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Research article

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

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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® 24 h 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.

1. Introduction

With a world population that is currently growing at 83 million per year, the pressure on food production will only increase. Rice (*Oryza sativa*) is one of the most important staple foods in the world, with a production of more than 730 million tons per year (Fao, 2016). Although not well-known because they cause mainly belowground symptoms, plant parasitic nematode infections contribute to major agricultural losses in rice production (Mantelin et al., 2017). The root-knot nematode (RKN) *Meloidogyne graminicola* is probably the most damaging root pathogen affecting – mainly aerobic – rice fields in Asia, and it was recently also detected in Italian rice fields (Fanelli et al., 2017; Mantelin et al., 2017). RKN induce the formation of 'giant cells' inside the root tissue, from which they withdraw plant metabolites for their nutrition, leading to the visible formation of root-knots (galls) (Mantelin et al., 2017). The control of RKN using conventional methods is challenging because of their wide host range, ability to survive in soil and weeds, and the low inherent level of resistance in rice against this nematode. An alternative to use of nematicides, activation of the plant innate immunity could be a more environmentally friendly control method (Conrath et al., 2015).

Through evolutionary history, plants have acquired complex defense or 'immunity' mechanisms towards biotic stress factors like bacteria, fungi, insects and nematodes. Plant innate immunity is based on recognition of pathogen-associated molecular patterns (PAMPs), activating basal immune responses. Plant immune responses typically include rapid physiological changes such as Ca^{2+} uptake and production of reactive oxygen and nitrogen species (ROS and RNS). After signal transduction, these changes induce production of secondary metabolites, including hormones (among which salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA)) and pathogenesis-related (PR) proteins. In case of PAMP-triggered immunity (PTI), the response is relatively small in magnitude but active against a broad range of pathogens (Jones and Dangl, 2006). The highly specific effector-triggered immunity (ETI) - that is activated upon recognition of pathogen effectors - is much stronger and often accompanied by a

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hypersensitive response (HR) – induced cell death – at the site of attempted host colonization (Jones and Dangl, 2006).

Triggering a defensive state in a plant can be achieved by applying chemical or biological molecules or micro-organisms. Traditionally, the distinction is being made between SAR (systemic acquired resistance (SAR)) that depends on the plant hormone SA, and the SA-independent induced systemic resistance (ISR), which is based on JA/ET activation (Pieterse et al., 2014). Strong activation of plant immunity can be energy-consuming, but a more energy-efficient mechanism to trigger plant acquired immunity, known as priming, has been described in the early 2000's. During priming, a treatment, such as a (minor) stress or application of a certain molecular agent or micro-organism, puts a plant in a state of increased alertness with no or minimal direct immune gene induction or hormone accumulation, and hence no energy and yield loss. Upon pathogen attack, a faster and more robust defense response is activated in the primed plants than in unprimed plants (Conrath et al., 2015). In the dicotyledonous model plant *Arabidopsis thaliana*, priming has been shown to involve (1) the accumulation of dormant mitogen-activated protein kinases; and/or (2) epigenetic modifications; and/or (3) accumulation of secondary metabolites (Conrath et al., 2015). In our research we are evaluating the potential and mode of action of priming to protect rice against RKN. In previous research, we have identified the activity and involved pathways for compounds such as beta-amino butyric acid (BABA), and thiamine (Huang et al., 2016; Ji et al., 2015). Thiamine treatment leads to increases in H_2O_2 production in rice (Huang et al., 2016). Increases in H_2O_2 and callose as well as lignification were observed in nematode-infected plants pretreated with BABA (Ji et al., 2015). Lignin precursors are formed by the phenylpropanoid pathway, in which enzymatic conversion of phenylalanine into trans-cinnamate, mediated by phenylalanine ammonia lyase (PAL), is the first and rate-limiting step. Next to monolignols, this plant-specific pathway, which is of significant importance to growth and development, can also convert phenylalanine into other secondary metabolites, such as flavonoids, salicylic acid, stilbenes and many other products playing a role in plant immunity (Vogt, 2010).

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

2. Materials & methods

2.1. Plant material and growth conditions

Rice (*Oryza sativa*) seeds of cultivar Nipponbare were provided by U.S. Department of Agriculture (GSOR-100). Seeds of the *Ospal4*-mutant (Tonnessen et al., 2015) and its wild-type IR64 were kindly provided by the lab of J. Leach (Colorado State University, CO, USA). Seeds were germinated on wet filter paper in a petri dish for 4 days at 30 °C. They were transplanted in in-house-made polyvinyl-chloride (PVC) tubes (height: 15 cm; diameter 3 cm) containing a mixture of fine sand and synthetic absorbent polymer (SAP) substrate (for more details see Nahar et al., 2011; Huang et al., 2016). The polymer used is Aquaperla® (DCM, Belgium). The plants were further kept in a growth room at 26 °C, 12 h/12 h light regime (150 μmol/m^{2}s) and relative humidity of 70–75%. The plants were maintained by supplying 10 ml Hoagland solution three times a week. To avoid possible effects induced by the photoperiod, all inoculations and samplings were done at the same moment of the day, 10 a.m., which is 2 h after sunrise.

2.2. Plant treatments

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

2.3. Infection experiments

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

Fifteen-day-old rice plants were inoculated with 250 juveniles (J2) of *M. graminicola* or mock inoculated with water. The infection level of the plants was evaluated at 14 days after inoculation by counting the number of galls and nematodes per plant. Individual root systems were removed from the substrate, gently washed and packed in a tissue bag (Miracloth, VWR). They were stained with acid fuchsin, which leads to intense pink staining of the galls: roots were boiled for 3 min in a solution of 0.8% acetic acid and 0.013% acid fuchsin. Nematode development inside the galls as well as giant cells can be observed when the acid fuchsin-stained root system is destained for approximately 4 d in acid glycerol. The development of nematodes until maturity (females) is considered as a measure of general nematode development in the root system. Galls and females were counted microscopically using a stereomicroscope (Leica S8 APO, Leica Microsystems, Diegem, Belgium).

2.4. Direct effect on nematodes

Approximately 50 J2s were placed into a 2.5-cm diameter well on a 12-well culture plate containing 1.5 ml of Fytosave or Fytosol at two different concentrations (1 and 0.5%) or 1.5 ml of distilled water for the mock treatment. The living and dead nematodes were counted at different time points under a stereomicroscope (Leica S8 APO, Leica Microsystems, Diegem, Belgium). Nematodes were considered dead if they were not moving and did not respond to being touched by a small probe. The experiment was performed three times with 6 replicates

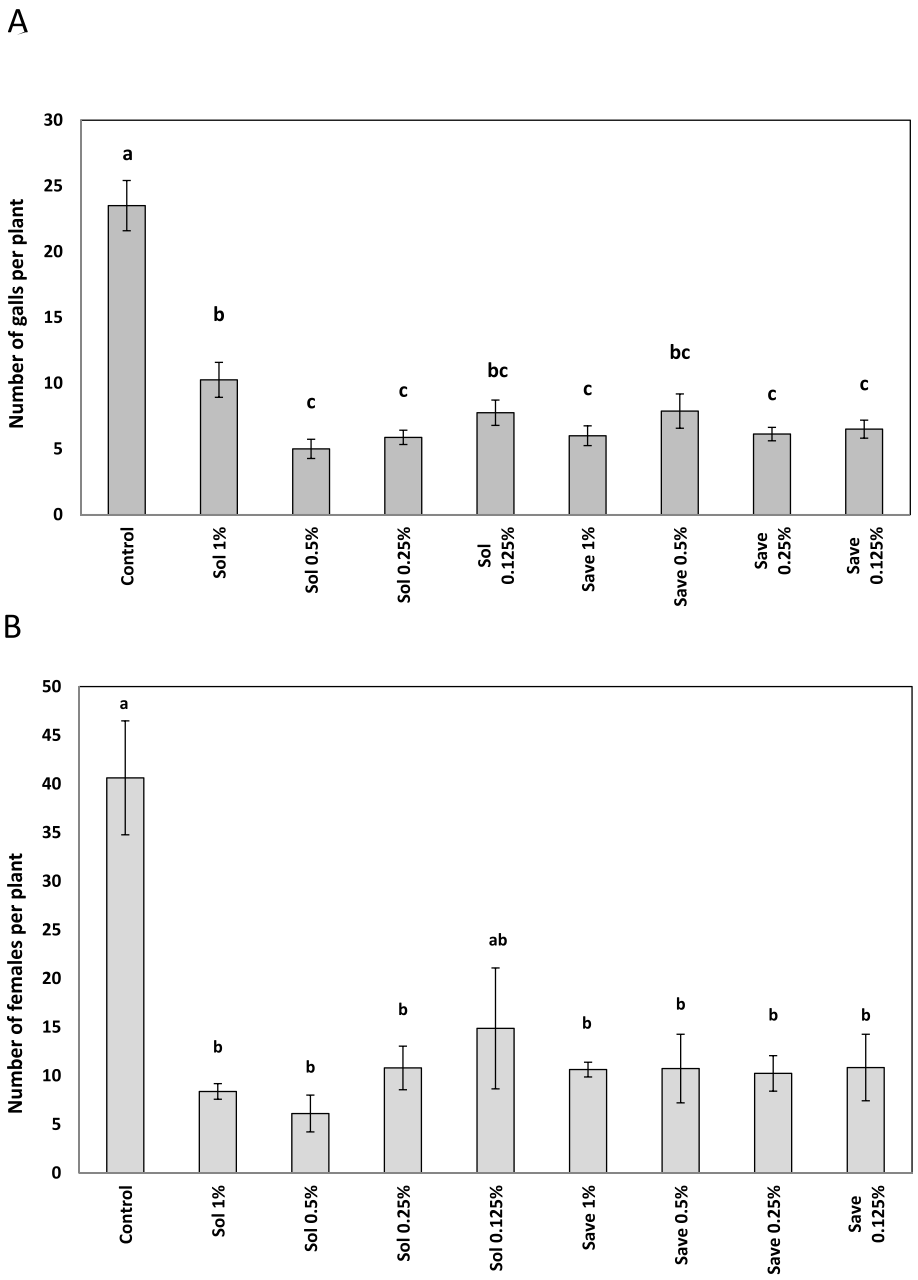


Fig. 1. Effect of COS-OGA formulations FytoSol (Sol) and FytoSave (Save) at different concentrations (0.1, 0.5, 0.25, 0.125% v/v) on rice susceptibility to the root-knot nematode *M. graminicola*. (A) Average number of root galls per plant counted at 14 dpi, (B) average number of females per plant counted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

each.

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

RNA was extracted using the Plant RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. For each treatment, 3 biological replicates were taken, consisting of a pool of at least 4 plants. qRT-PCR was performed and analyzed as described in Huang et al., 2016. Expression levels were normalized using three reference genes, *OsEIF5C*, and *OsEXP*. Primer pairs are listed in Huang et al., 2016 and Tonnessen et al. (2015).

2.6. Hormone measurements

For hormone measurement root and shoot tissues were collected and were homogenized using liquid N_2, and 100 mg of ground material was extracted at −80 °C using the modified Bieleski solvent. After filtration (30 kDa Amicon® Ultra centrifugal filter unit), solvent evaporation and extract reconstitution, chromatographic separation was performed on a U-HPLC system (Thermo Fisher Scientific) with a Nucleodur C18 column (50 × 2 mm; 1.8 µm d_p). The detailed procedure is described in Haeck et al. (2018). For each treatment, 5 biological replicates, each consisting of a pool of at least 3 plants, were measured.

2.7. PAL-activity measurement

PAL-activity was measured as described in Camacho-Cristobal et al. (2002). For each treatment, 4 biological replicates, each consisting of a pool of 3 plants, were sampled. From each replicate, 100 mg of shoot or 100 mg of root samples were ground in liquid nitrogen and dissolved in

800 µl of 50 mM sodium phosphate as assay buffer containing 2% (w/v) poly-vinylpolypyrrolidone (PVPP), 2 mM EDTA, 18 mM-mercaptoethanol and 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 7168 g, at 4 °C for 10 min. In different 2 ml tubes, 135 µl of reaction buffer, 50 µl of 5 mM of L-phenlyalanine, and 20 µl of supernatant were mixed. Absorbance was measured using a spectrophotometer at 290 nm. The reaction was started by incubating the samples in a water bath for 30 min at 40 °C. To stop the reaction, 10 µl of hydrochloric acid was added and the sample was mixed for 10 min, after which PAL activity was assayed by measuring the formation of trans-cinnamic acid at 290 nm. One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nmol trans-cinnamic acid per hour. Control assays had no L-phenylalanine as substrate.

2.8. Data collection and statistical analyses

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

3. Results

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

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

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

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

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

The involvement of SA and JA in COS-OGA induced defense was further evaluated by investigating a set of mutants in the SA and JA-pathway: the SA-deficient transgenic *NahG*-line, SA-signaling deficient *WRKY45*-RNAi line, and JA biosynthesis mutant *hebiba*. Results, presented in Fig. 4, show that all three lines are more susceptible to RKN, as expected based on previously shown importance of these genes for basal defense against RKN (Nahar et al., 2011; Ji et al., 2015). However, COS-OGA systemic induced defense is still active in these three lines (Fig. 4A and B), demonstrating that this phenomenon acts independently of SA-levels, SA-signaling and JA biosynthesis.

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

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

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

4. Discussion

Due to the current ban on chemical nematicides, the pressure to present alternative nematode control strategies is increasing. Next to the use of resistant varieties, crop rotation, flooding or other agronomic prevention strategies, the activation of plant innate immunity against nematodes and other pathogens gains more and more attention (Oka et al., 1999; Cohen et al., 2016; Asif et al., 2017; Medeiros et al., 2017). Although 100% resistance is not achievable with products acting as plant defense elicitors, they are very useful as one of the prevention methods in a well-designed integrated pest management plan, and as such can replace one or more pesticide applications in a seasonal program of plant protection (Walters et al., 2013). Interestingly, the

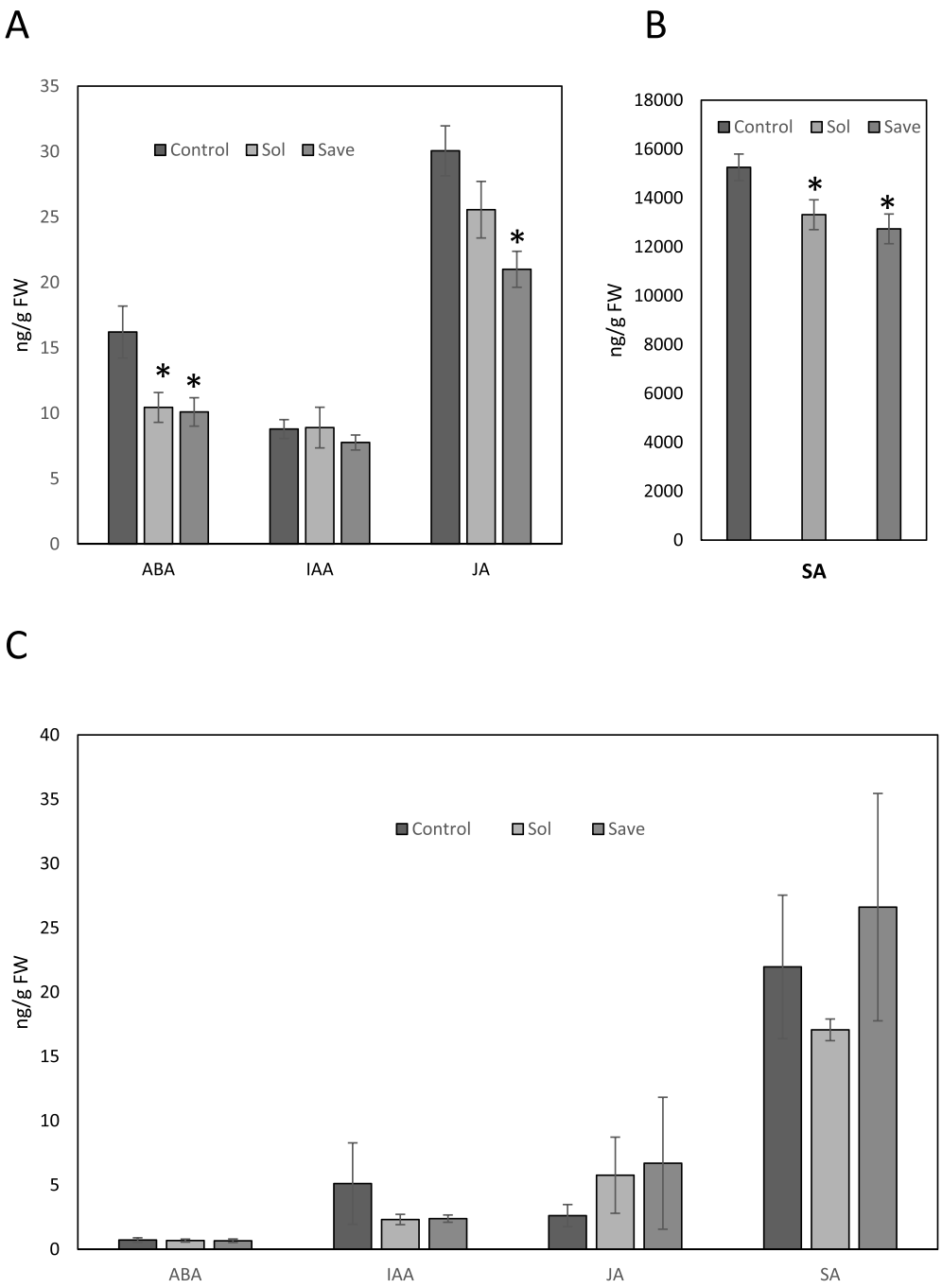


Fig. 2. Hormone levels in COS-OGA (0.5% v/v) treated rice plants in comparison with mock-treated control plants, measured 24 h after foliar application. (A) Abscisic acid (ABA), indole-3-acetic acid (IAA), and jasmonic acid (JA) content in the shoots of treated and control plants. (B) Salicylic acid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

protection conferred by these elicitors is often not specific and can potentially provide broad-spectrum protection against diseases and pests (Sharathchandra et al., 2004). For example, BABA application provides protection against nematodes as well as many bacterial and fungal diseases (reviewed by Cohen et al., 2016). Similarly, chitosan-induced resistance has a.o. been shown to protect eggplants from *M. inognita* infection (Asif et al., 2017) as well as *Pinus patula* against *Fusarium circinatum* infection (Fitza et al., 2013).

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

In order to elucidate the mode-of-action of COS-OGA systemic induced defense against RKN, hormone measurements were executed on roots and shoots of rice plants, 24 h after treatment. ABA-levels in shoots of treated plants were significantly reduced in comparison with untreated plants, while ABA root levels were unaffected. In previous research we found that foliar ABA application leads to increases in root

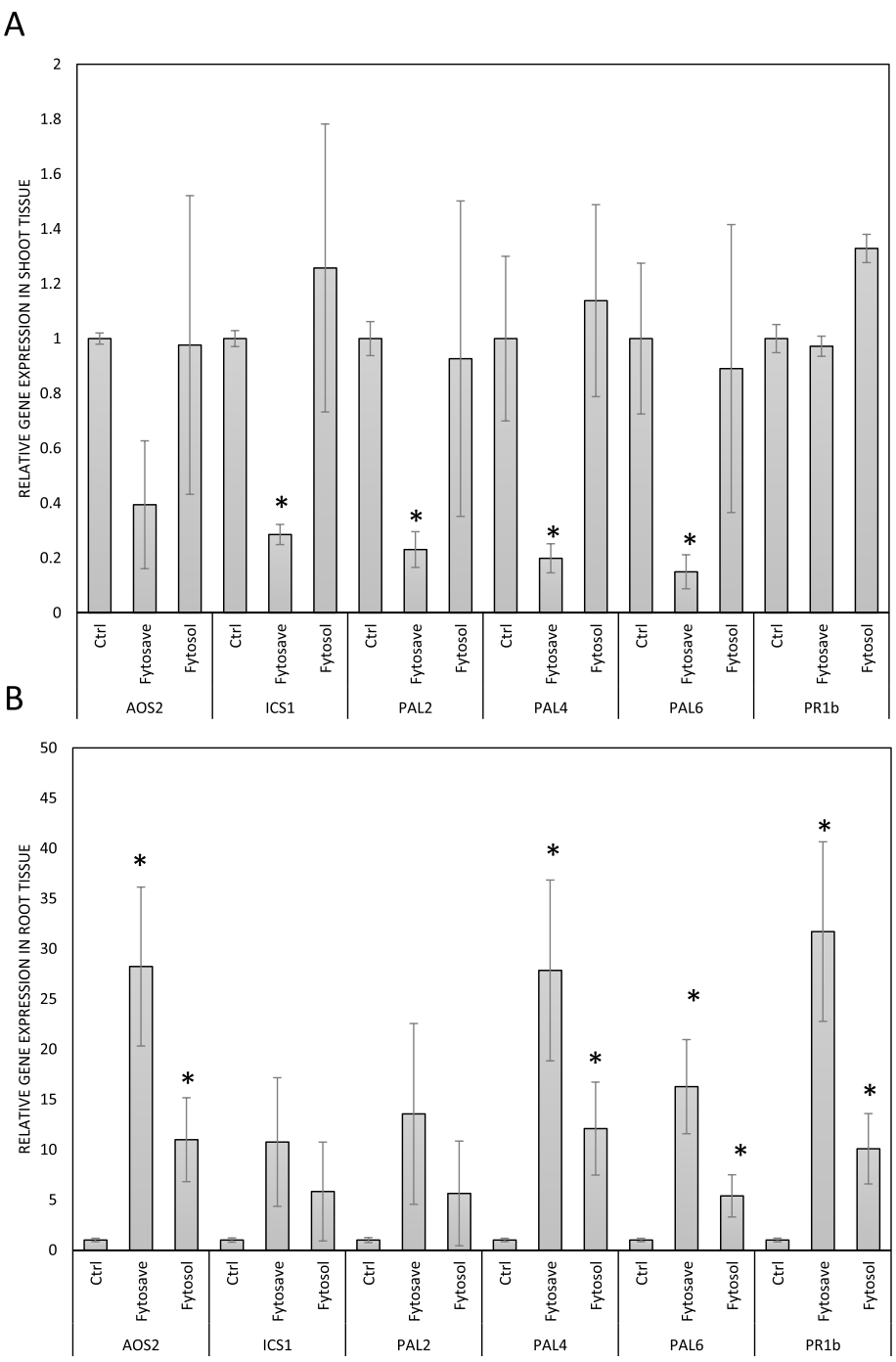
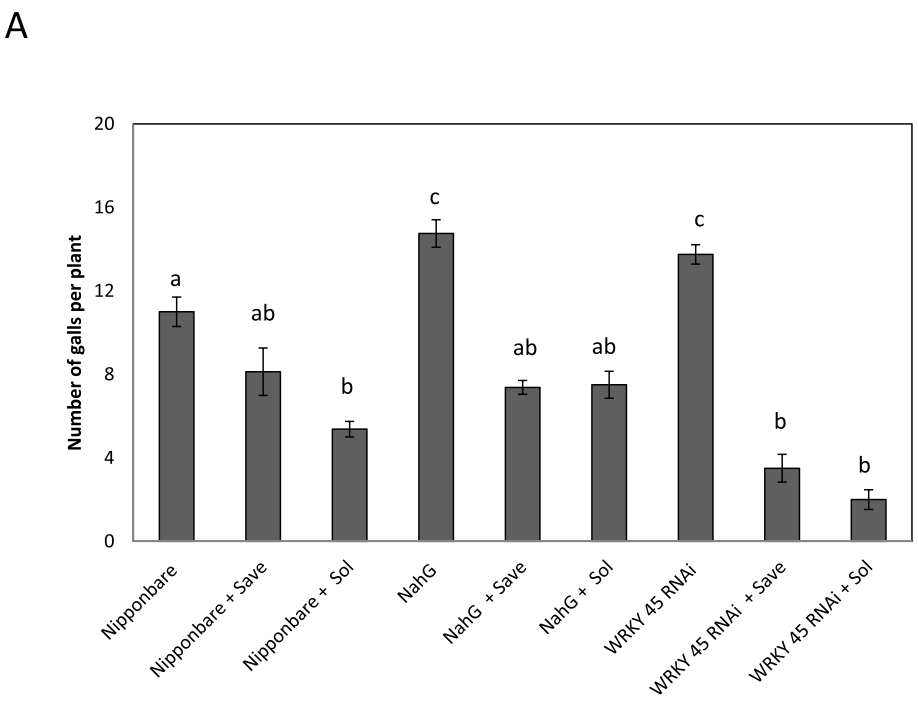


Fig. 3. Gene expression analysis with qRT-PCR on shoots (A) and roots (B) of COS-OGA treated rice plants. The relative expression levels of JA biosynthesis *OsAOS2,* SA-biosynthesis gene *OsICS1*, PAL-encoding genes *OsPAL2*, *OsPAL4*, *OsPAL6* and the general plant defense gene *PR1b*, were analyzed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

ABA levels and enhanced susceptibility to RKN through a negative antagonistic interaction with jasmonate-based defense (Kyndt et al., 2017). In combination with the current data showing that FytoSave and FytoSol treatment do not affect root ABA levels, ABA seems unlikely to be responsible for COS-OGA systemic induced defense.

It has been demonstrated that FytoSave-induced defense is based on activation of the SA pathway in tomato leaves starting after the second COS-OGA spraying (Van Aubel et al., 2016). In tomato, leaf proteomic analysis of plants sprayed twice with COS-OGA showed accumulation of Pathogenesis-Related proteins (PR), especially subtilisin-like proteases, and qRT-PCR confirmed upregulation of PR-genes and SA-related genes (Van Aubel et al., 2016). Here, PR1b expression was not activated in the locally treated tissue (shoots), but showed activation in the roots. Enhanced activation of PR1 has previously been correlated with enhanced RKN-resistance in tomato (Molinari et al., 2014; Medeiros et al., 2017). Although induction of this gene is generally correlated with SA, our observations do not show a clear role for SA in COS-OGA induced defense. The experiments with one single spraying on rice showed that shoot SA levels were significantly lower 24 h after COS-OGA treatment in rice, while root SA levels were unaffected and the transcripts of the SA biosynthesis gene *OsICS1* were not significantly induced or even slightly repressed in shoots or roots upon COS-OGA treatment. In addition, COS-OGA was still inducing systemic defense in the SA-deficient *NahG* line. Rice shoot tissue is well-known to contain very high basal levels of SA, which do not significantly rise upon pathogen inoculation, although activation of SA-signaling can activate defense responses against for example *Magnaporthe oryzae* (Shimono et al., 2007). Hence, one could reason that while actual SA-levels are not important, the SA-



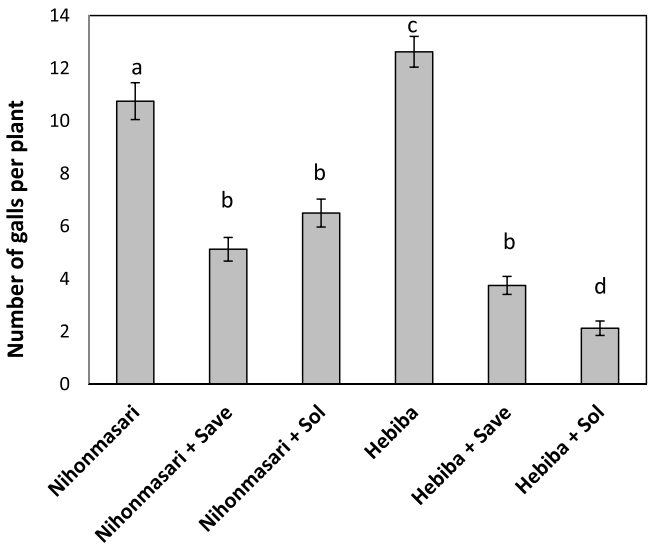


Fig. 4. Role of SA and JA in the COS-OGA systemic induced defense against RKN in rice. (A) Activity of COS-OGA against nematodes in the SA-deficient *NahG* line and *OsWRKY45* RNAi line and their corresponding wild-type ('Nipponbare'). Plants were treated with 0.5% FytoSave or FytoSol at 24 h before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

signaling pathway could still play a role in COS-OGA systemic induced defense. However, contradicting this hypothesis, FytoSave and FytoSol application were still fully active in the *OsWRKY45* RNAi line. From these observations we conclude that the SA–dependent defense pathway is not the main driver of COS-OGA systemic induced defense in rice against RKN.

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

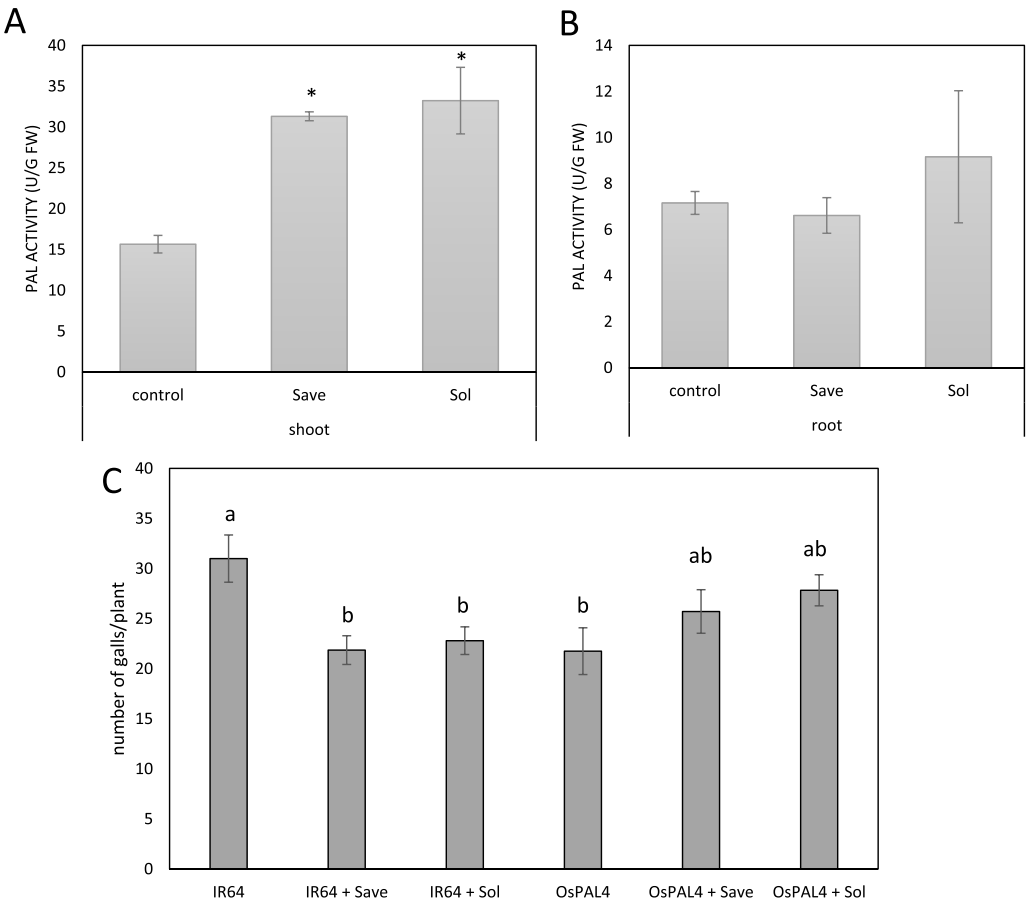


Fig. 5. Role of the phenylpropanoid pathway in the COS-OGA systemic induced defense against RKN in rice. (A) PAL enzymatic activity in the shoots of treated and control plants. (B) PAL enzymatic activity in the roots of treated and control plants. Values presented are means ± SE of 4 biological replicates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

related genes and in JA accumulation was observed in the COS-OGA treated plants and seeing the fact that both COS-OGA formulations were still effective in the JA-deficient *hebiba* mutant, JA-biosynthesis seems not to be required for FytoSol and FytoSave systemic induced defense.

However, our data demonstrate that activation of *OsPAL4* is essential for COS-OGA systemic induced defense. Similar to these observations, *PAL* expression has been shown to correlate with thiamine-induced systemic defense against *M. graminicola* in rice (Huang et al., 2016) as well as chitosan-induced defence against *Fusarium* in *Pinus* (Fitza et al., 2013). *OsPAL4* was found to be upregulated upon infection in the *M. graminicola*-resistant rice cultivar Vandana, while no differences in expression were observed in the susceptible cultivar Pusa (Kumari et al., 2016). In addition, the phenylpropanoid pathway is at least partially responsible for resistance against the foliar nematode *Ditylenchus angustus* in the rice genotype 'Manikpukha' (Khanam et al., 2018). Despite these observations and the fact that the *OsPAL4*-mutant is highly susceptible to rice blast (Tonnessen et al., 2015), this mutant was here found to be less susceptible towards RKN, which would indicate a role for this gene in rice susceptibility towards RKN. However, previous observations with the general PAL-inhibitor AOPP, showed that PAL inhibition does not significantly influence rice susceptibility towards RKN (Ji et al., 2015). Transcriptome analyses have shown that the phenylpropanoid pathway is generally suppressed in RKN-induced feeding sites in rice (Kyndt et al., 2012). This, together with the fact that PAL-family contains many paralogues and leads to a complex variety of metabolites, complicates interpretation of these data. Nevertheless, upon induced defense by COS-OGA, the RKN might not be able anymore to overcome the activated plant immune response.

While our data demonstrate that activation of *OsPAL4* is essential for COS-OGA systemic induced defense, it remains to be determined which metabolite produced in the shoot by the phenylpropanoid pathway determines RKN resistance in the roots. The fact that COS-OGA activity against RKN is not dependent on SA biosynthesis and signaling is an indication that other products derived from the phenylpropaonid pathway could be responsible for the observed lower nematode susceptibility.

The phenylpropanoid pathway can contribute to the biosynthesis of many defense-related compounds, such as phenolics, lignins, stilbenes, phytoalexins and isoflavonoids (Vogt, 2010). Recent findings show that the phenylpropanoid pathway is also involved in the induction of resistance in other pathogen-plant interactions, although no systemic effects have ever been investigated. For example, BABA-induced resistance against downy mildew (*Plasmopara viticola*) in grapevine was associated with the primed deposition of, among others, phenylpropanoid-derived phenolics in the treated tissue (Hamiduzzaman et al., 2005). Foliar silicon application to the rose (*Rosa hybrida*) cultivar Smart increased the expression of phenylpropanoid pathway genes and the concentration of antimicrobial phenolic acids and flavonoids (rutin and quercitrin), and this was correlated with increased plant protection against infection by rose powdery mildew in the leaves (*Podosphaera pannosa*) (Shetty et al., 2011). Concerning nematodes (Fujimoto et al., 2015) found that induced resistance against *M. incognita* in Arabidopsis by root sclareol treatment was correlated with higher transcript levels of *PAL1*, cinnamoyl 4-hydroxylase (*C4H*) and cinnamoyl-CoA reductase (*CCR2*). Similarly, root benzothiadiazole (BTH) application led to induced expression of phenylpropanoid biosynthesis genes, and this was correlated with significant changes in the monomer composition of lignin in RKN-induced galls in tomato roots (Veronico et al., 2018). Whether the flavonoid and/or lignin composition of rice roots is affected by COS-OGA treatment remains to be studied in follow-up research. Additionally, the observation that PAL-activity is mainly induced in the shoots of the treated plants, while phenylpropanoid gene expression and plant defense against nematodes is observed in the root system raises the question which PAL4-dependent signal is transported

from the shoot to the root system.

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

Conflicts of interest

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

Contribution

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 Aubel 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.003.

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