Research Article

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Influence of colour type and previous cultivation on secondary metabolites in hypocotyls and leaves of maca (*Lepidium meyenii* Walpers)

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Abstract

BACKGROUND: Maca is an Andean crop of the Brassicaceae family which is mainly known for its fertility-enhancing properties following consumption. The hypocotyls display various colours ranging from white to black. Each colour has different biological effects. The aim of this study was to analyse the concentrations of major secondary metabolites in hypocotyls and leaves of maca in a controlled planting experiment in the Peruvian Andes at 4130 m above sea level. The effects of colour type and of previous cultivation of the field were examined.

RESULTS: In the hypocotyls, the colour type effect was significant for most secondary metabolites; exceptions were β-sitosterol and campesterol. The lead-coloured, yellow and violet maca hypocotyls were rich in glucosinolates, macaene and macamides, respectively. Previous cultivation affected macaene, campesterol and indole glucosinolate concentrations. Effects on metabolite concentrations in the leaves were minor. Hypocotyls were richer in macaene, macamides and glucosinolates than were leaves, and were poorer in β-sitosterol and total phenols.

CONCLUSION: Colour type has to be considered in maca production, as colour associates with variations in concentrations of distinct bioactive metabolites. Leaves may be interesting for animal nutrition purposes as they contain essentially the same secondary metabolites as the hypocotyls but in clearly lower concentrations.

Keywords: *Lepidium meyenii*; glucosinolate; macamide; macaene; phytosterol

INTRODUCTION

Plants' secondary metabolites are gaining in interest owing to their presumed health benefits in human nutrition. Among the many plants rich in these metabolites, maca (*Lepidium meyenii* Walpers; sometimes called *Lepidium peruvianum* Chacón), a traditional Andean crop and a member of the Brassicaceae family, is viewed as particularly promising[1] because of its potential fertility-related effects.[2] The compounds of interest in maca, primarily macaene and macamides, are specific to this plant, but maca also contains other bioactive compounds such as phytosterols like campesterol and β-sitosterol, as well as a range of glucosinolates (GL). These secondary metabolites alone or in certain combinations may explain the exhibition of biological effects attributed to the consumption of maca.[2]

Maca is predominantly cultivated for its hypocotyls and seeds. The hypocotyls, which are the edible part of the plant, can be found in a variety of different colours ranging from cream-coloured through yellow, pink, violet and lead-coloured to black. The differently coloured hypocotyls seem also to correlate with differences in concentrations of secondary metabolites[3] and thus in their biological effectiveness. For instance, beneficial effects on spermatogenesis appear to be more pronounced with black maca compared to reddish and yellow maca,[4,5] and a reduction of the ventral prostate size in rats has been reported as a response to reddish maca.[6] The leaves of maca are usually left on the field after

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harvest of the hypocotyls. However, dried leaves are sometimes fed to livestock and guinea-pigs. If leaves can be shown to have similar beneficial biological effects to those seen for hypocotyls, maca leaves may prove to be a valuable supplementary feed for livestock.

Maca grows best at altitudes between 3500 and 4500 m above sea level (a.s.l.). When cultivated at lower altitudes it no longer contains significant amounts of macamides.[7] The traditional cultivation area is situated on a plateau at 4200 m a.s.l. around Lake Chinchaycocha (Lake Junín) in Peru. In 1998, the demand for maca increased considerably,[1] resulting in new cultivated areas in other high-altitude regions such as Cusco and the Lake Titicaca area.[8,9] For this reason, maca can be found repeatedly planted in the same soils, sometimes in rotation with different crops and occasionally with fertilization.[9] Alternatively, in response to the new demand, it may appear in soils in which it had not been previously cultivated. These differences in soil features may result in differences in maca quality, as its cultivation is generally perceived as a great exploitation of the soil resources,[9] and quite long fallow periods of 4 years,[10] or even 8–10 years and more,[8,9,11] are recommended. The producers' experience, as reported by Humala-Tasso and Combelles,[9] is that the sensory quality of maca (form, flavour before and after cooking, absence of cavities) is higher when maca is produced on newly cultivated terrains. This has not yet been scientifically confirmed; however, maca batches from different producers have been observed to vary significantly in the concentrations of its characteristic constituents,[3,12,13] suggesting that at least part of the variation is due to environmental factors.[3]

In the present study, an extensive planting experiment was carried out in the Peruvian Andes to test the hypothesis that hypocotyl colour type and previous form of cultivation have an effect on the level of major secondary metabolites found in maca hypocotyls and leaves. Additionally, it was examined whether these two factors of influence interact.

MATERIAL AND METHODS

Fields and seeds

As an experimental site, Alpacayán (latitude 10° 92′ 13″ S, longitude 76° 05′ 75″ W, altitude 4130 m a.s.l.), department of Pasco, Peru, was chosen as it is situated in an area traditionally used for maca cultivation. Two terrains were selected, one which had never been cultivated before with any crop and one that had been cultivated with maca 2 years previously, followed by a fallow period. The distance between the two terrains was 40 m, which ensured that the microclimates and soils were similar. The two terrains were tilled first with a plough and then with a hoe, and fenced in November. Fields were not fertilized and not irrigated, following common practice. Soil samples were collected twice on the two experimental terrains: once before tilling in October and again directly after harvest in July of the following year. Twenty subsamples, each comprising the soil without roots, were taken (after removal of the thin grass-containing layer) from an approximately 3–20 cm depth, following a zigzag gradient across each terrain. The subsamples were homogenized to one composite sample per terrain and date (total $n = 4$). The analysed soil characteristics of the two terrains are described in Table 1.

Maca seeds of four different colour types (yellow, collection number CR95S2-22-4; pink, RO95S2-306-8; violet, MOS2-142-1; lead-coloured, PLS2-284-20) were obtained from the germplasm collection of one of the authors (DD Ponce Aguirre, UNDAC, Cerro de Pasco, Pasco, Peru). This collection had been established in a program carried out over several years. At first hypocotyls of yellow, pink, violet and lead had been selected from different land races on the basis of their colour. As maca reproduces autogamously and is partially cleistogamous,[8] it was assumed that this parent material was already quite homozygous. These hypocotyls were transplanted to separated plough furrows to produce seeds in a strictly autogamous way. This procedure was followed over two generations (S1 and S2) in order to further increase homozygosity. The seeds used in the present planting experiment originated from one plant per colour of this second generation of auto-fertilized seeds. The seeds were cleaned with water, and stored for 24 h in a gibberellic acid solution. They were then placed on Petri dishes and allowed to germinate in a germinator (G30, Conviron, Winnipeg, Canada) for 1 week at 18 °C at the Centro Internacional de la Papa (CIP) in Lima. The germinated seeds were transplanted into jiffy pellets (diameter 42 cm; Jiffy Products (NB) Ltd, Shippagan, Canada) and grown for 1 month in a greenhouse (19 ± 1 °C; $80\% \pm 12\%$ humidity). In December, the plantlets were transported to the experimental sites and planted into the soil.

Table 1. Soil characteristics

Time in relation to experiment	Never cultivated		Cultivated with maca 2–3 years ago	
	Before	After	Before	After
Texture				
Sand (g g^{-1})	0.74	0.62	0.70	0.56
Silt (g g^{-1})	0.22	0.32	0.26	0.30
Clay (g g^{-1})	0.04	0.06	0.04	0.14
Organic matter (g kg^{-1})	4.5	4.8	4.6	4.4
Available P (mg kg^{-1})	30.2	33.6	33.1	24.1
Available K (mg kg^{-1})	64	57	46	34
pH	5.0	4.4	5.0	5.0
Electrical conductivity (dS m^{-1})	0.13	0.10	0.18	0.09
Cation exchange capacity (meq kg^{-1})	144	120	176	125
Exchangeable ions (meq kg^{-1})				
Ca^{2+}	13.7	27.6	51.9	60.8
Mg^{2+}	2.7	5.5	3.3	5.8
K^{+}	1.3	4.4	0.7	3.7
Na^{+}	2.1	1.6	1.0	1.8
$Al^{3+} + H^{+}$	16.0	24.0	6.0	9.0
Base saturation (%)	14	33	32	58

Experimental design

Terrains were divided into 16 plots 1 m apart, giving four replicates per maca colour and terrain. Each plot consisted of four rows (with 50 cm spacing between the rows) containing 25 plants per row (20 cm spacing between plants). Overall this resulted in $2 \times 4 \times 4 = 32$ plots and required 3200 individual plantlets. The plots were randomly arranged for colour type on the terrains. The harvest took place in July of the second year at the typical growth stage, i.e., when some leaves had turned yellow and flowering had begun. At that time, yield was judged to be normal by accustomed maca workers. Nevertheless, the hypocotyl yield of the four plots of pink maca in the terrain never previously cultivated was insufficient to allow further analysis. All undamaged

and potentially marketable hypocotyls as well as all intact leaves per plot were collected for later analysis. The hypocotyls were placed in a special instrument developed for maca drying, called the 'Fitotoldo', for 3 weeks in the plant of a maca distributor situated close to the experimental site. This equipment consisted of a black impermeable plastic sheet which was laid on the soil. The hypocotyls were placed on the sheet and covered by a transparent plastic cover supported, tent-like, by two poles at both ends allowing the air to circulate. Each morning, the cover was removed and then replaced in the evening to ensure that variation in temperature was low between day and night. Finally, samples were transported to Lima, where they were coarsely milled and placed in an industrial oven for final drying at $<60\,^{\circ}C$, as also practised by maca distributors. The samples were then milled with a 20-mesh (0.85 mm) filter. The leaves were kept under a roof at the same distribution plant and then also ground with a 20-mesh filter when dry.

Laboratory analyses

All analyses were carried out in duplicate. The individual soil samples were analysed using established standard methods:[14,15] sedimentation in water for texture, Walkley and Black for organic matter, 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^{+}. Base saturation, describing the exchangeable base fraction of the cation exchange capacity, was calculated.

Gross nutrient composition was also determined based on standard protocols. Briefly, dry matter (DM) and ash were analysed automatically with a TGA-500 (Leco Corporation, St Joseph, MI, USA). Crude protein (CP) was measured as $6.25 \times$ N by an automatic analyser (LECO C/N-Analyzer Type FP-2000, Leco Corporation, St Joseph, MI, USA), operated according to the Dumas method. Neutral (NDF) and acid detergent fibre (ADF) were determined according to Van Soest *et al.*[16] using the Fibertec System M (Tecator, 1021 Cold Extractor, Höganäs, Sweden). For NDF analysis, samples were treated with α-amylase (termamyl 120L, type S, Novozymes A/S, Bagsvaerd, Denmark) but not with sodium sulfite. Data on NDF and ADF were corrected for ash.

Glucosinolates (GLs) were extracted and analysed in duplicate by high-performance liquid chromatography (HPLC) based on the ISO certified method (ISO 9167-1) developed for canola seed, with small modifications.[17] This procedure includes internal standards and determination of response factors. Briefly, 0.3 g samples were added to centrifugation tubes together with 0.2 mL of sinigrin (20 mmol L^{-1}; Fluka, Buchs, Switzerland), an internal GL standard. The extracts were transformed into eluates of desulfo-GL according to ISO 9167-1,[17] which were mixed before the subsequent reversed-phase HPLC analysis (Waters Alliance Model 2695, Milford, MA, USA). A variable UV detector was used at 229 nm with a Lichrosorb standard column (SunFire™ C18, 5 µm, 4.6 × 150 mm column, Waters). Elution was carried out for 1 min with purified water (step A) before switching to the second mobile phase (B: acetonitrile in water, 0.2 L^{-1} v/v). Step B included a linear gradient starting with zero B, reaching exclusively B after 20 min, returning to zero B after 5 min, followed by another 5 min eluting with A for equilibration. Standard canola seed (ERM®-BC367) was analysed by the same method to allow peak identification. Gluconasturtiin (2-phenylethyl GL), glucoaubrietin (4-methoxybenzyl GL)[18,19] and glucolimnanthin (3-methoxybenzyl GL)[20,21] co-eluted off the column. The results obtained for the gluconasturtiin standard eliminated this peak as gluconasturtiin. However, it was not possible to definitely assign peaks to either glucoaubrietin or glucolimnanthin. These GL with concentrations being consistently <0.1 µmol g^{-1} DM were considered as trace and were not statistically evaluated for treatment effects. However, they were still included in the calculation of total GL.

Concentrations of macaenes, macamides and phytosterols were determined following the analytical protocols of Ganzera *et al.*[12] with minor modification in the composition of the mobile phase and sample size.[22] Sample amounts of 500 mg were sonicated in 2.5 mL of methanol for 20 min and then centrifuged for 15 min at $2900 \times g$. The solutions were injected in triplicate into the same HPLC pump system as used for GL, but equipped with a photodiode array detector (model 996; Waters) and a Synergi Max RP column (150 × 4.6 mm; 4 µm particle size; Phenomenex, Torrance, CA, USA). Temperature was maintained at 40 °C. The mobile phase consisted of high-purity water (2.5 ml L^{-1} trifluoroacetic acid) (C) and acetonitrile (2.5 ml L^{-1} trifluoroacetic acid) (C) at a flow rate of 1.0 mL min^{-1}. The gradient program was to change linearly from 0.5 C/0.5 D to 0.07 C/0.93 D within 55 min. This was followed by washing for 5 min exclusively with acetonitrile and re-equilibration with C/D as 1/1 for 15 min. *N*-Benzyl palmitamide (later called 'macamide 1'), *n*-benzyl-9-oxo-12*Z*-octodecenamide ('macamide 3') and *n*-(*m*-methoxybenzyl)-hexadecanamide ('macamide 4') were measured at a wavelength of 210 nm, while 5-oxo-6*E*, 8*E*-octadecadienoic acid ('macaene') and *n*-benzyl-5-oxo-6*E*, 8*E*-octadecadienamide ('macamide 2') were measured at 280 nm. Macamides 1–4 are the major representatives of this group, while the others are minor in concentration, are not specific to maca or are unstable. The chemical structures of the major macamides and of macaene are shown in Fig. 1. The peaks were assigned by comparisons of retention times by UV spectra. Macaene and macamide standard compounds were isolated at the National Centre for Natural Product Research. Their identity and purity were confirmed by chromatographic (thin-layer chromatography, HPLC) methods, by the analysis of the spectral data (infrared, 1D and 2D nuclear magnetic resonance, high-resolution electrospray mass spectrometry) and through comparison with published spectral data.[23,24] Data were analysed with Waters Empower software (Milford, MA, USA). For calculating total macamides, those found at <0.1 µmol g^{-1} DM were considered as well.

For the analysis of selected phytosterols, the same HPLC pump system was used, but equipped with a Sedex 75 ELS detector (SEDERE, Alfortille, France) and a Luna C8 column (150 × 4.6 mm; 5 µm particle size; Phenomenex, Torrance, CA, USA) maintained at 30 °C. The mobile phase consisted of distilled water (E) and methanol (F) at a flow rate of 1.0 mL min^{-1}. The isocratic elution was 0.1 E/0.9 F in 20 min. A 5 min washout period exclusively with methanol followed and the column was then equilibrated for 15 min with 0.1 E/0.9 F. The probe temperature of the ELS detector was set to 40 °C and a gain of 9. Pressure of nitrogen as the nebulizer gas was 3.5 bar. Test rungs of authentic standards (campesterol, Chromadex, Santa Ana, CA, USA; β-sitosterol, Sigma, St Louis, MO, USA) confirmed that peaks of interest were well separated and did not interfere with other peaks. Linear calibration curves in the range of 10–1000 µg mL^{-1} were established. Accuracy was $<$ 5.0% of residual standard deviation and therefore well within the predefined limits of acceptability. The limits of detection and quantification for campesterol and β-sitosterol obtained for signal-to-noise ratios of 3 and 10, respectively, were 3.0 and 10 µg mL^{-1}. Data were again analysed with Waters Empower software.

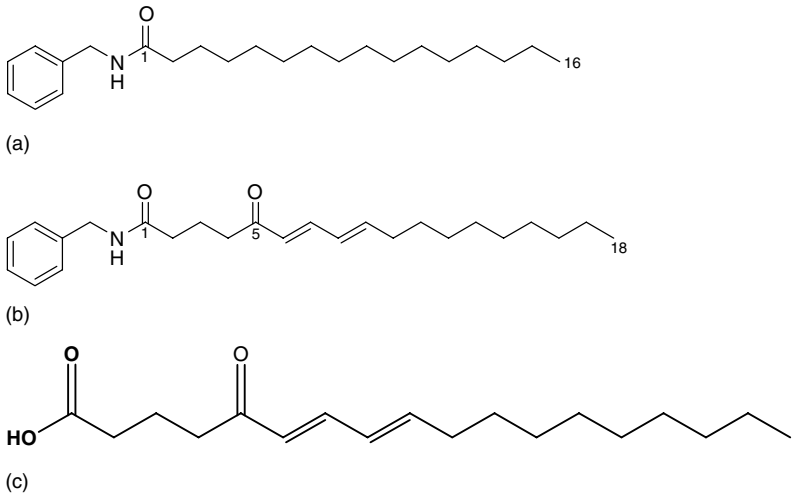

Figure 1. Chemical structure of the three major maca-specific compounds found: (a) *n*-benzyl palmitamide (macamide 1); (b) *n*-benzyl-5-oxo-6*E*, 8*E*-octadecadienamide (macamide 2); and (c) 5-oxo-6*E*, 8*E*-octadecadienoic acid (macaene).

Table 2. Effects of plant organ (O, hypocotyl/leaf), type of terrain (T: −, never cultivated; +, cultivated with maca 2–3 years ago) and colour type (C: Y, yellow; P, pink; V, violet; L, lead-coloured) on the concentrations of nutrients (mg g^{-1} dry matter) in maca

		Hypocotyls								Leaves												
		Colour				P^a	*P* across terrains[b]			Colour				P^a	*P* across terrains[b]				Overall P^c			
	T	Y	P	V	L	C	C	T	C × T	Y	P	V	L	C	C	T	C × T	SEM[d]	C	T	O	C × O	T × O
Dry matter[e]	−	909	–	916	907	×			**	845	–	860	866		×			2.6	*		***		×
	+	909b	908b	908b	919a	*				843	851	845	857										
Organic matter	−	953	–	949	955		*			935	–	853	804					12.1	×		***	×	
	+	956	952	951	956					876	849	909	850										
Crude protein	−	186	–	190	194					197	–	210	180					4.9			*	×	
	+	173	192	167	197					192	220	198	203										
Neutral detergent fibre	−	116	–	119	106		*			276	–	268	273					3.1			***		
	+	114	119	116	109					279	269	281	275										
Acid detergent fibre	−	97	–	93	90		*			226	–	231	225					2.6	×		***		
	+	94a	100a	95a	88b	**				230	216	246	225										

LS means within organ carrying no common letter are significantly different ($P < 0.05$). Significance of effects: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; × $P < 0.1$ (approaching significance). –, missing data.
[a] *P*-values for each row applying Model 1: $y_{ij} = \mu + C_i + \varepsilon_{ij}$.
[b] *P*-values for each variable within organ applying Model 2: $y_{ijk} = \mu + C_i + T_j + C \times T_{ij} + \varepsilon_{ijk}$.
[c] *P*-values for each variable applying Model 3: $y_{ijkl} = \mu + C_i + T_j + O_k + C \times O_{ik} + T \times O_{jk} + \varepsilon_{ijkl}$.
[d] SEM, standard errors of the mean.
[e] In original substance.

Total phenolic compounds were analysed according to the Folin–Ciocalteu method.[25]

Statistical analysis

The SAS procedure MIXED (SAS Institute, Inc., version 9.1.3) was used for analysis of variance applying the following models:

Model 1: $y_{ij} = \mu + C_i + \varepsilon_{ij}$
Model 2: $y_{ijk} = \mu + C_i + T_j + C \times T_{ij} + \varepsilon_{ijk}$
Model 3: $y_{ijkl} = \mu + C_i + T_j + O_k + C \times O_{ik} + T \times O_{jk} + \varepsilon_{ijkl}$

where C = colour type effect, T = terrain effect, O = organ (hypocotyl *vs.* leaf) effect and ε = residual error. Model 1 was used to test colour type effects within terrain, Model 2 served to analyse colour type, terrain and their interaction. The third model was applied to evaluate the effect of all three factors and their interactions. After testing all interactions, $C \times D$ and $C \times D \times O$ were excluded, as they were almost never significant. As mentioned above, data on pink maca was only available for one terrain, leaving pink maca data only for evaluation with Model 1. Multiple comparisons among colour type means were performed and adjusted with Tukey's method. The tables give least square (LS) means, standard errors of the LS means (SEM) and *P*-values for colour type, terrain and organ.

RESULTS

After drying, hypocotyls and leaves had DM concentrations of approximately 910 and 850 g kg^{-1}, respectively (Table 2).

Table 3. Effects of plant organ (O, hypocotyl/leaf), type of terrain (T: −, never cultivated; +, cultivated with maca 2–3 years ago) and colour type (C: Y, yellow; P, pink; V, violet; L, lead-coloured) on the concentrations of glucosinolates (μmol g^{-1} dry matter) in maca

		Hypocotyls								Leaves													
		Colour				P^a	*P* across terrains[b]			Colour				P^a	*P* across terrains[b]				Overall P^c				
	T	Y	P	V	L	C	C	T	C × T	Y	P	V	L	C	C	T	C × T	SEM[d]	C	T	O	C × O	T × O
Total GL	−	28.42b	–	34.30ab	56.00a	*	**			6.16	–	5.39	3.70	×				2.457	**		***	**	
	+	37.23	44.10	33.22	54.78					5.16	5.08	4.71	4.45										
Total aromatic GL	−	27.65b	–	33.39ab	55.06a	*	**			5.78	–	4.97	3.34	×				2.442	**		***	**	
	+	36.39	43.09	32.39	53.77					4.80	4.72	4.20	4.08										
Glucotropaeolin	−	23.60b	–	29.51ab	50.13a	*	**			4.56	–	3.85	2.66	×				2.280	**		***	**	
	+	31.43	37.78	28.30	48.84					3.71	3.63	3.14	3.23										
Glucolimnanthin	−	2.68	–	2.78	3.24					0.85	–	0.84	0.46					0.137			***	×	
	+	3.20	3.61	2.70	3.35					0.70	0.77	0.76	0.60										
Glucosinalbin	−	1.35	–	1.09	1.69	×	×			0.37	–	0.29	0.22	×	×			0.968	×		***	×	
	+	1.76	1.70	1.38	1.57					0.39	0.33	0.30	0.26										
Total indole GL	−	0.07	–	0.09	0.10		*	*		0.02	–	0.03	0.02					0.005	*		***	*	*
	+	0.10b	0.08b	0.09b	0.15a	**				0.01	0.01	0.02	0.02										
4-Hydroxyglucobrassicin	−	0.03	–	0.04	0.03					0.01	–	0.00	0.01					0.002			***		
	+	0.04b	0.02b	0.04ab	0.05a	**				0.00	0.01	0.00	0.00										
Tot. alkylthioalkyl GL	−	0.51	–	0.60	0.64		*			0.19	–	0.22	0.19		×			0.024	×		***	*	
	+	0.53	0.69	0.51	0.66	×				0.16	0.22	0.32	0.18										
Glucoalyssin	−	0.35	–	0.38	0.46		×			0.12	–	0.17	0.13		×			0.022			***	*	
	+	0.38	0.53	0.34	0.49	×				0.11	0.15	0.26	0.13	×									
Glucoraphanin	−	0.13	–	0.18	0.14					0.05	–	0.03	0.04					0.007			***		
	+	0.12	0.13	0.14	0.14					0.04	0.04	0.05	0.04										
Total olefin GL	−	0.19	–	0.22	0.20					0.16	–	0.17	0.15					0.009			***		
	+	0.21	0.23	0.23	0.20					0.19a	0.12b	0.17ab	0.16ab	*									

LS means within organ carrying no common letter are significantly different ($P < 0.05$). Significance of effects: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; × $P < 0.1$ (approaching significance). –, missing data.

[a] *P*-values for each row applying Model 1: $y_{ij} = \mu + C_i + \varepsilon_{ij}$.

[b] *P*-values for each variable within organ applying Model 2: $y_{ijk} = \mu + C_i + T_j + C \times T_{ij} + \varepsilon_{ijk}$.

[c] *P*-values for each variable applying Model 3: $y_{ijkl} = \mu + C_i + T_j + O_k + C \times O_{ik} + T \times O_{jk} + \varepsilon_{ijkl}$.

[d] SEM, standard errors of the mean.

Although there were some significant differences among colour types in terms of nutrient composition of the hypocotyls, variation was small in magnitude. Colour type had no effect on nutrient composition of the leaves and there was no terrain effect in either plant organ. The hypocotyls were richer in organic matter, but poorer in CP and especially in fibre than the leaves. The interactions occurring among the factors analysed were minor.

The total, and most individual, GLs were much higher in concentration in the hypocotyls than in the leaves (Table 3; factor of 6 for total GL). Colour type effects were frequent in individual GL (often significant for the hypocotyls, approaching significance for the leaves). The concentration of many GLs was particularly high in lead-coloured hypocotyls, while it varied for the other colours. Significant terrain effects were found only for the hypocotyls and in the indole GL group. Pink hypocotyls on the terrain previously cultivated with maca showed a tendency to have higher GL concentrations than yellow and violet maca hypocotyls. In the leaves, the highest GL concentration was found in yellow maca ($P < 0.1$; especially in the never cultivated terrain), but the colour trends were less clear than for the hypocotyls. Further representatives of the total of 13 GL found included glucobrassicin, glucoiberin, progoitrin, epigoitrin, gluconapoleiferin, gluconapin and glucobrassicanapin, but these were only present in trace amounts.

Colour effects were significant for macaene and macamides, but mostly only for the hypocotyls (Table 4). In the previously cultivated terrain where pink maca was available, hypocotyls tended to have the highest macaene levels (not significant), whereas in the terrain never cultivated before the yellow hypocotyls were significantly richer in macaene that the other two colours (significant also across both terrains). The yellow hypocotyls were poor in total macamide concentration, whereas violet maca hypocotyls were rich. Pink maca had a high concentration as well (but not significantly different from the other colours). Macaene concentration in hypocotyls was higher on the terrain previously cultivated with maca. The organ effect was very pronounced, and additional colour $\times$ organ interactions were found, mainly due to the response to colour only in the hypocotyls. Across colours and terrains, macaene and total macamides were higher in concentration by factors of 9 and 24 for hypocotyls compared to leaves, respectively. Only macamides 1 and 2 were found to be above the detection limit in concentration. These two representatives followed the trends found for total macamides.

The level of the phytosterol campesterol in the hypocotyls was higher in plants grown on the never-cultivated terrain but were not affected by either colour type or organ. In contrast, β-sitosterol showed a higher concentration in the leaves than in the hypocotyls but did not respond to the other factors. The colour effect again was significant for total phenolic compounds in the hypocotyls, with a trend tor the highest concentrations occurring in yellow maca. Total phenolic concentrations found in the leaves, where colour effects were less pronounced, were about threefold higher than those of the hypocotyls.

DISCUSSION

Effect of colour type

There were certain colour type differences in nutrient contents of the hypocotyls originating from four corresponding accessions. This affected fibre especially. Colour type differences in concentrations of secondary metabolites were larger. The lead-coloured hypocotyls were clearly richer than the yellow hypocotyls for concentrations of total GL, aromatic GL, indole GL and alkylthioalkyl GL. To the authors' knowledge lead-coloured maca was never specifically analysed for GL, which is why this finding requires further confirmation. In a preliminary study,[3] where different maca colour types had been phenotypically selected from producers, a low GL concentration of yellow maca compared to pink (red) and violet maca was noted, different from the present study. The yellow hypocotyls were richest in macaene in the never-cultivated terrain and in total phenolic compounds in both terrains as well as across both terrains. The violet hypocotyls were generally high in macamides 1 and 2. This is consistent with the preliminary findings,[3] except for macamide 2. The lack of response in campesterol and β-sitosterol concentrations confirmed earlier results,[3] where there was only a difference between black maca (not investigated here) and three other colour types.

Overall, colour type effects were quite frequent in the hypocotyls but were rarely found in the leaves of these plants. This means that generating accessions of certain colour types was probably also associated with generating clear metabolic differences in synthesis processes of underground biomass. The general availability of a variety of distinctly different maca hypocotyl colours demonstrates that colour has been a selection criterion for farmers and breeders. As the only known germplasm collection, UNDAC, Peru, is able to provide maca of different colour types for planting, though without knowing the underlying differences in genotype. It could be that the genes responsible for traits of colour and those for some secondary metabolites are associated to a certain degree when they would be situated on the same chromosome. However, publications sometimes do not even differentiate accurately among colours, especially not between black and lead-coloured maca and between reddish-types of maca which are either pinkish or violet.

The characteristic colour pigments are present only in the thin outer layer of the hypocotyls,[8] except for yellow maca where this colour is also present in the inner part, while all others are plain white inside. The colours investigated can be attributed to different pigments such as carotenoids, anthocyanins and some of their intermediate products.[26] Yellow colour can be associated with carotenoid pigments[27] or intermediate products of anthocyanin biosynthesis.[26] A yellow pigmented part typically lacks anthocynanins,[28] whereas reddish and blue colours are typically determined by anthocyanins.[27] No explicit relationship was observed between the metabolites analysed and the increasing intensity of pigmentation from pink to violet and black.[27] The anthocyanins (from the group of the flavonoids) probably do not represent a significant part of the ground maca hypocotyls. These compounds are obviously almost completely degraded even by simple air-drying.[29] Therefore they are unlikely candidates to contribute to the colour type differences in the biological effects described.[4] Flavonoids are the end product of biosynthetic pathways,[26,30] which differ from pathways resulting in polyunsaturated fatty acids/amides (macaene/macamides), phytosterols and sulfur-containing GL. The repeated reports of major colour type differences in secondary metabolite concentrations indicate that, despite the lack of a clear breeding goal, colour is not just a casual attribute and gene mapping might help to characterize the underlying mechanisms.

Effect of the terrain

As anticipated from the proximity of the two fields, the soils did not differ much in their characteristics. The most obvious

Table 4. Effects of plant organ (O, hypocotyl/leaf), type of terrain (T: −, never cultivated; +, cultivated with maca 2–3 years ago) and colour type (C: Y, yellow; P, pink; V, violet; L, lead-coloured) on the concentrations of macaene, macamides and phytosterols (μmol g^{-1} dry matter) and total phenolic compounds (mg gallic acid equivalent g^{-1}) in maca

		Hypocotyls								Leaves											
		Colour				P^a	P across terrains[b]			Colour				P^a	P across terrains[b]				Overall P^c				
	T	Y	P	V	L	C	C	T	C × T	Y	P	V	L	C	C	T	C × T	SEM[d]	C	T	O	C × O	T × O
Macaene	−	6.69a	–	4.86b	4.93ab	*	*	*		0.66	–	0.80	0.53	×				0.213	*	**	***	×	*
	+	7.21	8.24	6.83	6.03					0.83	0.86	0.88	0.71										
Macamide 1	−	0.47b	–	0.79a	0.65ab	**	***		×	0.09	–	0.08	0.07					0.320	***		***	***	
	+	0.49b	0.62ab	0.92a	0.40b	**				0.07	0.08	0.07	0.07										
Macamide 2	−	1.40	–	1.80	1.94	×	***		×	0.06	–	tr.	tr.				×	0.062	***		***	***	
	+	1.30b	2.15ab	2.28a	1.78ab	*				0.02	0.02	0.02	0.05										
Total macamides	−	1.87	–	2.60	2.59	*	***		×	0.15a	–	0.08b	0.07b	*			×	0.087	***		***	***	
	+	1.79b	2.77ab	3.21a	2.18ab	**				0.09	0.10	0.10	0.12										
Campesterol	−	0.14	–	0.08	0.07			*		0.13	–	0.09	0.12					0.016					
	+	0.07	0.01	0.06	0.05					0.11	0.12	0.06	0.14										
β-Sitosterol	−	0.23	–	0.14	0.01					0.43	–	0.52	0.44					0.025			***		
	+	0.13	0.14	0.17	0.10					0.45	0.34	0.41	0.43										
Total phenols	−	5.65	–	4.61	4.89	×	**			16.84	–	15.27	15.61					0.298	*		***		
	+	5.85	5.72	5.21	4.91	×				16.19	15.38	15.70	14.73										

LS means within organ carrying no common letter are significantly different ($P < 0.05$). Significance of effects: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; × $P < 0.1$ (approaching significance). –, missing data; tr, trace; i.e. detected under limit of quantification.

[a] P-values for each row applying Model 1: $y_{ij} = \mu + C_i + \varepsilon_{ij}$.

[b] P-values for each variable within organ applying Model 2: $y_{ijk} = \mu + C_i + T_j + C \times T_{ij} + \varepsilon_{ijk}$.

[c] P-values for each variable applying Model 3: $y_{ijkl} = \mu + C_i + T_j + O_k + C \times O_{ik} + T \times O_{jk} + \varepsilon_{ijkl}$.

[d] SEM, standard errors of the mean.

difference was that the soil cultivated with maca before had a higher sum of cations (mostly due to Ca^{2+}) and bases, as well as a higher base saturation. This was most probably due to the higher clay concentration and not the result of previous cultivation. In general, previous cultivation with maca had only occasionally effects on the composition of maca hypocotyls and leaves. Macaene and campesterol concentrations especially were respectively higher and lower when grown on the terrain planted previously with maca. Further studies are needed to confirm these findings and to clarify why soil properties would influence the concentration of these compounds. The present results do not support the recommendation for a long fallow period subsequent to repeated cultivation with maca.[8–11] The terrain that had been recently planted with maca even yielded hypocotyls with a higher concentration of the characteristic metabolite macaene. Plant health also does not seem to have been compromised on the terrain previously cultivated with maca.

Effect of the plant organ

The two plant organs investigated – hypocotyls and leaves – were significantly different in concentrations of all nutrients and most secondary metabolites analysed, except for campesterol. This finding for leaves was expected, as they are typically more fibrous compared to the hypocotyls, which serve as storage organs rich in reserve carbohydrates. Across all colours and terrains, the hypocotyls were richer in concentrations of GL, macaene and macamides, while higher concentrations of β-sitosterol and total phenols were found in the leaves. This is in agreement with a previous finding of a higher concentration of β-sitosterol in the leaves than in the storage organ of *Alchornea cordifolifolia*.[31] The concentration of total phenols found in the hypocotyls was comparable to that found in a range of fruits,[32] while that of the leaves was higher; however, the latter was still rather low when compared to concentrations found in many tropical fodder shrubs.[33]

The organ difference in the concentration of GL could be the result of GL translocation from the leaf to other plant organs. Chen and Andreasson[34] proposed that the GLs synthesized in the leaves follow the principle of assimilate translocation and are transported via the phloem from source to sink. In the present case, the sinks would be the hypocotyls and not the seeds as suggested by Chen and Andreasson,[34] as the harvest happened before flowering. The dried hypocotyls showed a higher GL concentration than those reported by Li *et al*.[19] and were even higher than those in fresh hypocotyls investigated in that study. Consistent with this, the GL concentration in the dried leaves in the present study was higher than that found by the same group of previous researchers[19] in fresh leaves. Differences between plant organs were larger than those caused by colour type in the hypocotyls.

Currently hypocotyls are almost exclusively consumed by humans, while leaves are sometimes fed to cattle or guinea-pigs as feed supplement, with the idea of benefiting from the same biological effects. However, these effects are first deduced from what is known about the consumption of the hypocotyls, where the secondary metabolites that are presumed to be responsible for the biological effects are present in a more concentrated form. Effects on fertility by β-sitosterol, the only metabolite found in higher concentration in the leaves than in the hypocotyls, are variable (ranging from negative[35] to positive[36]). This means that quite a large intakes of leaves will be required to be able to profit from any potential fertility-enhancing effects in livestock.

Interactions among factors of influence

Significant interactions were most frequent between colour type and plant organ. The major reason for this was that the leaves tended not to follow the colour type differences of the hypoctyls. Exceptions were yellow maca hypocotyls that had the lowest concentration of total GL and macamides, whereas this was opposite in the corresponding leaves. Due to the generally low importance of previous cultivation with maca, interactions among colour type and terrain were absent and those of organ and terrain were rare. This indicates that colour type effects would not differ much when maca is cultivated on different terrains. Further studies are needed to show whether soil type and climate cause these types of interactions to be expressed.

CONCLUSIONS

The present study demonstrated that the secondary metabolites investigated were sensitive to colour type and plant organ, giving a clear advantage in terms of concentration to the air-dried hypocotyls over the air-dried leaves. The associations with colour type have to be confirmed in other accessions, but the results suggest that it could be interesting to investigate the nature of the associations of colour and metabolites in full detail. As farmers tend to cultivate more than one colour type on the fields, a certain standardization of the product for larger markets or even for export has to be achieved. A good starting point would be to initiate a strategic breeding programme based upon the accessions used in the present study. First attempts to do this include analyses of batches for macaene and macamides.[37] Our results suggest that this should be extended by determining at least total glucosinolates and selected phytosterols. This is important as the active principle in maca that facilitates fertility and health has not yet been firmly established. Depending on the metabolite(s) that eventually do turn out to be most bioactive, different colour types are likely to be preferred.

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