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Evidence does not support a role for gallic acid in Phragmites australis invasion success

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EVIDENCE DOES NOT SUPPORT A ROLE FOR GALLIC ACID IN *Phragmites australis* INVASION SUCCESS

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26   **Abstract**—Gallic acid has been reported to be responsible for the invasive success of nonnative

27   genotypes of *Phragmites australis* in North America. We have been unable to confirm previous

28   reports of persistent high concentrations of gallic acid in the rhizosphere of invasive *P. australis*,

29   and of high concentrations of gallic acid and gallotannins in *P. australis* rhizomes. The half-life

30   of gallic acid in nonsterile *P. australis* soil was measured by aqueous extraction of soils and

31   found to be less than 1 day at added concentrations up to 10,000 µg g^{-1}. Furthermore, extraction

32   of *P. australis* soil collected in North Carolina showed no evidence of gallic acid, and extractions

33   of both rhizomes and leaves of samples of four *P. australis* populations confirmed to be of

34   invasive genotype show only trace amounts of gallic acid and/or gallotannins.   The detection

35   limits were less than 20 µg gallic acid g^{-1} FW in the rhizome samples tested, which is

36   approximately 0.015% of the minimum amount of gallic acid expected based on previous

37   reports. While the occurrence of high concentrations of gallic acid and gallotannins in some

38   local populations of *P. australis* cannot be ruled out, our results indicate that exudation of gallic

39   acid by *P. australis* cannot be a primary, general explanation for the invasive success of this

40   species in North America.

41   **Key Words**—*Phragmites australis*, allelopathy, gallic acid, invasive species, novel weapons
42   hypothesis.

43

44   INTRODUCTION

45

46   *Phragmites australis* (Cav.) Trin. ex Steud., a common marsh grass in wetlands in the U.S., has

47   become a serious invasive species. Its abundance has increased greatly over the past 150-200

48   years, and Saltonstall (2002) found that invasive populations of *P. australis* possessed a non-

49 native, Eurasian genotype and that these plants had displaced those of a native North American

50 lineage, especially in New England and along the Atlantic Coast.

51

52 It has recently been reported (Rudrappa et al. 2007) that gallic acid is released and maintained in

53 extremely high concentrations (>10,000 µg g^{-1} soil) beneath invasive *P. australis* genotypes and

54 may be responsible for the invasive success of this plant. Rudrappa et al. (2007) found that root

55 exudates of invasive *P. australis* plants were more toxic than native genotypes to test plants, and

56 gallic acid (3, 4, 5-trihydroxybenzoic acid) was identified as the principal phytotoxin of the root

57 exudates. Gallic acid was found to trigger a "suicidal cell death cascade" in test plants through

58 the generation of toxic levels of reactive oxygen species (Rudrappa and Bais 2008). In

59 subsequent work, Bains et al. (2009) reported that rhizomes of invasive *P. australis* plants

60 contained elevated levels of gallotannins along with free gallic acid, and that native plants and

61 microorganisms produced high levels of tannase that resulted in the release of high amounts of

62 gallic acid in the root zone of *P. australis* by hydrolysis of these gallotannins. Bains et al. (2009)

63 proposed that this represents a previously unobserved extension of the novel weapons hypothesis

64 (Callaway and Ridenour 2004), whereby neighboring plants and native microorganisms activate

65 a less toxic precursor exuded by roots of an exotic invader to the detriment of the invaded

66 community. Rudrappa et al. (2009) reported that ultraviolet light resulted in the photo-

67 degradation of gallic acid in the *P. australis* rhizosphere and that the degradation product

68 mesoxalic acid was toxic to seedlings through a mechanism similar to gallic acid.

69

70 Gallic acid has previously been reported as one of the compounds responsible for the allelopathic

71 effects of the perennial shrub *Polygonella myriophylla* (Small) Horton, which grows in the

72   Florida sand pine scrub community (Weidenhamer and Romeo 1989, 2004). *P. myriophylla* is

73   one of several fire-sensitive shrubs in this community that use allelopathic mechanisms to deter

74   invasion of native and exotic grasses and herbs that could provide fuel for fires (Weidenhamer

75   and Romeo 2005). Previous work has shown that when gallic acid was added to Florida scrub

76   soils at concentrations as high as 400 µg g^{-1}, it completely disappeared within three days in non-

77   sterile soils, but persisted for the length of the study (nine days) in sterile soils (Weidenhamer

78   and Romeo 2004). Other bioassay work established that spiking soil with gallic acid in

79   combination with hydroquinone, the other suspected inhibitor produced by *P. myriophylla*, did

80   cause long-term inhibitory effects on plant growth despite their rapid disappearance, possibly

81   due to the formation of more toxic and persistent breakdown products (Weidenhamer et al.

82   1989).

83

84   The marked difference in the reported persistence of gallic acid in *P. australis* soil compared to

85   what has previously been observed in Florida scrub soils suggested to us that the stability of this

86   phenolic acid may be greatly extended in saturated soils. The initial objective of this study was

87   therefore to characterize the breakdown of gallic acid in sterile and nonsterile *P. australis* soils.

88   Once field-collected soils were found to contain undetectable levels of gallic acid and the rapid

89   breakdown of gallic acid in nonsterile *P. australis* soils was established, a phytochemical

90   investigation of *P. australis* rhizomes was undertaken and both rhizomes and leaves of four

91   populations in Ohio and North Carolina USA were analyzed for gallic acid content.

92

93   METHODS AND MATERIALS

94

Soil Samples. Soil was collected from beneath *Phragmites australis* and from an adjacent

brackish marsh that did not have any *P. australis* in the Outer Banks of North Carolina, USA

(35.8448 N, 75.5660 W). Samples were stored frozen (-20°C) for approximately six months

prior to the start of this study. Before use, soils were air-dried, passed through a 2 mm screen to

remove roots and other large debris. The soil was then stored frozen until use. A subsample of

the *P. australis* soil was analyzed for basic soil parameters by the STAR laboratory (Ohio

Agricultural Research and Development Center, Ohio State University) and for soil texture

(Table 1).

103

Soil sample preparation. Initial extractions of the *P. australis* soil described above (thawed and

extracted before air drying) found no detectable gallic acid. Therefore, this soil was used for all

studies of the behavior of gallic acid in soil. Soil was treated with six concentrations of gallic

acid: 250, 500, 1000, 5000, 10,000, and 20,000 μg gallic acid g^{-1} soil. The upper concentrations

used in this study were chosen to be in the range of free gallic acid concentrations reported in *P.*

australis soil by Rudrappa et al. (2007) and Bains et al. (2009). In order to add these high

concentrations of gallic acid to soil, gallic acid was dissolved in absolute ethanol and added in

250 μl portions to 25 g soil. The soil was allowed to dry and was mixed well after each portion

was added. The total amount of ethanol added was constant for all treatments (4 ml per 25 g

soil). Complete evaporation of ethanol was confirmed by weighing. One gram of soil was then

weighed into a 15×125 mm test tube, and 0.75 ml of deionized water was added to create a

saturated aqueous environment. Finally, test tubes were sealed with Parafilm® and placed in the

116   dark at ambient laboratory temperature until extraction and analysis. Triplicate samples of each

117   treatment were extracted at 0, 0.5, 1, 1.5, 2, 3 days for rates of 10,000 μg gallic acid g^{-1} soil and

118   below. The initial sampling interval was 0.5 days based on preliminary experiments showing

119   very rapid breakdown of gallic acid in nonsterile soil. Soil treated with 20,000 μg gallic acid g^{-1}

120   was extracted at 0, 1, 2, 3, 5, 7 and 9 days, based on preliminary experiments indicating longer

121   persistence of gallic acid at very high concentrations. The initial analysis (0 days time point)

122   was done immediately after sample preparation. Sterilized soils were prepared in the same way,

123   but autoclaved three times at 48 hr intervals (Dalton et al. 1989). Triplicate samples of each

124   treatment were extracted at 0, 5 and 9 days.

125

126   *Soil extraction method.* The soil extraction method followed that of Weidenhamer and Romeo

127   (2004). Samples were either extracted immediately or frozen to terminate gallic acid breakdown

128   and stored for up to 10 days at -20°C until analysis. Extractions of replicate treated samples

129   extracted immediately and after storage confirmed that concentrations of frozen samples did not

130   change over this length of time. Soil samples were extracted for one hr with 2 ml of distilled

131   water, well mixed by briefly vortexing three times over this period. To insure complete

132   extraction of gallic acid, the extraction volume was increased to 4 ml for sterile soils spiked with

133   concentrations of 5,000 μg g^{-1} and above, and for nonsterile soils spiked with 20,000 μg g^{-1} at 0,

134   1 and 2 days. After centrifugation, extracts were filtered through a 0.22 μm nylon syringe filter.

135   When necessary, an appropriate dilution was made with water before HPLC analysis.

136

137 *Genotyping of P. australis.* Samples of *P. australis* were collected from two populations in Ohio

138 (Site 1: 41.1655 N, 81.8590 W; Site 2: 41.1355 N, 81.8058 W) and two populations in North

139 Carolina (Site 1: 35.8448 N, 75.5660 W; Site 2: 36.1696 N, 75.7569 W), USA. Each sample

140 was genotyped according to the restriction fragment-length polymorphism (RFLP) assay

141 published by Saltonstall (2003), which distinguishes between the native North American and

142 invasive Eurasian genotypes. DNA was isolated from young *P. australis* shoots using a 2%

143 CTAB (cetyl tri-methylammonium bromide) extraction procedure modified from Doyle and

144 Doyle (1987).

145

146 Two regions of chloroplast DNA were amplified using polymerase chain reaction (PCR) with the

147 following primer pairs: *rbcL* forward (5' TGTACAAGCTCGTAACGAAGG) & reverse (5'

148 GATTTGTCAAGTCTCATGATCGT), and *trnL* forward (5' TCTACCGATTTCGCCATATC)

149 & reverse (5' GGAGAAGATAGAATCATAGC). Each PCR reaction mixture consisted of

150 approximately 400 ng of DNA, 7.5 pmol of a forward and reverse primer, 25 μl One*Taq* DNA

151 polymerase 2X master mix (New England Biolabs, Ipswich, MA) with standard buffer, and

152 DEPC water up to a final volume of 50 μl. Reactions were amplified in a thermal cycler (Bio-

153 Rad DNA Engine Peltier thermal cycler, Hercules, CA) using the following conditions: 2 min. at

154 94˚C, followed by 35 cycles of 94˚C for 45 sec., 52˚C for 45 sec., and 72˚C for 1 min. 30 sec.,

155 and finished by a final extension of 72˚C for 2 min. Ten μl of the PCR products amplified with

156 the *rbcL* and *trnL*b primer pairs was digested individually with the enzymes *Rsa*I and *Hha*I (New

157 England Biolabs) according to the manufacturer's protocol. The digest products were run on 3%

158 TAE agarose gels with a 50 bp DNA ladder (Fermentas, Glen Burnie, MD). Digestion of *rbcL*

159   amplification products by *HhaI* are indicative of invasive strains while digestion of *trnLb*

160   amplification products by *RsaI* indicate a native strain (Saltonstall 2003).

161

162   *P. australis foliar and rhizome extracts.* Hydrolyzed extracts of *P. australis* foliage and rhizomes

163   from each of the four populations were prepared according to Harborne (1984) to examine the

164   phenolic aglycones present. Duplicate samples of fresh or frozen leaves or rhizomes (1-3 g fresh

165   weight) were covered with 2 M HCl in a 20 mL vial and placed in a boiling water bath for 30-40

166   min. After cooling, the aqueous phase was filtered through glass wool and extracted four times

167   with a one fourth volume of ethyl acetate. The ethyl acetate fraction was washed with water,

168   dried with granular anhydrous sodium sulfate, and evaporated with gentle warming in a fume

169   hood. Moisture content of the rhizomes and leaves was determined by drying for 48 hr at 60°C.

170   Extracts were analyzed by HPLC to confirm presence or absence of gallic acid. The retention

171   time of gallic acid was established using an authentic standard (see HPLC analytical methods

172   below). Triplicate samples of gallic acid and methyl gallate (2, 10 and 25 mg) were also

173   subjected to the acid hydrolysis and extraction procedure, followed by HPLC analysis, to verify

174   the success of the mild acid hydrolysis for bound forms of gallic acid as well as the stability and

175   recovery of gallic acid.

176

177   A bulk extraction of *P. australis* rhizomes (140 g) using the above methods was undertaken to

178   identify the major phenolic and flavonoid compounds. Initial separation was achieved by

179   preparative thin layer chromatography (TLC) on cellulose using 6% acetic acid as the mobile

180   phase. Further separation was carried out by preparative TLC on silica gel GF using ethyl

181 acetate: benzene (45:55, v/v) or acetic acid: dichloromethane (1:9, v/v) as the mobile phase.

182 Isolated fractions were analyzed by HPLC and by high field NMR. GC-MS analyses were

183 carried out on an Agilent 6890/5975 gas chromatograph/ quadrupole mass spectrometer, using a

184 30 m (250 μm inner diameter x 0.25 μm film thickness) HP-5MS column. Starting column

185 temperature was $40^{o}C$ (3 min hold), and increased at $12^{o}C$ per min to 260°. The Agilent

186 ChemStation software included the NIST/EPA/NIH Mass Spectral Library (version 2.0). LC-

187 MS analysis was carried out on a Thermo QExactive Orbitrap MS with a Thermo Accela 1250

188 UPLC, using the HPLC column and mobile phase detailed below.

189

190 *HPLC analytical method.* Samples were analyzed using a Restek Ultra C18 column (150 ×

191 4.6mm, 5μm particle size) on an Agilent 1100 HPLC system which included an autosampler and

192 diode array detector. Samples were eluted isocratically with water/ methanol/acetic acid

193 (97.9/2.0/0.1,v/v/v) for the first 12 min, followed by gradient to 95% methanol with 5% of water/

194 methanol/acetic acid (97.9/2.0/0.1,v/v/v) over 12-20 min. The mobile phase remained at this

195 composition until 25 minutes, before switching back to the initial conditions. There was a four

196 min equilibration time between runs to allow the system to stabilize. The injection volume was

197 25 μl, and the detection wavelength was 272nm for gallic acid. All samples were injected in

198 duplicate. Linearity of gallic acid response was established for both peak area vs. concentration

199 of authentic standards (R^2=0.9998) and log peak area vs. log concentration (R^2=0.999) over a

200 range of 1 to 500 mg l^{-1}. The quantitation limit was conservatively estimated at 0.25 mg l^{-1}, the

201 concentration of the lowest standard used, which corresponds to a concentration in soil of less

202 than 0.5 μg g^{-1}.

203

204 *Nuclear Magnetic Resonance Spectra.* NMR spectra were obtained on a JEOL ECS 400 MHz

205 spectrometer equipped with a 5mm proton/multi-frequency, autotune and match-ready, z-axis

206 pulsed field gradient, high sensitivity NMR probe. The operating frequencies were 399.782 MHz

207 for ^{1}H and 100.525 MHz for ^{13}C. Standard JEOL acquisition experiments (^{1}H, ^{13}C, COSY,

208 HMQC, and HMBC) were used without modification other than varying the number of scans.

209 The spectral assignments were obtained in methanol-d_4 using instrument referencing relative to

210 TMS.

211

212   RESULTS

213

214 ***Gallic acid breaks down rapidly in nonsterile P. australis soil.*** Gallic acid rapidly degraded in

215 nonsterile soil (Figure 1). The half-life of gallic acid increased somewhat with concentration, but

216 remained less than 1 day up to 10,000 µg g^{-1} (Table 2). At 20,000 µg g^{-1} the persistence of gallic

217 acid increased significantly, but increased variability was seen between replicates. Several

218 presumed microbial breakdown products of gallic acid were observed (Figure 2), but these are as

219 yet unidentified. All of the breakdown products eluted prior to gallic acid, indicating that they

220 were more polar than gallic acid. Gallic acid was much more persistent in autoclaved soil

221 (Figure 3), but extractable gallic acid did diminish significantly over time, particularly at the

222 lowest applied rates.

223

224   ***RFLP analysis of Ohio and North Carolina P. australis samples indicate that all are invasive***

225   ***strains.*** Polymorphisms in the chloroplast genome were used to determine whether *P. australis*

226   specimens used in this study were native or invasive. A PCR approach was used to amplify

227   these polymorphic regions and sequences were identified by restriction enzyme digest. PCR

228   amplification of the *rbcL* and *trnL*b regions of our purified *P. australis* DNA resulted in 350 bp

229   fragments as expected (Figure 4). The amplified *rbcL* regions from all four of our collection

230   sites were cut by *HhaI*, but not by *RsaI*. Neither restriction enzyme cut our amplified *trnLb*

231   regions. These results indicate that plant material from all four collection sites are invasive

232   strains of *P. australis* (Saltonstall 2003).

233

234   ***Gallic acid and gallotannins are not major components of P. australis leaves or rhizomes.***

235   Hydrolysis and extraction of known samples of gallic acid and methyl gallate demonstrated that

236   gallic acid is stable in the extraction procedure, and that methyl gallate (used as a proxy for

237   gallotannins) is effectively hydrolyzed (Table 3). Recovery decreased somewhat with

238   concentration, but reproducibility was good and all recoveries averaged above 70%. Application

239   of this procedure to rhizome and foliage samples from four *P. australis* populations recovered

240   undetectable to trace levels of gallic acid (Table 4). Only three of eight rhizome samples had

241   detectable gallic acid. These peaks were too small to give an acceptable ultraviolet spectrum to

242   compare with gallic acid, so identification was based on peak retention time. None of the

243   rhizome samples showed gallic acid concentrations above the estimated quantifiable detection

244   limit. Gallic acid was detected in seven of eight foliage samples, but again the amounts found

245   were quite low.

246

247 In HPLC monitoring at 272 nm, rhizome samples contained two major components that eluted

248 after gallic acid at approximately 15.9 and 16.5 min (Figure 5), however the relative amounts of

249 these two compounds varied from one population to another and in one case (data not shown)

250 from sample to sample. Monitoring at 360 nm indicated another strongly absorbing component

251 eluting at approximately 19.7 min (data not shown).

252

253 ***Characterization of other P. australis constituents.*** Initial efforts focused on the identification

254 of the major extract component eluting at 15.9 min by HPLC (Figure 5). This compound was

255 isolated from the bulk extraction by preparative TLC and characterized by NMR and mass

256 spectroscopy. ^{1}H NMR in methanol-d$_4$ clearly showed evidence of an aldehyde proton ($\delta = 9.51$

257 ppm, s), two aromatic protons ($\delta = 7.36$ ppm, d, J=3.2 Hz; and 6.56 ppm, d, J=3.2 Hz), and a

258 hydroxymethyl ($\delta = 4.59$ ppm, s) substituent. High resolution LC-MS showed a mass of

259 127.0389 (M+1), consistent with a molecular formula of $C_6H_6O_3$ for the parent compound. The

260 GC-MS spectrum showed major m/z fragments at 126, 97, 69, and 51, consistent with 5-

261 hydroxymethyl-2-furancarboxaldehyde (= 5-HMF, 5-hydroxymethylfurfural, $C_6H_6O_3$) in the

262 NIST mass spectral database (Supplemental Figure 1). This structure was also consistent with

263 the exact mass obtained by LC-MS. In addition, 2D NMR data (COSY, HMQC and HMBC

264 spectra) supported this structural assignment. The identification was further confirmed by

265 comparison with the ^{1}H NMR and ^{13}C NMR spectra of an authentic sample of 5-HMF (Sigma

266 Chemical Co., St. Louis, MO). GC-MS analysis of the fraction containing 5-HMF also

267 tentatively identified 2-methoxy-4-vinylphenol as a trace component in the extract

268 (Supplemental Figure 2).

269

270 5-HMF is a common constituent of dried fruits and baked goods, and can be formed as a result of

271 the Maillard reaction occurring when sugars degrade with heating in an acidic environment

272 (Ramírez-Jiménez et al. 2000; Gaspar and Lucena 2009). This suggested the possibility that this

273 compound might be an artifact of the extraction procedure used. To examine this, duplicate 1 g

274 samples of fresh foliage of three species (Sugar maple, *Acer saccharum* Marsh.; corn, *Zea mays*

275 L.; and kohlrabi, *Brassica oleracea* var. *gongylodes*) were carried through this procedure and

276 analyzed by HPLC. All samples were found to contain significant amounts of 5-HMF, verified

277 by both retention time and UV spectrum of the 5-HMF peak. Because of this, our finding of 5-

278 HMF in rhizome and leaf extracts of *P. australis* must be regarded as an artifact of the extraction

279 procedure.

280

281   DISCUSSION

282

283 *Gallic acid degradation in sterile and non-sterile soil.* The rapid degradation of gallic acid in

284 non-sterile soil is in agreement with previous work (Weidenhamer and Romeo 2004) and with

285 numerous studies showing that microorganisms are very effective in degrading simple phenolic

286 compounds and other allelochemicals (Schmidt 1988; Blum 1998; Blum et al. 2000; Inderjit

287 2005; Kaur et al. 2009; Inderjit et al. 2010). While the effect of frozen storage on the microbial

288 populations of the *P. australis* soil used in this study is unknown, the soil microbial community

289 present clearly was capable of rapidly degrading very large doses of gallic acid. The increased

290 persistence of gallic acid at the largest applied rate (20,000 µg g^{-1}) may be due to general

291 antimicrobial effects of this very high dose or to the formation of antimicrobial degradation

292 products.

293

294 Phenolic acids have been found to sorb very quickly to soil (Cecchi et al. 2004; Schmidt et al.

295 2012). Depending on the compound, organic matter and clays can either reversibly or

296 irreversibly sorb phenolic acids. Dalton et al. (1989) showed that the binding of ferulic acid

297 increased with soil organic matter content, and clays had a strong capacity for sorbing ferulic

298 acid. Schmidt et al. (2012) found that sorption of poylphenolic compounds increased with the

299 molecular weight and that hydrophobic compounds were sorbed more strongly than polar ones.

300 Sorption of non-tannin phenolics such as gallic acid was lower than it was for tannins in soils

301 amended with various organic carbon sources (Halvorson et al. 2012). Our results in sterile soil

302 (Figure 3) differ significantly from those of Weidenhamer and Romeo (2004), who found that

303 gallic acid applied at rates of 100-400 µg g^{-1} soil was stable in sterile soils. While we did not test

304 the autoclaved soil in this study to verify that it was sterile, autoclaving greatly extended the

305 persistence of gallic acid in this soil as would be expected if microbes capable of degrading

306 gallic acid were eliminated. The *P. australis* soil used in this study has a high sand content

307 (82%, Table 1) and a much higher organic matter content (11.74%, Table 1) than the Florida

308 scrub soils studied by Weidenhamer and Romeo (no more than 1%), and sorption to organic

309 matter thus seems the most likely explanation for the greater sorption of gallic acid observed

310 here.

311

312   While some researchers have argued that the ability of microorganisms to rapidly degrade many

313   allelochemicals will prevent the manifestation of allelopathy in many field situations (e.g.

314   Schmidt 1988; Kaur et al. 2009), some compounds do show resistance to degradation (Sosa et al.

315   2010) and others degrade to compounds that are more resistant to breakdown, such as the

316   formation of the more toxic and more persistent 2-amino-3*H*-phenoxazin-3-one (APO) from

317   microbial degradation of benzoxazinones released by roots of cereals such as rye (Krogh et al.

318   2006). In addition, it has recently been shown that both polar and non-polar phytotoxins can be

319   transported through soil via the common mycorrhizal network of fungal mycelia that permeate

320   soil, thus providing a potential conduit for these compounds away from microbial hot spots in the

321   rhizosphere (Barto et al. 2011). Other plant systems may continuously produce and release large

322   amounts of allelochemicals over time, regardless of whether the compounds persist in the soil

323   environment for long periods of time (Bertin et al. 2009). However, our studies suggest that it is

324   highly unlikely for gallic acid to be responsible for the allelopathic effects of invasive *P.*

325   *australis*, given the absence of significant levels of gallic acid and gallotannins in the suspected

326   source plant, and the absence of gallic acid in the *P. australis* soils that were tested.   Previous

327   bioassay work with gallic acid suggests that persistent degradation products with residual

328   phytotoxicity may be formed from gallic acid (Weidenhamer et al. 1989), but this would require

329   regular inputs of significant amounts of gallic acid into the soil and this does not appear to be the

330   case. Our detection limits for gallic acid in soil were less than 0.5 μg g^{-1}, compared to a lowest

331   concentration of more than approximately 70 μmol g^{-1} in fresh soil (approximately 12,000 μg

332   g^{-1}) beneath natural stands of invasive *P. australis* reported by Rudrappa et al. (2007). Bains et

333   al. (2009) reported soil concentrations of 7.2-22.5 μmol gallic acid g^{-1} (= 1225-3828 μg gallic

334   acid g^{-1}) in the rhizosphere of invasive *P. australis* growing in the Delmarva peninsula (USA).

335   Rudrappa et al. (2007) and Bains et al. (2009) used 2.5 mM potassium phosphate buffer (pH =

336   2.5) for their soil extractions. While different extraction methods for soil phenolics may recover

337   some reversibly bound material (Schmidt et al. 2012; Blair et al. 2005, 2009), the differences

338   here are orders of magnitude, and in addition we have found no evidence of significant gallic

339   acid production by the supposed source plant. We note that neither Rudrappa et al. (2007) nor

340   Bains et al. (2009) report data on soil blank extractions or recovery of gallic acid from spiked

341   soil, both of which are crucial for investigations of potential allelopathic activity (Blair et al.

342   2009). Bains et al. (2009) reported concentrations of gallic acid that were as much as 10-fold

343   less than those seen by Rudrappa et al. (2007), but this difference was not noted as significant

344   and no data on the seasonal or other dynamics of gallic acid concentrations over time have

345   subsequently been reported by these authors.

346

347   ***Gallic acid content of P. australis leaves and rhizomes.*** Bains et al. (2009) reported that

348   rhizomes of invasive *P. australis* contained 12.2-48.8 µmol gallic acid g^{-1} FW (= 2075-8302 µg

349   gallic acid g^{-1} FW), and some 194-265 µmol gallotannin g^{-1} FW. Gallotannin was quantified

350   using commercial tannic acid as a standard, which is a mixture of condensed and/or hydrolysable

351   tannins obtained from the source plant(s) for the extract (Hagerman et al. 1992; Kinraide and

352   Hagerman 2010). Typically, depending on the content of these tannins, 1 µmol of tannin will

353   yield 4-12 µmol of gallic acid (Kinraide and Hagerman 2010). Assuming a conservative yield of

354   4 µmol of gallic acid per µmol tannin, 194-265 µmol gallotannin g^{-1} FW could yield 132-180 **mg**

355   gallic acid g^{-1} FW. Combined with the reported amounts of free gallic acid, the expected

356   minimum total extractable gallic acid content in invasive *P. australis* rhizomes is 134-188 mg

357   gallic acid g^{-1} FW (equivalent to 13.4-18.8 weight %).   By contrast, we found only trace to

358   undetectable amounts of gallic acid in our samples (Table 4). Our detection limits were less than

359   20 μg gallic acid g^{-1} FW in the rhizome samples tested, which is approximately 0.015% of the

360   minimum amount of gallic acid expected.

361

362   Our results agree with those of Hendricks et al. (2011), who did not quantify gallic acid

363   specifically, but reported low amounts (approximately 0.12% dry mass) of total phenolics in

364   various extracts of *P. australis.* Hendricks et al. (2011) isolated one or more moderately polar

365   compounds that deterred grazing by the abundant salt marsh herbivore *Littoraria irrorata*. The

366   very low concentrations of phenolics isolated from *P. australis* tissues in their study suggested

367   that these are not the source of herbivore deterrence. The thin layer chromatography

368   characteristics of the isolated defense compound(s) were also not consistent with gallic acid.

369   The results from Hendricks et al. (2011) and our current data both suggest that compounds other

370   than gallic acid are involved in the chemical defense of *P. australis* against herbivores and

371   neighboring plants.

372

373   ***Other chemical constituents of P. australis.*** Among the compounds reported from *Phragmites*

374   *communis* (an early name for *P. australis,* see Mal and Narine (2004)) rhizomes are the anti-algal

375   compound ethyl acetoacetate (Li and Hu 2005) and methyl gallate (Choi et al. 2009). Rudrappa

376   et al. (2007) reported that they were unable to find evidence of ethyl acetoacetate in their root

377   secretion abstracts, and we also found no evidence of this compound by GC-MS analysis of

378   methanol extracts of *P. australis* rhizomes (data not shown). The presence of methyl gallate may

379   explain the trace amounts of gallic acid present in some rhizome and foliar extracts. We have

380   not yet tested for possible allelopathic effects of other compounds in *P. australis* rhizome and

381   foliar extracts, and so cannot comment on whether any of these may play an allelopathic role for

382   this species. Thus far, 5-HMF is the only major component of the *P. australis* rhizome and leaf

383   extracts that has been characterized. Further investigation of the phytochemistry of *P. australis*

384   is continuing.

385

386

387   ***Implications of this work for the hypothesis of gallic acid-mediated allelopathy as the***

388   ***mechanism for invasive success of P. australis.*** Our findings show: (1) Free gallic acid was not

389   detected in *P. australis* soil from North Carolina, in contrast to previous reports of persistent

390   levels of gallic acid in excess of 1000 μg g^{-1} in *P. australis* soils (Bains et al. 2009) to more than

391   10,000 μg g^{-1} (Rudrappa et al. 2007). (2) Gallic acid rapidly degrades in non-sterile soil, even at

392   very high doses, in contrast to the reported persistence of this compound in soil (Rudrappa et al.

393   2007; Bains et al. 2009). (3) The rapid degradation of gallic acid in non-sterile soil implies that

394   high amounts of gallic acid could therefore be maintained in *P. australis* soil only through the

395   regular influx of large quantities of gallic acid. However, our results show that only trace

396   amounts of gallic acid were found in samples of rhizomes and foliage of four different *P.*

397   *australis* populations. Bains et al. (2009) reported very high levels of gallotannins in addition to

398   the free gallic acid present in the plant. Our hydrolysis and extraction procedure was validated

399   using known samples of gallic acid to verify its stability and methyl gallate as a proxy for

400   gallotannins to verify the effectiveness of the acid hydrolysis procedure to cleave bound forms of

401 gallic acid. The amounts of gallic acid which we found in the *P. australis* rhizomes we tested

402 were less than 0.015% of the minimum amount expected based on previous reports. All four

403 populations sampled have been verified to be of the invasive Eurasian genotype previously

404 identified as possessing both high free gallic acid and gallotannin content (Rudrappa et al. 2007,

405 2009; Rudrappa and Bais 2008; Bains et al. 2009). (4) Most investigations of the toxicity of

406 gallic acid indicate that it is not particularly toxic to other plants. While Rudrappa et al. (2007)

407 reported that gallic acid inhibited the growth of *Spartina alterniflora* at concentrations of 0.05-

408 0.15 mM, other data in the literature indicate that gallic acid requires higher concentrations to

409 exert toxic effects. Reigosa et al. (1999) tested the effects of six phenolic acids including gallic

410 acid on the germination and growth of six weed species. Concentrations of 10, 1, 0.1 and 0.01

411 mM were used, and in only one case did gallic acid show any inhibitory activity below a

412 concentration of 10 mM. Chung et al. (2002) also found gallic acid to be less active than several

413 other phenolic acids tested in bioassays against barnyardgrass. Our data indicate that gallic acid

414 concentrations in soil will not reach even the 0.05 mM level at which Rudrappa et al. (2007)

415 reported effects against *Spartina alterniflora* and other species.

416

417 In summary, our results do not support the reported role for gallic acid in the invasive success of

418 *P. australis.* Because we have not examined plant populations and soils sampled in previous

419 studies by Rudrappa, Bains and coauthors, we cannot rule out the occurrence of high

420 concentrations of free gallic acid and gallotannins in the populations they tested. Gallotannin

421 concentrations are known to vary seasonally (Salminen et al. 2001), and in response to various

422 environmental factors (Laponen et al. 1998; Agrell et al. 2000; Gross 2003), but we are not

423 aware of any reports showing intra-species variation in gallotannin concentration of the

424   magnitude which would explain the difference between our results and those of Bains et al.

425   (2009). Our plant populations were located in both freshwater and brackish wetlands, but it is

426   unclear whether the Delaware and Georgia sampling sites of Rudrappa et al. (2007) and Bains et

427   al. (2009) were freshwater, brackish, or marine wetlands. However, experiments reported by

428   these authors included root exudates collected in hydroponic culture and plants grown in pots,

429   with no indication of any differences in chemistry based on water salinity. Saltonstall (2003)

430   notes that the invasive *P. australis* genotype has spread along the North American Atlantic coast

431   as well as inland, suggesting that a common mechanism is responsible for the invasive success of

432   this genotype independent of wetland type.   Based on the nearly complete absence of gallic acid

433   in the four populations of invasive genotype that we sampled, we conclude that gallic acid cannot

434   be a primary, general explanation for the invasive success of *P. australis* in North America.

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TABLE 1. PHYSICAL AND CHEMICAL CHARACTERISTICS OF *P. AUSTRALIS* SOIL

Parameter	Value
pH	6.96
P, $\mu g\ g^{-1}$	54.8
K, $\mu g\ g^{-1}$	156
Ca, $\mu g\ g^{-1}$	2812
Mg, $\mu g\ g^{-1}$	268
Cation exchange capacity, meq $100\ g^{-1}$	16.7
Organic matter, %	11.74
Sand content, %	82%

TABLE 2. DEGRADATION RATE CONSTANT, K, AND HALF-LIFE, $T_{1/2}$, FOR

BREAKDOWN OF GALLIC ACID IN NONSTERILE SOIL

Concentration ($\mu g \ g^{-1}$)	r^2	k (day^{-1} ± 1 SE)	$t_{1/2}$ (days ± 1 SE)
500	0.95	2.17 (± 0.13)	0.32 (± 0.02)
1000	0.89	2.16 (± 0.19)	0.32 (± 0.03)
5000	0.91	2.29 (± 0.15)	0.30 (± 0.02)
10,000	0.95	0.76 (± 0.04)	0.91 (± 0.04)
20,000	0.80	0.22 (± 0.02)	3.13 (± 0.27)

TABLE 3. STABILITY AND RECOVERY OF GALLIC ACID FROM ACID HYDROLYSIS

PROCEDURE

Amount	Gallic acid (% of expected ± S.D.)	Methyl gallate (% of expected ± S.D.)
2 mg	98.6 ± 1.2	84.0 ± 8.9
10 mg	83.0 ± 2.3	73.3 ± 1.0
25 mg	72.4 ± 5.4	70.6 ± 1.8

TABLE 4. GALLIC ACID CONTENT OF RHIZOMES AND LEAVES FROM FOUR

POPULATIONS OF *P. AUSTRALIS*

	Rhizome sample 1		Rhizome sample 2	
Population[a]	µg g^{-1} FW found[b]	detection limit[c]	µg g^{-1} FW found	detection limit
OH-1	ND	10	ND	6.3
OH-2	ND	11	ND	7.8
NC-1	ND	17	TR	13
NC-2	TR	7.4	TR	5.2
	Foliage sample 1		Foliage sample 2	
	µg g^{-1} FW found	detection limit	µg g^{-1} FW found	detection limit
OH-1	ND	14	TR	14.6
OH-2	20	18	28	16
NC-1	TR	14	TR	13.1
NC-2	TR	13	TR	17.6

[a]OH = Ohio; NC = North Carolina. See methods for information on sample collection.

[b]ND = not detected; TR = trace level (below quantitation limit but definite peak at retention time for gallic acid)

[c]Detection limits calculated assuming quantitation limit of 0.25 mg l^{-1} and 70% recovery of gallic acid through hydrolysis and extraction procedure.

FIGURE LEGENDS

FIG. 1. Degradation of exogenously applied gallic acid in nonsterile _P. australis_ soil.
Standard errors for all data points in Fig. 1A were less than 3% and are not shown. In Fig. 1B, bars indicate standard error based on triplicate determinations.

FIG. 2. Gallic acid breakdown in soil monitored by HPLC. Chromatograms are based on absorbance at 272 nm, and show unidentified microbial breakdown products (marked by *) observed after addition of gallic acid (500 µg g^{-1}) to nonsterile _P. australis_ soil. (A) Initial extraction immediately after spiking soil; (B) 0.5 days; and (C) 1.5 days.

FIG. 3. Stability of exogenously applied gallic acid in sterile _P. australis_ soil. Standard errors for all data points in Fig. 3 were less than 4% and are not shown.

FIG. 4. RFLP analysis of Ohio and North Carolina _P. australis_ samples. Shown are ethidium bromide stained 3% agarose gels containing PCR products of two chloroplast regions, _rbcL_ and _trnLb,_ from the four collection sites used in this study. The _rbcL_ region of invasive strains contains an HhaI restriction enzyme recognition site while the _trnLb_ region of native strains contains an RsaI restriction enzyme site. The presence of two reduced band sizes in the HhaI digested rbcL products indicates that all four collection sites contained invasive strains.

FIG. 5. Separation of major components of _P. australis_ rhizomes and foliage.
Chromatograms are based on absorbance at 272 nm and show separation of major components of rhizomes and foliage: (A) Ohio population 2 rhizome; (B) Ohio population 2 foliage (inset shows apparent gallic acid peak); (C) North Carolina population 1 rhizome; (D) North Carolina population 1 foliage.   The peak eluting at 15.9 min (marked by an asterisk in each chromatogram below) was identified as an artifact of the acid hydrolysis extraction procedure (5-hydroxymethylfurfural). The other compounds in the extract are yet to be identified. The difference in retention time for gallic acid compared to Fig. 2 is due to aging of the HPLC column.

Supplemental FIG. 1. Mass spectrum of 5-hydroxymethylfurfural isolated from _P. australis_ extract (A) compared to NIST library spectrum (B).

Supplemental FIG. 2. Mass spectrum of suspected 2-methoxy-4-vinylphenol isolated from _P. australis_ extract (A) compared to NIST library spectrum (B).
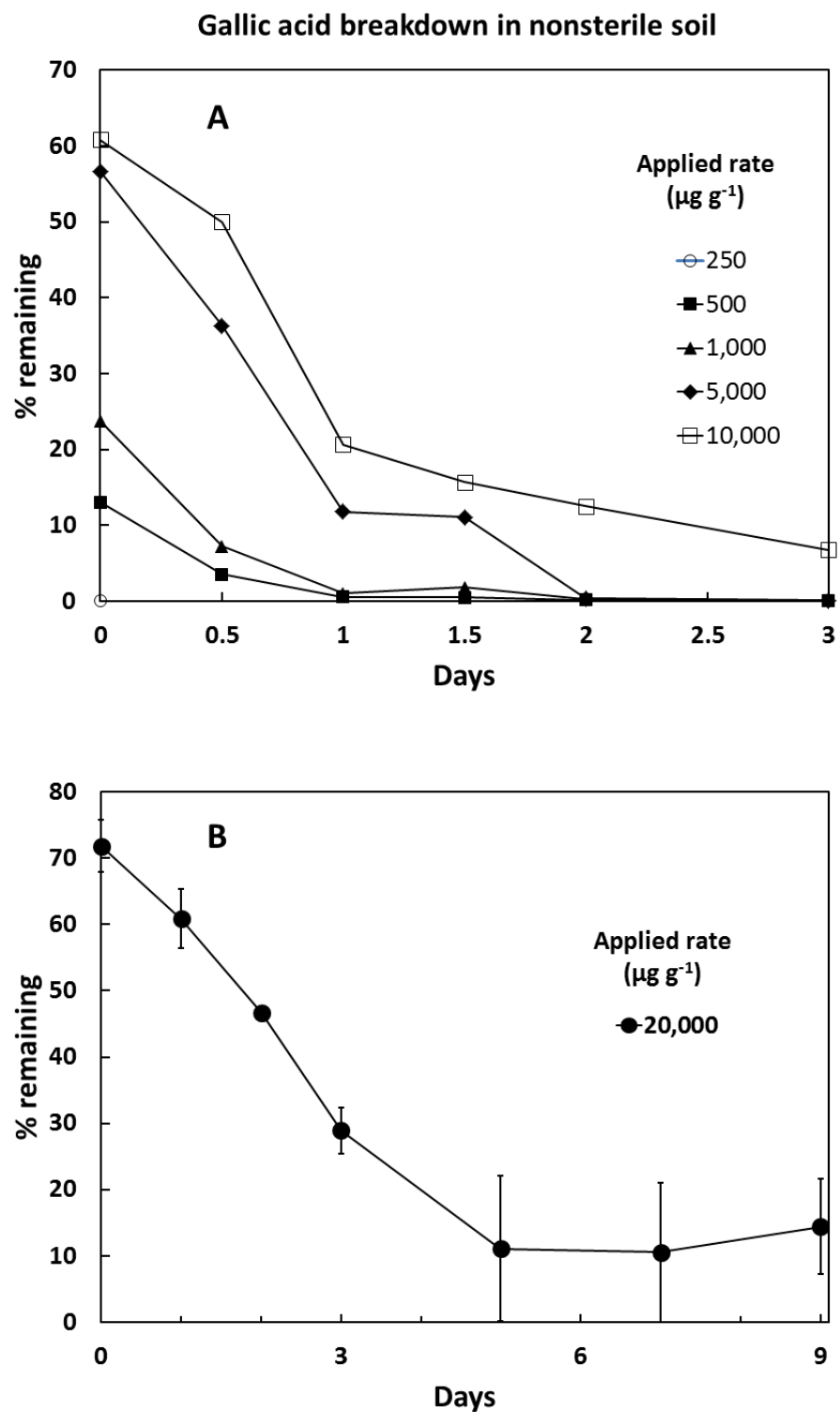
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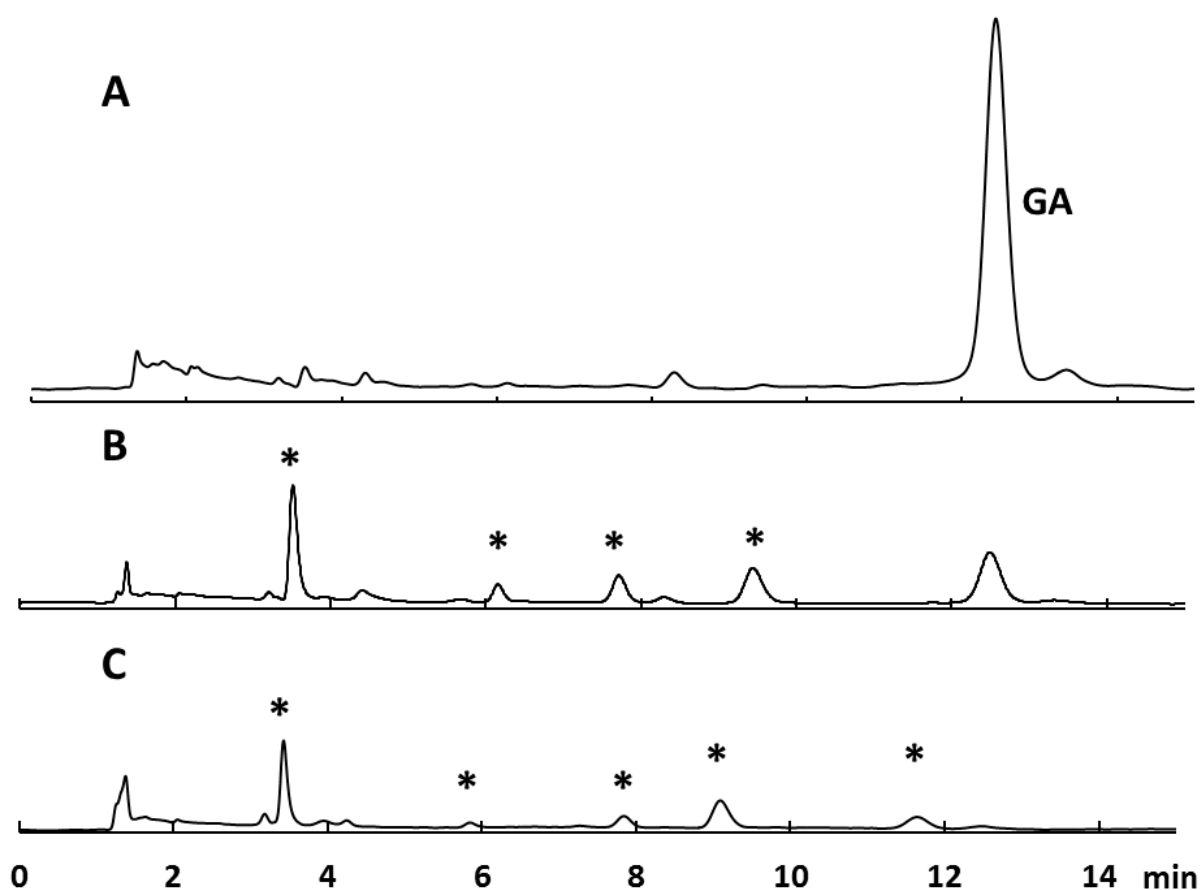bars indicate standard error based on triplicate determinations.

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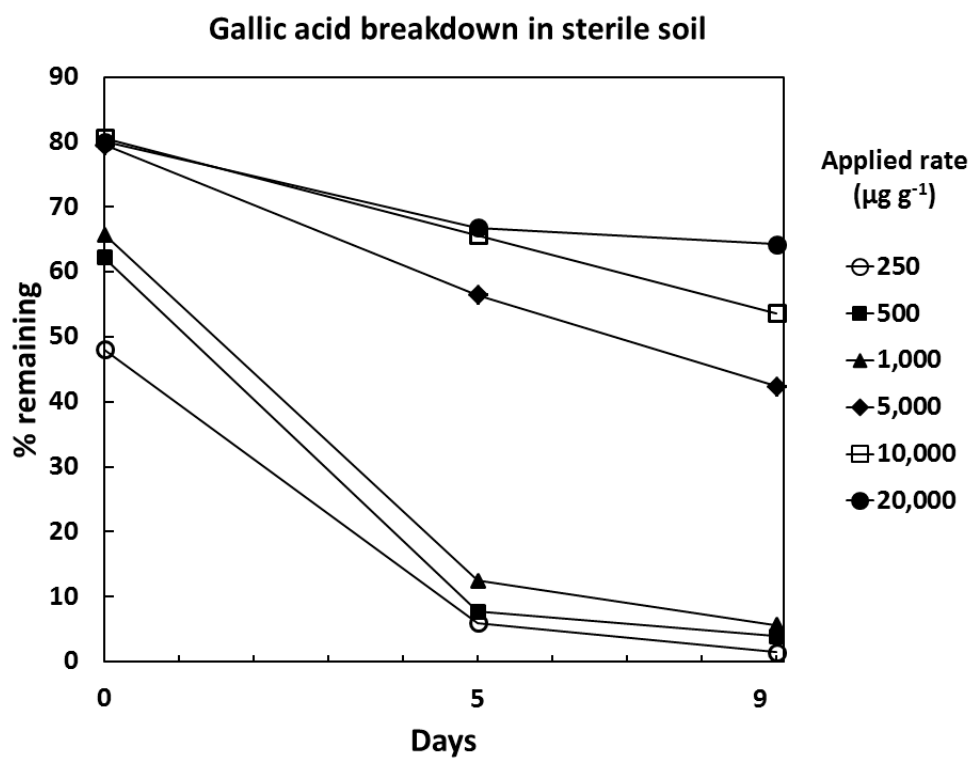
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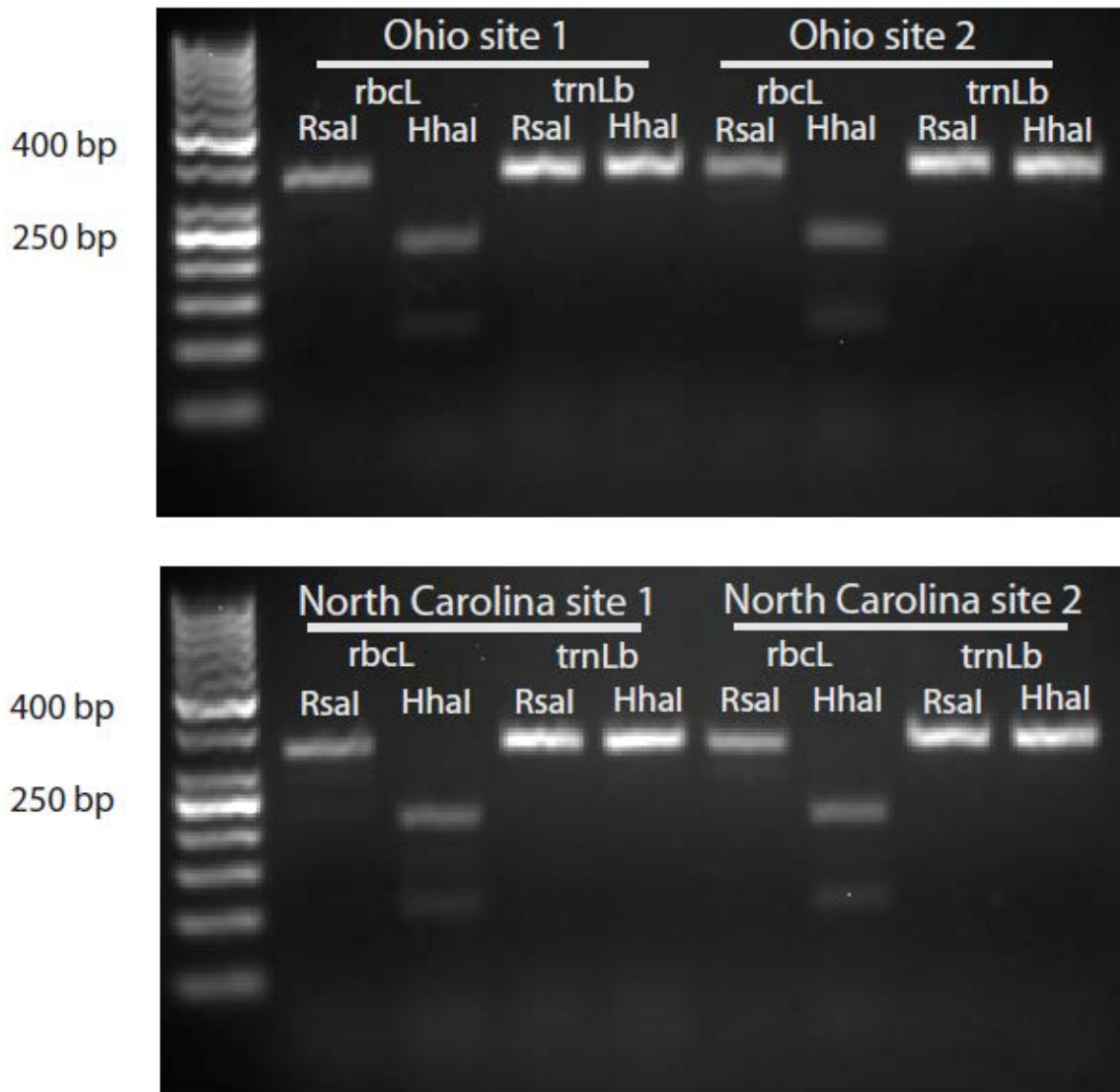
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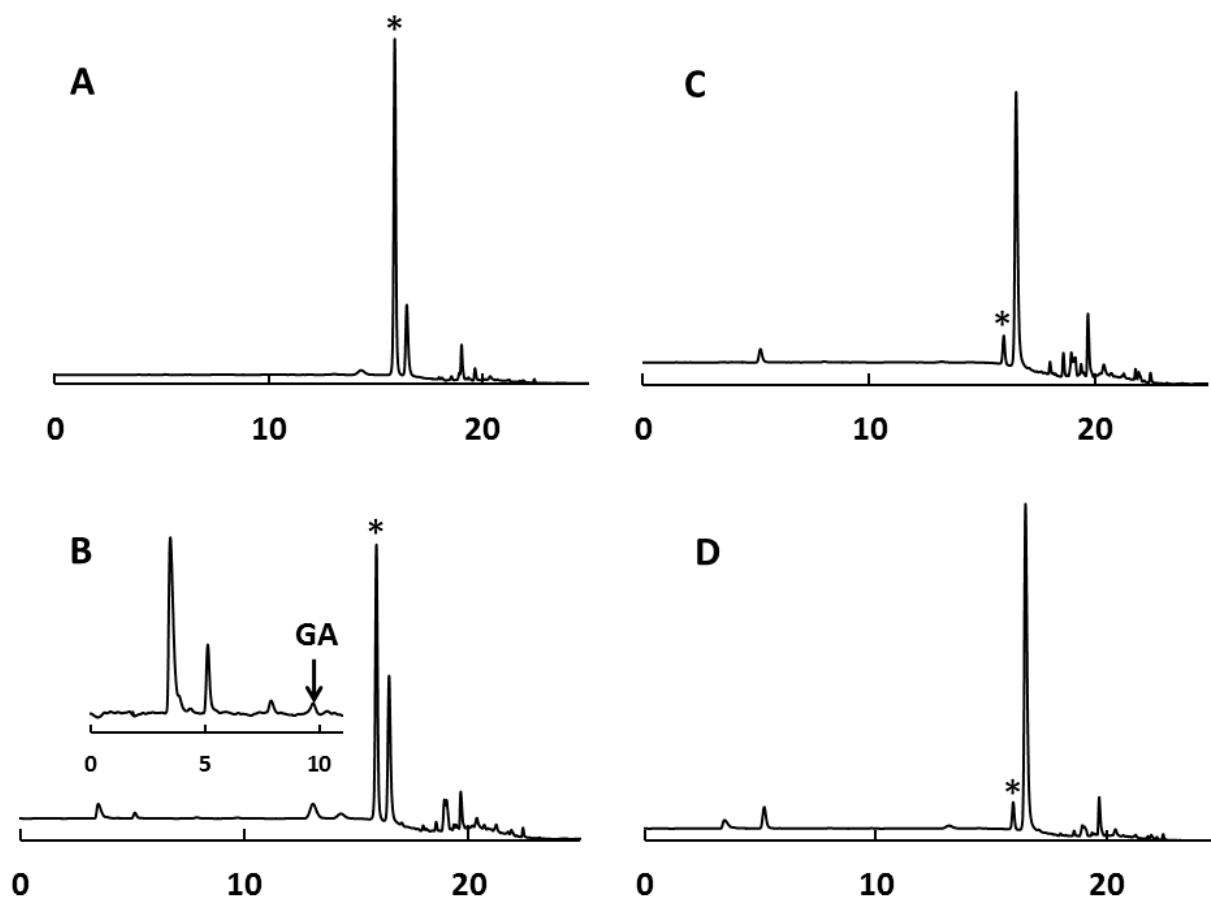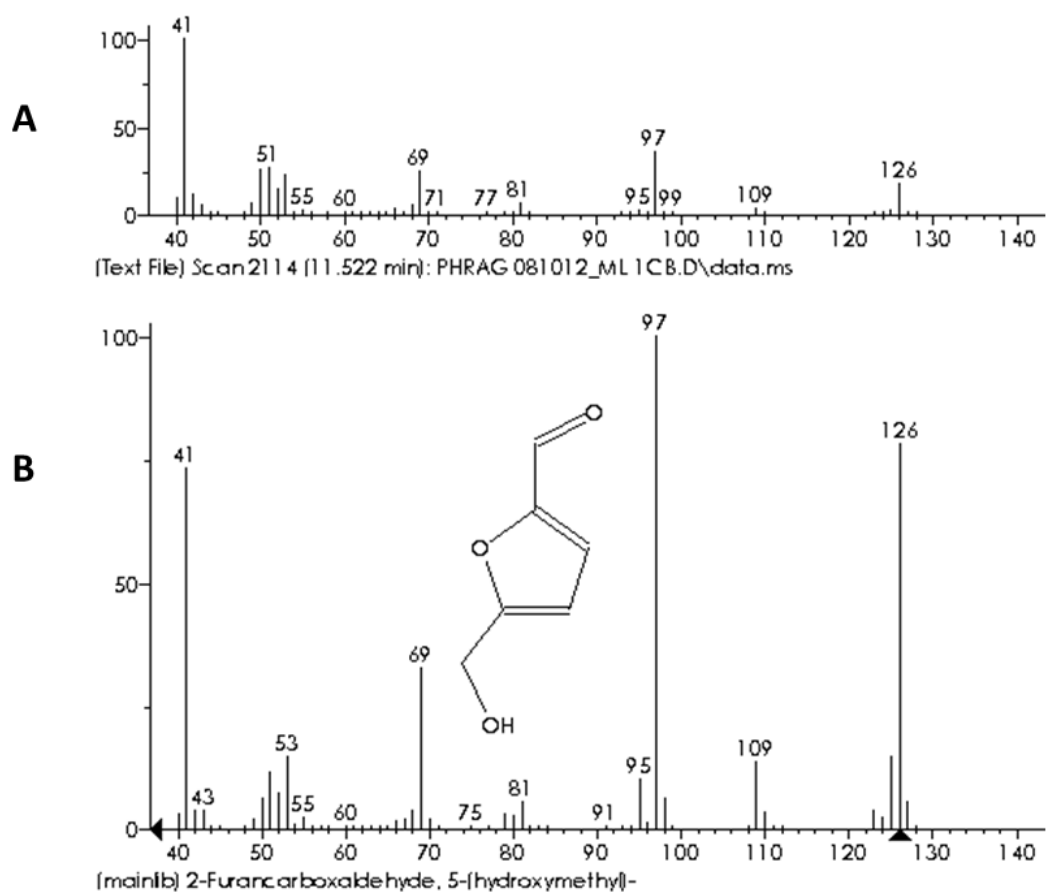
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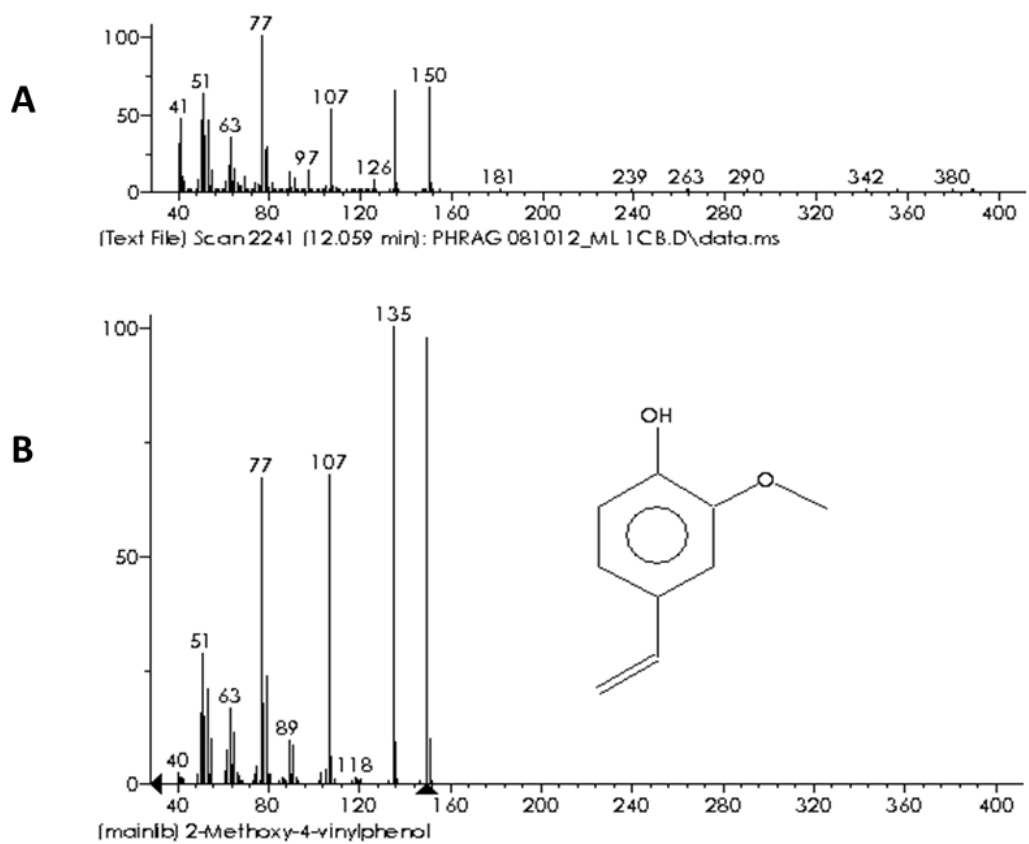
Supplemental Figure 2. Mass spectrum of suspected 2-methoxy-4-vinylphenol isolated from *P. australis* **extract (A) compared to NIST library spectrum (B).**