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Research article

Trypsin inhibitors in seeds and pods of *Phaseolus vulgaris*/*coccineus*: A comparative study of shaking and ultrasonic extraction methods ☆

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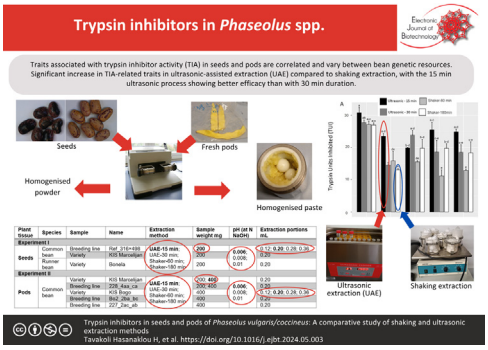
GRAPHICAL ABSTRACT



Trypsin inhibitors in *Phaseolus* spp.

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ABSTRACT

Background: Different methods for the extraction of trypsin inhibitors in beans (*Phaseolus* spp.) were investigated. Two randomised complete laboratory experiments were performed, one on the seeds and one on the pods. In the first, the seeds of common bean variety KIS Marcelijan, breeding line Ref_316 × 498 and runner bean variety Bonela were examined. In the second, the fresh pods of five common beans (three breeding lines, two varieties) were analysed. Four extraction methods were used, including ultrasonic-assisted extraction (UAE) for 15 and 30 min and shaking-assisted extraction for 60 and 180 min.

Results: The results showed a significant increase in trypsin inhibitor activity-related traits in UAE compared to shaking extraction, with the 15 min ultrasonic process showing better efficacy than the one with 30 min duration. In the seed experiment, the breeding line Ref_316 × 498 showed the highest Trypsin Units Inhibited (TUI) and TUI/mg sample after a 15 min UAE. In the pod experiment, the breeding line 228_4aa_ca also showed the highest TUI and TUI/mg sample after a 15 min extraction with UAE. These results underline the potential of UAE to maximise trypsin inhibitor content. In addition, remarkable correlations between TUI, TUI/mg sample and the percentage of trypsin inhibition (%TIn) were observed in both experiments.

☆ Audio abstract available in Supplementary material.

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Conclusions: These results provide valuable insights into the relationship between bean genetic resources, extraction methods and trypsin inhibitor content in bean pods and seeds and serve as a basis for refining extraction protocols. The study encourages further research on the practical implications of investigated protocols for breeding programmes and agricultural practices.

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1. Introduction

The common bean (*Phaseolus vulgaris* L.) and the runner bean (*Phaseolus coccineus* L.), which are staple foods in many global diets, are known for their high nutrient content. These bean species serve as an excellent source of protein, calories and essential micronutrients [1,2]. However, the uptake of these nutrients is subject to numerous variables, including the presence of anti-nutrients such as trypsin inhibitor, phytic acid, tannins and others, as well as the methods used in processing and the physicochemical state of the nutrients themselves [3]. Although trypsin inhibitor is commonly considered an anti-nutritional agent because it can interfere with nutrient absorption, it also has an interesting dual role. Apart from its effects on nutritional factors, trypsin inhibitor has shown a remarkable ability to serve as a natural defence mechanism for plants against various pests. Plants have evolved several effective mechanisms to defend themselves against herbivores, and one of these mechanisms is the production of protease inhibitors (PIs) that interfere with insect digestion [4]. PIs are the first proteins to be activated in plants as a defence mechanism against pathogens and promote resistance [5]. For example, PIs competitively bind to the catalytic site of insect proteases and block the proteolysis of ingested food proteins, leading to a reduction in amino acid assimilation in the insect gut and retarding their growth and development, which in turn leads to lower fecundity and survival rate [6]. Kunitz trypsin inhibitor has been shown to be effective in defence against spider mites (*Tetranychus urticae*) [7] and pod borer (*Helicoverpa armigera*) larvae in chickpea [8]. In addition, several studies have shown that extracts of trypsin inhibitors from common beans have successfully reduced the populations of *H. armigera* and *Spodoptera litura* during the larval growth phase [9].

Trypsin inhibitors are proteins that are involved in various biological processes, in particular the regulation of proteolysis. These enzymes effectively inhibit the activity of trypsin, an important digestive enzyme. By modulating trypsin activity, trypsin inhibitors contribute to the regulation of protein digestion, immune response and other important physiological functions. They are key enzymes in protein digestion as they inhibit pancreatic serine protease [3]. Understanding the activity of trypsin inhibitors in legumes is important for various purposes, including food processing, evaluation of anti-nutritional factors, and prevention of pest infestation in grain legumes [10]. The pods of common beans commonly known as French, snap, green or string beans are important vegetables and part of the plant susceptible to diseases, emphasising the importance of determining their trypsin inhibitor content. While several experiments have focused on the determination of trypsin inhibitor concentration in legume seeds, the trypsin inhibitor concentration in plant leaves has only been investigated to a limited extent [11]. To date, there have been few studies on common bean seeds [12,13,14], so it is important to investigate trypsin inhibitor levels in the pods to gain a comprehensive understanding of their distribution and potential impact on disease resistance.

Although methods already exist for measuring trypsin inhibitor activity (TIA) in legumes [15,16], these often need to be modified to take into account the specific characteristics of *Phaseolus* spp. bean seeds. Many of the modified methods for determining TIA are primarily designed for soybean seeds [15,17,18], while only shaking-assisted techniques are available for common bean seeds [13,14,19]. In recent years, ultrasonic-assisted extraction (UAE) has become a superior alternative to traditional shaking methods in sample preparation, especially in food processing [20]. This method has proven itself in a number of large-scale processes, including emulsification, homogenisation, extraction, crystallisation and more [20,21]. It solves the time-consuming nature of conventional extraction and offers rapid isolation of various compounds from food and environmental samples. This efficiency rivals established classical techniques and emphasises the central role of UAE in modern methods [20]. Ultrasonic techniques offer significant advantages over conventional methods such as manual or rotary shaking, Soxhlet extraction and steam distillation. These advantages include reduced solvent consumption, accelerated extraction, simplicity, cost-effectiveness, high extraction efficiency, rapid mass transfer, the use of environmentally friendly solvents and the ability to extract tightly bound residues that are difficult to dissolve using conventional methods [20,22]. The predominant methods for extracting trypsin inhibitor from plants are known to be shaking the samples for varying lengths of time [13,23,24] or magnetic stirring [25,26]. However, no results are available for the extraction of trypsin inhibitors using UAE methods.

To address the unique characteristics of common and runner bean pods and seeds, customised extraction methods were developed to provide more accurate results. Our research covered various aspects, including sample preparation techniques and optimised test conditions. These factors are crucial in overcoming the challenges associated with determining the traits associated with TIA in common and runner beans. The aim of this study was to contribute to the further development of TIA determination in these important legumes, in particular by investigating and refining extraction methods (UAE vs. shaking). It is of paramount importance to fill the knowledge gap and provide a comprehensive insight into the changes required to accurately measure the traits associated with TIA such as the percentage of trypsin inhibition (%TIn), Trypsin Units Inhibited (TUI), TUI/mg sample and μg Trypsin Inhibited/mg sample in *Phaseolus vulgaris*/*coccineus* pods or seeds.

2. Materials and methods

2.1. Plant material

In 2023, two laboratory experiments were conducted at the Agricultural Institute of Slovenia to evaluate different methods for the extraction of trypsin inhibitors in three replicates. First, the air-dried seeds of common bean (*P. vulgaris* L.) variety KIS Marcelijan, common bean breeding line Ref_316 × 498 and runner bean (*P. coccineus* L.) variety Bonela were investigated. Secondly, the fresh pods at the stage of technological maturity of five genetic resources of common beans (three breeding lines – 228_4aa_ca,

227_2ac_ab, bo2_2ba_bc; and two varieties – KIS Marcelijan, KIS Bogo) with unique pod characteristics were analysed. KIS Bogo is a candidate variety currently undergoing the registration process at the Community Plant Variety Office (CPVO). All bean samples were produced in the fields of the Infrastructure Centre Jablje (46°30′17.4″ N, 15°37′34.6″ E; 320 m a.s.l., subalpine climate) according to standard cultivation practices. Four extraction methods were used in both experiments, *i.e.* ultrasonic-assisted extraction (UAE) for 15 min, UAE for 30 min, shaker extraction for 60 min and shaker extraction for 180 min. The detailed experimental design for both experiments is shown in Table 1.

2.2. Chemicals and solutions

The chemicals used were predominantly purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and included the following: i) Trypsin from bovine pancreas (TPCK Treated, essentially salt-free, lyophilised powder, ≥10.000 BAEE units/mg protein); ii) Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA); iii) dimethyl sulfoxide (DMSO); iv) calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$); v) Tris base (hydroxymethyl methane); and vi) acetic acid. Sodium hydroxide (NaOH) was procured from Merck (Germany).

2.2.1. NaOH solution

0.1 g, 0.08 g and 0.06 g of NaOH were dissolved in 250 mL H_2O to obtain 0.01 N, 0.008 N and 0.006 N solutions, respectively. These solutions were used for the extraction of trypsin inhibitors from bean pods and seeds.

2.2.2. Tris-buffer solution with $CaCl_2 \cdot 2H_2O$ (50 mM)

6.05 g of Tris was dissolved in 900 mL of water. The pH of the solution was adjusted to 8.2 with HCl. Then, 2.94 g $CaCl_2 \cdot 2H_2O$ was dissolved in the Tris-HCl solution, taking care not to add Tris and $CaCl_2 \cdot 2H_2O$ simultaneously. The pH adjustment was performed after the dissolution of Tris and before the addition of $CaCl_2 \cdot 2H_2O$. A shift in pH occurred after the addition of $CaCl_2 \cdot 2H_2O$, emphasising the need to adjust it back to 8.2 with HCl.

2.2.3. BAPNA solution with DMSO

A total of 2 mL of DMSO was mixed with 40 mg of BAPNA at room temperature. This solution was stored at −20°C, where it remains stable over a longer period of time. For daily use, the BAPNA-DMSO solution was stored at room temperature in a dark environment. Once the solution had liquefied, it was vortexed vigorously to ensure thorough mixing. Subsequently, 2 mL of the BAPNA-DMSO solution was added to 98 mL of TRIS-buffer solution.

2.2.4. Trypsin solution (20 mg/L in 0.001 m HCl)

A total of 20 mg of trypsin was dissolved in 1000 mL of 0.001 M HCl and stored at 5°C. The solution was freshly prepared and used within one week.

2.2.5. 30% acetic acid solution

A total of 31.25 mL of 96% acetic acid was diluted with water to obtain 100 mL of 30% acetic acid.

2.3. Pods and seed extraction optimisation

To initiate the procedure, five air-dried seeds with a moisture content below 8% and three representative fresh pods at the stage of technological maturity were homogenised using a laboratory ball mill (Retsch MM400, GmbH, Germany). Subsequently, 200 mg of the pulverised seeds and well as 200 and 400 mg of the homogenised pod paste were used for the determination of the traits associated with TIA. The breeding line 228_4aa_ca and the variety KIS Marcelijan were used to determine which amount, either 200 or 400 mg, has a satisfactory %TIn in the range of 30 to 70% [22]. Consequently, a sample of 400 mg was selected for pod extraction (Table 2). The extraction of the homogenised samples was performed in 15 mL Falcon tubes. To ensure that the pH values of the extract samples were optimal for efficient extraction of the trypsin inhibitors, a series of experiments were performed in which extract samples were prepared with different concentrations of NaOH solution. Specifically, 10 mL of 0.006, 0.008 and 0.01 N NaOH solutions were added and the mixture was thoroughly mixed with NaOH using a vortex. For optimal homogenisation, a rotary shaker (Vibromix, 311 EVT, Tehtnica, Slovenia) with 8000 rpm for 60 and 180 min and an ultrasonic treatment (PRO, 40 Hz, ASonic Ultrasonic cleaning bath, Slovenia) for 15 and 30 min at room temperature were used.

The sample extracts were centrifuged at 17,500 g for 20 min at 4°C and then filtered in a further step using a Whatman™ syringe filter with a pore size of 0.45 μm. Since the pH must be maintained within the critical range of 8.4 to 10 [15], the pH of the extracts was measured and it was found that the seed and pod extracts treated with 0.006 N NaOH solution were within the desired range (Table 3). Indeed, through careful observation and measurement, it was found that the extract samples treated with a NaOH concentration of 0.006 N provided the most reliable and stable pH values over several trials. These samples were used for the determination of the trypsin inhibitors.

2.4. Trypsin inhibitor activity assay

To determine the traits associated with TIA, four portions of the sample extracts (0.12, 0.20, 0.28, 0.36 mL) were first prepared, pipetted into test tubes and the final volume was adjusted to 0.4 mL with distilled water. Liu [25] suggested that the sample extract suspension should be diluted with distilled water so that 1 mL of a diluted sample extract will cause 30–70% trypsin inhibition after correcting the blank values. Here, the pods of KIS Bogo variety and the seeds of Ref_316 × 498 breeding line were selected and the %TIn in the extracts was determined. The results showed

Table 1
Experimental design for the determination of trypsin inhibitor activity (TIA) in common bean (*P. vulgaris* L.) and runner bean (*P. coccineus* L.) samples.

Plant tissue	Species	Breeding line/ Variety	Name	Extraction method	Sample weight, mg	pH (at N NaOH)	Extraction portions, mL
Experiment I							
Seeds	Common bean	Breeding line	Ref_316 × 498	Ultrasonic-15 min;	200	0.006; 0.008; 0.01	0.12; 0.20; 0.28; 0.36
		Variety	KIS Marcelijan	Ultrasonic-30 min;	200		0.20
	Runner bean	Variety	Bonela	Shaker-60 min;	200		0.20
				Shaker-180 min			
Experiment II							
Pods	Common bean	Variety	KIS Marcelijan	Ultrasonic-15 min;	200; 400	0.006; 0.008; 0.01	0.20
		Breeding line	228_4aa_ca	Ultrasonic-30 min;	200; 400		0.20
		Variety	KIS Bogo	Shaker-60 min;	400		0.12; 0.20; 0.28; 0.36
		Breeding line	Bo2_2ba_bc	Shaker-180 min	400		0.20
		Breeding line	227_2ac_ab		400		0.20

Table 2
Influence of sample weight on the percentage of trypsin inhibition (%TIn) in selected common bean pods.

Plant tissue	Species	Breeding line/ Variety	Name	Extraction method	Sample weight, mg	%TIn
Pods	Common bean	Breeding line	228_4aa_ca	Ultrasonic-15 min	200	23.53 ± 3.85
					400	54.34 ± 3.79
				Ultrasonic-30 min	200	14.73 ± 1.89
					400	44.47 ± 3.73
				Shaker-60 min	200	9.72 ± 1.73
					400	48.60 ± 1.77
				Shaker-180 min	200	10.64 ± 1.28
					400	42.31 ± 2.51
		Variety	KIS Marcelijan	Ultrasonic-15 min	200	12.55 ± 2.45
					400	46.63 ± 1.27
				Ultrasonic-30 min	200	7.84 ± 2.45
					400	56.04 ± 6.28
				Shaker-60 min	200	12.00 ± 3.61
					400	32.67 ± 0.58
				Shaker-180 min	200	11.33 ± 3.51
					400	29.33 ± 1.15

Data are mean ± SD.

Table 3
pH values of different NaOH extraction solutions.

Plant tissue	Species	Breeding line/ variety	Name	Extraction method	pH		
					0.006 N NaOH	0.008 N NaOH	0.01 N NaOH
Pods	Common bean	Breeding line	228_4aa_ca	Ultrasonic-15 min	8.97 ± 0.18	9.93 ± 0.29	10.21 ± 0.60
				Ultrasonic-30 min	8.95 ± 0.41	10.01 ± 0.39	10.19 ± 0.20
				Shaker-60 min	8.89 ± 0.95	10.01 ± 0.25	10.20 ± 0.27
				Shaker-180 min	8.96 ± 0.24	10.03 ± 0.23	10.31 ± 0.26
			Bo2_2ba_bc	Ultrasonic-15 min	9.20 ± 0.50	10.26 ± 0.65	10.55 ± 0.77
				Ultrasonic-30 min	9.21 ± 0.75	10.30 ± 0.68	10.58 ± 0.38
				Shaker-60 min	9.29 ± 0.96	10.34 ± 0.97	10.64 ± 0.59
				Shaker-180 min	9.25 ± 1.04	10.36 ± 1.53	10.76 ± 0.98
			227_2ac_ab	Ultrasonic-15 min	8.83 ± 0.27	10.37 ± 0.72	10.66 ± 0.84
				Ultrasonic-30 min	8.93 ± 0.40	10.45 ± 0.21	10.64 ± 0.21
				Shaker-60 min	8.98 ± 1.29	10.45 ± 1.02	10.69 ± 1.52
				Shaker-180 min	8.98 ± 0.71	10.47 ± 0.96	10.76 ± 0.68
		Variety	KIS Bogo	Ultrasonic-15 min	9.18 ± 0.26	10.21 ± 0.48	10.52 ± 0.67
				Ultrasonic-30 min	9.28 ± 0.50	10.29 ± 0.38	10.50 ± 0.62
				Shaker-60 min	9.33 ± 0.51	10.29 ± 0.38	10.51 ± 0.62
				Shaker-180 min	9.34 ± 0.69	10.31 ± 0.56	10.62 ± 0.83
			KIS Marcelijan	Ultrasonic-15 min	9.20 ± 0.38	10.14 ± 0.36	10.26 ± 0.62
				Ultrasonic-30 min	9.24 ± 0.56	10.16 ± 0.81	10.24 ± 0.74
				Shaker-60 min	9.38 ± 0.55	10.20 ± 0.79	10.29 ± 0.72
				Shaker-180 min	9.35 ± 0.62	10.19 ± 0.35	10.31 ± 0.47
Seeds	Common bean	Breeding line	Ref_316 × 498	Ultrasonic-15 min	9.60 ± 0.24	10.14 ± 1.01	10.24 ± 0.25
				Ultrasonic-30 min	9.07 ± 0.27	10.08 ± 0.23	10.26 ± 0.23
				Shaker-60 min	8.86 ± 0.18	10.38 ± 0.27	10.59 ± 0.29
				Shaker-180 min	8.94 ± 0.18	10.17 ± 0.30	10.25 ± 0.23
		Variety	KIS Marcelijan	Ultrasonic-15 min	9.36 ± 0.04	10.21 ± 0.09	10.39 ± 0.10
				Ultrasonic-30 min	8.54 ± 0.06	10.29 ± 0.07	10.69 ± 0.04
				Shaker-60 min	9.18 ± 0.07	10.33 ± 0.04	10.79 ± 0.05
				Shaker-180 min	8.93 ± 0.06	10.46 ± 0.39	10.90 ± 0.11
	Runner bean	Variety	Bonela	Ultrasonic-15 min	9.40 ± 0.09	10.05 ± 0.13	10.40 ± 0.05
				Ultrasonic-30 min	8.77 ± 0.36	10.34 ± 0.12	10.61 ± 0.21
				Shaker-60 min	9.05 ± 0.06	10.14 ± 0.30	10.84 ± 0.09
				Shaker-180 min	9.01 ± 0.20	10.29 ± 0.10	10.49 ± 0.02

Data are mean ± SD.

that trypsin inhibition values between 30 and 70% were obtained when 0.2 mL of the extracts were used (Table 4). To proceed, 0.2 mL distilled water was added to the 0.2 mL extracts (from seeds and pods) and transferred to a water bath (Semič 40–8333, Kambič, Slovenia) at 37°C. 0.4 mL trypsin and 1 mL pre-warmed BAPNA (at 37°C) were added to the solution, which was vortexed after each addition and incubated at 37°C for 10 min. In addition, 0.2 mL of 30% (v/v) acetic acid was added to the reaction.

Trypsin activity was determined by measuring absorbance at 410 nm using a spectrophotometer (Agilent technologies Cary 8454 UV–Vis) calibrated with a blank solution. For the blank solution, a tube containing water was prepared instead of the sample extract using the same procedure as for the sample extract assay. However, the BAPNA solution was only added after the reaction had been terminated by the addition of acetic acid. As a control, a tube containing trypsin, BAPNA and acetic acid was prepared under the same conditions as the sample extract. The only difference was that no sample extract or water was added to the control.

Various traits associated with TIA, such as the %TIn, TUI, TUI/mg sample and μg Trypsin Inhibited/mg sample (μg TId/mg sample), were calculated using the following equations [25]:

$$\%\mathrm{TIn} = \frac{Acontrol - Asample}{Acontrol} \times 100 \quad (1)$$

Table 4
Percentage of trypsin inhibition (%TIn) in different extraction portions.

Plant tissue	Species	Breeding line/ Variety	Name	Extraction method	Extraction portions, mL	%TIn
Pods	Common bean	Variety	KIS Bogo	Ultrasonic-15 min	0.12	74.90 ± 1.80
					0.20	51.33 ± 2.08
					0.28	26.27 ± 2.45
					0.36	16.88 ± 4.45
				Ultrasonic-30 min	0.12	72.94 ± 3.11
					0.20	44.67 ± 1.15
					0.28	19.22 ± 2.45
					0.36	10.97 ± 2.64
				Shaker-60 min	0.12	80.78 ± 1.80
					0.20	47.06 ± 7.07
					0.28	26.27 ± 1.36
					0.36	4.64 ± 0.73
				Shaker-180 min	0.12	77.65 ± 2.35
					0.20	49.67 ± 1.13
					0.28	21.57 ± 1.80
					0.36	17.30 ± 3.65
Seeds	Common bean	Breeding line	Ref_316 × 498	Ultrasonic-15 min	0.12	75.76 ± 0.75
					0.20	59.30 ± 1.10
					0.28	26.84 ± 4.92
					0.36	15.49 ± 1.41
				Ultrasonic-30 min	0.12	77.92 ± 1.30
					0.20	60.61 ± 2.92
					0.28	17.75 ± 5.41
					0.36	11.27 ± 3.07
				Shaker-60 min	0.12	83.98 ± 0.75
					0.20	57.73 ± 0.45
					0.28	7.36 ± 1.98
					0.36	5.16 ± 1.77
				Shaker-180 min	0.12	80.95 ± 1.98
					0.20	32.44 ± 2.14
					0.28	11.69 ± 3.44
					0.36	11.27 ± 1.41

Data are mean ± SD.

$$\mathrm{TUI} = \frac{Acontrol - Asample}{0.02} \quad (2)$$

$$\mathrm{TUI/mg\,sample} = \frac{Acontrol - Asample}{0.02} \div (mg\ sample\ in\ the\ assay) \quad (3)$$

$$\mu\mathrm{g\,TId/mg\ sample} = \frac{Acontrol - Asample}{0.03} \div (mg\ sample\ in\ the\ assay) \quad (4)$$

where Acontrol is the reference measured value and Asample is the measured value of the extracted sample. TIA is measured as TUI/mg sample. Alternatively, TIA can also be expressed as μg TId/mg sample as described by Liu [25] and Liu et al. [27].

2.5. Data analysis

The statistical analysis was performed with the R package "agricolae" within the software RStudio. The results were subjected to an analysis of variance (ANOVA) with a significance level of $p < 0.05$ to determine significant differences between various parameters. In addition, pairwise comparisons were performed using the Least Significant Difference (LSD) test to determine significant differences in the mean values, with the significance level set at $p < 0.05$. The figures were created using the R package "ggplot2" in RStudio. The correlations between the variables were determined using the Pearson correlation coefficient, which is facilitated by the R package "psych" in RStudio.

3. Results and discussion

3.1. Trypsin inhibitor in Phaseolus vulgaris/coccineus seeds

Several traits associated with TIA in *Phaseolus* spp. seeds were measured after ultrasonic-assisted extraction (UAE) and shaker extraction methods (Fig. 1). Significant variations in several traits associated with TIA (TUI, % TIn, TUI/mg sample and μg TId/mg sample) could be noticed when using the UAE method, providing a more comprehensive insight into the behaviour of the enzyme in this specific context. The results demonstrate the effectiveness of the UAE method over the shaker extraction method for trypsin inhibitor extract.

For all three tested bean genetic resources, namely Ref_316 × 498, KIS Marcelijan and Bonela, the results showed that TUI, TUI/mg sample and μg TId/mg were higher with the 15 min UAE method. However, the Ref_316 × 498 breeding line showed the highest TUI (56.33), TUI/mg sample (0.28) and μg TId/mg (0.19) when subjected to the 15 min UAE method (Fig. 1A–C). This suggests that the specific bean genetic resource responds particularly well to the 15 min UAE method, highlighting its potential importance for optimising the extraction results of Ref_316 × 498. This phenomenon of higher TUI and TUI7 mg sample after 15 min compared to a 30 min UAE could be due to several factors: (i) a shorter extraction time may have been sufficient to release and extract a higher concentration of trypsin inhibitors from the seeds of breeding line Ref_316 × 498, (ii) prolonged exposure to ultrasound energy or extraction solvents could potentially lead to the degradation or denaturation of trypsin inhibitors or other proteins present in the seeds, iii) each breeding line may have a unique biochemical composition, including the content and stability of trypsin inhibitors. In contrast, the shaker extraction methods mostly

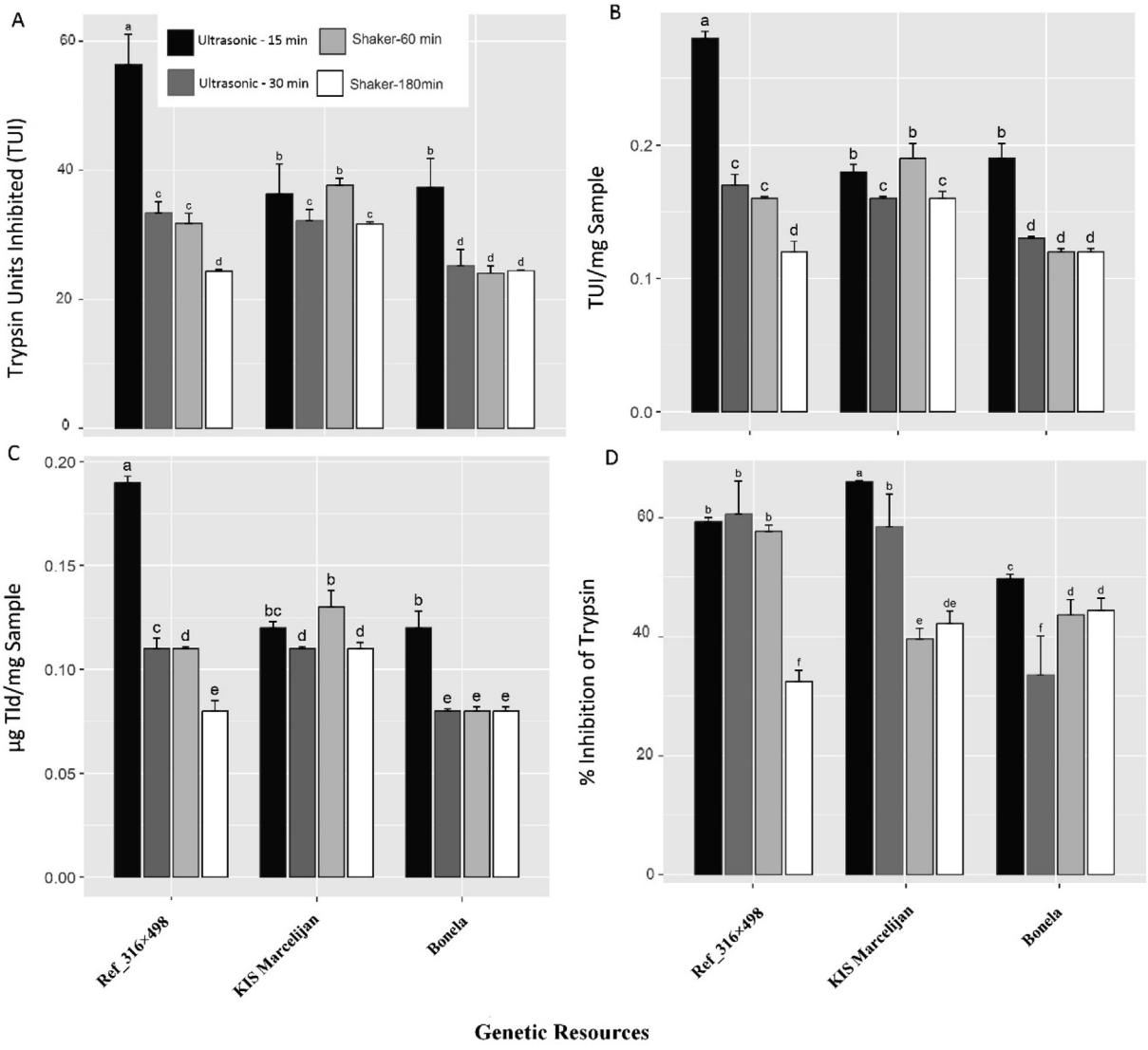

Fig. 1. The variation of TUI (A), % inhibition of trypsin (TIn) (B), TUI/mg sample (C) and µg TId/mg sample (D) in seeds from different bean genetic resources. Data represent the means of three replicates, and the bars indicate the standard deviation. Significantly different treatments ($p < 0.05$) are indicated by different letters.

yielded lower TUI, TUI/mg sample and µg TId/mg. This result is in line with the results of Gualberto et al. [28], who found that UAE provides optimal conditions for the extraction of antioxidants, total phenolic content and total flavonoids from acerola, guava and genipap plants compared to shaker extraction. Under the conditions tested, the Bonela variety showed the lowest values for TUI (24.017 and 24.433, respectively), TUI/mg sample (0.120 and 0.122, respectively) and µg TId/mg (0.080 and 0.081, respectively) for shaker extraction of 60 and 180 min (Fig. 1). However, these values were comparable to those observed for the Bonela variety exposed to UAE for 30 min, underlining the importance of considering not only the extraction method but also its duration when optimising results for specific plant species.

The results of TUI, TUI/mg sample and µg TId/mg sample show a clear pattern: a 15 min UAE consistently outperformed a 30 min UAE, suggesting that shorter exposure times with ultrasonic support can lead to optimal results [29]. This is in line with the results of Blicharski and Oniszczuk [30], who found that the use of UAE proved to be faster and more reliable than the mix-stirring technique. The crucial aspect of ultrasonic extraction is the duration of the process. As the extraction time increases, the plant cells are gradually broken up by the acoustic cavitation effect, which increases the efficiency to a certain extent. However, if the time is extended excessively, this leads to increased destruction of the cell components, which in turn leads to a decrease in overall efficiency [29].

Among the tested bean genetic resources, Ref_316 × 498 breeding line showed sensitivity to %TIn, with values ranging from about 32 to 61% (Fig. 1D). However, UAE consistently yielded higher %TIn values compared to shaker extraction, indicating a clear interaction between extraction methods and the potential for trypsin inhibition. The highest %TIn (66.06%) was achieved when the KIS Marcelijan variety was treated with the UAE method for 15 min. In contrast, Ref_316 × 498 breeding line showed the lowest %TIn value when subjected to shaker extraction for 180 min, resulting in a value of 32.44%. However, these values were not significantly different from the %TIn value of the Bonela variety when subjected to the UAE method for 30 min (Fig. 1D). The sensitivity of %TIn in Ref_316 × 498 with consistently higher values when using the UAE method indicates an interaction between extraction procedure and TIn potential. The genetic diversity observed in TUI, % TIn, TUI/mg sample and µg TId/mg sample among different bean

genetic resources could have implications for breeding programmes aimed at improving nutritional quality and pest resistance of *P. vulgaris*/*coccineus*. However, due to the variability of trypsin purity, accurate measurement is critical when modifying a TIA assay procedure. This is particularly relevant when the same method is used but the TIA is calculated as μg TId/mg sample using the %TIn method [25].

The results of correlation analysis showed that TUI, TUI/mg sample and μg TId/mg sample were positively correlated. In addition, the %TIn had a positive correlation with TUI, TUI/mg sample and μg TId/mg sample, which was statistically significant (Fig. 2). The results of this study are consistent with previous studies emphasising the importance of accurate measurement of TIA in legumes. Studies by Kakade et al. [15] and Smith et al. [16] have established basic methods for assessing TIA, particularly in soybean. However, as highlighted in this study, adapting these methods to the specific characteristics of *P. vulgaris*/*coccineus* seeds and pods remains a critical endeavour.

3.2. Trypsin inhibitor in P. vulgaris pods

Several traits associated with TIA in common bean pods were also determined in shaker extraction and UAE methods. The results of the study revealed a significant increase in TUI, TUI/mg sample and μg TId/mg in the UAE method compared to shaker extraction (Fig. 3). The 15 min UAE gave better results than the 30 min extraction method. On the other hand, a 180 min shaker extraction showed higher efficacy than a 60 min extraction. This pattern was consistent in four studied common bean genetic resources, including breeding lines, with the exception of the variety KIS Marcelijan.

The study showed significant variations in TUI, TUI/mg sample and μg TId/mg between different genetic bean resources, extraction methods and time periods. Breeding line 228_4aa_ca exhibited the highest TUI (30.76), TUI/mg sample (0.08) and μg TId/mg (0.05) after being subjected to a 15 min UAE process. Both the bo2_2ba_bc breeding line and the KIS Bogo variety exhibited lower TUI (11.15 and 12.83, respectively), TUI/mg sample (0.038 and 0.032, respectively) and μg TId/mg (0.017 and 0.020, respectively) when subjected to a 60 min shaker extraction. These values were comparable to the TUI, TUI/mg sample and μg TId/mg of breeding line 227_2ac_ab when subjected to shaker extraction for 180 min (Fig. 3A–C). In shaker extraction, complete rotation of the falcon tube in a controlled environment increases the solvent permeability in the sample [31]. It seems that a longer shaking duration, e.g. 180 min, was more effective than only 60 min. During this longer duration, the penetration of the solvent into the sample increased.

The variety KIS Bogo and the breeding line bo2_2ba_bc exhibited the lowest TUI, TUI/mg sample and μg TId/mg sample when subjected to shaker extraction for 60 and 180 min. In a previous study on different varieties of grass pea seeds, TIA variations were found to have significant differences between the grass pea accessions studied, with values ranging from 26.7 to 90.3 TUI/mg [32]. Higher values for TUI, TUI/mg sample and μg TId/mg were observed in the KIS Marcelijan variety when subjected to the 30 min UAE. This observation suggests that genetic factors may play a crucial role in influencing TIA. Similarly, studies by Shivakumar et al. [33] and Nagl et al. [32] have shown that different varieties have different trypsin inhibitor activities, further emphasising the genetic influence on these activity factors. In addition, a difference was found when comparing KIS Marcelijan pods and seeds: higher levels of TUI, TUI/mg sample and μg TId/mg were reported during the 15 min UAE for the seeds, while the highest levels were observed in KIS Marcelijan pods during the 30 min UAE. This observation is consistent with studies supporting the assumption that the physical state of the plant material can significantly influence extraction efficacy. For example, studies on the UAE of antioxidants from *Rosmarinus officinalis* showed that extraction from dried leaves yielded different results than extraction from fresh leaves [34]. The presence of water in fresh herbs was cited as a possible reason for this difference [34,35].

Among the common bean genetic resources, the breeding line bo2_2ba_bc showed sensitivity to %TIn, with inhibition values ranging from about 44 to 69% (Fig. 3D). This particular breeding

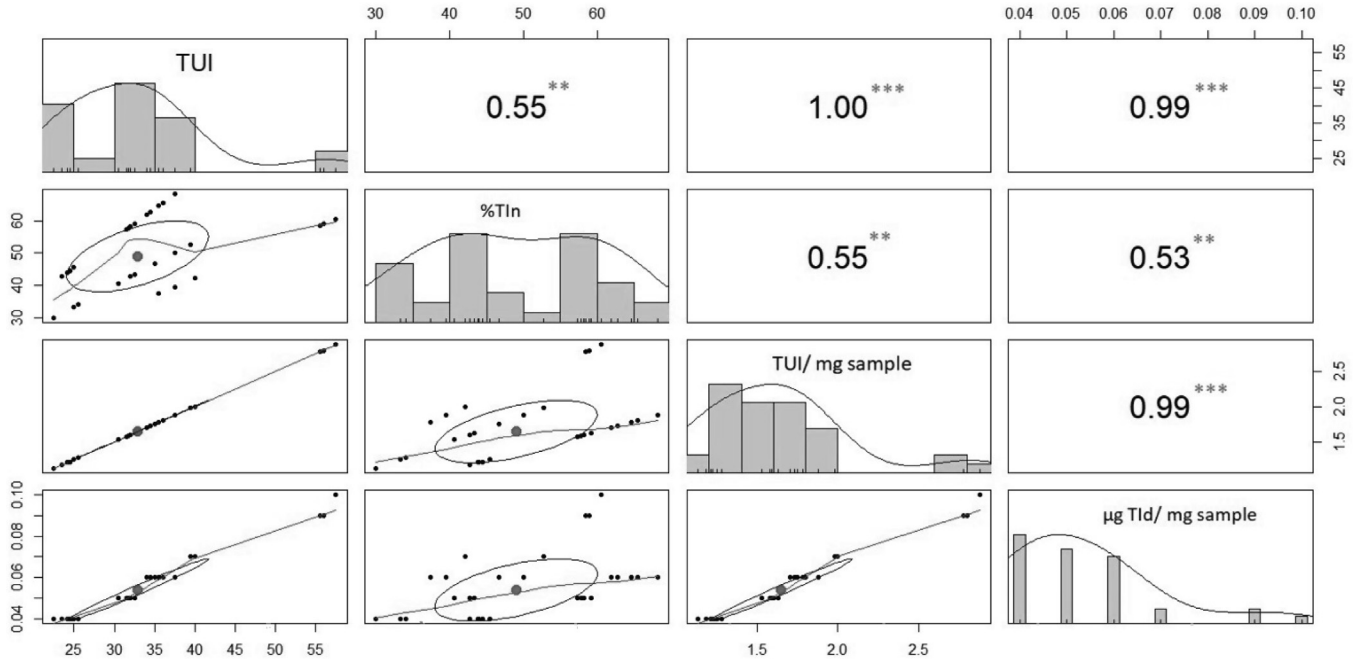

Fig. 2. Correlation matrix plot showing significant difference between, TUI, %TIn, TUI/mg sample and μg TId/mg sample in seeds. Each significance level is represented by a symbol: *p*-values 0.001 (***), 0.01 (**), 0.05 (*).

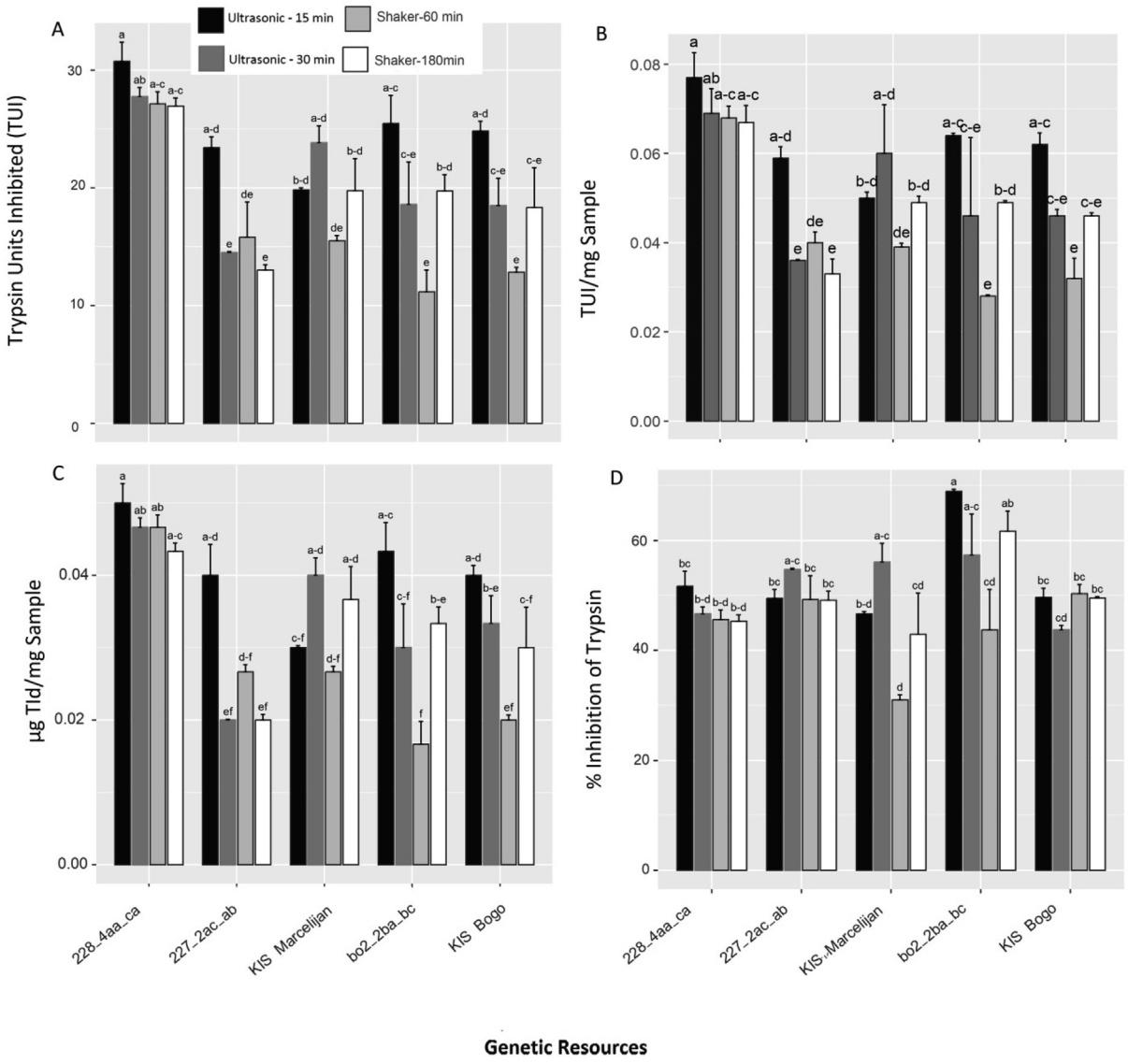

Fig. 3. The variation of TUI (A), % inhibition of trypsin (TIn) (B), TUI/mg sample (C) and µg TId/mg sample (D) in pods from different bean genetic resources. Data represent the means of three replicates, and the bars indicate the standard deviation. Significantly different treatments ($p < 0.05$) are indicated by different letters.

line consistently showed the highest %TIn values for almost all extraction methods, except for 30 min treatment with UAE. In addition, UAE yielded higher %TIn values compared to shaker extraction methods, which is consistent with its well-documented efficacy in various large-scale processes [20,21]. This trend was observed for almost all genetic resources except KIS Bogo, highlighting the significant impact of extraction method on %TIn potential. The results also indicated that the KIS Marcelijan variety had a lower %TIn value (31.00%) when subjected to shaker extraction for 60 min (Fig. 3D). This highlights the variability of trypsin inhibition responses between different genetic resources [32], suggesting that the optimal extraction method and duration may vary between varieties [35].

Based on the collected data, it is clear that there are certain correlations between the traits associated with TIA (Fig. 4). In particular, TUI, TUI/mg sample and µg TId/mg sample showed a positive correlation with each other. The %TIn displayed a positive and significant correlation with TUI, TUI/mg sample and µg TId/mg sample (Fig. 4). The positive correlations between TUI/mg sample and %TIn in seeds and pods (Fig. 2 and Fig. 4) are in agreement with the results of Liu et al. [27] and confirm the comprehensive approach of this study. The focus on the investigation of bean pods and seeds complements the research of Patriota et al. [11], who advocated a broader exploration of the distribution of trypsin inhibitors in different plant parts. The study of pods, a critical but often overlooked component, contributes significantly to our understanding of trypsin inhibitor distribution within the plant. The main objective was to develop robust methods for the determination of TIA in common bean seeds and pods for breeding purposes. We are now continuing the research within the public breeding programme with the practical application of these established methods. Considering how the observed genetic diversity in TIA concentrations could be practically exploited in breeding programmes or agricultural practices would be a valuable next step.

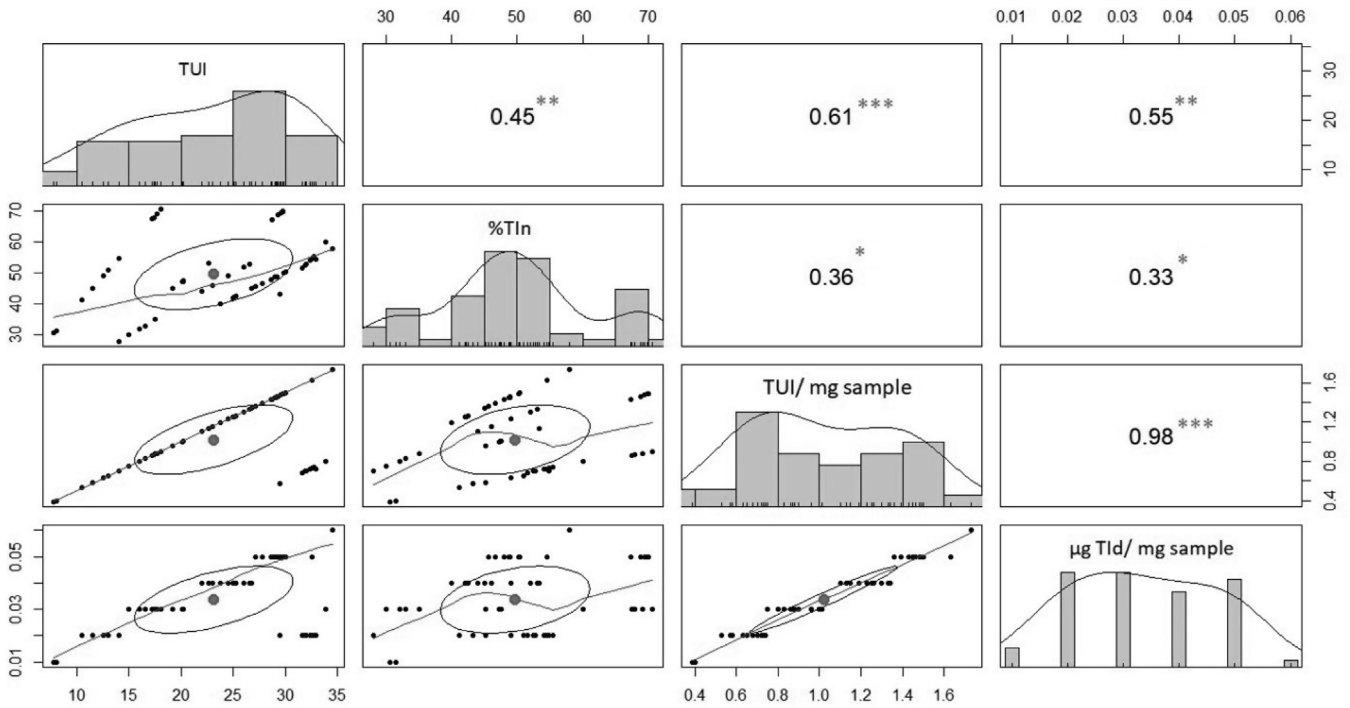

Fig. 4. Correlation matrix plot showing significant difference between, TUI, %TIn, TUI/mg sample and µg TId/mg sample in pods. Each significance level is represented by a symbol: *p*-values 0.001 (***), 0.01 (**), 0.05 (*).

4. Conclusions

In the study, TIA in *Phaseolus* spp. pods and seeds were analysed using shaker and UAE methods, with the UAE method providing the best results using the 15 min extraction procedure. Genetic diversity had a significant effect on TUI values, with the pods of breeding line 228_4aa_ca and the seeds of Ref_316 × 498 showing the highest activity, indicating that they are less susceptible to bruchid disease. The study provides valuable insights into the interplay between genetic bean resources, extraction methods, and TIA levels, highlighting the need for tailored approaches to specific genetic resources and ways to optimise extraction protocols. The focus was on establishing robust methods for TIA determination in bean seeds and pods to be used in the public breeding programme. Exploring practical applications of these results in agriculture and breeding programmes holds great promise for advancing TIA research. This study contributes to our understanding of trypsin inhibitor distribution, particularly in the pods of common bean, an important but often overlooked vegetable crop.

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Conflict of interest

None.

CRediT authorship contribution statement

Hourieh Tavakoli Hasanaklou: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Barbara Pipan:** Data curation, Formal analysis, Writing – review & editing. **Vladimir Meglič:** Funding acquisition, Supervision, Writing – review & editing. **Nevena Nagl:** Conceptualization, Writing – review & editing. **Lovro Sinkovič:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Supplementary material

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