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# Effect of Limiting Concentrations of Growth Factors in Mass Rearing Diets for *Ceratitis capitata* Larvae (Diptera: Tephritidae)

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**ABSTRACT** Effects of growth factors on *Ceratitis capitata* (Wiedemann) larval development were investigated using *C. capitata* #1 diet as a control diet. *C. capitata* #1 diet is a chemical base diet with only one nonchemical bulking agent, corncob. It contains 10 exogenous vitamins in addition to those trace amounts already present in corncob, and it is capable of supporting normal larval development. Removal of all 10 growth factors from *C. capitata* #1 diet resulted in a prolonged developmental period, decreased pupal recovery, pupal weight, and adult emergence, but normal flight ability and egg hatch. *C. capitata* #1 diet lacking exogenous nicotinic acid resulted in complete second-instar mortality. In the absence of exogenous riboflavin, larvae reached the third instar and pupated, but the rate of development was slower. However, the percent pupal recovery was not significantly different. Pupal weight and adult emergence were significantly decreased, whereas flight ability and egg hatch remained within normal ranges. The effects of omitting exogenous pantothenic acid were similar to those for riboflavin compared with the control diet. The omission of other exogenous vitamins, such as thiamine, pyridoxine, biotin, folic acid, *p*-amino benzoic acid, inositol or choline, had no significant effects on the rate of larval development and survival. Exogenous nicotinic acid was determined to be indispensable (requires a minimum of 2 ppm), whereas riboflavin and pantothenic acid appear to be required (at least >2 ppm) for normal growth and development of *C. capitata* larvae. Addition of exogenous ascorbic acid phosphate or  $\alpha$ -tocopherol in *C. capitata* #1 diet did not improve *C. capitata* development or growth. Wheat germ is not necessary as a mass rearing diet additive because two of its major nutrients ( $\alpha$ -tocopherol and choline) showed no impact.

**KEY WORDS** *Ceratitis capitata*, vitamins, growth factors, diet

GROWTH FACTORS, ORGANIC micronutrients required in small amounts in the diet of human beings and most animals for proper growth and function, are essential for the action of many enzymes (coenzymes) and therefore play a vital role in cell metabolism as coenzymes. Micronutrients in insect nutrition can be grouped into two classes, water-soluble and lipid-soluble. Water-soluble factors include the B vitamins (thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, *p*-amino benzoic acid, and folic acid), the lipogenic factors (choline and inositol), and ascorbic acid (vitamin C). Lipid-soluble factors include vitamins A, D, E, and K.

The vitamin requirements of insects have prompted much research on different orders of insects (Baker 1975, Dadd 1985, Reinecke 1985, Anand and Pant 1988), concluding that most species of insects require thiamine, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid (Friend and Patton 1956). Kumar and Anand (1992) demonstrated that deletion of either thiamine, nicotinic acid, pantothenic acid, or choline from *Dacus dorsalis* (Handel) larval diets resulted in zero pupal recovery, whereas omission of riboflavin, pyridoxine, inositol, biotin, or folic acid caused a large reduction in pupal recovery. Friend and Patton (1956) studied the vitamin requirements of onion maggot,

*Delia antiqua* (Meigen), larvae under aseptic conditions and found that omission of vitamin B12, thioctic acid, or coenzyme A delayed larval development slightly; fewer larvae pupated and the ratio of male to female flies was high.

Studies on optimizing diet quality for mass rearing *Ceratitis capitata* (Wiedemann) have been ongoing for over half a century. The diets used currently throughout the world were developed by combining ingredients selected mostly on the basis of availability, physical properties, and economics with little knowledge of nutrient value. The needed information on nutritional requirements for *C. capitata* is still lacking because of the inability to rear larvae on a completely purified diet. Here we investigated the effect of limiting concentrations of growth factors in *C. capitata* larval diet by using *C. capitata* #1 diet (contains thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folic acid, *p*-amino benzoic acid, choline, and inositol) as the control diet.

## Materials and Methods

**Insects.** *C. capitata* eggs were obtained from colonies of Maui med 93 adults maintained at the U.S. Pacific Basin Agricultural Research Center (USPBARC),

Table 1. Ingredient composition of *C. capitata* #1 diet

Constituents	mg per 50 g diet
Amino acid mixture	1,600.80
Ribonucleic acid	100.00
Sugar	2,000.00
Inositol	10.00
Choline chloride	20.00
Cholesterol	40.00
Vitamin mixture	6.35
McCormick & Davis salt mixture No. 185	100.00
Streptomycin	50.00
Tetracycline 343	5.00
Methylparaben	100.00
Sodium benzoate	100.00
Citric acid	500.00
Corncob 30/80	12,000.00
Water	33,000.00

USDA-ARS, Honolulu, HI, for  $\approx 60$  generations on the standard millfeed larval diet (Tanaka et al. 1969).

**Diet.** *C. capitata* #1 diet is a chemical base diet with only one nonchemical bulking agent, corncob (30/80) (Mt. Pulaski Products, IL). This diet was based on ARS standard millfeed larval diet ingredients (Tanaka et al. 1969). All chemicals used in this study were purchased from ICN Pharmaceuticals (Costa Mesa, CA). *C. capitata* #1 diet was used as a control diet throughout this study. The composition of this diet is listed in Tables 1, 2, and 3 for ingredients, amino acids, and vitamins, respectively. Growth factors in *C. capitata* #1 diet are referred to as exogenous or added vitamins to differentiate them from those endogenous in corncob.

In general, all diets were prepared as follows: a mixture of 18 amino acids (Table 2) and the vitamin mixture (Table 3) were each prepared in bulk. They were combined with other nutrients (sugar, McCormick & Davis (Aurora, OH) salt mixture No. 185, inositol, cholesterol, methylparaben, p-amino benzoic acid, sodium benzoate, streptomycin, tetracycline, citric acid). All dry nutrients with 12 g of corncob bulking agent were weighed into 80-ml sterile polyethylene

Table 2. Amino acid composition of *C. capitata* #1 diet

Amino acids	mg per 50 g diet
Essential amino acids:	636.0
L-arginine	106.8
L-histidine	45.6
L-isoleucine	56.4
L-leucine	108.0
L-lysine	58.8
L-methionine	27.6
L-phenylalanine	70.8
L-threonine	54.0
L-tryptophan	28.8
L-valine	79.2
Non-essential amino acid:	964.8
L-alanine	78.0
L-aspartic acid	112.8
L-cysteine	40.8
L-glutamic acid	392.4
L-glycine	90.0
L-proline	124.8
L-serine	78.0
L-tyrosine	48.0

Table 3. Vitamin composition of *C. capitata* #1 diet

Vitamins	mg per 50 g diet
Thiamine (Vit. B <sub>1</sub> )	1.00
Riboflavin (Vit. B <sub>2</sub> )	1.00
Nicotinic acid	1.00
Pantothenic acid (Coenzyme A)	1.00
Pyridoxine (Vit. B <sub>6</sub> )	1.00
Folic acid	0.25
p-amino benzoic acid	1.00
Biotin	0.10

sampling bags (16 by 10 cm) and mixed in a Stomacher laboratory blender (400 ml capacity) (Daigger and Company, Lincolnshire, IL) at normal setting for 60 s. Ribonucleic acid (stored at 4°C) and choline chloride (stored dry) were added into this diet mixture with 33 ml of hot distilled water (55°C) and mixed in the Stomacher blender for an additional 120 s at high speed. Diet mixtures in the sampling bags were labeled and stored in a refrigerator for later use. Before use, diets were moved from refrigeration to a  $24 \pm 1^\circ\text{C}$ ,  $65 \pm 1\%$  RH room and mixed at normal setting for 60 s.

**Bioassays.** Diets containing 10 exogenous growth factors (including eight B vitamins and two lipogenic factors) in addition to vitamins from corncob were tested by the following methods: (1) deletion of all exogenous growth factors, (2) individual omission (deletion of each added growth factor separately), (3) identifying the optimal concentrations for individual factors shown to be "essential" or "required" by method (2) (concentration ranges from 0.01 to 16 mg/50 g of diet). In addition to the 10 growth factors, ascorbic acid phosphate and vitamin E ( $\alpha$ -tocopherol), which originally were not included in the *C. capitata* #1 diet, were also evaluated in the similar manner.

Larval developmental period (number of days from the day of egg hatch to pupation), pupal recovery (percent pupal production from 50 larvae), pupal weight (average weight per pupa), percent adult emergence, and flight ability were used as evaluation parameters for determining specific growth factor requirements of *C. capitata*.

Fifty newly hatched larvae were transferred onto a strip of blotting paper. Each strip was placed on 50 g of test diet inside the sterile polyethylene bag and fed ad libitum. Four bags for each tested diet were prepared individually. Two hours after seeding larvae on the blotting paper, larvae left on the blotting paper (presumably unhealthy) were removed and replaced with live larvae. Each bag of diet was stapled closed on the creases of the bag and placed in a room maintained at  $24 \pm 1^\circ\text{C}$  and  $65 \pm 1\%$  RH. When larvae reached third instar, the polyethylene bags were cut down 10 cm from the top and the staples were removed to ease larval departure from the old open bag. The cut bags were placed in a 1-liter waxed cup with vermiculite serving as a pupation medium. Pupae were counted, weighed, and recorded daily. Total pupae recovered from each diet were expressed as percent pupal recovery. Daily pupal weights were totaled and divided by the total number of pupae from each diet. The

Table 4. Effect of absence of exogenous growth factors in diet compared to mass rearing diet on growth and development of *C. capitata*

Omitted exogenous growth factor	Larval period (days)	Pupal recovery (%)	Pupal weight (mg)	Adult emergence (%)	Flier (%)	Egg hatch (%)
Rearing control	8.07e	97.50a	9.70a	92.81a	61.44a	92.06a
None ( <i>C. capitata</i> #1 control)	9.60d	95.50a	10.05a	93.13a	46.23ab	94.12a
Thiamine	10.10d	95.00a	9.75a	92.14a	35.70ab	96.38a
Nicotinic acid	No survivors	—	—	—	—	—
Riboflavin	14.71b	85.50a	7.83bc	78.95c	32.28ab	90.46a
Pantothenic acid	13.06c	87.50a	8.40b	83.52bc	30.79b	82.50a
Pyridoxine	9.97d	95.50a	9.83a	91.16ab	47.86ab	84.63a
Folic acid	9.71d	95.00a	9.85a	93.18a	34.85ab	90.63a
<i>p</i> -Amino benzoic acid	9.11de	86.50a	10.13a	94.75a	41.57ab	86.00a
Inositol	9.76d	94.50a	9.98a	94.24a	38.46ab	91.75a
Choline	9.22d	91.50a	10.00a	97.23a	40.47ab	93.50a
Biotin	9.77d	96.50a	9.55a	90.01ab	24.29b	85.55a
10 growth factors	18.13a	49.50b	7.33c	83.45bc	48.38ab	88.09a
<i>F</i>	164.40	18.78	44.69	3.99	2.65	1.88
<i>df</i>	36	36	36	36	36	180
<i>P</i>	<0.0001	<0.0001	<0.0001	0.0008	0.0134	0.0448

Within a column, means followed by different letters were statistically different ( $\alpha = 0.05$ , ANOVA test).

larval developmental period was measured from larval hatch to the first day of pupation. The mean larval developmental period was calculated using the weighed arithmetic mean (the sum of the daily pupal collections times the number of days to pupation divided by total number of pupae) (Sanders 1990, p. 108.). Adult emergence and flight ability were determined according to Boller et al. (1981). A black Plexiglas tube (8.25 cm i.d., 20 cm long) was fitted into a black-painted petri dish (9 cm diameter) that was lined with a 1 cm wide paper strip. The insides of tubes were lightly coated with talcum powder to prevent the flies from climbing out (tubes were tapped on a firm surface to remove excess talc, and the talc was wiped off of the bottom 1 cm of the tube to provide resting places for newly emerged flies). Two days before adult emergence, pupae were placed in the petri dish. Flies were either trapped to "Tat" (fly paper, Walco-Linck, Valley Cottage, NY) and died or captured for further tests. Five days after emergence, insects remaining in the dish were categorized as follows: unemerged, partial emergence, emerged but malformed (including unexpanded wings), and nonfliers. Percent fliers were derived from the total number of pupae minus the number of unemerged, partially emerged, emerged but malformed, and nonfliers divided by total number of emerged flies and multiplied by 100.

All flight ability tests were performed in a metal cage (24.5 by 24.5 by 27.5 cm) held in a room with  $24 \pm 1^\circ\text{C}$  and  $65 \pm 1\%$  RH. Four replicates for each diet in this study were performed.

**Statistical Analysis.** Data are reported as means  $\pm$  SE. Differences among means with each diet were determined by analysis of variance (ANOVA), with the honestly significant difference value calculated as a Tukey statistic at  $\alpha = 0.05$  (SAS Institute 1996).

## Results and Discussion

**Effect of All Ten Growth Factors Deletion.** *C. capitata* #1 diet (includes 10 added or exogenous vitamins in addition to the trace amounts already present in the

corn cob) was able to sustain normal larval development. Total deletion of all 10 exogenous growth factors from the *C. capitata* #1 diet resulted in a prolonged developmental period, decreases in percent pupal recovery, pupal weight and percent adult emergence. However, the egg hatch and flight ability were not affected (Table 4). These findings were different from those of other research with Diptera. Kumar and Anand (1992) reported that removal of all B-complex vitamins from *D. dorsalis* larval diet terminated growth completely. Eymann and Friend (1985) also found that omission of a vitamin mixture allowed no development past the third stadium of onion maggot, *D. antiqua*. Here, deletion of 10 exogenous growth factors significantly affected all evaluated parameters, except egg hatch and flight ability, but did not result in zero survivorship. A larval developmental period of 18.13 d was required for 49.5% of the larvae to reach pupation, in comparison with 9.40 d for 95.5% pupation occurring in the control diet (*C. capitata* #1 diet). Pupal weight and adult emergence were significantly reduced (Table 4). These results allow some speculation: (1) trace amounts of vitamins from corn cob may be able to partially support growth, (2) vitamins synthesized by microorganisms originally present in the diet may nourish larval growth to reach 49.5% survivorship, (3) both (1) and (2) supplement the vitamins for growth. Corn cob alone could not support growth (unpublished data). Therefore, (4) amino acids (the main nutrient other than sugar and corn cob, which provide energy) may be responsible for larval development in the diet lacking added growth factors. To clarify this point, a completely defined chemical diet and further biochemical research would be needed.

**Effect of Omission of Individual Growth Factors.** Of the 10 exogenous growth factors studied, larvae reared on diets without added nicotinic acid suffered 100% mortality in the second instar (Table 4). In the absence of exogenous riboflavin, larvae reached the third instar, but the rate of development was slower and the developmental period was more scattered than that of the control (Fig. 1A). Pupal recovery, egg hatch, and

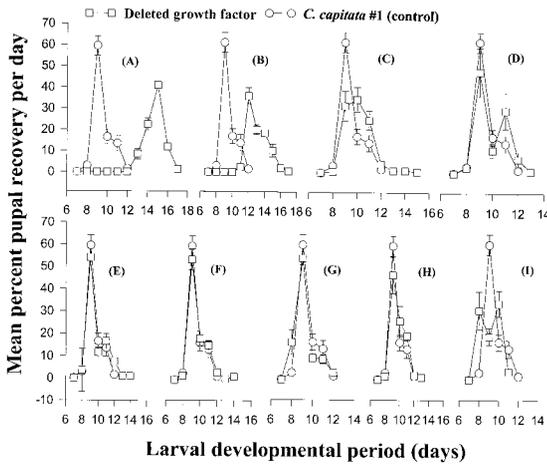


Fig. 1. Mean daily percent pupal recoveries from *C. capitata* #1 diet and diets without exogenous riboflavin (A), pantothenic acid (B), thiamine (C), pyridoxine (D), inositol (E), folic acid (F), choline (G), biotin (H), or p-amino benzoic acid (I).

flight ability remained unaffected; however, significant decreases were observed for pupal weight and adult emergence when compared with the control diet. The effects of omitting pantothenic acid were similar to those of riboflavin. The omission of thiamine, pyridoxine, folic acid, p-amino benzoic acid, biotin, inositol, or choline had no significant effect on the rate of development and survival of the larvae (Table 4; Fig. 1).

Larvae of tephritids require nicotinic acid, riboflavin, and pantothenic acid, thiamine, choline chloride, pyridoxine, folic acid, biotin, and inositol in the diet for normal development as with other insects (House 1962). However, the effect of an individual growth factor on development varies quantitatively and qualitatively with insect species (Baker 1975).

**Nicotinic Acid.** *C. capitata* larvae reared on the diet without added nicotinic acid caused zero develop-

ment before the third instar. No pupation occurred. Increasing the concentrations of exogenous nicotinic acid to equal or exceed 0.1 mg/50 g of diet (2 ppm) resulted in normal development (Table 5). Any concentrations of nicotinic acid from 1.0 to 16 mg in a 50 g of diet (20–320 ppm) show neither beneficial nor detrimental effects on *C. capitata* development. These results indicate that although the required amount of nicotinic acid in larval diet for proper development is relatively small (0.1 mg/50 g diet or 2 ppm), it is indispensable. This finding also suggests that diet containing only endogenous nicotinic acid (from either corncob, synthesized by microorganisms or from both corncob and microorganisms) lacks a sufficient concentration to promote normal growth. Furthermore, these low nicotinic acid levels could create imbalances of other nutrients, producing toxic substances that interfere with the coordination of enzyme systems within larval metabolism.

Comparatively, it is interesting to note the discrepancy of larval development and survivorship between one diet containing no added nicotinic acid and another where all 10 exogenous growth factors have been deleted. Ironically, development and survivorship was improved under circumstances where all 10 exogenous growth factors were absent and nonexistent in the absence of a single exogenous growth factor, nicotinic acid. This finding is quite different from those of Pant and Anand (1985), Anand and Pant (1986), Kumar and Anand (1992), and Nijjima (1993). A possible explanation is that on the diet lacking all 10 growth factors, larval development was slowed to a point where the endogenous growth factors could supply an adequate balance of vitamins to allow the enzyme systems to stay coordinated. Larval death on the diet in which nicotinic acid was not added probably resulted from dietary and metabolic imbalances where the enzyme systems requiring the other vitamins were proceeding at a rate that the nicotinic acid dependent system could not match. The mode of action is still unclear and further investigation is ongoing.

Table 5. Effect of different levels of nicotinic acid, riboflavin and pantothenic acid on growth and development of *C. capitata*

Diets (50g)	Larval period (days)	Pupal recovery (%)	Pupal weight (mg)	Adult emergence (%)	Flier (%)	Egg hatch (%)
Nicotinic acid						
0.00 mg	—	—	—	—	—	—
0.01 mg (0.2 ppm)	—	—	—	—	—	—
0.10 mg (2.0 ppm)	9.46a	78.00a	9.43a	83.45a	30.86a	88.04b
1.00 mg (control)	9.32a	82.50a	9.58a	88.47a	32.39a	91.73ab
Riboflavin						
0.00 mg	14.21a	82.00a	8.53b	86.97ab	32.02a	93.11b
0.01 mg (0.2 ppm)	12.72b	84.00a	8.53b	79.05b	31.02a	97.76a
0.10 mg (2.0 ppm)	9.68c	92.50a	10.15a	88.24ab	34.08a	94.49ab
1.00 mg (control)	9.54c	96.50a	9.90a	88.40ab	32.39a	91.73b
Pantothenic acid						
0.00 mg	11.76a	85.50a	8.43c	89.55b	34.01a	95.51a
0.01 mg (0.2 ppm)	10.75b	87.00a	9.13b	97.70a	37.52a	94.81a
0.10 mg (2.0 ppm)	8.80c	96.00a	9.85a	93.34ab	28.51a	94.88a
1.00 mg (control)	8.78c	97.50a	9.75a	93.86ab	39.69a	92.44a

Within a column, means of each diet followed by different letters were statistically different ( $\alpha = 0.05$ , ANOVA test).

**Riboflavin.** The omission of riboflavin from *C. capitata* #1 diet did not terminate the development completely. Instead, it caused a longer developmental period than that of the *C. capitata* #1 defined diet. The peak of pupal recovery occurred as late as 14.71 d (9.60 d for control) (Fig. 1A; Table 4), although the total pupal recovery was not affected. Pupal weight from larvae reared on the diet without exogenous riboflavin was 7.83 mg in comparison with 10.05 mg for *C. capitata* #1 diet and the percentage of larvae reared on the diet without riboflavin that became adults (adult emergence) was significantly lower than that of the control diet (Table 4). At a concentration of 0.1 mg of riboflavin in a 50 g of diet (2 ppm), *C. capitata* was able to develop normally, whereas concentrations  $\geq 0.01$  mg (0.2 ppm) exhibited the same effects as those from the diet without added riboflavin (Table 5). This is in contrast to the report by Friend and Patton (1956), who found that a riboflavin-free diet would cause zero survivorship in onion maggots. Again, this could be caused by the trace amounts of riboflavin in corn cob (0.24 ppm) or byproducts from microorganisms that support larval growth.

**Pantothenic Acid.** On a diet lacking exogenous pantothenic acid, *C. capitata* survived quite normally except there was an extended development time (Fig. 1B) and reduced pupal weight (Table 4). This result contrasts with those found by Kumar and Anand (1992) and Srivastava et al. (1977) that both *D. dorsalis* and *D. cucurbitae* (Coquillett) larvae would not be able to survive on pantothenic acid-free diet. Friend and Patton (1956) also reported that *D. antiqua* reared on pantothenic acid-free diet, produced only 14.7% third instar and that no pupation occurred. In this study, the required amount of pantothenic acid for proper development of *C. capitata* was found to be 0.1 mg (2 ppm) in comparison to the control diet (Table 5).

**Thiamine.** *C. capitata* larvae reared on the diet lacking exogenous thiamine developed normally. Careful observation of development found that pupation rate was different from the control. Pupae developed evenly scattered within 3 d (days 9, 10, and 11) and there was no pupation peak (Fig. 1C). There was no significant difference in pupal weight. In contrast, there was no survival from *D. cucurbitae* larvae reared on the thiamin-omitted diet (Srivastava et al. 1977).

**Pyridoxine.** The removal of pyridoxine from a diet caused no significant effect on development except showing two pupation peaks on days 9 and 11 (Fig. 1D).

**p-Amino Benzoic Acid.** Similar results were shown as those from pyridoxine except 1 d earlier (Fig. 1I).

In the absence of inositol (Fig. 1E), folic acid (Fig. 1F), choline (Fig. 1G), or biotin (Fig. 1H) in the diet, there was no significant effect on growth and development (Table 4), therefore studies to identify minimum concentrations required were not done.

**Ascorbic Acid 2-Phosphate.** Ascorbic acid 2-phosphate, a phosphate derivative of L-ascorbic acid, has been shown to increase pupal recovery of *Bactrocera latifrons* (Handel) with 5 mg/50 g of diet (Chang and

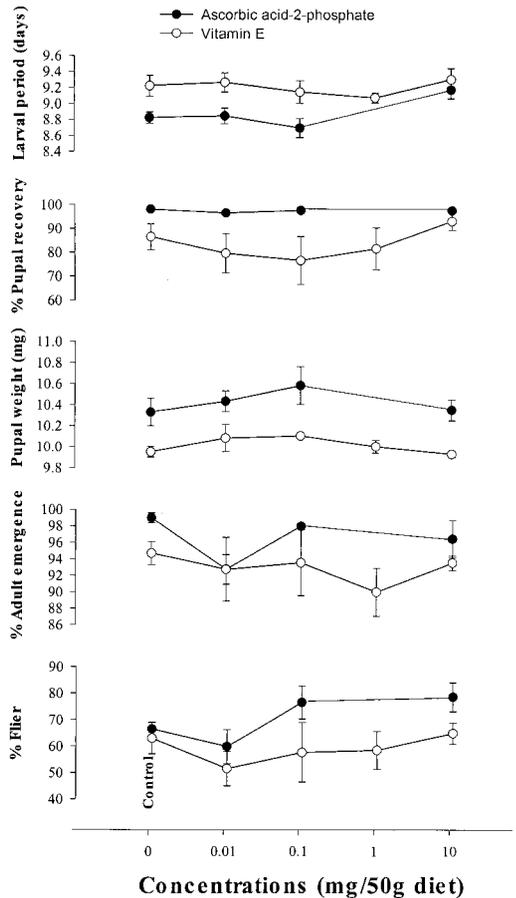


Fig. 2. Effect of graded doses of ascorbic acid-2-phosphate and vitamin E on larval period (days), percent pupal recovery, pupal weight (mg), percent adult emergence, percent flier of *C. capitata*.

Kurashima 1999). Results of this study showed that there were no differences among the various concentrations (0, 0.01, 0.1, 1, or 10 mg/50 g of diet) for all evaluation parameters (Fig. 2). This is probably caused by pantothenic acid sparing activity of ascorbic acid in larval nutrition (Kaul and Saxena 1975).

**$\alpha$ -Tocopherol (Vitamin E).** Addition of  $\alpha$ -tocopherol at 0, 0.01, 0.1, 1, or 10 mg/50 g of diet of *C. capitata* #1 diet did not improve *C. capitata* development and growth (Fig. 2). Because vitamin E and choline are the two major ingredients in wheat germ (United States-Canadian Tables of Feed Composition 1982) and their presence in the diet does not improve larval *C. capitata* development, the use of wheat germ in mass-rearing diets is not necessary.

In this study we used *C. capitata* #1 diet as the control diet. *C. capitata* #1 diet is a chemical based diet with one nonchemical bulking agent, corncob. Therefore, it is not a true chemically defined diet, although the nutrients from the corncob are trace amounts (1 g of corncob contains 0.007 mg nicotinic acid, 0.001 mg

riboflavin, 0.0038 mg pantothenic acid, and 0.0009 mg thiamine) (United States–Canadian Table of Feed Composition 1982). It is not the best diet to investigate the nutritional requirements for *C. capitata*. However, the purpose of this research was to select a purified diet to study nutritional requirements for *C. capitata* mass rearing. Before a completely chemically purified diet is developed, we should take advantage of this very similar recipe to gain important information for mass rearing of this economically important insect.

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