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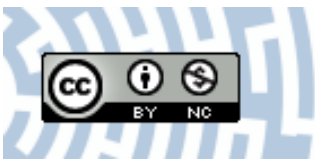

RESEARCH

The Glycolytic Enzymes Activity in the Midgut of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) adult and their Seasonal Changes

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ABSTRACT. The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is an important pest of maize. The diet of the *D. virgifera* imago is rich in starch and other polysaccharides present in cereals such as maize. Therefore, knowledge about enzymes involved in digestion of such specific food of this pest seems to be important. The paper shows, for the first time, the activities of main glycolytic enzymes in the midgut of *D. virgifera* imago: endoglycosidases (α-amylase, cellulase, chitinase, licheninase, laminarinase); exoglycosidases (α- and β-glucosidases, α- and β-galactosidases) and disaccharidases (maltase, isomaltase, sucrase, trehalase, lactase, and cellobiase). Activities of α-amylase, α-glucosidase, and maltase were the highest among assayed endoglycosidases, exoglycosidases, and disaccharidases, respectively. This indicates that in the midgut of *D. virgifera* imago α-amylase, α-glucosidase and maltase are important enzymes in starch hydrolysis and products of its digestion. These results lead to conclusion that inhibition of most active glycolytic enzymes of *D. virgifera* imago may be another promising method for chemical control of this pest of maize.

Key Words: α-amylase, glycosidase, midgut, western corn rootworm, *Diabrotica virgifera*

In the United States, economic loss resulting from a presence and an eradication of the pest—western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) were estimated to $1 billion, annually (Metcalf 1986, Gray 2000). In Europe, until the 1990s the most important pest of maize (*Zea mays*) was the European corn borer (*Ostrinia nubilalis*). In 1992, a new pest of maize, *D. virgifera* has been accidentally brought from North America to the former Yugoslavia (Bača 1994, Kuhlmann et al. 1998, Vidal et al. 2005). Until nowadays, it has not been explained why this, probably not the first accidental introduction of *Diabrotica* to Europe, ended up its successful spreading across the old continent. Its geographical expansion continues from year to year, hence, currently *D. virgifera* is becoming one of the most important maize pests. In 2011, its presence was noted in Eastern France, Northern Italy, and Central and Eastern Europe (Sahajdak et al. 2006, EPPO 2014).

The largest losses in maize crop are caused by the larvae of *D. virgifera*. Feeding in the soil presents problems to control them by chemical methods. In Central Europe, *D. virgifera* is an univoltine species. Females, after fertilization, from middle of July to late autumn lay eggs—more than 1,000 eggs each into the soil. The most of them will diapauses to following vegetative season. Although number of survived eggs strongly depends on winter severity. Next year larvae hatch in the soil from May to July and feed on corn roots (from Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie (BBCH) 09 to BBCH 51-59, according to BBCH-scale introduced in Europe to identify the phenological development stages of a plant) (Zadoks et al. 1974). The third instars larvae feeding inside maize roots cause damage to their supporting and transporting tissue functions, and leads to disturbances in the development of the aboveground parts of the plant. In addition, adverse weather, strong wind and heavy rain may cause lodging of maturing plants and then their harvest is more difficult. The adult beetles start to appear in July (BBCH 51-59) and feed until the first ground frost (BBCH 97). They mainly feed on pollen, maize silk, kernels and finally on the leaves in late summer. When adult beetles occur in large amount their feeding causes damage—the maize cobs are deformed with poorly filled grain (Onstad et al. 2001, Hammack and Petroski 2004, Bernklau and Bjostad 2008, Hibbard et al. 2008, Bereś and Sionek 2010).

One of the factors that determine insects' survival in the environment is ability to effective assimilation of food. Due to the fact that larvae of *D. virgifera* provide the highest damage the most of biochemical studies of digestive enzymes of *D. virgifera* have focused mainly on the larval instars, predominantly proteolytic enzymes, as the main target of the use of protease inhibitors to effective pest control (Koiwa et al. 2000, Titarenko and Chrispeels 2000, Bown et al. 2004, Kaiser-Alexnat 2009). Hence, we are interested in specific digestive enzymes activity, both selected glycolytic enzymes (in this paper) and proteolytic enzymes (data not shown) of *D. virgifera* imago feeding on European maize varieties (or other plants). Therefore, it can be assumed that any reduction in the number of imago also by disorder digestive enzymes activity will result in a decrease in larvae population size in the following years.

Taking into account that with the growth of maize *D. virgifera* imago feeds on maize silk and later on the soft maize kernels (~80% of the maize kernels mass are carbohydrates, mainly starch) the glycolytic enzymes (mainly α-amylase, maltase) should play an essential role in the digestive processes of *D. virgifera* imago (Myers et al. 2000, Titarenko and Chrispeels 2000, Silva et al. 2001, Jbilou and Sayah 2008, Bennetzen and Hake 2009).

This paper describes the activity of five endoglycosidases, among them α-amylase (EC 3.2.1.1) which hydrolyzes α-1,4-glycosidic bond of starch and related carbohydrates. Given the presence of various polysaccharides in the insect's diet and the occurrence of many symbionts in the digestive tract, the paper also considers the importance of other digestive polysaccharides. Therefore, for examination were taken: cellulase (EC 3.2.1.4)—hydrolysis of β-1,4-glycosidic bond of cellulose; chitinases (EC 3.2.1.14)—conversion of chitin to smaller oligosaccharides; laminarinase (EC 3.2.1.39)—hydrolysis laminarin and β-D-glucans with

β-1,3-glycosidic bonds and licheninase (EC 3.2.1.73)—split β-1,3 and 1,4-glycosidic bonds of lichenin and β-D-glucans, which occur abundant in the grass (Terra and Ferreira 1994, 2005, Genta et al. 2006, Jbilou and Sayah 2008).

Among exoglycosidases activity of: α-glucosidase (EC 3.2.1.20)—hydrolysis of terminal, nonreducing α-1,4-linked glucose residues of dextrins, disaccharides, oligosaccharides with releasing α-D-glucose was determined. This activity is responsible for the final starch digestion. Then: β-glucosidase (EC 3.2.1.21)—cleaves of cellobiose, oligosaccharides, glycoproteins, glycolipids, and glycosidic derivatives to β-D-glucose; α-galactosidase (EC 3.2.1.22)—hydrolysis terminal, nonreducing α-1,4-linked glucose residues from oligosaccharides (melibiose, raffinose, stachyose widely present in plants, mainly in grains rich in lipids), galactomannans, galactolipids (mainly in the leaves); β-galactosidase (EC 3.2.1.23)—cuts β-D-galactose from oligosaccharides, hemicelluloses, glycoproteins. As opposed to α-amylase these enzymes act in an ectoperitrophic space of midgut (Terra and Ferreira 1994, Ferreira et al. 1998, Grossmann and Terra 2001, Ferreira 2003, Terra and Ferreira 2005).

There was also tested the specific disaccharidases activity: maltase (EC 3.2.1.20), isomaltase (EC 3.2.1.10), sucrase (EC 3.2.1.48), trehalase (3.2.1.28), lactase (3.2.1.23), cellobiase (3.2.1.21) hydrolyze glycosidic bonds, respectively: α-1,4- of maltose, α-1-6- of isomaltose, α,β-1,2- of sucrose, α-1,1- of trehalose, β-1,4- of lactose, β-1,4- of cellobiose (BRENDA, The Comprehensive Enzyme Information System 2012).

So far there is no essential information neither about the profile of glycolytic enzymes of *D. virgifera* imago and larvae nor precise biochemical properties including the pH optimum and incubation time. If the activity of the main glycolytic enzymes of *D. virgifera* will reveal biochemical similarity to other beetles (pests of cereal) obtained results of this paper should help in searching for efficient methods of inhibition (e.g., plant α-amylase inhibitors—α-AI, Kunitz-like α-amylase inhibitors, lectin-like α-amylase inhibitors from bean, RNAi) of glycolytic enzymes activity as a potential tool of pests population control (Bellincampi et al. 2004, Alves et al. 2010). Currently, studies of selected α-amylase inhibitors in our laboratory are carried out.

The paper also reports the correlation of selected enzymes activity of *D. virgifera* imago versus maize growing season (according to BBCH-growth scale). It can be supposed that intensive feeding and increased digestive processes should correlate with the maize maturation phases, the availability of pollen and soft grains. The knowledge of relation between feeding behavior and digestive processes could be significant information for agricultural practices.

Material and Methods

Insects. Adults of *D. virgifera virgifera* LeConte (different age, both sexes) were caught by bucket traps with pheromone and floral baited dispenser (Medchem, Piaseczno, Poland; Csalomon company, Budapest, Hungary) and also were directly collected from the plant, mainly from maize silks. The collections were carried out on experimental plots of maize crops in the Institute of Plant Protection—National Research Institute, Experimental Stations (Southern Poland) since the second half of July to the end of September. The insects for general investigations of enzymes activity were collected in August. Immediately after collecting the insects were anaesthetized on ice and transported in low temperature to the laboratory. There the insect were dissected and isolated midgut's with contents were homogenized on ice for 3 min in 0.15 M NaCl. The midguts collected from 10 to 25 specimens constituted one sample. The size of such samples ($N = 6$) allowed to define different enzymes activity and determined correlations between them. Samples were centrifuged (Jouan centrifuge MR 22i, Thermo Fisher Scientific Inc., Waltham, USA) at 10,000 *g* for 10 min at 4°C. The supernatants were divided into subsamples and stored at −70°C from 1 wk to several months, until enzyme activity determinations. Control determination did not indicate differences in their activity in relation to storage sample.

Enzymes Activity Assays. For analyses of the pH optimum for enzymes activity 50 mM buffers were used as follows—the α-amylase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8), Tris-HCl (pH 7.8–9.0); the α-glucosidase: citrate-phosphate (pH 2.6–7.0); the β-glucosidase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8); the α-galactosidase: citrate-phosphate (pH 3.4–6.2); the β-galactosidase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8); the isomaltase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8), Tris-HCl (pH 7.8–9.0). The linear dependence of enzymes activity and incubation time in the range 0–360 min for the maltase and trehalase was also examined. All analyses were performed using a modified standard methods cited below (also summarized in Nakonieczny et al. 2006).

Endoglycosidases activity assay was based on Noelting and Bernfeld method (1948) that allows quantitative indication of reducing groups of disaccharides resulting from enzymatic hydrolysis of substrates, using a standard curve prepared for maltose. The following substrates were used: α-amylase—soluble starch, cellulase—sodium carboxymethylcellulose, chitinase—colloidal chitin (prepared according to Hsu and Lockwood 1975), laminarinase—laminarin from *Laminaria digitata*, licheninase—lichenin from *Cetraria islandica*. Samples were incubated (KS 4000i control incubator, IKA Laboratory Technology, Staufen, Germany) at 30°C (± 1°C) for 24 h, except the α-amylase, which was incubated for 40 min. The activities were measured colorimetrically at 540 nm, with a Helios Aquamate Ultraviolet-visible spectrophotometry (UV/VIS) spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) in the 620 µl of reaction mixture: 50 mM citrate-phosphate buffer (pH 4.2), 20 µl of homogenate (10 µl for α-amylase), 200 µl of 3,5-dinitrosalicylic acid reagent used for reaction termination and 300 µl H_2O. The unit activity was expressed as the µmol maltose·mg^{-1} protein·min^{-1}.

The analyses of the glycosidases hydrolyzing particular *p*np-glycopyranosides (α- and β-glucosidase, α- and β-galactosidase) were performed according to Terra et al. (1979) method description. The yellow product (*p*-nitrophenol) is released from the substrate, which consists the appropriate monosaccharide and *p*-nitrophenol linked with α- or β-glycosidic bond. The amount of released product was measured colorimetrically as above and calculated on the basis of a standard curve prepared for the different concentration of *p*-nitrophenol. The following substrates were used: α-glucosidase—*p*-nitrophenyl α-glucopyranoside, β-glucosidase—*p*-nitrophenyl β-glucopyranoside, α-galactosidase—*p*-nitrophenyl α-galactopyranoside, β-galactosidase—*p*-nitrophenyl β-galactopyranoside. Samples were incubated at 30°C (± 1°C) for 40 min, except for the α-glucosidase which was incubated 20 min. The reaction mixture contained 540 µl of 50 mM citrate-phosphate buffer (pH 5.0) (for β-galactosidase: pH 4.2) and 45 µl of homogenate. After incubation 450 µl of 1% sodium dodecyl sulfate (SDS) in 100 mM bicarbonate buffer (pH 10.4) was added. The activities of the glycosidases hydrolyzing *p*np-glycopyranosides were expressed as µmol of *p*-nitrophenol·mg^{-1} protein·min^{-1}.

The analyses of disaccharidases activity were performed according to the modified method of Dahlqvist (1968), which quantifies glucose released from particular disaccharide. Glucose concentration was determined in the reaction mixture using a diagnostic kit "Liquick Cor-Glucose" (PZ CORMAY S.A., Łomianki, Poland) according to Barham and Trinder (1972) method. Absorbance was measured at 500 nm with a Tecan Infinite F200 Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The amount of released glucose was calculated from the standard curve. The following substrates were used: maltose, isomaltose, sucrose, lactose, trehalose, and cellobiose (0.056 M). The reaction mixture (370 µl) contained: 10 µl of 50 mM citrate-phosphate buffer (pH 5.0) with the appropriate substrate, except for isomaltase and cellobiase: citrate-phosphate buffer (pH 5.4 and 3.8, respectively) and 10 µl of homogenate. Samples were incubated for 120 min at 30°C (± 1°C), (maltase—30 min). After incubation (KS 4000i control incubator, IKA Laboratory Technology, Staufen,

Germany) 200 µl of 5% trichloroacetic acid (TCA) was added. From this mixture 20 µl was collected and added to 350 µl of working solution of the glucose kit and incubated for 15 min at 37°C. The activities of disaccharidases were calculated per 1 molecule of glucose released from hydrolysis of particular disaccharide and expressed as µmol glucose·mg^{-1} protein·min^{-1}.

All enzymatic assays from the same stock subsamples were performed. The protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Enzymes Activity Versus Maize Growing Season. To clarify whether the activity of glycolytic enzymes of the *D. virgifera* imago depend on maize growing season the assays of the most active enzymes, according to obtained results were chosen—α-amylase, maltase, isomaltase, and sucrase. The investigations were performed in the two following years: 2010 and 2011. The insects for these determinations were collected in each maize growing season, at three chosen periods of maize development in July, August, September:

- 2010—I. BBCH 69–71 (the phase from the end of flowering to kernel's development with the consistency of watery), II. BBCH 83 (the phase from the beginning of kernels wax maturity), III. BBCH 85 (the phase of full kernels wax maturity);
- 2011—I. BBCH 67 (the phase of full flowering), II. BBCH 75–83 (the phase from the full kernels milky maturity to the beginning of kernels wax maturity), III. BBCH 85–87 (the phase from the full kernels wax maturity to physiological maturity, the kernels contain 60% dry matter).

Statistical Analysis. Statistical analysis of the obtained data of α-amylase and disaccharidases activities of *D. virgifera* imago versus maize growing season (2010 and 2011 year) was performed using the analysis of variance (ANOVA), post hoc Least Significant Difference (LSD) test ($P < 0.05$) (STATISTICA, data analysis software system), version 10 (StatSoft Inc. 2011).

Results

Glycolytic Activities. The highest α-amylase activity in *D. virgifera* imago midgut was stated in the acidic pH (citrate-phosphate buffer at pH 4.2) and it was relatively high up to pH 6.2 (Fig. 1). The α-amylase activity was the highest among all polysaccharidases assayed in this study. Specific activities of the other—laminarinase, licheninase, chitinase, and cellulase were at least about 270 times less.

For exoglycosidases (α- and β-glucosidase, α- and β-galactosidase) also optimal activity at low pH were stated. The highest activity of α-glucosidase, α-galactosidase, and β-glucosidase was stated at pH 5.0, and of β-galactosidase at pH 3.8 in citrate-phosphate buffer. All glycosidases activity, with the exception of β-galactosidase, showed broad pH optima (Fig. 2). The enzymes ordered according to decreasing activities are as follows: α-glucosidase, β-galactosidase, α-galactosidase, and β-glucosidase (Table 1).

Among the enzymes activity hydrolyzing particular disaccharides as the substrate the highest activity for maltase was stated. For other disaccharidases: lactase, trehalase, isomaltase, cellobiase lower activities were found (Table 1). For isomaltase activity the pH optimum at 5.4 was determined (Fig. 3). Figure 4 shows an effect of incubation time on maltase and trehalase activities in the midgut of *D. virgifera* imago. The activity of maltase increased rapidly with incubation time 0–360 min, in contrast to the trehalase activity.

The seasonal changes in selected glycolytic enzymes activity. The dependence of enzymes activity determined with corn growing season, in the two following years (2010, 2011) showed significant statistical differences for activities of α-amylase and maltase in 2010, as well as maltase and sucrase in 2011 year (Figs 5 and 6). Within disaccharidases the maltase was the most active enzyme regardless of year or the maize growing season. Among these enzymes in both years only for isomaltase the same pattern of enzymes activity was stated.

Discussion

Acidic pH occurs in midgut of the majority of Coleoptera larvae and adults (Wolfson and Murdock 1990, Terra and Ferreira 1994, Silva et al. 1999, Hosseininaveh et al. 2007, Lopes et al. 2010). This optimal pH is characteristic both for α-amylase and cysteine proteases (the main group of proteolytic enzymes) of *D. virgifera* larvae (Kaiser-Alexnat 2009). A relatively wide range of α-amylase activity at acidic pH for *D. virgifera* imago was stated. It is similar to those obtained for other herbivorous insects such as the maize weevil (*Sitophilus zeamais*) imago and two members of the family Chrysomelidae *Chrysolina pardalina*, *Chrysolina herbacea* with a pH range of 5.0 to 7.0 (Nakonieczny 2007, Lopes et al. 2010). Wide pH optimum of α-amylase activity is probably a result of the occurrence of several isoforms of the enzyme. Probably it is an evolutionary adaptation to the presence of secondary plant metabolites in their diet, including enzymes inhibitors and toxins (Titarenko and Chrispeels 2000, Hosseininaveh et al. 2007).

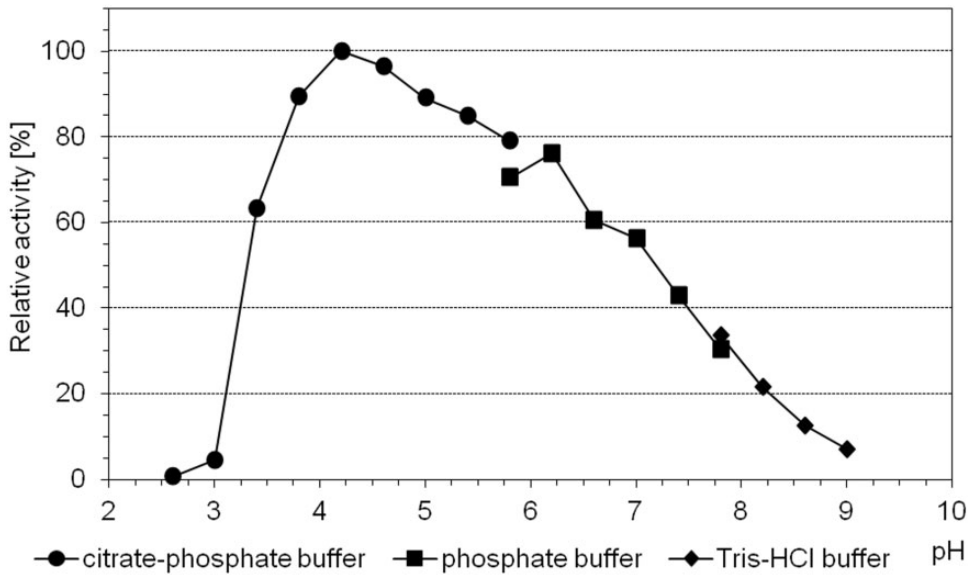

Fig. 1. The effect of pH on α-amylase activity in the midgut of *Diabrotica virgifera* imago.

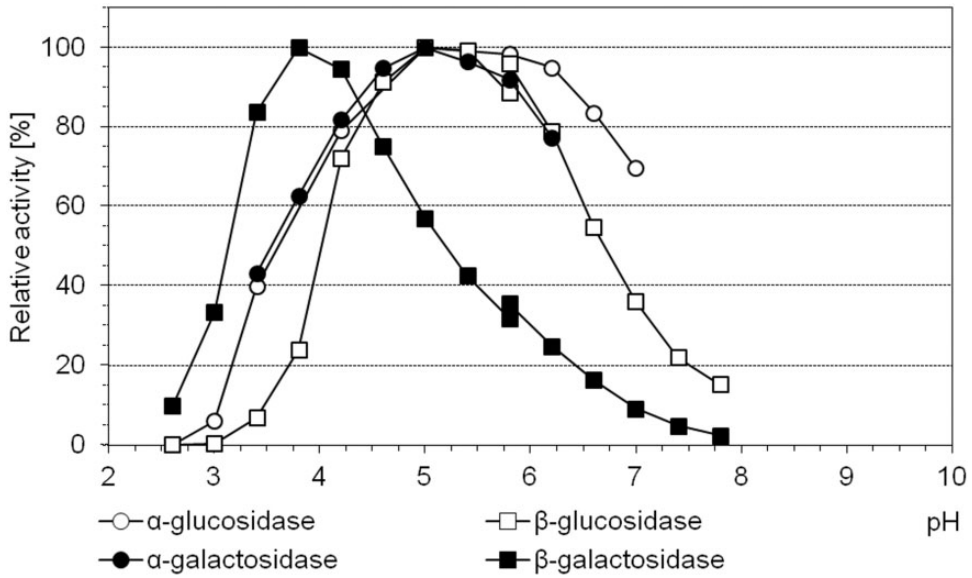

Fig. 2. The effect of pH on activities of exoglycosidases in the midgut of *D. virgifera* imago.

Table 1. The glycosidases activity in the midgut of *Diabrotica virgifera* imago

Glycolytic activity: final produkt, λ, [unit]

Enzyme:	Substrate, concentration	Activity	SD
Endoglycosidases: maltose, 540 nm, [µmol maltose·mg protein^{-1}·min^{-1}]			
α-amylase	Soluble starch in H_2O, 0.50%	149.275	38.040
Laminarinase	Laminarin (from *Laminaria digitala*), 0.25%	0.553	0.139
Licheninase	Lichenin (from *Cetraria islandica*), 0.25%	0.390	0.067
Chitinase	Colloidal chitin, 0.50%	0.112	0.043
Cellulase	Carboxymethyl cellulose (CMC), 0.25%	0.053	0.017
Disaccharidases: glucose, 500 nm, [µmol glucose·mg protein^{-1}·min^{-1}]			
Maltase	Maltose, 0.056 M	41.397	8.602
Sucrase	Sucrose, 0.056 M	18.639	4.905
Lactase	Lactose, 0.056 M	11.779	2.839
Trehalase	Trehalose, 0.056 M	7.883	3.091
Isomaltase	Isomaltose, 0.056 M	3.746	2.692
Cellobiase	Cellobiose, 0.056 M	0.676	0.357
Exoglycosidases: *p*-nitrophenol, 420 nm, [µmol *p*-nitrophenol·mg protein^{-1}·min^{-1}]			
α-glucosidase	*p*NPαGLU, 20 mM	1.506	0.189
β-glucosidase	*p*NPβGLU, 20 mM	0.775	0.223
α-galactosidase	*p*NPαGAL, 8 mM	0.413	0.080
β-galactosidase	*p*NPβGAL, 20 mM	0.822	0.085

Different profiles of glycolytic activities of insect's species express the adaptation of digestive processes to their diet. Imago of *D. virgifera* are polyphagous—initially, they feed on maize pollen, rich in digestible nitrogen and carbon compounds including free amino acids (mainly proline, serine, alanine), proteins, but also carbohydrates such as glucose, maltose, sucrose or stachyose. *Z. mays* has pollen rich in starch (starchy pollen) (Barker 1977, Baker and Baker 1979, Mullin et al. 1997, Hollister and Mullin 1999, Zona 2001, Bennetzen and Hake 2009). The analyses of glycolytic activities in the midgut of *D. virgifera* imago showed that the most active enzyme was α-amylase, followed by α-glucosidase and maltase, among polysaccharidases and disaccharidases, respectively. These enzymes belong to the starch metabolism pathways that hydrolyze molecules of poly-, oligo- and disaccharides with α-glycosidic bonds. The diet of *D. virgifera* imago, mentioned earlier, explains this profile of the glycolytic activities. Also, the studies of carbohydrate digestion in the Mexican bean weevil (*Zabrotes subfasciatus*) and the bruchid beetle (*Callosobruchus maculatus*) feeding on seeds of legumes have shown that the most active glycolytic enzymes were α-amylase, α-glucosidase, and maltase (Silva et al. 1999).

In our study, the lower activities of other polysaccharidases may result from much less content of compounds with β-1,3-, β-1,4- and β-1,3-1,4-glycosidic bonds in the diet of adult *D. virgifera*. Usually high activity of laminarinase in insects feeding on fungi is observed. Microorganisms which possess licheninase and laminarinase, occurring in the midgut, serve rather in the detoxification processes of plant toxic aglycones than in food digestion (Scrivener et al. 1997, Azevedo et al 2003, Terra and Ferreira 2005, Genta et al. 2006).

The glycosidases in the midgut of *D. virgifera* imago hydrolyzing α- and β-glucosidic, α- and β-galactosidic bonds showed maximum activities in acidic pH. For α- and β-glucosidases and α-galactosidase relatively wide pH optima of reaction were determined. Measurements of enzymes activities hydrolyzing *p*np-glycopyranosides as a substrates in the midgut of *D. virgifera* imago have shown the highest activity of α-glucosidase against NPαGlu. This enzyme is also responsible for the final phase of starch digestion (Silva et al. 1999). An insect α-glucosidases are structurally and functionally variable. In *Musca domestica* larvae two isoforms of α-glucosidase were identified. The smaller one (Molecular Weight = 72.7 kDa) hydrolyses preferably oligosaccharides longer than maltotetrose. The larger form (230 kDa) prefers smaller substrates such as maltose, maltotriose, maltotetrose (Jordão and Terra 1991, Fonseca et al. 2010). In *Z. subfasciatus* and *C. maculatus* α-glucosidases which can react with at least five

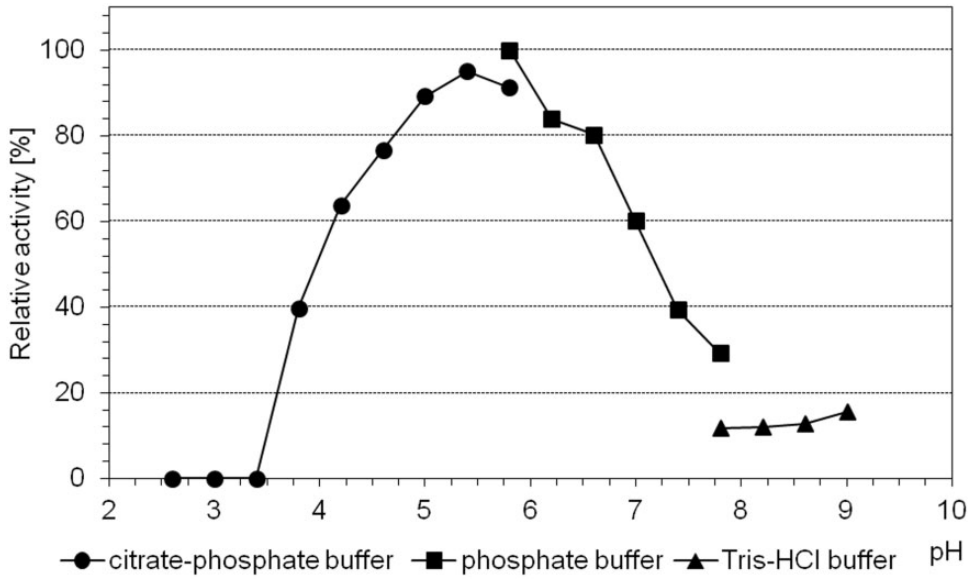

Fig. 3. The effect of pH on isomaltase activity in the midgut of *D. virgifera* imago.

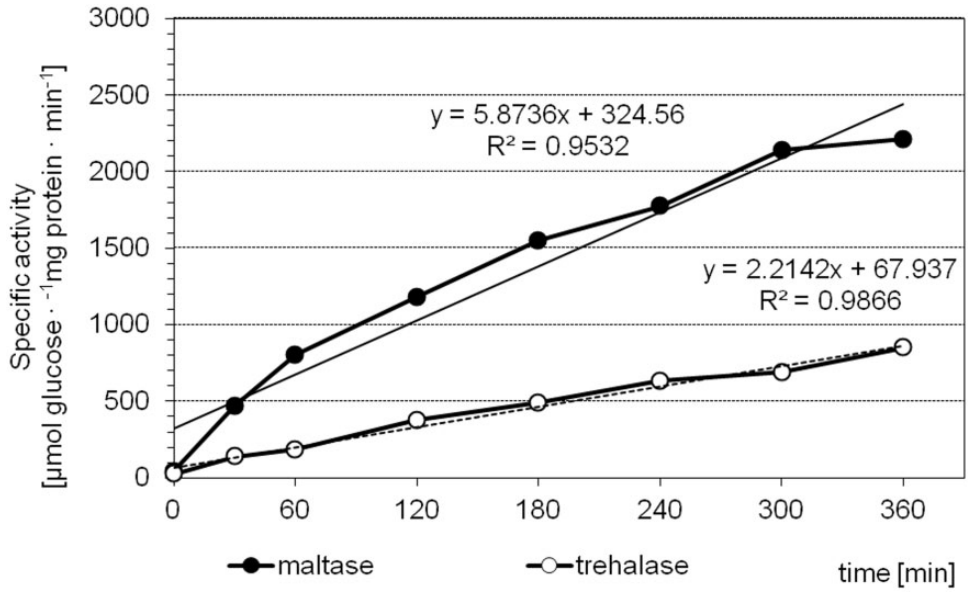

Fig. 4. The effect of incubation time on maltase and trehalase activities in the midgut of *D. virgifera* imago.

substrates, both for maltooligosaccharides and synthetic substrates NPαGlu were identified (Silva et al. 1999).

The activities of enzymes hydrolyzing NPαGlu as a substrate differed significantly from activities against other *p*-nitrophenyl substrates. In the midgut of the *D. virgifera* imago they were about two times higher than against NPβGlu and NPβGal. This may indicate a lower content of food components with β-glycosidic bonds in their diet or presence of toxic compounds which are not effectively digested. The β-glycosidases, occurring in the midgut of insects, may come both from plant food and midgut microflora (including symbionts). The β-glycosidases mainly hydrolyze oligosaccharides, but also disaccharides, derived from cellulose and hemicelluloses which contain β-1,3-, β-1,4- and β-1,6-glycosidic bonds (Terra and Ferreira 1994, Ferreira 2003). The low β-glycosidases activities also may result from a lower level of glycolipids in the diet, especially membranes lipids such as monogalactosyldiglycerides and digalactosyldiglycerides. The last one is hydrolyzed by β-galactosidase only after removing one residue of galactose by α-galactosidase (Grossmann and Terra 2001, Azevedo et al. 2003, Ferreira 2003). In the midgut of the *D. virgifera* the level of α-galactosidase activity among all tested glycosidases was the lowest against the *p*-nitrophenyl β-galactopyranoside as substrate.

Analyses of the disaccharidases activity among β-glycosidases showed higher activity against lactose than cellobiose as a substrate. Lower activity of cellobiase may result from low levels of disaccharides and oligosaccharides with β-glucosidic bonds in the diet. But higher level of lactase activity involved in hydrolysis of β-glucosidic bonds indicate that hydrophilic substrates with β-galactosidic bonds (like oligosaccharides, hemicelluloses, and glycoproteins), common in the tissues of green plants, may be efficiently hydrolyzed by *D. virgifera* imago (Ferreira et al. 1998). In *Tenebrio molitor* β-glycosidase has two active sites. This multifunctional enzyme is proposed as an adaptation to plant glycosides, because their hydrolysis generates toxic aglycones (Ferreira et al. 1998, Ferreira 2003).

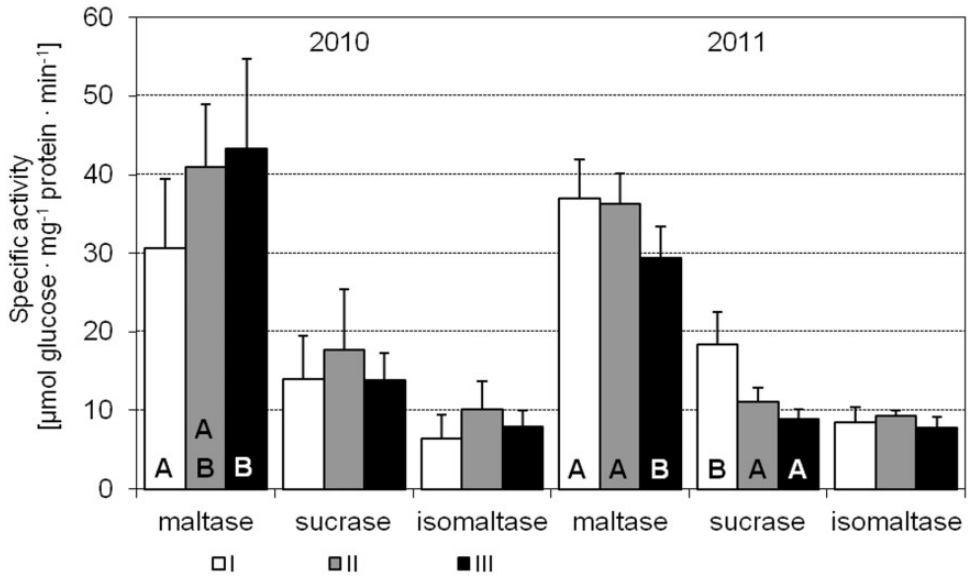

Fig. 5. The dependence of disaccharidases activities of *D. virgifera* imago versus corn growing season (2010 and 2011 years). The different letters inside bars indicate significant statistical differences (ANOVA, LSD test, $P < 0.05$). Error bars ± SEM. Abbreviations: I, II, III—three periods of corn growing season according BBCH-scale—see the M&M chapter.

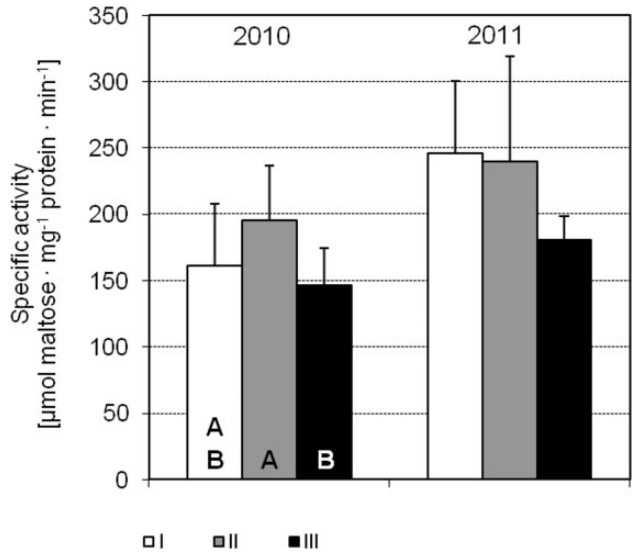

Fig. 6. The dependence of α-amylase activity of *D. virgifera* imago versus corn growing season (2010 and 2011 years). The different letters inside bars indicate significant statistical differences (ANOVA, LSD test, $P < 0.05$). Error bars ± SEM. Abbreviations: I, II, III—three periods of corn growing season according BBCH-scale—see the M&M chapter.

Disaccharidases activity in the midgut of *D. virgifera* imago showed that the most active enzymes are maltase and sucrase, what seems to be obvious results related to the diet of this beetle. Maltose is a product of starch hydrolysis and occurs in large amount in maize kernels, silk, and pollen (Shivanna and Sawhney 1997, Bennetzen and Hake 2009). The high activity of sucrase may indicate that the beetle diet is also high in sucrose and stachyose (Barker 1977). Among the examined enzymes isomaltase is the third one, involved in starch hydrolysis.

The starch digestion to glucose requires the presence of several enzymes which hydrolyze both the α-1,4- and α-1,6-glycosidic bonds and among them the isomaltase is responsible for cleavage of α-1,6-glycosidic bond (Kanehisa 1996). Its low activity in the *D. virgifera* midgut may result from the low expression of this enzyme because starch contains a few α-1,6-glycosidic bonds or due to the overall small amounts of compounds with this bond in the diet of *D. virgifera* beetle.

Trehalase is involved in the hydrolysis of α-1,1-glycosidic bond of trehalose. This disaccharide diffuse from the hemolymph into the midgut and trehalase helps to keep a proper gradient of glucose in the epithelium of the gut. But it does not play a significant role as a digestive enzyme in the midgut of *D. virgifera* imago (Applebaum 1985, Terra and Ferreira 1994, Nation 2002).

The diverse enzymatic profile of *D. virgifera* imago in the 2 yr and three periods of maize development may result from different weather conditions (air temperature, rainfall) and thereby the different availability of food in each year. For *D. virgifera* imago, maize is the main host plant, but after its senescence, the insects also feed on other plants. It was documented that the imago can also feed on plant species belonging to at least 29 families (e.g., Poaceae, Asteraceae, Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Malvaceae) (Hollister and Mullin 1998, Vidal et al. 2005). In particular, the fertilized females for the normal development of eggs need the food rich in amino acids. The changes in enzymes activity during the maize phenology reflect beetle adaptation to plant age or feeding on other plants (Elliot et al. 1990, O'Neal et al. 2002). The results may indicate that the weather conditions may significantly influence the daily activity of *D. virgifera* imago (Isard et al. 1999), and thus the intensity of feeding, which consequently can affect the digestive processes. Perhaps a further correlation of obtained results with weather data prove the changes of particular enzymes activity (inhibition or stimulation).

In summary, analyses of glycolytic enzymes activity in the midgut of *D. virgifera* imago shown that the most important enzymes in the hydrolysis of carbohydrates are the α-amylase, α-glucosidase, maltase and subsequently sucrase and lactase. The profile of glycolytic enzymes seems to be the most suitable for the insect, having a starch-rich diet, like it was proved for grain-storage pests.

Obtained results led to conclusion that inhibition of most active glycolytic enzymes of *D. virgifera* adults may be another promising method of the pest chemical control. Studies of prospective candidates for such inhibitors are currently carried out in our laboratory.

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