The Pharma Innovation Journal 2023; 12(8): 2136-2141

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(8): 2136-2141

www.thepharmajournal.com
Received: 12-06-2023
Accepted: 20-07-2023

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Unleashing the potential of endophytic *Beauveria bassiana* as a biocontrol agent against rice leaf folder (*Cnaphalocrocis medinalis*)

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Abstract
The presence and environmental condition is very important factor on agricultural production especially manage rice growth. The culture from the National Research Centre for banana, Tiruchirappalli was sub cultured and inoculated in the soil. The present experiment was conducted for isolation and inoculation of endophytic fungus, *Beauveria bassiana* in soil as well as in rice seedlings by differential application methods to ensure the availability of this fungus in the plant system. Experiment conducted for whether this fungus is in available in the plants system and also causing the impacts on the *Cnaphalocrocis medinalis* larva in rice. The source of leaf folder was collected from the field near by the Anbil Dharmalingam Agricultural College & Research Institute, Tiruchirappalli. The rice leaf folder making their incidents in all seasons of rice period in all over the world. The pathogenicity of *B. bassiana* tested against by using the 2 x 10^6 ratio of conidial suspension. The bio assay con ducted in pot culture revealed this fungus giving the mortality against the leaf folder in all the treatments. Even though the foliar spray, root dip method and seed treatment having the high mortality after the ten days inoculation in the plants as well as eco-friendly to the environment. Hence, endophytic *B. bassiana* present in the rice plants can be largely exploited for the controlling of rice leaf folder in eco-friendly manner.

Keywords: *Beauveria bassiana*, endophytes, rice seedlings, *Oryza sativa*, rice leaf folder, *Cnaphalocrocis medinalis*

1. Introduction
Rice is an edible starchy cereal grain that is produced by the grass plant (family Poaceae), *Oryza sativa*. In terms of production, rice was the second-most significant cereal crop after maize (FAO, 2013). The combined contribution of rice and wheat to human energy consumption is roughly 21% (Khush GS, 2005) [10]. Nearly all of East and Southeast Asia and around half of the world's population depend entirely on rice as a staple meal, and humans consume 95% of the world's rice production. The most significant food crop in India, rice accounts for about one-fourth of all farmed land and feeds almost half of the country's population. Over the past 45 years, Punjab has made significant advancements in the productivity and output of rice. The state of Punjab has earned the moniker "Rice Bowl of India" thanks to the usage of high yielding cultivars and modern technology. Due to its output of 519.3 million tonnes over an area of 650 million hectares, it is the main staple food crop in the world. Nearly 90% of global production is located in Asia. According to FAOSTAT, 2021, India produced 122 million metric tonnes of rice, which took up 40 to 43% of the country's land (FAOSTAT, 2021). Tamil Nadu is one of the main rice producing states in India, farming rice since ancient times due to the state's advantageous meteorological circumstances. For rice production to keep up with demand and global population growth by 2030, it must expand by at least 25% (Seck, Diagne, Mohanty, & Wopereis, 2012) [26]. Insects typically have higher populations, shorter generational cycles, fecundity, environmental adaptation, selection behaviour, and habitats that easily adjust to changing climates. The life stages of insects are affected by these environmental changes, and as a result, their diversity, abundance, and distribution quickly change. This turns out to be a key factor in the abrupt appearance of insect pests that are impacted by temperature changes since India is a tropical nation, faces several different climate challenges.
For more than 20% of global calorie consumption, rice (Oryza ap) is one of the most significant cereal crops for human and livestock use.

Despite its significance, a variety of pests and diseases restrict its output. Series pests have an impact on rice output both in the field and after harvest. Nearly 70 types of insect pests attacked Pakistan's rice crop, and stem borers are the most harmful and costly of these pests. The rice leaf roller, *Cnaphalocrocis medinalis* Guenee (Lepidoptera: Pyralidae), has developed the reputation of being a serious pest that may result in 30–40% leaf infection and 20–30% yield losses to the rice crop (Wakil W *et al.,* 2001; Pathak M D *et al.,* 1994; Prakash A *et al.,* 2008) [34, 20, 21] and the most recorded damage to rice farming in a single year was more than 30,000 hectares (Kushwaha *et al.,* 1984) [11]. The rice leaf folder (RLF), *C. medinalis* Guenée, is extensively spread in the rice-growing regions of humid tropical and temperate nations (Khan *et al.,* 1988) [9], and the RLF's developmental time shortens with an increase in temperature (Part *et al.,* 2014) [18]. RLF has emerged as one of the most significant insect pests of rice farming as a result of global warming (Bodlah *et al.,* 2017) [2]. The rice leaf folder (RLF) was once regarded as a significant insect pest. Because they defoliate and remove the chlorophyll content from the rice leaves. It harms rice plants from the time they are young until they are harvested, although it is most common during the crop's reproductive phase (Litsinger *et al.,* 2006) [13]. China, India, Japan, Korea, Malaysia, Sri Lanka, and Vietnam all saw frequent outbreaks of this pest (Khan *et al.,* 1988; Luo, 2010; Li *et al.,* 2012; Sun *et al.,* 2013) [9, 14, 30]. RLF is a longest distance travelling migratory pest (Ahn *et al.,* 2014) [18]. RLF was regarded as being of modest consequence, but as its population grew throughout the 1980s, it became a substantial and emergent pest in numerous regions of India. RLF was deemed to be of modest concern, but as its population grew in the 1980s, it became a big and developing pest in a number of Indian regions. *C. medinalis* was shown to be the most prevalent and dominating of the three species identified, which are *C. medinalis* (Guenee), *Marasmia patnalis* Bradley, and *Marasmia ruralis* (Walker). A significant rise in the use of fertilisers, the adoption of high yielding cultivars, and ongoing pesticide use caused the population of *C. medinalis* to relocate, resulting in the pest epidemic in India. Multiple rice leaves were injured by a single RLF larva, which interfered with photosynthesis and eventually decreased rice output. Leaf folders have been blamed for production losses ranging from 63 to 80% in sensitive high yielding or hybrid rice cultivars. The intensive cultivation of rice throughout both the Kharif and Rabi seasons has led to regular occurrences of biotic stress, which have developed into significant production restrictions. The timing of the rice crop's sowing is crucial for three main reasons. First of all, it makes sure that vegetative development takes place during a time when the temperature is suitable and there is a lot of solar radiation. Second, the ideal sowing window for each cultivar makes sure the cold-sensitive stage happens when the lowest nighttime temperatures are historically the warmest. Thirdly, timely planting ensures that grain filling takes place when warmer fall temperatures are more expected, resulting in high grain quality (Farrell *et al.,* 2003) [6]. The efficient pesticides that are available to deal with the occurrence of insect pests are not a long-term solution because to their influence on health and environmental concerns, exposure dangers, lingering persistence, and development of As a result, *C. medinalis* has redirected its attention in recent years to biological control. Previous studies hinted to the possibility of using *Beauveria bassiana* Vuill successfully.

The entomopathogenic fungus (EPF) is a special member present in the fungus, which infects the insects and other arthropods by enter in to the body by penetrating the cuticle and other openings present in the susceptible host from the soil or present in the any other susceptible host. Endophytes acting as a antagonist against plant diseases, Promoting the plant's growth and colonize in the rhizosphere soil and gives benefits to the plants from the stress conditions. Occurrence of EPF completely depends on the soil type, plant species and cultivation practice. The soil can protect the EPF from the UV light, other biotic and abiotic stresses. As per the climate changes all the IPM strategies are mostly going to the downward side. EPF is the one of the biocontrol organisms to manage the insect pests present in the field and also helps in to improve the IPM strategies. The fungus can enter in to the dead and alive insects. The incident, fungus entering in to the dead insect is called as saprophagous and entering in to the alive insect is called as entomophagous. Entomopathogenic fungi persist in the environment as they can develop on cadavers and thus recycle inoculum (Moore *et al,*). All over the world, different types of entomopathogens are presenting. The common and mostly presenting entomopathogens are *Beauveria bassiana*, *Metarhizium anisopliae, Metarhizium rileyi, Aspergillus* spp., *Nomuraea rileyi, Isaria fumosorosea, Verticillium lecanii, Pecilomyces lilacinus, Purpureocillium lilacinum* against the lepidopteran pests and other some of the arthropod pests. Basidiomycota and Entomophthoromycota both are causing problems in almost 280 known species of arthropods. *B. bassiana* (Bb), is a filamentous fungus (Hypocreales) that can be considered as both an entomopathogen and an endophyte. Its usage as a biopesticide for the biological control of insect pests is what makes it more well-known and an asymptomatic associate of plants. As a possible substitute for chemical control, endophytes have drawn more and more attention. Higher plants include endophytic microbes that occupy the leaves, stems, and roots asymptomatically and without obvious damage to the plant. Due to the secondary metabolites, they generate, which have a variety of potential uses in agriculture, endophytic fungi are significant. *B. bassiana* can act as a saprophyte by living in the organic matter and also act as endophytes by lives in the several plant species. The age-old plant or oldest part in the plant containing high number of fungal colonies compared to the youngest plants and plant parts. *B. bassiana* cause death in the host by activates the cellular and humolar responses in the body of pest. In host body *B. bassiana* at first the immune signalling pathways remained unstimulated but entered an acute alarm at 48h, resulting in the death of the host after some days. After post infection the dead individuals became melanization (Zhang *et al.,* 2013) [30]. Agostino Bassi discovered *B. bassiana* from silkworm corpses in the 19th century, and it has now spread to more than 200 insect species across six orders and 15 families. (Nakahara *et al.,* 2009) [16]. It vigorously grows, generating a range of toxins that cause external infections. (Chelico and Khachatourians, 2008; Naqqash *et al.,* 2016) [4, 17]. Studying the role of *B. bassiana* as a significant entomopathogenic fungal against the rice leaf folder *C. medinalis* is the aim of the research.

2. Materials and methods

2.1. Culture

B. bassiana culture was obtain from the National Research

Centre for Banana, Tiruchirappalli-27. The culture was sub cultured by using the PDA media and PD broth. The sub cultured fungi were inoculated in to the soil and as well as rice plant by root feeding method. Then the endophytic *Beauveria bassana* was isolated from the following methods.

2.2. Soil Sample collection
Soil samples collected from the rice growing pot which already having entomopathogenic fungi. Soil Samples collected by the sterile stainless steel from the rhizosphere region of the rice plant. Soil has been taken between the 5-20cm depth the soil in the polybags. Collected soils were shade dried in the Entomology laboratory until the moisture goes. The shade dries soil samples were kept in the refrigerator for keeping the microbes alive in the soil.

2.3. Isolation of *B. bassiana* from the soil
The method used to isolate the fungus from the soil is got from the Safavi (2010) [24] with the small modification as per the availability of sources. 0.2g of soil sample has been taken in the sterile centrifuge tube and 1.3 ml of Tween® 80 surfactant was added. Sample was vortexed for 15 minutes to mix the soil sample with the Tween® 80. Mixed sample was kept in a place for 10 minutes to settle down the soil particles in the lower portion of the centrifuge tube. Then the well mixed sample was gone under the serial dilution method up to the 10^{-1} under the Laminar Air Flow Chamber. After that 50µl sample was smeared over the PDA medium (Almjalawi *et al.*, 2023) and incubated for the 14 days in the 25°C.

2.4. Media Preparation
PDA medium was used for the culturing of fungus (Camara *et al.*, 2022). The medium was prepared by following the protocol mention by the Camara *et al.*, 2022.

2.5. Inoculation
These all was done under the Laminar Air Flow Chamber to decline the contamination. Sterilized PDA media pour in the sterile Petri plates and 50 µl of 10^{-1} Solution from the serial diluted sample was poured in the Petri plates containing PDA. Plates were incubated for 14 days under darkness in the 25°C for the colony development. Well-developed colonies were used for the morphological identification under microscopic view.

2.6. Conidial suspension Preparation
The conidial suspension used in the study was formulated in accordance with the Parsa *et al*. methodology with little bit modification. The isolated fungus culture again sub cultured in the Petri dishes containing PDA media along with the streptomycin. The inoculated culture was incubated for 14-18 days for getting the fully matured fungal spores. Fully matured cultures are rinsed with the sterile distilled water and allowed for some times for getting the matured spores. After that again it rinsed with the sterile distilled water and scraped by sterile glass slide for harvesting the spores along with the mycelia. Then it filtered through the muslin cloth for removing the mycelia and agar. Conidial density was standardized by using the improved Neubauer haemocytometer and adjusted to the 2 x 10^6 conidia/ml with sterile distilled water containing Tween® 80. The viability of the conidia was evaluated by taking a 100 µl sample and spread it on the PDA containing streptomycin and incubated at 25 °C. Conidia germination was assessed after 24 hours of incubation. Under a light microscope, the percentage of conidia germinating was determined. The assumption of conidium germination was made when hyphae could be observed or when the germ tube length was approximately twice the conidium length. The viability of each strain was evaluated by averaging three replicates, with the final inoculum being used for the seed treatment and for all other experiments.

2.7. Growing of rice plants for greenhouse studies
The seeds for the TRY 5 were sourced from ADAC & RI (Department of Plant Breeding ADAC Genetic) Tiruchirappalli. The seeds were divided into two parts. One part was used for seed treatment with the fungal spore suspension, while the other part was immersed in water overnight for pre-germination. After a day of soaking the seeds in both the spores suspension and water, the seeds were filtered and allowed to pre-germinate for a period of 12-18 hours. The fungal -surrounded seeds were planted in seed treatment pots, while the water-surfaced seeds were planted in soil drenching, foliar sprays and controls. Additionally, some of the grown seedlings from the control were used for seedling root dip methods. The seedlings were selected from the pots, and the roots were submerged in the fungal suspension overnight, before being transplanted to the pots.

2.7.1. Pot culture
Completely Randomized Design is followed for the studies conducted on the RLF against *Beauveria bassiana*. For this design totally 4 treatments and 4 replications were maintained. Each mud pots can contain 10 kg soil. The pots were filled with the soil + vermicompost (4.5 kg + 4.5 kg) one day before taking the transplanting.

2.7.2. Seed Treatment of *Beauveria bassiana* in Rice seeds
The sterilization technique was obtained from the Martin *et al.*, 2009 and Chase *et al.*, 1986 with the little bit modifications. The seeds of the TRY 5 were surface sterilized by the using the sodium hyphochloride 3% for 3 minutes and 70% ethanol for 2 minutes and seeds were rinsed with the sterile distilled water and dries under the laminar air flow chamber by using the filter paper. The dried 50 g seeds were soaked in the 10 ml of 2 x 10^6 conidial suspension for the overnight and suspension were dried and seeds were kept in a wet condition for the pre germination for 12 hours. Then the seeds were sowed in the pot culture arranged in the Completely Randomized Design (CRD) on 4 pots present in the single treatment.

2.7.3. Soil drenching and root dip method
Fungal suspension was prepared by same as for the seed treatment. For the soil drenching 100 ml of the fungal suspension poured in the soil containing the vermicompost. After pouring of spore suspension soil was thoroughly mixed using the sterilized stainless steel. After one hour of settled down, the rice seedlings which do not inoculated with the spore suspension transferred in to the 4 pots present in that treatment. After 3 days of planting, the soil was drenched with fungal culture 100 ml per pot. The pot containing a 9 kg of soil mixture (Greenfield *et al*. 2016) [7]. After one hour of settled down, the rice seedlings which do not inoculated with the spore suspension transferred in to the 4 pots present in that

treatment.

For the root dip method Saragih *et al.*, 2019 [25] procedure was followed and little bit modified for the crop, the seedling was dipped in the spore suspension having the 2 x 10^6 number of spores. After the dipping of seedlings, it maintained for the 6 hours. Then the seedlings were transplanted in to the Pots having a soil along with the vermicompost.

2.7.4. Foliar spray

The seedlings of rice were sprayed 3rd day after transplanting in to the pots. A hand sprayer was used to spray the spore suspension. Totally 50 ml of spore suspension was used to spray for each plant present in that treatment. Before spraying of suspension, the ground portion of the plants were fully covered by using the aluminium foil and small opening was given for the plants growth to avoid the leaching of the spore suspension from the plant surface to soil in the pots. Because of covering the soil portion the creation of soil drenching method was avoided. For increasing the humidity to the plants, whole plants were fully covered by using the poly bags.

2.7.5. Control

On control treatment, the seeds were soaked in the sterile distilled water. And the soils were fully mixed with the sterile distilled water before transplanting. After 3rd day after transplanting, sterile distilled water was sprayed and covered with the poly bags same as happened in the foliar spray method. To all the treatments, irrigation was done thrice a day to maintain the optimum growth.

2.8. Mass rearing of *Cnaphalocrocis medinalis*

The larvae and adults were collected from the nearby field of ADAC & RI, Tiruchirappalli. The larvae were released in to the rice plants which maintained in the cafeteria of Department of Entomology, ADAC & RI, Tiruchirappalli. The adults were maintained in the cage, which fully inner side covered by plain sheet and honey solution were given to the adults by dipping them in the cotton. The eggs laid by the adults were collected by using the sterile spatula and they transferred in the to container which fully sterilized and kept under the 25 °C for 12 hours dark and 12 hours light. The hatched larvae were transferred in another container which having wet paper and along with the rice leaf. The wet paper was used for maintaining the leaf rice freshly. The leaves were daily removed and replaced by the new leaved. The larvae was incubated at 26 °C for the better growth and developments.

The field released larvae also used for the studies. The pupae transferred to the cage containing the sugar syrup. The third instar larvae from the both lab and fields are used for the research works.

2.9. Evaluation of Endophytic *Beauveria bassiana* presence in the rice plants

The protocol was obtained from the Tkaczuk *et al.*, 2008 [32] and Selvaraj *et al.* 2012 [28] with little bit modification as per the availability of the sources. Plant samples were collected from the pot culture 7 days after inoculating the *B. bassiana* in the seedlings. The plants samples were maintained separately. All plants were washed thoroughly in the tap water to remove the soil from the roots and also remove the dust from the plants. The samples were subjected to a surface sterilization process in which they were submerged in a solution of 96% ethanol for one minute, followed by six minutes in a solution of 6% sodium hypochlorite, and finally a thirty-second immersion in the same 96% ethanol solution. After sterilization the plant parts were placed on the 90mm Petri dishes containing the PDA media (250 g potato, 20 g agar agar, 20 g dextrose). The petri dishes were incubated at 25 °C under the dark condition for 7 days for getting the *B. bassiana* growth. After 14 days fully matured spores can get from the Petri dishes for the further morphological identifications.

2.10. Re-isolation of *B. bassiana* from the soil

Soil samples were collected from the pot culture used for the soil drenching method after the 7 days of fungal inoculation. 0.2 g of soil was collected in the sterilized centrifuge tube, followed by the addition of 1.3 mL of surfactant. The sample was then vortexed for a period of 15 minutes to combine the sample with the surfactant, resulting in a well-mixed sample. The mixed sample was left to settle in a designated location for a period of 10 minutes, allowing the soil particles to settle into the lower part of the tube. Subsequently, the well-mixture sample was subjected to a serial dilution process, culminating in a dilution of the sample to a concentration of 10-1, which was then subjected to a laminar air flow chamber.

Fig 1: Subculture after re-isolation

2.11. Field release of leaf folder larvae

The larvae were collected from both the crop cafeteria and the laboratory-maintained culture. Subsequently, a total of 10 larvae were released for each replicate to be subjected to bioassays on rice plants which had been infected with *B. bassiana*o. After release, the larvae began to fold the leaves and also began to feed.

2.12. Data analysis

The data obtained from investigations were subjected to the analysis of variance using AGRESS software. Wherever the treatment difference was found significant, critical differences (CD) were worked out at 5% level of significance.

3. Results and discussion

The colonisation of the rice plant tissues by *B. bassiana* varied with time and was dependent on the inoculation method employed. Four different inoculation methods were

employed to colonise the *B. bassiana* in to the rice plants.

3.1. Isolation of Endophytic *B. bassiana* from soil and rice seedlings

After inoculation of the biologically active ingredient (Bb EPF 22) MK834817 in the pot culture, the colonisation of entamopathogenic fungi was observed in both plant tissues and soil of the pot culture.

3.2 Status of pathogenicity of endophytic *B. bassiana* against target insect pest RLF

Treatments	Initial No. of Larvae	3DAT	5 DAT	7DAT	10DAT	Mean
Seed treatment	10.0	9.67	8.67	8.00	7.67	8.50
Soil drenching	10.0	10.00	9.67	9.00	8.33	9.25
Seedling root dip	10.0	10.00	8.33	8.00	7.67	8.50
Foliar spray	10.0	9.67	8.33	8.00	7.33	8.33
TreatedControl	10.0	10.00	9.67	9.67	9.67	9.75
				T	D	TxD
			Sed	0.57	0.51	1.13
			CD(0.05)	1.14	NS	NS

Evolution of endophytic *B. bassiana* for the pathogenicity against the Rice Leaf Folder, *Cnaphalocrocis medinalis* is presented on the Table 1. There is 10 larave were released for the containing the bio assay against the *B. bassiana*. At three days after releasing of the larvae in to the treated seedlings treatment 1 and 4 having the same value of 9.67 which is the value having the highest mortality value after the three days B. bassisna application. After 5 days happened the treatment values were compared with the treated control, foliar spray and seedling root dip method having the same value of 8.33 and followed by seed treatment 8.67, soil drenching as well as treated control 9.67. Foliar spray, seedling root dip method and seed treatment having the same value of 8.00 and mortality followed by the soil drenching 9.00 after the 7 days fungal inoculation. After 10 days happened the treatments mortality values were compared with the untreated control. The is significant difference happened between the treated control and other all the treatments. The foliar spray treatment having the lowest mean value (7.33) and the values are followed by seed treatment and seedling root dip method (7.67) and soil drenching having the mortality mean 8.33. From day 5 to till 10 th day the treated control having the same mean value of 9.67 which it is showing the mortality didn't happen after that.

The initial larval count of 10 decreased over time, and a larval count was conducted three, five, seven and ten days after treatment. The results indicated that foliar spray was the most effective treatment, with the lowest larval count among the other treatments (8.33). Seed Treatment was the next treatment, with a larval count of 8.50, comparable to the larval count of seedling root dip of 8.50. The larval count reached a maximum of 9.75 with the treated control, and soil drenching resulted in a larval count higher than that of the treated control (9.25). The statistical analysis revealed that there is a significant difference between the treatments and registered non-significant (NS) with days after treatment and interaction among them.

4. Conclusion

The results of the study indicated the efficacy of the endophytic entomopathogenic fungus (*B. bassiana*) as an alternative to *C. medicalis* larvae. The larvae are under the control of the *B. bassiana* fungus. However, in order for the fungus to be successful in any environmental conditions, certain criteria must be fulfilled. Further field research is required to evaluate their effectiveness against the larva of *C. medialis* for long-term utility in biological control programme.

4. Acknowledgement

The authors are gratefully acknowledged for the sharing of knowledge about endophytes against the rice and carry out this research. We also extend the acknowledge to Department of Entomology, ADAC&RI, TNAU, Tiruchirappalli as well as National Research centre for Banana, Tiruchirappalli.

5. Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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