Secondary Metabolites in Maca as Affected by Hypocotyl Color, Cultivation History, and Site

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ABSTRACT

Maca (*Lepidium meyenii* Walpers) hypocotyls are reported to have a favorable biological activity in man. The presumed bioactive constituents seem to vary among maca hypocotyl color types, but the residual variation is large. This study tested the hypothesis that, apart from color types, environmental factors have a distinct influence on the concentration of characteristic secondary metabolites in maca. In a field experiment at 4100 m altitude (Peru), maca of four hypocotyl color types was evaluated at two sites with different soil types. At each site, experimental areas were either never cultivated or cultivated with maca 2 to 3 yr ago followed by a fallow period. Applying four color replicates per site and area resulted in 64 plots (100 plants plot^{-1}). Especially color type largely influenced concentrations of macaene, macamides, β-sitosterol, campesterol, and glucosinolates. Site (also clearly affecting growth yield) was weaker in effect on these metabolites but still significantly influenced concentrations of some constituents, while the effect of cultivation history was widely absent. Macaene, macamides, and β-sitosterol were negatively correlated with glucosinolates. This shows that environmental conditions and color type have to be considered in producing maca with high concentrations of distinct bioactive metabolites.

Maca is a traditional Andean crop of the Brassicaceae family that is grown at altitudes between 3500 and 4500 m a.s.l. in the Peruvian Highlands. When cultivated at low altitude, it lacks most macamides (Melnikovova et al., 2008), a group of compounds characteristic for maca. In Peru, maca is traditionally grown in the "meseta del Bombón", a plateau situated at 4200 m a.s.l. around the lake Chinchaycocha in the Departments of Junín and Pasco. In the early 1990s, only 50 ha of maca were grown in Peru and it was considered as a species in danger of going extinct. Interest in maca drastically increased around 1998 (Hermann and Bernet 2009), and its cultivation was extended to other high altitude areas of Peru such as Cusco and Lake Titicaca (Quirós and Aliaga Cárdenas, 1997; Humala-Tasso and Combelles, 2007). Quality is generally better in Junín and Pasco. In terms of land use, maca is currently produced on areas that either never produced maca before or areas fallowed without cultivation. When grown where maca had been grown before, it is sometimes grown in rotation with different crops (Humala-Tasso and Combelles, 2007).

The general perception is that maca exploits soil resources (Humala-Tasso and Combelles, 2007). Therefore, the recommended fallow period ranges from 4 (Chacón de Popovici, 2001) to 8–10 (Córdova Herrera, 2003) to > 10 yr (Quirós and Aliaga Cárdenas, 1997; Humala-Tasso and Combelles, 2007). When grown in rotation, plots sometimes have added fertilizer. In addition, maca is preferentially produced on sandy soils with less production on heavy soils for quality reasons (Humala-Tasso and Combelles, 2007).

The edible part of maca, the hypocotyl, has different colors with cream-colored, yellow, pink, violet, lead-colored and black the most common. Maca of different color type may differ in biological effect. Gonzales et al. (2006a) demonstrated that black maca is more beneficial than reddish (pink/violet) and yellow maca for spermatogenesis. Gasco et al. (2007a) described similar effects. In addition, Gonzales et al. (2007) and Gasco et al. (2007b) found that reddish maca might reduce ventral prostate size in rats.

Maca contains various secondary metabolites. The secondary metabolites macaene and macamides are only found in this plant. Others include campesterol and β-sitosterol, the most common phytosterols, and various glucosinolates (GL). Some (i.e., the aromatic GL glucotropaeolin) or all of these secondary metabolites, or a distinct combination of them, could play a role in the exhibition of biological effects of maca, especially the improvements in fertility reported (e.g., Gonzales et al., 2006a, 2006b, 2007). Here, in particular, the aromatic GL glucotropaeolin (benzyl GL) was often mentioned (Johns, 1981; Li et al., 2001; Gonzales et al., 2007). It has been observed that maca batches from different producers significantly vary in concentrations of macaene, total macamides, sterols, and GL (Ganzera et al., 2002; McCollom et al., 2005; Clément et al., 2007). However, only part

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Abbreviations: DM, dry matter; GL, glucosinolates; OM, organic matter; SEM, standard error of the mean.

of this variation results from differences found among maca color types (Clément et al., 2007). The environmental factors influencing this variation and their relative importance are still unknown. Humala-Tasso and Combelles (2007) reported producer's experience that the sensory quality of maca (form, flavor before and after cooking, absence of cavities inside of the hypocotyl) was improved when cultivated on an area cultivated before. This is the first study, to the authors' knowledge, investigating the influence of environmental factors on secondary metabolites in maca.

The hypothesis to be tested in the present study was that, apart from color type, cultivation history and site (soil type) have a significant effect on the concentration of the major secondary metabolites of maca and that these factors may interact. Influences of hypocotyl color, site, and cultivation history on secondary metabolites in maca were investigated in a field experiment.

MATERIAL AND METHODS

Plant Material and Preparation

Seeds of four distinct accessions characterized by different hypocotyl colors and originating from the germplasm collection held by Universidad Nacional Daniel Alcides Carrión, Cerro de Pasco, Pasco, Peru, were used. This included yellow (collection number CR95S2–22–4), pink (RO95S2–306–8), violet (MO95S2–142–1), and lead-colored maca (PL95S2–284–20). These accessions had been obtained after a controlled self-pollination to increase homozygosity, as maca is known to reproduce predominantly autogamously (Quirós and Aliaga Cárdenas, 1997). The seeds used were from the second generation of the selected accessions and had been harvested in 1995. The seeds were washed, put on Petri dishes and let germinate for 1 wk. Afterward, the germinated seeds were transplanted into jiffy pellets (42 cm diam.; Jiffy Products (N.B) Ltd. Shippagan, Canada) and grown for 1 mo in a greenhouse (18.6 ± 1.3°C; 80 ± 12% humidity).

Fields

The experiment took place at Patalá (12°20′899′′ S, 75°07′86′′ W, altitude 4086 m a.s.l.), department of Junín, Peru, and Alpacayán (10°92′129′′ S, 76°05′748′′ W, altitude 4130 m a.s.l.), department of Pasco, Peru. At each location, two experimental areas were selected, one which had never been cultivated before with any agricultural crop and one cultivated with maca 2 to 3 yr ago, followed by a fallow period. The distance between the two areas was 50 m at Patalá and 40 m at Alpacayán ensuring that microclimate and soil were similar. The four areas were tilled by hoe. At Alpacayán, the areas were also plowed and fenced in November 2006. Consistent with local practice, neither fertilization nor irrigation was used in the investigation.

Experimental Design

The seedlings were transplanted at the experimental sites in the period of 5 to 8 Dec. 2006. Each area was divided into 16 plots being 1 m apart, resulting in four replicates for each maca color. Each plot consisted of four rows (with 50 cm remaining between the rows) containing 25 plants per rows (20 cm between plants). Overall this resulted in $2 \times 2 \times 4 \times 4 = 64$ plots and 6400 individual experimental plantlets being transplanted. The experiment was based on a split-plot design. To avoid location effects, the arrangement of the plots on the areas was random with respect to the four replicates of each color type. Due to increasing risk of flooding, the harvest at Patalá had to be performed on 25 and 26 June 2007, before maca hypocotyls were really large. Harvest at Alpacayán took place on 10 and 11 July 2007, at a stage where some leaves turned yellow and flowering started. At that time, hypocotyl size was typical at Alpacayán but smaller than typical at Patalá. The hypocotyls were harvested and individually weighed, and total yield and average weight of the hypocotyls were calculated for each of the 64 plots. From the harvest of each plot, all undamaged, potentially marketable, hypocotyls were composited, put into paper bags and transported to the drying facility on the day after harvest for later analysis. The hypocotyls were cut into approximately 1 cm thick slices and immediately frozen at –20°C. Subsequently they were lyophilized (Series 216688, Labconco Corp., Kansas City, MO) and ground using a 20 mesh (0.85 mm) filter (Mini-Mill 383L60, Thomas Scientific, Swedesboro, NJ). Due to the small amounts harvested from some plots at Patalá, analytical data of only 60 of the 64 plots were available for statistical analysis. Concentrations of crude protein, macaene, macamides, and phytosterols were available only from 55 plots.

Soils and Soil Analyses

Soil samples were collected twice in the four experimental areas, first before tillering in October 2006 and then directly after harvest in June/July 2007. Twenty subsamples of the soil without roots were taken from approximately 3 (after removal of the thin grass-containing layer) to 20 cm depth following a zigzag pattern across each area. Subsamples were mixed resulting in a homogenous composite sample per area and date (total $n = 8$). The soil samples were analyzed by established standard methods (Klute 1986; Sparks 1996): sedimentation in water for texture, Walkley and Black for organic matter (OM), Olsen for available P, ammonium acetate at pH 7.0 for available K, a 1:1 water-soil mixture for conductivity and pH, ammonium acetate at pH 7.0 for exchangeable cations and with KCl for exchangeable Al^{3+} and H^{+}. The base saturation is equivalent to the exchangeable base cation fraction of the cation exchange capacity. The soil characteristics of the four areas found were analyzed both pretillering and postharvest.

Gross Nutrient Analysis

Dry matter (DM) and OM (DM minus total ash) were analyzed automatically by heating in duplicate of 1-g samples of the maca hypocotyls to 105 and 550°C, respectively (TGA-500, Leco Corp., St. Joseph, MI). Nitrogen concentrations were determined by a C/N-Analyzer (Leco-Analyzer Type FP-2000, Leco Corp., St. Joseph, MI). Crude protein was calculated as 6.25 × N. Starch and sugars (determined as the sum of glucose, fructose, and sucrose) were analyzed after extraction with perchloric acid (Hargreaves and ap Rees, 1998; Critchley et al., 2001; Smith and Zeeman, 2006), respectively, using a spectral photometer (UV-160 A, Shimadzu Kyoto Japan). The above method was modified with an extraction time of 30 min using 90 mg of sample.

Analysis of Macaene and Macamides

The analytical protocol followed was that of Ganzera et al. (2002) applying a minor modification in the composition of the mobile phase and sample size (cf. also Avula et al., 2008). Hypocotyl samples (500 mg) were sonicated in 2.5 mL of methanol for 20 min and then centrifuged for 15 min at 2900 *g*. Then sample solutions were prepared and injected in triplicate into a HPLC

system (Alliance Model 2695, model 2695, Waters, Milford, MA, USA) equipped with a photo diode array detector (model 996). A Synergi Max RP column (150 by 4.6 mm; 4 μm particle size; Phenomenex, Torrance, CA) was used. Temperature was maintained at 40°C. The mobile phase consisted of high purity water (0.025% trifluoroacetic acid) (A) and acetonitrile (0.025% trifluoroacetic acid) (B) at a flow rate of 1.0 mL min^{-1}. The gradient program linearly changed from 50% A/50% B to 7% A/93% B within 55 min. Subsequently, a washing step with 100% acetonitrile was performed for 5 min. Samples were then re-equilibrated with A/B as 1/1 for 15 min. N-benzyl-palmitamide ('macamide-1'), n-benzyl-9-oxo-12Z-octodecenamide ('macamide-3'), n-(m-methoxybenzyl)-hexadecanamide ('macamide-4') were detected at a wavelength of 210 nm, while 280 nm were observed for 5-oxo-6E,8E-octadecadienoic acid ('macaene') and n-benzyl-5-oxo-6E,8E-octadecadienamide ('macamide-2'). These four macamides are the most prominent in maca. Others are either minor in concentration or not specific to the maca plant or unstable. The peaks were assigned by a comparison of retention time and UV-spectra. Macaene and macamides standard compounds were isolated at NCNPR, their identity and purity was confirmed by chromatographic (TLC, HPLC) methods, by the analysis of the spectral data (IR, 1D- and 2D-NMR, HR-ESI-MS) and through comparison with published spectral data (Zhao et al., 2005). Waters Empower software (Milford, MA) was used for data collection and analysis. The calculation of total macamides comprised the results of macamides being below of < 0.1 μmol g^{-1} DM as well.

Phytosterol Analysis

A reversed-phase HPLC (Waters Alliance Model 2695, Milford, MA) was used which was equipped with a Sedex 75 ELS detector (SEDERE, Alfortille, France) and a Luna C8 column (150 by 4.6 mm; 5 μm particle size; Phenomenex, Torrance, CA). Temperature was maintained at 30°C. The mobile phase consisted of water (A) and methanol (B) used at a flow rate of 1.0 mL min^{-1}. The isocratic elution was 10% A/90% B in 20 min. This was followed by washing with 100% methanol for 5 min and re-equilibration with 10% A/90% B for 15 min. Probe temperature of the ELS detector was set to 40°C and a gain of 9. The nebulizer gas N adjusted to 3.5 bar. The chromatogram of the standard and of prepared samples showed that the peaks of interest were separated from each other and that there were no interfering peaks. The calibration curve was correlated in a linear manner between concentration and peak area ($r^2 > 0.999$) over the injected concentration of standard compounds in the ranges between 10 and 1000 μg mL^{-1}. Accuracy (% RSD) of the obtained results fell well within the predefined limits of acceptability (< 5.0%). The limits of detection and quantification obtained for a signal-to-noise ratio of 3 and 10, respectively, were 3 and 10 μg mL^{-1} for campesterol and β-sitosterol when using calibration graphs. Peaks were assigned to campesterol and β-sitosterol by comparing retention time and spiking with campesterol (Chromadex, Santa Ana, CA) and β-sitosterol standards (Sigma, St. Louis, MO). Waters Empower software (Milford, MA) was used for data collection and analysis.

Glucosinolate Analysis

Based on the ISO certified method (ISO 9167–1) to determine GL in canola seed (ISO, 1995), which includes internal standards and determination of response factors, GL were extracted and analyzed in duplicate by HPLC applying small modifications. Briefly, samples of 0.3 g of the hypocotyls were weighed into centrifugation tubes. The GL sinigrin (20 mmol L^{-1}; Fluka, Buchs, Switzerland) was added as an internal standard at an amount of 0.2 mL. The extracts were transformed into eluates of desulpho-GL according to ISO 9167–1 (ISO, 1995). The eluate was thoroughly mixed before being used for the subsequent chromatography with the same HPLC as applied for phytosterol analysis. Different from that, a variable UV detector was used and set to 229 nm and a lichrosorb standard column (SunFire C18, 5 μm, 4.6- by 150-mm column, Waters, Milford, MA) was inserted. The elution with purified water (step A) was done for 1 min before switching to the second mobile phase (acetonitrile in water, 20% (v/v). Step B included a linear gradient starting with 0% B, reaching 100% B after 20 min, returning to 0% B after 5 min, followed by another 5 min eluting with A to reach the equilibrium. For peak identification, a sample of standard canola seed (ERM- BC367, European Reference Materials, Geel, Belgium) was analyzed by the same method. Gluconasturtiin (2-phenylethyl GL), glucoaubrietin (4-methoxybenzyl GL) (Johns 1981; Li et al., 2001), and glucolimnanthin (3-methoxybenzyl GL) (Dini et al., 1994; Piacente et al., 2002) are eluated at the same peak site. Despite this correspondence with time and the fact that *Lepidium sativum* contains gluconasturtiin as well (Fahey et al., 2001), a noncorrespondence in the wavelength chromatogram with the gluconasturtiin standard peak basically excludes that the peak was gluconasturtiin. On the basis of our analysis and equipment, it was not possible to definitely assign results to either 3- or 4-methoxy benzyl GL (glucoaubrietin or glucolimnanthin). The GL that were consistently showing results < 0.1μmol g^{-1} DM were considered as trace and were consequently not statistically evaluated for treatment effects but were still included in the calculation of total GL.

Statistical Analysis

The SAS procedure MIXED (Version 9.1.3 for Windows from 2005, SAS Institute Inc., Cary, NC) was used for analysis of variance applying the following models:

$$\text{Model 1: } y_{ij} = \mu + \alpha_i + \varepsilon_{ij};$$

$$\text{Model 2: } y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + \varepsilon_{ijkl};$$

where α = color type, β = cultivation history, γ = site were fixed effects and ε = residual error. The interaction αγ was considered fixed, whereas the interaction βγ was considered random due to the low number of repetitions of cultivation history and site. Model 1 was used to test color type effects within area while the second model, based on a split-plot design, considered the four areas (interaction βγ) as main unit and tested the effects within and between the areas. After previous testing of all interactions, αβ and αβγ were excluded as they were almost never significant. Multiple comparisons among color type means were performed and adjusted with Tukey's method. The tables give LSmeans, standard errors of the mean (SEM) and *P* values for color type, cultivation history, and site. Pearson correlation coefficients were calculated between maca yield and concentrations of secondary metabolites as well as among concentrations of different secondary metabolites.

RESULTS

Differences between soils from the two sites and between areas were quite large as far as cation exchange capacity is concerned (Table 1). The variation in the other traits was lower. Generally, changes in soil characteristics occurring during the experiment were quite low.

Total yield and weight of individual hypocotyls were mostly affected by site (low at Patalá compared to Alpacayán; Table 2). Additionally there were some differences between color types, with yellow maca showing a trend for relatively higher yield except for the previously cultivated area at Patalá. Though not significant, yield was higher on the previously cultivated area than on the previously never cultivated areas in six out of the eight cases.

The maca hypocotyls were characterized by substantial proportions of starch and sugars (Table 2). Sugars were represented mostly by sucrose with on average 816 mg g^{-1} total sugars, followed by glucose and fructose with 109 and 75 mg g^{-1}, respectively; data not shown in table). Protein concentrations varied from about 150 to 250 mg g^{-1} DM. In general there were only few significant color type effects. One prominent example was the sugar concentration of maca harvested at Patalá from the previously never cultivated area (low in yellow maca, high in violet and lead-colored maca, intermediate in pink maca). This difference did not occur on the previously cultivated area and at the other planting site, there were interactions ($P < 0.1$) between color and cultivation history only at that site. Differences in nutrient concentrations between hypocotyls harvested from different sites were not significant, but there was a certain trend that maca at the Patalá site was higher in crude protein and lower in sugar (color × site interaction, $P < 0.1$) and starch concentration.

Concerning macaene and macamides, color effects occasionally were significant, especially at the Alpacayán site (Table 3). Levels showed a trend to be higher with reddish colored maca (pink, violet) than in yellow and lead-colored maca, particularly at Alpacayán Effects of site were present in macamides only, whereas color × site interactions were pronounced for macaene and macamides, showing a different color ranking of the concentrations of these metabolites at the two sites. Maca hypocotyls from Patalá were low in macamides compared to those from Alpacayán where, on average across colors and areas, levels were higher by factors of 5 and 13 for total macamides. Only two macamides, n-benzyl-palmitamide and n-benzyl-5-oxo-6E,8E-octadecadienamide, were found to be above the detection limit, but not n-benzyl-9-oxo-12Z-octodecenamide and n-(m-methoxybenzyl)-hexadecanamide. Concerning the phytosterols, campesterol did not differ in maca originating from the two different sites, but was affected by color at Alpacayán similarly to that of β-sitosterol. However, the latter was also higher in maca from Alpacayán than from Patalá. In both phytosterols a color × site interaction was found.

Maca from Patalá contained more total (3.3-fold) and more of most individual GL than at Alpacayán (Table 4). Cultivation history was not significant for any GL but showed a trend to be higher in concentration in the never cultivated area principally in pink (glucosinalbin, total alkylthioalkyl GL) and violet maca (glucoalyssin, total alkylthioalkyl GL). Additionally, there were frequent color type effects. Accordingly, the concentration of many GL was particularly high in lead-colored maca in Patalá, whereas in Alpacayán the yellow maca was poor in GL. The order of maca of the other colors varied between GL and also within GL but between areas. The effects of the experimental factors became less clear with GL (glucoraphanin and total olefine GL) present only in small concentrations. Further representatives of the total of 13 GL found included glucobrassicin, glucoiberin, progoitrin, epigoitrin, gluconapoleiferin, gluconapin,

Table 1. Soil characteristics at the two experimental sites, including cultivation history (cultivated or never cultivated) and time in relation to the experiment (before or after).

	Patalá				Alpacayán			
	Never cultivated		Cultivated†		Never cultivated		Cultivated†	
	Before	After‡	Before	After	Before	After	Before	After
Texture								
Sand, g 100 g^{-1}	78	–§	68	–	74	62	70	56
Silt, g 100 g^{-1}	20	–	24	–	22	32	26	30
Clay, g 100 g^{-1}	2	–	8	–	4	6	4	14
Organic matter, g 100 g^{-1}	1.94	1.45	1.76	1.57	0.45	0.48	0.46	0.44
Available P, mg 100 g^{-1}	0.99	0.79	1.28	0.79	3.02	3.36	3.31	2.41
Available K, mg 100 g^{-1}	20.9	5.8	9.1	6.2	6.4	5.7	4.6	3.4
pH	4.8	4.7	5.0	5.1	5.0	4.4	5.0	5.0
Electrical conductivity, dS m^{-1}	0.13	0.07	0.18	0.14	0.13	0.10	0.18	0.09
Cation exchange capacity, meq 100 g^{-1}	54.7	37.6	68.0	57.6	14.4	12.0	17.6	12.5
Exchangeable ions, meq 100 g^{-1}								
Ca^{+2}	4.80	2.54	3.90	17.64	1.37	2.76	5.19	6.08
Mg^{+2}	1.49	0.46	1.39	0.85	0.27	0.55	0.33	0.58
K^{+}	0.48	0.16	0.22	0.18	0.13	0.44	0.07	0.37
Na^{+}	0.56	0.53	0.22	0.45	0.21	0.16	0.10	0.18
Al^{3+} +H^{+}	1.50	1.00	0.50	0.40	1.60	2.40	0.60	0.90
Base saturation, %	13	11	8	33	14	33	32	58

† Cultivated last 2 to 3 yr ago with maca followed by a fallow period.

‡ Before is equivalent to pre-tillering, after is equivalent to postharvest.

§ Missing data.

Table 2. Effect of color type (C; Y = yellow, P = pink, V = violet, L = lead-colored), type of cultivation history [area (A): –, previously never cultivated; +, cultivated 2–3 yr ago with maca] and site (S, Patalá/Alpacayán) on yield of and nutrient concentrations in maca hypocotyls.†

		Patalá					Alpacayán						Overall P§				
		Color				P‡	Color				P‡						
	A	Y	P	V	L	C	Y	P	V	L	C	SEM§	C	A	S	CS	AS
		Yield, g wet weight															
Total per plot	–	381	135	188	188		1522a	283b	862ab	827ab	*	149.6	*			¶	
	+	130	76	520	351		1894	1019	1251	1187							
Hypocotyl weight	–	7.0	3.4	3.2	4.1		25.4	17.8	25.1	16.4		2.04			¶		
	+	4.7	2.3	9.7	8.8		31.2	26.7	24.1	28.9							
		Nutrients, mg g^{-1} dry matter															
Starch	–	198	187	178	245		300	237	259	232		14.9					
	+	283	256	316	247		309	276	281	292							
Sugars	–	211c	295a	260b	317a	***	273	292	280	284		7.9	**			¶	
	+	303	305	252	283		293	287	309	319	¶						
Crude protein	–	235	238	253	244		159b	186a	174ab	195a	**	13.7					
	+	182	181	171	181		157	181	156	164							
Total ash	–	52	51	44	38		41a	47b	42ab	43ab	*	1.9					
	+	36	39	40	40		37	44	44	41	¶						

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† LSMeans within area carrying no common superscript are significantly different ($P < 0.05$). CS = interaction of C and S; AS = interaction of A and S. Standard errors of the mean (SEM) refer to Model 2.

‡ *P* values applying Model 1 (cf. Materials and Methods).

§ *P* values and SEM applying Model 2 (cf. Materials and Methods).

¶ $P < 0.1$ (approaching significance).

Table 3. Effect of color type (C; Y = yellow, P = pink, V = violet, L = lead-colored), type of cultivation history [area (A): –, previously never cultivated; +, cultivated 2–3 yr ago with maca) and site (S, Patalá/Alpacayán) on the concentrations of macaene, macamides, and phytosterols in maca hypocotyls.†

		Patalá					Alpacayán						Overall P§				
		Color				P‡	Color				P‡						
	A	Y	P	V	L	C	Y	P	V	L	C	SEM	C	A	S	CS	AS
		μmol g^{-1} dry matter															
Macaene¶	–	0.34	0.30	0.17	0.44		0.83	1.91	1.48	0.80		0.198	**			***	
	+	0.27	0.37	0.07	0.28		0.83b	2.67a	1.78ab	1.73ab	*						
Macamide 1#	–	0.10	0.07	0.06	0.02		0.66	1.01	0.81	0.47		0.084	*			*	
	+	0.07	0.14	ND††	0.06		0.37	0.88	0.91	0.69							
Macamide 2‡‡	–	ND	ND	ND	ND		0.46	0.97	0.74	0.44		0.083	**		§§	**	
	+	0.06	0.11	ND	ND		0.22b	0.98a	0.96ab	0.77ab	*						
Total macamides	–	0.10	0.07	0.06	0.20		1.12	1.98	1.55	0.90		0.146	**		*	**	
	+	0.13	0.25	ND	0.06		0.59	1.86	1.87	1.46	§§						
Campesterol	–	0.20	0.20	0.22	0.26		0.11b	0.22a	0.17ab	0.13ab	*	0.025	§§			*	
	+	0.17	0.16	0.07	0.14		0.10	0.21	0.16	0.16							
β-sitosterol	–	0.26	0.19	0.28	0.28		0.27	0.43	0.37	0.21		0.064				*	
	+	0.10	0.04	0.03	0.17		0.20	0.41	0.43	0.46	*						

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† LSMeans within area carrying no common superscript are significantly different ($P < 0.05$). CS = interaction of C and S; AS = interaction of A and S. Standard errors of the mean (SEM) refer to Model 2.

‡ *P* values applying Model 1 (cf. Materials and Methods).

§ *P* values and SEM applying Model 2 (cf. Materials and Methods).

¶ 5-oxo-6E,8E-octadecadienoic acid.

N-benzyl-palmitamide.

†† Not detected.

‡‡ N-benzyl-5-oxo-6E,8E-octadecadienamide.

§§ $P < 0.1$ (approaching significance).

and glucobrassicanapin, but these GL were only present in trace amounts (< 0.1 μmol g^{-1} DM). The effect of cultivation history was not significant for any of the secondary metabolites.

The correlations between maca yield (total hypocotyls and average hypocotyl size) and concentrations of many constituents were significant (Table 5). Frequently, this was the result of the large yield differences between sites which, in the secondary metabolites, resulted in smaller or even contrary correlations within the two experimental sites. Correlations between GL and the other secondary metabolites analyzed (except campesterol), were significant and negative, whereas significant positive correlations were found between macaene, macamides, and β-sitosterol (Table 6). These correlations were mostly similar when considering only the Alpacayán site, but often smaller or absent at Patalá.

DISCUSSION

Level and Profile of Nutrients and Secondary Metabolites

Concentrations of DM and ash were comparable to those reported by Dini et al. (1994) for maca hypocotyls, whereas crude protein concentrations were higher in the present study. The average concentrations of macaene and total macamides ranged from 0.17 to 2.67 and 0 to 1.98 μmol g^{-1} DM, respectively. The values reported by Ganzera et al. (2002) and McCollom et al. (2005) (only macamides) fell within these ranges. The concentration of total GL (1.4–46.3 μmol g^{-1} DM) was similar to that reported by Clément et al. (2007), while campesterol (0.07–0.26 μmol g^{-1} DM) and β-sitosterol (0.03–0.46 μmol g^{-1} DM) were lower than analyzed in that study. The GL profile of the maca hypocotyls roughly resembled that described in Clément et al. (2007).

Macaene and macamides, compounds originating from the same biochemical pathway, were closely correlated in concentration. The correlations were also high with β-sitosterol, but not with campesterol. Nevertheless, there was a positive correlation between these two phytosterols. This was partially consistent with Amar et al. (2008) but not with Hamama and Starner (2003).

Effect of Color Type

It seems that maca has been selected for color for quite some time by farmers and plant breeders as the large number of distinct color types demonstrates. Still, the majority of the producers cultivate maca having different colors together, possibly to reduce the risk of losses. It is important to note that color associations are sometimes inaccurate or misleading in publications. Differentiation is particularly difficult between black and lead-colored maca and for reddish maca (there is not one single red maca, but pinkish and violet color types). When selecting from the accessions held at the germplasm collection we therefore carefully looked for seeds from plants with unmistakably yellow, pink, violet, and lead-colored hypocotyls.

There were no really consistent color type effects on either maca yield or the size of the hypocotyls across all environmental conditions. If any, there was a slight advantage for the yellow maca. The obvious color pigments (most likely anthocyanins) are present only in the thin outer layer of the hypocotyls (Quirós and Aliaga Cárdenas, 1997), except in yellow maca where also the inner part is yellowish and not plain white as in the other color types.

Consumers prefer some color types over others. Sweetness and size are important (Quirós and Aliaga Cárdenas, 1997). Yellow maca appears to have a competitive advantage with Peruvian consumers (Quirós and Aliaga Cárdenas, 1997), but in the present experiment hypocotyls of this color type were not larger and even contained less sugar than other color types. Apart from that, maca of different color type was similar in gross nutrient composition. Accordingly, size rather than color seems to determine its sugar concentrations as more sugar occurred in smaller hypocotyls.

Overall, pink maca was richest in macaene and macamides, especially in relation to yellow maca. This was different in the study of Clément et al. (2007) where yellow maca was richest in macaene and violet maca had the highest concentration of macamide-1. Concerning the phytosterols investigated, there was only a trend ($P < 0.1$) of a color effect in campesterol and no effect in β-sitosterol. This indicates that variation caused by color type might have been smaller in the phytosterols than in other secondary metabolites. Under unfavorable growth conditions (Patalá), lead-colored maca hypocotyls were clearly richer in total GL, aromatic GL, and indole GL than those of yellow, pink, and violet maca. The present study confirms the low GL concentration of yellow maca, compared to pink (red) and violet maca, as reported by Clément et al. (2007). Another obvious color difference was that violet maca was particularly rich in alkylthioalkyl and olefine (especially glucoalyssin) GL.

The clear differences on secondary metabolite concentrations in the color types studied support findings of differences in biological effects among color types of maca consumed by man and animal. Since lead-colored maca differed from the other color types (at least in GL), while differences were found with plain black maca earlier (Clément et al., 2007), it seems that the favored black maca (Gonzales et al., 2006a, 2006b, 2007) was probably actually lead-colored. Still, the active principles responsible for maca's bioactivity are not yet identified. Due to the lack of clear breeding goals, the question also remains whether distinct genetic color type differences have developed that go beyond the color itself. The anthocyanins as such are not likely to make up a significant proportion of the entire maca hypocotyls, and were shown to be largely destroyed (up to −85%) even by simple air-drying (Kwok et al., 2004). Furthermore, flavonoides (anthocyanins) are resulting from different biosynthetic pathways (Grisebach, 1982; Wittstock and Halkier, 2002) than macaene/macamides (i.e., polyunsaturated fatty acid/amides), phytosterols, and GL (i.e., sulfur-containing compounds). Still the repeated observation of major color type differences in secondary metabolite concentration suggests that color is not just a casual attribute that sometimes results in different concentration of secondary metabolites. It could be that the genes responsible for traits of color, macaene/macamides, and GL are associated (e.g., located on the same chromosome). Mapping of these genes in maca would help to clarify this assumption.

Effect of the Cultivation History

Cultivation history (either never cultivated or cultivated with maca 2–3 yr ago followed by a fallow period), had no significant influence on secondary metabolites and on nutrient composition though some trends were obvious. If real, such trends might be explained by a lower plant diseases pressure on previously never cultivated land compared to land with a previous maca monoculture, or by soil nutrient depletion or changes in other properties caused by previous cultivation. The most obvious difference in the soils was the higher sum of cations (mostly due to Ca^{2+}) and bases as well as the higher base saturation in the previously cultivated

Table 4. Effect of color type (C: Y = yellow, P = pink, V = violet, L = lead-colored), type of cultivation history [area (A): –, previously never cultivated; +, cultivated 2–3 yr ago with maca] and site (S, Patalá/Alpacayán) on the on the concentrations of glucosinolates (GL) in maca hypocotyls.†

		Patalá					Alpacayán						Overall P§				
		Color				P‡	Color				P‡						
	A	Y	P	V	L	C	Y	P	V	L	C	SEM	C	A	S	CS	AS
		μmol g^{-1} dry matter															
Total GL	–	18.73b	29.37ab	21.72b	45.22a	**	1.67	4.72	3.89	3.32	¶	1.717	***		*	***	
	+	27.31ab	23.67b	30.79ab	46.29a	*	1.36	2.23	2.68	2.88							
Total aromatic GL	–	18.10b	28.27ab	17.75b	44.23a	**	1.21	4.11	2.87	2.89		1.749	***		¶	***	
	+	26.60ab	22.61b	28.30ab	45.35a	*	1.07	1.75	1.81	2.37							
Glucotropaeolin	–	15.01b	23.75b	24.44b	40.71a	**	0.84	2.87	2.21	2.12		1.743	***		¶	***	
	+	23.47ab	19.82b	25.64ab	41.64a	*	0.74	1.10	1.30	1.72							
Glucolimnathin#	–	1.65	2.06	1.83	1.99		0.21	0.83	0.46	0.44	*	0.106	¶		*		
	+	1.89	1.75	1.79	2.41		0.17	0.41	0.32	0.38							
Glucosinalbin	–	1.44	2.50	1.48	1.52	¶	0.17	0.43	0.20	0.34	¶	0.176	*				
	+	1.24	1.03	0.87	1.30		0.16	0.24	0.19	0.27							
Total indole GL	–	0.12b	0.15b	0.19b	0.27a	***	0.03	0.06	0.04	0.05	¶	0.012	***		¶	***	
	+	0.15	0.17	0.25	0.26	*	0.02	0.02	0.03	0.04							
4-hydroxy-glucobrassicin	–	0.09b	0.12b	0.16ab	0.22a	**	0.02	0.04	0.04	0.03		0.008	***		¶	***	
	+	0.11b	0.13ab	0.21a	0.20a	*	0.02	0.01	0.02	0.02							
Tot. alkylthio-alkyl GL	–	0.35b	0.79b	3.32a	0.52b	***	0.18b	0.36b	0.81a	0.23b	***	0.074	***		¶	***	
	+	0.42b	0.71b	2.00a	0.49b	***	0.12	0.29	0.44	0.20							
Glucoalyssin	–	0.25b	0.60b	3.22a	0.39b	***	0.05b	0.21b	0.71a	0.12b		0.073	***		¶	***	
	+	0.30b	0.58b	1.88a	0.40b	***	0.05	0.20	0.33	0.08							
Glucoraphanin	–	0.07	0.15	0.06	0.08		0.10	0.11	0.06	0.08		0.013					
	+	0.08	0.10	0.08	0.06		0.05	0.06	0.08	0.09							
Total olefine GL	–	0.16	0.17	0.46	0.20		0.25	0.20	0.17	0.16		0.043	*				
	+	0.14b	0.18ab	0.24a	0.19ab	**	0.16	0.17	0.41	0.27							

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† LSMeans within area carrying no common superscript are significantly different ($P < 0.05$). CS = interaction of C and S; AS = interaction of A and S. Standard errors of the mean (SEM) refer to Model 2.

‡ *P* values applying Model 1 (cf. Materials and Methods).

§ *P* values and SEM applying Model 2 (cf. Materials and Methods).

¶ $P < 0.1$ (approaching significance).

Alternatively: glucoaubrietin.

area compared to the never cultivated area. These differences are likely due to the higher soil clay concentration in the selected cultivated areas, as clay is typically rich in micronutrients, and probably not the result of previous cultivation. Soil nutrients (e.g., the concentration of exchangeable cations like Ca^{2+}) may play a major role for hypocotyl yield and size which showed a trend to be higher on the areas that were previously cultivated.

In summary, the present results do not support the statements recommending a fallow period after cultivating maca going beyond 2 to 3 yr (Quirós and Aliaga Cardenas 1997; Chacón de Popovici, 2001; Córdova Herrera, 2003; Humala-Tasso and Combelles, 2007). Especially plant health does not seem to have been compromised on the area previously cultivated with maca. This could be different, when areas were cultivated with maca again in the same or in the following year.

Effect of Growing Site

The site had a significant effect on the concentrations macamides and GL. Though not always significant, the favorable growing conditions at Alpacayán seem to have led to higher concentrations of the maca specific compounds (macaene, macamides) while, at the same time, the GL were significantly lower concentrated than in maca from Patalá. Site effect was largely overriding the effect of cultivation history. Thus site was probably a major factor explaining the differences noted in hypocotyls from different producers (Clément et al., 2007). It seems that the site effects either favor the macaene/macamides/β-sitosterol group or the GL, but not both at the same time. Accordingly, site was specifically responsible for the close correlations of maca yield with secondary metabolites, while this was different for nutrient concentrations.

Variations among sites included several environmental factors, especially soil characteristics and climatic conditions. Effects of pest attacks on metabolite concentration were excluded in this

Table 5. Pearson correlation coefficients between growth yield and the concentrations of nutrients and secondary metabolites of the hypocotyls (*n* = 55, 28 and 27 for total, Patalá, and Alpacayán).

	Total hypocotyl yield			Average hypocotyl weight		
	Total	Patalá	Alpacayán	Total	Patalá	Alpacayán
Starch†	0.65***	0.46*	0.70***	0.60***	0.47	–0.60***
Sugar†	–0.15	–0.43*	–0.39*	–0.06	–0.36*	–0.43*
Crude protein	–0.60***	–0.43*	–0.48**	–0.71***	–0.61**	–0.44*
Total glucosinolates	–0.40**	0.28	–0.16	–0.47***	0.31	–0.10
Macaene	0.29*	–0.47*	–0.17	0.50***	–0.48**	–0.01
Total macamides	0.27*	–0.36‡	–0.37‡	0.50***	–0.41*	–0.25
Campesterol	–0.34*	–0.42*	–0.24	–0.25‡	–0.50**	0.05
β-sitosterol	0.22	–0.17	–0.03	0.40**	–0.24	0.29

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† Total no. of observations = 60.

‡ $P < 0.1$ (approaching significance).

study by not considering damaged hypocotyls. The soils of Patalá and Alpacayán were both acidic and Patalá had higher OM. Available P level was threefold higher at Alpacayán than at Patalà, but was still sufficiently high at the latter site to exclude P as a major limiting factor. Available K, being low at both sites, was twofold higher at Patalà that at Alpacayán, and cation exchange capacity was even more than threefold higher at Patalá. Despite these differences, it seems likely that soil characteristics were not the only determinant of the overall "site effect". It was not possible to measure microclimate due to technical problems, but this could have been relevant since the two sites were 217 km apart (air-line distance), and landscape also differed. Alpacayán was situated in a plain around the lake Junín, and the Patalá site was in a mountainous area. Temperature at 4100 m a.s.l. was probably mostly low at both sites, but precipitation might have differed contributing to differences in growth yield and composition. Additionally, the Patalá planting site experienced partly flooding. The higher soil OM at Patalá is consistent with poor drainage. Whatever the reason, maca plants at Patalá suffered from environmental stress while this was not the case at Alpacayán. Stress is known to have a major effect on secondary metabolites as for instance shown in tannin concentrations and properties (compiled in Tiemann 2008). Different from that, Hamama and Starner (2003) found no influence of the growing site on phytosterol concentrations in rapeseed, and Amar et al. (2008) described a high heritability but a low environment impact on phytosterols. The latter is consistent with the present results for campesterol but not for β-sitosterol.

A major response to site, and therefore environmental stress, as found in the present study, seemed to be related to increased GL concentrations. Consistent with the present results, Velasco et al. (2007) found that olefine GL (called aliphatic GL by them) in kale (*Brassica oleracea acephala*), another member of the Brassicaceae family, are less susceptible than indole GL to environmental variations. However, considering the higher yield at the Alpacayán site, the lower GL concentration at that site might also simply have been an effect of dilution by the increasing amount of nutrients (carbohydrates) incorporated into the hypocotyls. By contrast, macaene and macamide synthesis was widely downregulated at Patalá like in the study of Melnikovova et al. (2008) where unsuitable environmental conditions (lowland) likely caused a similar response. This suggests that a high concentration of these specific maca constituents requires conditions optimal for the growth of this plant (e.g., high altitude).

Interactions Among Factors of Influence

Color and site effects were often significant and their interaction was significant for almost all secondary metabolites. This highlights that color differences were expressed differently at the two sites. At Patalá, the lead-colored maca was richer in GL while at Alpacayán yellow maca was particularly poor in GL. Pink maca was richer in macaene and macamides at Alpacayán while no clear dominant maca color could be distinguished at Patalá. Obviously, site conditions provided room for the expression of color differences at both sites, but these were not very systematic across variables. In contrast, the interactions of cultivation history × color and cultivation history × site were never significant. Considering also the magnitude of the effects of color and site, it seems that the prediction of differences caused by distinct main factors is mostly not possible without considering interactions among these factors.

Table 6. Pearson correlation coefficients between concentrations of different secondary metabolites in the hypocotyls (n = 55, 28 and 27 for total, Patalá [P] and Alpacayán [A]).

	Total macamides			Macaene			Campestrol			β-sitosterol		
	Total	P	A	Total	P	A	Total	P	A	Total	P	A
Total glucosinolates	–0.70 ***	–038 *	–0.40 *	–0.60 ***	0.04	–0.40 *	–0.03	–0.11	–0.29	–0.50 ***	–0.10	–0.46 *
Total macamides				0.92 ***	0.58 **	0.84 ***	0.03	0.03	0.48 *	0.60 ***	–0.09	0.62 ***
Macaene							0.17	0.42	0.59 **	0.62 ***	0.24	0.62 ***
Campesterol										0.41 **	0.45 *	0.74 ***

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

CONCLUSIONS

The present study demonstrated that maca is far from being a standardized product and has no constant composition concerning its potentially bioactive components. The presence of differences among color types claimed previously could be confirmed. Additionally, the large residual "farm" effect described by Clément et al. (2007) could be resolved to be mainly a site effect. Further changes can be expected from processing inclusive of heating (Clément et al., 2007). Therefore, at least larger batches of maca to be sold on the market or designed for export should be analyzed for their concentration. This should not only include macaene and macamides, which are already established quality markers (Zheng et al., 2000), but also total glucosinolates and maybe selected phytosterols. These groups of secondary metabolites were responding not always in the same way to the factors of influence investigated as macaene and macamides. The active principle(s) in maca with respect to human fertility and health still await identification.

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