5

77

BARD

FINAL REPORT

PROJECT NO. 1-921-85

Mineral Nutrition and Physiological Disorders in Horticultural Crops

A. Bar-Akiva (Deceased), R. Salomon, T.J. Facticeau
E.A. Mielke, P.M. Chen

20

630.72
BAR/SAL
2nd copy

1991

34617

000000176194

80

Standard BARD Cover Page for Scientific Reports

Date 6.12.90

BARD
P.O. Box 6
Bet Dagan, ISRAEL

BARD Project No. IS-0921-85R

Title of Research Project:

Mineral Nutrition and Physiological Disorders in Horticultural Crops

Names of Investigators:

Principal Investigator: Raphael Salomon

Cooperating Investigator(s): T.J. Facticeau, Eugene Mielke and P.M. Chen

Name and Address of Affiliated Institutions:

Principal Institution: ARO, Volcani Center, Bet Dagan 50250, Israel

Cooperating Institution(s): Oregon State University, Mid Columbia
Experiment Station, Hood River, Oregon, 97031-9512, U.S.A.

Project's starting date: October 1986

Type of Report: 1st Annual _____ 2nd Annual _____ Final X

Signatures:

Principal Investigator

Institution's Authorizing Official

Raphael Salomon

הספרייה המרכזית
למדעי החקלאות
8-11-81

630.72 ; 634.13 ; 634.3 ; 581.133.5 ;

BAR/SAL 581.192.5

2nd copy

Table of Content	Page
Summary	1
Introduction	2
Pyruvate Kinase Activity	4
Results	7
Conclusions	9
Tables and Figures	10
Pyruvate Kinase: A potential indicator of calcium, level in citrus leaves and fruit	20
Fruit nutrient surveys	24
Pears and Apples	
Postharvest drenching in calcium	
Pears and Apples	27
Supplemental calcium application	28
Delay in Storage and fruit warming	31
Physiological studies	32
Significant findings	34
Tables and Figures	36
Quantative changes in RuBPCase under cation imbalance	70
Polypeptides separation by SDS-PAGE	74
Discussion	75
References	78
Figures	81
List of Publications	83

Summary

The BARD Project was undertaken to study some biochemical and molecular methods for the detection of nutrient element imbalance between K^+ , Mg^{+2} and Ca^{+2} which have been associated with some fruit blemishes. The Israeli team concentrated on citrus while the U.S. collaborators devoted their research to pears and apples. *C. volkamariana* cuttings which had been grown in complete nutrient solutions were compared with cuttings grown in K^+ , Mg^{+2} and Ca^{+2} deficient nutrient solutions. The activity response of two enzyme systems was investigated under these conditions and the same methodology applied to field grown trees in both countries. The enzymes ribulose 1,6 di phosphate carboxylase (RuBPcase) and pyruvate Kinase (PK) response to various nutritional regims was investigated. Profiles anlayzed by PAGE-SDS were also conducted. RuBPcase activity in leaves of plants grown under the nutrient deficient regimes was lower than that found in leaves of control plants. Evaluation of the amount of this protein by absorbancy scan, showed a decrease of about a 1/3 in Ca^{+2} deficiency as compared to control, in which this protein comprises about 35% of the total soluble proteins.

When the amount of PuBPcase in the different nutrient regimes was compared by ELISA similar effect was noticed, and a slight reduction under K^+ and Mg^{+2} deficient plants. The PK activity in low Ca^{+2} flaveds or in citrus leaves grown under Ca^{+2} deficiency was high, as a result of reduced Ca concentration which is an inhibitor of this enzyme. Deprivation of Ca^{+2} from the nurtrient solution increased the accumulation of 23Kd and 32Kd polypeptides. This accumulation was not induced by K^+ , Mg^{+2} deficiencies. The increased appearance of those polypeptides was also noticied in pears with cork spot (CS). Leaves analysis of the trees bearing those fruits showed Ca^{+2} deficiency. The results of the present research indicated that changes in enzymes activity and protein patterns exists with relation to three major cations K^+ , Mg^{+2} and Ca^{+2} imbalance that affect fruit quality and therefore should be followed from the early stages of the fruit development.

Introduction

Many of the physiological disorders affecting leaves and fruits have been associated with mineral nutrition problems. Most of them are related to the Ca^{+2} content of the tissues. Some of the best known calcium related disorders (CRD) are bitter pit in apples and cork spot in pears. They appear mostly during postharvest stage. In many other crop physiological disorders such as flavedo breakdown in citrus, blossom end rot in tomato, and green blotch in pears appear earlier in the preharvest stage.

Calcium (Ca) malnutrition may develop, either from Ca^{+2} deficiency, inefficient distribution or from impaired balance between the major cations (K^{+} , Mg^{+2} and Ca^{+2}) in plant tissue. In citrus fruits, either low or high K/Ca ratios may cause physiological disorders.

In some cases a high nitrogen to calcium ration (N/Ca) and any excess of N increases the need for Ca and may cause the problem. Nitrogen in the nitrate increase Ca-oxalate formation which is physiologically inactive. Ammonia (NH_4^{+}) ions on the other may interfere with Ca uptake. Nonconventional forms of N (such as glutamate can possibly improve Ca metabolism by regulating Ca availability in the fruit tissue while partially satisfying the nitrogen needs of the plant. Some evidence has shown the oxalic acid itself may be the causative agent of some CRD and the role of calcium is to eliminate the destructive effects of oxalic acid.

If a fruit quality-oriented nutritional program is to be successful, a reliable diagnostic method is essential. Leaf analysis which has been widely used as a diagnostic device in fruit orchards faces some difficulties with regard to fruit disorders as leaves do not necessarily reflect physiological levels of the mineral needed for fruit tissues. Furthermore, mineral analysis measure the total concentration of the nutrients and not the physiologically

active concentration (calcium may be in the form of calcium oxalate which can be inactive metabolically). The determination of the balance between Ca, K and Mg is subject to empirical evaluation.

The introduction of fruit analysis instead of foliar analysis in applies has improved forecast of CRD incidence in storage, but it has not yet been proved to be a reliable method for necessary corrective measures to be taken in the orchard in the early stage of the fruit development, or for programming fruit storage times. This prognosis is of special importance with respect to blemishes which are not apparent during the preharvest stage, but show up either in storage or in the packed box.

The biological approach which advocates the measurements of metabolic processes, like enzyme activities that are directly affected by mineral elements may be a more accurate measure to fruit quality. The use of enzyme activities have been useful in distinguishing between the active and inactive pools of nutrients in plant tissue. This approach is suggested for the prediction of potential Ca related disorders in fruit tissue caused by imbalances between K^+ Mg^{+2} and Ca^{+2} .

The experimental application to diagnosis of cation imbalance were the measurement of the activity and the amount of RuBPCase including serological analysis. This protein which is the most abundant protein in leaves comprise about 35% of the soluble protein in citrus leaves. Another enzymatic approach was by studying the effects of Ca^{++} , Mg^{++} and K^+ on PK activity in leaves and fruit tissues.

The effect of K^+ , Mg^{+2} and Ca^{+2} deficiencies on citrus as reflected in the soluble protein profile was also studied. Total soluble proteins from leaves of *Citrus volkamenaria* Pasq. grown in nutrient solutions were extracted and fractionated by PAGE SDS (Laemmli 1970; Salomon 1978, 1989). In leaves of plants

grown under K^+ , Mg^{+2} and Ca^{+2} deficient regimes the large subunit of PuBPcase was reduced. The most significant reduction was evident in Ca^{+2} deficient leaves. On the other hand two polypeptides of 31Kd and 23Kd accumulated in Ca^{2+} deficient leaves. Their appearance was associated with deprivation of Ca. They did not appear in K^+ or Mg^{+2} deficient leaves. In addition, a very low molecular weight polypeptide (12 Kd) also was found in Ca^{+2} deficient leaf extracts. When the PAGE SDS separation was utilized to analyze pear soluble proteins a similar increase in the 31Kd polypeptide was noticed in CS fruits. The results obtained in CS affected pear support the results from citrus leaves.

Pyruvate Kinase Activity: Dependence on K^+ , Mg^{+2} and Ca^{++} in citrus extracts, leave and fruit.

In plant kingdom a variety of physiological processes are regulated by changes in the tissue concentration of K^+ , Mg^{2+} and Ca^{2+} . Many enzymes are specifically activated by these inorganic cations (Williams 1970). Among them pyruvate kinase is known to require both K^+ and Mg^{2+} for activation (Boyer 1962, Besford 1978) and is known to be inhibited by Ca^{+2} (Tomlinson and Turner 1973). This enzyme catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP and is a key enzyme in the glycolytic pathway. There is a growing interest in the study of pyruvate kinase as a sensitive indicator to the concentration of K^+ and Mg^{2+} in plants (Besford 1975, Outlow and Lowry 1979). Ca^{2+} is an effective inhibitor of PK, new studies have been conducted taking into account the concentration of Ca^{2+} present in the assayed tissue. Because of Ca^{2+} inhibition, PK may respond differently to K^+ and Mg^{2+} presented in optimal quantities. Bar Akiva et al. (1976) have shown in a preliminary communication that deficiencies of Mg^{2+} and K^+ decreased PK activity in Eureka lemon leaves and Ca^{2+} deficiency caused an increase of the enzyme activity. Many

physiological disorders affecting storage organs such as bitter pit in apples are related to the Ca^{2+} distribution (Shear 1975). Recently, more attention has been given to the ratios of potassium and magnesium to calcium which are more closely related to the bitter pit development than the effect of cation separately. The present study compares in detail the combined effect of K^+ , Mg^{2+} and Ca^{2+} on PK activity and whether the reduced or enhanced activities in citrus leaves or apple and pear, subjected to different nutrient deficiencies results in any alteration of enzyme activity.

Material and methods

Plant material

Rough Lemon (*Citrus Volkameriana* Pasq.) plants from cuttings were grown in a modified aerated full nutrient solutions as described by Hewitt (1966), containing 115.5 ppm N, 24.7 ppm P, 46.0 ppm k, 141 ppm Ca, 21.2 ppm Mg, 28.2 ppm S and 18.4 ppm Na. Plants were transferred to deficient regimes after 4-6 weeks. When Ca was omitted, it was replaced by an equivalent amount of Mg when Mg was omitted, it was replaced by K and when K was omitted it was replaced by Mg. Plants were grown in deficient regimes for at least 6 weeks until deficiency symptoms were observed.

Enzyme preparation and assay

Pyruvate kinase was extracted from mature leaves. Leaves were deribbed and cut into small pieces. Four grams of leaf pieces were homogenized in a cold mortar and pestle with 20 ml grinding medium consisted of 10 mM imidazole - HCl buffer pH 7.0 containing 20% (v/v) glycerol and 2 mM DTT with small amount of acid washed sand and 1g of insoluble PVP (Ireland et al. 1974). The extracts were filtered through Miracloth and centrifuged at 37000xg for 20 min. The resulting supernatant was taken for crude enzyme assay. For cation activation studies, K^+ ,

Mg^{2+} and Ca^{2+} were removed from crude enzyme preparations by gel filtration on a column (1x10cm) of Sephadex G-75. seven ml of the supernatant were eluted through the column. The buffer used for equilibrating the column and eluting proteins was the same buffer used as a grinding medium. K^{+} dependence curves were run in the presence of 5mM MgCl_2 and the concentrations of K^{+} were 0, 0.5, 10.0, 20.0, 40.0, 80.0 and 100 mM. Mg^{2+} dependence curves were run in the presence of 50mM KCl and the concentrations of Mg^{2+} were: 0, 0.5, 1.0, 2.5, 5.0 and 10.0 mM. Ca^{2+} dependence curves were conducted with 50mM KCl and 5mM MgCl_2 . The concentrations of CaCl_2 in the assays were 0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mM.

PK activity was assayed using the colorimetric method of Kachmar and Boyer 1953 determining ADP dependent formation of pyruvate from PEP. The standard reaction was performed in a final volume of 1.0 ml and contained in addition to the enzyme preparation, 20mM Imidazole-HCl buffer with 25 μM Na_2MoO_4 adjusted to pH 7.0, 0.5 mM PEP, 2mM ADP, 50 mM KCl and 5mM MgCl_2 . Reactions were started by the addition of enzyme and ADP, and carried out for 15min at 37°C and terminated by the addition of 1 ml 0.0125% 2,4-dinitrophenyl- hydrazine in 2N HCl. For each assay an assay without ADP was included and subtracted from the activity with ADP to correct for unspecific phosphatase activity. Enzyme blanks contained boiled enzyme or reaction mixtures without PEP. Pyruvate was determined after further incubation for 15 min at 37°C and after adding 2 ml of 2M NaOH.

Precipitates of proteins or $\text{Mg}(\text{OH})_2$ were removed by centrifugation. PK activity was expressed as nmol pyruvate formed per μg protein per 15 min. Protein was determined according to Lowry et al. (1951) with BSA as a standard.

Concentrations of K^{+} , Mg^{2+} and Ca^{2+} in plant extracts were analysed by atomic absorption analyser.

Results

Effect of molybdate on inhibition of phosphatases

Molybdate was found to inhibit effectively the activity of phosphatases (Callagher et al. 1982). Pyruvate kinase in crude extracts of control leaves in the presence of K^+ and Mg^{2+} was only slightly affected by 25 μ M molybdate (2% inhibition) but the reaction in the absence of ADP which is considered as phosphatase activity was strongly inhibited (56% inhibition) (Table 1). When external cations were not added to the reaction mixture the activity of PK was very low because as molybdate inhibited the reactions in the presence or absence of ADP almost to the same level.

PK activity as a function of pH

The pH curves of fractions eluted from Sephadex G-75 column are shown in figure 1. PK activity was measured over a pH range of 5.5-9.0 in three buffers systems; Tris HCl, pH 7.0-, 9.0, Imidazole HCl pH 6.0-8.0 and Maleate NaOH pH 5.5-7.0. Activity was measured in the presence of 50 mM KCl, 5 mM $MgCl_2$ and 25 μ M Na_2MoO_4 . Activity was the highest at pH 7.0 in all the three buffer systems. In the absence of ADP the activity of unspecific phosphatases at pH 7.0 was very low (Fig. 2). On the other hand their activity increased markedly below or above pH 7.0, suggesting the presence of acid and alkaline phosphatases. Activity of PK from crude extracts over a pH range of 5.0-8.0 is shown in Figure 2. The optimal pH for crude extracts either from control, K^+ -deficient, Mg^{2+} -deficient and Ca^{2+} -deficient leaves was pH 7.0. Inhibition of activity became evident below and above this pH. The activities at pH 7.5 were lower from the optimal values by 12%, 24%, 36% and 23% for the control, K^+ -deficient, Mg^{2+} -deficient and Ca^{2+} deficient leaves, respectively.

Pyruvate kinase activity of crude extracts

When the activity of PK in crude extracts was dependent solely on endogenous cations it was very low in control and K^+ deficient leaves and dropped to zero in Mg^{2+} deficient leaves (Table 2). However, in Ca^{2+} deficient leaves it was significantly high and increased about 7-fold over that of the control. The addition of either K^+ or Mg^{2+} or both of them to the assay mixtures caused an increase in activity in all treatments (Table 2). The addition of Ca^{2+} was inhibitory (Figs 3,4,5,6). In the presence of added K^+ alone, stimulation of activity in K^+ deficient leaves was 4-fold while the activity of Mg^{2+} -deficient leaves was hardly stimulated. In the presence of added Mg^{2+} alone the activity of Mg^{2+} deficient leaves was highly stimulated while only slight stimulation was observed in K^+ deficient leaf extracts. Addition of both K^+ and Mg^{2+} brought about optimal activity reaching the highest value in Ca^{2+} -deficient leaf extracts, where it was 47% higher from the control leaf extracts.

Internal concentrations of K^+ , Mg^{2+} and Ca^{2+}

The concentrations of K^+ , Mg^{2+} and Ca^{2+} in the extracts were expressed in mM and varied according to the deficiency imposed (Table 3). Concentrations of Mg^{2+} and Ca^{2+} in K^+ deficient extracts were increased 2 and 3 fold, respectively, over the control. K^+ concentration was slightly reduced in Ca^{2+} deficient extracts but Mg^{2+} concentration was increased 2-fold. The concentration of these cations dropped to 0.1-0.3 mM in the various treatments after gel filtration.

Cation activation studies

PK activity of desalted extracts from control, K^+ -deficient, Mg^{2+} -deficient and Ca^{2+} -deficient leaf extracts were assayed in the presence of different concentrations of K^+ , Mg^{2+} and Ca^{2+} . Cation dependence curves show that PK

activity was dependent on the presence of both potassium and magnesium.

Exclusion of all cations following desalting procedure markedly reduced enzyme activity (Figs. 2,3,4,5,6). It is also obvious from these curves that the ADP

independent assays were not affected by different ion concentrations. Specific

activity was promoted by 5 and 10 mM KCl and reached its peak at 20mM KCl.

Higher concentrations of KCl (40, 80 and 100mM) affected only slightly PK

activity (Fig. 3). Magnesium dependence curves showed the highest promotion at

5.0 mM MgCl₂. Higher concentrations had only a slight effect. Calcium

dependence curves showed inhibition of activity over the entire range of Ca²⁺

concentrations. Inhibition by 0.2 mM Ca²⁺ was 5% and was increased up to 81% by

10mM Ca²⁺.

Conclusions

The results of the experiments with PK show that the differences in this enzyme activity are cation dependent.

Ca²⁺ inhibit significantly PK activity, while K⁺ and Mg²⁺ stimulate it.

Furthermore the elimination of all cations from the plant extracts show that the basic level of the enzyme is the same in leaves from citrus plants under various nutritional regimes.

PK activity as an indicator of Ca²⁺ concentration in leaves and fruits under field conditions was studied and reported in the following publication. Activity of PK was also examined in apple and pear fruits in cold storage. Fruit samples were harvested from problematic plots, from trees under various treatments and from post-harvest treated fruit (the U.S. collaborators report).

List of figures

Fig. 1. Effect of pH on the activity of PK in desalted fractions from control leaves of *C. Volkameriana*. Assays were performed with 20 mM Tris-HCl buffer, 20 mM Imidazole buffer and 20 mM Maleate buffer in the presence of 25 μ M Na_2MoO_4 , 50mM KCl and 5mM MgCl_2 .

Fig. 2. The influence of pH on the activity of PK from crude extracts in the presence of 20 mM Imidazole, 25 μ M Na_2MoO_4 , 50 mM KCl and 5 mM MgCl_2 . Control ($\bullet$), K^+ -deficient ($\square$), Mg^{2+} -deficient ($\circ$), and Ca^{2+} -deficient (Δ) leaves. Vertical bars denote SE of three replications.

Fig. 3. The influence of various concentrations of KCl, MgCl_2 and CaCl_2 on pyruvate kinase extracted from control leaves of *C. Volkameriana* after elution from Sephadex G-75 column. Experiments were repeated 3 times and representative values from one experiment are given.

Fig. 4. Legend as for Fig. 3 for K^+ deficient leaves.

Fig. 5. Legend as for Fig. 3 for Mg^{2+} -deficient leaves.

Fig. 6. Legend as for Fig. 3 for Ca^{2+} -deficient leaves.

Tab. 1. Effect of 25 μM Na_2MoO_4 on PK activity of *C. volkameriana* leaves in the presence or absence of ADP. Assays were performed in crude extracts of leaves grown in full nutrient solution. Activity is expressed as nmol pyruvate/ μg protein/ 15 min. Values represent the mean $\pm$ S.E. (n=3).

Assay	Specific activity				% inhibition by Mo	
	- uo		+ uo		+ ADP	- ADP
	+ ADP	- ADP	+ ADP	- ADP		
K^+	0.82 $\pm$ 0.08	0.60 $\pm$ 0.11	0.38 $\pm$ 0.02	0.12 $\pm$ 0.01	54	80
Mg^{2+}	0.90 $\pm$ 0.12	0.47 $\pm$ 0.08	0.74 $\pm$ 0.12	0.31 $\pm$ 0.01	18	34
$\text{K}^+-\text{Mg}^{2+}$	1.13 $\pm$ 0.12	0.50 $\pm$ 0.09	1.11 $\pm$ 0.11	0.22 $\pm$ 0.01	2	56
no added cations	0.84 $\pm$ 0.11	0.79 $\pm$ 0.16	0.23 $\pm$ 0.01	0.14 $\pm$ 0.02	73	82

Tab. 2. Specific activity of PK in crude extracts of *C. volkameriana*. Activities were analysed with no added cations and in the presence of K^+ , Mg^{2+} or $K^+ Mg^{2+}$. Activity is expressed as nmol pyruvate/ug protein/15 min. Values represent the mean $\pm$ S.E. (n=7). Values followed by different letters are significantly different ($P < 0.05$).

Nutrient solution	no added cations	KCl (50mM)	$MgCl_2$ (5mM)	KCl (50mM) $MgCl_2$ (5mM)
Control	$0.077 \pm 0.027_b$	0.283 ± 0.029^b	0.620 ± 0.065^b	0.787 ± 0.081^{bc}
K^+ -def.	0.066 ± 0.019^b	0.251 ± 0.013^b	0.144 ± 0.030^c	0.683 ± 0.050^c
Mg^{2+} -def.	0 ± 0	0.033 ± 0.028^c	0.754 ± 0.047^{ab}	0.899 ± 0.038^b
Ca^{2+} -def.	0.541 ± 0.055^a	1.040 ± 0.104^a	0.781 ± 0.067^b	1.154 ± 0.105^a

Tab. 3. Contents of K^+ , Mg^{2+} and Ca^{2+} in crude and desalted extracts of *C. volkameriana* leaves. Values represent the mean $\pm$ S.E. (n=3). Values followed by different letters are significantly different ($P < 0.05$).

Treatment	Crude extracts			Desalted extracts		
	K^+	Mg^{2+}	Ca^{2+}	K^+	Mg^{2+}	Ca^{2+}
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)
Control	23.6 $\pm$ 1.19 ^b	6.2 $\pm$ 0.34 ^b	9.3 $\pm$ 1.93 ^b	0.1 $\pm$ 0.02	0.2 $\pm$ 0.02	0.2 $\pm$ 0.06
K^+ -def.	9.1 $\pm$ 0.29 ^b	13.0 $\pm$ 13.8 ^a	30.5 $\pm$ 2.99 ^a	0.1 $\pm$ 0.04	0.2 $\pm$ 0.04	0.2 $\pm$ 0.03
Mg^{2+} -def.	30.0 $\pm$ 3.30 ^a	0.6 $\pm$ 0.04 ^c	8.3 $\pm$ 0.23 ^{bc}	0.2 $\pm$ 0.02	0.1 $\pm$ 0.04	0.2 $\pm$ 0.03
Ca^{2+} -def	19.3 $\pm$ 0.45 ^a	15.4 $\pm$ 1.31 ^a	2.5 $\pm$ 0.23 ^c	0.2 $\pm$ 0.07	0.2 $\pm$ 0.05	0.2 $\pm$ 0.05

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

List of Publications Resulting from this project

Ruth Lavon, Raphael Salomon and Eliezer E. Goldschmidt (1988) Pyruvate Kinase: A potential indicator of calcium level in citrus leaves and fruit, Proceeding of International Society of citriculture. Vol. 1:541-545.

Ruth Lavon (1991). Disorders resulting from K^+ , Mg^{+2} and Ca^{+2} imbalance in citrus. Ph.D. Thesis in preparation to be submitted to the Hebrew University, Jerusalem.