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Does catechin make the Mediterranean palm tree Chamaerops humilis L. an unsuitable host for Rhynchophorus ferrugineus (Olivier)?

Fernando Monroy, Paolo Curir, Virginia Lanzotti, Laura Grauso, Enrico Busato,

Lindsay Karen Hernández Nova, and Chiara Ferracini

Entomologia Generalis, 2021

DOI: 10.1127/entomologia/2021/1164

Can catechin make the Mediterranean palm tree *Chamaerops humilis* L. an unsuitable host for *Rhynchophorus ferrugineus* (Olivier, 1790)?

Short title

Toxic effect of catechin against *R. ferrugineus*

Fernando Monroy[1], Paolo Curir[1], Virginia Lanzotti[2], Laura Grauso[2], Enrico Busato[3], Lindsay Karen Hernández Nova[3], Chiara Ferracini[3*]

[1]CREA – Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Corso degli Inglesi 508, 18038 Sanremo, Italy

[2]Department of Agricultural Sciences, University of Napoli, Via Università 100, 80055 Portici, Italy

[3]Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

***Corresponding author**

Chiara Ferracini, chiara.ferracini@unito.it

Abstract

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), is an insect pest native to Southeast Asia, which has become the major threat of palms in the Mediterranean Basin, mainly due to the movement of infested planting material. Once infested, palms are difficult to manage and often die. In the literature, *Chamaerops humilis* palms are reported as resistant to RPW, but until now, no investigation has been performed on

C. humilis tissues to detect the possible presence of toxic endogenous metabolites on RPW. In the present study, we focused on the potential toxic effect of catechin against RPW larvae. In laboratory bioassays, the purified catechin was provided to two- and five-year old RPW larvae at three different concentrations. Our data showed how catechin can impair the survival of RPW, causing toxicity at concentrations as lower as 0.03 µg g^{-1}. Larval mortality was dose-dependent, and furthermore larval age influenced the effect of catechin, being older larvae more susceptible than younger ones. The observed toxicity of catechin on RPW larvae agrees with the hypothesis that this compound is a component of the chemical defense mechanism of *C. humilis* against this insect pest.

Keywords

Red palm weevil, Curculionidae, exotic insect pest, toxicity, palm resistance

Introduction

The invasive curculionid *Rhynchophorus ferrugineus* (Olivier, 1790), otherwise known as the Red Palm Weevil (RPW), has become in the last three decades the most important pest affecting palms all over the world (El-Mergawy and Al-Ajlan 2011). This insect, native to Southeast Asia, has quickly colonized wide geographical areas in Asia, Africa, America and Europe and now its diet encompasses more than 40 different palm species, almost all Arecaceae, although this pest originally subsisted on just four species in its native range (Nirula 1956).

Damage to host plants is mainly caused by the larval stage feeding within the trunk of palms and destroying the vascular system. Infestation can adversely affect fruit yield, lower the growth rate of the palm, and eventually cause its collapse and death (Blumberg 2008). A variable degree of tolerance to RPW has been observed in host palm species, ranging from a

51 minimum in *Phoenix canariensis* Chabaud (Barranco et al. 2000) to a significant degree in
52 *Washingtonia filifera* (Lindl.) H.Wendl. (Cangelosi et al. 2016). *C. humilis*, native to Europe,
53 is considered resistant to RPW (Barranco et al. 2000) and for this palm species, an antixenotic
54 mechanism of resistance has been postulated (Dembilio and Jacas 2012). According to this
55 hypothesis, the leaf rachis –a place frequently chosen by the females to lay their eggs—
56 would be too tough and fibrous, being therefore unsuitable for oviposition. This sole
57 antixenosis-based mechanism proved to be, in some cases, not enough to prevent RPW from
58 invading palm tissues (Dembilio et al. 2009), suggesting the presence of a supplementary
59 defensive mechanism in *C. humilis*. In a similar way, in *W. filifera* a complementary
60 antibiosis defensive mechanism was first postulated (Dembilio et al. 2009) and then
61 demonstrated (Cangelosi et al. 2015). Until now, however, no investigation has been
62 performed on *C. humilis* tissues to detect the possible presence of endogenous metabolites
63 with a significant degree of toxicity on RPW.
64 The aim of this study was to characterize part of the chemical mechanism that is
65 supposed to contribute to the observed resistance of *C. humilis* against *R. ferrugineus*. To do
66 this, we analysed the chemical composition of the leaf tissues of *C. humilis* looking for
67 compounds with a potential toxic effect against the larvae of *R. ferrugineus*. Due to its
68 structural similarity to the chalconoid compound that is supposed to contribute to the
69 chemical resistance mechanism in *W. filifera* (Cangelosi et al. 2015), we focused on the
70 potential toxic effect of catechin. We used several chromatographic techniques in order to
71 separate and purify this compound from the base of the leaves of *C. humilis*. The purified
72 catechin was provided in laboratory bioassays at diverse concentrations to RPW larvae.
73 Finally, we discussed the observed results in the context of the emerging importance of the
74 chemical resistance of palms against this invasive insect pest.
75

Material and Methods

Plant material

Leaves of *C. humilis* were collected from healthy individuals of about 20 years old located in the historical garden of Villa Ormond, Sanremo, Italy (43°49'20'' N, 7°47'24'' E). The leaf basal part, i.e., the first 25 cm from leaf insertion on the palm stem, was harvested and immediately submitted to the laboratory. The basal part of the leaves is considered a target zone where the RPW females can lay their eggs (Dembilio and Jacas 2012). Some samples were kept as vouchers at –20 °C in the biochemistry laboratory of the CREA at Sanremo.

Extraction, isolation and quantification of the selected compound

Fresh leaf material (1 Kg) from several leaves was cut in small pieces (0.8-1.0 cm^3) and extracted with H_2O kept boiling for 3 h. The extracts were then concentrated to 100 mL at reduced pressure, filtered on Whatman paper and kept at −20 °C until needed. Aliquots (5 mL) of the aqueous extract of *C. humilis* leaf basal tissues were chromatographed through a column (30 × 5 cm) filled with silica gel RP-18, packed with 200 mL MeOH and eluted according to a linear gradient elution profile from 90% to 0% A in B, where solvent A (150 mL) was HCOOH 5% in H_2O and solvent B (150 mL) was absolute EtOH. Fractionation of the elution was performed by a Gilson 203 Micro Fraction Collector. The obtained fractions (3 mL volume) were checked for purity on Merck precoated cellulose 20 × 20 cm glass TLC plates, eluted with propanol:H_2O:acetic acid (20:80:1, v/v/v) or propanol:H_2O (20:80, v/v) according to Vovk et al. (2005). TLC plates were inspected under visible and UV light at 254 and 366 nm wavelength.

Chromatographic fractions presumably containing catechin were pooled and its UV spectrum was obtained using a Hitachi 150-20 spectrophotometer. The concentration of catechin in the analysed tissues of the palm was initially calculated via its molar attenuation coefficient (ε), by dissolving known amounts of the purified compound in MeOH.

101 The purity of the isolated catechin fraction was checked via reversed-phase liquid
102 chromatography (RP-HPLC). The analysis was performed using a Supelco Discovery C18
103 column 25 cm × 4.6 mm, 5µm particle size (Sigma Aldrich, USA). Solvents used were 0.1%
104 H_3PO_4 in water (A) and acetonitrile (B); the solvent gradient method was 0-30 min from 10%
105 to 58% B, 1 min from 58% to 90% B, 5 min isocratic 10% A / 90% B, 1 min from 90% to
106 10% B and 25 min isocratic 90% A / 10% B. Sample injection volume was 20 µL; solvent
107 flow rate was 1 mL min^{-1}. The exact identity of the isolated catechin was further verified via
108 nuclear magnetic resonance (NMR), following the procedure used by Cangelosi et al. (2015).
109 *Insect colonies*
110 Adults of *R. ferrugineus* were collected from infested palm trees in Sanremo (Italy) and
111 transferred to the laboratory of the DISAFA of the University of Torino. The insects were
112 surface sterilized with 2% formalin (37% formaldehyde) and left to dry in Petri dishes (15 cm
113 diameter). Then they were transferred to rearing cages (20 cm length × 13 cm width × 12 cm
114 height) in order to set up a mass rearing. All the insects were fed on banana (*Musa*
115 *paradisiaca* L., Chiquita®'s organic banana) and apple slices (*Malus domestica* Borkh.,
116 Golden Delicious® organic apple) and kept in climatic chamber (29 ± 1.5 °C, 65 ± 0.8% RH,
117 16:8 L:D, 4000 lux illumination). The artificial diet was replaced every two days, carefully
118 dissected, and the eggs removed according to the methods described by Weissling and Giblin-
119 Davis (1994). The eggs were cleaned with 2‰ formalin solution for 10 min to prevent viral
120 disease (Singh *et al.*, 1985) and then were transferred to Petri dishes (9 cm diameter) lined
121 with moistened filter paper, using deionized water, sealed with parafilm, and stored in
122 climatic chamber (29 ± 1.5 °C, 65 ± 0.8% RH, 16:8 L:D, 4000 lux illumination) until larval
123 emergence.
124 *Laboratory bioassays*

125 Two- and five-week old RPW larvae were individually placed in sterilised Petri dishes lined
126 with humidified filter paper and sealed with Parafilm®. Larvae were fed with cylinder-shape
127 pieces (1 cm height × 2.5 cm diameter) of organic apple, dipped for 1 min in a water solution
128 of the selected flavonoid at three different concentrations. To do this, the pooled fraction
129 containing the isolated compound was evaporated under reduced pressure; the resulting
130 powder was resuspended in deionized water and adjusted to an initial concentration of 0.12
131 µg mL^{-1} (42 mM). Afterwards, two-fold dilutions in demineralized water were carried out in
132 order to get the other two experimental concentrations, namely 0.06 and 0.03 µg mL^{-1} (21 and
133 10.5 mM, respectively). Controls were represented by RPW larvae fed with apple cylinders
134 dipped for the same time duration in a filtered raw extract of basal leaf parts of *P. canariensis*,
135 since this extract has no negative effect on the growth and survival of *R. ferrugineus* larvae
136 (Cangelosi et al. 2016).
137 The effect of each concentration was tested on the two selected cohorts of RPW larvae for
138 192 h (n = 10). Every 24 hours the number of dead larvae was recorded. The test was carried
139 out in controlled conditions at 29 ± 1.5 °C, 65 ± 0.8% RH, 16:8 L:D, 4000 lux illumination.
140 *Statistical analysis*
141 Data were analysed using the R environment (R Core Team, 2019). The relationship between
142 exposure time and larval survival at different concentrations for each cohort was determined
143 via generalized linear models (GLMs) with binomial error distribution (dead / alive), using
144 the function glm( ). Lethal times (LT_{50} and LT_{95}) of catechin for two- and five-week old *R.*
145 *ferrugineus* larvae were calculated from the respective glm models using the function dose.p(
146) from the MASS package. Statistical difference between model slopes and lethal times at
147 different catechin concentrations for each larval age was calculated by means of z-tests (Zar
148 2010). The statistical significance of the observed differences in lethal times between the two

149 age groups of larvae was calculated using a *t*-test. Data presented through the text are means ±
150 SE.
151
152 **Results**
153 *Occurrence of catechin in the leaves of* C. humilis
154 TLC spots of the purified catechin fraction were uncoloured under visible light and appeared
155 as dark violet areas under UV light (254 nm). No colour was observed under UV light at 366
156 nm wavelength. Its UV spectrum in MeOH revealed a main peak of absorbance at 274 nm.
157 The NMR analysis of the molecular structure of the purified fraction confirmed that it was the
158 widespread flavan-3-ol catechin ($C_{15}H_{14}O_6$; molar mass = 290.271 g mol-1; IUPAC name
159 (2*R*,3*S*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol). Catechin can be
160 found in some plants as two enantiomers, called (+)-catechin (2*R*,3*S*) and (-)-catechin (2*S*,3*R*),
161 which differ significantly in their biological activity (Bais et al. 2002). Since our study did not
162 consider the use of chiral chromatography, it was no possible to determine the relative amount
163 of the two enantiomers in the leaf samples of *C. humilis*. Based on a log ε value, calculated in
164 MeOH at 274 nm wavelength, the overall concentration of catechin within *C. humilis* leaf
165 basal tissues was estimated to amount up to 0.238 μg g^{-1} fresh material, a concentration in the
166 same order of magnitude of those we used in the laboratory bioassays with RPW larvae,
167 considering a correspondence between g and mL. Previous HPLC analyses carried out on leaf
168 basal tissue extracts of both RPW-resistant *W. filifera* and RPW-susceptible *P. canariensis*
169 individuals revealed that catechin was more abundant in *C. humilis* tissues (data not shown).
170 *Laboratory bioassays*
171 Purified catechin from *C. humilis* had a significant negative effect on the survival of RPW
172 larvae (Table 1). Larval mortality was dose-dependent, being doses 1 and 2 (42 and 21 mM,
173 respectively) more toxic than dose 3 (10.5 mM, Figg. 1A-B). Larval age also influenced the

174 negative effect of catechin; overall, older larvae were more susceptible than younger larvae,
175 with an estimated LT_{50} 1.9 times shorter (Welch two sample *t*-test, $t = 5.073$, df = 2.74, $P =$
176 0.009, see Table 1). Average LT_{95} was also significantly shorter in five-week old larvae,
177 where catechin led to 95% mortality 2.1 times faster than in the two-week old group (Welch
178 two sample *t*-test, $t = 3.978$, df = 2.05, $P = 0.028$, see Table 1). Accordingly, GLMs slopes
179 were significantly steeper in the group of five weeks old larvae (Welch two sample *t*-test, $t =$
180 4.122, df = 3.90, $P = 0.008$, Figg. 1A-B).
181
182 **Discussion**
183 The impact of exotic pests varies considerably depending on the host plant species and the
184 area being invaded. Control strategies, namely chemical, cultural, and biological strategies are
185 often time- and cost-effective, and for many insect pests inapplicable due to the endophytic
186 habit during the early stages of infestation and the potential adverse effects on the
187 environment and public health.
188 In the search for alternative solutions to face pest outbreaks, the interest in studying plant
189 species compound as a defense tool against pests has increased. Secondary plant metabolites,
190 such as alkaloids, glycoalkaloids, terpenoids, organic acids or alcohols, are regarded as
191 promising sources of plant-protecting substances (Chowański et al. 2016). Literature reports
192 have indicated that many of these compounds have important implications in the agricultural
193 and forestry environment. For example, the pesticidal importance of saponins in higher plants
194 has been reported for *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae), an
195 invasive leafminer of *Aesculus* spp. (Ferracini et al. 2010), as well as the antifeedant activity
196 of *Ginkgo biloba* secondary metabolites against *Hyphantria cunea* (Lepidoptera: Arctiidae)
197 larvae (Pan et al. 2016), and the antifeedants compounds isolated from the bark of the
198 lodgepole pine effective against the pine weevil *Hylobius abietis* (L.) (Coleoptera:

Curculionidae) (Bratt et al. 2001). In this study, we found that catechin, an abundant phenolic compound present in the raquis of the *C. humilis* palm, can impair the survival of RPW larvae. Since this compound significantly contributes to the leaf phenolic profile of *C. humilis*, its observed toxic effect strongly points at its involvement in the chemical defense mechanism of this palm against *R. ferrugineus*. In our leaf samples, catechin was present at concentrations higher that previously reported by other Authors in *C. humilis* (0.08 – 0.16 µg g^{-1}, Delle Monache et al. 1972), although we found that this compound can cause toxicity in RPW larvae at concentrations as lower as 0.03 µg g^{-1}. Considering its relative abundance in the basal part of the leaves, this compound likely makes part of a constitutive, unspecific defence mechanism of this palm species against herbivore insects and pathogens in general. Actually, catechin has been reported to cause feeding deterrence and/or toxicity not only in curculionid species (Khatun et al. 2011; Hammerbacher et al. 2019), but also in insect species belonging to the Pyralidae, Chrysomelidae, Scarabeidae, Tenebrionidae and Culicidae families (Potter and Held 2002; Pavela 2007; Barboza-Silva et al. 2009; Khatun et al. 2011; Elumalai et al. 2016). In addition, catechin is known to display antimicrobial activity against plant pathogenic fungi (Yamaji and Ichihara 2011; Ullah et al. 2017). Although our leaf samples were collected from apparently healthy individuals, variation in catechin concentration as a plant response to the presence of insect eggs or larval stages cannot be excluded and needs to be checked in further studies.

The chemical structure of catechin significantly resembles that of the other metabolite hitherto isolated from palms and likely related to their resistance to *R. ferrugineus*, the chalconoid filiferol (Cangelosi et al. 2015). Contrary to filiferol, catechin cannot induce protein precipitation (Hagerman and Butler 1978), excluding direct inactivation of digestive enzymes and access limitation to ingested proteins as its mechanism of action. In addition, many insect species show protective mechanisms, such as alkalinisation of the gut fluids and the presence

of surfactants, as adaptations to prevent protein binding in the gut lumen (Martin et al. 1985; Zimmer 1997). The mechanism of action of catechin might instead rely on its oxidation under alkaline conditions to form *o*-benzoquinones (Han et al. 2019), that can lead to the production of reactive oxygen species (ROS) with significant cytotoxic and immunotoxic effects (Bolton and Dunlap 2017). Although catechin is also present in RPW susceptible species, such as *Phoenix dactylifera* L. and *P. canariensis* (Ziouti et al. 1996; Hifnawy et al. 2016), little is known about the distribution of this compound in the different parts of the plants and about its availability as a function of the type of tissue. In addition, there is evidence that the performance of herbivore insects does not necessarily follows a linear and dose-dependent relationship with the concentration of potentially toxic metabolites produced by plants (Lehrman et al. 2012), suggesting that catechin toxicity might depend on the presence of other compounds. These hypothesized interactions would support our previous idea that chemical defense of palms against RPW is likely based on a diversity of substances and mechanisms (Cangelosi et al. 2016).

The delayed mortality of young larvae treated with catechin revealed an unexpected pattern of response to a plant toxic compound. Except for some cases (Sáenz-de-Cabezón Irigaray et al. 2005), old and large larvae are usually more resistant to toxicants than young larvae (Schoonhoven et al. 2005). This tolerance is related to factors such as age-related changes in the physiology of the digestive apparatus (Schultz and Lechowicz 1986; Keller et al. 1996) and the ability to immobilize toxic compounds in storage tissues (Nishida, 1994). Our results indicate that in RPW larvae none of these mechanisms operate to reduce the toxic effect of catechin, suggesting that *R. ferrugineus* is poorly adapted to the toxicity caused by this kind of compound. The delayed mortality in young RPW larvae points also to a post-ingestive mode of action of catechin, excluding toxicity effects by contact. This kind of exposure effects depends on the body surface area / volume ratio and are typically stronger in

young/small individuals (Traas and Van Leeuwen 2007). The observed feeding rate of the larvae during our experiments also excluded the functioning of catechin as a feeding deterrent. Since at the subcellular level catechins are known to accumulate not only in the chloroplasts of the soft palisade cells, but also in the vessel wall (Liu et al. 2009), we hypothesize that age-related differences in food digestion efficiency may determine the greater susceptibility of older larvae to catechin. In RPW larvae, the relative sizes of the gut parts change and the overall gut length increases with age (Monroy and Ferracini, unpublished data), suggesting that increased gut transit times with age may favour the absorption of cell wall-bound phenolic compounds. As far as we know, no studies addressing this possible factor are available neither for *R. ferrugineus* nor for other insect larvae.

In conclusion, the observed toxicity of catechin on RPW larvae agrees with the hypothesis that this compound is a component of the chemical defense mechanism of *C. humilis* against this insect pest. Based on their relative abundance in the analysed leaf tissues, catechin may be considered as a constitutive defense compound, and therefore its concentration likely depends on biotic (sex, age) and abiotic (environment) factors. Finally, the identification of different toxic compounds to *R. ferrugineus* in different palm species supports the view of a rich, so far hidden array of chemical resistance mechanisms against this pest in some of its potential hosts.

Acknowledments

The authors thank Alberto Alma for his valuable comments which helped to considerably improve the quality of the manuscript, Claudio Littardi and the Municipality of Sanremo for allowing the collection of plant material from Villa Ormond at Sanremo (Italy), and Cosimo Graniglia for technical assistance during the field sampling of RPW adult individuals.

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391 **Figure legend**
392 Figure 1. Negative effect of three catechin concentrations on the survival of two-week-old
393 larvae (A) or five-week old larvae (B) of *R. ferrugineus* (n = 10). The control treatment
394 consisted of a non-toxic extract of leaf basal tissues from the palm *P. canariensis*, a
395 susceptible, non-resistant host of *R. ferrugineus*. Dose 1: 42 mM; dose 2: 21 mM; dose 3: 10.5
396 mM.

Table 1. Comparison of dose-response model slopes and lethal times (LT_{50} and LT_{95}) of the toxicity tests with (±)-catechin from *C. humilis* on 2- and 5-week-old *R. ferrugineus* larvae. Data are presented as estimates ± *SE*. Different letters indicate statistically significant differences at $\alpha = 0.05$ using a *z*-test.

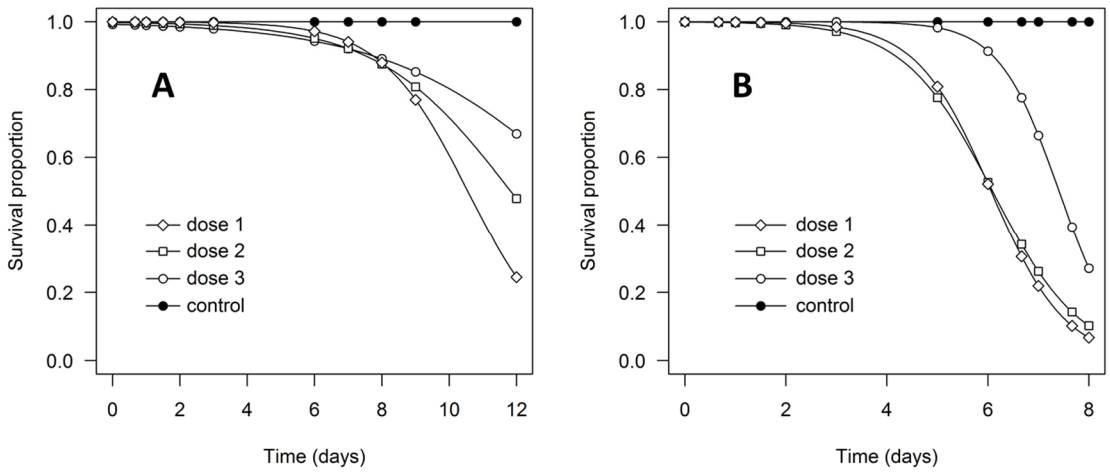
2-week-old larvae

Dose	Model slope	LT_{50} (days)	LT_{95} (days)
1 (42 mM)	− 0.779 ± 0.202 [a]	10.55 ± 0.62 [a]	14.33 ± 1.39 [a]
2 (21 mM)	− 0.509 ± 0.145 [ab]	11.83 ± 1.07 [b]	17.62 ± 2.53 [b]
3 (10.5 mM)	− 0.349 ± 0.121 [b]	14.02 ± 2.21 [c]	22.45 ± 4.96 [c]

5-week-old larvae

Dose	Model slope	LT_{50} (days)	LT_{95} (days)
1 (42 mM)	− 1.357 ± 0.303 [ab]	6.06 ± 0.25 [a]	8.23 ± 0.48 [a]
2 (21 mM)	− 1.140 ± 0.242 [a]	6.09 ± 0.27 [a]	8.67 ± 0.56 [ab]
3 (10.5 mM)	− 1.673 ± 0.471 [b]	7.41 ± 0.21 [b]	9.17 ± 0.59 [b]

402 Figure 1



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