Combining Mutualistic Yeast and Pathogenic Virus - a Novel Method for Codling Moth Control

Running title: Codling moth yeast and granulovirus

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Abstract

The combination of a pathogenic virus and mutualistic yeasts isolated from larvae of codling moth *Cydia pomonella* is proposed as a novel insect control technique. Apples were treated with codling moth granulovirus (CpGV) and either one of three yeasts, *Metschnikowia pulcherrima*, *Cryptococcus tephrensis* or *Aureobasidium pullulans*. The combination of yeasts with CpGV significantly increased mortality of neonate codling moth larvae, compared with CpGV alone. The three yeasts were equally efficient in enhancing the activity of CpGV. The addition of brown cane sugar to yeast further increased larval mortality and the protection of fruit against larvae. In comparison, without yeast, the addition of sugar to CpGV did not produce a significant effect. A field trial confirmed that fruit injury and larval survival were significantly reduced when apple trees were sprayed with CpGV, *M. pulcherrima* and sugar. We have shown earlier that mutualistic yeasts are an essential part of codling moth larval diet. The finding that yeast also enhances larval ingestion of an insect-pathogenic virus is an opportunity for the development of a novel plant protection technique. We expect the combination of yeasts and insect pathogens to essentially contribute to future insect management.

Keywords

Plant-microbe-insect-interaction, herbivory, mutualism, chemical communication, semiochemicals, apple, granulovirus

Introduction

Microorganisms interface insects and other animals with plants. In parallel with rapidly expanding research on the human microbiome (Fierer et al., 2012) and aided by more powerful and more affordable molecular tools, insect-plant-microbe interactions are a current research focus (Anderson et al., 2012; Berendsen et al., 2012; Raman et al., 2012; Vasquez et al., 2012, Davis et al., 2013). Few attempts have been made to bring this knowledge to application in plant protection, although the role of microbial mutualists and symbionts in insect ecology and evolution has long been recognized, for example in bark beetles (Farrell et al., 2001; Mueller et al., 2005).

Larval feeding of codling moth, *Cydia pomonella* (L.) (Lepidoptera, Tortricidae) in the flesh and core of pome fruits is a major factor affecting the design of integrated pest management programs in apple and pear. Although public concern and regulatory actions drive the adoption of environmentally safe technologies, few biological methods are available for codling moth control, besides phermone-mediated mating disruption (Knight, 2008; Witzgall et al., 2008)

Cydia pomonella granulovirus (CpGV) has received considerable attention as a microbial insecticide, owing to its specificity for codling moth and its safety to nontarget organisms. CpGV has been registered in Europe and the US and is used on 150,000 ha annually (Cross et al. 1999, Lacey and Shapiro 2008, Lacey et al. 2008). However, CpGV is not efficient enough to be used as a stand-alone control method against codling moth and is therefore often combined with mating disruption.

Unfortunately for pest managers, *C. pomonella* oviposits on or adjacent to fruit and neonates do not actively feed prior to cutting a hole through the skin of the fruit. Once inside the fruit, codling moth larvae are physically isolated from subsequent management action (Hoerner, 1925; Gilmer, 1933; Jackson 1979). This larval behavior makes codling moth a difficult pest to manage with CpGV, which requires ingestion to be effective (Jacques et al., 1987). While CpGV is highly virulent to *C. pomonella* larvae when mixed with artificial insect diet, practical field use is limited by short residual life, owing to the susceptibility of the virus to UV light (Lacey et al., 2007).

Efforts to improve the performance of CpGV have attempted to increase virus exposure and ingestion through attractants and feeding stimulants, before larvae penetrate the fruit (Lacey et al., 2007, 2008; Ballard et al. 2000a,b). However, plant volatiles which attract codling moth neonates (Sutherland and Hutchins, 1972; Knight and Light, 2001) or larval feeding stimulants, molasses or sugars, have shown only limited or no effect (Ballard et al., 2000b; Arthurs et al., 2007; Schmidt et al., 2008; Light and Knight, 2011).

The recent discovery that codling moth larvae are associated with yeasts has renewed the interest in developing larval attractants for codling moth management. The yeasts, *Metschnikowia andauensis*, *M. pulcherrima* and *Cryptococcus tephrensis*, and the yeast-like fungus *Aureobasidium pullulans* were isolated from field-collected codling moth larvae in Washington State, USA and Scania, Sweden (Witzgall et al., 2012). These microorganisms are commonly found in the phyllosphere of unsprayed fruit crops (Schisler et al., 2010). Interestingly, codling moth yeasts and related species have been studied and some commercialized as biocontrol agents for plant pathogens (Sharma et al., 2009).

Phylloplane microorganisms also influence insect behavior. Oviposition by European corn borer females on maize is deterred by an epiphytic yeast, *Sporobolomyces roseus* (Martin et al., 1993), whereas adult codling moths are attracted to yeast volatiles (Witzgall et al., 2012). Lepidopteran larvae, including codling moth larvae, are known to respond to plant volatiles and sex pheromones (Sutherland and Hutchins, 1972; Knight and Light, 2001; Becher and Guerin, 2009; Piesik et al., 2009; Poivet et al., 2012). However, yeasts as attractants or feeding stimulants for insect larvae have not been investigated.

In the laboratory, yeast was isolated from codling moth larvae feeding in apples only when the apple surface was inoculated with yeast; on surface-sterilized apples, yeast was not found in larval feeding galleries (Witzgall et al., 2012). In the field, *C. pomonella* larvae wander on the surface of fruits for up to a few hours before penetrating the skin of the fruit (Hall, 1934). These observations, taken together with the importance of yeast for codling moth larval survival and growth (Witzgall et al., 2012), points towards a possible role of volatile yeast metabolites in larval behavior and consequently raises the question whether yeasts could enhance the use of insect pathogens for management of codling moth.

Herein, we report laboratory and field studies on the use of yeasts associated with codling moth in combination with CpGV. We found that the addition of yeasts to CpGV enhanced larval mortality and improved protection of fruit against larval infestation. The combined use of yeast-based attractants or feeding stimulants and insect pathogens is proposed as a novel insect control technique.

Materials and methods

Laboratory bioassays

‘Red Delicious’ apples without codling moth injury were picked on August 2011 from an unsprayed orchard situated 15 km east of Moxee, WA (46° 33’N, 120° 20’W) at the USDA Research Farm. Apples, prior to use in the bioassays, were sterilized with 5% NaOH in a 3-L beaker for 25-30 min. Apples were then dried and rinsed with 70% ethanol and again dried with a paper towel. Apples were rinsed one final time with distilled water and air-dried under a hood.

The following microorganisms, isolated from codling moth larvae, were used: *Metschnikowia andauensis* Molnár & Prillinger, *M. pulcherrima* Pitt & Miller (Ascomycota, Saccharomycetes), *Cryptococcus tephrensis* Vishniac (Basidiomycota, Tremellomycetes), *Aureobasidium pullulans* (de Bary) Arnaud (Ascomycota, Sordariomycetes). They were grown on YPD medium (YPD Broth Mix, Research Products International, Mt. Prospect, IL; ph 6.5 to 7) containing 10 g yeast extract, 20 g dextrose and 20 g peptone/L with purified water (18.2 ohms). Broth medium was autoclaved for 20 min at 121°C. Broth (100 mL) was poured into 250 mL sterilized flasks and inoculated with one colony forming unit (1-2 mm diameter; grown on YPD agar plates), and placed on an incubating shaker for 48 h at 25°C. The content of the flask was transferred to a 1-L beaker and the total volume was increased to 500 mL with the addition of purified water.

Treatments prepared for the laboratory studies included a water control; cane sugar, yeasts, and CpGV applied alone; CpGV plus sugar; CpGV plus yeast; and CpGV plus sugar and yeast. Cane sugar (C&H Dark Brown Cane Sugar, Domino Foods, Yonkers, NY) was tested at 1.2 or 3.6 g/L. Yeasts were tested at these same two rates. The density of yeast cells was estimated with a hemacytometer (EMS, Hatfield, PA) to be between 6 x 10^7 and 10^8 cells/mL at the higher rate of yeast tested. CpGV (Cyd-X, Certis, Columbia, MD) was tested at a single rate, 3.8 x 10^7 occlusion bodies/L (39.1 µl Cyd-X/500 mL).

Bioassays were conducted with sterilized apples dipped five times into a 500 mL treatment solution and placed on a paper towel in a fume hood to dry. A single gelatin capsule (8.0 mm diameter, Snap-fit, Ted Pella Inc., Redding, CA) was attached with paraffin to the shoulder of each fruit. One end of the capsule was cut-off with a razor blade and the second part of the capsule was then slid over the cut end. One black-headed codling moth egg on a < 15 mm^2

piece of wax paper was placed inside each gelatin capsule next to the fruit. Apples were placed inside of 350 ml clear plastic cups and closed with a lid. Cups were placed in a room maintained at 25^{o}C and a 18:6 L:D photoperiod for 14 d. The larva was collected from each apple, scored as alive or dead, and its location on the surface or the depth of its penetration into the fruit was measured. Each replicate included ten gelatin capsule assays. The number of replicates for each treatment varied among experiments and ranged from four to ten.

Field study

A completely randomized field study with five single-tree replicates of four treatments was conducted at the USDA Research Farm (Yakima, WA) during 2012. The test orchard was a 0.4-ha block of apples (cv. Delicious) planted on 3.7 x 5.5 m within and between row spacing with 500 trees/ha. Mean tree canopy height was 2.7 m. Replicates were separated by 10 m. Treatments included an untreated control; a grower standard of three sprays of chlorantraniliprole (Altacor, 350 g AI/kg WG, E.I. Dupont de Nemours Co.), on June 15 and 29 and on July 13, and three sprays of spinetoram (Delegate, 250 g AI/kg WG, Dow AgroSciences, Indianapolis, IN) on July 27 and August 13 and 27; CpGV (Cyd-X, 3 x 10^{13} occlusion bodies/L) applied alone and with *M. pulcherrima* and cane sugar on June 15, 22, and 29, July 6, 13, and 27, August 7 17, and 27 and on September 6. CpGv was applied at 3.8 x 10^{7} occlusion bodies/L (78.0 µl Cyd-X/L). The rate of CpGV was increased 3-fold on the last three spray dates to accomodate for the increased canopy volume during late season. Both sugar and *M. pulcherrima* were applied at 3.6 g/L. The treatment concentrations of insecticides were 0.12 g AI/L for spinetoram and 0.10 g AI/L for chlorantraniliprole.

Sprays were applied with a gasoline-engine powered, diaphragm-pump sprayer (Rear's MFG, Eugene, OR) at 689 kPa with a handgun sprayer equipped with a D-6 nozzle (GunJet, Model 43, TeeJet Technologies, Wheaton, IL). Sprays applied through July 13 were applied at 1.2 L/tree (623 L/ha). This spray rate was increased to 1.9 L/tree (935 L/ha) beginning on July 27 to improve coverage. The untreated control trees were not sprayed.

All fruits were picked from trees on September 21 and stored at 2^{o}C for up to 1 month until inspected for injury. Fruit were scored as free of injury (clean) or injured by *C. pomonella*, tortricid leafroller larvae, or San Jose scale, *Quadraspidiotus perniciosus* (Comstock). Leafroller fruit injury was presumed to be caused by *Pandemis pyrusana* Kearfott, which was trapped in large numbers in kairomone-baited traps during the season. A subsample of 30 codling moth-injured fruits was selected for further dissection to score larvae as live or dead in each replicate. Fewer fruits (2 to 9) were inspected from replicates treated with the standard insecticide program due to the low level of fruit injury in this treatment. Larval exit holes in fruit were scored as live larvae. Dead larvae were scored as having caused a 'sting' or an 'entry' depending on the extent of its feeding in the fruit; stings not penetrating further than 4 mm into the mesocarp of the fruit were defined as a dead larvae.

Statistical Analyses

Numbers and proportions were transformed to square root and arcsine (square root), respectively, prior to analysis of variance (ANOVA). Data were analysed with the Shapiro-Wilks test of normality (Statistix 9, Analytical Software, Tallahassee, FL). Data which were not able to be normalized with these transformations were analysed with the non-parametric Kruskal-Wallis ANOVA of ranks. Means were separated in significant ANOVA's at $P < 0.05$.

Results

Laboratory bioassays

The addition of each of the three yeasts *C. tephrensis*, *A. pullulans* or *M. pulcherrima* to CpGV, at 1.2 and 3.6 g/L, significantly increased codling moth larval mortality in apples, compared to treatments with CpGV alone. Treatment of apples with CpGV alone produced significantly higher levels of larval mortality than the water control. The addition of cane sugar at either rate to CpGV did not increase the proportion of larval mortality (Figure 1).

The difference in larval mortality between the three yeasts or different concentrations of yeast was not significant. The combination of CpGV with sugar (1.2 g/L) and either *C. tephrensis or A. pullulans* at 3.6 g/L significantly increased the proportion of dead larvae, over CpGV alone. All larvae were killed with a combination of CpGV, *C. tephrensis* and cane sugar (Figure 1). Increasing the rate of sugar to 3.6 g/L increased larval mortality only slightly and this difference was not significantly different from the lower rate of sugar (data not shown). No direct toxic effects (mean proportional mortality < 0.10) against larvae were found in assays in which fruit was dipped in water, in aqueous solutions of two rates of sugar, or in two rates of each of the yeasts.

Field trial

High levels of fruit injured by *C. pomonella*, the tortricid leafroller *Pandemis pyrusana* Kearfott and San Jose scale *Quadraspidiotus perniciosus* (Comstock) occurred in the experimental orchard in 2012 (Table 1). The CpGV treatment did not significantly reduce the level of codling moth fruit infestation, compared with the untreated control. Similarly, numerically lower, but not significant reductions in fruit injury from leafrollers and scale occurred in the CpGV treatment compared to the untreated control. The addition of *M. pulcherrima* and sugar to CpGV did not reduce fruit injury from *C. pomonella* relative to the CpGV treatment; however, injury was significantly lower than in the untreated control (Table 1).

Similarly, adding *M. pulcherrima* and sugar to CpGV did not reduce leafroller or scale injury compared with the CpGV alone treatment, but these injury levels were significantly lower than those in the untreated control. The level of *C. pomonella* injury in the conventional insecticide treatment was substantially lower than in the untreated or CpGV treatments. The level of leafroller and San Jose scale injury in the insecticide treatment was not different from the CpGV plus sugar and yeast treatment, but significantly lower than CpGV alone and the untreated control (Table 1).

Significant differences were found among treatments in the relative proportion of dead and live larvae in injured fruit (Table 2). The proportion of dead larvae was highest in the CpGV plus the yeast and sugar treatment and lowest in the untreated control. Similarly, the lowest proportion of live larvae remaining in the fruits was found with the addition of yeast and sugar to CpGV. The distribution of dead larvae (stings or entries) differed between CpGV alone and with the yeast and sugar added to CpGV. The proportion of stings was similar, but the addition of the yeast and sugar resulted in a higher proportion of dead larvae scored as entries than with CpGV alone. The untreated control and the CpGV alone treatments had the highest proportion of fruits with exit holes (Table 2).

Discussion

We have recently shown that codling moth, a typical insect herbivore, is associated with yeasts, but did not fully recognize the importance of these mutualistic yeasts for insect management. The behavioral response of adult moths suggested the prospective use of yeast volatiles as attractants, for flight detection and population monitoring, or mass-trapping (Witzgall et al., 2012).

Since yeast strongly enhances larval fitness, it is conceivable that not only adults but also larvae sense and respond to yeast metabolites. This is indeed the most plausible explanation for the results obtained in laboratory (Figure 1) and subsequent field tests (Tables 1, 2), showing that the efficacy of CpGV can be enhanced by yeasts which are associated with codling moth.

The laboratory assay demonstrates that yeast significantly augmented the efficacy of the virus treatment (Figure 1). In the orchard, spraying a combination of CpGV, yeast and sugar significantly increased larval mortality and decreased fruit damage, compared to control (Table 1). Larval mortality was significantly higher with the virus, yeast and sugar treatment, compared to virus alone (Table 2). Low population densities and fruit infestation rates impede experimentation in commercial orchards and future work aims at the development of field formulations on a larger scale.

Biological control comprises three sectors: beneficials, pathogens and semiochemicals. The release of natural enemies mainly concerns greenhouse environments, while pathogens and semiochemicals are used in field crops and orchards (Witzgall et al. 2010, Chandler et al. 2011). Attempts to combine pathogens and semiochemicals into attract-and-kill techniques have not been very fruitful, because adult insects, which are targeted by commercially available semiochemicals, are not sufficiently susceptible to pathogens. Larvae, on the other hand, which are more susceptible to pathogens, cannot easily be manipulated with plant semiochemicals on their food plants. Manipulation of larval behavior for improved exposure to pathogens becomes now possible through combined use of pathogens with mutualistic yeasts that co-occur in close association with insect larvae.

Enhancing the effect of codling moth granulovirus (CpGV)

The key biological factor that constrains the effectiveness of CpGV is the uptake by codling moth larvae before they penetrate the fruit. However, combinations of CpGV with feeding stimulants and larval attractants has not decisively improved performance (Arthurs et al., 2007; Lacey et al. 2008, Ballard et al. 2000a,b).

Host plant volatiles, such as pear ester (*E*,*Z*)-2,4-decadienoate and (*E,E*)-α-farnesene, which attract codling moth neonates, appear to mediate host location rather than feeding (Sutherland and Hutchins, 1972; Knight and Light, 2001; Hughes et al., 2003; Light and Beck, 2010, 2012). Addition of α-farnesene slightly improved the effect of CpGV (Ballard et al. 2000b), pear ester reduced injury in walnuts (Light and Knight, 2011), but not in apple and pear (Arthurs et al., 2007; Schmidt et al., 2008).

Larval feeding stimulants, such as molasses, sugars and non-nutritive sugar substitutes including monosodium glutamate, have also been combined with CpGV (Ballard et al., 2000b). Field applications of sugary adjuvants with CpGV may promote the growth of native phylloplane yeasts or other microbes, and consequently ingestion of the virus. High rates of molasses and sorbitol reduced the incidence of fruit entries, but induced also secondary infections with sooty mould *Cladosporium spp*. (Ballard et al., 2000b).

Yeast and codling moth larval behavior

We currently are investigating the question of how yeasts, in combination with host plant cues, affect the behavior of codling moth larvae, and whether volatile or non-volatile cues, or both, are involved. The high mortality of the yeast-virus combination in our laboratory assay (Figure 1) suggests that yeasts on apple elicited larval feeding, and possibly also larval attraction.

Plant volatiles play an important role in adult codling moth reproductive behaviour (c.f. Trona et al., 2013) and effect also larval behavior (Sutherland and Hutchins, 1972; Landolt et al., 1999; Knight and Light, 2001; Jumean et al., 2005). Merely the expression of a subset of olfactory receptors genes in larval olfactory sensory neurons (Fishilevich et al., 2005; Kreher et al., 2005; Poivet et al., 2013) suggests some similarity in the responsiveness of adult and larval antennae to odorants. This is conceivable, since insect females and larvae locate the same food source for oviposition and feeding, respectively. In addition to volatile compounds, apple fruit and leaf sugars stimulate codling moth oviposition (Lombarkia and Derridj, 2008) and non-volatile apple or yeast metabolites may well stimulate larval feeding.

Ongoing research on the attractant and phagostimulatory effect of yeasts and host plant metabolites in codling moth larvae includes a comparative analysis of yeasts found with codling moth, *Metschnikowia* and *Cryptococcus*, in comparison with baker's yeast, *Saccharomyces cerevisiae*. The headspace of *Metschnikowia* yeasts contains several volatiles eliciting an antennal response in adult codling moths (Witzgall et al., 2012). The ongoing deorphanization of codling moth olfactory receptors (Bengtsson et al., 2012) will, after screening the expression of olfactory receptors in larvae, contribute to an identification of the compounds that guide the behavior of neonate larvae.

CpGV and codling moth management

Conventional apple and pear growers in the U.S. have recently transitioned from the use of broad-spectrum organophosphates to more selective classes of insecticides used in rotation to manage codling moth and to avoid selection for resistance. Unfortunately, growers have encountered new pests such as aphids, San Jose scale and phytophagous mites, because some of these new insecticides are still not sufficiently selective for pests and disrupt biological control (Martinez-Rocha et al., 2008; Jones et al., 2009).

Pest managers of European pome fruit orchards experience a different situation. Very few insecticides are available to the growers, which has accelerated resistance to the remaining products (Knight et al., 1994; Sauphanor et al., 2000). Integrated programs based on sex pheromone-mediated mating disruption, CpGV and reduced use of synthetic insecticides have been widely adopted to combat the evolution of resistance to the few available conventional insecticides (Charmillot and Pasquier, 2003). These programs are highly selective for codling moth and can minimize the secondary disruption of biological control agents (Lacey et al., 2008). Unfortunately, the failure to carefully manage the susceptibility of codling moth to CpGV has led to high levels of resistance and product failures in some regions (Fritsch et al., 2005; Sauphanor et al., 2006).

New strategies are needed to build sustainable programs based on CpGV and mating disruption. The finding that larval behavior can be manipulated with mutualistic yeasts to enhance exposure to or ingestion of an insect pathogen provides us with a new perspective of species-specific and sustainable control of codling moth.

Yeasts could perhaps also be combined with other killing agents. Several recently registered insecticides for control of codling moth are considered to be selective due to their reduced impact on biological control agents. *Bacillus thuriengiensis* does not provide efficient control of codling moth, but is used against other orchard insects, including noctuid and tortricid moths. Combinations of yeast and chemical insecticides or formulations of *Bacillus thuriengiensis* needs to be tested.

Orchard applications of yeast may, on the other hand, be constrained by the continual application of pesticides, including fungicides, during the season (Gildemacher et al., 2004). Yeasts are susceptible to pesticides (Slaviková and Vadkertiova, 2003; Walter et al., 2007) and differences in the diversity and abundance of yeast epiphytes have been found under apple spray programs characterized as organic or conventional (Granado et al., 2008). Residual effects of spray programs on the growth and survivorship of the yeasts need to be studied for the development of applied programs.

Conclusion

The combination of mutualistic yeasts, attracting larvae of associated insects for feeding, and a pathogenic virus is a novel insect control technique. Yeasts stimulate larval feeding and target ingestion of the virus. The method is environmentally safe and species-specific, through the choice of the virus.

Attract-and-kill techniques have earlier been designed to combine semiochemicals - for attraction of adult insects - with various killing agents, including insect pathogens. Using live yeast permits to target larvae, which is a novel and powerful approach.

Insect pathogens show promise for sustainable insect control, but need to become more efficient for widespread use. We expect this novel techniques of baiting insect-pathogenic viruses with insect-associated, attractant yeasts to essentially contribute to future insect control.

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Legend

Figure 1. Effect of codling moth *C. pomonella* granulovirus (CpGV) alone and in combination with cane sugar and yeasts, *Cryptococcus tephrensis*, *Aureobasidium pullulans* or *Metschnikowia pulcherrima* on codling moth larval mortality (%) on apples in the laboratory. CpGV was tested at 3.8×10^7 occlusion bodies/L, yeast and sugar were applied at two rates, 1.2 and 3.6 g/L (large and small circles), apples were dipped into treatment solutions. Controls (water and CpGV alone) shown in background, for each of three consecutive experiments (left to right). Column means within each experiment followed by a different letter were significantly different ($P < 0.05$).

Table 1. Mean proportion of fruit injury by codling moth (CM), leafroller (LR), and San Jose scale (SJS) in single tree plots (N = 5) of 'Delicious' in September 2012 following spray programs evaluating the effect of adding the yeast, *M. pulcherimma* (*Mp*) with brown cane sugar (S) to codling moth granulosis virus (CpGV) compared with an untreated control, CpGV alone, and a standard insecticide program.

	Mean (SE) proportion of fruit injury from		
Treatment [a]	CM	LR	SJS
Untreated control	0.48 (0.05)a	0.36 (0.04)a	0.45 (0.06)a
CpGV	0.34 (0.04)ab	0.22 (0.04)ab	0.34 (0.07)ab
CpGV + *Mp* + S	0.22 (0.03)b	0.10 (0.02)bc	0.15 (0.04)bc
Standard insecticide	0.02 (0.04)c	0.05 (0.05)c	0.09 (0.07)c
ANOVA:	$F_{3, 16} = 39.88$	$F_{3, 16} = 13.68$	$F_{3, 16} = 6.57$
Kruskal-Wallis	$P < 0.0001$	$P < 0.0001$	$P < 0.01$

Column means followed by a different letter were significantly different, $P < 0.05$.

[a] CpGV (78.0 µl Cyd-X/L) was applied alone and with *M. pulcherrima* and cane sugar ten times between June 15 and September 6, 2012. Three sprays of chlorantraniliprole (Altacor, 0.10 g AI/L) were applied between June 15 and July 13, and three sprays of spinetoram (Delegate, 0.12 g AI/L) were applied between July 27 and August 27, 2012. The rate of CpGV was increased 3-fold on the last three spray dates. Both sugar and *M. pulcherrima* were applied at 3.6 g/L. Sprays applied through 13 July were applied at 1.2 L/tree (623 L/ha). The spray rate was increased to 1.9 L/tree (935 L/ha) beginning on 27 July to improve coverage. The untreated control trees were not sprayed.

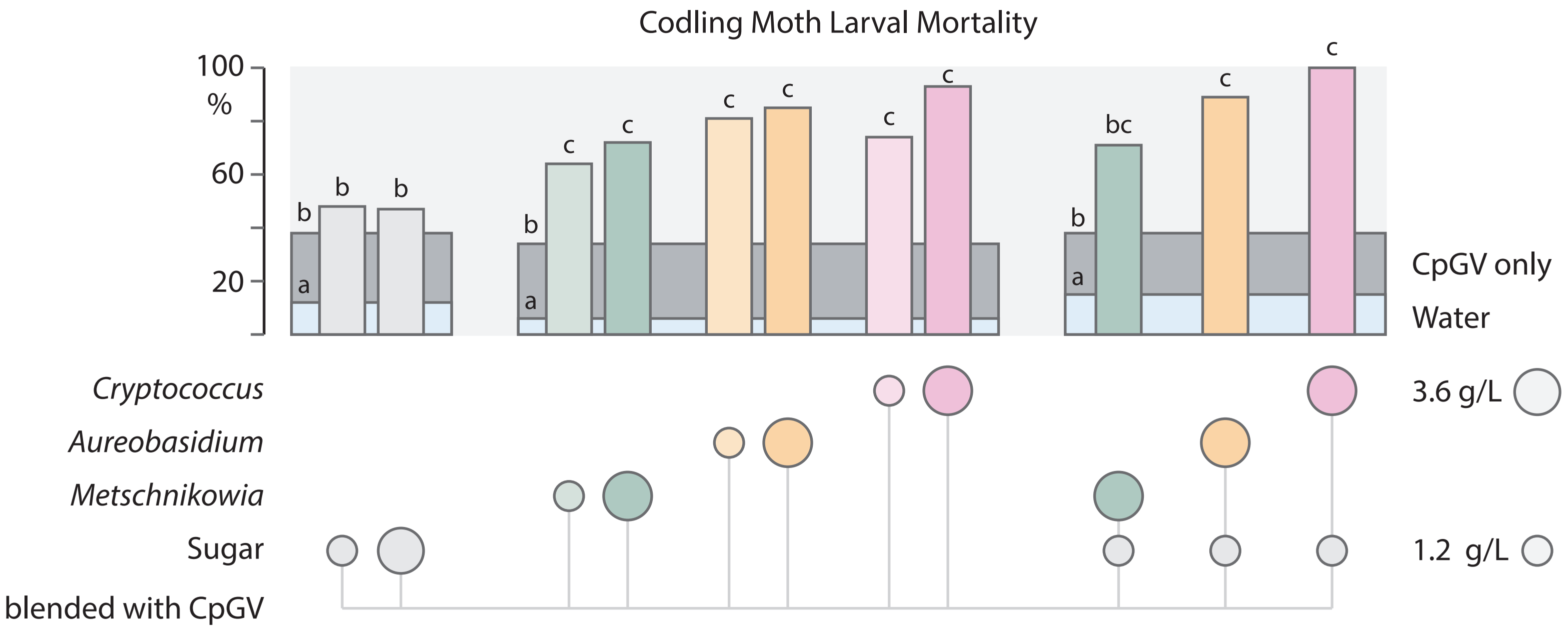
Table 2. Characterization of fruit injury by codling moth in single tree plots (N = 5) of ‘Delicious’ in September 2012 following spray programs evaluating the effect of adding the yeast, *M. pulcherima* (*Mp*) with brown cane sugar (S) to codling moth granulosis virus (CpGV) compared with an untreated control, CpGV alone, and a standard insecticide program.

Treatment [b]	Mean (SE) proportion CM injury [a]					
	Dead larvae			Live larvae		
	Sting	Entry	All	Entry	Exit	All
Untreated control	0.14 (0.03)b	0.00 (0.00)b	0.14 (0.03)b	0.41 (0.03)a	0.45 (0.04)a	0.86 (0.03)a
CpGV	0.39 (0.03)ab	0.04 (0.01)b	0.43 (0.04)b	0.19 0.02)b	0.38 (0.03)a	0.57 (0.04)a
CpGV + Mp + S	0.61 (0.03)a	0.20 (0.03)a	0.81 (0.02)a	0.04 (0.01)c	0.15 (0.01)b	0.19 (0.02)b
Standard insecticide	0.48 (0.18)ab	0.00 (0.00)b	0.48 (0.18)ab	0.30 (0.10)ab	0.22 (0.10)b	0.52 (0.18)ab
ANOVA: Kruskal-Wallis	$F = 5.18$	$F = 29.43$	$F = 10.98$	$F = 15.12$	$F = 8.55$	$F = 10.98$
df = 3, 16	$P < 0.01$	$P < 0.0001$	$P < 0.001$	$P < 0.0001$	$P < 0.001$	$P < 0.001$

Column means followed by a different letter were significantly different, $P < 0.05$.

[a] Dead larvae were scored as a ‘sting’ if the penetration was ≤ 4.0 mm or ‘entry’. Live larvae were scored as an ‘entry’ inside the fruit or ‘exit’ for larvae having left the fruits.

[b] CpGV (78.0 µl Cyd-X/L) was applied alone and with *M. pulcherrima* and cane sugar ten times between June 15 and September 6, 2012. Three sprays of chlorantraniliprole (Altacor, 0.10 g AI/L) were applied between June 15 and July 13, and three sprays of spinetoram (Delegate, 0.12 g AI/L) were applied three times between July 27 and August 27, 2012.). The rate of CpGV was increased 3-fold on the last three spray dates. Both sugar and *M. pulcherrima* were applied at 3.6 g/L. Sprays applied through 13 July were applied at 1.2 L/tree (623 L/ha). This spray rate was increased to 1.9 L/tree (935 L/ha), beginning on July 27 to improve coverage. The untreated control trees were not sprayed.



Codling Moth Larval Mortality
100
%
60
20
b
b
b
a
b
c
c
c
c
c
c
a
b
bc
c
c
a
CpGV only
Water
Cryptococcus
Aureobasidium
Metschnikowia
Sugar
blended with CpGV
3.6 g/L
1.2 g/L