Foliar Application of Sugar Alcohol Zinc Increases Sugar Content in Apple Fruit and Promotes Activity of Metabolic Enzymes

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Abstract. Application of sugar alcohol zinc (SA-Zn) spray to apple trees at certain developmental stages can improve fruit quality. Increasing the Zn concentration of fruit can improve nutritional content and promote human health. We conducted foliar application of SA-Zn to 13-year-old 'Fuji' apple trees at different developmental stages. The effects of SA-Zn application on Zn concentration, reducing sugar content, and carbohydrate metabolism-related enzyme activity in fruit were investigated. The foliar treatment increased Zn and reducing sugar concentrations significantly in mature fruit. Sorbitol dehydrogenase activity was higher in the fruit of trees treated before budbreak and 3 weeks after flowering compared with the control at the early fruit stage and was higher during fruit expansion in plants treated after termination of spring shoot growth. Mature fruit of trees treated during the fruit expansion stage showed higher sorbitol dehydrogenase activity than the control. Foliar SA-Zn treatment did not have a significant effect on sorbitol oxidase activity in apple fruit. Treatment before budbreak and at 3 weeks after flowering led to a significant increase in the activity of sucrose synthase and acid invertase at the early fruit stage. Treatment during the fruit expansion stage significantly increased the activity of acid invertase at maturity but had no effect on the activity of neutral invertase. Our results indicate that foliar SA-Zn application resulted in biofortification of Zn in apples, which led to higher activity of carbohydrate metabolism-related enzymes and accumulation of sugars.

Zinc is an essential trace element required by all organisms. As a regulating factor in the structure and function of more than 300 enzymes (Andreini and Bertini, 2012), Zn plays a key role in plant physiological processes, including protein synthesis, carbohydrate metabolism, and cell division (Hacisalihoglu et al., 2003). Apple production has been increasing worldwide but fruit quality is a limiting factor in achieving the economic potential of apple crops. Sugar is

an important index for evaluating fruit quality and flavor (Shü et al., 2001). Sucrose and sorbitol are the main forms of assimilated carbohydrate (photosynthetic products) transported from apple leaves to fruit with sorbitol accounting for ≈80% of the carbohydrate (Loescher et al., 1982). Sorbitol dehydrogenase (SDH) and sorbitol oxidase (SOX) catalyze the metabolism of sorbitol to fructose and glucose, respectively, in apple fruit (Park et al., 2002; Yamada et al., 1998). Various researchers have examined changes in these enzymes during fruit development under natural (field) conditions and they increase with the development time (Archbold and Nosarszewski, 2004; Yamaguchi et al., 1996; Yamaki, 1986). Sucrose, a major carbohydrate in many plants, plays a similar metabolic role in apples. Sucrose synthase (SS) and sucrose invertases, including acid invertase (AI) and neutral invertase (NI), catalyze the conversion of sucrose to fructose and glucose in apples as in other plants (Koch, 2004; Sturm and Tang, 1999).

Foliar Zn spray was shown to be effective in increasing grain Zn concentration (Phattarakul

et al., 2012). Application of Zn spray to apple trees was reported to increase fruit Zn content and to improve apple quality (Nielsen et al., 2005; Zhang et al., 2013), but the mechanisms underlying these improvements are not clearly understood. There are also no published data for apple regarding the relationship between foliar Zn spray and the activity of enzymes involved in carbohydrate metabolism. Here, we report on field experiments using apple trees to which SA-Zn was applied as a foliar spray at different development stages in Shandong Province, China. To our knowledge, this is the first study to demonstrate the effects of the timing of SA-Zn spray on enzyme activity.

Materials and Methods

Experimental design. The experiment was conducted in 2012 in an orchard (lat. 36°14' N, long. 116°50' E) in Feicheng City, Shandong Province, China, using 13-year-old Fuji apple trees (*Malus ×domestica* Borkh. 'Red Fuji'/*M. hupehensis* Rehd.). Trees were selected according to uniformity of size and the leaf Zn concentration of the chosen trees was (mean ± SD) 14.3 ± 1.2 mg·kg⁻¹ dry weight, and the trees showed no symptoms of Zn deficiency. They were sprayed at different development stages with SA-Zn [sugar alcohol-based zinc liquid fertilizer containing 3% (m/m) nitrogen, 10% (m/m) sugar alcohol, and 7% (m/m) Zn; Beijing Xinhefeng Agrichemical Co., Beijing, China]. The trees were arranged in a randomized block design with five replicates per treatment (n = one tree per replicate) with five to seven guard apple trees located between the treatments. Five treatments were used: 1) control, no SA-Zn; 2) foliar application of SA-Zn before budbreak (19 Mar.); 3) foliar application of SA-Zn 3 weeks after flowering (21 May); 4) foliar application of SA-Zn at termination of spring shoot growth (10 July); and 5) foliar application of SA-Zn during fruit expansion stage (17 Aug.). We applied spray to branches before budbreak with a 0.7% (v/v) aqueous solution of SA-Zn until runoff. After budbreak, a 0.1% (v/v) aqueous solution of SA-Zn was sprayed onto leaves until runoff. We randomly selected and picked 20 apples from the outer branches of each tree at the same canopy height 10 d after flowering (DAF); 10 fruits were collected from each tree at monthly intervals thereafter. We removed the 10 or 20 fruit peel from fruit entirely. After removing the core, the flesh was cut into pieces and mixed well per replicate. The samples weighed 10.0 g and were immediately frozen in liquid nitrogen and then enzyme activity was determined. The samples for determination of sugar contents after 1 d were stored at -80 °C.

The time at which each development stage of apple trees begins and ends differs among years according to meteorological conditions. We defined fruit developmental stages according to Shu et al. (1993) as follows: early stage, 10 to 80 DAF; fruit

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expansion stage, 80 to 160 DAF; mature stage, 160 to 190 DAF.

Determination of zinc concentration. The collected apples were peeled, cut into pieces, and oven-dried at 45 °C. The dried samples were acid digested in a 50-mL tube and analyzed for Zn content using an SP9-400 atomic absorption spectrophotometer (Pye, Cambridge, U.K.).

Determination of reducing sugar content. The samples were taken from the freezer and homogenized in 5 mL distilled water and centrifuged at 12,000 × g for 20 min. The precipitate was resuspended in distilled water and shaken, centrifuged a second time at 12,000 × g for 20 min, and the supernatants were pooled. The extract was filtered into a 50-mL volumetric flask and the concentration of total reducing sugar was determined by 3,5-dinitrosalicylic acid colorimetry.

Enzyme extraction and assays. Invertase, SDH, and SOX were extracted according to Yamaki (1986) with modifications. Immediately after harvest, a 10.0-g composited sample from at least 10 apples was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was further homogenized in 0.2 M potassium (K)-phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT), 10 mM K-ascorbate, 0.1% Triton X-100, and 10% of Polyclar AT. The homogenate was squeezed through a nylon cloth and centrifuged at 25,000 × g for 20 min. Protein was precipitated by adding ammonium sulfate to the supernatant; most enzyme activity was obtained from 40% to 80% saturation. A 5-mL aliquot of the supernatant was dialyzed for 12 h against 500 mL 0.01 M Tris-HCl buffer (pH 7.0) containing 1 mM DTT at 2 °C.

The activity of sorbitol enzymes in dialyzed samples was determined according to Yamaki (1986). For SDH, assays were conducted at 30 °C in 0.33 mL of a reaction medium consisting of 30 mM Tris-HCl buffer (pH 9.6), 1 mM nicotinamide adenine dinucleotide (NAD⁺), and 235 mM sorbitol for 60 min. The increase in absorbance at 345 nm resulting from the reduction of NAD⁺ was measured by spectrophotometry (ultraviolet-2550; Shimadzu, Kyoto, Japan). Assays for the determination of SOX activity were performed at 30 °C for 2 h in 1.7 mL of a reaction medium consisting of 59 mM acetate buffer (pH 4.0) and 235 mM sorbitol. To stop the reaction, 0.5 mL of the solution was withdrawn and boiled. After adding 1 mL of 0.1 M Tris-HCl buffer (pH 8.0), the glucose produced was analyzed enzymatically using yeast hexokinase (Sigma), grade-I yeast glucose-6-phosphate dehydrogenase (Sigma), adenosine-5'-triphosphate, MgCl₂, and NADP. For invertase, AI activity was determined in a mixture of 47 mM acetate buffer (pH 4.0) and 40 mM sucrose at 30 °C for 30 min. After boiling to stop the reaction, aliquots of sample were obtained for enzymatic analysis. For determination of NI activity, assays were performed at 30 °C for 30 min in a reaction medium consisting of 40 mM sucrose, 50 mM K-phosphate buffer (pH 7.5), 5 mM MgCl₂,

and 1 mM Na-EDTA. A blank containing no substrate was included with each determination of enzyme activity.

Sucrose synthase was extracted using the method of Hubbard et al. (1989) with modifications. Frozen fruit tissues stored in liquid nitrogen were ground in a cold mortar in buffer containing 50 mM HEPES-NaOH (pH 7.5), 5 mM MgCl₂, 1 mM EDTA, 2.5 mM DTT, 0.05% (v/v) Triton X-100, and 0.5 mg·mL⁻¹ bovine serum albumin (BSA). The homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant was desalted immediately by centrifugal filtration at 1 °C on Sephadex G-25 columns (Sigma) equilibrated with 50 mM MOPS-NaOH (pH 7.5), 5 mM MgCl₂, 2.5 mM DTT, and 0.5 mg·mL⁻¹ BSA. The activity of SS related to sucrose cleavage was assayed by the quantity of uridine diphosphate (UDP)-glucose coupled to the reduction of NAD⁺ in the presence of excess UDP-glucose dehydrogenase according to a slight modification of the procedure described by Morell and Copeland (1985).

Statistical analysis. Significant differences among the treatments and the control were determined through an analysis of variance using SAS (Version 8.1; SAS Institute, Cary, NC) followed by the Fisher's least significant difference test. The mean values were separated using Fisher's least significant difference test at the *P* < 0.05 level of significance.

Results

Effects of foliar SA-Zn application on changes of Zn concentration in fruit. The

concentration of Zn in apple fruit increased up to 70 DAF and then decreased with fruit enlargement (Table 1). Application of SA-Zn spray before budbreak and 3 weeks after flowering led to an increased Zn concentration at all stages. Application of SA-Zn spray after termination of spring shoot growth led to a significantly higher Zn concentration at 100 DAF. All treatments led to a significant increase in Zn concentration at maturity with the largest increases occurring in plants treated after termination of spring shoot growth and during the fruit expansion stage (Table 1).

Effects of foliar SA-Zn application on the concentration of reducing sugars in fruit. The concentration of reducing sugars in apples increased throughout fruit development (Table 2). There were no significant differences between the treatments and control during the early fruit stage. However, application of SA-Zn spray led to increased sugar concentrations at 100 to 190 DAF with the most significant increase during the fruit expansion stage.

Effects of foliar SA-Zn application on activity of sorbitol metabolism-related enzymes in fruit. Sorbitol dehydrogenase activity was very low during the early fruit stage (Table 3). When SA-Zn spray was applied before budbreak and 3 weeks after flowering, SDH activity was increased significantly during the early fruit stage compared with the control; however, these differences were no longer significant at maturity. Application of SA-Zn spray after termination of spring shoot growth led to

Table 1. Effects of foliar application of sugar alcohol zinc (SA-Zn) at different development stages on Zn concentration in Fuji apple fruit.

Treatment stage ^x	Days after flowering (DAF) ^y					
	10 d	40 d	70 d	100 d	130 d	160 d
	Zn concentration in fruit ^z (mg·kg ⁻¹)					
CK	7.11 b	8.79 b	9.80 c	9.26 b	7.02 b	3.51 b
T1	8.92 a	10.66 a	10.54 b	9.73 a	7.42 a	4.19 a
T2		10.02 a	11.11 a	9.84 a	7.53 a	4.46 a
T3				10.25 a	8.05 a	3.98 a
T4						4.12 a

^xEarly stage, 10 to 80 DAF; fruit expansion stage, 80 to 160 DAF; mature stage, 160 to 190 DAF.

^yDifferent letters in the same column indicate significant differences (*P* < 0.05) (Fisher's least significant difference test).

^zCK = no SA-Zn; T1 = sprayed before budbreak; T2 = sprayed at 3 weeks after flowering; T3 = sprayed at termination of spring shoot growth; T4 = sprayed at fruit expansion stage.

Table 2. Effects of foliar application of sugar alcohol zinc (SA-Zn) at different development stages on the concentration of reducing sugars in Fuji apple fruit.

Treatment stage ^x	Days after flowering (DAF) ^y					
	10 d	40 d	70 d	100 d	130 d	160 d
	Reducing sugar contents in fruit ^z (mg·kg ⁻¹)					
CK	9.12 a	17.10 a	25.14 a	40.33 b	58.40 c	76.14 c
T1	9.58 a	18.59 a	26.87 a	47.05 a	69.43 b	88.20 b
T2		19.84 a	28.86 a	49.65 a	65.47 b	89.78 b
T3				48.30 a	71.72 a	88.03 b
T4						94.74 a

^xEarly stage, 10 to 80 DAF; fruit expansion stage, 80 to 160 DAF; mature stage, 160 to 190 DAF.

^yDifferent letters in the same column indicate significant differences (*P* < 0.05) (Fisher's least significant difference test).

^zCK = no SA-Zn; T1 = sprayed before budbreak; T2 = sprayed at 3 weeks after flowering; T3 = sprayed at termination of spring shoot growth; T4 = sprayed at fruit expansion stage.

increased SDH activity at 100 and 130 DAF. Foliar SA-Zn applied during the fruit expansion stage was associated with a significant increase in SDH activity. SA-Zn application had no significant effect on SOX activity (Table 3).

Effects of foliar SA-Zn application on SS activity in fruit. Sucrose synthase activity decreased with fruit development (Table 4). Application of SA-Zn spray before budbreak and 3 weeks after flowering led to a significant increase in SS activity during the early fruit stage. SA-Zn application had no influence on SS activity after 70 DAF.

Effects of foliar SA-Zn application on sucrose invertase activity in fruit. The activities of AI and NI in fruit increased during the early development stage and then declined with development from 100 DAF (Table 5). SA-Zn treatment before budbreak and 3 weeks after flowering led to a significant increase in AI activity from 10 to 100 DAF. Treatment during the fruit expansion stage was also associated with a significant increase in AI activity. SA-Zn application had no effect on NI activity (Table 5).

Discussion

Foliar application of SA-Zn provides an efficient means of achieving biofortification of cereal grains (Cakmak, 2008). Research on brown rice (*Oryza sativa* L.) found that soil application of SA-Zn failed to cause a substantial increase in Zn concentration in the grain but that foliar application led to fortification of this nutrient (Wissuwa et al., 2008). Foliar SA-Zn application has consistently and significantly contributed to increased grain Zn in rice irrespective of cultivar, environmental conditions, and management practices in China, India, Laos, Thailand, and Turkey (Phattarakul et al., 2012). Foliar application of SA-Zn could be an effective strategy in biofortifying legumes with Zn (Pandey et al., 2013). Our results demonstrated that application of SA-Zn spray to branches and leaves of apple trees at different growth stages resulted in an increased concentration of Zn in fruit.

Sugars produced by photosynthesis are transported to sink organs as sucrose and sorbitol; sorbitol is the main form transported to fruit in apple trees (Loescher et al., 1982). In fruit, sucrose and sorbitol are transformed into reducing sugars, including fructose and glucose, by various enzymes (e.g., AI, NI, SS, SDH, and SOX) (Berüter et al., 1997; Loescher et al., 1982). In the present study, foliar application of SA-Zn had no significant effect on reducing sugars during the early fruit stage, probably because fructose and glucose were used in tissue building (Koch, 2004). However, all of the SA-Zn treatments were associated with a significant increase in the concentration of reducing sugars in mature fruit.

In fruit, sorbitol is transformed into fructose by SDH and into glucose by SOX for storage or participation in other metabolic processes (Yamaguchi et al., 1996). Foliar

SA-Zn application led to increased SDH activity, which contributed to the increasing of reducing sugar in fruit. The results showed SOX activity exhibited low levels in fruit. This is probably because SOX accounts for only one-fifth of SDH activity, and more than 80% of SOX exists in bound form (Chong, 1971). The transformation of sucrose into fructose and glucose is converted by SS, AI, and NI in fruit, leading to differences in sugar concentration between fruit and phloem that drive sucrose unloading (Wind et al., 2010).

Foliar application of SA-Zn had a significant effect on SS activity during the early stages but no effect during later stages of fruit development, probably because SS activity is generally higher in early stages than in mature stages of growth (Li et al., 2002). During early growth, fruit require more UDP-glucose (produced from sucrose by SS catalysis) for synthesis of base materials (Koch, 2004).

In fruit, many sugar-metabolizing enzymes (e.g., SDH, SOX, SS, AI, and NI) are closely related to sink strength and

Table 3. The effects of sugar alcohol zinc (SA-Zn) spraying at different development stages on the changes of sorbitol dehydrogenase (SDH) and sorbitol oxidase (SOX) activity in Fuji apple fruit.

		Days after flowering (DFA) ^z						
		10 d	40 d	70 d	100 d	130 d	160 d	190 d
Enzyme	Treatment stage ^x	Activities ^y (mmol·h ⁻¹ ·g ⁻¹)						
SDH	CK	0.26 b	0.58 c	2.36 b	5.33 b	4.75 b	5.99 b	5.24 b
	T1	0.95 a	1.26 b	3.30 a	6.21 a	5.06 b	6.57 b	5.54 b
	T2		1.79 a	3.36 a	6.27 a	5.18 b	6.49 b	5.53 b
	T3				6.97 a	5.80 a	6.54 b	5.56 b
	T4						7.24 a	6.29 a
SOX	CK	1.39 a	2.45 a	0.88 a	1.15 a	1.45 a	1.51 a	1.53 a
	T1	1.61 a	2.57 a	1.08 a	1.29 a	1.57 a	1.66 a	1.65 a
	T2		2.64 a	1.13 a	1.33 a	1.56 a	1.63 a	1.64 a
	T3				1.35 a	1.59 a	1.66 a	1.64 a
	T4						1.71 a	1.66 a

^zEarly stage, 10 to 80 DAF; fruit expansion stage, 80 to 160 DAF; mature stage, 160 to 190 DAF.

^yDifferent letters in the same column indicate significant differences ($P < 0.05$) (Fisher's least significant difference test).

^xCK = no SA-Zn; T1 = sprayed before budbreak; T2 = sprayed at 3 weeks after flowering; T3 = sprayed at termination of spring shoot growth; T4 = sprayed at fruit expansion stage.

Table 4. The effects of sugar alcohol zinc (SA-Zn) at different development stages on the changes of sucrose synthase (SS) cleavage activity in Fuji apple fruit.

Treatment stage ^x	Days after flowering (DAF) ^z						
	10 d	40 d	70 d	100 d	130 d	160 d	190 d
Activities ^y (mmol·h ⁻¹ ·g ⁻¹)							
CK	8.62 b	6.68 b	2.74 c	3.32 a	2.44 a	2.21 a	1.34 a
T1	9.68 a	7.49 a	4.25 a	3.79 a	2.69 a	2.64 a	1.66 a
T2		7.70 a	3.51 b	4.59 a	2.86 a	2.68 a	1.82 a
T3				4.00 a	2.91 a	2.62 a	1.87 a
T4						2.54 a	1.71 a

^zEarly stage, 10 to 80 DAF; fruit expansion stage, 80 to 160 DAF; mature stage, 160 to 190 DAF.

^yDifferent letters in the same column indicate significant differences ($P < 0.05$) (Fisher's least significant difference test).

^xCK = no SA-Zn; T1 = sprayed before budbreak; T2 = sprayed at 3 weeks after flowering; T3 = sprayed at termination of spring shoot growth; T4 = sprayed at fruit expansion stage.

Table 5. The effects of sugar alcohol zinc (SA-Zn) spraying at different development stages on changes of acid invertase (AI) and neutral invertase (NI) activity in Fuji apple fruit.

		Days after flowering (DAF) ^z						
		10 d	40 d	70 d	100 d	130 d	160 d	190 d
Enzyme	Treatment stage ^x	Activities ^y (mmol·h ⁻¹ ·g ⁻¹)						
AI	CK	8.13 b	9.18 b	11.69 b	13.35 b	9.14 b	8.06 b	8.29 b
	T1	9.13 a	10.14 a	12.98 a	14.25 a	9.46 ab	8.41 ab	8.57 ab
	T2		10.33 a	13.33 a	14.31 a	9.47 ab	8.57 ab	8.50 ab
	T3				14.63 a	9.71 a	8.62 ab	8.67 ab
	T4						9.05 a	9.45 a
NI	CK	6.21 a	6.91 a	9.66 a	9.49 a	6.63 a	5.61 a	5.23 a
	T1	6.57 a	7.21 a	9.87 a	9.51 a	6.59 a	5.89 a	5.49 a
	T2		7.26 a	10.18 a	9.53 a	6.55 a	5.80 a	5.49 a
	T3				9.55 a	6.68 a	5.94 a	5.41 a
	T4						6.02 a	5.65 a

^zEarly stage, 10 to 80 DAF; fruit expansion stage, 80 to 160 DAF; mature stage, 160 to 190 DAF.

^yDifferent letters in the same column indicate significant differences ($P < 0.05$) (Fisher's least significant difference test).

^xCK = no SA-Zn; T1 = sprayed before budbreak; T2 = sprayed at 3 weeks after flowering; T3 = sprayed at termination of spring shoot growth; T4 = sprayed at fruit expansion stage.

activity; the activity of SDH, SS, and AI largely determines the sink strength of fruit (Hockema and Etxeberria, 2001; Nosarszewski et al., 2004; Zhou et al., 2006). In the present study, foliar SA-Zn application led to increased activity of SDH, AI, and SS. Therefore, the increased concentration of reducing sugars was probably a result of enhanced activity of these enzymes induced by SA-Zn application, which led to large sink strength.

Interestingly, we found that the effect of foliar SA-Zn application on enzyme activity becomes less obvious with increasing time after application. Repeated foliar treatment with SA-Zn may be required to obtain continuous effects.

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