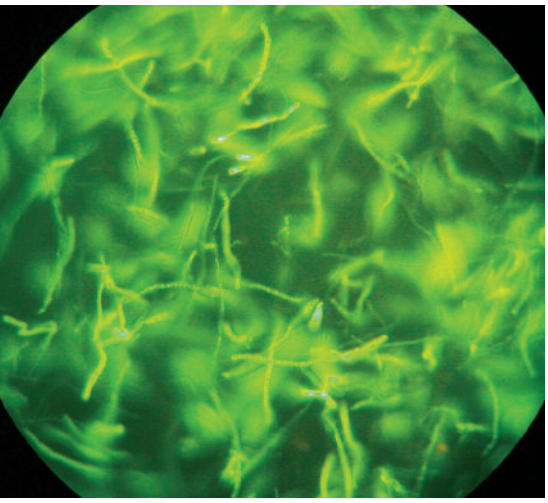
2005

HARC

Hawaii Agriculture Research Center



ANNUAL REPORT

On the Inside

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Background Image:

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

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Director's Letter

Stephanie A. Whalen,
President and Director since 1994

The year 2005 was the 111th year of continuous research and service performed by the Hawaii Agriculture Research Center's research and support staff for Hawaii's agricultural sector. Driven by a problem-solving institutional culture developed since inception, the dedicated scientists and their assistants strive to determine and deliver efficient, cost effective solutions to Hawaii's agricultural producers.

In recent annual reports, we've been reflecting on happenings 100 years ago. In 1905, there were 71 plantations which produced 427,365 tons of raw sugar from 95,443 acres of sugarcane. In 2005, there were two plantations which produced 252,343 tons from 21,735 acres. This increased productivity has been the result of cooperating entrepreneurs investing in research and technology such as, the sugarcane breeding program which was initiated in 1905. This program continues today and remains the backbone of the sugarcane research program. While the program's focus has been sugar yield increases combined with disease resistance, other traits related to fiber have also been explored. Also reported one hundred years ago was the industry's strong influence and efforts in reforestation for watershed protection.

This year's feature article is on progress in sugarcane molecular biology and the challenges for increasing yields. This report includes an update on the breeding and selection programs and further studies with the *Sugarcane yellow leaf virus* P0 protein and other viral suppressor proteins to advance our understanding of biological processes and help overcome the silencing of introduced high value proteins. Also reported is the successful transformation of sugarcane for resistance to the sugarcane yellow leaf virus and information on the antibody produced to monitor the virus.

In papaya, several areas are reported on: molecular approaches to reduce diseases caused by *Phytophthora palmivora, Oidium caricae* and *Collectotrichum gloeosporioides*; chromosome walking of the male specific Y region and determining the importance of the centromere in the evolution of this sex chromosome; cloning of organ-identity genes in flower development; differential expression of the cloned lycopene ß-cyclase gene; and the creation of organ-specific promoters.

The coffee program's first Mokka hybrid families are demonstrating cupping improvements over yellow Catuai and Catimore. Work continues to identify QTL markers controlling tree morphology and fruit quality which will ultimately lead to a more efficient marker-assisted selection program. To advance the understanding of factors affecting quality, a collaboration with Dr. Ishihara from Ochanomizu University was established. This work focuses on the metabolic pathways and concentrations of caffeine and trigonelline, two major alkaloids in coffee with quality, flavor and health impacts. To date, chemical markers have been identified that can discriminate among the various environments and conditions coffee is grown in, but so far cannot discriminate for quality.

A renewed interest in cacao production and a timely collaboration between HARC and the USDA-ARS/SHRS to preserve and co-locate individual trees with good quality traits is leading to a cacao breeding program. Molecular analysis by SHRS and tasting by an established chocolatier suggested that Hawaii could be a source of high quality chocolate. Identifying superior genotypes from existing stands, reproducing them vegetatively and establishing a germplasm base has been accomplished in the first steps for a cacao breeding program.

Recognizing the growing export cut flower industry as an important contributor to Hawaii's agriculture, HARC has partnered with the USDA Pacific Basin Agricultural Research Center and the Hawaii Anthurium Grower Association to tackle the two main problems faced by anthurium growers: bacterial blight and the burrowing nematode. This collaboration has resulted in optimizing an *Agrobacterium*-mediated transformation system and the development of several individual transgenic lines for greenhouse testing. HARC also partnered with the University of Hawaii to overcome quarantine barriers for cut flowers exploring methods to mitigate irradiation injury. Irradiation sensitivity varies from species to species but successful protocols are reported.

Based on the 2004 *Acacia koa* family variation to *Fusarium* wilt, surveys are ongoing throughout Hawaii to determine the frequency and geographic distribution of natural resistance of koa. Artificial inoculation techniques were developed for resistance screening and 332 *Fusarium* isolates have been obtained, which are being maintained in a special collection for future use and their virulence identified.

This year, HARC again faced a significant challenge: the potential loss of its leased Oahu Kunia field experimental site when its owner put the property on the market. This station is considered vital to the continuity of HARC's research in leeward environments not to mention the existing infrastructure investment on the site. The decision was made to sell the Aiea facility to provide the funds to purchase the field station and co-locate the laboratory and field portion of HARC's activities. The organization is hopeful that in 2006 the many factors necessary to implement this action will be achieved.

I want to recognize HARC's loyal and hardworking staff that not only are instrumental in bringing in research dollars but contribute countless hours volunteering their time and expertise in explaining the role of agriculture and agricultural research to society.

I want to thank HARC's supportive Board of Directors, their dedication to science and their recognition of its place in their companies' struggle to remain competitive in a very tough global agricultural market.

Respectfully,

Stephanie A. Whalen
President and Director

100 Years Ago: 1905

The Proceedings of the Twenty Fifth Annual Meeting of Hawaiian Sugar Planters' Association reported that, during the 1905 season, 71 plantations on four islands produced a total of 427,365 tons of sugar on 95,443 acres. This was a yield of 4.48 tons sugar per acre harvested as a one-year crop. The main varieties in production were Lahaina, Rose Bamboo, and Yellow Caledonia. These were imported *Saccharum officinarum* cultivars, not the *Saccharum* hybrids grown today. According to A.J. Mangelsdorf (1953) it was in 1905 that Hawaii first began a breeding program. The first lot of 5000 seedlings resulted in the noble cultivar H-109, a progeny of Lahaina and an unknown parent. This cultivar was greatly superior and eventually became the leading Hawaiian commercial cultivar.

The HSPA, Experiment Station, Division of Entomology reported on their mission to Australia and Fiji to collect parasitic insects in an attempt to control the sugarcane leaf hopper and cane borer. Many thousands of insects were shipped by steamship from Australia, but "it is unlikely that one individual in a thousand reached this country alive." Nonetheless, some of the beneficial parasites were successfully reared and released on plantations. This was one of the first examples of searching for parasitic insects for the control of insect pests.

1905 path laboratory

Dr. N.A. Cobb reported on the creation of a new HSPA Division of Pathology and Physiology of which he was the first Director. A new building was constructed that "excited much interest in its unusual construction features." A great deal of time and effort were put into the establishment of a microscope room, an "illustration room" where drawings could be made, and a "dark room" which was apparently not used for photography, but for concentrating natural light into microscopes since high-powered electric microscope lights were not yet available. They also started a three-quarter acre experimental field at the corner of Alexander and Bingham Streets. This location would now be next to or under the H1 freeway.

Mr. Hosmer, reporting for the Territorial Board of Agriculture and Forestry, said that five forest reserves with a total of 210,322 acres were created during 1905; two on Hawaii and one each on Kauai, Maui, and Oahu. He expected that in the near future more forest would be set aside at Ka'u and Kohala on Hawaii, at Ewa, Waialua, and Waianae on Oahu, in the West Maui Mountains, and in the Puna district of Kauai. He also mentioned the successful celebration on November 3rd of the first Arbor Day to be officially observed in Hawaii. "A good share of interest was aroused throughout the community which can but lead to good results."

A report on the Hawaiian rubber industry listed the importation of thousands of rubber plant seeds for the Nahiku and Koolau rubber companies. About half were of the Ceara type rubber and half of the finer quality Hevea rubber. These seeds came from Paris, Ceylon, and Singapore. Although there was great difficulty in keeping them alive for the length of time it took to ship by steamship, they expected to have about 100,000 plants in the field by January, 1906.

- S. Schenck

Sugarcane Research

Progress in Sugarcane Molecular Biology: Towards an Understanding of Gene-to-Crop Continuum

In August of 2004, the Sugar Research and Development Corporation of Australia funded an international workshop on sugarcane physiology to identify the critical gaps in our understanding of plant and crop physiology that, if addressed by research, could lead to advances in the economic and environmental sustainability of sugarcane production. The keynote address at this meeting, given by Paul Moore (PBARC, USDA at HARC), focused attention on the problems of scale and organizational complexity that must be integrated if advanced sugarcane industries such as those of Hawaii and Australia are to break through the current sugar yield ceiling. The proceedings of that meeting were published in a special issue of Field Crops Research (Volume 92, 2005) and a summary of Dr. Moore's paper (pp 119-135) is presented below to stimulate discussion within Hawaii's sugarcane industry.

We have a working knowledge of the general biology of sugarcane and are fairly efficient in crop production, but are only beginning to produce the detailed biochemical and genetic information that will be needed to continue the increases in sugarcane yields achieved over the last 50 years. Crop yields can be considered from at least four distinct production situations: actual, attainable, potential, and theoretical. Actual yields are those reached under the yield-limiting constraints of various pests, diseases and soil nutrient deficiencies for which ameliorations are generally available. Attainable yields are constrained by the prevailing environment that might include suboptimal factors such as limitations of water, radiation, temperature and soil inhibitory factors. Potential yield is the yield that is achieved when the crop is grown with an ample supply of water and nutrients, absence of pests, and optimal environmental factors. The general crop science literature indicates that yields obtained by the better farmers in favorable areas or at experiment station maximum yield trials are not far below the theoretical physiological limit. If the present yield ceilings of high-input agriculture are to be broken, it seems unlikely to be achieved through additional management and technical inputs. Nor is it likely that future yield increases will come from traditional genetic improvements. Instead, future advances will likely be from areas previously unattainable because of their genetic complexity. For example, we might see genetic improvements in photosynthetic efficiency, partitioning of photosynthates among metabolic pools and quantitative resistance to pests or pathogens.

In 1888, the Dutch established an innovative breeding and selection program in Java to incorporate the disease resistance, hardiness and tillering capacity of *Saccharum spontaneum* into the sugar producing germplasm of *S. officinarum*. The key event of this effort was the release in 1921 of the first of the nobilized hybrid cane cultivars, POJ 2725 and POJ 2878. The spectacular early successes of the newly developed hybrids encouraged most sugarcane-producing countries to establish sugarcane experiment stations to produce locally adapted cultivars and to develop cultural practices for optimizing production. The result of these efforts provided steadily increasing sugar yields of cane for a period of about 60 years. This jump in yields has been followed by a gradual slowing of yields that has been recorded in Hawaii and Australia for perhaps the last 20 years. One explanation for this is that the yields attained are such a

large fraction of the physiological yield possible that the percentage gain of each generation of crop improvement is small. In other words, yields are approaching a yield ceiling.

Sucrose synthesis and accumulation in higher plants are the product of a very large network of interactions that can be analyzed from several perspectives. There is increasing evidence that sucrose is involved in signaling to modulate expression of genes controlling cell division and differentiation, transporters and storage proteins, induction of flowering, differentiation of vascular tissue, seed development and accumulation of storage products. Each of the reactions involved is controlled by activation of specific genes by and interaction among the genotype of the plant, the environment under which it is growing, and its developmental stage at that instant. Modern sugarcane cultivars are interspecific hybrids that, under ideal conditions, are capable of storing sucrose in the parenchyma tissues of the stem up to 62% of the dry weight or 25% of the fresh weight. Multiple pathways involved in sucrose accumulation in which there might be rate-limiting physiobiochemical reactions include: photosynthetic rate, sucrose synthesis, phloem translocation and unloading, sucrose metabolism, genetic and developmental controls, and signal transduction, to name a few. The new sciences of systems biology and bioinformatics will be incorporated into sugarcane research and will further our knowledge of sucrose accumulation systems.

The application of DNA markers to genetic mapping for crop improvement in sugarcane began about a decade ago. The basic chromosome numbers of *Saccharum* have been resolved, knowledge of the genetic diversity and structure of the sugarcane genome is significantly advanced and early generations of linkage maps have been developed. Co-linearity has been used to evaluate the correspondence of quantitative trait loci (QTLs) affecting related traits in sugarcane and other grasses. Corresponding QTLs controlling plant height and flowering were found in sorghum and sugarcane. The QTLs of the sugar metabolic pathway might be candidate genes for controlling sugar content in sugarcane. Current large-scale genome projects on a variety of plants, animals and microbes are making available vast amounts of information in the form of genomic sequences and expressed sequence tags.

The ultimate goal of biological science is to understand life forms in sufficient detail to allow predictions about how the system will perform when it is either placed in a different environment or genetically changed. Although this goal has not been achieved, progress is being made. Successful physiological analysis requires an understanding of the functional interactions between the key components of cells, organs and systems within the living organism. This information resides neither in the genome nor even in the individual proteins encoded by the genes, but rather at the level of protein interactions within the context of subcellular, cellular, tissue, organ and system structures.

- S. Schenck

Developing Transgenic Sugarcane for SCYLV Resistance

Sugarcane yellow leaf virus (SCYLV) is present and widespread in susceptible cultivars in Hawaii. It is spread through infected cane seed and from plant to plant by an aphid vector. The leaf yellowing symptoms are not always evident, but usually appear under certain environmental stress conditions, especially cool temperatures and drought. However, preliminary tests in Hawaii and Louisiana indicated that the infection may reduce growth and yields even when the plants are symptomless. The reduction in growth rate, tillering and sucker formation appears to be greater in young plants. Since all plants of the susceptible cultivars in plantation fields contain the virus, no clear-cut comparison of infected and virus-free plants of the same cultivar at the same location have so far been possible in Hawaii.

This project was undertaken for the production of transgenic sugarcane lines resistant to SCYLV. Transgenic sugarcane lines were tested for resistance in the greenhouse by inoculation with viruliferous aphids. Virus resistant sugarcane lines will be employed in further studies for quantification of the effects of the virus on yield as well as in research into the physiological effects of the virus on the plants.

The inoculated transgenic plants were scored for the presence of virus and compared to controls. The parent line H62-4671 plants were virus-free at the start of the trial, but became infected when inoculated. The resistance levels of the seven transgenic lines varied from complete to slight resistance. The control transgenic plants, containing only the *NPT II* selectable marker tested positive, indicating that the *NPT II* transgene alone does not improve plant resistance to virus.

We have also developed semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) methods to determine virus titer in infected plants. For semi-quantitative RT-PCR, 50 µg of total RNA was treated with DNAase and purified using a phenol method. Reverse transcription was performed using 5 µg of total RNA. For each PCR reaction, the cycle number was optimized in order to remain in the exponential phase. The mRNA levels of SCYLV were also determined in parallel by RT-PCR. Primers for the sugarcane endogenous gene, TPI, were used as an internal control. The results of semi- and quantitative-PCR (qPCR) indicated that virus titer varied between the transgenic lines. They also indicated that virus titer was lowest in the lines which frequently gave negative results in the tissue blot immunoassays. The quantitative virus titre measurements will be compared to the starch and sugar content measurements in these lines.

- Y.J. Zhu, H. McCafferty, G. Osterman, C. Moritomo, R. Agbayani, A. Lehrer, S. Schenck and P. Moore (USDA ARS PBARC)

Characterization of the P0 Protein of Sugarcane Yellow Leaf Virus: A Suppressor of Gene Silencing

Plants use gene silencing to block the replication of infecting viruses; viruses in turn have evolved suppressor proteins which block the host posttranscriptional gene silencing (PTGS) mechanisms. We have previously seen that the *Sugarcane yellow leaf virus* (SCYLV) P0 protein acts to suppress PTGS in corn and in the experimental plant *Nicotiana benthamiana*. We have now further characterized this suppressor activity and found that P0 suppresses both local and systemic PTGS when silencing is triggered by a single-strand RNA molecule, but it does not suppress PTGS triggered by a double-stranded RNA. Additionally, in *N. benthamiana,* cells infiltrated with agrobacterium carrying a P0 expression construct undergo cell death. P0 proteins from dicot-infecting viruses *(Beet western yellows virus, Cucurbit aphid-borne virus,* and *Potato leaf roll virus)* suppress local PTGS, but do not suppress systemic PTGS, and do not induce cell death in the *N. benthamiana* system.

We have not yet determined whether the cell death we observe is necrosis or programmed cell death (PCD). There are several ways in which PCD could be involved in a plant-virus interaction. One example is the hypersensitive response (HR), which is a type of PCD, to *Tobacco mosaic virus* in tobacco. It is conceivable that SCYLV P0 triggers HR in *N. benthamiana,* but there are several alternative possibilities.

We are working to define those regions of the P0 protein which contain the functional domains required for suppressor activity. To do this we have made 17 deletions of different regions of the protein; most of these deletions lose all detectable suppressor activity, however two short deletions at the carboxy terminus of the protein retain some or all activity. A deletion of two amino acids retains all three of the activities of the full-length P0. Deleting 15 amino acids, however, retains only the suppression of local PTGS activity, while the systemic suppressor and cell death activities are lost. This is a first important step towards identifying the structures involved in P0 activity.

Viral suppressors of PTGS may be applied for protecting transgenes from gene silencing, however, this remains a long shot. At this point our goal is to do the basic research elucidating several critical roles for PTGS in plant biology, including plant development, gene regulation, and pathogen defense.

- T. Mangwende, M-L. Wang, S. Ancheta, T.E. Mirkov (Texas A&M), P.H. Moore (USDA ARS PBARC) and H. Albert (USDA ARS PBARC)

Production of Recombinant Proteins in Sugarcane

The challenges of containing transgenes and transgene products, and public perceptions of these challenges, are major barriers to development of plants as biofactory systems for production of high value recombinant proteins. Sugarcane has several significant advantages for transgene/product containment that may position this crop as a very "secure" platform for this purpose. Commercial sugarcane is propagated vegetatively from stem pieces. In Hawaii, commercial cultivars do not normally flower in production fields, so there is little chance of pollen drift or production of viable seed. If viable seed were produced, it would not become mixed with seed supplies because commercial fields are planted with stem pieces, not seed. Sucrose, the food commodity derived from sugarcane, is sold as a refined crystal that is essentially free of protein, rather than a whole fruit or vegetable.

In the unlikely event that sugarcane producing a recombinant protein were mixed into the food supply, the food product (refined sucrose) would remain unaffected. Hawaii has a combination of assets (a climate ideally suited for growing sugarcane, many farmers with extensive experience in growing this crop, and processing infrastructure) that together have produced world record yields. Due to the low world prices for sugar, Hawaii currently has excess capacity for growing sugarcane and this could be available for biofactory applications. Utilizing Hawaii's natural advantages and resources to produce high value proteins provides a great economic opportunity for the state and simultaneously meets critical human health needs with minimum risk to human food supplies or the environment.

For these reasons, we tested sugarcane for transgenic production of recombinant proteins. However, at our current expression levels, it is not economically feasible to use sugarcane for high value protein production. A major limiting factor for recombinant protein accumulation in sugarcane is transgene silencing. Avoiding or controlling gene silencing may result in significant increases in transgene product accumulation and thus increase the feasibility of using sugarcane as a biofactory.

Plants, and other eukaryotic organisms, use several RNA silencing pathways to regulate endogenous gene expression, to maintain proper chromatin structure, and to defend against "foreign" nucleic acid invaders, including viruses, transposons, and transgenes. Many, but not all, of the components of these pathways are shared. Identifying step(s) that are unique to transgene silencing may provide a tool for countering transgene silencing without affecting normal plant development. During co-evolution with their host, viruses have developed suppressors of RNA silencing as a counterdefensive strategy. Suppressors from various viruses target different steps of the RNA silencing pathway.

We are testing the effect of four different viral suppressors of gene silencing on recombinant protein production in transgenic sugarcane plants. The four suppressors are: 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 genes of these suppressors have been co-bombarded with a recombinant protein gene (either granulocyte macrophage-colony stimulating factor, GM-CSF, or GUS) into sugarcane calli. At the end of 2005, we have regenerated more than 70 independent lines that contain one of the suppressor genes. We expect more transgenic plants will be regenerated. The goals for 2006 will be molecular analyses of the transgenic plants including examining the expression levels of both the suppressor and the recombinant proteins. Since silencing suppressors may affect endogenous gene regulation, the growth and development of transgenic plants will also be observed.

- S. Ancheta, J. Hu (UH), T.E. Mirkov (Texas A&M) and M-L. Wang

Commercial sugarcane field

Production of an Antibody to Sugarcane Yellow Leaf Virus

Sugarcane yellow leaf virus (SCYLV) infects many sugarcane cultivars around the world. It has been classified as a Polerovirus in the *Luteoviridae* group. Antibody to the virus is needed for diagnosis of infection and for research purposes. Therefore, a project was undertaken that made use of published information on other Luteovirus coat protein structures, estimates of possible antigenic epitopes, sequencing of the capsid protein gene from a Hawaiian isolate, and testing of antibodies to them for detection and specificity to SCYLV.

From the amino acid sequences deduced to be on the capsid surface, two 14-amino acid peptides suggested by Sigma-Genosys as probably strongly antigenic were TVDDLKANSTGILK and KITSFPVKRNAKKV. Serum from inoculations of the first peptide did not yield any antibody reaction to tissue blots of SCYLV. However, the second peptide proved to react strongly with tissue blots of SCYLV-infected leaves. The tissue blots were made from cut sugarcane leaf midribs and the positive reactions were observed to be in the location of the phloem elements in the vascular bundles. There was no reaction to the surrounding tissue nor was there any reaction to tissue blots made from uninfected sugarcane leaves.

Membranes with tissue blots sent from USDA Canal Point, Florida, were assayed with the new antiserum. Results with infected cultivars Green German and CP65-357 were positive. Cultivars CP72-1210 and CP57-603 that gave negative reactions with antiserum made by Dr. B.E. Lockhart also tested negative with the new antiserum. The new antiserum reactions also agreed with Lockhart's antiserum when used to evaluate tissue blot membranes sent from CIRAD Montpellier, France. These tissue blots were made from a series of worldwide sugarcane cultivars from the French Sugarcane Quarantine Station at Montpellier and included SCYLV isolates from Brazil, Colombia, Florida and Hawaii, Mauritius, Réunion, South Africa, Martinique, Peru, and Senegal.

The active Hawaii peptide sequence was compared to the sequences from the same region of the capsid protein of 54 SCYLV isolates listed in GenBank. The 14-amino acid sequences were all identical except for three. These three were identical to each other and differed by only two amino acids from the other 51 sequences. One of these was from GenBank accession AF369928, a Colombian SCYLV isolate (C3) from sugarcane cultivar CC85-96. The other two, AJ582777 (CHN1) and AF582270 (CUB2) both came from a Chinese cultivar CYZ71-95 and were isolated in Réunion and entered in GenBank by E. Mirkov. The new antibody was not tested against the two Réunion isolates which could not be obtained, but reacted positively with a tissue blot of the Colombian isolate C3.

In addition, the new antibody was tested against dot blots of plant sap infected with other Luteoviruses and Poleroviruses. None of these gave positive reactions. It appears likely that antibody produced from short peptide sequences would be less apt to cross react with related viruses than antibody made to entire virus particles. There was little homology between the SCYLV CP sequence and those reported for other *Luteoviridae*. The new SCYLV antibody, at a dilution of 1:1000 proved to successfully detect all SCYLV isolates tested by tissue blot immunoassay without interfering reactions to other sugarcane leaf proteins or to other related *Luteoviridae*.

- S. Schenck and M-L. Wang

Yellowleaf symptoms

Transformation of Ustilago scitaminea with a GFP gene

Breeding sugarcane resistant to the smut fungus, *Ustilago scitaminea* would be more cost effective if susceptible cultivars could be pinpointed and eliminated sooner, before the appearance of whips. It is possible to detect systemic infections by sectioning, staining and microscopic observations of infected meristematic tissue, but this is too labor intensive for the Hawaii sugarcane breeding program to undertake. Our goal was to develop a fast, easy diagnostic technique using a fungus transformed with a GFP gene. Other pathogenic fungi including *Fusarium graminearum* (Scadsen and Hohn, 2004) and *Ustilago maydis* (Spellig et al., 1996) have been transformed with the GFP reporter gene to facilitate microscopic observation within plant tissue for infection experiments. Transformation of *U. scitaminea* will allow researchers to observe the fungus in systemically infected sugarcane meristem tissue under UV light.

Cultures of plus and minus mating type haploid sporidia were obtained from dilution plates of suspensions of germinating teliospores. Teliospores were obtained from whips of the Maui and Oahu pathovars. Fungal cultures were stored on V8 juice agar and were grown on YEPS liquid medium for transformation. Haploid sporidia were first treated with lytic enzymes from *Trichoderma* sp. to produce spheroplasts (Wang et al., 1988). The expression vector was pOTEF-SG that contains the GFP gene under control of the artificial OTEF promoter obtained from Dr. Jan Schirawski, University of Marburg, Germany. The vector also contained an *hpt* I cassette conferring resistance to Hygromycin. The plasmid pOTEF-SG (Spellig, 1996 #1205) containing the SGFP-TYG gene under the control of the synthetic OTEF promoter was introduced by polyethylene glycol mediated transfection. Selection was carried out on YEPS media containing Hygromycin B. Transgenic colonies were identified that produced a strong green fluorescence when excited by UV light at 470 nm.

Transformed haploid cells of opposite mating types were crossed and formed infectious dikaryon mycelium that was also transformed and could be observed under UV light. Transformed haploid sporidia were also mixed and placed as a "paste" on sugarcane stalk buds. They formed dikaryon and infected the meristem tissue, eventually producing whips. However, the mycelium has not, so far been observed within the plant meristematic tissue. This research is continuing and cultures of fluorescent haploid sporidial cells are being maintained on slants containing hygromycin agar.

- S. Schenck and H. Albert (USDA ARS PBARC)

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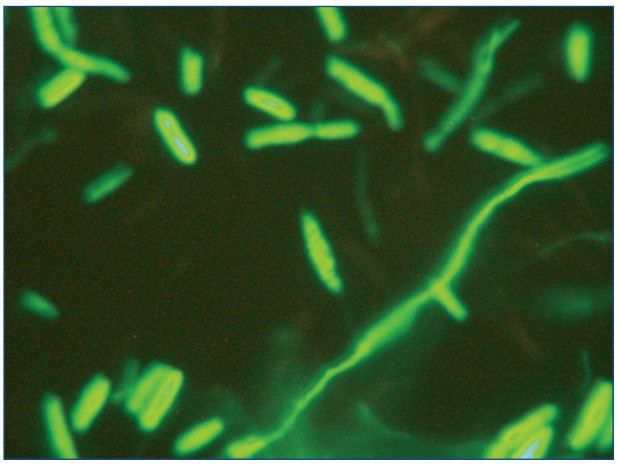

Transformed fungal cells

Sugarcane Breeding and Selection

Sugarcane breeding is an essential part of HARC's activities and developing new cultivars remains vital to Hawaii's sugarcane industry. Disease pressure and potential markets in biofuels and other products are challenges that require ongoing introduction of new cultivars. Collaboration with sugarcane breeding stations in the United States and around the world has allowed the Genetics and Pathology program to introduce new germplasm into the current breeding stock at HARC's Maunawili substation. New technologies and methods are evaluated and put into practice in order to gain efficiency over current practices.

Sugarcane breeding began Nov. 29, 2005 and was completed in the first week of January, 2006. Eight biparental crosses of energy (high fiber) canes were made in response to a renewed interest in high fiber canes by the industry. Thirty-five other biparental crosses were made using current commercial or previously commercial parents. Polycrosses (575) were made from 1,925 tassels of 218 breeding clones. In 2005, we evaluated 377 seedling clones in 23 yield tests (FT7), of which 26% were advanced for further yield trials. During the year, 20 new FT7 tests were installed to evaluate seedling clones selected in 2004.

There were 51,984 seedlings raised from true seeds (fuzz) transplanted into FT1 trials in 2005. Of 66,384 clones planted in 2003 and ratooned in 2004, 2,821 were advanced to FT4 trials in 2005. Of the FT4 clones installed in 2004, 915 were advanced to FT5 in 2005. The top five commercial varieties in 2005, ranked by planted acreage, were H65-7052, H78-7750, H78-3567, H77-4643, and H78-4153.

H78-7750 was the leading cultivar in the state from 2000 through 2003, reaching a peak of 48.3% of total acreage in 2002. By 2005, H65-7052 had replaced H78-7750 as the leading variety due to susceptibility of the latter to leafscald disease. In 2005, H65-7052 occupied 14,626 acres or 37.1% of the state's total cane area. H78-7750 ranked second in total acres with 23.2% of the state's sugarcane area with planting of new acres drastically reduced. H78-3567 has increased for the second consecutive year to rank third in 2005 with 15.3% of the state's total area, much of this increase on Maui. H77-4643, Kauai's dominant variety has dropped from third to fourth in 2005 with 7.9% of the state's total acres. It continues to be reduced due to uprooting which began in 2003 and subsequent poor yields, evidence that it may be experiencing yield decline after being the dominant variety in Kauai for 10 years.

New clones with commercial potential are H86-3792 and H92-5867 for leeward makai areas in Maui, H93-4068 for leeward makai in Kauai, and H87-4094 for leeward mauka areas in Kauai. New release H87-5794 has advanced out of block testing and is being evaluated in ratoon crops. New release H95-4655 is currently in block testing.

- A. Arcinas

Sugarcane inspection

Tropical Fruit Research

Papayas Resistant to Bangladesh Strain of Papaya Ringspot Virus

Papayas are a very good source of beta carotene (as well as other nutrients) and can be raised in the warmer climates of Southeast Asia and Africa where Vitamin A deficiency (VAD) prevails. Beta carotene, or pro-vitamin A, is considered to be non-toxic. It is also stored in the human liver and, as a precursor to vitamin A, can be accessed by our bodies to synthesize vitamin A whenever needed. Present in leafy green vegetables and in orange and yellow fruits such as papaya, beta carotene provides a safe source for the human body to make its own retinol as needed.

Papaya ringspot virus (PRSV) is a virulent pathogen of which 20 known strains from around the world have been identified. Wherever PRSV is prevalent, it severely damages the crop and makes profitable, large-scale production impossible. This project was designed to offer a solution to VAD problems in targeted areas of Bangladesh and Sub-Saharan Africa by restoring commercial production of the beta carotene-rich papaya crop.

University of Hawaii at Manoa (UHM), USDA, and HARC researchers produced transgenic papaya lines, genetically engineered to incorporate the coat protein (cp) gene of Hawaiian PRSV, which successfully prevented infection by our local strain of this virus. Subsequent research to study the effectiveness of coat protein mediated protection (CPMP) determined that shorter segments of the cp gene were also effective in conferring resistance to PRSV infection in papaya. Using this new data, shorter sequences common to all 20 known strains of PRSV worldwide, from both the conserved and variable regions of the cp gene were synthetically made and cloned into separate new constructs, which also contained the Neomycin Phosphotransferase II (NPTII) antibiotic selection gene.

Three papaya cultivars: Khakdum, Kapoho, and Sunrise were selected for transformation with the new constructs containing the shortened synthetic cp gene sequences. Genes were incorporated biolistically into somatic embryogenic calli from seedling hypocotyls of each of the three cultivars with gene gun bombardment. Bombardments were performed in August 2004, December 2004, May 2005, and June 2005. Antibiotic selection for putative transformants was determined using geneticin at 100 mg/L in the callus culture medium. More stringent selection at higher concentrations of geneticin are currently being used because many lines of Khakdum calli that were surviving on geneticin at 100 mg/L, were NPTII gene-negative when assayed for detection of that gene with polymerase chain reaction (PCR).

All PCR-positive lines will be inoculated in the greenhouse trial. Current counts for transformants based on PCR analysis are as follows: NPTII-positive: 17 lines, Gene of Interest (GOI)-positive: 12 lines

Cv. Khakdum: 5 NPTII-positive lines, 2 GOI-positive (conserved region)
Cv. Kapoho: 5 NPTII-positive lines, 2 GOI-positive (conserved region), 1 GOI-positive (variable region)
Cv. Sunrise: 7 NPTII-positive lines, 1 GOI-positive (conserved region), 6 GOI-positive (variable region)

These PCR-positive lines are being regenerated and multiplied in regeneration/proliferation medium, and rooted in vermiculite. After sufficient shoot and root growth, they will be repotted in commercial potting mix, and undergo greenhouse acclimatization for subsequent greenhouse inoculation trials at New York's Cornell University. Additional molecular characterization will be performed on these PCR-positive lines using Southern Hybridization and Detection methods.

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Identification and Characterization of Disease Resistance Genes in Papaya

Recognition of invading pathogens and subsequent activation of defense responses is controlled by resistance genes in plants. These genes in resistant plants recognize specific elicitor compounds produced by certain pathogenic organisms. The elicitors trigger active defense responses in the plant that ultimately inhibit the pathogen. A peptide of 13 amino acids (Pep-13) derived from a cell wall glycoprotein of *Phytophthora sojae* has been identified as being sufficient to elicit defense responses, and homologous genes and proteins are present in other *Phytophthora* species including *P. palmivora*. The Pep-13 motif is highly conserved in different members of the *Phytophthora* species and mutations abolish elicitor activity.

The Pep-13 gene was cloned from *P. palmivora,* the most important cause of fungal disease in papaya. This gene was inserted into a vector containing the green fluorescent protein (GFP) gene as a reporter. The *Agrobacterium* expression system will be used as a tool for the identification of disease resistance genes.

Preliminary leaf infiltration experiments using Kapoho wild-type plants showed that the Pep-13 gene can be expressed locally as determined by RT-PCR. Experiments are currently being carried out to determine the effect of the Pep-13 gene on *NPR*1 gene and PR-1 gene expression.

In addition, we are interested in understanding the signal transduction network that controls the activation of defense response in papaya. It was reported that some defense responses are activated by signal transduction networks that require jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) as signal molecules. Different pathogens are limited to different extents by SA-dependent responses and by JA/ET-dependent responses. Research is planned to elucidate the relative sites of action of the elicitor, Pep-13, in papaya.

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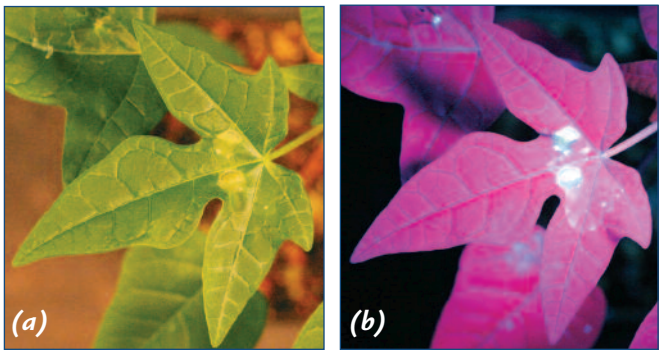

Fig. 1: Kapoho leaves infiltrated with Agrobacterium strain C58C1 transformed with a construct containing the Phytopthora elicitor Pep-13 fused with the GFP gene. Pictures were taken 12 days post-inoculation (a) white light (b) UV light.

Improvement of Papaya Pest Resistance through Genetic Engineering

Insects are a major cause of yield loss for papaya growers in Hawaii. The most serious damage is caused by leafhoppers of the *Empoasca* species and mites including the broad mite, *Hemitarsonemus latus,* the carmine spider mite, *Tetranychus cinnabarinus* Boisduval and a relatively new invader, the papaya leaf edgeroller mite, *Calacarus brionese.* The aim of this work is to improve pest resistance of papaya through plant transformation.

A biolistic gene gun procedure was used to introduce two genes which have already been incorporated into a number of different plants and have given resistance to sap-sucking insects and nematodes. The first gene encodes an insect chitinase (MSCH) from the tobacco hornworm, *Manduca sexta.* The protein from this gene is thought to offer resistance by disrupting insect digestion and chitin metabolism. The other gene encodes a lectin, GNA, from snowdrop, *Galanthus nivalis,* and this protein is also thought to affect insect digestion.

A number of independent papaya lines expressing either the MSCH or GNA proteins have been examined. Molecular analysis has been carried out to detect the presence of the transgenes. Western blotting and reverse transcription polymerase chain reaction (RT-PCR)

have indicated expression of the transgene. Differences in the level of protein expression were obtained with both GNA and MSCH transformed lines.

Five transformed lines expressing MSCH were selected for further study. A quantitative chitinase activity assay showed that the MSCH protein is biologically active. A laboratory assay using the carmine spider mite as a subject showed that mite feeding and reproduction was decreased when fed on MSCH-expressing plants. Also, there was a negative correlation between the amount of chitinase expression and mite survival.

Six GNA lines were selected. The biological activity of recombinant GNA protein was shown using a hemagglutination test with rabbit blood. In a laboratory test using carmine spider mites it was found that feeding on GNA-expressing lines caused a reduction in mite survival and reproduction. Lines calculated to have the highest expression of GNA appeared to have the most deleterious effect on the mite population.

This study indicates that both the insect chitinase and the lectin protein when expressed in papaya increase its tolerance to predation by carmine spider mites. In 'taste tests' where mites had a choice of plants, it was found that they preferred to feed on the wild type Kapoho plants and populations on those plants were higher than for the transgenic lines. It remains to be seen if other sap-sucking insects are also affected by MSCH and GNA proteins.

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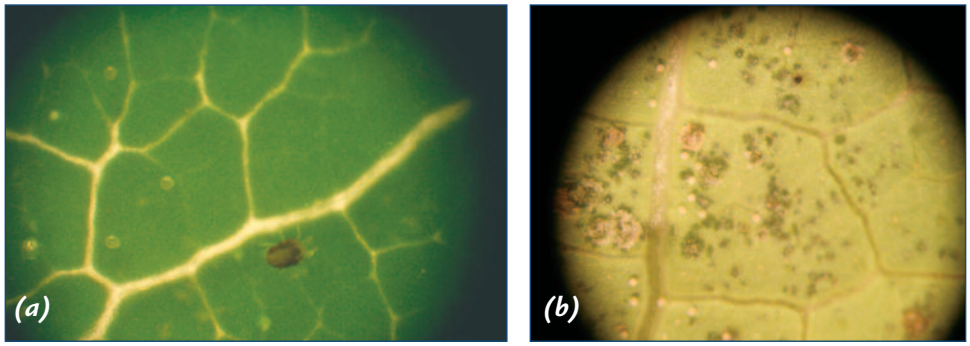

Fig. 1: Carmine spider mites on papaya leaves

a) Adult mite with eggs

b) Mite eggs on bleached leaf surface. Areas of damage due to mite feeding can be seen as dark stippled areas and brown necrotic patches.

Systemic Acquired Resistance in Papaya

*NPR*1 (non-expresser of pathogenesis-related genes) is the key regulatory gene controlling the onset of the systemic acquired resistance (SAR) signaling pathway in plants. Overexpression of the *Arabidopsis NPR*1 gene (*AtNPR*1) gave *Arabidopsis* plants enhanced resistance to certain bacterial pathogens. We found that the SAR pathway in papaya is similar to that of *Arabidopsis* and cloned an *NPR*1 gene homolog, *CpNPR*1. Like the *AtNPR*1, *CpNPR*1 has 4 exons and 3 introns. The overall *CpNPR*1 amino acid sequence is 67% similar to *AtNPR*1. Moreover, all 3 functional domains identified in *AtNPR*1 are conserved in *CpNPR*1 suggesting that *CpNPR*1 has similar function and role demonstrated in *Arabidopsis*. *NPR*1 or its papaya homolog could be the basis of an ideal approach for engineering broad-spectrum disease resistance in papaya.

The objective of this study is to enhance the immunity of papaya by over-expressing a key regulator of the SAR signaling pathway, *NPR*1. Transformations of papaya cv. Kapoho embryogenic calli using constructs containing either *AtNPR*1 or *CpNPR*1 were carried out

using gene gun bombardment.

Transgenic papaya plants expressing the *AtNPR*1 gene and plants over-expressing the *CpNPR*1 gene were produced. Southern blot was carried out to confirm transgene integration of the *AtNPR*1 gene. Presence of the selectable marker gene, *NPTII,* was detected by PCR and gene expression by RT-PCR in transgenic plants over-expressing *CpNPR*1. Bioassays with leaf discs from transgenic plants containing the *AtNPR*1 gene showed smaller necrotic lesions compared to Kapoho wild-type plants indicating that resistance in papaya has been improved. Quantitative RT-PCR showed that *NPR*1 expression is slightly elevated and PR1-d expression was up-regulated in *Phytophthora palmivora* inoculated *CpNPR*1 transgenic plants. Experiments are currently being carried out to compare results of the *AtNPR*1 with over-expression of *CpNPR*1.

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Differential Expression of Protein Profile of Papaya Responding to a Pathogen Inoculation

The goal of this proposed research is to identify proteins involved in the quantitative resistant interaction of Hawaii's most tolerant cultivar, Kamiya, to its major root-rot pathogen, *Phytophthora palmivora,* so strategies can be developed to improve resistance in papaya and other related species to fight off the root-pathogen. We propose to utilize a proteomic approach to a) identify and characterize the differentially expressed proteins in a partial resistant cultivar, Kamiya, as compared to a highly susceptible cultivar, SunUp, b) identify and characterize the differentially induced proteins in responding to *Phytophthora* during a time course post-inoculation, and c) further characterize the selected genes by cloning and Northern blot analysis. Identification of differentially regulated proteins can provide a systematic approach to elucidate the mechanisms of resistance mediated by the resistant proteins in future experiments.

Proteomics is the study of proteins and one of the major goals of proteomics is to identify protein population. The methodology consists of protein separation followed by peptide extraction and mass spectrometry. Sample separation is an important step to be considered before any method of protein separation applied because, plant tissues contain many compounds like lipids, polysaccharides, nucleic acids, salts and proteases that interfere with 2DE (2-dimensional electrophoresis).

Hawaii papaya, in general, is very susceptible to *P. palmivora,* which causes wilting and root rot. We inoculated 3-month-old SunUp plants by drenching the roots with a suspension of *P. palmivora* zoospores. Leaves and roots of SunUp plants were harvested at 24 h, 48 h, 72 h and 120 h post inoculation to extract proteins for 2D gel analysis. 2DE analysis showed a significant difference in expression of proteins between the inoculated samples and the uninoculated control. Mass spectrometric evaluations were carried out to identify the proteins. This research is continuing.

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Molecular Characterization of Disease Resistance Loci to Phytophthora in Carica papaya

Papaya diseases such as root rot and aerial blight caused by *Phytophthora palmivora* reduce yields and increase production costs. *Phytophthora* diseases are particularly devastating because there is relatively little genetic resistance to the pathogen that becomes established in wet, rainy weather when fungicide applications are ineffective.

In this project, we are using an F2 population derived from an F1 of the most tolerant Hawaiian cultivar, Kamiya, crossed with a highly susceptible cultivar, SunUp, to evaluate their progeny's reaction to *Phytophthora* infection. Plant reaction to the pathogen will be accomplished initially by using a laboratory leaf inoculation bioassay followed by a greenhouse plant assay. Ultimately, we will evaluate plant responses in field trials. These same individuals will be scored for DNA markers associated with resistance using methods of amplified fragment length polymorphism (AFLP) in an attempt to identify *Phytophthora* resistance loci that can be applied in breeding programs to accelerate the selection process.

The parental DNA polymorphism survey has been completed. Our goal is to produce a minimum of 300 polymorphic AFLP markers that will be mapped to nine papaya linkage groups (x=9). Papaya DNA extraction has been done as described previously (Steiger et al. 2002). AFLP analyses is being performed on a Li-Cor IR2 Automated DNA Sequencer (Li-Cor, Lincoln, NE) with selective amplification prepared from fluorescent labeled *Eco*R I or *Pst* I primers and *Mse* I primers as described by Kim et al. 2002. Selective amplifications are carried out using various combinations of two to four nucleotide extensions to *Eco*R I (E-) or *Pst* I (P-) primers with *Mse* I (M-) primers (E-2/M-2, E-2/M-3, E-2/M-4, E-3/M-3, E-3/M-2, P-3/M-2, P-3/M-3 and P-3/M-4), as optimized in previous studies (Ma et al. 2004). So far, we have screened 137 pairs of primers to obtain 300 polymorphic markers. The average polymeric bands per primer pair is about 2.1, which is somewhat lower than expected based on earlier work by Hao Ma in our laboratory. Research is projected to produce a fine map for the loci of the more important resistant genes. If multiple loci, quantitatively involved in resistance (quantitative trait loci), are found, they will offer the possibility of increasing resistance to *Phytophthora* by using marker-assisted selection in a traditional breeding program to accumulate multiple resistance genes. Alternatively, future efforts could use neighbor markers to screen a Bacterial Artificial Chromosome (BAC) library for cloning the genes responsible for *Phytophthora* resistance and using these for genetic engineering resistance. This research is a collaborative effort between University of Hawaii, HARC and USDA, supported by a USDA tropical and subtropical agriculture research grant.

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Cloning and Characterization of APETALA3 and PISTILLATA Orthologs in Papaya

Papaya (*Carica papaya* L.) is a polygamous species with three basic sex forms: female, male, and hermaphrodite. Although those three sex forms are genetically determined, the phenotypic sex expression of papaya is influenced by environmental factors, including temperature, nutritional status, and moisture. Instability of papaya flower sex expression is common, and sex reversal occurs in all three sex forms of papaya flowers, especially in hermaphrodite and male flowers. Incomplete sex reversal results in a continuous graded series of flower types. Thus, papaya provides a unique opportunity to study flower development in plants. On the other hand, the instability of papaya flowers can result in unmarketable, malformed fruit. Cloning major genes controlling flower development in papaya is the first step towards solving this problem.

According to the widely accepted ABCE model, B class organ-identity genes act in the second and third whorls of the flower to control petal and stamen identity. Papaya has all three sex forms so its flowers might be ideal for testing the function of B class genes in all three types of flowers. There are two B class genes in *Arabidopsis: APETALA3 (AP3)* and *PISTILLATA (PI)*. Sequences of the two B class genes were used to design degenerate primers for obtaining homologs of these two B class genes from papaya. Total RNA, isolated from different sized male papaya flowers, was used to synthesize cDNA. The degenerate primers of the B class genes amplify cDNA and the amplified fragments were transferred to nylon membrane for Southern hybridization using the *Arabidopsis AP3* and *PI* genes as probes. Portions of the cDNA with homology to the *AP3* and *PI* genes were cloned into vectors and used to screen the papaya BAC and cDNA libraries. Single BAC clones were chosen for each gene, confirmed by Southern hybridization, and then directly sequenced to acquire the full genomic sequence of the *Carica papaya* orthologs, *cpAP3* and *cpPI*.

The papaya gene *cpAP3* shares 60% amino acid sequence identity and 75% similarity with the tomato ortholog TDR6, but it shares only 30% identity and 65% similarity with *Arabidopsis AP3*. Similarly, *cpPI* shares 70% identity and 85% similarity with the *Betula pendula* PI ortholog, but only shares 60% identity and 80% similarity with *Arabidopsis PI*.

Functional analysis of the *cpAP3* and *cpPI* has been studied using quantitative RT-PCR and *in situ* hybridization. Expression data generated by quantitative RT-PCR showed the expression of *cpAP3* and *cpPI* was at the same level between the different sex types. In situ hybridization results revealed that both *cpAP3* and *cpPI* mRNA was detected in the floral organ primordia of papaya flowers. In the late stage of papaya flower development, *cpAP3* and *cpPI* mRNA was also detected at a very high level in the stamen and anther tissue, but not in the petals, sepals, or the surrounding vegetative tissues.

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Tissue Differential Expression of Lycopene ß-cyclase in Papaya

Carotene pigments in flowers and fruits are distinct features related to fitness advantages such as attracting insects for pollination and birds for seeds disposal. In papaya, two flesh colors, yellow and red (Figure 1), are controlled by a single gene with yellow color as dominant. Lycopene and ß-carotene are fat-soluble carotenoids essential to all higher plant species. Lycopene gives many red-fleshed fruits their brilliant color and is found in the chloroplasts and chromoplasts of tomatoes, watermelon, guava, pink grapefruit, and papaya. It has been previously documented that the red color of papaya fruit is due to the accumulation of lycopene; the yellow color is the result of converting lycopene to ß-carotene and ß-cryptoxanthin.

Fig. 1: Flesh color of SunUp (pink) and Kapoho (yellow)

To elucidate the carotenoid biosynthesis pathway in papaya, we took a candidate gene approach to clone the lycopene ß-cyclase genes, *LCY-B*. Degenerate primers were designed from conserved regions of this gene from multiple species to amplify the target gene using papaya genomic DNA as a template. The papaya *LCY-B* ortholog, cp*LCY-B*, was successfully identified from both cDNA and bacterial artificial chromosome (BAC) libraries and complete genomic sequence was obtained from the positive BAC including the promoter region. To obtain the complete genomic sequence of cp*LCY-B*, direct sequencing of positive BAC clone 68A06 through primer walking started from primers designed from the TIGR sequence. The total genomic sequence from BAC 68A06 of red-fleshed genotype SunUp reached 4118 bp, including 1281 bp of coding sequence, 2311 bp of upstream and 526 bp of downstream sequences. The full promoter region was covered as a TATA box was identified at 1503 bp upstream of the start codon. An almost equal length of genomic sequence was obtained from the yellow-fleshed genotype Kapoho. This cp*LCY-B* shared 80% amino acid identify with citrus *LCY-B*. However, full genomic sequences from both yellow- and red-fleshed papaya were identical.

Quantitative real time PCR (qPCR) was conducted using fruit samples from both SunUp and Kapoho immature fruit at three different sizes and mature fruit at color break, 30%, and 100% ripening stages with three replications. The papaya endogenous actin gene was used as a control. The expression of cp*LCY-B* was at the same level between the yellow- and red- fleshed genotypes and among all fruit developing and ripening stages as indicated by analysis of variance. Further expression analyses of cp*LCY-B* by applying qPCR to papaya leaves, flowers, and three different ripening stages of fruit tissues revealed significant difference among leaves, flowers, and fruits. The expression of cp*LCY-B* in leaves and flowers were 16 and 12 fold higher, respectively, than in fruits, suggesting that cp*LCY-B* is down-regulated during the fruit ripening process.

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Organ-Specific Promoter in Papaya

The goal of this project is to produce a database of papaya organ-specific genes and then to isolate a set of promoters corresponding to a selected subset of the differentially expressed genes. The organ-specific promoters developed by this research will provide a method for directing gene expression in the leaf, stem, root, flower, fruit, and seed. Our new promoters can be used for basic research and to manipulate traits having economic significance to the papaya industry. Leaf- and root-specific promoters could be used to direct resistance gene expression in organs attacked by the pathogens or pests without altering fruit quality or production. Flower-specific promoters might be useful in the study of flower development and sexual organ differentiation. Strong fruit-specific promoter(s) would be useful in ripening control. The use of organ-specific promoters will address some public concerns regarding genetically modified (GM) crops. In the draft guidance for industry intending to use plants as biofactories for pharmaceuticals, the Food and Drug Administration encouraged the use of tissue-specific expression to reduce the likelihood of unintended exposure. Similar strategies should also be applied to transgenic plants producing disease- or pest-resistant products. Furthermore, the organ-specific genes identified in this research would provide insight into plant development and can serve as the basis for future research.

The specific objectives of the research include: identification of papaya organ-specific cDNA fragments using amplified fragment length polymorphism (cDNA-AFLP), verification of differential expression patterns, and cloning of organ-specific promoter by screening papaya bacterial artificial chromosome (BAC) library using these cDNA fragments as probes.

We have synthesized cDNAs from 15 organs/developmental stages and completed 166 primer sets of AFLP analyses. Over 300 organ-specific cDNA-AFLP bands have been identified. We plan to screen 60 more primer sets to cover 12.5% of the transcriptome. In the meantime, the expression patterns of the organ-specific fragments will be verified using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and northern hybridization.

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The Role of the Centromere in the Evolution of the Primitive Y Chromosome in Papaya

The origin of sex chromosomes from a pair of autosomes has been confirmed by comparative genomic analyses between mammals and chicken, among species of Drosophila and Silene, and by the recently discovered primitive Y chromosome in papaya. It has been postulated that sex chromosomes evolved from a male sterile or female sterile mutation followed by suppression of recombination at the sex determination locus that led to the degeneration of the Y chromosomes. Suppression of genetic recombination has been documented on primitive to advanced sex chromosomes. Suppression of recombination that is characteristic of sex chromosomes occurs by at least two different mechanisms: chromosomal inversion as in human and Silene and chromosomal translocation as in Drosophila. Sex chromosomes evolved independently, even within the same genus, many times in animals and more recently in plants. Diverse mechanisms, in addition to rare chromosomal rearrangements for suppression of recombination at the sex determination locus, might have contributed to the origin of sex chromosomes in other species.

We previously demonstrated that severe suppression of recombination occurred in the male specific region of the papaya Y chromosome (MSY). We postulated that this region

might be associated with the centromere because it was centrally located in the linkage group. Our recent results showed that the MSY is located near the centromere. The chromosomal location of the MSY strongly suggests a possible mechanism for suppression of recombination that triggered the cascade of events leading to the evolution of papaya's sex chromosomes. Despite their recent origin and primitive state, papaya's X and Y chromosomes have already degenerated extensively, even within the conserved region. The chromosomal features (i.e., gene-poor and low recombination rate) associated with the centromeric location of the MSY appears to have accelerated the degeneration of the Y chromosome. Only two confirmed functional genes were detected in about 1 Mbp of DNA from five fully sequenced and 12 partially sequenced BACs of the MSY region. This extreme gene paucity is the consequence of rapid Y chromosome degeneration in the gene-poor pericentromeric region. In addition, we documented a trend of more extensive degeneration of the MSY in the region closer to the centromere than in the region more distally located. The spread of sequence degeneration from the centromere leads to the conclusion that the centromere has played an essential role in sex chromosome evolution in papaya.

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Genetic Transformation of Pineapple with Nematode Resistance and Flowering Control

We set out to improve the genetic transformation techniques for pineapple in order to solve two serious problems faced by the industry - nematode damage and precocious flowering. We are utilizing a rice gene encoding a proteinase inhibitor, cystatin (H. Atkinson, Leeds University, UK), to interfere with the digestive system and reproductive development of the nematodes. For synchronization of flowering and, in turn, fruit ripening, our strategy is to suppress the production of ethylene, a flowering inducing plant growth regulator. This will be achieved by suppressing the expression of a gene encoding a key enzyme in ethylene biosynthesis, aminocyclopropane-1-carboxylic acid (ACC) synthase.

Our effort in 2005 was focused on improving the transformation protocol by varying several parameters for *Agrobacterium* infection, co-cultivation, and selection. After manipulating over 23,000 leaf bases and 7,000 stem segments, we now have a fast and repeatable transformation method via the adventitious bud pathway. Transgenic plants have been regenerated from both leaf bases and stem segments infected with agrobacteria carrying either the cystatin or the anti-sense ACC synthase construct. The transformation efficiency from the best protocol was 0.17% and 0.26% for leaf bases and stem segment, respectively. Transgenic plants can be identified visually and by polymerase chain reaction (PCR) as early as 3 months after transformation. In all, 20 and 7 independent PCR-positive lines were produced for nematode-resistance and flowering control. Approximately 300 plants each of the first transgenic line containing the cystatin gene (Line 101) and non-transformed control plants were transferred to Dr. B. Sipes (UH) for nematode bioassays. We are currently propagating the other transgenic lines. Our goal for 2006 will be to complete the molecular analyses of the transgenic lines, such as western blot analyses to detect the production of cystatin protein, and southern blot analyses to examine the transgene integration in the pineapple genome.

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Coffee Research

Biosynthesis of Caffeine and Trigonelline during Growth and Ripening of Coffea arabica and Coffea canephora Fruits

Caffeine and trigonelline are major alkaloid compounds in coffee beans. These compounds have recently been studied from the point of view of quality and flavor of coffee beverage and health issues. We investigated the accumulation of these two alkaloids during a six-month period of fruit and seed development in two major beverage coffee species, *Coffea arabica* and *Coffea canephora*. Growth stages are specified by letters, A to G. They correspond to (A) the bean pinhead and small stages, (B) rapid expansion and pericarp growth, (C) endosperm formation, (D) early dry matter accumulation, (E) mature (green), (F) ripening (pink), and (G) fully ripened (red) stages.

Plant biosynthetic activity leading to caffeine accumulation, was estimated by means of incorporation of [8-^{14}C]adenine into purine alkaloids. High biosynthetic activity was found in whole fruits (perisperm and pericarp) in stages B and C, and in developing seeds (endosperm) in stages D and E. Likewise, caffeine and trigonelline content were seen to increase in stages D and E of seed development. Biosynthetic activity for caffeine was reduced in both pericarp (pulp) and seeds (beans) in stages F and G. The concentrations of caffeine in ripe seeds (stage G) of *C. arabica* and *C. canephora* seeds were respectively 1.0% and 1.9% dry weight.

In *C. arabica* var. Mokka and in *C. canephora*, the transcripts of *CmXRS1, CTS2* and *CCS1 N*-methyltransferase genes for caffeine biosynthesis, and also of methionine synthase gene were detected in every stage of growth, although the amounts of these transcripts were significantly less in stage G. The pattern of expression of genes for caffeine synthesis during growth is roughly related to the *in situ* synthesis of caffeine from adenine nucleotides, although exceptions were found in the very early and later stages of fruit growth. The amounts of the transcripts of *CmXRS1, CTS2* and *CCS1,* which encode three *N*-methyltransferases, were higher in seeds than in the pericarp, but the reverse was true for MS transcripts in developing coffee fruits. Similarly, caffeine synthase (*N3*-methyltransferase) activity was higher in seeds than in pericarp.

Concentrations of trigonelline in ripe seeds (stage G) of *C. arabica* var. Mokka, *C. arabica* var. Catimor and *C. canephora* were about 1.3%, 1.0% and 1.4% of dry weight, respectively. High biosynthetic activity leading to trigonelline accumulation was found in young fruits (stages A-C) and in the pericarp of developing fruits (stage E). The biosynthetic activity was reduced markedly in seeds at stages F and G, the harvesting stage of fruits. These results suggest that active trigonelline biosynthesis occurs in the pericarp of coffee fruits. Although the final concentration of caffeine and trigonelline varied in the three *Coffea* types, the overall patterns of fluctuations in caffeine and trigonelline biosynthetic activity were similar.

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Morphological Characterization of a True F2 Population for QTL Markers in Arabica Coffee

A segregating mapping population of Arabica coffee (*Coffea arabica L.*) was developed from a cross between the two varieties, Mokka Hybrid (MA2-7) and Catimor (T 5175-7-1). Mokka Hybrid and Catimor are distinctively different in cupping quality as well as tree and bean morphology. Difference in cupping quality of the two parents was confirmed by a professional coffee cupper. The results of cupping tests on coffee beans harvested in 2004 showed Mokka Hybrid as "almost premium coffee with sweet/mellow flavor" and Catimor as "no premium quality with bland/astringent flavor" (M. Alves, Coffee Lab International).

The first Arabica genetic map was constructed using a pseudo F2 population with 61 plants and amplified fragment length polymorphism (AFLP) markers (HARC Annual Report 2001-4). A true F2 population was generated from two F1 plants, 00-20-25 and 00-20-41, each producing 75 trees. This F2 population was planted at Kunia substation and phenotypic data were collected on tree height and width, branch angle, leaf characteristics. Cherry and green bean weight were also collected. Cherry/green bean characteristics showed normal distribution. No difference in distribution patterns was found between two sub-populations originated from two F1 trees. The variations of the true F2 population were larger than those of the pseudo F2 population as expected. These quantitative data on the population will be used to map QTLs controlling tree morphology and fruit quality when the linkage-map of this population is complete in 2006.

- C. Nagai, M.R. Jones, D. Adamski and R. Ming

New Coffee Hybrid Families for Hawaii

A coffee breeding and selection program designed to develop unique coffee cultivars for Hawaii was initiated in 1997 with the support of the Hawaii Coffee Growers' Association (HCGA) and the State Department of Agriculture. Our target traits for unique coffee include high yielding and larger bean size cultivars with superior cupping quality.

Hawaii has the only commercially grown Mokka variety of Arabica coffee. It was originally from Yemen. The cupping quality of the Mokka hybrid is considered excellent (Kaanapali Coffee, unpublished data), however its bean size is very small (about 30-40% of Red Catuai beans). More than 200 hybrid families have been produced between the selected cultivars including several Mokka hybrid selections made in 1999. About 1,500 progeny resulting from these crosses were evaluated for their tree morphology and fruit and seed characteristics, as well as cupping quality. Yield, cherry/bean characteristics, and cupping quality of the selected parental trees were also evaluated in the fall of 2001 (HARC Annual Report 2003, p. 21-22). Twelve families were selected including larger-bean-sized Mokka hybrids and higher yielding Margogipe hybrids.

The first field trial was initiated at Kauai Coffee plantation in 2004 in order to evaluate field performance and coffee quality of 12 new hybrid families including the Mokka progeny. New hybrid families were planted in Randomized Complete Blocks with 14 trees per plot at two different locations. The current Hawaiian commercial cultivars, Catuai and Typica, were the controls. Morphology and performance of trees were evaluated at six months and one year after planting. Vigorous hybrid families and individual trees are selected at this stage. The first significant cherry production is expected in the fall of 2006.

Cupping was conducted by the Kauai Coffee cupping panel in 2004 and 2005. The selected hybrid families (the parents of Kauai field trials) harvested at Kunia in 2004 were judged to have higher quality than Yellow Catuai and Catimor cultivars.

The F2 generation of the original crosses in 1999 (H99-) produced cherries at Kunia in fall of 2005. Large differences were observed in both tree and cherry morphology. Data analysis for cherry and bean characteristics are underway. We are expecting that other coffee growers in Hawaii will initiate field trials at various locations in 2006. Promising hybrid families and trees are expected to vary between locations due to the interaction of genotypes with environment.

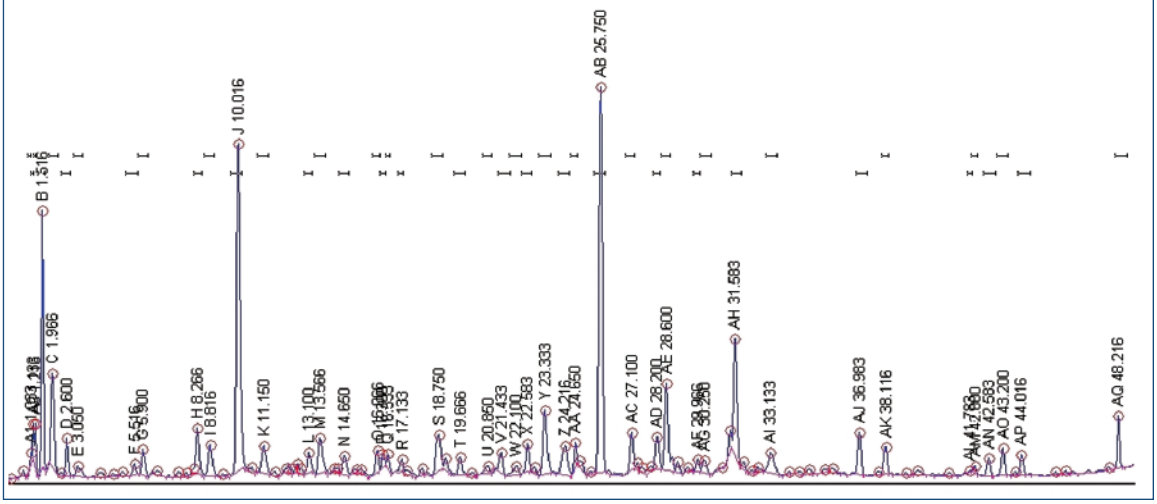
- C. Nagai, D. Adamski, R. Loero (Kauai Coffee) and G. Williams (Kauai Coffee)

Quality Aspects of Shade Grown Coffee

A USDA TSTAR-funded project was initiated in late 2003, and is ongoing. The aim of this project is to determine the effects of various shade regimes on the yield and quality of coffee. The experiment was run in two locations: Kona, Hawaii and Kunia, Oahu. Shade treatments included full sun, 40% black shade cloth, 40% aluminum shade cloth, kaolinite sprayed on the trees after fruit set, and near full shade under macadamia nut trees.

A first harvest has now been made and yield, bean size, cupping, and chemistry data have been taken. As expected, shade caused a decrease in yield and an overall increase in average bean size. However, cupping data showed no statistically significant difference in cup quality between treatments. HARC's role in this project has been to provide and maintain the Kunia test site and to provide the analytical services required for the chemical profiles of the harvested coffee samples. Analysis was undertaken using the same roasted coffee samples that had been cupped. It was decided to use a solid phase microextraction (SPME) technique, employing a coated fiber placed in the head-space above the coffee, brewed in the same way as that for cupping. Volatile compounds were sampled and adhered compounds separated by GC. The profile below shows a typical GC chromatogram of the separated head-space components.

GC CHROMATOGRAM PROFILE



Of the more than 80 compounds separated by GC, 43 were selected for discriminate analysis, so that based upon GC profile, coffees could be grouped by treatment and by location. Using a statistical technique of canonical analysis, coffee samples were correctly grouped by treatment using as few as 13 compounds, which collectively accounted for approximately 97% of the variation seen. When samples were analyzed to predict location, the data allowed for 100% correct classification with only four compounds. Unfortunately, chemical profile could not yet be correlated with cupping, as the cupping data were not able to discriminate between the coffees.

This technique has proven to be very powerful in discriminating between coffees with only relatively minor differences in environment (shade). It can also be used to track down the compounds specifically involved in the plant's physiological response to environment. In this study, the identity of the compounds involved have not been determined, but may be in the future. A second harvest of the coffee is now underway and will provide further discriminatory data that may finally be able to correlate chemistry with cupping.

- M.C. Jackson

Forestry Research

The Black Twig Borer, Xylosandrus compactus (Coleoptera: Scolytidae): A Serious Impediment to Acacia koa Reforestation

The black twig borer (BTB) is a serious pest of agriculture, forestry, and native Hawaiian plants. It was first discovered in Hawaii in 1931, and was determined to be naturalized in 1960. It now has a pantropical distribution which includes the southeastern US. The black twig borer is a typical ambrosia beetle that bores into the host and inoculates the galleries with an ambrosia fungus (*Fusarium solani*) (Daehler & Dudley 2002, Dudley et al. 2004). This fungus grows in the galleries to provide the only food source for the larvae, but is also a pathogen known to cause cankers, root rot, and wilt. The host list for this beetle is extensive and contains at least 108 tree and shrub species in Hawaii, including several listed as threatened and endangered. Koa (*Acacia koa*) is one of the most important of these host species in terms of cultural and economic values (Dudley et al. 2004).

Our approach focused on reforestation of koa plantations to evaluate attractants and repellents to help monitor, control, or prevent black twig beetle damage. Positive results will be applied to other tree species, with particular emphasis on restoration of endangered species in the state of Hawaii.

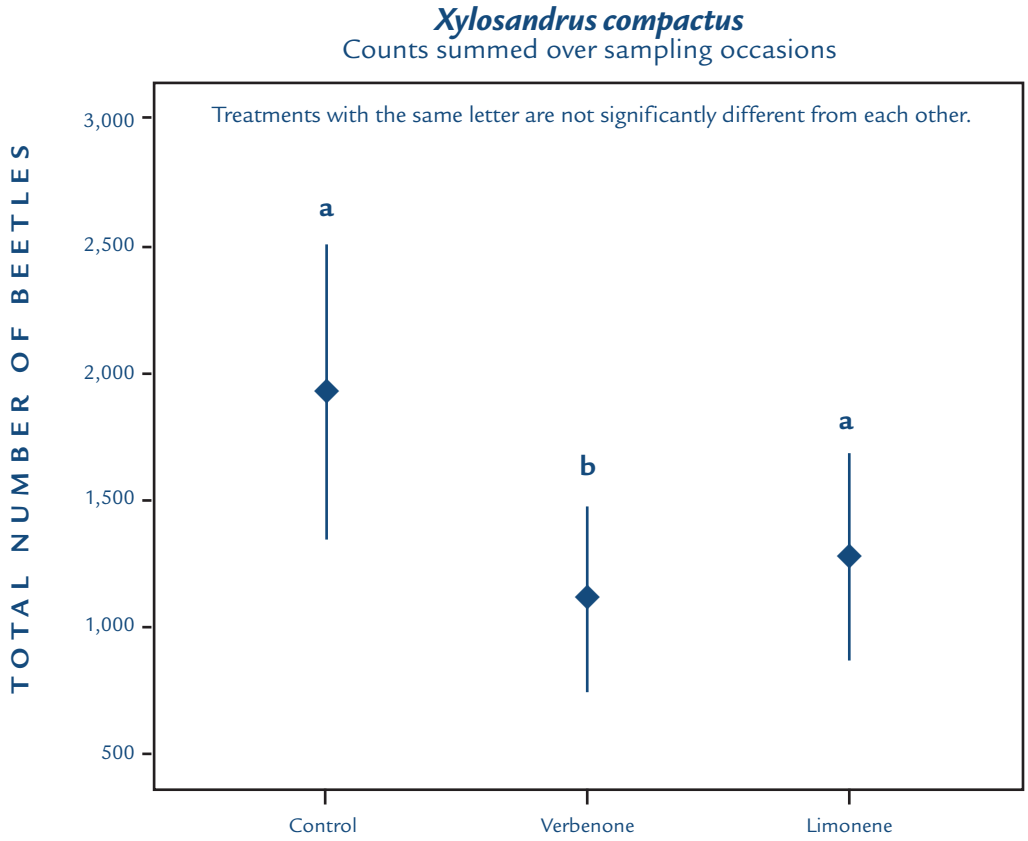
Previous research suggested that ethanol might be a useful attractant for BTB, but early tests were conducted with large, bulky multiple funnel traps that are difficult to deploy in tropical forests. We proposed to test the efficacy of ethanol-baited Japanese beetle traps, which are small, compact, and much cheaper than funnel traps. Once efficacy of these traps was demonstrated, we proposed to challenge the attraction of that trapping system using potential inhibitory or repellent pheromones. Our choice of verbenone and limonene was based on their favorable regulatory status and their demonstrated efficacy in repelling other insect species, especially beetles in the family *Scolytidae*. Once the most effective repellent is identified, we will develop a pheromone release system appropriate for use in Hawaiian forest plantations and ecosystems.

The study was located in a six year old *Acacia koa* reforestation site on the windward side of the island of Oahu. We had earlier demonstrated that ethanol-baited Japanese beetle traps were an effective means of trapping BTB, so we used that trap/bait combination as the positive control. That system also effectively trapped two nontarget pest species, *Xylosandrus crassiusculus* and *Xyleborinus saxeseni* (both Coleoptera: *Scolytidae*), so results for those two species were tallied as well. Ethanol lures were prepared by filling 15-ml low-density polyethylene vials with ethanol, then stoppering them with cotton to minimize the evaporation rate. Limonene lures consisted of 15-ml low-density polyethylene vials filled with 15 ml R-(+)-limonene (Phero Tech, Inc, Delta, BC, Canada) and verbenone lures consisted of polyethylene bubblecaps filled with 800 mg neat S-(-)-verbenone with 1% UV stabilizer (Phero Tech, Inc, Delta, BC, Canada).

Treatments, each replicated six times, were as follows:

- Ethanol-baited control traps
- Traps baited with ethanol and verbenone bubblecaps
- Traps baited with ethanol and limonene vials

Eighteen green-colored Japanese beetle traps (Trécé Company, Salinas, California) were placed in a grid at 15-m intervals and were suspended from the koa trees to a height of 1.5 m. Trap collection cups each contained one half of a Vaportape II pesti-



cide strip (Hercon Environmental, Emigsville, PA) to prevent predation of trapped beetles. Treatments were randomly assigned to the traps, and the ethanol, limonene, and verbenone lures were attached to the Japanese beetle traps using copper wire. Traps were monitored for four weeks, with collections made on a weekly basis. The ethanol vials were refilled after the second week to keep release rates consistent throughout the study. We did replace the limonene vials, however the verbenone bubblecaps were not replaced during the test because their release rates have shown to remain constant for longer than the study's duration. Beetles were counted and identified to species level for statistical analysis.

The ethanol-baited positive controls trapped extremely large numbers of BTB

Fig. 1: Ethanol-baited Japanese beetle trap

(nearly 500/trap/week, on average) (Fig. 1). Lower numbers of the other two species were trapped (roughly 80 beetles/trap/week for *X. crassiusculus*, and roughly 220/trap/week for *X. saxeseni*). Verbenone had a significantly lower trap catch of all

three beetle species. Limonene had a lower absolute number of beetles trapped for all three species, but only for *X. crassiusculus* was this difference significant. These results clearly demonstrate the efficacy of ethanol-baited, green Japanese beetle traps for monitoring of BTB in Hawaiian forest restoration plantings. This trapping system may also prove useful for the two nontarget pests, *X. crassiusculus* and *X. saxeseni*, but it is not clear how these numbers relate to absolute population densities of those two nontarget pests.

- N.S. Dudley, T. Jones and N. Gillette (USDA FS PSW)

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Daehler C, and N Dudley. 2002. Impact of the black twig borer, an introduced insect pest, on *Acacia koa* in the Hawaiian Islands. Micronesica Supplement 6:35-53.

N. Dudley, R. Anderson, R.A. Sniezko, A. Oguchi. 2004. Family Variation in Field Survival of Acacia koa: Prototype testing for variation in Genetic Resistance to Koa Wilt (*Fusarium oxysporum* f. sp. *koae*). International Union of Forestry Research Organizations, Division 2. Nov. 1-5, Charleston, SC.

Selecting for Genetic Resistance to Fusarium Wilt in Koa for Conservation, Restoration, and Utilization in Hawaii

Koa (*Acacia koa*) is the most important native tree in the State of Hawaii from both an economic and environmental perspective, but young trees are having severe problems at low elevations due to a wilt caused by *Fusarium oxysporum* f. sp. *koae*. This project was designed to utilize natural genetic tree improvement techniques to evaluate natural disease resistance and develop a hazard rating system that could have major positive effects in managing this important disease. Surveys are ongoing to determine the frequency and geographic distribution of natural resistance of koa to *F. oxysporum* f. sp. *koae* throughout Hawaii. Field validation tests are also installed to monitor durability of resistance.

Artificial inoculation techniques were developed for resistance screening using a small subset of putatively resistant and non-resistant seedling families. Seed was collected from forest stands for the tests and seedlings were germinated. They are being inoculated with *Fusarium* for evaluation of the rate and severity of disease. This prototype will help confirm the utility of short-term testing. An artificial inoculation procedure using perlite/cornmeal inoculum rather than the previously used technique (seedling root dipping in spore suspensions) was completed. Eleven *Fusarium* isolates from four species (Table 1), which were obtained from diseased koa trees, koa seeds or soil, were evaluated for virulence over a four-month trial. Criteria used to evaluate isolate virulence included seedling mortality, and effects on seedling height, diameter, root volume, and overall vigor as manifested by foliar disease symptoms. Although seedling mortality was low during the test (Table 1), significant reductions of seedling growth and vigor were recorded by many tested isolates when compared to controls. Some isolates of *F. oxysporum*, and one isolate each of *F. solani* and *F. semitectum* were the most virulent.

Seed collections are being made for the range-wide resistance testing with emphasis on natural koa populations. Resistant trees around the islands are located with GPS, tagged for future visits, and distribution of the trees is mapped. Dead and diseased trees are being evaluated to determine the possible causes of koa mortality. Pathogenic fungi are isolated from these trees for identification. Collection goals for

2005 and 2006 were to obtain seed from 25 koa accessions from each of the four major islands (Hawaii, Oahu, Kauai, Maui) for a total of about 100 accessions. To date, 15 accessions have been collected from Oahu and 10 collected from the island of Hawaii. Further seed collections are scheduled for Kauai and Maui. All mother trees are being referenced via GPS and collections are made from both disease-symptomatic and non-symptomatic trees.

Existing field trials were monitored throughout 2005 for disease-associated mortality. In addition, samples were collected from several diseased seedlings and trees as well as seeds and seedpods throughout 2005. More than 170 samples were processed in the laboratory for associated *Fusarium* isolates. *Fusarium oxysporum* and *F. solani* were isolated most frequently from most of the samples. *Fusarium oxysporum* was most commonly isolated from roots and *F. solani* was frequently isolated from stems and branches of diseased trees. Fourteen species of *Fusarium* have so far been isolated from diseased koa, seeds/seedpods, and soil adjacent to roots of diseased trees (Table 2). From these species, 332 isolates have been obtained and are being maintained in a special collection for future use (Table 2).

Future work includes completing the seed collections for range-wide testing of disease resistance, outplanting surviving seedlings at Maunawili, Oahu and initiating field validation trials to monitor durability of resistance. Resistant trees found in the inoculation studies will be planted in moderate to high disease-hazard sites for further evaluation.

Table 1.

Effects of selected Hawaiian *Fusarium* isolates on *Acacia koa* seedlings following artificial inoculations during spring-summer 2005.

ISOLATE NUMBER	*FUSARIUM* SPECIES[1]	PERCENT MORTALITY	AVERAGE HEIGHT	AVERAGE DIAMETER	AVERAGE ROOT WEIGHT[2]	AVERAGE VIGOR RATING[3]
0421A	FSUB	0	30.6 BC	0.38 B	0.63 AB	0.91 DC
0421J	FSUB	0	22.9 E	0.30 E	0.53 BC	0.96 BDC
0424E	FSOL	0	29.2 CB	0.33 DE	0.52 BC	0.54 E
0424I	FSOL	0	23.8 E	0.30 E	0.48 CD	1.23 AB
0425K	FOXY	4.2	24.3 DE	0.35 CD	0.55 BC	0.79 DE
0429K	FOXY	4.2	35.6 A	0.42 A	0.73 A	0.59 E
0430B	FOXY	0	27.2 CD	0.32 DE	0.52 BC	1.22 ABC
0431B	FOXY	8.3	22.6 E	0.29 E	0.39 D	1.36 A
0433D	FOXY	8.3	31.2 B	0.38 BC	0.61 AB	1.05 BCD
0431J	FSEM	25.0	28.3 BC	0.36 BC	0.52 BC	1.18 ABC
—	CONTROL	0	38.8 A	0.42 A	0.60 BC	0.0 0F

[1]*Fusarium* species: FSUB = *F. subglutinans* [isolated from seeds/seedpods]; FSOL = *F. solani* [isolated from diseased seedlings]; FOXY = *F. oxysporum* [isolated from soil (0429K, 0430B) and diseased seedlings/roots]; FSEM = *F. semitectum* [isolated from diseased seedling].

[2]Based on 24 seedlings inoculated per isolate.

[3]Height & diameter = cm; roots = g [oven-dry weight]; numerical vigor rating: [0 = no disease symptoms; 1 = seedling with less than 50% of its crown with dieback/wilt symptoms; 2 = seedling with greater than 50% of its crown with dieback/wilt symptoms, but still alive; 3 = seedling dead [entire crown with disease symptoms]. Means followed by the same capital letter are not significantly different (P=0.05) using the Waller-Duncan K-ratio t test.

Table 2.

Isolates of *Fusarium* spp. obtained from Hawaiian *Acacia koa* within culture collection.

FUSARIUM SPECIES	NUMBER OF ISOLATES IN COLLECTION
Fusarium oxysporum	128
Fusarium solani	91
Fusarium subglutinans	26
Fusarium semitectum	22
Fusarium equiseti	18
Fusarium graminearum	11
Fusarium avenaceum	11
Fusarium proliferatum	10
Fusarium acuminatum	8
Fusarium sporotrichioides	2
Fusarium sambucinum	2
Fusarium poae	1
Fusarium chlamydosporum	1
Fusarium decemcellulare	1
All *Fusarium* Species	332

- N. Dudley

Miscellaneous Crops

Characterization and Evaluation of Cacao

Cacao trees, occurring in the wild in the Amazon basin and tropical areas of South and Central America, belong to the genus *Theobroma*. Of the more than 20 species in the genus, only the cacao tree (*Theobroma cacao*) is extensively cultivated. The three major types of cacao include Criollo, Forastero, and Trinitario. The Forastero type is further divided into Amelonado, and Upper and Lower Amazon Forastero varieties, based largely on pod morphology. Trinitario is the only type not found in the wild, and originated from a cross between Criollo and Forastero.

Commercial production of cacao in Hawaii is increasing and this trend is expected to continue. The increased acreage is being planted with seedlings from uncharacterized cacao populations from at least three initial introductions of cacao into the islands. High yield and superior quality will be necessary for cacao production in Hawaii to be profitable.

Dole Fresh Fruit Co. planted 20,000 cacao seedlings on 17 acres of former Waialua Sugar Plantation in 1998. This field was rejuvenated in 2004 and commercial cacao production was initiated. Molecular analysis by R. Schnell identified the population as Upper Amazon Forastaro (UAF) x Trinitario type with high levels of genetic diversity (Schnell et al. 2005). Large variation in pod (fruit) morphology was also observed among trees (Fig. 1a-c). Preliminary evaluation of pod characteristics was performed as a primary step to select superior genotypes among these trees.

The variation in pod color observed at Waialua varies, ranging from red and ripening to red/orange, red/purple, or red/green. Pods also vary from green, ripening to lime green, green/yellow, and green/orange. Additional pod characteristics include a bottleneck (basal constriction) that ranges from a pronounced bottleneck to none and either a very pointed or rounded bottom. The pod's exterior texture ranges from very smooth to extremely warty. In 2005, we evaluated various pod and bean traits and pod index, for the 20 previously selected trees. Average pod weight (range = 299.0 - 1062.3 g, coefficient of variation, CV = 46.4%) and average seed weight (R = 86.2 – 245.8 g, CV = 38.2%) showed the greatest variation. Less variation was found in the average number of seeds per pod (R = 36.5-55.7 seeds, CV = 13.5%) and seed weight to pod weight ratio (R = 0.17 - 0.39, CV = 22.7%).

An additional 17 trees were selected based on phenotypic variation in pod types. Pods from nine of these trees were micro-fermented by Dole Company and sent for chocolate quality and taste evaluation. Preliminary results show high quality and unique flavor for four different trees. We will select superior individual trees based on pod index data. F1 hybrids will also be produced by cross pollination in 2006.

- D. Adamski, C. Nagai, E. Seguine (Guittard Chocolate), M. Conway (Dole Hawaii) and R.J. Schnell (USDA-ARS)

Fig. 1a-c: Phenotypic variation of cacao fruits (pods) in Dole Waialua Field:

a) Lower Amazon Type Forestero (LAF)

b) Trinitario Type

c) Upper Amazon Type Forestero (UAF)

Vegetative Propagation of Cacao

Cacao is widely propagated by seed when the original trees' genotypes are uniform. Vegetative propagation is necessary, however, when commercially desirable genotypes are either hybrids or genetically heterozygous. A majority of cacao trees in Hawaii were characterized by DNA markers as heterozygous (Schnell et al. 2005), such as Trinitario and Trinitario/Upper Amazon Forastero crosses.

The vegetative propagation of cacao (*Theobroma cacao*) by grafting was initiated to develop an efficient protocol to clonally preserve the selected genotypes of cacao in Hawaii's commercial fields. Planting material used for field trials will be produced by grafting selected trees. Bud-grafting is the technique we selected. Initially both bud-grafting and side-cleft grafting were attempted, but we could not produce successful grafts by the latter method. Two vigorous Trinitario type trees, K25 and K43, from HARC's Kunia substation were used for root stocks. Seeds grown for rootstock had a 95% germination rate, and seedlings could be used for grafting in approximately 3-4 months. Budwood was collected from vigorous branches of selected trees from Dole's field at Waialua, Oahu, and Opihikao, Hawaii, and grafted onto rootstock within 24 hours. Rootstocks which had similar stem size as grafting buds were used for grafting.

Various factors affected the efficiency of grafting. Budwood size and vigor, and matching the diameter of rootstock to budwood pieces were most important. Overall grafting efficiency was 38%. To date, 28 genotypes of selected trees have been grafted. Little difference in grafting efficiency was found between budwood sources Opihikao (42%) and Waialua (35%). Overall grafting success of individuals varied from 19% to 63%, becoming more efficient as individuals' techniques improved.

Once successfully grafted, plants were ready for planting in the field in approximately five months. A germplasm field was established at HARC's Maunawili substation that included clones of 25 genotypes. Grafting of selected trees is ongoing, and successfully grafted trees will be added to the germplasm.

- D. Adamski, J. Rockie, J. Buenafe, T. Menezes (Hakalau Farm and Nursery) and C. Nagai

Marketing Hawaii's Value-Added Agricultural Products

A project was undertaken in 2003 to explore the possibilities for exporting Hawaii's specialty agricultural products to foreign markets. Recent changes in Chinese world trade regulations have created new opportunities which were not previously available. The Chinese economy is growing. In the large coastal cities, affluent consumers with increasing per capita incomes are becoming interested in the available high-end imported specialty products. Hawaiian growers now produce a very large and diverse number of high value, low volume crops and value-added products. The critical issue for many island growers is the lack of export market outlets and an efficient, statewide collection, shipping and marketing contact system. This project was designed to investigate the barriers to entering the Chinese market, the difficulties and regulations needed to be overcome, and to assess the interest of Chinese buyers in our products.

The project collaborators took samples of Hawaiian value-added products to several trade shows and they were very well received. The Chinese buyers are interested in Hawaii and it was found that the Hawaiian theme is attractive to them. Certain colors and package designs are preferred. High-end specialty products that can be given as gifts, particularly when packaged as a gift basket, can command high prices. We therefore, determined to pack-

age our products in koa bowls with inserts explaining each product and a message from the governor attesting to their authenticity.

Overcoming these problems proved to be more complex than at first imagined. It entailed ensuring that proper standards were met, labeling and registration requirements were followed, Chinese promotion and sales agents found, locating and targeting the most promising high-end market, determining packaging and shipping procedures, and sending initial shipments into China. All of these problems took two years to solve, but a group of five products, uniquely Hawaiian, were selected for a pilot shipment. Labels and package designs were created. Once registered and their labeling approved, the same products may continue to be sent into China via a contracted agent.

The marketing team, consisting of a private consultant, University of Hawaii, State Department of Agriculture representatives and HARC personnel, plans to apply for further business funding for establishment of a marketing consortium of Hawaiian producers of value-added products. The core group of producers already registered in China will continue to sell their products either as a group or individually. Eventually, a website may be developed for further sales.

This project was supported by the USDA Foreign Agricultural Service, Emerging Markets Program.

- S. Schenck

Gift package in koa bowl

Impact of Irradiation on the Vase Life of Hawaii's Cut Flowers and Treatments to Mitigate the Injury

The export cut flower industry is an important and growing segment of Hawaii's agriculture. There is a potential for further growth if problems concerning import quarantine regulations against possible infestations of insects could be overcome. The development of an irradiation facility has provided the means of overcoming these quarantine barriers if methods can be found for mitigating irradiation injury to cut flowers.

Irradiation sensitivity of cut flowers varies from species to species. Flowers with low sensitivity to 250 Grays (Gy) of radiation were red gingers, bird of paradise and Oncidium orchid sprays. Ruscus, leatherleaf fern and baby eucalyptus foliage also have low sensitivity to irradiation. Medium sensitive flowers were Dendrobium 'Royal Purple' orchid and 'Red Stricta' heliconia. Flowers that showed high sensitivity to 250 Gy were 'Keanae' heliconia, 'Beehive', 'Barbatus', and Indian Head gingers, Lycopodium and bracken fern.

Dendrobium spray vase life is reduced by exposure to 100 Gy which induces premature flower drop. Treatment of sprays with sucrose, which had been shown to protect chrysanthemum from damage, did not mitigate the damage in Dendrobium. Pretreatment with benzyladenine (BA) also did not help, but pretreatment with the ethyl-

ene inhibitor 1-methylcyclopropene (MCP) significantly extended vase life of the orchids. The MCP treatment can be carried out in the carton during shipment to the irradiation plant, provides great flexibility in handling and packing and removes the need for a fumigation chamber.

Although bird of paradise flowers have low sensitivity to irradiation, they do sustain some damage to the inflorescence bract base and vase life is reduced by two days. Hot water treatment at 40°C for 30 minutes or at 47.5°C for 20 to 30 minutes has potential to reduce the injury. When treated with 200 ppm BA, they were able to withstand 250 Gy. MCP and glucose pulse treatments were less effective. Vase life of red ginger and ruscus foliage was also increased by BA treatments.

Irradiation at 250 Gy induced bract darkening in 'Red Stricta' and 'Keanae' heliconias and reduced vase life by half. A hot water treatment at 42.5°C to 50°C for 20 minutes effectively reduced irradiation injury.

The inflorescences of the sensitive ginger flowers showed short vase life, bract desiccation and cessation of flower development upon irradiation. None of the treatments tried had any effect in reducing irradiation injury for ginger flowers.

- R.E. Paull (UH) and S. Schenck

This research was supported in part by the USDA FAS Technical Assistance for Specialty Crops program.

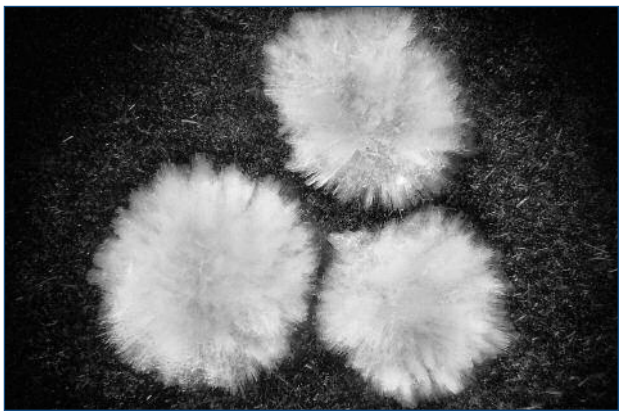
Analyzing Sweetener Compounds from Stevia

Stevia rebaudiana L. Bertoni, is native to Paraguay, where it grows as an annual. The leaves of this plant have traditionally been used in South America to sweeten beverages such as mate.

There are a number of stevia extracts currently on the market. But, while stevia extracts represent the only non-sugar natural sweetener available, they have a taste profile that is not as "clean" as sucrose. This is because removal of bitter tasting components in stevia extracts has proven extremely difficult on a commercial scale. There are two major glycoside components in commercially available stevia extracts. They are stevioside, reported to be about 100-fold sweeter than sucrose on a weight basis and rebaudioside A, reported to be about 300-fold sweeter than sucrose. While rebaudioside A has a desirable taste profile with minimum lingering bitter notes, stevioside is much more bitter. This is not the only factor contributing to stevia extracts' undesirable bitterness. This lab has identified a yellow oily compound in all commercial extracts so far analyzed, which has an extremely bitter taste profile.

The aims of this project were to attempt to develop a method suitable for commercial preparation of pure rebaudioside A from commercially available stevia extracts. In 2005, the last experiments were completed and patent applications filed that describe a commercially acceptable method for producing a stevia extract that contains a minimum of 99.6% rebaudioside A. In addition, this method has now been scaled up to the commercial pilot production scale (1,000 Kg) and repeated a number of times, and has been successful in reproducing those results found in the laboratory. Efforts are now underway to transfer this technology to industry.

- M.C. Jackson



Purified rebaudioside

Control of Apple Snails in Taro

The golden apple snail (*Pomacea canaliculata*) continues to be one of the major pests affecting local taro production. It has a prodigious appetite and rate of reproduction. The snail was introduced into Hawaii, Japan and many other countries in Southeast Asia from South America as a source of food in the early 1980s. However, after its commercial markets had failed, discarded and escaped snails invaded taro and rice ecosystems and have been causing significant economic damage. In Hawaii, these snails were also purposely introduced into taro paddies (lo`i), the reasoning being that they could be harvested for food. However, the consequences of this action were not fully understood at the time. The snails are voracious, fast growing and have a huge reproductive potential. A single female can produce as many as 15,000 offspring per year, and can thrive in water at a density of 1,000 snails per square meter. They mature within 60 to 85 days and spawn at weekly intervals and have been described as the most damaging pest ever to hit neotropical areas. The snails very quickly spread throughout taro lo`i via the extensive and interconnected irrigation systems.

In 2005, a project funded by the Hawaii Department of Agriculture was initiated to study the effects of ferric phosphate on apple snail mortality, feeding behavior and reproductive rate. In previous years, a test system was devised to allow test compounds to be studied in a limited area within a lo'i. This comprises a 4-foot diameter circle with an inner 1-inch mesh circle standing approximately 18 inches high and an outer plastic circle about 12 inches high. The outer circle fits tightly around the inner circle. The circle contains four plants in commercial production and is seeded with 20 snails (15 female, 5 male). This facilitates containment of the test snails and the test compound. In this study the lo'i was drained to an average 1 inch depth and ferric phosphate (1% formulated into bait pellets) was applied to replicate test areas at rates ranging from 1/2 pound/1,000 square feet to 5 pounds/1,000 square feet. Snail mortality, feeding on the taro plants and number of egg clusters were counted at intervals during the following 21 days. No significant differences were found in egg laying or feeding behavior over the observation period, compared with controls. However, a doubling of the mortality rate to 45% was observed in the 5 pounds/1,000 square feet rate of application, compared with the untreated controls.

In addition, the scope of the project was widened to include testing of two additional botanical extracts supplied by the USDA ARS in Mississippi. One was an extract from the mugwort (*Artimesia douglisiana*) containing a highly purified vulgarone B, and the other was an extract from the yucca (*Yucca schigidera*). Using the same test setup, both of these extracts were effective in killing apple snails in a very short period of time. The mugwort extract at a concentration of 75 µM killed 85% of the test snails within four days. Similarly a 120 ppm concentration of the yucca extract caused 73% mortality within four days. One difference in experimental approach between these tests and the ferric phosphate tests, was that the snails were piled together on one side of the test ring, rather than distributing the snails evenly through the test area. When the first observations were made (four days after test substance application), it was noted that with both extracts, the snails had not moved from the pile, indicating that the snails were almost immediately affected, and did not have time to disperse.

Efforts are now under way to determine the feasibility of seeking an EPA registration for either or both of these extracts, so that they can be used to control apple snails in taro lo'i.

- M.C. Jackson

Apple snail test set-up

Transformation of Anthurium for Improved Disease and Pest Resistance

Two main production problems faced by Hawaii's anthurium growers are losses due to bacterial blight, caused by *Xanthomonas axonopodis* pv. *dieffenbachiae,* and the burrowing nematode *Radopholus similis.* A project designed to evaluate biotechnology for overcoming these economic problems of Hawaii's floral industry was created by HARC, USDA-PBARC, and the Hawaii Anthurium Industry Association on the Big Island. The aim of the study is to first optimize protocols for transformation of anthurium and then to use the methods we develop to evaluate candidate genes in new lines engineered for increased resistance to blight and nematode diseases.

Although limited tolerance to blight and burrowing nematodes exists in a few lines of anthurium, it is lacking in the more important cultivars. Two commercially significant lines of standard form anthurium having good breeding potential, Marian Seefurth, with a uniform pink color, and Midori, with a uniform, medium-green spathe that lasts for weeks in floral arrangements, were selected for transformation. Tissue cultures were established from leaf material and transformations were carried out on these cultures using two *Agrobacterium* strains. Genes that produce proteins that have been shown by other researchers to have antibacterial or nematicidal properties were transformed into both cultivars. Over 600 putatively transformed lines of callus were produced (Figure 1a) and have been regenerated into plants (Figure 1b). Our transformation efficiency, that is, the number of putative transgenics recovered per gram of plant tissue inoculated, was considerably improved from previously published results. Preliminary molecular analysis of the plants by the polymerase chain reaction (PCR) shows the presence of the transgene, whereas reverse transcriptase PCR (RT-PCR) and western blotting indicate that the recombinant protein is being expressed in the plant tissue. Whether the level of expression is adequate for resistance is not known. Plants exhibiting positive molecular analysis are being hardened off. These will be used in a laboratory bioassay to test for any improvement in disease or pest resistance. These are normally propagated by cuttings and tissue culture. Thus, phenotype floral traits which segregate out with traditional breeding will be conserved.

With increased resistance to bacterial blight and nematodes, Hawaiian anthurium floral production may increase as will income from sales of our anthurium blossoms around the world. Big Island growers will be able to eliminate costly surveillance for blight and the costs incurred for expensive control treatments should be lowered or eliminated.

- H. Albert (USDA ARS PBARC), M. Fitch (USDA ARS PBARC), D. Gonsalves (USDA ARS PBARC), H. McCafferty, P. Moore (USDA ARS PBARC), T. Leong, J. Suzuki (UH) and Y.J. Zhu

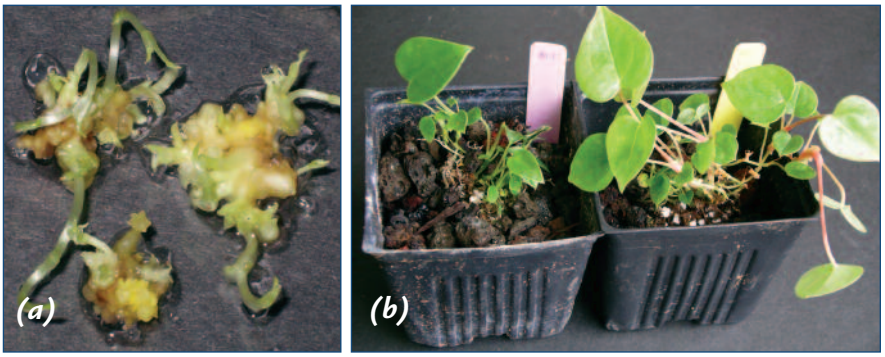


Fig. 1: Anthurium plants grown under laboratory conditions.
a) Transformed callus after 3 months antibiotic selection regenerates into plantlets.
b) Plants regenerated from transformed callus

Services

Facility Administration

The Experiment Station's long-term building superintendent retired necessitating a shuffle in job responsibilities. Standard operating procedures were written to facilitate the guidance and training of individuals involved. The asbestos abatement program at the Experiment Station was completed. A health insurance provider conducted their first annual on-site "HealthPass" program for the benefit of member employees and management. Over half of the building's T12 fluorescent lamps and corresponding ballasts have been replaced with more energy-efficient T8 lamps and electronic ballasts. The exterior of the building was painted with an elastomeric coating to mitigate spalling and moisture penetration. Floor space was measured to allow area calculations according to the industry standards of the Building Owners and Managers Association (BOMA). Corresponding computer aided drawings were generated. Two additional tenants leased rooftop space for their communication operations. HARC's multi-purpose building at our Kunia substation was renovated to accommodate a tenant's requirements.

- B. Vance

Computer System Administration

Office software productivity software for users was upgraded from StarOffice version 7 to 8.

- B. Vance

Quality Assurance Unit (QAU)

Several in-phase portions of sugarcane field trials were inspected and their subsequent reports audited for compliance with the Environmental Protection Agency's Good Laboratory Practices (GLP) as promulgated in the Federal Insecticides, Fungicides and Rodenticides Act. One of our sponsors sent a representative of their Quality Assurance Unit to conduct a facility inspection, which HARC passed. A GLP training session was conducted for employees.

- B. Vance

Sugar Quality Control Technical Services

Raw sugar and molasses from the plantations were routinely analyzed for quality. Samples were taken weekly when the sugar arrived at the terminal and again when the shipment arrived at C&H refinery in California. The shipments carried about 34,000 tons sugar every six weeks. Sugar samples were analyzed for Pol, moisture content, dextran, sulfated ash, color and crystal size. Molasses was analyzed for Brix and total sugars. Cane samples from the breeding FT-7 trials were also analyzed at each harvest. Cane samples are taken, crushed, and analyzed for fiber, Pol and solids.

- B. Somera

B. Somera analyzing sugar

Kunia Substation

HARC's substation at Kunia on Oahu is the site for many HARC research projects as well as for contracts for projects for other Hawaii and mainland companies. During 2005, there were four acres of coffee in place for the coffee breeding program. There were also four and one half acres of papaya being used for research projects and also for transgenic seed production. Corn seed is also an important part of the substation work. Winter seed increases and crossing is done for mainland clients and HARC produced Supersweet 10A hybrid corn seed for the University of Hawaii.

Rice growouts and seed increases were carried out for a mainland company as well as Protea variety testing for the University of Hawaii. Sugarcane clones for the HARC breeding program are tested at Kunia for smut disease resistance. In addition, a sugarcane quarantine field is located at Kunia for any sugarcane varieties imported into the state. Many of the HARC pathology projects are planted at Kunia either in the field or in the greenhouse.

Plant production for a water phytobioremediation project was carried out in the greenhouse for Marine AgriTech company. The plants were taken from the Kunia greenhouse directly to the Ala Wai canal for cleanup of water pollution.

A complete weather station is maintained at Kunia substation and measures radiation, air temperatures, rainfall, wind speed and relative humidity.

- L. Santo

Banana Micropropagation

Production of micropropagules is important to Hawaii's banana industry as a control measure of Banana bunchy top virus (BBTV). In addition, micropropagated plants of uniform size have an advantage for initiation of new plantings. We produced micropropagules for banana growers in Hawaii using the protocol developed at HARC (Annual Report 1997, p. 25).

The initial cultures were established from shoot explants (1.5- to 2-inch length by 0.5- to 0.75-inch diameter) of William and Apple banana cultivars provided by the growers. All the explants were confirmed as BBTV-free before initiating the cultures. A total of 27,000 plants of William and 17,000 of Apple banana were produced in 2005. Besides these two major banana types, cultures of "Sava" cooking banana were successfully initiated. Approximately 1.0% of total plants produced showed some morphological variation, which included shortened plants and narrowed leaves.

- J. Buenafe, M. Pascua, N. Rosete and C. Nagai

Micropropagated banana at nursery

Administration and Support Staff

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Kuo Kao Wu, Vice President and Assistant Director
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Florida Chow, Human Resources
Patrick Ching, Buildings and Grounds Assistant
Becky Clark, Bookkeeper
Elon Clark, Buildings and Grounds Superintendent
Gloria Duncan, Housekeeping
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Ladislao Gonzalez, Watchman, Maintenance
Anthony Lannutti, Secretary-Treasurer, Controller
Alyson Manuel, Office Clerk
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Julie Pinget, Housekeeping
Cynthia Pinick, Executive Secretary
Blake Vance, Assistant Administrator

Staff

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Mel Jackson, Director of Product Development and Services
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Chifumi Nagai, Plant Breeder, Biotechnologist
Michael Poteet, Assistant Agronomist
Lance Santo, Agronomist/Field Coordinator
Susan Schenck, Plant Pathologist
Ben Somera, Sugar Technologist
Ming-Li Wang, Molecular Biologist
Kuo Kao Wu, Sugarcane Breeder
Aileen Yeh, Hawaii Coordinator
Qingyi Yu, Plant Molecular Biologist
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Sachiko Saito, Laboratory Assistant
George Yamamoto, Special Projects Assistant

Kunia and Maunawili Substations

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Anna Ancheta, Field Worker
Rudy Dizor, Mechanical Operator
Angel Galvez, Mechanical Operator
Roland Fernandez, Experimentalist
John Rockie, Experimentalist
Roger Styan, Experimentalist, Supervisor

Maui Substation

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Artemio Bacay, Field Worker
Teodoro Bonilla, Field Worker
Romeo Cachola, Field Worker
Orlando Castres, Field Worker
Luis Dela Cruz, Weighing Machine Operator
Wilson Galiza, Foreman
Domingo Vallecera, Field Worker

Kauai Substation

Fernando Garcia, Field Worker
Narciso Garcia, Field Worker

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Erin Yafuso, Biological Aide, PBARC

Sugar Company Production

COMPANY	ACRES HARVESTED	TONS RAW SUGAR (96°)	TONS SUGAR PER ACRE
2001			
Gay & Robinson, Inc.	4,193	54,691	13.04*
Hawaiian Commercial & Sugar Co.	15,101	191,512	12.68
Totals & average	19,294	246,203	12.76**
2002			
Gay & Robinson, Inc.	4,754	54,196	11.40*
Hawaiian Commercial & Sugar Co.	16,557	215,888	13.04
Totals & average	21,311	270,084	12.67**
2003			
Gay & Robinson, Inc.	4,191	55,267	13.19
Hawaiian Commercial & Sugar Co.	15,660	205,742	13.14
Totals & average	19,851	261,009	13.15**
2004			
Gay & Robinson, Inc.	4,903	59,111	12.06
Hawaiian Commercial & Sugar Co.	16,887	198,755	11.77
Totals & average	21,790	257,866	11.83**
2005			
Gay & Robinson, Inc.	5,096	59,612	11.70
Hawaiian Commercial & Sugar Co.	16,639	192,730	11.58
Totals & average	21,735	252,342	11.61**

* Includes Kekaha salvage cane

** Weighted average

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