Factors Affecting the Production of Ethylene by Penicillium digitatum[1]

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Introduction

Ethylene production by the green mold of citrus, *Penicillium digitatum* Sacc., has been investigated by a number of workers (1), but its pathway of biosynthesis and importance in fungal metabolism have yet to be defined. Factors affecting growth of the fungus have been studied intensively and a satisfactory medium has been developed (2, 7). The importance of zinc and yeast extract as growth factors in the medium was demonstrated by Pratt (7) and later workers (3, 9). In these studies, ethylene production and growth were assumed to be directly related. More recently, Spalding and Lieberman (8) reported that yeast extract exerts a much greater stimulatory effect on ethylene production than on growth.

The present report will consider the effect of yeast extract components on ethylene production by *P. digitatum*. Variation found to be associated with the source of fungal inoculum and cultural conditions will also be discussed.

Materials and Methods

Preparation of Medium. The modified Pratt's medium used contained the following: 18.0 g glucose, 4.0 g NH_4NO_3, 13.61 g KH_2PO_4, 1.23 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.5 g yeast extract (Difco), and demineralized distilled water up to 1 liter. The pH was adjusted to 4.5 with 0.5 N NaOH before autoclaving for 20 minutes at 15 psi.

Preparation of Inoculum. Single spores of *P. digitatum* ATCC No. 10030 were isolated with the aid of a Chambers' micromanipulator, and were allowed to germinate and grow in the modified Pratt's medium in small moist chambers. When visible growth had developed, a small amount of the mycelium was transferred to 50-ml Erlenmeyer flasks containing 10 ml of modified Pratt's medium. The flasks were then sealed and placed in an incubator at 25°. The single-spore isolate yielding the most ethylene was used to prepare a large number of slant cultures that were used as the source of inoculum. The inoculum for a given test was prepared by swabbing spores from a culture stored at 5° over the surface of plates of Difco malt extract agar to which 0.3 % Difco yeast extract and 0.5 % agar had been added. After growth and sporulation at 25°, spores were removed by gently rubbing the surface with a moist sterile cotton swab. With the aid of a Spencer hemacytometer, a spore suspension of ca. 250,000 spores per ml was prepared in sterile 0.01 % Tween 20 (polyoxyethylene sorbitan monolaurate).

Preparation of Test Samples. Test flasks were prepared in triplicate or quadruplicate. One ml of the inoculum was pipetted aseptically into a 250-ml Erlenmeyer flask containing 40 ml of a given medium. Loss of ethylene through cotton plugs was prevented by sealing the flasks with 2-hole rubber stoppers containing 10 cm lengths of 6 mm glass tubing filled with cotton and stoppered with rubber serum caps. The flasks were incubated in still culture at 25°. Shake cultures were incubated in a water bath at 25° and shaken at 120 2.5 cm strokes per minute. Because of the inhibitory effects of high CO_2 and low O_2 levels on both growth and ethylene production, the gaseous contents of the flasks were analyzed daily and the atmosphere was replaced by flushing for 4 minutes with air drawn through cotton filters of the flasks with a water aspirator pump. Growth, in terms of dry weight, was determined, as indicated in figures of the text, by centrifuging the cultures at 16,000 rpm for 15 minutes, and drying the pellet at 60° for 48 hours before weighing. Additional details of methods of culture are described in the text.

Gas Analyses. CO_2 and O_2 in the atmosphere of the flasks were determined with a Fisher Gas Partitioner, Model 25 V. CO_2 was separated on a 70 cm × 0.6 cm diam column packed with 30 % hexamethylphosphoramide on chromosorb P (60–80 mesh). O_2 was separated on a 16.25 cm × 0.5 cm diam column of specially treated molecular sieve 13X (40–60 mesh). Samples were swept through with helium at 80 ml per minute. This instrumentation is sensitive to ca. 0.01 % O_2 or CO_2.

Ethylene analyses were made with a 60 cm × 0.6 cm diam glass column packed with activated alumina (60–80 mesh) and immersed in a water bath at 30°. The flame ionization detector (5) was supplied with air for combustion at a rate of 300 ml per minute. The eluant gas, a 1 : 1 mixture (v/v) of Seaford nitrogen and hydrogen, was supplied at 40 ml per minute. The detector output was amplified (610A Electrometer, Keithley Instruments) and recorded on a 1-milliamp galvanometric recorder. This instrumentation is sensitive to ca. 0.015 ppm or 0.005 μg ethylene.

Results

Variability of Cultures of P. digitatum. Twelve single spore cultures of *P. digitatum* after 7 days of growth in a closed system varied in ethylene production from virtually none (0.1 μg) to very high (36

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μg). However, growth in these 12 cultures was approximately the same. Spores harvested from a 5-day culture of Isolate No. 11, a high producer of ethylene, were used in the present experiments as a standard inoculum.

Relation of Growth to Ethylene Production. Time-course studies of growth and ethylene production by *P. digitatum* grown in modified Pratt's medium, with and without yeast extract, were made for a 3-week period (fig 1). In these experiments, an open system was used. The culture flasks were plugged with cotton and were sealed only for 4 or 5 hours to accumulate a gas sample. The medium containing yeast extract gave a faster growth rate and a greater total growth. Growth preceded ethylene production in either the presence or absence of yeast extract. The lag period for ethylene production, however, was 6 days without yeast extract, but only 2 days in its presence. The highest rate of ethylene production did not occur until the growth rate had passed its maximum. Ethylene production ceased only after mycelial mats began to lose weight. This decline in growth was probably caused by lack of nutrients, and autolysis might have caused the weight loss.

Growth and ethylene production in shake and still cultures of *P. digitatum* are compared in table I. Ethylene production was not proportional to growth. The still cultures, with only about one-fourth as much growth as shake cultures, produced 20 times as much ethylene. Shake cultures produced practically no ethylene after 7 days. Fergus (3) noted the low ethylene production in shake cultures and suggested that shaking may alter the metabolism of the fungus and thus suppress ethylene production. The low ethylene production in shake cultures was associated with submerged ball-like growth. This type of growth and the lack of ethylene production could not be reversed by placing the shake cultures, after 7 days, in still culture for 5 days or longer. High ethylene production appeared to be related to surface growth and was associated, in still cultures, with the development of a surface mat. Accidental submersion of this mat usually reduced ethylene production.

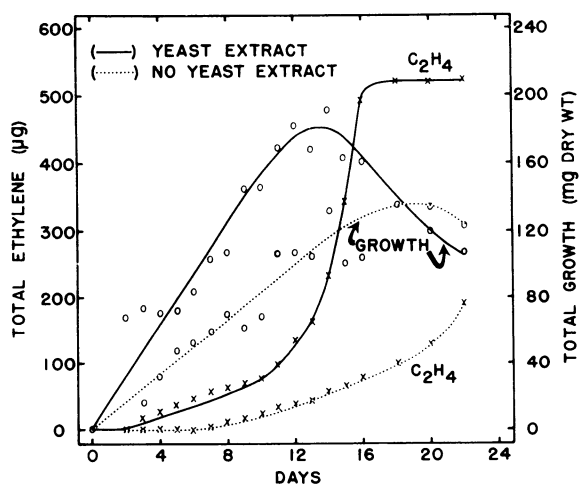

FIG. 1. Growth and ethylene production of *Penicillium digitatum* in still culture at 25° for 22 days in an open system containing modified Pratt's medium, with and without yeast extract. Each day 3 different flasks were flushed, sealed for 4 to 5 hours, sampled for ethylene, CO_2 and O_2 and dry weights determined as described in text. The values obtained for ethylene were converted to production on a 24-hour basis and the daily values were added to give a cumulative figure for total ethylene.

Table I. *Ethylene Production by Shake and Still Cultures of* Penicillium digitatum

Cultures were grown in modified Pratt's medium in a closed system, flushed daily, for 7 days at 25°. These data represent the means of 1 trial run in pentuplicate. Means not followed by letters in common are significantly different at the 1 % level.

Culture	Initial pH	Final pH	Total growth (mg dry wt)	Total ethylene (μg)
Still	4.5	3.5	125 a	168 a
Shake	4.5	2.9	488 b	8 b

Relation of Respiration to Growth and Ethylene Production. The rise in respiration of *P. digitatum*, as in the case of growth (fig 1), preceded the marked rise in ethylene production (fig 2). The highest ethylene production rate was not observed until the respiration rate had decreased. This phenomenon was especially marked in the cultures lacking yeast extract. These data (fig 2) also show the system to have an RQ of ca. 2. Similar RQ levels were shown by Gibson (4) in a *P. digitatum* system continuously swept with air. This level of CO_2 production, relative to O_2 consumption, does not, there-

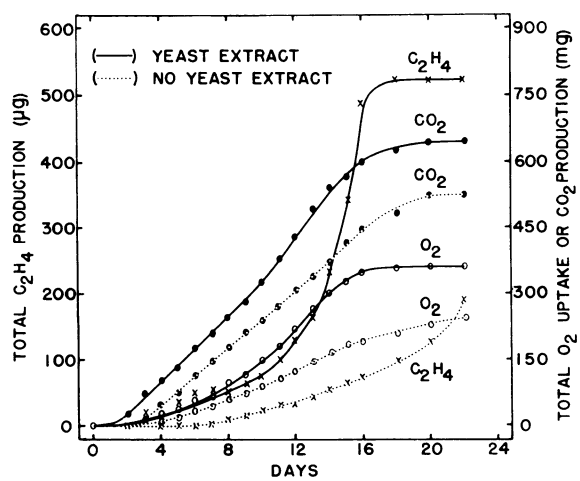

FIG. 2. Respiration and ethylene production of *Penicillium digitatum* in still culture at 25° in an open system containing modified Pratt's medium, with and without yeast extract.

fore, seem to be an unusual phenomenon or to inhibit either growth or ethylene production.

Relation of Yeast Extract to Growth and Ethylene Production. Addition of yeast extract to the basal medium increased growth by a factor of ca. 2 and ethylene production by ca. 15 (table II). This disproportionate increase in ethylene production, relative to growth, indicates that some factor or factors in yeast extract sharply stimulate ethylene production. The effects of known components of yeast extract (Difco) were, therefore, tested separately. According to a typical analysis supplied by Difco, its yeast extract consists mainly of amino acids, vitamins, and mineral salts. Table II shows the results when these components, separately and in different combinations, were added to cultures. The vitamins and amino acids were added to the basal medium in approximately the same concentrations as present in a typical sample of yeast extract. None of the combinations of vitamins and amino acids added to the basal medium, however, equaled the stimulatory effect of yeast extract. At the 5 % level of certainty, biotin significantly increased growth and ethylene production, whereas thiamin and the combination of thiamin plus biotin had no effect. Thiamin and biotin appear to be antagonistic, although the complete vitamin mixture significantly increased ethylene production without a significant increase in growth. The ethylene stimulation by the vitamin mixture was significant even at the 1 % level. On the other hand, a mixture of the amino acids did not significantly increase ethylene, but did cause a significant increase in growth. When a combination of amino acids and vitamins was used, there was a significant difference in both growth and ethylene. These data again emphasize the relative independence of growth and ethylene production.

Effect of Heat on Yeast Extract Activity. The yeast extract was usually autoclaved with the other components of the medium. In order to test the heat stability of the active components in the yeast extract, however, autoclaved yeast extract and yeast extract sterilized by passage through a Seitz filter pad were compared. Yeast extract that was not autoclaved showed greater stimulation of ethylene production. The lag period, however, was the same with either autoclaved or "Seitzed" yeast extract. The ethylene-stimulating capacity of yeast extract was reduced 10 to 30 % by autoclaving.

The factors in yeast extract that stimulate ethylene production were found to be destroyed by ashing and were largely removed by passage through a Dowex 50 cation exchange resin in the H form.

Comparison of an Open Versus a Closed System for Growth and Ethylene Production. Although the closed system used in these experiments was convenient for following the respiration and ethylene production of *P. digitatum,* the question arose as to whether the buildup of CO_2 and other gases and the depletion of O_2 interfered with fungal growth and ethylene production. In a time-course study, however, even when the CO_2 increased to 14 % and the O_2 decreased to 10 % in 24 hours, no change in the fungal growth rate was observed. If, at this point, the system was not flushed and the atmosphere renewed, the rate of fungal growth would decline with subsequent decrease in ethylene production. Accordingly, the system must be flushed at least once every 24 hours in order to maintain maximum growth

Table II. *Effect of Yeast Extract (Difco) Components on Growth and Ethylene Production by* Penicillium digitatum

Cultures were grown in still culture and a closed system flushed daily, for 16 days at 25°.

Additions to basal medium* (μg/ml × 10^3)	Total growth (mg dry wt)**	Total ethylene (μg)
None	80 a	53 a
Thiamine • HCl (1.5)	98 ab	161 ab
Biotin (0.5)	108 b	192 ab
Thiamine • HCl (1.5) and biotin (0.5)	80 a	64 ab
Vitamins: Thiamine • HCl (1.5), biotin (0.5), riboflavin (6.7) Pryidoxal phosphate (6.7), and nicotinic acid (140)	93 ab	261 b
Amino acids: Asparagine (0.0025), DL-Aspartic acid (0.025) L-Glutamic acid (0.0032), Glycine (0.012) L-Histidine (0.0047), L-Isoleucine (0.014) L-Leucine (0.018), L-Lysine (0.020) L-Methionine (0.004), L-Phenylalanine (0.011) L-Threonine (0.017), L-Tryptophan (0.0044) L-Tyrosine (0.0030), and L-Valine (0.017)	111 b	154 ab
Combination of above vitamins and amino acids	116 b	174 b
Yeast extract (500,000)	170 c	768 c

* Basal medium contained all the components of the modified Pratt's medium except yeast extract.

** Growth and ethylene data represent the means of 4 replicates. Means not followed by letters in common are significantly different at the 5 % level.

and ethylene production. Nevertheless, because fungi are normally grown in systems plugged with cotton, allowing gas diffusion and the maintenance of a near normal atmosphere, ethylene production was compared in the open and closed systems. Although total growth was not significantly different, total ethylene production was significantly higher in the closed system. The different atmospheric conditions apparently influence ethylene production or the accumulation of ethylene, in a closed system, may have an autocatalytic effect on ethylene production.

Discussion

Variability in ethylene production by *P. digitatum* is a major problem that has not been emphasized sufficiently in published reports. Cultures from individual spores appear to vary in ethylene production from zero to relatively high rates, therefore, spore differences may be a source of considerable variability and should be considered. All the tests of Pratt (7) and Fergus (3) were run in triplicate to avoid errors due to biological variability. This degree of replication appears to be a minimum requirement.

Ethylene production by *P. digitatum* has been assumed to be closely associated with growth (3, 6, 7). Our data, however, show that abundant growth did not necessarily indicate high ethylene production. In shake cultures, the metabolism of the fungus was apparently altered, with respect to ethylene production, although growth and respiration rates were very high. According to the data from our time-course studies of growth and ethylene production by *P. digitatum*, the period of greatest rate of ethylene production occurs during the time that the growth rate starts to decline. Ethylene production reached its maximum when the culture was entering a period of senescence and when weight loss suggested that the culture was beginning to autolyze. The very sharp increase in ethylene production at this time suggests that an antagonism might exist between active growth and ethylene biosynthesis.

One may recognize 4 phases in the production of ethylene by *P. digitatum*, as follows: 1) a lag phase with no production, 2) a phase of low but slowly rising production coinciding with the period of most active growth, 3) a phase of very rapid ethylene production coinciding with a period of declining growth, and 4) a final phase with no production coinciding with loss of weight in the culture. This sequence was observed both with systems containing yeast extract and with systems lacking yeast extract. However, in the latter case the complete sequence was not followed through phase 4.

Yeast extract has been assumed to stimulate ethylene production as a result of its stimulation of growth of *P. digitatum* (3, 7). Our data show that addition of yeast extract to the medium increased average ethylene production 15-fold, although growth was only doubled. The pronounced reduction in the lag phase in ethylene production suggests that yeast extract may supply precursors or cofactors for the synthesis of ethylene. The amino acids of yeast extract do not appear to be responsible for the stimulation of ethylene production. The vitamins, on the other hand, were found to increase ethylene production significantly and may play a role in ethylene synthesis by *P. digitatum*.

Summary

Wide variation in ethylene-producing capacity of different single spore cultures of *Penicillium digitatum* Sacc. was observed. Ethylene production was not proportionate to growth, but as expected respiration and growth rates were closely associated. The submerged ball-like growth in shake culture produced only traces of ethylene, even though growth was much more abundant than in still culture. Ethylene production was associated with surface growth, and the amount of ethylene was greatly stimulated by the inclusion of yeast extract in the medium. Ethylene production increased 15-fold in the presence of yeast extract, whereas growth was only doubled. The stimulatory effect of yeast extract could not be duplicated with amino acids or vitamins, alone or combined, and was partially destroyed by autoclaving. More ethylene was produced in a closed, than in an open system.

Acknowledgments

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