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Characterization of apple cultivar susceptibility to Neofusicoccum parvum Brazilian strains

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1 **Characterization of apple cultivar susceptibility to *Neofusicoccum parvum* Brazilian strains**

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16

17 **Abstract**

18 Due to climate change, *Neofusicoccum parvum* is currently emerging as a new pathogen of Rosaceae

19 plant species. This increases the need of gaining knowledge on the pathogenicity of this fungus,

20 particularly on apple cultivars of considerable economic importance. In this study, the virulence and

21 temperature dependence of *N. parvum* isolates was assayed on 'Gala' and 'Fuji' apple fruit and wood.

22 At 25°C and 30°C, 'Gala' fruits showed a higher susceptibility to all fungal strains than 'Fuji',

23 whereas low infection severity was shown at 15°C and 20°C on both cultivars. Infrared spectroscopy

24 revealed that 'Fuji' fruit tissues displayed a higher content of phenolic compounds and pectin

25 esterification degree, as factors possibly correlated with fruit susceptibility. When strain virulence

26 was assayed on wood, no significant difference in susceptibility was detected, as also shown by SEM

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27 analysis. The virulence of N. parvum was also tested by assaying the activity of lytic enzymes such
28 as cellulase, polygalacturonase, polymethylgalacturonase, and xylanase.
29
30 **Keywords:** *Botriosphaeriaceae* – *Malus domestica* (L. Borkh.) - Lytic enzymes – SEM observation
31 – IR Spectroscopy
32
33 1. Introduction

34 *Botryosphaeriaceae* (Theiss & P. Syd.) are cosmopolitan fungal species pathogens of a broad range
35 of both annual and perennial plant hosts (Slippers and Wingfield 2007). The taxonomy of
36 *Botryosphaeriaceae* is complex and distinction of members based on morphological aspects is very
37 difficult due to the high similarity among species (e.g. *Botryosphaeria* (Ces. & De Not.)) and
38 *Neofusicoccum* (Crous, Slippers and Phillips) species produce very similar conidia (Phillips et al.
39 2013). For this reason, molecular analyses are often required for species identification (Slippers et al.
40 2013). On the other hand, pathogens belonging to this family can grow fast on fruit crops, causing
41 significant economic losses worldwide (Coakley et al. 1999). Among *Botryosphaeriaceae* species
42 reported to be pathogenic in fruit crops, *Neofusicoccum parvum* is one of the common species, in
43 warmer geographical areas (Úrbez-Torres 2011; Sakalidis et al. 2013). Few years ago, Sakalidis et
44 al. (2013) confirmed the widespread diffusion of *N. parvum* showing its virulence on about 90 plant
45 hosts worldwide. Generally, *N. parvum* is reported as the causal agent of grapevine dieback (Úrbez-
46 Torres 2011; Taylor et al. 2005; Luque et al. 2009; Spagnolo et al. 2014) causing plant losses up to
47 50% (Bertsch et al. 2013). In particular, *N. parvum* symptoms are associated with orange/brown
48 stripes located in the longitudinal direction in the outer xylem of wood plants. These symptoms are
49 often associated with the foliar symptoms (Mugnai et al. 1999) suggesting that the pathogen is capable
50 of producing phytotoxic metabolites which can migrate systemically into the host plant.
51 *Botryosphaeriaceae* can cause severe symptoms also in apple fruit consisting of a soft light-colored
52 rot called “white rot” (Khon and Hendrix 1983). In Brazil, *N. parvum* is recognized as an emerging

53 pathogen for apple crops, raising concerns about protection strategies (Melzer and Berton 1986;
54 Hansen et al. 2007). Nevertheless, its ecological role in plant communities is poorly studied (Slippers
55 et al. 2007).
56 In order to gain knowledge on the infection mechanism of this fungus in apple tree and fruit we set
57 out to study the virulence of five *N. parvum* Brazilian strains at different temperatures and on different
58 cultivars by *in vitro* and *in vivo* assays respectively. Scanning Electron Microscopy (SEM) was
59 performed on apple twigs artificially infected with the pathogen in order to assess the infection
60 timings. The activities of major lytic enzymes were quantified by *in vitro* agar plate assays and
61 infrared spectroscopy analysis of infected apples was performed to detect metabolic profiles likely
62 associated with cultivar susceptibility.
63
64 **Materials and methods**
65
66 **Pathogen**
67 After field observations, *N. parvum* strains were isolated from cankers of internal wood necrosis of
68 'Gala' apple trees, located in different experimental orchards of the State University of Mid-Western
69 of Paraná (Brazil) (Supplemental Fig. 1). Wood necrotic parts were inoculated on Potato Dextrose
70 Agar (PDA, 39 g L^{-1} of distilled water) (Sigma, St. Louis, MO, USA) at 25°C for 5 d. Suddenly, fungi
71 were morphologically selected, purified and grown on Malt Extract Agar plates (MEA, 50 g L^{-1} of
72 distilled water) (Sigma, St. Louis, MO, USA). From each experimental orchard, 3 pure cultures were
73 isolated.
74 The strains NP1, NP2, NP3, NP4, and NP5 were selected for their high virulence on apple fruit and
75 identified by morphological analysis and sequencing of ribosomal DNA ITS regions. PCR was
76 performed using universal primers ITS1 (GCCGTAGGTGAACCTGCGG) and ITS4
77 (GCCTCCGCTT ATTGATATGC) directly from mycelia in pure culture, using the protocol
78 described by Iotti and Zambonelli (2006). PCR reactions were conducted in 50 µL volume reactions

79 containing 10 mM TrisHCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 150 μM of each dNTP, 300 μM
80 of each primer, and 1.5 U of TaKaRa Taq DNA polymerase (Takara, Otsu, Japan). Twenty
81 micrograms of Bovine Serum Albumin (BSA) (Fermentas, Vilnius, Lithuania) were added to the
82 reaction tubes containing the mycelial fragments before adding the other PCR reagents. The
83 amplification reactions were carried out in a T gradient thermal cycler (Biometra, Gottingen,
84 Germany) with an initial denaturation at 95°C for 6 min followed by 30 cycles of 94°C for 30 s, 55
85 °C for 30 s, 72 °C for 1 min and a final step of 72 °C for 10 min. PCR products were run on 1%
86 agarose gels, stained with ethidium bromide and visualized under UV light. The amplified DNA was
87 purified by Nucleospin Extracts II Kit (Macherey–Nagel, Germany) and finally sequenced by
88 Eurofins Genomics (Luxemburg). The ITS sequences were compared to those of the GenBank
89 database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN search.
90
91 **Culture substrates**
92 NP1, NP2, NP3, NP4, and NP5 strains were grown on MEA, Apple Agar (AA, 15 g agar technical
93 and 250 mL of a commercial apple juice per 1000 mL of distilled water), PDA in Petri dishes (90
94 mm) with/without four autoclaved woody sticks (70 mm) (Supplemental Fig. 2) and incubated at
95 25°C with 12-h dark and 12-h light cycles for 8 d, to verify the most suitable condition for conidia
96 production. For each medium, 10 plates (replicates) were considered.
97
98 **Wood and fruit**
99 Hardwood twigs of one year old (50 cm) were collected from 'Fuji' and 'Gala' plants of Bologna
100 University experimental fields located in Cadriano (Bologna, Italy) in February 2018, stored at 5°C
101 and used for experiments within 5 d from collection. 'Fuji' and 'Gala' apples (*Malus domestica* L.
102 Borkh) were obtained from an orchard located in Sala Bolognese (Bologna, Italy). Harvested fruits
103 were stored at 0°C and used for experiments within 5 d after harvest. Fruits were wounded by a sterile
104 nail (3 × 3 × 3 mm) on the equator (one wound per fruit).

105105

106 ***In vivo* experiment: virulence in wood**

107 Onehundredfifty twigs of ‘Gala’ and ‘Fuji’ apples were cut into 30 cm long segments, rinsed with tap
108 water, surface disinfected with 70% alcohol and air dried. Three wounds were made between buds
109 position on each twig using a sterile blade and a mycelium agar plug (4 mm in diameter) from a 8 d-
110 old colony of each strain was placed in the wound and wrapped with sterile cotton wetted by 5 mL of
111 sterile water and covered with Parafilm® (Pechiney Plastic Packaging, USA) (Fig. 1 a). For each
112 apple cultivar, 15 twigs were inoculated with the above mentioned five fungal strains. Negative
113 control was constituted by no inoculated twigs. Twigs were soaked in 200 mL of sterile water in
114 upright position and incubated at room temperature (20°C), with 12-h dark and 12-h light at 60% of
115 relative humidity (RH). The lesions were evaluated 12 d after the inoculation and measured by the
116 software ImageJ 1.x (Wisconsin, USA) (Schneider et al. 2012). Conidia from inoculated twigs of
117 both apple cultivars were collected with 2 mL of distilled water and counted through a microscope
118 (Nikon Eclipse TE2000-E). The experiment was repeated twice.

119119

120 **Scanning Electron Microscope observation in wood**

121 The virulence of *N. parvum* strain NP1 on wood was investigated with SEM observations of
122 inoculated ‘Gala’ and ‘Fuji’ twigs as previously described. The control twigs were not inoculated.
123 The SEM observations were carried out 3, 6, and 12 d after the twigs inoculation. A little portion of
124 tissue (3 × 3 × 3 mm), corresponding to the inoculated wound, was excised from treated twigs and
125 observed under SEM (Zeiss DSM 962 microscope at 30 kV). Three samples from each twig were
126 fixed in a phosphate buffer (pH 7.4) containing glutaraldehyde (3%). Before observation, the fixed
127 tissues were rinsed three times with phosphate buffer (pH 7.4), dried by washing with increasing
128 concentrations of ethanol, stuck on aluminium stubs, and coated with gold palladium (Di Francesco
129 et al. 2017). Nine samples derived from three twigs of the same cv were examined for each sampling

130 time (for a total of 54 samples).

131131

132 ***In vivo* experiment: virulence in apple fruit**

133 Fungal virulence displayed by the isolate NP1 was also tested on apple fruit and the influence of
134 different temperatures was assayed to evaluate the effect on the strain virulence on apples measured
135 as lesion diameter.

136 'Gala' and 'Fuji' fruits were inoculated with *N. parvum* NP1 conidia suspensions (1×10^4 conidia mL^{-}
137 ^{1}). Conidial suspensions were prepared by washing *N. parvum* pycnidia, artificially inoculated on
138 'Gala' and 'Fuji' apple twigs, with sterile distilled water containing 0.05% (v/v) Tween 80, quantified
139 with a hemocytometer and diluted to the required concentration.

140 Each fruit was wounded with a sterile needle and inoculated with 20 µL of a pathogen suspension.
141 The control consisted of inoculated fruit with sterile water. Apples were kept at 15°C, 20°C, 25°C
142 and 30°C at 90–95% of RH. The lesion diameters were measured after 7 d from the inoculation. The
143 experiment was conducted with 15 fruits per temperature and cultivar and was repeated twice (for a
144 total of 480 fruits).

145145

146 ***Neofusicoccum parvum* pathogenesis enzyme activity**

147 Cellulase (endo-1,4-β-glucanase), pectic enzyme (polygalacturonase and polymethylgalacturonase)
148 and xylanase enzyme activities of *N. parvum* were evaluated 3, 9, 24, and 48 h after the fungus
149 inoculation.

150 Assays were performed in Petri dishes containing modified agar medium (Di Francesco et al. 2018).
151 Three mycelium plugs (4 mm) from 8 d-old fungal colony were placed equidistant in the medium.
152 Control plates for each enzyme-pathogen combination were inoculated with MEA plugs without
153 mycelium. After incubation at 25°C, enzymes activities were determined with specific colorimetric
154 methods visualizing a clear zone of substrate degradation around the mycelium plugs. The diameter

155 of degradation halos (mm) was measured with a ruler. For each incubation time for pathogen and
156 control, four plates (replicates) were considered.
157 For cellulase activity, the agar medium consisted in PYE (peptone: 0.5 g; yeast extract: 0.1 g; agar:
158 16 g L^{-1}) supplemented with 0.5% Na-carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO, USA)
159 (Mugnai et al. 1997). The halo produced by endo-1,4-β-glucanase activity of each fungus was
160 visualized by staining plates with a 0.2% Congo red water solution for 15 min, then discolored with
161 NaCl 1 M. Polygalacturonase activity was determined through the Eriksson and Pettersson culture
162 medium (Eriksson and Pettersson 1975) enriched with the sodium salt of polygalacturonic acid
163 (Sigma-Aldrich) from citrus fruit (0.5%) and agarized with low calcium Oxoid No. 1 agar (2%,
164 Oxoid, Basingstoke, UK). The medium was sterilized following the procedure of Ayers et al. (1966)
165 and then the pH was adjusted to 5 and 8 respectively by adding NaOH 1M. Polymethylgalacturonase
166 activity was determined through the above cited culture medium enriched with Sigma citrus pectin
167 (0.5%) and agarized with calcium-rich Oxoid No. 3 agar (2%). The growth media was sterilized
168 following the procedure of Durrands and Cooper (1988) and the pH was adjusted to 8 by adding HCl
169 1 M. The halo produced by pectic enzyme activities was visualized by 1% cetylmethyl ammonium
170 bromide (CTAB) dissolved in distilled water, heated to 30 °C. For the xylanase assay, the medium
171 contained 0.5% beech wood xylan (Sigma-Aldrich) in a minimal medium consisting of $NaNO_3$ 0.3%;
172 KH_2PO_4 0.1%; $MgSO_4$ 0.05%; yeast extract 0.1%; agar 1.2% (St Leger et al. 1997). The clearing halo
173 was visualized by staining with Congo red, then destained with NaCl and measured as previously
174 described. Each assay was repeated twice.
175175

176 **ATR Spectroscopy**

177 Three sterile flasks containing 50 mL of Malt Extract Broth (MEB, 50 g L^{-1} of distilled water) were
178 inoculated each with 15 plugs of *N. parvum* NP1 mycelium 8 d-old, and incubated at 25°C in a rotary
179 shaker (250 rpm) for 8 d. The culture was centrifuged at 5000 ×g for 20 min at 4 °C and the mycelium

180 was washed with distilled water and filtered through two layers of miracloth. Suddenly, ‘Gala’ and
181 ‘Fuji’ fruit slices (70 mm thickness) were inoculated with 1 g of *N. parvum* mycelium.
182 After 3, 9, 24, and 48 h, *N. parvum* mycelium was removed and apple slices were stored at -80°C and
183 suddenly lyophilized (FD-10 Freezing Dryer, Lab kits, H.K.) for 10 d to avoid water spectroscopic
184 interferences. The control consisted in not inoculated apple slices.
185 The IR/ATR spectra of fruits at different times of both cultivars were compared in order to ascertain
186 how *N. parvum* infection affected the chemical composition of fruits. Moreover, the IR/ATR spectra
187 of the pulp of apple fruits displayed increasing differences compared to their control references with
188 the inoculation time, therefore, only the longest inoculation time spectra are discussed.
189 IR spectra were recorded with a Bruker ALPHA series FT-IR spectrophotometer (Bruker, Ettlingen,
190 Germany) equipped with a crystal diamond cell for attenuated total reflectance (ATR). The spectra
191 were collected from 4000 to 400 cm^{-1} and averaged over 100 scans (resolution = 4 cm^{-1}) on three
192 different samples for each inoculation time.
193193

194 Statistical analysis

195 Data were subjected to a one-way analysis of variance (ANOVA). Separation of means was
196 performed using the least significant difference (LDS) test, at $P < 0.05$. Data were reported as mean
197 values ± standard error (SE) of two independent experiments. All analyses were performed with the
198 software Statgraphic Plus Version 2.1 (Statistical Graphics Corp., USA 1996).
199199

200 Results

201201

202 Pathogen

203 The sequence analysis of the ITS rDNA regions identified the five pathogen strains used in this study
204 as *N. parvum* with a sequence homology percentage of 99% with the relative accession number

205 KY053054. The results of molecular characterization were compared with *N. parvum* morphological
206 identification keys, showing a significant match.
207207

208 Conidia production

209 *Neofusicoccum parvum* strains used in our tests, showed the ability to grow on different culture
210 substrates, such as MEA, PDA, and Apple Agar. On these substrates, fungi grew very fast reaching
211 complete colonization of the plate (90 mm) in only 5 d, however they did not produce pycnidia, in
212 any of the different substrate (Table 1), even after 15 d (data not shown). On the other hand, when
213 grown for 12 d on 'Gala' and 'Fuji' apple twigs (Fig. 1 a), all the strains produced pycnidia, but no
214 statistical difference was detected among their concentrations, except for NP1 strain grown on 'Gala'
215 apple twigs (Fig. 1 b and c and Table 1). At this incubation time (12 d), apple twigs showed great
216 lesions only in correspondence of the fungus inoculation point and a large amount of black pycnidia
217 appeared under the Parafilm layer (Fig. 1 a, b). Pycnidia derived from wood lesions contained conidia
218 of an average length of 14 µm, without showing statistically significant differences between the two
219 apple cultivars (data not shown). On the contrary, statistically significant difference was shown
220 between conidia concentration detected on 'Gala' and 'Fuji' twigs (Table 1), with the first cultivar
221 ('Gala') resulting the most suitable substrate for the pathogen strains (3.07×10^4 conidia mL^{-1} on
222 average). The obtained conidia were inoculated on MEA plate and the mycelium growth and
223 morphology were very similar to those of the original *N. parvum* isolates inoculated on twigs. After
224 8 d of incubation in MEA plates, mycelium plugs were placed on artificial wounds of new apple twigs
225 treated as above and similar wood lesions after 12 d were detected containing pycnidia, fully
226 satisfying Koch's postulates, and supporting protocol reproducibility. The *N. parvum* isolates
227 similarly colonized woody tissues of the two tested apple cultivars. After 12 d, blackish-brown
228 discolorations were observed on woody tissues of both apple cultivars twigs (Fig. 1 c). Lesions were
229 equally extended (about 20 mm^2) in both cultivars. No clear and significant pathogenicity/virulence

230 difference in the inoculated twigs was detected among the tested strains, and for this reason, one strain
231 (NP1) for subsequent analyses was chosen.
232232

233 **SEM analysis**

234 Scanning Electron Microscopy (SEM) observations of 'Gala' and 'Fuji' apple twigs inoculated with
235 *N. parvum* NP1, revealed that after 3 d of incubation at 25°C the pathogen started to germinate and
236 colonize the tissue surface (Fig. 2Aa). At 6 d (Fig. 2 Ab) after inoculation the SEM observations
237 displayed a great increase of the mycelium with respect to the 3th day. In addition, the scanning
238 observation at higher magnification (500 ×) emphasized the difference between the healthy and the
239 tissue colonized by the fungus (Fig. 4 **panel)
240 Six days later (Fig. 2 Ac), the pathogen hyphae already severely invaded the twigs tissues (cross
241 section). No differences were observed between 'Gala' and 'Fuji' twigs about the *N. parvum*
242 virulence (Fig. 2B (a, b, c)).
243243

244 **Virulence on apple fruit**

245 After 7 d, the strain NP1 did not show any disease symptoms at 15°C on both apple cultivars, whereas
246 at 30°C the fungal pathogen displayed the highest severity (60 mm and 71 mm for 'Fuji' and 'Gala'
247 respectively) (Fig. 3). Moreover, NP1 displayed a higher virulence in 'Gala' apples with respect to
248 'Fuji' both at 25°C and 30°C. The fungus pathogenicity was limited at 20°C.
249249

250 **Enzymes activity**

251 The pectinase, polygalacturonase, xylanase, and cellulase pathogenesis enzymes activity of *N.*
252 *parvum* strain NP1 was evaluated after 3, 9, 24 and 48 h of the fungal growth. All the examined fungal
253 enzyme activities started to be detectable at 3 h from the inoculation on the substrate, gradually
254 increasing in time. At 24 and 48 h from the inoculation, the fungal strain showed the highest enzymes
255 activity (Fig. 4). Xylanase resulted the highest enzymatic activity with respect to the other, displaying

256 a very intense and large halo areas, already from 3 h of inoculation (200 mm^2). Only cellulase activity
257 appeared to decrease after 48 h from the inoculation. On the other hand, pectinase resulted in the
258 tested fungal time of growth the lowest enzymatic activity with respect to the others CWDEs.
259259

260 **ATR Spectroscopy**

261 IR/ATR spectra gave useful information to gain insight the chemical variation of affected fruits. In
262 particular, the ratio between the peaks areas centered at 1730 and 1610 cm^{-1} was used to monitor the
263 esterification degree of pectins (Supplemental fig. 3). Figure 5 shows the spectrum of 'Gala' slices
264 after 48 h from inoculation (in black) and the spectrum difference between this and the control at the
265 same time (in blue). Positive peaks in the difference spectrum can be correlated to an increased
266 concentration of phytochemical compounds, while a negative peak is related to a decreased
267 concentration. Actually, several peaks related to the esterification degree of pectins turned out to be
268 positive in the difference spectrum: 1737, 1370, 1280, 1231, 1119, 991 and 799 cm^{-1}. More in detail,
269 the peaks at 1737 and 1119 cm^{-1} are typical of acetylated pectins, whose concentration increases in
270 infected 'Gala' apples compared to control. Moreover, other bands attributable to pectin vibrations
271 changed their relative intensities or their wavenumber (Synytsya et al. 2003): 1406, 1343, 1252, 1141,
272 1080 and 964 cm^{-1}, indicating chemical changes into pectin structure induced by the fungal presence.
273 On the other hand, peaks related to phenolic compounds (i.e. flavonoids, lignins and ferulate esters)
274 showed a marked decrease with respect to the control: in particular the bands at 1594 and 1182 cm^{-1}
275 (Heneczowski et al. 2001).
276 'Fuji' apples' spectrum after 48 h from inoculation, showed other variations in the difference
277 spectrum (blue spectrum, Fig. 6), indicating different chemical variations induced by fungal attack.
278 For example, the esterification degree of pectins decreased (Synytsya et al. 2003): 1739, 1274, 1117,
279 989, 929 and 834 cm^{-1} bands showed a negative peak. Some of these peaks indicate that pectin in this
280 cv is mainly methylated: i.e. the 1739 and the 989 cm^{-1} bands are indicative of methyl esterification
281 (Barros et al. 2002). As in 'Gala', other pectin-related bands are affected by inoculation: 1365, 977,

282 918 and 834 cm^{-1}. Conversely, typical peaks related to phenolic compounds increased after fungal
283 inoculation (Bonora et al. 2009): 1578 and 1184 cm^{-1}. Other features typical of the IR/ATR spectra
284 of inoculated 'Fuji' slices are the increase in intensity of bands attributable to hemi-cellulose (1058
285 and 815 cm^{-1}) and those related to free monosaccharides, in particular to glucose moieties (i.e. 1294,
286 885 and 777 cm^{-1}) (Schulz et al. 2007).

287

288 Discussion

289 The increase of temperatures and the wide variety of commercialized apple cultivars (Di Francesco
290 et al. 2019) are contributing factors in the emergence of different fungal pathogens such as
291 *Botriosphaeraceae* spp. in Europe (Carlucci et al. 2009; Spagnolo et al. 2011). Between
292 *Botryosphaeraceae* species, N. parvum and Neofusicoccum luteum resulted the most aggressive
293 (Pérez et al. 2010; Adesemove et al. 2014; Chen et al. 2014; Delgado-Cerrone et al. 2016).
294 Our study aimed to characterize the infection mechanism of *N. parvum* as causal agent of fruit and
295 wood diseases of apple. This fungus is representing a real concern in South America and Europe
296 particularly in Trentino Alto Adige region (Italy), causing severe losses for apple production
297 (Produrotti et al. 2012; Javier-Alva et al. 2009; Molina-Gayosso et al. 2012; Marques et al. 2013).
298 The present study showed that the pathogenicity of the strain NP1 in apple fruit and wood was highly
299 influenced by the temperature (25°C/30°C) and the host chemical composition, especially considering
300 the fruit. In effect, NP1 strain did not show differences of virulence between cultivars in the apple
301 wood colonization. These results can be partially confirmed by Yang et al. (2017), who demonstrated
302 that shoots inoculated with conidia derived from an *in vitro* production method had larger lesions than
303 those inoculated with conidia from *in planta* production. In fact, as Amponsah et al. (2008), we could
304 not produce spores by *in vitro* assays from our *N. parvum* isolates but only through the twig
305 inoculation.
306 Conversely between apple fruit cultivars, where 'Gala' apples showed a higher susceptibility to *N.*
307 *parvum* with respect to "Fuji", displaying lesion diameters 1.5-fold larger. This finding can be related

308 to a different biochemical response to fungal attack as revealed by ATR/IR spectroscopy. In fact,
309 'Fuji' apples showed a higher content of phenolic compounds compared to the control fruits, while
310 'Gala' showed a decrease of the corresponding IR bands. Boyer and Liu (2004) reported a higher
311 total phenolic and flavonoid content in 'Fuji' compared to 'Gala' apples. Several reports highlighted
312 the role of phenolic compounds such as ferulic acid and its derivatives in fruit defense response
313 against fungi attack in different crops (Bonora et al. 2009; Lambert et al. 2012; Srivastava et al. 2013).
314 Moreover, the two apple cultivars showed an opposite trend of the pectins esterification degree that
315 is reported to be affected by the fungal attack (Bonora et al. 2009): usually the esterification degree
316 decreases in response to fungal infection by the action of pectin methyl esterases secreted by fungi
317 (Kikot et al. 2009; Vallette-Collet et al. 2003); in fact de-esterification increases the susceptibility of
318 the cell wall structure by favoring the spreading of the attack (Lionetti et al. 2012). Our results showed
319 that 'Gala' preferentially displayed the typical peaks of acetylated pectins that increase with time after
320 inoculation, therefore a less effective response to fungi, while 'Fuji' showed the typical peaks of
321 methylated pectins. In fact, it is known that the main commercial apple cultivars displayed different
322 susceptibility to pathogens supporting previous data reported by Spotts et al (1999), where 'Fuji' and
323 'Granny Smith' apple were the most resistant varieties to apple fungal pathogens with respect to
324 'Gala' and 'Golden' cvs. Overall, significant differences on susceptibility to *Botriosphaeriaceae*
325 fungi were detected among stone fruit cultivars by Beckman and Reilly (2005) with the cv
326 'Summergold' as the most susceptible and the 'Redskin' the least, but no significant differences in
327 virulence between fungal strains were observed in accordance with our results.
328 Doubtless, *N. parvum* NP1 strain phytopathogenic behaviour is related to the high production of
329 degradative and oxidative enzymes, often simultaneously secreted (Esteves et al. 2014) such as
330 cellulases and xylanases. Fungi associated with wood diseases may be related to different fungal
331 activities such as the production of phytotoxic compounds (Stempien 2017), and enzymes. As the
332 latter, the enzymatic activities could be associated with the fungal behaviour: low extracellular
333 cellulolytic activities are indicative of a symbiotic lifestyle and low polysaccharide degrading activity

334 accompanied by high proteolytic and lipolytic activities may reflect an adaptation to a non-pathogenic
335 lifestyle (Esteves et al. 2014). In our case, *N. parvum* was able to degrade the carbohydrate
336 components of cell walls and exhibit mainly cellulase and xylanase activities starting from 3 h after
337 the inoculation, explaining its high and rapid wood decay capability. Furthermore, results showed
338 that our strain particularly exerted xylanase enzyme activity, known to be responsible for the
339 degradation of polysaccharides by *Phaeoacremonium minimum* (esca disease) (Stempien 2017).
340 These results are confirmed by the increase of IR bands related to free monosaccharides (in particular
341 glucose) that were found in both apple cultivars. Moreover, the increase of hemicellulose bands in
342 'Fuji' spectra could be related to an increased resistance of this cultivar to fungal xylanase activity.
343 On the contrary, pectic and polygalacturonic enzymatic activities, principally involved in the
344 degradation of pectic polymers of fruits and consequence of soft-rot symptoms, were less effective
345 than the above-mentioned cellulase and xylanase enzymes. This fact is probably due to *N. parvum*
346 ability to shift between endophytic and pathogenic phases (Amponsah et al. 2011; Sakadilis et al.
347 2011; Baskarathevan et al. 2012; Ramírez-Suero et al. 2012; Fernandes et al. 2014; Abou-Mansour
348 et al. 2015). Mugnai et al. (1999) also described how some fungal secreted compounds in the wood
349 could be translocated to the leaves and fruits, inducing the typical white rot symptoms. Therefore,
350 more investigations on different apple fruit and wood cultivars are necessary also to better clarify the
351 relationship between the increase of fungal virulence and the climate change to prevent the real risk
352 of new epidemics in several geographical regions.

353353

354 **Ethical standards**

355 This work was not financed by grants. The authors declare no conflict of interest. This article does
356 not contain any work conducted on animal or human participants.

357357

358 **References**

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table

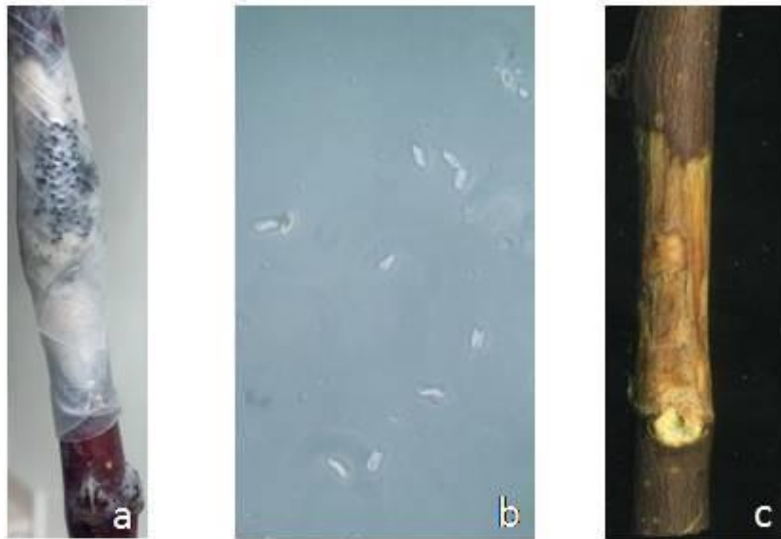
Table 1. Culture substrates used to induce *Neofusicoccum parvum* strains to produce conidia (MEA: Malt Extract Agar, PDA: Potato dextrose agar, AA: Apple Agar, SS: Sterile Sticks, 'Gala' Twigs, 'Fuji' Twigs

	Culture substrate							
Strain	MEA	PDA	AA	MEA+S.S.	PDA+S.S.	AA+S.S.	Gala twigs	Fuji twigs
NP1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.10×10^{4} cell $mL^{-1}\pm$ 6.2bA	1.53×10^{4} cell $mL^{-1}\pm$ 3.3aB
NP2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.07×10^{4} cell $mL^{-1}\pm$ 0.6aA	1.52×10^{4} cell $mL^{-1}\pm$ 5.2aB
NP3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.07×10^{4} cell $mL^{-1}\pm$ 8.9aA	1.47×10^{4} cell $mL^{-1}\pm$ 8.8aB
NP4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.06×10^{4} cell $mL^{-1}\pm$ 9.9aA	1.44×10^{4} cell $mL^{-1}\pm$ 9.0aB
NP5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.07×10^{4} cell $mL^{-1}\pm$ 5.2aA	1.47×10^{4} cell $mL^{-1}\pm$ 8.3aB

n.d.= not detected

Conidia concentration values are the means of 15 repetitions (3 wounds) each per strain. Values within the same column followed by different lowercase letters are significantly different ($P < 0.05$) according to the LSD test. Values within the same row followed by different uppercase letters are significantly different ($P < 0.05$) according to the LSD test.

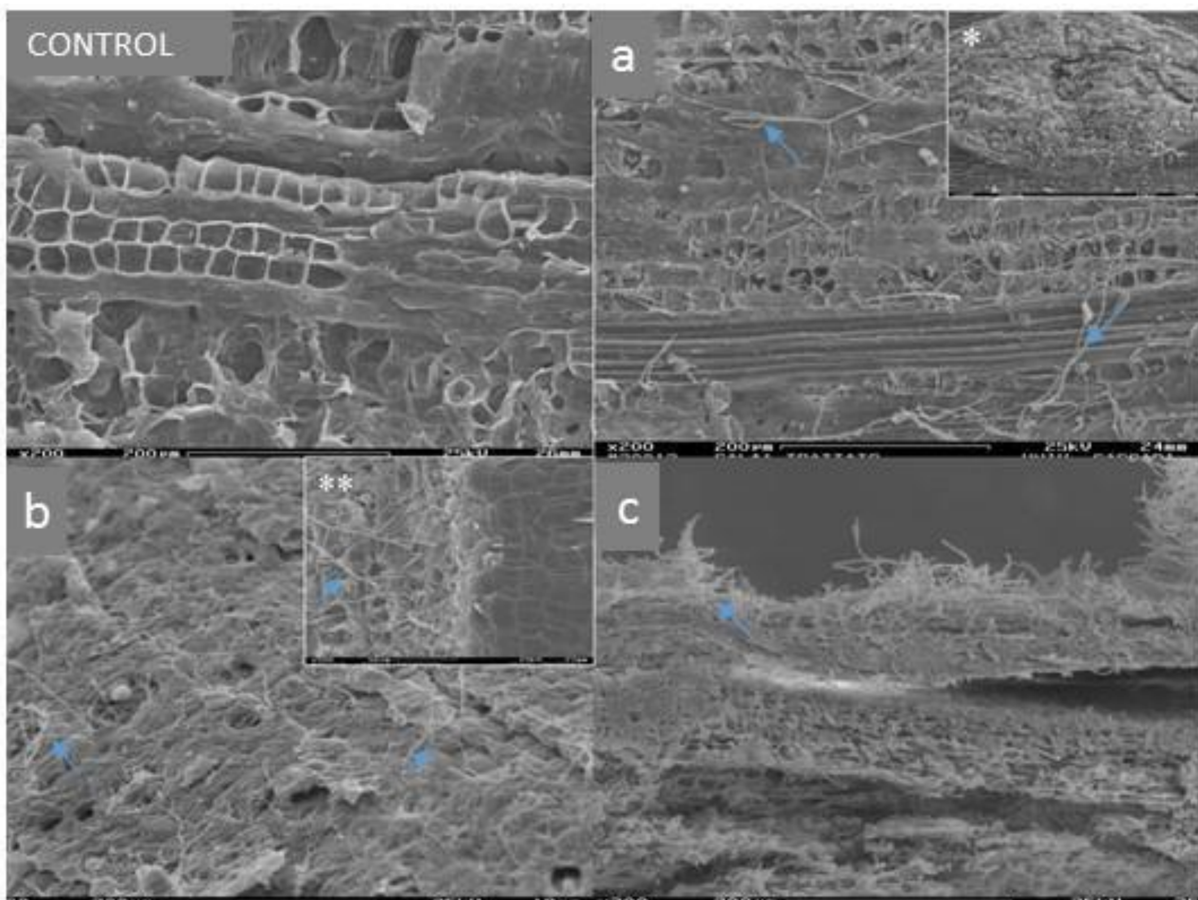
Fig. 1 'Gala' apples twigs were wounded and inoculated with Neofusicoccum parvum (NP1 strain) mycelium agar plug, wrapped with sterile cotton wetted by 5 mL of sterile water and covered with Parafilm. After 12 d from the artificial inoculation are noticeable: pycnidia (a), conidia (14 µm length on average) (b) and symptoms (c).



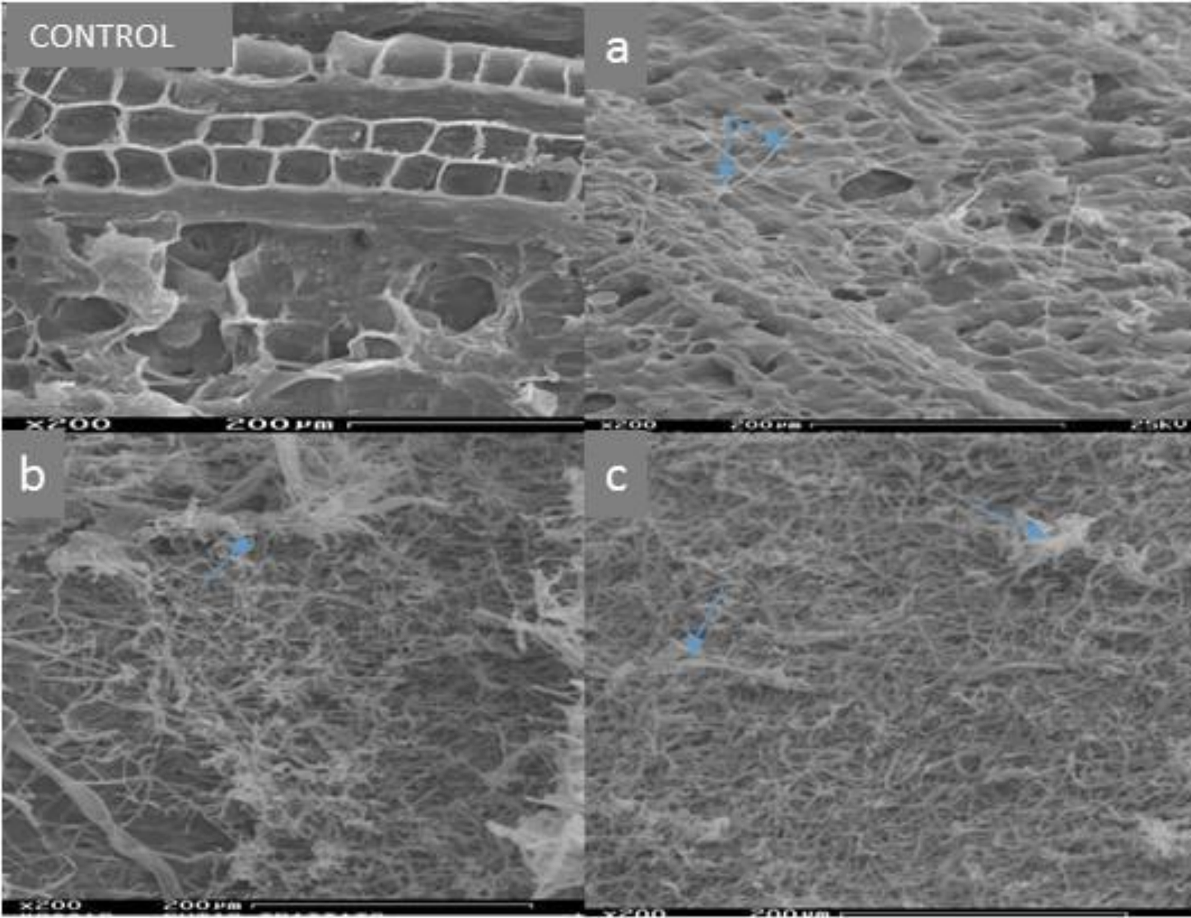
line figure

Fig. 2 Scanning electron micrographs (SEM) of ‘Gala’ (A) and ‘Fuji’ (B) twigs (surface and cross section) inoculated and not (Control) with *Neofusicoccum parvum* NP1 strain at different times from the inoculation (3d (a), 6d (b), 12d (c)). SEM shown, 200 x magnification in all images. *Specifics of the hyphae fungus penetration in twig tissue at 3 d from the artificial inoculation.**Specifics (500 ×) of the hyphae fungus colonization in twig tissue at 6 d from the artificial inoculation. Light blue arrows pointed NP1 mycelium.

A)

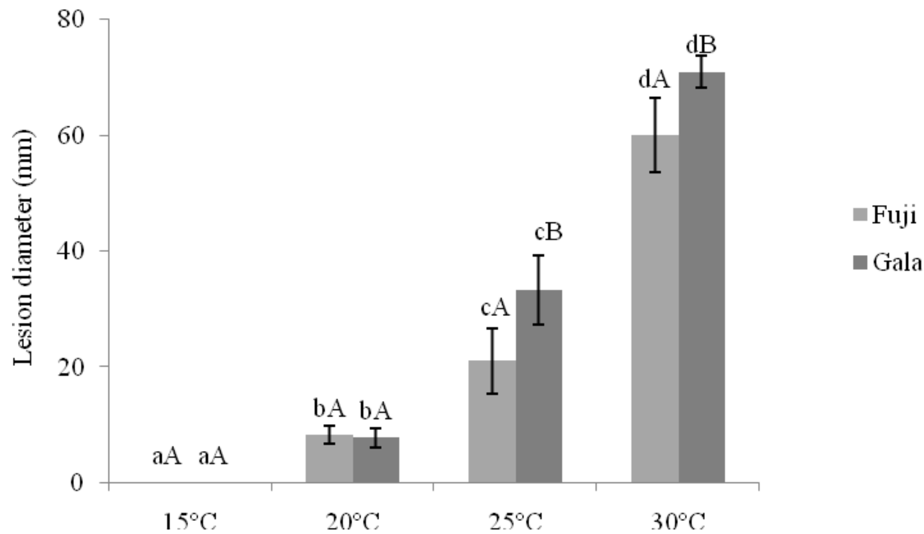


B)


CONTROL
a
b
c
x200
200 µm

line figure

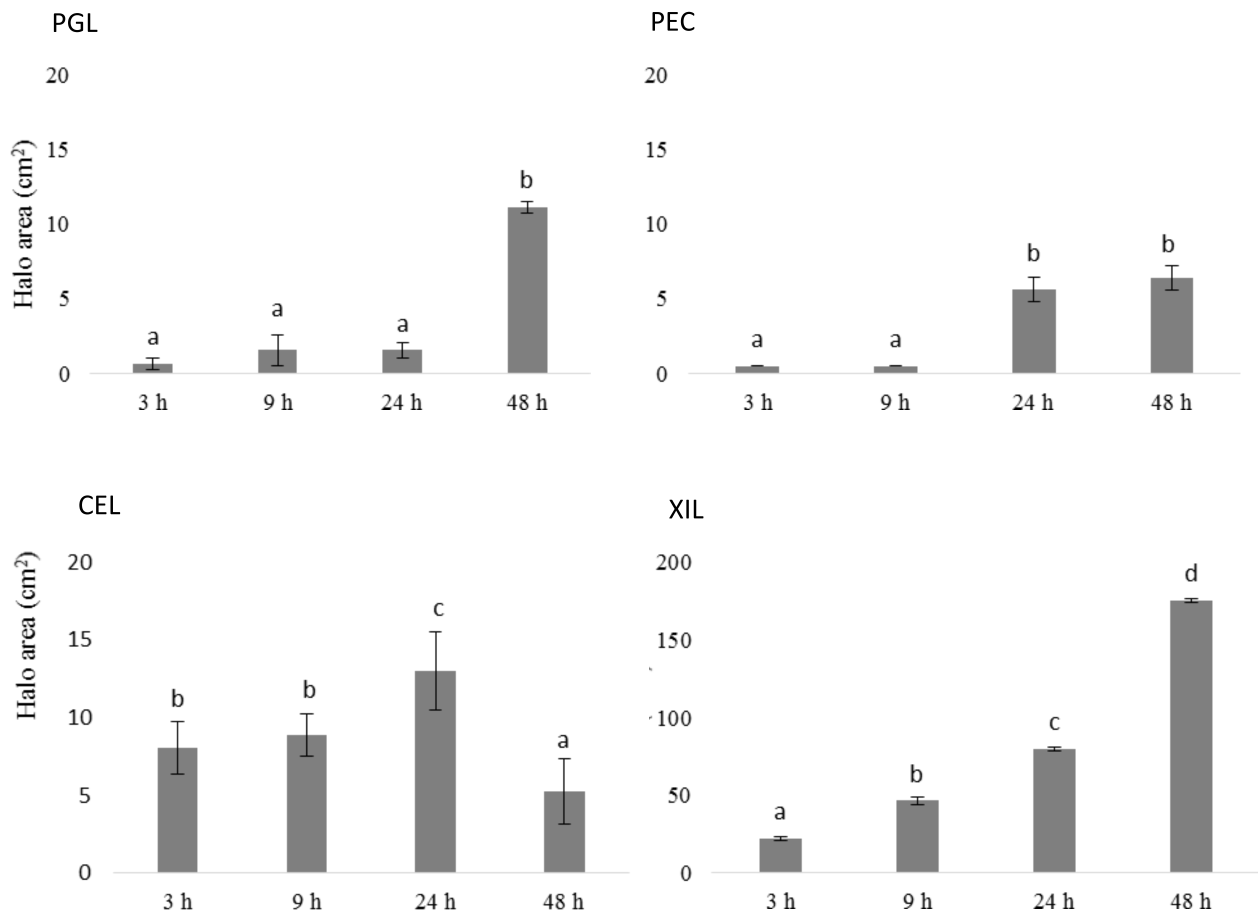
Fig. 3 Lesion diameter (mm) of *Neofusiccoccum parvum* NP1 strain on apple fruit artificia lly inoculated after one week.



Fruit wounds were inoculated with 20 µL of *N. parvum* conidia suspension (10^4 conidia mL^{-1}). Fruits were kept at 15°C, 20°C, 25°C and 30°C for 7 d. Data are the means of fruits for each incubat io n temperature. Lower case: analysis per cultivar - Upper case: analysis between cultivar; different letters indicate significant differences according to LSD test, $P < 0.05$.

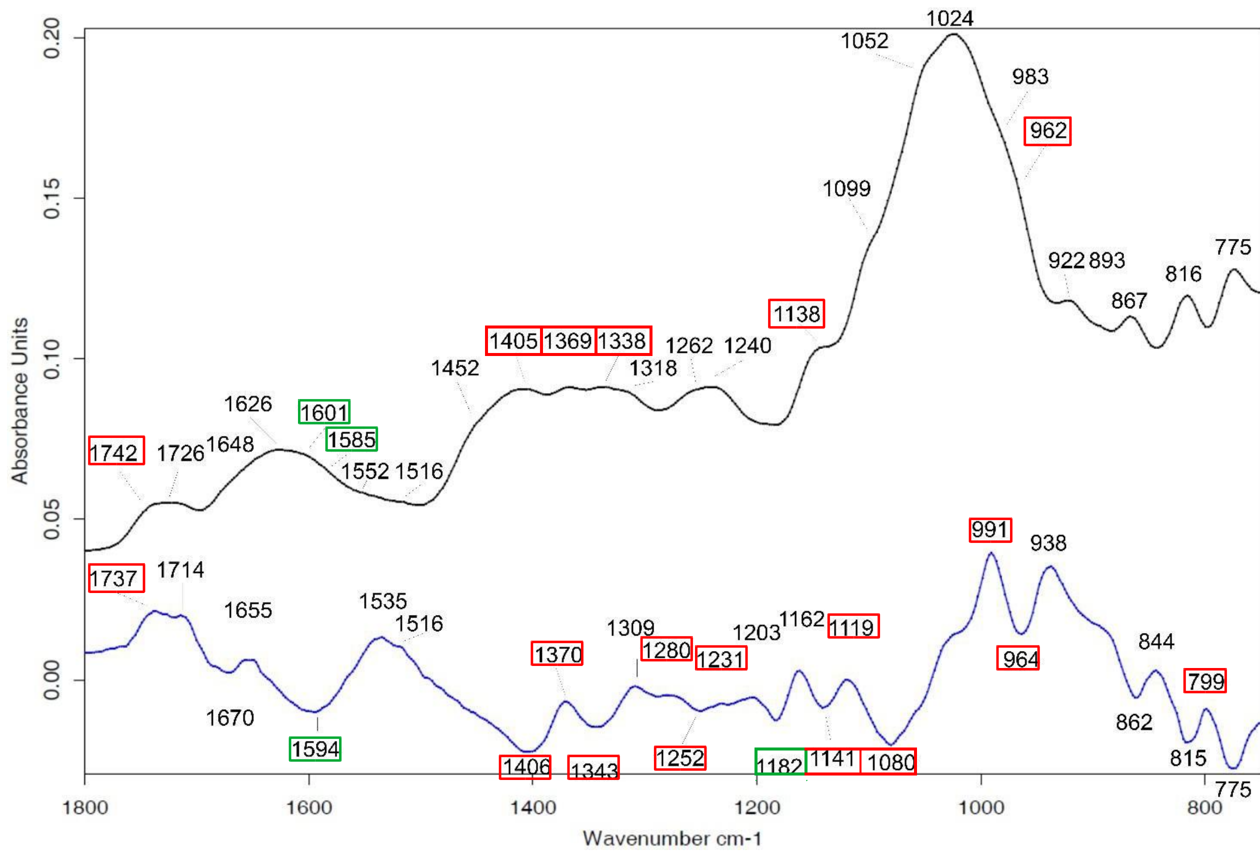
line figure

Fig. 4 Polygalacturonase (PGL), Pectinase (PEC), Cellulase (CEL) and Xylanase (XIL) activity (halo-cm^2) of *Neofusicoccumparvum*NP1, after 3h, 9 h, 24 h and 48 h of incubation at 25°C. Each value is the mean of three replicates ± standard errors. Data were analyzed for significant differences (P < 0.05) by analysis of variance (ANOVA) followed by LSD test. Within the same enzyme the same letters represent no significant differences according to LSD test (P <0.05).



line figure

Fig. 5 Infrared spectrum of 'Gala' apple fruit at 48 h after inoculation (black spectrum). The blue spectrum is the difference between the inoculated sample at 48 h and its control. Red boxes: peaks attributed to pectin; green boxes: peaks attributed to phenolics.



line figure

Fig. 6 Infrared spectrum of 'Fuji' apple fruit at 48 h after inoculation (black spectrum). The blue spectrum is the difference between the inoculated sample at 48 h and its control. Red boxes: peaks attributed to pectin; green boxes: peaks attributed to phenolics; purple boxes: peaks attributed to hemicellulose; black boxes: peaks attributed to glucose.