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RESEARCH ARTICLE

Design and selection of an artificial diet for the coconut black-headed caterpillar, *Opisina arenosella*, based on orthogonal array analysis

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Abstract

Opisina arenosella has been an outbreak pest of coconut trees in southern China since 2013. To develop efficient control methods for this invasive pest, adequate rearing protocols are desirable. In this study, an orthogonal array of artificial diets with 11 factors at 3 levels was deployed for both 2nd–4th and 5th–6th instar larvae of *O. arenosella*. Biological parameters including survival time of larvae, development time from larva to pupa, pupation rate, emergence rate, and pupal weight were monitored to reveal the most important components in the diet formulas. Biological parameters in *O. arenosella* were most affected by brewer's yeast, sucrose, ascorbic acid, and wheat germ. Statistical analysis indicated that different diet combinations supported optimum performance of biological parameters for 2nd–4th and 5th–6th instar larvae. The validity of the optimization predicted by the orthogonal array analysis was confirmed in a follow-up bioassay with similar optimized diets for both 2nd–4th and 5th–6th instar larvae. The optimal artificial diet has great potential for the mass rearing technique, and can provide valuable results for using parasitoids in biological control of *O. arenosella*.

Keywords: *Opisina arenosella*, orthogonal analysis, diet optimization, mass rearing

1. Introduction

Coconut is a major tropical crop and is one of the most commercially important palms worldwide. Coconut palms are used in landscapes for aesthetic value, as windbreaks in forests, and are cultivated in urban areas along streets, walkways, and seaside coasts, forming an indispensable component of the tropical and subtropical landscape in southern China. However, coconut trees are vulnerable to, and easily infested by, pests (Borowiec *et al.* 2010; Jin *et al.* 2014; Dembilio *et al.* 2015). The coconut black-headed caterpillar, *Opisina arenosella* Walker (Lepidoptera: Xyloryctidae), is the most destructive leaf-eating pest on coconut palm in South Asia, including India, Sri Lanka, Bangladesh, and Myanmar (Nasser and Abdurahiman 2001; Mohan *et al.* 2010). This species has gradually become one of the most harmful coconut pests in China since it spread to Hainan Island in 2013 (Lü *et al.* 2013; Lu 2013).

O. arenosella attacks coconut palms during all stages, from seedlings to maturity. Larvae remain concealed inside a gallery woven with silken thread and excreta and bitten or chewed leaf bits (Mohan *et al.* 2010). The larvae feed on the upper side of leaf tissues and disregard the lower surface of leaves. Severe damage results in leaf dryness and defoliation, a reduction in the rate of production of

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flower spikes, retarded growth and even major declines in yield (Mohan *et al.* 2010). Most chemical sprays are inefficient and waste labor because the palm trees have high and straight trunks and *O. arenosella* remains hidden under the leaves (Lü *et al.* 2013). Therefore, attempts to control this pest rely primarily on cultural or biological control. Over 40 parasitoids and 20 predators attack *O. arenosella* at nearly all growth stages (Pillai and Nair 1993), and if a successful mass rearing technique could be developed and implemented for this pest, mass production of these potential biocontrol agents could provide strategies for managing the coconut black-headed caterpillar.

Generally, colonies of *O. arenosella* are maintained on coconut leaflets in the laboratory, and it is necessary to constantly provide a large quantity of fresh coconut fronds for mass rearing (Nasser and Abdurahiman 2001; Mohan *et al.* 2010). The fronds required for healthy growth of coconut trees are quite expensive, and landholders limit the number that can be obtained. The development of artificial diets facilitates the mass rearing of this herbivorous pest. These diets save labor, time, space, and the costs associated with growing host plants in the laboratory. For decades, considerable and intensive research has been conducted on formulating main components and supplementary components, and on rearing substrate material for artificial diets of lepidopteran pests (Grisdale 1973; Cao *et al.* 2014; Hervet *et al.* 2016). Most diets combine purified natural products such as wheat, corn meal, and sugar with pure nutritional chemicals. The diet formulas are simplified to rear most primarily pestiferous species based on measures of insect fitness at different life stages. However, the nutritional quality and variety of formulations can be manipulated for specific insect species, according to the research aims, including for insect development, bioassay use, or mass production, among others. The principal purpose of the present study was to rear *O. arenosella* in the laboratory on available diet components to avoid the often-costly effort necessary to maintain fresh coconut leaves.

The orthogonal array design (OAD) is a fractional factorial design approach for analysis of optimal levels of combined parameters in multi-parameter and multi-level experiments, which requires fewer treatments than in a conventional parametric design (Sharma *et al.* 2005). An OAD analysis indicates representative combinations of factors and levels for laboratory experiments and can also be used to aid in the design of an optimized artificial diet for an insect based on the combination of ingredients (Bian *et al.* 2014; Lü *et al.* 2014). In the present study, initial successes using primary nutrition sources such as wheat germ, corn meal, coconut frond powder and brewer's yeast to feed *O. arenosella* led to development of an experimental OAD with eleven factors at three levels. Different combinations of ingredients were fed to *O. arenosella*, and parameters including survival time of larvae, development time from larva to pupa, pupation rate, emergence rate, and pupal weight were monitored to reveal the most important ingredients and optimize diet composition. A follow-up bioassay was used to assess the optimized formula. The final artificial diet formula can be used to optimize artificial rearing of *O. arenosella* in order to study effective control measures of this invasive pest.

2. Materials and methods

2.1. Insects

The population of *O. arenosella* tested in this experiment was originally collected in August 2015 from coconut palms in Danzhou, Hainan Province, China (109°17′E, 19°39′N). Approximately 1 000 adults were transported with their host leaves to the quarantine facilities of the Chinese Academy of Tropical Agriculture Sciences in Danzhou. The colony was reared at (25±2)°C under a 16 h L:8 h D photoperiod with (70–80)% RH on coconut palm leaves in the laboratory. The adults were supplied with 20% honey as a nutrition supplement and fresh coconut leaves for laying eggs. Newly hatched larvae were separated daily and provided with fresh new coconut leaves as food in a plastic container for the experiment.

2.2. Optimization of artificial diet using an OAD

Several ingredients prepared for the diet test were based on the McMorran formula for Lepidoptera larvae (McMorran 1965). Twelve ingredients were included in the artificial diet for *O. arenosella*: wheat germ, sucrose, corn meal, brewer's yeast, coconut frond powder, Wesson's salt, cholesterol, choline chloride, inositol, sorbic acid, ascorbic acid, and agar. Agar is a thickener, and different concentrations might affect the physical condition and shape of the diet, which could create side effects in the biological parameters of the test insect; therefore, the agar concentration was set at 1% in all diet treatments. An $L_{27}(3^{11})$ OAD was employed to assign the other 11 ingredients (factors from A to K) at 3 concentrations (levels from 1 to 3) (Table 1), with 27 tests conducted according to the matrix given in Table 2. Each row of the orthogonal array was a specific set of factor levels to be tested.

The ingredients of the basic artificial diets used in this study are given in Table 1. Sterile water was added as a supplement to reach a total of 100 g for all formulas. To prepare artificial diets, each treatment used 1 g of agar that was completely dissolved in boiling sterile water. Then, the

Table 1 Factors and levels in an orthogonal array design (OAD) to optimize an artificial diet for *Opisina arenosella* larvae

Level	Factor (g)[1)]										
	A	B	C	D	E	F	G	H	I	J	K
1	5	2	3	2	3	0.5	0.05	0.01	0.01	0.2	0.2
2	7.5	3	4.5	3	4.5	0.75	0.075	0.03	0.025	0.35	0.4
3	10	4	6	4	6	1	0.1	0.05	0.04	0.5	0.6

[1)] A, wheat germ; B, sucrose; C, corn meal; D, brewer's yeast; E, coconut frond powder; F, Wesson's salt; G, cholesterol; H, choline chloride; I, inositol; J, sorbic acid; K, ascorbic acid.

primary nutrients, including wheat germ, sucrose, corn meal, brewer's yeast, and coconut frond powder, were added, and the fluid solution was heated at 80°C for approximately 2 min. Complementary nutrients, including Wesson's salt, cholesterol, choline chloride, inositol, sorbic acid, and ascorbic acid, were added to the fluid solution when it cooled to 60°C. The entire fluid solution was stirred continuously until all ingredients were completely dissolved. All solid formula of artificial diets was stored at 4°C.

2.3. Rearing method and biological parameters assessed

To evaluate the efficacy of the proposed diet for different stages of larvae, 20 individuals of 2nd–4th or 5th–6th instar larvae were placed in separate plastic containers (15 cm× 10 cm×10 cm) with feed according to each formula in Table 1. During the rearing process, the numbers of living larvae, pupae, and adults were counted daily. Pupal weight was obtained on the first day after pupation. Survival time of larvae, development time from larva to pupa, pupation rate, and emergence rate were calculated as follows:

Survival time of larvae=Days until death or pupation–Days fed on artificial diet (1)

Development time from larva to pupa=Days until pupation –Days fed on artificial diet (2)

Pupation rate (%)=Number of pupae/Total number of larvae observed per trial×100 (3)

Emergence rate (%)=Number of adults/Total number of pupae observed per trial×100 (4)

High values of survival time of larvae, pupation rate, and emergence rate and a low value of development time from larva to pupa were considered characteristic of an optimized diet for the *O. arenosella* population.

2.4. Data analyses

The orthogonal array (L_{27}) was designed and analyzed using SPSS 17.0 software (Statistical Package for the Social Sciences, Chicago, IL, USA) (Bian *et al.* 2014; Lü *et al.* 2014). All biological parameters were analyzed in each artificial diet trial with different combinations of the eleven ingredients (Table 2). Range analysis was used to demonstrate the effect of each factor and to determine optimal levels of the various factors. The range (R_j) was defined according to the following equation (Deng *et al.* 2012; Lü *et al.* 2014):

$$R_j=\max(\bar{k}_{ji})-\min(\bar{k}_{ji}) \quad (5)$$

Where, j is the factor letter (j=A, B, C, D, E, F, G, H, I, J, K), i is the factor level (i=1, 2, 3), and $\bar{k}_{ji}$ indicates the mean value of the sum of the evaluation indices of all levels for each factor; this equation was used to determine the theoretically ideal level and the optimal combination of factors (Wu *et al.* 2011). A relatively large R_j value indicates that the ingredient is a more suitable factor, and the optimal level for each factor is indicated by the largest value of $\bar{k}_{ji}$ (Cui *et al.* 2010). The range analysis cannot distinguish whether the difference between the data at each factor level was caused by experimental variables or by experimental errors, so analysis of variance (ANOVA) was employed to analyze the OAD results to assess the effect of the factors (ingredients) on biological parameters (Lü *et al.* 2014); differences among mean values were considered significant at P<0.05.

The most similar optimized formulas between the 2nd–4th and 5th–6th instar larvae were selected for the second bioassay. Each diet had 3 replicates, and each replicate had a total of 20 individuals for the test. The data were analyzed with a one-way ANOVA to test for significant differences. Mean values were separated using Tukey's test. In all experiments, differences among mean values were considered significant at P<0.05.

3. Results

3.1. Optimization of artificial diet using OAD

According to the L_{27} orthogonal array, 27 separated treatments were analyzed for the 2nd–4th and 5th–6th instar larvae, with the biological parameters shown in Table 2. Variations in the biological parameters indicated that *O. arenosella* larvae could complete development on all 27 formulas. Range analyses of the artificial diets for the 2nd–4th and 5th–6th instar larvae are shown in Tables

Table 2 Orthogonal array design (OAD) for three levels of eleven factors used for diet optimization with the corresponding biological parameters of *Opisina arenosella* larvae

Trial	Factor[1)]											2nd–4th instar larvae				5th–6th instar larvae			
	A	B	C	D	E	F	G	H	I	J	K	Survival time of larvae (day)	Development time from larva to pupa (day)	Pupation rate (%)	Emergence rate (%)	Pupal weight (mg)	Survival time of larvae (day)	Development time from larva to pupa (day)	Pupation rate (%)	Emergence rate (%)	Pupal weight (mg)
T1	1	3	1	3	3	3	3	1	3	2	2	18.1	27.5	59.0	90.0	35.0	10.1	12.3	70.0	60.0	42.1
T2	3	3	3	3	1	2	3	1	2	1	1	16.9	32.3	44.4	75.0	53.9	13.2	17.4	70.0	57.1	22.1
T3	2	3	3	1	1	2	2	2	3	2	3	29.6	34.5	38.0	83.0	50.3	8.2	10.6	70.0	60.0	30.5
T4	3	2	3	2	3	1	2	1	1	2	2	24.6	34.6	50.0	90.0	44.1	14.3	18.7	75.0	83.3	36.5
T5	1	2	1	2	2	2	2	1	2	3	3	22.9	37.9	31.6	100.0	41.5	10.8	11.9	90.0	100.0	26.9
T6	2	1	1	3	1	1	2	3	3	3	2	17.4	34.9	43.0	30.0	53.4	10.8	11.6	62.0	50.0	26.1
T7	2	2	1	1	2	2	3	3	1	2	1	22.8	32.2	35.0	67.0	40.2	6.8	12.5	52.0	40.0	28.3
T8	3	1	2	3	3	2	2	3	1	1	3	26.3	32.7	70.0	85.7	45.1	6.3	15.7	30.0	50.0	30.8
T9	3	1	1	2	1	1	3	2	2	2	3	19.5	29.2	55.0	52.0	60.1	8.7	12.5	30.0	50.0	25.0
T10	1	1	3	3	2	3	2	3	2	2	1	23.2	28.9	44.4	75.0	36.6	8.9	12.1	70.0	80.0	33.4
T11	2	1	3	2	2	3	3	2	1	1	2	28.7	32.1	50.0	50.0	45.2	9.2	16.5	55.0	75.0	45.1
T12	2	1	2	1	3	2	1	1	2	2	2	30.3	31.9	43.0	82.0	42.1	10.4	11.3	50.0	90.0	38.5
T13	2	3	1	2	3	3	1	3	2	1	3	23.6	30.9	52.0	40.0	35.6	9.8	12.3	52.0	60.0	33.3
T14	2	3	2	3	2	1	3	1	1	3	3	25.1	32.2	55.0	75.0	34.3	14.3	14.6	60.0	90.0	35.4
T15	1	3	2	1	2	1	2	2	2	1	2	24.8	36.2	42.9	100.0	40.8	10.4	16.5	60.0	83.0	64.3
T16	1	3	3	2	1	2	1	3	1	3	2	28.4	33.0	78.9	100.0	31.7	10.6	17.0	60.0	83.3	31.9
T17	3	2	2	1	1	3	3	3	2	3	2	28.1	31.3	50.0	100.0	41.4	6.9	19.8	70.0	50.0	30.5
T18	3	3	1	1	3	3	2	2	1	3	1	18.8	28.8	55.6	90.0	55.3	6.9	14.5	44.4	50.0	33.7
T19	3	2	1	3	2	2	1	2	3	1	2	25.5	42.4	27.8	60.0	32.5	13.1	29.2	80.0	75.0	33.3
T20	2	2	2	2	1	3	2	1	3	1	1	18.5	42.2	26.3	60.0	39.3	11.1	11.9	70.0	85.7	27.3
T21	3	3	2	2	2	1	1	3	3	2	1	22.4	29.5	52.6	80.0	51.6	6.0	14.3	62.0	100.0	27.1
T22	1	2	2	3	1	3	1	2	1	2	3	21.0	32.1	47.4	44.4	43.4	8.4	12.7	60.0	80.0	44.9
T23	1	1	1	1	1	1	1	1	1	1	1	30.5	32.0	20.0	75.0	41.2	7.1	8.6	60.0	50.0	53.0
T24	2	2	3	3	3	1	1	2	2	3	1	25.1	37.9	50.0	72.0	40.7	14.1	16.2	53.0	70.0	43.0
T25	1	2	3	1	3	1	3	3	3	1	3	25.9	34.3	44.0	43.0	58.0	7.1	17.9	40.0	100.0	46.9
T26	3	1	3	1	2	3	1	1	3	3	3	21.7	31.5	64.7	81.8	79.2	5.6	12.5	40.0	25.0	39.1
T27	1	1	2	2	3	2	3	2	3	3	1	21.1	26.5	10.5	0.0	52.6	7.6	10.8	77.8	57.1	52.4

[1)] A, wheat germ; B, sucrose; C, corn meal; D, brewer's yeast; E, coconut frond powder; F, Wesson's salt; G, cholesterol; H, choline chloride; I, inositol; J, sorbic acid; K, ascorbic acid. 1, 2 and 3 indicate the different levels in OAD, respectively. The exact amount of the three levels are shown in Table 1.

3 and 4, respectively. A large R_j value indicates that a factor was considered more suitable at the different levels, and the significance of each ingredient for the different biological parameters was shown as follows. First, brewer's yeast (D) was the most important ingredient and coconut frond powder (E) was the least important ingredient for survival time of both 2nd–4th instar and 5th–6th instar larvae. Sucrose (B) had the strongest effects on development time from larva to pupa and emergence rate of both 2nd–4th instar and 5th–6th instar larvae. Pupation rate of 2nd–4th instar and 5th–6th instar larvae was most affected by ascorbic acid (K) and sucrose, respectively. Wheat germ (A) was the most important factor on pupal weight of both 2nd–4th instar and 5th–6th instar larvae.

Based on the $\overline{k}_{ji}$ values of each factor, the artificial diets theoretically yielding optimum biological parameters are shown in Table 3 and were the following: the highest survival time,

A2B1C3D3E3F2G1H3I1J1K2 (2nd–4th instar) and A2B2C3D3E3F1G2H1I2J3K2 (5th–6th instar); the shortest development time from larva to pupa, A1B1C2D1E3F1G3H3I1J2K1 (2nd–4th instar) and A2B1C1D1E1F3G2H1I2J2K1 (5th–6th instar); the highest pupation rate, A3B3C3D3E3F3G1H3I1J3K3 (2nd–4th instar) and A1B2C1D2E2F2G2H1I3J3K2 (5th–6th instar); the highest emergence rate, A3B3C3D1E2F2G2H1I2J2K2 (2nd–4th instar) and A1B2C2D2E2F1G2H1I2J2K2 (5th–6th instar); and the heaviest pupal weight, A3B1C3D1E1F1G3H2I3J3K3 (2nd–4th instar) and A1B1C2D1E3F1G1H2I1J1K2 (5th–6th instar).

The ANOVA results for the primary factors affecting different biological parameters are shown in the supplementary material. ANOVA indicated that wheat germ significantly affected development time from larva to pupa (P=0.04) and pupal weight (P=0.021) of 5th–6th instar larvae; sucrose significantly affected development time from larva to pupa of both 2nd–4th and 5th–6th instar larvae (P=0.008 and P=0.048, respectively); brewer's yeast significantly affected survival time of larva at the 5th–6th instar (P=0.039); and cholesterol and sorbic acid significantly affected development time from larva to pupa of 2nd–4th instar larvae (P=0.03 and P=0.028, respectively). ANOVA confirmed the results of the range analysis: wheat germ, sucrose, and brewer's yeast influenced survival time, development time, and pupal weight, respectively.

3.2. Verification of the optimized artificial diet for both 2nd–4th and 5th–6th instar larvae

The ideal diet formula for both 2nd–4th and 5th–6th instar larvae of *O. arenosella* was identified as follows. Diet 1 (A2B1C3D3E3F2G1H3I1J1K2) and diet 2 (A2B2C3D3E3F1G2H1I2J3K2) had identical levels of 5 ingredients, with the composition A2C3D3E3K2, and diet 3 (A3B3C3D1E2F2G2H1I2J2K2) and diet 4 (A1B2C2D2E2F1G2H1I2J2K2) had identical levels of 6 ingredients, with the composition E2G2H1I2J2K2. Therefore, these four formulas were selected for the second bioassay test, and the results are shown in Fig. 1. Among the four diets, for the 2nd–4th instar larvae, no significant differences were detected in average development time from larva to pupa (30.33–36.2 days), average pupation rate (28.3–63.3%), or average emergence rate (68.3–84%). For the 5th–6th instar larvae, no significant differences were detected in survival time of larva (9.03–10.85 days), average emergence rate (62.7–95%) or pupal weight (31.7–65.3 mg). However, a significantly lower survival time of larva (15.53 days) with diet 3 and pupal weight (26.3–36.7 mg) with diets 2–4 were detected for 2nd–4th instar larvae, and the lowest development time from larva

Table 3 Range analysis of artificial diets for 2nd–4th instar larvae of *Opisina arenosella* tested in the orthogonal array design (OAD) experiment[1)]

Biological parameter[2)]	Survival time of larva (d)				Development time from larva to pupa (d)				Average pupation rate (%)				Average emergence rate (%)				Pupal weight (g)			
	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j
Factor																				
A	24	24.6	22.6	1.9	32	34.3	32.5	2.3	42.1	43.6	52.2	10.2	69.7	62.1	79.4	17.3	42.3	42.3	51.5	9.2
B	24.3	23.8	23.1	1.2	31.1	36.1	31.6	5	44.5	40.2	53.2	12.9	59.1	70.7	81.4	22.4	50.6	42.3	43.2	8.3
C	22.1	24.2	24.9	2.8	32.8	32.7	33.2	0.5	42.1	44.2	51.6	9.5	67.1	69.7	74.4	7.3	43.9	43.4	48.8	5.5
D	25.8	23.3	22.1	3.8	32.5	32.9	33.4	0.9	43.7	45.2	49	5.3	80.2	63.6	67.5	16.6	49.8	44.6	41.7	8.2
E	23.3	24.1	23.7	0.8	33.5	33.6	31.7	2	44.8	44.9	48.2	3.4	68.8	76.5	65.9	10.7	46.1	44.6	45.4	1.4
F	23.9	24.9	22.4	2.5	33.4	33.7	31.7	2	45.8	42.1	49.9	7.8	68.6	72.5	70.1	4	47.1	43.3	45.7	3.8
G	25.4	22.9	22.9	2.5	33.4	34.5	30.8	3.7	48.5	44.6	44.8	3.9	70.6	79.3	61.3	18	44.2	45.1	46.7	2.5
H	23.2	23.8	24.2	1	33.5	33.3	31.9	1.6	43.8	41.9	52.2	10.3	81	61.3	69	19.7	45.6	46.8	43.7	3
I	25.1	23.8	22.2	2.9	32.2	32.9	33.7	1.5	51.3	45.9	40.7	10.7	75.2	77.3	58.6	18.7	42.3	43.6	50.2	7.9
J	24.5	23.5	23.2	1.4	35	31.1	32.6	3.8	41.9	47.2	48.8	6.9	65.4	73.7	72.1	8.3	43.5	44.8	47.8	4.3
K	22.1	25.1	24	2.9	32.2	33.8	32.8	1.5	37.7	49.4	50.9	13.2	66	78	67.2	12	45.7	40.7	49.7	9
Order of factors	D>K>I>C>G>F>A>J>B>H>E				B>J>G>A>F>E>H>K>I>D>C				K>B>I>H>A>C>F>J>D>G>E				B>H>I>G>A>D>K>E>J>C>F				A>K>B>D>I>C>J>F>H>G>E			
Optimized medium levels	A2B1C3D3E3F2G1H3I1J1K2				A1B1C2D1E3F1G3H3I1J2K1				A3B3C3D3E3F3G1H3I1J3K3				A3B3C3D1E2F2G2H1I2J2K2				A3B1C3D1E1F1G3H2I3J3K3			

[1)] $\bar{k}_{j1}$, $\bar{k}_{j2}$ and $\bar{k}_{j3}$ indicate the mean value of the sum of the evaluation indices of levels 1, 2, and 3 for each factor, respectively; R_j indicates the difference value between the maximum $\bar{k}_{ji}$ and the minimum $\bar{k}_{ji}$ for each factor.

[2)] A, wheat germ; B, sucrose; C, corn meal; D, brewer's yeast; E, coconut frond powder; F, Wesson's salt; G, cholesterol; H, choline chloride; I, inositol; J, sorbic acid; K, ascorbic acid.

Table 4 Range analysis of artificial diets for 5th–6th instar larvae of *Opisina arenosella* tested in the orthogonal array design (OAD) experiment[1)]

Biological parameter[2)]	Survival time of larva (d)				Development time from larva to pupa (d)				Average pupation rate (%)				Average emergence rate (%)				Pupal weight (g)			
	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j	$\bar{k}_{j1}$	$\bar{k}_{j2}$	$\bar{k}_{j3}$	R_j
Factor																				
A	9	10.5	9	1.5	13.3	13	17.2	4.1	65.3	58.2	55.7	9.6	77.1	69	60.1	17	44	34.2	30.9	13.1
B	8.3	10.3	9.9	2	12.4	16.7	14.4	4.3	52.8	65.6	60.9	12.8	58.6	76	71.5	17.4	38.1	35.3	35.6	2.9
C	9.3	9	10.1	1.1	13.9	14.2	15.4	1.5	60	60	59.2	0.8	59.4	76.2	70.4	16.8	33.5	39	36.5	5.5
D	7.7	9.8	11	3.3	13.8	14	15.8	2	54	63.5	61.7	9.5	60.9	77.2	68	16.3	40.5	33.9	34.6	6.6
E	9.5	9.5	9.6	0.1	13.6	15.6	14.4	2	61.3	63.2	54.7	8.5	62.9	74.2	68.9	11.3	32.4	37	39.7	7.3
F	10.3	9.7	8.5	1.8	14.5	15.2	13.8	1.3	55.8	64.4	59	8.6	75.1	68.1	62.9	12.3	39.7	32.7	36.6	7
G	9.4	9.7	9.3	0.4	14.9	13.7	14.9	1.2	57.4	63.5	58.3	6	70.4	71.3	64.4	7	38.2	34.4	36.4	3.8
H	10.8	9.6	8.1	2.6	13.2	15.5	14.8	2.3	65	58.9	55.3	9.7	71.2	66.7	68.1	4.6	35.7	41.3	32	9.3
I	9.3	10.4	8.8	1.5	14.5	14.5	14.6	0.1	55.2	60.6	63.5	8.4	66.9	71.1	68.1	4.3	37.7	35.2	36.1	2.5
J	9.7	9.1	9.7	0.6	16.2	13	14.3	3.2	57.4	59.9	61.9	4.5	70.7	71.5	63.9	7.5	39.6	34	35.4	5.5
K	9.1	10.6	8.8	1.8	13.2	17	13.4	3.8	62.1	64.7	52.4	12.2	65.6	72.2	68.3	6.6	35.6	38.7	34.8	3.9
Order of factors	D>H>B>K>F>A>I>C>J>G>E				B>A>K>J>H>E>D>C>F>G>I				B>K>H>A>D>F>E>I>G>J>C				B>A>C>D>F>E>J>G>K>H>I				A>H>E>F>D>J>C>K>G>B>I			
Optimized medium levels	A2B2C3D3E3F1G2H1I2J3K2				A2B1C1D1E1F3G2H1I2J2K1				A1B2C1D2E2F2G2H1I3J3K2				A1B2C2D2E2F1G2H1I2J2K2				A1B1C2D1E3F1G1H2I1J1K2			

[1)] $\bar{k}_{j1}$, $\bar{k}_{j2}$ and $\bar{k}_{j3}$ indicate the mean value of the sum of the evaluation indices of levels 1, 2, and 3 for each factor, respectively; R_j indicates the difference value between the maximum $\bar{k}_j$ and the minimum $\bar{k}_j$ for each factor.

[2)] A, wheat germ; B, sucrose; C, corn meal; D, brewer's yeast; E, coconut frond powder; F, Wesson's salt; G, cholesterol; H, choline chloride; I, inositol; J, sorbic acid; K, ascorbic acid.

to pupa (11.1 days) was found with diet 1 for 5th–6th instar larvae. Thus, diet 1 was superior to diets 2–4 for rearing both 2nd–4th and 5th–6th instar larvae. The following ingredients were selected for the optimal artificial diet because this combination had the best performance for larvae in all stages: 7.5 g wheat germ, 2 g sucrose, 6 g corn meal, 4 g brewer's yeast, 6 g coconut frond powder, 0.75 g Wesson's salt, 0.05 g cholesterol, 0.05 g choline chloride, 0.01 g inositol, 0.2 g sorbic acid, 0.4 g ascorbic acid, 1 g agar, and 72.04 mL sterile water.

4. Discussion

In this study, we developed an artificial diet for *O. arenosella* larvae. The results identified the roles of various ingredients and the optimal ingredients based on an orthogonal experimental analysis. The order of factors revealed that: (1) brewer's yeast was the primary ingredient affecting the survival time of larvae; (2) sucrose was the key ingredient affecting the development time of larvae and the emergence rates; (3) sucrose and ascorbic acid were the primary ingredients affecting pupation rate; and (4) wheat germ was the key ingredient affecting pupal weight. However, the optimal formula for each biological parameter was different between the 2nd–4th and 5th–6th instar larvae. Diet 1 was verified in the follow-up bioassay to have the most optimal biological parameters in common and therefore was suitable to rear both 2nd–4th and 5th–6th instar larvae of *O. arenosella*. This mixed diet led to a high survival rate that was similar to rearing on natural leaves. This diet has been used for successful mass rearing this insect in our laboratory for over three generations. However, it is worth noting that the physical conditions of the diet are slightly sticky and caused some mortality of smaller individuals observed in our laboratory. Therefore, we suggest that the first instar of *O. arenosella* should be reared on fresh coconut leaves. Further, keeping rearing conditions in a dry or air-circulating environment is one of the most important factors of the system. These conditions are necessary because larvae of *O. arenosella* in nature are found in the space covered with their chewed leaf bits and excreta, which are easily dried by atmospheric circulation on the palm trees. Air-circulating conditions in the laboratory similarly dried excreta and provided a comfortable niche for this insect.

The McMorran diet has been widely used to rear Lepidoptera and many other insect species for decades (McMorran 1965; Hervet *et al.* 2016). This formulation was subsequently modified or more ingredients were added to reduce the incidence of deformities or to improve the biological fitness of different species (Hervet *et al.* 2016). Our original ingredients for the *O. arenosella*

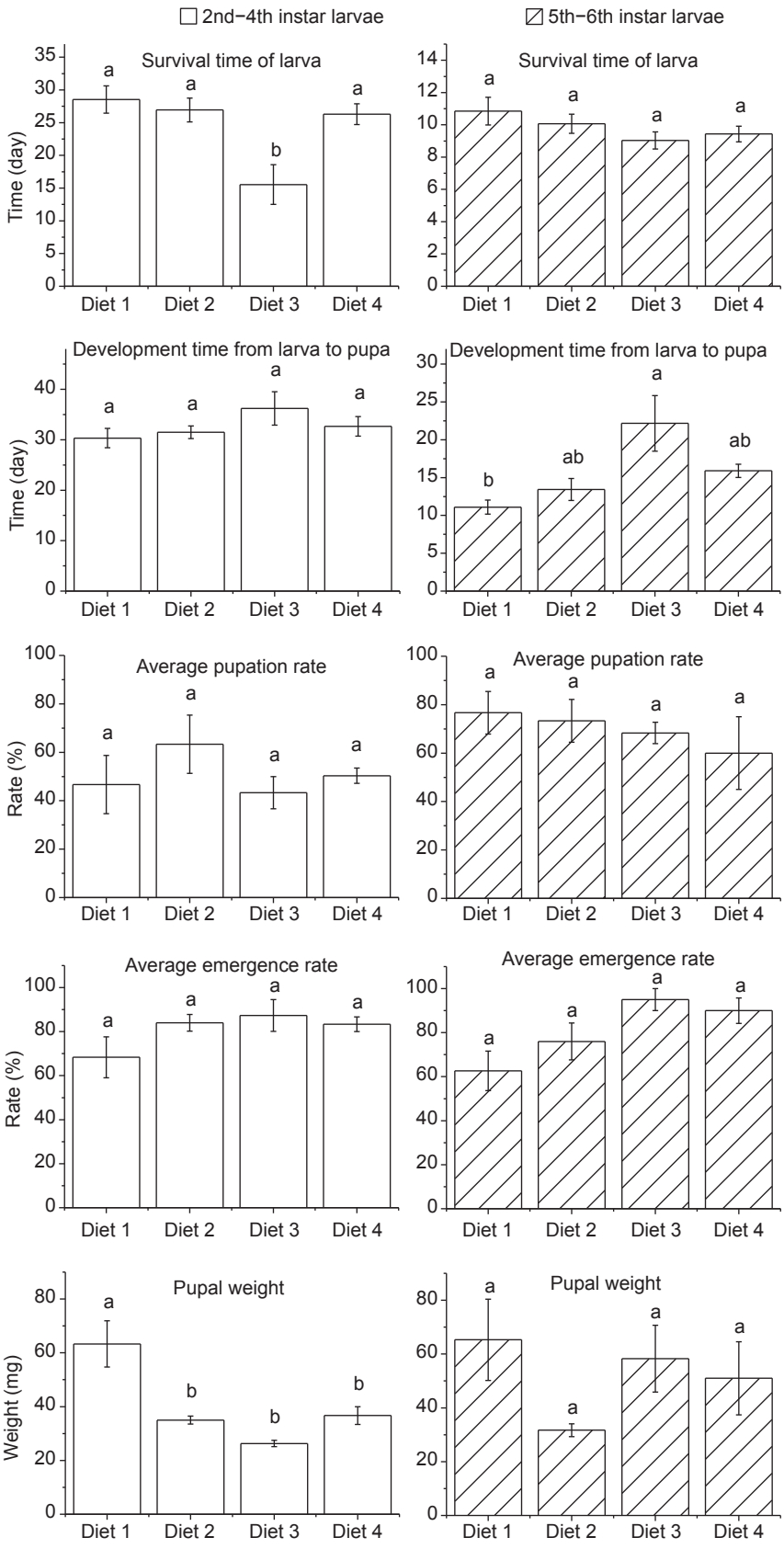

Fig. 1 Biological parameters of *Opisina arenosella* reared on artificial diets 1–4. Mean values with different letters are significantly different (Tukey's test: $P<0.05$). Error bars indicate SE.

diet were based on the McMorran diet but were modified according to published literatures (McMorran 1965; Cao *et al.* 2014; Hervet *et al.* 2016) and our previous rearing experience. Because coconut leaf is the primary natural food for this insect, including coconut frond powder as a factor was an important consideration in the design of an optimal formulation. However, no significant correlation was found between coconut frond powder and the biological parameters we monitored. Thus, coconut frond powder was not a key factor affecting the biological fitness of *O*. *arenosella*. In some formulations, linseed oil is added to the diet to reduce wing deformities in Lepidoptera (Grisdale 1973). However, linseed oil makes the diet more viscous and sticky, and because the larvae of *O*. *arenosella* hidden in palm leaves likely prefer a drier environment, linseed oil or other oils were not considered for the design of this artificial diet.

The primary nutrition sources were not easily determined in our study because different dietary components may satisfy the same nutritional components. For example, both brewer's yeast and casein have an enriched protein component and provide a highly nutritional substance for artificial diets of insects, and nearly all formulations have one or both of these ingredients (Vanderzant 1974; Hervet *et al.* 2016). Our data showed that the survival time of *O*. *arenosella* larvae was significantly affected by the concentration of brewer's yeast and indicated that 4% brewer's yeast should be adopted for the artificial diet. Sucrose, or another sugar, is an indispensable ingredient in artificial diets for Lepidoptera (Hervet *et al.* 2016), Diptera (Vanderzant 1974; Tachibana and Numata 2001; Chen *et al.* 2014), and Coleoptera (Vanderzant 1974; Ichiki *et al.* 2009; Tan *et al.* 2015). In the present study, sucrose was the primary ingredient affecting the development time of larvae, pupation rate, and emergence rate of *O*. *arenosella*, similar to the results found for *Ceratitis capitata* (Nestel and Nemny-Lavy 2008), *Drosophila melanogaster* (Rovenko *et al.* 2015), and *Chilo suppressalis* (Han *et al.* 2012). Wheat germ provides carbohydrates, proteins, fatty acids, and sterols that are required by insects. As a primary constituent, wheat germ has been used in most artificial diets for over 200 species of insects for many years (Vanderzant 1974; Hervet *et al.* 2016). Our results showed that wheat germ affected pupal weight in *O*. *arenosella*, similar to the results found for *Pectinophora gossypiella* (Adkisson *et al.* 1960), *Ceratitis capitata* (Vargas *et al.* 1994), and *Diatraea saccharalis* (Roe *et al.* 1982).

A nutritionally complete diet must contain all or most of the substances required by an insect. Therefore, some trace complementary ingredients are indispensable to balance the nutritional requirements of insects. Based on the current state of knowledge, plant-feeding insects likely require complementary nutrients such as cholesterol, inositol, sorbic acid, ascorbic acid, and trace amount of other ingredients in artificial diets (Vanderzant 1974; Chippendale 1975; Hervet *et al.* 2016). Our results showed that the biological fitness of *O*. *arenosella* was not significantly influenced by the complementary supplements, with the exception of ascorbic acid (Vitamin C), which was the primary ingredient that affected pupal weight. Ascorbic acid is a feeding stimulant and is an essential nutrient for the growth for *Diatraea grandiosella* (Chippendale 1975), *Lymantria dispar* (Roth *et al.* 1994), and *Nilaparvata lugens* (Pan *et al.* 2014), among other insect species. Therefore, the complementary nutrient ascorbic acid and other additional trace elements should be included in formulations and modified to improve the biological fitness of *O*. *arenosella* when reared in the future.

OAD is often used to design experiments with multiple factor levels to minimize assay numbers, time and experimental cost, and the optimum parameters determined in the laboratory can be utilized at larger scales of production (Oles 1993). OAD has been used previously to design and analyze combinations of factors and levels for the optimization of artificial diets for other insect species (Assemi *et al.* 2012; Lü *et al.* 2014). The present study demonstrated that orthogonal array analysis is a useful method to obtain optimal combinations of ingredients for artificial diets to rear larvae of *O*. *arenosella*. The most reasonable formula was selected for both early and late instars after verification from a follow-up bioassay. This new formulation allows us to have successive indoor populations, which may be useful for mass production of *O*. *arenosella*.

5. Conclusion

The following artificial diet with the best performance was to rear larvae of *O*. *arenosella*: 7.5 g wheat germ, 2 g sucrose, 6 g corn meal, 4 g brewer's yeast, 6 g coconut frond powder, 0.75 g Wesson's salt, 0.05 g cholesterol, 0.05 g choline chloride, 0.01 g inositol, 0.2 g sorbic acid, 0.4 g ascorbic acid, 1 g agar and 72.04 mL sterile water. Our study indicates that orthogonal array analysis is a useful method to obtain the best composition of an artificial diet for insects.

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