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Field evaluation of native white grub bio-agent, *Bacillus cereus* strain WGPSB-2 in Uttarakhand Himalayas and its impact on soil microbiota

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Abstract
A microbial pathogen, *Bacillus cereus* strain WGPSB-2 isolated from mid hills of Uttarakhand Himalayas (1000-1500 amsl) had potential as a biocontrol agent against white grubs of the region. Thus, the pathogen's ability to suppress grubs was investigated under field conditions over a period of five years in five villages of the region. A single dose annual application of WGPSB-2 as talc based formulation ($1x10^{10}$ spores/g) at the rate of 5kg/ha showed a sharp decline in grub population over the years in all the targeted villages. Pit sampling data showed maximum grub reduction during initial years of treatment in all villages and found significant for time, villages and time X village interactions in repeated measures ANOVA. Twenty four months post-inoculation resilience took place in micropits tested for bioagent impact on micro-floral densities, resulting in similar/ positive structures of tested microbial populations. The substantial numerical reduction of grub density coupled with no negative impacts on soil inhabitant microbiota, signifies WGPSB-2 as a vibrant technological option in this ecologically sensitive region.

Keywords: *Bacillus cereus*, management, microbiota, Uttarakhand Himalayas, white grubs

Introduction
"White grub" is a collective term used for root feeding scarab larvae of the families Scarabaeidae, Dynastidae, Rutelidae and Cerambycidae in order Coleoptera. They cause significant damage to many agricultural and horticultural crops, ornamentals, plantation crops, lawn, turf, pasture and forest trees around the world and are major limiting factor of agricultural production in India [17]. Though white grub incidence has been documented in different agro-ecological regions of India, their prevalence is highly pronounced in the North-Western Indian Himalayan region comprising the states and union territories of Uttarakhand, Himachal Pradesh, Jammu and Kashmir and Ladhakh. As per available records, this region harbours nearly 78 species of phytophagous white grub species [19]. However, the mid hills (1000-1500 amsl) of Uttarakhand, where the study was undertaken harbour *Anomala dimidiata* (Hope), *H. seticollis* Moser and *H. longipennis* as predominant species [16, 18].

Keeping in view, the environmental contamination by chemical pesticides especially in the dynamic hilly ecosystem, biocontrol can be considered as ecofriendly alternative [4, 12]. The association of variety of native microbial entomopathogens with white grubs, innate hardiness to prevailing climate [7], their governance in natural regulation of pest [21] etc offers promises in successful pest management. Moreover, in order to formulate new biological control programme, evaluation of the bio-agent interacting dynamics among the entomopathogenic organism, host and environment is necessary [2].

Our exploration studies yielded an entomopathogenic bacterium, *Bacillus cereus*, strain WGPSB-2 having high pathogenicity against first and second instars of predominant species Uttarakhand Himalayas, *Anomala dimidiata* and *Holotrichia seticollis* [19]. However, its practical utility comes from potential performance under field conditions. With a view to ascertain its potential as augmentative bio-control, we conducted field trials to determine the relative effects of this bio-agent on the suppression of white grubs and its impact on other soil microbiota for ecological compatibility.

Materials and Methods

Field Evaluation of WGPSB-2

Site selection

Based on random survey on cropping pattern and white grub sampling in the mid hills (1000-1500 amsl) of Uttarakhand Himalayas five villages *viz*., Chausali, Tunakot, Daulaghat, Govindpur and Manan (detailed in Table 1) in Almora district were selected for testing of WGPSB-2 in 'adopted village' concept. Dhaspad, a non-adopted village where no application of the bioagent, WGPSB-2 was done served as control. All the villages have an initial white grub population of one grub per 30x30x20 cm pit at all ten random locations covering the entire village. In each village, four individual fields (200 m^2) were tagged for data collection.

Application of WGPSB-2

WGPSB-2 has a strong ability to colonize on different compost substrates. So, the application was planned in such a way that talc based formulation ($1x10^{10}$ spores per gram) was initially applied to compost pits at a rate of 1kg/tonne for colonization [19]. After one month, WGPSB-2 enriched compost was evenly applied in the field during land preparation at the rate of 5 tons/hectare. The same procedure was followed in every year on community basis covering every field in entire village during first week of June, which coincides with the beetle emergence.

Data recording and statistical analysis

Pit sampling technique was used to monitor grub populations in the pre-tagged four fields in each village for four months from June to October for five years. During second week of each month, 30x30x20 cm pits were dug at three random locations and mean grub population of all the four fields per village was taken (grub population/month/village). The data obtained clearly showed that in any selected year, natural selection process directed the population density of white grubs to a seasonal decreasing trend from June to October. So, comparison of the mean grub population was made over the years in individual months as a base. The means were subjected to repeated measures ANOVA to compare grub reduction over the period, within the villages and interactive effects of village x time in SPSS software (version 10.0.1). The grub population densities of the adopted (Manan) and non-adopted village (Dashpad) in July were also compared using two sample t-test.

Impact of WGPSB-2 on the soil microbiota

The experiment was conducted at ICAR-Vivekananda Parvatiya Krishi Anusandhana Sansthan (VPKAS), Experimental Farm, Hawalbagh (1250 amsl), in a Completely Randomized Design (CRD) in $1m^3$ galvanized sheet walled micro-pits with three doses of the bio-agent *viz*., 1x, 2x, 4x (x is the recommended dose) and a control (no application of the bio-agent) with four replications. The treatment dose was mixed with top 15cm soil in each pit and sampling was done at monthly intervals up to 24 months. All the samples were analyzed for total bacteria, pseudomonads, free living nitrogen fixers and total fungal counts by using a standard soil-plate dilution technique as described by Seeley *et al.* [14]. Appropriate dilutions of sample were plated on selective medium and incubated at 28ºC for 24-48hrs for bacteria and 72-96hrs for fungal counts. The impact of WGPSB-2 was determined using principal component analysis (PCA) using XLSTAT software (XLSTAT 2010) to display the correlations and affinities between different doses for tested micro biota.

Results and Discussion

Field Evaluation of WGPSB-2

Soil bacteria such as *Bacillus thuringiensis*, *Paenibacillus popilliae*, *P. lentimorbus*, *Micrococcus* sp., *Serratia entomophila, S. proteamaculans* and *Yersinia entomophaga* etc [5, 7, 12) predisposes the subterranean white grubs to a large array of diseases. An entomopathogenic bacterium, *B. cereus* stain WGPSB-2 native to Uttarakhand Hills reported to have biocontrol potential against predominant white grubs of the region [19]. The tangible usage of any entomopathogen comes from its sustainable field management of targeted pest under given ecological conditions. In any selected year, particularly under Uttarakhand mid hills condition, the period between June to August was considered as peak activity period of white grubs. Bonferroni pair wise comparison of pit sampling data evidently showed a significant numerical reduction in grub population in all the adopted villages over years in only two months *viz*., June ($1^{st}/4^{th}$ and $2^{nd}/4^{th}$ years with mean difference of 1.156 (P=0.047) and 0.190 (P=0.044), respectively) and July ($1^{st}/2^{nd}$, $1^{st}/3^{rd}$, $1^{st}/4^{th}$ and $1^{st}/5^{th}$ years with mean difference of 0.934 (P=0.028), 0.924 (P=0.021), 0.992 (P=0.009) and 0.963 (P=0.020), respectively). August month also showed numerical reduction in grub population but not significant. Despite of the tested five months, this significance reduction in only two months can be attributed to specific activity of WGPSB-2 against early instars [19] and reduced susceptibility of later instars [15] coupled with their movement into deep soil profiles for pupation which was beyond the sampling pit depth i.e. 20cm. This is more evident in September and October months with erratic variations over years (Figure 1). The maximum reduction of grub population within a year (Figure 1) and subsequent maintenance of stumpy populations shows the viability of WGPSB-2 as both a preventive and curative biological control agent as applications targeted against the first instars are essentially preventive [11]. This type of stage specific bioactivity is also pronounced in white grub pathogenic nematodes [7, 8, 9 10, 11] and fungi [20, 22]. Moreover, the susceptibility of developmental stage and time taken to kill the host are key points in developing a control strategy [1, 22]. Moreover, the annual and prophylactic applications (prior to incidence of grub) of WGPSB-2 have additive effects with residual population of previous year which is evidenced by maximum grub reduction during initial years.

With respect to individual villages, maximum reduction of grub population was recorded in Manan (94.2%) and Chausali (89.3%) in June and July, respectively over five years period. Repeated measures ANOVA of grub density over years showed a significant interaction between time (years) ($F_{4,16}$ = 26.47, 51.22 and 13.68), villages ($F_{1,4}$= 28.25, 68.64 and 19.74) and time X villages ($F_{1,4}$ = 79.56, 199.27 and 94.28) in June, July and August. By considering all the villages, an average reduction of 85.2 and 84.4% reduction in grub population is achieved over a five years period. A representative grub population in Manan (Table 2) also showed a significant reduction of more than 85% by fifth year and variability between individual months of test years. These minor non significant fluctuations in grub density are also reported in other villages which can be attributed to differences in their topographic structures, biodiversity and proximity to the forest areas. A t-test (n=12) comparison (Table 3) of grub populations between Manan (adopted) and Dashpad (non-adopted) also showed significant difference

between them with an equilibrium in grub density in latter (Figure 2).

Impact of WGPSB-2 on soil microbiota

Besides pathogenicity, environmental competency and compatibility of the bio-agent were key factor for any potential microbial control agent against soil pests [6] in order to have sustained management. A classical microbiological numbering technique was used to analyse soil microbial perturbations induced in soil over a period of 24 months. The data on tested microorganisms in both inoculated and non-inoculated micropits over a period of two years was presented in table 4. Introduction of WGPSB-2 resulted in a significant increase of bacterial and cultivable fungi populations from one and 6 month post-inoculation, respectively until the end of the experiment. However, only a slight increase is noticed in case of Pseudomonads populations, which became significant after 6 months. These results showed that variations of the numbers of viable micro-organisms due to the introduction of WGPSB-2 were less important than the ‘‘non intentional’’ variations due to the experimental conditions and also observed in the non-inoculated soil. Studies also reported these type of non-intentional variations under the control of indigenous microbial communities, sampling time [3], field site, plant growth stage and microenvironment [13].

The positions of total bacterial, *Pseudomonas*, and fungal counts in different months in the four zones of ordinate biplot of principal component analysis (PCA) are depicted in Figure 3. The PCA comprising two principal components (F1 65.3% and F2 23.8%) accounted for 89.1% of variance. The interrelationship among doses of WGPSB-2 and microbial counts was more evident through the projection of F1 and F2. The F1 and F2 had a cluster of microbial counts TBC (1X, 4X), TPC (2X $ 4X and TBC control) with large positive loading for the first and second components and were distributed in the right upper side of biplot and had high positive loading for the first component, and negative loading for the second component. Other treatments occupied positions either on the left upper or left lower side of the biplot. Of these different treatments, some had a negative first component and others showed a positive second component but none of the treatment dose showed negative effect for both the components.

Table 1: Details of the villages selected for study

Sl. No.	Village	Altitude (m amsl)	Block	Geographical identity
1	Chausali	1133	Hawalbagh	29.5592°N, 79.5997°E
2	Tunakot	1065	Tarikhet	29.5765°N, 79.4698° E
3	Daulaghat	1285	Hawalbagh	29.4806°N, 79.2611°E
4	Govindpur	1310	Hawalbagh	30.19°N, 78.04°E
5	Manan	1350	Takula	30.7726°N, 79.4953°E
6	Dashpad	1370	Dhauladevi	29.5988°N, 79.6579°E

Table 2: Grub population in tagged fields of the adopted village, Manan (1350 m amsl)

Experimental fields	Mean number of grubs/30x30x20cm pit ± SD															Reduction in grub population (%)*			
	1st Year			2nd Year			3rd Year			4th Year			5th Year						
	June	July	Aug	June	July	Aug	June	July	Aug	June	July	Aug	June	July	Aug	2008	2009	2010	2011
Field-1	1.8±1.0 (1-4)	1.2±0.6 (0-2)	0.7±0.8 (0-2)	0.3±0.7 (0-2)	0.1±0.3 (0-1)	0.1±0.3 (0-1)	0.2±0.4 (0-1)	0.2±0.4 (0-1)	0.1±0.3 (0-1)	0.1±0.31 (0-1)	0.1±0.31 (0-1)	0.05±0.22 (0-1)	0.15±0.37 (0-1)	0.2±0.41 (0-1)	0.1±0.31 (0-1)	91.7	83.3	91.6	88.89
Field-2	1.2±0.4 (0-2)	0.9±0.4 (0-2)	0.0±0.0 (0-0)	0.2±0.4 (0-1)	0.2±0.4 (0-1)	0.1±0.3 (0-1)	0.0±0.0 (0-0)	0.2±0.4 (0-1)	0.1±0.3 (0-1)	0.0±0.0 (0-0)	0.1±0.31 (0-1)	0.05±0.22 (0-1)	0.0±0.0 (0-0)	0.10±0.31 (0-1)	0.0±0.0 (0-0)	77.8	77.8	88.8	91.67
Field-3	1.4±0.5 (1-2)	0.8±0.5 (0-1)	0.0±0.0 (0-0)	0.1±0.3 (0-1)	0.2±0.4 (0-1)	0.0±0.0 (0-0)	0.1±0.3 (0-1)	0.1±0.3 (0-1)	0.1±0.3 (0-1)	0.0±0.0 (0-0)	0.15±0.37 (0-1)	0.1±0.31 (0-1)	0.1±0.31 (0-1)	0.2±0.41 (0-1)	0.1±0.31 (0-1)	75.0	87.5	81.3	85.71
Field-4	1.7±1.1 (0-2)	1.1±0.6 (0-2)	0.4±0.5 (0-1)	0.1±0.3 (0-1)	0.1±0.3 (0-1)	0.2±0.4 (0-1)	0.2±0.4 (0-1)	0.2±0.4 (0-1)	0.1±0.3 (0-1)	0.1±0.31 (0-1)	0.15±0.37 (0-1)	0.1±0.31 (0-1)	0.1±0.31 (0-1)	0.15±0.37 (0-1)	0.15±0.37 (0-1)	90.9	81.8	86.4	91.18

Figures in parentheses are range.

*Reduction in grub population calculated based on first year grub data.

Table 3: Comparison of mean grub populations in July between Dashpad (Non-adopted) and Mannan (Adopted village)

Village	Mean grub population ±SE				
	2007	2008	2009	2010	2011
Dashpad	2.50±0.31 (1-4)	2.92±0.23 (2-4)	2.83±0.37 (1-4)	2.83±0.24 (2-4)	2.83±0.21 (2-4)
Mannan	1.25±0.22 (0-2)	0.21±0.11 (0-1)	0.21±0.11 (0-1)	0.17±0.11 (0-1)	0.25±0.13 (0-1)
t value	3.27	10.58	6.85	10.03	10.55
P value	0.002	<0.001	<0.001	<0.001	<0.001

Figures in parentheses are range.

Table 4a: Indigenous microbial population densities assessed using culture plating method in soil before inoculation of entomopathogenic bio-agent *Bacillus cereus* strain WGPSB-2

Microorganisms	Pits initial microbial population densities			
	1	2	3	Control
Total Bacterial counts	4.5×10^6	6.4×10^6	5.4×10^6	6.1×10^6
Total Fluorescent Pseudomonads	2.3×10^5	2.4×10^5	2.8×10^5	2.3×10^5
Total Diazotrophic Bacteria	1.0×10^5	1.0×10^5	1.2×10^5	1.0×10^5
Total Fungal counts	1.2×10^5	9.0×10^4	9.0×10^4	9.0×10^4

Table 4b: Indigenous microbial population densities assessed using culture plating method in soil after inoculation of entomopathogenic bio-agent *Bacillus cereus* strain WGPSB-2

Doses	Time interval (months)				
	1st	6th	12th	18th	24th
	Total bacterial population (x10^6 cfu/g of oven dry soil)				
T1 (1x)	5.7	13.9	8.02	20.0	2.75
T2 (2x)	6.1	37.8	4.69	22.5	1.25
T3 (4x)	3.7	6.7	4.94	29.0	3.50
T4 (control)	5.5	13.2	3.97	22.5	2.10
	Total fluorescent Pseudomonads (x10^{5}cfu/g of oven dry soil)				
T1 (1x)	6.56	262	36.2	28.0	40.0
T2 (2x)	5.24	188	51.5	23.0	27.5
T3 (4x)	4.63	178	63.9	28.7	35.0
T4 (control)	2.79	94	71.2	30.0	30.5
	Total Diazotrophic Bacteria (10^{5}cfu/g of oven dry soil)				
T1 (1x)	1.3	4.5	25.2	2.8	2.5
T2 (2x)	1.1	4.0	13.6	2.8	1.9
T3 (4x)	1.4	4.4	25.3	3.8	4.4
T4 (control)	1.4	4.4	29.8	3.4	3.5
	Total Fungi (x10^{4}cfu/g of oven dry soil)				
T1 (1x)	7.0	20.8	7.55	27.0	40.0
T2 (2x)	4.0	31.4	5.75	8.5	40.0
T3 (4x)	3.0	25.9	8.54	6.8	45.5
T4 (control)	8.0	19.7	7.29	13	20.0

*Pits were inoculated with 1x, 2x and 4x doses of WGPSB-

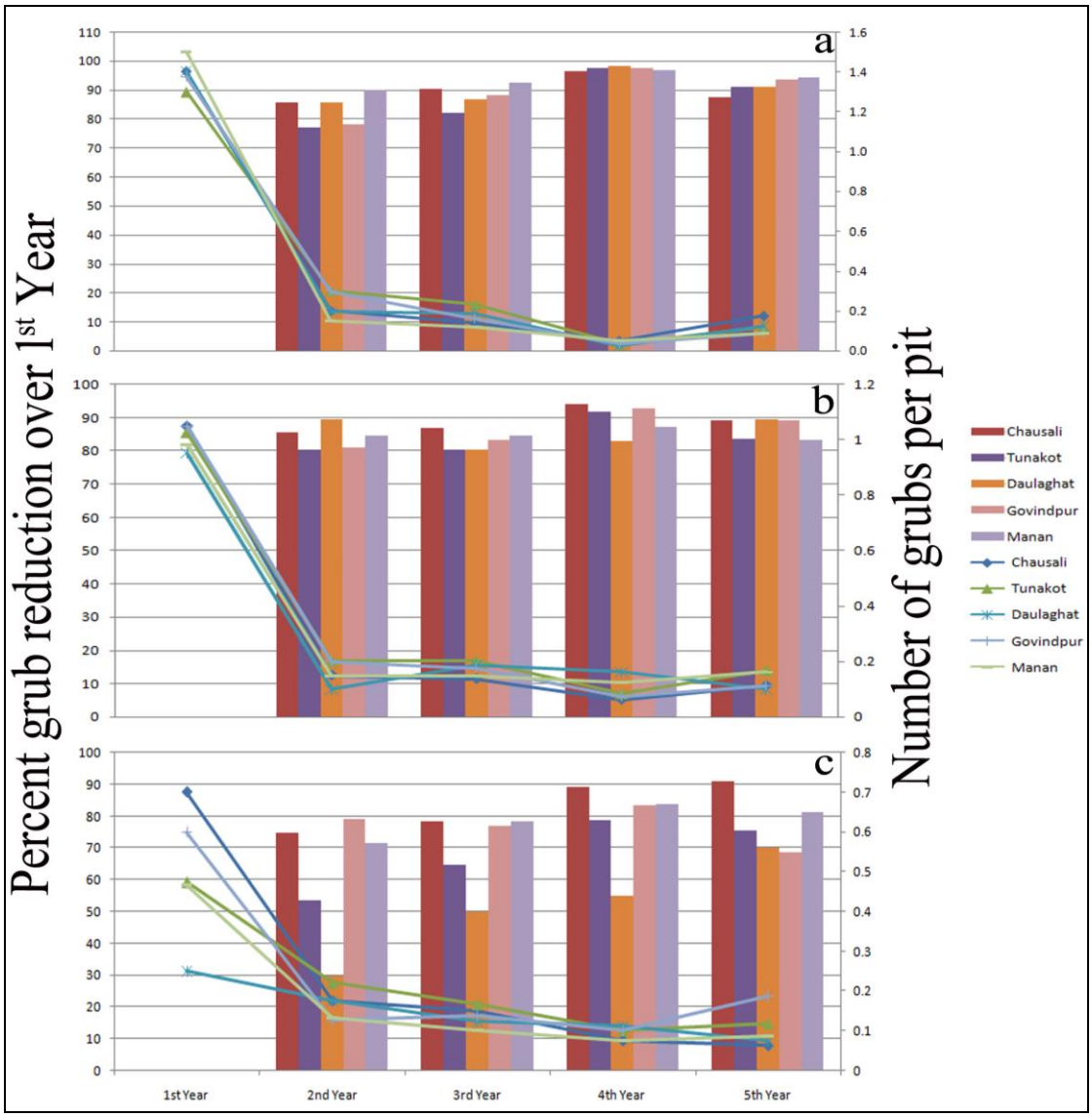


Fig 1: Grub population reduction of targeted villages in different months for the month of June, (b) For the month of July, (c) For the month of August

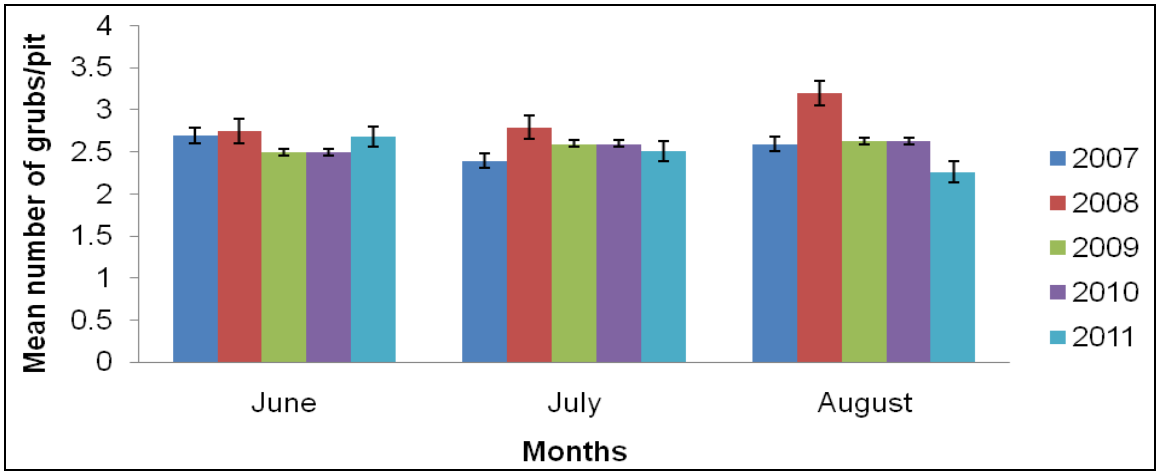
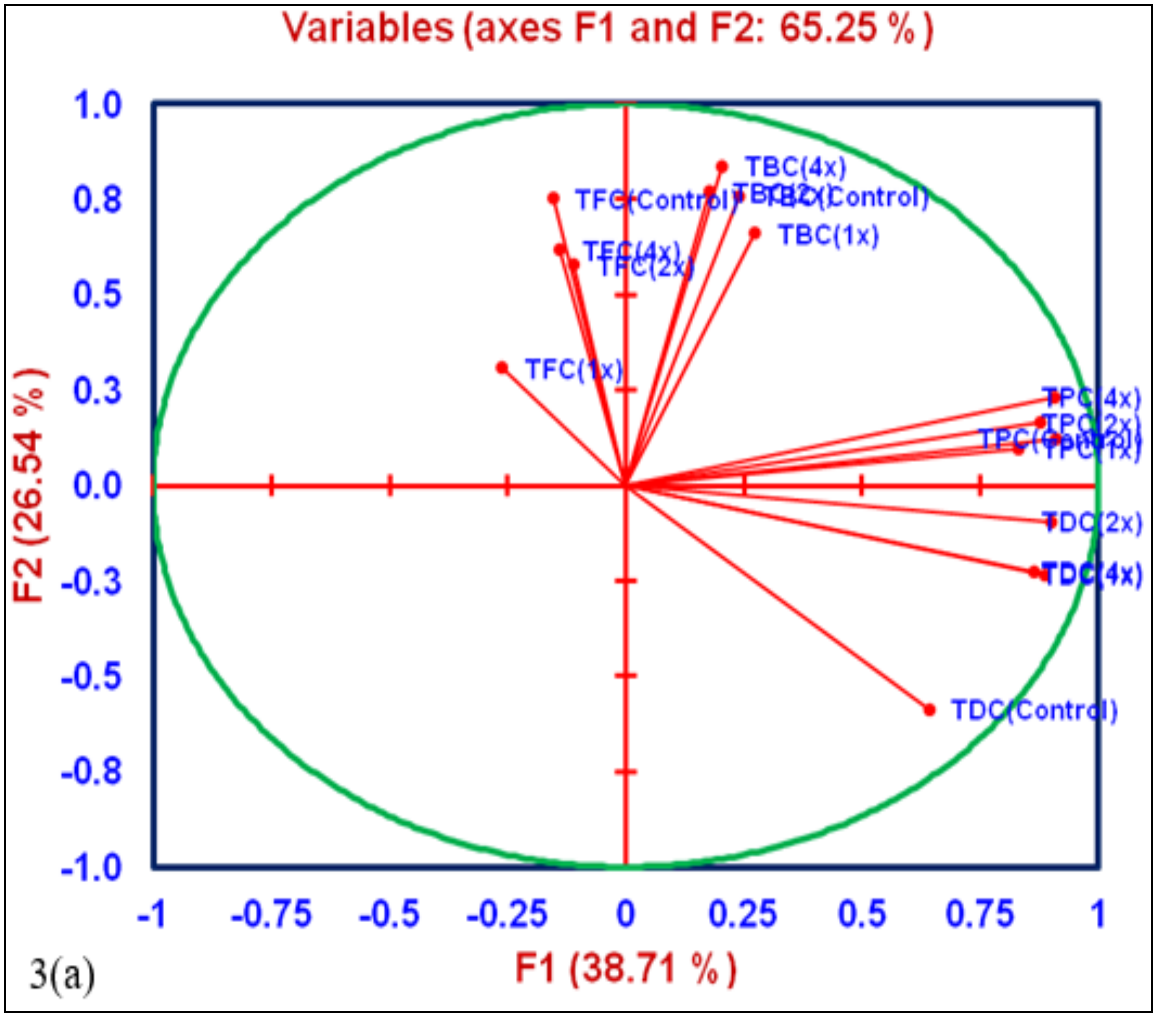


Fig 2: Grub populations in non adopted village, Dashpad



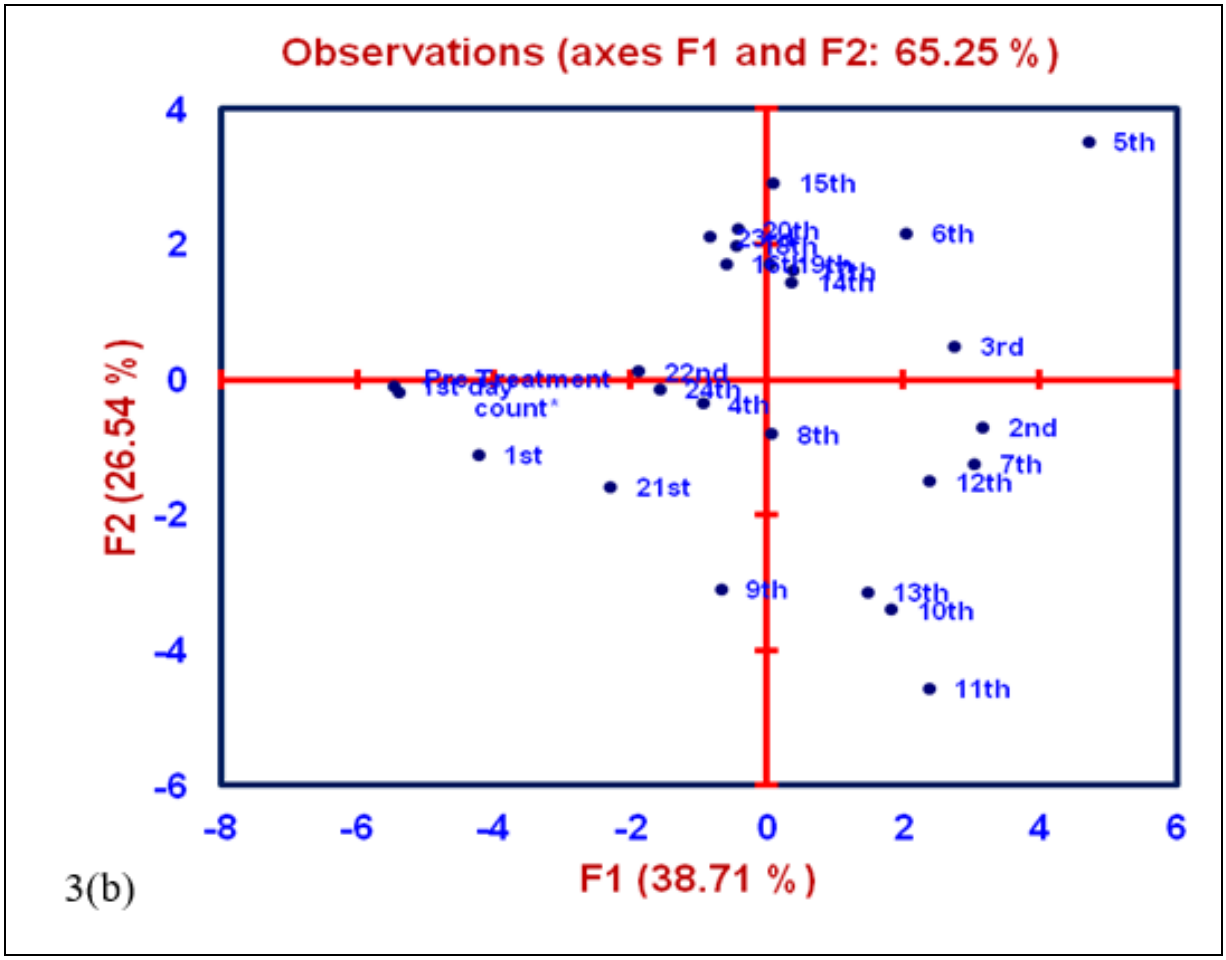


Fig 3: Multifactorial comparison of the field experiment using PCA. (a) Correlation between indigenous microbial population densities, (b) Correlation between four treatments and time interval. *Parameter codes:* TBC- Total bacterial population; TFP- Total fluorescent Pseudomonads; TDB- Total diazotrophic bacteria and TFC- Total fungal counts

Conclusions

The present investigation on white grub management through talc based formulation of a native isolate of entomopathogen, *B. cereus* strain WGPSB-2 has provided eco-friendly, cost effective and sustainable technology option. The high degree of management provided by the bio-agent may be attributed to the nativity of the pathogen to the region. However, its wide spread usage comes from its performance over the different agro climatic conditions and its pathogenicity against other species of white grubs.

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