1 **Different responses in mandarin cultivars uncover a role of cuticular waxes in the**

2 **resistance to citrus canker**

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ABSTRACT

'Okitsu' is a mandarin cultivar showing substantial resistance to *X. citri* subsp. *citri* (*X. citri*). We have previously shown that this cultivar has significantly lower canker incidence and severity than cultivar 'Clemenules', particularly during early stages of leaf development in the field. This differential response is only seen when the leaves are inoculated by spraying, suggesting that leaf surface contributes to resistance. In this work, we have studied structural and chemical properties of leaf surface barriers of both cultivars. Ultrastructural analysis showed a thicker cuticle covering epidermal surface and guard cells in young 'Okitsu' leaves than in 'Clemenules'. This thicker cuticle was associated with a smaller stomatal aperture and reduced cuticle permeability. These findings correlated with an accumulation of cuticular wax components, including primary alcohols, alkanes and fatty acids. None of these differences were observed in mature leaves, where both cultivars are equally resistant to the bacterium. Remarkably, mechanical alteration of cuticular thickness of young 'Okitsu' leaves allows canker development. Furthermore, cuticular waxes extracted from young 'Okitsu' leaves have higher antibacterial activity against *X. citri* than 'Clemenules'. Taken together, these data suggest that a faster development of epicuticular waxes in 'Okitsu' leaves play a central role in its resistance to *X. citri*.

INTRODUCTION

Plants have evolved multiple mechanisms to defend against pathogen invasion. The first line of defense is the plant surface composed by preformed structural barriers such as the cuticle, considered a specialized lipidic modification of the epidermal cell wall (Domínguez et al. 2011; Samuels et al. 2008; Yeats and Rose 2013). The cuticle covers the aerial parts of plants, which is dominated by the leaves forming the phyllosphere, and it serves as a key interface between plant and environment, protecting against invading pathogens and abiotic stresses (Aragón et al. 2017; Yeats and Rose 2013; Ziv et al. 2018). The plant cuticle is mainly composed of cutin, a lipid-derived polyester, and waxes, which are either embedded in the cutin matrix (intracuticular waxes) or deposited on its outer surface (epicuticular waxes) (Aragón et al. 2017; Domínguez et al. 2011; Samuels et al. 2008). The architecture and composition of the cuticle varies between plant species, organs and developmental stages. The molecular basis of cuticle biosynthesis, export and regulation has been extensively studied in the model plant *Arabidopsis*, as well as in crop plants, including tomato, maize, rice, citrus and *Brassica napus* (Lee and Suh 2015; Liu et al. 2015; Samuels et al. 2008; Wang et al. 2014; Wang et al. 2016).

The cuticle is the first contact with bacteria when they land on the leaf surface. In order to cope with this defensive barrier, many foliar pathogenic bacteria have evolved the ability to adhere and develop biofilm on the host surface before gaining access into the intercellular spaces of the mesophyll tissue through stomata (Melotto et al. 2008; Rigano et al. 2007; Vojnov and Marano 2015). Plant genotype play a major role in determining the structure of the phyllosphere, interfering with the ability to develop biofilm, a key factor of bacterial pathogenicity (Bodenhausen et al. 2014; Favaro et al. 2014; Schlechter et al. 2019; Whipps et al. 2008). It has been demonstrated that thicker and less permeable cuticles might interfere with epiphytic bacterial colonization,

71   inhibiting the wetting of the leaf surface and limiting solubilization and diffusion of

72   nutrients from the leaf (Bodenhausen et al. 2014; Lindow and Brandl 2003; Schlechter et

73   al. 2019). Moreover, stomatal density and structure could also play an important role as

74   preformed physical barriers against bacterial infection (Gonçalves-Zuliani et al. 2016;

75   Wang et al. 2011).

76   The second line of plant defense is triggered by the perception of conserved

77   pathogen-associated molecular patterns (PAMPs) by cell surface pattern-recognition

78   receptors (PRRs) in the plasma membrane (Couto and Zipfel 2016). Activation of PRR-

79   mediated response results in stomatal closure, limiting bacterial proliferation at early

80   stages of infection. However, pathogenic bacteria can reverse the stomatal-based defense

81   and fully colonize the host plant (Chiesa et al. 2019; Melotto et al. 2017). A further level

82   of induced defense is initiated by host recognition of effectors that are secreted into cells,

83   resulting in the triggering of multiple responses that lead to the arrest of bacterial growth

84   (Cesari 2018; Toruño et al. 2016).

85   *Xanthomonas citri* subsp. *citri* (*X. citri*) is the causal agent of Asiatic citrus canker,

86   a disease that seriously affects most of the world's commercial citrus cultivars. Infected

87   fruits have decreased commercial quality, compromising the acceptance by most markets

88   (Ference et al. 2018). Evaluations in field and controlled conditions suggest that several

89   types of citrus and closely related genera, including *C. ichangensis*, *C. junos*, *C. medica*,

90   *C. mitis*; *C. unshiu*, 'Dalan Dalan' (a cultivar similar to *C. paradisi*), ~~*Citro fortunella*~~

91   *Citrofortunella* spp., *Fortunella* spp., and 'Lakeland' limequat (*C. aurantifolia* x *F.*

92   *japonica*) are resistant to *X. citri* (Chen et al. 2012; De Carvalho et al. 2015; Deng et al.

93   2010; Favaro et al. 2014; Gochez and Canteros 2008; Gonçalves-Zuliani et al. 2016:

94   Graham et al. 1992; Lee et al. 2009; Shiotani et al. 2009; Viloria et al. 2004; Wang et al.

95   2011). However, in some of these pathosystems quantitative resistance present in field is

96   fully or partially broken down when invasive inoculation methods, such as pressure

97   infiltration or pin prick inoculation, are used. This suggests that preformed defenses at

98   the plant surface might be involved in the resistance to bacterial invasion (Favaro et al.

99   2014; Gonçalves-Zuliani et al. 2016; Graham et al. 1992; Wang et al. 2011).

100   The mechanisms underlying plant preformed defense and its relevance for limiting

101   bacterial pathogen entry to the apoplast remain poorly understood. It has been reported

102   that smaller stomatal density, size and aperture, and also higher epicuticular wax content

103   contribute to the differential response to *X. citri* infection between the resistant kumquat

104   genotype 'Meiwa' and the susceptible navel orange genotype 'Newhall' (Wang et al.

105   2011). In addition, a smaller stomatal aperture was associated with a lower susceptibility

106   to *X. citri* in 'Pera' IAC orange but no differences were observed in the stomatal density

107   between this genotype and the more susceptible cultivar 'Washington navel' (Gonçalves-

108   Zuliani et al. 2016).

109   Previously, we have shown that the resistance to *X. citri* in *C. unshiu* 'Okitsu' was

110   associated with a faster phenological development of the leaf during the period of

111   maximal susceptibility to *X. citri* infection, which might be coincident with a rapid cuticle

112   development (Favaro et al. 2014). In addition, the resistance to *X. citri* was evident in

113   bacterial spray-inoculated plants but not in those inoculated by infiltration, suggesting

114   that the leaf surface contributes to quantitative resistance, limiting bacterial epiphytic

115   fitness and biofilm formation.

116   In this work, we have studied the structural and chemical properties of leaf surface

117   barriers of two mandarin cultivars to shed light on the differences that lead to resistance

118   or development of citrus canker disease. Our findings highlight the multiple functions of

119   the thicker 'Okitsu' cuticle in limiting bacterial establishment, including small stomatal

120   aperture, low water permeability and a fast development of cuticular waxes. ~~Furthermore,~~

121 ~~the amount of cuticular waxes is associated with a strong antimicrobial activity against *X.*~~

122 ~~*citri.*~~ Furthermore, the amount of cuticular waxes in the early stage of leaf development

123 is associated with antimicrobial activity against *X. citri.*

124

MATERIAL AND METHODS

Plant material and bacterial inoculation

127 One-year old 'Clemenules' (*C. clementina* Hort. ex Tan.) and 'Okitsu' (*C. unshiu*

128 Marc.) plants, grafted onto *Poncirus trifoliata* (L.) Raf. rootstocks were kept in a growth

129 chamber, with a temperature range of 25 to 28°C, high humidity (> 95%), a 14 h

130 photoperiod and a light intensity of 150-200 μE m^{-2} s^{-1}. New shoots of approximately 1

131 cm size with at least 5 leaves were selected after pruning the plants. All the leaves of the

132 same shoot were considered to be of the same ontological age. Experiments described in

133 this work were done in young (18-day-old) and mature (36-day-old) leaves (Fig. 1A).

134 These phenological development stages showed maximal and minimal differences

135 between cultivars to canker disease (Favaro et al. 2014).

136 Bacterial suspensions of *X. citri* strain A^{E28} (10^7 CFU ml^{-1}) were prepared in 10

137 mM MgCl$_2$ and inoculated spraying mandarin leaves (Favaro et al. 2014). Inoculated

138 plants were kept in a growth chamber. ~~Bacterial populations were isolated from the leaves~~

139 ~~according to the method described by Rigano et al. (2007). Disease progression was~~

140 ~~monitored phenotypically, registered by using a stereomicroscope MVX10 as described~~

141 ~~in Roeschlin et al. (2017) and through analysis of bacterial growth curves (Favaro et al.~~

142 ~~2014).~~

143

Stomatal density and stomatal aperture analysis

145   Stomatal density, spatial distribution and aperture size were evaluated in abaxial

146   epidermis imprints of the leaf, obtained using cyanoacrylate adhesive, according to

147   Chiesa et al. (2019). Stomatal density was determined as the number of stomata per square

148   millimeter. Spatial distribution of stomata was evaluated considering the predominant

149   stomatal type and the relation between the number of large and small stomata.

150   To evaluate stomatal aperture, mandarin plants were exposed to light for at least

151   3 h at 150-200 $\mu E\,m^{-2}\,s^{-1}$, 70% humidity and temperatures ranging from 25 to 28°C,

152   before beginning the experiment. Leaves were inoculated with *X. citri* suspensions. As

153   control of stomatal closure and opening, 20 μm ABA (mixed isomers; Sigma-Aldrich, St.

154   Louis, MO, USA) and water were used, respectively.

155   Imprints of abaxial epidermis (sample) were observed under a light microscope

156   (BH2; Olympus Optical Ltd. Company). For the different treatments and times,

157   photographs were taken of at least 10 random zones per sample. Eighteen samples were

158   obtained from 6 leaves collected from 2 different plants. ~~Three independent experiments~~

159   ~~were repeated with similar results.~~Three independent experiments were conducted

160   yielding similar results. The width of 50 random stomatal apertures was measured for

161   each treatment and time point, using the software Image J v 1.41 (National Institutes of

162   Health, Bethesda, MD, USA).

163

Cuticular permeability

165   For assessing the permeability of mandarin leaf surfaces to water-soluble

166   molecules, ~~T~~toluidine blue staining was adapted from a previously described protocol

167   (Bessire et al. 2007) with some modifications. Tissue samples (5 pieces of 1 cm^2 per leaf)

168   were decolorized in 95% ethanol, equilibrated in 0.2 M $NaPO_4$ (pH 9.0) for 1 h, and

169   incubated in 0.05% (w/v) toluidine blue solution for up to 6 hours. Tissue samples treated

170 with water and processed as described above were used as controls. Dye penetration to

171 cells was examined with an Olympus BX50F4 microscope (Olympus Optical Ltd.

172 Company, Shinjuku, Tokyo, Japan). Toluidine blue is a polychromatic dye, therefore the

173 color observed depends on the tissue staining (O'Brien et al. 1964). The samples were

174 photographed in a Molecular Imager ChemiDoc TM XRS+ Imaging System (BIO-RAD,

175 USA) and quantification of permeability was performed using a standard dye curve and

176 the Quantity One software (BIO-RAD, USA). Each experiment involved at least 15

177 samples per treatment, obtained from 3 different leaves, each one collected from different

178 plants. ~~Three independent experiments were repeated with similar results.~~Three

179 independent experiments were conducted yielding similar results.

180

181 **Transmission electron microscopy (TEM) ~~assays~~**

182   The ultrastructure of the abaxial leaf cuticle was analyzed by TEM. Leaf sections

183 (samples) from both mandarin cultivars were prepared and observed according to

184 Roeschlin et al. (2017). Cuticle thickness was measured at three points of the epidermis

185 using the Software Image J v 1.41 (National Institutes of Health, Bethesda, MD, USA).

186 At least 6 different photographs were analyzed per leaf section. Samples from 3 different

187 leaves, obtained from different plants were analyzed for each leaf age and genotype.

188

189 **Total wax extraction**

190   Cuticular wax from leaves surface was extracted as described by Beattie and

191 Marcell (2002) with minor modification. A pool of ~~Y~~young or mature leaves (150 cm^2, ~

192 20 to 15 leaves according to phenological development) randomly selected from 3

193 different 'Okitsu' and 'Clemenules' plants were fully submerged in 50 ml of chloroform

194 (Merck, Darmstadt, Germany). After stirring for 1 min at room temperature, the solvent

195   was evaporated under a gentle stream of nitrogen. Total wax concentration was expressed

196   in µg per cm^2 of leaf area. The obtained samples were processed according to Wang et al.

197   (2014) either for GC-MS analysis or antibacterial activity assays. Control (blank) samples

198   were prepared in the same way as other samples, except that no leaves were added. ~~Three~~

199   ~~independent experiments were repeated with similar results.~~ Three independent

200   experiments were conducted yielding similar results.

201

202   **Wax analysis by gas chromatography–mass spectrometry (GC–MS)**

203   Wax composition of cuticular waxes of mandarin leaves ~~GC-MS analysis~~ was

204   ~~performed~~ analyzed according to Wang et al. (2014), using capillary GC (Agilent 7890B,

205   Agilent Technologies, Santa Clara, CA, USA), coupled with MS detector (5977A,

206   Agilent Technologies) and a HP-5ms UI capillary column (30 m, 0.25 mm inner diameter,

207   0.25 µm film thickness, Agilent Technologies). Helium was used as a carrier gas at a flow

208   rate of 1 ml min^{-1}. The following parameters were employed: inlet temperature 250°C,

209   MS transfer line temperature 280°C, ion source temperature 230°C, electron impact (EI)

210   70 eV, *m/z* range 50-750. Wax components were identified using a mass spectral data

211   base (NIST MS Library SW Kit, 2011b, Agilent Technologies). The relative percentage

212   of each compound was determined by dividing the integrated area of the peak of the

213   specific ion for the compound in question (×100), by the summed value for the areas of

214   all peaks (Chen et al. 2014). ~~Three independent experiments were repeated with similar~~

215   ~~results.~~ Three independent experiments were conducted yielding similar results.

216

217   **RNA isolation and expression analysis of cuticle-associated genes**

218   The transcriptional levels of 7 genes related to wax biosynthetic pathways were

219   analyzed by quantitative polymerase chain reaction (qPCR). Total RNA from mandarin

220   leaves (100 mg) was isolated according to the manufacturer's instructions (NucleoSpin®

221   RNA, Macherey-Nagel, Dueren, Germany). Reverse transcription was performed by M-

222   MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with 1 µg DNase-treated

223   total RNA and oligo-dT12-18 as primers. Synthesized cDNA was used for qPCR

224   ~~quantitative polymerase chain reaction (qPCR).~~ Seven candidate genes potentially

225   involved in mandarin cuticle formation were selected from the *C. clementina* genome

226   database (www.phytozome.net) based on the homology to corresponding Arabidopsis

227   genes with known functions in leaf cuticular wax biosynthesis. The gene locus, function

228   of gene product, primers sequences and PCR conditions are given in Table 1. The

229   reactions were carried out with real-time PCR master mix (Biodynamics SRL, BA,

230   Argentina), and monitored in a Mastercycler® ep realplex system (Eppendorf, Hamburg,

231   Germany). Relative transcript abundance between samples was normalized against

232   *histone H4* (Shiotani et al. 2007) as an internal standard using the $\Delta\Delta Ct$ method (Livak

233   and Schmittgen 2001). 'Clemenules' leaves served as the reference sample. Each assay

234   was performed with 3 different samples, involving 3 different leaves from 3 different

235   plants. ~~Three independent experiments were carried out with similar results.~~ Three

236   independent experiments were conducted yielding similar results.

237

238   Mechanical removal of 'Okitsu' cuticular waxes and canker development

239   Epicuticular waxes were removed from the abaxial surface of 'Okitsu' 18-day-old

240   leaves by cotton swab and with gum arabic, according to Marcell and Beattie (2002) and

241   Gniwotta et al. (2005), respectively. For gum arabic treatment, an aqueous solution of the

242   adhesive was applied onto the entire abaxial surface of the leaves using a small

243   paintbrush. After 1 to 2 h, the gum arabic solution formed a dry and stable polymer film

244   in which wax crystals were embedded and the film was extracted without damaging the

245   leaves. ~~Leaves subjected to both treatments and untreated 'Okitsu' and 'Clemenules' leaves~~

246   ~~were inoculated with *X. citri* suspensions. Fifteen days post-inoculation (dpi) samples~~

247   ~~were processed for TEM analysis (Roeschlin et al. 2017). All plant inoculations involved~~

248   ~~a minimum of two shoots with at least 5 leaves from each plant and four plants for each~~

249   ~~mandarin cultivar.~~   Leaves subjected to both treatments and untreated 'Okitsu' and

250   'Clemenules' leaves were inoculated with *X. citri* suspensions (10^7 CFU ml^{-1}) by spraying

251   onto the abaxial epidermis of mandarin leaves, according to Favaro et al. (2014).

252   Inoculated plants were kept for 30 days in a growth chamber. All plant inoculations

253   involved a minimum of two shoots with at least 5 leaves from each plant and four plants

254   for each mandarin cultivar. Disease progression was monitored phenotypically and

255   through analysis of bacterial growth curves. Fifteen days post-inoculation (dpi) samples

256   were processed for TEM analysis (Roeschlin et al. 2017). For determination of bacterial

257   population, three leaf disks of 1 cm^2 were selected randomly from the inoculated leaves.

258   The disks were immersed in 500 µl of 10 mM MgCl$_2$ in Eppendorf microfuge tubes.

259   Bacterial cells were collected by homogenization of tissue with a plastic pestle. The

260   suspension was stirred at room temperature for 5 min and serial dilutions of this

261   suspension were plated on 1.8% agar NYG medium (5 g l^{-1} peptone extract, 3 g l^{-1} yeast

262   extract and 20 g l^{-1} glycerol) supplemented with 100 μg ml^{-1} ampicillin, to estimate the

263   total bacterial numbers. Leaf samples were taken during 1 week to determine bacterial

264   population sizes on the leaves. At each time point, samples were determined from three

265   separate experiments and each experiment was measured in triplicate. Three independent

266   experiments were conducted yielding similar results.

267

268   **Antibacterial activity assays of cuticular waxes**

269   Microbicidal properties of cuticular waxes from mandarin leaves were tested *in*

270   *vitro* against *X. citri*. A pool of ~~Y~~young leaves (150 cm^2, ~ 20 leaves) randomly selected

271   from 3 different 'Okitsu' and 'Clemenules' plants were fully submerged in 50 ml of

272   chloroform (Merck, Darmstadt, Germany) and processed as described above. The

273   extracted cuticular waxes were dissolved in 200 µl of chloroform (Merck, Darmstadt,

274   Germany) and used in the screening of antibacterial activity on NYG agar plates. Spots

275   of approximately 50 µl of each wax extract (corresponding to the wax present in 37.5 cm^2

276   of leaf) were deposited in triplicate onto 1.8% agar NYG supplemented with 100 µg ml^{-1}

277   ampicillin. As control, spots of 50 µl of chloroform were deposited onto agar plates. Ten

278   microliters of *X. citri* suspensions (10^4 CFU ml^{-1} in 10 mM MgCl$_2$) were inoculated over

279   the dry wax extracts and control. The plates were incubated at 28°C for 72 h and the

280   colonies grown on each spot were counted using the Software Image J v 1.41 (National

281   Institutes of Health, Bethesda, MD, USA). ~~Three independent experiments were repeated~~

282   ~~with similar results.~~ Three independent experiments were conducted yielding similar

283   results.

284   For determination of bactericidal activity in NYG liquid medium, the assay was

285   adapted from the method described by Golus et al. (2016). Cuticular waxes from young

286   leaves (150 cm^2) were extracted and processed as described above. The extracted

287   cuticular waxes were dissolved in 15 µl of dimethylsulfoxide (DMSO, Merck, Darmstadt,

288   Germany), and 2 µl of this extract was mixed with 200 µl of *X. citri* suspension (10^6 CFU

289   ml^{-1} in 10 mM MgCl$_2$) and deposited in triplicate on 96-well Clear Flat

290   Bottom Polystyrene TC-treated Microplates (Corning, USA). As control of bacterial

291   growth, 2 µl of DMSO was mixed with 200 µl of *X. citri* suspension (10^6 CFU ml^{-1} in 10

292   mM MgCl$_2$). The plate was incubated at 28°C, and bacterial growth was assessed by

293   microplate reader (Bio Tek Synergy 2 Multi-Detection, USA) at 600 nm. ~~Three~~

294 ~~independent experiments were repeated with similar results.~~ Three independent

295 experiments were conducted yielding similar results.

296

Statistical analyses

298 Data were analyzed according to Student´s *t* test ($P < 0.05$) through InfoStat

299 Software v2017 (Di Rienzo et al. 2017), excepting stomatal aperture and bacterial

300 population data that were subjected to a three-way ANOVA (cultivar, time, treatment)

301 and one-way ANOVA, respectively. In both cases the means were analyzed using

302 Tukey´s test ($P < 0.05$).

303

304 **RESULTS**

305 **The stomatal pore aperture is smaller in young-'Okitsu' leaves than in 'Clemenules'**

306 ~~In previous work, we have shown that 'Okitsu' is more resistant to *X. citri* than~~

307 ~~'Clemenules', particularly during early stages (18-day-old) of leaf development and~~

308 ~~exclusively when the leaves were inoculated by spraying, a noninvasive method (Favaro~~

309 ~~et al. 2014). However, mature (36-day-old) leaves showed similar resistance to canker~~

310 ~~disease.~~

311 In order to investigate the role of stomatal density and size in the resistance to *X.*

312 *citri*, imprints of abaxial epidermis of 18-day-old leaves of both mandarin cultivars were

313 analyzed (Fig. 1A). No apparent qualitative differences were observed in the disposition

314 of guard cells in relation to the subsidiary cells, showing both cultivars the same stomatal

315 complex (anomocytic type, Fig. 1B). Moreover, the relation between the number of large

316 and small stomata was similar to that reported for other *Citrus* species (Graham et al.,

317 1992). In addition, no differences were observed in the stomatal density, indicated by the

318   number of stomata per square millimeter between 'Okitsu' (943 ± 64) and 'Clemenules'-

319   (1004 ± 41). (943 ± 64 and 1004 ± 41, respectively, $P < 0.05$ Student's *t* test).

320   In order to investigate the role of stomatal response to *X. citri* in both mandarin

321   cultivars during the different phenological leaf stages (young and mature leaves),

322   bacterial promotion of stomatal closure was analyzed at 1 and 4 h post-inoculation (hpi).

323   ABA and water were used as control of stomatal closure and opening, respectively

324   (Melotto et al. 2008; Chiesa et al. 2019). Interestingly, stomatal aperture was significantly

325   lower smaller in 'Okitsu' than in 'Clemenules' at 1hpi in 18-day-old leaves, independently

326   of bacterial inoculation or water and ABA treatments either for bacterial inoculation or

327   control treatments (Fig. 1C). Moreover, at 4 hpi *X. citri* reversed stomatal closure in

328   'Clemenules' whereas 'Okitsu' stomatas remained closed. Nevertheless, in mature leaves

329   (36-day-old), stomatal aperture and response to bacterial infection or treatments were

330   similar in both mandarin cultivars (Fig. 1C).

331   These findings suggest that a smaller stomatal aperture in young 'Okitsu' leaves may

332   be one component of the surface barriers that contributes to the resistance to *X. citri*

333   infection.

334

335   **'Okitsu' show reduced cuticle permeability in young leaves**

336   We previously demonstrate that young 'Okitsu' leaves interfere with plant-

337   associated bacterial biofilms required for *X. citri* pathogenicity and canker development

338   (Favaro et al. 2014). We hypothesized that the inhibition of bacterial adhesion and biofilm

339   formation may be associated with a reduced cuticle permeability limiting solubilization

340   and diffusion of nutrients from the leaf in this mandarin cultivar. To test this, permeability

341   of the cuticle of 18-day-old leaves was assessed with toluidine blue and monitored over

342   6 h. After 30 min, only 'Clemenules' epidermal tissue showed accumulation of the dye

343 (blue staining) within the guard cells and cell wall junctions (Fig. 2). Six hours after

344 incubation with the staining solution, 'Clemenules' showed a deep penetration of the dye

345 to subepidermal leaf tissue, indicated by the characteristic reddish staining of

346 parenquimatic tissue. The percentage of cuticular permeability to the dye was 1.5-fold

347 higher in 'Clemenules' than in 'Okitsu' ($58.0 \pm 4\%$ and $37.2 \pm 3\%$, respectively, $P < 0.05$

348 Student's *t* test), suggesting a negative relation between permeability and resistance to *X.*

349 *citri*. As expected, the percentage of dye penetration was similar in 36-day-old leaves of

350 both mandarin cultivars ($31.8 \pm 4\%$ and $35.6 \pm 2\%$, respectively).

351

352 **A thick and deeper extending cuticle covers epidermal surface and guard cells in**

353 **young 'Okitsu' leaves**

354 To gain insight into the relationship between cuticular permeability and thickness

355 in the resistance to *X. citri* the ultrastructure of the cuticle in both mandarin cultivars was

356 analyzed by transmission electron microscopy (TEM) during the main phenological

357 stages of the leaf that show clear differences in susceptibility to *X. citri* infection (Fig. 3).

358 Eighteen-day-old 'Okitsu' leaves showed a thicker cuticle than 'Clemenules', which covers

359 the pavement epidermal cells and formed stomatal edges in the guard cells (Fig. 3A). In

360 addition, in some areas 'Okitsu' cuticle penetrates deeply in the epidermal cell wall

361 junctions, leading to the cuticularization of the anticlinal cell wall, generating a flat

362 continuous layer on the epidermis. Conversely, in 'Clemenules', a thin cuticle covers the

363 surface of epidermal cells, leaving depressions between epidermal cells junctions,

364 designing a sinuous topography in the epidermal layer (Fig. 3A). In contrast, no

365 differences in cuticle thickness were observed in mature (36-day-old) leaves between

366 both mandarin cultivars (Fig. 3B).

367   Taken together, our data suggest that a faster cuticle development takes place in

368   young 'Okitsu' leaves, where it may play a substantial role as a preformed physical barrier

369   against *X. citri* infection.

370

371   **The thick cuticle in young 'Okitsu' leaves is associated with an early accumulation of**

372   **cuticular wax components**

373   The plant cuticle waxes are predominantly composed of a mixture of aliphatic very

374   long chain fatty acid (VLCFA) and their derivatives, as well as cyclic compounds

375   including triterpenoid and sterols (Samuels et al. 2008). To evaluate if there is a

376   relationship between wax amount and resistance to *X. citri*, we studied the levels of total

377   waxes in young and mature leaves from 'Okitsu' and 'Clemenules'.

378   In accordance with the increase of cuticular thickness, 18-day-old 'Okitsu' leaves

379   showed significantly higher accumulation of total waxes per leaf unit area (13.9 ± 0.9 µg

380   cm^{-2}) than 'Clemenules' (3.7 ± 0.3 µg cm^{-2}). Nevertheless, the chemical analysis of wax

381   components of these leaf samples through GC-MS shows that wax profile shares high

382   similarities between 'Okitsu' and 'Clemenules' (Table 2). The most abundant cuticular wax

383   fraction in both mandarin cultivars was the primary alcohols, representing 96.6% in

384   'Okitsu' and 95.7% in 'Clemenules' of the GC-MS-detected compounds. They were

385   accompanied by minor amounts of n-alkanes (2.4 and 3.2% for 'Okitsu' and 'Clemenules',

386   respectively) and fatty acids (0.5 and 0.6% for 'Okitsu' and 'Clemenules', respectively). In

387   the primary alcohol fraction, even-number homologs prevailed, such as hexacosanol (C_{26}),

388   tetracosanol (C_{24}) and octacosanol (C_{28}). The second group was integrated by alkanes

389   between C_{22} and C_{31}, such as docosano (C_{22}), tricosano (C_{23}) untriacontano (C_{31}),

390   heptacosano (C_{27}), nonacosano (C_{29}) and pentacosano (C_{25}). Tetracosanoic (C_{24}),

391   octadecanoic (C_{18}), hexadecanoic (C_{16}) and docosanoic (C_{22}) fatty acids established the

392   third group in abundance of aliphatic lipids. Considering the differences between the total

393   amounts of waxes per leaf unit area among the mandarin cultivars, the quantity of all these

394   compounds was higher in 'Okitsu' than in 'Clemenules'. On the other hand, at 36-day-old

395   leaves the wax levels increased notably in 'Clemenules' (16.4 ± 0.5 µg cm^{-2}), reaching

396   similar accumulation than in 'Okitsu' (18.2 ± 0.2 µg cm^{-2}). In this developmental stage, the

397   predominant compounds were similar to those found in 18-day-old cuticle leaves and new

398   compounds corresponding to aliphatic lipids of longer carbon chains were present in trace

399   amounts in both cultivars, suggesting changes in the wax composition during leaf

400   development.

401   The higher accumulation of waxes in 18-day-old 'Okitsu' leaf in comparison with

402   'Clemenules', suggests differences in expression of wax biosynthesis pathways between

403   both mandarin cultivars. To further investigate this, the expression of candidate genes

404   related to cuticular wax-biosynthesis was analyzed (Table 1; Fig. 4). Remarkably, the

405   relative expression of *CER6,* a β-ketoacyl- CoA synthase (KCS), key in catalyzing the first

406   step in VLCFAs formation was 4.6-fold higher in young 'Okitsu' leaf than in 'Clemenules'.

407   Furthermore, the expression of *CER1* and *CER3*, which are involved in VLC alkane

408   synthesis, was increased in the resistant cultivar compared to 'Clemenules' (induced by 5-

409   fold and 4.5-fold, respectively). In the same way, the level of expression of *WIN1*, a

410   transcription factor that regulates the expression of *CER1*; and *CER7,* a regulatory

411   transcription factor of *CER3* (Hooker et al. 2007), was also elevated in the resistant cultivar

412   (induced by 2.6-fold and 1.8- fold, respectively). Interestingly, the expression levels of

413   VLC alcohol-forming genes, such as *CER4* and *FAR2* were higher in young 'Clemenules'

414   leaves than in 'Okitsu'.

415 Taken together, these data show that resistance to *X. citri* correlates with a higher

416 wax deposition, in accordance with the thicker cuticle shown in TEM analysis rather than

417 differences in chemical cuticular composition.

418

419 **Mechanical alteration of 'Okitsu' cuticle thickness allows *X. citri* colonization and**

420 **canker development**

421 To examine the hypothesis that the inhibition of bacterial survival on young

422 'Okitsu' leaf surface might be due to a higher wax accumulation, we analyzed the effect

423 of reducing abaxial cuticle thickness on disease development. Epicuticular waxes from

424 the leaf surface were selectively removed without damage to the epidermal cells using

425 either cotton swabs or gum arabic (Gniwotta et al. 2005; Marcell and Beattie 2002).

426 Ultrastructural analysis showed that gum arabic and cotton swab treatments reduced

427 cuticular thickness in epidermal pavement and guard cells from 18-day-old 'Okitsu'

428 leaves. Cuticular thicknesses were 0.29 ± 0.09 μm and 0.18 ± 0.08 μm, for gum arabic

429 and cotton swab treatments, respectively, compared with 1.24 μm for untreated leaves

430 (Fig. 5A). Untreated 18-day-old 'Okitsu' and 'Clemenules' leaves were used as controls.

431 Both treated and untreated leaves were inoculated by spraying with *X. citri*. The integrity

432 of the epidermal layer and mesophyll tissue in untreated 'Okitsu' leaves remained

433 unaltered after bacterial inoculation (Fig. 5B). The population size of *X. citri* began to

434 decline 3 dpi and no canker symptoms were observed after 20 days, as expected from

435 previous work (Favaro et al. 2014) (Fig. 5C). Notably, at 15 dpi surface-treated 'Okitsu'

436 leaves, whose epicuticular waxes were removed, showed the presence of bacteria

437 invading the intercellular space of a hypertrophied mesophyll tissue. Moreover, these

438 samples showed similar ultrastructural changes by bacterial colonization to 18-day-old

439 'Clemenules' leaves (Fig. 5B). ~~Comparison of the bacterial numbers *in planta* revealed no~~

440   ~~differences in~~ Similar bacterial growth was observed between the surface-treated 'Okitsu'

441   and untreated 'Clemenules' leaves. In these tissue samples *X. citri* population gradually

442   increased more than two orders of magnitude over the monitoring period that was

443   correlated with the canker symptoms developed at 20 dpi (Fig. 5C).

444   These results confirm that 'Okitsu' cuticle interferes with *X. citri* fitness affecting

445   early events required for bacterial infection and consequently mesophill colonization.

446

447   **Cuticular waxes inhibit *X. citri* growth**

448   In order to determine if cuticular waxes from young 'Okitsu' leaves also act as an

449   inhibitor of pathogen survival, we performed *in vitro* antibacterial activity assays. In an

450   attempt to reproduce the conditions *in planta*, *X. citri* was exposed to the cuticular waxes

451   on solid medium. Cuticular waxes extracted from both 18-day-old young 'Okitsu' and

452   'Clemenules' leaves have a potent inhibitory effect on the growth of *X. citri* compared

453   with the control (Fig. 6A). Notwithstanding, 'Okitsu' waxes have an antibacterial activity

454   50% higher than 'Clemenules', according to the level of cuticular waxes per leaf unit area

455   (Fig. 6A). In order to follow the waxes effect on the bacterial growth over a 12 h time

456   course, the cells were propagated in liquid medium supplemented with 'Okitsu' or

457   'Clemenules' cuticular waxes. This study confirmed that the higher amount of cuticular

458   waxes per cm^2 of leaf area in 'Okitsu' compared to 'Clemenules', results in a greater

459   antibacterial activity in the resistant cultivar (Fig. 6B). These results indicate that the

460   increase of wax accumulation play a role in the resistance against *X. citri,* contributing

461   not only as preformed defenses but also as an antimicrobial agent.

462

463   **DISCUSSION**

464   The plant leaf surface, or the phyllosphere, is one of the most important natural

465   habitats for microorganisms. It has been demonstrated that epiphytic bacterial populations

466   are directly influenced by certain environmental conditions of the phyllosphere such as

467   fluctuating temperature, radiation, relative humidity, presence of free water, and the

468   availability of plant-leached metabolites at the leaf surface, so that only adapted bacteria

469   can survive (Aragón et al. 2017; Schlechter et al. 2019; Schreiber et al. 2005; Whipps et

470   al. 2008). In previous studies, we showed that in young 'Okitsu' leaves, surface defense

471   barrier impedes epiphytic growth and biofilm formation of *X. citri*, conferring resistance

472   to canker disease (Favaro et al. 2014). In this work, we investigated the physical and

473   chemical characteristics of this defense barrier.

474   ~~Stomatal movement is~~Changes in stomata aperture size are function of both guard

475   and epidermal cell turgor, regulated by signalling components in guard cells in response

476   to environmental conditions, including abiotic and biotic stress (Melotto et al. 2017). Our

477   results indicate that a thicker cuticle is correlated with a smaller stomatal aperture and

478   less permeable stomatal cuticular edges. *Arabidopsis* mutants that are unable to

479   synthesize cutin have diminished cuticular projections surrounding the stomatal pore

480   resulting in increased susceptibility to pathogens (Li et al., 2007). Considering that the

481   range of stomatal aperture in young 'Okitsu' leaves (0.3 to 0.6 µM) and the *X. citri* size

482   (1.5-2.0 x 0.5-0.75 μm; Goto 1992), we could hypothesize that the stomatal cuticular

483   edges enhance stomatal defense by reducing bacterial entry into the mesophyllic tissue.

484   The absence of both bacterial proliferation and hypertrophy in the mesophyll tissue after

485   bacterial inoculation of 'Okitsu' young leaves support this hypothesis.

486   We demonstrated that 'Okitsu' cuticle in young leaf is almost 2-fold thicker

487   compared to 'Clemenules'. No differences in cuticle thickness between cultivars were

488   observed in mature (36-day-old) leaves. This result indicates that 'Okitsu' cuticle develops

489 rapidly during the leaf expansion process, which is the period of optimal susceptibility to

490 *X. citri* infection. A thicker 'Okitsu' cuticle was related with the fortification of epidermal

491 anticlinal cell wall, which generates a smooth surface, whereas, the leaf cuticle has a

492 sinuous or rough surface in 'Clemenules'. It has been demonstrated that these cavities

493 formed in epidermal cell wall junctions are protected sites where phytopathogenic

494 bacteria survive (Lindow and Brandl 2003; Schlechter et al. 2019; Whipps et al. 2008).

495 Furthermore, we found a negative relationship between the cuticular thickness and water

496 permeability on 'Okitsu' leaf, which may be associated with a low availability of water

497 and nutrients on leaf surface, preventing also the epiphytic growth of *X. citri*. It has been

498 extensively demonstrated that changes in cuticle permeability influence plant–bacterial

499 interactions (Aragón et al. 2017; Tang et al. 2007; Xiao et al. 2004; Yeats and Rose 2013;

500 Ziv et al. 2018). Moreover, a number of epiphytic (pathogenic and non-pathogenic)

501 bacteria have been shown to increase cuticular permeability, enhancing solubilization and

502 diffusion of nutrient from the leaf to improve epiphytic fitness on the leaf surface (Lindow

503 and Brandl 2003; Schreiber et al. 2005; Vacher et al. 2016; Whipps et al. 2008).

504 The cuticular thickness of 'Okitsu' and its consequence over leaf permeability could

505 be explained considering a greater quantity of total cuticular waxes. In young 'Okitsu'

506 leaves, the ~~induction~~ higher expression of genes involved in the first step of VLCFA

507 formation, coupled with the up regulation of genes involved in VLC alkane production,

508 resulting in a higher accumulation of wax constituents, may indicate that the wax

509 biosynthetic pathways are induced earlier in this resistant cultivar compared to

510 'Clemenules' in the same phenological stage. Although primary alcohols dominated the

511 wax mixture in leaves of both mandarin cultivars, the absolute amounts of these

512 compounds (referred as $\mu g\ cm^{-2}$ of the leaf) differ between them. We could speculate that

513 the higher expression levels of VLC alcohol-forming genes, such as *CER4* and *FAR2* in

514 young 'Clemenules' leaves might means an enhanced synthesis, to reach similar levels to

515 'Okitsu' in mature leaves. Riederer and Schneider (1990) also found a higher quantity of

516 primary alcohols in *C. aurantium* as leaves age increased. The increase of cuticular wax

517 production during leaf development has been also reported in other plant species (Lee and

518 Suh 2015; Yeats and Rose 2013; Zhu et al. 2018).

519   It has been proposed that waxy broad-leaved plants support lower populations of

520 culturable bacteria in the phyllosphere due to avoidance of water stagnation on the plant

521 surface (Marcell and Beattie 2002; Whipps et al. 2008). Thus, the abundance of

522 epicuticular waxes has been associated to a self-cleaning mechanism known as the lotus

523 effect, which repels water avoiding pathogen establishment (Yeats and Rose 2013).

524 Hydrophobicity of the epicuticular waxes depends on the nature of the chemical groups

525 exposed on the surface (Marcell and Beattie 2002). Although there are no significant

526 differences in cuticular wax compositions between 'Okitsu' and 'Clemenules', the larger

527 amount of cuticular waxes in young 'Okitsu' leaves could be associated with a greater

528 hydrophobicity compared to 'Clemenules', and consequently, with a greater water-

529 repellent surface which interfere with the proliferation of *X. citri* in the epiphytic phase.

530 Glossy maize mutants (*gl1*, *gl3* and *gl5/gl20*) which produce less epicuticular waxes and

531 have a less hydrophobic surface than wild-type, support greater epiphytic growth of the

532 pathogenic bacteria *Clavibacter michiganensis*, presumably due to the increased leaching

533 of nutrients from mesophill (Marcell and Beattie 2002). According to our results, the

534 mechanical removal of epicuticular waxes of the abaxial surface of young 'Okitsu' leaf

535 become it susceptible to *X. citri* infection, indicating that waxes are involved in the

536 resistance mechanism. In accordance with our results, a high association between

537 epicuticular wax content and resistance to *X. citri* was shown in 'Meiwa' *Fortunella*

538 cultivar (Wang et al. 2011).

539 Over recent years, significant progress has been made to understand the biological

540 role of cuticular waxes in the susceptibility or resistance to fungal infection (Aragón et

541 al. 2017; Batista dos Santos et al. 2019; Hansjakob et al. 2010). However, there is no data

542 about the activity of the *Citrus* leaf cuticular waxes against plant bacterial pathogens.

543 Here, we show that cuticular waxes of both mandarin cultivars have antibacterial activity

544 *in vitro* against *X. citri.* Nevertheless, cuticular waxes from young 'Okitsu' leaf have a

545 greater inhibitor effect than 'Clemenules' in the same developmental stage, which is

546 correlated with its ~~major~~ higher amount of waxes. The most abundant wax components

547 in both cultivars are primary alcohols, whose mix is known as policosanol. Further

548 research is necessary to understand the importance of these compounds as antibacterial

549 agents.

550 In conclusion, we provide evidence for the 'Okitsu' resistance to *X. citri* infection

551 by physiological, biochemical and ultrastructural analysis of its cuticle. The presence of

552 a higher amount of cuticular waxes, particularly epicuticular ones, in the beginning of

553 'Okitsu' leaf development would lead to a less susceptibility to *X. citri* infection in this

554 genotype. Plant cuticle reinforcement in young leaves could be then used as a functional

555 trait to manage foliar bacterial diseases.

556

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558

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567

568

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741

742 **Table 1. Primers of mandarin cuticle-associated genes involved in wax biosynthesis used for qPCR**
743 **analysis**.

744

Gene	Gene product	Arabidopsis accession No.[a]	*C. clementina* accession No.[b]	Primer sequence (5´→ 3
CER1	VLC-aldehyde decarbonylase involved in alkane-forming pathway	At1g02205	Ciclev10019279m	TCACAGTTTCCACCAAATGA CGTAACCACATTCGTGTTCG
CER3 [c]	VLC-acyl-CoA reductase involved in aldehyde and alkane-forming pathway	-	-	CAAGCAGCTCAACATTCCAA ATCGTCAGGCAATCTCATGG
CER4	Fatty acyl-CoA reductase involved in alcohol- forming pathway	At4g33790	Ciclev10028507m	GCTTCCTTGGAGACGTGAAG CGGTAGGCGTAATCCTGAAG
CER6	β-keto acyl-CoA synthase involved in fatty acid elongation	At1g68530	Ciclev10031329m	AGCTCGTAATCTTCTCCGCC TGCAGCCCATACCCGAAAG
CER7	3'-5' exoribonuclease involved in the regulation of total wax loads	At3g60500	Ciclev10015159m	TAGGAGGCCTGAATGCTCAC GCTTCTTCGTGGTGAGTTGG
FAR2[d]	Fatty acyl-CoA reductase involved in alcohol- forming pathway	-	-	GAAAGTCAGTAGAGCAAGCGAAGC TTCCAGTCAATGCTCCCCAC
WIN1	AP2-EREBP-type transcriptional factor that activates cuticular wax biosynthesis by up-regulation of *CER1* and other genes	At1g15360	Ciclev10027305m	GTCATCACCAACGGAGAAGG TGAGGGATGGAGATGGAGAC
H4[e]	Histone H4	-	Ciclev10029640m	AGGCAAGGGATTGGGAAAGG AGAGCGTAAACGACGTCCATC

745 [a]Arabidopsis genome database (http//www.arabidopsis.org). [b]*C. clementina* genome database
746 (http//www.phytozome.net); [c]Matas et al. (2010); [d]Liu et al. (2015); [e]Shiotani et al. (2007).

747 *CER1*, eceriferum 1*; CER3*, eceriferum 3; *CER4*, acyl-CoA reductase; *CER6*, β-ketoacyl- CoA synthase
748 KCS6/ eceriferum 6*; CER7*, eceriferum 7; *FAR2*, acyl-CoA reductase; *WIN1*, wax inducer 1 transcription
749 factor. qPCRs were performed for 40 cycles according to the following conditions: denaturation at 95°C
750 for 15 s, annealing at 57°C for 30 s and extension at 72°C for 40 s. After amplification, melting-curves
751 analysis were performed to exclude artefactual amplifications.

752

753   **Table 2. Cuticular waxes and their composition in 'Clemenules' and 'Okitsu' leaves**

	18-day-old leaves		36-day-old leaves	
	'Okitsu'	'Clemenules'	'Okitsu'	'Clemenules'
Wax coverage (μg cm^{-2})	13.90	3.70 *	18.20	16.40 n.s.[a]

Wax composition (%)

Primary Alcohols				
Hexacosanol (C$_{26}$)	58.55 $\pm$ 0.055	49.38 $\pm$ 0.031	49.34 $\pm$ 0.729	40.10 $\pm$ 0.817
Tetracosanol (C$_{24}$)	23.94 $\pm$ 0.054	32.50 $\pm$ 0.021	21.65 $\pm$ 0.094	27.45 $\pm$ 0.128
Octacosanol (C$_{28}$)	9.36 $\pm$ 0.045	8.51 $\pm$ 0.046	10.86 $\pm$ 0.024	9.72 $\pm$ 0.047
Pentacosanol (C$_{25}$)	1.94 $\pm$ 0.007	2.18 $\pm$ 0.034	2.28 $\pm$ 0.076	2.62 $\pm$ 0.073
Heptacosanol (C$_{27}$)	1.26 $\pm$ 0.010	1.16 $\pm$ 0.050	1.69 $\pm$ 0.056	1.83 $\pm$ 0.078
Docosanol (C$_{22}$)	0.75 $\pm$ 0.000	1.40 $\pm$ 0.520	1.01 $\pm$ 0.087	1.79 $\pm$ 0.178
Triacontanol (C$_{30}$)	0.60 $\pm$ 0.038	0.18 $\pm$ 0.042	3.09 $\pm$ 0.059	1.36 $\pm$ 0.066
Tricosanol (C$_{23}$)	0.11 $\pm$ 0.029	0.24 $\pm$ 0.129	0.17 $\pm$ 0.022	0.36 $\pm$ 0.057
Nonacosanol (C$_{29}$)	0.06 $\pm$ 0.008	0.04 $\pm$ 0.066	0.30 $\pm$ 0.036	0.17 $\pm$ 0.033
Dotriacontanol (C$_{32}$)	0.04 $\pm$ 0.005	0.13 $\pm$ 0.007	3.01 $\pm$ 0.179	1.87 $\pm$ 0.131
Untriacontanol (C$_{31}$)	0.01 $\pm$ 0.000	0.02 $\pm$ 0.047	0.19 $\pm$ 0.024	0.09 $\pm$ 0.035
Tritriacontanol (C$_{33}$)	-	-	0.07 $\pm$ 0.011	0.04 $\pm$ 0.008
Tetracontanol (C$_{34}$)	-	-	0.04 $\pm$ 0.036	0.06 $\pm$ 0.031
Hexatriacontanol (C$_{36}$)	-	-	0.01 $\pm$ 0.005	-
Alkanes				
Untriacontano (C$_{31}$)	0.54 $\pm$ 0.008	1.03 $\pm$ 0.066	2.27 $\pm$ 0.016	4.86 $\pm$ 0.038
Pentacosano (C$_{25}$)	0.52 $\pm$ 0.000	0.52 $\pm$ 0.256	-	0.02 $\pm$ 0.000
Heptacosano (C$_{27}$)	0.35 $\pm$ 0.021	0.57 $\pm$ 0.232	0.23 $\pm$ 0.007	0.44 $\pm$ 0.023
Docosano (C$_{22}$)	0.35 $\pm$ 0.144	0.46 $\pm$ 0.399	-	-
Nonacosano (C$_{29}$)	0.32 $\pm$ 0.014	0.18 $\pm$ 0.114	0.69 $\pm$ 0.034	0.59 $\pm$ 0.037
Tricosano (C$_{23}$)	0.30 $\pm$ 0.003	0.11 $\pm$ 0.010	0.12 $\pm$ 0.000	1.69 $\pm$ 0.000
Octacosano (C$_{28}$)	-	-	0.05 $\pm$ 0.007	0.08 $\pm$ 0.003
Dotriacontano (C$_{32}$)	-	-	0.10 $\pm$ 0.011	0.60 $\pm$ 0.018
Tetratriacontano (C$_{34}$)	-	0.20 $\pm$ 0.025	0.16 $\pm$ 0.015	1.95 $\pm$ 0.051
Tetracosano (C$_{24}$)	-	0.26 $\pm$ 0.111	0.01 $\pm$ 0.000	0.01 $\pm$ 0.000
Fatty acids				
Tetracosanoic acid (C$_{24}$)	0.24 $\pm$ 0.016	0.36 $\pm$ 0.005	0.53 $\pm$ 0.055	0.50 $\pm$ 0.058
Octadecanoic acid (C$_{18}$)	0.10 $\pm$ 0.061	0.13 $\pm$ 0.000	0.68 $\pm$ 0.123	1.68 $\pm$ 0.123
Hexadecanoic acid (C$_{16}$)	0.06 $\pm$ 0.018	0.13 $\pm$ 0.000	0.43 $\pm$ 0.062	1.07 $\pm$ 0.048
Docosanoic acid (C$_{22}$)	0.06 $\pm$ 0.005	-	0.09 $\pm$ 0.010	0.05 $\pm$ 0.006
Hexacosanoic acid (C$_{26}$)	0.11 $\pm$ 0.000	0.05 $\pm$ 0.002	0.26 $\pm$ 0.015	0.20 $\pm$ 0.017

754   Quantification and composition of cuticular waxes per unit of leaf area of both cultivars and in different
755   development stages are expressed as means from three independent experiments. The dataset marked with
756   an asterisk is significantly different as assessed by Student´s *t* test ($P < 0.05$). [a]Not significant.

757

758

759 **CAPTIONS**

760 **Fig. 1. Comparison of stomatal distribution and aperture size between 'Okitsu' and**

761 **'Clemenules' leaves. A,** Phenological stages of young (18-day-old) and mature (36-day-

762 old) 'Okitsu' and 'Clemenules' leaves. **B**, Dried-gel imprint of intact mandarin epidermis

763 showing stomatal distribution in young leaves. ls, large stomata; ss, small stomata. **C**,

764 Quantification of stomatal aperture at 1 and 4 h post-inoculation (hpi) in leaves exposed

765 to *X. citri* infection and abscisic acid (ABA) or water treatments by spraying. Values are

766 expressed as the means ± SD from three independent experiments (n = 50 stomata).

767 Different letters above the bars indicate significant differences at $P < 0.05$ [three-way

768 analysis of variance (ANOVA), Tukey's test].

769

770 **Fig. 2. Cuticle permeability is reduced in young 'Okitsu' leaves compared to**

771 **'Clemenules'.** The permeability of the cuticle was assessed using toluidine blue and the

772 leaves were photographed under white light. Permeability to the dye is visualized in 18-

773 day-old 'Clemenules' epidermal tissue within the stomatal ledges, guard cells of stomata

774 (s) and cell wall junctions (cw) after 30 min of exposure to the dye (blue staining), and in

775 all subepidermal tissue after 6 hours (red staining). Epidermal tissue of 18-day-old of

776 'Okitsu' remained unstained. Control: leaves not treated with the dye. A minimum of 15

777 samples for each leaf age from 3 plants per mandarin cultivar were analyzed. ~~Three~~

778 ~~independent experiments were repeated with similar results.~~ Three independent

779 experiments were conducted yielding similar results.

780

781 **Fig. 3. Epidermal structure in 'Okitsu' and 'Clemenules' leaves under transmission**

782 **electron microscopy.** Cuticle of abaxial epidermis of 18-day-old leaves (**A**) and 36-day-

783 old leaves (**B**). The thickness of the leaf cuticle (cu) and the stomatal cuticular edges are

784   indicated by black arrows, the cuticularization of anticlinal cell walls (cw) and the

785   depressions of cell junctions are indicated by white and grey arrows, respectively. Scale

786   bar: 2 μm. gc, guard cells; n, nucleus; v, vacuole. The Table indicates the measurement

787   of the cuticle thickness in micrometers (μm). A minimum of 6 images ~~was~~ were analyzed

788   per leaf section. Three different leaves, obtained from different plants were observed per

789   genotype and age leaf. Similar results were observed in three separate experiments. The

790   dataset marked with an asterisk is significantly different as assessed by Student´s *t* test (*P*

791   < 0.05). n.s. Not significant.

792

793   **Fig. 4. ~~Differential e~~Expression analysis of genes involved in cuticular wax-**

794   **biosynthesis.** Quantitative ~~reverse transcription~~ polymerase chain reaction analysis of β-

795   ketoacyl- CoA synthase *KCS6/* eceriferum 6 *(CER6),* eceriferum 1 *(CER1),* eceriferum 3

796   *(CER3),* wax inducer 1 transcription factor *(WIN1),* eceriferum 7 *(CER7),* fatty acyl-CoA

797   reductase *(CER4)* and fatty acyl-CoA reductase *(FAR2)* mRNAs, measured at 18-day-old

798   leaves. The relative gene expression (ΔΔCt) fold change was performed considering

799   'Clemenules' leaves as reference samples and a *histone H4* transcript as an endogenous

800   control. Values are expressed as means ± SD from three independent assays. The dataset

801   marked with an asterisk is significantly different as assessed by to Student´s *t* test (*P* <

802   0.05).

803

804   **Fig. 5. Ultrastructural and phenotypic features of young 'Okitsu' leaves inoculated**

805   **with *Xanthomonas citri* after removal of epicuticular wax. A**, Overview of uninfected-

806   leaf tissue showing cuticle (cu) structure of pavement cells, guard cells (gc) and

807   mesophyll tissue before and after mechanical removal of 'Okitsu' epicuticular waxes.

808   Differences in cuticle thickness between leaves treated with cotton swab or gum arabic

809 and untreated leaf (black arrow). Scale bar: 1 μm. **B**, Ultramorphological changes of *X.*

810 *citri*-infected tissues 15 days post-inoculation (dpi). Scale bar: 1 μm. b, bacteria; cw, cell

811 wall; ch, chloroplast; n, nucleus; is, intercellular space; sg, starch granule; v, vacuole. **C**,

812 *In vivo* growth of *X. citri* on treated mandarin leaves, whose epicuticular waxes were

813 extracted, and untreated mandarin leaves. Values are expressed as means ± SD of

814 triplicate measurements from three independent experiments. Bacterial populations in

815 untreated 'Okitsu' leaves significantly differed from those found in 'Clemenules' and

816 treated 'Okitsu' leaves from 3 to 7 dpi [one-way analysis of variance (ANOVA), Tukey's

817 test, $P < 0.05$]. Symptom development induced by *X. citri* strain on lower surfaces of

818 treated and untreated mandarin leaves 20 dpi. c, canker lesions.

819

820 **Fig. 6. Antimicrobial activity of cuticular wax extracts isolated from young 'Okitsu'**

821 **and 'Clemenules' leaves against *Xanthomonas citri*. A**, Growth of *X. citri* over

822 cuticular wax- and control- spots in NYG agar plate. The graph shows the number of

823 bacterial colonies per mandarin cuticular wax spot. **B**, *X. citri* growth in NYG broth

824 supplemented with cuticular wax extract. Values are expressed as means ± SD of three

825 independent experiments. The dataset marked with an asterisk is significantly different

826 as assessed by Student's *t* test ($P < 0.05$).