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Full length Research paper

Growth Characterisitics And Pathogenecity Of Indigen -ous *Beauveria Bassiana* Isolates Against The Nyambe -ne Tea Weevil(*Sphrigodes Mixtous*) In Kenya

[*1]Evelyn Cheramgoi, [2]Samuel Muluka, [1]Irene Cherono [3] Robert Nyukuri and [2]Jackline Chepkemoi

[1]Crop Improvement Programme, Integrated Pest and Disease Management (IPDM)Section, Tea Research Foundation of Kenya(TRFK), P.O. Box 820-20200, Kericho
[2] Department of Medical and Applied Sciences Rift Valley Technical Training Institute,P.O. Box 244 ,30100, Eldoret
[3]Department of Biological Sciences, School of Science, University of Eldoret, P.O. Box 1125, Eldoret, Kenya.

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22 Local isolates of Beauveria bassiana were characterised on their radial growth, sporulation, germination and assayed for insecticidal activities against adult Nyambene tea weevils. The isolates were coded according to the different places they were isolated from. They include Bb Ke 1-5 recently isolated from Kericho County, TRFK Timbilil Tea Fields and isolates Bb Ke 6a and Bb Ke 6b isolated in 2006 from within Kericho County, TRFK Timbilil tea Estate Fields, Bb Gi7 series which were isolated from soils and assorted weevils from Giciaro farm in Nyambene, Meru County, Bb Ch 8 and Bb Ch c(1-5) isolated from Chepkoilel soils in Eldoret, Uasin Gishu County and Bb Mu 9 series which was isolated from Mununga, Kirinyaga County. The growth characteristics were determined by measuring the radial diameter in mm for seven days of the isolates growing on Potato Dextrose Agar (PDA), sporulation was determined after 2 weeks using a Neubauerheamacy to meter under a light microscope (40X) and germination of conidia after 16hrs of inoculation on freshly prepared PDA plates. A few representatives of the isolates were chosen to determine their efficacy against the Nyambene weevils (Sphrigodes mixtous) by dipping in conidia concentration of 10^5, $10^4$and 10^3. The radial growth, germination and sporulation varied in the different isolates. Bb Ke 4 (51mm) and Bb Ke 5(31.33mm) exhibited higher radial growth, Bb Ch c4 (17216.70) had high sporulation whereas Bb Ke 6a (75.67%), Bb Ch c3 (70%) and Bb Ch c4 (68%) showed high number of germinating conidia. Other isolates exhibited slow growth rates i.e. isolates Bb Gi 7f (8.33mm), Bb Gi 7c (8.67mm), Bb Gi 7a (9mm) and Bb Ke 3 (9.33mm). Isolates Bb Ke 5 (925), Bb Ke 4 (1058.3), Bb Gi 7d (1375), Bb Ke 1Bb Ke 1(1800) and Bb Ke 2(1483.30) showed low sporulation. Low number of germinating conidia was seen in Bb Ke 1 (13.67%), Bb Gi 7f (19%), Bb Ke 3 (20%), Bb Ch 8b (30.67%) and Bb Ke 4 (24.33%) isolates. Isolate Bb Gi 7a showed higher insecticidal activity against the tea weevils compared to the other few selected isolates. High negative correlation was noted between some variables like radial growth and spore count. A positive correlation between the cfu and germinating conidial count was noted as well as between spore count and germinating conidia.

Key words: *Beauveria bassiana***,** radial, germination, sporulation, efficacy, tea, *Sphrigodes mixtous,* isolates, spore, microscope.

INTRODUCTION

Several insect pests have been recorded on tea affecting production and prominent among which is the tea weevils ((Anon, 2002). Tea weevils reported to occur in Kenya are Kangaita/Kimari Weevil (*Entypotrachelus meyeri*) [micans/Kolbe], Nyambene Weevils (*Sphrigodes mixtous*) and *Systates spp.* Weevils (Benjamin, 1968). Adult weevils damage tea by defoliating nursery, newly established and more often on mature tea orchards; in addition, they occasionally girdle roots, the stem just above ground level and feeding on the bark of twigs and branches of tea (Benjamin and Demba, 1968; Muraleedharan and Chen, 1997). All these damages lead to decline in productivity, although the effect on

Correspondence Author's Email: cherameve@yahoo.com

productivity has not been studied, effect on tea production must be substantial.

The development of insect resistance to chemical insecticides and the concern over the deleterious effects of chemicals on environmental and human safety have provided a strong impulse to the development of microbial control agents for use in integrated control of insect pests. As part of increasing emphasis on the use of entomopathogenic fungi in biological control programmes, the potential of several species of class Hyphomycetes as microbial insecticides has been reviewed by many authors (Hajek and St. Leger, 1994; Boucias and Pendland, 1998; Butt and Goettel, 2000). Hypomycetes include the most promising fungal species employed against a variety of different insect pests in different agroecosystem. Several taxa including *B. bassiana, Metarhiziumanisopliae* and *Verticilliumlecanii* have demonstrated excellent capacity of reducing pest populations. *B. bassiana* is the causative agent of the white muscardine disease of many insect species (Tanada and Kaya, 1993) and under certain climatic conditions causes epizootics among field insects and soil borne pests. *B. bassiana* has been isolated from a variety of mites and soil insects e.g. grub, lepidopteran pupae, termites, ants, etc. (Keller and Zimmermann. 1989; Feng*et al*., 1994) and also grows on soil as a saprophyte (Bidochka*et al.,* 1998). The entomopathogenic fungus, *B. bassiana* contains a diverse assemblage of genotypes and probably comprises species complexes. Therefore it is conceivable to have individual isolates or pathotypes which exhibit a substantially restricted host range (Inglis*et al.*, 2001). The entomopathogenic fungi have attracted substantial attention as biological control agents of insect pests. It is widely distributed throughout the world and can be isolated from insects, mites, and soil. This fungus can infect a wide range of hosts and shows potential for commercial development as a biological control agent of agricultural insect pests.

The isolation of indigenous isolates or strains of mycopathogens from different hosts or localities provides control programmes with available and specific tool of controlling certain indigenous pests. Therefore isolating entomopathogens from soils provides insight into the natural occurring pathogens biodiversity and provide a pool of potential control agents. The present work aimed at evaluating the relationship between the radial growth, germination sporulation of local *B. bassiana* isolates and screening for their insecticidal activities against the Nyambene tea weevil pests.

MATERIALS AND METHODS

Rearing of *Galleria mellonella*

The study involved first isolation of the *B. bassiana* fungus from the soil. The insect bait method was used for the isolation of *B. bassiana* isolates. Trap larvae of the waxy moth (*Galleria mellonella*L (Lepidoptera: pyralidae)) were used as insect baits to capture entomopathogenic fungi from soil samples collected from different localities. The trap insect technique (Bedding and Akhurst, 1975) was originally used for the isolation of entomopathogenic nematodes from soil. A colony of *Galleria mellonella* L which was originally obtained from Kenya Agricultural Research Institute (KARI), National Horticultural Research Centre (NHRC) in Thika, was maintained in the laboratory at room temperature (20°C ± 3). The larvae were reared on a Galleria diet which consists of 307g maize meal, 225g honey, 45g bees wax and 90g yeast. The diet was prepared by first boiling the bees wax, once melted it was mixed with honey. The mixture of maize meal and yeast was poured into the melted bees wax and honey and stirred while cooking on a Bunsen flame until it becomes firm and evenly mixed. The mixture was then placed on a bowl with a perforated lid (Plate 1) and left to cool overnight. Then followed by the introduction of the larvae of *G. mellonella.*

Moist soil was then placed in a Petri dish (should not be moist to the point of forming clumps or moist at approximate field capacity) approximately 10 medium sized larvae were placed into the soil. The dishes were regularly turned in the beginning of baiting period (first week) to make bait insects penetrate as much soil as possible while they are still vigorous. The larvae were left in the soil until a fungus growth is seen on it growing (Plate 2). Whole infected larvae that already showed hyphal growth on their bodies was first surfaced sterilised with 70% ethyl alcohol prior to incubation on potato dextrose agar (PDA) to prevent external saprophytic fungi from growing.

The larvae were incubated at room temperature until adequate growth of fungus was observed, and then the fungus was transferred to fresh PDA medium and incubated for at least 7 days under the same conditions. PDA culture is known to be among the cultures that has induced the best linear growth for *B. bassiana* according to the findings of Santa *et al.,* (2005). After sporulation, microscopic examinations of the fungus were done. Whole living larvae that might be infected with entomopathogenic fungi were surface-sterilised by dipping consequently in 70% ethyl alcohol and sterile distilled water; each for 3 seconds. Insects were then cultured under the same conditions as mentioned above.

Fungal growth Characteristics

Radial growth

The 22 isolates of *B. bassiana* obtained from different sites were each cut aseptically 2mm diameter from the edge of the growing mycelium and placed in the centre of freshly prepared PDA plates then sealed with parafilm. The experiment design was complete random with three

Plate I: Bowl with perforated lid with Galleria diet

Plate 2: Sporulated isolate of *B. bassiana* grown on PDA cultures

replicates on each isolate. The inoculated PDA cultures were then incubated at room temperature (18°C) for 24hrs then the diameter of the growing colony was measured daily for 7 days on a pre-marked line with a ruler.

Spore count

B. bassiana isolates were allowed to grow for 2 weeks to sporulate (Plate 2). A conidial suspension of each isolate was made using 10mls of sterile distilled water by scrapping off the conidia using a sterile glass slide which was then poured into a sterile test tube as the stock solution. The conidia were then counted under a light microscope (40X) using a Neubauer haemocytometer.

Colony count

In three replicates, 0.1ml of aqueous conidial suspensions (10^{-3}) of each isolate were spread-plated on PDA in Petri dishes then sealed with a parafilm and incubated at 18°C for 16hrs. Viability was determined by examining 100 conidia per plate using a light microscope (40X). A conidium was considered germinated if the germ tube was at least as long as the swollen conidium.

Colony forming unit (cfu)

In each isolate, 0.1ml of aqueous conidial suspensions (10^{-3}) were spread-plated on PDA in Petri dishes, in three replicates, sealed with a parafilm and incubated at 18°C for 48hrs then the cfu were counted.

Laboratory Bioassay-Susceptibility of weevils to the fungal isolates

Bioassays of *Beauveria bassiana* against the Nyambene tea weevils on five (5) isolates were performed. The weevils were collected from the tea fields. Active adult Nyambene tea weevils (plate 3) were used for the bioassays. The isolates used in the bioasays were taken from pure cultures in long term storage. PDA cultures of the isolates were flooded with 10ml of sterile water containing 0.1% tween 20 solution and the spores were removed by gentle agitation with a sterile loop. The spores were counted with the help of a haemocytometer and the spore concentrations were adjusted to 10^5, 10^4 and 10^3 conidia/ml accordingly in sterile distilled water. The bioassays were set up in a completely randomized design with three replicates of 6 adult weevils per replicate. The adult weevils were exposed to the fungus by the dipping technique, where they were placed in a large petri-dish (125 mm diameter) containing 30 ml of each fungal suspension for 1 minute. Sterile-distilled water containing 0.1% of tween 20 was used instead of fungal suspension for control treatment. Each batch of adult weevils was transferred to filter paper lined in a Petri dish on the laboratory bench for 1 h to dry and then transferred or placed in perforated rearing jars and offered appropriate and equal pieces of tea shoots

Plate 3: Active Adult Nyambene weevils (*Sprigodesmixtous)*

Plate 4: Perforated rearing jars with small beakers planted with pieces of tea shoots for the adult weevils to feed on

Plate 5: Dead Cadavers of the Nyambene weevils

planted in small beakers to feed on (Plate 4).Weevils' mortalities were recorded up to 30 days post-exposure or up to 100 % mortality. The eaten tea leaves were replaced with fresh tea leaves every 3-4 days and the area eaten was measured and calculated using a graph paper. Dead weevils were counted, removed and incubated on damp filter paper within Petri dishes (20 °C) and inspected for the presence of *B.bassiana* mycelium on the cadavers (Plate 5)

STATISTICAL DATA ANALYSIS

SAS version 9.0 was used for all statistical ANOVA analyses on radial growth, sporulation, germination and weevils' mortalities Correlation analysis was used to measure the linear relationship between two variables (Bewick*et al.,* 2003).

RESULTS

Radial growth of the 22 *B. bassiana* isolates

Bb Ke 4 had the highest growth rate followed by Bb Ke 5 while Bb Chch 8d, Bb Ch c3, Bb Ch c5, Bb Mu 9, Bb Ch c1, Bb Ch c4, Bb Chch 8a, Bb Ke 6a and Bb Chch 8c had moderately high growth rates. Bb Ke 1, Bb Gi 7e, Bb Ke 2Bb Ke 2, Bb Ch c2, and Bb Ke 6b also had moderately

Table 1: Growth parameters of the 22 *Beauveriabassiana* isolates

Isolate	Radial growth for 7 days(mm)	Non germinating(%)
Bb Ke 1	8.05def (2.04)	86.33a
Bb Ke 2	7.62ef (1.92)	58.67ef
Bb Ke 3	5.86fg (1.85)	83.33ab
Bb Ke 4	22.05a (2.74)	75.67c
Bb Ke 5	17.86b (2.67)	61.33e
Bb Ch c1	10.81c (2.28)	37.00j
Bb Ch c2	6.24fg (1.92)	42.67i
Bb Ch c3	11.62c (2.32)	30.00l
Bb Ch c4	10.71c (2.29)	32.00lk
Bb Ch c5	11.24c (2.31)	47.67h
Bb Ke 6a	10.19cd (2.23)	24.33m
Bb Ke 6b	6.57fg (1.91)	51.00gh
Bb Gi 7a	5.24g(1.75)	54.33fg
Bb Gi 7c	4.8g (1.73)	49.00h
Bb Gi 7d	6.57fg (1.88)	60.67e
Bb Gi 7e	7.00fg (2.03)	66.00d
Bb Gi 7f	5.1g (1.67)	81.00b
Bb Ch 8a	10.52cd(2.23)	49.33g
Bb Ch 8b	5.62hij(1.88)	69.33d
Bb Ch 8c	10.24cd(2.04)	60.00e
Bb Ch 8d	11.71c(2.34)	48.00g
Bb Mu 9	9.48cde (2.14)	36.33jk
CV%	**8.60**	**4.74**
LSD(P≤0.5)	**0.11**	**4.28**

**The figures in parenthesis are log transformed ln(x+1). Means followed by the same letter in the column are not significantly different at P< 0.05.*

low growth rates. Bb Gi 7d, Bb Ke 3, Bb Gi 7a, Bb Gi 7c and Bb Gi 7f had the lowest growth rates (Table 1).

Spore count

Bb Ch c4 had the highest number of spores followed by Bb Ke 6a, Bb Mu 9, Bb Ch c3, Bb Ke 6b, Bb Ch c2 and Bb Chch 8d whereas Bb Ke 1, Bb Ke 2Bb Ke 2, Bb Gi 7d, Bb Ke 4 and Bb Ke 5 had the least number of spores. Bb Ch c5, Bb Ch c1, Bb Gi 7c, Bb Chch 8a and Bb Gi 7a had moderately high number of spores while Bb Ke 3, Bb Chch 8c, Bb Chch 8b, Bb Gi 7f and Bb Gi 7e had moderately low number of spores (table 1 and figure 1a).

Germination counts

Bb Ke 6a, Bb Ch c3, Bb Ch c4, Bb Mu 9, Bb Ch c1 and Bb Ch c2 had high numbers of germinating conidia

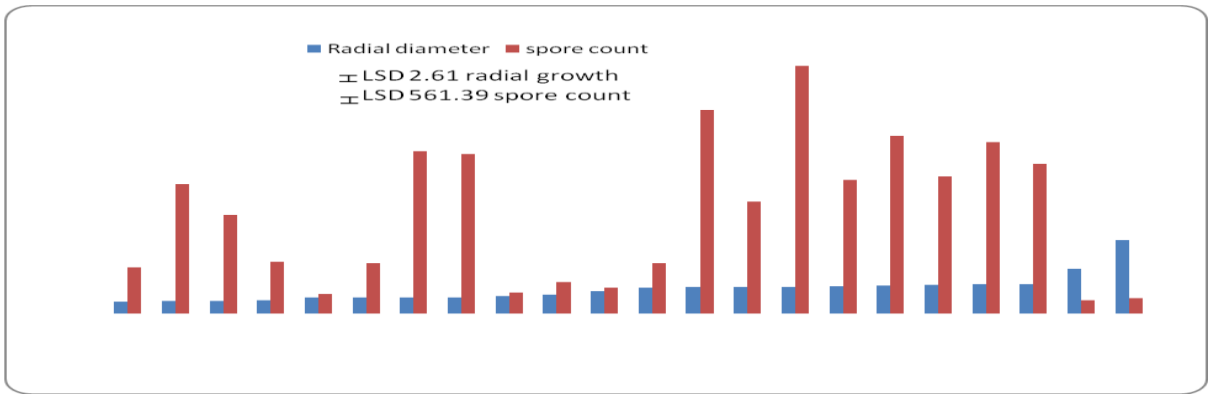

Figure 1a: Spore count of *B. bassiana*isolates under a light microscope and radial diameter in mm on day 7

There was a degree of negative correlation between the radial diameter and spore count of the isolates (Figure 1b).

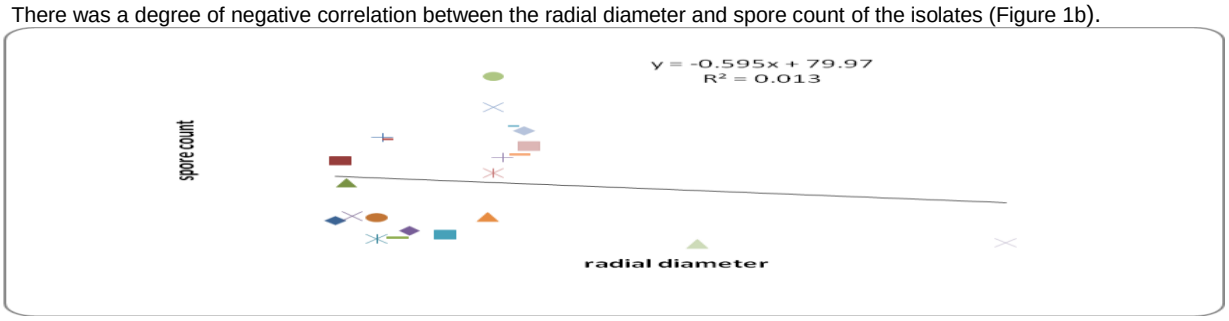

Figure 1b: Correlation between radial diameter and spore count

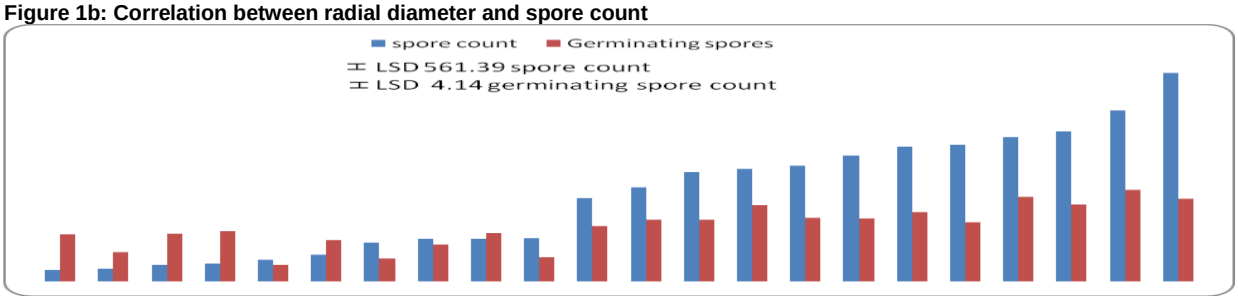

Figure 2a: Germination and spore count of *B. bassiana* isolates after 16hrs under a light.

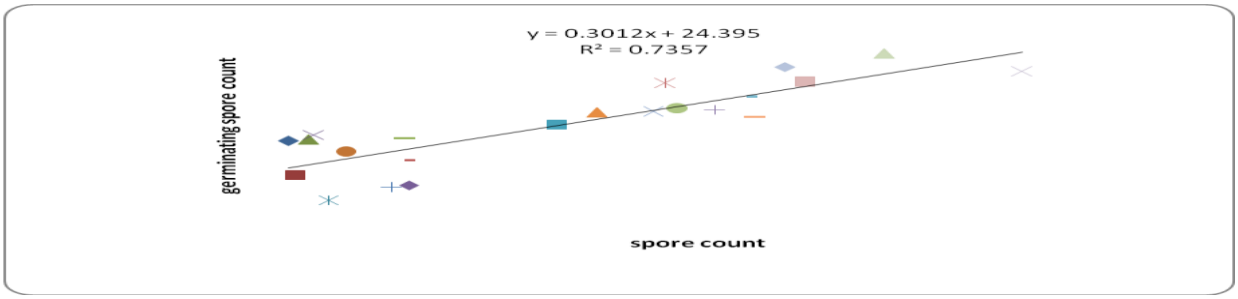

Figure 2b: Correlation between spore count and germinating spores

whereas Bb Ke 1, Bb Gi 7f, Bb Ke 3, Bb Chch 8b and Bb Ke 4 had the lowest number of germinating conidia. Bb Ch c5, Bb Chch 8d, Bb Gi 7c, Bb Chch 8a, Bb Ke 6b and Bb Gi 7a had moderately high number of germinating conidia while Bb Ke 2, Bb Chch 8c, Bb Gi 7d, Bb Ke 5 and Bb Gi 7e had moderately low number of germinating conidia.

A high positive correlation between the spore count and the germinating spores of the isolates was noted.

Colony forming units

Bb Ke 6a had the highest number of colony forming units followed by Bb Ch c5, Bb Ch c3, Bb Ch c1, Bb Ch c4, Bb Chch

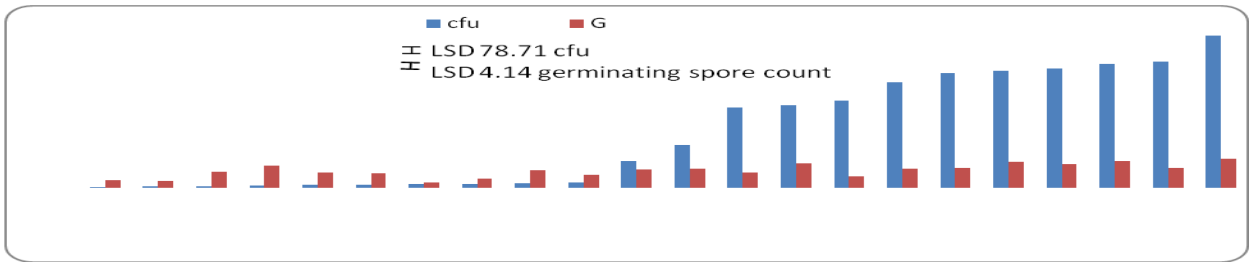

Figure 3a: Germination and cfu counts of *B. bassiana* isolates after 16hrs and 48hrs respectively under a light microscope

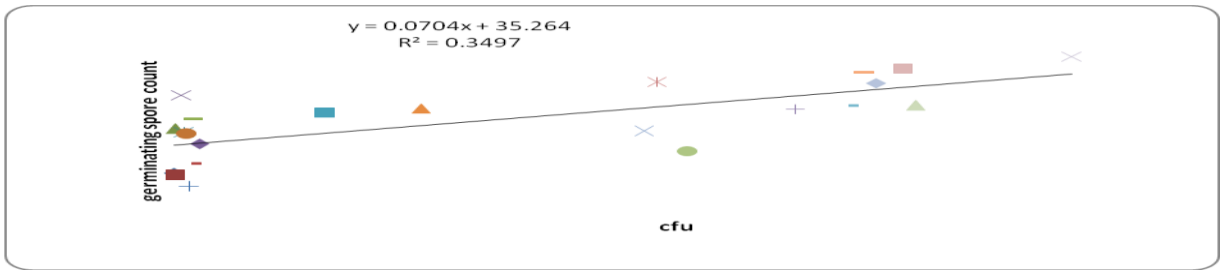

Figure 3b: Correlation between cfu and germinating spore counts

Figure 4: Effect of five *BeauveriaBassiana* isolates on mortality of adult tea weevils over time.

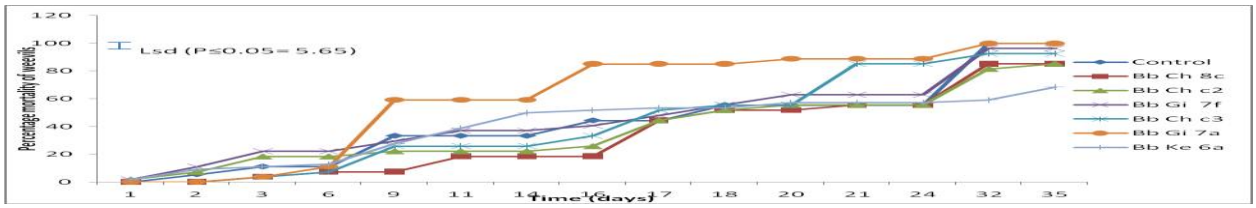

Table 2: Effect of *B. Beauveria* isolates on feeding of the tea weevil (Area eaten in cm^2)

Isolates	**Concentration**			**area of leaf eaten (cm^3)**
	10^5	10^6	10^7	
Bb Gi 7a	2.13	1.39	1.11	1.55
Bb Ch c2	1.59	2.19	1.70	1.72
Bb Gi 7f	2.30	2.00	2.23	2.18
Bb Ch c3	3.78	2.02	3.48	3.09
Bb Ch 8c	2.37	2.72	2.03	2.37
Bb Ke 6a	2.21	1.69	2.04	1.98
Control	2.80	2.80	2.80	2.80
CV (%)				49.70
LSD (p≤0.05)				
Isolates				**1.08**
Concentration				**NS**

8d, Bb Chch 8a, Bb Chch 8b, Bb Mu 9 and Bb Chch 8c. The isolates with the lowest number of colony forming units include Bb Ke 3, Bb Gi 7f, Bb Ke 2, BbCh c2, Bb Gi 7d, Bb Ke 5, Bb Ke 1, Bb Ke 4, Bb Gi 7a and Bb Gi 7e. Bb Ke 6b and Bb Gi 7c had moderately high numbers of colony forming units.

There was a positive correlation between cfu and germinating spore count of the isolates (Figure 3b).

Effect of *B. bassiana* on population of the tea weevils

Isolate Bb Gi 7a proved to have higher insecticidal activity against the tea weevils compared to the other isolates (Figure 4). This study also indicates that the effect of the insecticidal activities of the isolates shows up between the 9thday and 15th day (Figure 4). There was no significant difference on the concentration of the Isolates

Effect of *B. beauveria* isolates on feeding of the tea weevil

According to the results obtained from the experiment there was a significance in the different isolates in area of the leaf eaten. Isolate Bb Ch c3 had the largest area eaten by the tea weevils but not significantly different to leaf area eaten bythe weevils in the controlled treatment. The isolate treatment with least eaten area was Bb Gi 7a followed by Bb Gi 7f, Bb Ch c2, Bb Chch 8c and they do not differ significantly (P≤0.05) (Table 3). .

DISCUSSION & CONCLUSION

There were significant differences in radial growth, germination, sporulation and colony count among the different *B. bassiana* isolates included in this study. The isolates obtained from Kericho had high radial growth but low sporulation and some have low number of germinating conidia such as Bb Ke 4 and Bb Ke 5. Isolates from Chepkoilel had high sporulators with a high number of germinating conidia such as Bb Ch c4. those isolates also had high numbers of colony forming units. Most of those isolated from Giciaro farm in Nyambene exhibited slow growth rates e.g. Bb Gi 7a, Bb Gi 7c and Bb Gi 7f, and had low colony counts. Isolates from Mununga also showed high growth rates, high spore count and high number of germinating conidia while one isolate from TRFK stock (Bb Ke 6a) showed high spore count, high numbers of germinating colonies and colony counts but had a moderate rate of growth.

The daily hyphal growth rate observed in this study was similar to that obtained by Fargues*et al.,* (1997), Ouedraogo*et al*., (1997), Thomas and Jenkins (1997), Varela and Morales (1996), and Vidal *et al.,* (1997) (between 1.0 and 6.0 mm/day). It is believed that fungal isolates with rapid germination and hyphal growth rates may have an advantage as biological control agents because host infection can potentially occur much more quickly (Hajek and St. Leger, 1994; Varela and Morales, 1996). In this study, isolates from TRFK stock, Chepkoilel, Mununga and those from Kericho had these traits thus may have an advantage as biological control agents.

Spore production by the *B. bassiana* isolates, in this study ranged from 9.25 x 10^2 to 1.7216 x 10^4conidia/cm2, which was quite different from that of Varela and Morales (1996) where production ranged from 2.2 x 10^8 to 1.6 x 10^9conidia/cm^2. Differences in fungal isolates and incubation temperatures (18^{0}C in our study vs. 27^{0}C in theirs) may have contributed to this discrepancy.

High negative correlation was noted between some variables like radial growth and spore count such that isolates with a high radial growth had no relationship with spore count. A positive correlation between the cfu and germinating conidial count was noted. Isolates with high spore count had high number of germinating conidia indicating a positive correlation as well.

It is evident from the results of this study that some *B. bassiana* isolates are most virulent on the tea weevil. Bb7a was found to be the most virulent on the tea Nyambene weevil. This observation may be attributed to the locality of the isolate which is the common habitat of the Nyambene weevil and therefore this may mean a local isolate is more promising as a control agent since it has adapted to the local conditions. The study also has shown that the virulent type affects the feeding rate of the weevils pest, this implies that the more the *B. bassiana* penetrates the body of an insect the weaker the insect becomes hence feed less. Therefore this study proves that the entomopathogenic fungus *B. bassiana* contains a diverse assemblage of genotypes and probably comprises species complexes and as reported by Inglis *et al* in year 2001, it is conceivable to have individual isolates or pathotypes which exhibit a substantially specificity on the host range.

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