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An Exploratory Study of the Nutritional Composition of Tanoak (*Lithocarpus densiflorus*) Acorns after Potassium Phosphonate Treatment

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Native American Pomo communities who live in the Northern Coastal range of California and consume acorns from tanoak trees as part of traditional diets are facing the potential loss of many culturally important trees to sudden oak death. Pomo and other Native American communities are reluctant to use the protective fungicide, potassium phosphonate, on trees used for acorn collection without information on how the treatment affects acorn properties. In this study, select macronutrients and polyphenolics were quantified in tanoak acorns to evaluate the influence of potassium phosphonate treatment on the composition and nutritional value of tanoak acorns. Of the fatty acids tested from C14:0 to C20:1, only C17:0 was significantly lower ($p < 0.05$) in the nontreated and treated acorns after the first year. There were no differences detected in total phenolic content, gallic acid content, or ellagic acid content. Protein, phosphorus, and potassium levels were not significantly affected by fungicide treatment. Soluble glucose and fructose levels were significantly higher ($p < 0.05$) in both nontreated and treated groups after the first year; soluble sucrose levels did not change. Total glucose, starch, and total nonstructural carbohydrates increased significantly ($p < 0.05$) in the nontreated group after the first year but not in the treatment group; however, the treatment group values did not differ significantly from the control group values at baseline. The lack of any negative significant differences between acorns from treated and untreated tanoak trees implies that sodium phosphonate application for the prevention of sudden oak death does not impact the predominant polyphenolics or macronutrient quality of tanoak acorns.

KEYWORDS: Acorn; phosphonate; phosphite; phenolic; lipid; carbohydrate; mineral; antioxidant; *Lithocarpus densiflorus*

INTRODUCTION

Sudden oak death is a disease characterized by lethal stem cankers on tanoak (*Lithocarpus densiflorus*) and some oak species (*1*). The causal agent, *Phytophthora ramorum*, is a funguslike microorganism now classified in the Chromista kingdom, which appears to have been introduced into native forests in coastal California from an unknown location (*2*). Since it was first recognized in the mid 1990s, the disease has spread to native forests from the Big Sur area on the central coast of California to Curry County in southern Oregon, although the distribution of the disease is patchy across this range (*2*). High levels of tanoak and oak mortality have been seen in many affected stands.

Currently, relatively few options are available for reducing the impact of sudden oak death. In 2003, the California Department of Pesticide Regulation approved potassium phosphonate (KH_2PO_3 and K_2HPO_3) as the only pesticide for use in preventing the spread of sudden oak death. This systemic fungicide does not eliminate established infections but can be used as a prophylaxis to prevent infection and may limit symptom development in very early stages of the disease (*3*). It is classified by the U.S. EPA as a "reduced risk" pesticide (*4*) and is exempt from residue tolerance on food crops (*5*) due to both low toxicity and the fact that phosphonate is eventually incorporated into plant tissues as a source of phosphorus.

Native American Pomo communities live in areas impacted by sudden oak death, and the loss of tanoaks due to this disease has begun to impact their use of tanoak acorns as part of traditional diets. Tanoak acorns once comprised upward of 50% of the diet of Northwestern coastal tribes in California (*6*). Although no longer a dietary staple, the acorn is still used by Pomo peoples in traditional dishes, such as acorn soup and dumplings. Although Pomo and other Native Americans would like to protect tanoaks traditionally used for acorn gathering from *P. ramorum*, they are also concerned that application of potassium phosphonate to tanoak trees may detrimentally affect

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the nutrient composition of the acorns produced by the trees. This project is critical to determine if acorns from Agri-Fos-treated trees are altered in any way that would impact tribal members' traditional uses of acorns as food and ultimately whether Agri-Fos treatment will be acceptable to use as a management tool for the Kashia Tribe.

Potassium phosphonate is a systemic fungicide, and it spreads through the xylem and phloem to all parts of the treated tree within hours (*7*). Potassium phosphonate has a complex mode of action; it acts directly on *Phytophthora* spp. to reduce growth, and it also recruits the plant's natural defenses to curb pathogen invasion (*8*). The mechanisms involved in the plant's natural defense system include upregulation of enzymes involved in phenylpropanoid biosynthesis, which are responsible for the production of polyphenolics, increased production of secondary metabolites such as phytoalexins, early production of ethylene, and early use of the pentose phosphate pathway (*9*).

The aim of the present study was to evaluate the content of moisture, protein, carbohydrates, fatty acids, total phenolics, the specific phenolics, gallic acid and ellagic acid, nitrogen, phosphorus, and potassium in acorns before and after treatment with potassium phosphonate.

METHODS AND MATERIALS

Reagents. Agri-Fos (potassium phosphonate) and Pentra-Bark were purchased from Agrichem (Medina, OH). Methanol, chloroform, hexane, sodium carbonate, and acetone were obtained from Fisher Scientific (Houston, TX). Formic acid, potassium chloride, potassium carbonate, 3 N methanolic hydrogen chloride, gallic acid, Folin−Ciocalteu's phenol reagent, and 2,6-di-*tert*-butyl-4-methyl-phenol were purchased from Sigma (St. Louis, MO). Ellagic acid and myricetin were purchased from Indofine Chemical Co. (Hillsborough, NJ). Fatty acid standards were purchased from Nu Chek Prep (Elysian, MN). Reagent-grade water was generated by a Barnstead E-pure deionization system (Dubuque, IA).

Acorns. On October 6, 2005, mature acorns were harvested from eight *L. densiflorus* trees from the Stewart's Point Rancheria in northwest Sonoma County, CA. Acorns are considered mature when they drop from tanoak trees. Trees were shaken, and the fallen nuts were pooled and collected on tarps. After collection, the acorns were selected to be free from visible defects and were refrigerated at 7 °C until they were delivered the next day to UC Davis, CA, where they were frozen at −20 °C. The pooled samples were resampled to obtain duplicate 50 g samples. Each 50 g sample was then deshelled, ground in a laboratory mill for 1 min, and divided for analysis.

The eight tanoak trees were paired on the basis of geographic proximity. Trees within pairs ranged from about 18 to 170 m apart. After the initial acorn collection, one tree from each pair was randomly assigned for treatment with Agri-Fos (45.8% mono- and dipotassium salts of phosphorous acid, also referred to as potassium phosphonate or potassium phosphite) (*10*), and the other served as a control and was not treated. Initial applications of Agri-Fos to the treated trees were made in December 2005 (one tree) and January 2006 (three trees). A second application was made to these four trees in May 2006. An aqueous solution of Agri-Fos systemic fungicide was mixed with Pentra-Bark surfactant (89% polyalkalene-modified heptamethyltrisiloxane and nonionic surfactants and 10% coupling agents) (*10*) according to label instructions for control of sudden oak death. Phosphonate was applied to the lower 3 m of the tanoak main stems using a calibrated low-pressure sprayer. The application volume was standardized so that each of the main stems from each tree received 9.4 g of active ingredient (6 g of phosphorous acid equivalent) per cm of stem diameter (measured at 137 cm height) at each application.

On October 10, 2006, mature acorns were harvested from all eight trees, transported, stored, and processed in the same manner as for the first harvest. Two of the control trees produced too few acorns to be considered robust, so acorn samples were harvested from replacement trees from the same populations that were within the distances of the original tree pairings.

Fatty Acid Analysis. A 0.1 g amount of ground acorn cotyledon was extracted and derivatized with methanolic HCl according to previously published methods (*11*, *12*). A 0.1 μL derivatized sample was injected onto a Varian 3800 Gas Chromatograph (Walnut Creek, CA) equipped with a 30 m × 0.25 mm i.d. and 0.25 μm DB-225MS column (Agilent, Santa Clara, CA). Helium was used as a carrier gas at 0.7 mL/min. The split ratio was set to 20:1. The injector temperature was 270 °C, and the flame ionization detector temperature was 280 °C. The column temperature program was 165−192 °C at 2.8 °C/min; 192−197 °C at 1.3 °C/min; and 197−210 °C at 3 °C/min with a 4.5 min hold. Fatty acid methyl esters were identified with known standards and quantified by peak area using internal standardization with heptadecenoic acid (C17:1), a fatty acid not normally synthesized de novo by eukaryotic organisms. The fatty acid concentration was calculated on a dry weight basis. Each sample was analyzed in duplicate and averaged.

Total Phenolics Analysis. Total phenolics were measured using a modified Folin−Ciocalteu method (*13*) from a 1 g sample of ground acorn cotyledon. Samples were read at 765 nm in a Shimadzu 1700 spectrophotometer (Kyoto, Japan). The total phenolic concentration was calculated on a dry weight basis as mg gallic acid equivalents (GAE) per g, and each sample was run in duplicate and averaged.

Gallic and Ellagic Acid Analysis. Two grams of ground acorn cotyledon was extracted according to a previous method (*14*). Chromatographic analyses were performed on a Waters Alliance 2695 LC (Milford, MA) monitoring at 255 nm. Separations were achieved using reversed phase LC with a 250 mm × 4.6 mm i.d., 5 μm Prodigy ODS column (Phenomenex, Torrance, CA) with previously published conditions (*14*). Gallic and ellagic acids were identified and quantified using a series of standard curves with myricetin as an internal standard. All samples were analyzed in duplicate and averaged.

Moisture Content Analysis. Two grams of ground acorn cotyledon was placed in a drying dish. Samples were dried at 65 °C for 5 h under partial vacuum at −20 in Hg. Constant weight was recorded. All samples were analyzed in duplicate and averaged.

Analysis of Protein, Carbohydrates, and Minerals. Twenty grams of acorn cotyledon was ground in a laboratory mill, dried at 65 °C for 5 h under partial vacuum at −20 in Hg, and delivered to the University of California Agriculture and Natural Resources Analytical Laboratory in Davis, CA, for analysis. Total nitrogen was measured using an induction furnace and thermal conductivity detector (*15*). Total protein was determined by multiplying total nitrogen by a conversion factor of 6.25 g protein/g N. Total potassium was extracted with 2% acetic acid and analyzed by atomic emission spectroscopy (*16*). Total phosphorus was measured by nitric acid/hydrogen peroxide microwave digestion and inductively coupled plasma atomic emission spectrometry (*17*, *18*). Soluble glucose, fructose, and sucrose were extracted with hot water and analyzed by high-performance liquid chromatography (HPLC) with mass selective detection (*19*). Total glucose was determined by HPLC after the sample was hydrolyzed with amyloglucosidase (*20*). Starch is the total glucose minus the free glucose multiplied by 0.9. Total nonstructural carbohydrates are the sum of total glucose, free fructose, and free sucrose. All samples were analyzed in duplicate and averaged.

Statistical Analysis. Data were analyzed with SigmaStat 3.5 software (Systat Software, San Jose, CA). Data were confirmed to be normal by the Kolmogorov−Smirnov normality test. Data were compared pairwise to determine differences due to treatment (treated trees in year 2 vs control trees in year 2) and year-to-year variability (control trees in year 2 vs all baseline trees in year 1) using one-way analysis of variance followed by the Tukey multiple comparison test. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

An array of macro- and micronutrient components was measured in tanoak acorns collected in 2005 from eight trees prior to fungicidal treatment and again in acorns collected in 2006, after four of the original eight trees were treated with

Table 1. Fatty Acid Composition of Acorns from Tanoak Trees on a Dry Weight Basis before and after Treatment with Potassium Phosphonate

		year 1[a]	
	baseline[b]	control	treated
C 14:0 (mg/g)	0.15 ± 0.09	0.29 ± 0.09	0.16 ± 0.13
C 16:0 (mg/g)	39 ± 4.2	33 ± 2.2	34 ± 4.9
C 16:1 *n*9 (mg/g)	1.2 ± 0.19	1.7 ± 0.43	0.91 ± 0.98
C 17:0 (mg/g)	0.20 ± 0.05	0.01 ± 0.03[c]	0.05 ± 0.10[c]
C 18:0 (mg/g)	5.0 ± 1.4	4.4 ± 0.76	5.6 ± 4.3
C 18:1 *n*9 (mg/g)	98 ± 16	94 ± 9.8	83 ± 10.9
C 18:2 *n*6 (mg/g)	39 ± 6.4	35 ± 2.6	33 ± 4.0
C 18:3 *n*3 (mg/g)	0.66 ± 0.20	0.93 ± 0.38	0.66 ± 0.27
C 20:0 (mg/g)	0.55 ± 0.19	0.64 ± 0.18	0.89 ± 0.40
C 20:1 *n*9 (mg/g)	0.25 ± 0.11	0.60 ± 0.15	0.81 ± 0.87

[a] Data from year 2 are presented as means ± standard deviations for $n = 4$. [b] Data from year 1 are presented as means ± standard deviations for $n = 8$. [c] Data within rows are significantly different from the year 1 control ($p < 0.05$).

potassium phosphonate. The macronutrients and phytochemicals analyzed were those of major nutritional significance and/or thought to have the greatest potential to be affected by any biochemical changes caused by the potassium phosphonate. The study design allowed for the comparison of nutritional components as affected by potassium phosphonate while controlling for year-to-year variation in the crop.

The fatty acid composition of acorns was determined in pre- and post-treated samples. Plant lipids are a means of energy storage in plants and function in cellular membranes and are involved in signaling and defense mechanisms (*21*). Fat is a critical macronutrient in acorns, comprising 20% of the total weight of the acorn (*22*). Fatty acids from 14 carbons to 20 carbons were measured by gas chromatography as fatty acid methyl esters (**Table 1**). The fatty acids found in highest concentration in the first year were oleic acid (98 ± 16 mg/g), palmitic acid (39 ± 4.2 mg/g), and linoleic acid (39 ± 6.4 mg/g). There were no detectable changes in fatty acids from acorns in either the control or the phosphonate-treated trees, except for margaric acid (C17:0). In acorns from both nontreated and phosphonate-treated trees, the concentration of margaric acid decreased from a baseline value of 0.20 ± 0.05 to 0.01 ± 0.03 mg/g in year 1 in the nontreated acorns and 0.05 ± 0.10 mg/g in the treated acorns ($p < 0.05$). Because the decrease in margaric acid was comparable in acorns from both treated and control trees, the differences can be attributed to year-to-year variation and not phosphonate treatment. Overall, after 1 year, phosphonate treatment did not significantly alter fatty acid levels in acorns.

Phenols are associated with defense responses of plants (*23*), and environmental and pathogenic stress play roles in the production of phenols during plant defense (*24*). Phosphonate has been shown to increase a plant's production of certain flavonoids as an antimicrobial defense (*9*). Acorns are a good source of polyphenolics (*25*), and in an earlier study, we established that the main polyphenolics in tanoak acorns are gallic acid and ellagic acid (*14*). **Table 2** demonstrates the levels of gallic acid, ellagic acid, and total phenolic activity in response to potassium phosphonate treatment. The total phenolic activity did not change significantly from baseline (71 ± 11 mg GAE/g) to year 1 (86 ± 30 mg GAE/g) or with phosphonate treatment (84 ± 11 mg GAE/g). Levels of gallic acid and ellagic acid did not change significantly over the year test period in acorns from control trees. The concentration of gallic acid at baseline was 0.23 ± 0.25 and 0.23 ± 0.09 mg GAE/g in year 1. The concentration of ellagic acid at baseline was 0.14 ± 0.13 and 0.12 ± 0.05 mg GAE/g in year 1. Although average levels of these two polyphenols were two-fold higher in acorns from phosphonate-treated trees than control trees in both years (0.42 ± 0.16 and 0.28 ± 0.09 mg/g, respectively), these changes were not significant due to the large standard deviations and small sample size.

Table 2. Moisture, Protein, Carbohydrate, Elemental, and Phenolic Composition of Acorns from Tanoak Trees on a Dry Weight Basis before and after Treatment with Potassium Phosphonate

		year 1[a]	
	baseline[b]	control	treated
moisture content (%)	36 ± 3.6	33 ± 4.4	37 ± 7.1
protein (%)	4.0 ± 0.43	4.5 ± 0.08	4.3 ± 0.11
total phosphorus (%)	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
extractable potassium (%)	0.72 ± 0.08	0.74 ± 0.08	0.70 ± 0.15
soluble glucose (%)	0.18 ± 0.14	0.58 ± 0.32[c]	0.86 ± 0.27[c]
soluble fructose (%)	0.08 ± 0.11	0.43 ± 0.29[c]	0.70 ± 0.24[c]
soluble sucrose (%)	1.7 ± 0.54	1.6 ± 0.85	1.6 ± 0.86
total glucose (%)	39 ± 3.3	48 ± 3.4[c]	44 ± 3.9
total nonstructural carbohydrates (%)	41 ± 3.4	50 ± 3.9[c]	47 ± 4.3
starch (%)	35 ± 3.0	42 ± 3.2[c]	39 ± 3.7
total phenolics (mg/g)	71 ± 10.6	86 ± 30.3	84 ± 22.7
gallic acid (mg/g)	0.23 ± 0.25	0.23 ± 0.09	0.42 ± 0.16
ellagic acid (mg/g)	0.14 ± 0.13	0.12 ± 0.05	0.28 ± 0.09

[a] Data from year 2 are presented as means ± standard deviations for $n = 4$. [b] Data from year 1 are presented as means ± standard deviations for $n = 8$. [c] Data within rows are significantly different from the year 1 control ($p < 0.05$).

Carbohydrates were also measured in response to phosphonate treatment (**Table 2**). Glucose, fructose, and sucrose are sugars that can be accumulated and mobilized to be metabolized or translocated to other plant parts (*26*). Glucose levels increased significantly ($p < 0.05$) in both the nontreated and the treated samples in year 1 (0.58 ± 0.32 and 0.86 ± 0.27%, respectively) as compared to the baseline value of 0.18 ± 0.14% for year 1. Fructose values increased significantly ($p < 0.05$) from 0.08 ± 0.11% at baseline to 0.43 ± 0.29% for the year 1 nontreated acorns and 0.70 ± 0.24% for the year 1 treated acorns. Although glucose and fructose levels were, respectively, more than four- and eight-fold higher in the treated acorns than baseline acorns, differences between the nontreated and the treated samples in year 2 were not statistically significant for either glucose or fructose. Soluble sucrose did not change significantly between years or treatment groups. Starch in seeds and tubers is stored for long periods during growth and used to support growth during the next growing season (*21*). Starch increased significantly in the year 1 nontreatment group vs the baseline group (42 ± 3.2 vs 35 ± 3.0%) but not for the year 1 treated group (39 ± 3.7%). A similar pattern was observed for total glucose (soluble glucose plus enzymatically hydrolyzed glucose) and total nonstructural carbohydrates (sum of total glucose, soluble fructose, and soluble sucrose). Total glucose increased significantly for the year 1 control group vs the baseline group (48 ± 3.4 vs 39 ± 3.3%) but not for the year 1 treated group (44 ± 3.9%). Similarly, total nonstructural carbohydrates increased from 41 ± 3.4% at baseline to 50 ± 3.9% in year 1 but did not change significantly in the treated acorns (47 ± 4.3%). Although total glucose, starch, and total nonstructural carbohydrate levels were higher in both nontreated and treated acorns in year 1 than at baseline, only nontreated trees had significant increases in these carbohydrates. These observations indicate that treatment of trees with potassium phosphonate may have reduced the production or accumulation of these carbohydrates in acorns, at least over this period. The underlying cause of the year-to-

year variation is unclear, although differences in rainfall and temperatures between the years may be involved. Observations in additional years would be needed to determine whether this treatment-associated effect is detectable across different levels of year-to-year variation.

Potassium and phosphorus, two main elements in potassium phosphonate, were also measured (**Table 2**). Potassium and phosphorus are essential plant macronutrients (*27*). After absorption, measurable increases in ions such as potassium can be found in distal portions of the plant (*28*). Phosphonate concentrations in planta have been directly correlated to application rate, but in planta phosphonate concentrations in treated plants also vary widely by species (*29*). Neither total phosphorus nor extractable potassium changed significantly from baseline to year 1 or due to potassium phosphonate treatment. Baseline phosphorus levels were 0.06 ± 0.01%, and year 1 levels were 0.08 ± 0.01% for the nontreated group and 0.07 ± 0.01% for the treatment group. Potassium levels at baseline were 0.72 ± 0.08% and in year 1 were 0.74 ± 0.08% for the nontreated group and 0.70 ± 0.15% for the treatment group. The lack of change in potassium and phosphorus levels in the acorns does not imply that the fungicide was not taken up by the plant. However, we can conclude that potassium phosphonate was not sequestered in acorn cotyledons to any appreciable level between application and acorn harvest.

The other assayed acorn constituents, total protein concentration and moisture content, did not change significantly from baseline to year 1 or in response to potassium phosphonate treatment (**Table 2)**. The protein content at baseline was 4.0 ± 0.43% and in year 1 was 4.5 ± 0.08% for the nontreated group and 4.3 ± 0.11% for the treatment group. Additionally, the acorn moisture content did not change signficantly between the years and was not detectably affected by potassium phosphonate treatment.

Although potassium phosphonate application may have affected the metabolism of nutrients in the tanoak trees directly after application of the fungicide, those changes did not substantially affect the nutritional composition of acorns harvested in the year that the fungicide was applied. The only nutritional components that were negatively affected by fungicide treatment were total glucose, starch, and total nonstructural carbohydrates, but those changes were within the range of values seen in nontreated trees in 2005. Overall, variation in acorn composition between individual trees and between years appears to be greater than variation associated with phosphonate application. This suggests that use of potassium phosphonate for preventing sudden oak death is not likely to noticeably alter the phytochemical and macronutrient quality of acorns from treated tanoak trees. However, a larger study with a greater sample size would be needed to provide more definitive results.

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