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Alberto Moreira da Silva Neto[1], Tatiana Rodrigues de Oliveira Santos[1], Vanessa Simões Dias[1], Iara Sordi Joachim-Bravo[1]*, Leandro de Jesus Benevides[2], Clicia Maria de Jesus Benevides[2], Mariangela Vieira Lopes Silva[2], Deise Cristina Campos dos Santos[3], Jair Virgínio[3], Givanildo Bezerra Oliveira[4], Julio Marcos Melges Walder[5], Beatriz Aguiar Jordão Paranhos[6], Antônio Souza do Nascimento[7]

[1]UFBA/IBIO – Depto. de Biologia Geral, Av. Ademar de Barros, s/n, Campus Ondina – 40170-115 – Salvador, BA – Brasil.

[2]UNEB – Depto. de Ciências da Vida, R. Silveira Martins, 2555 – 41150-000 – Salvador, BA – Brasil.

[3]MOSCAMED – Biofábrica Moscamed Brasil, Quadra D-13, Lote 15, Distrito Industrial do São Francisco – 48900-000 – Juazeiro, BA – Brasil.

[4]UFRB/Centro de Ciências da Saúde, Av. Carlos Amaral, 1015 – 44570-000 – Santo Antônio de Jesus, BA – Brasil.

[5]USP/CENA – Lab. de Irradiação de Alimentos e Radioentomologia, Av. Centenário, 303, C.P. 96 – 13416-000 – Piracicaba, SP – Brasil.

[6]Embrapa Semiárido, BR 428, Km 152, Zona Rural, C.P. 23 – 56302-970 – Petrolina, PE – Brasil.

[7]Embrapa Mandioca e Fruticultura, R. Embrapa, s/n – 44380-000 – Cruz das Almas, BA – Brasil.

*Corresponding author <iara_bravo@yahoo.com.br>

ABSTRACT: *Ceratitis capitata* is one of the most important pests of fruits for exportation, and Sterile Insect Technique (SIT) has been the most efficient and environmental friendly technique used to control fruit fly populations around the world. A key goal in achieving a successful SIT program is a mass rearing system producing high quality insects at low cost. Providing adults with an artificial diet containing hydrolysed protein has been the major obstacle for bio-production facilities in Brazil, because it is expensive and has to be imported. Two other commercial products, autolysed yeast (AY) and yeast extract (YE), of domestic origin and low cost, were tested as substitutes of the imported hydrolyzed protein. To compare their efficiency we observed the female fecundity, adult survival and egg viability of flies raised on diets containing one of each of the different protein products. Flies reared on the domestic yeast products had equivalent or superior performance to the flies reared on imported protein. Both AY and YE can be a possible substitute for imported hydrolyzed protein for *C. capitata* mass-rearing, as they are cheaper and are readily available in the national market.

Keywords: *Ceratitis capitata*, protein, artificial diet, rearing facility

Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most serious agricultural pests in the world. One of the principal techniques for controlling populations of *C. capitata* is the Sterile Insect Technique (SIT) (Hendrichs et al., 2002). The SIT allows government agencies to control the population levels of many pests, and has found large scale use in many parts of the world (Hendrichs et al., 2002). This technique uses males from specific laboratory lines that are often generated in large scale in bio-production facilities and subsequently sterilized using irradiation. Then, they are released en masse as an attempt to force females in the environment to mate with sterile males only and thus eradicate wild populations or significantly reduce the number of offspring they can produce (Hendrichs et al., 1995).

Brazil currently occupies third place in the world's commercial production level of fruits, although their export to several countries has been impeded due to quarantine restrictions (Santos, 2003). The Brazilian northeastern region has shown a true vocation for natural fruit production, mainly fruits adapted to tropical conditions, and the submedian São Francisco Valley region [Juazeiro (Bahia State) / Petrolina (Pernambuco)] is currently the largest irrigated fruit producing region in this region (Santos, 2003).

Due to the economic importance for this region, a bio-production facility was recently established to supply mass-produced sterile males to combat *C. capitata* infestations and uses large quantities of artificial food for raising both larva and adults. Artificial diets must supply the insects with all of the nutrients they will need for increase fecundity, longevity and for sexual success (mating success and production of viable eggs), and must include a protein source, an energy source (normally carbohydrates), B complex vitamins, and mineral salts (Dadd, 1985). Kaur and Srivastava (1991) have demonstrated that diets without B complex vitamins, folic acid, or biotin will reduce fecundity and eclosion rates in fruit flies. The larval diet for mass-rearing of *C. capitata* is well-defined and fulfills the technical and cost necessities of the bio-production facility. The adult diet, however, uses an enzymatically hydrolyzed protein product that is imported at great cost (Manrakhan and Lux, 2006).

In the bio-production facility, 88.86 g of hydrolyzed protein are required to produce one million *C. capitata* pupae, at a cost of approximately US$ 14.00, only for this ingredient. Usually, the protein used for adult diets in fruit fly facilities around the world is derived from yeast which is treated to produce a source of hydrolyzed protein as well as lipids, B complex vitamins (especially thiamine, riboflavin, niacin and pantothenic acid) and salt (Schulz and Oslage, 1976; Chang, 2009).

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These yeast-based diets are used in large-scale commercial production of larval and adult fruit-flies throughout the world (Aluja et al., 2001; Rohlfs and Hoffmeister, 2005). However, in a large scale production (200 million pupae per week), the quantity needed to attend whole Vale do São Francisco-Brazil, it is the most expensive item used in the facility, pushing us to search for new options of hydrolyzed protein source, which could be found in local markets, with high nutritional quality, and lower cost for both fruit fly facility and fruit producers (consumers of the SIT flies).

This study tested the viability of using a low-cost extract and commercially available yeast products produced in Brazil as a substitute for the imported material currently used in the adult diet of *C. capitata*.

Materials and Methods

The *C. capitata* flies used in this study were of strain Vienna 8 *tsl* (temperature sensitive lethal), a genetic sexing strain, from a population maintained by bio-production facility in Juazeiro, Bahia (BA) state, Brazil (09°34′ S; 40°21′ W), since 2005. The biological parameters used to evaluate the protein sources were: fecundity (evaluated in both small and large-scale experiments), adult survival and egg viability. The two diets tested used protein sources derived from either a yeast extract designated here as YE, or an autolyzed yeast designated here as AY; both are produced in Brazil and are used as food supplements in human diets. The control diet used an imported hydrolyzed protein source, designated here as IHP. Refined sugar was added to all of the diets in the proportion of 3:1 (sugar: protein). Recently emerged flies were divided into three groups receiving one of the three diets on a daily basis throughout the experiment (in addition to water *ad libitum*). Small-scale tests of fecundity and survival were undertaken under laboratory conditions using a 12 h photophase. The large-scale tests of fecundity and egg viability were performed using 24 h of light. All tests were performed at 25 ± 3 °C and 65 ± 5 % relative humidity.

Small-scale fecundity tests

In these experiments, five recently emerged virgin females were placed in a plastic cage (10 × 5 cm), covered with voile screen at the top, containing one of the three diets. During the period of the tests the flies received the respective diet and water *ad libitum*. The females' fecundity (estimated by the numbers of eggs laid by these flies) was verified daily for 10 days after initiating ovipositioning. Daily, females oviposited on the screen and the eggs were collected using a scalpel. All collected eggs were deposited on a Petri dish with wax and were counted. Some eggs oviposited inside the cage were registered too. Twenty replicates were performed for each diet tested with flies from different groups of our Vienna 8 *tsl* flies.

Large-scale fecundity tests

Large-scale fecundity experiments were undertaken using the same cages (71 cm × 80 cm × 7.7 cm) employed in the mass production lines of the bio-production facility in Juazeiro-BA. Each cage contained 150 mL of female pupae (white) and 150 mL of male pupae (brown), totaling 300 mL of pupae. Each mL of pupae contained an average 60 pupae, totaling an average of approximately 18,000 pupae per cage with an adult emergence estimate of 70 %, according bio-production facility information. Each cage - lined with voile fabric - was supplied with a tray containing filtered water for collecting the eggs.

The eggs deposited on voile fabric were removed from the cages two times in the morning (09h00 and 12h00) and two times in the afternoon (14h00 and 17h00). The positions of the cages were changed each day to avoid any possible effects of subtle variations in the light intensity or temperature in the culture room. The eggs were always collected in the same sequence from the same cages (independent of their shifting positions) to eliminate the possibility that the females in the last cage handled would have more time to lay eggs. All of the trays were carefully washed after the morning collections to avoid leaving residual eggs. The eggs were collected early in the morning and transferred successively to smaller flasks (after five minutes of settling followed by decantation of the excess water) until the residual volume was transferred to a graduated cylinder (with an open bottom covered by cheese cloth to filter the water and retain the eggs) and measured in milliliters. The eggs were collected on a daily basis for 10 days after the first observed ovipositioning. Three replicates (cages) were made for each diet tested. Inside each cage, a different batch from the same population of Vienna 8 *tsl* flies was used to ensure sampling of flies with more genetic diversity.

Tests of egg viability

During each experimental test of fecundity under large-scale egg production conditions, sub-samples of eggs were removed for viability tests. Small quantities of eggs from each of the cages were placed into ten Petri dishes containing a piece of wet sponge cloth (to avoid desiccation) covered by a piece of dark cloth (to contrast with the eggs and facilitate counting them). Any dry or obviously damaged eggs were removed from the sub-samples at the beginning of the tests, and the remaining eggs were counted. The number of eggs considered to be unable to hatch was determined by adding those not hatched at 96 h to those removed as dry or damaged eggs. This value was then used to determine the relative percentages of total eggs that were hatched and not hatched.

Survival tests

In these experiments, recently emerged virgin males or females were individually maintained in round plastic cages (10 × 5 cm) containing one of the three diets and water *ad libitum*. The cages were monitored

on a daily basis for 20 days, noting the numbers of dead or living flies. The observation period was limited to 20 days as this represents the maximum time the flies are held in their cages during rearing at the bio-production facility. Thirty replicates were performed for each diet and for each sex.

Analyses of diet composition

The analyses of the compositions of the diets (proteins, lipids, ash, humidity, and carbohydrates) were performed according to the following methodology: (i) Proteins: it was based on determination of total nitrogen (Kjeldahl) which evolves steps of digestion, distillation and titration of nitrogen. The conversion factor for the N protein was 6.25; (ii) Lipids: continuous extraction with petroleum ether type in the Soxhlet apparatus, followed by removal of solvent emissions and lipids quantified gravimetrically; (iii) Ash: samples obtained by calcination in muffle at 550 °C; (iv) Humidity: direct heating of the sample at 105 °C and calculated by gravimetry; and (v) Carbohydrates: The percentage of carbohydrate was determined by difference.

Statistical analyses

All data were examined for normality using the Kolmogorov and Smirnov test, and for variance of homogeneity using Bartlett's method. The data that were not normally distributed were transformed to be compared by parametric tests. The data of small-scale fecundity tests were analyzed by one-way ANOVA with Tukey-Kramer post-test to compare their averages. The data of large-scale fecundity and egg viability tests were analyzed by two-way ANOVA (concerning possible interactions between female age and diet during the experiment) with Bonferroni multiple comparisons analyses post-test. The male and female survival on different diets survival was analyzed by using the Kaplan-Meyer survival test method. The statistical tests were performed using GraphPad Prism version 5.04 software program for Windows (GraphPad Software, San Diego, California, USA). An alpha value of 5 % was used in all statistical tests.

Results and Discussion

In small-scale fecundity tests, the mean of eggs laid per female during ten days of the experiment was greater for females fed with YE compared with those fed with IHP diet (one-way ANOVA, F = 5.015, df = 2, p = 0.00099 – Figure 1A). Regarding AY diet, there was no difference compared with the other diets. In large-scale tests, the total production of eggs was similar among the three diets (Figure 1B, one-way ANOVA, F = 2.937, df = 2, p = 0.1290).

In small-scale tests, the egg production (eggs per female per day) was greater for YE group comparing with the two other diet groups, independent of flies' age (two-way ANOVA with Bonferroni multiple com-
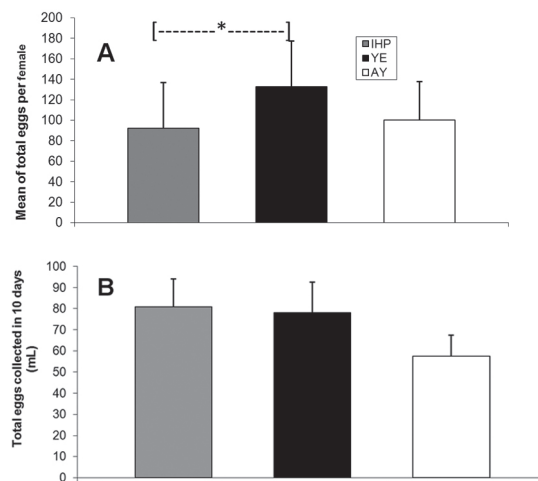

Figure 1 – Comparison of egg production for diets containing each protein source. **(A)** Total eggs per female produced during the 10 days of small-scale fecundity tests (mean ± SD; n = 20). Asterisk represent difference between IHP *vs.*YE; one-way ANOVA (Tukey-Kramer post-test), F = 5.015, df = 2, p = 0.00099. **(B)** Total eggs collected (mL) during 10 days of large-scale fecundity tests (mean ± SD; n = 3). There is no difference between diets; one-way ANOVA, F = 2.937, df = 2, p = 0.1290. IHP: imported hydrolyzed protein, YE: yeast extract and AY: autolyzed yeast.

parisons post test, F = 6.811, df = 17.82, p < 0.05 – Figure 2A). In large-scale tests, the egg production (mL eggs per day) did not differ between the three tested diets and there was no interaction between the factors age and diet (two-way ANOVA with Bonferroni multiple comparisons post test, F = 0.1132, df = 18, p > 0.05 – Figure 2B). The great variance (SD) in egg production observed in small and large scale tests is likely due to natural individual variation of flies, because in each replicate we used a different group of flies. Also, in large scale tests there were different numbers of flies in each treatment and replicate (because of the method used) and so this could have contributed to the amount of variation found in large scale data. However, there was a consistent result concerning the similar efficiency of diets with regard to this parameter.

The equivalence of egg production among the diets, and the superiority of YE in small scale tests, represented excellent results in that SIT requires mass rearing with high fecundity rates and the production of millions of insects every week (Chang, 2009). Adult female fecundity in *C. capitata* is related to protein intake in larval stage (Cocareli et al., 1988) but principally determined by protein ingestion in the adult phase, especially after the pre-oviposition period (the fifth day of adult life) (Cangussu and Zucoloto, 1995). Not only the quantity, but also the quality of the protein sources will affect reproductive rates in *C. capitata* (Chang, 2004).
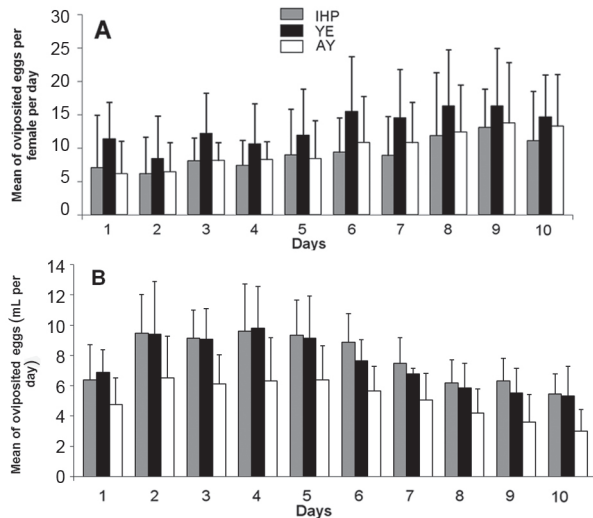
Figure 2 – Daily fecundity in small and large-scale tests. (**A**) Daily fecundity in small-scale tests (mean ± SD; n = 20). There is difference between IHP *vs.* YE and YE *vs.* AY (two-way ANOVA with Bonferroni post test, F = 6.811, d.f = 17.82, *p* < 0.05). (**B**) Daily fecundity (mL per day) in large-scale tests (mean ± SD; n = 3). There is no difference in egg production (mL per day) among the three diets: IHP = 7.82 ± 1.61 (mean ± SD of mean daily production- mL of eggs in 10 days); YE = 7.54 ± 1.70 (mean ± SD of mean daily production- mL of eggs in 10 days); AY = 5.16 ± 1.25 (mean ± SD of the mean daily production- mL of eggs in 10 days) (two-way ANOVA with Bonferroni post test, F = 0.1132, d.f = 18, *p* > 0.05). IHP: imported hydrolyzed protein, and YE: yeast extract and AY: autolyzed yeast.

The compositional analysis of the three diets indicated that they were all very similar in terms of the majority of their components, and that the protein contents (above 50 %) of the diets tests were all adequate (with the YE diet having a greater protein content than the control diet) (Table 1). The data from the compositional analyses corroborated the results of the fecundity rates in the small-scale tests that demonstrated greater fecundity on the YE diet.

The eclosion percentages of the eggs were similar (means around 60-70 %) for the three diets tested and there was no interaction between the factors age and diet (two-way ANOVA with Bonferroni multiple comparisons post test, F = 0.1099, df = 18, *p* > 0.05 – Figure 3). The equivalence between the three diets in relation to egg viability was an important result because the quality, not just the quantity, of the eggs is fundamental to the success of the SIT technique (Chang, 2009).

Regarding fly survival, after 20 days on each diet, the survival curve for females (Figure 4A) and males (Figure 4B) analyzed by Kaplan-Meyer method did not show differences in mortality patterns between the flies from different diets. Females - Log-rank (Mantel-cox) test: X2 = 5.615, d.f = 2; *p* = 0.0603. Males - Log-

Table 1 – Comparison of approximate composition of proteins, lipids, ash, moisture and carbohydrate between the three diets (mean ± SD; n = 3). IHP: imported hydrolyzed protein, AY: autolyzed yeast and YE: yeast extract.

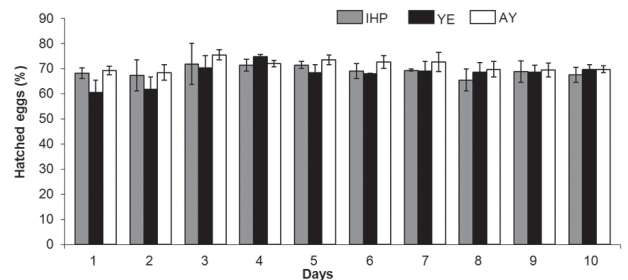| Diet	Protein	Lipid	Ash	Moisture	Carbohydrate
			%		
IHP	58.1 ± 1.3	7.6 ± 1.8	10.0 ± 0.2	7.3 ± 0.2	16.86
AY	51.5 ± 0.9	6.9 ± 0.4	6.5 ± 0.5	6.46 ± 0.2	28.47
YE	63.0 ± 0.4	8.5 ± 0.2	15.1 ± 0.2	4.6 ± 0.2	8.79

Figure 3 – Comparison of egg viability for diets containing each protein source. Egg eclosion percentage of each diet group in each day is shown (mean ± SD; n = 3). There is no difference among the three diets (two-way ANOVA with Bonferroni multiple comparisons post test, F = 0.1099, d.f = 18, *p* > 0.05). IHP: imported hydrolyzed protein, AY: autolyzed yeast and YE: yeast extract.
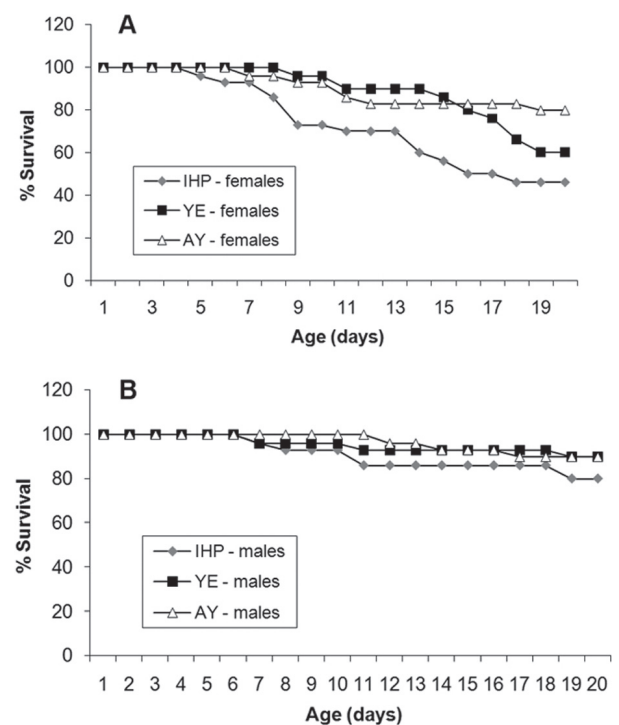

Figure 4 – Survival curves of females (**A**) and males (**B**) fed on each diet (for each sex, diets were tested across 30 replicates). There were no differences in mortality patterns between the flies from different diets. Females - Log-rank (Mantel-cox) test: X2 = 5.615, d.f = 2; *p* = 0.0603. Males - Log-rank (Mantel-cox) test: X2 = 1.727, d.f = 2; *p* = 0.4217.

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rank (Mantel-cox) test: $X2 = 1.727$, d.f $= 2$; $p = 0.4217$. On the twentieth day (the end of experiment) around 80-90 % of males and 45-80 % of females of all diets were still alive. The means ($\pm$ SD) of survival percentage show a greater variation for females' survival fed on IHP diets (73.9 $\pm$ 20.68) compared with other diets (YE $= 89.00 \pm 13.64$; AY $= 90.25 \pm 8.10$). These data could explain the no significant result in Long-rank (Mantel-cox) test ($p = 0.0603$) for females' survival comparison. Among males there was a more uniform pattern of survival results as shown also by means ($\pm$ SD): IHP $= 91.15 \pm 7.12$; YE $= 95.4 \pm 3.50$; AY $= 96.55 \pm 4.23$).

Some lines of *C. capitata* supplied with low concentration protein diets in their adult phase demonstrate longevity reductions (Cangussu and Zucoloto, 1997; Niyazi et al., 2004). Kaspi and Yuval (2000) noted that after 24 h of fasting, *C. capitata* adults that had been fed on high protein diets demonstrated much higher mortality rates than adults that had been raised on low protein diets. In another study, Muller et al. (1997) reported that the mortality rates of *C. capitata* decreased when the adults were fed on protein diets. Others authors do not show any strict link between the amount of protein ingestion in their adult phase and longevity (Shelly and Kennelly, 2002; Shelly and McInnis, 2003). Despite the fact that longevity can be an important parameter in evaluating the quality of any diet (Chang et al., 2001), the most important considerations for large scale commercial producers are that the females produce large numbers of viable eggs near the peak phase of ovipositioning and that the males are active and able perform copulation so that there will be no drop in fertility.

Survivorship beyond 20 days will not result in any significant benefits to breeding these fruit flies, as it has been established that senescence has strong negative effects on the fertility and survival rates of most insects, including *C. capitata* (Carey et al., 1998; Burger et al., 2007). The Brazilian commercial yeast products tested here demonstrated very good results in terms of male and female longevity when compared to insects raised on imported hydrolyzed protein, thus eliminating any concerns about this parameter.

In terms of economic viability, the IHP feed currently costs approximately 122.00 US$ kg^{-1}, while the two domestic products YE and AY have much lower costs: approximately 3.00 US$ kg^{-1}, and 2.00 US$ kg^{-1}, respectively - which represents a huge savings in terms of the costs of commercial mass production of *C. capitata*.

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