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Improved methodology of *in vivo* mass multiplication of entomopathogenic nematodes: A remarkable bio agent for soil arthropod pests management

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Abstract
In the process of *in vivo* multiplication of EPN, the Infective Juvenile (IJs) enters into the body of host insect; *Galleria mellonella* (wax moth) larvae through natural openings and cuticle. They release symbiotic bacteria *Photorhabdus luminescens* in insect body. The bacteria multiply in hemocoel, cause septicemia disease and kills insect within 48 hours, multiplies and complete 2-3 generations inside host insect body. With the proven effectiveness demonstrated in recent years, the demand of EPNs is increasing day by day. The production of EPNs has been mainly restricted up to laboratory level only due to the low recovery rats of IJs in the existing method. Recovery of EPN by the existing method is labor intensive, time consuming and inconvenient for mass multiplication of EPN at large scale. The standardizations of technology with equipment modifications have been optimized for multiplication of EPN at small scale entrepreneurs. The higher recoveries 241424 IJs/GC in it as compared to existing white trap methodology 197445 IJs/GC. This is the first scalable system in India for mass production entomopathogenic nematode; *In vivo* methodology based on LOTEK. In present study we describe an improved version of it in which bulk conditioning, harvesting, separations are automated to reduce labor costs.

Keywords: Entomopathogenic nematode, *in vivo*, Mass production, *Heterorhabditis indica*

Introduction
The entomopathogenic nematodes (EPNs) are the best alternative of chemicals against most soil insect pests. EPNs are mostly used against major insect pests in farming systems of economic importance, *viz.* fruit orchards, vegetable garden, nurseries and greenhouses crop protection. EPNs can be considered good candidates for commercialization as biological control agents as they can rapidly kill the insect host; have a wide pest host range; have active searching behavior; they can be mass produced; have potential for application in integrated pest management programs; and are considered safe for vertebrates and most non-target invertebrates. Ehlers in 2001, stated that the most important requirement for successful and economically reasonable usage of EPNs in crop protection is their production on large scale at competitive cost within a short time. The use of EPNs involved a step-by-step scientific and technical development mechanism. *In vivo* production is based on Dutky *et al.* (1964) [4] adaptation of the White trap (White, 1927) [13] in which EPN-killed larvae are placed above a water reservoir. This system is appropriate for laboratory bench scale production of inoculums for experiments, but its labor-intensive nature makes it inefficient for large-scale production. Bains in 1993 reported that scale-up is the process of taking a method from the bench up to a production level which is commercially useful. In the present study we describe an improved version of LOTEK (Gaugler and Brown, 2001) [6], the first scalable *in vivo* system for entomopathogenic nematode mass production.

Materials and Methods
In vivo methodology is a simple, economic and time saving process of EPN multiplication in live host. This methodology consists of host insect rearing, maintenance of EPN culture, inoculation of host insect larvae, conditioning of cadavers, harvesting & separation of IJs.

All the steps in this process completed at ambient (27+2 °C) temperature in FARMER laboratory Ghaziabad, Uttar Pradesh, India.

Host insect rearing

Rearing of host insect is very important and initial kick to start the process. The hosts used *in vivo* methods must be susceptible, have high multiplication potential, and reared easily using economical materials. The choice of host species and nematode for *in vivo* production should depend on nematode yield per cost of insect and the suitability of the nematode for the pest target (Costa *et al.* 2007) [3]. The most common insect host used for *in vivo* production is the last instar wax moth *Galleria melonella* (L.) (Lepidoptera: Pyralidae). Mass scale rearing of *G. mellonella* was done on modified artificial diet (Jagpal Singh *et al.* 2019) [9] to cater the need of fully grown healthy 5th instar larvae (3.5- 4.0 mg weight) for EPN multiplication.

Maintenance of EPN culture

EPN *H. indica* strains isolated from local soil cultured in laboratory and their viability is checked on 5th instar *G. mellonella* larvae according to Riazuddin *et al.* in 2020 [12].

Results

Inoculation of host insect larvae

The Pipette method (Dutky *et al.*, 1964) [4] is followed for inoculation. Fifth instar *Galleria* larvae are infected by using@50 IJs EPN *H. indica* /larva and kept in dark up to 48 hrs for obtaining satisfactory level of infection. Infected GC @500 was put in to the perforated stainless steel holding trays for conditioning. In large scale inoculation of host larvae; the perforated trays loaded with 5th instar larvae of wax moth, and dipped in another tray containing 1.0 cm deep 1 liter EPN suspension having suitable number of IJs for few seconds with gentle agitation, repeat dipping at least twice with 30 seconds interval for proper inoculation. A fresh suspension was prepared after a maximum of 10 dips. This process infects more than 95% of wax moth larvae with minimum labour inputs.

Conditioning of cadavers

The trays containing EPN infected GCs were transferred in to the conditioning apparatus for completing the life cycle of EPNs. Humidity plays an important role in proper multiplication of EPN. To maintain humidity inside Acrylic Chamber, the chamber is attached with the attachment fitted with one air pump, two plastic containers of one-liter capacity each; one for water storing and passing air through water and another to withhold extra water flows with wet air. Both containers and Acrylic chamber are inter-connected with 5 mm diameter thick plastic tube to pass on wet air into the chamber. A control panel with sensor is also attached to this chamber to monitor humidity. After keeping all the GC loaded 50 trays in the chamber and power switch is on maintain humidity up to 5-7 days. At 70-75% relative humidity maximum multiplication of IJs inside the *Galleria mellonella* cadaver was recorded.

Fig 1: Standardizations of technology for mass multiplication of entomopathogenic nematodes (EPN) *in vivo*

Harvesting of IJs

Once EPN completes their life cycle in insect body (GC); IJs starts emerging from GC body after 8-9 days of infecting. Moisture is essential for proper harvesting (recovery) of IJs. The GC loaded steel trays hanged on steel arms of equipment in vertical stacking. Digital Timer is set to release water to nozzles fitted in equipment for spraying water on GCs for fixed time duration and at a fixed time interval. Making provision of "Digital Timer in Harvester, to regulate quantity and flow of water into nozzles for spraying on (GC) placed in trays for fast emergence of IJs from GC and ensure maximum recovery of EPN (up to 95% and more). Making provision of Canopy in Harvester, covering trays loaded with GC to prevent any escape pilferage of EPN in water spraying on trays during harvesting. Making provision of "Light" in Harvester, in back side of Canopy to monitor proper functioning of nozzles. The Harvester equipment ensures higher percentage of recovery of EPN (95% or more).

Separation of IJs

The IJs aqueous suspension from Harvester Apparatus is poured into the IJs reservoir with the help of funnel. The EPN reservoir tank providing constant gravity flow through distribution manifold facilitates the flow into 17 several small water flow openings @ of 1059 ml / minute (184 ml concentrated IJS and 875 ml spent water) by each water flow opening. The water flow opening directed the IJs suspension on to a 600 mesh sieve oriented at 40 degree angle. This sieve separates the IJs from the water and dense slurry of IJs moves slowly by 2^0 angle drain pipe. IJs slurry is washed at least 3 times by the separator. After collecting of EPN in a water tank attached to Harvester, the IJs mixed water is transferred to Deflector for separation/filtration. The IJs mixed water is transferred to the water tank of Deflector and the water mixer is released to tray fitted with 600 meshes by pouring through manifolds steel tube. Water flows downward to the next tray attached below with outlet for collecting extra water out for collection in a tank and IJs remains suspended on lower lid of upper tray. These are collected with the help of soft brush.

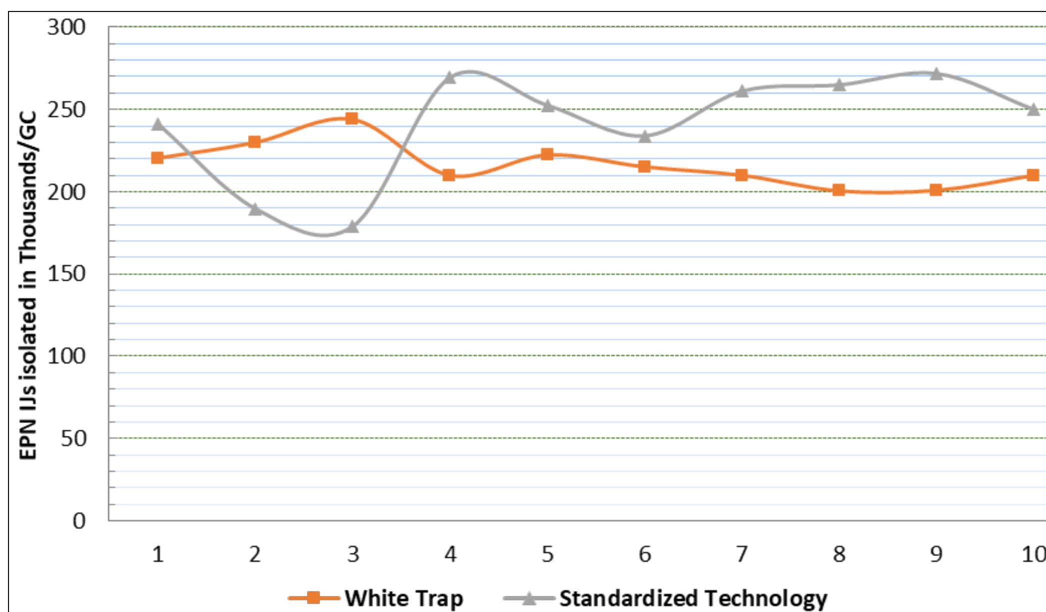

Fig 2: Number of IJs harvested from *Galleria* cadavers in White Trap methodology and in improved methodology

Discussion

Entomopathogenic nematodes multiplication methodology consists of host insect rearing, as number of IJs multiplication depends upon the body mass of host larvae; healthy culture with maximum body mass suitable for *in vivo* multiplication of EPN. *Galleria mellonella* rearing on standardized artificial diet (Jagpal Singh *et al.* 2019) [9] is suitable for healthier and heavier larvae production. The number of IJs per larvae used in inoculation also affects the numbers of IJs multiplication in it. According to Riazuddin *et al.* (2020) [12] 5 IJs is sufficient to kill the *Galleria* larvae but in maximum multiplication of IJs @50 IJs/Larva is recommended dose. In the present study humidity also optimized for proper conditioning of cadavers it is 70 -75%. The conditioning, harvesting and separation of IJs are time consuming process. In the present methodology by using modified LOTEK model more than 500 larvae can be infected once, conditioning of 25000 cadavers done at a time and harvesting of maximum IJs (241424), separation of IJs by using 600 meshes done easily with less efforts and time. Entomopathogenic nematodes have emerged as important biological control agents against soil-dwelling and plant-boring insects. Recent advances in the discovery of numerous isolates/strains and their mass production with the appeal of reducing pesticide usage, has resulted in a flow of scientific and commercial interest in these entomopathogenic nematodes. Recently, some cottage production has come into view that produces EPN *in vivo* local farmers. *In vivo* nematode production is based on the White trap method; the method involves the natural migration of IJs away from the infected host cadaver into a surrounding water layer, from where it can be harvested. This method was devised, reconstructed and later on modified by several workers (White1927, Dutky *et al.* 1964, Poinar 1979, Woodring and Kaya, 1988, Abdel Razek and Abd-elgawad 2007, Lindegren *et al.* 1993) [4, 13, 11, 14, 1, 10]. Gaugler *et al.* (2002) [7] developed LOTEK system consists of perforated trays to secure insects, harvesters with misting nozzles and a continuous deflection separator for washing and concentrating IJs. Generally, the conditioning of EPN infected GC is done by keeping GC in Petri Plates or in trays covered with lid in ambient conditions, whereas in this process the conditioning of GC is done in controlled conditions by regulating humidity with the mechanical infusion of wet air into the chamber.

Conclusion

Several factors as the weight of host insect larvae, no of IJs/lava at the time of inoculation, temperature, humidity plays major role in the multiplication of IJs of EPN, simultaneously more IJs recovery by less efforts with time saving technology is an important prerequisite towards their successful commercialization in present day scenarios. The substantial outcome of the new innovation is to provide an economically viable, easily adaptable, cost effective and more scientifically innovative solution for *in vivo* mass multiplication and production of EPN to the rural entrepreneurs for providing to farmers locally. This study embodies suitable innovative design of equipments and processes to sustainably maximum IJs harvest from GC. Each approach has its advantages and disadvantages relative to production cost, technical know-how required, economy of scale, and product quality (Grewal *et al.*2005) [8] and each approach can be improved further.

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