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

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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

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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^{0}C 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^{TM} 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)

		Amino Acid								
Source	d.f.	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 $P > 0.05$

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

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

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

Table 3. (Continued)

Source	d.f.	Amino Acid							
		Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Treatment (Trt)	2	**	NS	**	**	NS	NS	NS	**
Experiment (Exp)	1	**	*	**	**	NS	NS	*	**
Determination (Exp)	2	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 $P > 0.05$

Table 4. Total amino acid concentration of seed.

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
Ala	1	45.3	2.61	39.8	3.03*	55.8*	2.69	7.1	0.13
	2	58.8	3.51	43.6*	2.67*	36.4*	2.69*	8.8	0.60
	$\bar{X}$	52.0	3.06	41.7*	2.85	46.1*	2.69	3.7	0.20
Arg	1	79.2	4.57	62.4	4.75	87.9	4.23	37.1	1.47
	2	86.6	5.17	69.8	4.26	61.6	4.55	46.1	2.97
	$\bar{X}$	82.9	4.87	66.1	4.51	74.8	4.39	19.1	1.07
Asx	1	34.5	1.99	26.1*	2.00	42.7*	2.01	3.4	0.71
	2	38.6	2.31	29.1	1.78	33.7	2.49	20.3	1.47
	$\bar{X}$	36.5	2.15	27.6*	1.89	38.2	2.27	6.6	0.53
Cys	1	40.6	2.34	20.0*	1.53*	37.6	1.81*	7.7	0.18
	2	14.2	0.85	33.5	2.05	40.3*	2.35	21.8	3.85
	$\bar{X}$	27.4	1.59	26.8	1.79	39.0*	2.09	7.5	1.24
Glx	1	286.8	16.52	219.2*	16.66	289.4	13.94*	42.7	1.66
	2	280.2	16.74	270.7	16.52	219.4	16.19	63.0	4.15
	$\bar{X}$	283.5	16.63	245.0*	16.63	254.4*	15.06*	24.5	1.44
Gly	1	222.1	12.79	161.5*	12.29	229.1	11.03*	23.8	0.85
	2	211.4	12.63	205.2	12.53	157.0*	11.58	19.9	1.43
	$\bar{X}$	216.7	12.71	183.3*	12.41	193.0*	11.31*	10.0	0.54

Table 4. (Continued)

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%^{a}	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
His	1	41.8	2.42	34.9	2.66	49.2	2.37	22.6	1.17
	2	45.4	2.71	37.5	2.29	42.0	3.09	30.4	2.26
	$\bar{X}$	43.6	2.56	36.2	2.47	45.6	2.73	12.2	0.82
Ile	1	54.7	3.15	42.9*	3.27	68.6*	3.31	6.5	0.46
	2	60.2	3.59	56.9*	3.47	55.0*	4.06*	2.8	0.14
	$\bar{X}$	57.4	3.37	49.9*	3.37	61.8*	3.68*	2.3	0.16
Leu	1	83.8	4.83	68.8*	5.23*	97.4*	4.69	13.3	0.36
	2	90.9	5.43	83.0*	5.06*	74.2*	5.48	2.9	0.15
	$\bar{X}$	87.4	5.13	75.9*	5.15	85.8	5.09	4.4	0.13
Lys	1	54.0	3.11	38.6*	2.94*	64.1*	3.09	7.6	0.08
	2	36.8	2.20	51.3*	3.13*	27.6*	2.04	5.8	0.38
	$\bar{X}$	45.4	2.65	45.0	3.03*	45.9	2.56	3.1	0.13
Met	1	79.0	4.55	37.5*	2.81	57.8	2.79	39.4	2.52
	2	63.8	3.82	65.6	4.00	70.4	5.20	19.1	1.39
	$\bar{X}$	71.4	4.18	51.5*	3.41	64.1	3.99	14.1	0.93
Phe	1	54.0	4.15	38.6*	4.74*	64.1*	4.21	7.6	0.15
	2	72.0	4.30	77.4*	4.72*	63.2*	4.66*	3.5	0.27
	$\bar{X}$	71.9	4.22	69.9	4.73*	75.3	4.44*	4.6	0.10

Table 4. (Continued)

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%^{a}	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
Pro	1	353.7	20.38	289.3*	21.99*	454.9*	21.90*	48.3	0.63
	2	314.3	18.78	355.6*	21.70*	249.8*	18.44	34.3	1.28
	X̄	334.9	19.58	322.5	21.85*	352.4	20.17	19.1	0.62
Ser	1	67.7	3.90	57.6	4.38	78.0	3.75	40.1	1.40
	2	65.0	3.88	63.7	3.89	60.1	4.44	54.7	3.73
	X̄	66.4	3.89	60.6	4.13	69.0	4.09	21.9	1.28
Thr	1	37.7	2.18	35.2	2.68*	52.3	2.52*	11.6	0.34
	2	44.2	2.64	36.3	2.21	35.2	2.60	9.9	0.73
	X̄	41.0	2.41	35.7*	2.44	43.7	2.56	4.9	0.26
Tyr	1	45.4	2.62	34.0*	2.58	44.8	2.16*	9.5	0.32
	2	43.5	2.60	43.6	2.66	37.6	2.77	9.2	0.68
	X̄	44.5	2.61	38.8*	2.62	41.2	2.47	4.3	0.24
VAL	1	137.9	7.95	85.6*	6.5*	280.6*	13.6*	29.5	1.42
	2	148.4	8.87	116.0	7.08	100.0	7.39	24.7	1.82
	X̄	143.2	8.41	100.8*	6.79*	190.3*	10.47*	12.4	0.74

Table 4. (Continued)

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
Total	1	1736.0	-	1315.9*	-	2077.7*	-	277.2	-
	2	1674.2	-	1638.7*	-	1363.6*	-	31.8	-
	$\bar{X}$	1705.1	-	1477.3*	-	1720.6	-	90.0	-

* Significantly greater or lesser than the control at $P \leq 0.05$

[a] Mol% = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) x 100

phenylalanine, proline, valine and total amino acid concentration were observed as compared to the control. For mol%, however, significant reduction in mol% cysteine, glutamate, glycine and tyrosine were observed.

Experiment 2. Spikes cultured on 1 mM LT had a significant reduction in alanine, isoleucine, leucine and total amino acid concentration (Table 4). A significant increase in lysine, phenylalanine and proline were observed. For mol%, significant reduction in alanine and leucine were noted while significant increases in mol% lysine, phenylalanine and proline were observed when compare to the control.

For spikes cultured in 1 mM lysine, threonine and methionine, significant reduction in alanine, glycine, isoleucine, leucine, lysine, phenylalanine, proline and total amino acid concentration were observed, while a significant increase was observed only in cysteine. For mol%, significant reduction in mol% alanine was observed while significant increases in mol% isoleucine and phenylalanine were observed.

Free amino acid concentration of seed

Combined analysis of experiment 1 and experiment 2 showed a significant treatment by experiment interaction for all free amino acids (Table 5) and for eight amino acids for free mol% (Table 6).

Experiment 1. Spikes growing in 1 mM LT media had significant increases in alanine, isoleucine, methionine, proline and threonine when compared to the

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

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

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

Table 5. (Continued)

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

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

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

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

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

Table 6. (Continued)

		Amino Acid							
Source	d.f.	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Treatment (Trt)	2	NS	**	*	**	NS	NS	*	NS
Experiment (Exp)	1	NS	**	*	NS	*	*	*	NS
Determination (Exp)	2	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 $P > 0.05$

control (Table 7). For mol%, significant increases in alanine, methionine and proline were observed. For spikes cultured in medium containing 1 mM lysine, threonine and methionine, significant increases in alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine and total amino acids concentration were observed (Table 7). Significant increases in mol% methionine and tyrosine were observed.

Experiment 2. Results for experiment 2 (Table 7) indicated that wheat spikes cultured in 1 mM LT media had significant reductions in alanine, arginine, glycine, methionine, proline and total amino acid concentration. A significant increase in lysine was observed when compared to the control. For spikes cultured in 1 mM lysine, threonine and methionine, significant reductions in alanine, arginine, methionine and proline were observed while significant increases in the concentration of aspartate, isoleucine, leucine, lysine, serine and threonine were noted. For mol%, significant reduction in arginine, methionine, and proline were observed while significant increase in aspartate, glycine, isoleucine, leucine and lysine were observed.

Table 7. Free pool amino acid concentration of seed.

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%^{a}	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
Ala	1	160.9	4.44	740.6*	7.80*	1343.5*	4.29	129.9	1.99
	2	637.0	6.64	217.5*	4.47*	579.9*	6.13	40.2	1.28
	$\bar{X}$	399.0	5.54	479.1*	6.13	961.7*	5.21	43.9	0.77
Arg	1	252.8	6.96	684.9	7.21	2197.7*	6.99	616.1	2.00
	2	1011.9	10.55	439.9*	9.03*	625.7*	6.62*	70.2	1.37
	$\bar{X}$	632.3	8.76	562.4	8.12	1411.7*	6.80*	200.1	0.78
Asx	1	747.7	20.64	1446.2	15.23	6134.2*	19.51	1338.6	12.29
	2	1809.5	18.85	817.3*	16.78	2400.1*	25.37*	136.1	2.47
	$\bar{X}$	1279.0	19.74	1132.0	16.00	4267.0*	22.44	434.1	4.04
Cys	1	57.1	1.56	130.2	1.38	217.0*	0.70	89.6	1.95
	2	99.7	1.03	95.0	1.95*	121.0	1.28	71.4	0.54
	$\bar{X}$	78.4	1.29	112.6	1.66	169.0*	0.99	37.0	0.65
Glx	1	131.0	3.61	174.7	1.84	1347.8*	4.30	238.4	1.87
	2	292.4	3.05	183.1	3.76	622.7	6.57	459.9	4.50
	$\bar{X}$	211.7	3.33	178.9	2.80	985.2*	5.43*	167.2	1.57
Gly	1	342.2	9.42	800.9	8.44	3328.8*	10.60	926.9	1.98
	2	849.9	8.84	385.2*	7.91*	888.0	9.39*	148.1	0.31
	$\bar{X}$	596.1	9.13	593.0	8.17*	2108.4*	9.99*	302.9	0.65

Table 7. (Continued)

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
His	1	62.8	1.72	148.0	1.56	589.6*	1.88	225.5	1.19
	2	171.8	1.79	95.3	1.96	200.5	2.12	174.9	1.62
	$\bar{X}$	117.3	1.75	121.6	1.76	395.0*	2.00	92.1	0.65
Ile	1	125.6	3.43	290.2*	3.06	993.0*	3.18	121.7	2.22
	2	321.2	3.35	237.4*	4.87*	401.4*	4.25*	17.9	0.66
	$\bar{X}$	223.4	3.39	263.8*	3.97	697.2*	3.71	39.7	0.75
Leu	1	231.7	6.34	360.8	3.80	1202.4*	3.84	269.9	3.62
	2	326.8	3.39	299.9	6.16*	416.6*	4.41*	76.3	0.44
	$\bar{X}$	279.2	4.87	330.4	4.98	809.5*	4.12	90.5	1.18
Lys	1	88.0	2.39	135.3	1.42	516.0*	1.66	179.6	3.73
	2	132.6	1.38	165.8*	3.41*	254.0*	2.69*	21.3	0.32
	$\bar{X}$	110.3	1.89	150.6	2.41	385.0*	2.17	58.4	1.21
Met	1	1461.3	8.54	2482.3*	17.20*	1984.5*	9.80*	285.9	1.19
	2	1775.8	10.75	1013.4	6.14*	523.7*	3.00*	753.5	2.97
	$\bar{X}$	1618.6	9.65	1747.8	11.67*	1254.1	6.40*	260.0	1.03
Phe	1	152.2	4.14	241.2	2.54	2640.4*	8.41	629.5	5.18
	2	188.2	1.96	224.2	4.59*	264.3	2.80	127.2	2.28
	$\bar{X}$	170.2	3.05	232.7	3.56	1452.3	5.60*	207.2	1.83

Table 7. (Continued)

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%^{a}	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
Pro	1	326.7	9.00	1229.2*	12.95*	2587.7*	8.23	658.7	3.14
	2	1092.8	11.35	490.2*	10.04*	863.0*	9.12*	165.0	1.28
	$\bar{X}$	709.7	10.17	859.7	11.50*	1725.3*	8.67*	219.1	1.09
Ser	1	173.5	4.76	438.0	4.62	1636.8*	5.20	639.2	2.30
	2	516.2	5.37	270.2*	5.55	567.6*	6.00	38.0	0.77
	$\bar{X}$	344.8	5.06	354.1	5.08	1102.2*	5.60	206.6	0.78
Thr	1	70.4	1.93	149.5*	1.58	468.4*	1.50	72.5	1.09
	2	144.0	1.51	127.1	2.61*	206.6*	2.19	63.0	1.01
	$\bar{X}$	107.2	1.72	138.3*	2.09	337.5*	1.84	31.0	0.48
Tyr	1	111.2	3.06	276.9	2.92	1147.9*	3.66*	250.9	0.17
	2	294.3	3.06	190.6*	3.91*	302.1	3.20	23.4	0.64
	$\bar{X}$	202.7	3.06	233.7	3.42*	725.0*	3.43*	81.31	0.21
Val	1	295.9	8.11	612.0	6.45	2008.5*	6.33	1462.1	4.37
	2	694.7	7.18	334.9	6.91	465.6	4.93	586.8	7.57
	$\bar{X}$	495.3	7.64	473.5	6.68	1237.0*	5.63	508.31	2.82

Table 7. (Continued)

Amino Acids	Exp.	Control		1LT		1LTM		LSD	
		Pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%	Pmol mg^{-1}	Mol%
Total	1	4791.0	-	10341.0	-	30344.0*	-	6981.7	-
	2	10358.6	-	5587.0*	-	9702.6	-	1957.0	-
	$\bar{X}$	7575.0	-	7964.0	-	20023.0*	-	2339.4	-

* Significantly greater or lesser than the control at $P \leq 0.05$

[a] Mol% = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) x 100

DISCUSSION

Spike culture

Wheat spike culture was a useful tool to study the effect of lysine and threonine on seed development. Our results (Table 1), however, do not show a significant reduction in seeds spike^{-1} or average seed weight for spikes cultured in 1 mM LT media as compared to the control. Spikes cultured in 1 mM LTM, however, had a significant 36% increase in seed spike^{-1} as compare to the control. These results suggest that the addition of methionine to the medium improved the number of seeds spike^{-1}.

Total amino acid concentration

Although significant treatment by experiment interactions were observed, spikes cultured in 1 mM LT medium showed significant reduction in isoleucine, leucine and total amino acids concentration and significant increase in mol% phenylalanine and proline for both the experiment 1 and 2 (Table 4). The average reduction in isoleucine and leucine concentration was 13%. While the average increase in mol% phenylalanine and proline was 12 and 11%, respectively. The combined analysis of experiment 1 and 2 for spikes cultured on 1 mM LT medium also showed significant reduction in the concentration of alanine, aspartate, glutamate, glycine, methionine, threonine and valine, however, no significant reduction in lysine was observed as compared to the control. For mol%, significant reduction in mol% valine was observed and significant increase in mol% lysine, phenylalanine and proline were observed.

For spikes cultured in 1 mM LTM, significant reductions in alanine, glutamate and glycine were observed. Significant increases in cysteine, isoleucine and valine were observed. For mol%, significant increases in the concentration of isoleucine, phenylalanine and valine were observed.

For protein%, spikes cultured in 1 mM LT, which had a low total amino acids concentration, also showed a significant reduction in protein% (Table 1). The average reduction in protein% was 13%.

Free amino acid concentration

Data for combined analysis for the free pool amino acid concentration (Table 7) indicated that spikes cultured in 1 mM LT medium had a significant 10% reduction in glycine mol%. Significant increases in alanine, isoleucine and proline were observed. For mol%, significant increases in methionine, proline and tyrosine were observed. The average increase for mol% methionine, proline and tyrosine were 20.9%, 13% and 12%, respectively.

For spikes cultured in 1 mM LTM significant increases in aspartate, isoleucine, leucine, lysine, serine and threonine were observed for both experiment 1 and 2, and combined analysis. Other increases in the combined analysis were alanine, arginine, cysteine, glutamate, glycine, histidine, proline, tyrosine valine and also the total amino acids concentration. For mol%, significant reduction in arginine (22%), methionine (33%) and proline (15%) were observed. While significant increase in mol% glycine (9%), phenylalanine (83%) and tyrosine (12%) were observed.

Results from this study indicated that inclusion of 1 mM of lysine and threonine into the liquid media caused reduction in ten of the total amino acids and this lead to a 13% reduction in the protein percentage when compared to the control. Inclusion of methionine, with lysine and threonine, in the liquid media enhanced grain development by increasing seed number per spike, and it also overcame the inhibitory effect of lysine and threonine on the accumulation of 7 amino acids. Inclusion of methionine also improved the concentration of cysteine, isoleucine and valine as compared to the control.

REFERENCES

Armstrong, T.A., T.S. Soong, and M.A.W. Hinchee. 1987. Culture of detached spikes and the early development of the fourth floret caryopsis in wheat. J. Plant Physiol. 131:305-314.

Bieleski, R.L. and N.A. Turner. 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Analytical Biochem. 17:278-293.

Bright, S.W.J., E.A. Wood, and B.J. Miflin 1978. The effect of aspartate derived amino acids (lysine, threonine and methionine) on the growth of excised embryos of wheat and barley. Planta 139:113-117.

Cohen, S.A., T.L. Tarvin, B.A. Bidlingmeyer, and G.E. Tarr. 1984. Analysis of amino acids using precolumn derivatization with phenylisothiocyanate. Amer. Lab., Aug. issue, pp. 48-59.

Corke, H. and D. Atsmon. 1988. Effect of nitrogen nutrition on endosperm protein synthesis in wild and cultivated barley grown in spike culture. Plant Physiol. 87:523-528.

Donovan, G.R., C.F. Jenner, J.W. Lee and P. Martin. 1983. Longitudinal transport of sucrose and amino acids in the wheat grain. Aust. J. Plant Physiol. 10:31-42.

Donovan, G.R. and J.W. Lee. 1977. The growth of detached wheat heads in liquid culture. Plant Sci. Lett. 9:107-113.

Furuhashi, K. and M. Yatazawa. 1970. Methionine-lysine-threonine-isoleucine interrelationships in the amino acid nutrition of rice callus tissue. Plant and Cell Physiology 11:569-578.

Frisch, D.A, B.G. Gengenbach, A.M. Tommey, J.M. Sellner, D.A. Somers, and D.E. Myers. 1991. Isolation and characterization of dihydropicolinate synthase from maize. Plant Physiol. 96:444-452.

Gamborg, O.L. and D.E. Eveleigh. 1968. Culture methods and detection of glucanases in suspension cultures of wheat and barley. Can. J. Biochem. 46:417-421.

Green, C.E. and R.L. Phillips. 1974. Potential selection system for mutants with increased lysine, threonine and methionine in cereal crops. Crop Sci. 14:827-830.

Heinrikson, R.L. and S.C. Meredith. 1984. Amino acid analysis by reverse-phase high-performance liquid chromatography : Precolumn derivatization with phenylisothiocyanate. Analytical Biochem. 136:65-74.

Manther, D.E. and H. Giese. 1984. Protein and carbohydrate accumulation in normal and high-lysine barley in spike culture. Physiol. Plant 60:75-80.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Sakano, K. and Komamine. 1978. Change in the proportion of two aspartokinase in carrot tissue in response to in vitro culture. Plant Physiol. 61:115-118.

Singh, B.K. and C.F. Jenner. 1983. Culture of detached ears of wheat in liquid culture: Modification and extension of the method. Aust. J. Plant Physiol. 10:227-236.

Wong, K.F. and D.T. Dennis. 1973. Aspartokinase from wheat-germ, isolation, characterization and regulation. Plant Physiol. 51:322-326.

CHAPTER II

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

In vitro Selection in Wheat to Recover Mutants which Overproduce Lysine and Threonine

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ABSTRACT

Lysine and threonine are the first and second limiting amino acids in wheat (Triticum aestivum L. em. Thell.) seeds for proper human and non-ruminant animal nutrition. This study was conducted to recover mutants in wheat for regulatory enzymes which control the biosynthesis of lysine and threonine. Experiments were conducted to study the effects of lysine and threonine (LT) at 0, 1, 2, 4 and 8 mM concentrations on the growth of wheat callus tissue. Based on these results, a large scale in vitro direct selection experiment was initiated with 0, 1, and 2 mM LT. A second step-wise selection strategy was conducted to attempt recovery of tolerant mutants to 1, 2, and 3 mM LT. Resistant calli capable of regenerating plants (R_0 plants), R_2 and R_3 seed from regenerated plants the direct selection experiments, and R_1 seed from the step-wise selection experiment were analyzed for free and total amino acid concentrations. Callus growth rates were significantly inhibited at 1, 2, 4 and 8 mM LT. Plants were regenerated from callus tolerant to 1, 2 mM LT (direct selection) and 3 mM LT (step-wise selection). Resistant calli had an average increase of 15.2 of mole percentage (mol%) lysine and 3.6 mol% threonine. Seven R_1 plants contained a significantly higher concentration of total lysine or threonine in the seed. The average increase for total lysine and threonine was 43% and 63%, respectively. Two R_2 plants had an average increase of 150% in total lysine and another two R_2 plants had an average increase of 60% in total threonine. Three R_0 plants, from the step-wise

selection strategy, had 57% increases in free lysine and two of the plants had 35% increases in free threonine.

INTRODUCTION

Lysine and threonine are essential for proper growth and development of animals, however, monogastric animals such as humans, pigs and chickens are unable to synthesize these amino acids. To fulfill the nutritional requirement, these amino acids must be included in the diet of these animals. Cereals such as maize, wheat and barley serve as a major source of amino acids, however, they are deficient in these essential amino acids. The average lysine composition of wheat is 1.98%, compared to 5.5% which is recommended by the World Health Organization (WHO) (Bright and Shewry, 1983). The composition of threonine in wheat is 2.74% which is again lower than the WHO recommended value of 4%. Lysine, threonine, methionine and isoleucine are members of the aspartate family of amino acids (Figure 1). The biosynthesis of these amino acids are regulated by feedback inhibition mechanisms.

The inhibitory effect of lysine and threonine (LT) on the growth of callus tissue and enzyme activity of aspartokinase and dihydropicolinate synthase have been reported by several researchers. Furuhashi et al. (1970) reported inhibitory effect of LT on rice callus tissue. Wong et al. (1973) reported LT feedback inhibition of wheat germ aspartokinase. Other researchers have also reported a similar inhibitory effect (Green and Philips, 1974; Sakano et al., 1978; Bright et al., 1978). The growth inhibition of LT can be overcome by the addition of either methionine, homoserine or homocysteine (Furuhashi et al., 1970; Henke et al., 1974; Bright et al., 1978).

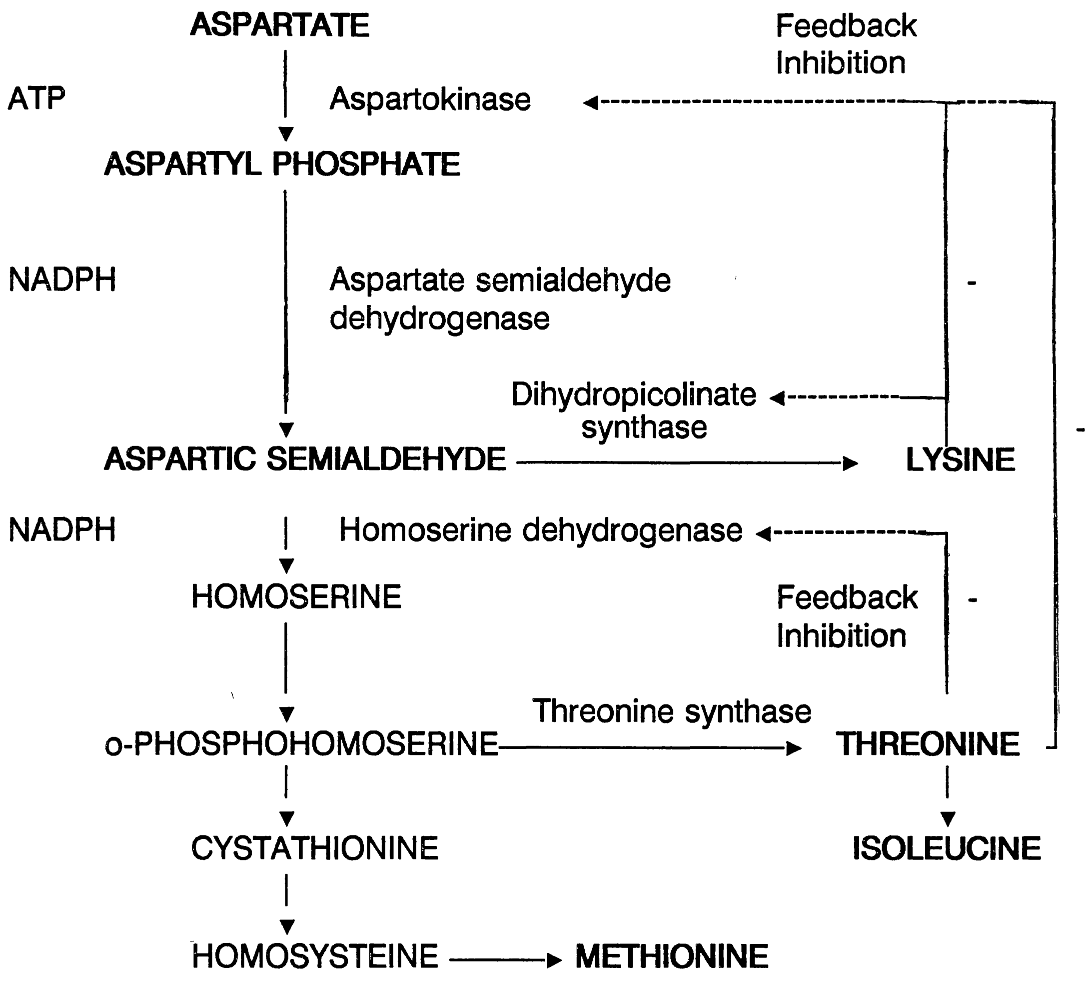

Fig. 1. Aspartate family of amino acids and regulatory enzymes involved in lysine and threonine biosynthesis.

Based on these feedback inhibition mechanisms, two selection strategies have been used to recover mutants which overproduce aspartate family amino acids. First was the use of amino acid analogs such as S-(2-aminoethyl) cysteine (AEC), delta hydroxylysine and ethionine (Widholm, 1976). The second strategy employs selection of plant cells which grow at inhibitory concentrations of lysine plus threonine whereby mutants in key enzymes such as aspartokinase and homoserine dehydrogenase are insensitive to feedback inhibition (Green and Philips, 1974). High concentrations of lysine and threonine cause synergistic inhibition of aspartokinase and homoserine dehydrogenase which result in growth inhibition and cell death due to methionine starvation (Green and Philips, 1974; Bright et al., 1978). Selection for mutant cells growing in the presence of an inhibitor can lead to the recovery of mutants with alterations in the control of several amino acid biosynthethic pathways and these mutations can lead to the overproduction of specific amino acids (Widholm, 1977; Gonzales et al., 1985).

Mutants resistant to LT have been recovered in maize (Hibberd et al., 1980), barley (Bright et al., 1982), carrot (Cattoir-Reynaerts et al., 1983), rice (Schaeffer and Sharpe, 1987) alfalfa (Binarova et al., 1989), sugarcane (Thai et al., 1989) and potato (Kuroda, 1990). Elevated levels in free threonine or lysine in the leaves or kernels of LT mutant lines were observed (Hibberd and Green, 1982; Miao et al., 1988; Diedrick et al., 1990; Dotson et al., 1990). The high lysine or threonine characteristic has been reported to be heritable (Hibberd and Green, 1982), the high lysine and threonine characteristic was reported to be controlled by dominant gene (Hibberd and Green, 1982; Bright et al., 1982) or by recessive gene (Singh and Axtell, 1973; Doll et al., 1973). The high free threonine phenotype was control by two unlinked, codominance genes (Diedrick et al., 1990).

Enzymology of LT mutants have been performed by several researchers. Rognes et al. (1983) isolated three independently regulated aspartate kinase isoenzymes from barley mutants resistant to LT. Aspartate kinase I was inhibited by threonine while aspartate kinase II and III were inhibited by lysine or lysine plus s-adenosylmethionine. Arruda et al. (1984) observed that aspartate kinase activity in extracts of leaves, root and maturing endosperm of barley double LT mutant line (Lt1b/Lt1b, Lt2/Lt2) was less sensitive to lysine inhibition as compared to the wild type. Frisch et al. (1991) found that lysine exerts strong allosteric feedback inhibition to purified dihydrodipicolinate synthase, the first enzyme specific to lysine biosynthesis. Dotson et al. (1990) indicated that aspartate kinase purified from the isogeneic heterozygous Ask and homozygous Ask 2 maize mutants requires much higher (25 and 760 uM) L-lysine concentrations for 50% inhibition as compared to 10 mM for the control.

Little progress has been made in improving the lysine and threonine concentration of wheat by conventional plant breeding methodologies. Entries from the world wheat collection were screened for high lysine concentration (Mattern et al., 1970) and the variability present was found to be nongenetic (Johnson et al., 1985). Improving the lysine and threonine concentration of wheat through selection for mutant cells growing at inhibitory concentrations of lysine and threonine has not been reported. Therefore, our research was conducted to study the effect of lysine and threonine on the growth of wheat callus and to recover wheat mutants which overproduce lysine and threonine. Two selection strategies were employed to recover mutants arising during tissue culture conferring tolerance to lysine and threonine. First strategy was direct selection to obtain mutants with possible point mutations, and step-wise selection to recover tolerance by gene amplication or possible point mutations. Increasing the lysine and threonine concentration of wheat will result in a higher nutritive value and hence provide a better diet for humans and other monogastric animals.

MATERIALS AND METHODS

Effect of lysine and threonine on wheat callus growth.

The wheat cultivar 'Bobwhite', which is well adapted to tissue culture, was used for this study. Plants were grown in a growth chamber at 20^0C with a 14 h photoperiod (300 umol m^{-2} s^{-1}). Immature seeds, 10 to 14 d after anthesis, were harvested.

Seeds were surfaced sterilized for 5 min in 70% (v/v) ethanol, followed by 15 min in 20% (v/v) clorox solution. Seeds were then rinsed twice with sterile distilled water. Embryos, 1 to 1.5 mm in length, were excised from seeds under sterile conditions and placed on agar medium similar to the one described by Sears and Deckard (1982). Medium was modified by using 1.25 mg L^{-1} 2,4-D for callus initiation, 0.5 and 0.75 mg L^{-1} 2,4-D for maintenance, and 0.25 mg L^{-1} 2,4-D for plant regeneration.

Four calli, each weighing 0.2 ± 0.02 gm were placed onto media containing lysine plus threonine in 25 x 100 mm petri dishes. The medium was similar to the one used for growth maintenance (0.5-0.75 mg $liter^{-1}$ 2,4-D), with the addition of lysine and threonine. Each gram of calli contained approximately a million cells.

Five different concentrations of lysine plus threonine (LT) were used. Treatments were: 0 (control), 1, 2, 4, and 8 mM LT. After callus transfer, petri dishes were sealed with parafilm and incubated at 25 ± 1^0 C with a 12 h photoperiod (50 umol m^{-2} s^{-1}).

Each petri dish was considered a replication and each treatment was replicated 6 times. Every 3 to 4 wk the callus was transferred onto similar fresh medium and relative growth rate was calculated (Singer and McDaniel, 1986). Relative growth rate = (ln final weight - ln initial weight)/ transfer days. Data were evaluated by analysis of variance.

At the beginning of each transfer period, if the total weight of a callus exceeded 0.5 g, the callus was trimmed back to 0.5 gm ± 0.02 g. After 15 wk, calli were transferred to shoot initiation media containing similar lysine plus threonine concentration, but with 0.25 mg L^{-1} 2,4-D.

Callus was also sampled for amino acid analysis. Shoots initiated were transferred to the rooting media as described above. Twenty ml of medium was poured into 25 x 150 mm culture tubes and single shoots were placed into individual tubes under sterile conditions for root initiation. The tubes were placed under a 12 h photoperiod (50 umol m^{-2} s^{-1}) at 25 ± 1^{0}C.

Recovery of mutants resistant to LT

Two in vitro selection strategies, direct selection and step-wise selection, were implemented. For both studies callus of the wheat cultivar 'Bobwhite' was used to recover mutants tolerant to lysine and threonine. Concentrations of 1 and 2 mM LT, which reduced growth rates by at least 20% were used to initiate in vitro selections. Based on relative growth rate, resistant calli were identified from the population by using a prediction interval (Steel and Torrie, 1980):

$$P\{ \bar{Y} - t_{0.025} \sqrt{s^2(n+1)/n} \leq Y \leq + t_{0\,025} \sqrt{s^2(n+1)/n} \} = 0.95$$

P is the prediction interval, s^2 is the sample variance, n is the number of observations, Y is the predicted sample growth rate, $\bar{Y}$ is the sample mean, $t_{0.025}$ is the value of two tailed student-t.

Direct selection to recover mutants resistant to LT. Callus from maintenance media was inoculated on 3 different types of media containing lysine plus threonine. The treatments were 0 (control), 1 and 2 mM LT. Four calli, each weighing 0.2 ± .02 gm were inoculated into petri dishes of each medium type. The petri dishes were sealed with parafilm. Each petri dish was considered a replication, and each treatment was replicated 20 times. For each treatment the initial population contained approximately 16 million cells. Tissue cultures were grown as described earlier. Every 3 to 4 wk, calli were transferred onto similar fresh media and fresh weighs were recorded. If the total weight of a callus exceed 0.5 ± .02 g, it was trimmed back to 0.5 ± .02 g at the beginning of the transfer period. Only cells that were capable of growing well were selected and maintained, susceptible cells which grew poorly were discarded. At the 4th transfer period, calli were transferred to shoot initiation media still containing the same concentration of LT. Shoots were transferred to root initiation medium. At the end of 7th transfer period, calli were sampled for amino acid analysis and stored at -70^0 C. R_1 seed from regenerated plants were used for seed increase and R_1 plants were grown in the greenhouse to recover R_2 seed. R_2 seed were subjected to amino acid analysis to determine the free pool and total amino acid concentration.

Step-wise selection to recover mutants resistant to LT. 'Bobwhite' wheat was used for this study. The tissue culture procedure employed on this step-wise selection was similar to the one described above for direct selection. For the step-wise selection 30 replications per treatment were used. The other difference in this method was that we began with two LT concentrations 0 and 1 mM. After two transfer periods, calli on 1 mM LT with relative growth rates equal to or greater than the growth rate of the control were transferred to a higher concentration of lysine and threonine, first to 2 mM LT and gradually stepped-up to 3 mM LT. This method was used in order to recover callus tolerant to higher concentrations of LT such as 2 and 3 mM. Other calli which were still growing but not as well as the control were not stepped-up, but were maintained on the same type of media. R_1 seeds produced by regenerated plants were then analyzed for the free and total amino acid concentration as described above.

Analysis of free amino acids in calli

Approximately 0.4 g fresh weight of callus from each sample was chopped into small pieces and placed in 5 ml chem tubes. Four nmol norleucine was added as an internal standard to each sample. Amino acids were extracted with 12:15:3 (v/v/v) methanol, chloroform and water, respectively (Bieleski and Turner, 1966). Top 2.5 ml of methanol-water-phase was removed from each sample and placed in a 5 ml cryotube. The samples were then dried by vacuum centrifugation (Speed-vac, Savant Instrument). Mild acid hydrolysis with 2N HCl at 100°C for 2 h was performed to convert asparagine and glutamine to aspartic and glutamic acid, respectively. After acid hydrolysis, samples were dried by vacuum centrifugation. Samples were then resuspend in 400 ul of 2:1 water:methanol and then transfered to 10,000 m.w. ultrafiltration device (Millipore Inc). One hundred ul of the filtrate was sampled for amino acid analysis. Sample preparation and derivatization was 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 conducted for each sample.

Analysis of total amino acids in seed

Approximately 0.01 g wheat meal from each seed sample was placed in 6 x 50 mm pyrex disposable culture tubes. One umol 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. Dried samples were redissolved in 1500 ul deionized water and transferred to a 1.5 ml microfuge tube. Samples were centrifuged at 10,000 *xg* for 30 min and filtered with 0.45 um nylon filter. Amino acid analysis was performed as described earlier.

Analysis of free amino acids in seed

Fifty seeds from each plant were ground with mortar and pestle. Approximately 0.1 g meal from each seed sample was place in a 5 ml tube. An internal standard of 50 nmol norleucine was added to each tube. Other preparation steps were similar to the analysis of free amino acids in calli as described above.

Seedling bioassay with lysine plus threonine

Fifty R_2 seeds (embryo half) from each R_1 plant were placed in a germination box consisting of foam, filter paper and 10 ml germination solution [1 g L^{-1} terracoat, 0.034 g L^{-1} gibberellic acid (GA_3), 0.1 g L^{-1} piperacillin and 10 ml L^{-1} dimethyl sulfoxide]. The seeds were placed on the filter paper and kept at room temperature for 24 h. Germination boxes were then transferred to 4^0C for 48 h. Germination boxes were then transferred to 25^0C for 4 d. After germination the endosperm was removed from each seedling. Twenty uniform seedlings were chosen and placed on a styrofoam float (20 x 30 x 1cm) with 1 cm square slot cut out of the foam and covered with polystyrene wire-mesh on the bottom. Each styrofoam float contained 48 slots, one seedling was placed in each slot. The styrofoam float was placed in a hydroponic solution in a 23 x 32 x 16 cm plastic container aerated with an aquarium pump. The hydroponic solution consisted of half-strength Hoagland solution (Hoagland and Arnon, 1950), 2 mM of lysine and threonine, and 0.1 g L^{-1} of piperacillin. The pH was adjusted to pH 7. The seedlings were grown in the bioassay solution for 10 d.

Water was added to the solution everyday to maintain a constant volume. Root and shoot length were measured at the beginning and end of the bioassay. Seedlings which had greater shoot/root growth, as compared to the control, were selected, transferred to vermiculite for a wk and finally to soil in the greenhouse. The R_3 seed from fertile selected plants were then analyzed for free and total amino acid concentration.

RESULTS

Effect of lysine and threonine on wheat callus growth

Callus relative growth rates were significantly inhibited by all LT concentrations after transfer 2 (T2) (Table 1). At T1 significant reductions in growth rate were also observed for callus grown on 2, 4 and 8 mM LT. The overall mean growth rate for the 7 transfer periods indicated a significant reduction in growth for callus on 1, 2, 4 and 8 mM LT when compared to the control. Figure 2 illustrates the observed inhibitory effect of lysine and threonine on callus relative growth rate at T1 (21 d). Callus growth rate was reduced by 18% at 1 mM LT concentration during T1.

Table 1. Relative growth rates of callus grown on varying concentrations of lysine and threonine for seven transfer periods (ca. 150 d).

	Lysine and threonine concentration (mM)				
Transfer	0	1	2	4	8
T1	0.022	0.018	0.014**	0.009**	0.009**
T2	0.018	0.008**	0.003**	0.003**	0.001**
T3	0.018	0.003**	0.003**	0.002**	0.002**
T4	0.035	0.011**	0.004**	0.004**	0.005**
T5	0.031	0.011**	0.009**	0.001**	0.001**
T6	0.027	0.005**	-0.001**	-0.002**	-0.004**
T7	0.028	0.005**	-0.0002**	0.002**	0.002**
Mean	0.027	0.009**	0.004**	0.002**	0.002**

** significant less than the control (0 mM LT), $P \leq 0.01$, respectively

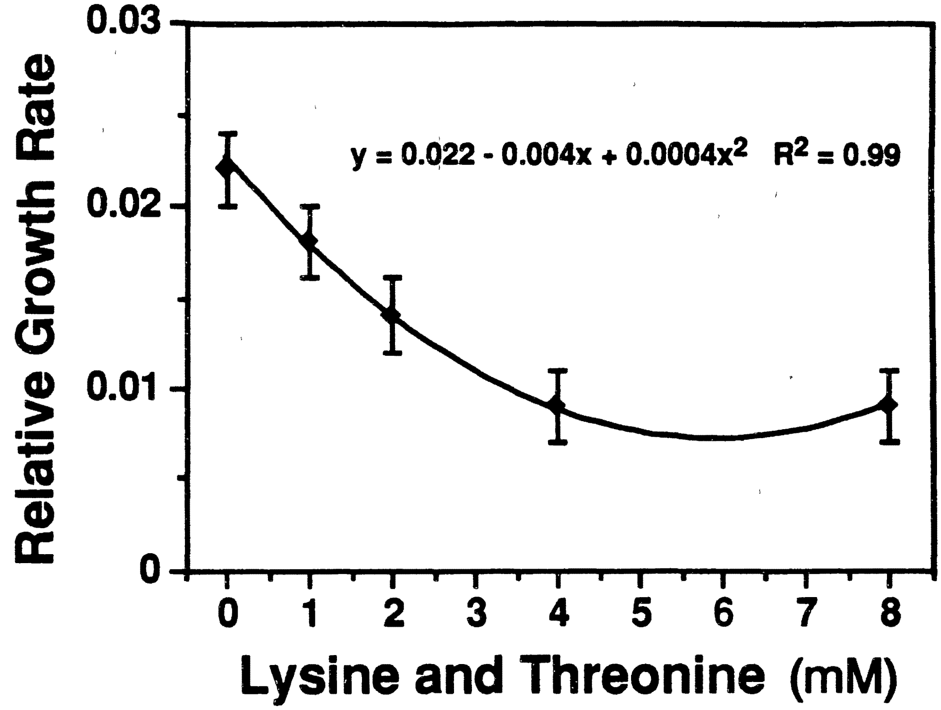

Fig. 2. Mean relative growth rate and standard error of calli growing on 0, 1, 2, 4 and 8 mM lysine plus threonine during transfer 1 (21 d).

In vitro selection for resistance to LT

Direct selection. Based on results from the growth inhibition study, the direct in vitro selection strategy was initiated at 1 and 2 mM LT. Results showed a similar reduction in callus relative growth rate as LT concentration increased (Table 2).

Table 2. Least square means of callus relative growth rate for direct in vitro selection strategy.

	Lysine and threonine concentration (mM)		
Transfer	0	1	2
T1	0.015	0.014	0.010**
T2	0.019	0.010**	0.008**
T3	0.020	0.014**	0.010**
T4	0.026	0.016**	0.010**
T5	0.028	0.016**	0.009**
T6	0.031	0.017**	0.008**
T7	0.034	0.024**	0.007**
Mean	0.023	0.016**	0.009**

** significant less than the control (0 mM LT), $P \leq 0.01$

Callus growth was significantly inhibited at 1 and 2 mM LT from T2 to T7 when compare to the control. For T1, significant reduction in callus growth rate was observed only at 2 mM LT. Resistant calli were identified by their ability to grow, produce shoots and the absence of injury symptoms. Figure 3 illustrates the distribution of callus relative growth rates for the direct selection strategy. As the concentration of LT increased, the distribution of calli growth rates was skewed to the left (slower growth rates) as compared to the control. The same

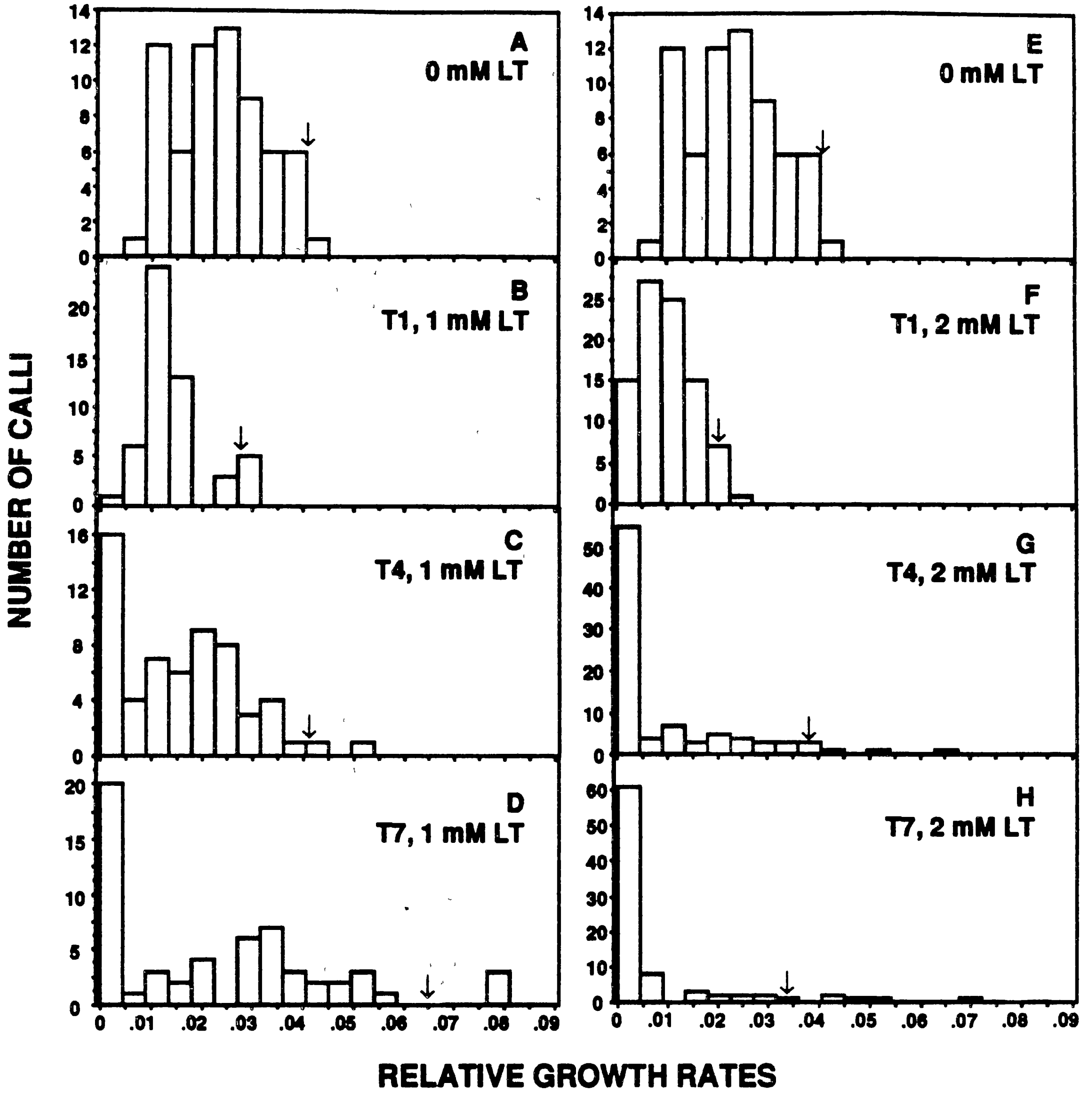

Fig. 3. Histrograms of relative growth rates of calli from direct selection strategy. Average relative growth rates for the control (0 mM LT) calli for 7 transfer periods (A and E). Calli growing on 1 mM LT at T1 (B), T4 (C) and T7 (D) and calli growing on 2 mM LT at T1 (F), T4 (G) and T7(H). Calli with relative growth rates exceeding the upper predicted interval lie to the right of the arrow.

response was observed as exposure time to LT media increased. Tolerant calli which had relative growth rates exceeding the upper predicted interval lie to the right of the arrow.

A total of 136 shoots were recovered from cell lines tolerant to 1 mM LT and 82 shoots were recovered from cell lines tolerant to 2 mM LT (Table 3). Five plants derived from cells tolerant to 1 mM LT produced selfed-seed, while 3 plants derived from cells tolerant to 2 mM LT produced selfed-seed. Five plants (derived from both 1 and 2 mM LT) were male and female sterile, other plants either did not form roots, died after transfer from tubes to vermiculite, or died early in development.

Step-wise selection. Step-wise selection was performed by gradually exposing the callus to higher concentrations of LT. Callus growth rates for the step-wise selection are shown in Table 4. Significant reduction in callus growth rate on 1 mM LT was observed at T1 and T2. After stepping-up to 2 mM LT, significant reduction was observed at T4. When calli were finally stepped-up to 3 mM LT media, significant reductions in relative callus growth rate were observed from T5 to T8 as compared to the control. Histograms of calli relative growth rates for this selection strategy are presented in Fig. 4. No differences were observed on selected calli growing on 1 or 2 mM LT as compared to the control, however, as calli were stepped up to 3 mM LT, the relative growth rate of calli was skewed to the left (slower growth). Tolerant individual calli with relative growth rates exceeding the upper prediction interval were identified at

1, 2, and 3 mM LT (Fig. 4). From the step-wise selection strategy a total of 34 shoots were recovered from 2 mM LT, and 40 shoots were recovered from 3 mM LT (Table 3). Three plants derived from cells tolerant to 2 mM LT produced selfed-seed, while 9 plants derived from cells tolerant to 3 mM LT produced selfed-seed.

Table 3. Regenerated plants recovered from callus tolerant to lysine plus threonine (LT).

LT	Initial number of Calli	Callus producing shoots	Shoots	Plants	Callus producing plants with selfed-seed	Plants producing selfed-seed
-mM-	-No.-	-No.-	-No.-	-No.-	-No.-	-No.-
<u>Direct selection</u>						
1	104	44	136	98	3	5
2	104	38	82	54	1	3
<u>Step-wise selection</u>						
2	120	18	34	10	3	3
3	120	22	40	11	5	9

Table 4. Relative growth rates of selected callus from step-wise in vitro selection experiment.

	Lysine and threonine concentration (mM)			
Transfer	0	1	2	3
T1	0.049	0.038**	-	-
T2	0.033	0.027**	-	-
T3	0.033	0.032	0.039	-
T4	0.035	0.035	0.030**	-
T5	0.035	-	0.032	0.030**
T6	0.030	-	-	0.015**
T7	0.029	-	-	0.012**
T8	0.028	-	-	0.010**
Mean	0.034	0.030	0.029	0.013**

** significant less than the control (0 mM LT), $P \leq 0.01$, respectively

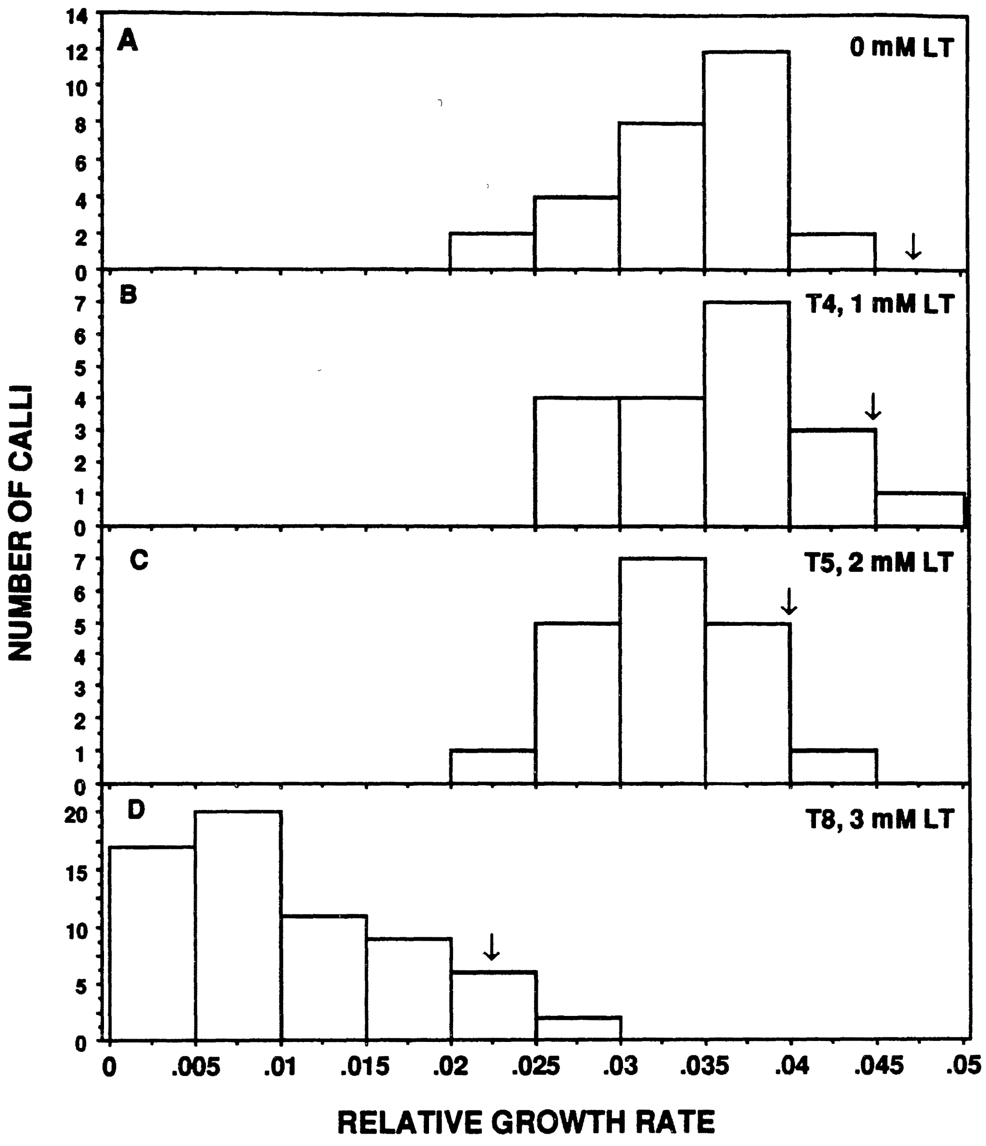

Fig. 4. Histograms of relative growth rates for step-wise selection. Average relative growth rates of control (0 mM LT) calli for 8 transfer periods (A). Last transfer period for calli growing on 1 LT (B), last transfer period for calli growing on 2 LT (C), and last transfer period for calli growing on 3 mM LT media (D). Calli with relative growth rates exceeding the upper predicted interval lie to the right of the arrow.

Free amino acids in resistant calli

Of the four resistant calli from the direct selection from which plants were regenerated, callus 87501-5 (tolerant to 1 mM LT) and callus 87511-11 (tolerant to 2 mM LT), both contained significantly greater amounts of free lysine when compared to the control (Table 5). Besides free lysine, callus 87501-5 also had a significant increase in the free threonine.

All four calli capable of regenerating plants had significant increases in mol% lysine and threonine as compared to the control. Beside lysine and threonine, callus 87515-25 also had a significant increase in the mol% methionine (Table 5 and 6). Callus 87515-20 also had significant increase in the mol% methionine.

Total amino acid concentration of R_2 seed

Seven of the ten R_1 plants selected on the basis of lysine and threonine concentration (Table 7) had a significant increase in total lysine in R_2 seed. The greatest increase was observed in plant 89A121-2 as compared to the control. Plants 89A122-5 and 89A124-11 had significant increases in lysine mol%. Seven of the ten plants selected had a significant increase in the total threonine. The highest concentration was in plant 89A124-6. The average increase in total threonine concentration for the seven plants was 36%. The same seven plants also had significant increases in the mol% threonine. The highest increase was in plant 89A127-1.

Besides lysine and threonine, significant increases in other aspartate

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

		Amino Acids										
Callus	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
87501-5	1	53.0	2.70	24.0	1.22	408.5*	20.79*	16.5	0.85	88.5*	4.51*	1963.6
87515-20	1	17.1	2.04	10.4	1.24	102.3	12.19*	10.8	1.30*	34.1	4.04*	849.0
87515-25	1	27.0	2.65	8.2	0.80	131.5	12.76*	15.6	1.48*	32.5	3.13*	1037.2
87511-11	2	29.8	2.48	12.2	1.01	178.4*	14.89*	3.0	0.25	32.4	2.70*	1200.5
Control	0	50.6	2.49	33.3	1.64	97.0	4.78	11.4	0.56	29.2	1.44	2031.7
LSD (0.05)		5.2	0.36	2.7	0.17	47.8	1.97	7.3	0.71	12.5	0.28	334.7

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

		Amino Acids						
Callus	LT	HIS		LEU		VAL		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
87501-5	1	19.9	1.04	47.6	2.42	56.9	2.90	1963.6
87515-20	1	13.1	1.56	18.2	2.17	20.7	2.45	849.0
87515-25	1	14.6	1.41	20.0	1.96	20.9	2.01	1037.2
87511-11	2	16.6	1.39	28.3	2.35	27.6	2.30	1200.5
Control	0	38.3	1.89	54.2	2.67	66.6	3.28	2031.7
LSD (0.05)		18.1	1.13	11.6	0.86	6.3	0.29	334.7

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

		Amino Acids										
R_2 Seed	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	nmol mg^{-1}	Mol%[a]	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}
89A121-2	1	45.8*	2.53	63.7*	3.51	42.2*	2.33	84.4*	4.65*	44.5	2.46	1814.4*
89A122-5	1	26.9	2.15	49.9	3.98	36.3*	2.90*	44.0	3.52	42.3	3.38	1255.6
89A122-8	1	34.4*	2.68*	52.6*	4.10	32.5	2.54	50.1	3.91	47.9*	3.73*	1283.7
89A127-1	1	40.2*	2.63*	59.4*	3.89	34.8	2.27	58.2*	3.81	65.1*	4.26*	1529.2*
89A124-2	2	29.8	2.10	69.3*	4.87*	35.6*	2.51	59.9*	4.22*	55.5*	3.90*	1422.6*
89A124-4	2	38.8*	2.60	62.3*	4.18	38.1*	2.56	59.3*	3.98	58.2*	3.90*	1491.2*
89A124-6	2	37.5*	1.98	80.3*	4.23*	40.0*	2.12	110.7*	5.86*	69.4*	3.66*	1697.2*
89A124-9	2	34.8*	2.72*	59.7*	4.66*	17.4	1.37	45.7	3.57	53.4*	4.17*	1280.7
89A124-10	2	38.9*	2.56*	62.8*	4.15*	39.2*	2.60	42.3	2.80	58.3*	3.84*	1518.2*
89A124-11	2	39.0*	2.93*	50.4	3.79	38.5*	2.90*	37.5	2.82	43.6	3.29	1329.0
Control	0	28.9	2.36	46.9	3.82	26.9	2.19	43.7	3.56	42.7	3.48	1227.4
LDS (0.05)		3.1	0.17	3.5	0.25	8.3	0.56	9.7	0.58	2.4	0.16	133.4

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

family amino acids were also observed. Plant 89A121-2 had the greatest amount of aspartate as compared to the control. The maximum values for isoleucine and methionine were observed in plant 89A124-6.

Significant increases in other essential amino acids were also observed (Table 8). Plant 89A124-10 had the highest mol% histidine while plant 89A121-2 had the greatest mol% valine. Six plants showed a significant increase in total amino acids. The greatest total was observed in plant 89A121-2. Data on protein % (Table 9), indicated that 8 of the 10 selected plants had significant increases in protein % as compared to the control.

Free amino acid concentration of R_2 seed

Of the ten plants selected for total lysine and threonine concentration, only R_2 seeds from R_1 plant 89A121-2 had a significant increase in free pool threonine (Table 10). Besides free threonine, plant 89A121-2 also had a significant increase in free pool histidine when compared to the control (Table 11). On the basis of free threonine mol%, R_2 seed from three plants, 89A121-2, 89A122-8 and 89A124-6 showed a significant increased in free mol% threonine as compared to the control.

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

		Amino Acids						
R_2 Seed	LT	HIS		LEU		VAL		Total[b]
	-mM-	nmol mg^{-1}	Mol%[a]	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}
89A121-2	1	43.8*	3.26*	82.5*	4.55	175.0*	9.64*	1814.4*
89A122-5	1	35.0	2.80	66.3	5.30	88.8	7.09	1255.6
89A122-8	1	41.3*	3.22*	63.0	4.91	101.3*	7.89*	1283.7
89A127-1	1	53.0*	3.46*	78.1*	5.11	111.7*	7.30	1529.2*
89A124-2	2	40.9*	2.88	81.9*	5.76*	118.9*	8.36*	1422.6*
89A124-4	2	44.7*	3.00	79.0*	5.30	114.4*	7.67	1491.2*
89A124-6	2	52.5*	2.76	98.4*	5.19	155.2*	8.18*	1697.2*
89A124-9	2	44.5*	3.47*	79.4*	6.20*	90.4	7.06	1280.7
89A124-10	2	65.4*	4.31*	88.1*	5.81*	99.2*	6.53	1518.2*
89A124-11	2	41.0*	3.08	74.8*	5.63*	81.5	6.13	1329.0
Control	0	35.8	2.92	66.0	5.38	88.4	7.20	1227.4
LSD (0.05)		3.8	0.19	1.7	0.20	8.9	0.52	133.4

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

R2 seed	LT	Av. seed wt.	Protein	Total AA
	-mM-	-gm--	--%---	nmol mg^{-1}
89A121-2	1	0.035	22.76*	1814.4*
89A122-5	1	0.047	16.00	1255.6
89A122-8	1	0.036	16.32*	1283.7
89A127-1	1	0.034	19.34*	1529.2*
89A124-2	2	0.031	18.00*	1422.6*
89A124-4	2	0.028	18.86*	1491.2*
89A124-6	2	0.039	23.82*	1697.2*
89A124-9	2	0.031	16.22	1280.7
89A124-10	2	0.032	19.39*	1518.2*
89A124-11	2	0.030	16.96*	1329.0
Control	0	0.039	15.51	1227.4
LSD (0.05)			0.71	133.4

* Significantly greater than the control at $P \leq 0.05$

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

		Amino Acids										
R_2 Seed	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
89A121-2	1	1323.8	10.60	309.5	2.48	437.2	3.50	293.1	2.35	366.3*	2.93*	12505
89A122-5	1	776.2	9.11	232.8	3.06	387.0	4.73	202.2	2.58	217.8	2.64	8207
89A122-8	1	597.2	7.89	327.1	4.32	319.3	4.22	245.0	3.24	212.5	2.81*	7576
89A127-1	1	1107.1	12.15	340.4	3.74	233.2	2.58	276.5	3.04	243.2	2.67	9102
89A124-2	2	1488.4	13.15	288.2	2.95	406.4	3.79	214.9	2.15	263.4	2.41	10780
89A124-4	2	1670.6	13.10	310.7	2.91	449.6	3.85	244.9	2.15	307.0	2.56	11923
89A124-6	2	662.9	8.14	318.7	4.15	339.1	4.42	215.8	2.81	221.1	2.88*	7679
89A124-9	2	1067.1	11.95	341.7	3.83	231.2	2.62	247.6	2.77	219.3	2.46	8933
89A124-10	2	1057.7	10.55	369.4	3.68	331.2	3.30	305.8	3.05	264.8	2.64	10040
89A124-11	2	1379.7	13.15	269.2	2.89	340.2	3.44	224.8	2.34	285.2	2.80*	10101
Control	0	1012.8	10.90	326.6	3.51	379.4	4.08	428.3	4.60	230.6	2.48	9310
LSD (0.05)		841.4	3.46	57.8	1.64	169.0	1.26	51.7	0.78	114.1	0.27	4227

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

		Amino Acids						
R_2 Seed	LT	HIS		LEU		VAL		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
89A121-2	1	441.5*	3.53	669.7	5.36	634.1	5.07	12505
89A122-5	1	277.9	3.33	535.6	6.86	509.1	6.69	8207
89A122-8	1	268.4	3.55	631.8	8.34	666.0	8.79	7576
89A127-1	1	297.3	3.27	408.1	4.49	768.9	8.45	9102
89A124-2	2	331.7	3.07	575.7	5.95	577.8	5.74	10780
89A124-4	2	420.9	3.58	548.7	5.06	746.7	6.72	11923
89A124-6	2	305.1	3.97*	563.1	7.34	600.2	7.82	7679
89A124-9	2	287.3	3.24	635.8	7.13	619.9	6.95	8933
89A124-10	2	326.1	3.24	659.0	6.57	655.7	6.54	10040
89A124-11	2	323.3	3.25	605.9	6.35	646.9	6.75	10101
Control	0	288.1	3.10	603.6	6.48	686.9	7.38	9310
LSD (0.05)		147.3	0.82	114.2	2.77	115.3	2.89	4227

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

Total amino acid concentration in the R_3 seed

R_3 seed from R_2 plant 90A104-14, showed a significant increase in total amino acids (Table 12 and 13). It also had significant increases in total lysine, threonine, aspartate, isoleucine and methionine. Other essential amino acids significantly increased in plant 90A104-14 were histidine, leucine, and valine.

Plant 90A104-14 had a significant increase in mol% aspartate. Plant 90A103-7, which had a 72% increase in total lysine, also had a significant increase in valine, mol% valine and mol% isoleucine. Plant 90A108-2 had a significant increase only in mol% aspartate while plant 90A135-19 had significant increases in total threonine and histidine. For the total amino acids, three of the eight plants had significant increases, the highest was in plant 90A104-14, second in plant 90A135-19 and finally plant 90A107-10. Results on protein% showed three of the eight plants had significant increase in protein% (Table 14).

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

		Amino Acids										
R_3 Seed	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	nmol mg^{-1}	Mol%[a]	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}
90A103-7	1	32.1	2.00	59.9	3.75*	49.5*	3.20	60.6	3.79	47.0	2.94	1599.2
90A104-14	1	116.7*	3.80*	91.2*	2.97	68.8*	2.24	97.2*	3.17	76.0*	2.48	3074.7*
90A107-10	1	44.3	2.37	61.3	3.29	35.9	1.90	69.3	3.73	48.3	2.58	1875.9*
90A108-2	1	52.4	3.29*	49.5	3.11	35.7	2.25	51.4	3.19	44.5	2.78	1592.2
90A113-1	1	36.8	2.81	46.7	3.58	20.1	1.54	38.9	2.99	30.5	2.34	1307.3
90A135-13	2	41.3	2.32	57.1	3.21	30.3	1.72	55.2	3.10	43.2	2.43	1782.5
90A135-19	2	54.2	2.82	62.0	3.24	44.0	2.29	63.4	3.31	56.0*	2.93	1915.5*
90A138-14	2	36.4	2.64	46.3	3.35	33.2	2.41	37.6	2.69	35.5	2.59	1379.5
Control	0	38.3	2.52	49.9	3.29	28.8	1.90	53.8	3.55	41.4	2.73	1518.8
LSD (0.05)		16.3	0.57	12.6	0.33	20.4	1.43	21.6	1.15	10.8	0.26	330.2

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* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

		Amino Acids						
R_3 Seed	LT	HIS		LEU		VAL		Total[b]
	-mM-	nmol mg^{-1}	Mol%[a]	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}
90A103-7	1	40.9	2.56	74.4	4.66	144.8*	9.03*	1599.2
90A104-14	1	92.9*	3.03	144.0*	4.69	182.0*	5.93	3074.7*
90A107-10	1	51.3	2.75	95.2	5.10	108.5	5.78	1875.9*
90A108-2	1	47.3	2.97	81.8	5.14	82.5	5.19	1592.2
90A113-1	1	40.2	3.08	70.9	5.43	75.2	5.75	1307.3
90A135-13	2	49.6	2.78	95.9	5.38	94.9	5.32	1782.5
90A135-19	2	57.1*	2.99	92.1	4.81	113.3	5.92	1915.5*
90A138-14	2	36.1	2.63	73.9	5.37	85.3	6.19	1379.5
Control	0	42.7	2.82	82.2	5.41	83.7	5.50	1518.8
LDS (0.05)		10.4	0.54	15.7	0.27	31.8	0.76	330.2

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

R_3 seed	LT	Av. seed wt.	Protein	Total AA
	-mM-	--gm--	--%--	-nmol mg^{-1}-
90A103-7	1	0.028	20.14	1599.2
90A104-14	1	0.023	39.80*	3074.7*
90A107-10	1	0.023	24.02*	1875.9*
90A108-2	1	0.019	20.55	1592.2
89A113-1	1	0.032	16.90	1307.3
90A135-13	2	0.021	23.03	1782.5
90A135-19	2	0.021	24.70*	1915.5*
90A138-14	2	0.026	17.58	1379.5
Control	0	0.040	19.47	1518.8
LSD (0.05)		-	4.13	330.2

* Significantly greater than the control, $P \leq 0.05$

Free amino acid concentration of R_3 seed

R_3 seeds from eight fertile R_2 plants which had survived the seedling bioassay were analyzed for their free amino acid concentration. R_3 seed from six of the eight plants had significant increases in free lysine when compared to the control (Table 15). The highest increase was observed in plant 90A104-14. However, only two of the six plants, plants 90A104-14 and 90A107-10, had significant increases in mol% lysine. For free threonine, two of the eight plants analyzed, 90A104-14 and 90A107-10 had significant increases in free threonine. Besides free pool lysine and threonine plant 90A104-14 also had significant increases in free aspartate, isoleucine, methionine, histidine, leucine and valine (Table 15 and 16). Plant 90A107-10 had significant increases in aspartate, isoleucine, histidine, leucine and valine.

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

		Amino Acids										
R_3 Seed	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
90A103-7	1	483.7	8.27	192.0	3.29	144.6*	2.50	235.8	4.03	92.3	1.57	5851.5*
90A104-14	1	3924.3*	24.84*	436.6*	2.77	525.4*	3.33*	732.7*	4.64	235.2*	1.49	15800.9*
90A107-10	1	1346.7*	15.61	211.0*	2.45	326.3*	3.78*	338.2	3.93	122.3*	1.42	8625.6*
90A108-2	1	634.8	11.21	194.9	3.52	156.0*	2.83	271.1	4.84	94.3	1.69	5611.8*
90A113-1	1	1068.1*	15.21	201.5	2.88	151.7*	2.18	498.9*	7.12	86.7	1.25	7012.7*
90A135-13	2	677.0	12.89	174.6	3.34	126.5	2.42	491.1*	9.39	89.2	1.71	5243.1
90A135-19	2	986.1*	16.23	205.7	3.39	166.4*	2.74	506.9*	8.34	94.9	1.57	6077.8*
90A138-14	2	604.5	13.35	166.8	3.70	134.9	2.99	359.8	7.97	44.7	0.98	4521.7
Control	0	662.9	14.41	186.4	4 06	106.9	2.33	403.9	8.79	79.4	1.73	4599.7
LSD (0.05)		195.0	1.96	20.5	0.75	36.5	0.91	40.3	0.70	36.3	0.61	713.0

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

		Amino Acids						
R_3 Seed	LT	HIS		LEU		VAL		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
90A103-7	1	126.7	2.17	315.9*	5.41	468.4*	8.01	5851.5*
90A104-14	1	746.3*	4.72*	651.2*	4.12	1213.9*	7.69	15800.9*
90A107-10	1	199.0*	2.32	458.5*	5.32	508.7*	5.90	8625.6*
90A108-2	1	155.8	2.81*	324.6*	5.84	421.7*	7.56	5611.8*
90A113-1	1	129.8	1.85	326.3*	4.67	352.0	5.03	7012.7*
90A135-13	2	109.6	2.09	282.8	5.42	398.1*	7.61	5243.1
90A135-19	2	152.8	2.52	352.2*	5.80	464.6*	7.65	6077.8*
90A138-14	2	99.4	2.20	263.5	5.84	340.8	7.54	4521.7
Control	0	77.4	1.69	273.0	5.94	353 2	7.68	4599.7
LSD (0.05)		93.0	0.85	24.9	0.99	24.2	0.71	713.0

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

In terms of free amino acid mol%, plant 90A104-14 had significant increases in aspartate and histidine. Other plants such as 90A113-1 and 90A135-19 had a significant increases in free pool lysine, aspartate, methionine and leucine. Plant 90A135-13 had significant increases in free methionine and valine as compared to the control, while plant 90A108-2 had significant increases in free leucine, valine and mol% histidine.

Plant 90A103-7 had significant increases in free lysine, leucine and valine. For the total free amino acids six, of the eight plants had significant increases when compared to the control. Plant 90A104-14 had the greatest amount of total free amino acids with a 87% increase as compared to the control. The average increase for the six plants was 77%.

Total amino acid concentration in R_1 seed produced by regenerated plants derived from step-wise selection

No significant differences were observed for lysine and threonine concentration in the seed of all four R_0 plants tested (Table 17 and 18). However, plant 89416-29A-1 had significant increases in aspartate and isoleucine. Plant 89417-14-1 had significant increase in mol% isoleucine only. Plant 89417-78-2 show significant increases in isoleucine, valine and mol% valine, while plant 89417-78-3 had significant increase in aspartate, isoleucine and total amino acid concentration. For protein% only plant 89417-78-3 had a significant increase as compared to the control (Table 19).

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

		Amino Acids										
R_1 Seed	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	nmol mg^{-1}	Mol%[a]	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}
89416-29A-1	2	57.1*	3.53	55.1*	3.41	23.1	1.43	41.9	2.59	43.6	2.70	1616.4
89417-14-1	3	35.4	2.59	52.5	3.83*	25.9	1.89	37.5	2.74	37.8	2.76	1371.7
89417-78-2	3	40.8	2.58	54.1*	3.43	24.7	1.57	64.0	4.06	36.8	2.33	1579.9
89417-78-3	3	58.7*	3.39	55.6*	3.21	36.4	2.11	51.7	2.99	45.5	2.63	1732.1*
Control	0	38.3	2.52	49.9	3.29	28.8	1.90	53.8	3.55	41.4	2.73	1518.8
LSD (0.05)		17.8	1.01	3.0	0.38	10.5	0.70	13.0	0.90	6.0	0.32	129.9

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

		Amino Acids						
R_1 Seed	LT	HIS		LEU		VAL		Total[b]
	-mM-	nmol mg^{-1}	Mol%[a]	nmol mg^{-1}	Mol%	nmol mg^{-1}	Mol%	nmol mg^{-1}
89416-29A-1	2	46.3	2.86	85.9	5.32	93.4	5.78	1616.4
89417-14-1	3	32.2	2.35	81.1	5.92	80.6	5.88	1371.7
89417-78-2	3	39.9	2.52	82.0	5.20	105.5*	6.69*	1579.9
89417-78-3	3	60.7	3.51	84.0	4.85	98.9	5.71	1732.1*
Control	0	42.7	2.82	82.2	5.41	83.7	5.50	1518.8
LSD (0.05)		23.4	1.36	6.0	0.53	17.9	1.15	129.9

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (nmol mg^{-1} amino acid / total nmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

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

R_1 seed	LT	Av. seed wt.	Protein	Total AA
	-mM-	--gm--	--%--	-nmol mg^{-1}-
89416-29A-1	2	0.027	20.85	1616.4
89417-14-1	3	0.029	17.62	1371.7
89417-78-2	3	0.036	20.31	1579.9
89417-78-3	3	0.018	22.39*	1732.1*
Control	0	0.040	19.47	1518.8
LSD (0.05)		-	1.69	129.9

* Significant greater than the control at $P \leq 0.05$

Free amino acid concentration in R_1 seed produced by regenerated plants derived from step-wise selection.

R_1 seed from three of the four R_0 plants analyzed (Table 20 and 21) showed an increase in free pool lysine. The average increase was 58%. Plant 89416-29A-1 had the highest amount (83% increase), followed by plant 89417-78-2 with 60% increase and finally plant 89417-14-1 with a 30% increase as compared to the control.

Plants 89416-29A-1 and 89417-14-1 also had significant increases in free pool threonine. Plant 89416-29A-1 had a 38% increase, while plant 89417-14-1 had a 32% increase. Plant 89416-29A-1, besides free lysine and threonine, also had significant increases in aspartate, isoleucine, leucine, valine and mol% aspartate, leucine and valine.

Plant 89417-14-1 had significant increases in isoleucine, leucine, valine and mol% valine. Plant 89417-78-2 had significant increases in free lysine and

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

		Amino Acids										
R_1 Seed	LT	ASX		ILE		LYS		MET		THR		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
89416-29A-1	2	1098.8*	16.40*	293.2*	4.42	195.7*	2.97	217.4	3.28	109.7*	1.64	6673.2*
89417-14-1	3	762.4	12.86	257.6*	4.36	139.1*	2.37	295.5	5.00	105.2*	1.78	5921.5*
89417-78-2	3	734.4	14.05	227.9*	4.37	171.1*	3.28*	191.7	3.67	65.3	1.25	5222.8
89417-78-3	3	1276.5*	18.67*	271.0*	3.96	118.4	1.73	942.9*	13.75*	85.9	1.26	6842.0*
Control	0	662.9	14.41	186.4	4.06	106.9	2.33	403.9	8.79	79.4	1.73	4599.7
LSD (0.05)		207.5	1.18	11.5	0.56	25.7	0.69	65.1	0.55	22.1	0.26	957.2

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

Table 21. Free pool histidine, leucine, and valine concentration of R$_1$ seed.

		Amino Acids						
R$_1$ Seed	LT	HIS		LEU		VAL		Total[b]
	-mM-	pmol mg^{-1}	Mol%[a]	pmol mg^{-1}	Mol%	pmol mg^{-1}	Mol%	pmol mg^{-1}
89416-29A-1	2	126.0	1.88	457.2*	6.89*	597.9*	9.00*	6673.2*
89417-14-1	3	99.2	1.67	381.7*	6.47	537.8*	9.09*	5921.5*
89417-78-2	3	108.8	1.93	380.4*	7.29*	489.2*	9.38*	5222.8
89417-78-3	3	158.0*	2.31	366.1*	5.35	481.8*	7.04	6842.0*
Control	0	77.4	1.69	273.2	5.94	353.2	7.68	4599.7
LSD (0.05)		66.4	0.77	33.8	0.70	34.0	0.87	957.2

* Significantly greater than the control at $P \leq 0.05$

[a] Mol % = (pmol mg^{-1} amino acid / total pmol mg^{-1} amino acids) X 100

[b] Total = Total free pool of twenty amino acids (trytophan was not quantified).

also mol% lysine. It also had significant increases in free isoleucine, leucine, valine, mol% leucine and valine. While plant 89417-78-3 had significant increases in aspartate, isoleucine, methionine, histidine, leucine, valine, mol% aspartate, and methionine. For total free amino acids three plants had significant increases with the highest being in plant 89417-78-3 followed by plants 89416-29A-1 and 89417-14-1. Table 22 lists the regenerated plants family histories for both the direct and step-wise selection stratergies whereby the plants can be traced back to the originating cell lines.

Discussion

Effect of lysine and threonine on wheat callus growth

Lysine and threonine clearly inhibited growth of wheat calli. After the second week, calli grown on media containing LT turned yellowish-brown. Green shoots (sectors) of the susceptible calli turned brown and died. The symptoms of injury were more severe on calli grown on higher LT concentrations such as 4 and 8 mM LT. Resistant calli on the other hand showed little injury and were capable of growing as well as the control. The frequency of resistant calli decreased as the concentration of lysine and threonine increased. As we can see from Table 1, at T4 an increase in callus growth rates were observed on the 1, 2, 4 and 8 mM LT, after a brief reduction in growth at T2. This increase in growth rate was due to some calli becoming tolerant to lysine and threonine. At lower concentrations of LT, such as 1 and 2 mM LT, the presence of the resistant calli was demonstrated by it's ability to

Table 22. Regenerated plants family histories.

LT	Cell line	Ro Plant	R_1 Family	R_2 Family
-mM-				
Direct selection				
1	87501-5	88B266	89A127	
1	87515-20	88B276	89A121	90A103
1	87515-25	88B262	89A122	90A104 90A107 90A108 90A113
2	87511-11	88B271	89A124	90A135 90A138
Stepwise selection				
2	89416-29A-1	89416-29A-1	-	-
3	89417-14-1	89417-14-1	-	-
3	89417-78-2	89417-78-2	-	-
3	89417-78-3	89417-78-3	-	-

produce green shoots and the absence of injury symptoms. Towards the end of T7, resistant calli with green shoots were only observed on the 1 and 2 mM LT concentrations. No calli with green shoots were observed growing on 4 and 8 mM LT concentration.

In vitro selection for resistance to LT

Direct selection. Results from direct selection indicate a similar trend of inhibition with increasing LT concentration as observed in the initial study (Table 2). Calli grown on the 1 and 2 mM LT showed a significant reduction in growth rate from T2 to T7. Beginning at T3, relative growth rates of calli growing on the 1 mM LT media began to increase from a slight reduction at T2 period. Increased callus growth rate at T3 was also observed for 2 mM LT. However, on 1 mM LT the relative growth rate continued to increase through T7, whereas, on the 2 mM LT the growth rate began to decrease at T5. The observed increase in growth rate at T3 was probably due to the accumulation of tolerant tissue.

From the initial study and also the direct selection screening, eight regenerated plants (R_0) that produced selfed seeds were recovered, five of the plants were derived from 1 mM LT concentration and 3 from the 2 mM LT concentration. Due to the insufficient amount of seeds produced by each plant, (2-58 seeds/plant), R_1 seed were used for seed increase.

Step-wise selection. The objective of the step-wise selection was to recover mutants tolerant to higher concentrations of LT. Therefore, calli which were performing poorly and not capable of regenerating plants were discarded from both the control and treatment. Due to selection for tolerant calli, no significant differences were observed for callus relative growth rates at 1 mM LT for T3 and T4. Similar response was observed for callus growing on 2 mM LT at T5. This indicates that tolerant callus was recovered which grew well on 1 and 2 mM LT. However, callus grown on 3 mM LT media showed significant reduction of growth rate beginning of T5 till T8 (Table 3).

Free amino acid concentration of resistant calli

All resistant calli which were capable of regenerating plants had significant increase in the mol% lysine and threonine. However, only cell line 87501-5 had significant increases in both free lysine and threonine with 321.1% increase in lysine and 201% increase in threonine. Callus line 87501-5, which was derived from 1 mM LT concentration, had the highest increase in mol% lysine and threonine when compared to other resistant calli (Table 5). Callus line 87511-11, which was derived on 2 mM LT medium had the second highest value in free and mol% lysine. Callus 87515-20 had the second highest value in mol% threonine. The increase in the lysine and threonine concentration of resistant calli might indicate altered regulation in the aspartate amino acid family pathways (Gonzales and Widholm 1985; Diedrick et al., 1990).

Amino acid concentration of seed produced by regenerated plants

Total amino acid concentration of R_2 seed. For total amino acid concentration, two of the ten plants had significant increases in mol% lysine, plant 89A122-5 and 89A124-11 both had a mol% of 2.9 (Table 7). For total lysine, seven of the ten selected plants had an average increase of 43% with the highest increase in plant 89A121-2 (57% increase). Lysine is the first limiting amino acid in cereals. Cereals have poor nutritional quality storage proteins (prolamins) which are low in lysine (Bhatia and Rabson, 1987). Improving the lysine concentration in wheat will result in better nutritional quality.

Results on total mol% threonine showed seven of the ten selected plants had significant increases in total mol% threonine. Two of the plants with the highest amount were 89A127-1 and 89A124-9. For total threonine, seven of the ten plants had an average increase of 36%. The highest increase was in 89A124-6, with a 63% increase. Threonine is the second limiting amino acid in wheat, increasing the amount of threonine in wheat grain will also provide better nutrition for humans and other monogastric animals. Increases in total amino acids were also observed for six of the ten selected plants, with plant 89A121-2 having the highest total amino acids. Data on protein% (Table 9) showed that eight of the ten selected plants had significant increases in protein%, the highest increase was observed on plant 89A124-6.

Comparing our results to other cereals with high lysine or threonine mutants indicates that our material does not demonstrate the elevated level of free lysine or threonine as reported in other cereals. Miao et al. (1988), reported an increase of 19 fold greater level of free threonine in LT resistant maize calli. Hibbed and Green (1982) observed a 75-100 fold increase in free pool threonine in the kernels of homozygous Ltr* 19, an LT resistant line. Dotson et al. (1990) reported a 174 fold increase of free threonine and 10 fold increase of free lysine in the kernel of homozygous Ask2 mutant maize line. They also reported a 29 fold increase in free threonine in bulked F_2 maize kernel samples. Bright et al. (1982) found in 4 fold increase in the concentration of free threonine and 2 fold increase in the concentration of methionine in the barley mutant tolerant to LT plant. In our material, significant increases were observed primarily in total lysine and threonine. Besides total lysine and threonine, increases in total aspartate, isoleucine, methionine of the aspartate family of amino acids and increases in other essential amino acid such as histidine, leucine, and valine were also observed in several plants (Table 7 and 8).

Free amino acid concentration of R_2 seed. Amino acid analysis of R_2 seed indicated three of the ten selected plants had significant increases in the free threonine mol% (Table 10). The plants were 89A121-2 (derived from callus line 87515-25 grown on 1 mM LT) plant 89A122-8 (derived from callus line 87515-25 also grown on 1 mM LT media) and plant 89A124-6 (derived from callus 87511-11 grown on 2 mM LT media).

Total amino acid concentration of R_3 seed. For total amino acid concentration, plant 90A104-14 was the best plant with significant increases in total lysine (139%) and threonine (83%). Plant 90A104-14 also had significant increases in other amino acids such as aspartate, isoleucine, methionine, histidine, leucine and valine. Increase in several other amino acids also increased the total amino acid concentration of plant 90A104-14 (Table 12 and 13). For protein% (Table 14), three of the eight plants had significant increase in protein% as compared to the control, again plant 90A104-14 had the greatest protein%.

Free amino acid concentration of R_3 seed. R_3 seed from six of the eight plants (which had gone through hydroponic bioassay) had significant increases in free lysine as compared to the control (Table 15). The average increase for the six plants was 129%. Two of the plants, 90A104-14 and 90A107-10 seem to be superior, both have significant increase in free pool lysine, mol% lysine and free pool threonine. Plant 90A104-14 had the highest increase in all 3 categories. Plant 90A104-14 had 392% increase in free lysine and 83% increase in free threonine, while plant 90A107-10 had 205% increase in free lysine and 54% increase in free threonine.

Total amino acid concentration in R1 seed from step-wise selection

For total amino acids, none of the plants showed significant increase in lysine or threonine (Table 17 and 18). Increased aspartate and isoleucine was observed in plant 89416-29A-1. Plant 89417-78-2 had significant increase in isoleucine, valine, mol% valine. Plant 89417-78-3 had significant increases in aspartate, isoleucine and total amino acids concentration. While plant 89417-14-1 had significant increase in isoleucine only. Plant 89417-78-3 which had the highest concentration of total amino acids also had the highest protein% (Table 19).

Free amino acid concentration in R_1 seed from step-wise selection

Plant 89416-29A-1, derived from 2 mM LT had the highest increase in free lysine (83% increase) and threonine (33% increase) in R_1 seed. However, plant 89417-78-2, derived from 3 mM LT was the only plant analyzed with significant increase in mol% lysine (Table 20).

LITERATURE CITED

Arruda, P., S.W.J. Bright, J.S.H. Kueh, P.J. Lea, and S.E. Rognes. 1984. Regulation of aspartate kinase isoenzymes in barley mutants resistant to lysine plus threonine. Plant Physiol. 76:442-446.

Bhatia, C.R. and R. Rabson. 1987. Relationship of grain yield and nutritional quality. Nutritional quality of cereal grains. Agronomy No. 28, ASA, CSSA, SSSA, Publishers, Madison, Wisconsin, USA, 11-43.

Bieleski, R.L. and N.A. Turner. 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Analytical Biochem. 17:278-293

Binarova, P., F. Novotny and B. Nedbalkova. 1989. Selection and characterization of alfalfa cell lines resistant to lysine + threonine and/or ethionine. Biochem. Physiol. Pflanzen 185:99-107.

Bright, S.W.J., B.J. Miflin, and S.E. Rognes. 1982. Threonine accumulation in the seeds of a barley mutant with an altered aspartate kinase. Biochem. Genet. 20: 229-243.

Bright, S.W.J., E.A. Wood, and B.J. Miflin. 1978. The effect of aspartate derived amino acids (lysine, threonine and methionine) on the growth of excised embryos of wheat and barley. Planta 139:113-117.

Bright, S.W.J. and P.R. Shewry. 1983. Improvement of protein quality in cereals. CRC Critical Reviews in Plant Science vol. 1(1):49-93.

Cattoir-Reynaerts, A., E. Degryse, I. Verbruggen, and M. Jacobs. 1983. Selection and characterization of carrot embroid cultures resistant to inhibition by lysine plus threonine. Biochem. Physiol. Pflanzen 178:81-90.

Cohen, S.A., T.L. Tarvin, B.A. Bidlingmeyer, and G.E. Tarr. 1984. Analysis of amino acids using precolumn derivatization with phenylisothiocyanate Amer. Lab., Aug issue, pp 48-59.

Diedrick, T.J., D.A. Frisch and B.G. Gengenbach. 1990. Tissue culture isolation of a second mutant locus for increased threonine accumulation in maize. Theor. Appl. Genet. 79:209-215.

Doll, H. 1973. Inheritance of the high lysine character of a barley mutant. Hereditas 74:293-294.

Dotson, S.B., D.A. Frisch, D.A. Somers, and B.G. Gangebach. 1990. Lysine insensitive aspartate kinase in two threonine-overproducing mutants of maize. Planta 182:546-552.

Frisch, D.A., B.G. Gengenbach, A.M. Tommey, J.M. Sellner, D.A. Somers, and D.E. Myers. 1991. Isolation and characterization of dihydropicolinate synthase from maize. Plant Physiol. 96:444-452.

Furuhashi, K. and M. Yatazawa. 1970. Methionine-lysine-threonine-isoleucine interrelationships in the amino acid nutrition of rice callus tissue. Plant and Cell Physiology 11:569-578.

Gonzales, R.A. and J.M. Wildhom. 1985. Selection of plant cells for desirable characteristics : Inhibitor Resistance. Plant Cell Culture A Practical Approach.IRL Press, Oxford.Washington D.C. 67-78.

Green, C.E. and R.L. Phillips. 1974. Pottential selection system for mutants with increased lysine, threonine and methionine in cereal crops. Crop Sci. 14:827-830.

Henke, R.R., K.G. Wilson, and J.W. McClure. 1974. Lysine methionine -threonine interactions in growth and development of Mimulus cardinalis seedlings. Planta. 116:333-345.

Heinrikson, R.L. and S.C. Meredith. 1984. Amino acid analysis by reverse-phase high-performance liquid chromatography : Precolumn derivatization with phenylisothiocyanate. Analytical Biochem. 136:65-74.

Hibberd, K.A. and C. E. Green. 1982. Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture. Proc. Natl. Acad. Sci. U.S.A. 79:559-563.

Hibbed, K.A., T. Walter, C.E. Green, and B.G. Gengenbach. 1980. Selection and characterization of a feedback- insensitive tissue culture of maize. Planta 148:183-187.

Hoagland, D.R. and D.I. Arnon. 1950. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Circ. 347:31-32.

Johnson, V.A., P.J. Mattern, C.J. Peterson, and S.L. Kuhr. 1985. Improvement of wheat protein by traditional breeding and genetic techniques. Cereal Chem 62(5): 350-354.

Kuroda, S. 1990. Induction, Selection and Characterization of Lysine-Resistant Mutants of Potato using Protoplast culture System. JARQ Vol. 23, No. 4:255-260.

Mattern, P.J., J.W. Schmidth, and V.A. Johnson. 1970. Screening for high lysine content in wheat. Cereal Science Today 15 (12):409-411.

Miao, S., D.R. Duncan and J. Widholm. 1988. Selection of regenerable maize callus cultures resistant to 5-methyl-DL-tryptophan, S-2-aminoethyl-L-cysteine and high levels of L-lysine plus L-threonine. Plant cell tissue and Organ Culture 14:3-14.

Rognes, S.E., S.W.J. Bright and B .J. Miflin. 1983. Feedback-insensitive aspartate kinase isoenzymes in barley mutants resistant lysine plus threonine. Planta 157:32-39.

Sakano, K. and A. Komamine. 1978. Change in the proportion of two aspartokinases in carrot tissue in response to in vitro culture. Plant Physiol 61:115-118.

Schaeffer, G.W. and F.T. Sharpe, Jr. 1987. Increased lysine and seed storage protein in rice plants recovered from calli selected with inhibitory levels of lysine plus threonine and S-(2-aminoethyl) Cysteine. Plant physiol 84:509-515.

Sears, R.G. and E.L. Deckard. 1982. Tissue culture variability in wheat: callus induction and plant regeneration. Crop Sci 22:546-550.

Singer, R.S. and C.N. McDaniel. 1986. Analyzing growth in cell cultures. II. Effect of initial cell mass on growth. Can. J. Bot. 64:238-241.

Singh, R. and J.D. Axtell. 1973. High lysine mutant gene (hl) that improves protein quality and biological value of grain sorghum, Crop Sci 13:535-539.

Thai, D.X., V.V. Tran, T.S. Tran, S.K. Ngo and U.V. Nguyen. 1989. Protein and amino acid composition in a high lysine, high-threonine tolerant sugarcane cell line. Can. J. Plant Sci. 69:625-629.

Widholm, J.M. 1977. Selection and characterization of amino acid analog resistant plant cell cultures. Crop Sci 17:597-600.

Widholm, J.M. 1976. Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine, and proline analogs. Can. J. Bot 54:1523-1529.

Wong, K.F., and D.T. Dennis. 1973. Aspartokinase from wheat-germ, isolation, characterization, and regulation. Plant Physiol. 51:322-326.

APPENDIXES

APPENDIX 1

Free amino acid concentration in resistant calli

Appendix 1. Free amino acids concentration in resistant calli

		Amino Acids										
Callus	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
87501-5	1	539.9	243.9	53.0	7.3*	118.4*	53.5	19.9	24.0	47.6	408.5*	16.5
87511-11	2	295.7	411.5	29.8	3.1	48.3	20.5	16.6	12.2	28.3	178.4*	3.0
87515-20	1	239.5	204.4	17.1	3.3	35.1	22.4	13.1	10.4	18.2	102.3	10.8
87515-25	1	221.8	354.1	27.0	3.0	35.8	20.0	14.6	8.2	20.0	131.5	15.6
Control	0	750.3	493.5	50.6	5.5	89.9	69.3	38.3	33.3	54.2	97.0	11.4
LSD		79.4	130.2	5.2	1.4	12.8	8.0	18.1	2.7	11.6	47.8	7.3

* Significantly greater than the control at $P \leq 0.05$

Appendix 1. (Continued)

		Amino Acids						
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
87501-5	1	20.1	185.3*	60.5	88.5*	20.0	56.9	1963.6
87511-11	2	9.7	47.3	25.6	32.4	10.7	27.6	1200.5
87515-20	1	8.0	82.4	18.6	34.1	8.8	20.7	849.0
87515-25	1	5.3	64.5	22.4	32.5	39.9*	20.9	1037.2
Control	0	24.7	141.6	51.1	29.2	25.6	66.6	2031.7
LSD		3.2	29.3	10.1	12.5	8.8	6.3	334.7

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 2

Mole percentages of free amino acids in resistant calli

Appendix 2. Mole percentages of free amino acids in resistant calli

		Amino Acids										
Callus	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
87501-5	1	27.51	12.39	2.70	0.38	6.04*	2.73	1.04	1.22	2.42	20.79*	0.85
87511-11	2	24.63	34.28*	2.48	0.26	4.03	1.71	1.39	1.01	2.35	14.89*	0.25
87515-20	1	28.29	23.65	2.04	0.40	4.15	2.64	1.56	1.24	2.17	12.19*	1.30*
87515-25	1	21.37	34.13*	2.61	0.29	3.45	1.93	1.41	0.80	1.96	12.76*	1.48*
Control	0	36.95	24.25	2.49	0.27	4.43	3.42	1.89	1.64	2.67	4.78	0.56
LSD		1.63	5.50	0.36	0.19	0.38	0.23	1.13	0.17	0.68	1.97	0.71

* Significantly greater than the control at $P \leq 0.05$

Appendix 2. (Continued)

		Amino Acids					
Callus	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
87501-5	1	1.03	9.44*	3.09*	4.51*	1.02	2.90
87511-11	2	0.81	3.94	2.14	2.70*	0.90	2.30
87515-20	1	0.96	9.94*	2.18	4.04*	1.03	2.45
87515-25	1	0.51	6.21	2.15	3.13*	3.84*	2.01
Control	0	1.21	6.97	2.51	1.44	1.26	3.28
LSD		0.32	0.42	0.39	0.28	0.31	0.29

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 3

Free amino acid concentration in R_1 seed

Appendix 3. Free amino acid concentration in R_1 seed

		Amino Acids										
R_1 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
8941629A-1	2	577.1	287.0*	1098.8*	115.3*	258.1*	660.4*	126.0	293.2*	457.2*	195.7*	217.4
89417-14-1	3	751.5*	178.9	762.4	84.9	186.0	592.9	99.2	257.6*	381.7*	139.1*	295.5
89417-78-2	3	375.5	301.6*	734.4	100.3*	184.7	484.8	108.8	227.9*	380.4*	171.1*	191.7
89F17-78-3	3	531.9	176.0	1276.5*	50.2	251.3*	499.0	158.0*	271.0*	366.1*	118.4	942.9*
Control	0	455.9	190.0	662.9	76.9	173.5	503.0	77.4	186.4	273.2*	106.9	403.9
LSD (0.05)		178.5	59.7	207.5	22.5	64.1	133.1	66.4	11.5	33.8	25.7	65.1

* Significantly greater than the control at $P \leq 0.05$

Appendix 3. (Continued)

		Amino Acids						
R_1 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
8941629A-1	2	273.9	803.0*	436.6	109.7*	116.1	597.9*	6673.2*
89417-14-1	3	217.7	738.4*	371.8	105.2*	221.0	537.8*	5921.5*
89417-78-2	3	261.8	637.7	361.0	65.3	154.7	489.2*	5222.8
89417-78-3	3	189.0	774.1*	391.6	85.9	278.4	481.8*	6842.0*
Control	0	153.6	480.5	271.5	79.4	151.6	353.2	4599.7
LSD (0.05)		30.1	197.6	73.2	22.1	129.4	34.0	957.2

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 4

Mole percentages of free amino acids in R_1 seed

Appendix 4. Mole percentages of free amino acids in R_1 seed

		Amino Acids										
R_1 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89416-29A-1	2	8.63	4.29	16.40*	1.75	3.86	9.87	1.88	4.42	6.89*	2.97	3.28
89417-14-1	3	12.65*	3.02	12.86	1.45	3.14	10.02	1.67	4.36	6.47	2.37	5.00
89417-78-2	3	7.20	5.78*	14.05	1.92	3.53	9.28	1.93	4.37	7.29*	3.28*	3.67
89417-78-3	3	7.78	2.58	18.67*	0.74	3.68	7.30	2.31	3.96	5.35	1.73	13.75*
Control	0	9.91	4.13	14.41	1.68	3.77	10.90	1.69	4.06	5.94	2.33	8.79
LSD (0.05)		1.97	0.70	1.18	0.45	0.79	0.92	0.77	0.56	0.70	0.69	0.55

* Significantly greater than the control at $P \leq 0.05$

Appendix 4. (Continued)

		Amino Acids					
R_1 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89416-29A-1	2	4.15	12.00	6.54	1.64	2.50	9.00*
89417-14-1	3	3.70	12.46	6.29	1.78	3.75	9.09*
89417-78-2	3	5.01*	12.22	6.90*	1.25	2.97	9.38*
89417-78-3	3	2.77*	11.32	5.73	1.26	4.06	7.04
Control	0	3.34	10.46	5.91	1.73	3.29	7.68
LSD (0.05)		0.87	2.26	0.96	0.26	2.02	0.87

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 5

Total amino acid concentration in R_1 seed

Appendix 5. Total amino acid concentration in R_1 seed

		Amino Acids										
R_1 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89416-29A-1	2	51.7	65.0	57.1*	12.7	359.7*	147.2	46.3	55.1*	85.9	23.1	41.9
89417-14-1	3	48.4	55.8	35.4	12.6	280.9	122.6	32.2	52.5	81.1	25.9	37.5
89417-78-2	3	47.3	65.3	40.8	13.1	310.2	149.5	39.9	54.1*	82.0	24.7	64.0
89417-78-3	3	63.2*	96.2*	58.7*	10.9	395.7*	182.9*	60.7	55.6*	84.0	36.4	51.7
Control	0	48.8	65.3	38.3	10.3	316.4	147.5	42.7	49.9	82.2	28.8	53.8
LSD (0.05)		5.5	28.7	17.8	6.3	32.4	14.6	23.4	3.0	6.0	10.5	13.0

* Significantly greater than the control at $P \leq 0.05$

Appendix 5. (Continued)

		Amino Acids						
R$_1$ Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89416-29A-1	2	64.0	292.7	110.2	43.6	66.9*	93.4	1616.4
89417-14-1	3	61.1	277.4	79.6	37.8	50.3	80.6	1371.7
89417-78-2	3	66.7	328.9*	86.0	36.8	65.1*	105.5*	1579.9
89417-78-3	3	61.7	269.8	100.2	45.5	60.1	98.9	1732.1*
Control	0	60.3	284.6	111.9	41.4	52.9	83.7	1518.8
LSD (0.05)		6.9	20.2	40.0	6.0	10.1	17.9	129.9

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 6

Mole percentages of total amino acids in R_1 seed

Appendix 6. Mole percentages of total amino acids in R_1 seed

		Amino Acids										
R_1 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89416-29A-1	2	3.20	4.01	3.53*	0.79	22.28	9.11	2.86	3.41	5.32	1.43	2.59
89417-14-1	3	3.53*	4.07	2.59	0.92	20.48	8.94	2.35	3.83*	5.92	1.89	2.74
89417-78-2	3	3.00	4.13	2.58	0.84	19.63	9.46	2.52	3.43	5.20	1.57	4.06
89417-78-3	3	3.65*	5.56	3.39	0.63	22.85*	10.55*	3.51	3.21	4.85	2.11	2.99
Control	0	3.22	4.31	2.52	0.69	20.84	9.71	2.82	3.29	5.41	1.90	3.55
LSD (0.05)		0.18	1.60	1.01	0.43	1.76	0.17	1.36	0.38	0.53	0.70	0.90

* Significantly greater than the control at $P \leq 0.05$

Appendix 6. (Continued)

		Amino Acids					
R_1 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89416-29A-1	2	3.97	18.13	6.81	2.70	4.14*	5.78
89417-14-1	3	4.45	20.23	5.81	2.76	3.67	5.88
89417-78-2	3	4.23	20.84*	5.43	2.33	4.13*	6.69*
89417-78-3	3	3.56	15.58	5.79	2.63	3.47	5.71
Control	0	3.98	18.74	7.37	2.73	3.49	5.50
LSD (0.05)		0.60	1.92	2.16	0.32	0.47	1.15

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 7

Total amino acid concentration in R_2 seed

Appendix 7. Total amino acid concentration in R_2 seed.

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A121-1	1	44.4	45.9	30.9	23.5	144.7	124.2	41.8*	66.0*	78.8*	29.0	55.4*
89A121-2	1	122.0*	55.3*	45.8*	58.1*	267.8*	174.1*	43.8*	63.7*	82.5*	42.2*	84.4*
89A121-4	1	97.9*	56.4*	55.3*	57.0*	281.9*	147.2*	48.2*	59.4*	81.7*	44.1*	78.3*
89A122-1	1	109.9*	60.2*	52.6*	54.5*	318.3*	185.5*	50.9*	67.9*	89.7*	37.9*	82.4*
89A122-2	1	44.5	43.5	29.3	19.5	183.7	125.6	38.7	61.2*	78.0*	27.6	45.8
89A122-3	1	105.6*	60.9*	40.7*	33.1*	367.9*	183.6*	48.6*	60.5	87.0*	44.3*	51.9
89A122-4	1	78.5*	55.2*	31.5	24.2	220.5*	158.3*	42.3*	55.3*	72.7*	32.4	55.0*
89A122-5	1	75.0	45.0	26.9	26.0*	200.6	89.4	35.0	49.9	66.3	36.3*	44.0
89A122-6	1	60.7	51.5*	35.4*	16.1	226.9*	120.9	48.0*	52.6*	68.9*	26.4	35.4
89A122-7	1	53.1	55.5*	39.8*	19.4	202.2	131.2	56.3*	69.0*	91.7	32.7	73.8*
89A122-8	1	65.2	51.9*	34.4*	21.1	199.2	119.3	41.3*	52.6*	63.0	32.5	50.1
89A122-9	1	54.9	50.5*	28.5	17.3	181.4	122.2	43.5*	69.9*	88.9*	31.2	46.0
89A122-10	1	74.0	50.5*	30.7	30.8*	205.7*	132.7	34.8	50.2	70.7*	33.6	61.6*

* Significantly greater than the control at $P \leq 0.05$

Appendix 7. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A122-11	1	136.7*	66.4*	48.8*	59.2*	291.4*	203.3*	43.1*	68.4*	95.7*	33.2	94.3*
89A122-12	1	47.2	49.8*	25.9	17.2	149.1	113.3	43.2*	58.4*	77.3*	25.1	48.0
89A122-13	1	53.3	47.3	35.9*	21.2	155.1	127.5	41.3*	66.2*	76.6*	32.7	58.3*
89A122-14	1	43.8	39.4	26.6	18.2	153.1	125.6	34.4	59.1*	74.9*	30.5	53.6*
89A124-2	2	59.7	56.2*	29.8	24.8	173.6	135.4	40.9*	69.3*	81.9*	35.6*	59.9*
89A124-3	2	70.1	49.0	28.1	24.7	166.0	133.4	37.0	54.4*	67.2	32.6	56.8*
89A124-4	2	66.3	54.7*	38.8*	22.4	212.9*	145.6*	44.7*	62.3*	79.0*	38.1*	59.3*
89A124-5	2	150.8*	56.4*	40.4*	77.9*	236.8*	179.4*	43.0*	71.0*	81.1*	44.2*	105.4*
89A124-6	2	82.5*	65.9*	37.5*	55.2*	212.5*	183.8*	52.5*	80.3*	98.4*	40.0*	110.7*
89A124-7	2	66.6	48.6	26.1	20.8	167.9	124.7	32.9	50.4	63.6	34.6	53.0
89A124-8	2	95.1*	56.1*	39.2*	31.2*	300.4*	159.4*	39.5	57.4*	89.3*	32.4	53.4
89A124-9	2	63.4	51.1*	34.8*	15.7	181.9	112.9	44.5*	59.7*	79.4*	17.4	45.7
89A124-10	2	60.1	80.6*	38.9*	22.2	227.0*	139.2	65.4*	62.8*	88.1*	39.2*	42.3

* Significantly greater than the control at $P \leq 0.05$

Appendix 7. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A124-11	2	71.6	47.7	39.0*	23.7	248.4*	129.3	41.0*	50.4	74.8*	38.5*	37.5
89A124-12	2	59.7	46.3	31.6	18.8	123.2	114.0	37.4	56.1*	66.0	33.5	54.4*
89A124-14	2	82.3*	56.78	55.6*	38.2*	346.3*	146.1*	48.4*	59.3*	86.8*	36.3*	59.1*
89A124-15	2	138.4*	67.1*	49.0*	60.9*	263.7*	200.3*	47.1*	63.7*	84.3*	47.4*	78.0*
89A127-1	1	86.5*	65.7*	40.2*	22.2	214.8*	139.3	53.0*	59.4*	78.1*	34.8	58.2*
89A128-M	0	71.4	47.1	28.9	21.3	188.1	120.7	35.8	46.9	66.0	26.9	43.7
LSD 0.05		6.2	2.6	3.1	4.3	16.2	20.8	3.8	3.5	1.7	8.3	9.7

* Significantly greater than the control at $P \leq 0.05$

Appendix 7. (Continued)

		Amino Acids						
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A121-1	1	65.5*	286.5*	42.3	48.0*	44.0	110.6*	1281.4
89A121-2	1	71.2*	362.6*	59.8	44.5	61.5*	175.0*	1814.4*
89A121-4	1	69.3*	309.5*	64.4*	50.8*	62.6*	152.7*	1716.7*
89A122-1	1	77.3*	376.3*	63.2	52.4*	64.2*	153.1*	1896.0*
89A122-2	1	67.1*	291.7*	40.3	44.9	41.3	113.1*	1295.9
89A122-3	1	74.3*	357.5*	69.7*	47.7*	49.0*	109.5*	1791.7*
89A122-4	1	60.8	357.0*	70.2*	54.8*	41.7	107.0*	1517.4*
89A122-5	1	59.7	268.4*	61.6	42.3	40.5	88.8	1255.6

* Significantly greater than the control at $P \leq 0.05$

Appendix 7. (Continued)

		Amino Acids						
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A122-6	1	57.2	231.4	69.4*	44.2	41.7	74.5	1261.2
89A122-7	1	73.0*	304.4*	85.3*	60.0*	44.0	119.4*	1510.8*
89A122-8	1	56.0	246.7	58.1	47.9*	43.0	101.3*	1283.7
89A122-9	1	73.1*	307.8*	70.2*	52.3*	38.0	118.2*	1393.8*
89A122-10	1	65.9*	301.1*	56.4	43.6	43.3	115.4*	1401.0*
89A122-11	1	88.7*	479.5*	84.6*	54.9*	77.6*	195.0*	2120.8*
89A122-12	1	66.4*	293.6*	59.0	45.9*	40.0	118.5*	1277.8
89A122-13	1	70.0*	295.8*	48.4	51.4*	46.6*	114.9*	1342.4
89A122-14	1	66.6*	290.9*	50.5	43.4	40.3	108.9*	1259.8
89A124-2	2	70.7*	309.6*	55.6	55.5*	45.3	118.9*	1422.6*
89A124-3	2	65.2*	297.0*	56.6	47.1*	42.9	112..4*	1340.5
89A124-4	2	69.9*	306.7*	71.2*	58.2*	46.8*	114.4*	1491.2*
89A124-5	2	74.5*	415.4*	57.7	47.4*	65.7*	213.7*	1960.7*

* Significantly greater than the control at $P \leq 0.05$

Appendix 7. (Continued)

		Amino Acids						
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A124-6	2	88.7*	418.2*	94.0*	69.4*	52.8*	155.2*	1697.2*
89A124-7	2	59.3	281.2	57.7	47.9*	41.2	109.6*	1286.2
89A124-8	2	75.8*	339.8*	64.7*	49.4*	47.1*	107.2*	1637.4*
89A124-9	2	62.1	251.6	78.3*	53.4*	38.4	90.4	1280.7.
89A124-10	2	72.8*	269.7*	110.0*	58.3*	41.6	99.2*	1518.2*
89A124-11	2	59.6	226.2	67.6*	43.6	48.7*	81.5	1329.0
89A124-12	2	64.2*	261.0*	56.4	45.9*	44.2	103.5*	1197.0
89A124-14	2	65.9*	296.7*	76.5*	47.9*	51.9*	119.6*	1673.5*
89A124-15	2	76.0*	426.6*	71.3*	54.3*	58.7*	159.1*	1945.7*
89A127-1	1	67.7*	294.5*	89.8*	65.1*	48.4	111.7*	1529.2*
89A128-M	0	59.9	239.3	59.1	42.7	41.3	88.4	1227.4
LSD 0.05		3.3	18.5	4.5	2.4	4.1	8.9	133.4

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 8

Mole percentages of total amino acids in R_2 seed

Appendix 8. Mole percentages of total amino acids in R_2 seed.

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A121-1	1	3.47	3.59	2.41	1.84	11.29	9.69	3.26*	5.15*	6.15*	2.27	4.33*
89A121-2	1	6.73*	3.05	2.53	3.20*	14.76	9.60	2.42	3.51	4.55	2.33	4.65*
89A121-4	1	5.70	3.29	3.22*	3.32*	16.43*	8.58	2.81	3.46	4.76	2.57	4.56*
89A122-1	1	5.80	3.18	2.78*	2.88*	16.78*	9.78	2.69	3.58	4.73	2.00	4.35*
89A122-2	1	3.44	3.36	2.26	1.50	14.18	9.69	2.99	4.73*	6.02*	2.14	3.54
89A122-3	1	5.90	3.41	2.27	1.85	20.53*	10.25	2.71	3.38	4.86	2.47	2.90
89A122-4	1	5.17	3.64	2.08	1.59	14.53	10.45	2.79	3.65	4.80	2.14	3.62
89A122-5	1	5.99	3.60	2.15	2.08*	15.99	6.98	2.80	3.98	5.30	2.90*	3.52
89A122-6	1	4.80	4.09*	2.81*	1.28	18.00*	9.59	3.81*	4.17*	5.46	2.09	2.81
89A122-7	1	3.51	3.76	2.64*	1.28	13.38	8.69	3.73*	4.57*	6.07*	2.17	4.89*
89A122-8	1	5.08	4.05	2.68*	1.65	15.52	9.29	3.22*	4.10*	4.91	2.54	3.91
89A122-9	1	3.94	3.62	2.05	1.25	13.01	8.77	3.13*	5.01*	6.38*	2.24	3.30
89A122-10	1	5.29	3.61	2.20	2.21*	14.69	9.47	2.48	3.59	5.05	2.41	4.40*
89A122-11	1	6.45*	3.13	2.31	2.79*	13.74	9.59	2.04	3.23	4.51	1.57	4.45*

* Significantly greater than the control at $P \leq 0.05$

Appendix 8. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A122-12	1	3.70	3.90	2.03	1.35	11.67	8.87	3.38*	4.57*	6.05*	1.97	3.76
89A122-13	1	3.97	3.53	2.68*	1.58	11.56	9.50	3.08	4.93*	5.71*	2.44	4.34*
89A122-14	1	3.48	3.13	2.11	1.45	12.15	9.97	2.73	4.70*	5.95*	2.42	4.26*
89A124-2	2	4.20	3.95	2.10	1.74	12.20	9.52	2.88	4.87*	5.76*	2.51	4.22*
89A124-3	2	5.24	3.66	2.10	1.84	12.38	9.98	2.76	4.07	5.01	2.44	4.24*
89A124-4	2	4.45	3.67	2.60*	1.51	14.28	9.77	3.00	4.18*	5.30	2.56	3.98
89A124-5	2	7.70*	2.88	2.06	3.97*	12.08	9.15	2.20	3.62	4.14	2.26	5.38*
89A124-6	2	4.35	3.47	1.98	2.91*	11.20	9.69	2.76	4.23*	5.19	2.12	5.86*
89A124-7	2	5.18	3.78	2.03	1.62	13.05	9.70	2.56	3.92	4.95	2.69	4.12
89A124-8	2	5.81	3.43	2.40	1.91	18.35*	9.74	2.41	3.51	5.45	1.99	3.26
89A124-9	2	4.96	3.99	2.72*	1.22	14.21	8.82	3.47*	4.66*	6.20*	1.37	3.57
89A124-10	2	3.96	5.32*	2.56*	1.47	14.93	9.17	4.31*	4.15*	5.81*	2.60	2.80
89A124-11	2	5.39	3.59	2.93*	1.79	18.69*	9.73	3.08	3.79	5.63*	2.90*	2.82
89A124-12	2	4.91	3.81	2.60*	1.55	10.13	9.37	3.07	4.61*	5.43	2.75	4.48*

* Significantly greater than the control at $P \leq 0.05$

Appendix 8. (Continued)

R_2 Seed	LT	Amino Acids										
		ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A124-14	2	4.92	3.39	3.33	2.28*	20.70*	8.73	2.89	3.54	5.19	2.17	3.53
89A124-15	2	7.11*	3.45	2.52	3.13*	13.55	10.30	2.43	3.28	4.34	2.44	4.01
89A127-1	1	5.66	4.30*	2.63*	1.45	14.05	9.11	3.46*	3.89	5.11	2.27	3.81
89A128-M	0	5.82	3.84	2.36	1.74	15.33	9.84	2.92	3.82	5.38	2.19	3.56
LSD (0.05)		0.44	0.22	0.17	0.30	0.70	1.40	0.19	0.25	0.20	0.56	0.58

* Significantly greater than the control at $P \leq 0.05$

Appendix 8. (Continued)

		Amino Acids					
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A121-1	1	5.11	22.36*	3.30	3.75*	3.44	8.63*
89A121-2	1	3.93	19.99	3.30	2.46	3.39	9.65*
89A121-4	1	4.04	18.03	3.75	2.96	3.65*	8.89*
89A122-1	1	4.08	19.85	3.34	2.77	3.39	8.08*
89A122-2	1	5.18*	22.51*	3.11	3.47	3.20	8.73*
89A122-3	1	4.15	19.96	3.89	2.67	2.74	6.11
89A122-4	1	4.01	23.53*	4.62	3.62	2.75	7.06
89A122-5	1	4.77	21.37*	4.93	3.38	3.22	7.09
89A122-6	1	4.54	18.36	5.51*	3.51	3.30	5.91
89A122-7	1	4.84	20.15*	5.65*	3.98*	2.91	7.91*
89A122-8	1	4.37	19.22	4.52	3.73*	3.35	7.89*
89A122-9	1	5.25*	22.09*	5.04	3.75*	2.72	8.47*
89A122-10	1	4.71	21.49*	4.03	3.12	3.09	8.24*

* Significantly greater than the control at $P \leq 0.05$

Appendix 8. (Continued)

		Amino Acids					
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A122-11	1	4.18	22.62*	4.00	2.59	3.66*	9.20*
89A122-12	1	5.20*	22.97*	4.62	3.59	3.13	9.27*
89A122-13	1	5.22*	22.04*	3.60	3.83*	3.47	8.56*
89A122-14	1	5.28*	23.09*	4.01	3.45	3.20	8.65*
89A124-2	2	4.97	21.76*	3.92	3.90*	3.19	8.36*
89A124-3	2	4.87	22.15*	4.22	3.51	3.20	8.38*
89A124-4	2	4.69	20.57*	4.78	3.90*	3.14	7.67
89A124-5	2	3.80	21.19*	2.95	2.42	3.35	10.90*
89A124-6	2	4.68	22.04*	4.95	3.66*	2.78	8.18*
89A124-7	2	4.62	21.87*	4.49	3.73*	3.21	8.52*
89A124-8	2	4.64	20.75*	3.95	3.02	2.88	6.55
89A124-9	2	4.85	19.65	6.12*	4.17*	3.00	7.06
89A124-10	2	4.80	17.76	7.31*	3.84*	2.75	6.53
89A124-11	2	4.49	17.02	5.09	3.29	3.67*	6.13

* Significantly greater than the control at $P \leq 0.05$

Appendix 8. (Continued)

		Amino Acids					
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A124-12	2	5.28*	21.46*	4.64	3.78*	3.64*	8.52*
89A124-14	2	3.94	17.73	4.57	2.86	3.10	7.14
89A124-15	2	3.91	21.92*	3.66	2.79	3.02	8.18*
89A127-1	1	4.44	19.26	5.88*	4.26*	3.17	7.30
89A128-M	0	4.88	19.50	4.81	3.48	3.36	7.20
LSD (0.05)		0.27	0.64	0.23	0.16	0.23	0.52

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 9

Free amino acid concentration in R_2 seed

Appendix 9. Free amino acid concentraation in R_2 seed

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A121-1	1	573.7	502.0	625.3	248.7	715.6	1044.6	286.4	309.2	708.3	355.8	249.4
89A121-2	1	884.1	861.7*	1323.8	276.1	1065.1	1811.6	441.5*	309.5	669.7	437.2	293.1
89A121-4	1	629.7	528.9	1374.2	432.4*	890.8	1440.1	342.0	293.9	625.0	378.9	225.0
89A122-1	1	1188.3*	572.5	1248.9	224.7	1090.4	1197.2	297.5	271.5	604.5	299.4	402.1
89A122-2	1	345.2	292.1	578.8	352.0	557.3	793.1	279.0	305.7	605.3	298.1	220.0
89A122-3	1	898.8	558.0	950.4	199.6	574.7	997.2	266.1	301.8	581.5	266.6	333.1
89A122-4	1	509.9	492.8	585.0	554.4*	586.0	830.7	300.3	372.0	539.2	370.7	265.4
89A122-5	1	554.0	488.3	776.2	235.3	695.9	1075.2	277.9	232.8	535.6	387.0	202.2
89A122-6	1	606.0	384.0	662.9	209.2	882.8	958.7	248.2	353.3	637.0	328.9	277.0
89A122-7	1	682.0	502.6	820.6	247.7	835.0	993.7	320.1	355.4	548.5	360.0	306.3
89A122-8	1	429.6	430.9	597.2	309.5	499.6	903.8	268.4	327.1	631.8	319.3	245.0
89A122-9	1	641.7	392.6	636.8	247.9	922.0	1015.7	260.6	362.9	511.0	300.7	293.9
89A122-10	1	910.2	629.2	1275.3	288.2	902.5	1671.1	408.2	290.5	625.9	461.7	286.3

* Significantly greater than the control at $P \leq 0.05$

Appendix 9. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A122-11	1	964.0	535.0	956.5	1093.8*	656.7	1091.9	285.1	310.0	676.2	277.5	643.6*
89A122-12	1	880.0	501.8	1191.3	279.3	1540.2*	1394.6	442.0*	360.7	593.5	310.0	381.6
89A122-13	1	721.8	565.2	1058.4	200.7	838.3	1441.2	349.7	311.2	410.9	471.0	269.3
89A122-14	1	468.1	360.4	496.4	290.0	670.0	979.4	251.7	290.1	667.4	246.2	255.4
89A124-2	2	1103.3	614.4	1488.4	223.3	776.0	1512.5	331.7	288.2	575.7	406.4	214.9
89A124-3	2	981.6	561.8	918.2	308.9	724.4	1690.3	394.9	297.2	600.9	458.6	232.1
89A124-4	2	897.1	733.1*	1670.6	391.5	797.5	1638.0	420.9	310.7	548.7	449.6	244.9
89A124-5	2	883.5	510.8	1259.3	346.5	662.4	1136.3	293.8	303.1	609.6	400.6	363.7
89A124-6	2	394.7	579.1	662.9	393.9	379.6	937.2	305.1	318.7	563.1	339.1	215.8
89A124-7	2	1017.1	559.3	1484.5	300.6	922.8	1696.2	351.5	280.6	609.1	400.7	254.1
89A124-8	2	698.4	451.4	1226.1	312.4	614.6	1069.1	269.9	310.8	553.7	438.2	553.4*
89A124-9	2	608.8	404.1	1067.1	167.8	756.0	1081.8	287.3	341.7	635.8	231.2	247.6
89A124-10	2	633.0	430.6	1057.7	188.0	1026.9	947.3	326.1	369.4	659.0	331.2	305.8

* Significantly greater than the control at $P \leq 0.05$

Appendix 9. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A124-11	2	516.6	571.8	1379.7	234.7	842.8	1364.5	323.3	269.2	605.9	340.2	224.8
89A124-12	2	457.5	530.4	700.4	276.6	639.4	1024.1	274.5	318.8	667.7	357.8	240.1
89A124-14	2	769.7	503.2	926.6	249.1	592.0	1178.8	271.5	335.4	689.7	376.9	280.6
89A124-15	2	1020.6	495.1	1067.4	265.5	605.9	1185.0	312.3	330.9	690.6	322.4	405.3
89A127-1	1	665.5	534.1	1107.1	196.3	763.7	1041.6	297.3	340.4	408.1	233.3	276.5
89A128-M	0	619.1	440.2	1012.8	261.6	628.2	1111.8	288.1	326.6	603.6	379.4	428.3
LSD (0.05)		508.1	264.2	841.4	147.4	568.7	839.8	147.3	57.8	114.2	169.0	51.7

* Significantly greater than the control at $P \leq 0.05$

Appendix 9. (Continued)

		Amino Acids						
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A121-1	1	407.4	1080.4	306.6	201.4	333.9	603.1	8852
89A121-2	1	397.6	1709.7	573.7	366.3*	450.5	634.1	12505
89A121-4	1	419.0	1717.8	552.5	277.8	393.5	721.0	11242
89A122-1	1	407.7	1889.9	484.2	250.9	355.4	543.7	11329
89A122-2	1	387.4	940.0	338.9	190.0	301.7	572.2	7357
89A122-3	1	355.8	1080.9	380.3	225.7	293.5	582.3	8847
89A122-4	1	491.4*	950.1	328.9	210.3	311.6	681.7	8380
89A122-5	1	328.3	1059.6	233.0	217.8	398.9	509.1	8207
89A122-6	1	414.1	1422.0	423.9	221.1	331.4	693.7	9054
89A122-7	1	404.7	1411.5	457.1	252.9	359.3	612.1	9469
89A122-8	1	380.9	765.4	307.2	212.5	282.0	666.0	7576
89A122-9	1	438.5*	1511.8	419.3	252.2	309.6	671.6	9189

* Significantly greater than the control at $P \leq 0.05$

Appendix 9. (Continued)

		Amino Acids						
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A122-10	1	392.1	1793.3	745.5*	296.5	411.6	640.5	12020
89A122-11	1	487.2*	1524.2	648.3	248.1	329.0	565.0	11292
89A122-12	1	453.1*	2352.7*	561.7	343.3	432.0	751.8	12770
89A122-13	1	384.7	1371.8	431.2	271.8	347.7	794.7	10239
89A122-14	1	416.9	1070.9	273.8	179.1	357.4	566.4	7840
89A124-2	2	404.5	1285.5	356.4	263.4	358.1	577.8	10780
89A124-3	2	443.1*	1304.6	505.6	267.3	330.4	638.3	10658
89A124-4	2	454.3*	1140.1	563.1	307.0	609.6*	746.7	11923
89A124-5	2	368.8	1163.2	395.1	228.5	303.3	574.3	9803
89A124-6	2	360.4	748.2	379.5	221.1	318.3	600.2	7679
89A124-7	2	403.8	674.3	442.5	301.5	392.3	665.2	10756
89A124-8	2	423.8*	1223.6	421.0	217.6	372.7	533.5	9690
89A124-9	2	412.3	1133.2	398.2	219.3	321.2	619.9	8933

* Significantly greater than the control at $P \leq 0.05$

Appendix 9. (Continued)

R_2 Seed	LT	Amino Acids						
		PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
89A124-10	2	466.9*	1536.5	447.8	264.8	392.9	655.7	10040
89A124-11	2	377.3	1254.7	483.3	285.2	380.2	646.9	10101
89A124-12	2	389.7	960.6	330.2	213.9	377.5	584.8	8344
89A124-14	2	422.7*	1322.0	439.8	233.1	355.7	637.9	9585
89A124-15	2	411.6	1236.3	419.9	240.4	339.7	577.1	9926
89A127-1	1	371.9	1123.4	442.6	243.2	288.0	768.9	9102
89A128-M	0	390.1	1077.5	478.1	230.6	347.2	686.9	9310
LSD (0.05)		31.6	827.1	258.7	114.1	141.0	115.3	4227

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 10

Mole percentages of free amino acids in R_2 seed

Appendix 10. Mole percentages of free amino acids in R_2 seed.

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A121-1	1	6.71	5.87*	7.31	2.91	8.37	12.25	3.35	3.62	8.28	4.16	2.92
89A121-2	1	7.07	6.89*	10.60	2.21	8.52	14.50	3.53	2.48	5.36	3.50	2.35
89A121-4	1	5.52	4.68	11.73	4.00	7.71	12.70	3.03	2.79	5.84	3.48	2.07
89A122-1	1	10.40*	5.05	10.90	2.01	9.54*	10.60	2.65	2.46	5.46	2.67	3.57
89A122-2	1	4.70	3.97	7.87	4.79	7.58	10.75	3.79	4.16	8.23	4.05	2.99
89A122-3	1	10.15*	6.31*	10.75	2.26	6.50	11.25	3.01	3.41	6.57	3.02	3.77
89A122-4	1	6.09	5.88*	6.99	6.62*	6.99	9.92	3.59	4.44	6.42	4.43	3.17
89A122-5	1	6.56	5.88*	9.11	3.09	8.10	12.85	3.33	3.06	6.86	4.73	2.58
89A122-6	1	6.69	4.25	7.32	2.31	9.73*	10.60	2.74	3.91	7.04	3.64	3.06
89A122-7	1	7.20	5.31	8.67	2.61	8.82	10.50	3.38	3.75	5.80	3.81	3.24
89A122-8	1	5.67	5.69*	7.89	4.06	6.59	11.95	3.55	4.32	8.34	4.22	3.24
89A122-9	1	6.98	4.28	6.93	2.71	10.03*	11.05	2.84	3.95	5.56	3.28	3.20
89A122-10	1	7.51	5.23	10.60	2.41	7.50	13.90	3.37	2.45	5.26	3.87	2.40
89A122-11	1	8.54	4.74	8.47	9.69*	5.82	9.67	2.53	2.75	5.99	2.46	5.70*

* Significantly greater than the control at $P \leq 0.05$

Appendix 10. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A122-12	1	6.71	4.17	9.11	2.17	11.70	10.44	3.61	3.03	5.04	2.63	3.06
89A122-13	1	6.86	5.49	9.88	2.12	7.81	13.75	3.36	3.40	4.29	4.67	2.82
89A122-14	1	5.97	4.60	6.33	3.70	8.55	12.50	3.22	3.70	8.51	3.15	3.26
89A124-2	2	9.83*	5.58*	13.15	2.48	7.03	13.45	3.07	2.95	5.95	3.79	2.15
89A124-3	2	8.87*	5.22	8.17	3.17	6.51	15.35*	3.72	3.08	6.03	4.36	2.31
89A124-4	2	7.20	6.00*	13.10	3.73	6.35	13.25	3.58	2.91	5.06	3.85	2.15
89A124-5	2	8.96*	5.21	12.85	3.55	6.77	11.60	3.02	3.10	6.23	4.04	3.71
89A124-6	2	5.14	7.54*	8.14	5.13*	4.95	12.25	3.97*	4.15	7.34	4.42	2.81
89A124-7	2	9.16*	5.10	13.25	3.00	8.12	15.35	3.19	2.80	5.97	3.75	2.44
89A124-8	2	7.21	4.67	12.65	3.16	6.36	11.00	2.84	3.23	5.79	4.43	5.72*
89A124-9	2	6.80	4.53	11.95	1.89	8.45	12.15	3.24	3.83	7.13	2.62	2.77
89A124-10	2	6.31	4.29	10.55	1.87	10.20*	9.44	3.24	3.68	6.57	3.30	3.05
89A124-11	2	4.98	5.56*	13.15	2.53	8.22	13.10	3.25	2.89	6.35	3.44	2.34
89A124-12	2	5.49	6.36*	8.41	3.31	7.68	12.30	3.30	3.83	8.00	4.28	2.88

* Significantly greater than the control at $P \leq 0.05$

Appendix 10. (Continued)

		Amino Acids										
R_2 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A124-14	2	8.03	5.26	9.67	2.60	6.17	12.25	3.15	3.50	7.20	3.94	2.93
89A124-15	2	10.30*	4.99	10.75	2.68	6.11	11.95	2.84	3.36	6.96	3.25	4.09
89A127-1	1	7.31	5.87*	12.15	2.16	8.39	11.45	3.27	3.74	4.49	2.58	3.04
89A128-M	0	6.66	4.73	10.90	2.81	6.75	11.95	3.10	3.51	6.48	4.08	4.60
LSD (0.05)		1.90	0.81	3.46	2.12	2.18	2.84	0.82	1.64	2.77	1.26	0.78

* Significantly greater than the control at $P \leq 0.05$

Appendix 10. (Continued)

		Amino Acids					
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A121-1	1	4.77	12.63	3.59	2.36	3.91	7.05
89A121-2	1	3.18	13.67	4.59	2.93*	3.60	5.07
89A121-4	1	3.90	15.00*	4.85	2.49	3.54	6.68
89A122-1	1	3.68	16.60*	4.24	2.22	3.15	4.86
89A122-2	1	5.27	12.78	4.61	2.59	4.10	7.78
89A122-3	1	4.03	12.22	4.30	2.55	3.32	6.58
89A122-4	1	5.87*	11.33	3.93	2.51	3.72	8.14
89A122-5	1	4.20	12.58	2.97	2.64	4.74	6.69
89A122-6	1	4.58	15.71*	4.68	2.44	3.66	7.67
89A122-7	1	4.28	14.91*	4.83	2.67	3.79	6.47
89A122-8	1	5.03	10.10	4.06	2.81*	3.72	8.79
89A122-9	1	4.77	16.46*	4.56	2.74	3.37	7.31
89A122-10	1	3.26	14.93*	6.07	2.47	3.43	5.37
89A122-11	1	4.32	13.50	5.75	2.20	2.92	5.00

* Significantly greater than the control at $P \leq 0.05$

Appendix 10. (Continued)

		Amino Acids					
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A122-12	1	3.81	17.80*	4.48	2.74	3.41	6.09
89A122-13	1	4.14	12.81	4.13	2.68	3.48	8.36
89A122-14	1	5.32	13.66	3.49	2.29	4.56	7.23
89A124-2	2	4.02	11.60	3.37	2.41	3.43	5.74
89A124-3	2	4.39	11.88	4.61	2.54	3.20	6.60
89A124-4	2	4.06	9.23	4.55	2.56	5.75*	6.72
89A124-5	2	3.77	11.87	4.04	2.34	3.10	5.89
89A124-6	2	4.70	9.74	4.95	2.88*	4.15	7.82
89A124-7	2	3.89	6.98	4.02	2.75	3.69	6.53
89A124-8	2	4.40	12.62	4.33	2.26	3.85	5.53
89A124-9	2	4.62	12.66	4.46	2.46	3.59	6.95
89A124-10	2	4.65	15.32*	4.46	2.64	3.92	6.54
89A124-11	2	3.92	12.13	4.77	2.80*	3.86	6.75
89A124-12	2	4.67	11.52	3.96	2.57	4.54	7.01

* Significantly greater than the control at $P \leq 0.05$

Appendix 10. (Continued)

		Amino Acids					
R_2 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
89A124-14	2	4.42	13.79	4.59	2.43	3.72	6.68
89A124-15	2	4.15	12.46	4.23	2.42	3.43	5.81
89A127-1	1	4.09	12.34	4.86	2.67	3.17	8.45
89A128-M	0	4.19	11.57	5.14	2.48	3.73	7.38
LSD (0.05)		1.52	3.14	1.14	0.27	1.49	2.89

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 11

Total amino acid concentration in R_3 seed

Appendix 11. Total amino acid concentration in R_3 seed

		Amino Acids										
R_3 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
90A103-7	1	49.5	65.0	32.1	18.1	198.8	171.9	40.9	59.9	74.4	49.5*	60.6
90A104-14	1	111.7*	166.3*	116.7*	36.2*	697.3*	304.7*	92.9*	91.2*	144.0*	68.8*	97.2*
90A107-10	1	65.0*	82.8*	44.3	27.4*	389.9*	197.3*	51.3	61.3	95.2	35.9	69.3
90A108-2	1	52.7	79.5*	52.4	12.8	351.1	153.3	47.3	49.5	81.8	35.7	51.4
90A113-1	1	41.9	65.5	36.8	10.9	296.3	132.0	40.2	46.7	70.9	20.1	38.9
90A135-13	2	62.9*	81.3*	41.3	11.0	445.9*	187.2*	49.6	57.1	95.9	30.3	55.2
90A135-19	2	69.9*	106.2*	54.2	14.8	422.6*	190.0*	57.1*	62.0	92.1	44.0	63.4
90A138-14	2	45.1	57.4	36.4	25.6*	307.6	143.1	36.1	46.3	73.9	33.2	37.6
Control	0	48.8	65.3	38.3	10.3	316.4	147.5	42.7	49.9	82.2	28.8	53.8
LSD (0.05)		12.4	12.8	16.3	12.4	60.8	36.5	10.4	12.6	15.7	20.4	21.6

* Significantly greater than the control at $P \leq 0.05$

Appendix 11. (Continued)

		Amino Acids						
R_3 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
90A103-7	1	81.6*	363.6	89.2	47.0	52.3	144.8*	1599.2
90A104-14	1	115.0*	517.1*	155.0*	76.0*	102.9*	182.0*	3074.7*
90A107-10	1	72.1	349.0	110.2	48.3	67.9*	108.5	1875.9*
90A108-2	1	61.1	251.6	128.2	44.5	56.9	82.5	1592.2
90A113-1	1	51.4	230.4	71.6	30.5	48.1	75.2	1307.3
90A135-13	2	68.4	303.2	91.2	43.2	63.7	94.9	1782.5
90A135-19	2	63.9	327.8	114.8	56.0*	63.7	113.3	1915.5*
90A138-14	2	55.9	244.8	86.9	35.5	28.8	85.3	1379.5
Control	0	60.3	284.6	111.9	41.4	53.0	83.7	1518.8
LSD (0.05)		17.0	81.4	24.4	10.8	14.7	31.8	330.2

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 12

Mole percentages of total amino acids in R_3 seed

Appendix 12. Mole percentages of total amino acids in R_3 seed

		Amino Acids										
R_3 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
90A103-7	1	3.09	4.13	2.00	1.15	12.38	10.75*	2.56	3.75*	4.66	3.20	3.79
90A104-14	1	3.64*	5.41*	3.80*	1.08	22.71*	9.90	3.03	2.97	4.69	2.24	3.17
90A107-10	1	3.48*	4.43	2.37	1.16	20.88	10.55*	2.75	3.29	5.10	1.90	3.73
90A108-2	1	3.30	4.99	3.29*	0.81	22.10*	9.64	2.97	3.11	5.14	2.25	3.19
90A113-1	1	3.21	5.00	2.81	0.84	22.66*	10.10	3.08	3.58	5.43	1.54	2.99
90A135-13	2	3.53*	4.57	2.32	0.62	25.03*	10.50*	2.78	3.21	5.38	1.72	3.10
90A135-19	2	3.65*	5.55*	2.82	0.78	22.07*	9.92	2.99	3.24	4.81	2.29	3.31
90A138-14	2	3.28	4.16	2.64	1.86*	22.29*	10.40*	2.63	3.35	5.37	2.41	2.69
Control	0	3.22	4.31	2.52	0.69	20.84	9.71	2.82	3.29	5.41	1.90	3.55
LSD (0.05)		0.21	0.79	0.57	0.67	1.08	0.41	0.54	0.33	0.27	1.43	1.15

* Significantly greater than the control at $P \leq 0.05$

Appendix 12. (Continued)

R_3 Seed	LT	Amino Acids					
		PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
90A103-7	1	5.18*	22.63*	5.59	2.94	3.25	9.03
90A104-14	1	3.74	16.84	5.05	2.48	3.35	5.93
90A107-10	1	3.84	18.66	5.90	2.58	3.64	5.78
90A108-2	1	3.81	15.85	8.05	2.78	3.57	5.19
90A113-1	1	3.94	17.64	5.46	2.34	3.68	5.75
90A135-13	2	3.84	17.01	5.12	2.43	3.58	5.32
90A135-19	2	3.34	17.10	6.00	2.93	3.33	5.92
90A138-14	2	4.05	17.75	6.32	2.59	2.09	6.19
Control	0	3.98	18.74	7.37	2.73	3.49	5.50
LSD (0.05)		1.03	1.34	0.98	0.26	0.39	0.76

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 13

Free amino acid concentration in R_3 seed

Appendix 13. Free amino acids concentration in R_3 seed

		Amino Acids										
R_3 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
90A103-7	1	770.2*	291.0*	483.7	98.9*	278.5	832.9	126.7	192.0	315.9*	144.6*	235.8
90A104-14	1	1568.4*	1332.9*	3924.3*	132.6*	1201.6*	1088.4	746.3	436.6*	651.2*	525.4*	732.7*
90A107-10	1	773.5*	1124.2*	1346.7*	137.6*	404.5*	926.4	199.0	211.0*	458.5*	326.3*	338.2
90A108-2	1	716.9*	250.0	634.8	84.2	254.7	753.9	155.8	194.9	324.6*	156.0*	271.1
90A113-1	1	609.4	754.8*	1068.1*	105.4*	349.5*	893.9	129.8	201.5	326.3*	151.7*	498.9*
90A135-13	2	481.9	244.8	677.0	172.5*	154.7	593.2	109.6	174.6	282.8	126.5	491.1*
90A135-19	2	618.3	318.9*	986.1*	136.3*	241.6	604.3	152.8	205.7	352.2*	166.4*	506.9*
90A138-14	2	457.0	190.1	604.5	64.8	176.4	586.8	99.4	166.8	263.5	134.9	359.8
Control	0	455.9	190.0	662.9	76.9	173.5	503.0	77.4	186.4	273.0	106.9	403.9
LSD (0.05)		205.4	79.1	195.0	19.7	114.3	180.2	93.0	20.5	24.9	36.5	40.3

* Significantly greater than the control at $P \leq 0.05$

Appendix 13. (Continued)

		Amino Acids						
R_3 Seed	LT	PHE	PRO	SER	THR	TYR	VAL	TOTAL
		nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}	nmol mg^{-1}
90A103-7	1	162.9	638.9*	356.6	92.3	362.3*	468.4	5851.5*
90A104-14	1	318.2*	716.3*	526.3	235.2*	450.9*	1213.9	15800.9*
90A107-10	1	239.8*	873.9*	283.1	122.3*	352.0*	508.7	8625.6*
90A108-2	1	178.4	539.1	331.1	94.3	250.5*	421.7	5611.8*
90A113-1	1	151.5	676.1*	433.6	86.7	223.5*	352.0	7012.7*
90A135-13	2	167.1	683.5*	276.3	89.2	120.2	398.1	5243.1
90A135-19	2	210.5*	472.6	292.1	94.9	253.6*	464.6	6077.8*
90A138-14	2	142.7	429.5	242.6	44.7	217.4	340.8	4521.7
Control	0	153.6	480.5	271.5	79.4	151.6	353.2	4599.7
LSD (0.05)		42.2	122.4	67.9	36.3	60.7	24.2	713.0

* Significantly greater than the control at $P \leq 0.05$

APPENDIX 14

Mole percentages of free amino acids in R_3 seed

Appendix 14. Mole percentages of free amino acids in R_3 seed

		Amino Acids										
R_3 Seed	LT	ALA	ARG	ASX	CYS	GLX	GLY	HIS	ILE	LEU	LYS	MET
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
90A103-7	1	13.15*	4.96*	8.27	1.70	4.78	14.2*	2.17	3.29	5.41	2.50	4.03
90A104-14	1	9.95	8.44*	24.84*	0.84	7.61*	6.89	4.72*	2.77	4.12	3.33*	4.64
90A107-10	1	8.97	13.00*	15.61	1.60	4.68	10.75	2.32	2.45	5.32	3.78*	3.93
90A108-2	1	12.65*	4.44	11.21	1.54	4.55	13.35*	2.81*	3.52	5.84	2.83	4.84
90A113-1	1	8.69	10.75*	15.21	1.51	4.98	12.75*	1.85	2.88	4.67	2.18	7.12
90A135-13	2	9.21	4.68	12.89	3.30*	2.92	11.30	2.09	3.34	5.42	2.42	9.39
90A135-19	2	10.15	5.25*	16.23	2.25*	3.98	9.94	2.52	3.39	5.80	2.74	8.34
90A138-14	2	10.10	4.20	13.35	1.44	3.88	12.95*	2.20	3.70	5.84	2.99	7.97
Control	0	9.91	4.13	14.41	1.68	3.77	10.90	1.69	4.06	5.94	2.33	8.79
LSD (0.05)		2.33	0.79	1.96	0.49	1.66	1.64	0.85	0.75	0.99	0.91	0.70

* Significantly greater than the control at $P \leq 0.05$

Appendix 14. (Continued)

		Amino Acids					
R_3 Seed	LT	PHE	PRO	SER	THR	TYR	VAL
		Mol%	Mol%	Mol%	Mol%	Mol%	Mol%
90A103-7	1	2.80	10.95	6.10	1.57	6.20*	8.01
90A104-14	1	2.02	4.54	3.33	1.49	2.86	7.69
90A107-10	1	2.78	10.15	3.29	1.42	4.08	5.90
90A108-2	1	3.25	9.53	5.92	1.69	4.50	7.56
90A113-1	1	2.18	9.64	6.17	1.25	3.20	5.03
90A135-13	2	3.20	13.00*	5.26	1.71	2.30	7.61
90A135-19	2	3.47	7.78	4.81	1.57	4.18	7.65
90A138-14	2	3.16	9.50	5.36	0.98	4.84*	7.54
Control	0	3.34	10.44	5.91	1.73	3.29	7.68
LSD (0.05)		1.08	1.68	0.76	0.61	1.49	0.71

* Significantly greater than the control at $P \leq 0.05$

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