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REGULAR ARTICLE

Efficiency and bioavailability of new synthetic strigolactone mimics with potential for sustainable agronomical applications

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Abstract
Purpose Arbuscular mycorrhizal fungi (AMF) play important roles in agriculture because of their ability to improve plant resilience against abiotic and biotic stresses. AMF as a technology to promote a more sustainable agriculture holds great potential, yet many factors affect the efficiency of this plant-microbe symbiosis leading to inconsistency in performance. The beneficial symbiosis between plants and AM fungi, also-known-as the mycorrhiza is promoted by strigolactones (SLs), carotenoid derivatives active as phytohormones and rhizosphere signals. Natural SLs are effective at extremely low concentrations, however their bioavailability in soil is scarce because their biosynthesis and exudation are plant-regulated, their degradation is fast and their mobility in soil is limited.
Methods Through a broad synthetic chemistry approach, we explored how structurally diverse SL derivatives could improve hyphal branching of *Gigaspora spp* AMF under laboratory conditions and thus possibly boost mycorrhization into soil.
Results We tested twenty-six different derivatives and we could highlight structural enhancements to promote hyphal branching of in vitro germinated AMF spores at equal, and in some cases higher levels compared to natural SLs. A subset of these derivatives was tested for bioavailability, but no clear correlation was found with their activity on hyphal branching.
Conclusion This study suggests that we could use a targeted, chemical-design approach to synthetize new SL derivatives to enable enhanced promotion of mycorrhization and potentially enhanced bioavailability compared to natural SLs. Due to the roles of AMF in crop production systems, these results highlight new innovative approaches to promote sustainable agriculture.

Keywords Strigolactone · Hyphal branching · Arbuscular mycorrhizal fungi · Structure activity relationship · PLEIOTROPIC DRUG RESISTANCE 1 · Synthetic strigolactone derivatives · SL mimics · Sustainable agriculture

This publication is dedicated to Prof. dr. em. Enrico Martinoia, for his kindness, curiosity, culture and for the great input he gave to the global scientific community on plant physiology and plant nutrition.

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Introduction

Strigolactones (SLs) are carotenoid derivatives synthetized in terrestrial plants and green algae (Lopez-Obando et al. 2015; Waters et al. 2017). They were recently characterized as phytohormones playing key roles in plant development (Sun et al. 2016; Bennett et al. 2016), plant

architecture (Brewer et al. 2016; Song et al. 2017) and biotic and abiotic stress resistance (Torres-Vera et al. 2014; Marzec 2016; Saeed et al. 2017). Once exuded from plant roots to the rhizosphere, SLs trigger mycorrhization and germination of parasitic weeds (Khosla and Nelson 2016; Lanfranco et al. 2018; Yoneyama et al. 2019), while their impact on soil bacterial diversity seems to be restricted (Carvalhais et al. 2019). SLs are natural stimulants and are potentially interesting molecules in basic science for the investigation of plant-rhizosphere interactions and in applied science for their use in modern agriculture to address crop productivity, resilience challenges and promote more sustainable agronomical practices (Mostofa et al. 2018; De Mesmaeker et al. 2019; Bouwmeester et al. 2019; Aliche et al. 2020). The SL synthesis and perception pathway are strongly conserved among several plant species and summarized in Fig. 1, based on the contributions of (Alder et al. 2012; Bennett and Leyser 2014; Zhang et al. 2014, 2018; Abe et al. 2014; Xie 2016; Zwanenburg et al. 2016; Moturu et al. 2018; Shabek et al. 2018; Marzec and Brewer 2019; Yoshimura et al. 2020; Marzec and Brewer 2019; Bürger and Chory 2020; Mashiguchi et al. 2021). At present, the hydrolysis of SL after binding to the receptor MAX2/D14 is interpreted as either the beginning or the end of the signaling pathway (Wallner et al. 2017; Walker et al. 2019; Machin et al. 2019). It is not known yet how SL signaling works in fungi, as no D14 or MAX2 sequence homologues were isolated in the up-to-date, sole sequenced mycorrhizal fungus *Rhizophagus irregularis* (Besserer et al. 2008; Bonfante and Genre 2015; Chen et al. 2018a).

SL synthesis and root exudation into the rhizosphere are induced by low-nutrient conditions (especially phosphate and nitrogen) in soil (Breuillin et al. 2010; Kretzschmar et al. 2012). Root-exuded SLs, as well as cutin monomers (Rich et al. 2017), jasmonic acid secreted into root exudates under high R/FR light (Nagata et al. 2016) and possibly flavonoids (Abdel-Lateif et al. 2012) were all reported to play a role in mycorrhization establishment. This soil nutrient / root exudation feedback-regulation increases the chance of high mycorrhization levels and likely enhances plant nutrient uptake, thus optimizing plant growth and biomass production also on not optimal soils and/or growth conditions (Liu et al. 2018a, 2018b). In the Solanacea *Petunia hybrida*, SL root exudation is actively regulated by the ATP-BINDING-CASSETTE (ABC) class G protein PLEIOTROPIC DRUG RESISTANCE1 (PhPDR1) (Kretzschmar et al. 2012; Sasse et al. 2015; Shiratake et al. 2019; Liu et al. 2019). The ectopic expression of PhPDR1 in *Petunia hybrida* showed that the enhanced exudation of endogenous SL also induced SL biosynthesis (Sasse et al. 2015), increased mycorrhization levels and plant biomass production on soils with low nutrient conditions (Liu et al. 2018a, 2018b; Liu et al. 2019). The ectopic expression of *PhPDR1* in *Arabidopsis thaliana* (PDR1-OE) surprisingly caused no visible phenotype, likely due to the alternative endogenous SL species synthesized in *Arabidopsis* in comparison to other plant species (see Fig. 1). PDR1 sequence homologues are present in several plant species but the closest hit in *Arabidopsis* is AtABCG40, an ABA transporter (Borghi et al. 2019). Still, PDR1-OE *Arabidopsis* seedlings, compared to their wildtype counterpart increased exudation rates of the synthetic SL mimic GR-24 and allowed seedling growth on GR-24 concentrations otherwise inhibiting seedling growth (Kretzschmar et al. 2012). At present, only a few groups characterized SL transporters in other plant species, such as *Nicotiana benthamiana* (Xie et al. 2015), *Zea mays* (Ravazzolo et al. 2019) and *Medicago truncatula* (Banasiak and Jasiński 2014; Banasiak et al. 2020).

Despite the fact that SLs exert several important functions in plant development, plant nutrition and rhizosphere signaling, research on SLs and their possible applications in agronomics are hampered by the low SL amounts produced by plants (Yoneyama et al. 2012; Guillotin et al. 2017; Rial et al. 2019), by SL short soil half-life and by hydrolytic instability (Yoneyama et al. 2009; Lumbroso et al. 2016; Halouzka et al. 2018; De Mesmaeker et al. 2019; Yoshimura et al. 2019, 2020). Cotton seedlings exude as low as 20 pg / plant over 24 h (Sato et al. 2014); Chinese milk vetch exudes higher amounts of SLs (Sorgomol) in the same time window, up to 100 ng per g of root in low nutrient conditions (Yoneyama et al. 2011). Low phosphate conditions increase SL exudation also in tomato, alfalfa, wheat, and marigold up to 1000X from control conditions (10 pg/ g of root). In water, the half-like of deoxystrigol was quantified as short as 9 h (Halouzka et al. 2018); in alkaline soils 1–3 days and in acidic soils 6–8 days (Zwanenburg and Pospíšil 2013). The chemical synthesis of new, stable and affordable SL mimics might allow new agricultural practices e.g., aimed at i) increasing plant biomass production on nutrient poor soil with lower fertilizing inputs, ii) fighting parasitic weeds by inducing suicidal germination or by application of mock competitors of naturally occurring SLs (Khosla and

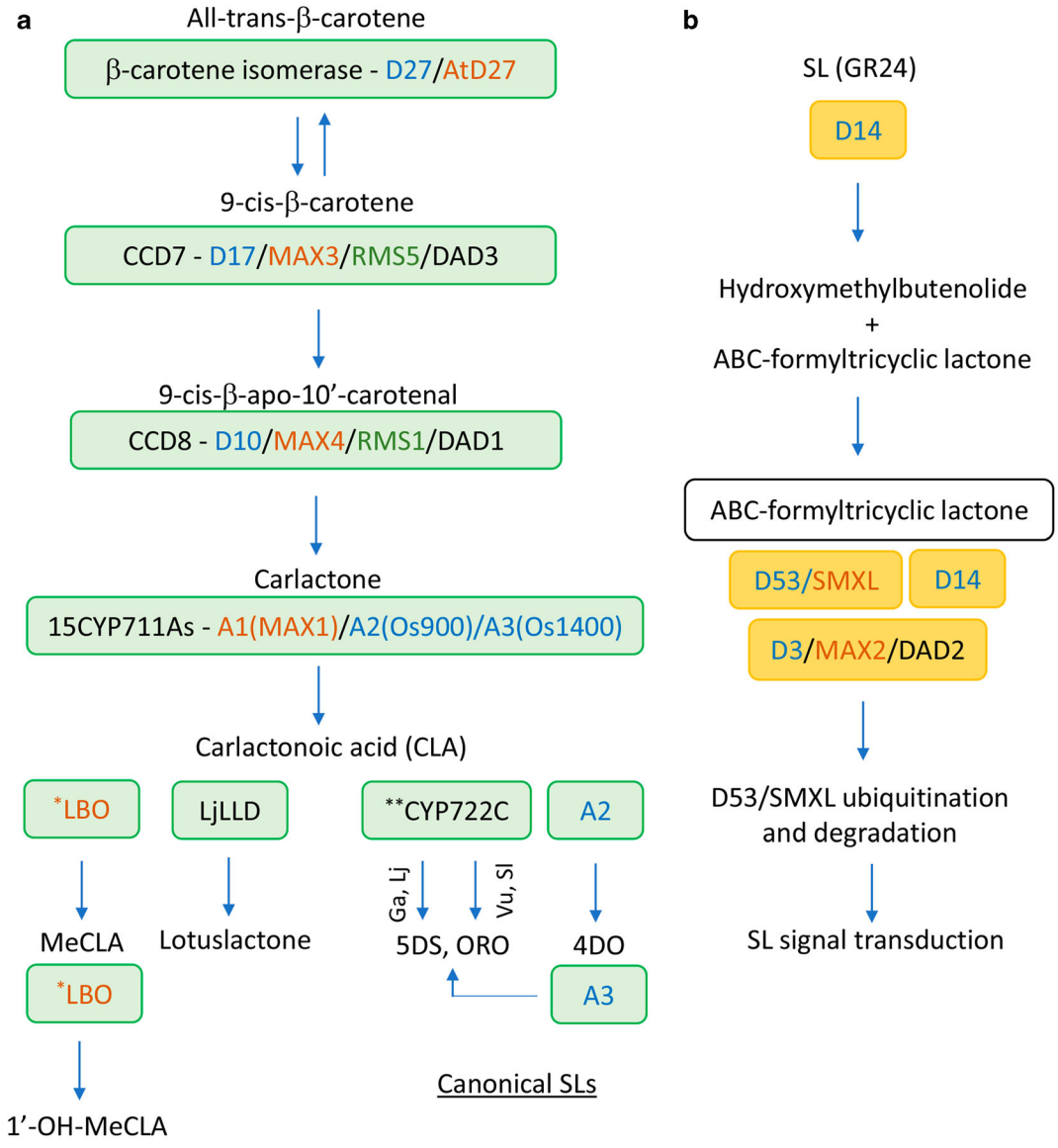

Fig. 1 Pathways of SL biosynthesis and signaling. Green boxes: enzymes active in SL biosynthesis. Orange boxes: enzymes and proteins active in SL signaling. Acronyms: DWARF14/17/27/53 (D14/17/27/53); MORE AXILLLARY GROWTH (MAX1/2/3/4); SMAX1-LIKE (SMXL); RAMOSUS1/5 (RMS1/5); DAD1/2/3 (DECREASED APICAL DOMINANCE1/2/3); LBO (LATERAL BRANCHING OXIDOREDUCTASE); LOTULACTONE-DEFECTIVE (LjLLD). * At (*Arabidopsis thaliana*), Zm (*Zea mays*), Sl (*Solanum lycopersicum*), Sb (*Sorghum bicolor);* ** Lj (*Lotus japonicum*), Ga (*Gossypium arboreum*), Vu (*Vigna unguiculata*), Sl (*Solanum lycopersicum*)

Nelson 2016; Yoneyama et al. 2019) and iii) stimulating Mycorrhizal Induced Resistance (MIR) (Pozo and Azcón-Aguilar 2007) with the vision of promoting more sustainable agricultural practices and reducing chemical inputs (e.g., pesticides and fertilizers).

Multiple synthesis approaches for SL mimics have been designed and evaluated in the past years (Cala et al. 2016; Lachia et al. 2012, 2014, Lachia et al. 2015; Lumbroso et al. 2016; Zwanenburg et al. 2016; Takahashi et al. 2016, Dieckmann et al. 2018;

Yoshimura et al. 2019, 2020). GR-24 is at present an affordable synthetic SL molecule and is the reference used in SL research (Johnson et al. 1981; Akiyama et al. 2010; Boyer et al. 2012). GR-24 is available on the market as racemic mixture as well as single enantiomers. GR-24 structure and stereochemistry follows that of naturally occurring SLs: an ABC-ring moiety bound to the D-ring (the butenolide group) via an enol-ether bridge. Previous studies (Tab. S1) revealed the structure-activity relationship (SAR) of SLs based on AtD14 reporter lines, parasitic weed germination, root hair development, shoot-lateral- and hyphal-branching bioassays (Akiyama et al. 2010; Boyer et al. 2012; Cohen et al. 2013; Umehara et al. 2015; Artuso et al. 2015; Sanchez et al. 2018). The core chemophore of natural and synthetic SLs consists of the enol-ether bridge between the lactone (or ketone) of the C-ring and the D-ring, plus the D-ring itself (Zwanenburg and Pospíšil 2013). Structural and stereochemical modifications of A- and B-rings might affect AMF branching but have low impact on other SL functions, e.g., weed germination is not affected by the stereochemistry of the methyl group on the A-ring (Zwanenburg and Pospíšil 2013). An additional critical point for the activity of SL mimics is the stereochemistry at the B/C-ring junction and in the D-ring. Two naturally occurring SL families are conceived at present, one with the stereochemistry of the BCD moiety as in orobanchol, the other as in strigol. In both families, natural variants show different substitution patterns on A- and B-rings. Exceptions are still present: e.g., the aromatic A-ring in root exudates of *Nicotiana tabacum* is to date unique in natural stimulants (Xie et al. 2007).

The successful synthesis of new SL mimics might open new research directions also targeted to avoid the issues connected to natural SLs, such as instability and low abundance. Moreover, optimization of chemical design of strigolactones derivatives can have strong impacts for agronomical applications (Screpanti et al. 2016). Imino analogs, where the enol-ether bridge is substituted by an oxime group were reported in the past as active stimulants for *Striga hermonthica* seeds (Kondo et al. 2007), although with weaker activity in the parasitic weed seed germination. More recently, carbamate analogs where the enol-ether bridge is substituted by a carbamate group have been reported to promote striga germination with exceptional femtomolar activity (Uraguchi et al. 2018). However, their activity is limited to parasitic weeds and only marginal effects were observed on AM fungi interaction; such compounds showed limited hydrolytic stability under mild alkaline conditions. SLs with an added hydroxyl group on A- or B-ring show very high activity towards parasitic weed germination (Brun et al. 2018). Bioassays on hyphal branching showed that the A-ring is necessary, e.g., compare GR-24 and GR-7 (Besserer et al. 2006). Instead, the repression of shoot lateral branching via SL mimics does not require A- or B-rings and is strongly boosted via a single, extra methyl group on the D-ring or via a thio group (Boyer et al. 2012; Pandey et al. 2016). Still, the difficulties for enhancing SL stability in natural substrates like soil seem to be many and hard to overcome. Alkaline pH and soil moisture greatly affect GR-7 and GR-24 stability (Zwanenburg and Pospíšil 2013). However, in acidic soils, the biological residual activity of GR-24 was observed up to 6–8 days (Babiker et al. 1987). The strigolactam analogue of GR-24 was reported to have a stronger activity than GR-24 for the induction of parasitic weed germination (Lachia et al. 2015). The synthesis of lactam analogues showed that their α-epimers are strong stimulants of seed germination while their corresponding β-epimers are inactive. However, GR-24, GR-28 and corresponding strigolactam derivatives are very unstable in active soil: their half-lives under lab conditions can be as short as 3 h or less (Lumbroso et al. 2016). New modifications are therefore needed to improve SLs for agronomical applications, particularly for soil applied solutions (e.g., seed treatment, in-furrow, direct spray on bare soil).

Here we report the results of a broad structure-activity relationship analysis specifically for both hyphal branching promotion and potential transport *in planta*, conducted via two approaches: i) a hyphal branching induction in vitro on *Gigaspora margarita* and *G. rosea AMF* adapted from (Akiyama et al. 2010) and ii) an affinity-to-PDR1 test (evaluation of molecular transport) via a toxicity bioassay adapted from (Kretzschmar et al. 2012). With these tests we aimed at quantifying the efficiency of new SL mimics on AMF and SL transport, assuming a PDR1-driven exudation system is present in most plants. The accumulation of soil- or foliar-supplied SLs in plants might induce SL-driven- beneficial as well as collateral effects on plant growth (Brewer et al. 2009; Kretzschmar et al. 2012), e.g., high PDR1-regulated SL accumulation in the rhizosphere and changes in the plant architecture such as reduction of lateral branching. The PDR1-based

quantification of exudation of exogenously applied SLs and SL-mimics from plant roots might therefore reveal important information for the application of SL mimics in agronomical environments. Our results show that a handful of the SL mimics we tested are at least as stable as GR-24 and a few are good candidates for root exudation. The toxicity bioassay revealed no SL mimic group that clearly scored high for both AMF branching and affinity-to-PDR1. Promising candidates with potentially improved stability and affordable synthesis are discussed.

Materials and methods

Plant material

Arabidopsis thaliana plants PDR1 OE (in *rdr6* background) and a vector control line in *rdr6* background seeds (referred to as wildtype, WT) from (Kretzschmar et al. 2012; Sasse et al. 2015) were sterilized and plated on Petri dishes, which were vertically placed into a climatized growth chamber with temperature of 21 °C and 60% humidity. *Arabidopsis rna-dependent rna polymerase6* (*rdr6*) is a mutant that allows stabilized ectopic expression of transgenes otherwise prone to silencing (Butaye et al. 2004). Germinated *Arabidopsis* seedlings were grown at 21 °C under long day conditions (16 h light, 8 h darkness) on ½ MS petri dishes.

AMF material

G. margarita and *G. rosea* spores were ordered at Mycagro Lab (Bretenière, France). Spore batches were sterilized (Kretzschmar et al. 2012) and plated on the same day of delivery. For the *Hyphal branching bioassay,* growth of AMF spores was carried out in a CO_2 incubator as described in (Liu et al. 2018a). In brief, AMF spores were quickly sterilized in a mild bleach/Triton X-100 buffer and rinsed several times in sterilized water. Single spores were then placed in the lower half of petri dishes containing a thin layer of phytagel (3.5 g / L, Sigma-Aldrich, Buchs, Switzerland) to give support and keep transparency for branching quantification. Petri dishes were partially sealed with micropore tape (3 M, Rüschlikon, Switzerland) and vertically placed in the incubator at 32 °C, 4% CO2 *v*/v. Five to seven days after incubation, sterilized filter paper discs (two, 0.4 cm of diameter) were placed aside the elongated primary hypha of the germinated spore. The treatments were run with molecular amounts of 10 pg, following the methods described in (Akiyama et al. 2010). 10 pg of each compound, dissolved in acetone were pipetted on the paper discs and 2nd and 3rd orders of lateral branches were quantified (visually counted under a binocular) at time zero and 48 h after incubation. The treatments included several replications (minimum 3) depending on the quality of spores and their germination. Independent tests (different spore batches) were carried out per treatment.

SL stocks were prepared in 100% acetone. Mock treatment consisted of acetone with equal volume (1 ul) applied for SL-mimic treatments. Data acquired from each biological replicate were normalized to each mock treatment.

Chemical synthesis of GR-24 and SL mimics

Rac GR-24, SL1, SL-8, SL-13 were prepared according to (Johnson et al. 1981). SL-2 was prepared according to (Dieckmann et al. 2018). SL-3, SL-5, SL-7, SL-22 were prepared according to (Lachia et al. 2012). SL-6 was prepared according to (Lachia et al. 2013). SL-4, SL-17, SL-19, SL-21, SL-26 were prepared according to (Villedieu-Percheron et al. 2013a). SL-18 was prepared according to (Villedieu-Percheron et al. 2013b). SL-10, SL-14, SL-24 were prepared according to (Lachia et al. 2015). SL-15 and SL-16 were prepared according to (De Mesmaeker et al. 2016). SL-9 was prepared according to (Davidson et al. 2018). SL-11 was prepared according to (Lumbroso and De Mesmaeker 2017). SL-12, SL-25 and SL-23 were prepared according to (Boyer et al. 2012).

Toxicity bioassay

Arabidopsis thaliana seedlings PDR1-OE and *rdr6* vector control line (Kretzschmar et al. 2012) were germinated on 1/2 MS mock plates (with 2.2 g l − 1 MS medium, 1% (*w*/*v*) sucrose) and transferred 3 days after germination from MS mock plates to MS treatment plates with 2.2 g l − 1 MS medium, 1% (w/v) sucrose plus specific amounts of rac-GR24 (10 μM or 20 μM, as indicated) and tested SL-mimics (10 μM) solved in the agar. Mock consisted in equal volume amounts of acetone. Although *Arabidopsis* germination was synchronized, only seedlings with equal root length were chosen for the bioassay to normalize main root growth and

optimize root measurements in the subsequent steps. Phytotoxicity was then quantified as inhibition of main root growth or growth arrest 5 days after treatment. Plates were digitally scanned, and main root growth of the previous 5 days was measured. Data was normalized on root length from mock plates for each replicate.

Statistical analyses

The quantification of the lateral branches for each treatment was used to calculate a single descriptive parameter that we called Hyphal Branching Index (HBI). The index is defined as follow:

$$\begin{aligned} &\textit{Hyphal Branching Index} \\ &= Log\ 1 + \left(2 \cdot \sum Branches^{2nd\ order}\right) \\ &+ \left(3 \cdot \sum Branches^{3rd\ order}\right) \\ &+ \left(4 \cdot \sum Branches^{3rd\ order} with\ Arbuscular\right) \end{aligned}$$

The multiplying factors in the HBI give higher scoring to newly formed hyphae with high branching orders. The rationale behind this strategy is that higher branching orders were reported as more sensitive to alterations in root exudates or soil nutrients (Nagahashi et al. 1996) and therefore, tightly reflecting changes in branching factors. The results were analysed using R version 4.03 (R Core Team, 2013) The effects of different chemical treatments on the HBI were analysed using one-ANOVA (Tab. S3) and the multi-comparison analysis were performed using a Tukey test.

Results

AMF branching bioassays

Twenty-six unique synthetic compounds were evaluated for their ability to induce hyphal branching in AMF (Fig. 2). The procedure followed the protocol developed in the seminal paper of Akiyama and colleagues (Akiyama et al. 2010). Two AMF varieties were initially tested: *Gigaspora margarita* and *Gigaspora rosea*. Hyphal branching induction via *rac-GR24* scored similar values in the two fungal varieties, still the batches of *G. margarita* we tested showed weak induction of hyphal branching (Fig. S1A, B). Most germinated *G. margarita* spores did not branch either with or without treatment (Fig. S1C, D), thus hampering consistent data acquisition. Because of that, a restricted number of compounds was tested, and a narrow number of treatments resulted to be statistically different compared to the mock. The results obtained with *G. margarita* followed a similar trend to *G. rosea:* SL4, SL15 and SL5 showed a strong and significant hyphal branching promotion effect compared to acetone other compounds. (Fig. S2 and Tab. S2)**.**

Due to higher spore vitality and more consistent germination, *G. rosea* was chosen as AMF species for further bioassays. As expected, GR-24 and its closely related structural isomer SL1 showed a significant hyphal branching promotion effect compared to the mock (Fig. 2 and Fig. 3). Another close derivative of GR-24, SL12 - with an additional methyl group in the C3 carbon of the D ring –was completely inactive.

The N-H strigolactams SL5 and SL7, as well as the N-methyl substituted analog SL22 were significantly less active than the corresponding lactones and not significantly different from the acetone treatment. The simplified analogs containing a B, C, D-tricyclic structure SL21 and SL17 and the corresponding lactones SL13 and SL8 were not significantly different from the acetone treatment (Fig. 2 and Fig. 3). It is noteworthy that all simplified tricyclic structures containing the B, C, D rings in the lactones and the lactams series were only numerically less active compared to their canonical analogs with four rings A, B, C, D and they displayed an activity similar to the acetone, except the simplified tricyclic lactam SL21, which was significantly less active than the GR-24 and SL1. Although the removal of the A ring can have an impact on the phys-chemical properties of the molecules, it did not appear to have any clear effect in promoting the hyphal branching. This was not the case for the promotion of *Orobanche cumana* seed germination (Lumbroso et al. 2016), with the simplified analogs being equally and sometimes even more potent than the corresponding tetracyclic ones. The replacement of the phenyl with a thiophen ring as in lactam derivative SL18 did not improve AMF hyphal branching compared to the close derivatives SL5 and SL7 (Fig. 2).

Interestingly, the N-acetyl strigolactam derivative SL3 was one of the most active compounds, equally active as GR-24. The addition of a N-acetyl group on the nitrogen atom of the C-lactam rings led to a significant improvement in hyphal branching compared to the close derivatives SL5, SL7 and acetone treatment. The

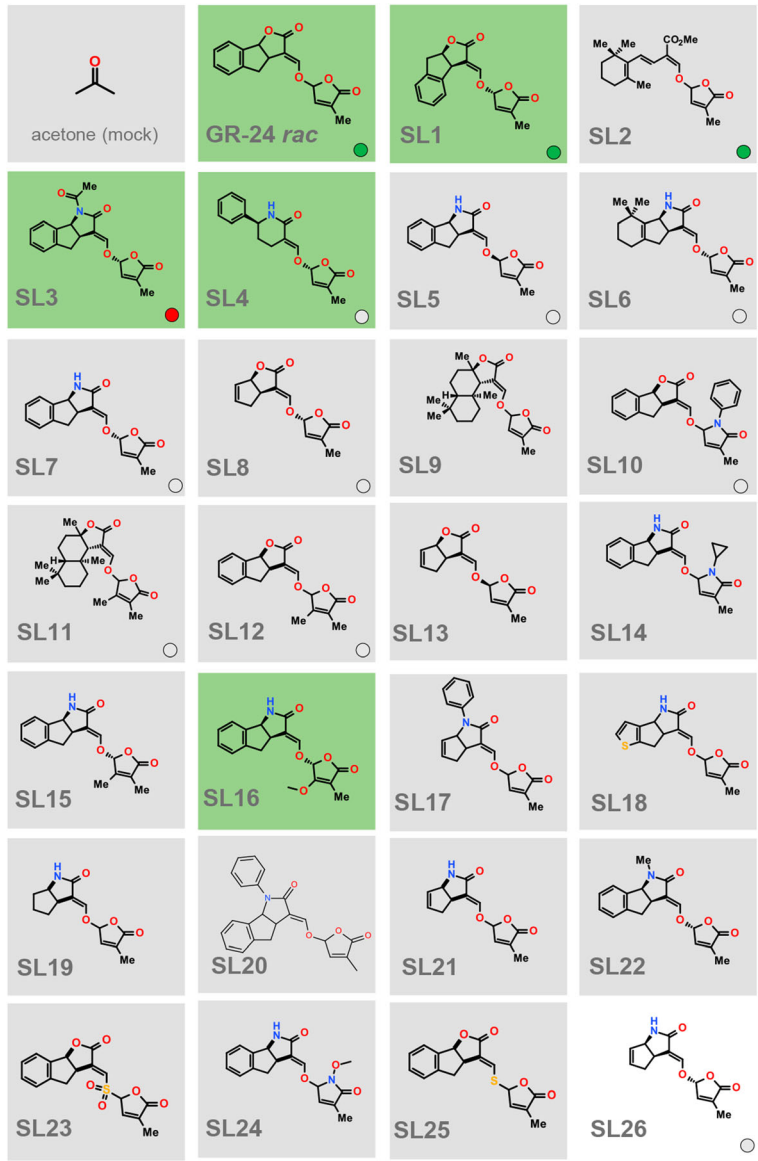

Fig. 2 Activity of SL mimics on AMF branching *(G. rosea)* compared to baseline acetone (mock). Average of hyphal branching index includes 2nd and 3rd order of lateral branches. Compounds that stimulated branching significantly different from acetone are in green, otherwise grey. Color coded circles (when present) indicate SL-molecules affinity to PDR1: green = affinity like GR-24; red = low affinity to PDR1; grey = affinity not possible to estimate. Panels without circles were not tested for affinity to PDR1

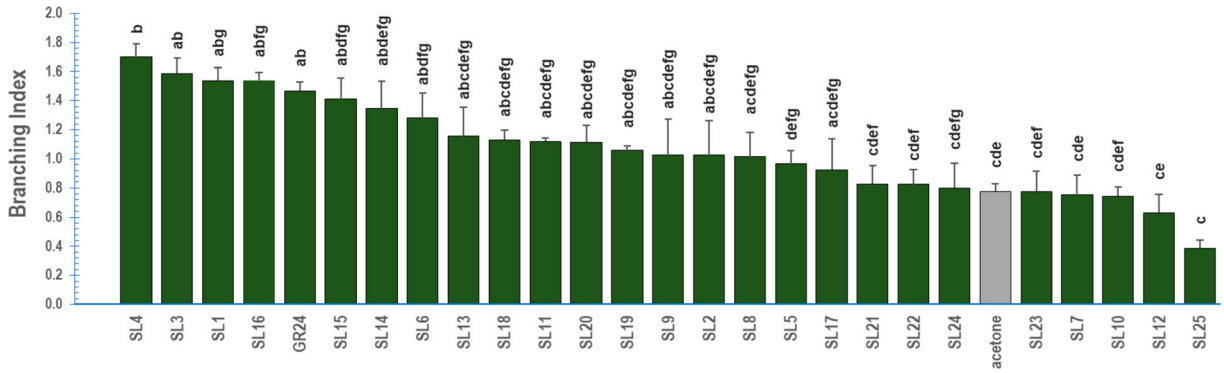

Fig. 3 Hyphal Branching Index on *G. rosea* with different SL derivatives. Different letters indicate significant differences between treatments (Tukey post-hoc test, $P < 0.05$). Bars represent standard deviation. The mock is indicated grey column

addition of a N-acetyl group in the lactam ring might have changed the local electrostatic potential by making it somewhat similar to the one of the C-ring to the lactone in GR-24. The contribution of a simple modification as the N-acetyl moiety in an ABC canonical strigolactone scaffold demonstrated the opportunity for chemical optimization around an AMF hyphal branching chemical inducer. The replacement of the aromatic A-ring of the strigolactam SL7 by the gem dimethyl cyclohexenyl moiety - present in several natural strigolactones – did not promote significant differences. Further substitution of the A, B, C-core structure of strigolactones by lipophilic residues as in SL9 and SL11 had little effect on hyphal branching compared to GR-24.

The introduction of an additional methyl group at the C3 carbon of the butenolide ring in the strigolactam SL15 did not result in a significant shift in the activity as it was observed for GR-24 and its close derivatives SL12. Moreover, the hyphal branching induction activity was significantly improved by the introduction of a methoxy substituent at C3 in the butanolide in SL16 (De Mesmaeker et al. 2016) compared to the parent compound SL7, reaching a similar activity as GR-24.

Non-canonical strigolactone derivatives have also been evaluated in the present study. For example, methyl carlactonoate SL2 (Dieckmann et al. 2018) showed a slightly higher BHI compared to acetone but not significant, this is somehow consistent with some previous work (Mori et al. 2016) where methyl carlactonate showed weak activity for hyphal branching at 1 to 3 magnitude higher concentrations than what we applied. In contrast, the most potent compound among the ones we investigated is, surprisingly, the N-H lactam SL4, with a 6-membered ring replacing the C-ring and a phenyl ring adjacent to the nitrogen atom of the lactam mimicking the A-ring of the strigolactam SL7. The high activity of SL4 compared to the inactive strigolactam SL7 is striking and arises most probably from higher intrinsic activity of SL4 which has very similar physicochemical and stability properties than SL7. This underlines again that the replacement of the lactone moiety by a lactam in the C-ring of SLs can be beneficial for the induction of hyphal branching, provided that the overall structure is optimized compared to the parent strigolactone. The influence of the stereochemistry at the D-ring on the induction of hyphal branching seems relatively moderate (Scaffidi et al. 2014; Umehara et al. 2015)(Scaffidi et al. 2014; Umehara et al. 2015), although hyphal branching induced by the β-epimers in SL5 and SL13 was not significant different compared to the corresponding α-epimers SL7 and SL8 (Fig. 2), respectively. Consequently, in an initial screening for the search of novel potential leads for hyphal branching induction, the mixture of α- and β-epimers could be used without compromising the quality of the initial ranking of their relative activity.

The replacement of the canonical D-ring lactone by a N-phenyl substituted lactam moiety in SL10 leads to the loss of activity compared to the parent compound GR-24. The replacement of the D-ring lactone of the strigolactam SL7 by a N-methoxy lactam in SL24 (a bis strigolactam derivative) does not restore hyphal branching. The bis strigolactam derivative SL14 containing a N-H lactam C-ring and a N-cyclopropyl lactam D-ring is, albeit not significantly, closer to rac-GR24 than the corresponding strigolactams SL5 and SL7.

The replacement of the enol ether link between the C- and D-rings of GR-24 by a thioether in SL25 or by a sulfone in SL23 leads to complete loss of biological activity (no hyphal branching induction), as already reported in the literature (Akiyama et al. 2010; Umehara et al. 2015).

Phyto-toxicity bioassays of SL mimics in Arabidopsis PDR1 OE and wildtype (rdr6) seedlings.

This bioassay (Fig. 4) is based on the quantification of phyto-toxicity (measured by primary root growth inhibition) of SL mimic compounds. From seminal studies on GR-24 effects on plant development, main root growth was reported to be one of the most sensitive developmental cues to exogenous SL treatments (Koltai 2011). PDR1 overexpression (PDR1-OE) was previously shown to be functional in *Arabidopsis* by quantifying amounts of a radiolabelled SL mimic (^{3}H-GR-24) that was exogenously applied on the root, let passively diffuse into the root and finally exuded by roots of PDR1 OE seedlings at higher amounts than the WT reference (Kretzschmar et al. 2012). In the same paper, the authors showed that PDR1 OE main root elongation was less affected by 10 μM GR-24, which on the contrary could inhibit WT main root development or even arrest it at concentrations as high as 20 μM (see also Fig. 4). Inhibition of main root growth of PDR1 OE *Arabidopsis* seedlings was therefore quantified against its WT reference as proxy for PDR1-driven, root exudation of GR-24 and its analogs. In case root growth inhibition after treatment was weaker in PDR1-OE seedlings than in the WT, we concluded that the compound might have been exuded from the plant root as substrate for PDR1. The bioassay showed its limitations, as no

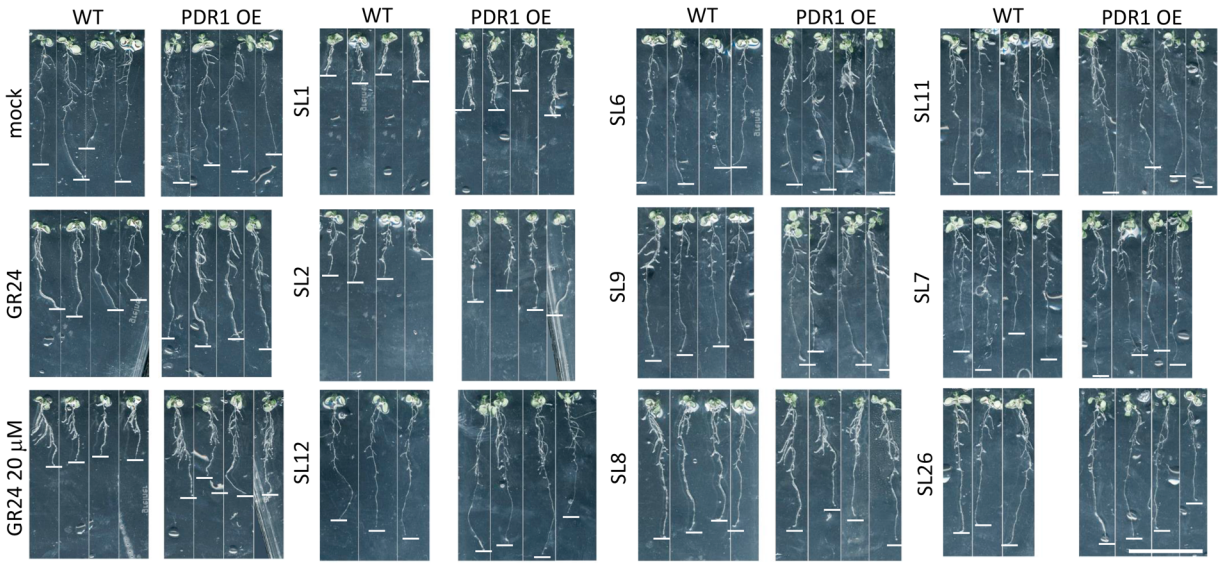

Fig. 4 Representative wildtype (WT) and PDR1-OE vertically grown seedlings treated with acetone (mock), GR24 (positive control) and SL mimics. Root tips are highlighted with a white bar. Concentrations, when not otherwise indicated, are 10 µM. Scale bar = 3 cm

phytotoxic effects were visible with the chosen concentration (up to 20 µM) in the following cases, among them several strigolactams: SL4, SL5, SL6, SL7, SL8, SL9, SL10 and SL11 (Fig. 1, Tab. S1 and Fig. 5). Strigolactams are indeed reported as not phyto-toxic (Lachia et al. 2015; Lumbroso et al. 2016) and we could not draw conclusions on their affinity to PDR1. For the same reason, affinity to PDR1 could not be evaluated for SL12, a GR-24 D-ring dimethyl derivative, like SL26. Still, the absence of the A-ring in SL26 and the not significant promotion of main root growth in PDR1-OE compared to WT suggests that the ABC ring, at least for the simplified N-H strigolactam derivative, is not necessary for affinity to PDR1. Strong phytotoxicities but no differences between *Arabidopsis* PDR1-OE and WT main root lengths were scored by SL3 and SL4, which therefore all both *bone fide* low affinity to PDR1. To summarize, the substrates with higher affinity to PDR1 were i) SL1, a structural isomer of GR-24 that showed similar activity to GR-24 on hyphal branching and ii) Me-CLA SL2, precursor of natural SLs (Figs. 2, 4, 5). Although we tested a broad range of strigolactone derivatives we found no clear correlation between affinity to PDR1 and induction of AMF hyphal branching. This, still considering that most of our compounds were not phytotoxic, suggests that there is no obvious conserved SAR (Structure Activity Relationship) between plants and fungi, between SL sensing and SL transport (Fig. 6).

Discussion

We report here the results of our strategy aimed at increasing GR-24 efficiency and bio-availability through the synthesis of new GR-24 related SL mimics. Through the quantification of AMF hyphal branching and the comparison of main root growth between *Arabidopsis* PDR1 OE and WT seedlings we could characterize new SL mimics (either as diastereomeric or enantiomeric mixtures) with similar efficacy to

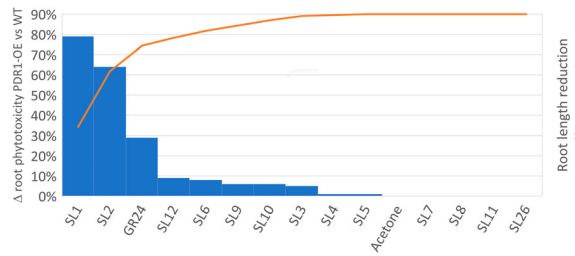

Fig. 5 Root phytotoxicity PDR1-OE vs wildtype (WT) (blue bars) and root length reduction effect vs mock (orange line). The delta root phytotoxicity indicates (in percent) how much longer the primary root of PDR1-OE seedlings is compared to their WT reference under the same treatment. The delta root phytotoxicity was calculated as follows: (length of the main root in PDR1-OE line – length of the main root in WT)/length main root in WT. The primary root length reduction indicates (in percent) length differences between the primary root of treated PDR1-OE seedlings compared to mock (acetone). Each SL mimic compound was applied at a concentration of 10 µM

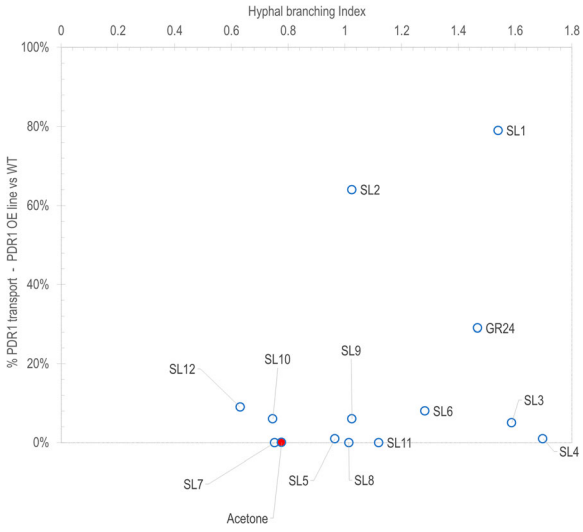

Fig. 6 Regression plot to visualize a relationship of a selection of strigolactone derivatives between hyphal branching promotion in *G. rosea* and PDR1 transport. The axes are expressed as normalized value (i.e. GR-24 hyphal branching promotion in *G. rosea* and root phyto-toxicity vs mock)

rac-GR24. Minor changes of the GR-24 structure, as in its isomer SL1, did not affect the ability to induce hyphal branching. SL1 as the "inverted form" of GR-24 induces AMF hyphal branching at even higher levels than GR-24. Recently, SL hydrolysis was suggested to arrest the SL signaling pathway rather than activating it (Seto et al. 2019). A possible way of action of SL1 might consist in keeping a SL receptor (not yet characterized in AMF) active for a longer time than its GR-24 counterpart. The introduction of further lipophilic residues on the core structure of GR-24, as in the case of SL9 was not favorable for hyphal branching. However, also more pronounced modifications of the GR-24 core structure by the replacement of the lactone C-ring with a N-H or N-methyl lactam led to the almost inactive compounds SL5, SL7 and SL22. These strigolactams have been shown to have somewhat improved physicochemical properties, as higher water solubility and lower logP and a slightly longer half-life in soil compared to GR-24 (Lachia et al. 2015; Lumbroso et al. 2016; De Mesmaeker et al. 2019). The ability to induce hyphal branching could be fully restored in the strigolactams by adding an acetyl group on the nitrogen atom of the lactam C-ring in SL3 as replacement of the lactone. The N-acetyl group decreased the electron density in the lactam moiety compared to the N-H, mimicking more accurately the corresponding lactone.

The replacement of the phenyl by a thiophene A-ring in the N-H lactam derivative SL18 slightly improved hyphal branching compared to its parent derivatives SL5 and SL7, even though these substitutions did not lead to a significant improvement compared to the acetone control. The simplified analogs displayed more attractive physicochemical properties as lower logP and higher water solubility compared to their A, B, C, D-rings derivatives (Lumbroso et al. 2016) but their impaired biological activity on hyphal branching made them less attractive for possible agronomical applications. In contrast, the simplified analogs previously displayed equally or even superior ability to induce *Orobanche cumana* seed germination. Simplification of the tetracyclic A, B, C, D-core structure could lead to potent hyphal branching induction ability as in SL4 with a 6-membered ring lactam and without B ring. This profound structural modification leading to good biological performance should stimulate, in the future, further broad variation of the GR-24 core.

There are several additional modifications of the tetracyclic strigolactam core structure which could be performed to improve their biological performance for hyphal branching. Modification of the canonical D-ring of N-H strigolactam by introduction of an additional methoxy group at the C3 carbon of the butenolide ring in SL7 increased, albeit not significantly its hyphal branching induction activity compared to the unsubstituted strigolactams SL5 and SL7, which was not the case for the corresponding lactones where the additional methyl group had a significant detrimental effect in SL12 being inactive compared to GR-24. The improved, albeit not significant biological performance of the methoxy-substituted D-ring derivatives SL15 might partially arise from a stabilization of the sensitive D-ring towards hydrolysis by to the electron-donating ability of these substituents (Lumbroso et al. 2016; Yoshimura et al. 2019, 2020). Taken together, these results demonstrate that the N-H strigolactam motive could be effectively modified in several positions of the tetracyclic core structure to reach promising hyphal branching induction activity, retaining the favorable physicochemical properties compared to their lactone analogs. Our results confirmed the importance of the enol-ether link between C and D rings of GR-24, given the complete loss of activity of the thioether SL25 and sulfone SL23 analogs, as also previously described (Akiyama et al. 2010; Umehara et al. 2015).

Methyl carlactonoate SL2 is the biosynthetic precursor of various strigolactones *in planta* (Alder et al. 2012; Abe et al. 2014). Therefore, we incorporated it in our present study on hyphal branching induction. In agreement with the previous work of (Mori et al. 2016), methyl carlactonoate SL2 did not show any strong activity in our assay, despite its high affinity to PDR1 (Figs. 2, 4, 5). Combined with its high instability towards hydrolysis in solution, in soil and probably *in planta* (Yoshimura et al. 2019, 2020), the use of methyl carlactonoate as precursor of strigolactones *in planta* (procide approach) seems to be rather unattractive to induce hyphal branching of AMF associated to field crops. Me-CLA SL2 and SL1 were here characterized as having the highest affinity to PDR1 together with rac-GR24. It was previously hypothesized that substrates to PDR1 might be not only orobanchol but also SL precursors, since CCD8/*DAD1* and *PhMAX1* are both expressed in petunia roots, the latter albeit within a narrow pattern (Shiratake et al. 2019). The presence of PDR1 in the root tip (Kretzschmar et al. 2012) is proposed to contribute to root tip unloading of SL and SL-related compounds, thus releasing a negative feedback loop on SL biosynthesis. The newly discovered affinity to PDR1 of Me-CLA and previously reported PDR1 expression patterns (Kretzschmar et al. 2012; Liu et al. 2019) suggest new investigation directions, aimed to understand a possible role of PDR1-driven transport of Me-CLA for regulating plant-mycorrhizal symbioses, shoot lateral bud outgrowth and root cell differentiation.

A pipeline for directly quantifying root exudation of SL mimic compounds in PDR1-OE and its WT reference has been envisioned in substitution to the indirect phenotypic quantification of main root length. However, the quantities of exuded compounds are likely too low for detection and radiolabeling might be the future choice for direct quantification of SL mimic import into the seedling and SL mimic exudation out of the seedling. Also, the quantification of SL in root exudates would not be informative about SL and SL mimic catabolism *in planta*. A molecular quantification of SL mimics in buffer based on plant extracts might be considered in the future as an additional approach to investigate SL half-life in plant tissue equivalents. It was recently published that the role of PDR1 in SL transport seems to be direct for SL root exudation but not for SL root-to-shoot translocation (Shiratake et al. 2019). Therefore, the exogenous function of PDR1 in *Arabidopsis* is likely exclusively associated to root exudation of GR-24 and SL mimic compounds, underlying the significance of the toxicity assay results here reported. Compounds that showed no phytotoxicity, but efficient induction of AMF hyphal branching are very promising for a follow up in semi-field analyses, as they might improve plant nutrient uptake via mycorrhization without negatively impacting plant development.

Finally, we found no clear correlation between affinity to PDR1 and induction of AMF hyphal branching (Fig. 6). PDR1 transport specificity seems to be high, as for example ABA is not transported by PDR1, despite the *Arabidopsis* ABA transporter AtABCG40 is the sequence closest homologue to PDR1 (Kretzschmar et al. 2012). The mechanisms behind SL uptake from soil, internal re-localization and signaling in AMF are largely unknown (Tsuzuki et al. 2016). We tested a broad chemistry scope and the results showed that there was no convergence (overlap) between the structure-activity relationships between AMF and transport.

Conclusion

The positive biological effects of SLs in promoting AMF colonization make this chemical class particularly interesting for application in agriculture, especially in relation to sustainable crop practices. Recent work hypothesized that mycorrhizae are functioning as keystone taxa (Banerjee et al. 2019) considering their contribution in the ecosystem functioning, microbial community and especially in crop resilience against some abiotic stresses like low nutrients or drought (Chen et al. 2018b). This work demonstrated that exploring canonical strigolactones with specific substitutions in the ABC-rings can be very effective not only to retain good hyphal branching induction but also to maintain bioavailability. Similarly, specific changes in the D-ring can contribute to overall activity. Efforts in optimizing high-performing AMF strains coupled with synthetic SL signals represent a new technological venue towards sustainable agriculture.

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Availability of data and material All data are available upon request to the corresponding author.

Code availability Not applicable.

Authors' contributions LB, AL and ML designed this study. LB and CG performed the experiments. AL and ML synthetized the molecules. LB and CS analyzed the data. LB, CS and ADM wrote this paper.

Declarations

Ethics approval This article does not contain any studies with human and/or animal participants performed by any of the authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of interest/competing interests The authors declare that they have no conflict of interest.

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