Indian Journal of Agricultural Sciences **87** (4): 473–8, April 2017/Article
https://doi.org/10.56093/ijas.v87i4.69355

Characterization and evaluation of *Bacillus thuringiensis* var. *kurstaki* based formulation for field persistence and insect biocontrol

SANGEETA PAUL[1], BISHWAJEET PAUL[2], MD. ASLAM KHAN[3], CHETANA AGGARWAL[4], MAHESHWAR SINGH RATHI[5] and SATYA PRAKASH TYAGI[6]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012

Received: 22 January 2016; Accepted: 28 September 2016

ABSTRACT

Commercial formulations of *Bacillus thuringiensis* do not deliver in the field trials, mainly due to short persistence and residual efficacy of bioagent. In the present investigation, different subspecies of *Bacillus thuringiensis* were evaluated for their larvicidal potential and using the most promising subspecies, *B. thuringiensis kurstaki*, Wettable powder (WP) formulation addressing these issues was developed by us. The laboratory developed formulation was evaluated for efficacy under laboratory and field conditions. WP formulation was observed to meet all the criteria set for physico-chemical parameters according to the FAO/WHO specifications. Spore counts and toxicity obtained in this formulation were considerably higher than those obtained in the commercial formulation of *B. thuringiensis kurstaki*. The developed formulation considerably improved the persistence of *B. thuringiensis kurstaki* under field conditions. Under field trial on cabbage (*Brassica oleracea* var. *capitata*), this formulation significantly reduced number of leaves damaged; and improved marketable yield of cabbage heads. It could effectively control pests in the field for 10 days after spray. It was very effective in the control of *Pieris brassicae*, *Spilarctia obliqua*, *Plutella xylostella* and *Spodoptera litura*. Thus, WP formulation of *B. thuringiensis kurstaki*, developed by us, has good potential for effective biocontrol of insect pests in the field, due to its longer persistence and delivery of higher dose of active ingredient on the crop.

Key words: *Bacillus thuringiensis kurstaki*, Cabbage, Field evaluation, Insect biocontrol, WP formulation

Biological control methods are being sought as alternatives to insecticides, to control pest menace, due to environmental and health hazards associated with chemical control measures; and development of insecticide resistance in insects (Karim *et al*. 2000). *Bacillus thuringiensis*, a well-known entomopathogen has a very high potential to control insects in a field with no deleterious effect to the nature and has been effectively used in the control of many species of lepidopteran borer and defoliator larvae. It has broad and effective entomocidal host range against larvae of Lepidopteran, Coleopteran and Dipteran insects (Sanchis and Bourguet 2008, Bibi *et al*. 2013). Commercial products based on *B. thuringiensis* have been available for 70 years and currently more than a hundred products are registered for the management of important lepidopteran insect pests (Glare and O'Callaghan 2000). However, over the past many years use of *B. thuringiensis* has encountered problems with acceptance by growers due to low field efficacy and need for repeated application for successful control of the pest population. Successful control of pests by *B. thuringiensis* is greatly dependant on its persistence, residual efficacy and delivery of optimum dose for insect control. Major problems associated with the commercial formulations are short persistence and residual efficacy of bioagent and lower storage stability (Moazami 2008). *B. thuringiensis* sprays have a short effective residual period, generally, five to seven days in spring and three to five days in summer (Sudarsan *et al*. 1994), mainly due to inactivation of active ingredients, viz. spores and toxins by solar radiation (Sánchez-Yáñez and Peña-Cabriales 2000, Shah *et al*. 2016). The efficacy of commercial *B. thuringiensis* formulations is extremely poor under field conditions and need to be applied many times, to control insect pests. These problems, with persistence, have limited the commercial use of *B. thuringiensis* as an insect biocontrol agent. Thus, development of better formulation of *B. thuringiensis* is urgently needed, which would be more effective and persistent under field conditions.

Keeping the various factors affecting the efficacy of *B. thuringiensis* biopesticide in view, in the present investigation different subspecies of *B. thuringiensis* were evaluated for

[1]Principal Scientist, Division of Microbiology (email: sangeeta_paul2003@yahoo.co.in, [5,6]Technical Officer, [4]Research Associate, [2]Principal Scientist (email: bishwajeet_paul@yahoo.com), Division of Entomology, Indian Agricultural Research Institute, New Delhi 11 0012. [3]Assistant Professor, Department of Biology, Jazan University, Saudi Arabia.

their larvicidal potential. The most promising subspecies *B. thuringiensis kurstaki* was used for the development of Wettable Powder (WP) formulation. The laboratory prepared (LP) formulation of *B. thuringiensis kurstaki* was evaluated under laboratory and field conditions using a commercial formulation as reference. The field evaluation of the LP formulation was carried out on cabbage (*Brassica oleracea* var. *capitata*), since important lepidopteran pests like *Pieris brassicae* L., *Plutella xylostella* L., *Spodoptera litura* F. and *Spilarctia obliqua* W. etc. cause considerable damage to this crop leading to heavy losses in yield (Singh and Gandhi 2012). The survival of the formulation on phyllosphere was also monitored to determine its persistence under field conditions.

MATERIALS AND METHODS

Different subspecies of *B. thuringiensis,* viz. *B. thuringiensis kurstaki, B. thuringiensis galleriae, B. thuringiensis sotto, B. thuringiensis entomocidus, B. thuringiensis thuringiensis* and *B. thuringiensis aizawai* were obtained from the culture collection of Division of Entomology, Indian Agricultural Research Institute, New Delhi, India. These were grown in 500 ml Erlenmeyer flasks containing nutrient broth supplemented with (g/L): Glucose, 10; $MgSO_4$, 0.2; $FeSO_4$, 0.2; $ZnSO_4$ 0.2 and $MnSO_4$, 0.1 (pH 7.0) (Thompson and Stevenson 1984). The flasks were maintained in an incubator shaker at 32±1°C for five days and then kept for another two days under static conditions for uniform sporulation.

S. obliqua colony was maintained in the laboratory, in cylindrical plastic containers, with fresh castor leaves as diet at 27±1°C, 65 ± 5% RH and 12:12-h (LD) light regime. The larvae were reared, using standard entomological practices. Larvicidal potential of the different *B. thuringiensis* subspecies against *S. obliqua* was determined on third instar laboratory-reared *S. obliqua* larvae by leaf-dip bioassay (Morse *et al*. 1986). Three replications, with 20 larvae each were maintained. Appropriate untreated controls were maintained. Larval mortality was scored until pupation and expressed as per cent mortality. *B. thuringiensis kurstaki* showing highest larval mortality was used for the development of biopesticide formulation.

For WP formulation preparation, broth culture of *B. thuringiensis kurstaki* was grown. The cells were centrifuged at 10000 rpm for 10 min in a Sigma centrifuge. A talc based WP formulation was prepared using 10% (w/w) dry powder of pelleted *B. thuringiensis kurstaki* as active ingredient and Congo red as UV protectant along with other adjuvants. Physico-chemical characterization of the formulation was carried out in accordance with FAO/WHO specifications (FAO/WHO 2010). Suspensibility of the formulation was determined, according to CIPAC method (1995). Suspension (0.1%) was prepared, poured into a measuring cylinder and allowed to stand undisturbed for 30 min, then top $9/10^{th}$ was drawn off with auto-pipette and the content of active ingredient in the bottom $1/10^{th}$ was determined, thereby allowing the content of the top $9/10^{th}$ to be calculated.

Wetting of the formulation was determined according to CIPAC method (1995). The formulation was dropped into a beaker containing water. The time taken by the formulation to get completely wet was recorded. Wet sieve test of the formulation was carried out according to CIPAC method (1995). The formulation was dispersed in water and the suspension formed was transferred to a 200 µm sieve and washed. The quantity of the material retained on the sieve was determined by drying and weighing. Persistent foam of the formulation was determined according to CIPAC method (1995), by preparing 0.1% suspension. The suspension was poured into a measuring cylinder, inverted 30 times and the amount of foam created and remaining after one minute was measured.

Water content and pH of the formulation was determined according to CIPAC method (2000) and presence of chemical impurities were determined by the method of Finney (1971), using *Drosophila melanogaster* Meigen as a test insect. Microbial contaminants were determined by spread plating suitable serial dilutions of the formulation on nutrient agar plates and visually observing for bacterial colonies morphologically different from those of *B. thuringiensis*. Total viable cell count and spore counts were determined in the LP WP formulation by the method of Thompson and Stevenson (1984).

Third instar laboratory-reared *S. obliqua* larvae were used to evaluate the insecticidal activity of the LP formulation by leaf-dip bioassay (Morse *et al*. 1986) using 0.02%, 0.04%, 0.06%, 0.08%, 0.1% and 0.12% concentrations of the laboratory prepared formulation. A commercial WP formulation ((Lipel®), was used as reference. Three replications per treatment, with twenty larvae each, were maintained. Larval mortality was scored until pupation and corrected for control mortality using Abbott's formula (Abbott 1925). The experiment was repeated thrice. Probit analysis (Finney 1971) was carried out to calculate LC_{50} values as well as 95% fiducial limits.

The LP WP formulation was evaluated for survival of *B. thuringiensis kurstaki*, on the phyllosphere of cabbage plants, under field conditions. The formulation was applied at the rate of 1 g/l and 30 ml of this was used per plant to completely drench it. The commercial WP formulation (Lipel®) was used as reference. Sixty replications for each product were maintained. Bacterial counts were taken every day, for a period of 12 days. For this, every day five treated plants were randomly selected and two outer leaves were plucked and bacterial population on phyllosphere was determined by serially diluting the sample and spread plating the appropriate dilutions on nutrient agar plates (Thompson and Stevenson 1984). The plates were incubated in BOD at 32±2°C for 48 hr and then the developed colonies were counted.

The efficacy of LP WP formulation of *B. thuringiensis kurstaki* was evaluated for two years (2011-12 and 2012-13), under mini-plot trials, at IARI research farms. The cabbage variety POI was used in the present study. Seeds were sown in the nursery and were transplanted, 40 days after sowing, in the mini-plots. The plot size was 3.2m ×

3.2m with seven lines per plot and spacing between the lines and between plants was 0.45m. The experiment was conducted by following randomized block design with three treatments; and six replications per treatment were maintained. The treatments undertaken were a) control, b) LP WP formulation of *B. thuringiensis kurstaki* and c) commercial WP formulation of *B. thuringiensis kurstaki* as reference. The crop was maintained, using standard agronomic practices, with furrow irrigation as, and when, required.

Visual observations were made every day for insect damage. When the insect population reached economic threshold level, the biopesticide was sprayed on the crop @ 1g/l/plot. Second spraying was carried out on the 15th day after 1st spray application when a fresh attack of the borer complex was observed. Observations were recorded, on total number of leaves (in the pre-heading stage), number of cabbage heads damaged, damage caused by the borer complex in percent, weight of total undamaged cabbage heads/plot and weight of total damaged cabbage heads/plot at the time of harvesting. The data was also recorded, periodically, on leaves damaged 0, 5, 10, 15 days after first spraying and again 5 and 10 days after second spraying. The crop was harvested 13 days after the second spray.

Viability data [number of colony forming units (CFU)/g of formulation] were transformed into log values and subjected to analysis of variance (ANOVA). Data generated was statistically analyzed, using OPSTAT statistical software. Probit analysis was carried out, to calculate LC_{50} values as well as 95% fiducial limit, using the EPA 1.5 software.

RESULTS AND DISCUSSION

Physico-chemical characterization of LP WP formulation

In the present investigation, the percent mortality observed with different subspecies of *B. thuringiensis* ranged between 72-85%. *B. thuringiensis kurstaki* with 85% mortality was observed to be the most potent and hence was used for the development of biopesticide formulation. The WP formulation, developed by us, was physico-chemically characterized based on FAO/WHO specifications (FAO/WHO 2010) (Table 1). To improve effectiveness of a biopesticide, it is essential to develop a formulation which can address the issues of stability during storage and meet all the specifications set by FAO/WHO (Brar *et al*. 2006). It was observed that LP WP formulation had near neutral (6.9) pH and possessed very good suspensibility. Wet sieve test indicated that the formulation was in the form of fine powder and was free from visible extraneous matter and hard lumps.

Table 1 Physico-chemical characterization of Wettable Powder formulation of *Bacillus thuringiensis kurstaki*

Relevant impurities and contaminants	
Microbial contaminants	Nil
Chemical impurities	Negligible
Water	7%
Physical and physico-chemical properties	
pH	6.9
Wet sieve test	0.3% of the formulation was retained on a 200 μm test sieve.
Suspensibility	98% of the product was in suspension after 30 min in Standard Water D at 30 ± 2°C
Wettability	The formulation was completely wetted in 20 seconds.
Storage stability	24 months at room temperature
Stability at elevated temperature	22 months at 10°C above ambient temperature

Tendency of powder particles to aggregate or agglomerate can lead to deterioration in their ability to suspend properly and thereby, can hamper spraying operations, resulting in reduced efficacy of biopesticide (Knowles 2005). Storage stability of the formulation was observed to be two years (total viable cell count, after 2 years > 10^9 cfu/g). Stability of the formulation, at elevated temperature, was observed to be 22 months (total viable cell count, after 22 months > 10^9 cfu/g). Thus, the developed formulation was able to meet all the specifications, according to the criteria set by FAO/WHO (2010).

Total viable cell and spore count and laboratory evaluation of formulation

Total viable cell count and spore count of the LP formulation were 1.62×10^{16} cfu/g and 1.48×10^{16} cfu/g respectively, which were considerably higher than that obtained in the commercial formulation (1.89×10^{14} cfu/g and 7.1×10^{12} cfu/g, total viable cell count and spore count, respectively). Thus, most of the active ingredient in the LP WP formulation was in the form of spores which might have resulted in its observed higher toxicity to *S. obliqua* third instar larvae as compared to the commercial formulation during laboratory evaluation (Table 2), since the toxicity of *B. thuringiensis* is associated with spore-protein complex (Paul *et al*. 2011). The LC_{50} value for LP WP formulation was 0.066 μg/ml.

Table 2 Toxicity of WP laboratory formulation and commercial formulation of *Bacillus thuringiensis kurstaki* against *Spilarctia obliqua* third instar larvae

Treatment	Df	Heterogeneity	Regression equation	LC_{50} (μg/ml)	Fiducial limit	
					Lower	Upper
Laboratory formulation	5	9.110	y=7.64 + 2.23x	0.066	0.059	0.074
Commercial formulation	5	4.876	y=6.05 + 1.28x	0.153	0.116	0.251

Field persistence studies

The laboratory formulation was evaluated for its effect on survival of *B. thuringiensis kurstaki* on the phyllosphere under field conditions, using a commercial formulation as reference (Fig 1). A drastic decline in phyllospheric population of *B. thuringiensis kurstaki* was observed within 24 hr after spraying the commercial formulation whereas, comparatively a very slow decrease was observed for LP WP formulation. *B. thuringiensis kurstaki* could survive on phyllosphere for 12 days in LP WP formulation as compared to the commercial formulation, where it survived for 6 days only. Thus, LP WP formulation considerably increased the survival of *B. thuringiensis kurstaki* on phyllosphere as compared to commercial formulation. LP WP formulation, developed by us, contained Congo red for providing UV protection to *B. thuringiensis* and thereby, increased their persistence under field conditions. Congo red has been reported to be effective in protecting spores and binary toxins of *Bacillus sphaericus neide* from UV-B radiation (Hadapad *et al*. 2009). Sánchez-Yáñez and Peña-Cabriales (2000) reported limited persistence of *B. thuringiensis* spores on maize and bean leaves, less than three days after application, due to their sensitivity to solar radiation and drying. Survival on the phyllosphere for sufficiently long periods improves the efficacy of biocontrol agent and reduces the need for repeated spraying of the biopesticide to control the pest population.

Field evaluation

The efficacy of WP formulation of *B. thuringiensis kurstaki* was evaluated under mini-plot trials for two years and commercial formulation was used as reference. Total number of leaves/plot in all the three treatments was statistically similar (Table 3). Highest number of leaves damaged/plot was recorded in control treatments (86.6%), followed by commercial formulation. LP WP formulation recorded a significantly lower number of leaves damaged/plot (18.4%).

Observations were also recorded on the number of leaves damaged per treatment, at regular interval of five days (Fig 2). A sudden increase, in number of leaves damaged,

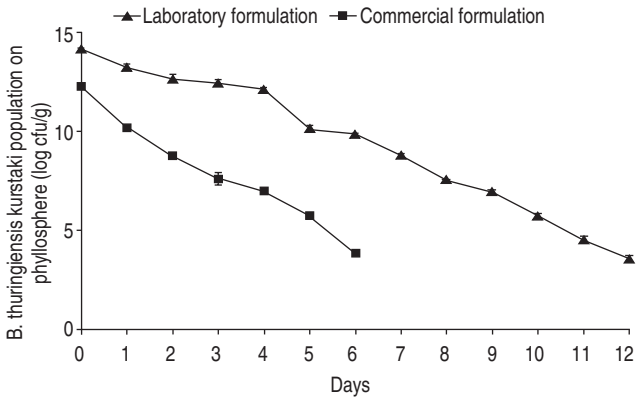

Fig 1 Survival of *B. thuringiensis kurstaki* in laboratory and commercial formulations on the phyllosphere under field conditions. Error bars represent the standard deviation of five replications.

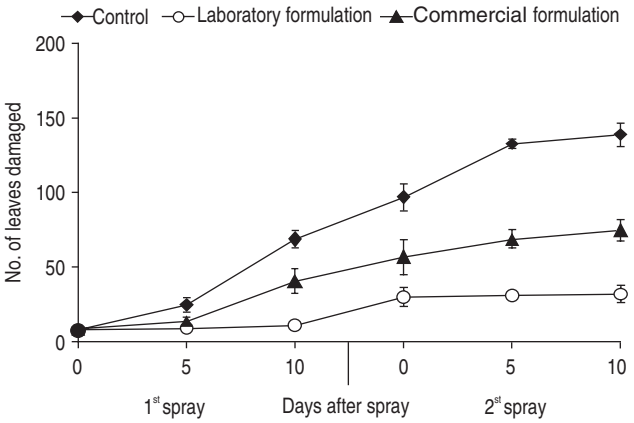

Fig 2 Temporal effect of laboratory and commercial formulations on leaf damage by pests. Error bars represent the standard deviation of six replications.

Table 3 Total number of leaves of cabbage damaged by insects (pooled data)

Treatment	Total no. of leaves/plot	No. of leaves/plot		Percent leaves	
		Undamaged	Damaged	Undamaged	Damaged
Control	545	73	472	13.4	86.6
Laboratory formulation	558	455	103	81.5	18.5
Commercial formulation	551	286	265	51.9	48.1
SE(m) ±	15.1	17.6	12.05		
CD (P=0.05)	NS	62.0	42.5		

Table 4 Insect wise damage of leaves of cabbage (percent damage) (pooled data)

Treatment	*P. xylostella*	*P. brassicae*	*S. litura*	*S. obliqua*
Control	25.3	65.6	7.4	13.6
Laboratory formulation	7.0	6.8	1.7	0.5
Commercial formulation	16.0	31.1	4.8	4.8
SE(m) ±	1.23	1.65	0.47	0.77
CD (P=0.05)	4.35	5.81	1.67	2.71

Table 5 Total marketable yield of cabbage obtained (pooled data)

Treatment	Total marketable yield (kg)/plot	Total no. of cabbage heads/plot		Weight per cabbage head (kg)	
		Undamaged	Damaged	Undamaged	Damaged
Control	39.8	39	10	1.02	0.30
Laboratory formulation	53.3	47	2	1.14	0.93
Commercial formulation	46.8	43	6	1.09	0.73
SE(m) ±	2.03	0.44	0.49	0.054	0.047
CD (P=0.05)	7.15	1.56	1.74	NS	0.164

was observed 15 days after the first spray in case of LP WP formulation, indicating that this formulation was effective in controlling insect pests in the field only for about 10 days, however, commercial formulation was observed to be effective for five days only. At that time second spraying of the crop was carried out. After the second spraying, again the LP WP formulation was found to be effective in pest control for 10 days. Increased duration of persistence of *B. thuringiensis kurstaki* in the formulation may have improved its performance as compared to the commercial formulation under field conditions. These results were also corroborated by its effectiveness in pest control in the field for about 10 days.

It was observed that maximum damage was caused by *P. brassicae* followed by *P. xylostella* (Table 4), in control treatments. *S. obliqua* and *S. litura* were the other pests which caused damage to the crop. The LP WP formulation was very effective in controlling *P. brassicae* and *S. obliqua*. However, commercial formulation was not observed to be as effective and could reduce the damage caused by pests by only 50% in most of the cases.

There was a considerable decrease in yield of marketable produce in the control treatment, due to damage caused by pests (Table 5). Spray of LP WP formulation considerably reduced the damage caused by insect pest to the crop; only 4% damage was recorded in plots treated with laboratory formulation. However, nearly 20% of the cabbage heads were damaged due to pest attack in control treatment. Lower percent of damaged leaf and the number of damaged heads; higher marketable yields were recorded with LP WP formulation as compared to commercial formulation. Even the extent of damage caused per head, as evidenced by weight of individual damaged cabbage head, was lower, thus, indicating that LP WP formulation was more effective in controlling the magnitude of damage caused by insects.

The results obtained, in the present investigation, indicate that performance of the *B. thuringiensis kurstaki* based biopesticide was improved by addressing the core issues, affecting the efficacy in the field, while formulating this biopesticide. By increasing the field persistence and dose of active ingredient in the developed formulation, we were able to increase its efficacy under field conditions.

REFERENCES

Abbott W S.1925. A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**(3): 265–7.

Bibi A, Ahmed K, Ayub N, Alam S.2013. Production of low cost *Bacillus thuringiensis* based-biopesticide for management of chickpea pod-borer *Helicoverpa armigera* (Huebn) in Pakistan. *Natural Science* **5**(11): 1 139–44.

Brar S K, Verma M, Tyagi R D and Valéro J R.2006. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process Biochemistry* **41**(2): 323–42.

CIPAC.1995. Physico-chemical methods for technical and formulated pesticides. *CIPAC Handbook, Volume F*, pp 1–179. Dobrat W and Martijn A (Eds). Collaborative International Pesticides Analytical Council, Harpenden.

CIPAC.2000. Analysis of technical and formulated pesticides. *CIPAC Handbook, Volume J*, pp1–131. Dobrat W and Martijn A (Eds). Collaborative International Pesticides Analytical Council, Harpenden.

FAO/WHO.2010. Manual on development and use of FAO and WHO specifications for pesticides. http://www.fao.org/fileadmin/templates/agphome/documents/Pests Pesticides / PestSpecs Manual2010.pdf

Finney D J.1971. *Probit Analysis*, 3rd edn, p 333. S. Chand and Co. Ltd, New Delhi.

Glare T R and O'Callaghan M.2000. *Bacillus thuringiensis: Biology, ecology and safety*, pp350. John Wiley and Sons, Chichester.

Hadapad A B, Hire R S, Vijayalakshmi N and Dongre T K.2009. UV protectants for the biopesticide based on *Bacillus sphaericus neide* and their role in protecting the binary toxins from UV radiation. *Journal of Invertebrate Pathology* **100**(3): 147–52.

Karim S, Zafar A U, Nasir I A and Riazuddin S.2000. Field efficacy of CAMB *Bacillus thuringiensis* biopesticide to control *Helicoverpa armigera* (Hübner) and *Earias vitella* (Fabricius) in okra crop. *Pakistan Journal of Biological Sciences* **3**(8): 1296–8.

Knowles A.2005. *New Developments in Crop Protection Product Formulation*: *Agrow Reports*, p 344. T&F Informa UK Ltd, London.

Moazami N.2008. Biopesticide production. *Industrial Biotechnology VI, Encyclopedia of Life Support Systems (EOLSS)*, pp 1–52. Doelle H W and Rokem S (Eds). Eolss Publishers, Oxford.

Morse J G, Bellows T S and Iwata Y.1986. Technique for evaluating residual toxicity of pesticides to motile insects. *Journal of Economic Entomology* **79**(1): 281–3.

Paul B, Paul S and Khan M A.2011. A potential economical substrate for large scale production of *Bacillus thuringiensis* var. *kurstaki* for caterpillar control. *Biocontrol Science and Technology* **21**(11): 1 363–8.

Sánchez-Yáñez J M and Peña-Cabriales J J.2000. Survival of spores of *Bacillus thuringiensis* in leaves of maize, beans and ground. *Terra* **18**(4): 325–31.

Shah J V, Lad S B, Yadav R K and Ingle S S.2016. Activity of two indigenous *Bacillus thuringiensis* isolates on lepidopteran insect pest *Amsacta albistriga* (Arctiidae). *International Journal of Applied Research* **2**(2): 20–3.

Singh A and Gandhi S.2012. Agricultural insect pest: Occurrence and infestation level in agricultural fields of Vadodara, Gujarat. *International Journal of Scientific and Research Publications* **2**(4): 1–5.

Sudarsan N, Suma N R, Vennison S J and Sekar V.1994. Survival of a strain of *Bacillus megaterium* carrying a lepidopteran-specific gene of *Bacillus thuringiensis* in the phyllospheres of various economically important plants. *Plant and Soil* **167**(2): 321–4.

Thompson P J and Stevenson K E.1984. Mesophilic spore forming aerobes. *Compendium of Methods for the Microbiological Examination of Foods*, pp 211–20. Speck M (Ed). American Public Health Association, Washington.