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Gamma Irradiation-Derived, Methionine-Enriched Mutant Lines of *Lathyrus sativus* L.

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ABSTRACT

Methionine (Met), an essential sulphur-containing amino acid, is supposed to protect from lathyrism disease caused by over-consumption of *Lathyrus sativus* (grass pea). There is a strong and positive association between moisture stress and the biosynthesis of the crippling lathyrism causative molecule, 3-(N-oxalyl)-L-2, 3-diamino propionic acid (β-ODAP). Seeds of *L. sativus* local cv. 'Debre Zeit' were irradiated using 40 kR gamma cell irradiation aimed at genetic alteration in favour of overproduction of Met-based seed proteins. The amino acid determination in seeds were analysed using HPLC with pre-column PITC derivatization. Three morpho-phenotypically distinct mutant lines were identified and selected from irradiated clusters, which displayed glittering deep green leaves, stem and pods, smaller leaf and plant size, and reasonable yield compared to the parental line. Observation of root growth response in ethionine media supported the phenotypic and chromatograph findings. Chemical analysis of seeds revealed that the putative mutants had 63% improved Met over the parent (control). This level shifts the Met supply capacity of grass pea from 25% in the parent line to 50% in the altered putative mutant lines.

Keywords: diet, irradiation, *Lathyrus sativus*, Met, phenotype
Abbreviations: DG, deep green; **DZARC**, Debre Zeit Agricultural Research Center; **HPLC**, High Pressure Liquid Chromatography; **Met**, Met; **ODAP**, 3-(N-oxalyl)-L-2, 3-diamino propionic acid; **PITC**, Phenyl isothiocyanate

INTRODUCTION

Ethiopia is the largest producer (85%) of *Lathyrus sativus* L. (grass pea) in Africa and is the most affected by undesirable toxicity of the crop (Fikre 2007). Grass pea is a good source of proteins, constituting about 28% of dry seed weight (Urga *et al.* 2005) but is limited in methionine (Met) and cysteine amino acids (Kuo *et al.* 1995), accounting for only a quarter of standard egg protein (Coates *et al.* 1985).

Lathyrism is an irreversible crippling disease that occurs as a consequence of over-consumption of grass pea. The causative molecule 3-(*N*-oxalyl)-L-2, 3-diamino propionic acid (β-ODAP) is a free amino acid (Lambein *et al.* 2001; Rao 2001) that is affected both by genetic and environmental factors (Fikre *et al.* 2011). The neurotoxicity mechanism of β-ODAP is counteracted by the antioxidant activity of Met (Getahun *et al.* 2005) that makes it a complete protein source (Molving *et al.* 1997).

Because cysteine is a reactant in Met biosynthesis, the internal concentration of cysteine may contribute to the regulation of Met biosynthesis (Kim *et al.* 1999). The internal concentration of *O*-acetyl serine (OAS) might regulate the relative abundance of the β-subunit of β-conglacynine (Kim *et al.* 1999). Thus strategically located mutation in the genes encoding either cystathionine γ-synthase or serine acetyl-transferase may promote Met overproduction and the genetic improvement of sulphur amino acid-deficient grass pea. The aim of the study was to mutate grass pea seeds to find Met-rich variants.

MATERIALS AND METHODS

A total of 3000 seeds of *Lathyrus sativus* landrace cv. "Debre Zeit" were irradiated with 40kR doses of gamma rays (at the Debre Zeit Agricultural Research Center (DZARC), Ethiopia) in 2003. Irradiated seeds (M_0) were planted along with parental checks in insect-proof plots and M_1 seeds derived from M_0 plants were harvested, bulked and subsequently planted. In M_2, three distinct mutant lines showing true breeding nature for a number of contrasting morphological traits (leaf color (degree of greenness), leaf size, plant size, shininess (i.e., how shining the plant is, referring to the leaf and stem)) versus their parental cultivar were identified and independently advanced to raise M_3 generation plants. M_3 selfed mutant lines had an average of 76% purity. These selections were isolated and independently harvested. Chemical analysis was undertaken on the M_3-derived lines referred to as M_4 plants. These M_4 lines were approx. 95% pure. The sulphur concentration and level of β-ODAP from randomly selected seeds (M_4) was determined using high pressure liquid chromatography (HPLC) following a standard procedure (Kuo *et al.* 2003) as indicated below.

Preparation of seed extracts for free amino acids analysis

200 mg of seed powder was weighed and mixed with 10 ml of 70% ethanol. To each sample, 50 µl of DL-allylglycine (100 µmol/ml, Sigma-Aldrich, USA) was added as an internal standard. The mixture was allowed to stand at 4°C overnight before centrifugation at 34,800 × *g* for 20 min. The supernatant was collected and the pellet was washed with 2 ml 70% ethanol and centrifuged as above. The pooled supernatant was concentrated to 0.5 ml of extract with a rotavapor under vacuum at 45°C. A 50-µl aliquot was used for derivatisation with phenyl isothiocyanate (PITC) for HPLC analysis.

Preparation of seed extract for protein amino acid analysis

100 mg of seed powder was weighed into a 2-ml glass vial designed for hydrolysis (Wheaton, USA). To each vial, 2 ml of 6N HCl, 0.01% β-mercaptoethanol and 100 µmol/ml (Sigma-Aldrich, USA) were added. The mixture was allowed to freeze by placing the glass vial designed for hydrolysis in dry ice (carbon glass) for 5-8 min. The vial was then connected to a vacuum system with

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gentle shaking for about 10 min. The evacuated vial was then sealed by a gas flame and placed in an oven for hydrolysis at 110°C. The hydrolysate was transferred to an Eppendorf centrifuge (Hawksley MBC, UK) and centrifuged at 38,000 rpm for 10 min at room temperature. The supernatant was transferred to a conical evaporatory flask and dried using a rotary evaporator under vacuum at 45°C. This procedure was repeated twice by adding distilled water after drying to remove the HCl. 2 ml of distilled water was added to the flask to dissolve the dried samples and the mixture was then centrifuged. The supernatant was collected and a 50-µl aliquot was used for derivatisation with PITC for HPLC analysis.

PITC derivatisation

Each 50-µl sample was first dried under vacuum in an Eppendorf Vacufuge concentrator (Model 5301) at 45°C. To each dried sample, 20 µl of coupling buffer (methanol: water: triethylamine; 2: 2: 1; v/v) was added, mixed then dried in the concentrator. Finally, 30 µl of PITC reagent (methanol: water: triethylamine: phenylisothiocyanate; 7: 1: 1: 1; v/v) was added and left to react at room temperature for 20 min before concentrating to dryness. To each PITC-derivatised sample, 500 µl of buffer A (0.1M ammonium acetate, pH = 6.5) was added, mixed well and centrifuged. The supernatant was filtered through a Millipore Millex filter (0.45 µm) and then a 20-µl aliquot was injected into the HPLC prior to analysis. A standard amino acid mixture (AA-S-18, Sigma-Aldrich, USA), L-(+)-homoarginine (99+%, Janssen Chimica, Belgium) and synthetic β-ODAP (obtained from Dr. SLN Rao, Department of Biochemistry, Osmania University, Hyderabad, India) were also derivatised and prepared as above and injected into the HPLC as standard.

HPLC for amino acid analysis

A Waters 625 LC system with a Waters 991 photodiode array detector was used as reported previously (Kuo *et al.* 2003). A gradient system with buffer A (0.1 MNH4OAc, pH = 6.5) and buffer B (0.1M NHOAc, containing acetonitrile and MeOH; 44: 46: 10; v/v; pH = 6.5) with flow rate of 1 ml/min was used to separate the amino acids for 50 min. An Alltima C18 column (250 × 4.6 mm I.D., 5 µm particle size, Alltech, USA) was used with a column temperature of 43°C during analysis. Absorbance was calculated at 254 nm and results were analysed by Millennium software (Waters, ver. 1.10). Negative selection was made on ethionine (12.5 and 50 ppm) screening media to reconfirm the findings. Ethionine is a synthetic analogue of Met that kills poor Met-containing plants.

Phenotypic markers

Procedures for the identification of morpho-phenotypic markers (Imsande 2001) were adapted to identify and characterize putative mutant lines in M_4.

RESULTS

Met-overproducing phenotypes of putative mutant lines were identified based on their qualitative trait at the second generation (M_2). Phenotypic distinctions exhibited by the selections include glittering or shining deep green leaves, stem, smaller plant size and pods (**Fig. 1**), which were comparable to those reported in *Glycine max* (Imsande 2001). They also exhibited smaller biomass, sub-erect, smaller leaf size, medium seed size and reasonable grain productivity. They were designated as deep greens (DG) A to C (DGA, DGB and DGC). Selection resulted in improved root growth (by about 50%) on ethionine screening medium compared to parental and other seeds (**Fig. 2**) that could be an indicator of Met enrichment (Imsande 2001) while poor Met plant roots showed a shock response and become short, curled, deformed and brown on ethionine media.

Phenotypically, parental lines have greater biomass and size than derived putative mutant lines (**Table 1; Fig. 1**). The selections with the highest DGA scores showed varied yield; these selections also flowered and matured two weeks earlier than their counterparts. Seed size was similar in both groups. DGB had poor economic yield following an intra-mutant comparison.

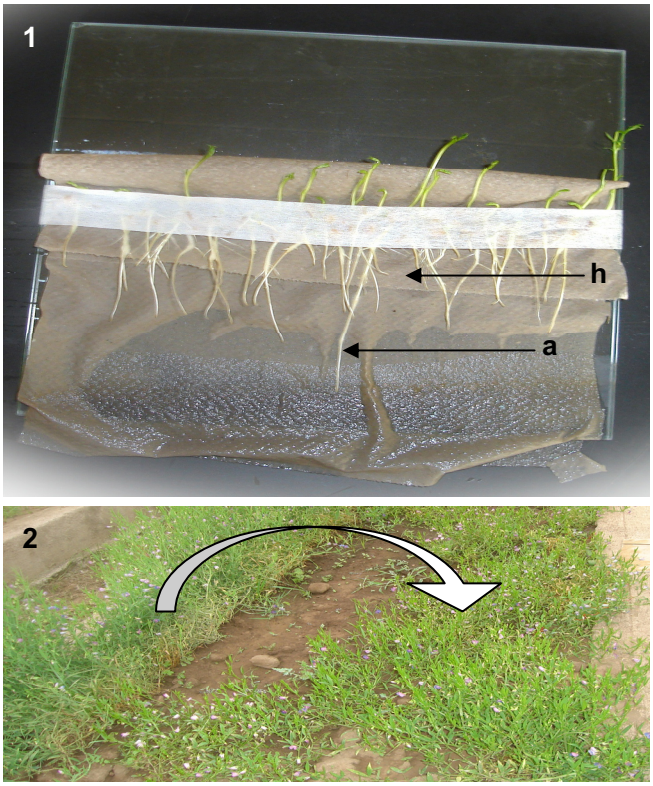

Fig. 1 Differences in roots reaction grown on ethionine (50 ppm) solution kept beneath seed placement, eight days after plantation. **Fig. 2** Phenotypic appearance of control (light green on the left) vs. mutant (deep green on the right) lines at flowering stage in the field.

The amino acid analysis showed that selected putative mutant lines had a 63% improved level of the sulphur-containing amino acid, Met over the parental line (**Table 1**). A fertility test for the mutant lines showed that the mutants were self fertile, fertilized by an external pollen source; however, the pollen source was *trans*-infertile, which is an interesting phenomenon and needs a further cytogenetic study.

DISCUSSION

In this work a successful method by Imsande (2001) for the selection of Met-enriched *Glycine max* by phenotypic indicators was adapted. Firstly, there were alterations in leaves and stem to produce a deep green colour induced by Met enrichment, which indicates a high level of leaf chlorophyll (Schumacher 2009). Secondly, root length in the presence of the phytotoxic Met analogue ethionine was analyzed. This is instrumental in the identification of Met-enriched genes throughout subsequent generations (M_1-M_4). The isolation of Met-overproducing lines both by phenotypic and ethionine screening procedures (Imsande 2001) allowed the sulphur concentration in seed plant to be determined. Schumacher *et al.* (2009) used the same procedure on different legumes. Unlike other legumes, the sulphur amino acid limitation of protein in grass peais accompanied with a neurodegenerative disease (Lambein *et al.* 2001; Getahun *et al.* 2005; Nunn *et al.* 2005) which results in irreversible crippling of patients' legs. On the other hand, the counteractive nature of Met against such oxidative diseases is well documented (Nunn *et al.* 2005; Al-Mayah 2006; Fikre *et al.* 2011).

Mutagenesis, which is reported for the first time, could improve the Met supply capacity of grass pea from one-fourth in the parents to one-half in mutant lines and eventually fulfill global standards (WHO 1985). This would imply improved antioxidation levels against toxicity of ODAP

Table 1 Descriptive statistics for some of characters of selected putative mutants (M_4) and parental line.

Variables	Means				Standard error of mean	Significance
	DGA	DGB	DGC	Parent		
Days to flowering	76	82	60	51	7.13	*
Days to maturity	115	115	116	106	2.35	*
Plant height (cm)	60	70	84	97	8.09	**
Pods/plant	230	147	253	265	26.59	**
100-seed weight (g)	7.4	6.7	6.7	7.6	0.24	*
Yield per plant (g)	42.8	23.7	26.8	34.1	4.25	**
Leaf area (cm^2)	1.21	1.69	1.60	3.43	0.49	**
Leaf number	1427	841	1430	2258	291.35	**
Leaf area index	3.82	3.16	5.08	17.20	3.32	**
Leaf width (cm)	0.61	0.87	0.74	1.12	0.11	**
Leaf length (cm)	3.4	3.7	1.8	4.7	0.60	**
Leaf perimeter	6.58	8.05	7.96	11.25	0.99	*
LL: LW	6	4.22	4.93	4.08	0.44	*
Leaf shape (LA/LP), 1 = circle	0.33	0.46	0.32	0.39	0.03	ns
β-ODAP (%)	0.38	0.22	0.36	0.35	0.04	*
Met (%)	0.18	0.19	0.16	0.11	0.02	*

*, **- Significance at $P = 0.05$ and 0.01 according to Duncan's multiple range test; ns = non-significance

present in seeds under Met limitation. As Met biosynthesis depends on sulphur fixation into the cysteine pathway (Molving *et al.* 1997), the mutagenic event might have altered gene/enzyme in favour of cysteine/Met biosynthesis. On the other hand, 80% of free Met is converged into S-adenosyl-methionine (AdoMet) (Ravanel *et al.* 2004). AdoMet plays an important role in chlorophyll biosynthesis (Bollivar 2006).

Enhancing Met levels via genetic engineering has shown to increase the nutritive value of seed (Molving *et al.* 1997). Met improvement *in vivo* potentially improves the nutritional quality towards a healthy food source. However, simultaneous reduction of toxicity during breeding needs to be considered. A sufficient Met source can improve the quality of a protein diet of pulses in general and lathyrism-protective efficiency in grass pea, in particular. The addition of a synthetic Met supplement improves the quality of protein in legumes (Molving *et al.* 1997) which produce of sulphur sources of amino acids, i.e., Met and cysteine, in sufficient amounts. Compared to the control, putative mutant lines are > 50% Met-rich, which implies an improved inherent nutritional quality. The new gene pools of grass pea could be further explored in subsequent breeding studies.

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