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Nutrient Utilization of *Macrophominia phaseolina*: A Chromogenic Isolate from Cantaloupe Fruit

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ABSTRACT

Carbon, nitrogen, vitamin, and pH requirements for mycelial growth were determined for the chromogenic cantaloupe fruit isolate *Macrophomina phaseolina* (TX-33) cultured in liquid and solid media. In liquid culture, the fungus best utilized, sucrose, maltose, fructose, mannose and starch as individual carbon sources when compared to glucose, the reference compound. In agar cultures, the fungus grew best on fructose, glucose, and sucrose. In regard to nitrogen sources, mycelial growth in liquid culture was greatest on glutamic acid, peptone, and asparagine when compared to the reference compound ammonium succinate. However, radial growth ranked highest on agar amended with ammonium succinate or peptone.

Vitamin treatments suppressed mycelial growth in liquid culture but had little effect in agar. Mycelial growth of the cantaloupe isolate in liquid culture was suppressed above pH 4.6. Microsclerotial production was generally greater in nutrient studies that had the best mycelial growth. Conversely, as microsclerotial production decreased, microsclerotial size increased. The results of this study demonstrate the importance of substrate composition which may strongly influence disease development by *M. phaseolina* both as a fruit rotter and as the cause of vine decline.

RESUMEN

Se determinaron los requerimientos de carbono, nitrógeno, vitaminas y pH para el crecimiento micelial del aislamiento cromogénico proveniente de fruto de melón de *Macrophomina phaseolina* (TX-33) cultivado en medios sólido y líquido. En cultivo líquido, el hongo utilizó mejor sacarosa, maltosa, fructosa, manosa y almidón en comparación con glucosa, el compuesto de referencia. En cultivos de agar, el hongo creció mejor en fructosa, glucosa y sacarosa. En lo referente a las fuentes de nitrógeno, el crecimiento micelial en el cultivo líquido fue mayor sobre ácido glutámico, peptona y asparagina en comparación con el crecimiento observado en el compuesto de referencia succinato de amonio. Sin embargo, el valor más alto de crecimiento radial se presentó en agar modificado con succinato de amonio o peptona. Los tratamientos con vitaminas suprimieron el crecimiento micelial en cultivo líquido pero tuvieron poco efecto en el crecimiento en agar. El crecimiento micelial del aislamiento de *M. phaseolina* en cultivo líquido fue suprimido a valores de pH mayores de 4.6. La producción de macroesclerocios fue generalmente mayor en los estudios de nutrientes que presentaron el mejor crecimiento micelial. Recíprocamente, a medida que la producción de microesclerocios decreció, se incrementó el tamaño de éstos. Los resultados de este estudio demonstraron la importancia de la composición del sustrato la cual puede influenciar tremendamente el desarrollo de la enfermedad causada por *M. Phaseolina* tanto como agente pudridor del fruto o como causa del declinamiento de la enredadera.

Macrophomina phaseolina (Tassi) Goid. is a widespread root, stem, and fruit pathogen of many economic crops (Dhingra and Sinclair, 1978). Considerable losses due to vine decline and/or fruit rot of cantaloupe (*Cucumis melo* L.) caused by *M. phaseolina* have been reported in India (Jhooty and Singh, 1971), USA (Bruton et al., 1985; Bruton et al., 1987; Carter, 1979), and Israel (Reuveni et al., 1982). Purkayastha and Roy (1974) showed that two strains of *M. phaseolina* from jute displayed differences in their amino acid requirements which correlated with virulence. Additionally, Gangopadhyay et al. (1974) demonstrated that nutrient availability in soybean varieties was correlated with resistance to *M. phaseolina*. Infection of cantaloupe roots, caused by *M. phaseolina*, occurs early in plant growth but the fungus causes little damage until fruit near maturity (Bruton et al., 1985; Bruton et al., 1987). During fruit maturation, distribution of nutrients change as nutrients are directed to the fruit (source-to-sink). Hughes et al. (1983) demonstrated that photosynthate from the leaves nearest the melon were the most important in supplying carbohydrates to the fruit. The associated physiological changes may be associated with the development of vine decline in cantaloupe. With the onset of vine decline, the crown leaves are the first to succumb, thus fruit quality is adversely affected. Therefore, nutrient availability and requirements of this fungal pathogen may be related to its virulence. Wide ranging nutritional requirements and their relationship to the host demonstrate the importance of defining specific nutritional needs for each host-pathogen relationship.

The purpose of this study was to determine the pH and nutritional (carbon, nitrogen, and vitamin) requirements of a chromogenic cantaloupe fruit rot pathogen (*M. phaseolina* TX-33) in agar and liquid media.

MATERIALS AND METHODS

The methods used for nutrient studies were essentially those of Taber, et al. (1968) and Bruton, et al. (1990) with minor modification. All nutrient sources were reagent grade or better. The commercial grades of mannitol and galactose were used without further purification and powdered methylcellulose was used rather than Whatman filter paper. Biotin and thiamin were not tested for contamination. All liquid culture treatments were harvested at 3 or 6 days using 4 replications/treatment in two different studies. Glucose, ammonium succinate, and all vitamins were used as reference compounds for mycelial growth on carbon, nitrogen, and vitamins, respectively. The reference pH was 4.6. Mycelial growth in each of the respective studies is reported as a ratio to the reference compound. Presence or absence of wine-red pigment in liquid culture was noted at the end of 6 days. Mean dry weights of fungal mycelial mats were determined by filtering fungal shake cultures through Whatman filter paper using a Buchner funnel. The fungal mats were dried in an incubator at ca. 50 C until dry weight was stabilized over several days and weighed immediately after removal from incubator.

For solid agar, 6 fourteen-day old cultures were used to obtain sclerotia enumeration and size. The culture was macerated in 100 ml of water using a semi-micro blender. A 1-ml aliquot from a final dilution of 1:1000 was counted. Size determinations were made using a microscope ocular micrometer. Radial growth measurements were taken daily for 3 days. All agar treatments consisted of 10 replications in each of two separate studies. ANOVA and Duncan's multiple range tests were used to determine significant differences (P=0.05) among treatments in both agar and liquid broth experiments. All studies were carried out at 25 C.

RESULTS

Carbon Source. The growth of *M. phaseolina* in liquid culture was optimum when sucrose was the carbon source (Table 1). Growth relative to glucose was enhanced by supplying fructose, maltose, mannose, or starch as a carbon source. The enhanced relative growth in response to sucrose, fructose, maltose, mannose, and starch was detected at both the 3- and 6-day harvest. Five of the 9 carbon sources examined in liquid culture resulted in growth superior to glucose, the reference carbon source. There was no significant (P=0.05) difference among galactose, mannitol, methyl cellulose, and the no-carbon treatments. In contrast to growth in liquid culture, only fructose enhanced radial growth relative to glucose on agar medium (Table 2). The increase was very slight and averaged 5%. After 14 days incubation, the number of microsclerotia on agar medium supplemented with sucrose was over twice that of the glucose standard. Microsclerotial density was also greater in the galactose, maltose and starch treatments when compared to the glucose standard. Methylcellulose appeared to inhibit radial hyphae growth when compared to the no-carbon treatment. No clear relationship could be established between the length and width of microsclerotia on specific carbon sources.

Nitrogen Source. The rankings of *M. phaseolina* growth response to nitrogen sources changed substantially in liquid culture (Table 3). Growth relative to ammonium succinate, the reference nitrogen source, was greater after 6 days in medium containing glutamic acid, peptone or asparagine. Liquid cultures were often pigmented after 6 days incubation. The pigmentation ranged from bright-red to dark-red but failed to occur in medium supplied with glutamic acid or no nitrogen (Table 3). However, bright-red pigmentation was consistently present in cultures supplemented with glycine or peptone.

Although significant (P=0.05) differences in the relative radial growth were observed, the magnitude of those growth differences on agar in response to the selected nitrogen sources were small (Table 4). Relative radial growth of *M. phaseolina* was greater on ammonium succinate and peptone than on any of the other nitrogen sources in agar. Phenylalanine and glycine were the only nitrogen sources yielding less than 90% of the growth obtained on ammonium succinate. Pigment synthesis was not observed in cultures on solid agar. The magnitude of responses to different nitrogen sources was also much greater in liquid than in agar. The entire range of growth responses to various nitrogen sources was 203% and 24% for liquid and solid media, respectively. The physical characteristics of microsclerotia were generally greater on the inferior nitrogen sources. Density of microsclerotia/cm3 appeared to be inversely proportional to microsclerotia length and width ratio. Potassium nitrate, leucine, phenylalanine and glycine were found to yield fewer microsclerotia/cm^3, however, the microsclerotia were significantly (P=0.05) larger in these nitrogen sources than the others tested.

Vitamin Source. Early growth at 3 days was enhanced in liquid culture by the addition of vitamins with the exception of calcium pantothenate, pyridoxine and thiamine (Table 5). In 6-day old cultures, inositol was the only vitamin that was not suppressive to growth. The vitamin requirements for optimum growth were not clearly defined when agar was the substrate (Table 6). No more than a 5% difference in relative growth was observed across all treatments. Microsclerotial development was relatively unaffected by vitamin treatments.

pH of Media. Mycelial growth at different pH's was variable. However, growth on both media, was optimum at 4.6 and suppressed at 7.1 (Table 7 and 8). Consequently, the growth of the cantaloupe isolate was favored by acidic cultural conditions. Robust microsclerotial development was observed in the slower growing treatments where microsclerotial densities were lowest (Table 8).

DISCUSSION

Mycelial growth of the *M. phaseolina* isolate from cantaloupe fruit was greatest in liquid media containing sucrose followed by maltose and fructose. Sucrose has been reported the best carbon source for both a tobacco (Singh, 1967) and

Table 1. Effect of carbon sources on mycelial growth of *Macrophomina phaseolina* in liquid culture relative to glucose.

Carbon Source	Growth ratio[z] 3 days	Growth ratio 6 days
Sucrose	3.54a	1.92a
D-(+)-Maltos	2.02b	1.74b
D-Fructose	1.88b	1.68b
D-(+)-Mannose	1.09c	1.26c
Starch	1.69c	1.19c
D-(+)-Glucose	1.00d[y]	1.00d[x]
D-Galactose	0.24e	0.17e
D-Mannitol	0.09e	0.09e
Methyl-cellulose	0.15e	0.05e
No carbon	0.05e	0.05e

[z]Fungal growth ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference. The absolute values can be calculated.

[y]Ratio of dry weight of mycelial mat to reference carbon (glucose): 50.2 mg.

[x]Ratio of dry weight of mycelial mat to reference carbon (glucose): 221.0 mg.

sugarbeet isolate (Singh et al, 1974). In the same study, growth of the tobacco and sugarbeet isolates on starch was comparable to sucrose. Starch was also the best carbon source for a potato (Paharia and Sahai, 1968) and cotton isolate (Luthra et al., 1940) of *M. phaseolina*; whereas, sucrose ranked much lower.

Cantaloupe fruits are relatively high in sucrose, fructose, and glucose but contain no starch (Hughes and Yamaguchi, 1983). The lack of starch in cantaloupe may explain the reduced capacity of this isolate to utilize starch as a carbon source. Singh and Chohan (1979) reported that cantaloupe fruit tissue infected by *M. phaseolina* was depleted of sucrose and fructose with over 75% reduction in original levels of glucose. On agar media, mycelial growth of *M. phaseolina* from cantaloupe fruit was greatest on fructose, glucose, and sucrose while starch was one of the poorest sources of carbon for growth. Growth of a sorghum *M. phaseolina* isolate on agar (Livingston, 1945) generally had the same carbon preferences as the cantaloupe isolate used in this study.

In general, isolates from different hosts, including our cantaloupe isolate, grew well in liquid media containing glutamic acid, peptone, or asparagine as nitrogen sources (Livingston, 1945; Paharia and Sahai, 1968; Singh et al, 1974). With the exception of a potato isolate (Paharia and Sahaai, 1968), the growth of most *M. phaseolina* isolates is relatively poor on urea which follows closely with results obtained from this study. Growth studies by Purkayastha and Roy (1974) indicated that amino acid utilization was a metabolic determinant of virulence in *M. phaseolina*. The virulent strain in their study grew most rapid on a medium supplemented with glutamic acid as a nitrogen source. Glutamic acid proved to be the best nitrogen source for our cantaloupe

Table 2. Effect of carbon sources on radial growth and microsclerotia development of *Macrophomina phaseolina* on agar media relative to glucose.

Carbon source	Growth ratio[z] 3 days	Microsclerotia[y] #/cm³	Length ratio	Width ratio
D-Fructose	1.05a	1.04e	0.89b	0.98b
D-(+)-Glucose	1.00b[x]	1.00e[w]	1.00a[v]	1.00b[u]
Sucrose	0.99b	2.11a	0.75cd	0.76c
D-(+)-Mannose	0.93c	0.69f	1.05a	1.14a
D-Galactose	0.88d	1.95b	0.62e	0.66e
D-Mannitol	0.86d	0.47g	0.70d	0.81c
D-(+)-Maltose	0.86d	1.72c	0.82bc	0.78c
Starch	0.71e	1.44d	0.79c	0.79c
No carbon	0.59f	0.16h	0.58ef	0.67d
Methyl-cellulose	0.46g	0.16h	0.53f	0.66d

[z]Ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference.

[y]Microsclerotia counts were made after 14 days of growth on specified carbon source.

[x]Radial growth ratio to reference carbon (glucose): 35.4 mm.

[w]Ratio of microsclerotial number to reference carbon (glucose): 26.6/cm³.

[v]Microsclerotial length ratio to reference carbon (glucose): 146.4 µm.

[u]Microsclerotial width ratio to reference carbon (glucose): 89.9 µm.

Table 3. Effect of nitrogen sources on mycelial growth and chromogenic activity of *Macrophomina phaseolina* in liquid culture relative to ammonium succinate.

Nitrogen	Growth ratio[z] 3 days	Growth ratio 6 days	Chromogenic[y] rating
L-Glutamic acid	0.96c	1.37a	-
Peptone	2.39a	1.12b	+
L-Asparagine	1.30b	1.08b	+
Ammonium succinate	1.00c[x]	1.00c[w]	+
Urea	0.66d	0.74d	+
L-Leucine	0.56d	0.57e	+
Potassium nitrate	0.43e	0.53f	+
Glycine	0.30f	0.48fg	+
L-Phenylalanine	0.36ef	0.46g	+
No nitrogen	0.11g	0.04h	-

[z]Fungal growth ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference. The absolute values can be calculated.

[y]Chromogenic activity was determined visually with red-wine pigmentation considered a + value.

[x]Ratio of dry weight of mycelial mat to reference nitrogen (ammonium succinate): 97.7 mg.

[w]Ratio of dry weight of mycelial mat to reference nitrogen (ammonium succinate): 438.8 mg.

isolate as well. The evidence linking amino acid utilization with virulence has gained additional support from a study showing that the level of glutamic acid was selectively reduced in cantaloupe fruit infected by *M. phaseolina* (Singh and Chohan, 1979).

Cantaloupe isolates from Texas (Carter, 1979) and Israel (Reuveni et al., 1982) were reported to be chromogenic, producing a red pigment in infected melon fruits. Ghosh and Sen (1973) obtained a deep blood red pigment when glycine, alanine, serine, aspartic acid, glutamic acid, sodium glutamate, asparagine, or ammonium acetate were used as a nitrogen source. In contrast, our cantaloupe isolate produced no pigment in the presence of glutamic acid with nconsistent pigment intensity in the remaining nitrogen sources. The significance of this chromophore and metabolic regulation are as yet undetermined (Dunlap and Bruton, 1986).

Mycelial growth of the cantaloupe isolate of *M. phaseolina* in liquid culture was suppressed by all vitamins except inositol. However, calcium pantothenate, pyridoxine and thiamine, were the most inhibitory to growth during the first 3 days in liquid culture. The agar media dramatically reduced the sensitivity to various vitamin treatments which indicates the importance of medium in assessment of certain nutrient requirements. Growth of isolates from other hosts has been promoted by thiamine (Sankhla and Mathar, 1967). Gough and Lilly (1955) demonstrated that a *M. phaseolina* isolate

Table 4. Effect of nitrogen sources on radial growth and microsclerotia development of *Macrophomina phaseolina* on agar media relative to ammonium succinate.

Nitrogen source	Growth ratio[z] 3 days	Microsclerotia[y] #/cm³	Length ratio	Width ratio
Ammonium succinate	1.00a[x]	1.00c[w]	1.00cd[v]	1.00cd[u]
Peptone	0.99a	1.20b	0.92de	0.98cd
L-Asparagine	0.96b	1.45a	0.85e	0.91d
Urea	0.96b	1.04c	1.06bc	1.07c
Potassium nitrate	0.94b	0.64d	1.27a	1.24b
L-Glutamic acid	0.94b	1.27b	1.13b	0.92d
L-Leucine	0.92c	0.65d	1.24a	1.23b
L-Phenylalanine	0.82d	0.46e	1.33a	1.39a
Glycine	0.78e	0.40e	1.35a	1.32ab

[z]Ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference.

[y]Microsclerotia counts were made after 14 days of growth on specified carbon source.

[x]Radial growth ratio to reference nitrogen (ammonium succinate): 39.4 mm.

[w]Ratio of microsclerotial number to reference nitrogen (ammonium succinate): 26.8/cm³.

[v]Microsclerotial length ratio to reference nitrogen (ammonium succinate): 142.2 µm.

[u]Mcrosclerotial width ratio to reference nitrogen (ammonium succinate): 84.6 µm.

was self sufficient with regard to thiamine, biotin, inositol, and pyridoxin since the rate of growth was the same on both vitamin-free and vitamin-containing media.

Considerable differences have been reported among isolates as to their tolerance to pH with the optimum in the 3.6 to 5.0 range (Dhingra and Sinclair, 1978). The growth of our cantaloupe isolate was significantly better at pH 4.6 than 7.1. As pointed out by Dhingra and Sinclair (1978), data must be interpreted carefully because most researchers did not use buffered media.

Microsclerotial production in the various nutrient studies was normally greater on those sources in which the most growth occurred. The same trend has been reported by other researchers working with various isolates (Ahmed and Ahmed, 1969; Singh et al., 1974; Vasudeva, 1937). Conversely, as microsclerotial production decreased, microsclerotial size increased. Inoculum densities of *M. phaseolina* have been positively correlated with root infection (Watanabe et al., 1967; Meyer et al., 1974). Microsclerotial population and size play an important role in inoculum potential. The number of cells were directly related to size of microsclerotia and size appeared to depend on the available

Table 5. Effect of vitamins on mycelial growth of *Macrophomina phaseolina* in liquid culture relative to vitamin treatment.

Vitamin source	Growth ratio[z] 3 days	Growth ratio 6 days
All vitamins **minus**:		
calcium pantothenate	1.27a	2.01a
pyroxidine hydrochloride	1.30a	1.96a
para-aminobenzoic acid	0.90bc	1.96a
thiamine	1.27a	1.82a
nicotinamide	1.01b	1.76ab
biotin	0.86bc	1.54bc
all vitamins	0.75c	1.41c
inositol	0.77c	1.09d
All vitamin	1.00b[y]	1.00d[x]

[z]Fungal growth ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference. The absolute values can be calculated.

[y]Ratio of dry weight of mycelial mat to the all vitamin reference: 45.1 mg.

[x]Ratio of dry weight of mycelial mat to the all vitamin reference: 219.8 mg.

Table 6. Effect of vitamins on radial growth and microsclerotia development of *Macrophomina phaseolina* on agar media relative to vitamin treatment.

Vitamin source	Growth ratio[z] 3 days	Microsclerotia[y] #/cm^3	Length ratio	Width ratio
All vitamins	1.00a[x]	1.00ab[w]	1.00ab[v]	1.00abc[u]
All vitamins minus:				
calcium pantothenate	0.99ab	0.99ab	1.04ab	1.00abc
thiamine	0.99abc	1.02ab	1.07ab	0.99abc
pyroxidine hydrochloride	0.99abc	0.98ab	1.12a	1.05ab
nicotinamide	0.98abc	1.02ab	1.08ab	0.98abc
all vitamins	0.98abc	0.96ab	1.03ab	0.96bc
inositol	0.97bcd	0.89b	1.05ab	1.08a
biotin	0.96cd	0.95ab	0.96b	0.93c
para-aminobenzoic acid	0.95d	1.10a	1.04ab	1.04abc

[z]Ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference.

[y]Microsclerotia counts were made after 14 days of growth on specified carbon source.

[x]Radial growth ratio to all vitamin reference: 36.6 mm.

[w]Ratio of microsclerotial number to all vitamin reference: 38.5/cm^3.

[v]Microsclerotial length ratio to all vitamin reference: 133.5 µm.

[u]Mcrosclerotial width ratio to all vitamin reference: 85.2 µm.

Table 7. Effect of pH on mycelial growth of *Macrophomina phaseolina* in liquid culture relative to pH 4.6.

pH	Growth Ratio[z] 3 days	Growth Ratio 6 days
4.6	1.00a[y]	1.00a[x]
5.6	0.70b	0.64b
6.6	0.57c	0.55c
7.1	0.57c	0.51c

[z]Fungal growth ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference. The absolute values can be calculated.
[y]Ratio of dry weight of mycelial mat to reference pH (pH 4.6): 82.6 mg.
[x]Ratio of dry weight of mycelial mat to reference pH (pH 4.6): 277.6 mg.

Table 8. Effect of pH on radial growth and microsclerotial development of *Macrophomina phaseolina* on agar media relative to pH 4.6.

	Growth ratio[z]	Microsclerotia[y]		
pH	3 days	#/cm³	Length ratio	Width ratio
4.6	1.00a[x]	1.00a[w]	1.00b[v]	1.00c[u]
5.6	1.00a	0.78b	1.05b	1.10b
6.6	0.99a	0.85b	1.02b	1.11b
7.1	0.77b	0.65c	1.18a	1.20a

[z]Ratios followed by a different letter are significant at the P=0.05 level as determined by Duncan's multiple range test, within columns. Growth of *M. phaseolina* is expressed as a ratio using glucose as a carbon reference.
[y]Microsclerotia counts were made after 14 days of growth on specified carbon source.
[x]Radial growth ratio to reference pH (pH 4.6): 37.1 mm.
[w]Ratio of microsclerotial number to reference pH (pH 4.6): 30.8/cm³.
[v]Microsclerotial length ratio to reference pH (pH 4.6): 132.8 µm.
[u]Mcrosclero

nutrients of the substrate on which the *M. phaseolina* propagules were produced (Short and Wyllie, 1978). In addition, large microsclerotia produce more germ tubes than do small microsclerotia on culture media. Chan and Sackston (1973) found differential sclerotial formation of virulent and avirulent isolates from plant tissue. They further proposed that sclerotial formation reflects the nutritional level of a given isolate on its substrate, as well as its genotypic relationship. Our study demonstrated that nutrients have an effect on microsclerotial production and size which may have a significant impact on inoculum potential.

The nutritional requirements of our cantaloupe isolate, grown on agar, were much less stringent than the same treatments in liquid media. After 6 days in liquid culture, mycelial growth was best in cultures amended with sucrose and glutamic acid as the carbon and nitrogen source, respectively. However, radial growth was best on agar media amended with fructose and ammonium succinate or peptone as the carbon and nitrogen source, respectively. The vitamin experiments demonstrated substantial differences in growth response as a function of medium. The growth on agar was essentially the same in the presence or absence of vitamins. In liquid culture, the entire vitamin complex suppressed the growth of 3-day old cultures with even more extensive suppression at 6 days. Consequently, nutrient composition and availability may play a major role in the onset of vine decline and fruit rot of cantaloupe caused by *M. phaseolina*.

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