Constituents of Orange Juice that Stimulate the Germination of Conidia of Penicillium digitatum

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ABSTRACT

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Conidia of *Penicillium digitatum* germinated 90% in a diluted dialysate of orange juice, but not in distilled water. Maximum germination was observed in 1% (v/v) orange juice dialysate at pH 3.5 - 8.0, but germ tube development was most vigorous at pH 4.0 - 5.5. The constituents of the dialysate which were adsorbed by an anion exchange resin (anionic fraction) were most active in stimulating germination, whereas the cationic and neutral fractions supported vigorous germ tube development, but did not initiate germination. A mixture of L-ascorbic, citric, and L-malic acids stimulated germination to essentially the same level as a dilution of the anionic fraction which contained the same concentration of these acids. Citric or L-malic acids, alone or combined, did not significantly increase germination. Both L-ascorbic and dehydroascorbic acid induced a higher level of spore germination than isoascorbic acid (D-ascorbic acid). Of the major carbohydrates present in the neutral fraction of orange juice, glucose (2.56 g/ 100 ml) alone supported 50-70% germination, whereas equivalent concentrations of fructose and sucrose were less effective and myo-inositol was inactive. A solution containing 76.8 mg glucose and 29.4 mg citric acid/ 100 ml, concentrations equivalent to 3% orange juice dialysate, resulted in 31% germination and more vigorous germ tube development than that observed in solutions containing higher concentrations of glucose alone. Several other organic acids likewise were stimulatory when combined with glucose, but succinic, fumaric, maleic, and acetic acids were inactive. The combination of glucose and citric acid produced higher germination at pH 4.5 than at pH 6.0. Relatively simple mixtures of nutrients stimulated a high rate of germination. Maximum germination was observed in spores incubated in (i) glucose combined with one of several organic acids, (ii) glucose combined with L-ascorbic acid, and (iii) citric acid combined with L-ascorbic acid. Exogenous nitrogen-containing nutrients were not required for maximum germination, but vigorous germ tube growth was observed only in solutions containing mixtures of amino acids and vitamins.

Additional key words: nutritional requirements for spore germination.

Penicillium digitatum Sacc. is the major cause of postharvest decay (green mold) of citrus fruits produced in arid subtropical regions. The fungus has not been reported to be pathogenic on other fruits. Conidia of *P. digitatum* are abundant in the atmosphere of citrus groves and packinghouses throughout the world and fruit infection is initiated by germination of spores in moist injuries in the peel of the fruit. Several investigators have demonstrated that the incidence of infection may be increased significantly by the addition of certain nutrients to spore suspensions used for inoculation of fruits (4, 18, 19). *Penicillium digitatum* has been cultivated on synthetic media, but mycelial growth is increased substantially by the addition of complex mixtures of nutrients to the minimal medium (14, 25, 26, 29). Rapid and complete germination of spores has been observed only in media containing extracts of citrus fruits or other materials (10, 19, 25), but no systematic study of the nutritional factors required for germination has been reported.

The present investigation was undertaken to identify the components of orange juice which are required for vigorous development of conidia of *P. digitatum* and to formulate a completely synthetic medium which would possess activity equivalent to orange juice in this respect.

MATERIALS AND METHODS

All chemicals were the purest available from commercial sources and the water was distilled twice; the second time in a glass apparatus. Conidia of *P. digitatum* (California isolate M6R of this laboratory) were harvested from decaying lemons which had been inoculated aseptically with a pure culture of the fungus. The conidia were brushed from the surface of the lemons, passed through a 75-μm sieve to remove any fruit debris and water droplets, and stored in a glass vial over anhydrous $CaSO_4$ (Drierite®) at room temperature (22-24 C). Dry conidia were suspended in sterile 0.05% (v/v) Tergitol XD (polyalkylene glycol ether, Union Carbide Chemicals Co., NY 10017) with the aid of a glass tissue homogenizer and the suspension was adjusted to contain approximately 10^6 conidia/ml. One ml of conidial suspension was added to 24 ml of nutrient medium after pH adjustment with dilute KOH or HCl. Triplicate 0.2 ml portions of the seeded nutrient solutions were placed in glass microbeakers (5 mm deep, 10 mm in diameter) and were incubated in a water-saturated atmosphere at 25 C for 16 hr. One hundred conidia in each of the triplicate

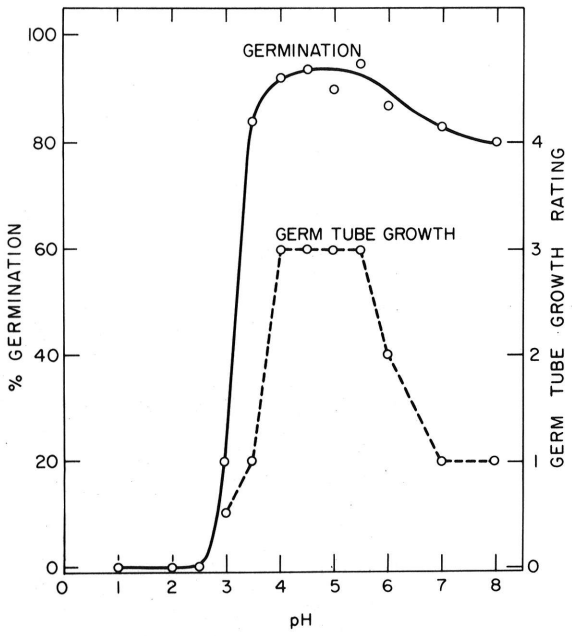

Fig. 1. Development of conidia of *Penicillium digitatum* in 1% (v/v) orange juice dialysate at several pH values.

microbeakers were inspected for the presence of a germ tube, the length of which was estimated with the aid of an ocular micrometer. A germ tube rating of 1 corresponded to a germ tube length of approximately 130 μm, but accurate measurements were impractical for this investigation owing to the ramification of the germ tubes.

Valencia orange juice concentrate ("Calfame" brand, Paramount Citrus Association, San Fernando, CA 91341) was reconstituted by the addition of three volumes of water and the resultant single-strength juice was filtered with the aid of Celite Analytical Filter Aid (Johns-Manville Corp., NY 10016). The clear filtrate was designated 100% orange juice and dilutions were made by volume with water. Orange juice dialysate was prepared by placing 15 ml of Valencia orange juice concentrate in cellulose dialysis tubing ("Visking" brand - 2.86 cm diameter, 0.03-mm wall thickness, average pore diameter 4.8 nm) and allowing dialysis to proceed against water at 1 C for 16 hr. The dialysate was concentrated to the original volume of the orange juice concentrate and stored at 1 C.

Orange juice dialysate was passed through a 2.8 × 46 cm bed of cation exchange resin (Dowex 50-X8, 150-300 μm particle size, H^+ form) and followed by 1 liter of water. The effluent was evaporated almost to dryness twice and diluted to 15 ml with water. This solution was designated the "noncationic" fraction. The cationic compounds were eluted from the resin by passage of 1 liter 2N NH_4OH

TABLE 1. Development of conidia of *Penicillium digitatum* in the neutral and ionic fractions of orange juice dialysate

Orange juice fraction[a]	Concentration equivalent to a dilution of orange juice (%, v/v)	Spore germination (%)	Germ tube growth
A. Cationic and noncationic fractions			
Orange juice	1	95	3.50
Orange juice dialysate	1	92	2.50
Orange juice dialysate	3	93	2.75
Cationic[b]	1	1	0.25
Cationic	3	13	0.25
Noncationic[c]	1	88	0.75
Noncationic	3	90	1.25
Cationic + noncationic	1	95	2.00
Cationic + noncationic	3	94	2.75
B. Cationic, anionic, and neutral fractions			
Orange juice dialysate	3	95	3.75
Cationic[b]		3[g]	...
Cationic	3	8	0.25
Anionic[d]	1	66	0.63
Anionic	3	90	0.75
Neutral[e]	1	3[g]	...
Neutral	3	35	0.38
Anionic + neutral	1	94	1.50
Anionic + neutral + cationic	1	95	1.75
Anionic + neutral + cationic	3	91	2.00

[a]Adjusted to pH 4.5 with KOH or HCl.
[b]Organic bases eluted from Dowex-50 resin with 2 N NH_4OH.
[c]Components of orange juice dialysate remaining after removal of cations.
[d]Acidic substances eluted from Dowex-1 resin with 1 N HCl.
[e]Components of orange juice dialysate remaining after removal of acidic and basic substances.
[f]Value of 1.00 = average germ tube length approximately 130 μm.
[g]Most of conidia swollen.

through the column followed by 1 liter of water. The effluent was evaporated to dryness under vacuum several times on a rotary evaporator to remove ammonia from the fraction. The dry sample was dissolved in 15 ml of water and designated the "cationic" fraction.

A portion of the noncationic fraction was passed through an anion exchange resin (Dowex 1-X8, 150-300 μm particle size, $CO_3^=$ form) and followed by 1 liter of water. The effluent was concentrated as above and identified as the "neutral" fraction. The anionic compounds were eluted from the Dowex-1 column by passage of 1 liter of 1.0 N HCl through the resin followed by 1 liter of water. The eluate and rinse were combined and concentrated as described above and designated the "anionic" fraction. Five ml of concentrated anionic fraction was diluted to 240 ml with water, acidified with HCl to pH 1.0, and then extracted with 750 ml diethyl ether in a Soxhlet apparatus for 72 hr. The ether solution was concentrated to 30 ml and extracted with five 100-ml portions of water. The water extracts were pooled, evaporated to dryness, and the residue was dissolved in 10 ml of water. This fraction was designated the "organic acids" fraction. The residual component of the anionic fraction after ether extraction was evaporated to dryness several times to remove HCl and the residue was dissolved in water to give the "sugar acids" fraction. The water-extracted ether was dried with anhydrous $MgSO_4$ and evaporated to dryness. The residue was dissolved in a few drops of 0.1 N KOH and diluted to 10 ml with water. This solution was labelled the "fatty acids" fraction.

Sugars in the neutral fraction were chromatographed on Whatman 52 paper with butanol:pyridine:water (2:2:1, v/v) and localized on the chromatogram with silver nitrate reagent. Acids in the anionic fraction were chromatographed on Whatman 52 paper with diethyl ether:formic acid:water (5:2:1, v/v) and localized with a solution of bromphenol blue. The amino acids in the cationic fraction were determined quantitatively by means of a Beckman 120 C automatic amino acid analyzer. Ascorbic acid in the sugar acids fraction was determined spectrophotometrically (21).

TABLE 2. The effects of components of the anionic fraction of orange juice upon the germination of conidia of *Penicillium digitatum*

Orange juice fraction[a]	Spore germination (%)	Germ tube growth[g]
Orange juice dialysate	90	3.50
Anionic[b]	85	0.75
Sugar acids[c]	50	0.50
Organic acids[d]	5	...
Fatty acids[e]	0	...
Neutral[f]	35	0.50
Sugar acids + organic acids	84	0.75
Sugar acids + organic acids + fatty acids	83	0.75
Sugar acids + neutral	82	1.25
Organic acids + neutral	84	0.63
Fatty acids + neutral	40	0.50

[a]Fractions tested at concentration equivalent to 3% orange juice and adjusted to pH 4.5.
[b]Acidic substances eluted from Dowex-1 resin with 1 N HCl.
[c]Components of the anionic fraction which, at pH 1, did not partition into ether.
[d]Ether-extractable and water-soluble acids of anionic fraction.
[e]Ether-extractable and alkali-soluble acids of anionic fraction.
[f]Components of orange juice dialysate remaining after removal of anions and cations.
[g]Value of 1.00 = average germ tube length approximately 130 μm.

RESULTS

Effect of ionic and nonionic fractions of orange juice on germination and development of conidia.—At pH 4.5, maximum germination and germ tube growth were observed in 1.0 - 1.5% orange juice (1.0 - 1.5 ml of orange juice diluted to 100 ml with water). The factors in orange juice responsible for germination and growth were dialyzable and had molecular weights below 1,000 as indicated by filtration through Sephadex G-10 gel. Maximum germination and growth in 1% orange juice dialysate occurred in the range pH 4.0 - 5.5 (Fig. 1). At pH 3, the conidia enlarged abnormally and the germ tubes were shorter and thicker than those of spores germinated in the optimum pH range. At pH 8, approximately 80% of the spores germinated, but germ tube growth also was poor.

Sugars and organic acids are the major components of the noncationic fraction of orange juice whereas the cationic fraction consists of amino acids, amines and cationic vitamins (5, 8, 9, 20, 27). The noncationic fraction at a concentration equivalent to 3% orange juice did not give a positive test with ninhydrin for amines or amino acids. Table 1 (Section A) shows that the noncationic fraction at 3% was almost as active as whole orange juice in stimilating spore germination, whereas the cationic fraction at an equivalent concentration induced only 13% spore germination. However, germ tube growth was improved considerably when the noncationic and cationic fractions were combined. The noncationic fraction appeared to contain all factors required for spore germination whereas vigorous development of the germ tubes depended upon additional nutrients which were present in the cationic fraction of orange juice.

The noncationic fraction of orange juice dialysate was resolved further into an anionic fraction and a neutral fraction by passage through an anion exchange resin. The major components of the anionic fraction were citric, malic, ascorbic, and phosphoric acids; the neutral fraction contained sucrose, glucose, fructose, and myo-inositol (8, 20, 27). Table 1 (section B) shows that the anionic fraction alone at a concentration equivalent to 1% orange juice dialysate stimulated 66% spore germination and, at a concentration of 3%, spore germination was only slightly lower than in orange juice dialysate. However, the germ tubes were short and thin on conidia germinated in the anionic fraction alone. The neutral fraction alone at 1% induced only 3% germination, but spores germinated in the combined neutral and ionic fractions produced germ tubes considerably more vigorous than those observed in the anionic fraction alone. The cationic fraction alone did not stimulate spore germination, but significantly increased the vigor of the germ tubes of spores when combined with the neutral and anionic fractions. Failure of the combined anionic,

neutral, and cationic fractions to support germination and growth equal to orange juice dialysate suggests that some constituents of the dialysate were not quantitatively recovered from the ion exchange resins.

Effect of the anionic fraction and its components upon spore germination.—The activity of the anionic fraction was reduced significantly by heating for 15 min at 125 C and one atmosphere gauge pressure and was destroyed completely by ashing the fraction. These results appeared to eliminate inorganic ions as the major factors involved in the stimulation of germination by orange juice.

The anionic fraction was resolved further into an organic acids fraction, a sugar acids fraction, and a fatty acids fraction by partitioning against water at two pH values (see Materials and Methods section). This procedure was expected to result in an organic acids fraction containing citric acid and malic acid and a sugar acids fraction containing ascorbic acid, sugar phosphates, and sugar carboxylates. Table 2 shows that the sugar acids fraction was more effective in stimulating germination than either the organic acids or the fatty acids fractions. The combination of the sugar acids and the organic acids fractions stimulated the development of conidia to essentially the same degree as the whole anionic fraction, whereas the fatty acid fraction did not show activity in any of the combinations tested. Combination of the neutral fraction with either the organic acids or the sugar acids fraction also resulted in a mixture of nutrients which stimulated spore germination to the same level as the whole anionic fraction.

The principal organic acids of orange juice (8) were assayed alone at concentrations equivalent to 3% orange juice dialysate (Table 3). Ascorbic acid alone could account for a substantial portion of the activity of the anionic fraction in stimulating spore germination. Citric acid and malic acid, either alone or in combination, had no significant effect upon spore germination. However, the combination of ascorbic acid and citric acid produced a substantial increase in germination over that supported by ascorbic acid alone. The combination of ascorbic, citric, and malic acids stimulated germination to essentially the same level as the anionic fraction. Heat treatment of solutions of ascorbic acid, with or without other organic acids, resulted in a loss in activity comparable to that observed when the anionic fraction was heated.

TABLE 3. Germination of conidia of *Penicillium digitatum* in solutions of organic acids and sugars

Nutrients[a]	Spore germination (%)
Orange juice dialysate	90
Anionic fraction[b]	85
Neutral fraction[c]	35
Neutral + organic acid fractions	85
Ascorbic acid	50
Citric acid	3
L−(−)malic acid	0
Ascorbic acid + citric acid	78
Ascorbic acid + malic acid	52
Ascorbic + citric + malic acids	93
Citric acid + malic acid	5
Glucose	10
Glucose + citric acid	31
Fructose	1
Sucrose	0
Sucrose + citric acid	9

[a]All fractions and compounds were tested at concentrations equivalent to 3% orange juice. Actual concentrations (mg/100 ml) - Ascorbic acid, 1.5; citric acid·H_2O, 29.4; l(−) malic acid, 4.8; glucose, 77; fructose, 77; sucrose, 141. pH adjusted to 4.5.

[b]Acidic substances eluted from Dowex-1 resin with 1 N HCl.

[c]Components of orange juice dialysate remaining after removal of acidic and basic substances.

Effect of the neutral fraction of orange juice and its components upon spore germination.—Table 3 shows that the neutral fraction of orange juice dialysate supported a moderate level of spore germination (35%). Combination of the neutral fraction with the organic acids fraction resulted in spore germination which was almost equal to that observed in orange juice dialysate. Paper chromatography of the neutral fraction confirmed the presence of sucrose, glucose, fructose, and myo-inositol as reported earlier (27).

Glucose, fructose, sucrose, and myo-inositol alone at a concentration equivalent to 3% orange juice (5, 20, 27) had little effect upon spore germination (Table 3). The combination of citric acid with glucose or fructose, but not sucrose, increased germination significantly over either compound alone.

Effect of concentration and combinations of active constituents of orange juice and related compounds upon spore germination.—Ascorbic acid, 2 mg/ 100 ml, exhibited maximum stimulation of spore germination in the pH range 4.3-4.5. At pH 4.5, the optimum concentration of L-ascorbic acid for stimulation of spore germination (80%) was in the range 5-10 mg/ 100 ml (Table 4). These concentrations are 10-20 times greater than in 1% orange juice, which provided maximum spore germination. None of the conidia germinated in a solution of 50 mg ascorbic acid/ 100 ml. The activity of L-ascorbic acid was compared with D-ascorbic acid (iso-ascorbic acid) and dehydroascorbic acid (oxidized form of L-ascorbic acid) at concentrations of 2.5 and 5.0 mg/ 100 ml. L-Ascorbic and dehydroascorbic acids stimulated germination of 75-80% of the spores, whereas D-Ascorbic acid supported only one-half as much germination.

Glucose (77 mg/ 100 ml) was combined with 11 other organic acids (0.42 meq/100 ml). D(+)−Malic, L(−)−malic, L(+)−tartaric, L(−)−tartaric, malonic, oxalic, and pyruvic acids were almost as effective as citric acid when combined with glucose, whereas succinic, fumaric, maleic, and acetic acids were inactive in this combination. Incubation of spores with glucose alone resulted in a shift from pH 4.5 to 6.0 during the 16-hr germination period, whereas the organic acids buffered the culture at pH 4.5. In the glucose-citric acid solution, spore germination was essentially the same (approximately 60%) at pH 4.0, 4.5, and 5.0, but was reduced to 14% at pH 6.0.

Combinations of glucose, ascorbic acid, and citric acids were assayed to determine the simplest mixture of these compounds which would provide maximum spore germination and growth. At concentrations equivalent to 3% orange juice, ascorbic acid was considerably more active than glucose in stimulating germination, but germ

tube growth was poor in ascorbic acid alone (Tables 3,4). Germ tube development was improved by supplementing ascorbic acid with either glucose or citric acid (Table 4). In fact, a high level of glucose (2.56 g/100 ml) combined with citric acid also supported a high level of germination and moderately vigorous germ tube development (Table 4).

Investigation of the influence of the cationic fraction of orange juice on the development of germ tubes.—Although the cationic fraction of orange juice alone had little effect upon the initiation of spore germination, the combination of the noncationic and cationic fractions usually resulted in a mixture of nutrients which stimulated spore germination to the same level as 1% orange juice dialysate (Table 1). However, this combination often did not match orange juice dialysate in supporting vigorous germ tube development. In these cases, the addition of KH_2PO_4, $MgSO_4$, and $Ca(NO_3)_2$ to the recombined orange juice fractions restored essentially all of the activity of orange juice dialysate, indicating that inorganic cations were not eluted quantitatively from the cation exchange resins by NH_4OH. However, the inorganic salts alone or in combination did not have a significant effect upon spore germination. Several partitions of the cationic fraction were prepared by ion exchange chromatography, paper chromatography, paper electrophoresis, and liquid-liquid extractions in an attempt to isolate all of the activity of the cationic fraction into one component which was less complex than the original mixture of cationic substances. Numerous fractions were isolated and tested in combination with the noncationic fraction of orange juice, but none of these fractions possessed all the activity of the cationic fraction.

The L-isomers of the following amino acids were evaluated alone at concentrations equivalent to 3% orange juice (9): proline, arginine, asparagine, glycine, phenylalanine, serine, isoleucine, leucine, threonine, alanine, α-aminobutyric acid, valine, cysteine, methionine, tryptophan, histidine, glutamine, tyrosine, glutamic acid, aspartic acid, and lysine. Other nitrogenous compounds screened for activity were: cystine, glutathione, γ-aminobutyric acid, β-aminobutyric acid, β-alanine, hydroxyproline, ornithine, citrulline, $(NH_4)_2SO_4$, NH_4NO_3, $(NH_4)_2HPO_4$, adenine, hypoxanthine, cytidine, cytosine and guanine. When combined with the noncationic fraction of orange juice, none of these compounds approached the activity of the cationic fraction. The possibility that the stimulation by the cationic fraction was due to the rather unique amines present in citrus juices (27) was evaluated by testing an ether extract of the alkalized cationic fraction. Neither this extract nor the amines reported as components of citrus fruits (choline, betaine, ethylamine, synephrine, octopamine, tyramine, and putrescine) had any visible influence upon germ tube development.

Formulation of a synthetic medium for germination and growth of conidia of Penicillium digitatum.—The amino acid composition of the cationic fraction was determined by ion exchange chromatography. Sixteen amino acids were identified and no major unknown bases were detected. Proline, serine, arginine, aspartic acid, and alanine were the principal amino acids in the cationic fraction of the orange juice samples examined, as previously reported (9). Mixtures of amino acids and vitamins were formulated to resemble the components of the cationic fraction of 1% orange juice (5, 9, 20, 27). The amino acid solution contained (mg/liter): L-proline, 9.5; L-arginine, 3.7; L-asparagine, 3.5; L-aspartic acid, 2.6; DL-serine, 1.1; DL-alanine, 0.71; L-glutamic acid, 0.62; DL-lysine, 0.55; L-glutamine, 0.47; DL-histidine $HCl \cdot H_2O$, 0.22; L-phenylalanine, 0.20; L-valine, 0.17; glycine, 0.14; L-tyrosine, 0.07; L-threonine, 0.07; DL-leucine, 0.07; L-isoleucine, 0.07; γ-aminobutyric acid, 0.07; and L-methionine, 0.03. Addition of this amino acid mixture to the noncationic fraction or the combined anionic and neutral fractions of orange juice dialysate did not stimulate germ tube development to the same extent as the cationic fraction in these same combinations (Table 5). Addition of the vitamins reported to be present in orange juice [(μg/liter); thiamine·HCl, 52.0; nicotinamide, 200; pyridoxine·HCl, 30.4; biotin, 0.40; riboflavin, 14.4; Ca-pantothenate, 120; and folic acid, 2.40] to the combined synthetic amino acid mixture and noncationic fraction resulted in germ tube development equal to that produced by the cationic fraction when combined with the noncationic fraction (Table 5). The vigor of germ tube growth was further increased by addition of the inorganic macroelements of orange juice (5, 20, 27) [(mg/liter): KH_2PO_4, 178; $MgSO_4 \cdot 7H_2O$, 149; and $Ca(NO_3)_2$, 400] to the synthetic mixture of amino

TABLE 4. The interaction of glucose, ascorbic acid, and citric acid in stimulating germination of conidia of *Penicillium digitatum*

Nutrients (units/100 ml)	Spore germination (%)	Germ tube growth[b]
3 ml orange juice dialysate	93	2.25
1.5 mg ascorbic acid[a]	43	0.25
5 mg ascorbic acid	80	0.25
10 mg ascorbic acid	81	0.25
5 mg ascorbic acid + 1.28 g glucose	88	0.88
5 mg ascorbic acid + 2.56 g glucose	87	0.87
1.5 mg ascorbic acid + 32 mg citric acid[a]	79	0.50
77 mg glucose[a]	14	0.25
2.56 g glucose	75	0.50
2.56 g glucose + 32 mg citric acid	88	1.25

[a]Approximate concentration of nutrients in 3% orange juice.
[b]Value of 1.00 = average germ tube length approximately 130 μm.

acids and vitamins. Addition of trace amounts of Zn, Mn, Cu, Co, Mo, and B had no visible influence upon germination or germ tube growth.

The amino acids were tested individually at the concentration in which they were present in 1% orange juice. Several amino acids appeared to be slightly superior to the others in supporting vigorous germ tube growth. On the basis of these tests, proline, asparagine, aspartic acid, glutamic acid, arginine, leucine, serine, methionine, cysteine, histidine, tyrosine, and γ-aminobutyric acid were deleted singly from the amino acid mixture. The activity of the amino acid mixture in stimulating germ tube development was not diminished by omission of any one amino acid. A similar experiment revealed that the stimulatory property of the vitamin mixture was not dependent upon any single vitamin in the mixture.

A completely synthetic medium similar in composition to 1% orange juice was prepared by addition of the sugars and organic acids present in orange juice (5, 8, 20, 27) to the mixture of amino acids, vitamins, and inorganic nutrients described above. The following sugars and organic acids were added to make the complete synthetic nutrient solutions (mg/liter): glucose, 256; fructose, 256; sucrose, 486; myo-inositol, 16; citric acid·H_2O, 98; malic acid, 8; and ascorbic acid, 5. This synthetic medium supported germination and germ tube growth to almost the same extent as orange juice dialysate (Table 5-B). The composition of this synthetic medium could be simplified somewhat without a significant loss of activity. Fructose and sucrose could be deleted from the medium if the concentration of glucose was doubled. Malic acid was not essential and the omission of myo-inositol resulted in only a slight reduction in the thickness of the germ tubes. The presence of $MgSO_4$ and the microelements in the medium was not essential for maximum germination or vigorous germ tube growth. The development of large-diameter, vigorous germ tubes was dependent upon KH_2PO_4 in the medium; neither Na_2HPO_4 nor KCl alone could substitute, although the combination of the latter salts gave results identical to KH_2PO_4. Elimination of glucose from the medium resulted in a 50% reduction in germination and a 75% reduction in germ tube growth. Omission of the amino acids and/or vitamins reduced germ tube growth about 50%, but did not have a significant effect upon germination per se. The amino acid mixture could be replaced, without a substantial loss in activity, by proline, arginine, or $(NH_4)_2SO_4$ alone at a concentration equivalent to the total nitrogen in orange juice.

DISCUSSION

Over 80% of the conidia of *P. digitatum* (California isolate M6R) germinated in 1% orange juice dialysate in the range pH 3.5 - 8.0, but germ tube growth was most vigorous at pH 4.0 - 5.5. Generally, these values agree with those reported by earlier investigators (10, 22, 25, 29), but the bimodal pH curves reported for germination of *P. digitatum* in orange albedo extract (10) and for mycelial growth in synthetic medium (29), were not observed for conidia in dilute orange juice in our study.

The anionic fraction of orange juice dialysate stimulated germination of conidia, but did not support vigorous germ tube growth. In contrast, the neutral and cationic fractions, at concentrations equivalent to 1% orange juice, did not induce germination but supported vigorous growth of the germ tubes in combination with the anionic fraction. The most effective compound in the anionic fraction in stimulating germination was L-ascorbic acid. Citric acid enhanced the activity of ascorbic acid, but was not active alone. L-ascorbic acid at 5-10 mg/100 ml, a concentration range greater than present in 1% orange juice dialysate, supported 80% germination

TABLE 5. Development of conidia of *Penicillium digitatum* in synthetic mixtures of amino acids and vitamins simulating orange juice

Orange juice fraction[a]	Supplement of amino acids, vitamins and minerals[b]	Spore germination (%)	Germ tube growth[d]
A. Orange juice dialysate (3%)	...	97	3.00
Anionic + neutral + cationic	...	86	2.00
Anionic + neutral	Amino acids	85	1.50
Anionic + neutral	Amino acids + vitamins	90	2.00
Noncationic	...	90	0.88
Noncationic + cationic	...	94	1.75
Noncationic	Amino acids[c]	88	1.25
Noncationic	Amino acids[c] + vitamins	96	2.25
Noncationic	Amino acids[c] + vitamins + macroelements	95	2.75
Noncationic	Amino acids[c] + vitamins + macroelements + microelements	95	2.75
B. 5% Orange juice dialysate	...	96	3.5
...	Synthetic medium 1×	92	2.0
...	Synthetic medium 5×	95	3.5

[a]Fractions in "A" tested at concentrations equivalent to 3% orange juice dialysate.
[b]Tested at 3× the concentration given in the text except as noted.
[c]Tested at 6× the concentration given in test.
[d]Value of 1.00 = average germ tube length approximately 130 μm.

whereas the same concentration of other organic acids present in orange juice had little effect upon germination when administered alone. A mixture of ascorbic, citric, and malic acids stimulated germination of two Florida isolates of *P. digitatum* to about the same extent as the California isolate, whereas ascorbic acid alone was much less effective in stimulating germination of the Florida isolates. Ascorbic acid has been reported to increase germination of spores of *Spinellus macrocarpus* (28) and *Colletotrichum trifolii* (24). Ascorbic acid could fill several roles in stimulating the germination process (1, 6, 13) – (i) a carbon source, (ii) a structurally nonspecific redox buffer in the medium or in the cell, and (iii) an electron transport carrier. The utilization of ascorbic acid by spores of *P. digitatum* solely as a source of energy seems improbable since ascorbic acid did not support growth of the germ tubes after germination. The observation that isoascorbic acid (D-ascorbic acid) was much less effective than L-ascorbic acid and dehydroascorbic acid does not lend support to the hypothesis that ascorbic acid functions as a nonspecific redox buffer. A more plausible mechanism for stimulation of germination is that L-ascorbic acid and dehydroascorbic acid are part of an electron transport system comprised of enzymes which are sterespecific for the reduced and oxidized forms of L-ascorbic acid (3).

Glucose was significantly more effective than the other carbohydrates tested in supporting germination and germ tube growth and it appeared to be the principal factor responsible for the activity of the neutral fraction of orange juice. Kavanagh and Wood (19) observed that 1% glucose stimulated germination of spores of *P. digitatum* suspended in phosphate buffer at pH 5.2; fructose and sucrose were only slightly less effective. However, less than 10% of the spores germinated in water solutions of these same sugars. Glucose was considerably more effective than fructose or sucrose in stimulating germination of spores of *P. atrovenetum* (17) and *P. griseofulvum* (15) suspended in a mineral salts medium. The superiority of glucose over fructose and sucrose in stimulating spore germination may result from more efficient transport of glucose across the cell membrane or more effective utilization of this sugar by constitutive enzymes of the spore (2, 7, 11). However, enzymes for the metabolism of sucrose must be present or readily induced in the hyphae of *P. digitatum* since this disaccharide is an excellent substrate for mycelial growth of this fungus (14, 26, 29). Previous work in our laboratory revealed that dry spores of *P. digitatum* contained substantial amounts of trehalose and polyols, but not detectable quantities of reducing sugars (12). The trehalose and polyols disappeared during germination, but apparently were not utilized in the absence of exogenous glucose or another sugar that supports germination. A rapid turnover of trehalose in yeast also is dependent upon the presence of a fermentable sugar (2).

A variety of organic acids (i.e., citric, L-malic, D-malic, L-tartaric, D-tartaric, malonic, oxalic, and pyruvic) at 0.42 meq/ 100 ml greatly increased the effectiveness of 0.08% glucose in supporting a high level of spore germination and vigorous germ tube development. Citric and malic acids have been reported to support mycelial growth of *P. digitatum* (14, 29) whereas fumaric, tartaric, acetic, and oxalic acids are poor substrates in cultures of this fungus. The diversity in chemical structure of the stimulatory organic acids, and the fact that they do not all serve as carbon sources for *P. digitatum*, suggest that these compounds might function to buffer the medium in the optimum pH range for spore germination. This buffer hypothesis is supported by the observation that the pH of a 0.08% glucose solution increased from pH 4.5 to 6.0 during a 16-hr incubation period with spores and less than 10% of the spores germinated. Citric acid (0.42 meq/ 100 ml) buffered this glucose solution at pH 4.5 and over 50% of the spores germinated. However, only 14% of the spores germinated in the glucose-citric acid solution adjusted to pH 6 with dilute NaOH. A possible interpretation of these interactions is that transport of glucose into the conidia is a limiting factor in germination and this process may be more efficient at pH 4.5 than at pH 6.0. The observation that a higher concentration of glucose (2.56 g/ 100 ml) stimulated germination equally at pH 4.5 and pH 7.5 is consistent with this hypothesis. However, citric acid further increased the effectiveness of the higher concentration of glucose, indicating either that pH 4.5 is beneficial to germination for reasons other than glucose utilization or that certain organic acids have a role other than pH control. The observation that solutions of glucose buffered at pH 4.5 with acetic or succinic acids do not stimulate germination does not support the buffer hypothesis. However, acetic acid may be toxic. Kavanagh and Wood (19) reported that glucose stimulated germination in the presence of phosphate or citrate buffers at pH 5.2, but not in acetate buffer.

Previous investigators (4, 26, 29) reported that orange juice or extracts of orange peel stimulated germination and growth of *P. digitatum*, but they were unable to isolate growth factors that were responsible for this activity. Our investigations indicate that the following components of orange juice, when combined, stimulate germination: (i) glucose and citric acid; (ii) glucose and ascorbic acid; (iii) ascorbic acid and citric acid. Exogenous nitrogen compounds were not required for germination, but amino acids and vitamins, when combined, substantially increased germ tube growth after germination. Davis and Smoot (10) reported also that exogenous amino acids were not essential for germination of their isolate of *P. digitatum* even though other investigators found that early growth (germ tube growth) in liquid cultures was stimulated by amino acids and vitamins (14, 26, 29). Other species of *Penicillium* appear to require both exogenous carbon and nitrogen sources for high percentages of germination of conidia (15, 17, 23).

A synthetic mixture of amino acids, vitamins, and other known components of orange juice was almost as effective as orange juice in supporting vigorous germ tube growth in *P. digitatum*. No single amino acid or vitamin, at the concentration in orange juice, promoted germ tube growth to the same extent as 1% natural orange juice and none of those compounds was uniquely essential for good growth. Both potassium and phosphate, but not magnesium or microelements, were essential for vigorous germ tube development. Gottlieb and Tripathi (17) observed that phosphate, but not potassium, was required for germination of *P. atrovenetum* and

magnesium had only slight effect upon this process. Potassium may stimulate phosphate uptake in *P. digitatum* as in the case of yeast (16). A complex synthetic medium containing most of the major constituents of natural orange juice was required to support germination and growth of *P. digitatum* conidia equivalent to that provided by natural orange juice. These observations lead to the conclusion that the activity of orange juice in supporting germination and development of conidia of *P. digitatum* is a summation of the interaction of many nutrients and growth factors rather than the stimulation by one or several unique constituents of orange juice.

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