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Effect of Continuous Exposure to Low Levels of Ethylene on Mycelial Growth of Postharvest Fruit Fungal Pathogens

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Abstract: Ethylene enhances the ripening and senescence of fruit with increased susceptibility to fungal decay a common feature of such changes. Most studies on the effect of ethylene have been in vivo where it is not possible to determine whether any effect due to ethylene arises from changes in metabolism of produce or from a direct effect on the pathogen. The few in vitro studies, that have been carried out, have been with very high ethylene levels, and did not identify the source of pathogens tested. This study examined the effect of air and ethylene, at 0.1 and 1 μL L^{-1}, on the growth of fungi isolated from five climacteric fruits (persimmon, pear, tomato, mango and papaya), and three non-climacteric fruits (orange, grape and blueberry). All fungi isolated from climacteric fruits had reduced mycelial growth when held in 0.1 and 1 μL L^{-1} ethylene but those from non-climacteric fruits showed no effect of ethylene. The finding was unexpected and suggests that fungi that colonise climacteric fruits are advantaged by delaying growth when fruits start to ripen. Since non-climacteric fruits do not exhibit any marked increase in ethylene, colonising pathogens would not need such an adaptive response.

Keywords: postharvest; fungi; ethylene; plant diseases

1. Introduction

Ethylene is a gaseous plant hormone that is well known to initiate postharvest ripening of climacteric fruit, and enhance the senescence of non-climacteric fruit and vegetables [1]. A common feature of such postharvest changes in fruits and vegetables is an increased susceptibility to develop fungal decay [2,3]. Published data on the effect of ethylene on fungal decay development have mostly been in vivo studies, where native or inoculated produce were exposed to atmospheric ethylene at a wide range of concentrations. Early interest in the role of ethylene arose from the commercial postharvest degreening process of citrus fruit where physiologically mature but green skinned fruit are exposed to relatively high temperatures (25 to 30 °C) in the presence of an ethylene concentration of about 5 μL L^{-1} to accelerate the loss of chlorophyll in the peel and thus 'degreen' the fruit [4,5]. The findings on the effect of such ethylene concentrations on postharvest decay of citrus fruit are inconsistent. Grierson and Newhall [6] found enhanced incidence of stem-end rot due to *Diplodia natalensis*, and Brown [7] found reduced green mould due to *Penicillium digitatum*; while Plaza et al. [8] found no effect on mould incidence for *P. digitatum*, and Porat et al. [9] showed decreased blue mould due to *P. italicum*. The effect of ethylene on the development of decay in other fruits include Zhu et al. [10] who reported that fumigation of 200 μL L^{-1} ethylene had no effect on grey mould incidence in grapes inoculated with *Botrytis cinerea*, Palou et al. [11] who found that

decay development from *Monilinia fructicola* inoculated into a range of stone fruits was not affected by exposure to ethylene up to 100 μL L^{-1}, and Lockhart et al. [12] who reported that exposure to ethylene at about 2000 μL L^{-1} inhibited development of apple rot caused by *Gloeosporium album*. Thus in vivo studies have shown variable effects of ethylene on fungal rot development of fruits. However, it is not possible from these studies to ascertain whether any enhanced mould growth induced by ethylene is a secondary effect arising from increased cell permeability that facilitates germination and growth of fungal spores [13], or any inhibition of mould growth is due to a defence response by tissues to ethylene [14].

An alternate action, that needs to be considered, is whether ethylene directly affects the growth of fungi present on the surface of produce as speculated by Sharon et al. [15] for *Botrytis cinerea*. It would seem necessary to conduct in vitro studies in order to determine if ethylene has a direct effect on fungal growth. However, few such studies have been reported. Lockhart et al. [12] reported in vitro growth of *Gloeosporium album* was not affected by exposure to 2000 μL L^{-1} ethylene while Kepczynski and Kepczynska [16] reported that germination of *B. cinerea*, *P. expansum*, *Gloeosporium perennans* and *Rhizopus nigricans* was stimulated by exposure to ethylene at 10 to 1000 μL L^{-1}. El-Kazzaz et al. [17] exposed 10 pathogens to ethylene at 1 to 1000 μL L^{-1} and found fungal growth rates were not significantly affected by ethylene for *Alternaria alternata*, *Botryodiplodia theobromae*, *B. cinerea*, *P. digitatum*, and *P. expansum* while ethylene showed a slight but significant increase between some ethylene concentrations for *Colletotrichum gloeosporioides*, *M. fructicola*, *P. italicum*, *Rhizopus stolonifera*, and *Thielaviopsis paradoxa*. None of these studies indicated the fruit source of the fungi. The only study to specify the fungal source was Brown and Lee [13] who found growth of *D. natalensis* isolated from Temple orange was inhibited by exposure to 55 μL L^{-1} ethylene.

Most of the in vitro and in vivo studies have been conducted by exposing produce to a much higher concentration of ethylene than would normally be encountered during commercial marketing situations which has been found to be invariably <2 μL L^{-1} and commonly about 0.1 μL L^{-1} [18,19]. While ethylene concentrations may be higher in internal produce tissue, many fungal pathogens reside on the surface of the produce and are therefore exposed to atmospheric concentrations of ethylene. Any action of abnormally high ethylene concentrations of ethylene (>2 μL L^{-1}) is of scientific interest but the response to lower ethylene concentrations would better demonstrate how fungal pathogens behave on fruit and vegetables in commercial supply chains. In this study, fungal spores were isolated from eight ripe fruits. Actively growing mycelia taken from rots on these fruits were sub-cultured onto a petri dish and continually ventilated at 20 °C with an air stream containing 0 (actually <0.001 μL L^{-1}), 0.1 and 1 μL L^{-1} ethylene. The growth of fungal mycelia was observed daily and measured when the mycelial diameter for each pathogen was about 50% of the petri dish diameter.

2. Materials and Methods

Persimmon, pear, tomato, mango, papaya, orange, grape, and blueberry fruit of commercial maturity with a visible rot were obtained from a retail outlet. The pathogen was collected from the rot area by cutting a disc (4 mm diam.) of flesh under the fruit skin at the outer edge of the lesion. The pathogen disc from each fruit was cultured on potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD, USA). PDA media (120 mL) was placed into each petri dish (90 mm diam.) using a liquid dispenser (OminispensePlus, Millville, NJ, USA). A petri dish was inoculated in the centre with the pathogen disc and incubated at 25 °C for 3 to 5 days to allow pathogen development. A sub-culture was then obtained from the edge of the mycelia with a core borer (6 mm diam.). The sub-sample for each pathogen was re-plated onto three petri dishes and incubated at 25 °C for two days to grow in ambient air before the petri dishes were transferred into ethylene treatments. However, the pathogen isolate obtained from tomatoes were transferred immediately to ethylene storage system without the pre-incubation process as the pathogen grew more rapidly than the other isolates. Groups of three petri dishes with the same fruit isolate constituted a replicate and were placed into three separate metal drums (60 L) that were fitted with inlet and outlet ports in the lid. Containers were placed into a

temperature controlled room at 20 °C. Each fruit pathogen was assessed with four to nine independent replicate trials at different times.

Containers were ventilated with humidified air (100 mL min^{-1}) containing the concentrations of ethylene of 0, 0.1 or 1 μL L^{-1}. The desired ethylene level was obtained by mixing ethylene from a gas cylinder (BOC Gases, Sydney, Australia) with compressed air that was made "ethylene-free" by passing through a jar containing potassium permanganate pellets, and humidified to approximately 95% RH by bubbling through water. Ethylene concentration flowing through the system was monitored daily by flame ionisation gas chromatography as described by Huque et al. [20] and to be the same inside the petri dish as in the surrounding atmosphere. The "ethylene-free" air contained <0.001 μL L^{-1} ethylene as this was the limit of detection of the analytical method. Growth of fungal mycelia was observed daily and measured when the mycelial diameter for each pathogen was in the range 30–50 mm which was about 50% of the petri dish diameter. On the designated assessment day, petri dishes were removed from the ethylene system and placed under sterile conditions where the diameter of mycelia was measured twice diagonally, and an average diameter calculated for each plate. The data for mycelial growth of each pathogen were subjected to analysis of variance using the statistical analysis system (SAS) 9.4 program. When the mean for growth of a fungal organism was found to be significantly different between ethylene levels, the least significant difference (LSD) at $P = 0.05$ was calculated.

Fungi associated with diseased lesions on each of the eight ripe fruit were isolated to agar media and identified to genus or species using conventional morphological taxonomy. In all cases a single fungus free of contamination was observed. Their identities were then confirmed or further characterised by comparison of their ITS rDNA sequences with GenBank accessions. Cultures were also submitted to the NSW plant pathogen herbarium (DAR). All identified fungi are known pathogens on the respective host fruit and are listed in Table 1.

Table 1. Mycelial growth of postharvest fungi isolated from eight fruit in the presence of low concentrations of ethylene.

					Mycelial Growth (mm diam)				
Pathogen	**Fruit**	**Cultivar/Type**	**Reps ***	**Days †**	**0**	**0.1**	**1 μL L^{-1} C_2H_4**	***P* Value**	***LSD* ‡**
		Climacteric Fruit							
Geotrichum candidum	Persimmon	Fuyu	8	13	50.3 [a]	45.9 [b]	45.9 [b]	0.003	*2.5*
Cladosporium herbarum	Pear	Packham	4	13	45.8 [a]	39.9 [b]	40.9 [b]	0.000	*3.0*
Rhizopus stolonifera	Tomato	Gourmet	5	2	67.9 [a]	63.8 [b]	63.6 [b]	0.041	*3.7*
Colletotrichum asianum	Mango	Kensington Pride	6	5	39.3 [a]	35.8 [b]	36.2 [b]	0.031	*2.8*
Phomopsis caricae-papayae	Papaya	Yellow papaw	6	13	49.1 [a]	44.6 [b]	43.0 [b]	0.004	*3.5*
		Non-Climacteric Fruit							
Penicillium waksmanii	Orange	Washington Navel	9	9	40.3	41.4	40.0	0.84	ns
Botrytis cinerea	Grape	Black seedless	8	3	32.5	30.4	30.6	0.09	ns
Colletotrichum simmondsii	Blueberry	OB 1	8	9	37.4	36.0	35.6	0.21	ns

* Number of replicated trials using the same batch of fungal spores. † Number of days at 20 °C before assessment. ‡ Least significant difference between means at $P = 0.05$.

3. Results and Discussion

Table 1 gives the time for each pathogen to grow to 30–50 mm and the effect of exposure to atmospheric ethylene at 0, 0.1 and 1 μL L^{-1} on in vitro mycelial growth at this time. The results show there was a significant effect of ethylene on the growth of five pathogens, *Geotrichum candidum*, *Cladosporium herbarum*, *Rhizopus stolonifera*, *Colletotrichum asianum*, and *Phomopsis caricae-papayae*. Mycelial growth of these fungi was greatest when they were in an ethylene-free (i.e., <0.001 μL L^{-1}) atmosphere. Pathogen growth was significantly decreased when held in an ethylene concentration of 0.1 μL L^{-1} but there was no further decrease in growth in the presence of 1 μL L^{-1} ethylene. The other three pathogens, *Penicillium waksmanii*, *Botrytis cinerea*, and *Colletotrichum simmondsii*, did not show any significant difference in mycelial growth in the ethylene concentrations.

The five pathogens that showed inhibited growth in the presence of exogenous ethylene were, respectively, isolated from persimmon, pear, tomato, mango and papaya (Table 1), which are all climacteric fruits. Climacteric fruit are defined by having a distinct increase in endogenous ethylene and respiration associated with ripening [1]. The three non-ethylene responsive pathogens were, respectively, isolated from orange, grape and blueberry (Table 1), which are all non-climacteric fruits and do not exhibit a climacteric rise in ethylene and respiration after harvest. This result was quite unexpected. It would seem logical that ethylene-tolerant pathogens would preferentially colonise climacteric fruits. The inhibitory effect of ethylene on growth of climacteric fruit pathogens raises an evolutionary question of what advantage does the organism derive from having its growth rate slightly inhibited when the fruit starts producing ethylene and hence begins ripening. A possible explanation needs to be viewed in the context that endogenous ethylene stimulates loss of cellular integrity of the fruit as part of the ripening process, and the loss of cellular integrity facilitates access by mycelia to nutrients in fruit tissues. A slight delay in fungal growth could be advantageous to allow greater degradation of fruit tissues to occur so that colonisation by the pathogen can occur more rapidly thereafter. The lack of any response by fungal pathogens that colonise non-climacteric fruit to an increase in ethylene production could reflect the lack of a need to adapt to a natural increase in ethylene production by such fruits early in the senescence process.

It would seem that exposure of climacteric fruit fungal pathogens to an ethylene concentration of 0.1 μL L^{-1} generates the greatest inhibition of fungal growth as no pathogen showed any further growth inhibition when exposed to 1.0 μL L^{-1} ethylene. An ethylene concentration of 0.1 μL L^{-1} was found by Wills et al. [18] to be a common background concentration of fruit and vegetables in a wholesale market. However, the magnitude of the reduction in mycelial growth of the climacteric fruit pathogens in the presence of 0.1 and 1.0 μL L^{-1} ethylene was only about 10% which would not be great enough to be of commercial significance. The longest postharvest life would still be attained by maintaining ethylene as low as possible as this will delay the loss of cellular integrity that enhances to the ability of fungi to grow.

It is recognised that only a limited number of fruits were examined in this report and an extended study on a greater range of fruits with exposure to a greater range of ethylene concentrations is warranted to test the universality of the findings. It is further recognised that some of the fungi tested in the study have a very broad host range, for example, *Botrytis cinerea* and *Geotrichum candidum* can invade both climacteric and non-climacteric fruit. Further research could examine the ethylene response to isolates of broad host fungi from different produce to see if there is a differential adaptive response to ethylene. Such studies could also examine if the ethylene response to pathogen mycelial growth also corresponds with infection potential by measuring parameters such as spore germination, appressorial formation and germ tube elongation or even to the ability of different fungi to metabolise ethylene.

4. Conclusions

The finding that pathogens isolated from all the climacteric fruits showed reduced growth when exposed to a low level of ethylene was quite unexpected. It suggests that pathogens colonising climacteric fruits derive some advantage by delaying growth early in the ripening sequence, possibly

related to enhanced loss of cellular integrity arising from increasing ethylene action on fruit tissues. The lack of an effect of ethylene on pathogens from non-climacteric fruit could reflect the lack of enhanced ethylene synthesis during the ripening of such fruits and thus there is no advantage in an adaptive response to ethylene.

Author Contributions: P.P. carried out the experiments and analysed the data, R.B.H.W. conceived the research hypothesis that led to the experiments, contributed to the experimental design and wrote the manuscript draft, L.T. identified the fungal organisms and provided the pathology laboratory, J.B.G. organised the conduct of the experiments and contributed to the experimental design. All authors reviewed the draft manuscript.

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