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TITLE:

Isolation and Selection of Entomopathogenic Fungi from Soil Samples and Evaluation of Fungal Virulence Against Insect Pests

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KEYWORDS:

entomopathogenic fungi; *Tenebrio molitor*; *Beauveria*; *Metarhizium*; effective conidia number; biological control agent

SUMMARY:

Here we present a protocol based on the mealworm (*Tenebrio molitor*)-bait system that was used for isolating and selecting entomopathogenic fungi (EPF) from soil samples. An effective conidia number (ECN) formula is used to select high stress tolerant EPF based on physiological characteristics for pest microbial control in the field.

ABSTRACT:

Entomopathogenic fungi (EPF) are one of the microbial control agents for integrated pest management. To control local or invasive pests, it is important to isolate and select indigenous EPF. Therefore, the soil bait method combined with the insect bait (mealworm, *Tenebrio molitor*) system was used in this study with some modifications. The isolated EPF were then subjected to the virulence test against the agricultural pest *Spodoptera litura*. Furthermore, the potential EPF strains were subjected to morphological and molecular identifications. In addition, the conidia production and thermotolerance assay were performed for the promising EPF strains and compared; these data were further substituted into the formula of effective conidia number (ECN) for laboratory ranking. The soil bait-mealworm system and the ECN formula can be improved by replacing insect species and integrating more stress factors for the evaluation of commercialization and field application. This protocol provides a quick and efficient approach for EPF selection and will improve the research on biological control agents.

INTRODUCTION:

Currently, entomopathogenic fungi (EPF) are widely used in the microbial control of agricultural, forest, and horticultural pests. The advantages of EPF are its wide host ranges, good environmental adaptability, ecofriendly nature, and that it can be used with other chemicals to show the synergistic effect for integrated pest management[1,2]. For the application as a pest control agent, it is necessary to isolate a large number of EPF from either diseased insects or the natural environment.

The sampling of these organisms from their hosts helps in understanding the geographic distribution and prevalence rate of EPF in natural hosts[3,4,5]. However, the collection of fungal infected insects are usually limited by environmental factors and insect populations in the field[4]. Considering that insect hosts will die after EPF infection and then fall into the soil, isolation of EPF from soil samples might be a stable resource[3,6]. For example, saprophytes are known to use the dead host as their resource for growth. The soil bait and selective medium systems have been widely used to detect and isolate EPF from the soil[3,4,7-10].

In the selective medium method, the diluted soil solution is plated onto a medium containing broad-spectrum antibiotics (e.g., chloramphenicol, tetracycline, or streptomycin) to inhibit the growth of bacteria[2,3,9,11]. However, it has been reported that this method may distort the strain's diversity and density and can cause an over- or under-estimation of many microbial communities[6]. Moreover, the isolated strains are less pathogenic and compete with saprophytes during isolation. It is difficult to isolate EPF from the diluted soil solution[3]. Instead of using a selective medium, the soil bait method isolates EPF from the infected dead insects,

which can be stored for 2–3 weeks, thereby providing a more efficient and standard EPF separation method[3,4,7,6]. Because the method is easy to operate, one can isolate a variety of pathogenic strains at a low cost[4]. Therefore, it is widely used by many researchers.

Upon comparing the different types of insect bait systems, *Beauveria bassiana* and *Metarhizium anisopliae* are the most common EPF species that are found in insects belonging to the Hemiptera, Lepidoptera, Blattella, and Coleoptera[6,12-14]. Among these insect baits, *Galleria mellonella* (order Lepidoptera) and *Tenebrio molitor* (order Coleoptera) show higher recovery rates of *Beauveria* and *Metarhizium* spp., when compared with other insects. Therefore, *G. mellonella* and *T. molitor* are commonly used for insect baiting. Over the years, the United States Department of Agriculture (USDA) has established an EPF Library (Agricultural Research Service Collection of EPF cultures, ARSEF) that contains a wide variety of species, including 4081 species of *Beauveria* spp., 18 species of *Clonostachys* spp., 878 species of *Cordyceps* spp., 2473 species of *Metarhizium* spp., 226 species of *Purpureocillium* spp., and 13 species of *Pochonia* spp. among others[15]. Another EPF Library was constructed by the Entomology Research Laboratory (ERL) from the University of Vermont in the United States for c.a. 30 years. It includes 1345 strains of EPF from the United States, Europe, Asia, Africa, and the Middle East[16].

To control local or invasion pests in Taiwan, isolation and selection of indigenous EPF is required. Therefore, in this protocol, we have modified and described the procedure of the soil bait method and combined it with the insect bait (mealworm, *Tenebrio molitor*) system[17]. Based on this protocol, an EPF library was established. Two rounds of screening (quantification of inoculation) were performed for the preliminary EPF isolates. EPF isolates showed pathogenicity to insects. The potential strains were subjected to morphological and molecular identifications and further analyzed by the thermotolerance and conidial production assay. Further, a concept of effective conidia number (ECN) was also proposed. Using ECN formula and principal component analysis (PCA), the potential strains were analyzed under simulated environmental pressure to complete the process of establishing and screening the EPF library. Subsequently, pathogenicity of promising EPF strains were tested for the target pest (e.g., *Spodoptera litura*). The current protocol integrates thermotolerance and conidial production data into the ECN formula and PCA analysis, which can be used as a standard ranking system for EPF related research.

PROTOCOL:

NOTE: The whole flowchart is shown in **Figure 1**.

1. Isolation and selection of potential Entomopathogenic fungi (EPF)

1.1. Collect the soil sample

1.1.1. Remove 1 cm of the surface soil, and then collect the soil within the 5–10 cm depth using a shovel from each sampling site.

NOTE: Sampling sites would be a mountain, forest, or sparsely populated areas to avoid the contamination of artificially sprayed EPF strains. Ensure that areas for the soil samples collection are covered with weed on the surface. Dry soil or damp soil is not suitable for this experiment.

1.1.2. Record the details of each sampling site, including GPS, elevation, type of field, annual temperature, yearly precipitation, collection time (season), soil type, and pH value.

1.1.3. Collect 100 g of the soil sample into a plastic bag and maintain it at room temperature if subjecting it to the fungal isolation protocol in the laboratory within 3 h.

NOTE: If the sample cannot be used within 3 h, store the soil at room temperature in dark conditions. If the experiment is not performed immediately, the soil sample can be stored at 4 °C for 1 week until the start of the protocol[18,19].

1.2. Bait and isolate the EPF with mealworm (*Tenebrio molitor*)

1.2.1. Place 100 g of the soil sample in a plastic cup (cap diameter = 8.5 cm, height = 12.5 cm), and then place 5 mealworms on the surface of the soil at room temperature in the dark for 2 weeks.

NOTE: Other types of plastic cup containers can also be used. If soils are too dry (cracked or sandy), spray sterilized ddH_2O (about 5–10 mL) on the soils. The body length c.a. 2.5 cm (14^{th} instar) of *T. molitor* larvae helps in fungal isolate screens[20].

1.2.2. Observe and record the larvae daily for mortality and mycosis; keep the dead larvae in the cup until 2 weeks for fungal isolation.

NOTE: The fungal conidia spore in the soil samples will attach to the mealworm larvae during the above process. The fungal mycosis will be observed as the hyphae grow from the intersegmental membrane, and then the whole body will be covered with the mycelium. Sporulation will start after 7 days and the color of the fungal infection will change to the color of the conidia mass.

1.2.3. Transfer the dead insects to a clean bench and use a sterile toothpick to collect the conidia. Streak them on a quarter strength Sabouraud dextrose agar medium (¼ SDA) plate (55 mm) in the laboratory[21]. Incubate the culture plate at 25 °C for 7 days to obtain the primary culture of fungi.

NOTE: The ¼ SDA plate is prepared as follows: Mix 1.5 g of Sabouraud dextrose broth and 3 g of agar in 200 mL of H_2O, and then sterilize for 20 min. Aliquot ¼ SDA into each 55 mm Petri dish before solidification. The solidified ¼ SDA plates are stored at 4 °C until use.

1.2.4. Re-streak each primary culture fungi on one 55 mm ¼ SDA plate in a laminar flow and incubate the culture plate at 25 °C for 7 days to obtain single colonies of fungi.

1.2.5. Repeat this re-isolation ~2–3 times and observe under light microscopy to obtain single and pure morphological fungal colonies.

NOTE: Isolate all EPF using *T. molitor*-bait and store as described in the following section. The separation, preservation, pure culture, and streaking must be performed in a laminar flow in the subsequent sections.

1.3. Store the EPF isolates

1.3.1. Cut 3 of the 5 mm agar blocks at the edge of each isolated pure fungal cultured plate with a cork borer and place it into a 1.5 mL micro-centrifuge tube as one replicate.

NOTE: Three replicates for each fungal isolate are recommended for the EPF strains' storage after 2nd virulence test. Molecular identification is also recommended if storage space is limited.

1.3.2. Add 250 µL of 0.03% surfactant solution (**Table of Materials**) and 250 µL of 60% glycerol into the 1.5 mL micro-centrifuge tube using a micropipette; then, vortex for 10 s.

1.3.3. Seal the 1.5 mL micro-centrifuge tube with paraffin film and precool it in a -20 °C refrigerator for 24 h. Then, transfer the precooled fungal stocks to a -80 °C refrigerator for cryopreservation.

NOTE: Extra pure fungal culture plates (aside from the cryo-preserved samples) were used in section 1.4.

1.4. 1st pathogenicity screen for fungal isolates

1.4.1. Place five *T. molitor* larvae directly on the surface of each pure fungal culture plate at 25 °C.

1.4.2. Observe and record the mycosis and mortality for 10 days. Select the fungal isolate for further analysis.

NOTE: Fungal isolates causing 100% mortality are selected for 2nd virulence test to confirm their virulence to *T. molitor* larvae. Alternatively, the researcher can adjust the criteria as per their own study.

1.5. 2nd virulence test of fungal isolates

NOTE: Based on the 1st pathogenicity screen, recover the selected fungal isolates from -80 °C for the 2nd virulence test. The purpose of the 2nd virulence test is to quantify the pathogenicity of the selected fungal isolates after the 1st round of screening.

1.5.1. Harvest conidia of each fungal isolate by vortexing for 1 min and count the number of conidia using a hemocytometer.

1.5.2. Adjust the conidia suspension to a concentration of 1 x 10^7 conidia/mL in a 0.03% surfactant solution (**Table of Materials**).

1.5.3. Spread 10 µL of the fungal suspension onto 55 mm ¼ SDA plates and grow for 7 days at 25 °C in the dark.

1.5.4. Place five *T. molitor* larvae directly on the surface of each pure fungal culture plate (c.a. 6 x 10^7 conidia). Seal the plates with paraffin film and incubate at 25 °C in the dark.

1.5.5. Observe and record the mycosis and mortality for 10 days.

1.5.6. Repeat the test (from step 1.51 to 1.5.5) in triplicate for each fungal isolate.

NOTE: Fungal isolates causing 100% mortality are selected for 3rd virulence test to confirm their virulence to target the pest.

1.6. 3rd virulence test of fungal isolates for target pest (*Spodoptera litura* as an example)

1.6.1. Repeat steps 1.5.2 to 1.5.6 with selected isolates from 2nd virulence to test virulence against target pest.

1.6.2. Calculate the LT_{50} of each fungal isolate[22].

NOTE: The LT_{50} of each fungal isolate was calculated through generalized linear models (GLMs) using R studio (version 3.4.1); the quasibinomial error distribution and a log link function can be used to account for overdispersion.

2. Molecular identification of EPF

2.1. Extraction of fungal genomic DNA

2.1.1. Collect c.a. 1 mm^2 EPF from the 7-day ¼ SDA plate.

2.1.2. Extract the fungal genomic DNA using a fungal genomic DNA extraction kit according to the manufacturer's instructions[23] (**Table of Materials**).

2.2. PCR amplification and DNA sequencing

2.2.1. Amplify Fungal ITS region by PCR of the DNA sample[21] using the PCR Master Mix (2x), ITS1F/ITS4R primer set[24] (**Table 1**) with the following PCR program: 94 °C for 1 min, and then 35

cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a 7 min final extension at 72 °C.

NOTE: The ITS1F/ITS4R primer set is for the genus level identification.

2.2.2. Sequence the PCR by commercial sequencing service.

2.2.3. Use NCBI BLAST search for similar fungi in the NCBI database and select the relative fungal type species for phylogenetic analysis.

NOTE: The fungal species belonging to the genus of *Metarhizium* or *Beauveria* should be further identified to the species level with tef-983F/tef-2218R primer set[25] (repeat steps 2.1.1 to 2.2.3). For fungi that do not belong to the genera *Metarhizium* or *Beauveria,* other molecular markers can be used to identify the species, including *DNA lyase* (*APN2*), *beta tubulin* (*BTUB*), *RNA polymerase II largest subunit* (*RPB1*), *RNA polymerase II second largest subunit* (*RPB2*), and *3' portion of translation elongation factor 1 alpha* (*TEF*)[25,26].

2.3. Phylogenetic analysis

2.3.1. Use ClustalX 2.1 software[27] to align the multiple sequences from steps 2.2.2 and 2.2.3. Trim the conserved sequences region manually with GeneDoc[28].

2.3.2. Perform the phylogenetic analysis by MEGA7 software[29] based on the minimum evolution (ME), Neighbor-Joining (NJ), and maximum likelihood (ML) methods.

NOTE: Performing all three methods can help to confirm and accurately conclude the classification status. The fungal isolates screened by the 1st pathogenicity screen are used for molecular identification at the genus level. The fungal isolates screened by the 2nd virulence test are used for the species level molecular and morphological identification.

3. Morphological identification of EPF

3.1. Observation of the fungal colony morphology

3.1.1. Use a camera to capture the fungal culture colony growth for 7 days, and record the growth, form (fluffy, firm), and color of the colonies.

3.2. Observation of conidia and conidiophores

3.2.1. Scrape conidia from the pure culture fungal colony with an inoculation loop and transfer the spores to a glass slide with 0.1% Tween 80 solution. Then, cover with a coverslip for light microscopic observation of conidia.

3.2.2. Use a scalpel to cut a 5 mm^2 agar block of the hyphal strand at the edge of the fungal colony, and then transfer the agar block to a glass slide.

3.2.3. Perform the cleaning as follows: Add the 0.1% Tween 80 solution on the agar block with a plastic dropper and wash off most of the excess conidia using tweezers. Then, cover it with a coverslip for light microscopic observation.

NOTE: 0.1% Tween 80 can be substituted with another more potent surfactant (**Table of Materials**) depending on fungal species and hydrophobicity.

3.2.4. Measure and record the width and length of the conidia and conidiophores to compare the differences between different fungal isolates.

3.2.5. Use Welch's ANOVA test and Games-Howell test (post-hoc test) to analyze the conidial width and length of each strain using R studio (version 3.4.1).

NOTE: Data analysis of morphological characters can be adjusted by cases. The fungal isolates screened with the 3rd virulence test are used for the physiological characterization and ECN ranking in sections 4 and 5.

4. Investigation of conidial productivity and thermotolerance

4.1. Conidial production assay

4.1.1. Culture the selected fungal isolate on ¼ SDA medium at 25 ± 1 °C in dark for 10 days.

4.1.2. Prepare 1 mL of the conidial suspension of the fungal isolate in 0.03% surfactant solution and adjust to 1 x 10^7 conidia/mL as described above.

4.1.3. Drop three droplets of 10 µL of conidial suspension on ¼ SDA and incubate at 25 °C in the dark for 7, 10, and 14 days to count the sporulation of fungi.

NOTE: 10 µL is the best volume to collect the 5 mm block with even fungal sporulation after fungal growth for 7–14 days.

4.1.4. Use the cork borer to detach 5 mm agar block from the center of the colony and transfer into 1 mL of 0.03% surfactant solution (**Table of Materials**) in a 1.5 mL micro-centrifuge tube at each time point.

4.1.5. Vortex the tube at 3,000 rpm at room temperature for 15 min and use a hemocytometer to count the number of conidia.

NOTE: The formula used for counting is the number of conidia per 25 squares of the smallest cell (size = 0.025 mm^2; chamber depth = 0.1 mm):

$$\text{Total No of conidia in 5 squares} \div 80 \times (4 \times 10^6)$$

4.1.6. Repeat three times for each isolate.

4.2. Thermotolerance assay

4.2.1. Culture the selected fungal isolate on ¼ SDA medium at 25 ± 1 °C in dark for 10 days.

4.2.2. Prepare 1 mL of the conidial suspension of fungal isolate in 0.03% surfactant solution and adjust to 1 x 10^7 conidia/mL as described above.

4.2.3. Vortex the conidial suspension and heat it in a 45 °C dry bath for 0, 30, 60, 90, and 120 min. Drop three droplets of 5 µL of the conidial suspension on 55 mm ¼ SDA medium at each time point post heat-exposure and incubate at 25 ± 1 °C for 18 h.

NOTE: Avoid spreading the fungal droplets to be able to better focus on the area.

4.2.4. Count the number of germinated conidia spores with five randomly selected fields under 200x light microscopy to determine germination rate.

4.2.5. Perform three replicates for each isolate.

5. Effective conidia number (ECN) ranking

5.1. ECN calculation

NOTE: Obtain the conidial production and thermotolerance data of each potential fungal strain before calculating the total ECN[21].

5.1.1. Calculate the fold-change (*FC*) of conidial production at each time point:

$$FC_x = n_{cp}/I$$

where, x = time point for data collection; n_{cp} = number of conidia after each day of growth; and I = initial number of seeded conidia.

5.1.2. Calculate the conidia number under the stress treatment at each time point using the following formula:

$$ECN_y = I \times FC_x \times TT_0 \times Stress\ coefficient(TT_Z)$$

Where, y = the ECN of the time point under treatment; TT_0 = the germination rate of conidia not undergoing heat stress (= germination rate of 0 min heat treatment); TT_Z = stress coefficient is the conidia germination rate at different times of heat treatment (z).

5.1.3. Calculate the total ECN using the following formula:

$$ECN_{sum} = \Sigma ECN_y = I \times FC_x \times TT_0 \times Stress\ coefficient(TT_Z)$$

5.1.4. Compare the ECN of each fungal strain.

5.2. Principal component analysis (PCA) of fungal strains

NOTE: The PCA analysis confirms the ranking of ECN and helps in understanding the correlation between the physiological character values. Compare the ECN values and select EPF isolates having higher ECN values.

5.3. Use R software to create PCA by coding:

```
#Input PCA data file
a = read.table("PCA.csv",sep=',',header=T)

5.3.1. # Processing sample data
row.names(a) <- c("NCHU-9","NCHU-11", "NCHU-64", "NCHU-69", "NCHU-95", "NCHU-113")
X=row.names(a)
df<- a[2:11]

5.3.2. #PCA calculation
pca <- prcomp(df, center = TRUE, scale = TRUE)
vars <- (pca$sdev)^2
pc1_percent = vars[1] / sum(vars)
pc2_percent = vars[2] / sum(vars)
value = pca$x

5.3.3. #Output PCA visualization file
png(file = 'pca.png', height = 2000, width = 2000, res = 300)
```

NOTE: Use 7 to 14 days conidial production and all thermotolerance data to execute principal component analysis (PCA) for confirming the ECN ranking.

5.4. Select the best-performing fungal strains based on ECN or PCA and perform the virulence test of target pests for further research.

REPRESENTATIVE RESULTS:

Isolation and selection of potential Entomopathogenic fungi (EPF)

By using the *Tenebrio molitor*-mediated Entomopathogenic fungi (EPF) library construction method, the number of fungi without insect-killing activity would be excluded; thus, the isolation efficiency and selection of EPF could be largely increased. During the application of this method, the information of sampling sites, soil samples, and the fungal germination rates were recorded (**Table 2**). Based on our previous data, a total of 101 fungal isolates were obtained from 172 soil samples, indicating a high isolation efficiency of 64%. Among the 101 fungal isolates, 26 isolates showed insecticidal activity against *T. molitor* (100% mortality) after the 1st pathogenicity screening, hence the elimination of fungal isolates was 26/101 = 25.7%. In the 2nd virulence test, the high virulence of the 26 fungal isolates against *T. molitor* was further

demonstrated, 12 of which showed high pathogenicity against the *T. molitor* larvae (100% mortality at 5 days post inoculation) (**Figure 2A**). These were used to evaluate the virulence test against the agricultural pest. Based on the data of the 3rd virulence test mortality and LT_{50}, a total of six fungal isolates (NCHU-9, 11, 64, 69, 95, and 113) revealed rapid insect-killing activity against *Spodoptera litura* (LT_{50} = 2.94, 2.22, 2.84, 2.57, 2.96, and 1.13), assessed using the physiological assay and effective conidia number (ECN) (**Figure 2B**).

Molecular identification of EPF

To better understand the fungal taxonomic positions, 26 isolates from the 1st pathogenicity screening were subjected to molecular analysis based on the ITS region (**Figure 3A**). The result showed that these fungal isolates could be clearly divided into seven genera, including *Beauveria*, *Clonostachys*, *Fusarium*, *Cordyceps*, *Penicillium*, *Purpureocillium*, and *Metarhizium* (**Figure 3A**). Based on the ITS1-5.8S-ITS2 region, the genus classification of EPF was accurately confirmed, while the species level is still indistinguishable. Therefore, the sequence of the *tef* region is used to clearly classify the species level for 12 promising EPF isolates from the virulence test against the agricultural pest. The molecular identification of the 12 isolates showed that 11 isolates belong to *Metarhizium* and contained four species, including *M. lepidiotae* (NCHU-9, NCHU-102), *M. pinghaense* (NCHU-10, NCHU-11, NCHU-30, NCHU-32, NCHU-64), *M. brunneum* (NCHU-27, NCHU-29), and *M. anisopliae* (NCHU-69, NCHU-95). The remaining isolate was identified as *B. australis* (NCHU-113) (**Figure 3B,C**). According to the above result, the sequence region of *tef* can effectively distinguish the genus *Metarhizium* at species level, while other species need to find other sequence regions as molecular markers to distinguish the species.

Morphological identification of EPF

Through the cleaning method (step 3.2.3) of fungi morphological observations, the structures of conidiophores could be seen clearly with 0.1% Tween 80 solution (**Figure 4A**), and these observations could serve as a benchmark to measure the size of the structure and take a photo record. The color, shape, and arrangement of the conidia can be seen through microscopic observations of the colony conidia (**Figure 4B,C**). After observation, the sizes of conidia and conidiophore shapes could be further measured and statistically compared (**Table 3**).

Investigation of conidial productivity, thermotolerance and ECN ranking

The ECN formula, which was proposed by the previous report[21], could help with the selection of a high stress-tolerance EPF based physiological character. The ECN combines the conidia production and thermotolerance data of each EPF (**Figure 5A,B**), which means high viability of

fungal strain when ECN value is high (**Figure 5**). Moreover, principal component analysis (PCA) visualization was used to verify the results from the ECN formula. The result revealed a high coordination between PCA and ECN, suggesting that the ECN formula could be used to evaluate the hierarchy of viability related parameters and development potential of fungi to field application and further commercialization (**Figure 5C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of *Tenebrio molitor*-mediated Entomopathogenic fungi (EPF) library construction. Part 1: Fungal isolation from soil sample; Part 2: Pathogenicity and virulence-based screening and fungal identification; Part 3. Physiological characterization and fungal ranking. ECN = Effective conidia number; and PCA = Principal component analysis.

Figure 2: Mortality of mealworms and *Spodoptera litura* for selection of promising Entomopathogenic fungi (EPF) isolates. (**A**) 26-selected fungal 2nd mealworm virulence test; The mycoses of 12 rapid killing and high virulence fungal isolates are shown in parallel. (**B**) 12 fungal isolates were selected for virulence test against *S. litura*. Test on each fungal strain was repeated three times. d.p.i. = days post inoculation. Modified figure and legend reproduced with permission of the Fronteris[21].

Figure 3: The phylogenetic analysis of Entomopathogenic fungi (EPF) strains at (A) Genus-level based on ITS region and (B–C) Species-level based on *tef*. The phylogenetic analysis of ITS region and *tef* were constructed using the maximum likelihood (ML), minimum evolution (ME), and neighbor-joining (NJ) methods. Bootstrap analyses were performed to evaluate the robustness of the phylogenies using 1,000 replicates, and bootstrap proportions greater than 50% are indicated above branches. Bold = Ex-type strains. The red and green arrows indicate the promising EPF. Modified figure and legend reproduced with permission of the Fronteris[21].

Figure 4: Microscopic examination of Entomopathogenic fungi (EPF) (*M. anisopliae*) morphology. Observation of conidiophores (**A**) after washing. (**B**) The observation of conidia shape and color. (**C**) Arrangement of conidia and bundles of spore strings (SS) of *M. anisopliae*; Cp = conidiophores; cc = cylindrical conidia. Scale bar = 10 µm.

Figure 5: Physiological characterization of six potential isolates and the effective conidia number (ECN) analysis. (**A**) Conidial production assay. (**B**) Thermotolerance assay. (**C**) The bar plot of ECN values. (**D**) Principal component analysis showed the distribution of each strain. Modified figure and legend reproduced with permission of the Fronteris[21].

Table 1: Primer pairs for fungal identification.

Table 2: The parameters recorded for *Tenebrio molitor*-mediated EPF library construction method are listed below. NIU = the campus of National Ilan University. NCHU = the campus of National Chung Hsing University. Modified table and legend reproduced with permission of the Fronteris[21].

Table 3: An example of morphological observations of six promising entomopathogenic fungal isolates. Statistical analysis was performed based on the Welch's ANOVA test by using R studio. Modified table and legend reproduced with permission of the Fronteris[21].

DISCUSSION:

Entomopathogenic fungi (EPF) have been used for insect control. There are several methods to isolate, select, and identify EPF[30-32]. Comparing the different types of insect bait methods, *Beauveria bassiana* and *Metarhizium anisopliae* were commonly found in insect baits[6,12-14]. Among these insect baits, *Galleria mellonella* and *Tenebrio molitor* showed high recovery rates of *Beauveria* and *Metarhizium* spp. As a matter of fact, the utility of the mealworm (*T. molitor*)-bait method was demonstrated as a convenience method for isolation of EPF from soil samples[17,21]. Nonetheless, it has been reported that used insects as bait would show the bias to isolate specific fungal species[3,33]. Therefore, combination of different insect species (i.e., *Galleria mellonella* and *T. molitor* together) to bait the EPF from the soil samples might increase the diversity of EPF[34].

To evaluate the insect killing activity, there are two rounds of screening by mealworm (*T. molitor*) in the current protocol to select the promising fungal strains for further study. Following this process, the number of fungal isolates, which are isolated from soil samples, could be dramatically reduced and restricted to fungal isolates that showed high insecticidal activity after the 1st and 2nd screening. Large reduction of the fungal isolates for the next round of screening save time cost and labor-consuming. It could be also noted that the selected fungal isolates showed similar trends of pathogenicity between mealworm (*T. molitor*) and tobacco cutworm (*Spodoptera litura*), demonstrating a consistent testing of pathogenicity of different insect species and could be further extended to other crop pests[21]. In addition, the calculation of the ECN based on the physiological tests could facilitate the selection of potential fungal strains to be used in crop fields. Similar selective methods were mentioned by other reports, while the factors such as abiotic and strain characteristics have not been included[12,8,31,35-37].

Therefore, these strains may be affected by environmental factors resulting in an influence on the performance of fungal germination and thereby difficulty to be commercialized. This consequence is also the main reason for the inapplicability of laboratory strains in the field[38].

From the morphological identification, further efforts should be directed toward finding the clear structure of conidiophore and observe the different characteristics of each fungal strain. Studies can include various methods for solving the difficult morphological identification[39]. However, the morphological identification fails to distinguish closely related fungal species; therefore, it is necessary to integrate the data of molecular identification. The internal transcribed spacer (ITS) region showed the genus level discrimination, while other molecular markers (i.e., *tef* for *Metarhizium*, *bloc* for *Beauveria*) are needed for the identification at the species level[26,40,41].

In the physiological characterization, to reduce the standard deviation of the experiments, either the machine or manual vortex for at least 10 min at max rpm is suggested[21]. Thoroughly mixed conidia suspension would increase the consistency of the results of thermotolerance assay and conidial production assay. On the other hand, in the conidial production assay, the counted conidia number under a fixed area (5 mm block in the central part of fungal colony in this study) on the cultured plate at different time growth raised the problem that different fungal species and even different strains revealed different performance of the growth compactness of the hyphae, which might lead to the non-representativeness of the sampling. Therefore, a rigorous test method for conidia production can improve the problem, such as accurately count the number of conidia of whole fungal colony and normalize with the growth area[42].

The effective conidia number (ECN) formula is designed based on the influence of environmental stress against EPF conidia, that is, simulate conidia under the condition of wild environment to select the promising fungal strains for commercialization[21]. In this protocol, the data of conidia production and heat treatment were used for the calculation of the total ECN value of each promising fungal strain, following the previous study[21]. Further, under a natural environment, except the temperature, other environmental stress, such as humidity, ultraviolet radiation (UV), and host could influence conidia germination. Especially, the UV stress is a main factor affecting conidia germination because the high-energy free radicals (such as peroxides, hydroxyl groups, and singlet oxygen) generated by UV may reduce pathogenicity and the persistence of microbial pesticides[43]. Thus, UV stress could be further included in the ECN formula in the future. To avoid UV stress, the formula should contain oil or sodium alginate to

increase the UV tolerance of conidia[44-46], which confirms that potential strains have practicality for pest control[47].

The present protocol provided a method for quick and precise isolation of potential EPF strains to construct *T. molitor*-mediated EPF library. This is the basis and is necessary for the development of biological control research. Moreover, the ECN formula could be flexibly improved to help researchers grasp the potential of EPF and develop it into a commodity for use in agricultural systems.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare there is no conflict of interest involved in this work.

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Figure1

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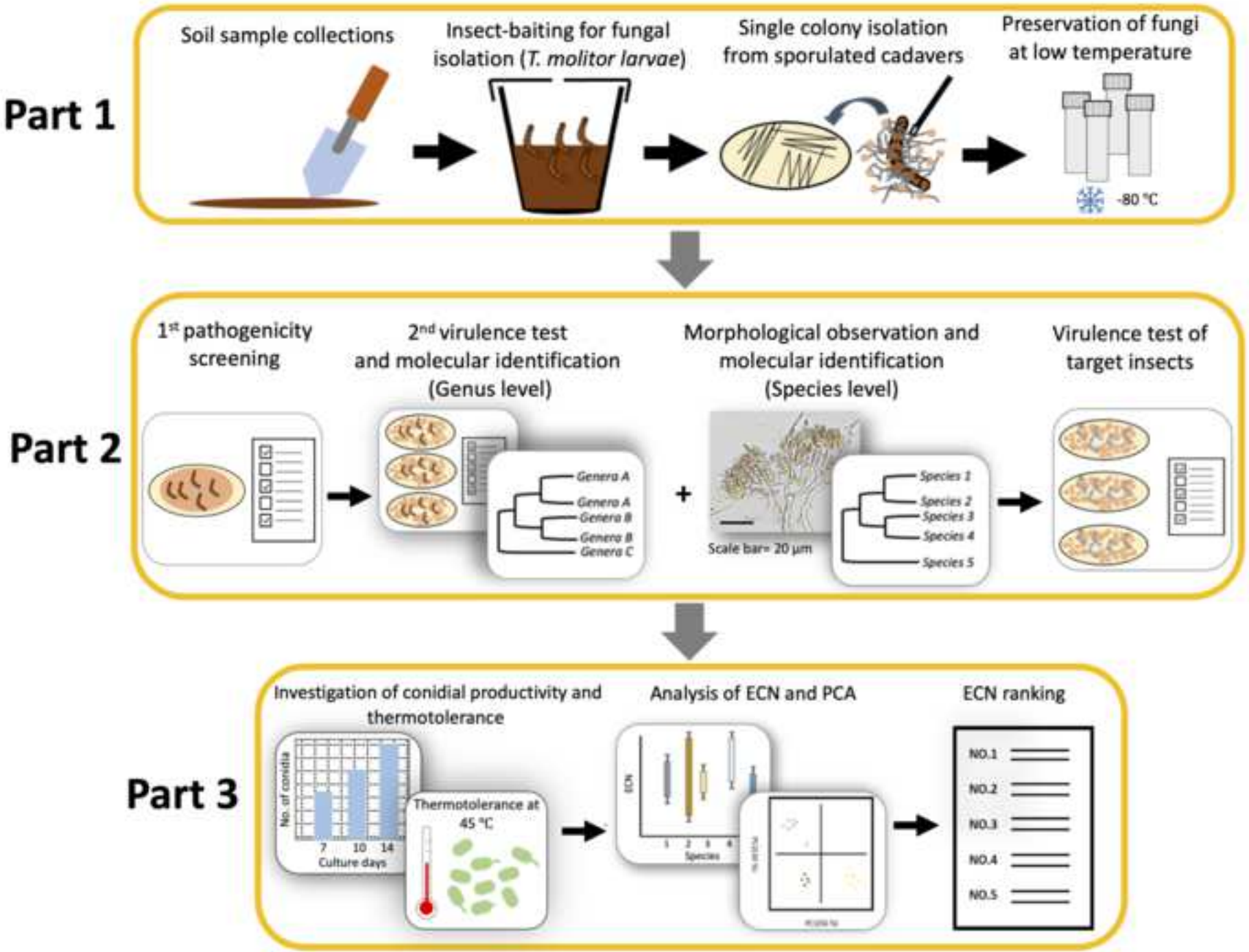

Figure2

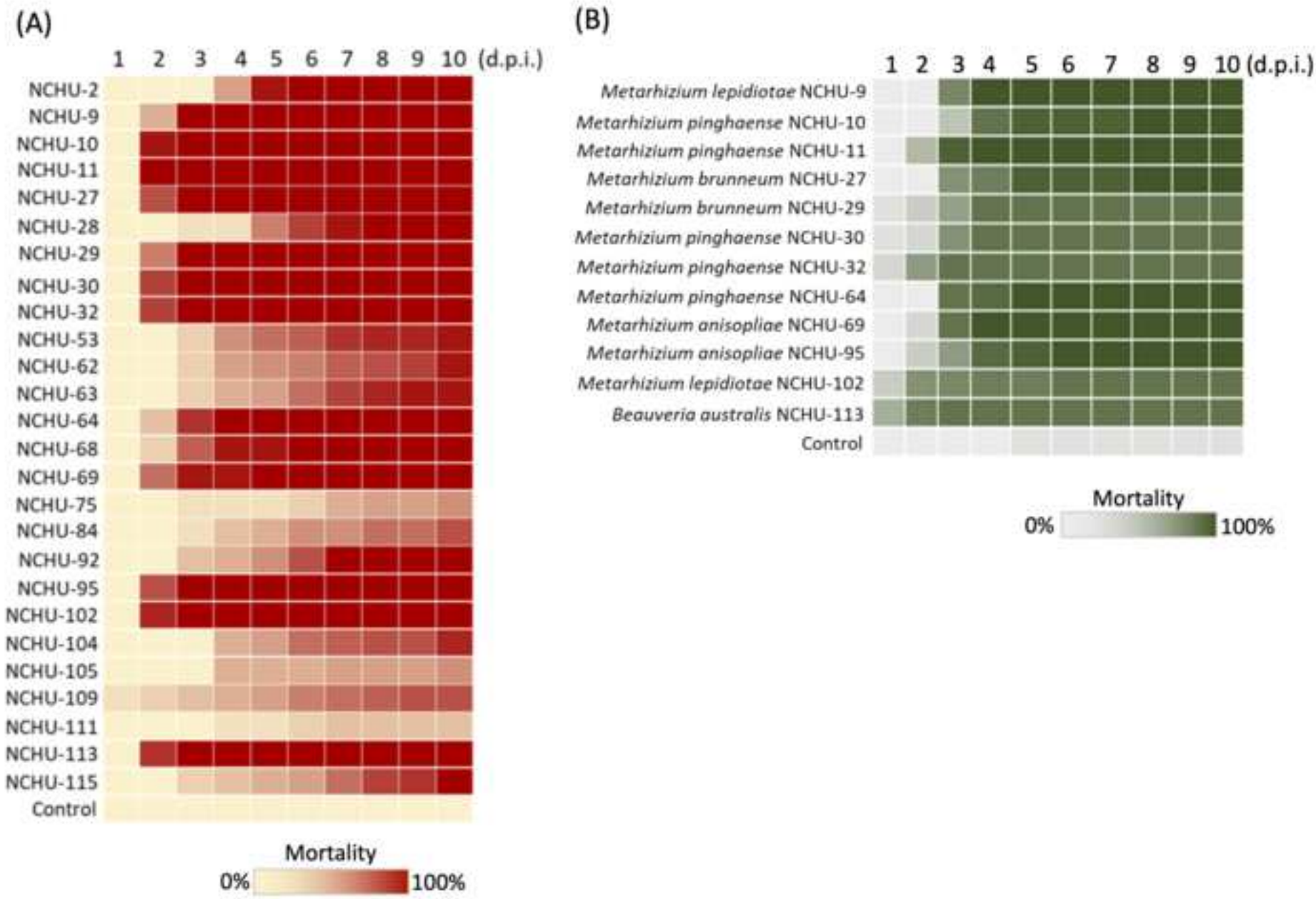

Figure 3

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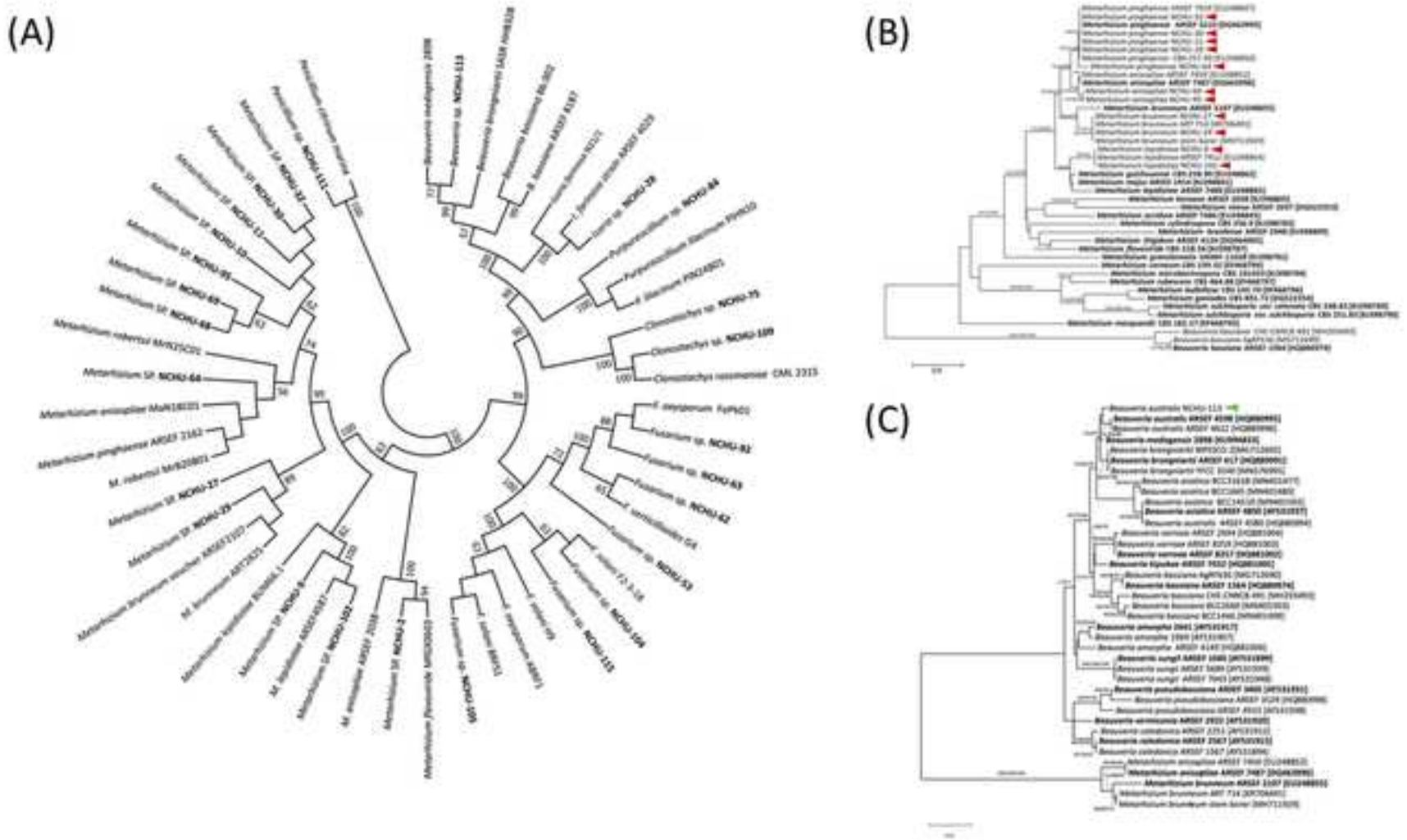

Figure4

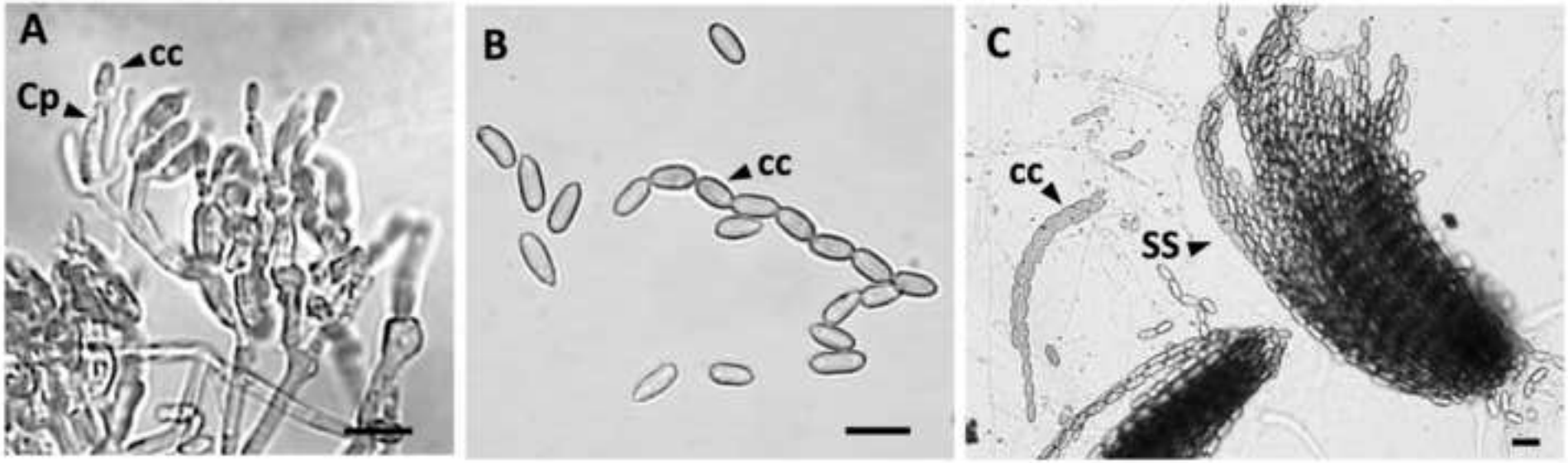

Figure5

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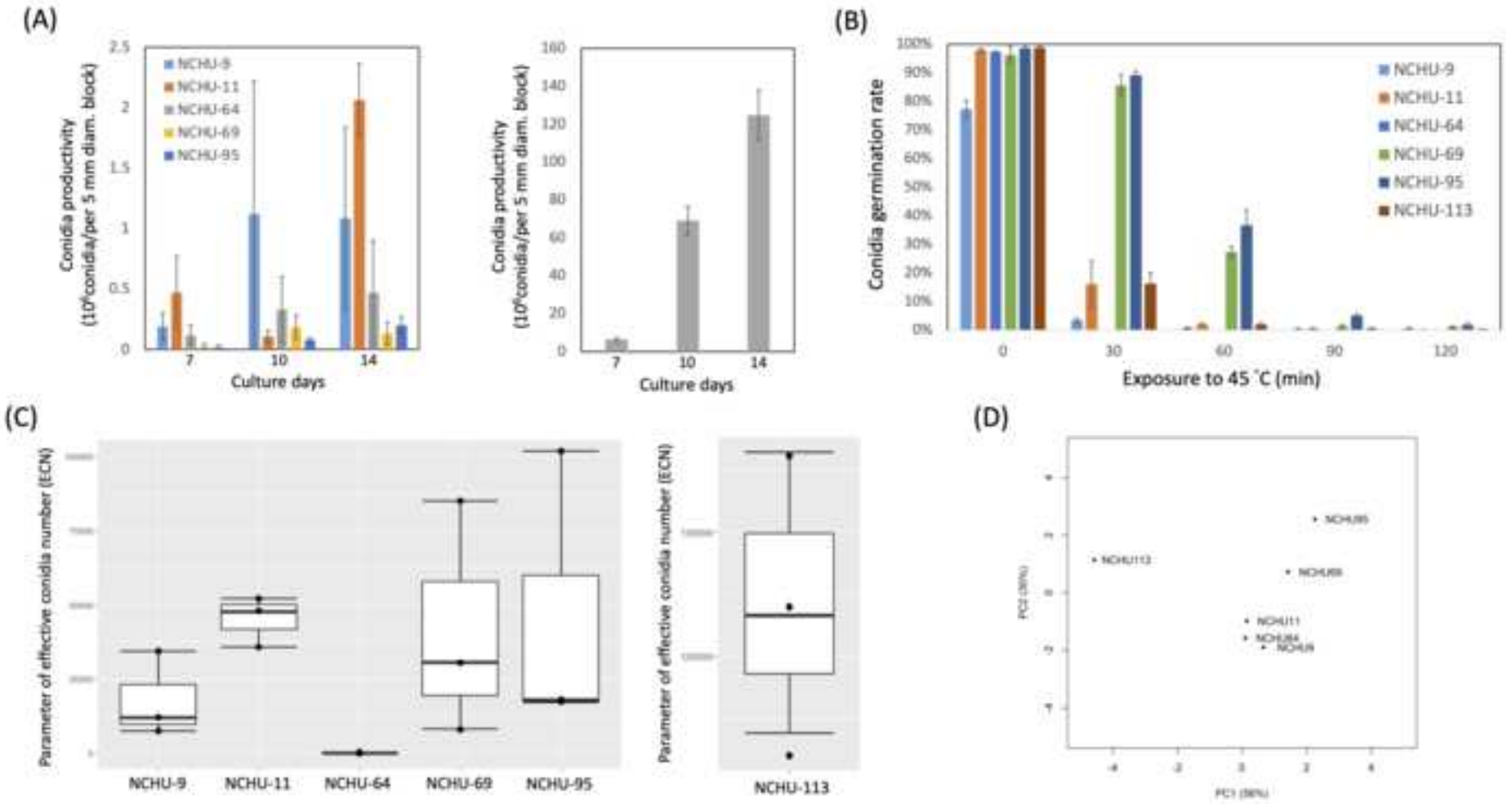

Table 1. Primer pairs for fungal identification.

Primer name	Amplicon Size (bp)	Sequence(5'-3')
ITS1F	550	TCCGTAGGTGAACCTGCGG
ITS4R		TCCTCCGCTTATTGATATGC
tef-983F	1000	GCYCCYGGHCAYCGTGAYTTYAT
tef-2218R		ATGACACCRACRGCRACRGTYTG

Region	Reference
ITS	25
tef	26, 27, 41, 42

Table 2. The parameters recorded for Tenebrio molitor -mediated EPF library construction method a

City	Sample site	Location (GPS)	Elevation (m)
Yilan	NIU	24°44'49.6"N 121°44'46.2"E	7
Yilan	JiaoXi (JX)	24°49'23.4"N 121°44'08.8"E	287
Yilan	SanXing (SX)	24°39'30.1"N 121°36'48.0"E	134
Yilan	Yuanshan (YS)	24°46'37.5"N 121°42'02.5"E	343
Taichung	WuLing (WL)	24°20'14.7"N 121°18'34.8"E	1970
Taichung	NCHU	24°07'11.9"N 120°40'33.9"E	95

NIU= the campus of National Ilan University

NCHU= the campus of National Chung Hsing University

* Modified table and legend reproduced with permission of the Fronteris (23).

are listed below*

Type of field	Annual Temperature (°C)	Precipitation/ per year (mm)	Season (Y/M/D)
Campus pasture	22	~2700	Autumn (2018/November/7)
Forest	22	~3076	Autumn (2018/November/9)
Forest	22	~2000	Autumn (2018/November/16)
Forest	22	~2828	Autumn (2018/November/30)
Forest	18	~2000	Winter (2018/December/11)
Campus Forest	23	~1642	Spring (2019/May/2)

Soil type	No. of Samples	Primary Germination% (No. of germination)
Loam soil	12	66.7 (8)
Silt soil	30	53.3 (16)
Silt soil	34	50.0 (17)
Silt soil	51	66.7 (34)
Clay soil	38	47.4 (18)
Loam soil	7	100.0 (7)
	Total= 172	Average= 64.02%

No. of isolates
13
5
8
41
20
14
Total= 101

Table 3. The example of morphological observations for the six promising entomop

Species	Strain	Phialides µm±SD*
Metarhizium lepidiotae	NCHU-9	Cylindrical, 7.3±0.67b×2.4±0.36a
Metarhizium pinghaense	NCHU-11	Cylindrical, 8.6±0.68ab×2.6±0.30a
Metarhizium pinghaense	NCHU-64	Cylindrical, 10.5±1.54a×2.4±0.32a
Metarhizium anisopliae	NCHU-69	Cylindrical, 9.4±1.58a×2.7±0.32a
Metarhizium anisopliae	NCHU-95	Cylindrical, 9.6±0.87a×2.6±0.29a
Beauveria australis	NCHU-113	Ellipsoid, NA×2.6±0.57

* Statistical analysis was performed based on the Welch's ANOVA test by using R st

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ɔathogenic fungal isolates§.

Conidia µm±SD*
Cylindrical, 6.5±0.45a×2.7±0.13b
Cylindrical, 6.3±0.41b×2.7±0.16b
Cylindrical, 6.8±0.53a×2.9±0.31a
Cylindrical, 6.3±0.34b×2.6±0.19b
Cylindrical, 6.0±0.82b×2.9±0.29a
Globose, 2.3±0.24×2.3±0.24

tudio.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Ans: Thank you for the formatting of this manuscript and we will use the attached file for revision.

2. Please address all the specific comments marked in the manuscript.

Ans: Ok, we have checked and addressed all the specific comments marked in the manuscript.

3. Please address all the reviewers' comments as well.

Ans: Ok, we have checked and addressed all the reviewers' comments.

4. Currently the language is compromising the science being presented. Please employ a professional copyediting service.

Ans: We have send the manuscript to English editing to improve the writing.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Suggest: The established protocol based on the mealworm (Tenebrio molitor)-bait system was used for "isolating and selecting" the entomopathogenic fungi (EPF) from soil samples.

Ans: We have corrected it.

Major Concerns:

The authors have made all corrections accordingly and the manuscript was much improved.

Ans: Thank you for your effort on the review of this manuscript.

Minor Concerns:

There are some minor concerns which needed to be justified:

L186: Why quantification screening was not performed at the beginning when conducting the 1st pathogenicity test?

Ans: The rationale of first round screening is narrow down the number of testing fungal isolates, therefore, there is only one plate (one replicate) after the fungal isolate (from soil sample to single fungal isolate). However, it still has the possibility

that mealworm might die not cause by fungal infection, thus, the second screening is to further confirm the virulence against mealworm.

L213: How sure are you with the concentration of the fungal plate (c.a. 6x10^7 conidia) as different fungi might have different growth rate. And whether the quantity 6x10^7 conidia is per plate or per worm?
Ans: The concentration of the fungal plate could be counted during the investigation of conidial productivity. Indeed, different fungi might have different growth rate. In our case, all the entomopathogenuc fungi subjected to the second round virulence test were belonging to *Metarhizium* or *Beauveria*, therefore, it could be estimated as c.a. $6x10^7$ conidia.

Reviewer #2:
Manuscript Summary:
The changes made by the authors are considered sufficient, and they are congratulated for their excellent work and contribution to the scientific community.
Ans: Thank you for your effort on the review of this manuscript.

Reviewer #3:
Manuscript Summary:
Thanks to authors for revising the Ms as per my advice. Now the Ms is more scientific and useful.so I recommend to publish the Ms in the esteemed journal
Ans: Thank you for your effort on the review of this manuscript.

Reviewer #4:
Manuscript Summary:
The authors describe a soil-bait method to isolate entomopathogenic fungi using mealworms. They then test the isolates on a target pest (Spodoptera litura), sequence the isolates, and examine production of conidia.

Major Concerns:
All of my comments have been addressed and the manuscript has greatly improved.
Ans: Thank you for your effort on the review of this manuscript.

Minor Concerns:
Improvements should be made to the overall language of the manuscript - I believe this can be handled during the copyediting process. Dividing the 'representative results' section into subsections with the same headings as the 'protocol' section

would make the section easier to read.

Ans: Thank you for our suggestions and we have send the manuscript to English editing to improve the writing. Besides, we have added the subtitles in the 'representative results' section as well.

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EDITOR'S SUMMARY REPORT

From your editor:

It has been a pleasure to edit your paper on the isolation and application of entomopathogenic fungi. It was engaging, and it is clear that you put a great deal of effort into writing it. I have carefully edited your manuscript, paying careful attention to grammar and phrasing. Please review my edits carefully before accepting or rejecting any changes. In addition, please review this summary report for some of my more general observations about your paper.

Thank you for giving me the opportunity to work on your manuscript. I wish you the best of luck throughout the publication process.

Yours sincerely,
Denise

Overall Summary	
• **Abstract**	A very good stand-alone passage of the study
• **Introduction**	The Introduction provides an adequate background with the rationale and research aims. Major revisions were only required in this section related to structure; please verify that the intent was retained or provide further details so I can edit it more accordingly.
• **Protocol**	The methods are described in sufficient detail and the protocol offers step-by-step guidance, which is highly advantageous for someone not used to the original methods. I would have liked to see the diagram though, but the method has been nicely explained.
• **Results**	The results have been presented for the protocol described and logically answers the research question.
• **Discussion**	You have analysed well your results and discussed within the context of your research. You have raised the limitations and provide a scope of direction.

Finally, I'd like to share an example of a style convention and how I applied it in your paper: Academic writing should be very concise and redundancies (that is, the unnecessary repetition of words and ideas) should be avoided. One way of doing this is by providing a concise alternative where you don't repeat words.

Original sentence: Entomopathogenic fungi (EPF) are currently wide-used microbial control agent to control agricultural, forest, and horticultural pests.

Edited sentence: Currently, entomopathogenic fungi (EPF) are widely used in the microbial control of agricultural, forest, and horticultural pests.