Variation in *Fusarium*-induced Ethylene Production among Tulip Cultivars

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Abstract

Ethylene evolution is a well-known consequence of *Fusarium* infection of tulip (*Tulipa gesneriana*) bulbs, yet little is known about the bulb-pathogen interactions involved in the induction or the time course of ethylene production in the infected bulb. Bulbs of 36 tulip cultivars were wounded, inoculated with a liquid *Fusarium* suspension (isolated from infected bulbs, and grown on agar plates) and held at 21°C. Control bulbs were wounded, but not inoculated. Ethylene production was monitored by headspace accumulation and gas chromatography. The results indicated that ethylene could increase rapidly after a lag phase of at least 8 days after inoculation, but there was a great variability in ethylene production among cultivars. Of the cultivars tested, the highest ethylene producer was 'Furand', which evolved more than 340 µl/kg fwt wt/h (ca. 250 µl/bulb/day) on the 12th day after infection. By 19 days, inoculated 'Furand' and 'Nashville' produced ethylene at ca. 800 µl/bulb/day. Approximately 40% of the cultivars produced ethylene at rates greater than 150 µl/bulb/day, and only 11% of them produced less than 5 µl/bulb/day. These results suggest high-ethylene producing tulips should be stored separately from other cultivars, or that increased ventilation should be maintained during storage or transportation. Knowledge of cultivar variation might also be useful in breeding programs.

INTRODUCTION

Tulip bulbs are subject to a variety of diseases with a range of effects, both direct and indirect. While always a threat, in the last 4-5 years, *Fusarium oxysporum* has emerged as a persistent and expensive problem in many tulip production regions. Although *Fusarium* is ubiquitous, the emergence of *Fusarium* as a larger problem may have a number of causes including a) changes in farming practices (e.g., farm size) and bulb handling equipment (e.g., "peeling" machines), b) regulatory changes affecting fungicide availability, c) buildup of spore and inoculum in the soil, and d) the appearance of one or more "new" *Fusarium* strains that may be more resistant to fungicides and/or more aggressive in their infection and spread (Miller, 2002).

While problematic as a plant pathogen, *Fusarium* has an additional and ultimately more significant effect, that being the production of ethylene after infection of the bulb. Although the phenomenon of *Fusarium* induced ethylene production in tulips is well known, relatively little work has been conducted in this area in the last two decades. Swart and Kamerbeek (1976, 1977) found that *Fusarium* isolates that are pathogenic on tulips produce at least 2,000 times more ethylene than nonpathogenic species or forms, and that ethylene production is highly oxygen dependent. The rate of ethylene production by infected tulips is highly temperature dependent, and is maximal at 21°C. At this temperature, infected 'White Sail' tulip bulbs produce ca. 140 µl/bulb/day, a value that appears to be the basis for industry standards for tulip ventilation requirements (de Munk, 1972).

Whether arising from *Fusarium* infection, or from external biological or mechanical sources, ethylene has many well-known effects on tulip bulbs, including

flower abortion (which may be induced nearly any time between G-stage and anthesis the following spring), reduced shoot growth, reduced or slower rooting, production of "twisted" appearance and root hairs, enhanced bulblet production in the field ("splitting"), polysaccharide eruption from the bulb's cells (gummosis), and increased respiration (De Munk and de Rooy, 1971; de Munk, 1971, 1972, 1973a, b; Kamerbeek et al., 1971; Kamerbeek and de Munk, 1976; De Hertogh and Le Nard, 1993; de Wild et al., 2002).

The main technique used by industry to remove ethylene from the atmosphere surrounding bulbs is forced ventilation, the goal being to keep ethylene levels surrounding the bulbs to 0.1 ppm (µl/L) or less (Kamerbeek and de Munk, 1976). Ethylene production during transportation in temperature-controlled shipping containers is of special concern, and the maximal ventilation rates of >100 m^3/h (De Hertogh and Le Nard, 1993) are less than optimal for complete removal of ethylene. The ventilation required is obviously affected by the degree of *Fusarium* infection in the cargo, and the rate of ethylene production by the bulbs.

Ethylene production by pathogenic fungi has been studied for some time, the classic example being green mold on citrus, *Penicillium digitatum*. Infection with this fungus promotes degreening in adjacent fruit, a phenomenon known to be ethylene-related (reviewed in Primrose, 1979). Fungal ethylene is produced by a different pathway than that in higher plants, and proceeds via glutamate or 2-oxoglutarate, with the ethylene arising from carbons 3 and 4 of the molecule (Chou and Yang, 1973) and Hottiger and Boller (1991) confirmed this for tulip strains of *Fusarium*. Although ethylene is produced by infected bulbs, the actual source of the ethylene (bulb vs. fungal tissue) is unclear.

The objectives of the present work were three-fold. First, to assess cultivar variability in ethylene production as a result of *Fusarium* infection, with our hypothesis being that cultivars vary greatly in their ability to support ethylene production, and second, to determine the time course of ethylene evolution after inoculation. The third objective was to determine the biological source of the ethylene produced by infected bulbs, and to test the hypothesis that both bulb and fungus contribute to ethylene production in the infected-bulb system.

MATERIALS AND METHODS

The basic procedure was to wound bulbs and then inoculate using a suspension made from cultures of *Fusarium* grown on potato dextrose agar (PDA) plates. Ethylene production was then followed over the following several weeks. The *Fusarium* culture originated from isolations performed by one of the authors (KSC) from *Fusarium*-infected bulbs originating in the Netherlands. Suspensions were prepared by homogenizing (in a blender) the fungal lawn from a 10-cm plate with ca. 150 ml water. The resulting suspensions were not further quantified in these experiments.

Experiment 1

Thirty-six cultivars were selected (Table 1). In late November, bulbs were wounded by piercing the bulb base (avoiding the root collar) 3 times with a 1 ml plastic pipette tip to a depth of ca. 0.5 cm. The tunic, if present, was removed prior to wounding. Bulbs were inoculated by dipping the bottom half of the bulb into the fungal suspension. After inoculation, bulbs were placed into 0.5 L jars and held at 21°C. Control bulbs were wounded, but not inoculated. Bulbs were kept dark except during experimental manipulations. There were two replicates per treatment, with three bulbs in a replicate. Ethylene production by the infected bulb system was determined at ca. 3 day intervals for 19 days. This experiment was repeated in early January using a subset of cultivars selected based on the initial results. In the repeat experiment, bulb wounding was much gentler than in the first experiment and was accomplished by gently scraping the bulb surface.

Experiment 2

This experiment was designed to determine if bulb tissue *per se* is involved in the ethylene generation of the infected bulb system. Bulbs of the cultivars ‘Prominence’, ‘Furand’, and ‘Friso’ were either living or heat killed by heating in a microwave oven for sufficient time to kill the tissue, as determined in preliminary experiments. The upper 1/4 of each bulb was removed, and ca. 1 cm^2 of fungal lawn from the *Fusarium* PDA cultures was placed on the cut surface. In the experiment, there were four treatments: killed bulbs + *Fusarium*, killed bulbs without *Fusarium*, living bulbs + *Fusarium*, and living bulbs without *Fusarium.* Other controls consisted of 10 cm^2 of PDA agar, with or without *Fusarium* inoculated as above. Each treatment had 3 replicates, each with two bulbs. Bulbs were held at 21°C, in 0.5 L glass jars, which were loosely capped to minimize external contamination of the bulb and fungal combinations. Prior to sealing for ethylene accumulation, open jars were placed in a laminar flow hood for 1 h to remove any previously accumulated ethylene.

Ethylene Determination

Ethylene was determined by head space analysis. The jars were sealed for 2 h, then a 1 ml gas sample removed with a syringe. Samples were injected onto a Hewlett Packard gas chromatograph equipped with a flame ionization detector, using standard methodology.

RESULTS AND DISCUSSION

Experiment 1

Large variations in ethylene production among tulip cultivars were observed (Table 1). Of the cultivars tested, the highest ethylene producer was ‘Furand’, which evolved more than 340 µl/kg fwt wt/h (ca. 250 µl/bulb/day) on the 12th day after infection. This rate of ethylene evolution was ca. 440-fold greater than in wounded, non-inoculated bulbs and ca. 1,300-fold greater than the ethylene production rate of non-wounded, non-inoculated bulbs (the native ethylene production rate of non-inoculated and unwounded bulbs was less than 0.36 µl/bulb/day for any cultivar, and was usually 0.1-0.2 µl/bulb/day). Other inoculated cultivars producing ethylene at rates exceeding 140 µl/bulb/day included ‘Mary Belle’, ‘Libretto’, ‘Nashville’, ‘Yonina’, ‘Friso’, ‘Librettto’, ‘Fusor’, and ‘Annie Schilder’.

Ethylene production continued through at least 19 days, when 14 cultivars produced ethylene at rates >150 µl/bulb/day (Table 1). The lag period from inoculation to the start of the logarithmic phase of ethylene production varied by cultivar from ca. 8 to 15 or more days. The changing ethylene production rates of the cultivars over time are illustrated by dividing them into three production categories – low, medium, and high (Table 2). The onset of ethylene production and the maximal ethylene rate obtained do not appear to be related. By the time this experiment ended, maximal ethylene production rates of more than 800 µl/bulb/day were obtained for ‘Nashville’, and 7 other cultivars had more than 500 µl/bulb/day (‘Furand’, 790; ‘Fusor’, 740; ‘Mary Belle’, 690; ‘Libretto’, 660; ‘Friso’, 640; ‘Prominence’, 610 and ‘Yonina’, 570) (Table 1).

A comparison of the time course of ethylene production by the 6 highest control bulbs (wounded, but non-inoculated) showed that ‘Louvre’ produced the most wound-induced ethylene (10.8 µl/bulb/day), but other cultivars were substantially less than this (Table 3). These values are in agreement with those of Kawa et al. (1991) who reported ethylene production of severely wounded bulbs to be 1-2 µl/bulb/day.

In the repeat experiment, ethylene production rates were much lower, with maximal rates of 250 and 140 µl/bulb/day being produced by inoculated ‘Evita’, and ‘Mary Belle’, respectively, but the basic ranking of cultivars in terms of ethylene production was similar. The reduced rate of ethylene production in the second experiment could be due to several factors, including different physiological/biochemical age of the bulbs, the much milder wounding technique used in the second experiment, or possibly

lower inoculum levels being used.

The maximal rates of ethylene production we obtained in the first experiment are much greater than the ca. 140 µl/bulb/day maximal ethylene rate published by de Munk (1972), which has been taken as the theoretical maximum ethylene production of a *Fusarium* infected tulip. We currently do not know if the values we have obtained are realistic in a commercial sense, or if they only reflect the ethylene production of an artificially (and massively) infected bulb. Further experimentation will be needed to explore this question.

Experiment 2

Ethylene produced by *Fusarium* infected bulbs could have two sources: the fungus or the bulb tissue itself. Heat-killed bulbs are no longer capable of metabolism as enzymes are denatured and membranes destroyed. However, the cellular contents used for fungal growth (amino acids, sugars and minerals) are fully available for fungal growth as the active defense systems of the bulb are destroyed. Ethylene production by heat-killed bulbs was negligible in the absence of *Fusarium*, and was very low in living, non-inoculated bulbs (Fig. 1). In the presence of *Fusarium*, heat-killed bulbs produced large quantities of ethylene, and, for the cultivars 'Friso' and 'Prominence', the quantity produced was more than 3-fold greater than produced by inoculated, living bulbs (Fig. 1). These data have two interpretations: 1) there exists a degree of active resistance to the fungus (the living bulb is inhibiting fungal growth), or 2) the heat-killed bulbs are a readily available source of nutrients for the fungus. While tulips are also known to contain a biofungicide, α-methylenebutyrolactone, or tulipaline-A (Bergman and Beijersbergen, 1968), and this may indeed be playing a role here, we hypothesize the greater availability of nutrients as a result of heat-induced cellular damage and membrane disruption to be more significant. From these data, we can tentatively conclude that metabolism of bulb tissue *per se* contributes very little, if any, to ethylene production in the infected bulb system. Most, if not all, of the ethylene emanating from *Fusarium*-infected tulip bulbs is produced by the fungus.

Both control treatments, inoculated or non-inoculated PDA, had very little to no ethylene produced (typically 0.5 µl/L accumulated in 2 h), compared with infected bulbs. The reasons for this are not clear, and we expected to find significant quantities from the inoculated PDA treatment. It is possible that our sampling times of 3-4 days missed the burst of ethylene production. Swart and Kamerbeek (1977) found that this burst could be relatively short-lived.

With 'Furand', inoculated, live bulbs produced ca. 25% more ethylene than inoculated, heat-killed bulbs, and therefore does not fully fit the pattern above.

While the relationship between *Fusarium* infection and ethylene production is well known, we believe this to be the first report to specifically show that the tulip bulb itself is *not* contributing to the ethylene. Our data suggest that all of the ethylene produced derives from the *Fusarium* mycelium. Ethylene production from living, inoculated bulbs varies greatly by cultivar, and leads to speculation on the reasons for this. Perhaps the cultivars that support the greatest ethylene production contain higher tissue concentrations of arginine, the amino acid that directly supports ethylene biosynthesis (Hottiger and Boller, 1991) or some other promoting factor. Alternatively, cultivars supporting lower ethylene levels may have greater levels of tulipaline-A, and therefore inhibit fungal growth and the development of a sufficiently mature mycelium to support maximal ethylene production (Swart and Kamerbeeek, 1977). Additional experiments are needed to follow these questions.

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Tables

Table 1. Ethylene production (μl/bulb/day) of 36 tulip cultivars 12 and 19 days after inoculating with a suspension of *Fusarium oxysporum.* Wounded, but non-inoculated control values for Day 19 are also shown. All bulbs were wounded (3 x 0.5 cm deep) before inoculation.

Cultivar	Control	12 days	19 days
Furand	0.4	483	793
Mary Belle	1.2	439	693
Nashville	2.8	384	833
Friso	0.7	318	637
Libretto	2.4	235	662
Fusor	0.2	214	737
Yonina	3.4	177	567
Annie Schilder	0.3	158	458
Prominence	0.5	131	615
Yellow Present	0.4	122	347
Pieter de Leur	1.5	59	207
Kikomachi	2.0	58	145
Synaeda Blue	3.2	54	200
Orange Princess	0.4	49	219
Mondial	1.4	39	203
Evita	2.2	36	148
Adamo	1.0	28	147
Sapporo	4.3	23	32
L. Figi	0.6	19	65
Jan van Nes	5.8	16	35
Coloeur Cardinal	1.2	11	67
Louvre	10.8	11	17
Angelique	3.0	8	40
World’s Favourite	3.6	7	18
Strong Gold	1.5	7	29
Sevilla	0.6	6	18
Yellow Flight	0.4	6	55
Purple Flag	0.7	4	7
Wirosa	2.4	4	5
Blue Ribbon	0.5	4	13
Bright Parott	0.5	3	25
Varinas	0.9	3	10
Calgary	1.0	3	4
The Mounties	1.2	2	10
Pretty Woman	2.1	2	5
Kees Nelis	0.3	2	3

Table 2. Categories of cultivar ethylene production (µl/bulb/day) for 8, 12, 15, and 19 days after inoculation.

Ethylene production (µl/bulb/day)	Days after inoculation			
	Day 8	Day 12	Day 15	Day 19
	Number of cultivars			
>150	0	1	8	14
5-150	11	20	19	18
<5	25	15	9	4

Table 3. Time course of ethylene production (µl/bulb/day) of wounded, non-inoculated tulip bulbs at 21°C. Data are presented for the 6 control cultivars with the highest ethylene evolution rates.

Cultivar	Days after wounding				
	1	8	12	15	19
Louvre	1.5	2.4	6.0	7.4	10.8
Jan van Nes	1.4	2.3	2.1	3.8	5.8
Sapporo	1.9	1.7	2.7	2.3	4.3
World's Favourite	2.9	2.7	2.8	2.7	3.6
Synaeda Blue	0.2	0.6	1.2	1.4	3.2
Angelique	1.0	1.2	1.5	1.9	3.0
30 remaining cultivars	0.5	0.7	0.8	0.9	1.1

Figures

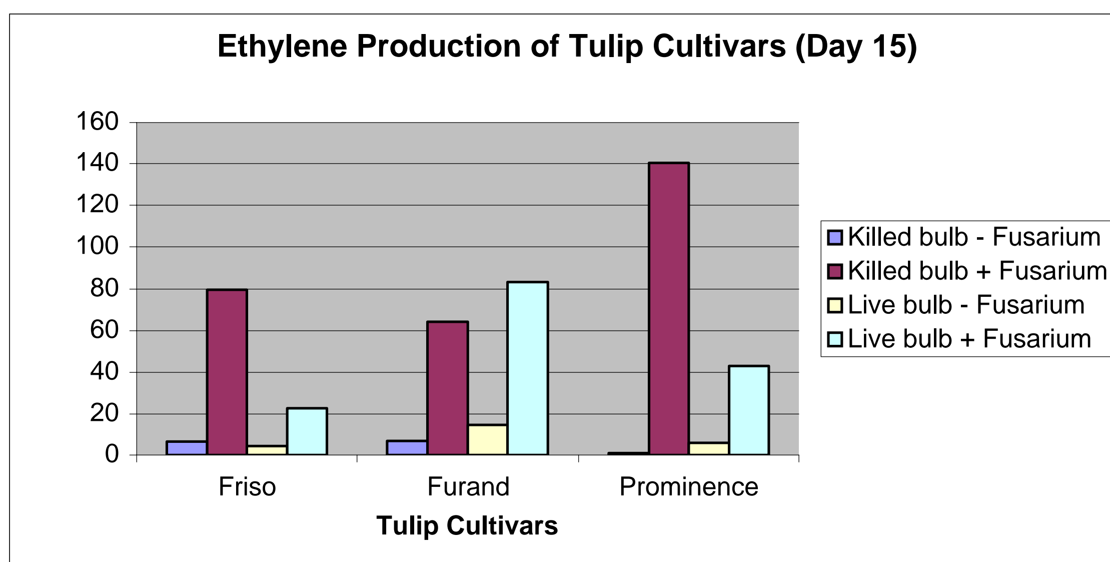

Fig. 1. Ethylene production by 3 cultivars of heat killed (microwave radiation) or live bulbs, 15 days after *Fusarium* inoculation. Ethylene production is given as µl/L ethylene accumulated by 2 bulbs for 2 hours.