UNIVERSITI PUTRA MALAYSIA

IN VITRO SELECTION IN WHEAT TO RECOVER MUTANTS WHICH OVERPRODUCE LYSINE AND THREONINE

MIHDZAR ABDUL KADIR

IN VITRO SELECTION IN WHEAT TO RECOVER MUTANTS WHICH OVERPRODUCE LYSINE AND THREONINE

By

MIHDZAR ABDUL KADIR

Bachelor of Science
Louisiana State University
Baton Rouge, Louisana
1980

Master of Science
Louisiana State University
Baton Rouge, Louisiana
1982

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfilment of
the requirement for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1991

ACKNOWLEDGEMENTS

I wish to express my deep appreciation to my major adviser, Dr. Arron C. Guenzi for his skillful guidance, invaluable advice and help throughout the course of my study. Appreciation is also extended to Dr. Charles M. Taliaferro, Dr. Becky C. Johnson and Dr. Robert A. Gonzales for their time, helpful suggestions and assistance as members of my graduate committee. I would also like to thank Dr. David Porter for his expertise in electrophoresis and Dr. Mike Anderson and Dr. Bjorn Martin for the use of their research equipment.

I would like to thanks my mother Rugaiyah, and brother Abdul Aziz for their encourgement, my beloved wife Aminah for her continued support and assistance, my daughter Farahiyah and my son Wafiq for their patience and understanding.

My special thanks are also extended to my firends Troy, Mark, John, Don, Jackie, Dave, Mohammad, Hirut and Kristi for their assistance.

Finally I wish to thanks my sponsors, University of Agriculture Malaysia, Public Service Department of Malaysia and Oklahoma State University for their financial support during my study.

TABLE OF CONTENTS

Chapter		Page
	INTRODUCTION	1
I.	THE EFFECT OF LYSINE, THREONINE OR METHIONINE ON SEED DEVELOPMENT IN WHEAT SPIKE CULTURE	3
	Abstract	4
	Introduction	5
	Material and Methods	7
	Spike culture	7
	Analysis of total amino acids	8
	Analysis of free amino acids	8
	Results	10
	Spike culture	10
	Total amino acid concentration of seed	10
	Free amino acid concentration of seed	20
	Discussion	30
	Literature cited	33
II.	IN VITRO SELECTION IN WHEAT TO RECOVER MUTANTS WHICH OVERPRODUCE LYSINE AND THREONINE	36
	Abstract	37
	Introduction	39
	Material and Methods	44

Chapter Page

Effect of lysine and threonine (LT) on wheat callus growth 44
Recovery of mutants resistant to LT 45
Analysis of free amino acids in calli 47
Analysis of total amino acids in seed 48
Analysis of free amino acids in seed 49
Seedling bioassay with lysine plus threonine 49

Results 51
Effect of lysine and threonine on wheat callus growth 51
In vitro selection for resistant to LT 53
Free amino acids in resistant calli 59
Total amino acid concentration in R_2 seed 59
Free amino acid concentration in R_2 seed 63
Total amino acid concentration in R_3 seed 68
Free amino acid concentration in R_3 seed 71
Total amino acid concentration in R_1 seed 74
Free amino acid concentration in R_1 seed 77

Discussion 80

LITERATURE CITED 88

APPENDIXES 92

APPENDIX 1 - Free amino acid concentration in resistant calli 93

APPENDIX 2 - Mole percentages of free amino acids in resistant calli 96

APPENDIX 3 - Free amino acid concentration in R_1 seed 99

APPENDIX 4 - Mole percentages of free amino acids in R_1 seed 102

APPENDIX 5 - Total amino acid concentration in R_1 seed 105

APPENDIX 6 - Mole percentages of total amino acids in R_1 seed 108

APPENDIX 7 - Total amino acid concentration in R_2 seed 111

APPENDIX 8 - Mole percentages of total amino acids in R_2 seed 118

Chapter Page

APPENDIX 9 - Free amino acid concentration in R_2 seed..... 125

APPENDIX 10 - Mole percentages of free amino acids in R_2 seed.... 132

APPENDIX 11 - Total amino acid concentration in R_3 seed..... 139

APPENDIX 12 - Mole percentages of total amino acids in R_3 seed.... 142

APPENDIX 13 - Free amino acid concentration in R_3 seed..... 145

APPENDIX 14 - Mole percentages of free amino acids in R_3 seed.... 148

LIST OF TABLES

Table Page

Chapter I

1. Combined data for seed per spike, seed weight and protein percentage for spike cultured in media supplemented with lysine, threonine or methionine..... 11

2. Combined analysis of variance for total free amino acids..... 12

3. Combined analysis of variance for total amino acid mole percentages..... 14

4. Total amino acid concentration of seed..... 16

5. Combined analysis of variance for free amino acids..... 21

6. Combined analysis of variance for free amino acid mole percentages..... 23

7. Free pool amino acid concentration of seed..... 26

Chapter II

1. Relative growth rates of callus grown on varying concentrations of lysine and threonine for seven transfer periods..... 51

2. Least square means of callus relative growth rates for direct in vitro selection strategy..... 53

3. Regenerated plants recovered from callus tolerant to lysine plus threonine..... 56

4. Relative growth rates of selected callus from step-wise in vitro selection strategy..... 57

Table | Page

5. Free amino acid concentration for aspartate family amino acids for resistance callus capable of plant regeneration..... 60

6. Free histidine, leucine, and valine concentration of callus tissue capable of regenerating plants..... 61

7. Concentration of aspartate family amino acids in R_2 seed..... 62

8. Concentration of other essential amino acids histidine, leucine, valine in R_2 seed..... 64

9. Average seed weight, protein concentration and total amino acid (AA) concentration of R_2 seed..... 65

10. Aspartate family of free amino acids in R_2 seed..... 66

11. Free histidine, leucine, and valine concentration of R_2 seed..... 67

12. Aspartate family of total amino acids in R_3 seed..... 69

13. Total histidine, leucine, and valine concentration of R_3 seed..... 70

14. Average seed weight, protein concentration and total amino acid (AA) concentration of R_3 seed..... 71

15. Aspartate family of free amino acids in R_3 seed..... 72

16. Free histidine, leucine, and valine concentration of R_3 seed..... 73

17. Aspartate family of total amino acids in R_1 seed from step-wise selection strategy..... 75

18. Total histidine, leucine, and valine concentration of R_1 seed..... 76

19. Average seed weight, protein concentration and total amino acid (AA) concentration of R_1 seed..... 77

20. Aspartate family of free amino acids in R_1 seed..... 78

Table | Page

21. Free pool histidine, leucine, and valine concentration of R_1 seed..... 79

22. Regenerated plants family histories..... 81

LIST OF FIGURES

Figure Page

Chapter II

1. Aspartate family of amino acids and regulatory enzymes involve in lysine and threonine biosynthesis..... 40

2. Mean relative growth rate and standard error of calli growing on 0, 1, 2, 4 and 8 mM lysine plus threonine during transfer 1 (21 d)..... 52

3. Histograms of relative growth rates of calli from direct selection strategy..... 54

4. Histograms of relative growth rates of calli for step-wise selection.... 58

INTRODUCTION

Each chapter in this thesis is a manuscript to be submitted for publication in Crop Science, a Crop Science Society of America publication.

CHAPTER I

THE EFFECT OF LYSINE, THREONINE OR METHIONINE ON SEED DEVELOPMENT IN WHEAT SPIKE CULTURE

The Effect of Lysine, Threonine or Methionine on Seed Development in Wheat Spike Culture

Mihdzar A. Kadir, and Arron C. Guenzi*

M.A. Kadir, A.C. Guenzi, Dep. of Agronomy, Oklahoma State Univ., Stillwater, OK 74078.
Contribution of the Oklahoma Agric. Exp. Stn., Journal No. _____ . Received _____ , 1991. * Corresponding Author.

ABSTRACT

Lysine and threonine cause feedback inhibition to aspartokinase, dihydropicolinate synthase, and homoserine dehydrogenase which result in growth inhibition due to lack of methionine. This research was conducted to study the effect of lysine, threonine and methionine on wheat seed development and amino acid content. Wheat spikes were grown in liquid media with three different concentrations of lysine, threonine or methionine. Concentrations used were 1 mM lysine and threonine (LT), 1 mM lysine, threonine and methionine (LTM) and without lysine, threonine or methionine (0 LTM). Spikes were cultured from anthesis for a period of 6 wk. Seed number per spike, seed weight and amino acid composition were analyzed. No significant effects on seed number per spike and seed weight were observed for spikes cultured in 1 mM LT medium. Spikes cultured in 1 mM LT medium had a significant reduction in total concentration of ten amino acids. The reductions in the 10 amino acids lead to a significant 13% reduction in protein percentage. Inclusion of methionine with lysine and threonine overcame the inhibitory effect of lysine and threonine on accumulation of 7 amino acids. Addition of lysine, threonine and methionine also enhanced grain development by increasing seed number per spike. Inclusion of methionine does not overcome the significant reduction in total alanine, glutamate and glycine. It did improve the concentration of cysteine, isoleucine and valine when compared to the control.

INTRODUCTION

Wheat spike culture is a useful tool in studying grain development. Spike are easily cultured in liquid medium consisting of basic nutrients and other elements of interest. Spike culture has been used to study the effects of various treatments on grain development. Wheat spike culture medium containing sucrose, amino acids, salts and minor organic elements was first developed by Donovan and Lee (1977). Their study indicated that media deficient in sucrose or amino acids lead to slow accumulation of dry weight. They also observed that radioactively labelled amino acids supplemented in the liquid media are rapidly incorporated into protein. The use of spike culture for studying grain development is very promising. Singh and Jenner (1983) found that wheat spikes can be cultured in liquid media from anthesis until grain maturity.

Amstrong et al. (1987) performed wheat spike culture in Murashige and Skoog media with 3% sucrose from anthesis for a period of 10 d. They reported a 3 fold increase in distal floret seed number of cultured spikes as compared to the intact control. Spike culture has also been used in barley to study protein and carbohydrate accumulation in normal and high-lysine barley (Manther and Giese, 1984). Corke and Atsmon (1988) used spike culture to study the effect of nitrogen nutrition on endosperm protein synthesis in wild and cultivated barley.

The inhibitory effect of lysine and threonine (LT) on the growth of callus

tissue and regulatory enzymes for the aspartate family of amino acids has been well documented (Furuhashi and Yatazawa, 1970; Wong and Dennis, 1973; Green and Phillips, 1974; Sakano and Komamine, 1978; Bright et al., 1978). However, to date no work has been conducted to study the effect of lysine and threonine on seed development. Therefore, this research was conducted to study the effect of lysine, threonine and methionine on seed development and their effect on the amino acid composition of the seed by utilization of spike culture.

MATERIALS AND METHODS

Spike Culture

'Bobwhite' wheat was used for this study. Plants were grown in the greenhouse at 20^0C with a 14 h photoperiod. The procedure employed was similar to the one described by Armstrong et al. (1987). After anthesis, spikes were removed from the plant by cutting the stem underwater just above the flag leaf. The peduncle portion of the spike was surface sterilize in 30% (v/v) clorox with 60 ul L^{-1} Triton-X. One cm from the base of the peduncle was cut-off under sterilized water after sterilization. The basic medium consisted of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), Gamborg B5 vitamins (Gamborg and Eveleigh, 1968), 3% sucrose, 0.15 g L^{-1} L-asparagine, and 0.25 g L^{-1} each of benlate, ampicillin and streptomycin. Three treatments of combinations of lysine, threonine or methionine were used for this study. The treatments were: 1) control without lysine, threonine or methionine (0LTM), 2) 1 mM lysine plus threonine (LT), and 3) 1 mM lysine, threonine and methionine (LTM). Each spike was cultured in 100 ml of medium in a 125 ml Erlenmeyer flask. It was held in place by a sterilized split foam plug. Each flask was considered a replication. Two experiments were conducted. In the first experiment each treatment was replicated 6 times while in the second experiment each treatment was replicated 5 times. Every 2 wk the spike was transferred into similar fresh medium. Spikes were cultured for a period of 6 wk. After 6 wk spikes were harvested and seed yield, seed $spike^{-1}$

and average seed weight were determined. Data were analyzed by analysis of variance.

Analysis of total amino acids

One-tenth gm wheat meal from each seed sample was placed in a 6 x 50 mm pyrex disposable culture tube. One thousand nmol norleucine was added as an internal standard to each sample. Samples were hydrolysed with 6 N HCl for 24 h at 100°C under nitrogen. After hydrolysis, samples were dried by vacuum centrifugation. Samples were redissolved with 3 washes of 500 ul of deionized water and filtered with a 0.45 um nylon filter. Further preparation and derivatization steps were adapted from the Pico-Tag™ procedures (Cohen et al., 1984). Amino acid derivatives were separated and quantified by reverse-phase high performance liquid chromatography (Heinrikson and Meredith, 1984). Two determinations were performed for each sample.

Analysis of free amino acids

Twenty seeds from each spike of the same treatment were pooled and ground by mortar and pestle. Experiments 1 and 2 were analyzed separately. Approximately 0.1 gram meal from each seed sample was place in a 5 ml tube. An internal standard of 50 nmol norleucine was added into each tube. Amino acids were extracted with 12:15:3 (v/v/v) methanol, chloroform and water (Bieleski and Turner, 1966). From each sample, 2.5 ml of methanol-water phase (top layer) was removed and transferred to a 5 ml cryotube. The

samples were then dried by vacuum centrifugation (Speed-Vac, Savant Instruments). Samples were then subjected to mild acid hydrolysis with 2 N HCl at 100°C for 2 h to convert asparagine and glutamine to aspartic and glutamic acid, respectively. After acid hydrolysis samples were dried by vacuum centrifugation. The sample was then resuspend in 400 ul of 2:1 water:methanol (v/v) and then transferred to a 10,000 m.w. ultrafiltration device (Millipore Inc.). One hundred ul of the filtrate was sampled and dried. Other preparation and derivatization procedures were similar to those described above for total amino acids analysis. Two determinations were conducted for each sample.

RESULTS

Spike culture

Spikes cultured in media containing 1 mM LTM showed significant increases in seed number spike^{-1} when compared to the control (Table 1). However there was no significant difference between the control and 1 mM LT for seed spike^{-1}. For average seed weight, no significant differences were observed between treatments and the control.

Total amino acid concentration of seed

An analysis of variance on combined data from experiment 1 and 2 showed significant treatment by experiment interactions for total amino acids (Table 2) and mole percentage (mol%) (Table 3), therefore each experiment was analyzed separately.

Experiment 1. Total amino acid concentration of seed (Table 4) from spikes cultured on 1 mM LT had a significant reduction in aspartate, cysteine, glutamate, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, valine and total amino acid concentration when compared to the control. For mol%, significant reductions in cysteine and lysine were noted, while significant increases in mol% for alanine, leucine, phenylalanine, proline and threonine were observed.

For spikes cultured on 1 mM lysine, threonine and methionine (LTM) significant increases in alanine, aspartate, isoleucine, leucine, lysine,

Table 1. Combined data for seeds per spike, seed weight and protein percentage for spikes cultured in media supplemented with lysine, threonine or methionine.

Treatment	Seed spike^{-1}	Seed wt.	Protein	Total AA
		-gm-	-%-	-nmol mg^{-1}-
Control	30.0	0.0178	21.5	1705.1
1 mM LT	33.0	0.0174	18.6*	1477.3*
1 mM LTM	41.0*	0.0180	21.6	1720.6
LSD (0.05)	6.9	NS	1.2	90.0

* Significantly higher or lower than control at $P \leq 0.05$

Table 2. Combined analysis of variance for total amino acids.

		Amino Acid								
Source	d.f.	Ala	Arg	Asx	Cys	Glx	Gly	His	Ile	Leu
Treatment (Trt)	2	**	NS	*	*	*	**	NS	**	**
Experiment (Exp)	1	NS	NS	NS	NS	NS	*	NS	*	NS
Determination (Exp)	2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Trt *Exp	2	**	NS	NS	**	**	**	NS	**	**
Error	4	-	-	-	-	-	-	-	-	-

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively
NS $P > 0.05$

Table 2. (Continued)

Source	d.f.	Amino Acid								
		Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val	Total
Treatment (Trt)	2	NS	*	NS	*	NS	*	NS	**	**
Experiment (Exp)	1	**	NS	NS	**	NS	NS	NS	**	**
Determination (Exp)	2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Trt *Exp	2	**	*	**	**	NS	**	*	**	**
Error	4	-	-	-	-	-	-	-	-	-

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively
NS $P > 0.05$