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1 **Title:** Systemic defense activation by COS-OGA in rice against root-knot nematodes depends
2 on stimulation of the phenylpropanoid pathway

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15 **Competing interests**

16 Pierre Van Cutsem and Geraldine Van Aubel are co-inventors of a granted patent on the
17 COS-OGA defense elicitor. They are employed by the company Fytofend, which
18 commercializes COS-OGA based products. The other authors declare no competing interests.
19 This research has not been financed by Fytofend.

20

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Abstract

Activation of induced plant resistance to control pests and diseases is regaining attention in the current climate where chemical pesticides are being progressively banned. Formulations of chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA), COS-OGA, have previously been described to induce resistance against fungal diseases in different crop plants. Here, we investigated their potential and mode-of-action as preventive measures to control root-knot nematode *Meloidogyne graminicola* infection in rice.

The results show a significant reduction in root-galling and nematode development in rice plants that were treated through foliar application with the COS-OGA formulations FytoSol® and FytoSave® 24h before nematode inoculation. Hormone measurements, gene expression analyses, corroborated by treatments on salicylic acid (SA) and jasmonic acid (JA)-mutants indicated that the systemic COS-OGA induced defense mechanism against nematodes is not based on SA or JA activation. However, phenylalanine ammonia lyase (*PAL*) gene expression in roots as well as enzymatic PAL activity in the shoots were significantly induced 24 h after foliar COS-OGA spraying in comparison with untreated plants. COS-OGA-induced systemic defense was abolished in the rice *OsPAL4*-mutant, demonstrating that COS-OGA-induced defense is dependent on OsPAL4 activation in rice plants.

Keywords: plant defense elicitor, *Meloidogyne graminicola*, *Oryza sativa*, hormone, phenylpropanoids.

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45 1. Introduction

46 With a world population that is currently growing at 83 million per year, the pressure on food
47 production will only increase. Rice (*Oryza sativa*) is one of the most important staple foods
48 in the world, with a production of more than 730 million tons per year (FAO, 2016).
49 Although not well-known because they cause mainly belowground symptoms, plant parasitic
50 nematode infections contribute to major agricultural losses in rice production (Mantelin et al.,
51 2017). The root-knot nematode (RKN) *Meloidogyne graminicola* is probably the most
52 damaging root pathogen affecting – mainly aerobic – rice fields in Asia, and it was recently
53 also detected in Italian rice fields (Fanelli et al., 2017; Mantelin et al., 2017). RKN induce the
54 formation of ‘giant cells’ inside the root tissue, from which they withdraw plant metabolites
55 for their nutrition, leading to the visible formation of root-knots (galls) (Mantelin et al.,
56 2017). The control of RKN using conventional methods is challenging because of their wide
57 host range, ability to survive in soil and weeds, and the low inherent level of resistance in rice
58 against this nematode. An alternative to use of nematicides, activation of the plant innate
59 immunity could be a more environmentally friendly control method (Conrath et al., 2015).
60 Through evolutionary history, plants have acquired complex defense or ‘immunity’
61 mechanisms towards biotic stress factors like bacteria, fungi, insects and nematodes. Plant
62 innate immunity is based on recognition of pathogen-associated molecular patterns (PAMPs),
63 activating basal immune responses. Plant immune responses typically include rapid
64 physiological changes such as Ca^{2+} uptake and production of reactive oxygen and nitrogen
65 species (ROS and RNS). After signal transduction, these changes induce production of
66 secondary metabolites, including hormones (among which salicylic acid (SA), jasmonic acid
67 (JA), ethylene (ET), and abscisic acid (ABA)) and pathogenesis-related (PR) proteins. In case
68 of PAMP-triggered immunity (PTI), the response is relatively small in magnitude but active
69 against a broad range of pathogens (Jones & Dangl, 2006). The highly specific effector-

70 triggered immunity (ETI) - that is activated upon recognition of pathogen effectors - is much
71 stronger and often accompanied by a hypersensitive response (HR) – induced cell death – at
72 the site of attempted host colonization (Jones & Dangl, 2006).

73 Triggering a defensive state in a plant can be achieved by applying chemical or biological
74 molecules or micro-organisms. Traditionally, the distinction is being made between SAR
75 (systemic acquired resistance (SAR)) that depends on the plant hormone SA, and the SA-
76 independent induced systemic resistance (ISR), which is based on JA/ET activation (Pieterse
77 et al., 2014). Strong activation of plant immunity can be energy-consuming, but a more
78 energy-efficient mechanism to trigger plant acquired immunity, known as priming, has been
79 described in the early 2000's. During priming, a treatment, such as a (minor) stress or
80 application of a certain molecular agent or micro-organism, puts a plant in a state of
81 increased alertness with no or minimal direct immune gene induction or hormone
82 accumulation, and hence no energy and yield loss. Upon pathogen attack, a faster and more
83 robust defense response is activated in the primed plants than in unprimed plants (Conrath et
84 al., 2015). In the dicotyledonous model plant *Arabidopsis thaliana*, priming has been shown
85 to involve (1) the accumulation of dormant mitogen-activated protein kinases; and/or (2)
86 epigenetic modifications; and/or (3) accumulation of secondary metabolites (Conrath et al.,
87 2015). In our research we are evaluating the potential and mode of action of priming agents
88 in the protection of rice against RKN. In previous research, we have identified the activity
89 and involved pathways for priming agents, such as beta-amino butyric acid (BABA), and
90 thiamine (Huang et al., 2016; Ji et al., 2015). Thiamine treatment leads to increases in H₂O₂
91 production in rice (Huang et al., 2016). Increases in H₂O₂ and callose as well as lignification
92 were observed in nematode-infected plants pretreated with BABA (Ji et al., 2015). Lignin
93 precursors are formed by the phenylpropanoid pathway, in which enzymatic conversion of
94 phenylalanine into trans-cinnamate, mediated by phenylalanine ammonia lyase (PAL), is the

95 first and rate-limiting step. Next to monolignols, this plant-specific pathway, which is of
96 significant importance to growth and development, can also convert phenylalanine into other
97 secondary metabolites, such as flavonoids, salicylic acid, stilbenes and many other products
98 playing a role in plant immunity (Vogt, 2010).

99 FytoSol and FytoSave are commercial formulations of a plant defense elicitor,
100 commercialized by the company FytoFend. FytoSol and FytoSave contain chitosan oligomers
101 (COS) combined with pectin-derived oligogalacturonides (OGA), aka COS-OGA. FytoSave
102 (12.5 g/L COS-OGA) has been described to increase the resistance of Cucurbitaceae
103 (cucumber, zucchini and melon), grapes and Solanaceae (tomato and sweet pepper) against
104 powdery mildew (Van Aubel et al., 2014), through a mechanism relying on the induction of
105 SA-related genes and proteins in tomato leaves (Van Aubel et al., 2016). FytoSave can also
106 alleviate late blight caused by the oomycete *Phytophthora infestans* in potato, and this
107 phenomenon is correlated with PR-gene activation (Clinckemaillie et al., 2017). FytoSol is a
108 new composition still under development by the company FytoFend. Recently, FytoSol was
109 shown to be even more effective at preventing late blight in potato under controlled
110 conditions (Van Aubel et al., 2018). Although FytoSave strongly increased the SA content, it
111 failed to induce sufficient protection against late blight, while FytoSol maintained or even
112 decreased the free SA content in the presence of *P. infestans* and was more effective. In this
113 manuscript, foliar application of FytoSave and FytoSol as potential activators of systemic
114 defense was evaluated against root-knot nematodes in rice. By using hormone measurements,
115 gene expression and biochemical analyses and rice mutants we investigated the involvement
116 of the plant defense hormones SA and JA and of the phenylpropanoid pathway in COS-OGA
117 induced root defense against RKN.

118 2. Materials & Methods

119 2.1. Plant material and growth conditions

120 Rice (*Oryza sativa*) seeds of cultivar Nipponbare were provided by U.S. Department of
121 Agriculture (GSOR-100). Seeds of the *Ospa14*-mutant (Tonnessen et al., 2015) and its wild-
122 type IR64 were kindly provided by the lab of J. Leach (Colorado State University, CO,
123 USA). Seeds were germinated on wet filter paper in a petri dish for 4 days at 30°C. They
124 were transplanted in in-house-made polyvinyl-chloride (PVC) tubes (height: 15 cm; diameter
125 3 cm) containing a mixture of fine sand and synthetic absorbent polymer (SAP) substrate (for
126 more details see Nahar et al., 2011; Huang et al., 2015). The polymer used is Aquaperla®
127 (DCM, Belgium). The plants were further kept in a growth room at 26°C, 12 h/12 h light
128 regime (150 $\mu\text{mol}/\text{m}^2\text{s}$) and relative humidity of 70-75%. The plants were maintained by
129 supplying 10 mL Hoagland solution three times a week. To avoid possible effects induced by
130 the photoperiod, all inoculations and samplings were done at the same moment of the day, 10
131 am, which is 2h after sunrise.

132

133 2.2. Plant treatments

134 FytoSol and FytoSave (patent: US2015045221 (A1), US8871923B2) are commercial
135 formulations containing 12.5 g/l oligosaccharide complex (chitosan fragments and pectin-
136 derived fragments: COS-OGA; Van Aubel et al., 2018). In the first experiment the product
137 was applied as foliar spray on 14-days-old rice plants, at different concentrations (1%, 0.5%,
138 0.25% and 0.125%, v/v) to evaluate the dose effect. In following experiments, the
139 recommended dose of 0.5% was used, which corresponds to 62.5 ppm COS-OGA in the
140 spray solution. In case of nematode infection experiments root inoculation was done 24 h
141 after foliar treatment.

142 **2.3. Infection experiments**

143 *M. graminicola* - originally isolated in the Philippines (Batangas) - was kindly provided by
144 Prof. D. De Waele (Catholic University, Leuven, Belgium). The nematode culture was
145 maintained on susceptible rice plants grown in potting soil, under light and temperature
146 conditions as described above. About 3 months after inoculation, infected roots were cut into
147 1 mm pieces and nematodes (second stage juveniles, J2s) were extracted using a Baermann
148 funnel (Luc et al., 2005). The nematode suspension was collected 48 hrs later and
149 concentrated by centrifugation for 10 minutes at 1500 rpm at room temperature. Nematodes
150 were counted under light microscopy to estimate the number of nematodes in the suspension.

151 Fifteen-day-old rice plants were inoculated with 250 juveniles of *M. graminicola* or mock
152 inoculated with water. The infection level of the plants was evaluated at 14 days after
153 inoculation by counting the number of galls and nematodes per plant. Individual root systems
154 were removed from the substrate, gently washed and packed in a tissue bag (Miracloth,
155 VWR). They were stained with acid fuchsin, which leads to intense pink staining of the galls:
156 roots were boiled for 3 min in a solution of 0.8% acetic acid and 0.013% acid fuchsin.
157 Nematode development inside the galls as well as giant cells can be observed when the acid
158 fuchsin-stained root system is destained for approximately 4 d in acid glycerol. The
159 development of nematodes until maturity (females) is considered as a measure of general
160 nematode development in the root system. Galls and females were counted microscopically
161 using a stereomicroscope (Leica S8 APO, Leica Microsystems, Diegem, Belgium).

162 **2.4. Direct effect on nematodes**

163 Approximately 50 J2s were placed into a 2.5-cm diameter well on a 12-well culture plate
164 containing 1.5 ml of Fytosave or Fytosol at two different concentrations (1 and 0.5%) or 1.5

165 ml of distilled water for the mock treatment. The living and dead nematodes were counted at
166 different time points under a stereomicroscope (Leica S8 APO, Leica Microsystems, Diegem,
167 Belgium). Nematodes were considered dead if they were not moving and did not respond to
168 being touched by a small probe. The experiment was performed three times with 6 replicates
169 each.

170 **2.5. RNA extraction, cDNA synthesis, and qRT-PCR**

171 RNA was extracted using the Plant RNeasy Plant Mini kit (Qiagen) following the
172 manufacturer's instructions. For each treatment, 3 biological replicates were taken, consisting
173 of a pool of at least 4 plants. qRT-PCR was performed and analyzed as described in Huang et
174 al.,2015. Expression levels were normalized using three reference genes, *OsEIF5C*, *OsEXP*
175 and *OsEXPNarsai*. Primer pairs are listed in Huang et al., 2015 and Tonnessen et al., 2015.

176 **2.6. Hormone measurements**

177 For hormone measurement root and shoot tissues were collected and were homogenized
178 using liquid N₂, and 100 mg of ground material was extracted at -80°C using the modified
179 Bieleski solvent. After filtration (30 kDa Amicon[®] Ultra centrifugal filter unit), solvent
180 evaporation and extract reconstitution, chromatographic separation was performed on a U-
181 HPLC system (Thermo Fisher Scientific) with a Nucleodur C18 column (50 x 2 mm; 1.8 µm
182 d_p). The detailed procedure is described in Haeck et al. (2018). For each treatment, 5
183 biological replicates, each consisting of a pool of at least 3 plants, were measured.

184 **2.7. PAL-activity measurement**

185 PAL-activity was measured as described in Camacho-Cristóbal et al., 2002. For each
186 treatment, 4 biological replicates, each consisting of a pool of 3 plants, were sampled. From

187 each replicate, 100 mg of shoot or 100 mg of root samples were ground in liquid nitrogen and
188 dissolved in 800 μ l of 50 mM sodium phosphate as assay buffer containing 2% (w/v) poly-
189 vinylpyrrolidone (PVPP), 2 mM EDTA, 18 mM-mercaptoethanol and 0.1% (v/v) Triton
190 X-100. The homogenate was centrifuged at 7168 g, at 4°C for 10 mins. In different 2 ml
191 tubes, 135 μ l of reaction buffer, 50 μ l of 5 mM of L-phenylalanine, and 20 μ l of supernatant
192 were mixed. Absorbance was measured using a spectrophotometer at 290 nm. The reaction
193 was started by incubating the samples in a water bath for 30 mins at 40°C. To stop the
194 reaction, 10 μ l of hydrochloric acid was added and the sample was mixed for 10 mins, after
195 which PAL activity was assayed by measuring the formation of trans-cinnamic acid at 290
196 nm. One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1
197 nmol cinnamic acid per hour. Control assays had no L-phenylalanine as substrate.

198 **2.8. Data collection and statistical analyses**

199 All statistical analyses were performed in SPSS. Normality of the data was checked by
200 applying the Kolmogorov-Smirnov test of normality ($\alpha = 0.05$). Homoscedasticity of the data
201 was checked by applying the Levene test ($\alpha = 0.05$). Since the assumptions of normality and
202 homoscedasticity of the data were found to be fulfilled in all cases, a Student's t-test or an
203 ANOVA and Duncan's multiple mean comparison test were applied ($\alpha = 0.05$). In case of
204 gene expression analysis, the REST2009-software, which is based on a data permutation test
205 was used.

206 3. Results

207 3.1. Foliar COS-OGA treatment reduces the number of galls and female nematodes in 208 rice roots

209 In a first experiment, four different concentrations of COS-OGA (2 formulations: FytoSol
210 and FytoSave) were applied to rice plants to assess the effect on subsequent nematode
211 infection. Rice cv. Nipponbare roots were inoculated with *M. graminicola* 1 day after foliar
212 spraying with COS-OGA, and the numbers of galls and female nematodes were counted at 14
213 days post-inoculation (dpi). Compared with control plants, pre-treatment with all
214 concentrations and both formulations of COS-OGA resulted in a significantly lower number
215 of root galls per plant at 14 dpi (Fig. 1A). In addition, a significant decline in number of
216 females was observed in roots of pre-treated plants. At 14 dpi, the number of adult females in
217 the treated plants was significantly lower than in control plants, for all formulations and
218 dilutions except 0.125% FytoSol (Fig. 1B). The treatments did not have any negative effect
219 on visual plant appearance (data not shown) or plant growth, based on an evaluation of shoot
220 and root fresh weight at 14 dpi (Supplementary Figures 1A and 1B). COS-OGA is not
221 directly nematicidal, as no increased mortality was seen after nematode incubation even up to
222 7 days in 1% of FytoSol or FytoSave in comparison with water incubation (data not shown).
223 These data demonstrate that foliar COS-OGA treatment one day before inoculation not only
224 hinders root infection by *M. graminicola*, indicating that COS-OGA induces systemic
225 defense against *M. graminicola* in rice. Based on these data, it was decided to continue all
226 further experiments with the recommended dose of 0.5% COS-OGA.

227

228 **3.2. COS-OGA induced defense acts independently of the major hormonal defense**
229 **pathways salicylate and jasmonate**

230 We hypothesized that COS-OGA might be activating the hormonal pathways involved in
231 plant defense. Therefore, SA, JA, ABA and IAA levels were measured inside shoots (Figures
232 2A and 2B) and roots (Fig. 2C) of treated and untreated rice at 24 h after foliar application,
233 which is the moment when nematodes are usually inoculated (although in this experiment the
234 plants were not infected). Results presented in Figure 2A and 2B show that foliar application
235 of both COS-OGA formulations resulted in decreased ABA and SA levels in the shoots at 24
236 h after treatment. FytoSave additionally led to significantly decreased JA levels in the shoots,
237 a trend which was not significant for Fytosol treated plants. No significant changes in
238 hormone levels were observed in the roots of treated rice plants at 24 h after treatment
239 (Figure 2C), although JA levels were slightly but insignificantly increased and IAA levels
240 decreased in roots of COS-OGA treated plants.

241 Gene expression analysis revealed only minor and very variable induction of the investigated
242 defense genes in the shoot tissue at 24h post treatment with Fytosol (Fig. 3A). For Fytosave
243 treatment, significant repression of *ICS1*, *PAL2*, *PAL4* and *PAL6* expression was observed in
244 the shoots. In the root tissue, clear induction of many defense genes was seen (Fig. 3B).
245 More specifically, both Fytosave and Fytosol induce the expression of *AOS2*, *PAL4*, *PAL6*
246 and *PR1b* in the roots at 24 after foliar treatment. Similar trends were seen for *ICS1* and
247 *PAL2*, although this was not statistically significant (Fig 3B).

248 The involvement of SA and JA in COS-OGA induced defense was further evaluated by
249 investigating a set of mutants in the SA and JA-pathway: the SA-signaling deficient
250 *WRKY45*-RNAi line, and JA biosynthesis mutant *hebiba*. Results, presented in Fig. 4, show
251 that all three lines are more susceptible to RKN, as expected based on previously shown

252 importance of these genes for basal defense against RKN (Nahar et al., 2011; Ji et al., 2015).
253 However, COS-OGA systemic induced defense is still active in these three lines (Fig. 4A &
254 B), demonstrating that this phenomenon acts independently of SA-levels, SA-signaling and
255 JA biosynthesis.

256 **3.3. COS-OGA induced defense against nematodes activates PAL-activity in shoots and** 257 **is dependent on the *OsPAL4* gene**

258 Based on the above-described results, the typical defense hormones seem not to be
259 underlying COS-OGA-induced defense against RKN. However, gene induction did confirm
260 enhanced expression of multiple *PAL*-genes in the rice roots of treated plants. Therefore, we
261 decided to focus on the phenylpropanoid pathway, a well-known biosynthesis pathway for
262 several defense-related metabolites. PAL-activity was measured in root and shoots of the
263 treated plants at 24h after treatment. Data shown in Fig. 5A reveal significant induction of
264 PAL-activity in shoots of COS-OGA treated plants, both for FytoSave and FytoSol. No
265 increase in PAL-activity was seen in the roots (Fig. 5B).

266 To confirm the involvement of *OsPAL*-enhancement in COS-OGA induced defense, the
267 *OsPAL4*-mutant was used in an infection experiment. The wild-type line 'IR64', which
268 belongs to the subspecies 'indica', is slightly less responsive to FytoSol and FytoSave
269 treatment than the 'japonica' cultivars. 'Nipponbare' and 'Nihonmasari' (> 50% reduction,
270 Fig. 1A; Fig. 4B), although still a significant reduction in gall number was seen in treated
271 IR64-plants (33% reduction, Fig. 5C). It deserves also to be noted that a negative effect on
272 root length was observed in the COS-OGA treated 'IR64' rice plants (Supplementary Fig. 2).
273 Interestingly, the *OsPal4* mutant is not responding to COS-OGA treatments while wild-type
274 IR64 does (Fig. 5C). These data demonstrate that the COS-OGA induced defense against
275 nematodes is dependent on *OsPal4*.

ACCEPTED MANUSCRIPT

277 **4. Discussion**

278 Due to the current ban on chemical nematicides, the pressure to present alternative nematode
279 control strategies is increasing. Next to the use of resistant varieties, crop rotation, flooding or
280 other agronomic prevention strategies, the activation of plant innate immunity against
281 nematodes and other pathogens gains more and more attention (Oka et al., 1999; Cohen et al.,
282 2016; Asif et al., 2017; Medeiros et al., 2017). Although 100% resistance is not achievable
283 with products acting as plant defense elicitors, they are very useful as one of the prevention
284 methods in a well-designed integrated pest management plan, and as such can replace one or
285 more pesticide applications in a seasonal program of plant protection (Walters et al., 2013).
286 Interestingly, the protection conferred by these elicitors is often not specific and can
287 potentially provide broad-spectrum protection against diseases and pests (Sharathchandra et
288 al., 2004). For example, BABA application provides protection against nematodes as well as
289 many bacterial and fungal diseases (reviewed by Cohen et al., 2016). Similarly, chitosan-
290 induced resistance has a.o. been shown to protect eggplants from *M. inognita* infection (Asif
291 et al., 2017) as well as *Pinus patula* against *Fusarium circinatum* infection (Fitza et al.,
292 2013).

293 In this paper, we demonstrate the activity of two such defense elicitor formulations, based on
294 COS-OGA, as a foliar spray to control RKN infection in rice roots. Depending on the
295 experiment and the rice cultivar, reductions in gall and female numbers ranging between 25
296 and 75% were observed in roots after one single foliar application of COS-OGA. The
297 systemic control provided by FytoSave and FytoSol was comparable for both products and
298 was similar to what has been observed in our previous research with BABA (Ji et al., 2015) ,
299 while thiamine gave less pronounced effects to control nematode infection in rice roots
300 (Huang et al., 2016).

301 In order to elucidate the mode-of-action of COS-OGA systemic induced defense against
302 RKN, hormone measurements were executed on roots and shoots of rice plants, 24 h after
303 treatment. ABA-levels in shoots of treated plants were significantly reduced in comparison
304 with untreated plants, while ABA root levels were unaffected. In previous research we found
305 that foliar ABA application leads to increases in root ABA levels and enhanced susceptibility
306 to RKN through a negative antagonistic interaction with jasmonate-based defense (Kyndt et
307 al., 2017). In combination with the current data showing that FytoSave and FytoSol treatment
308 do not affect root ABA levels, ABA seems unlikely to be responsible for COS-OGA systemic
309 induced defense.

310 It has been demonstrated that FytoSave-induced defense is based on activation of the SA
311 pathway in tomato leaves starting after the second COS-OGA spraying (Van Aubel et al.,
312 2016). In tomato, leaf proteomic analysis of plants sprayed twice with COS-OGA showed
313 accumulation of Pathogenesis-Related proteins (PR), especially subtilisin-like proteases, and
314 qRT-PCR confirmed upregulation of PR-genes and SA-related genes (Van Aubel et al.,
315 2016). Here, PR1b expression was not activated in the locally treated tissue (shoots), but
316 showed activation in the roots. Enhanced activation of PR1 has previously been correlated
317 with enhanced RKN-resistance in tomato (Molinari et al., 2014; de Medeiros et al., 2017).
318 Although induction of this gene is generally correlated with SA, our observations do not
319 show a clear role for SA in COS-OGA induced defense. The experiments with one single
320 spraying on rice showed that shoot SA levels were significantly lower 24 h after COS-OGA
321 treatment in rice, while root SA levels were unaffected and the transcripts of the SA
322 biosynthesis gene *OsICS1* were not significantly induced or even slightly repressed in shoots
323 or roots upon COS-OGA treatment. In addition, COS-OGA was still inducing systemic
324 defense in the SA-deficient *NahG* line. Rice shoot tissue is well-known to contain very high
325 basal levels of SA, which do not significantly rise upon pathogen inoculation, although

326 activation of SA-signaling can activate defense responses against for example *Magnaporthe*
327 *oryzae* (Shimono et al., 2007). Hence, one could reason that while actual SA-levels are not
328 important, the SA-signaling pathway could still play a role in COS-OGA systemic induced
329 defense. However, contradicting this hypothesis, FytoSave and FytoSol application were still
330 fully active in the *OsWRKY45* RNAi line. From these observations we conclude that the SA-
331 dependent defense pathway is not the main driver of COS-OGA systemic induced defense in
332 rice against RKN.

333 Gene expression analysis showed a clear activation of *OsPAL4* and *OsPAL6*-gene expression
334 in root systems of COS-OGA treated plants, similar to the observations reported by Fitza et
335 al. (2013) in chitosan-treated *Pinus patula*. PAL is the committed step into the
336 phenylpropanoid pathway, that involves a complex series of branching biochemical pathways
337 to provide plants with structural cell components (lignin, suberin and other cell wall-
338 associated phenolics), pigments (flavonoids, anthocyanins), SA and toxins (coumarins and
339 furanocoumarins) (Vogt, 2010). Our data show that *OsPAL4*-activation is essential for COS-
340 OGA systemic induced defense, as the *OsPal4*-mutant was insensitive to COS-OGA
341 treatments. PAL-activity measurements confirmed its enzymatic activation in the shoots,
342 although gene expression of different *PAL*-paralogues was negatively affected in this tissue.
343 PAL has been shown to be tightly metabolically regulated through negative feedback by
344 cinnamic acid on *PAL* transcription and on enzyme activity (Blount et al., 2000). Based on
345 our data, we propose that the COS-OGA induced PAL-activity lead to negative feedback
346 control of *PAL*-gene expression in shoot tissue. Hormone data revealed a minor accumulation
347 of JA in roots of COS-OGA treated plants, while shoots levels were significantly reduced 24h
348 after FytoSave-treatment. The jasmonate pathway is known to play a central role in immunity
349 against RKN in (Nahar et al., 2011; Gleason et al., 2016) and it is known that the
350 phenylpropanoid pathway is positively regulated by JA (Pauwels et al., 2008; Taheri &

351 Tarighi, 2010). However, since only a small change in expression of JA-related genes and in
352 JA accumulation was observed in the COS-OGA treated plants and seeing the fact that both
353 COS-OGA formulations were still effective in the JA-deficient *hebiba* mutant, JA-
354 biosynthesis seems not to be required for FytoSol and FytoSave systemic induced defense.

355 However, our data demonstrate that activation of *OsPAL4* is essential for COS-OGA
356 systemic induced defense. Similar to these observations, *PAL* expression has been shown to
357 correlate with thiamine-induced systemic defense against *M. graminicola* in rice (Huang et
358 al., 2015) as well as chitosan-induced defence against *Fusarium* in *Pinus* (Fitza et al., 2013).
359 *OsPAL4* was found to be upregulated upon infection in the *M. graminicola*-resistant rice
360 cultivar Vandana, while no differences in expression were observed in the susceptible
361 cultivar Pusa (Kumari et al., 2016). In addition, the phenylpropanoid pathway is at least
362 partially responsible for resistance against the foliar nematode *Ditylenchus angustus* in the
363 rice genotype 'Manikpukha' (Khanam et al., 2017). Despite these observations and the fact
364 that the *OsPal4*-mutant is highly susceptible to rice blast (Tonnessen et al., 2015), this mutant
365 was here found to be less susceptible towards RKN, which would indicate a role for this gene
366 in rice susceptibility towards RKN. However, previous observations with the general PAL-
367 inhibitor AOPP, showed that PAL inhibition does not significantly influence rice
368 susceptibility towards RKN (Ji et al., 2015). Transcriptome analyses have shown that the
369 phenylpropanoid pathway is generally suppressed in RKN-induced feeding sites in rice
370 (Kyndt et al., 2012). This, together with the fact that PAL-family contains many paralogues
371 and leads to a complex variety of metabolites, complicates interpretation of these data.
372 Nevertheless, upon induced defense by COS-OGA, the RKN might not be able anymore to
373 overcome the activated plant immune response.

374 While our data demonstrate that activation of *OsPAL4* is essential for COS-OGA systemic
375 induced defense, it remains to be determined which metabolite produced in the shoot by the

376 phenylpropanoid pathway determines RKN resistance in the roots. The fact that COS-OGA
377 activity against RKN is not dependent on SA biosynthesis and signaling is an indication that
378 other products derived from the phenylpropanoid pathway could be responsible for the
379 observed lower nematode susceptibility.

380 The phenylpropanoid pathway can contribute to the biosynthesis of many defense-related
381 compounds, such as phenolics, lignins, stilbenes , phytoalexins and isoflavonoids (Vogt,
382 2010)). Recent findings show that the phenylpropanoid pathway is also involved in the
383 induction of resistance in other pathogen-plant interactions, although no systemic effects
384 have ever been investigated. For example, BABA-induced resistance against downy mildew
385 (*Plasmopara viticola*) in grapevine was associated with the primed deposition of, among
386 others, phenylpropanoid-derived phenolics in the treated tissue (Hamiduzzaman et al., 2005).
387 Foliar silicon application to the rose (*Rosa hybrida*) cultivar Smart increased the expression
388 of phenylpropanoid pathway genes and the concentration of antimicrobial phenolic acids and
389 flavonoids (rutin and quercitrin), and this was correlated with increased plant protection
390 against infection by rose powdery mildew in the leaves (*Podosphaera pannosa*) (Shetty et al.,
391 2011). Concerning nematodes (Fujimoto et al., 2015) found that induced resistance against
392 *M. incognita* in Arabidopsis by root sclareol treatment was correlated with higher transcript
393 levels of *PAL1*, cinnamoyl 4-hydroxylase (*C4H*) and cinnamoyl-CoA reductase (*CCR2*).
394 Similarly, root benzothiadiazole (BTH) application led to induced expression of
395 phenylpropanoid biosynthesis genes, and this was correlated with significant changes in the
396 monomer composition of lignin in RKN-induced galls in tomato roots (Veronico et al.,
397 2018). Whether the flavonoid and/or lignin composition of rice roots is affected by COS-
398 OGA treatment remains to be studied in follow-up research. Additionally, the observation
399 that PAL-activity is mainly induced in the shoots of the treated plants, while phenylpropanoid

400 gene expression and plant defense against nematodes is observed in the root system raises the
401 question which PAL4-dependent signal is transported from the shoot to the root system.

402 In conclusion, we have shown that foliar COS-OGA applications can effectively protect rice
403 roots from RKN infection. We demonstrate for the first time that the effect of COS-OGA is
404 systemic and its systemic mode-of-action is not based on the traditional SA or JA defense
405 hormones, but on activation of the phenylpropanoid pathway.

406

407

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412 **Figure legends**

413 **Figure 1.** Effect of COS-OGA formulations FytoSol (Sol) and FytoSave (Save) at different
414 concentrations (0.1, 0.5, 0.25, 0.125% v/v) on rice susceptibility to the root-knot nematode
415 *M. graminicola*. (A) Average number of root galls per plant counted at 14 dpi, (B) average
416 number of females per plant counted at 14 dpi. The bars are the means \pm standard error (SE)
417 of 8 individual plants per treatment. Different letters indicate significant differences (Duncan;
418 $\alpha = 0.05$). The whole experiment was independently repeated with similar results.

419
420 **Figure 2.** Hormone levels in COS-OGA (0.5% v/v) treated rice plants in comparison with
421 mock-treated control plants, measured 24 h after foliar application. (A) Abscisic acid (ABA),
422 indole-3-acetic acid (IAA), and jasmonic acid (JA) content in the shoots of treated and
423 control plants. (B) Salicylic acid (SA) content in the shoots of treated and control plants. (C)
424 ABA, IAA, JA and SA content in the roots of treated and control plants. Values presented are
425 means \pm SE of 5 biological replicates (each a pool of 3 individual plants) per treatment.
426 Asterisks indicate statistically significant differences (t-test; $\alpha = 0.05$).

427
428 **Figure 3.** Gene expression analysis with qRT-PCR on shoots (A) and roots (B) of COS-OGA
429 treated rice plants. The relative expression levels of JA biosynthesis *OsAOS2*, SA-
430 biosynthesis gene *OsICS1*, PAL-encoding genes *OsPAL2*, *OsPAL4*, *OsPAL6* and the general
431 plant defense gene *PR1b*, were analyzed using qRT-PCR at 24h after treatment. Values
432 presented are means \pm SE of 3 biological replicates (each a pool of 4 individual plants) per
433 treatment. Gene expression levels were normalized using two internal reference genes,
434 *OsEXP* and *OsEif5C*. Data are shown as relative transcript levels in comparison with the
435 control plants (expression level set at 1). Asterisks indicate significant differential expression
436 (REST-analysis; $\alpha = 0.05$).

437

438 **Figure 4.** Role of SA and JA in the COS-OGA systemic induced defense against RKN in
439 rice. (A) Activity of COS-OGA against nematodes in the SA-deficient *NahG* line and
440 *OsWRKY45* RNAi line and their corresponding wild-type ('Nipponbare'). Plants were treated
441 with 0.5% FytoSave or FytoSol at 24 h before inoculation. Number of galls per plant were
442 counted at 14 dpi. (B) Activity of COS-OGA against nematodes in the JA-deficient *hebiba*
443 mutant and its corresponding wild-type ('Nihonmasari'). Plants were treated with FytoSave
444 or FytoSol at 24 h before inoculation. Number of galls per plant were counted at 14 dpi. The
445 bars are the means \pm SE of 8 individual plants per treatment. Different letters indicate
446 statistically significant differences (Duncan; $\alpha = 0.05$). The whole experiment was
447 independently repeated with similar results.

448

449 **Figure 5.** Role of the phenylpropanoid pathway in the COS-OGA systemic induced defense
450 against RKN in rice. (A) PAL enzymatic activity in the shoots of treated and control plants.
451 (B) PAL enzymatic activity in the roots of treated and control plants. Values presented are
452 means \pm SE of 4 biological replicates (each a pool of 4 individual plants) per treatment.
453 Asterisks indicate statistically significant differences from control plants ($\alpha = 0.05$). (C)
454 Activity of COS-OGA against nematodes in the *OsPAL4*-mutant line and its corresponding
455 wild-type ('IR64'). Plants were treated with FytoSave or FytoSol at 24h before inoculation.
456 Number of galls per plant were counted at 14 dpi. The bars are the means \pm SE of 8
457 individual plants per treatment. Different letters indicate statistically significant differences
458 (Duncan; $\alpha = 0.05$). The whole experiment was independently repeated with similar results.

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463 **Supplementary information**

464 **SI.** Effect of COS-OGA formulations FytoSol (Sol) and FytoSave (Sav) at different
465 concentrations (0.1, 0.5, 0.25, 0.125% v/v) on rice cv. 'Nipponbare' shoot (A) and root (B)
466 fresh weight under root-knot nematode *M. graminicola* infected conditions, measured at 14
467 dpi. The bars are the means \pm SE of 8 individual plants per treatment. No significant
468 differences were observed (Duncan; $\alpha = 0.05$).

469 **SII.** Effect of COS-OGA formulations FytoSol (Sol) and FytoSave (Sav) at 0.5% v/v on rice
470 cv. 'IR64' root length under root-knot nematode *M. graminicola* infected conditions,
471 measured at 14 dpi. The bars are the means \pm SE of 8 individual plants per treatment.
472 Different letters indicate significant differences (Duncan; $\alpha = 0.05$).

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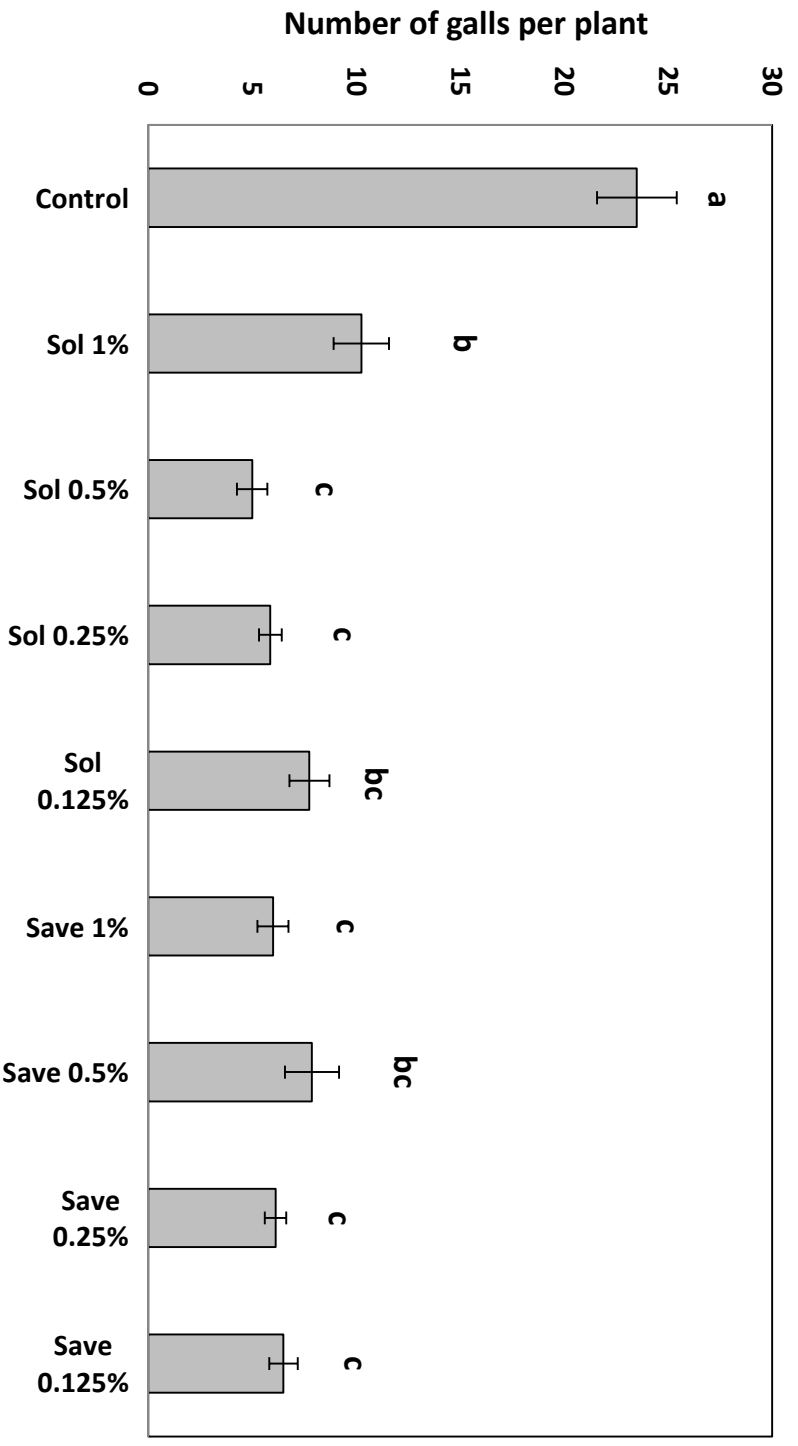
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Figure 1

A



B

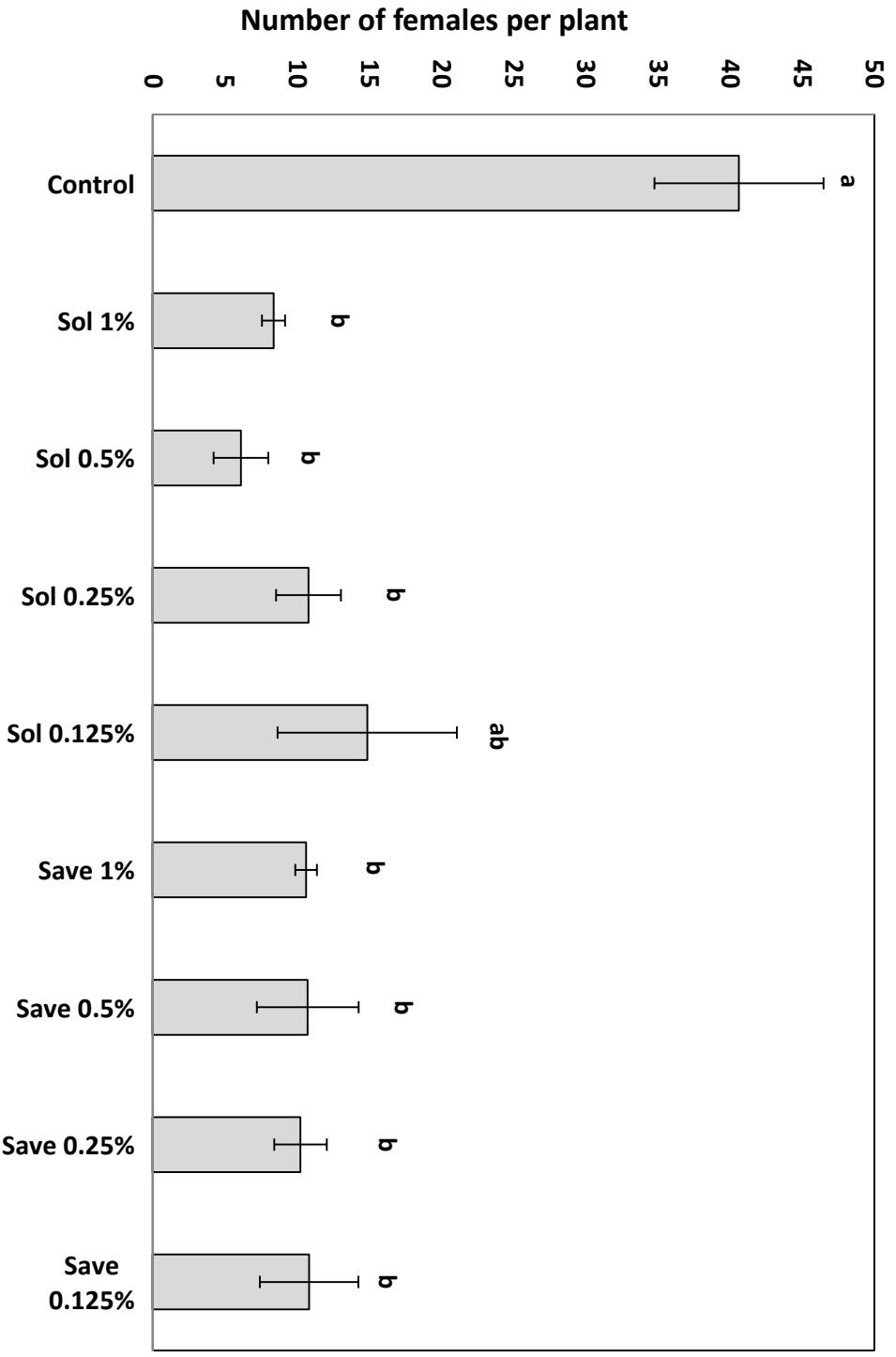
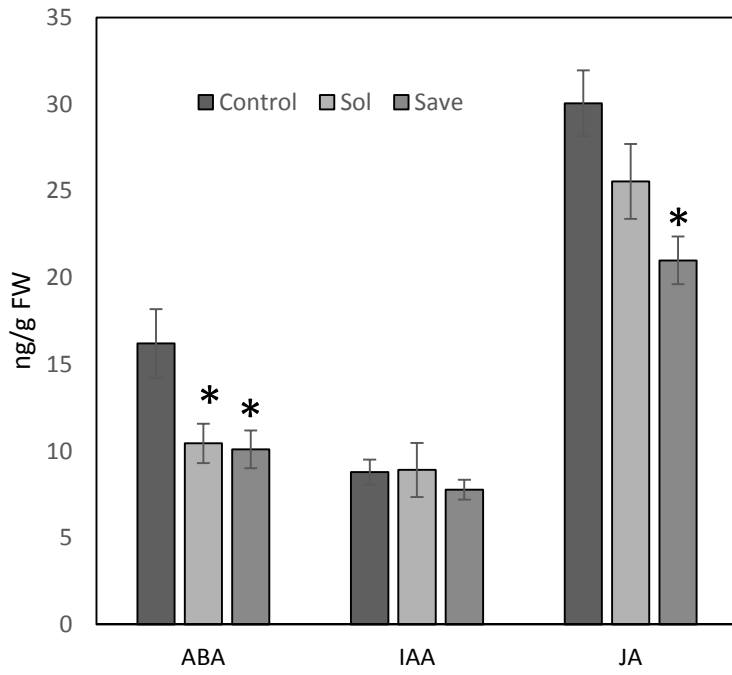
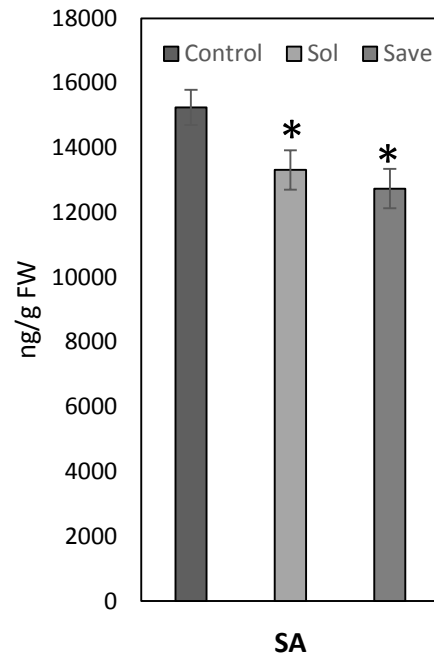


Figure 2

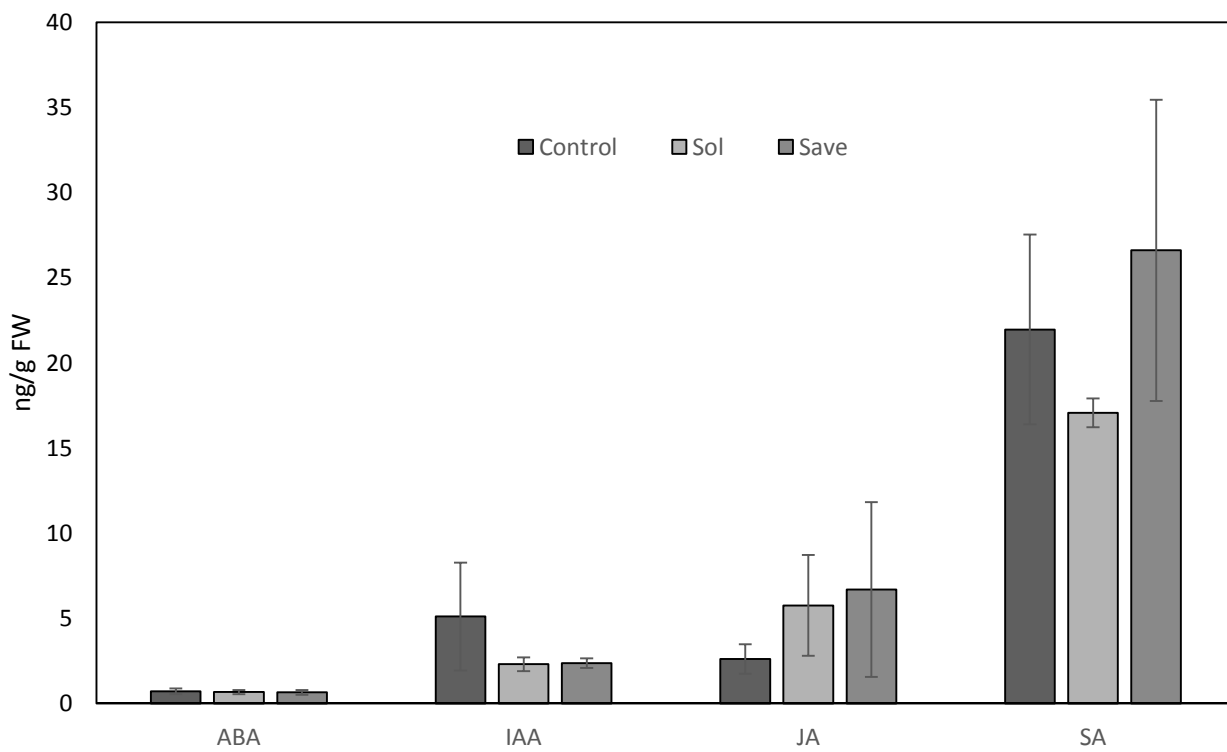
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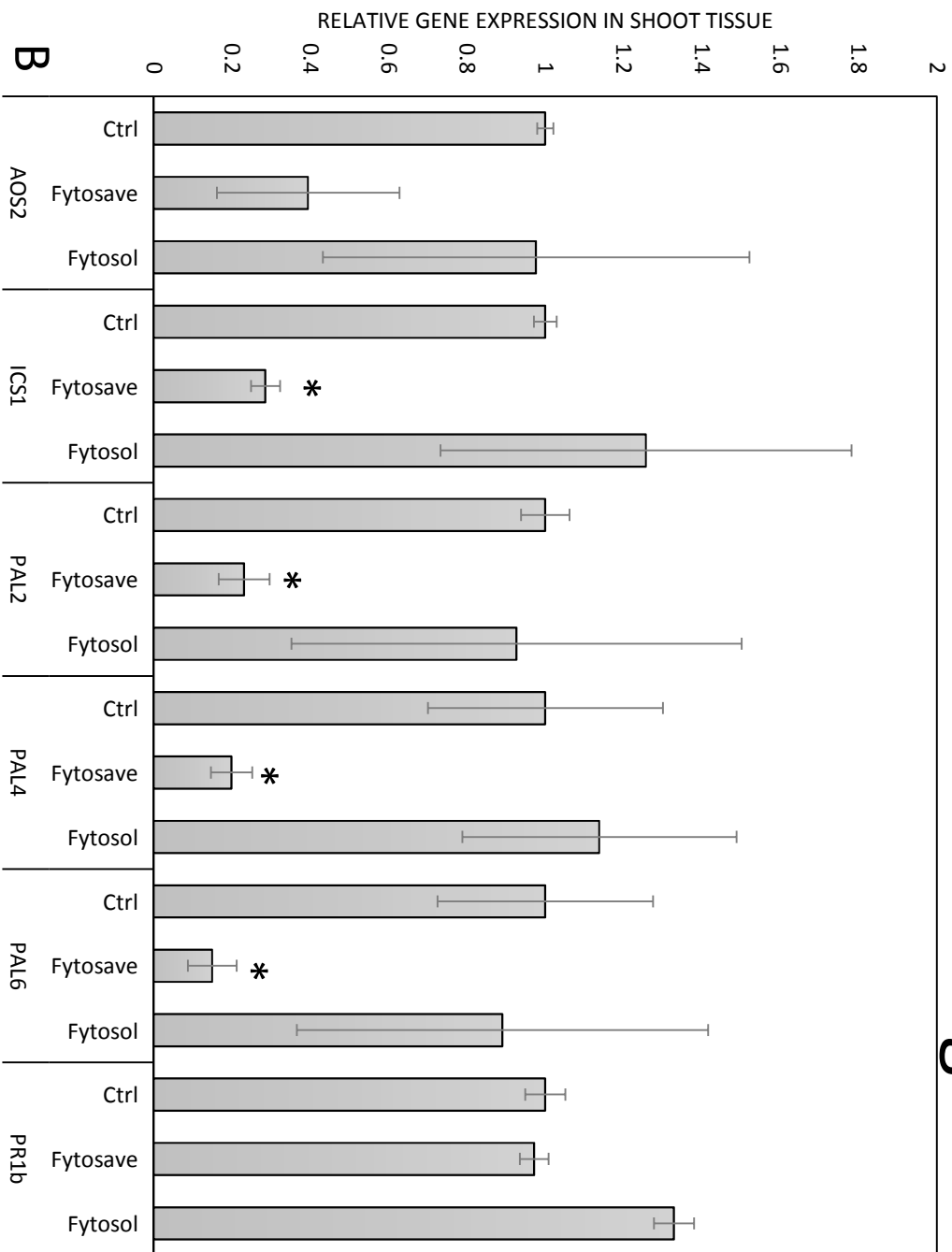
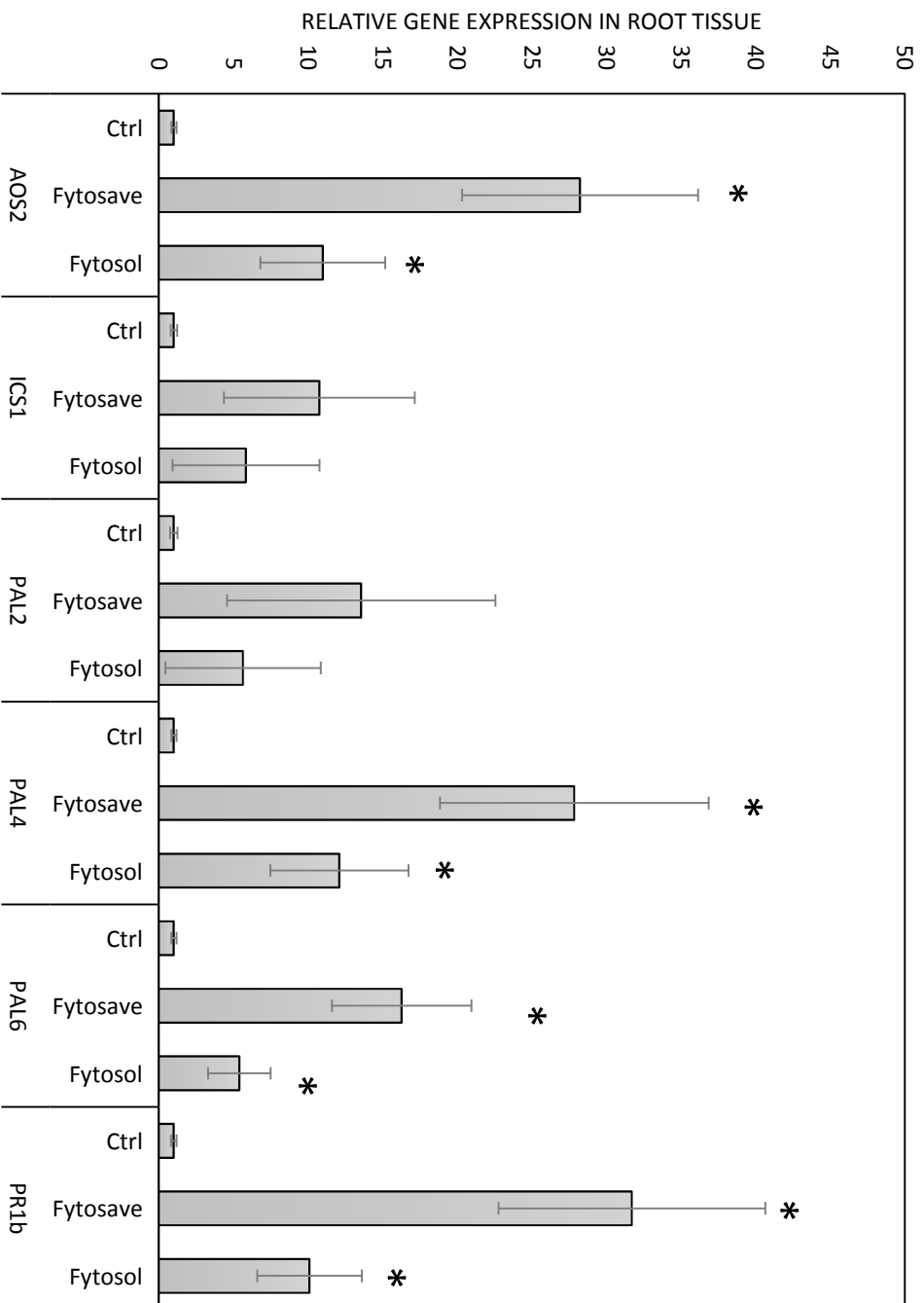


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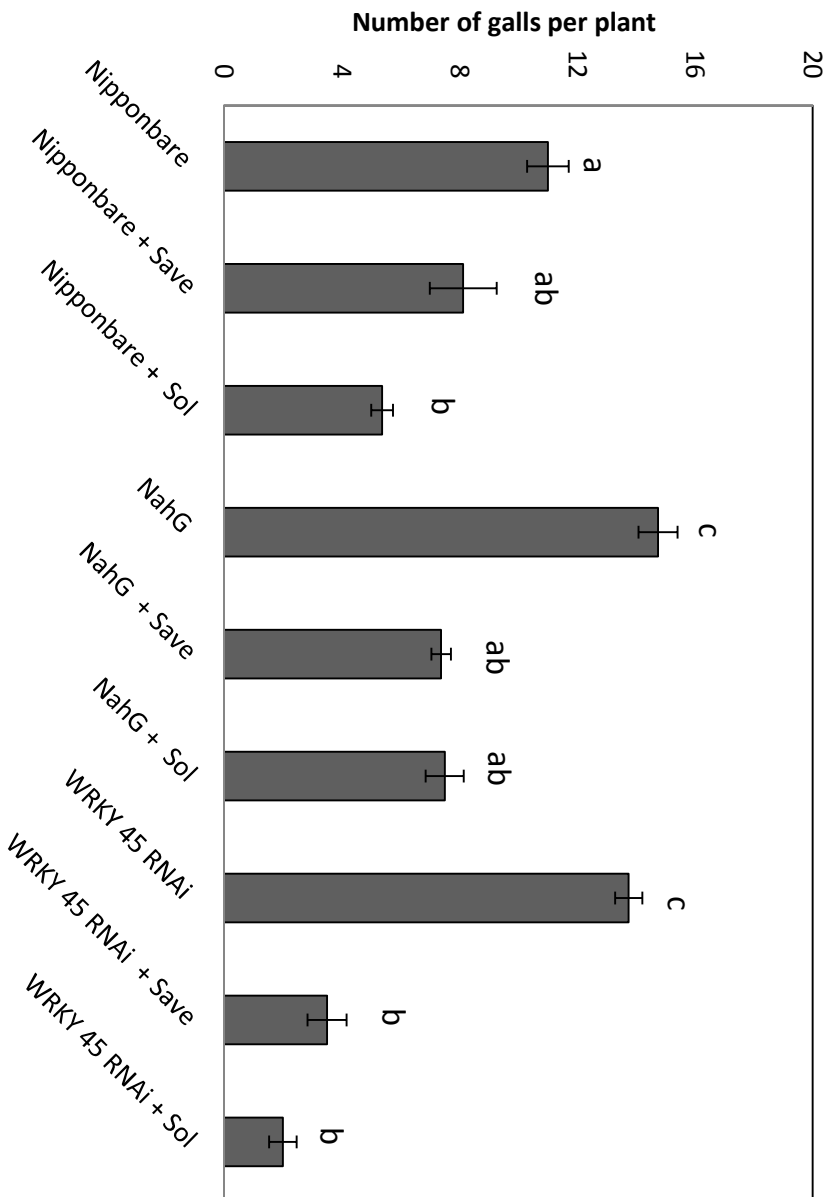


C



A**Figure 3**

A



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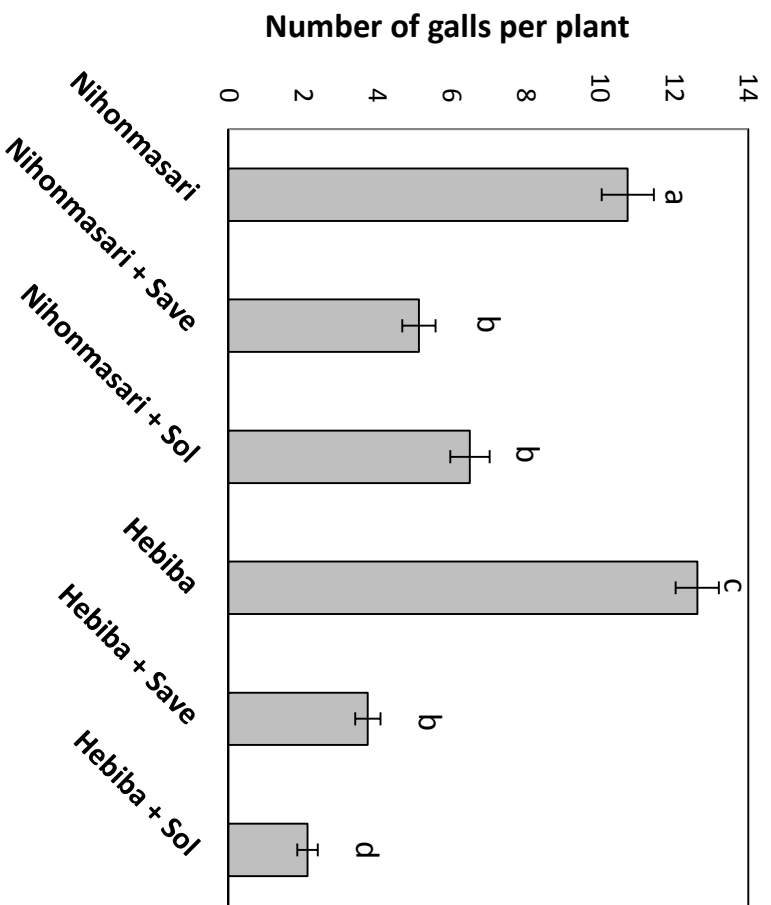
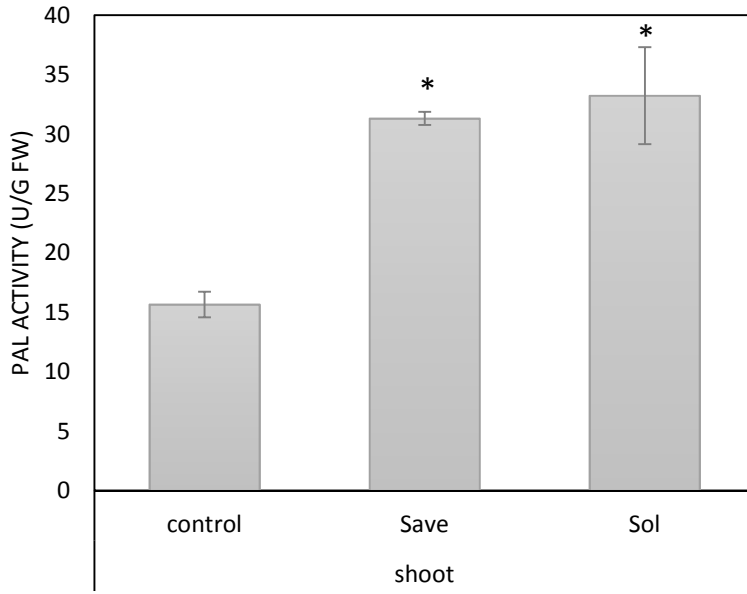


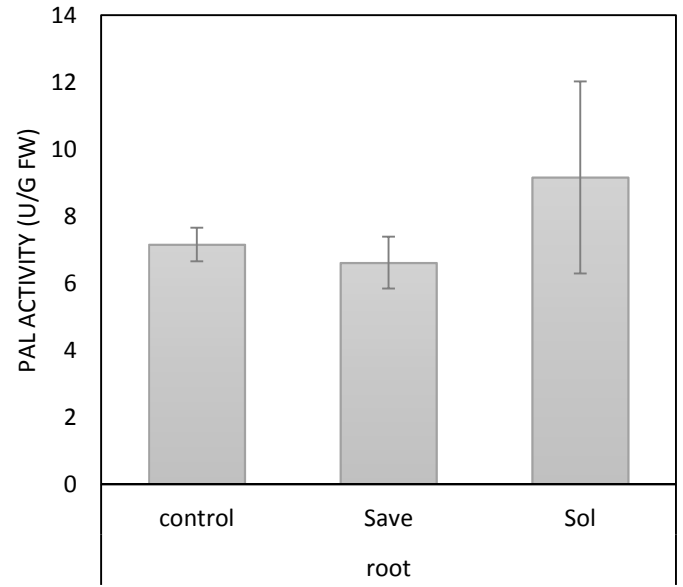
Figure 4

Figure 5

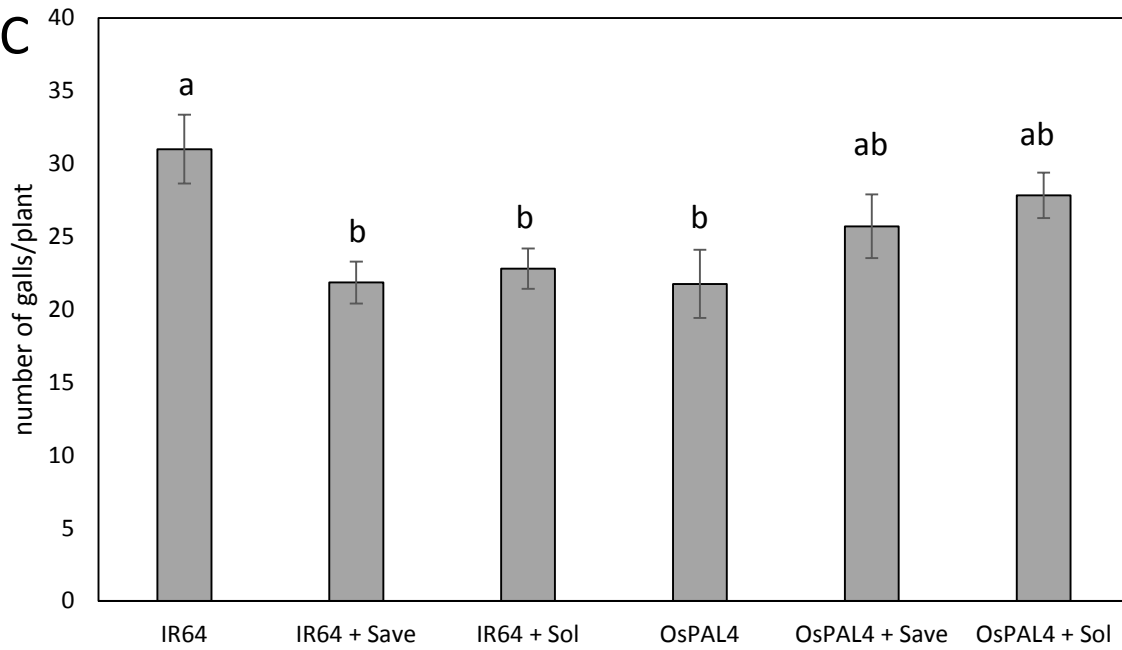
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C



Highlights

- Foliar application of two formulations of chitosan oligomers and pectin-derived oligogalacturonides reduces nematode infection by more than 30% in rice roots, showing for the first time a systemic effect of these defence elicitors.
- Systemic defence activation is not correlated with defence hormone accumulation in the rice shoots and roots.
- The systemic defence against root-knot nematodes is dependent on stimulation of the phenylpropanoid pathway.

B. Chinnasri did the infection experiments on wild-type and mutant plants. L. De Smet executed the direct nematicidal assays. R.R. Singh performed the PAL-measurements and the experiments on the PAL4-mutant and wrote the manuscript. A. Haeck and K. Demeestere executed the hormone measurements. P. Van Cutsem and G. Van Aobel formulated the defence elicitors. G. Gheysen and T. Kyndt designed and supervised the experimental set-up and provided extensive corrections on the draft manuscript. All authors have read and approved the manuscript before submission.