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New bioassays reveal susceptibility of stone-fruit rootstocks to *Capnodis tenebrionis* larvae

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Abstract

Larvae of *Capnodis tenebrionis* (L.) (Coleoptera Buprestidae) feed and develop in roots of stone-fruit trees, thereby decreasing their efficiency, which can lead to plant death. The control of these larvae is critical, due to their localization in the root, and the management of this pest is focused on adults, mainly by using non-specific synthetic insecticides. Less susceptible *Prunus* rootstocks might be applied as a preventative management of larval infestation by this pest. The current research investigated the susceptibility to *C. tenebrionis* larvae of the most commonly used rootstocks by combining two bio-assays during two-year trials: development of larvae assayed on semi-artificial substrates containing rootstock bark flour; infestation by neonate larvae on rootstock twigs. The rearing assay on semi-artificial substrates made it possible to distinguish (1) a rootstock cluster (Montclar and GF677) in which larvae developed faster and heavier and produced larger adults, (2) a cluster (Adesoto, CAB6P, Colt and MaxMa60) in which larval growth was less efficient as well as adult size, and (3) a cluster (Garnem and Myrabolan 29C) with intermediate responses in larval development and adult size. The twig infestation assay by neonates showed the most infested (Colt) and least infested (Barrier, MaxMa60 and Marianna 26) rootstocks. When the results of both assays are combined, GF677 and Myrabolan 29C appear more susceptible, while Adesoto and MaxMa60 less susceptible to *C. tenebrionis* larvae, although Barrier and Marianna 26 require further investigation. The experimental model applied in the current trials can enable processing of a large number of tests on different rootstocks, thereby allowing the accumulation of a large quantity of data on the potential susceptibility of rootstocks. The possibility of rearing larvae on a substrate can allow comparison of additional compounds that could interact with larval growth.

Key words: peach flat headed root borer, larval rearing, semi-artificial diet, twig infestation assay.

Introduction

Capnodis tenebrionis (L.) (Coleoptera Buprestidae) is a stone-fruit pest, mainly infesting apricot, peach, plum, cherry and almond (Ben-Yehuda *et al*., 2001). It is common in the Mediterranean European countries, Northern Africa and western Asia, in commercial and ornamental orchards as well as on some wild *Prunus* (Ben-Yehuda *et al.*, 2000; Gindin *et al.*, 2014). Adults appear in spring, when temperatures rise, starting their feeding on twigs and young branches, usually causing mild defoliation and often damaging buds and twig bark (Bonsignore *et al.*, 2008). Each female lays up to 2,000 eggs in summer (Rivnay, 1946b), usually in cracks of dry soils, at collar bark and under stones (Mendel *et al*., 2003). The new larvae crawl through the soil to locate the host roots, in which they penetrate to develop endophytically (Marannino *et al*., 2007). Root efficiency is affected due to the large sinuous galleries produced by larval drilling (Rivnay, 1946a). The greatest damage is observed on young plants, but a few larvae can also kill a fruit-producing tree in a short time (García del Pino and Morton, 2005; Bonsignore, 2012). Few tools are available to manage this pest. Enemies of immature stages and adults are rare in nature, and the efficacy and application of some microbiological control agents, such as entomopathogenic nematodes (del Mar Martinez de Altube *et al*., 2008; Morton and García del Pino, 2009), fungi (Marannino *et al*., 2006; 2008; Ment *et al*., 2020), and bacteria (Gindin *et al*., 2014) are still under investigation. Currently, mainly non-specific synthetic insecticides are used against adults (Ben-Yehuda *et al*., 2000; Gindin *et al.*, 2014), while the control of larvae still remains critical due to their localization in the root (Ben-Yehuda *et al*., 2000; Mendel *et al.*, 2003). Exploitation of genetic resistance in plant material would be a promising eco-friendly and complementary approach within the integrated control strategy of this pest (Gindin *et al.*, 2014). Several authors have suggested different levels of resistance in *Prunus* spp. rootstocks to *C. tenebrionis* larvae (Mulas, 1994; Ben-Yehuda *et al*., 2001; Soler *et al.*, 2014). Brahimi and Djazouli (2017) have demonstrated that larval activity was influenced by physico-chemical composition of *Prunus* spp. rootstocks. The vigour, nutritional quality and secondary compounds of the infested plant appear to play an important role in successful development of the larvae (Mendel *et al*., 2003). The presence of cyanogenic compounds in roots is thought to be related to larval-host relationships, but the pathway has not been completely clarified yet (Malagón and Garrido, 1990; Mulas, 1994; Dicenta *et al*., 2002; 2006; Mendel *et al.*, 2003).

The current study aims to meet the demand from growers and operators in nursery activities for less susceptible rootstocks, which might be applied as a preventative tactic of pest management, in order to reduce the potential impact of larval infestation. Therefore, this research investigated the susceptibility to *C. tenebrionis* larvae of the most commonly used rootstocks, combining two assays which evaluated the development of larvae, reared on semi-artificial substrates, and the infestation rate by neonate larvae on particular microcosms.

Materials and methods

Adult collection and rearing to obtain neonates

Adults of *C. tenebrionis* were collected from infested apricot orchards in the Districts of Taranto, Matera and Bari (southern Italy), Imola and Faenza (northern Italy). Beetles were caught by hand from March to June 2017 and from April to early August 2018. The most active and healthy males and females were selected and reared together (in groups of 10-15 specimens) in metal net cages (30 × 30 × 30 cm) at room temperature in Battistini Vivai (Cesena, Italy) and D.i.S.S.P.A. (University of Bari, Bari, Italy). Adults with broken legs and antennae, as well as those with plane mandibles, were discharged. Considering the life cycle of this beetle in the collecting areas, adults were emerged one year before their collection. The adults were fed with fresh apricot twigs. The cages were inspected every 5-7 days to renew twigs with fresh ones and remove faeces and dead beetles. Adults were left to mate and females laid eggs on eggs-laid arena; from one to three were provided per cage, and consisted of a Petri dish bottom (10 cm in diameter) holding a cellulose disc covered by a thin layer of fine soil. Agricultural soil sieved through a 20-mesh sieve was used. The eggs adhering to the cellulose disc were collected daily, and incubated in a thermostatic refrigerator chamber (DAS 37000, Intercontinental®, Roma, Italy) (27 ± 2 °C, 60 ± 10% RH), in darkness, until they hatched.

Bark flour and substrate preparation

The semiartificial substrates were prepared as described by Gindin *et al.* (2009). Roots of 2 to 3-year-old plants in their vegetative stage (June-July), provided by Battistini Vivai (Cesena, Italy) and Vivai Fortunato (Sammichele di Bari, Bari, Italy), were shredded in bark flour. The roots were washed with tap water to remove debris, soaked in 10% commercial bleach solution for 2 hours, then washed with distilled water and dried at room temperature. The bark was removed using a knife and placed in an oven at 70 °C for 24 hours (Kokici *et al.*, 2020). Finally, the dried bark was shredded, and the obtained flour was stored in PVC jars at −20 °C and used for preparing fresh substrate as required each time. The substrate was prepared using 5% of bark flour per each rootstock and was stored in PVC jars at −20 °C. Before being used in rearing, the substrate was left to warm at room temperature for a short time. Eight rootstocks were tested, selecting those most commonly used by growers and those of commercial interest to industrial partners (table 1). Each rootstock represented a treatment.

Rearing of larvae on semi-artificial substrate

The trial was repeated for two years, starting with neonate larvae obtained from adults collected in July 2017 and in August 2018. Neonate larvae (24 hours-old at most) were randomly selected among those hatched from eggs laid by the reared female population. These larvae could not be assigned to a single female because the need of synchronizing the trial. The larvae were placed singly, on the same day, onto a Petri dish (3.5 cm in diameter) containing one of the eight substrates prepared as above (Gindin *et al.*, 2009). The number of larvae used for each rootstock in the current study and for the statistical analysis is reported in table 2. Each larva was coded to identify the single larva from hatching to adulthood. Larvae were randomly assigned to each treatment and were maintained on the same substrates, in a dark chamber at 27 ± 1 °C until the appearance of pupae. Every two weeks the dishes were inspected, the substrate was renewed, and the weight of each larva was recorded up to pupation. Larvae were categorized as "mature" at the last inspection before moulting into a pupa (*i.e.*, two weeks before moulting). The appearance of pupae was recorded, and each pupa was transferred into a clean new Petri dish, with no substrate, and kept in darkness at 27 ± 2 °C until the emergence of the adults. The new emerging adults were separated according to sex, and their body length and pronotum width were measured using a slide caliper with an accuracy of 0.02 mm.

Susceptibility of rootstock twigs to larval infestation

Host preference assay was repeated at Battistini Vivai (Cesena, Italy) during 2017 and 2018. Nine rootstocks were tested: those most commonly used by growers and those with commercial interest to the industrial partners (table 1). The plant material was collected from 2-3-year-old trees during 2017, and from 6-12-month-old trees during 2018. Each rootstock represented a treatment.

Table 1. Rootstocks used in the current experiments.

Botanical names	Rootstocks used for larval infestation of twigs assay	Rootstocks used in preparing substrates for larval rearing
Prunus domestica subsp. *insititia* (L.) Bonnier et Layens	Adesoto	Adesoto
P. persica (L.) Batsch *x P. davidiana* (Carrišre) Franch.	Barrier	-
P. cerasus L.	CAB6P	CAB6P
P. avium L. *x P. pseudocerasus* Lindl.	Colt	Colt
P. persica (L.) Batsch *x P. dulcis* Webb	Garnem	Garnem
P. persica (L.) Batsch *x P. dulcis* Webb	GF677	GF677
P. cerasifera Ehrh *x P. munsoniana* W. Wight et Hedrick	Marianna 26	-
P. mahaleb L. *x P. avium* L.	MaxMa60	MaxMa60
P. persica (L.) Batsch	-	Montclar
P. cerasifera Ehrh.	Myrabolan 29C	Myrabolan 29C

Figure 1. Experimental microcosms used for the evaluation of the rootstock susceptibility to *C. tenebrionis* neonate larvae.

The following microcosm was arranged for carrying out the assays on the susceptibility of rootstocks to *C. tenebrionis* neonate larvae (figure 1) (modified by Azoulay, 2017). Twigs partially lignified with a few buds (10-15 cm long, 3.5-4.3 mm in diameter) were taken from each plant (the number of assayed twigs per each rootstock is reported in the related table) and each twig represented a replication. The basal part of the twig was passed through a tiny polyethylene film and inserted in a hole, made in the bottom of a cylindrical cup (35 mm in diameter × 51 mm tall), protruding for 3-4 cm from the cup bottom. The first cup was introduced into a second cup (40 mm in diameter × 60 mm tall) containing water for approximately 2.5 cm of its height in order to enable the twig to obtain water. The first cup was filled with fine dry soil (sieved through a 0.4 mm mesh net). Neonate larvae (24 hours old at most) were transferred singly into the topsoil near the twig, using a fine brush (1 larva per twig). The twigs were left in natural environmental conditions, and were examined after 10-11 days to verify infestation success, larval health and the presence of drilling on twigs. The number of untouched and infested twigs were recorded and expressed as a percentage of the total number of twigs. Healthy twigs or those with minor erosions and no larval gallery (figure 2A) were categorized as untouched twigs, and those with dead or live larvae in the galleries they had bored, were categorized as infested twigs (figure 2B, 2C). The mean length of galleries was recorded only for the most infested treatments (CAB6P, Colt, GF677, Myrabolan 29C). Correlation between twig size and gallery length was not assessed because of the small range in the diameter of the used twigs.

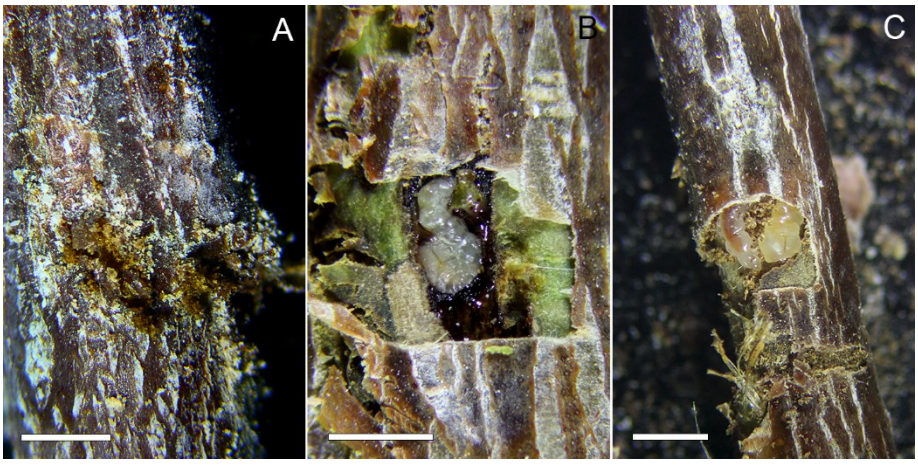

Figure 2. Neonate larval action on twigs: (**A**) erosion without larval penetration; (**B**) dead larva surrounded by gum under the bark, (**C**) live larva under the bark. Scale bar: 1 mm.

Table 2. Mean size (± SE) of *C. tenebrionis* larvae and emerged adults separated for rearing on substrates containing bark flour of eight stone-fruit rootstocks.

Rootstocks	Number of initial larvae	Larval weight at the 23rd week (g)	Weight of mature larvae (g)	Length of adults' body (mm)	Width of adults' pronotum (mm)
Montclar	36	0.59 ± 0.02 a	0.55 ± 0.02 a	19.86 ± 0.21 a	7.54 ± 0.11 a
GF677	37	0.55 ± 0.03 ab	0.54 ± 0.02 ab	19.58 ± 0.25 ab	7.44 ± 0.12 ab
Garnem	21	0.43 ± 0.05 bc	0.50 ± 0.03 abc	18.90 ± 0.40 abc	7.27 ± 0.15 abc
Myrabolan 29C	30	0.49 ± 0.02 abc	0.48 ± 0.02 abc	19.20 ± 0.29 abc	7.09 ± 0.13 abc
Colt	23	0.37 ± 0.03 c	0.47 ± 0.02 abc	18.66 ± 0.28 abc	6.96 ± 0.11 bc
MaxMa60	25	0.51 ± 0.02 abc	0.46 ± 0.02 bc	18.62 ± 0.28 bc	6.98 ± 0.11 bc
Adesoto	31	0.40 ± 0.03 c	0.46 ± 0.02 bc	18.72 ± 0.23 bc	6.99 ± 0.10 c
CAB6P	35	0.41 ± 0.03 c	0.45 ± 0.02 c	18.42 ± 0.28 c	7.00 ± 0.11 bc
Significance		$F_{(1,7)} = 2.72$ $P = 0.0001$	$F_{(1,7)} = 4.06$ $P = 0.0003$	$F_{(1,7)} = 4.22$ $P = 0.0002$	$F_{(1,7)} = 4.62$ $P = 0.0008$

Means ± SE followed by the same letter within a column do not differ significantly (Tukey's test: $P \leq 0.05$).

Data analysis

The normality of increase in larval weight was confirmed by Q-Q plot and, then, ANOVA analysis was performed, followed by Tukey's post-hoc comparison test ($P \leq 0.05$). The weight of mature larvae, the body length and pronotum width of adults were normally distributed, according to the Shapiro-Wilks test, and their variances resulted homogenous, according to Bartlett test. These data were processed by applying factorial ANOVA analysis, followed by Tukey's post-hoc comparison test ($P \leq 0.05$). One-way ANOVA analysis, followed by Tukey's post-hoc comparison test ($P \leq 0.05$) was performed on the length of galleries produced by larvae on twigs (twig infestation trials), confirming the normal distribution of data and homogeneity of variance using tests by Shapiro-Wilks and Bartlett, respectively. Statistical analysis on the rate of twig infestation was performed with non-parametric Yates corrected χ^2 test ($P \leq 0.05$). All the tests were carried out using Statistica 10 software (StatSoft, 2010).

Results

Rearing of larvae

A total of 238 larvae reared on the eight substrates developed into pupae, and 205 adults were obtained. All larvae increased their weight regularly during the assay. Montclar and GF677 showed a significantly greater mean weight of all instar larvae than that of the other treatments at the inspection two weeks before the appearance of the first pupa (23rd week from the beginning of the assay). Conversely, larvae reared on Adesoto, CAB6P, Colt and Garnem substrates recorded the lowest mean weights (table 2).

The mean weights of all mature larvae ranged between 0.55 g and 0.45 g among the treatments (table 2). The highest values were recorded for larvae grown on a substrate containing Montclar and GF677 bark flour, whereas the lowest mean weight of mature larvae was observed for CAB6P, Adesoto and MaxMa60 treatments (table 2). Indipendently of the treatments, the weight of all mature larvae producing females was significantly higher than that males (table 3).

The data on adult size showed significant differences among treatments and between sexes, whereas no effects of interaction were observed between rootstock and sex (data not shown). The body of all adults that emerged from Montclar and GF677 treatments was significantly longer than that of all adults reared on Adesoto, CAB6P and MaxMa60 substrates (table 2). Similarly, the pronotum width of all adults from Montclar and GF677 treatments was significantly larger than that of all adults from Adesoto, CAB6P, Colt and MaxMa60 treatments (table 2). For all the treatments, the size (body length and pronotum width) of the new emerging females was significantly larger than that of the male specimens (table 3).

Susceptibility of rootstock twigs to larval infestation

A total of 716 twigs were tested. No differences were observed among the treatments during 2017, although MaxMa60 was the least infested and Colt was the most infested. Differences were observed in the 2018 trials, whit Colt and CAB6P presenting the highest percentage of infested twigs (table 4), and Barrier, MaxMa60 and Marianna 26 displaying the lowest percentage of infested twigs (table 4).

Additionally, of the rootstocks with the highest larval

Table 3. Mean size (±SE) of all emerged adults and weight of all mature larvae of *C. tenebrionis* separated by sex.

Sex	Number of emerged adults	Length (mm)	Width (mm)	Weight of mature larvae (mg)
Females	92	19.34 ± 2.02 a	7.29 ± 0.76 a	0.51 ± 0.05 a
Males	113	18.78 ± 1.77 b	7.08 ± 0.66 b	0.47 ± 0.04 b
Significance		$F_{(1,1)} = 9.28$, $P = 0.002$	$F_{(1,1)} = 8.00$, $P = 0.005$	$F_{(1,1)} = 6.87$, $P = 0.009$

Means ± SE followed by the same letter within a column do not differ significantly (Tukey's test: $P \leq 0.05$).

Table 4. Percentage of twigs infested by *C. tenebrionis* neonate larvae on nine different stone-fruit rootstock twigs.

Rootstocks	Number of tested twigs	Infested twigs (%) in 2017	Number of tested twigs	Infested twigs (%) in 2018
Colt	54	68.5	30	73.4 a
CAB6P	40	45.0	30	70.0 a
Myrabolan 29C	60	68.0	30	53.4 ab
GF 677	60	55.0	30	43.3 b
Adesoto	40	50.0	30	40.0 b
Garnem	45	55.6	30	36.6 abc
Marianna 26	40	52.5	30	26.7 abcd
MaxMa60	55	43.6	30	13.3 cd
Barrier	52	55.8	30	10.0 d
Significance		$\chi^2 = 13.27$, $P > 0.05$		$\chi^2 = 49.64$, $P < 0.05$

Percentages followed by the same letter within a column do not differ significantly (Yates corrected χ^2 test: $P \leq 0.05$).

infestation in 2018, CAB6P presented a significantly greater mean gallery length than Myrabolan 29C, which had the lowest mean gallery length (table 5).

Discussion and conclusions

A few previous studies had investigated the susceptibility of different *Prunus* spp. rootstocks to *C. tenebrionis* larvae, and almost all of them were carried out by infesting planted saplings, which were sacrificed at the end of the experiments in order to obtain infestation data. The pros and cons of these experimental models should be considered and compared with those of the current experiment.

Our results showed that larvae reared on semi-artificial substrates containing Montclar and GF677 bark flour, grew faster and better than those on the other substrates. Meanwhile, Adesoto, CAB6P, Colt and MaxMa60 showed a lower mean weight of mature larvae and a smaller adult size compared to the other treatments. Garnem and Myrabolan 29C seemed to induce intermediate larval responses between those given by the previous two rootstock clusters. These results appear to be similar to those found in our earlier preliminary study (Kokici *et al.*, 2020), suggesting that Montclar and GF677 rootstocks favoured the postembryonic development of flatheaded woodborer, meanwhile Adesoto, Colt, CAB6P, Garnem and MaxMa60 negatively affected larval growth and development.

Table 5. Mean length (±SE) of galleries produced by *C. tenebrionis* larvae during the infestation trials on nine different stone-fruit rootstock twigs, referred to 2018 trials.

Rootstocks	Mean length of galleries (mm)
CAB6P	18.70 ± 2.11 a
GF 677	15.55 ± 1.92 ab
Colt	12.64 ± 1.45 ab
Myrabolan 29C	11.90 ± 1.40 b
Significance	$F_{(3)} = 3.32$, $P = 0.03$

Means ± SE followed by the same letter within a column do not differ significantly (Tukey's test: $P \leq 0.05$).

Our results on twig infestation by neonate larvae indicated Colt as the most infested rootstock in both years of the trial, whereas Barrier, MaxMa60 and Marianna 26 showed the lowest rate of infestation in 2018, with the same results for MaxMa60 also in 2017.

Combining the results of larval rearing on the semi-artificial substrates with those of twig infestation by neonate larvae, it can be observed that Adesoto and MaxMa60 were relatively less susceptible, and GF677 and Myrabolan 29C were relatively more susceptible to *C. tenebrionis* larvae. This is quite consistent with a frequent spread of beetle infestation in Apulia and on the Ionian side of Basilicata, where GF677 and Myrabolan 29C are more commonly used as rootstocks in apricot orchards (personal observations). The current data are consistent also with previous studies, which indicated GF677, and apricot in some cases, as the most affected rootstocks among the tested ones (Mulas *et al.*, 1989; 1992; Mulas, 1994; Soler *et al.*, 2014). According to Soler *et al.* (2014), Garnem rootstock recorded the lowest level of damage and this is mostly consistent with our results, too. Vice versa, Colt and CAB6P gave contrasting data, since larval growth was lowest in the semi-artificial substrate containing the bark flour of these two rootstocks, but neonate larvae infested a higher percentage of twigs in the twig infestation trials.

In the attempts to identify the factors hindering the beetle, cyanogenetic glucosides, which are very common in Rosaceae, have been considered as being involved in plant-beetle interactions. Prunasin is contained in all tissues of stone-fruit rootstocks and amygdaline is mainly located in seeds (Ben-Yehuda *et al.*, 2001). Prunasin becomes toxic when degraded into cyanide due to the insect's chewing activity, and this compound is assumed to be involved in rootstock resistance to *C. tenebrionis* larvae (Mulas, 1994; Malagón and Garrido, 1990). Owing to its higher prunasin and amygdalin contents, bitter almond was considered for decades to be less susceptible to *C. tenebrionis* (Mferrej and Sharaf, 2011). However, other authors found that rootstock resistance was inversely proportional to the roots' cyanide content, suggesting that prunasin was not involved in *Prunus* spp. roots' resistance to *C. tenebrionis* (Ben-Yehuda *et al.*, 2001; Mendel *et al.*, 2003). Later, larval survival was

observed to depend also on other root compounds, such as water-soluble proteins, amount of total sugar in *P. domestica*, or proline in *P. cerasus*, and on wood hardiness (Brahimi *et al.*, 2017). All these results create confusion in understanding the resistance mechanisms to *C. tenebrionis* in *Prunus* taxa, and additional procedures are needed to obtain larger amounts of data in a simpler way.

The twig infestation assays and the larval rearing on a semi-artificial substrate that were carried out in this study confirmed the possibility of performing laboratory tests on *C. tenebrionis* larvae in a simple and inexpensive way, enabling the detection of a series of morphological and biological parameters, including the length, number and duration of larval instars, the effects of temperature and different substances on the larval development and adult size. Using twigs instead of roots of stone fruits avoids destroying the plants in order to exploit the ability of larvae to infest twigs, as previously showed by Rivnay (1946a). Further studies might focus on the existence of a correlation between the diameter of the twigs and the rate of infestation. Our unpublished personal observations have shown that thicker twigs can be more easily infested by neonate larvae. The differences in age of the plants from which the twigs were collected in 2017 and 2018 trials (trees were 2-3 years old in 2017, 0.5-1 years old in 2018) might explain the reduced differences in the infestation rate observed among the treatments. This difference could be related to the chemical composition of the plant tissue, since cyanogenic compounds are differently spread in plants of the same species, depending on age, phenological stage, tissues and organs (Usai and D'hallewin, 1990; Ohtsubo and Ikeda, 1994; Emmett *et al.*, 2014; Brahimi and Djazouli, 2017). The possibility of rearing larvae on a substrate in which compounds could be modified seems an interesting approach, because it can allow comparison of compounds which could directly (e.g., cyanogenic compounds and their derivatives; quantities of water, proteins, fibres, etc.) and indirectly (antibiotic compounds) interact with larval growth. In this sense, the study of the interactions of these compounds with the larval microbiota can offer an increasing number of suggestions (Barak *et al.*, 2019).

In conclusion, the current study confirmed the existence of differences in rootstock susceptibility to *C. tenebrionis* larval infestation. The results obtained in the assays indicated GF677, Colt and Myrabolan 29C as the most susceptible rootstocks, whereas MaxMa60 and Adesoto seem to be more promising as they appear less susceptible. The relevance of Barrier and Marianna 26 as less susceptible rootstocks requires further investigation. In addition, the different methods used in larval rearing can enable processing of a large number of tests on different rootstocks, making it possible to accumulate a large quantity of data on the potential susceptibility of rootstocks. These data should be considered indicative, but the method can offer a preliminary and rapid approach to the screening of the available rootstock clones, which could have a certain interest for nurseries or their associations, and also for plant breeders. Obviously, all these data require field validation.

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