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Effect of Sodium Nutrition on Chlorophyll *a*/*b* Ratios in C_4 Plants

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Abstract

Methods for the determination of chlorophyll were compared in *Amaranthus tricolor*, *Kochia childsii* and *Chloris gayana*. From sequential extraction data, 96% ethanol appeared to be more efficient than 80% acetone in extracting chlorophyll from these plants.

The chlorophyll *a*/*b* ratio was significantly lower in sodium-deficient compared to normal C_4 plants. Of the group I elements, only sodium, irrespective of the salt supplied to deficient cultures, restored the chlorophyll *a*/*b* ratios to the value observed in normal plants. The concentration of sodium required to increase the chlorophyll *a*/*b* ratio in leaves of sodium-deficient plants was similar to that required to bring about the growth responses. The increase of the chlorophyll *a*/*b* ratio occurred at an early stage during recovery from sodium deficiency preceding the increase in chlorophyll concentration and the growth response. It is therefore likely that the low chlorophyll *a*/*b* ratio may be intrinsically associated with the condition of sodium deficiency.

Introduction

Sodium is a unique micronutrient in that it is required only by plants possessing the C_4 system (Brownell and Crossland 1972, 1974). Preliminary phaeophytin data suggested that the chlorophyll *a*/*b* ratio was lower in sodium-deficient than normal C_4 plants. Three types of C_4 plants have been described on the basis of the predominant C_4 acid decarboxylase present (Gutierrez *et al.* 1974; Hatch *et al.* 1975). In the NADP–ME-type species, the chlorophyll *a*/*b* ratio of the bundle sheath cells is greater than that of the mesophyll cells whereas, in the NAD–ME-type species, the opposite has been found (Mayne *et al.* 1974). In PEP–CK-type species, the mesophyll and bundle sheath cells have similar chlorophyll *a*/*b* ratios (Mayne *et al.* 1974).

High chlorophyll *a*/*b* ratios are, in general, associated with low chlorophyll/P_{700} ratios, low delayed light emission and low Hill reaction activity which suggests relatively more cyclic to non-cyclic electron flow and thus a potentially higher ratio of ATP/NADPH production (Edwards *et al.* 1976; Edwards and Huber 1981). It is therefore possible that a lower chlorophyll *a*/*b* ratio in sodium-deficient plants could result in an imbalance in the energy production of the mesophyll or bundle sheath cells or both.

Although the balance between cyclic and non-cyclic electron flow can be estimated from photochemical data, pseudocyclic electron flow should also be considered. Changes in the balance between cyclic, non-cyclic and pseudocyclic electron flow give chloroplasts flexibility to meet the specific energy requirements for carbon assimilation (Edwards *et al.* 1976).

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The suggestion that the chlorophyll *a/b* ratio is lower in sodium-deficient plants was inconsistent with the findings of Boag and Brownell (1979), who concluded that the chlorophyll *a/b* ratio was not affected by sodium nutrition. Boag and Brownell (1979) reported that the chlorophyll *a/b* ratio was 3·8 in both sodium-deficient and normal plants of *Kochia childsii* and 3·4 in sodium-deficient and 3·3 in normal plants of *Chloris barbata* when extracted with 80% (v/v) acetone. This inconsistency may be due to the different methods used for the chlorophyll determination. This paper compares two widely used methods of chlorophyll determination and describes the effects of sodium nutrition on the chlorophyll *a/b* ratio in C_4 plants.

Materials and Methods

Plant Material

Amaranthus tricolor L. (non-pigmented form), *Chloris gayana* Kunth and *Kochia childsii* Hort. were chosen as representatives of the three types of C_4 plants. *Atriplex hastata* L. and *Lycopersicum esculentum* Miller, cv. Red cloud (tomato) were chosen as representative C_3 species.

Growth Conditions

The procedures for the germination and growth of plants under conditions of low sodium have been described previously (Brownell 1979). The concentration of sodium as an impurity in the complete culture solution was approximately 0·08 μM. Normal plants were obtained by supplying appropriate cultures with NaCl to give a final concentration of 0·1 mM except where otherwise specified. Where the other group I elements were used, they were supplied as chlorides. The sodium as an impurity contributed by these salts gave sodium concentrations less than 0·02 μM in the final culture solution.

Plants were grown in 2-litre culture vessels containing eight plants each. Experiments were carried out in a naturally illuminated growth cabinet supplied continuously with filtered air to minimize sodium contamination. The maximum photon irradiance was approximately 2500 μmol m^{-2} s^{-1}.

Harvesting Procedure

The age of plants at harvest was between 20 and 42 days. Dry weights were obtained after drying to constant weight at 90°C.

Determination of Chlorophyll

Chlorophyll was extracted from 1–2 g of young fully expanded leaf tissue with either 80% (v/v) acetone or 96% (v/v) ethanol. After harvesting, the tissue was immediately weighed and ground with 20 ml of solvent in a mortar and pestle in the presence of a small quantity of acid washed sand and approximately 0·4 g of $CaCO_3$ per gram fresh weight of tissue. The homogenates were centrifuged at 10 000 *g* for 10 min and the pellets were re-extracted with 10 ml of solvent. Samples were extracted four or five times, until all colour was removed.

The combined supernatants were made up to volume and appropriately diluted to give a maximum absorbance reading not exceeding 0·8 units. All extraction procedures were carried out in the dark or at very low light intensities at 0–5°C. Prior to spectrophotometric measurements, the samples were equilibrated at room temperature in the dark for about 5 min. Spectrophotometric measurements were made using a Beckman DB-G spectrophotometer which was calibrated against a didymium standard. Occasionally, a Varian series 634 spectrophotometer was used to confirm the results obtained with the Beckman DB-G instrument. Both instruments gave the same results. Chlorophyll was determined using the equations of Mackinney (1941) for the 80% (v/v) acetone extracts and those of Wintermans and De Mots (1965) for the 96% (v/v) ethanol extracts. The absorbance of the samples was measured at 740 nm to correct for any possible turbidity in the extracts. The extraction procedures were carried out rapidly and the spectrophotometric measurements were made within 3 h of harvesting the leaf tissue.

Results and Discussion

With normal plants of *K. childsii*, *A. tricolor* and *C. gayana* the extraction of chlorophyll *a*, and to a lesser extent chlorophyll *b*, was achieved with fewer extractions with 96% ethanol than 80% acetone (Figs 1*a*, 1*b* and 1*c*). The chlorophyll *a/b* ratio of

the cumulative 96% ethanol extracts showed little change with sequential extractions (Figs 1*d*, 1*e* and 1*f*). However, with the cumulative 80% acetone extracts, the chlorophyll *a/b* ratio increased up to at least the third extraction. With four extractions the chlorophyll *a/b* ratios of the cumulative extracts were significantly greater ($P<0{\cdot}01$, *t*-test) with 96%

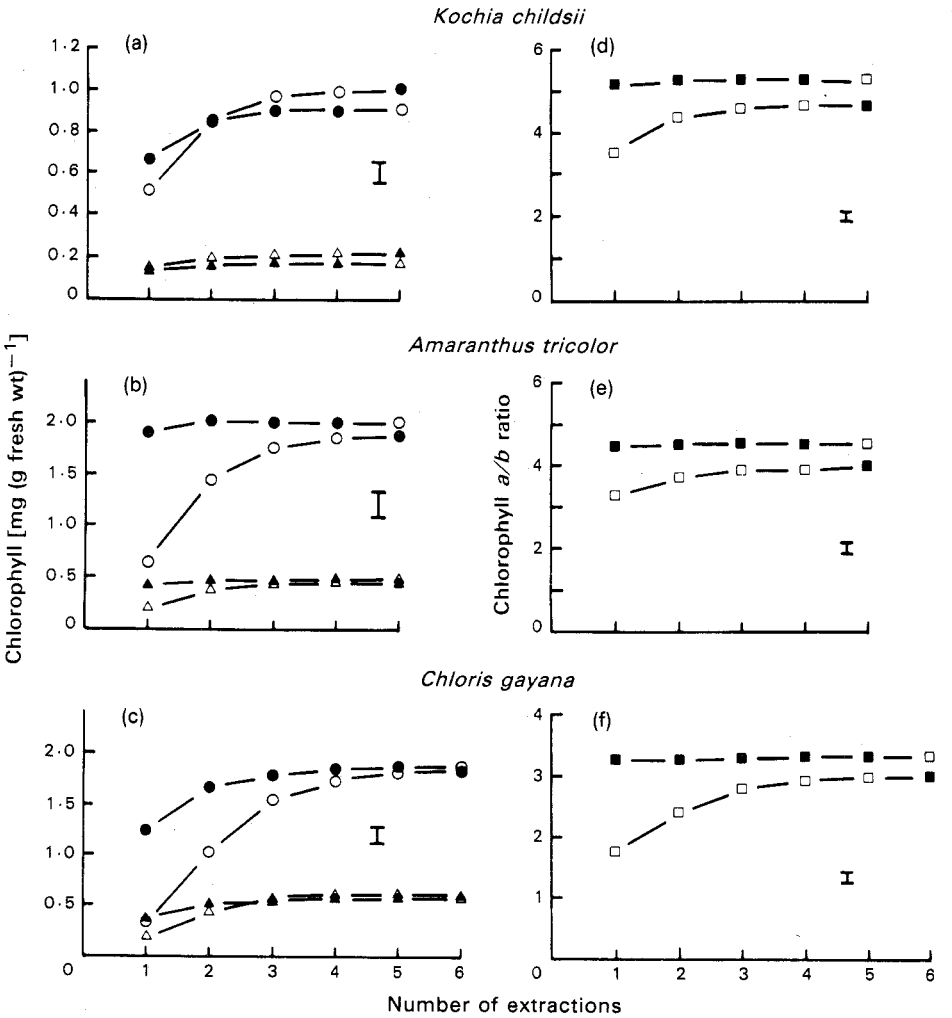


Fig. 1. The comparison of 96% ethanol and 80% acetone for the sequential extraction of chlorophyll from C_4 plants. (*a*), (*b*) and (*c*) The cumulative concentrations of chlorophyll *a* and *b* with sequential extractions. (*d*), (*e*) and (*f*) The chlorophyll *a/b* ratio of cumulative extracts obtained from sequential extractions. Bars indicate twice the maximum s.e.m., $n = 4$. The extracting solvents were exchanged for the final extraction. ● Chlorophyll *a* extracted with 96% ethanol. ○ Chlorophyll *a* extracted with 80% acetone. ▲ Chlorophyll *b* extracted with 96% ethanol. △ Chlorophyll *b* extracted with 80% acetone. ■ Chlorophyll *a/b* ratio extracted with 96% ethanol. □ Chlorophyll *a/b* ratio extracted with 80% acetone.

ethanol than 80% acetone for the three species. This was not due to incomplete extraction of chlorophyll, as substituting the extracting solvents for each other for the final extraction failed to yield any additional chlorophyll or alter the chlorophyll *a/b* ratios. Wickliff and Arnoff (1962) and Wintermans (1969) suggested that 80% acetone extracted compounds

that interfere with the determination of chlorophyll *a*. This could account for the differences in the chlorophyll *a/b* ratios observed using the two extraction solvents.

Leaves of sodium-deficient and normal plants of *K. childsii* were extracted with both 96% ethanol and 80% acetone. Both methods gave similar results for the chlorophyll *a/b* ratio in sodium-deficient plants (Fig. 2). However, in normal plants, the chlorophyll *a/b* ratio was significantly greater ($P<0.001$, *t*-test) when extracted with 96% ethanol than with 80% acetone. With both methods, the chlorophyll *a/b* ratio was lower ($P<0.001$, *t*-test) in sodium-deficient than normal plants.

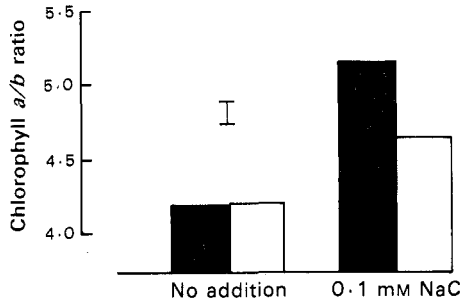


Fig. 2. The chlorophyll *a/b* ratio of sodium-deficient and normal plants of *K. childsii* extracted with 96% ethanol (shaded) and 80% acetone (unshaded). Bar represents twice the maximum s.e.m., $n = 4$.

The above data suggest that 96% ethanol is a more efficient solvent than 80% acetone for the extraction of chlorophyll in these plants. This is in agreement with the findings of Wintermans (1969). Consequently, 96% ethanol was used in subsequent experiments.

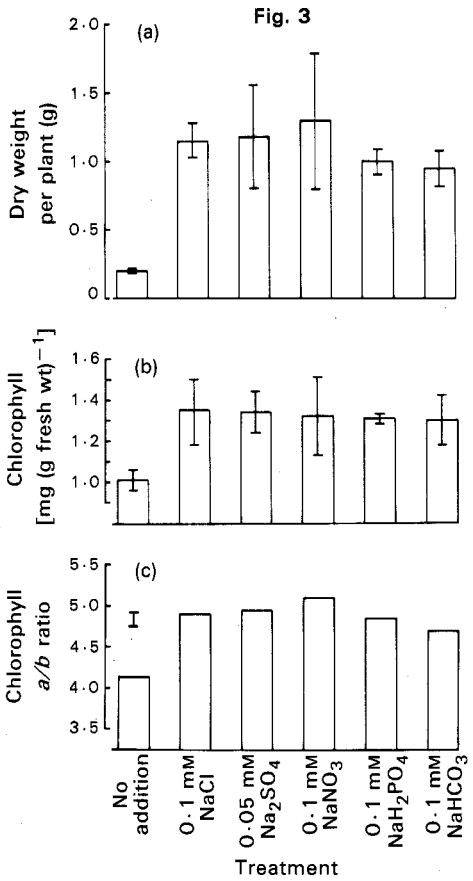
A decreased chlorophyll *a/b* ratio in sodium-deficient compared to normal plants was observed in *K. childsii*, *C. gayana* and *A. tricolor* representing each of the three types of C_4 plants (Table 1). This finding is inconsistent with that of Boag and Brownell (1979),

Table 1. Effect of sodium nutrition on chlorophyll *a/b* ratios
Significance of differences between adjacent values: n.s., not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$

	Chlorophyll *a/b* ratio		
	No addition		0·1 mM NaCl
	C_4 species		
NADP-ME-type			
Kochia childsii	4·19	***	5·16
PEP-CK-type			
Chloris gayana	3·49	*	3·73
NAD-ME-type			
Amaranthus tricolor	3·47	**	4·84
	C_3 species		
Lycopersicum esculentum	3·75	n.s.	3·90
Atriplex hastata	3·66	n.s.	3·69

who reported no significant difference between the chlorophyll *a/b* ratio of sodium-deficient and normal plants of *K. childsii* and *Chloris barbata*. This inconsistency may be attributed to the differences observed between 96% ethanol and 80% acetone as chlorophyll extracting solvents. The C_3 species *L. esculentum* (tomato) and *Atriplex hastata* showed no difference in chlorophyll *a/b* ratio when grown with or without added sodium (Table 1). As only plants possessing the C_4 appendage have been shown to require sodium as a micronutrient (Brownell and Crossland 1972, 1974) and the chlorophyll *a/b* ratio of only the C_4 species was affected by sodium nutrition, it is likely that these differences in chlorophyll *a/b* ratios are related to sodium nutrition.

Equivalent concentrations of sodium supplied to cultures of sodium-deficient plants as the chloride, sulfate, nitrate, dihydrogen orthophosphate or bicarbonate gave significant increases ($P<0.01$, *t*-test) in yield, chlorophyll concentration and chlorophyll *a/b* ratios (Fig. 3). Of the group I elements supplied as LiCl, NaCl, KCl, RbCl, and CsCl to give a final concentration of 0·1 mM to cultures of sodium-deficient *Amaranthus tricolor*, only sodium significantly increased ($P<0.001$, *t*-test) the yield, chlorophyll concentration and chlorophyll *a/b* ratio (Fig. 4). These results demonstrate that the increase in chlorophyll *a/b* ratio is due specifically to sodium.



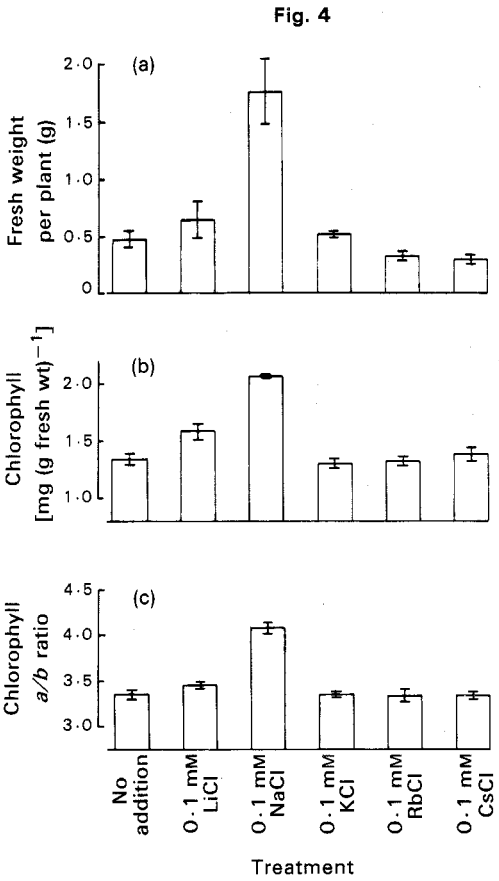


Fig. 3. The effect of different sodium salts on the yield (*a*), chlorophyll concentration (*b*) and chlorophyll *a/b* ratio (*c*) of *A. tricolor*. (*a*) Mean ± s.e.m., $n = 2$. (*b*) Mean ± s.e.m., $n = 3$. (*c*) Bar represents twice the maximum s.e.m., $n = 3$.

Fig. 4. The effect of different group I elements on yield (*a*), chlorophyll concentration (*b*) and chlorophyll *a/b* ratio (*c*) of *A. tricolor*. (*a*) Mean ± s.e.m., $n = 3$. (*b*) and (*c*) Mean ± s.e.m., $n = 4$.

When plants of *A. tricolor* were grown in increasing concentrations of sodium chloride, the yield, chlorophyll concentration and chlorophyll *a/b* ratio increased sharply, reaching a plateau at approximately 50 μM sodium chloride (Fig. 5). This indicates that the chlorophyll concentration and the chlorophyll *a/b* ratio are closely involved in the systems affected by sodium nutrition.

The chlorophyll *a/b* ratio of *A. tricolor* increased rapidly during the recovery from sodium deficiency (Fig. 6*a*). Within 1 day of receiving sodium, the recovering plants had significantly greater ($P<0.01$, *t*-test) chlorophyll *a/b* ratios than the sodium-deficient plants.

After an additional day, the chlorophyll *a/b* ratio of the recovering plants did not differ significantly from the normal plants. The increase in chlorophyll *a/b* ratio in recovering plants preceded the increases in chlorophyll concentration and yield. The chlorophyll concentration of recovering plants was not significantly greater than that of sodium-deficient plants until 2 days after the addition of sodium (Fig. 6*b*). Differences in yield between recovering plants and sodium-deficient plants were not apparent until 3–5 days after the addition of sodium (Fig. 6*c*). The rapid increase in chlorophyll *a/b* ratio in response to the sodium treatment may represent a key step in the recovery from sodium deficiency.

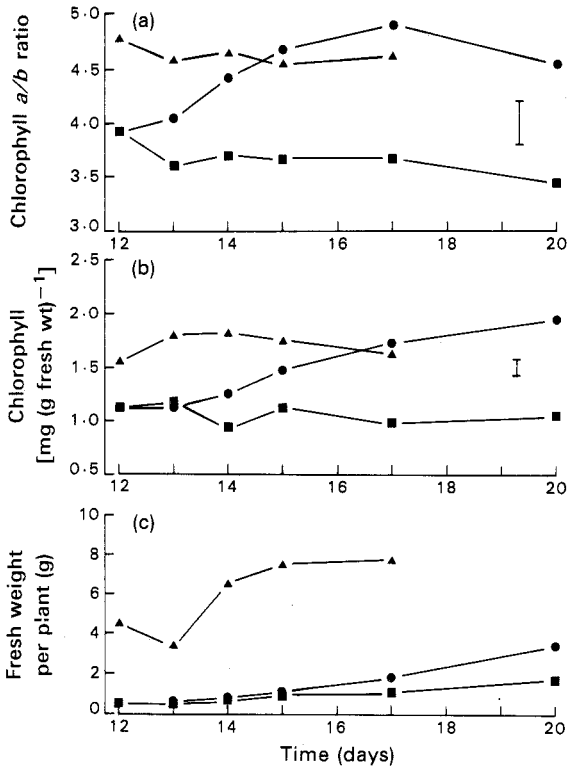


Fig. 5. The effect of concentration of sodium chloride upon yield (*a*), chlorophyll concentration (*b*) and chlorophyll *a/b* ratio (*c*) of *A. tricolor*. (*a*) Mean ± s.e.m., $n = 2$. (*b*) and (*c*) Mean ± s.e.m., $n = 3$.

In general, high chlorophyll *a/b* ratios suggest relatively more cyclic to non-cyclic electron flow and thus a potentially higher ratio of ATP/NADPH production (Edwards *et al.* 1976; Edwards and Huber 1981). The lower chlorophyll *a/b* ratio could result in a lower ratio of ATP/NADPH production in sodium-deficient compared to normal plants. If the chlorophyll *a/b* ratio is lower in the mesophyll cells, this could lead to a decrease in the production of ATP. As the regeneration of phospho*enol*pyruvate from pyruvate in mesophyll chloroplasts requires ATP (Hatch 1976), the rate of this process could be limited in sodium-deficient plants. This would support the suggestion of Nable and Brownell (1984) that the observed increased alanine concentration in sodium-deficient plants is associated with a corresponding increase in the concentration of pyruvate due to a limitation in the conversion of pyruvate to phosph*enol*pyruvate in sodium-deficient plants.

Acknowledgments

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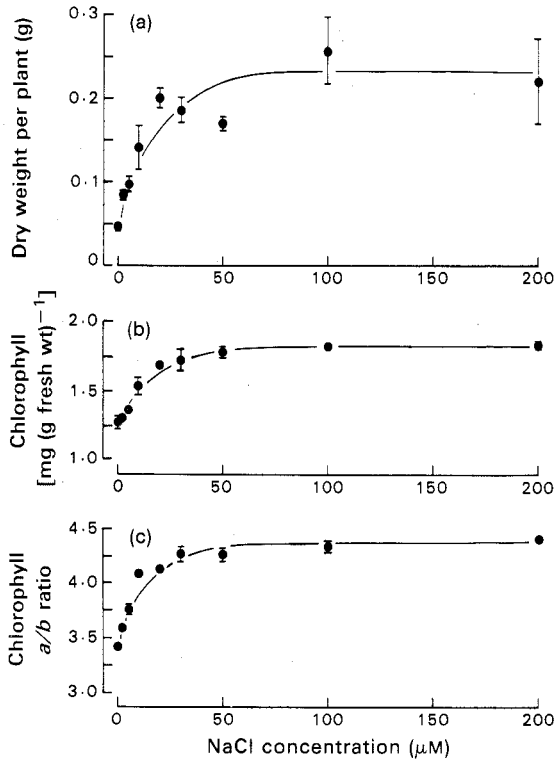

Fig. 6. Changes in chlorophyll *a/b* ratio (*a*), chlorophyll concentration (*b*) and yield during the recovery from sodium deficiency in *A. tricolor* (*c*). Bars indicate twice the maximum s.e.m. ■ No addition. ▲ 0·1 mM NaCl day 0. ● 0·1 mM NaCl day 12.

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