Arsenite Reduces Acid Content in *Citrus* Fruit, Inhibits Activity of Citrate Synthase but Induces Its Gene Expression

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ABSTRACT. Arsenic compounds generate diverse effects in all living organisms. In citrus (*Citrus* L. sp.), they reduce acidity and improve fruit quality by unknown mechanisms. The major organic acid in citrus fruit is citric acid, which begins accumulating early in fruit development, reaches a peak in middle-sized fruit and then, in most species, declines as the fruit matures. In an attempt to understand the basis of the effect of arsenite, it was applied to 'Minneola' tangelo (*Citrus paradisi* Macf. x *C. reticulata* Blanco) ≈6 weeks postanthesis, and a detailed analysis of total titratable acidity and citric acid concentration was performed throughout fruit growth. Within 35 days after arsenite application, total acid content and citrate concentration were slightly lower compared with the controls, and this difference persisted throughout fruit development. The concentrations of other organic acids were not reduced by the treatment. Sodium arsenite reduced the citrate concentration in 'Eureka' lemon callus [*Citrus limon* (L.) Burm.] also, without affecting tissue growth. Extractable activity of citrate synthase in treated fruit was inhibited within 1 day following arsenite spray, but recovered to a normal level a few days later. In contrast, gene expression was remarkably induced 1 day following treatment, which might explain the recovery in enzyme activity. Data suggest that reduction in acid accumulation may not be related to the initial inhibition of citrate synthase activity.

Pulp acidity of citrus fruit (*Citrus* sp.) is correlated with the citric acid concentration, and is a major factor in determining fruit maturity and quality (Sinclair, 1984). Citrate begins to accumulate during the second phase of fruit development, when the fruit and its juice vesicle cells enlarge rapidly (Erickson, 1968). The accumulation continues for a few weeks, reaching a peak when the fruit volume is ≈50% of its final value, and then, in most citrus species, declines gradually as the fruit matures. Citrate concentration in the juice usually reaches a peak of 0.1 to 0.2 M (3% to 4% total titratable acidity), and it decreases to 20 to 50 mM (about 1% total titratable acidity) in mature fruit (Sinclair, 1984).

The biosynthesis of citric acid in the mitochondria is catalyzed by citrate synthase, which condenses the four-carbon acid, oxaloacetate, with the two-carbon molecule, acetyl-CoA. Next, citrate is isomerized into isocitrate by aconitase. It has long been hypothesized that a metabolic block in the mitochondrial aconitase plays a major role in citrate accumulation (Bogin and Wallace, 1966). Indeed, we have shown recently that activity of this enzyme in 'Eureka' lemon (*Citrus limon*) is greatly reduced early in fruit development (Sadka et al., 2000). Following synthesis, citrate is transported and stored in the vacuole (Brune et al., 1998; Canel et al., 1995; Echeverria and Valich, 1988).

In many citrus species, high acid content in mature fruit reduces quality or delays harvest. Management tools that influence acidity are limited. For instance, a few rootstocks can partially reduce acid content at harvest time but, in many cases, they also have negative characteristics (Davies and Albrigo, 1994). Several arsenic com-

pounds, including lead arsenate, calcium arsenate, sodium arsenite, and para-aminobenzenearsonic acid, in the soil or as a foliar spray, have been found to reduce the acid content in citrus fruit (Erner et al., unpublished; Miller et al., 1933; Wilson and Obreza, 1988; Yamaki, 1990a). In an attempt to understand the mechanism of the arsenate effect, Vines and Oberbacher (1965) treated mitochondria from 'Shamouti' orange [*Citrus sinensis* (L.) Osbeck] fruit pulp with lead arsenate, and showed that treatment uncoupled phosphorylation while having little effect on oxidation. It has also been shown that lead arsenate reduces citrate synthase activity and acetyl-CoA concentration by 20% to 30% during the 5 weeks following the spraying of 'Satsuma' mandarin [*Citrus unshiu* (Mak.) Marc.] (Yamaki, 1990a, 1990b). The effect of arsenite, which also reduces fruit acidity, has not been studied previously in citrus. Similar to arsenate, it is probably taken up by the phosphate transport system (Lenartowicz, 1990; Meharg and Macnair, 1992; Yompakdee et al., 1996). However, unlike arsenate, which acts as a phosphate analogue, inhibits phosphorylation-dependent processes and competes with phosphate as an electron acceptor, arsenite binds to vicinal thiol groups and inhibits enzymes which require SH groups for their catalytic activity (Lenartowicz, 1990; Lopez et al., 1990; Narayan and Nair, 1989). Therefore, arsenite is considered to be more toxic than arsenate, and it may affect fruit acidity through a different mechanism from that of arsenate.

Arsenic compounds, including sodium arsenite, are prohibited for commercial use. Therefore, we initiated a study to elucidate the mechanism of the arsenite action, in order to develop alternative tools to improve fruit acidity. We present herein the first detailed study of changes in the kinetics of citric acid accumulation and total titratable acidity, which follow sodium arsenite treatment. It is demonstrated that arsenite effects become detectable immediately following a lag in acid accumulation early in fruit development. We also show that an initial and a temporal inhibition of citrate synthase activity, which lasts for a few days following arsenite application, is accompanied by induction of its gene expression.

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Materials and Methods

PLANT MATERIAL AND TISSUE CULTURES. Fruit of 'Minneola' tangelo trees (*Citrus paradisi* × *C. reticulata*) grafted on sour orange rootstocks (*Citrus aurantium* L.) grown in the central-coastal region of Israel were used in this study. Tissue cultures originating from 'Eureka' lemon (*C. limon*) fruit juice sacs were also used. The lemons were surface sterilized with 70% ethanol and cut into halves, and the juice sacs, including the stalks, were placed in 50-mL glass tubes containing 10 mL of a medium described by Erner and Reuveni (1981), except for omission of orange juice. The explants were incubated in the dark at 28 °C, and calli were formed within 4 to 6 weeks. Pieces weighing about 100 mg were cut from the callus under aseptic conditions, transferred to fresh medium and allowed to grow for an additional 4 weeks. At least two cycles of growth were performed before calli were used in experiments.

SODIUM ARSENITE TREATMENTS. Whole tree arsenite sprays were applied during 1997 and 1998. In 1997, arsenite was applied 14 July, ≈12 weeks postanthesis when fruit diameters were 25 to 30 mm. In 1998, the treatment was applied 3 June, about 6 weeks postanthesis, when fruit diameters were 10 to 15 mm. Aqueous solutions containing 0, 42, 85 or 170 mg·L⁻¹ of sodium arsenite were adjusted to pH 3.5 with HNO₃, and sprayed in the presence of 0.025% Extravon surfactant (Novatis AG, Basel, Switzerland). In both years, the experiments were performed on four replicates in randomized blocks, each containing four trees, with a border row between the blocks. Ten west-sided fruit from each block, located about 1.5 m above ground level, were collected at various times following the spray treatments, as indicated in Fig. 1. Fruit diameters in a few sampling dates were: 20 to 25 mm 28 d after treatment (7 July), 40 to 45 mm 84 d after treatment (8 Aug.), and 80 to 90 mm 210 d after treatment (30 Dec.). Following harvest, fruit were placed on ice for further analysis in the laboratory. Pieces of 'Eureka' lemon calli weighing about 100 mg were placed on fresh media containing 0, 0.085, 0.425 or 0.850 mg·L⁻¹ sodium arsenite and allowed to grow as described above for 4 to 6 weeks before analysis.

TOTAL TITRATABLE ACID AND ORGANIC ACID ANALYSES. Total titratable acid content of the fruit pulp was determined by titration (Sinclair, 1984). Depending on the juice content of the fruit, either 200 to 500 mg of juice sacs (young fruit), or 0.5 mL of juice sac extracts was analyzed for organic acids with a 330 gas chromatograph (Varian, Walnut Creek, Calif.) according to Erner and Reuveni (1981). We used a Megabore DB17 column (J & W Scientific, Folsom, Calif.) with the following conditions: N₂ flow-through, 10 mL·min⁻¹; N₂ makeup, 20 mL·min⁻¹; H₂, 30 mL·min⁻¹; air, 400 mL·min⁻¹. Detection was performed with a 4290 Integrator (Varian). When calli were used for analyses, they were extracted similarly to young fruit.

CITRATE SYNTHASE ACTIVITY. Depending on the size of the fruit and the juice content of the sacs, 0.6 to 2.0 g of juice sacs from 10 or 30 fruit, at similar developmental stages, were divided evenly between two tubes, and kept in an ice bath. In order to prevent enzyme inhibition by

the low pH of the vesicles, we routinely made a trial extraction from half of the juice sacs. The contents of one tube were homogenized in a Polytron (Kinematica AG Littau, Luzern, Switzerland), in 0.3 to 1.0 mL of extraction buffer containing 50 mM HEPES-NaOH, pH 8.5, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.1% Tween-20, 0.5 mM phenyl-methylsulfonylfluoride, and 10% glycerol (Landschutze et al., 1995). The homogenate was adjusted to pH 7 by addition of a measured volume of 5 M KOH, and the same volume was then added to the extraction buffer prior to its addition to the second tube. Following quick extraction in an ice bath, cell debris was removed by centrifugation at 5,000 g_n for 10 min at 4 °C, and the supernatant was assayed for enzyme activity. The enzymatic assay was performed by the 5'5'-dithiobis-(2-nitrobenzoate) method at 412 nm, as described by Srere (1969).

RNA EXTRACTION AND ANALYSIS. RNA was extracted from juice vesicles by means of the phenol-chloroform method of Ausubel et al. (1988), with modifications. About 5 g of tissue was ground with liquid nitrogen, and added to a tube containing 17 mL of extraction buffer and 8 mL of phenol saturated with 0.2 M Tris-HCl (pH 8.2),

Fig. 1. Effect of sodium arsenite sprays on (A) total titratable acidity and (B) citric acid concentration of 'Minneola' tangelo fruit. Trees of 'Minneola' tangelo were sprayed with various concentrations of sodium arsenite on 3 June 1998, ≈6 weeks postanthesis (10 to 15 mm fruit). Fruit were collected at different times, as indicated, and analyzed for titratable acidity and citric acid concentration. Mean values of four independent replicates ± SE. Legend in A applies to both figures. Inset (in B), citric acid concentration on an expanded scale.

Fig. 2. Effect of sodium arsenite concentration on citric acid concentration (columns) and growth (■) of 'Eureka' lemon callus. Small pieces of callus (50 to 100 mg) originating from lemon juice vesicles were placed on solid media containing various sodium arsenite concentrations, as indicated. After 6 weeks, the calli were weighed and analyzed for citric acid concentration. Results are means of three independent experiments. The linear component of the treatment effect on citric acid concentrations was highly significant ($P = 0.0046$), whereas the deviation from linearity was nonsignificant ($P = 0.611$). Effect of arsenite treatments on callus growth was not significant ($P = 0.877$).

0.1 M LiCl and, 5 mM EDTA. The mixture was blended by means of a Polytron (Kinematica AG Littau), and 8 mL of chloroform was added to it, followed by a 30-min incubation at 50 °C with occasional shaking. The mixture was centrifuged at 10,000 g_n for 20 min, and the upper phase was reextracted with one volume of phenol-chloroform (1:1, v/v), and recentrifuged. Lithium chloride was then added to the upper phase to a final concentration of 2 M, followed by overnight incubation at 4 °C and centrifugation at 10,000 g_n for 20 min at 4 °C. The pellet was dissolved in 0.5 mL H₂O, and centrifuged briefly to remove nonprecipitated material. Northern blot analyses (Sambrook et al., 1989) were performed with [³²P]dCTP-radiolabeled probe for pummelo citrate synthase cDNA (Canel et al., 1996), provided by Mikeal Roose, Riverside, Calif. The membranes were autoradiographed with X-ray film, and were also scanned with a Fujifilm BAS-1500 Phosphorimager (Fugi Photo Film Co., Tokyo, Japan). The images were quantified with the aid of TINA2 software (Fugi Photo Film Co., Tokyo, Japan) and the ethidium bromide (EtBr)-stained RNA was scanned and quantified with the same software.

STATISTICAL ANALYSIS. Two-way analysis of variance for arsenite concentrations and experiments were carried out on citric acid and calli growth (Fig. 2) followed by partitioning the concentration sum of squares into linear and deviation from linear components. One-way analysis of variance for arsenite concentrations and citrate synthase activity (Fig. 3) was followed by Tukey's Studentized range test.

Results

Results from 1997 and 1998 were very similar, therefore, only 1998 results are presented. Total titratable acidity (Fig. 1A) decreased slightly during the first 21 d after arsenite application. Between 21 and 56 d after treatment, total acidity of control fruit increased 10 fold, before decreasing to about three times the

initial level toward fruit maturation (210 d after treatment). The pattern of acid accumulation in the arsenite-treated fruit was similar to that in the controls, but the acid concentration was significantly lower on each sampling date, starting from 35 d after treatment. On a few sampling dates, 56, 112, 140, 196, and 210 d after treatment, a dose response of arsenite concentration was usually found: increasing the arsenite concentration resulted in a larger reduction in the total titratable acidity. However, on the other sampling dates there was no difference in total acidity among the arsenite concentrations. By 196 d after treatment, the acid content of treated fruit was 1% to 1.2%, allowing commercial

Fig. 3. Effects of sodium arsenite concentration on citrate synthase activity of 'Minneola' tangelo fruit harvested at various intervals following application. Citrate synthase activity was measured in treated fruit as described in Fig. 1, and collected (A) 1, (B) 2, and (C) 7 d following application. Lower case letters denote mean separation by Tukey's Studentized range test ($P < 0.001$).

Table 1. Effect of sodium arsenite concentration on the concentrations of organic acids in 'Minneola' tangelo fruit 35 d following sodium arsenite sprays (n = 4, means $\pm$ SE).

Arsenite concn (mg·L ⁻¹)	Organic acids content (mg·g ⁻¹ fresh wt)			
	Malonic	Fumaric	Succinic	Citric
0	0.0303 $\pm$ 0.0053	0.0193 $\pm$ 0.003	0.0193 $\pm$ 0.003	8.156 $\pm$ 0.881
42	0.0388 $\pm$ 0.0038	0.0266 $\pm$ 0.006	0.0242 $\pm$ 0.003	4.962 $\pm$ 1.318
85	0.0280 $\pm$ 0.0018	0.0196 $\pm$ 0.002	0.0177 $\pm$ 0.004	3.640 $\pm$ 1.107
170	0.0280 $\pm$ 0.0018	0.0196 $\pm$ 0.002	0.0177 $\pm$ 0.004	4.382 $\pm$ 0.408

harvest, while the acid content of control fruit was 1% higher and therefore too high for harvesting.

The pattern of citrate accumulation in the control fruit was similar to that of total titratable acidity. There was little accumulation the first 21 d after treatment, more than a 100-fold increase between 21 and 84 d, and a decline toward fruit maturation. The decrease in citrate concentration continued for 14 d longer than that in total acidity. The effect of arsenite treatment on citric acid concentration was also very similar to its effect on total titratable acidity. The inhibition became evident only when the major increase in citric acid concentration was detected after 35 d. Although there was no difference between control and treated fruit in the time of maximum titratable acid accumulation (Fig. 1A), the citric acid concentration peaked 28 d later in the treated fruit than in the control fruit (Fig. 1B). Only at 112 d after treatment was the reduction in citrate level correlated with the increase in arsenite concentration. On the other dates, 84, 140, 168, and 196 d after treatment, the two highest arsenite concentrations gave similar values of citrate concentrations. On the other sampling dates, the difference between the arsenite concentrations was not significant.

The effect of arsenite on total titratable acidity appeared to be correlated primarily with its effect on citric acid, since it had no effect on the concentrations of other organic acids found in the fruit (Table 1). Growth of 'Eureka' lemon callus was unaffected by arsenite, up to 0.85 mg·L⁻¹, in the culture medium (Fig. 2). However, there was a gradual reduction in the citric acid concentration in callus as the sodium arsenite concentration increased. The highest concentration (0.85 mg·L⁻¹) resulted in a 2.5-fold decrease in acid level, as compared with the controls. Higher concentrations of sodium arsenite in the medium greatly inhibited callus growth (data not presented).

A 3- to 4-fold decrease in citrate synthase activity was observed in fruit treated with the two higher sodium arsenite concentrations, within 1 d after application (Fig. 3A). After an additional day (Fig. 3B), the activities started to recover approaching that of the controls, and after 7 d, no difference

was detected (Fig. 3C). No differences in citrate synthase activity between treated and control fruit were detected during the remainder of the fruit development period.

Citrate synthase mRNA was undetectable in young control fruit (Fig. 4A). However, in the arsenite-treated fruit, expression was induced within 1 d, with the greatest increase evident at the two higher arsenite concentrations. A further increase was detected after 7 d. Although 42 mg·L⁻¹ sodium arsenite did not inhibit activity of citrate synthase (Fig. 3), it induced citrate synthase mRNA to levels similar to that of the other treatments after 7 d. Gene expression was also induced in callus grown in the presence of 0.85 mg·L⁻¹ sodium arsenite (Fig. 4B).

Fig. 4. Effects of sodium arsenite treatment on expression of citrate synthase gene in (A) 'Minneola' tangelo fruit and (B) 'Eureka' lemon callus. RNA was extracted from treated 'Minneola' tangelo fruit as described in Fig. 1, and collected 1 or 7 d following application, or from 'Eureka' lemon calli grown for 6 weeks in the presence of 0.85 mg·L⁻¹ sodium arsenite. Standardization of the results was performed with (A) 18S RNA, which was used as a probe, or with (B) ethidium bromide (Et-Br)-stained total RNA.

Discussion

In spite of the broad-spectrum effects of arsenic compounds in all living organisms, the concentrations of sodium arsenite used in the present study did not alter fruit growth and development (data not presented). Even tissue cultures, exposed continuously to sodium arsenite, up to $0.85 \text{ mg} \cdot \text{L}^{-1}$, grew normally. In addition, sugar accumulation was unaffected in the fruit following arsenite spray (data not presented), suggesting that metabolic pathways, other than acid accumulation, were not altered. We describe herein two effects of arsenite treatment. First, a reduction in citric acid accumulation that was detected 35 d following treatment and which proceeded throughout fruit development; and second, a transient decrease in citrate synthase activity that occurred 1 to 2 d following treatment and was accompanied by induction of gene expression.

Pulp acidity of citrus fruit is thought to be dependent on two mechanisms: the accumulation of citric acid in the vacuoles of the juice sac cells (Brune et al., 1998; Canel et al., 1995; Sadka et al., 2000), and acidification of the vacuole by a tonoplastic H^+ -ATPase, which creates a proton influx and reduces vacuolar pH to about 2.5 (Müller et al., 1996, 1997). Although the patterns of citric acid concentration and total titratable acidity (representing the vacuolar pH) were similar, slight differences between them might indicate that these two mechanisms are independent. For instance, total titratable acidity decreased slightly during the first 21 d following treatment, while there was a minor increase in citric acid concentration. This might indicate that following its transport into the vacuole, citrate is protonated (Müller et al., 1996) and slightly reduces the total titratable acidity. Similarly, the increase in citric acid concentration between 56 and 84 d might have caused the reduction in total titratable acidity during that time. The two mechanisms are most likely coregulated and inhibiting one of them may inhibit the other. Indeed, in the present study the inhibitory effects of sodium arsenite on total titratable acidity and citrate concentration became evident at the same time, 35 d after treatment, and affected both parameters almost simultaneously throughout the season. On most sampling dates during the acid decline stage, the two highest arsenite concentrations usually elicited the largest reductions in total titratable acidity and citrate concentration. On the other dates, especially during the acid accumulation stage, the sample size was probably not large enough to show differences among the various arsenite treatments, in citrate level or total titratable acidity.

Citrate synthase activity was reduced transiently following application of arsenite at the two higher concentrations. It is possible that the lowest arsenite concentration also reduced the activity, but that the recovery time was shorter than 24 h; by the following sampling dates, activity of citrate synthase in the sodium arsenite-treated fruit was similar to that in the controls. The long delay between enzymatic inhibition, which lasted 7 d following arsenite application, and the onset of the effect on acid accumulation, which was detected only after 35 d, lessen the possibility that the two phenomena are directly related. The detection method should, in principal, be able to detect differences even with the low acid concentrations found during the first 21 d after treatment, as small differences were detectable in the tissue cultures. In addition, the lowest arsenite concentration ($42 \text{ mg} \cdot \text{L}^{-1}$) reduced acid accumulation without a detectable effect on the enzyme activity, unless the latter recovered sooner than 24 h. It is therefore concluded that sodium arsenite does not reduce fruit acidity through a direct effect on the activity of citrate synthase.

This is in contrast to the effect of lead arsenate, which caused a reduction in the activity of citrate synthase during the 5 weeks following treatment, in parallel to the reduction in acid level, and also inhibited enzymatic activity when added to the assay medium (Yamaki, 1990a).

It is well established that arsenite inhibits activity of pyruvate dehydrogenase (PDH), which requires an SH group for its activity (Hu et al., 1998; McKay et al., 1988). PDH catalyses synthesis of acetyl-CoA, used for citrate synthesis, and this might be the reason for the reduction in acid level in citrus fruit. A reasonable scenario, which might explain our results, distinguishes between the short- and long-term effects of sodium arsenite. Immediately following application, activity of citrate synthase is inhibited, probably by indirect effects of arsenite, and it returns to the normal level quite rapidly, because of gene induction (see below). The activity of PDH is also inhibited following treatment, but it remains relatively low, resulting in a reduced supply of acetyl-CoA. Citrate synthase is operated, in vivo, under lower rates without a detectable effect on its extractable activity, and so causing the long-term effect on citrate accumulation. Interestingly, it has been reported that lead arsenate also reduces the concentration of acetyl-CoA, in parallel to the reduction in citrate synthase activity (Yamaki, 1990b). However, it should be considered that lead arsenate was used at a concentration of $3000 \text{ mg} \cdot \text{L}^{-1}$, and if it contained even a minor amount of arsenite, this might have caused the effect. It should also be considered that prokaryotes and eukaryotes can metabolize one arsenic form into another, pump it out of the cell, or detoxify it by methylation (Kuroda et al., 1998; Mass and Wang, 1997; Silver and Phung 1996). This raises the possibility that even when a particular material is used on citrus, conversion or detoxification mechanisms may be operating to change it to a different form.

Reduction in citrate synthase activity was followed by a major increase in its mRNA accumulation, which could already be detected 1 d after treatment. Arsenite induced the expression of a few genes belonging to the heat-shock family in *Arabidopsis thaliana* (L.) Heynh., but this probably reflected a general stress response (Miloni and Hatzopoulos, 1997). In citrus, the increase in citrate synthase gene expression most likely played a role in the fast recovery of enzyme activity. The mechanism of arsenite action on gene induction might be either indirect, through the accumulation of citrate synthase substrate (Avsian-Kretchmer et al., 1999), or direct, as in the case of methotrexate, which inhibits the activity of dihydrofolate reductase (DHFR), but induces its mRNA level through gene amplification (Schimke, 1984). Interestingly, arsenite resistance in *Leishmania tarentolae* cell line TarIIWT has also been associated with amplification of a few genes (Grondin et al., 1997).

In summary, arsenite treatment was very effective in reducing total fruit acid concentration to a desired level of $1\% \approx 2$ weeks sooner than in the controls, thus allowing early harvest. Since use of this chemical is prohibited, a better understanding of its mode of action might lead to development of a nonhazardous alternative means to reduce fruit acidity. Although the work described herein does not answer the question of the mechanism of arsenite effect, it raises interesting possibilities for future research.

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