KWANTLEN POLYTECHNIC UNIVERSITY
INSTITUTE FOR SUSTAINABLE HORTICULTURE

Project Title:

Native *Beauveria bassiana* pilot products for controlling Lepidoptera in grape and other crops

Final Report

To:

Wine Grape Council

And

BC Investment Agriculture Foundation

From:

Institute for Sustainable Horticulture (ISH)

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SUMMARY

In the current research, four isolates of *Beauveria bassiana*, two Okanagan isolates (OKA-372, OKA-373) and two coastal isolates (ISH-189, ISH-190), were studied for efficacy against cabbage looper, CL (*Trichoplusia ni*) and green peach aphid, GPA (*Myzus persicae*) on plants. In addition, direct and residual toxicity studies were carried out on non-target organisms; an aquatic plant (duckweed, *Lemna minor*) and a beneficial parasitoid wasp, *Trichogramma sibericum,* to provide data for eventual registration. Production optimization trials were continued for registration purposes. In addition to the isolates as treatments, BotaniGard and 0.1% Tween 20 were used as positive and negative controls, respectively. In the field, second instar larvae of CL were sprayed using 4×10^8 spores/ml concentration of OKA-373 and ISH-189. For the greenhouse GPA bioassay, the aphids on cabbage plants were sprayed twice using all treatments (using 1×10^8 spores/ml for all isolates the first time and 4×10^8 spores/ml for ISH-189, OKA-373 and BotaniGard and 1×10^8 spores /ml for ISH-190 and OKA-372 the second time). On the same day as the treatments to the CL and GPA were applied, a control trial was performed for each application in the lab. A residual contact test for GPA in the lab was also carried out. All treated insects were incubated in a rearing room at 25 ± 1°C, and 16L: 8D. Number of live, dead and sporulated insects were recorded consistently. Four trials were carried out with duckweed using a range of concentrations of isolates, as well as, dead and live spores. For the *Trichogramma* test, ten one-day old females of *T. sibericum* were introduced onto each leaf disc treated with the isolates at 2×10^5 to 2×10^8 spores/ml concentration. Treated duckweed and *T. sibericum* were kept in a rearing room at 24 ± 1°C and L: D 16:8. The quantity of normal and abnormal duckweed leaves and live and dead *Trichogramma* were assessed. To compare mass production of the four isolates, the spores were cultured on rice grains or barley flakes, after being cultured in a liquid media (glucose, KNO_3, $K_2H_2PO_4$, $MgSO_4$, $CaCL_2$, yeast extract). In the second mass production trial, rice grains were used with a different liquid media (brewer's yeast, sucrose).

The results indicated that mortality and sporulation of CL were higher and occurred earlier in the lab compared to the field. OKA-373 showed the lowest LT_{50} and, significantly, the highest rate of mortality and sporulation of cabbage looper larvae in comparison with ISH-189 and BotaniGard in the field. After a week in the lab, OKA-373, ISH-189 and BotaniGard caused 100% mortality of CL larvae and no significant difference was observed between them. A significantly higher rate of mortality for GPA was observed for ISH-190 a week after the first application than for other isolates. Two weeks after the second application, ISH-190 was still responsible for the highest rate of mortality,; however, the rate was not significantly higher than the Okanagan isolates. In the lab control of the second trial, almost 100% mortality and 85-97% sporulation were observed and there was no significant difference between the isolates and BotaniGard. In the residual test, there was no significant difference between isolates 8 days post exposure. There was no consistent relationship between ability of duckweed to produce new leaves or the amount of chlorosis with increasing concentration of *B. bassiana.* The chlorosis of duckweed was significantly higher in both dead and live spores than the treatments without spores. The number of new leaves in the

treatment without spores and Tween was higher than the other treatments containing Tween-20. There was no significant difference for the effects on *T. sibericum* between the treatments for each isolate. In the first trial of mass production, OKA-373 produced on rice and barley had significantly the highest number of spores per gram of substrate; however, it did not show any significant difference between ISH-190 and ISH-189 produced on rice or barley. The quantity of spores for isolates produced on rice and barley were statistically similar. In the second trial, the highest rate of spores per gram of rice was for OKA-373; however, the number of spores for OKA-372 was very close to OKA-373.

In summary, these isolates are effective against the pests and are safe on non-target organisms. Further studies are needed however, for the optimization of isolate efficacy. In addition, microsclerotia production systems using liquid culture fermentation (LCF) is promising as a new and economic method for mass production of *B. bassiana*.

GENERAL MATERIALS AND METHODS

Fungal Isolates

Two native Okanagan isolates of *Beauveria bassiana* (OKA-373, OKA-372) and two coastal isolates (ISH-189, ISH-190) were selected as treatments (Table 1). BotaniGard® 22WP (*B. bassiana* strain GHA, label concentration: 4.4×10^{13} spores/kg, BioWorks Inc.) was included as the positive control.

Table 1. Origin and collection sites of *Beauveria bassiana* isolates

B. bassiana isolate	Substrate or insect host	Location of origin
OKA-372	Cutworm	Okanagan valley
OKA-373	Cutworm	Okanagan valley
ISH-189	Aphid	Fraser valley
ISH-190	Aphid	Fraser valley

Product Sources

Prior to beginning the trials, production experiments were conducted to obtain a sufficient amount of spores; but because of the experimental nature of these production runs, the quantities of harvested spores were not always adequate. Therefore, only two isolates were included in some trials as there was sufficient product remaining from 2016 production for the cabbage looper trial (Objective 1).

For the other trials (Green Peach Aphid, Duckweed and *Trichogramma*), spore suspensions were made from isolates that had been cultured on aluminum cookie sheets in the lab. Before performing the trials, viability and spore concentration (spores/gram) were determined using hemocytometer counts.

Culturing Spores on Aluminum Cookie Sheets

Aerial spores were harvested from cultures of each *Beauveria* isolate growing on Potato Dextrose Agar (PDA) and used to inoculate 250 ml flasks containing 150 ml of sterile liquid media (20g/L brewer's yeast, 20g/L sucrose, 1ml/L Gentamycin). Inoculated flasks were placed on an orbital shaker at 27°C, 150 rpm for 4 days. Then, each flask of liquid media was poured over the surface of an aluminum cookie sheet with a 1cm layer of 50% PDA. A sterile tray was inverted and placed over each inoculated tray. Trays were incubated at 27°C for 10 days. After 10 days, each tray was flooded with 50 ml of 0.1% Tween 20. Spores were gently dislodged from the media using a sterile metal cell spreader.

Statistical Analysis:

Virulence of the isolates against the target pests was compared using One-Way Analysis of Variance (ANOVA) followed by a comparison of means using the Tukey's honest significant difference (HSD) test (P _ 0.05) and a statistical software, PASW Statistics 18 (SPSS Inc. 2002).

To calculate Lethal Time (LT_{50}) of isolates, mortality recorded in treatments was corrected using Abbott's formula (1925). If Control mortality exceeded 20%, the trial was rejected. When this value was between 5% and 20%, the treatment mortality was corrected.

Corrected mortality (%) = $\frac{x-y}{100-y} \times 100$

X= Percent mortality in treatment, *Y*= Percent mortality in control

Probit analysis, LdP Line, was used to calculate lethal times of the isolates with 95% confidence limit, CL (Finney, 1971).

Objective 1: To determine Efficacy of the 4 (OKA-373, OKA-372, ISH-190, ISH-189) *B. bassiana* isolates against Cabbage looper on brassica plants in raised bed plots out of doors

This trial was the next step after the in vitro and small-scale lab efficacy studies in 2016.

Materials and Methods

Treatments

Treatments consisted of OKA-373, ISH-189, 0.1% Tween-20 solution (negative control) and BotaniGard® (positive control). The isolate solutions were prepared at a concentration of 4×10^8 spores/ml. The BotaniGard solution was made according to the label provided on the bag. OKA-372 and ISH-190 were not included.

Cabbage Looper: Cabbage looper eggs, *Trichoplusia ni* (Hübner), were purchased from Benzon Research Inc. and reared on broccoli leaves that were grown at KPU and surface sterilized with 10% bleach. The leaves were placed in disinfected containers (30 cm ×18 cm × 6 cm) with mesh windows on the lid which allowed for ventilation (Fig. 1, a). Egg sheets were attached inside the lid (Fig. 1, b) so that the first instar larvae dropped to the food source without being handled. The containers were kept in an insect rearing room at 23 ± 1°C and L: D 16:8 (Fig. 1. c).

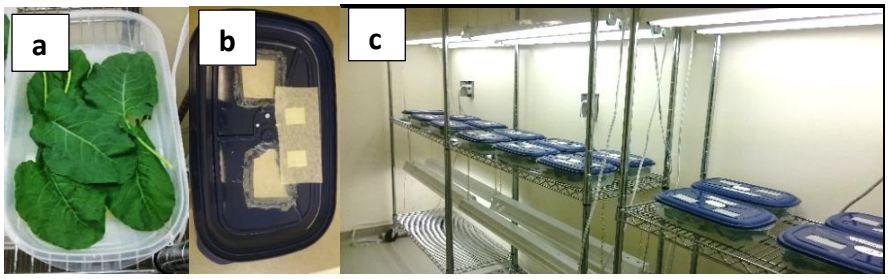


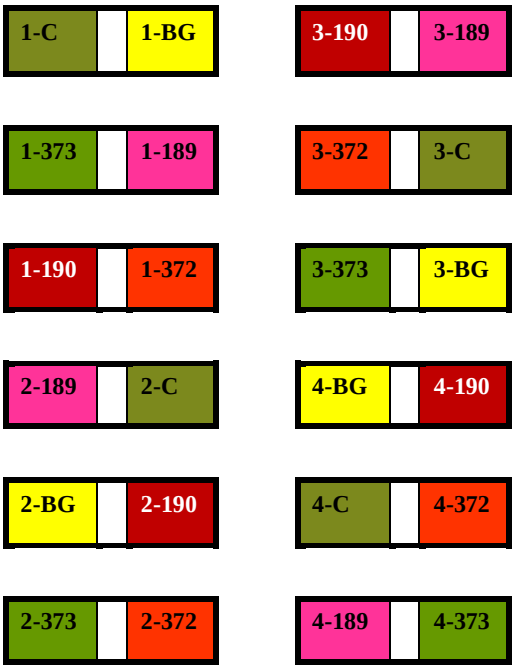
Fig. 1. Rearing container (a), egg sheets attached inside of the lid (b), insect rearing room (c)

Fig. 2. Bed divided to two plots using a plastic wall

Host Plant and Trial Preparation: In August 2017, 12 wooden raised beds (1.2 m X 2.4 m x 23 cm) were used for transplanting the seedlings of broccoli. The raised bed soil was fertilized using granular fertilizer 15-8-11 (TerraLink) to provide sufficient nutrition for the plants based on pH and EC measurements. Hoop houses were constructed on the raised beds and a drip irrigation line installed that was controlled by an Argus System. To have 24 plots, each bed was divided to two plots using a plastic wall (Fig. 2). Two trays of 3-week old seedlings of broccoli, *Brassica oleracea* var. Everest (West Coast Seeds) were grown for the tr at the

For the trial at the KPU Tsawwassen Farm School; and six seedlings were transplanted to each plot (half a bed). The beds were covered with Reemay® cloth to prevent negative effects of rain and to avoid the loss of cabbage looper larvae to natural enemies (Fig. 3).

Fig. 3. Beds covered with Reemay® cloth



The 24 plots were randomly assigned and labeled for 6 treatments (four *B. bassiana* isolates, BotaniGard and negative control) and 4 replicates when it was believed that sufficient spores of each isolate would be available for inoculation (Fig. 4); however the spores for OKA-372 and ISH-190 were not adequate and so only 4 treatments were applied.

Three weeks following transplanting, each plant was infested with ten second-instar looper larvae by placing the ten into a solo cup and hanging the cup from each plant stem (Fig. 5 a, b). The cups were assessed the following day to ensure that all larvae had left the cup. If dead larvae were observed in the cups, they were replaced with live larvae.

Fig. 4. 24 plots assigned for 6 treatments and 4 replicates.

Fig. 5. Placing 10 larvae on each plant

Application of Treatments: Hand pressure sprayers (Hudson 60136 Rose N Garden 1/2 Gallon Sprayer Poly) were calibrated and used to apply treatments (one sprayer per treatment) (Fig. 6). Each plot was sprayed with 130 ml of treatment (4×10^8 spores/ml). First, the upper sides (blue) were sprayed for 12 seconds. The same was done to the bottom sides (red) and the top of each row was sprayed for 3 seconds per row for a total of 30 seconds per plot. (Fig. 7).

Fig. 6. Hand pressure sprayer, Hudson

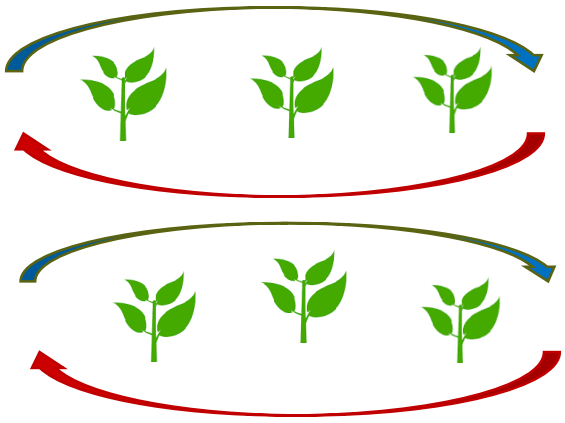


Fig. 7. Spraying pattern of each plot

Two days later, the broccoli plants were harvested and transferred to the lab. The larvae were placed individually into a 2 Oz Solo cup and provided with treated broccoli leaves from their respective plots. The cups were incubated at 25 ± 1°C, and 16L: 8D.

Lab Control Trial: On the same day as the field application, 1 µl of each treatment (4×10^8 spores/ml) was applied to individual larvae in the lab which were then individually placed in 2 Oz Solo cups and fed treated broccoli leaves from the field (Fig. 8). Four replicates of 4 larvae were treated for each treatment. The cups were maintained at 25 ± 1°C, and 16L: 8D.

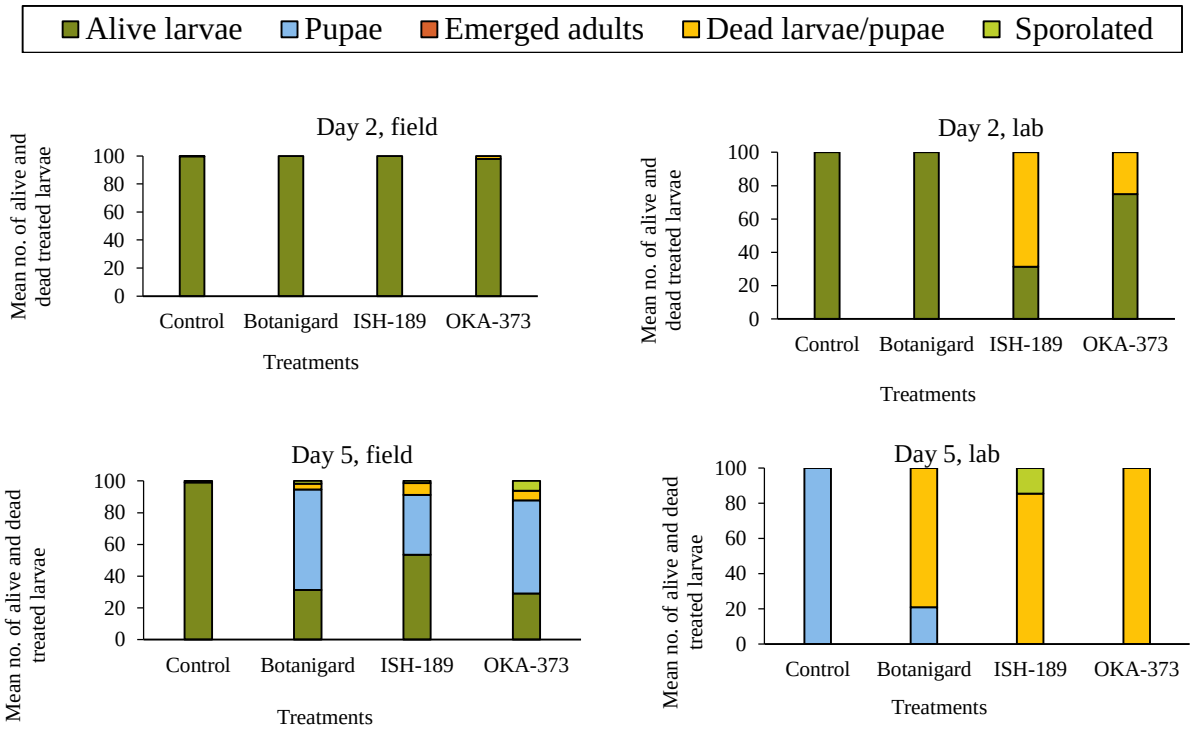
For both the field trial and lab trial, larvae were assessed on day 2, 5, 8, 14 and 19 and live larvae were fed with fresh, treated leaves (from their treatment plants) as needed. Assessment included the number of dead larvae, live larvae and larvae exhibiting sporulation.

Fig. 8. Treated cabbage looper larva kept in cup in the lab

Results

Figure 9 shows the mean number of live larvae, live pupae, emerged adults, dead larvae/pupae and sporulated larvae/pupae that were exposed to tap water as a negative control, BotaniGard as the positive control and *Beauveria* isolates, ISH-189 and OKA-373, at a concentration of 4×10^8 spores/ml in the field and lab on the same day.

Larvae killed by the isolates were observed earlier in the lab compared to the field. Comparison of the larvae treated in the lab and in the field on the days 2, 5, 8, 14 and 19 post exposure showed the percentages of mortality and sporulation of the dead larvae/pupae were higher and occurred earlier in the lab as well (Fig. 9). Accordingly, the number of adults emerged was higher in the field compared to the lab.



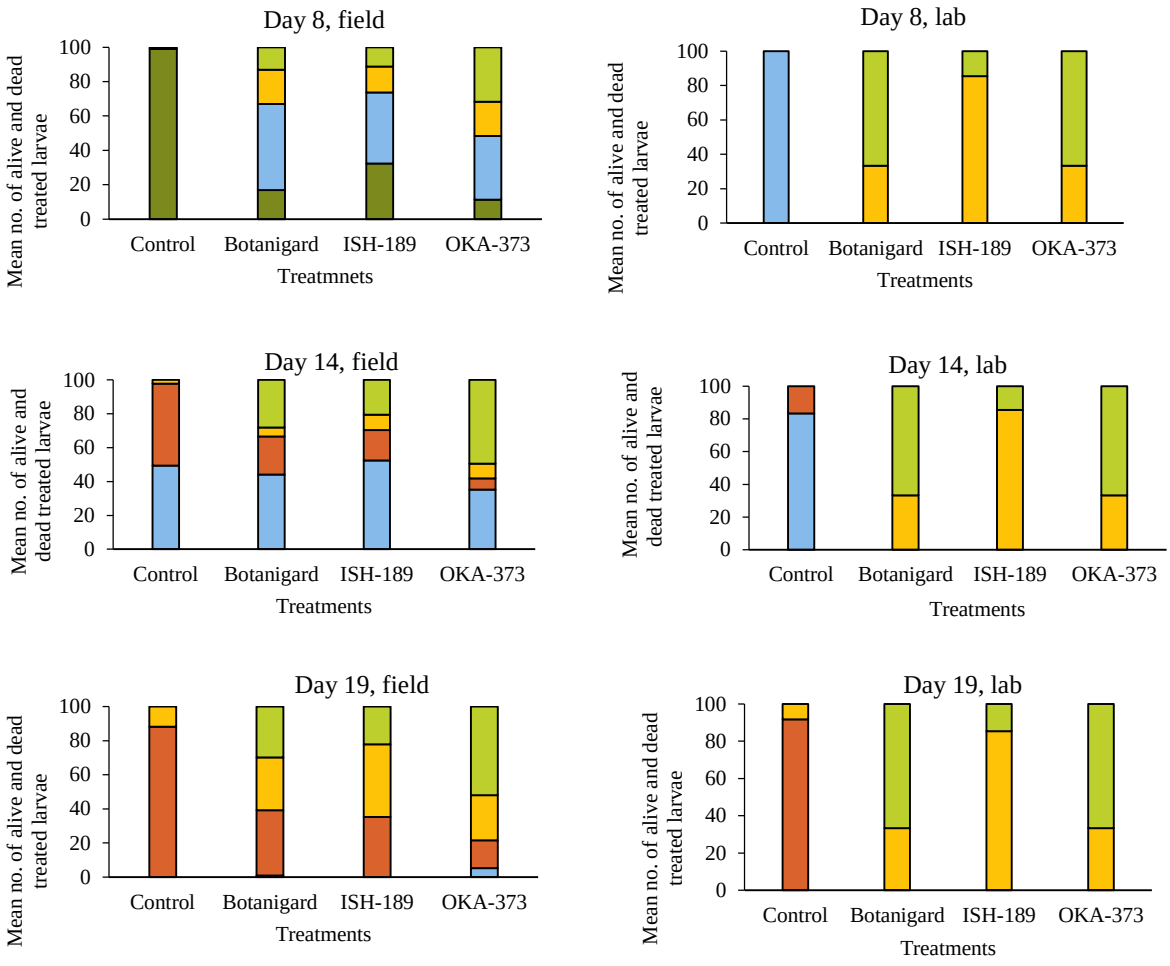


Fig. 9. Comparison of *B. bassiana* isolates virulence against second instar larvae of cabbage looper in the field and laboratory

Table 2 shows the percent mortality of cabbage looper larvae exposed to *B. bassiana* isolates in the field and in the laboratory.

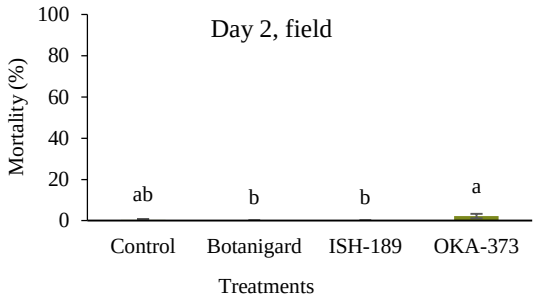
On the 2nd day post exposure, low percent mortality was observed in the field; however, in the lab, mortality rates were 68.75% and 25% for ISH-189 and OKA-373, respectively. On day 5 in the field, mortality was 5.4%, 8.9% and 12.1% for BotaniGard, ISH-189 and OKA-373, respectively. Meanwhile, in the lab on day 5, mortality caused by BotaniGard was 79.2% and for ISH-189 and OKA-373 mortality was 100%. On the 8th day, OKA-373 showed the highest rate of mortality (51.7%) in comparison to ISH-189 (26.3%) and BotaniGard (33.1%) in the field. However, these three isolates had all caused 100% mortality in the lab. Field mortality of larvae and pupae between day 14 and 19 post exposure increased from 33.4 % to 60.9% for BotaniGard, 29.6% to 64.7% for ISH-189 and 58.2% to 78.5% for OKA-373. Only 11.9% mortality was observed for the larvae exposed to just water (negative control) on day 19. OKA-373 caused significantly higher mortality

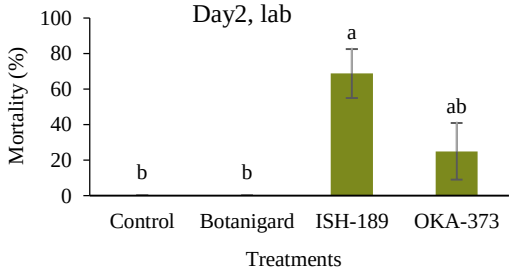
earlier than ISH-189 and BotaniGard which was apparent for the first two weeks after exposure. However, no significant difference was observed between OKA-373 and ISH-189 by day the 19th day. No mortality was observed in the negative control in the lab trial (Fig. 10). When comparing the mortality rates of the larvae in the lab from this study (2017) to our previous assay (2016), mortality rates in 2017 were higher than 2016; 5 days after exposure, OKA-373 and ISH-189 showed 100% mortality, whereas last year, these number were 61% and 93%, respectively.

Similarly, 79% and 55% mortality was observed for BotaniGard in 2017 and 2016, respectively. This difference for BotaniGard may be the result of a lower concentration of BotaniGard (1×10^8 spores/g) used in 2016 as opposed to 4×10^8 spores/g in 2017. This was not intended since the label claimed 4.4×10^{10} spores/g. however, in 2016, the actual spore concentration was checked in our lab. In 2017, we completed our own spore count using a hemocytometer, as well as determined viability of spores. The number of spores per gram counted was actually 1.1×10^{10}. Therefore, for the 2017 trials we adjusted the concentration of the product up to reflect the label claim.

Table 2. Mortality (%) (mean ± SE) of cabbage looper larvae exposed to *B. bassiana* isolates at 4×10^8 spores ml^{-1} concentration via direct contact toxicity

Treatments	Trial site	Days after exposure				
		2	5	8	14	19
OKA-373	Field	2.27 ± 1.14	12.13 ± 2.1	51.69 ± 5.39	58.16 ± 5.47	78.53 ± 3.3
	Lab	25 ± 15.96	100	100		
ISH-189	Field	0	8.9 ± 1.7	26.31 ± 2.63	29.65 ± 2.62	64.74 ± 3.63
	Lab	68.75 ± 13.77	100	100		
BotaniGard®	Field	0	5.4 ± 2.0	33.06 ± 4.03	33.4 ± 3.7	60.87 ± 4.32
	Lab	0	79.17 ± 12.5	100		
0.1% Tween20	Field	0.42 ± 0.42	0.93 ± 0.64	0.93 ± 0.64	2.3 ± 1.11	11.86 ± 3.1
	Lab	0	0	0		
ANOVA statistics	Field	F_3=3.184 P=0.028	F_3=7.739 P=0.000	F_3=33.464 P=0.000	F_3=40.649 P=0.000	F_3=48.487 P=0.000
	Lab	F_3=9.469 P=0.002	F_3=57.889 P=0.0			





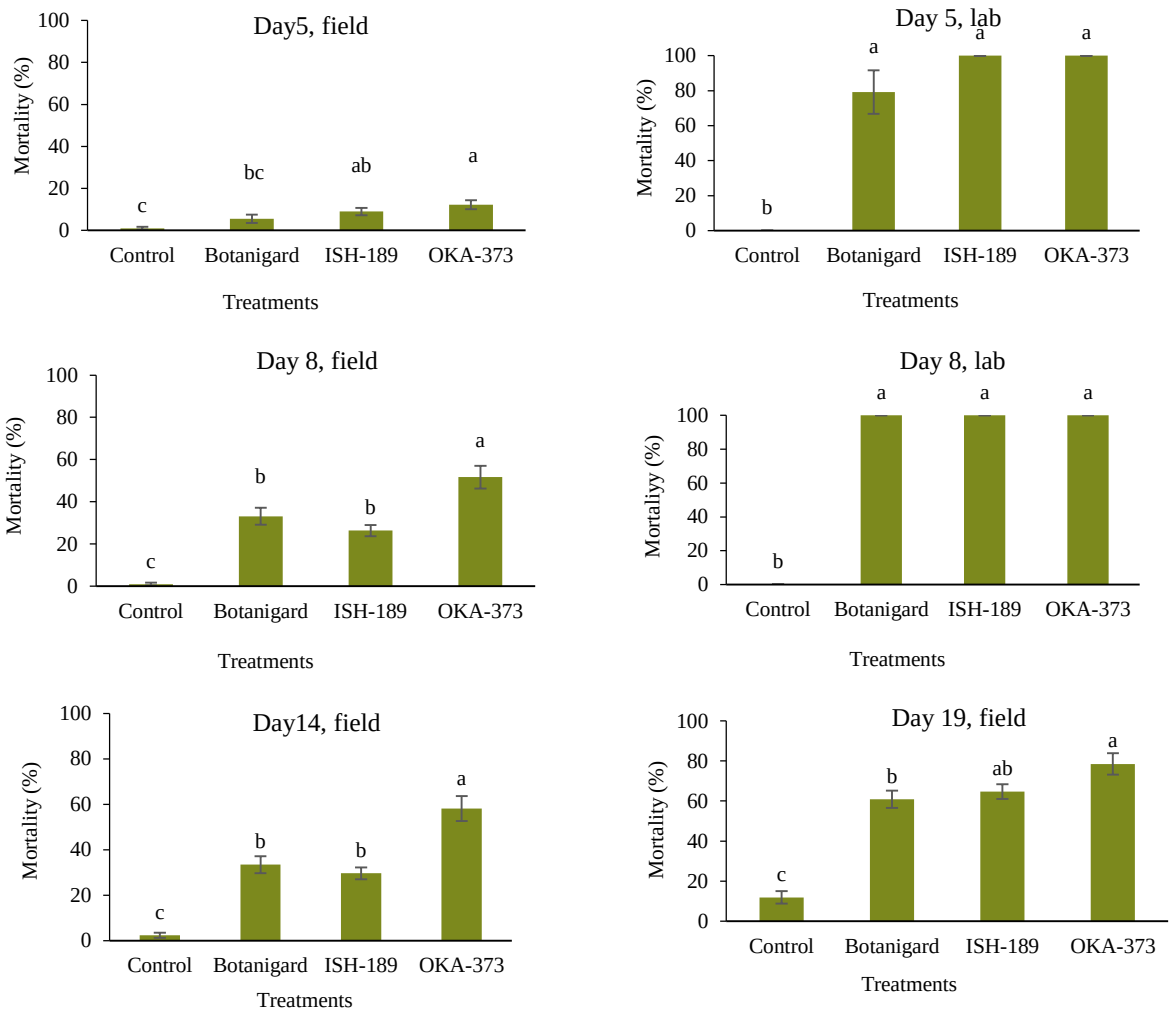


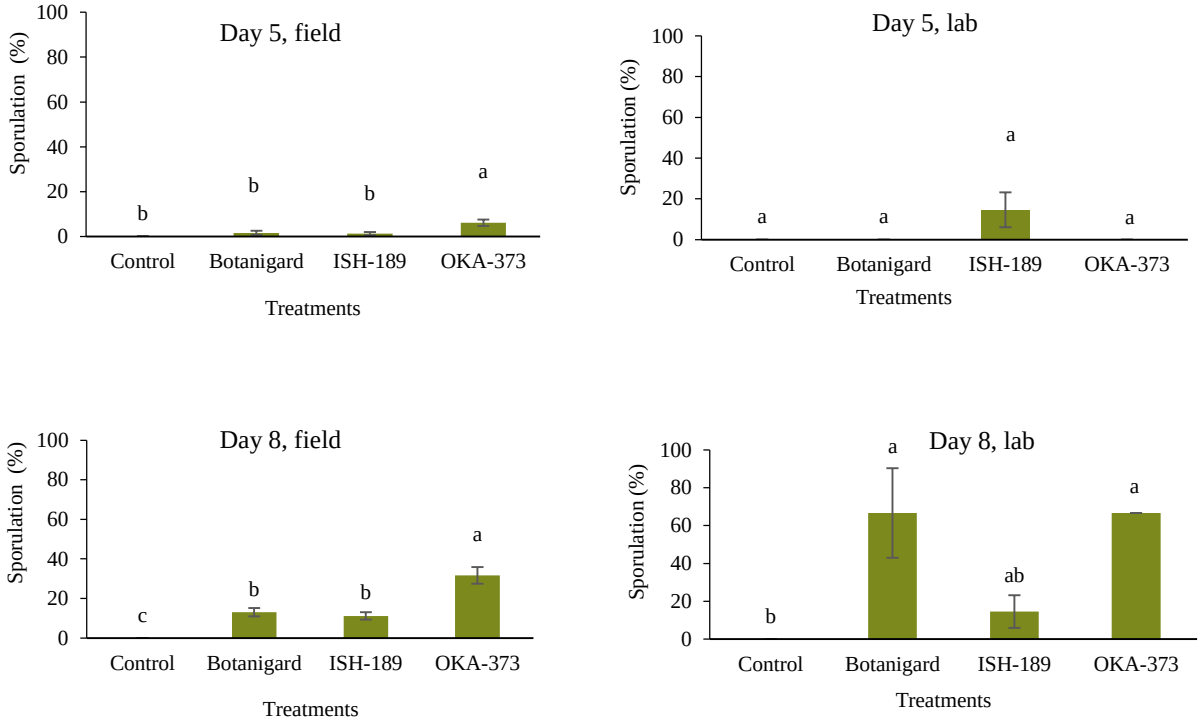
Fig. 10. Comparison of mortality (%) of cabbage looper larvae exposed to *B. bassiana* isolates at 4 $\times 10^8$ spores ml^{-1} concentration in the field and lab; Values with the same letter are not significantly different in Tukey's HSD at P=0.05

Sporulation of cabbage looper larvae caused by *B. bassiana* isolates are displayed in Table 3 and Figure 11. On the 5th day after exposure, the highest rate of sporulation on the dead larvae or pupae was caused by OKA-373 (6.18 %) in the field and ISH-189 (14.58 %) in the lab, however, there was no significant difference between isolates in the lab. Eight days post exposure, OKA-373 showed the highest rate of sporulation (31.71 %) in the field. In the lab, all isolates showed sporulation and no significant difference was observed between them. On day 8, 100% sporulation was observed in the lab. Two weeks post exposure, OKA-373 had a significantly higher rate of sporulation (49.45 %) in the field than the other isolates; the rate of sporulation increased to 52 % by day 19. No sporulation was observed in either of the negative controls in the lab or field.

A comparison of lethal time values (LT_{50}), the lethal time to kill 50% of larvae, showed that mortality of isolates in the lab occurred faster (smaller number for LT_{50}) compared to the field (Table 4). This was to be expected and despite the lower mortality rate in the field, the two isolates performed reasonably well in both environments.

Table 3. Sporulation (%) (mean ± SE) of cabbage looper larvae exposed to *B. bassiana* isolates at 4×10^8 spores ml^{-1} concentration via direct contact toxicity

Treatments	Trial place	Days after exposure			
		5	8	14	19
OKA-373	Field	6.18 ± 1.42	31.71 ± 4.23	49.45 ± 4.71	52.0 ± 4.59
	Lab	0	66.67 ± 0	66.67 ± 0	
ISH-189	Field	1.35 ± 0.75	11.18 ± 1.79	20.61 ± 3.21	22.1 ± 3.18
	Lab	14.58 ± 8.59	14.58 ± 8.59	14.58 ± 8.59	
BotaniGard®	Field	1.67 ± 0.94	13.05 ± 2.15	28.06 ± 3.77	29.87 ± 3.93
	Lab	0	66.67 ± 23.57	66.67 ± 23.57	-
Control (0.1% Tween 20)	Field	0	0	0	0
	Lab	0	0		
ANOVA statistics	Field	F_3=8.313 P<0.0001	F_3=26.860 P<0.0001	F_3=25.71 P<0.0001	F_3=439.579 P<0.0001
	Lab	F_3=2.882 P =0.08	F_3=7.694 P =0.004		



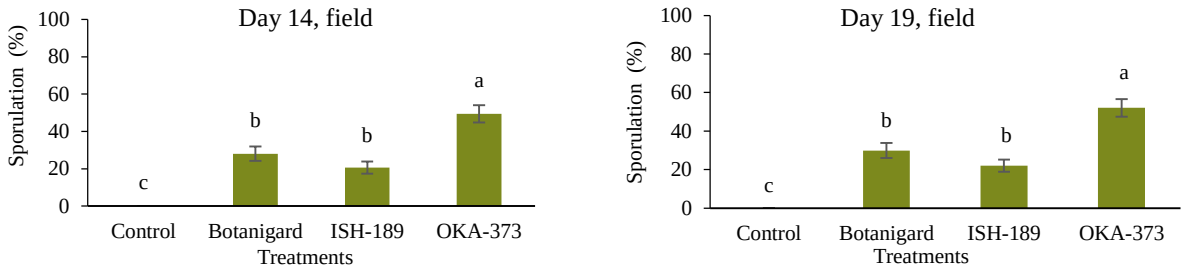


Fig. 11. Comparison of sporulation (%) of cabbage looper larvae exposed to *B. bassiana* isolates at 4×10^8 spores ml^{-1} concentration in the field and lab; Values with the same letter are not significantly different in Tukey's HSD at *P*=0.05

Table 4. Lethal time (LT_{50}) for *B. bassiana* isolates on the larvae of cabbage looper via direct contact toxicity

Isolates	LT_{50} (day)		
	2017		2016
	Field	Lab	Lab
OKA-373	10.56	2.8	3.45
ISH-189	16.76	1.28	2.55
BotaniGard®	17.56	3.7	4.52

Conclusion and Discussion

In the lab, mortality and sporulation occurred faster and at higher rates compared to the field. This is likely due to higher humidity in the individual solo cups in which the larvae were kept in the lab. The average temperature in the lab was constant at 25 ºC but in the field and under the hoop houses it was variable from 6.9 ºC to 47.8 ºC.

In the lab trial of the current study, LT_{50}s for OKA-373, ISH-189 and BotaniGard were 2.8, 1.28 and 3.7 days, respectively; although these amounts concluded in 2016 were 3.45, 2.55 and 4.52 days. The speed of kill (LT_{50}) obtained in 2017 were more favorable than those obtained in 2016 in the lab, but in the same general range. In order to calculate the exact values of LT_{50}, the trial should be repeated a few more times. Due to insufficient product availability at the time, this trial was not repeated in 2017.

Some isolates may show better efficacy against pests or ability to produce spores when re-isolated from the original host insects.

Overall, OKA-373 showed the quickest time to kill (i.e. lowest LT_{50}) and the highest rate of mortality and sporulation of cabbage looper larvae in comparison with ISH-189 and BotaniGard in the field. However, ISH-189 caused mortality of the larvae, as well as BotaniGard.
Further trials are needed to study optimum, maximum and minimum temperatures and relative humilities for the growth and sporulation of each isolate.

Objective 2. Determine efficacy of *B. bassiana* isolates against green peach aphid on cabbage in the greenhouse

Materials and Methods

Treatments: OKA-373, OKA-372, ISH-189, ISH-190, BotaniGard® (positive control) and 0.1% Tween-20 solution (negative control) were applied as treatments. The BotaniGard® solution was made according to spore concentration (spores/gram) which was calculated to verify the concentration stated on the label. The number of spores per gram for BotaniGard® was calculated as 1.1×10^{10} per gram whereas the label stated there to be 4.4×10^{10} spores per gram.

Green Peach Aphid: *Myzus persicae* (Sulzer) was obtained from (Applied Bio-Nomics Ltd) in the winter of 2016 and lab reared lab for around 40 generations (Fig. 12).

Fig. 12. Green peach aphid in different stages

Host Plants and Preparation of the Bioassay: In August 2017, two trays of 3-week old seedlings of cabbage Caraflex (West Coast Seeds Ltd. Delta, BC) were grown for the trial at the KPU Tsawwassen Farm School and transplanted into 4 inch pots and maintained in the room described above. Two months later, the cabbage plants were transplanted into 6 inch pots, infested with aphids, placed in a 250µm mesh cages (75cm x 39cm) and kept in greenhouse at 20 ºC, L: D 12; 12 and RH 55% (Fig. 13, a). The pots were watered using a drip irrigation system controlled by a programmable Argus System.

Twenty four cages were randomly labeled as one of 6 treatments and one of 4 replicates. When the aphid population was sufficiently high (average 50-100 aphids per leaf), three leaves of each plant were tagged with different color ribbon (red, blue and yellow) (Fig. 13, b) for post spray monitoring.

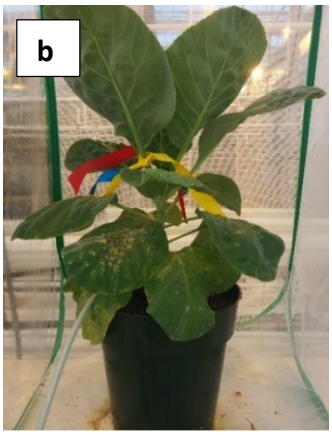


Fig. 13. a, Aphid infested cabbage pots placed in the cages and kept in the greenhouse; **b,** cabbage tagged with ribbon

Application

Six plastic spray bottles were calibrated, one for each treatment (Fig. 14). On November 1st, 2017 twenty ml of each treatment at a concentration of 1×10^8 spores/ml was sprayed onto each cabbage plant as the first application. Prior to this application, counts of aphids (wingless; adult and nymph) on each tagged leaf were completed.

Fig. 14. Plastic spray bottles used to apply treatments to cabbage plants with GPA

Fig. 15. Treated leaf in 12 Oz cup

A week later, on November 8th, 2017 a second application was sprayed with suspensions at a concentration of 4×10^8 spores/ml for ISH-189 and OKA-373, and BotaniGard, and a concentration of 1×10^8 spores/ml for ISH-190 and OKA-372. Six days following the second application, the treated leaves were harvested and placed in separate 12 Oz plastic cups. The cups were covered with a voile mesh cloth and sealed using a rubber band. These cups were then transferred to the lab and the aphid population was counted. Petioles of leaves were then wrapped in cotton and held in 2.5 ml tube and filled with tap water. The

leaves were placed in a growth chamber at 25 ± 1°C , RH 85% and L:D 16:8. Live, dead and sporulated wingless adults and nymphs were recorded every second day until sporulation was observed.

Lab Control Bioassay, Application 1: In the lab during the first application day (November 1st, 2017) replicate leaves infested with aphids were treated with each isolate and placed in plates with mesh windows on the lid for ventilation. The leaf petioles were wrapped with wet cotton and the

plates were sealed with parafilm. The number of aphids (wingless; adult and nymph) on each leaf was counted and recorded prior to application. Each treatment was sprayed on the leaves at a concentration of 1×10^8 spores/ml, mirroring the first application in the greenhouse trial (Fig. 16, a).

Lab Control Bioassay, Application 2: The trial was performed as described above, excluding the mesh windows on the plate lid. The isolate suspensions had concentrations of 4×10^8 spores/ml for ISH-189 and OKA-373 and 1×10^8 spores/ml for ISH-190 and OKA-372, mirroring the second application in the greenhouse trial (Fig. 16, b, c).

Residual Contact Test: Suspensions of the isolates were prepared using 14-day old culture plates. Four cabbage leaf discs were dipped in each suspension (4×10^8 spores/ml) for 6 seconds and placed on a paper towel to dry. Then, each leaf disc was transferred individually to a 9 centimeter plate. Five wingless adult aphids reared on cabbage in the lab were transferred to each leaf disc, then, the plates were sealed with parafilm.

All plates were maintained in a rearing room at 24 ± 1°C and L: D 16:8. The numbers of live, dead and sporulated aphids (wingless) for each tagged leaf were recorded approximately every second day until sporulation was observed.

Fig. 16. Treated aphid for the first lab control trial (a), and second trial (b,

Results

Field trial: Figure 17 shows the mean number of live aphids in the greenhouse over first and second applications and two weeks following. A week after the first application, percentage of live aphids treated with ISH-190 was significantly less than the others and mortality was the highest (Table. 5, Fig. 18). However, there were no significant difference between the percentages of live aphids treated with the isolates on the days 13 and 21 post application (Table 5). On day 13, there was no significant difference between isolates on percent mortality of aphids. On day 20, ISH-190 caused the highest rate of mortality, however, the rate was not significantly higher than the Okanagan isolates (OKA-373 and OKA-372). It is important to note that the concentration of ISH-190 and OKA-372 was ¼ the concentration of ISH-189 and OKA-373 in the second application.

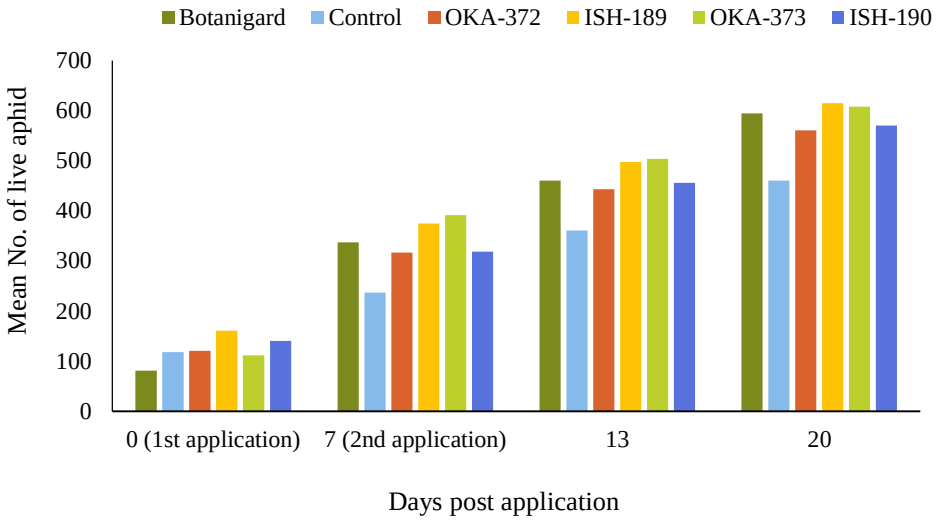


Fig. 17. Mean number of live aphids exposed to the all isolates at 1×10^8 spores/ml concentration in the first application day (day 0) and at 4×10^8 spores/ml concentration for OKA-373, ISH-189, and BotaniGard and 1×10^8 spores/ml concentration for OKA-372 and ISH-190 at second application (day 7) in ISH greenhouse

Table 5. Percentage of live aphids one week after the first application (day 7) and two weeks after the 2nd application

Treatments	Days post application					
	7 (2nd application)		13		20	
BotaniGard	99.92	a	99.64	ns	97.94	Ns
Control	99.95	a	99.83	ns	99.30	Ns
OKA-372	99.73	a	99.72	ns	98.77	Ns
ISH-189	99.78	a	99.68	ns	98.99	Ns
OKA-373	99.95	a	95	ns	98.42	Ns
ISH-190	97.79	b	97.97	ns	96.26	Ns
ANOVA statistics	F_5= 5.149 P<0.0001		F_5=0.922 P=0.473		F_5=1.673 P=0.153	

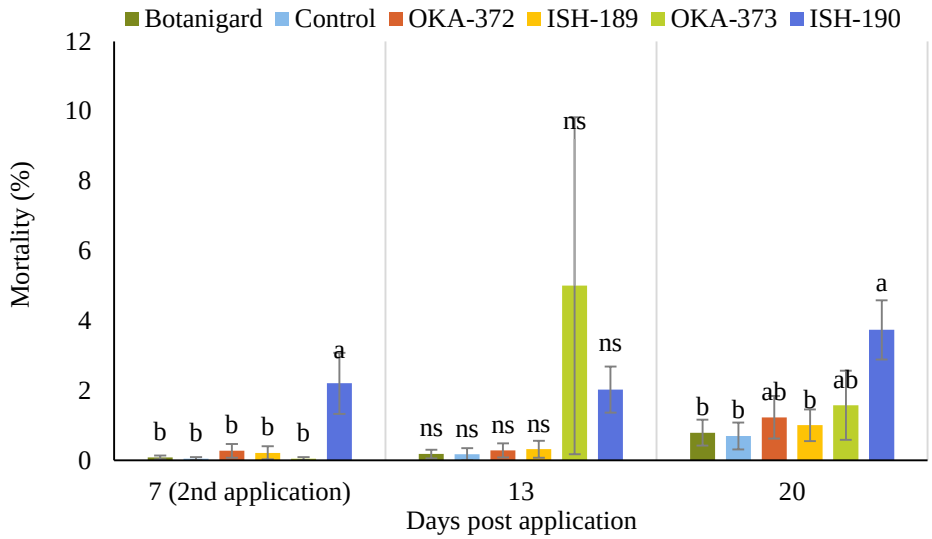


Fig. 18. Mortality (%) of aphids exposed to the all isolates at 1×10^8 spores/ml concentration in the first application, and at 4×10^8 spores/ml concentration for OKA-373, ISH-189 and BotaniGard, and 1×10^8 spores/ml concentration for OKA-372 and ISH-190 in the second application in ISH greenhouse Day7, $F_{5=}5.149$, $P<0.0001$; Day 13, $F_{5=}0.941$, $P=0.46$; Day 20, $F_{5=}3.033$, $P=0.016$; Values with the same letter are not significantly different in Tukey's HSD at $P=0.05$; **n. s.,** no significant difference from group

I Lab control bioassay (1st application): Mortality of aphids in the lab (treated at the same time as the first application in the greenhouse) was higher in ISH-190 (14.99%) on day 8. There was no significant difference in sporulation a week after application and the rate was low ($F_5=1.285$, $P=0.313$).

II Lab control bioassay (2nd application): Mortality and sporulation rates of aphids were high in aphids treated with the isolates and there was no significant difference between isolates and BotaniGard (Table 6).

Table. 6. Mortality (%) and sporulation (%) of the aphids exposed to *B. bassiana* isolates in the lab in the sealed Petri dishes (II lab trial) a week post application

Treatments	Concentration (spores/ml)	Mortality (%)	Sporulation (%)
BotaniGard	4×10^8	68.67	79.42
ISH-189	4×10^8	97.78	89.22
OKA-373	4×10^8	98.57	89.46
ISH-190	1×10^8	100	85.59
OKA-372	1×10^8	100	96.82
Control	-	0	0

Residual Contact Test: 5 days after application, high rates of mortality were observed in all treatments even negative control (Tween), therefore the mortality percentages were not statistically analyzable.

Five days after application, no significant difference was observed between the sporulation rates of the treatments (F_5=1.502, P=0.238); eight days post application, the highest rate of sporulation was for ISH-189, however there was no significant difference between ISH-189 and the other isolates, including BotaniGard (F_5=3.032, P=0.037; Fig. 19).

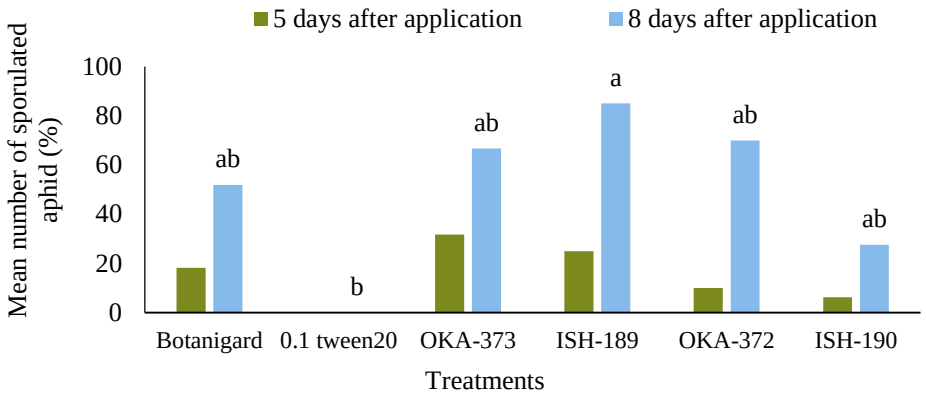


Fig. 19. Sporulation (%) of green peach aphid exposed to *B. bassiana* isolates at 4×10^8 spores/ml concentration via residual contact toxicity; Values with the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

Conclusion and Discussion

Overall, % mortality of aphids was very low in the greenhouse trial and this may have been due to lack of sufficient relative humidity (Average: 54.7%, Max: 70.2%, Min: 2.22%) and temperature (Average: 18.5°C, Max: 21.4°C, Min: 16.4°C) in the greenhouse during this trial in November. It could also have been because the aphid population was much higher than it would be in a commercial crop, so new aphids were being added to the population rapidly.

In the lab, the first trial mortality was not significant. This may have been due to a deficiency of humidity in the Petri dishes which had mesh lids. In the second lab trial, the rates of mortality and sporulation were high as well as in the residual test due to high relative humidity using sealed Petri dishes. This highlights the importance of humidity in infection of aphids with *B. bassiana*. The formulation used in these trials was simply water, and nothing was added to improve conditions for penetration of the fungus through the cuticle as there can be in commercial formulated products. Frequently better results have been obtained using oil formulations rather than water (N. Jenkins pers. Comm. 2017). Future work with these isolates will include different formulations to optimize infection. It is good news that even without adjuvants or formulation, they were able to cause mortality to both aphids and looper larvae in field and greenhouse trials.

Objective 3. Non-target toxicity studies for the top 4 isolates with *Lemna minor* (aquatic plant) and *Trichogramma sibericum* (parasitic wasp) (residual contact)

Lemna minor

Materials and Methods

Duckweed: This plant was purchased from a private seller as the season had passed when it could be collected locally. Uniform plantlets were separated into individual wells in a plastic 12-well plate and allowed to grow (Rumsey and Carter, 2014). The media used for growing duckweed was a UTCC formulation of Hoagland's E-Medium (Acreman, 2017; Fig. 20).

Fig. 20. Duckweed grown in media

Preliminary Test: While fungal isolate suspensions are made with 0.1% Tween 20 to allow for their suspension in water, the media for growing duckweed was made with Reverse Osmosis (RO) water. On November 7th, 2017 preliminary test were conducted to determine how susceptible duckweed was to Tween toxicity and to discern any impacts on nutrient amount in the media should fungal isolate suspensions be mixed with the media. Six treatments with varying combinations of Tween and media were prepared, and each treatment included 4 replicates (Table 7).

Table 7. Amount of 0.1% Tween 20 and culture medium in each treatment of preliminary test

Treatments	Percentage of 0.1% Tween 20	Percentage of culture medium
A	100	0
B	80	20
C	60	40
D	40	60
E	20	80
F	0	100

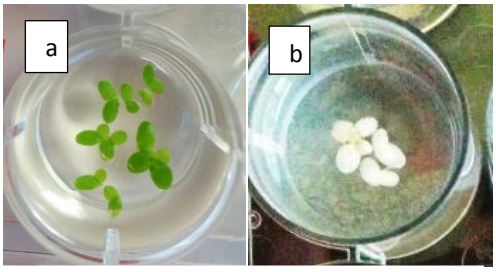


Fig. 21 Duckweed fronds in a cell of a 12–well tray before (a) and after (b) application

Three dark green fronds with 2-4 identical attached leaves were selected for each replicate and gently placed on a paper towel to remove extra moisture. Then, all were immediately weighted using an accurate milligram balance and transferred into one of 12-wells of the cell culture plates, containing 4 ml of each treatment (Fig. 21). Initial fresh weight and the number of leaves for each

replicate were recorded. One week later, counts of leaves, fresh weight (produced biomass) and dry weight were recorded.

Duckweed Trial I: On November17th, 2017 the first trial of duckweed was conducted.

Treatments: OKA-373, ISH-190, and BotaniGard® (positive control) were applied at concentrations of 4×10^8, 4×10^7, 4×10^6 and 4×10^5 spores/ml. Based on the result from the preliminary test, duckweed needs a medium with 100% nutrients to grow. Suspensions of isolates were prepared in 0.1% Tween 20; therefore, each cell was filled with 4 ml of a blend of media and suspension, 2 ml of double rate nutrients of duckweed media and 2 ml of double rate concentration of isolates or BotaniGard or Tween 20 for negative control (no spore treatment). To obtain a 2 ml suspension of 4×10^8 spores/ml in each cell with 4 ml final solution, a double rate suspension of 8×10^8 spores/ml was made per isolate using 14-day old cultured plates. Then, dilutions were prepared to yield suspensions of 8×10^7, 8×10^6 and 8×10^5 spores/ml for each isolate. 4 cells (replicates) were assigned to each specific concentration (Fig. 22). Counts of green and colorless leaves were conducted for each cell on day 5, 8 and 12 using a magnifying glass.

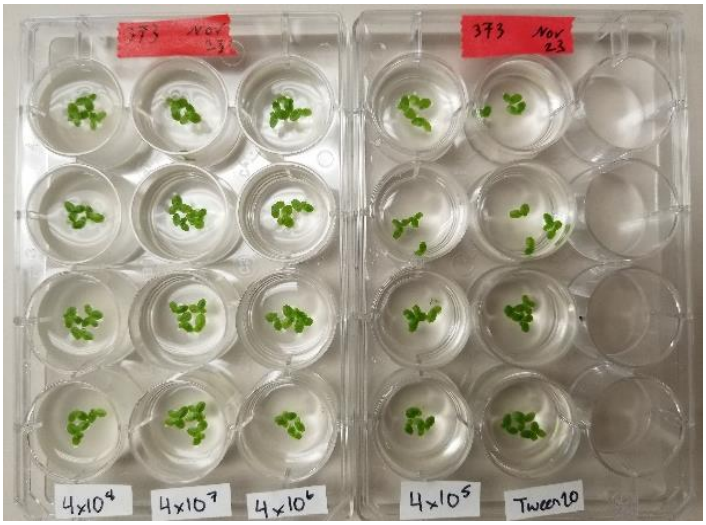


Fig. 22. 4 replicates assigned per concentration

Duckweed Trial II: On November 23rd, 2017 the second duckweed trial was carried out.

Treatments: OKA-373, OKA-372, ISH-189, ISH-190, BotaniGard® (positive control) were applied at varying concentrations of 2×10^8, 2×10^7, 2×10^6, 2×10^5 spores/ml, and 0.1% Tween 20 was used for the negative control.
To have the same condition in the BotaniGard treatments as the other isolates, solutions were made in water and also in 0.1% Tween 20. The suspensions were made using the same protocol as was used in the first trial. Four replicates (cells) were used for each concentration. The cells were kept in a rearing room at 24 ± 1°C and L: D 16:8. Numbers of healthy and chlorotic leaves were assessed on days 2 and 8.

Duckweed Trial III: On November 29th, 2017 the third duckweed trial was conducted using the same treatments and concentrations as the second trial but with an additional treatment (pure

duckweed media, a mix of nutrients in RO water, no spores & no Tween 20). Numbers of healthy and chlorotic leaves were assessed on days 6 and 8. The cells were kept in a rearing room at 24 ± 1°C and L: D 16:8 (Fig.23).

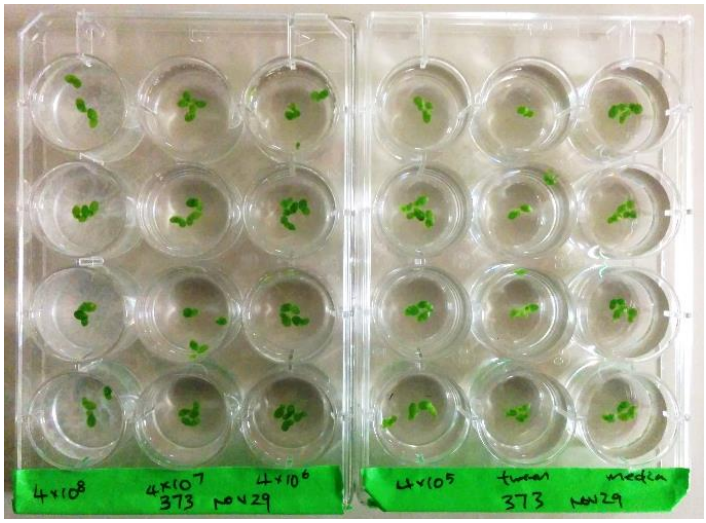


Fig. 23. Duckweed trial using a range of concentrations of each isolate and 0.1% Tween 20 (no spores) and mix of nutrients in RO water (no spores & no Tween 20)

Duckweed Trial IV: Live spores and dead (autoclaved) spores of ISH-190 at a concentration of 2×10^8 spores/ml were applied as treatments and a mix of nutrients and 0.1% Tween 20 as the no-spore control and a mix of nutrients and RO water as the no-spore & no-Tween 20 control. Four replicates (cells) were used for each concentration. The cells were kept in a rearing room at 24 ± 1°C and L: D 16:8. Numbers of healthy and chlorotic leaves were assessed on day 5.

Results

Preliminary test: Figure 26 shows mean number of new leaves, percentage of chlorotic leaves, biomass weight (g) and dry weight (%) of duckweed (*Lemna minor*) 7 days after treated using 0.1% Tween-20, a mix of Tween-20 and duckweed media or pure duckweed media. Number of new leaves and biomass produced were significantly higher in the treatments containing media (Fig. 24. A, B). Duckweed leaves showed the highest rate of chlorosis in the Tween-20 treatments without media (Fig. 24. C). There was no significant difference between dry weight amounts of treatments (Fig. 24. D). Based on this result, subsequent trials continued using 50% Tween-20 and 50% media (a mix of 2 ml 0.1% Tween-20 containing spores of isolates and 2 ml media per well cell).

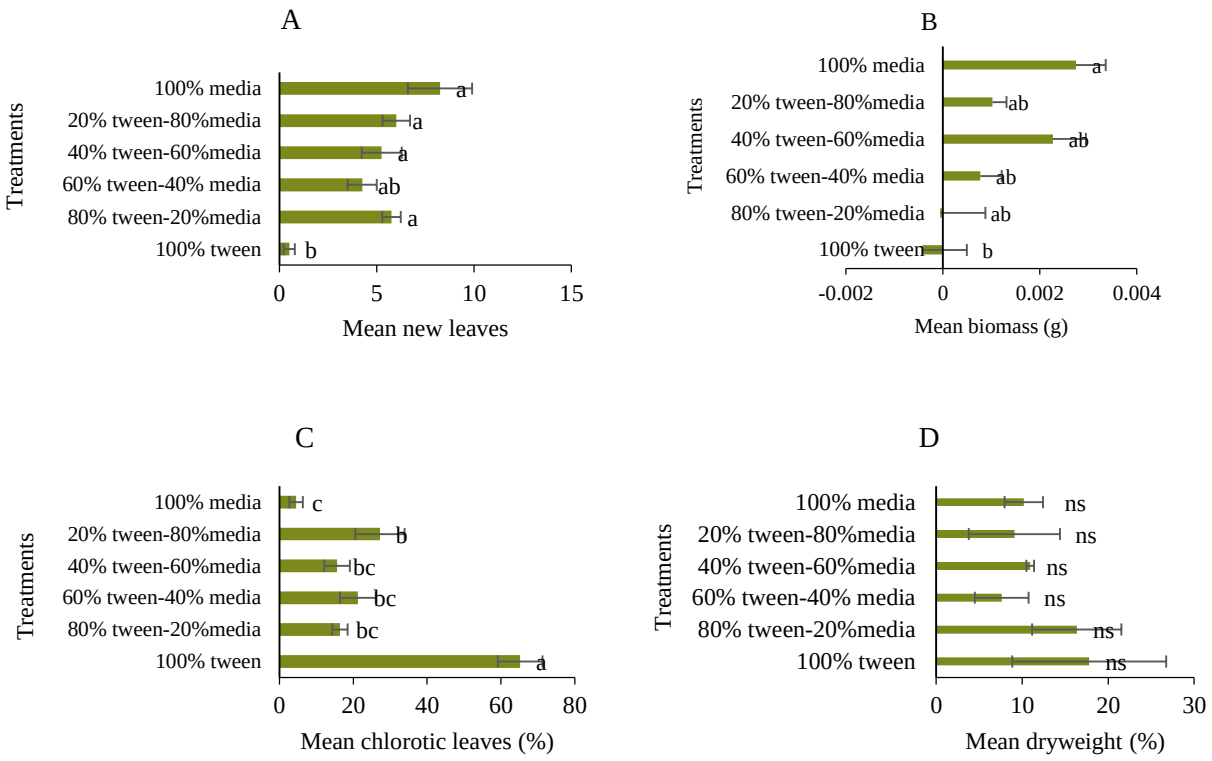


Fig. 24. Effect of 0.1% Tween 20 on growth factors of duckweed; A, New leaves, F_5=7.665, P=0.001; B, Biomass, F_5= 3.334, P=0.026; Chlorotic leaves, F_5= 021.411, P =0.5000; D, Dry weight, F_5= 0.8676, P =0.647; Values followed by the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

Duckweed trials

Figure 25 shows the duckweed leaves one week post exposure to the different concentrations of the isolate. The leaves lose chlorophyll a week after application

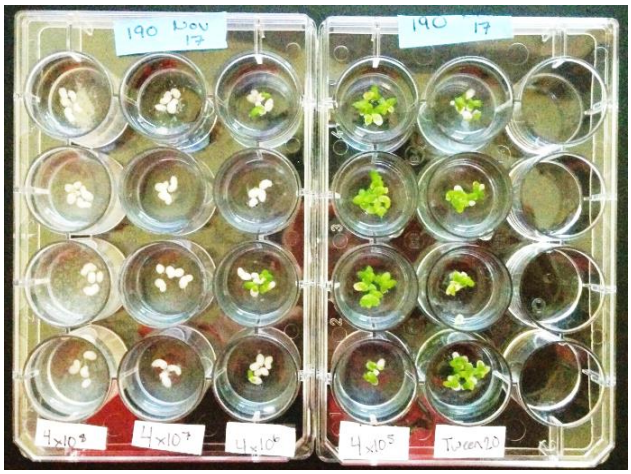
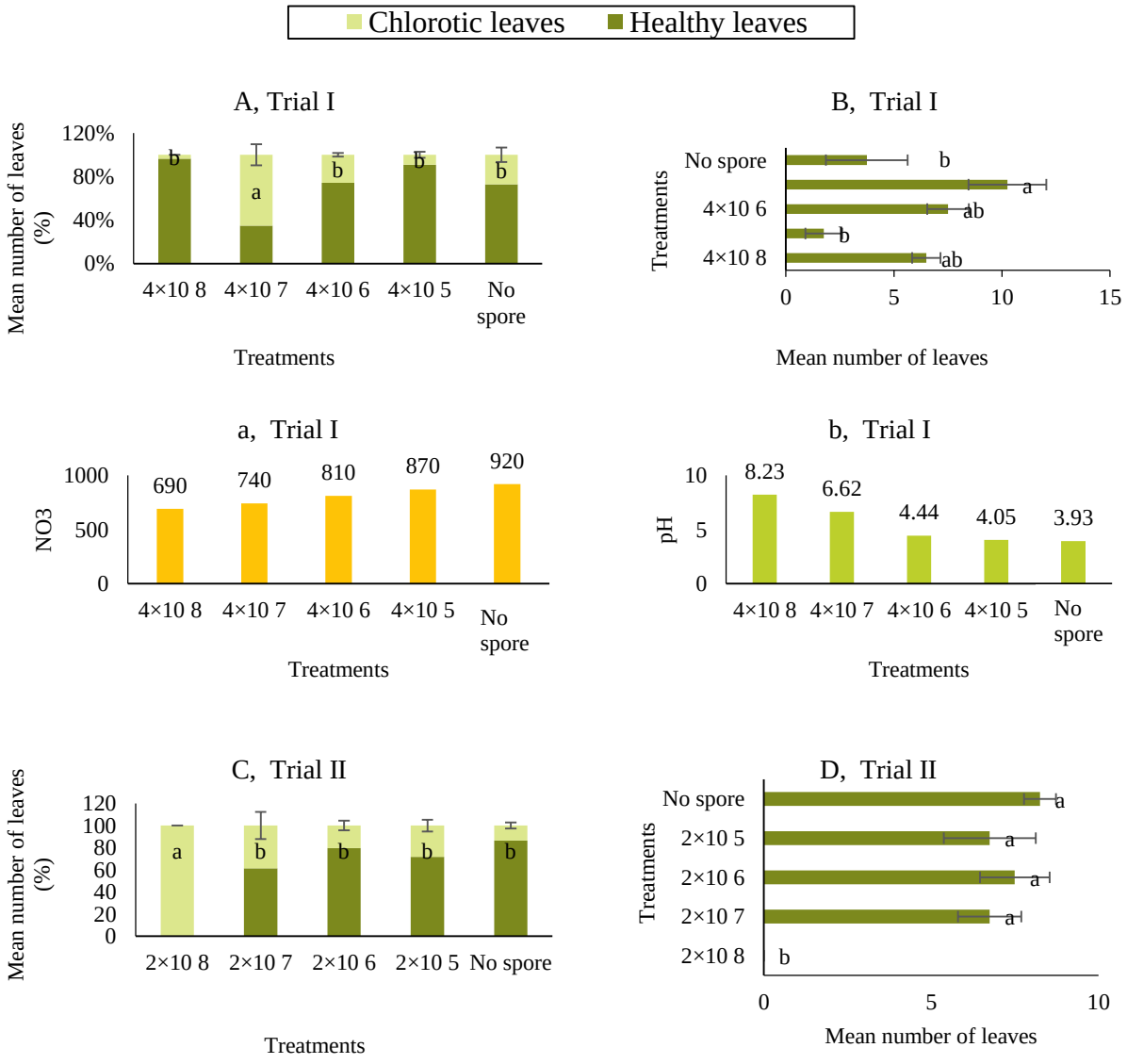


Fig. 25. Chlorosis of the leaves a week after exposure to various concentrations of *B. bassiana* isolate

OKA-373: The highest rate of chlorosis and the least amount of new leaves of duckweed were for 4×10^7 and 2×10^8 spores/ml in Trials I and II, respectively (Fig. 26. A, B, C, D).

In Trial III, the treatments, i.e. no-spores, 2×10^7 and 2×10^8 spores showed the highest rate of chlorosis; however, there was no significant difference among concentrations (Fig. 26. E). The treatments containing Tween-20 showed the lowest number of new leaves 8 days after application (Fig. 26. F); therefore it was concluded that Tween-20 suppressed growth of duckweed.

In the first trial, as the concentrations of the isolates increased, the amount of nitrate in the solution decreased and the pH increased (Fig. 26. Trial 1). In the 2nd trial, this pattern was not observed (Fig. 26. c, d). The value of pH for the treatment without spores which showed the highest number of new leaves was statistically similar to treatment 2×10^8 which caused the most chlorosis (Fig. 26. F). Therefore, pH and nitrate values did not appear to have a direct effect on the growth of duckweed.



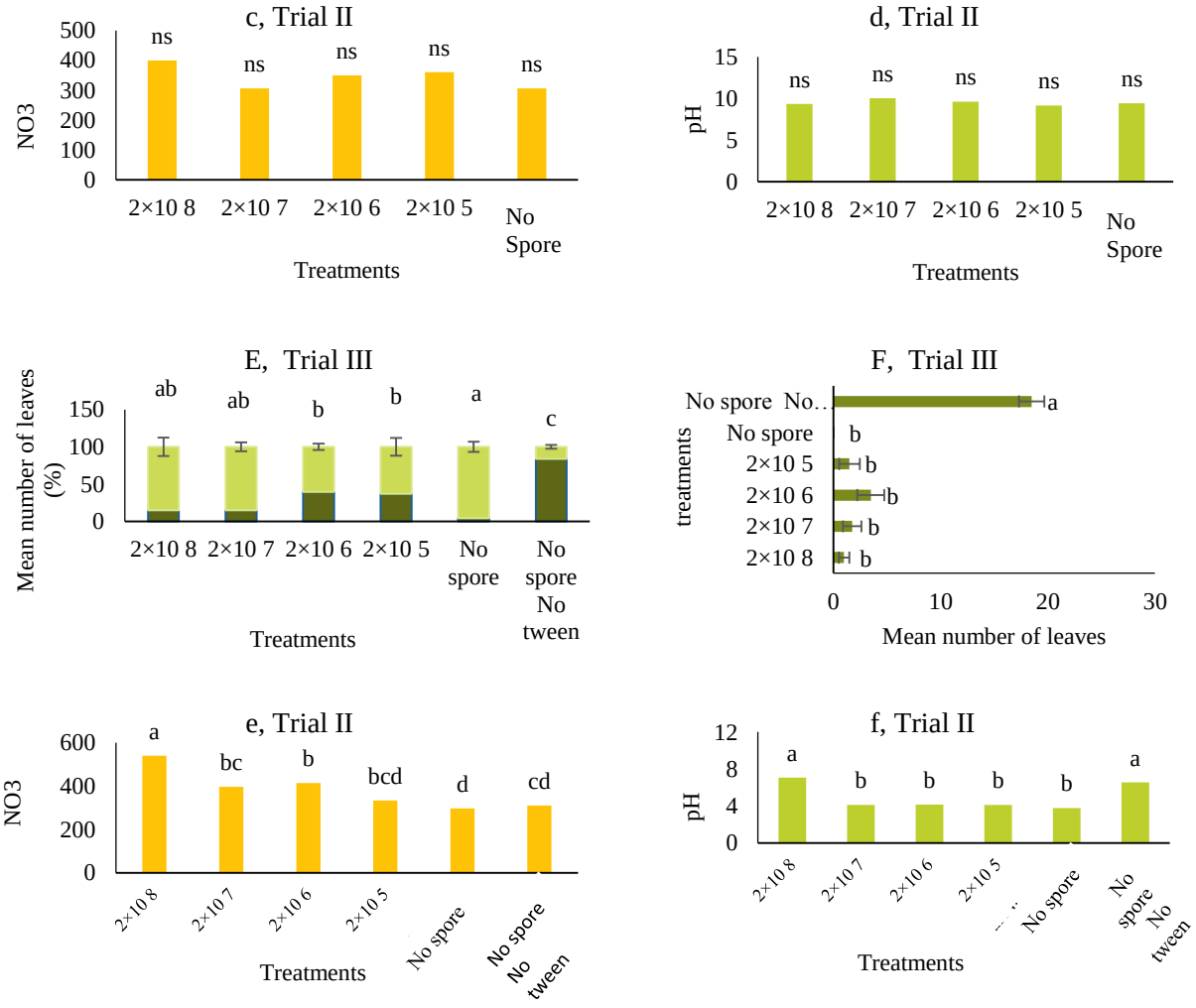


Fig. 26. Effect of isolate OKA-373 at different concentrations on percentage of healthy and chlorotic leaves and new leaves of duckweed 8 days after application; A, F_4=16.616, P<0.0001; B, F_4=6.166, P=0.004; C, F_4=29.696, P<0.0001; D, F_4=13.5, P<0.0001; c, F_4=2.144, P=0.15; d, F_4=1.493, P=0.276; E, F_5=13.512, P<0.0001; F, F_5=61.167, P<0.0001; e, F_5=21.597, P<0.0001; f, F_5=83.807, P<0.0001; Values with the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

OKA-372: In trial II, the highest rate of chlorotic leaves was observed in treatment 2×10^8; however, concentration 2×10^7 also caused a high rate of chlorosis. In these two concentrations the new leaves were fewer than the other treatments (Fig. 27. A, B). There was no significant difference among treatments containing Tween-20 (no-spores and all concentrations of the isolate) (Fig. 27. D).

Nitrate values for all concentrations were statistically similar and there was no significant difference between values of pH observed (Fig. 27. a, b). Overall, pH and amounts of nitrate did not appear to influence duckweed growth while Tween 20 was clearly responsible for suppressing the growth of leaves.

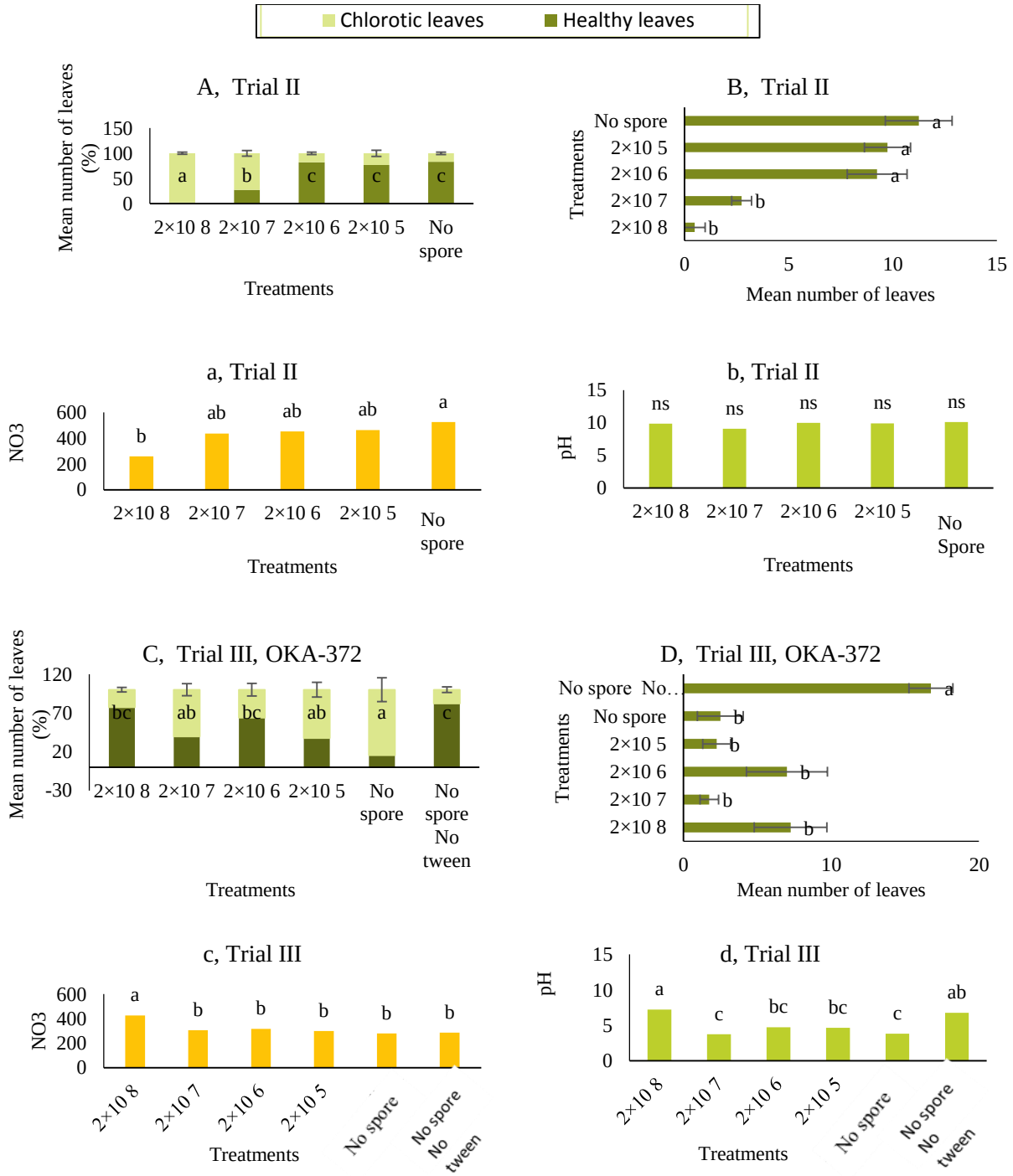
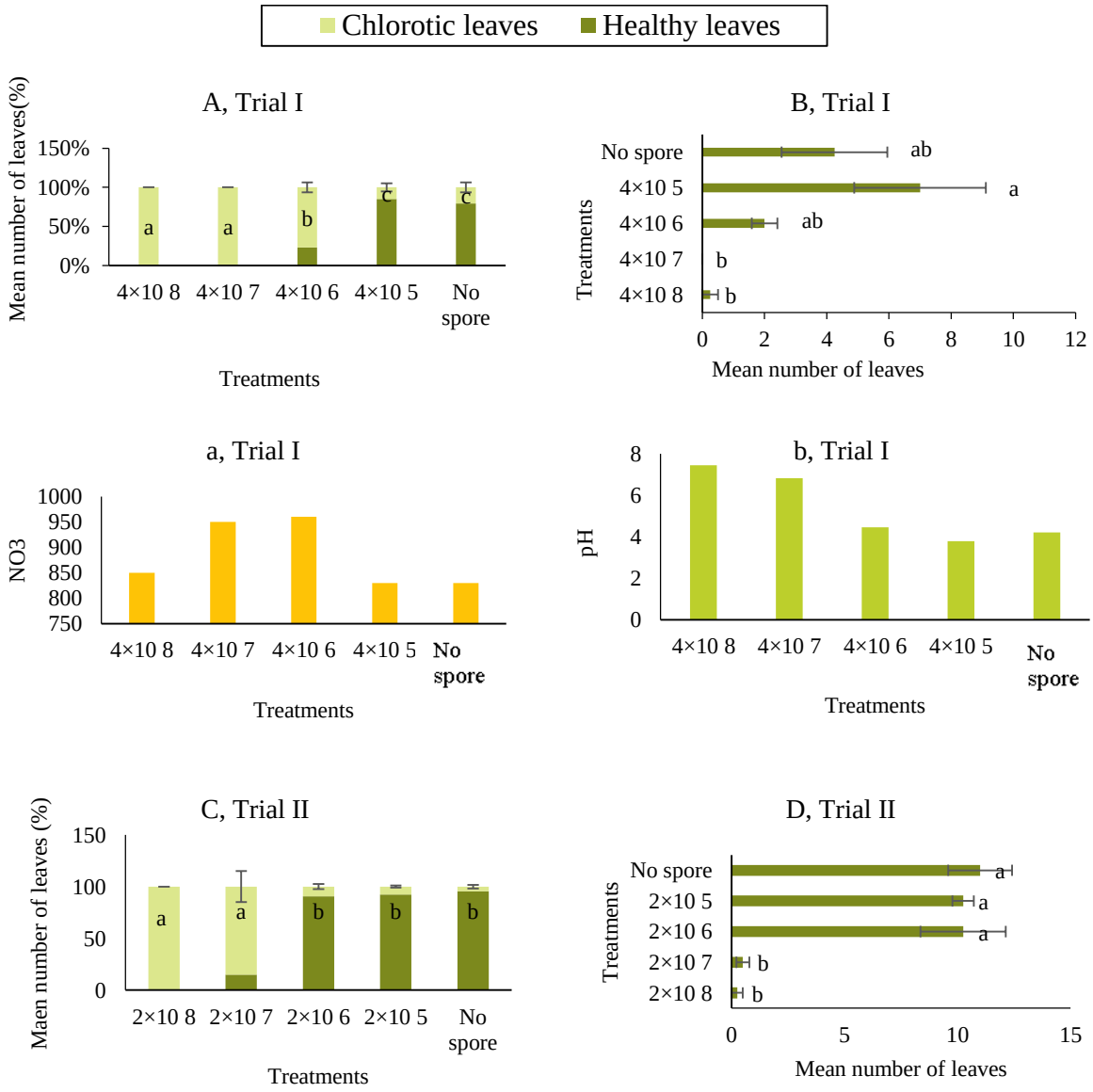


Fig. 27. Effect of isolate OKA-372 at different concentrations on percentage of healthy and chlorotic leaves and new leaves of duckweed 8 days after application; A, F_4=883.439, P<0.0001; B, F_4=17.872, P<0.0001; a, F_4=5.104, P=0.017; b, F_4=0.839, P=0.503; C, F_5=8.617, P<0.0001; D, F_5=9.977, P<0.0001; c, F_5=16.862, P<0.0001; d, F_5=10.468, P<0.0001; Values with the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

ISH-190: In the first trial, the highest rate of chlorosis was observed in 4×10^8 and 4×10^7 concentrations (Fig. 28. A). In the 2nd trial, the highest rate of chlorosis and the lowest number of new leaves were caused by treatments 2×10^8 and 2×10^7 (Fig. 28. C, D). In the 3rd trial, the highest rate of chlorosis and the lowest number of new leaves were in 4×10^7, 4×10^6, 4×10^5 and the treatment without spores (Fig. 28. E, F).

There was no significant difference in values of nitrate and pH for treatments in the 2nd trial (Fig. 28. c, d). In the 2nd trial, the values of nitrate and pH for 2×10^7 that showed the highest chlorosis rate and the fewest new leaves were similar to the treatment without spores and Tween 20 that displayed the lowest rate of chlorosis and the highest rate of new leaves (Fig. 28. e, f).

pH and nitrate did not change the ability of duckweed to grow; in contrast, Tween 20 and high concentrations of suspension reduced the growth of leaves.



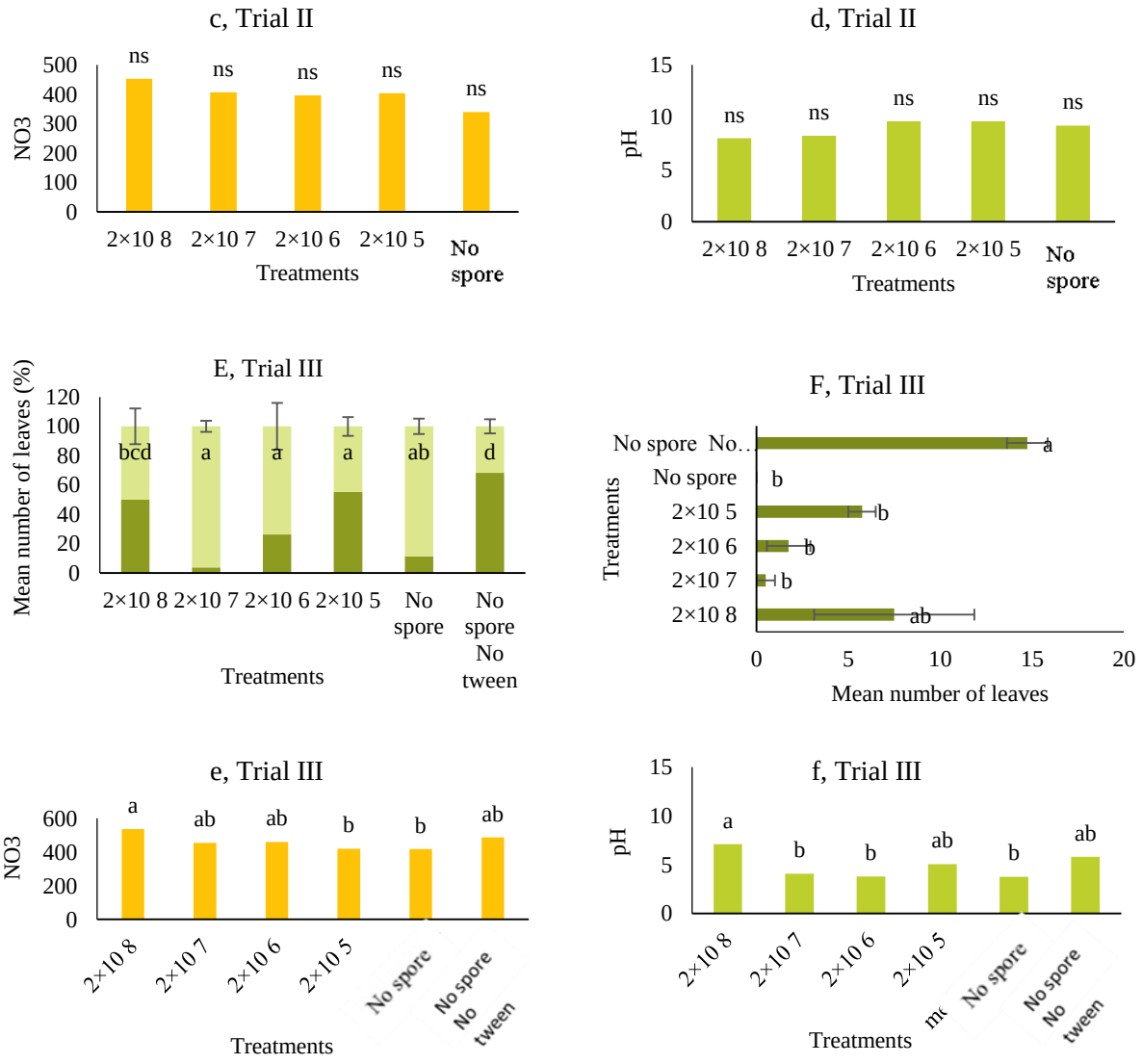


Fig. 28. Effect of ISH-190 isolates at different concentrations on percentage of healthy and chlorotic leaves and new leaves of duckweed 8 days after application; A, F_4=79.413, P<0.0001; B, F_4=5.684, P=0.005; C, F_4=47.2, P<0.0001; D, F_4=25.984, P<0.0001; c, F_4=1.103, P=407; d, F_4=2.342, P=0.125; E, F_5=7.899, P<0.0001; F, F_5=8.402, P<0.0001; e, F_5=3.547, P=0.034; f, F_5=7.295, P=0.002; Values with the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

ISH-189: In trial II, the highest concentration (2×10^8) showed the highest amount of chlorosis while the treatment without spores showed a low rate of chlorosis (Fig. 29. A). No significant difference was observed between treatments for the number of new leaves (Fig. 29. B). Similarly, there was no significant different between concentrations in the third trial (Fig. 29. C). The nitrate value for 2×10^8 was similar to the nitrate value of the no-spore treatment; while their pH values varied significantly (Fig. 29. a, b). The nitrate values of treatment without spores and Tween 20,

as well as 2×10^8, that caused the lowest amount of chlorosis and highest number of new leaves were significantly different; however, their pH values were similar (Fig. 29. c, d).

The results showed that the concentrated suspensions decrease the growth of duckweed and pH and nitrate were not responsible for this decrease.

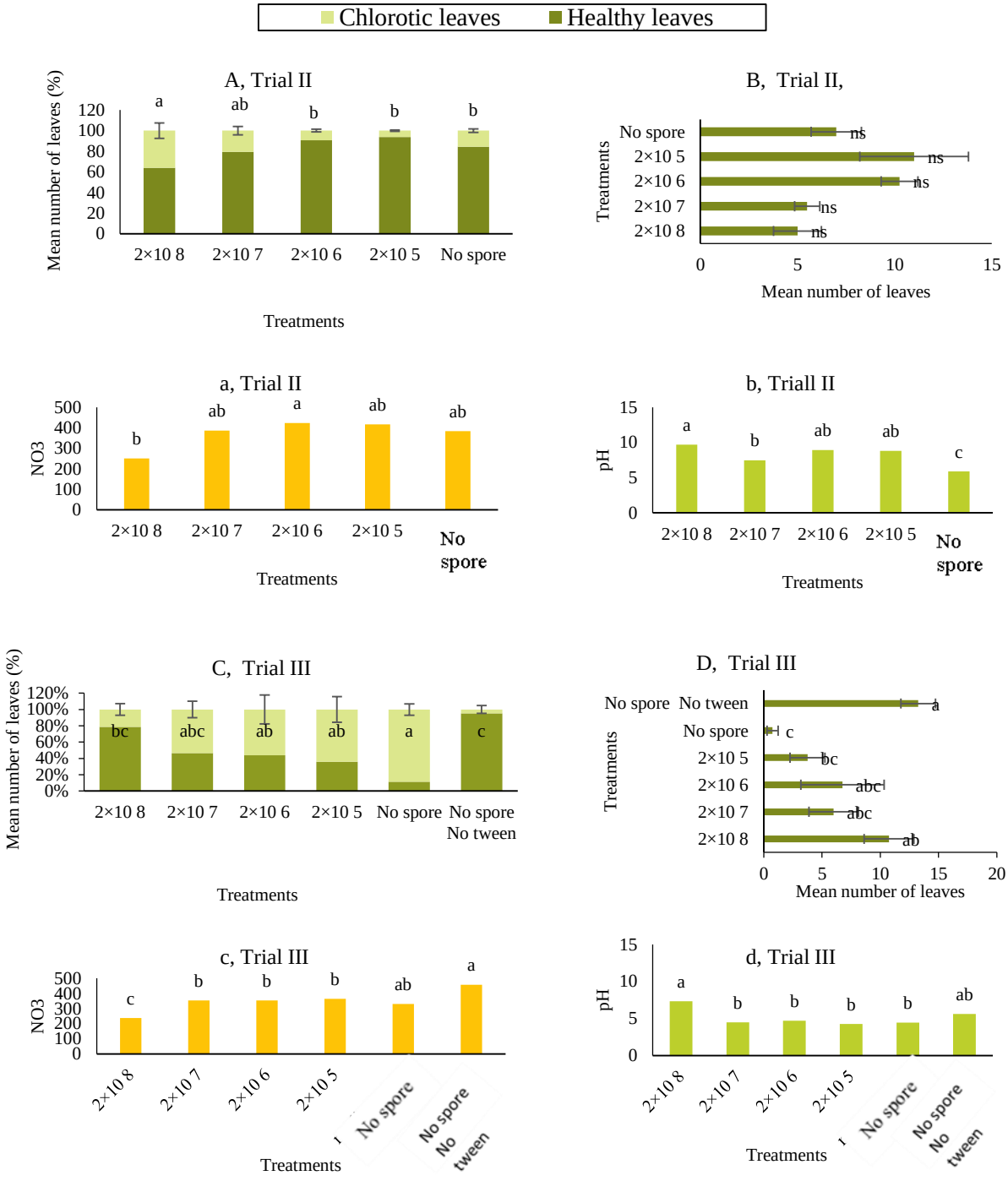


Fig. 29. Effect of isolate ISH-189 at different concentrations on percentage of healthy and chlorotic leaves and new leaves of duckweed 8 days after application; A, F_4=9.006, P=0.001; B, F_4=3.046, P=0.05 ; a, F_4=3.755, P=0.041; b, F_4=22.496, P<0.0001; C, F_5=7.004, P=0.001; D, F_5=4.7, P=0.006; c, F_5=13.814, P<0.0001; d, F_5=6.29, P=0.004; Values followed by the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

BotaniGard: When BotaniGard was diluted in RO water (according to the label), the chlorosis was quite low for all concentrations, even lower than the control (no-spores) (Fig. 30. A, C); however, new leaves grown in the highest spore treatment in the 1st trial (4×10^8) was statistically similar to the control (no-spores) (Fig. 30. B). In the 2nd trial no new leaves grew in 2×10^8 (Fig. 30. D); this may have occurred due to the dark color of inert ingredients in the BotaniGard formulation. Dark water may have obstructed some light from reaching the leaves in the water. In the third trial, the rate of chlorosis and new leaves grown were similar for the all treatments (Fig. 30. G, H).

BotaniGard did not appear to be the cause of chlorosis or low growth of duckweed.

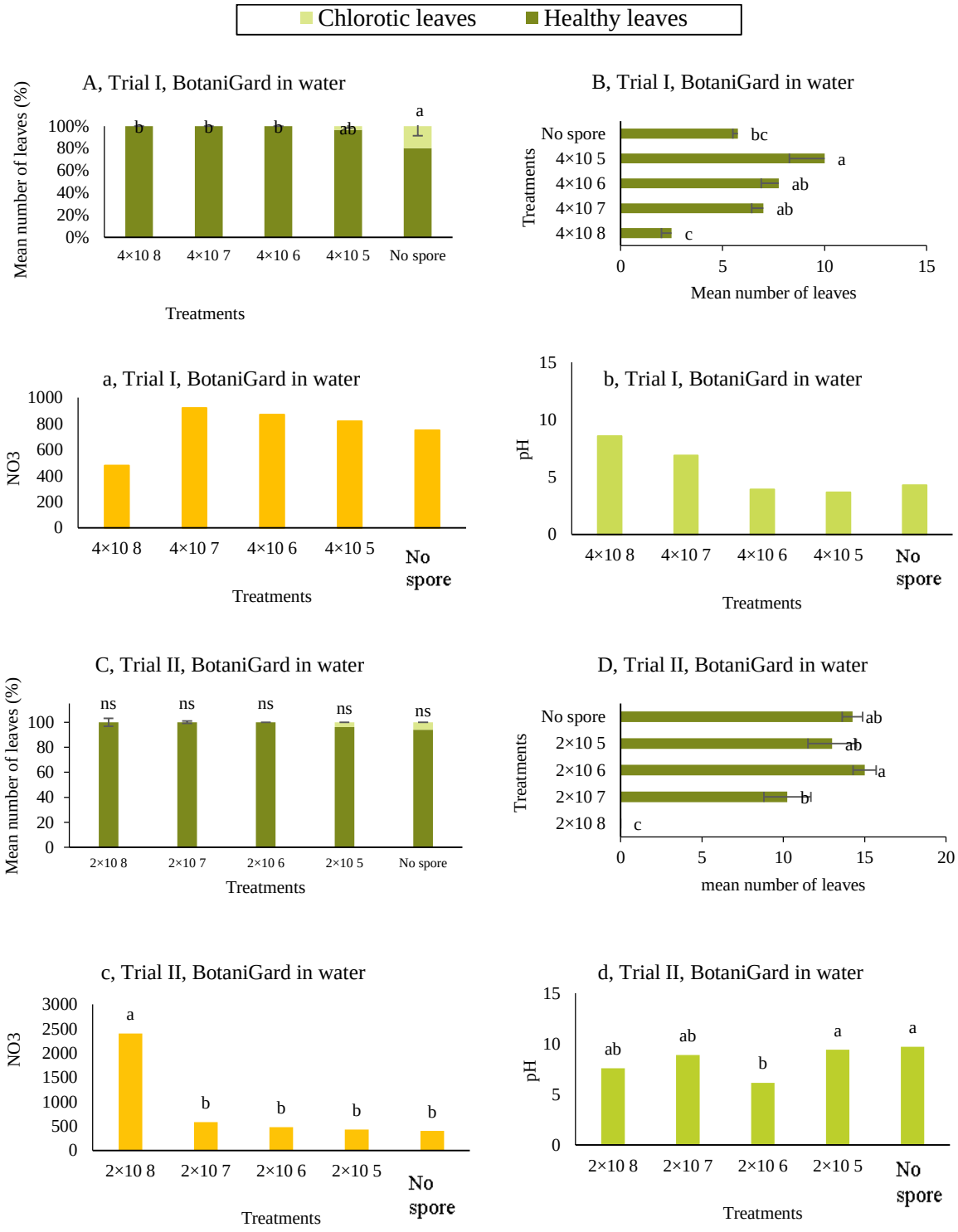
When 0.1% Tween-20 was used in place of RO water to prepare BotaniGard solutions as in the previous trial, there was no significant difference in chlorosis among treatments (Fig. 30. E). The number of new leaves for the highest spore concentration treatments, 2×10^8 and 2×10^7 was low (Fig. 30. F), this may have occurred because of the dark brown color of those suspensions (Fig. 31). In the third trial, the highest amount of chlorosis and the lowest number of new leaves were found in the no-spore plus Tween 20 treatment, followed by 2×10^6 (Fig. 30. I, J).

When the effect of pH and nitrate was assessed in the 2nd trial, in both water and Tween-20, the value of nitrate for 2×10^8 was very high, but the pH values and the amount of chlorosis for all treatments were similar. The number of new leaves was lowest in the highest BotaniGard concentration 2×10^8 (Fig. 30. c, d, e, f).

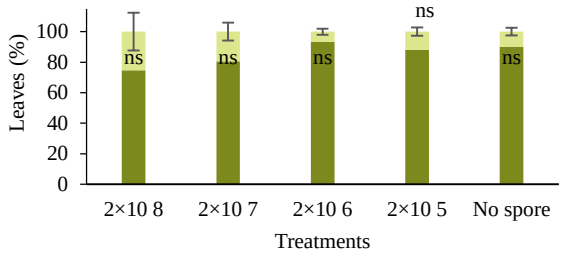
In the 3rd trial, as for the 2nd trial, the nitrate value for 2×10^8 was the highest, while the number of new leaves for this concentration was similar to the no-spore treatment (Fig 30. g, h). Chlorosis ratings for 2×10^8 and the no-spore no Tween 20 were low; while, the nitrate value for 2×10^8 was the highest. Nitrate in the no-spore treatment with 100% chlorosis and no new leaves was the same as the no-spore treatment with Tween 20 (Fig 30. i, j).

It was concluded therefore, that pH and NO3 were not responsible for the chlorosis and growth suppression observed.

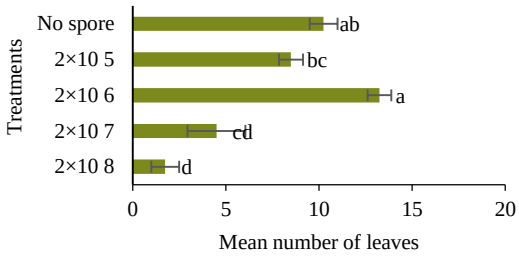
The effects on duckweed of suspensions of BotaniGard made in RO water or Tween-20 differed. More chlorosis and fewer new leaves were recorded when BotaniGard was prepared in Tween 20. These results agree with previous trials using unformulated spores with and without Tween 20. Overall, these trials strongly support the conclusion that Tween 20 changes the growth rate of duckweed and is the reason for the chlorosis.


Chlorotic leaves
Healthy leaves
A, Trial I, BotaniGard in water
Mean number of leaves (%)
100%
80%
60%
40%
20%
0%
b
b
b
ab
a
4×10 8
4×10 7
4×10 6
4×10 5
No spore
Treatments
B, Trial I, BotaniGard in water
Treatments
No spore
4×10 5
4×10 6
4×10 7
4×10 8
bc
a
ab
ab
c
0
5
10
15
Mean number of leaves
a, Trial I, BotaniGard in water
NO3
1000
800
600
400
200
0
4×10 8
4×10 7
4×10 6
4×10 5
No spore
Treatments
b, Trial I, BotaniGard in water
pH
15
10
5
0
4×10 8
4×10 7
4×10 6
4×10 5
No spore
Treatments
C, Trial II, BotaniGard in water
Mean number of leaves (%)
ns
ns
ns
ns
ns
100
80
60
40
20
0
2×10 8
2×10 7
2×10 6
2×10 5
No spore
Treatments
D, Trial II, BotaniGard in water
Treatments
No spore
2×10 5
2×10 6
2×10 7
2×10 8
ab
ab
a
b
c
0
5
10
15
20
mean number of leaves
c, Trial II, BotaniGard in water
NO3
3000
2500
2000
1500
1000
500
0
a
b
b
b
b
2×10 8
2×10 7
2×10 6
2×10 5
No spore
Treatments
d, Trial II, BotaniGard in water
pH
15
10
5
0
ab
ab
b
a
a
2×10 8
2×10 7
2×10 6
2×10 5
No spore
Treatments

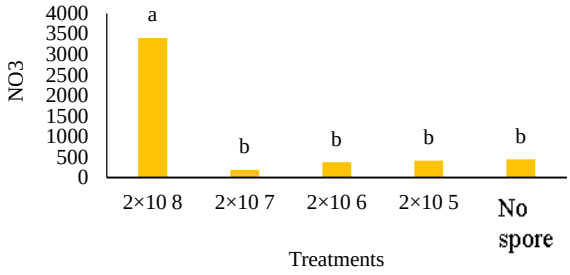
E, Trial II,BotaniGard in tween



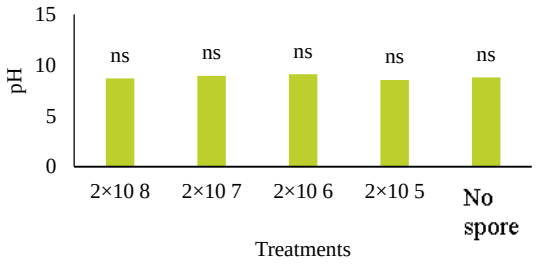
F, Trial II, BotaniGard in tween



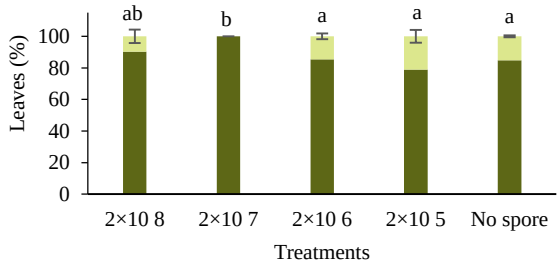
e, Trial II,BotaniGard in tween



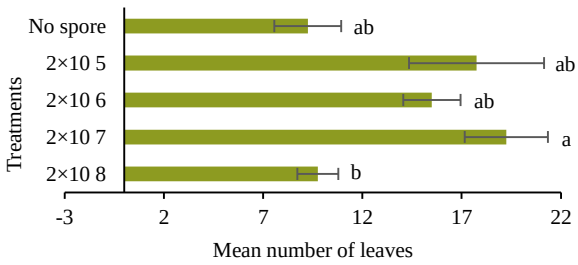
f, Trial II,BotaniGard in tween



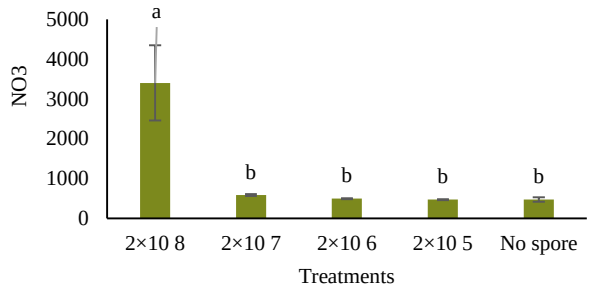
G, Trial III, BotaniGard in water



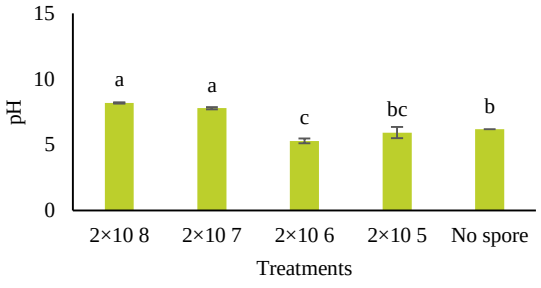
H, Trial III, BotaniGard in water



g, Trial III, BotaniGard in water



h, Trial III, BotaniGard in water



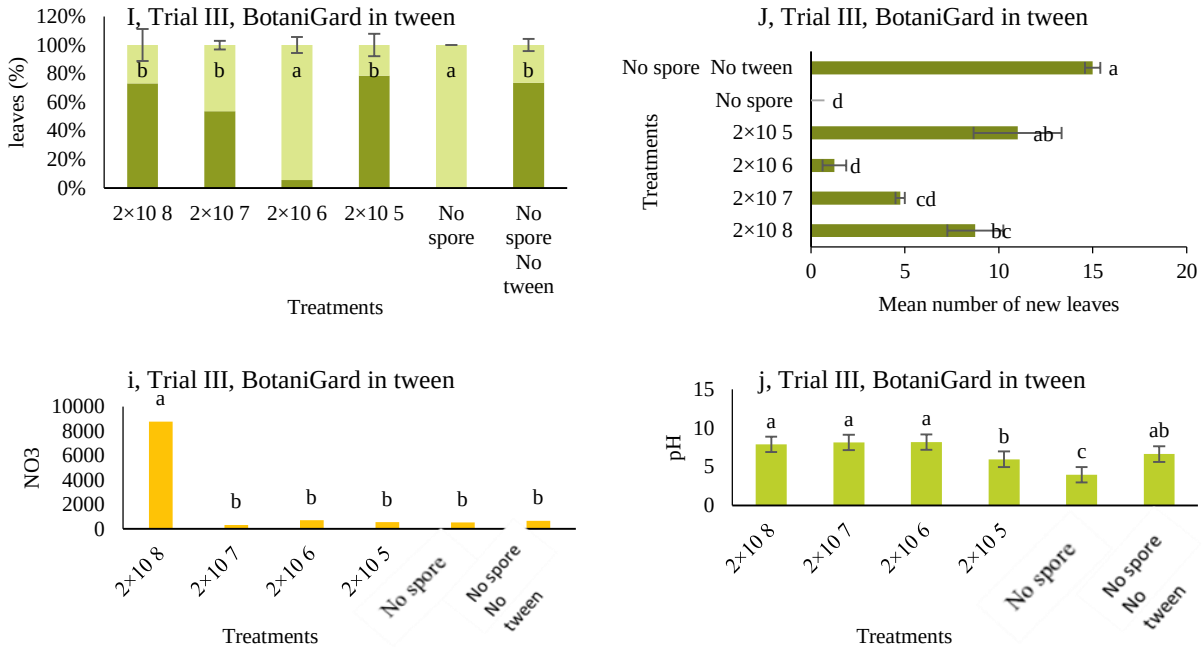


Fig. 30. Effect of BotaniGard at different concentrations diluted using tween or water on percentage of healthy and chlorotic leaves and new leaves of duckweed 8 days after application; A, F_4=4.74, P=0.011; B, F_4=8.736, P=0.001; C, F_4=3.643, P=0.029; D, F_4=36.799, P<0.0001; c, F_4=18.176, P<0.0001; d, F_4=5.328, P=0.015; E, F_4=1.386, P=00286; F, F_4=23.933, P<0.0001; e, F_4=75.203, P<0.0001; f, F_4=0.22, P=0.921; G, F_4=78.1334, P=0.0011; H, F_4=3.2849, P=0.04202; g, F_4=9.43035, P=0.0021; h, F_4=32.8159, P<0.0001; I, F_5=31.199, P<0.0001; J, F_5=24.408, P<0.0001; i, F_5=62.482, P<0.0001; j, F_5=21.368, P<0.0001; Values with the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

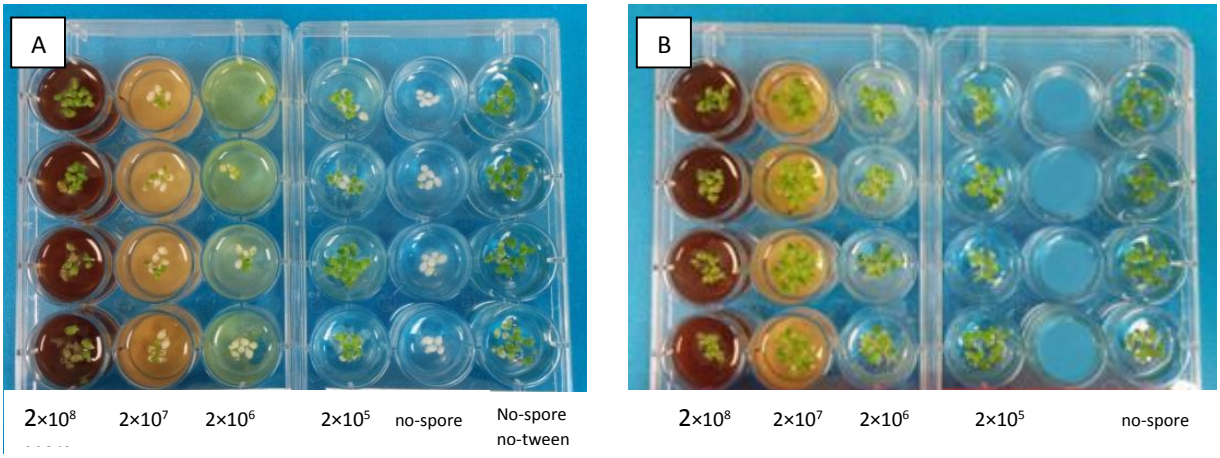


Fig. 31. Suspensions of BotaniGard using 0.01 % Tween-20 (A) and RO water (B)
Note dark colour of treatments in far left rows which were the highest concentration of BotaniGard

Duckweed IV Trial: Chlorosis was significantly higher for both suspensions containing live and dead spores than those treatments without spores; and there was no significant difference between suspensions of live and dead spores (Fig 32. 33. A). The number of new leaves in the treatment without Tween-20 (no-spores & no-Tween 20) was higher and there was no significant difference among treatments containing Tween i. e. live spores, dead spores and no-spores (Fig 33. B). Figure C shows growth of duckweeds up to 5 days post application, the growth of leaves in media without spores and Tween 20 was higher than the other.

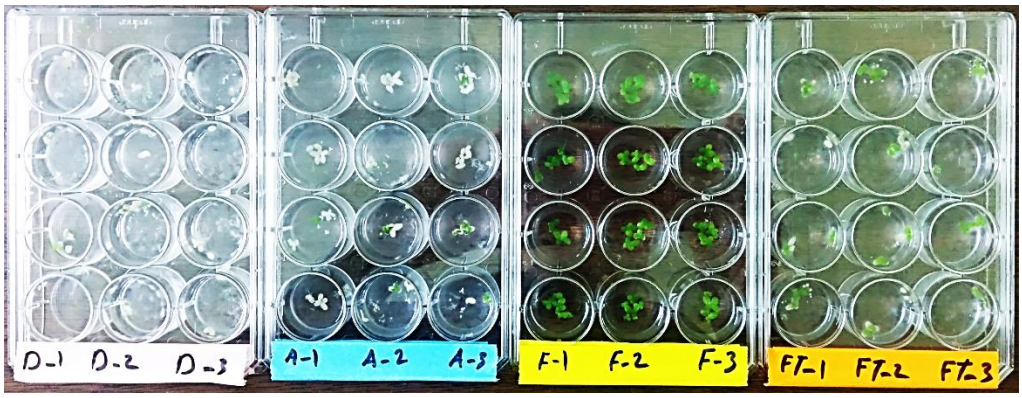
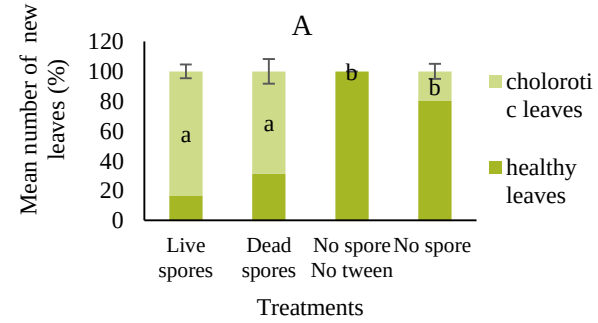
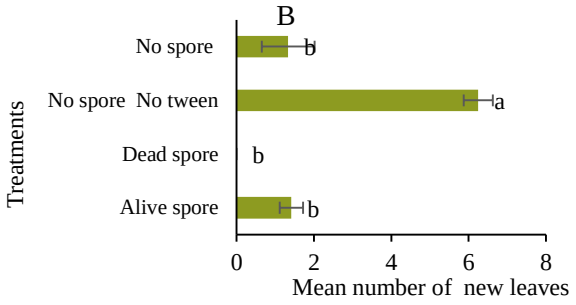


Fig. 32. Duckweed leaves treated with alive (A) and dead spores (D), no spore & no Tween (F) and (FT) 5 days after application





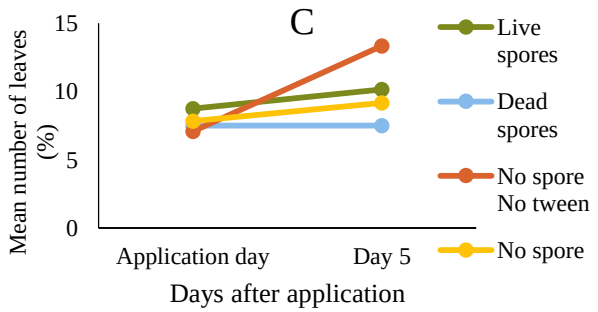


Fig. 33. Effect of live and dead spores of *B. bassiana* isolates, ISH-190, at concentrations of 2×10^8, spores/ml on percentage of healthy and chlorotic leaves and new leaves of duckweed 5 days after application in the fourth trial; A, F_3=54.916, P<0.0001; B, F_3=42.957, P<0.0001; Values followed by the same letter are not significantly different in Tukey's HSD at P=0.05; **n. s.,** no significant difference from group

Conclusion and Discussion

Duckweed survives at pH values between 5 and 9 but optimal growth happens in a range of 6.5-7.5 and duckweed needs a source of nitrogen to grow (Van den Berg *et al*., 2015). Therefore, pH values and amounts of nitrate could reasonably be expected to affect duckweed growth in the current research. Our results however, showed there was no consistent or logical relationship between amounts of nitrate or pH values and ability of duckweed to produce new leaves or lose chlorophyll (chlorosis). On these trials, pH and nitrate did not affect growth of duckweed.

The results showed that high concentrations of spores of these isolates, as well as Tween 20 appeared to be the reasons for the high rate of chlorosis and low rate of growth in duckweed. A comparison of suspensions of BotaniGard in RO water and Tween-20 showed that chlorosis in suspensions with Tween 20 was higher than RO water. In addition, numbers of new leaves grown in Tween 20 was lower than in water. The last experiment supported the hypothesis that the suspensions applied were responsible for chlorosis and the decreasing ability of duckweed to grow and produce new leaves; since the effect of the suspensions containing live and dead spores on chlorosis of duckweed was similar. The effect could have been mechanical or physical. Two hypotheses exist: 1- the mass of spores block the stomata under leaves and prevent absorption of carbon dioxide; 2- the mass of spores made the water cloudy, changing its color and preventing sunlight from reaching the plants. It was clear in these trials that Tween 20 was responsible for loss of chlorophyll and suppressing the growth of duckweed.

***Trichogramma sibericum* Trial**

T. sibericum was reared on *Ephestia kuehniella* eggs for approximately 20 generations in the lab (since the last passage through their natural host *Rhopobota naevana*, Cranberry fireworm) (Fig. 34).

Fig. 34. *Trichogramma* wasp

Material and Methods

Treatments: OKA-373, OKA-372, ISH-189, ISH-190, and BotaniGard® (positive control) were applied at varying concentrations of 2×10^8, 2×10^7, 2×10^6, 2×10^5 spores/ml. For comparison 0.1% Tween 20 was used as the trial negative control.

Serial dilutions were made from a suspension of 4×10^8 spores/ml for each isolate.

Bioassay for *T. sibericum*: Four leaf discs of organic cabbage plant, var Caraflex, per treatment were dipped in each concentration for 6 seconds, allowed to dry on a paper towel and placed in a tube (5 cm × 2 cm) containing a small droplet of diluted honey as a food source. Then, 6 one-day old females of *T. sibericum* were introduced onto each leaf disc and allowed contact with the treated leaf for up to one week. The tubes were sealed with their lid and incubated in a rearing

room at 24 ± 1°C and L: D 16:8. The health of the *Trichogramma* wasps was assessed on days 2, 4 and 7 post application.

Fig 35. *T. sibericum* trial

Results

No significant difference was observed in survival of T. sibericum exposed to any isolate (Table 8). Therefore, all the concentrations of isolates studied were safe for *T. sibericum.*

Table 8. Effect of different concentrations of *B. bassiana* isolates on mortality (%) of *T. sibericum* 7 days after application

Concentrations	*B. bassiana* isolates									
	OKA- 373		**OKA-372**		**ISH-190**		**ISH-189**		**BotaniGard**	
0	12.1 ± 1.85	ns	15.1 ± 2.07	ns	8.33 ± 4.8	ns	8.333 ± 4.81	ns	8.33 ± 8.33	ns
4×10^{5}	4.17 ± 4.17	ns	4.17 ± 4.17	ns	4.17 ± 4.2	ns	4.167 ± 4.17	ns	4.17 ± 4.17	ns
4×10^{6}	8.33 ± 4.81	ns	8.33 ± 8.33	ns	4.17 ± 4.2	ns	4.167 ± 4.17	ns	0	ns
4×10^{7}	4.17 ± 4.17	ns	4.17 ± 4.17	ns	4.17 ± 4.2	ns	4.167 ± 4.17	ns	8.33 ± 4.81	ns
4×10^{8}	4.17 ± 4.17	ns	4.17 ± 4.17	ns	8.33 ± 4.8	ns	4.167 ± 4.17	ns	8.33 ± 8.33	ns
ANOVA statistics	F_4= 0.806 P=0.540		F_4= 0.902 P=0.487		F= 0.265 P=0.5896		F_4= 0.187 P=0.941		F_4= 0.387 P=0.814	

n. s., no significant difference from group

Conclusion and Discussion

The test on *T. sibericum* showed that usage of *B. bassiana* in a pest management programs is safe for this natural enemy. Many researchers indicated that *B. bassiana* could be used in conjunction with *Trichogramma* to control pests (Potrich, *et al*., 2015) and our results corroborate these conclusions.

Objective 4. Continue production trials with amendments to substrate or media to optimize production protocol for at least one of the Okanagan isolates

Materials and Methods

First Mass Production Trial Using Rice and Barley Flakes as Solid Substrate: In 26 June, 2017 two solid substrates, rice grain (A.A.A Premium Scented Rice, Rooster) and barley flakes, were used to produce *B. bassiana* isolates, OKA-372, OKA-373, ISH-190 and ISH-189, in 3 replications. First, mycelia of *Beauveria* isolates were propagated in 150 ml of a blend of CSYE and broth (Table 9) that had been sterilized. After cooling, the media was inoculated by aseptic transfer of 3 x 6 mm diameter plugs from a sporulating culture to each flask. The flasks were agitated on an orbital shaker at 24-27°C, 110 rpm for 4 days. Five hundred grams of solid substrate were placed in each polypropylene vented spawn bag (Unicorn Bags, Plano Texas) with 300 ml Reverse Osmosis water. The solid substrate was sterilized in bags by autoclaving at 121 °C for 55 minutes then cooled at room temperature in a bio safety cabinet. Solid substrate was inoculated with *B. bassiana* by adding the entire contents of one flask of liquid media to each bag and sealing it with an Impulse™ Heat sealer. Bags were shaken to homogeneously mix the mycelia with the substrate, placed on a tray and fermented at 24-27 °C for 14 days. Then material was transferred into brown paper bags and allowed to air dry for 10 days. Spores were harvested using a RoTap Sieve Shaker for 20 minutes per bag.

Table 9. Liquid media ingredients for making 150 ml of media

Ingredients	**Amount**
Glucose	6 g
KNO_3	1.5 g
K_2H_2PO4	0.75 g
MgSO4	0.3 g
$CaCL_2$	0.0075
Yeast extract	0.375 g
Gentamycin	150 µl

Second Mass Production Trial Using Rice as Solid Substrate: Aerial spores were harvested from cultures of each *Beauveria* isolate growing on Sabaraud Dextrose Agar (SDA) or Potato Dextrose Agar (PDA) and used to inoculate 250 ml flasks (1 per isolate) containing 150 ml of sterile liquid media (20g/L brewer's yeast, 20g/L sucrose, 1ml/L Gentamycin). Inoculated flasks were placed on an orbital shaker at 27°C, 150 rpm for 4 days. For each flask of inoculated liquid media, 1 kg of rice was prepared for solid state fermentation. For each kg of rice, 300 ml Reverse Osmosis water and 20 ml vegetable oil were added and rice was 'parboiled' over low heat until all liquid was absorbed. Rice was then distributed into polypropylene vented spawn bags (1kg/bag)

(Unicorn Bags, Plano Texas) and autoclaved at 121°C for 45 minutes. Once the rice was cooled to room temperature, each bag was inoculated with 1 flask of 4-day old liquid culture. The substrate and liquid media were mixed thoroughly by massaging the bag. Bags were sealed using an Impulse™ Heat sealer. Inoculated bags were incubated at 27°C for 10 days. After 10 days, each spawn bag was cut open and divided between 2 paper bags. The substrate was dried using a fan at 27°C for 5 days. Spores were harvested using a RoTap Sieve Shaker for 20 minutes per bag.

Viability and spore concentration (spores/gram) were determined and the harvested spores were then stored in a dark bottle at 4°C until used in trials.

Results

The mother culture plates of the isolates that were used in mass production trials came from 4°C and -86°C for the first and second trials, respectively.

First mass production trial using rice and barley flakes as solid substrate: The results of the test repeated twice show that OKA-373 on rice and barley produced the highest quantity of spores per gram of substrate, 1.48×10^{10} and 1.41×10^{10}, respectively; however, it was not statistically difference from the quantity of spores produced by ISH-190 and ISH-189 on rice or barley. The quantity of spores for isolates produced on rice and barley were statistically similar; whereas the quantity of spores per gram of rice was higher than barley (Table 10; Fig. 36). Hadapad and Zebitz (2006) have indicated that rice was the most appropriate solid substrate for growth and sporulation of *B. brongniartii* in comparison with the other substrates tested. The highest number of conidia was 1×10^{9} conidia/g with 50% humidity 14 days after incubation at 25 ºC. Nelson *et al*., (1996) have found rice was better solid substrate compared to wheat and barley to produce conidia of *B. bassiana*. Amandeep and Neelam (2014) have applied rice, wheat, maize, sorghum, mini potato tubers and rice stew as solid substrate to mass production of *B. bassiana* and reported that rice is the most suitable substrate. Karanja *et al*., (2010) have indicated that maximum conidia yields of *B. bassiana* strain M58 and M60 were on broken maize (8.03g and 7.36g, respectively) and rice (5.43g and 6.68g, respectively).

Table 10. Spore count and weight of harvested spores of *B. bassiana* isolates produced on rice grain and barley flaks in the first production

***B. bassiana* isolate**	**Solid substrate**	**Quantity of spores by mass (g)/substrate (Kg)**	**Spore count/ gram of substrate**
ISH-189	Barley	0.396	8.15×10^{9}
	Rice	0.744	9.25×10^{9}
ISH-190	Barley	0.396	4.7×10^{9}
	Rice	0.56	7.38×10^{9}
OKA-372	Barley	0.334	2.04×10^{9}
	Rice	0.45	2.81×10^{9}
OKA-373	Barley	0.516	1.41×10^{10}
	Rice	0.716	1.48×10^{10}

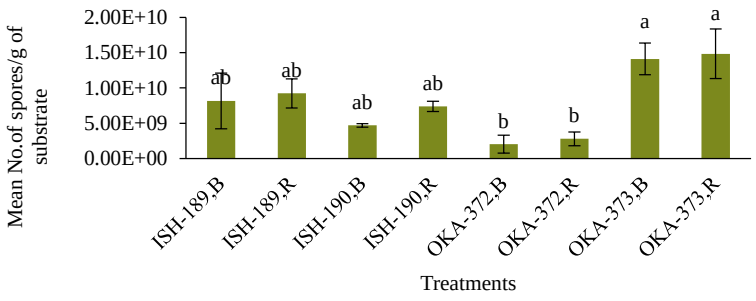


Fig. 36. Mean number of spores per gram of substrate produced on grain of rice (R) or barley flaks (B); F_7=4.529, P=0.006; Values with the same letter are not significantly different in Tukey's HSD

Second Mass Production Trial Using Rice as Solid Substrate: The highest rate of spores per gram of rice was for OKA-373; however, the number of spores for OKA-372 was very close to OKA-373 (Table 11).

Table 11. Number of harvested spores of *B. bassiana* isolates produced on rice in the second trial

B. bassiana isolate	**Spore count/ gram of substrate)**
ISH-189	5.56×10^9
ISH-190	8.0×10^9
OKA-372	1.39×10^{10}
OKA-373	5.5×10^{10}

When the isolates were cultured on PDA in cookie sheets, the highest rate of spores per gram of ml of suspension was for ISH-190, followed by OKA-373, ISH-189 and OKA-372 (Table 12).

Table 12. Number of viable spores per ml of suspension of *B. bassiana* isolates cultured on PDA in cookie sheets (used in aphid and duckweed trials)

B. bassiana isolate	Viability %	Spores /ml	Mean Viable spores
OKA-372	94.8	8.7×10^7	6.484×10^7
OKA-373	96.7	8.3×10^8	8.026×10^8
ISH-189	93.9	5.7×10^8	5.35×10^8
ISH-190	94.5	1.03×10^9	1.003×10^9

Conclusion and Discussion

A comparison of the number of spores per gram and quantity of spores per kg of substrate for *B. bassiana* isolates obtained from trials in 2017 and 2016 shows that more spores were produced in 2016 than in 2017; however, the media used were different. Table 13 shows the spore count of 2017 and 2016 from different liquid and solid media. Clearly more work is needed to optimize production of these isolates.

Table 13. Comparison of spore count of *B. bassiana* isolates from trials in 2017 and 2016

B. bassiana isolate	2017, I trial		2017, II trial		2016, I trial		2016, II trial	
	Substrate *	Spores count	Substrate	Spores count	Substrate	Spores count	Substrate	Spores count
ISH-190	Barley	4.7×10^9	Solid: Rice Liquid: brewer's yeast, sucrose	4×10^9	Solid: Wheat Liquid: SDB+ Peptone +Dextrose	5.43×10^{10}	-	
	Rice	7.38×10^9					-	
ISH-189	Barley	8.15×10^9		2.8×10^9		6.38×10^{10}	-	-
	Rice	9.25×10^9					-	-
OKA-373	Barley	1.41×10^9		5.5×10^{10}		1.06×10^{11}	SDB, Barley flakes	1.23×10^{11}
	Rice	1.48×10^9					PD, Barley flakes	9.38×10^{10}
							SDB, Wheat	1.33×10^{11}
							PD, Wheat	1.22×10^{11}
OKA-372	Barley	2.04×10^9		7×10^9		8.05×10^{10}	SDB, Barley flakes	6.44×10^9
	Rice	2.81×10^9					PD, Barley flakes	7.0×10^9
							SDB, Wheat	1.56×10^{10}
							PD, Wheat	3.18×10^{10}

* Liquid: blend of CSYE Glucose, KNO_3, K_2H_2PO4, MgSO4, $CaCL_2$, Yeast () and broth
SDB, Sabouraud dextrose broth
PD, Peptone Dextrose

Sporulation is affected by nutritional factors, i. e. carbon concentration, C: N ratios, combinations of carbon and nitrogen sources, and environmental factors, i. e. pH, water potential, temperature, and light (Gao, 2011). *B. bassiana* isolates are also variable in their response to high temperature and low water availability (Fargues *et. al*., 1997; Fargues *et. al*., 1992; Fargues and Remaudiere, 1977). Further studies are needed to optimize the factors (nutritional and environmental) that affect the traditional mass production protocol for these particular isolates.

A promising alternative production method has recently been developed: liquid culture fermentation (LCF) to produce microsclerotia. The technique is promising as a new and economic protocol for mass production of entomopathogenic fungi e. g. *B. bassiana*.

Microsclerotia produced by entomopathogenic fungi have been shown to be effective to control a broad range of economically important species of insects that are active in the soil and plant canopy (Jaronski 2014). Microsclerotia are compact melanized hyphae, 50–200 μm in size, that are especially durable, desiccation tolerant and long-lasting during storage. They are air-dried to low moisture levels which gives them excellent shelf-life at room, as well as low temperatures. In addition, microsclerotia can be sized in formulations which are compatible with conventional particular granular pesticide applicators. They are able to be applied in soil, on grass, or plants and are also able to produce infective spores under optimal environmental conditions (Suszkiw 2015; Jaronski, 2014; Jackson and Jaronski 2007).

As Suszkiw (2015) explained, the researchers added *B. bassiana* and *Trichoderma harzianum* as two fungal biocontrol agents to their list of LCF successes, in 2014. Mortality of silver leaf whitefly

nymphs was 25 percent faster when *Beauveria* microsclerotia were used with just 1/4 the quantity of spores than would be necessary produced by traditional production methods. Based on the studies by Jackson, LCF produces microsclerotia in 2-3 days while producing conidia using traditional solid-substrate culture methods takes 10-14 days. The cost of production is estimated to be reduced by 80-90 percent using low-cost cottonseed flour medium in LCF compared to expensive nitrogen sources such as hydrolyzed casein in traditional methods. Microsclerotial fermentations can be simply filtered and air dried. When microsclerotia are rehydrated, they rapidly conidiate and can thus be made into conidiogenic granules (Jackson and Jaronski, 2012).

OVERALL CONCLUSION

Efficacy of *B. bassiana* isolate on Cabbage Looper

Comparison of the larvae of cabbage looper treated in the lab and in the field on the days 2, 5, 8, 14 and 19 post exposure showed that the percentages of mortality were higher and sporulation of the dead larvae/pupae occurred earlier in the lab, which was as expected.

Overall, OKA-373 showed the lowest LT_{50} and the highest rate of mortality and sporulation of cabbage looper larvae in comparison with ISH-189 and BotaniGard in the field. However, ISH-189 caused reasonable mortality of the larvae, as did BotaniGard (due to insufficient product availability, OKA-372 and ISH-190 were unfortunately not tested on cabbage looper).

Efficacy of *B. bassiana* isolates on Green Peach Aphid

The results obtained from greenhouse and lab trials on Green Peach Aphid showed that when the relative humidity was sufficient for growth of the *Beauveria* isolates, mortality and sporulation occurred for all isolates in a high rate on the target insects. The high sporulation rate of *Beauveria* isolates helps the spores remain in soil and environment to attack the same pests, as well as the other pests, in the same season or future growing seasons; accordingly, this would help to manage the insect pest populations in the greenhouse and field without any extra costs.

Effects of *B. bassiana* isolates on non-target organisms

There was no consistent relationship between nitrate or pH of the media and growth of duckweed. It was concluded therefore, that changes in the pH or nitrate were not the reason for loss of chlorophyll in duckweed. Results demonstrated that high concentrations of *B. bassiana* isolates and Tween 20 were responsible for decreasing duckweeds ability to photosynthesize and grow. Spores might have mechanical or physical effects on the leaves. Masses of spores could decrease absorption of CO_2 by clogging up the stomata under leaves or preventing sunlight from reaching the leaves by making the water cloudy. In trials with formulated BotaniGard, RO water and Tween 20 the cause of chlorosis and low production of leaves was not the BotaniGard product, but the presence of Tween 20. It was concluded therefore, that Tween 20 has a negative effect on duckweed growth, increasing chlorosis and suppressing leaf production.

The tests on *T. sibericum* showed that including *B. bassiana* in pest management programs is safe for this natural enemy and potentially other non-target insects that come into residual contact with the fungus. The combination of *Trichogramma* species and *B. bassiana* has been suggested and is common in biological control programs.

Mass production

In traditional mass production trials with these isolates, sporulation was apparently affected by some nutritional and environmental factors in complicated ways; therefore, further studies are needed for the optimization of these factors. Alternately, investigation and study of these isolates in microsclerotia production systems using liquid culture fermentation (LCF) is promising as a new economic method for mass production of *B. bassiana*.

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