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Project Title: Epidemiology and management of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi*

Keywords: Bactericides, Biological controls, and Systemic Acquired Resistance (SAR) compounds

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JUSTIFICATION/ BACKGROUND

Olive knot caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi (Psv)*, is a significant pathogen of olives worldwide including California. The bacterium is an opportunistic wound pathogen but also exist as an epiphyte on olives. Disease symptoms include tumors, galls, or knots that may lead to tree defoliation and branch diebacks with subsequent reductions in yield, fruit size, and oil quality. Historically, the most susceptible olive cultivars are: Manzanillo, Sevillano, Ascolano, and Mission, but many of the newer oil varieties are also highly susceptible which include: Arbequina, Arbosana, and Koroneiki.

The pathogen survives on olive leaves and stems but high populations mainly reside within actively growing knots. Bacterial cells are exuded to the knot surface during periods of wetness and are readily water splash/wind-dispersed or may be disseminated by insects or birds. It is not known if inoculum production varies at different times of the year but exudation relies on free water. Leaf and blossom scars as well as injuries to the twig bark caused by any biological (e.g., other diseases), environmental (e.g., freeze cracks), or physical (e.g., mechanical harvesting, pruning) means may serve as sites of infection. Twig injuries occurring at harvest are major potential entry points. The period of susceptibility of injuries is not known and is currently under evaluation. In California, infection occurs mostly during the rainy season (late fall, winter and spring) but the knots do not develop until new vegetative growth begins in the spring. Infections can occur at fairly low temperatures (5-10 C) and thus, wetness and availability of susceptible wounds are the main limiting factors for the disease.

Sanitation and prevention are the most successful strategies for management of olive knot. Any horticultural practice that promotes plant health, minimizes tree stress, and results in less leaf drop (i.e., control of peacock spot) will reduce infections. Pruning and removal of knots during dry periods (i.e., summer and early fall) reduces inoculum and avoids re-infection at pruning sites. Because the bacteria may be carried on pruning shears, frequent disinfection of equipment is necessary. Painting galls with Gallex® is an effective therapeutic treatment but is very labor intensive. Spray applications of copper-containing bactericides have been very effective in minimizing the disease, but they often may need to be repeated to protect new wounds. A minimum of two applications is usually necessary: one in the fall before the rainy season starts and one in the spring when most leaves have been shed. New copper formulations have been developed to reduce the metallic copper equivalent while maintaining the efficacy of the treatment. Our evaluations of copper sensitivity in populations of the olive knot pathogen indicated a reduced sensitivity in all isolates, but higher rates of copper were still effective in preventing bacterial growth. Because olive knot infections occur mostly during the rainy period, knowledge on the persistence of treatments is critical. Thus, we will determine the efficacy of copper alone and in mixtures with materials to increase its persistence by inoculating treated twig wounds at selected times after application and overhead irrigation. Rainfall will be simulated using overhead sprinklers established in orchards at UC Davis.

We have been instrumental in the development of the new agricultural antibiotic kasugamycin (commercial name Kasumin) for several bacterial diseases of agronomic crops in the United States. Kasugamycin has high activity against *Erwinia* and *Pseudomonas* species and moderate activity against *Xanthomonas* species and other plant pathogenic bacteria. We found it to be the most promising new treatment for preventing olive knot in our field studies, including in a commercial application to inoculated branches and we are continuing its evaluation. Kasugamycin is currently federally registered on pome fruit crops (e.g., apples and pears), whereas use on olives was approved as an "A" priority by IR-4 for the 2015 season. We will be involved with the IR-4 residue studies in the spring of 2015. Several systemic acquired resistance (SAR) compounds (e.g., Actigard, Regalia, quinoxyfen - Quintec, and USF2018A) were also effective in some studies, but not equivalent to copper or kasugamycin. SAR treatments need to be applied several days in advance of favorable disease conditions (or inoculation) but they potentially have a longer lasting effect on plant health and may provide sustainable treatments that could be developed for an integrated approach with other treatments. Initial studies on the use of biocontrols were not successful, but further evaluation with other biocontrol agents may provide disease control.

We have also been working on sanitation treatments for orchard equipment as part of an integrated olive knot management program. We demonstrated that quaternary ammonia compounds, guanidine, and chlorhexidine were highly toxic against the olive knot pathogen in laboratory studies. Citrox, a natural product derived from citrus extracts, and the quaternary ammonia sanitizers were also highly effective in disinfecting hard surfaces that were contaminated with *Psv*. The quaternary ammonia sanitizers are volatile compounds that do not leave residues and are not corrosive to equipment. Deccosan 321 has a federal label and is used in Florida for disinfesting field equipment to prevent the spread of bacterial diseases on citrus and tomatoes caused by *Xanthomonas* species. Deccosan 321 was submitted to the State of California as a Section 24c, Special Local Need, registration on harvesting and pruning equipment used by the olive industry. Registration is expected in the spring of 2015. We will test additional parameters that may affect the efficacy of the sanitizer including inoculum concentration and post-inoculation treatment time. Field evaluations of the material as an equipment sanitizer will be accomplished once registration is approved.

RESEACH OBJECTIVES

1. **Epidemiology – pathogen variability, inoculum availability and period of susceptibility of selected injuries (leaf scars, pruning injuries, etc.) to infection**
    a. Evaluate genetic pathogen variability using DNA markers
    b. Monitor galls for production of inoculum over time
    c. Duration of susceptibility of injuries under different environmental conditions (wetness and temperature)
2. **Evaluate populations of the pathogen for laboratory sensitivity to chemicals**
3. **Test the performance of an equipment sanitizer (e.g., quaternary ammonium) under field conditions once registration has occurred.**
4. **Evaluate the efficacy of protective treatments such as new copper formulations, antibiotics (Kasumin, Mycoshield), captan, dodine, SAR compounds (acibenzolar-S-methyl - Actigard, PM-1, quinoxyfen, ProAlexin, Regalia), and combination treatments**
    a. Field trials with and without adjuvants
    b. Timing studies: Protective (pre-infection) vs. post-infection activity of treatments; proper timing of SAR compounds; treatment at spring leaf drop or after harvest.
    c. Persistence of different copper treatments with and without the addition of lime or other additives under simulated rain conditions.
    d. Develop copper activity-enhancing materials such as mancozeb and other products
5. **Systemic infection of Psv**
    a. Investigate potential factors leading to systemic movement of Psv and twig dieback as was observed in evaluations of field trials done in the fall of 2013.

MATERIALS and METHODS

1. Epidemiology.

a. *Evaluation of Psv genetic variability.* Olive knots were collected in collaboration with farm advisors and PCAs. Knots were surface-sterilized with sodium hypochlorite, internal tissue was removed, suspended in sterile water, and the suspension was plated onto KMB medium. Single bacterial colonies were cultured and

species identity was verified using primers that target the IAA-lysine synthase gene (Penyalver et al., 2000). Genetic variability of our current collection of Psv strains was evaluated using REP primers in PCR reactions as described previously.

b. ***Monitor galls for production of inoculum over time.*** This objective was studied in the previous funding season and additional experiments are planned for 2015.

c. ***Duration of susceptibility of injuries and effect of inoculum concentration on development of olive knot.*** In field studies on the effect of inoculum concentration on knot development, leaf scar and lateral twig wounds of cv. Arbequina twigs were inoculated in June 2014 with a copper-sensitive or a -tolerant strain of Psv using selected inoculum concentrations ranging from $2x10^5$ to $2x10^8$. The incidence of knot formation was evaluated in October of 2014.

2. ***Evaluation of pathogen populations for sensitivity to copper, antibiotics, and sanitizers in the laboratory.*** The effect of exposure time to Deccosan 321 or sodium hypochlorite on viability of Psv was tested in a laboratory study. For this, Psv was suspended in 5 ppm Deccosan 321 or 5 ppm sodium hypochlorite. After exposure for 15, 30, 45, or 60 seconds, the suspensions were diluted 1:1000 with sterile distilled water and spiral-plated onto King's B medium. Bacterial colonies were enumerated after 2 to 3 days of incubation at 25C. Psv recovery (cfu/mL) for each exposure time was calculated as percentage of the water control and was the average of three replicates.

3. ***Test the performance of an equipment sanitizer (e.g., quaternary ammonium) under field conditions once registration has occurred.*** These studies are planned for 2015.

4. ***Evaluation of protective treatments in greenhouse and field studies.*** In the fall of 2013, greenhouse studies on cv. Manzanillo were done at UC Riverside. Field trials on cvs. Arbequina and Manzanillo were done at UC Riverside, UC Davis, and in a commercial orchard in the fall of 2013 and spring of 2014. Twigs were wounded laterally with a razor blade or leaves were removed to create leaf scar wounds. Wounds were either first inoculated and then treated; or first treated, air-dried, and then inoculated (see Figures of the Results). Inoculations were done by hand-spraying of bacterial suspensions (2×10^7 to 1×10^8 cfu/ml). Treatments of bactericides and SAR compounds were done by spraying the wound sites to run-off. Additionally, a trial was done at UC Davis on cv. Manzanillo on the persistence of selected treatments (Kasumin, Kocide 3000, Kocide with lime and zinc, and Kasumin with Kocide) after simulated rain application. Wounds were first treated, allowed to dry, subjected to simulated rain for 1 h, and then inoculated. Plants were evaluated for the presence of knot formation in the spring of 2014 or fall 2014. All data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.4.

5. ***Investigate potential factors leading to systemic movement of Psv and twig dieback.*** Environmental data (precipitation, temperature) were obtained from a CIMIS station in proximately to our field trials, graphed out, and visually analyzed for differences between the 2013/14 and previous winter seasons. To simulate field conditions, in an initial controlled temperature study, wounds of cvs. Arbequina and Manzanillo olive plants were inoculated and then exposed to freezing temperatures (-5°C) for 4 to 12 h. Plants were then moved to the greenhouse and observed for disease development.

RESULTS

1. Epidemiology

a. ***Evaluation of Psv genetic variability***. Based on rep-PCR, two main genotypes (comprising >95% of the strains evaluated) were identified in our current collection of 120 Psv strains from northern California. Thus, variability was found to be very limited and additional genetic markers will be tested in 2015.

b. ***Monitor galls for production of inoculum over time.*** This objective was studied in the previous funding season and we showed that after external application of water to galls, inoculum was immediately produced at very high concentrations. Galls continued to produce inoculum with continued wetness duration. Thus, short wetness periods in the field can result in a high potential for new infections to occur. Additional experiments are planned for 2015.

c. ***Duration of susceptibility of injuries and effect of inoculum concentration on development of olive knot.*** Studies on susceptibility of injuries of cv. Manzanillo olives under different wetness conditions (overhead irrigation to simulate rain or no irrigation) were conducted in the fall season of 2013 and data were presented

in our July 2014 interim report. Our results indicated that wounds were less susceptible to infection when provided with simulated rain as compared to no rain and this was explained by possible washing off of inoculum from the wounding sites by excess simulated rain. Injuries that received no irrigation remained susceptible to Psv even after 17 days (the maximum wound healing time tested) while irrigated wounds had very low incidence of olive knot after 10 days.

In previous studies on cv. Arbequina, susceptibility of wounds declined significantly after only 10 days and there were no differences between irrigated and non-irrigated trees. To find out if differences between the two studies were due to the olive cultivar used, comparative studies with cvs. Manzanillo and Arbequina are planned for 2015 under controlled conditions in the greenhouse.

The effect of inoculum concentration on knot induction on cv. Arbequina olive was tested in a field study. Fewer knots developed on leaf scar injuries than on lateral wounds when inoculum concentration was reduced for the two Psv strains tested (Fig. 1A). This suggests that leaf scar wounds in this study were less sensitive to infection than lateral wounds. For lateral and leaf scar wounds, a significant difference between the two strains in the incidence of knots was only observed at the lowest inoculum concentration of 2×10^5 cfu/ml and fewer knots developed using the Cu-sensitive strain, indicating that this strain may be less virulent. For the leaf scar wounds, the incidence of knot formation decreased with decreasing inoculum concentration for both strains (Fig. 1B).

Improved control of olive knot may be achieved by understanding threshold inoculum concentrations that are needed for disease development. Different inoculum levels (disease pressure) may require different treatment strategies. Thus, SAR compounds may only provide adequate control when disease pressure is low and may require integrated use with conventional treatments such as Kocide and Kasumin.

2. *Evaluation of pathogen populations for sensitivity to copper, antibiotics, and sanitizers in the laboratory.* Laboratory studies were conducted on the in vitro sensitivity of more than 100 strains of Psv to potential bactericidal treatments (e.g., oxytetracycline, streptomycin, and kasugamycin as well as different copper formulations) and results were presented in our July 2014 interim report. Briefly, there was a range of sensitivities to the three antibiotics, but all strains were all considered sensitive. Most strains showed reduced copper sensitivity in the 10- to 20-ppm range, however, one strain was considered copper-resistant with growth at 50 ppm MCE. We are currently sampling this site. Thus, copper resistance does occur in the pathogen population at some locations and its spread from overuse of copper products has to be minimized. This emphasizes the need for alternative treatments and for new copper-enhancing alternatives.

In other in vitro assays we tested several strains against new copper products (e.g., Magna Bon) and copper additives (thiadiazoles, dodine, etc.) with very promising results. Copper additives (enhancers) were evaluated due to EPA mandated registration restrictions of mancozeb. At the same metallic copper equivalent (MCE), MagnaBon was more effective in inhibiting bacterial growth in laboratory assays than copper sulfate.

Addition of a thiadiazole (ATD) to copper improved copper activity against copper-sensitive strains (when using copper at a concentration where sensitive strains were still viable), but was not effective against a copper-tolerant strain. In work with copper-tolerant strains of the walnut blight pathogen *Xanthomonas arboricola* pv. *juglandis*, this mixture improved copper activity. Dodine inhibited growth of copper-tolerant and -sensitive Psv strains. Mixtures of copper and dodine are currently being tested.

In vitro direct contact assays indicated an exponential decrease in *Psv* viability with increased exposure duration to Deccosan 321 at very low rates (5 ppm) (Fig. 2). As compared to the water control, there was an 84% reduction after a 15-sec exposure and a 97% reduction after a 60-sec exposure. Deccosan 321 at 25 ppm completely inactivated *Psv* after 15 sec. In comparison, 5 ppm sodium hypochlorite completely inhibited growth at any of the exposure times tested. Thus, Deccosan 321 is a very effective sanitizer and is not corrosive to equipment as chlorine is and performed better than chlorine in the presence of an organic load (i.e., plant debris) as we previously showed in hard-surface disinfestation assays.

3. *Test the performance of an equipment sanitizer (e.g., quaternary ammonium) under field conditions once registration has occurred.* In collaboration with the registrant, we submitted the quaternary ammonium compound Deccosan 321 for a special local need registration for use in California, and approval is expected for March 2015. Once a Section 24C is in place, we plan to test the material on harvesting and pruning equipment in the field.

4. *Evaluation of protective treatments in greenhouse and field studies.*

a. Copper and copper alternatives with adjuvants. In greenhouse trials conducted in the fall of 2013, the effect of two adjuvants, NuFilm-P and Washgard (a carnauba-based adjuvant), on the performance of Kasumin and copper treatments was evaluated. Overall, there was some inconsistency in results among treatments and between lateral wounds and leaf scar wounds. Washgard numerically increased the activity of Kasumin and of Kasumin-Kocide, but not of Kocide in lateral wound inoculations (Fig. 3). For NuFilm-P, there was a trend for reduced activity for the Kasumin and Kasumin-Kocide treatments. For leaf scar wounds, there was a trend of improved activity with Washgard for the Kasumin and Kasumin-Kocide treatments, and a trend for reduced activity with NuFilm-P for the Kasumin and Kocide treatments. The activity of Kasumin-Kocide, however, was significantly improved with the addition of NuFilm-P (Fig. 3B). Thus, these results warrant further studies.

In the fall of 2013, field trials were done at UC Davis and in several commercial cv. Manzanillo and Arbequina groves. In evaluating these trials in the spring of 2014, we noted disease symptoms that we did not observe previously, even at study sites that were used in previous years. On cv. Manzanillo, and to a much lesser extent also on cv. Arbequina, many of the inoculations resulted in major shoot dieback and blistering on the inoculated as well as neighboring branches. Symptoms on non-inoculated neighboring branches are an indication of bacterial movement inside the host, and this was substantiated by bacterial isolation from these distal points. With this severe disease development, performance in our bactericide efficacy studies was often compromised and treatment efficacy was generally very poor. Thus, results are only presented for some of these trials, and mostly for trials on cv. Arbequina because this cultivar appeared to be much less susceptible to Psv infection than cv. Manzanillo.

In a commercial cv. Manzanillo orchard, severe dieback was observed on untreated, inoculated controls and on copper- and copper-mixture-treated trees. Still, treatments with kasugamycin, streptomycin, and oxytetracycline significantly reduced dieback and systemic movement was reduced indicating that these treatments may be more effective than copper.

On cv. Arbequina, Kocide performed mostly well in reducing the incidence of knots on lateral and leaf scar wounds when using a copper-sensitive strain for the inoculations (Fig. 4A, 5, 6A) and was moderately effective when using a copper-tolerant strain (Fig. 4B, 7B). Kasumin treatments resulted generally in very good (Fig. 4A, 6A) to moderate (Fig. 4B, 5A, 7A) control when using a copper-sensitive strain for inoculation; and very good (Fig. 6B, 7B) control when using a copper-tolerant strain. Addition of Syllit or Captan to Kasumin did not improve Kasumin efficacy in one trial where this was studied (Fig. 4).

Efficacy of streptomycin ranged from excellent (Fig. 4A, 5A, 6, 7A), to moderate (Fig. 7B), to ineffective (Fig., 4B, 5B); whereas oxytetracycline had excellent (Fig. 6, 7), moderate (Fig. 4A), or no (Fig. 4B, 5) activity. Trials where these antibiotic treatments had low efficacy were at sites with cold injury. In comparison of copper treatments, Kocide 3000 generally performed similar to Badge X2, and these treatments performed numerically or statistically better than MagnaBon or Previsto at the rates used (Figs. 6, 7). MagnaBon was used at a very low rate (100 MCE) based on the manufacturer's recommendation and based on the risk of phytotoxicity that was observed in our trials with MagnaBon on other crops. The bacterial membrane disruptor Ceragenin was not or only slightly effective in reducing olive knot (Figs. 6, 7). Additionally, ATD that we found to enhance copper activity for some bacterial diseases of other plants did not improve copper activity in our studies on olive. Mancozeb is registered for management of bacterial diseases of some tree crops in California such as walnut where it increases efficacy of copper when disease is caused by copper-resistant strains of the pathogen. Mancozeb, however, cannot be registered on olives. Therefore, we are evaluating alternatives. Quinoxyfen has been reported to improve copper performance against diseases caused by *Xanthomonas* species in Florida and we are planning to apply this compound in mixture with other bactericides for *Psv* control (we previously tested quinoxyfen by itself).

In summary, although reduction in disease by bactericidal treatments was not as high as in previous trials, several treatments still significantly reduced the disease as compared to the control. Higher inoculum levels were used in these studies as compared to previous years and environmental conditions were highly favorable for olive knot, and this may have impacted symptom development and efficacy of treatments. Under these severe conditions, antibiotic treatments were able to significantly reduce dieback providing some measure of control against *Psv* infection.

Overall, we made significant progress in understanding the management of olive knot with chemicals. Copper sensitivity surveys in Psv populations provide information on potential of copper treatments, but also indicate the risk of copper resistance and the need for alternatives. Antibiotics were identified as the most effective alternatives. Our post-infection timing studies indicated that when olives are treated immediately after injury occurs, olive knot development can be reduced significantly to low levels. Thus, under commercial conditions, treatments should be applied as soon as possible after harvest or after injuries from frost or hail occur. Using an equipment sanitizer will reduce inoculum spread during orchard maintenance and harvest.

b. SAR Compounds. Field trials in the fall of 2013 using SAR compounds (i.e., Regalia, ProAlexin, Stout, Actigard, and Quintec) resulted in little or no control of *Psv* when a foliar application was done three days before wound-inoculation. As in other trials conducted over the winter of 2013/14, branch dieback and systemic movement of the bacterium was observed on cv. Arbequina. In previous studies, significant reductions in disease were found using Regalia, quinoxyfen, Actigard, and Stout. Thus, SARs may be effective under specific conditions including timing and application method used. We conducted a new trial using these SAR compounds in October of 2014 on cvs. Manzanillo and Arbequina. We used different inoculum concentrations to find out if SARs possibly are more effective under lower disease pressure. Our goal is to incorporate SAR compounds into an effective spray program along with conventional treatments.

c. Persistence of treatments. Field trials to test the persistence of treatments under simulated rain conditions at UC Davis on cv. Manzanillo olives were performed in the fall of 2013. There was severe systemic movement of the pathogen in all treatments (Kasumin, Kocide 3000, Kocide-lime-zinc, and Kasumin-Kocide) with symptoms including knots, bumps, and blistering at and distant from the initial inoculation point. Disease conditions were highly favorable and we suspect cold injury effected the results of this trial preventing any interpretation of persistence. Trials are ongoing with adjuvants that have been reported to increase the persistence of copper in other crops (e.g., carnauba-based adjuvants, pinolene-based adjuvants).

5. *Investigate potential factors leading to systemic movement of Psv and twig dieback.* The occurrence of symptoms of apparent systemic infections in the fall/winter 2013/14 field trials created new challenges for research on olive knot control and prompted investigations to elucidate the cause. Weather data for the winter 2013/14 season obtained from CIMIS stations in proximately to our field trial locations were compared to those of previous years. It was noted that low temperatures and rainfall were recorded earlier in the winter of 2013/14 as compared to 2012/13. The resulting frost injuries of the olive trees and subsequent rainfall may have led to the dissemination of residual inoculum and infection.

Thus, an initial low temperature study was performed in growth chambers in an attempt to reproduce symptoms of apparent systemic infection. Observations in this study indicated that plants exposed to low temperatures (-5°C) for 4 to 12 hours showed severe responses which included heavy leaf drop and branch dieback. Cvs. Arbequina and Manzanillo plants that were wounded and inoculated before low-temperature exposure showed similar symptoms as observed in the field trials. It is unknown at this time whether Psv was migrating internally within olive tissue (i.e., systemic movement) leading to new infection sites (nodules) or if frost damage created microscopic wounds that were entry points for an external source of Psv inoculum.

Treatments that were applied in the fall may have been compromised by the cold environments. Possibly some inoculum was inadvertently applied to areas that were not covered by chemical treatments and was able to persist and then infect wounds created by the later occurring frost. All chemical treatments tested are contact materials and good coverage of susceptible tissue is critical for control. Frost may have created new susceptible wounds that were not well protected leading to poor control of olive knot. These hypotheses need further investigation, and thus, chemical treatments before and after occurrence of frost damage will be evaluated in future growth chamber studies. Previously, our wounding studies indicated that the disease could be controlled if copper was applied within 24 h of when the injury occurred. Additional testing with antibiotics will be done under low-temperature conditions because they provided some measure of control against the apparent systemic infection. The goal is to provide an acceptable measure of disease control under these severe conditions.

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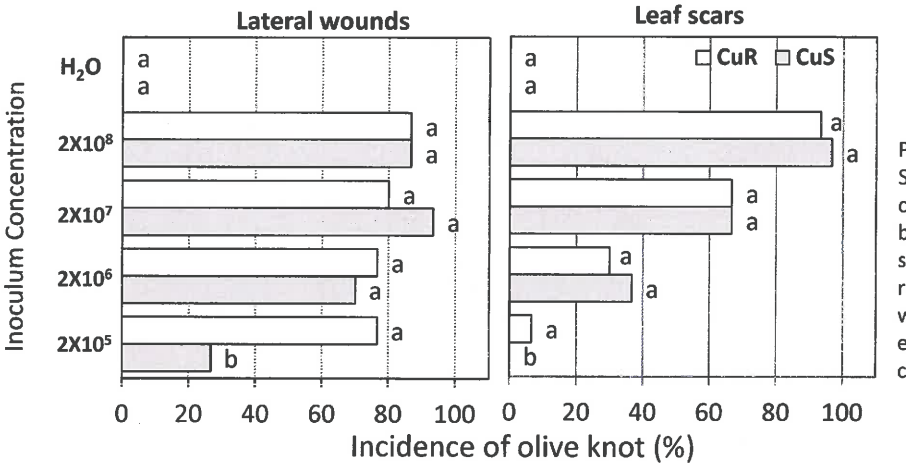
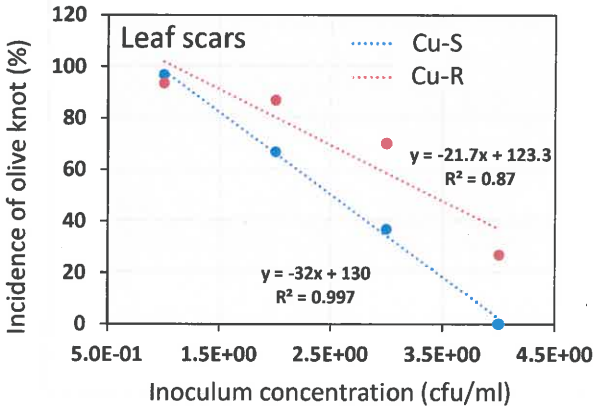
Fig. 1. Effect of inoculum concentration on development of olive knot in field studies at UC Riverside

A. Incidence of olive knot caused by Cu-sensitive and Cu-resistant isolates of Psv using selected inoculum concentrations.



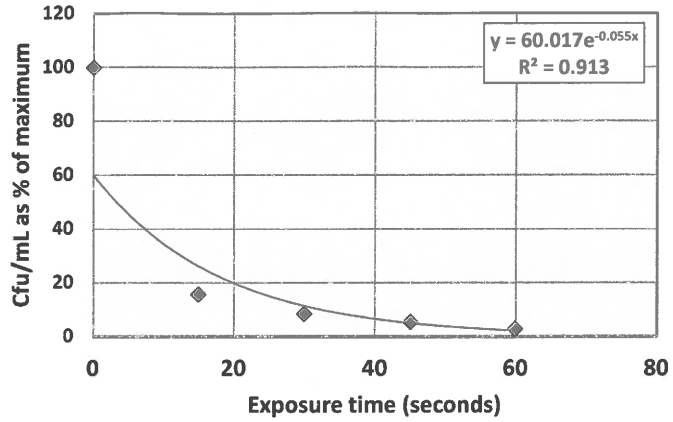
Pairwise Statistical comparisons between Cu-sensitive and Cu-resistant isolates were made for each inoculum concentration.

B. Regression of inoculum concentration on incidence of olive knot caused by Cu-sensitive and Cu-resistant isolates of Psv



In June of 2014, leaf scar and lateral twig wounds of cv. Arbequina twigs were inoculated with either of 2 strains of Psv at selected inoculum concentrations ranging from $2x10^5$ to $2x10^8$ at UC Riverside. Knot incidence recorded in October of 2014.

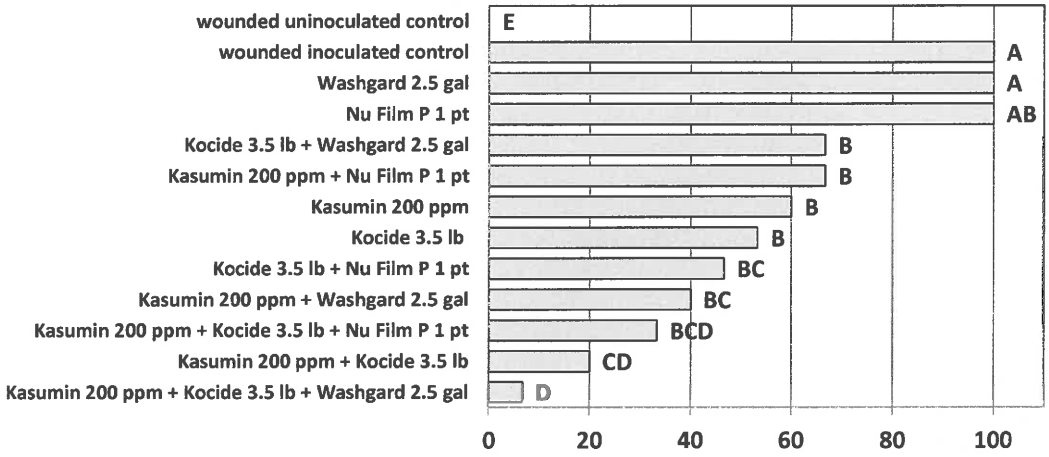
Fig. 2. Viability of Psv after selected exposure times to Deccosan 321 in a direct contact assay



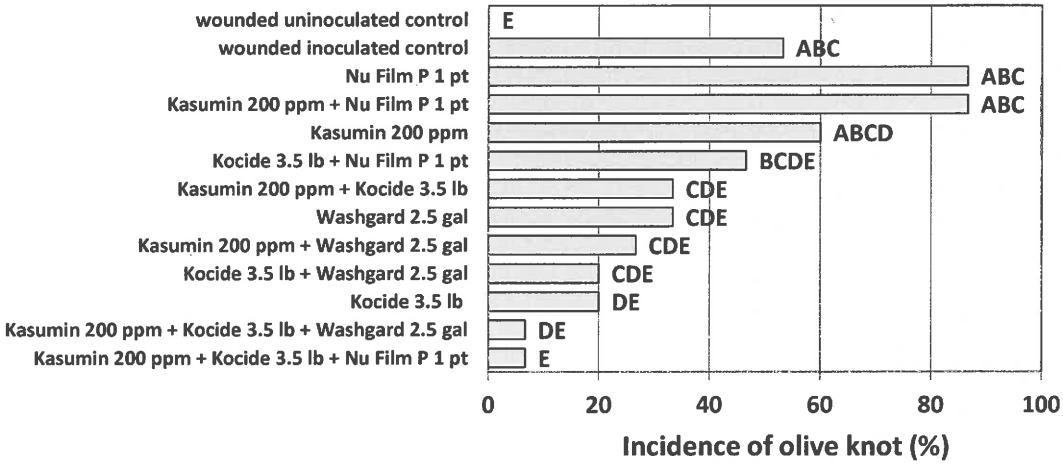
Psv was suspended in 5 ppm Deccosan 321. After 15, 30, 45, or 60 seconds, the suspension was diluted 1:1000 with sterile water and plated onto King's B medium for bacterial enumeration. *Psv* recovery (cfu/mL) for each exposure time is calculated as percentage of the water control and is the average of three replicates.

Fig. 3. Greenhouse trial on the effect of selected adjuvants on the efficacy of copper and kasugamycin against olive knot

A. Treatment and inoculation of lateral wounds

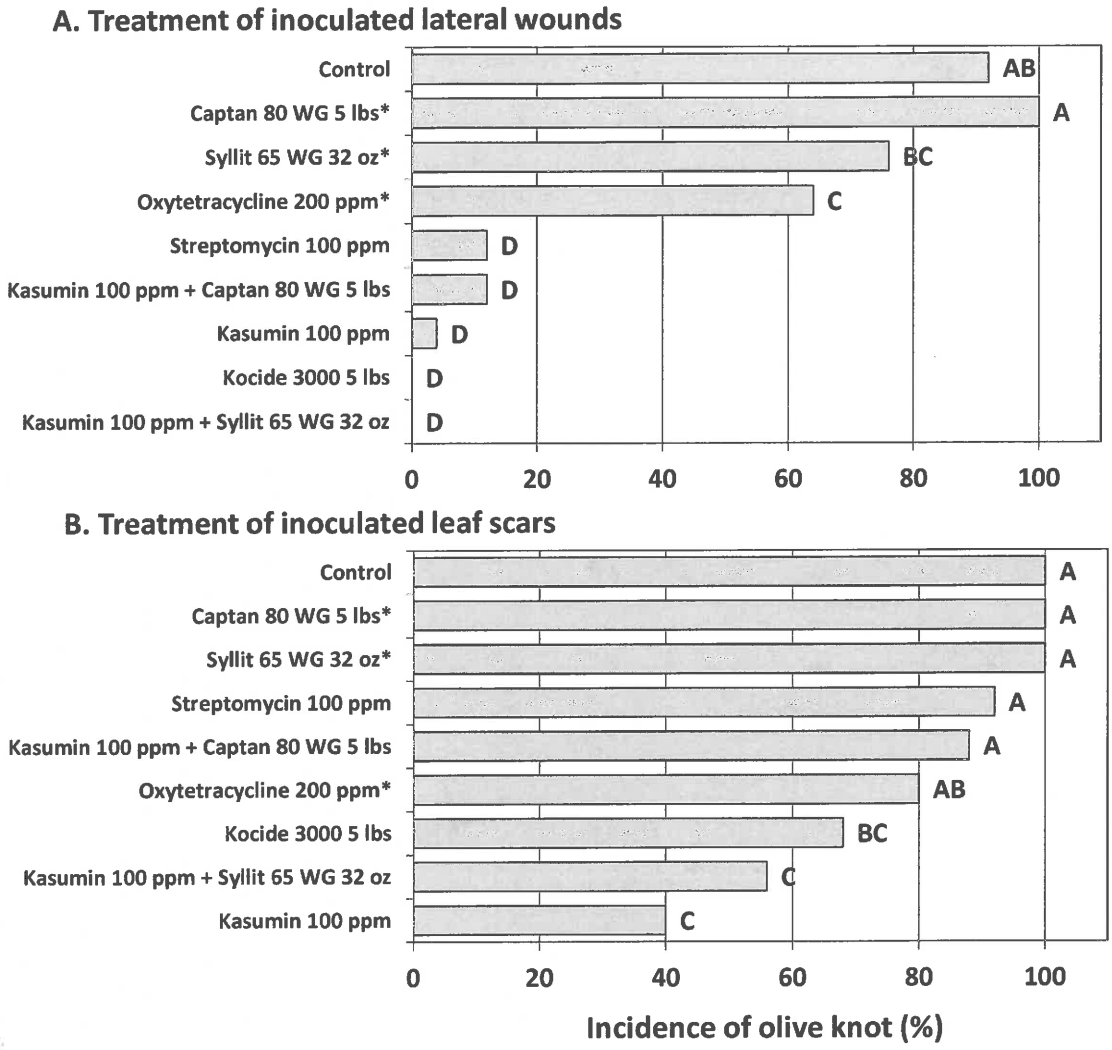


B. Treatment and inoculation of leaf scars



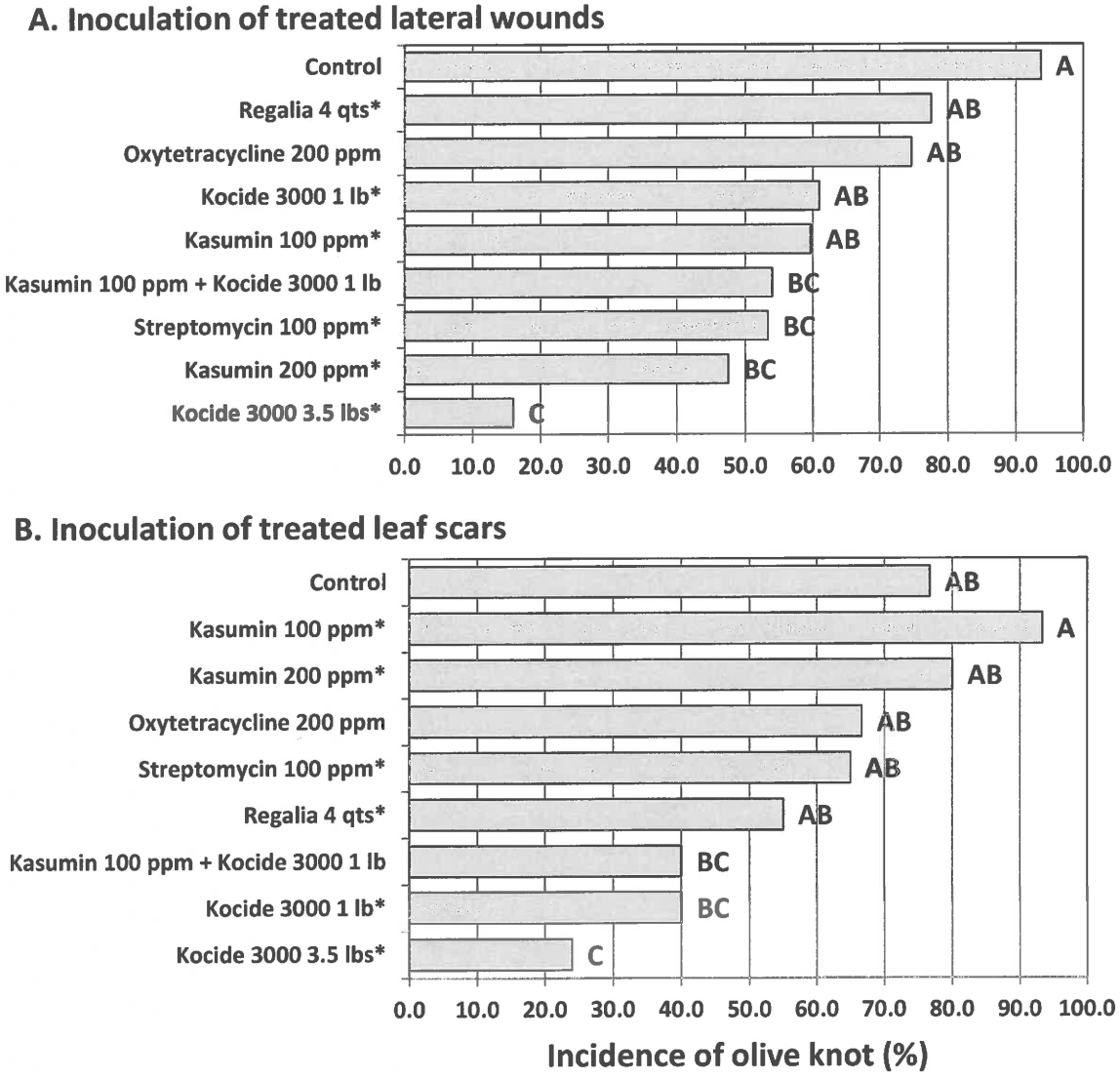
Cv. Manzanillo twigs were wounded (lateral or leaf scar wounds), treated to run-off using a hand sprayer, allowed to air-dry, and then inoculated with a suspension of a copper sensitive isolate of *Psv* ($2x10^7$). Rates specified are per acre/100 gal. Trial performed in late August and incidence of knots recorded in early October.

Fig. 4. Efficacy of bactericide treatment against olive knot in a field trial at UC Davis



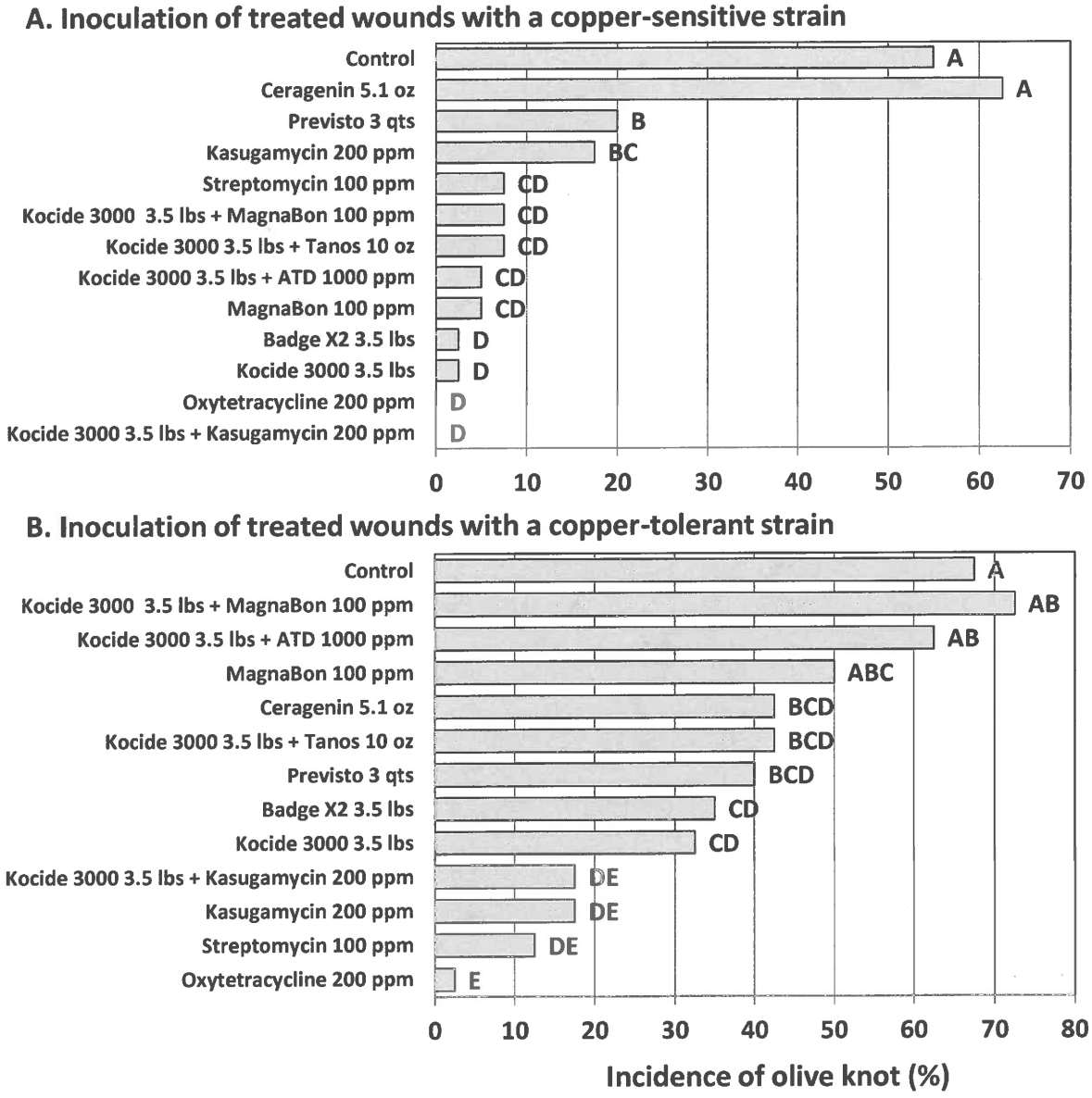
In November 2013, cv. Arbequina olive twigs were wounded, inoculated with a copper-sensitive strain of *Psv* ($1x10^8$), allowed to air-dry, and treated by hand-spraying to run-off. Disease was evaluated in April 2014. * Indicates treatments where some replicates showed symptoms of apparent systemic infection. Rates specified are per acre/100 gal. Knot incidence recorded in May of 2014.

Fig. 5. Treatment efficacy against olive knot in a field trial in a commercial olive orchard after inoculation with a copper-sensitive strain of *Psv*



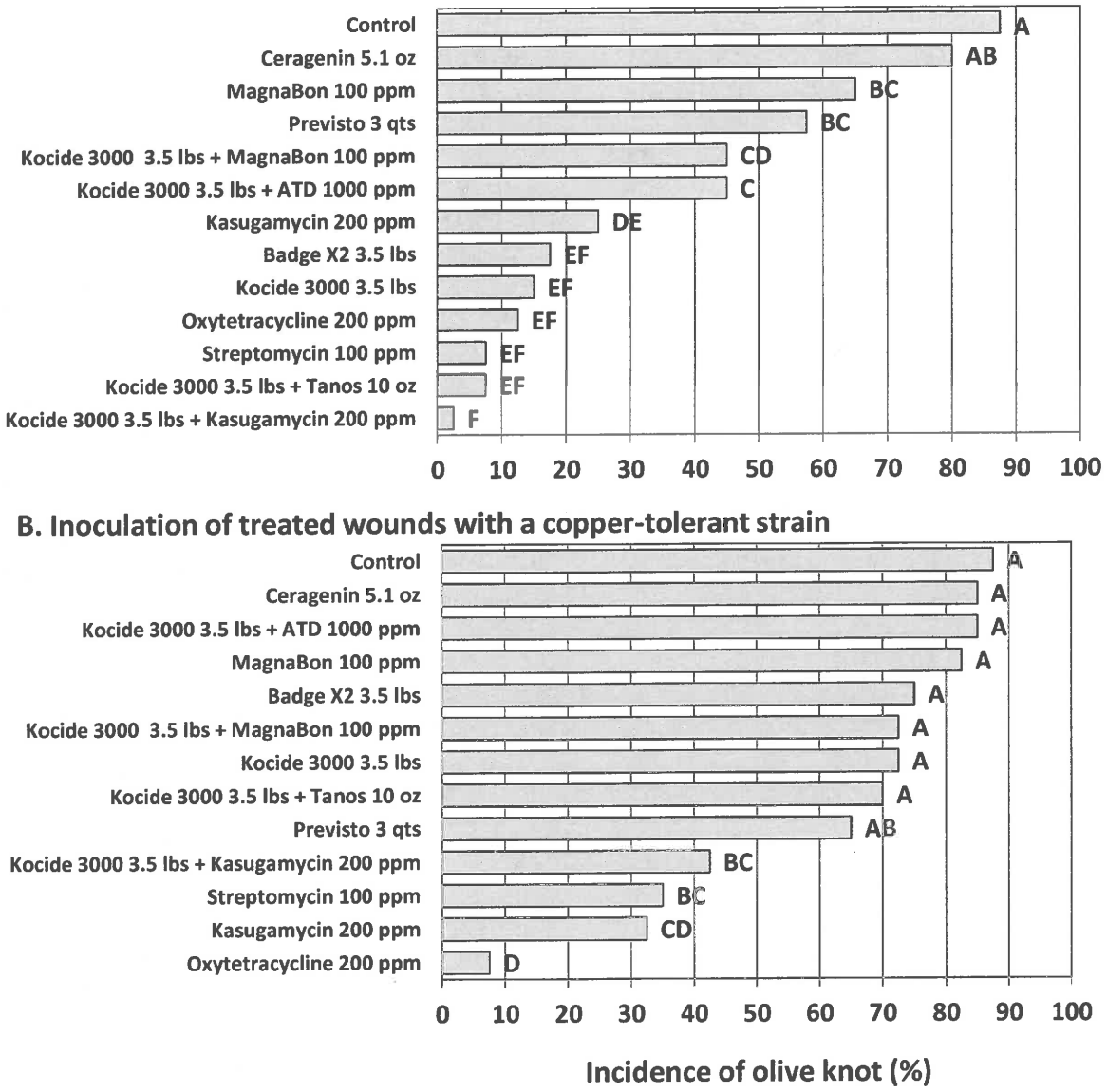
In October 2013, cv. Arbequina olive twigs were wounded, treated, allowed to air-dry, and inoculated with a copper-sensitive strain of *Psv* ($1x10^8$). * Indicates treatments where some replicates showed symptoms of an apparent systemic infection. Rates specified are per acre/100 gal. Knot incidence recorded in early June of 2014.

Fig. 6. Treatment efficacy against olive knot in a field trial at UC Riverside using a copper-sensitive or -tolerant *Psv* strain for inoculation of leaf scar wounds



In late May of 2014, leaf scars on cv. Arbequina were treated, allowed to air-dry, and inoculated with a copper-sensitive or -tolerant strain of *Psv* ($2x10^7$). Rates specified are per acre/100 gal. Knot incidence recorded in early October of 2014.

Fig. 7. Treatment efficacy against olive knot in a field trial at UC Riverside using a copper-sensitive or -tolerant *Psv* strain for inoculation of lateral twig wounds



In May of 2014, lateral twig wounds on cv. Arbequina olive were treated, allowed to air-dry, and inoculated with a copper-sensitive or -tolerant strain of *Psv* ($2x10^{7}$). Rates specified are per acre/100 gal. Knot incidence recorded in early October of 2014.

Department of Plant Pathology
Relevant AES/CE Project No.: 11103864

University of California
Division of Agricultural Sciences
INTERIM PROJECT/RESEARCH PROGRESS REPORT
July, 2014

Project Year: 2014

Project Leader:
Dr. J. E. Adaskaveg, Professor
Department of Plant Pathology
University of California, Riverside
Riverside, CA 92521
(O) 951-827-3880 FAX: 951-827-7577 (M) 951-288-9312
jim.adaskaveg@ucr.edu

Title: Epidemiology and management of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi (Psv)*

2014 Research Objectives:

1) Epidemiology – pathogen variability, inoculum availability and period of susceptibility of selected injuries (leaf scars, pruning injuries, etc.) to infection
    a. Evaluate genetic pathogen variability using DNA markers
    b. Monitor galls for production of inoculum over time
    c. Duration of susceptibility of injuries under different environmental conditions (wetness and temperature)
2) Evaluate populations of the pathogen for laboratory sensitivity to chemicals
3) Test the performance of an equipment sanitizer (e.g., quaternary ammonium) under field conditions once registration has occurred.
4) Evaluate the efficacy of protective treatments such as new copper formulations, antibiotics (Kasumin, Mycoshield), captan, dodine, SAR compounds (acibenzolar-S-methyl - Actigard, PM-1, quinoxyfen, ProAlexin, Regalia), and combination treatments
    a. Field trials with and without adjuvants
    b. Timing studies: Protective (pre-infection) vs. post-infection activity of treatments; proper timing of SAR compounds; treatment at spring leaf drop or after harvest.
    c. Persistence of different copper treatments with and without the addition of lime or other additives under simulated rain conditions.
    d. Develop copper activity-enhancing materials such as mancozeb and other products

Summary of progress in 2014 including ongoing studies:

1a. Evaluation of *Psv* genetic variability of our current collection of strains using REP primers identified two main genotypes, comprising >95% of the 120 isolates from Northern California evaluated. Thus, variability was found to be very limited and additional genetic markers will be tested.

1b. In studies on inoculum production from galls over time, we showed that after wetting the galls, they can immediately produce inoculum at very high concentrations. Galls continued to produce inoculum with continued wetness duration. Thus, short wetness periods in the field can result in a high potential for new infections to occur.

1c. In fall 2013 field trials, we tested the susceptibility of injuries of cv. Manzanillo olives under different wetness conditions (overhead irrigation to simulate rain or no irrigation). Our results indicate that wounds were less susceptible to infection when provided with simulated rain as compared to no rain.

This result was unexpected. An explanation considered was that inoculum was washed off from the wounding sites by excess simulated rain. Injuries that received no irrigation remained susceptible to *Psv* even after 17 days (the maximum wound healing time tested). This is in contrast to our previous repeated studies on cv. Arbequina olives where inoculation of 10-day old injuries (leaf scar and lateral wounds) resulted in a very low incidence of knot formation. To determine if wound healing of cv. Manzanillo is different from cv. Arbequina, comparative studies will be done under controlled conditions in the greenhouse using both cultivars.

2. Laboratory studies were conducted on the in vitro activity of potential bactericidal treatments. We previously showed that systemic acquired resistance (SAR) compounds have no in vitro effect against *Psv*. Screening of the majority of our *Psv* collection (>100 strains) to the antibiotics oxytetracycline, streptomycin, and kasugamycin as well as different copper formulations has been accomplished. Strains showed a range of sensitivities to the three antibiotics but were all considered sensitive. Most strains in our collection showed reduced copper sensitivity in the 10- to 20-ppm range, however, one strain in our collection is considered copper-resistant with growth at 50 ppm MCE. Thus, copper resistance does occur in the pathogen population and its spread from overuse of copper products has to be minimized. This emphasizes the need for alternative treatments. In other vitro assays we tested several strains against new copper products (e.g., Magna Bon) and copper additives (thiadiazoles, dodine, etc.) with very promising results. These materials were evaluated due to EPA mandated registration restrictions of mancozeb (*see* 4d below).

3. In collaboration with the registrant, we submitted Deccosan 321, a quaternary ammonium compound, for a special local need registration for use in California, and approval is still pending. We are currently performing in vitro assays to compare the performance of Deccosan 321 to sodium hypochlorite using direct contact, as well as hard surface tests. Different concentrations and exposure durations are being compared for their effect on *Psv* viability. Results of direct contact tests indicated an exponential decrease in *Psv* viability with increased exposure duration to Deccosan 321 at very low rates (5 ppm) with an 84% reduction at 15 sec and a 97% reduction at 60 sec exposure. Deccosan at 25 ppm completely inactivated *Psv* after 15 sec. Thus, Deccosan 321 is a very effective sanitizer. Once a Section 24C is in place, we plan to test the material on harvesting and pruning equipment in the field.

4a. In the fall of 2013, field trials were done at UC Davis and in several commercial cv. Manzanillo and Arbequina olive groves. In evaluating these trials in spring 2014, we noted disease symptoms that we did not observe previously, even at study sites that were used in previous years. On cv. Manzanillo, and to a much lesser extent also on cv. Arbequina, many of the inoculations resulted in major shoot dieback and blistering on the inoculated as well as neighboring branches. Symptoms on non-inoculated neighboring branches are an indication of bacterial movement inside the host, and this was verified by bacterial isolation. We are currently conducting comparative greenhouse and field studies on both cultivars in an attempt to identify the cause of this dieback and systemic movement. Assays include using different inoculum concentrations, long periods of high humidity and wetness, and wrapping wounded and non-wounded olive branches with parafilm after inoculation. Observations made in past studies revealed that inoculated wounds that were parafilm-wrapped developed similar symptoms of a systemic infection. These studies will provide new information on the etiology of olive knot.

The severe disease development in Manzanillo and Arbequina experimental and commercial groves in the spring of 2013 compromised some of our bactericide efficacy studies. Severe dieback was observed on untreated, inoculated controls and on copper and copper-mixture treated trees in the commercial cv. Manzanillo orchard. Still, treatments with kasugamycin, streptomycin, and oxytetracycline significantly reduced dieback and had some control of systemic movement, caused by *Psv*. Kocide performed well at reducing the incidence of knots formed on lateral and leaf scar wounds when using a copper-sensitive

strain for the inoculations and was moderately effective when using a copper-resistant strain. Kasugamycin gave moderate control when using a copper-sensitive strain and good control with a copper-resistant strain on lateral wounds, but leaf scar wounds were not well protected.

In a separate trial on cv. Arbequina olives, Kasumin, Kasumin-Syllit mixtures, and Kocide gave outstanding control of olive knot development on lateral wounds and moderate control on leaf scars. Streptomycin resulted in excellent control of *Psv* on lateral wounds but not on leaf scars. This trial also exhibited systemic infections but not on plants treated with Kasumin, Kasumin-Syllit, Kocide, or streptomycin. Syllit alone gave some control of *Psv* on lateral wounds but not on leaf scars but has great potential when used in mixtures with other treatments (i.e., Kasumin). In summary, although reduction in disease by bactericidal treatments was not as high as previously, treatments still significantly reduced the disease from the control. Higher inoculum levels were used in these studies as compared to previous years and this may have impacted symptom development and efficacy of treatments. Under these conditions, antibiotic treatments were able to significantly reduce dieback providing some measure of control against severe symptoms caused by *Psv* infection.

4b. Field trials in the fall of 2013 using SAR compounds (i.e., Regalia, ProAlexin, Stout, Actigard, and Quintec) resulted in little or no control of *Psv* when a foliar application was done 3 days before wound-inoculation. Again, branch dieback and systemic movement of the bacterium was observed on cv. Arbequina. In previous studies, significant reductions in disease were found using SARs (e.g., Regalia, quinoxyfen, Actigard, Stout). Thus, other timings of these treatments and application methods may be effective and will be considered in future trials.

4c. Field trials on cv. Manzanillo olives were performed in the fall of 2013 to test the persistence of treatments under simulated rain conditions at UC Davis. Treatments included Kasumin, Kocide 3000, Kocide with lime and zinc, and Kasumin with Kocide. There was severe systemic movement in all treatments with symptoms such as knots, bumps, and blistering occurring at and distant from the initial inoculation point. Therefore, these treatments did not persist and additional additives need to be tested. Trials are ongoing with products reported to increase the persistence of copper in other crops (carnauba-based adjuvants, Pinolene-based adjuvants).

4d. Evaluation of cv. Arbequina field trials at UC Riverside that were initiated in the spring of 2013 revealed that Kocide alone and in mixtures with Manzate performed similarly well as in previous studies against *Psv* on both leaf scar and lateral wounds. Badge X2 and a combination of different coppers were similarly effective, but Kasumin and Kasumin-Manzate mixtures did not perform well in this study but performed well in previous trials. Inoculum concentrations used were higher than previously and thus, limits to the antibiotic may occur under high disease pressure. In discussions with regulatory officials, EPA indicated that the Manzate label cannot be expanded to allow use on other crops. Kasugamycin is a pending IR-4 project once the pome fruit label is approved (e.g. for fire blight). In late spring of 2014 a field trial was initiated to test the efficacy of several copper formulations, as well as the antibiotics streptomycin, oxytetracycline, and kasugamycin. Kocide alone and in mixtures with several new promising copper activity-enhancing compounds are also being tested along with a novel compound (ceragenin) with a unique mode of action against bacteria. In field trials at UC Riverside we did not observe *Psv* systemic movement and severe dieback that we found in trials at UC Davis and in Sutter Co. on cv. Manzanillo and to a lesser extent on cv. Arbequina. Again, our planned studies on the etiology of disease establishment will help to explain differences in symptom expression which could provide useful insights for developing better management strategies.

UNIVERSITY OF CALIFORNIA, DAVIS
Accounting Office
Extramural Accounting
Contractor's Invoice

Please include the following claim number on your remittance advice.
Claim No. XB05196

INVOICE TO

CA OLIVE COMMITTEE
770 E SHAW, SUITE 310
FRESNO, CA 93710

e-mail: denise@calolive.org

Invoice Number: 28022-2
Date: October 20, 2014
Amount: $9,990.00

Questions regarding this invoice should be directed to Eli Berici @ (530) 752-5618 or eberici@ucdavis.edu

Federal Employee ID # 94-6036494

Contract/Grant/Agreement/Purchase Order **# 201402161**

Period Billed: From **02/28/14** To **12/31/14**

Project Title: **Propagating Dwarfing Olive Rootstocks and Establishing a Long Term Orcha**
PI /Director: **Louise Ferguson**
Department: **Plant Sciences**

Description of Services			CURRENT
PER TERMS OF AGREEMENT			

Award Amount: **$24,975.00**

20% Due no later than July 15, 2014
The budget for the first 20% of this project has been spent on Labor and Materials

40% Due upon receipt of progress report $9,990.00

40% Due upon receipt of final report

Please Return Invoice Copy with Check **PAY THIS AMOUNT>>>>>** **$9,990.00**

Remarks:
OUTSTANDING INVOICES

Barbara Gerbrich for
James Ringo/eb Division Manager

Make Check Payable and Mail To:
The Regents of The University of California
Cashier's Office
University of California, Davis
P.O. Box 989062
West Sacramento, CA 95798-9062

To the best of my knowledge and belief this report is correct and complete and all outlays are for the purposes set forth in the award documents.

CALIFORNIA OLIVE COMMITTEE

CONCEPT PROJECT PLAN/ 2ND INTERIM REPORT

Workgroup/Department: Olive / Plant Sciences, UC Davis

Project Year 2014 Anticipated Duration of Project: One Year

Project Title:
Propagating Dwarfing Olive Rootstocks and Establishing a Long Term Orchard

Project Leaders:

Louise Ferguson, Extension Specialist, Department of Plant Sciences, 2037 Wickson Hall, Mail Stop II, UC Davis, 1 Shields Ave., Davis CA 95616, (530) 752-0507 [Office], (559) 737-3061 [Cell], LFerguson@ucdavis.edu

Dr. John Preece, Curator, USDA National Clonal Germplasm Repository, Davis CA. JAMiles@ucdavis.edu.Edu
Dr. Tziano Caruso, University of Palermo, Palermo, Italy: Tziano.Caruso@unipa.it
Dr. Guilana Marino, University of Palermo, Palermo, Italy: giulia.marino@unipa.it

Commodity: Olive Relevant AES/CE Project No.

Year Initiated: 2014 Current Funding Request: **24,975.00**

Problems and Significance:
To facilitate mechanical harvesting the newest table olive orchards are planted in hedgerows and require regular mechanical pruning to keep the trees small.

Such plantings and mechanical harvesting could be facilitated if, rather than cultivars propagated by cuttings and grown on their own roots, they could be grafted on dwarfing rootstocks. This could have favorable impacts on productivity in a manner similar to apples grafted onto dwarfing rootstocks.

The National Clonal Germplasm Repository for Tree Fruits, Nut Crops, and Grapes is a USDA-ARS facility that operates in collaboration with UC Davis and maintains the national collection of olives. The collection consists of more than 200 genetically different olives, some with slow growth and potential as dwarfing rootstocks.

Among those olives with promise for use a dwarfing rootstocks are: Nikitskaya (there are 60 rooted cuttings), *Olea cuspidate* (65 rooted cuttings), Verticillium Resistant Oblonga Seedling (68 rooted cuttings) and Dwarf D (20 rooted cuttings, more rooting is necessary). Additionally, our cooperators at University of Palermo and in University of Cordoba in Spain are willing to contribute promising cultivars.

We propose to propagate these rootstocks and test them for their dwarfing potential with 'Manzanillo' to produce a tree that is more amenable to mechanical harvesting.

Progress thus far:

Objective I: Propagation of trees:

Dr. John Preece successfully propagated Nikitskaya, *Olea cuspidate* Verticillium Resistant Oblonga Seedling and Dwarf D.

Objective II: Planting the orchard:

The orchard was planted May 19-21, on the north side of Hutchinson Road the UC Davis campus. The block was split in to 4 replications of 13 tree rows of the following rootstocks, Nikitskaya, *Olea cuspidate,* Verticillium Resistant Oblonga Seedling and Dwarf D. Two rows of Manzanillo were also included, one to be self grafted with Manzanillo and another to remain ungrafted. The purpose is to determine if grafting has any effect on dwarfing. An excel map is attached. Sevillano pollinizer rows are planted between each N-S replication and E-W between the two different spacing plots and in all the border rows. The trees were staked, tree wraps installed and an irrigation system with 1, 2 gallon per hour dripper per tree installed. Figure 1 is the orchard on August 8, 2014.

The trees will be grown through the summer and fall of 2014 and grafted in the spring of 2015 by Dr. John Preece's staff.

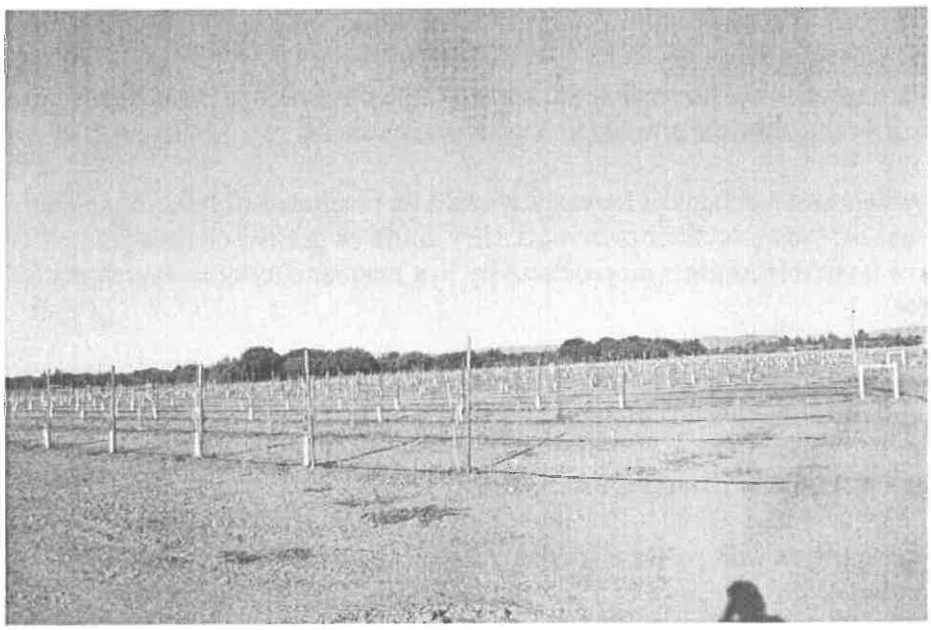


Fig. 1. Dwarfing olive orchard on August 8, 2014.

UNIVERSITY OF CALIFORNIA, DAVIS
Accounting Office
Extramural Accounting
Contractor's Invoice

Please include the following claim number on your remittance advice.
Claim No. XB05193

INVOICE TO

CA OLIVE COMMITTEE
770 E SHAW, SUITE 310
FRESNO, CA 93710

e-mail: denise@calolive.org

Invoice Number: **28003-2**
Date: October 15, 2014
Amount: **$7,294.80**

Questions regarding this invoice should be directed to Eli Berici @ (530) 752-5618 or eberici@ucdavis.edu

Federal Employee ID # 94-6036494

Contract/Grant/Agreement/Purchase Order **# 201402127**

Period Billed: From **01/15/14** To **12/31/14**

Project Title: **Investigation of High Temperature Effects on Olive Fruit Set: Structural and Developmental Aspects of Pollen Tube Growth**
PI /Director: **Judy Jernstedt**
Department: **Plant Sciences**

Description of Services			CURRENT
PER TERMS OF AGREEMENT			

Award Amount: **$18,237.00**

20% Due no later than July 15, 2014
The budget for the first 20% of this project has been spent on Supplies and Expenses

40% Due upon receipt of progress report $7,294.80

40% Due upon receipt of final report

Please Return Invoice Copy with Check **PAY THIS AMOUNT>>>>>** **$7,294.80**

Remarks:
OUTSTANDING INVOICES

Barbara Gold for
James Ringo/eb Division Manager

Make Check Payable and Mail To:
The Regents of The University of California
Cashier's Office
University of California, Davis
P.O. Box 989062
West Sacramento, CA 95798-9062

To the best of my knowledge and belief this report is correct and complete and all outlays are for the purposes set forth in the award documents.

Progress Report - July 2014

Project Leader: Judy Jernstedt, Professor Location: 212 Hunt Hall

Mailing Address: Plant Sciences, Mail Stop 1, UC Davis, Davis, CA 95616

Phone: 530-752-7166 FAX: 530-752-4361 E-mail: jjernstedt@ucdavis.edu

Project Title: Investigation of high temperature effects on olive fruit set: Structural and developmental aspects of pollen tube growth (Research Priority: PGRs, with Focus on Pistils)

Cooperating Personnel: **Louise Ferguson**, Extension Specialist, Department of Plant Sciences, 2037 Wickson Hall, Mail Stop II, UC Davis, 1 Shields Ave., Davis CA 95616, (530) 752-0507 [Office], (559) 737-3061 [Cell], LFerguson@ucdavis.edu

Keywords: pistils, style, stigma, pollen, pollen tube, fruit set

Spring 2014: Beginning 21 April 2014 and continuing through 1 May 2014, we emasculated and bagged perfect flowers of six mature Manzanillo olive trees located on Hutchison Drive, Davis, CA 95616. A total of 180 tissue paper bags were attached, each containing 1or 2 emasculated flowers. At the end of the bagging, an unseasonably hard rain hit Davis and most of the bags were torn open by subsequent strong wind. Emasculated flowers in the eight surviving intact bags were pollinated with Manzanillo pollen.

At intervals during the first half of May, hand-pollinated and open-pollinated Manzanillo flowers were collected, chemically fixed, and dehydrated in an ethanol dilution series. They are now ready to be processed for embedding and sectioning. In addition, multiple flowers at six developmental stages (unopened bud, open flower with closed anthers, anthers split open, etc.) were collected from a single tree of Morcal (tree A-9-3) olives growing at the USDA Clonal Germplasm Repository Wolfskill orchard in Winters, CA. These samples were fixed and dehydrated, and are now ready to be processed for embedding in resin for sectioning and staining (histochemical and immunocytochemical staining). The undergraduate lab assistant is working 10-15 hours per week to accomplish the sectioning and staining part of the project. The goal is to have some results and photomicrographs by the middle of August 2014.

Sub Fund Summary by Consolidation (FIS55)

DaFIS Decision Support >> Fund Source Summary Reports >> Sub Fund Summary by Consolidation >> Report Output

Jump to account: 3-9228003 | 3-28003UB | 3-APSPO12

Through Fiscal Period: October, 2014
Chart(s): 3,L,S
OP Fund: 28003

Previous Month Next Month
Totals On This Report Are: Year To Date (click to toggle)

Sub Fund Group	OP Fund Account	Obj. Consol			Appropriation	Expenditure	Encumbrance	Balance
PRCONT - PRIVATE CONTRACTS								
28003 - CALIFORNIA OLIVE COMMITTEE-201402127		OP Fund Award #: 201402127		Award End Date: 12/31/2014 Fund Manager: DAWA,ROBERT HENRY				
	Originating Office:OVCR-201402127							
	3-9228003							
	Account Manager: DAWA,ROBERT HENRY	INC:CALIFORNIA OLIVE COMM		PI: JERNSTEDT,JUDY				
	Higher Ed.:	NIH:	Pmt. Method: 50	ICR: D000				
	Acct. Award #: 201402127	Acct. Award End Date: 12/31/2014		LOC Group:				
		INC0	INCOME		18,237.00	3,647.40CR	0.00	14,589.60OD
	Total Account:				18,237.00	3,647.40CR	0.00	14,589.60OD
	3-28003UB							
	Account Manager: DAWA,ROBERT HENRY	UBA:CALIFORNIA OLIVE COMM		PI: JERNSTEDT,JUDY				
	Higher Ed.:	NIH:	Pmt. Method: 50	ICR: D000				
	Acct. Award #:	Acct. Award End Date: 12/31/2014		LOC Group:				
		BLSH	BALANCE SHEET		0.00	0.00	0.00	0.00
	Total Account:				0.00	0.00	0.00	0.00
	3-APSPO12							
	Account Manager: CLEARWATER,KERRY P	PS: COC: 13-14: JERNSTEDT		PI: JERNSTEDT,JUDY				
	Higher Ed.: ORES	NIH:	Pmt. Method: 50	ICR: B110				
	Acct. Award #: 201402127	Acct. Award End Date: 12/31/2014		LOC Group:				
		SUBG	GENERAL ASSISTANCE		10,000.00CR	289.56	0.00	9,710.44CR
		SUB3	SUPPLIES AND EXPENSE		6,000.00CR	2,687.42	865.17	2,447.41CR
		SUB5	TRAVEL		300.00CR	0.00	0.00	300.00CR
		SUB6	EMPLOYEE BENEFITS		130.00CR	3.76	0.00	126.24CR
	Account Direct Costs Sub-Total:				16,430.00CR	2,980.74	865.17	12,684.09CR
		INDR	INDIRECT COSTS		1,807.00CR	327.89	0.00	1,479.11CR
	Total Account:				18,237.00CR	3,308.63	865.17	14,063.20CR
	Total Fund 28003:				0.00	338.77CR	865.17	526.40OD
Total Sub-Fund Group:					0.00	338.77CR	865.17	526.40OD
Total Groups:					0.00	338.77CR	865.17	526.40OD

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Billing For Fixed Installment Payment

JV # 34098967

PR-

Template: /DecisionSupport/financial/subfundsummary.cfm ($Revision: 1.7.2.9 $$Date: 2013/05/22 23:28:19 $)
Page Generated At: 15-Oct-14 03:50 PM

DS Release 7.6

Determination of Ethanol in Table Olives as a Simple, Rapid and Dependable Method to Detect Low-Quality Olives and Development of a Standard to be used by the USDA Inspectors

Alice Tang, Selina C. Wang

Funding Source: California Olive Committee

Abstract

It has been found that US consumers have low acceptance to vinegary, alcohol and gassy sensory notes in black ripe table olives. These sensory notes are suspected to be related to fermentation products, such as ethanol. Ethanol is proposed to be an indicator for defects in black ripe table olives. A SPME-GC-MS method is developed and optimized to determine the ethanol concentration in black ripe olive. The ethanol concentrations are below the quantification limit (21.0µg/g) in the samples with defective sensory notes. This suggested that ethanol may not be an appropriate marker for the defects in black ripe olives. In the attempt to find other possible chemical indicators for defective sensory notes, volatile profiles are determined and evaluated. It is found that high concentrations of nonanal (1385-3218 ng myrcene /kg olive) and no styrene are detected in the normal samples, while significantly lower concentrations of nonanal (201-615 ng myrcene /kg olive) and high level of styrene (372-6502 ng myrcene /kg) are found in the defective samples. These results suggest that nonanal can be a positive standard for black ripe olives, and styrene should be monitored to differentiate defective samples.

Introduction

Black ripe table olives are an important product in Mediterranean diet. It is also a widely-used ingredients used in pizza, salad or as snacks. United States Department of Agriculture (USDA)'s standard for the quality of black ripe olives is vaguely defined as "reasonably good flavor". The enforcement of the "good flavor" relies on the individual inspector's sensory judgment. Lack of adequate tools to screen the quality of black ripe olives limits the ability of the agency to identify defective products sold in US. A sensory panel seems to be a good solution for this issue. However, the trained sensory panel is expensive and time-consuming. A chemical marker the most promising rapid and inexpensive tool for USDA to regulate the quality of black ripe table olives.

Flavor is the main reason for the consumers to purchase or reject the product. It has been shown that US consumers generally dislike imported black ripe olives with vinegary, alcohol, metallic, gassy and soapy/medicinal flavors (Lee 2012). Sansone-Land (2014) has identified seemingly fermentation-derived volatiles in imported black ripe olives, such as ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutyl acetate, ethyl hexanoate, Z-3-hexenyl acetate, hexyl acetate. Ethanol is typical fermentation compound. Its level is significantly elevated after olive fermentation (Sabatini, 2007, Sabatini, 2009). The hypothesis in this study is that ethanol is an indicator for defective black ripe olives. The goal is to develop and optimize the method to determine the concentration of ethanol in black ripe olives, and correlate the level of ethanol to the sensory evaluation.

Method

Sample Preparation for Ethanol Determination

30g olive samples are accurately measured and added to 50mL Milli-Q water. 75µL of internal standard (1mL of D_6-Ethanol in 9ml of water, corresponding to an internal standard concentration of 90mg/mL) is added into the olive and water mixture. The mixture is blended in Waring Blender for exactly 1 minute. The olive slurry was transferred to a 500mL amber bottle. The blender was rinsed with 100mL Milli-Q

water and transferred to the bottle. After 54g (saturated) NaCl (previously heated at 120ºC for 5 hours to remove volatiles) was added to the slurry, the mixture was stirred for 5 minutes to dissolve the salt. 50mL slurry was transferred to a 150mL amber screw-top headspace bottle, and sealed with a PTFE-silicon septum. A SPEM fiber (DVB/CAR/PDMS, 2cm) was inserted into the headspace through septum. The SPEM was performed for at room temperature 60 minutes. Then the fiber is injected in the gas chromatography coupled with a mass spectrometry (GC-MS)

Sample Preparation for Volatile Profile

The preparation procedure follows the sample preparation for ethanol determination. The only difference is that 25µg myrcene (25 µL of 10 µg/mL myrcene solution) are added as internal standard.

GC-MS Detection

A DB-1 (60 m × 0.25 mm i.d.) fused silica capillary column was employed. The oven temperature was programmed from 40ºC (5 min isothermal) to 220ºC at 5 °C/min (hold for 5 min), and then raised to 300ºC at 20 °C/min (final hold for 5 min). The system utilized helium gas at a flow rate of 1.0 ml/min. Splitless injection was used, and the injection temperature is 260 ºC.

The ion trap MS was operated in electron impact mode at 70 eV. A scan range of m/z 40–400 at 0.4 scans/s was employed. m/z 45 and 49 are used to quantify ethanol and D_6-ethanol, respectively.

Identification of table olive volatile compounds

Volatile compounds are identified by comparing the compound's mass spectra and experimental Linear Retention Index (LRI) with that of authentic standards. When the standards are not available, the identification are assigned based on the spectra reported in NIST Mass Spectral library 2005, and the LRI reported in the literature.

Quantification for table olive volatile compounds

For the quantification of ethanol, mass to charge ratio m/z 45 and 49 are the most abundant peak for ethanol and D_6-ethanol, respectively. Therefore, there are chosen to represent ethanol and D_6-ethanol, respectively. The quantification of ethanol is calculated as follows:

$$\text{Concentration of ethanol} = \frac{Area\ of \frac{m}{z}45}{Area\ of \frac{m}{z}49} \times concentration\ of\ D6 - ethanol$$

For the quantification of other volatile compounds, the ratio of volatile compounds/internal standard (myrcene) ratio is used to calculate the concentration of volatile compounds.

Result and Discussion

Method validation for ethanol determination

SPEM Time The effect of five SPEM absorption times are tested in this study: 10, 30, 60, 90 and 120 minutes. At 10mins, the peak to noise ratio is below 3, indicating it is below quantification limit. The results of 30, 60, 90 and 120 minutes are shown in the Figure 1. It is clear that 1hour gives the best yield of ethanol. Therefore 1 hour is selected to use in the final method.

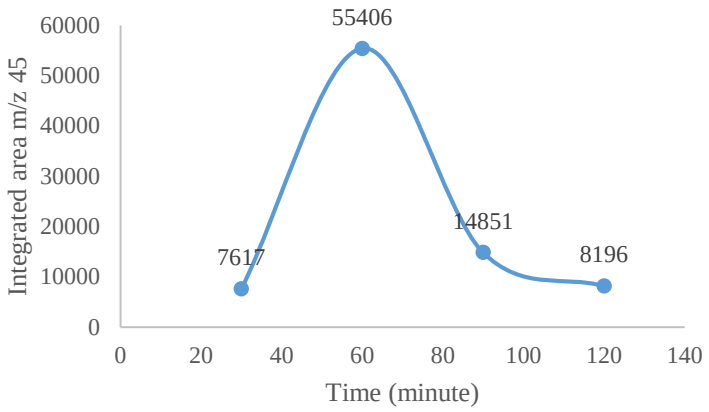


Figure 1. The effect of time on the m/z 45 integrated area

SPEM Temperature The effect of four temperatures are investigated: 20, 30, 45 and 65°C. The effect of temperature is shown in Figure 2. It is clear that in the tested range, as the temperature increases, the integrated area of m/z 45 decreases, indicating decreasing amount of ethanol is absorbed by the SPEM fiber. Therefore 20ºC (room temperature) is selected to use in the finalized method.

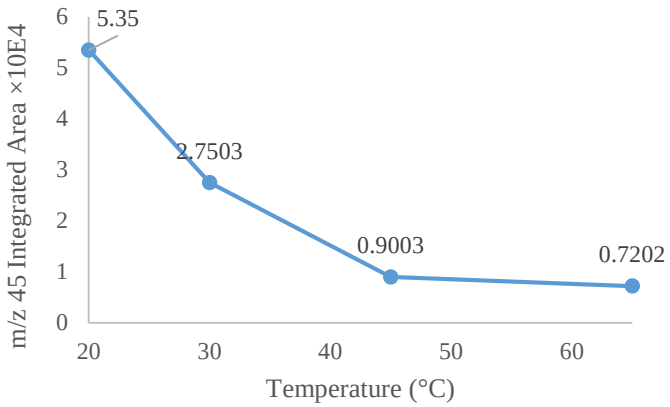


Figure 2. The effect of temperature on the area of m/z 45

Effect of Salt The effect of two salts are investigated in this study: NaCl and Na_2SO_4. Both salts are added to the olive slurry such that they reach the saturation level (54g NaCl and 28g Na_2SO_4, respectively). The "salt-out" effect of salts releases volatiles from the olive cell. The results are shown in Figure 3, which shows that NaCl yields 4.25 times more of ethanol than Na_2SO_4. As a result, NaCl is chosen for the final method.

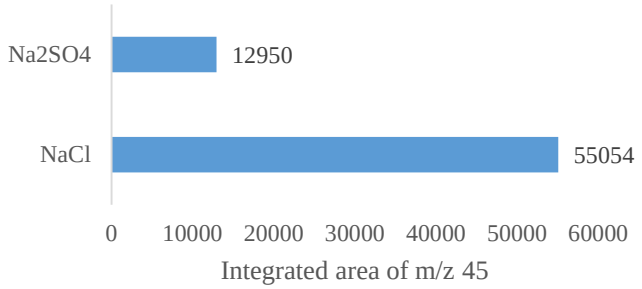
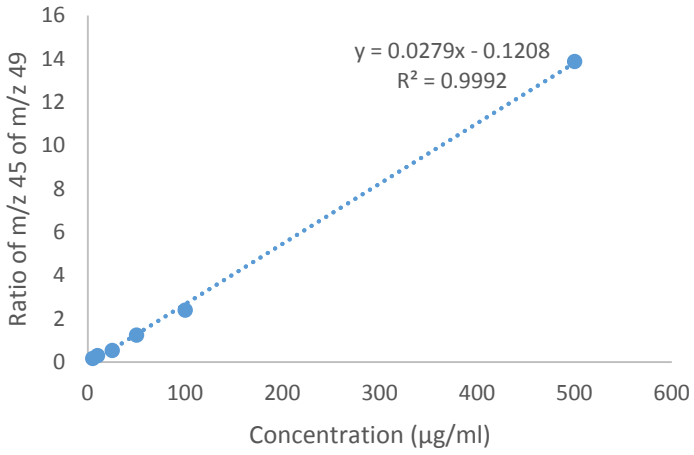


Figure 3. The effect of salts on the integrated area of m/z 45

Preliminary Accuracy and Repeatability The preliminary accuracy is determined by spiking 0.3mL of ethanol stock solution (2.77mg) to the olive slurry, and comparing the ethanol concentration before and after the spike. The result shows that the accuracy of the method is 90%.

The preliminary repeatability is determined by using 3 replicates of standard solution. It is found out the relative standard deviation is 0.2%.

Calibration curve The calibration curve is shown in Fig 4. The coefficient is 0.992. The curve is linear is in the range of 5-500 µg/ml.



Figureure 4. The calibration curve

LOD and LOQ The instrumental LOD and LOQ are defined as the amounts of ethanol at S/N ratio 3:1 and 6:1. Based on this definition, the LOD and LOQ are 2.1 µg/ml and 4.2 µg/ml. The method LOD and LOQ are 10.5 µg/g and 21.0 µg/g.

Determination of ethanol in black ripe table olives

10 black ripe tables are obtained from US supermarket. Ethanol in the 10 black ripe table olive samples were determined using the method described above. The results are shown in Table 1. Not detected indicates that the ethanol level is below LOD. Detected indicated that the ethanol level is above LOD but below LOQ. No ethanol levels in the 6 olive samples exceeds the LOQ.

Olive Type	Ethanol level (µg/g)	Sensory
Early California Large Sample 1, black ripe	Not detected*	Normal
Early California Large Sample 2, black ripe	Not detected	Normal
UC Davis, black ripe	Not detected	Normal
Pearls Medium, black ripe	Not detected	Normal
Early California Medium, black ripe	Not detected	Normal
Spanish Black Ripe	Detected**	Winey, vinegary
"Oloves" black ripe olives, chili and garlic	82.06	Chili, oily
365 sliced Black Olives	Detected	Fusty
"Sun Valley" Black Ripe Olives	Detected	Fusty, earthy
Whole Olives, green olives	102.67	Winey, acetic acid

*Not detected indicates the concentration of ethanol is lower than LOD.
**Detected means the concentration of ethanol is lower than LOD and higher than LOQ

Table 1. Ethanol level in 6 black ripe table olives.

Whole Olives are the only green olive, while the rest of the olives are black ripe olives. As shown in the Table 1, "Oloves" black ripe olives contains 82.06 µg/g of ethanol. However, due to the fact flavors and chili stuffing was added to "Oloves", the amount of ethanol doesn't necessarily generate from the olive. Whole Olives is a fermented green olives. Lactic acid bacteria is introduced to the olives, and forms significant amount of ethanol during fermentation. This result agrees with the research conducted by Sabatini (2007). The concentrations of ethanol in 5 olive samples are lower than the limit of detection (Early California Large Sample 1 and 2, UC Davis Black ripe, Pearls Medium and Early California Medium). None of the 5 samples show any defective sensory note. In Spanish Black ripe, 365 sliced black olives and "Sun Valley" black ripe olives, the concentration of ethanol is higher than limit of detection, but lower than limit of quantification, indicating the concentrations are between 10.5 µg/g and 21.0 µg/g. Though defective sensory notes can be observed in those samples, the low concentration of ethanol indicates that ethanol may not be the final products in fermentation, and ethanol may not be an appropriate chemical marker for fermentation defect in black ripe olives.

Determination of other possible fermentation products

In the attempt of finding possible volatile fermentation products, 7 samples are evaluated for their volatile profiles. The 7 black ripe olives are either obtained from the US market or kindly given by the producers. The sensory notes and the volatile concentrations of each sample are shown in Table 2. Among the 7 samples, no sensory defects are observed in the Sample 5 and Sample 7, while the other samples show some of vinegary, moldy, musty and fusty sensory notes. In a consumer study on the black ripe olives conducted by Lee (2012), it was found that US consumers have low acceptance to vinegary flavor. Fusty and musty are common defects in olive oil. Except for Sample 6, styrene is not detected in Sample 5 and 7, which has no sensory defect, while it is present in the Sample 1, 2, 3 and 4 in the concentration of 372-6502 ng myrcene /kg olive. This indicate that styrene can be a possible indicator of the defective samples. Nonanal concentration in the normal samples are 1385 and 3218 ng myrcene /kg olive, while in the defective samples, the concentration of nonanal is from 201 to 615 ng myrcene /kg olive. The significant difference in nonanal between the normal and defective samples suggest that nonanal can be used to differentiate the normal from defective samples.

Compound	LRI	LRI ref	Concentration (ng myrcene/kg of table olive)						
			S 1	S 2	S 3	S 4	S 5	S 6	S 7
			a little vinegary	moldy, musty	vinegary, musty	vinegary, fusty, moldy	normal	fusty, moldy	normal
Acetaldehyde	<700	400	22	57	246	29	156	54	113
2-propanol (Tentative)	<700	475	1143	1079	7212	1570	9497	2401	6179
3-methylpentane (tentative)	<700	576	79	111	1381	73	120	112	125
3-methylbutanal	<700	636	304	ND	162	161	345	557	ND
2-methylbutanal	<700	646		ND		118	728		21
E-2-butenol	<700	643	ND	ND	167	ND	ND	ND	17
Toluene	734	748	ND	40	67	24	1159	20	ND
Dimethyl-Silanediol	748	Unknown	ND	369	1075	207	ND	217	88
5-methyl-1-heptanol	767	Unknown	ND	ND	233	ND	67	ND	ND
Octane	778	800	78	713	6675	288	5266	338	1427
Furfuryl alcohol	810	837	5	10	ND	25	33	ND	ND
Xylene	838	857	5	45	69	61	81	ND	95
Styrene	855	873	372	6502	1464	691	ND	ND	ND
Heptanal	861	876	ND	ND	ND	ND	ND	1217	ND
Benzaldehyde	920	926	1687	10832	8287	2531	3363	1942	2007
2-pentylfuran	961	977	ND	ND	492	ND	133	ND	ND
Myrcene (I. S.)	961	981	833	833	833	833	833	833	833
Hexyl acetate	968	997	58	92	100	ND	450	267	300
α-terpinene	971	1006	ND	ND	ND	44	ND	ND	ND
Limonene	995	1020	31	109	356	39	100	ND	94
Beta-E-ocimene	1021	1041	ND	ND	233	ND	ND	ND	ND

Octanol	1037	1062	ND	ND	92	ND	ND	ND	ND
2-methoxyphenol	1050	1063	ND	34	377	33	ND	ND	22
Methyl benzoate	1056	1067	ND	ND	2551	ND	ND	ND	ND
Nonanal	1065	1083	201	247	475	427	3218	615	1385
E-4,8 dimethyl-1,3,7-nonatriene	1081	1106	117	19	5033	344	457	ND	675
4-propyl pyridine	1099	1024	ND	61	ND	28	ND	42	41
Ethyl benzoate	1134	1143	ND	ND	12295	72	233	873	40
4-methylguaiacol	1149	1164	67	ND	0	103	334	ND	ND
Hexyl butyrate (Tentative)	1158	1175	ND	34	86	ND	66	ND	112
Ethyl octanoate	1164	1182	13	261	655	81	109	ND	9
Decanal (Tentative)	1174	1184	40	13	8019	251	9788	ND	5647
2-decenal	1214	1236	ND	ND	306	ND	249	ND	86
Propyl benzoate	1222	1242	ND	ND	3408	ND	ND	ND	ND
Vitispirane A	1233	1263	ND	20	ND	14	106	50	ND
cyclosativene	1334	1367	9	ND	ND	48	66	ND	ND
α-copaene	1341	1374	180	68	641	423	427	ND	150
ethyl-decane (Tentative)	1361	Unknown	ND	15	ND	ND	52	ND	ND
α-muurolene	1457	1492	20	26	132	49	69	ND	ND

Table 2. The sensory notes and volatile concentrations in 7 black ripe olives

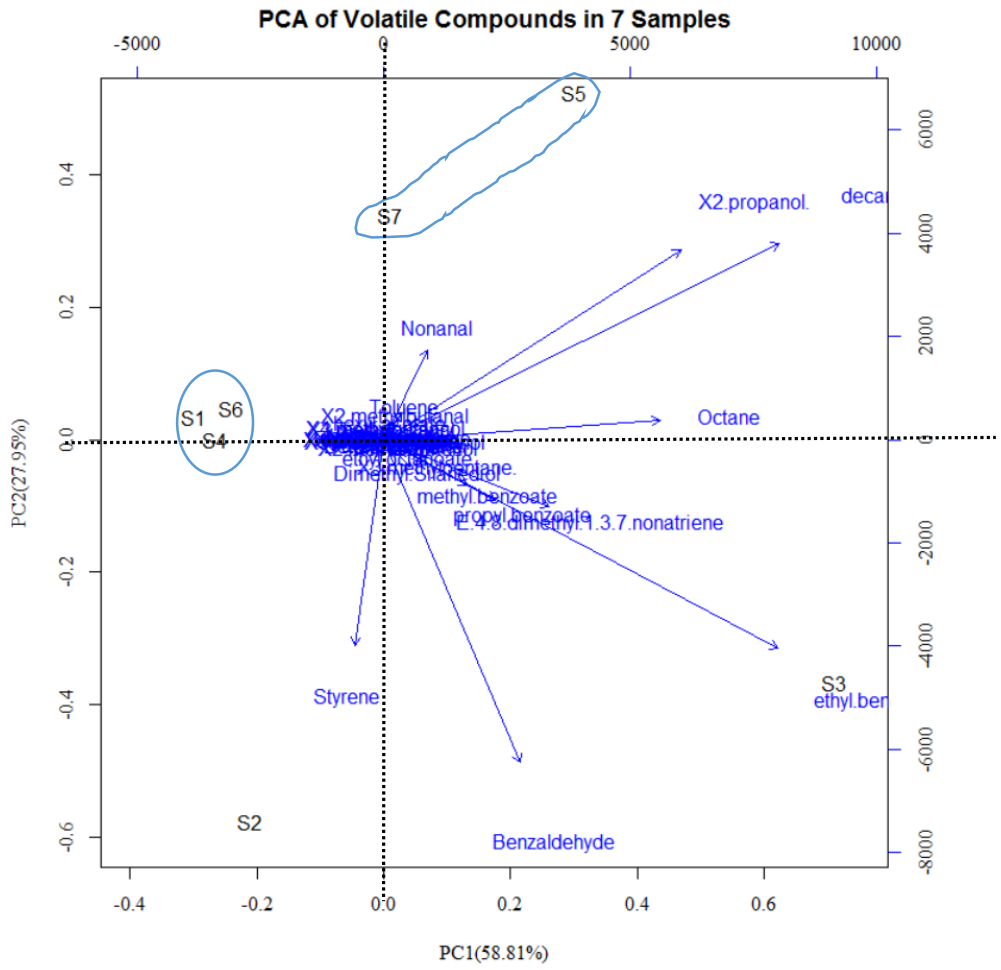


Figure 4. Principle Component Analysis on the 7 black ripe olives

A Principle Component Analysis (PCA) is applied to the volatile profiles in the 7 black ripe olive samples. The first and second principle components cover 58.81% and 27.95% of variance. As is shown in the Figure 4, S1, S4 and S6 are very close on the plot, indicating that their volatile profiles are similar. Similarly, S5 and S7 can be grouped together. Normal samples are present in the first quadrant, and the defective samples appear in the rest of the quadrants. Vector of nonanal, 2-proponol, octane, decanal and octane are in the first quadrant, which the normal samples are located. Nonanal directly points towards the normal samples, which suggests that it can be potentially used for positive standard of normal samples. The vector of nonanal and styrene points at opposite direction, indicating that they are reversely related to each other. Sensory note of styrene is described as gassy and pungent plastic-like, which has been found in the consumer study to be a disliked attributes among US consumers. Styrene can possibly be a marker for defective samples.

We are currently working on obtaining more olive samples, especially defective ones in order to confirm our findings mentioned above.

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