STUDENT RESEARCH REPORT – REEU PROGRAM 2022

Survey for Entomopathogenic Predator Populations of Plum Curculio in Apple Orchards of Western Massachusetts

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Abstract

Plum curculio (*Conotrachelus nenuphar*) (PC) is an economically devastating pest to pome and stone fruit production. Recommended pest-control methods (such as insecticide application and pheromone-based trapping) and cultural controls work to great effect managing this pest but can impose an important financial and logistical burden on farmers. As an alternative, some entomopathogenic nematode (EPN) species are effective biological control agents for PC, parasitizing the developing larvae as it pupates underground. By using established aggregation techniques, designated trap trees can focus PC activity and larvae in a specific area for the use of insecticide and EPNs to curb the current and future population. By sampling the soil of grafted trees that attract higher traffic of PCs, I aim to monitor the presence of native EPNs that may target PCs in Massachusetts. Soil from unmanaged trees, subject to organic practices, were also surveyed to observe whether or not practices that allow for more insect activity could influence native presence of entomopathogenic predators. Specifically, I hypothesized that natural soil biota, including native EPNs, can be detected from soil traps inoculated with *Galleria mellonella L.* (wax moth) larvae, a species susceptible to EPN attack. To test my hypothesis, I collected soil samples from underneath grafted and ungrafted trees from managed apple trees at the UMass Cold Spring Orchard (Belchertown, MA) and unmanaged trees from Small Ones Farm (Amherst, MA). Either, *Steinernema riobrave* or water were used as positive and negative controls, respectively. Then, I monitored the mortality of wax moth larvae introduced into each soil sample. Significantly greater levels of wax moth larval mortality was recorded in soil from unmanaged trees compared to managed orchards. My findings will inform growers on the presence of natural PC enemies relative to the management practices and cultural controls implemented in their orchard.

Introduction

Apple growing is the largest fresh fruit industry in the United States, generating an estimated 3.2 billion USD for farmers and generating an estimated 23 billion USD in downstream (latent) revenue [1]. The plum curculio (PC) (*Conotrachelus nenuphar*) is an economically significant pest as it hinders pomes and stone fruit production (e.g. apples, pears, peaches, cherries, and blueberries) [2-4]. Native to North America, PC overwinters in the ground and emerges in April [2]. From early-May to June, PC adults migrate into apple orchards to feed and mate [2]. After mating, adult female PC deposit their eggs in fruitlets, causing highly characteristic, crescent-

shaped scars [2]. As PC larvae complete all four instars and develop inside the fruit, feeding and causing the fruit to fall, often before they reach 3cm in diameter [2,5,6]. PC larvae will spend 16 days feeding and developing in the fruit before entering the soil from the fallen fruitlet [8]. Burrowing 2-8 cm into the soil, larvae will use body movement to create a pupation chamber and may spend up to an additional 16 days as larva before pupating [2,8]. PC pupae will develop in 10-12 days, meaning PC spends up to 30 days in the soil before the new adult generation emerges to feed on fallen fruit [2,8]. PC adults then leave the orchard to over winter underneath leaf litter around late September and October [2]. Injured fruits that do not fall from the tree become more structurally deformed over time with variations between cultivars [7]. Thus, oviposition by PC is economically significant as it leads to fruit falling, larval feeding, and the development of the next PC generation which, if left unmanaged, can result in up to 85% loss in fruit yield [7, 8].

To find ecologically and financially viable solutions, Integrated Pest Management (IPM) implements alternatives to reduce the impact of PC. In IPM, pest control strategies emphasize monitoring and ecologically-friendly management practices to keep pest-related injury below the economic threshold, after which the value of the crop destroyed outweighs the costs incurred to manage the pest [9]. Current IPM strategies involve directly monitoring pest populations in the farm and using various forms of population controls when these thresholds are exceeded [4, 10-12]. For instance, to deal with PC, certain perimeter trees are equipped with pheromone-based lures (designated to aggregate pest activity; also known as trap trees). This practice is implemented to attract PC to certain areas of the orchard perimeter where it is easier to spot oviposition scars [4, 10-12]. If the presence of PC is estimated to exceed the economic threshold of the orchard, IPM strategies advise growers to apply insecticides strategically: once on the whole block, then routinely on the perimeter [8, 11]. Chemical approaches are time-sensitive as they should only occur during late bloom in order to avoid unintentional harm to pollinators during initial bloom and allow ample time for PC populations to increase before fruit set [8, 11]. Due to sporadic population growth, the high activity that coincides with flower bloom periods, and a dwindling selection of effective chemical insecticides, PC is a difficult pest to manage [8].

Outside of chemical insecticides, cultural controls are another important management technique. These practices revolve around sanitation, pruning, and cultivar selection [2, 8]. Cultivar selection can prominently impact pest activity by either choosing highly attractive fruit varieties to increase PC infestation or by choosing less attractive fruit varieties to decrease PC activity [2]. Recently, multiple, highly attractive fruit varieties have been grafted to existing trap trees, which are thought to attract PC populations to these areas where population control strategies can be implemented.

Other promising alternatives to insecticides and cultural controls include combinations of the aggregation pheromone (grandisoic acid) and naturally-derived plant volatiles such a benzaldehyde [4, 13]. The combination of grandisoic acid and benzaldehyde has been shown to concentrate PC population for targeted insecticide application, an approach referred to as an ‘Attract and Kill’ strategy [13, 14]. These types of ‘Attract and Kill’ systems rely on a combination of lures that becomes less attractive after petal fall and are single use, which can incur a significant cost to the growers if used routinely [13]. Selected perimeter-row trees that are grafted with six cultivars become very attractive to insect pests. Those grafted trees are termed ‘trap trees’ and have been established at the UMass Cold Spring Orchard (CSO) in Belchertown,

MA since 2018. Recent trials of this model have incorporated commercially available entomopathogenic nematodes (EPN) as biological controls of PC larvae in the soil [10]. Together, a multi-stacked IPM approach that targets PC at the various ecological niches it occupies throughout its development may offer growers a powerful alternative to excess pesticide use, known to detriment human, orchard, and ecosystem health [15, 16].

Just like any biological control, certain EPNs must be implemented at a particular junction during the pest's life cycle to best control a population [17]. By using a readily available host such as *Galleria mellonella L.* (wax moth) larvae to study EPNs, published studies have shown that, in terms of PC mortality rate, the most effective EPN families are Steinernematidae and Heterorhabditidae [18-20]. These EPNs kill by having free living infective juveniles (IJs) infect hosts through natural openings in the mouth, anus, eyes, and sometimes the cuticle [21-23]. Once inside, a species-specific bacteria symbiont is then released into the host intestines to kill the host and break down host tissue [21-23]. The nematodes feed off of the processed organic matter which provides sustenance for the nematodes to reproduce and carry out their lifecycle [9]. A single host promotes the generation of up to three generations of EPNs until the host no longer provides enough nutrients to sustain, at which point the third-generation of IJs will leave their host and seek out another host to infect [21,22]. As the third-generation is the only free moving IJ generation, it will leave the deteriorated host in search of a new host, thereby restarting its life cycle [23].

Overall, I sought out to determine if designated trap trees that were grafted with six apple cultivars that are attractive to PC would also harbor EPNs in the soil area underneath the canopy of managed trees. Soil samples were also collected from unmanaged trees at Small One's Farm (Amherst, MA) to determine if different management practices could impact these predators' population levels. Unmanaged was defined as trees which were not harvested, cultural maintenance was no longer conducted, and chemical pesticides were no longer used. Both grafted and unmanaged trees were incorporated into the study because grafted and unmanaged apple trees are expected to attract the most PC activity, which could potentially attract more hosts for EPNs (unpublished observations). I hypothesized that natural PC enemies, including native EPNs, can be detected from soil traps that receive *Galleria mellonella L.* (wax moth) larvae as a sentinel host, a species susceptible to EPN parasitism. To test my hypothesis, soil samples were collected from managed grafted trap trees and managed non-grafted trees. Then, mimicking pre-established EPN baiting techniques, wax moth larvae were selected as a susceptible model for EPN infection. *Steinernema riobrave* and water were applied to the evenly divided soil samples to assess EPN lethality and entomopathogenic activity [25]. Soil samples that were collected exhibited low mortality rates compared to high mortality positive control groups with little demonstrable differences between 'Grafted' samples and 'Non-grafted' samples. However, samples collected from unmanaged sites where organic practices were being implemented demonstrated higher levels of mortality compared to managed non-grafted sites.

Materials and Methods

1a. Soil Sample Collection and Preparation

The assessment for native EPNs took place at two fruit farms in Western Massachusetts; the UMass Amherst Cold Spring Orchard (CSO) located in Belchertown, MA and Small One's Farm

(SOF) located in Amherst, MA. The UMass Amherst Cold Spring Orchard is a research orchard under standard management; it is here that the aforementioned grafted trap trees have been established in two different blocks (X-block and Empire-block). Small One's Farm, included for their organic practices and unmanaged trees, was designated as Plum Brook-block.

I collected soil samples from 5 sites beneath the foliage of sampled trees. Sites were chosen randomly, ranging from near the trunk to the furthest branch. Soil samples were collected by digging at a targeted depth of 8 to 10 centimeters which is the greatest estimated depth at which PC larvae pupate at [2]. Prior to digging, the top layer of grass and visible organic matter were removed to minimize organic debris. Then, the 5 samples were poured back and forth in two large buckets 3 times to mix thoroughly. 1 liter of mixed sampled soil was stored in the shade to prevent drying. At CSO, soil samples were collected from two blocks that included 4 grafted trees and 4 randomly selected non-grafted trees in the interior of the orchard zone to account for possible PC penetration into the orchard. At SOF, soil was collected from a single organic block that contained 3 large unmanaged Macintosh trees. Soil samples at SOF were collected using the same methodology as the soil collected at CSO. In between each tree sampled, shovels and containers were disinfected with a 10% chloride solution and dried with paper towels. Notably, due to the unkempt nature of the trees at SOF, there was a high number of unmanaged weeds and grasses on the surface of the site, which was not the case at CSO. All soil samples were collected 24 hours post rainfall.

The samples were evenly split into two sanitized containers and labeled to later record mortality data (Table 1). Samples were then stored at room temperature and 10mL of distilled water were applied to each sample to prevent drying before treatment application.

Table 1. Label Key for Soil samples collected underneath grafted and non-grafted trees at the UMass Amherst Cold Spring Orchard, as well as unmanaged trees at Plum Brook. Soil samples were treated with either a water control or diluted sample of *S. riobrave* entomopathogenic nematodes.

Block	Tree#	Application
[X] X-block, CSO	[NG] Non-grafted	[C] Water Control
[E] Empire-block, CSO	[G] Grafted	[Sr] Positive Control, *S. riobrave*
[P] Plum Brook-block, SOF	[#] Replication	

1b. Treatment Application

5 million *Steinernema riobrave*, purchased from Arbico Organics (Oro Valley, AZ), were diluted to the density of 5,000 per 1 mL of distilled water. A small sample of the diluted infective juveniles (IJs) were examined underneath a dissecting scope to assess viability. Then, 1.9 mL of distilled water and diluted IJs were applied to the water control and positive control soil samples, respectively. The specific volume was calculated to attain the most effective IJs concentration, 100 IJ/cm^2, in Petri dishes which had an area of 9.5 cm^2 [10]. Control applications were spread evenly over the area of the soil sample. Immediately after sample treatment, twenty waxed

moth larvae, purchased from BestBait (Marblehead, OH) were counted into sets of 20 and applied to the soil. To promote nematode activity, post-treatment soil samples were stored at 24-26°C [25]. Mortality was documented at three time intervals: 24, 48, and 72 hours post-application. Among other observations, the change in body color were noted. Cadavers and live larvae were exhumed at each interval for accurate counting and then placed back so infective juveniles could continue to leave the host and invade other living larvae.

2. Data analysis

Color was qualitatively categorized, so statistical analysis focused on the mortality data between positive versus water controls; grafted versus non-grafted; and conventional versus organic trees. Resulting graphs and statistical analyses were conducted using Microsoft Excel (Microsoft Suite, v.2207) and Statistica v.13 (TIBCO Software Inc., Palo Alto, CA. To compare between two groups (e.g. grafted trees from E Block vs grafted trees from X Block), I ran two-tailed T-tests in Excel. To compare multiple groups, (e.g. grafted and ungrafted trees from E Block and X block versus unmanaged soil), I ran analyses of variance (ANOVA) with a post-hoc multiple comparison test in Statistica. Prior to these tests, the data, which was processed as proportions, was Arcsine-transformed.

Results

1a. Wax Moth Larvae Mortality: X block

All samples from grafted and non-grafted sites treated with *S. riobrave* resulted in 100% mortality within 24 hours (Fig. 1). Of these dead larvae, 88.75% attained a 'black' color and only 11.25% became 'tan' 72 hours post-treatment (Fig. 1).

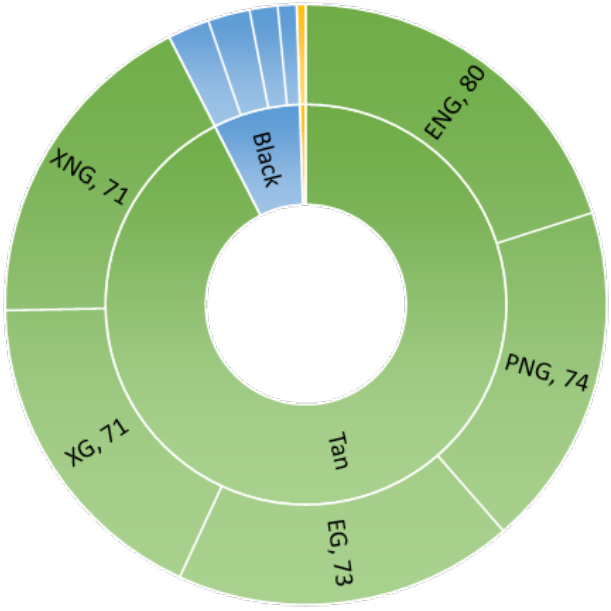

Figure 1. Positive Control Color Distribution. Detailing the entire color distribution for each block sample set with positive control applied. 'Tan'-colored was the majority of recorded cadavers. 'Black' was found in every sample set aside from ENG. 'Other' had cadavers only from Plum Brook-Block.

All samples from grafted and non-grafted sites treated with water showed significantly lower rates of mortality over the measured time when compared to the samples treated with *S. riobrave* (Single-tailed T-test; T_7=1.895, $p < 0.001$) (Fig. 2). At 72 hours, sampled grafted sites ranged from 0-7 dead larvae ($\bar{x} = 4 \pm 2.55$ S.D.) while sampled non-grafted sites ranged from 2-6 dead larvae ($\bar{x}= 3.75 \pm 1.48$ S.D.). These samples both saw dead larvae to be 83.87% 'black' and 16.13% 'tan' (Fig. 3).

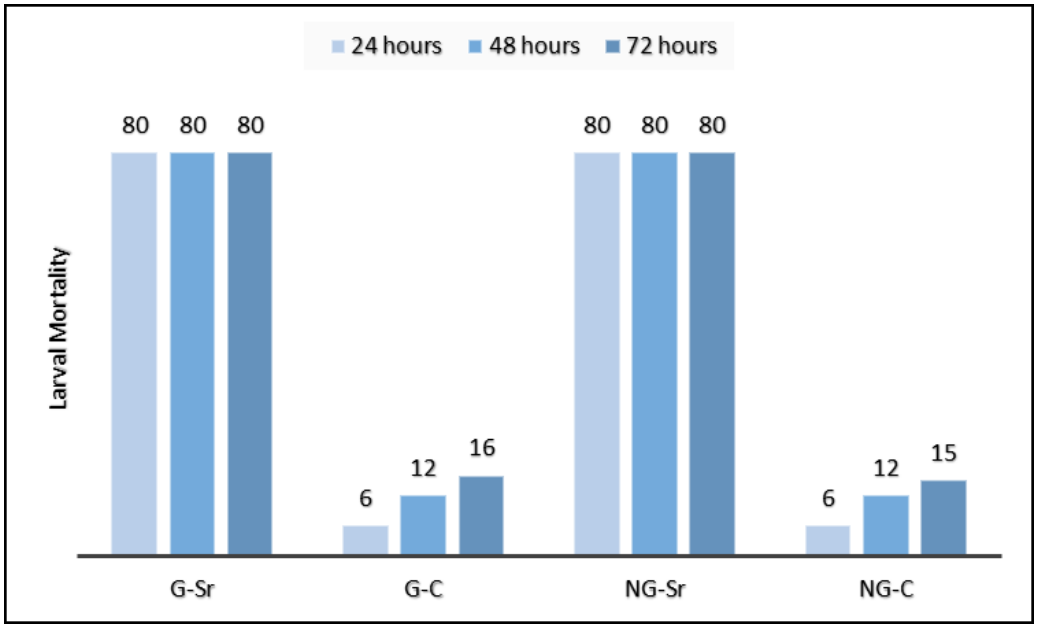

Figure 2. Mortality in Managed Block (X). Larvae mortality rates from X block. Sum of every replication per each sample set (G-Sr, G-C, NG-Sr, NG-C) are quantified and their mortality rates are distributed over the set 24, 48, and 72 hour intervals. Color of larvae is not represented by this figure.

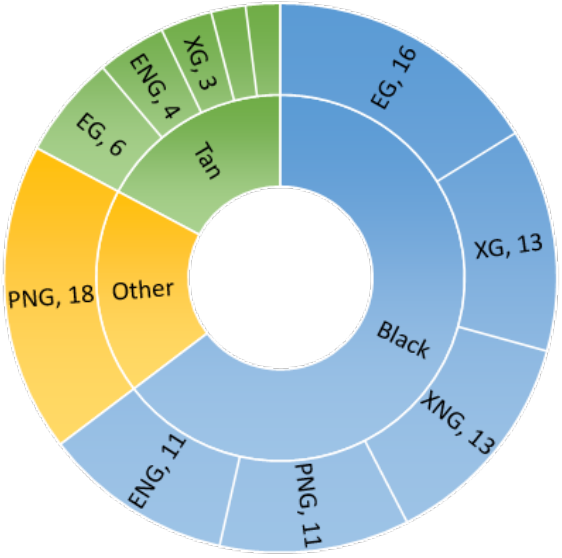

Figure 3. Water Control Color Distribution for Wax Moth larvae cadavers. Color distribution of all wax moth larvae cadavers by block's sample sets for water-treated soil samples. 'Black' was the leading color exhibited. Both 'Black' and 'Tan' were found in samples from each block.

2. Wax Moth Larvae Mortality in a Managed Orchard (E-Block)

All grafted and non-grafted sites sampled saw a collective 100% mortality within 48 hours when applied with the positive control, *S. riobrave* (Fig. 4). The distribution of color was 96.25% and 3.75% 'tan' and 'black' respectively at the final measured interval (Fig. 3).

Larvae mortality rates for non-grafted and grafted water control samples were still significantly lower than the positive controls (Single-tailed T-test; T_7=1.895, $p < 0.001$) [fig. 5]. Compared to non-grafted samples, grafted samples saw increased mortality rates: 133.33%, 150%, and 46.67% differences across the 24, 48, 72 hour intervals respectively (Fig. 4). At 72 hours grafted sites saw a range of 2-9 dead larvae ($\bar{x}$= 5.5, ± 2.5) and non-grafted sites had a range of 2-5 dead larvae ($\bar{x}$= 3.75, ± 1.09). At 72 hours these samples were composed of dead larvae 72.97% 'black' and 27.03% 'tan' , 0% 'other' was recorded (Fig. 2).

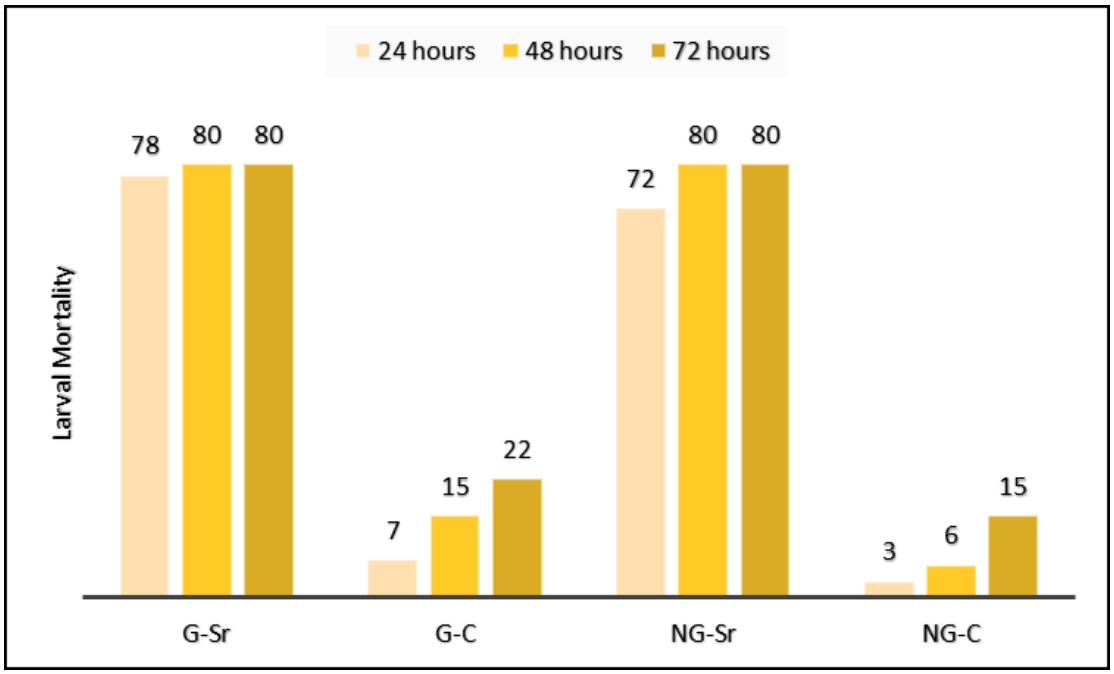

Figure 4. Mortality in Managed Block (E). Larvae mortality rates from E block. Sum of every replication per each sample set (G-Sr, G-C, NG-Sr, NG-C) are quantified and their mortality rates are distributed over the set 24, 48, and 72 hour intervals. Color of larvae is not represented by this figure.

3. Wax Moth Larvae Mortality in an Unmanaged Orchard (Plumbrook)

Non-grafted-tree positive control samples collectively saw a 97.5% mortality rate within 24 hours, climbing to 100% over 72 hours (Fig. 5). The distribution of color consisted of 'Tan' at 92.5% and 'Black' at 7.5% of dead larvae (Fig. 3).

Larvae mortality rates for the water control sample set were significantly lower in comparison to the positive control, (Single-tailed T-test; T_3=2.353, $p < 0.001$) (Fig. 5). At 24 hours, larvae mortality was recorded at 7 (Fig.5). At 48 hours, the larvae mortality increased by 314.29% to 29 (Fig. 5). At 72 hours larvae mortality increased by 6.9% to 31. The control sample

set saw a range of 6-11 dead larvae ($\bar{x}$= 7.75, ± 1.92) at 72 hours. At 72 hours 'black' accounted for 35.48% of dead larvae, 'tan' accounted for 6.45%, and 'other' accounted for 58.06%. Only trials: P-1-C, P-2-C, and P-4-C displayed these 'other' colored larvae (Fig. 2).

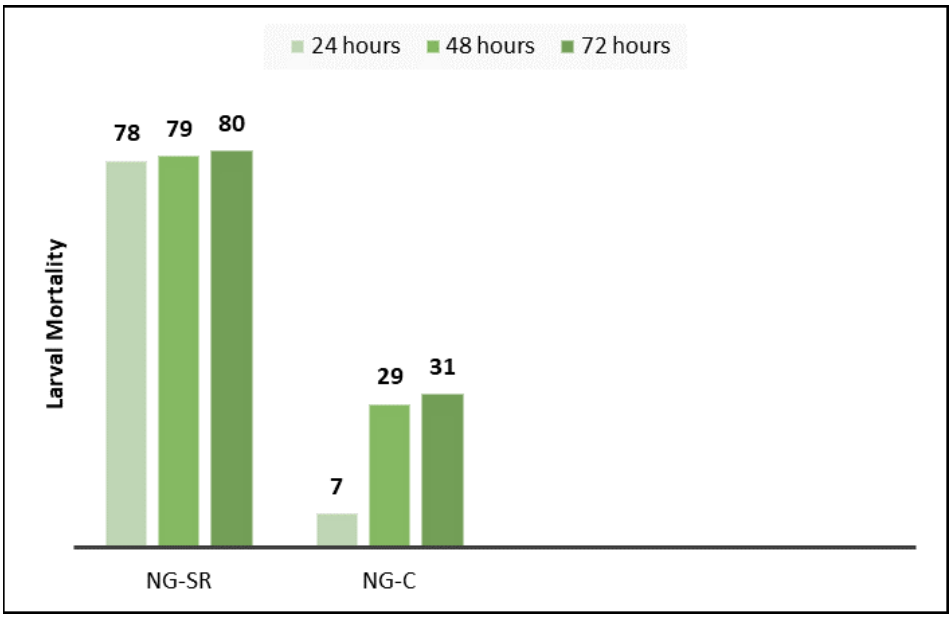

Figure 5. Mortality in Unmanaged Block (P). I compared larvae mortality rates from P-block. The sum of every replication per each sample set (NG-Sr and NG-C) are quantified and their mortality rates are reported 24, 48, and 72 hours post-treatment.

4. Grafted and Non-grafted Comparison

Between grafted samples applied with the water control and non-grafted samples applied with the water control, I did not observe a significant difference between mortality rates of the grafted and non-grafted sites sampled (Two-tailed T-test; T_{14}=0.925; p = 0.37) at 72 hours.

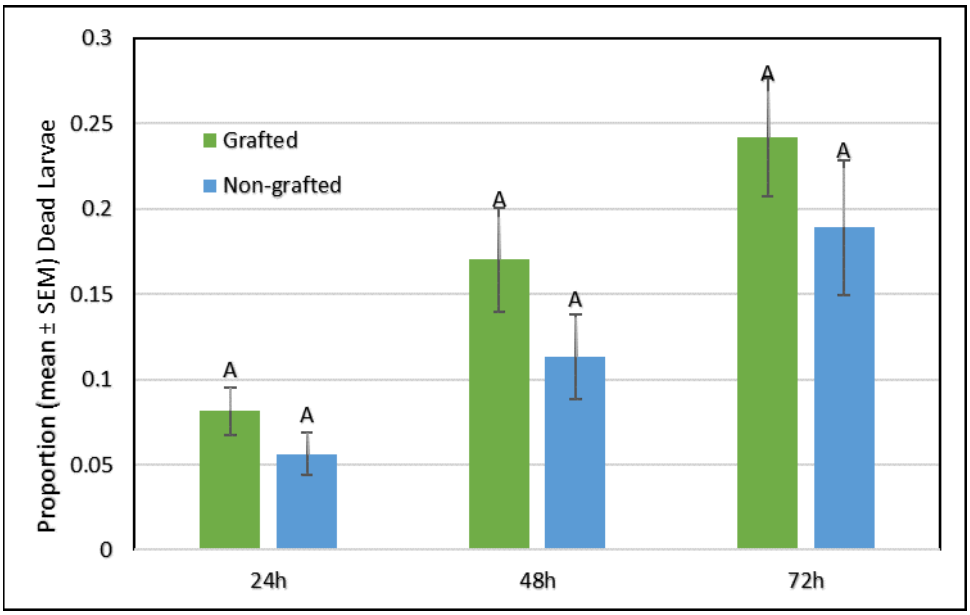

Figure 6. Mortality Comparison Between Grafted and Non-Grafted Sites. I compared the proportion of wax moth mortality in water-treated samples from non-grafted and grafted sites of the UMass Cold Spring Orchard. Grafting type mortality rates are shown for 24, 48, and 72 hours post-treatment. Standard errors are shown by 'whiskers'. Different letters represent grouped data with statistically significant differences between groups.

5. Managed vs. Unmanaged

Non-significant differences in mortality rates were recorded for water control grafted samples from the managed blocks X and E (Two-tailed T-test; T_6=2.447, p = 0.494) at 72 hours.

Mortality rates of larvae associated with non-grafted samples treated with water control found significantly higher mortality in the unmanaged orchard (Plumbrok, = P-block) at 48 hours (ANOVA; F_9=9.537, $p < 0.05$) as well as at 72 hours (ANOVA; F_9= 6.619, $p < 0.05$) when compared to larval mortality recorded in the two managed blocks (X and E).

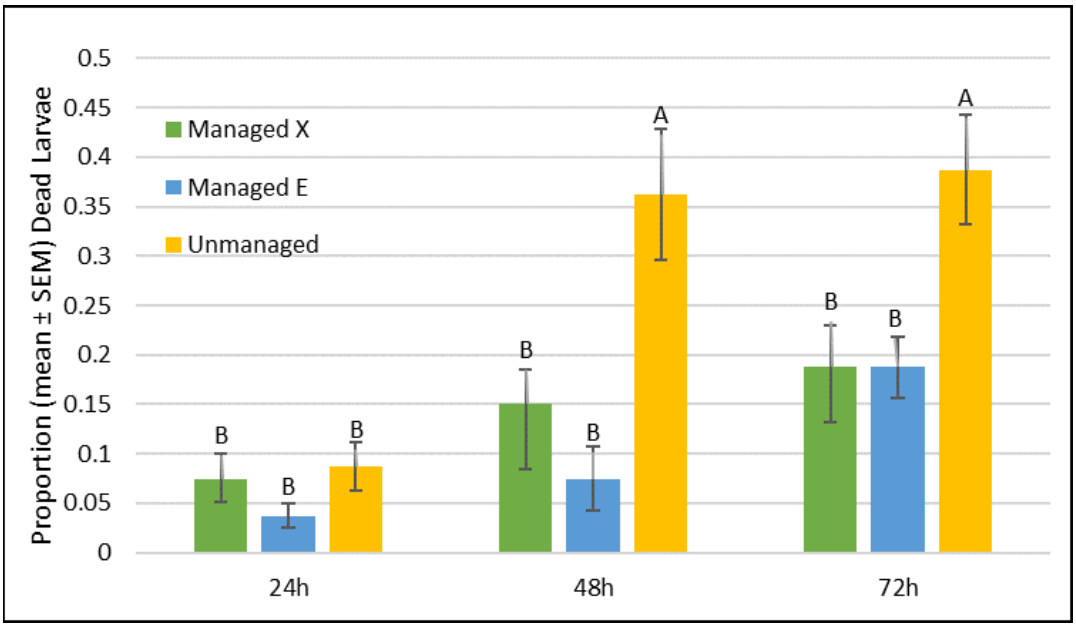

Figure 7. Comparison of Mortality Between Managed and Unmanaged Sites in Massachusetts. I compared the proportion of wax moth mortality in water-treated samples from X-block, E-block, and P-block. Block mortality rates are shown for 24, 48, and 72 hours post-treatment. Standard errors are shown by 'whiskers'. Different letters represent grouped data with statistically significant differences between groups.

Discussion

When compared to the positive control (which demonstrated rapid and complete wax moth larvae mortality using *Steinernema riobrave*), there were low to moderate mortality levels in the water-applied samples from trap trees in X, E, and P blocks. These water control samples also saw maximum mortality rates at 72 hours, compared to the positive controlled samples which had already reached peak mortality (where all larvae had died) at 24 hours. Such fluctuation could be attributed to low population levels of entomopathogenic predators. It should be noted that EPNs specifically tend to go through multiple generations in a host before infective juveniles leave and seek a new host [18].

I expected higher mortality levels from grafted trees when compared to non-grafted trees, but did not detect significant differences between the two. This may be because the grafted branches (which are expected to aggregate PCs) have only begun producing fruit in 2020, and a strong PC response was only documented in 2021. Therefore, at present time (2022), it is likely that the PC aggregation has not yet promoted the growth of natural populations of EPNs and other microorganisms.

My comparison of larval mortality between non-grafted and unmanaged trees revealed that, in water-applied samples, the unmanaged block (P-block) exhibited significantly more mortality than the managed blocks X or E after 48 hours, and this trend remained true 72 hours post-treatment. Higher larvae mortality levels may be indicative of higher population density of EPNs or the presence of other, lethal entomopathogenic predators. Key differences between

these blocks would be that the managed block (P-block) is unmanaged and surrounded by an organic orchard. These factors may promote the growth of EPNs or other predators of soil-dwelling pests. Notably, P-block was the main block that exhibited 'other' colors in the wax moth cadavers.

It is important to recognize that wax moth cadavers were collected and counted after the last interval of 72 hours. However, this may not have allowed ample time for entomopathogenic predators to attack the host and reproduce, as most baiting procedures dictate that you should check every 2-3 days for wax moth larvae cadavers [26]. However, I stopped collecting data at the same time that cadavers had begun to decompose and were difficult to distinguish. Future studies should take note of this fact to plan for cadaver retrieval and counting. For most of the water control samples, peak mortality was observed at the final interval recorded and sharp increases could be noted. All these results suggest that entomopathogenic species may have: 1. Different life cycles, 2. Different effects on the host and 3. Different lethality rates.

Color is an important indicator of different bacterial symbionts which are mutualistic to specific species of EPNs [26]. Compared to the positive control of applied *Steinernema riobrave* which exhibited the prescribed 'tan', water controls exhibited mostly 'black' and 'other' colors. However, shades of color may also be indicative of different bacterial symbionts and thus different species of EPNs, so the categories 'tan', 'black', and 'other' may have been too limiting and under-represented diversity of EPN populations in the water applied sample set [26].

At the time that the soil samples were collected (June 13th), PC activity had already begun around May 20th (based on degree day models for the season) but not many oviposited fruits had yet fallen. Further, given that entomopathogenic predators can remain in the host for 1-4 weeks, relying on reserves of bacteria cells and processed host tissue, it is possible that soil samples were collected too early to capture sufficient free living infective juveniles that had infected and emerged from new hosts [27]. It is also possible that my data collection occurred before the EPN population levels could adequately increase with the sudden increase in available host, under-representing EPN predators in the soil [23]. This notion was supported by the low mortality levels in water-controlled soil samples 72 hours after translocation of wax moth larvae acting as abundant health prey.

Failure to accurately represent the actual population structure of EPNs could be in part due to inadequate soil sampling and baiting procedures. While it is suggested that PC larvae remain and congregate at a maximum depth of 8-10 cm, due to lack of overwhelming PC larvae present in soil at the time of sampling, EPNs could have been distributed deeper in the soil. To capture the most representative EPN populations soil should have been examined at a depth of at least 15 cm, with 3 sub-samples taken [26]. To best promote EPN interactions with insect bait, Orozco, et. al (2014) suggested that containers are flipped upside down to ease the movement of EPN towards insect baits with the aid of gravity, that the samples are to be kept in darkness, and that insect cadavers are to be removed and replaced with healthy living insect baits [25]. These methods conflict with my own, which were devised for ease of data acquisition and to heighten the probability of spreading EPN infection spread.

Another limitation to this study was the presence of saprophytic flies. An unidentified species of flies infested the experimental wax moth larvae, potentially introducing a confounding variable that could have affected larval mortality rates, observed cadaver color, and possible causes of mortality.

Conclusion

My results indicate that, two years after the initial grafting year, grafted trap trees still do not show heightened mortality for soil-dwelling larvae when compared to non-grafted trees. This finding can only be exclusively applied to grafted trap trees of western Massachusetts and must be replicated across the region for a conclusive correlation between trap trees, which are expected to concentrate soil-dwelling pests, and the presence of soil-dwelling predators. Significantly greater levels of wax moth larval mortality was recorded in soil from unmanaged trees compared to managed orchards.

Further research is needed to better understand how farm practices may affect EPN populations as there was a significant difference between the samples from an organic and unmanaged orchard compared to a conventional and maintained orchards. Isolated EPN populations must be identified through classical and modern means to aid in piecing together the soil ecology of western Massachusetts.

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