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Evaluation of imidacloprid and entomopathogenic fungi, *Beauveria bassiana* against the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

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Abstract
The Red Palm Weevil is a serious problem of the date palm in Pakistan. The sole and combine application effects of *Beauveria bassiana* (Balsamo) Vuillemin (Hyphomycetes) and sub-lethal dosages of Imidacloprid against the second and fourth instar larvae of *Rhynchophorus ferrugineus* Olivier was investigated in laboratory. Additionally, the *in vitro* hyphal growth of *B. bassiana* amended with three different concentrations (50, 250, 500 µl L^{-1}) of Confidor® 200 SL was also assessed. The results showed significantly higher mortality for the second and fourth instar larvae of RPW when *B. bassiana* and Imidacloprid applied together compared to their sole application. The highest mortality level (100%) for the second instar was recorded after 15 days of treatment with *B. bassiana* (1×10^6 conidia/ml) and Imidacloprid (1 µl L^{-1}) in combination, while for the fourth instar larvae it was achieved after 20 days of application. However, the presence of Imidacloprid did not significantly affect the hyphal growth of *B. bassiana* on Sabouraud Dextrose Agar (SDA), and greater colony sizes compared to untreated control were recorded in all cases. The maximum number of sporulation (173, 159.83 conidia ml^{-1}) and percentage (89.15, 79.4%) of mycosed *R. ferrugineus* cadavers was observed at lower dose rate (1×10^6 conidia/ml) of *B. bassiana*, exhibited the potential to be exploited for the control of RPW.

Keywords: Entomopathogenic fungi, Imidacloprid, Mortality, Mycosis, Sporulation.

1. Introduction
The invasive Red Palm Weevil (RPW) *Rhynchophorus ferrugineus* Olivier (Curculionidae: Coleoptera), is considered as economically important and destructive pest insect of date palm in Pakistan [46]. It was first reported in 1992 from Khairpur Sindh [3], from where the pest spread to other date palm growing areas of Pakistan. At present, the pest has successfully invaded many areas including Asia, Africa, Oceania, and Europe [18], and more recently it has been reported from California and Australia [32, 36]. The female RPW lay their eggs singly in the apertures made with their rostrum at the leaf base [35]. On hatching the larvae made their way into the center of palm and make galleries by feeding inside the trunk, the mature larvae made their cocoons from the chewed fiber and pupate at the leaf base [22]. The adult emerged from the cocoon, resided on the same tree and reproduced till the death of host [13].
The existing methods of RPW management largely rely on the IPM strategies, which include: phyto sanitation, use of conventional insecticide, pheromone traps and bio-control agents. The chemical control method against RPW, include repeated fumigation, spraying and injecting of synthetic insecticides into infected palms [19]. These approaches are not enough as under this category the use of certain chemicals causes the development of resistance in the pest and further have negative environmental and human impacts [1]. Neonicotinoids, comparatively new group of synthetic insecticides, similar to natural product nicotine present in the insect nervous system, agonists the nicotinic acetylcholine receptors (nAChR) [30]. The peculiar mode of action and Chemorational ability of the neonicotinoid insecticides make them a better choice in pest management, especially in case of concealed borers. Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine] possess both systemic and contact mode of action and is compatible with different application methods for example foliar application, seed treatment [45], soil drench and stem application in different crops and trees [10].

Imidacloprid causes irreversible blockage of postsynaptic nicotinergic acetylcholine receptor of the central nervous system [34]. Laboratory and semi-field trials of Imidacloprid (SL) against RPW has been reported by Cabello *et al*. [11] and Kaakeh, [29] which strengthen the case for its further evaluation against RPW having different geographical origin.

An additional and alternate tool for RPW control is entomopathogenic fungi. Upon contact with the host, entomopathogenic fungi penetrate the cuticle of the host, germinate the spores, and overcome the host immune system. The blastopores proliferate producing conidia in cadavers of the host [51], hence the host can be infected either by direct contact, horizontal transmission from infected cadavers to healthy host or through the germination of appressoria from dead cadavers [31, 38]. Entomopathogenic fungi are considered unique due to the above mentioned characteristic among the other biological control agents, especially in case of borer insect. The eggs and other developmental stages of *R. ferrugineus*, except the adult stage, occur in the tree trunk, and entomopathogenic fungi is more likely to infect the adult stage of the host insect [23], and thus serve as an inoculum for the trunk inhabiting stages of RPW. The potential of hypocerealen species *Metarhizium brunneum* (Petch) (Hypocreales: Clavicipitaceae), *Metarhizium anisopliae* (Metsch.) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Clavicipitaceae) as biocontrol agents against several cryptic insect species has been investigated by various researcher [43, 40].

Several studies have been conducted to study the combined effects of entomopathogenic microbes with sub-lethal doses of synthetic insecticides, proved to augment the effectiveness of entomopathogenic organisms [2], specifically against the invasive pests, for e.g. longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Cerambycidae: Coleoptera), when Imidacloprid was used in combination with *M. brunneum*. Chemical induces nutritional stresses, which increases susceptibility of economically important pest against their pathogens [7, 15, 21, 24]. Successful control against different insect pest has increased the combine use of sub-lethal dosages of Imidacloprid with other entomopathogenic microbes.

The present study was designed with the aim to examine the sole and combine use of *B. bassiana* and Imidacloprid against the second and fourth instar larvae of *R. ferrugineus* and to study the post-mortal sporulation and mycosis.

2. Materials and methods

Test insect

The larvae of RPW used in the experiment were obtained from Integrated Pest Management laboratory, Department of Entomology, University of Agriculture, Faisalabad, Pakistan. Both larvae and adult of *R. ferrugineus* were reared on clean, fresh and uninfested sugarcane stems. The laboratory conditions maintained at 26±2 ºC, 70±5% RH, and a photoperiod of 12:12 D: L hours for rearing purpose [29].

Insecticide and Fungal isolation

Imidacloprid commercial liquid formulation (Confider® 200 SL), obtained from Bayer Crop Sciences, Limited, Pakistan which contain 200g/L active ingredients was used in the experiment. The fungal strain *B. bassiana* strain used in the experiment was originally isolated from the infected pupae of RPW by the single spore method, modified by Choi *et al.* [12], and later the fungus was identified based on the morphology according to Barnett and Hunter [4]. Conidia was picked from the surface of 14-days old well sporulating culture using a sterilized scalpel. The14-days old well sporulating culture was previously subculture and maintained on Potato Dextrose Agar (PDA) and incubated at 26 °C and 75% RH. The harvested conidia were then added in sterilized water containing 0.05% Tween 80. After serial dilution, required conidial suspensions of 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1} were prepared and enumerated under hemocytometer. *B. bassiana* produced more than 95% viable spores in a preliminary bioassay.

Radial growth test

The conical flasks containing Sabouraud Dextrose Agar (SDA) media were autoclaved at 121 ºC for 25-30 minutes, upon cooling the three concentrations (50, 250, 500 µl L^{-1}) of Imidacloprid were added to these flasks. For control treatment the same SDA media without any concentration of insecticide was used. After gently shaking the flasks for 2-3 minutes, the media was poured into Petri plates (9 cm), about 15ml amended media was transferred into each Petri plate. Eleven Petri plates were used for each concentration. To check the effect on radial growth, three mm diameter cores of *B. bassiana* from fresh unsporulated fungal cultures were picked and placed in the center of individual Petri plate. Radial colonies growth was measured by using two cardinal diameters in every two days up to 15 days [50]. The whole experiment was replicated three times.

Bioassay

Seven treatments were tested in the bioassays each for *B. bassiana* alone (1×10^6, 1×10^7, 1×10^8 conidia ml^{-1}), Imidacloprid alone (1 µl L^{-1}) and Imidacloprid with the higher, intermediate and lower dose of *B. bassiana*, respectively. To treat the second and fourth instar larvae of *R. ferrugineus*, a batch of 10 larvae for each instar were dipped directly in *B. bassiana* conidial suspension of for 60s [14], and were air dried for 10 minutes in sterilized Petri plate (2.5 cm diameter) lined with moist filter paper [33]. The treated larvae were provided with vertically cut sugarcane pieces (1×4 cm), previously dipped in the specific concentration of Imidacloprid (1 µl L^{-1}). In case of alone treatments, one batch (n=10) dipped in fungal suspensions was air dried and then fed on fresh untreated sugarcane pieces, while another fresh batch (n=10) was only fed on Imidacloprid treated sugarcane pieces to represent the fungal alone and Imidacloprid alone treatments, respectively. For the control group, the larvae were dipped in distilled water having 0.01% Tween 80. In each group, larval mortality rate was calculated after 5, 10, 15 and 20 days of treatment. The experiment was repeated for three times with six replicates for each treatment.

Mycosis and Sporulation

To measure the sporulation and mycosis data, dead larvae of *R. ferrugineus* were collected every day after each mortality count and placed on an autoclaved Petri plate which was immediately transferred into refrigerator at 5-6 ºC. For surface sterilization, these cadavers were washed with a 75% ethanol solution for 2 minutes, rinsed with deionized water and then the surface sterilized dead larvae were placed on Sabouraud

Dextrose Agar (SDA) and incubated for 9-10 days at 25±2 ºC and 75% RH. The number of larvae showing external fungal growth was measured directly under the stereo microscope. Sporulation was calculated by stirring mycosed insects in a beaker containing 0.05% Tween 80. After thorough stirring of the solution for 10 minutes, hemocytometer and compound microscope were used to count the number of conidia in the solution [48].

Statistical analysis

The data was subjected to statistical analysis, mortality means were corrected by Abbott's formula. However, throughout the experiment, control mortality was very low, therefore mortality data was excluded from the analysis, and mortality data was analyzed using two-way analysis of variance (ANOVA) having treatment and interval as the main factors while mortality was the response variable for each larval instar. The means were compared by using Tukey's test at significance level of α= 0.05%, while the entire analysis was carried out using Minitab 13.2.

3. Results

Radial growth test

In *in vitro* trials, vegetative growth of *B. bassiana* on Sabouraud dextrose agar (SDA) media was not affected significantly by the different concentration of Imidacloprid and greater colony sizes were witnessed on all three different dose rate of Imidacloprid as compared to control treatment (Fig.1). (D3: F3, 47=7.44; *P*<0.01, D5 F3, 47=3.30; *P*<0.01, D7 F3, 47=12.2; *P*<0.01, D9 F3, 47=14.1; *P*<0.01, D11 F3, 47=10.7; *P*<0.01, D13 F3, 47=25.7; *P*<0.01, D15 F3, 47=16.3; *P*<0.01).

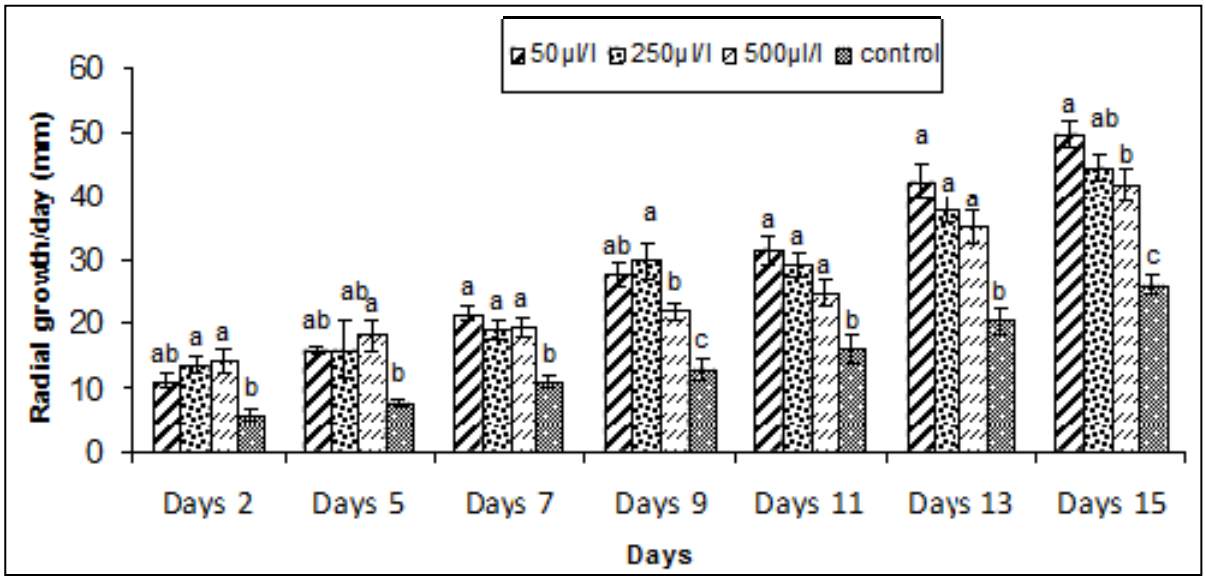

Fig 1: Radial growth rate (mm/days ±SE) of *B. bassiana* on SDA amended with 50, 250 and 500 µl/L imidacloprid (Imd) (within each day means followed by the same letter are not significantly different

Larval mortality of *R. ferrugineus*

For the second instar larvae of *R. ferrugineus*, the mortality was significantly affected by the treatment, interval ($F_{6,41}$ =18.4; *P*< 0.01; $F_{6,41}$=20; *P*< 0.01; $F_{6,41}$ =22; *P*< 0.01; $F_{6,41}$ =26.6; *P*< 0.01) and their associated interaction treatment × interval $F_{18,167}$ =2.72; *P*< 0.01). Initially at day 5, highest larval mortality was achieved at the lowest dose rate of *B. bassiana* 3.33% and at the single alone dose of Imidacloprid 37.19%. During the experiment the highest mortality (100%) rate was recorded when (1 × 10^6 conidia ml^{-1}) of *B. bassiana* was applied in combination with 1ppm of Imidacloprid after 20 days (Table 1).

Larval mortality of the fourth instar larvae of RPW was also significantly affected the main effect ($F_{6,41}$ =18.4; *P*< 0.01; $F_{6,41}$ =20; *P*< 0.01; $F_{6,41}$ =22; *P*< 0.01; $F_{6,41}$ =26.6; *P*< 0.01) and their associated interaction ($F_{3,167}$ =2.28; *P*< 001). Among the four used alone treatment, the highest larval mortality 17.67% was observed for single and alone dose rate of imidacloprid than that of applying *B. bassiana* 2.50%. Similarly after 20 days, mixed application of *B. bassiana* (1 × 10^6 conidia ml^{-1}) and Imidacloprid (1ppm) gave significantly higher mortality 82.14% (Table 2).

Mycosis and sporulation

Treatments significantly affected the mycosis (2^{nd} instar: $F_{6, 41}$ =21.1, P< 0.01; 4^{th} instar: $F_{6, 41}$ =21.9, P< 0.01 and sporulation (2^{nd} instar $F_{6, 41}$ =44.2; *P*< 0.01; 4^{th} instar $F_{6, 41}$ =47.7, *P*< 0.01) in the cadavers of *R. ferrugineus*. Maximum mycosis (89.15, 79.4%) and sporulation (173, 159.83 conidia ml^{-1}) was recorded where *B. bassiana* (1×10^6 conidia/ml) was applied at its lower dose rate against the 2^{nd} and 4^{th} instars larvae of *R. ferrugineus*, respectively. However reduced rate of mycosis (10.83, 5%) and sporulation (28, 13 conidia ml^{-1}) was observed for treatments where *B. bassiana* was combined with Imidacloprid (Fig. 3).

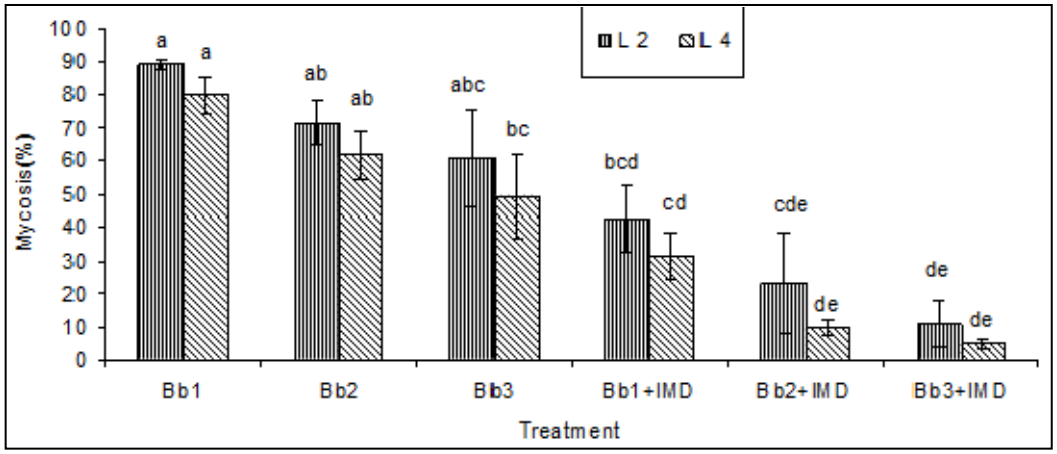

Fig 2: Mycosis (% SE) in cadavers of *R. ferrugineus* treated with *B. bassiana* alone and combined with imidacloprid (Bb1:1×10^6, Bb21×10^7: Bb3: 1×10^{8}conidia/ ml; Imd: 1µl/L); means with the same letters are not significantly different; Tukey-Kramer test at 5% significance

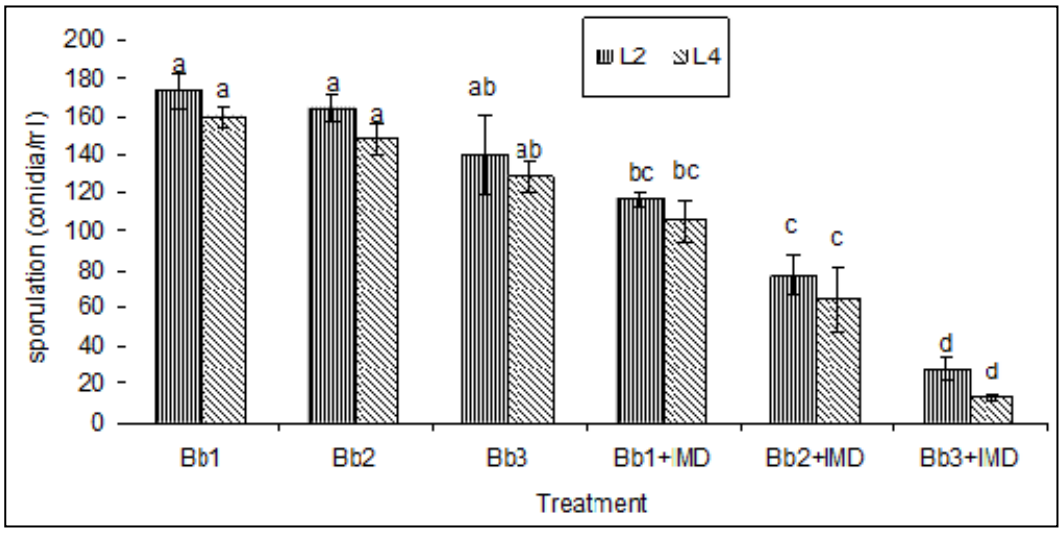

Fig 3: Sporulation (conidia/ml) on cadavers of *R. ferrugineus* treated with *B. bassiana* alone and combined with imidacloprid (Bb1: 1×10^6 Bb2 1×10^7 Bb3: 1×10^8 conidia/ml; Imd: 1 µl/L); means with the same letters are not significantly different; Tukey-Kramer test at 5% significance

Table 1: Mean mortality (±SE) of 2nd instar larvae of *R. ferrugineus* larvae after different interval with one dose rate of Imd (1 ppm), three- dose rates of *B. bassiana* (Bb1, 1×10^6; Bb2, 1×10^7; Bb3, 1×10^8) within each treatment followed by the same letter are not significantly different; HSD test at P= 5%

	Mortality(±SE)			
Treatment	**Days 5**	**Days 10**	**Days 15**	**Days 20**
Bb1	3.33±1.66e	10.96±3.94e	23.64±5.08d	54.16±4.41d
Bb2	5.83±2.38de	18.50±5.07de	39.73±4.97d	63.46±4.53cd
Bb3	11.00±1.57cde	32.93±5.34cde	46. 49±5.54bc	77.80±6.08bc
Imd	16.93±1.67bcd	39.69±4.61bcd	71.09±7.36cd	84.07±3.93ab
Bb1+Imd	21.88±3.22bc	48.20±4.95bc	72.80±7.80b	100±0.00a
Bb2+Imd	28.72±4.50ab	61.66±7.02ab	80.48±5.10ab	100±0.00a
Bb3+Imd	37.19±3.65a	74.47±3.83a	100±0.00a	100±0.00a

Table 2: Mean mortality (±SE) of 4th instar larvae of *R. ferrugineus* larvae after different interval with one dose rate of Imd (1 µl/L), three- dose rates of *B. bassiana* (Bb1, 1×10^6; Bb2, 1×10^7; Bb3, 1×10^8) within each treatment followed by the same letter are not significantly different; HSD test at P= 5%

	Mortality(±SE)			
Treatments	**Days 5**	**Days 10**	**Days 15**	**Days 20**
Bb1	2.50±1.70c	9.21±2.99E	20.26±4.62d	46.53±4.01e
Bb2	4.16±2.00c	16.00±4.14de	31.27±5.15cd	55±4.9de
Bb3	8.37±2.77bc	21.93±4.72de	42.28±3.62c	68.64±3.26cd
Imd	12.58±5.11bc	32.10±4.67cd	65.17±2.60b	79.51±7.37bc
Bb1+Imd	17.67±5.09abc	44.03±4.94bc	67.71±5.57ab	82.14±3.89abc
Bb2+Imd	25.30±5.58ab	57.50±6.48ab	73.59±6.27ab	94.07±2.74ab
Bb3+Imd	32.89±4.44a	68.55±3.22a	85.57±1.62a	100.00±0.00a

4. Discussion

The present study suggests that the simultaneous use of a chemical insecticide enhance the pathogenicity of entomopathogenic fungi against *R. ferrugineus*. It is already established that the synthetic insecticides impose physiological changes or chemical stresses in insects, rendered them more vulnerable to hyphomycetes, subsequently fungal spore penetrate easily into cuticle of insect and successful penetration results extensive vegetative growth of fungi which drain the nutrition and ultimately cause death [8, 27, 47, 5, 20, 25-26]. Prior to evaluate the integrated effect of Imidacloprid and *B. bassiana* against test insect species, the compatibility test of both compounds revealed no inhibitory effect of Imidacloprid on the radial growth of *B. bassiana*. During a similar study, Jaramillo *et al*. [28] found that the conidial or hyphal growth of *M. anisopliae* increased positively in Imidacloprid treated and blank formulation group compared to control untreated group. Higher colony sizes were observed in *M. anisopliae*, when treated with amended neonicotinoids insecticides in a laboratory experiment [37]. Contrary to this, a significantly reduced vegetative growth of *B. bassiana* has been reported by Anderson *et al*. [2] with five different non neonicotinoid insecticides. Likewise, Saenz-de-Cabezón Irrigaray *et al*. [44] reported inhibitory effect of triflumuron on the mycelial growth of *B. bassiana*.

The mixed application of synthetic insecticides with entomopathogenic organisms is not only the way to enhance their insecticidal effect but this is also an alternate approach to reduce resistance development in different insects as in combinations they synergize the effect of each other thus allowing to be used at lower concentrations [6]. Imidacloprid causes irreversible blockage of postsynaptic nicotinergic acetylcholine receptor of the central nervous system [34] and proved to be effective against various sucking insect pests from different orders include: Diptera, Coleoptera, and Lepidoptera, and [7].

In our bioassays, significantly higher mortalities of RPW larvae were observed at combined use of *B. bassiana* with a low concentration of Imidacloprid as compared to their sole applications. Several factors like poor nutrition, overcrowding, weakened immune system and chemical stress predisposed the insects to entomopathogenic diseases [27]. The synergistic phenomenon first reported by Easwaramoorthy *et al*. [16], where two insecticides augmented the efficacy of *Verticillium lecanii* used against coffee green scale *Coccus viridis* Green (Coccidae: Hemiptera). Later on, the combined application of *B. bassiana* with sub-lethal dose of abamectin, triflumuron and carbaryl insecticides caused maximum mortality of Colorado potato beetle [2]. Another successful use of the synergistic effect of Imidacloprid and two entomopathogens against sugar-cane rootstock borer weevil *Diaprepes abbreviatus* (Cuculionidae: Coleoptera) [39], and adult guava weevil *Conotrachelus psidii* Marshall (Curculionidae: Coleoptera) [9]. The similar findings in present study could be explained by the fact that sub-lethal dose of Imidacloprid may act as a behavioral modifier or physiological stressor that reduce the mobility of test insects thus not allowing them to remove the attached conidia from their skin. Thus, insecticide intoxication augmented the pathogenicity of fungi leading to easy conidial attachment, cuticle penetration, spore germination, or devastating the immune system of host [21].

Sub-lethal concentration of Imidacloprid not only enhanced the effectiveness of *B. bassiana* but also curtailed the amount of fungal inoculums required to cause high level of larval mortality of *R. ferrugineus*. In this experiment, significantly higher mortality was observed in the combined application of *B. bassiana* with imidacloprid against second instar as compared to fourth after 20 days which are in congruent with the results of Jaramillo *et al.* [28] who observed similar result when *M. anisopliae* and imidacloprid applied against the subterranean burrower bug *Cyrtomenus bergi* Froeschner (Cydnidae: Hemiptera).

The infection of entomopathogenic fungi cab be disseminated into the healthy population through the post-mortal sporulation and mycosis in the dead ones [42]. In the current bioassays, maximum number of sporulation and mycosis in cadavers of *R. ferrugineus* was recorded at the lowest and sole concentration of *B. bassiana* as compared to all other treatments. Similar trend in mycosis and sporulation were reported by Riasat *et al*. [41] for the cadavers of *R. dominica* (Bostrychidae: Coleoptera) and Tefera and Pringle [48] for *Chilo partellus* (F.) (Pyralidae: Lepidoptera), respectively. The possible reasons of maximum mycosis and sporulation at low conidial concentration as compared to high concentration could be due to the fact that the rapid death of the specimen occurred when treated with high concentration and thus just a few could be available for sporulation. Moreover, the high dose rate of fungus also cause the conidial self-inhibition scenario [48], Contrary to this, Vandenberg [49] found higher rate of sporulation and mycosed percentage in the cadaver's of leaf cutting bee *Megachile rotundata* (F.) (Hymenoptera: Megachilidae) at intermediate concentration of *B. bassiana* instead of its lower and higher dose rate.

5. Conclusions

To our knowledge, this might be the first study in which the combine effect of entomopathogenic fungi and Imidacloprid has been tested against *R. ferrugineus*. These laboratory trials, exhibited the potential of this integrated approach for the successful IPM of *R. ferrugineus* and it can be exploited for the control of other insect species of date palms in combination with certain other reduced risk insecticides.

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