Demonstrating Pathogenicity of *Enterobacter cloacae* on Macadamia and Identifying Associated Volatiles of Gray Kernel of Macadamia in Hawaii

K. A. Nishijima, M. M. Wall, and **M. S. Siderhurst,** Pacific Basin Agricultural Research Center (PBARC), USDA-ARS, P.O. Box 4459, Hilo, HI 96720

ABSTRACT

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Gray kernel is an important disease of macadamia (*Macadamia integrifolia*) that affects the quality of kernels, causing gray discoloration and a permeating, foul odor. Gray kernel symptoms were produced in raw, in-shell kernels of three cultivars of macadamia that were inoculated with strains of *Enterobacter cloacae*. Koch's postulates were fulfilled for three strains, demonstrating that *E. cloacae* is a causal agent of gray kernel. An inoculation protocol was developed to consistently reproduce gray kernel symptoms. Among the *E. cloacae* strains studied, macadamia strain LK 0802-3 and ginger strain B193-3 produced the highest incidences of disease (65 and 40%, respectively). The other macadamia strain, KN 04-2, produced gray kernel in 21.7% of inoculated nuts. Control treatments had 1.7% gray kernel symptoms. Some abiotic and biotic factors that affected incidence of gray kernel in inoculated kernels were identified. Volatiles of gray and nongray kernel samples also were analyzed. Ethanol and acetic acid were present in nongray and gray kernel samples, whereas volatiles from gray kernel samples included the additional compounds, 3-hydroxy-2-butanone (acetoin), 2,3-butanediol, phenol, and 2-methoxyphenol (guaiacol). This is believed to be the first report of the identification of volatile compounds associated with gray kernel.

Additional keywords: anaerobic storage, bacterial fermentation, *Enterobacteriaceae*, food safety, spoilage, water activity

Macadamia (*Macadamia integrifolia* Maiden & Betche) ranks among the four top commodities in Hawaii (10) with a farm value of $44.4 million (2005 to 2006 season) (21). Gray kernel of macadamia is an important disease in Hawaii that affects the quality of kernels with a uniformly gray color, off-flavor, and a permeating, foul odor (14). Gray discoloration and odor usually occur together on affected kernels, but may occur separately. During periods of high rainfall, up to 10% of a harvested crop in commercial orchards may be affected by the disease (14), although 0.7 to 4% occurrence is typical of most years (A. Yamaguchi, *personal communication*). Gray-discolored kernels are usually detected and removed during the culling stage of commercial processing. However, when kernels exhibiting only the foul odor pass through culling and reach holding bins, healthy kernels become contaminated with the odor and entire batches become unmarketable. Although several bacterial species were isolated from both gray and foul-smelling kernels, a bacterial cause of the disease was not clearly established because of difficulties in consistently reproducing the disease or fulfilling Koch's postulates (14). The most frequently isolated bacterial strain was *Enterobacter cloacae* (Jordan) Hormaeche & Edwards (14).

E. cloacae, a member of the family *Enterobacteriaceae*, is ubiquitous in nature and commonly found on or in plants, insects, and many sources in our environment such as water, sewage, or soil (11,15,17,18,25,27,28). It is one of the most frequently isolated *Enterobacter* species in man and animals (25,28), and although generally not known to be an enteric pathogen, it is an opportunistic pathogen in humans (25). *E. cloacae* also is pathogenic to plants, affecting elm trees, mung bean sprouts, coconut, orchid, corn, and onion bulb (1,2,4,7,20,26,30,35). It causes two additional plant diseases that occur in Hawaii, internal yellowing of papaya fruit (*Carica papaya* L.) (23) and rhizome rot of ginger (*Zingiber officinale* Roscoe) (22). In papaya, recovery of *E. cloacae* from flowers suggested this as a site of infection and fruit fly as a possible vector of the pathogen (23). In ginger, *E. cloacae* is an opportunistic pathogen that causes foliar symptoms and rhizome rot when environmental conditions favor bacterial growth or host susceptibility. Atypical symptoms consisting of gray discoloration and putrid odor can also occur in infected rhizome tissue under low oxygen conditions created by vacuum-sealing rhizome segments (22) or by growing ginger plants under prolonged (2 months) waterlogged soil conditions (K. A. Nishijima, *unpublished data*). The low oxygen and high moisture atmospheres associated with gray tissue in infected ginger rhizomes are similar to the environmental conditions of naturally occurring gray kernel of macadamia (14) and may provide clues to the etiology of the disease. In addition, virulence among *E. cloacae* strains from different hosts or sources can vary (22,23), and an understanding of strain differences may help develop bioassay methods for cultivar screening for resistance in macadamia or other hosts.

Detection of volatiles for diagnosis of food spoilage by microorganisms is an emerging field (5,9,16,34). Many volatile compounds are released during microbial growth, and gas chromatographic techniques have been used to signal the presence of bacteria or fungi by detecting specific volatiles (16). For example, gas-sensor array technology was used to detect and monitor the growth of several food spoilage bacteria (*Serratia marcescens*, *S. proteamaculans*, and *Pseudomonas putida*) in milk by analyzing the type and amount of volatile compounds (gas profiles) that were produced (9). Since an unpleasant odor is usually associated with macadamia nut graying, headspace volatile analysis could be used to determine differences in volatile profiles between healthy and gray nuts that may be used in the future to detect spoiled batches of nuts.

This investigation focused on *E. cloacae* because it was identified as the most frequently isolated bacterial strain from gray kernels and the most likely causal agent among 29 isolated bacterial strains by Kaneshiro and others (14). It also occurs in Hawaii on at least two plant hosts (22,23), one of which is edible ginger, a ground crop. Finally, as an enteric coliform bacterium, it has food safety implications for the macadamia industry. The objectives of our investigation were to (i) demonstrate that *E. cloacae* is one of the causal agents of

Corresponding author: K. A. Nishijima
E-mail: kate.nishijima@ars.usda.gov

Current address of M. S. Siderhurst: Eastern Mennonite University, 1200 Park Road, Harrisonburg, VA 22802-2462.

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gray kernel of macadamia by consistently reproducing disease symptoms in inoculated nuts and fulfilling Koch's postulates, (ii) identify some abiotic and biotic factors that may influence the production of gray kernel in artificially inoculated nuts, and (iii) identify some of the major volatiles associated with infected gray kernels.

MATERIALS AND METHODS

Macadamia inoculations. Macadamia nuts of cultivars Keauhou (HAES 246), Kau (HAES 344), and Kakea (HAES 508) were harvested from trees or the ground from commercial orchards located on the island of Hawaii (first experiment, Keaau; second experiment, Keaau and Kau). The two orchard locations were compared in the second experiment because climatic conditions for Keaau are considered wet (336 cm, median annual rainfall from 1999 to 2003), while conditions for Kau are dry (117 cm, median annual rainfall from 1999 to 2003) (19). Husks were removed from nuts within 24 h of harvest.

The husked nuts were washed in distilled water, surface-disinfected for 2 min in 0.5% sodium hypochlorite, then air-dried without rinsing. An inoculation hole (approximately 1 mm diameter) was drilled through the shell of each nut at a marked site for all treatments except the "no treatment controls" (nuts that were neither drilled nor inoculated). The drill bit was disinfected with 70% ethanol or 0.5% sodium hypochlorite, then flamed before each nut was drilled. The holes were wiped clean with 0.5% sodium hypochlorite and then covered with transparent tape to prevent cross-contamination or infection by other microorganisms. Nuts were individually labeled with cultivar and harvest location (tree or ground) information, then stored in paper sacks and refrigerated at 4°C in buckets with lids until used in inoculation experiments conducted within 1 week.

Nuts used in bacterial treatments in the first inoculation experiment were inoculated with *E. cloacae* strains ATCC 13047 (type strain, human) (23), B193-3 (ginger strain) (22), Dd-18 (fruit fly strain) (22,23), KN 04-2 (macadamia strain from HAES 508), or LK 0802-3 (macadamia strain from HAES 246). Strain LK 0802-3, which had a unique "swarming" colony character (feathery, irregular margin), was graciously provided by Lisa Keith (USDA-ARS-PBARC, Hilo, HI). Both macadamia strains were isolated from nuts with gray kernel symptoms that were collected from an orchard in Keaau, HI. Bacterial treatments in the second inoculation experiment included the two most virulent *E. cloacae* strains, LK 0802-3 and B193-3, based on results of the first experiment. All *E. cloacae* strains were 3 to 5 days old, grown at 30°C in a Fisher Scientific low temperature incubator on PT-M4 agar medium (22) (1.8% bacto-agar [Difco], 1% peptone, 0.5% yeast extract, 0.25% sodium chloride, and 0.001% triphenyltetrazolium chloride [Sigma Chemical Co., St. Louis, MO] which is added to sterile molten agar prior to pouring).

The inoculation procedure consisted of injecting bacterial suspensions of *E. cloacae* strains or sterile distilled water (SDW) into the raw (unprocessed) nuts through a previously drilled hole in the shell. Noninoculated controls consisted of nuts that were drilled only (not inoculated, NI) or not treated (neither drilled nor inoculated, ND/NI). Five to 10 nuts of each cultivar harvested from the tree or ground were used for each bacterial treatment (*E. cloacae* strain or control [SDW or noninoculated]).

Bacterial suspensions were prepared for each *E. cloacae* strain by scraping two to three loopsful of cells from cultures grown on PT-M4 medium, suspending in 30 ml SDW, and adjusting the cell density to 0.4 to 0.5 $A_{600\ nm}$ (equivalent to bacterial concentration of approximately 10^9 CFU/ml) using a Turner SP-830 spectrophotometer. A tuberculin syringe fitted with a 23-gauge needle was used to inject approximately 0.2 ml of bacterial suspension or SDW into each nut through the drilled hole in the shell. The surface area was disinfected with 0.5% sodium hypochlorite, and the opening was covered with autoclavable laboratory tape.

Incubation and storage conditions. Nuts of all bacterial treatments (*E. cloacae* strains and controls) were grouped together by individual cultivar and either tree or ground harvest, and stored in sterile Mason canning jars (approximately 1 to 2 liters in volume) with lids fitted with a rubber septum for analyzing relative humidity and gases (carbon dioxide and oxygen) throughout the storage period of 58 to 60 days. Half the jars in the first inoculation experiment contained 50 ml SDW (six jars), while the other half did not contain water (six jars) to determine the necessity of free water for gray kernel development. All 12 jars in the second inoculation experiment contained SDW. Nuts in each jar were placed on a section of wire screen that was bent to support the nuts above the jar bottom and water level (if present). The jars were incubated at 30°C in a VWR 2020 or Fisher Scientific low temperature incubator.

Symptom evaluation and bacterial reisolation. Kernels were evaluated for gray kernel symptoms (discoloration and putrid odor) after 2 months (58 to 60 days) incubation in Mason jars. Nuts were surface-disinfected in 0.5% sodium hypochlorite for 2 min, then drained on clean paper towels before shells were cracked and kernels removed. For full development of gray discoloration, kernels were incubated an additional 2 days at 30°C on paper food trays then evaluated for presence of foul odor and gray discoloration. Kernels were evaluated for color intensity according to a rating scale consisting of nongray (cream color), light gray, medium gray, and dark gray. Additionally, selected kernels (at least 17) of each nongray or gray intensity rating category were analyzed for lightness (L*) and chroma (C*) using a Minolta Chroma Meter (Model CR-300, Ramsey, NJ). L* ranges from 0 (black) to 100 (white), while C* is on a scale ranging from 0 to 60, with full saturation at 60.

Isolations were attempted from nongray and gray kernels to determine the presence of *E. cloacae*. Kernel tissues were dissected aseptically, surface-disinfected in 0.5% sodium hypochlorite solution (plus trace of Liquinox detergent) 30 s to 1 min, drained on clean Kimwipes, then crushed aseptically in 5 ml SDW in test tubes using a sterile glass rod. After about 30 min, 20-µl aliquots of the tube contents were streaked on PT-M4 agar plates. Plates were incubated at 30°C and observed for bacterial growth.

Purified strains (single-colony isolates streaked consecutively at least twice) were inoculated in tubes containing oxidative-fermentative (OF) basal media (Difco) with 1% glucose and incubated under aerobic and anaerobic conditions (presence or absence of sterile mineral oil layered over inoculated media) to determine OF reactions. Selected (based on colony morphology) facultative anaerobes were identified using API 20E strips (bioMerieux, Inc., USA office, Durham, NC) incubated at 30°C, 18 to 24 h.

Jar atmosphere measurements. Jar atmospheres of both inoculation experiments (jars with or without free water in the first experiment and jars with free water in the second experiment) were monitored at approximately 20-day intervals during the 2-month incubation period for percent relative humidity (% RH), carbon dioxide (CO_2), and oxygen (O_2) concentrations. Percent RH was measured using a HOBO data logger (H8 Pro Series, Onset Computer Corp., Bourne, MA) placed and sealed with Parafilm over the opening of the jars containing the nuts. Readings were measured for 5 min per jar. Oxygen and CO_2 concentrations were measured with an Oxygen/Carbon Dioxide Analyzer (Model 6600, Illinois Instrument, Inc., Johnsburg, IL). Air samples from each jar were withdrawn through the rubber septum of the jar lid using a sterile 25-gauge needle that was attached to a sampling wand connected to the instrument.

Kernel analysis. Percent moisture content (% MC) and water activity (a_w) of noninoculated (control) kernels were determined at the end of the 2-month incubation when nuts were removed from sealed Mason canning jars. Samples consisting of approximately 10-g portions of corresponding halves of five to 10 kernels (shells removed) were used for each set of measurements (% MC and a_w). Moisture

content calculations were based on initial and final weights of kernel samples dried over a period of 3 days in a 60°C oven (Lindberg/Blue UT150). Water activity was determined on kernel samples held in a sealed container equipped with a Rotronic AW-Dio water activity sensor (Huntington, NY).

Volatiles analysis. Headspace analyses of volatiles from infected gray kernel and noninfected healthy kernel samples were conducted with nuts that were previously incubated in Mason jars for 2 months at 30°C. Sampling was conducted using solid phase microextraction (SPME) and Porapak Q trapping methods. Two different SPME fibers were used for volatiles sampling: polydimethylsiloxane (PDMS; film thickness 100 µm; Supelco Inc., Bellefonte, PA) and carbowax/divinylbenzene (CW/DVB; film thickness 70 µm; Supelco). SPME fibers were conditioned (250°C) before use in a GC injector (Agilent Technologies 6890N) for 30 min. The SPME needle containing the fiber was inserted through the septa of lids of Mason jars (0.97 liter) containing the gray or nongray kernel samples (10 g). The fiber was exposed for 10 min to adsorb volatiles, then withdrawn from the collection jar and inserted into the GC injection port to desorb the volatiles.

Headspace sampling was also conducted using Porapak Q (50/80 mesh; Alltech, Deerfield, IL). The adsorbent (1 g) was packed between glass wool in a Pasteur pipet, preconditioned at 70°C for 16 h, washed with dichloromethane, and then dried under carbon-filtered argon. Kernel samples (30 g) were placed inside a 50-cm length glass tube equipped with airtight inlet and outlet attachments for volatile collection. Carbon-filtered argon (1 liter/min) was used to sweep volatiles from the tube for 1 h. Volatiles were eluted from the adsorbents with 1 ml of dichlomethane, then 10 µg of 2-heptanone and eicosane (internal standards) were added, and the collection was stored at minus 70°C. Collections were concentrated under a purified argon stream before analysis.

GC/MS analysis was performed on an Agilent Technologies 6890N gas chromatograph interfaced with a Hewlett-Packard 5973 Mass Selective Detector equipped with an HP-5MS column (30 m × 0.25 mm ID, 0.25-µm film thickness). The oven temperature program was 45°C (1 min) then ramped to 240°C at 10°C/min, then held for 13 min. The injector temperature was set at 250°C using helium as a carrier gas (1.1 ml/min). Compounds from the kernel samples were identified on the basis of mass spectra (NIST98 mass spectral database) and comparison with authentic standards. All synthetic compounds were purchased from Sigma-Aldrich (St. Louis, MO), with compound purities >98% based on GC (FID) analysis.

Experimental design. Two inoculation experiments were conducted in which inoculation treatments and incubation protocols varied slightly. Experimental variables consisted of bacterial treatments, tree or ground harvests, macadamia cultivars, orchard locations, and presence or absence of free water in storage jars. The experiments were repeated at least once. In total, 120 and 240 nuts per *E. cloacae* strain were inoculated in the first and second experiments, respectively.

The first inoculation experiment was a 2 × 3 × 2 × 8 factorial experiment arranged in a split-plot design where the main plot effects were moisture environment in the jars (presence or absence of SDW), macadamia cultivar (HAES 246, 344, or 508), and nut harvest location (tree or ground), and the subplot effect was bacterial treatment (five *E. cloacae* strains and three controls [SDW, NI, or ND/NI]) with treatment replicates consisting of five nuts. Each jar, with or without water, contained 40 nuts of a single cultivar and a single harvest location that were treated with the bacterial treatments (five nuts per treatment). The design of the second inoculation experiment differed slightly in that nuts were harvested from two geographic orchard locations (Kau or Keaau), the bacterial treatments utilized the two most virulent *E. cloacae* strains (LK 0802-3 and B193-3), treatment replicates consisted of 10 nuts, and all nuts were incubated in jars with SDW. Other variables (cultivar and nut harvest location) remained the same as the first experiment.

The jar atmosphere analyses of O_2 and CO_2 concentrations included the effect of duration (days-interval) because these gas measurements were conducted at approximately 20-day intervals over the 2-month incubation period. Assay experiments (gas atmospheres, volatiles, kernel analyses) were conducted at least twice.

Data analysis. All data (disease incidence, kernel color analysis, gas atmosphere, kernel moisture) were analyzed using the general linear models (GLM) procedure of SAS version 8.0 (SAS Institute, Inc., Cary, NC). Arcsine-square root transformations were performed before analysis on proportion of kernels with gray kernel symptoms in the second inoculation experiment (nuts from Kau and Keaau

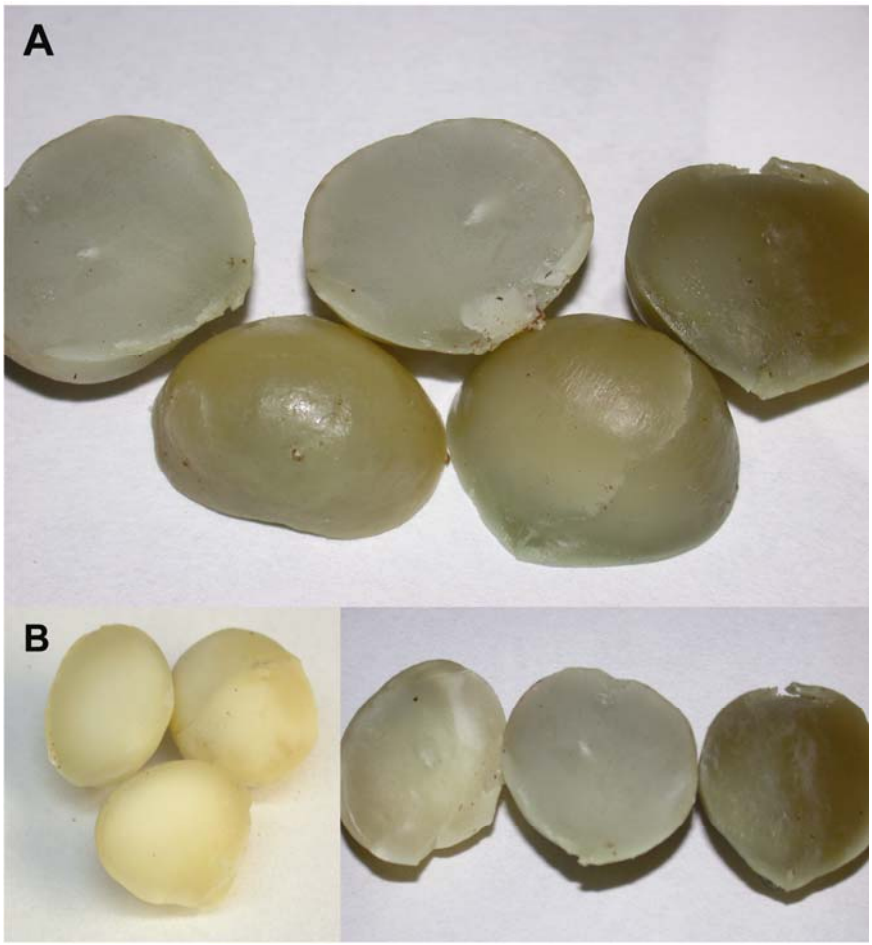

Fig. 1. Typical gray kernel symptoms produced on raw macadamia nuts artificially inoculated with *Enterobacter cloacae* strains. **A,** Uniform gray discoloration and different shades of gray of internal and external surfaces of infected kernels; note the inoculation site near the center area of the kernels. **B,** Kernel color intensity categories consisted of: nongray (cream color: left, cluster of three kernels), light gray (right, first kernel), medium gray (right, second kernel), and dark gray (right, third kernel).

orchards) according to protocols described in Snedecor and Cochran (29). Data were presented as percent incidence of gray kernel in tables and plotted figures. Means separation (where appropriate) were performed by Fisher's protected LSD test, at $P = 0.05$ (SAS version 8.0).

RESULTS

Disease incidence and gray color intensity. Typical gray kernel symptoms of kernel discoloration (Fig. 1A) and foul odor were observed in inoculation experiments. The first experiment consisted of nuts from Keaau that were stored in jars with or without the presence of free water. Analysis of variance indicated significant ($P < 0.001$) differences in percent incidence of gray kernel between these moisture environments, among bacterial treatments (*E. cloacae* strains or no bacteria), and the interaction of these two effects. Data were sorted into separate datasets (presence or absence of water in jars) for further analysis. Analysis of variance indicated significant differences only in incidence of gray kernel among bacterial treatments ($P < 0.0001$), but not among nut cultivars ($P = 0.2411$) or harvest locations (tree or ground) ($P = 0.6806$) for nuts stored in jars with water (Table 1). Macadamia strain LK 0802-3 caused the highest incidence of gray kernel (65.0%) followed by ginger strain B193-3 (40.0%) among the bacterial treatments (Table 1). There were no significant ($P > 0.05$) differences in gray kernel incidence among bacterial treatments, cultivars, or harvest locations for nuts stored in jars without water (Table 1).

The second inoculation experiment compared nuts obtained from Kau and Keaau orchards. Analysis of variance of transformed data indicated a significant difference between the two orchards ($P = 0.0189$), among the cultivars ($P = 0.0037$), and among the bacterial treatments ($P < 0.0001$) (Table 2). *E. cloacae* strain LK 0802-3 had the highest incidence of gray kernel (46.3%) among the bacterial treatments, while Keauhou (HAES 246) had the lowest incidence of gray kernel (8.3%) among the macadamia cultivars (Table 2).

Lightness (L*) and chroma (C*) analyses of nongray and gray color intensity categories (Fig. 1B) revealed significant differences among means ($P = 0.0031$ for L*, $P = 0.0169$ for C*) (*analysis not shown*). L* values, which range from 0 to 100 indicating dark to light color, were highest for nongray kernels (L* = 67.61), not different between light and medium gray kernels (L* = 58.20 and 57.21, respectively), and lowest for dark gray kernels (L* = 49.89), according to Fisher's LSD at $P = 0.05$. C* values, which indicate color saturation from low to full (0 to 60), followed a similar trend and means were 16.62, 10.29, 11.37, and 8.01 for nongray and light, medium, and dark gray kernels, respectively, according to Fisher's LSD at $P = 0.05$. Light and medium gray ratings were indistinguishable by Chroma Meter analysis, indicating that these gray intensity categories can be combined in future evaluations of gray kernel symp-

Table 1. Percent incidence of gray kernel of macadamia nuts inoculated with *Enterobacter cloacae* strains (ATCC 13047, B193-3, Dd-18, KN 04-2, or LK 0802-3) or sterile distilled water (SDW) or not inoculated (NI, ND/NI) and incubated in Mason canning jars with or without 50 ml SDW for 60 days at 30°C[y]

		% Incidence of gray kernel	
Effect	**Rep.**	**Water (mean[z])**	**No water (mean)**
Cultivar			
Keauhou (HAES 246)	32	15.3	2.3
Kau (HAES 344)	32	14.7	2.3
Kakea (HAES 508)	32	26.9	0.8
Harvest			
Tree	48	20.2	2.6
Ground	48	17.7	1.0
Bacterial treatment			
E. cloacae: LK 0802-3	12	65.0 a	2.1
E. cloacae: B193-3	12	40.0 b	4.2
E. cloacae: KN 04-2	12	21.7 bc	0.0
E. cloacae: Dd-18	12	11.7 cd	2.1
E. cloacae: ATCC 13047	12	8.3 cd	4.2
No *E. cloacae*: SDW	12	1.7 d	2.1
No *E. cloacae*: NI	12	1.7 d	0.0
No *E. cloacae*: ND/NI	12	1.7 d	0.0

Analysis of variance

Source	**df**	***F* value**	***F* value**
Cultivar (C)	2	1.92	0.49
Harvest (H)	1	0.19	1.10
Test (T)	1	0.26	5.98
Bacterial treatment (B)	7	12.13***	0.95
C × H	2	0.65	1.46
C × B	14	0.74	0.88
H × B	7	0.13	1.93
C × H × B	14	0.23	0.68

[y] Nuts of cultivars Keauhou (HAES 246), Kau (HAES 344), and Kakea (HAES 508) were harvested from trees or the ground from a macadamia orchard in Keaau, island of Hawaii. A total of 120 nuts per bacterial strain were inoculated in the repeated experiment.

[z] Means in columns followed by the same letter are not significantly different according to the LSD test of percent disease incidence at $P = 0.05$. *** = F value for effect significant at $P = 0.001$.

Table 2. Percent incidence of gray kernel of macadamia nuts inoculated with *Enterobacter cloacae* strains (B193-3 or LK 0802-3) or sterile distilled water (SDW) or not inoculated (NI, ND/NI) and incubated in Mason canning jars with 50 ml SDW, for 2 months at 30°C[w]

	% Incidence of gray kernel[x]	
Effect	**Rep.**	**Mean[y]**
Location of orchard		
Kau	60	18.5 a
Keaau	59	12.2 b
Cultivar		
Keauhou (HAES 246)	40	8.3 b
Kau (HAES 344)	39	20.3 a
Kakea (HAES 508)	40	17.8 a
Harvest		
Tree	59	15.8
Ground	60	15.0
Bacterial treatment		
E. cloacae: LK 0802-3	24	46.3 a
E. cloacae: B193-3	24	20.4 b
No *E. cloacae*: SDW	24	4.6 c
No *E. cloacae*: NI	24	5.0 c
No *E. cloacae*: ND/NI	23	0.0 c

Analysis of variance (transformed data)

Source	**df**	***F* value**
Location (L)	1	7.56*[z]
Cultivar (C)	2	9.76**
Harvest (H)	1	0.17
Test (T)	1	8.51*
Bacterial treatment (B)	4	39.21***
L × C	2	1.28
L × H	1	0.16
C × H	2	0.25
L × C × H	2	0.48
L × C × B	8	0.54
L × H × B	4	0.77
L × B	4	1.26
C × B	8	1.46
H × B	4	0.24
C × H × B	8	0.61
L × C × H × B	8	0.39

[w] Nuts of cultivars Keauhou (HAES 246), Kau (HAES 344), and Kakea (HAES 508) were harvested from trees or the ground from macadamia orchards in Kau or Keaau, island of Hawaii. A total of 240 nuts per bacterial strain were inoculated in the repeated experiment.

[x] Data were transformed (arcsine-square root) for analysis by GLM procedure then back-transformed for presentation.

[y] Means in columns, by effect, followed by the same letter are not significantly different according to the LSD test of percent disease incidence at $P = 0.05$.

[z] *, **, *** = F value for effect significant at $P = 0.05$, $P = 0.01$, or $P = 0.001$, respectively.

toms. Incidences among the gray intensity categories also were analyzed to determine if there were virulence differences among the *E. cloacae* strains as indicated by higher incidences in the darker gray categories. No single bacterial strain showed a significantly higher incidence in the dark gray category compared to the other categories; however, strain LK 0802-3 had the highest incidence in the medium gray category for nuts from the Keaau orchard in both inoculation experiments (20.8 to 40.0%) (Fig. 2A and B). Incidences among the intensity categories were similar or not significantly different for strain B193-3 (Fig. 2A and B).

Reisolation of bacterial strains from gray kernels. Reisolations were successful for *E. cloacae* strains that produced gray kernel symptoms. All five *E. cloacae* strains (ATCC, B193-3, Dd-18, KN 04-2, and LK 0802-3) were reisolated at least once from gray kernels resulting from inoculation with the respective strains, and were identified as *E. cloacae* by API 20E strips. Strains LK 0802-3 and B193-3 were the most readily recovered strains with 80% (8 of 10 attempts) and 75% (6 of 8 attempts) recovery, respectively. In addition, the distinctive colony form of strain LK 0802-3 (characterized by swarming margins) was observed in the reisolated strain.

Isolations were attempted from nuts that were not inoculated with *E. cloacae* (SDW, NI, and ND/NI treatments) but were associated with gray kernel symptoms or were healthy (Tables 1 and 2). Bacteria were not recovered from 12 discolored (brown or gray) kernels, or from eight healthy kernels of the control treatments. *E. cloacae* was isolated from one healthy kernel (Kakea [HAES 508], tree-harvested) that was not drilled or inoculated (ND/NI treatment).

Relative humidity, oxygen, and carbon dioxide concentrations in jars. Relative humidity in the storage jars of both experiments was constant at 100% (±3%) throughout the 2-month incubation, regardless of presence or absence of free water. Oxygen was depleted (0%) within the first 20 days (Table 3). Carbon dioxide concentrations did not significantly differ ($P = 0.2389$) in jars with or without free water in the first experiment, and data were combined for analysis of % CO_2. Carbon dioxide levels in both experiments increased during the 2-month incubation, especially at the 41 to 60 days period. Whereas % CO_2 was 40.8 and 46.9% by 40 days incubation, the gas significantly increased to 64.0 and 56.0% (experiments 1 and 2, respectively) by 60 days incubation (Table 3).

Moisture content (% MC) and water activity (a_w) of kernels. The effect of presence or absence of free water in storage jars was significant for data for kernel moisture content ($P = 0.0002$), water activity ($P = 0.0069$), and incidence of gray kernel (*E. cloacae* inoculation treatments) ($P = 0.0034$) of nuts harvested from Keaau in the first experiment (*analyses not shown*). The effect of tree or ground harvest location was slightly significant ($P = 0.0487$) for kernel moisture content (26.3% MC for tree-harvested nuts compared with 23.1% MC for ground-harvested nuts). Nuts incubated in jars with water had significantly higher % MC, a_w, and gray kernel incidence compared with nuts stored in jars without water (Table 4).

Data for kernel moisture content and water activity for nuts in the second experiment (storage in jars with water) were combined for Kau and Keaau orchards after analyses of variance indicated orchard location effect was not significant ($P > 0.05$). Means for % MC and a_w were not significantly different ($P > 0.05$) for tree or ground harvest and among cultivars (*data not shown*). Overall % MC and a_w for the second experiment were 26.7% and 0.96, respectively. Incidence of gray kernel for *E. cloacae* inoculation treatments was

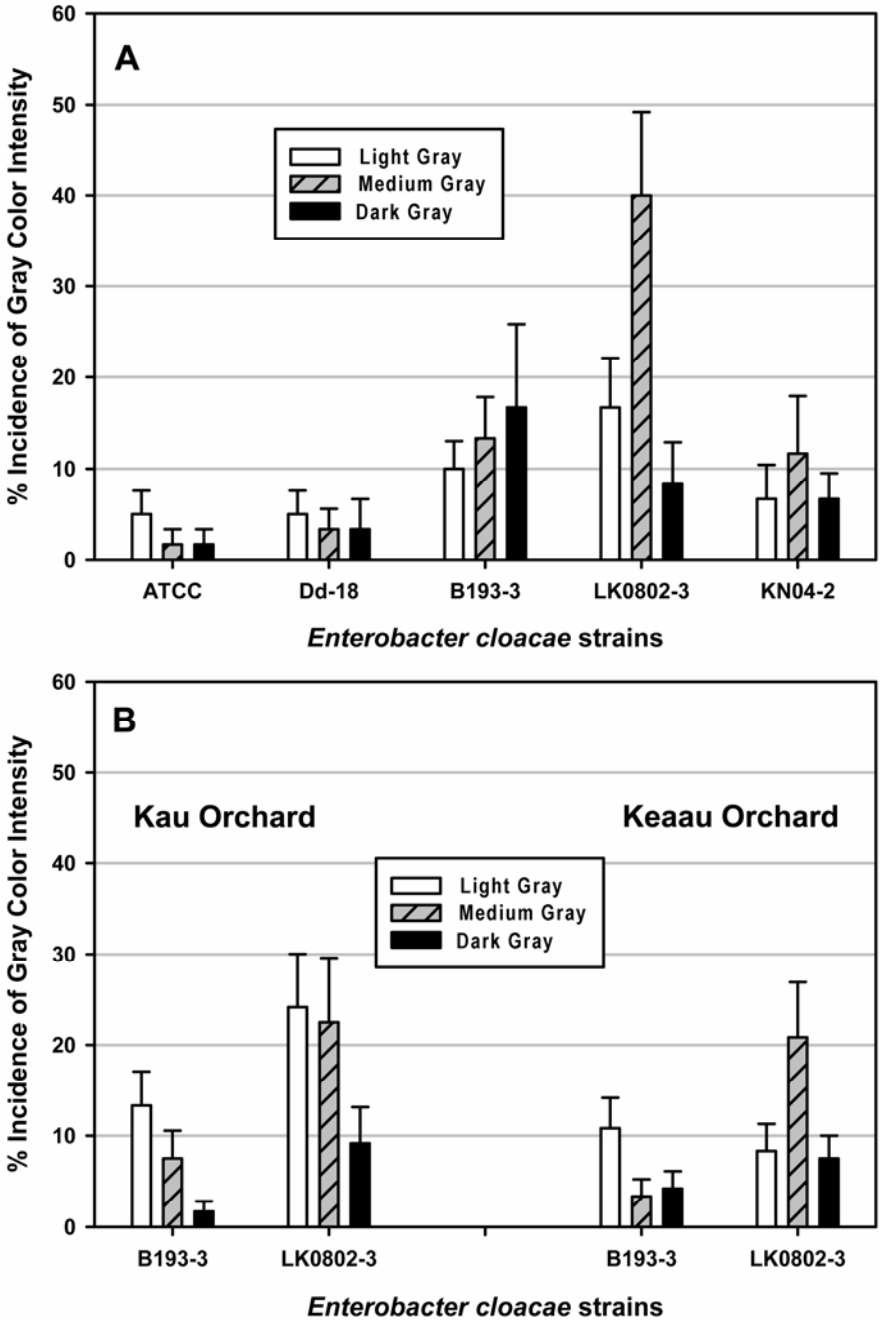

Fig. 2. Mean percent incidence of light, medium, and dark gray color intensity categories of macadamia nuts: **A,** inoculated with *Enterobacter cloacae* strains ATCC 13047, Dd-18, B193-3, LK 0802-3, or KN 04-2. Nuts were harvested from trees or the ground from a macadamia orchard in Keaau on the island of Hawaii; and **B,** inoculated with *E. cloacae* strains B193-3 or LK 0802-3. Nuts were harvested from trees or the ground from macadamia orchards located in Keaau or Kau on the island of Hawaii. Nuts were incubated in Mason canning jars with 50 ml of sterile distilled water for approximately 60 days at 30°C. Bars represent standard error of the means.

33.4% (mean of both orchard locations). These results were similar to results in the first experiment for nuts incubated in jars with water (Table 4).

Identification of volatiles from gray and nongray kernels. After 2 months incubation in jars at 30°C, infected, gray kernels were associated with a pungent, rancid, fermenting odor, while nongray kernels had a musty, fermenting odor. Six major volatiles from kernels, collected by SPME and Porapak Q trapping, were identified by GC/MS analysis. Control nuts (nongray kernels) were found to emit predominately ethanol and acetic acid. Infected nuts (gray kernels), by contrast, emitted at least four additional compounds, 3-hydroxy-2-butanone (acetoin), 2,3-butanediol, phenol, and 2-methoxyphenol (guaiacol) (Table 5).

The primary purpose of the headspace analysis was to identify differences in volatiles produced by nongray and gray kernels. However, comparisons of GC peak area measurements (total ion chromatogram, TIC) to internal standards were conducted to obtain relative compound concentrations in the Porapak Q collections for gray kernel samples. Trapping time and the mass of kernels analyzed were used to calculate the amounts of each compound emitted ($ng \cdot h^{-1} \cdot g^{-1}$). The calculations were based on GC peak areas (TIC) and the assumption that all components have the same response with the detector, which is a reasonable approximation for most compounds. Compounds 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) were highest in concentrations of the four additional compounds identified in gray kernel samples (Table 5).

Table 3. Oxygen (O_2) and carbon dioxide (CO_2) concentrations in Mason canning jars (approximately 1 to 2 liter vol) containing macadamia nuts after 0 to 20, 21 to 40, and 41 to 60 days incubation at 30°C

Days interval	Experiment 1[w] (Keaau orchard)			Experiment 2[x] (Kau and Keaau orchard)		
	Reps.	% O_2	% CO_2[y]	Reps.	% O_2	% CO_2[y]
0 to 20	10	0	37.4 b[z]	26	0	25.6 c
21 to 40	3	0	40.8 b	26	0	46.9 b
41 to 60	15	0	64.0 a	25	0	56.0 a

[w] Analysis of variance of gas atmosphere results for jars incubated with or without water in experiment 1 were not significantly different ($P = 0.2389$), and data were combined.
[x] All jars in experiment 2 were incubated with water.
[y] Data (percent CO_2) for each experiment were analyzed by the GLM procedure at $P = 0.05$.
[z] Means in columns followed by the same letters are not significantly different according to the LSD test of percent CO_2 at $P = 0.05$.

Table 4. Effect of water (50 ml sterile distilled water [SDW]) or no water in Mason canning jars on percent moisture content (% MC) and water activity (a_w) of macadamia nuts harvested from trees or the ground from an orchard in Keaau, island of Hawaii, and on percent incidence of gray kernel of macadamia nuts inoculated with strains of *Enterobacter cloacae* (ATCC 13047, B193-3, Dd-18, KN 04-2, or LK 0802-3)[x]

Water treatment in jars	Reps.	% MC	a_w	Reps.	% Gray kernel[y]
SDW	15	26.8 a[z]	0.96 a	60	29.3 a
None	11	21.9 b	0.93 b	60	2.5 b

[x] Kernels were evaluated after storage for 60 days at 30°C.
[y] Results were for bacteria-inoculated treatments only.
[z] Means in columns followed by the same letters are not significantly different according to the LSD test of percent MC, a_w, or percent disease incidence at $P = 0.05$.

Table 5. Volatiles identified from nongray (control) and gray macadamia kernels inoculated with *Enterobacter cloacae* strains (B193-3 or LK 0802-3) or inoculated with sterile distilled water (SDW), or not inoculated (control treatments)[z]

Compound	SPME		Porapak Q ($ng \cdot h^{-1} \cdot g^{-1}$)	
	Nongray	Gray kernels	Nongray	Gray kernels
Ethanol	+	+	ND	ND
Acetic acid	+	+	ND	ND
3-Hydroxy-2-butanone (acetoin)	–	+	–	38
2,3-Butanediol	–	+	–	80
Phenol	–	+	–	8
2-Methoxyphenol (guaiacol)	–	+	–	2

[z] Nuts were incubated in Mason jars with SDW for 60 days at 30°C; then kernels were removed from shells, aerated 1 to 2 days, and evaluated for gray kernel symptoms. Headspace analysis of kernel samples (10 g in 0.97 liter vol jars) was conducted using solid phase microextraction (SPME) and Porapak Q trapping with identification by GC/MS. Concentrations ($ng \cdot h^{-1} \cdot g^{-1}$) of selected compounds were also determined for Porapak Q collections of gray kernel samples (ND designates compounds not done). Compounds present (+) or absent (–) are listed.

DISCUSSION

Gray kernel symptoms were produced consistently on nuts inoculated with *E. cloacae* strains. However, virulence differences were apparent among the strains and corroborated similar findings for *E. cloacae* strains from different hosts or sources (22,23). Macadamia strain LK 0802-3 was the most virulent strain in the two replicated inoculation experiments with 46.3 to 65.0% gray kernel incidence. Colony morphology (swarming appearance) of this strain was characteristically different from the other *E. cloacae* strains and was easily recognizable in reisolations from kernel tissues. Ginger strain B193-3 also caused gray kernel in two experiments (20.4 to 40.0%), adding to its host list, which includes ginger, papaya, and onion (22). Macadamia strain KN 04-2 caused 21.7% gray kernel, but disease incidences for ATCC 13047 (human strain) and Dd-18 (fruit fly strain) were not significantly different from the controls. The fulfillment of Koch's postulates for *E. cloacae* strains LK 0802-3, B193-3, and KN 04-2 demonstrates that *E. cloacae* can be a major cause of gray kernel of macadamia.

Other bacteria or abiotic factors may also cause similar symptoms in macadamia (14). *Phyllobacterium* sp. and *Sphingomonas* sp. have been implicated in gray kernel because symptoms were produced in inoculated nuts, but Koch's postulates were not fulfilled for these bacterial species, demonstrating the difficult nature of this disease (14). The focus here on *E. cloacae* does not preclude the role or involvement of other bacterial species in the disease.

Reisolation of *E. cloacae* from gray kernel samples was successful in approximately 60% of isolation attempts (or 75 to 80% recovery for the most virulent strains) despite prompt processing of samples. Other investigators have also encountered difficulties in reisolation attempts from gray kernel. Kaneshiro et al. (14) was not able to consistently reisolate *E. cloacae* from gray kernels inoculated with the bacterium. It is unknown why reisolation of *E. cloacae* sometimes is not successful, but the bacterium has been reported in a "viable but nonculturable" (VBNC) state on leaves and in soil (24), and this may be the case with macadamia. Bacteria also were not recovered from gray or brown discolored control nuts (not inoculated or SDW inoculated) and may be attributed to VBNC bacterial species, including *E. cloacae*, or physiological causes (3). Nut deterioration resulting from oxidation and lipolysis can occur during postharvest storage under high moisture conditions (3). The isolation of *E. cloacae* from a healthy

kernel that was not drilled or inoculated may have been the result of a naturally infected kernel that was asymptomatic. It is unknown how this tree-harvested nut became infected with *E. cloacae*.

Some abiotic conditions that were conducive to production of gray kernel symptoms were identified. Moisture was previously identified as a critical requirement for disease development (14). Results in this study confirmed that under anaerobic storage environments, the presence of free water was an important factor in the development of gray kernel symptoms. A significantly higher incidence of gray kernel was produced in jars containing water (29.3%) than in jars without water (2.5%). For the most virulent strain (LK 0802-3), this difference was greatest (65.0% compared to 2.1%). Kernel moisture and water activity parameters conducive to development of gray kernel were also identified. Higher kernel % MC and a_w (26.8 and 0.96, respectively) were associated with nuts incubated for 2 months in jars with water compared with lower results for nuts stored in jars without water (21.9% MC and 0.93 a_w). In comparison, moisture content of fresh (nonprocessed) kernels from the Puna district (Keaau orchard) were reported at 22.5 to 23.6% MC for cultivars HAES 246, 344, and 508 (33). Water activity reflects the amount of "free water" in a product available for biological and chemical reactions. Water activity of 0.96 for kernels stored under conditions favorable for gray kernel development is within the conducive a_w range (0.95 to 1.00) for bacterial activity in food and food products (6). More studies are needed to confirm the relationship between a_w and gray kernel disease.

Incubation period (duration) under high moisture conditions was also important for gray kernel symptom development. Kaneshiro and others (14) identified a long incubation period as an important condition for producing gray kernel symptoms in inoculated macadamia. Gray kernel incidence was also observed to increase with increasing duration (up to 2 months) of incubation during this study. Similarly, 56 days incubation was required for development of gray tissue and rhizome rot in ginger plants inoculated with *E. cloacae* and grown under waterlogged soil conditions (K. A. Nishijima, *unpublished data*). Carbon dioxide concentrations in storage jars containing inoculated and noninoculated nuts continuously increased from 20 to 60 days incubation. This indicated that anaerobic respiration and metabolic processes such as fermentation were occurring during this time period. These findings suggest that macadamia grown under high rainfall conditions need to be collected from the ground and dried and processed as soon as practical to reduce disease incidence and ensure kernels of optimum quality. Waterlogged soil likely results in low oxygen conditions for macadamia that fall to the ground and become embedded in soil and leaf debris. Along with a harvest interval that does not exceed 1 month (3), implementing cultural practices that increase drainage or aeration could also help reduce incidence of gray kernel.

Gray color intensity seemed to be affected by kernel exposure to air after storage under anaerobic and high moisture environments. The color of most gray kernels initially was faint immediately after removal from shells, but intensified as the kernels were exposed to air, especially when incubated at 30°C. Also, gray coloration sometimes faded with prolonged (more than 7 days) exposure to air. These observations are consistent with previous observations that gray or nongray kernels may or may not be associated with the foul odor (14). It is suspected that light gray-colored kernels faded and eventually became indistinguishable from healthy kernels, while the foul odor symptom either dissipated or persisted.

Biological factors such as nut maturity may have affected incidence of gray kernel in inoculated macadamia. The significantly higher incidence of gray kernel in nuts harvested from Kau (18.5%) compared with Keaau (12.2%) in the second experiment may have been related to nut maturity. In 2005, the Kau orchard had an extended (late flowering) season compared with the Keaau orchard, which had a short flowering season. This likely resulted in nuts harvested in December from Kau that were more immature than nuts from Keaau (A. Yamaguchi, *personal communication*). Tree- and ground-harvested nuts at the two locations did not differ significantly in incidence of gray kernel, and nut maturity may have been similar. Host or tissue maturity affecting susceptibility to *E. cloacae* infection was reported in papaya fruit (23) and ginger rhizomes (22), and more studies are needed in macadamia to determine if nut maturity affects susceptibility to gray kernel disease.

Macadamia cultivars have different flowering patterns, and crop maturity can vary during the harvest season (3). Nuts of three macadamia cultivars were inoculated to determine cultivar susceptibility to gray kernel. Macadamia cultivars did not differ in gray kernel incidence in the first experiment, but differed in the second experiment where Keauhou (HAES 246) had the lowest disease incidence among the three cultivars. These results are contrary to field observations that Keauhou (HAES 246) usually has the highest occurrence of gray kernel (A. Yamaguchi, *personal communication*). Cultivar screening to identify highly resistant macadamia cultivars is needed, but nut maturity, kernel biochemistry (such as carbohydrate composition), and nut morphology (especially shell thickness and micropyle openings) can vary among cultivars (3,33) and need to be considered in evaluations.

Some volatile compounds associated with infected gray kernels were identified for the first time. The volatile compounds ethanol and acetic acid produced by nongray and gray kernels are byproducts of fermentation and indicate that the nuts were fermenting when they were held at 30°C under anaerobic, saturated atmospheres. Additional bacterial fermentation byproducts acetoin (3-hydroxy-2-butanone) and 2,3-butanediol (9,34) were associated with infected gray kernel samples, but not with nongray samples in our studies. Bacteria associated with food and food products reportedly produce acetoin (3-hydroxy-2-butanone) (9,13,34), 2,3-butanediol (13,34), phenol, and guaiacol (2-methoxyphenol) (5,12). These volatile compounds have been implicated in food spoilage and are manifested as off-odors and/or off-flavors (5,12,13). The findings in this study indicate that *E. cloacae* is capable of spoilage in macadamia kernels, and the volatile compounds acetoin and 2,3-butanediol are potential indicators of nut spoilage, including gray kernel. Volatile monitoring to check food quality is an emerging field, and it may be possible to detect infected nuts in storage by headspace sampling or other volatile monitoring techniques (9,16,32,34).

Volatiles associated with infected gray kernels were produced under artificial inoculation and anaerobic storage conditions. Although the identified compounds are likely present in naturally infected gray kernels, other compounds may also be present under natural conditions. Hydrogen sulfide (H_2S), which forms from the breakdown of sulfur-containing compounds (such as benzyl isothiocyanate) by *E. cloacae* in papaya (31), also may occur in macadamia and results in the acrid odor associated with gray kernel (14). However, H_2S was not among the compounds identified in our investigation using GC/MS. Hydrogen sulfide was also poorly or not detected by mass spectrometry of volatiles in spoiled, "hydrogen sulfide-smelling", cold-smoked salmon and was attributed to the high volatility of the compound, possibly having dissipated during sample preparation (13).

The etiology of the pathogen(s) of gray kernel is unknown. However, considering the ubiquitous nature of *E. cloacae*, the pathogen likely occurs in the soil and leaf debris, and possibly gains entry to the kernel through open micropyles (14) or damaged husks and shells caused by field equipment or insects. The southern green stink bug, *Nezara viridula* (L.), is one of the major insect pests of macadamia nuts, causing up to 50% damage in some orchards (8). Puncture wounds through husk, shell, and to the kernel by *N. viridula* (8) may serve as inoculation sites for the bacterial pathogen(s) as well as other micro-

organisms such as fungi. Future etiological studies are needed to determine if there is a relationship with insect feeding wounds (*N. viridula* or others) that occur on nuts on trees and the ground (8), and the presence of *E. cloacae*.

Whether *E. cloacae* or other bacterial species previously isolated from gray kernels (14) enter via flowers, similarly to the route suggested for *E. cloacae* that causes internal yellowing of papaya (23), is also worthy of investigation. In a preliminary survey conducted in a commercial orchard to identify microorganisms associated with macadamia racemes, neither *E. cloacae* nor *Phyllobacterium* sp. or *Sphingomonas* sp. (14) were recovered from 18 flower racemes or 53 immature fruit (K. A. Nishijima, *unpublished data*). The small sample size of the survey limited likelihood of finding these bacterial species associated with gray kernel, and a more comprehensive survey is needed to conclusively determine whether macadamia flowers are points of entry for the pathogen(s) of gray kernel.

In summary, gray kernel symptoms were consistently produced in raw macadamia nuts artificially inoculated with strains of *E. cloacae*, demonstrating that this bacterium is one of the causal agents of gray kernel disease. This acknowledges that gray kernel is a food quality as well as a food safety concern for the macadamia industry. Inoculation protocols were developed so that disease-affecting factors and cultivar resistance can be further investigated. Some conditions conducive to the development of gray kernel symptoms in inoculated kernels were identified as: anaerobic, saturated atmospheres, incubation temperature of 30°C, incubation period of at least 2 months, and air exposure of 1 to 2 days at 30°C after kernels are removed from shells. In addition, headspace analysis was conducted and major volatiles were identified for the first time from infected gray nuts. The information may eventually establish a "volatile profile" that could help identify contaminated batches of kernels in storage bins using volatile-sensing technology.

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LITERATURE CITED

1. Bishop, A. L., and Davis, R. M. 1990. Internal decay of onions caused by *Enterobacter cloacae*. Plant Dis. 74:692-694.
2. Carter, J. C. 1945. Wetwood of elms. Ill. Nat. Hist. Surv. Bull. 23:407-448.
3. Cavaletto, C. C. 1983. Macadamia nuts. Pages 361-397 in: Handbook of Tropical Foods. H. T. Chan, Jr., ed. Marcel Dekker, New York.
4. Cother, E. J., and Dowling, V. 1986. Bacteria associated with internal breakdown of onion bulbs and their possible role in disease expression. Plant Pathol. 35:329-336.
5. Eisele, T. A., and Semon, M. J. 2005. Best estimated aroma and taste detection threshold for guaiacol in water and apple juice. J. Food Sci. 70:267-269.
6. Fennema, O. R. 1996. Water and Ice. Pages 52-53 in: Food Chemistry, 3rd ed. O. R. Fennema, ed. Marcel Dekker, New York.
7. George, M., Potty, V. P., and Jayasankar, N. P. 1976. Association of *Enterobacter* with coconut root (wilt) disease. Curr. Sci. 45(18):677-678.
8. Golden, M., Follett, P. A., and Wright, M. G. 2006. Assessing *Nezara viridula* (Hemiptera: Pentatomidae) feeding damage in macadamia nuts by using a biological stain. J. Econ. Entomol. 99(3):822-827.
9. Haugen, J. E., Rudi, K., Langsrud, S., and Bredholt, S. 2006. Application of gas-sensor array technology for detection and monitoring of growth of spoilage bacteria in milk: A model study. Anal. Chimica Acta 565:10-16.
10. Hawaii Agricultural Statistics Service (HASS). 2005. Statistics of Hawaii Agriculture 2004. Summaries. Hawaii Department of Agriculture, Agricultural Development Div., Honolulu, HI, and USDA-National Agricultural Statistics Service.
11. Jang, E. B., and Nishijima, K. A. 1990. Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). Environ. Entomol. 19(6):1726-1731.
12. Jensen, N., Varelis, P., and Whitfield, F. B. 2001. Formation of guaiacol in chocolate milk by the psychrotrophic bacterium *Rahnella aquatilis*. Lett. Appl. Microbiol. 33:339-343.
13. Joffraud, J. J., Leroi, F., Roy, C., and Berdague, J. L. 2001. Characterisation of volatile compounds produced by bacteria isolated from the spoilage flora of cold-smoked salmon. Int. J. Food Microbiol. 66:175-184.
14. Kaneshiro, W. S., Cavaletto, C. G., Tang, C. S., and Alvarez, A. M. 2003. Gray kernel disease of macadamia nut: Are bacteria involved? Online publication. Plant Health Progress doi:10.1094/PHP-2003-0825-01-HN.
15. Ladha, J. K., Barraquio, W. L., and Watanabe, I. 1983. Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. Can. J. Microbiol. 29:1301-1308.
16. Linton, C. J., and Wright, S. J. L. 1993. Volatile organic compounds: Microbiological aspects and some technological implications. J. Appl. Bacteriol. 75:1-12.
17. Marchini, D., Rosetto, M., Dallai, R., and Marri, L. 2002. Bacteria associated with the oesophageal bulb of the medfly *Ceratitis capitata* (Diptera: Tephritidae). Curr. Microbiol. 44:120-124.
18. Mirza, M. S., Ahmad, W., Latif, F., Haurat, J., Bally, R., Normand, P., and Malik, K. A. 2001. Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane *in vitro*. Plant Soil 237(1):47-54.
19. ML Macadamia Orchards L. P. 2004. Nut Annual Report (2004). Edgar® Online, http://sec.edgar-online.com/2004/03/17/0001104659-04-007786/Section2.asp.
20. Murdoch, C. W., and Campana, R. J. 1983. Bacteria species associated with wetwood of elm. Phytopathology 73:1270-1273.
21. National Agricultural Statistics Service (NASS). 2006. Hawaii Macadamia Nuts – Final Season Estimates. August 1, 2006. NASS, USDA, Hawaii Field Office, Honolulu, HI. http://www.nass.usda.gov/hi/fruit/mac-fin.htm.
22. Nishijima, K. A., Alvarez, A. M., Hepperly, P. R., Shintaku, M. H., Keith, L. M., Sato, D. M., Bushe, B. C., Armstrong, J. W., and Zee, F. T. 2004. Association of *Enterobacter cloacae* with rhizome rot of edible ginger in Hawaii. Plant Dis. 88:1318-1327.
23. Nishijima, K. A., Couey, H. M., and Alvarez, A. M. 1987. Internal yellowing, a bacterial disease of papaya fruits caused by *Enterobacter cloacae*. Plant Dis. 71:1029-1034.
24. Pedersen, J. C., and Leser, T. D. 1992. Survival of *Enterobacter cloacae* on leaves and in soil detected by immunofluorescence microscopy in comparison with selective plating. Microb. Releases 1:95-102.
25. Richard, C. 1984. Genus VI. *Enterobacter* Hormaeche and Edwards 1960, 72; Nom. Cons. Opin. 28, Jud. Comm. 1963, 38. Pages 465-469 in: Bergey's Manual of Systematic Bacteriology. Vol. 1. N. R. Kreig and J. G. Holt, eds. Williams & Wilkins, Baltimore, MD.
26. Rosen, H. R. 1922. The bacterial pathogen of corn stalk rot. Phytopathology 12:497-499.
27. Rossiter, M. C., Howard, D. J., and Bush, G. L. 1983. Symbiotic bacteria of *Rhagoletis pomonella*. Pages 77-84 in: Fruit Flies of Economic Importance. R. Cavalloro, ed. A. A. Balkema, Rotterdam.
28. Sanders, W. E., and Sanders, C. C. 1997. *Enterobacter* spp.: Pathogens poised to flourish at the turn of the century. Clin. Microbiol. Rev. 10:220-241.
29. Snedecor, G. W., and Cochran, W. G. 1989. Statistical Methods, 8th ed. Iowa State University, Ames.
30. Takahashi, Y., Takahashi, K., Sato, M., Watanabe, K., and Kawano, T. 1997. Bacterial leaf rot of *Odontioda* orchids caused by *Enterobacter cloacae*. Ann. Phytopathol. Soc. Jpn. 63:164-169.
31. Tang, C. S., Bhothipaksa, K., and Frank, H. A. 1972. Bacterial degradation of benzyl isothiocyanate. Appl. Microbiol. 23:1145-1148.
32. Triqui, R. 2006. Sensory and flavor profiles as a means of assessing freshness of hake (*Merluccius merluccius*) during ice storage. Eur. Food Res. Technol. 222:41-47.
33. Wall, M. M., and Gentry, T. S. 2007. Carbohydrate composition and color development during drying and roasting of macadamia nuts (*Macadamia integrifolia*). LWT 40:587-593.
34. Wang, J. C., and Wang, H. H. 2002. Fermentation products and carbon balance of spoilage *Bacillus cereus*. J. Food Drug Anal. 10(1):64-68.
35. Wick, R. L., Rane, K. K., and Sutton, D. K. 1987. Mung bean sprout disease caused by *Enterobacter cloacae*. (Abstr.) Phytopathology 77:123.