STUDIES ON THE SODIUM REQUIREMENT OF C_4 PLANTS

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ABBREVIATIONS

CAM	Crassulacean Acid Metabolism
d	day
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FeCN	potassium ferricyanide
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MV	methyl viologen
NAD-ME	NAD-malic enzyme
NADP-ME	NADP-malic enzyme
NR	nitrate reductase
PEP	phospho*enol*pyruvate
PEP-CK	phospho*enol*pyruvate-carboxykinase
3PGA	3-phosphoglyceric acid
PVP	polyvinylpyrrolidone
RGR	relative growth rate
RH	relative humidity
RuBP	ribulose-1,5-bisphosphate

CONTENTS

INTRODUCTION

What is the essential elements of plants ? It will be difficult to begin this thesis on the Na requirement of C_4 plants without making some references to the term essentiality.

Although most of the naturally occurring mineral elements are found in plant tissues, many of these elements are thought to be present merely because of the absorption from the soil and are not operative in plant metabolisms. In accordance with the Arnon's proposal (1950), it is generally accepted that the essential elements should satisfy following criteria composed of three main parts: (1) the element is absolutely required for plant growth, and the plant is unable to complete its normal growth and reproduction without the element, (2) it is not possible to replace the function by other elements, and (3) the element has a direct or indirect action in plant metabolism. So far, sixteen elements has been firmly established to be essential nutrients which satisfy above criteria. These comprise the macronutrients: carbon, hydrogen, oxygen, nitrogen, phosphorous, potassium, calcium, magnesium and sulphur, and micronutrients: iron, manganese, zinc, copper, molybdenum, boron and chlorine. On the other hand, there are so-called 'beneficial elements' which stimulate plant growth only under certain conditions or compensate the toxic effects of other elements but not fulfill the criteria of essentiality.

Many plant species respond positively to Na, however, Na is defined as one of the beneficial elements, because the Na effect is usually observed under condition where K supply is limited and is considered to be a partial substitution for the role of K as

an essential element. Since Na ion normally tends to accumulate to higher concentrations in the vacuoles than in the cytoplasm, if substantial amounts of Na are taken up by plants under low-K condition, Na would be transported into vacuoles in exchange for vacuolar K, thereby substituting for K in its contribution to the leaf solute osmorality or increasing the availability of K in the cytoplasm. However, Na cannot displace the role of K as an enzyme activator. Thus, Na will substitute for K in some, but not all its roles in the plants, and the degree to which its substitution occurs depends upon the extent of Na absorption and transport within any particular species (Flowers and Läuchli 1983). In general, crops can be divided into one of two classes each of which was subdivided into two groups with regard to their responses to Na (Pirson 1955, Marschner 1972). The responses of plants to Na application can be summarized as Table 1, although there was some variability in specific results.

Table 1. Responses of several crops to Na application under low-K and sufficient-K condition (Flowers and Läuchli 1983).

Condition	Response to Na	Crop
Low-K	slight to none	maize, lettuce, onion
	slight to medium	barley, oat, mustard
Sufficient-K	slight to medium	cabbage, pea, oat
	large	beet, celery

On the other hand, there is a quite distinct role of Na from that as a the beneficial element, *i.e.,* essential function in metabolism of certain plant species. Brief history of the studies on the Na requirement of plants are presented below.

Sodium was first shown to be essential for cyanobacteria *Anabaena cylindrica* cells, when it was demonstrated that 0.22 mM (5 ppm) Na was required and no other monovalent cations could substitute for Na (Allen and Arnon, 1955). In Na-deficient *Anabaena* cells, the activity of N_2-fixation decreased and the levels of nitrate reductase activity were decontrolled (Brownell 1979). Recently, Na was shown to be required to maintain nitrogenase activity of *Anabaena torulosa* cells (Apte and Thomas 1983). In *Anabaena variabilis* (Reinhold *et al.* 1984) and *Synechococcus leopoliensis* cells (Miller *et al.* 1984, Miller and Canvin 1987), extracellular Na was shown to be required for attainment of high rates of photosynthesis and high affinity transport of HCO_3^- across plasmamembrane at low concentrations of dissolved inorganic carbon in the medium. On the other hand, 0.1 mM (2.3 ppm) Na was shown to be essential for angiosperm, *Atriplex vesicaria* (Brownell and Wood 1957). Subsequently, it was demonstrated that Na was generally required by the plants having C_4 photosynthetic pathway but not by species with C_3 pathway (Brownell and Crossland 1972). In addition, it was reported that facultative CAM plant, *Bryophyllum tubiflorum* responded to Na when performing CAM but not when photosynthesizing via C_3 pathway (Brownell and Crossland 1974). The correlation between the possession of C_4 pathway and the requirement for Na would be

indicative of a possible role of this element in the C_4 dicarboxylic acid pathway. Therefore, a great deal of experiments has been carried out in this view point (Boag and Brownell 1979, Brownell 1979, Shormer-Ilan 1979), however, involvement of Na in C_4 pathway is still uncertain. In attempts to elucidate the physiological mechanism of the Na requirement of C_4 plants, the fact that not all C_4 plants seem to require Na (Hewitt 1983) should not be overlooked. In other words, it is possible that Na may affect some metabolism other than C_4 pathway or may be involved in a part of metabolisms not yet defined which occurs in C_4 but not in C_3 plants.

On assumption that there may be a target site of Na in C_4 plants' metabolism besides C_4 dicarboxylic acid pathway, I started my investigation on the Na requirement of C_4 plants using a NAD-ME type C_4 plant, *Amaranthus tricolor* L. cv. tricolor. At first, the effects of Na on the growth of *A. tricolor* plants were studied (Chapter 1). Next I studied on the metabolic responses of the plants during the recovery from Na deficiency and found that the increase in the level of nitrate reductase activity was one of the most rapid responses. Since it was considered that the increased nitrate reductase activity may have significant contribution to the growth enhancement during the recovery from Na deficiency, the effects of Na application on NO_3^- assimilation of *A. tricolor* plants were investigated (Chapter 3). In Chapter 4, I report on the Na-stimulated NO_3^- uptake in *A. tricolor* plants and discuss a causal relation between the Na-stimulated NO_3^- uptake and the enhancement of the level of nitrate reductase

activity by Na application. These studies revealed that nitrate reductase activity level increased in consequence of the stimulation of NO_3^- uptake by Na application, in turn NO_3^- assimilation was then promoted resulting in the growth enhancement. Finally, I report on the effects of Na on the growth and the levels of nitrate reductase activities of several monocotyledonous C_4 plants (Chapter 5).

CHAPTER 1

EFFECTS OF SODIUM APPLICATION ON *AMARANTHUS TRICOLOR* L. CV. TRICOLOR PLANTS

Section 1. Effect of Na Application on Growth of *Amaranthus tricolor* L.

Many plant species show positive growth response to Na. The effect of Na, however, has been ascribed to a poor supply of K, that is, Na can in part compensate for the shortage of K (Flowers and Lauchli 1983). Brownell and Wood (1957) first demonstrated the essentiality of Na for the growth of *Atriplex vesicaria*. Subsequently Brownell and Crossland (1972, 1974) demonstrated that the Na requirement was remarkable only in C_4 and CAM plants, and not in C_3 plants. Moreover, in two genera of Chenopodiaceae, *Atriplex* and *Kochia*, which contain both C_3 and C_4 species, Na was shown to be essential only for C_4 species (Brownell 1979). Although these evidences may indicate a possible role of Na in CO_2 assimilation by C_4 dicarboxylic acid pathway, physiological basis for the Na requirement of C_4 plants has not been established.

Taking into account that not all C_4 plants have been shown to require Na (Hewitt 1983), it seems reasonable to assume that metabolic process other than C_4 pathway may necessitate Na for its operation. This means that a research on the Na requirement of C_4 plants must be begin with a quite new view point. That is to say, every sort of experimental results must be given careful

consideration once again.

For this purpose, I have studied the effects of Na on growth (Section 1) and CO_2 assimilation of a NAD-ME type C_4 plant, *Amaranthus tricolor* L. (Section 2).

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Seeds of *Amaranthus tricolor* L. Tricolor were purchased from Takii Seeds Co., Ltd., Kyoto, 605. Japan, in may 1983. The seeds used in the growing seasons of 1984 were obtained from plants grown under Na-deficient conditions in 1983 in a green house. The seeds (1 g) were washed with distilled and deionized water five times each for 5 min. The seeds were sown on a sheet of cheesecloth covering acid-washed polyethylene beads (diameter 5 mm, packed in a 20×25×3 cm polyethylene container) wetted with distilled water, and were kept at 30 °C under continuous illumination (8000 lux). After germination, a half-strength culture solution (see below) was given and just before the second leaf pair appeared, the seedlings were transplanted to water culture, using 3 L plastic pots. The solution was aerated for 30 min every two hours without air purification. The pots were kept in a greenhouse, where they were dipped into a water bath with circulating water of 20 °C. The standard culture solution contained 1 mM KCl, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.25 mM $(NH_4)_2HPO_4$ and 1 mM $Ca(NO_3)_2 \cdot 4H_2O$. The salts were purified by recrystallization from ethanol-water, except for calcium nitrate. The micronutrient composition was

that of Arnon's solution cited by Hewitt (1966) except that all the iron was supplied as ferric citrate. The culture solutions were prepared using distilled and deionized water. Sodium was supplied as NaCl or Na_2SO_4 and in reference treatments, KCl or K_2SO_4 was supplemented to give the same anion concentration. Every pot had four or two seedlings and the solution was changed every four or two days, as the plant growth proceeded.

The growth experiments were carried out for four times in the summer of 1983 and 1984. In the 1983 experiments, the plants were allowed to grow until their seed formation stage. The K-treated plants had only 20 % seeds compared with the Na-treated plants, but the germination efficiency was not different.

Analysis. The plants were dissected into leaves, stems and roots, and the parts were washed with distilled water, blotted dry, weighed and dried in an oven at 70 °C. The materials were finely ground using a ball mill and appropriate amounts were digested with a nitric acid-sulfulic acid mixture (10:1) at 150 °C. Contents of Na and K were determined by emission spectrophotometry and Ca, Mg, and Fe by atomic absorption spectrophotometry. Total N and P contents were determined colorimetrically using the indophenol blue method (Weatherburn 1967) and the molybdenum blue method (Murphy and Riley 1962), respectively, after Kjeldahl digestion. Chlorine was extracted with boiling water and measured by a colorimetric method (Iwasaki *et al.* 1952). Chlorophyll content was estimated in 80 % (V/V) acetone-water extracts (Arnon 1949) and betacyanin contents in 67 % (V/V) methanol-water extracts (Elliott 1979) prepared from the fresh leaves.

RESULTS

Growth. Dry matter production by *A. tricolor* plants under various growth conditions is shown in Fig. 1. Growth of the plants treated with Na salts were about 300% of those grown without Na salts. As the reference treatment plants received K

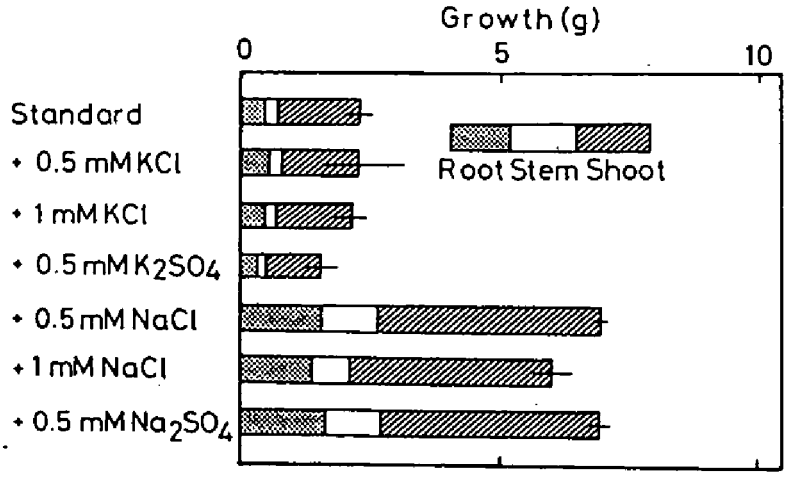

Fig. 1 Dry matter production of *Amaranthus tricolor* plants grown with or without sodium salts. The seeds were sown on May 4, 1984. Sodium salts were given for 27 days and the plants were harvested on July 9, 1984. The results are shown as an average of three plants and the bars show standard deviations. The growth experiment was repeated three times with similar results.

salts instead of Na salts to give the same anion concentrations, the growth stimulation induced by the application of Na salts was a Na specific effect and was due neither to compensation of K shortage nor to the accompanying anions.

Mineral Contents. Mineral contents of *A. tricolor* plants grown under various conditions are shown in Table 1 and 2. For elements other than Na and K, only the contents in the leaves are presented. The plants supplied with K_2SO_4 at a concentration of 0.5 mM showed poorer growth (Fig. 1) and lower Cl and K contents (Table 1) than the K-treated plants. Sodium contents in the leaves increased 40- to 50-fold with addition of Na salts at

Table 1 Contents of sodium, potassium and chlorine in *A. tricolor* plants under various conditions

Culture conditions	Na (ppm)			K (%)			Cl (ppm)
	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves
Standard	32.6	48.9	110	6.69	12.7	9.03	5,940
+0.5 mM KCl	41.8	54.3	93.9	7.30	13.9	11.3	6,740
+1 mM KCl	32.5	38.9	68.8	6.53	12.9	10.8	7,070
+0.5 mM K_2SO_4	59.6	56.2	54.3	4.38	7.46	5.58	3,350
+0.5 mM NaCl	1,700	6,500	6,930	4.80	9.26	5.19	9,150
+1 mM NaCl	2,430	10,500	6,300	4.08	7.25	5.29	8,570
+0.5 mM Na_2SO_4	2,650	12,400	7,520	4.30	9.16	5.86	8,220

The values are the means of three replicates.

Table 2 Contents of nitrogen, phosphorus, calcium, magnesium and iron in *A. tricolor* plant leaves under various conditions

Culture conditions	N (%)	P (%)	Ca (%)	Mg (%)	Fe (ppm)
Standard	5.91±0.249	0.743±0.010	2.84±0.100	1.12±0.058	128±5.50
+0.5 mM KCl	6.20±0.370	0.855±0.058	2.85±0.064	1.21±0.067	N.D.
+1 mM KCl	5.99±0.050	0.807±0.071	2.54±0.50	1.08±0.130	249±12.4
+0.5 mM K_2SO_4	5.50±0.189	0.509±0.044	3.33±0.162	1.01±0.025	213±10.0
+0.5 mM NaCl	5.20±0.161	0.722±0.058	3.01±0.020	1.08±0.052	N.D.
+1 mM NaCl	5.28±0.117	0.628±0.023	3.20±0.016	1.12±0.027	331±12.5
+0.5 mM Na_2SO_4	5.10±0.116	0.641±0.006	3.17±0.067	1.16±0.063	205±4.49

The values are the means of three replicates±standard errors. N.D., Not determined.

0.5 or 1 mM, but the distribution of this element among the organs was almost the same as in the K-treated plants. *A. tricolor* plants responded to Na but the Na content and distribution in the Na-treated plants were not different from those of other crop plants grown under ordinary conditions, for which the Na requirement has not been reported (Flowers and Läuchli 1983). The Cl content (Table 1) was somewhat higher in the Na-treated plants, but even that of the reference plants far

exceeded the critical content for Cl deficiency (Broyer *et al.* 1954).

Comparing the mineral contents between the Na-treated and the K-treated plants, significant differences were found also for N, K and Ca contents (Table 1, 2). Nitrogen and K contents were higher in the Na-treated plants. These results suggest that Na improves the efficiency of N and K utilization. Phoshorous contents did not differ significantly between the Na-treated and the K-treated plants. Calcium contents were generally higher in the Na-treated plant leaves but no difference were found in stems and roots. Magnesium and Fe contents in the leaves were also determined (Table 2), since the K-treated plants easily suffered from chlorosis. Although significant fluctuations were observed in the Fe contents, magnesium contents were not different.

Betacyanin. Brownell and Crossland (1972) demonstrated that the Na-deprived *A. tricolor* plants showed the deficiency symptoms of chlorosis and necrosis. In my experiment, symptoms of Na

Table 3 Effect of sodium application on the chlorophyll and betacyanin contents in the leaves of *A. tricolor* plants

Treatment	Chlorophyll (mg/g fr. wt.[b]) (Chlorophyll a/b ratio)	Betacyanin (μmoles/g fr. wt.[b])
Without sodium (+0.5 mM KCl)[a]	1.49 (3.41)	0.305
With sodium (+0.5 mM NaCl)[a]	2.30 (3.72)	1.23

[a] 0.5 mM KCl or 0.5 mM NaCl was supplemented to the standard culture solution.
[b] Leaves just reaching their maximum sizes were used.
Determinations were carried out six times using different plants.

deficiency such as pale leaf color and low betacyanin contents were observed (Table 3), but usually necrosis was not found.

Medium pH Changes. Figure 2 shows the medium pH changes. The pH decrease was faster in the Na-containing medium. Although

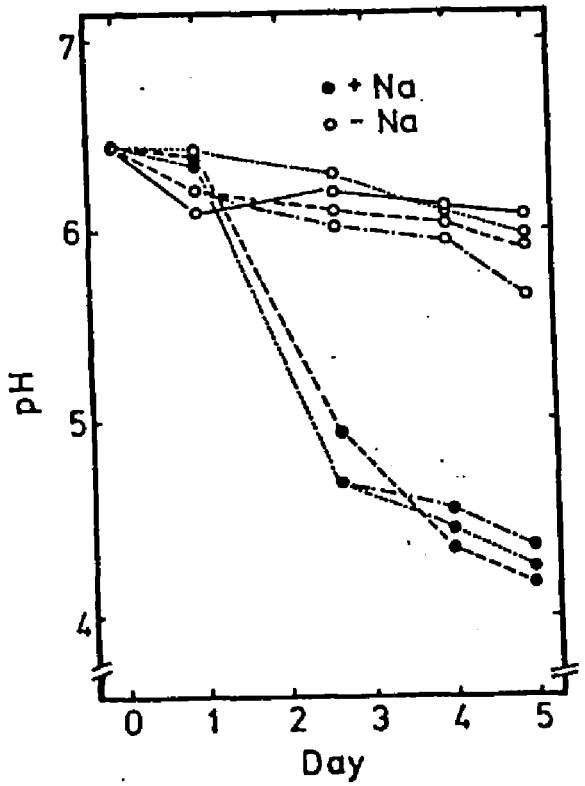

Fig. 2 Acidification of the *Amaranthus tricolor* rooting medium by sodium salt application. The medium pH changes were traced in the 1984 growing period using the same plants as in Fig. 1. Determinations were done from the 17th day after sodium salt application. Standard culture solution (-○-); standard culture solution containing 0.5 meq./liter chloride salts (·····) of sodium (●) and potassium (○), the same at 1 meq./liter cation concentration (-·-·-); the same at 1 meq./liter cation concentration, but given as sulfate salts (----).

this difference was reproducible, it is not clear at present whether Na has a direct effect on this pH decrease or not. Since the Na-treated plants grow vigorously, the faster pH drop in the Na-containing medium may be due to a greater imbalance of uptake between cations and anions, that is, the secondary effect

of Na. Another possibility is involvement of Na in the proton extrusion by the roots.

DISCUSSION

Under my growth conditions, the contaminating Na in the culture solutions was less than 20 ppb, and the Na contents in the leaves of plants grown without added Na were 30 to 60 ppm (Table 1) although in the stems and roots they were generally higher. According to Brownell (1979), his group reduced the concentration of contaminating Na in the culture solutions to less than 2 ppb, but the Na content in their *Atriplex vesicaria* leaves, showing Na deficiency symptom, was 10 mmol·kg^{-1} dry matter, equaling 230 ppm. Kushizaki and Yasuda (1964) reported that when sugar beet plants showed Na deficiency symptoms, the internal Na contents was 6 mmol·kg^{-1} dry matter. Comparing these findings indicates that the critical concentration of Na at which plants suffer Na deficiency may differ from species to species.

Brownell (1979) also pointed out that the Na requirement of the C_4-*Atriplex* plants was met by application of about 0.1 meq. l^{-1} of Na. My results (Fig. 1) indicate that application of 0.5 meq. l^{-1} of Na satisfies the Na requirement of *A. tricolor* but the precise threshold concentration of Na for the plants was not determined. Assuming that all the Na in the leaves was free, the Na concentrations were calculated to be about 0.2 mM in the K-treated leaves and about 20 mM in the Na-treated leaves on the tissue water bases. Accordingly, if the system(s) which requires Na occurs in the leaves, the internal threshold

concentration of Na is in a mM range.

Nunes *et al.* (1983) demonstrated a NaCl-stimulated proton efflux from sugar beet leaf discs. When the leaf discs absorbed Na, stoichiometric release of K and proton occurred under light. They concluded that sugar beet has a Na-stimulated proton pump and discussed the possibility of the pump being a Na-activated ATPase. As *A. tricolor* belongs to the same family, Chenopodiaceae, as the sugar beet, there might be some similar response to Na as to proton efflux in *A. tricolor* plants.

Elliott (1979) investigated cytokinin effects on betacyanin biosynthesis in dark-grown *A. tricolor* seedlings and his intensive studies revealed that there is a Na-K synergism for the cytokinin-dependent betacyanin synthesis. Elliott (1979) suggested involvement of a Na-K ATPase activity, but its occurrence in higher plants has yet been demonstrated. Our results (Table 3) indicate a four fold increase in the betacyanin contents in mature leaves. As cytokinin is known to participate in many diverse aspects of plant cell metabolism, Na may operate through the cytokinin functions.

Section 2. Effect of Na on $^{14}CO_2$ Assimilation of *Amaranthus tricolor* L.

Positive correlation between the possession of C_4 photosynthetic pathway and the requirement for Na (Brownell 1979) make us expect a possible role of this element in the C_4 pathway. However, the involvement of Na in the C_4 dicarboxylic acid pathway has never been demonstrated.

In this Section, I report on a study carried out to determine whether Na actually affects CO_2 assimilation of *Amaranthus tricolor* L. plants or not.

MATERIALS AND METHODS

Plant Materials. Plants of *Amaranthus tricolor* L. were grown with either 0.5 mM NaCl or 0.5 mM KCl for 30 days in a greenhouse as described in Section 1. A large excess of leaf discs (diameter 6 mm) were prepared from the youngest fully expanded leaves of the Na-treated and the K-treated plants.

$^{14}CO_2$ Fixation by Leaf Discs. The rates of $^{14}CO_2$ fixation by leaf discs were determined basically according to Atkins and Canvin (1970). To achieve high and reproducible rates of $^{14}CO_2$ fixation, leaf discs had to be stored in distilled and deionized water for 1 h (for the Na-treated plants) or 2 h (for the K-treated plants) in darkness. This pretreatment may relate to stomatal responses of the freshly cut tissues (Atkins and Canvin 1970). After the

dark pretreatment period, a sample of twenty leaf discs was selected, and placed with leaf surface down onto distilled and deionized water in a petri dish (5 cm in diameter). The tissue was then transferred to the photosynthesis chamber (250 ml total volume). After 20 min of preillumination under low-CO_2 condition, leaf discs were exposed to $^{14}CO_2$ air (5 μCi/mg CO_2, flow rate 150 ml/min) for each period (1, 2, 4 and 6 min) in an illuminated chamber. Illumination was provided with white incadescent projection lamp (2000 $\mu E\ m^{-2}\ s^{-1}$). The low-CO_2 condition was obtained by passing normal air through 4 N KOH solution.

Analysis. After feeding of $^{14}CO_2$, the tissues were plunged into liquid nitrogen and kept at this temperature until extraction. Tissues were extracted with 67 % (V/V) methanol-water and fractionated into the acidic (organic acids and phosphate esters), the basic (mainly amino acids) and the neutral fraction (mainly sugars) by using ion exchange resins (Atkins and Canvin 1970). The basic fraction was applied to 20×20cm cellulose sheet (Merck, cellulose plate Art. 5552) and developed in 88 % (W/W) phenol-water:water:acetic acid:0.2 M EDTA (83:15:1:0.5, V/V). After drying samples were run with methanol:NH_4OH:H_2O (7:1:1, V/V) at right angles to the first solvent. Radioactive areas were located by autoradiography, collected and counted in 0.5 ml distilled water and 5 ml Triton based scintillator solution (Dotite, Scintisol EX-H). The radioactivity of all samples were determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Figure 3 shows the higher rate of $^{14}CO_2$ fixation into 67 % methanol-soluble fraction of the Na-treated plants compared with that of the K-treated plants. The incorporation of ^{14}C into the 67 % methanol-insoluble fractions were about 10 % (K-treated plants) and 15 % (Na-treated plants) of total fixation during 6 min of $^{14}CO_2$-feeding period. The net photosynthetic rates

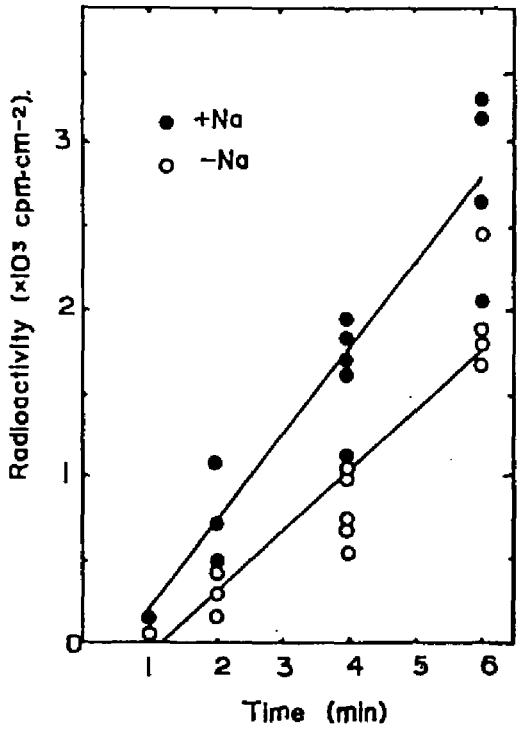

Fig. 3. Effect of Na on the $^{14}CO_2$ fixation by the leaves of *Amaranthus tricolor*. A sample of twenty leaf discs (diameter 6 mm) from the K-treated (O) or the Na-treated plants (●) was exposed to $^{14}CO_2$ air (5 μCi/mg CO_2) for each period in a 250 ml chamber at light intensity of 2000 μE m^{-2} s^{-1}.

calculated were 8.1 mg CO_2 dm^{-2} h^{-1} for the Na-treated plants and 4.1 for the K-treated plants. These values were comparable to the

$^{14}CO_2$ assimilation rates recorded in a NAD-ME type C_4 plant, *Portulaca orelacea* (Hatch 1975).

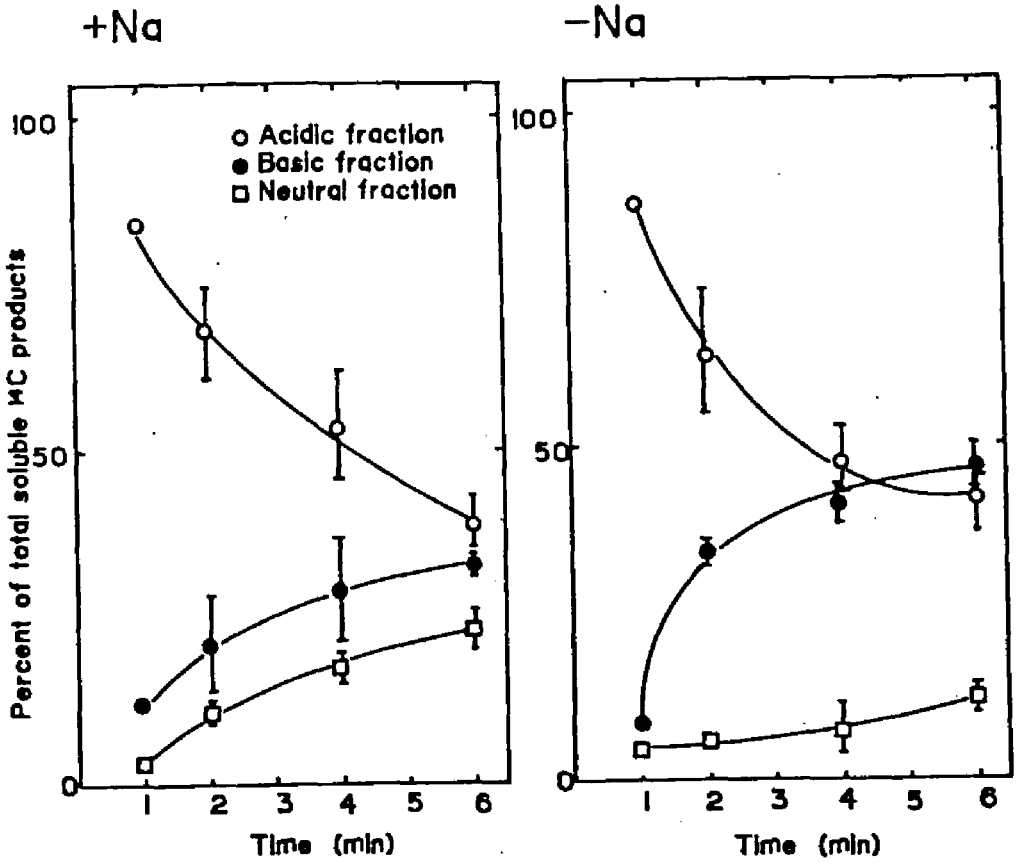

Fig. 4. Effect of Na on the ^{14}C labeling of the acidic (○), the basic (●) and the neutral fractions (□) from the leaves of *Amaranthus tricolor*. Samples were the same as those in Fig. 3. Data are the means and SD of four replicates.

Time course of distribution of ^{14}C among the neutral, the acidic and the basic fractions of leaf discs were shown in Fig. 4. In the Na-treated plants, faster increase in radioactivity in the neutral fraction (sugars) and slower increase in the basic fraction (amino acids) compared with those in the K-treated plants were observed.

From these results, it can be deduced that the Na application promoted CO_2 assimilation into sugars resulting in the growth enhancement of *A. tricolor* plants. Brownell (1979) also reported that greater amount of ^{14}C from the $^{14}CO_2$ being incorporated into the sugar fraction in normal than Na-deficient *Atriplex vesicaria* plants indicated that they were photosynthesizing more rapidly.

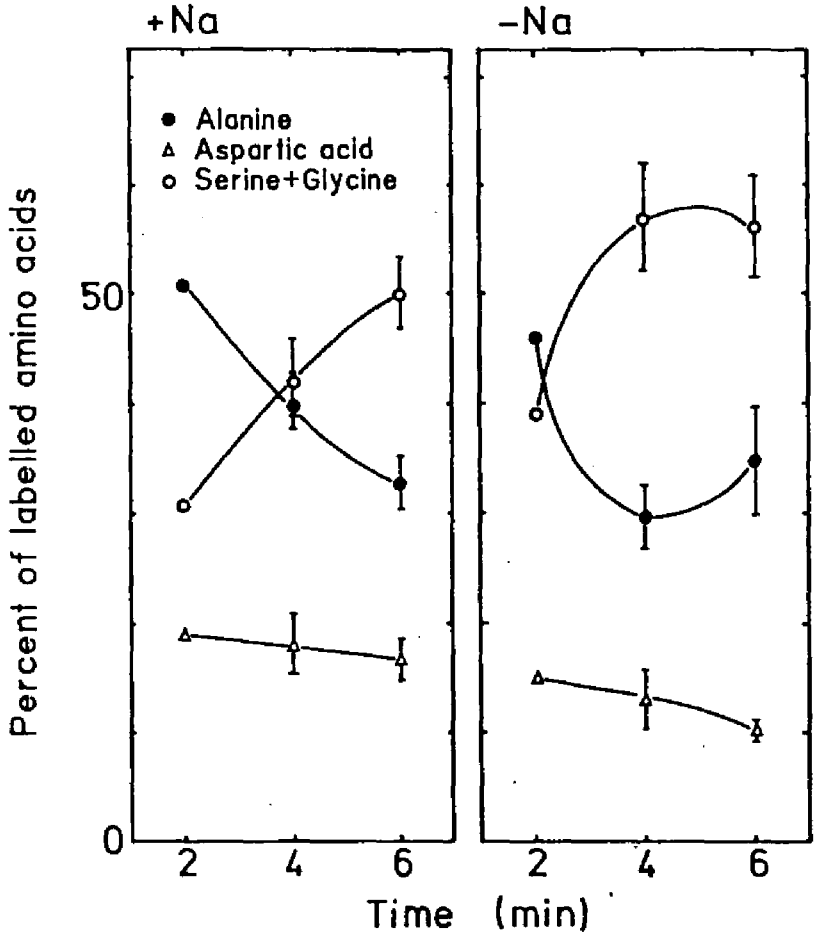

Fig. 5. Distribution of ^{14}C in the basic fraction from the leaves of *Amaranthus tricolor* grown with or without Na. The basic fraction: alanine (•), aspartic acid (▲) and serine plus glycine (○), were separated as described in 'Materials and Methods'. Samples were the same as those in Fig. 4. Data are the means and SD of four replicates.

As it was suggested that the lower rate of $^{14}CO_2$ assimilation into sugars of the K-treated plants was due to the greater amount of ^{14}C retained in the basic fraction (Fig. 4), the basic fraction was further analyzed (Fig. 5). It was revealed that the basic fraction was composed of aspartic acid, alanine and serine plus glycine (not separately determined) in the both treatments, and the higher radioactivity in the basic fraction of the K-treated plants mainly came from greater amount of ^{14}C retained in serine plus glycine compared with that of the Na-treated plants.

Kennedy and Laetsch (1973, 1975) suggested that a direct incorporation of $^{14}CO_2$ into pyruvate and alanine in a C_4 plant, *Portulaca orelacea* (NAD-ME type). However, Hatch (1975) concluded that the label found in alanine was derived from the ^{14}C initially incorporated into C_4 acids via the interchange of the label between 3PGA and PEP via 3PGA mutase and enolase or via the C-4 to C-1 randomization of malate via the action of fumarase. Nable and Brownell (1984) reported that photosynthetically active pool of alanine in the leaves of *A. tricolor* was greater in the Na-deficient than in the Na-sufficient plants. They proposed that Na is necessary for pyruvate, Pi dikinase activity *in vivo*, thereby pyruvate and alanine accumulated whilst PEP was depleted in the Na-deficient plants. If this was the case, all the C_4 plants should require Na. In practice, however, some C_4 plants, such as sugarcane and maize, seem not to require Na (Hewitt 1983, Ohta *et al.* 1988 C). Although it is possible that the critical concentrations of Na in C_4 plants may be different from species to species, I consider

that the alanine accumulation is one of results of the disturbed metabolisms by Na deficiency rather than the direct involvement of Na in the maintenance of the *in vivo* activity of pyruvate, Pi dikinase.

At present, the pathway of C_3 amino acids labeling in my experiment is unknown. One of the possibilities is the carbon flow via photorespiratory process, although the capacity of photorespiration in the bundle sheath cells seemed to be inhibited or suppressed, in spite of the presence of peroxisomes and glycolate pathway enzymes in C_4 plants (Canvin 1979). Johnston *et al.* (1984 B) have reported that a high ambient CO_2 concentration increased the yield of the Na-deficient C_4 plants and discussed a possible increased leakiness of bundle sheath to CO_2 by the deficiency of Na. At any rate, it is considered that the normal carboxyl donation from amino acids to sugars does not proceed smoothly under Na-deficient condition, leading to the lower photosynthetic rate of the K-treated plants.

In this Chapter, I demonstrated that Na application of 0.5 meq. l^{-1} to *A. tricolor* plants grown under Na-deficient condition brought about a three-fold increase in dry matter production compared with those of the K-treated plants. This growth stimulation was due to Na itself and not a supplementary effect of Na on K shortage nor to the accompanying anion (Section 1). In Section 2, it was revealed that Na promoted CO_2 assimilation. However, the plants used have been grown with or without Na throughout the experiment. Therefore, it is essential hereinafter to discriminate the immediate effects from the cumulative ones by Na on plant metabolism.

CHAPTER 2

EARLY RESPONSES OF SODIUM-DEFICIENT *AMARANTHUS TRICOLOR* L. PLANTS TO SODIUM APPLICATION

Since Na seems to be required only by the plants having C_4 photosynthetic pathway, it has been proposed that there may be a possible role of this element in the process of CO_2 fixation by the C_4 pathway (Brownell 1979). However, no information on the involvement of Na in any cellular metabolism is currently available. This must be because too much attention have been paid to the positive correlation between the possession of C_4 pathway and Na requirement.

In this Chapter, I report on the early responses of Na deficient *Amaranthus tricolor* L. plants to Na application. Rapid responses of the plants to Na can represent the primary steps of recovery from Na deficiency, providing a clue to understanding of the function of Na in C_4 plants.

MATERIALS AND METHODS

Plant Materials. Seeds of *Amaranthus tricolor* L. cv. Tricolor plants used in this experiment were obtained from the plants grown under Na-deficient conditions in a greenhouse (Matoh *et al.* 1986). The seedlings were raised as described previously (Chapter 1), unless otherwise stated. Briefly, the low-Na seeds were germinated on a sheet of cheesecloth covering acid-washed polyethylene beads. After germination, the seedlings were supplied with a half strength of the culture solution and just

before the second leaf pair appeared, the seedlings were trasferred to a water culture solution using 1.5 L plastic containers. Every container had nine plants and the culture solution was renewed every day at the end of the light period. The standard culture solution (pH 6.0) contained 1 mM KCl, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM $(NH_4)_2HPO_4$, and 0.5 mM $MgSO_4$ $7H_2O$. The salts were purified by recrystallization from ethanol-water, except for calcium nitrate. The micronutrient composition was that of Arnon's solution cited by Hewitt (1966) except that all the iron was supplied as ferric citrate. Sodium concentration as an impurity in this culture solution was assayed to be less than 20 ppb using emission spectrophotometry, which was 10 times higher than that in the Brownell's culture solution (Brownell 1979). However, as the value obtained by emission spectrophotometry should be overestimated due to positive interferences from ions in the culture solution (Brownell 1979), actual Na contamination in the culture solution should be lower than the obtained values. Throughout this study, a growth chamber (LPH-200 RDS, Nippon Medical and Chemical Instruments Co. Ltd., Osaka, Japan) was used under the following conditions: RH 80%, photo period 15 h, and temperature 30°C for whole growth period. The light intensity was approximately 150 $\mu E\ m^{-2}\ s^{-1}$ at the top of the seedlings. NaCl was supplied to 30-d-old Na-deficient seedlings to reach a final concentration of 0.5 mM at the end of the dark period and in the reference treatment KCl was supplied at the concentration of 0.5 mM. The 30-d-old seedlings had nine leaves under the current growth conditions. The leaf position was counted from the base to the tip of the shoot.

Relative Growth Rate. Changes in the fresh weight were determined at the end of the dark period every day using 10 to 16 identical plants. RGR was calculated from the fresh weight values according to the equation:

$$RGR = (\ln W_1 - \ln W_2)/(t_1 - t_2)$$

where W_2 is the fresh weight at time t_2 and W_1 is the fresh weight at time t_1.

Light-Dependent Oxygen Evolution and Dark Oxygen Uptake. Leaf discs 2 mm in diameter (0.2 g) were vacuum infiltrated with 50 mM Hepes-Tris buffer (pH 7.2) containing 1 mM $CaCl_2$ under reduced pressure. The discs were washed with 10 ml of the same buffer three times and immediately subjected to O_2 assay using a Hansatech O_2 electrode (King's Lynn, Norfork, England). A batch of 10 leaf discs were placed in the sample cuvette containing 1 ml of the same buffer at 30°C. After 5 min of preillumination (white incandescent projection lamp, 2000 $\mu E\ m^{-2}\ s^{-1}$), the reaction was started by adding 15 µl of 0.75 M of $KHCO_3$. Dark O_2 uptake was determined by shading the sample cuvette with a black cloth.

Photosynthetic Electron Transport. To determine the effects of Na on the rate of photosynthetic electron transport by thylakoids, the leaves were harvested at 24 h after the treatments. Chloroplasts were isolated according to the method of Jensen and Bassham (1966) with slight modifications. The blending medium contained 50 mM Hepes-Tris (pH 7.6), 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, 0.5 mM KH_2PO_4 and 2 mM ascorbate. Thylakoid samples were washed once with the blending medium

omitted ascorbate and resuspended in the same medium. Oxygen evolution or consumption were followed polarographycally using a Hansatech oxygen electrode at 25 C basically according to Izawa (1980). Illumination was provided by a slide projection lamp that produced as incident quantam flux density of at least 2000 $\mu E\ m^{-2}\ s^{-1}$ at a reaction cell. The basic reaction mixture contained 50 mM Hepes-Tris (pH 7.6), 2 mM $MgCl_2$, 10 mM KCl and 20-25 μg Chl/ml. The rate of electron transport from water to FeCN was measured in the basic reaction mixture supplemented with 1 mM FeCN and 5 mM NH_4Cl. Electron transport from water to MV was determined in the basic reaction mixture containing 0.1 mM MV, 1 mM NaN_3 and 5 mM NH_4Cl.

Enzyme Activity. The 30-d-old Na-deficient seedlings were supplied with either 0.5 mM of NaCl or KCl at the end of the light period, and after 24 h, some C_4 photosynthetic enzymes were assayed as follows. The changes in the NR activity were traced in detail. The day/night period was changed to 12 h/12 h. The 5th to the 8th leaves (1 g) from five different seedlings were sampled for the enzyme extraction.

For measurement of RuBP carboxylase, the tissue was ground in 10 ml of ice-cold buffer containing 100 mM Hepes-KOH (pH 7.4), 10 mM $MgCl_2$, 5 mM DTT, 2 mM EDTA, 10% (W/V) insoluble PVP. After filtration through four layers of cheesecloth, the extract was centrifuged at 4°C and 10,000g for 10 min. Subsequent steps were carried out at room temperature. The supernatant was desalted and activated using a Sephadex G-25 column (Pharmacia, PD-10) which was equilibrated with 100 mM Bicine-KOH (pH 8.0), 30 mM $MgCl_2$, 10 mM $KHCO_3$, 20 μM 6-P-gluconate, and 2.5 mM DTT. The enzyme was

assayed basically according to Lorimer *et al.* (1977) at 25°C. The assay mixture (0.5 ml) contained 100 mM Bicine-KOH (pH 8.2), 1 mM EDTA, 30 mM $MgCl_2$, 20 mM $KH^{14}CO_3$ (0.1 mCi/mmol). The reaction was started by adding 0.5 mM RuBP and stopped after 60 s by adding 0.1 ml of 12 N HCOOH. Pyruvate, Pi dikinase was assayed according to Edwards *et al.* (1980) with slight modifications. My grinding medium contained 5 mM DTT, 2.5 mM K_2HPO_4, 2.5 mM pyruvate, and 0.5% (W/V) ascorbate. The desalting buffer was the same as the grinding medium but without ascorbate. NAD-malic enzyme (Hatch *et al.* 1982), PEP carboxylase (Osmond and Greenway 1972), aspartate aminotransferase and alanine aminotransferase (Hatch 1973) were assayed according to the referred sources.

For NR determination, the crude enzyme was prepared basically according to the recommendations given by Notton and Hewitt (1979). Samples were ground in ice-cold medium containing 50 mM K-phosphate (pH 7.5), 0.01 mM DTT, 1 mM EDTA, and 10% (W/V) insoluble PVP using a chilled mortar and pestle. The homogenate was passed through four layers of cheesecloth. After centrifugation at 4°C and 20,000g for 10 min, the supernatant was desalted using a Sephadex G-25 column (Pharmacia, PD-10) equilibrated with the grinding medium and used for the enzyme assay at 30°C. All the extraction procedures were carried out at 4°C. NR activity was determined according to the method of Nakagawa *et al.* (1984).

Enzyme activities are expressed as μmol of product formed or substrate utilized /min, or /h mg Chl, or /g fresh weight.

Sodium and Potassium. Sodium and K contents in the plant

materials were determined by emission spectrophotometry using a boiling-water extract (10 min) and the concentrations were expressed on the basis of tissue water contents (Matoh *et al.* 1986).

Chlorophyll. Total Chl contents and Chl *a/b* ratios were determined according to the method of Arnon (1949) using the leaf discs prepared for the determination of the light-dependent O_2 evolution assay.

RESULTS

Growth. Following the addition of NaCl at a concentration of 0.5 mM, the Na-deficient *Amaranthus tricolor* seedlings acquired significantly higher growth rate than the seedlings which

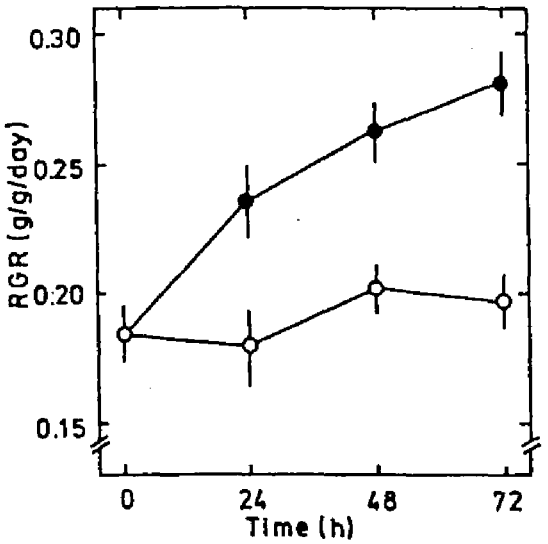

FIG. 1 Changes in the relative growth rate of sodium-deficient *A. tricolor* plants during recovery from sodium deficiency. Thirty-day-old sodium-deficient *A. tricolor* seedlings were supplied with 0.5 mM NaCl (●) or 0.5 mM KCl (○) at the end of the dark period (at time 0 h) and the relative growth rate was calculated from the daily changes in the fresh weights. Data are the means and SD.

received KCl within 24 h (Fig. 1). The relative growth rate gradually increased until 72 h, and thereafter reached a constant value. The value for the plants with an adequate supply of sodium was 0.295 ± 0.033, while that for the deficient plants was 0.243 ± 0.034.

Sodium and Potassium Concentration. Since the Na concentration in the roots of both treatments did not change during the experiments (data not shown), it was considered that the Na taken up by the plants is transported mainly to the shoots. In the stems of the plants which received Na, the Na concentration was lower than that of the leaves and it remained at about 1.5 mM even after 48 h (Fig. 2). Sodium concentration increased more

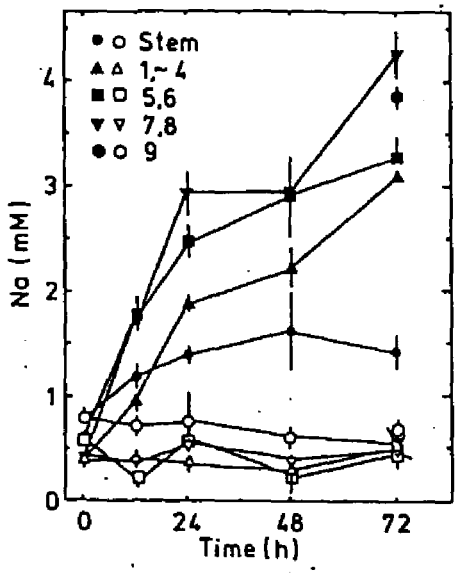

FIG. 2. Changes in the sodium concentration of leaves and stems of 30-d-old *A. tricolor* seedlings during the recovery from sodium deficiency. Leaf position was counted from the base to the tip of the shoot. The seedlings were supplied with 0.5 mM NaCl (●) or 0.5 mM KCl (○) at the end of the light period (at time 0 h). Data are the means and SD of determinations of four individual plants. SD are not shown when smaller than the symbols.

Table 1 *Changes in the Potassium Concentration of Leaves and Stems of 30-d-old A. tricolor Seedlings during the Recovery from Sodium Deficiency*

		Potassium Concentration	
		mM	
0 h	Stem	183 ± 38.3[a]	
	Leaf 1–4[b]	163 ± 53.2	
	Leaf 5, 6	105 ± 40.5	
	Leaf 7, 8	97.7 ± 34.2	
		+ 0.5 mM KCl	+ 0.5 mM NaCl
12 h	Stem	204 ± 15.1	213 ± 16.5
	Leaf 1–4	163 ± 7.85	154 ± 8.58
	Leaf 5, 6	142 ± 24.5	150 ± 18.5
	Leaf 7, 8	145 ± 30.3	140 ± 13.4
24 h	Stem	238 ± 13.5	223 ± 20.5
	1–4	140 ± 12.4	173 ± 16.6
	5, 6	163 ± 21.5	150 ± 15.3
	7, 8	152 ± 15.4	140 ± 22.4
48 h	Stem	207 ± 12.2	209 ± 28.5
	1–4	153 ± 16.8	154 ± 18.4
	5, 6	145 ± 28.4	138 ± 14.5
	7, 8	156 ± 12.4	110 ± 24.8
72 h	Stem	170 ± 19.5	171 ± 11.9
	1–4	163 ± 27.4	126 ± 25.4
	5, 6	173 ± 26.3	129 ± 28.9
	7, 8	135 ± 12.7	137 ± 21.8

[a] Potassium concentrations (mM) were calculated on tissue water base. [b] Leaf position and treatments are the same as in the legend for Figure 2. Data are the means and SD of determinations of four individual plants.

rapidly in the younger than in the older leaves, and during the first 24 h it increased faster than during the following 24 h. In the plants to which KCl was supplied, the Na concentration remained constant at the initial value of about 0.4 mM in the leaves and 0.8 mM in the stems (Fig. 2). Potassium concentration was nearly the same between the treatments (Table 1) suggesting that the K uptake was not affected by sodium application.

Chlorophyll. Chl content per leaf area which was not significantly affected by the Na treatment within the first 24 h increased markedly in the leaves of the recovering plants within 48 h of receiving Na (Fig. 3A). The Chl *a/b* ratio increased appreciably within the first 24 h of receiving the Na treatment (Fig 3B).

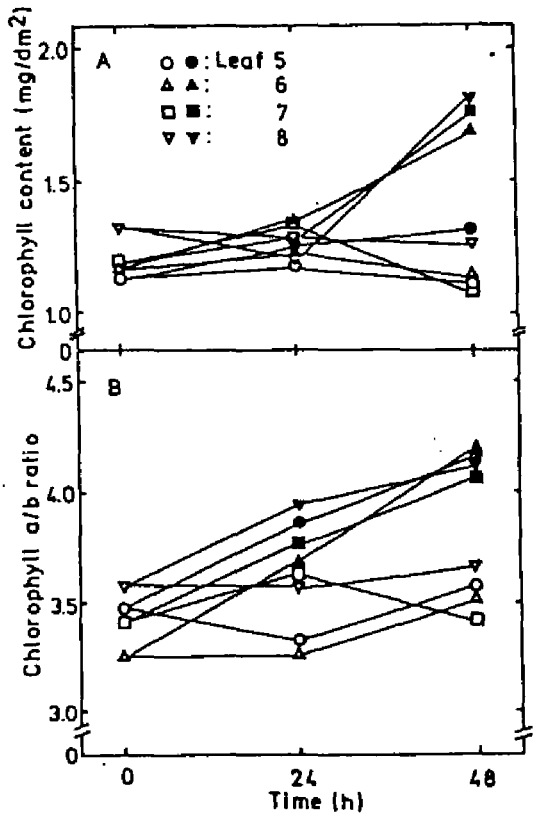

FIG. 3. Changes in the Chl content (A) and Chl *a*/*b* ratio (B) of the leaves of 30-d-old *A. tricolor* seedlings during the recovery from sodium deficiency. Leaf position and treatments are the same as in the legend for Figure 2. Data are the means of determinations of two samples of three plants. Curves are the representatives of three independent determinations.

Oxygen Evolution and Uptake. The rate of the light-dependent O_2 evolution determined by using the leaf discs was enhanced by Na application and the extent of the stimulation differed from leaf to leaf (Fig. 4). Within 24 h of receiving the Na treatment, the O_2 evolution greatly increased in leaves 5 and 6, which were almost fully expanded. The values recorded in leaf 7, which was just developing, and in leaf 8, about 5 d after its initial appearance, increased linearly during the experimental period. At 48 h, the rate of O_2 evolution in leaves 7 and 8 still

increased while that in leaf 5 decreased gradually and the rate of O_2 evolution in leaf 6 remained constant. These results indicate that the effect of Na on the light-dependent O_2

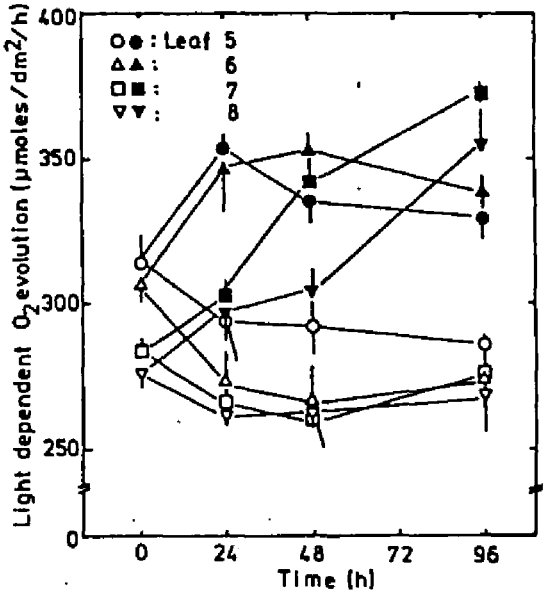

FIG. 4. Changes in the light-dependent O_2 evolution of the leaf discs of 30-d-old *A. tricolor* seedlings during the recovery from sodium deficiency. Leaf position was counted from the base to the tip of the shoot. Treatments are the same as in Figure 1. Data are the means and SD of determinations of three samples of four plants.

evolution was greater in the developing leaves than in the mature leaves. It is also suggested that Na enhanced photosynthesis irrespective of the degree of stomatal opening, because, in the method for the measurement of photosynthesis using an O_2 electrode, CO_2 was absorbed mainly through the cut surface of the leaf tissues. It is therefore unlikely that the stomatal diffusion resistance limited the CO_2 fixation (Pitman *et al.* 1975).

Dark O_2 uptake rates in the leaf discs also responded

positively to Na application within 24 h (Table 2). Brownell and Jackman (1966) who reported that the O_2 uptake rates of the leaves were stimulated by feeding of Na to cut shoots of Na-

Table 2 *Effects of Sodium Application to Sodium-Deficient A. tricolor Seedlings on Dark O_2 Uptake Rates of the Leaf Discs*

Leaf position, treatments, and preparation of leaf discs are the same as in the legend for Figure 2. O_2 uptake rates of the leaf discs were determined at 0 and 24 h after the supply of salts. Data are the means and SD of determinations of three samples of four plants.

Leaf Position	Dark O_2 Uptake		
	Control (0 h)	+0.5 mM KCl (24 h)	+0.5 mM NaCl (24 h)
		$\mu mol/dm^2 \cdot h$	
Leaf 5	54.5 ± 3.77	56.3 ± 4.13	69.3 ± 3.83
Leaf 6	56.5 ± 0.835	58.1 ± 7.96	79.2 ± 4.23
Leaf 7	54.8 ± 1.60	63.9 ± 0.505	73.5 ± 3.54
Leaf 8	58.1 ± 0.290	61.8 ± 2.14	74.8 ± 1.81

deficient *Atriplex* plants suggested that Na may be involved in the glycolytic stages of respiration.

The threshold concentration of internal Na which elicited the stimulation of O_2 evolution was considered to be below 3 mM (Fig. 2); however, I failed to stimulate the O_2 evolution by infiltrating NaCl (5 mM) into the leaf discs of the youngest fully expanded leaves which were Na deficient (data not shown).

Photosynthetic Electron Transport. The rates of photosynthetic electron transport from water to FeCN and to MV increased within 24 h of the addition of Na (Table 3). Increase in the electron transport activities by the addition of Na were more evident in the younger leaves than in the older leaves.

Table 3 Effect of Na application on the rates of photosynthetic electron transport by thylakoids of A. tricolor plants.

Data are the means and SE of at least three determinations of pooled samples of 20 to 30 leaves per sample.

Reaction	Oxygen exchange rate			
	Leaf 6*		Leaf 8	
	+Na	-Na	+Na	-Na
	μmole O_2/mg Chl/h			
$H_2O \longrightarrow$ FeCN	268 ± 10.8	226 ± 15.4	308 ± 15.4	251 ± 9.89
	**(521 ± 20.9)	(501 ± 34.1)	(587 ± 29.3)	(479 ± 18.8)
$H_2O \longrightarrow$ MV	263 ± 8.82	243 ± 6.51	304 ± 24.3	249 ± 6.08
	(508 ± 16.9)	(538 ± 14.3)	(580 ± 46.2)	(475 ± 11.6)

* On the day 30 after germination Na-deficient A. tricolor plants were supplied either 0.5 mM of NaCl (+Na) or KCl (-Na) at the end of the light period. Leaves were harvested during the last 5h of the light period on d 31. Leaf positions were counted from the base to the tip of the shoots.

** Values in the parenthesis represent the rates of electron transport of thylakoids per leaf area (μmol $O_2/dm^2/h$).

Enzyme Activity. Measurable increase was not detected in the activities of PEP carboxylase, RuBP carboxylase, pyruvate, Pi dikinase, NAD-malic enzyme, aspartate aminotransferase, and alanine aminotransferase by the Na treatment within 24 h (Table 4). The activity levels of NAD-malic enzyme and pyruvate, Pi dikinase were lower than the typical values (Hatch 1976, Hatch *et al.* 1982). As the activities reached the comparable values in the leaves of 50-d-old plants, these low activities may be due to

Table 4 *Effects of Sodium Application on the Extractable Enzyme Activities of Leaves of the 30-d-old A. tricolor Seedlings*

The 30-d-old sodium deficient *A. tricolor* seedlings were received either 0.5 mM of NaCl or KCl at the end of the light period and the enzyme activities were measured after 24 h of the treatments. See "Materials and Methods" for detail. Data are the means and SD of at least three replications. Enzyme activities are expressed as μmol of product formed or substrate utilized/min·mg Chl.

Enzyme	+ 0.5 mM KCl	+ 0.5 mM NaCl
	units/mg Chl	
PEP carboxylase	17.6 ± 3.19	17.8 ± 1.84
RuBP carboxylase	8.70 ± 0.25	8.20 ± 0.204
NAD-Malic enzyme	6.23 ± 0.723	6.78 ± 0.526
Pyruvate, Pi dikinase	1.68 ± 0.285	1.62 ± 0.149
Aspartate aminotransferase	61.0 ± 5.01	57.2 ± 8.51
Alanine aminotransferase	20.3 ± 2.59	19.4 ± 3.16

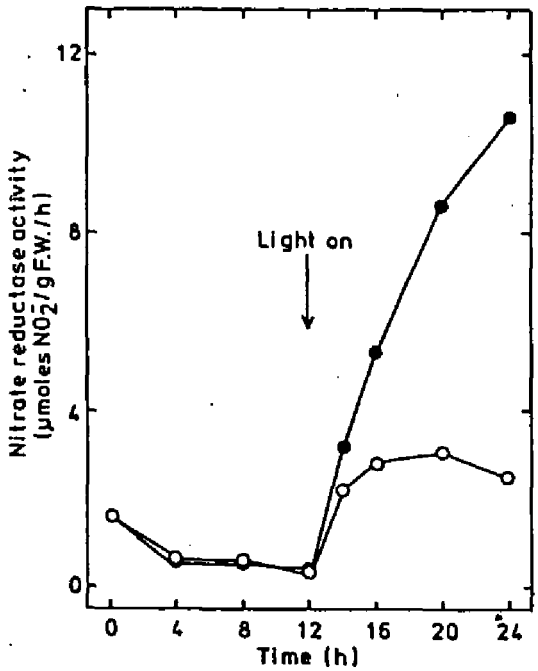

FIG. 5. Effects of sodium application on the induction of NR activity in sodium-deficient *A. tricolor* leaves. The 30-d-old seedlings received either 0.5 mM NaCl (●) or 0.5 mM KCl (O) at the beginning of the dark period (at time 0 h) and the changes in the NR activity were followed. Curves are the representatives of four independent experiments.

the immaturity of the sampled leaves.

Extractable NR activity in the *A. tricolor* leaves increased significantly in response to the Na application (Fig. 5). The activity was not different between the treatments at the end of the dark period; however, in the light period, the activity began to increase, more rapidly in the Na-supplied leaves. Twelve h later, the activity reached 10.6 μmol of nitrite formed/g fresh weight/h in the Na-supplied leaves while 3.06 in the Na-deprived leaves.

DISCUSSION

Growth increase in the Na-deficient *Amaranthus tricolor* seedlings occurred within 24 h of the addition of the Na salt. Although the RGR values were calculated from the daily changes in the fresh weight, since the water content per fresh weight of the plants did not vary appreciably during this experiment, it is assumed that the changes in fresh weight closely reflected well the daily changes in dry matter production.

The rates of O_2 evolution determined in the leaf discs were found to increase within 24 h of receiving the Na treatment. Accordingly, the improvement of the growth was intrinsically correlated with the stimulation of photosynthesis by Na application. However, since there were not significant increase in the enzyme activities responsible for C_4 photosynthesis within the same period, it was considered that photosynthesis was improved by the Na application without altering the enzyme levels of C_4 photosynthetic pathway. Boag and Brownell (1979)

demonstrated that the C_4 pathway was operating even in the Na-deficient *Kochia* and *Chloris* plants, that is, the ^{13}C value, CO_2 compensation point and percentage of ^{14}C label in C_4 dicarboxylic acid in short-term photosynthesis were similar in the Na-deficient and the normal plants.

On the other hand, the rates of electron transport by thylakoids increased within 24 h of the addition of Na, indicating that the stimulation of photosynthesis may come out in terms of the enhancement of photochemical energy supply. In the current experiments, the increase in the rate of O_2 evolution of leaf discs preceded the increase in the total Chl content, suggesting that the recovery of photosynthesis occurred prior to the increase in the total Chl content. Johnston *et al.* (1984 B) and Matoh *et al.* (1986) reported higher Chl *a/b* ratios in the *Amaranthus* plants with as adequate supply of Na than in the Na-deficient plants. In addition, it was confirmed that the Chl *a/b* ratio increased prior to the increase in the total Chl content and reached the values typical of C_4 plants within 48 h of receiving the Na treatment, while in the Na-deficient leaves the values remained lower (Fig. 3). The increase in the ratio was ascribed to the faster recovery to the normal levels of the Chl *a* content than that of Chl *b* (data not shown), as was reported by Brownell and Jackman (1966) in the Na-deficient *Atriplex* leaves. In general, the C_4 plants have a higher Chl *a/b* ratio and P_{700}/Chl total ratio than C_3 plants suggesting that the PSI activity is higher in C_4 plants (Black and Mayne 1970). This characteristic confers a higher capacity of ATP supply required for the regeneration of PEP from pyruvate in the mesophyll

chloroplasts of C_4 plants (Edwards and Huber 1981). Taken together, the rapid stimulation of the light-dependent O_2 evolution by the Na treatment could be due to the fast recovery of the photochemical activity by means of the increase in the Chl *a/b* ratio to the normal level in the Na-supplied leaves.

Edwards (1974) reported that high levels of PSI would be reflected in high Chl *a/b* ratio in C_4 plants. It has been reported that PSI components seemed to be synthesized in preference of Chl and PSII components during the early stage of iron-nutrition mediated chloroplast development in sugar beet (Nishio and Terry 1983). It is possible that there may be a similar chloroplast development sequence during the recovery from Na-deficiency in *A. tricolor* plants. Effects of Na on the photosystem should be examined further.

Allen and Arnon (1955) first demonstrated that Na was essential for the growth of *Anabaena cylindrica* and later Brownell and Nicholas (1967) showed that in the Na-deficient *A. cylindrica* cells, the NR activity was much higher than that in the normal cells and that the higher NR activity induced a large accumulation of nitrite resulting in detrimental effects on the metabolism. Smith (cited by Brownell 1979) also reported the decontrol of NR in the same species. In *Anabaena tolurosa*, Apte and Thomas (1983) reported that Na-deficient cells lacked the nitrogen-fixing ability and the effect of Na was replaced by the addition of combined inorganic or organic nitrogen to the culture medium. In higher plants, Brownell (1979) reported that no such effects of Na deficiency on NR activity were observed in a C_4

plant, *Echinochloa utilis*. However, in the Na-deficient *A. tricolor* plants, NR activity increased in the same way as was recorded for the light-dependent O_2 evolution within 24 h by the addition of Na. The enhanced activity of NR which recorded a value of 10.6 μmol nitrite/g fresh weight/h is comparable to that recorded for other C_4 plants (Hewitt *et al.* 1979) and in the case of *A. tricolor*, the activity seemed to be restored to a normal level rather than to be decontrolled by the Na treatment. As the extractable NR activity reflects well the absolute amounts of this enzyme protein (Somers *et al.* 1983), it is considered that the increase in the extractable NR activity is due to the *de novo* synthesis of NR. However Na would not be involved directly in the NR protein synthesis, because Na requirement is confined to only some C_4 plants. In higher plants, the level of NR is regulated by many factors including substrate nitrate, molybdenum, phytohormones, and light conditions (Beevers and Hageman 1980, Duke and Duke 1984). Therefore, Na may have an effect on these regulating factors to produce the NR protein. It is also possible to consider that Na promotes the nitrate availability for NR induction system, thereby affecting the levels of NR activity.

I previously showed that betacyanin content was lower in the Na-deficient *A. tricolor* plants than in the normal plants (Chapter 1). Elliott (1979) reported that the synthesis of betacyanin in *A. tricolor* seedlings is a cytokinin- and phytochrome-dependent reaction, and that Na stimulated the synthesis. As cytokinin has been known to influence NR activity (Knypl 1979) and induce *de novo* synthesis of NR (Kende and Chen

1972), the increase in the NR activity could be due to the stimulation of the cytokinin-dependent process by Na.

In spite of the extensive studies carried out by Brownell's group, the role of Na in higher plants has not yet been elucidated (Brownell 1979). In this Chapter, I demonstrated that Na application to the Na-deficient *A. tricolor* plants increased not only photosynthesis but also the extractable NR activity within 24 h. These results suggest that the Na-sensitive metabolic process is not confined to photosynthesis. Following Chapters will focus on the actual site of Na by means of the effects of Na on the NR activity as an indicator of the Na effect.

CHAPTER 3

EFFECT OF SODIUM APPLICATION ON NITRATE ASSIMILATION IN *AMARANTHUS TRICOLOR* L. PLANTS

Rapid responses to the Na treatment are important in attempts to understand the function of Na in C_4 plants. In Chapter 2, I presented evidence that the enhancement of NR activity level might have significant effects on the growth of *Amaranthus tricolor* L. plants during the recovery from Na deficiency. In this Chapter, I report on the studies carried out to determine to what extent the NO_3^- assimilation process is affected by the Na application.

MATERIALS AND METHODS

Plant Materials. Seedlings of *Amaranthus tricolor* L. cv. Tricolor were cultured under Na deficient condition as described previously (Ohta *et al.* 1987). The basal culture solution (pH 6.0) prepared in distilled and deionized water contained 2 mM KNO_3, 1 mM $CaCl_2$, 0.25 mM $(NH_4)_2HPO_4$ and 0.5 mM $MgSO_4 \cdot 7H_2O$. The micronutrients composition was that of Arnon's cited by Hewitt (1966) except that all the iron was supplied as ferric citrate. The concentration of Na as an impurity in the culture solution was estimated to be less than 5 ppb using atomic absorption spectrophotometry. Throughout this study, a controlled environment chamber was used under following conditions: temperature 30 °C for whole growth period, RH 80 %, photoperiod 15 h-light/9 h-dark cycle, light intensity 350 $\mu E\ m^{-2}\ s^{-1}$. Ten plants were grown in a 3 L plastic container. Culture solution

were changed every 2 days and aerated continuously.

For a comparison of RGR on different nitrogen sources, Na-deficient *A. tricolor* plants were transferred from the basal culture solution to the NO_3^--N culture solution consisting of 2 mM KNO_3, 1 mM $CaCl_2$, 0.25 mM KH_2PO_4 and 0.5 mM $MgSO_4$ or the NH_4^+-N culture solution consisting of 2 mM KCl, 1 mM $(NH_4)_2SO_4$, 1 mM $CaCl_2$, 0.25 mM KH_2PO_4 and $MgSO_4$ at the end of the light period on d 27 after germination. These solutions were supplemented with the Arnon's micronutrients described above. At the end of the light period on d 30 (at 72 h after the transfer), the plants were supplied with either 0.5 mM NaCl or 0.5 mM KCl. Tungstate treatment was carried out as follows. On d 32, a batch of the Na-treated and the K-treated plants grown in the basal culture solution were supplied with 0.2 mM potassium tungstate at the beginning of the dark period.

Fresh weights of the identical plants were determined at the end of the light period on d 32 and d 33 and RGRs were calculated (Ohta *et al.* 1987). NR activities of the leaves except cotyledons were determined at 6 h after the onset of the light period on d 32.

NR Assay. On d 30 after germination, *A. tricolor* plants were supplied with either 0.5 mM NaCl or 0.5 mM KCl at the beginning of the dark period. During the following 60 h, leaves except cotyledons from five plants were sampled at 6 h or 12 h intervals, and NR activity was assayed as described previously (Ohta *et al.* 1987).The enzyme activity is expressed as μmol of NO_2^- formed g^{-1} fresh weight h^{-1}. In this experiment, light/dark

cycle were changed to 12 h/12 h.

$^{15}NO_3^-$ Uptake and Assimilation. At the beginning of the dark period on d 30, a batch of the Na-deficient *A. tricolor* plants received either 0.5 mM of NaCl or KCl.

(1) At 48 h after the treatments (at the beginning of the dark period on d 32), plants were transferred from the basal culture solution in which they had been grown to the uptake solution where 2 mM $^{14}NO_3^-$ was replaced by 2 mM $^{15}NO_3^-$ (99 atom % of ^{15}N). The plants were kept in the uptake solution during the following 9 h-dark period and sampled at the beginning of the light period.

(2) At the beginning of the light period on d 33 (at 57 h after the treatments), the plants were transferred to the uptake solution. The plants were sampled at the end of the 15 h-light period.

Immediately after harvest (5 plants each), the shoots were weighed and ground with 80 % (V/V) ethanol water in a mortar and pestle. The homogenate was centrifuged at 5000 g for 15 min. The pellet was re-extracted with 80 % ethanol five times. The combined supernatant was made up to volume. Nitrate content in the supernatant was determined colorimetrically (Association of Official Analytical Chemists 1980).

For determination of total N and ^{15}N in the supernatant and the pellet, both fractions were digested with salicylic acid using Kjeldahl procedure (MacKenzie and Wallace 1954) after removal of ethanol by evaporation. NH_4^+ was recovered as ammonium sulfate by steam distillation and determined colorimetrically (Weatherburn 1967). The atom % ^{15}N were determined by mass spectrometry (committed to Shōkō Tsūshō Ltd.,

Tokyo 105, Japan). Data are the means and SD of three replicates.

RESULTS

Sodium-deficient *A. tricolor* plants were supplied with either 0.5 mM NaCl or 0.5 mM KCl at the beginning of the dark period on d 30 after germination. During the following 12 h-dark period, there was no difference in the levels of leaf NR activity,

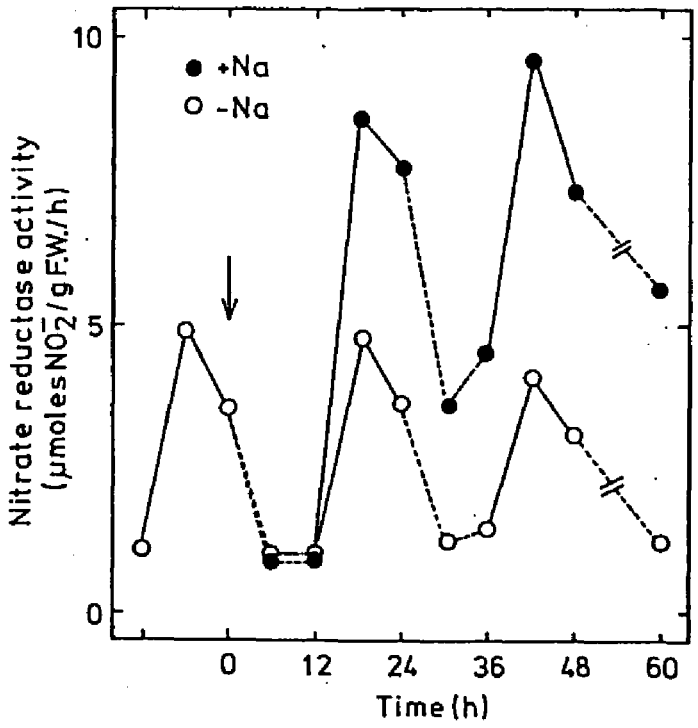

Fig. 1. Effects of Na application on the levels of nitrate reductase activity of the Na-deficient *A. tricolor* plants. On d 30 after germination, the Na-deficient *A. tricolor* seedlings were supplied with either 0.5 mM NaCl (●) or 0.5 mM KCl (○) at the end of the dark period (indicated by an arrow). Changes in the NR activity during the light period and the dark period are shown by the full lines and the dotted lines, respectively. Data is the representatives of three independent experiments.

however, the activity began to increase more rapidly in the Na-treated plants from the onset of the light period and after 6 h it reached 9.2 μmole nitrite formed g^{-1} F.W. h^{-1} while 4.96 in the K-treated plants (Fig. 1). Daily changes in the NR activity were observed both in the Na-treated and the K-treated plants. Increase in the levels of the NR activity by the addition of Na was not a transient phenomenon but the higher levels of the activity were maintained in the Na-treated plants thereafter. NR activity was not detected in the root tissues.

To determine if the growth stimulation by Na application was due to the enhanced levels of NR activity, comparison of RGRs and NR activities of the plants grown under different nitrogen sources was carried out (Table 1). The greatest stimulation of RGR by the Na treatment was observed when the plants were grown in the culture solution where NO_3^- was a sole nitrogen source. On the other hand, Na did not affect the NR activity levels and RGRs when the plants were grown with NH_4^+ as a sole nitrogen source. When the plants were supplied with 0.2 mM potassium tungstate, NR activities decreased to the same levels as those of the plants grown with NH_4^+ within 24 h and there was not a significant difference in RGR between the treatments.

These results suggest that Na affects growth through enhancement of NR activity levels. Therefore, NO_3^- assimilation rates of the Na-treated and the K-treated plants were traced using $^{15}NO_3^-$. When the plants were exposed to $^{15}NO_3^-$ for 9 h-dark period, although most of the ^{15}N present in the shoots remained in the soluble fraction, the total uptake of $^{15}NO_3^-$ by

Table 1 Effects of Na application on the relative growth rates and the levels of nitrate reductase activities of A. tricolor seedlings grown with different nitrogen sources.

Data are the means and SD of 6 replicates.

N-source*		RGR	NRA
		(g/g/day)	(μmole NO_2^-/g F.W./h)
NO_3^- + NH_4^+	-*	0.24 ± 0.03	5.79 ± 0.11
	+	0.30 ± 0.03	9.60 ± 0.11
NO_3^-	-	0.20 ± 0.02	ND
	+	0.31 ± 0.10	ND
NH_4^+	-	0.13 ± 0.02	2.12 ± 0.24
	+	0.15 ± 0.05	1.45 ± 0.07
NO_3^- + NH_4^+ + WO_4^{-**}	-	0.13 ± 0.01	1.37 ± 0.08
	+	0.15 ± 0.01	1.78 ± 0.12

* On the day 27 after germination, Na-deficient A. tricolor plants established in the basal culture solution containing 2 mM NO_3^- and 0.5 mM NH_4^+ were transferred to the NO_3^-- or the NH_4^+-solution at the end of the light period (see Materyals and Methods). After 72 h of the transfer, the seedlings received either 0.5 mM of NaCl (+) or KCl (-). Fresh weights of the plants were determined at the end of the light period on d 32 and d 33. Relative growth rates were calculated from the changes in the fresh weights of the seedlings and the levels of nitrate reductase activity were also determined on d 33.

** Seedlings maintained in the basic culture solution received K-tungstate at a concentration of 0.2 mM at 48 h after the addition of Na^+ or K^+ (on d 32).

ND: Not determined.

Table 2. Accumulation of ^{15}N in the insoluble- and soluble-nitrogen of the shoots of A. tricolor plants during 9h-dark or 15h-light $^{15}NO_3^-$-exposure period.

Data are the means and SD of 3 replicates.

Exposure period		Total N	Insoluble N	Soluble N	(NO_3^--N)
			mg ^{15}N/g F.W.		
9h-dark	+*	0.244 ± 0.02	0.012 ± 0.001	0.232 ± 0.019	
			(3.27 ± 0.06)**	(1.23 ± 0.01)	(0.63 ± 0.01)
	-	0.096 ± 0.02	0.002 ± 0.004	0.095 ± 0.011	
			(2.81 ± 0.02)	(1.19 ± 0.06)	(0.84 ± 0.02)
15h-light	+	0.839 ± 0.04	0.384 ± 0.017	0.449 ± 0.018	
			(3.98 ± 0.08)	(1.16 ± 0.01)	(0.51 ± 0.02)
	-	0.531 ± 0.03	0.148 ± 0.012	0.380 ± 0.013	
			(3.09 ± 0.01)	(1.46 ± 0.02)	(0.93 ± 0.04)

* On day 30 after germination, Na-deficient A. tricolor plants received either 0.5 mM NaCl (+) or KCl (-) at the beginning of the dark period. The plants were transferred to a culture solution labelled with 99.5 atom % ^{15}N (2 mM NO_3^-) at the the beginning of the dark period on d 32 or at the beginning of the light period on d 33, and five plants each (one replicate) was harvested.

** Values in the parenthesis represent the contents of insoluble-, soluble and nitrate-N (mg N/g F.W.).

the Na-treated plants was 254% of the K-treated plants (Table 2), indicating that NO_3^- uptake was stimulated by the Na treatment. There was not a significant difference in the soluble-N contents between the treatments, however, NO_3^--N content was higher in the K-treated plants. In the second experiment, the plants were

exposed to the $^{15}NO_3^-$ solution during 15 h-light period (Table 2). Total ^{15}N uptake of the Na-treated plants was about 158% of that of the K-treated plants. Contents of total-N of the Na-treated plants was only 128% of the K-treated plants; however, insoluble ^{15}N content of the shoot of the Na-treated plants was about 259% of that of the K-treated plants, indicating greater capacities for NO_3^- assimilation of the Na-treated plants. About 72% of the total ^{15}N in the shoot remained in the soluble fraction of the K-treated plants and about 54% of the Na-treated plants. Although soluble-^{15}N content was higher in the Na-treated plants, contents of total soluble-N and nitrate-N of the K-treated plants were about 126% and 182% of the Na-treated plants, respectively. Rufty *et al.* (1987) reported that ^{15}N was readily assimilated into insoluble macromolecules following $^{15}NO_3^-$ reduction without staying in soluble reduced nitrogen in soybean plants. It is, therefore, considered that much more $^{15}NO_3^-$ remain in the K-treated plants than in the Na-treated plants.

DISCUSSION

Within 24 h of the addition of Na, NR activity in the leaves of the Na-deficient plants increased to the comparable level which were reported for other C_4 plants (Hewitt *et al.* 1979) and the enhanced level was maintained thereafter. Therefore, I examine the possibility that the increased NR activities are responsible for the growth stimulation by Na. By Na application, RGR was most affected when the plants utilized NO_3^--N as a sole nitrogen source, and application of tungstate (Heimer *et al.* 1969) canceled the stimuratory effect of Na on the NR

activity and RGR. These results suggest that Na is functional when NO_3^--N is utilized. The $^{15}NO_3^-$ studies revealed that NO_3^- assimilation was hindered by Na deficiency. To sum up, Na-deficient *A. tricolor* plants suffer from nitrogen deficiency and Na stimulates the growth through enhanced NO_3^- assimilation.

Beevers and Hageman (1980) have summarized the process of the transport and storage of NO_3^- in the plant tissues as follows; (a) the movement of nitrate in and out of the cytoplasm is regulated at the plasmamembrane and tonoplast, (b) NO_3^- appears to enter the cytoplasm more readily from the apoplast than from the vacuoles and its presence in the cytoplasm is transient. Results have been reported so far indicate that availability of NO_3^- at the induction and assimilation sites plays a major role in regulating the level of NR activity and the rate of NO_3^- reduction (Heimer and Filner 1971, Shaner and Boyer 1976 A, 1976 B, Udayakumar *et al.* 1981, Soualmi-Boujemaa *et al.* 1985). Judging from the lower capacity for NO_3^- assimilation and the lower NR activity level in the K-treated plants, the NO_3^- concentration in the cytoplasm of the Na-deficient plants would be lower than that of the Na-sufficient plants. In other words, it is possible that the Na treatment may enhance the level of NR activity by increasing the NO_3^- availability for the induction of the enzyme. This possibility was further investigated (Chapter 4).

CHAPTER 4

SODIUM-STIMULATED NO_3^- UPTAKE IN *AMARANTHUS TRICOLOR* L. PLANTS

I have demonstrated that the Na treatment increased the NR activity level of the Na-deficient *Amaranthus tricolor* plants (Chapter 2) and promoted the growth by stimulating NO_3^- assimilation (Chapter 3). However, it remains to be clarified whether the increase in NR activity level by the Na treatment is only one of consequences of the stimulation of metabolism by Na or Na directly affects the induction process of the enzyme.

Here, I report on the Na-stimulated NO_3^- uptake of *A. tricolor* plants and discuss a causal relation between the Na-stimulated NO_3^- uptake and the enhancement of NR activity level by the Na application.

MATERIALS AND METHODS

Plant Culture. Seedlings of *Amaranthus tricolor* L. cv. Tricolor were cultured under Na-deficient condition until 30 days after germination as described previously (Chapter 1, 2). The standard culture solution (pH 6.0) prepared in distilled and deionized water contained 1 mM KCl, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM $(NH_4)_2HPO_4$ and 0.5 mM $MgSO_4 \cdot 7H_2O$. The micronutrient composition was that of Arnon's solution cited by Hewitt (1966) except that all the iron was supplied as ferric citrate. The concentration of Na as an impurity in the culture solution was estimated to be

less than 5 ppb using atomic absorption spectrophotometry. Throughout this study, an environment chamber (NS280FHW, Takayama Seisakusho, Kyoto 610, Japan) was used under following conditions: temperature 30 °C for whole growth period, RH 80 %, photo period 15 h and light intensity 350 μE m^{-2} s^{-1}. Culture solutions were renewed every 3 days and continuously aerated.

Ion Uptake Studies. Half-strength standard culture solution, adjusted with 0.1 M HCl to pH 6.0, was used as an uptake solution.

Experiment 1. Roots of seedlings grown under the Na-deficient condition for 30 days after germination were rinsed with the uptake solution, and then four seedlings each were transferred to 60 ml of the uptake solution supplemented with either 0.5 mM NaCl or 0.5 mM KCl at the end of the dark period. Uptake of ions by the seedlings was determined by following the disappearance of the ions from the uptake solution.

Experiment 2. The following experiment was carried out to evaluate the effects of intracellular Na^+ the NO_3^- uptake of *A. tricolor* plants. The 30-d-old Na-deficient seedlings were treated with 0.5 mM NaCl during the dark period (9 h), and the seedlings were transferred to the uptake solution at the end of the dark period. They were allowed to stand for 10 min, then four seedlings each were placed in 60 ml of the uptake solution supplemented with either 0.5 mM NaCl or 0.5 mM KCl and ion fluxes were determined at 3 h intervals during 6 h of the light period.

The amount of water lost from the uptake solutions due to evapotranspiration was determined from the decrease in the weight of vessels containing the experimental seedlings and water was

added to return the vessels to their initial weight. The uptake solutions were continuously aerated throughout the experiment and were renewed at every sampling. All the data are the means of at least three replications and are represented on the g fresh weight basis of roots unless otherwise stated.

Analysis. Nitrate, Cl^-, and SO_4^{2-} were determined using ion chromatography (CDD-, LC-6A, Shimazu Co., Ltd., Kyoto 604, Japan). Phosphate (Murphy and Riley 1962) and NH_4^+ (Weatherburn 1967) were determined colorimetrically. Sodium, K^+, Ca^{2+} and Mg^{2+} were determined using atomic absorption spectrophotometry. Sodium concentrations in the plant materials are presented on a tissue water basis.

Proton release was measured with a H^+ electrode and calculated on a basis of quantity of hydroxide required to return the solution to the initial pH.

Concentration of NO_3^- in Xylem Sap. The 30-d-old Na-deficient *A. tricolor* seedlings received either 0.5 mM NaCl or 0.5 mM KCl at the beginning of the 9 h-dark period and collection of xylem sap was started at the beginning of the following light period (at time 0 h). Briefly, the plants were transferred to a 100 ml glass vessel containing the culture solution, which was placed in a pressure chamber, at intervals during the light period. The shoots were excised at about 5.0 mm above the root/shoot junction. A protruding stem of the decapitated plant above a steel lid was fixed with a sealing plug of silicon rubber and a silicon tube was inserted over the cut end for collection of the sap (about 10 µl). Xylem sap were obtained by applying

pressure to the roots of decapitated plants (Munns 1985). Exudate collection was completed within 15 min of root pressurization at 0.2 MPa. Higher pressures could not be applied without risk of tissue injury and leakage.

NR Assay. For NR assay, the 30-d-old seedlings grown under the Na-deficient condition were treated with the same manner as 'Experiment 1'. All the leaves except the cotyledons were sampled and the NR activities were determined as described previously (Ohta *et al.* 1987, Chapter 2).

To determine whether NR activity level respond to enhancement of NO_3^- translocation or not, Na or NO_3^- was loaded through cut stems. The 30-d-old Na-deficient *A. tricolor* plants were excised at the base about 5 mm above the root/shoot junction under distilled and deionized water, and the cut ends of the shoots were dipped in a solution containing either 10 mM NaCl or 10 mM NaCl plus 10 mM KNO_3, and in the reference treatments, either 10 mM KCl or 10 mM KCl plus 10 mM KNO_3 was supplied. Then the cut shoots were placed in a growth chamber conditioned as described above. After 10 h of continuous illumination, the youngest fully expanded leaves (3 g) were harvested from ten different shoots and extractable NR activities were determined. When the cut shoots were loaded with fuchsine, it took about 3 to 4 h for the dye to develop over the leaves. Therefore 10 h of the treatment period was considered to be sufficient to determine the effect of the test solutions on the NR activity levels. The enzyme activity is expressed as μmole of NO_2^{-1} formed g^{-1} fresh weight h^{-1}.

RESULTS AND DISCUSSION

Within 30 min of application of Na at a concentration of 0.5 mM, the seedlings showed significantly higher NO_3^- uptake capacities (4.51 ± 0.06 μmol/g F.W. root/h) than seedlings which received 0.5 mM KCl (1.90 ± 0.56 μmol/g F.W. root/h) (Fig. 1A). After 4 h, the difference in the NO_3^- uptake rate further increased. The total amount of NO_3^- taken up by the Na-treated seedlings during the experimental period was about twice of that by the K-treated seedlings (Fig. 1B).

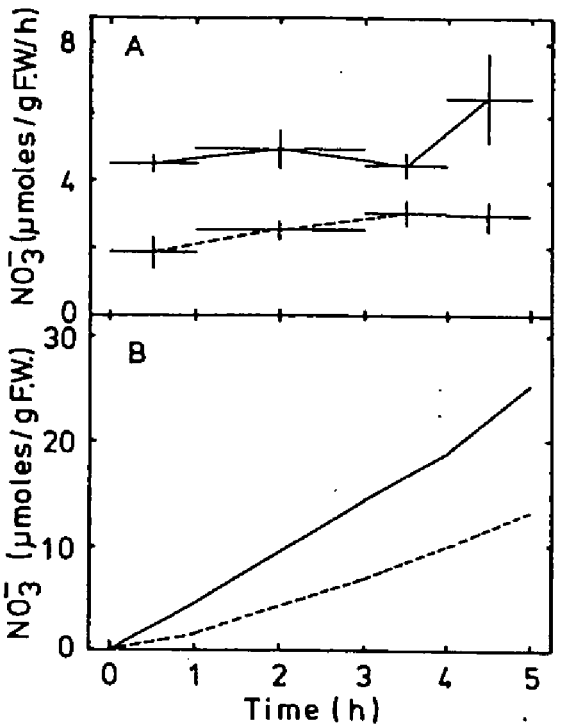

FIG. 1. Effects of Na^+ application on NO_3^- uptake of *A. tricolor* seedlings. The 30-d-old Na^+-deficient *A. tricolor* seedlings were placed in the uptake solution supplemented with either 0.5 mM NaCl or 0.5 mM KCl at the end of the dark period (at time 0 h). A, Changes in the NO_3^- uptake rates with time; B, cumulative NO_3^- uptake. Results are represented by solid lines for the Na^+-treated seedlings and by dotted lines for the K^+-treated seedlings. Data are the means and SD of three replications of each four plants and are expressed as μmol g^{-1} root FW.

Figure 2 shows changes in the Na concentration in the Na-treated seedlings. Following the Na application, Na concentration in the shoots increased linearly with time from an initial value of 0.06 mM to 0.35 mM after 5 h. On the other hand, that in the roots increased from 0.35 mM to 0.60 mM within 1 h, and then leveled off.

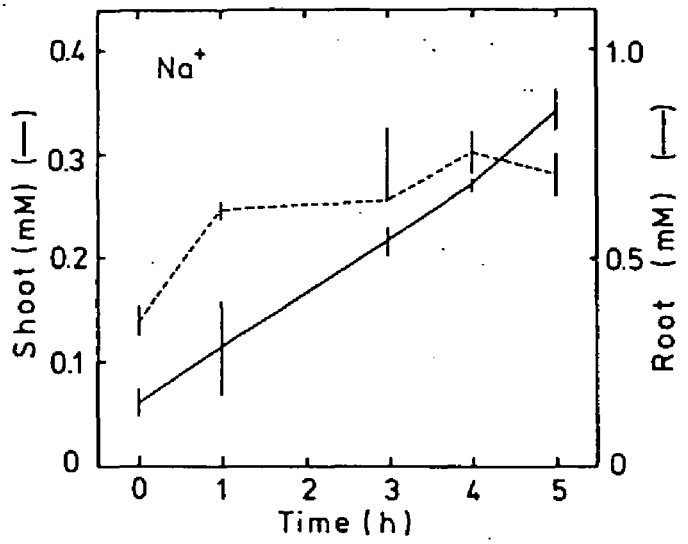

FIG. 2. Changes in Na^+ concentrations in the shoots (——) and the roots (- - -) of the 30-d-old *A. tricolor* seedlings following Na^+ application. The Na^+-deficient *A. tricolor* seedlings were transferred to the uptake solution containing 0.5 mM NaCl at the end of the dark period (at time 0 h) and were sampled at specified intervals during 5 h of the light period. Data are the means and SD of determinations of four individual seedlings. Sodium concentrations (mM) were calculated on the basis of tissue water contents.

Table 1 shows a summary of fluxes of Na^+, K^+, Cl^- and NO_3^-. The Na-treatment stopped the K^+ and Cl^- uptake and significant amounts of K^+ and Cl^- were released. Taking into account the K^+ and Cl^- contents of the plants in long-term growth experiment (Chapter 1), these effluxes were transient phenomena. Uptake rates of HPO_4^{2-}, SO_4^{2-}, NH_4^+, Ca^{2+} and Mg^{2+} did not differ

Table 1. *Fluxes of Na^+, K^+, Cl^-, and NO_3^- Affected by Na^+ Application to A. tricolor Seedlings*

Values represent net influxes (+) and effluxes (−). Sodium influx was calculated from the data of Figure 2. Net fluxes of K^+ and Cl^- during 5 h of the uptake period were taken from the identical experiment presented in Figure 1. Data are the means and SD of three replications.

Element	Na^+-Treated Seedlings	K^+-Treated Seedlings
	μmol/g FW root/5 h	
Na^+	+2.3	
K^+	−1.2 ± 1.7	+10.3 ± 3.9
NO_3^-	+26.6 ± 4.2	+12.6 ± 1.7
Cl^-	−3.5 ± 2.3	+35.2 ± 18.4
H^+[a]	−14.5 ± 0.6	−6.1 ± 1.8

[a] Amount of OH^- required to return the medium pH to the initial value.

significantly between the treatments during the experimental period (data not shown). The anion uptake exceeded the cation uptake more in the K-treated seedlings, and the excess mainly consisted of Cl^-. Acidification of the medium was faster in the Na treatment.

How is Na involved in the enhancement of NO_3^- uptake ? The uptake of NO_3^- is about 11-fold higher than that of Na^+ (Table 1) and Na^+ in the uptake solution had no significant effect on the NO_3^- uptake and acidification of the medium when the plants were pre-loaded with Na (Table 2). Moreover, the Na-preloaded seedlings released Na^+ into the uptake solution supplemented with 0.5 mM KCl. Accordingly the Na^+ influx was not essential to the NO_3^- uptake process in the roots but intracellular Na stimulated the NO_3^- uptake. These results argue against the Na^+/NO_3^- symport which appeared in the report of the Na-dependent NO_3^- uptake by a marine diatom *Phaeodactylum tricornutum* (Rees *et al.* 1980).

Table 2 Effects of intracellular Na^+ on the NO_3^- uptake of 30-d-old Na^+-preloaded A. tricolor seedlings.

Values are the means and SD of five replications of each four plants.

		0-3h[a]	3-6h[a]
		(μmol/g F.W. root/h)	
NO_3^- uptake	+[a]	21.5 ± 3.74	25.3 ± 2.59
	-[a]	22.1 ± 5.69	29.5 ± 3.38
Na^+ release	+	ND[b]	ND
	-	0.16 ± 0.07	0.05 ± 0.01
H^+ release[c]	+	2.64 ± 0.12	3.33 ± 0.40
	-	2.52 ± 0.25	2.91 ± 0.20

[a]Sodium-preloaded seedlings were placed in the uptake solution supplemented with either 0.5 mM NaCl (+) or 0.5 mM KCl (-) at the beginning of the light period (at time 0 h). The NO_3^- uptake and Na^+ release were determined at 3 h intervals. [b]ND: Not determined. [c]Amount of OH^- required to return the medium pH to the initial value.

It is well known that NR activity is induced by its substrate NO_3^- and in turn NR activity then influences NO_3^- uptake from the external medium (Beevers and Hageman 1980, Guerrero *et al.* 1980). Therefore NR activities in the leaves were measured at the

same time as NO_3^- uptake using the same batch of the plants (Fig. 3). Enhancement of the NR activity became appreciable after 1 to 3 h of the addition of Na, *i. e.* the stimulation of NO_3^- uptake by the roots preceded the increase in

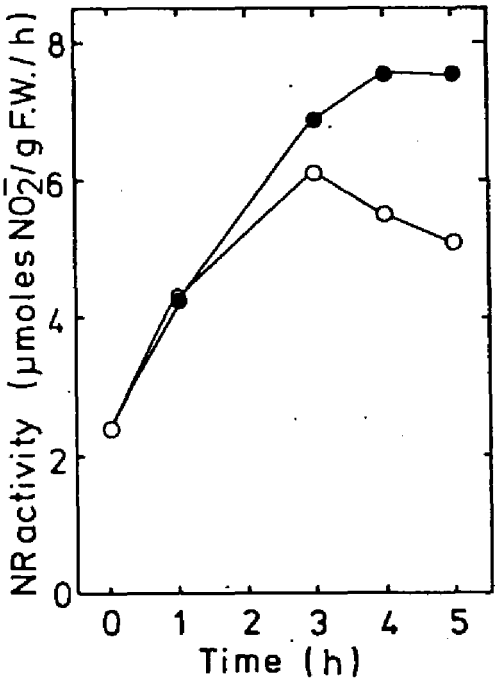

FIG. 3. Effects of Na^+ application on NR induction in the Na^+-deficient *A. tricolor* leaves. The 30-d-old seedlings were placed in the uptake solution supplemented with either 0.5 mM NaCl (●) or 0.5 mM KCl (○) at the beginning of the light (at time 0 h), and the changes in the extractable NR activity in the light period were followed. Curves are the representatives of three independent experiments.

NR activity in the leaves (Fig. 1 and 3). In addition, NR activities were not detected in the roots. Accordingly, it seemed difficult to correlate the stimulation of NO_3^- uptake by Na with the increased levels of NR activity.

Concentration of NO_3^- in xylem sap of the Na-treated plants was higher than that of the K-treated plants even at the beginning of

the light period (at time 0 h), and the difference between the treatments became greater with time (Fig. 4). At 12 h of the

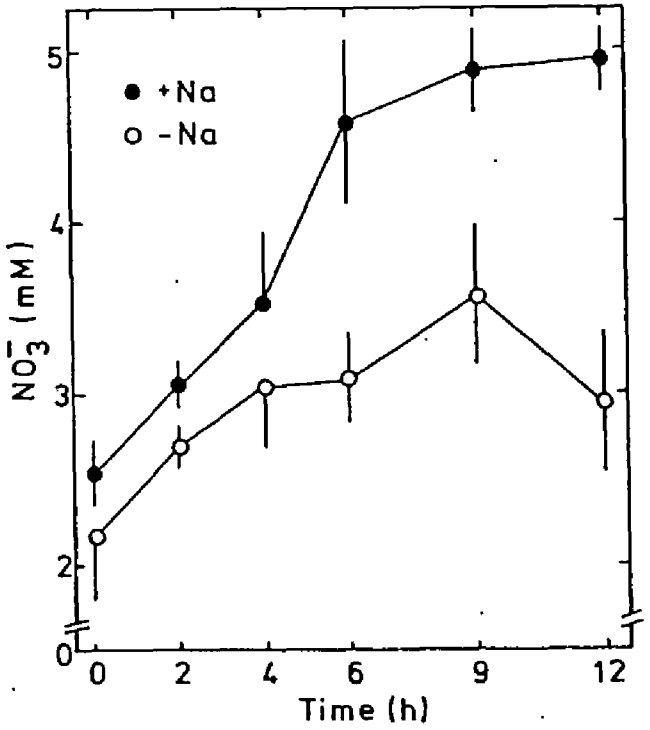

Fig. 4. Changes in the NO_3^- concentration of xylem sap of *Amaranthus tricolor* seedlings following Na application. The 30-d-old Na-deficient *Amaranhtus tricolor* seedlings received either 0.5 mM NaCl (●) or 0.5 mM KCl (O) at the beginning of the 9 h-dark period on the day 30 after germination. Xylem sap were collected at required intervals from the beginning of the following light period (at time 0 h). Data are the means and SD of at least three replicates.

light period, NO_3^- concentration of the Na-treated plants was about 160 % of that of the K-treated plants. These results indicated that Na stimulated NO_3^- uptake by the roots, thereby the NO_3^- translocation to the shoots was also stimulated. In plant tissues, it is considered that electrogenic proton pumps located at the plasmamembrane of epidermal and cortical cells provide a means of the ion fluxes from outside to the symplast

(Pitman 1977, Poole 1978). Recently it has been reported that electrogenic proton pumps working at the xylem/parenchyma symplast interface mediate ion exchange between xylem and surrounding tissue, then facilitate the upward transport of ions (DeBoer *et al.* 1983, DeBoer *et al.* 1985). It should be noted here that stimulation of H^+ efflux was observed simultaneously with the stimulation of NO_3^- uptake (data not shown).

When the cut shoots of the Na-deficient plants were supplied with K^+ plus NO_3^- via the xylem stream, leaf NR activity level

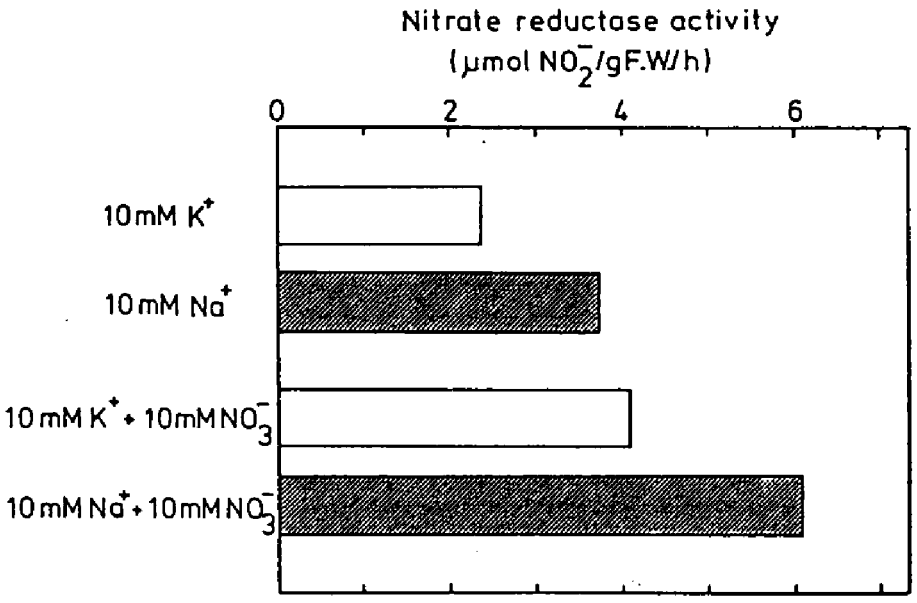

Fig. 5. Effect of Na- or NO_3^--loading via xylem stream on the NR activity level of the cut shoots of *Amaranthus tricolor*. The 30-d-old Na-deficient *A. tricolor* seedlings were excised at about 5 mm above the root/shoot junction and the cut ends of the shoots were dipped in the test solution containing either 10 mM NaCl or 10 mM NaCl plus 10 mM KNO_3, and in the reference treatments, 10 mM KCl or 10 mM KCl plus 10 mM KNO_3 was supplied. After 10 h under continuous illumination, the youngest fully expanded leaves were harvested from ten different shoots from each treatment and extractable NR activities were determined.

increased by about 180 % of that of the plants supplied with K^+ (Fig. 5). On the other hand, the level of NR activity of the shoots loaded with Na^+ was comparable to that of the shoots supplied with K^+ plus NO_3^-, and the loading of Na^+ plus NO_3^- was most effective in the enhancement of NR activity level (Fig. 5).

It is considered that there are two pools for NO_3^- called the metabolic (presumably in the cytoplasm) and storage pools (presumably in vacuoles), respectively, and the former pool size is closely related to the capacity for NR induction (Beevers and Hageman 1980). Heimer and Filner (1971) first demonstrated that the decline of NR activity occurred even though substantial amounts of NO_3^- were present in the tissues, indicating that most of the NO_3^- was sequestered in the storage pools and relatively unavailable for reduction. The maintenance of NR in corn leaves has been shown to be more closely associated with the flux of NO_3^- to the leaves from the roots than to the existing NO_3^- concentrations in the leaves (Shaner and Boyer 1976 A, 1976 B). Subsequently, a close relationship between concurrent absorption and xylem transport has been demonstrated (MacKown *et al.* 1981, Rufty and Volk 1986). It is evident, therefore, that provision of NO_3^- by the root system for transport in the xylem is a important determinant of the rate of NO_3^- reduction in illuminated leaves (Rufty *et al.* 1987).

In conclusion, the presented results indicate that delivery of NO_3^- to leaves was promoted by the application of Na in terms of the stimulation of NO_3^- uptake, thereby the levels of NR activity increased. Also it is possible that Na may increase

the NO_3^- supply from the storage pool to the NR induction site, since the Na-treatment of the cut shoots enhanced the levels of NR activity without additional supply of NO_3^- to the shoots (Fig. 5). From Figures 2 and 3, the stimulatory threshold of Na concentration in the leaves for the enhancement of the NR activity is presumed to be in a range of 0.1 mM to 0.2 mM on a basis of whole cell water, although intracellular localization of Na is probable. Taking into account that growth reduction in the Na-deficient *A. tricolor* plants was attributable to the lowerling of the capacity for NO_3^- assimilation (Chapter 3), it is considered that the Na requirement of *A. tricolor* plants is due to the involvement of this element in the NO_3^- uptake, hence in keeping the NO_3^- availability in leaf tissues, for which is enough to maintain the normal NR acitvity level and NO_3^- assimilation capacity. While it remains to be determined whether Na stimulates the NO_3^- uptake and NR induction in other C_4 plants or not, the reason why C_4 plants require Na for growth may be explained at least in part by the study on the Na-stimulated ion transport.

CHAPTER 5

SODIUM REQUIREMENT OF MONOCOTYLEDONOUS C_4 PLANTS

Now that I have demonstrated that the Na requirement of a NAD-ME type C_4 plant, *Amaranthus tricolor* L. is due to the involvement of this element in the NO_3^- assimilation process (Ohta *et al.* 1988 A, B), it should be determined whether C_4 species other than *A. tricolor* plants require Na for the NO_3^- assimilation or not.

For this purpose, I studied on the effects of Na application on the NR activity levels and growth of several monocotyledonous C_4 species (Ohta *et al.* 1988 C).

MATERIALS AND METHODS

Seven species of C_4 plants representing three C_4 sub-types: NADP-ME type, *Zea mays* cv. Golden Crossbantam Bell and *Echinochloa crus-galli*; NAD-ME type, *Panicum miliaceum, Panicum coloratum* cv. Kabulabula and *Panicum dichotomiflorum*; and PEP-CK type, *Panicum maximum* var. Trichoglume and *Chloris gayana* plants were grown under Na-deficient condition. Seeds were germinated on a sheet of cheesecloth covering acid washed polyethylene beads. After germination, seedlings were supplied with the basal culture solution (Matoh *et al.* 1986, Chapter 1) containing 1 mM KCl, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM $(NH_4)_2HPO_4$, and 0.5 mM $MgSO_4 \cdot 7H_2O$ and Arnon's micronutrients cited by Hewitt (1966) except that all the iron was supplied as ferric citrate. The

basal culture solution was adjusted with 0.1 N HCl to pH 6.0. Sodium concentration as an impurity in this culture solution was estimated to be less than 5 ppb using atomic absorption spectrophotometry. When the seedlings became approximately 5-10 cm in heights, ten seedlings each was transferred to a 3 L plastic container containing the basal culture solution. After 3 days of the transfer, either 0.5 mM of Na_2SO_4 or K_2SO_4 were supplemented to the culture solution. Culture solution was continuously aerated and renewed every 2 days. Throughout this study, a controlled environment chamber was used under following conditions: RH 70 %, photo period of 15 h-light/9 h-dark cycle, and temperature 30 °C for whole growth period. The light intensity was 300 $\mu E\ m^{-2}\ s^{-1}$.

Fresh weights of the identical plants were determined at the end of the light period on day 5 and day 6 after the treatments and RGR was calculated from the fresh weight values using a equation described in Chapter 2. Although the RGR values were calculated from the changes in the fresh weight, since the water content per fresh weight of the plants did not vary appreciably during this period, it is assumed that the changes in the fresh weight closely reflected the daily changes in dry matter production.

For determination of NR activities, shoots of the plants were harvested on d 5 after the treatments and leaf laminae were ground in a pre-chilled mortar and pestle with 50 mM K-phosphate buffer (pH 7.5) containing 1 mM EDTA, and 10 % (W/V) insoluble PVP. The concentration and kind of the sulphydryl-protectant used were changed according to species (See Table 3). The

homogenate was squeezed through 4 layers of cheesecloth and centrifugated at 20,000 g for 10 min. The supernatant was desalted using a Sephadex G-25 column (Pharmacia PD-10) equilibrated with the grinding medium and used for the enzyme assay at 30 °C. All the extraction procedures were carried out at 4 °C. NR activities were determined as described previously (Ohta *et al.* 1987).

Total chlorophyll content and chlorophyll *a/b* ratio were determined according to the method of Arnon (1949).

RESULTS AND DISCUSSION

Plants of *Echinochloa crus-galli* (NADP-ME), *Panicum coloratum* (NAD-ME) *P. dichotomiflorum* (NAD-ME), *P. maxumum* (PEP-CK) and *Chloris gayana* (PEP-CK) which had been grown under Na-deficient condition for 10 to 15 days after germination showed poor growth and had leaves of yellow green color. Within five days of the addition of 0.5 mM Na_2SO_4, these symptoms disappeared and the plants seemed to have almost recovered from Na deficiency, while the addition of 0.5 mM K_2SO_4 had no effect. Relative growth rates of the Na-deficient plants of these species were significantly higher than those of the K-treated plants (Table 1). Especially, RGRs of *P. coloratum* and *P. maximum* increased by about 300 % and 200 % by the addition of Na, respectively. On the other hand, *Z. mays* (NADP-ME) and *P. miliaceum* (NAD-ME) plants grown without Na had dark green leaves and could not be distinguished from those grown with Na by their appearance, and there were not significant differences in RGRs between the Na-

Table 1 Effects of Na application on the relative growth rates of C_4 monocotyledonous plants grown under Na-deficient condition.

Data are the means and SD of 6 replicates.

Species	age at treatment	+ K_2SO_4*	+ Na_2SO_4
	days	g/g/day	
NADP-ME			
Zea mays	9	0.23 ± 0.02	0.23 ± 0.01
Echinochloa crus-galli	15	0.30 ± 0.02	0.36 ± 0.01
NAD-ME			
Panicum miliaceum	15	0.23 ± 0.03	0.23 ± 0.01
P. coloratum	15	0.10 ± 0.01	0.30 ± 0.02
P. dichotomiflorum	15	0.33 ± 0.02	0.41 ± 0.03
PEP-CK			
P. maximum	15	0.14 ± 0.02	0.29 ± 0.03
Chloris gayana**	25	0.40 ± 0.01	0.47 ± 0.01

* Seedlings grown under Na-deficient condition in a growth chamber were supplied with 0.5 mM of Na_2SO_4 or K_2SO_4. Relative growth rates were calculated from the changes in the fresh weight values determined after 5 to 6 days of the treatments.

treated and the K-treated plants. Although the threshold concentrations of Na in the tissues to promote the healthy growth may be different from species to species, *Z. mays* and *P. miliaceum* plants judged not to require Na for growth at least under my culture condition.

Chlorophyll contents of the plants which showed Na-requirement, *i.e., E. crus-galli, P. coloratum, P. dichotomiflorum, P. maximum* and *C. gayana*, markedly increased by the Na application (Table 2). However, increase in the Chl *a/b*

Table 2 Chlorophyll contents and chlorophyll a/b ratio of monocotyledonous C_4 plants grown with or without Na.

Data are the means and SD of at least three replicates.

Species		Chlorophyll	Chlorophyll a/b
		mg/dm^2	
NADP-ME			
Zea mays	-*	3.45 ± 0.11	3.31 ± 0.11
	+	3.25 ± 0.12	3.21 ± 0.06
Echinochloa crus-galli	-	2.45 ± 0.19	3.58 ± 0.07
	+	3.50 ± 0.32	3.62 ± 0.06
NAD-ME			
Panicum miliaceum	-	2.81 ± 0.17	3.68 ± 0.01
	+	2.84 ± 0.17	3.71 ± 0.04
P. coloratum	-	0.73 ± 0.12	3.19 ± 0.13
	+	1.58 ± 0.32	3.68 ± 0.06
P. dichotomiflorum	-	1.22 ± 0.05	3.79 ± 0.25
	+	1.66 ± 0.11	3.69 ± 0.09
PEP-CK			
P. maximum	-	1.15 ± 0.12	3.22 ± 0.08
	+	1.92 ± 0.09	3.53 ± 0.04
Chloris gayana	-	1.88 ± 0.11	3.01 ± 0.12
	+	3.39 ± 0.18	3.22 ± 0.05

* Plants grown under Na-deficient condition received either 0.5 mM of Na_2SO_4 or K_2SO_4 when the seedlings became 5 cm in heights. The treatments were carried out for 5 days.

ratios were observed only in *P. coloratum* and *P. maximum* plants which seemed most susceptible to Na deficiency (Table 1). Johnston *et al.* (1984 B) proposed that the chlorophyll concentration and the Chl *a/b* ratio are closely involved in the systems affected by Na nutrition. Johnston *et al.* (1984 B) also reported that Chl *a/b* ratios of Na-deficient C_4 species including *C. gayana* plants increased by Na application. It is possible that the responses of Chl *a/b* ratios to Na in C_4 plants may be different according to plant species, varieties and culture conditions.

The correlation between the possession of the C_4 photosynthetic pathway and the essentiality of Na may indicate a possible role of Na in the C_4 dicarboxylic acid pathway (Brownell 1979), however not all C_4 plants require Na for growth (Hewitt 1983). The growth responses of C_4 plants to Na application were observed irrespective of the C_4 photosynthetic subgroups to which the plants belong (Table 1). Therefore, it is unlikely that Na is solely involved in the C_4 dicarboxylic acid pathway. Now it has been made clear that Na in involved in the NO_3^- uptake process (Ohta *et al.* 1988 B, Chapter 4), thereby affected NO_3^- assimilation in the leaves of *A. tricolor* (Ohta *et al.* 1988 A, Chapter 3). Present results suggest that the function of Na in the maintenance of the levels of NR activity exists not only in *A. tricolor* plants but also in the C_4 plants which require Na for growth except for *E. crus-galli* (Table 3). There were not differences between the levels of NR activities of the Na-treated and the K-treated plants of *Z. mays* and *P. miliaceum*. At present it is uncertain whether Na directly affects NR activity

Table 3 Effects of Na application on extractable nitrate reductase activities in the shoots of Na-deficient monocotyledonous C_4 plants grown with or without Na.

Values are the means and SD of three replicates.

Species	age at harvest	+ K_2SO_4*	+ Na_2SO_4
	days	µmole NO_2^-/g F.W./h	
NADP-ME			
Zea mays	14	10.0 ± 1.18	12.5 ± 1.13
Echinochloa crus-galli	20	14.8 ± 1.93	15.3 ± 0.93
NAD-ME			
Panicum miliaceum	20	13.2 ± 1.66	13.9 ± 1.40
P. coloratum	20	8.1 ± 0.93	15.0 ± 1.15
P. dichotomiflorum	20	13.8 ± 0.38	22.8 ± 1.57
PEP-CK			
P. maximum	20	2.6 ± 0.38	8.8 ± 0.81
Chloris gayana	30	17.2 ± 0.27	21.6 ± 0.55

To determine the effects of Na application on the levels of nitrate reductase activities of the shoots, plants receiving 0.5 mM of Na_2SO_4 or K_2SO_4 for 5 days were harvested after 6h from the beginning of the light period.

levels or not; however, it is sure that the increased NR activities could contribute to the growth enhancement. The NR activity of the *E. crus-galli* plants was not influenced by Na nutrition, while RGR of the plants grown under Na deficient condition increased in response to the addition of Na (Table 1). Therefore there must be some target site(s) of Na other than NO_3^- uptake and C_4 pathway. The fact that C_4 photosynthetic pathway has arisen in relatively few families of terrestrial plants suggests that this special photosynthetic pathway is only one of many features which contribute to ecological success in different environment (Osmond *et al.* 1982), and Brownell (1979) pointed out that Na may affect some part of metabolism not yet defined which occurs in C_4 and CAM plants but not in C_3 plants. It is possible that C_4 plants may have acquired metabolism besides the C_4 pathway, during the evolutionary process, which would be affected by Na, such as the Na-facilitated NO_3^- uptake process in *A. tricolor* plants (Ohta *et al.* A).

CONCLUSION

I have investigated the Na requirement of C_4 plants using a NAD-ME type C_4 plant, *Amaranthus tricolor* L. cv. tricolor and found that *A. tricolor* plants requires Na for assimilation of NO_3^-.

When the Na-deficient *A. tricolor* plants were supplied with 0.5 mM NaCl, the relative growth rate increased within 24 h. Since metabolic responses which can be detected prior to the growth enhancement are considered to be the primary steps of recovery from Na deficiency, studies were concentrated on the responses of the plants within 24 h of the Na treatment and following sequence of metabolic responses was demonstrated.

Within 30 min of the addition of 0.5 mM NaCl, NO_3^- uptake was stimulated by about 240 % of that of the plants treated with 0.5 mM KCl. Consequently, translocation of NO_3^- to the shoots was also stimulated. At 1 to 3 h of the Na treatment, the level of NR activity began to increase and after 24 h it reached about 350 % of that of the K-treated plants. By the ^{15}N studies, it was demonstrated that the capacity for NO_3^- assimilation of the Na-treated plants was about 260 % of that of the K-treated plants. On the other hand, photosynthetic oxygen evolution by leaf discs increased within 24 h of the Na treatment; however, there were not detectable increase in the levels of the C_4 photosynthetic enzymes, whereas the photosynthetic electron transport by thylakoids increased significantly.

From these results, I concluded that *A. tricolor* plants require Na because the NO_3^- uptake process necessitates this element. As the Na requirement of several monocotyledonous C_4 species were observed irrespectively of the C_4 subgroups to which the plants

belong, it is undoubtedly that Na is not involved in the main pathway of the C_4 photosynthesis but there may be a metabolism, which require Na for its functioning, other than C_4 pathway. This idea implies that C_4 plants might have acquired metabolism besides the C_4 pathway, which do not occur in C_3 plants, during the evolutionary process. One of the possibilities is that the involvement of Na in solute transport system which is indispensable for normal growth, such as the Na-stimulated NO_3^- uptake, may be prevailing in C_4 plants.

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