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Structural, biochemical, and physiological characterization of photosynthesis in two C₄ subspecies of *Tecticornia indica* and the C₃ species *Tecticornia pergranulata* (Chenopodiaceae)

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Abstract

Among dicotyledon families, Chenopodiaceae has the most C₄ species and the greatest diversity in structural forms of C₄. In subfamily Salicornioideae, C₄ photosynthesis has, so far, only been found in the genus *Halosarcia* which is now included in the broadly circumscribed *Tecticornia*. Comparative anatomical, cytochemical, and physiological studies on these taxa, which have near-aphyllous photosynthetic shoots, show that *T. pergranulata* is C₃, and that two subspecies of *T. indica* (*bidens* and *indica*) are C₄ (Kranz-tecticornoid type). In *T. pergranulata*, the stems have two layers of chlorenchyma cells surrounding the centrally located water storage tissue. The two subspecies of *T. indica* have Kranz anatomy in reduced leaves and in the fleshy stem cortex. They are NAD-malic enzyme-type C₄ species, with mesophyll chloroplasts having reduced grana, characteristic of this subtype. The Kranz-tecticornoid-type anatomy is unique among C₄ types in the family in having groups of chlorenchymatous cells separated by a network of large colourless cells (which may provide mechanical support or optimize the distribution of radiation in the tissue), and in having peripheral vascular bundles with the phloem side facing the bundle sheath cells. Also, the bundle sheath cells have chloroplasts in a centrifugal position, which is atypical for C₄ dicots. Fluorescence analyses in fresh sections indicate that all non-lignified cell walls have ferulic acid, a cell wall cross-linker. Structural–functional relationships of C₄ photosynthesis in *T. indica* are discussed. Recent molecular studies show that the C₄ taxa in *Tecticornia* form a monophyletic group, with incorporation of the Australian endemic genera of Salicornioideae, including *Halosarcia*, *Pachycornia*, *Sclerostegia*, and *Tegicornia*, into *Tecticornia*.

Key words: C₃ plants, C₄ plants, Chenopodiaceae, chloroplast ultrastructure, *Halosarcia*, immunolocalization, NAD-ME type, photosynthetic enzymes, phylogeny, *Tecticornia*.

Introduction

In the family Chenopodiaceae, which has C₃ and C₄ species, all C₄ genera occur in subfamily Chenopodioidae (*Atriplex*) and in a succulent clade made up of three
subfamilies: Suaedoidae (Suaeda and Bienertia), Salsoloideae (various genera), and Salicornioideae (Halosarcia) (Carolin et al., 1975; Pyankov, 1991; Akhani et al., 1997; Jacobs, 2001; Pyankov et al., 2001a; Kadereit et al., 2003; Kapralov et al., 2006; Akhani and Ghasemkhani, 2007). This family has the largest number of C4 species and also the greatest diversity in leaf anatomy among dicot families, including C4 Kranz and C4 single-cell type species, as well as C3 type species (Carolin et al., 1975; Sage et al., 1999; Edwards et al., 2004). Six C4 types of Kranz anatomy (atriplicoid, kochioid, salsoloid, halosarcoid, and, in the genus Suaeda, salsina and schoberia types) and five C3 types (axyroid, corispermoid, austrobossioid, neokochioid, and sympegmoid) have been described among species of this family, mostly in corresponding genera (Carolin et al., 1975, 1982; Voznesenskaya, 1976b; Voznesenskaya and Gameley, 1986; Jacobs, 2001; Kadereit et al., 2003). Recently, leaf anatomy in representative Chenopodiaceae species was further revised with the description of 15 C4 types (Kadereit et al., 2003). The C4 types of anatomy vary in the structure and arrangement of the two-layered chlorenchyma adjacent to the vascular bundles, and by the presence or absence of water storage (WS) tissue, hypodermal cells, and selerenchyma, and whether they have continuous or interrupted Kranz tissue.

Species in subfamily Salicornioideae are hydrohalophytic plants which belong to the most salt-tolerant angiosperms inhabiting salt marshes and inland saline habitats. In this subfamily, only one species, Halosarcia indica, has been identified as C4 on the basis of its anatomy and C4-type carbon isotope composition, while 11 species of this genus have C3-type carbon isotope composition (Wilson, 1980; Carolin et al., 1982; Akhani et al., 1997).

Carolin et al. (1982) studied the anatomical structure in several representatives of the genus Halosarcia. Species with C3-type carbon isotope values had 2–3 layers of chlorenchyma tissue surrounding WS parenchyma, while several subspecies of H. indica had C4-type isotope values and Kranz anatomy. Unlike the salsoloid type of Kranz anatomy, an unusual occurrence of colourless cells within Kranz anatomy and ultrastructure of chlorenchyma, and the phylogenetic position of these representatives of Tecticornia in subfamily Salicornioideae was also evaluated.

The aim of the present study was to characterize the anatomy and ultrastructure of chlorenchyma, and the unusual occurrence of colourless cells within Kranz anatomy, to identify the C4 biochemical subtype, and analyse features of CO2 fixation in T. indica (using two subspecies which occur on different continents and are visibly different, bidens and indica). Comparative analyses were made with the C3 species T. pergranulata. The phylogenetic position of these representatives of Tecticornia was placed in subfamily Salicornioideae.

Materials and methods

Plant material

Seeds of T. pergranulata (J. M. Black) K. A. Sheph. & Paul G. Wilson subsp. pergranulata and T. indica subsp. bidens (Nees) K. A. Sheph. & Paul G. Wilson were provided by G Barrett, Greg Barrett & Associates, Darlington, Western Australia. Seeds of T. indica (Willd.) K. A. Sheph. & Paul G. Wilson subsp. indica were collected by H Akhani from Pakistan, 40 km NW of Karachi (H. Akhani 16537). Seeds were stored at 3–5 °C prior to use, then germinated on moist paper in Petri dishes in a growth chamber at 30/25 °C and a photosynthetic photon flux density (PPFD) of 75 μmol m−2 s−1 with a 14/10 h light/dark photoperiod. The seedlings were then transplanted to 10 cm diameter pots with commercial potting soil and grown for 3 d under the same regime. Established plants were then transferred to a growth chamber (model GC-16; Enconoir Ecological Chambers Inc., Winnipeg, Canada) and grown under ~400 PPFD with a 16/8 h light/dark photoperiod and 25/18 °C day/night temperature regime. For microscopy and biochemical analyses, samples of mature segments were taken from ~2.5- to 3-month-old plants.


Light and electron microscopy

Hand-cut sections of fresh stems were placed in water and studied under a light stereo microscope. The area of chlorenchyma tissue external to the central cylinder, and of WS tissue, as a percentage of the total cross-sectional area was determined from digital images (on ~10 cross-sections taken from two different plants) using UTHSCSA, Image Tool for Windows, version 3.00, University of Texas Health Science Center, San Antonio, TX, USA.

For microscopy on fixed material, samples were taken from 2–3 plants (5–6 samples from 2–3 branches of each plant). Samples for structural studies were fixed at 4 °C in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), post-fixed in 2% (w/v) OsO4, and then, after a standard acetone dehydration procedure, embedded in Spurr’s epoxy resin. Cross-sections were made on a Reichert Ultracut R ultramicrotome (Reichert-Jung GmbH, Heidelberg, Germany). For light microscopy, semi-thin sections were stained with 1% (w/v) toluidine blue O in 1% (w/v) Na2B4O7. Ultra-thin sections were stained for transmission electron microscopy with 2% (w/v) uranyl acetate followed by 2% (w/v) lead citrate. Hitachi H-600 (Hitachi Scientific Instruments, Mountain View, CA, USA) and JEOL JEM-1200 EX (JEOL USA, Inc., Peabody, MA, USA) transmission electron microscopes were used for observation and photography.
For scanning electron microscopy (SEM), leaf samples were fixed at 4 °C in 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 0.05 M PIPES buffer, pH 7.2. The samples were dehydrated with a graded ethanol series and embedded in London Resin White (LR White, Electron Microscopy Sciences, Fort Washington, PA, USA) for 1 h. After rinsing, the sections were ready for analysis by light microscopy. CWs and starch stained bright red, while other elements of the cells (cytoplasm) remained unstained. Controls lacking the periodic acid treatment (required for oxidation of the polysaccharides giving rise to Schiff’s-reactive groups) showed little or no background staining (not shown).

Western blot analysis

Total proteins were extracted from leaves by homogenizing 500 mg of tissue in 1 ml of extraction buffer [100 mM TRIS-HCl, pH 7.5, 5 mM MgSO4, 10 mM dithiothreitol, 5 mM EDTA, 0.5% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 1 mM phenylmethylsulphonyl fluoride, and 25 μg ml−1 each of aprotinin, leupeptin, and pepstatin]. After centrifugation at high speed for 3 min in a microcentrifuge, the supernatant was collected and the protein concentration was determined by Bradford protein assay (Bio-Rad) using BSA as a standard. Protein samples (10 μg) were separated by 12.5% SDS–PAGE, blotted onto nitrocellulose, and probed with anti-A. hypochondriacus NAD-ME (1:5000), anti-Z. mays NADP-malic enzyme (NADP-ME), and anti-PPDK (1:5000), anti-Z. mays PEPC (1:10 000), anti-Z. mays PPDK (1:5000), anti-Urochloa maxima phosphoenolpyruvate carboxykinase (PEP-CK), courtesy of RC Legood, and anti-Spinacia oleracea Rubisco LSU (1:10 000) overnight at 4 °C. Goat anti-rabbit IgG–alkaline phosphatase conjugate antibody (Bio-Rad) was used at a dilution of 1:5 000 for detection. Bound antibodies were localized by developing the blots with 20 mM nitroblue tetrazolium and 75 mM 5-bromo-4-chloro-3-indolyl phosphate in the detection buffer (100 mM TRIS-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl2).

Acidity

Plant samples were collected just before the beginning of the light period, in the middle of the day, and in the late afternoon just before the beginning of the dark period. Samples of known fresh weight (between 0.2 g and 0.5 g) were ground in 2 ml of distilled water.

In situ immunolocalization

Leaf samples were fixed at 4 °C in 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 0.05 M PIPES buffer, pH 7.2. The samples were dehydrated with a graded ethanol series and embedded in London Resin White (LR White, Electron Microscopy Sciences, Fort Washington, PA, USA) acrylic resin. Antibodies used (all raised in rabbit) were anti-Spinacia oleracea L. Rubisco LSU IgG (courtesy of B McFadden), commercially available anti-Zea mays L. phosphoenolpyruvate carboxylase (PEPC) IgG (Chemicon, Temecula, CA, USA), anti-pyruvate, Pi dikinase (PPDK) IgG (courtesy of T Sugiyama), anti-L. mitochondrial NAD-malic enzyme (NAD-ME) IgG (courtesy of J Berry), which was prepared against the 65 kDa α-subunit (Long and Berry, 1996), and anti-Pismum sativum L. glycine decarboxylase (GDC) against the α-subunit (Ruzin, 1999). The periodic acid–Schiff’s procedure (PAS) was used for staining starch in sectioned materials. Sections, 0.8–1 μm thick, were made from the same samples used for immunolocalization, dried onto gelatin-coated slides, incubated in periodic acid [1% (v/v)] for 30 min, washed, and then incubated with Schiff’s reagent (Sigma, St Louis, MO, USA) for 1 h. After rinsing, the sections were ready for analysis by light microscopy. CWs and starch stained bright reddish pink, while other elements of the cells (cytoplasm) remained unstained. Controls lacking the periodic acid treatment (required for oxidation of the polysaccharides giving rise to Schiff’s-reactive groups) showed little or no background staining (not shown).

Staining for polysaccharides

The periodic acid–Schiff’s procedure (PAS) was used for staining starch in sectioned materials. Sections, 0.8–1 μm thick, were made from the same samples used for immunolocalization, dried onto gelatin-coated slides, incubated in periodic acid [1% (v/v)] for 30 min, washed, and then incubated with Schiff’s reagent (Sigma, St Louis, MO, USA) for 1 h. After rinsing, the sections were ready for analysis by light microscopy. CWs and starch stained bright reddish pink, while other elements of the cells (cytoplasm) remained unstained. Controls lacking the periodic acid treatment (required for oxidation of the polysaccharides giving rise to Schiff’s-reactive groups) showed little or no background staining (not shown).

Fluorescence of chloroplasts and cell walls, and lignification

Hand-cut sections of leaves or stems were placed on slides in distilled water and examined under UV light [with a 4',6-diamidino-2-phenylindole (DAPI) filter] with a Zeiss LSM 510 META (Jena, Germany) microscope. For comparison, similar sections were treated with 0.1 M NH4OH to reveal the presence of CW-bound ferulic acid. According to Harris and Hartley (1976, 1980), Hartley and Harris (1981), and Rudall and Caddick (1994), if the tissue contains CW-bound ferulic acid, an increase of the pH (to ~10.3) will change the blue fluorescence of CWs to blue-green by ionization of the phenol OH group. This treatment does not change the autofluorescence of CWs in lignified or suberized tissues. To detect the position of lignified tissue, sections were treated for 1 h with phloroglucinol (2% in 10% HCl), which stains lignin-containing CWs red, while for detection of suberization, sections were stained with Sudan IV in 70% alcohol, which stains suberized CWs dark red (Ruzin, 1999).

C4 photosynthesis in the genus Tecticornia

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The sample was titrated with 0.01 M NaOH to a pH 7 end point using a pH meter, and the µeq acid per g fresh weight was calculated.

**Measurements of rates of photosynthesis**

Rates of photosynthesis in response to light were measured with a CO2 analyser (ADC LCPro+, ADC BioScientific Ltd, Hoddesdon, UK) operating in a differential mode. The air temperature was 25±0.5°C (stem temperature was 25–27°C), the minimum humidity was 12.0±0.5 mbar, and the flow rate was 200 µmol s⁻¹. The local average barometric pressure, as determined by the CO2 analysing system, was 922.3±3.4 mbar.

For each experiment, part of a branch of an intact plant (3–4 months old) was enclosed in the conifer chamber designed for terete or semi-terete leaves. The branch was illuminated with 920 PPFD under 370 µbar CO2 until a steady-state rate of CO2 fixation was obtained (generally 40–50 min). For varying light experiments at 370 µbar CO2, measurements were made beginning at 1380 PPFD, followed by decreasing increments of light intensity at 4 min intervals.

For measurement of the response of photosynthesis to varying CO2 at 2% and 21% O2, and for determining the CO2 compensation point based on Rubisco carboxylase/oxygenase activity (Γ*), gas exchange was measured with the FastEst gas system (see Laik and Edwards, 1997; Sun et al., 1999). A branch was enclosed in a small leaf chamber (4 cm³ × 0.5 cm) with an open gas flow rate of 0.5 mmol s⁻¹. The chamber temperature was maintained at 25°C, with the water jacket of the chamber connected to a thermostated water bath. Both sides of the branch were illuminated with a PPFD of 900 µmol quanta m⁻² s⁻¹ (measured with a Li-Cor 185 quantum sensor) at the glass window by fibreoptics with a Schott KL1500 source (H Walz, Effeltrich, Germany). Relative humidity in the leaf chamber was controlled by diverting part of the air flow stream through air that was equilibrated with water at 50°C. CO2 and O2 partial pressures were obtained by mixing pure CO2, O2, N2, and CO2-free air with the help of capillaries. The pressure difference in the capillaries was stabilized by manostats (tubes with open ends submerged in water to adjustable heights). The water vapour pressure was measured with a psychrometer. CO2 exchange was measured with a MK3-225 IR gas analyser (ADC, Hoddesdon, Hertfordshire, UK) or a Li-6251 analyser (Li-Cor, Lincoln, NE, USA). Data were recorded by computer using an A/D board ME-30 and a RECO program, and analysed by computer programs ANAL and SYNT. The programs RECO and ANAL were written by V Ova (University of Tartu, Estonia) in Turbo-Pascal. The intercellular CO2 concentration in the leaf was calculated with inputs for the rate of photosynthesis, the CO2 concentration in the air, and the diffusive resistance of CO2 from the atmosphere to the intercellular space. The latter was calculated by determining the diffusive resistance to water by measuring transpiration, and the water vapour concentration difference from the leaf to air (for a description see Ku et al., 1977; von Caemmerer and Farquhar, 1981). The Γ*, where the rate of CO2 uptake equals photorespiratory loss of CO2, was determined by taking the co-ordinates of the intersection of CO2 response curves measured at different light intensities (Brooks and Farquhar, 1985).

The area of tissue exposed to incident light was calculated by taking a digital image of the branch that was enclosed in the chamber, and then determining the exposed branch area using an image analysis program (Image Tool for Windows).

**δ¹³C values**

Measures of the carbon isotope composition (δ¹³C values) were made at Washington State University on leaf and stem samples taken from plants using a standard procedure relative to PDB (Pee Dee Belemnite) limestone as the carbon isotope standard (Bender et al., 1973). Plant samples (from plants growing in the Washington State University School of Biological Sciences growth chamber) were dried at 80°C for 24 h, milled to a fine powder, and then 1–2 mg were placed in a tin capsule and combusted in a Eurovector elemental analyser. The resulting N2 and CO2 gases were separated by gas chromatography and admitted into the inlet of a Micromass isoprobe mass spectrometer (IRMS) for determination of ¹³C/¹²C ratios (R). δ¹³C values were determined where δ=1000×(Rsample/Rstandard)−1.

**Statistics**

Where indicated, standard errors were determined, and analysis of variance (ANOVA) was performed with Statistica 7.0 software (StatSoft, Inc.). Tukey’s HSD (honest significant difference) tests were used to analyse differences between cell types. All analyses were performed at the 95% significance level.

**Results**

**General features including the stem surface**

Plants of all three representatives are prostrate to erect shrubs and subshrubs with stems comprised of segments with intercalary growth. These plants have reduced opposite leaves (~1 mm in length) at the distal (top) end of each segment (Fig. 1B, G, L). Photosynthesis is accomplished in the fleshy cortex of the articulated shoots. Under the growth conditions used, *T. pergranulata* (Fig. 1A) was fast growing, having bright-green stems which were 2–3 mm in diameter (Fig. 1B), *T. indica* subsp. *indica* (from Pakistan) had thicker stems (diameter 4–5 mm) with dark- or purple-green colour (Fig. 1F), while *T. indica* subsp. *bidens* (from Australia) had thin stems with a bright-green colour (Fig. 1J), resembling *T. pergranulata*. In *T. indica* subsp. *indica*, the segments in the vegetative branches are compact with formation of a cylindrical jointed stem (Fig. 1F), in contrast to *T. indica* subsp. *bidens*, whose stems are longer and narrower towards the base, resulting in a moniliform jointed stem (Fig. 1K). Figure 1E shows plants of *T. indica* subsp. *indica* in a natural habitat in Pakistan.

All three taxa have morphology which is typical for members of subfamily Salicornioideae, including short internodes and nearly aphyllous shoots with scale-like leaves (Fig. 1). The cylindrical stem has a fleshy cortex with chlorenchyma on the periphery, which is characteristic of all species in the subfamily. Sunken anomocytic stomata are mostly distributed in vertical rows on the epidermis of the fleshy cortex of the segments, alternating with rows of cells without stomata, with their long axis oriented perpendicular to the axis of the stem (Fig. 1C, H, L, M, light bands, and D, I). In all species, stomata are located throughout the epidermis of the fleshy cortex of the segment and leaf, but they are absent in the transparent leaf marginal area and on the abaxial
epidermis along the leaf main rib (Fig. 1F, G, K, L). In C₄ T. indica, stomata are located only in the epidermal cells which are external to the groups of chlorenchyma cells (Fig. 2K).

**Light microscopy**

The stem tissue of *T. pergranulata* has C₃ anatomy, with two layers of mesophyll chlorenchyma surrounding the periphery of the cortex with WS tissue in the centre (Fig. 2A–D). In the reduced leaves of *T. pergranulata*, the chlorenchyma tissue occurs only on the abaxial side (results not shown, but similar to that of *Salicornia fruticosa*; see Fahn and Arzee, 1959).

In the stems of *T. pergranulata*, most of the peripheral vascular bundles are located in WS tissue one cell apart from the chlorenchyma cells, and they are distributed with the phloem facing towards the chlorenchyma, with the central cylinder in the centre of the stem. There are large intercellular air spaces beneath the stomata (also see Carolin et al., 1982). In this species, chlorenchyma tissue comprises ~35% and WS tissue ~60% of the total area of stem cross-section. Starch grains are abundant throughout all chlorenchyma cells, with the highest density in the outermost layer (Fig. 2D).

In both subspecies of *T. indica*, analysis of cross-sections of the young shoot segments showed that the main volume of fleshy cortex is comprised of WS tissue (Fig. 2E, L). In subspecies *indica* (Fig. 2E), the peripheral chlorenchyma tissue is 15–20% while the WS parenchyma is 70–75% of the total area of the stem cross-section. In *T. indica* subsp. *bidens* (Fig. 2L), the stems are thinner, and the tissue in the chlorenchymatous rings is 20–30% of the total area of the stem segment (depending on the position of the section from the node). As in *T. pergranulata*, chlorenchyma tissue occurs only on the abaxial side of the reduced leaves in both subspecies of *T. indica* (Fig. 2M). In the stems of *T. indica*, small peripheral vascular bundles are distributed directly under the bundle sheath cells (BSCs).

Both subspecies of *T. indica* have two layers of chlorenchyma, which are characteristic of C₄ species with Kranz anatomy, an outer layer of palisade MCs and an...
inner layer of BSCs (Fig. 2E–G, J, L). The unusual feature of these species is the presence of large colourless MCs separating groups of chlorenchymatous palisade MCs (Fig. 2F–I, L). Observations of paradermal sections show that the islands of chlorenchyma cells are surrounded by a network of large colourless MCs which consist of 1–3 cells across (Fig. 2H, I). In hand-cut paradermal sections, it was also noticed that there is no green colour in BSCs in some regions where there are colourless MCs between the chlorenchyma cells (Fig. 2H). More careful studies showed that there are sparse, nearly empty cells in the layer of BSCs which are located under colourless MCs (Fig. 2J); colourless BSCs were observed more often in T. indica subsp. indica. The colourless BSCs located under groups of colourless MCs appear to have no, or limited, contact with the neighbouring chlorenchymatous MCs. There are rather large intercellular air spaces between the epidermal and chlorenchyma cells beneath the stomata (substomatal cavity), while between stomata the MCs are closely associated with epidermal cells (Fig. 2J, K). Larger intercellular air spaces also occur between the distal ends of MCs in both subspecies of T. indica; whereas, at the proximal ends, all MCs are close to each other with little or no intercellular air space,
depending on the subspecies studied: in subsp. bidens there are more intercellular air spaces where the bundle sheath (BS) CW faces the MCs, and, in general, cells are more tightly packed in subsp. indica. Starch granules are abundant in BSC chloroplasts of both subspecies (results shown only for subsp. indica, Fig. 2K).

Development of the two-layered chlorenchyma tissue in T. indica subsp. bidens is shown in the longitudinal section of the shoot tip (Fig. 2M) and the young segment (Fig. 2N–P). Both layers of photosynthetic cells evidently originate from one layer of pre-chlorenchyma cells during leaf development (Fig. 2M) and during formation of the cortex chlorenchyma in the internodal meristem (Fig. 2N). In the outer row of chlorenchyma, there are cells with different levels of development, with some having a lower cytoplasmic content which could be distinguished at a rather early stage (Fig. 2O, P). Presumably, these cells with lower cytoplasmic content are precursors to the formation of the colourless MCs.

Fluorescence of chloroplasts and cell walls, and lignification

For all three representative taxa, fresh hand-cut sections placed in water have red fluorescence from chloroplasts in the outer chlorenchyma layers, with lower intensity red fluorescence coming from the pith, and from parenchyma tissue between the central cylinder and a suberized layer (which is considered periderm, e.g. see Discussion, and Arcihowskii, 1928; Vosnesenskaya and Steshenko, 1974). In all three taxa there was very bright blue fluorescence of CWs under UV light (Fig. 3A, D, G). Since it is known that lignified and suberized CWs have bright blue fluorescence, the sections were treated with phloroglucinol to test for lignification; the results are shown in Fig. 3B, E, H. Staining with phloroglucinol changed the colour of xylem, sclerenchymatous tissue, and mechanical extraxylary fibres to dark red, showing the presence of lignification only in these tissues (Fig. 3B, E, H). The blue fluorescing CWs of WS tissue did not change their colour. Several cell layers outside the central cylinder, having especially bright light-blue fluorescence in sections placed in water, changed their colour slightly to red with phloroglucinol treatment. Staining of sections with Sudan IV changed the colour of CWs outside the central cylinder to dark red, showing the presence of suberin (not shown). Thus, the blue fluorescence of CWs of WS and other cells is not related to lignification or suberization in these species.

Sections were then treated with NH₄OH to check for the presence of bound ferulic acid. In all three representatives, under alkaline conditions the blue fluorescence of all non-lignified CWs became more intense and changed colour to green, demonstrating the presence of CW-bound ferulic acid. In contrast, the colour of CWs of all xylem vessels in the central cylinder and in small vascular bundles, sclerenchymatous tissue in the central cylinder, and rare mechanical fibres outside the suberized layer remained bright blue under alkaline conditions (Fig. 3C, F, I), indicative of lignified or suberized CWs.

In all three Tecticornia representatives, the most intensive blue fluorescence of CWs in sections in water was in the epidermis, WS tissue, the 2–3 layers of thick-walled peridermal cells outside the central cylinder, and mechanical tissues surrounding vascular bundles, together with the xylem (Fig. 3A, D, G). Furthermore, in both C₄ subspecies, BS and colourless mesophyll CWs fluoresce more intensively than chlorenchymatous mesophyll CWs. Morphometrical study of CW thickness showed that all three taxa have a rather thick outer epidermal CW, which was thickest in T. indica subsp. indica (Table 1). Chlorenchyma MCs have thin CWs (~0.07–0.08 μm) in all three representatives. BSCs of both T. indica subspecies have rather thick CWs (~0.8 μm in subsp. indica and ~0.5 μm in subsp. bidens). The thickness of CWs in WS tissue and colourless MCs is similar to the thickness of BS CWs for both T. indica subspecies, with greater thickness in subsp. indica. The thickness of the CW in WS tissue in T. pergranulata is also more than twice that of the mesophyll CW, but much lower than in the two subspecies of T. indica (Table 1). Thus, the higher fluorescence in the CW of the outer epidermal, BS, and WS tissue appears related to the greater thickness of CW in these tissues.

Electron microscopy

Stems of all three Tecticornia taxa are covered by a thick cuticle which has a structure typical of many desert chenopods, with a rather well-formed outer lamellated layer of cuticle proper, followed by the inner cuticular layer with intensive development of reticulated polysaccharide microfibrils, also called dendrites (Fig. 4A, E). The thickness of the cuticle layer depends on the age of the segment, but, in general, the thickest cuticle was found in T. indica subsp. indica (~2 μm) while the other two taxa, T. pergranulata and T. indica subsp. bidens, have rather similar cuticle thickness of ~0.7 μm (Table 1). The thickness of the outer epidermal CW varies from 1.1 μm in T. indica subsp. bidens, to 1.7 μm in T. pergranulata, to 3 μm in T. indica subsp. indica (Table 1).

In chlorenchyma cells of T. pergranulata, the chloroplasts, which are located mostly towards the intercellular spaces, have grana consisting of 8–10 thylakoids (Fig. 4B). Mitochondria are rather small (~0.4 μm, Table 2), and have falciform cristae, which is typical for C₃ species. MCs have a thin CW, 0.08 μm (measured between two adjacent MCs divided by 2, Table 1), which is similar to that measured at the intercellular air space (not shown).
Fig. 3. Blue-green fluorescence under UV light and lignification in the hand-cut cross-sections of *T. pergranulata* (A–F) and *T. indica* subsp. *indica* (G–I). (A, D, G) Blue autofluorescence of the CW in fresh sections placed in water. (B, E, H) Staining with phloroglucinol changes the colour of mechanical tissues and xylem to dark red, showing the presence of lignified CWs. (C, F, I) Light-green fluorescence of CWs in sections placed in 0.1 M NH₄OH. C, chlorenchyma; CC, central cylinder; M, mesophyll cells; SL, suberized layer; WS, water storage tissue. Scale bars: 350 μm for A and C; 500 μm for B and H; 150 μm for D–F; 200 μm for G and I.

Table 1. *Thickness of the cuticle and cell walls in Tecticornia species* (μm)

Analyses were made by one-way ANOVA with Tukey’s HSD. Means followed by a different lower-case letter within a row indicate a significant difference between cell types (*P* ≤ 0.05). Means followed by a different upper-case letter within a column indicate a significant difference between species (*P* ≤ 0.05).

<table>
<thead>
<tr>
<th>Species</th>
<th>Cuticle</th>
<th>Cell wall</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Outer epidermal</td>
</tr>
<tr>
<td><em>T. pergranulata</em></td>
<td>0.65±0.04 A</td>
<td>1.71±0.04 Aa</td>
</tr>
<tr>
<td><em>T. indica</em> subsp. <em>indica</em></td>
<td>2.18±0.07 B</td>
<td>3.07±0.18 Ba</td>
</tr>
<tr>
<td><em>T. indica</em> subsp. <em>bidens</em></td>
<td>0.74±0.01 A</td>
<td>1.14±0.08 Aa</td>
</tr>
</tbody>
</table>

⁸ The thickness of two adjacent cell walls was measured and divided by 2. The average number of partial cell profiles/sections examined was 29.
Very often, two neighbouring mesophyll CWs are not very tightly appressed to each other, having the intercellular space filled with fibrillar material (Fig. 4C). Plasmodesmata are more often found in the tangential (periclinal) CW between two MCs rather than in the radial (anticlinal) CW; but, in both cases, they are located in the local thickening of the CW (Fig. 4D). All WS cells are interconnected by plasmodesmata, which are also located in a thickened area of the CW (not shown).

The ultrastructure of palisade MCs and Kranz BSCs in both subspecies of *T. indica* is similar in general features. The chloroplast size (based on length) in the chlorophyllous and colourless MCs and BSCs is ~4–6 μm, with little to no difference in size between the cell types, and from that in MCs of *T. pergranulata*. The thylakoid system in the mesophyll chloroplasts consists of sparse grana which have short thylakoids with a high degree of stacking and numerous, long intergranal thylakoids (Fig. 4G, subsp. *indica*). Mesophyll mitochondria in the two subspecies are rather small (~0.4 μm) and comparable in size with mitochondria in MCs of *T. pergranulata* (Table 2). MCs usually are packed rather tightly on their
Table 2. *Size of mitochondria and chloroplasts in Tecticornia species (μm)*

Analysis was by one-way ANOVA with Tukey’s HSD. Means followed by a different lower-case letter within a row indicate a significant difference between cell types; comparison was made independently for chloroplast and mitochondria sizes (*P* ≤ 0.05). Means followed by a different upper-case letter within a column indicate a significant difference between species (*P* ≤ 0.05). The average number of organelle sections examined in each case was 35 for chloroplasts and 20 for mitochondria. M, mesophyll; BS, bundle sheath.

<table>
<thead>
<tr>
<th>Species</th>
<th>Chloroplast length</th>
<th>Mitochondria small diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorophyllous M</td>
<td>Colourless M</td>
</tr>
<tr>
<td><em>T. pergranulata</em></td>
<td>4.98±0.12 A</td>
<td>–</td>
</tr>
<tr>
<td><em>T. indica</em> subsp. <em>indica</em></td>
<td>5.05±0.18 Aa</td>
<td>5.08±0.29 Aa</td>
</tr>
<tr>
<td><em>T. indica</em> subsp. <em>bidens</em></td>
<td>6.23±0.15 Ba</td>
<td>3.94±0.31 Bb</td>
</tr>
</tbody>
</table>

Fig. 5. Western blots for C₄ enzymes and Rubisco from total proteins extracted from green shoots of *T. pergranulata*, and *T. indica* subsp. *indica* and subsp. *bidens*. Blots were probed with antibodies raised against PEPC, PPDK, NAD-ME, NADP-ME, and Rubisco, respectively. Numbers on the left indicate the molecular mass in kilodaltons. Western blots were replicated a minimum of three times with each antibody with similar results.

BS CWs and similar in thickness to CWs of colourless MCs (Table 1). The thick CWs in WS parenchyma, colourless MCs and BSCs have a similar undulated distribution of cellulose microfibrils (Fig. 4P), which is not observed in other tissues. In WS tissue, the cells are interconnected with plasmodesmata, which are located in a thickened area of the CW (not shown, but similar to that in Fig. 4D). As noted earlier, the small peripheral bundles are often directly adjacent to BSCs, and one of the most interesting features of this genus is that the phloem side of the bundles is facing chlorenchyma tissue (Fig. 4Q).

**Western blots**

SDS–PAGE blots of total proteins extracted from leaves were probed immunologically to test for C₄ enzymes and Rubisco LSU (Fig. 5). The molecular masses of the immunoreactive bands corresponded to the expected mass of the different polypeptides. The results show a strong
immunoreactive band for Rubisco LSU at 56 kDa in all species. Strong immunoreactivity was observed for PEPC and PPDK in the two C₄ subspecies. With antibodies to C₄ acid decarboxylases, there was immunolabelling for NAD-ME (65 kDa) in both subspecies of *T. indica*, with extremely low labelling for NADP-ME (62 kDa) (Fig. 5), and no labelling for PEP-CK in any of the species (not shown). In the C₃ species *T. pergranulata*, there were very low immunoreactive bands for all C₄ enzymes, i.e. PEPC, PPDK, NAD-ME, and NADP-ME (Fig. 5), and no labelling for PEP-CK (not shown).

**Immunolocalization of enzymes and starch distribution**

In the C₃ species *T. pergranulata*, immunolabelling for Rubisco occurs in chloroplasts of all chlorenchyma cells (Fig. 6A), similar to the distribution of starch grains (Fig. 2D). The distribution of *in situ* immunolabelling for several photosynthetic enzymes in the C₄ *T. indica* subsp. *indica* is shown at light microscopy (Fig. 6B, C) and electron microscopy levels (Fig. 7) (also see Table 3 for comparison of the density of labelling for different photosynthetic enzymes in different cell types for the two subspecies). There was strong labelling for Rubisco LSU in chloroplasts in BSCs (Fig. 6B, Table 3) which also store starch (Fig. 2K), and the few chloroplasts found in WS cells also show labelling for Rubisco (not shown). Labelling for PEPC is high in MCs (Fig. 6C) and is confined to the mesophyll cytosol (Fig. 7C, Table 3). Transmission electron microscopy studies of the two subspecies of *T. indica* show immunolabelling for NAD-ME and GDC in BSC mitochondria (Fig. 7B).

To study the possible function of colourless MCs, immunolabelling was performed at the electron microscopy level (see results Table 3). For both *T. indica* subspecies, the labelling for PEPC is highest in the cytosol of MCs (Fig. 7C, Table 3); however, the colourless MC also showed substantial labelling (though significantly lower than in MCs) (Fig. 7D, Table 3). Labelling for PPDK was shown to be localized predominantly in chloroplasts of MCs, with lower, but significant, labelling in chloroplasts of colourless MCs. Labelling for PPDK in BS chloroplasts and for PEPC in BS cytosol was similar to background (Table 3). Starch distribution, in general, followed the pattern of Rubisco localization, with higher starch content in BSCs in comparison with MCs, but the largest starch granules are localized in the colourless MCs and colourless BSCs in *T. indica* subsp. *indica* (Fig. 4H, J, M).

**Gas exchange measurements, carbon isotope composition, and titratable acidity**

Similar responses of photosynthesis to varying light were observed for the C₃ plant *T. pergranulata* and C₄ species *T. indica* subsp. *indica* and subsp. *bidens*. In all three taxa, photosynthesis saturates at relatively high light intensity, ~1200 PPFD, but *T. pergranulata* reaches a higher maximum rate than the C₄ species (Fig. 8). The rate of photosynthetic CO₂ fixation was measured at varying...
intercellular levels of CO₂ (Cₗ) under atmospheric (21%) and low (2%) concentrations of O₂. Under varying CO₂ and ambient O₂, the C₃ species T. pergranulata has lower carboxylation efficiency, and increasing rates of CO₂ fixation up to a Cₗ of 900 μmol mol⁻¹ (Fig. 9A), whereas the two Kranz-type subspecies show a similar, relatively rapid increase in photosynthesis with increasing Cₗ up to ~600 μmol mol⁻¹ (Fig. 9B, C). A higher level of O₂ was inhibitory for photosynthesis rates under varying CO₂ in T. pergranulata (Fig. 9A), while both T. indica subspecies had no inhibition of photosynthesis by O₂ (Fig. 9B, C). The Γ* was determined for the three taxa (Table 4) by analysis of the intercept of CO₂ response curves at different light intensities (as illustrated in Fig. 9D for T. indica subsp. bidens). Γ* is much lower in the Kranz-type C₄ species than in the C₃ species. Both T. indica subspecies have C₃-type δ¹³C values (subsp. indica -13.7‰, and subsp. bidens -15.2‰), while T. pergranulata has C₄-type values (-31.4‰). Titratable acidity tests did not reveal any changes in pH of cell sap during the diurnal cycle (Table 4).

**Discussion**

There has been a strong interest in the evolution of C₄ photosynthesis in the family Chenopodiaceae, due to its unusually high diversity, with different Kranz and non-Kranz C₄ leaf types as well as variation in C₃ leaf types (Monteil, 1906; Carolin et al., 1975; Fisher et al., 1997; Jacobs, 2001; Pyankov et al., 2001a, b; Schütze et al., 2003; Kapralov et al., 2006). Halosarcia, as traditionally defined, has been shown to be paraphyletic in relation to other Australian Chenopodiaceae genera (Shepherd et al., 2004; Kadereit et al., 2006; this study). The monophyly obtained from molecular studies has been supported based on morphological characters which show a high level of homoplasy (Shepherd et al., 2005; Shepherd and Wilson, 2007). Because of this, these genera have all recently been reorganized into a more broadly defined Tecticornia (Shepherd and Wilson, 2007), which is accepted here. While this clade of species is predominantly Australian in distribution, it is also found on other continents, including southern Asia (Malaysia, Sri Lanka, India, and Pakistan) and tropical East Africa along coastal and inland saline areas. Interestingly, the only species previously described as having C₄ photosynthesis, T. indica, is also one of the few Australian chenopod lineages also to be found outside of the continent. Carolin et al. (1982) identified four subspecies of Halosarcia (= Tecticornia) indica (bidens, indica, julacea, and leiostachya) as C₄ plants. From molecular phylogeny based on nuclear DNA internal transcribed spacer (ITS) data, T. indica and most of its subspecies form a strongly supported clade with undescribed entities previously
referred to as ‘Yanneri Lake’ (Shepherd and Wilson, 2007), which has been suggested also to be C₄ (K Shepherd, personal communication). This may indicate a single origin of C₄ photosynthesis in Salicornioideae. However, T. indica subsp. julacea is not part of the T. indica clade in the ITS tree (Kadereit et al., 2006), only in the chloroplast DNA trnL tree of Shepherd et al. (2004). The phylogenetic positions of the C₃ and C₄ taxa utilized in this study were verified by comparison with some other species in genus Tecticornia and related genera in subfamily Salicornioideae using ITS as a marker and maximum likelihood analysis (Fig. S1 and Table S1 in Supplementary material available at JXB online). Obviously, a more detailed analysis of T. indica subspecies is needed. The differences between two subspecies of T. indica, subsp. indica and subsp. bidens, including their different habit (subsp. indica is a prostrate dwarf shrub up to 50 cm, rarely to 1 m, versus subsp. bidens which is an erect shrub up to 2 m tall) (Wilson, 1980), macroscopic, microscopic, and genetic differences described in this paper, and their different geography, collectively support specifically different entities. This needs to be clarified in the re-evaluation of the taxonomic status of this complex in a broad geographical context. These results, and a similar case already discussed in the genus Bienertia (Akhani et al., 2005), indicate that the taxonomy of several critical groups of Chenopodiaceae needs to be reassessed using multidisciplinary approaches.

**Table 4. CO₂ compensation point, carbon isotope discrimination (δ¹³C), and test for CAM in Tecticornia species**

For determination of Γ* see Fig. 9. For δ¹³C and titratable acidity n=2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Γ* (μmol mol⁻¹)</th>
<th>δ¹³C (‰)</th>
<th>Titratable acidity (μeq g FW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>End of the night</td>
</tr>
<tr>
<td>T. pergranulata</td>
<td>34.2</td>
<td>-31.4±0.05</td>
<td>1.82±0.23</td>
</tr>
<tr>
<td>T. indica subsp. indica</td>
<td>5.2</td>
<td>-13.7±0.01</td>
<td>2.42±0.57</td>
</tr>
<tr>
<td>T. indica subsp. bidens</td>
<td>4.8</td>
<td>-15.2±0.01</td>
<td>3.99±0.10</td>
</tr>
</tbody>
</table>

**Fig. 9.** Rates of CO₂ fixation in response to varying intercellular levels of CO₂ at 25°C and 900 PPFD in T. pergranulata (A), T. indica subsp. indica (B), and subsp. bidens (C). The results represent the average that was taken of ambient to low CO₂ response, and ambient to high CO₂ response, from separate measurements on 2–3 branches. (D) Illustration of calculation of Γ* from CO₂ response curves at 25°C under four light intensities with T. indica subsp. bidens: 90 (line 1), 170 (line 2), 260 (line 3), and 360 (line 4) PPFD. Each light level is the response to two replications.
Anatomical features

The structure of chlorenchyma in C₃ *T. pergranulata*, consisting of two layers of elongated MCs around the periphery of cylindrical leaves or aphyllous stems, is rather typical for different C₃ representatives of Salsolioideae and Salsoloideae; in aphyllous species, small reduced scale-like leaves have similar chlorenchyma tissue only on their abaxial side. This type of structure, with a peripheral position of chlorenchyma and a network of small vascular bundles, and a central cylinder (in stems) or main vascular bundle (in leaves) in the centre, has been called ‘centric’ (Metcalfe and Chalk, 1950), ‘sympegmoid’ (Carolin et al., 1975), or ‘arco-vascular’ (Vasilevskaya and Butnik, 1981). It has also been described in Salsoloideae as having peripheral vascular bundles with the xylem side facing the chlorenchyma (Carolin et al., 1975; also see figs 7, 8 and 11 in Pyankov et al., 1997; fig. 2C, D in Voznesenskaya et al., 2001; figs 2, 5, 7 in Voznesenskaya et al., 2003). Although not perfect, this is a convenient way of identifying this anatomical type when it occurs, and we consider that the term ‘centric’ reflects well all features of C₃ anatomy in these cases. The characteristic feature distinguishing *T. pergranulata* from C₃ Salsoloideae species which have a similar structure is the positioning of peripheral vascular bundles. In *Tecticornia*, the phloem side of the small peripheral bundles faces towards the chlorenchyma tissue. Also, all peripheral bundles in C₃ *T. pergranulata* are separated from the chlorenchyma tissue by one layer of large WS cells, while in C₃ or C₃–C₄ *Salsola* species, the peripheral vascular bundles are separated from chlorenchyma cells by rather small parenchyma cells representing parenchymatous BS around peripheral vascular bundles (Pyankov et al., 1997; Voznesenskaya et al., 2001; Akhani and Ghasemkhani, 2007; EV Voznesenskaya, unpublished results). In other species of the Australian Salsolioideae such as *Tecticornia* s. str. and *Pachycornia* and *Sarcocornia*, many of the vascular bundles are adjacent to chlorenchyma (Carolin et al., 1982). While species in the genus *Salicornia* have a similar positioning of the phloem to that in *Tecticornia*, the peripheral bundles are often adjacent to the chlorenchyma tissue (personal observation of EV Voznesenskaya and NK Koteyeva, unpublished results). The structure and position of peripheral vascular bundles in C₃ *T. pergranulata* represent a rather distinctive feature, which may also be characteristic for some other Salsolioideae. Accepting this type of anatomy as C₃ centric, as a minimum two variants should be mentioned according to the positioning of peripheral vascular bundles.

In the two C₄ subspecies of *T. indica*, chlorenchyma tissue consists of two cell layers, elongated MCs and roundish BSCs, on the periphery of the stems and rudimentary leaves. The present observation of paradermal sections revealed that the islands of chlorenchyma cells are surrounded by sections of large, colourless MCs with thick CWs, which consist of one to three cells across, as previously reported (Carolin et al., 1982). Kadereit et al. (2003) distinguished the anatomical type in *T. indica* as Kranz-halosarcoid, based on the presence of colourless MCs and the centrifugal position of chloroplasts in BSCs. An additional distinguishing anatomical feature of this Kranz type is the position of peripheral vascular bundles directly adjacent to BSCs, with the phloem facing the chlorenchyma tissue, as was also observed in C₃ *T. pergranulata*. This differs from salsoloid-type C₄ species, where the xylem in the peripheral vascular bundles faces the chlorenchyma tissue (see Olesen, 1974; Voznesenskaya, 1976a, b; figs 7, 8, 11, 12 in Pyankov et al., 1997; fig. 2A, B in Voznesenskaya et al., 2001; figs 2, 5, 7 in Voznesenskaya et al., 2003). Interestingly, a similar positioning of peripheral vascular bundles, with their phloem side towards the chlorenchyma, was only previously mentioned in the ‘single-cell functioning’ C₄ species, *Suaeda (=Borszczowia) aralocaspica* (Freitag and Stichler, 2000). It was thought (Olesen, 1974) that such positioning of vascular bundles could facilitate the transport of assimilates and water, but this idea needs further investigation. Thus, the type of chlorenchyma structure in C₄ *Tecticornia* represents a unique variation of Kranz anatomy with discontinuous chlorenchyma, interrupted by the thick-walled colourless cells in both layers, mesophyll and BS, centrifugally arranged organs in BSCs, and positioning of peripheral vascular bundles with their phloem side to the chlorenchyma. This type of anatomy was designated Kranz-halosarcoid by Kadereit et al. (2003) according to the previous name of the genus, which is now changed to Kranz-tecticornoid type. In general, this type of anatomy can be described as Kranz centric discontinuous with the specific position of vascular bundles and chloroplasts in BSCs.

According to Carolin et al. (1982), Fahn and Arzee (1959), and Al-Turki et al. (2003), in all species of subfamily Salsolioideae studied, the network of vascular bundles in the fleshy cortex is derived from the leaf bundle of the upper internode. The type of venation in reduced leaves and cortex was classified as *Salicornia-Arthrocnemum* type (Fahn and Arzee, 1959), where the descending network venation of the cortex was derived only from lateral branches of the leaf strands. The study of venation in C₃ and C₄ *Tecticornia* species also showed this type of venation and origin of peripheral bundles, with certain differences in the structure of the primary vascular system between *T. pergranulata* and *T. indica* subsp. *bidens*; in general, the *Salicornia-Arthrocnemum* type of venation was suggested to be more advanced in comparison with venation in *Kochia-Bassia* or *Rhagodia-Atriplex* types (Bisalputra, 1962). There has been an extensive discussion of the origin of the fleshy cortex in articulated Chenopodiaceae species (see Fahn and Arzee,
From examination of the origin of the cortex during plant development, it was concluded that the assimilating cortex is not a product of leaf fusion and adnation to the stem, but rather is a result of simultaneous growth of the leaf basis and cortex. It was shown that the development of the reduced leaves in species with such shoots is similar to that of ordinary foliar leaves (Vasilevskaya, 1955; Werker and Fahn, 1966) and that the fleshy tissue external to the central cylinder of these plants develops as a result of intercalary growth at the base of each internode and should be regarded as true cortex (Vasilevskaya, 1955; Fahn and Arzee, 1959). The relationship between positioning of the small peripheral bundles and transport of assimilates in different Chenopodiaceae species needs additional study.

It is interesting to note that chenopods with a fleshy cortex have a special form of secondary growth and peridermal formation which was previously studied in the genus *Haloxylon*. The secondary cambium, as well as the peridermal cambium, originates in the pericycle, which is internal to the endoderm, and usually after formation of the periderm the outer fleshy chlorophyllous cortex withers and dries up (Archihovskii, 1928; Vosnesenskaya et al., 1974). While the process of secondary growth was not studied in *Tecticornia*, light microscopy images show very similar secondary growth to that in *Haloxylon*.

**Tecticornia indica: C₄ biochemical subtype and enzyme compartmentation**

The high levels of C₄ cycle enzymes PPDK, PEPC, and NAD-ME in *T. indica* are indicative of C₄ photosynthesis, as compared with the very low levels of these enzymes in the C₃ species *T. pergranulata*. Analysis by western blots for C₄ acid decarboxylases shows that *T. indica* is an NAD-ME-type C₄ species. Generally, consistent results have been obtained in subtyping C₄ species by immunodetection versus enzymatic assay of C₄ decarboxylases (Walker et al., 1997; Wingler et al., 1999; Pyankov et al., 2000; Vosnesenskaya et al., 2002). Immunolocalization studies show selective compartmentation of PPDK and PEPC in MCs, and Rubisco in BSCs, in the two subspecies of *T. indica*, characteristic of C₄ plants. High levels of starch accumulate in the BSC chloroplasts compared with MC chloroplasts (see also Carolin et al., 1982). Also, NAD-ME and GDC are selectively localized in mitochondria of BSCs, as expected for NAD-ME-type C₄ species.

**Ultrastructural features of photosynthetic tissue**

The ultrastructural characteristics of chlorenchyma cells in *T. pergranulata* are typical of other C₃ species, with chloroplasts and mitochondria around the periphery of MCs. In the two C₄ subspecies of *T. indica*, there is differentiation of chloroplasts and mitochondria between MCs and BSCs. There are numerous mitochondria in BSCs which, along with chloroplasts, are predominantly located in the centrifugal position, as was also observed by Carolin et al. (1982) and Jacobs (2001). The mitochondria in BSCs are ~50% larger than in MCs, while the chloroplasts in the two cell types are similar in size. However, the mesophyll chloroplasts have a reduction of grana with prevalence of intergranal thylakoids compared with BS chloroplasts. The abundance of mitochondria in BSCs and the reduction of grana in mesophyll compared with BS chloroplasts are typical of NAD-ME-type C₄ species (Carolin et al., 1975; Voznesenskaya, 1976a, b; Gamalev, 1985; Voznesenskaya and Gamalev, 1986; Fisher et al., 1997).

In most NAD-ME-type C₄ species, including dicots and monocots, the chloroplasts are in a centrifugal position (Gutierrez et al., 1974; Hattersley, 1987; Dengler and Nelson, 1999). However, there are established cases of NAD-ME-type species having BS chloroplasts in the centrifugal position. With respect to dicots, the centrifugal position of chloroplasts in BSCs of *T. indica* is similar to that found in *Suaeda* species having a schoberia leaf type with NAD-ME C₄ photosynthesis (Schiitze et al., 2003; Voznesenskaya et al., 2007). This also occurs in *Trianthema triquerta*, family Aizoaceae, which has atriplicoid leaf anatomy and an unspecified biochemical subtype, but with ultrastructural features characteristic of NAD-ME species (Carolin et al., 1978). With respect to monocots, the centrifugal position of BS chloroplasts has been found in several NAD-ME-type species: in spp. of *Panicum* sect. Dichotomiflora, in *Eragrostis*, and in *Enneapogon* (Ohsgui et al., 1982; Hattersley, 1987). Whether there is functional significance to the chloroplast position, or whether it is only indicative of alternative forms of C₄, is unknown.

Many C₄ species have BSCs with thickened CWs (see Sage and Monson, 1999). Among C₄ NAD-ME species in family Chenopodiaceae, it is possible to distinguish two groups according to the thickness of their BS CWs. Most *Atriplex* and *Suaeda* species have rather thin CWs, while representatives studied from tribe Caroxyloneae with NAD-ME-type anatomy, *Climacoptera* transoxana, *Halocharis hispida*, and *Salsola rigida* (= *Caroxylon orientale*) (Akhan et al., 2007), have very thick BS CWs (Voznesenskaya, 1976b), similar to those in *C₄ Tecticornia*. The most interesting feature of BSC structure in the C₄ *T. indica* subspecies is the presence of intercellular connections by plasmodesmata, not only in the outer tangential CW (between BSCs and MCs), but also in the inner tangential CW, between BSCs and WS tissue, and between BSCs and vascular bundle parenchyma cells. This feature suggests symplastic transport of assimilates from chlorenchyma to the vascular tissue in these C₄ species. Also, in *T. indica*, the WS cells have rather thick CWs which are interconnected by plasmodesmata.
**Fluorescence of chloroplasts and cell walls, and lignification**

When excited by UV radiation, leaves of all plants have intensive red fluorescence from all chlorophyll-containing cells. Obviously, the most intensive red colour in sections of stems was in the chlorenchyma tissue in the outer cortex layers, with lower red fluorescence from several other chloroplast-containing parenchymatous tissues including the pith, xylem, and phloem parenchyma, and the peridermal parenchyma (the phelloderm) which is located just outside the central cylinder. Possible functions of the internal chlorophyll-containing tissues were studied in some Salicornioideae species by Redondo-Gómez et al. (2005).

Certain groups of green plants exhibit a genuine blue fluorescence from their CW due to accumulation of phenolic substances, especially lignins and/or suberins. In stem sections of the Tecticornia taxa studied, the brightest blue fluorescence was emitted from lignified fibres, sclerenchyma, and xylem elements, and from the suberized layers outside the central cylinder representing the periderm. The blue fluorescence of non-lignified CWs (i.e. those that have a negative phloroglucinol-HCl test) changes to green with increasing intensity after treatment with 0.1 M NH₄OH, indicating the presence of bound ferulic acid (Rudall and Caddick, 1994). According to previous studies, families of monocotyledons can be divided into two groups depending on the UV fluorescence behaviour of their CW and presence or absence of bound ferulic acid (Harris and Hartley, 1976; Harris and Hartley, 1980). In dicots, wall-bound ferulic acid has only been found in the order Caryophyllales, and was previously shown for eight species of family Chenopodiaceae (Hartley and Harris, 1981).

In *T. pergranulata* and *T. indica* subspecies, the non-lignified CWs of assimilating organs fluoresce blue under UV radiation and change colour to intense green after NH₄OH treatment, indicating the presence of CW-bound ferulic acid. Intense fluorescence following NH₄OH treatment was found in all three representatives and, thus, it does not depend on photosynthetic type. There was differential distribution of fluorescence intensity in different tissues, with maximum green fluorescence after NH₄OH treatment in epidermal and WS tissues. In both C₄ subspecies, the walls of BSCs fluoresce more intense green than the chlorenchymatous MCs, with the highest intensity in the thick-walled, colourless MCs. In C₃ and C₄ Tecticornia, the intensity of green fluorescence following NH₄OH treatment tended to correspond to CW thickness. The epidermis has very thick CWs, and the WS tissue has much thicker CWs than the MCs in both species. In the C₄ subspecies of *T. indica*, the WS tissue, BSCs, and colourless MCs have higher fluorescence and much thicker CWs than the MCs. Carolin et al. (1975) mentioned different staining of the mesophyll and BS CW by electron microscopy; however, no differences were observed in the present study. Nevertheless, most of the thickened CWs in *T. indica*, including BSCs, colourless MCs, and WS tissue, have a specific undulating distribution of cellulose microfibrils which is absent in all other tissues.

With respect to the possible functions of CW ferulic acid, it has been suggested that, in certain groups of plants (in particular in Poaceae), ferulic acid in the walls of epidermal cells absorbs UV-B radiation and protects the photosynthetic apparatus (Lichtenhaler and Schweiger, 1998). Wakabayashi et al. (1997) showed that increased levels of ferulic acid led to decreased CW extensibility and to significantly increased mechanical strength of tissues. In some desert plants (e.g. *Tecticornia*), in which the stem is the main carbon-assimilating organ, there is little tissue to give mechanical support; thus, the presence of ferulic acid may provide strength to the CW to support the stems. It has been shown that the quantity of ferulic acid increases under water and osmotic stresses, which was suggested to facilitate adaptation to dry and saline environments (Wakabayashi et al., 1997; Fan et al., 2006).

For function of C₄ photosynthesis, there needs to be resistance to loss of CO₂ from sites of C₄ acid decarboxylation in BSCs, in order for it to be assimilated effectively by Rubisco, and a number of factors contribute to this to varying degrees, depending on the species and C₄ subtype (von Caemmerer and Furbank, 2003). The BS CWs may contribute to this resistance, depending on thickness and composition, and it has long been recognized that BSCs in some C₄ species have a suberized lamella, which is thought to contribute to diffusive resistance. In *T. indica*, fluorescence and histochemical analyses indicate that BSCs lack lignin and suberization, and that the higher apparent content of ferulic acid in BSCs corresponds to a thicker CW. In the two C₄ subspecies, the BS CW is 7- to 10-fold thicker than the MC CW; thus, the thicker CW may contribute to the resistance to leakage of CO₂ from BSCs. The chloroplasts in BSCs of *T. indica* are predominantly located in a centrifugal position, which would reduce diffusive resistance through the liquid phase, and increase potential for leakage from sites of decarboxylation to the exterior of the cell. However, the mitochondria in BSCs, which are the site of C₄ acid decarboxylation via NAD-ME, are positioned internal to the chloroplasts, which is favourable for refixation of CO₂ by Rubisco in the BS chloroplasts.

**Photosynthetic CO₂ exchange, carbon isotope composition, and titratable acidity**

The two C₄ subspecies of *T. indica* have C₄ type δ¹³C values (subsp. indica −13.7‰ and subsp. bidens −15.2‰) and low Γ* values (subsp. indica 5.2 and subsp bidens 4.8 µmol CO₂ mol⁻¹, Table 4) which indicates the efficiency of function of C₄, while *T. pergranulata* has C₃...
type $\Gamma^*$ (34.2 $\mu$mol mol$^{-1}$) and $C_3$ type $\delta^{13}C$ values ($-31.4$‰). The $\delta^{13}C$ values of these species are consistent with earlier results of Carolin et al. (1982), who obtained values of $-12.2$‰ to $-14.2$‰. The $CO_2$ response curves under $2\%$ versus $21\% O_2$ show that $CO_2$ assimilation in $T. indica$ is insensitive to $O_2$, which is characteristic of $C_3$ plants. $CO_2$ assimilation in $T. pergranulata$ is inhibited by $21\% O_2$ under limiting $CO_2$ due to photorespiration and lack of a $CO_2$-concentrating mechanism. The results show that the $C_4$ Tecticornia would have an advantage under conditions where $CO_2$ is limiting. $C_3$ plants often have a lower light saturation of photosynthesis than $C_4$ plants. However, the light response curves were similar for the $C_3$ and $C_4$ Tecticornia species, which may be related to the thick stems requiring high light to saturate photosynthesis.

The absence of nocturnal acidification of cell sap in all three representatives of this genus indicates that they do not have the CAM type of photosynthesis.

Possible functions of unique colourless cells

In the stem tissue of $C_4 T. indica$, there is a wreath of photosynthetic tissue near the periphery. However, this is interrupted by an unusual co-occurrence of colourless MCs and BSCs within the layers of chlorenchyma, characteristic of Kranz anatomy. The area of the colourless MCs in the longitudinal plane appears to be greater than that of the colourless BSCs. The very few plastids which occur in these cells have high levels of starch, although the Kranz BSCs are the main sites of starch storage. Analysis of the enzyme composition of the colourless MCs also showed they did not have mesophyll-type specialization for $C_4$ photosynthesis. Colourless MCs were not observed in the $C_3$ species $T. pergranulata$, which raises the question as to whether this feature may have co-evolved with evolution of $C_4$ photosynthesis in the genus.

There has been speculation as to how windows in some succulent species may influence photosynthesis (see Egbert and Martin, 2002). One possible function of these colourless areas within Kranz anatomy is to distribute some of the incident radiation on the tissue inside the stem. As direct sunlight is received from one side of the stem, the colourless areas may increase penetration of light to the opposite side, which could increase efficiency of photosynthesis in densely growing shoots. Recently, it was noted that windows may influence photosynthesis in some plants by illuminating the chlorenchyma from two sides, inside and outside; also, in some CAM species, there is evidence that windows increase infrared radiation inside the tissue, possibly functioning to optimize leaf temperature (C Martin, personal communication).

Another function may be mechanical, contributing to stem strength, since the multicellular network of colourless MCs have much thicker CWs than the MCs. Similar structures have been observed in many xerophytic species, where the patches of green mesophyll are interrupted by colourless cells, which can occur as fibre strands (in orders Fabales and Asterales), by modified thick-walled chlorenchyma or parenchyma sheath cells, elongated perpendicular to the surface (in Restionaceae) (Böcher and Lyshede, 1972; Fahn and Cutler, 1992), or by separate fibres or tracheids in Arthrocennum and Salicornia (Chenopodiaceae) (SaadEddin and Doddema, 1986; Fahn and Cutler, 1992; Keshavarzi and Zare, 2006). This type of structure was thought to have a supporting function, preventing collapse of soft chlorenchyma tissue during water stress, or this compartmentation may help prevent spread of fungal infection from one patch of chlorenchyma to others.

There is also a distinctive colourless region of cells at the tips of the reduced leaves in both the $C_3 T. pergranulata$ and $C_4 T. indica$ subspecies. This feature may increase penetration of light into the photosynthetic cortex of the tissue. Also, in Suaeda monoica, two translucent gaps have been observed at the edges of leaves (Shomer-Ilan et al., 1975; Schütze et al., 2003).

Conclusions

Family Chenopodiaceae has many $C_4$ species occurring in three subfamilies, Chenopodioidae, Salsoleioideae, and Suedoideae. However, in species of subfamily Salicornioideae, which have stems as the major photosynthetic organ, Tecticornia indica s. 1. and an undescribed taxon Tecticornia ‘Yanneri Lake’ form a single well-supported clade which appear to be the only $C_4$ lineages in the subfamily (this study; K Shepherd, personal communication; Carolin et al., 1982). Tecticornia indica has an unusual type of Kranz anatomy with a network of colourless MCs surrounding the patches of MCs within the outer layer of chlorenchyma. These colourless cells, which have thick CWs and a few chloroplasts with limited development for photosynthesis, may function to give a more optimum distribution of incident radiation in the photosynthetic tissue. Tecticornia indica is an NAD-DE $C_4$ plant having chloroplast structural features, and abundance of mitochondria in BSCs, typical of this $C_4$ subgroup. $C_3$-type $\delta^{13}C$ values, low $\Gamma^*$, and $O_2$ insensitivity of carbon assimilation indicate effective function of $C_4$ photosynthesis. The positioning of the mitochondria, which is the site of $C_4$ acid decarboxylation, internal to the centrifugally located BS chloroplasts, and the thickened BS CWs may support efficient donation of $CO_2$ to Rubisco. This study describes a unique $C_4$ structural type of anatomy, Kranz-tecticornoid, in the genus Tecticornia. Further research is needed on Tecticornia species and subspecies to determine if there is more diversity in forms of photosynthesis in the genus (i.e. other $C_4$ species, or $C_3$–$C_4$ intermediates), and to
determine through structural and phylogenetic studies how C_4 may have evolved in this subfamily.

**Supplementary material**

The Supplementary material available at JXB online consists of one figure and one table. They show the phylogenetic position within Tecticornia of taxa analysed in this study based on ITS sequence data. Table S1 lists the taxa sequenced and Fig. S1 shows a phylogram.

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**References**


