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

3 **Running title:** Codling moth yeast and granulovirus

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18

## 18 Abstract

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

## 33 Keywords

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

## 36 Introduction

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

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

50 *Cydia pomonella* granulovirus (CpGV) has received considerable attention as a microbial  
51 insecticide, owing to its specificity for codling moth and its safety to nontarget organisms. CpGV  
52 has been registered in Europe and the US and is used on 150,000 ha annually (Cross et al.  
53 1999, Lacey and Shapiro 2008, Lacey et al. 2008). However, CpGV is not efficient enough to be  
54 used as a stand-alone control method against codling moth and is therefore often combined  
55 with mating disruption.

56 Unfortunately for pest managers, *C. pomonella* oviposits on or adjacent to fruit and neonates do  
57 not actively feed prior to cutting a hole through the skin of the fruit. Once inside the fruit,  
58 codling moth larvae are physically isolated from subsequent management action (Hoerner,  
59 1925; Gilmer, 1933; Jackson 1979). This larval behavior makes codling moth a difficult pest to  
60 manage with CpGV, which requires ingestion to be effective (Jacques et al., 1987). While CpGV  
61 is highly virulent to *C. pomonella* larvae when mixed with artificial insect diet, practical field use  
62 is limited by short residual life, owing to the susceptibility of the virus to UV light (Lacey et al.,  
63 2007).

64 Efforts to improve the performance of CpGV have attempted to increase virus exposure and  
65 ingestion through attractants and feeding stimulants, before larvae penetrate the fruit (Lacey et  
66 al., 2007, 2008; Ballard et al. 2000a,b). However, plant volatiles which attract codling moth  
67 neonates (Sutherland and Hutchins, 1972; Knight and Light, 2001) or larval feeding stimulants,  
68 molasses or sugars, have shown only limited or no effect (Ballard et al., 2000b; Arthurs et al.,  
69 2007; Schmidt et al., 2008; Light and Knight, 2011).

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

78 Phylloplane microorganisms also influence insect behavior. Oviposition by European corn borer  
79 females on maize is deterred by an epiphytic yeast, *Sporobolomyces roseus* (Martin et al.,  
80 1993), whereas adult codling moths are attracted to yeast volatiles (Witzgall et al., 2012).  
81 Lepidopteran larvae, including codling moth larvae, are known to respond to plant volatiles and  
82 sex pheromones (Sutherland and Hutchins, 1972; Knight and Light, 2001; Becher and Guerin,  
83 2009; Piesik et al., 2009; Poivet et al., 2012). However, yeasts as attractants or feeding  
84 stimulants for insect larvae have not been investigated.

85 In the laboratory, yeast was isolated from codling moth larvae feeding in apples only when the  
86 apple surface was inoculated with yeast; on surface-sterilized apples, yeast was not found in  
87 larval feeding galleries (Witzgall et al., 2012). In the field, *C. pomonella* larvae wander on the  
88 surface of fruits for up to a few hours before penetrating the skin of the fruit (Hall, 1934). These  
89 observations, taken together with the importance of yeast for codling moth larval survival and

90 growth (Witzgall et al., 2012), points towards a possible role of volatile yeast metabolites in  
91 larval behavior and consequently raises the question whether yeasts could enhance the use of  
92 insect pathogens for management of codling moth.

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

## 98 **Materials and methods**

### 99 *Laboratory bioassays*

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

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

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

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

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

### 133 *Field study*

134 A completely randomized field study with five single-tree replicates of four treatments was  
135 conducted at the USDA Research Farm (Yakima, WA) during 2012. The test orchard was a 0.4-  
136 ha block of apples (cv. Delicious) planted on 3.7 x 5.5 m within and between row spacing with  
137 500 trees/ha. Mean tree canopy height was 2.7 m. Replicates were separated by 10 m.  
138 Treatments included an untreated control; a grower standard of three sprays of  
139 chlorantraniliprole (Altacor, 350 g AI/kg WG, E.I. Dupont de Nemours Co.), on June 15 and 29  
140 and on July 13, and three sprays of spinetoram (Delegate, 250 g AI/kg WG, Dow AgroSciences,  
141 Indianapolis, IN) on July 27 and August 13 and 27; CpGV (Cyd-X,  $3 \times 10^{13}$  occlusion bodies/L)  
142 applied alone and with *M. pulcherrima* and cane sugar on June 15, 22, and 29, July 6, 13, and  
143 27, August 7 17, and 27 and on September 6. CpGv was applied at  $3.8 \times 10^7$  occlusion bodies/L  
144 (78.0 µl Cyd-X/L). The rate of CpGV was increased 3-fold on the last three spray dates to  
145 accomodate for the increased canopy volume during late season. Both sugar and *M. pulcherrima*  
146 were applied at 3.6 g/L. The treatment concentrations of insecticides were 0.12 g AI/L for  
147 spinetoram and 0.10 g AI/L for chlorantraniliprole.

148 Sprays were applied with a gasoline-engine powered, diaphragm-pump sprayer (Rear's MFG,  
149 Eugene, OR) at 689 kPa with a handgun sprayer equipped with a D-6 nozzle (GunJet, Model 43,  
150 TeeJet Technologies, Wheaton, IL). Sprays applied through July 13 were applied at 1.2 L/tree  
151 (623 L/ha). This spray rate was increased to 1.9 L/tree (935 L/ha) beginning on July 27 to  
152 improve coverage. The untreated control trees were not sprayed.

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

### 164 *Statistical Analyses*

165 Numbers and proportions were transformed to square root and arcsine (square root),  
166 respectively, prior to analysis of variance (ANOVA). Data were analysed with the Shapiro-Wilks

167 test of normality (Statistix 9, Analytical Software, Tallahassee, FL). Data which were not able to  
168 be normalized with these transformations were analysed with the non-parametric Kruskal-Wallis  
169 ANOVA of ranks. Means were separated in significant ANOVA's at  $P < 0.05$ .

## 170 Results

### 171 *Laboratory bioassays*

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

177 The difference in larval mortality between the three yeasts or different concentrations of yeast  
178 was not significant. The combination of CpGV with sugar (1.2 g/L) and either *C. tephrensensis* or *A.*  
179 *pullulans* at 3.6 g/L significantly increased the proportion of dead larvae, over CpGV alone. All  
180 larvae were killed with a combination of CpGV, *C. tephrensensis* and cane sugar (Figure 1).

181 Increasing the rate of sugar to 3.6 g/L increased larval mortality only slightly and this difference  
182 was not significantly different from the lower rate of sugar (data not shown). No direct toxic  
183 effects (mean proportional mortality  $< 0.10$ ) against larvae were found in assays in which fruit  
184 was dipped in water, in aqueous solutions of two rates of sugar, or in two rates of each of the  
185 yeasts.

### 186 *Field trial*

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

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

202 Significant differences were found among treatments in the relative proportion of dead and live  
203 larvae in injured fruit (Table 2). The proportion of dead larvae was highest in the CpGV plus the

204 yeast and sugar treatment and lowest in the untreated control. Similarly, the lowest proportion  
205 of live larvae remaining in the fruits was found with the addition of yeast and sugar to CpGV.  
206 The distribution of dead larvae (stings or entries) differed between CpGV alone and with the  
207 yeast and sugar added to CpGV. The proportion of stings was similar, but the addition of the  
208 yeast and sugar resulted in a higher proportion of dead larvae scored as entries than with CpGV  
209 alone. The untreated control and the CpGV alone treatments had the highest proportion of fruits  
210 with exit holes (Table 2).

## 211 Discussion

212 We have recently shown that codling moth, a typical insect herbivore, is associated with yeasts,  
213 but did not fully recognize the importance of these mutualistic yeasts for insect management.  
214 The behavioral response of adult moths suggested the prospective use of yeast volatiles as  
215 attractants, for flight detection and population monitoring, or mass-trapping (Witzgall et al.,  
216 2012).

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

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

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

### 238 *Enhancing the effect of codling moth granulovirus (CpGV)*

239 The key biological factor that constrains the effectiveness of CpGV is the uptake by codling moth  
240 larvae before they penetrate the fruit. However, combinations of CpGV with feeding stimulants



241 and larval attractants has not decisively improved performance (Arthurs et al., 2007; Lacey et  
242 al. 2008, Ballard et al. 2000a,b).

243 Host plant volatiles, such as pear ester (*E,Z*)-2,4-decadienoate and (*E,E*)- $\alpha$ -farnesene, which  
244 attract codling moth neonates, appear to mediate host location rather than feeding (Sutherland  
245 and Hutchins, 1972; Knight and Light, 2001; Hughes et al., 2003; Light and Beck, 2010, 2012).  
246 Addition of  $\alpha$ -farnesene slightly improved the effect of CpGV (Ballard et al. 2000b), pear ester  
247 reduced injury in walnuts (Light and Knight, 2011), but not in apple and pear (Arthurs et al.,  
248 2007; Schmidt et al., 2008).

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

#### 255 *Yeast and codling moth larval behavior*

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

260 Plant volatiles play an important role in adult codling moth reproductive behaviour (c.f. Trona et  
261 al., 2013) and effect also larval behavior (Sutherland and Hutchins, 1972; Landolt et al., 1999;  
262 Knight and Light, 2001; Jumean et al., 2005). Merely the expression of a subset of olfactory  
263 receptors genes in larval olfactory sensory neurons (Fishilevich et al., 2005; Kreher et al.,  
264 2005; Poivet et al., 2013) suggests some similarity in the responsiveness of adult and larval  
265 antennae to odorants. This is conceivable, since insect females and larvae locate the same food  
266 source for oviposition and feeding, respectively. In addition to volatile compounds, apple fruit  
267 and leaf sugars stimulate codling moth oviposition (Lombarkia and Derridj, 2008) and non-  
268 volatile apple or yeast metabolites may well stimulate larval feeding.

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

#### 277 *CpGV and codling moth management*

278 Conventional apple and pear growers in the U.S. have recently transitioned from the use of  
279 broad-spectrum organophosphates to more selective classes of insecticides used in rotation to

280 manage codling moth and to avoid selection for resistance. Unfortunately, growers have  
281 encountered new pests such as aphids, San Jose scale and phytophagous mites, because some  
282 of these new insecticides are still not sufficiently selective for pests and disrupt biological control  
283 (Martinez-Rocha et al., 2008; Jones et al., 2009).

284 Pest managers of European pome fruit orchards experience a different situation. Very few  
285 insecticides are available to the growers, which has accelerated resistance to the remaining  
286 products (Knight et al., 1994; Sauphanor et al., 2000). Integrated programs based on sex  
287 pheromone-mediated mating disruption, CpGV and reduced use of synthetic insecticides have  
288 been widely adopted to combat the evolution of resistance to the few available conventional  
289 insecticides (Charmillot and Pasquier, 2003). These programs are highly selective for codling  
290 moth and can minimize the secondary disruption of biological control agents (Lacey et al.,  
291 2008). Unfortunately, the failure to carefully manage the susceptibility of codling moth to CpGV  
292 has led to high levels of resistance and product failures in some regions (Fritsch et al., 2005;  
293 Sauphanor et al., 2006).

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

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

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

### 311 *Conclusion*

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

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

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

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- 488

488 **Legend**

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



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

| Mean (SE) proportion of fruit injury from |                    |                    |                   |
|---|--------------------|--------------------|-------------------|
| Treatment <sup>a</sup>                    | 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 + <i>Mp</i> + 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                            | $P < 0.0001$       | $P < 0.0001$       | $P < 0.01$        |

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

7 <sup>a</sup> CpGV (78.0 µl Cyd-X/L) was applied alone and with *M. pulcherrima* and cane sugar ten  
 8 times between June 15 and September 6, 2012. Three sprays of chlorantraniliprole (Altacor,  
 9 0.10 g AI/L) were applied between June 15 and July 13, and three sprays of spinetoram  
 10 (Delegate, 0.12 g AI/L) were applied between July 27 and August 27, 2012. The rate of  
 11 CpGV was increased 3-fold on the last three spray dates. Both sugar and *M. pulcherrima* were  
 12 applied at 3.6 g/L. Sprays applied through 13 July were applied at 1.2 L/tree (623 L/ha). The  
 13 spray rate was increased to 1.9 L/tree (935 L/ha) beginning on 27 July to improve coverage.  
 14 The untreated control trees were not sprayed.

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

| Treatment <sup>b</sup> | Mean (SE) proportion CM injury <sup>a</sup> |              |               |               |              |               |
|------------------------|---|--------------|---------------|---------------|--------------|---------------|
|                        | 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             | $P < 0.01$                                  | $P < 0.0001$ | $P < 0.001$   | $P < 0.0001$  | $P < 0.001$  | $P < 0.001$   |

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

5 <sup>a</sup> Dead larvae were scored as a ‘sting’ if the penetration was  $\leq 4.0$  mm or ‘entry’. Live larvae were scored as an ‘entry’ inside the fruit  
 6 or ‘exit’ for larvae having left the fruits.

7 <sup>b</sup> 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,  
8 2012. Three sprays of chlorantraniliprole (Altacor, 0.10 g AI/L) were applied between June 15 and July 13, and three sprays of  
9 spinetoram (Delegate, 0.12 g AI/L) were applied three times between July 27 and August 27, 2012.). The rate of CpGV was increased  
10 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  
11 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  
12 untreated control trees were not sprayed.

Codling Moth Larval Mortality

