ROOT ORGANIC ACID EXUDATION AS A PHYSIOLOGICAL CAUSE FOR SOIL PHOSPHORUS DEFICIENCY TOLERANCE IN *VIGNA UNGUICULATA* (L.) WALP (COWPEA) LINES

A Senior Scholars Thesis

by

PIERCE GREGORY YOUNG

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Plant and Environmental Soil Science

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Approved by:

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Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Root Organic Acid Exudation As A Physiological Cause For Soil Phosphorus Deficiency Tolerance In *Vigna Unguiculata* (L.) Walp (Cowpea) Lines. (May 2012)

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Phosphorus (P) fertilizers are not readily available in underdeveloped countries, and excessive P application can lead to problems such as eutrophication. The nonrenewable nature of P, mined for use in fertilizers, raises concerns that P fertilizers may become more expensive and less accessible. The objective of this study was to measure root organic exudation in *Vigna unguiculata* (L.) Walp. (cowpea) in response to soil P deficiency. Eight cowpea lines were planted in coarse silica sand and given three treatments: a nutrient solution with P, a nutrient solution without P, and a nutrient solution without P but with rock phosphate included in the sand. It is suspected that organic acid exudation will increase in plants treated with solutions without P. Six organic acids were identified in the root exudates samples, but no difference was observed in the type of organic acids exuded among different cowpea lines. Succinic acid and maleic acid were identified as the two most likely candidates for exudation as a physiological response to low soil P conditions. Lines that have been found to exhibit

increased soil P deficiency tolerance could be exuding larger quantities of these organic acids than lines that exhibit low or moderate soil P deficiency tolerance.

DEDICATION

I would like to dedicate this to my parents, John and Diane Young. All of your love and support have made this possible.

ACKNOWLEDGMENTS

First of all, I would like to thank Dr. Dirk Hays for acting as my advisor. I had worked in Dr. Hays's lab for a couple of years before he convinced me to apply to the Undergraduate Research Scholars program. This has been an invaluable experience, and only in the years to come will I be able to understand the full benefits. I am grateful for the experience both as a student worker as an undergraduate researcher.

I would also like to thank Julie Rothe for her guidance and assistance throughout this project. She acted as a mentor for the duration of the project especially in the absence of Dr. Hays during the spring semester. Julie took the time to ensure that I was progressing with the experiment and to edit my thesis throughout the semester.

Dr. Linda Dykes also deserves recognition for her assistance with the UPLC portion of the experiment. Her knowledge of the machine was an integral portion of the method development process. I would have been unable to proceed without her expertise.

NOMENCLATURE

ATP	Adenosine triphosphate
DAP	Days after planting
DNA	Deoxyribonucleic acid
P	Phosphorus
P^+	Treatment with phosphorus
P^-	Treatment without phosphorus
PO_4^{2-}	Phosphate
RNA	Ribonucleic acid
RP	Treatment with rock phosphate
UPLC	Ultra performance liquid chromatography

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CHAPTER I

INTRODUCTION

Vigna unguiculata (L.) Walp. (cowpea), an annual warm weather legume, is also referred to as black-eyed pea, cream pea, and crowder pea. Similar to most legumes, it exhibits a symbiotic relationship with *Rhizobium* bacteria. The *Rhizobium* reside in root nodules of cowpea and fix atmospheric nitrogen, which is unusable by the plant, into ammonium which is usable by the plant. As a result, little nitrogen fertilizer is required for cowpea growth. Cowpea is generally drought tolerant and shade tolerant. It also performs well in a wide variety of soils and soil conditions, although it performs best in well-drained sandy loams at a pH of 5.5 to 6.5.

The leaves and seeds of cowpea are edible. The seeds are high in protein, especially the amino acids lysine and tryptophan. However, the seeds are relatively deficient in the amino acids methionine and cystine when compared to animal meat. Cowpea can therefore act as a nutrient supplement to cereal crops and an extension animal protein. Fresh green seeds can be boiled, canned or frozen. Dry mature seeds can be boiled and canned. In addition to human consumption, cowpea is also often used as fodder for livestock.

This thesis follows the style of *Journal of Experimental Botany*.

Cowpea is commonly grown in Africa and Brazil and to a lesser extent in the United States. Phosphorus fertilizers are affordable and accessible in the United States, but farmers in Africa have less access to phosphorus fertilizers. The research conducted for this thesis is intended to primarily benefit farmers in developing areas such as Africa where fertilizers are less accessible. However, this research can benefit cowpea farmers globally. By understanding the nature of phosphorus deficiency tolerance in cowpeas, breeders can more effectively produce cowpea cultivars that require less phosphorus. Farmers can then produce crops using less phosphorus fertilizer.

All plants require phosphorus (P) for proper growth and development. P is classified as a macronutrient and composes approximately 0.2% of plant dry weight. P is an integral component of many cellular components: phospholipids that make up the plant cell membranes, nucleotides that are involved in energy transfer such as ATP, and in the nucleotides that make up DNA and RNA. P deficiency results in stunted growth, dark coloration of the leaves, and necrotic spots.

Plants acquire P from the soil. The pH of the soil plays a large role in determining the availability of the P. In acidic soils, phosphate ions become adsorbed to the insoluble oxides of iron, aluminum, and manganese and become unavailable for plant uptake. In alkaline, calcareous soils, soluble $H_2PO_4^-$ ions react with calcium ions to produce monocalcium phosphate. The monocalcium phosphate reacts with calcium carbonate to produce first dicalcium phosphate then tricalcium phosphate thereby decreasing the

solubility and plant availability of the P. The range of lowest phosphate fixation and highest plant availability occurs at a pH of about 6.0 to 7.0.

P is a non-renewable resource and must be mined for use in fertilizers. Apatite found in rock phosphate deposits serves as the primary source for P fertilizers. Apatite is treated with strong acids to yield plant available forms of P.

Excess P fertilizer applied to the soil can become runoff that pollutes bodies of water. The sudden availability of P allows microorganisms in the water to rapidly proliferate in a process known as eutrophication. The algal blooms that arise as a result of eutrophication can kill fish living in the water by removing oxygen from the water.

Plants employ a variety of techniques to acquire P from P deficient soils. White lupin (*Lupinus albus*) has been shown to enhance P mobilization by root exudates (Wang *et al.*, 2008). White lupin exudes more malic acid, citric acid, and succinic acid in soils with deficient amounts of P than in soils with sufficient amounts of P (Johnson *et al.*, 1996). Wheat (*Triticum aestivum*) increases the root-to-shoot ratio, and cotton (*Gossypium hirsutum*) increases phosphatase activity in the rhizosphere (Wang *et al.*, 2008). Many plants engage in a symbiotic relationship with certain types of fungi to create mycorrhizae that act as extensions to the plant roots. The mycorrhizae increase the root surface area allowing for increased P acquisition.

This research project is an ongoing study at Texas A&M University to understand the physiological response of cowpea to soil P deficiency. As mentioned previously, plants employ a wide variety of physiological methods to increase P acquisition. Prior study has indicated that organic acid exudation from the roots is a likely method utilized by cowpea. Organic acids can decrease the pH of the soil in the rhizosphere. By decreasing the pH of the soil, insoluble tricalcium phosphate can be converted to more soluble monocalcium phosphate. Prior to this study, it was unknown which organic acids cowpea exudes. The organic acid exudation data for individual cowpea lines can be compared to soil phosphorus deficiency tolerance data that has been previously collected for the same lines.

CHAPTER II

METHODS

Eight cowpea lines were selected for analysis: IT98K-476-8, IT98K-1092-1, TX2028-1-3-1, Aloka, CB-46, Dan Ila, GEC, and Big John. The plants were grown in a greenhouse with a 12 hour/ 12 hour day/ night cycle. Supplemental lighting was applied if the photon flux density fell below 700 W/m^2. Daytime temperatures were kept between 28 ^oC and 31 ^oC, and nighttime temperatures were kept between 26 ^oC and 28 ^oC. Three treatments were applied to each line: plants grown with phosphorus (P^+), plant grown without phosphorus (P^-), and plants grown with rock phosphate (RP) as the only source of applied phosphorus. Three repetitions were completed for each treatment for a total of seventy-two pots prepared. Pots were watered every two to three days with the appropriate nutrient solution. The P^+ solution, adapted from Johnson et al. (1994), contained 3.0 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 0.5 mM $Ca(H_2PO_4)_2$, 1.0 mM $MgSO_4$, 12.0 μM FeEDTA, 4.0 μM $MnCl_2$, 22.0 μM H_3BO_3, 0.4 μM $ZnSO_4$, 0.05 μM $NaMoO_4$, and 1.6 μM $CuSO_4$. The P^- solution did not contain the 0.5 mM $Ca(H_2PO_4)_2$. The pH was adjusted to 5.5 for both solutions. The P^+-treated plants received the P^+ solution. The P^- and RP-treated plant received the P^- solution.

Pot preparation

Four-inch pots were each filled with 1.0 kg of silica sand. To prevent loss of sand, heavy duty solar screen (SunTex 90, Phifer, Inc., Tuscaloosa, AL) was cut into approximately

35 cm x 35 cm pieces and placed into each pot. For the RP treatment, 0.65 g of RP was added to 1.0 kg of silica sand. Mixing was done in a gallon size Ziploc bag with a small amount of water. Two seeds with *Rhizobium* were added to each pot, and the pots were thinned to one plant per pot shortly after emergence.

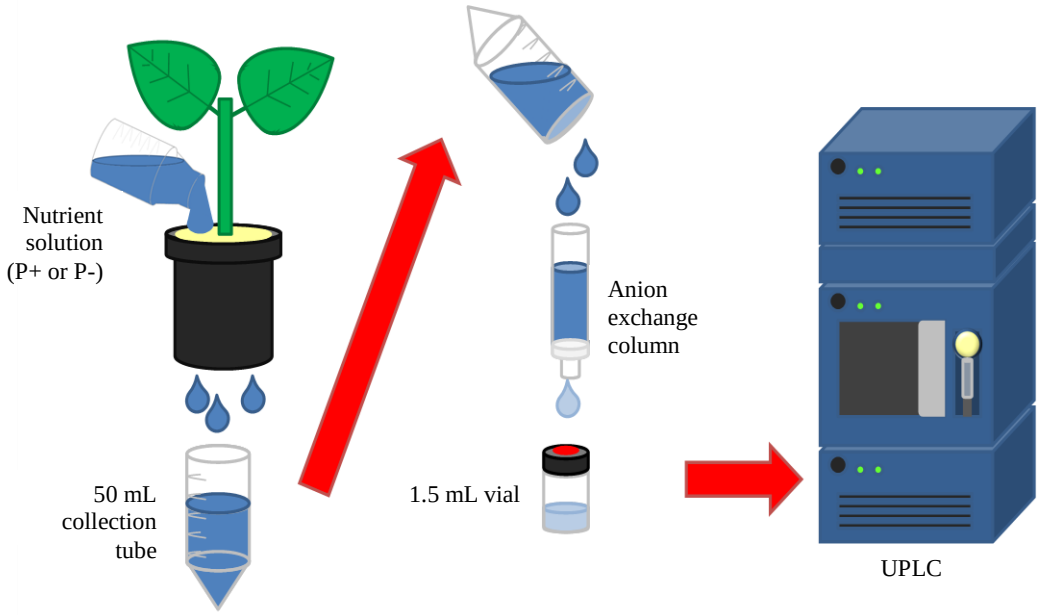

Fig 1. Schematic of the methods. The plants are watered with the appropriate nutrient solution, and the eluate was collected in 50 mL tubes. The organic acids were separated from the rest of the eluate using anion exchange chromatography. The resulting sample was analyzed using UPLC.

Sample Collection

The eluate from watering the pots was collected for analysis at 10, 12, 14, 16, 19, 21, 28 and 35 DAP. To collect the eluate, each pot was propped above a 6 inch diameter weigh boat. Each pot was then watered with approximately 175 mL of the appropriate nutrient solution. The eluate was divided in half between two 50 mL sample tubes for two storage methods. One tube of each sample was stored at room temperature at 25 ^{o}C, and the other tube was stored at -20 ^{o}C.

Analysis of organic acids

The organic acids were separated from the eluate using strong anion exchange chromatography (Johnson et al., 1996). The column (HyperSep SAX, Thermo Scientific, Waltham, MA) was pre-conditioned with 2 mL of 100% methanol (MeOH) followed by 4 mL of 50% ethanol (EtOH). The entire sample was then loaded onto the column. The column was then washed with 4 mL of 50% EtOH (basic and neutral fractions). The acid fraction was collected by eluting the column with 3 mL of glacial acetic acid. Samples were then placed in auto evaporator (AES2000 with vapor net, Savant Instruments, Inc., Farmingdale, NY) to dry.

The organic acids were separated using an Acquity UPLC equipped with a binary solvent manager and a sample manager (Waters Corporation, Milford, MA). The separation of organic acids was performed using a bonded phase column (Acquity UPLC HSS C18 Column, 2.1 mm X 100 mm, 1.8 μm, Waters Corporation, Milford, MA). The

column was equipped with a VanGuard Pre-Column (Waters Corporation, Milford, MA). The injection volume was 5 μL with a flow rate of 0.4 ml/min. The A solvent was 0.05% formic acid in LCMS grade water, and the B solvent was 100% LCMS grade methanol. The solvent gradient was as follows: 0-0.2 min, 5% B; 0.2-1.0 min, 5-90% B; 1.0-1.5 min, 90% B; 1.5-1.8 min, 90-5% B; 1.8-4.8 min, 5% B. Column temperature was 45 ^{o}C. The instrument was operated in negative ionization mode. The detector was a tandem quadrupole detector (TQD) mass spectrometer. Capillary voltage was 3.0 kV. Source temperature was 150 ^{o}C. Desolvation temperature was 400 ^{o}C. Desolvation gas flow was 800 L/hr. Cone gas flow was 50 L/hr. The collision gas was argon (AOC, Bryan, TX).

Ten organic acids were selected for observation: pyruvic acid, oxalic acid, L-(+)-lactic acid, malonic acid, maleic acid, succinic acid, DL-malic acid, L-(+)-tartaric acid, *trans*-aconitic acid, and citric acid. Standards of each organic acid were used to determine the appropriate collision energy and cone voltage for detection. Quantification of the organic acids is not a current concern of the project, and so standard curves were not created. Instead the presence or absence of each organic acid was determined.

CHAPTER III

RESULTS

Samples were analyzed for the presence of organic acids. An emphasis was placed on identification rather than quantification. In other words, the UPLC machine was used to identify the various organic acids, but no standard curves were developed for quantification.

Succinic acid was detected the most frequently and gave a strong signal for nearly all of the samples. Maleic acid was frequently detected, but the signal strength was sometimes weak. L-(+)-lactic acid gave a weak signal for several samples on all days except for 12 DAP where no lactic acid was detected. *Trans*-aconitic acid was detected and gave strong signals in many of the samples on 12 DAP, but it was not detected on any other dates. Malic acid was occasionally detected and gave a strong signal for CB-46 P-A on 21 DAP and for IT98K-1092-1 on 21 DAP. However, signal strength is not an accurate means to quantify and compare different compounds. Pyruvic acid, oxalic acid, L-(+)-tartaric acid, and citric acid were never detected.

Tables 1, 2, 3, 4, and 5 show the results for 12, 14, 16, 19, and 21 DAP, respectively. The results listed are incomplete as the experiment is still currently in progress.

Table 1. Organic acids detected in samples collected on 12 DAP

Line	**Sample**	**Organic acid detected**					
		L-(+)-lactic acid	Malonic acid	Maleic acid	Succinic acid	DL-malic acid	Trans-aconitic acid
Big John	RPA		X	X	X	X	
Big John	P-A			X	X		
Big John	P+A		X	X	X	X	X
IT98K-476-8	RPA		X	X	X		X
IT98K-476-8	P-A		X		X		
IT98K-476-8	P+A		X	X	X	X	X
TX2028-1-3-1	RPA		X		X		
TX2028-1-3-1	P-A		X	X	X		X
TX2028-1-3-1	P+A				X	X	X
CB-46	RPA		X	X	X		X
CB-46	P-A				X		X
CB-46	P+A		X	X	X	X	X
Dan Ila	RPA			X	X		X
Dan Ila	P-A				X		X
Dan Ila	P+A		X		X		
IT98K-1092-1	RPA				X		X
IT98K-1092-1	P-A				X		X
IT98K-1092-1	P+A				X	X	
GEC	RPA			X	X		
GEC	P-A			X	X		X
GEC	P+A		X	X	X	X	X
Aloka	RPA			X	X	X	X
Aloka	P-A				X	X	X
Aloka	P+A		X		X	X	X

The presence of an organic acid is indicated with an "X." If a given organic acid was not detected for a sample, the corresponding cell was left blank.

Table 2. Organic acids detected in samples collected on 14 DAP

Line	**Sample**	**Organic acid detected**					
		L-(+)-lactic acid	Malonic acid	Maleic acid	Succinic acid	DL-malic acid	Trans-aconitic acid
Big John	RPA	X	X	X	X		
Big John	P-A	X	X	X	X	X	
Big John	P+A	X	X	X	X	X	
IT98K-476-8	RPA	X		X	X		
IT98K-476-8	P-A	X	X	X	X		
IT98K-476-8	P+A			X	X	X	
TX2028-1-3-1	RPA	X	X	X	X	X	
TX2028-1-3-1	P-A	X	X	X	X		
TX2028-1-3-1	P+A		X	X	X	X	
CB-46	RPA		X	X	X	X	
CB-46	P-A	X	X	X	X		
CB-46	P+A	X	X		X		
Dan Ila	RPA	X	X	X	X	X	
Dan Ila	P-A				X		
Dan Ila	P+A		X	X	X	X	
IT98K-1092-1	RPA	X	X	X	X	X	
IT98K-1092-1	P-A			X	X		
IT98K-1092-1	P+A			X	X	X	
GEC	RPA		X	X	X		
GEC	P-A			X	X		
GEC	P+A			X	X	X	
Aloka	RPA		X	X	X		
Aloka	P-A				X		
Aloka	P+A	X		X	X	X	

The presence of an organic acid is indicated with an “X.” If a given organic acid was not detected for a sample, the corresponding cell was left blank.

Table 3. Organic acids detected in samples collected on 16 DAP

Line	Sample	Organic acid detected					
		L-(+)-lactic acid	Malonic acid	Maleic acid	Succinic acid	DL-malic acid	Trans-aconitic acid
Big John	RPA		X	X	X	X	
Big John	P-A	X		X	X		
Big John	P+A	X	X	X	X	X	
IT98K-476-8	RPA	X		X	X	X	
IT98K-476-8	P-A	X		X	X		
IT98K-476-8	P+A			X	X	X	
TX2028-1-3-1	RPA			X	X		
TX2028-1-3-1	P-A	X		X	X		
TX2028-1-3-1	P+A	X	X	X	X	X	
CB-46	RPA	X	X	X	X		
CB-46	P-A			X	X		
CB-46	P+A		X	X	X		
Dan Ila	RPA	X		X	X	X	
Dan Ila	P-A	X		X	X		
Dan Ila	P+A	X		X	X	X	
IT98K-1092-1	RPA	X		X	X		
IT98K-1092-1	P-A		X	X	X		
IT98K-1092-1	P+A		X	X	X		
GEC	RPA	X		X	X		
GEC	P-A	X		X	X		
GEC	P+A		X	X	X	X	
Aloka	RPA			X	X		
Aloka	P-A	X		X	X		
Aloka	P+A	X		X	X		

The presence of an organic acid is indicated with an “X.” If a given organic acid was not detected for a sample, the corresponding cell was left blank.

Table 4. Organic acids detected in samples collected on 19 DAP

Line	Sample	Organic acid detected					
		L-(+)-lactic acid	Malonic acid	Maleic acid	Succinic acid	DL-malic acid	Trans-aconitic acid
Big John	RPA	X	X	X	X	X	
Big John	P-A	X	X	X	X	X	
Big John	P+A	X		X	X		
IT98K-476-8	RPA		X	X	X		
IT98K-476-8	P-A		X	X	X		
IT98K-476-8	P+A		X		X		
TX2028-1-3-1	RPA	X	X	X	X		
TX2028-1-3-1	P-A	X		X	X		
TX2028-1-3-1	P+A	X	X	X	X	X	
CB-46	RPA	X	X	X	X		
CB-46	P-A	X	X	X	X	X	
CB-46	P+A				X		
Dan Ila	RPA	X	X	X	X	X	
Dan Ila	P-A	X	X	X	X		
Dan Ila	P+A					X	
IT98K-1092-1	RPA		X		X		
IT98K-1092-1	P-A	X		X	X		
IT98K-1092-1	P+A	X	X	X	X		
GEC	RPA		X	X	X	X	
GEC	P-A		X	X	X		
GEC	P+A	X		X	X	X	
Aloka	RPA	X	X	X	X		
Aloka	P-A	X	X	X	X	X	
Aloka	P+A	X		X	X	X	

The presence of an organic acid is indicated with an “X.” If a given organic acid was not detected for a sample, the corresponding cell was left blank.

Table 5. Organic acids detected in samples collected on 21 DAP

Line	Sample	Organic acid detected					
		L-(+)-lactic acid	Malonic acid	Maleic acid	Succinic acid	DL-malic acid	Trans-aconitic acid
Big John	RPA			X	X		
Big John	P-A			X	X		
Big John	P+A	X		X	X		
IT98K-476-8	RPA			X	X		
IT98K-476-8	P-A			X	X		
IT98K-476-8	P+A		X	X	X		
TX2028-1-3-1	RPA		X	X	X		
TX2028-1-3-1	P-A			X	X		
TX2028-1-3-1	P+A		X	X	X		
CB-46	RPA		X	X	X		
CB-46	P-A		X	X	X	X	
CB-46	P+A	X	X	X	X	X	
Dan Ila	RPA			X	X		
Dan Ila	P-A		X	X	X		
Dan Ila	P+A		X	X	X		
IT98K-1092-1	RPA			X	X	X	
IT98K-1092-1	P-A		X	X	X		
IT98K-1092-1	P+B			X	X		
GEC	P-A	X	X	X	X		
GEC	P+C	X	X	X	X	X	
GEC	P+A			X	X		
Aloka	P+A		X	X	X		
Aloka	RPA	X	X	X	X	X	

The presence of an organic acid is indicated with an “X.” If a given organic acid was not detected for a sample, the corresponding cell was left blank.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Succinic acid and maleic acid were the most frequently detected organic acids. In general, succinic acid gave stronger signal peaks than maleic acid, but standard curves need to be generated to quantitatively compare these compounds. A total of six organic acids were identified. In addition to succinic acid and maleic acid, L-(+)-lactic acid, malonic acid, DL-malic acid, and *trans*-aconitic acid were detected. Of these, only *trans*-aconitic acid gave strong signals, but *trans*-aconitic acid was only detected 12 DAP.

Standard curves need to be generated before any further insights can be made. Given the current trend, succinic acid and maleic acid are the most likely candidates of an organic acid physiological response in cowpea to low soil P conditions. It is possible that the quantity of one or more of these organic acids that cowpea exudes could vary from line to line. More specifically, lines that have been found to exhibit increased soil P deficiency tolerance could potentially exude more organic acids than lines that exhibit low or moderate soil P deficiency tolerance.

The addition of a P gradient among pots in the applied nutrient solutions could also provide more insight. By varying the amount of P applied to plants in a range from sufficient to deficient, clearer evidence for P deficiency tolerance could be observed, and a critical P level for cowpea plants could be determined. This tolerance would be evident

in biomass data as well as in root organic acid exudation data. When a critical P level is identified for each line, this data could be useful for application of P fertilizers.

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