FORM 2

THE PATENT ACT 1970

(39 of 1970)

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The Patents Rules, 2003

COMPLETE SPECIFICATION

(See section 10 and rule 13)

1. TITLE OF THE INVENTION:

"A NOVEL TECHNIQUE FOR MASS PRODUCTION AND FORMULATION OF ENTOMOPATHOGENIC NEMATODES"

2. APPLICANT(S):

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3. PREAMBLE TO THE DESCRIPTION

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

4. DESCRIPTION:

TECHNICAL FIELD

[001] The present disclosure generally relates to the field of biocontrol agents. More particularly, the present disclosure relates to the field of mass culturing of entomopathogenic nematodes for use as a biocontrol agent against insect pests.

BACKGROUND

[002] Biological control is a potential, non-chemical and ecofriendly approach for managing insect pests. Biocontrol agents are being used worldwide for suitable management of various foliar and soil-borne insects. These are acclaimed as effective, ecofriendly and cheap, nullifying the ill effects of chemicals. Further, biological control strategy is highly compatible with sustainable agriculture and has a major role to play as a component of integrated pest management (IPM) programme. Large scale production of biocontrol agents along with good shelf life and establishment of biocontrol agents in targeted niche, determine the success of biological control.

[003] Entomopathogenic nematodes (EPN) can play a significant role as biocontrol agents in management of soil inhabiting insects. Entomopathogenic nematodes of families Steinernematidae and Heterorhabditidae are unique in their action and potential. They are considered to be the most suitable entomopathogens for managing a wide variety of insect pests particularly soil inhabiting ones. As the only biocontrol agent available for many soil insects, entomopathogenic nematodes should be poised for wider use. However, mass rearing techniques of EPN are expensive and currently not feasible at farm level. Therefore cost effective large scale production at farm level and good shelf life of the formulation are the primary concerns.

[004] Entomopathogenic nematodes are currently mass produced by both in-vitro and in-vivo methods. In-vivo mass production of EPN is one of the most appropriate

technologies for grower cooperatives (Gaugler and Han, 2002). Currently, mass production system is based on the White trap method (White, 1929), which is a simple process of culturing specific EPN on live insect hosts at laboratory level. However, mass production of EPN in large scale becomes expensive in view of the huge requirement of glasswares and labour. Moreover, in-vivo mass production of EPN in White trap method requires skilled personnel to monitor EPN from escaping from the petriplates and to prevent the insect larvae from escaping infection. Periodic inspection is required to replace the escaping larvae on to the bottom of the petriplate to ensure infection by EPN. Also, by the labour intensive White trap method, only 8 to 10 larvae can be infected in each petriplate and to produce 0.5 to 1 billion infective juveniles (IJ) of EPN that are required for biocontrol of one acre, large number of petriplates and space are required. In the White trap technique, if the infective IJs are not harvested from 6th day after inoculation of IJs, crowding effect ensues leading to high mortality rates of IJs.

[005] In the light of aforementioned discussion, there exists a need for development of an in vivo method for cost effective, large scale production of EPN that can be used at the farm level as well as at the commercial level to serve as biocontrol agents, particularly in the context of huge costs involved in in vitro mass production technology. The new technique disclosed in the present invention is low cost, and can be easily adopted both by farmers and commercial entities for mass production of EPNs.

BRIEF DESCRIPTION OF THE DRAWINGS

[006] Referring to FIG.1A, it represents a schematic illustration of a low cost container with insect larvae that are inoculated with the infective juveniles of EPN and is a non limiting exemplary embodiment of the present disclosure.

[007] Referring to FIG. 1B, it represents a schematic illustration of a polythene bag 105 containing the biocontrol formulation against insects and is a non limiting exemplary embodiment of the present disclosure.

BRIEF SUMMARY

[008] The following presents a simplified summary of the disclosure in order to provide a basic understanding to the reader. This summary is not an extensive overview of the disclosure and it does not identify key/critical elements of the invention or delineate the scope of the invention. Its sole purpose is to present some concepts disclosed herein in a simplified form as a prelude to the more detailed description that is presented later.

[009] Exemplary embodiments of the present disclosure are directed towards a cost effective method for mass production of entomopathogenic nematodes comprising: inoculating insect larvae with a first sodium alginate gel suspension comprising IJ of EPN; placing a sponge sheet over the inoculated insect larvae; applying a second sodium alginate gel suspension comprising IJ of EPN over the sponge sheet; incubating the insect larvae at a temperature of about 25 to 27 degrees C for about 10 days, thereby enabling the emerging infective juveniles from the insect larvae to migrate into the sponge; and harvesting the IJ of EPN, wherein the sponge sheet with the migrated IJ is removed and sealed in a plastic bag to be used as a biocontrol formulation.

[010] Another exemplary embodiment of the present subject matter is directed towards a biocontrol formulation for managing insect pests, comprising: a plurality of infective juveniles of entomopathogenic nematodes embedded in a sponge sheet of a predetermined thickness moistened with a sodium alginate gel suspension, preferably 1% sodium alginate gel suspension.

[011] Exemplary object of the present invention to develop a cost effective in vivo method for mass production of EPN that can be used as effective biocontrol agents.

[012] It is an object of the present invention to develop an integrated, unified method for mass production of EPN and preparation of the EPN formulation. The sponge sheet used in the production of EPNs is directly used as carrier material for the EPN formulation and can be directly supplied for field use.

[013] It is an object of the present invention to get a higher yield of about 25-50% of infective juveniles of EPN when compared to the White trap method.

[014] Yet another exemplary object of the present invention to develop a method for mass production of EPN that is simpler, easily scalable and can be easily adopted at both farm level and commercial level.

[015] It is another exemplary object of the present invention to develop a cost effective method for mass production of EPN that is not labour intensive, requires less capital investment and does not have a huge requirement of glassware or space. The present invention does not require skilled personnel or periodic inspection and can be easily adopted by farmers at farm level.

[016] It is another object of the present invention to develop a cost effective method for mass production of EPN, wherein the crowding effect and consequent mortality of IJs as seen in the White trap method is overcome by ensuring required moisture level for the IJs. The harvesting of all the EPNs can be done in a single day that is on the 10th day after inoculation of IJs.

[017] It is another object of the present invention to develop a biocontrol formulation comprising infective juveniles of EPN with a good shelf life.

DETAILED DESCRIPTION

[018] It is to be understood that the present disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The present disclosure is capable of other embodiments and of being practiced or of being carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of “including”, “comprising” or “having” and variations thereof herein is meant to encompass the items listed thereafter and equivalents thereof as well as additional items. The terms “a” and “an” herein do not denote a limitation of quantity, but rather denote the presence of at least one of the referenced item. Further, the use of terms “first”, “second”, and “third”, and the like, herein do not denote any order, quantity, or importance, but rather are used to distinguish one element from another.

[019] According to a non limiting exemplary embodiment of the present disclosure, a cost effective, in vivo method for mass production of EPNs viz. Steinernema Carpocapsae, Steinernema glasari or Heterorabhditis indica is disclosed. Mass production of other EPNs can also be carried out by this method without limiting the scope of the present disclosure.

[020] Entomopathogenic nematodes disclosed herein were isolated from rhizospheric soil samples collected from plots cultivated with vegetable crops in Telangana, India. The insect hosts were obtained from pressed honey combs collected from National Institute of Rural Development and Panchayati Raj (NIRD), Rajender nagar, Hyderabad, Telangana, India.

[021] Mass production of EPN by adopting the method disclosed herein makes the process simpler and cost effective and lends itself to production at both farm level and commercial level. The method given in the present disclosure is an excellent

alternative to the existing White trap method which is costly, requires skilled manpower, periodic inspections, more space and more labour for mass multiplication.

[022] Referring to FIG.1A, it is a schematic illustration of a low cost container with insect larvae that are inoculated with the infective juveniles of EPN, wherein a low cost container 103 having insect larvae inoculated with IJs of EPN 101 is shown. A sponge sheet 102 is spread over the insect larvae inoculated with IJs of EPN 101 and a muslin cloth 104 is covered over the low cost container 103 and kept at 25-27 0C temperature for about 10 days.

[023] Referring to FIG. 1B, it is a schematic illustration of a polythene bag 105 containing the biocontrol formulation against insects, wherein the formulation comprising the IJs of EPN is embedded in a sponge sheet.

[024] According to a non-limiting exemplary embodiment of the present disclosure, Insect larvae were inoculated with infective juveniles (IJ) of EPN in a suitable low cost container 103. The larvae of Corcyra cephalonica, Galleria melonella, Tribolium molitor, Tribolium castinium or any other insect can be inoculated with IJs of EPN. In each low cost container 103, a plurality of insect larvae was infected by applying a suspension of infective juveniles of EPN, made in a gel solution such as a sodium alginate solution or a silica gel solution. After inoculation, a sponge sheet 102 of a predetermined thickness was spread over the insect larvae carefully, preventing escape of the insect larvae, maintaining sufficient moisture level required for the emergence of IJs and preventing crowding effect as seen in the White trap method. In accordance with a non-limiting exemplary embodiment of the present subject matter, some more suspension of IJs of EPN in a gel solution was applied over the sponge sheet 102 that is spread over the inoculated larvae. In a particular embodiment of the invention, a suspension of IJs of EPN in sodium alginate solution was applied over the sponge sheet 102 though any gel solution that would provide sufficient moisture level to sustain the IJs can be used without limiting the scope of the present

disclosure. The low cost container 103 is then covered with a muslin cloth 104 though any cover that would prevent contamination from dust or microbes can be used without limiting the scope of the present disclosure. Though the IJs begin to emerge early, the low cost containers 103 were maintained without disturbance for about ten days to ensure that sufficient numbers of infective juveniles were harvestable. The IJs usually begin to emerge from the cadaver of the insect larvae on 3th to 5th day after inoculation and by about 10th day, maximum number of IJs will emerge and migrate into the sponge sheet 102, where sufficient moisture level is maintained because of the presence of the gel substance. After 10 days, harvesting of IJs was done. The above described method of mass production of entomopathogenic nematodes is hereinafter termed as the SS technique. It is to be understood that the SS technique is not limited in its application to the details of construction and the arrangement of components set forth in this paragraph. The SS technique is capable of other embodiments and of being practiced or of being carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting.

[025] In accordance with a non-limiting exemplary embodiment of the present disclosure, early kill of larvae was found when inoculated with a dosage of 100 IJs per larva. The emergence of IJs from the larva started within three days of inoculation whereas it takes six days in the case of White trap method. In this method, all the larvae get killed simultaneously preventing their escape from the tub. The larvae come in contact with the IJs everywhere because of the presence of the sponge sheet 102.

[026] In yet another non-limiting exemplary embodiment of the disclosure, about 500 larvae were inoculated with IJs of EPN in a low cost container 103. In the White trap method, each Petri plate of 2 inch radius measuring 12.5 sq. inches in surface area can accommodate only 10 larvae and 5 Petri plates can accommodate 50 larvae occupying a total surface area of 62.5 sq. inches. However, on the same surface area of the low

cost container, the SS technique can accommodate more number of larvae. According to an exemplary embodiment of the present disclosure, one plastic tub of six inch radius measuring about 113.1 sq. inches in surface area can accommodate 500 larvae, whereas in the White trap method 50 Petri plates will be required. The present method provides an exponential and efficient utilization of space when compared to the White trap method. The space utilization efficiency is 11 times more than the White trap method.

[027] According to a non-limiting exemplary embodiment of the present disclosure, in the method disclosed in the present disclosure, each infected larvae yielded up to 1.5 lakh IJs of EPN when compared to the White trap method which yields up to 1 lakh IJs of EPN per infected larva. The production efficiency of IJs is 25-30% more than the regular method.

[028] In accordance with a non-limiting exemplary embodiment of the present disclosure, a biocontrol formulation against insect pests, comprising entomopathogenic nematodes mass produced by the SS technique is disclosed. The disclosed formulation has a good shelf life of up to three months with retention of more than 75% survival of IJs.

[029] In accordance with a non-limiting exemplary embodiment of the present subject matter, the entomopathogenic nematodes mass produced by the SS technique, are directly used as a bio control formulation against insect pests. The IJs that emerge from the cadaver of the larvae migrate into the sponge sheet due to the availability of moisture. Thus, harvesting of IJs of EPN is simpler in the sense that there is no need to harvest the IJs from the tub separately and the sponge sheet 102 itself can act as a carrier material and can retain the infectivity of the IJs for upto three months. At about the 10th day after inoculation of the larvae with the EPN, the cover of the low-cost container 103 is removed and the entire sponge sheet 102 with the IJs of the EPN, is folded at least once after which all the contents in the low cost container 103

were transferred into a suitable bag 105 of appropriate size ensuring that all the EPN suspension remaining in the container was also poured into the bag 105 and sealed. This sealed bag 105 with the bio control formulation can be directly used for field foliar and soil application. The IJs of the EPN present in one sponge sheet 102 will be sufficient to spray in an area of one acre and remain infective for about three months in polyurethane sponge sheet 102.

[030] The White trap method of EPN mass production requires harvesting of EPN by skilled personnel and also requires carrier material for storage and transport. The current method shortens the time, reduces the investment and eases the procedure required for production of the EPN making it cost effective, easily adaptable and ecofriendly.

Example 1:

A method for mass production of EPN by SS and subsequent preparation of the biocontrol formulation:

[031] A round plastic tub of about 16 inches diameter was washed, dried thoroughly and then used for the inoculation of insect larvae with EPN. About 500 Corcyra larvae were placed in the tub. The larvae were inoculated with an EPN suspension prepared in about 30 ml of 2-3% sodium alginate solution at the rate of approximately 70 IJs per larva. A sodium alginate solution from about 2% to about 3% can be used to prepare the suspension. A round sponge sheet 102 of polyether polyurethane foam with a thickness of about 4 mm and a diameter of at least half inch more than that of the bottom surface of the tub was spread over the inoculated larvae to ensure that they do not migrate and escape from the inoculation. This sponge sheet 102 adhered to the side wall of the tub aided by sodium alginate gel (5-7.5% with 100 IJs). On top of the sponge sheet 102 which is spread to cover the larvae, about 25 to 30 ml of a 1% sodium alginate solution with about 30 IJs per larva was sprayed or poured. Sufficient care was taken to ensure that the IJ solution was poured in areas where the larvae

were located in the tub. This tub was then covered with a muslin cloth 104 to avoid contamination with dust or microbes. The inoculated culture was incubated for a maximum period of 10 days at 25 – 27 0C temperature.

[032] On 10th day after inoculation, the muslin cloth 104 covering the tub was removed and the entire sponge sheet 102 was folded twice along with the contents of the tub and placed into a polythene bag 105 and fastened with a zipper lock. Thus, harvesting of IJs of EPN is simpler and the contents of the tub can be directly packed to constitute the biocontrol formulation.

[033] Although the present disclosure has been described in terms of certain preferred embodiments and illustrations thereof, other embodiments and modifications to preferred embodiments may be possible that are within the principles and spirit of the invention. The above descriptions and figures are therefore to be regarded as illustrative and not restrictive.

[034] Thus the scope of the present disclosure is defined by the appended claims and includes both combinations and sub combinations of the various features described herein above as well as variations and modifications thereof, which would occur to persons skilled in the art upon reading the foregoing description.

REFERENCES

Gaugler, R., and R. Han. 2002. Production technology. Pp. 289–310 in R. Gaugler, ed. Entomopathogenic nematology. New York, NY: CABI

White, G.F. 1927. A method for obtaining infective nematode larvae from cultures. Science, 66: 302-303.

5. CLAIMS:

We claim:

1. A method for mass production of entomopathogenic nematodes comprising:

inoculating insect larvae with a first sodium alginate gel suspension comprising infective juveniles of entomopathogenic nematodes in a low cost container;

placing a sponge sheet over the inoculated insect larvae;

applying a second sodium alginate gel suspension comprising infective juveniles of entomopathogenic nematodes over the sponge sheet;

incubating the container at a temperature of about 25 to 27 degrees C for about 10 days thereby enabling infective juveniles of entomopathogenic nematodes emerging from the insect larvae to migrate into the sponge; and

harvesting the infective juveniles, wherein the sponge sheet with the infective juveniles is removed from the container and stored in a suitable bag.

2. The method as claimed in claim 1, wherein the first gel suspension has 2% to 3% sodium alginate.

3. The method as claimed in claim 1, wherein the second gel suspension has 1% sodium alginate.

4. The method as claimed in claim 1, wherein the concentration of the infective juveniles in the first gel suspension is about 100 IJs/ larva.

5. The method as claimed in claim 1, wherein the concentration of the infective juveniles in the second gel suspension is about 30 IJs/larva.

6. The method as claimed in claim 1, wherein the sponge sheet is a polyether polyurethene foam sheet.

7. The method as claimed in claim 1, wherein the yield is about 1.5 lakh IJs/infected larva.

8. The method as claimed in claim 1, wherein the insect larva is *Corcerya cephalonica, Galleria melonella, Tribolium molitor* or *Tribolium castinium.*

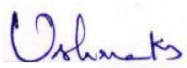
9. A biocontrol formulation for managing insect pests, comprising: infective juveniles of entomopathogenic nematodes embedded in a sponge sheet moistened with a sodium alginate gel suspension.

10. The formulation as claimed in claim 9, wherein the gel suspension has 1% sodium alginate.

11. The formulation as claimed in claim 9, wherein the sponge sheet is a polyether polyurethene foam sheet.

6. DATE AND SIGNATURE:

Dated this 24 Jan 2019,

Authorized Patent Agent's Signature: 

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PROMETHEUS PATENT SERVICES

7. ABSTRACT

A NOVEL TECHNIQUE FOR MASS PRODUCTION AND FORMULATION OF ENTOMOPATHOGENIC NEMATODES

Exemplary embodiments of the present disclosure are directed towards methods for in vivo mass culturing of EPN, comprising: inoculating insect larvae with a first sodium alginate gel suspension comprising IJ of EPN; placing a sponge sheet over the inoculated insect larvae; applying a second sodium alginate gel suspension comprising IJ of EPN over the sponge sheet; incubating the insect larvae at a temperature of about 25 to 27 degrees C for about 10 days, thereby enabling the emerging infective juveniles from the insect larvae to migrate into the sponge; and harvesting the IJ of EPN, wherein the sponge sheet with the migrated IJ is removed and sealed in a plastic bag to be used as a biocontrol formulation. This insecticidal formulation is easy to prepare at both farm level and commercial level and has a good shelf life of up to three months.

Fig. 1A