TRYPSIN INHIBITORY ACTIVITY AND FRACTIONATION OF PHENOLIC COMPOUNDS AND DEVELOPING PODS AND SEEDS OF COMMON BEAN (*PHASEOLUS VULGARIS* L.)

BY

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ABSTRACT

The cultivation of common bean Bronco variety was performed in the Agricultural Research Station - Mallawy to conduct this experiment in order to know changes in the percentage of moisture, trypsin inhibitory activity and the quantity of total phenolic compounds and relation of the seeds development by collection the pods after 10, 20, 30, 40, 50 and 60 days after the anthesis. Our results showed that the percentage of moisture be in the maximum value in the seeds and pods after 10 days of the anthesis as well as the trypsin inhibitory activity low begins in pods and seeds after 10 days of the anthesis and then increase up to a maximum value in the maturity stage and the quantity of total phenolic compounds begins to low in 10 days after the anthesis and then begin to increase until it reaches a maximum value in the maturity stage.

Key words: TIs, HPLC, fractionation of phenolic compounds, TPCs, common bean, salicylic acid (SA), secondary metabolites.

1. INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a food legume grown in many countries throughout the world since it is an important source of vegetable protein. It is also important source of several B-complex vitamins, minerals and fiber. On the other hand Common bean contain some antinutritional factors that may have adverse effects on human nutrition as it is the case in all legumes **(Rehman *et al.*, 2001; Beatriz *et al., 2013*; Awoyinka *et al.*, 2016).**

Common bean (*Phaseolus vulgaris* L.) is an important role in human nutrition since they are rich source of protein, calories, certain minerals and vitamins. In African diets, legumes are the major contributors of protein and calories for economic and cultural reasons **(El Maki *et al.*, 2007; Tako and Glahn, 2011).** These proteins might have physiological functions in the plants as well. Changes in the expression of the genes encoding them, for example, have been correlated with alterations in flower morphology, plant growth rate and seed development (**Hartl *et al.*, 2010; Awoyinka *et al.*, 2016**).

Protease inhibitors are widespread anti-nutrient substances which block either trypsin or chymotrypsin, thereby reducing digestibility **(Adebowale *et al.*, 2005; Li Wang *et al.*, 2013).** Trypsin inhibitors (TI), which inhibit the proteolytic activity of the digestive enzyme trypsin, can lead to reduced availability of amino acids and reduced growth.

A trypsin inhibitor was also isolated from *Phaseolus vulgaris* L. with a molecular weight of 13000 Da **(Jacob and Pattabiraman, 1986)** but their activity during seed formation and in seed parts as well as pods has not been investigated. In *Phaseolus vulgaris* L. the changes in trypsin inhibitory activity were assessed at different stages of seed development (including flowers, string bean and ripe bean) **(Sotelo and Lucas, 1998).** There are also studies on changes in some bioactive proteins during seed development of other legumes **(Harsulkar *et al.*, 1997; Kokiladevi *et al.*, 2005; Kumar *et al.*, 2005 and Awoyinka *et al.*, 2016).** However, there is a lack of more comprehensive investigation which could include determination of trypsin inhibitory activity in different seed parts and pods of red kidney bean at different stages of seed development.

The main objectives of the present work:

(1) assay the some secondary metabolites such as Trypsin Inhibitors (TI) and Total Phenolic Compounds (TPCs) in the seeds**.**

(2) to study the relation of the developments in pods, seeds and levels of Trypsin Inhibitors (TI) and fractionation of Phenolic Compounds (TPCs).

2. MATERIALS AND METHODS

2.1. Plant material

The common bean (*Phaseolus vulgaris* L.) of the Bronco variety has grown in agricultural research station, Mallawy – Agricultural Research Center (ARC). The pods were collected of different ages of 10, 20, 30, 40, 50 and 60 days and then dry mature seeds were dried drying antenna, then dried at 60° for 24 hours and then raise the temperature 105° for two hours to determine the percentage of moisture and then grinding the dried pods and dry seeds to estimate the trypsin inhibitory activity and Total phenolic compounds (TPCs).

2.2. Determination of moisture:

Common bean samples were firstly dried at 60°C for 6 hours and the temperature was increased to be 105°C until a constant weight was reached **(AOAC; 1975).**

Figure 1: Seed development in Common bean. From left to right; seeds and pods at 10, 20, 30, 40, 50, 60 days after flower fall and at the mature dry seed stage.

2.3 Preparation of Dry Defatted Meal:

Mature bean seeds and dried pods were ground to a fine flour, then blended with ice cold acetone, the defatted material was air-dried over night at room temperature and stared in a closed container at 4ºC until used.

2.4. Assay of Trypsin Inhibitory activity: -

The trypsin inhibitor activity (TIA) was determined by the procedure of **Hamerstrand *et al.*, (1981),** with minor modification with respect to the initiation of the TIA assay, i.e. trypsin was added last component to the inhibitor-substrate mixture **(Stauffer, 1993).**

Nα-Benzoyl-L-arginine-p-nitroanilide hydrochloride (BAPNA) was used as synthetic substrate for trypsin which hydrolysis of this substrate according to the following equation:-

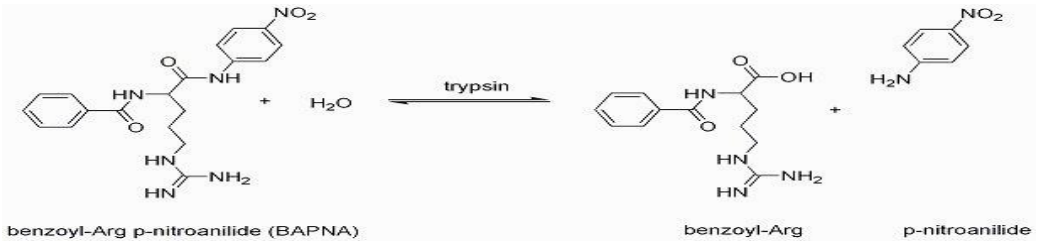

Figure 2: Equation describes the reaction between trypsin enzyme and BAPNA (the substrate).

Preparation of stock solutions:-

1.Tris -buffer:-

Tris (hydroxymethyl-amino methane) (1.21 g) and 0.59 g of $CaCl_2.2H_2O$ were dissolved in 180 ml of distilled water. The pH was adjusted to 8.2 with 1N HCl (10-15 drops) and made up to 200 ml with distilled water. This solution, prewarmed to 37°C for BAPA formulation, stable up to 8 hr.

2. Substrate Solution (BAPNA):-

BAPNA (0.080 g) was dissolved in 2 ml of dimethylsulfoxide and diluted to 200 ml with buffer. The solution was stable up to 4 hr.

3. Trypsin solution:-

Trypsin (0.004 mg) was weighed into a 200 ml volumetric flask and diluted to 200 ml with 0.001N HCl. A fresh solution was made with each run.

Procedure:-

To each of four test tubes, 2 ml aliquot the diluted sample extract (which inhibits 40-60% of trypsin activity) were added with a wide-tip pipette. A fifth tube was prepared for the trypsin standard by adding 2 ml of distilled water. To three of the four tubes containing the sample extract and the tube containing distilled water, 5 ml of BAPA solution was added, and the tubes were placed in a constant temperature bath (37°C) for 10 min.. Two milliliters trypsin solution was rapidly flown into each tube. The contents were stirred immediately on a vortex mixer, and the tubes were replaced in the constant temperature bath. The reaction was terminated exactly 10 min. later by flowing in 1 ml of 30 % acetic acid with immediate mixing with a vortex mixer. A sample blank (the four tube containing sample extract) was prepared by the same procedure except that the trypsin solution was added after the reaction was terminated by addition of acetic acid. The absorbance of each solution was determined at 410 nm against the blank sample values obtained from each of the three sample extracts were subtracted from the trypsin standard. These values were averaged, and the trypsin inhibitor content was determined from the following relationship:-

$$\text{TI,mg / g of sample} = \frac{\text{Differential absorbance x Dilution factor}}{0.019 \times 1000}$$

2.5. Extraction and determination of Total Phenolic Compounds (TPCs):-

TPCs was extracted from defatted sample (0.5g) by refluxing with 30 ml of methanol containing 1 % HCl for 10 min, the extract was centrifuged at 5.000 r.p.m. for 10 min. The concentration of total phenolic compounds in the methanolic extracts expressed as gallic acid equivalents. According to the methods of **Singleton and Rossi (1965)** with some modifications. One milliliter of sample was mixed with 1 ml of Folin and Ciocalten's phenol reagent. After 3 min. 1 ml of saturated Na_2CO_3 (~ 35%) was added to the mixture and this was made up to 10 ml by adding distilled water. The reaction was kept in the dark for 90 min, after which its absorbance was read at 725 nm.

A calibration curve was constructed with different concentrations of gallic acid (0.01 – 1 mM) as standard.

2.6. Separation and quantification of phenolic compounds

Separation and determination of common bean and malt phenolics were performed by reverse phase HPLC (RP-HPLC)/diode array detection (DAD) (Hewlett Packard 1050) using a column Alltima C18, 5mm (150mm x 4.6mm id) with a guard column Alltima C18, 5mm (Alltech). The solvent system used was a gradient of A (CH3COOH 2.5%), B (CH3COOH 8%) and C (acetonitrile). The best separation was obtained with the following gradient: at 0min, 5% B; at 20min, 10% B; at 50min, 30% B; at 55min, 50% B; at 60min, 100% B; at 100min, 50% B and 50%C; at 110min, 100% C until 120min. The solvent flow rate was $1 ml min^{-1}$ and separation was performed at 35°C. The volume injected was 10ml. Phenolic compounds were assayed by external standard calibration at 280nm and expressed in $mg g^{-1}$ dry matter of equivalent (+)-catechin for flavan-3-ols, equivalent coumarin for apolar aromatic compounds and equivalent quercetin-3-rutinoside for flavonols. A variability of 8% was determined on five extractions of phenolics from the same Esterel common bean sample. All values were the mean of two injections.

Procyanidins and prodelphinidin were purified by fractionation of common bean EA extract using a column (300mm x 26mm id) filled with Fractogel Toyopearl HW-40(s), particle size 0.025±0.040 (TOSOH, Merck). Elution was carried out with MeOH at a flow rate of $50 ml\ h^{-1}$. Fractions (7ml) were collected and detection was performed at 280nm. Monomeric and polymeric flavan-3-ols were visualized by spraying spots corresponding to each fraction laid on cellulose plate with 1% vanillin in HCl 12N. After evaporation to 1ml under N_2, the fractions of flavan-3-ols were analysed by analytical RP-HPLC to compare their retention times with commercial standards ((+)- catechin and (-)-epicatechin), and by HPLC+MS and thioacidolysis carried out at IPV-INRA (Montpellier, France).

Thioacidolysis, performed according to the method described by **(Rigaud *et al.*,1991)** allowed us to distinguish terminal units released as flavan-3-ols and extension units as benzylthioethers. Total extracts and isolated fractions were analysed by liquid chromatography coupled with ion spray mass spectrometry in negative ion mode.

3. RESULTS AND DISCUSSION

3.1. Visible changes in pods and seeds of Common bean (*Phaseolus vulgaris* L.) during seed development and with maturity stages:

The size of common bean of the variety Bronco seed increased more than fourfold from 10 days after flower fall to maturation (data not shown). The colour of immature seeds was green up to 40 days after flower fall and then started changing to white**.** Overall, the water contents of developing seeds increased up to 15 days after flower fall and then started decreasing significantly **(Table 1)**. Water contents in pods, seeds coats and whole seeds dropped to very low levels at day 60 (the seed maturation stage).

Table 1: The percentage of water in developing pods, seeds Coats and Whole Seeds of Common bean (10-60 days after flower fall).

Seed parts and pod	Percentage of water in developing pods, Seeds Coats and Whole Seeds (%) with maturity stages					
	10	20	30	40	50	60
Whole seed	81.21	80.35	65.24	45.32	20.55	11.30
Seed Coat	80.11	78.52	76.32	60.34	35.25	7.52
Pods	90.85	90.11	84.52	70.55	30.32	8.54

3.2. Changes in Trypsin Inhibitory Activity (TIA) of Common bean (*Phaseolus vulgaris* L.) during developing of the pods, Seeds Coats and Whole Seeds:

In the Pods, the result of the level of TI increased from 5.6 mg/g sample at 10 DAA to 8.4 mg/g sample at 20 DAA and continue to increase reached to be 38.2 mg/g sample in the maturity stage **(Figure 3)**. In the seeds coat, TI was detected at the early stages of seeds coats development at a level of 10.5 mg/g sample at 10 DAA to 39.8 mg/g in the maturity stage **(Figure 3)**. In the whole seeds, the level of TI increased from 15.5 mg/g at 10 DAA to 38.5 mg/g in the maturity stage **(Figure 3)**.

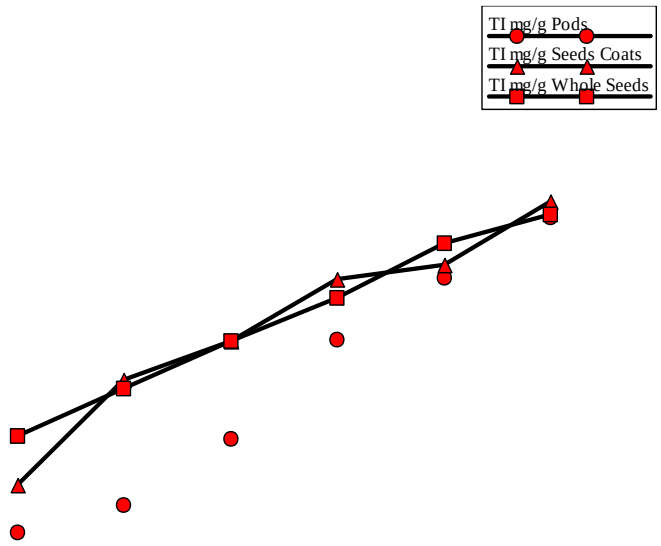

Figure 3: Changes in Trypsin Inhibitory Activity (TIA) of Common bean (*Phaseolus vulgaris* L.) during developing of the pods, Seeds Coats and Whole Seeds.

3.3. Changes in Total Phenolic Compounds (TPCs) during developing of the pods, Seeds Coats and Whole Seeds:

In the seeds coat, TPCs was detected at the early stages of seeds coats development at a level of 30 mg/100g sample at 10 DAA to 330 mg/100g in the maturity stage **(Figure 4)**. In the Pods, the result of the level of TPCs increased from 25 mg/100g sample at 10 DAA to 50 mg/100g sample at 20 DAA and continue to increase reached to be 320 mg/100g sample in the maturity stage **(Figure 4)**. In the whole seeds, the level of TI increased from 45 mg/100g at 10 DAA to 380 mg/100g in the maturity stage **(Figure 4)**.

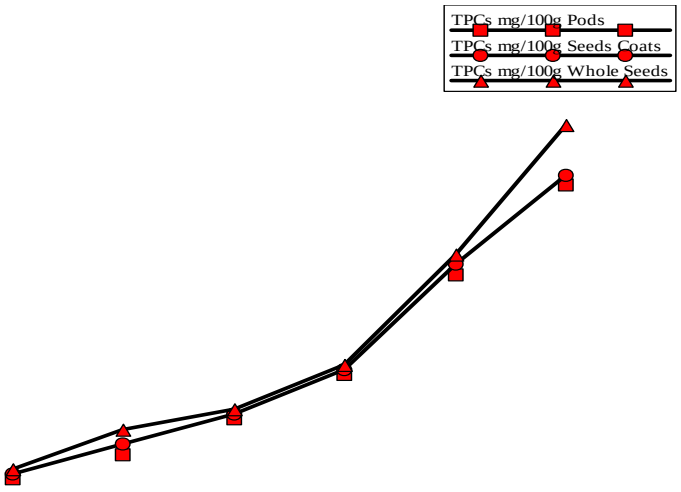

Figure 4: Changes in Total Phenolic Compounds (TPCs) of Common bean (*Phaseolus vulgaris* L.) during developing of the pods, Seeds Coats and Whole Seeds.

3.4. Fractionation of phenolic acids (PAs) in common bean (*Phaseolus vulgaris* L.)seeds:

Results of HPLC fractionation of common bean dry seeds are given in Table (2). PAs fractionation revealed to existence of 15 phenolic acids.

Table (2): Concentrations of Pas (mg/g) in the seeds of common bean (*Phaseolus vulgaris* L.)

Phenolic acids	Concentrations
Gallic acid	2.24
Benzoic acid	23.90
4-Amino-benzoic acid	1.01
Protocatchuic acid	6.26
Chlorogenic acid	4.25
Caffeic acid	0.43
Vanillic acid	1.46
e-vanillic acid	25.48
Ferulic acid	0.85
Iso-ferulic acid	1.04
Ellagic acid	---
3,4,5-Methoxy-cinnamic	0.75
α-cumaric acid	1.64
p-Cumaric acid	1.82
Cinnamic acid	0.35
Total of PAs	71.48
Total of phenols	300.86
Salicylic acid	4.80
Total of PAs + Phenols + SA	377.14

The concentrations of PAs ranged from 0.45 to 25.48 (mg/g). Caffeic acid is less abundant acid whereas, e-vanillic acid is the highest abundant one followed by benzoic acid and Protocatchuic acid. The individual of PAs in common bean seeds are synthesized through shikimic acid cycle which mentioned the defensive roles of these acids against pathogens and pest insects.

3.5. Fractionation of phenols (mg/g) in common bean (*Phaseolus vulgaris* L.)seeds:

HPLC-fractionation of phenols revealed seven compounds listed in Table (3) and the results indicate that pyrogallol is more predominant and the concentration is 282.15 mg/g in the common bean seeds. Coumarin concentration ranged from less one 0.2 mg/g.

Table (3): Concentrations of phenols (mg/g) in common bean (*Phaseolus vulgaris* L.)

Phenols	Concentrations
Pyrogallol	282.15
Catchol	11.28
Catechein	3.41
Epicatechein	1.77
Caffeine	1.44
Reversetrol	0.61
Coumarin	0.20
Total Phenols	300.86

The phenolic acids determined by HPLC in common bean seeds play an important role as antioxidative compounds. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activity of these phenolic compounds **(Ross and Kasum 2002; McMue and Shetty 2004).**

3.6. Salicylic acid (SA) levels in common bean (*Phaseolus vulgaris* L.)Seeds:-

Salicylic acid concentration in common bean seeds are 4.80 mg/g. SA is plant hormone in several organs of plant and plays an important in both local and systemic resistance. SA is an endogenous regulator of disease resistance. Two key enzymes involved in SA biosynthesis and metabolism: benzoic acid 2-hydroxylase. Progress in enzymology and molecular biology of SA biosynthesis and metabolism will provide a better understanding of signal transduction pathway involved in plant disease resistance. The importance of SA as a component of a signal transduction pathway in disease resistance and as a regulator of thermogenesis has stimulated interest in its biosynthesis and metabolism **(Lee *et al.*, 1995)**.

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نشاط مثبط أنزيم التربسين وتفريد المركبات الفينولية وتطور القرون والبذور في الفاصوليا

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الملخص العربى

تم زراعة الفاصوليا الصنف برنكو في محطة البحوث الزراعية بملوي وذلك لأجراء هذه التجربة بغرض معرفة العلاقة بين التغيرات في نسبة الرطوبة ونشاط مثبط إنزيم التربسين وكمية المركبات الفينولية الكلية وتطور البذور والقرون بواسطة جمع القرون بعد 10 – 20 – 30 – 40 – 50 – 60 يوم بعد العقد. وأظهرت نتائجنا أن نسبة الرطوبة تكون في أقصى قيمة لها في البذور والقرون بعد 10 أيام من العقد وكذلك مثبط إنزيم التربسين يبدأ منخفض في البذور والقرون بعد 10 أيام من العقد ثم تزيد وتصل لأقصاها في مرحلة نضج البذور وكذلك كمية المركبات الفينولية الكلية تبدأ منخفضة بعد 10 أيام من العقد ثم تبدأ في الزيادة حتى تصل لأقصاها في مرحلة نضج البذور.