INHIBITION OF DIGESTIVE A-AMYLASE FROM *BACTEROCERA OLEAE* GMELING (DIPTERA: TEPHRITIDAE) BY THE PROTEINACEOUS EXTRACTS OF *PHASEOLUS VULGARIS* L. AND *VIGNA UNGUICULATA* L.

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Abstract: In the current research, effects of the two proteinaceous inhibitors were studied on digestive α-amylase of *Bacterocera oleae* Gmelin, a severely economic pest of olive fruits around the world. The two proteinaceous inhibitors were extracted from *Phaseolus vulgaris* L. (white bean) and *Vigna unguiculata* L. (cowpea) using 20, 40, 60 and 80% concentrations of ammonium sulfate precipitations. The highest amylolytic inhibitions were obtained in the samples extracted by 80 and 60% concentrations of ammonium sulfate for white bean and cowpea, respectively. IC_{50} values of white bean and cowpea were calculated 0.9 and 0.201 mg/ml, respectively. The highest inhibitions were observed at temperature of 25^0C and pH of 5 for both inhibitors. Meanwhile, Kinetic studies in control and treated enzymes revealed lower V_{max} and higher K_m indicating mixed inhibition. Uunderstanding of enzymatic reactions in media containing inhibitors could be useful to have a better viewpoint in control of agricultural pests via plant breeding programs.

Keywords: *Bacterocera oleae, α-amylase inhibitor, white bean, cowpea*

INTRODUCTION

The Olive fruit fly, *Bacterocera oleae* Gmelin (Diptera:Tephritidae), is the most serious pest of olive in the world. The pest causes severe damages in southern Europe, North Africa, India, western Asia and Middle East (Richard et al. 2003; Zalom et al. 2009; Mirrahimi et al. 2008). Females lay their eggs (one egg per fruit) by ovipositor in the hollows below the skin of fruit. Hatched larvae initially dig a tunnel and move deeper into flesh to drop off the fruits and infection by bacteria, yeasts and molds (Richard et al. 2003). Control methods rely on chemical battle and trapping against adults, biological control, sterilized male techniques and pheromones (Richard et al. 2003).

Plants and insects perform ecological, physiological and biochemical mechanisms during evolution to obtain biological benefits. More precisely, plants demonstrate an eco-physiological feature against digestive enzymes of insects by increasing inhibitory systems in their tissues (Rakwal et al. 2001). α-Amylase inhibitors are divided into proteinaceous and non-proteinaceous (Franco et al. 2002). Non-proteinaceous inhibitors contain organic compounds such as acarbose, isoacarbose, acarviosine-glucose, hibiscus acid and the cyclodextrins (Franco et al. 2002), while protein compounds of α-amylase inhibitors are found in microorganisms, plants and animals (Franco et al. 2000; Iulek et al. 2000). In plants,

proteinaceous inhibitors of α-amylase are found in cereals and legumes (Franco et al. 2002; Payan 2003; Sivakumar et al. 2006; Iulek et al. 2000). These molecules are classified by their tertiary structure into six classes including lectin-like, knottin-like, cereal-type, Kunitzlike, γ-purothionin-like, and thaumatin-like used in pest control (Franco et al. 2002) that perform several important roles in plant defense against pests and pathogens (Koiwa et al. 1997; Franco et al. 2000, 2002; Payan, 2003). They disrupt growth, development and reproduction of insects via discrepancies in digestion and metabolic processes (Gatehouse & Gatehouse 1998; Franco et al. 2000, 2002; Sadasivam & Thayumanavan 2003). In our previous study, amylolytic activity was determined and characterized in the midguts of *B. oleae* larvae (Delkash-Roudsari et al. 2014). It was found that the larvae utilize α-amylases for carbohydrate metabolism like many other phytophagous insects. So, inhibition of the enzyme might lead to disturbance of larval digestive physiology.

The aims of this study were to determine inhibitory effects of *Phaseolus vulgaris* L. (White bean) and *Vigna unguiculata* L. (Cowpea) against α-amylase of *B. oleae*.

MATERIAL AND METHODS

Insect rearing

Larvae were reared on the olive fruits of *Arbequina* variety in containers of 20×12 cm. Laboratory conditions were 25±1^{0}C, 70% of RH and 16L: 8D. Rearing containers were daily checked and cleaned to remove any contaminations.

Sample preparation and enzyme assays

Third larval instars were randomly selected, separated from fruits and their midguts were dissected and homogenized in distilled water using a hand-held glass homogenizer. Samples were centrifuged at 13,000 rpm for 20 min at 4^{0}C. The obtained supernatants were kept at -20^{0}C for biochemical analyses.

Determination of α-amylase activity

α-Amylase activity was assayed according to a method described by Bernfeld (1955) using dinitrosalicilic acid (DNS) as the reagent and 1% solution of starch as substrate. Reaction mixture consisted of 50µl phosphate buffer (0.02 M, pH 7), 20µl starch and 10µl enzymatic sample. The reaction was stopped after 30 min by addition of 50µl DNS and heating in boiling water for 10 min prior to read absorbance at 545nm.

Extraction of α-amylase inhibitor from *P. vulgaris* and *V. unguiculata*

Inhibitors were extracted from seeds of *P. vulgaris* and *V. unguiculata* as described by Baker (1987) and Melo et al. (1999). Powdered seeds (15g each) were separately mixed by distilled water for two hours. Then, those were centrifuged at 5000 rpm for 20 min. The supernatant was incubated at 7^{0}C for 20 min to inactivate major endogenous enzymes. Fractions of the supernatant were precipitated using 20, 40, 60 and 80% concentrations of ammonium sulfate followed by centrifugation at 5000 rpm for 20 min at 4^{0}C. The pellets containing the highest fraction of α-amylase inhibitors were used as a source of amylase inhibitors in enzymatic assays.

Inhibition of α-amylase by different concentrations inhibitors

To find possible inhibition of α-amylase, 50µl phosphate buffer (20 mM, pH 7), 20µl starch 1%, 15 µl different concentrations of *P. vulgaris* and *V. unguiculata* (0.05, 0.2, 0.5, 1, 1.5 and 2 mg/ml) and 10µl of the enzyme were incubated for 30 min prior to adding 50µl of

DNS and heating in boiling water for 10 min. After all, the absorbance was read at 545 nm. Blank contains buffer, starch 1% and each concentration of white bean and cowpea, separately.

Kinetic parameters of α-amylase and kinetic of inhibition in *B.oleae*

Kinetic parameters of α-amylase inhibition were determined using different concentrations of starch (0.1, 0.2, 0.3, 0.5, 0.8 and 1mM), 50µl of phosphate buffer, 20µl of IC_{50} concentration of each inhibitor and 10µl of the enzyme. The reaction was stopped by addition of 50µl DNS and heating in boiling water for 10 min prior to read absorbance at 545 nm.

Effect of pH on α-amylase inhibition

Effects of pH on α-amylase inhibition were determined at different pH values using universal buffer (20mM) and pH set of 3-10. The α-amylase activity was assayed after incubation of the reaction mixture containing universal buffer, starch 1%, enzymatic sample and IC_{50} concentrations of the given inhibitors

Effect of Temperature on α-amylase inhibition

The reaction mixture containing phosphate buffer, starch 1%, inhibitors (IC_{50} concnetrations) and enzyme were incubated at different temperature set (15, 20, 25, 30, 35 and 40^0C) and effect of temperature on α-amylase inhibition was found.

Protein assay

Protein concentrations were measured according to the method described by Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

The data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test when significant differences were found at $p \leq 0.05$ using SAS program.

RESULTS

In the current study, α-amylase inhibition was assayed using 20, 40, 60 and 80% concentrations of samples precipitated by ammonium sulfate (Table 1). The highest inhibitions were observed in 80 and 60% concentrations of ammonium sulfate for *P. vulgaris* and *V. unguiculata*, respectively. Other precipitated samples by ammonium sulfate had high protein concentration, but no enzymatic inhibition was observed on digestive α-amylase of *B. oleae* (Table 1). High concentration of proteins in the samples might be due to presence of other proteins that they are precipitated using ammonium sulfate during precipitations. Inhibition of α-amylase in *B. oleae* was obtained by using different concentrations of extracted inhibitors from seeds of *P. vulgaris* and *V. unguiculata* (Fig. 1). Inhibitors IC_{10}, IC_{30} and IC_{50} were also shown in Table 1. Values of IC50 in *P. vulgaris* and *V. unguiculata* were calculated as 0.9 and 0.201mg/ml by POLO-PC software, respectively (Table 2). Kinetic parameters of α-amylase inhibition by *P. vulgaris* and *V. unguiculata* extracts were measured by increasing concentrations of starch (0.1, 0.2, 0.3, 0.5, 0.8 and 1mM) in association of IC_{50} concentration of each inhibitor (Fig. 2 and Table 3). In the treated samples, maximal velocities (V_{max}) were significantly decreased while amount of affinity (K_m) increased as well (Table 3). Concentrations of IC_{50} to *P. vulgaris* and *V. unguiculata* had negative effects on *B. oleae* α-amylase in different ranges of pH and temperature. The highest inhibitions were observed at pH 5 (Fig. 3) and 25^0C for *P. vulgaris* and *V. unguiculata*, respectively (Figure 4).

DISCUSSION

α-Amylases (1,4-α-D-glucan-4-glucanohydrolase; EC3.2.1.1) are a family of endoglycosidases that catalyze hydrolysis of internal α-1, 4-D-glucosidic linkages in starch and glycogen. These enzymes play some key functions in carbohydrate metabolism of microorganisms, plants and animals (Ishimoto & Kitamura 1989). Previous studies have shown inhibition of insect α-amylases by *P. vulgaris* that is due to presence of defensive proteins against phytophagous insects (Marshal & Lauda 1975; Powers & Whitaker 1977). Mehrabadi et al. (2010) have shown dose-dependent effect of inhibitor from Triticale (T-αAI) on α-amylase of *E. integriceps* Puton (Hemiptera:Scutelleridae). α-Amylase of *Callosobruchus maculatus* Fabricius (Coleoptera:Bruchidae) larvae was inhibited by proteinaceous extract from cowpea seeds (Melo et al., 1999). Valencia et al. (2000) reported that an inhibitor from *Amaranthus cruentus* caused 80% inhibition of α-amylase from coffee berry borer. Borzouei et al. (2013) found that proteinaceous extracts of wheat seeds inhibited α-amylase of *Leptinotarsa decemlineata* (Say) (Coleoptera:Chyrsomelidae). Suzuki et al. (1994) found that *P. vulgaris* inhibited digestive α-amylases of *C. maculatus* and *C. chinensisand* and *Zabrotes subfasciatus* Boheman (Coleoptera:Chrysomelliade).

Results of kinetic studies indicated mixed inhibition of α-amylase by the two given inhibitors. This inhibition is a combination of competitive and uncompetitive processes that might be resulted to decrease in V_{max} value and lower affinity of enzyme to substrate. Mixed inhibition has been reported for other amylase inhibitors, *i.e.* inhibition of PPA by α-amylase inhibitor from *P. vulgaris* seeds (LeBerre-Anton et al. 1997), *Rhyzopertha dominica* α-amylase by wheat α-amylase inhibitors (Priya et al. 2010) and α-amylase of *Chilo suppressalis* larvae by *Citrulus colocynthis* L. inhibitor (Valizadeh et al. 2013).

Several studies have revealed correlation between inhibition and pH (Marshall & Lauda 1975; Powers & Whitaker 1977; Valencia et al. 2000). Mehrabadi (2010) found the highest α-amylase inhibition at pH 5 and temperature of 35^0C in *E. integriceps* from *Triticale*. Valencia et al. (2000) reported an optimal pH of 5 for inhibition of coffee berry borer (*Hypothenemus hampei*) by amylase inhibitor (αAI-1) from the common bean, *P. vulgaris* and *Amaranthus* sp. Dastranj & Bandani (2012) have shown the highest α-amylase inhibition at pH 8 in *Helicoverpa armigera* Hubner (Lepidoptera:Noctuidea). LeBerre-Anton et al. (1997) reported that temperature had a moderate effect on the activity of pancreatic alpha-amylase (PPA) by *P. vulgaris.* Marshall & Lauda (1975) reported a 10-fold increase in activity of α-amylase inhibitor when the temperature of the reaction was raised from 25 to 37^0C. A proper inhibitor should block the insect enzyme at a low concentration and at the pH found in the insect gut. This might cause a type of resistance to attack by insect gut proteases (Valencia et al. 2000).

In summary, this work shows potentials of *P. vulgaris* and *V. unguiculata* for inhibition of *B. oleae* α-amylase. Determination of these molecules and their nature is the fundamental to include them in host plant development. These days, many studies have been conducted to introduce toxic molecules to host plants providing resistance to pests. Since, digestive enzyme inhibitors do affect nutrition of insects; their involvements in resistant varieties will lead to have a sustainable and safe pest control.

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Table 1. Inhibitory effects of samples extracted by ammonium sulfate precipitations on α-amylase of *B. oleae*

Inhibition	Concentrations of ammonium sulfate	Relative activity
P. vulgaris	Control (%)	100±14.34 c
	20	206.22±14.59 a
	40	139.92±14.47 b
	60	56.77±19.05 d
	80	3.29± 2.28 e
V. unguiculata	Control (%)	100±6.68 c
	20	284.92±13.86 a
	40	138.97±18.53 b
	60	49.26±7.70 d
	80	140.44± 29.38 b

Table 2. Inhibitory concentrations of *P. vulgaris* and *V. unguiculata* on α-amylase of *B.oleae*

	IC_{10}	IC_{30}	IC_{50}	Slope ± SE	X^2	df
P. vulgaris	0.047	0.35	0.9	2.23±0.037	34.23	4
V. unguiculata	0.02	0.078	0.201	1.283±0.0107	25.347	4

*. Calculation was made by POLO-PC software.

Table 3. Kinetic parameters of digestive α-amylase from *B. oleae* larvae in control and treated enzyme by proteinaceous extracts

Treatment	V_{max}	K_m
Control	0.595±0.069a	0.28±0.018c
P. vulgaris	0.192±0.34b	2.52±0.87a
V. unguiculata	0.219±0.29b	1.93±0.62b

*. Statistical differences have been shown by various letters ($p \leq 0.05$)

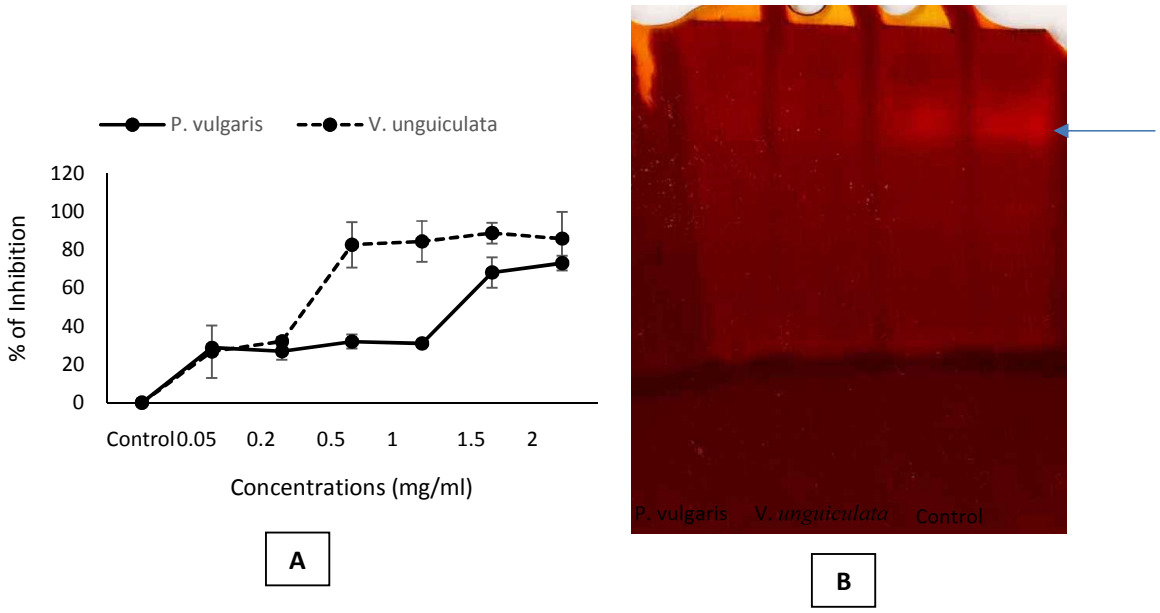


Figure 1. Inhibition of *B. oleae* α-amylase by different concentrations (mg/ml) of *P. vulgaris* and *V. unguiculata* proteinaceous inhibitor
A) in biochemical assay; B) in native-polyacrylamide gel electrophoresis;

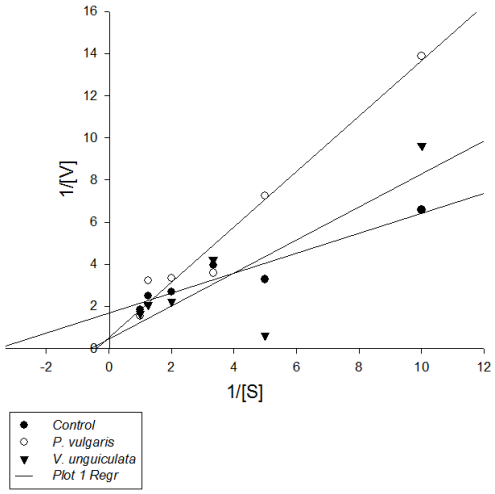
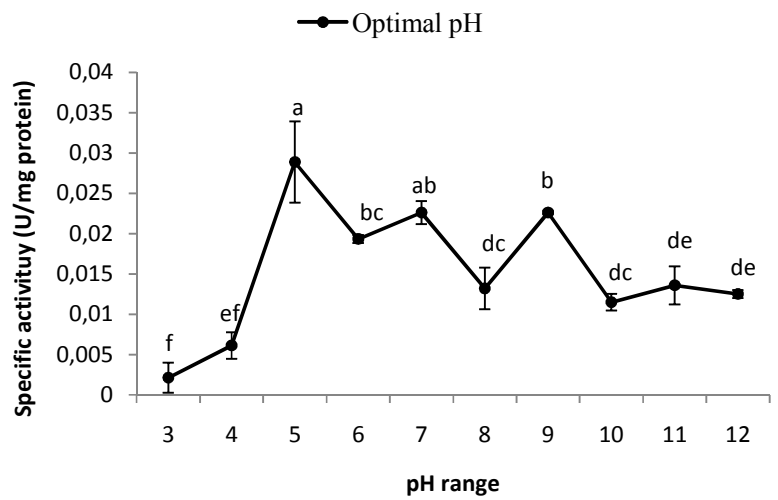
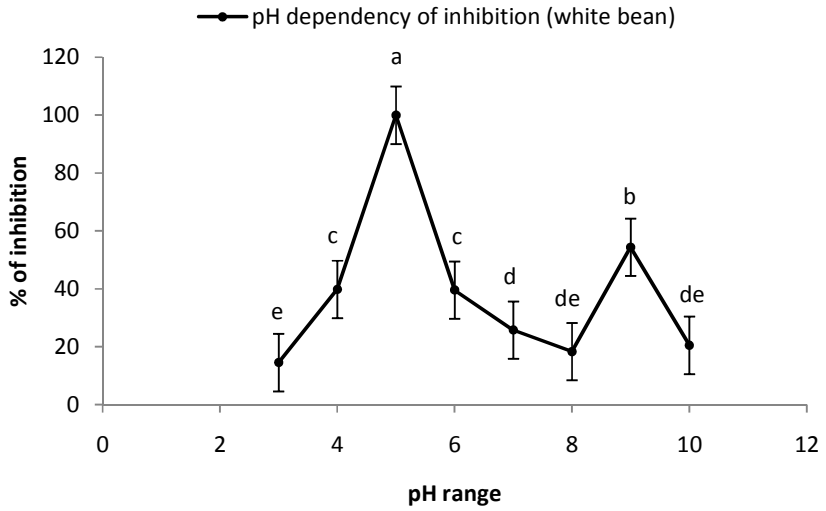


Figure 2. Double reciprocal plots to show the kinetic parameters of α-amylase inhibition from *B. oleae* by proteinaceous inhibitors of *P. vulgaris* and *V. unguiculata*
($1/V_{max}$= intercept on the $1/V_0$ ordinate, $-1/K_m$= intercept on the negative side of the 1/[S] abscissa)





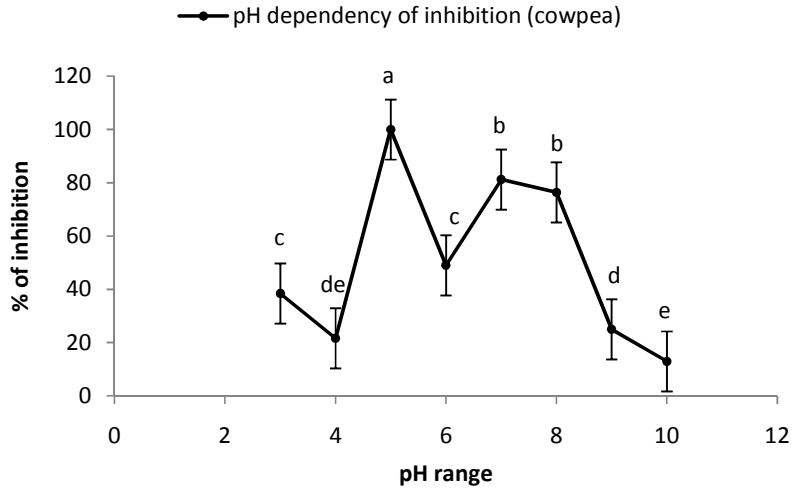
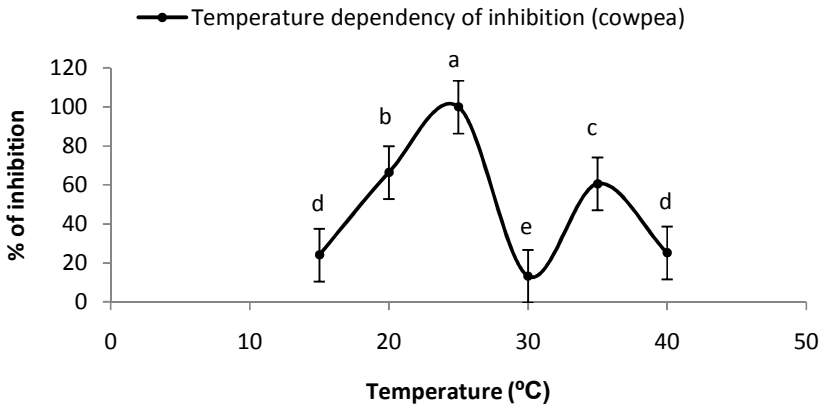
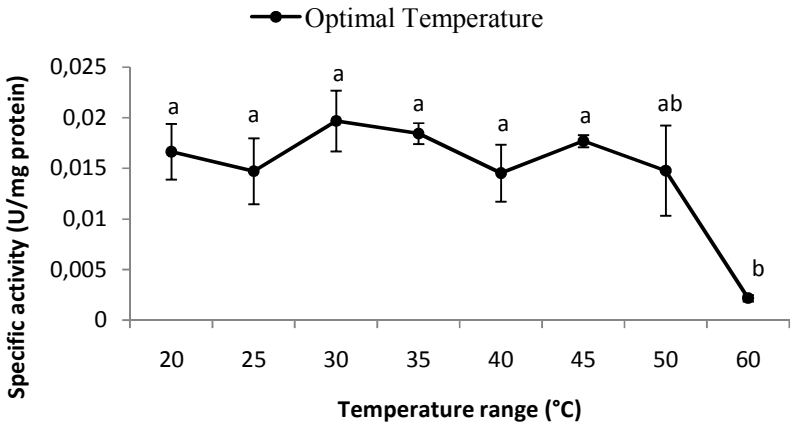


Figure 3. pH dependency of inhibition by *P. vulgaris* and *V. unguiculata* on α-amylase of *B.oleae*. Statistical differences have been marked by various letters (Tukey test, $p \leq 0.05$)





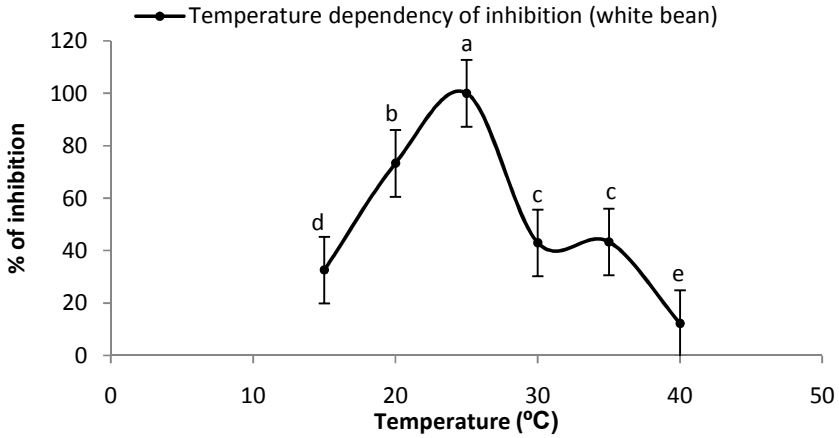


Figure 4. Temperature dependence of inhibition by *P. vulgaris* and *V. unguiculata* on α-amylase of *B.oleae*.
Statistical differences have been marked by various letters (Tukey test, $p \leq 0.05$)