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Drought stress increases the production of 5-hydroxynorvaline in two C_4 grasses

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ABSTRACT

Plants produce various compounds in response to water deficit. Here, the presence and identification of a drought-inducible non-protein amino acid in the leaves of two C_4 grasses is first reported. The soluble amino acids extracted from the leaves of three different species were measured by high-performance liquid chromatography of derivatives formed with *o*-phthaldialdehyde and β-mercaptoethanol. One amino acid that increased in amount with drought stress had a retention time not corresponding to any common amino acid. Its identity was determined by metabolite profiling, using ^{1}H NMR and GC–MS. This unusual amino acid was present in the dehydrated leaves of *Cynodon dactylon* (L.) Pers. and *Zoysia japonica* Steudel, but was absent from *Paspalum dilatatum* Poir. Its identity as 2-amino-5-hydroxypentanoic acid (5-hydroxynorvaline, 5-HNV) was confirmed by synthesis and co-chromatography of synthetic and naturally occurring compounds. The amount of 5-HNV in leaves of the more drought tolerant C_4 grasses, *C. dactylon* and *Z. japonica*, increased with increasing water deficit; therefore, any benefits from this unusual non-protein amino acid for drought resistance should be further explored.

1. Introduction

Water deficit is one of the most important factors affecting plant growth, development and survival. In particular in Mediterranean climates, which are characterized by long hot and dry periods, water availability is the major limitation to plant productivity (Turner, 2004). To exacerbate the problem, aridity is expected to increase in many areas of the globe (Petit et al., 1999). Thus, the efficient management of water resources by agricultural and recreational systems is a priority. Therefore, there is increasing pressure to improve the efficiency of water use by plants, namely through the use of species and varieties better adapted to drought conditions.

Plant adaptability to unfavourable environments may involve a number of morphological, physiological, biochemical and molecular adjustments. Some features associated with plant resistance to drought are constitutive rather than adaptive and result from the selection of traits conferring better fitness to arid environments (Chaves et al., 2003). Plant species may adapt to extreme environments through the acquisition of novel biosynthetic capacities. For instance, in the extremophile *Thellungiela halophila*, compared to its relative *Arabidopsis thaliana*, stress-inducible compounds were present in higher amounts in the absence of stress and increased to a greater extent under salt stress (Gong et al., 2005).

Metabolic adjustments in response to the adverse environment conditions may result in production of different types of organic compounds (Shulaev et al., 2008). Metabolome and transcriptome data, namely the identification of metabolites and genes that show increased or decreased amounts and/or expression under drought, should be considered when defining targets for genetic engineering of drought tolerance (Seki et al., 2007). In forage and turf grasses, the molecular basis for stress tolerance remains largely unknown (Zhang et al., 2006).

Warm-season C_4 grasses perform better than their C_3 counterparts at high temperatures and irradiance levels (Brown, 1999). Their efficient assimilation of CO_2 in combination with lower transpiration rates results in high water use efficiency (Edwards et al., 1985). In warm dry summer conditions, C_4 grasses produce higher yields than C_3 grasses (Gherbin et al., 2007), but better drought resistance in C_4 relative to C_3 grasses is not always observed (Ripley et al., 2007).

Three classical variants of C_4 photosynthesis are named after the main decarboxylating enzyme: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and phospho*enol*pyruvate carboxykinase (PEPCK). The bio-geographical distribution of C_4 species demonstrates that NADP-ME species are positively correlated with annual rainfall, whereas NAD-ME species

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predominate in arid zones (e.g., Hattersley, 1992; Cabido et al., 2008). Enhanced water use efficiency in NAD-ME species (Ghannoum et al., 2002) may contribute to explain this distribution. However, as observed by Taub (2000), the distribution of the grass subfamilies Panicoideae and Chloridoideae is also strongly correlated with the precipitation gradients. Thus, characteristics other than the biochemistry of photosynthesis may be responsible for the geographical distributions, and may reflect divergent patterns associated with the multiple origins of C_4 grasses (Kellogg, 1999).

The free amino acids contained in the leaves were investigated in three different C_4 grasses exposed to drought stress: *Paspalum dilatatum* Poir. (NADP-ME), *Cynodon dactylon* (L.) Pers. (NAD-ME) and *Zoysia japonica* Steudel (PEPCK). Dallisgrass (*P. dilatatum*), bermudagrass (*C. dactylon*) and zoysiagrass (*Z. japonica*) are perennial species used for forage and turfgrass purposes throughout the world and especially in tropical and subtropical regions (Brown, 1999). *P. dilatatum* belongs to the subfamily Panicoideae, while *C. dactylon* and *Z. japonica* belong to the subfamily Chloridoideae (Watson and Dallwitz, 1992). Previous studies under rapidly-imposed (Carmo-Silva et al., 2007) and slowly-induced water deficit (Carmo-Silva et al., 2008) suggested better drought tolerance for *C. dactylon* and *Z. japonica* than for *P. dilatatum*. Gradually-induced drought conditions caused changes in amounts of many of the readily identified amino acids (Carmo-Silva et al., 2008, 2009), but one compound that increased with leaf dehydration was not immediately identified. The characteristics of this unusual component and its ultimate identification by metabolite profiling are described.

2. Results and discussion

This investigation arose from more extensive studies of the effects of drought on leaves and carbon metabolism in C_4 grasses. Soluble amino acids were measured to provide an indication of the effects of drought on photorespiration by *P. dilatatum*, *C. dactylon* and *Z. japonica*. The results showed that photorespiration was slow even in drought-stressed leaves (Carmo-Silva et al., 2008), but the analysis also demonstrated increases in many amino acids as a consequence of drought, including a component that was

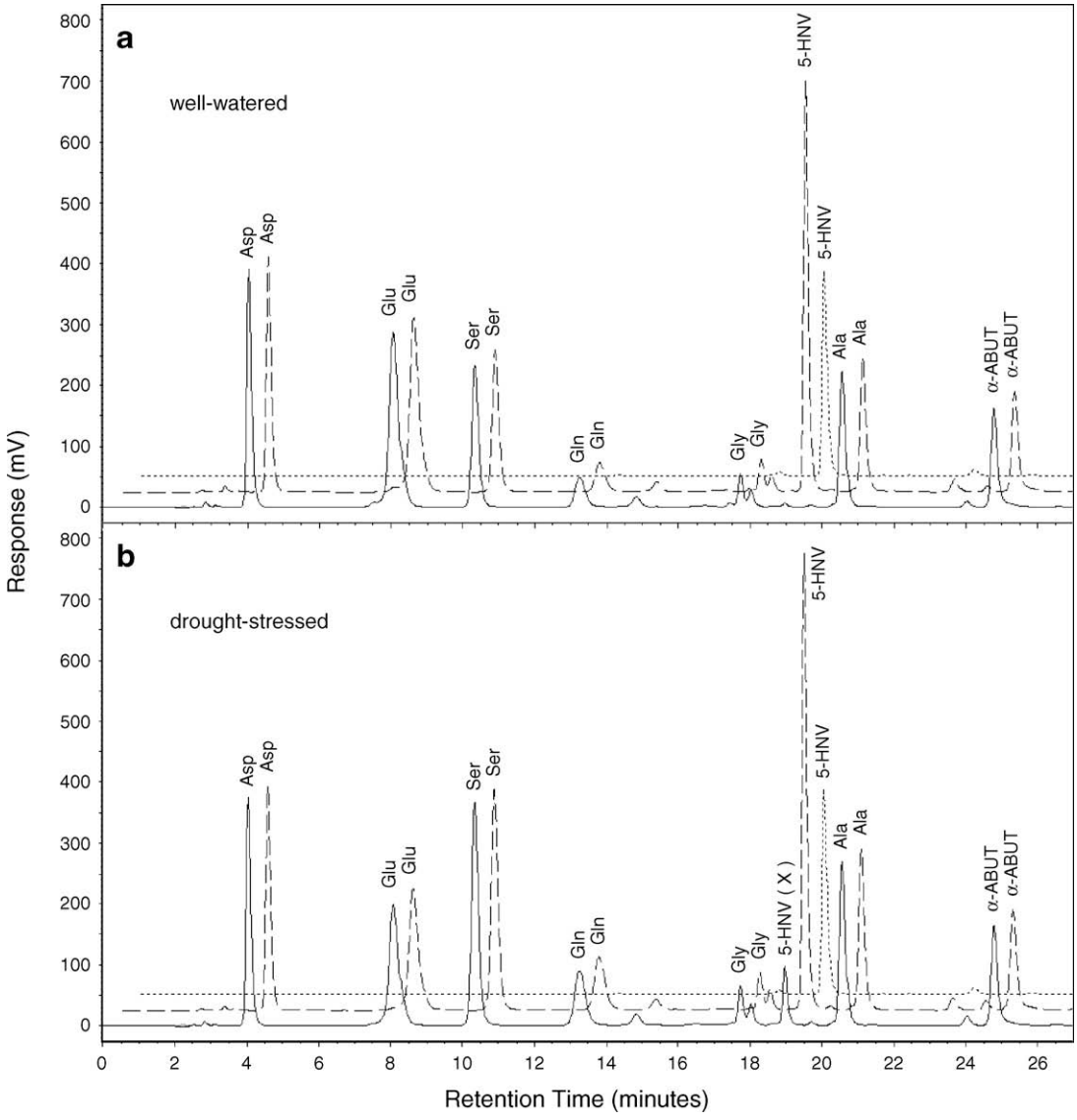

Fig. 1. Reversed-phase high-performance liquid chromatograms of amino acids present in the leaves from well-watered (a) and drought-stressed (b) plants of *Cynodon dactylon* (solid traces). Co-chromatography of each sample with 40 mM 2-amino-5-hydroxypentanoic acid (5-hydroxynorvaline **1**, 5-HNV) resulted in co-elution with the unknown (X) present in the drought-stressed sample (dashed traces). Chromatography of 20 mM 5-HNV (**1**) alone is also shown (dotted traces).

Table 1
Retention times (RT) for various amino compounds as OPA derivatives in reversed-phase HPLC.

Amino compound	RT (min)
GSH + GSSG	2.8
Aspartate	4.0
Glutamate	8.2
Asparagine	10.3
Aminoadipic acid	13.0
Serine	13.2
Methioninesulphoximine	13.9
Glutamine	14.8
Homoserine	16.7
2-Aminopimelic acid	17.0
Histidine	17.3
Citruline	17.5
Glycine	17.8
X	19.0
Ornithine	19.5
Taurine	19.7
β-Alanine	19.8
Alanine	20.6
2-Aminoisobutyric acid	20.9
γ-Aminobutyric acid	21.4
3-Aminoisobutyric acid	21.5
ACC	22.6
Ethanolamine	23.9
α-Aminobutyric acid	24.8
Ammonia	25.0
Tryptophan	26.3
Methionine	27.0
Valine	27.7
Phenylalanine	28.2
Isoleucine	29.9
Leucine	30.4
Putrescine	35.4

not a common protein amino acid. Several amino acids, and especially proline, increased by more than tenfold in the leaves of the C_4 grasses, particularly *C. dactylon* and *Z. japonica*, in response to gradually-induced drought stress (Carmo-Silva et al., 2009). It became a reasonable objective therefore to identify the non-protein amino acid that increased with drought in *C. dactylon* and *Z. japonica* but not in *P. dilatatum*.

Chromatograms of *o*-phthaldialdehyde (OPA) derivatives showed the presence of an unusual compound in the drought-stressed leaves of *C. dactylon* (Fig. 1), whereas the amount present in control leaves was very low or negligible. Leaves of *Z. japonica* from both drought-stressed and well-watered plants contained this compound, but it was not detected in *P. dilatatum* (chromatograms not shown). This unknown component, with a retention time of 19 min, accounted for up to 7% of the total amino compounds measured through high-performance liquid chromatography (HPLC). Besides protein amino acids, many other amino compounds have been reported to be present in the leaves of grasses and some of these vary in amount upon exposure to changing environment conditions (Jones, 1985). An OPA adduct can only be formed with substances having primary amine groups (Stoney-Simons and Johnson, 1978). Table 1 shows the retention times of compounds analysed in attempts to identify the unknown component, shown as "X".

Since X had at first only been characterized as an OPA derivative, its detection by other methods of analysis involved comparison of components in leaf extracts from drought-stressed and well-watered plants of *C. dactylon*. After passage of the leaf acid extracts through a Dowex-50 column, bound components were eluted with 0.05 M NH_4OH. HPLC analysis showed that the component of interest appeared in fractions containing the main amino acids. The purification procedure was applied to extracts from leaves of both well-watered and drought-stressed plants of *C. dactylon*.

^{1}H NMR spectroscopic analysis (Fig. 2) indicated the presence of a new compound in the drought-stressed plant sample. Complex multiplets were observed in the 1D spectrum at δ 1.64 and δ 1.91, which were not present in the ^{1}H NMR spectrum of well-watered plants. A [^{1}H–^{1}H] COSY experiment indicated correlations between these peaks and suggested further correlations to peaks at δ 3.64 and δ 3.72. The signal at δ 3.72 was characteristic of a proton on C_2 of an amino acid, while a spin-system of three bond couplings in the COSY spectrum (δ 3.72–1.91, δ 1.91–1.64 and δ 1.64–3.64) indicated a chain of four hydrogen-bearing carbon

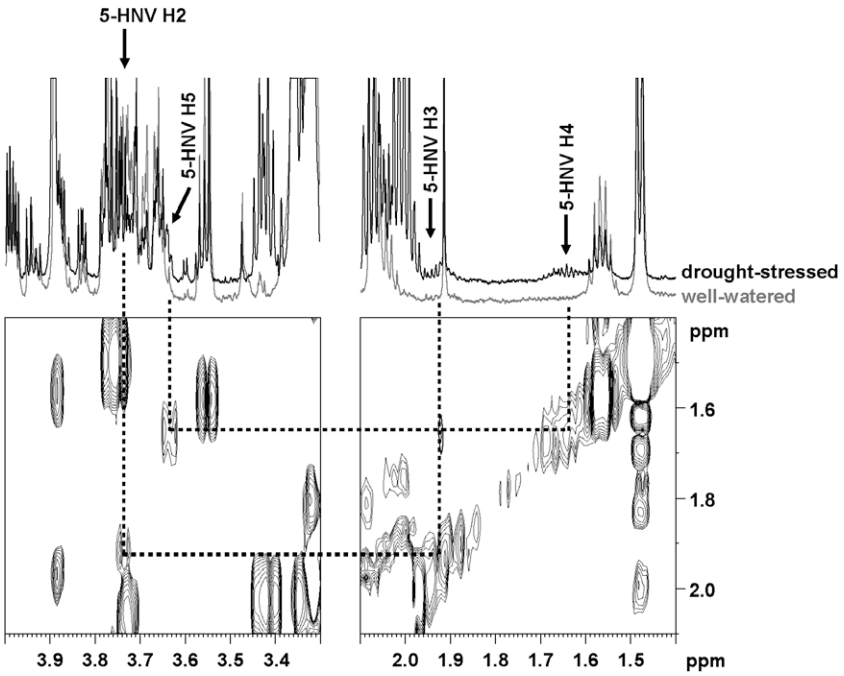

Fig. 2. Sections of ^{1}H NMR spectra and [^{1}H–^{1}H] COSY correlations of leaves from well-watered (grey traces) and drought-stressed (black traces) plants of *Cynodon dactylon*.

atoms, with the chemical shift of the terminal methylene (δ 3.64) being suggestive of an electronegative substituent such as a hydroxyl group.

GC–MS analysis (Fig. 3) of the trimethylsilyl (TMS) derivatives of components from extracts of drought-stressed and well-watered leaf samples indicated an additional component with a retention time of 7.35 min in the drought-stressed material. Analysis of its mass spectrum indicated an M^+ of 349 with fragments consistent with those expected from a TMS derivative of 5-hydroxynorvaline **1** (5-HNV). Comparison of the mass spectra of endogenous 5-HNV **1** to that synthesized in the laboratory showed that there was good agreement between the two spectra. Thus, by a combination of the structural information from ^{1}H NMR (Fig. 2) and GC–MS (Fig. 3) analyses, the unusual amino acid was identified as 2-amino-5-hydroxypentanoic acid (5-hydroxynorvaline **1**, 5-HNV) (Fig. 4). Co-chromatography of the synthetic and the naturally occurring compound (Fig. 1) further confirmed its identity.

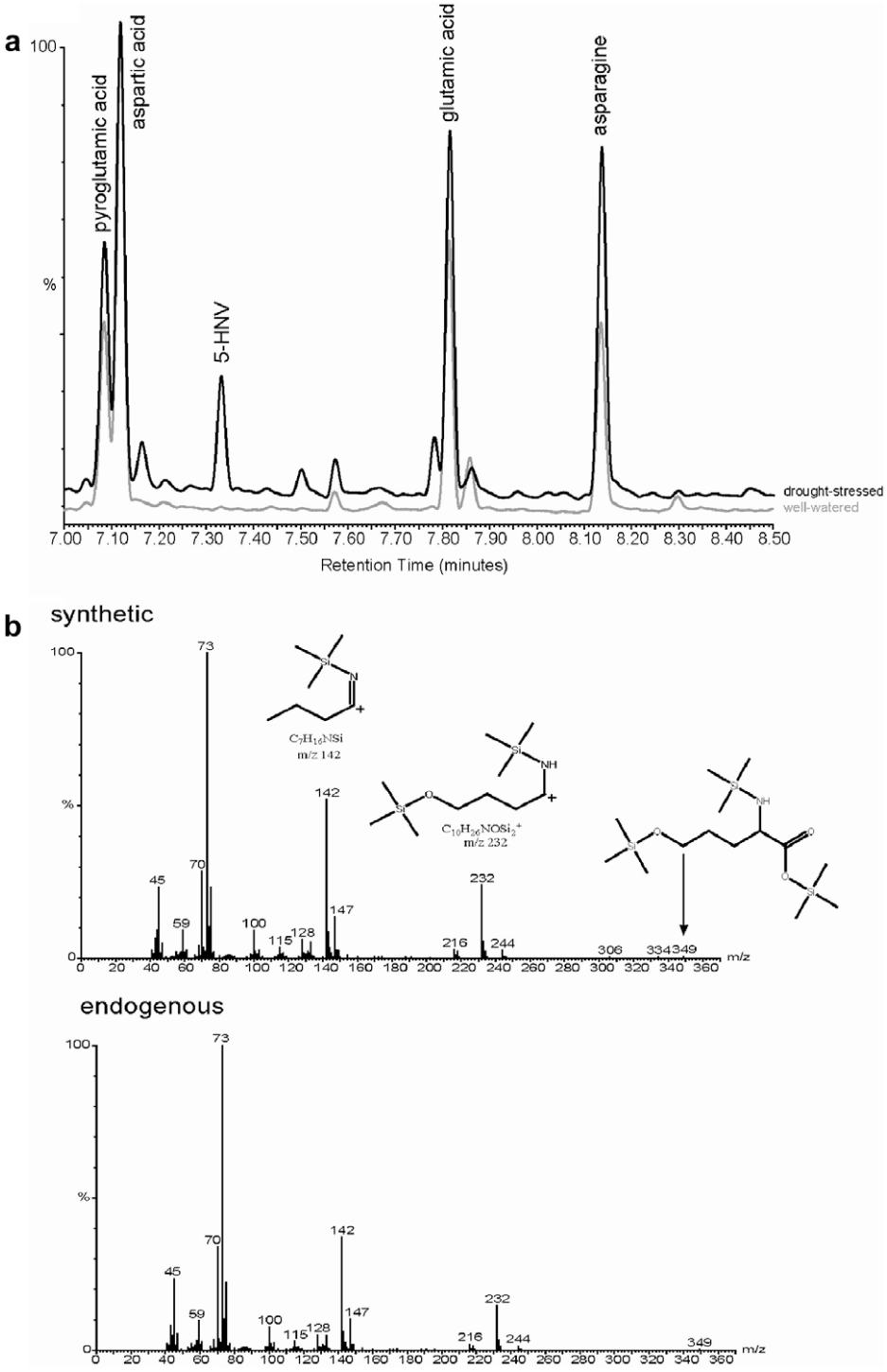

Fig. 3. GC–MS analysis of trimethyl silylated derivatives of components in leaf extracts from well-watered (grey trace) and drought-stressed (black trace) plants of *Cynodon dactylon* (a) and comparison of mass spectra for the derivatives of synthetic 2-amino-5-hydroxypentanoic acid (5-hydroxynorvaline **1**, 5-HNV) and the endogenous compound (b). Structures giving rise to fragments with *m*/*z* 142, 232 and 349 are shown.

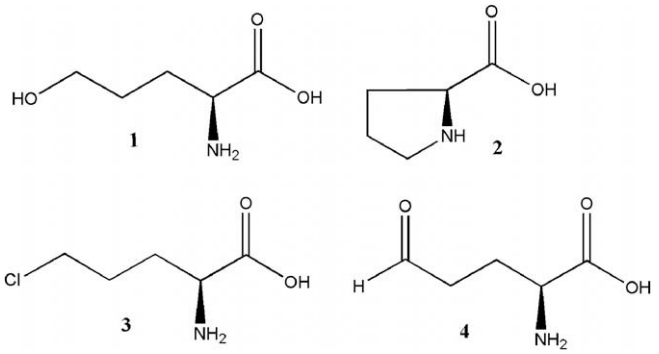

Fig. 4. Structures of the 2-amino-5-hydroxypentanoic acid (5-hydroxynorvaline, 5-HNV) and related compounds mentioned in the text: 5-hydroxynorvaline (**1**), proline (**2**), 5-chloronorvaline (**3**) and glutamic γ-semialdehyde (**4**).

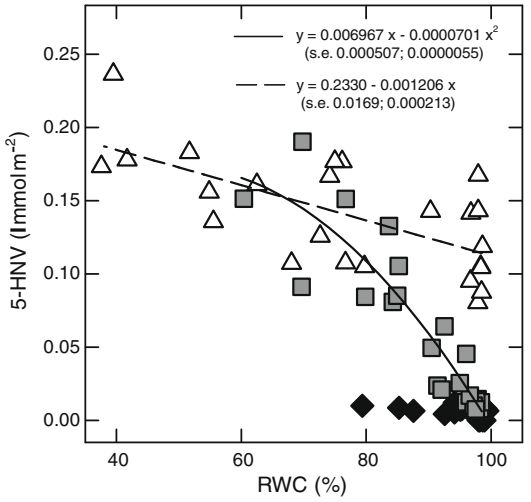

Fig. 5. Variation of the content in 5-hydroxynorvaline (**1**) (5-HNV) with the relative water content (RWC) in the leaves of *Paspalum dilatatum* (black diamonds), *Cynodon dactylon* (grey squares, solid line) and *Zoysia japonica* (open triangles, dashed line). Each data point corresponds to one sample, with 24 samples per species. Regression lines were fitted when the RWC effect was significant ($P < 0.05$; $R^2 = 0.898$, $s^2 = 0.03151$, d.f. = 68, $P < 0.001$).

The non-protein amino acid 5-HNV (**1**) was present in all leaves of *Z. japonica* and in leaves from drought-stressed plants of *C. dactylon*, whereas no significant ($P > 0.05$) amounts were found in *P. dilatatum* (Fig. 5). The content of 5-HNV (**1**) increased with leaf dehydration in both *C. dactylon* and *Z. japonica*, but the increase was much greater in the former species, attaining values similar to those found in *Z. japonica* at *ca.* 65% RWC. To the best of our knowledge, the presence of 5-HNV (**1**) in plant leaves has not previously been reported, although it is a known constituent of legume seeds (Thompson et al., 1964; Dunnill and Fowden, 1967). Some molecular responses commonly observed during the maturation and desiccation of seeds may also be observed in vegetative tissues upon exposure of plants to drought conditions (Bray, 1993). Preliminary analyses of leaves from well-watered and drought-stressed plants of both wheat (C_3) and maize (C_4, NADP-ME) showed no significant amounts of 5-HNV (**1**) to be present. 5-HNV (**1**), however, accumulated in cultures of pyridoxine auxotrophs of *Escherichia coli* B (Hill et al., 1993), and an enzyme from *Neurospora crassa* catalysed the reversible conversion of 5-HNV **1** to glutamic γ-semialdehyde **4** (Yura and Vogel, 1959). Hill et al. (1993) discussed some conflicting evidence for metabolism of 5-HNV (**1**) in *Streptomyces* sp.

The structural relationship of 5-HNV (**1**) to precursors in the biosynthesis of proline (**2**) and ornithine have made it of special interest in the past (Thompson et al., 1964). However, there seems to be no evidence for a metabolic process directly involving 5-HNV (**1**) in either the synthesis or degradation of proline (**2**) and the conversion of 5-HNV (**1**) to proline (**2**) *in vitro* requires harsh conditions. Gaudry (1951) reported the synthesis of DL-proline (**2**) from DL-5-HNV (**1**) by prolonged heating with HCl. Subsequently 5-HNV (**1**) was identified as a product of HCl hydrolysis of proteins that had been chemically modified. Thomas et al. (1983) observed the production of 5-HNV (**1**) through borohydride reduction of a thiol ester of a glutamate residue present in the transmembrane human C_3 protein, followed by HCl hydrolysis of the modified protein. Depending on the conditions, proline (**2**) was also formed through the formation of 5-chloronorvaline (**3**) as an intermediate. Jennings and Anderson (1987) also reacted exposed carboxyl groups in a transmembrane protein with Woodward's reagent K, and reduced the adducts formed with borohydride; hydrolysis with HCl released 5-HNV (**1**) formed from a glutamate residue and this 5-HNV (**1**) was partly converted to proline (**2**) under the strong acidic conditions. Oxidation of several proteins by metal catalysis in the presence of O_2, followed by reduction with borohydride and HCl hydrolysis, produced 5-HNV (**1**) derived from proline (**2**) and arginine residues (Amici et al., 1989). The presence of 5-HNV (**1**) in protein hydrolysates made after reduction with borohydride is a good indicator of prior oxidation by active oxygen species (Ayala and Cutler, 1996). One possible advantage of proline (**2**) accumulation in plants under stress is the effective scavenging of reactive oxygen species both by the free amino acid and by proline (**2**) residues in proteins (Matysik et al., 2002).

Conserved plant responses under adverse environmental conditions may involve synthesis of compounds of primary metabolism, while specific divergent responses are more likely to involve products of secondary metabolism (Sanchez et al., 2008). The drought-induced accumulation of proline in C_4 grasses (Jones, 1985) was observed in *P. dilatatum*, *C. dactylon* and *Z. japonica*, with a similar variation of this amino acid with leaf dehydration in all three grasses (Carmo-Silva et al., 2009). Conversely, there is a clear distinction of the response of 5-HNV (**1**) in the three C_4 species (Fig. 5). Previous studies with these grasses suggested that *C. dactylon* and *Z. japonica* were better adapted to conditions of water scarcity than *P. dilatatum*, in agreement with the geographical distribution of grasses of the C_4 subtype NADP-ME (Hattersley, 1992; Cabido et al., 2008) and the subfamily Panicoideae (Taub, 2000) in areas with good rainfalls. *Z. japonica* shows constitutive characteristics of adaptation to xeric environments that enable it to resist drought conditions (Carmo-Silva et al., 2009). *C. dactylon* responds to decreased water availability by quickly buffering the adverse effects of the stress event, and thus keeping an active photosynthetic metabolism, through efficient stomatal control that delays water loss (Carmo-Silva et al., 2008). The presence of 5-HNV (**1**) in leaves of *Z. japonica* before exposure to water deficit, together with the drought-inducible increase in the production of 5-HNV (**1**) both in leaves of *Z. japonica* and *C. dactylon*, suggests a possible link with an adaptive drought stress response. Neither the biosynthetic pathway of 5-HNV (**1**), nor its subsequent metabolism, is known in plants. The presence and drought-induced increase of 5-HNV (**1**) in leaves of C_4 grasses with greater drought resistance needs further investigation in order to understand if this response is common to other grass species and if it plays an important role in stress defensive mechanisms.

3. Concluding remarks

A non-protein amino acid present in the leaves of two grasses was identified as 5-hydroxynorvaline (**1**). This compound seems not to have been reported previously in leaf tissue. The amounts

present were increased by water deficit in two C_4 grasses that were previously shown to be more drought tolerant.

4. Experimental

4.1. Plant material

The C_4 grasses *P. dilatatum* Poir. cv. Raki, *Cynodon dactylon* (L.) Pers. var. Shangri-Lá and *Zoysia japonica* Steudel 'Jacklin Sunrise Brand' (produced by Jacklin Seed Company, Post Falls, ID, USA) were grown from seeds in pots with peat-free compost in a glasshouse as previously described (Carmo-Silva et al., 2008). Artificial light was provided whenever the natural light was below a photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1} during a 16 h photoperiod. Temperature was maintained at *ca.* 25 °C during the day and 18 °C during the night. All plants were well-watered until the beginning of the drought stress treatment. Pots were placed according to a split-plot design and water deficit was imposed by ceasing to provide water to the 'stress' pots. Young fully expanded leaves of five-week old plants of *P. dilatatum* and *C. dactylon* and nine-week old plants of *Z. japonica* were collected in the growth environment 4–5 h after the beginning of the photoperiod. Three control and five non-watered pots were used per species per day for three consecutive days, corresponding to 8–12 days without watering (Carmo-Silva et al., 2008), making a total of 24 samples per species (nine control and 15 non-watered pots). A second leaf sample was collected from each pot for determination of the leaf relative water content (RWC) (Catsky, 1960).

4.2. High-performance liquid chromatography of o-phthaldialdehyde derivatives

The preparation of acid extracts of leaves, derivatisation with *o*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol, and HPLC were carried out essentially as described by Noctor and Foyer (1998). Analysis used a Waters Alliance 2695 Separation Module, a 474 Scanning Fluorescence Detector and a Waters Symmetry C_{18} 3.5 μm 4.6 × 150 mm column (Waters, Milford, MA, USA). The autosampler was set to mix and pre-incubate 10 μL of sample with 15 μL of OPA reagent for 2 min at 25 °C immediately before injecting this mixture onto the column. The OPA reagent (40 mM OPA, 0.28 M 2-mercaptoethanol, 0.45 M H_3BO_3 adjusted to pH 9.2 with NaOH) was prepared *ca.* 12 h before each run and used within the following three days.

A linear elution gradient was formed using solution A (80% 50 mM NaOAc pH 5.9, 19% MeOH, 1% THF) and solution B (80% MeOH, 20% 50 mM NaOAc pH 5.9) and was initiated with 100% A for the first minute and continued with increasing B to 10% from 6 to 10 min, to 45% from 16 to 20 min, to 100% from 32 to 40 min and then decreasing to 0% from 41 to 46 min, at a flow rate of 0.8 mL min^{-1}. The column temperature was 30 °C. Separations by similar methods are described by Hunt (1991) and Brückner and Westhauser (2003).

4.3. Acid extraction of leaves

Leaf samples (50–200 mg fr. wt.) were placed in liquid N_2 and stored at −80 °C. Each sample was ground to a fine powder in liq. N_2 and mixed with 0.1 M HCl (1.4 mL). Each mixture was ground further during thawing to produce a fine suspension. The homogenate so obtained was centrifuged for 10 min (16,000*g*) at 4 °C. Samples (100 μL) of each supernatant were then mixed with 120 μM α-amino-*n*-butyric acid (100 μL) as an internal standard and 1 mL of H_2O to give an HCl final concentration of 0.017 M. Mixtures were stored at −20 °C, then later thawed and centrifuged for 40 min (16,000*g*) at 4 °C. Supernatants were filtered using 0.2 μm syringe filters into 2 mL HPLC autosampler vials. Standard solutions of α-amino-*n*-butyric acid, and other amino compounds were prepared in 0.1 M HCl and diluted as needed for introduction into the column from the autosampler in 0.017 M HCl (final concentration). Amino acids in leaf samples were quantified by reference to standards using Waters Millenium^{32} software (Waters, Milford, MA, USA).

4.4. Purification and concentration of the amino acid fraction from leaf acid extracts

Samples of leaf acid extracts (1 mL) were applied to Dowex-50 NH_4^+ columns (0.6 mL). These were eluted individually with 0.05 M NH_4OH, with 1 mL fractions collected. Fractions containing amino acids were combined, and solutions were freeze-dried.

4.5. ^{1}H NMR analyses of a concentrated amino acid fraction from leaf acid extracts

^{1}H NMR spectra were recorded at 600.05 MHz with 64k complex data points over a sweep width of 12 ppm on a Bruker Avance 600 spectrometer (Bruker BioSpin, Coventry, UK) at a temperature of 300 K (*ca.* 28 °C). The freeze-dried amino acid fractions were dissolved in CD_3OD in D_2O (1:4, v/v) and referenced to 0.05% w/v 2,2,3,3-d_4-sodium 3-(trimethylsilyl) propionate (d_4-TSP) at δ 0.00. The residual HOD peak was eliminated by a pre-saturation pulse during the 5 s relaxation delay. Parts of the [^{1}H] spectrum of the new amino acid could be discerned by comparison of the spectra of drought-stressed and well-watered *C. dactylon* samples (multiplets at δ 1.64 and δ 1.91). The remainder of the signals of the new amino acid (δ 3.64 and δ 3.72) was observed in a [^{1}H–^{1}H] COSY spectrum of the mixture, that was recorded with 128 t1 increments of 2048 data points. The spectrum was acquired with 32 scans over a sweep width of 9 ppm; the residual HOD peak was eliminated by a pre-saturation pulse during the 1.5 s relaxation delay.

4.6. GC–MS analyses of TMS-amino acids

A 50 μL sample solution was transferred to a 1.1 mL high recovery vial and evaporated to dryness *in vacuo*. To the residue obtained, was added methoxyamine hydrochloride in anhydrous pyridine (50 μL, 16 mg mL^{-1}). The sample was heated at 30 °C for 90 min with continuous agitation at 1400 rpm in a Thermomixer Comfort (Eppendorf AG, Cambridge, UK). A volume of 70 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) + 1% trichloromethylsilane (TMCS) was then added and the sample was heated for a further 30 min at 37 °C with continuous agitation (1400 rpm).

GC–MS analysis was carried out using a Pegasus III time of flight mass spectrometer (Leco Corp., St. Joseph, MI, USA) coupled to an Agilent 6890N gas chromatograph system (Agilent Technologies, Palo Alto, CA, USA) fitted with an MPS2 autosampler (Gerstel, GmbH, Mülheim an der Ruhr, Germany) and a DB-5 ms capillary column (15 m × 0.18 mm ID × 0.18 μm d.f. with 5 m integrated guard column). An aliquot (0.5 μL) of each liquid sample was injected using a splitless injection technique with an inlet temperature of 280 °C and helium gas carrier flow of 1.4 mL min^{-1}, constant flow. The oven was set to an initial temperature of 70 °C for 2 min, then ramped at 17 °C min^{-1} to 350 °C and held for 1.5 min. The GC interface and source temperatures were 310 and 245 °C, respectively, and EI+ (electron ionization) mass spectra were acquired from 40 to 800 amu at 70 eV from 3.75 to 20 min with an acquisition rate of 20 spectra s^{-1}. Spectra of known amino acids were assigned by reference to an in-house spectral library and the NIST library. The novel amino acid TMSi derivative gave an EI spectrum with *m*/*z* (%) 349 [0.6, M^+], 334 (0.4, M–CH_3), 232

(24, M–CO_2TMSi), 147(914), 142(53), 100(9), 75(22), 73(100), 70 (28), 59(9) and 45(24).

4.7. Chemical synthesis

5-Hydroxynorvaline (**1**) was synthesized from methyl glutamic acid by adaption of the method of Thompson et al. (1964). Methyl glutamic acid (1 g) was added in small portions under dry N_2, with stirring, to a 2 M solution of $LiBH_4$ in THF (Sigma–Aldrich, Gillingham, UK; 12.5 mL). The solution was stirred overnight, and then heated under N_2 until reflux began. This being maintained for 6 h. After cooling, the reaction mixture was poured into ice-cold MeOH–H_2O (60:40, v/v) and stirred for 40 min. The solution was acidified to pH 5.0 with glacial HOAc and then concentrated *in vacuo* to approximately 10 mL. Examination by TLC (*n*BuOH–HOAc–H_2O, 4:1:1; ninhydrin detection) showed the presence of two products. Aliquots (1 mL) of this solution were applied to cation exchange resin cartridges (10 × 200 mg, SCX, Extract-Clean, Alltech Co., Ltd., Tokyo, Japan) washed with H_2O with the 'neutral' flow-through collected each time. The target compound (lower TLC spot) was contained in the flow-through with the eluent for each separation combined (*ca.* 20 mL). The compound responsible for the upper TLC spot was retained on these cartridges and then eluted with 2 M NH_4OH. Final purification of 5-hydroxynorvaline (**1**) (lower TLC spot) was achieved by elution of a 1 mL (from 20 mL) portion through another SCX cartridge, where it was retained and subsequently eluted with 2 M NH_4OH. Evaporation of the solution gave 5-hydroxynorvaline **1** (6 mg) containing 10% proline **2** as an impurity, as estimated by NMR spectroscopic and GC–MS analyses. HREI–MS [$(TMSi)_3$ derivative] *m*/*z* 349.1933, [M^+, 0.6%] (calc. for $C_{14}H_{35}NO_3Si_3$, 349.1925): *m*/*z* (%) 334 (0.4, M–CH_3), 232 (24, M–CO_2TMSi), 147(914), 142(53), 100(9), 75(22), 73(100), 70(28), 59(9) and 45(24); HREI–MS [$(tBuMe_2Si)_3$ derivative] *m*/*z* 475.3336, [M^+] (calc. for $C_{23}H_{53}NO_3Si_3$, 475.3333). ^{1}H NMR (D_2O:CD_3OD, 4:1): δ 1.64 (2H, *m*, 4-H_2), 1.91 (2H, *m*, 3-H_2), 3.64 (2H, *dt*, J = 6.3 and 1.3 Hz, 5-H_2) and 3.72 (1H, *dd*, J = 6.7 and 5.4 Hz, 2-H). This compound was identical (GC–MS and NMR) to the compound identified in the natural amino acid extract.

4.8. Statistical analysis

Regression analysis was applied to the variation of the content of 5-HNV with RWC in the leaves of the C_4 grasses, using GenStat® 9.2, 2005 (Lawes Agricultural Trust, Rothamsted Research, Harpenden, UK). The non-significantly different parameters (*t*-tests, $P < 0.05$) of the fitted model (*F*-test, $P < 0.05$) were amalgamated in order to attain parsimony. The residuals were checked and found to conform to the assumptions of the analysis.

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