1 **A. Ros-Barceló, L.V. Gómez-Ros, M.A. Ferrer and J.A. Hernández**

2

3

4 **The apoplastic antioxidant enzymatic system in the wood-forming**

5 **tissues of trees**

6

7

8

9 **A Ros Barceló (✉ ☏)· L.V. Gómez-Ros ·M.A. Ferrer**

10 Department of Plant Biology (Plant Physiology),

11 University of Murcia, E-30100 Murcia, Spain

12 **J.A. Hernández**

13 Department of Plant Breeding and Physiology,

14 CEBAS-CSIC, E-30100 Murcia, Spain

15 ✉ e-mail: rosbarce@um.es

16 ✉ Fax: + 34 968 363 963

17 ☏ Tel.: + 34 968 364 945

18 **Abstract**. The complete apoplastic enzymatic antioxidant system, composed by
19 class I ascorbate peroxidases (class I APXs), class III ascorbate peroxidases (class
20 III APXs), ascorbate oxidases (AAOs), and other class III peroxidases (PRX), of
21 wood-forming tissues has been studied in *Populus alba*, *Citrus aurantium* and
22 *Eucalyptus camaldulensis*. The aim was to ascertain whether these enzymatic
23 systems may regulate directly (in the case of APXs), or indirectly (in the case of
24 AAOs), apoplastic H_2O_2 levels in lignifying tissues, whose capacity to produce and
25 to accumulate H_2O_2 is demonstrated here. Although class I APXs are particularly
26 found in the apoplastic fraction of *P. alba* (poplar), and class III APXs are
27 particularly found in the apoplastic fraction of *C. aurantium* (bitter orange tree), the
28 results showed that the universal presence of AAO in the extracellular cell wall
29 matrix of these woody species provokes the partial or total dysfunction of apoplastic
30 class I and class III APXs, and of the whole plethora of non-enzymatic redox
31 shuttles in which ascorbic acid (ASC) is involved, by the competitive and effective
32 removal of ASC. In fact, the redox state (ASC/ASC+DHA) in IWFs of these woody
33 species was zero, and thus strongly shifted towards DHA (dehydroascorbate), the
34 oxidized product of ASC. This imbalance of the apoplastic antioxidant enzymatic
35 system apparently results in the accumulation of H_2O_2 in the apoplast of secondary
36 wood-forming tissues, as can be experimentally observed. Furthermore, it is
37 hypothesized that since AAO uses O_2 to remove ASC, it could regulate O_2
38 availability in the lignifying xylem and, thorough this mechanism, AAO could also
39 control the activity of NADPH oxidase (the enzyme responsible for H_2O_2 production
40 in lignifying tissues) at substrate level, by controlling the tension of O_2. That is, the
41 presence of AAO in the extracellular cell wall matrix appears to be essential for
42 finely tuning the oxidative performance of secondary wood-forming tissues.

43 **Keywords**: apoplast · ascorbate oxidase · ascorbate peroxidase · ascorbic acid ·
44 H_2O_2 · lignification · peroxidase
45

46 **Introduction**

47 The tree life form imposes several different physiological and morphological
48 constraints compared with those pertaining to herbaceous plants. Many of the
49 processes that distinguish trees from herbs take years to fully develop and express
50 themselves, as it is the case of wood formation. Trees and shrubs form hard, long-
51 lasting structures that are distinct from the soft stems and branches of herbs,
52 especially annuals. The lignocellulosic cell walls of trees and shrubs are critical for
53 their survival, stature, competitive ability and provision of habitat; they have a
54 dramatic influence on ecosystem cycles (Boehle et al. 1996). Trees and shrubs are
55 found intermixed with herbaceous plants in many phylogenetic groups within the
56 angiosperms (Groover 2005), showing that the tree growth habit has been lost or
57 acquired many times during evolution (Boehle et al. 1996). The herbaceous life
58 form is thus considered to be the derived state, evolving numerous times from tree-
59 like ancestors.

60 If there is something that characterises the tree life form it is secondary
61 growth and wood formation (Mellerowicz et al. 2001). Wood formation is initiated
62 in the vascular cambium, where cambial derivatives develop into xylem cells
63 through the process of division, expansion, secondary cell wall formation,
64 lignification and, finally, programmed cell death (Mellerowicz et al. 2001).
65 Lignified cells thus constitute most wood, the importance of lignification being
66 underlined by the fact that lignins account for about 25% of plant biomass (Ros
67 Barceló 1997).

68 Lignin is a complex hydrophobic network of phenylpropanoid units
69 derived from *p*-hydroxycinnamyl alcohols, which are cross-linked by xylem class III
70 peroxidases (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) (Christensen et al. 1998), in
71 a reaction strictly dependent on H_2O_2 (Nose et al. 1995; Ros Barceló and Pomar
72 2001; Pomar et al. 2002a). This need for H_2O_2 before cell wall lignification can start
73 explains why lignifying xylem tissues are universally able to sustain H_2O_2
74 production (Czaninski et al. 1993; Olson and Varner 1993; Schopfer 1994; Ros
75 Barceló 1998a, 1998b and 2005). However, the ability of the lignifying xylem to
76 produce H_2O_2 is not confined to this vascular tissue. Indeed, most plant tissues are
77 able to produce H_2O_2, either constitutively or under stress situations, and the result
78 is not always net H_2O_2 accumulation (Hernández et al. 2001; Mittler et al. 2004).
79 The reason for this last observation is that plant cells also contain a whole battery of
80 enzymatic antioxidant systems capable of removing H_2O_2 from the apoplast
81 (Vanacker et al. 1998; Pignocchi and Foyer 2003). Class I APX (L-ascorbate: H_2O_2
82 oxidoreductase, EC 1.11.1.11) is one of such enzymatic systems, and its substrate,
83 ascorbic acid, is by far the most abundant low-molecular-weight antioxidant in the
84 apoplast (Pignocchi and Foyer 2003).

85 Class I APXs are involved in the removal of H_2O_2 in a reaction strictly
86 dependent on ASC (de Gara 2004). Class I APXs are widely distributed in plant
87 cells, where they are present in the cytosol, chloroplasts, microbodies, mitochondria
88 and the cell wall, in different isoenzymatic forms which, in turn, are diversely
89 regulated (Nakano and Asada 1981; de Gara and Tommasi 1999; Jiménez et al.
90 1998; de Leonardis et al. 2000; Córdoba-Pedregosa et al. 2003; Hernández et al.
91 2004). Class I APXs are different from class III peroxidases in several aspects. The
92 latter are the classical secretory plant peroxidases, and are responsible for cell wall

93 lignification and other cell wall stiffening processes which conclude in the
94 maturation of the cell wall (Passardi et al. 2004). Both types of peroxidase are not
95 easily distinguishable since it is known that some class I APXs are able to oxidize
96 phenolic compounds (Chen and Asada 1989; Jiménez et al. 1998), whereas some
97 class III PRXs (those that should be renamed as class III APXs to avoid confusion)
98 show great specificity for ASC (Vianello et al. 1997; de Gara 2004). Most of the
99 knowledge on this type of APX have been obtained in tea, where a class III APX
100 (TcPOD I) has been purified and characterized (Kvaratshelia et al. 1997). TcPOD I
101 is a glycosylated protein which oxidizes phenols and ASC to the same extent, and is
102 probably an extracellular enzyme secreted through the conventional default pathway
103 (Kvaratshelia et al. 1997). Nevertheless, both types of peroxidases can be
104 distinguished by the use of thiol reagents, such as *p*-chloromercuribenzoate (*p*-
105 CMB), which specifically inhibits class I APXs (Chen and Asada 1989; Jiménez et
106 al. 1998).
107 The developmental regulation of the activity/expression of class I APXs
108 and class III PRXs seems to be different (de Pinto and de Gara 2004; Córdoba-
109 Pedregosa et al. 2004 and 2005). Class I APXs are generally associated with
110 meristematic and actively growing young tissues, whereas class III PRXs are
111 generally expressed in non-growing tissues, where cell wall stiffening processes are
112 emerging. This situation is further complicated by the fact that differentiating
113 xylem cells are able to produce nitric oxide (NO), which is a potent endogenous
114 regulator of the activity of these hemeproteins (Gabaldón et al. 2005, Neil 2005).
115 Both class I APXs and class III PRXs use H_2O_2 as their electron
116 acceptor, and therefore compete for the same substrate when they are present in the
117 same cellular compartment. Class I APXs could be responsible for H_2O_2

118 detoxification, preventing its accumulation in the apoplastic space when it is
119 produced in excess (de Gara 2004). For the efficient removal of H_2O_2 by class I
120 APXs, ASC levels in the apoplastic space should be sustainable. ASC sustainability
121 in cell walls is regulated by different mechanisms: for example, apoplastic ASC
122 pools may be regenerated by a plasma membrane cytochrome *b* type protein
123 (Horemans et al. 2000), through which electrons shuttle from cytoplasmic ASC to
124 apoplastic ascorbic free radical (AFR), the first product of the oxidation of ASC by
125 APXs. On the other hand, ASC and DHA (the product of the dismutation of AFR)
126 may also be transported by specific plasma membrane carriers, which transport ASC
127 from the cytosol to the cell wall, and DHA from the cell wall to the cytosol, where
128 the DHA is promptly reduced to ASC (Horemans et al. 2000).

129 However, to complete the picture of the antioxidant enzymatic system in
130 cell walls, it is necessary to mention the main enzyme which consumes ASC in the
131 apoplastic space, AAO (L-ascorbate: O_2 oxidoreductase, EC 1.10.3.3). This is a cell
132 wall localized glycoprotein belonging to the family of blue copper oxidases (de
133 Tullio et al. 2004), which catalyzes the aerobic oxidation of ASC to AFR which, in
134 turn, rapidly disproportionates to ASC and DHA. Although its biological function in
135 the cell wall is not totally understood (de Tullio et al. 2004), it has been
136 demonstrated that AAO regulates the apoplastic ascorbate pool and therefore the
137 redox state (ASC/ASC+DHA), reducing apoplastic ASC levels to values which, in
138 certain cases, are below the detection threshold (Sanmartin et al. 2003; Pignocchi et
139 al. 2003). That is, the presence of AAO in cell walls seems to provoke the partial or
140 total dysfunction of apoplastic class I and class III APXs, and all the plethora of
141 non-enzymatic redox shuttles in which ASC is involved (Takahama 1993), by the
142 competitive and effective removal of ASC.

143 To date, most of the data concerning apoplastic APXs and AAOs have
144 been obtained from leaves, etiolated (poorly lignifying) hypocotyls, and roots, from
145 several herbs (Vanacker et al. 1998; Hernández et al. 2001; Pignocchi and Foyer
146 2003; de Pinto and de Gara 2004; Liso et al. 2004; Córdoba-Pedregosa et al. 2005),
147 but there are no data available on APX and AAO levels in the apoplast of wood-
148 forming tissues. In this report, we study APX and AAO levels in the apoplast from
149 young branches of trees to ascertain whether these enzymatic systems regulate
150 directly (in the case of APX), or indirectly (in the case of AAO), apoplastic H_2O_2
151 levels in secondary wood-forming tissues. This is especially important to describe
152 the oxidative performance of secondary wood-forming lignifying xylem cells, since
153 the presence of APXs in the lignifying xylem would keep H_2O_2 levels to minimum
154 values, while the presence of AAOs in the lignifying xylem would protect H_2O_2
155 levels, since it is known (Sanmartin et al. 2003; Pignocchi et al. 2003) that the
156 apoplastic ASC pool is partially or totally removed by the presence of this enzyme.
157 For this task, we chose as a model tree, poplar (*Populus alba*), since this is one of
158 the few trees that may be genetically transformed and regenerated (Brunner et al.
159 2004). Studies were extended to two other trees (*Citrus aurantium* and *Eucalyptus*
160 *camaldulensis*), and to an annual herb (*Zinnia elegans*).
161

162 **Materials and methods**

163 *Plant material*

164 Young branches of *Populus alba* (poplar), *Eucalyptus camaldulensis* (eucalyptus)
165 and *Citrus aurantium* (bitter orange tree) were harvested in April and May from
166 trees at least 10 years old which were growing in the University Campus. Seedlings
167 of *Zinnia elegans* L (cv. Envy) were grown for 30 days in a greenhouse under

168 daylight conditions at 25 °C on Humus King™ (type 3) (Impra S.L., El Ejido,
169 Almería, Spain) containing 30 % organic C, 0.5 % organic N, and 52 % total
170 organic material, pH 5.5-6.0. Fertilizers present in the humus were 120-160 mg/L N,
171 100-130 mg/L P_2O_5 and 150-200 mg/L K_2O.

172

173 *Histochemical stains for monitoring lignins and H_2O_2 localization/production*

174 Lignins were detected using the Wiesner test by soaking 0.5 mm-thick sections in
175 1.0 (w/v) phloroglucinol in 25:75 (v/v) HCl-ethanol for 10 to 15 min (Pomar et al.
176 2002b). H_2O_2 localization/accumulation was monitored by staining 250-500 μm
177 thick sections with the KI/starch reagent, composed of 4 % (w/v) starch and 0.10 M
178 KI (Olson and Varner, 1993; Ros Barceló 1998a), adjusted to pH 5.0 with KOH.
179 Areas of H_2O_2 localization/accumulation were monitored by observing the
180 development of a dark stain on the cut surface over a period of 1 to 10 hr. Controls
181 were performed in the presence of 200 U/ml catalase.

182 For monitoring H_2O_2 production we used the 3,5,3',5'-
183 tetramethylbenzidine (TMB) endogenous peroxidase-dependent method (Ros
184 Barceló 1998b). For this, sections were directly incubated for 10 min at 25 °C in a
185 staining solution composed of 0.1 mg/ml TMB-HCl in 50 mM Tris-acetate buffer
186 (pH 5.0). Controls were performed in the presence of 0.1 mM ferulic acid (Ros
187 Barceló et al. 2000), a competitive inhibitor of peroxidase, whose oxidation is
188 strictly dependent on H_2O_2.

189

190 *Isolation of intercellular washing fluids (IWFs)*

191 To obtain IWFs, 5 mm-thick sections were washed three times with deionized water,
192 and subsequently vacuum-infiltrated for 10 periods of 30 s at 1.0 kPa and 4 ºC with

193 50 mM Na-acetate buffer (pH 5.0) containing 1 M KCl, 50 mM $CaCl_2$ and 1 mM
194 ASC. Later, the sections were quickly dried and subsequently centrifuged in a 25-ml
195 syringe barrel placed within a centrifuge tube at 900 g for 5 min at 4 ºC. IWF
196 samples were desalted and concentrated using the Centricon 10^{TM} system (Amicon
197 Inc., Beverly, MA, USA).

198 Contamination by cytoplasmic constituents, as monitored by the activity
199 of glucose-6-phosphate dehydrogenase (Ros Barceló 1998a), was always less than
200 0.1 % with regard to that found in the cytosolic fraction. Further confirmation of the
201 absence of noticeable symplastic contamination in this apoplastic fraction was
202 obtained by protein fingerprint analysis. SDS-PAGE analyses of the major proteins
203 in the symplastic fractions showed the presence of specific proteins, which were
204 almost totally absent from the respective apoplastic fractions (López-Serrano et al.
205 2004). Using the same method, the recovery of IWFs was extremely high since
206 apoplast specific proteins were absent from symplastic (IWF-extracted tissue)
207 fractions (López Serrano et al. 2004).

208

209 *Determination of the (ASC/ASC+DHA) ratio in IWFs*

210 To obtain IWFs for ASC/DHA analysis, sections were vacuum-infiltrated for 10
211 periods of 30 s at 1.0 kPa and 4 ºC with 2% metaphosphoric acid (w/v). The rest of
212 the protocol was as described above for the extraction of enzymes. ASC and DHA
213 contents in the IWFs were directly determined by HPLC according to Hernández et
214 al. (2001).

215

216 *Determination of peroxidase, ascorbate peroxidase and ascorbate oxidase activies*
217 *in IWFs*

218 PRX activities were determined in IWFs in assay media containing 50 mM Na-

219 acetate buffer (pH 5.0) and 500 µM H_2O_2, using as electron donors 1.0 mM 4-
220 methoxy-α-naphthol (ε_{595} = 21,600 $M^{-1}cm^{-1}$), 0.1 mM coniferyl alcohol (ε_{262} = 9,600
221 M^{-1} cm^{-1}) or 320 µM TMB-HCl (ε_{652} = 39,000 M^{-1} cm^{-1}). Controls were carried out
222 in the absence of H_2O_2.
223 APX activities were measured in a reaction medium containing 50 mM
224 K-phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 0.5 mM ASC (Amako et al. 1994),
225 by monitoring the decrease in absorbance at 290 nm (ε_{290} = 2.8 mM^{-1} cm^{-1}). Class I
226 APX activity was distinguished from class III APX activity by pre-incubation of
227 plant extracts for 10 min with 50 µM of Na-*p*CMB, a specific suicide inhibitor of
228 class I APXs (Amako et al. 1994).
229 AAO activities were determined (Moser and Kanellis 1994) in a reaction
230 medium containing 35 mM K-phosphate buffer (pH 5.3), 0.002% (w/v)
231 metaphosphoric acid, and 0.15 mM ASC, by monitoring the changes in absorbance
232 at 265 nm (ε_{265} = 9246 M^{-1} cm^{-1}). Controls were performed in the presence of 0.1
233 mM sodium azide.
234
235 *Extraction of symplastic enzymes*
236 After removal the IWFs, sections were frozen in liquid nitrogen, and homogenized
237 in 100 mM K-phosphate buffer (pH 7.8), containing 2% polyvinylpolypyrrolidone,
238 0.5% Triton X-100, and 5 mM ASC (Polle et al. 1996). The homogenate was
239 centrifuged at 48000 g for 20 min, and the supernatants were desalted using a
240 Sephadex G-25 (PD-10 columns, Pharmacia, Germany) column equilibrated with 50
241 mM K-phosphate buffer (pH 7.0), containing 1 mM ASC.
242
243 *Isolation of cell walls and assay for ASC oxidation*

244 Cell walls were prepared and purified through a Triton X-100 washing procedure, as
245 described by Ros Barceló (1998b). To monitor ASC oxidation by isolated cell walls,
246 an amount of purified cell walls, equivalent to 1.0 g FW, was incubated with stirring
247 for 1 h at 25°C in an assay medium (10.0 mL) containing 35 mM K-phosphate
248 buffer (pH 5.3), 0.002% (w/v) metaphosphoric acid, and 0.15 mM ASC. Controls
249 were performed in the presence of 0.1 mM sodium azide, in the presence of a heat
250 (100°C)-denatured cell wall fraction, and in the presence of catalase (200 U/mL).
251 After this time, the cell walls were centrifuged at 1100 g for 5 min at 5ºC, the
252 amount of ASC being determined by the decrease in absorbance at 290 nm (ε_{290} =
253 2.8 mM^{-1} cm^{-1}).
254
255 *Isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE) and staining*
256 *of enzymatic activities*
257 IEF and staining of peroxidase isoenzymes with 4-methoxy-α-naphthol was
258 performed as described (Ferrer et al. 1990). Controls were carried out either in the
259 absence of H_2O_2, or in the presence of 0.5 mM Na-*p*-CMB, an inhibitor of class I
260 APXs (Chen and Asada, 1989). In the last case, a 20 min pre-incubation of the gels
261 in the presence of the inhibitor was performed before peroxidase staining.
262 Proteins were subjected to discontinuous PAGE under non-denaturing
263 and non-reducing conditions essentially as described (Pomar et al. 2002a), except
264 that the gels were supported with 10% (w/v) glycerol (Mittler and Zilinskas 1993).
265 In the case of APX and AAO activities, polyacrylamide gels were pre-run for 30
266 min in the carrier buffer containing 2 mM ASC, prior to the application of the
267 protein samples (Mittler and Zilinskas 1993).
268 Staining for APX activity was performed in successive steps. First, gels

269 were pre-equilibrated for 30 min the gels in 50 mM Na-posphate buffer (pH 7.0),
270 containing 2 mM ASC. Then, the gels were incubated for 20 min in 50 mM Na-
271 phosphate buffer (pH 7.0), containing 4 mM ASC and 2 mM H_2O_2. Finally, the gels
272 were washed with buffer for 1 min, and stained with 50 mM Na-phosphate buffer
273 (pH 7.8), containing 28 mM N,N,N',N'-tetramethylethylenediamine (TEMED) and
274 2.45 mM nitrotetrazolium blue chloride (NBT). APX activities were observed as
275 achromatic bands on a purple-blue background. Controls for class I APXs were
276 performed in the presence of 0.5 mM Na-*p*-CMB.

277 Staining for AAO activity was performed as reported for APX activities,
278 but using 0.1 M Na-phosphate buffer (pH 5.3), containing 4 mM ASC, and in the
279 absence of H_2O_2. After washing, the gels were stained in the same staining solution
280 described above containing TEMED and NBT. AAO activities were observed as
281 achromatic bands on a purple-blue background. Controls were performed in the
282 presence of 1 mM sodium azide.

283

284 *Chemicals*

285 TMB-HCl, KI, ferulic acid, horseradish peroxidase C, *Cucurbita sp.* AAO, NBT,
286 TEMED, and catalase (from bovine liver, EC 1.11.1.6) were purchased from Sigma
287 (Madrid, Spain). Ascorbic acid and sodium azide were from Merck (Barcelona,
288 Spain). The other chemicals were of the highest purity available.

289

290 **Results**

291 *Histochemical localization of H_2O_2 production/accumulation in the secondary*
292 *xylem*

293 Re-initiation of sustainable secondary growth is one of the characteristic of trees
294 during spring, when the axillary buds elongate and develop into lateral branches.

295 Secondary growth of young branches during spring is easily monitored in poplar and
296 bitter orange tree by light microscopy. In these cases, secondary growth arises from
297 the periclinal (tangential plane) division of elongated fusiform cambial initial cells,
298 which generate the wood elements, vessels, parenchyma cells and fibres, on the
299 inner side, and phloem cells (sieve tubes, parenchyma cells and fibres) on the outer
300 side.

301 During spring, the xylem and phloem from young branches of poplar
302 begin to lignify (Fig. 1a). Such tissue specificity in the case of lignification is also
303 showed for H_2O_2 production, since cytochemical probes with the TMB reagent
304 revealed that only lignifying tissues show a strong stain reaction (Fig. 1b).
305 Nevertheless, H_2O_2 is only accumulated in the secondary xylem, as is revealed by
306 the KI-starch reagent (Fig. 1c) and not in the phloem. Controls in the presence of
307 ferulic acid (Fig. 1d) support the peroxidase/H_2O_2-dependent nature of the staining
308 reaction described in Figure 1b.

309 Similar patterns for H_2O_2 production were also found in bitter orange tree
310 (Figs. 2a-d) and eucalyptus (Figs. 2e-g), where H_2O_2 production was restricted to a
311 few cell layers of the differentiating (lignifying) secondary xylem, as was the case in
312 bitter orange tree (Fig. 2b, arrowheads), or to a large number of cells layers of the
313 differentiating (lignifying) secondary xylem, as was the case in eucalyptus (Fig. 2f).
314 In bitter orange tree, the pattern found for H_2O_2 production (Fig. 2b) was similar to
315 that obtained for H_2O_2 accumulation (Fig. 2c). Interestingly, no H_2O_2 production
316 was observed in the phloem of bitter orange tree or eucalyptus, despite the lignifying
317 nature of these vascular tissues (Figs. 2a,e). Controls in the presence of ferulic acid
318 (Fig. 2d,g) lent weight to the peroxidase/H_2O_2-dependent nature of the staining
319 reaction described in Figures 2 b,f.

320 Since the TMB reagent is able to reveal H_2O_2-production at short times
321 (Ros Barceló 1998b and 2005), while the KI-starch reagent is able to reveal H_2O_2
322 accumulation at longer times (Olson and Varner 1993, Ros Barceló 2005), it seems
323 that H_2O_2 is not only produced by the secondary xylem (Figs. 1b, 2b and 2f), but is
324 also accumulated in the secondary xylem (Figs. 1c, 2c), at the very same time and
325 place that lignification is beginning.
326
327 *Nature of the peroxidase isoenzyme complement in the secondary xylem*
328 The results shown in Figures 1b, 2b and 2f, obtained with the TMB-endogenous
329 peroxidase dependent probe, revealed that H_2O_2 is produced by the secondary xylem
330 of trees. It has been shown (Ros Barceló 1998b) that for this staining to be
331 successful, peroxidase must be localized in the same secondary tissues. To confirm
332 this tissue distribution of peroxidase, exogenous H_2O_2 (0.1 mM) was added to the
333 TMB staining medium. However, addition of H_2O_2 does not reveal further staining
334 in other tissues, the only difference being that the reaction of the secondary xylem
335 was faster and, with time, stronger. These results clearly indicate that peroxidase is
336 only localized in H_2O_2-producing vascular tissues. For this reason, the nature of
337 peroxidase activity and the nature of the peroxidase isoenzyme complement were
338 studied in IWFs from these species, the research being extended to the herb, *Zinnia*.
339 Results show that the IWFs of all these species contained PRX activities
340 capable of oxidizing 4-methoxy-α-naphthol, TMB and coniferyl alcohol (Table 1),
341 the last a substrate for lignin biosynthesis. In the case of citrus orange tree,
342 eucalyptus and *Zinnia*, oxidation rates of 4-methoxy-α-naphthol and coniferyl
343 alcohol by IWFs were measured both in the absence and in the presence of
344 exogenous H_2O_2 (Table 1), the rates in its absence never exceeding 10 % of the rates

345 measured in its presence. However, in the case of poplar, the IWFs show an
346 appreciable substrate oxidation rate in the absence of H_2O_2, particularly when
347 assayed with 4-methoxy-α-naphthol (Table 1), suggesting that an oxidase activity
348 independent of H_2O_2 is also present in this species.

349 This observation was confirmed by IEF analysis of the peroxidase
350 isoenzymes present in this apoplastic fraction. In fact, IEF analyses of IWFs from
351 poplar revealed the presence of a protein with oxidase activity, whose activity was
352 not stimulated by H_2O_2 (Fig. 3a, lanes 1 and 2, white arrow). Such enzymatic
353 activities have been described in poplar previously (Ranocha et al. 1999), but were
354 not found in IWFs of bitter orange tree (Fig. 3b), eucalyptus (Fig. 3c) or *Zinnia* (Fig.
355 3d), species in which all the bands showing phenol-oxidizing activity were most
356 intense in the presence of H_2O_2 (lanes 1 and 2). This was particularly the case with
357 the most basic PRX isoenzymes (Fig. 3, arrowheads). In poplar, IWFs also showed
358 peroxidase isoenzymes, whose activity was inhibited by the inhibitor of class I
359 peroxidases, *p*CMB (Fig. 3a, lanes 2 and 3, arrows). This result clearly demonstrates
360 the presence of class I peroxidases in the IWFs of this species.

361

362 *Determination of APXs in IWFs*

363 The presence of class I peroxidases in IWFs from poplar was confirmed by
364 measuring *p*CMB-sensitive APX activities, since ASC is the prototype natural
365 substrate of these enzymes. The results (Table 2) showed that APX activities
366 measured in IWFs from poplar were inhibited by *p*CMB. In bitter orange tree IWFs,
367 APX activities were not inhibited by *p*CMB, whereas both eucalyptus and *Zinnia*
368 showed no APX activity in IWFs. For comparative purposes, Table 2 also shows
369 APX activities in the symplastic fraction of these species, in which most of

370 symplastic APX activities were sensitive to *p*CMB. These results suggest that IWFs
371 from poplar contained class I (*p*CMB-sensitive) APXs, while IWFs from bitter
372 orange tree contained class III (*p*CMB-insensitive) APXs. The IWFs from
373 eucalyptus and *Zinnia* did not contain either type of APX. These results also
374 illustrate the variability found in the apoplastic antioxidant enzyme pattern between
375 woody species.
376 The presence of class I APXs in IWFs from poplar was confirmed by
377 anionic PAGE, which showed the presence of one highly mobile APX isoform (Fig.
378 4a, lane 1, arrow), whose activity was inhibited by *p*CMB (Fig 4a, lane 2, arrow).
379 By anionic PAGE, this *p*CMB-sensitive APX isoform showed the same mobility as
380 a peroxidase isoform stained with 4-methoxy-α-naphthol (Fig. 4a, lane 3, arrow),
381 supporting the results previously obtained by IEF (Fig. 3a), which showed the
382 existence in poplar of 4-methoxy-α-naphthol peroxidases sensitive to *p*CMB. In the
383 case of bitter orange tree, the only APX isoform detected (Fig. 4b, lanes 1 and 2,
384 arrow) was not sensitive to *p*CMB (Fig. 4b, lanes 3 and 4, arrow), supporting the
385 previous observation that only class III APX activities are presents in the IWFs of
386 this species (Table 2). This is not surprising since the prototype class III peroxidase,
387 horseradish peroxidase C, also shows APX activity after migration by anionic
388 PAGE (Fig. 4c, lanes 1 and 2). In the case of eucalyptus (Fig. 4d) and *Zinnia* (Fig
389 4e), no APX (either class I or class III) activities were detected by anionic PAGE.
390
391 *Determination of AAOs in IWFs*
392 As in the case of APX activities, AAO activities were detected in the apoplastic
393 fraction of poplar and bitter orange tree, but not in eucalyptus or *Zinnia* (Table 3). In
394 the species where AAO was measured, levels of AAO activity were of the same

395 order as found for APX activities (Table 2), suggesting that both enzymatic
396 activities contribute equally to the apoplastic enzymatic antioxidant system. In the
397 case of eucalyptus and *Zinnia*, although it was not possible to determine AAO
398 activities in the IWF fraction, cell walls isolated from both species were able to
399 aerobically oxidize ASC. In fact, amounts of cell walls equivalent to 1 g FW were
400 able to oxidize 15 μmol ASC in less than 1 h in the presence of catalase. This
401 oxidation was exclusively due to AAO activities since the addition of H_2O_2, in the
402 absence of catalase, does not stimulate the ASC oxidation rate
403 Analyses by anionic PAGE of AAO isoforms in the IWFs from poplar
404 showed three azide-sensitive achromatic bands, one of low mobility and two of high
405 mobility (Fig. 5a, lanes 1 and 2, arrows). In bitter orange tree, three azide-sensitive
406 achromatic bands of medium mobility were observed (Fig. 5b, lanes 1 and 2,
407 arrows). The low mobility of the azide-sensitive achromatic band in poplar is not
408 surprising, since AAO from *Cucurbita* spp shows a similar low mobility by anionic
409 PAGE (Fig. 5a and 5b, lanes 3). Again, no AAO activity was detected by anionic
410 PAGE in IWFs from eucalyptus (Fig. 5c) or *Zinnia* (Fig 5d).
411
412 *Determination of the ascorbic acid redox state (ASC/ASC+DHA) in IWFs*
413 Analysis by HPLC of ascorbate species (ASC and DHA) in IWFs from poplar, bitter
414 orange tree, eucalyptus and *Zinnia* showed that ASC levels were, in all the cases,
415 below the threshold for detection, and only DHA could be detected. The redox state
416 (ASC/ASC+DHA) was thus zero, and therefore strongly shifted towards DHA, the
417 oxidized product of ASC.
418
419 **Discussion**

420 The secondary xylem of poplar, bitter orange tree and eucalyptus are not only able
421 to produce H_2O_2, but also to accumulate during the phase of active lignification
422 which follows the re-initiation of secondary growth during spring it (Figs. 1 and 2).
423 Such H_2O_2 production and accumulation has previously been observed in *Zinnia*
424 (Ros Barceló 1998a, 2005), where evidence suggests that H_2O_2 is produced by an
425 NADPH oxidase-like enzyme, broadly similar to the phagocytic NADPH oxidase of
426 mammalian cells (Ros Barceló 1998a). The evidence obtained in *Zinnia* also
427 suggests (Ros Barceló 2005) that this H_2O_2 is produced on the outer-face of the
428 plasma membrane of both differentiating thin-walled xylem cells and non-lignifying
429 xylem parenchyma cells, from where it diffuses, through the continuous cell wall
430 space, to differentiated lignifying xylem vessels**.**
431 However, there is a gap in our knowledge of why lignifying xylem
432 tissues are able to accumulate H_2O_2, since some plant tissues, which are able to
433 produce H_2O_2 constitutively, do not shown net H_2O_2 accumulation (Hernández et al.
434 2001; Mittler et al. 2004). The results obtained here suggest that the reason for such
435 H_2O_2 accumulation is an imbalance of the apoplastic antioxidant enzymatic system
436 of the secondary xylem provoked by the presence of AAO, which triggers the
437 apoplastic redox state (ASC/ASC+DHA) until zero or nearly zero values.
438 It is now accepted (de Gara 2004) the presence in the apoplast of three
439 H_2O_2 detoxifying systems which use ASC as a source of reducing equivalents: class
440 I APXs, class III APXs, and class III peroxidases, which are unable to oxidize ASC
441 directly, but which use a redox phenol shuttle to carry out this task. The use of ASC
442 as reducing substrate for removing H_2O_2 by all these enzymatic systems offers
443 several advantages for the plant cell, since the ASC oxidation does not produce toxic
444 or reactive molecules. In fact, AFR, the first product of ASC oxidation by these

445 enzymes, shows insignificant reactivity with O_2 (de Gara 2004), unlike phenolic
446 radicals (Pomar et al. 2002a), and, therefore, does not trigger O_2^- production, thus
447 avoiding the emergence of a possible $O_2^-/H_2O_2/OH^\bullet$ cascade in the apoplast (Mittler
448 et al. 2004; de Gara 2004). Moreover, both AFR and DHA, the final product of ASC
449 oxidation in the apoplastic space, may be continuously reduced to ASC, using the
450 cytoplasmic pyridine nucleotide and glutathione pools (de Gara and Tommasi
451 1999).

452 The first of these ASC-dependent antioxidant systems is constituted by
453 class I APXs, which are specific for ASC. Although some class I APX genes are
454 expressed in vascular tissues (Fryer et al. 2002), class I APXs are generally
455 associated with meristematic and actively growing young tissues (de Pinto and de
456 Gara 2004; Córdoba-Pedregosa et al. 2005), and this could explain their absence
457 from lignifying tissues in three of the four species studied (Table 2). In fact, class I
458 APXs were only detected in poplar (Fig. 4a, Table 2), and are generally absent from
459 the apoplast of most mature plant tissues (Polle et al. 1990; Hernández et al. 2001).
460 For this reason, it is hard to imagine that class I APXs constitute the central pivot of
461 the H_2O_2 detoxifying system in the apoplast of lignifying tissues.

462 The second ASC-dependent antioxidant system in the apoplast is
463 constituted by class III APXs, which are only moderately specific for ASC. Class III
464 APXs are generally expressed in non-growing tissues (de Pinto and de Gara 2004),
465 where the cell wall stiffening process is emerging. Class III APXs were only
466 detected in the apoplastic fractions of bitter orange tree (Table 2 and Fig. 4), and
467 therefore neither appears as a possible candidate for constituting the core of the
468 H_2O_2 detoxifying system in the apoplast of lignifying tissues.

469 The third ASC-dependent antioxidant enzymatic system in the apoplast is

470 constituted by class III peroxidases, which, although unable to oxidize ASC directly,
471 can perform this task when they are coupled by means a phenolic/phenolic radical
472 shuttle (Takahama 1993; Otter and Polle 1994). In this mechanism, phenolic
473 radicals ($R^{\bullet}$), which have been generated by a H_2O_2-dependent peroxidase-catalyzed
474 reaction ($2\ RH + H_2O_2 \rightarrow 2\ R^{\bullet} + H_2O$), may act as diffusible oxidants to oxidize a
475 secondary substrate, such as ASC ($2\ R^{\bullet} + ASC \rightarrow 2\ RH + DHA$). This type of
476 reaction has been described for the oxidation of ASC mediated by the coniferyl
477 alcohol radical ($R^{\bullet}$) (Takahama 1993; Otter and Polle 1994), and may have
478 important physiological connotations in the detoxification of H_2O_2 in the apoplast of
479 lignifying tissues, since peroxidases unable to oxidize ASC, such as those present in
480 the apoplast of eucalyptus and *Zinnia* (Table 2) could oxidize this substrate using
481 coniferyl alcohol as redox shuttle. The only condition that these peroxidases must
482 fulfil is an intrinsic capacity for oxidizing coniferyl alcohol, and this condition is
483 completely fulfilled by the peroxidases present in the apoplast of eucalyptus and
484 *Zinnia*, and even by the peroxidases present in the apoplast from bitter orange tree
485 and poplar (Table 1).

486 In fact, the thermodynamic driving force for the two-electron transfer
487 reaction, which constitutes the basis of this shuttle, is given by the difference
488 between the oxidation/reduction potentials of coniferyl alcohol/coniferyl alcohol
489 radical ($R^{\bullet},H^{+}$ /RH, E = + 0.810 V) and ASC/DHA (E = + 0.06 V), $\Delta E = E$
490 ($R^{\bullet},H^{+}$/RH) - E (DHA/ASC) = + 0.804 V, which gives a change of free energy for
491 the reaction, ΔG [$\Delta G = -n\ F\ \Delta E$] of - 155.17 kJ mol^{-1}, and which is responsible for
492 the spontaneous nature of the reaction. This theoretical consideration suggests that
493 class III peroxidases, which paradoxically do not show APX activity, may be the
494 better candidates for constituting the core of the ASC-dependent H_2O_2 detoxifying

495 system in the apoplast of lignifying tissues, since they are largely able to oxidize
496 coniferyl alcohol (Table 1), the redox mediator, which is present in μM amounts in
497 lignifying tissues (Hosokawa et al. 2001).

498 However, all these apoplastic H_2O_2 detoxifying systems, which use ASC
499 as source of reducing equivalents, may either be partially non-functional or
500 dysfunctional in the lignifying cell wall in the presence of AAO. AAO oxidizes
501 ASC at the expense of O_2, and contrarily to class I APXs, is exclusively localized in
502 the cell wall of plant tissues and, more specifically, in the cell wall of vascular
503 tissues (Liso et al. 2004). AAOs are present in IWFs from poplar and bitter orange
504 tree (Table 3 and Fig. 5) and, although they are not present in IWFs from both
505 eucalyptus and *Zinnia*, it is known that blue copper oxidases mRNAs are
506 differentially expressed in the secondary xylem of poplar (Mellerowicz et al. 2001),
507 eucalyptus (Paux et al. 2004) and *Zinnia* (Demura et al. 2002). The reason why
508 AAOs are not detected in the IWFs of eucalyptus and *Zinnia* probably resides in the
509 fact that these enzymes are covalently-bound to the cell wall, a situation already
510 described for the laccase-type blue copper oxidases in *Zinnia* (Liu et al. 1994), since
511 isolated cell wall fractions from eucalyptus and *Zinnia* are able to oxidize ASC
512 aerobically.

513 Although the biological function of AAOs in the cell wall remains to be
514 totally understood (de Tullio et al. 2004), it has been demonstrated that AAO
515 regulates the apoplastic ascorbate pool and therefore the redox state
516 (ASC/ASC+DHA), reducing apoplastic ASC levels to values below the detection
517 threshold (Sanmartin et al. 2003; Pignocchi et al. 2003). Under these circumstances,
518 the apoplastic redox state is zero, or near to zero, apparently due to the fact that
519 when apoplastic AAO activity is present, the rate of ASC oxidation far exceeds the

capacity of DHA transport to the cytosol (Pignocchi and Foyer 2003).

In fact, the effectiveness of this ASC removal system compared with class I APXs is such that the presence of AAO in the apoplastic space of non-growing tissues provokes the total shift of the redox state (ASC/ASC+DHA) towards DHA (de Pinto and de Gara 2004), the oxidized product of ASC. This does not occur in the apoplast of young tissues, where class I APXs predominate (de Pinto and de Gara 2004). Zero values were found for the redox state of ascorbic acid in IWFs from poplar, bitter orange tree, eucalyptus and *Zinnia*, and similar results (with values of zero or close to zero) have been reported in the xylem of *Larix* during the early stages of lignification (Antonova et al. 2005). In other words, it is conceivable that the presence of AAO in cell walls provokes the partial or total dysfunction of apoplastic class I and class III APXs, and of all the plethora of non-enzymatic redox shuttles in which ASC is involved (Takahama 1993), through the competitive and effective removal of ASC, allowing the accumulation of H_2O_2 in the apoplast of lignifying plant tissues, as can be observed experimentally (Figs. 1 and 2).

Furthermore, since AAO uses O_2 to remove ASC, it could regulate O_2 availability in the lignifying xylem (Tullio et al. 2004). Thorough this mechanism, AAO could also control the activity of NADPH oxidase (the enzyme responsible for H_2O_2 production in lignifying tissues) (Ogawa et al. 1997; Ros Barceló 1998a) at substrate level, by controlling the tension of O_2. That is, AAOs, through a double mechanism, appear to be essential for the fine control of the oxidative performance of lignifying secondary wood-forming tissues.

Acknowledgments

This work was supported by grants from the MCYT (HI2001-0064 and BOS2002-

546 03550). LVGR hold a FPI fellowship from the MCYT.

547

548 **References**

549 Antonova GF, Chaplygina IA, Varaksina TN, Stasova VV (2005) Ascorbic acid and
550 xylem development in trunks of the Siberian larch trees. Russ J Plant Physiol
551 52:97-107

552 Amako K, Chen GX, Asada K (1994) Separate assays specific for ascorbate
553 peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic
554 isozymes of ascorbate peroxidase in plants. Plant Cell Physiol 35:497-504

555 Böhle U-R, Hilger HH, Martin WF (1996) Island colonization and evolution of the
556 insular woody habit in *Echium* L. (Boraginaceae). Proc Natl Acad Sci USA
557 93:11740-11745

558 Brunner AM, Busov VB, Strauss SH (2004) Poplar genome sequence: functional
559 genomics in an ecologically dominant plant species. Trends Plant Sci 9:49-56

560 Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: Occurrence of two
561 isozymes and their differences in enzymatic and molecular properties. Plant Cell
562 Physiol 30:987-998

563 Christensen JH, Bauw G, Welinder KG, van Montagu M, Boerjan W (1998)
564 Purification and characterization of peroxidases correlated with lignification in
565 poplar xylem. Plant Physiol 118:125-135

566 Córdoba-Pedregosa MC, Córdoba F, Villalba JM, Gonzáles-Reyes JA (2003)
567 Differential distribution of ascorbic acid, peroxidase activity, and hydrogen
568 peroxide along the root axis in *Allium cepa* L. and its possible relationship with
569 cell growth and differentiation. Protoplasma 221:57-65

570 Córdoba-Pedregosa MC, Villalba JM, Córdoba F, González-Reyes JA (2005)

571 Changes in intracellular and apoplastic peroxidase activity, ascorbate redox
572 status, and root elongation induced by enhanced ascorbate content in *Allium*
573 *cepa* L. J Exp Bot 56:685-694
574 Czaninski Y, Sachot RM, Catesson AM (1993) Cytochemical localization of
575 hydrogen peroxide in lignifying cell walls. Ann Bot 72:547-550
576 de Gara L (2004) Class III peroxidases and ascorbate metabolism in plants.
577 Phytochemistry Rev 3:195-205
578 de Gara L, Tommasi F (1999) Ascorbate redox enzymes: a network of reactions
579 involved in plant development. Recent Res Devel Phytochem 3:1-15
580 de Leonardis S, Dipierro N, Dipierro S (2000) Purification and characterization of
581 an ascorbate peroxidase from potato tuber mitochondria. Plant Physiol Biochem
582 38:773-779
583 de Pinto MC, de Gara L (2004) Changes in the ascorbate metabolism of apoplastic
584 and symplastic spaces are associated with cell differentiation. J Exp Bot
585 55:2559-2569
586 de Tullio MC, Liso R, Arrigoni O (2004) Ascorbic acid oxidase: an enzyme in
587 search of a role. Biol Plantarum 48: 161-166
588 Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N, Minami
589 A, Nagata-Hiwatashi M, Nakamura K, Okamura Y, Sassa N, Suzuki S, Yazaki J,
590 Kikuchi S, Fukuda H (2002) Visualization by comprehensive microarray
591 analysis of gene expression programs during transdifferentiation of mesophyll
592 cells into xylem cells. Proc Natl Acad Sci USA 99:15794-15799
593 Ferrer MA, Calderón AA, Muñoz R, Ros Barceló A (1990) 4-Methoxy-α-naphthol
594 as a specific substrate for kinetic, zymographic and cytochemical studies on
595 plant peroxidase activities. Phytochem Anal 1:63-69

596 Fryer MJ, Oxborough K, Mullineaux PM, Baker NR (2002) Imaging of photo-
597 oxidative stress responses in leaves. J Exp Bot 53:1249-1254
598 Gabaldón C, Gómez-Ros L, Pedreño MA, Ros Barceló A (2005) Nitric oxide
599 production by the differentiating xylem of *Zinnia elegans*. New Phytol 165: 121-
600 130
601 Groover AT (2005) What genes makes a tree? Trends Plant Sci 10: 210-214
602 Hernández JA, Ferrer MA, Jiménez A, Ros Barceló A, Sevilla F (2001) Antioxidant
603 systems and $O_2^{\cdot -}/H_2O_2$ production in the apoplast of pea leaves. Its relation with
604 salt-induced necrotic lesion in minor veins. Plant Physiol 127:817-831
605 Hernández JA, Rubio M, Olmos E, Ros Barceló A, Martínez-Gómez P (2004)
606 Oxidative stress induced by long-term plum pox virus infection in peach (*Prunus*
607 *persica*). Physiol Plantarum 122:48-495
608 Horemans N, Foyer Ch, Asard H (2000) Transport and action of ascorbate at the
609 plasma membrane. Trends Plant Sci 5:263-266
610 Hosokawa M, Suzuki S, Umezawa T, Sato Y (2001) Progress of lignification
611 mediated by intercellular transportation of monolignols during tracheary element
612 differentiation of isolated *Zinnia* mesophyll cells. Plant Cell Physiol 42:959-968
613 Jiménez A, Hernández JA, Ros Barceló A, Sandalio LM, del Río LA, Sevilla F
614 (1998) Mitochondrial and peroxisomal ascorbate peroxidase of pea leaves.
615 Physiol Plantarum 104:687:692
616 Kvaratskhelia M, Winkel C, Thorneley RNF (1997) Purification and
617 characterization of a novel class III peroxidase isoenzymes from tea leaves. Plant
618 Physiol 114:1237-1245
619 Liso R, de Tullio MC, Ciraci S, Balestrini R, La Rocca N, Bruno L, Chiappetta A,
620 Bitonti MB, Bonfante P, Arrigoni O (2004) Localization of ascorbic acid,

621 ascorbic acid oxidase, and glutathione in roots of *Cucurbita maxima* L. J Exp
622 Bot 55:2589-2597
623 Liu L, Dean JFD, Friedman WE, Eriksson KEL (1994) A laccase-like
624 phenoloxidase is correlated with lignin biosynthesis in *Zinnia elegans* stem
625 tissues. Plant J 6:213-224
626 López-Serrano M, Fernández MD, Pomar F, Pedreño MA, Ros Barceló A (2004)
627 *Zinnia elegans* uses the same peroxidase isoenzyme complement for cell wall
628 lignification in both single-cell tracheary elements and xylem vessels. J Exp Bot
629 55:423-431
630 Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001) Unravelling cell wall
631 formation in the woody dicot stem. Plant Mol Biol 47:239-274
632 Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen
633 gene network of plants. Trends Plant Sci 9:490-498
634 Mittler R, Zilinskas B (1993) Detection of ascorbate peroxidase activity in native
635 gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium.
636 Anal Biochem 212:540-546
637 Moser O, Kanellis A (1994) Ascorbate oxidase of *Cucumis melo* L. var. reticulatus:
638 purification, characterization and antibody production. J Exp Bot 45:717-724
639 Nakano Y, Asaka K (1981) Hydrogen peroxide is scavenged by ascorbate-specific
640 peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880
641 Neil S (2005) NO way to die-nitric oxide, programmed cell death and xylogenesis.
642 New Phytol 165: 5-7

643 Nose M, Bernards MA, Furlan M, Zajicek J, Eberhardt TL, Lewis NG (1995)
644 Towards the specification of consecutive steps in macro-molecular lignin
645 assembly. Phytochemistry 39:71-79
646 Ogawa K, Kanematsu S, Asada K (1997) Generation of superoxide anion and
647 localization of CuZn-superoxide dismutase in the vascular tissue of spinach
648 hypocotyls: their association with lignification. Plant Cell Physiol 38:1118-1126
649 Olson PD, Varner JE (1993) Hydrogen peroxide and lignification. Plant J 4:887-892
650 Otter T, Polle A (1994) The influence of apoplastic ascorbate on the activity of cell
651 wall-associated peroxidases and NADH oxidase on needles of Norway spruce
652 (*Picies abies* L.). Plant Cell Physiol 35:1231-1238
653 Passardi F, Penel C, Dunand C (2004) Performing the paradoxical: how plant
654 peroxidasas modify the cell wall. Trends Plant Sci 9: 534-540
655 Paux E, Tamasloukht MB, Ladouce N, Sivadon P, Grima-Pettenati J (2004)
656 Identification of genes preferentially expressed during wood formation in
657 *Eucalyptus*. Plant Mol Biol 55:263-280
658 Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the
659 regulation of cell signalling. Curr Opinion Plant Biol 6:379-389
660 Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, Foyer CH (2003) The function
661 of ascorbate oxidase in tobacco. Plant Physiol 132:1631-1641
662 Polle A, Chakrabarti K, Schürmann W, Rennenberg H (1990) Composition and
663 properties of hydrogen peroxide decomposing systems in extracellular and total
664 extracts from needles of norway spruce (*Picea abies* L., Karst.). Plant Physiol
665 94:312-319
666 Polle A, Kröniger W, Rennenberg H (1996) Seasonal fluctuations of ascorbate-
667 related enzymes: Acute and delayed effects of late frost in spring on

668 antioxidative systems in needles of norway spruce (*Picea abies* L.). Plant Cell
669 Physiol 37:717-725
670 Pomar F, Caballero N, Pedreño MA, Ros Barcelò A (2002a) H_2O_2 generation during
671 the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly
672 conserved class III peroxidase involved in lignin biosynthesis. FEBS Lett
673 529:198-202
674 Pomar F, Merino F, Ros Barceló A (2002b) *O*-4-linked coniferyl and sinapyl
675 aldehydes in lignifying cell walls are the targets of the Wiesner (phloroglucinol-
676 HCl) reaction. Protoplasma 220:17-28
677 Ranocha P, McDougall G, Hawkins S, Sterjiades R, Borderies G, Stewart D,
678 Cabanes-Macheteau M, Boudet AM, Goffner D (1999) Biochemical
679 characterization, molecular cloning and expression of laccases – a divergent
680 gene family – in poplar. Eur J Biochem 259:485-495
681 Ros Barceló A (1997) Lignification in plant cell walls. Int Rev Cytol 176:87-132
682 Ros Barceló A (1998a) The generation of H_2O_2 in the xylem of *Zinnia elegans* is
683 mediated by an NADPH-oxidase-like enzyme. Planta 207:207-216
684 Ros Barceló A (1998b) Hydrogen peroxide production is a general property of the
685 lignifying xylem from vascular plants. Ann Bot 82:97-103
686 Ros Barceló A (2005) Xylem parenchyma cells deliver the H_2O_2 necessary for
687 lignification in differentiating xylem vessels. Planta 220: 747-756
688 Ros Barceló A, Pomar F (2001) Oxidation of cinnamyl alcohols and aldehydes by a
689 basic peroxidase from lignifying *Zinnia elegans* hypocotyls. Phytochemistry
690 57:1105-1113
691 Ros Barceló A, Pomar F, Pedreño MA (2000) Competitive inhibitor-dissected
692 histochemistry of the peroxidase responsible for syringyl lignin biosynthesis in

693 *Zinnia elegans* xylem. Aust J Plant Physiol 27:1101-1107
694 Sanmartin M, Drogoudi PD, Lyons T, Pateraki I, Barnes J, Kanellis AK (2003)
695 Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco
696 results in altered ascorbate and glutathione redox states and increased sensitivity
697 to ozone. Planta 216:918-928
698 Schopfer P (1994) Histochemical demonstration and localization of H_2O_2 in organs
699 of higher plants by tissue printing on nitrocellulose paper. Plant Physiol
700 104:1269-1275
701 Takahama U (1993) Regulation of peroxidase-dependent oxidation of phenolics by
702 ascorbic acid: Different effects of ascorbic acid on the oxidation of coniferyl
703 alcohol by the apoplastic soluble and cell wall-bound peroxidases from epicotyls
704 of *Vigna angulari*s. Plant Cell Physiol 34:809-817
705 Vanacker H, Harbinson J, Ruisch J, Carver TLW, Foyer CH. (1998). Antioxidant
706 defences of the apoplast. Protoplasma 205:129-140
707 Vianello A, Zancani M, Nagy G, Macrì F (1997) Guaiacol peroxidase associated to
708 soybean root plasma membranes oxidise ascorbate. J Plant Physiol 150:573-577
709

710

711 **Fig. 1 a**) Histochemical localization of lignins in poplar using the phloroglucinol/HCl
712 reagent. **b**) Histochemical localization of H_2O_2 production in poplar using the TMB
713 reagent. **c**) Histochemical localization of H_2O_2 accumulation in poplar using the
714 KI/starch reagent. **d**) Control for the histochemical localization of H_2O_2 production in
715 poplar using the TMB reagent, performed in the presence of 0.1 mM ferulic acid. Bar =
716 300 μm.

717

718 **Fig. 2** Histochemical localization of lignins in **a**) bitter orange tree and **e**) eucalyptus
719 using the phloroglucinol/HCl reagent. Histochemical localization of H_2O_2 production in
720 **b**) bitter orange tree and **f**) eucalyptus using the TMB reagent. Histochemical
721 localization of H_2O_2 accumulation in **c**) bitter orange tree using the KI/starch reagent.
722 Controls for the histochemical localization of H_2O_2 production in **d**) bitter orange tree
723 and **g**) eucalyptus using the TMB reagent, performed in the presence of 0.1 mM ferulic
724 acid. Bar = 600 μm.

725

726 **Fig. 3** Isoenzyme pattern obtained by isoelectric focusing in 3.5-10.5 pH gradients (top,
727 acidic; bottom, basic) of IWF proteins from **a**) poplar, **b**) bitter orange tree, **c**)
728 eucalyptus and **d**) *Zinnia*, stained with 4-methoxy-α-naphthol in the absence (1) and in
729 the presence (2) of H_2O_2, and in the presence of H_2O_2 after pre-incubation with *p*CMB
730 (3). The black arrows mark class I APXs, the white arrows mark H_2O_2-independent
731 oxidases, and the black arrowheads mark the strongly basic H_2O_2-dependent
732 peroxidases.

733

734 **Fig 4 a)** Isoenzyme pattern obtained by native PAGE of IWF proteins from poplar after

735 staining for APX activity in the absence (1) and in the presence (2) of *p*CMB, and after
736 staining with 4-methoxy-α-naphthol/H_2O_2 (3). **b)** Isoenzyme pattern obtained by native
737 PAGE of IWF proteins from bitter orange tree after staining for APX activity in the
738 absence (1) and in the presence (3) of *p*CMB, and after staining with 4-methoxy-α-
739 naphthol/H_2O_2 in the absence (2) and in the presence (4) of *p*CMB. **c)** Isoenzyme pattern
740 obtained by native PAGE of horseradish peroxidase c after staining for APX activity
741 (1), and after staining with 4-methoxy-α-naphthol/H_2O_2 (2). **d)** Isoenzyme pattern
742 obtained by native PAGE of IWF proteins from eucalyptus after staining for APX
743 activity (1). **e)** Isoenzyme pattern obtained by native PAGE of IWF proteins from
744 *Zinnia* after staining for APX activity (1), and after staining with 4-methoxy-α-
745 naphthol/ H_2O_2 (2)
746
747 **Fig 5 a)** Isoenzyme pattern obtained by native PAGE of IWF proteins from poplar after
748 staining for AAO activity in the absence (1) and in the presence (2) of azide. **b)**
749 Isoenzyme pattern obtained by native PAGE of IWF proteins from bitter orange tree
750 after staining for AAO activity in the absence (1) and in the presence (2) of azide. **c)**
751 Isoenzyme pattern obtained by native PAGE of IWF proteins from eucalyptus after
752 staining for AAO activity (1). **d)** Isoenzyme pattern obtained by native PAGE of IWF
753 proteins from *Zinnia* after staining for AAO activity (1). Lanes 3 in **a**) and **b**) is the
754 isoenzyme pattern obtained by native PAGE of *Cucurbita* AAO after staining for AAO
755 activity.
756

Table 1. Peroxidase activities in IWFs of young branches (*C. aurantium, P. alba* and *E. camaldulensis*) and stems (*Z. elegans*) from different plant species, in the absence and in the presence (0.1 mM) of H_2O_2. Values are the mean ± SE (n). n, number of determinations.

Species	**Peroxidase activity (nmol S oxidized min^{-1} g^{-1} FW)**					
	4-Methoxy-α-naphthol		**3,3',5,5'-Tetramethylbenzidine**		**Coniferyl alcohol**	
	- H_2O_2	**+ H_2O_2**	**- H_2O_2**	**+ H_2O_2**	**- H_2O_2**	**+ H_2O_2**
P. alba	15.74 ± 0.04 (3)	125.2 ± 11.63 (3)	0 ± 0 (3)	1124 ± 158 (3)	2.53 ± 0.96 (3)	668 ± 79 (3)
C. aurantium	18.80 ± 0.03 (6)	320.3 ± 18.45 (6)	0 ± 0 (6)	2300 ± 180.0 (6)	0.360 ± 0.015 (6	616 ± 45 (6)
E. camaldulensis	0.143 ± 0.034 (3)	23.18 ± 5.76 (3)	0 ± 0 (3)	79.86 ± 12.65 (3)	0.01 ± 0.00 (3)	98.2 ± 9.54 (3)
Z. elegans	0.56 ± 0.00 (4)	6.23 ± 0.85 (5)	0 ± 0 (4)	104.2 ± 1.94 (4)	0.59 ± 0.20 (4)	53.73 ± 6.13 (4)

Table 2. APX activities in IWFs and symplastic fractions of young branches (*C. aurantium, P. alba* and *E. camaldulensis*) and stems (*Z. elegans*) from different plant species, in the absence and in the presence (50 µM) of *p*CMB. Values are the mean ± SE (n). n, number of determinations. [a]Differences were significant at P <0.01 according to Duncan´s Multiple Range Test

Species	**APX activity (µmol ascorbic acid oxidized min^{-1} g^{-1} FW)**					
	IWF			**Symplast**		
	- *p*CMB	**+ *p*CMB**	**Δ *p*CMB**	**- *p*CMB**	**+ *p*CMB**	**Δ *p*CMB**
P. alba	0.081 ± 0.021 (14)[a]	0.033 ± 0.009 (10)[a]	0.048	3.26 ± 0.36 (8)[a]	0.70 ± 0.22 (5)[a]	2.56
C. aurantium	0.405 ± 0.026 (15)	0.422 ± 0.029 (10)	0	9.77 ± 0.70 (6)[a]	4.22 ± 0.35 (5)[a]	5.55
E. camaldulensis	0 ± 0 (11)	0 ± 0 (9)	0	1.91 ± 0.09 (10)[a]	0 ± 0 (10)[a]	1.91
Z. elegans	0 ± 0 (10)	0 ± 0 (10)	0	0.70 ± 0.02 (10)[a]	0.22 ± 0.01 (10)[a]	0.48

Table 3. AAO activities in IWFs of young branches (*C. aurantium, P. alba* and *E. camaldulensis*) and stems (*Z. elegans*) from different plant species, both in the absence and in the presence (0.1 mM) of azide. Values are the mean ± SE (n). n, number of determinations. [a]Differences were significant at P <0.01 according to Duncan´s Multiple Range Test

Species	**AAO activity (nmol ascorbic acid oxidized min^{-1} g^{-1} FW)**	
	- Azide	+ Azide
P. alba	56 ± 20 (10)[a]	7 ± 5 (6)[a]
C. aurantium	866 ± 78 (10)[a]	15 ± 5 (5)[a]
E. camaldulensis	0 ± 0 (9)	0 ± 0 (5)
Z. elegans	0 ± 0 (6)	0 ± 0 (5)

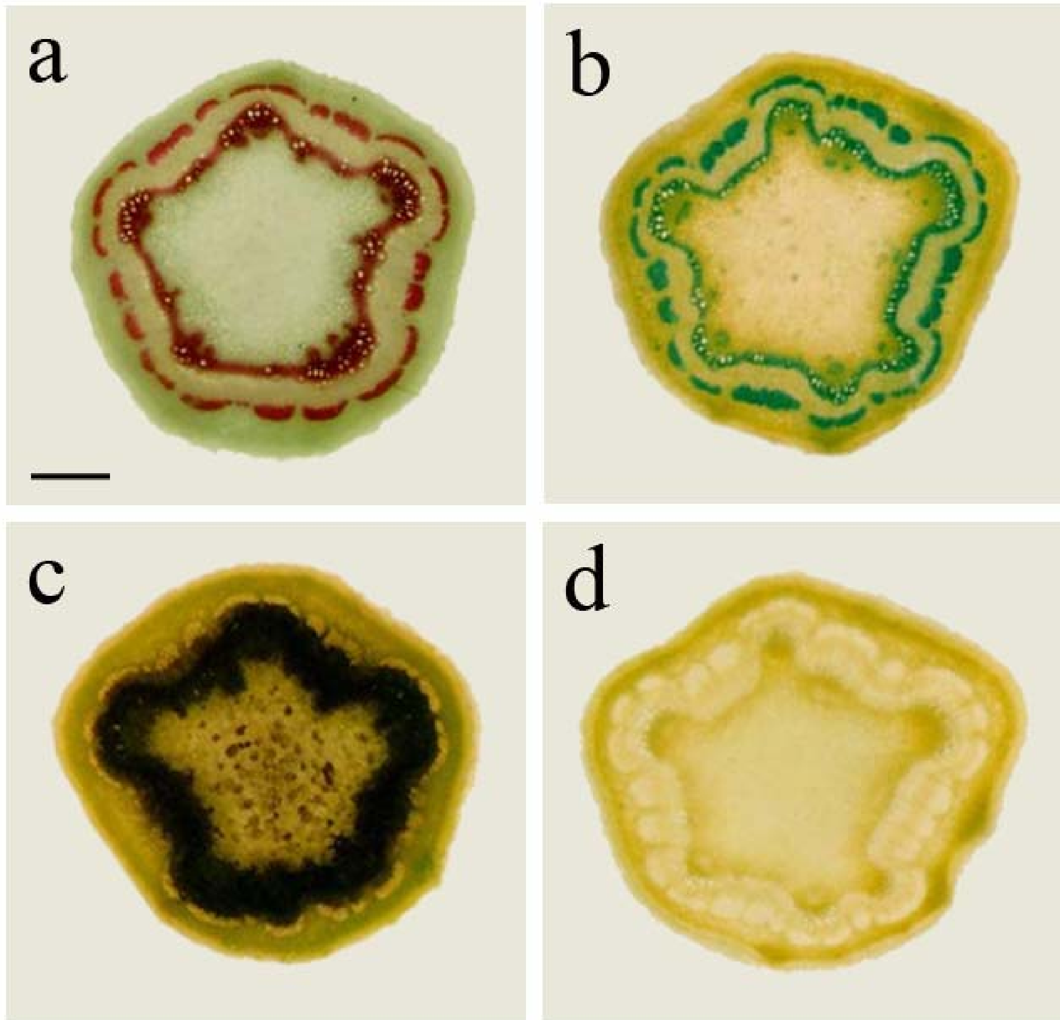

Figure 1

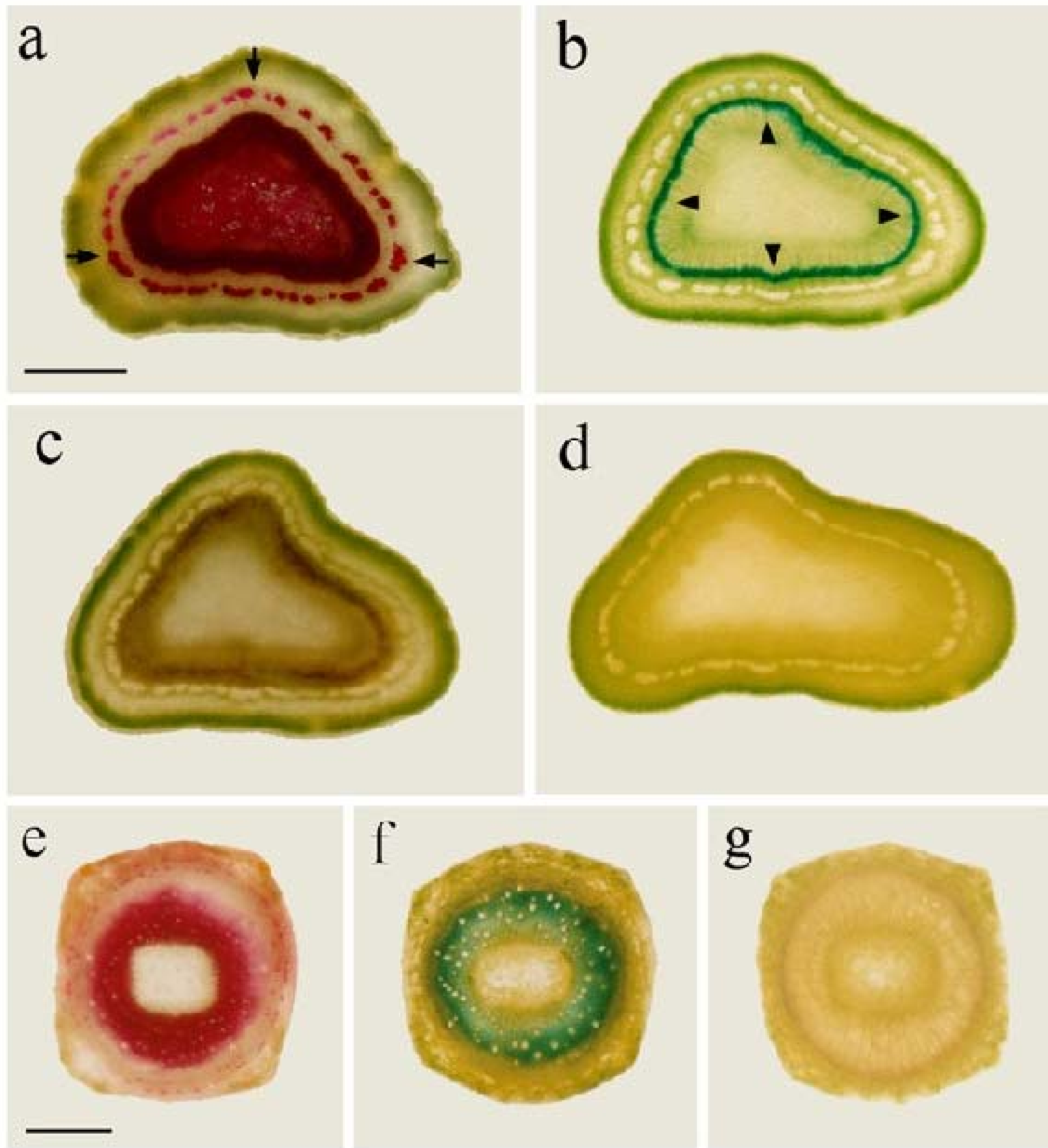

Figure 2

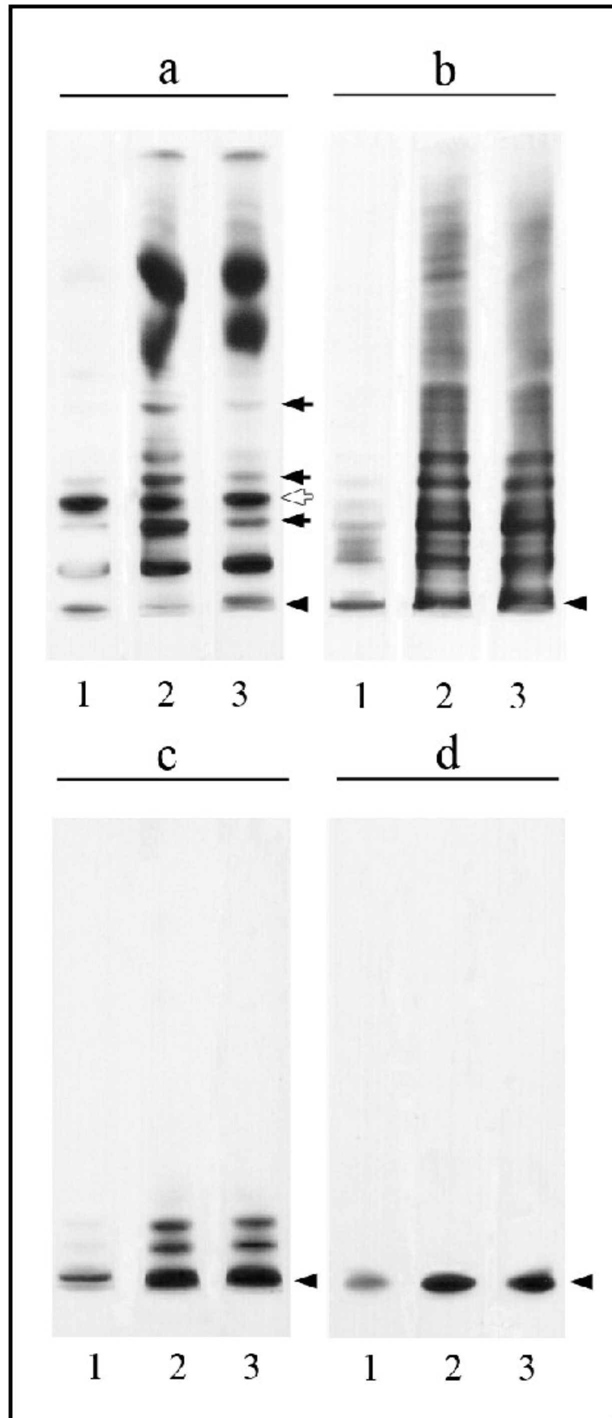

Figure 3

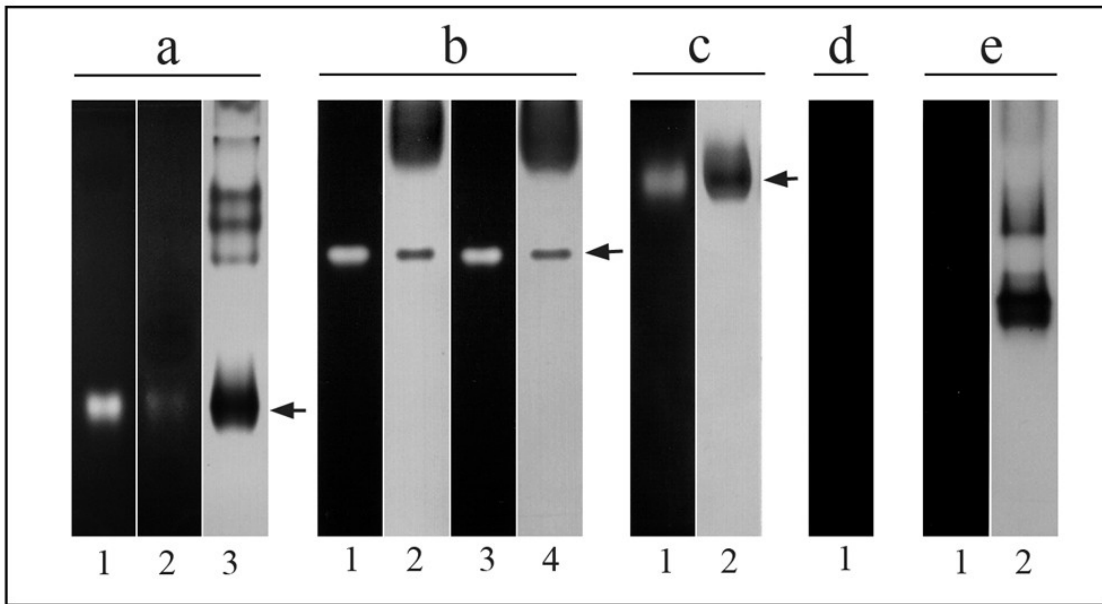

Figure 4

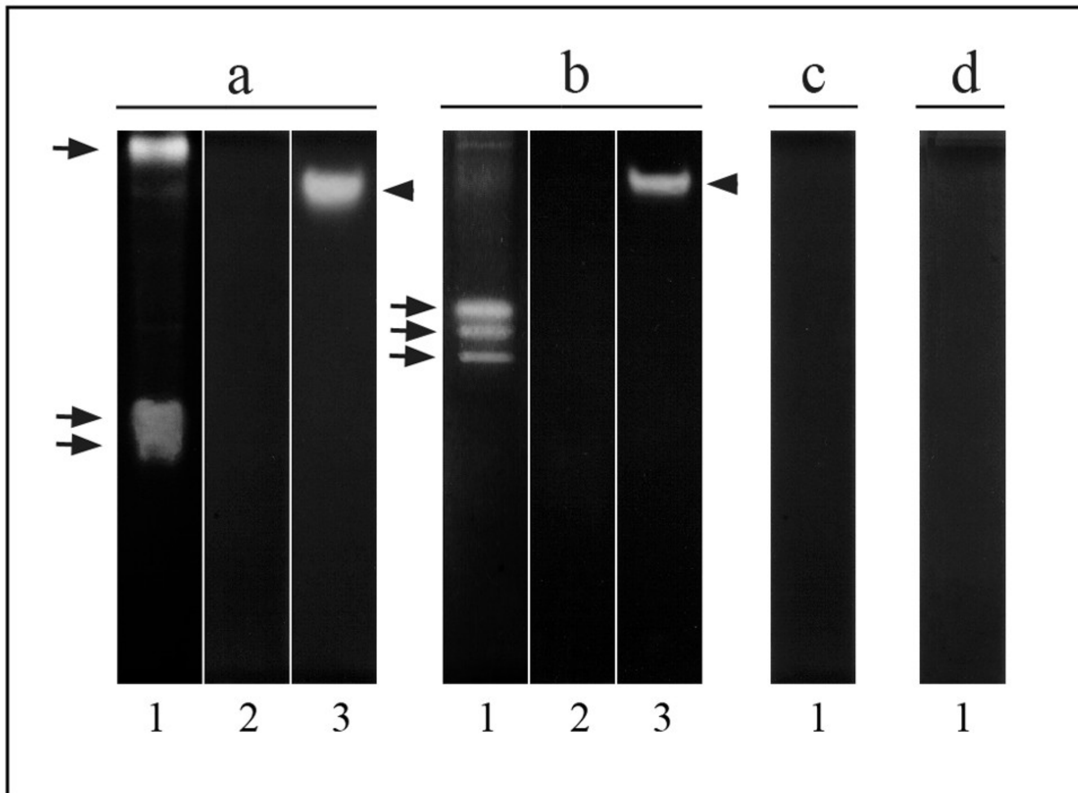

Figure 5