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RELACS
IMPROVING INPUTS FOR ORGANIC FARMING

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1. Executive Summary

This document addresses the validation on-farm of novel pesticide compounds and strategies proposed for the gradual phase-out of mineral oil. It reports the description of their applications in two different contexts: (Part A) in a greenhouse (FEM-UNITN, Italy) against the greenhouse whitefly (GW), *Trialeurodes vaporariorum* on tomato plants and (Part B) in a Citrus orchard (CIHEAM-Bari, Italy) to control the populations of the orange spiny whitefly (OSW), *Aleurocanthus spiniferus*. The optimal schedules, the adopted protocols as well as the technical features of the used devices and installation specifics both in field and greenhouse are reported.

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2. State of the Art of Essential Oils and Vibrations to control Whiteflies

Trials previously conducted in Task 2.3 proved a significant effect of Disturbance Vibrational Signals (DVS), alone and in combination with a mixture of plant derived products (namely, 030-S-1-D (Orange Essential Oil, OEO) and BPA044I (extract of *Clitoria ternatea*, CT)), in reducing the GW population on both tomato and zucchini grown in the greenhouse. DVS were developed based on the results of tasks 2.2 add 2.3, in which the candidate disturbance signals was designed to mask the main peak of frequency of the GW mating signals. The selected DVS was then tested for its capacity to affect the fitness and thus to reduce the infestation rates of GW in greenhouse conditions by disrupting intraspecific communication. We concluded that the adoption of a strategy to control GW in greenhouse based on the synergistic use of DVS and the tested products can significantly reduce the GW population similarly to the reference pyrethroid commercial products. In addition, it seemed to maintain the GW suppression effect for a longer time than the positive control.

3. Part A: Application of DVS and a mixture of BPA04411 and 030-S-1-D against the greenhouse whitefly *Trialeurodes vaporiarorum* on tomato plants in the greenhouse

3.1. Aim of the Study

The aim of the study was to evaluate the effectiveness of the combination of DVS + BPA044I and 030-S-1-D against GW. The trials were performed in commercial-like conditions after manual infestation of GW on two different levels: high and low.

3.2. Materials and Methods

3.2.1. Insects and plant material

Adults and immature stages of the greenhouse whitefly (GW) have been acquired by Bioplanet srl (Cesena, Italy) in October 2021 and they were used to establish a mass rearing in San Michele all'Adige, TN, Italy. The insects were reared inside two cages (Bugdorm 30x30x60 cm, MegaView Science Co., Ltd., Taiwan) provided with tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum* var. Cuor di Bue), and bean (*Phaseolus vulgaris*) plants. The cages were kept in a greenhouse at 25 ± 2 °C, 70 ± 5% RH, and 16:8 L:D. Tomato plants were all grown from seed in a greenhouse at the same conditions of insects' rearing and used for the trials.

3.2.2. Characteristics of the Disruptive Vibrational Signal

DVS were designed specifically to mask the GW mating signals frequency pattern (150-350 Hz) and were developed in task 2.2 and 2.3. The minimal DVS amplitude, measured from the plants was 30 µm/s, which is considered sufficient to mask the GW signal.

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3.2.3. Combination of plant derived products

The mixture of BPA044I and 030-S-1-D products was diluted in tap water and applied to the crop with standard spray devices used for conventional pesticides at the dose of at 20 and 4 mL/L, respectively.

3.2.4. Setup and DVS application to the plants

Four cages (BugDorm-6M1010 100x100x100 cm, MegaView Science Co., Ltd., Taiwan) were placed each individually on a wood panel (100x100x 0.5 mm) and each panel on a bench inside the greenhouse (Fig.1)

For each experiment, two cages were used for the treatment plants and two for the control plants. The wood panels of the cages with the treatment plants were placed on top of five 'vibroplates', whereas the wood panels of the control cages were placed above plastic cups with the same height of the 'vibroplates'. This ensured that the space between the bench and the wood panel was the same for treatment and control cages. In each cage and for each experiment, 30 tomato plants were used (height at the beginning of each experiment 20±5 cm).

Before the beginning and at the end of each experiment, the intensity and the spectral parameters of the DVS played back through the vibroplates was monitored by recording the vibrations on the leaf of ten tomato plants for each treated cage with a laser vibrometer (PDV-100, Polytec GmbH, Germany) with a sampling rate of 8 kHz and 16-bit resolution and stored on a laptop computer using LAN-XI data acquisition hardware and BK Connect software (Brüel and Kjær Sound and Vibration A/S, Nærum, Denmark).

3.2.5. Experiment 1 – High level infestation

Before the insects' release, treated plants were sprayed with a mixture of BPA044I extract and 030-S-1-D, and control plants with the same amount of water. Once the plants were positioned inside the cages, 15 leaves from each cage were randomly collected and observed under stereoscope to ensure that they were GW free. Then, 100 GW adults were released in each cage to simulate a high infestation level. After two weeks, all plants were sprayed with the same extract mixture or water depending on the group.

Plants were monitored to assess the GW presence by sampling 30 leaves from each cage after four, five and six weeks from infestation. All leaves were observed under a stereoscope and the number of eggs and immature stages (nymphs+pupae) was counted.

As for the adults, a yellow sticky trap (10x15 cm) was placed in each cage after five weeks from the infestation and removed one week later.

In total the experiment lasted six weeks after the initial infestation and the GW population was monitored three times.

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3.2.6. Experiment 2 – Low level infestation

The initial preparation of the cages was the same as in experiment 1. In this case, 30 GW adults were released in each cage. The first appearance of immature stages was observed after two weeks from the infestation. At this moment, the extract mixture used in experiment 1 was sprayed on treated plants. Control plants were sprayed with water the same day. The spray was repeated after three additional weeks (five weeks after infestation).

The infestation level was assessed following the same protocol used in experiment 1, but leaves were sampled and observed under the stereoscope every week beginning starting from two weeks after the infestation.

The yellow sticky traps were deployed in each cage seven weeks after infestation and removed one week later to count the number of GW adults.

In total the experiment lasted eight weeks after the initial infestation and the GW population was monitored seven times.

3.2.7. Statistics

To test if the treatment significantly affected the GW population, the number of GW eggs and immature stages were analysed by means of Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) followed by post–hoc pairwise comparisons to assess significant differences between groups (Anderson 2001). Treatment (mixture+DVS) and week were considered as fixed factors and the cages as blocks. For adults, we have performed a Chi^2 test (likely ratio test) with multiple pairwise comparisons between the 4 cages. All analyses and figures were carried out in R environment using Vegan: Community Ecology Package (Oksanen et al 2013); Figures were built using "dyplr", "tidyr" and "ggplot" packages.

3.2.8. Results

3.2.9. Experiment 1

The GW population trend was increasing for the control in terms of eggs and especially immature stages during the three weeks of observations. On the contrary, the treated cages did not show any clear trend, as the GW population stayed quite constant during the full period (Fig.2). Permanova analysis (Table 1) showed a significant difference of number of eggs (mean±SD: Control = 91.0 ± 55.4; Treatment = 22.7±1.5) between treatments ($F = 17.2$; $p = 0.001$), whereas we did not observe a difference between weeks ($p = 0.97$) nor any interaction (treatment x week; $p = 0.49$). A similar result was observed with immature stages, where the number of individuals was significantly different ($F = 17.5$; $p = 0.001$) between treatment (81.0 ± 55.0) and control (220.7 ± 159.8), while no difference was found between weeks ($p = 0.16$) nor the interaction (treatment x week) was significant ($p = 0.20$). Finally, the number of adults found after 6 weeks of experiments was significantly different between cages ($Chi^2 = 126$, $df = 3$, $p < 0.001$), although the difference was due to one cage of the control (C1 =327) and one of the

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treatments (T1 = 100), which were significantly higher and lower, respectively, than the other two cages (C2 = 190; T2 = 210) (Fig.4).

3.2.10. Experiment 2

During the 8 weeks of experiment, we found a peak of GW eggs at the 6th week in both control and treatment cages, while the number of immatures continued to increase with the time (Fig.3). However, Permanova analysis (Tab.1) revealed that the number of eggs was significantly different ($F = 21.8$; $p = 0.001$) between treatment and control (Control = 107.5 ± 94.9; Treatment = 33.5 ± 35.5). This time we found also a different between weeks ($F = 17.7$; $p = 0.001$), in special between week 6 and all the others ($p < 0.05$). In addition, we found a significant effect of interaction between treatment and week ($p = 12.4$; $p = 0.003$; Figure 5). Similar results were observed with immature stage, where the number of individuals (Control = 193.6 ± 205.6; Treatment = 58.0 ± 58.3) was significantly different between treatments ($F = 30.57$; $p = 0.001$), weeks ($F = 66.6$; $p = 0.001$) and also the interaction of treatment and week was also significant ($F = 25.7$; $p = 0.001$). For the immature stages the weeks with significantly more individuals than the others were 7 and 8 ($p < 0.05$). Finally, the number of adults found after 8 weeks of experiments was significantly different between control and treatment cages ($Chi^2 = 339$, $df = 3$, $p < 0.001$) (C1 = 332; C2 = 249; T1 = 55; T2 = 49; Fig. 5).

3.3. Conclusions

Our results confirm that the adopted strategy, consisting of the combined use of a mixture of plant derived products (BPA04411 and 030-S-1-D) and DVS, can reduce the population of the greenhouse whitefly (GW), *Trialeurodes vaporariorum*, on tomato plants. In particular, our trials were conducted in semi-field conditions, in a commercial-like greenhouse, where groups of 30 plants were treated starting from an identical GW manual infestation. These trials were, in fact, a scale-up in comparison with previous experiments conducted in task 2.3, where groups of 4 plants were simultaneously treated. In these trials, all GW stages (eggs, immatures and adults) were consistently found lower during the weeks of tomato rearing and even if we did not measure the damage on plant tissues, we observed a clear reduced leaf surface negatively affected by GW punctures. More specifically, we observed a more distinct gap in terms of GW populations between treatment and control in experiment 2, which was associated with a lower starting GW infestation. This seems to indicate that this strategy works better when the GW infestation is still at an early stage, whereas the efficacy could be lower or null in case of high populations occurring on the crops. Our advice, therefore, is to: 1) transmit DVS from the beginning and throughout the crop cultivation and 2) spray with the product mixture at the first appearance of GW immature stages.

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4. Part B: On-farm evaluation of the impact of either the combined use of a mixture of plant derived products (BPA04411 + 030-S-1-D) or and vibrational signals on a citrus

4.1. Aims of the study

Approaches alternative to paraffinic oils and allowed in organic agriculture were tested on a field scale at the premises of CIHEAM-Bari to assess whether plant derived products and DVS might be used to control the orange spiny whitefly *Aleurocanthus spiniferus* (OSW).

4.2. Materials and Methods

4.2.1. DVS and plant-based products

The DVS were developed based on the results of task 2.3, which demonstrated that specific disruptive vibrations may reduce the fitness and infestation rates of whiteflies in greenhouse conditions. The DVS were the same ones used in the trials conducted in the greenhouse and described in the Part A of the present document. Similarly, the plant derived products (i.e., EOE, 030-S-1-D and the CT extract, BPA044I), were prepared here as in Part A.

4.2.2. Treatments

The citrus orchard consisted of eight lanes of eight young plants each. In January 2022, two lanes were treated with the mixture of 030-S-1-D and BPA044I, two lanes with the mixture and vibrations, and two lanes with only DVS. Two further lanes were used as a negative control (no treatments). To reduce the role of positional effects, the treatments were not delivered to two consecutive lanes, but they were alternated with other treatments (Fig.6). The DVS were continuously transmitted throughout the cropping season by means of shakers that vibrated poles placed at the top, end, and middle of each lane; wires in direct contact with the poles allowed the transmission of DVS to the plants (Fig.7). The correct transmission of the signals was assessed by recording the signal with laser vibrometers (PDV-100, Polytec GmbH, Germany) and by comparing the recorded vibrations with the original signals and the expected spectral features (i.e., the spectrum of the signals used under greenhouse conditions). Energy supply to the shakers was provided by solar panels that were placed at the edges of the orchard (Fig.7). The mixture of derived plant products was delivered once by directly treating the plants with hand sprays.

4.2.3. Insects and Measurements

OSW were not inoculated in the citrus orchard, given that the latter was already heavily infested by the pest. Surveys were performed before the treatments started to assess that OSW was present on all the plants within each lane, although the rate of infestation differed.

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After the treatments, OSW populations were weekly measured (five times, from 9/3/22 to 21/4/22), in that five plants per lane were selected, and OSW nymphs on 10 leaves per plant (n = 10) were counted and ranked either as immature or mature stage depending on their size.

4.2.4. Statistics

To test whether the treatment significantly affected the OSW populations, the number of immature and mature stages collected at each sampling date were compared across the treatments by Kolmogorv-Smirnov test to assess whether the data distribution and consequently the population trend was different. All analyses were carried out with the software PAST.

4.3. Results

Our samplings revealed that the lanes with DVS treatment had higher OSW infestation at the beginning of the trials, whereas all other lanes had similar GW populations both in terms of immatures and adults. During the experiment, however, these differences did not change significantly, and we observed a similar trend both for immatures and adults (Fig.8). Statistics did not reveal any significant difference between treatments and control in the trend of population densities, neither for immature nor adult stages. In a general, we observed a reduction of the presence of immature stages and a stable presence of adults throughout the period of observation without any significant divergence between treatments (Kolmogorov-Smirnov test; $p > 0.05$).

4.4. Conclusions

Our results indicate that the treatment with DVS and plant products did not significantly influence the OSW population in the field. Further studies should address this subject, by developing a species-specific signal (the one used here was selected to control *Trialeurodes vaporariorum* in the greenhouse) and understanding the role of vibrational signals in the OSW biology. Another possible issue was the occurrence of a natural OSW infestation that did not allow us to define with precision the infestation dynamics. We must also consider that in the field, the dispersion and abundance of the pest can be conditioned by many parameters that are not under our control and that can determine relevant differences between plants. In addition, our tests, because of the late availability of the functioning system (explained in the Deviations from Annex 1) were conducted in winter and early spring, thus covering only a minimal part of the OSW life cycle. The experimental period has presumably negatively influenced the final outcome given that the continuous transmission of the DVS seems to be associated to long term effects, especially when the mating is occurring (as we have seen in the greenhouse). Therefore, trials during late spring and summer should better address our questions.

Overall, the experiment demonstrated that it is possible to transmit disruptive signals through entire lanes of a citrus orchard. Considering that DVS do not affect the life cycle of beneficial arthropods (see D2.3), we consider it important to further test this method to better assess its validity against OSW in field conditions.

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5. General Conclusions

The combined use of vibrations and plant derived products seems to be a promising tool to replace contentious inputs for pest control in organic farming systems. We developed a prototype device, which was reliable in transmitting DVS endowed with those spectral characteristics that negatively affected the mating communication of the greenhouse whitefly. We demonstrated that DVS improve the effects of the mixture of OEO and CT in greenhouse conditions on tomato and zucchini. DVS seemed to work as synergists of pesticide products and it will be interesting to test them also in combination with other kinds of strategies and products. Working in the greenhouse was comparatively easier than in the field, because we had the full control of environmental factor and we defined the pest infestation in terms of time and quantity. In fact, when we applied the same strategy in the field, we found more problems, due to the higher difficulty in making a reliable set-up and also in guaranteeing the energy supply for the total experimental period. This happened because the development of an efficient and reliable device, even if at a prototype stage, requires long sessions of testing in different conditions. As much as we finally realized a device able at transmitting DVS to the Citrus plants, however we could not make a large-scale vibrational landscape (= vibroscape) analysis, to cover all the treated plants. In fact, the presence of plant portions under the required amplitude threshold would determine shelters where the pest can proliferate. Conversely, in the greenhouse we had a plentiful control of the vibroscape to which corresponded successful results. Another important possible reason of the unsuccess of the field experiments was likely the lack of specificity of the DVS. In fact, we used the signal selected for the GW to control the OSW. In the future, it will be important to study in deep the role of the vibrational signals in OSW and define their role in mating and characterize their spectral and temporal features before making a new attempt.

To conclude, we think that the use of vibrational signals as semiophysicals could become soon a tool for pest control to be used in synergy with other methods to replace contentious inputs. DVS are environmentally safe and do not constitute any hazard for human health. The specific technology to make DVS working in the greenhouse is already available while it needs to be further developed for field applications.

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6. Figures

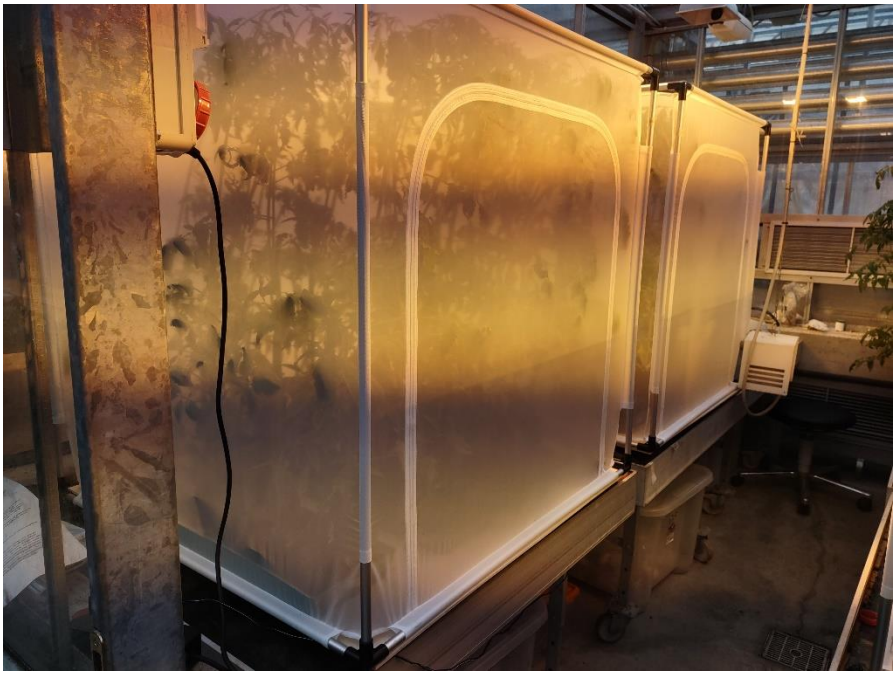

Figure 1: Cages used for experimental trials. In each cage, a basal wood panel sustained 36 tomato potted plants. Five vibroplates were placed under the panel and emitted DVS for the full experiment duration (Photo of Valerio Mazzoni, FEM, Italy).

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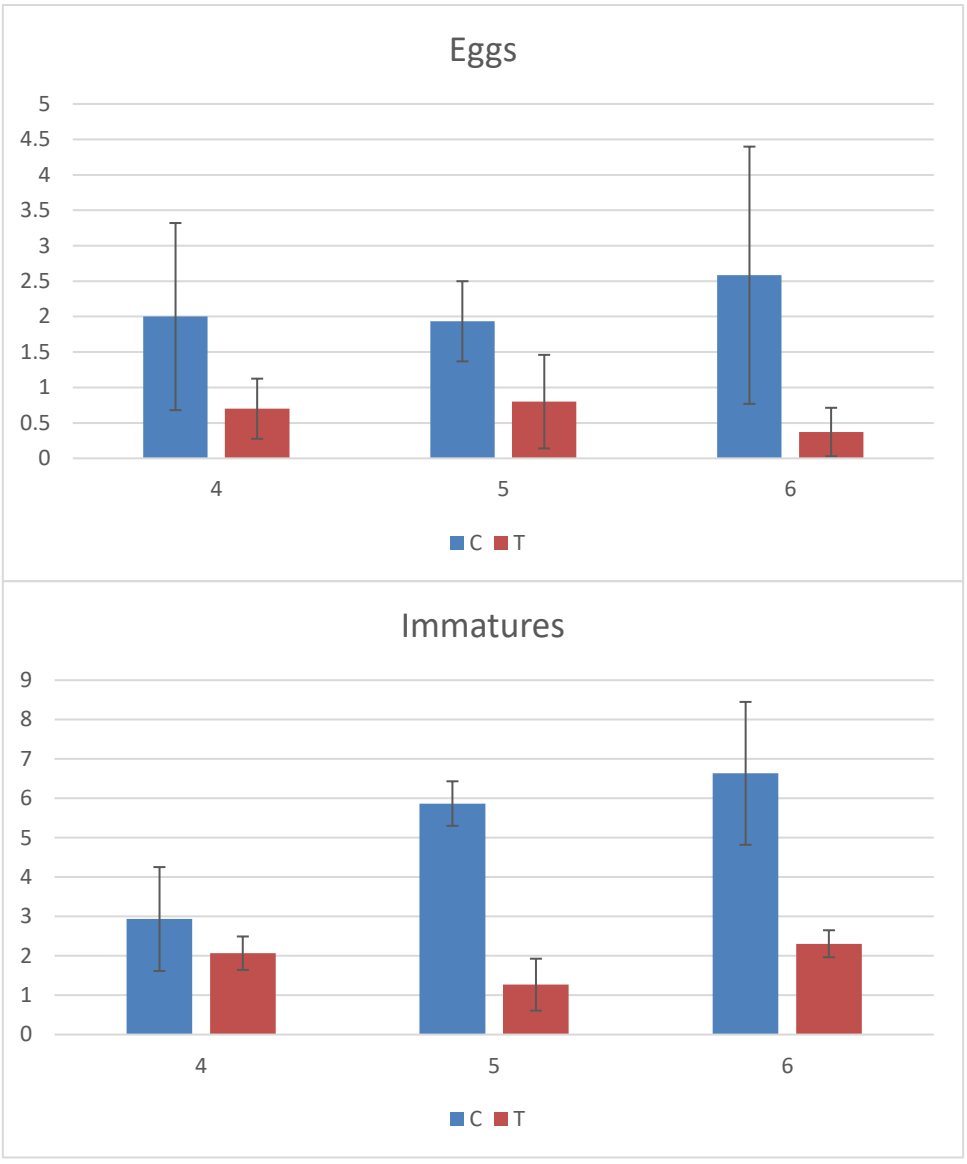

Figure 2: Mean number (±SD) of eggs (above) and immature stages (below) found per week (from 4 to 6) in the control (C) and treatment (T) cages during experiment 1.

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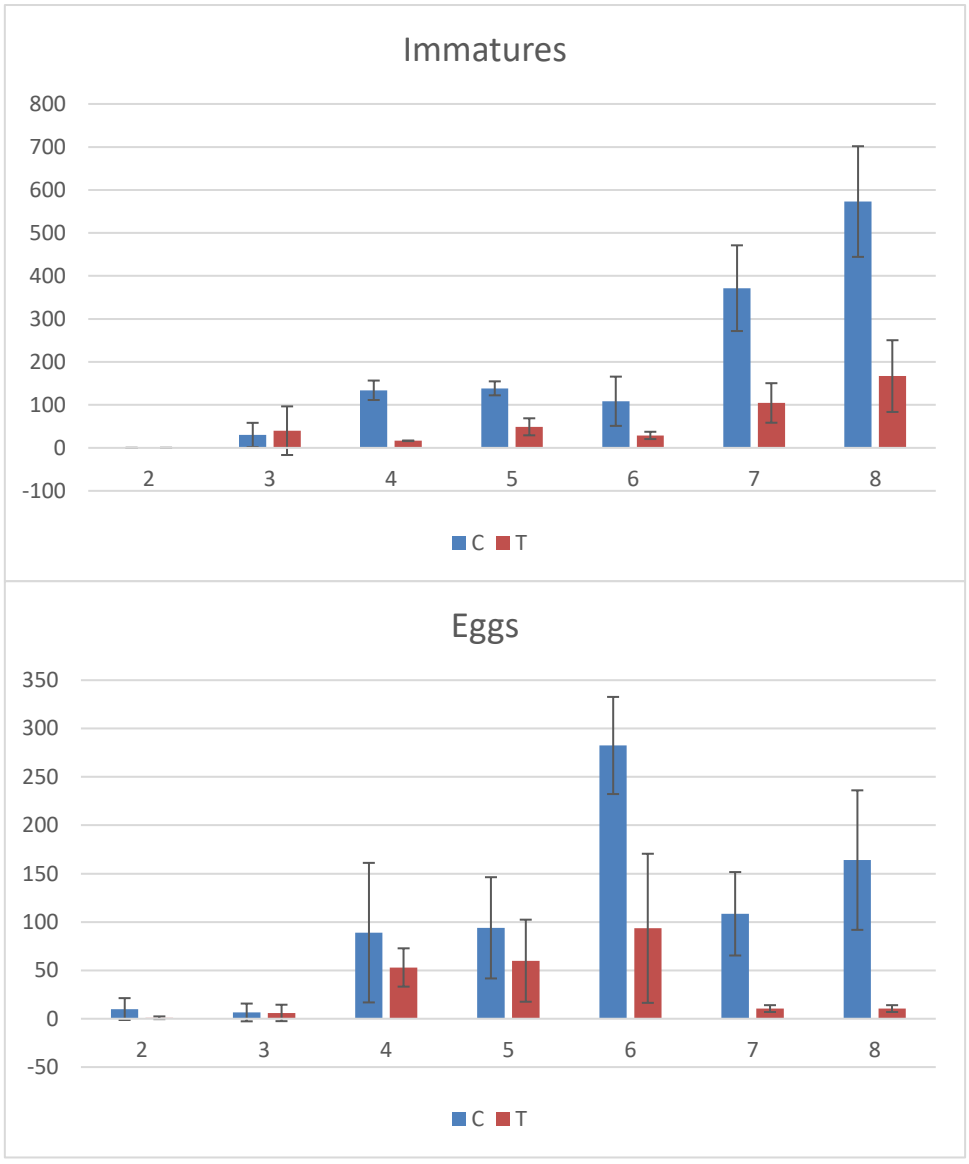

Figure 3: Mean number (±SD) of eggs (above) and immature stages (below) found per week (from 4 to 6) in the control (C) and treatment (T) cages during experiment 2.

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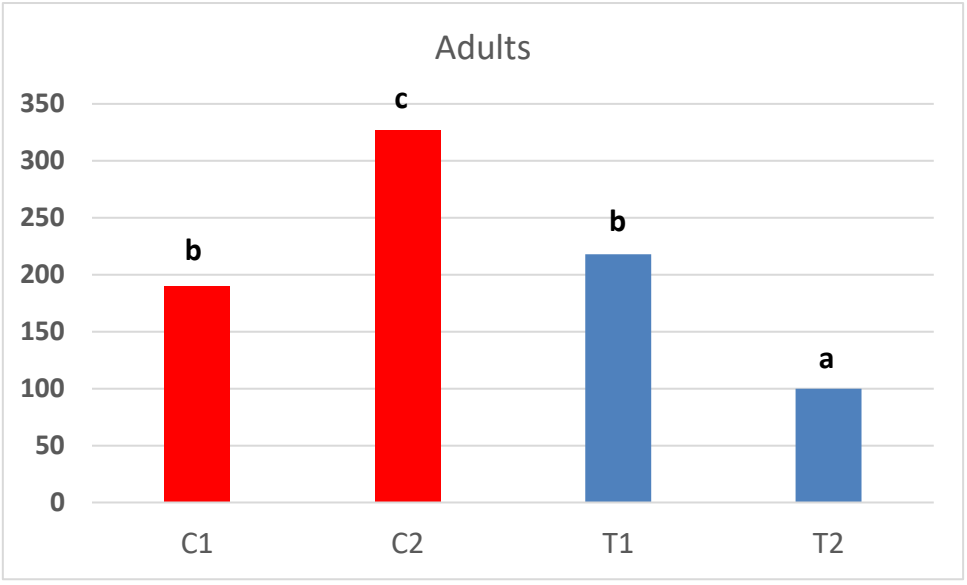

Figure 4: Number of adults captured with yellow sticky traps in the tomato cages after 6 weeks from the beginning of experiment 1. C and T are Control and Treatment, respectively.

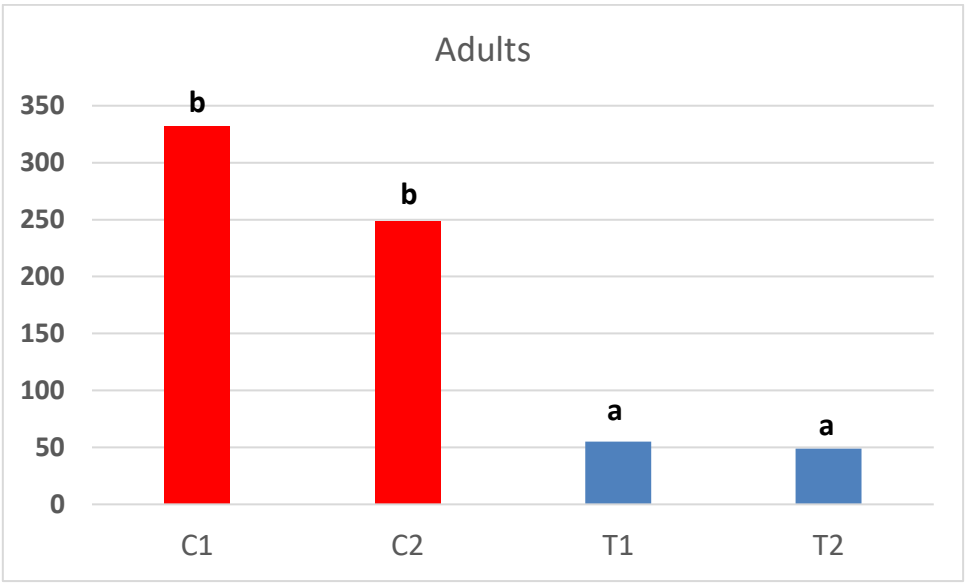

Figure 5: Number of adults captured with yellow sticky traps in the tomato cages after 8 weeks from the beginning of experiment 2. C and T are Control and Treatment, respectively.

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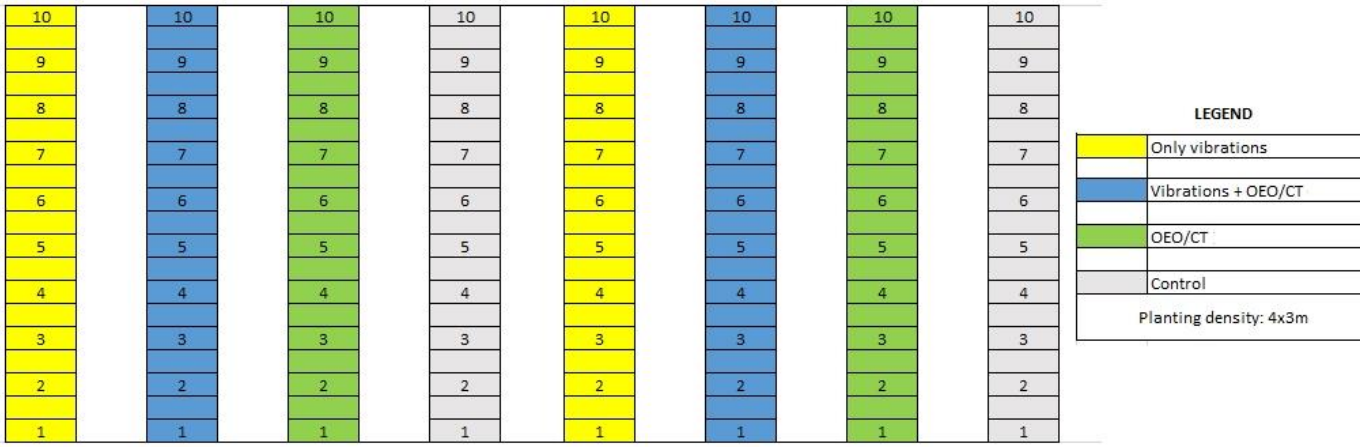

Figure 6: The structure of the citrus orchard infested by OSW. Each lane was composed of 10 plants and was subjected to a specific treatment (i.e., vibrations (DSV), mixture of plant products (OEO/CT) and combinations of DVS and plant products)

Figure 7: Left: the mini shaker is connected to the pole and the wires in contact with the plants. Right: citrus plants in direct contact with the wires that transmit the vibrations received from the pole and powered by solar panels (Photo of Sabina Avosani, CIHEAM, Bari)

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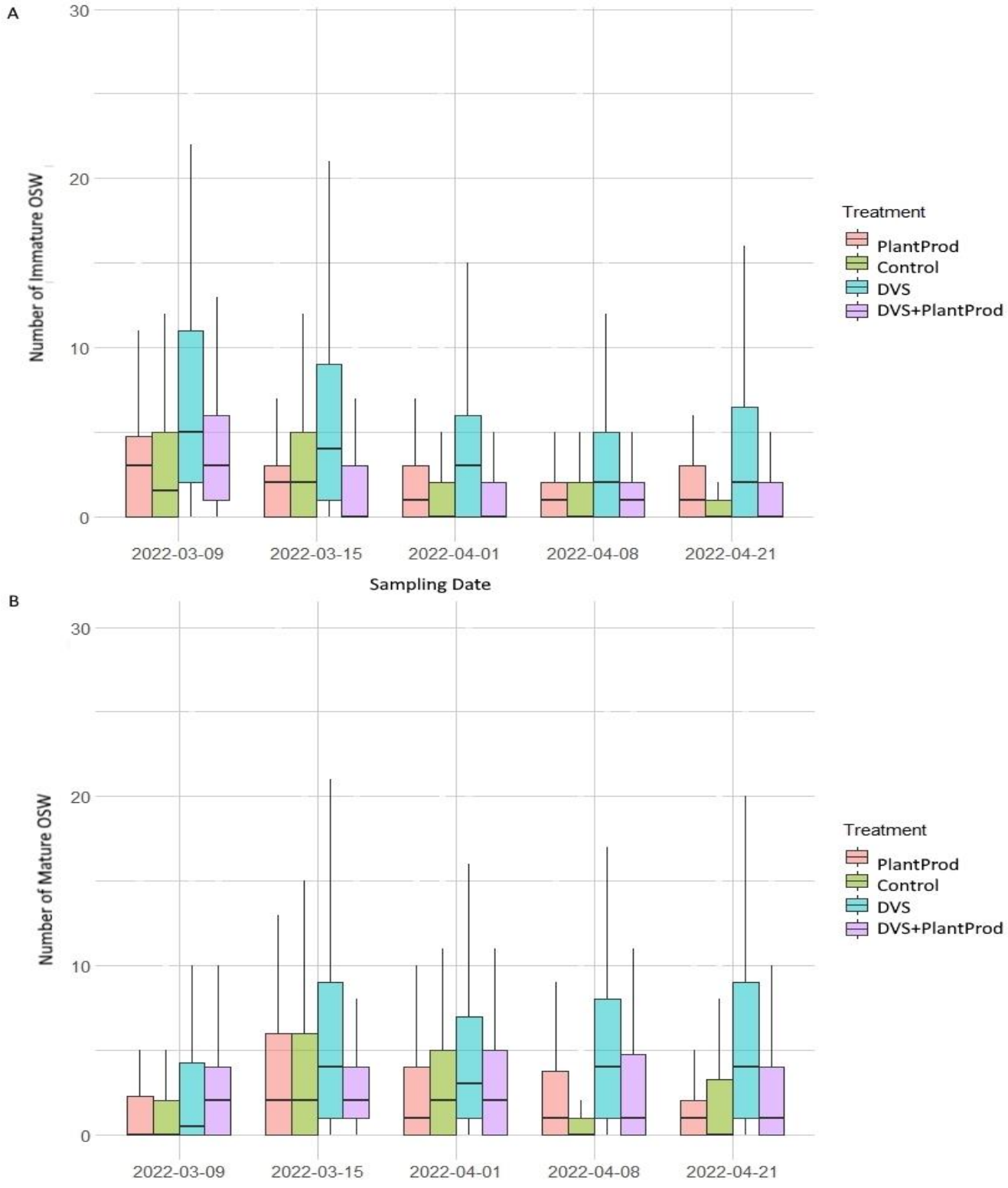

Figure 8: Average number (±SD) of immature and mature (i.e., adults) of OWU, sampled during the experimental period in the Citrus orchard.

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Experiment	Stage	PERMANOVA	F	p
1	Eggs	Treatment	17.2	0.001 ***
		Week	0.09	0.968
		Treatment*Week	0.73	0.489
	Pre-adults	Treatment	17.5	0.001 ***
		Week	1.8	0.17
		Treatment*Week	1.7	0.2
2	Eggs	Treatment	21.9	0.001 ***
		Week	17.8	0.001 ***
		Treatment*Week	12.3	0.003 ***
	Pre-adults	Treatment	34.6	0.001 ***
		Week	87.5	0.001 ***
		Treatment*Week	28.8	0.001 ***

Table 1: Summary of the results of PERMANOVA analysis for experiment 1 and 2.