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L-Homoarginine Accumulation in Grass Pea (*Lathyrus sativus* L.) Dry Seeds. A Preliminary Survey

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ABSTRACT

Grass pea (Lathyrus sativus L.) has great agronomic potential as grain and forage legume, and presently is considered as a model crop for sustainable agriculture. However, the development into an important food legume has been hindered by the presence of the neurotoxic amino acid β-N-Oxalyl-α, β-diaminopropionic acid (β-ODAP). Recent studies reported that homoarginine (Har) can counteract this toxic action. This research was undertaken to shed light on the variation of Har amount within grass pea. The influence of the environment and of the year-to-year variation of climatic conditions was also investigated. Seven Italian grass pea ecotypes were evaluated for two subsequent growing seasons in two locations of southern Italy. In contrast with previous studies, collected data evidenced a significant variation of Har amount among the tested ecotypes. Moreover, a significant positive correlation between Har and ODAP level was observed. The effect of year-to-year variation of temperature and rainfall quantity is also discussed.

Keywords: *Capillary Zone Electrophoresis, Ecotype, Genetic Variation, Lathyrus sativus, Non-Protein Amino Acids, Rainfall Amount*

1. Introduction

Over 300 non-protein amino acids naturally occur in plants. In large part, they are intermediates in both synthesis and catabolism of protein amino acids and usually occur in different plant tissues, free or as 4-glutamyl derivatives [1,2]. Legumes contain not only high concentrations of non-protein amino acids but also a more diverse range than any other plant species. Seeds are generally the most concentrated sources of these compounds [3]. Previous studies demonstrated that non-protein amino acids plays different roles such as intermediates in the synthesis of protein amino acids, defensive agents [4], multifunctional hormones [5] or osmoregulators [6]. Some dietary non-protein amino acids are implicated in poisonings, diseases and disorders [7] while others can have beneficial effects [8]. The toxicity of non-protein amino acids has been observed in insects, laboratory and farm animals and in humans, but there are striking differences among the species in their sensitivity to these compounds. For example, it has been demonstrated that in both mammalians and birds, the biochemical versatility for amino acid detoxification is considerably reduced [9].

The relatively high concentration of non-protein amino acids in seeds is a major factor limiting exploitation of alternative grain legumes, such as *Lathyrus sativus* and *Vicia sativa*, as protein sources for poultry and other non-ruminant animals [9,10]. Grass pea (*Lathyrus sativus* L.) is an important annual crop for human and animal consumption because it may be the only affordable food in times of food shortage or under adverse environmental conditions. Unfortunately, consumption of large quantities or prolonged eating can give rise to the neurolathyrism [2,7]. It has been demonstrated that all *Lathyrus* species which synthesise β-N-Oxalyl-α, β-diaminopropionic acid (β-ODAP), the toxin responsible of neurolathyrism, also accumulate L-homoarginine (Har). β-ODAP and Har are the major free non-protein amino acids present in grass pea seeds [11]. Together they make up about 90% of ninhydrin-reacting compounds in the 70% ethanol extracts [12]. There are contrasting opinions about the impact of Har on human and animal diets. It is considered by some to be a positive factor because can be converted into lysine by the mammalian liver [13].

However, Breitner *et al.* [14] suggested that the presence of Har in gene activator-repressor histones may be a direct cause of most cancer types. Dawson *et al.* [15] reported that Har is a modulator of the biosynthesis of nitric oxide (NO) which, in turn, reduces the excitation of neuronal receptors [13]. Finally, it has been proposed that Har could modulate to some extent the toxicity of *β*-ODAP [16,17].

Since non-protein amino acids occur in legume seeds in unconjugated forms, their extraction with solvents or aqueous solutions is easy and quantitative. HPLC or capillary zone electrophoresis (CZE) methods have been developed to analyse the extracts. Sample preparation required by these techniques is different. HPLC methods necessitate of long derivatization times before analysis [18-23], while CZE allows selective and rapid measurements without the off-line derivatization step and brought less waste [11,24]. Although Har could play a positive effect in contrasting neurolathyrism, very little attention has been devoted to evaluate the effect of grain yield, location and growing season on its storage in seeds. The aim of this contribution was provide information on this topic by comparing different genotypes cultivated for two subsequent years in two environments of Southern Italy characterised by different soil properties and unlike climatic conditions.

2. Materials and Methods

The material utilised in the present study consisted of 6 ecotypes of grass pea (*Lathyrus sativus* L.), traditionally cultivated in small areas located in three regions (Apulia, Basilicata and Campania) of southern Italy, and the line n. 3151 belonging to the "Iannelli Germplasm Collection" held at University of Basilicata (Potenza, Italy) (**Table 1**). The field trials were conducted, for two consecutive years (2006-2007 and 2007-2008), at Battipaglia, a village situated at 7 km from the coast of Tirrenium sea (40˚36'N 14˚58'E, 65 m asl, Campania region) and at Guardia Perticara, a village placed in a hilly area along the Appenninic ridge (40˚22'N 16˚2'E, 720 m asl, Basilicata region). Intensive agricultural systems are predominant in the open plain of Battipaglia, while Guardia Perticara is located in hilly zones where traditional agriculture is still practiced. Moreover, the locations differ for soil type (Battipaglia - clay Gleyic Luvisol type, sand - 31%, silt - 29.2% and clay - 39.8%; Guardia Perticara – clay sandy soil, sand - 46.9%, silt - 19.2% and clay - 33.9%) and climatic conditions (**Figure 1**). The layout of the field experiments was a randomised complete block design. The ecotypes were sown in plots 4 m^2 with 40 cm between rows and 4 cm within the row for a density of 62.5 seeds per square meter. Sowing and harvesting date between growing seasons and locations were: I decade November 2006 - II decade June 2007; II decade October 2007 - II decade July 2008 at Battipaglia; II decade November 2006 - II decade July 2007; II decade November 2007 - I decade July 2008 at Guardia Perticara. Seed samples were dried at 105 °C until constant weight, so to ormalize grain yield reported in **Table 1** at 13% moisture.

2.1. Homoarginine Extraction

Approximately 150 g of dry seeds were taken from the bulk material harvested for each sample. Seeds were ground using a Cyclotec 1093 mill Tecator (Sweden). The non-protein amino acids were extracted from meal with a mixture ethanol-water (6:4, v/v) by tumbling the capped plastic tubes for 1h at room temperature [11]. The slurries were centrifuged at 2800 g for 20 min and the supernatant recovered. Then fresh aqueous ethanol was added to the pellet to a second extraction step. The pooled supernatants were stored at −20˚C until analysed by capillary zone electrophoresis. The extraction of each sample was repeated twice, and each extract was analysed in triplicate by CZE and the content was expressed as mean value.

2.2. Capillary Zone Electrophoresis (CZE) Analyses

A P/ACE MDQ Beckman-Coulter (USA) was used to analyse the seed extracts. The CZE analyses were performed according to Zhao *et al.* [11] with slight modifycations. Separations were achieved using uncoated fused-silica capillaries 55 cm long (42 cm to detector) with 50 μm ID. The capillary temperature was kept at 30˚C and a constant voltage of 25 kV was applied for 6 min. The peak detection was performed at 200 nm. Beckman Karat 32 software was used for acquiring, storing and analyzing the electrophoregrams. The separation buffer was 18.5 mM $Na_2B_4O_7$ (pH 9.2) and 10 mM Na_2SO_4 dissolved in 18 MΩ cm distilled and deionized water (Milli-Q water system Millipore, USA). All the chemicals were of analytical reagent grade. A known amount of L-homoarginine, purchased from Fluka (St. Louis, USA), was dissolved in an ethanol-water mixture (6:4) and termed the stock standard solution. Homoarginine peak was identified by the increase of peak area after the addition to the sample extracts of a known volume of the standard solution. Various concentrations of L-homoarginine were prepared by diluting the stock standard solution to obtain the calibration plot. Statistica version 6.0 (Statsoft, Tulsa, OK, USA) was used for the statistical analyses.

3. Results and Discussion

In southern Italy there is a long lasting tradition of cultivation and consumption of grass pea for human use as

Table 1. Ecotype name, L-homoarginine and ODAP amounts, and grain yields recorded at the two locations for the tested ecotypes. The growing seasons are coded: I - 2006-2007; II - 2007-2008.

Ecotype name	Moisture ($g \cdot kg^{-1}$)		Homoarginine ($g \cdot kg^{-1}$ dm)		ODAP ($g \cdot kg^{-1}$ dm)*		Grain yields ($g \cdot m^{-2}$)*	
	I	II	I	II	I	II	I	II
Battipaglia								
Apulia region								
Altamura	99	97	14.16	16.15	1.21	3.50	341	251
Bari	103	88	8.14	13.80	1.52	2.96	350	242
Campania region								
Castelcivita	101	92	10.60	13.92	1.30	2.94	385	277
Grottaminarda	99	97	9.08	15.22	1.35	2.88	371	245
Basilicata region								
Line n. 3151	103	87	8.20	14.09	1.58	2.71	351	258
Palazzo S. Gervasio	99	92	9.18	14.48	1.32	3.16	318	208
S. Rufo	99	97	6.26	14.68	1.28	3.50	386	304
mean	100 *aA*	93 *bA*	9.38 *aA*	14.62 *bA*	1.37 *aA*	3.09 *bA*	357 *aA*	255 *bC*
Guardia Perticara								
Apulia region								
Altamura	91	82	14.80	20.97	1.66	4.13	177	230
Bari	91	83	10.68	18.79	1.82	6.48	187	267
Campania region								
Castelcivita	91	90	10.44	20.90	1.55	4.13	198	285
Grottaminarda	91	79	10.38	20.22	1.39	2.96	168	256
Basilicata region								
Line n. 3151	92	80	9.05	18.46	1.48	4.52	227	235
Palazzo S. Gervasio	94	82	11.88	20.28	1.41	4.51	128	271
S. Rufo	92	80	12.31	17.91	1.62	3.00	166	279
mean	92 *aB*	83 *bB*	11.36 *aA*	19.65 *bB*	1.56 *aB*	4.25 *bB*	179 *aB*	260 *bC*

Means with the same letter are not significantly different (P = 0.05). Capital letters refer to comparison between locations; lowercase letters refer to comparison among years at the same location. *Reference [31].

well as animal feed. In the last decade organic farmers have been rediscovering this pulse both as model crop for sustainable agriculture and traditional local product [25,26]. A few autochthonous ecotypes, still under cultivation in marginal areas of Southern Italy, were compared in this study. Field trials were carried out at Battipaglia and Guardia Perticara, two locations characterised by different agro-ecological conditions. Moreover, these locations were outside the areas where the studied ecotypes traditionally grown. This experimental design was adopted to allow the enhancement of the adaptation bias on the Har storage in seeds.

According to the literature [11,22], CZE analyses of alcoholic extracts of tested ecotypes revealed that, in addition to β-ODAP, Har was always the most abundant non-protein amino acid. This is a feature specific for grass pea because Har was undetectable in pea and bean seeds [27], while its presence in seeds of some *Lens* species has been recently reported by Rozan *et al.* [28,29]. Within the *Lens* genus the highest amounts were detected in *Lens ervoides* accessions (ca. 0.06 mg g^{-1}), but the values were significantly lower than those typical of

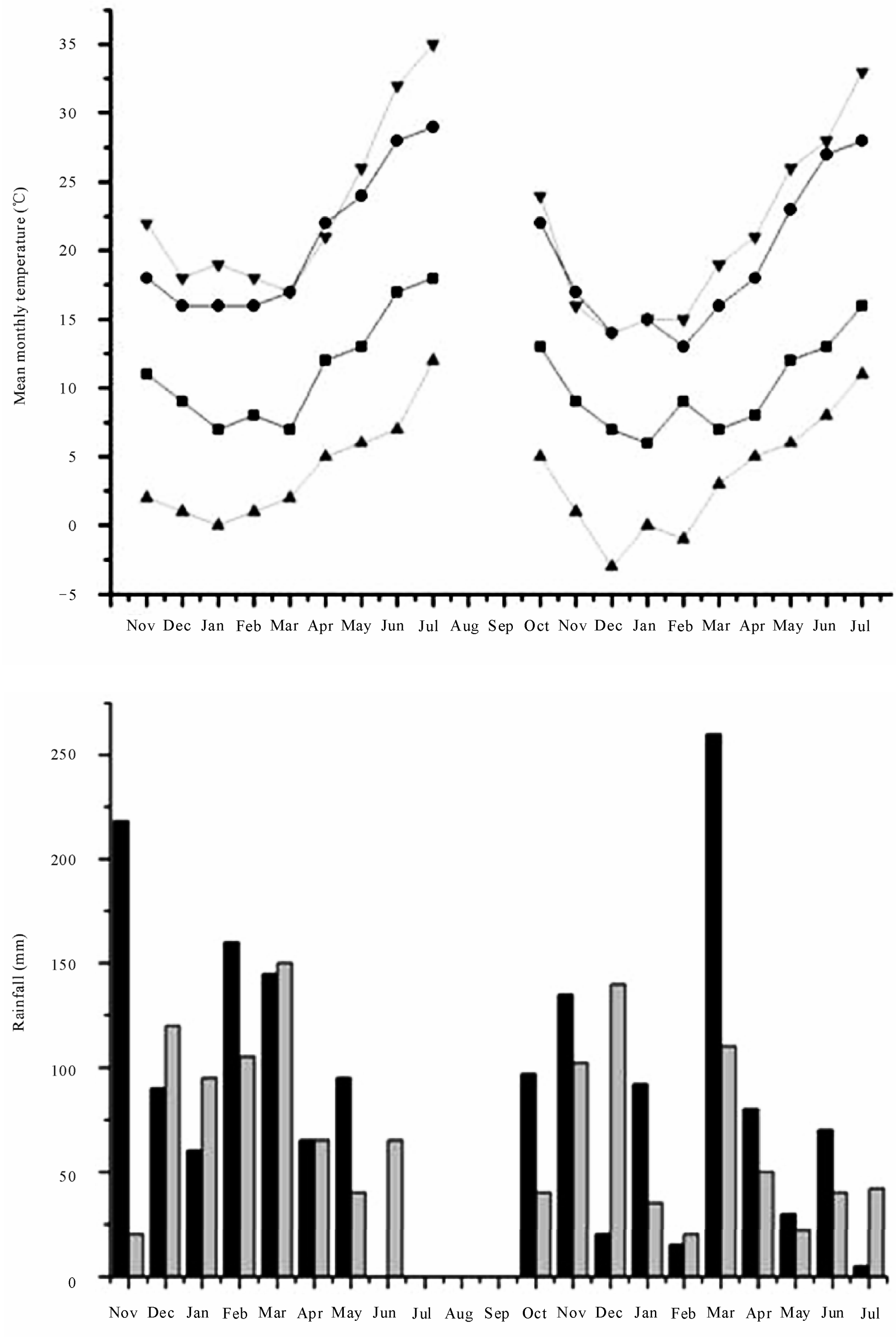

Figure 1. Top—Mean temperatures recorded from November 2006 to July 2008 at Battipaglia (■ minimum mean values; ● maximum mean values) and Guardia Perticara (▲ minimum mean values; ▼ maximum mean values). Bottom—Amount of rainfall recorded from November 2006 to July 2008 at Battipaglia and Guardia Perticara (black and grey bars, respectively).

grass pea [11,21,30].

The analysis of experimental data reported in **Table 1**, evidenced a threefold variation of Har amount between the extreme values (6.26 vs 20.97 g kg^{-1}). The lowest value was recorded during the first growing season at Battipaglia for S. Rufo ecotype, while the highest one was observed in the second growing season at Guardia Perticara for Altamura ecotype. This wide variation did not agree with Fikre *et al.* [23], who analysing 9 genotypes with different origin, concluded that the variation of Har level within grass pea (0.68% - 0.86%) is not significant. Other episodic studies available in the literature refer to a few genotypes grown in disparate environments (*i.e.* Mediterranean-type climate, under rainfall conditions, high salinity of soil, etc.) generally for a single growing season. A narrow variation (5.3 - 6.7 mg g^{-1}) was reported by Yan *et al.* [21], while Zhao [11] recorded a wider range (3.2 - 10.6 mg·g^{-1}). Based on these data, the ecotypes analysed in the present study did not rank within these ranges.

In this study, statistically significant differences ($P < 0.05$) were observed at each location between the growing seasons (**Table 1**). Overall, for all the ecotypes it was observed a trend towards increasing Har values in the second season. Moreover, the Har amounts of the material grown at Battipaglia was always inferior than those recorded in the same growing season for the harvests from Guardia Perticara. However, only for the second growing season the difference between the values recorded at the two locations resulted statistically signifycant ($P < 0.05$). The grass pea from Altamura attracted the attention not only because it showed invariably the highest Har values, but also for a not significant year-to-year variation at Battipaglia (14.16 vs 16.15). At the opposite, the widest year-to-year variation was recorded for S. Rufo ecotype and the line n. 3151 at Battipaglia and Guardia Perticara, respectively.

Recently, it has been published a paper dealing with the evaluation of nutritional and technological quality of seeds belonging to Italian grass pea ecotypes [31]. This last study and the present one share a part of tested ecotypes, so matching the results, interesting consideration on factors affecting the Har storage can be done. A wide variation between locations as well as growing seasons was observed also for grain yield and ODAP [31]. As shown in **Table 1**, for each ecotype, the yield recorded in the first growing season at Battipaglia was about two times that registered at Guardia Perticara, while in the subsequent season grain yields recorded at the two locations resulted comparable. However, this year-to-year variation of grain yield did not affect the Har storage because correlation analysis did not evidence a signifycant relationship between these traits. As concerns ODAP, differences statistically significant were detected between growing season and location. If Har could really modulate to some extent the toxicity of β-ODAP, a positive correlation between the amounts of these amino acids might be highly desirable. In actual fact, correlation analysis of Har and ODAP amounts shown in **Table 1**, evidenced a statistically significant relationship between these amino acids ($R = 0.816$, $P < 0.05$).

Table 2 gives the results of ANOVA analysis. The effects of both growing season and location of cultivation were found important in determining the Har amount, though the significance level resulted different. Conversely, only the effect associated with the growing season was important in determining the ODAP storage. These findings indicate that, from a statistical point of view, the Har storage in seeds changed from one year to the next, and that the impact of growing location on this trait was likewise statistically significant. Although the present study cannot allow drawing unequivocal decisions on the relative importance of soil composition, yield, sowing and harvesting date, environmental conditions of growing location, genotype x environment interaction, etc. some working hypotheses can be devised. For example, grain yield should have a minor role in determining Har storage for the reason that correlation analy-

Table 2. Results of the analysis of variance for Har and ODAP amount, and grain yield. Mean square and significance are reported.

Sources of variation	Df	Homoarginine	ODAP	Grain yields
Years (Y)	1	320.3566**	34.0783**	750.8929
Locations (L)	1	86.1354*	3.1894	52 548.89**
Ecotypes (E)	6	7.4526	0.4211	1458.988
Y x L	3	140.8832**	12.9586**	37 546.61**
Y x E	13	28.8202*	2.9259*	929.3984
E x L	13	10.3306	0.6341	4902.937

Df—degrees of freedom; *—significant at 5%; **—significant at 1%.

sis did not evidenced a significant relationship between these traits. Similarly, in consequence of the large overlap of sowing and harvesting periods between growing seasons at each location, it is predictable that weather conditions might have a major influence in regulating the Har accumulation. This is not surprising because it is well known that the amounts of some antinutritional and toxic factors stored in legume grains are modulated by rainfall quantity and/or average temperature during specific vegetative stages [32-34]. Graphical representations of both minimum and maximum average temperatures (calculated on monthly base) and the amount of rainfall recorded in the field trials are given in **Figure 1**. The plots revealed that, at each location, the variation of temperature between the growing seasons were not sta- tistically significant, and that Battipaglia was character- rised by a lower gap between minimum and maximum mean temperatures. In regard to the precipitation value, a higher stress was suffered by plants at Guardia Perticara, being been registered at this location in the second growing season the lowest rainfall amount (489 mm) over the trial. It is interesting to underline that, for all the ecotypes, the highest Har levels were actually recorded at Guardia Perticara in the second growing season (**Table 1**). Based on these evidences it can be inferred that, similarly to other secondary metabolites of pulses, also Har appear to be affected by the amount of rainfall. How much the environmental conditions can affect grass pea seed quality traits has been reported in recent studies dealing with morphological and compositional seed traits [26,31].

As a consequence of the increasing interest towards *Lathyrus* species as alternative pulses, it has been agreed that grass pea breeding should be focused, besides to the ODAP reduction and the plant architectural ideotype, also towards the increase in seeds of the level of Har and methionine in consideration of potential beneficial effects of on human health of these amino acids [16,17,35]. The results presented in this paper give preliminary in- formation not only on the variation of Har amount within grass pea but also on some features affecting its storage. This provides an useful starting point to program new field trials involving a higher number of genotypes and a wider range of climatic conditions.

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