JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Different Organic Farming Methods on the Concentration of Phenolic Compounds in Sea Buckthorn Leaves

MERJA HEINÄAHO,* JYRKI PUSENIUS, AND RIITTA JULKUNEN-TIITTO

Department of Biology, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland

The effects of different cultivation methods on the amount of phenolic compounds in leaves of 1-year-old seedlings of two Finnish sea buckthorn (*Hippophae rhamnoides* L. ssp. *rhamnoides*) cultivars 'Terhi' and 'Tytti' were studied in a field experiment established at coastal area in Merikarvia, western Finland. The cultivation methods included different fertilizers (suitable for organic cultivation), mulches (organic and plastic), and land contours (flat vs low hill surface). Two experiments were conducted. The first allowed the estimation of the effects of cultivar, fertilizer, surface contour, and all their interactions, while the other allowed the estimation of the effects of mulches, land contours, and their interactions for the cultivar 'Tytti'. Eleven different hydrolyzable tannins, pentagalloylglucose, and 14 other phenolic compounds were detected by chemical analysis with high-performance liquid chromatography (HPLC). The amount of phenolic compounds varied between different land contours and mulches. The concentrations of gallic acid, pentagalloylglucose, quercetin-3-rhamnoside, monocoumaroyl astragalin A, total hydrolyzable tannins, and condensed tannins were significantly higher on the flat surface than on the low hill surface. The plastic mulch decreased the concentration of gallic acid, hydrolyzable tannins, and condensed tannins compared to the other mulches used. These results suggest ways to cultivate sea buckthorn to produce large amounts of valuable chemicals, especially tannins in the leaves.

KEYWORDS: ***Hippophae rhamnoides*; *Hippophae rhamnoides* cultivars; *Hippophae rhamnoides* leaves; farming methods; fertilizers; land contours; mulches; phenolic compounds**

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L., Eleagnaceae) is a medium-sized deciduous tree or large bush with thorny branches. It grows naturally from central Asia to Europe and is cultivated mainly in Europe and North America. It is classified into nine subspecies, two of which are native European subspecies (*H. rhamnoides* L. ssp. s*inensis* Rousi and *H. rhamnoides* L. ssp. *rhamnoides*) (*25*, *29*). These two are most abundant and are usually used for commercial farming purposes (*25*). Sea buckthorn is best known for its nutritional and medicinal berries. Also, its bark and leaves contain many compounds that may be anticarcinogens and antioxidants (*28*), such as nutrients, flavonoids, and phenolic acids (*8*, *33*). According to pharmacological studies, phenolic compounds lower the risk of coronary heart disease and lung cancer (*19*, *22*). Phenolic compounds may protect plants against ultraviolet light, fungi, bacteria, viruses, herbivores, and plant competitors, that is, allelopathy (*2*, *27*). They also inhibit decay of plant materials and may thus affect nitrogen release and formation of organic matter in the soil (*3*).

Because of all these potentially important biological activities of the tissues of sea buckthorn, the need of research on its chemical composition has increased. In particular, there is interest in organically farmed sea buckthorn. The various methods used to organically cultivate sea buckthorn may induce changes in the chemical composition for the plant tissues. Many phenolic derivatives, such as quercetin, kaempferol, isorhamnetin, ellagic acid, and gallic acid, have been isolated from the berries or leaves of sea buckthorn (*12*, *21*, *32*), but less attention has been focused on the effects of farming methods on the occurrence and the amount of different phenolic compounds.

Generally, cultivation methods applied in organic farming include different organic fertilizers, mulches, and surface profiles. The basic idea is to increase resource availability for the cultivated plants. Fertilizing directly increases the amount of available resources. Mulching controls soil temperature and water conditions and increases availability of other resources (such as nutrients) by decreasing their uptake by competitors. Mulching may also increase the soil microorganism population. Surface profiles (e.g., hills) are more airy and warmer than flat profiles. The roots have more space to grow and develop well. Hills may also increase the activity of symbiotic organisms in rhizosphere and thus nutrient availability (*20*, *24*).

* To whom correspondence should be addressed. Phone: +358-50-3595165; fax: +358-2-5501471; e-mail: merja.heinaaho@netti.fi.

10.1021/jf061018h CCC: $33.50
Published on Web 09/07/2006

Table 1. Statistical Tests for High-Performance Liquid Chromatography (HPLC) Compound Groups and Condensed Tannins in Mulched Tytti ($n = 4$ = Number of Blocks)[a]

compound group	transformation	mulch *F*	P	contour *F*	P	mulch *F*	contour *P*
1 total HPLC phenolics	none	6.20	**0.001**	8.16	**0.008**	3.11	**0.032**
2 gallic acid	none	4.47	**0.007**	6.45	**0.017**	3.16	0.030
3 hydrolyzable tannin 1	none	13.76	**<0.001**	14.54	**0.001**	2.85	0.043
4 hydrolyzable tannin 2	$\ln(x+1)$	2.72	0.051	1.91	0.178	1.54	0.220
5 hydrolyzable tannin 3	$\ln(x+1)$	3.30	0.025	2.85	0.103	1.75	0.167
6 hydrolyzable tannin 4	none	6.47	**0.001**	13.55	**0.001**	2.64	0.056
7 hydrolyzable tannin 5	none	7.79	**<0.001**	14.20	**0.001**	3.10	0.032
8 hydrolyzable tannin 6	none	2.02	0.121	0.63	0.434	3.56	0.019
9 hydrolyzable tannin 7	none	3.63	**0.017**	2.05	0.164	3.95	0.012
10 hydrolyzable tannin 8	none	10.77	**<0.001**	13.47	**0.001**	1.00	0.424
11 hydrolyzable tannin 9	none	11.29	**<0.001**	17.14	**<0.001**	4.22	0.009
12 hydrolyzable tannin 10	none	2.36	0.078	4.23	0.050	2.96	0.038
13 hydrolyzable tannin 11	none	9.66	**<0.001**	8.93	**0.006**	4.05	0.011
14 ellagic acid	$\ln(x+1)$	1.05	0.400	1.09	0.306	0.24	0.912
15 rhamnetin diglucoside	none	0.27	0.893	0.39	0.537	1.14	0.357
16 quercetin-3-galactoside*		0.88	0.349	<0.01	>0.999	<0.01	>0.999
17 pentagalloylglucose	none	3.99	**0.011**	10.53	**0.003**	1.90	0.139
18 kaempherol-3-glucoside	$\lambda = -5.0$	1.89	0.141	0.06	0.808	0.21	0.931
19 quercetin-3-rhamnoside	$\ln(x+1)$	1.82	0.153	0.51	0.479	0.90	0.477
20 isorhamnetin-3-glucoside	$\mathrm{sqrt}(x+0.5)$	0.77	0.552	0.13	0.724	0.70	0.600
21 monocoumaroyl astragalin A	$\ln(x+1)$	2.04	0.117	0.07	0.790	1.17	0.347
22 astragalin derivative	$\ln(x+1)$	1.30	0.295	0.30	0.590	0.57	0.688
23 isorhamnetin derivative 1	$\ln(x+1)$	1.92	0.135	0.15	0.699	1.77	0.165
24 isorhamnetin derivative 2	$\ln(x+1)$	2.12	0.106	0.53	0.473	1.32	0.288
25 isorhamnetin derivative 3*		2.80	0.094	0.12	0.729	0.04	0.836
26 isorhamnetin derivative 4	none	9.13	**<0.001**	5.55	0.026	2.30	0.084
27 condensed tannins	$\ln(x+1)$	16.67	**<0.001**	17.78	**<0.001**	6.91	**0.001**

[a] In the cases denoted by asterisks, generalized linear model with binomial distribution was fitted and the test values given are deviances with 1 df. *F*-values denote the ratio of variance because of a treatment and error variance. *P*-values denote the probability of rejecting a true null hypothesis. The *P*-values smaller than 0.05, after a correction using false-discovery rate procedure, are marked using bold.

Three well-known often cited hypotheses predict allocation of resources to growth versus secondary chemistry in conditions with changing resource availability. All of these hypotheses, carbon/nutrient balance (CNB) (*5*), growth differentiation balance (GDB) (*10*), and protein competition (PCM) (*13*), predict that fertilization with nitrogen should decrease the concentration of carbon-based secondary metabolites such as phenolic compounds. These hypotheses provide an appropriate theoretical framework to study the effects of organic fertilizers, mulches, and land contours on the concentrations of secondary compounds in plant tissues.

The first aim of this study was to test the effects of different organic farming methods on phenolic composition of sea buckthorn leaves. The second aim was to find the farming methods producing the highest phenolic content to leaves. The low nitrogen fertilizers were chosen to test their suitability for this plant, which typically grows in symbiosis with nitrogen-fixing bacteria (*30*). The mulches were chosen because they prevent effectively weed growth and most of them decompose quite well delivering more nutrients for the plants. In addition, they are easily available and affordable for farmers. The potential effect of land contours on plant secondary metabolism has not been addressed in earlier studies. The results of this study should provide useful information for sea buckthorn cultivation and utilization of its leaves for herbal medicines.

MATERIALS AND METHODS

Experimental Design and Plant Material. The Finnish sea buckthorn cultivars 'Terhi', 'Tytti', and 'Tarmo' used in this study descend from the wild sea buckthorn strains originated from the Baltic Sea region in Finland (*15*). The cultivars were grown at the study field in a coastal area in Merikarvia, western Finland (61°52′N, 21°30′E). The study field comprised 560 seedlings. The seedlings were arranged in rows of five individuals such that the first seedling in each row was a male (cultivar 'Tarmo') and the four remaining ones were females. Nine blocks were formed into the study area, such that each of the blocks randomly received either of the two female cultivars Terhi (four blocks) and Tytti (five blocks). In each block, four different fertilizers (control, apatite, bioapatite, test fertilizer) and two land contours (flat surface, low hill surface) were randomized according to a fully crossed design among eight rows of four seedlings in each block. In addition, each of these rows was covered with plastic mulch. The blocks with Tytti had eight additional rows of four seedlings, which were randomized to fully crossed combinations of the two land contours and five different mulches (control, straw, dry grass, conifer chips, plastic mulch). These rows also received test fertilizer. Thus, in practice, two experiments were conducted. The first allowed evaluation of the effects of cultivar, fertilizer, contours, and their interactions, while the other one allowed assessment of the effects of mulches, contours, and their interactions for the cultivar Tytti. The rows with plastic mulch and test fertilizer were included in both fertilizer and mulch experiment. To keep the experimental design balanced, only four randomly chosen Tytti blocks were taken to this study. The chemical composition of commercial fertilizers used in the fertilization experiment and their added amounts were as follows: apatite (0:14:0 NPK, corresponds to 1000 kg/ha), bioapatite (0:2:4 NPK, corresponds to 5000 kg/ha), and test fertilizer (3:3:15 NPK, at 1670 kg/ha). The chemical composition of mulches was measured in Viljavuuspalvelu Oy in Mikkeli, Finland. The contents were straw (N 7.5 g/kg, P 1.4 g/kg, K 20 g/kg), dry grass (N 16 g/kg, P 2.2 g/kg, K 19 g/kg), and conifer chips (N 0.86 g/kg, P 0.1 g/kg, K 0.7 g/kg). In the control treatment of the mulch experiment, the area was not mulched, but grass growing in the area was pulled out and was left to decompose. Low hills of the land contour treatment were 30-cm high. Leaf samples were collected from the cultivars at the end of the first growing season from August 4 to 6, 2004. Five random leaves were taken from one seedling and all leaves from the seedlings grown in the same row were put together into the same paper bag and were analyzed as a single sample. Totally, 96 samples were taken from the study area. They were air-dried in open paper bags at room temperature and were stored at −20 °C until analyses.

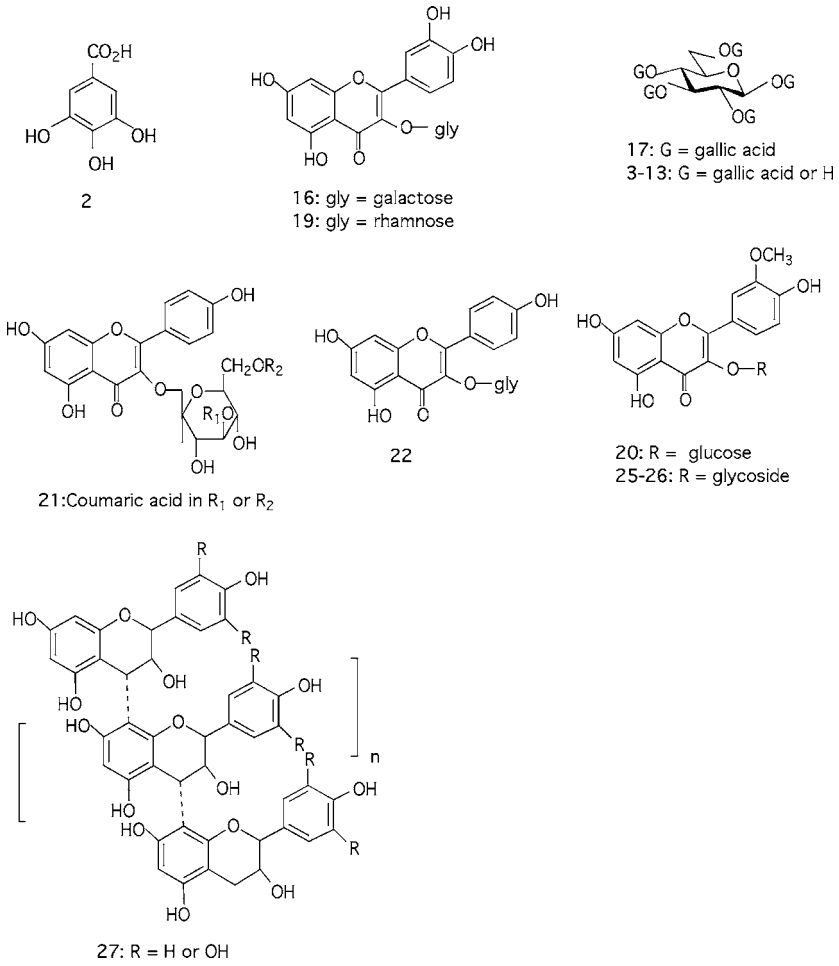

Figure 1. Structural formulas of phenolic compounds from sea buckthorn leaves. Compound **2** is gallic acid. Compounds **3**–**13** are hydrolyzable tannins. Compound **16** is quercetin-3-galactoside. Compound **17** is pentagalloylglucose. Compound **19** is quercetin-3-rhamnoside. Compound **20** is isorhamnetin-3-glucoside, **21** is monocoumaroyl astragalin A, and **22** is astragalin derivative. Compounds **25**–**26** are isorhamnetin derivatives. Compound **27** is condensed tannin. Only those compounds affected by the experimental treatment are included. Refer to those in **Table 1**.

Sample Preparation. Samples for analyses of methanol soluble phenolic compounds were taken from the dried leaves using a cork borer. Leaf disks (25 mm in diameter, 20 mg of dry weight) were crushed with a glass rod and were homogenized for 2 min in 600 μL of methanol. Samples were allowed to stand on ice for 15 min before they were centrifuged for 3 min (13 200 rpm). The residues were re-extracted four more times (1 min per time) in 600 μL of methanol. The combined extracts were dried under nitrogen. For HPLC analyses, the dried samples were dissolved in methanol:water (1:1, v/v) and were analyzed as described in Julkunen-Tiitto and Sorsa (*14*).

HPLC Analyses. The phenolic compounds were analyzed by RP-HPLC. The system used was Hewlett-Packard (Avondale, PA) instrument with a quaternary pump (HP 1050), an autosampler (HP 1050), and photodiode array detector (HP 1040A) controlled by HP Chem Station Software. A 3-μm HP Hypersil ODS column (60 × 4.6 mm ID) was used. The gradient elution systems consisted of aq 1.5% tetrahydrofuran + 0.25% *o*-phosphoric acid (=A) and 100% methanol (=B). The samples were eluted as follows: 0−5 min 100% A; 5−10 min 85% A, 15% B; 10−20 min 70% A, 30% B; 20−30 min 65% A, 35% B; 30−50 min 50% A, 50% B; 50−55 min 100% B; 55−60 min 100% A. The flow rate was 2 mL/min. The injection volume was 15 μL. The injector and column temperature were 25 and 30 °C, respectively. The phenolic compounds were identified using their retention times and the UV spectra. The compounds were monitored at 220, 270, and 320 nm and were quantified by comparisons with reference compounds as follows: gallic acid (Aldrich, Steinheim, Germany) for gallic acid derivatives, pentagalloylglucose, and other hydrolyzable tannins and ellagic acid; quercetin-3-galactoside (Roth, Karlsruhe, Germany) for isorhamnetin derivatives, rhamnetin diglucoside, quercetin derivatives; kaempherol-3-*O*-glucoside (Extrasynthetase, Genau, France) for kaempherol-3-*O*-glucoside, astragalin derivative, and monocoumaroyl astragalin A. The amount of condensed tannins was determined both from the methanol extract and from the dried residue using the butanol−HCl test according to Hagerman (*9*). Total tannin content was the sum of the extracted and the residual tannins.

Statistical Tests. The experiment included (1) a split-plot design with two female cultivars (main plot factor) and combinations of two land contours and four fertilizers (split-plot factors) as well as (2) a randomized block design for the cultivar Tytti with combinations of two land contours and five mulches in each block. The data from these experiments were analyzed using appropriate models of analysis of variance (ANOVA). To meet the requirements of ANOVA, if the chemical data was not normally distributed, it was log- or square-root-transformed. The multiple comparisons with mulches were made using Tukey's HSD test. In a few cases, quercetin-3-galactoside, isorhamnetin-3-glucoside, and astragalin derivative in design 1 and quercetin-3-galactoside and isorhamnetin derivative 3 in design 2, there were some samples with zero concentration, that is, below limit of detection, and other samples with concentrations larger than zero, and the data was scored to zeros (the compound was not detected) and ones (the compound was detected). A generalized linear model was then fitted to the data. Appropriate model contours, that is, analogous to those of the ANOVAs, were used. Because multiple tests were performed from the same data, the significance values of each of the main effects and interactions were adjusted by the false discovery rate procedure of Benjamini and Hochberg (*1*). The number of tests for each of the studied effects corresponded to the number of detected compounds and included both ANOVAs and generalized linear models. The hypothesis concern-

Table 2. Statistical Tests for High-Performance Liquid Chromatography (HPLC) Compound Groups and Condensed Tannins in Fertilized Terhi and Tytti ($n = 4$ = Number of Blocks)[a]

compound group	transformation	cultivar (Cu)		fertilization (Fe)		contour (Co)		Cu Fe		Cu Co		Fe Co		Cu Fe Co	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
1 tot. HPLC phenolics	**none**	0.02	0.881	1.68	0.185	38.54	**<0.001**	0.09	0.963	0.42	0.518	0.82	0.493	0.73	0.540
2 gallic acid	none	2.70	0.152	1.73	0.175	16.39	**<0.001**	3.50	0.024	6.23	0.017	0.83	0.483	0.50	0.685
3 hydrolyzable tannin 1	**ln(x + 1)**	14.76	**0.009**	0.15	0.932	39.67	**<0.001**	0.57	0.638	4.06	0.050	2.32	0.089	2.32	0.090
4 hydrolyzable tannin 2	**ln(x + 1)**	11.02	**0.016**	0.37	0.77 2	24.30	**<0.001**	0.17	0.914	0.07	0.800	0.62	0.607	0.90	0.448
5 hydrolyzable tannin 3	none	0.72	0.429	0.14	0.936	30.01	**<0.001**	0.07	0.974	0.00	0.969	0.92	0.439	0.28	0.837
6 hydrolyzable tannin 4	**ln(x + 1)**	10.45	**0.018**	3.48	0.024	40.89	**<0.001**	0.07	0.978	1.56	0.219	1.32	0.280	0.74	0.533
7 hydrolyzable tannin 5	**ln(x + 1)**	26.15	**0.002**	1.24	0.308	39.01	**<0.001**	0.14	0.935	0.00	0.993	1.10	0.360	0.41	0.746
8 hydrolyzable tannin 6	ln(x + 1)	6.92	0.039	2.71	0.057	8.83	**0.005**	0.72	0.545	2.26	0.140	0.60	0.619	2.72	0.056
9 hydrolyzable tannin 7	**ln(x + 1)**	14.39	**0.009**	0.73	0.538	24.23	**<0.001**	0.02	0.998	0.46	0.502	1.35	0.271	0.27	0.848
10 hydrolyzable tannin 8	none	1.79	0.229	4.42	0.009	40.44	**<0.001**	0.63	0.603	0.44	0.513	0.73	0.542	0.91	0.444
12 hydrolyzable tannin10	sqrt(x + 0.5)	196.62	**<0.001**	1.17	0.332	42.98	**<0.001**	0.34	0.797	0.17	0.683	0.57	0.639	0.56	0.643
13 hydrolyzable tannin11	**none**	125.58	**<0.001**	1.78	0.166	35.89	**<0.001**	0.46	0.715	0.10	0.750	0.32	0.814	0.15	0.927
16 quercetin-3-galactoside*		17.55	**<0.001**	0.87	0.344	1.26	0.262	<0.01	>0.9 9	<0.01	>0.99	<0.01	0.94	<0.01	>0.99
17 pentagalloylglucose	ln(x + 1)	77.45	**<0.001**	0.40	0.752	15.53	**<0.001**	1.04	0.385	0.47	0.495	0.89	0.454	0.99	0.407
18 kaempherol-3-glucoside*		2.83	0.092	0.69	0.19	0.19	0.667	1.63	0.202	1.23	0.267	1.64	0.201	1.90	0.168
19 quercetin-3-rhamnoside	none	0.01	0.920	0.40	0.755	6.36	**0.016**	2.85	0.049	0.16	0.687	2.29	0.092	2.44	0.078
20 isorhamnetin-3-glucoside	$\lambda = -0.41$	55.23	**<0.001**	2.31	0.090	0.97	0.331	2.928	0.045	3.42	0.072	2.06	0.120	1.56	0.214
21 monocoumaroyl astragalin A	ln(x + 1)	6.16	0.048	0.86	0.469	4.99	**0.031**	1.10	0.360	2.50	0.121	1.02	0.395	2.12	0.112
22 astragalin derivative*		32.19	**<0.001**	0.41	0.520	0.51	0.475	<0.01	>0.99	<0.01	>0.99	0.40	0.526	<0.01	>0.99
24 isorhamnetin deriv. 2	ln(x + 1)	6.99	0.038	0.52	0.670	0.20	0.657	0.44	0.728	0.31	0.580	0.99	0.407	0.16	0.920
25 isorhamnetin deriv. 3*		7.88	**0.005**	0.19	0.667	7.88	**0.005**	0.094	0.759	0.28	0.597	2.45	0.117	5.68	0.017
26 isorhamnetin deriv. 4	ln(x + 1)	0.07	0.794	0.81	0.494	3.82	0.057	0.18	0.913	1.01	0.320	1.12	0.353	0.17	0.913
27 condensed tannins	sqrt(x + 0.5)	2.81	0.145	3.06	0.038	23.22	**<0.001**	0.30	0.826	2.40	0.129	0.99	0.409	1.83	0.157

[a] The transformations used for ANOVA are given. In the case of isorhamnetin-3-glucoside, box-cox transformation was used. In the cases denoted by asterisks, generalized linear model with binomial distribution was fitted and the test values given are deviances with 1 df. *F*-values denote the ratio of variance because of a treatment and error variance. *P*-values denote the probability of rejecting a true null hypothesis. The *P*-values smaller than 0.05, after a correction using false-discovery rate procedure, are marked using bold.

ing the effects of the experimental factors on the total concentration of phenolic compounds was considered as a separate from that concerning the individual compounds. Therefore, the false discovery rate procedure was not applied for it. The ANOVAs were analyzed with SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, 2000), and generalized linear models were run with R 2.1.1 for Windows (R Project for Statistical Computing).

RESULTS

In fertilized cultivars, 23 phenolic compounds were found in Terhi and 24 compounds were found in Tytti. In mulched Tytti, 25 phenolic compounds were found. In addition, both cultivars had also condensed tannins. Among the compounds identified were 11 hydrolyzable tannins and 4 isorhamnetin derivatives (**Table 1**, **Figure 1**). Because of the different number of phenolic compounds between cultivars, only those 22 compounds which were found in both cultivars were compared in the fertilization experiment (**Table 2**). The concentration of total phenolic compounds found by HPLC in plants grown on the flat surface was greater than that of those grown on low hills. The concentration of total phenolic compounds was not affected by fertilization or cultivar. Among the individual compounds, there were significant differences found between the cultivars and contours but no fertilization effect (**Table 2**). The contents of **3**, **4**, **6**, **7**, and **20** were significantly higher in Terhi than in Tytti (**Figure 2**). Furthermore, the concentration of **16** was more often above the detection level in Terhi (in 32 of 32 samples) than in Tytti (in 21 of 32 samples). On the other hand, there were significantly more **9**, **12**, **13**, and **17** in Tytti than in Terhi (**Figure 2**). Moreover, the concentrations of **22** and **25** were more often above the detection level in Tytti (in 32 of 32 samples, in 30 of 32 samples, respectively) than in Terhi (in 14 of 32 samples, in 22 of 32 samples, respectively). The concentrations of **2**, **3**−**13**, **17**, **19**, **21**, and **27** were higher in seedlings grown on the flat surface than in those grown on the low hills (**Figure 2**). On the other hand, the concentration of **25** was more often above the detection level on the low hills (in 30 of 32 samples) than on the flat surface (in 22 of 32 samples).

In the mulch experiment (**Table 1**), the total concentration of phenolic compounds detected by HPLC was affected by mulch and land contour. However, the significant mulch × contour interaction indicated that the effects of these factors depended on each other. On the flat surface, the total concentration of phenolic compounds did not differ between the mulches. On the low hills, the total concentration of phenolic compounds was significantly higher in straw, dry grass, and conifer chips compared to plastic mulch treatment. In addition, plants grown with straw had significantly higher total phenolic concentration compared to plants grown with no mulch (Tukey, $P < 0.05$) (**Figure 3**). The mulch × contour interaction was significant for **27** (**Table 1**). On the flat surface, the plants with plastic mulch had lower concentration of condensed tannins than those with other mulches. On the low hills, the plants mulched with straw or dry grass had higher concentration of condensed tannins than those with plastic mulch or no mulch. In addition, mulching with dry grass seemed to yield more condensed tannins than mulching with conifer chips (Tukey, $P < 0.05$). However, of the studied treatment combinations, flat surface and mulching with conifer chips seemed to yield the highest concentration of condensed tannins (**Figure 3**).

Of the other individual compounds, the concentrations of **2**, **3**, **10**, **11**, and **13** were significantly higher in control, straw, dry grass, and conifer chips treatments than in the plastic mulch treatment (**Figure 3**) (Tukey multiple comparisons, $P < 0.05$). The concentration of 7 was higher in straw, dry grass, and conifer chips treatments than in the plastic mulch treatment (**Figure 3**) (Tukey multiple comparisons, $P < 0.05$). Straw and dry grass treatments increased the concentration of 6 compared to plastic mulch (**Figure 3**) (Tukey multiple comparisons, $P < 0.05$). **9** and **17** were induced only by straw compared to plastic

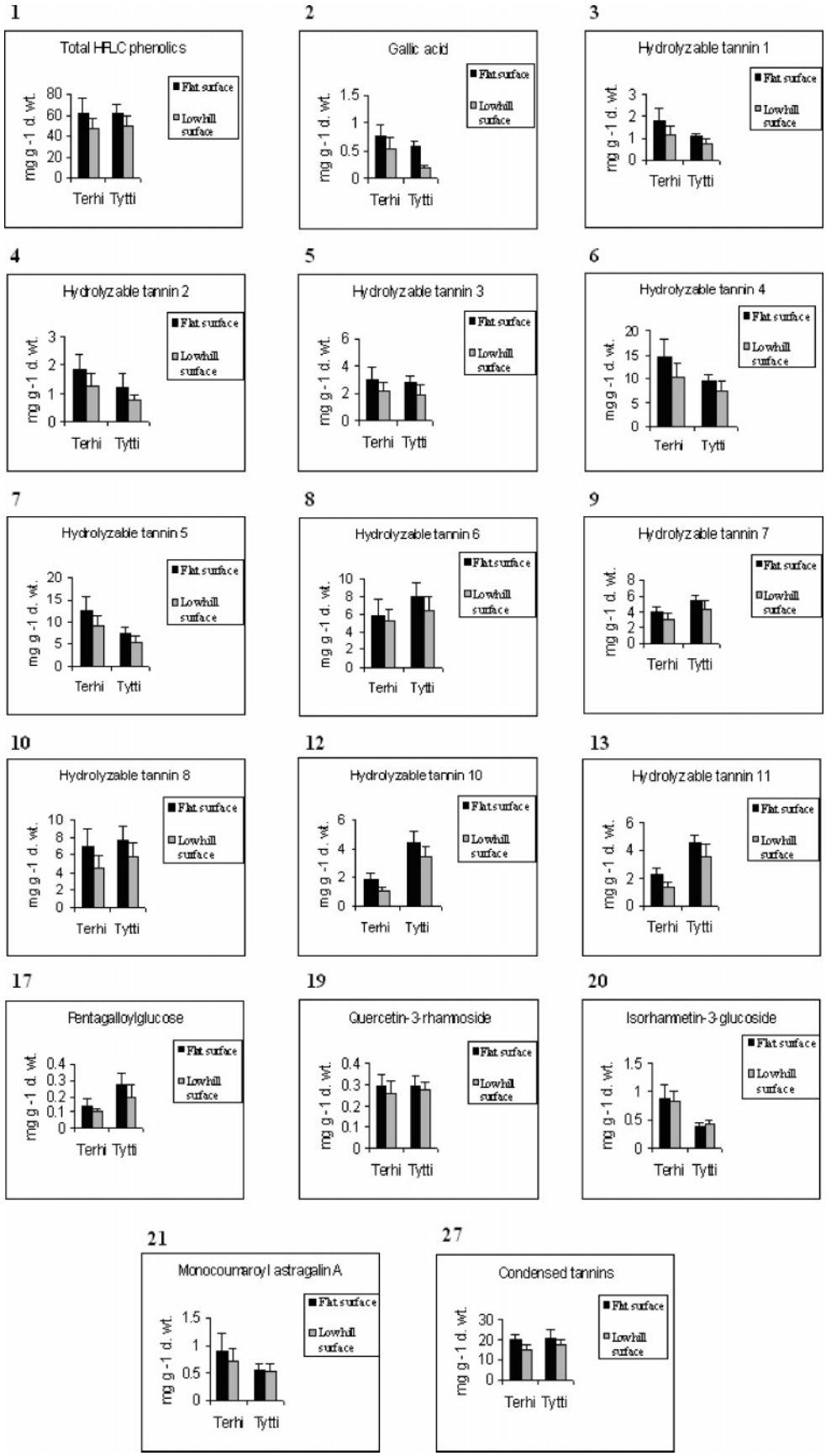

Figure 2. Effects of cultivar and land contour on concentrations of phenolic compounds in the fertilization experiment. Only compounds with significant effects are included. Fertilization did not have any significant effects and is thus omitted.

mulch (**Figure 3**) (Tukey multiple comparisons, $P < 0.05$). Only **26** was induced by plastic mulch compared to other mulches used (**Figure 3**) (Tukey multiple comparisons, $P < 0.05$). The concentrations of **2**, **3**, **6**, **7**, **10**, **11**, **13**, and **17** were higher in

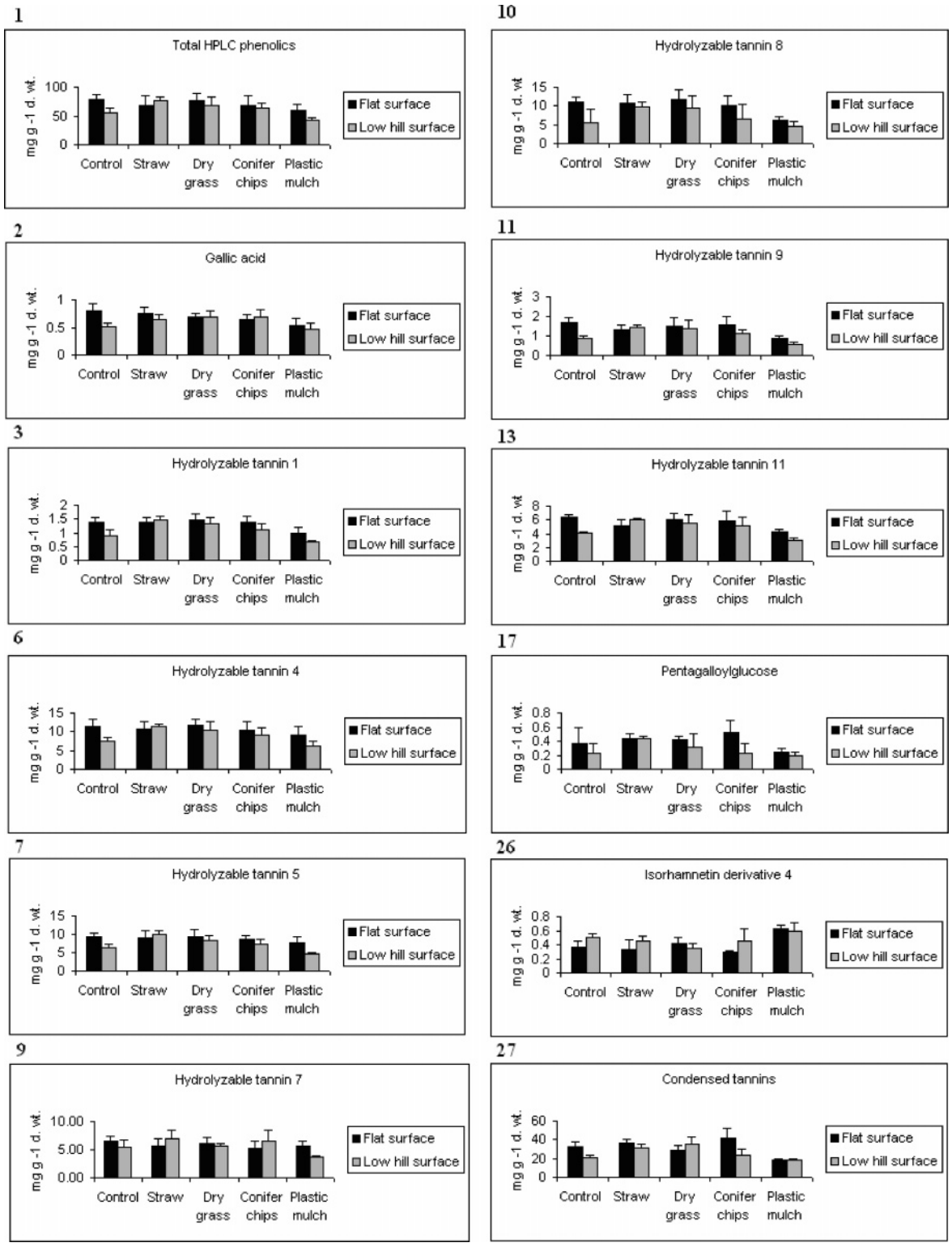

Figure 3. Effects of mulch and contour on the concentrations of phenolic compounds in the mulch experiment. Only compounds with significant effects are included.

plants grown on the flat surface than in those grown on the low hills (**Figure 3**). Although mulch × contour interactions were not significant for these compounds after the false discovery rate correction, it seems that the effect of contour was often less clear when straw was used as a mulch.

DISCUSSION

Most of the phenolic compounds we found, including gallic acid, hydrolyzable tannins, isorhamnetin, kaempherol, and quercetin derivatives, have been identified in earlier studies of sea buckthorn leaves using chromatographic methods. However, we did not find myricetin, which was found in earlier studies (*8*, *32*). Here, we report for the first time the differences in phenolic composition between two cultivars of sea buckthorn. We examined the occurrence and concentrations of several phenolic compounds in the mature leaves of Terhi and Tytti cultivars grown under various conditions. The number and concentrations of individual compounds was generally higher

in Tytti, but there were also several phenolic compounds with higher concentrations in Terhi. The differences between the cultivars are most likely a result of differences in genotype.

Farming methods had significant effects on the concentrations of several phenolic compounds in the leaves of sea buckthorn. In the mulch experiment, the concentrations of many phenolic compounds were significantly lower when the plants were mulched with plastic compared to control and other mulch treatments. This applies to the concentrations of gallic acid, several hydrolyzable tannins, and condensed tannins. The concentration of one hydrolyzable tannin, 7, was higher in straw, dry grass, or conifer chips treatments compared to plastic mulch treatment. Straw or dry grass treatments increased the concentration of **6** while **9** and **17** were increased only by straw mulch. The organic mulches used in this study contained different amount of nutrients. According to chemical analyses, straw and dry grass had the highest levels of N, P, K, and Ca. The concentrations of nitrogen in straw and dry grass were 7.6 g/kg and 16 g/kg, respectively. Straw provides nitrogen at the beginning of its decomposition, but it decomposes quickly (*11*). Nitrogen concentration in the conifer chip treatment was only 0.86 g/kg. Conifer chips decompose slowly, and they may utilize nitrogen during decomposition rather than release it (*11*, *18*, *24*). The control plots were not free of added fertilizer, as grass growing in the plots was weeded out and left to release nutrients while decomposing (*11*, *18*).

In previous studies, it has been noted that fertilizers, especially nitrogen, decrease the concentration of carbon-based secondary compounds such as tannins (*6*, *16*, *17*). These observations provide most of the support for hypotheses that seek to explain plant secondary product accumulation on the basis of nutrient status, such as the CN balance hypothesis. The results of our experiment seem to contradict this pattern. However, there is evidence that high levels of nitrogen in soil have negative effects on the activity of nitrogen-fixing symbionts (*26*, *30*). It may be that the nitrogen release from organic mulches disturbed the function of the nitrogen-fixing bacteria typically found in symbiosis with sea buckthorn. Thus, effects on symbiosis could explain the high concentrations of some hydrolyzable and condensed tannins in control and mulched plants. Plants with nitrogen-fixing symbionts may in general be inadequately described by the existing hypotheses for the interaction between nutrient levels and secondary product accumulation.

Nitrogen stress triggers the gene expression of flavonoid pathway enzymes and may boost production of condensed tannins (*4*, *23*). Hovewer, some phenolic compounds such as flavonoid glycoside (kaempherol, quercetin) do not seem to be affected to the same extent (*5*, *6*, *16*), and phenolic compound levels may vary independent of nutrition levels (*6*, *31*). In our study, **26** was independent of nutrient level when grown with plastic mulch.

Generally, the cultivars produced more phenolic compounds when grown on the flat surface than when grown on the low hills. Especially in the fertilization experiment, the concentration of several hydrolyzable tannins and condensed tannins were high in the plants grown on the flat surface. In the mulch experiment, the pattern was similar. The observed pattern is likely related to the different environmental conditions on the flat versus low hill surface. The ground was more poorly aerated and colder on the flat surfaces than on the low hills. The low hills were formed using an excavator, which made the soil smooth and well aerated. Because the low hills were about 30-cm high, their soil also collected more solar heat than flat surface. Hills offer better growing conditions to roots and symbiotic microorganisms of sea buckthorn (*20*). On the flat surface, root growth and nitrogen fixing may have been inhibited by soil structure. Therefore, the differences in the environmental conditions for nitrogen-fixing symbionts and thus nitrogen availability between the treatments likely explain our results.

This study shows that the chemical composition of sea buckthorn leaves depended on both cultivar and the cultivation method applied. Cultivar, land contours, and mulches caused specific and different responses in different types of phenolic compounds in sea buckthorn leaves. Overall, hydrolyzable and condensed tannins were the dominant phenolic compound groups. In the mulch experiment, tannin levels were increased by growth with organic mulches on flat surfaces. In the fertilization experiment, tannin levels were increased by growth on flat surfaces. These results suggest ways to cultivate sea buckthorn to produce large amounts of valuable chemicals, especially tannins in the leaves. Other tissues besides berries of sea buckthorn are rarely utilized in spite of their high potential value. Because the leaves of sea buckthorn have these valuable phenolic compounds, they have low cost compared to berries and they do not compete at market with other sea buckthorn products. We suggest that with appropriate cultivation methods, sea buckthorn leaves may be a valuable commodity for the health and natural supplements markets.

ACKNOWLEDGMENT

We thank Juha and Katri Viikilä for providing their field for this experiment and Sinikka Sorsa for help with the laboratory work. Dr. Saila Karhu and Pirjo Kivijärvi and Prof. Ann Hagerman provided comments on the manuscript.

LITERATURE CITED

(1) Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate — a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* **1995**, *57*, 289−300.

(2) Bennick, A. Interaction of Plant Polyphenols with Salivary Proteins. *Crit. Rev. Oral Biol. Med.* **2002**, *13*, 184−196.

(3) Benoit, R. E.; Starkey, R. Enzyme inactivation as a factor in the inhibition of decomposition of organic matter by tannins. *Soil Sci.* **1968**, *105*, 203−208.

(4) Bongue-Bartelsman, M.; Phillips, D. A. Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. *Plant Physiol. Biochem.* **1995**, *33*, 539−546.

(5) Bryant, J. P.; Chapin, F. S., III; Klein, D. R. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* **1983**, *40*, 357−368.

(6) Bryant, J. P. Feltleaf willow-snowshoe hare interactions: plant carbon/nutrient balance and floodplain succession. *Ecology* **1987**, *68*, 1319−1327.

(7) Bryant, J. P.; Clausen, T. P.; Reichardt, P. B. et al. Effect of nitrogen fertilization upon secondary chemistry and nutritional value of quaking aspen tortrix (*Choristoneura conflictana*) (Walker). *Oecologia* **1987**, *73*, 513−517.

(8) Guliyev, V. B.; Gul, M.; Yildirim, A. *Hippophae rhamnoides* L.: chromatographic methods to determine chemical composition, use in traditional medicine and pharmacological effects. *J. Chromatogr. B* **2004**, *812*, 291−307.

(9) Hagerman, A. E. *Tannin analysis*; Department of chemistry, Miami University: Miami, OH, 1995; pp 24−25; http://www.users.muohio.edu/hagermae/.

(10) Herms, D. A.; Mattson, W. J. The dilemma of plants: to grow or defend. *Q. Rev. Biol.* **1992**, *67*, 283−335.

(11) Jaakkola, S. *Organic soil covering on cultivated lands and green areas*; Publications of Work Efficiency Institute: Huhmari, Finland, 1996, 12; p 4 (in Finnish).

(12) Jeppson, N.; Gao, X. Changes in the contents of kaempherol, quercetin and L-ascorbic acid in sea buckthorn berries during maturation. *Agric. Sci. Finl.* **2000**, *9*, 17−22.
(13) Jones, C. G.; Hartley, S. E. A protein competition model of phenolic allocation. *Oikos* **1999**, *86*, 27−44.
(14) Julkunen-Tiitto, R.; Sorsa, S. Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. *J. Chem. Ecol.* **2001**, *27* (4), 779−789.
(15) Karhu, S.; Prokkola, S. Sea buckthorn cultivars to Finnish conditions. In *Meeting of Agricultural Science*, 2004; Hopponen, A., Rinne, M., Eds.; Publications of Agricultural Society of Finland, 2004; 19, p 4 (in Finnish).
(16) Keinänen, M.; Julkunen-Tiitto, R.; Mutikainen, P.; Walls, M.; Ovaska, J.; Vapaavuori, E. Trade-offs in phenolic metabolism of silver birch: effects of fertilization, defoliation, and genotype. *Ecology* **1999**, *80*, 1970−1986.
(17) Keski-Saari, S.; Julkunen-Tiitto, R. Resource allocation in different parts of juvenile mountain birch plants: effect of nitrogen supply on seedling phenolics and growth. *Physiol. Plant.* **2003**, *118*, 114−126.
(18) Kivijärvi, P.; Heiskanen, L.; Dalman, P. *Cultivation technique of organically grown currant. Literature review*; Publications of Agricultural Research Centre of Finland: Jokioinen, Finland, 1999; A 19, p 43 (in Finnish).
(19) Knekt, P.; Järvinen, R.; Seppänen, R.; Heliövaara, M.; Teppo, L.; Pukkala, E.; Aromaa, A. Dietary flavonoids and the risk of lung cancer and other malignant neoplasm. *Am. J. Epidemiol.* **1997**, *146*, 223−230.
(20) Koivunen, T. Tricks of farmed garden. In *Suomalainen piha ja puutarha*, 6th ed.; Tapola, S., Ed.; Gummerus: Jyväskylä, Finland, 2003; pp 229−363 (in Finnish).
(21) Krolikowska, M. Flavonoid glycosides from common sea buckthorn berries *Hippophae rhamnoides*. *Planta Med. Dec.* **1972**, *22* (4), 418−427.
(22) Kuchta, G. M. M. Dietary Flavonoids and Risk of Coronary Heart Disease. *Physiol. Res.* **2001**, *50*, 529−535.
(23) Lavola, A.; Julkunen-Tiitto, R. The effect of elevated carbon dioxide and fertilization on primary and secondary metabolites in birch, *Betula pendula* (Roth). *Oecologia* **1994**, *99*, 315−321.
(24) Lehtonen, U. Mulch farming. In *Organic farming in home garden*, 2nd ed.; WS Bookwell Oy: Porvoo, Finland, 2004; pp 25−29 (in Finnish).
(25) Li, T. S. C.; Schroeder, W. R. Sea Buckthorn (*Hippophae rhamnoides* L.): A Multipurpose Plant. *HortTech.* **1996**, *6*, 370−380.
(26) Li, T. S. C. Sea buckthorn: New crop opportunity. In *Perspectives on new crops and new uses*; Janick, J., Ed.; ASHS Press: Alexandria, VA, 1999; pp 335−337.
(27) Mallik, A. U. Effect of phenolic compounds on selected soil properties. *For. Ecol. Manage.* **1997**, *92*, 11−18.
(28) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, *2*, 152−159.
(29) Rousi, A. The genus *Hippophae* L. A taxonomic study. *Ann. Bot. Fenn.* **1971**, *8*, 177−227.
(30) Saario, M. Tyrni. Palatable fruits and berries. In *Suomalainen piha ja puutarha*, 6th ed.; Tapola, S., Ed.; Gummerus: Jyväskylä, Finland, 2003; pp 365−479 (in Finnish).
(31) Waterman, P. G.; Mole, S. Extrinsic factors influencing production of secondary metabolites in plants. In *Insect-Plant Interactions*; Bernays, E. A., Ed.; CRC Press: Boca Raton, FL, 1989; Volume I, pp 107−134.
(32) Yoshida, T.; Tanaka, K.; Chen, X-M.; Okuda, T. Tannins from *Hippophae rhamnoides. Phytochemistry* **1991**, *30*, 663−666.
(33) Zeb, A. Important Therapeutic Uses of Sea Buckthorn (*Hippophae*): A Review. *J. Biol. Sci.* **2004**, *4*, 687−693.

Received for review April 11, 2006. Revised manuscript received August 8, 2006. Accepted August 13, 2006. This project was funded by the EU Leader project and the Finnish Cultural Foundation.

JF061018H