# 2007-2008

HARC

# Hawaii Agriculture Research Center


# ANNUAL REPORT

# On the Inside



**Front cover images, clockwise, from top right corner:**

(1) Coffee flowers with bees
(2) Crew collecting soil from US Army firing range
(3) Anthurium flowers
(4) Cover crop project field day with Sunn hemp in foreground
(5) Papayas in market display
(6) Lance Santo planting lettuce trial

**Background Image:**

Cultivars of Hawaiian sugarcane (*Saccharum officinarum*) - photo courtesy of Maui Nui Botanical Gardens, Kahului, Maui


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# Director's Letter

**Stephanie A. Whalen,**
*President and Director since 1994*

To our Board of Directors, Members, Clients and Friends,

This years' 2007 and 2008 report marks a milestone event in this organization's 114 year history. During this two-year period Gay and Robinson, Inc., one of two remaining sugar companies in Hawaii, struggled to find a suitable partner to go forward as a sugarcane/energy business. While at times looking favorable, the collapse of the ethanol market and the economy put an end to this effort. In October 2008, Gay and Robinson, Inc. announced its closure and terminated its support for Hawaii Agriculture Research Center (HARC). The remaining company, the Hawaiian Commercial & Sugar, Co. farms over 34,000 acres in sugarcane on the island of Maui. While providing renewable energy for decades and continuing to maintain an active interest in other forms of renewable energy, viable alternatives remain elusive. Sugar yields have suffered at both companies over the past two years due to a prolonged drought in the islands, high costs of inputs, and labor shortages.

HARC has moved to evolve its business structure from a trade organization to a non-profit scientific organization with a mission focused on the greater agricultural community rather than just one commodity sector. It continues to provide sugarcane crop improvement but is expanding that effort to include breeding for the national sugarcane program. HARC is fortunate to have its breeding station located where crosses can occur naturally without the aid of expensive environmental chambers as required by other national and international programs. Thus with minimal inputs, the number of annual crosses can far exceed those from other locations, potentially accelerating the opportunity for variety development not only for sugar yield but also for biomass production. This report provides an update on HARC's efforts to increase high-value protein expression for improvement of the economics of co-products from sugarcane.

We are continuing with the 100 years ago report. Don't miss it! In 1907, many significant events occurred including the organization's concern over deforestation. It hired Dr. Harold L. Lyon to begin reforestation projects many of which remain in place today. There was the first successful sugarcane seedling production, immigration of Spanish labor and the start of the University of Hawaii to name a few. In 1908, Hawaiian paniolo (cowboys) made history in world competition steer roping. Hawaii sugar tonnage increased by ~16% over the previous record; mocha coffee seeds were imported; and the Pineapple Growers' Association was organized.

Efforts in coffee improvement continued with the growers input and three top varieties were selected. These varieties were planted in grower's field for observation and further evaluation. Nematode resistant coffee plants were imported and continued to show resistance in trials compared to susceptible local varieties. These imported varieties will be used as rootstock for the current commercial varieties in field trials. Two genes, CaPOP1 and CaPOP2, related to coffee organ size have been cloned. Work with these suggests that the expression difference observed between the large bean and the small bean varieties is caused by an upstream regulatory mechanism.

Commercial production of cacao in Hawaii is increasing from trees previously introduced into the islands. However, the quality and yield are variable and unpredictable. Continuing the collabora-

tion with the USDA-ARS/SHRS, molecular analysis is being used to identify cacao genotypes across the state to assist farmers in selection of superior cultivars based on their parentage pedigrees. This work has established that there is a diverse variety of cacao germplasm in Hawaii that can be used for development of superior cultivars. Preliminary work in fermentation studies have led to promising microorganism cocktails that result in reproducible uniquely flavored chocolate. Chocolate from these trials have been judged by expert tasters to be commercially desirable.

Collaboration with the USDA Pacific Basin Agricultural Research Center (PBARC) and the Hawaii Anthurium Grower Association continues with transformed plants being tested in the greenhouse for nematode and bacterial wilt resistance. Micropropagation improvements are being developed to speed the availability of any new resistant cultivars developed.

HARC's forestry work has generated interest, especially regarding Fusarium wilt. Highly virulent isolates of the pathogen are being used to screen koa seedlings in a search for resistant trees. Non-pathogenic strains that extensively colonize koa roots may prove useful for biological control. Significant progress continues to be made in identifying naturally resistant families throughout the state that can be used for reforestation and commercial operations.

Included in this year's report are efforts to increase local produce production for lettuce by demonstrating the availability of *Tomato spotted wilt virus* resistant varieties and to improve the efficiency of banana micropropagation for production of *Banana bunchy top virus*-free starting plants.

Biofuels have long been of interest to the sugar industry and the state. Preliminary efforts are reported on several potential crops.

Several papaya projects concern the continuing effort to find molecular solutions for disease and pest problems. Alternative planting methods to reduce costs and increase production are being evaluated.

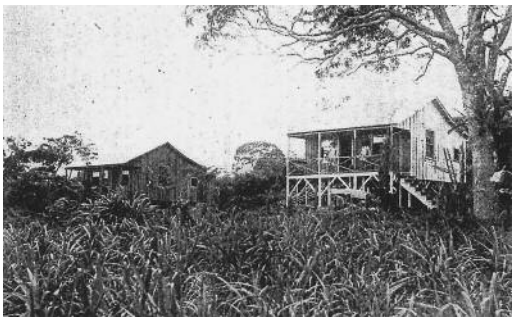
Thanks to HARC's staff and their continuing commitment to science and its role in improving the business of agriculture in Hawaii. Thank you to HARC's Board of Directors and their commitment to agriculture and the future of this organization. Without these two committed groups of supporters this organization would not be able to continue to serve agriculture as it evolves and diversifies.

Respectfully,

Stephanie A. Whalen
President and Director

# 100 Years Ago: 1907

The year 1907 saw the consolidation of the Hawaiian Sugar Planters' Association Experiment Station departments into one entity and the establishment of the sugarcane library which eventually became the largest sugarcane library in the world. The initial library collection contained 1250 volumes and numerous other papers and pamphlets. It would require the continued dedicated efforts of the first HSPA librarian, Mr. Kirkaldy, to organize and catalogue the materials.



The Experiment Station at that time consisted of the Agriculture and Chemistry Department, headed by Mr. Noel Deerr, and the Pathology and Physiology Department, headed by Dr. Nathan A. Cobb. That year Dr. Cobb resigned to take a position with the USDA and was replaced by Mr. L. Lewton-Brain. A new botanist, Dr. Harold L. Lyon, was hired who subsequently became renowned in the state for his efforts in preserving the native flora and forests of Hawaii. Lyon Arboretum was named for him.

Improvements in sugar crystallization and refining were made. Previously, cane juice was stored in a series of tanks and dried with wood fires to evaporate it down to sugar. But this was slow and used up a great deal of wood. Mills began adopting the "crystallization in motion tanks," a much more efficient method. That year also saw the establishment of the first sugar refinery in the Territory of Hawaii at Honolulu Plantation in Aiea. It successfully produced the first run of white granulated sugar.

Of special interest to sugarcane breeders was the first successful production of seedlings from biparental crosses. Five hybrid seedlings were obtained from this "exceedingly delicate operation".

It was reported that the Hawaiian Mahogany Lumber Co. had contracted to produce 500,000 railroad ties for the Atchison, Topeka, and Santa Fe Railroad Co. These ties were to be cut from native Hawaiian Ohia forests. It seems incredible today that stands of these large native hardwood trees could have been cut in such quantity for such a use. However, even at that time the Report of the Committee of Forestry at the 27th annual meeting of the HSPA expressed its grave concern over the loss of Hawaiian native timber trees, the shortage of wood for the islands, and the damage caused to watersheds. It concluded that the only possible solution was the immediate establishment of reforestation projects in the state. Dr. Lyon became the leading botanist for Hawaiian reforestation.

The Territory of Hawaii and sugarcane representatives were visited by a party of US Congressmen and by Secretary Straus of President Theodore Roosevelt's cabinet. It was hoped that "a better understanding of Hawaii's necessities should prevail with the powers that be at Washington, and it is to be earnestly hoped that some good will result".

Over two thousand Spanish immigrants from Malaga arrived in the islands to work on plantations. Housing was provided for them (see photo).

The College of Agriculture and Mechanic Arts was founded. It was renamed the University of Hawaii in 1919 and is now our state university.

The Maui courthouse was built in Wailuku, Maui and is still standing. The Coca Cola bottling company was established in Honolulu and the Iwilei pineapple cannery was completed by Dole.

*- S. Schenck*

# 100 Years Ago: 1908

The "Tunguska Event" meteor impact in Siberia explosion was equivalent to 1,000 Hiroshima bombs. On June 30, 1908, a ball of fire exploded about six miles above the ground in the sparsely populated region. The blast released 15 megatons of energy and flattened 770 square miles (2,000 square kilometers) of forest.

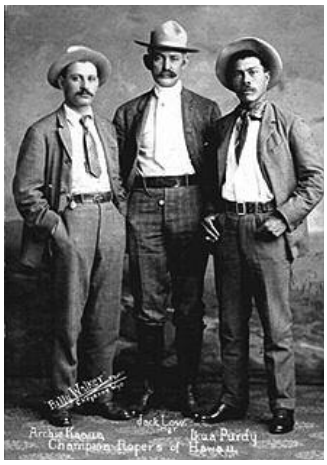
The Great New York to Paris round-the-world automobile race, an epic test of man and machines, took place in 1908. It was won by the American Thomas Flyer automobile driven by George Schuster.

This was a very successful year for the sugar industry with record yields. The total raw sugar produced was 521,123 tons, while the previous high was less than 450,000 tons. The yields were thought to be due mainly to judicious field work and to soil treatments with lime and fertilizer. There was also better use of advanced milling and boiling equipment. “Too much praise cannot be given to the continued good work accomplished by the Experiment Station - -” . The Division of Entomology continued with its successful campaign of controlling injurious insects by means of their natural enemies. In addition to its work with sugarcane, the Division of Pathology and Physiology initiated research into pineapple and forest tree diseases.

The Hawaiian Pineapple Growers' Association was organized for the improvement of methods of cultivation and marketing of pineapples. A campaign was undertaken to popularize consumption of the fruit.

Over 1000 mocha coffee seeds were brought to Hawaii from Mexico and planted by the Board of Agriculture and Forestry.

The US Congress decided to establish a naval base at Pearl Harbor.



Three Hawaiian paniolo, Ikua Purdy, Jack Low and Archie Kapua, carried off top prizes in the world famous Cheyenne Frontier Days Rodeo in Wyoming. The crowds went wild. The events leading up to the 1908 Cheyenne Frontier Days rodeo, the competition itself and the Hawaiians' victory have fostered legends. Ikua Purdy, secured the 1908 title of World Champion Steer Roper when he stunned his mainland opponents and 20,000 spectators by snaring a steer in a record 56 seconds.
Also of note: the Ford Model T was introduced, SOS became the standard radio distress symbol and Mother's Day was celebrated for the first time.

*- S. Schenck*

# Sugarcane Research

## *2007-08 Sugarcane Breeding and Selection*

Sugarcane breeding remains a vital and necessary activity for the long term success of Hawaii's sugarcane industry. HARC continues to develop and test new cultivars for commercial production in a breeding program that dates back to 1905. During this long period new cultivars have helped the sugarcane industry to survive major disease outbreaks, drought, and other factors that contribute to yield decline. Weather conditions and and skyrocketing costs of inputs have resulted in the decline of the sugar industry in Hawaii and the mainland US. This has led to renewed emphasis on collaboration among US domestic breeding stations. The HARC sugarcane breeding program will be increasingly integrated into a national system with shared facilities, personnel, and germplasm. There remains strong interest in collaborative basic research for the development of sugarcane as an alternative biofuel.

Between November 26, 2007 and January 7, 2008, commercial sugarcane breeding was conducted at the Maunawili Experiment Station. There were a total of 2,016 tassels cut during this period using 178 high yielding cultivars as parents. There were 487 unique polycrosses from five separate melting pots. In addition, there were 21 bi-parental crosses made from commercial or near commercial cultivars. From these crosses there were 67,632 hybrid seedlings raised from true seed (fuzz) and transplanted to FT1 trials at the HARC variety station located on the Hawaii Commercial and Sugar plantation in Maui. FT4 selections in 2008 numbered 5,091 which were combined from ratooned seedlings originally crossed in 2005-06 and plant seedlings from the 2006-07 crossing season to eliminate one stage of selection. In 2008, there were 925 FT5 clones selected from the previous year FT4 trials. There were seven installations of FT6 stage trials in 2008. The total number of FT7 yield trials installed at both plantations was 17, totaling 434 plots. These are scheduled to be harvested in 2010. There were 201 new clones evaluated in a total of 16 FT7 yield trials harvested in 2008, of which 22% were advanced to further testing.

The leading cultivar in the state was H65-7052 which rapidly increased in acres during 2006-07 at Gay and Robinson due to the yield decline of H77-4643. The shift in production acres to H65-7052 led to a return to long term historical average of about 12.2 TSA in 2007. For harvest year 2008, at Hawaii Commercial and Sugar, H87-4319 had the highest TSA yield of 9.6 among the commercial cultivars. High smut infestation in the seed fields of H78-3567 and yield decline of H78-4153 had led to a reduction of planted acres in these two cultivars. New commercial cultivars H95-4655, H87-5794, and H87-4319 have increased as a result.

The prolonged and severe drought on the island of Maui combined with reduced age of the crop has led to a significantly reduced yields at HC&S. All commercial cultivars have been affected. The continuing challenge will be to find drought tolerant cultivars and fast maturing cane that can produce high yields in these adverse conditions.

*-A. Arcinas*

## *Real-time PCR-based Quantification of Sugarcane yellow leaf virus (SCYLV) in Susceptible and Resistant Sugarcane Cultivars*

*Sugarcane yellow leaf virus* has been reported to cause yield loss in sugarcane worldwide. Current detection methods using tissue blot immunoassays (TBIA) are fast and reliable, but are not quantitative. We have developed a real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) assay for the detection and relative quantification of SCYLV in susceptible and resistant cultivars in Hawaii. This method was also shown to be more sensitive and could detect lower virus titres in infected plants. We observed positive RT-PCR and qRT-PCR reactions in cultivars previously thought to be immune to the virus.

Leaves from ten Hawaii sugarcane cultivars (H65-7052, H77-4643, H78-3567, H78-4153, H78-7750, H87-4094, H87-4319, H87-5794, H93-4068 and H95-4655) were used in the three assays (TBIA, conventional RT-PCR and qRT-PCR) to evaluate degree of infection with SCYLV. Mature leaves were collected from field-grown plants on Oahu and on Maui. Midribs of freshly-collected leaves were used for TBIA. Leaf midribs from the same leaves were combined and stored at -80°C until used for RNA extraction and RT-PCR testing.

The results of qRT-PCR assay showed a variation in the amounts of RT-PCR product amplified from virus RNA over a range of 106 fold. Based on the results of qRT-PCR, we have arranged the commercial cultivars into three susceptibility groups; high (H87-4094 and H93-4068), intermediate (H77-4643, H95-4655, H87-4319 and H65-7052), and low (H87-7750, H87-5794, H87-3567 and H78-4153). The group low levels of qRT-PCR product was on average 103-fold lower than the intermediate group, which in turn had consistently ten times lower qRT-PCR product than the high group. We hypothesize that the amount of virus RNA RT-PCR product detected in these analyses correlates with the actual SCYLV particle titre in the leaves based on the linear correlation of qRT-PCR product with dilution factor of each virus RNA extract (data not shown).

Real-time qRT-PCR detection also correlated with conventional RT-PCR and TBIA results. The conventional RT-PCR showed a relative lower intensity of bands with the low group (H87-7750, H87-5794, H87-3567 and H78-4153). The RT-PCR band intensities of the intermediate group (H77-4643, H95-4655, H87-4319 and H65-7052), were similar to those for the high group (H93-4068, H87-4094) due to the saturation limitation of dynamic range of conventional PCR method. The groups with low, intermediate and high RT-PCR products also correlated with the groups with few, medium and high percentages of positive leaf blots with TBIA.

Cultivar H65-7052 was selected for further detailed analysis because this cultivar frequently shows the typical SCYLV symptoms in field plots. Three methods were used for virus detection in symptomatic and asymptomatic plants: TBIA, conventional RT-PCR and qRT-PCR.

The data obtained from TBIA and conventional RT-PCR from mature H65-7052 sugarcane showed a strong correlation between YLS-symptoms and RT-PCR products. YLS symptomatic and non-symptomatic leaves were sampled over a six-month period in test plots on the island of Maui and TBIA was performed with the midribs of these samples. Overall, we observed a positive association between conventional RT-PCR and qRT-PCR results with SCYLV symptom expression in leaves of H65-7052. This held true with other plant parts tested: new leaves, mature lamina and midribs of cultivar H65-7052. Based on the qRT-PCR data, symptomatic sugarcane leaves contained about 30-fold higher virus RNA com-

pared to non-symptomatic tissue. Mature sugarcane lamina and midrib exhibited a difference of about 104 and 105-fold, respectively.

Our results indicate that qRT-PCR was a more sensitive detection method for SCYLV compared to the other two methods tested and that it measured quantitative differences in virus titre. It will be a useful tool for screening for SCYLV infection in sugarcane fields and for conducting research in studies of SCYLV resistance mechanisms in various sugarcane cultivars.

*- S. Lim, S. Schenck, A. Arcinas, and Y.J. Zhu*

## *Transgene Expression and Silencing in Sugarcane*

Integrating high-value protein production into a bioenergy crop such as sugarcane could greatly improve its economic viability. Sugarcane is a secure platform for high-value recombinant protein production in terms of transgene containment. We previously showed the feasibility of producing a biologically active pharmaceutical protein in sugarcane and determined that transgenic silencing is one of the limiting factors for recombinant protein accumulation in sugarcane.

In order to utilize suppressors of gene silencing from viruses to improve protein accumulation, we produced sugarcane transgenic lines that express one of four viral silencing suppressors: P0 from *Sugarcane yellow leaf virus* (SCYLV), HC-Pro from *Sorghum mosaic virus* (SrMV), p25 from *Potato virus X* (PVX), and p38 from *Turnip crinkle virus* (TCV). The goal is to find a suppressor to prevent transgene silencing without interfering with plant growth and development and to expand our knowledge of gene silencing in sugarcane. Expression data from P0 and HC-Pro transgenic plants showed that Hc-Pro and P0 had no obvious effect on the expression of five endogenous genes nor on the accumulation of five different miRNAs. We have not observed obvious developmental abnormalities in any of the four suppressor transgenic lines. However, accumulation of the recombinant protein did not improve.

We are now testing whether modifying the pharmaceutical protein gene (usually a human gene) to become more similar to a sugarcane gene would improve the level of protein accumulation. We are also testing a new method for sugarcane transformation to speed up transgenic plant regeneration for protein production.

*-S. Ancheta, W. Borth (UH), J. Hu (UH), T.E. Mirkov, (Texas A & M), H. Albert (USDA ARS and currently Pioneer Hi-Bred), and M-L. Wang*

## *Enhanced Atrazine Degradation in Hawaii's Sugarcane Soils*

Hawaiian sugarcane growers have used atrazine for pre- and post-emergent broadleaf weed control for over 30 years. Recent work from the United States Department of Agriculture's Agriculture Research Service in Fort Collins, CO and Stoneville, MS has indicated that intensive use of atrazine and other triazines may lead to more rapid degradation by soil microbial populations, reducing the efficacy of the herbicides. HARC began discussions with this research group at the 2007 Weed Science Society of America's Annual Meetings in San Antonio, TX. Upon learning of the research being conducted by USDA-ARS on enhanced atrazine degradation, Hawaii's sugarcane growers determined that a submission of soils to the Fort Collins research station may provide valuable information on improving weed control practices.

Soils were collected in April 2007 from Maui and Kauai sites in areas with noted broadleaf weed problems, and samples were sent to ARS in May. The sample sites included locations that had recently received the first broadcast application of atrazine, the second broadcast application of atrazine, areas with no applications made in over 20 months, and areas with no prior use history of atrazine. Results from the analysis of Hawaii's sugarcane soils showed that the half-life of atrazine in the most-recently treated soils was less than half of the half-life for the soils with no use history. These initial tests have provided information that will help develop fully-structured and replicated research projects to determine the magnitude of enhanced atrazine degradation in Hawaii soils. Plans are in place for expanded research into this phenomenon in sugarcane soils of Hawaii, Louisiana, and Florida.

*- M. Poteet*

# Tropical Fruit Research

## *Molecular Characterization of Disease Resistance Loci to Phytophthora palmivora in Carica papaya using AFLP Marker Analysis*

In Hawaii, papaya losses due to the fungal pathogen *Phytophthora palmivora* can be devastating, especially during the rainy season. Of the Hawaiian papaya cultivars, Kamiya is more tolerant to *P. palmivora* than the susceptible SunUp cultivar (Figure 1.). In an effort to understand disease tolerance and to improve papaya resistance to *P. palmivora,* amplified fragment length polymorphism (AFLP) molecular markers were identified between SunUp and Kamiya. The two cultivars were crossed, seeds from hermaphrodite F1 plants were sown, and the F2 progeny was grown and scored for *P. palmivora* disease response using a leaf disk assay. It was found that the *P. palmivora* tolerance in the F2 had a normal distribution, indicative of quantitative trait loci (QTL). Sixty of the most tolerant and most susceptible F2 plants were selected and analyzed using the developed AFLP markers. The markers showed that there may be polymorphisms linked with disease tolerance and disease susceptibility. Further research will be to sequence and identify the location of those markers to determine candidate genes involved in *C. papaya* tolerance and susceptibility to *P. palmivora*. These sequence characterized amplified regions (SCARs) will then be used to screen a backcross population of papayas for *P. palmivora* QTLs.

Mapping of QTLs is the first step toward identifying genes that are involved in *P. palmivora* tolerance. This approach of studying genetic regions that confer resistance to *P. palmivora* would make it possible to breed and/or use biotechnological approaches to grow papaya varieties that are more tolerant to the fungus. This in turn will reduce crop loss and fungicide costs to farmers as well as the stress those chemicals have on Hawaii's ecosystem. Additionally, knowledge of the molecular biology will contribute to our overall objective of understanding the plant-pathogen interactions between *C. papaya* and *P. palmivora*.

*- M. Noorda-Nguyen, B. Porter, Q. Yu, W. Nishijima (UH) and Y.J. Zhu*

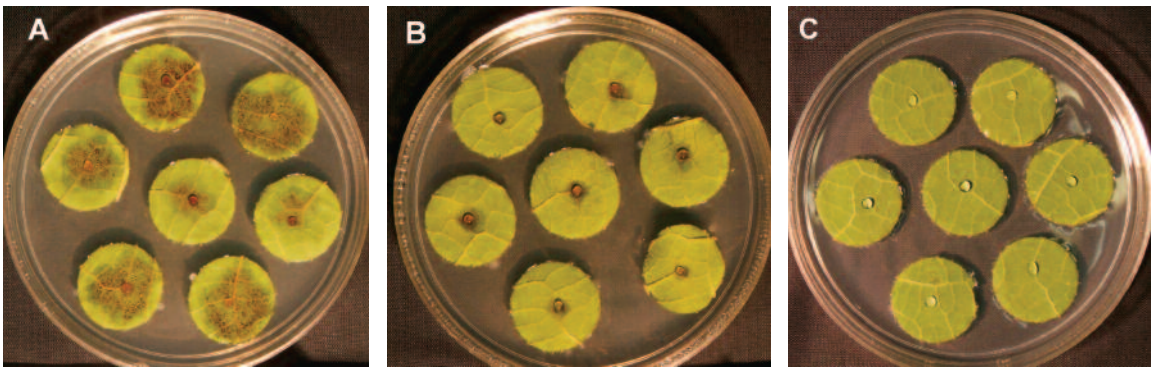



*Figure 1. Leaf disks of SunUp (A), Kamiya (B) and a tolerant F2 (C) 3 days after P. palmivora inoculation.*

## Papaya transformed with Galanthus nivalis GNA gene shows spider mite control

The aim of this project was to improve pest resistance of a commercial papaya cultivar, 'Kapoho'. A biolistic gene gun method was used to introduce the GNA gene from snowdrop (*Galanthus nivalis*) into papaya callus tissue. Plants were regenerated in the presence of Geneticin Disulfate salt (G418) to select for the Kanamycin selectable marker gene. Molecular analysis was performed on multiple lines to test for incorporation and expression of the transgene. Six lines were selected and further analyzed.

The level of recombinant GNA protein in each line was determined directly using an enzyme-linked immunosorbent assay (ELISA). Statistical analysis using the Statistix 7.0 program, showed that GNA expression in four of the lines was significantly different to the control Kapoho ($p < 0.05$). These four lines with the highest level of GNA expression were used in a laboratory bioassay using carmine spider mites, *Tetranychus cinnabarinus* maintained on leaf disks. It was found that mites feeding on disks from GNA-expressing plants showed a significantly lower reproductive ability ($p < 0.05$). Damage to leaf disks, as measured by amount of chlorophyll left in the leaf, was significantly lower compared to Kapoho ($p < 0.05$). Together this data suggests that expression of GNA in the leaves is affording some protection against mite feeding.

*- H. McCafferty, P. Moore, and Y.J. Zhu*

*Leaf disk with two adult mites and eggs.*

## Papaya Breeding Research Projects

**Production of Kapoho backcrosses:** The cultivar evaluation project included several papayas of interest for different purposes. Transgenic, PRSV virus-resistant papayas, introduced in 1998, saved the Hawaiian papaya industry. Rainbow papaya, an F1 hybrid between between transgenic SunUp and untransformed Kapoho, currently occupies about 90% of the papaya acreage on the Big Island. The seed is produced by hand pollination of female SunUp with Kapoho pollen. Kapoho bears the risk of virus infection and constant surveillance is carried out to rogue infected trees.

We backcrossed a Rainbow F2 seedling with Kapoho (named Poamoho Gold, patent #xxx) and backcrossed four more times. Kapoho BC5 seed was obtained that contained the virus resistance transgene. Seedlings of Kapoho BC4 planted in our field test showed growth and fruit characteristics similar to Kapoho, although Kapoho was the latest to flower and tallest before buds appeared. The fruit were similar in shape, firmness, color, and flavor to Kapoho.

**Production of virus-resistant Kamiya and Maunawili Sweet hybrids:** Two large-fruited papayas, Kamiya and Maunawili Sweet, were most likely derived from the University of Hawaii cultivar Waimanalo (X77). Each had been crossed with virus resistant Rainbow F2 seedlings to create hybrids for Oahu. The Kamiya hybrid, named Laie Gold was patented, and has been developed into a popular local market

favorite. Virus resistant Kamiya BC3 lines were developed to produce large (700-800 g), round fruit with sweet, thick, deep orange or pale pink flesh. Unfortunately, Kamiya BC3 lines were susceptible to powdery mildew and sometimes had large lesions on fruit and loss of leaves.

Maunawili Sweet F1 was micropropagated and planted in several small field trials. This virus-resistant hybrid produces large fruit and is valued by at least one grower because it was slightly offset from other cultivars like Laie Gold in its period of winter sterility. Only male flowers that make no fruits are produced during the cool season with consequent loss of marketable fruit. The Maunawili Sweet clone produced fruit while other trees had gaps and went through its sterile phase when other trees produced fruit.

**Evaluation of papaya cultivars for differences in the incidence of blemishing:**

Blemished papaya fruit are often observed in supermarket displays. Although most of the blemishes, often called freckles, do not develop into disease lesions, they may not appeal to the consumer when they occur in high density. Documentation of fruit blemishing was undertaken in December 2006. Seventeen Hawaiian cultivars and two Australian cultivars known to produce nearly blemish-free fruits were compared.

Monthly growth data were collected and the fruit harvested were scored for number of blemishes (freckles). These were differentiated from mechanical injuries caused by the wind and weather. The Australian fruit were indeed low-blemishing, especially on young trees. The cultivar Richter was generally unblemished but its fruit did not have a pleasant taste. The K7 hybrid was somewhat better but it requires additional selection/inbreeding/crossing because the flavor was mild to bland and fruit shape was elongate and often carpellodic.

The greatest blemish damage was the result of pathogen-attack. Powdery mildew (*Oidium caricae*) lesions were observed on Rainbow inbreds and Kamiya backcrosses that appeared to be highly susceptible to the disease. Kapoho showed mild blemishing as compared to SunUp and Sunrise. The latter had freckles and sunburn damage, presumably as a result of leaf losses from root rot and/or powdery mildew. Kapoho, its two backcrosses, and Richter fruits still had large leaf canopies 14 months after transplanting, suggesting stronger root systems. Richter trees had remarkably clean roots with no root-knot nematode damage as is common on other cultivars. In conclusion, blemishes differed among the seventeen cultivars and lines observed during the trial. Kapoho and its two inbred lines showed the least amount of freckling. The Australian lines Richter and K7 also had generally blemish-free skin compared to the Hawaiian cultivars. Extensive breeding and selections will have to be made before fruit suitable for Hawaii's growers and consumers is developed.

**Papayas Resistant to Worldwide Strains of Papaya ringspot virus:**

Because papayas (*Carica papaya*) are recognized as good sources of beta carotene, they offer one solution to eliminating global vitamin A deficiency. Previous research has succeeded in transforming PRSV-resistance into papayas using the virus coat protein gene (CPG). But these transformed plants are only resistant to specific, local PRSV strains, of which there are many worldwide. For instance, transformed Thai papaya varieties are only resistant to the Thai strain of PRSV and would succumb to the Hawaiian PRSV strain.

Papaya varieties resistant to all 20 strains of PRSV that exist around the world would be of great value to producers. Toward this objective four gene constructs containing synthetically made portions of the CPG common to

all 20 strains were produced. Each of the four constructs was successfully inserted into calli from different papaya cultivars: 'Kapoho', 'Khak Dum', 'Khak Nual', and 'Sunrise'. Both the gene gun and *Agrobacterium* gene transformation methods were used. *Agrobacterium* transformation is known to be a faster and gentler method that is more likely to transfer the entire gene construct without breakage.

Sixty-four papaya lines that are PCR positive for both selection gene and one of the four coat protein gene constructs (genes of interest) started as undifferentiated, transformed callus tissue. These 64 lines have been placed on proliferation medium which promotes leaf and stem production from the callus stage. Additional PCR analyses indicated that there were 32 hermaphrodite and 2 female papaya lines among the transformed lines. All transformed lines that successfully develop into potted plants will be selfed. The goal for this project is to produce ten independent lines by the end of 2009 that are ready for field-testing.

**Breeding Papayas Resistant to Enterobacter cloacae:**

*Enterobacter cloacae* is a coliform bacterium like *Escherichia coli* and, thus, poses problems with human consumption. There are some reports of *E. cloacae* causing distress in patients with compromised immune systems. Fresh fruit preparations are subjected to a zero tolerance policy that hinders development of commercial offerings of flash-frozen papaya cubes and related products. An earlier study showed that the papaya cultivar Kapoho was highly susceptible and cultivar Sunrise was highly resistant to infection by *E. cloacae*. Infection results in a disease in papaya called Internal Yellowing (IY) that is characterized by fluorescent yellow, malodorous seed cavities that give the fruit a bad, sour taste. Using the bioassay developed in the study, we determined that Rainbow papaya, an F1 hybrid of Kapoho and a sibling line of Sunrise named SunUp, had intermediate resistance to IY and SunUp itself was highly resistant. We finally discovered a population of Rainbow segregants that was highly resistant to IY with pure breeding, yellow-fleshed fruit that, if adopted, could help the papaya cube processors avoid the coliform problem.

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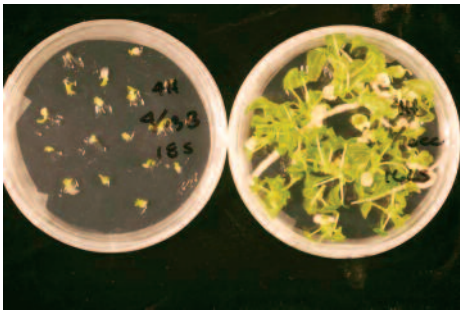
## *Re-isolation of Papayas from Greenhouse Grown Plants to Increase the Rate of Micropropagation*

Papayas are typically grown by sowing multiple seeds and are thinned to a single hermaphrodite plant per planting hole after three to five months when the papaya plants flower and the sex of the tree can be determined. Clonally propagated hermaphrodite papaya plants eliminate wasted time and plants. The clonally propagated plants produce earlier fruit lower on the fruit column and have higher yields than thinned seedlings. We have produced and sold about 17 acres of cloned papayas since 2002.

Problems with microbial contamination of the micropropagation stocks have hindered further output despite efforts to decontaminate the tissue cultures. Consequently, it was necessary to reisolate and repropagate the plant lines from the original plants. All of our plant lines were repropagated with the goal of increasing the production rate of cloned hermaphrodites available for sale to the growers.

The re-isolated, aseptic papaya plants showed improved growth and root forma-

tion and new protocols are continuously being attempted to further increase output. Recent Improvements in micropropagation include increased ventilation of vessels. The ventilation resulted in better plant survival and rooting. Other improvements are also being investigated. These improvements in clonal papaya propagation are enabling us to produce greater numbers of plants for growers. The current output of seeds by the papaya industry is adequate, but growers are also looking toward alternative means for field establishment as costs for seeds, fertilizer, crop protection chemicals, and labor continue to increase.



*Papaya in petri dishes*

Six of the seven original Rainbow hermaphrodite clones were successfully repropagated. Fourteen of the original Laie Gold hermaphrodite clones selected by Mr. Ken Kamiya were successfully repropagated along with three newer selections from Mr. Kamiya's and Mr. Clyde Fukuyama's Laie Gold commercial fields. In addition, a Kamiya backcross 1 (~75% Kamiya) with exceptionally large fruit and another Laie Gold selection made by Mr. Jimmy Song were cloned and added to the cultivar collection. Finally, for growers interested in non-GMO red-fleshed fruit, four Sunrise cultivar selections with low-blemishing skin and red, flavorful flesh were put into culture and are being micropropagated for spring of 2009.

*- A. Yeh and M. Fitch*

## *Isolating Phytophthora palmivora resistance genes from Vasconcellea goudotiana and Carica papaya 'Kamiya'*

Our objective is to isolate naturally occurring resistance genes from *Carica papaya* 'Kamiya' and its wild relative, *Vasconcellea goudotiana*, that are effective in protecting papaya against the pathogenic fungus *Phytophthora palmivora*. These genes will be used to transform the susceptible cultivar 'SunUp', providing a new, resistant variety for producers. *P. palmivora* zoospore drench inoculation experiments suggest that *Vasconcellea goudotiana* and *C. papaya* 'Kamiya' possess resistance to *P. palmivora* that is not found in *Carica papaya* 'SunUp' (unpublished data, Fig. 1 at right).

The *Carica papaya* genome survey sequence is providing a valuable resource for genome analysis and comparison. Because *Phytophthora* resistance genes have already been cloned from potato and soybean, homology-based sequence comparison was initiated to search for highly related genes in papaya that may afford resistance to *P. palmivora*.

*3 days*

*7 days*

*Figure 1. Root drench experiments of 'SunUp', 'Kamiya', Vasconcellea parviflora, and Vasconcellea goudotiana 3 and 7 days post-inoculation, respectively, with 1 x 103 Phytophthora palmivora zoospores/ml.*


*Figure 2. Blastp comparison of Rpi-blb1 to predicted R-gene homologs from the Carica papaya 'SunUp' genome database and Rps1-k-1 and Rps1-k-1 from soybean. Domains were predicted using Pfam (http://pfam.sanger.ac.uk/). Red bars depict the location of leucine-rich-repeat domains (LRR).*

Comparison of Rpi-blb1 with the *Carica papaya* 'SunUp' genome database revealed highest similarity to a gene from Supercontig 10, designated 10.212. Other genes from the same and other supercontigs also showed high sequence homology (Fig. 2).

Cloning and sequence analysis of a putative induced gene from of *V. goudotiana* revealed a single gene that is related to but diverged from its counterpart in *Carica papaya* 'SunUp'. Multiple deletions and nucleic acid differences between *V. goudotiana* and *Carica papaya* 'SunUp' predicted gene 10.215 suggest a unique gene that, if induced, could possibly afford resistance to *P. palmivora*.

In addition to the identification of an induced gene from *V. goudotiana*, R-gene splice variants were identified from 'Kamiya' and 'SunUp'. Although R-gene splice variants have been previously identified, the abundance of splice variants in *Carica papaya* may explain the fewer number of R-gene homologs in papaya relative to other plant species such as arabidopsis and rice. Perhaps generous use of splice variants and other post-transcriptional modifications, such as RNA editing, may explain how *Carica papaya* is able to "get away with" only a relative handfull of R-genes. Splice variants allow for more than one transcript from a single gene. Why splice variants are not seen for genes from Supercontig 16 is unknown. Perhaps they are less abundant, non-existent, or the primers used failed to amplify these transcripts. In summary, the presence of abundant splice variants is an exciting finding that deserves further investigation.

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## *Clonal Propagation of Papaya: Micropropagation and Rooted Cuttings*

Papayas are typically grown by sowing multiple seeds per hole and the plants (female or hermaphrodite) are thinned to a single hermaphrodite plant three to five months later when the papaya plants flower, and the sex of the tree can be determined. The lack of available seed for the *Papaya ringspot virus* (PRSV) -resistant Rainbow papaya is a problem for some Hawaiian farmers. Clonally propagated hermaphrodite papaya plants eliminate waste incurred in the traditional method. Alternatively, the use of tissue-cultured and rooted cuttings of hermaphrodites allows the farmer to plant one tree per hole, reduce fertilizer use, and eliminate the need for thinning out unwanted seedlings. This practice will greatly reduce the numbers of seeds needed. The clonally propagated plants produce fruit earlier, lower on the fruit column, and have higher yields than thinned seedlings.

Hermaphrodite Rainbow and Laie Gold (Kamiya-type) have been micropropagated for several years to try to develop two new procedures that local companies could adopt to enhance the papaya industry. While micropropagation is expensive in terms of a skilled labor force, facilities, and consumables, rooted cuttings offer a simple, greenhouse/shadehouse technique that farmers and/or nursery people could adopt in any location. In 2006-2007, several improvements to both micropropagation and rooted cuttings protocols were implemented. Improvements in micropropagation initiated recently include increased ventilation of vessels. The ventilation resulted in some increased plant survival and rooting. Other improvements are also being investigated.

Field-grown cuttings were also used for production of rooted cuttings. Our experiments in Hilo with field-grown cuttings have not been encouraging because of contaminated field-grown lateral branches, but several growers used their own field materials and reported limited success. On Oahu in springtime, about 20% of the field cuttings from well-sprayed trees rooted. Further experiments to reduce contamination of field-grown material could result in increased yields of rooted plants.

Micropropagated Laie Gold and cuttings initially serve as stock plants from which the shoot is removed, rooted, and sold to growers. The basal portion is allowed to re-grow a shoot and is also sold. A population of about 200 large stock plants is currently maintained in a Hilo greenhouse built with Hawaii County funding. The stock plants continuously provide clean shoots for rooting. The project also serves as an educational tool for training interested growers.

The re-isolated, aseptic papaya plants show improved growth and root formation and new protocols are continuously being attempted to further increase output. Countries like Brazil and China are reported to produce greenhouse rooted papaya cuttings for large commercial plantings. These improvements in clonal papaya propagation will also enable us to produce greater numbers of plants for growers. The current output of seeds by the papaya industry is adequate, but growers are also looking toward alternative means for field establishment as costs for seeds, fertilizer, crop protection chemicals, and labor continue to increase.

*- A. Yeh and M. Fitch*

## Molecular Genetic Basis for Papaya Fruit Flesh Color: Cloning and Characterization of a Chromoplast-Specific Lycopene Beta-cyclase Gene in Papaya

Papaya breeders have long recognized papaya fruit flesh color to be controlled by a single gene as segregating populations show a 3:1 inheritance of yellow: red fruit flesh color. Previous attempts to identify this gene were unsuccessful. Using a papaya BAC library, physical map and the newly available papaya whole genome shotgun sequence, a putative chromoplast-specific lycopene ß-cyclase gene, *CpCYC-b*, was identified. *CpCYC-b* along with four other genes in the carotenoid biosynthesis pathway was examined for relative gene expression during fruit development and ripening between the papaya cultivars yellow-fleshed 'Kapoho' and red-fleshed 'SunUp.' During fruit development *CpCYC-b* expression in 'Kapoho' increases to 11.5-fold higher expression in mature green fruit over leaf tissue whereas expression in 'SunUp' remains relatively stable. Sequence analysis of a 6.3 kb segment spanning the *CpCYC-b* coding region, 4 kb upstream region and 758 bp downstream region reveals 98.9% sequence identity between 'Kapoho' and 'SunUp.' A 2 bp insertion found in the 'SunUp' *CpCYC-b* coding region results in a frame-shift and early stop codon. Transformation of a lycopene-accumulating bacterial line with 'SunUp' *CpCYC-b* failed to reduce lycopene while transformation with 'Kapoho' *CpCYC-b* resulted in a color change from red (lycopene) to yellow (ß-carotene) confirming lycopene ß-cyclase activity. It is likely that both regulation of *CpCYC-b* and functional activity of the gene product affect final fruit flesh color in papaya.

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## Construction of a Sequence-Tagged High-Density Genetic Map of Papaya for Comparative Structural and Evolutionary Genomics in Brassicales

A high-density genetic map of papaya (*Carica papaya* L.) was constructed using microsatellite markers derived from BAC end sequences and whole-genome shotgun sequences. Fifty-four F2 plants derived from AU9 and SunUp cultivars were used for linkage mapping. A total of 707 markers, including 706 microsatellite loci and the morphological marker fruit flesh color, were mapped into nine major and three minor linkage groups. The resulting map spanned 1069.9 cM with an average distance of 1.5 cM between adjacent markers. This sequence-based microsatellite map resolved the very large linkage group 2 (LG 2) of the previous high-density map using amplified fragment length polymorphism markers. The nine major linkage groups of our map represent papaya's haploid nine chromosomes with LG 1 of the sex chromosome being the largest. This map validates the suppression of recombination at the male-specific region of the Y chromosome (MSY) mapped on LG 1 and at potential centromeric regions of other linkage groups. Segregation distortion was detected in a large region on LG 1 surrounding the MSY region due to the abortion of the YY genotype and in a region of LG 6 due to an unknown cause. This high-density sequence-tagged genetic map is being used to integrate genetic and physical maps and to assign genome sequence scaffolds to papaya chromosomes. It provides a framework for comparative structural and evolutionary genomic research in the order Brassicales.

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## Low X/Y Divergence in Four Pairs of Papaya Sex-Linked Genes

Sex chromosomes in flowering plants, in contrast to those in animals, evolved relatively recently and only a few are heteromorphic. The homomorphic sex chromosomes of papaya show features of incipient sex chromosome evolution. We investigated the features of paired X- and Y-specific bacterial artificial chromosomes (BACs), and estimated the time of divergence in four pairs of sex-linked genes. We report the results of a comparative analysis of long contiguous genomic DNA sequences between the X and hermaphrodite Y (Yh) chromosomes. Numerous chromosomal rearrangements were detected in the male-specific region of the Y chromosome (MSY), including inversions, deletions, insertions, duplications and translocations, showing the dynamic evolutionary process on the MSY after recombination ceased. DNA sequence expansion was documented in the two regions of the MSY, demonstrating that the cytologically homomorphic sex chromosomes are heteromorphic at the molecular level. Analysis of sequence divergence between four X and Yh gene pairs resulted in an estimated age of divergence of between 0.5 and 2.2 million years, supporting a recent origin of the papaya sex chromosomes. Our findings indicate that sex chromosomes did not evolve at the family level in *Caricaceae* and reinforce the theory that sex chromosomes evolved at the species level in some lineages.

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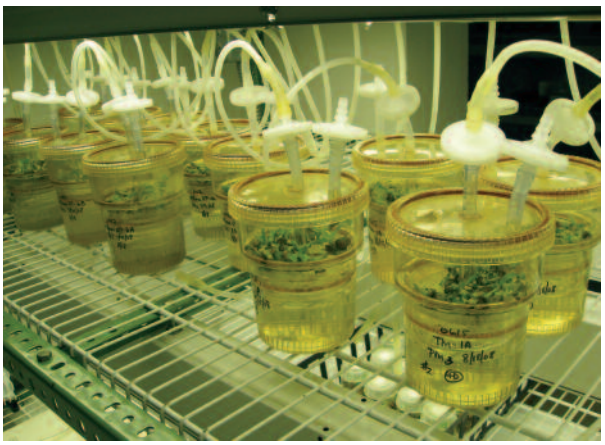
## Partial Automation of Banana Micropropagation

Hawaii is the only significant commercial producer of banana (*Musa* sp.) in the US. Banana production in Hawaii totaled 10,450 tons in 2005. However, the Hawaiian banana industry suffers serious losses from *Banana bunchy top virus* (BBTV). Production of micropropagules is an important method for production of disease-free planting material. Uniformly sized micropropagated plants also have an advantage for new plantings. During the past ten years, HARC produced over 250,000 banana micropropagules using stationary liquid micropropagation protocol for all three commercial types of banana including Williams, Apple, and Saba cooking banana. Cost of production, however, is high and many small farmers cannot afford to plant micropropagules.

We developed a more efficient protocol of banana micropropagation using partial automation in a large culture container system, Rita® (VITROPIC, Saint-Mathieu-Treviers, France). This method uses filtered air to push liquid medium every 4 - 6 hours into an upper chamber where propagules are placed. The HARC protocol was established by modifying the protocols used in Costa Rica and France.



*Banana micropropagation*

In vitro culture of banana was established using HARC's stationary liquid culture system (Annual Report 1997 p.25). When the propagules started multiplying (2-3 months from culture initiation), they were placed in the Rita containers for partial immersion. We successfully established shoot multiplication in Rita® for three varieties: Saba, Apple, and William. Comparison of propagation efficiency was conducted for all three varieties. Total fresh weight and propagule numbers produced in various culture media with BA 0-10 mg/L clearly showed that the Rita system resulted in more propagules within six weeks. PM2 and PM4 media produced the most efficient propagule production. The Rita system produced 30-40% higher number of shoots than those in the stationary liquid Magenta boxes. Twice as much root mass per plant was obtained in root induction medium from Rita-derived propagules as compared to those from the stationary liquid method. Comparisons of Rita and liquid Magenta cultures clearly showed that the Rita system produced higher numbers of propagules and greater fresh weight in both Apple and Williams varieties.

We planted propagules of both Apple and Williams in the nursery of a commercial operation. No difference was found in survival rate (range: Apple 89%-100%, Williams 100%) or plant growth between the stationary liquid Magenta box and the Rita methods for either variety. No variant mutants were observed among the propagules multiplied in various culture media in the Rita system.

The Rita method successfully lowered production cost estimates, since most of the cost of propagule production is the labor. Cost of production by the Rita system was about 30-40% less than the earlier method. Although careful handling of medium changes is required for Rita, the overall labor requirement is significantly reduced. Rita culture system however has its limitation in size. We tested 20-60 propagules per Rita container (200 mL capacity) as starting propagules. When higher numbers of propagules were placed in the container, lower multiplication and more contamination were observed. However, once scaling up of the proven Rita banana culture system is achieved, higher multiplication and overall more efficient propagule production are expected with further cost reduction.

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## *Genetic Transformation of Pineapple*

Previously we reported the development of an improved protocol for pineapple transformation (HARC 2005 Annual Report). Adventitious buds were induced directly from *Agrobacterium*-infected leaf bases and stem discs of in vitro grown plants. Since we bypassed the establishment of callus cultures, the time required for production of transgenic pineapple plants was significantly reduced. Now we report the confirmation of transgene integration in these pineapple plants via Southern hybridization.

The presence of the transgene was tested on twelve lines transformed with a modified rice cystatin gene and seven lines transformed with a pineapple aminocyclopropane synthase (ACS) gene in the antisense orientation. These two constructs are for nematode-resistance and flowering control, respectively. We detected the respective transgene in all nineteen transgenic lines. Most of the transgenic lines contained one to four copies of the transgene. The random banding patterns suggested that the transgenic lines were generated from independent events.

This is the first time that the genetic transformation of pineapple was confirmed in our lab by Southern blot analysis. We found that 1) high quality of extracted

DNA was very important for a successful Southern blot analysis, 2) the SDS DNA extraction protocol modified from the method of Lin. et al. (2001) was appropriate for extracting DNA from pineapple leaves, although the method could be further improved, 3) enzyme *Eco*RI worked better than *Sac*I, and 4) Amersham non-radioactive Southern blot analysis kit worked well for direct labeling and detection. In addition, the results in two cystatin lines 101 and 107 were consistent in two experiments, proving that this method was repeatable.

In summary, a total of 42 independent transgenic lines were produced for the two constructs and two pineapple cultivars from 27 transformation experiments. No significant difference in transformation efficiency (number of transgenic lines/explants) was observed between cultivars or constructs. Transformation efficiency was significantly higher for stem discs than for leaf bases. However, since approximately three times more leaf bases can be obtained from each in vitro grown plant, a greater total number of transgenic plants were produced from leaf bases (25 lines) than from stem discs (17 lines).

Reference: Lin, R.C., Z.S. Ding, L.B. Li, and T.Y. Kuang, 2001 A rapid and efficient DNA minipreparation suitable for screening transgenic plants. Plant Molecular Biology Reporter 19:379a-379e.

*- M.-L. Wang, C. Nagai, X. He, G. Uruu, and K. Cheah (UH)*

# Beverage Crop Research

## *Coffee Genomics*

### FLP linkage map

Hawaiian coffee has a long-standing reputation of high quality, but breeding programs to improve available cultivars are needed to continue producing a superior product. Construction of a *Coffea arabica* genetic map is the first step towards marker assisted selection for traits such as high yield, large bean size and superior cupping quality. An Arabica cross was made between Mokka Hybrid (MA2-7) and Catimor (T 5171-7-1) varieties to develop a segregating mapping population. These varieties are divergent in cupping quality as well as in tree and bean morphology. Two F1 plants (00-20-25 and 00-20-41) from the progeny were used to produce a true F2 population which was planted at Kunia in 2005.

Amplified fragment length polymorphism (AFLP) markers were used to produce the genetic map from 61 individuals of the 00-20-25 F2 population. In total, 663 polymorphic markers were generated and of these, 572 markers were linked to 14 major linkage groups and 14 minor ones. Six of the major linkage groups are Mokka dominant and eight are Catimor dominant. Recombination did not occur in any of the linkage groups. An additional 119 markers are currently being evaluated for addition to the map.

In addition to the genetic data, phenotypic data for tree height and width, branch angle, leaf characteristics, cherry weight, and green bean weight have been collected from a larger F2 population of 259 individuals. Once the linkage map is finalized, it will be used in conjunction with the phenotypic data to identify quantitative trait loci that will help farmers to identify quality cultivars.

### Mokka BAC library

Bacterial Artificial Chromosome (BAC) libraries are an extremely useful tool for studying plant genomes. These libraries can be basic resources used for genome sequencing, cloning genes of interest, chromosome walking, and identifying Quantitative Trait Loci (QTLs). Used in collaboration with the coffee linkage map, it may be possible to identify loci that influence cupping quality, tree and bean morphology and disease resistance. A BAC library was constructed using genomic DNA from the small bean, high cupping quality Arabica variety Mokka Hybrid. Arabica coffee (*C. Arabica*) was chosen as the best candidate for the library because it is the most commercially important species in the genus, accounting for 70% of the world's coffee production.

The genomic DNA was partially digested with the restriction enzyme Hind III and ligated into the pindigo BAC vector. The final library consists of 52,416 clones with an average insert size of 94 kb which is estimated to cover 4x coffee genome equivalents. High-density filters have been made for further characterization of this BAC library using plastid specific probes and coffee genes. This Arabica BAC library will be used for cloning genes of interest to be utilized for coffee improvement. In addition to the applied research, this large insert genomic library will be essential for basic research such as understanding the basis for evolution of this allotetraploid genome from its two progenitor diploid genomes of *C. canephora* and *C. eugenioides*.

### Recent speciation event of allotetraploid *Coffea arabica* revealed by orthologous BAC sequence comparison

*Arabica coffee* (*Coffea arabica* L.) is a perennial allotetraploid (2n=4x=44) species and self pollinated, whereas Robusta coffee (*C. canephora* L.) is a perennial diploid (2n=2x=22) species and self incompatible. Arabica and Robusta coffee account for almost all of the world's coffee supplies. It has been shown by molecular and cytogenetic evidences that *C. arabica* was derived

from a spontaneous hybridization between two *Coffea* species; i.e. *C. canephora* and *C. eugenioides*. To investigate the patterns and degree of DNA sequence divergence between Arabica and Robusta coffee genomes, we identified orthologous bacterial artificial chromosomes (BACs) from *C. arabica* and *C. canephora* and compared their sequences to trace their evolution history. Although the high level sequence similarity was found between the BACs from *C. arabica* and *C. canephora*, numerous chromosomal rearrangements were detected, including inversions, deletions, and insertions. DNA sequence identity between *C. arabica* and *C. canephora* orthologous BACs ranged from 94-98%. Analysis of ten orthologous gene pairs resulted in the estimated ages of divergence from 0.36 to 1.01 million years, indicating a recent origin of the allotetraploid species *C. arabica*.

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## *Advancement of Selection and Propagation of New Coffee Hybrids*

In 1998, HARC and HCGA, Hawaii Coffee Growers Association, started a coffee breeding program to develop new coffee cultivars with uniquely Hawaiian quality and adapted to Hawaii's growing conditions. Cultivars are being developed by traditional breeding and selection along with biotechnology techniques, such as propagation via tissue culture and DNA markers, which will shorten the time to accomplish our goals.

Three new hybrid families, H99-153, H99-150 and H99-160 were selected from a Kauai field trial (Elele, Kauai, HARC Annual Report 2006) based on tree performance and beverage quality over the previous three years (2006-2008). Cherries were wet processed to obtain green beans for evaluation of bean characteristics and cupping quality. Cherry and green bean yield of H99-153 was not different from the two commercial cultivars, KA17 (Yellow Catuai) and KO34 (Typica). Yields of H99-150 and H99-160 were significantly lower. Green bean size of the three selected hybrid families were compared using the coffee industry standard (ISO4150-1980). The mean screen sizes of two varieties, H99-153 and H99-150, were not different from those of KA17 and KO34, whereas the bean size of H99-160 was significantly smaller.

*Coffee visitors*

Beverage quality evaluations were conducted by the Kauai Coffee Team and Hawaii Coffee Growers' Association (HCGA) members, and cuppers of the UCC Ueshima Coffee Co (Kobe, Japan). The cuppers gave various evaluations for beverage quality as expected, although many agreed that the H99-160 family (progeny of Maui mokka) had distinct flavors.

Seeds from selected hybrid trees were distributed to HCGA growers for further field trials on other islands with different growing environments. It is expected that growth and quality of these promising hybrid families will vary in the different locations due to interaction of genotypes with environment.

Rapid production of superior selected cacao genotypes is underway by propagation via somatic

embryos and the Rita® system (see Banana Micropropagation). Seven genotypes selected from a Kauai trial of 4,000 seedlings were placed in tissue culture during 2006-2007, and all the genotypes produced somatic embryos. The three selected genotypes mentioned above, H99-153, H99-150 and H99-160, were given top priority. Somatic embryos of these three genotypes were placed in the Rita Bioreactor system. Thirty to forty plantlets were harvested and transferred to rooting medium and those with roots were transferred to soil. Currently we are conducting experiments to regenerate plants efficiently in Rita. The optimum size for germinated (regenerated) plants is being evaluated in order to achieve efficient acclimation to soil.

Funding for the development of coffee cultivars was provided by the Hawaii Coffee Growers' Association and the Hawaii State Department of Agriculture.

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## *Isolation and Characterization of Genes Related to Organ Size Control in Coffee*

Coffee (*Coffea arabica* L.) cultivars 'Tall Mokka' (MA2) and 'Kona-Typica' (KO34) produce high quality beans with distinct flavors. MA2 differs from KO34 in having smaller organs, including leaves, fruits, and seeds, resulting in lower yield. Comparison of leaf epidermal cell size and number between the two cultivars indicated that cell size is probably the major organ size determinant. Our goal is to identify genes related to organ size control in these two cultivars.

Using potato cDNA microarrays from TIGR as a cross-species platform, we identified forty-five genes with ≥ two-fold difference in expression between the two cultivars. Homologous coffee sequences were identified for 28 of these genes in the publicly available databases. The differential expression pattern was confirmed for only one of these 28 genes using qRT-PCR. This gene is homologous to prolyl oligopeptidase (POP) from *Arabidopsis*. POP expression is higher in MA2 as well as two other *arabica* coffee varieties with small organs, ´Laurina´ and ´Mokka´, as compared with KO34. In both MA2 and KO34, the expression of POP in leaf tissue increases as the leaves mature but decreases during the first eight weeks of fruit development. The difference in POP expression between MA2 and KO34 is greater at the very early stages of development for both leaves and fruit.

We have cloned two POP genes, *CaPOP1* and *CaPOP2*, from the MA2 Bacterial Artificial Chromosome (BAC) library and from KO34 genomic DNA. The major difference between these two genes is in their promoter regions that differ by two insertion-deletions (in-del). *CaPOP1* and *CaPOP2* share 91% similarity excluding the deleted fragment. Identification of these two POP genes supports the alloploid nature of *Coffea arabica*. Its genome was formed by hybridization between *C. canephora* and *C. eugenioides*. No difference was detected in the *CaPOP* sequences from MA2 and KO34, suggesting the expression difference is caused by upstream regulatory mechanism.

This study will increase our understanding of the role of POP in plant growth and development. The gene(s) controlling organ size may be used in coffee breeding to improve bean size and yield.

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## Nematode-Resistant Ethiopian Coffee

Nematodes are one of the greatest long-term threats to coffee worldwide. In Kona, Hawaii, 80% of coffee farms are infested with *Meloidogyne konaensis*, the Kona root-knot nematode, resulting in the replacement of entire plantations and great economic hardship for growers. No nematicides are currently cleared by the EPA for coffee, so growers are using *Coffea liberica* rootstocks to graft with high quality typica *Coffea arabica* trees.

*Greenwell grafter*

Recently, nematode-resistant genotypes have been identified among Ethiopian arabica collections at CATIE, Costa Rica (C. Astorga, CATIE unpublished data). Ten genotypes of selected Ethiopian arabica, including genotypes with nematode resistance, were imported into Hawaii in 2006. A project was initiated to evaluate these Ethiopian arabica trees for nematode resistance and for commercially desirable characters.

A total of 512 seedlings were released from Hawaii Department of Agriculture Plant Quarantine facility in December 2007. These plants were used for the various evaluations. Ten trees per variety were directly planted in a field at HARC's Kunia Substation. Another 20 trees per variety were grafted with typica and Red Catuai and the grafted seedlings were planted at Greenwell Farms in Kona.

A bioassay for resistance to Kona root-knot nematodes was conducted at the University of Hawaii Whitmore Greenhouse. Typica, Red Catuai, and Fukunaga rootstocks were used as susceptible controls and compared with the ten Ethiopian varieties. The seedlings (12 plants per variety) were each inoculated with 3,000 eggs of *M. konaensis*. Eight months after inoculation the plants were destructively assayed. Roots were gently washed free of soil and rated for galling. The roots were removed and macerated in a NaOCl solution to extract nematode eggs. The numbers of eggs and juvenile nematodes were counted and *M. konaensis* numbers were compared. The bioassay clearly showed that nematode reproduction was extremely low in all the ten Ethiopian varieties (400-2,200/20 g roots) compared to two controls (25,000/20 g roots-typica, 47,000/20 g roots-Red Catuai). The seeds from these Ethiopian arabica trees will be harvested in the fall of 2009 at Kunia for further evaluation.

*- C. Nagai, S. Aoki (UH), B. Sipes (UH), R. Heinig, P. Miranda (Greenwell Farms)*

## Selection of High-Yielding Cacao Trees for Hawaii

Cacao production in Hawaii is becoming an important industry and is now grown on the five major islands. Quality and bean yield are the two major characteristics to select for since disease resistance is not an issue so far. Molecular marker studies showed that there are a wide range of cacao types in Hawaii within the major Criollo, Trinitario and Upper Amazon Forastero (UAF) groups and their hybrids.

We initiated a selection program for superior trees with high yields and qualities such as large seed size and good liquor quality. The aim is to develop cacao cultivars suited to Hawaii's environment. Dole Co. at Waialua, Oahu has about 10,000 cacao

trees which were identified by molecular analysis as Upper Amazon Forastero (UAF) x Trinitario hybrids. Data were collected over two years (2007-2008) for pod and bean characteristics from over 9000 pods from 300 trees. All the beans from the pods were micro-fermented and dried along with commercial beans.

*Cacao selection*

The Waialua population has a large variation for commercially important pod and bean traits such as pod index (number of pods/kg of beans) and bean size. We were looking for a low pod index and large bean size as well as trees that produced a large number of pods. An average of thirty three pods were harvested per tree. The top 5% of the trees (15) produced over 70 pods per tree each. Pod index average was 35.1 with a range of 13-124, and 5.7% of trees (16) had less than 25 pods each. Large, dry bean weight (1.0 gram or greater) was recovered from 3.6 % of the trees (10). The third-year data set will be collected in 2009. The most productive trees will be multiplied by both grafting and seed propagation for further selection of superior genotypes.

Superior trees with high pod production and trees with low production were selected for a study of marker assisted selection (MAS). Leaf samples were collected from these trees for molecular analysis at USDA/ARS, Miami, Florida. Application of genetic markers may permit the selection of new superior cultivars adapted to local growing conditions for long-term cacao improvement for Hawaii.

*- C. Nagai, R. Heinig, J. Buenafe, A. Lewis, N. Rosete and R.J. Schnell (USDA-ARS)*

## *Genetic Diversity of Cacao in Hawaii*

Commercial production of cacao in Hawaii is increasing, and this trend is expected to continue. The increased acreage is being planted with seedlings from genetically uncharacterized cacao populations from various introductions of cacao into the islands. Quality and yields from these cacao populations are variable and unpredictable.

A molecular analysis was initiated in 2006 to identify genotypes of cacao in Hawaii (HARC Annual Report 2006) in order to assist cacao farmers in selection of superior cultivars based on their parentage pedigrees. More than 100 leaf samples were collected from Kauai, Oahu, and Hawaii, and Simple Sequence Repeat (SSR) analysis was used to determine the genotypes of each sample. Based on the results of this analysis, the trees were classified into four genetic groups: Criollo, Upper Amazon Forastero (UAF), and Trinitario. Trinitario cacao is a hybrid of UAF and Criollo groups and backcrosses of Trinitario with UAF produced the fourth group, Trinitario-UAF hybrid.

Twelve trees from Kona and Kauai were classified as Criollo. All the trees were homozygous for the eleven SSR loci analyzed. Criollo is a desirable type of cacao, but there are few areas in the world where it is cultivated due to its susceptibility to

disease. According to University of Hawaii records, there were two Criollo trees at the Poamoho Station in the 1950s (H.C. Bittenbender, personal communication). We identified a yellow-pod Criollo group on Kauai and a red-pod group at Kona which appear to have been propagated from these two original trees.

Eleven of the trees analyzed were classified as UAF. The samples came from five locations on Hawaii and Oahu. Every sample was unique for the loci tested, indicating a high level of genetic diversity within the group.

The largest and most widely represented group was Trinitario, with 47 samples collected from 14 different locations on all three islands. This group was highly variable; 70% of the samples were genetically unique at all 11 loci.

The Trinitario-UAF hybrid group consisted of 33 genetically unique samples. These samples were collected from multiple locations on Hawaii and Oahu. Twenty-four samples collected from a Dole Co. Waialua field were in this group.

These results indicate that a diverse variety of cacao germplasm is available in Hawaii for selection and development of superior cultivars. The presence of Criollo, Trinitario, Forastero and Trinitario-Forastero hybrids in multiple locations throughout the islands is of great advantage to the state, since it is unlikely that new germplasm will have to be introduced for breeding. So far, Hawaii has none of the major cacao disease problems found in other countries so restrictions on imports are necessary. The availability of this genotype information gained through molecular technology will provide growers with a useful tool for predicting and selecting superior cultivars for their growing environments. They will be able to select superior genotypes based on genetic information as well as from morphological charactieristics and yield performance.

*- R. Heinig, C. Nagai, and R.J. Schnell (USDA-ARS)*

## *Development of Hawaiian Cacao Fermentation Standard Methods*

Production and processing of cacao beans is a promising agricultural industry for Hawaii. Cacao trees grow well in the subtropical climate and a range of genetic types already exist in the islands. The crop could increase greatly if a standardized high quality product could be exported to chocolate manufacturers. Genetic typing of cacao trees is underway for selection of preferred cultivars. But another variable is in the fermentation process itself.

Cacao pods are harvested from trees when ripe and the beans inside are surrounded by a white mucilage. The beans and mucilage are placed in wooden containers and allowed to ferment for about five days at somewhat elevated temperatures. Several different fermenting microorganisms are involved at different stages of the fermentation process. Under natural conditions the fermentation takes place with whichever yeasts or bacteria happen to be present in the air or on the beans. But the final taste of the chocolate can vary considerably with different fermenting microorganisms. Some give an off flavor that is unacceptable. So to ensure constant export quality to buyers, it is necessary to standardize the fermenting microorganisms that give the best flavors.

During the fermentation process the temperature and acidity of the beans increases and the mixture starts to become anaerobic. It is necessary to turn and mix the beans

each day. As conditions change in the fermentation box different microorganisms become dominant. It was reported that by inoculating the beans at the start of fermentation with a mixture of yeast and bacteria, the desirable microorganisms were present during the whole process. Therefore, we isolated microorganisms occurring naturally from fermenting beans at different Hawaiian locations hoping to get a series of the best ones for our purposes.

Samples of mucilage were taken daily from fermentation boxes on Oahu and Big Island and a dilution series made in sterile water. The dilute solution was plated on selective media for yeasts, *Lactobacillus*, and *Acetobacter* species. From the surface of the agar plates, individual colonies were selected and grown on media suitable for each. Five yeast species from three genera were selected. The anaeroic gram positive bacteria *Lactobacillus* and the aerobic gram negative acetic acid bacteria *Acetobacter* were also isolated in pure culture.

The yeast isolates were identified to genus and species using API-Biomérieux diagnostic strips for yeasts. The *Lactobacilli* were identified using API-Biomérieux diagnostic strips for anaerobic bacteria and the *Acetobacter* isolate was sent to Microbial ID, Newark DE for identification. The isolates were grown in liquid culture for producing the cocktails for cacao inoculations.

Various combination cocktails of a yeast, *Lactobacillus* and the *Acetobacter* isolates were used to inoculate batches of cacao beans for fermentation. The fermented beans were dried and sent to Guittard Chocolate Company, San Francisco, CA for chocolate production from each separate sample. Their chocolate samples were tested by their expert tasters and scored for flavor types and overall quality. There was considerable variation, but all of the Hawaiian samples tended to have fruity flavors and several were judged of very high quality.

*- S. Schenck, J. Clayton, M. Jackson, and L. Gautz (UH)*

*Cacao pods*

# Forestry Research

## *Comparative Virulence of Hawaiian Fusarium oxysporum Isolates on Acacia koa Seedlings*

*Fusarium oxysporum* Schlecht. is a taxon containing a complex of morphologically-similar fungal species that cause important plant diseases worldwide. Strains may vary widely genetically, although they usually cannot be distinguished morphologically. Pathogenic strains of *F. oxysporum* are often quite host-specific and those capable of eliciting disease on particular hosts are designated by a sub-specific taxon called a formae specialis. There are also saprophytic strains incapable of causing diseases which cannot be differentiated from pathogenic strains unless either tested on susceptible hosts or separated on the basis of specific genetic markers associated with virulence genes.

We have been investigating an important *Fusarium* wilt/dieback disease of *Acacia koa* in Hawaii. The primary cause of wilt is infection with virulent pathogenic strains of *F. oxysporum* f.sp. *koae*. A genetic analysis of several pathogenic isolates from diseased koa plants indicated potentially low genetic diversity, which may indicate recent introduction(s) of the pathogen into Hawaii. Subsequent genetic characterization of a much larger population of *F. oxysporum* from different sized hosts and islands indicated that the overall population within this species complex is likely quite diverse; at least six genetically-distinct clades were identified within a population representing more than 100 isolates from throughout Hawaii.

We initiated greenhouse tests to determine pathogenic potential of selected *F. oxysporum* isolates in an effort to identify specific isolates that might be used to screen koa families for potential disease resistance and to quantify the prevalence of potential virulence within fungal populations. Sixty one fungal isolates were selected and inoculum prepared by growing the fungi on a mixture of Perlite, cornmeal and 1% water agar. For each tested isolate, four replications of six seedlings were evaluated. Two fully replicated sets of 24 seedlings each were included as controls.

Only about 15% of the tested isolates exhibited high- or moderately-high virulence on young koa seedlings under our greenhouse inoculation conditions. More than half of the isolates exhibited low virulence or were considered non-pathogenic. The other tested isolates were somewhere in between. As expected, isolates classified as highly-virulent caused extensive disease and seedlings survived only for short periods. Differences in average seedling height among the six virulence categories were inconsistent. Our results indicated that the majority of the tested *F. oxysporum* isolates were not highly-virulent strains and therefore probably not responsible for the wilt/dieback disease of koa.

We found that all tested isolates, even those considered non-pathogenic, always infected roots of inoculated seedlings. Roots inoculated with non-pathogenic isolates exhibited no noticeable necrosis or discoloration; they were white and appeared completely healthy. However, they were extensively colonized by inoculated isolates, even to the point where no other fungi were detected during root assays. We suspect that non-pathogenic isolates were unable to successfully colonize vascular systems and thus spread systemically throughout inoculated seedlings. Such isolates may have been restricted to root cortical cells where they existed as endophytes and did not adversely affect seedling health.

Specificity of pathogenic strains of this fungus on different genetic populations of koa is unknown. For example, the closely-related *Acacia koaia* may have its own specific strains of *F. oxysporum* that are only virulent on this species. Much more testing will be required to elucidate the specificity of virulent isolates. The high percentage of non-pathogenic isolates identified in our tests could prove to be useful in developing biological control agents for control of the highly-virulent strains of *F. oxysporum*.

*- N.S. Dudley, R.L. James (US Forest Service) and A. Yeh*

## *Survey of Hawaiian Tree Nurseries for Occurrence of Fusarium on Acacia koa*

*Acacia Koa* (koa) and *Metrosideros polymorpha* (syn. *Metrosideros collina*) (ohia) are the two major native tree species in Hawaii from both an economic and environmental perspective. Koa and ohia are often the dominant overstory species within many different forest areas.

Both wild and planted *Acacia koa* trees are severely affected at mid and low elevations by a wilt/dieback disease caused by pathogenic strains of the fungus *Fusarium oxysporum* (designated *F. oxysporum* f.sp. *koae*). The pathogen also causes disease on *Acacia koaia* (koaia), *Acacia confusa* (Formosan koa), and may affect other *Acacia* species. Our survey goal was to determine extent, distribution and identity of *Fusarium* spp. on *A. koa* seeds and seedling stock being produced at important tree nurseries on the four main Hawaiian islands: Kauai, Maui, Oahu and Hawaii. Each nursery location was geo-referenced and digital maps were developed. Selected nurseries included Federal, State and privately-owned nurseries.

Seedlings of *Acacia koa* and *A. koaia* were inspected for disease symptoms, including top wilting, foliar chlorosis and necrosis. Symptomatic seedlings were quantified for each particular seedlot within the nursery. Symptom severity for individual seedlings was numerically determined using a rating system: [0 = no disease symptoms; seedling appears healthy; 1 = seedling with minor symptoms (some foliar chlorosis or necrosis); 2 = seedling with moderate to severe symptoms including wilting and mortality. Seedlings adversely affected by cultural or environmental factors, such as irregular irrigation, were not selected. Selected seedlings were carefully extracted from soil or their containers, their roots thoroughly washed to remove soil or growing media, and transported to the laboratory for analysis.

Roots of all seedlings were sampled for *Fusarium* colonization; stems and/or branches were sampled from only about 40% of the seedlings. Roots and stem/branch sections were dissected into pieces about 5 mm in length. Randomly-selected pieces were surface sterilized in 0.525% aqueous sodium hypochlorite (10% bleach solution), rinsed in sterile water, and placed on a selective agar medium for *Fusarium* and closely-related fungi.

Eleven different *Fusarium* species were isolated from seedlings. The most commonly-isolated species by far was *F. oxysporum*; it was found on about 78% of all sampled seedlings. We found *F. oxysporum* commonly on the roots of many seedlings that displayed little or no disease symptoms. All sampled roots appeared healthy with no indications of necrosis or discoloration.

Vascular discoloration was noted within stems of some, but not all, seedlings from which *F. oxysporum* was isolated.

The other most common *Fusarium* species isolated from koa seedlings were *F. solani*, *F. semitectum*, and *F. subglutinans*. *Fusarium solani* is a common soil-borne fungus capable of causing several types of plant diseases, including root decay. We found it most commonly within the interior portions of large roots, stems and branches of koa trees exhibiting wilt/dieback symptoms. *Fusarium solani* is often associated with activity of black twig borers (*Xylosandrus compactus*), which commonly infest seedlings as well as larger trees. Both *F. semitectum* and *F. subglutinans* are commonly isolated from *Acacia koa* seed. The other seven *Fusarium* species isolated from koa seedlings were found at very low levels. We suspect that few, if any of these are capable of causing koa diseases. Most of them may be non-pathogenic endophytes that do not adversely affect their hosts.

In conclusion, we confirmed that *F. oxysporum* commonly colonizes *Acacia koa* seedlings from commercial Hawaiian nurseries. However, nursery growers need not necessarily be concerned unless the majority of isolates within their nurseries are pathogenic. Determining pathogenic potential of *F. oxysporum* populations usually requires expensive, time-consuming inoculation tests. However, in some cases specific molecular genetic markers have been developed to differentiate pathogenic from non-pathogenic isolates. With such markers, characterization of fungal populations may be determined much more quickly and at less cost. Using such techniques to characterize *Fusarium* populations associated with koa seedlings will greatly improve our understanding of the potential impacts of these fungi on nursery crops.

*- A. Yeh, N.S. Dudley and R.L. James (USDA Forest Service)*

## *Surveys of Hawaiian Nurseries for Occurrence of Puccinia psidii on Myrtaceae Seedlings*

In April of 2005, a rust disease caused by *Puccinia psidii* was first reported on Oahu. Since that time, this disease has been found on the islands of Hawaii, Maui, Molokai, Lanai, Oahu and Kauai. The rust fungus affects primarily plant species in the Myrtaceae family, particularly plants in the genera *Metrosideros*, *Eugenia*, *Syzygium*, *Psidium, Rhodomyrtus* and *Myrtus*. This includes ohia (*Metrosideros polymorpha* syn. *Metrosideros collina*), one of the two major native tree species in Hawaii. Impacts of this disease could be catastrophic if it spreads to many related species grown in Hawaii. *P. psidii* is endemic in Brazil, Florida, the Caribbean, and portions of Central and South America; it is commonly called *Eucalyptus* rust, or guava rust in these areas. Our goal was to determine the extent and distribution of *P. psidii* on plant species within the family Myrtaceae grown in Hawaii nurseries. In addition, we wanted to determine effects of elevation, rainfall, and surrounding vegetation on disease severity.

Confirmation of presence or absence of the *P. psidii* was based on observation of pustule production on above-ground plant tissues, particularly leaves and stems. Samples in question were submitted to plant pathologists at the Cooperative Extension of the University of Hawaii for microscopic identification. Since symptoms may not appear for two weeks after a plant is infected, nurseries were contacted that disseminate plants. These nurseries were located on the islands of Hawaii,

Maui, Kauai, and Oahu only, although Ohia rust has also been documented on Molokai and Lana'i.

A list of all species in the order Myrtales was sent to various nurseries. The Worldwide *P. psidii* host list was also included for the nurseries to review. when looking for signs of the rust. They were then contacted by phone or email to find out what plants were grown and whether they were willing to allow us to visit their nurseries.

On Hawaii Island, rust was observed on ohia in natural areas around Volcano Village, but not in the ohia collection at the Volcano Experiment Station. In the nurseries surveyed on the Big Island, rust pustules have been observed on *Metrosideros. polymorpha, Myrtus communis, Eugenia paniculata, E. uniflora, Eucalyptus dunnii, Callistemon viminalis*, and *Melaleuca quinquenervia*.

Eight nurseries on Maui were visited. Species affected by the rust were *E. reinwardtiana* and *E. paniculatum*. Rust pustules were also observed on *M. polymorpha, E. paniculata, M. quinquenervia*, and *Callistemon* spp at one of the higher nurseries. Spots were very few, small, and not noticeable. *Syzigium jambos* in outside areas was severely impacted, while *Rhodomyrtus tomentosa* and *E. uniflora* were reported to be affected in the Kahului area by State Forestry personnel and community members.

On Kauai, eight nurseries were visited. Ohia rust was present at all nurseries except the highest (3700 ft) where small purple spots were observed on some of the Ohia leaves, but no pustules were seen. Nurseries reported that the varieties of *M. polymorpha* having small silvery and/or furry leaves appear to be more resistant to the rust. The more glabrous type plants appear to be most susceptible to the rust.

On Oahu, rust infections were more severe on ohia from wet areas, with smooth, non-hairy leaves. Nurseries in the drier areas of Hawaii Kai did not appear to have problems with the rust. One nursery reported that their yellow ohia seemed to be more resistant to the rust than other colors, but again the glabrous leaf types from the wetter areas appear to be more susceptible.

It was suggested that removal of host plants, such as paperback and eucalyptus might help cut down on innoculum levels. Areas which surround the lower elevation nurseries on Kauai have large populations of *S. jambos, R. tomentosa*, and *Psidium guajava* that may also act as reservoirs for the rust innoculum. Efforts were also made to determine how different nurseries were handling outbreaks of ohia rust. The majority of nurseries reported an increase of rust in the spring. A few of the commercial nurseries contacted have decided to select for resistant strains of Ohia for propagation, rather than attempt to control the rust by continuous spraying of fungicides.

*- A. Yeh, N.S. Dudley and R.L. James (USDA Forest Service)*

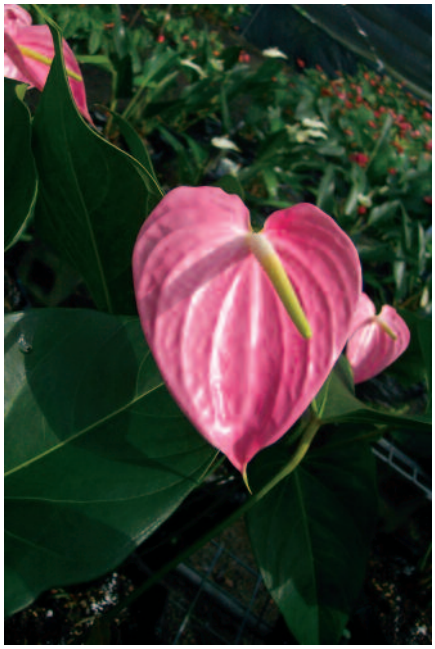
# Miscellaneous Crops

## *Testing the Antimicrobial Activity of Recombinant Proteins in Anthurium*

An *Agrobacterium*-mediated transformation system was used to transform two commercial cultivars of anthurium, Marian Seefurth and Midori. A number of different constructs were used with the aim of improving resistance of the plants to bacterial blight which is caused by *Xanthomonas campestris* pv. *dieffenbachiae*. The constructs used contained genes for the proteins attacin, cecropin and T4 lysozyme. Each of these has been shown to have general antimicrobial properties. Expression of these genes was under the control of a constitutive 35S promoter. However, there were two constructs used which incorporated a wound-inducible promoter, PIN2. One was a dual construct containing both attacin and T4 lysozyme while the other contained only cecropin. Use of all these different constructs allows a comparison of promoters as well as proteins to be made. A number of lines have been generated with each construct. Molecular analysis including PCR, RT-PCR and western blotting indicated incorporation and expression of the antimicrobial proteins in these lines.

A test was carried out to determine whether there was a difference in efficacy of the three antimicrobial proteins against *Xanthomonas campestris* pv. *dieffenbachiae*. It was found that the lowest amount of protein which caused a reduction in bacterial growth was for attacin followed by cecropin and finally T4 lysozyme. From this it would appear anthurium lines expressing attacin might be more resistant to bacterial blight than plants expressing the other two proteins.

A laboratory assay was set up using detached leaves to test activity of the proteins *in planta*. Leaves of plants expressing the recombinant proteins were infiltrated with a bacterial suspension of known $OD_{600}$. After a two week incubation, leaves were photographed and bacteria re-isolated from the leaves. Serial dilutions of the bacteria were made, plated on nutrient media and the resulting colonies counted. All 36 lines tested were shown to produce fewer colonies than the control non-transformed plants. This suggests that expression of the recombinant proteins in detached leaves is sufficient to inhibit growth of the bacteria.



*Anthurium "Marian Seefurth"*

Work is currently underway to inoculate potted plants with a bacterial suspension. It remains to be seen whether lines which appeared most promising in the detached leaf assay also show the best resistance when the whole plant is challenged. Concurrently a field trial for bacterial blight resistance is being undertaken on the Big Island of Hawaii. This is in cooperation with USDA personnel and the anthurium growers.

*- H. McCafferty and Y.J. Zhu*

## Anthurium Micropropagation

Commercial production of anthuriums would benefit from an ample supply of planting materials for new and replanted greenhouses and shadehouses. A project was initiated to determine whether the rate of micropropagation and the number of plants that could be produced within a certain time interval could be increased. Our existing tissue culture stocks of control plants of Marian Seefurth were used as starting material. About 240 plates of Marian Seefurth were inventoried and served as stocks from which small shoot tip cuttings were used to produce new plants. Within six months, we produced about 5000 shoots from the plant clusters. Four to five months after shoot tip cuttings were prepared, about 900 of the shoots were ready to pot in the greenhouse. Losses as a result of contamination were approximately 20%, but we were able to decrease contamination by replenishing them more frequently. In early April 2008, we shipped the first samples to commercial growers to determine if the plants were suitable for planting. Without increasing the original number of stock plates, at least 10,000 shoots could be generated in one year. Increasing the stock plate number should result in even larger yields of separated shoots. Different growth media formulations are being evaluated to determine whether rooted shoots can be developed in less than four to five months.

*- M. Fitch*

## Transformation of Anthurium for Increased Disease Resistance

*Anthurium andraeanum* Hort. cultivars for the cut flower market are highly susceptible to bacterial blight caused by *Xanthomonas axonopodis* pv. *dieffenbachiae*. They are also susceptible to damage from the burrowing nematode, *Radopholus similis*, and the root-knot nematode, *Meloidogyne javanica*. About 800 independently transformed lines of two anthurium cultivars, Marian Seefurth and Midori, were obtained by our group. About 300 of the transgenic lines were shipped under USDA permit to Hilo where the tissue cultured plants were acclimatized to greenhouse conditions for screening for resistance to the pests. *Xanthomonas* testing commenced in late March 2008 starting with 15 Marian Seefurth clones containing genes for an *Arabidopsis* nonpathogenesis-related protein (NPR1). NPR1 controls the cascade of gene activation events in response to pathogen attack. Clones also contained the gene attacin, a lytic peptide from the giant luna moth *Hyalophora cecropia*, and an attacin + T4 lysozyme gene. T4 lysozyme is a lytic protein from the T4 phage virus. Three control Marian Seefurth clones were also tested. We expect that some of these lines will be resistant to bacterial blight. Nematode resistance tests will be carried out later on plants in cinder beds infested with nematodes.

In total, four transgenic lines with the T4 lysozyme gene (T4 1-4), two transgenic lines with the NPR1 gene (XQ1 and XQ2), and two transgenic lines with the cystatin gene (CY1 and CY2) were tested by PCR and Southern blot analyses. All transgenic lines showed positive PCR results using primers specific for the nptII gene. Lines T4 1-3 showed positive PCR results using primers specific for the lysozyme gene, line XQ2 showed positive PCR results using primers specific for the NPR1 gene, and line CY1 showed positive PCR results using primers specific for the cystatin gene. However, lines T4-4, XQ1 and CY2 did not amplify specific bands using primers specific for the gene of interest. The line T4-2 gave a positive result with Southern blot analysis using the nptII gene as a probe. A single band was found in line T4-2, indicating a single-copy transgene insertion into the anthurium genome. This

was the first promising Southern blot result for transgenic anthurium lines produced in our lab. A major reason that other lines did not show a positive Southern blot result could be because of a problem with the quality of the extracted DNA. The DNA extraction protocol will be further modified and more Southern blot analyses are planned.

Of the 800 lines we obtained from transformation of anthurium, about three quarters or about 650 contained a gene for bacterial resistance while about 150 contained one or two nematode resistance genes (rice cystatin and/or cowpea trypsin inhibitor genes). Prior to the bioassays, molecular characterization was accomplished using PCR for selection and resistance genes, enzyme linked immunosorbent assay (ELISA) for expression of the selection gene neomycin phosphotransferase II, western hybridization for expression of the rice cystatin gene, and Southern hybridization for integration of selection genes.

*- M. Fitch, H. McCafferty, J. Zhu, L. Keith (PBARC), D. Gonsalves (PBARC), T. Leong, and X. He*

## *HARC Continues its Jatropha curcas Agronomy Trials*

In late-2006, HARC initiated the first test plot in Hawaii of *Jatropha curcas*, a tropical tree that has received a great deal of attention for its potential to provide vegetable oils for the biofuel industry.

HARC's jatropha research began with the establishment of trees from seed of Madagascar origin. More field plots were established using seeds of Indian origin. The test plantings were designed to monitor plant density as the trees mature. The first year of growth provided a field laboratory to begin evaluating the growth of jatropha in Hawaii's leeward environments. We have been able to achieve year-round flower and fruit production through supplemental irrigation and minimal fertilization. Two significant insect pests were identified in the test plantings: the papaya mealybug (*Paracoccus marginatus*) and the castor semilooper (*Achaea janata*). There also appear to be other viral and fungal pathogens that may affect jatropha's growth and reproduction cycles.

Hawaii's leeward climate zones appear capable of producing sapling jatropha trees that can begin to produce fruit within the first five months of growth. Although the yields are not economically significant, this rapid growth and reproduction will allow HARC researchers and their collaborators the opportunity to rapidly make improvements through traditional breeding procedures. One of the most significant needs for research in jatropha is the development of uniform morphology, development and yield. Early plantings suggest that there is extreme genetic variability across populations. Selections and isolated plantings of superior materials are in planning stages to increase the availability of uniform seed material for further research efforts. Uniformity in established plantings will directly affect the ability to automate field operations and harvesting.

Our staff continued looking for ideal growth characteristics by evaluating trees in several research plots that date back to late 2006. Additionally, there has been a great deal of early success in determining water use parameters and production cycles for the trees. A field with three origins of seed and two irrigation rates was established in late 2007 and monitored closely throughout 2008. Across all seed origins the lower irrigation rate produced more than twice as much fruit than the trees grown under higher irrigation rates. The high irrigation rate

plots received more than 28.5" of rainfall plus irrigation in 2008, and the low irrigation rate plots received just over 19" of rainfall plus irrigation for the year.

There were two substantial fruiting cycles in the first year of growth, with one occurring in late spring and the second occurring in early-to-mid autumn. The second cycle produced about 30% more fruits per plot across all seed origins, but no conclusions can be drawn from this data as trees had not yet reached maturity during the first spring cycle. The trees from Madagascar and India sources performed very similarly, but the trees from the Oahu-origin seed produced less than half the number of fruits.

Another positive development from this project was the insight gained into tree architecture and how it may affect the strategies for designing mechanical harvesters. The ideal row spacing of the trees had been determined from work done in 2007 and this has given greater uniformity in tree architecture from all origins seed sources. The architecture of the tree will dictate the method of harvest that will need to be employed. Trees with bunched branches where fruits may be densely arranged could be harvested with over-the-top machines similar to those used in coffee or blueberries. Trees that develop in an orchard-like manner may be better suited to having fruits harvested off-the-ground by using equipment similar to that used in the macadamia nut industry.

HARC will work to further refine water requirements for jatropha under drip-irrigation systems. A field established in late 2008 has two different rates of irrigation from those used in this experiment. The new field was also planted with seed from five different sources. including seeds from Honduras and the Island of Hawaii that were added to our germplasm collection. The first pruning demonstrations in Hawaii for jatropha grown with drip irrigation are planned for 2009. Next year will also see the continuation of individual tree surveys and selections to begin developing a breeding program for this potential crop.

This research has been supported by the Hawaii Farm Bureau Federation, the Hawaiian Electric Company, and the Hawaii Department of Agriculture.

*- M. Poteet*

*14 month old jatropha trees at HARC's Kunia research station*

## *HARC Scientists Take International Trip to Study Jatropha curcas*

In October 2007, Assistant Agronomist Mike Poteet and retired HARC Vice-President Dr. Robert V. Osgood traveled to multiple international locations to collect information on the research and production of *Jatropha curcas*. The trip was part of a project between HARC and the University of Hawaii's College of Tropical Agriculture and Human Resources (CTAHR) for start-up research into tropical oilseed-bearing trees. The project entitled 'Terrestrial Oilcrops for Biodiesel Production' was granted $150,000 by the Hawaii Department of Agriculture. The international trip was intended to gain knowledge of historic production areas and recent efforts at commercialization and also to meet with other researchers at their field stations.

The first visit on the trip was to the African island-nation of Cape Verde. Cape Verde has a long history of small-scale production of jatropha oil for local use and export to European markets. Those limited supplies of oil were used for soap production in Europe and for lighting the small towns and cities of Cape Verde. Although the majority of commercial production ended in the 1970's, jatropha trees are still looked to as a resource in reforestation efforts. Cape Verde's national agricultural research station, INIDA, served as hosts for the visit and will be supplying some materials for future research projects in Hawaii.

Following the visit to Cape Verde, HARC's staff visited D1 Oils in London and the University of Hohenheim in Stuttgart, Germany. D1 Oils is a world leader in the commercial development of jatropha research and production with operations in Africa, South America, and Asia. The University of Hohenheim is home to Dr. Klaus Becker, an international leader in jatropha research in tropical regions over the last two decades.

From Europe, Dr. Osgood and Mr. Poteet traveled to India for a week of visits. Starting in southern India at Tamil Nadu Agricultural University (TNAU), tours were given of field trials and extraction equipment. TNAU has been declared the Centre of Excellence for Biofuels by the Indian government. Their ties with local growers, processors and industry representatives provided an excellent opportunity to learn more about India's growing interest and investment into jatropha as a biofuel feedstock. While in Tamil Nadu, visits were also made to D1 Oils' regional research station and Bannari Amman's industrial site where jatropha oil extraction and transesterification are taking place.

From southern India, HARC scientists traveled to central India to visit several field sites of CleanStar Energy. CleanStar Energy is a private firm developing their own research and commercialization ventures to determine the economic feasibility of jatropha production in marginal lands across India. CleanStar served as hosts throughout the entire India trip, providing invaluable assistance in networking and travel.

Lastly, there was a brief tour of Haryana Agricultural University's Bawal Research Station. Researchers at Bawal have been studying jatropha as part of an agro-forestry system for nearly ten years. The most potential for inter-cropping within jatropha and other oilseed-bearing trees comes within the first two to three years of growth when tree size is still relatively small and yields have not reached their full potential. Fully mature trees (~10 years old) were observed in an area that could be described as having extreme climate conditions (115°F in summer and <10°F in winter). This climate also dictates that superior trees be selected based on number of fruit clusters produced per branch per year. By isolating their most important production characteristic, Bawal scientists have

been able to manage their selection efforts more effectively.

The final stop of the trip was at the University of the Philippines Los Baños (UPLB). UPLB has recently begun to collect jatropha planting materials from across the country to evaluate at their main campus in Laguna. Through government and private industry support, UPLB has plans to expand their research efforts in field production, oil extraction, and transesterification. Jatropha has been used by villagers in the Philippines for various purposes over many decades. Utilizing information from parts of the world on the tree's varied uses will help develop research that identifies valuable co-products to support the possible commercial production in Hawaii and elsewhere.

The remainder of the HDOA project with the University of Hawaii will select multiple test sites for different oilseed-bearing trees. Other trees of interest include *Elaies guineensis* (African oil palm), *Aleurites moluccana* (kukui nut), *Moringa oleifera* (kalamungay), and *Pongamia pinnata* (karanj). Plantings of these species will be made across the State to compare climates and soils and their effects on the potential productivity of each species. These test plots will help lay the foundation for the necessary research of perennial oilcrops in Hawaii.

*- M. Poteet and R.V. Osgood*

## *Identifying Potential Co-Products and Biochemical Isolates from Jatropha curcas*

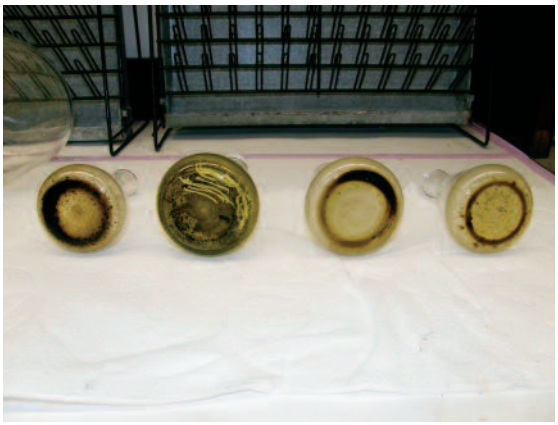
HARC worked with Alternative Aviation Fuels, Inc. in 2008 on a USDA Small Business Innovation Research (SBIR) Phase I grant. The project sought to identify additional possible income streams from commercial production of jatropha. Co-product revenue streams are critically important to the economic viability of any biofuel business models, and jatropha presents unique problems compared to many commercial oilcrops like soybean and canola.

The most common co-product from oilseeds like jatropha is livestock feed made from the seedcake remaining after the oil is expelled from the seeds. This is a high-protein feed additive useful in finishing livestock. Jatropha may not be suitable for such uses due to the presence two different toxic compounds found in its seeds – the protein curcin and phorbol esters. HARC developed screening methods for each of these compounds. The curcin is identified using a bioassay that can only provide a range of the actual concentration through a series of dilutions. Phorbol esters were quantified using standardized HPLC scans. The presence of both compounds confirms that further processing of the seedcake will be present if the varieties currently available are to be commercialized.

Jatropha exudes a milky latex-like material when its branches or leaves are damaged. This material is believed to be the substance that gives jatropha branches their flexibility and resistance to breakage. HARC simulated a harvest practice that would remove branch segments from outside a defined radius from the trees' trunks. The harvested biomass was separated from the fruits to attempt latex extractions. The latex extracted was very similar to that of the rubber found in the *Para* genus of South America, but the volumes present were not great enough to offer a viable co-product stream.

A final possible co-product from jatropha commercial plantings could be in the form of carbon offsets. By taking land out of intensive agricultural tillage and replanting operations, the use of perennial crop species may sequester, or store, more organic carbon by reducing the release of $CO_2$ from soils. We found that one acre of 18-month old trees with a planting density of 1,000 trees per acre can sequester up to 3 MT of carbon, which is the equivalent of about 11 MT of $CO_2$. Further work will be needed to determine additional income streams for future commercial producers of jatropha.

*- M. Poteet, M. Jackson, and J. Clayton*



*Natural rubber forms a thin film on the bottom of flasks after latex extraction*

## *Sweet Sorghum as a Possible Biofuel Crop in Hawaii*

*Sorghum bicolor* L. Moench is an underutilized crop that is beginning to draw attention as a possible biofuel feedstock. Sorghum is produced as either a grain crop that is grown specifically for its seeds or as a forage or silage crop grown for high sugar-content biomass. Both types of sorghum can be used for traditional fermentation ethanol production, either of the harvested grains or extractable sugars, based on type. Sorghum is often promoted in dryland agricultural systems.

HARC (formerly HSPA) participated in two significant sweet sorghum research programs in the 1960's and 1980's. Recently, renewed interest in sorghum by sugarcane producers and other landowners in Hawaii led HARC to seek out old varieties that showed superior adaptation to our special growing conditions. After harvests of over 16 T/acre of dry biomass each from one planting and two ratoon cycles in late 2007 and early 2008, it was determined that further research may be warranted. Sorghum will continue to be evaluated as a promising feedstock crop for biomass and sugar production for conversion into energy and ethanol.

*- M. Poteet*

## *HARC Works with Sandia National Laboratory to Create Biofuel Models*

HARC collaborated on a project with Sandia National Laboratory in Albuquerque, NM, in 2008 as part of a larger Department of Defense's Defense Advanced Research Projects Agency, or DARPA, contract focusing on developing feedstocks for conversion into a biofuel form of JP-8 military-grade jet fuel. Sandia was cooperating with UOP-Honeywell to develop the models of possible crop development strategies and the processing of possible vegetable oils into a biofuel to meet the DOD's rigorous standards. HARC's contribution was to assist in developing production parameters for tropical and subtropical species that would be possible to produce in Hawaii.

HARC staff helped Sandia's team isolate important production inputs and capabilities for hypothetical agricultural operations in Hawaii. These inputs were part of a larger Systems Dynamics model that was created to show the relationships between the multitude of production, processing, conversion, and transportation factors that could impact the feasibility of any new oilcrop operations. Considerations such as land costs, operating costs, co-product development and value, crop selection, impact of crop improvement programs and experience, and soil quality contributed to the inputs provided for the models.

HARC identified 19 separate locations in the State, based on available soil and land use data, from Kauai to Hawaii islands that may have potential for long-term development of a biofuel operation. These locations were selected based on details such as land ownership, infrastructure available and climatic conditions. Each location was assigned a range of yield potentials based on each crop selected for the model. Using these assigned values and parameters, the model could begin to make predictions as to profitability of operations by adjusting certain variables.

GIS soil mapping overlays with input from HARC staff, the final graphical representations from the project suggested that there may be more than 400,000 acres of land in Hawaii that would be suitable for oilcrop production. From these 400,000 acres, over 100 million gallons of oil could conceivably be produced based on yield estimates from the highest yielding species or combination of species for each of the 19 identified areas. This project provided data to federal and state government officials for future use as a decision-making tool in biofuel development strategies.

*- M. Poteet*

## HECO Funds Oilcrop Development Programs

Hawaiian Electric company, Inc. (HECO) began funding the research of potential oilcrops in 2007. These funds are for the establishment of research programs into tropical oilcrops. HARC is administering three projects to be carried out by HARC and the University of Hawaii's Manoa and Hilo campuses. HECO continued support for the projects in 2008 with continued support likely in 2009 and beyond as they work to reduce their dependence on imported petroleum for power generation.

HARC's portion of the projects has consisted of evaluation of *Moringa oleifera* as an additional perennial oilcrop that may produce oils for biodiesel conversion. Moringa is a drought-tolerant species that is well-adapted to Hawaii's leeward climate zones and has been naturalized in Hawaii for decades. HARC's initial work with moringa has shown that although it is well-adapted to our climate and can be productive at very early stages of its life cycle, the trees can grow to unmanageable heights without significant management. Coppicing, or pruning back the tree's main trunk, within the first eight months of growth should improve the viability of the crop in the long-term. The long pods produced by the trees after only a few months' growth contain many small oil-rich seeds. The pod shape and tree architecture will present difficulties to commercialization efforts in Hawaii that would require mechanical harvesting. Moringa will continue to be evaluated, but its short-term prospects appear to be limited as a biofuel crop.

*HARC summer intern Jamey Smith counts seedpods on a young moringa tree*

The University of Hawaii campuses have been working on two other possible oilcrops of interest to Hawaii. The UH-Manoa research objective has been to develop a protocol for tissue culture of *Jatropha curcas*. Early results show problems from contamination and poor cell division, but new leaf growth has been identified as the material most likely to form viable cultures.

The UH-Hilo team has been working to establish *Elaies guineensis*, or African oil palm. After visiting Costa Rica to acquire germplasm which may be better adapted to Hawaii's subtropical climates, the Hilo team imported three varieties totaling 10,000 seeds to begin variety-by-environment test plots on the island of Hawaii. Oil palm requires over 14 months of gestation time in greenhouse conditions, and the seedlings will be transplanted into field trials in late 2009. Each of these oilcrops may be part of an integrated vegetable oil production sector in Hawaii as the demand for feedstocks increases in coming years.

*- M. Poteet*

## Adoption of Cover Crop Technology

Although the benefits of cover cropping to improve soil and reduce erosion have a long and proven history, very few farms in Hawaii employed this cultural practice prior to 2000. Since the mid 1900s, large monoculture plantations were being replaced by smaller, diversified agricultural operations. The new crops used bare ground fallow as a way of managing soil-borne pests and weed competition. This practice increased the risk of soil erosion and runoff of pollutants such as nitrogen and phosphorus into streams and coastal waters.

Interest in, and support for cover crops is growing among grower support organizations. Since 1999, projects funded by SARE, the State of Hawaii Department of Health, American Farmland Trust and National Resource Conservation Service (HRCS) have demonstrated the utility of cover crops. To date, about 1600 acres in Hawaii have converted from bare ground fallow to cover crops.

Currently, a project is underway to further advance the adoption of cover crop technology. It is sponsored by NRCS and involves researchers from Resource Conservation and Development (RC&D), Crop Care Hawaii, HARC, USDA, and the University of Hawaii. Many Hawaiian agriculture companies and individual farmers on all islands are collaborating by having demonstration projects installed on their land.

The demonstration projects are about one half acre in size and include replicated plots of Sunn hemp, buckwheat, oats, combination of these and bare ground. Sunn hemp is a legume plant that contributes nitrogen to the soil and is inhibitory to plant parasitic nematodes. Oats grow quickly, suppress weeds and are not a plant host for the parasitic reniform nematode. Buckwheat loosens topsoil, makes phosphorus and potassium more accessible to crops and chokes out weeds. The major point to be made to farmers is that covercropping is cost effective and can be adapted to various crops and soil types. The project demonstrates that the technique works without additional fertilizer or irrigation.

Before and after cover crop planting in each of the demonstration plots, the soils will be tested for nutrient levels, organic matter, soil compaction, microbial activity and biomass production. Natural enemies of insect pests will be identified and are expected to increase, especially with buckwheat. The populations of plant parasitic nematodes will be counted. The impact on erosion, water infiltration and degree of ground cover will be measured and a cost and benefit analysis made. All of the information will be made available to the farm community with field days and educational materials.

It is hoped that these innovative ideas will appeal to small and large growers and that the adoption of cover crops will improve their economic bottom line as well as conserve soil and reduce pollution of streams and the ocean.

*- S. Schenck, J. McHugh (Crop Care Hawaii), L. Constantinides (Crop Care Hawaii) and M. Johnson (Oahu Resource Conservation & Development*

## *Selection and Improvement of Noni germplasm in Hawaii*

Noni (*Morinda citrifolia*) is a South Pacific tree species. Its fruits and foliage have been used for medicinal purposes for centuries, but just over the past eight years, noni has quickly become a worldwide, billion-dollar industry. The Hawaiian noni industry is just beginning to gain ground in world markets and this necessitates standardized, quality products from juice of measurable quality. Extraction and processing of the juice needs to be standardized for active ingredients and general quality control standards need to be established.

One of the problems facing noni processors and marketers is consistent quality of fruit and products. Noni is harvested from trees growing in a variety of environments and under a variety of nutritional conditions, but so far no efforts have been made to select trees adapted to particular growing conditions. Neither has there been any research into the potential variability of fruit composition. This is of particular importance for noni processors who are faced with variable product yields due to the heterogeneity of the juice. If Hawaii is to compete successfully in the world noni market, it must differentiate itself from the bulk of that market by producing consistently high quality products that are based upon well characterized inputs. This can be achieved by putting in place a program to identify and collect superior noni trees that have high yield, disease resistance and desirable chemical characteristics.

*Figure 1. Variegated leaves of a Molokai variety*

*Unique leaves of a Hawaii Island variety*

*Figure 2. Noni trees hardening off*

*Noni trees in Maunawili field*

The first objective was to locate and record noni plantings around the islands in various altitudes, soil types and environments. This data was obtained from the islands of Oahu, Maui, Molokai, and Kauai. It was found that noni grew in a variety of shade conditions (40% to 100% shade), various altitudes (2 to 427 ft) and in a variety of soil conditions ranging from dry soil / lava rock (Molokai), to extremely wet conditions on steep cliffs (Maui). Both shade and soil conditions were measured by observation, while altitude data was obtained by GPS. We found significant morphological variation within our preliminary collections, including variation in fruit size and shape as well as a putatively seedless variety. There are still areas not yet sampled, including an area reputed to have a pinkish noni (Laupahoehoe, Big Island). Only trees that were apparently healthy and vigorous and had a high fruit load were sampled. It is hoped that this will ensure high yielding and vigorous trees in the final accession.

A collection of accessions has been established in field plots at Maunawili, Oahu. We currently have 19 Noni trees of various phenotypes (Figure 1). This is exactly half of the original 38 noni trees planted. Factors such as insects, mold, draught, animal damage, and wind damage caused the loss of the other 19 trees (Figure 2). The surviving trees represent noni plants from the islands of Oahu (two different genotypes), Maui (five different genotypes), Hawaii, (five different genotypes) and Molokai (one genotype).

In addition to these genotypes, we currently have 12 more genotypes from five Hawaiian islands hardening off in pots (Figure 2). Once these plants have become acclimatized, it is our goal to plant them in both of our fields. This will give us a complete collection at our Maunawili site representing genotypes from five Hawaiian islands, as well as a partial duplicate planting in Kunia representing most of the genotypes. This is being done to protect our collection if one of the fields should suffer weather damage. It is our hope that these plants will survive and subsequent cuttings can be made from them. The location of the Maunawili field is between two sets of honey bee hives in hope that some interesting fruit will be produced by cross pollination. It is this genetic diversity that may give rise to disease and pest resistance as well as desirable chemical characteristics.

Methodology was developed for chemical profiling of noni fruit, leaves and wood. It must be noted that it was decided that chemistry profiles for noni wood samples would have no practical application to farmers due to the fact that the tree itself would have to be trimmed to such an extent that it would reduce fruit producing limbs. Three different assays were conducted on 117 samples to determine the percentage of polysaccharides and phenolics found in noni leaves, both ripe and unripe fruit, as well as the activity level of anti-oxidants found in noni fruit. All samples underwent two different extraction protocols for a) polysaccharides and b) phenolics and anti-oxidants.

*- M. Jackson, S. Nelson (UH), and J. Clayton*

## *Fungal Impregnated Gel for Decontamination of PCBs*

Polychlorinated biphenyls (PCB) are a class of compounds found in insulating materials and other industrial chemicals. PCBs are very slowly degraded in nature and can remain in soil or on surfaces for years. They contaminate the interior metal surfaces of ship hulls and present a problem when ships are decommissioned and scrapped. This project was designed to investigate the development of a spray carrier for fungi that are natural degraders of PCBs and that could be used for decontamination of metal surfaces.

The fungal species that degrade PCBs are known as white rot fungi and include several different species of polypore basidiomycetes that grow on dead wood in moist forest habitats. The fungal mycelia grow throughout the rotting logs and produce their sporulating basidiocarps during wet, cool weather. They degrade the wood lignin, hemicellulose, and cellulose components. We collected three different species that had been shown in previous trials to degrade PCBs. The basidiocarps were brought to the lab and pure *in vitro* cultures isolated.

A carrier substance was also needed to keep the fungi alive and moist while applying them to metal surfaces. The substance had to be non-toxic to the fungi, remain moist for several days, and be able to stick to vertical surfaces. We attempted to use water agar, but it dried out too quickly unless covered. We also tried a 2% alginate gel with calcium citrate solidifier that worked somewhat better. Another carrier tried was psyllium husk powder obtained in a health food store. It forms gels of various levels of solidity depending on its concentration in water. Various combinations and concentrations of alginate, calcium citrate, and psyllium were tried until we finally settled on a formulation composed of 3% psyllium.

One of the fungi was a good degrader of PCBs in previous trials in liquid culture, but did not grow rapidly or cover the metal plate adequately with mycelium. Another grew extensively, but was not a very effective PCB degrader. The third species with extensive growth and good penetration of the gel as well as being an effective PCB degrader was selected as the test fungus in further trials.

For eventual commercialization, the fungi will have to be grown in large quantity. Future work will continue with assays of the fungus and carrier on PCB-contaminated metal surfaces to measure the rate of PCB degradation.

*- S. Spengler (Pacific Hydrogeological), S. Schenck, and J. Clayton*

*Bioremediation fungi*

## *Evaluating New Lettuce Varieties for Resistance to Tomato spotted wilt virus*

Tomato spotted wilt disease, caused by *Tomato spotted wilt virus* (TSWV) has one of the widest host ranges known for any plant virus. There are over 500 known host species, mostly in tropical or subtropical environments. The disease causes severe losses in tomatoes, lettuce and other crops. It is a major limiting factor in lettuce production in Hawaii where the disease has caused 50 - 90% losses. This has resulted in an over-dependence on lettuce imports into the state. The Hawaii market share of lettuce averaged only about 12% from 2001 through 2005 (Statistics of Hawaii Agriculture).

Symptoms of TSWV on lettuce in older plants are brown necrotic spots on leaves and petioles. Infection usually affects one side of the plant, which results in a twisted appearance. Early infections often lead to plant death. TSWV is transmitted by several species of thrips. There are at least five species known to be present in Hawaii that are vectors of TSWV and there is a significant correlation between thrips numbers and incidence of TSWV in lettuce.

Because of its wide host range and its presence in perennial ornamentals and weeds, it is extremely difficult to eliminate the disease. Control of thrips with insecticides is not effective, apparently because the virus is transmitted before residual chemicals on the plant can kill the thrips. The use of resistant varieties offers the most effective and environmentally sustainable long-term control of TSWV. New lettuce varieties are available and a trial was installed to test for resistance to TSWV and suitability to Hawaii's growing conditions.

Twenty-three lettuce varieties of Romaine, Leaf, and Iceberg lettuce were imported from Western Pacific Seed Co. in California. They were planted in a randomized complete block with three replications each as well as replicate plots of four standard Hawaiian varieties of known susceptibility to TSWV as check plants. The first trial planting was followed by a second trial in an adjacent area and then a third trial. The successive trials allowed thrips to multiply and by the third trial a high inoculum level of TSWV was present. Continual monitoring with insect traps showed the thrips numbers to increase rapidly. Out of the 23 lettuce varieties, several were observed to be more resistant to TSWV than the controls.

*- S. Schenck, J. McHugh (Crop Care Hawaii) and L. Constantinides (Crop Care Hawaii)*

*Lettuce TSWV*

## *Bioremediation of Soils Contaminated with Explosive Compounds Residues*

The ability to maintain a well-trained military in the Pacific is threatened by the public's concern about the environmental impact associated with the Army's live-fire exercises. The energetic compounds RDX, HMX, and DNT have been detected in soils and pore water in live-fire training areas. These compounds are mildly soluble in water, but easily bond with soil particles and so persist in both soil phases. Although slowly degraded by soil microorganisms, they can potentially leach into ground water. The purpose of this project was to demonstrate methods for increasing the rate of degradation and removing RDX and HDX from the soil environment in a cost effective way.

The project was planned to proceed in two phases. In 2008, a greenhouse study was carried out with measured amounts of RDX in order to measure the rate of degradation under different treatments. The second, larger scale, field trial phase will be located in a live-fire training area.

The greenhouse trial consisted of 3-gal pots filled with soil from the training area. RDX was added at the same rate to each of the pots. There were six different treatments applied to the pots with nine pots per treatment. The objective was to measure the reduction of RDX over a 12-week period using Guinea grass (Panicum maximum) as phytoremediation with additions of molasses as soil amendments. Previous work had shown that Guinea grass (a common, widespread weed in Hawaii) was effective in taking up contaminants. Molasses soil amendments are known to supply carbon to soil and to increase microbial metabolic activity of soil microorganisms resulting in increases in population levels. Treatments were: A - irrigation water alone, B - no irrigation or other applications, C - Guinea grass with irrigation, D - molasses at a 1:20 dilution applied biweekly, E - molasses at a 1:40 dilution applied biweekly, and F - molasses at 1:20 and Guinea grass. At 4-week intervals, three pots of each treatment were removed and soil of each pot analyzed for RDX content.

*Makua soil in pots*

Few microorganisms can break down RDX into intermediary compounds, but these products can then be metabolized by many more microorganisms into carbon dioxide and simple nitrogenous compounds. The initial degraders use RDX as a sole source of nitrogen. Addition of molasses to soil increases the carbon/nitrogen (C/N) ratio and promotes the activity of the RDX degraders as well as other bacteria and fungi. Certain RDX degraders are known to be anaerobic (*Morganella morganii, Providencia rettgeri*) whereas others are aerobic (*Phanerochaete chrysosporium, Stenotrophomonas maltophilia*, *Rhodococcus rhodocrous*, and *Rhizobium rhizogenes*). Six RDX-degrading bacteria from the training area soil were isolated by rinsing 1-gram (g) soil samples several times in sterile water and incubating the samples in liquid media containing only RDX as a nitrogen source. After three to five days, aliquots were diluted and plated on agar containing RDX. Individual colonies were selected and transferred several more times on RDX

agar. Subsequently, DNA was extracted from the isolates for the purpose of identifying the bacterial species.

In the greenhouse trial, small soil samples were taken biweekly with a cork borer from each pot. Multiple samples from each treatment were mixed and a 1-g subsample was placed in 100-mL of sterile water and shaken for 30 min. A series of dilutions were made and 200-µL of the 1 x $10^{-4}$ and 1 x $10^{-5}$ dilutions were spread on glucose-peptone agar plates. After 5-days the number of colonies were counted and number of live bacteria per gram soil calculated. This method did not measure the RDX degraders which would have been in too low numbers to count, but allowed estimates of any bacteria multiplying on the plates. The estimates gave an indication of general bacterial population increases with each treatment. As can be seen in Table 1, the bacterial counts in the three treatments with molasses increased over the trial period, whereas the counts in treatments without molasses did not. Numbers in Treatment F (Guinea grass with molasses) remained higher longer, probably due to increased water holding capacity or to root exudates. The first soil samples for RDX analysis were taken at four weeks and are shown in Table 2. At that time, the RDX had virtually disappeared in the molasses treatments. The RDX concentration remained about the same for the other three treatments for the duration of the 12-week project. RDX disappeared to the same degree over the same time with either molasses at a 1:40 or 1:20 concentration. Guinea grass alone did not remove RDX in the greenhouse trial.

**Table 1. Bacterial colonies per gram soil x $10^7$**

| Treatment | 2 weeks | 4 weeks | 6 weeks | 8 weeks | 12 weeks | 14 weeks |
|---|---|---|---|---|---|---|
| A. irrigation alone | 3.3 | 0.6 | 0.97 | 2.6 | 1.3 | 2.8 |
| B. no irrigation | 0 | 0.2 | 2.3 | 1.7 | 0.3 | 0.6 |
| C. Guinea grass | 1.4 | 0.5 | 1.8 | 1.8 | 2.2 | 3.7 |
| D. molasses 1:20 | 64.0 | 113.0 | 324.0 | 256.0 | 326.0 | 25.0 |
| E. molasses 1:40 | 9.5 | 162.0 | 238.0 | 241.0 | 36.8 | 23.5 |
| F. molasses 1:20, grass | 34.0 | 74.0 | 141.0 | 424.0 | 370.0 | 343.0 |

**Table 2. RDX mg / kg soil in greenhouse trial at four weeks**

| Treatment | Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|
| A. irrigation | 0.66 | 0.50 | 0.62 |
| B. no irrigation | 0.88 | 0.37 | 0.61 |
| C. Guinea grass | 0.46 | 0.54 | 0.38 |
| D. molasses 1:20 | 0 | 0 | 0.08 |
| E. molasses 1:40 | 0.02 | 0.18 | 0 |
| F. molasses 1:20, with Guinea grass | 0 | 0 | 0 |

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## *Control of Apple Snail in Taro Lo'i*

As reported previously, research is underway to find an inexpensive, environmentally sustainable, effective control measure for apple snails in Hawaiian taro. These snails (*Pomacea canaliculata*) are not native to Hawaii, but were introduced into Hawaii and several other countries for aquariums and possible food source. However, instead of being confined to isolated wet locations and feeding harmlessly on plant refuse, they spread rapidly and began to destroy taro crops. As a result, local taro production was heavily impacted and control measures were sought.

Control of these snails is extremely difficult. A single female can produce as many as 15,000 eggs per year, can thrive in water at a density of 1,000 snails per square meter and can survive buried in mud for several months when the lo'i is drained and left fallow. As a result of the snails spreading throughout the state, production of taro declined from approximately 60,000 lb in 1992 to only 10,000 lb in 1996. Since that time, research projects have targeted several possible control agents for testing.

In 2003, two botanical extracts were tested that had previously shown promise. One of these was an extract from the neem tree and resulted in an average snail mortality rate of greater than 90%. Even though it is a natural extract and not a synthetic compound, the EPA ruled that a full registration study would be required. Since this would be prohibitively expensive, the study was terminated without clearance for use of neem in taro lo'i.

Further studies were undertaken with two more botanical extracts. One was an extract from the mugwort (*Artimesia douglisiana*) and the other an extract of yucca (*Yucca schigidera*). The yucca extract caused 73% mortality within four days. Snail egg clusters were also counted. Unfortunately, these extracts were so toxic that they impacted other animals in addition to the snails and so could not be released into the environment.

In 2005, a project was funded by the Hawaii Department of Agriculture to study ferric phosphate on apple snails. Since this is already cleared for use on terrestrial snails as a commercial product (Sluggo Slug Bait), it is hoped that clearance for use in wet taro will not be difficult.

Counts of live snails and dead snails were taken at one month and two months following treatment applications. The percent of dead snails was significantly higher with ferric phosphate treatment as compared to the untreated control with both 1/2 and full concentration of chemical. At 5X concentration, the percentage dead snails was even higher. The feasibility of EPA clearance for ferric phosphate use in taro is being investigated further.

*- M. Jackson and J. Clayton*

*Apple snail test setup*

# Services

## *Kunia Substation*

Rainbow papaya seeds were crossed, harvested and processed for the Hawaii Papaya Industry Association. The papaya ring spot virus infected all of the Kapoho trees making it impossible to continue Rainbow seed production at Kunia. Seeds were harvested until mid-April from the crossed SunUp trees. The University of Hawaii '10a' sweet corn is still being sold by HARC to local farmers. The last planting was in 2006. The University of Hawaii intended to continue to produce the '10a' as of mid-2007. HARC's supply of the '10a' seeds was expected to last until mid-2008. Corn crops were grown for General Mills, Hoegemeyer and Fiscus. These trials were grow-outs, seed increases and winter nurseries. Coffee crosses and selections were made and collected for variety improvement. Rice was planted for two clients for grow-out and some seed collection.

Conservation projects included constructing diversions, settling basins, rock dams, and grassed waterways in the upper fields at Kunia. Vetiver strips were planted to slow runoff and vetiver was also provided to several farmers. All of the upper fields were ripped/subsoiled to increase water infiltration to minimize runoff. Trees were planted to provide windbreak.

Five hundred sixty-eight new sugarcane cultivars were screened for smut disease susceptibility. The seedpieces were dipped in water and smut fungus spores, planted in the field and ratooned after four months. The cultivars were rated as to susceptibility based on smut symptom severity relative to older sugarcane varieties of known ratings.

Velpar herbicide trials on sugarcane varieties were installed at Kunia. Rates of 0.5, 1.0 and 1.5 lb a.i. per acre of hexazinone were applied over three cultivars. Visual ratings and cane yield will be measured to determine varietal susceptibility to Velpar damage.

Coffee was sprayed with GoalTender and Goal 2XL to evaluate potential crop phytotoxicity. Damage to coffee was minimal so an IR4 project is being initiated to get EPA registration for over the top application of oxyfluorfen. Several rice varieties were sprayed with glyphosate to evaluate crop tolerance to that herbicide.

Three trials were installed at Kunia, Maunawili and Waialua to evaluate several bamboo varieties for use as windbreaks. Another replicated trial was installed to evaluate 18 varieties of papaya for fruit blemishes from agricultural sprays.

Routine ongoing tasks included weather station calibration and a new weather station was installed at the Maunawili Substation. The weather data were placed online via the Internet. Coffee trees at Kunia and Maunawili were pruned. Consultations for nutrient and weed control were performed for Kauai Coffee. HARC also assisted the State of Hawaii Department of Agriculture in evaluation of agriculture leases currently under the control of the State of Hawaii Department of Land and Natural Resources. Properties on the islands of Kauai, Oahu, Maui and Hawaii were inspected.

*- L. Santo*

*Vitiver strips across waterway*

## Computer System Administration

A new file server was established to make the papaya genome database available to the public. After field testing a few PCs using Windows Vista as their operating system, wider deployment on the local area network was begun. Two open-source applications are being evaluated as potential replacements for current applications. Dormant channels in our T-1 line were activated as backup for Internet access. A new ISP was engaged to provide greater bandwidth and better uptime. All PCs were reconfigured to accommodate the change. Fifteen new computers and two printers were added to the LAN. New users were provided basic training and all users were required to pass an on-line information technology (IT) security test. A discrete server for relocated administration personnel was set up. Many accumulated PCs, monitors, printers, etc. were responsibly disposed of through the University of Hawaii's e-Waste collection program.

*- B. Vance*

## Quality Assurance Unit (QAU)

HARC's QAU was engaged in the inspection of three sugarcane field trials as part of the Good Laboratory Practice pesticide registration studies in 2008. HARC's GLP operations were also inspected by one of its sponsors.

*- B. Vance*

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Angel Galvez, Experimentalist
Roland Fernandez, Experimentalist
John Rockie, Experimentalist
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Teodoro Bonilla, Field Worker
Romeo Cachola, Field Worker
Luis Dela Cruz, Weighing Machine Operator
Wilson Galiza, Foreman
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## Kauai Substation

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