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Trypsin inhibitor activity in grass pea seeds (*Lathyrus sativus* L.)

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Summary: Grass pea (*Lathyrus sativus*) is a valuable grain legume known for its high protein content and rich essential amino acid profile. Its exceptional characteristics such as drought tolerance, high adaptability to extreme conditions, disease resistance, and low cultivation inputs, make it particularly suitable for cultivation by resource-poor farmers. However, the potential use of grass pea is limited due to the presence of anti-nutritional factors, including protease inhibitors, especially trypsin inhibitor. This study aimed to develop a rapid and reliable method for measuring TI activity in seeds of grass pea and to investigate the influence of genotype and environment on trypsin inhibitor (TI) activity in seeds of grass pea. A set of 25 grass pea accessions from seven European countries was cultivated in Slovenia at the Agricultural Institute of Slovenia, and a set of 12 grass pea accessions from the Serbian gene bank was grown in Serbia at the Institute of Field and Vegetable Crops. The TI activity varied considerably among the grass pea accessions studied, with values ranging from 26.7 to 90.3 TUI/mg. To further evaluate the effects of environmental conditions on TI activity, eight grass pea accessions originating from Serbia were grown in both Slovenia and Serbia. The TI activity of the accessions grown in Slovenia ranged from 26.7 to 81.0 TUI/mg, while the activity of the accessions grown in Serbia ranged from 40.3 to 57.0 TUI/mg. The correlation of TI activity between grass pea accessions grown in Slovenia and those grown in Serbia was 0.39, with genotype diversity being the largest contributor (55.9%). This study provides a valuable insight into the variability of TI activity in grass pea and shows the possible influence of environmental conditions on this trait. However, since the data are only from a one-year field trial, further studies are needed to fully evaluate the influence of different environmental factors on TI activity.
Key words: grass pea, seeds, *Lathyrus sativus*, trypsin inhibitor activity, accessions, genebanks

Introduction

Grass pea (*Lathyrus sativus* L.) is a grain legume known for the exceptional nutritional value of its seeds, which are high in protein and rich in essential amino acids (Ennenking, 2011). It is cultivated in the Indian subcontinent and in various parts of Africa, Australia and Asia (Hanbury et al., 2000). In the last two decades, interest in grass pea cultivation has increased in Europe, along with other less cultivated grain legumes such as chickpea, lentil, and vetch (Mikić et al., 2011; Petrova & Chipilski, 2020). Grass pea is valued for its high nutritional value, excellent adaptability to extreme environmental conditions, disease resistance, and low requirement for cultivation inputs. In addition, grass pea exhibits

remarkable drought tolerance and remains unaffected by excessive rainfall, making it suitable even for cultivation in flood-prone areas (Urga et al., 2005). Like other grain legumes, grass pea seeds contain anti-nutritional factors, including trypsin inhibitor (Guillamo et al., 2008). This trypsin inhibitor, belonging to the serine proteinase inhibitors (serpins), can negatively affect seed nutritional quality by reducing protein digestibility and adsorption, thereby inhibiting growth. Trypsin inhibitor has long been considered as an important anti-nutrient factor (Gatel, 1994), but recently some of them have been found to be beneficial to humans and animals (Kennedy, 1998; Ashton-Rickardt, 2012) or can be used as food processing agents (Boudida et al., 2016; Singh & Benjakul, 2018). In addition, trypsin inhibitor has been found to be partially associated with resistance to the bruchid or pulse beetle (*Callosobruchus chinensis*) in many legume species, including grass pea (Deepika et al., 2020).

Trypsin inhibitor (TI) activity is routinely measured in seeds of grain legumes, proteinaceous foods, and feeds, usually using two methods (ISO, 2012; Liu, 2019; Liu et al., 2021), which have a range of modifications depending on the plant species or type of sample studied. The aim of the present study was to develop a rapid and reliable method for measuring TI activity in seeds of grass pea and (i) to determine the content of TI activity in seeds of grass pea accessions from different European countries. In addition, (ii) to verify whether genotype and environment have a significant influence on TI activity.

Materials and Methods

Seed samples of 25 grass pea (*Lathyrus sativus* L.) accessions from seven European countries were grown in 2019 in Slovenia at the Agricultural Institute of Slovenia (Jablje; 302 m a.s.l.; 46.08°N 14.33°E) and of 12 accessions in Serbia at the Institute of Field and Vegetable Crops (Rimski Šančevi; 84 m a.s.l.; 45.20°N 19.51°E) according to the production technology established in each country. Grass pea accessions were from seven different genebanks: 1 from Greece, 4 from Portugal, 7 from Bosnia and Herzegovina, 2 from Romania, 12 from Serbia, 2 from Bulgaria, and 1 from Slovenia, as shown in Table 1. To ensure uniformity and accuracy of analysis, dry seeds of each accession were homogenised using a laboratory ball mill (Retsch MM400) before being used for determination of TI activity by hydrolysis of BAPNA (N_{α}-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride) with trypsin.

Table 1. List of studied grass pea (*Lathyrus sativus* L.) accessions

Origin	No. of ACC	ACC name	Genebank	Grown in Slovenia	Grown in Serbia
Greece	1	GR1	Faculty of Crop Science, University of Athens	yes	no
Portugal	4	ISOP1176	ISOPlexis-Germobanco, University of Madeira	yes	no
		ISOP1177		yes	no
		ISOP1189		yes	no
		ISOP1190		yes	no
Bosnia and Herzegovina	7	GB00954	Institute of Genetic Resources, University in Banja Luka	yes	no
		GB00999		yes	no
		GB01000		yes	no
		GB01001		yes	no
		GB01002		yes	no
		GB01003		yes	no
		GB01004		yes	no
Romania	2	SVGB19385	Banca de Resurse Genetice Vegetale 'Mihai Cristea',	yes	no
		SVGB20803		yes	no

			Suceava		
Serbia	12	KL1	Institute of Field and Vegetable Crops Novi Sad	yes	yes
		KL2		yes	yes
		KL3		no	yes
		KL4		yes	yes
		KL5		yes	yes
		KL6		no	yes
		KL7		yes	yes
		KL8		yes	yes
		KL9		yes	yes
		KL10		yes	yes
		KL11		no	yes
		KL12		no	yes
Bulgaria	2	BGR40415	Institute of Plant Genetic Resources 'Konstantin Malkov', Sadovo	yes	no
		BGR43334		yes	no
Slovenia	1	SRGB5486	Agricultural institute of Slovenia, Ljubljana	yes	no

ACC, accession.

Preparation of buffers and solutions

Buffers and solutions were prepared according to Kakade et al. (1974). Tris-buffer (0.05M, pH 8.2) contained 0.02M $CaCl_2$ (6.05 g Tris (hydroxymethyl) aminomethane and 2.94 g $CaCl_2$:$2H_2O$) dissolved in 900 mL water. The pH was adjusted to 8.2 and the volume was brought to 1 L. The substrate solution was prepared by dissolving 40 mg BAPNA in 1 mL dimethyl sulfoxide (DMSO). The BAPNA solution was then diluted with prewarmed Tris-buffer at 37 °C to a final volume of 100 mL. The BAPNA solution was prepared daily and stored at 37 °C during use. For the trypsin solution, 4 mg of porcine trypsin (freeze-dried, salt-free) was dissolved in 200 mL of 0.001M HCl. The trypsin solution proved stable and maintained its activity when stored in the refrigerator for 2 to 3 weeks.

Preparation of sample solutions

Sample solutions were prepared using homogenised raw grass pea seeds as starting material according to the method described by Liu & Markakis (1989). To extract the trypsin inhibitor, 200 mg of homogenised seeds were mixed with 20 mL of distilled water. The extractions were performed by mechanical shaking at a speed of 200 rpm on a rotary shaker (Heidolph Unimax 1010, Germany) for 1 h. The sample suspensions of trypsin inhibitor were completed by re-suspending with 20 mL of assay buffer. The sample suspensions were shaken vigorously for 2–3 min before being filtered through a No. 2 Whatman paper (Sigma-Aldrich, USA). The resulting filtrate was then used to prepare a series of dilutions with distilled water (0.2, 0.4, 0.6, and 0.8 mL of filtrate in 1 mL of final solution) to obtain a final sample solution containing 30–70% trypsin inhibition.

Trypsin inhibitor activity assay

The procedure for the TI activity assay was performed using the microplate method, with assay conditions described by Liu & Markakis (1989) and Župunski et al. (2016). Aliquots of 45 µL of the final sample solution and 22.5 µL of the trypsin solution were added to eight wells of each row of the microtiter plate to prepare the sample reaction mixtures. Another four wells of each row were used to

prepare the positive and negative control reaction mixtures and were filled with 45 µL of distilled water instead of the sample and 22.5 µL of trypsin solution. Preincubation was performed at 37 °C for 10 min. 90 µL of BAPNA solution was added to each well and incubated at 37 °C for 30 min. The positive control reaction mixture resulted in a non-inhibited reaction of enzyme (trypsin) and substrate (BAPNA), while the negative control reaction mixture was used as a reagent blank, as 45 µL of 30% acetic acid was added immediately after the trypsin solution to stop the reaction. The purpose of the negative control reaction mixture was to measure the absorbance not associated with the reaction of trypsin and BAPNA substrate. The absorbance of the samples and the positive and negative control reaction mixtures was measured in triplicate with light at a wavelength of 405 nm using the Multiskan Ascent microplate photometer (Thermo Fisher Scientific, USA).

Trypsin inhibitor activity measurement

Because the TI activity has been shown to deviate from linearity at high inhibitory concentrations and values are highly scattered when trypsin inhibition is less than 30%, the TI activity was calculated only when trypsin inhibition was between 30–70% (Liu & Markakis, 1989). The TI activity was expressed in number of trypsin units inhibited (TUI) per milligram of sample (TUI/mg), taking into account that one trypsin unit is defined as 0.01 increase in absorbance units at 405 nm under the assay conditions (Kakade et al., 1974; Page et al., 2000).

Data analysis

Descriptive statistics parameters, i.e. mean, standard deviation (SD), range and coefficient of variation (CV %), were calculated using Infostat software (Di Rienzo et al., 2011). To compare trypsin inhibitor activity between the different genotypes, the Tuckey test and least significance test (LSD) at $p<0.001$ were used. In addition, two-way analysis of variance (ANOVA) was used to separate the main effects contributing to trypsin inhibitor activity at two different sites.

Results and Discussion

The study focused on investigating the presence of anti-nutritional compounds, especially TI activity, in grass pea seeds from different European countries. In particular, grass peas grown in Slovenia showed a wide range of TI activity, ranging from 26.7 TUI/mg to 90.3 TUI/mg (Table 2). As shown in Table 2, in Slovenia it was possible to distinguish a group of eight grass pea accessions with high TI activity (> 72 TUI/mg). The order of these accessions, ranked by TI activity frequency, from highest to lowest values, was as follows: ISOP1177 (90.3 TUI/mg) > KL5 > (81.0 TUI/mg) > GB01002 (78.0 TUI/mg) > ISOP1190 (78.0 TUI/mg) > GB0999 > (74.7 TUI/mg) > ISOP1176 (73.7 TUI/mg) > GB01003 (73.3 TUI/mg) > GB01001 (72.3 TUI/mg). Conversely, there was another group of four grass pea accessions with low TI activity (< 40 TUI/mg) and the order of accessions was KL10 (26.7 TUI/mg) < KL1 (34.0 TUI/mg) < BGR40415 (35.3 TUI/mg) < SVGB20803 (38.7 TUI/mg). Compared to previous studies, Wang et al. (1998) reported lower TI activity (mean 28 TUI/mg) for nine grass pea lines tested in Canada. Similarly, Starzynska-Janiszewska & Stodolak (2011) reported lower TI activity (22 TUI/mg) in raw grass pea seeds of Krab variety grown in Poland. In addition, an Italian study examining 13 genotypes of grass pea from three growing seasons also revealed generally lower TI activity (11.6–33.1 TUI/mg) compared to our data (Piergiovanni et al., 2011). The substantial differences in TI activity among different accessions and geographic regions suggest the influence of various environmental and genetic factors. Understanding these differences is crucial for selecting accessions with lower TI activity, which could be beneficial for improving the nutritional quality of grass pea seeds and promoting their safe consumption. Moreover, this study highlights the importance of regional diversity of grass pea accessions and provides valuable insights for further research and crop improvement strategies.

TI activity of grass pea accessions grown in Serbia varied from 40.3 TUI/mg to 57.0 TUI/mg (Table 3). As shown in Table 3, in Serbia it was possible to distinguish the two grass pea accessions with high TI activity (> 55 TUI/mg), namely KL7 57.0 TUI/mg) and KL4 (55.5 TUI/mg). In addition, it was also possible to distinguish two grass pea accessions with low TI activity, namely KL1

(42.0 TUI/mg) and KL8 (40.3 TUI/mg). The TI activity showed higher variability at the site in Slovenia (CV = 27.1%, Table 2), where 25 grass pea accessions were tested, compared to 12 grass pea accessions tested in Serbia (CV = 12.9%, Table 3). Sinkovič et al. (2021) previously reported high genetic diversity and differences in seed traits in grass pea accessions from south-eastern Europe.

Table 2. Trypsin inhibitor (TI) activity in seeds of grass pea accessions grown in Slovenia.

ACC name	TI activity (TUI/mg)	ACC name	TI activity (TUI/mg)
GR1	50.0 ± 5.9 $^{e-h}$	SVGB20803	38.7 ± 1.2 ^{hi}
ISOP1176	73.7 ± 7.8 $^{a-c}$	KL1	34.0 ± 1.6 ^{hi}
ISOP1177	90.3 ± 0.5 ^{a}	KL2	63.7 ± 3.9 $^{b-g}$
ISOP1189	65.3 ± 5.8 $^{b-f}$	KL4	51.3 ± 5.2 $^{d-h}$
ISOP1190	78.0 ± 5.4 $^{a-c}$	KL5	81.0 ± 7.3 ^{ab}
GB00954	66.0 ± 5.7 $^{b-f}$	KL7	63.7 ± 4.5 $^{b-g}$
GB00999	74.7 ± 1.2 $^{a-c}$	KL8	68.8 ± 5.0 $^{b-e}$
GB01000	64.7 ± 3.3 $^{b-g}$	KL9	70.5 ± 2.2 $^{b-d}$
GB01001	72.3 ± 1.7 $^{a-c}$	KL10	26.7 ± 3.1 ^{i}
GB01002	78.0 ±3.6 $^{a-c}$	BGR40415	35.3 ± 4.5 ^{hi}
GB01003	73.3 ±1.2 $^{a-c}$	BGR43334	69.0 ± 7.0 $^{b-e}$
GB01004	48.7 ± 4.8 $^{f-h}$	SRGB5486	45.3 ± 2.9 $^{g-i}$
SVGB19385	60.3 ± 3.7 $^{c-g}$		
LSD (0.01)		19.7	
CV (%)		27.1	

Data are means ± SD (n=3). Mean values with different letters (a-i) are significantly different ($p<0.001$).

Table 3. Trypsin inhibitor (TI) activity in seeds of grass pea accessions grown in Serbia.

ACC name	TI activity (TUI/mg)
KL1	42.0 ± 1.4 ^{cd}
KL2	44.7 ± 2.5 $^{a-d}$
KL3	50.0 ± 2.9 $^{a-d}$
KL4	55.5 ± 3.6 ^{ab}
KL5	54.0 ± 4.1 $^{a-c}$
KL6	43.0 ± 2.8 $^{b-d}$
KL7	57.0 ± 0.8 ^{a}
KL8	40.3 ± 3.4 ^{d}
KL9	51.7 ± 2.5 $^{a-d}$
KL10	44.7 ± 3.7 $^{a-d}$
KL11	45.7 ± 0.5 $^{a-d}$
KL12	45.2 ± 4.0 $^{a-d}$
LSD (0.01)	12.6
CV (%)	12.9

Data are means ± SD (n=3). Mean values with different letters (a-d) are significantly different ($p<0.001$).

Eight grass pea accessions (KL1, KL2, KL4, KL5, KL7, KL8, KL9, and KL10) originating from the Serbian genebank were cultivated in two different locations: Slovenia and Serbia. Analysis of TI activity of these accessions showed relatively low correlation (0.39) of TI activity between grass pea accessions grown in Slovenia and those grown in Serbia. Two-way analysis ANOVA indicated that the total variability of TI activity was significantly influenced by genetic factors (55.9%). The location of cultivation also had a noticeable influence, accounting for 9.9% of the total variability. In addition, the interaction between genotype and location (genotype × location) was responsible for a substantial proportion (34.1%) of the total variability in TI activity (Table 4). In comparison, Piergiovanni et al.

(2011) reported that no significant interaction with growing season and location was observed in 13 ecotypes of grass pea from Italy. However, the genotype effect remained significant for TI activity, suggesting that genetic factors play a crucial role in determining TI activity in different accessions. The observed variation in TI activity among sites and the significant influence of genetic factors and genotype × location interaction highlights the complexity of regulating TI activity in grass pea accessions. Understanding these factors is important for making informed decisions in breeding programs aimed at improving the nutritional quality of grass pea plants. Moreover, this study highlights the importance of considering both genetic diversity and growing location when evaluating anti-nutritional compounds such as TI activity in grass pea accessions.

Table 4. Two-way analysis of variance (ANOVA) for eight grass pea accessions grown at two locations (Slovenia and Serbia).

Source	df	MS
Genotype	7	736.1**
Location	1	914.4**
Genotype × Location	7	449.4**
Error	32	21.3

Fisher test, **p<0.001

Conclusions

This study is the first investigation of TI activity in grass pea seeds from several European countries. The results shed light on the influence of both genetic variation among accessions and environmental factors on TI activity in grass pea. The study showed a wide variation in TI activity among grass pea accessions grown in Slovenia, with eight accessions having high TI activity and four accessions having low TI activity. These differences suggest that location and genetic factors have a significant influence on TI activity in different geographic regions. Understanding these factors is critical for selecting accessions with lower TI activity and thus improving the nutritional quality and safety of grass pea seeds. In addition, the study highlighted that genetic factors had the greatest influence on the variability of TI activity, followed by cultivation location. The interaction genotype × location also played an important role in determining the TI activity, adding to the complexity of TI activity regulation in grass pea accessions. These results highlight the importance of considering both genetic diversity and cultivation site in future breeding programs for grass pea. When these factors are considered, breeders can make informed decisions to develop improved varieties with higher nutritional quality. Overall, this study provides valuable insights into understanding and controlling TI activity in grass pea accessions and paves the way for further advances in sustainable agriculture and food security.

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Aktivnost inhibitora tripsina u semenu sastrice (*Lathyrus sativus* L.)

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Sažetak: Sastrica (*Lathyrus sativus*) je zrnena mahunarka koja je cenjena zbog svog visokog sadržaja proteina, te bogatog i raznolikog sadržaja aminokiselina. Zbog osobina kao što su tolerantnost na sušu, visoka adaptabilnost na ekstremne uslove sredine, otpornost na bolesti i niski troškovi gajenja, sastrica predstavlja jedan od useva koji se može gajiti u siromašnim regionima. Međutim, potencijalna eksploatacija sastrice je ograničena, usled prisustva anti-nutritivnih faktora, kao što su inhibitori

proteinaza, uključujući i tripsin inhibitor. Cilj istraživanja je bio razvoj metode za određivanje aktivnosti inhibitora tripsina (TI) u semenu sastrice, kao i ispitivanje uticaja genotipa i spoljašnje sredine na aktivnost TI. Set od 25 genotipova poreklom iz sedam evropskih zemalja je gajen u Sloveniji, u Poljoprivrednom institutu Slovenije, a set od 12 genotipova poreklom iz Srbije je gajen u Srbiji, u Institutu za ratarstvo i povrtarstvo. TI aktivnost je značajno varirala između ispitivanih genotipova sastrice, krećući se od 26,7 do 90,3 TUI/mg. Osam genotipova poreklom iz Srbije je gajeno u obe zemlje. TI aktivnost genotipova gajenih u Sloveniji se kretala od 26,7 do 81,0 TUI/mg, dok je aktivnost TI kod genotipova gajenih u Srbiji iznosila od 40,3 do 57,0 TUI/mg. Korelacija TI aktivnosti između genotipova gajenih u Sloveniji i onih gajenih u Srbiji je iznosila 0,39, pri čemu je u njoj najveći udeo imala genetička raznovrsnost (55,9%). Ova istraživanja predstavljaju značajan uvid u aktivnost TI kod sastrice i ukazuju na mogućnost uticaja uslova sredina na ispoljavanje ovog svojstva. Međutim, pošto su ovo rezultati jednogodišnjeg poljskog ogleda, potrebno je nastaviti ispitivanja da bi se uticaj sredine na aktivnost TI mogao u potpunosti definisati.

Ključne reči: sastrica, seme, *Lathyrus sativus*, tripsin inhibitori, genotip, banka gena