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Quantification of viable spore load of oil based formulation of *Beauveria bassiana* Bals. (Vuill) delivered through different delivery equipments on onion and chilli

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Abstract
Some of the main issues in the successful use of entomopathogenic fungi for pest control include the application, infectivity and persistence of their inoculum in the environment. In this connection, suspending entomopathogenic fungal conidia in oil frequently improves their environmental persistence and virulence against insects, compared to water suspensions. In microplot, the quantification of viable spore load (in terms of CFU per sq.cm leaf disc) of oil based formulation of *Beauveria bassiana* Bals. (Vuill) (Bb 112) delivered through different delivery equipments revealed that the CFU load was invariably higher in onion (fully opened 4th leaf) (fully opened 4th leaf) and top leaves of chilli plants sprayed with CDA sprayer (Controlled Droplet Applicator) (434.23 and 453.21 CFU cm^{-2}) on the day of application. This was followed by Avenger ULV sprayer > Aspee Maruyama Engine Sprayer > Aspee Knapsack Hand Sprayer > Aspee Hitech Hand Sprayer > Aspee Battery Sprayer.

Keywords: Onion, Chilli, Oil based formulation, *Beauveria bassiana*, CDA sprayer

1. Introduction
Onion (*Allium cepa* L.) is a bulbous plant, with cylindrical green leaves, belongs to the family Alliaceae, cultivated throughout the world for its food, therapeutic and medicinal value. The onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) is the major polyphagous pest of vegetables that causes extensive economic losses in greenhouse and open-field plant production (Diaz-Montano *et al.,* 2011 [5]; Reitz *et al.,* 2011 [24]). The damage to onion is caused by both adults and nymphs in green plant tissues (Trdan *et al.,* 2005) [29]. Damaged areas become dessicated causing a silvery flecked appearance and the bulbs become undersized (Diaz-Montano *et al.,* 2011) [5]. In addition to the direct quantitative and qualitative damage caused by its feeding, *T. tabaci* also act as the main vector of Iris yellow spot virus (Family : Bunyaviridae; Genus : Tospoviruses, IYSV), which can significantly reduce the bulb size (Gent *et al.,* 2004) [8]. The pest status of onion thrips can be attributed to its polyphagous nature, high reproductive rate, short generation time, high survival of cryptic instars, ability to reproduce without mating (parthenogenesis), ability to transmit plant pathogens and development of resistance to insecticides (Morse and Hoddle, 2006 [18]; Diaz-Montano *et al.,* 2011 [5]). Failure to control this pest by timely and effective means causes yield loss up to 50 per cent.
Chilli (*Capsicum annum* L.), is one of the important crop grown for green and ripe dry fruits throughout the tropics and warm temperate regions of the world. According to a survey by the Asian Vegetable Research and Development Committee, *Scirtothrips dorsalis* Hood (Thripidae: Thysanoptera) is one of the most important limiting factors for chilli production in India. This crop mainly suffers from leaf curl symptoms, which affect the pod yield (Rai *et al.,* 2009) [23] to the tune of 60 to 74 per cent (Patel and Gupta, 1998 [21]; Patel *et al.,* 2009 [20]).
Use of conventional synthetic insecticides to manage thrips population poses great difficulties owing to their cryptic nature and hence, led to growing interest in novel and effective alternatives like microbial bio-control agents. Microbial Biological control with entomopathogenic fungi has potential for thrips management in developed countries (Arthurs *et al.,* 2013) [1]. Biopesticide formulations based on *Beauveria bassiana* Bals. (Vuill) (Hypocreales: Cordycipitaceae), a wide host range insect pathogenic fungus are being used in insect pest management programmes worldwide, for the control of many agricultural and

horticultural pests of economic importance. Entomopathogenic fungi (EPF) are unique for controlling insect pests that feed by sucking plant or animal juices (Fuxa and Tanada, 1987 [7]; Lacey and Goettel, 1995 [10]; St. Leger and Roberts, 1997 [28]; Roy and Cottrell, 2008 [25]) since, they are able to infect through the host's integument.

Besides, the main issues in the successful usage of fungi for pest control under field conditions include delivery techniques, infectivity and persistence of their inoculum in the environment (Moore and Prior, (1993) [17]. Many studies have reported the foliar application of *B. bassiana* conidia. Although, little is known about the survival of conidia of *B. bassiana* in epigeal habitat. In general, the conidia of entomopathogenic fungi are strongly hydrophobic and difficult to suspend in water. Neverthless, the conidia of entomopathogenic fungi, *viz.*, *Beauveria* and *Metarhizium* spp. suspend easily in oils and in field applications, oil prevents small droplets from evaporating before reaching the target insect (Prior *et al.*, 1988 [22]; Bateman *et al.*, 1993 [4]; McClatchie *et al.*, 1994 [15]; Vimaladevi and Prasad, 1996 [30]; Bandam and Esmailpour, 2006 [2]). Furthermore, oil affords protection to fungal conidia from the UV rays (Moore *et al.*, 1993) [16]. Thus, the success of any formulation is decided based on their persistence over time on foliage. The main objective of this study is to quantify the viability of spore load of oil based formulation of *B. bassiana* (Bb 112) on onion and chilli using different delivery equipments.

2. Materials and methods

2.1 Spore production by diphasic liquid-solid fermentation technique

The aerial conidia of *B. bassiana*, which is best suited to be formulated in oil was produced by diphasic liquid – solid fermentation technique developed by LUBILOSA (Lutte Biologique contre les Locustes et Sauteriaux, www.lubilosa.org) project to produce *Metarhizium flavoviride* (Lomer *et al.*, 1997) [13]

2.2 Preparation for vegetative phase

The first stage in diphasic technique is to encourage the production of hyphal bodies and mycelium of an isolate that can be used for inoculation into the second (solid) stage of production. The SMY broth was used as substrate for vegetative phase of fungal growth.

Seventy five ml of SMY broth was taken in two hundred and fifty ml conical flasks. The flasks were plugged with non absorbent cotton wool, covered with aluminium foil and kept for sterilization at 121° C (15 psi) for 20 minutes.

After cooling, broth was inoculated with spore suspension of Bb 112 containing approximately 6 x 10^6 spores ml^{-1} and kept in a shaker at approximately 150 rpm for three days at room temperature (~25 to 30° C)

2.3 Inoculation of solid media (rice) for solid state fermentation

Broken white rice is often the preferred substrate for second phase, since individual rice particles provide large surface area and optimum aeration which could favour the formation of conidia.

Five hundred gram of broken rice sprinkled with 100 ml sterile distilled water was filled in polythene bags (45 x 35 cm; 300 gauge). The rice bags were then autoclaved at 121°C (15 psi) for 30 minutes. One flask of liquid inoculum (75 ml) for each 500 g bag of rice was utilized. After cooling, each rice bags were inoculated with 75 ml of liquid inoculum of the fungal isolate prepared as per sec. 3.1.3. The bag was massaged gently from outside to evenly distribute the inoculum over the rice grains. The top of the bag was folded loosely to aerate the substrate as the fungus grows. The bags were incubated at room temperature (~25 to 30° C) for about 10 to 15 days for sporulation.

After sporulation, the bag was opened and the rice was allowed to dry by spreading the grains in sterile stainless steel trays of uniform size (20 x 15 x 4 cm), covered by sterile muslin cloth under aseptic condition for seven to ten days depending on the temperature and humidity in the environment. Once dried, the spores were extracted from rice by manual sieving with 36 micron mesh sieve. After sieving, the strength of the spore powder was assessed using Neubauer haemocytometer. Then the spores were kept in sterile vials and stored in refrigerator for further experimental purposes.

2.4 Preparation of Oil based formulation of *B. basssiana* (Bb 112)

Oil based formulation of *B. bassiana* (Bb 112) was prepared as per the protocol developed by Sangamithra (2015) [26]. Oil based formulation was prepared by dissolving 1 g of pure conidia (10^{10} conidia g^{-1}) of Bb 112 in 100 ml of paraffin oil, along with adjuvants to enhance the efficacy of the formulation.

2.5 Quantification of viable spore load of oil based formulation of *B. bassiana* (Bb 112) delivered through different delivery equipments on onion and chilli

Microplot experiments were conducted at Insectary, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore to quantify the viable spore load of the oil based formulation of *B. bassiana* (Bb 112) that were reaching the onion and chilli foliage through different delivery equipments. Oil based formulation of *B. bassiana* (Bb 112) @ 10^8 spores ml^{-1} was sprayed over the onion (var. Co 1) and chilli (Hyb. Bullet) plants raised in microplots using different delivery equipments *viz.*, ASPEE Maruyama Engine sprayer, Avenger ULV sprayer, ASPEE Battery sprayer, ASPEE Knapsack hand sprayer, ASPEE Hitech hand sprayer and CDA (Controlled Droplet Applicator) sprayer. After spraying, fully opened (4th leaf on 45 DAP) leaves of onion plants and top, middle, bottom leaves of chilli plants from different treatment plots were detached separately and brought to the laboratory. From each leaf samples, one sq.cm leaf disc was taken and dipped separately in ten ml sterile distilled water and shaken well. From each suspension, one ml was plated separately in SMAY medium for assessing the Colony Forming Units (CFU). CFU was assessed on 0, 1, 3, 5 and 7 DAS and expressed as CFU cm^{-2} leaf area.

The recorded CFU were subjected to square root (X+0.5) and *arc sine transformation* and the analysis of variance for different experiments was carried out in AGRES and the means were separated by least significant difference (LSD) available in the package

3. Results and discussion

The results revealed that the spore load (in terms of CFU per sq.cm leaf disc) was higher in both onion and chilli plants sprayed with oil formulation of Bb 112 through CDA sprayer followed by Avenger ULV sprayer. In onion (on fully opened 4th leaf), the CFU load (CFU per sq.cm) was found to be high (434.23 CFU cm^{-2}) on the day of application through CDA sprayer and least in Aspee Battery sprayer (136.71 CFU cm^{-}

^{2}). Invariably in all the sprayers, the CFU load was found to be higher in top leaves of chilli than middle and bottom leaves. In chilli, it was as high as 453.21 CFU cm^{-2} in plants sprayed with Bb 112 through CDA sprayer and least in Bb 112 sprayed with Aspee Battery sprayer (102.36 CFU cm^{-2}) on the day of application. The other sprayers in the order of CFU load were Aspee Maruyama Engine Sprayer> Aspee Knapsack Hand Sprayer > Aspee Hitech Hand Sprayer> Aspee Battery Sprayer (Table 1 and Table 2).

The results showed that the mean number of colony forming units retrieved per plant decreased with increase in days of exposure. The present findings were in agreement with Mathews (1992) [14] who reported that the rotary atomisers are the most appropriate means of achieving the narrow droplet spectrum required. He also stressed the relevance of controlled droplet application and this approach was field-tested with Green Muscle® and was proved to be effective against a wide range of locusts and grasshoppers (Lomer *et al.*, 1999) [12] at volume application rates (VARs) of as little as 0.5 l ha^{-1} (Langewald *et al.*, 1999) [11]. Sangamithra (2015) [26] have reported that among the different formulations tested, against onion thrips, *Thrips tabaci* Lindeman which includes *B. bassiana* (Bb 112) oil based, talc based and crude formulations were evaluated in comparison with imidacloprid (standard insecticide check), oil based formulation of *B. bassiana* (Bb 112) recorded the highest persistence as evidenced by the number of CFU per plant (31.63 x 10^{7} ml^{-1}) on 7 days after treatment (DAT). The present findings are also in accordance with Naiak (2008) [19] who reported that the persistence of oil based formulation of *B. bassiana* on okra foliage was high (0.207 x 10^{4} CFUs per 5 leaves) compared to wettable powder formulation (0.109 X 10^{4} CFUs per 5 leaves) on 10 DAT. Saranya (2012) [27] reported that the *B. bassiana* + corn oil + skimmed milk recorded higher mean persistence of 0.313 x 10^{5} CFU per leaf on 7 DAT. Therefore, the enhanced persistence of conidia in oil based formulation can increase their performance under field conditions. Guinossi *et al.*, (2012) [9] also have reported that *Metarhizhium anisopliae* (Metschnikoff) Sorokin deposited on soybean leaves immediately after application were 101 CFU cm^{-2} in sprayed plots, with fungal dispersion up to 100 m and no significant differences were observed at days 3,7,9 and 11 after application. FAO (1994) [6] also reported that all droplets driven out through CDA contained viable spores, typically in the order of 100 to 10000 conidia (>10^{12} spores l^{-1}) per droplet in the size range of 40 to 120µm. The highest CFU load coupled with less risk of drift associated with CDA sprayer makes it as an effective delivery system for the oil based mycopesticides.

Table 1: Quantification of spore load (CFU) of oil based formulation of *B. bassiana* (Bb 112) delivered through different delivery equipments on onion.

Delivery Equipments	DAT (CFU cm^{-2})*				
	0	1	3	5	7
CDA Sprayer	434.23 (20.88)^{a}	320.55 (17.90)^{a}	272.22 (16.49)^{a}	222.00 (14.89)^{a}	198.33 (14.08)^{a}
Avenger ULV Sprayer	278.92 (16.70)^{b}	255.55 (15.98)^{b}	226.66 (15.05)^{b}	197.50 (14.05)^{b}	165.00 (12.84)^{b}
Aspee Maruyama Engine Sprayer	203.45 (14.26)^{c}	194.44 (13.94)^{c}	145.55 (12.06)^{c}	132.00 (11.48)^{c}	126.66 (11.25)^{c}
Aspee Knapsack Hand Sprayer	174.36 (13.20)^{d}	165.55 (12.86)^{d}	132.22 (11.49)^{d}	128.00 (11.31)^{c}	83.33 (9.12)^{d}
Aspee Hitech Hand Sprayer	154.89 (12.44)^{e}	148.88 (12.20)^{e}	111.11 (10.54)^{e}	108.00 (10.39)^{d}	78.33 (8.85)^{e}
Aspee Battery Sprayer	136.71 (11.69)^{f}	128.88 (11.35)^{f}	106.66 (10.32)^{e}	91.70 (9.57)^{e}	56.66 (7.52)^{f}

Figures in parentheses are square root ($\sqrt{x+0.5}$) transformed values
* Mean of four replications
# CFU assessed from the top fully opened leaf (*i.e.* 4th leaf on 45 DAP)

Table 2: Quantification of spore load (CFU) of oil based formulation of *B. bassiana* (Bb 112) delivered through different delivery equipments on chilli.

Delivery Equipments	DAT (CFU cm^{-2})*					
	Leaf position	0	1	3	5	7
CDA sprayer	Top	453.21 (21.28)^{a}	370.00 (19.23)^{a}	265.00 (16.27)^{a}	227.50 (15.08)^{a}	170.00 (13.03)^{a}
	Middle	440.43 (20.98)^{a}	352.50 (18.77)^{a}	217.50 (14.74)^{a}	210.00 (14.49)^{a}	168.75 (12.99)^{a}
	Bottom	432.98 (20.80)^{a}	347.50 (18.64)^{a}	202.50 (14.23)^{a}	181.25 (13.46)^{a}	145.00 (12.04)^{a}
Avenger ULV Sprayer	Top	257.34 (16.04)^{b}	228.75 (15.12)^{b}	175.00 (13.22)^{b}	165.00 (12.84)^{b}	145.00 (12.04)^{b}
	Middle	246.57 (15.70)^{b}	225.00 (15.00)^{b}	150.00 (12.24)^{b}	146.88 (12.11)^{b}	124.30 (11.15)^{b}
	Bottom	234.67 (15.31)^{b}	207.50 (14.40)^{b}	142.50 (11.93)^{b}	133.13 (11.53)^{b}	106.87 (10.33)^{b}
Aspee Maruyama Engine Sprayer	Top	209.23 (14.46)^{c}	195.00 (13.96)^{c}	172.50 (13.13)^{b}	157.50 (12.55)^{c}	132.50 (11.51)^{c}
	Middle	176.45 (13.28)^{c}	152.50 (12.34)^{c}	147.50 (12.14)^{b}	143.75 (11.98)^{b}	105.00 (10.24)^{c}
	Bottom	169.00	132.50	122.50	117.50	71.87

		(13.00)^{c}	(11.51)^{c}	(11.06)^{c}	(10.84)^{c}	(8.47)^{c}
Aspee Knapsack Hand Sprayer	Top	197.34 (14.04)^{d}	172.50 (13.13)^{d}	121.25 (11.01)^{c}	111.25 (10.54)^{d}	102.50 (10.12)^{d}
	Middle	145.67 (12.06)^{d}	120.00 (10.95)^{d}	108.75 (10.42)^{b}	95.00 (9.74)^{c}	72.50 (8.51)^{d}
	Bottom	129.34 (11.37)^{c}	125.00 (11.18)^{c}	93.75 (9.68)^{d}	85.62 (9.25)^{d}	60.00 (7.74)^{d}
Aspee Hitech Hand Sprayer	Top	167.89 (12.95)^{e}	156.25 (12.50)^{e}	92.50 (9.61)^{d}	81.25 (9.01)^{e}	45.00 (6.70)^{e}
	Middle	132.34 (11.50)^{e}	147.50 (12.14)^{c}	65.00 (8.06)^{c}	58.75 (7.66)^{d}	37.50 (6.12)^{e}
	Bottom	110.21 (10.49)^{d}	101.25 (10.06)^{d}	72.50 (8.51)^{e}	52.50 (7.24)^{e}	35.00 (5.91)^{e}
Aspee Battery Sprayer	Top	102.36 (10.11)^{f}	95.00 (9.74)^{f}	77.50 (8.80)^{e}	67.50 (8.21)^{f}	45.00 (6.70)^{e}
	Middle	85.64 (21.28)^{f}	72.50 (8.51)^{e}	60.00 (7.74)^{d}	47.50 (6.89)^{e}	32.50 (5.70)^{f}
	Bottom	75.93 (8.71)^{e}	68.75 (8.29)^{e}	57.50 (7.58)^{f}	51.37 (7.16)^{e}	24.37 (4.93)^{f}

Figures in parentheses are square root ($\sqrt{x+0.5}$) transformed values
*Mean of four replication

4. Conclusion

Oil based formulations of conidia seem to have several advantages over dust formulations for field applications. The persistence of oil based formulation of *B. bassiana* (Bb 112) is highly noted in the foliage sprayed with the CDA sprayer. According to Bateman and Alves (2000) [3] CDA represents a very specialised delivery system for oil formulations which can only be used with specialised application equipments (often rotary atomisers). In this context, the highest CFU load coupled with less risk of drift associated with CDA sprayer makes it as an effective delivery system for the oil based mycopesticides. However, more extensive studies are needed regarding the physiological interactions of oils at the foliage and conidia of entomopathogenic fungi in order to improve their field persistence for pest management.

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