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## New insights into the specificity and processivity of two novel pectinases from *Verticillium dahliae*

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1           **New insights into the specificity and processivity of two novel**  
2                                   **pectinases from *Verticillium dahliae***

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21

22 **Abstract**

23 Pectin, the major non-cellulosic component of primary cell wall can be degraded by  
24 polygalacturonases (PGs) and pectin methylesterases (PMEs) during pathogen  
25 attack on plants. We characterized two novel enzymes, VdPG2 and VdPME1, from  
26 the fungal plant pathogen *Verticillium dahliae*. VdPME1 was most active on citrus  
27 methylesterified pectin (55-70 %) at pH 6 and a temperature of 40 °C, while VdPG2  
28 was most active on polygalacturonic acid at pH 5 and a temperature of 50 °C. Using  
29 LC-MS/MS oligoprofiling, and various pectins, the mode of action of VdPME1 and  
30 VdPG2 were determined. VdPME1 was shown to be processive, in accordance with  
31 the electrostatic potential of the enzyme. VdPG2 was identified as endo-PG releasing  
32 both methylesterified and non-methylesterified oligogalacturonides (OGs).  
33 Additionally, when flax roots were used as substrate, acetylated OGs were detected.  
34 The comparisons of OGs released from *Verticillium*-susceptible and partially resistant  
35 flax cultivars identified new possible elicitor of plant defence responses.

36

37 **Keywords:** Pectin, polygalacturonase, pectin methylesterase, flax,  
38 oligogalacturonides, *Verticillium dahliae*

39

## 40 **1. Introduction**

41 The plant cell wall is a complex and dynamic structure of proteins and  
42 polysaccharides, consisting of a hydrogen-bonded network of cellulose microfibrils  
43 and hemicelluloses embedded in a matrix of pectins [1]. Pectins are the most  
44 complex combination of plant cell wall polysaccharides and are found in the middle  
45 lamella and primary cell walls of dicotyledonous plants, where they can contribute up  
46 to 30 % of dry cell mass [2]. The composition of pectin differs depending on plant  
47 species and organs, but generally consist mainly of homogalacturonan (HG),  
48 rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) domains. HG, which  
49 is a linear homopolymer of  $\alpha$ -1,4-linked galacturonic acids (GalA), is the most  
50 abundant pectic domain representing up to 65 % of pectin [3]. HG can be  
51 methylesterified at the C-6 carboxyl and can be O-acetylated at O-2 or O-3. HG  
52 chains can be modified by different enzyme families, including pectin acetyltransferase  
53 (PAEs; EC 3.1.1.6), pectin methylesterases (PMEs; CE8, EC 3.1.1.11),  
54 polygalacturonases (PGs; GH28, EC 3.2.1.15, EC 3.2.1.67, EC 3.2.1.82), pectate  
55 lyases (PLs; EC 4.2.2.2) and pectin lyase (PNLs, EC 4.2.2.10). While these enzymes  
56 are endogenously produced by plants to fine-tune pectin structure during  
57 development, they are also secreted by some bacteria, fungi, insects and nematodes  
58 during plant infestation [4–8]. PMEs act on methylesterified GalA chain where they  
59 hydrolyse the O6-ester linkage between methyl group and GalA. PAE play similar  
60 role by hydrolysing the O2-acetylated linkage. Demethylesterification makes HG  
61 more susceptible to degradation by pectin-degrading enzymes such as PLs, PNLs  
62 and PGs [9]. PMEs may differ with respect to their pH optimum, substrate specificity  
63 and salt requirements, and have an optimum activity between pH 4-6 for fungal  
64 PMEs and pH 6-8 for plant PMEs [10]. It has been shown that plant and bacterial  
65 PMEs remove long stretches of methyl groups in a processive manner before  
66 dissociating from the HG chain, whereas fungal enzymes act more randomly and are  
67 considered as non-processive PMEs [11]. PGs are a family of hydrolases that cut the  
68 HG chain releasing oligogalacturonides (OGs) of different degree of polymerisation  
69 (DP) and methylesterification/acetylation. Depending on their mode of action, PGs  
70 are subdivided into endo-PGs (EC 3.2.1.15), which randomly hydrolyse internal sites  
71 and exo- PGs (EC 3.2.1.67, EC 3.2.1.82) that hydrolyse HG from non-reducing end,  
72 creating monomers or dimers of GalA. These two types of PGs preferably act on

73 partially demethylesterified HG chains and have been identified in various fungal  
74 species where they can be a determinant of the pathogenicity [12]. Indeed, various  
75 fungi secrete pectinases, including PGs to degrade plant cell walls, and invade cells.  
76 For instance, it has been shown that Botrytis PG1 and Verticillium PG1 are important  
77 virulence factors in tomato and cotton [13,14] respectively. *Verticillium dahliae* Kleb  
78 soil-borne vascular fungus, which attack a broad range plants and has increasing  
79 effects on flax species, also produces a number of pectinases for degrading cell wall.  
80 Infection of *V.dahliae* occurs at the root surface levels, later invading xylem vessels  
81 with progression in acropetal direction [15,16]. To date, without effective chemical  
82 control, there is no flax cultivar totally resistant to Verticillium wilt [16], thus it appears  
83 of prime importance to understand the pathogenicity of this fungus by characterizing  
84 pectinases such as PGs and PME. Such a characterisation is indeed required to  
85 devise strategies useful to control or inhibit pathogenic activity and must occur on  
86 multiple levels: from the characterisation of the protein mechanism of action to the  
87 enzymatic structures and dynamics, which provide information on substrate  
88 specificity and processive tendencies of different PMEs and PGs. Considering the  
89 large number of protein isoforms and ubiquitous presence of PMEs and PGs across  
90 plants, bacterial and fungi, relating the functional behaviour and the structural  
91 features of PGs and PMEs is challenging. The lack of knowledge for these enzymes  
92 is aggravated by the relatively scarce number of resolved structures, compared to the  
93 whole number of PGs and PMEs isoforms. More recently the *in-silico* comparison of  
94 PMEs electrostatic potentials has underscored the relation between protein  
95 electrostatics and substrate specificity, highlighting the importance of balancing  
96 electrostatic vs. hydrophobic interactions within the binding groove of PMEs to  
97 promote fine-tuning of substrates specificity and different processivity profiles [17].  
98 Although the fold of PGs and PMEs is conserved, with a binding groove able to  
99 allocate approximately 10 saccharide units [18] in as many subsites, subtle  
100 differences exist between the chemical microenvironments of different subsites,  
101 which can either bind, with different affinities, negatively charged, carboxy-methylated  
102 or O-acetylated saccharide units. The structural and biochemical description of newly  
103 identified PGs and PMEs, with the combination of *in-vitro*, *ex-vivo* and *in-silico*  
104 methods, thus become powerful tools to characterize and contextualize the action of  
105 these enzymes within plant physiology and pathology.

106 The aims of this study are to characterize two novel enzymes from *V. dahliae*,  
107 VdPME1 (VDAG\_05799) and VdPG2 (VDAG\_04977) using biochemical and  
108 computational approaches. In particular, their mode of action can be determined using a  
109 recently developed SEC-MS technique [19], which enables analysis of the OGs  
110 generated from commercial pectin as well from flax roots. Our hypothesis is that we  
111 can relate the structure of enzymes to their processivity and this can have impact on  
112 the OG produced during plant-pathogen interactions.

## 113 **2. Material and methods**

### 114 **2.1. Bioinformatical analysis**

115 *V. dahliae* PGs and PMEs were identified using publicly available genome data  
116 (<ftp.broadinstitute.org/>). Sequences were checked for signal peptide using SignalP-  
117 5.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). Glycosylation sites were  
118 predicted using NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and  
119 NetOGlyc 4.0 Server (<http://www.cbs.dtu.dk/services/NetOGlyc/>). The sequence  
120 alignments were performed using MEGA multiple sequence alignment program.  
121 UCSF Chimera (<http://www.cgl.ucsf.edu/chimera/>) was used for creation of graphics.

### 122 **2.2. Modelling, calculations and comparison of protein electrostatic**

123 VdPME1 and VdPG2 models were created using I-TASSER structure prediction  
124 software (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The linearized version  
125 of the Poisson-Boltzmann equation was used to solve the electrostatic potentials of  
126 the PMEs from *Verticillium dahliae*, *Dickeya chrysanthemi*, *Citrus sinensis* and  
127 *Daucus carota* after that radii and partial charges were assigned to each atom of the  
128 structures according to the parameters from the AMBER99 force field [20] by using  
129 PDB2PQR (version 2.1.1, [21]).

130 The PMEs structures were superimposed to fairly compare the electrostatic potential  
131 differences across the calculated grids, and the electrostatic potentials were  
132 calculated using the APBS software version 3 [22]. The potentials were computed at  
133 pH 7, which is the pH used for the activity tests of VdPME1, with protonation states of  
134 single residues assigned using the PROPKA software, version 3.3 [23]. Poisson-  
135 Boltzmann equation was solved by discretizing the molecule on a 19.3 nm<sup>3</sup> grid (grid  
136 spacing equal to 6x10<sup>-2</sup> nm) centred on the C<sub>α</sub> atom of one of the PMEs catalytic  
137 aspartic acid residues, conserved across PMEs. The computation of the electrostatic

138 potential was carried out considering a dielectric term of 78.5 for the solvent, in order  
139 to account for an aqueous environment, with solute dielectric set to 4.0 and  
140 temperature set at 298.15 K.

141 Electrostatic potentials were numerically compared by calculating electrostatic  
142 similarity indices [24,25]. We calculated the cross-product between two electrostatic  
143 potentials calculated at each grid point as follows:

144

$$145 \quad SI_{a,b}^H = \frac{2\phi_a(i,j,k)\phi_b(i,j,k)}{(\phi_a^2(i,j,k) + \phi_b^2(i,j,k))}$$

146 Where  $\phi_a^2(i,j,k)$  and  $\phi_b^2(i,j,k)$  are the electrostatic potentials calculated at the grid  
147 points  $i,j,k$  for proteins  $a$  and  $b$  [24,25].

### 148 **2.3. Fungal strain and growth**

149 *V.dahliae* was isolated from CALIRA company flax test fields (Martainneville, France)  
150 and was kindly provided by Linéa-Semences company (Grandvilliers, France).  
151 Fungus was grown in flasks containing 50 mL M3 medium [26] enriched with different  
152 carbon source: polygalacturonic acid sodium salt (PGA, P3850, Sigma) and pectin  
153 esterified potassium salt from citrus fruit (55-70 % methylesterified, P9436, Sigma) at  
154 10 g.L<sup>-1</sup> in order to stimulate *PG* and *PME* expression and secretion. Flasks were  
155 kept 15 days in dark condition under 25°C, 80 rpm agitation, and mycelium was  
156 harvested by vacuum filtration using Buchner flask. Fresh mycelium was frozen in  
157 liquid nitrogen, lyophilized and ground using mortar and pestle. Total RNA isolation  
158 cDNA synthesis was as previously described in Lemaire et al. 2020 [27].

## 159 2.4. Cloning and heterologous expression of VdPG2 and VdPME1

160 One PG, VDAG\_04977 (hereafter named VdPG2, gene ID: 20706440) and one  
161 PME, VDAG\_05799 (hereafter named VdPME1, gene ID: 20707262) were amplified  
162 using cDNA and gene-specific primers listed in **Table 1** excluding the signal peptide.

163 **Table 1. Primers used for cloning *Verticillium dahliae* enzymes into pPICZαB expression**  
164 **vectors.** VdPG2: Polygalacturonase; VdPME1: Pectin methylesterase. Restriction enzymes sites for  
165 *EcoRI*, *PstI*, *NotI* are underlined, added bases are written in *italics*.

Enzyme	Gene ID	Forward 5'- 3'	Reverse 3'- 5'
VdPG2	20706440	TCTAAGA <u>AATTC</u> ACAACCCCTCTTCCCGCCAAG	TGCACGCGGCCCGCAGAGCACGCGGCAGGG
VdPME1	20707262	TCTAACTGCAGGAGCCACGAGGACCTCG	TGCACGCGGCCCGCCATGTACGAAGCGTCATAGTAG

166

167 Amplified genes were gel purified with gel extraction kit (Neo biotech, Nanterre,  
168 France), ligated to pPICZαB (Invitrogen, Carlsbad, California, United States) in frame  
169 with His-tag, previously digested with *PstI* and *NotI* for VdPG2 and *EcoRI* and *NotI*  
170 for VdPME1, and used for transformation of *E. coli* TOP10 (Invitrogen). After  
171 sequencing, linearized vector was used to transform *Pichia pastoris* X33 strain as  
172 described in the instruction manual EasySelect Pichia Expression Kit manual  
173 (Invitrogen).

## 174 2.5. VdPG2 and VdPME1 expression, purification and enzyme analysis

175 VdPG2 and VdPME1 were produced in *Pichia pastoris* as described in the  
176 EasySelect Pichia Expression Kit manual (Invitrogen) and in Lemaire et al. (2020).  
177 After purification, enzyme buffer was changed to reaction buffer using PD Spintrap G-  
178 25 column (GE Healthcare). Determination of protein concentration, glycosylation  
179 patterns, enzyme purity and molecular weight were as described in Lemaire et al.  
180 2020 [27].

## 181 2.6. VdPG2 and VdPME1 biochemical characterization

182 The substrate specificity of VdPG2 was determined with the DNS method [28] using  
183 polygalacturonic acid (81325, Sigma); Citrus pectin with degree of  
184 methylesterification (DM) 20-34 % (P9311, Sigma), DM 55-70 % (P9436, Sigma) or  
185 DM >85 % (P9561, Sigma); apple pectin with DM 70-75 % (76282, Sigma); sugar



186 beet pectin with DM 42 % and degree of acetylation 31 % (DA, CP Kelco, Atlanta,  
187 United States) using  $2.45 \cdot 10^{-3} \mu\text{g} \cdot \mu\text{L}^{-1}$  VdPG2 as described in Habrylo et al. 2018 [4].  
188 Results were expressed as  $\text{nmol of GalA} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$  of proteins.

189 The substrate specificity of VdPME1 was determined using above-mentioned  
190 substrates and the alcohol oxidase assay [29,30] with modifications as described in  
191 L'Enfant et al. 2019 [31]). VdPME1 concentration was  $2.3 \cdot 10^{-3} \mu\text{g} \cdot \mu\text{L}^{-1}$ . Results were  
192 expressed as  $\text{nmol of MeOH} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$  of proteins.

### 193 **2.7. VdPG2 and VdPME1 temperature, salt and pH dependency assay**

194 The VdPG2 optimum temperature was determined by incubating the enzymatic  
195 reaction from 20 to 70 °C during 60 min using polygalacturonic acid (0.4 %, w/v)  
196 diluted in 50 mM ammonium acetate buffer (pH 5). The VdPG2 pH optimum was  
197 determined between pH 3 and 8 using sodium acetate buffer (pH 3 to 5) and Tris-HCl  
198 buffer (pH 6 to 8) and PGA as a substrate at 0.4 % (w/v) final concentration. The  
199 VdPME1 pH optimum was determined by mixing citrus pectin DM 55-70 % (P9436,  
200 Sigma) at 0.4 % (w/v) final concentration using sodium acetate buffer (pH 3 to 5) and  
201 Tris-HCl buffer (pH 6 to 9). The VdPME1 optimum temperature was determined by  
202 incubating the enzymatic reaction from 10 to 60 °C using citrus pectin DM 55-70 %  
203 (P9436, Sigma) at 0.4 % (w/v) diluted in 50 mM sodium phosphate buffer (pH 7) as  
204 mentioned above. VdPME1 activity was calculated using a standard curve between  
205 saponified and non-saponified samples as  $\text{nmol of MeOH} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$  of proteins. All  
206 experiments were conducted in triplicate.

### 207 **2.8. Determination of $K_m$ , $V_{max}$ , and specific activity**

208 The VdPG2 kinetic parameters were calculated using GraFit7 software (Michaelis-  
209 Menten/Hill; Erithacus Software, Horley, Surrey, UK) using PGA as a substrate. The  
210 reactions were performed using 1 to 20  $\text{mg} \cdot \text{mL}^{-1}$  PGA concentrations at 50 mM  
211 sodium acetate (pH 5) during 10 min at 50 °C. The same procedure was used for  
212 VdPME1 with 1 to 20  $\text{mg} \cdot \text{mL}^{-1}$  pectin DM 55-70 % concentrations in 50 mM sodium  
213 phosphate buffer (pH 7) during 20 min at 30 °C.

### 214 **2.9. Oligoprofiling of digested commercial pectins**

215 Oligogalacturonides (OGs) released after digestions by recombinant VdPG2 and  
216 VdPME1 were identified as described in Voxeur et al. 2019 [19]. Briefly, citrus pectin

217 of DM 24-30 % (P9311, P9436, Sigma), DM 55-70 % (P9436, Sigma) or DM >85 %  
218 (P9561, Sigma) were prepared at 0.4 % (w/v) final concentration in 50 mM  
219 ammonium acetate buffer (pH 5) and incubated with VdPG2 at  $2.45 \cdot 10^{-3} \mu\text{g} \cdot \mu\text{L}^{-1}$   
220 concentration during 15 min, 45 min, 90 min, 180 min and overnight at 40 °C. To  
221 analyse the processivity of VdPME1, citrus pectins DM 55-70 % (P9436, Sigma)  
222 were digested with VdPG2 for 2 h (to obtain OG of various DP and DM) as  
223 mentioned above. Resulting OGs were lyophilised, and resuspended in 90  $\mu\text{L}$  50 mM  
224 Tris-HCl buffer (pH 7). 10  $\mu\text{L}$  of VdPME1 at  $2.3 \cdot 10^{-3} \mu\text{g} \cdot \mu\text{L}^{-1}$  concentration was added  
225 and the reaction was incubated for 15 min and overnight at 40 °C. The rest of the  
226 procedure was as previously described. Pellets were resuspended in 200  $\mu\text{L}$  dH<sub>2</sub>O.

## 227 **2.10.Oligoprofiling of digested cell wall pectins from flax roots**

228  
229 Flax seeds from two different cultivars, Évéea (partially resistant to *Verticillium* wilt)  
230 and Violin (more susceptible to *Verticillium* wilt) were kindly provided by Linéa-  
231 Semences (Grandvilliers, France). Seeds were sterilized using Triton 0.01 % (w/v,  
232 T8787, Sigma) diluted in ethanol 70 % (w/v) and dried overnight. Seeds grew during  
233 three days on wet towel tissue at 21 °C, 16 h/8 h light/dark. Forty roots were cut and  
234 placed into ethanol 100 % (w/v) for 24 h. They were washed two times for 5 min with  
235 acetone 100 % (w/v) and left to dry 24 h. Roots were rehydrated in 140  $\mu\text{L}$  50 mM  
236 ammonium acetate pH 5 during 2 h at room temperature and digested with VdPG2 at  
237  $2.45 \cdot 10^{-3} \mu\text{g} \cdot \mu\text{L}^{-1}$  concentration, using the above-mentioned protocol.

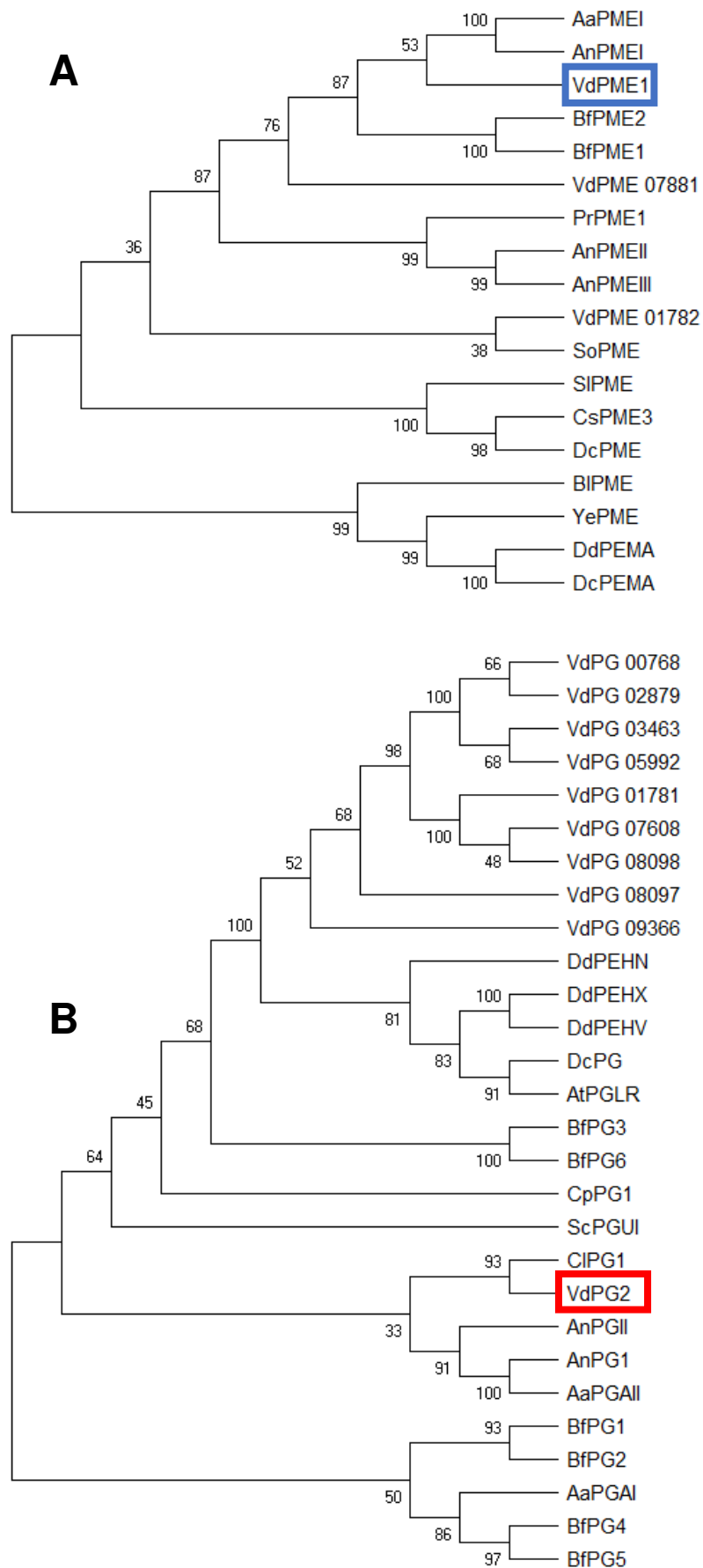
## 238 **2.11.Ultra-performance size-exclusion chromatography (UP-SEC)** 239 **coupled with electrospray ionization high-resolution mass spectrometry (ESI-** 240 **HRMS)**

241 OGs produced from above-mentioned commercial pectins and flax roots after  
242 digestion were subjected to chromatographic separations and MS-detection as  
243 described in Hocq et al. 2020 [32]. We have determined, according to Voxeur et al.  
244 2019 [19], the relative amount of each oligogalacturonide (OG) comparing its peak  
245 area to the peak area of total OGs (sum of all areas) detected for each sample. Only  
246 the most abundant OGs, present as more than 1% of the total OGs, were plotted.  
247 With this method we cannot compare OGs between them, but we can emphasize the  
248 comparison, for a given OG, between different conditions such as substrates,  
249 cultivars as well as enzymes used for digestions.

## 250 **3. Result and discussion**

### 251 **3.1. Sequence analysis and phylogeny**

252 *V. dahliae* encodes more than 40 putative pectinolytic enzymes. Among them, 30  
253 PLs and PNLs (belonging to PL1, PL3 and PL9 families), 9 PGs (including putative  
254 endo and exo) and 4 PMEs. We first performed phylogenetic analysis and compared  
255 Verticillium PGs and PMEs protein sequences with selected bacterial, fungal, insect  
256 and plant enzymes. The 18 PMEs clustered into five clades with plant PMEs (carrot  
257 DcPME, orange CsPME3 and tomato SIPME) in a distinct clade, as well as insect  
258 rice weevil PME (SoPME) (**Fig. 1A**). VdPME1 appears to be closely related to the  
259 two other PMEs from *Aspergillus* species, which are recognized as non-processive  
260 PMEs in their mode of action. VdPME1 showed 51.23 % and 51.08 % sequence  
261 identity with AaPMEI [33] and AnPMEI [34], respectively. As shown on **Fig. 1B** PGs,  
262 comprising 28 sequences, which clustered in seven clades allowing clear separation  
263 between putative endo and exo PGs from *V.dahliae* (VdPG 02879, VdPG 03463,  
264 VdPG 05992, VdPG 07608 VdPG 00768, VdPG 01781, VdPG G08089) with endo  
265 VdPG2 (04977). The plant PGs from *D. carota* (DcPG) and *A. thaliana* PGs  
266 (AtPGLR) clustered in a separated clade as well as PGs from yeast *S. cerevisiae*  
267 (ScPGUI) and fungal *C. purpureum* PG1 (CpPG1). VdPG2 forms an independent  
268 clade with fungal PGs from *C. lupini var. setosum* (CIPG1. 68.14 % sequence  
269 identity) which was shown to be endo-PGs [35].



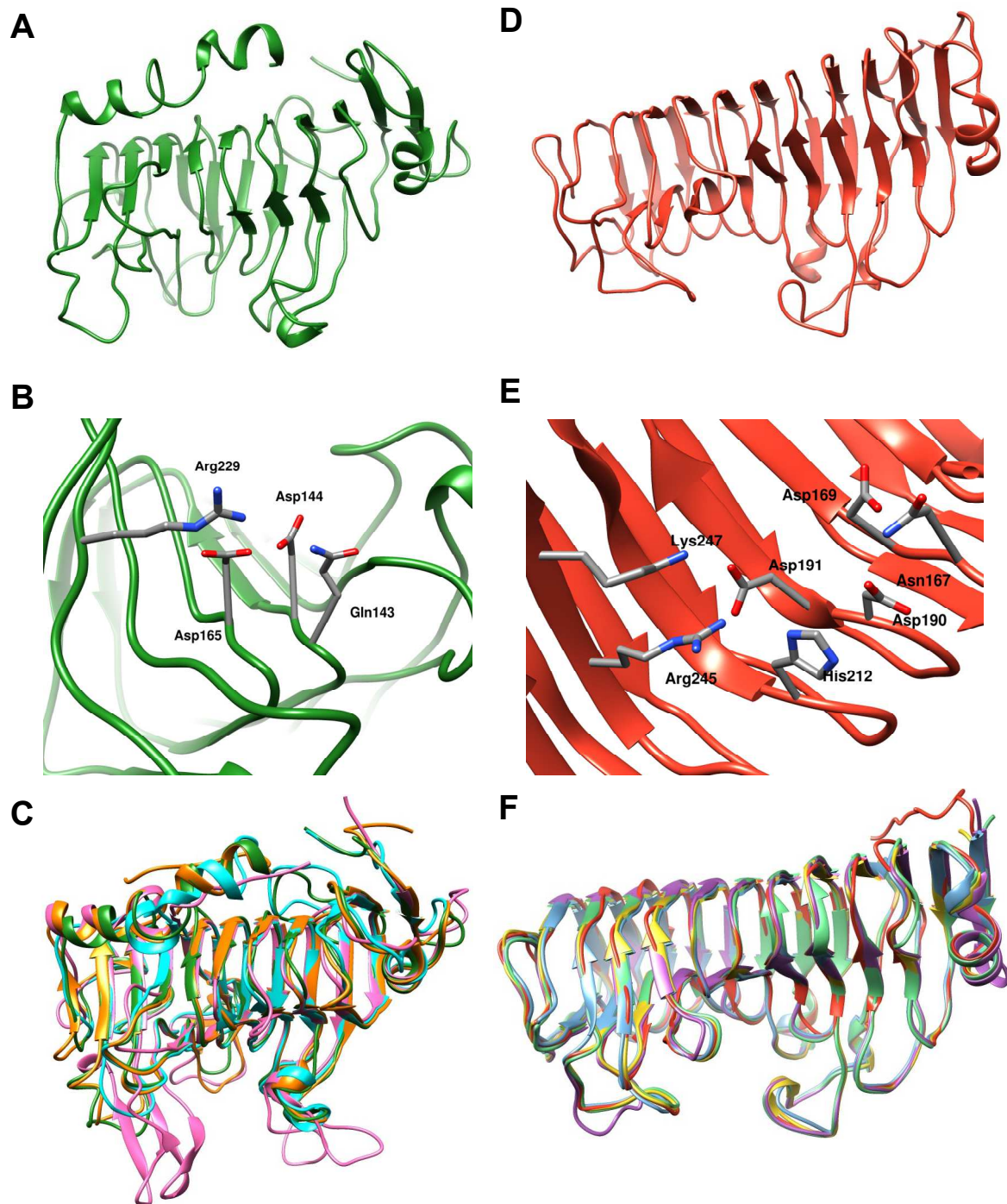
270

271 **Fig. 1. Phylogenetic relationships of VdPME1 and VdPG2 with selected fungal and bacterial**  
 272 **enzymes.**

273 Phylogenetic tree for PMEs (A) and PGs (B) *V. dahliae* PG2 (VdPG2, **VDAG\_04977**) and PME1  
274 (VdPME1, **VDAG\_05799**) are red and blue-boxed, respectively. Amino acids sequences used are the  
275 following: *V. dahliae* PMEs (VDAG\_07881, VDAG\_1782) and *V. dahliae* PGs (VDAG\_00768,  
276 VDAG\_01781, VDAG\_02879, VDAG\_03463, VDAG\_05992, VDAG\_07608, VDAG\_08097,  
277 VDAG\_08098, VDAG\_09366). *B. licheniformis* PME (Q65F39). *D. dadantii* PEHN (E0SDX9), PEHX  
278 (E0SKR4), PEHV (E0SKR2), PEMA (P0C1A9). *D. chrysanthemi* PEMA (P0C1A8). *Y. enterocolitica*  
279 PME (A1JJ76). *C. purpureum* PG1 (P79074) *B. fuckeliana* PGI (Q4G496), PG2 (Q4G495), PG3  
280 (Q9Y7V9), PG4 (Q9Y7W0), PG5 (Q9Y7W1), PG6 (Q9Y7W2), PME1 (A0A384JQ57), PME2  
281 (A0A384JCI5). *A. aculeatus* PGAI (O74213), PGAI (Q70HJ4), PME1 (Q12535). *A. niger* PGI  
282 (P26213), PGAI (P26214), PME1 (P17872), PMEII (G3YAL0), PMEIII (A0A345K402). *P. rubens* PME1  
283 (B6HGX6). *C. lupini var. setosum* PG1 (A1E266). *S. cerevisiae* PGUI (P47180). *S. oryzae* PME  
284 (E7CIP7). *D. carota* PME (P83218), PG (Q75XT0). *S. lycopersicum* PME (P14280). *C. sinensis* PME3  
285 (P83948). *A. thaliana* PGLR (Q9LYJ5). The maximum-likelihood tree was deduced from the genetic  
286 distances between aligned amino-acid sequences using MEGA. UniProt accession numbers were  
287 used.

### 288 **3.2. Homology modelling and structure analysis**

289 To fully understand the structural features of VdPG2 and VdPME1, their structures  
290 were modelled using the I-TASSER server for protein structure prediction. VdPME1  
291 model was created using *D. carota* PME (P83218, PDB:1GQ8, [36]) as the template,  
292 with 31.42 % amino acid (AA) identity and 46.6 % similarity. Although VdPME1  
293 doesn't share a high degree of identity with its template, the modelling remarkably  
294 provided a structure with a root mean square deviation (RMSD) of only  $4.1 \pm 2.8 \text{ \AA}$ . The  
295 model consisted of 314 AA, without the 16 AA of the signal peptide. Modelling shows  
296 that the enzyme is a right handed  $\beta$ -helix (**Fig. 2A**) structure that shares four highly  
297 conserved regions, as well as key active site residues characterized in other PMEs.  
298 These four regions comprise one N terminal region (Gly47, Ser48, Tyr49, Ala50 and  
299 Glu51), two internal regions (Tyr166-Phe166 (mutation in VdPME1) Gly167, Asp168,  
300 Thr169 and Asp165, Phe166, Ile167, Phe168 and Gly169) and one C-terminal region  
301 (Leu227, Gly228, Arg229, Pro300 and Trp301) [37]. The AA of the active site are  
302 Gln143, Asp144, Asp165 and Arg229 (**Fig. 2B, Fig. S1**) where the two Asp act as a  
303 general acid/base in the catalytic mechanism [18,38]. The VdPME1 showed high  
304 structural superposition with *D. dadantii*, *A. niger* and *D. carota* PMEs (PDB code  
305 2NT6, 5C1C, 1GQ8, **Fig. 2C**).



306

307 **Fig. 2. Homology modelling and structural comparison of VdPME1 and VdPG2.**

308 (A) VdPME1 homology model created using I-TASSER. (B) Structure of the active site of VdPME1.  
 309 Gln-143, Asp-144, Aps-165 and Arg-229 (VdPME1 numbering) are coloured in grey. (C) Structural  
 310 alignment of VdPME1 with crystalized enzymes from *D. carota* (DcPME, orange, PDB code 1gq8), *D.*  
 311 *dadantii* (DdPME, pink, PDB code 2nt6) and *A. niger* (AnPME, cyan, PDB code 5c1c). (D) VdPG2  
 312 homology model created using I-TASSER. (E) Structure of the active site of VdPG2. Asn-167, Asp-  
 313 169, Aps-190 and Aps-191, His-212, Arg-245, Lys-247 (VdPG2 numbering) are colored in grey. (F)  
 314 Structural alignment of VdPG2 PGs with crystalized enzymes from *A. niger* (AnPGII, bleu, PDB code

315 1czf), *A. niger* (AnPGI, green, PDB code 1nhc), *A. acuelatus* (AaPG, purple, PDB code 1ia5) and *C.*  
316 *lupini* (CIPG1, yellow, PDB code 2iq7, A1E266).

317 The VdPG2 model was created using *A. acuelatus* PGAI (O74213, PDB:1IA5, [39] as  
318 a best template with an estimated RMSD of  $5.6 \pm 3.5 \text{ \AA}$ . VdPG2 exhibits lower  
319 sequence identity with AaPGAI (55.74 %) compared to CIPG1 (68.14 %) but higher  
320 structural homology. The final model consists of 352 AA without the 18 AA of the  
321 signal peptide. The structure is a right handed  $\beta$ -helix with 10 complete turns (**Fig.**  
322 **2D**). Members of GH28 family, including endo and exo PGs have conserved AA  
323 motifs, including Asn167, Thr168, Asp169 (NTD) Asp189, Asp190 (DD), Gly211,  
324 His212, Gly213 (GHG), Arg244, Ile245, Lys246 (RIK, **Fig. 2E**, **Fig. S2**, [40,41]) in  
325 VdPG2. Structural alignment with published PGs structures [35,39,42] confirmed that  
326 VdPG2 is likely to be an endo-PG with a tunnel like active cleft (**Fig. 2F**).  
327 Furthermore, while the catalytic amino acids are strongly conserved, tunnel like  
328 active cleft having the same shape differ in size, specifically regarding the size of the  
329 substrate which could be accepted [42].

### 330 **3.3. Cloning, expression and purification of VdPG2 and VdPME1**

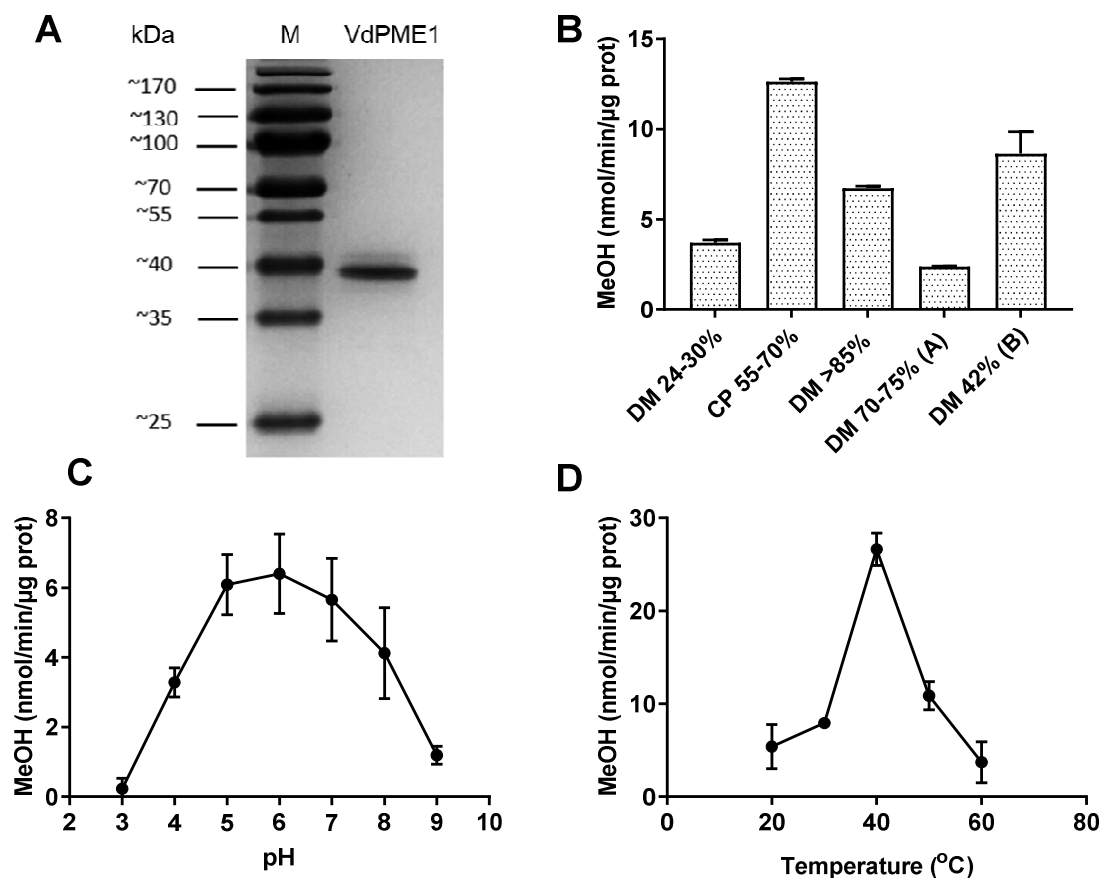
331 The *VdPG2* (VDAG\_04977) and *VdPME1* (VDAG\_05799) genes consisting of 1113  
332 and 993 bp, respectively; were amplified using gene specific primers without the  
333 signal peptide (**Table 1**), ligated in pPICZ $\alpha$ B vector, expressed in *P. pastoris* and  
334 secreted in the culture media. The open reading frame consisted of 384 and 343 AA  
335 for VdPG2 and VdPME1, respectively, containing the poly-histidine tag for affinity  
336 chromatography purification. Following purification, VdPME1 and VdPG2 were  
337 resolved on SDS-PAGE, having an apparent molecular mass of ~ 39 and 50 kDa,  
338 respectively (**Fig. 3A, 4A**). The observed molecular mass is slightly higher compared  
339 to the predicted mass of 36.5 and 39.7 kDa, which could be related to the presence  
340 of two (Asn84, Asn201) and five (Asn122, Asn199, Asn220, Asn232, Asn331)  
341 potential N-glycosylation sites in VdPME1 and VdPG2, respectively. Additional O-  
342 glycosylation sites were predicted in VdPME1. The occurrence of glycosylation was  
343 confirmed through digestion with PNGase, leading to the expected shift in molecular  
344 mass for both enzymes (**Fig. S3**).

### 345 **3.4. Biochemical characterization of VdPME1**



346 Although PME<sub>s</sub> from different species catalyse the same reaction, they can differ in  
347 their pH and temperature optima, substrate specificities and processivity [43].  
348 VdPME1 exhibited the highest activity on moderately methylesterified citrus pectin  
349 (DM 55-70 %, **Fig. 3B**), albeit an activity was also detected on a wide range of pectic  
350 substrates that varied in their DM. VdPME1 activity was 68 % of the maximum on  
351 sugar beet pectin of DM 42 %, 53 % on citrus pectin of DM >85 % and 29 % on citrus  
352 pectin of DM 30 %; suggesting that VdPME1 acts preferably on moderate DM. This is  
353 in accordance with the results described for PME from *A. niger* [44] and *B.*  
354 *licheniformis* PME [45]. Furthermore, while citrus (DM 55-70 %) and apple (DM 70-75  
355 %) pectins have slightly different DM, the differences in activity could result from  
356 distinct patterns of methylesterification or xylose linkages (higher amount in apple  
357 pectin,[46]) which could reduce the accessibility to the substrate [47]. When using  
358 citrus pectin DM 55-70 % as substrate, VdPME1 was active over a broad range of pH  
359 (**Fig. 3C**). While the maximum relative activity is at pH 6, residual activities were 95  
360 % and 72 % at pH 5 and 7, respectively. pH optimum contrasted with that of fungal  
361 PME<sub>s</sub>, AaPMEI (pH 4.5) [33], AnPMEII (pH 4.5) [38], plant DcPME (pH 7.5) [48], and  
362 orange CsPME3 (pH 7) [49]. Temperature optimum, assessed using citrus DM 55-70  
363 %, was 40 °C (**Fig. 3D**). The activity of VdPME1 drastically declined when not at  
364 optimal temperature with 40 % and 30 % of maximum activity at 50 °C and 30 °C,  
365 respectively. Similar values were reported for *P. chrysogenum* F46 PME (40 °C) [50],  
366 *A. niger* ZJ5 PME (45 °C) [44] and CsPME3 at (50 °C) [49]. Salt dependency of  
367 VdPME1 activity was determined using 0 to 300 mM NaCl (**Fig. S4**). Non-substantial  
368 effect was observed when using 0 to 50 mM concentration, while the residual activity  
369 was 50 % and 24 % at 100 mM and 300 mM NaCl, respectively. In that respect, as  
370 NaCl had no positive effect on activity, VdPME1 appears to be a salt-independent  
371 PME [43], as opposed to previously reported fungal AaPMEI [33] where salt  
372 increased the activity. Therefore, although of fungal origin, VdPME1 is more similar to  
373 Valencia orange peel PME where no salt is needed for the activity [51]. The  
374 enzymatic parameters were determined using citrus pectin DM 55-70 % as a  
375 substrate.  $K_m$  and  $V_{max}$  were  $3.27 \pm 0.16$  mg. mL<sup>-1</sup> and  $89.91 \pm 1.39$  nmol of  
376 MeOH.min<sup>-1</sup>. µg<sup>-1</sup> of proteins respectively (**Table S1**). These  $K_m$  values show a high  
377 affinity for the substrate, and are comparable to that of *A.niger* ZJ5 (AnPMEIII,  $3.27$   
378 mg.mL<sup>-1</sup>) [44] with higher  $V_{max}$  values ( $5.63$  nmol of MeOH.min<sup>-1</sup>.µg<sup>-1</sup>). In contrast, the  
379  $V_{max}$  are much lower than AaPMEI ( $5500$  nmol of MeOH.min<sup>-1</sup>.µg<sup>-1</sup>) [33].





381

382 **Fig. 3. Biochemical characterization of VdPME1.**

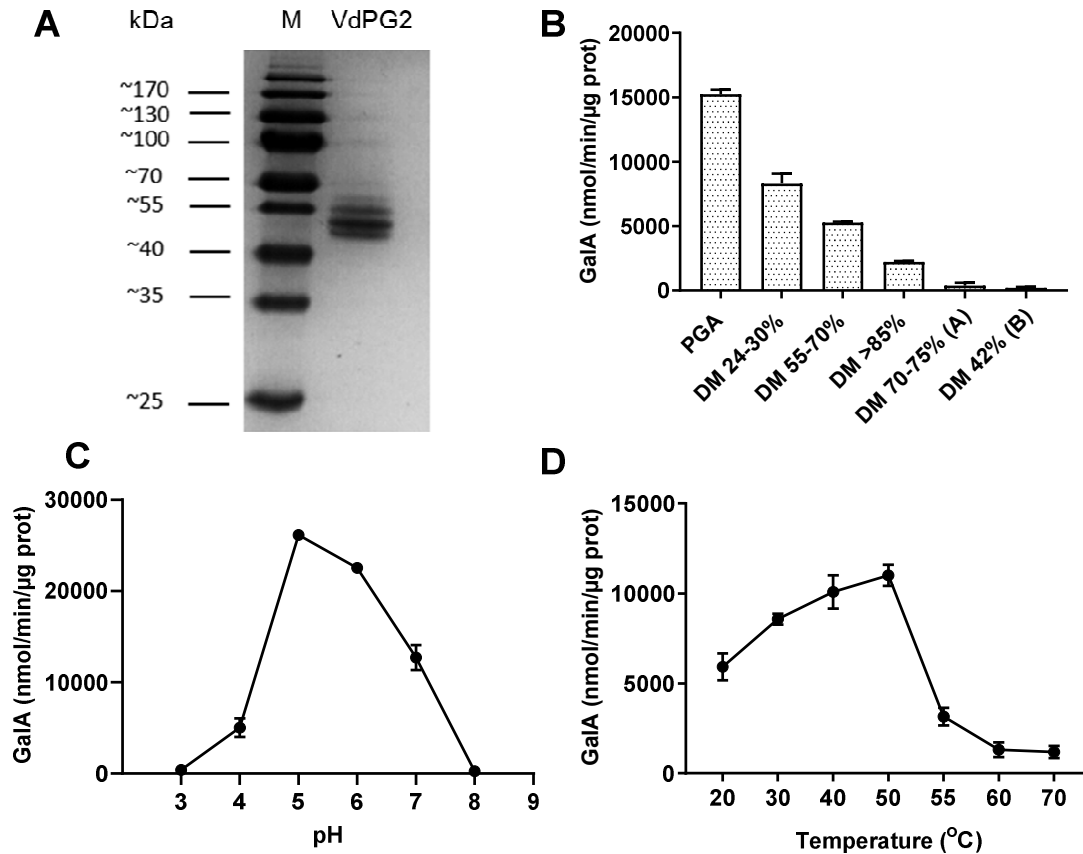
383 (A) SDS-PAGE analysis of VdPME1 following His-tag affinity purification. The gel was stained with  
 384 Coomassie blue. The band at  $\pm 34$  kDa, corresponds to His-tagged and multiple bands corresponds to  
 385 differently N-glycosylated forms of the purified enzyme. (B) Substrate specificity of VdPME1: Activity of  
 386 VdPME1 was measured on pectic substrates of distinct DM and DA. Activity was measured at pH 7.5  
 387 using the alcohol oxidase coupled assay (C) Influence of the pH on VdPME1 activity. Activity was  
 388 measured using sodium acetate (pH 3-5) and Tris-HCl (pH 6-9) buffer. (D) Influence of the  
 389 temperature on VdPME1 activity. Activity was measured on pectins 55 %-70 % DM, pH 7.5 using the  
 390 alcohol oxidase assay. The values are calculated as a MeOH released in as nmol of MeOH.min<sup>-1</sup>.μg<sup>-1</sup>  
 391 of proteins. Data represent mean  $\pm$  SD of three replicates.

392 **3.5. Biochemical characterization of VdPG2**

393 VdPG2 was most active on PGA (**Fig. 4B**) and its activity was negatively correlated  
 394 with increasing the DM of pectins. A number of fungal, bacterial and plant PG show  
 395 similar trend [52–54]. VdPG2 residual activity was 57 % on citrus pectin DM 30 %,  
 396 and 34 % on citrus pectin DM 55-70 %. Similar biochemical characteristics were

397 described for *A. luchuensis* PGA B [55] and *P. occitanis* PG2 [56]. The activity of  
398 VdPG2 was close to null on sugar beet pectin which could be explained by the  
399 overall structure and acetylation patterns of the substrate, which could impair PG  
400 activity [4]. Using PGA as a substrate we showed that the optimal VdPG2 activity was  
401 at pH 5 (**Fig. 4C**), while at pH 6 and pH 7 only 14 % and 49 % of the activity  
402 measured at pH 5 were detected, respectively. This is similar with the previously  
403 reported fungal acidic pectinase BfPG1 and BfPG2 (pH 4.2 and 4.5) that also  
404 showed high sequence similarity to VdPG2 [54]. In contrast, the optimal pH for  
405 VdPG2 was slightly higher to that of *C. pteridis* PG activity on (pH 4) [57], *A.*  
406 *acuelatus* PG (pH 4.5) [58] and *S. purpureum* PG (pH 4.5) [59]. Temperature  
407 optimum was at 50°C (**Fig. 4D**), with 90 % and 78 % residual activities at 40 °C and  
408 30 °C, respectively. Above 50 °C there was a sharp decline in activity. These  
409 temperatures optimums (40-50 °C) were previously reported for a number of fungal,  
410 insect and plant PGs [4,32,55]. In contrast *F. palustris* and *S. purpureum* PGs were  
411 most active at 60 °C [59,60]. The enzymatic parameters were calculated using PGA  
412 as a substrate (**Table S1**).  $K_m$  and  $V_{max}$  were  $8.34 \pm 0.74$  mg. mL<sup>-1</sup> and  $40.28 \pm 1.2$   
413  $\mu\text{mol of GalA min}^{-1} \cdot \mu\text{g}^{-1}$  of proteins respectively. This is in the range of previously  
414 reported values for fungal PGs [12].

415



416

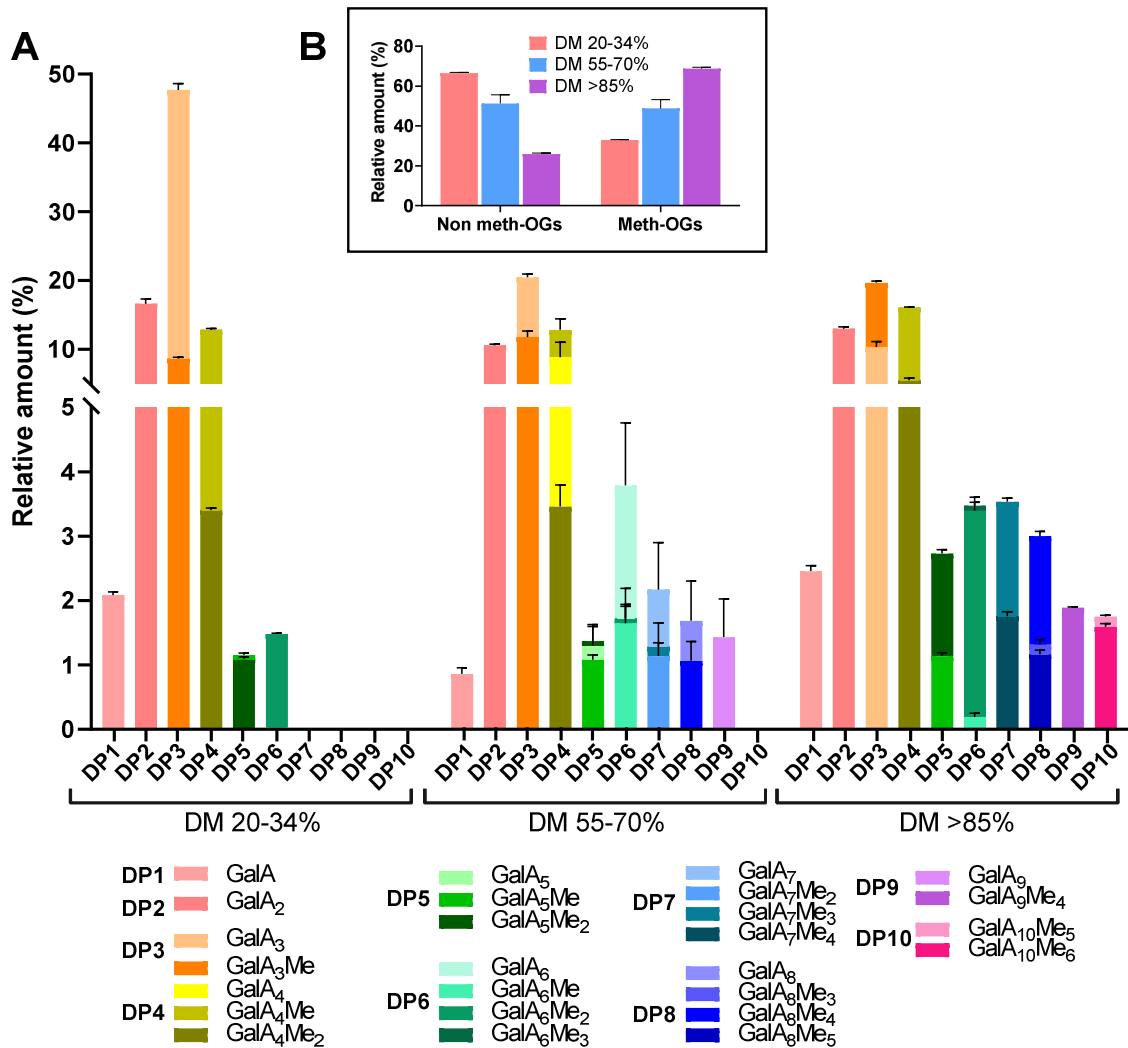
417 **Fig. 4. Biochemical characterization of PG2.**

418 (A) SDS-PAGE analysis of VdPG2 following His-tag affinity purification. The gel was stained with  
 419 Coomassie blue and the bands at 38 kDa corresponds to His-tagged and multiple bands correspond  
 420 to differently N-glycosylated forms of the purified enzyme. (B) Substrate specificity of VdPG2. Activity  
 421 of VdPG2 was measured on PGA and pectic substrates of distinct DM and DA. Activity was measured  
 422 at pH 5 using the DNS method. (C) Influence of the pH on VdPG2 activity. Activity was measured on  
 423 PGA at 50 °C using the DNS method (D) Influence of the temperature on VdPG2 activity. Activity was  
 424 measured on PGA, pH 5 using the DNS method. The values are calculated as a nmol of GalA min<sup>-1</sup>.μg<sup>-1</sup>  
 425 of proteins. Data represent mean ± SD for three replicates.

426 **3.6. Identification of the OGs released VdPG2**

427 The analysis of the digestion products of PG is a key to understand the diversity of  
 428 this family of enzymes and assess the potential role of the various isoforms. Using a  
 429 recently developed LC-MS approach [19], OGs released by VdPG2 were identified  
 430 after digestion of citrus pectin of various DM. When digesting citrus pectin of DM 20-  
 431 34 % (**Fig. 5A, DM 20-34 %, Fig. S6A**) the majority of released OGs were non-  
 432 methylesterified OGs, of DP 3 and 2 (GalA<sub>3</sub> 47.67 % and GalA<sub>2</sub> 16.63 % of total OGs

433 detected). Moreover, GalA<sub>3</sub> and GalA<sub>2</sub> were not cleaved and accumulated during the  
434 reaction, as in order to hydrolyse them, the enzyme would have to fold into  
435 unfavourable conformation [61]. Other OGs were methylesterified GalA of DP 3 and 4  
436 (GalA<sub>4</sub>Me 12.88 % and GalA<sub>3</sub>Me 8.70 %). Altogether, these four OGs represented  
437 85.89 % of all detected OGs. GalA<sub>6</sub>Me<sub>2</sub>, was the OG with the highest DP detected.  
438 This shows that VdPG2 act as an endo enzyme, in contrast to exo-PGs which  
439 release mostly GalA products [40]. When using a more methylesterified substrate  
440 (DM 55-70 %), the GalA<sub>3</sub> was still the most abundant OG (20.49 %) with a relative  
441 abundance of GalA<sub>4</sub>Me and GalA<sub>3</sub>Me of 12.82 % and 11.82 % (**Fig. 5A, DM 55-70**  
442 **%, Fig. S6B**), respectively. Furthermore, additional methylesterified OGs of DP7 and  
443 DP8 were detected (GalA<sub>7</sub>Me<sub>2</sub>, GalA<sub>7</sub>Me<sub>3</sub>, GalA<sub>8</sub>Me<sub>4</sub>). Interestingly, when digesting  
444 the citrus pectin DM 55-70 % increase of non-methylesterified OGs is observed,  
445 notably GalA<sub>4</sub> (8.88 %) and GalA<sub>6</sub> (3.78 %). This could be due to the random  
446 distribution of methyls in HG chain coupled with single-attack (non-processive) nature  
447 of VdPG2 which could lead to increased release of non-methylesterified OGs.  
448 GalA<sub>3</sub>Me, GalA<sub>4</sub>Me and GalA<sub>2</sub> were the most abundant OGs identified after digestion  
449 of citrus pectins of DM >85 %, with 19.66 %, 16.07 % and 13 %, respectively (**Fig.**  
450 **5A, DM >85 %, Fig. S6C**). In contrast the relative amount of GalA<sub>3</sub> was drastically  
451 reduced to 10.37 % (of total OGs detected), and 74.14 % of all OGs were  
452 methylesterified. Overall VdPG2 released 66.56 % of non-methylesterified OGs from  
453 citrus pectin DM 20-34 % and 25.86 % from pectins of DM >85 % (**Fig. 5B**). These  
454 OGs represent the final products after 24 h digestion and it can be assumed that they  
455 cannot be further hydrolysed due to unfavourable methyl substitutions. This was  
456 tested by analysing the OGs released following 15, 45, 90 and 180 min incubation,  
457 with DM 24-30 %, where no differences between various incubation times were  
458 observed (**Fig. S5**).



459

460 **Fig. 5. HP-SEC-MS analysis of OGs released by VdPG2 after over-night incubation with citrus**  
461 **pectins.**

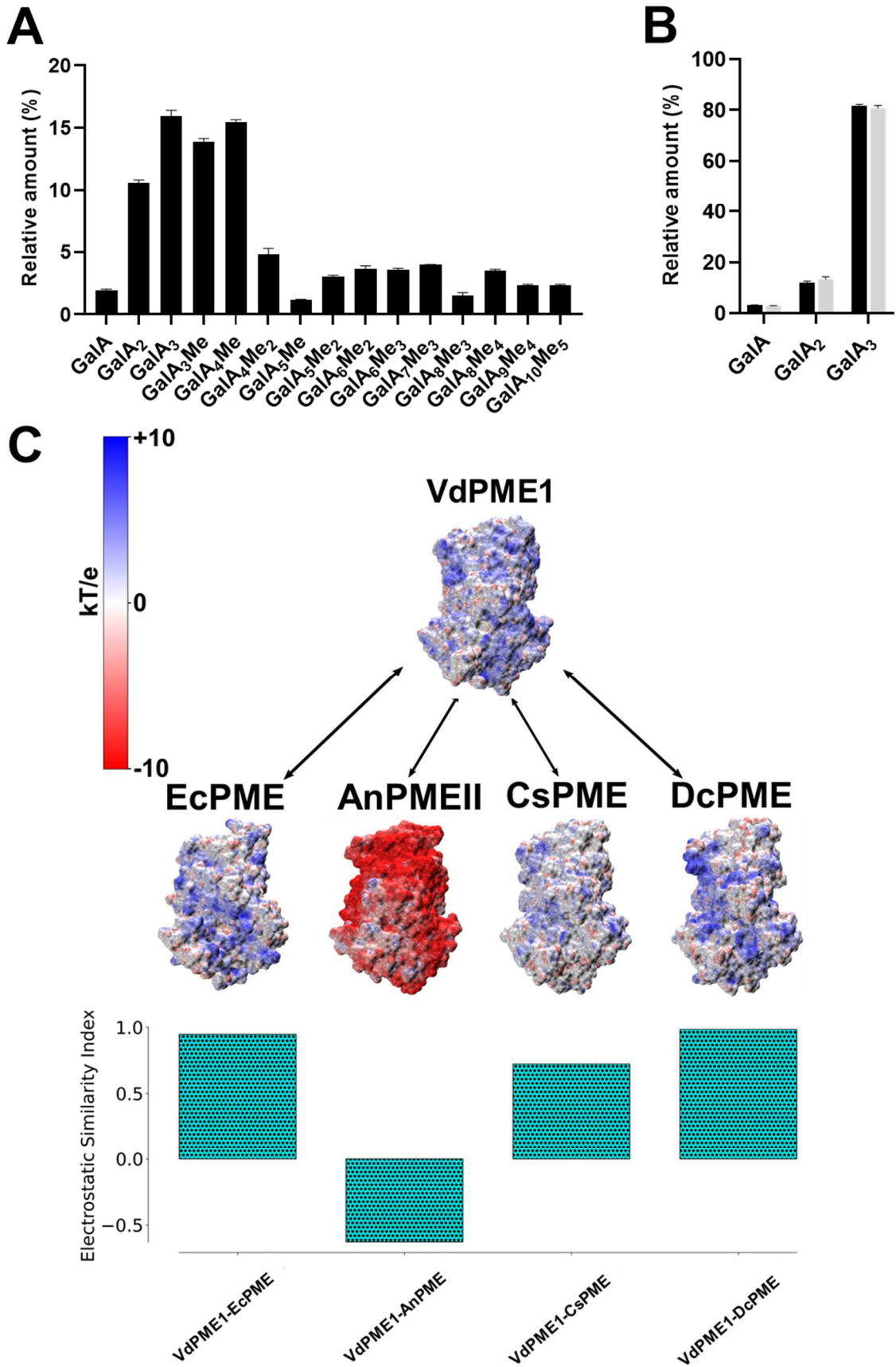
462 (A) OGs released by activity of VdPG2 on pectins of distinct DM (20-34 %, 55-70 % and >85 %) were  
463 separated using SEC and analysed using MS/MS. OGs are represented according to their DP and  
464 DM. Figure shows 88.5 % (DM 20-34 %), 80 % (DM 55-70 %) and 82.6 % (DM >85 %) of total OGs  
465 detected. (B) Total of the non-methylesterified and methylesterified OGs. Data are means  $\pm$  SD; n =  
466 3. Subscript numbers indicate the DP and DM.

467 As a means of comparing the mode of action of VdPG2 with previously characterized  
468 PG, the digestion profile of AaPGM2, a commercial endo-PG M2 from *Aspergillus*  
469 *aculeatus*, was compared with that of VdPG2. Citrus pectin with DM 20-34 % and DM  
470 55-70 % were digested during 2 hours with each of the enzymes and the released  
471 OGs were compared (**Fig. S7**). Overall, both enzymes released the same OGs in  
472 different relative quantities. Differences came from the abundance of non-

473 methylesterified GalA<sub>3</sub> (17 % for AaPGM2 vs 47 % VdPG2) and the absence of  
474 GalA<sub>4</sub>Me<sub>2</sub> detected following AaPGM2 digestion of pectin DM 20-34 % (**Fig. S7A**).  
475 Higher differences were detected with pectin DM 55-70 % (**Fig. S7B**), where  
476 AaPGM2 releases OGs of higher DP with complex methyl substitutions. This further  
477 reinforce the previous statement that VdPG2 has less affinity for pectins with complex  
478 methylesterification patterns compared to AaPGM2. The mode of action of these two  
479 enzymes differ and probably these differences are likely to be dependent on the  
480 active site subsites, as reported for AnPGII, where the Glu252Ala mutation increased  
481 the activity of the enzyme on partially methylesterified substrate [62]. That specific AA  
482 is different for two enzymes (Asp259, VdPG2 numbering) which could explain this  
483 distinct behaviour, but AA at other subsites should be also considered when  
484 determining enzyme specificity.

### 485 **3.7. VdPME1 has a processive mode of action**

486 As a way to determine the mode of action of VdPME1, we first digested moderately  
487 methylesterified substrate citrus pectin DM 55-70 % for 2 hours using VdPG2 (**Fig.**  
488 **6A**). This led to the release of methylesterified and non-methylesterified OGs. This  
489 pool of OGs was subsequently used as substrate for determining the mode of action  
490 of VdPME1 at pH 7 after 15 min and overnight digestion. LC-MS/MS oligoprofiling  
491 showed that for the two incubation times, only non-methylesterified GalA, GalA<sub>2</sub> and  
492 GalA<sub>3</sub> were detected (**Fig. 6B**).



493

494 **Fig. 6. Mode of action and electrostatic potential of VdPME1**

495 (A) Population of OGs of various DP and DM generated by action of VdPG2 during 2 h at 37 °C on  
496 pectin DM 55-70 % (B) OG produced from the “VdPG2-population” after 15 min (black) and overnight  
497 incubation (grey) with VdPME1. Only mono-, di-, tri- galacturonic acids were identified. Data are  
498 means  $\pm$  SD; n = 3. Subscript numbers indicate the DP and DM. (C) Electrostatic potentials, projected  
499 on the enzyme surface, calculated for *Verticillium dahliae* (VdPME1), *Dickeya dadantii* (DdPME),  
500 *Citrus sinensis* (CsPME) and *Daucus carota* (DcPME) PMEs at pH 7.0. Positive and negative regions  
501 of the potential are coloured from blue to red with a scale ranging between -10 and +10 kT/e. Pairwise  
502 similarity indices calculated to understand the resemblance of electrostatic potentials between the  
503 PME from *Verticillium dahliae* (VdPME1) and the other PMEs are shown as bars in the lower panel. A  
504 similarity index of +1.0 shows correlated electrostatic potentials (high similarity – see methods for  
505 more details), whereas a value of -1.0 shows anti-correlated potentials. Any value in between shows  
506 different to no correction (similarity index = 0).

507 Together, these data show that VdPME1 can produce long stretches of non-  
508 methylesterified GalA that have been, in the context of the experiment, further  
509 hydrolysed by the still-active VdPG2. Processive PMEs have been reported to have a  
510 neutral to alkaline optimal pH while non-processive PMEs are active at acidic pH [38].  
511 It has been shown that *A. niger* PMEII, (AnPMEII) that has an optimal pH at 4.5 is a  
512 non-processive, while orange (CsPME), carrot (DcPME) and *D. dadantii* (DdPME)  
513 PMEs were shown to be processive at pH 7.5 [38,63]. Considering the importance of  
514 the electrostatic properties in defining substrate specificity and processivity [17], we  
515 have performed a quantitative analysis of the electrostatic properties of the modelled  
516 VdPME1 and compared those to what is known for other well-characterised PMEs.  
517 As shown on the **Fig. 6C** the comparison highlights a clear difference with the  
518 acidophile AnPMEII with electrostatics characterising the binding grooves being of  
519 opposite signs (a strong negative electrostatic potential for AnPMEII, and a strongly  
520 positively charged group for VdPME1 at pH 7.0). The electrostatic potential of  
521 VdPME1 is similar to that of the CsPME and DcPME, known to be processive at  
522 neutral pH, with an overall positive charge and a similarity index close to 1.0. This  
523 contrasted with the negative electrostatic potential of AnPMEII which can facilitate the  
524 dissociation of the enzyme-substrate complex for highly de-methylesterified  
525 substrates, which have a strong negative charge and anticorrelated similarity index.  
526 The relation between the electrostatic properties of the binding groove and  
527 processivity could be explained on the basis of simple electrostatics, with the  
528 repulsion between carboxylate groups of HGs and negatively charged residues in the  
529 binding groove being a determinant for low affinity. Nevertheless, residue-specific



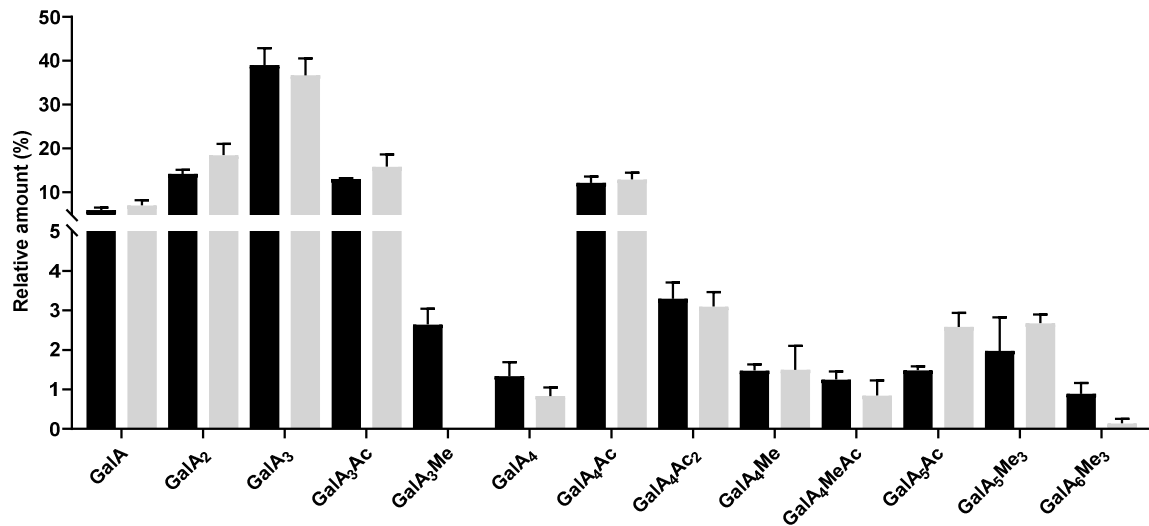
530 preferences for different methylation or acetylation states for the substrate in specific  
531 subsites along the binding groove are most likely important to quantitatively tune  
532 processivity among PME's showing processive behaviour.

### 533 **3.8. Two *Verticillium*-sensitive and tolerant cultivars differ in their root** 534 **pectin structure**

535 Phytopathogenic fungi use pectinolytic enzymes, including PGs, PME's, PLLs during  
536 infection to degrade pectins [64]. *V. dahliae* as a soilborne pathogen attacks flax root  
537 to infect the plants. In order to determine the contribution of VdPG2 in degrading flax  
538 cell wall we digested roots cell wall of two flax cultivars Évéea (*Verticillium*-partially  
539 resistant), and Violin (*Verticillium*-susceptible) with VdPG2. VdPG2 showed activity  
540 on flax roots and OGs up to DP6 were detected (**Fig. 7**). In particular, VdPG2  
541 released non-methylesterified (GalA, GalA<sub>2</sub>, GalA<sub>3</sub>, GalA<sub>4</sub>) and methylesterified  
542 (GalA<sub>3</sub>Me and GalA<sub>4</sub>Me, in both cultivars, approx. 1.5 %) OGs which have been  
543 previously identified from commercial pectic substrates. In addition, VdPG2 released  
544 acetylated substrates, GalA<sub>3</sub>Ac, GalA<sub>4</sub>Ac, GalA<sub>4</sub>Ac<sub>2</sub>, showing that the enzyme is able  
545 to hydrolyse acetylated pectins. GalA<sub>4</sub>MeAc was the only methylesterified and  
546 acetylated OG detected. It appears that VdPG2 has lower affinity for pectic  
547 population having acetyl and methyl groups. In particular, the distribution of methyl  
548 and acetyl groups on HGs was shown to have an impact on enzyme substrate  
549 interaction, as acetylation of HGs strongly change the association of enzyme on  
550 substrate [65]. Overall, following digestion by VdPG2, both genotypes, Évéea and  
551 Violin, produced similar diversity and relative abundance of OGs (**Fig. 7**) except for  
552 GalA<sub>3</sub>Me, which was specifically detected in Évéea, the *Verticillium*-partially resistant  
553 cultivar. This specific OG only produced from the roots of the resistant cultivar  
554 suggests that its cell wall structure differed to that of the susceptible cultivar. When *V.*  
555 *dahliae* infests flax roots, pectins digestion by PGs such as VdPG2 can lead to  
556 releasing OGs similar to the above-mentioned one. This could play a role in triggering  
557 distinct signalling events between resistant and susceptible cultivars. Indeed,  
558 previous reports show that small OGs, especially trimers could be involved in  
559 activation of genes involved in defence and phytohormone signalling, as well in the  
560 down-regulation of genes involved in growth regulation and development [66]. In  
561 addition, this OG could act as danger-associated molecular patterns (DAMPs) in flax

562 which would lead to activation of defence-related pathways, thus reducing the  
563 susceptibility to pathogen infection [67].

564



565

566 **Fig. 7. Analysis of OGs released by VdPG2 from flax roots.**

567 VdPG2 was incubated overnight with roots from Évéa (spring flax, partially resistant to *Verticillium* wilt,  
568 black) and Violin (winter flax, more susceptible to *Verticillium* wilt, grey). Data are means  $\pm$  SD; n = 3.  
569 Subscript numbers indicate the DP and DM.

570

#### 571 4. Conclusion

572 We characterized two novel enzymes from *V.dahliae*: VdPME1 and VdPG2. They  
573 show high activity on pectic substrates but also, for VdPG2, on cell wall pectins from  
574 flax roots. Biochemical characteristics were determined for both enzymes which  
575 showed, VdPG2 is an endo PG that can release methylesterified, non-  
576 methylesterified and acetylated OGs, with preference for unsubstituted and slightly  
577 substituted pectic populations. This, together with homology modelling suggests that  
578 small differences in the enzyme structure could be of importance to determine the  
579 substrate specificities. Model for VdPME1 shows similar structural features to that  
580 reported for fungal and plant PMEs, but a surprising processive behaviour commonly  
581 observed for plants PMEs. This processive behaviour could relate to the electrostatic  
582 potential of the protein. The processive mode of action of VdPME1 could enable

583 release of pectins for which VdPG2 has a high affinity. This study shows that HP-  
584 SEC-MS can be used as a method of choice for the detection and quantification of  
585 OGs released by the action of VdPG2 and VdPME1 from commercial pectin and flax  
586 root cell wall pectins. Moreover, synergistic properties of these two enzymes suggest  
587 that VdPG2 and VdPME1 are important pectinolytic enzymes in *V.dahliae* arsenal.  
588 This study may lead to new approach for the protection of crops against pathogens.

589

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## 595 **CRedit authorship contribution statement**

596 **Josip Safran:** Conceptualization, Data curation, Formal analysis, Investigation,  
597 Methodology, Writing - original draft. **Olivier Habrylo:** Formal analysis, Investigation,  
598 Methodology. **Mehdi Cherkaoui:** Methodology. **Sylvain Lecomte:** Methodology.  
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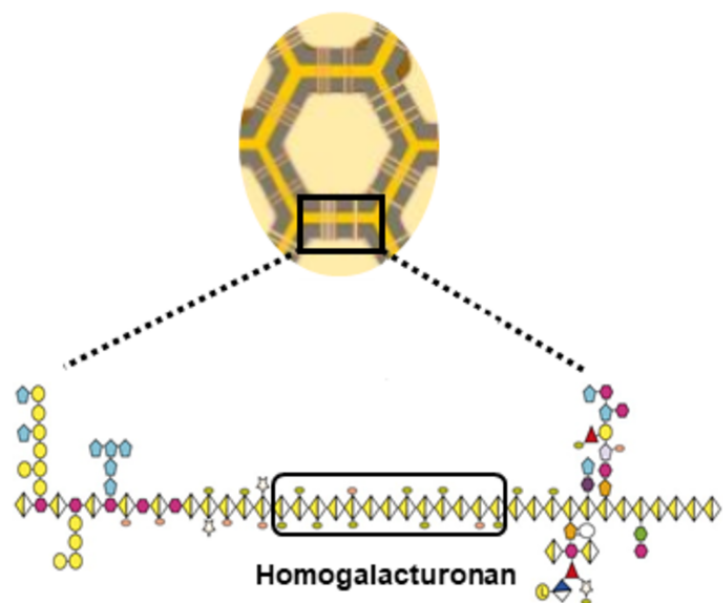
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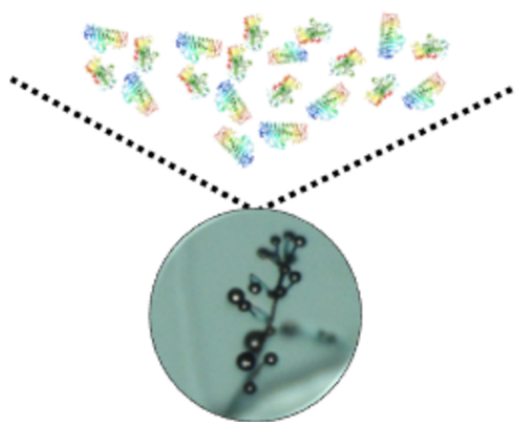
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863

## PRIMARY CELL WALL

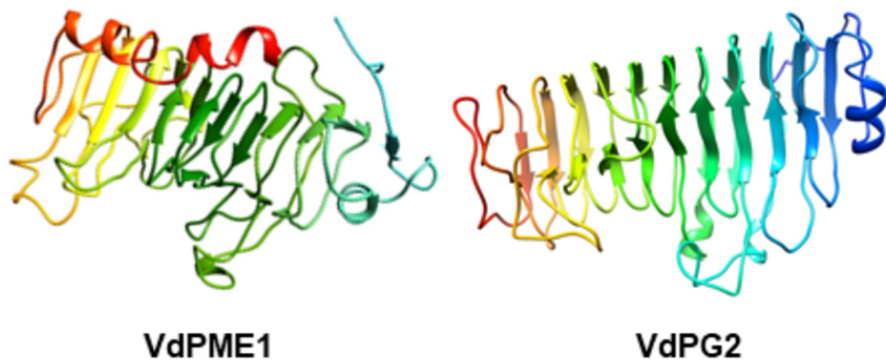


## Homogalacturonan degrading enzymes

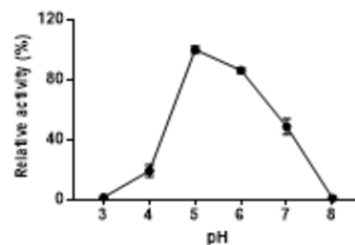
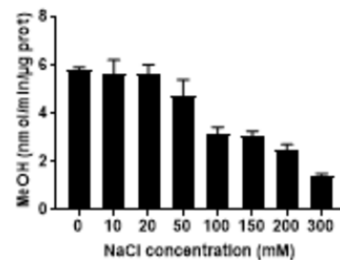
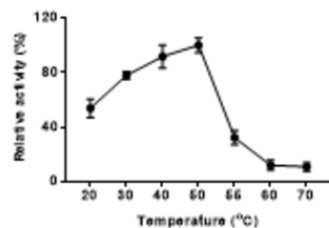
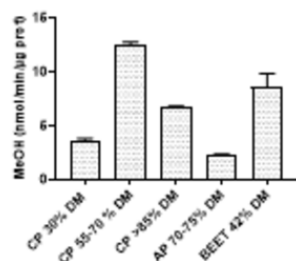


**VERTICILLIUM DAHLIAE**

## HOMOLOGY MODELING



## BIOCHEMICAL CHARACTERIZATION



## MODE OF ACTION

