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Effect of Temperature on Growth, Germination, Sporulation and Phathogenicity of *Beauveria Bassiana* BB1 Isolated from Black Pepper Soil against Root Mealybug *Formicococcus* sp. (Homoptera: Pseudococidae) in Dak Lak Province of Viet Nam

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Abstract: Selection of potential entomophathogenic fungi isolates for biocontrol control need base on the assessment their biological attributes to determine that may be better suited for the local environment. This study aimed to evaluate the effect of temperature on growth, conidia germination, and phathogencityof the fungal strains Beauveria bassiana BB1 isolated from black pepper soil against root mealybug Formicococcus sp.As a result of the provided analyses, it was shown that the optimum temperature growth was between 25°C to 27°C, while the growth stops was at 35°C. However, conidia harvested from different temperature levels (20^{o}C, 22^{o}C, 25^{o}C, 27^{o}C, 30^{o}C, 32^{o}C)for culture has no effect on mortality of adult mealybugs. It was concluded that Beauveria bassiana BB1 is active within temperature ranges experienced in black pepper fields in Dak Lak province of Viet Nam.

Keywords: black pepper, root mealybug, entomophathogenic fungi, Beauveria bassiana.

I. Introduction

In recent years, the sharping increase in area, intensification and excessive use of chemical pesticides have caused the pepper production industry in Vietnam to face many challenges related to pests (Lesueur et al., 2022). Root mealybugs Formicoccus sp. (Homoptera: Pseudococcidae) currently become an economically important soil- borne insect pest in the production of black pepper (Piper nigrum L.) in Dak Lak province of Viet Nam. Using pesticides to control black pepper root mealybugs is not high effective because root mealybugs often live in thick mycelium layers formed by combining them with a special fungus. Therefore, development a microbial control agent in regulation of black pepper root mealybug is necessary for sustainable production of black pepper.

Beauveria bassiana is a well-known species of entomophathogenic fungi with thousands of isolates have been collected from different parts of the world (Mascarin and Jaronski, 2016). It can infect about 700 species of hosts belonging to all Acari and Insecta order (Zimmermann, 2007). There are many registered commercial products based on *B. bassiana* for use in biological control against various arthropod pests around the world (Mascarin and Jaronski. 2016). *B. bassiana* has been reported that can apply in management of root mealybug on enset in Ethiopia (Lemawork *et al*., 2011), coffee in Brazil (Andaló *et al*., 2004) black pepper in India (Ummer and Kurien, 2021).

Temperature has been confirmed by many studies that was an important role in the success of entomophathogenic fungi as a bioagent in integrated pest management (Acheampong *et al*., 2020; Kumar *et al*., 2021; Poidatz *et al*., 2019; Teja and Rahman. 2016; Tumuhaise *et al*., 2018; Yeo *et al*., 2003). In general, most entomopathogenic fungi can tolerate in a wide range of temperature from 0^{0}C to 40^{0}C (Lacey *et al*., 2001) However, each strain entomophathogenic fungi usually require the different optimal temperature for conidial germination, mycelium growth, sporulation and infection on target insect pest (Acheampong *et al*., 2020; Cabanillas and Jones. 2009). Therefore, the study to determine the appropriate temperature for each indigenous entomophathogenic fungi strain is always necessary in order to select the potential strain for development as a microbial control agent.

Our study focused on investigating the effect of temperature onthe rate of germination, the radial growth of the colony, the conidial production, and the phathogenicity of *B. bassiana* BB1 against *Formicococcus* sp. with seven temperatures levels (20, 22, 25, 27, 30, 32, and 35^{o}C). The result of this study is one of the criteria to decide whether to selection *B. bassiana* BB1 as biological control agent for black pepper root mealybug in

II. Material and Method

Source of fungi and mealybug

A fungal strain (*B. bassiana* BB1) was isolated from a soil sample of the black pepper rhizosphere in Cao Thanh village, Eakao commune, Buon Ma Thuot city, Dak Lak province. *B. bassiana* BB1 strain was identified by molecular analysis and submitted to Genbank with Accession Number (OQ618225). Pure fungal strain were cultured on PDA slant tube stored in refrigerator at 4°C and sub-cultured monthly (Sevim *et al*., 2010)

Formicoccus sp. was collected from black pepper at the above location and identified by morphology and molecular analysis with the support of Dr. Dao Thi Hang, (Vietnam Plant Protection Institute). Mealybug was reared with fresh pumpkin at 25–28°C, 70% - 80% RH, in the dark. Second generation, 3-day-old adult female mealybugs were used in the experiments.

Preparation of Conidial Suspensions

*B. bassiana*BB1was activated on petri dishes containing PGA medium and incubated for at 25° and 12:12 (L:D). After 14 days, conidia were scraped from the surface of the PGA medium and suspended in falcon tubes in sterile distilled water containing 0.05% Tween 80. The suspension was vortexed and passed through double layer of cloth to collect a homogeneous conidial suspension (Kumar *et al*., 2021). A Neubauer Improved Hemocytometer was used to measure the number of conidia in suspension and adjust them to suitable concentrations for each experiment.

Effect of temperature on germination of fungal conidia and sporulation in vitro

100 µl conidial suspension (10^3cfu.ml^{-1}) were spread on PGA in 9 cm - petri dishes and incubated at 20, 22, 25, 27, 30, 32, and 35^{o}C, 65% RH and 12:12. (L:D). Ten replicate dishes were done for each temperature level. The number of the colonies on petri dish were counted to calculate the germination rate after 12, 24, 48, 60 and 72 hours. A square plug (1cm^2) was randomly taken with the help of a cork borer after 14 days. Each plug was placed in 10 ml of 0.02% Tween 80 solution and vortexed to suspend the spores. Spore yield was determined using a Neubauer Improved Hemocytometer (Mustafa and Kaur. 2009). Log transformation was used to normalize the numbers of B. bassiana spores.cm-2 before analysis using statistical analysis software.

Effect of temperature on colony growth and pathogenicity of fungi in vitro:

A circular plug (5mm diameter) was cut from non-sporulating mycelia on 3-day-old culture dishes then transfered to the center of a new dish of PDA medium. Ten dishes for each temperature were placed in each incubators maintained at 20, 22, 25, 27, 30, 32, and 35^{o}C, 65% RH and 12:12. (L:D). The radial of colony growth was measured every 5 days until 20 days after culture. After 20 days, conidia were harvested for bioassay root mealybugs. Inoculum from different temperature level was assayed against the adult mealybugs. 30 adult female mealybugs were dipped in conidial suspension (1 x 10^7cfu.ml^{-1}) for 60 seconds. The inoculated mealybugs were placed on surface sterilized pumpkin in laboratory at room temperature. Inoculated mealybugs were checked daily and the mortality was recorded.

The AUDPC (Area Under Disease Progress Curve) of the germination, radial colony growth and pathogenicity of *B. bassiana* BB1 in vitro were calculated for each temperature and each replicate (Campbell and Madden, 1990; Poidatz *et al*., 2019)

Data Analysis

The Statistical Package for the Social Sciences 20 (SPSS 20) software is used for all analyses. The values were analyzed using simple variance analysis (ANOVA) to compare average values ($p \leq 0,01$ was considered significant).

III. Results and Discussion

The results of the study showed that the conida of the *B. bassiana* BB1 strain did not germinate at 35^{o}C. The germination rate of *B. bassiana* BB1 conidia at six temperature levels was statistically significant at all the period given (**Table 1**). The optimum temperatures for the germination of *B. bassiana* BB1was 25^{o}C (AUDPC 72 hours after inoculation was 183, 33) and 27^{o}C (AUDPC 72 hours after inoculation was 187, 25). The lowest value of AUDPC 72 hours after inoculation was recorded at 32^{o}C and 20^{o}C (89, 50 and 92, 30 respectively). The study of Ahmad*et al.* (2016) reported that *B. bassiana* B2 and *B. bassiana* B4 strains both gave the highest germination rate at 25^{o}C. Moldovan *et al.* (2022) also confirmed that *B. bassiana* CNMN-FE-01 strain gave the highest germination rate at 25°C.

Table 1. Effect of temperature on germination rate of *B. bassiana* BB1conidia

Temprature	**Germination rate (%)**						**AUDPC**
(°C)	**12h**	**24h**	**36h**	**48h**	**60h**	**72h**	
20	0,00	8,50	9,60	66,80	69,30	69,30	92,30[d]
22	19,30	32,30	61,40	75,00	77,50	77,50	134,40[c]
25	52,40	67,60	95,10	95,10	95,10	95,10	183,33[a]
27	56,80	74,90	96,30	96,30	96,30	96,30	187,25[a]
30	10,00	72,30	80,30	80,30	80,30	80,30	158,60[b]
32	7,60	51,40	43,80	43,80	43,80	43,80	89,50[d]
35	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Fvalue							185,49
Pr > F							<0,001
CV%							7,11
Sig.							**

*In the AUDPC column within the same superscript letter(s) are not significantly different, based on Duncan's multiple range test, ** p < 0,01.*

The radial growth of the *B. bassiana* BB1was given in **Table 2 (**the *B. bassiana* BB1 did not grow at 35°C)**.** The growth of *B. bassiana* BB1 was fastest at 25°C and 27°C (AUDPC 20 days after inoculation was 367,19 at 25°C and 372,23 at 27°C), and slowest at 32°C **(**AUDPC 20 days after inoculation was only 54,14). Optimum temperatures for growth of four *B. bassiana* strains in study by Acheampong *et al*. (2020) ranged from 26, 19 to 28, 31°C. Amad *et al*. (2016) showed that the colony growth for *B. bassiana* was faster at 25°C. Research by Parveen and Jeyarani. (2023) reported that all four strains of *B. bassiana* (Bb111), *B. bassiana* (Bb112), *B. bassiana* (Bb113) and *B. bassiana* (Bb114) was the highest radial growth at 25°C.

Table 2. Effect of temperature on mycelia radial growth of the *B. bassiana* BB1

Temprature	**Mycelial Radial growth (mm)**						**AUDPC**
(°C)	**1 day**	**5 days**	**10 days**	**15 days**	**20 days**	**Average/day**	
20	2,70	6,40	10,17	12,95	16,25	0,58	158,46[c]
22	2,72	6,67	11,10	15,31	19,70	0,72	183,42[b]
25	2,91	11,60	20,28	31,28	43,75	1,72	367,19[a]
27	2,92	11,71	20,87	31,43	44,29	1,74	372,23[a]
30	2,66	6,09	9,11	10,84	14,36	0,50	137,95[d]
32	2,53	3,09	3,65	4,15	4,64	0,09	54,14[e]
Fvalue							3995,84
Pr > F							<0,001
CV%							3,05
P							**

*In the AUDPC column within the same superscript letter(s) are not significantly different, based on Duncan's multiple range test, ** p < 0,01.*

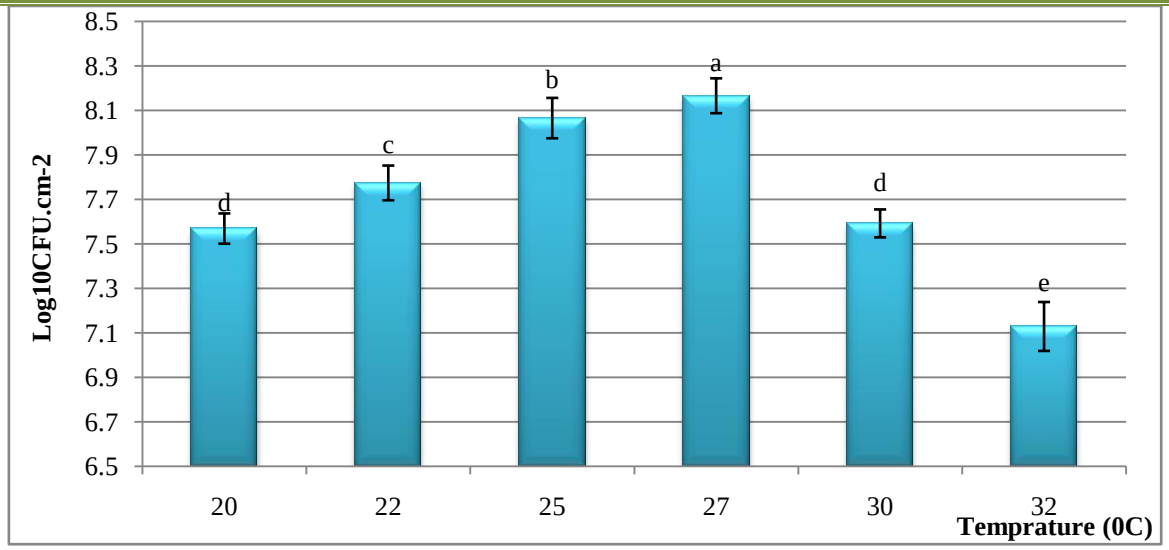

Figure 1. The sporulation of B.bassiana BB1 at different temprature levels. Different letters are used to indicate statistically significant differences between the values of temperature levels.

The temperature hassignificant impacton the sporulation of *B. bassiana* BB1**(Figure 1)**.The number of conidia/cm^2 produced by *B. bassiana* BB1 at the 27^{o}C and 25^{o}Cwas higehst compared to other temperature levels. Log$_{10}$CFU's cm^2 acounted for over 8,0 (8,17 at 27^{o}C and 8,06 at 25^{o}C), which was significantly higher than at 20, 22, 30 and 32^{o}C (only reached 7,57; 7,77; 7,59 and 7,13). The results of this study are similar to those of Dhar *et al* (2016) (Dhar *et al*., 2016), Amad et al (2016) (Ahmad *et al*., 2016), and Acheampong et al (2020) (Acheampong *et al*., 2020).

Table 3. Effect of temperature on phathogencity of *B. bassiana* BB1against adult mealybugs in vitro

Temprature (^{o}C)	**Mortality of adult mealybugs (%)**					**AUDPC**
	1 day	**3 days**	**7 days**	**11 days**	**14 days**	
20	0,0	22,2	71,1	88,9	100,0	812,2
22	0,0	20,0	64,4	87,8	100,0	775,0
25	0,0	20,0	72,2	88,9	100,0	810,0
27	0,0	18,9	73,3	92,2	100,0	822,8
30	0,0	20,0	66,7	88,9	100,0	787,8
32	0,0	18,9	64,4	86,7	100,0	767,8
F value						1,72
Pr > F						0,20
CV%						3,69
P						ns

In the AUDPC column within the same superscript letter(s) are not significantly different, based on Duncan's multiple range test, ns: non-significant differences.

Conidia harvested from its production different temperature levelshas no effect on mortality of adult mealybugs (**Table 3).** After 14 daysinoculated, mortality of *Formicoccus* sp. adult mealybugs at different temperatures levels reached 100%. The AUDPC of mortality of adult mealybugs after 14 days of inoculation was from 767,8 to 822,8 at all temperature levels.

Many previously reported that laboratory evaluation of temperature effect on germination, growth and sporulation and phathogencity of each entomophathogenic fungi strain is necessary to consider whether it has a potential in developing a mycoinsecticide for control of target insect (Acheampong *et al*., 2020; Wu *et al*., 2020; Yeo *et al*., 2003). Selection entomophathogenic fungi isolates has based on a range of characteristics including the ability to grow and germinate quickly in vitro at a temperature corresponding to the local temperature at which these isolates strain will be used (Yeo *et al*., 2003). In this study, the isolated *B. bassiana* BB1was able to germinate, grow and produce conidia in all six temperature levels (20, 22, 25, 27, 30, 32^{o}C) and with an optimum at 25°C and 27°C. The average temprature in Dak Lak province of Viet Nam is around 25^{o}C and

ranged between 13°C to 37°C. For these reasons, *B. bassiana* BB1may be the preferred isolate selected for controlling *Formicoccus* sp.mealybug species in this locality.

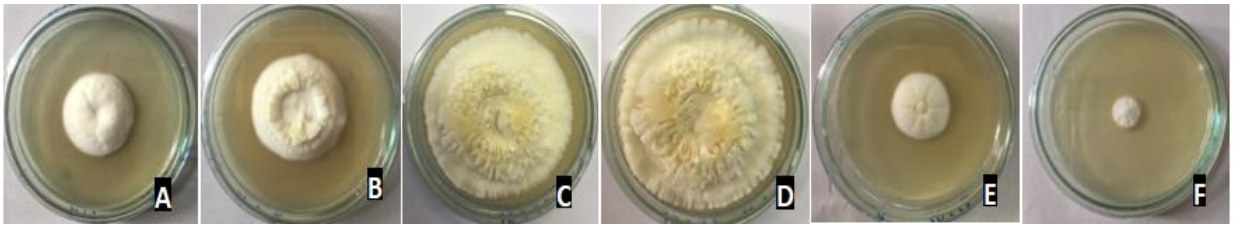

Figure 2.the growth of the BB1after 20 days: 20^{o}C (A), 22^{o}C (B), 25^{o}C (C), 27^{o}C (D), 30^{o}C (A), 32^{o}C (F)

Figure 3. *Formicococcus* sp. adult mealybugs: uninfected adults (A), infected BB1 after 3 days (B), infected BB1 after 5 days (C)

IV. Conclusions

Our results concluded that fungal strains *B.bassiana* BB1 isolated from black pepper soil require temperature range similar to local environment in which was root mealybug *Formicococcus* sp. (Homoptera: Pseudococidae). Therefore, further investigation is necessary to be able to develope *B.bassiana* BB1 as a promising biological control agent in the integrated management of black pepper root mealybugs in the Dak Lak province of Viet Nam.

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