

“DEVICE FOR DEVELOPING GERMFREE AND GNOTOBIOTIC MOSQUITOES”

The following specification particularly describes the invention and the manner in which it is to be performed.

5 TECHNICAL FIELD:

The present disclosure relates to the field of rearing germfree and gnotobiotic mosquitoes and other similar insects as well. Specifically, the present disclosure provides a novel device for rearing insects such as mosquitoes and others like beetles, butterflies, and bees. Also, the present invention provides a process for
10 rearing germfree and gnotobiotic mosquitoes.

BACKGROUND ART:

Mosquitoes are insect vectors that can carry and spread many types of diseases that are caused by parasites, viruses, etc. Mosquito-transmitted infections are considered to be the emerging public health problem globally. Malaria, Japanese encephalitis,
15 Dengue, Chikungunya, Lymphatic filariasis, Yellow fever, and Zika are responsible for huge morbidity and mortality of humans around the globe. Several millions of rupees have been spent on preventing and controlling the diseases transmitted by mosquitoes. Mosquito control is one of the major challenging tasks for many countries including India. Commonly mosquitoes are controlled by spraying
20 mosquitocides and by using mosquito repellants, and by personal protection. However, these methods or steps do not provide a permanent solution to the problem stated above. In other words, these methods are not completely successful in controlling mosquitoes, which is a threat to mankind. As there is no commercial vaccine available for treatment to many vector-borne diseases (VBDs), vector
25 control remains the only available option for the control. These notorious vector mosquitoes are often controlled by the wide application of larvicides and adulticides especially during the outbreak of VBDs. The two main vector control interventions are long-lasting insecticidal nets and indoor residual spraying. Chemical larvicidal and environmental management are also important control parameters. However, the
30 spread of insecticide resistance is becoming a threat to control interventions.

Scientists rear mosquitoes in laboratory for different research purposes. Mosquitoes can be reared in an insectary with controlled conditions that mimic natural conditions. It may be a separate building, a room or section of the room, usually modified or remodeled to suit the condition required for rearing. Mosquito
5 insectaries vary widely in their sophistication and cost. The goal of organized mosquito rearing is to provide reliable, affordable sources of high-quality mosquitoes for their many important purposes.

The microbiota of mosquito, that are present on the surface area, in the gut and other
10 possible areas play several important roles in mosquito development and fitness, providing a promising avenue for novel mosquito control strategies. It is therefore desirable to develop a device that can be used for the rearing of germfree and gnotobiotic mosquitoes to understand the biology of vector mosquitoes especially their relationship with microorganisms. This process for rearing germfree and
15 gnotobiotic mosquitoes involves the complete development of mosquitoes i.e., from egg stage to adult stage while maintaining a completely sterile condition. It is also desirable that a device which is better suited to wider climatic environment /conditions since there are different species of mosquitoes which are active during the day, evening or nighttime. Further, the device should endeavor to control the
20 disease transmitting mosquitoes to prevent and control of malaria, dengue, chikungunya, and other mosquito-borne diseases.

U.S. Pat. No. 3,727,580 discloses an apparatus for rearing silkworms on horizontal trays which are conveyed through the apparatus during the rearing process in which
25 each of the rearing units being composed of a feeding tray and a net superposed on the feeding tray. U.S. Pat. No. 2,539,633 discloses a device for breeding insects in which a vertical breeding tray is suspended by hooks and eyes from the top of the device. The breeding tray is constructed of wood or metal, has wire screening sides and contains grain. U.S. Pat. No. 3,750,625 discloses a plastic insect breeding tray
30 with multiple cup-like compartments and with egg receiving recesses connected to the cups by crawl passages. In the closest prior art, the Ethiopian Public Health

Institute issued a guideline titled “Anopheles mosquito rearing and insectary handling guideline” which provides users with detailed standard operating procedures of Anopheles mosquito rearing and introduces a way of designing and handling of insectary. But in their standard operational procedures, there was no provision for maintaining sterility in the cage and those are not meant for growing germfree and gnotobiotic mosquitoes.

The present invention overcomes one or more limitations stated above or any other limitations associated with the prior art by providing a self-contained unit for the mosquito rearing and incubation of a germfree and gnotobiotic mosquitoes in a complete microbe’s free sterile environment. The present invention further relates to device and method for the development of mosquitoes introduced known species of microbes to understand the role of each species of microbes in the rearing of mosquitoes. It is to be noted that complete sterilization of the whole device is not known in the art however in the presently disclosed invention, the whole unit can be kept inside the autoclave and total sterilization can be achieved.

OBJECTIVE OF THE PRESENT INVENTION:

The primary objective of the present invention is to provide a device for developing germfree and gnotobiotic insects in a controlled environment.

In a further objective, the present invention provides a methodology for rearing germfree and gnotobiotic mosquitoes.

SUMMARY OF THE INVENTION:

The present disclosure overcomes one or more shortcomings of the prior art and provides additional advantages discussed throughout the present disclosure. Additional features and advantages are realized through the techniques of the present disclosure. Other embodiments and aspects of the disclosure are described in detail herein and are considered a part of the claimed disclosure.

In one non-limiting embodiment of the present disclosure, a device for insect rearing germfree and gnotobiotic insect is disclosed. The device comprises a support structure having an interior space, a non-porous cover configured to cover the support structure and a closure device configured to close an opening of the non-porous cover. Further, the support structure is provided with a neck like provision for fixing a porous element configured to close the opening of said support structure and allowing sterilized air to passively pass through it.

In another non-limiting embodiment of the present disclosure, the support structure is cuboid in shape provided with a wireframe structure and is made of stainless steel.

In still another non-limiting embodiment of the present disclosure, the non-porous cover is an autoclavable polyethylene cover to firmly cover the outer surface of the support structure firmly and to maintain the sterility i.e. germ-free environment inside the device defined by it.

In yet another non-limiting embodiment of the present disclosure, the non-porous cover is reusable.

In another non-limiting embodiment of the present disclosure, the non-porous cover is transparent and is also used to monitor the activities of the mosquitoes or any other insect inside the device.

In still another non-limiting embodiment of the present disclosure, said support structure has a rigid plate configured at the bottom.

In yet another non-limiting embodiment of the present disclosure, the rigid plate is made of stainless steel.

In yet another non-limiting embodiment of the present disclosure, the porous element is a cotton plug. Further, the porous element is covered with a meshed cloth to close the opening of the neck region.

In another non-limiting embodiment of the present disclosure, the porous element is configured to prevent an inflow of contaminated air inside the device to maintain sterility.

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Further, a method of rearing a germfree and gnotobiotic insect is disclosed. The method includes the device for rearing and incubation of germfree and gnotobiotic insects. The disclosed device is sterilized such that the device is subjected to a high temperature and pressure steam sterilization. The device comprises a support
10 structure having an interior space, a non-porous cover configured to cover the support structure and a closure device configured to close an opening of the non-porous cover. Further, the support structure is provided with a neck like provision for fixing the porous element configured to close the opening of the said support structure and allowing sterilized air to pass through it.

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The method of rearing the germfree and gnotobiotic insect further includes an aseptic larva-rearing reservoir configured for placing surface-sterilized eggs into it for pupation in a sterile condition. Alternatively, the mosquito can be grown till its pupal stage in a sterile conical flask, covered by sterile cotton plug by providing sterile
20 larval food. There are two petri dish are provided inside the support structure, wherein the first petri dish is configured to keep a pupa into it and the second petri dish is configured to keep a sugar-soaked cotton ball into it.

The method of rearing the germfree and gnotobiotic insect further includes transferring the aseptically grown pupae to the first petri dish. Further, sterile sugar-
25 soaked cotton balls are provided in the second petri dish. The pupae are allowed to develop into adult insects and feeding sterile sugar solution to the adult insects. In the end, sterile de-fibrinated blood-soaked cotton balls are provided to the adult mosquitoes. Only female either before or after mating, go for blood-feeding. After blood-feeding, the gravid female mosquitoes will take specific period for the
30 development of eggs. The mating behavior and the interactions of germ free and gnotobiotic mosquitoes can be observed directly through the transparent cover.

Lastly, the developed adult germfree/ gnotobiotic mosquitoes are collected for microscopic observation and further studies.

5 The foregoing summary is illustrative only and is not intended to be in any way limiting. In addition to the illustrative aspects, embodiments, and features described above, further aspects, embodiments, and features will become apparent by reference to the drawings and the following detailed description.

BRIEF DESCRIPTION OF DRAWINGS

10 The novel features and characteristics of the disclosure are set forth in the appended claims. The disclosure itself, however, as well as a mode of use, further objectives, and advantages thereof, will best be understood by reference to the following detailed description of an embodiment when read in conjunction with the accompanying drawings. One or more embodiments are now described, by way of
15 example only, with reference to the accompanying drawings wherein like reference numerals represent like elements and in which:

Figure 1 shows a schematic view which illustrates the structural details of a device for growing germ-free and gnotobiotic mosquitoes.

20 **Figure 2** shows a flowchart of the process of growing germfree and gnotobiotic mosquitoes in the present disclosure.

Figure 3 shows the time required for each stage mosquitoes grown under normal vs. sterile conditions.

Figure 4a shows the percentage of egg hatching under normal vs. sterile conditions.

25 **Figure 4b** shows the percentage of pupa emergence under normal vs. sterile conditions.

Figure 4c shows the percentage of pupae to adult conversion under normal vs. sterile conditions.

Figure 5 shows the bacterial growth of larvae and adults grown both under germfree conditions using the disclosed device and normal conditions.

Figure 6 shows the larvae grown in normal conditions and in germ-free conditions.

5 DETAILED DESCRIPTION OF INVENTION

It is to be understood that the figures and descriptions of the present invention have been simplified to illustrate elements that are relevant for a clear understanding of the invention. The detailed description will be provided herein below with reference to the attached drawings.

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The invention relates to a device (10) which is simple and cost-effective and designed for rearing germfree and gnotobiotic insects. The device (10) comprises a support structure (11) having an interior space (12), a non-porous cover (13) configured to cover the support structure. A closure device (14) configured to close
15 an opening (15) of the non-porous cover (13). The support structure (11) is provided with a neck like provision (16) for fixing a porous element (17) which is configured to close the opening of the said support structure (11). The porous element (17) further allows a sterilized air to pass through it. The rearing of insects takes place in the controlled sterilized environment. Therefore, insects are protected from external
20 environmental microbes and are raised in clean optimal conditions.

Referring now to the drawings, there is shown an illustrative embodiment of the device designed for developing germfree and gnotobiotic insects. It should be understood that the invention is susceptible to various modifications and alternative
25 forms; specific embodiment thereof has been shown by way of example in the drawing and will be described in detail below. It will be appreciated as the description proceeds that the invention may be realized in different embodiments.

Before describing in detail embodiments, it may be observed that the novelty and inventive step that are in accordance with the present invention reside in the construction of the device designed for developing germfree and gnotobiotic insects, accordingly, the drawings are showing only those specific details that are pertinent
5 to understanding the embodiments of the present invention so as not to obscure the disclosure with details that will be readily apparent to those of ordinary skill in the art having benefit of the description herein.

The terms “comprises”, “comprising”, or any other variations thereof, are intended
10 to cover a non-exclusive inclusion, such that a setup, device that comprises a list of components does not include only those components but may include other components not expressly listed or inherent to such setup or device. In other words, one or more elements in a system or apparatus preceded by “comprises... a” does not, without more constraints, preclude the existence of other elements or additional
15 elements in the system or apparatus.

Growing animals under the germfree (microbe-free) condition is an invaluable technique to analyze the impact of normal microbiota associated with. It is not possible to determine the role of each and every microbial species an animal harbors
20 without growing it under germ-free conditions. After providing one or more species of known microbes, the animal called gnotobiotic (gnoto=known; biotic=organism) that will show the impact of the microorganisms supplied. Gnotobiotic animals include animals of only known biological species such as axenic animals bred and reared so as to be free of contamination by other biological species, particularly
25 microorganisms. Such animals, particularly small animals, are bred and reared under carefully controlled sterile conditions in isolators and constitute valuable produce useful in medical and biological research.

The following paragraphs describe the present disclosure with reference to FIGs. 1 to 6. In the figures, the same element or elements which have similar functions are
30 indicated by the same reference signs.

FIG. 1 illustrates the assembly of the device (10) designed for developing germfree and gnotobiotic insects. The device (10) comprises of the support structure (11) having the interior space (12) defined by a wireframe structure (18). The non-porous cover (13) is configured to cover the support structure. The closure device (14) is configured to close the opening (15) of the non-porous cover (13) wherein, said support structure (11) is provided with the neck like provision (16) for fixing the porous element (17). The porous element (17) is configured to close the opening of the said support structure (11) and allowing sterilized air to pass through it. The support structure (11) is cuboidal in shape and is made up of stainless steel with the neck like provision (16) provided for fixing the porous element (17) as shown in FIG. 1. In one of the embodiments of the invention, the porous element (17) is a cotton plug. The reason to select cuboidal shape is that it mimics the normal insect breeding cages where insects are grown successfully in colonies. Therefore, insects easily adapt to the cuboidal or rectangular cages.

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An autoclave is used to sterilize the support structure (11) at elevated temperature and pressure. Therefore, stainless steel is used as a material for the support structure, such that it can withstand inside the autoclave. A rigid plate (21) made of stainless steel is configured at the bottom of the support structure (11) to keep an insect pupa for emergence in a first petri dish and to keep the sugar-soaked cotton balls for feeding the adult insect in a second petri dish. An entrance near the neck section in the support structure (11) is provided slightly above the center of the support structure (11) for easy handling of pipettes or micropipettes.

The non-porous cover (13) is provided which is configured to cover firmly an outer surface (19) of the support structure (11). The non-porous cover (13) further maintains the sterility i.e. microbe-free environment inside a cavity (20) defined by it. In one of the embodiments of the invention, the non-porous cover (13) is an autoclavable polyethylene cover. The non-porous cover (13) is transparent and can be used to clearly monitor the activities of the insects. The non-porous cover (13) is reusable and can be used for more than 10 times without any damage or breach in

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sterility. Since the non-porous cover (13) is autoclavable, the device (10) after keeping inside the autoclave, can be sterilized as normally done in microbiological culture procedures.

- 5 The closure device (14) is configured to close the opening (15) of the non-porous cover (13) tightly. In one of the embodiments of the present invention the closure device (14) is a ring with screw adjustment. The porous element (17) covered with a meshed cloth (22) is configured to close the opening of the support structure (11) near the neck region. In one of the embodiments of the present invention, the porous
- 10 element (17) is a cotton plug which is covered with a gauze cloth. The porous element (17) is configured to prevent the inflow of contaminated air inside the device (10) to maintain sterility and acts as a barrier between the cavity (20) of the support structure (11) covered with the non-porous cover (13) and outside air.
- 15 The air passes through the porous element (17) is sterile since the air suspended microbes get filtered in the porous element (17). As per the embodiment of the present invention, the device (10) is used to maintain sterility inside it and also to provide a facility for the passive passage air (with oxygen) through the porous element (17) at the same time. Further, the observer can monitor the activity of the
- 20 insect or any other insect inside the device (10) through the transparent non-porous cover (13).

The following examples serve to illustrate certain embodiments and aspects of the present disclosure and are not to be considered as limiting the scope thereof.

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Example:

- The device (10) along with the process for developing germfree and gnotobiotic insect is described below in detail after considering an example for the present embodiment in which the rearing is done to develop germfree and gnotobiotic
- 30 mosquitoes.

The support structure (11) with the dimension 18x16x15.5 cm, which is configured to hold up to 50 mosquitoes are considered for developing germfree and gnotobiotic mosquitoes. The dimensions of the support structure (11) can be increased further as per the requirement as well as after considering the size of the autoclave available in the lab. Firstly, the device (10) is prepared to keep the germfree mosquitoes by keeping the first petri dish inside the interior space (12) of the device (10). After that, the device (10) is covered with the non-porous cover (13) and the opening (15) of the non-porous cover (13) is clamped tightly using the closure device (14) through the neck like provision (16) configured in the device (10). The step further comprises, inserting the porous element (17) along with the meshed cloth (22) to close the opening of said device (10). The disclosed device (10) is then sterilized for 20 minutes at 121°C inside the autoclave. After sterilization, the device (10) is brought and kept inside a laminar air-flow chamber, under ultraviolet light for 30 minutes. Now the device (10) is ready to keep germfree (sterile) pupae of mosquitoes inside it.

Germfree mosquitoes are developed up to the pupal stage by surface sterilization of mosquito eggs using mercuric chloride and washing them with sterile distilled water in the laminar airflow chamber (biosafety cabinet). The surface sterilized and washed eggs are placed in the sterile tap/sterile distilled water, which is kept in cotton plugged aseptic larva rearing reservoir in a laminar airflow chamber. The cotton plug is used to allow microbes' free oxygen to enter the larva rearing reservoir and CO₂ to move out through it. After this step, the sterilized feed is provided to the growing larvae in a laminar airflow chamber. The aseptic larva rearing reservoir with larva is kept at room temperature for incubation till the conversion of a larva into pupae. Lastly, the pupae grown in sterile (germfree) condition are transferred inside the disclosed device (10).

The pupae transferred inside the disclosed device (10) are developed as Germfree adult mosquitoes after following these steps: A sterile cotton ball soaked in sterile sucrose solution is kept inside the device (10) by opening the porous element (17),

before the Bunsen burner flame, inside the laminar airflow chamber. Cotton balls soaked in defibrinated, microbe-free goat or sheep blood is kept inside the device (10) by opening the porous element (17), before the Bunsen burner flame, inside the laminar airflow chamber. The adult mosquitoes grown in the sterile condition are collected for microscopic observation and other analyses in the same way in the laminar airflow chamber.

Finally, the gnotobiotic mosquitoes are developed after following these steps: Pure culture of selected bacterial species isolated from the normal mosquito are mixed in the sucrose solution and allowed to feed by germfree adult mosquitoes, otherwise in other embodiment mixed in larval feed to feed larvae. The effect that occurred in the larval morphology, behavior and survival ability is analyzed. The larvae are further allowed to pupate in a Petri dish or beaker in the disclosed device (10). The emergence and adult behavior, its morphological characters are studied. FIG. 2 shows a complete flowchart of the process of growing germfree and gnotobiotic mosquitoes explained in this example and used for the present disclosure.

Results

The equipment had been tested for its use in generating germ-free and gnotobiotic mosquitoes. The *Aedes aegypti* mosquitoes were grown in the germfree device (10) were compared with the control group which was grown under normal condition. The morphometric analysis of both groups was done to see the difference in growing the mosquito under normal vs. germfree conditions. The larvae grown in aseptic conditions showed slow growth, low egg hatching, low pupae emergence from larvae and poor conversion of pupae to adults. FIG. 3 and FIG. 4 shows the developmental patterns of larvae grown in the normal and sterile condition in the germfree device (10). Sterile larvae took longer time for the conversion of each stage and their Morphometric outcome is also poor.

FIG. 5 shows the bacterial growth in nutrient agar plates inoculated with larvae and adults grown both under germfree conditions using the disclosed device and normal

conditions wherein FIG. 5A and FIG. 5B show adult mosquitoes, FIG. 5C and FIG. 5D shows adult mosquitoes' body teased using a sterile needle, FIG. 5E and FIG. 5F show Larvae of the mosquitoes in observation, and FIG. 5G and FIG. 5H show mid-gut in observation. It is to be noted that FIGs. 5A, 5C, 5E, and 5G corresponds to a mosquito grown in germfree condition whereas FIGs. 5B, 5D, 5F, and 5H corresponds to a mosquito grown in normal condition. Figures 6A and 6B show the larvae grown in normal conditions and in germ-free conditions. It is to be noted that the difference in the morphology and size of the growth is clearly visible. These results provide evidence that the device and the method selected for developing the mosquito, described by the present disclosure is capable of producing a set of germfree and gnotobiotic mosquito.

The disclosure has been described herein, with reference to certain embodiments, in order to enable the reader to practice the disclosure without undue experimentation. However, a person having ordinary skill in the art will readily recognize that many of the components and parameters may be varied or modified to a certain extent or substituted for known equivalents without departing from the scope of the disclosure. It should be appreciated that such modifications and equivalents are herein incorporated as if individually set forth. In addition, titles, headings, or the like are provided to enhance the reader's comprehension of this document and should not be read as limiting the scope of the present disclosure.

ADVANTAGES OF INVENTION

Some of the advantages of the present invention are as under:

- The presently disclosed invention is used to produce germ-free (free from any microbes) insects.
- The device can be kept in a normal lab or room condition for growing germfree or gnotobiotic insects while maintaining the sterility inside the device.

- The presently disclosed invention is to maintain sterility inside the device. It also provides a facility for the passive passage of air (with oxygen) through the porous element. One can also monitor the activity of the mosquito or any other insect inside the device through the transparent non-porous cover.
- 5 • The presently disclosed invention does not require electricity or any other power source to operate.
- Apart from mosquitoes, the presently disclosed invention can also be used for other insects such as beetles, butterflies, bees, etc. with an increase of size according to the size of the insects.
- 10 • The presently disclosed invention is cost-effective since the non-porous cover can be used several times after ensuring that the non-porous cover is airtight.
- The presently disclosed invention does not use any expensive filters for filtering the contaminating microbes from the air to maintain the sterility inside the device. Instead, the presently disclosed invention uses the porous element (17) which is nothing, but a cotton plug covered by cotton gauze.
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LIST OF REFERENCE NUMERALS

Description	Reference Number
Device	10
Support structure	11
Interior space	12
Non-porous cover	13
Closure device	14
Opening	15
Neck like provision	16
Porous element	17

Wireframe structure	18
Outer surface	19
Cavity	20
Rigid plate	21
Meshed cloth	22

The Claims:

1. A device (10) for rearing and incubation of a germfree and gnotobiotic insects, wherein said device (10) comprises:
5 a support structure (11) having an interior space (12);
a non-porous cover (13) configured to cover the support structure;
a closure device (14) configured to close an opening (15) of the non-porous cover (13);
wherein said support structure (11) is provided with a neck like provision
10 (16) for fixing a porous element (17) configured to close the opening of the said support structure (11) and allowing sterilized air to pass through it.
2. The device (10) as claimed in claim 1 wherein the support structure (11) is
15 cuboid in shape provided with a wireframe structure (18) and is made of stainless steel.
3. The device (10) as claimed in claim 1, wherein the non-porous cover (13) is
an autoclavable polyethylene cover to firmly cover an outer surface (19) of
the support structure (11) and to maintain the sterility i.e. germ-free
20 environment inside the device (10) defined by it.
4. The device (10) as claimed in claim 1, wherein the non-porous cover (13) is
reusable.
- 25 5. The device (10) as claimed in claim 1, wherein said non-porous cover (13) is transparent and is also used to monitor the activities of the mosquitoes or any other insect inside the device (10).
- 30 6. The device (10) as claimed in claim 1 wherein said support structure has a rigid plate (21) configured at the bottom.

7. The device (10) as claimed in claim 1 wherein the rigid plate (21) is made of stainless steel.
- 5 8. The device (10) as claimed in claim 1, wherein the porous element (17) is a cotton plug, said porous element (17) is covered with a meshed cloth (22) to close the opening (15) of the neck region.
- 10 9. The device (10) as claimed in claim 1, wherein the porous element (17) is configured to prevent an inflow of contaminated air inside the device (10) to maintain sterility.
10. A method of rearing a germfree and gnotobiotic insect, the method comprising the steps of:
- 15 providing a device (10) for rearing and incubation of germfree and gnotobiotic insect;
- sterilizing said device (10) such that the device (10) is subjected to a high temperature and pressure steam sterilization, the device (10) comprising:
- 20 a support structure (11) having an interior space (12);
- a non-porous cover (13) configured to cover the support structure;
- a closure device (14) configured to close the opening (15) of the non-porous cover (13);
- wherein said support structure (11) is provided with a neck like provision (16) for fixing a porous element (17) configured to close the opening of the said support structure (11) and allowing sterilized air to pass through it.
- 25 11. A method of rearing germfree and gnotobiotic insect as claimed in claim 10 further comprising the steps of:

providing an aseptic larva-rearing reservoir;

placing surface-sterilized eggs into the said aseptic larva-rearing reservoir for pupation in a sterile condition;

5 providing a first petri dish and a second petri dish inside the support structure, wherein the first petri dish is configured to keep a pupa into it and the second petri dish is configured to keep a sugar-soaked cotton ball into it.

12. A method of rearing germfree and gnotobiotic insect as claimed in claim 10 and claim 11 further comprising the steps of:

10 providing aseptically grown pupae to said first petri dish;
providing a sterile sugar-soaked cotton ball in a said second petri dish;
allowing said pupae to develop into adult insects and feeding sterile sugar solution to the adult insects;
15 providing a sterile defibrinated blood-soaked cotton ball to the adult insects; and
collecting the reproduced adult gnotobiotic insects for microscopic observation.