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Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets

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ABSTRACT

The pine sawyer *Monochamus galloprovincialis* (Olivier, 1795) is the vector of the introduced pine wood nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) in Portugal, and until recently was considered a secondary insect pest. In this study eight artificial diets were tested with the purpose of rearing this insect under laboratory conditions with reduced costs and high efficiency. Tested diets included various artificial mediums commercially available for other insects and mediums based on dried or fresh pine phloem. Among the eight tested variations, diet #5 was the most efficient with 80% eclosions of adult beetles, while diet #6 allowed the fastest development, with the first adult emerging within 53 days. The best rearing method, the diet ingredients and the prices of the diets are discussed, resulting in a list of essential ingredients for the artificial rearing of the pine sawyer.

Key words: Artificial mediums, laboratory rearing, *Pinus pinaster, Bursaphelenchus xylophilus,* pine wilt disease.

INTRODUCTION

The use of artificial diets for maintaining insects in laboratory colonies is now a routine procedure worldwide (Alfazairy *et al*., 2012; Assemi *et al*., 2012). Artificial diets allow the continuous rearing of a large number of insects throughout the year, with the purpose of studying their behaviour, physiology, biochemistry, taxonomy or control, among others. Other objectives of using artificial diets include production of live material for food supply of other animals, either invertebrates or vertebrates. Artificial diets are especially useful in the case of insects whose biology, length of life cycle and habits make their study difficult in nature, such as bark and wood-boring beetles of the families Buprestidae and Cerambycidae, which usually spend a significant part of their lives under the bark and/or inside the wood. Several diets have been successfully developed for a variety of species of those families (*e. g*., Gardiner, 1970; Payne *et al*., 1975; Cannon and Robinson, 1982; Viedma *et al*., 1985).

In the case of the beetles of the genus *Monochamus* Dejean, 1821, worldwide recognised as the most important vectors of *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934), the pine wood nematode (PWN) (Linit, 1988), a few diets have been developed and tested on both the North American and Asian species (Alya and Hain, 1987; Kosaka and Ogura, 1990; Aloo and Katagiri, 1994; Necibi and Linit, 1997). The pine sawyer *Monochamus galloprovincialis* (Olivier, 1795) was found to be the vector of the PWN in Portugal (Sousa *et al.*, 2001), causing heavy losses and damages to the local forestry sector (Fig.1a). Numerous aspects of the insect's bio-ecology and interaction with the PWN have already been studied (Naves *et al.*, 2005, 2007b, 2008; Petersen-Silva *et al*., 2012), but the lack of an artificial medium to rear the immature life stages of the beetle causes limitations to some studies. Therefore, the main objective of this study is to develop and compare artificial diets intended for mass rearing of the pine sawyer *Monochamus galloprovincialis*, testing and adapting pre-existing diets chosen by the simplicity of manufacturing and low-cost of ingredients (Carle, 1969; Alya and Hain, 1987; Kosaka and Ogura, 1990; Aloo and Katagiri, 1994; Necibi and Linit, 1997; Dubois *et al*., 2002).

Fig. 1. Adult *Pinus pinaster* affected by the pine wilt disease a), diets #1 to #3 inside the climatic chamber b), wood boxes with *P. pinaster* prepared for *M. galloprovincialis* oviposition c), detail of *M. galloprovincialis* larvae feeding on the artificial medium d), *M. galloprovincialis* larval stages in diets #6 to #8 reared inside climatic chamber e), detail on the diets #1 to #4 (f).

MATERIAL AND METHODS

Diet preparation

Eight diets were tested, numbered #1 to #8 (Table 1). The phloem-cambium was obtained from two healthy adult pine trees (*Pinus pinaster* Aiton, 1789), which were felled and divided into small logs. In the INIAV laboratory the outer bark was removed and the phloem-cambium layer was peeled off and dried at room temperature before use. Fresh green pine needles were also removed from the pine branches and dried in an oven at 60ºC for four hours. The dried material was chopped into small pieces, and blended with a mixer followed by hand.

Diets #1 to #3 differed in the insect culture medium selected, which corresponded to around ¼ of the composition. Diets #4 and #5 excluded the culture medium, but incorporated cellulose powder as key ingredient (Fig.1b,e).

Diets #6 to #8 (Fig.1e) included an autoclaved pre mixture (pre-mix) (Table 2) with ascorbic and propionic acids added to prevent contamination with fungus or with other life forms and were adapted from the *Anoplophora glabripennis* (Motschulsky, 1854) (Coleoptera: Cerambycidae) diet developed by Dubois *et al.* (2002). In these diets the pre-mix and all the pre-autoclave ingredients were prepared in a large and sterile recipient, to which the phloem cambium was added later. All the ingredients were then autoclaved for 15 minutes at 121ºC. After sterilization, all procedures were conducted in a biological safety chamber to avoid contaminations. The methyl paraben, ascorbic and propionic acids were added to the mixture inside a fume hood using anti-acid gloves and safety mask. A total of 240 glass Petri-dishes (90mm diameter) were sterilized and filled with 50cm^3 of diet each.

Production price comparison

The price of production for each diet was calculated based on a virtual 100% survival rate for the diets, not considering the real survival observed. The values of production were calculated based on the reagent catalogue of reference chemistry international companies (Sigma-Aldrich ©, Merck © and Roche ©).

Collection of adult *M. galloprovincialis* and obtaining neonates

Adult maritime pine trees colonised by *M. galloprovincialis* were felled in February 2012 at Comporta, Portugal, and taken to the INIAV laboratory to allow the emergence of adult beetles, which were kept in groups of 25 individuals (15 ♀♀ and 10 ♂♂) in large netted boxes (80x60x40cm) with *Pinus pinaster* branches and logs under natural photoperiod conditions (±25ºC, 70% Relative humidity, 14/10 light/dark cycle) (Fig.1c). Every two days, the adults were fed with fresh *Pinus pinaster* branches and one or two-day old eggs were collected from the branches and removed from the boxes. The eggs were immersed in 70% ethanol for 10 seconds, followed by 10 seconds immersion in distilled water and placed in a Petri dish with a moistened filter paper in a rearing chamber at 25ºC 65% relative humidity (RH) and 0:24 light/dark cycle (LD), until hatching.

Table 1. Ingredients (quantities in g or ml) used to prepare 1 kg of each diet.

Ingredients	Diet (#)							
	1	2	3	4	5	6	7	8
Agar (g)	10	10	10	10	30	-	-	-
Dried yeast (g)	49	49	49	49	40	-	-	-
Potassium sorbate (g)	1	1	1	1	-	-	-	-
Culture medium* (g)	230	230	230	-	-	-	-	-
Pinus dried needles (g)	30	30	30	30	-	-	-	-
Pinus dried phloem-cambium (g)	200	200	200	200	-	108.8**	108.8***	-
Distilled water (ml)	480	480	480	480	671	641.17	641.17	641.17
Cellulose powder (g)	-	-	-	230	160	-	-	108.8
Sucrose (g)	-	-	-	-	20	-	-	-
Potato starch (g)	-	-	-	-	20	-	-	-
Soybean powder (g)	-	-	-	-	50	-	-	-
Soybean oil (ml)	-	-	-	-	4	5.86	5.86	5.86
95% Ethanol (ml)	-	-	-	-	3	-	-	-
Propionic acid (ml)	-	-	-	-	1	1.67	1.67	1.67
Citric acid (ml)	-	-	-	-	1	-	-	-
Pre-mix (g)	-	-	-	-	-	240	240	240
Ascorbic acid (g)	-	-	-	-	-	2.50	2.50	2.50

*Culture mediums: Diet #1: Painted lady *Vanessa cardui* (Linnaeus, 1758) (Lepidoptera: Nymphalidae); Diet #2: confused flour beetle *Tribolium confusum* Jacquelin du Val, 1863 (Coleoptera: Tenebrionidae); Diet #3: silkworm *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) medium, all commercially-available mediums acquired from Carolina Biological Supply Company®, USA. ** Phloem-cambium extracted 1 day after the felling of an adult maritime pine tree; *** Phloem-cambium extracted 2 weeks after the felling of an adult maritime pine tree.

Ingredients to mix before autoclave (exclusive for diets #6 to #8)

Ingredients to mix after autoclave (exclusive for diets #6 to #8)

Table 2. Ingredients (in g) used in the Pre-mix of diets #6 to #8.

Ingredients	Quantity
Potato starch	25.01 g
Sucrose	25.01 g
Defatted soybean flour	60.03 g
Casein	14.01 g
Cholesterol	1.50 g
Wesson's salt mixture	4.05 g

Ingredients	Quantity
Dried yeast	45.03 g
Cellulose	70.01 g
Agar	35.02 g
Citric Acid	2.00 g
Sorbic Acid	2.50 g
Methyl Paraben	2.50 g

Rearing and handling of *M. galloprovincialis* larvae until eclosion

A two mm round hole was created on the diet's surface to insert the recently-hatched larvae, which were placed using a paintbrush. The number of larvae tested on each diet was 30. Only one larva was used by Petri dish, as the individuals tend to be aggressive to each other (Victorsson and Wikars, 1996; Dodds *et al*., 2001). The Petri dishes were kept in rearing chambers with constant temperature and humidity (25ºC, 65% RH, 15:9 LD) until pupae were obtained (Fig.1d), being then removed from the diets and placed in a clean Petri dish with moistened filter paper. Each individual was observed once a day after reaching the pupal stage. Three days after becoming an adult, the sex and weight were recorded. Diets were evaluated and compared based on the development time, number of adults obtained (herein referred as "eclosion"), and their weight. Diets were not replaced by fresh ones, although they were humidified to prevent excessive dehydration when considered necessary. To avoid contaminations, diets #6 to #8 were never re-hydrated or opened until the pupal stage.

Comparison with pine-reared *M. galloprovincialis*

To obtain adults reared in their natural pine hosts, two adult dead *P. pinaster* trees with confirmed presence of *M. galloprovincialis* were cut in Comporta and taken to the INIAV Lab, where 30 last-instar larvae were removed from the xylem galleries with the help of a vertical chain saw. The non-feeding mature larvae were placed individually in Petri dishes with moistened filter paper and kept at 25ºC 65% RH 15:9 LD until eclosion. Adult sex and weight were recorded and compared with diet-reared *M. galloprovincialis*.

Statistical analysis

Means were compared with the nonparametric Kruskal-Wallis test followed by the Tukey HSD test, with $p \leq 0.05$. Values are presented as means ± standard error (SE) in the text and tables, unless otherwise stated. Statistical analyses were performed with the software Statistica 6.0 (StatSoft Inc. 2003).

RESULTS

Diet efficiency

Adult *M. galloprovincialis* were obtained from all tested diets. Diets #1, #5 and #7 showed the highest survival rate, with a maximum of 80% in diet #5 (Table 3). In general, mortality was higher during the first days after the larvae were placed in the diet, and lowest for subsequent larval instars.

Due to the low number of available adults (eclosion rates below 25%), diets #2, #3 and #8 were excluded from subsequent statistical analysis. When comparing the time required to obtain adult insects, a mean of 107 ± 2.5 days was registered for the other diets. Diet #6 allowed the fastest development, with the eclosion of the first insect only 53 days after egg laying, (Table 3) differing in 29 days from the diet with the longest development time (Kruskal-Wallis test: $\chi^2=15.76$, d.f.=4, P=0.0034). Regarding the weight of the adult insects, this was also statistically conditioned by

the diet (Kruskal-Wallis test: χ^2=14.11, d.f.=4, P=0.0069), with diet #6 providing the heaviest adults and diet #4 the lightest individuals. Concerning the sex of the reared *M. galloprovincialis*, the overall proportion of males and females was balanced, with a ratio of 0.91 and slightly dominated by males.

Table 3. Number of adults (percentage of eclosion), development time (days), adult weight (grams), sex ratio (male/female) and estimated production price (€) for each diet.

	Diet (#)							
	1[1]	2	3	4[1]	5[1]	6[1]	7[1]	8
Nb of adults (% eclosion)	16 (53%)	5 (16%)	3 (11%)	8 (28%)	24 (80%)	14 (46%)	17 (57%)	3 (11%)
Development time (days) (mean ± SE, range)	124±6.0a (86–162)	101±5.6 (77–110)	101±5.1 (74–96)	102±3.1ab (77–113)	107±2.7ab (84–133)	95±6.7b (53–147)	106±8.7ab (59–165)	123 (99– 147)
Adult weight (g) (mean ± SE)	0.36±0.10a	0.39±0.13	0.65±0.19	0.33±0.10a	0.39±0.12ab	0.47±0.12c	0.45±0.12bc	0.29
Sex ratio (male/female)	0.78	na	na	na	0.50	1.13	0.33	na
Estimated price per adult (€) (100% virtual survival)	1.39	0.83	1.11	1.15	1.56	1.93	1.93	1.93

[1] Mean within each line followed by the same letter do not differ, P≤0.05. na - not applicable.

Production price comparison

The prices of manufacturing the diets had significant variations, with two groups of diets that could be distinguished. The first five diets had lower prices, being the last three the most expensive (Table 3).

Comparison with field-collected *M. galloprovincialis*

The mean weight of the field collected insects was 0.26 ± 0.02g, being significantly lower than the weight of adults reared from diets #5, #6 and #7 (Kruskal-Wallis test: X^2=24.01, d.f.=5, P=0.0002). All insects, both field collected and lab reared, were able to lay viable eggs on pine logs.

DISCUSSION

Three of the diets allowed the attainment of more than 50% of adults, with a maximum of 80%. According to Singh (1983), an ideal diet for an insect mass rearing programme should produce an average yield of adults of at least 75% from the initial viable eggs, with development rates and adult size similar to those in nature. Considering our results, we observe that diet #5 fulfils all those requirements.

Furthermore, if this diet is used to rear older larvae (instead of neonate individuals), the survival can be even higher, as found by Naves *et al*. (2007b), and also supporting previous observations by other authors which found that high mortality on the diets primarily affects the newly emerged and first instars larvae (Viedma, *et al.*, 1985; Kosaka and Ogura, 1990; Allo and Katagiri, 1994; Hernández, 1994).

The absence of plant tissues on the diets apparently did not influence the duration of larval development or their survival. Our results contradict previous observations, as according to Gardiner (1970) the presence of plant tissues on the diets act as

phago-stimulants for the young larvae and improve their acceptance by the insects. Allo and Katagiri (1994) also found that the inclusion of host plant tissue on *Monochamus alternatus* Hope, 1843 (Coleoptera: Cerambycidae) diets had a significant benefit influence on larval growth and development.

With the diets, adult insects could be obtained with a minimum mean of just 95 days, which implies a significant reduction of the normal larval development time, thus allowing the rearing of two or three sequential generations per year instead of the single annual generation observed under field conditions (Naves *et al*., 2008).

The disparity of prices between the two groups of diets is justified by the presence of the costly methylparaben and the propinoic and ascorbic acids in diets #6 to #8. While diets #1 to #5 were consecutively contaminated with fungi, the last 3 ones did not present any sign of degradation or contamination when stored for various months, suggesting that adding these components is important when storing the diets for a long period. Nevertheless, when considering the tentative prices presented, it has to be considered that they exclude the cost of obtaining the phloem-cambium and/or the pine needles incorporated in all diets, except #5 and #8. As the phloem-cambium almost comprehends 25% of the total weight of some diets, its extraction from the pine trunk is time and labour-consuming, increasing its price. Although diet #2 has the lowest price of production, with approximately 0.83€ per larvae, the low number of obtained adults with this diet constitute a major setback. However, the diet with the highest success did not require this ingredient, suggesting that this ingredient appears to be facultative.

Additional studies to compare the vigour and fecundity of the diet and tree-emerged insects are needed to confirm the absence of differences between them. Furthermore, the development of an artificial oviposition substrate would substitute the pine material (logs and branches) currently used to obtain *M. galloprovincialis* eggs on the laboratory colonies, thus diminishing the labour and environmental impact of the diet. The permanent availability of insects from diets, allowing the continuous laboratory rear of multiple annual generations, would be a useful contribution for research lines requiring large numbers of *M. galloprovincialis* of all life stages year round.

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