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Occurrence and stress response of *N*-methylproline compounds in *Tamarix* species

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Abstract

A number of *N*-methylproline analogues have been found to accumulate in different species of *Tamarix*. These include *N*-methyl-L-proline (MP), *trans*-4-hydroxy-*N*-methyl-L-proline (M4HP) and *trans*-3-hydroxy-*N*-methyl-L-proline (M3HP). The three compounds appeared in all species but their relative and absolute levels depend upon species, ecotype and level of applied salt stress. A salt-conditioned ecotype of *T. jordanis* (Sodom) dramatically increased its accumulation of all proline analogues when subject to salt stress whereas a non-saline ecotype (Gilboa) showed little effect. The levels of M4HP and M3HP in *T. meyeri* increased with increasing salt stress whereas MP levels remained almost constant.

Keywords: *Tamarix*, Salt stress; Proline analogues; Compatible solutes; NMR

1. Introduction

Tamarix is a genus belonging to a group of woody plants native to the arid and semi-arid regions of the world. The various species within the genus have diverse life forms, different crown architecture, different leaf structures and most of them are deciduous, only *T. aphylla* being evergreen. Because of the fast growth and the high salt tolerance of most species of *Tamarix* the trees are planted as dune stabilizers and windbreaks in coastal areas. In such habitats, the trees would be expected to have some protective mechanisms to combat the high salinity levels of their environment.

One of the mechanisms that enables adaptation to dry and saline habitats is the accumulation of compatible solutes (Waisel, 1972, 1991; Poljakoff-Mayber et al., 1987; Aspinall and Paleg, 1981). Indeed, it has been shown that various woody plants can accumulate proline and proline analogues in response to environmental stress (Naidu et al., 1987, 2000). For example, various species of *Melaleuca* accumulate *N*-methyl-L-proline (MP), *trans*-4-hydroxy-*N*-methyl-L-proline (M4HP) and *trans*-4-hydroxy-*N*,*N*′-dimethyl-L-proline (DMHP) (Jones et al., 1987).

The occurrence of mono-*N*-methylated hydroxyproline derivatives in plants has not been widely reported although they are found in certain algae (Sciuto et al., 1983) and in *Croton gubouga* (Goodson and Clewer, 1919) in addition to Melaleucas (Naidu et al., 2000). On the other hand, the non-*N*-methylated hydroxyprolines are ubiquitous in nature, occurring as the free amino acids in low concentrations in plants and in combination with proline in plant cell wall

Abbreviations: MP, (*N*-methyl-L-proline); M4HP, (*trans*-*N*-methyl-4-hydroxy-L-proline); M3HP, (*trans*-*N*-methyl-3-hydroxy-L-proline); DMHP, (*trans*-4-hydroxy-*N*,*N*′-dimethyl-L-proline).

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matrices and in animal cartilage. Cartilage is a rich source of 3-hydroxyproline, a proline derivative also found in relatively low concentrations in the seeds and young shoots of *Delonix regia* (Sung and Fowden, 1968, 1971). Whilst the non-*N*-methylated hydroxyproline analogues appear to have no protective role in plants the *N*-methylated compounds have been shown not only to accumulate in plants as a result of salt stress and water stress (Naidu et al., 1987), but to protect the activity of Rubisco, isolated from various ecotypes of *T. jordanis*, when plants were exposed to high levels of NaCl (Solomon et al., 1994). As a result of the latter study, *trans*-3-hydroxy-*N*-methyl-L-proline (M3HP) was identified in the same taxa of *Tamarix* (Jones et al., 1995) in amounts sufficient to protect the in vivo activity of Rubisco. In the current study, several species and/or ecotypes of *Tamarix* were investigated and characterised for: (a) their ability to accumulate proline analogues and (b) the effect of salt stress on endogenous levels of such substances in the shoots.

2. Results and discussion

MP, M4HP and M3HP have been identified and measured in *Tamarix* extracts by ^{1}H NMR spectroscopy using the N–CH_3 peak in each compound for quantitation. This peak occurs at 2.99 ppm for MP, 3.09 ppm for M4HP and 3.06 ppm for M3HP (pH 1.0, D_2O). These compounds were found in all the investigated taxa although the relative amounts varied (Table 1).

Additional unidentified compounds in trace quantities as well as choline (Ch) were also seen in the NMR spectra (*). Low levels of M3HP were found in *T. aphylla* whilst *T. jordanis* (S) and *T. meyeri* had the highest levels. This also coincided with high levels of MP and M4HP. The data presented in Table 1 are from field grown trees where little reliable information is available about water availability, partial contributions of roots at different soil horizons and salt levels at these horizons. Some information about potential salt stress can be gained from gross measures of sodium and chloride ion concentrations in the root zones in the particular areas where the samples were collected (Table 2). However, comparisons of salt concentrations in the root zone of plants grown under field and laboratory conditions is not an easy task and should be regarded cautiously.

It is clear that Qalya, Sodom and Acre are all high salinity areas whereas Gilboa, the Waite Arboretum and the Melbourne Botanic Gardens are low salinity areas. However, differences in these measures of proline analogue concentrations and those of the same tree material grown under controlled conditions are not necessarily comparable and some of the high levels measured could be due to the fact that the trees were suffering from a range of environmental stresses.

When grown in hydroponic culture, the ecotype of *T. jordanis* (S), which grows in areas of high salinity, showed large increases in the concentration of all three proline analogues in response to an increase in NaCl concentration in the rootgrowing medium whereas the levels of these analogues remained similar to that of the control in the Gilboa ecotype when subjected to the same elevated levels of NaCl (Table 3).

When young plants of *T. meyeri* were subjected to salt stress both M3HP and M4HP levels increased with increases in salinity levels but MP levels fluctuated much less, and erratically (Table 4). The fact that these analogues are accumulated as a result of salt stress is illustrated by their levels in both *T. jordanis* and *T. meyeri* when sub-

Table 1
Concentration[a] of *N*-methylproline analogues identified in various species of field grown *Tamarix* trees collected from various regions

Species	Origin	MP	M4HP	M3HP
T. jordanis: Gilboa	Israel	38.4 ± 3.2	55.8 ± 1.6	36.6 ± 3.7
T. jordanis: Qalya	Israel	25.9 ± 1.1	23.1 ± 3.8	18.1 ± 5.2
T. jordanis: Sodom	Israel	27.2 ± 1.7	67.6 ± 10.1	48.1 ± 12.1
T. nilotica: Mikhmoret	Israel	22.1 ± 0.8	32.3 ± 1.2	22.4 ± 0.6
T. meyeri: Kishon	Israel	33.2 ± 1.3	78.9 ± 3.6	48.8 ± 3.2
T. aphylla	Waite	15.7 ± 1.0	40.1 ± 4.7	8.7 ± 0.8
T. aphylla	Melbourne	10.0 ± 0.9	34.4 ± 1.1	3.5 ± 0.2
T. parviflora	Melbourne	8.7 ± 1.2	11.9 ± 0.1	17.7 ± 2.8
T. ramossisima	Melbourne	6.5 ± 1.2	21.8 ± 1.8	24.0 ± 2.0

Trees were mature, 2–4 m high with the scale-like leaves and green stem parts collected.

[a] Expressed as μmol/g dry wt. Measurements were made in duplicate with standard errors given.

Table 2
The salt content of the soils of the various habitats

Location	Sodium ion (meq/L)	Chloride ion (meq/L)
Qalya	173–250	190–227
Sodom	168–232	184–378
Mikhmoreth	17–22	19–36
Acre	158–287	177–206
Mt. Gilboa	12–18	17–22
Kishon	15–67	11–66
Waite Arboretum	3–16	6–16
Melbourne Botanic Gardens	3–19	6–22

Estimated values for the salt concentrations recorded in the root zone, summed over different soil horizons and during various seasons of the year are listed. Sodium ion concentrations were determined by flame photometry and chloride ion with a chloridimeter.

Table 3
Changes in *N*-methylproline analogue concentration[a] in *Tamarix jordanis* ecotypes grown in hydroponic solution as a result of applied salt stress

Ecotype		Treatment	MP	M4HP	M3HP
Sodom	Control	(1.5 mM NaCl)	27	65	60
Sodom	Salt stress	(101.5 mM NaCl)	82	131	92
Gilboa	Control	(1.5 mM NaCl)	22	75	62
Gilboa	Salt stress	(101.5 mM NaCl)	25	89	69

[a] Expressed as μmol/g dry wt. A single determination for each treatment, only, was made.

Table 4
Changes in *N*-methyl proline analogue concentration[a] in *Tamarix meyeri* (Acre ecotype) grown in hydroponic culture as a result of salt stress

Treatment	MP	M4HP	M3HP
0 mM NaCl (control)	16.8 (±5.5)	27.9 (±4.1)	25.8 (±6.2)
100 mM	6.1 (±1.6)	39.2 (±4.1)	39.7 (±5.7)
200 mM	10.4 (± 2.7)	54.6 (±1.7)	54.7 (±2.1)

[a] Expressed as μmol/g dry wt. Results are the means of three replicates with standard errors given in parentheses.

jected to saline media with increased salt concentration. The results with *T. jordanis*, however, would suggest that those ecotypes which are conditioned to higher salinity levels in their native habitats, they have a greater ability to accumulate the proline analogues than those that originated in salt-free habitats. Exposure to increased levels of salinity also caused increased levels of both M4HP and M3HP, but not of MP, in *T. meyeri*. It is suggested, therefore, that the plants with low internal concentrations of *N*-methylprolines have a disadvantage in growth/viability under high salinity conditions.

The biosynthetic pathways that lead to the formation of *N*-methylated hydroxyproline analogues are not fully understood with the sequence of hydroxylation and *N*-methylation not known. In animal systems the formation of both 3- and 4-hydroxylated proline has been shown to occur through the incorporation of proline into polypeptides which is then hydroxylated via an O_2-dependent reaction. Specific hydroxylases have been shown to produce *trans*-3- and *trans*-4-hyroxyproline residues as a function of sequence specificity in proline-rich peptides (see Adams and Frank, 1980). In plants, both insoluble cell wall-bound proteins (Lamport and Miller, 1971) and soluble proteins rich in hydroxyproline (Mani and Radhakrishnan, 1974) have been reported. The process of hydroxylation has been shown to again occur through the action of hydroxylases on protein-bound prolyl residues although the hydroxylases are thought to be different from animal hydroxylases based on sequence specificities (Sadava and Chispeels, 1971; Adams and Frank, 1980). The prolyl hydroxy groups in these proteins are predominantly glycosylated with arabinose. Alternatively, free hydroxyprolines have been shown to be formed by the enzymatic hydoxylation of free proline in a number of plants; *cis*-4-hydroxy-L-proline being formed in Sandalwood (Kuttan and Radhakrishnan, 1970) and *trans*-3-hydroxy-L-proline in the germinating seeds of *Delonix regia*. (Sung and Fowden, 1971). Whilst the accumulation of free proline has been shown to have a beneficial effect on plant growth, particularly in situations where the plants are subject to drought stress (Aspinall and Paleg, 1981) the presence of the free hydroxyprolines appears to be detrimental in some plants. Avena coleoptile growth was shown to be inhibited by free *trans*-4-hydroxyproline (Cleland, 1967) as was the radical growth of mung bean seedlings by *trans*-3-hydroxyproline (Sung and Fowden, 1968). *N*-methylation of hydroxyprolines in some plants may provide a method for their detoxification akin to glycosidation in other plant systems. It is unlikely that this alone would explain the accumulation of high concentrations of *N*-methylated hydroxyprolines in plants such as *Tamarix* and *Melaleuca* unless large quantities of hydroxyprolines are produced, possibly by the breakdown of hydroxyproline-rich peptides in the plants.

N-methylation of proline in alfalfa has been reported to occur via a sequential methylation of free proline via the intermediate NMP to produce stachydrine although NMP itself was not detected (Essery et al., 1962). Stachydrine also accumulates in various *Medicago* species but the presence of NMP was not detected and it does not accumulate in this taxa (Naidu et al., 1992). In *Tamarix*, however, only mono-*N*-methylated species are found; no *N*,*N*′-dimethyl derivatives have yet been observed, indicating that these compounds are probably not biosynthetic intermediates. It is possible that in *Tamarix* the initial product formed is MP from the action of a mono-*N*-methyl transferase on free proline. This compound may then be the intermediate in the formation of M4HP and M3HP by specific hyroxylases acting on the MP, the relative concentrations of MP and its hydroxylated forms depending upon specific enzyme activities. It is important to note that MP was present in all *Tamarix* extracts investigated. In *Melaleuca* species, on the other hand, this is not the case. *M. lanceolata* and related species appear to accumulate only M4HP whilst others accumulate MP as well as M4HP and a number of species also accumulate DMHP. This suggests that different *N*-methyl transferases are active in *Tamarix* and in some *Melaleucas* species as well as different hydroxylases, and that these systems may be useful for in vivo studies of solute biosynthesis.

We have attempted to determine the localisation of proline analogues in *Tamarix* leaves. Attempts to detect these compounds in isolated chloroplasts failed (data not shown) in contrast to the finding of glycinebetaine in the isolated chloroplasts of species that accumulate betaines in their leaves (Robinson and Jones, 1986). It is possible that *N*-methylproline analogues do not accumulate in *Tamarix* chloroplasts or that due to the weaker charge properties of these tertiary amines compared to betaines they leak out of the chloroplasts during isolation by passive diffusion and are not observed.

3. Experimental

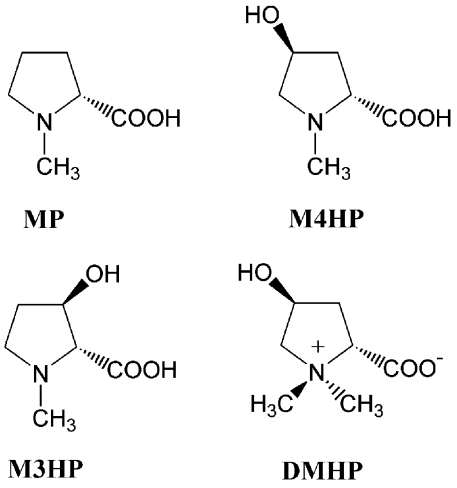
3.1. Plant material

Young green shoots of three ecotypes of *T. jordanis* Boiss were collected from three habitats in Israel: (1) the saline flats of Sodom (S), Dead Sea region; (2) near Mt. Gilboa (G), a low salinity habitat in the upper Jordan Valley (Northern Israel); and (3) high salinity wet sites near Qalya, on the southern end of the Jericho Valley. Samples of shoot tissues of *T. nilotica* (Ehrenb.) Bge., and *T. meyeri* Boiss., were collected in the Coastal Plain of Israel, one site

near Mikhmoreth which had low saline levels and the other from the salt marshes near Acre. All trees were 2–4 m high and amply branched. Samples of *T. aphylla* (L.) Karst were obtained from the Waite Arboretum (Adelaide), *T. parviflora* DC, *T. aphylla* and *T. ramosissma* Ledeb. from the Melbourne Botanic Gardens (Melbourne) both of which have low saline levels. A comparison of salt content of the soils in the various habitats is given in Table 2. They are estimated values for the the salt concentrations recorded in the root zone, at different soil horizons and during various seasons of the year. All samples were collected in summer. Approximately 10 g of green shoot tissue was sampled, air dried and then stored at 4 °C until analysed. For the salt stress experiments, woody shoots of *T. jordanis* and *T. meyeri* were collected from the Sodom and Gilboa, and Acre sites, respectively, and were then grown up in hydroponic culture. Plants were grown for 4–6 weeks in the hydroponic solution. Salinity treatments were applied after an establishment period of approximately 2 weeks. In addition, approximately 400 g of *T. ramosissima* were collected from the foreshore of Tumby Bay (South Australia) for the extraction and purification of M3HP for analytical measurements.

3.2. Extraction of tissue for estimation of solutes

0.5 g of air-dried tissue was extracted into aqueous methanol and the basic solutes partially purified by cation exchange chromatography (Jones et al., 1986). The column was eluted with 4 N HCl which was then removed on a rotary evaporator. The residue was washed with water which was evaporated on a rotary evaporator to remove the residual hydrochloric acid. The resulting residue was dissolved in 1000 μL of D_2O for NMR analysis (Jones et al., 1986). Extractions and analyses were done in duplicate. The method does not determine non-charged species such as polyols and sugars which may be present in the tissue (see Scheme 1).



Scheme 1.

3.3. ^1H NMR analyses

^1H NMR measurements were made in 5 mm tubes using a Varian Unity 300 spectrometer operating at 300 MHz at 25 °C. A spectral width of 4500 Hz, a pulse width of 4.5 μs (45°) and a recycle delay of 5 s were used. Routinely, the data from 16 acquisitions were accumulated into 16 K data space with a line broadening of 0.3 Hz applied prior to Fourier transformation. *t*-Butanol (2 μmol) was added as an internal reference for quantitation and for peak positions (δt-butanol = 1.245 ppm).

3.4. Salt stress experiments

Woody cuttings of *T. jordanis* were collected from the Sodom and Gilboa ecosystems and grown in hydroculture using half-strength Hoagland's solution (Hoagland and Arnon, 1950) supplemented with 1.5 mM NaCl (control) or 101.5 mM NaCl (salt stressed). The solutions were continuously aerated. Shoots were collected when the plants were 4 months old.

An additional experiment was conducted with *T. meyeri*. Woody cuttings were grown as for *T. jordanis* , in triplicate, for 4 months and then transferred to jars containing 0, 100 and 200 mM NaCl. After 7 days the green shoots were harvested, dried and later analysed for proline analogues.

3.5. Isolation of analytically pure trans-3-hydroxy-N-methyl-L-proline

Four hundred grams of fresh *T. ramosissima* shoots were extracted with two 1.5 L portions of 70% aqueous methanol, draining and pressing the shoots in a muslin bag. The extraction solvent was reduced in volume to approximately 400 ml (also removing all the methanol) and centrifuged at 4000 rpm in a bench top centrifuge. The clarified supernatant was then loaded on to an Amberlite IR-120 cation exchange column (30 mm dia × 650 mm long) in the H^+ form. After washing the column with water (2 L) the amino acid derivatives were eluted from the column with 1 L of 4 N HCl. The acid eluent was dried yielding approximately 12 g of a reddish brown solid. The solids were taken up in 50 mL of ethanol leaving behind about 2 g of a white crystalline solid (shown to be inorganic salts). The ethanol was removed on a rotary evaporator and the residue dissolved in approximately 20 mL of water. This solution was loaded on to a similar size column as above but containing Dowex 50X4–200 resin in the H^+ form. After washing the column with 2 L of water a solvent reservoir was connected to the column via a peristaltic pump and a fraction collector was connected to the column outlet. The column was eluted with a linear gradient of HCl ranging from 0 to 1 M using a total solvent volume of 2.5 L. Thirty millilitre fractions were collected with the compounds detected by thin layer chromatography of the individual fractions (silica gel plates; solvent 85% ethanol, 15% conc. ammonia; detection, iodine vapour). MP was

eluted in fractions 18–26, M3HP in fractions 29–40 and M4HP in fractions 37–46. The fractions containing only M3HP were pooled, dried on a rotary evaporator, and recrystallised from methanolic solution by the addition of anhydrous ether and cooling, yielding 1.3 g white crystals as the hydrochloride. Crystals suitable for X-ray diffraction studies were grown from the same solvent system (Jones et al., 1995).

A small aliquot of the hydrochloride was converted to the free base on an Amberlite IR 120 H column by elution with 2 M NH_4OH. The free base gave the following ^1H, ^{13}C NMR and FAB-MS data: ^1H NMR (300 MHz, D_2O, pH 5.5); 4.64 ppm, *m*, 1p.; 3.89 ppm, *m*, 2p; 3.40 ppm, *m*, 1p; 3.03 ppm, *s*, 3p; 2.13 ppm, *q*, 2p. Proton-decoupled ^{13}C NMR (75.13 MHz, D_2O, pH 5.5); 173.36 ppm (–COOH), 80.88 ppm (C-2), 76.77 ppm (C-3), 57.42 ppm (C-5), 44.91 ppm (N–CH_3), 35.05 ppm (C-4). The FAB-MS gave a molecular ion peak $(M - H)^+$ at 146.0.

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