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Growth conditions modify the concentrations of bioactive caffeic acid derivatives, amino acids and the structure of *Plantago* leaves

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ABSTRACT

Plantago species are widely used in natural medicine, but the effect of growth conditions on the chemical content of leaves is poorly understood. Three species of *Plantago* and three varieties of *Plantago major* L. were grown in greenhouse and outdoors to investigate effects on the content and composition of three bioactive caffeic acid derivatives (BCAD) (plantamajoside, iso-plantamajoside and verbascoside), free amino acids (FAA) and leaf texture of the plants. High Performance Thin Layer Chromatography (HPTLC) was used for the quantification of the BCAD, amino acids were determined by High Performance Liquid Chromatography (HPLC) and a histochemical technique was used to study the physical structure of the plants. Plants grown outdoors were significantly richer in BCAD compared with greenhouse grown plants. The highest content was seen in outdoor grown *Plantago major* L. (45.15 ± 4.36 mg/g DW) and the lowest in greenhouse grown *Plantago major 'Frills'* (7.74 ± 0.96 mg/g DW). Conversely, amino acid concentrations were significantly greater for greenhouse grown plants compared with outdoor grown plants. The highest concentration was found in greenhouse grown *P. major* L. and *Plantago major* '*Rubrifolia*' (8.66 ± 0.64 mg/g DW and 7.94 ± 0.97 mg/g DW, respectively), whereas the lowest was in *Plantago lanceolata* L. grown outdoors (0.73 ± 0.09 mg/g DW). The leaf texture of the plants grown outdoors and in the greenhouse was significantly different. This study underlines the importance of environmental and growing conditions for plants in order to obtain high concentrations of bioactive compounds either from a nutritional point of view or for use in natural medicine.

Keywords: Biochemical analysis, histochemistry, HPLC, HPTLC, plantamajoside, verbascoside.

INTRODUCTION

The *Plantago*-genus represents about 275 species in the Plantaginaceae family (Ravn et al., 2015). *Plantago* species have worldwide distribution and are widely used in natural medicine (Jdey et al., 2017; Ogbiko et al., 2018). They are often employed in the treatment of diseases related to the skin, respiratory, reproductive and digestive organs and against inflammations and infections (Ravn et al., 2015). *Plantago major* L. and *P. asiatica* L. have strong market demands in

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Asia and *P. major* is emerging as a medicinal crop in horticulture in Europe. The aerial parts of *P. asiatica* L. are used as the crude drug 'Plantago Herba' (Shazenso) in China, Korea and Japan (Kyokai, 1986).

The major active constituents of these medicinal plants are the bioactive caffeic acid derivatives (BCAD) plantamajoside, iso-plantamajoside and verbascoside, which are important antioxidants (Amakura et al., 2012; Ravn et al., 2015; Jdey et al., 2017). The aerial parts of *Plantago media* L., *P. major*, *P. lanceolata* L., *Plantago depressa* (Willd.) and *P. asiatica* have been analyzed for the content of plantamajoside and verbascoside (Olennikov et al., 2011; Gonda et al., 2013). Bioactive plant components are well known from many plant species (Byamukama et al., 2015; Ogwuche et al., 2015; Adagba et al., 2017; Sawadogo et al., 2017).

Unlike BACD, there are little data on the free amino acid content of *Plantago* species. The few reported studies relate to individual species, such as *P. major* (Mohamed et al., 2011), *Plantago ovata* Forssk. (Romero-Baranzini et al., 2006), *P. lanceolata* (Grange and West, 1994) and *Plantago japonica* Franch. & Sav. (Sagisaka et al., 1988).

This aim of this study was to conduct a comparative investigation of three BCAD (plantamajoside, iso-plantamajoside and verbascoside), free amino acid composition and physical structure as biomarkers in three species and three selected varieties of *Plantago* grown under greenhouse contra outdoor environmental conditions, in order to verify the importance of environmental conditions for the content and composition of the plants.

MATERIALS AND METHODS

Plant and soil material

Seeds of *P. major*, *P. media*, *P. lanceolata* were obtained from HerbiSeed, Twyford, UK and the varieties *P. major* '*Frills*', *P. major* '*Rosularis*' and *P. major* '*Rubrifolia*' were obtained from Plant World Seeds, Devon, UK. The seeds were sown in a greenhouse on April 10 2013. On May14 2013 half of the plants were placed outdoors until June 24 2013, when all the leaves of the plants were harvested and stored at -20 °C until freeze-drying. The soil used for cultivation both in the greenhouse and outdoors was 2/3 professional substrate, Kekkilä Brown 525W, Kekkilä Garden, Garta, Odense, Denmark (Brown sphagnum peat H2-von post, 5-25 mm, Addition: 1.0 kg/m^3 N-P_2O_5-K_2O 14-16-18+TE, Dolomite limestone wetting agent, pH=5.5-5.6 and 1/3 ordinary beach sand. The plants were watered regularly until harvest.

Standards and identification of plantamajoside, iso-plantamajoside and verbascoside

Standards of plantamajoside and verbascoside (Figure 1) were isolated from *P. major* subsp. *pleiosperma* according to a previously published protocol (Ravn et al., 1988) and plantamajoside used as standard for the quantification of iso-plantamajoside. Identification of the three compounds was optimised using different HPTLC plates, eluents and reagents to enhance UV detection and colour reactions (Ravn et al., 1988).

Sample preparation for BACD

Leaves (six replicates) from the different *Plantago* species and varieties were placed in individual paper bags and lyophilized. The plant material was crushed and 6 x 50.00 mg greenhouse plants and 6 x 25.00 mg outdoor plants per ml of 80% ethanol/water were extracted in an ultrasonic bath for two hours. The temperature in the ultra-sonic bath was maintained at less than 40 °C during the extraction to avoid decomposition of the BCAD. The samples were filtered through a Whatman filter GMF w/GMF 0.45 µm and analysed by for HPTLC.

HPTLC analysis of BACD

The analysis was performed using HPTLC-plates with silicagel 60 (Merck 1.005547.0001) (20 x 10 cm). The application volume was 5 µl of both extracts and standards. Samples were added to the plates as a band of 10 mm using an ATS-4 CAMAG applicator. Fresh eluent was prepared daily, consisting of ethyl acetate: 40% formic acid

(2:1). The development time was 56.4 ± 1.4 min (N=18). The plates were air-dried and BCAD were detected under UV light at 366 nm using auto-fluorescence of the compounds. They were also detected using 1% diphenylboric acid-2-aminoethyl ester (Fluka) in 5% polyethyleneglycol 4000 (PEG_{4000}) in 96% ethanol (366 nm under UV-light). The equipment used for the analysis was from CAMAG (Muttenz, Switzerland) and comprised an ATS-4 applicator and a Reprostar 3 cabinet with DXA 252 camera (lens Computar, focal length 16 mm, aperture f4.0). Camag software, WinCats (version 1.4.2.8121) and VideoScan (version 1.01.00) were used to analyse the digital images obtained from the photographs of the HPTLC plates with both extracts and the standard solutions on each plate.

Sample preparation for amino acids

For each repetition, 50 mg of each lyophilized sample were weighed in pre-weighed 2 ml Eppendorf tubes and stored at -20 °C until analysis. A previously optimized (Hjorth, et al., 2006) extraction method was used: 1 or 1.20 ml (depending on the volume of the lyophilisate) of 10% (v/v) ethanol in water was added to the tubes and the amino acids were extracted for two hours in an ultrasonic bath. Ice packs were added to the bath to ensure that the temperature remained below 40°. The samples were then diluted 5-fold with freshly distilled water for further analysis.

HPLC analysis of amino acids

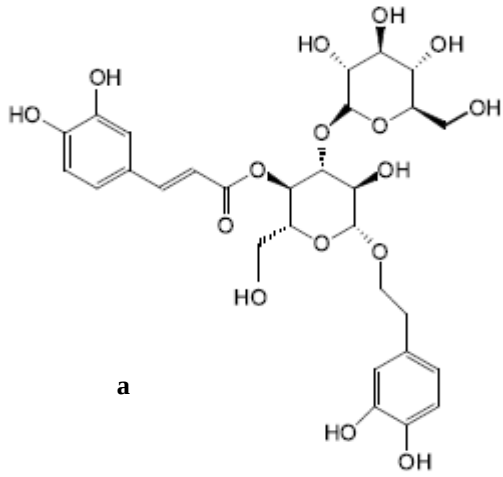
Samples were analysed according to a previously published method (Kelly and Larroque, 2010) with some modifications. Specifically, mobile phase A contained 2.5% acetonitrile and 1% tetrahydrofuran in 0.05 M acetate buffer (pH 6.5) and mobile phase B consisted of acetonitrile: methanol [1:1] to obtain complete separation of the critical pair histidine and glycine. The in-house *o*-phthaldialdehyde (OPA) reagent solution used in the previous paper was replaced by a proprietary *o*-phthaldialdehyde-mercaptoethanol solution (PN 5061-3335) obtained from Agilent (Agilent Technologies Massy, France). Gradient and derivatisation conditions and the method of quantification were as previously described.

Histochemical technique

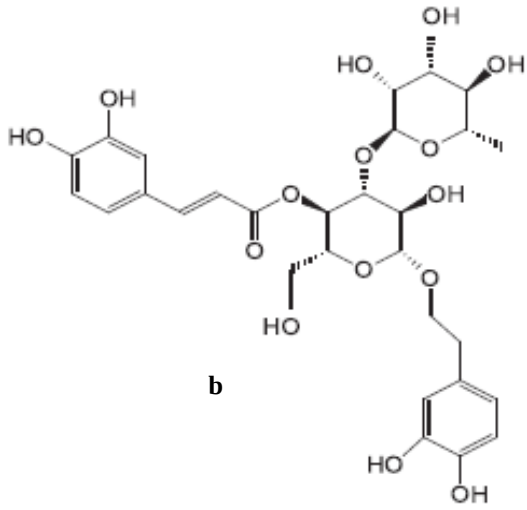
For each lyophilized sample, a 4-6 mm portion of the leaf center was used from six replicate plants. They were embedded in 3% agarose (type II EEO, Panreac) before cutting for histochemical examination. Transverse sections (40 µm) were obtained using a Leica VT 1000S vibrating blade microtome (frequency 7, speed 2). For auto-fluorescence observation, transverse sections were mounted in distilled water without any reagent. Transverse sections of specimens were viewed under a light microscope (Nikon Optiphot) with UV light (filter UV-1A: 365 nm excitation filter). Leaf transverse sections were then observed after treatment with a specific reagent that enhanced the fluorescence of the BCAD, containing 0.25% diphenylboric acid-2-aminoethyl ester (DPBA) and 0.02% Triton-X-100 (v/v). The leaf sections were placed on a microscope slide and 5µl of the DPBA-solution was distributed evenly on each. Under these conditions, BCAD were visible as a greenish-white fluorescence (Mondolot-Cosson et al., 1997). Photographs were taken with a digital Nikon Coolpix 4500 camera.

Statistical analysis

All data were reported as means ± standard deviations of six replicates for each treatment. Statistical analysis was performed using ANOVA in order to test for differences in content of plantamajoside, iso-plantamajoside, verbascoside, total content of BCAD and amino acids between plants grown outdoors and in the greenhouse. Principal component analysis (PCA) was based on a matrix with six species/varieties and their content of the compounds. The PCA was based on correlations among variables in order to place equal weight on the variables included in the analysis.



a



b

Figure 1: Chemical structure of a) plantamajoside (3,4-dihydroxy-β-phenethyl-O-β-D-glucopyranosyl-(1→3)-4-O-caffeoyl-β-D-glucopyranoside) and b) verbascoside (3,4-dihydroxyphenyl)ethyl 3-O-(6-deoxy-a-L-mannopyranosyl)- β -D-glucopyranoside).

RESULTS

Plantamajoside, iso-plantamajoside and verbascoside

The location and separation of the BCAD (plantamajoside, iso-plantamajoside and verbascoside) on the HPTLC-plates were indicated as Rf-values (Table 1). The content and composition of the BCAD were significantly different between plants grown in the greenhouse and outdoors (Table 2). The content of plantamajoside, iso-plantamajoside and verbascoside were, in most cases, higher in the outdoor grown plants compared with the greenhouse grown plants. Overall, the total content of BCAD was two- to four- times greater for plants grown outdoors compared with plants grown in the greenhouse.

The differences in the total content of BCAD in the different species were minor compared with differences produced by growth conditions: *P. major* had the highest content of iso-plantamajoside, whereas *P.*

lanceolata and *P. media* had the highest concentrations of verbascoside, for both greenhouse and outdoor grown plants. The total content of BCAD between the varieties *P. major* '*Rosularis*', *P. major* '*Rubrifolia*' was almost identical whereas the total content in *P. major* '*Frills*' was significantly lower. *P. major* '*Rosularis*' and *P. major* '*Rubrifolia*' mainly contained plantamajoside whereas *P. major* '*Frills*' principally contained verbascoside for both greenhouse grown and outdoors.

The highest content of plantamajoside was found in *P. major* '*Rosularis*' and *P. major* '*Rubrifolia*' grown outdoors (36.54 ± 4.90 mg/g DW and 34.88 ± 5.04 mg/g DW, respectively) and lowest for *P. media* (0.21 ± 0.25 mg/g DW for the plants grown in the greenhouse and 0.33 ± 0.44 mg/g DW for the plants grown outdoors). The highest content of iso-plantamajoside was observed in *P. major* grown outdoors (21.01 ± 3.19 mg/g DW) and lowest for *P. major* '*Rosularis*' grown in the greenhouse (0.22 ± 0.22 mg/g DW). Iso-plantamajoside was not found in *P. lanceolata*, *P. media* and *P. major* '*Frills*', neither in plants grown in greenhouse nor outdoors. The highest content of verbascoside was detected in *P. lanceolata* and *P. media* grown outdoors (38.79 ± 10.53 mg/g DW and 34.95 ± 9.36 mg/g DW, respectively) and the lowest in *P. major* '*Rubrifolia*' and *P. major* '*Rosularis*' (not detected in both plants grown in the greenhouse) and (0.07 ± 0.18 mg/g DW and 0.07 ± 0.17 mg/g DW for the plants grown outdoors). The highest total content of all three BCAD was found in *P. major* and *P. lanceolata* (45.15 ± 2.55 mg/g DW and 40.31 ± 9.91 mg/g DW, respectively, for the plants grown outdoors) and lowest for *P. major* '*Frills*' grown in the greenhouse (7.74 ± 0.96 mg/g DW).

Histochemical analysis confirmed HPTLC results: Higher BCAD concentrations were observed for the plants grown outdoors compared with those grown in the greenhouse. Interestingly, the mesophyll in the outdoor grown plants was more dense and homogenous than that of the plants grown in greenhouse, and the BCAD were distributed in both the palisade and spongy parenchyma (Figure 2). This was particularly obvious in *P. major* where the highest content of BCAD was detected (Figure 2a). In *P. major* '*Rubrifolia*', this was also visible at greater magnification (Figure 2b). The BCAD were almost exclusively confined to the palisade parenchyma in greenhouse grown plants (Figure 2a(ii) and 2b(ii)), whereas these compounds were present in the entire mesophyll in outdoor grown plants (Figure 2a(i) and 2b(i)).

Free amino acids

The FAA and the biogenic amines, histamine and tyramine were determined High Performance Liquid Chromatography (HPLC). The total FAA content was greater in greenhouse grown plants (Figure 3) and interestingly, histamine was more abundant in outdoor grown plants. There were statistically significant differences both among species and varieties and between plants grown outdoors and in greenhouse. Amongst the outdoor grown plants, *P. major* was the richest in most FAA with 8.66 ± 0.64 mg/g DW, whereas *P. media* had the lowest content with 3.36 ± 0.57 mg/g DW (Table 3). Aspartate was the most abundant amino acid in the majority of plants with a concentration of 5.40 ± 0.87 mg/g DW. The total content of glutamine at 6.25 ± 0.62 mg/g was skewed by the fact that *P. major* '*Rubrifolia*' was remarkably rich in this compound (2.70 ± 0.23 mg/g DW), whereas concentrations varied from 0.31 ± 0.07 mg/g DW for *P. media* to 1.26 ± 0.11 mg/g DW for *P. major*. Other predominant amino acids were, in order of decreasing abundance: alanine, glutamate, GABA, glutamine, serine and asparagine.

The PCA of the amino acid concentrations showed a clear distinction among species and varieties and illustrated that the content was higher in greenhouse grown plants (Figure 4). The fi rst axis describing 59% of the variation showed a clear separation among the species. The content of the individual FAA were positively correlated with the first axis, which demonstrates that some species notably *P.*

major and *P. major* '*Rubrifolia*' were particularly rich in amino acids, whereas concentrations in *P. lanceolata* and *P. media*, were relatively low. *P. major* '*Frills*' had high concentrations of glutamate and aspartate when grown in the greenhouse, which was expressed on the second axis explaining 12% of the variation.

Table1: Rf-values of bioactive caffeic acid derivatives: plantamajoside, iso-plantamajoside and verbascoside as standards or in extracts.

Compound name	In extract/standard	Rf-values	Number of replicates
Plantamajoside	standard	0.63±0.03	39
Plantamajoside	In extract	0.62±0.03	125
Iso- plantamajoside	In extract	0.67±0.02	54
Verbascoside	standard	0.78±0.02	39
Verbascoside	In extract	0.78±0.02	106

HPTLC-analysis, development time: 56.4 min ± 1.4min, N=18.

Table 2: Bioactive cafeic acid derivatives (mg/g DW) in *Plantago* species.

Species/varieties	Growth condition	Plantamajoside	Iso-plantamajoside	Verbascoside	Total
P. major	Greenhouse	4.33 ± 0.52	12.12 ± 1.86	3.65 ± 0.71	20.10 ± 2.55
P. major	**Outdoors**	**14.82 ± 0.78**	**21.01 ± 3.19**	**9.32 ± 1.15**	**45.15 ± 4.36**
P. major 'Rubrifolia'	Greenhouse	8.60 ± 1.98	*0.63 ± 0.55*	0.00 ± 0.00	9.23 ± 2.32
P. major 'Rubrifolia'	**Outdoors**	**34.88 ± 5.04**	***1.25 ± 1.02***	**0.07 ± 0.18**	**36.20 ± 8.98**
P. major 'Rosularis'	Greenhouse	11.45 ± 2.23	*0.22 ± 0.22*	0.00 ± 0.00	11.67 ± 2.15
P. major 'Rosularis'	**Outdoors**	**36.54 ± 4.90**	***1.00 ± 0.92***	**0.07 ± 0.17**	**37.61 ± 9.14**
P. lanceolata	Greenhouse	0.42 ± 0.30	*0.00 ± 0.00*	9.96 ± 1.09	10.38 ± 1.09
P. lanceolata	**Outdoors**	**1.52 ± 0.81**	***0.00 ± 0.00***	**38.79 ± 10.53**	**40.31 ± 9.91**
P. media	Greenhouse	*0.21 ± 0.25*	*0.00 ± 0.00*	10.26 ± 1.44	10.47 ± 1.47
P. media	**Outdoors**	***0.33 ± 0.41***	***0.00 ± 0.00***	**34.95 ± 9.36**	**35.28 ± 9.12**
P. major 'Frills'	Greenhouse	*0.35± 0.34*	*0.00 ± 0.00*	7.39 ± 0.82	7.74 ± 0.96
P. major 'Frills'	**Outdoors**	***0.35 ± 0.46***	***0.00 ± 0.00***	**25.04 ± 5.82**	**25.39 ± 5.59**

Values are means ± SD of replicates, values in italics are significantly different.

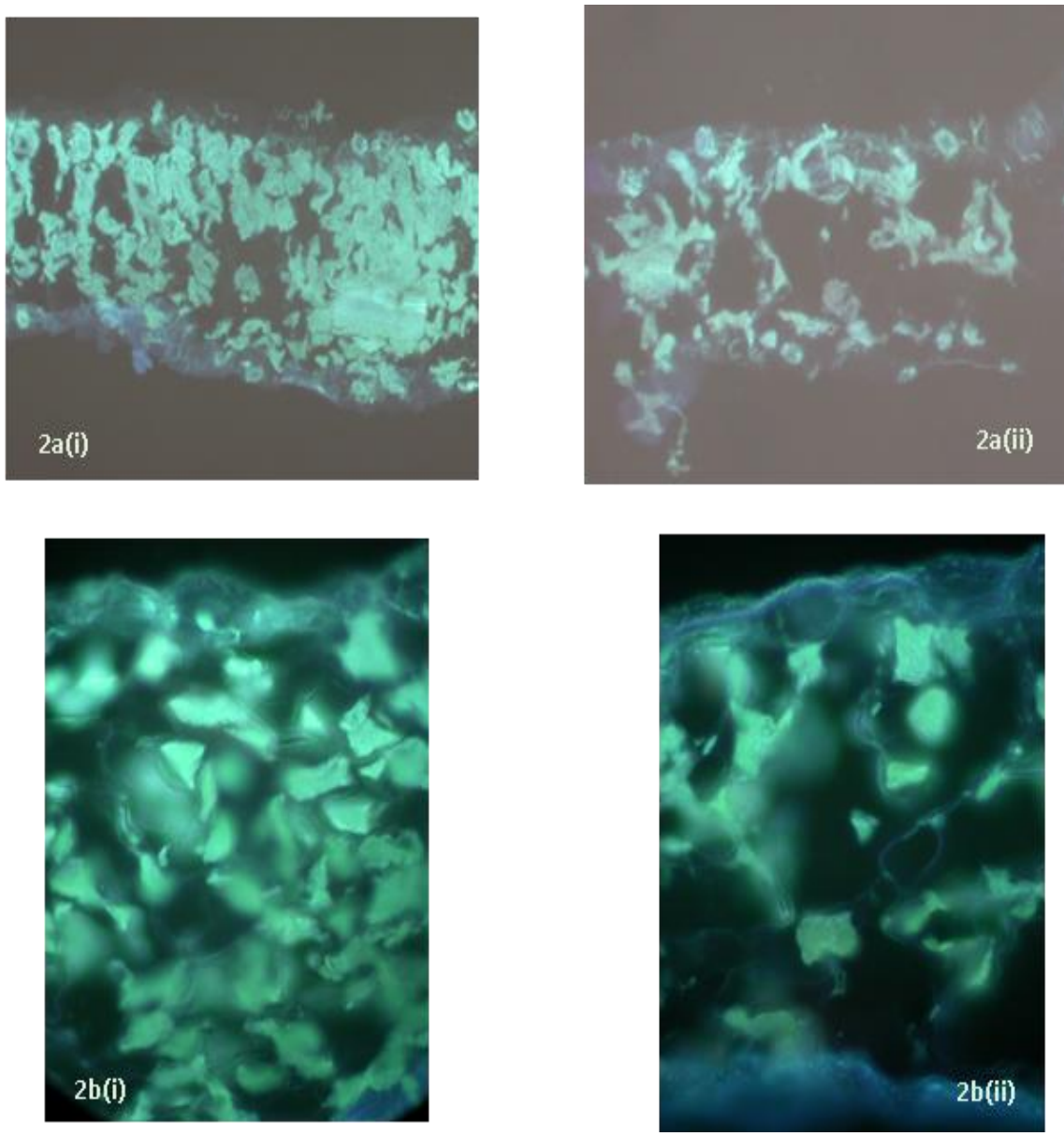


Figure 2: Histochemical analysis of *Plantago* leafs from plants cultivated in greenhouse and outdoors. 2a(i): *P. major* x 100, outdoors. 2a(ii) *P. major*, x 100, greenhouse. 2b(i): *P. major 'Rubrifolia'* x 400, outdoors. 2b(ii): *P. major 'Rubrifolia'* x 400, greenhouse. The greenish-white fluorescence of bioactive caffeic acid derivatives in the outdoor grown plants was more intense and present in almost all mesophyll cells compared to the plants grown in the greenhouse, in which the cells were also more dispersed. At this magnification, it can be observed that in the indoor grown plants the fluorescence was only present in some palisade parenchyma cells. In the plants grown outdoors the bioactive caffeic acid derivatives were present in high concentrations and present in almost all mesophyll cells.

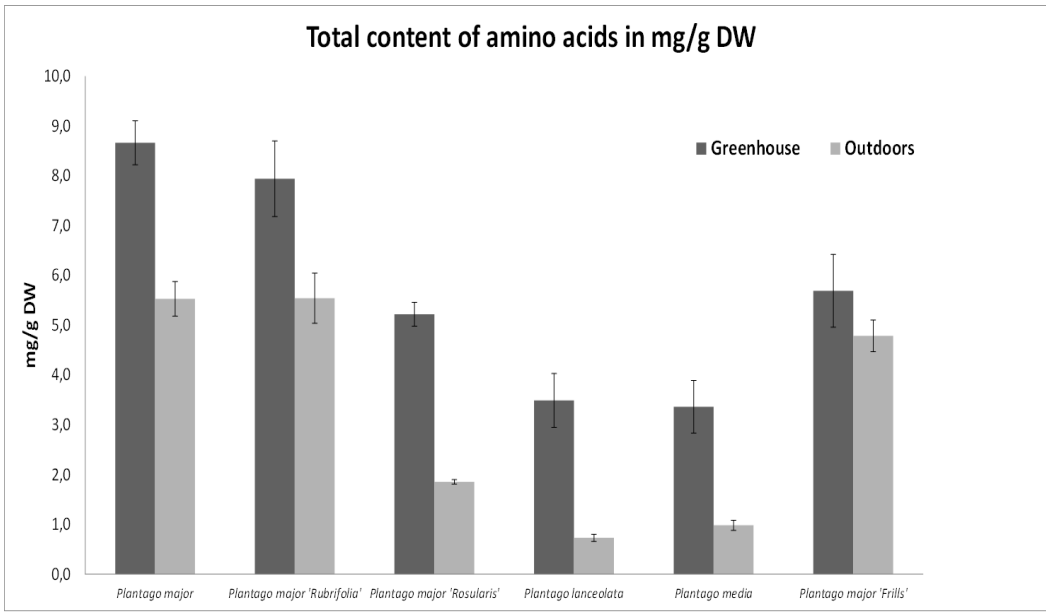


Figure 3: Total content of free amino acids in mg/g dry weight in plants grown in the greenhouse (dark grey) and outdoors (light grey).

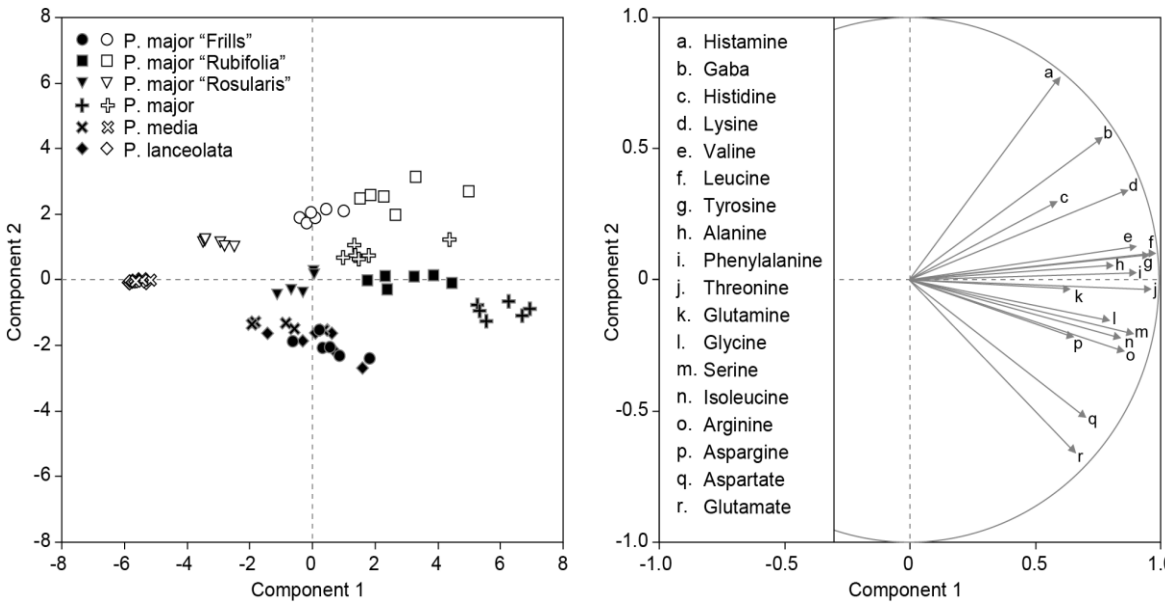


Figure 4: PCA diagram of total amino acid content of six *Plantago* species/varities grown in greenhouse (open signatures) and outdoors (filled signatures). Ala: alanine, Arg: arginine, Asn: asparagine, Asp: aspartate, GABA: gamma-aminobutyric acid, Glm: glutamine, Glu: glutamate, Gly: glycine, Him: histamine, His: histidine, Ile: isoleucine, Leu: leucine, Lys: lysine, Phe: phenylalanine, Ser: serine, Thr: threonine, Tyr: tyrosine, Val: valine.

Table 3: Content of free amino acids, histamine in *Plantago* grown in greenhouseand outdoors.

Species/varieties	Growth conditions	Asp	Glu	Asn	Ser	Glm	His	Gly	Thr	Arg	Ala	
P. major	Greenhouse	1.04 ± 0.06	0.71 ± 0.03	1.07 ± 0.17	0.90 ± 0.03	1.26 ± 0.11	0.02 ± 0.00	0.05 ± 0.00	0.17 ± 0.03	0.12 ± 0.01	*1.23 ± 0.04*	
P. major	**Outdoors**	**0.31 ± 0.02**	**0.31 ± 0.02**	**0.05 ± 0.00**	**0.23 ± 0.02**	**0.52 ± 0.12**	**0.02 ± 0.00**	**0.03 ± 0.00**	**0.09 ± 0.01**	**0.05 ± 0.00**	***1.20 ± 0.10***	
P. major 'Rubrifolia'	Greenhouse	0.99 ± 0.21	0.59 ± 0.08	0.19 ± 0.02	0.52 ± 0.04	2.70 ± 0.23	0.04 ± 0.01	*0.05 ± 0.02*	0.12 ± 0.01	0.12 ± 0.01	*0.83 ± 0.09*	
P. major 'Rubrifolia'	**Outdoors**	**0.41 ± 0.05**	**0.25 ± 0.02**	**0.08 ± 0.01**	**0.28 ± 0.03**	**0.78 ± 0.11**	**0.00 ± 0.01**	***0.05 ± 0.00***	**0.10 ± 0.01**	**0.09 ± 0.02**	***0.81 ± 0.07***	
P. major 'Rosularis'	Greenhouse	0.82 ± 0.11	0.55 ± 0.04	0.07 ± 0.01	0.36 ± 0.03	0.87 ± 0.05	0.01 ± 0.01	0.03 ± 0.00	0.09 ± 0.00	0.06 ± 0.01	0.84 ± 0.05	
P. major 'Rosularis'	**Outdoors**	**0.06 ± 0.01**	**0.12 ± 0.01**	**0.02 ± 0.00**	**0.07 ± 0.01**	**0.05 ± 0.00**	**0.00 ± 0.00**	**0.02 ± 0.01**	**0.05 ± 0.00**	**0.03 ± 0.01**	**0.24 ± 0.01**	
P. lanceolata	Greenhouse	0.64 ± 0.14	0.71 ± 0.12	0.08 ± 0.02	0.23 ± 0.04	0.35 ± 0.08	*0.00 ± 0.00*	0.04 ± 0.01	0.08 ± 0.02	0.12 ± 0.02	0.54 ± 0.10	
P. lanceolata	**Outdoors**	**0.05 ± 0.01**	**0.14 ± 0.01**	**0.01 ± 0.00**	**0.04 ± 0.00**	**0.01 ± 0.00**	***0.00 ± 0.00***	**0.02 ± 0.00**	**0.02 ± 0.00**	**0.01 ± 0.00**	**0.19 ± 0.02**	
P. media	Greenhouse	0.55 ± 0.07	0.69 ± 0.10	0.05 ± 0.02	0.21 ± 0.03	0.31 ± 0.07	*0.00 ± 0.00*	0.04 ± 0.00	0.07 ± 0.02	0.06 ± 0.01	0.66 ± 0.10	
P. media	**Outdoors**	**0.05 ± 0.03**	**0.13 ± 0.05**	**0.01 ± 0.01**	**0.04 ± 0.03**	**0.03 ± 0.04**	***0.00 ± 0.01***	**0.02 ± 0.01**	**0.02 ± 0.01**	**0.01 ± 0.01**	**0.32 ± 0.07**	
P. major 'Frills'	Greenhouse	1.35 ± 0.28	0.77 ± 0.08	0.09 ± 0.01	0.39 ± 0.04	0.76 ± 0.09	0.00 ± 0.00	0.05 ± 0.00	*0.09 ± 0.03*	0.06 ± 0.01	1.05 ± 0.13	
P. major 'Frills'	**Outdoors**	**0.05 ± 0.01**	**0.13 ± 0.02**	**0.01 ± 0.00**	**0.04 ± 0.00**	**0.03 ± 0.00**	**0.00 ± 0.00**	**0.02 ± 0.00**	***0.02 ± 0.00***	**0.01 ± 0.00**	**0.32 ± 0.04**	

Species/varieties	Growth conditions	GABA	Tyr	Val	Met	HisNH2	Phe	Ile	Leu	Lys	Tyramine	TOTAL
P. major	Greenhouse	0.95 ± 0.06	0.09 ± 0.01	0.12 ± 0.01	0.00 ± 0.00	*0.51 ± 0.04*	0.08 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	*0.09 ± 0.01*	0.04 ± 0.01	8.66 ± 0.64
P. major	**Outdoors**	**0.94 ± 0.07**	**0.05 ± 0.00**	**0.09 ± 0.01**	**0.01 ± 0.00**	***0.65 ± 0.06***	**0.04 ± 0.00**	**0.05 ± 0.00**	**0.06 ± 0.00**	***0.07 ± 0.00***	**0.01 ± 0.00**	**4.79 ± 0.45**
P. major 'Rubrifolia'	Greenhouse	0.87 ± 0.10	0.06 ± 0.01	0.10 ± 0.01	0.01 ± 0.00	0.49 ± 0.06	0.05 ± 0.01	*0.06 ± 0.03*	0.08 ± 0.01	0.07 ± 0.01	0.01 ± 0.00	7.94 ± 0.97
P. major 'Rubrifolia'	**Outdoors**	**1.39 ± 0.11**	**0.08 ± 0.01**	**0.11 ± 0.01**	**0.00 ± 0.01**	**0.74 ± 0.06**	**0.07 ± 0.01**	***0.07 ± 0.02***	**0.10 ± 0.02**	**0.11 ± 0.02**	**0.02 ± 0.01**	**5.54 ± 0.63**
P. major 'Rosularis'	Greenhouse	0.75 ± 0.04	*0.04 ± 0.01*	0.07 ± 0.01	0.01 ± 0.00	0.46 ± 0.04	*0.03 ± 0.00*	*0.05 ± 0.00*	*0.04 ± 0.01*	*0.05 ± 0.01*	0.01 ± 0.00	5.22 ±0.42
P. major 'Rosularis'	**Outdoors**	**0.60 ± 0.04**	***0.04 ± 0.00***	**0.06 ± 0.00**	**0.00 ± 0.00**	**0.32 ± 0.01**	***0.03 ± 0.01***	***0.05 ± 0.00***	***0.04 ± 0.01***	***0.05 ± 0.00***	**0.00 ± 0.00**	**1.85 ± 0.14**
P. lanceolata	Greenhouse	0.24 ± 0.05	0.05 ± 0.01	0.09 ± 0.01	*0.00 ± 0.00*	*0.05 ± 0.01*	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	3.49 ± 0.67
P. lanceolata	**Outdoors**	**0.05 ± 0.01**	**0.02 ± 0.00**	**0.02 ± 0.00**	***0.01 ± 0.01***	***0.05 ± 0.01***	**0.02 ± 0.00**	**0.02 ± 0.01**	**0.02 ± 0.00**	**0.02 ± 0.00**	**0.01 ± 0.00**	**0.73 ± 0.09**
P. media	Greenhouse	0.33 ± 0.08	0.05 ± 0.01	0.07 ± 0.02	0.00 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.00 ± 0.00	3.36 ± 0.57
P. media	**Outdoors**	**0.12 ± 0.05**	**0.02 ± 0.01**	**0.03 ± 0.01**	**0.00 ± 0.00**	**0.09 ± 0.04**	**0.01 ± 0.01**	**0.02 ± 0.01**	**0.02 ± 0.01**	**0.02 ± 0.01**	**0.01 ± 0.00**	**0.97 ± 0.43**
P. major 'Frills'	Greenhouse	0.71 ± 0.09	*0.05 ± 0.01*	0.08 ± 0.01	0.00 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.01	0.06 ± 0.01	0.00 ± 0.00	5.69 ± 0.81
P. major 'Frills'	**Outdoors**	**0.12 ± 0.01**	***0.02 ± 0.00***	**0.03 ± 0.00**	**0.00 ± 0.00**	**0.09 ± 0.01**	**0.01 ± 0.00**	**0.02 ± 0.00**	**0.02 ± 0.00**	**0.02 ± 0.00**	**0.01 ± 0.00**	**0.98 ± 0.12**

Valuesare means±Sdof six replicate. Values in italics are not significanty diferent.

DISCUSSION

It is well known that environmental factors, such as exposure to sunlight, temperature, wind, water/moisture and soil composition can influence the presence and content of natural compounds in plants.

UV-absorbing phenolic compounds, such as flavonoids are particularly important for plants to protect their vegetative organs from harmful UV radiation from the sun (Murai et al., 2009). Both plantamajoside and verbascoside were found in the leaves of *P. asiatica* in Mt. Norikura in Japan (Murai et al., 2009). Verbascoside was not detected in plants at low altitude, where the intensity of UV radiation is lower that at higher elevations. This is in agreement with the results of the present study where lower contents of all BCAD were found in plants grown in the greenhouse, where UV radiation is low compared with outdoors. The UV absorbing aspect is directly correlated to the antioxidative activity of the compounds and the antioxidant activity of verbascoside is potentially stronger than that of plantamajoside (Nishibe, 2002). Other studies of this nature have been described for *Plantago* spp. under different environmental conditions. The top DW and content of verbascoside were greater for *P. lanceolata* grown at 15 °C/10 °C day/night temperature compared with 20 °C/18 °C day/night temperature (Tamura et al., 2001). Plants grown under shading had a lower number of leaves per plant, top dry matter weight and top dry matter content, though they were greater in height. The content of verbascoside was significantly lower in plants grown in the shade.

In any growing season, UV exposure and other environmental factors have an influence on the composition and content of the BCAD and amino acids. In two cultivars of *P. lanceolata*, verbascoside concentration increased from 3.4 to 7.1% in Grassland Lancelot cultivar and from 1.5 to 4.1% in Ceres Tonic cultivar over the course of the growing season after which concentrations declined steadily to 2.5% in Grassland and 1.9% in Ceres Tonic (Tamura and Nishibe in 2002). These data suggested that mid-autumn was an appropriate time to harvest plantain for medical use by ensuring a greater concentration of verbascoside.

The concentration of bioactive compounds steadily decreased in the initial stages of drying both under natural climate conditions and at 60 °C. In our study, all the *Plantago* plants were sown and harvested at the same time and once only during the season for both the greenhouse and the outdoor cultivated plants. However, it would be useful to study the effect of greenhouse and outdoor growing conditions throughout the growing season as the differences in content of BCAD and amino acids between the greenhouse and outdoor grown plants may vary during the growing period.

Nitrogen application enhanced the growth of *P. lanceolata* cultivars, especially the top fresh weight and it significantly diminished the top dry-matter content. The amount of verbascoside was apparently lower in the plants treated with nitrogen as opposed to non-fertilized plants and shade repressed the growth and accumulation of both compounds (Tamura, 2001).

The genetic composition of *P. major* is an important determinant for the composition of plantamajoside and verbascoside in the plants. The composition of three BCAD in the different *Plantago* species was genetically determined (Mølgaard, 1986), who observed that plantamajoside was found in both *P. major* subsp. *major* and *P. major* subsp. *pleiosperma*, but verbascoside was only found in *P. major* subsp. *pleiosperma*. The synthesis of caffeic acid esters is determined by a single gene with complete dominance for production of caffeoyl glucose, as found in plantamajoside (Mølgaard et al., 1980), although some population samples may indicate the genetics may be more complex (Mølgaard, 1986; Rohilla et al., 2012a,b).

Histochemical analysis and the HPTLC methods complement each other perfectly. The former can provide qualitative analysis and locate the BCAD compounds visually, both with and without reagents in the different plant cells and HPTLC can be used both for

qualitative visual detection of compounds with or without reagent and quantification by separating the BCAD chromatographically into plantamajoside, iso-plantamajoside and verbascoside.

There are a few reported studies on the determination of individual FAA in *Plantago*, for example, Mohamed et al. (2011) reported the amino acid profile of *P. major*. Sagisaka et al., (1988) described the role of amino acids and inorganic ions in osmoregulatory responses of *Plantago japonica* L. and another study investigated the effect of fungicide treatment on the amino acid content of *P. lanceolata* leaves (Grange and West, 1994). Two studies were also carried out on the amino acid content of *Plantago ovato* (Romero-Baranzini et al., 2006). The content of FAA determined in this study is distinguished from previous reports, since experiments were carried out on protein hydrolysates (Romero-Baranzini et al., 2006). None of the previous studies investigated the effect of environmental conditions on the amino acid content in these *Plantago* species. However, it is interesting to note that in these studies the most abundant amino acids were glutamate, aspartate, glutamine, asparagine and alanine, as was the case in the present study.

There are reports on the effect of solar radiation on the production of mycosporine-like amino acids in several species. Research has shown that UVB/UVA exclusion in the field reduced nitrate reductase (NR) activity in leaves of silver birch seedlings (Krywult et al., 2002). It was confirmed by other observations that enhanced UVB inhibits growth and reduces NR activity in dragon spruce needles (Yao and Liu, 2007) and in young crop seedlings (Rajendiran and Ramanujam, 2006). Nitrate reductase is a key enzyme of N metabolism, which converts nitrate into nitrite in the metabolic pathway leading to the formation of amino acids (Canovas et al., 2007).

Of particular interest to the present paper is a study in 2006 on the influence of solar radiation on NR and the amino acid content of Scots pine needles in subarctic ecosystems (Krywult et al., 2008). It was found that the exclusion of both UVA et UVB light lead to an increase in the amino acid content of the pine needles, an increase that the authors attributed to a lower demand for stress proteins and/or a higher demand for enzyme proteins involved in the saplings' antioxidant scavenging metabolism, given that UV stress is known to generate reactive oxygen species (Turunen and Latola, 2005). The present study also found that amino acid content in outdoor grown plants was significantly lower than in the same plants cultivated under identical conditions in the greenhouse. This has never before been observed in *Plantago* species, but these findings confirm the observation of Krywult et al. (2008), although it should be stressed that they did not report the effects on individual amino acids.

Aromatic amino acids such as phenylalanine and tyramine are important intermediates for the biosynthesis and production of the BCAD. They are involved in the shikimic acid biosynthesis of aromatic caffeoyl esters (Abe et al., 2002) and therefore there is a strong relationship between the two groups of compounds. However, the contents of the BCAD are much higher than the amino acids in all the *Plantago* spp., and especially the aromatic FAA. The FAA, are the products of primary metabolism, are primary metabolites, whereas BCAD are secondary metabolites, often produced as a reaction to stress in plants. It is interesting to note that results in this study clearly demonstrated that BCAD concentrations in plants grown outdoors were significantly greater than those in greenhouse grown plants, whereas the converse was true for FAA. The results of this study underline the importance of looking into more than one group of compounds when environmental studies are performed.

Furthermore a comparative study demonstrating environmental effects on BCAD and amino acids simultaneously has not previously been reported for *Plantago* species. Since the BCAD are important natural compounds, outdoor grown plants with greater amounts of plantamajoside, iso-

plantamajoside and verbascoside would be preferred for the production of Plantago and other plants used for their medicinal properties.

Conclusion

The content of bioactive compounds in plants change with respect to growth conditions. *Plantago* plants grown outdoors were significantly richer in BCAD compared with greenhouse grown plants where UV radiation is lower. In contrast FAA concentrations were significantly richer for greenhouse grown plants. Histochemical analysis clearly illustrated major differences in leaf texture; notably in the plants grown outdoors the BCAD were present at high concentrations in almost all mesophyll cells, but were only detected in some palisade parenchyma in the greenhouse grown plants. This study underlined the importance of growing conditions for plants in order to obtain high concentrations of bioactive compounds either from a nutritional point of view or for the anti-inflammatory properties of BCAD in natural medicine. It also highlights the importance of environmental conditions from an agricultural perspective where plants are used as a food source.

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AUTHORS CONTRIBUTIONS

HPTLC analyses were carried out by HWR, histochemistry by LM, amino acid analysis by MTK and statistical tests and analysis by AML. All authors contributed to writing and editing.

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