

Controlled Mass Rearing of Cochineal Insect (Hemiptera: Dactylopiidae) Using Two Laboratory-Scale Production Systems in Peru

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The research was conducted at the Laboratory of Bioprocess Research (LBR), Department of Pharmacy, Bio-Chemistry and Biotechnology, Universidad Católica de Santa María (UCSM), Arequipa, Perú. The insects collected in field were maintained under laboratory-controlled conditions ($27 \pm 0.5^\circ\text{C}$, $40 \pm 5\%$ RH, and a photoperiod of 16:8 (L:D) h).

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Abstract

The carmine cochineal (*Dactylopius coccus* Costa) has high economic value as it is a natural source of carminic acid, an organic chromophore used in a wide range of sectors including pharmaceuticals, food, and cosmetics. High demand is fuelling the search for innovative production techniques in order to move away from dependence on the prickly pear, which carries a number of limitations. The aim of this study was to establish cochineal colonies and breed and mass-produce the insects using two laboratory-scale production systems. The first system (STC-01) comprised a prismatic acrylic box with three compartments; synthetic matrices were placed vertically inside the box to provide support and a source of nutrients for the cochineal, and the system was lit artificially during fixed daylight periods. The second system (STC-02) comprised an automated micro-tunnel allowing the insects to move towards the sunlight, containing synthetic matrices arranged horizontally. There was a significant difference in yield between the two systems in a cochineal total life cycle of 120 d (80–90 d harvest period in both cases), with STC-01 being superior and producing a maximum yield of 4.86 ± 0.68 g fresh weight per day per square metre compared with 3.20 ± 0.14 g fresh weight per day per square metre production yield in STC-02. We conclude that cochineal production under controlled artificial conditions is feasible and sustainable, removing the need for natural and biological support and overcoming the environmental limitations posed by traditional production methods.

Key words: artificial rearing device, Peruvian cochineal, collagen synthetic matrix, natural host-free, increased fresh weight

In all the world, and specifically in Peru and other peripheral countries, a large variety of edible insects are harvested in wild conditions since several centuries ago but a few insect species have could be domesticated in controlled environments. Nowadays, due to the huge demand for products of biological origin, there has been an increase in the use of insects to produce commercially-valuable products including proteins and fats (Gasco et al. 2020), oils (Kierończyk et al. 2018), margarines (Smetana et al. 2020), and insect gut enzymes have also been used for the bioengineering of proteases (Kannan et al. 2019). Therefore, it is important to automate the rearing, production, harvesting, and processing of insects to make this sector more attractive, profitable, and competitive (Kröncke et al. 2020). Carmine cochineal *Dactylopius coccus* Costa (DCC) is a sap-sucking insect belonging to the order Hemiptera that feeds on the cactus *Opuntia ficus-indica* (L.) Miller (OFI) commonly known

as the prickly pear or barbary fig. This insect is commercially and economically very valuable because it is a natural biological source of carminic acid, a red dye from the anthraquinone group with a vast range of industrial uses and applications in sectors including pharmaceuticals, food, and cosmetics (Méndez-Gallegos et al. 2010, Campana et al. 2015, Serrato et al. 2017). On the other side, this cactus specie also has huge socio-economic value, producing nutritional fruits and young edible cladodes that are used as vegetables (Cota-Sánchez 2015). In addition, mucilage from the cladodes has a number of uses, mainly in the environmental sector, for instance, as a natural flocculant, emulsifier, and stabilising agent (Bouaouine et al. 2019, de Andrade Vieira et al. 2021). However, maintenance and production of this cactus specie are affected by biotic (invasive) factors including the carmine cochineal, which is parasitic to the plant and sucks its sap. This, lead to chlorosis and other damage to the

cladodes, including necrosis and eventually the death of the plant, causing serious agro-industrial problems (Palacios-Mendoza et al. 2004, Flores-Hernández et al. 2006, Rodríguez-Leyva et al. 2010).

Cochineal insect has a natural binomial symbiotic relationship comprising a host and parasite (plant and insect) with specific affinity and a particular preference for *Opuntia ficus-indica* (Falcão et al. 2013), therefore, leads to a natural symbiosis wherein *O. ficus-indica* cladodes provides support, shelter protection, and nutrition for the breeding and growth of this scale insect, target of this study, as well as serving as a site for copulation and oviposition, which ultimately leads to necrosis of the plant (Méndez-Gallegos et al. 2010, Serrato et al. 2017). Studies of the mass-rearing released from the natural hosts of various commercially-valuable insects (parasites) have considered ways to reduce and completely eliminate damage to the host plant species. Current review in this topic reports some successful systems, both traditional and modern, for artificially rearing different insects in various parts of the world, using synthetic nutrients and substrates. These allow mass production of parasites like *Hypothenemus hampei* (Portilla et al. 2006, 2018), *Sitotroga cerealella* (Palma et al. 2011), and *Anastrepha fraterculus* (Flores et al. 2012), including some bio-control agents like Ladybird *Coleomegilla maculata* (Riddick and Zhixin 2015), at various stages without the need for a plant host; however, no reports have been found related to unnatural or artificial systems for breeding cochineal insects that do not use their common and natural host (*Opuntia sp.*).

Owing to the trend for natural and environmentally friendly products, the past two decades have seen an increase in demand for natural dyes, which is expected to continue (Lokeshwari et al. 2010) so the improved natural breeding of the cochineal insect has prompted efforts to design and run large-scale high-yield and rearing systems without leaving the natural host (OFI) in different configurations since several years ago (Aldama-Aguilera and Llanderal-Cázares 2003, Campos-Figueroa and Llanderal-Cázares 2003, Tovar et al. 2005, Flores-Hernández et al. 2006, Diodato et al. 2009, Méndez-Gallegos et al. 2010, Paul-Fils et al. 2020). Such systems enable optimal control of the environmental parameters (temperature, relative humidity, daylight, etc.) that influence the establishment and growth of *Dactylopius coccus* Costa, while minimising antagonists such as extreme temperatures, dehydration, and adverse weather conditions such as rain, wind, and excessive solar radiation. Mexico and Peru have reported the use of alternative DCC mass-rearing methods using greenhouses for the infestation and establishment of cochineal colonies on cut stems hung vertically. These methods, which were intended to resolve the recurring biotic and abiotic problems that inhibit normal development of the insects, have met with

relative success (Campos-Figueroa and Llanderal-Cázares 2003). Alternatively, micro-tunnels (Aldama-Aguilera et al. 2005) and the called ‘Nopalotecas’ (Hernández et al. 2017, Zacarías-Alvarado et al. 2020) are outstanding and have been used to protect infested *Opuntia ficus* cladodes, and have produced excellent results with respect to the carminic acid content of the final harvest ensuring a large fresh weight of the reared cochineal (Rodríguez et al. 2005, Alonso et al. 2013, Flores-Alatorre et al. 2014, Zacarías-Alvarado et al. 2020); however, the dependence of these systems on OFI cladodes result in serious problems with the preservation of this cacti specie. This study, therefore, reports the use of artificial supports for the establishment and growth of DCC rather than natural cladodes in two engineered controlled systems. These devices used synthetic matrices made of hydrolysed collagen that replaced OFI’s natural cladodes taking advantage of its permeability, gelling, texture, thickness, and water-binding properties (Gómez-Guillén et al. 2011). The objective of this investigation was to establish, rear, and mass-produce carmine cochineal colonies in two laboratory-scale production systems to efficiently substitute natural rearing in prickly pear or barbary fig in open field defining a good quality and concentration of carminic acid with that produced in natural conditions by ensuring a highest value of *D. coccus* fresh weight using artificial and functional synthetic medium (Hendrikson and Merkle 2014).

Materials and Methods

Source of Clean and Infested Cladodes

Cladodes were collected from *Opuntia ficus-indica* plants of between 1 and 2 years old from the region of Tiabaya (16° 29′ 32.80″ S, 71° 50′ 54.07″ W, altitude 1,513 m) in Arequipa (Peru) as a natural source of both host and parasite (Fig. 1A); healthy samples (host only) and infested samples (host + parasite) were collected from different plants in a typical production zone in this geographic locality (Fig. 1B). Cladodes were harvested by cutting the base of the plant, leaving a clean and healthy stump, then transported to the laboratory and stored whole in metal cages (60 cm wide × 60 cm long × 30 cm high) before being covered with transparent polypropylene sheets and kept at 27 ± 0.5 C and 40 ± 5% RH (Fig. 2).

Taxonomic Identification of DCC

Wild insects were harvested from the infested cladodes and preserved in 70% ethanol, before being mounted on slides using the method for coccidian species proposed by Solís (1993) and the keys described by De Lotto (1974) related on the status and identity of



Fig. 1. Location of the study area in La Joya region, Arequipa-Peru (A) Google Earth (GE) image. (B) Landscape view of the sampled area.



Fig. 2. Collected cladodes in field. (A) Transport system used in the study. (B) Clean and healthy cladodes. (C) Infested cladodes.

the native cochineal insects at the considered geographical location in South America. Taxonomic identification of these cochineal insects was performed by the Entomology Department of the Faculty of Biological Sciences at Universidad Nacional de San Agustín de Arequipa based on the study of the main morphological features distributed along the observed Tiabaya region in Peru, who studied the physical anatomy of three adult females using a stereoscope and optical microscope.

Biological Characteristics

The growth cycle, biological cycle, preoviposition period, oviposition ability, postoviposition, and egg viability of insects were all examined to obtain a description of insect behaviour, based mainly on movement, copulation, and feeding in 12 clean and cut stems of *O. ficus-indica* in laboratory ($27 \pm 0.5^\circ\text{C}$, $40 \pm 5\%$ RH, and photoperiod of 16:8 (L:D) h). In each cladode, fastened by an ironmade hook in vertical inverted position, 10 adult and egg-filled females of *D. coccus* collected from original infested samples were put into a small folded paper envelope (Zhang 2017).

Physical and Chemical Composition of OFI Stems (Cladodes)

An uninfested stem was washed, cut, and peeled to produce two fractions: skin and flesh. The first fraction was subjected to scanning electron microscopy (SEM) to determine its structural morphology and surface microstructure using a scanning electron microscope VEGA II LSH; the second fraction was homogenised in order to determine its physical and chemical properties including pH, total titratable acidity (TTA) expressed as a percentage of malic acid (MA), conductivity (G, mS/cm), total dissolved solids (TDS, ppm), oxidation-reduction potential (ORP, mV), and heavy metals, as described by Terán et al. (2015) and Hernández-Carranza et al. (2019). Finally, the second fraction was separated in a solid and liquid fraction for use as precursors of the mucilage (liquid phase) and pulp solids (solid phase).

In Vitro Mass-rearing of DCC on an Artificial Medium

Live cochineal were reared in vitro using the artificial medium described by Hendrickson and Merkle (2014), free of predators, and under optimal growth laboratory conditions at $27 \pm 0.5^\circ\text{C}$, to obtain clean and egg-filled females free of dust, sericins, remains of exuviae, male cocoons, or another environmental contaminant (Aldama-Aguilera and Llanderal-Cázares 2003). The

synthetic base medium was prepared by mixing 1.125 ml water, 37 g agar-agar, and 175 ml corn syrup. The mixture was sterilised at 121°C and 15 psi for 20 min and then cooled to 40°C , before 80 mg methyl paraben was added as an antifungal agent, ensuring it was fully dissolved. Finally, 15 g mucilage, 15 g pulp solids, and 15 g pulverised skin obtained from the uninfested cladodes were added to the base medium as additives and attractants to stimulate its appeal to the insects and achieve normal and optimal growth. The medium was then poured into six 10 cm Petri dishes and left to cool completely and harden at laboratory temperature. Each plate was inoculated under sterile conditions with 5 egg-filled females (approximately 0.40–0.50 g fresh weight) removed from the infested cladodes harvested in the field; the cultures were then incubated under controlled conditions at $27 \pm 0.5^\circ\text{C}$ for 70–90 d, $40 \pm 5\%$ RH with 16 h light per day, until harvested near infested cladodes anchored in laboratory in order to prove the artificial diet.

Preparing Three-dimensional (3-D) Permeable Hydrolysed Collagen Support Matrices

To elaborate 3-D permeable hydrolysed collagen support as hosts for biomimetic controlled production of cochineal, were manufactured synthetic membranes (1–2 cm thick) using a modified synthetic medium proposed by Hendrickson and Merkle (2014), replacing the agar with food-grade hydrolysed collagen and adding a hardening solution (50% water, 25% formaldehyde, 15% ethanol v/v), then combining 1.125 ml agar-free Hendrickson medium with 35 g hydrolysed collagen powder type I (2,000–4,000 Da) and 25 ml hardening solution. This modified medium was poured into transparent acrylic moulds measuring 10 cm wide \times 25 cm long \times 2.5 cm thick, then left to gelify for 2 h at $27 \pm 0.5^\circ\text{C}$ under completely sterile conditions. The 3-D support matrices were then removed from the moulds and used to construct the cochineal mass-rearing systems. In addition, for the purposes of comparative statistical analysis, blank permeable 3-D matrices were prepared without the natural OFI additives (mucilage, pulp solids, and skin obtained from healthy cladodes).

Experimental Configuration of the Cochineal Mass-rearing Systems

Two different controlled engineered systems were used to house the artificial host (3-D hydrolysed collagen matrices) and parasites (insects) to encourage infestation and mass breeding.

System 1 (STC-01)

This system comprised a prismatic box made of transparent acrylic and measuring 40 cm long × 30 cm wide × 25 cm high with a metal support structure. The box was divided into three 10 cm chambers (I, II, and III) separated by a metal mesh, such that the chambers were linked but the insects could not invade or cross the barriers to contaminate a different chamber.

The structure also included four 0.64 cm stainless steel cross-bars for holding the hydrolysed collagen matrices in the V position (vertically) in each of the three chambers; one of the chambers (III) contained the blank matrices. The unit was fitted with two 45-W high-pressure sodium lights (F20 T12-PL, GRO, and SHO P.L., General Electric) to provide 16 h light per day controlled by a simple on-off system; a humidity, temperature, and carbon dioxide concentration (CO₂) datalogger (Extech Mod. RHT-50), and a container of a supersaturated potassium carbonate solution to provide constant relative humidity of 45%, prevent the collagen matrices from drying out, and generate the modified atmosphere needed for establishment of the insect colonies.

System 2 (STC-02)

This system comprised a micro-tunnel 15 cm wide × 20 cm long with a 316-L stainless steel base, a finely textured hemispherical cupula of tulle net, an automatically-controlled electric servomotor, an Arduino UNO microcontroller, PWM communication, LDR photoresistors, and BPW34 photodiodes to determine the path of maximum luminosity during the day and for use as sunlight-tracking sensors, in order to reduce energy consumption and the use of sodium lights to provide the required 16 h of light per day.

Unlike the first system, this structure included tracking of the sun as a source of natural light to optimise energy consumption. In addition, the 3-D hydrolysed collagen membranes were arranged as a single layer in the H position (horizontally), with the same instruments for monitoring RH (%), temperature (°C), and CO₂ (ppm).

Start Up, Inoculation, and Induced Infestation in STC-01 and STC-02

The experiment used a single STC-01 unit and a set of five STC-02 units so that the predetermined operational and functional parameters could be studied in quadruplicate. In STC-01, four hydrolysed collagen membranes were placed in the V position in chambers I and II, and four blank membranes were placed in chamber III.

For STC-02, four 3-D hydrolysed collagen membranes were placed in the H position in each unit, with one unit used for the blank membrane.

Infestation was induced using nests of sterile filter paper containing 10 clean egg-filled females (Diodato et al. 2009) in an optimal state, bred in laboratory from the in vitro culture.

Four nests were placed in each of the chambers of the STC-01 unit, ensuring an equal ratio of the **number of inoculated insects to membrane to total number of usable surfaces** (NII:M:NUS) of 40:4:8 or 10:1:2, i.e., 10 insects per membrane with both sides used, until the end of their total life cycle. The infesting nest was placed in the same location on each membrane and in each chamber to avoid introducing an additional variable.

For STC-02, one 3-D hydrolysed collagen membrane was placed on the stainless steel base in the H position, creating a single surface for infestation. One infesting nest of 20 insects was placed in each of the STC-02 units to ensure the same NII:M:NUS ratio as that used in the STC-01 unit, given that the STC-02 units had just one available surface per hydrolysed collagen membrane.

Once the systems had been set up and inoculated using the process described by Diodato et al. (2009), the colonies were left to grow and infest for a total period of 120 d.

Carmine Cochineal Insects Production Using the STC-01 and STC-02 Systems Under Controlled Conditions

Once infestation had been established in all STC-01 and STC-02 units under controlled conditions protected from biotic and abiotic factors, the colonies were monitored to avoid cross-contamination until the gravid female phase was reached (Tekelenburg 1995). The cochineal were then collected, the total number of insects and fresh weight per 3-D hydrolysed collagen membrane in each system were recorded, and the average yield was calculated using the following equation (Feng et al. 2010):

$$R_{NHC} = \frac{NHC}{t * S * n}$$

where:

R_{NHC} is the production yield per system, i.e., the number of insects per day per square metre;

NHC is the number of adult females harvested;

t is the time to reach the gravid phase in days;

S is the membrane surface area (m²); and

n is the number of surfaces exposed to infestation.

Likewise, the yield was calculated in terms of fresh weight (R_{WHC}), replacing NHC by WHC (the fresh weight of adult females harvested, g fresh weight per day per square metre).

Statistical Analysis

The results obtained for yield (R_{NHC} and R_{WHC}) in terms of gravid and non-gravid insects harvested on the two different systems (STC-01 and STC-02) were subjected to Barlett and Kolmogorov tests to determine the normality and homogeneity of variance needed for analysis of variance. One-way analysis of variance (ANOVA) was conducted to assess the effect of the STC-01 chambers I and II and four STC-02 units on yield (NHC and WHC) results. Post hoc multiple comparison, Games Howell's method, was conducted to evaluate differences among means for those data due to unequal variances. All set of experimental data were analyzed with Microsoft SPSS software version 26 (IBM SPSS Statistics 2019). In this study a P-value < 0.05 was considered statistically significant.

Results

Taxonomic Identification and Biological Characteristics of DCC

The Entomology Laboratory of the Faculty of Biological Sciences at Universidad Nacional de San Agustín de Arequipa produced a taxonomic identification for the collected biologic units of *Dactylopius* sp. (report no. 179-2016-LE-UNSA), reported the individuals belong to Family *Dactylopiidae*, Genus *Dactylopius* and Species *Dactylopius coccus* Costa 1835.

In all cut stems of *O. ficus-indica* used in laboratory were observed that eggs emerged irregularly, occurring over a period of 3–11 d of initial infestation (7.15 ± 2.32 d; $F = 1.01$; $df = 9,11$; $P \leq 0.2896$), observed incubation period before they hatched was less than 30 min in all cases and the hatching process was similar to that described by Guerra and Kosztarab (1992) for other species of this genus and characteristic of *D. coccus* specie. There were no

statistically significant differences in developmental periods for first instars (15.00 ± 2.83 d; $F = 1.05$; $df = 9,11$; $P \leq 0.2488$), second instars (19.25 ± 2.18 d; $F = 0.99$; $df = 9,11$; $P \leq 0.3497$), or adult stage (69.66 ± 18.8 d; $F = 1.09$; $df = 9,11$; $P \leq 0.2133$) among the twelve cut stems (Table 1) and an interesting moult was observed from second-instar to adult stage between 25 and 40 d. Complete adult and mature was verified around 70 d with harvest capacity and the initial oviposition stage before 90 d and their complete death after 120 d. The main biological parameters of the DCC evaluated population were also determined and, expressed as average observed periods where the experimental growth life cycle in this study was, approximately, between 75 and 90 d in laboratory (Fig. 3).

Furthermore, *Opuntia sp.* shoots, were identified by Herbarium Arequipensis (report no. 069-2016-HUSA) and belong to Family *Cactaceae*, Tribe *Opuntiae*, Genus *Opuntia*, and Species *Opuntia ficus-indica*. The skin fraction of the *O. ficus-indica* stems was characterized by SEM analysis and high-resolution micrographs with an accelerating voltage of 25 KV at 5 μ m scale (Fig. 4), which revealed a characteristic porous and permeable surface cell microstructure with long fibrous microcapillaries similar to those reported by Rykaczewski et al. (2016) in OFI that also shows fissures in the cuticle. Observed size, shape, and a random thin microfibrils distribution in electronic view at 12,000 \times magnifications indicated their potential use as a natural attractant and support of cochineal insect because they can apparently consist of a compact dispersed arrangement of slender cellulosic and pectin microfibrils (0.1 mm long, 4–6 μ m diameter) with very small lumens used by the insect to hook tightly and suck the sap of natural cladodes (Falcão et al. 2013). The compositional cationic spectrogram shows inorganic metal quality content, where magnesium was the most abundant element, followed by aluminium, sodium, potassium, calcium, and iron, well characterized as cofactors of the metabolic production of carminic acid in DCC (Zacarias-Alvarado et al. 2020).

Table 2 shows no significant differences among physical and chemical properties of the mucilage and indicate a normal pH acid value (3.65 ± 0.02) and a characteristic TTA expressed by 0.84 ± 0.03 g MA/100 ml and a TDS value of 355 ± 5.66 ppm according with the literature (Hernández-Carranza et al. 2019); ORP and G values ($+509 \pm 1.45$ mV and 8.16 ± 0.21 mS/cm, respectively) showing the succulent and fluid nature of the plant material, along to high ionic and nonionic contents (de Andrade Vieira 2021, Barazarte et al. 2017, Terán et al. 2015, Falcão et al. 2013), and provide a good capacity for nutritional functional compounds exchange and mobility for the development and mass-rearing of the cochineal insect.

Table 1. Main developmental times(days) of *Dactylopius coccus Costa 1835* reared in cut inverted cladodes ($27 \pm 0.5^\circ\text{C}$, $45 \pm 5\%$ RH, and a photoperiod of 16:8 (L:D) h)

Stages	Mean \pm SD	df	F value	P value
Eggs ^a	7.15 \pm 2.32	9,11	1.01	0.2896
First-instar nymph	15.00 \pm 2.83	9,11	1.05	0.2488
Second-instar nymph	19.25 \pm 2.18	9,11	0.99	0.3497
Adult	69.66 \pm 18.8	9,11	1.09	0.2133
Growth life cycle	74.75 \pm 5.51	9,11	0.91	0.3754
Pre-oviposition period	42.75 \pm 2.18	9,11	0.98	0.3521
Oviposition period	91.33 \pm 6.80	9,11	0.88	0.4725
Post-oviposition period	102.75 \pm 11.47	9,11	1.32	0.2875

^aObserved period in which eggs appeared in infested cladodes (incubation period < 30 min in all cases).

In-vitro Mass Rearing of DCC on Hendrickson and Merkle Synthetic Medium

Experimental values from the mass breeding of DCC in laboratory were completely satisfactory since the success of the procedure (Fig. 5) for obtaining native and owned insect germplasm from contaminated cladodes to inoculate the Petri dishes and, finally with the very overcrowded cultured plates after 84 d, with an impoverished medium that has obviously lost its initial organoleptic properties but where the inoculum of insects seeded in vitro increased to 3.28 ± 0.41 g fresh weight ($n = 6$; $F = 3.86$; $P < 0.01$) that served to infest used controlled systems in this study.

Schematic and experimental structure of STC-01 (Fig. 6) presents the three chambers housed 12 hydrolysed collagen membranes in the vertical position (V); likewise, schematic and actual structure of STC-02 (Fig. 7) presents a final laboratory configuration with hemispherical cupula of tulle net to avoid external contamination with a microtunnel shape. Then, the main variables evaluated in this study between STC-01 and STC-02 were the operation mode (stationary and mobile, respectively) and the matrices position (V and H) in their configurations.

The induced inoculation in both experimental set-ups was described at baseline (time, $t = 0$), then maintained under controlled conditions with constant observation during 120 d. Table 3 presents the operational and test conditions for each type of mass-rearing units in STC-01 and STC-02 systems ($N = 120$) determining that both systems worked under similar operating conditions, so they had the property to be compared statistically.

The operational behavior presented a normal variation of temperature ($27\text{--}32^\circ\text{C}$ for STC-01 and $25\text{--}31^\circ\text{C}$ for STC-02), relative humidity ($42\text{--}46\%$ for both cases) and a photoperiod of 16:8 h as control and operational parameters; and concentration of carbon dioxide generated in-situ (CO_2) in STC-01 (stationary mode) and STC-02 (mobile mode) as a response variable for a total life period of 120 d study of growth and controlled massification of DCC (Fig. 8). The CO_2 profile shows the increasing pattern of metabolic concentration of carbon dioxide (ppm) produced as a waste product of cellular respiration as a result of growth and mass-rearing of the DCC on the two experimental systems defining the insect respiratory system as responsible for delivering sufficient oxygen to all cells of the DCC bodies and for removing generated carbon dioxide to ensure growth and development of biomass. Both systems behaved similarly up to 80–85 d (preoviposition period); however, STC-01 recorded a slightly higher CO_2 concentration (1,200–1,500 ppm) compared with STC-02 (800–1,200 ppm) from 55 d (adulthood and mature stage) that was magnified at final life cycle recording peaks of up to 5,000 ppm that indicated a high cellular and metabolic insect activity after oviposition and before its final death in this experimental configuration.

Once both mass-rearing systems (STC-01 and STC-02) were infested, the life cycle and reproduction period of the insects were determined based on the gravid female phase (Tekelenburg 1995). This phase was chosen because the insects at this stage are easily identifiable owing to their growth and their external red carmine colouration, together with the almost complete disappearance of the white waxy shell that protects the insects from the first and second instar nymphs until adulthood.

At harvest, each of the systems was dismantled and the life cycles and reproduction periods were recorded, and the NHC and WHC per 3D hydrolysed collagen membrane were determined to calculate R_{NHC} and R_{WHC} . For STC-01 and STC-02, the gravid stage was reached after 81 d (1,944 h) and 88 d (2,112 h), respectively; this was when the maximum growth of the mass-reared insects was

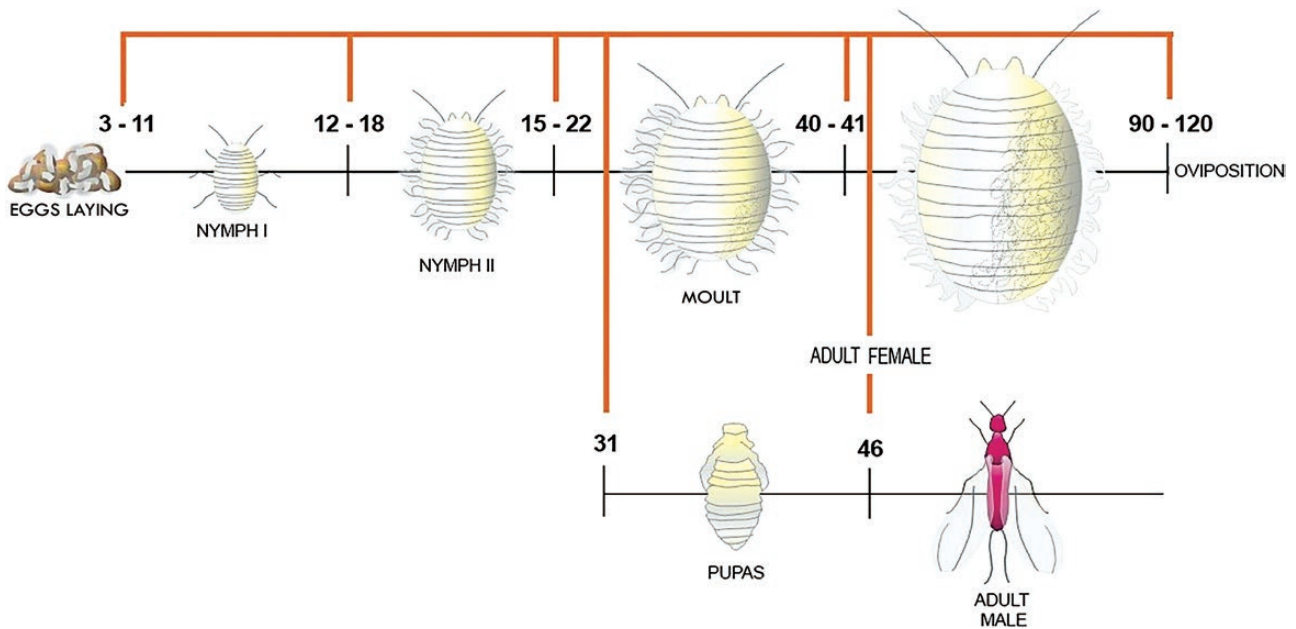


Fig. 3. Peruvian *Dactylopius coccus* Costa – total life cycle (days) obtained as a mean description of insect behaviour, based mainly on movement, copulation, and feeding in 12-cut stems of *O. ficus-indica* in laboratory ($27 \pm 0.5^\circ\text{C}$, $40 \pm 5\%$ RH, a photoperiod of 16:8 (L:D) h.).

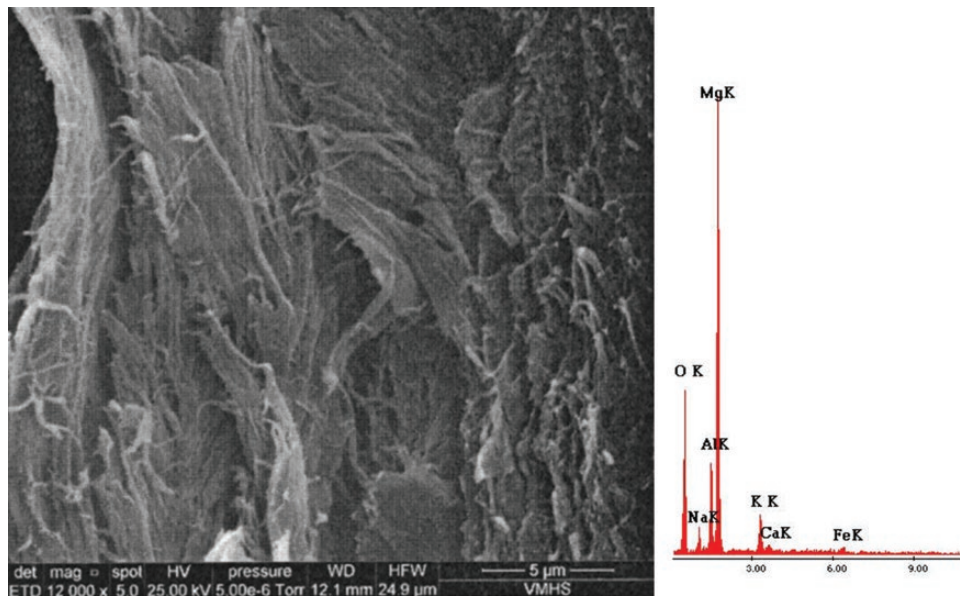


Fig. 4. Scanning electron micrograph at 12,000 \times magnification (5 μm) with compositional cationic spectrogram showing superficial microstructure of the skin fraction of *O. ficus-indica* stems.

observed previous oviposition Final harvest of DCC insects obtained and withdrawn from STC-01 (19.69 ± 2.77 g fresh weight) was significantly higher to STC-02 results (8.45 ± 0.37 g fresh weight) in, approximately, 100% in number and fresh weight desired of cochineal insects was achieved with the same operational conditions. NHC and WHC result in chambers I, II, and III in STC-01 and in each of one STC-02 units followed a normal distribution but with significantly different variances when the Barlett and Kolmogorov test was applied. Box-and-whisker diagrams for the NHC and WHC results from chambers I and II (STC-01) and from STC-02 units

show statistical differences between systems (Fig. 9). Means and variances for chambers I and II were similar ($n = 4$; $P > 0.05$) but were so different with STC-02 ($n = 4$; $P < 0.001$).

NHC was significantly influenced by the system structure used in this study ($F_{2,9} = 51.84$, $P < 0.01$) and the same for WHC ($F_{2,9} = 52.626$, $P < 0.001$); the highest NHC ($F_{1,6} = 0.21$, $P > 0.05$) and WHC ($F_{1,6} = 0.005$, $P > 0.05$) values were obtained from STC-01 in chambers I and II compared with the results from STC-02 (Tables 4 and 5).

Experimental values were used to calculate an average final yield for STC-01 unit of $R_{NHC} = 22.81 \pm 2.04$ adult females per day per

exposed square metre and $R_{WHC} = 4.85 \pm 0.50$ g fresh weight per day per exposed square metre; the blank membrane produced only 5.74 ± 0.58 adult females per day per exposed square metre and 1.14 ± 0.19 g fresh weight per day per exposed square metre (Table 4), that is, a difference of more than 75% in each case showing significant difference ($n = 4$; $P < 0.05$) determined, conclusively, by the absence of attractant additives in their elaboration.

For STC-02, the average final yield was 14.96 ± 1.36 adult females per day per exposed square metre and 3.20 ± 0.54 g fresh weight per day per exposed square metre (Table 5); the blank membrane produced 6.82 adult females per day per exposed square metre, and 0.81 g fresh weight per day per exposed square metre, that is, a difference in yield of more than 45% NHC and 25% WHC.

Discussion

In this study was found that a typical Peruvian cochineal insect characterized as *Dactylopius coccus* Costa 1835 obtained in open field from infested cactus *Opuntia ficus-indica* (L.) Miller could be conveniently reared and massified in two different controlled systems

Table 2. Physical and chemical properties of the mucilage fraction of clean cladodes of *O. ficus-indica* harvested in Tiabaya region (Peru)

Parameter	Unit	Mean \pm SD ($n = 2$) ^a
pH	–	$3.65 \pm 0.02b$
TTA	g MA/100 ml	$0.84 \pm 0.03cd$
G	mS/cm	$8.16 \pm 0.21a$
TDS	ppm	$355 \pm 5.66bc$
ORP	mV	$+509 \pm 1.45bc$
Aluminum	ppm	$0.31 \pm 0.03d$
Iron	ppm	$0.09 \pm 0.01d$

^aMeans \pm SD followed by same letter in last column are not significantly different ($P < 0.05$, Tukey's test).

(STC-01 and STC-02) without using the natural host in laboratory conditions (Tables 4 and 5); however, it is unknown whether the concentration, quality, and yield of carminic acid are different from that produced under natural conditions. Therefore, to promote normal and efficient induced growth in artificial hydrolyzed collagen supports, three different fractions of the natural cladodes of *O. ficus* (skin, pulp solids, and mucilage) were used in the synthetic growth medium with components of specific function such as corn syrup as a carbon source, because they can be added to facilitate the growth of insects, as well as to promote crop health, insect health and potentially shorten the life cycle of insects to increase carminic acid/carmine yield (Hendrickson and Merkle 2014).

Obtained values of biological characteristics of the DCC population are comparable to that found by Guerra and Kosztarab (1992) for first and second instars nymphs and Zhang (2017) for nymphs and adult stages developed normally (Table 1) under laboratory conditions ($27 \pm 0.5^\circ\text{C}$, $40 \pm 5\%$ RH, and 16 h light per day) defining a specific *Dactylopius* genus like carminic acid producer since this *D. coccus* specie is reported as a source of this red dye when hosted and reared in cactus of *O. ficus-indica* specie (Rodríguez et al 2005, Hernández et al. 2017). Zacarias-Alvarado et al. (2020) reported that the concentration of carminic acid and the fresh weight of the reared cochineal increase proportionally, when one of the two variables is increased to a certain limit; suggesting that *D. coccus* has specific thresholds for optimal development; in addition, Rodríguez et al. (2005) determined that cochineal with the highest fresh weight had the lowest carminic acid concentration, even though they can have more eggs, which means an inverse relationship between carminic acid concentration and fecundity. In this study, the harvest was carried out before 91.33 ± 1.96 d when was observed the oviposition period (Table 1); 81 d for STC-01 (Table 4) and 88 d for STC-02 (Table 5) ensuring high fresh weights values and adult females in a period prior to oviposition in both systems.

Total dissolved solids (355 ± 5.66 ppm) and pH (3.65 ± 0.02) values of *O. ficus* cladode mucilage (Table 2) and aluminium, calcium, and magnesium content in the microstructure of skin (Fig. 4) and mucilage (Table 2) obtained from our study are in accordance to



Fig. 5. In-vitro rearing method for *D. coccus* Costa using modified artificial medium suggested by Hendrickson and Merkle (2014). (A) Rearing Petri-Dishes for egg-filled cochineal females (0.40–0.50 g fresh weight). (B) Egg-filled cochineal females obtaining procedure in laboratory. (C) Petri-Dishes after 84 d at $27 \pm 0.5^\circ\text{C}$, $40 \pm 5\%$ RH, and a photoperiod of 16:8 (L:D) h (3.28 ± 0.41 g fresh weight).

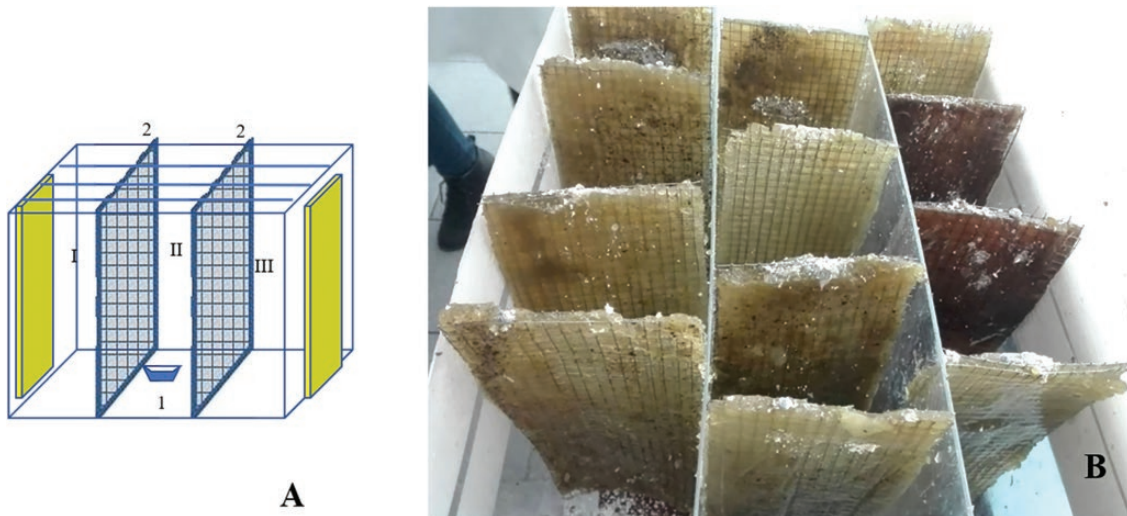


Fig. 6. Schematic (A) and experimental structure (B) of system STC-01. Notice the configuration of three chambers housed 12 hydrolysed collagen membranes in the vertical position (V).

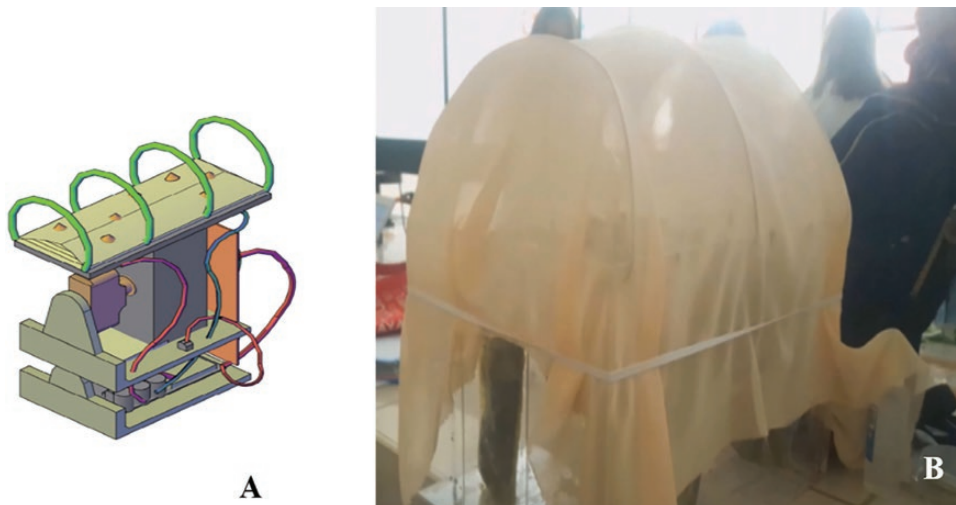


Fig. 7. Schematic (A) and experimental structure (B) of system STC-02. Notice the configuration with hemispherical cupula of tulle net to avoid external contamination with a microtunnel shape.

Table 3. Operational conditions^a for each type of mass-rearing experimental systems

System	n^b	Light per day, ^c h	Temperature, ^c °C	Relative humidity, ^c %
STC-01	1	16 ± 0.23ab	30.08 ± 2.33ab	45.21 ± 2.87ab
STC-02	5	16 ± 0.10ab	28.55 ± 3.04ab	47.35 ± 1.09ab

^aBoth systems under the same environmental and operational conditions.

^bUnits used in experimental study in each system.

^cValues are mean ± SD in both systems during 120 d of operation and monitoring (followed by the same letter in each column are not significantly different; $P < 0.05$, Tukey's test).

that reported by Rykaczewski et al. (2016), Barazarte et al. (2017) and Hernández-Carranza et al. (2019) provided a nutritionally well-balanced diet in the modified synthetic medium of Hendrickson and Merkle (2014) used to build their aluminum complex carmine,

precursor of the anthraquinone carminic acid that would have metabolized our Peruvian cochineal insect during their total life cycle evidenced by a typical and intense red colour in the adult females bodies collected.

It is important to clarify that although two different systems were compared, both laboratory-scale production systems are free of natural host and use a stationary in V-membrane position (STC-01) and mobile in H-membrane position (STC-02) operation mode for rearing *D.coccus*. Our data showed that the production NHC and WHC estimated from the collected period was statistically higher ($n = 4$; $F = 3.52$, $P < 0.05$) in STC-01 (19.63 ± 2.03 g fresh weight, 0.025 m², 81 d, Fig. 9B) with respect to STC-02 (8.45 ± 0.37 g fresh weight, 0.03 m², 88 d, Fig. 9B) and, between STC-01 chambers I and II there was no significant difference so this system behaved much more homogeneously. Aldama-Aguilera and Llanderal-Cázares (2003) noted that to produce one kilogram of fresh cochineal, a mean of 100 cladodes were needed in 120 d; however, results obtained in our study indicate, comparatively, a use of only 23 cladodes in 81

d (STC-01) and 32 cladodes in 88 d (STC-02) achieving four harvests per year providing all the biotic and abiotic key factors (operation mode, carbon source, and cladode fractions added in synthetic medium, membrane position, environmental constant parameters) necessary for the insects to complete their life cycle in continuous generations, successfully replacing the prickly pear cactus (OFI) in field as reported by other authors included other insect species (Sprugeon and Brent 2016, Morales-Ramos et al. 2015, Ramírez-Cruz et al. 2008, Chaudhury and Alvarez 1999, Barriga-Ruiz 1994). The largest cochineal population was obtained with STC-01, which used constant daylight times (Table 4), it probably resulted in smaller temperature fluctuations (Fig. 8A) and no movement requirement for its operation compared to the other system. This meant a better establishment and development of migratory and fixed nymphs in the first system since synthetic permeable matrices were used in a vertical position and their infestation was allowed on both sides simulating natural growth (Aldama-Aguilera et al. 2005). Therefore, our method could be adaptable for scale-up and start-up

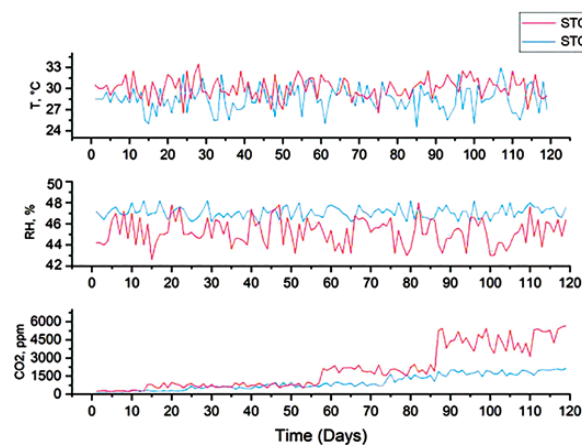


Fig. 8. Temperature (T, °C), relative humidity (%), and concentration of carbon dioxide generated by insect metabolic respiration (CO₂, ppm) variation profiles in STC-01 (static mode) and STC-02 (mobile mode) for a total life period of 120 d under study of growth and controlled massification of *D. coccus* Costa.

in continuous production mode owing were found that the cochineal presented a similar characteristic feeding behavior; similar digestive and nutritional demands, and similar reproductive and gas exchange requirements; Veldkamp et al. (2012) reported that is technically feasible to produce insects on a large scale in artificial containers under controlled conditions. Used synthetic matrices (V-position) in STC-01 produced larger populations of cochineal owing to their uniform structure and composition, avoiding the issues associated with use of a natural support (OFI) and plant age; cladode colour and age can have a negative impact on cochineal growth because of variations in nutritional content (Rodríguez et al. 2005).

Obtained experimental results allowed to warn that there was a statistically significant difference ($N = 4$; $F = 2.45$; $P < 0.05$) in fresh weight and number of insects values obtained in the blank membranes used in both systems that did not contain skin, pulp solids, and mucilages in their composition compared to those membranes evaluated during the total life cycle of cochineal, which would mean that these fractions are a key factor affecting the production yield of a mass rearing insect colony, 1.14 ± 0.19 g gravid insects/day/m² in STC-01 (Table 4) and 0.81 ± 0.08 g gravid insects/day/m² in STC-02 (Table 5) resulting in 76.54% and 74.68% lower than the maximum R_{WHC} obtained, respectively.

There are no published mass rearing systems for *D. coccus* without natural host (*O. ficus*) nor using artificial diets; therefore, the results obtained in this study were compared to the fresh weight production of cochineal insect (*Dactylopius coccus* C.) (Hemiptera: Dactylopiidae) using natural and induced host. Fresh weight and number of reared insects harvested from our study were, in all cases, higher than those reported and found in open field with native and transplanted plants (Santibañez 1992, Barriga-Ruiz 1994, Tovar et al. 2005, Feng et al. 2010), in greenhouses using cut cladodes (Aldama-Aguilera and Llanderal-Cázares 2003, Campos-Figueroa and Llanderal-Cázares 2003, Alonso et al. 2013), in microtunnel greenhouses (Aldama-Aguilera et al. 2005), and in 'Nopaloteca' systems (Hernández et al. 2017, Zacarias-Alvarado et al. 2020).

In this context, based in the obtained results, is referring to that using proposed facilities, STC-01 specifically, up to four cochineal harvests could be produced per year, compared with two or three obtained in open field or greenhouses that used natural host. Santibañez (1992), Barriga-Ruiz (1994), and Tovar et al. (2005) reared *D. coccus* species using natural *O. ficus-indica* in open and

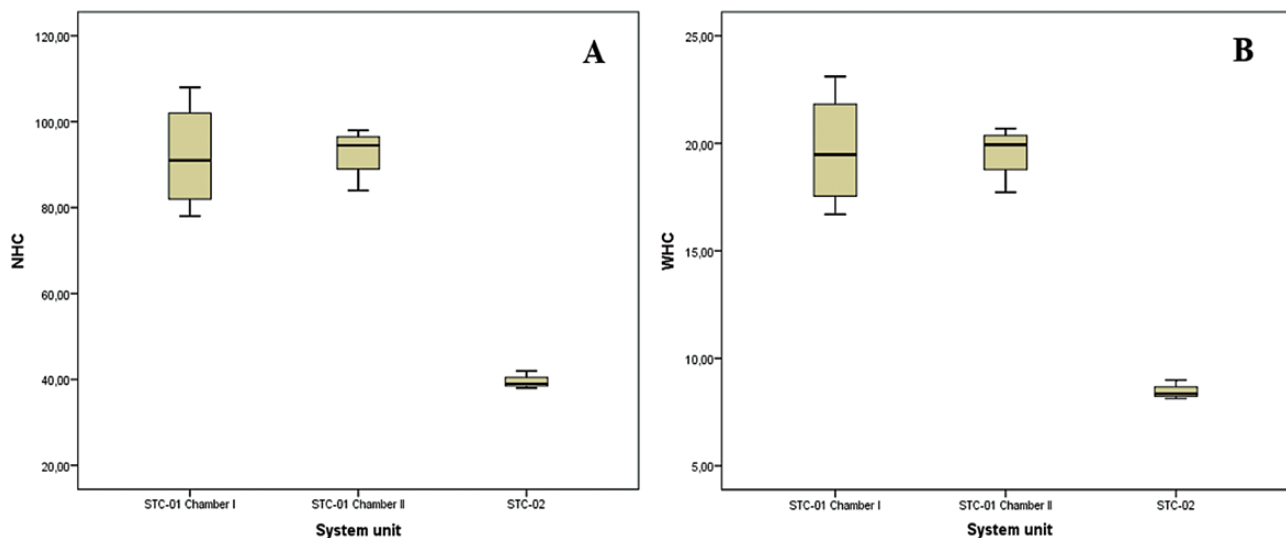


Fig. 9. Box-and-whisker diagrams for (A) NHC values from chambers I and II (STC-01) compared with STC-02 results. (B) WHC values in both systems.

Table 4. Final population number (NHC) and fresh weight (WHC) of *Dactylopius coccus* Costa at harvest in STC-01 system^a (30.08 ± 2.33°C, 45.21 ± 2.87% RH, and a photoperiod of 16:8 (L:D) h)

Position	Chamber					
	I		II		III ^d	
	NHC, adult females	WHC, g fresh weight	NHC, adult females	WHC, g fresh weight	NHC, adult females	WHC, g fresh weight
Membrane 1	86	18.40	95	20.05	22	4.38
Membrane 2	78	16.69	84	17.72	20	3.98
Membrane 3	96	20.54	94	19.83	22	4.38
Membrane 4	108	23.11	98	20.69	29	5.77
Mean ± SD (n = 4)	92.00 ± 12.96ab	19.69 ± 2.77cd	92.75 ± 6.08ab	19.57 ± 1.28cd	23.25 ± 3.95e	4.63 ± 0.79e
R _{NHC} ^b	22.72 ± 2.77ab	–	22.90 ± 1.30ab	–	5.74 ± 0.84c	–
R _{WHC} ^c	–	4.86 ± 0.68ab	–	4.83 ± 0.32ab	–	1.14 ± 0.19c

^aMembrane area = 0.025 m²; Harvest period (oviplane phase) = 81 d; Number of used infestation surfaces = 2.

^bProduction yield reported as number of adult females per day per square metre.

^cProduction yield reported as g fresh weight per day per square metre.

^dBlank values; Mean ± SD followed by the same letter are not significantly different ($P < 0.05$, Tukey's test).

Table 5. Final population number (NHC) and fresh weight (WHC) of *Dactylopius coccus* Costa at harvest in STC-02 system^a (28.55 ± 3.04°C, 47.35 ± 1.09% RH, and photoperiod of 16:8 (L:D) h)

Replicate	NHC, adult females	WHC, g fresh weight
Blank	18	2.13
R _{NHC} ^b	6.82	–
R _{WHC} ^c	–	0.81
1	42	8.99
2	39	8.35
3	38	8.13
4	39	8.35
Mean ± SD (n = 4)	39.50 ± 1.73	8.45 ± 0.37
R _{NHC} ^b	14.96 ± 1.36	–
R _{WHC} ^c	–	3.20 ± 0.54

^aMembrane area = 0.03 m²; Harvest period = 88 d; Number of used infestation surfaces = 1.

^bProduction yield reported as number of adult females per day per square metre.

^cProduction yield reported as g fresh weight per day per square metre.

reported harvest at 120–150 d; similar results are found in greenhouses with glass or plastic covering (cut cladodes and microtunnels) with 90–120 d of harvest periods (Aldama-Aguilera and Llanderal-Cázares 2003, Feng et al. 2010, and Alonso et al. 2013); however, Hernández et al. (2017) and Zacarias-Alvarado et al. (2020) reared cochineal using Nopalotecas in 66–90 d with temperatures around 30–31°C, 16 h of light, 95% relative humidity, and extreme ventilation collected when 50% of females had initiated oviposition for ensure high carminic acid concentration and obtained four expected harvests per year compared with results of Campos-Figueroa and Llanderal-Cázares (2003), Diodato 2009, and Tekelemburg (1995).

In summary, the observed properties of permeability and porosity of the hydrolysed collagen membranes used to replace the natural process were promising to achieve the establishment and development of insects in both systems, but the V position turned out to be more significant to define this variable in the systems under

analysis. STC-01 used in this study provided full protection from biological and physical contamination and ensure optimal levels of biotic and abiotic factors but the uncontrolled factors such as stray sounds or vibrations (STC-02) influence negatively the rearing process as reported by Choi and Park (2019).

Currently, controlled environment monitored in this study was found superior to natural conditions because the biological growth of cochineal may be limited by meteorological factors such as heavy rain, sub-zero temperatures, direct sunlight, and strong winds, especially during the early stages of development of cochineal (Flores-Hernández et al. 2006, Diodato et al. 2004). In addition, a controlled environment protects the insects from exposure to parasitic and predatory insects that feed on them, including Hymenoptera, Diptera, Coleoptera, Lepidoptera, and Neuroptera (Cohen 2018, Zimmermann et al. 1979).

The economic value of OFI and DCC can be multiplied through the use of controlled systems, with box and stationary system (STC-01) proving superior to mobile and dynamic system with membranes in H-position (STC-02). South America is largely arid, and the farming of OFI for infestation with DCC is a widespread practice requiring vast areas of farmland that could be used for other economic purposes. Growing DCC under controlled conditions means a host is no longer required, thus freeing up extensive areas of farmland. Finally, no or very little research has yet focused on the establishment of cochineal insects in this type of controlled or semi-controlled systems free of OFI; this is one of the innovative aspects of the current study. However, additional investigation is needed that examines the potential of the proposed methods and innovative controlled production systems to evaluate quality, yield, and concentration of carminic acid in order to verify no detrimental changes of DCC reproduction with this important response.

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Author Contribution

The author contributed to the conceptualization, writing, review and editing of this paper.

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