

Nicotinamide in Relation to Dietary Nicotinic Acid and Nine Other Vitamins and Larval Development of *Ceratitis capitata* (Diptera: Tephritidae)

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This study was undertaken to understand why *Ceratitis capitata* larvae reared on a diet fortified with nine vitamins except nicotinic acid had 100% mortality, while those reared on a 10-vitamin-free diet had 66% survival (Chang, C. L.; Li, Q. X. *Ann. Entomol. Soc. Am.* **2004**, *97*, 536–540). Our results showed that nicotinamide was detected at a level of 0.07 $\mu\text{g/g}$ in second-instar larvae reared on the 10-vitamin-free diet and 0.30 $\mu\text{g/g}$ in the corresponding spent diet, while it was not detected in either the larvae reared on the diet fortified with 707 $\mu\text{g/g}$ of nine vitamins (nicotinic acid absent) or the corresponding spent diet. Nicotinamide was detected at concentrations of 0.13 and 0.15 $\mu\text{g/g}$ in the larvae fed the diets that were fortified with 707 $\mu\text{g/g}$ of nine other vitamins and 2 and 20 $\mu\text{g/g}$ of nicotinic acid, respectively, but it was not found in the larvae fed the 0.2 $\mu\text{g/g}$ of nicotinic acid diet. Nicotinamide was detected at concentrations of 0.44, 0.52, and 0.55 $\mu\text{g/g}$ in the spent diets that were fortified with the nine vitamins (707 $\mu\text{g/g}$) and 0.2, 2, and 20 $\mu\text{g/g}$ of nicotinic acid, respectively. Nicotinamide adenine dinucleotide (NAD) was in the live larvae, but not in the dead larvae. These findings indicate that dietary nicotinic acid is converted to nicotinamide, which, in turn, is used to synthesize NAD, and suggest a positive relationship between *C. capitata* larvae survival rates and concentrations of dietary nicotinic acid and nicotinamide in the larvae as well as in the spent diets. The result shows that nicotinamide derived from supplemental nicotinic acid is essential for the development and survival of *C. capitata* larvae. Nicotinamide may be a biomarker for larval survival and development.

KEYWORDS: Larvae (*Ceratitis capitata*); diet; nicotinic acid; nicotinamide; essential nutrients; biomarker

INTRODUCTION

Like many other insects, larvae of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), require several vitamins, including nicotinic acid (niacin), thiamin, riboflavin, pantothenic acid, pyridoxine, folic acid, biotin, inositol, choline chloride, and *p*-aminobenzoic acid, from their diet for normal development (1–5). Nicotinic acid in particular plays an important role in larval growth and survival (6–9). It belongs to the hydrophilic portion of the vitamin B complex and is a component of nicotinamide adenine dinucleotide (NAD). Although nicotinic acid and nicotinamide have similar functions as vitamins, they have different pharmacological effects (10). Pantothenic acid, pyridoxine, *p*-aminobenzoic acid, and choline chloride are absolutely essential in the artificial diet of *Lasio-derma serricornis* (Fab.) larvae (4). The importance of riboflavin, nicotinic acid, and possibly thiamin and folic acid was also

shown to some extent (4). Nicotinic acid in combination with the other nine vitamins as a group has been proven to be indispensable in insect rearing, especially the rearing of fruit flies (3, 5).

Larvae of *C. capitata* reared on a nicotinic acid-free diet, but fortified with 70.7 ppm or higher of nine other vitamins, suffered 100% mortality in the second instar, while there was 66% pupal recovery from larvae reared on a diet lacking all 10 vitamins (including nicotinic acid) (9). Chang and Li (9) proposed that high doses of the other vitamins do not interact with nicotinic acid when lower doses (≤ 0.2 ppm) of nicotinic acid are present in the diet, resulting in toxic or other adverse metabolic effects. Niacin along with the nine other B vitamins as a group is proved indispensable for *C. capitata*. The larvae are not able to survive on a nicotinic acid-free diet, probably due to imbalances between nicotinic acid and the nine other vitamins in the diet or metabolic pathways in which the enzyme systems require the other vitamins to interact at a rate that matches with nicotinic acid-dependent systems (8, 11, 12). To find the cause of the interactive dosage effect between nicotinic

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Table 1. Vitamin Composition of *C. capitata* #1 Meridic Larval Diet (8)

vitamins	mg/50 g diet	vitamins	mg/50 g diet
nicotinic acid	1.00	folic acid	0.25
thiamine	1.00	biotin	0.10
riboflavin	1.00	inositol	10.00
pantothenic acid	1.00	choline chloride	20.00
pyridoxine	1.00	<i>p</i> -aminobenzoic acid	1.00

acid and the other vitamins on larval development of *C. capitata*, the nutritional aspects of the interaction between various doses of nicotinic acid and the other B vitamins were studied with a meridic fruit fly larval diet previously developed for *C. capitata* (Wiedemann) (9). However, the biochemical aspect of the interactions among dietary nicotinic acid and nine other vitamins and larval development of *C. capitata* (Diptera: Tephritidae) were not studied. The biological steps affected by deletion of nicotinic acid can be pinpointed if the levels of enzyme cofactors in larvae fed a nicotinic acid-free diet can be determined. The biological functions of nicotinic acid in insects may be elucidated.

The purpose of this work was to compare metabolite profiles in the spent diet (the leftover or waste diet after larval consumption for 7 days) and fruit fly larvae reared on diets of different nutrient combinations, to clarify the relationship between dietary nicotinic acid levels and larval mortality rates and to explore the possible cause of mortality.

MATERIALS AND METHODS

Diet and Larval Sample Preparations. Insects and diets were prepared according to the procedure set forth in the previous work (9). Ingredient compositions of all the diets in this study are the same as those of *C. capitata* #1 diet (8), except the vitamin compositions (Tables 1 and 2). *C. capitata* #1 diet was used as a control. At 7 days after the eggs hatched, *C. capitata* larvae were collected from each testing diet using fine-pointed forceps and rinsed with distilled water to separate larvae from diet materials. The dead larvae were collected from testing diets under a microscope because the larvae were very small. Larvae in the nicotinic acid-free diets (diet D) developed very slowly as compared with the control and died within 7 days after the eggs hatched.

One gram of larvae were rinsed with distilled water and homogenized with 5 mL of 0.01% TFA solution in distilled water for 3 min using an Ultraturax homogenizer. The homogenate was then centrifuged for 10 min at 17 000g and the supernatant was obtained through a 0.45 μ m nylon filter. Each 2 g of composite powder collected from each diet was dissolved in 10 mL of 0.01% TFA solution followed by sonication for 3 min. After the larval and diet extracts were frozen at -80 °C, they were lyophilized (temp, -50 °C; pressure, 130×10^{-3}

mbar) for 6 h. The residues were dissolved in 1 mL of 0.01% TFA solution and filtered through a 0.45 μ m nylon membrane filter prior to HPLC analysis.

Chemicals. All of the following chemicals were of analytical grade or high performance liquid chromatography (HPLC) grade: acetonitrile, methanol, 1-hexanesulfonic acid sodium salt, glacial acetic acid, hydrochloric acid, phosphoric acid, ammonium hydroxide (Fisher ChemiAlert), trifluoroacetic acid (TFA) (Aldrich), nicotinic acid, thiamine hydrochloride, riboflavin, *D*-pantothenic acid, pyridoxine hydrochloride, folic acid, *D*-biotin, *D*-*myo*-inositol, choline chloride, *p*-aminobenzoic acid, nicotinamide hydrochloride, β -NAD (ICN Bio-medical Inc.), and nicotinamide (Sigma). Ultrapure water was prepared with a Milli-Q filter system (Millipore, Bedford, MA). Nicotinic acid solutions (0.2, 2, 20 μ g/g) and a mixture of nine vitamins (707 μ g/g) for making diets were prepared according to the procedure set forth in the previous work (9). Standard solutions for HPLC analysis were 10.0 μ g/mL of nicotinic acid, nicotinamide, or NAD in 0.1 N HCl.

Equipment. Analyses were carried out on a Dionex BioLC system consisting of a 100 photodiode array (PDA) detector, AS50 autosampler, GP50 gradient pump, and column oven, which were controlled by Chromeleon software. Beckman Coulter Gold HPLC with a 168-PDA detector, 126-solvent module, and 508-autosampler (32Karat) was also used for the analysis. Fluorescence of β -NAD was measured with a PTI fluorospectrophotometer (PhotoTechnology Int'l LCD, London, Ontario, Canada).

An Agilent 1100 liquid chromatograph–mass spectrometer (LC/MS) consisted of a vacuum degasser, a binary pump, an autosampler, a column thermostat, and a PDA with an atmospheric pressure chemical ionization (APCI) source controlled by an Agilent ChemStation. The corona discharge electrode was 2.0 kV and the APCI probe temperature 450 °C. The APCI heater gas (nitrogen) was set to 400 L/h, and the nebulizer gas (nitrogen) to 80 psi. The sample fragmentary voltages were -40 , -80 , and -120 V. Mass-to-charge (m/z) data were obtained at 124.1 for nicotinic acid and 123.2 for nicotinamide. Mass spectra were recorded at a scan range of 100–800 m/z and the protonated molecular ions ($[M + H]^+$) were measured.

An Ultraturax homogenizer (Cole Parmer) with Omnitip disposable generator probe (Model CTH-115), ultrasonicator, lyophilizer (Lab-conco, Kansas city, MO), Sorvall RC-5C ultracentrifuge, Nalgen reusable filter holders, Nalgen 0.45 μ m nylon filter, and syringe filters for HPLC application (0.2 μ m) were used for sample preparation, extraction, and cleanup.

HPLC Conditions. *HPLC Method 1 for Detection of Components in Larvae and Diet.* The same Dionex BioLC instrument as described above was used: injection volume, 50 μ L; flow rate, 1.0 mL/min; column temperature, 40 °C; wavelength, 280 nm; column, Inertsil ODS-2, 5 μ m, 4.6 \times 250 mm; mobile phase, 0.01% TFA in methanol.

HPLC Method 2 for Detection of Nicotinic Acid, Nicotinamide, and NAD in Larvae and Diet. The same Dionex BioLC System as described above was used: injection volume, 20 μ L; flow rate, 1.5 mL/min;

Table 2. Concentrations of Dietary Nicotinic Acid and Nicotinamide in 7-Day-Old *C. capitata* Larvae and the Corresponding Spent Diets

diet ^a	concentration ^b (μ g/g ww ^c) \pm standard deviation				ratio of concn of nicotinamide in spent diet to that in larvae	larval mortality ^d (%)
	spent diet		larvae			
	nicotinic acid	nicotinamide	nicotinic acid	nicotinamide		
A: 20 μ g/g of nicotinic acid + 707 μ g/g of the nine other vitamins	2.16 \pm 0.05	0.55 \pm 0.02	0.08 \pm 0.03	0.15 \pm 0.04	3.7	0
B: 2 μ g/g of nicotinic acid + 707 μ g/g of the nine other vitamins	nd	0.52 \pm 0.04	nd	0.13 \pm 0.02	4.0	6
C: No nicotinic acid nor the nine other vitamins	nd	0.30 \pm 0.03	nd	0.07 \pm 0.02	4.3	34
D: No nicotinic acid but 707 μ g/g of the nine other vitamins	nd	nd	nd	nd	0	100
E: 0.2 μ g/g of nicotinic acid + 707 μ g/g of the nine other vitamins	nd	0.44 \pm 0.07	nd	nd	∞	100

^a Ingredient compositions of diets A–E are the same as that of *C. capitata* #1 diet, except the B vitamin compositions. Diet A as a control is the *C. capitata* #1 diet (8). In the text, the larvae fed the diets are referred correspondingly to as larval samples A, B, C, D, and E and as spent diets A, B, C, D, and E, accordingly. Spent diet is defined as the leftover or waste diet after 7 days. ^b Values are averages of triplicates. ^c ww = wet weight. ^d Data are from ref 9.

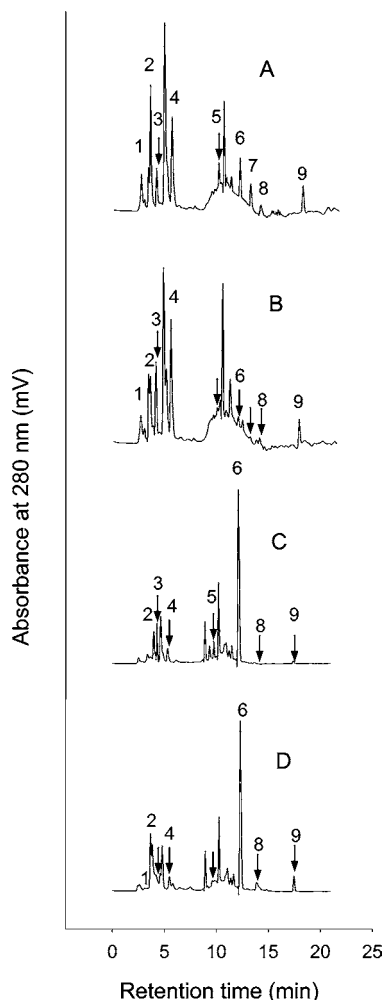


Figure 1. HPLC chromatograms (method 1) of metabolites in the extracts of live larvae **A** (**Figure 1A**) and the corresponding spent diet **A** (**Figure 1C**), and dead larvae **D** (**Figure 1B**) and the corresponding spent diet **D** (**Figure 1D**) (second instar, 7-day-old larvae). Diet **A** contained 20 $\mu\text{g/g}$ of nicotinic acid and 707 $\mu\text{g/g}$ of nine other vitamins. Diet **D** contained no nicotinic acid but 707 $\mu\text{g/g}$ of the nine other vitamins. 1, citric acid; 2, *p*-aminobenzoic acid; 3, nicotinic acid; 4, pyridoxine; 5, nicotinamide; 6, folic acid; 7, riboflavin; 8, pantothenic acid; and 9, inositol.

column temperature, 40 $^{\circ}\text{C}$; wavelength, 254 nm; column, PRP-X100, 7 μm , 4.1 \times 250 mm; mobile phase, 2% glacial acetic acid in water.

RESULTS AND DISCUSSION

Differences of Vitamin Profiles between Live Larvae and Dead Larvae. Figure 1 shows the optimal conditions for HPLC analysis of the 10 vitamins in larval extracts. HPLC method 1 allows complete separation of all the 10 vitamins in standard solutions (not shown). However, there were only seven of the 10 fortified vitamins (*p*-aminobenzoic acid, nicotinic acid, pyridoxine, folic acid, riboflavin, pantothenic acid, and inositol) and nicotinamide detected in the larvae and diet samples (**Figure 1**). Thiamin, choline chloride, and biotin were not detected in extracts from live and dead larvae. There were distinct differences in nicotinamide and riboflavin levels in the extracts from the live (**Figure 1A**) and dead larvae (**Figure 1B**). Nicotinamide and riboflavin were detected at levels of 0.14 $\mu\text{g/g}$ and 0.12 $\mu\text{g/g}$, respectively, in 7-day-old larvae reared on diet **A** (20 $\mu\text{g/g}$ of nicotinic acid and 707 $\mu\text{g/g}$ of the nine other vitamins) (**Figure 1A**) but not detected in dead larvae reared on diet **B** (nicotinic acid-free) (**Figure 1B**). Nicotinic acid and nicotinamide

Table 3. HPLC Parameters for the Analysis of Nicotinic Acid, Nicotinamide, and NAD in the Extracts of Larvae and Spent Diets^a

compound	retention time (min)	range of linearity (ng)	R^2	limit of detection ($\mu\text{g/g}$)
nicotinic acid	6.26	2–20	0.932	0.02
nicotinamide	3.80	2–20	0.993	0.02
NAD	11.86	4–20	0.929	0.05

^a Fresh corncob diet was used to rear the larvae.

were found in diet **A** (**Figure 1C**), while they were not detected on diet **B** (**Figure 1D**). This shows that, in the live larvae, some quantity of dietary nicotinic acid (>0.79 $\mu\text{g/g}$) may be converted to nicotinamide (0.14 μg) by amidation (13). The results support that niacin and riboflavin are integral to larval health and development (3, 6). Like nicotinic acid, a precursor to NAD and NADP, riboflavin is a precursor to flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), enzyme cofactors that are involved in energy metabolism.

Profiles of Nicotinic Acid, Nicotinamide, and NAD in Larval Extracts. HPLC method 2 showed a good linearity and accuracy for the analysis of nicotinic acid, nicotinamide, and NAD in larvae and spent diets (**Table 3**). The detector response was linear in the tested ranges of 2 or 4–20 ng/injection ($R^2 = 0.929$ – 0.993). The retention times for nicotinic acid, nicotinamide, and NAD are 6.26, 3.80, and 11.86 min, respectively. The limit of detection was 0.02 $\mu\text{g/g}$ for nicotinic acid and nicotinamide and 0.05 $\mu\text{g/g}$ for NAD (**Table 3**).

Figure 2 shows HPLC chromatograms of nicotinic acid, nicotinamide, and β -NAD in the standard mixture and the extracts of live larvae (**A**–**C**) and dead larvae (**D**). The nicotinamide (HCl form) was detected in all live larvae fed with the 10-vitamin-fortified diets (e.g., 0.15 $\mu\text{g/g}$ in diet **A**, **Figure 2A**), but not in the extracts from the dead larvae reared on diet **D** (nicotinic acid-free) (**Figure 2D**). It is interesting that a low level (0.07 $\mu\text{g/g}$) of nicotinamide was found in larvae fed a 10-vitamin-free diet (diet **C**) (**Figure 2C**).

Table 2 also shows the concentrations of nicotinamide found in the spent diets in which fruit fly larvae were fed for 7 days. The concentrations of nicotinamide were 0.55, 0.52, and 0.30 $\mu\text{g/g}$ in spent diets **A**, **B**, and **C**, respectively (**Table 2**). However, no nicotinamide was detected in spent diet **D** (nicotinic acid-free) (**Table 2**). While all larvae fed diets **A** and **B** survived in the second instar, larvae fed diet **D** were all dead in the second instar. It is interesting that nicotinamide was found in the larvae and the corresponding spent diet **C** (**Table 2**), which indicates that nicotinamide derives from nicotinic acid in corncob. Corncob contains nicotinic acid (7 $\mu\text{g/g}$), thiamine (9 $\mu\text{g/g}$), pantothenic acid (3.8 $\mu\text{g/g}$), and riboflavin (1 $\mu\text{g/g}$) (8).

Nicotinic acid was only detected at a level of 0.08 $\mu\text{g/g}$ in the larvae fed the control diet (**A**). However, nicotinamide was detected at concentrations of 0.15, 0.13, and 0.07 $\mu\text{g/g}$ in the larvae fed the diets **A**, **B**, and **C**, respectively. The results may indicate the specific biological conversion of nicotinic acid to nicotinamide in *C. capitata* larvae (14–16). Nicotinamide was detected neither in the larvae fed diet **D** (nicotinic acid-free) nor the corresponding spent diet **D**. We speculate that the larval mortality is due to imbalanced ratios of nicotinic acid and the other nine vitamins, which may interrupt the formation of NAD or NADP from nicotinic acid at its trace levels. This is evidenced by the detection of nicotinic acid in fresh corncob (data not shown), but no nicotinamide detected in larvae **D** and spent

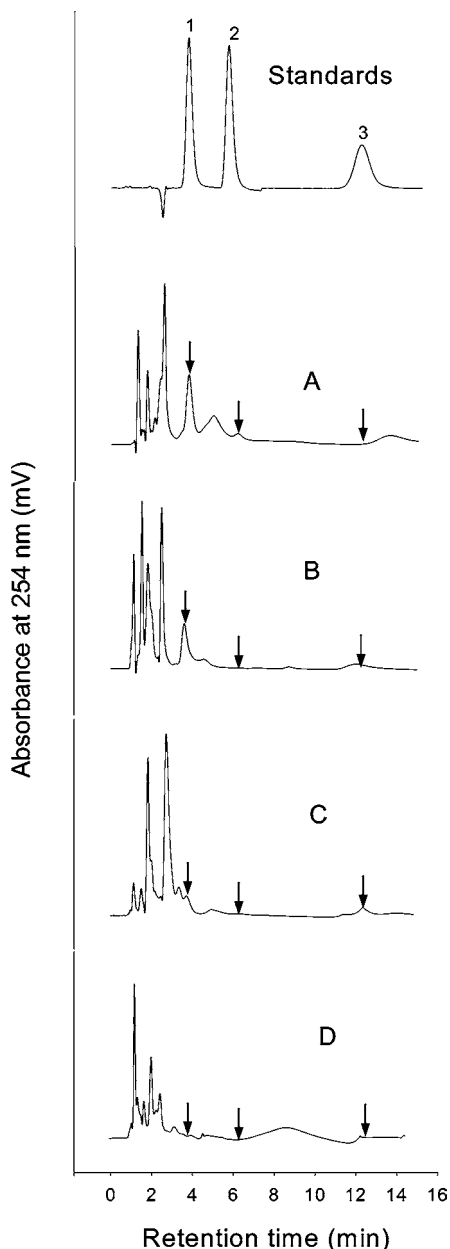


Figure 2. HPLC chromatograms (method 2) of nicotinic acid, nicotinamide, and NAD in the extracts of live (A, B, and C) and dead larvae (D). Standards: a mixture of standard nicotinamide (1), nicotinic acid (2), and NAD (3) at each $10 \mu\text{g/mL}$, injection volume of $20 \mu\text{L}$. Larvae were reared on diets A (A, $20 \mu\text{g/g}$ of nicotinic acid + $707 \mu\text{g/g}$ the nine other vitamins), B (B, $2 \mu\text{g/g}$ of nicotinic acid + $707 \mu\text{g/g}$ of the nine other vitamins), C (C, no nicotinic acid nor the nine other vitamins), and D (D, no nicotinic acid but $707 \mu\text{g/g}$ of the nine other vitamins) as described in Table 2.

diet D samples (Table 2, Figure 2D). Identification of nicotinamide in live larvae was confirmed with LC/MS. It is interesting to note that the ratios of the concentration of nicotinamide detected in the spent diet to that in the corresponding larvae correlate with the larval mortality (Table 2). When the ratios were 3.7, 4, and 4.3, the larval mortality percentages were 0%, 6%, and 34%, respectively. No larvae survived when the ratio was zero or infinitely large. The results suggest that a balanced ratio of all vitamins is essential for larval growth and development.

Levels of NAD in live larvae reared on diet A were measured and compared with those of dead larvae reared on diet D (not nicotinic acid-fortified). No NAD was detected in the dead

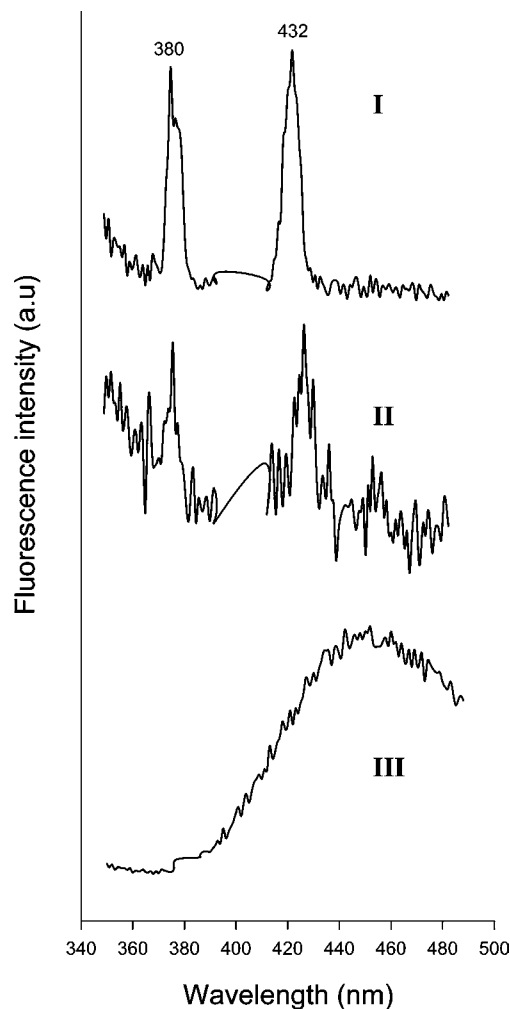


Figure 3. Fluorescence spectra of NAD standard solution (I, 10 ppm), NAD in the extract of live larvae reared on diet A (II), and dead larvae reared on diet D (III). Excitation wavelength was at 380 nm and emission at 432 nm.

larvae, but it was detected in the live larvae (Figure 3). The nicotinamide moiety of NAD and nicotinamide adenine dinucleotide phosphate (NADP) serves as the transient intermediate carrier of a hydride ion, which is enzymatically removed from a substrate molecule by the action of certain dehydrogenases (17). It is known that both nicotinamide and nicotinic acid can be converted into NAD (18). Nicotinic acid can be metabolized to nicotinuric acid through nicotinyl Co A by glycine conjugation or to nicotinamide (19) that can then be deamidated to nicotinic acid and ammonia (20). Nicotinamide is a favored precursor in NAD biosynthesis relative to nicotinic acid, because of its longer half-life in vivo (16). The results of this study indicate that dietary nicotinic acid is converted to nicotinamide, which, in turn, is used to synthesize NAD (Figure 4). Nicotinic acid may also be converted to NAD via nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide (Figure 4). However, this requires confirmation. A study is in progress to elucidate specific biosynthesis pathways of NAD from nicotinamide and nicotinic acid in *C. capitata* larvae.

Correlation between Larval Mortality Rate and Nicotinamide Concentrations. Figure 5 shows an inverse correlation between larval mortality rates and concentrations of nicotinamide in both larvae and diets. The results show biochemical evidence that nicotinic acid, involved in NAD biosynthesis, is essential in *C. capitata* larval development and growth.

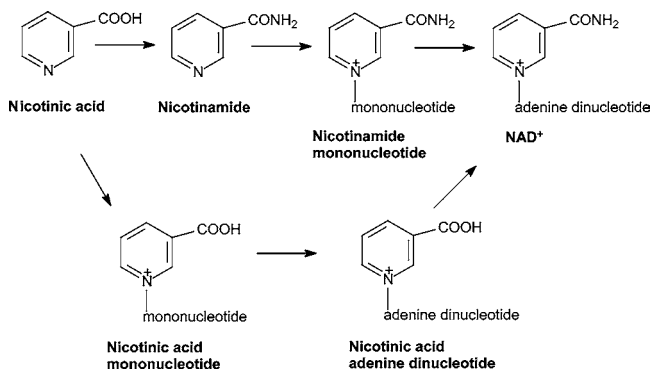


Figure 4. Proposed biosynthesis pathways of nicotinamide adenine dinucleotide (NAD) from nicotinamide and nicotinic acid in *C. capitata* larvae.

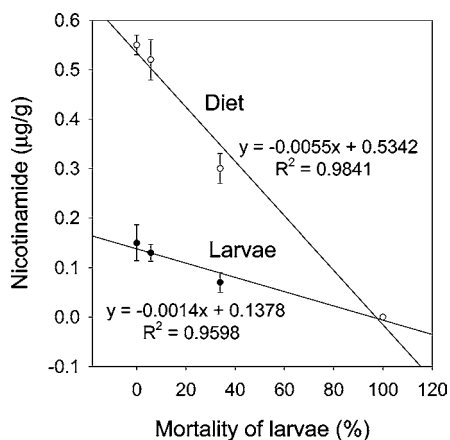


Figure 5. Correlation between nicotinamide concentration and larval mortality rate.

Nicotinic acid is probably converted to nicotinamide prior to incorporation into NAD. Therefore, nicotinic acid is very important to be included in the diets along with nine other vitamins for *C. capitata* rearing.

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