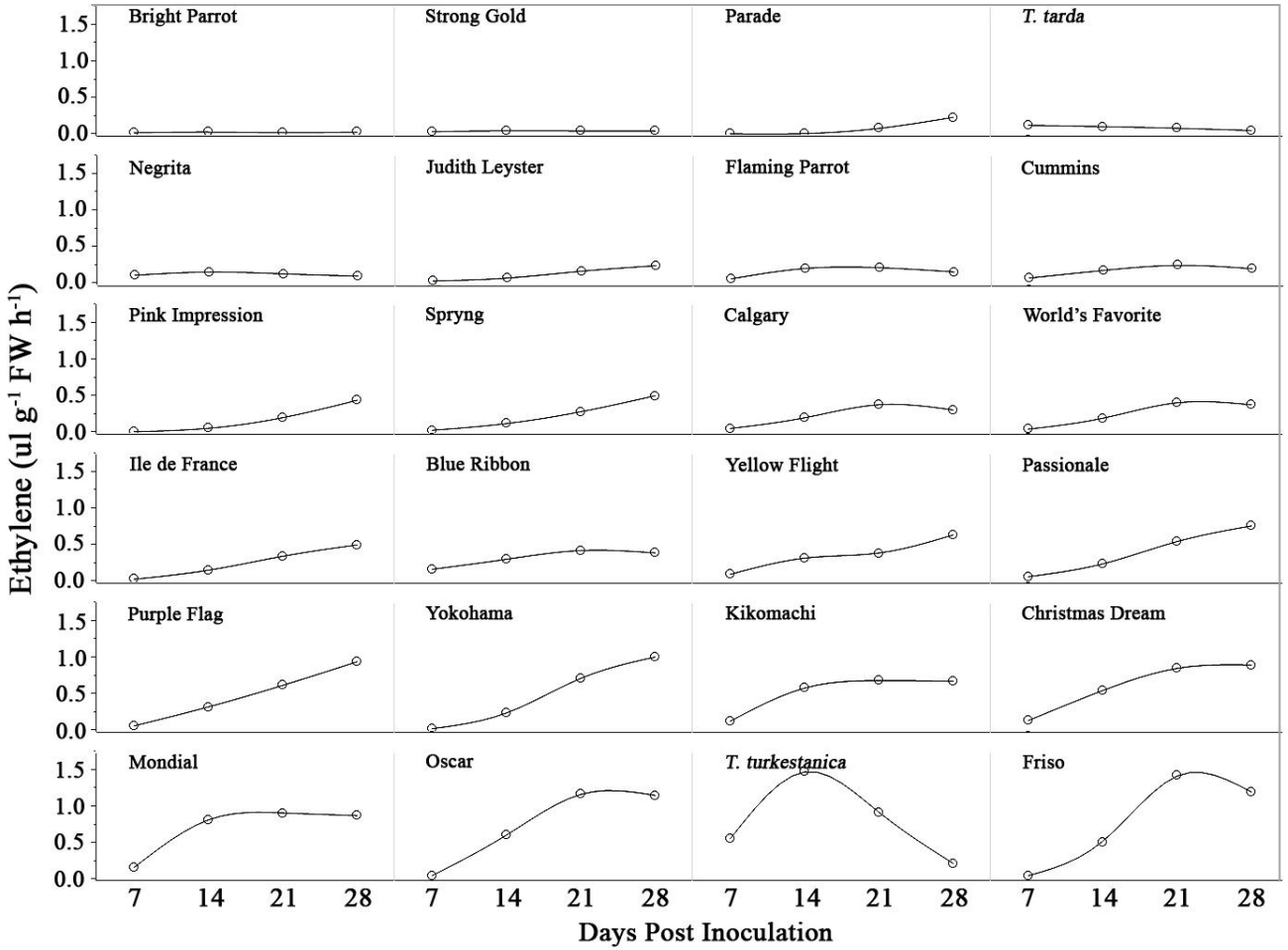

Figure 5. 4. Time course of ethylene production by F.o.t. in 22 cultivars and two tulip species. Data points are predicted values with n=16.

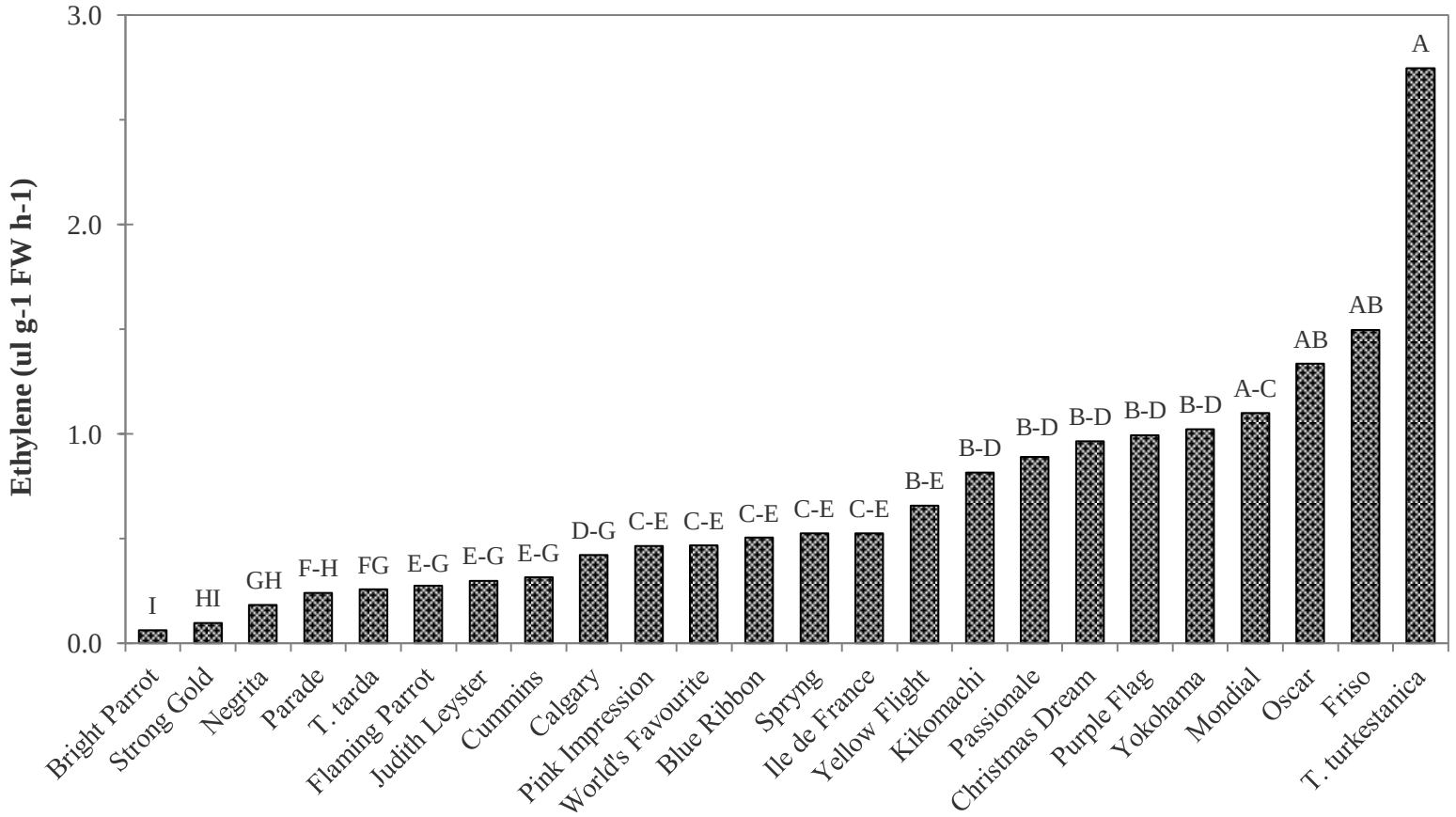

Figure 5. 5. Maximal ethylene values in 22 cultivars and 2 species. Bars with the same pattern belong to an individual lineage. Cultivars not connected by the same letter are significantly different ($p < 0.0001$) using Tukey's HSD with n=16.

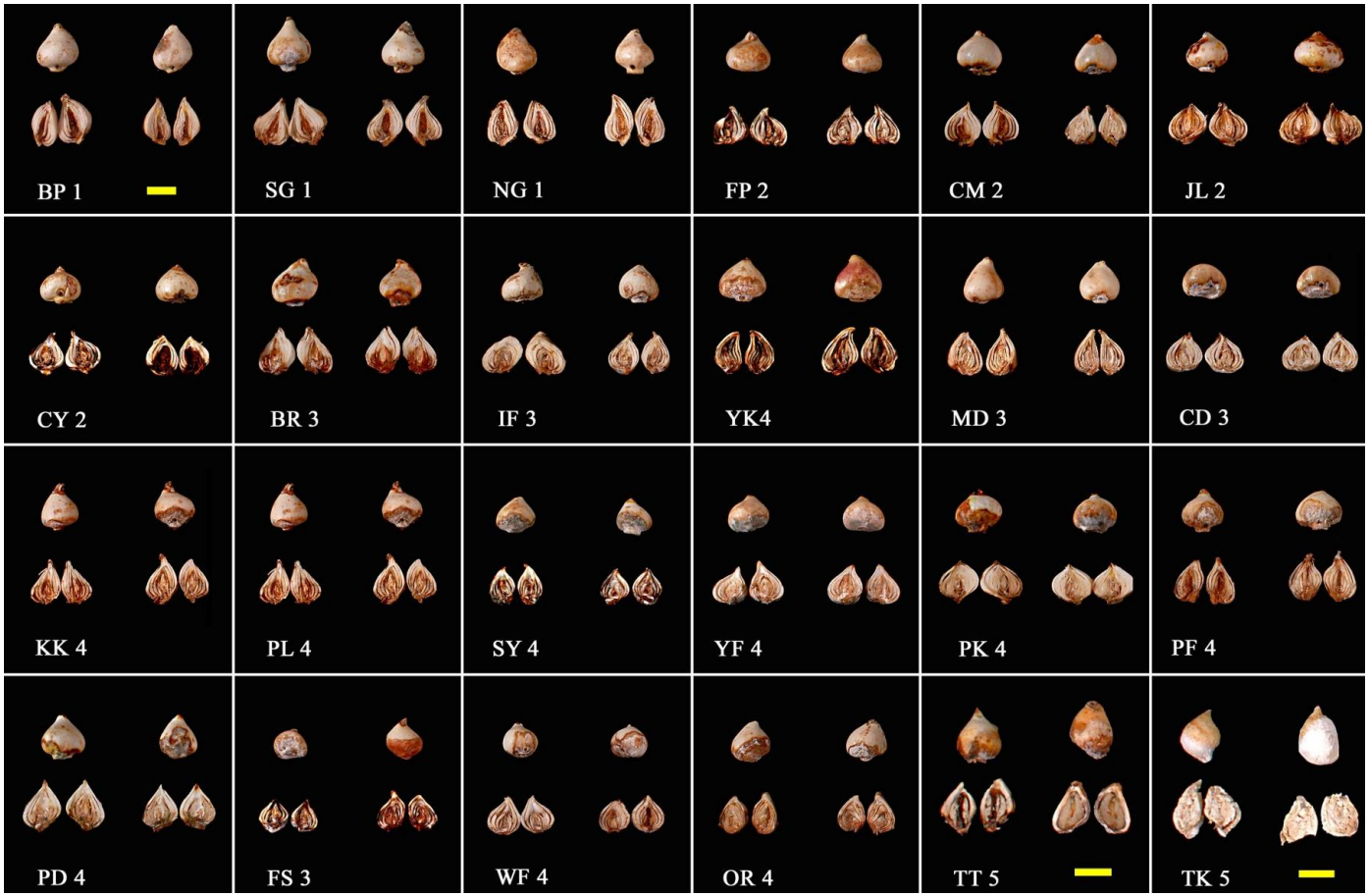

Illustration 5. 3 *Fusarium* cover on tulip bulbs at 28 DPI. Legend at the bottom of each frame depicts the cultivar name , (Table 5. 2, page 80); and numbers depict the cover rating. Yellow scale bars correspond to 2.5 cm.

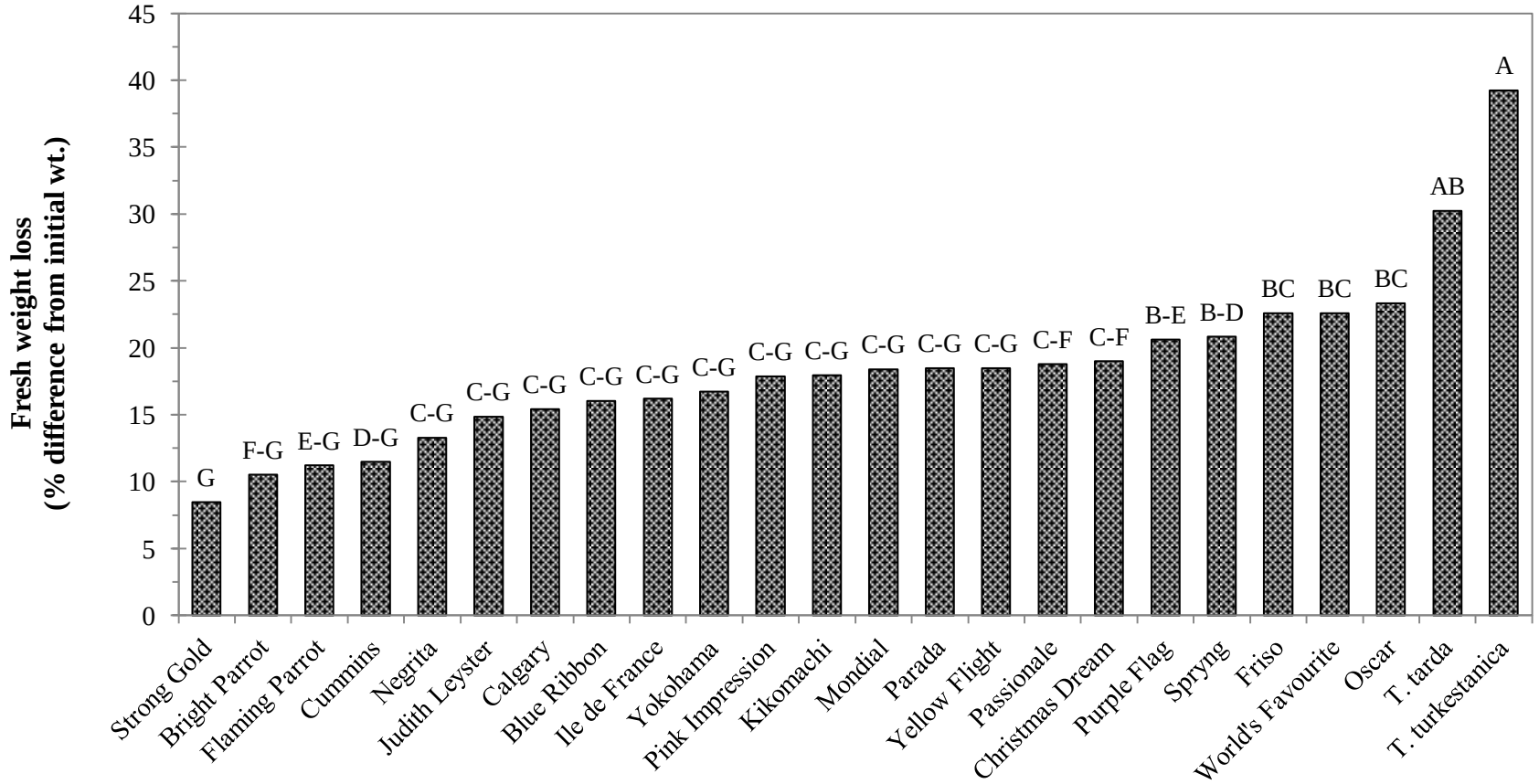

Figure 5. 6. Fresh weight loss at 28 days post inoculation in all tulip cultivars and species tested. Bars not connected by the same letter are significantly different ($p < 0.0001$) using Tukey's HSD with n=16.

Percentage fresh weight loss

The difference in fresh weight (Figure 5. 6) between 0 and 28 days after inoculation ranged from 8.5% ('Strong Gold') to ca. 40% (*T. turkestanica*). Data were highly variable across cultivars which was seen in the means separation where 18 out of the 24 tulips tested were not significantly different ($p < 0.0001$).

Correlation between Fusarium infected area and maximal ethylene production

When plotting the predicted ordinal *Fusarium* cover on the bulb versus the maximum ethylene production by cultivar (Figure 5. 7), the relationship around the linear fit line was 0.36, which was similar to that observed in the lineages experiment. Cluster analysis revealed six clusters, and the resulting correlation within clusters was significantly higher than the linear fit model (Table 5. 5).

Table 5. 5. Cultivars grouped by cluster, showing the correlation between ordinal *Fusarium* cover vs. maximal ethylene.

Cluster	Cultivars	Correlation (r^2) of ordinal *Fusarium* cover vs. maximal ethylene
1	Bright Parrot, Negrita, Strong Gold	0.52
2	Blue Ribbon, Calgary, Cummins, Flaming Parrot, Ile de France, Judith Leyster	0.91
3	Chritmas Dream, Kikomachi, Mondial, Pasionale, Purple Flag, Yokohama,	0.60
4	Parade, Pink Impression, Spryng, *T. tarda*, World's Favourite, Yellow Flight	0.65
5	Friso, Oscar	-1.0
6	*T. turkestanica*	-

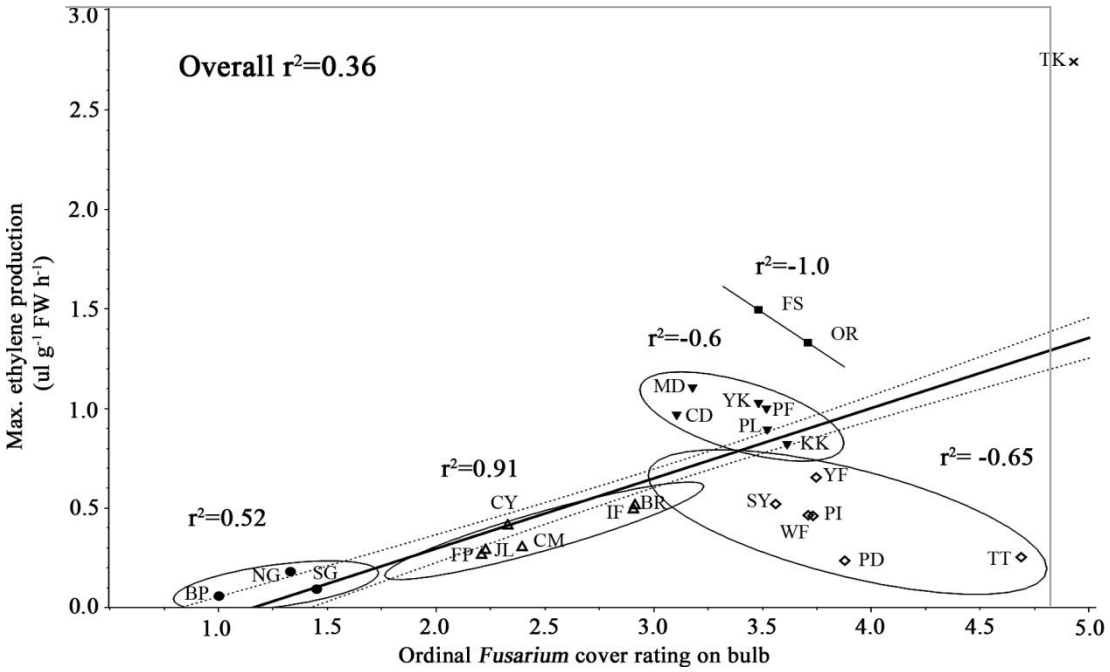

Figure 5. 7. Correlation between *Fusarium* cover rating and maximal ethylene production. Diagonal line represents fitted model; dashed lines show 95% confidence interval of the linear fit; tulips of the same cluster are grouped with lines or circles.

Combined cover rating and ethylene correlation of two experiments

The maximum ethylene and *Fusarium* cover data from both experiments were pooled to explore relationships in a larger group of cultivars and the two species (Figure 5. 8). The overall correlation of cover to ethylene production in the 40 cultivars and 2 species was 0.32.

Cluster analysis using predicted maximum ethylene production and ordinal *Fusarium* bulb cover ratings grouped tulips into six groups with a wide range of correlation between them (Table 5. 6).

Tulips in cluster 5 had low relative ethylene production compared to the cover rating. If this group was removed from the chart, the overall linear correlation increased from 0.32 to 0.65, and to 0.74 with a quadratic function. This suggests that cultivars in this cluster may have a different ethylene-*Fusarium* cover response than the rest of the cultivars tested.

Disease Severity Index

Exploratory analysis of variables to explain disease severity in experiment two showed that the predicted ordinal *Fusarium* cover rating on bulb and the predicted % FW loss had the highest correlation ($r^2 = 0.85$). The original cover rating values (ordinal values) were multiplied by the % FW loss at 28 DPI to obtain a continuous variable called Disease Severity Index (DSI) which was log transformed and subject to analysis of variance, using the same mixed model to analyze ethylene production. After back-transforming the values, the resulting data were subject to cluster analysis and five *Fusarium* categories were defined (Figure 5. 9). Since the last two groups were composed of a single species *T. tarda* and *T. turkestanica*, respectively, we grouped these species into a single cluster resulting in four categories: 1) highly resistant, 2) resistant, 3) susceptible, and 4) highly susceptible.

The three highly resistant cultivars were ‘Bright Parrot’, ‘Strong Gold’ and ‘Negrita’. Resistant cultivars (6) include ‘Flaming Parrot’, ‘Cummins’, ‘Judith Leyster’, ‘Calgary’, ‘Blue Ribbon’ and ‘Ile de France’. Thirteen cultivars fell into the susceptible category ‘Yokohama’, ‘Mondial’, ‘Chistmas Dream’, ‘Kikomachi’, ‘Passionale’, ‘Yellow Flight’,

'Pink Impression, 'Purple Flag', 'Parade', 'Spryng', 'Friso', 'World's Favourite' and 'Oscar'. Finally the two tulip species (*T. tarda*, and *T. turkestanica*) were placed in the highly susceptible group.

Grouping of 40 tulip cultivars and two tulip species by maximum ethylene production of Fusarium.

The maximum ethylene production from all tulip cultivars and species in the two experiments were combined. Cluster analysis was used to group tulips into five categories (Table 5. 7) as follows: cluster 1) low ethylene sustaining category (ethylene production ≤ 0.33 ul g^{-1} FW h^{-1}) containing 10% of all the tulips with nine cultivars and *T. tarda*; cluster 2) medium-low ethylene group (0.4-0.66 ul ethylene g^{-1} FW h^{-1}) composed of fourteen cultivars (33%); 3) medium ethylene production (0.79-1.10 ul ethylene g^{-1} FW h^{-1}) with 12 cultivars (29%); cluster 4) includes three tulips cultivars (7%) with medium-high ethylene production (1.33-1.72 ul g^{-1} FW h^{-1}); cluster 5) two cultivars and *T. turkestanica* (≥ 2.1 ul g^{-1} FW h^{-1}), (7%).

Tulip lineages fell into the following clusters: 'Wirosa' group) 2 cultivars in cluster 1 ('Top Lips', 'Pink Star') and one cultivar ('Wirosa') in cluster 2; the five cultivars in the 'Couleur Cardinal' lineage fell into cluster 2; members of the 'Apeldoorn' family were distributed in cluster 2 ('Banjaluka') and cluster 3 ('Golden Apeldoorn', and 'Apeldoorn'); cluster 3 contained all cultivars of the 'Yellow Present' and 'Ad Rem' lineages; finally the 'Leen van der Mark' family cultivars were ranked as medium-high ('Down Jones') and high ('Leen van der Mark' and 'Markant').

Development of Fusarium infection and fungal predation by tulip mites

The infection process of *Fusarium* on several tulip cultivars was recorded on two time-lapse movies. Six cultivars studied by Miller *et al.* (2005) were chosen for their ability to sustain high and low *Fusarium* ethylene to make Video 5.1. Low ethylene-sustaining cultivars

are: ‘Strong Gold’, ‘Calgary’ and ‘Kees Nelis’. High ethylene-sustaining cultivars are ‘Prominence’, ‘Friso’ and ‘Mary Belle’. The cultivars from left to right are: ‘Strong Gold’, ‘Calgary’, ‘Prominence’, ‘Friso’, ‘Kees Nelis’ and ‘Mary Belle’. Columns show two views of the same cultivar. The series of events that take place in Video 5.1 span for 28 days and are described in Table 5. 8.

Video 5. 2. shows the visual symptoms of non-inoculated and inoculated bulbs of ‘Ad Rem’ (AR) and ‘Strong Gold’ (SG) over 32 days. Video 5.3. is a close up of video 5. 2 and shows the inoculated bulbs of ‘Ad Rem’ and ‘Strong Gold’. The series of events that take place in Video 5.2 and 5.5 are listed in Table 5. 9.

Table 5. 6. Grouping of all 40 cultivars and 2 species into clusters according to their relationship between cover rating and max. ethylene production.

Cluster	Tulips	Correlation (r^2) of ordinal *Fusarium* cover vs. maximal ethylene	Range of max. ethylene production range in cluster (ul g^{-1} FW h^{-1})	Cover rating in cluster
1	Bright Parrot, Negrita, Strong Gold	0.52	< 0.2	1
2	Cummins, Calgary, Flaming Parrot, Judith Leyster, Pink Star, Top Lips, Wirosa	0.27	0.27-0.42	2
3	Blue Ribbon, Couleur Cardinal, Hermitage, Ile de France, Pricess Irene, Prinses Margriet, Rococo	0.69	0.41-0.63	3
4	Ad Rem's Beauty, Ad Rem, Chistmas Dream, Dow Jones, Friso, Kikomachi, Mondial, Oscar, Passionale, Purple Flag, Red Present, Yellow Present, Yokohama	-0.14	0.82-1.72	3-4
5	Apeldoorn, Banjaluka, Golden Apeldoorn, Parade, Pink Impression, Spryng, *T. tarda*, World's Favourite, Yellow Flight	-0.004	0.24-0.88	4-5
6	Leen van der Mark, Markant, *T. turkestanica*	0.85	2.1-2.75	4-5

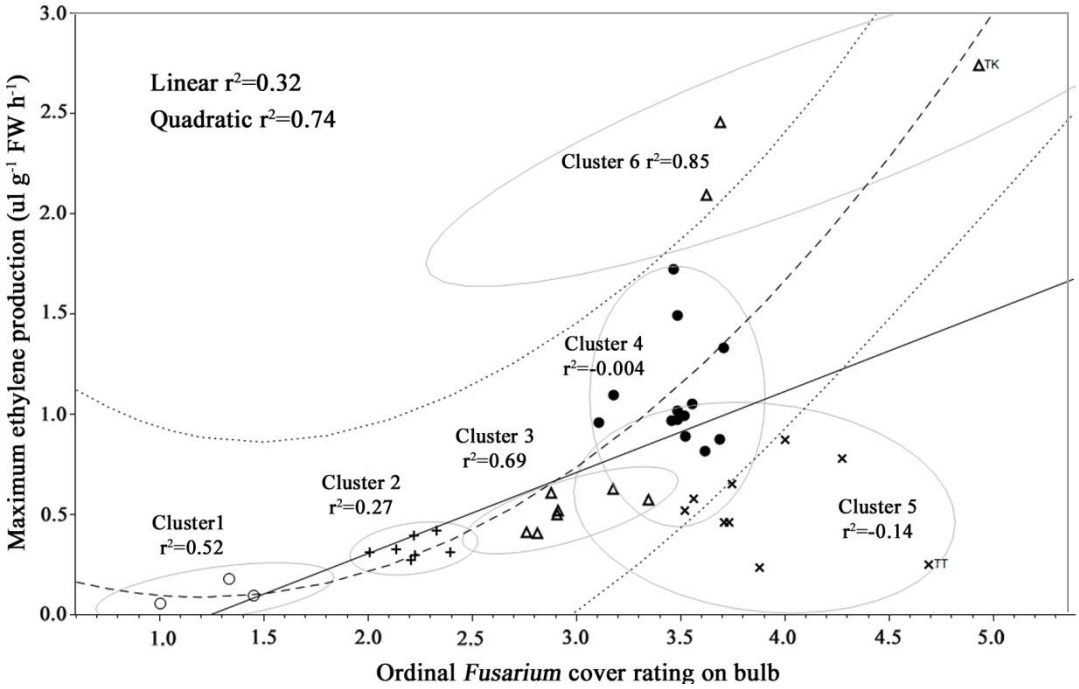

Figure 5. 8. *Fusarium* cover rating and maximum ethylene production plot. Circles enclose tulip cultivars and species into Clusters. Diagonal line represents fit of linear model. Dashed line shows the quadratic fit after removing Cluster 5; dotted lines show 95% confidence interval of quadratic fitted line.

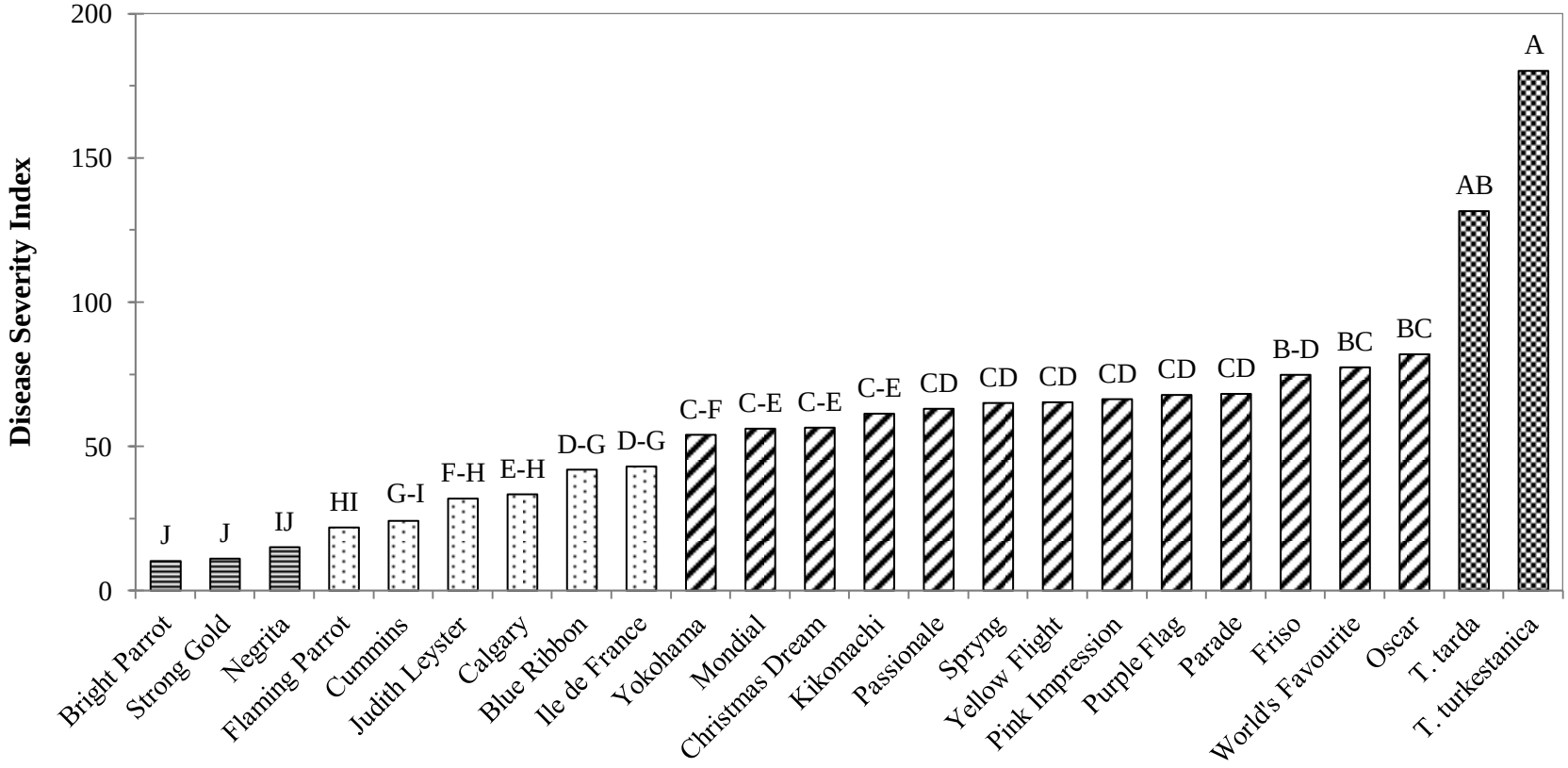

Figure 5. 9. Disease Severity Index for 20 tulip cultivars and two species inoculated with F.o.t. Cultivars with the same pattern share the same resistance level to F.o.t; from left to right: highly resistant (3 cultivars), resistant (6 cultivars), susceptible (13 cultivars), highly susceptible (2 cultivars). Cultivars not connected with the same letter are significantly different ($p<0.0001$) according to Tukey's HSD test. Each bar is composed of n=16.

Table 5. 7. Tulip cultivars and species grouped in clusters by maximal ethylene production.

Category		Cultivars and species	Max. ethylene (ul g^{-1} FW h^{-1})	Max. ethylene average (ul g^{-1} FW h^{-1})	St. Dev.	95% Confidence Interval	
						Low limit	High Limit
1 Low ethylene	1	Bright Parrot	0.06	0.24	0.09	0.17	0.30
	2	Strong Gold	0.10				
	3	Negrita	0.18				
	4	Parade	0.24				
	5	*T. tarda*	0.26				
	6	Flaming Parrot	0.27				
	7	Judith Leyster	0.30				
	8	Cummins	0.31				
	9	Top Lips	0.31				
	10	Pink Star	0.33				
2 Medium-low ethylene	1	Wirosa	0.40	0.51	0.088	0.464	0.565
	2	Prinses Margriet	0.41				
	3	Couleur Cardinal	0.42				
	4	Calgary	0.42				
	5	Pink Impression	0.46				
	6	World's Favourite	0.47				
	7	Blue Ribbon	0.50				
	8	Spryng	0.52				
	9	Ile de France	0.53				
	10	Banjaluka	0.59				
	11	Hermitage	0.64				
	12	Prinses Irene	0.61				
	13	Rococo	0.63				
	14	Yellow Flight	0.66				

Table 5.7. (Continued)

Category		Cultivars and species	Max. ethylene (ul g^{-1} FW h^{-1})	Max. ethylene average (ul g^{-1} FW h^{-1})	St. Dev.	95% Confidence Interval	
						Low limit	High Limit
3 Medium ethylene	1	Golden Apeldoorn	0.79	0.94	0.092	0.88	1.00
	2	Kikomachi	0.82				
	3	Apeldoorn	0.88				
	4	Red Present	0.88				
	5	Passionale	0.89				
	6	Christmas Dream	0.96				
	7	Yellow Present	0.97				
	8	Ad Rem	0.98				
	9	Purple Flag	0.99				
	10	Yokohama	1.02				
	11	Ad Rem's Beauty	1.06				
	12	Mondial	1.10				
4 Medium-high ethylene	1	Oscar	1.33	1.52	0.195	1.03	2.00
	2	Friso	1.50				
	3	Dow Jones	1.72				
5 High ethylene	1	Leen van der Mark	2.10	2.44	0.324	1.63	3.24
	2	Markant	2.46				
	3	*T. turkestanica*	2.75				

Table 5. 8. Description and sequence of events that take place in Video 5.1.

Second	Event
0-12	No fungal growth in any of the bulbs
13-25	*Fusarium* starts growing on 'Mary Belle', and then it develops in various degrees in all the cultivars. *Fusarium* protrudes out of the base plate of 'Prominence', 'Friso', and 'Mary Belle', while it grows weakly on 'Strong Gold', 'Calgary', and 'Kees Nelis'.
25	Mites are initially noticeable in 'Prominence' and eventually migrate to all the cultivars, on which infestation differs in extent.
26-39	Fungal growth reaches its maximum and mycelium starts senescing, which is characterized by shrinkage, predation by bulb mites, and color change to brown-red. Mycelium shrinkage is visible in all cultivars, but is particularly noticeable in 'Strong Gold', 'Calgary', and 'Kees Nelis'. *Fusarium* changes color in 'Friso', while invasion by secondary fungi occurs in 'Calgary', 'Friso' and 'Mary Belle'.

Table 5. 9. Description and sequence of events that take place in Video 5.2 and 5.3.

Second	Real time point	Event
0-3	Day 3	Mycelium is visible in the inoculated wound of 'Strong Gold', while in 'Ad Rem' the fungus is less dense but it grows faster into the tissue and out of the inoculated area
3-10	Day 4	A few bulb mites feed on the mycelium of 'Strong Gold', and sporodochia starts forming around the wound of 'Ad Rem'
6-49	Days 5-31	*Penicillium* colonies are seen both in inoculated and non-inoculated bulbs. Soon after *Penicillium* appears, bulb tissue starts browning around it, similar to a hypersensitive response. The same is true for the tissue surrounding the wounded areas of wounded and non-inoculated bulbs which turn brown but are not infected with *Fusarium*.
10-20	Days 7-13	Tissue surrounding the mycelium in 'Strong Gold' turns brown and the fungus slows down its growth. At the same time, mite numbers start increasing and feed exclusively on mycelium of 'Strong Gold'. *Fusarium* has extended well out of the wound in 'Ad Rem' while abundant sporodochia has formed around and below the wound site.
20-22	Days 13-15	Tissue around *Fusarium* in 'Strong Gold' is completely brown and the fungus has been devoured by bulb mites. During the same period bulb tissue turns light-brown around fungal growth in 'Ad Rem', *Fusarium* stops growing, and mites start feeding on it from the outer edge of the fungus towards the center.
27	Day 17	*Fusarium*-infected tissue expands next to the bulb scar on the base plate and starts forming sporodochia three days later.
39	Day 25	*Fusarium* on the outside of the 'Ad Rem' bulb has been highly destroyed by bulb mites, but it keeps colonizing the flower bud, but not in 'Strong Gold'.
40-49	Days 26-31	As mites have consumed most of *Fusarium* around the inoculation wound they migrate to the base plate where the tissue around the fungus has started turning brown and as the fungus stops growing mites start feeding on the sporodochia. This process continues until day 31 when the video finishes.

DISCUSSION

Ethylene production

Inoculation of 42 cultivars of *Tulipa gesneriana* and 2 tulip species with F.o.t. showed a wide range of ethylene production and fungal infection. In most cultivars, ethylene of *Fusarium* origin had a lag phase of 7 days and reached maximum levels between 21 and 28 days post inoculation. The only exception was *T. turkestanica* in which the lag phase took less than one week and maximum readings were observed 14 days post inoculation. The lowest producing cultivar was 'Bright Parrot' (0.06 ul g^{-1} FW h^{-1}), while the highest ethylene value (2.75 ul g^{-1} FW h^{-1}) was in *T. turkestanica*, a 45-fold difference.

In previous experiments, the maximal ethylene production in 'Leen van der Mark' inoculated in the base plate (Chapter 4, Table 4. 3,Figure 4. 4) was approximately 1.0 ul g^{-1} FW h^{-1} which is less than observed in experiment two (2.1 g^{-1} FW h^{-1}) of this chapter. The differences between both experiments might be due to the fact that in Chapter 4 data held variability due to 'day post inoculation', while in this Chapter data variability due to 'day post inoculation' was removed by choosing only the highest values from each bulb replicate. The later data analysis ultimately provided a more precise means separation.

The maximum amount of *Fusarium*-derived ethylene registered in the 40 tulip cultivars and two species was analyzed by cluster analysis to define five categories of ethylene-sustaining tulip bulbs (Table 5. 7). In our experiments we used 13 out of the 36 cultivars observed by Miller (2005). The cultivar rankings between the two experiments differ (Table 5. 10), possibly due to experimental differences and data analysis.

Table 5. 10. Relative ranking comparison between Miller *et. al.* (2005) and results of experiment two. Tulip cultivars are ranked according to the amount of ethylene produced by F.o.t. 19-28 days post inoculation, where 1 represents the highest ethylene value and 13 the lowest value.

Cultivar	Ranking Miller *et. al.* (2005)	Ranking experiment 2
Friso	1	1
Yellow Present	2	4
Kikomachi	3	5
Mondial	4	2
Couleur Cardinal	5	10
World's Favourite	6	8
Strong Gold	7	12
Yellow Flight	8	6
Purple Flag	9	3
Wirosa	10	11
Blue Ribbon	11	7
Bright Parrot	12	13
Calgary	13	9

Tulip sports are a result of single point mutations that show a different phenotype (flower color, shape, etc.) from the cultivar from which they arose. Since the gene pool in these cultivars does not recombine, successive mutant clonal generations would be expected to show identical phenotypes (e.g. ethylene production by *Fusarium*). This was observed in experiment one, where cultivars of the same lineage did not differ (statistically) in ethylene production pattern (Figure 5. 1) and maximal ethylene production (Figure 5. 2), however, as expected, significant differences ($p < 0.0001$) were found between lineages (Table 5. 3).

The tulip industry has essentially zero tolerance for ethylene in storage rooms (De Wild et al. 2002) due to detrimental physiological responses to ethylene. Thus standard air exchange rates are > 100 m^3 h^{-1} per m^3 tulip bulbs, in an attempt to maintain ethylene levels below 0.1 ul l^{-1} or 100 ul m^3 (De Munk 1971; Kamerbeek et al. 1971; De Munk 1973b, 1973a, 1975; Kamerbeek and De Munk 1976; De Hertogh et al. 1980).

Latent *Fusarium* infection in tulip bulbs in storage (Bergman and Bakker-van der Voort 1979) may develop into fusariosis which is the most important source of ethylene in

this stage (Kamerbeek and De Munk 1976). Bergman (1965) collected bulbs of susceptible cultivars to F.o.t. at harvest and recorded the time to appereance and frequency of infection symptoms. After 49 days of storage the percentage of bulbs infected with *Fusarium* was: 'Enterprise' 4.2%, 'Red Giant' 7.7%, and 'Mantilla' 6.5%; of all the infected bulbs, at least 50% showed *Fusarium* infection symptoms during the first two weeks after harvest.

One kilogram of tulip bulbs (approx. 30 bulbs size 12+ cm of most cultivars) infected with F.o.t. in each of the five ethylene-sustaining categories (Table 5. 7) has the potential to produce the following average amounts of ethylene: low) 240 ul h^{-1}, medium low) 510 ul h^{-1}, medium) 940 ul h^{-1}, medium high) 1,520 ul h^{-1}, high) 2,440 ul h^{-1}. If these amounts were to be replicated in a storage room, one kilogram of infected bulbs from these categories in one hour would realize ethylene concentrations of at least 0.1 ul in a volume of 2.4 m^3 (for the low category) to 24.4 m^3 (for the high category). It is possible that during the first weeks after harvest, ventilation may be insufficient to remove enough ethylene from *Fusarium* infected bulbs of susceptible cultivars, and as a consequence, the concentration of this gas in the atmosphere may reach above thresholds (Kamerbeek et al. 1971) that cause gummosis. This may explain why growers commonly observe gummosis in ethylene-sensitive cultivars upon arrival of bulb consignments. The information that we provide, in conjunction with other studies (De Wild et al. 2002; Liou 2006) could be used to develop strategies to design more efficient ventilation rates and reduce energy costs.

Quantification of resistance to Fusarium

The extent of *Fusarium* infection, as measured by cover ratings, was equivalent among cultivars of the same lineage, except in the 'Yellow Present' group. These results were expected because pathogen resistance is the result of additive gene action (van Eijk et al. 1979), which as explained above, does not change in color sports.

Two methods were investigated to determine resistance to *Fusarium* infection. The first method consisted of ploting the predicted logistic values of the ordinal *Fusarium* cover

rating on the bulb versus the predicted values of maximal ethylene production for cultivars in the two experiments. In the second method, a Disease Severity Index was calculated (experiment two).

The correlation coefficient of the fit model using cover rating and maximal ethylene production of all cultivars in experiment one (Figure 5. 3) was 0.29, in experiment two (Figure 5. 7) was 0.36, and for the combined tulips from the two experiments (Figure 5. 8) was 0.32. The correlation between clusters in the 40 cultivars and two species (Figure 5. 8), shows most cultivars of Clusters 1 to 4 fitted along the regression line, however, there are contrasting results between tulips that become heavily infected and produce high amounts of ethylene (Cluster 6, $r^2 = 0.85$) and those that show high infection levels but produce low amounts (Cluster 5, $r^2 = 0.14$). After removing cultivars of Cluster 5 (Figure 5. 8) the correlation among all cultivars had a quadratic function with a higher correlation (r^2 0.74), indicating that the response (phenotype) to *Fusarium* infection and ethylene production across cultivars in Cluster 5 differers from the rest of the tulips studied. These results indicate that cover rating is not an appropriate predictor of ethylene production, and that the combination of both are not good indicators of tulip bulb resistance to *Fusarium*.

Although ordinal cover rating provides a good visual quantitative value of tulip resistance to *Fusarium*, the Disease Severity Index (DSI) uses two parameters of *Fusarium* virulence: ordinal cover rating and % FW loss observed 28 days after inoculation. The DSI is a continuous variable which can be analyzed with the standard least squares method and allows mean separation between cultivars. The DSI model fit r^2 was 0.787 and detected highly significant differences between cultivars ($F=48.65$, $p <0.0001$). The results (Figure 5. 9) show three highly resistant cultivars, six resistant cultivars, thirteen susceptible cultivars, and the two species as highly susceptible.

The screening method for *Fusarium* resistance reported by van Eijk (1978) is applicable to infection under soil conditions, however, the results are subject to variability due to environmental elements. The DSI proposed in this Chapter is valid for controlled

conditions to enable screening of a large number of cultivars in less than one month while reducing experimental variability. DSI results should be compared with the model from van Eijk (1978) to validate the resistance reliability and reproducibility of cultivars under field conditions.

In Chapter 4, condensation occurred in the jars holding inoculated bulbs, which in the case of experiment 2 of this chapter, may have increased the variability of the results leading to poor mean separation (Figure 5. 6).

In Chapter 4 ethylene evolution and change in fresh weight in 'Leen van der Mark' (a high ethylene producer) were positively correlated (Figure 4. 10). In this chapter when screening several cultivars and species using the same parameters (data not shown), we observed that the overall correlation was highly influenced by individual cultivar or species response (similar to the observed by Cluster 5 in Figure 5. 8). We conclude that DSI is an appropriate tool that can be used to determine *Fusarium* resistance in tulip bulbs of various cultivars and species because its outcome is not influenced by individual cultivar or species variations.

Fusarium resistance and infection symptoms

Symptoms of *Fusarium* infection were documented for all screened tulips (Illustration 5. 2 and Illustration 5. 3) and are similar to those observed by Mukobata *et. al.* (1982) in resistant and susceptible cultivars. Highly resistant cultivars (cover score 1, DSI < 20) showed either minimal fungal growth around the inoculation wound (eg. 'Strong Gold'), or no fungal growth at all (eg. 'Bright Parrot', 'Negrita') and had low ethylene production. In resistant and some susceptible cultivars with cover scores of 2 and 3 (DSI 20-57), *Fusarium* colonized the base plate partially or completely, however, in most cases the external infection was contained by dark necrotic tissue surroundung the fungal growth area. In susceptible and highly susceptible cultivars with scores 4 and 5 (DSI > 60) *Fusarium* was observed at the end of the

experiment as a white ('Yellow Flight') or reddish ('Friso') mycellium growing on 'healthy' (*T. turkestanica*) or brown tissue ('Parade', *T. tarda*).

Resistance to *Fusarium* in flower bulbs may be achieved by structural or chemical mechanisms (Baayen 1992). Saniewska (2004) observed changes in the cell wall of tulip bulbs cv. 'Apeldoorn' inoculated with F.o.t., however, these apparent defense responses did not stop the invasion of the fungus. In experiment two, the edge of fungal growth in 'Apeldoorn' bulbs (cover rating 4) showed a delimited brown area (Illustration 5. 2). This symptom was also expressed in resistant cultivars such as 'Calgary', 'Cummins' and 'Ile de France' (Illustration 5. 3). In 'Strong Gold' fungal development was halted four days after inoculation (Video 5.3, second 5) and by 7 days (second 10) the appereance of brown tissue around the inoculation completely suppressed *Fusarium* infection. Cell death and the activity of polyphenyl oxidase results in tissue browning (van Rossum et al. 1997), which was observed around *Penicillium* in both inoculated and control bulbs of 'Ad Rem' and 'Strong Gold'(video 5.3). This response has the characteristics of a defense hypersensitive response (Lamb and Dixon 1997; Podwyszynska et al. 2001). Previous studies with explants of tulip bulbs *in-vitro* noticed that tissue browning was correlated with high tulipaline content (5 days after dissection, but not related to initial concentration) and non-regenerating tissue rates (van Rossum et al. 1998).

Upon pathogen recognition, plants are able to deploy a series of signals and defense mechanisms including activation of ion channels of the plasma membrane, oxidative burst (the first step of the hypersensitive response), and accumulation of phytoalexins (low lolecular weight antimicrobial compounds) in the cells surrounding the avirulent pathogen (VanEtten et al. 1994; Lamb and Dixon 1997; Zimmermann et al. 1997; Blumwald et al. 1998). It is known that tuliposides (the precursors of tulipaline) are able liberate tulipaline both enzymatically (at pH above 5.5), and chemically - slowly at pH 5.5, and quick ly at pH 7.5 (Beijersbergen and Lemmers 1972). Tulipaline is lethal to F.o.t. in concentrations >100 ppm (Bergman and Beijersbergen 1968; Beijersbergen and Lemmers 1972) and it has been suggested that it might

be stored in vesicles (van Rossum et al. 1998), however, it is unknown if upon pathogen attack the concentration of this lactone increases from tuliposide synthesis, or if it is produced *de novo*. Further evidence is needed to define if tulip bulb cells utilize tulipaline as a chemical defense tool against pathogen infection.

There is a group of tulips that show a high degree of infection (4-5), but are low in *Fusarium*-ethylene production. This group of bulbs is observed in Cluster 5 of Figure 5. 8. In particular *T. tarda* and 'Parade' showed high DSI (132 and 68, respectively) but ranked low on ethylene production (≤ 0.33 ul g^{-1} FW h^{-1}). Leaves of *T. tarda* showed absolute resistance to *Botrytis* infection and developed necrotic spots around inoculation sites (Straathof et al. 2002) indicating pathogen recognition and defense responses by the plant. The DSI of *T. tarda* and appareance of the bulb illustrate that the necrotic tissue was accompanied by large changes in FW. It is diffifult to identify the exact mechanism behind the responses of cultivars in cluster 5, and in specific *T. tarda* and 'Parade', however, we present two possible scenarios: the first example may be an extreme hypersensitive response, in which the bulb may undergo a cascade of toxic chemical reactions for both the tulip tissue (causing necrosis) and the fungus which may grow minimally (on a biomass basis); high transpiration rates from fungal activity (thus, low ethylene biosynthesis) along with uncontrolled water loss by the tissue may result. In the second scenario the bulb tissue does not show a hypersensitive response but possibly has low levels of arginine and proline, which are the ethylene substrates in the *Fusarium* ethylene-biosynthesis pathway (Hottiger and Boller 1991); in contrast to scenario one, *Fusarium* may colonize the bulb but low ethylene levels are produced in cultivars of Cluster 5 when compared to cultivars that show the same degree of infection but produce ca. 10 fold more ethylene (i.e. 'Leen van der Mark', *T. turkestanica*).

Resistance of tulips to *Fusarium* is of polygenic nature (van Eijk et al. 1979), and conferred by resistance (*R*) genes that provide the deployment of defense responses to initial stages of *Fusarium* infection. This possibly occurs by sensing ethylene and detection of microbe-associated molecular patterns (MAMPs, eg. chitin) with Pattern-Recognition

Receptors (Ecker and Davis 1987; Boller and He 2009), however, more work is needed to substatiate this.

Tulip bulbs possess lectins, which are proteins that reversibly bind to specific mono or oligo saccharides (Cammue et al. 1986; Oda and Minami 1986; van Damme and Peumans 1989; Peumans and Van Damme 1995). Lectins act as carbohydrate receptors and although it is still to be determined if they play a role in pathogen recognition (Cambi et al. 2005), it is known that they are involved in plant defense mechanisms against pathogens, insects, and animal predators (Sequeira 1978; Chrispeels and Raikhel 1991; Peumans and Van Damme 1995; Delatorre et al. 2007). The highest level of tulip lectin in cultivar 'Atilla' is mainly found in the scales of the bulb (10-30% of the total protein) at the time of planting and its concentration decreases (except in the outer scale of the bulb) as the plant develops. Since the fluctuation of lectin concurs with the resting period of the bulb and the growing phase of the plant, it is thought that this protein serves as a nitrogen reserve (van Damme and Peumans 1989), however, it has not been stablished whether it plays a role in tulips against pathogens.

There are two other known resources against pathogen attack in tulips. Chitinases were found in four species of *Tulipa* and some cultivars of *T. gesneriana*. Out of six isoforms of chitinases, TBC-2 and TBC-3 were detected in *T. tarda* and TBC-4, TBC-5 in *T. turkestanica* (Yamagami et al. 1998). Another defense system of tulip bulbs against pathogens, and specifically versus fungi are antimicrobial peptides (*Tu*-AMP 1 and *Tu*-AMP 2) which have 5-10 times more inhibiting growth activity against *F. oxysporum* than to bacteria (Fujimura et al. 2004).

Even though the *Fusarium*-tulip pathosystem has been documented from the mid-1900's, there is limited understanding of the resistance and susceptibility mechanisms in tulip bulbs, and the cues that trigger defense responses. Promising results have been observed in bulbs of 'Apeldoorn' that showed enhanced resistance to *Fusarium* when treated with D,L-*β*-aminobutyric acid (Jarecka and Saniewska 2007; Luzzatto-Knaan and Yedidia 2009).

The ethylene production data and tulip groups in this chapter should be useful to growers and bulb handlers who can use the information to make informed decisions on ventilation rates or bulb groupings during storage. Simmilarly, the information should be useful for breeders to open the way for breeding of low ethylene producing cultivars.

There is a wide range of approaches for future research which could expand on the genetic, molecular, and biochemical mechanisms involved in the defense responses by the bulbs which confer resistance or susceptibility to *Fusarium*, together with the metabolites involved in ethylene production by the fungus.

CONCLUSION

The capacity of *Fusarium* to infect and produce ethylene in tulip bulbs depends on the genetic resistance of tulip bulbs. The response to ethylene production and resistance to F.o.t. in 18 sport cultivars provided almost identical results to the original parents, and generational distance did not alter the expression of these traits. Tulips can be divided into five categories based on their potential to sustain ethylene production from *Fusarium* origin: low, medium low, medium, medium high, and high.

Ethylene production is not related to *Fusarium* resistance, however, the product of fresh weight loss and tissue colonization by *Fusarium* (Disease Severity Index) is useful to determine resistance to this fungus which can be broken down into: highly resistant, resistant, susceptible, and highly susceptible.

The biological mechanism of resistance in tulips to F.o.t. is not completely understood, nevertheless, we observed that defense responses versus *Fusarium* and other fungi might have components equivalent to a hypersensitive response.

CHAPTER FIVE APPENDIX

Table 5. 11 Compiled results of tulip cultivars and species inoculated with F.o.t.

Cultivar	Max. ethylene (ul g^{-1} FW h^{-1})		Most likely cover coding	Ordinal Fusarium cover	% FW Loss		DSI	
T. turkestanica	2.75	A	5	4.9	39.2	A	180.1	A
Friso	1.50	AB	4	3.5	22.6	BC	74.8	B-D
Oscar	1.33	AB	4	3.7	23.4	BC	81.9	BC
Mondial	1.10	A-C	3	3.2	18.4	C-G	56.1	C-E
Yokohama	1.02	B-D	4	3.5	16.7	C-G	54.0	C-F
Purple Flag	0.99	B-D	4	3.5	20.6	B-E	67.8	CD
Christmas Dream	0.96	B-D	3	3.1	19.0	C-F	56.4	C-E
Passionale	0.89	B-D	4	3.5	18.8	C-F	63.0	CD
Kikomachi	0.82	B-D	4	3.6	18.0	C-G	61.3	C-E
Yellow Flight	0.66	B-E	4	3.7	18.5	C-G	65.2	CD
Ile de France	0.53	C-E	3	2.9	16.2	C-G	43.0	D-G
Spryng	0.52	C-E	4	3.6	20.8	B-D	65.0	CD
Blue Ribbon	0.50	C-E	3	2.9	16.0	C-G	41.9	D-G
World's Favourite	0.47	C-E	4	3.7	22.6	BC	77.3	BC
Pink Impression	0.46	C-E	4	3.7	17.9	C-G	66.3	CD
Calgary	0.42	D-G	2	2.3	15.4	C-G	33.3	E-H
Cummins	0.31	E-G	2	2.4	11.5	D-G	24.1	G-I
Judith Leyster	0.30	E-G	2	2.2	14.9	C-G	31.8	F-H
Flaming Parrot	0.27	E-G	2	2.2	11.2	E-G	21.8	HI
T. tarda	0.26	FG	5	4.7	30.2	AB	131.5	AB
Parade	0.24	F-H	4	3.9	18.5	C-G	68.1	CD
Negrita	0.18	GH	1	1.3	13.3	C-G	15.0	IJ
Strong Gold	0.10	HI	1	1.5	8.5	G	11.0	J
Bright Parrot	0.06	I	1	1.0	10.5	FG	10.3	J

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CHAPTER SIX: AMINO ACIDS AND TULIPALINE A IN TULIP BULB ORGANS OF TWO TULIP CULTIVARS DO NOT INFLUENCE ETHYLENE FROM FUSARIUM ORIGIN AND ERGOSTEROL CONTENT

ABSTRACT

A time course study of *Fusarium oxysporum* f. sp. *tulipae* (F.o.t.) infection of tulip bulb organs of 'Leen van der Mark' *in-vitro* showed that the flower bud was the fastest organ to be colonized, and sustained the highest ethylene values (on a per gram basis) at 13 days post inoculation. Organs from whole inoculated bulbs had values with a similar trend from the *in-vitro* system but were more susceptible to contamination from other fungal species as age of the bulb increased.

Ethylene production in bulb organs of 'Leen van der Mark' (high ethylene sustaining cultivar) was several fold higher than in 'Strong Gold' (low ethylene sustaining cultivar). Although the amino acid content between organs of the two cultivars was similar, the flower bud and the base plate contained the highest amount of amino acids; arginine and proline were the most abundant. Fungal biomass in organs of each cultivar (expressed as ergosterol content) was primarily located in tissue with visible fungal growth. We hypothesized that ethylene production was correlated with tulipaline-A (Tul-A) content, however, our data did not fulfill this hypothesis. Preformed Tul-A in 'Leen van der Mark' was almost twice as high as 'Strong Gold', however, ergosterol in the later cultivar was up to 5 times lower than in 'Leen van der Mark'.

Tul-A doubled in potassium phosphate buffer after ten hours of extraction, but remained unchanged in water extracts. This result together with observations from live bulbs of the two cultivars lead us to believe that determination of this compound in crude water extracts of bulb tissue is not an indicator of the total potential of tulipaline content of the bulb,

and that in live tissue this compound may increase to fungitoxic levels in resistant cultivars such as 'Strong Gold'. Future experiments should: 1) compare Tul-A change in live tissue when challenged with *Fusarium*, and 2) compare water extracts with extracts made with buffer at pH 6.5 to induce tuliposide breakdown into tulipalines

INTRODUCTION

As with many plants, tulip bulbs employ certain physical and chemical mechanisms to overcome pathogen invasion. Bulb susceptibility or resistance to *Fusarium* infection results from the interaction between 1) the pathogen's arsenal of virulence genes which encode effector proteins, and 2) presence of resistance (R) genes of the host, which provide ability to recognize Pathogen Associated Molecular Patterns (PAMPs) and deploy defense responses (van Eijk et al. 1979; McDowell et al. 2003; Boller et al. 2009; Stergiopoulos et al. 2009).

Several species of the Liliaceae family contain antimicrobial and antifungal compounds, tuliposides A and B. Upon exposure to pH above 5.0 or enzymatic activity, they yield tulipalines - Tul-A and Tul-B (Beijersbergen 1969; Beijersbergen et al. 1972; Slob et al. 1975; Kato et al. 2009; Shiguetomi et al. 2010). In tulips, Tul-A (α-methylene-γ-butyrolactone) occurs in various proportions with Tul-B. Depending on the species and cultivar, total Tul-A can account for 0.15-1.5% (FW) of the flower bud (mainly in the pistils), and 0.1-0 and 0.4% (FW) of the white skin and the outer cells of the first scale. *Fusarium oxysporum* f.sp. *tulipae* is insensitive to Tul-B, and depending on the strain, its growth is fully inhibited by Tul-A at 100-300 ppm (Bergman 1966; Bergman et al. 1968; Beijersbergen 1969; Bergman et al. 1971; Beijersbergen and Lemmers 1972; Schönbeck et al. 1972; Shoji et al. 2005). There has not been conclusive indication if (and how) tulipalines are formed in tissues under fungal attack, however, if they result from mechanisms mentioned above they would be considered phytoanticipins (VanEtten et al. 1994; Osbourn 1999; Dixon 2001).

In higher plants, methionine is the precursor of ethylene, the gaseous hormone involved in many developmental phases of plants, and also in plant-pathogen interactions (Yang et al. 1984; Chen et al. 2003). In microorganisms, ethylene biosynthesis proceeds from two precursors 1) methionine via α-keto-γ-methylthiobutyric acid (KMBA); 2) glutamate via 2-oxoglutarate (Tudzynski et al. 2002). During infection to tulip bulb tissue, F.o.t. is able to produce ethylene. Like plants, ethylene biosynthesis in *Fusarium* is oxygen dependent but proceeds from the fungal pool of glutamate/2-oxoglutarate, and may require arginine as an enzymatic cofactor. Although proline stimulates higher ethylene yield at a faster rate than arginine, its role has not been fully clarified (Hottiger et al. 1991). Little is known about the amino acid content in tulip bulbs and their distribution in different organs. It has been reported that during storage of tulip bulbs, the most abundant amino acids in tulip pistils were arginine, glutamic acid, and proline; during 10 weeks of storage arginine increased, proline remained relatively unchanged, but glutamate decreased dramatically (Lukaszewska et al. 1989; Tonecki et al. 1990).

In axenic and *in-vitro* conditions, ethylene production by F.o.t. increased when the exponential phase of the fungus slowed down, and the dry-weight stopped increasing (Swart et al. 1977). One way of estimating fungal biomass in infected plants is by analyzing ergosterol, which is a compound unique to fungal cell membranes (Montgomery et al. 2000; Bååth 2001) .

In chapter five, we showed that F.o.t. inoculated on 'Strong Gold' bulbs caused minor infections and produced low amounts of ethylene, in contrast, high amounts of ethylene were produced and bulbs were fully colonized in 'Leen van der Mark'. In this chapter we studied the time course of ethylene production of F.o.t. inoculated onto detached and dissected organs of 'Leen van der Mark' *in-vitro*, and in organs of whole bulbs dissected 21 days post inoculation. We tested the hypothesis that different amino acid abundances and tulipaline content in bulb organs of 'Strong Gold' and 'Leen ven der Mark' lead to differential growth (fungal biomass) and ethylene production by *Fusarium*.

MATERIALS AND METHODS

Experiment one: In-vitro assay to study ethylene production by FOT in tulip bulb organs

Fifteen bulbs of 'Leen van der Mark' (a high-ethylene producing cultivar when inoculated with *Fusarium)* were dissected into individual organs on September 19, 2008. Bulb scales were cut into strips 1-2 cm wide by 3-5 cm long (≤ 2.1 g); while the flower bud and the base plate containing the root collar were not further dissected. Organs were surface sterilized by a 3 minute immersion in 70% ethanol, then 10 minutes in 10% v/v commercial bleach (0.62% final concentration of sodium hypochlorite, with 0.01% Tween® 80), and triple rinsed (2 minutes each) in sterile water. In this chapter, all control inoculations, solutions, analytical extractions and determinations were made with ultrapure water (18.2 MΩ·cm resistivity).

Five days prior to bulb dissection, 15 ml of (8 g l^{-1}) plant culture agar (Phytotech Laboratories, Shawnee Mission, KS) was poured as a slant into tissue culture test tubes (25 X 150 mm, Sigma Aldrich, St. Louis, Mo., Part. No. C-5916). One set of 60 tubes was inoculated with 24 ul of a 5 x 10^5 ml^{-1} F.o.t. conidia suspension (1.2 x 10^4 conidia $tube^{-1}$), incubated at 21C and 12h fluorescent light; 24 ul of sterile distilled water was added to 30 control tubes and kept under the same conditions. One explant of each of the tulip organs was placed in a test tube (n=10 inoculated, n=5 non-inoculated) and incubated in a complete randomized fashion as described above. All tubes were capped with a modified rubber stopper (Figure 6. 1) with a (0.785 cm^2) piece of Milliwrap® (Millipore corp.) which allowed gas exchange and kept water vapor inside the tube. Ethylene evolution was analyzed at intervals for 33 days. Prior to ethylene analysis, the internal tube atmosphere was flushed for 30 seconds with moist filtered-sterile air (1 l min^{-1}) introduced with a 20-gauge hypodermic needle through the rubber stopper. The centrifuge tube section of the rubber stopper was

closed with a screw cap for 30 minutes; then 1 ml sample of the headspace was collected to analyze ethylene as previously described. Between ethylene samplings, the screw cap was removed to allow air exchange. Each bulb explant was weighed before placing it inside the test tube and this value was used to calculate ethylene production (ul g^{-1} FW h^{-1}); maximal ethylene values were used to separate treatment means. All values were transformed with log + 0.001 for statistical analysis. Lastly, photographs of representative treatments were taken at 14 days post inoculation.

Data analysis

Data from most experiments in this chapter were analyzed with JMP 9.0 (SAS Institute Corp. Cary, NC.). Where indicated, ANOVA and mean separations were performed with Tukey's HSD test using the Standard Least Squares method.

Experiment two: Ethylene production by individual bulb organs from whole bulbs

The *in-vitro* assay (above) had several variables (e.g. large wound surface area, sterile conditions, and detachment of organs from the bulb) that differ from the conditions that a non-dissected bulb would have. In order to study the ethylene production of *Fusarium* infecting tulip organs in a whole living bulb, a second experiment was conducted.

On November 26, 2008 six bulbs each of cultivars 'Leen van der Mark' and 'Strong Gold' were inoculated with a conidia suspension of F.o.t. and incubated as previously described (Chapter 4). 'Leen van der Mark' and 'Strong Gold' are high and low-ethylene producing cultivars, respectively, when infected with *Fusarium*. At fourteen and 21 days post-inoculation, bulbs were dissected into individual organs, placed into a 1 pint (473 ml) glass jar, and weighed before analyzing for ethylene accumulation in the headspace (after 30 minutes). Unlike previously described in this dissertation, where ethylene production on a FW basis was calculated using the bulb weight at time zero, in this experiment FW at the time of sampling was used in the calculations.

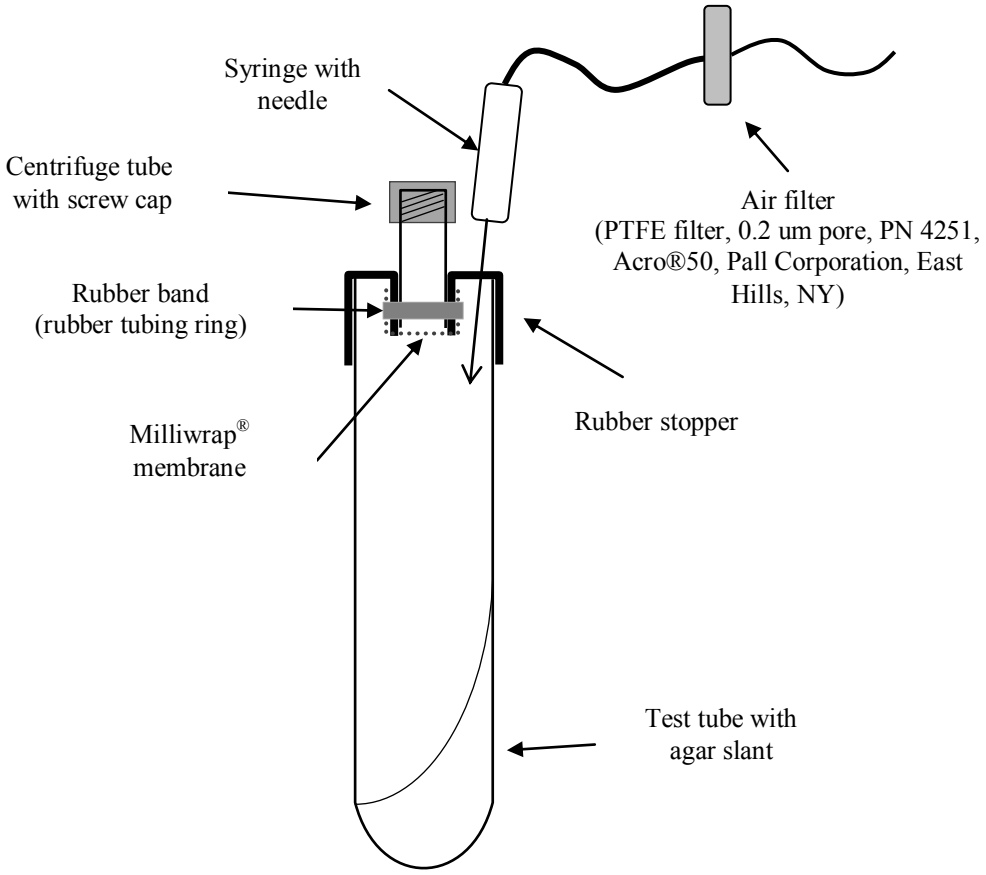

Figure 6. 1 Test tube with modified rubber stopper (not to scale) for *Fusarium* inoculation studies of tulip bulb explants *in-vitro*.

Experiment three: Determination of amino acids, Tul-A, ethylene, and ergosterol in inoculated and non-inoculated tulip bulb organs

In January 10, 2009 tulip bulb organs from six bulbs of cultivars 'Leen van der Mark' and 'Strong Gold' were freeze-dried and ground (20 mesh) for determination of amino acid, Tul-A, and ergosterol content. At the same time, a set of intact bulbs was inoculated (n=12) or non-inoculated (n=3) using sterile water. Amino acid and Tul-A content were determined from tissue only at time zero; ergosoterol was analyzed from tissue collected at 0 and 21 days post inoculation, and ethylene production was measured at 21 DPI. Ethylene determinations were made from whole bulbs, which were then dissected into individual organs. Each organ was further divided into tissue showing signs of fungal growth (any visible growth of *Fusarium*, contaminating fungi, or a mixture of both on the tissue) or completely healthy looking. *Penicillium* was consistently found growing with *Fusarium* on the same colonization area. Samples with fungal growth were kept and stored individually, whereas the tissue fraction from all organs of the same bulb with no fungal growth was pooled into a single sample. Ethylene was analyzed once more (2-3 hours later) then tissue samples were weighed, freeze-dried, and weighed again before determining ergosterol content.

Amino acid extraction

Amino acids were extracted following adjustments to a published protocol (Redgwell 1980). The main adjustments made to the Redgwell procedure were: 1) elimination of formic acid in the solvent (MCW) used for tissue extraction, 2) use of norleucine as internal standard, 3) fractionation of amino acids in a Strata X-C column instead of Sephadex ion-exchange resins, and 4) thorough column washing with water to remove sugars which interfered in the first 3 minutes of the chromatograms with the elution of arginine. The final procedure used is as follows: Tulip bulb tissue (30 ±0.5 mg DW) was placed into a 15 ml disposable centrifuge tube, to which 2.0 ml of MCW (methanol/ chloroform/water - 12:5:3 v/v) and 18 ul of 5

mMolar norleucine (internal standard, previously dissolved in 0.1N HCl) were added; the norleucine recovery rate was used to correct results. Tubes were briefly vortexed, sonicated (10 minutes), extracted in a water bath at 30C (30 minutes), and centrifuged (3,000 g for 5 minutes). The supernatant was decanted into a second disposable centrifuge tube. After extracting the tissue with MCW two more times, 1.0 ml of chloroform and 1.5 ml water were added to the combined extract solution which was vortexed and centrifuged (3,000 g for 10 minutes) to split it into two phases (chloroform remained at the bottom of the tube).

A 1.5 ml aliquot of the upper (aqueous) phase extract was added to a Strata X-C column (33 um 30 mg/3ml, Phenomenex, Inc. Torrance, Ca.) pre-conditioned with 2 ml methanol, then 2 ml water. The extract was eluted under vacuum and discarded. The column was washed with 3 ml of water and the eluate discarded. A second 1.5 ml tulip extract aliquot was added to the column and eluted as before. The column was washed twice with 3 ml water and the eluate discarded. The amino acid fraction was eluted by washing with 3 x 2 ml of 2.5 N NH_4OH in MeOH (approx. 5% NH_4), and 2 x 1 ml water. Once the amino acid fraction was dried under vacuum at 45C on a rotary evaporator, it was reconstituted with 6 ml water. Depending on the amino acid content, reconstituted samples were diluted 4 or 20 times with ultrapure water, and analyzed with the Amino Acid Analysis-Direct method using a Dionex 500 High Performance Anion Exchange Chromatograph with Pulsed Amperometric Detection (Dionex, Corp. Sunnyvale, Ca.) following a gradient (Hanko et al. 2004) program (See appendix).

Adaptation of tulipaline (Tul-A) extraction methods and HPLC analysis

Tulipaline extraction from tulip tissue is relatively simple and has been achieved by using either water (Christensen et al. 1999) or 0.1M potassium-phosphate buffer (PB) in 10% methanol at pH 5.2 (van Rossum et al. 1998). The tulipaline yield using these two solvents was evaluated as well as the stability of tulipaline in the extracts over time.

Freeze-dried tissue (40 mg, n=3) from a pooled sample of ten 'Friso' bulbs (susceptible to *Fusarium*) or 'Calgary' (*Fusarium* resistant) were extracted with 4 ml of water or PB in 10% MeOH at pH 5.2. The powder suspension was vortexed, sonicated for 20 minutes, centrifuged at 3,000 g for 3 minutes, and filtered with a 10 um syringe filter before analysis.

Each treatment was extracted separately and analyzed immediately to maintain incubation time at minimum. Twenty-six hours after extraction one PB and one water extract sample of each cultivar was analyzed again to determine whether tulipaline content had changed. Tulipaline values in water extracts were practically the same, nonetheless, an increase (12% 'Friso' and 20% in 'Calgary') was observed in PB extracts (data not shown). A second experiment was conducted to confirm this observation.

Determining tulipaline change in water or phosphate buffer extracts

In this experiment only tissue from 'Calgary' was used. Samples (20 mg, n=2) were extracted in either water or PB as described above. An authentic Tul-A standard diluted in water (25 nano moles ml^{-1}) was used as control. All samples were placed in an HPLC carrousel at the same time and each vial was periodically analyzed for more than 16 hours.

Final extraction method

In the previous experiment tulipaline increased in PB extracts after approximately 10 hours, but remained nearly unchanged in water. Further extractions were made using 20 mg of tissue and 2 ml of water as extractant.

Tulipaline (Tul-A) analysis

At time zero, tulip organs from intact tulip bulbs of 'Leen van der Mark' and 'Strong Gold' (n=6) were dissected, freeze-dried, extracted with water, and filtered with a 0.2 um syringe filter (Acrodisc® 25mm, 0.2 um; Pall Life Sciences, Ann Arbor, MI). Tulipaline was

analyzed by RP-HPLC using a Waters 2695 HPLC equipped with a photodiode detector, model 2996 (Waters Corp. Milford, MA). Twenty microliters of the extract were injected and separated with an Atlantis T3 C18 reverse phase column (5um, 250 x 4.8 mm, Waters Corp.). Solvent A (90:10 water:methanol) and solvent B (60:40 water:methanol) were used in a gradient as follows: 0-6 min 100% A, 6-35 min linear to 100% B, 35-40 min linear to 100% A, 40-50 min 100% A. The flow rate was 0.8 mL min^{-1}. Tulipaline A was detected at 208 nm and eluted at 15.2 min (SD = 0.373 min)

Tulipaline LC-MS analysis

Tulipaline identity was confirmed by HPLC-MS/MS analyses on a Quantum Access triple quadropole system (ThermoFisher LLC, San Jose, CA). Compounds were separated on a ThermoFisher Accela HPLC equipped with an Atlantis T3 C18 reversed phase column using the solvent gradient described above. The MS detector was equipped with an electro spray ionization (ESI) probe operated under the following conditions: spray voltage 4.5 kV, capillary temperature 300°C, sheath gas (N2) pressure 30 arbitrary units, auxiliary gas (N2) pressure 55 arbitrary units. Mass spectra were recorded in positive mode between m/z 100 and m/z 1200 to determine molecular ions [M+H]+. The Tul-A fragment pattern was analyzed by collision-induced dissociation (CID energy 15 V, CID gas (Ar) pressure 1.5 m Torr) of selected molecular ions and compared with those of authentic Tul-A standard (Sigma-Aldrich, St. Louis, MO).

Ergosterol analysis

Ergosterol was extracted and analyzed following an established protocol (Bååth 2001). Freeze-dried tulip tissue (40 mg) was weighed and placed in a 15 ml centrifuge tube, then 5 ml of 1.5M KOH in methanol was added and sonicated for 15 min. After incubating in a 70 C water bath for 90 min, 1 ml of water plus 2 ml of cyclohexane were added to each sample and vortexed before centrifuging at 3,000 g for 2 minutes. The upper phase (cyclohexane) was

transferred to a second test tube. Two ml of cyclohexane was added to the residue and water, and the tissue re-extracted. The combined extract was evaporated with N_2 at 40C, then 1.5 ml of methanol was added, sonicated for 10 minutes, passed through a 0.2 um syringe filter (Acrodisc® 13mm, 0.2 um; Pall Life Sciences, Ann Arbor, MI) and analyzed.

Ergosterol was determined with reverse phase HPLC (RP-HPLC) using a Waters 2695 HPLC equipped with a photodiode detector, model 2996 (Waters Corp. Milford, MA). Forty micro liters of the extract were injected and separated with a Nova-Pak column C18 (3.9 x 150 mm, Waters) using an isocratic MeOH gradient for 10 minutes at 25C. Ergosterol was detected at 282 nm and eluted at 6.1 minutes. An authentic ergosterol standard (Alfa Aesar, Ward Hill, MA) was used to generate standard curves.

Freeze-dried f.o.t. mycelium (grown in a 250 ml Erlenmeyer flask with 50 ml of 2% w/v Czapek-Dox broth shaking at 150 rpm), and freeze-dried pooled tulip tissue were either extracted (2 and 6 day-old, n=3), or spiked (4 day old, n=3) with ergosterol (18.75 ug sample^{-1}) to determine ergosterol:fungal biomass, or recovery rates.

RESULTS

Experiment one: In-vitro assay to study ethylene production by FOT in tulip bulb organs

In non-inoculated explants, the highest rate of ethylene production was $\leq$0.004 ul g^{-1} FW h^{-1} (Figure 6. 2). While ethylene evolution in most organs remained rather stable for eighteen days, a short-lived burst of ethylene was observed in the flower bud on day 12. Except for an incremental trend in the base plate at 27 days, no major changes were seen in all other organs.

In inoculated organs (Figure 6. 3), ethylene production by F.o.t. was highest when growing on buds after thirteen days of inoculation (4.4 ul g^{-1} FW h^{-1}, 1,100 fold higher than non-inoculated tissues), but decreased rapidly afterwards. Ethylene evolution in the remaining

organs increased linearly over 27 days. Values of the base plate and the 1st scale were lower than in the 2nd, 3rd and 4th scales and bud.

Data analysis (of only maximal ethylene values during the experiment) showed that the highest values were obtained in inoculated flower buds (5.16 ul g^{-1} FW h^{-1}), while no significant differences were observed between the rest of inoculated organs (Table 6. 1). Non-inoculated organs were significantly lower than the inoculated treatments.

Table 6. 1 Maximal ethylene values (ul g^{-1} FW h^{-1}) from bulb organ explants inoculated or non-inoculated *in-vitro* with *Fusarium oxysporum* f. sp. tulipae

Bulb organ	**Non-inoculated**	**Inoculated**
1st scale	0.002^{cd}	1.68^{b}
2nd scale	0.002^{d}	2.91^{ab}
3rd scale	0.002^{cd}	3.17^{ab}
4th scale	0.002^{cd}	3.40^{ab}
Base plate	0.004^{cd}	1.60^{b}
Bud	0.007^{c}	5.16^{a}
Sum	0.019	17.92

Values not connected by the same letter are significantly different ($p<0.0001$) using Tukey's HSD test with n=5 for controls, and n=10 for inoculated treatments

Images of bulb explants 14 days after dissection placed on inoculated or non-inoculated agar are shown in Figure 6. 4. No fungal growth was observed in non-inoculated organs, however, the tissue around the wound site of non-inoculated organs was surrounded by a brown line (i.e. 1st-3rd scale). *Fusarium* mycelium is visibly growing in inoculated organs and is less abundant in the 1st, 2nd scale and base plate compared with the 3rd, 4th scale and bud.

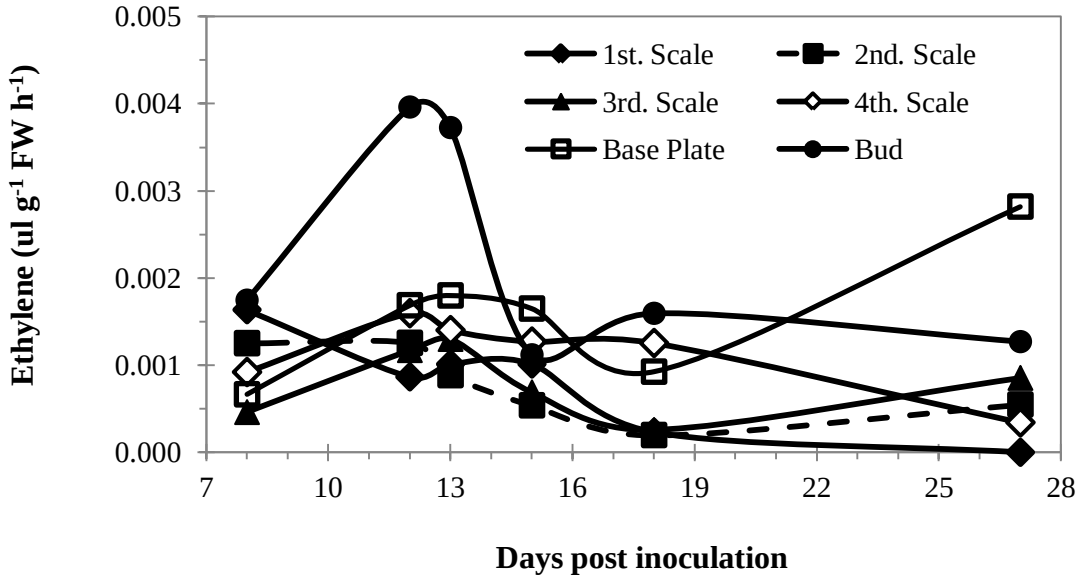

Figure 6. 2 Ethylene evolution in non-inoculated tulip bulb explants of 'Leen van der Mark'

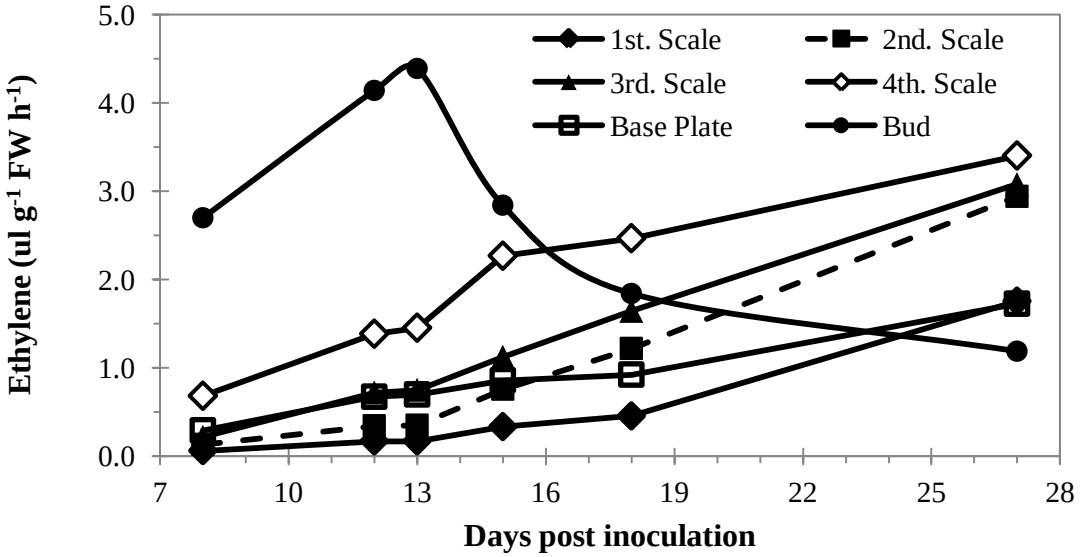

Figure 6. 3 Ethylene evolution in inoculated tulip bulb explants of 'Leen van der Mark'

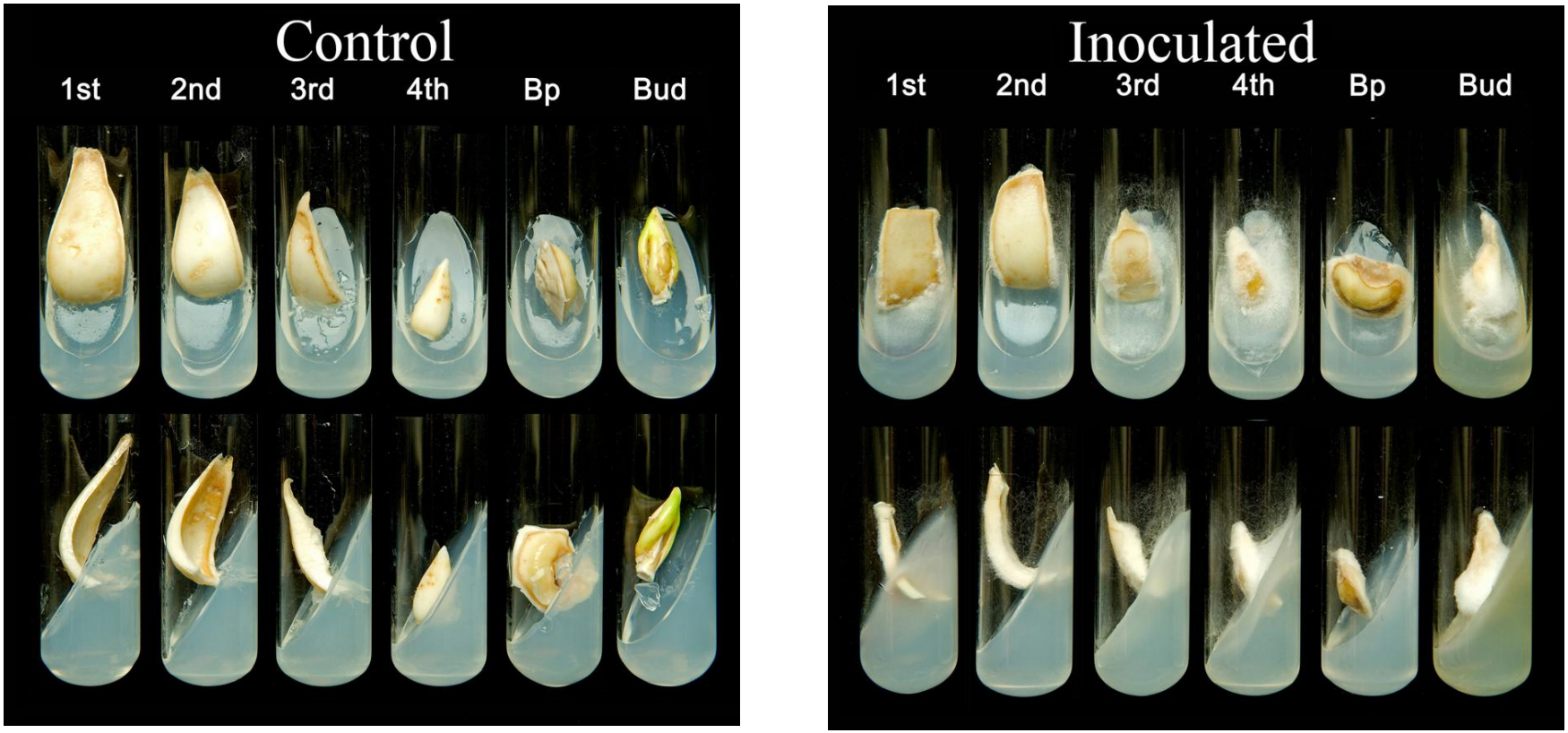

Figure 6. 4 Visual aspect of tulip bulb explants of 'Leen van der Mark' 14 days after dissection and placing onto non-inoculated (left) or F.o.t.-inoculated agar (right). From left to right: 1st scale, 2nd scale, 3rd scale, 4th scale, base plate, and flower bud.

Experiment two: Ethylene production by individual bulb organs from whole bulbs

Ethylene production in organs dissected from inoculated bulbs of 'Leen van der Mark' and 'Strong Gold' (dissected at 14 and 21 days post inoculation) was highly variable (Table 6. 2). *Fusarium* produced much less ethylene in 'Strong Gold' than in 'Leen van der Mark' in both ethylene per organ, and ethylene per gram of organ. Within 'Strong Gold' there was twice as much ethylene after 14 dpi than at 21 dpi for total ethylene production. In most organs of 'Leen van der Mark' ethylene values per organ, and per gram rose at different rates in between 14 and 21 DPI.

In terms of weight, the first scale was the largest organ (>7.5 g) in both cultivars comprising one-third of the bulb mass, nonetheless, at 21 DPI the ethylene produced in that organ represented only 5% (0.01 ul h^{-1}) and 2% (0.77 ul h^{-1}) of the total bulb ethylene in 'Strong Gold' and 'Leen van der Mark' respectively. The flower bud had the highest ethylene production in both cultivars, on a per gram basis, but values in 'Leen van der Mark' were more than 80 times higher than 'Strong Gold'.

Table 6. 2 Bulb organ FW and ethylene produced by *Fusarium oxysporum* f. sp *tulipae* in 'Strong Gold' and 'Leen van der Mark'

Strong Gold

Bulb organ	Organ FW (g)[y]	% organ FW relative to bulb	Ethylene per organ (ul h^{-1})		Ethylene organ FW (ul g^{-1} FW h^{-1})	
			14 dpi	21 dpi	14 dpi	21 dpi
1st. scale	7.55^{a}	34%	0.002^{c}	0.011^{bc}	0.001^{b}	0.003^{b}
2nd. scale	5.95^{b}	27%	0002^{c}	0.004^{bc}	0.001^{b}	0.002^{b}
3rd. scale	3.45^{c}	16%	0.004^{bc}	0.009^{bc}	0.002^{b}	0.004^{ab}
4th. scale	1.12^{e}	5%	0.112^{ab}	0.004^{bc}	0.085^{a}	0.004^{ab}
Base plate	2.62^{d}	12%	0.244^{a}	0.023^{bc}	0.083^{a}	0.010^{ab}
Bud	1.28^{e}	6%	0.112^{ab}	0.067^{abc}	0.083^{a}	0.073^{ab}
Sum	**21.95**	**100%**	**0.51**	**0.22**		
ul ethylene g^{-1} bulb FW			**0.02**	**0.01**		

Leen van der Mark

1st. scale	7.60^{a}	36%	0.15^{e}	0.77^{de}	0.02^{e}	0.10^{de}
2nd. scale	6.48^{a}	30%	3.84^{bcd}	9.08^{b}	0.57^{cde}	1.41^{bcd}
3rd. scale	3.44^{b}	16%	5.52^{bc}	10.95^{a}	1.47^{bc}	3.33^{ab}
4th. scale	0.70^{d}	3%	3.18^{bcd}	2.12^{cde}	4.02^{ab}	3.48^{ab}
Base plate	2.15^{c}	10%	4.57^{bcd}	6.62^{bc}	2.50^{bc}	2.61^{abc}
Bud	0.98^{d}	5%	2.38^{cde}	4.61^{bcd}	1.91^{bc}	6.12^{a}
Sum	**21.35**	**100%**	**19.64**	**34.13**		
ul ethylene g^{-1} bulb FW			**0.92**	**1.60**		

Values not connected by the same letter are significantly different ($p<0.0001$) using Tukey's HSD test with n=5 for controls, and n=10 for inoculated treatments.

[y] Averaged organ weight of 14 and 21 d.p.i

Experiment three

Method adaptation for Tulipaline-A extraction

Water extraction of 'Calgary' and 'Friso' yielded 26% and 12% more Tul-A than PB extraction (Figure 6. 5). Although the PB extract contained less Tul-A (which eluted at ca. 14.8 minutes), the chromatograms showed more abundant early-eluting peaks (retention time of 3-5 minutes) in the PB extract than in the water extract (see appendix).

In subsequent experiments, it was found that Tul-A levels changed over time in phosphate extracts starting ca. 8 hours after extraction. Tul-A levels doubled in 18 hours in PB; but did not change in water extracts (Figure 6. 6). (The consistency of the autosampler of the instrument was validated injecting a solution of Tul-A standard (0.5 nMoles per 20 ul injection) which showed constant and unchanged values during 20.5 h.

These results showed that water extraction was the most suitable method to maintain unchanged levels of Tul-A in the extracts for up to 20 hours, which represents the time that a set of samples would remain in the HPLC carrousel before analysis. Therefore, we chose water extraction for the following analyses.

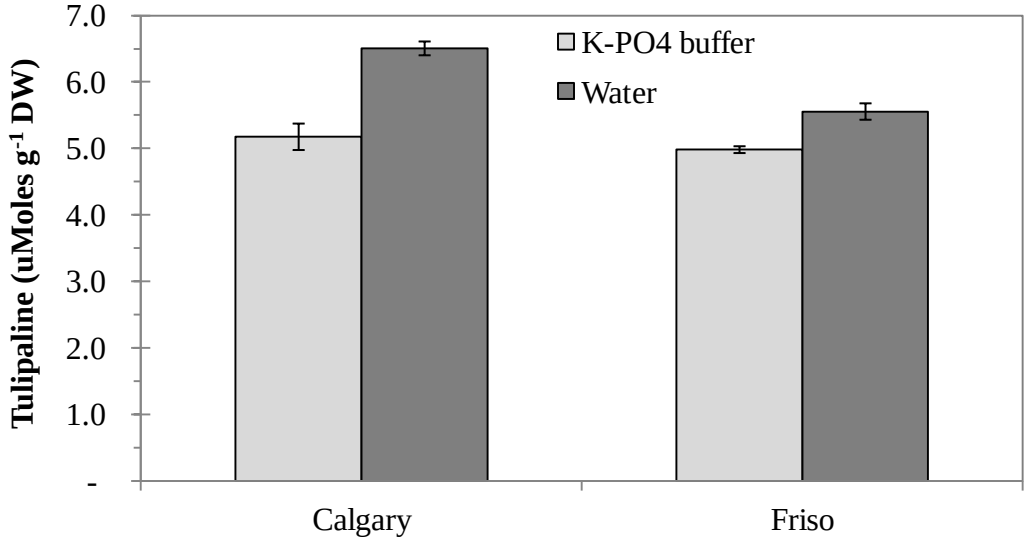

Figure 6. 5 Tul-A extracted from freeze-dried tulip tissue of 'Calgary' and 'Friso' with either phosphate buffer or ultra pure water. Error bars indicate one standard deviation (n=3)

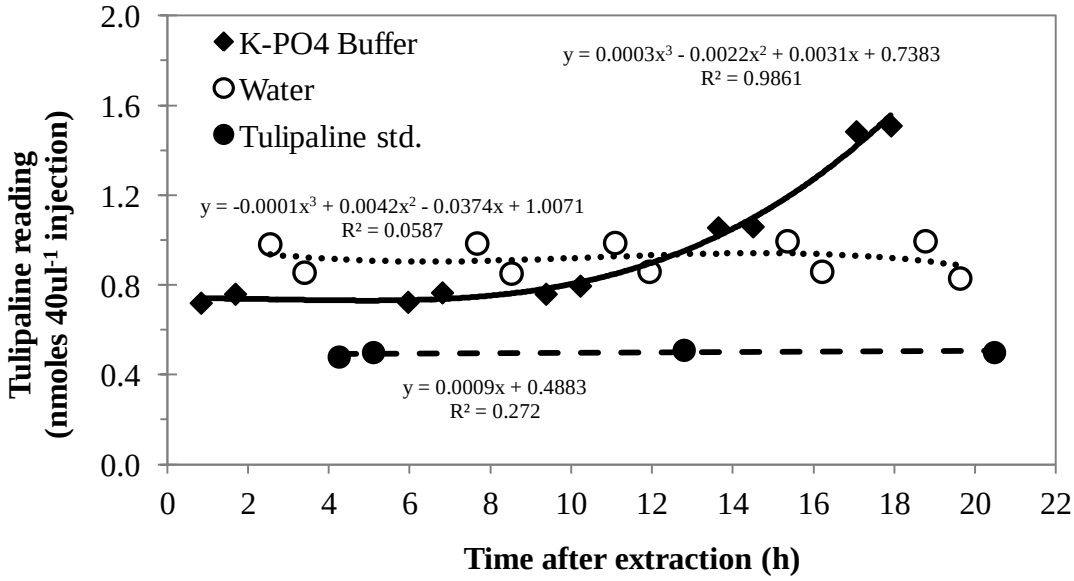

Figure 6. 6 Time course of Tul-A levels in tulip bulb extracts of 'Calgary' with potassium phosphate buffer or water. Tul-A standard (0.5 nmoles injection^{-1}) was used as control.

Tulipaline A in bulb organs

Tul-A was separated and determined by LC-MS. The ms-ms fragmentation pattern [M+1] of the authentic standard were 99, 81, and 53. These values matched with the fragmentation obtained from the tulip extract peak eluting at 15.3 min and detected at 208 nm. Although the Atlantis T3 C18 column was able to resolve Tul-A in the tulip extract, it was not able to resolve other tuliposides and tulipalines such as those seen in chromatograms by Christensen and Kristiansen (1999).

When summed across all organs, 'Leen van der Mark' bulbs had double the Tul-A content of 'Strong Gold' bulbs but they were not statistically different ($p < 0.05$). Compared with 'Strong Gold', 'Leen van der Mark' tended to have higher levels of Tul-A in 3rd and 4th scales, in the base plate (BP) and bud, however no differences were detected between the buds.

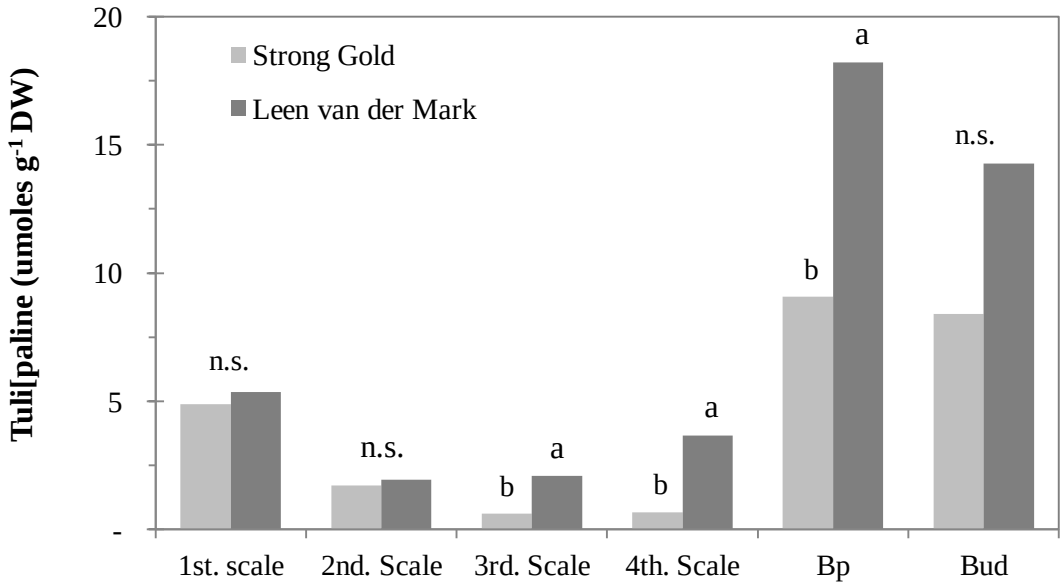

Figure 6. 7 Tulipaline content (umol g^{-1} DW) of tulip bulb organs of 'Strong Gold' and 'Leen van der Mark'
Values connected by the same letter are statistically different according to Student's t test ($p<0.05$). Non-significance is denoted n.s.

Amino acid content in bulb organs

Individual amino acids in bulb organs of 'Strong Gold' (Table 6. 3) and 'Leen van der Mark' (Table 6. 4) were similar in presence and abundance.

The sum of amino acids on a dry weight basis, across all tulip organs was 10% higher in 'Strong Gold' (524 umol g^{-1} DW) than in 'Leen van der Mark' (481 umol g^{-1} DW). The most abundant amino acids in both cultivars were arginine, proline and glutamine. Only five amino acids showed levels above 20 umol g^{-1} DW in 'Leen van der Mark', while in 'Strong Gold' there were eight. Arginine was the most abundant of all amino acids with 45% and 40% of the total amino acid content of 'Leen van der Mark' and 'Strong Gold' respectively. Interestingly, methionine (the ethylene amino acid precursor in higher plants) levels were twice as high in 'Strong Gold' (3.7 umol g^{-1} DW h^{-1}) than in 'Leen van der Mark' (1.8 umol g^{-1} DW h^{-1}) and represented only 0.4 to 0.8% of the total amino acid content of bulbs.

Buds contained 32% and 38% of the total content of amino acids in 'Leen van der Mark' and 'Strong Gold'. Each of the other organs of 'Leen van der Mark' contained less than 15% of the total amount. In 'Strong Gold' the results were similar, although the base plate contained 20% of the amino acid content of the bulb, and the bud contained 38%.

Table 6. 3 Amino acid content (umol g^{-1} DW) of tulip organs of 'Strong Gold'

Amino acid	1st scale	2nd scale	3rd scale	4th scale	Base Plate	Bud	Sum
Ala	4.77 ± 0.21	4.97 ± 0.97	4.03 ± 1.33	3.70 ± 1.84	4.83 ± 0.90	4.50 ± 0.56	26.8
Arg	18.57 ± 1.12	17.43 ± 3.19	13.50 ± 3.21	8.20 ± 1.84	25.73 ± 1.80	103.2 ± 14.14	186.7
Asn	0.10 ± -	0.10 ± -	0.10 ± 0.00	- ± -	0.10 ± 0.00	0.13 ± 0.06	0.5
Asp	0.07 ± 0.06	- ± -	- ± -	- ± -	0.13 ± 0.06	0.47 ± 0.15	0.7
Cys	0.10 ± 0.00	- ± 0.00	0.03 ± 0.06	0.05 ± 0.07	0.13 ± 0.06	0.20 ± 0.00	0.5
Gln	7.10 ± 1.13	6.63 ± 2.75	6.83 ± 2.42	5.25 ± 1.77	13.57 ± 3.44	10.77 ± 5.62	50.2
Glu	0.15 ± 0.21	- ± -	0.10 ± -	- ± -	0.33 ± 0.15	0.77 ± 0.35	1.4
Gly	0.20 ± 0.00	- ± -	4.00 ± -	- ± -	0.20 ± 0.00	0.25 ± 0.07	4.7
His	2.83 ± 0.32	2.30 ± 0.10	2.57 ± 0.21	2.20 ± 0.28	3.67 ± 0.78	3.83 ± 0.49	17.4
Ile	6.13 ± 0.55	4.07 ± 0.06	3.50 ± 0.89	2.65 ± 0.64	7.10 ± 0.10	6.93 ± 1.41	30.4
Leu	6.17 ± 0.70	4.80 ± 0.17	4.17 ± 1.12	3.05 ± 1.20	4.27 ± 0.64	2.50 ± 1.92	25.0
Lys	3.50 ± 0.78	2.60 ± 0.17	2.67 ± 0.80	1.90 ± 0.71	3.07 ± 0.60	2.87 ± 2.57	16.6
Met	1.13 ± 0.06	0.60 ± 0.00	0.47 ± 0.15	0.45 ± 0.21	0.83 ± 0.12	0.23 ± 0.06	3.7
Nor	2.43 ± 0.12	2.57 ± 0.12	2.50 ± 0.17	2.80 ± 0.42	2.47 ± 0.15	2.47 ± 0.25	*
Phe	3.57 ± 0.57	3.27 ± 0.15	3.13 ± 0.50	2.80 ± 0.85	3.80 ± 0.70	3.13 ± 0.92	19.7
Pro	7.90 ± 0.36	5.90 ± 0.61	4.73 ± 1.29	3.40 ± 0.28	12.73 ± 0.31	22.93 ± 1.21	57.6
Thr	2.90 ± 0.61	0.97 ± 0.35	0.53 ± 0.25	0.50 ± 0.00	3.47 ± 0.68	5.43 ± 1.16	13.8
Try	1.90 ± 0.20	1.60 ± 0.10	1.77 ± 0.15	1.85 ± 0.35	1.87 ± 0.25	1.53 ± 0.49	10.5
Tyr	3.40 ± 0.40	2.97 ± 0.15	3.00 ± 0.40	2.60 ± 0.71	2.40 ± 0.46	1.27 ± 0.64	15.6
Val	6.00 ± 0.78	4.63 ± 0.06	3.55 ± 1.63	2.25 ± 0.92	6.60 ± 0.53	4.63 ± 1.29	27.7
Total*	76.5	62.8	58.7	40.9	94.8	175.6	509.3

* Excludes Norleucine which was used as internal standard

± Indicate one standard deviation

Table 6. 4 Amino acid content (umol g^{-1} DW) of tulip organs of 'Leen van der Mark'

Amino acid	1st scale	2nd scale	3rd scale	4th scale	Base Plate	Bud	Sum
Ala	3.10 ± 0.00	2.63 ± 0.40	2.37 ± 0.35	1.60 ± 0.42	2.80 ± 0.66	4.30 ± 0.95	16.8
Arg	23.93 ± 3.67	29.97 ± 5.66	27.13 ± 4.44	32.05 ± 5.16	20.60 ± 1.87	74.6 ± 10.73	208.3
Asn	3.10 ± -	- ± -	- ± -	- ± -	0.10 ± -	0.13 ± 0.06	3.3
Asp	0.10 ± -	0.20 ± -	- ± -	- ± -	0.10 ± 0.17	0.95 ± 0.35	1.4
Cys	0.33 ± 0.06	0.10 ± 0.00	0.17 ± 0.12	0.15 ± 0.07	0.53 ± 0.06	0.80 ± 0.20	2.1
Gln	6.77 ± 0.25	5.33 ± 1.62	4.17 ± 0.91	4.10 ± 0.57	8.63 ± 1.60	5.80 ± 0.20	34.8
Glu	0.33 ± 0.31	0.10 ± -	0.00 ± -	0.00 ± -	0.15 ± 0.07	2.00 ± 0.44	2.6
Gly	0.15 ± 0.07	0.40 ± -	- ± -	- ± -	- ± -	0.27 ± 0.06	0.8
His	2.10 ± 0.53	2.00 ± 0.50	2.43 ± 0.71	2.75 ± 0.64	2.17 ± 0.61	3.50 ± 0.87	15.0
Ile	2.07 ± 0.64	2.63 ± 0.55	2.87 ± 0.76	2.65 ± 0.78	2.47 ± 0.49	3.27 ± 0.12	16.0
Leu	1.57 ± 0.55	3.50 ± 0.69	4.17 ± 1.10	4.40 ± 0.99	1.17 ± 0.42	3.57 ± 0.59	18.4
Lys	3.27 ± 1.07	2.93 ± 0.15	2.90 ± 0.85	4.65 ± 0.64	2.75 ± 1.06	8.40 ± 5.63	24.9
Met	0.33 ± 0.15	0.33 ± 0.06	0.30 ± 0.10	0.40 ± -	0.10 ± 0.00	0.30 ± 0.10	1.8
Nor	2.53 ± 0.15	2.50 ± 0.10	2.47 ± 0.12	2.60 ± -	2.67 ± 0.21	2.33 ± 0.12	*
Phe	1.67 ± 0.64	2.17 ± 0.45	2.37 ± 0.42	2.60 ± 0.57	1.60 ± 0.30	2.73 ± 0.25	13.1
Pro	5.57 ± 1.36	4.17 ± 0.47	3.73 ± 0.97	2.75 ± 0.07	7.40 ± 0.82	19.67 ± 0.42	43.3
Thr	3.77 ± 0.68	0.90 ± 0.10	0.90 ± 0.17	1.35 ± 0.50	5.23 ± 1.47	8.77 ± 1.51	20.9
Try	1.47 ± 0.46	1.30 ± 0.46	1.67 ± 0.57	2.90 ± 1.13	1.27 ± 0.32	1.93 ± 0.40	10.5
Tyr	1.40 ± 0.87	1.60 ± 0.62	1.93 ± 0.85	3.05 ± 1.34	0.93 ± 0.50	1.90 ± 0.17	10.8
Val	3.20 ± 1.30	3.40 ± 0.76	3.30 ± 1.30	2.10 ± 0.85	2.77 ± 0.80	4.67 ± 0.31	19.4
Total*	64.2	63.7	60.4	67.5	60.8	147.6	464.1

* Excludes Norleucine which was used as internal standard

± Indicate one standard deviation

Ethylene production

Ethylene values per bulb did not differ before or after dissection (after summing production from individual parts, data not shown). At 21 dpi, bulb organs from non-inoculated treatments showed zero or insignificant amounts of ethylene compared to inoculated treatments, which were several fold higher (Table 6. 5). On a per gram basis, *Fusarium* produced less ethylene in 'Strong Gold' bulbs than in 'Leen van der Mark', however, there were few significant differences between organs. Pooled tissue with no visible fungal growth did not produce ethylene, with the exception of traces in inoculated 'Leen van der Mark'.

Percent infected tissue from whole bulb

Most of the tulip bulbs showed various degrees of dehydration (wrinkling) and incidence of brown spots at the beginning of the experiment (January 10) due to the prolonged storage period. As the incubation time progressed (under humid conditions), it became common to observe contamination on the scales with other fungi (mostly *Penicillium*). These anomalies were not visible in experiments conducted before January.

The fresh weight fraction of the entire bulb with visible fungal growth in non-inoculated bulbs of 'Leen van der Mark' was approximately 35% compared to 80% in inoculated bulbs (Figure 6. 8). In contrast, the fraction of tissue with visible fungi (mostly contaminant fungi) in 'Strong Gold' was 42%, which did not differ significantly between inoculated or non-inoculated treatments.

Ergosterol content

The ergosterol:fungal biomass ratio was not influenced by the age of the fungus. Ergosterol concentration in 2 and 6 day old *Fusarium* mycelium was not significantly different, 3.96 and 4.22 ug ergosterol mg^{-1} DW mycelium, respectively (average 4.09 ug mg^{-1} DW).

When *Fusarium* mycelium or tulip bulb tissue were spiked with ergosterol (0.25 ug ml^{-1} extract), the recovery rate was 107.8% (±2.3 st. dev.), and 94.8% (±1.7 st. dev.) respectively.

The ergosterol content (mg g^{-1} DW) in healthy tissue of all tulip organs of the two cultivars collected at day zero was around 0.04 mg g^{-1} DW (Table 6. 6). We believe that these low readings are artifacts resulting from instrument noise, solvent contamination, or non-visible fungal growth since the non-infected tissue at day 21 showed almost null values. At 21 dpi the fungal growth in tissue of wounded but non-inoculated organs increased several fold in both cultivars (except in the 4^{th} scale and bud of 'Leen van der Mark'). Ergosterol content did not differ between the two inoculation treatments of 'Strong Gold'; similar results were observed in 'Leen van der Mark' except in the 4^{th} scale, base plate, and bud, which were significantly different than the non-inoculated treatments. Non-infected tissue of the non-inoculated and inoculated treatments showed almost no ergosterol compared to the rest of the organs of either cultivar.

Table 6. 5 Ethylene production (ul g^{-1} FW h^{-1}) from non-inoculated and inoculated tulip bulb organs 21 days post inoculation with or without visible fungal growth.

		Non-inoculated		Inoculated	
		SG	LV	SG	LV
Tissue with fungal growth	1st scale	0.000^{a}	0.006^{a}	0.005^{ef}	0.610^{abc}
	2nd scale	0.000^{a}	0.001^{a}	0.013^{def}	0.846^{ab}
	3rd scale	0.000^{a}	0.000^{a}	0.051^{cde}	2.060^{a}
	4th scale	0.000^{a}	0.003^{a}	0.035^{de}	1.304^{ab}
	Base Plate	0.000^{a}	0.000^{a}	0.134^{bcd}	2.697^{a}
	Bud	0.000^{a}	0.000^{a}	0.20^{a-d}	2.183^{a}
	Tissue with no fungal growth	0.000^{a}	0.000^{a}	0.000^{f}	0.002^{ef}
	Sum	**-**	**-**	**0.441**	**9.7**

Values connected by the same letter within a column are not significantly different according to Tukey's mean separation ($p<0.05$) with n=3 for controls and n=12 for inoculated treatments.

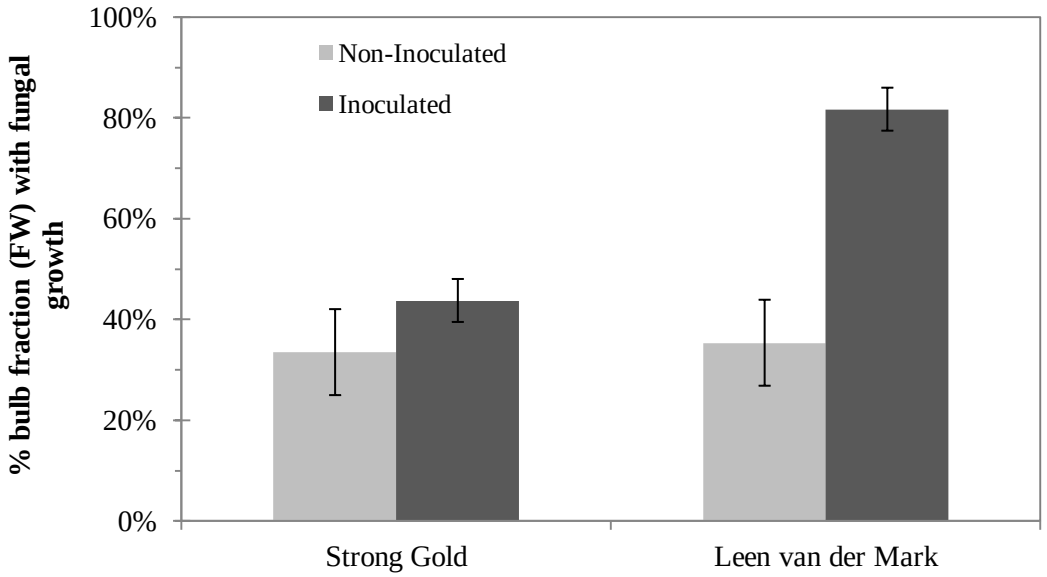

Figure 6. 8 Fraction of tulip bulb tissue from the entire bulb (wt/wt) of 'Leen van der Mark' or 'Strong Gold' with visible fungal colonization.
Error bars represent one standard error of n=3 for non-inoculated and n=12 for inoculated treatments

Table 6. 6 Ergosterol content (mg g^{-1} FW) in tissue fraction with visible fungal growth, which included contaminating fungi.

	Strong Gold			Leen van der Mark		
	0 DPI[y]	21 DPI		0 DPI	21 DPI	
Organ	Intact bulb tissue	Non-Inoculated	Inoculated	Intact bulb tissue	Non-Inoculated	Inoculated
1st scale	0.0445[a]	0.132[a]	0.162[a]	0.043[a]	0.311[bcd]	0.209[cd]
2nd scale	0.043[a]	0.171[a]	0.216[a]	0.043[a]	0.102[cde]	0.305[bcd]
3rd scale	0.042[a]	0.068[a]	0.122[a]	0.042[a]	0.108[cde]	0.377[bc]
4th scale	0.042[a]	0.278[a]	0.138[a]	0.042[a]	0.015[e]	0.914[ab]
Base plate	0.042[a]	0.314[a]	0.310[a]	0.042[a]	0.110[cde]	0.970[ab]
Bud	0.042[a]	0.267[a]	0.315[a]	0.043[a]	0.039[de]	1.580[a]
Non-infected[z]	---	0.000[b]	0.000[b]	---	0.000[f]	0.001[f]

Values connected by the same letter are not significantly different ($p<0.05$) according to Tukey's mean separation with n=3 for non-inoculated and n=12 for inoculated treatments

[y] Bulbs at the beginning of the experiment did not show visible signs of fungal colonization

[z] Non-infected refers to the pooled bulb tissue with no visible fungal growth

Correlation between ethylene production and bulb ergosterol content

The correlation between ethylene production (Log ul g^{-1} bulb FW h^{-1}) and ergosterol content (mg g^{-1} bulb FW) in inoculated and non-inoculated treatments of 'Strong Gold' was weak (r^2=0.16 and r^2=0.05 respectively, Figure 6. 9). Inoculated bulbs of 'Leen van der Mark' (Figure 6. 10) showed a cubic correlation (r^2=0.57); ethylene production peaked at 0.12 mg ergosterol g^{-1} bulb FW and decreased sharply beyond that point. While the values of non-inoculated treatments showed a linear trend (r^2=0.93) and were almost as high as the inoculated 'Strong Gold' they were much lower than the inoculated 'Leen van der Mark'.

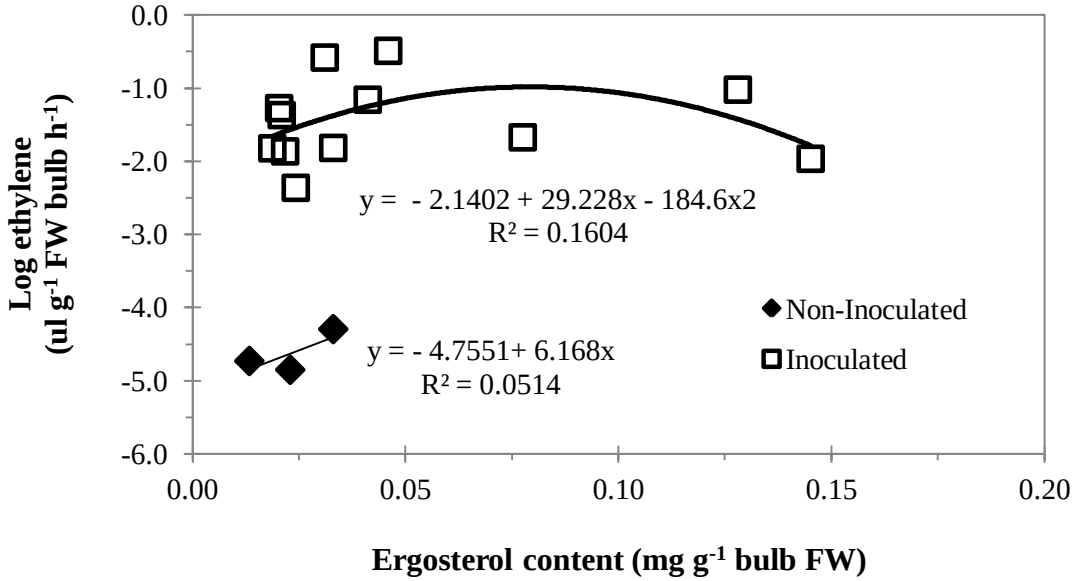

Figure 6. 9 Ergosterol content in non-inoculated and inoculated bulbs of 'Strong Gold'

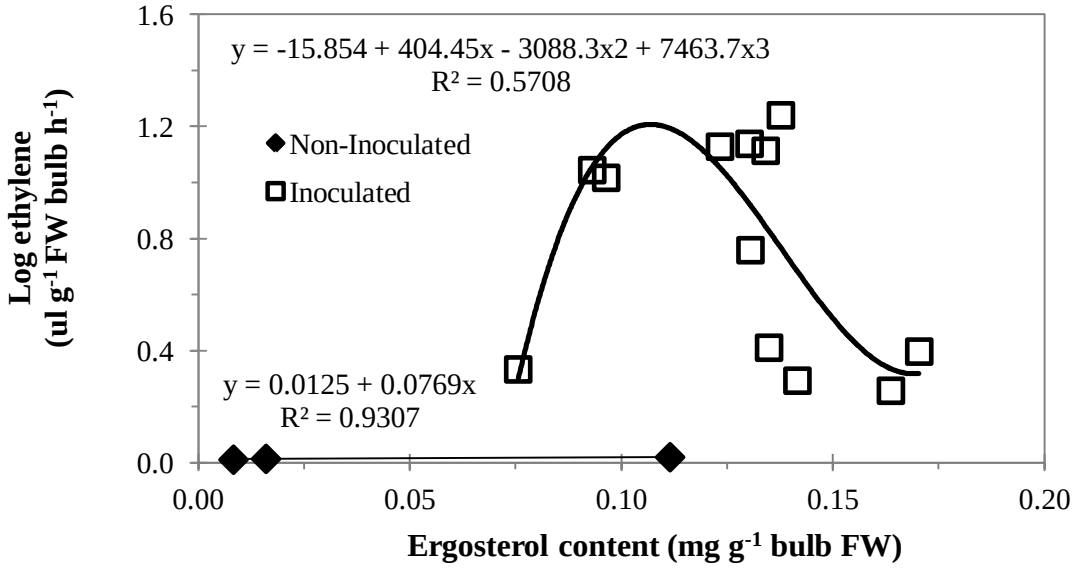

Figure 6. 10 Ergosterol content in non-inoculated and inoculated bulbs of 'Leen van der Mark'

DISCUSSION

Experiments one and two

Tulip bulb organs of 'Len van der Mark' were colonized by *Fusarium* at an unequal rate (Figure 6. 4). In experiment one, both ethylene production and visual colonization were fastest in the flower bud, whereas slowest in the two outermost scales and base plate. Periodic headspace analysis helped determine and analyze separately both ethylene evolution and maximum ethylene produced by *Fusarium* in each organ. Time course ethylene evolution showed that the inoculated flower bud produced 25 times more ethylene than the first scale at 13 dpi (Figure 6. 3); however, these differences were only 3 fold when analyzing maximum ethylene values these differences are not evident when comparing results of maximum ethylene values (Table 6. 1).

Large differences in amount and timing of ethylene production between non-inoculated and inoculated tulip bulb organs of 'Leen van der Mark' (experiments 1 and 2) were consistent with previous experiments (Chapter 4, Figure 4. 2).

As observed in Table 6. 2 the ethylene contribution of each organ to the pool of the whole bulb depends on its characteristics to sustain fungal growth and provide substrates for ethylene biosynthesis. Although the flower bud of 'Leen van der Mark' had the highest ethylene production on a per gram basis at 21 dpi (6.12 ul g^{-1} FW h^{-1}), accounting for 13.5% of the total ethylene production, it represented only 5% of the bulb weight. In contrast, the third scale produced about half the ethylene (fresh weight basis) of the flower bud but because it was three times larger, it produced more than twice the ethylene of the bud.

The *in-vitro* assay provided two main advantages over experiments with whole bulbs: 1) lack of microbiological contamination, and 2) ability to observe the progression of fungal colonization on the same tissue without further disturbance. Ethylene results of the *in-vitro* assay were analogous but not identical to experiments 2 and 3. Bulb explants had a large wound area, and due to surface sterilization washes the cellular content on the perimeter was

'washed off' (shown as white tissue delimited by brown line in the control scales, Figure 6. 4). Beijersbergen et al. (1971) noticed that tulipaline increased in macerated tissue, possibly to protect from microbial infection. It is likely that the "washed off" wounded cells on the bulb tissue lacked the capacity to increase tulipaline, providing a head start to the fungus. Variations to the *in-vitro* system can be made (container type, explant size, inoculation site, etc.) to design focused experiments such as real-time histological observations, time course photography, etc.

Experiment three

In experiment three, the levels of Tul-A were highest in the flower bud and the base plate of both cultivars with concentrations 2-13 times higher than in scales. The amino acid abundance between the two cultivars was similar and no differences were found in tulipaline content, however, ethylene and ergosterol were highly dissimilar. Preformed levels of Tul-A did not play any role in ethylene produced by the fungus. Tul-A resulting from depolymerization of tuliposides may play a role in fungal growth and ethylene produced by the fungus.

Amino acids

Because of the lack of amino acid differences between the two cultivars, differences in ethylene production between the two cultivars were not due to availability or scarcity of the amino acids involved in the *Fusarium* ethylene biosynthesis pathway (Table 6. 3 and Table 6. 4).

Interestingly, the amino acid content in 'Strong Gold' (low ethylene producer) was almost 10% higher than 'Leen van der Mark' (high producer). Hottiger and Boller (1991) fed *Fusarium* with several amino acids and saw twice as much ethylene produced with proline feeding than with arginine, while glutamate did not cause significant changes. Although proline concentration (on a dry wt basis) in 'Strong Gold' was 33% higher than 'Leen van der

Mark' glutamate and arginine were 50% and 11% below the later cultivar. Approximately >30% of the amino acid fraction of the bulb was found in the flower bud of both cultivars, where glutamate, arginine, and proline (up to 55% of the bulb content) may have contributed to the high ethylene produced in that organ. Based on the ethylene conversion rates of individual amino acids reported by Hottiger and Boller, the arginine:proline ratio in freeze-dried flower buds of 'Strong Gold' and 'Len van der Mark' (3:1 and 5:1 respectively) suggest that most of the ethylene synthesized by *Fusarium* may proceed from these amino acids.

Tulipaline

Because 'Strong Gold' does not support high level of ethylene production upon *Fusarium* infection, we hypothesized that intact bulb organs of 'Strong Gold' would have higher levels of Tul-A than 'Leen van der Mark'. Contrary to what we expected, the levels of tulipaline in 'Leen van der Mark' were twice as high (although not statistically different) as 'Strong Gold' (Figure 6. 7). This finding did not support our hypothesis. No ethylene was detected in tissue with visible fungal growth in non-inoculated treatments (Figure 6. 8). Inoculated bulbs of 'Strong Gold' had equivalent fungal growth fraction (mostly *Penicillium*) as the non-inoculated, but diverged in the amount of ethylene produced (Table 6. 4). In contrast, inoculated 'Leen van der Mark' bulbs had twice as much tissue with fungal growth and each organ produced between 10-100 times more ethylene than those of 'Strong Gold'.

Tulipaline in 'Friso' and 'Leen van der Mark' (high ethylene producing cultivars) was higher than low ethylene producing cultivars ('Calgary' and 'Strong Gold'). During the Tul-A extraction experiment, Tul-A in extracts of both 'Friso' (high ethylene producing) and 'Calgary' (low ethylene producing) increased over time. In 'Calgary' extracts, Tul-A remained stable for 10 hour then started increasing, and doubled by 18 hours (Figure 6. 6). This change is likely due to tuliposides cleaving into tulipalines. This process takes place by: 1) chemical breakdown at pH above 5.0, and 2) enzymatic activity with half activity pH 5.5, and maximal activity pH 6.5 at 25°C (Beijersbergen and Lemmers 1972; Kato et al. 2009).

Although we do not have additional data to fully explain these phenomena, we presume that the phosphate buffer (with 10% MeOH) allowed slow chemical cleavage and/or enzymatic activity. Apparently, 10% methanol concentration in the buffer was insufficient to inhibit the conversion.

Botrytis tulipae has mechanisms that make it a specialized pathogen of tulips. Tulip pistils inoculated with *B. cinerea* had higher cell permeability and tuliposide increase than with *B. tulipae*. While *B. cinerea* has shown high sensitivity to tuliposides, *B. tulipae* is insensitive up to 2.5 mM. When tuliposides were added to *B. cinerea* cultures, they were almost completely cleaved into tulipaline A and B, however, no change occurred in *B. tulipae*. Tuliposides were found in healthy tissue but not in tissue infected with *B. tulipae*; the transition zone had less tuliposide than the healthy tissue. Only when tuliposides were leached out of the pistils *B. cinerea* was able to colonize the tissue (Schönbeck and Schroeder 1972; Shiguetomi et al. 2011). The degree of infection of *B. tulipae* on tulip plants is dependent on the cultivar and developmental stage. Straathof et al. (2002) found that tulip leaves inoculated with *B. tulipae* after flowering were much more prone to infection than before anthesis; while *T. tarda* was completely resistant, 'Leen van der Mark' was very susceptible.

Similar to *B. tulipae*, susceptibility or resistance of tulip cultivars to *F.o.t.* pathogens is cultivar-dependent and polygenic in nature (van Eijk et al. 1979). Plants can detect and respond to infecting microorganisms by sensing pathogen associated molecular patterns - PAMPs (Boller and He 2009), which are likely produced as *Fusarium* conidia germinate (4-6 hours in water agar). Several species of pathogenic fungi, including *B. tulipae,* have the ability to detoxify phytoalexins via enzymatic degradation (Pedras et al. 2005; Shiguetomi et al. 2011), however, there are few reports on the biochemical (Beijersbergen 1969; Shiguetomi et al. 2011) and molecular specialization features of F.o.t.

When combined in intact tissue, tuliposides (serving as storage compounds) and tulipalines work as phytoanticipins against a broad range of microorganisms. Similar to what happens with amino acids (Tonecki and Gorin 1990), starch (Gorin et al. 1985), lectins (van

Damme et al. 1989), and polyamines (Kollöffel et al. 1992), changes in the tuliposide:tulipaline ratio is influenced by environmental cues and orchestrated by phenological stages of the bulb. The suspected increase in tulipalines synthesized from tuliposide upon pathogen invasion is another feature of phytoanticipins (Bergman and Beijersbergen 1971; Beijersbergen and Lemmers 1972; Schönbeck and Schroeder 1972; Shoji et al. 2005).

In our experiments, 'Strong Gold' appeared to detect and react to F.o.t. while 'Leen van der Mark' did not. We have repeatedly observed that a few days after infection 'Strong Gold' suppresses *Fusarium* growth, and this concurs with a drop in ethylene readings (Table 6. 2, and Chapter 5: Figure 5. 4, videos 5.1 and 5.2).

Previous work and our own results of Tul-A content in PB extracts indicate that the level of Tul-A may change rapidly, and that the amounts present at the time of infection are not related to the likelihood of *Fusarium* to cause infection. In order to identify the full (converting) potential of tulipaline in tulip tissues, future analyses should compare tulipaline in water extracts vs. extracts made in PB at pH 6.5 and incubated at 30°C to maximize enzymatic conversion of tuliposides into tulipalines (Beijersbergen and Lemmers 1972; Kato et al. 2009). Further evidence is needed to determine if tulipaline changes *in-vivo* during *Fusarium* infection. Additional time course studies should determine how F.o.t. triggers, avoids, or escapes defense responses of resistant and susceptible cultivars.

Ergosterol

Montgomery et al. (2000) used a correction formula to compensate for the ergosterol recovery (40-77%) from *Fusarium oxysporum* mycelium. We omitted this step because the recovery rate from ergosterol-spiked tulip tissue was approximately 95%. Fungal biomass (expressed as ergosterol content) was mainly located in the tissue with visible fungal growth (Table 6. 6), which is where ethylene emanated almost exclusively. The ergosterol:fungal biomass ratio that we obtained (244.5 ug ergosterol ug^{-1} *Fusarium* dry wt.) is similar to values reported by Montgomery et al. (2000).

As the age of the bulbs increased through the fall storage season, so did the incidence of superficial fungal contamination. At 21dpi, ergosterol content was highly affected by contaminating fungi, as can be seen in non-inoculated organs of both cultivars (Table 6. 6) and previous experiments (videos in Chapter 5). In contrast, tissue with no visible fungal growth of non-inoculated and inoculated organs of 'Strong Gold' or 'Leen van der Mark' had minute amounts of ergosterol. Since ergosterol determination is not selective for specific fungus species, the *in-vitro* assay may be a better approach to determine ergosterol from *Fusarium* and to remove the contamination variable.

Correlation between ethylene production and ergosterol content

Previous experiments showed that the exponential phase of ethylene production was reached at 21 dpi, therefore sampling on this date was chosen to study *Fusarium* growth. Although no correlation was observed when plotting ergosterol content vs. log ethylene in 'Strong Gold' (Figure 6. 9) there was a weak binomial relationship between ergosterol and log ethylene in inoculated 'Leen van der Mark' (Figure 6. 10). The results of inoculated 'Leen van der Mark' might be explained by the work of Swart and Kamerbeek (1977) who showed that maximal ethylene production in F.o.t. *in-vitro* coincided with the deceleration of the exponential phase of fungal biomass (or onset of senescence). Interestingly, they did not find a correlation between total ethylene production and fungal biomass. Our results show that the

asynchronous ethylene production that we have continuously observed can be attributed to the different physiological stages of *Fusarium* development of senescence.

Final remarks

The Tul-A and amino acid data from the organs of the two cultivars do not provide sufficient evidence to explain the disparity in ergosterol and ethylene values (Table 6. 5).

It is known that the pistils contain substantial amounts of tuliposide B, and the presence of this compound in several tissues of tulip species is concomitant with various amounts of tuliposide A (Beijersbergen and Lemmers 1972; Schönbeck and Schroeder 1972; Slob et al. 1975; Shoji et al. 2005). We can hypothesize that as the fungus colonized the flower bud and used up all the amino acids, the (possibly immobile) localization of tulipaline A in the pistils may have not had any effect on ethylene production by the fungus. Since the HPLC column that we used did not resolve tuliposides it is hard to determine if 'Strong Gold' could have high amounts of tuliposides which could eventually be converted into tulipalines upon fungal recognition.

CONCLUSION

Ethylene produced by F.o.t. at a particular time is dependent on the physiological phase of the fungus and extent of colonization in any given organ, but it is not related to fungal biomass. The potential of *Fusarium* to infect organs of a susceptible ('Leen van der Mark') and a resistant ('Strong Gold') cultivar was influenced by: the genetic ability of the tulip cultivar to detect the fungus and their defense responses. The content of substrates (mainly proline and arginine) involved in stimulating the fungal ethylene biosynthesis pathway were not decisive factors for infection or total ethylene production between the two cultivars. However, total ethylene production per organ in the susceptible cultivar was determined by the ethylene production rate (ul g^{-1} FW h^{-1}) and the total weight of the organ (g).

Tul-A extracted from bulb tissue with phosphate buffer at pH 5.2 in 10% methanol changed after 10 hours, but remained stable when water is used as solvent. Analyses must be done within 20 hours after extraction to avoid artifact values due to sudden chemical change or enzymatic cleavage.

Determination of preformed Tul-A in organs of intact bulbs does not provide a robust measure of the inhibitory capacity of the bulb to halt *Fusarium* colonization. However, ergosterol content of bulbs can be a useful tool to determine fungal biomass. The *in-vitro* assay that we developed reduces biological contamination and can be a useful tool to study fungal colonization in tulip tissue.

Further biochemical and molecular evidence is needed to better characterize the Tulip-*Fusarium* pathosystem.

CHAPTER SIX APPENDIX

Program to analyze amino acids with HPAEC-IC using a Dionex 500

Pressure.LowerLimit = 200 [psi]
Pressure.UpperLimit = 3000 [psi]
%A.Equate = "Millipore Water"
%B.Equate = "250mM Sodium Hydroxide"
%C.Equate = "1.0M Sodium Acetate"
%D.Equate = "0.1N Acetic Acid
Pump_InjectValve.State LoadPosition
Cell = On
Data_Collection_Rate = 1[Hz]
Temperature.nominal 30 [°C]
Electrode = pH
pH.LowerLimit = 10.0
pH.UpperLimit = 13.5
Waveform Time = 0.00, Potential = 0.13
Waveform Time = 0.04, Potential = 0.13
Waveform Time = 0.05, Potential = 0.33
Waveform Time = 0.21, Potential = 0.33, Integration = Begin
Waveform Time = 0.22, Potential = 0.55
Waveform Time = 0.46, Potential = 0.55
Waveform Time = 0.47, Potential = 0.33
Waveform Time = 0.56, Potential = 0.33, Integration = End
Waveform Time = 0.57, Potential = -1.67
Waveform Time = 0.58, Potential = -1.67
Waveform Time = 0.59, Potential = 0.93
Waveform Time = 0.60, Potential = 0.13

-0.900 Pump_Relay_1.Closed
Duration=138.00
Flow = 0.250 [ml/min]
%B = 4.0 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 5

0.000 Autozero
ECD_1.AcqOn
Pump_InjectValve.InjectPosition
Duration=30.00
8.000 Flow = 0.250 [ml/min]
%B = 4.0 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 5

14.0 Flow = 0.250 [ml/min]
%B = 36.0 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 8

17.0 Flow = 0.250 [ml/min]
%B = 36.0 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 8

24.0 Flow = 0.250 [ml/min]
%B = 20.0 [%]
%C = 40.0 [%]
%D = 0.0 [%]
Curve = 8

27.0 Flow = 0.250 [ml/min]
%B = 16 [%]
%C = 40.0 [%]
%D = 0.0 [%]
Curve = 5

29.0 Flow = 0.250 [ml/min]
%B = 16 [%]
%C = 70 [%]
%D = 0.0 [%]
Curve = 8

45.9 Flow = 0.250 [ml/min]
%B = 16 [%]
%C = 70 [%]
%D = 0.0 [%]
Curve = 5

46.0 Flow = 0.250 [ml/min]
%B = 0 [%]
%C = 0 [%]
%D = 100.0 [%]
Curve = 8

48.0 Flow = 0.250 [ml/min]
%B = 0 [%]
%C = 0 [%]
%D = 100.0 [%]
Curve = 5

48.1 Flow = 0.250 [ml/min]
%B = 80 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 8

50.1 Flow = 0.250 [ml/min]
%B = 80.0 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 5

50.2 Flow = 0.250 [ml/min]
%B = 4.0 [%]
%C = 0.0 [%]
%D = 0.0 [%]
Curve = 8

81.0 ECD_1.AcqOff
Flow = 0.250 [ml/min]
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%C = 0.0 [%]
%D = 0.0 [%]
Curve = 5

End

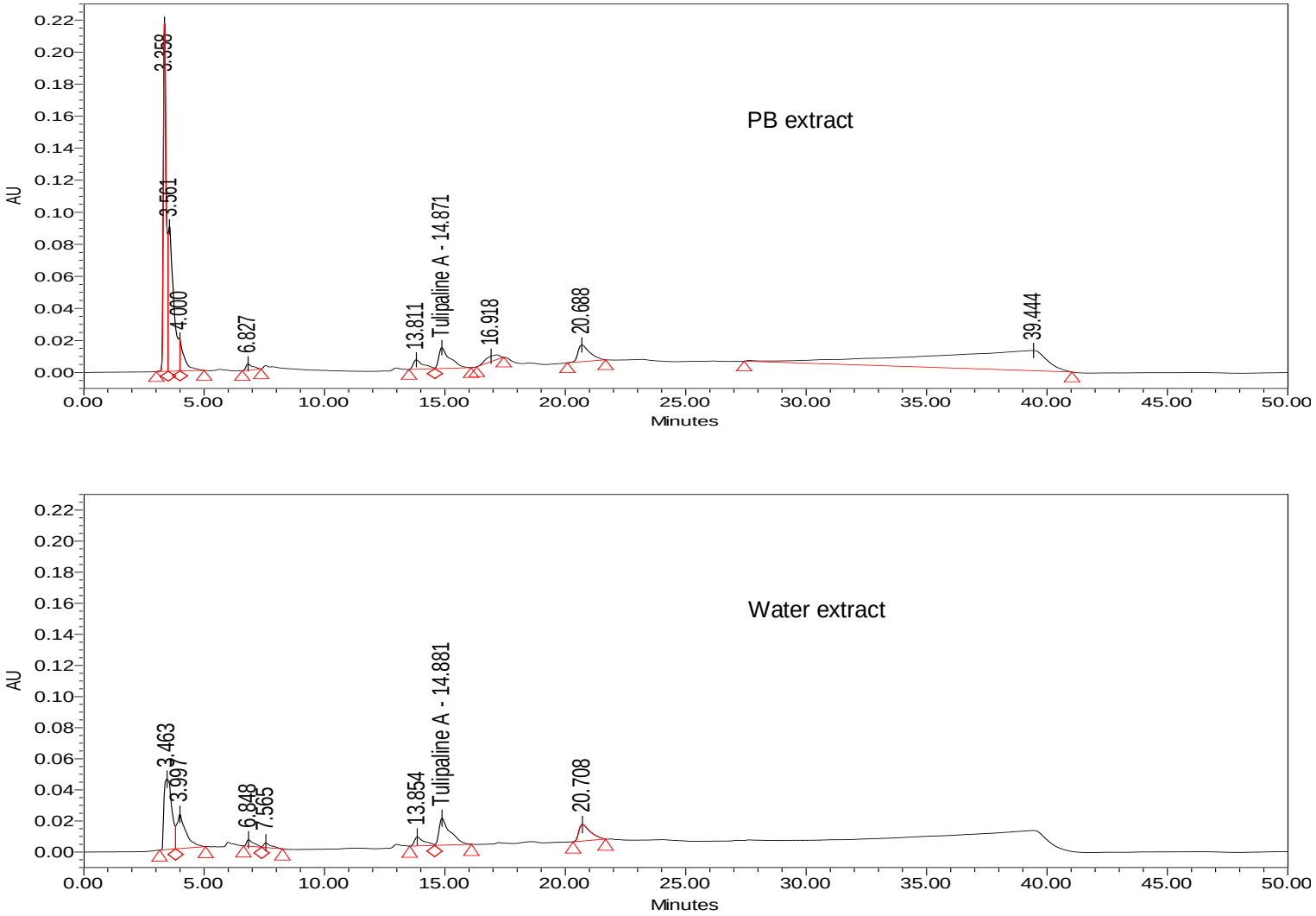

Figure 6. 11 Tulipaline chromatograms showing compound profile obtained from PPB and water extracts of 'Calgary'

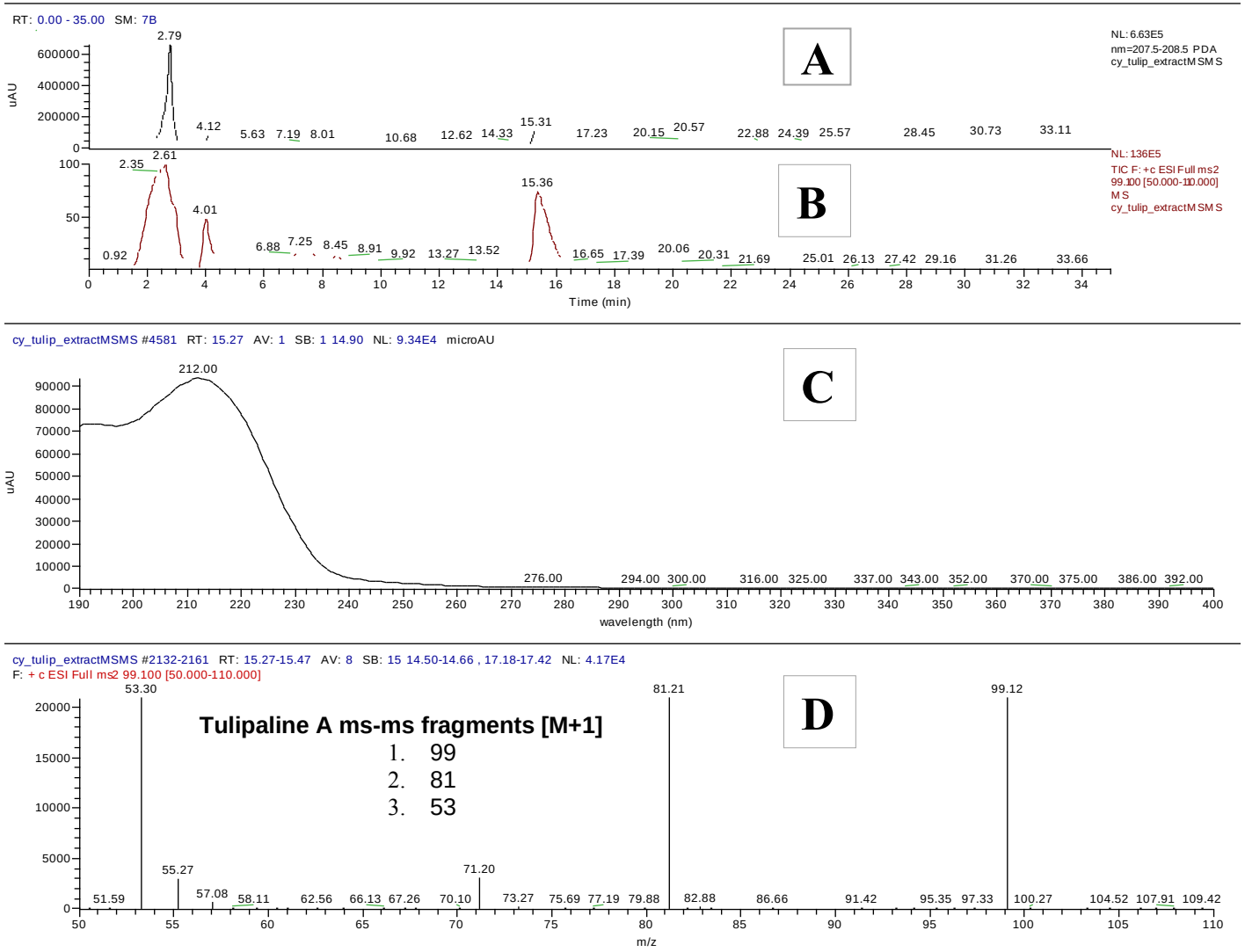

Figure 6. 12 LC-MS characterization of tulipaline-A: A and B) Chromatograms of tulipaline-A standard, C) UV extinction spectra of tulipaline-A, D) tulipaline-A ms-ms fractionation

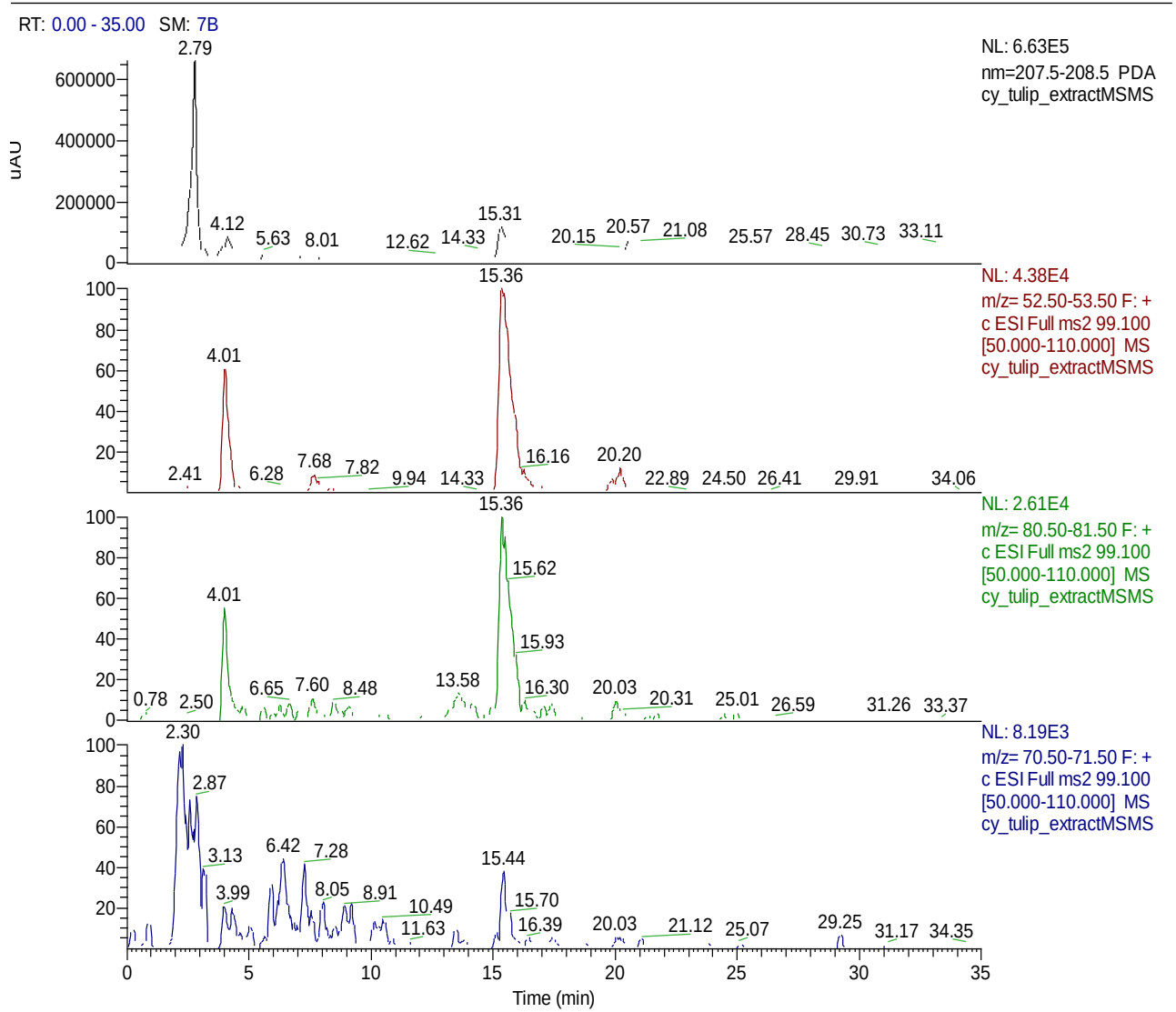

Figure 6. 13 Chromatogram (207.5-208.5 nm) and mass spectra of tulipaline A (15.36 min.) showing different m/z profiles

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