PROJECT DELIVERABLE REPORT
D.4.3 Technical documentation for application of entomopathogenic fungi FF OFF-Season-IPM tool made available for WP6

Fruit Flies In-silico Prevention & Management

FF•IPM

Project Title:

In-silico boosted, pest prevention and off-season focused IPM against new and emerging fruit flies ('OFF-Season' FF-IPM)

SFS-2018-2

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1	July 22, 02022	EB	Approval of EB Members

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1. Summary

The control of fruit flies (Diptera: Tephritidae) is mainly based on insecticide treatments targeting epigean adults. Targeting the soil- dwelling stages by soil treatment with a strain of entomopathogenic fungus could be a strategy to be considered. The main objective of the present deliverable was to examine the effectiveness of a commercially available mycoinsecticide product based on strains of the *Beauveria* entomopathogenic fungus as a tool for soil application in orchards against the Mediterranean fruit fly (medfly), *Ceratitis capitata*, in spring and/or in autumn (Off-Season).

This deliverable presents the results of screening process starting with five commercial products (Naturalis®, Botanigard® WP22, Ostrinil®, Serenisim® and Betel®) against medfly, *C. capitata*, in laboratory studies with the selection of the most effective product to be used in the field experiments, especially for an Off-Season application.

GHA strain, corresponding to Botanigard® WP22 was selected because: i/it was the most virulent in the pathogenicity screening test carried out by dipping larvae in an inoculum of 10^6 and 10^8 conidia/ml of five registered *Beauveria* strains corresponding to five commercial products on *C. capitata* and two other fruit flies, *Bactrocera dorsalis* and *Zeugodacus cucurbitae*, on their late third instar larvae; and ii/ because this product is the one with the highest spore density, which makes it possible to treat soils with the highest spore quantity. The proof of concept that the use of this product in soil treatment was able to control the soil-dwelling stages was achieved using treated sand with Botanigard® WP22 at the dose of 10^7 conidia/g before depositing a larva of *C. capitata* or *B. dorsalis*. This treatment allowed, whatever the fly species, to reduce the rate of emergence. It induced a high rate of mortality and mycosis on the cadavers of late third instar larvae, pupae but also adults contaminated during their emergence. The growth of the GHA strain of Botanigard® WP22, modelled as a function of temperature, allowed to validate that the soil temperatures in Italy, where the field trials were planned, were compatible with those allowing the growth and survival of the fungus. Finally, the laboratory test of the temperature and dose effects on the pathogenicity and virulence induced by sands treated with Botanigard® WP22 showed that it induced a mortality of *C. capitata* significantly different from the control whatever the dose (10^5 to 10^7 conidia/g) and at all the temperatures tested (10, 15, 20 and 25°C). *C. capitata* mortality was positively correlated with the dose of Botanigard® WP22, whereas it was negatively correlated with the temperature. This was probably due to the fact that the insect did not develop or very slowly at low temperature while the fungus remained active. This suggests that the impact of the treatment may be greater off- season, in spring, and/or in autumn, when temperatures are lower and insect development is slow while the fungus remains active.

For the in-situ field testing in apple and peach orchards in Italy, off- Season (in spring and autumn) and on season (in summer) was carried out. For practical reasons of implementation, it was applied by drenching Botanigard® WP22 to the soil surface at the rate of 29 g/2 m^2, corresponding to the equivalent of 10^7 conidia/g of soil in the first 5 cm. Tests performed after one-year revealed that the fungus was able to maintain itself in the soil, being pathogenic and able to reduce emergence of *C. capitata* for at least one year.

Our results showed that the soil treatment with Botanigard® WP22: i/ may have the potential to reduce emergence and kill *C. capitata* OFF-season and ii/ the soil temperatures of the apple and peach orchards in Italy where we will perform the field tests are compatible with those that allow the growth and survival of the fungus. Botanigard® WP22 applied off-season or on early season before medfly population build-up could provide a useful tool for a sustainable environmental-friendly way to control the medfly. Implementing this strategy in WP6, is an opportunity to evaluate this strategy in large scale. Application of Botanigard® WP22 can be performed with simple drenching with standard sprayer equipment beneath the canopy in the orchards but we recommend if possible to incorporate the product in spring at 10^7 conidia/g in the first 5cm horizon of soil for a better repartition and protection of the inoculum. However, costs at the dose tested in the field are very high and not sustainable in IPM practice, even if the product is applied

only under the tree canopy. Using lower dose might be more economically sustainable. The use of Botanigard® WP22 against medflies might be constrained by efficacy and by costs.

2. Introduction

Fruit flies (Diptera: Tephritidae) are major pests of fruit and vegetable crops. Damage is caused by the larvae that develop inside the fruit. Today, the control of these pests is mainly based on insecticide treatments targeting the epigean adults, the only accessible stage. Soil treatment with a strain of an entomopathogenic fungi of the genus *Beauveria* has been used in Reunion island for more than 30 years to control the larva of a sugarcane chafer. This strategy could also be considered to control fruit flies by targeting the soil-dwelling stages; about twenty publications, including more than ten on *Ceratitis capitata*, have dealt with this subject and have shown the potential of this strategy (Buron-Musseau 2020). At the late third instar larvae, the larvae jump out of the fruit to get into the soil (about 5 cm) where it will pupate. During the soil-dwelling phase (late third instar larvae, or just emerged adult), the insect could be infected by spore of entomopathogenic fungi.

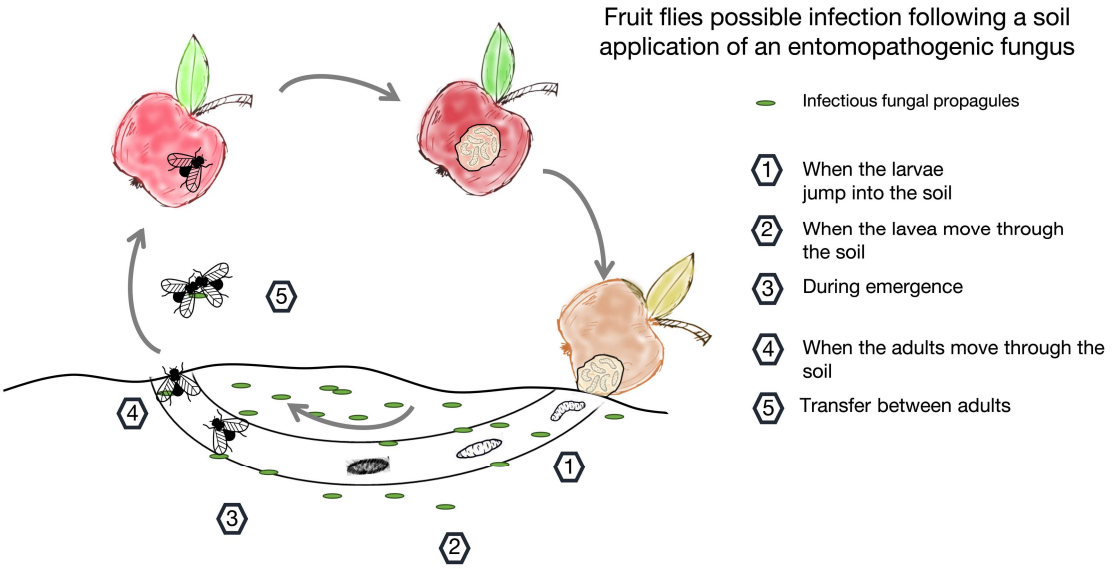

Figure 1 - Entomopathogenic fungi soil treatment targeted medfly stages or events - Mature larvae while jumping out and burying into the soil and Adults while emerging and leaving the soil.

2.1. Purpose and Scope

The main purpose and scope of D4.3 was to provide to WP6 a technical documentation for the application of the FF OFF-Season-IPM tool for entomopathogenic fungi-based biocontrol tool with technical/biological description, estimate its effectiveness, applicability and limitations, application protocol, and (d) estimate of application that could be applied against medfly populations off-season in late autumn or early spring.

This deliverable D4.3 presents the results of screening process starting with five commercial products of *Beauveria* strains (Naturalis®, Botanigard® WP22, Ostrinil®, Serenisim® and Betel®) against medfly, *C. capitata*, in laboratory studies with the selection the most effective product to be used in the field experiments, especially for an Off-Season application. All those products are used for controlling insects but had never been tested against fruit fliesas soil treatments). Three main tasks were undertaken:

i/ Laboratory screening of the pathogenicity of the different recorded *Beauveria* strains on three very different fruit fly species: *C. capitata, Bactrocera dorsalis* and *Zeugodacus cucurbitae* at the third larval stage corresponding to the "jumping" stage and selection of the commercial product corresponding to the most pathogenic strain and the most suitable formulation for soil treatment. *B. dorsalis* and *Z. cucurbitae* have an overlapping host range with *C. capitata*. In areas where these three flies are present, especially in the

outermost regions of Europe such as Reunion Island, they must be controlled simultaneously. As *B dorsalis* and *Z cucurbitae* represent a major threat for continental Europe. These two flies have been included in this screening.

ii/ Using the best selected commercial product to realize in laboratory conditions the proof of concept for the soil treatment strategy with this mycoinsecticide to control soil-dwelling stages and testing for the effective temperature range and doses of the selected commercial product to control the soil-dwelling stages of *C. capitata*;

iii/ In-situ field testing of the selected commercial product at the most effective dose identified in laboratory on *C. capitata* to evaluate its potential and durability in apple and peach orchards in Italy.

Finally, a technical documentation for application of the best selected commercial entomopathogenic fungi product is delivered.

3. Materials and Methods

3.1. Laboratory experiments conducted in Reunion Island

3.1.1. Insects

Fruit flies, *C. capitata* and two other fruit flies *B. dorsalis* and *Z. cucurbitae* were reared in climatic chambers as described by (Rohrlich et al. 2018) for dipped larvae pathogenicity tests, for sand treated pathogenicity test *C. capitata* and *B. dorsalis* were reared respectively on organic pepper and banana fruits. Only late third instar larvae that had already jumped were collected for pathogenicity tests.

3.1.2. Commercial mycoinsecticides and their *Beauveria* strains

Commercial products based on *B. bassiana*: Naturalis® (strain ATCC 74040), Botanigard® (strain GHA), Ostrinil® (strain I-2960), Serenisim® (strain I-2961), and *B. hoplocheli*: Betel® (strain B507) were obtained from their producing companies or their distributor. Strains were isolated from commercial products grown on potato dextrose agar (PDA) and stored at -80°C. The number of viable conidia indicated by the manufacturer was checked every month to ensure the stability of the product during the different experiments.

3.1.3. Development of an individual monitoring device

We have developed and used for all the pathogenicity tests, an individual rearing device that optimizes rearing operations, adult longevity, and avoid cross contamination between individuals (Figure 2a). A small box integrated in this device allowed the late third instar larvae to continue their development (pupation) and the imago to emerge directly into the adult monitoring chamber (Figure 2b).

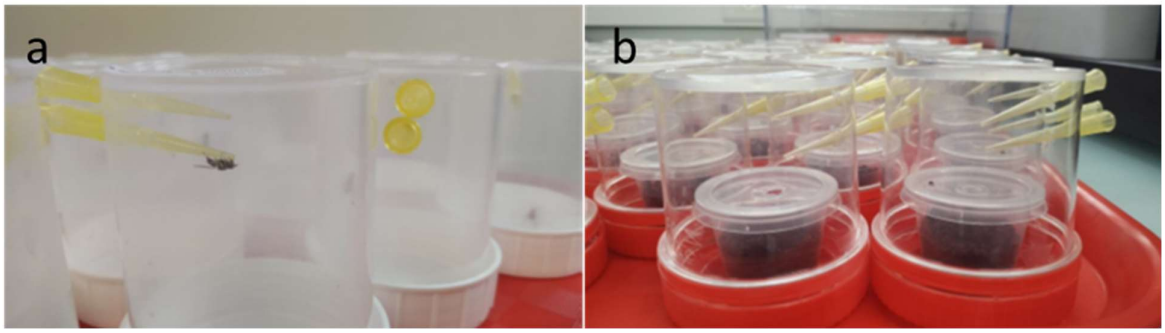

Figure 2 - "Double cones" adult stage individual monitoring/rearing device used in Réunion Alone (a) or associated with the emergence device (b).

3.1.4. Dipped larvae pathogenicity tests

Inoculum were prepared by scraping the surface of the spore-forming cultures and then suspending the conidia in 0.05% solution of Tween® 80. A first screening was done using conidial suspensions adjusted to 10^6, 10^7 and 10^8 conidia/ml to contaminate late third instar larvae of *C. capitata*, *B. dorsalis* and *Z. cucurbitae*. Each of the three repetitions contained 30 larvae for each modality ((five fungal strains+ control) x two doses of conidia x three species = 36 modalities =1080 individuals). As the first screening was only repeated once with a small number of individuals for each modality it, to confirm observed effects, a second screen was done using conidial suspensions adjust 10^7 conidia/ml for the strain GHA (Botanigard and B507 (Betel®) to contaminate larvae of *C. capitata* and *B. dorsalis* ((two fungal strains+ control) x one dose of conidia x two species = 6 modalities =180 individuals). After contamination, the larvae were isolated in the monitoring device and placed in a climatic chamber. Adult mortality was recorded daily for 30 days. The cadavers of all stages were placed in wet chamber to check for mycosis. External fungal growth was recorded daily for 10 days.

3.1.5. Proof of concept with sand treated with commercial product

In order to evaluate the pathogenicity of the strains by contact with contaminated soil, commercial products Betel® (strain B507) and Botanigard® WP22 (strain GHA) were incorporated into autoclaved sand at 10^7 conidia/g. Each of the three repetitions contains 30 late third instar larvae of *C. capitata* and *B. dorsalis*. The bioassays were carried out in the device allowing contamination with sand containing the mycoinsecticide and individual monitoring of the insects. Mortality of individuals and mycosis were monitored as described in 3.1.5.

3.1.6. Evaluation of the effect of temperature on the growth of the fungus and comparison to soil temperatures in Campomarino and Paliano (from 15 years of meteorological data)

In order to check if the soil temperatures of the apple and peach orchards in Italy where we will conduct the field tests would be compatible with those that allow the fungus to grow and survive we first studied the effect of temperature on the growth of the GHA strain that composes Botanigard WP22. The growth of the GHA strain was studied at constant temperatures of 5, 10, 15, 20, 25, 30 and 35°C. Three replicates of eight Petri dishes were made for each temperature.

Soil temperatures of the Italian regions of Campomarino and Paliano were retrieved for the period 2002-2019 in 1h steps from the Copernicus database, Era Land5 (https://cds.climate.copernicus.eu). This was the temperature in the middle of the so-called level 1 soil layer between 0 and 7 cm.

3.1.7. Evaluation of the effects of the conidia doses and temperature on the pathogenicity of Botanigard® WP22

Sand was contaminated with the commercial product Botanigard® WP22. Four doses of Botanigard® WP22 were evaluated: 0, 10^5, 10^6 and 10^7 conidia.g^{-1} of sand at four temperatures 10, 15, 20 and 25°C. For each experiment, the four doses of the product were tested for a given temperature. We performed 12 experiments, the four temperatures being repeated three times. Each modality (dose-temperature) contained 30 late third instar larvae, i.e. a total of 1440 *C. capitata* larva. All the other experimental settings followed those described in 3.1.6.

3.2. Field experiments (UNIMOL, Italy)

3.2.1. Insects

Ceratitis capitata infested fruits were collected in the areas of Paliano and Campomarino in December 2020 and October 2021 and transferred in indoor ambient condition. From F2 to F6 laboratory generations, were used. The fruits were placed in sterile sand and sifted at regular intervals (at least one a week) to collect the pupae. Pupae remained in the same conditions until adult emergence. Upon emergence, adults were placed in plastic-screen cages with ad libitum access to adult diet (sugar: yeast in ratio 4:1) and water. Flies were allowed to oviposit on banana fruits and the larvae completed their development on them.

The day before the release of the larvae into the experimental units, late third instar larvae were taken with a soft-bristled brush by dissecting banana fruits that had previously been left to incubate for 10 days (at 26°C) after oviposition. The late third instar larvae were then placed, until the required number was reached, on a portion of the banana cut to form a cup. The piece of banana containing the larvae was then placed in jars and transported to the field for release into the experimental unit (one infested banana piece per experimental unit).

3.2.2. Field experimental units

The experimental unit consisted of a white mesh net fixed in the shape of a cone, at the top of which was placed a funnel that directed the emerging *C. capitata* adults from the soil into a transparent bottle (Figure 3). Experimental units were placed on the ground with the lower part of the net inserted into the soil to a depth of about 15 cm and supported by cables in a vertical position. Each unit covered an area of soil of approximately 1 m^2 (circle of approx. 1.13 m of diameter) to intercept the emerging *C. capitata* adults.

The experimental units were placed in rows between two plants. The ground projection of the canopies occupied the area of the traps.

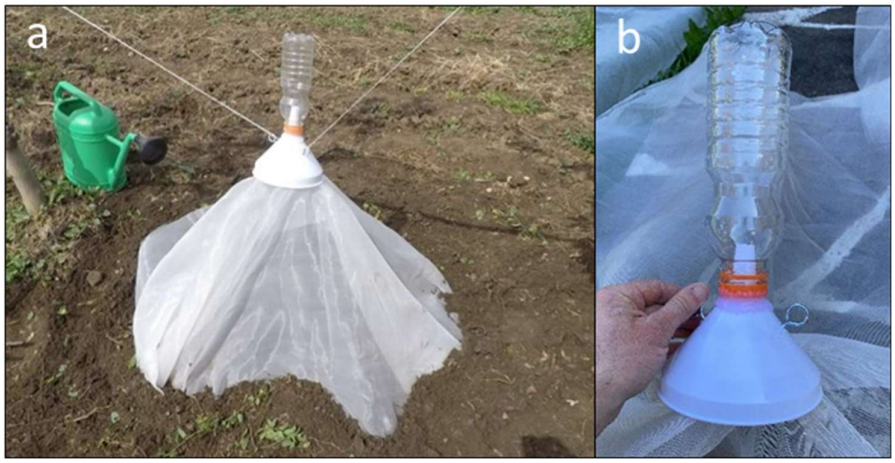

Figure 3 – Field experimental unit
Experimental unit (a) and detail of the top part, funnel and bottle (b).

3.2.3. Evaluation of life span of collected adults in the field

Living flies collected in the experimental units were placed in an individual rearing device to assess their life span. This device consisted of a wide-necked vial (125 ml volume) into which a water-soaked cotton pad with sugar and yeast had also been placed (Figure 4a). To ensure air exchange, a series of small holes were punched in the vial screw caps. Dead flies were checked daily and transferred in moist chamber to check for mycosis.

3.2.4. Evaluation of adult contamination in the field

Non-mycotic dead samples collected in the experimental units or in the individual rearing device were transferred in moist chamber to check for mycosis following this protocol: fly surface was sterilized in 70% ethanol for 3 seconds, rinsed in sterile distilled water twice for 5 seconds, and placed on sterilized filter paper moistened with 200 µl of sterile distilled water in a 55 mm Petri dish. These dishes were then parafilmed and placed at 25°C and 75% RH. Fungal growth was monitored up to 10 days after death (Figure 4b).

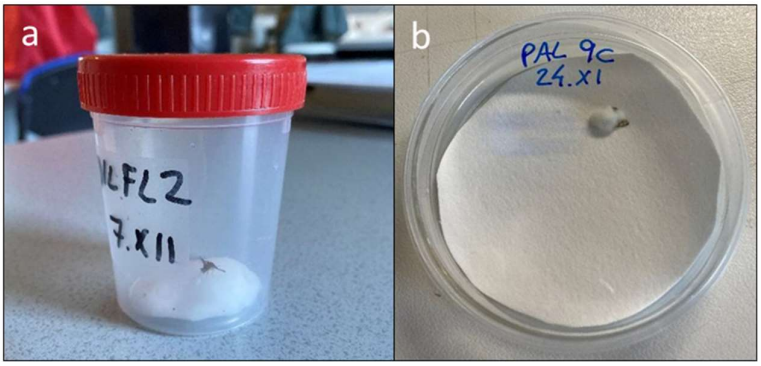

Figure 4 – Individual rearing device used in Italy- (a) and moist chamber with *Beauveria bassiana* contaminated adult (b).

3.2.5. Experimental design

3.2.5.1. OFF-Season –Botanigard soil treatment and effect on late third instar larvae in spring

The experimentation was set in April-May 2021 (OFF-Season - spring). Thirty-six experimental units were installed in the six pilot site's farms with six experimental units per farm. For technical reason of applicability in the field, it was decided not to incorporate the product in the soil but to use the product Botanigard® WP22 by putting the powder in suspension in water and watering the soil surface. A dose of 14.5 g/m^2 was chosen because it would correspond to the equivalent to the dose of conidia of 10^7 conidia/g used in the laboratory to contaminate the sand, which would be distributed homogeneously in the first 5 cm of the soil horizon. Half of the experimental units were treated with 29 g of commercial product Botanigard® 22WP (*B. bassiana* GHA strain) mixed in five litres of water (*Beauveria* April units). The solution was applied to the soil occupied by the experimental unit and around (2 m^2). The other half was treated with water as control (control units). After each application, 30 late third instar larvae ready to jump were released on the ground into the experimental unit.

For the Campomarino pilot site, the experimental units were installed on 16 April. For the Paliano pilot site, the units were installed on 27 April. The experimental units were serviced twice a week. *C. capitata* adults caught in the units were removed, counted and examined in order to assess the duration of life and the presence of the fungus on the body.

3.2.5.2. ON-Season – effect of spring and summer Botanigard® WP22 soil treatments on late third instar larvae in summer

In July-September 2021 (ON-Season - summer). Three "new" experimental units per farm were installed in addition to the units previously installed in the first trial that were maintained in the same position, for a total of 54 experimental units. In the "new" experimental units, the soil for 5 cm deep occupied by the unit and around was treated with 29 g of Botanigard® 22WP mixed in 5 litres of water (*Beauveria* July units).

In detail, for the ON-Season modality, nine experimental units were present in each farm: three untreated experimental units (control units), three experimental units in which the fungus was applied in mid-April (*Beauveria* April units), and tree experimental units in which the fungus was applied in July (*Beauveria* July units). Fifty late third instar larvae ready to jump were released on the ground within the area occupied by the experimental units. Trials started on 8 July in the three farms in Campomarino and on 27 July in the three farms in Paliano.

The experimental units were serviced twice a week. *C. capitata* adults caught in the units were removed, counted and, for the Paliano pilot site trials, examined in order to assess the duration of life and the presence of the fungus on the body.

3.2.5.3. OFF-Season - autum

In October-December 2021 (OFF-Season - autumn), no units were added and no Botanigard treatments were repeated. On 13 October for the three farms in Campomarino and on 21 October for the three farms in Paliano, 50 late third instar larvae ready to jump were released on the ground within the area occupied by the experimental unit.

The experimental units were serviced twice a week. *C. capitata* adults caught in the units were removed, counted and examined in order to assess the duration of life and the presence of the fungus on the body.

3.2.6. Entomopathogenic activity of soil one year after Botanigard WP22 soil treatments

Galleria mellonella, being very sensitive to entomopathogens is often used to "catch" entomopathoenic fungi in the soil or to control that pathogenic activity of a mycoinseticid treatment in a strategy called "Galleria bait" (Meyling 2007). *Galleria mellonella* larvae were purchased the day before the beginning of the trial. Two laboratory tests were carried out to assess the persistence in the soil and efficacy of the fungus *B. bassiana* against *G. mellonella* and *C. capitata* larvae by taking soil in May 2022 from the treated experimental units in April and July 2021.

3.2.6.1. *Galleria mellonella* trial

Four different soil treatments were evaluated: (1) control (soil untreated, taken from the not treated experimental unit); (2) *Beauveria* treated soil from April (taken from the experimental unit in which fungus was applied in April 2021); (3) *Beauveria* treated soil from July (taken from the experimental unit in which fungus applied in July 2021); (4) *Beauveria* Lab treated soil (soil taken from the treated experimental unit and treated with fungus in the laboratory). The *Beauveria* Lab soil was treated with Botanigard® 22WP mixed in water at a concentration of 6 g/l on the trial day.

For each treatment 1000 ml of soil was placed in aluminium containers (21x14x4 cm). Tap water was added the day before the trial (50 ml) and on the trial day (50 ml). Eight *G. mellonella* larvae were released in the container for each treatment. After larvae release the containers were closed with pitted aluminium film and stored at room temperature (21-25°C). Three replicates per treatment were performed.

The containers were serviced at weekly interval.

3.2.6.2. *Ceratitis capitata* trial

The same soil treatments as described for the *G. mellonella* trials were used.

For each treatment 250 ml of soil was placed in glass jar (diameter 8 cm, height 13 cm). Tap water was added the day before the trial (12.5 ml) and on the trial day (12.5 ml). Thirty *C. capitata* ready to jump larvae were released in the jar for each treatment. After larvae release the jars were closed with pitted lid and stored at room temperature (21-25°C). Three replicates per treatment were performed.

The jars were serviced at weekly interval.

3.2.7. Meteorological data

At the beginning of each trial, two dataloggers, equipped with a double probe, were placed (one in Campomarino pilot site and one in Paliano pilot site) to record the air temperature and the soil temperature measured at approximately 7 cm below the ground.

3.3. Data analysis

Growth rate of the GHA strain isolated from the Botanigard® WP22 product, was monitored for the seven experimental temperatures (5, 10, 15, 20, 25, 30, 35°C). These data allow the modelling of the growth curve of the fungus strain as a function of the temperature adjusted with the cardinal model with inflection (CTMI) the R software version 4.0.5.

Modelling of the survival curves, of the time required to obtain a mortality rate of 50% (LT50) and the calculation of the mean survival time were carried out using the Kaplan-Meier estimator. To test, the effects of the spore doses and temperature on the virulence for each variables, we used a logistic regression based on a generalized linear model (GLM) with Firth's penalized likelihood calculation in order to model data in near complete separation using SAS 9.3.

One-Way ANOVA was used to determine the effect of Botanigard® WP22 soil treatment on the number of emerging adults, treatment on the number of dead larvae (for *G. mellonella*) and emerging adults (for *C. capitata*). The life span data were analysed by using a two-way ANOVA with treatment and contamination as main effect. Before the analysis, counts were transformed to natural log (x + 1), to normalize variances and standardize means. Means were separated by the Tukey HSD test at 0.05 probability. All statistical analyses were performed separately for each trial.

Chi-square (χ^2) test was used to statistically analyse the contamination of adults collected in the experimental units. The Yates correction was applied when the sample had low expected frequencies (below 50).

All analyses were performed using SPSS v.26.0 (SPSS Inc., Chicago, IL, USA).

4. Results

4.1. Laboratory testing of different strains of the entomopathogenic fungi *Beauveria bassiana* (Reunion Island)

4.1.1. Evaluation of the pathogenicity of commercial strains

In order to select among commercial products, a preliminary pathogenicity test was performed with the strains of *B. bassiana* ATCC 74040, GHA, I-2960, I-2961 and the strain of *B. hoplocheli* B507 corresponding respectively to the five commercial products Naturalis®, Botanigard® WP22, Ostrinil®, Serenisim® and Betel®. *C. capitata, B. dorsalis* and *Z. curcurbitae* late third instar larvae were dipped in a suspension containing 10^6 and 10^8 conidia/ml. Results are reported in Figure 5 and Table 1.

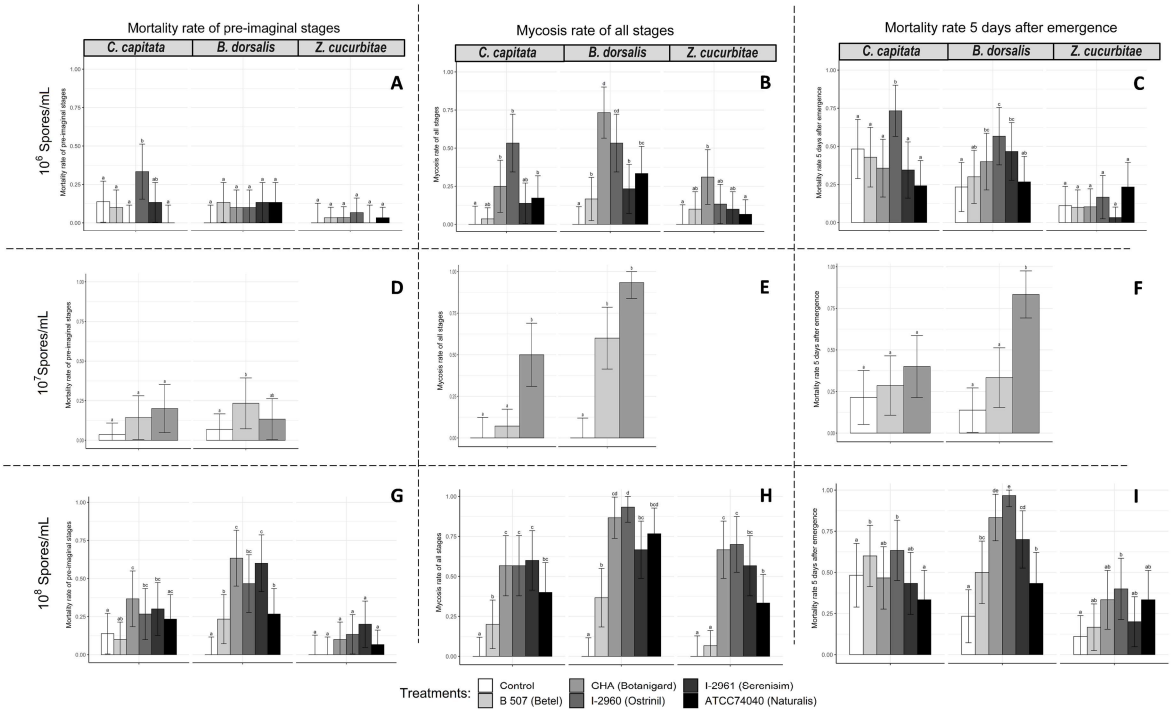

Figure 5 – Pathogenicity screening of *Beauveria hoplocheli* strain B507, and *Beauveria bassiana* strains I-2960, I-2961, ATCC 74040 and GHA using suspensions of 10^6, 10^7 10^8 conidia/mL on at late third instar larvae « jumping stage » *C. capitata*, *B. dorsalis* and *Z. cucurbitae*.

Mortality rate at emergence (A, D, G), mycosis rate (B, E, H), and mortality at five days after emergence (C, F, I) of late third instar larvae « jumping stage » for *C. capitata*, *B. dorsalis* and *Z. cucurbitae* treated with suspensions of 10^6 (A, B, C), 10^7 (D, E, F), 10^8 (G, H, I) conidia/mL. Data presented are means and the asymptotic or exact 95% confidence interval (30 insects per modality). For each modality a logistic regression based using a generalized linear model (GLM) with Firth's penalized likelihood was fitted and pairwise differences between treatments were tested, different letters above the 95% confidence interval of the mean, indicate significant differences between treatments ($P < 0.05$).

Table 1 - Results of the statistical tests looking at the effect of the five different strains of the fungi used in the pathogenicity screening test (figure 5) on three variables: mortality at emergence, mycosis and mortality five days after emergence

Dose (conidia /ml)	Variable	*Ceratitis capitata*			*Bactrocera dorsalis*			*Zeugodacus cucurbitae*		
		DF	χ2	P-value	DF	χ2	P-value	DF	χ2	P-value
10^{6}	Mortality at emergence	5	14.5848	0.0123	5	3.6222	0.605	5	1.7664	0.8804
	Mycosis	5	23.4867	0.0003	5	30.3825	<.0001	5	9.2457	0.0997
	Mortality 5 days after emergence	5	15.056	0.0101	5	14.9884	0.0104	5	4.9504	0.422
10^{7}	Mortality at emergence	2	2.1523	0.3409	2	6.5578	0.0377			
	Mycosis	2	18.3537	0.0001	2	20.0552	<.0001			
	Mortality 5 days after emergence	2	0.7672	0.6814	2	26.3355	<.0001			
10^{8}	Mortality at emergence	5	12.0763	0.0338	5	33.7267	<.0001	5	6.8238	0.2341
	Mycosis	5	21.0383	0.0008	5	27.6823	<.0001	5	30.5794	<.0001
	Mortality 5 days after emergence	5	9.8861	0.0785	5	46.2166	<.0001	5	8.432	0.134

This screening test revealed that the five commercial strains were pathogenic to the three fruit fly species as they all induced the development of mycosis on cadavers in the pre-imaginal and imaginal stages (Figure 5 B, E, H and Table 1).

Mortality at emergence is presented in figure 5 (A, D, G). Analysis of the emergence rates revealed that the fungi strain effect (GHA, ATCC 74040, I-2960, I2961, B507 and Tween (control)) (Chisq = 9.6; DF = 4; p = 0.048), the dose (10^6, 10^7 and 10^8 conidia / mL) (Chisq = 22.59; DF = 2; p <0.0001), the species effect (*C. capitata*, *B. dorsalis* and *Z. cucurbitae*) (Chisq =11.29; DF = 2; p =0.0035), and interaction treatment x dose (Chisq = 12.3; DF = 5; p = 0.03) were significant. For a dose of 10^6 conidia / mL, I-2960 on *C. capitata* was the only strain that significantly reduced emergence compared to the control (Figure 5A). For a dose of 10^7 conidia / mL B507reduced significantly the emergence of *B. dorsalis* compared to the control (Figure 5D). For a dose of 10^8 conidia / mL on *C. capitata,* except for B507, all the strains reduced significantly the emergence compared to the control, and the GHA strain reduced the emergence significantly more than the other strains. For a dose of 10^8 conidia / mL on *B. dorsalis,* all the strains reduced significantly the emergence compared to the control, and the GHA and I-2960 strains reduced the emergence significantly more than the other strains (Figure 5G).

For mortality five days after emergence figure 5 (C, F, I), treatment effect (GHA, ATCC 74040, I-2960, I2961, B507 and Tween (control)) (Chisq = 9.6; DF = 4; p = 0.048), dose (10^6, 10^7 and 10^8 conidia / mL) (Chisq = 22.59; DF = 2; p <0.0001), species (*C. capitata*, *B. dorsalis* and *Z. cucurbitae*) (Chisq =11.29; DF = 2; p =0.0035), and interaction treatment x dose (Chisq = 12.3; DF = 5; p = 0.03) were significant. The main result was that the maximum emergence reduction was observed for the GHA strain at 10^8 conidia / mL on *B. dorsalis*. For a dose of 10^6 conidia / mL, I-2960 on *C. capitata* was the only strain that significantly reduced emergence compared to the control (Figure 5B). For a dose of 10^7 conidia / mL GHA reduced significantly emergence of *B. dorsalis* compared to the control. For a dose of 10^8 conidia / mL on *C. capitata,* the strain effect was not significant (Table 1). For a dose of 10^8 conidia / mL on *B. dorsalis,* all the strain reduced significantly the emergence compared to the control, and the GHA and I-2960 strains reduced the emergence significantly more than the other strains.

The GHA strain was the most pathogenic regarding to the mortality of preimaginal, mycosis or mortality 5 day after emergence or imaginal stages (Figure 6).

We selected the Botanigard® WP22, the commercial product corresponding to the strain of the GHA product, for further experimentation on the bases of:

i/ the screening pathogenicity test that revealed that GHA was among the most pathogenic strains

ii/ the fact that Botanigard® WP22 is the product with the highest spore density (4.4 x 10^{10} versus 5 x 10^{8} conidia/g for Ostrinil® and Serenisim® or 2 x 10^{7} conidia/ml or conidia/g for Naturalis® and Betel®), which will allow to inoculate soils on a wider range of spore doses.

iii/the fact that Botanigard® WP22 is widely distributed in Europe.

The Betel® product was also selected for further experimentations as a control because this product has already been widely used as soil treatments in Reunion Island where the strain B507 was able to remain active and settle in soils.

Figure 6 - *Beauveria* mycosis - *Beauveria* mycosis on *Ceratitis capitata* adult stage (left). External (top right) and internal (bottom right) mycosis on a pupa of *Bactrocera dorsalis*.

4.1.2. Proof of concept of soil-dwelling stages control of fruit fly using *Beauveria* species

To achieve proof of concept that the use of these *Beauveria* products as soil treatments will have the potential to control the soil-dwelling stages of fruit flies, we treated sand with Botanigard® WP22 or Betel® at a rate of 10^{7} conidia/g before depositing a late third instar larvae of *C. capitata* or *B. dorsalis*.

This experiment demonstrated that these treatments significantly reduced emergence compared to the control for all fly species. Emergence rates dropped significantly ($p<0.05$) from over 90% for the controls to 64% (±16%) for Botanigard® WP22 and 47% (± 19%) for Betel®. Botanigard® WP22 and Betel® induced significantly higher mortality rates ($p<0.05$) than controls. For *C. capitata*, mortality ranged from 86.7% (Betel®) to 88.9% (Botanigard) (± 7%). For *B. dorsalis*, rates ranged from 98.3% (Betel®) to 98.9% (Botanigard®WP22) (± 3%) (Figure 7). *Beauveria*-contaminated sands induced significantly ($p<0.05$) more mycosis in *B. dorsalis* individuals (95.9 ± 3.6% mycosis) than in *C. capitata* (72.2 ± 13%), (Figure 8). Botanigard® WP22 induced significantly more mycosis than Betel® ($p<0.05$).

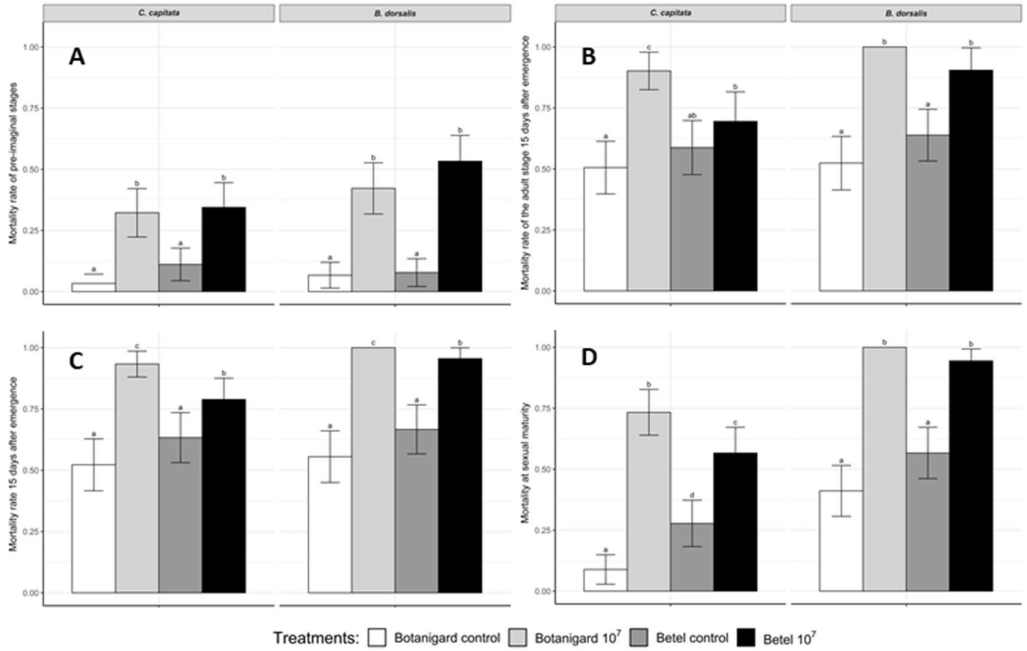

Figure 7 - Mortality rate of *Bactrocera dorsalis* and *Ceratitis capitata* placed at late third instar larvae « jumping stage » on sand treated with Botanigard WP 22 for a dose of 0 (Control) and 10^7 conidia/g of sand at 25°C.

Mortality at emergence (A), at the adult stage 15 days after emergence (B), for all stages at 15 days after emergence (C) for all stages at sexual maturity, (four days after emergence for *C. capitata*, 15 day for *B. dorsalis*) (D). Data presented are means and the asymptotic or exact 95% confidence interval, with three replicates of 30 insects for each treatment and each species. A logistic regression based using a generalized linear model (GLM) with Firth's penalized likelihood was fitted and pairwise differences between treatments were tested, different letters above 95% confidence interval of the mean, indicate significant differences between treatments ($P < 0.05$).

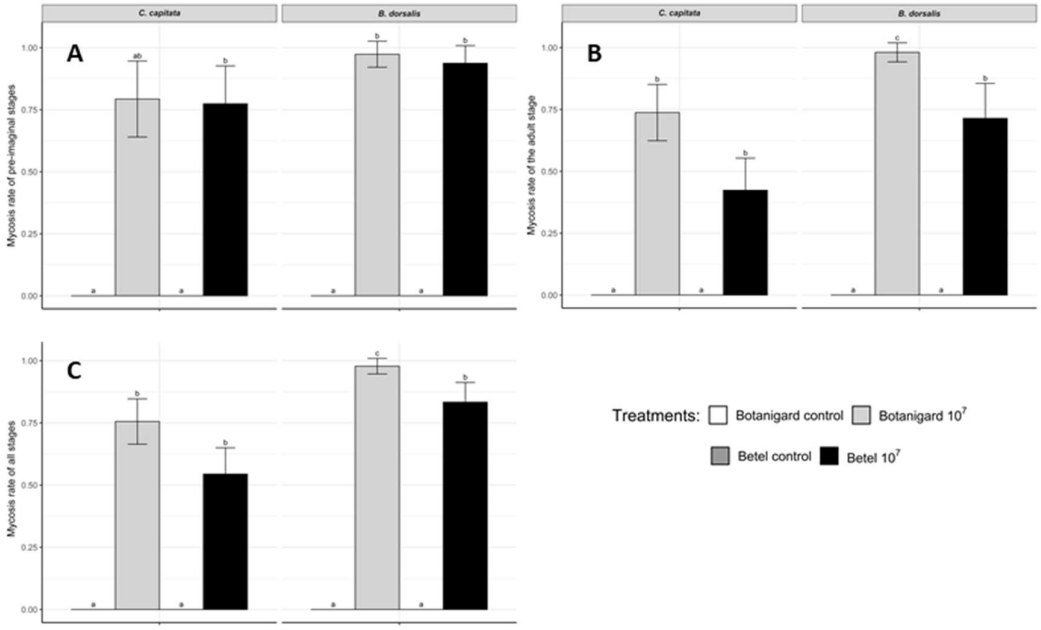

Figure 8 - Mycosis rate of *B. dorsalis* and *Ceratitis capitata* placed at late third instar larvae « jumping stage » on sand treated with Botanigard WP 22 for a dose of 0 (Control)and 10^7 conidia/g of sand for at 25°C.

Mycosis of pre-imaginal stages (A), at the adult stage (B), for all stages (C). Data presented are means and the asymptotic or exact 95% confidence interval, with three replicates of 30 insects for each treatment and each species. A logistic regression based using a generalized linear model (GLM) with Firth's penalized likelihood was fitted and pairwise differences between treatments were tested using a Z test. Different letters above the standard error of the mean bar, indicate significant differences between treatments ($P < 0.05$).

4.1.3. Effect of temperature on the growth rate of GHA strain of *Beauveria bassiana*

Another question was whether the soil temperatures of the apple and peach orchards in Italy where we will conduct the field tests would be compatible with those that allow the fungus to grow and survive. For this purpose, we studied the effect of temperature on the growth of the GHA strain that composes Botanigard WP22.

The optimum growth of the GHA strain of *B. bassiana* isolated from Botanigard® WP22 was 25.8°C [EC 95%: 25.6-27.0]. The estimated minimum growth temperature was 6.5°C [EC 95%: 6.1-6.8] and the maximum temperature was 35°C [EC 95%: 34.9-35.0] (Figure 9).

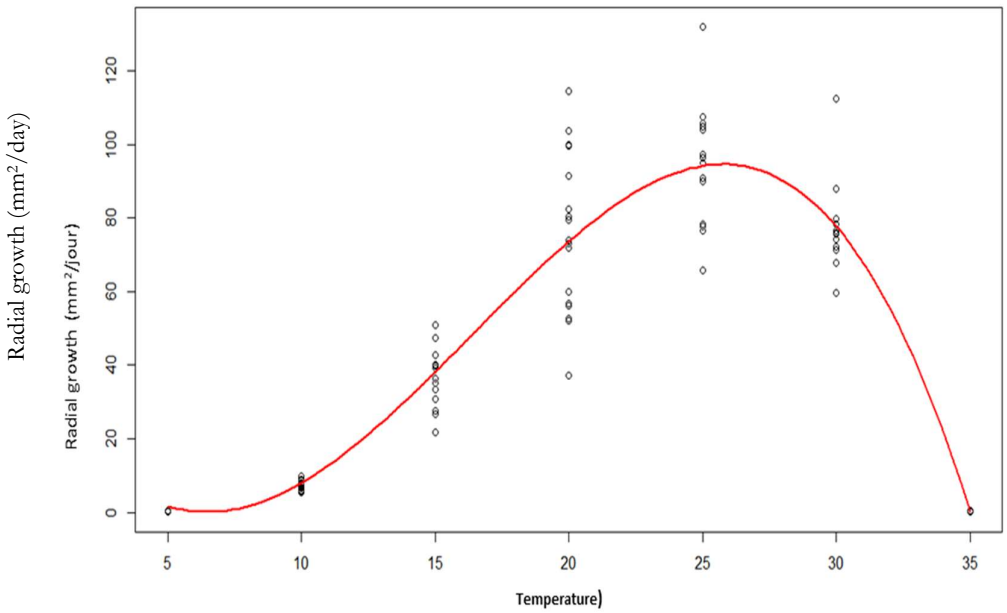

Figure 9 - Effect of temperature on vegetative growth of *Beauveria bassiana* GHA strain isolated from Botanigard® WP22
Raw data are identified by the white dots and the red curve represents the CTMI model.

Then we placed the temperature range of growth of the GHA strain on the observed temperatures for the period 2002-2019 in the middle of the soil layer called level 1 between 0 and 7 cm for the sites of Campomarino and Paliano where the field trials in apple orchard will take place (Figure 10). We noted that the fungus would be able to develop between the end of February and the beginning of March until the end of November to mid-December. It should be noted, however, that temperatures can occasionally exceed 35°C and exceptionally reach 42°C. It is not sure that the fungi can resist to such temperatures.

These results, coupled with *C. capitata* development thresholds of 9.7°C (Tassan et al. 1990) and their thermal limits (Vargas et al. 1996; Duyck et Quilici 2002), allowed the selection of four temperatures for the next pathogenicity tests: 10, 15, 20 and 25°C.

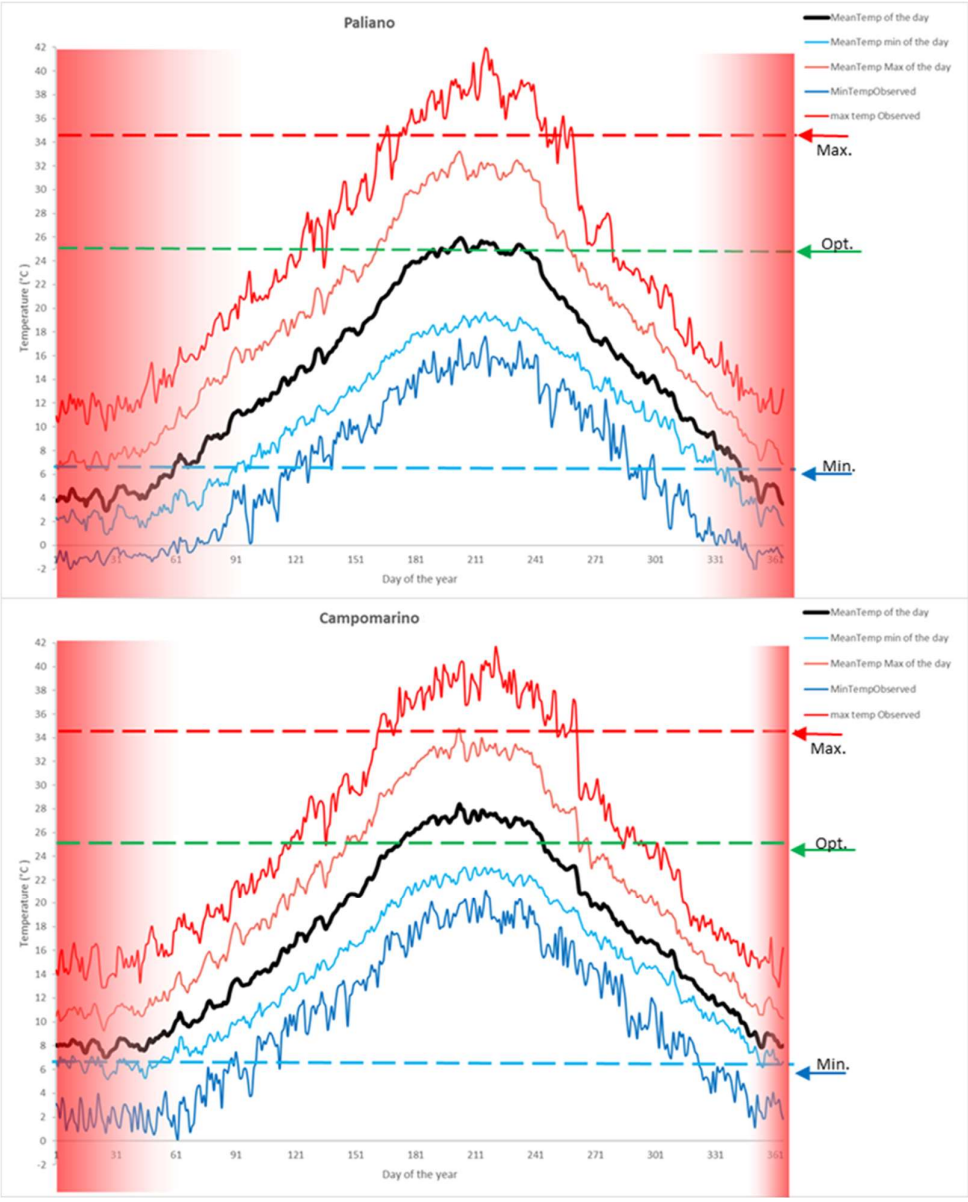

Figure 10 - Maximal, optimal and minimal growth temperatures of the *Beauveria* GHA strain of Botanigard® WP22 positioned on daily average of past (2002-2019) soil temperature in Paliano and Campomarino

The dotted lines represent respectively the maximal, the optimal and the minimal growth temperatures of the *Beauveria* GHA strain of Botanigard® WP22. The soil temperatures of the Italian regions of Campomarino and Paliano were retrieved for the period 2002-2019 in 1h steps from the Copernicus database, Era Land5 (https://cds.climate.copernicus.eu). It is the temperature in the center of the so-called level 1 soil layer between 0 and 7cm.

4.1.4. Effect of spore doses depending on the temperature on pathogenicity and virulence of the Botanigard® WP22 against soil-dwelling stages of *Ceratitis capitata*

The objective of this experiment conducted with late third instar larvae of *C. capitata* was to determine the effects of temperature (10, 15, 20 and 25°C) and doses (0 (Control), 10^5, 10^6, and 10^7, conidia/g) of Botanigard® WP22 on pathogenicity and virulence.

4.1.4.1. Mortality of pre-imaginal stages

At 10°C, all individuals died in the pre-imaginal stages in both the control and Botanigard modalities. The logistic regression applied to mortality of pre-imaginal stages of *C. capitata* (Figure 11a) revealed a significant dose effect at 25°C (χ^2= 8.8; DF=3; p<0.05) and highly significant at 20°C (χ^2= 29.5; DF=3; p<0.0001) and at 15°C (χ^2= 47.7; DF=3; p<0.0001). At 20°C, the fungus significantly killed pre-imaginal stages whatever the dose of Botanigard® WP22 used to contaminate the sand. At 25°C, only the treatment with sand contaminated with 10^7 conidia.g^{-1} was significantly different from the control for mortality rates of 21.7% and 6.8%, respectively.

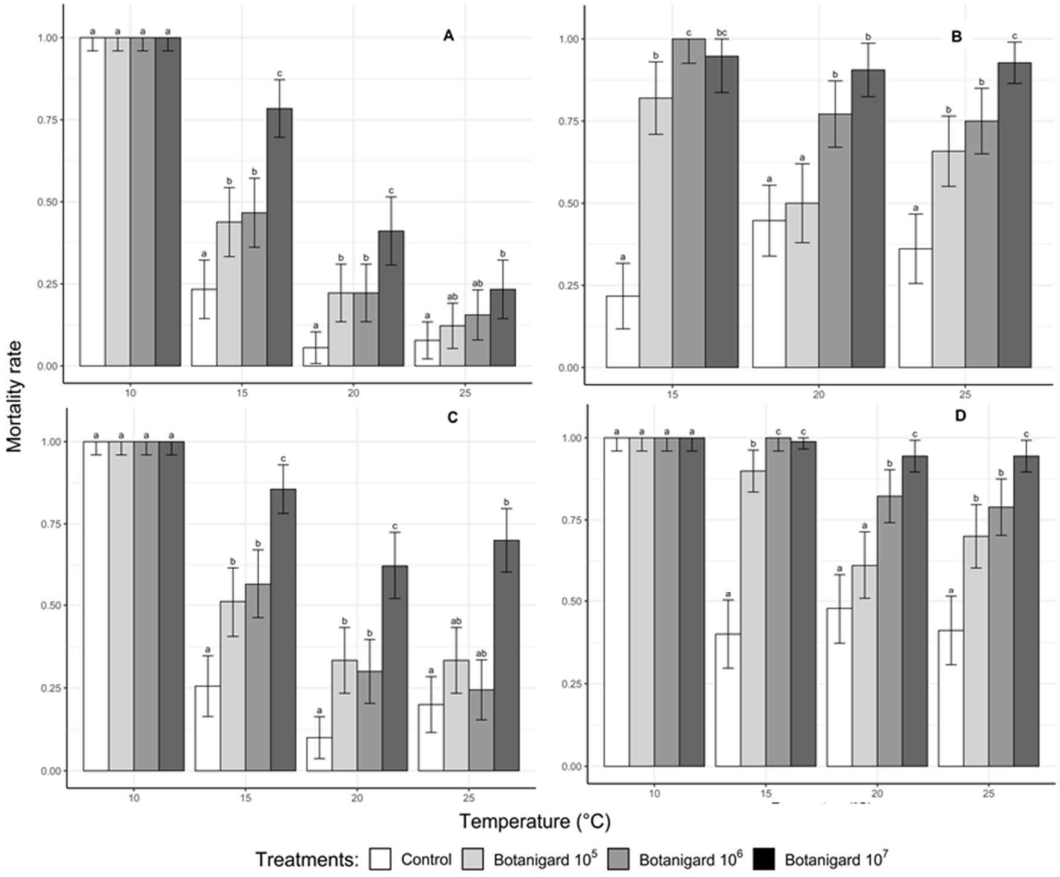

Figure 11 - Mortality rate of *Ceratitis capitata* placed at late third instar larvae « jumping stage » on sand treated with Botanigard WP 22 for doses of 0 (Control), 10^5, 10^6, and 10^7 conidia/g of sand for four temperature: 10, 15, 20 and 25°C

Mortality at emergence (A), at the adult stage 15 days after emergence (B), for all stages at sexual maturity, four days after emergence (C), for all stages at 15 days after emergence (D). Data presented are means and the asymptotic or exact 95% confidence interval, with three replicates of 30 insects for each temperature, each treatment and each species. For each temperature, a logistic regression based using a generalized linear model (GLM) with Firth's penalized likelihood was fitted and pairwise differences between treatments were tested. Different letters above 95% confidence interval of the mean, indicate significant differences between treatments (P < 0.05).

4.1.4.2. Mortality of the adult stage

The logistic regression applied to mortality of the adult stage, 15 days after emergence (Figure 11b), revealed highly significant dose effect all temperature with at 25°C, χ^2= 46.3 (DF=3; p<0.0001), at 20°C χ^2= 34.3 (DF=3; p<0.0001) and at 15°C χ^2= 51.3; (DF=3; p<0.0001). The fungus significantly killed adult stages whatever the dose of Botanigard® WP22 used to contaminate the sand except for the modality at 20°C treated with Botanigard® WP22 for the dose of 10^5 conidia.g^{-1}.

The analysis of adult survival revealed a highly significant (P< 0.0001) dose effect for temperatures 15, 20 and 25°C, on adult survival. With the exception of the modality at 20°C treated with Botanigard® WP22 for the dose of 10^5 conidia.g^{-1}, all the multiple comparison tests adjusted with Sidak's correction revealed that the survival of adults were significantly affected by all concentrations being effective in reducing the life expectancy of flies (p< 0.05). Thus, at this temperature, LT50 was 18 days in the control and 16 days for flies treated with sand with a concentration of 10^5 conidia.g^{-1}; but drops to 8 days for those treated with sand with a concentration of 10^6 conidia.g^{-1} and to 6 for those treated with sand with a concentration of 10^7 conidia.g^{-1} (Figure 12).

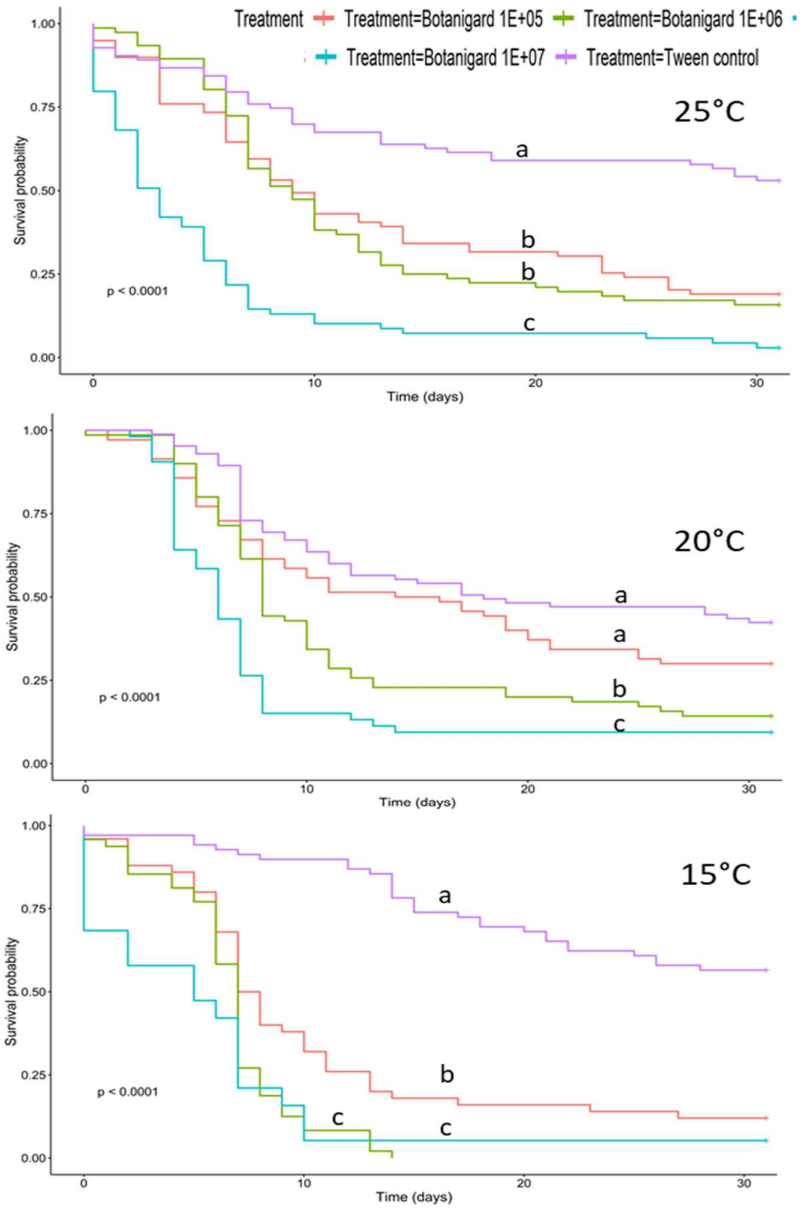

Figure 12 - Kaplan-Meier survival curves of adults of *Ceratitis capitata* when placed at the late third instar larvae « jumping stage on sand treated with Botanigard WP 22 for doses of 0 (Control), 10^5, 10^6, and 10^7 conidia/g of sand for temperature: 15, 20 and 25°C.

Different letters indicate significant differences between treatments (log-rank test, P < 0.05 after Sidak's correction). Crosses at the end of each curve indicate censored data.

4.1.4.3. Mortality before flies can reproduce and Kaplan-Meier survival curves of adults

The logistic regression model applied to mortality at sexual maturity (Figure 11C) of *C. capitata* (4 days after emergence) revealed that the dose effect of Botanigard® WP22 was highly significant at 20°C (χ^2=23.9 DF=3; p<0.0001) and 25°C (χ^2= 52.1; DF=3; p<0.001). At 15 and 20°C, the fungus significantly killed individuals before their sexual maturity regardless of the dose of Botanigard® WP22 used to contaminate the sand. At 25°C, only treatments with sand contaminated with a concentration of 10^7 conidia.g^{-1} induced a mortality significantly different from the control.

4.1.4.4. Mortality 15 days after emergence

The logistic regression applied to mortality of the adult stage 15 days after emergence (Figure 11D) revealed highly significant dose effect all temperature with at 25°C, χ^2= 51.6 (DF=3; p<0.0001), at 20°C χ^2= 46.6 (DF=3; p<0.0001) and at 15°C χ^2= 67.5; (DF=3; p<0.0001). The fungus significantly killed *C. capitata* whatever the dose of Botanigard® WP22 used to contaminate the sand except for the modality at 20°C treated with Botanigard® WP22 for the dose of 10^5 conidia.g-1.

4.1.4.5. Mycosis of soil-dwelling and adult stages

The mycosis rates of dead individuals for soil-dwelling stages (Figure 13 A), adults (Figure 13 B) or overall stage (figure 13 C) were over 0.50. Whatever the dose of conidia in the sand, the mycosis rate was significantly different from the control for all temperatures tested. The conidial content of the sand had very little effect on the fungal mycosis rate in the different insect stages.

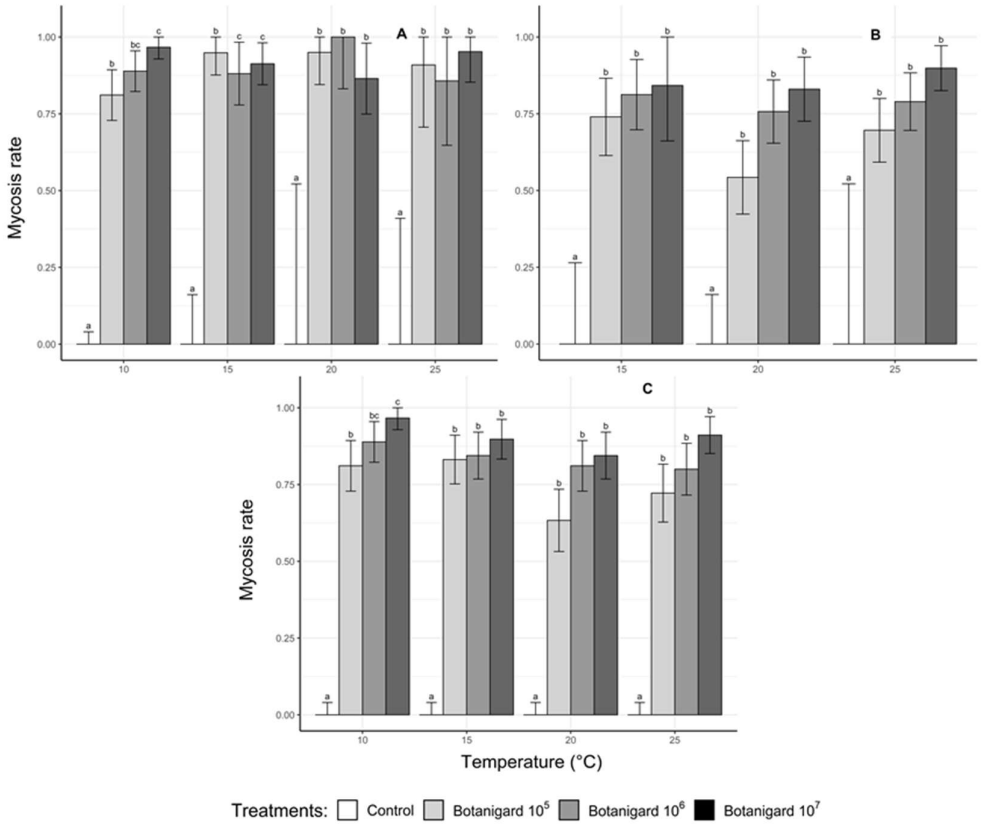

Figure 13 - Mycosis rate of *Ceratitis capitata* placed at the late third instar larvae « jumping stage on sand treated with Botanigard WP 22 for doses of 0 (Control), 10^5, 10^6, and 10^7 conidia/g of sand for four temperature: 10, 15, 20 and 25°C.

Mycosis of pre-imaginal stages (A), at the adult stage (B), for all stages (C). Data presented are means and the asymptotic or exact 95% confidence interval, with three replicates of 30 insects for each temperature, each treatment and each species. For each temperature, a logistic regression based using a generalized linear model (GLM) with Firth's penalized likelihood was fitted and pairwise differences between treatments were tested using a Z test. Different letters above the standard error of the mean bar, indicate significant differences between treatments (P < 0.05).

In conclusion, in laboratory, the treatment of sand with Botanigard® WP22 induced a mortality of *C. capitata* significantly different from the control whatever the dose and at all temperatures tested. *C. capitata* mortality was positively correlated with the dose of Botanigard® WP22, whereas it was negatively correlated with the temperature.

4.2. Field experiments (Italy)

4.2.1. Climatic data

Records of air and soil temperatures during the trial periods (from the day of larval release until the day of the last emerged adult) are reported in figures 14-16.

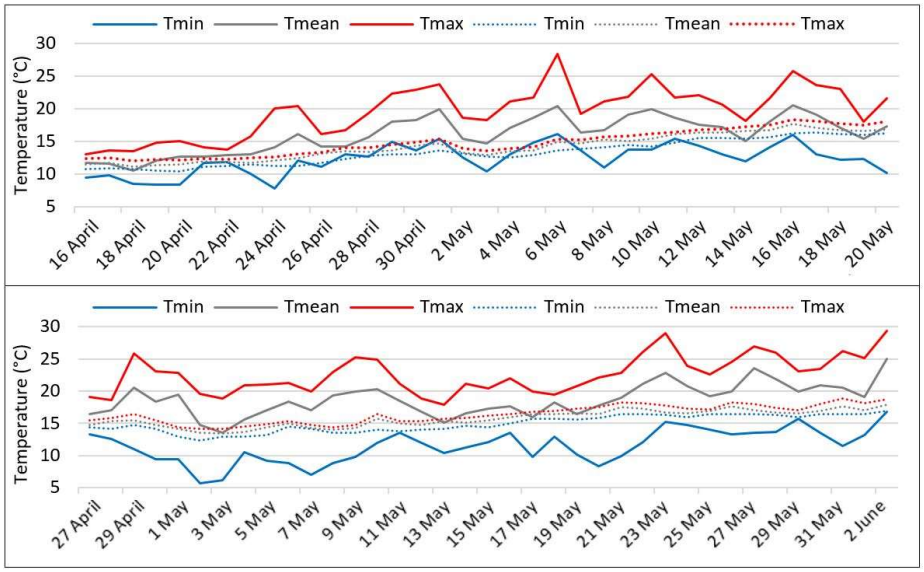

Figure 14 – OFF-Season – spring: minimum, mean and maximum recorded temperature during the field experiment

OFF-season spring: minimum (Tmin), mean (Tmed) and maximum (Tmax) temperature recorded on air (continuous line) and approx. 7 cm below ground (dotted line) in Campomarino from 16 April to 20 May (A) and Paliano from 27 April to 2 June (B) pilot sites.

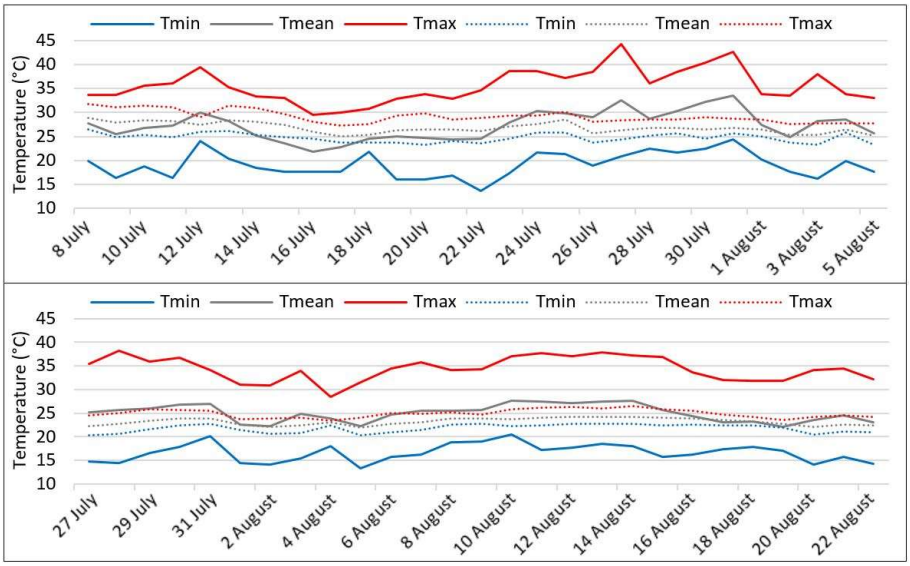

Figure 15 - On-Season – summer: minimum, mean and maximum recorded temperature during the field experiment

ON-Season – summer: minimum (Tmin), mean (Tmed) and maximum (Tmax) temperature recorded on air (continuous line) and approx. 7 cm below ground (dotted line) in Campomarino from 8 July to 5 August (A) and Paliano from 27 July to 22 August (B) pilot sites.

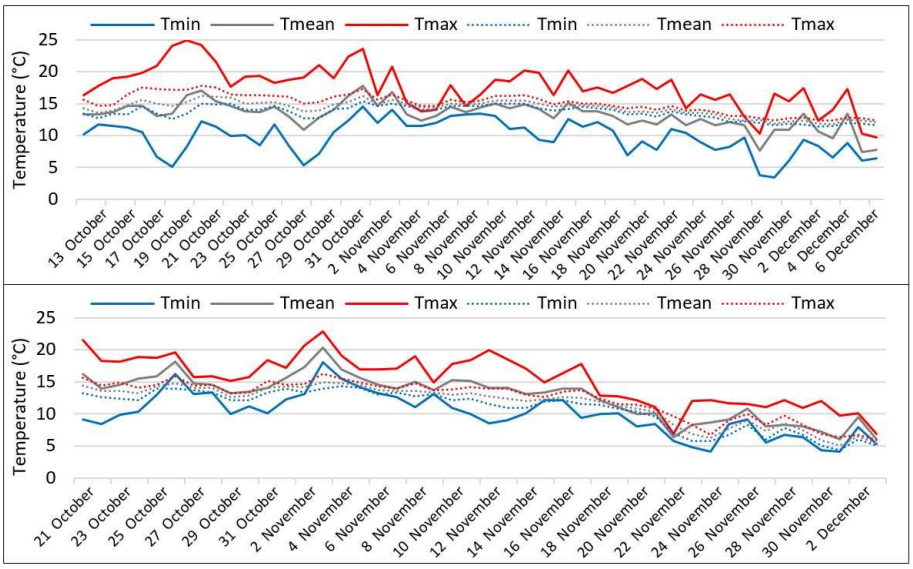

Figure 16 - OFF-Season – Autumn minimum, mean and maximum recorded temperature during the field experiment

OFF-Season – autumn: minimum (Tmin), mean (Tmed) and maximum (Tmax) temperature recorded on air (continuous line) and approx. 7 cm below ground (dotted line) in Campomarino (A) and Paliano (B) pilot sites.

In all cases, it can be noted that soil temperatures follow the same trend of the air temperature, but the peaks in daily maximum and minimum temperatures were much more moderate.

4.2.2. Flies emergence and contamination of adults at emergence

For the OFF-Season – spring modality, the first adults were recorded 35 days (in Campomarino pilot site) and 22 days (in Paliano pilot site) after the release of late third instar larvae. A total of 44 adults (4.1% of released larvae) were collected, 27 (5% of released larvae) from control units and 17 (3.1% of released larvae) from *Beauveria* treated units (Figure 17A). No statistical differences were reported between them (ANOVA, F = 0.392, df = 1, $p = 0.536$). No mycosis contamination was detected in the flies collected in control units, whereas 5 (29.4% of samples) from the *Beauveria* units developed the fungus (Figure 17B). As resulted by the χ^2 test (Table 1), the number of contaminated flies was significantly higher for adults emerged from *Beauveria* treated units ($\chi^2 = 6.29$, df = 1, $p < 0.05$).

For the ON-Season – summer modality, the first adults were recorded 12 days (in Campomarino pilot site) and 13 days (in Paliano pilot site) after the release of the larvae. A total of 247 adults (9% of released larvae) were collected, 76 (8.4% of released larvae) from control units, 79 (8.7% of released larvae) from *Beauveria* April treated units and 92 (10.2% of released larvae) from *Beauveria* July treated units (Figure 18A). No statistical differences were reported between different treatments (ANOVA, F = 0.204, df = 2, $p = 0.816$). Mycosis contamination was detected in the flies collected in all units. 13.3% of flies emerged from control units, 76.9% of the flies emerged from *Beauveria* April units, and 62.5% of the flies emerged from

Beauveria July units developed the fungus (Figure 18B). As resulted by the χ^2 test, the contamination was significantly higher for flies emerged from *Beauveria* treated units ($\chi^2 = 9.95$, df = 2, $p < 0.01$), for detail see Table 1.

For the OFF-Season – autumn modality, the first adults were recorded 30 days after the release of the larvae in Campomarino pilot site. No emergence was observed in the trial conducted in the Paliano pilot site. This could be due to a significant drop in soil temperature (6 °C) at the end of November. A total of 135 adults (5 % of released larvae) were collected, 50 from control units, 49 from *Beauveria* April treated units and 36 from *Beauveria* July treated units (Figure 19A). No statistical differences were reported between differently treated experimental units (ANOVA, F = 1.529, df = 2, $p = 0.240$). Mycosis contamination was detected in the flies collected in all units. 16.3% of flies emerged from control units, 68.7% of flies from *Beauveria* April units, and 67.6% of flies from *Beauveria* July units developed the fungus (Figure 19B). As resulted by the χ^2 test, the contamination was significantly higher in flies emerged from *Beauveria* treated units ($\chi^2 = 30.38$, df = 2, $p < 0.005$), for detail see Table 1.

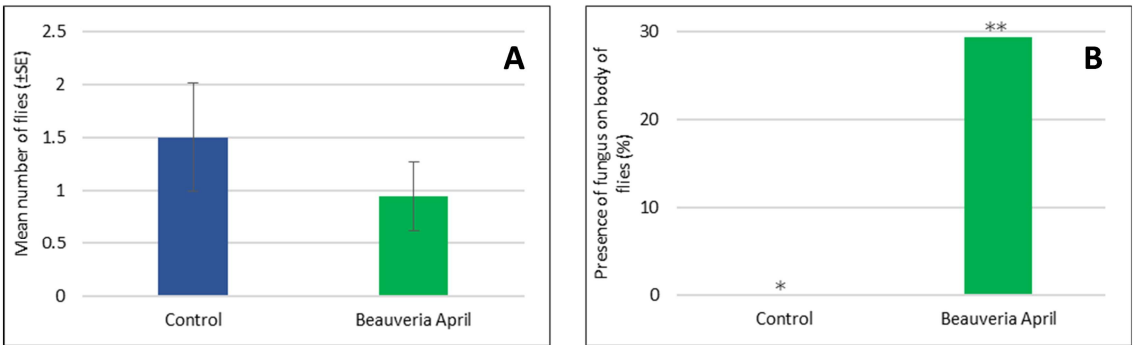

Figure 17 – OFF-Season – spring: mean number of flies collected in the experimental units (A) and percentage of presence of *B. bassiana* on body of flies after the emergence (B)
In the B figure, above each histogram, different stars indicate significant differences (χ2 test, p < 0.05).

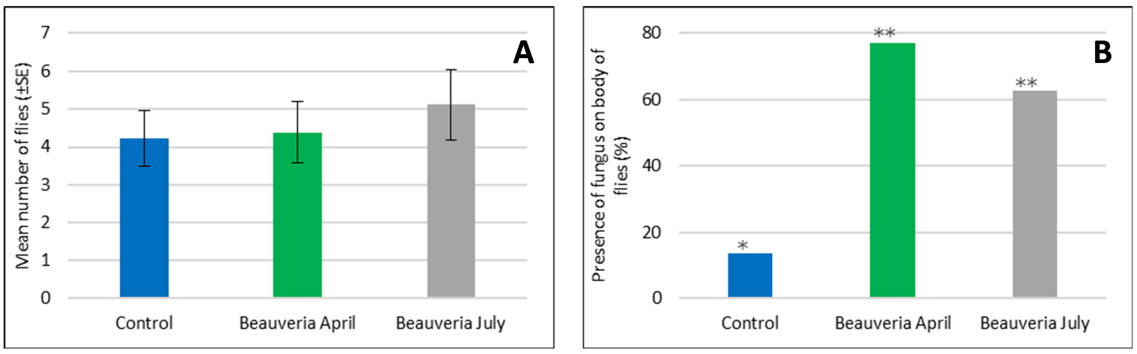

Figure 18 – ON-Season – summer: mean number of flies collected in the experimental units (A) and percentage of presence of *B. bassiana* on body of flies after the emergence (B)
In the B figure, above each histogram, different stars indicate significant differences (χ2 test, p < 0.05).

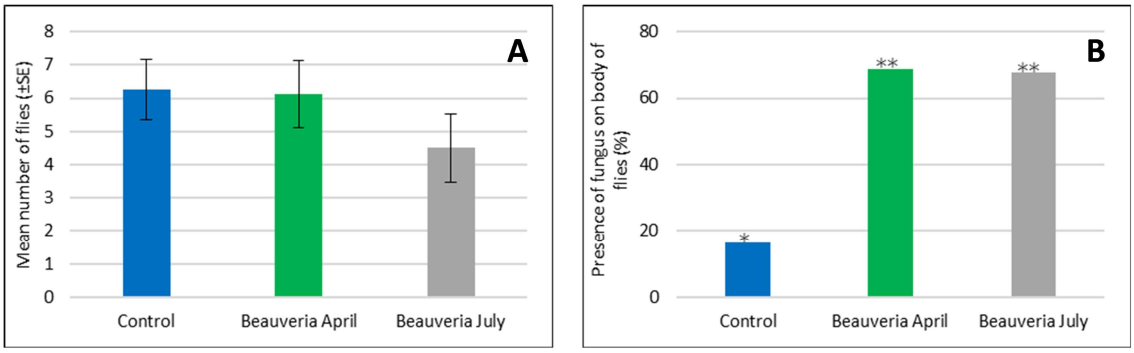

Figure 19 – OFF-Season – autumn: mean number of flies collected in the experimental units (A) and percentage of presence of *B. bassiana* on body of flies after the emergence (B)

In the B figure, above each histogram, different stars indicate significant differences ($\chi 2$ test, $p < 0.05$).

	First trial (OFF-Season – spring)			Second trial (ON-Season – summer)			Third trial (OFF-Seasor autumn)	
	χ^2	df	*P*-Value	χ^2	df	*P*-Value	χ^2	df
General				9.95	2	0.007	30.38	2
***Beauveria* April vs *Beauveria* July**				0.60	1	0.440	0.01	1
***Beauveria* April vs Control**	6.29	1	0.012	11.26	1	<0.001	26.66	1
***Beauveria* July vs Control**				7.48	1	0.006	21.16	1
***Beauveria* April + *Beauveria* July vs Control**				538.51	1	<0.001	4342.49	1

Table 2 – Results of Chi-square ($\chi 2$) analyses on contamination of adults collected in the experimental units.

4.2.3. Evaluation of the life span of emerged adults

For the OFF-Season – spring modality, in both pilot sites no adult was taken alive from the experimental units.

For the ON-Season – summer modality, in Paliano pilot site few adults were collected alive (5 samples in total, 2 from control units, 1 from *Beauveria* April units and 2 from *Beauveria* July units). The mean lifespan was 19.4 days but no data analysis was done, given the low number of samples.

For OFF-Season – autumn modality, in Campomarino pilot site, 52 emerged flies were collected alive, 19 from control units, 17 from *Beauveria* April units and 16 from *Beauveria* July units. Table 2 reported the average life span of collected flies divided per units and contamination after death. ANOVA was not significant for treatment (control, *Beauveria* April and *Beauveria* July), contamination and their interaction (ANOVA, $F = 0.570$, $df = 5$, $p = 0.723$).

Table 3 – Average life span (±SE) of collected flies in Campomarino pilot site during the third trial

Treatment	Average life span in days (±SE)	
	Fungus on the body	**No fungus on the body**
Control trap	36.4 ± 10.7	31.2 ± 5.7
***Beauveria* April**	33.6 ± 6.4	22.8 ± 10.1
***Beauveria* July**	23.4 ± 5.6	16.3 ± 13.8

(OFF-Season – autumn).

4.2.4. Entomopathogenic activity of soil OFF-Season - spring (N+1) season after Botanigard WP22 soil treatments

4.2.4.1. *Galleria mellonella* trial

The Figures 20 show the mortality rate observed after one week and after two weeks of contact with the different soil treatments.

After one week, all larvae in the *Beauveria* Lab treatment were dead. The analysis of mortality rates after one week revealed that the effect of the treatment was significant (ANOVA, $F = 12.612$, $df = 3$, $p = 0.002$). No statistical difference was observed between *Beauveria* April and Control but the Control is statistically different with the other two treatments (*Beauveria* July and *Beauveria* Lab).

After two weeks, all larvae in *B. bassiana* contaminated soils were dead. The analysis of mortality rates after two week revealed that the effect of the treatment was highly significant (ANOVA, $F = 79.252$, $df = 3$, $p < 0.001$).

Mycosis contamination (Figure 21) was detected in all larvae in *B. bassiana* contaminated soils and in 45% of larvae in Control treatment. As resulted by the χ^2 test, the contamination was significantly higher on *G. mellonella* larvae in *Beauveria* treated units ($\chi^2 = 10.36$, $df = 3$, $p = 0.001$).

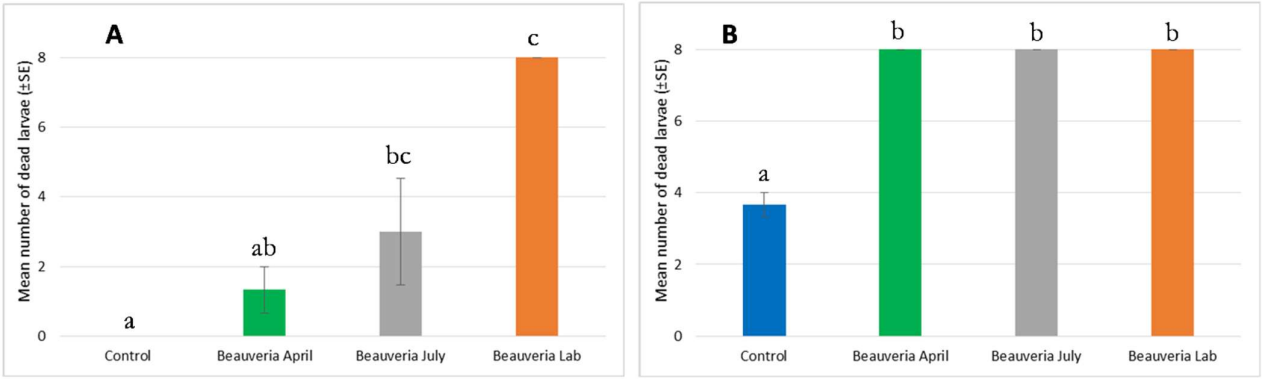

Figure 20 – Mortality rates after one week (A) **and two weeks** (B) **of *G. mellonella* in laboratory trials using different soil treatments.**

The data presented are means (±SE). Above each histogram, different letters indicate significant differences (Tuckey HSD test, $p < 0.05$).

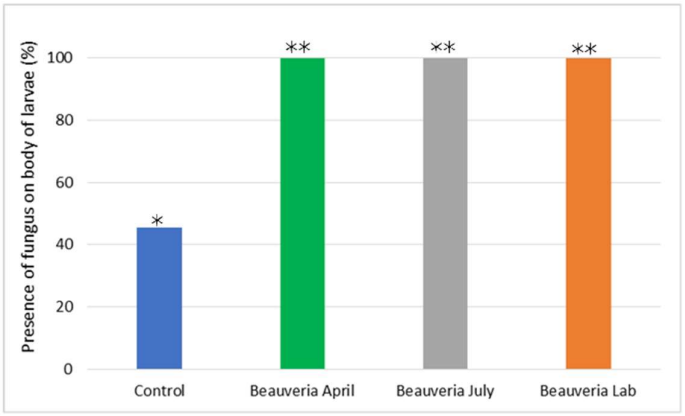

Figure 21 – Percentage of presence of *B. bassiana* on body of larvae in laboratory trials using different soil treatments.

Above each histogram, different stars indicate significant differences (χ^2 test, $p < 0.05$).

4.2.4.2. *Ceratitis capitata* trial

A total of 104 adults (28.9% of released larvae) were emerged, 48 (53.3% of released larvae) from Control treatment, 29 (32.2% of released larvae) from *Beauveria* April treatment, 27 (30.0% of released larvae) from *Beauveria* July treatment. No adults emergence were found in *Beauveria* Lab treatment (Figure 22A).

Statistical differences were reported between different treatments (ANOVA, F = 130.109, df = 3, $p < 0.001$). No statistical difference was observed between *Beauveria* April and Control but the Control is statistically different with the other two treatments (*Beauveria* July and *Beauveria* Lab).

Mycosis contamination was detected in the flies collected in *Beauveria* April (48.3%) and *Beauveria* July (74.1%) treatments (Figure 22B). As resulted by the χ^2 test, the contamination was significantly higher in flies emerged from *Beauveria* treated units ($\chi^2 = 43.73$, df = 2, $p < 0.001$), for detail see Table 4.

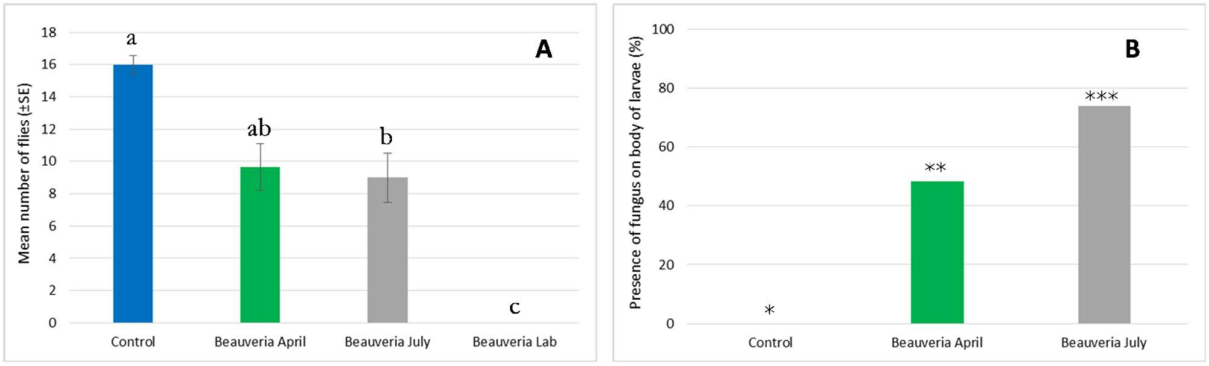

Figure 22 – Mean number of flies emerged in laboratory trials using different soil treatments (A) and percentage of presence of *B. bassiana* on body of flies after emergence (B).

Above each histogram: different letters indicate significant differences (Tuckey HSD test, p< 0.05) (left); different stars indicate significant differences (χ2 test, p < 0.05) (right).

Table 4 – Results of Chi-square (χ^2) analyses on contamination of adults collected in the laboratory trial

	χ^2	df	*p*-Value
General	43.73	2	<0.001
***Beauveria* April vs *Beauveria* July**	4.23	1	0.039
***Beauveria* April vs Control**	19.15	1	<0.001
***Beauveria* July vs Control**	43.09	1	<0.001
***Beauveria* April + *Beauveria* July vs Control**	4502.90	1	<0.001

5. Conclusions

Botanigard® WP22, the commercial product corresponding to the strain GHA was selected on the bases of i/ the results of the screening pathogenicity test that revealed that GHA was the most pathogenic strain, ii/ the fact that Botanigard® WP22 is the product with the highest spore density (4.4 x 10^{10} versus 5 x 10^{8} conidia/g for Ostrinil® and Serenisim® or 2 x 10^{7} conidia/ml or conidia/g for Naturalis® and Betel®), which will allow to inoculate soils on a wide range of spore doses iii/the fact that Botanigard® WP22 is widely available in Europe.

We demonstrated the proof of concept that treatment of a soil with the commercial products Botanigard®WP2 and Betel® at 10^{7} conidia/mL is able to kill *C capitata* and *B. dorsalis* reducing the emergence rate and longevity of adults. Growth tests of the GHA *B. bassiana* strain used in the commercial product Botanigard® WP22 and historical climatic data confirmed that the fungus has the potential to be active from March to November and survive in the soils of Paliano and Campomarino, Italy. In laboratory, the treatment of sand with Botanigard® WP22 induced a mortality of *C. capitata* significantly different from the control whatever the dose and at 15, 20 and 25°C. A 10°C, all late third instar larvae died. Botanigard® WP22 was able to induce mycosis of flies over the whole range of temperatures (10, 15, 20 and 25°C) and concentrations tested (10^{5}, 10^{6} and 10^{7} conidia/g). *C. capitata* mortality was positively correlated with the applied dose of Botanigard® WP22, whereas it was negatively correlated with the temperature. This was probably due to the fact that the insect did not develop or very slowly at low temperature while the fungus remained active. This suggests that the impact of the treatment may be greater OFF-Season, in Spring, and/or in Autumn when temperatures are lower with slow insect development while the fungus remains active.

In the pilot test in apple and peach orchards in Italy *B. bassiana* contamination was detected in the flies collected in all experimental units. The flies caught in the *Beauveria*-treated experimental units showed significantly higher percentages of contamination compared to those in the control units. This means that the *B. bassiana* was active in the soil and able to contaminate the larva, pupae or adults at the time of emergence. The finding in December of *Beauveria*-infected flies from units treated in April means that *B. bassiana* remained active in the soil for at least 8 months (despite the summer heat). A few *Beauveria*-infected flies were collected from control units in both pilot sites, suggesting that *B. bassiana* was naturally present in the soil and able to contaminate adult flies at emergence (13% in Paliano July and 16% in Campomarino October of emerged flies, respectively).

In the field trial, there was no difference in the emergence rate of *C. capitata* adults in the different treatments (Control, *Beauveria* April and *Beauveria* July experimental units) in any modality (OFF-Season – spring, ON-Season – summer, and OFF-Season – autumn). This result suggested that Botanigard applied to the soil at a rate of 14.5 g/ m^2 was not effective in reducing *C. capitata* emergence. Because this was observed at different application periods (modalities), it means that soil and air temperatures did not affect the results. In these field trials the rate of emergence of medfly adults was low (between 5 % in spring trial to 11.2 % in autumn trial). Although, this has been observed by others (Eskafi & Fernandez 1990), the fact that we observed 80% emergence in the laboratory when we used soil from these orchards confirmed that we had an experimental bias between what we observed in the laboratory and what we observed in the field. This could be due to the effect of natural enemies but also to thermal shock during the release of larvae, especially for the spring trial. OFF-Season - spring (N+1) season after Botanigard WP22 treatments soil presented a significant entomopathogenic activity against *G. mellonella* and significantly reduced *C. capitata* emergence.

Laboratory experiments using the biological insecticide Botanigard® WP22 *(B. bassiana* strain GHA), provided proof of concept that soil treatment significantly reduced emergence and increased mortality of *C. capitata. C. capitata* mortality was positively correlated with the dose of Botanigard® WP22, whereas it

was negatively correlated with the temperature suggesting this strategy could be particularly well adapted for OFF-Season, in Spring, and/or in Autumn to control *C. capitata*. The field trial revealed that the fungus is able to maintain itself in the soils of apple and peach orchards tested in Italy, being pathogenic and able to reduce emergence for at least one year in the soil. Botanigard® WP22 applied off-season or on early season before medfly population build-up could provide a useful tool for a sustainable environmental-friendly way to control the medfly. Implementing this strategy in WP6, is an opportunity to evaluate this strategy in large scale. Application of Botanigard® WP22 can be performed with simple drenching with standard sprayer equipment beneath the canopy in the orchards but we recommend if possible to incorporate the product in spring at 10^7 conidia/g in the first 5cm horizon of soil for a better repartition and protection of the inoculum. The use of Botanigard® WP22 against medflies might be constrained by efficacy and by costs.

6. Technical documentation and recommendations for use of entomopathogenic fungi in orchards for IPM applications

General description

- This tool consists of soil applications of a product formulating conidia of the *Beauveria bassiana* GHA strain, an entomopathogenic fungus to control the Mediterranean fruit fly by targeting its soil-dwelling life phase.
- Conidia adhering to the host germinate and produce enzymes that attack and dissolve the cuticle, allowing *B. bassiana* to penetrate, releasing toxins and grow inside the insect's body (manufacturers sell sheet[1]).
- Possible infections following a soil application:

1 When the larvae jump into the soil
2 When the larvae move through the soil
3 During emergence
4 When the adults move through the soil
5 Transfer between adults

Table 5 - Targeted medfly stages

Eggs in the fruit	
Larvae developing inside the fruit	
Late third instar larvae while jumping out and burying into the soil	x
Pupae	
Teneral adults while leaving the soil	x
Adults (females) active in the orchard	
Adults (females) present but not active (non-flying) in the orchard	

Directions for use (practices; how to apply, precautions)

- Botanigard® WP22 is a wettable powder formulation. The powder added to water can be sprayed on the soil application. For practical reasons of implementation, this strategy was chosen to apply Botanigard® WP22 to the soil surface at the rate of 14.5 g/m^2 in the first field trial in Italy in WP4.3. At this dose, the fungus is able to maintain in the soils, being pathogenic and able to reduce emergence for at least one year in the soil. Furthermore, results obtained in WP4.5 suggest that Botanigard® WP22 treatment associated with the cover crop *Festuca arundinacea* could reduce the emergence of *C. capitata* compared to the control with the cover crop alone.
- Apply BotaniGard 22WP using hand-held, ground spray equipment and/or low-volume application equipment e.g. herbicides sprayer (@10 hectolitre per ha).
- For future experimentation, even if the implementation seems more difficult, the incorporation of Botanigard® WP22 powder directly in the 0-5cm soil horizon should be considered, this strategy has the advantage of protecting the conidia from UV, of distributing the conidia in the soil where the larvae will crawl and where the adult will emerge. This strategy is used to apply Betel in sugarcane field in Reunion Island for the control of a white grub.

[1] https://bioworksinc.com/wp-content/uploads/products/botanigard-22wp/botanigard-22wp.pdf

Precautions

- If sprayed, a continuous agitation during application is required.
- Do not mix more Botanigard 22WP than can be sprayed in one day.

Timing of application

- Treating the soil early in the season targeting the first larvae jumping out the fruits into the soil could be a strategy to limit the increase of *C. capitata* populations in the orchard. We suggest treating the soil two weeks before the expected date of the first report of *C. capitata* in the area.

Frequency of application

- The field trial revealed that the fungus remained in the soils of apple and peach orchards in Italy and remained pathogenic for at least one year. We suggest treating the soil once a year, less if a laboratory test of soil pathogenicity against *C. capitata* reveals that the soil is still pathogenic.

Commercial distributers

- The material is available as a commercial product but for a different usage.
- Botanigard® WP22 is a registered trademark *LAM International*, a subsidiary of Certis USA the manufacturer. Different companies, depending on the country, distribute the product.
- Soil application, as being proposed, is not included in the product label, so any application in commercial orchards would require prior authorization from national authorities.

Compatibility and interactions with other treatments (our tool fungi, predators, pesticides)

Pesticides:

- Botanigard® WP22 is compatible with most chemical insecticides. However, some insecticide formulations can kill the fungal conidia. Botanigard® WP22 can be used a stand-alone, as a tank-mix partner or in rotation with other insecticides (Manufacturer label[2]).

Fungicides:

- Botanigard® WP22 is compatible in tank mix with some fungicides[3].

Compatibility with other biocontrol agents:

- https://bioworksinc.com/wp-content/uploads/20200303_BCA_Compat.pdf?_ga=2.54539691.1872145694.1644860389-1517584871.1626170316

Nematodes:

- It is OK to apply Botanigard® WP22 with nematodes, namely *Steinernema feltiae* and *Steinernema carpocapsae* (Manufacturers compatibility sheet).

Ground predators:

- *B. bassiana* is compatible with several predators. See compatibility sheet. and WP 4.5 deliverable.

Harvest and post-harvest sanitation:

- Compatible.

Efficacy

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[2] https://bioworksinc.com/wp-content/uploads/products/botanigard-22wp/botanigard-22wp-label.pdf

[3] Contact LAM International or your dealer for specific instructions on using BotaniGard 22WP with fungicides. https://bioworksinc.com/wp-content/uploads/products/botanigard-22wp/botanigard-22wp-label.pdf

- In the pilot laboratory test in Italy, Botaniguard® WP22 is persistent, pathogenic in the soil and able to reduce emergence for at least one year (field soil pathogenicity evaluated in laboratory conditions).
- In the laboratory, distributed at doses of 10^7, 10^6 and 10^5 conidia.g^1 of sand were evaluated and significantly reduced emergence and killed *C. capitata*. The higher the dose, the higher the treatment-increased the mortality rate.
  - At 15ºC: 10^7conidia.g^1 dose is the most efficient one, 10^6 dose is the second more efficient and 10^5 dose presents lower results.
  - At 20ºC and 25ºC: the results are significant lower. Nevertheless, in all scenarios 10^7 conidia.g^1 dose is the most efficient one.

Factors effecting efficacy

- Efficacy is related to soil temperature, depends on the nature of the soil.
- The time curve of fungal activity in the soil probably depends in part on the growth capacity of the fungus in relation to soil temperature.
- Growth limit temperatures of the *Beauveria* strain of Botanigard® WP22: below 6.5°C and above 35°C.

Application costs

- Cost of the product per ha of soil assuming that it has to be treated at 5 cm deep (or sprayed with the corresponding dose), knowing the cost of BotaniGard 22WP in 2022: 166 euros/kg.

High dose: 10^7 conidia/g of soil:

Soil with density 1.3; 5 cm deep treated = 650 000 kg of soil/Ha
650 000 x 10^{10} = 6.5 10^{15} conidia/ha
6.5 10^{15} / 4.4x10^{13}= 148 kg Botanigard WP22/ ha
148 x 166 =24568 euro/ha

Moderate dose: 10^6 conidia/g of soil:

14.8 kg Botanigard WP 22/ ha
2457 euro/ha

Low dose: 10^5 conidia/g of soil:

1.48 kg Botanigard/ ha
246 euro/ha

- The application of Botanigard WP22 could be done by a drench application to the soil around trees where the larvae would drop from the fruits.
- To prepare Botanigard WP22 drench, suspend the product in the needed water volume.
- No special equipment is needed other than what is commonly used by farmers namely tank sprayers (or backpack sprayers for small farms), so these are not included in the application costs.
- The calculations assume that area to be treated under the tree is around 7 m^2.
- Considering an average of 350 trees per ha (around 300 to 400 trees per ha), we estimate that the area to be treated per ha is around 2450 m^2 per ha.
- Labour costs vary from country to country: from 3.5 to 13.5 euros per hour. We assume an average of 8.5 euros per hour.

- Application could be once per season (but persistence of the fungus will have to be re-checked according to different soil types).
- Applications can be made at a low dose or a moderate dose but we recommend high dose for maximum efficacy in WP6 (Table 6).

Table 6 – Cost per hectare depending on the scenario

	Scenario	Once per season spread under the canopy		
		Low dose	**Moderate dose**	**High dose**
Botanigard WP 22	Cost per hectare treated	246	2457	24568
	Doses (kg/ha)	1.48	14.8	148
	m^2 to treat per ha under the canopy	2450	2450	2450
	Kg per ha (under the canopy)	**0.36**	**3.6**	**36.3**
	Costs/ha (under the canopy)	**60**	**602**	**6019**
Labour	Labour hours per ha	30	30	30
	Labour cost (€/h)	8.5	8.5	8.5
	Labour costs	**255**	**255**	**255**
	TOTAL COSTS/HA	**315**	**857**	**6274**

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