NARDI FUNDULEA, ROMANIA ROMANIAN AGRICULTURAL RESEARCH, NO. 34, 2017
www.incda-fundulea.ro Print ISSN 1222-4227; Online ISSN 2067-5720

INTERACTION BETWEEN *DIABROTICA VIRGIFERA VIRGIFERA* AND HOST PLANTS DETERMINED BY FEEDING BEHAVIOR AND CHEMICAL COMPOSITION

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ABSTRACT

The western maize rootworm, *Diabrotica virgifera virgifera* (Le Conte) (Insecta: Coleoptera: Chrysomelidae), is the one of the most dangerous maize pest as in the USA and Europe. This invasive species exhibits different feeding behaviours in different ecosystems and geographical areas. Though the immature stages of this pest are relatively stationary, feeding only on the roots of the maize, the adults may migrate considerable distances and feed on different host plants. The aim of the study was to identify the profile of plant chemicals that was attractive to adults and rank their preferences. Using specially constructed cages, the insects were fed with known and potential host plants. The following substances were quantified in samples of plants (3×20) and insects (3×10): glucose, fructose, caffeic acid, quercetin, flavone rutoside, total phenols, antioxidant activity, lutein, zeaxanthin and total carotenoids. We found that only five main species plants were consumed by the insects (*Cucurbita pepo*, *Phaseolus vulgaris*, *Glycine max*, *Zea mays* and *Helianthus annuus*). Other plants were consumed; however, the statistical results were not significant. Small-scale investigations, as in this study, are important to better understand the possible adaptations of this insect on a large scale.

Key words: invasive insect, *Diabrotica virgifera virgifera*, maize plants, chemical composition.

Abbreviations: AOAC: Association of Official Analytical Chemists, ICRS: International Chemical Reference Substances, Ph. Eur: The European Pharmacopoeia, CtS: solar type cage.

INTRODUCTION

Different hypotheses have been developed concerning the feeding behaviour of *Diabrotica virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae) adults. The expansion of its population in Europe exposes this insect to new environmental conditions that could influence the species' ecology, especially its feeding behaviour. This insect has a long history of study, particularly in regard to its environmental behaviour (Spencer et al., 2009). It has been 145 years since the first report on this insect and its scientific determination and 103 years since its recognition as a species harmful to crops (Gillette, 1912). Unfortunately, there is no known ancestral host species, which makes it difficult to understand the insect's behaviour in the context of evolution (Moeser and Hibbard, 2005).

From its origin in the US maize belt, where it is known to be harmful to maize crops (Metcalf, 1986; Spencer et al., 2005), it has expanded across the American continent and is now present in Europe (Edwards and Kiss, 2007; Ciosi et al., 2009). There are predictions that from Europe, the pest will easily spread to Africa and Asia (Hummel, 2007). The feeding biology of this insect is highly plastic and depends on three factors: the impact of the habitat, the phenological evolution of the plant and sex of the specimen (Moeser and Vidal, 2003). It is known that the phylogeny, biogeography, chemistry, and intra- and interspecies variations influence the selection and specificity of host plants by insects (Becerra, 1997). The ages of insects

Received 8 October 2015; accepted 7 February 2017. First online: March, 2017. DII 2067-5720 RAR 2017-144

and plants are other factors that can influence feeding and survival. Elliot and Gustin (1990) showed that beetle survival decreased with the age of the plant. Adults are present in the crops from the end of June to the middle of October. Krysan et al. (1980) reported that the maximum population occurred during July and August, but this depended heavily on zone and climatic factors. The phenology of the host plant can also influence the damage level and the development of the insect (Hibbard et al., 2008). Another factor involved in the insect-host plant interaction is the longevity and type of food. The influence of diet on adult longevity was reported by Mullin et al. (1991), who showed that adults fed one type of diet, had reduced longevity compared with those fed alternative or mixed diets. Additionally, Campbell and Meinke (2006) conducted an adult feeding behavior study on different plants under natural conditions.

Adults of *Diabrotica virgifera virgifera* prefer maize plants; though it has not yet been proven, it has been shown that changing the diet from maize profoundly influences the insect phenophase (Moeser and Vidal, 2003). The leaves, silk, pollen and maize cobs are the generative organs of the maize, which provides basic nutrition for the beetles (OEPP/EPPO, 2004). In addition to maize, adults can feed on different cultivated plants when alternative sources of pollen are available (Sivcev et al., 2012). By the end of the crop growing season, adults are attracted to blooming plants, notably to wild plants of the genera Amaranthus, Chenopodium and Ambrosia (Moeser and Vidal, 2005).

It is known that the adults fly from mature maize to other crops at flowering. Cucurbits are considered attractive plants, but adults can also be found in crops of alfalfa, clover, rape, soybean and sunflower (OEPP/EPPO, 2004). Adults are primarily observed on the flowers of soybean, alfalfa, cucurbits and sunflower (Mabry et al., 2004; Tallamy et al., 2005).

Adults have been observed feeding on flowers of several other plant species; however, oviposition has not been confirmed in plants other than maize (Siegfried et al. 1990). Other research has shown that when maize silk is present, adults consume less than when maize leaves are at the initial stages of formation (O'Neal et al., 2002).

There are various questions regarding the feeding behavior of these insects. Adult preference for reproductive or vegetative stages of maize has not been verified, though adults are observed before, during and after flowering (O'Neal et al., 2002). One explanation is that most insects are attracted to the yellow color (Kisimoto, 1968; Webb et al., 1994), a common color of flowers and the most visible; therefore, the majority of traps used for capturing insects are yellow (Toth et al., 2006). There have been studies on the chemical composition of host plant species for *Diabrotica virgifera virgifera*, though they were performed only in the roots that support the immature stages. These studies showed the presence of phytosterols in the larval body (Moeser and Vidal, 2004).

Chemical content in aerial organs has been determined in other plant species for development and production, but there has been no correlation with insect feeding (Chourkova, 2012). Considering that the adults are attracted to several host plants belonging to several botanical groups, we attempted to determine what made the host plant attractive to the insect. To examine this, adults were subjected to controlled diets including crops that are listed in the literature as host plants for this species.

The objective of this experiment was to quantify the compounds or chemical properties that that were attractive to adults and rank their preferences. Because this is a harmful pest, it is important to investigate why some plants are more attractive than others.

MATERIAL AND METHODS

Field plants and insects

Experimental design. The experiment was conducted in 2010 and 2011 at a location in western Romania (Timisoara, Timis County, 45°44′58″ N, 21°13′38″ E/45.749444, 21.227222).

Mixed cultures, potential hosts for adults of *Diabrotica virgifera virgifera*, were planted in this location. The experimental area was divided into three plots, and the plots were seeded with different plant species: *Zea mays* (4 or 5 plants), *Helianthus annuus* (4 or 5 plants), *Phaseolus vulgaris* (2 plants/planting hole), *Glycine max* (2 plants/planting hole), *Cucurbita maxima* (2 plants/planting hole), *Cucurbita pepo* (2 plants/planting hole), *Cucumis sativus* (2 plants/planting hole), *Cucumis melo* (2 plants/planting hole) and *Citrullus lanatus* (2 plants/planting hole).

The plants were organized differently according to the experimental factors, in three rows, placed at a distances of 25 or 50 cm/1 m. Unique experimental techniques were used for each plant subject to observation, taking into account the biology of the insect and the phenology of the plant. Experimental species were sown over time in different cultures, so the adult insects could have access to the flowering phenophase. We developed a specific methodology for the *Diabrotica virgifera virgifera* species. Generally, there were specific times necessary for the development and successions of the main phenophases of the plants in this study (Krysan and Miller, 1986).

Observation cages. Each species was sown in special observation cages called solar type cages (CtS) that could accommodate both medium (beans, soybean, cucumber, watermelon, muskmelon, white squash and pumpkin), and tall plants (maize and sunflower). Cages were constructed of wood covered with dense mesh (holes of 2 mm in diameter), so that the plants and insects had access to light and air (dimensions of the cages were 2 m high, 2 m wide and 2.5 m long). The cages also had easily accessible doors, placed on one side, to facilitate plant observation and collection, insect introduction, and other maintenance. The placement of the observation cages in experiment was such that the introduced insects could not escape, and immediately after placement in the cages, a safe attachment was made to the substrate, further preventing escape.

Sampling of biological material

Collection of insects. Collection of adults occurred in early July, corresponding to the pre-flowering and flowering stages of the studied plants and to the first flight of *Diabrotica virgifera virgifera* adults. Beetles were collected from a neighboring experimental maize culture, usually in the evening between 18:00 and 20:00 hours. Each cage was filled with 200 individuals. Collection was performed immediately after installing cages in the experimental field (collection amount depended largely on the availability of the insect population from nearby cultures).

Collection of samples (plants and insects) for chemical analysis. After repeated observations of the plants inside the cages, whole small leaves, small portions of large leaves, flowers and inflorescences were collected. In each cage, 50 insects were also collected, representing one-third of the total number of insects initially present in the cage. The collection of the insects was conducted manually, with the help of some special plastic boxes.

Preparation of the biological material. The biological material (plants and insects) were prepared for chemical analysis. The plant samples were naturally dried, and the insect samples were frozen.

The study in the observation cages

Establishing the injury level of the plants. To estimate the injury level and indirectly measure the food consumed by *Diabrotica virgifera virgifera* adults, a percentage rating system based on the frequency and intensity of damaged plants was employed. Attack frequency was quantified by damage to the aerial organs of the plant and was calculated by the total number of leaves attacked relative to the total number of leaves per plant, or the total number of flowers attacked (or inflorescences) compared with the total number of flowers analyzed. A leaf or flower was considered to be attacked when one or more parts of its tissue had been consumed. The data from these observations were centralized and compared to a method of damage calculation for leaf beetles and other arthropods using a rating scale from 0 to 4 (Peterson et al., 1984; Baker and Robinson, 1985; Hoffman et al., 1986; White, 1990).

Quantitative chemical analysis

Glucose, fructose, coffee acid, quercetin, flavone rutoside, total phenols, antioxidant activity, lutein, zeaxanthin and total carotenoids were determined according to the accepted standards (Ph. Eur. Reference Standards [http://www.edqm.eu/en/Ph-Eur-Reference-Standards-627.html], Retrieved 2011/10/16, WHO: International Chemical Reference Substances. [http://www.edqm.eu/en/WHO-International-Chemical-Reference-Substances-ICRS-1393.html] (ICRS) 2010 Retrieved 2011/10/18, ISO–14502–1:2005. AOAC 941.15. AOAC, 2003).

Statistical analysis

The system of linear equations was solved using Cramer's method (Cramer's rule in linear algebra), and the calculation of the determinants was made using Microsoft Excel (Insert Function/Math&trig/Mdeterm).

RESULTS

The feeding behavior. Diabrotica virgifera virgifera adults were observed, in the case of maize, feeding only on the leaves, flowers, and silk. O'Neal et al. (2002) stated that adults are present on the plants during the period of flowering; therefore, adult individuals were placed in cages prior to the pre-flowering stage. Observations of adult feeding behavior began approximately 1 week after their introduction in the cages, so the insects would have time to adapt to the new space and food resources. All of the plants in the study were consumed by the beetles, although the amount and type of damage varied.

The injury level of plants. Weekly observations during July and August showed that the plants from the mixed crops were injured in different ways. Thus, depending on the intensity of the attack on the leaves and flowers, the preferences of adults were ranked based on the injury level.

The number of leaves and flowers (silk) injured was different among the plants because the plants have specific morphological characteristics: some species have a larger number of leaves than others. The linear-shaped leaves had longitudinal injuries between the nervures (maize), while the round- and oval-shaped (bean, soybean, sunflower) leaves permitted the tearing of vegetal tissue through circular perforations. The evaluation of the intensity of attack showed that it was influenced by the foliar or flower surfaces (silk) (Table 1).

Table 1. Injury level of leaves of host plants by *D. virgifera virgifera* adults using a valuation scale of 0:4

Name of plant	Leaves (no/plant)					Average/ injured leaves
	No damage	1–25%	26–50%	51–75%	76–100%	
Phaseolus vulgaris	46.8	24.5	12.57	9.428	5.71	13.05
Cucurbita pepo	33.28	11.428	3.857	1.428	3.285	4.999
Zea mays	9.857	6	4.428	3.285	1.71	3.855
Glycine max	51.71	1.33	2.33	2.33	2	1.997
Citrullus lanatus	22.85	3.14	1.285	0.285	0	1.177
Cucumis sativa	18.25	1	0.57	0.142	0	0.428
Cucurbita maxima	35.71	0.57	0	0	0	0.142
Cucumis melo	9.16	0.166	0.166	0.166	0	0.124
Helianthus annuus	9.16	0.166	0.166	0.166	0	0.124

The means were calculated using the formula AVERAGE (A_1: A_n)/MO in Excel.

For the plants with a large vegetal surface, the 0-4 scale had smaller values (≤1.177), and for plants with smaller leaves, the values were significantly larger (13.05-1.997). Most of the leaves and flowers (silk) had injury values between 1 and 2, (1-25% and 26-50%, respectively). For the inflorescences, the intensity of the injury had smaller values (≤0.787) and was present in most plants from the experiment compared with the values obtained for the leaves. Most affected inflorescences had values with three damage levels: 1:1-25%, 2: 26-50% and 3:76-100% (Table 2).

Table 2. Injury level of flowers (silk/ligulae) of host plants by *D. virgifera virgifera* adults using a 0-4 evaluation scale

Name of plant	Flowers/silk/ligulae flowers (no/plant)					Average/injured flowers/silk
	No damage	1–25%	26–50%	51–75%	76–100%	
Glycine max	5.857	5	3.714	2.428	2.714	3.464
Phaseolus vulgaris	0.571	1.66	2.66	3.5	5.66	3.370
Cucurbita pepo	8.428	5.714	2.285	0.857	2.428	2.821
Helianthus annuus	11.5	2.33	0.66	0.16	0	0.787
Cucumis melo	11.5	2.33	0.66	0.16	0	0.787
Cucumis sativa	2.75	0.857	1.714	0.428	0.142	0.785
Zea mays	0.714	0.857	0.428	0.285	0.714	0.571
Cucurbita maxima	2.285	0.571	0.857	0.571	0	0.499
Citrullus lanatus	14.5	1.285	0.428	0.142	0	0.463

The means were calculated using the formula AVERAGE (A1: An)/MO in Excel.

Ranking the host plants according to the level of injury of the leaves and flowers resulted in four favoured species: *Phaseolus vulgaris* (bean), *Glycine max* (soybean), *Zea mays* (maize) and *Cucurbita pepo* (white squash). The squash, beans and maize presented a somewhat constant curve of injuries, with maximum values at the minimum level of injury (1-25%) and small values at the maximum level of the scale (76-100%). However, for soybean, the evolution curve was different; with small values at the minimum level of 1-25% and the highest values at the maximum level of 76-100%. Usually, there were larger values for injuries in leaves than for the flowers; soybean was the exception because the flowers were affected much more.

Ranking the host plants according to the level consumed by insect

The interaction of *Diabrotica virgifera virgifera* with the host plant may be expressed by the percentage of consumption of the vegetal tissue (leaves, flowers, silk) by the adults. To have a baseline for comparison, chemical analyses were conducted in advance for both plants and insects. The plant samples (leaves, flowers, silk) from the three mixed crops (MixC_1, MixC_2 and MixC_3) were analysed for their chemical contents and presented different values. High glucose values (3.06-4.0 g/L) and fructose values (3.4-4.4 g/L) were obtained for the samples of white squash, soybean, watermelon, muskmelon, maize and sunflower (Table 3).

The spectrophotometric analysis of the alcoholic extracts emphasized a significant content of flavonoids compounds in the 3×10 analyzed species. The plants can bio-accumulate and biosynthesis a wide spectrum of phenolic compounds as a physiological response to stress. These compounds appear as a result of diverse ways of modifying the metabolism of the plant and not from the outer environment because the epidermis (the cells and the outer walls) are intact. On some regions of the leaves, there were necrotic areas with dissolved cells that occurred as a result of the accumulation of phenolic compounds. The highest values were observed for sunflower samples, most likely due to the high number of C=C links and of the C–H distortion vibrations of the double-tied carbon. These results can also be explained by the content of fatty acids in the sunflower samples. The sunflower samples contained 68% of linoleic acid (C18:2), 19% of oleic acid (C18:1) and only 12% of saturated acids (C16:0 and C18:0). High values were obtained for the soybean samples as well. This can be explained by the high content of Omega–3 fatty acids and the low content of saturated fats. The main characteristics of the soybean samples was the high contents of nutritive substances and the superior quality of proteins due to large quantities of essential amino acids (lysine, methionine, threonine, histidine, valine, isoleucine, leucine, phenylalanine, tryptophan and arginine). In addition to its over 30% nutritive substances, soybean has a high oil (17-25%) content of exceptional quality. Other bioactive compounds that can explain the antioxidant potential are protease inhibitors, phytates, phytosterols, saponins, isoflavones (1 g of soybean protein containing 1.2-1.7 mg isoflavones), phenols, and lecithin. Soybean also contains genistein, an isoflavone that is similar to estrogen (phytoestrogen). It is known that sunflower samples contain various carotenoids such as lycopene and β-carotene. The highest content of lutein was determined in muskmelon, with a concentration of 8.43 mg/kg dry matter (dm), followed by maize with 7.89 and 7.31 mg/kg (dm) and zucchini with 6.89 mg/kg (dm). The zeaxanthin content from the samples ranged from 2.70 mg/kg and 8.56 mg/kg. Comparing the carotenoid concentration distributed in all samples, nearly all of the samples had high amounts of zeaxanthin, 6.4-8.56 mg/kg, except for cucumber where the concentration was 2.70 mg/kg. Samples of beetle individuals subjected to chemical analysis revealed the presence of all substances present in the plant samples. The values of glucose and fructose were 5.33 g/L and 6.43 g/L, respectively, polyphenol content 2.2 mg% dm expressed in caffeic acid, 7.23 mg% dw expressed in quercetin and 19.63 mg% dw rutoside (Table 3). Additionally, high phenol contents were observed in the insect samples (reaching 67.33 mC/ mM).

Table 3. Average contents of substances of different plant samples and insects collected from cages

Plant samples/ Average content of substances determined in different stages of vegetation	Cucumber	Pumpkin	Squash	Bean	Sunflower	Muskmelon	Watermelon	Maize (leaves)	Maize (silk and pollen)	Soybean (leaves and flowers)	Insect collected from mixed cultures (1,...,10)
	(leaves and flowers)										
Glucose (g/L)	1.3	2.867	3.067	2.067	4	3.4	3.7333	2.533	2.333	3.6	5.333
Fructose (g/L)	1.8	2.3	3.467	2.633	3.733	4.4	4.467	3.533	3.333	4.2	6.433
Caffeic acid (mg% dw)	1.015	1.0467	0.986	1.609	1.307	1.152	1.301	1.134	1.224	1.507	2.203
Quercetin (mg% dw)	5.157	4.57	3.95	5.9	6.347	3.733	3.407	5.43	5.39	6.356	7.237
Flavone rutoside (mg% dw)	17.277	15.103	14.313	17.323	19.203	16.15	16.02	16.45	17.343	18.32	19.63
Total phenols (mC/mm)	45.667	41.433	35.4	50	62.2	31.6	41.1	44.367	47.067	54.5	67.333
Antioxidant activity (mM Trolox/mL alcoholic extract)	25.867	18.8	16.567	23.633	37.533	14.433	17.7667	18.167	25.967	29.733	41
Lutein (mg/kg)	3.013	6.21	6.983	6.467	7.597	8.4333	3.807	7.3167	7.897	4.956	10.953
Zeaxanthin (mg/kg)	2.703	6.547	6.557	7.627	6.4	6.9333	6.353	8.563	8.503	6.413	10.733
Total carotenes (mg/% dw)	25	32.533	34.667	41.5	59.767	52.233	27.267	51.567	56.766	46.866	23.7

g/L − gram/liter, mg%dw − milligram (dry weight), mC/mm − micro-curie per millimeter, mg/kg – milligram per kilogram.

It was also observed that the lutein and zeaxanthin contents had approximately the same values (10.73 mg/kg and 10.95 mg/kg, respectively). A system of equations was solved to determine the consumption matrix; more precisely the values x1, x2, ...x10 in the table associated with the figure 1 (data were grouped hierarchically in order of consumption).

There were only five samples consumed; the rest did not meet the conditions of negativity ($x_i \geq 0$, i = 1, …, 10) (Figure 1). Determined separately from other samples, the proportion of consumption for white squash was 71.77%, followed by beans (18.53%), soybean (6.90%) and maize (2.23%). Sunflower was the last in rank, with 0.55% consumption.

Name of plants/sample	Hierarchy plant samples	Value	Percentage consumption (%)
Squash (leaves and flowers)	X3	6.81	71.77
Bean (leaves and flowers)	X4	1.76	18.53
Soybean (leaves and flowers)	X10	0.66	6.91
Maize (silk and pollen)	X9	0.21	2.23
Sunflower (leaves and flowers)	X5	0.05	0.56
Watermelon (leaves and flowers)	X7	-0.25	0
Cucumber (leaves and flowers)	X10	-0.63	0
Maize (leaves)	X8	-1.97	0
Muskmelon (leaves and flowers)	X6	-2.28	0
Pumpkin (leaves and flowers)	X2	-2.71	0

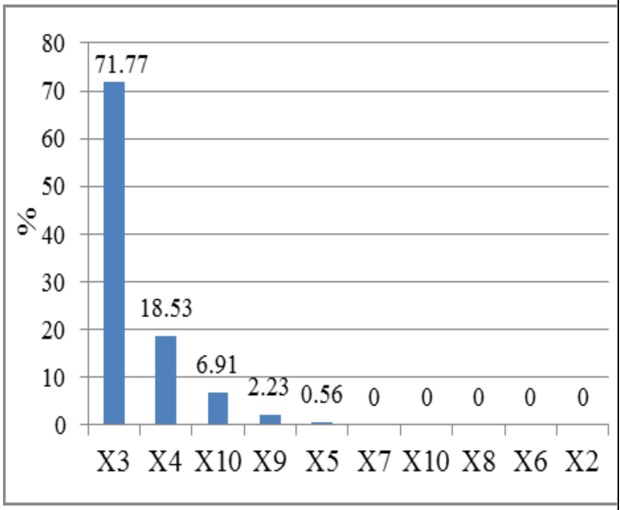

Figure 1. Hierarchy plant samples based on level of consumption

DISCUSSION

The ability of *Diabrotica virgifera virgifera* adults to seek out and feed on alternate host plants may contribute to its potential expansion and has significant implications for the integrated management of this pest (Moeser and Vidal, 2004). However, given the adults' high mobility and their olfactory abilities, we can hypothesize that adults are able to explore larger areas and adapt to new sources of food. In trophic terms, *Diabrotica virgifera virgifera* adults are considered phytophagous consumers. In the absence of their preferred host plant (maize), they can migrate in search of other plants to ensure their survival. It has been observed that if maize is combined with other known or potential host plants, adults were especially attracted to the other host plants (soybean, white squash, beans and sunflower). Muskmelon, cucumber, watermelon and pumpkin were less attractive as host plants but had obvious injuries. If we compare the food source attractiveness in a controlled closed area (observation cages) with an open area (open field), we can see that they orient to nearby plots containing the above-mentioned plants. The order of feeding in an open field would certainly be dependent on distance and not preference. It is normal for an insect that is limited to a small and controlled area to feed more aggressively on available plant material and to even adapt to new fragrances, colors and flavors.

Analyzing the results for the level of injury, we observed that host leaves and flowers had similar values, though there were higher values for leaves. This is because the number of leaves/plant is higher than the number of flowers/plant. The studied flowers were larger but present in a smaller number, and the leaves were smaller, situated on different levels of the plant. The bright colors (yellow, white) of the plants studied, played an important role in initially attracting insects. Immediately after the introduction of the adult insects into the cages, we observed a focus on the inflorescences, and only after a few hours did the insects spread to other aerial organs of the plant (leaves).

The chemical analysis of the samples was absolutely necessary for the association between the levels of injury imposed by the adult insect on the host plant with the level of consumption of vegetable samples. All plants and insects surveyed presented lower or higher values of chemical substances determined in different phases of vegetation. If the main source of energy for *Diabrotica* *virgifera virgifera* is represented by catabolism of carbohydrates and the main metabolic pathway of glucose is the bio-oxidation of the cell, we can conclude that glucose and fructose are the principal substances in the hierarchy of host plants. Lower values of glucose and fructose were obtained in some samples, thought this was most likely due to a harvest in an early stage of maturity. The hierarchy of host plants by the percentage of consumption (determined as a result of the chemical analysis) highlighted five favored plants: *Cucurbita pepo* (white squash), *Phaseolus vulgaris* (bean), *Glycine max* (soybean), *Zea mays* (maize) and *Helianthus annuus* (sunflower). It is interesting to note that, according to other studies, the preferred host plant species is maize (Moeser and Vidal, 2003; OEPP/EPPO, 2004; Hibbard et al., 2008); however, without a deeper chemical determination and correlation between levels of injury and insect consumption level, this hypothesis cannot be verified.

The bio-analytical method combined with multivariate statistical analysis techniques can provide an effective analysis regarding costs for the routine monitoring of carbohydrates in samples, as quality indicators and selected biomarkers, for the prevention of damage caused by *Diabrotica virgifera virgifera*.

CONCLUSIONS

The adult stage of the invasive insect, *Diabrotica virgifera virgifera* Le Conte, may also be an economically important stage. The beetles' capacity for adapting to different food sources was demonstrated by the level of damage to the plants and through chemical tests which made it possible to rank the level of consumption. The injuries produced on leaves and flowers (silk), in terms of frequency and intensity, were used as indicators of conditions in the field, and illustrated the insects' preference for beans, soybean, maize and white squash. Through chemical laboratory tests of the host plants and *Diabrotica virgifera virgifera* beetles, it was possible to establish a hierarchy of preference. Therefore, the most favoured host plants were white squash, soybean, maize and sunflower, with values that satisfy negativity conditions. Through chemical determinations and direct observations of trophic behaviour, it was possible to establish an interaction between these insects and host plants.

Acknowledgement

We wish to thank the team of workers from the Didactic Resort BUASVMT, for their support in maintaining the experimental field.

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