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THE EFFECT OF A STRIGOLACTONE MIMIC ON GROWTH AND COLONY MORPHOLOGY IN PHYTOPATHOGENIC FUNGI

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Abstract

The paper investigates the effect of a strigolactone mimic, 3-Methyl-5-(benzo[de]isoquinoline-1,3-dione-2-yloxy)-5H-furan-2-one, bearing a naphthylamide core linked by an ether group to the furan-2-one ring, on hyphal branching and radial fungal growth of two plant pathogenic fungi, Colletotrichum acutatum CBS 112980 and Sclerotinia minor DSM 63016. Our results demonstrated that the tested mimic compound, which is easier to synthetize comparing tostrigolactone analogues, have the same effect as the synthetic analogue GR24, inducing hyphal branching stress response and inhibiting phytopathogen growth. The results are discussed considering their importance for both fundamental studies (role and receptor of SLs D-ring in plant pathogenic fungi) and practical applications - shaping plant rhizomicrobiome.

Key words: *strigolactones, exo-signal, strigolactone mimics, plant pathogenic fungi.*

INTRODUCTION

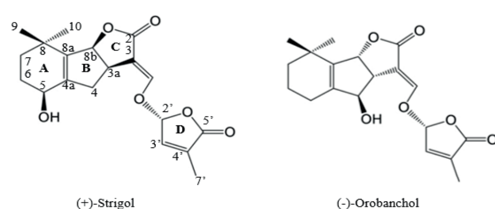
Plants, being sessile organisms, rely mainly on chemical compounds (ecomones/ semiochemicals) for their interactions with above- and below ground species (López-Ráez et al., 2017). Plants use these chemical signals to fight against biotic and abiotic stress and to establish associations below ground with microorganisms such as bacteria and fungi (Morgan et al., 2005; Raaijmakers et al., 2009). In the same time, the ecomonesreleased by plant roots are hijacked by herbivores and plant parasiticorganisms to detect/trace plants presence (Padje et al., 2016).

A particular case of such chemical signals are strigolactones (SLs), apocarotenoids which belong to carotenoid-derivative metabolites that include phytohormones, signaling molecules, and volatile compounds (Cheng et al., 2017; Hou et al., 2016). SLs act as both rhizosphereexosignals and hormonal endo-signals (Machin et al., 2020). As rhizosphericsemiochemicals (SLs) have a dual action. SLs got their name from the scientific name of parasitic plant genera, *Striga*, and lactone (Cook et al., 1966). Initially these carotenoids derivatives were considered as being mainly a signal (cue) for parasitic seed germination (Matusova et al., 2005). Almost in the same time it was demonstrated that SLs are bioactive molecules that can stimulate the branching and metabolism of pre-symbiotic hyphae in arbuscularmycorrhizal fungi (AMF) (Akiyama et al., 2005; Besserer et al., 2006).

The recruitment of beneficial symbiotic microorganisms is not limited to AMF. Strigolactones promote also nodulation and rhizobia symbiosis (Foo & Davies, 2011; Rehman et al., 2018).

More than 25 SL and SL-like compounds have been identified so far in the root exudates of several plant species (Kramna et al., 2019). They all share a conserved tricyclic lactone

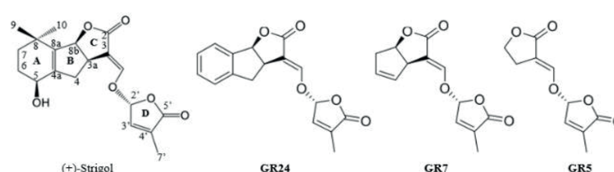
structure containing rings referred to as ABC rings, linked via an enol-ether bridge to an invariable α, β-unsaturated furanone moiety named D ring (Wang & Bouwmeester, 2018). The structures of naturally occurring SLs are presented in Scheme 1. It can be observed that there are two isomeres, strigol- and orobanchol-type SLs, with different stereochemistry of the C-ring [(3aR,8bS) in strigol and (3aS,8bR) in orobanchol], but the D-ring always has R configuration (Butler, 1994).



Scheme 1. Chemical structures of naturally occurring SLs

Zwanenburg et al. (2009) observed that the seed germination of root parasites, under alkaline conditions, is induced by the cleavage of enol ether bridge, the C-D ring moiety (Zwanenburg et al., 2009).

Due to their complex structure, naturally occurring SLs cannot be synthesised on a multi-gramme scale (Zwanenburg et al., 2016). In order to study the SLs effect on biological processes, model compounds were designed and prepared. These synthetic SLs should have a more simple structure, but almost the same bioactivity as natural SLs. Therefore, it was necessary to identify the part of the molecule, named bioactiphore, which is primarily responsible for bioactivity. If A-ring from strigol becomes aromatic, a new compound is obtained. It is named GR24, after its inventor Gerald Rosebery, and is used as standard for parasitic seed germination. These synthetic compounds, which have structural analogy with natural occurring SLs, were called SLs analogues (Johnson et al., 1981). If A-ring is removed, another compound, GR7 is formed. From GR7, by cutting the B-ring, GR5 is obtained (Scheme 2). All three GR molecular species are considerably active germination stimulants for parasitic weeds seeds. The biological activity is lost if the C-ring is removed. The bioactiphore is due to the CD parts of SLs.



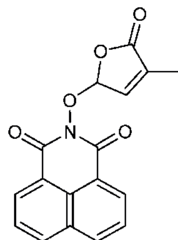
Scheme 2. GR compounds derived from SL structure

Beside these SL analogues, SL mimics, simple derivatives of SLs D-ring, were also discovered (Fukui et al., 2011).

There are very few studies regarding the effects of synthetic SLs, as a proxy for the plant rhizosphere signals, on plant pathogenic fungi. According to Dor et al. (2011), the growth of phytopathogenic fungi was strongly modulated by the synthetic SL analogue GR24. The response of tested phytopathogenic fungi showed an increase in hyphal branching and a decrease in colony diameter. This response differs from the response of symbiotic AMF. In the case of AMF, SLs stimulate not only the branching activity, but also the growth of hyphae. Cell proliferation was observed, increasing the nucleus number per mycelial length and gene expression due to mitochondrial activity and increased respiration (Buee et al., 2000; Besserer et al., 2006). In the case of plant pathogenic fungi, the response is a stress response and not a stimulatory one.

The experiments made by Belmondo et al. (2017) on wild strain *B. cinerea* B05.10 in the presence of different concentrations of GR24, using the same biological assay described by Dor et al. (2011), showed a denser hyphal network, with an increased branching level. In the case of control and acetone treatment, 2nd order branching was obtained while in the presence of GR24 the branching order increased to 4 (Belmondo et al., 2017).

There is only one study which evaluates the effect of strigolactone mimics on phytopatogenic fungi. We have previously investigated the effects of several newly synthesized SL mimics (SL3, SL5 and SL7) on the development pattern of some phytophatogenic fungi (Oancea et al., 2017). The behaviour of SL5,3-Methyl-5-(benzo[de]isoquinoline-1,3-dione-2-yloxy)-5H-furan-2-one (see chemical structure in Scheme 3) was the most similar to GR24 in the assay on plant pathogen development pattern.



Scheme 3. Structure of SLs mimics SL5

In this study we investigated the activity of SL5 (denoted from this point throughout this paper SL mimic 5) and GR24 on two other phytopathogenic fungi, *Colletotrichum acutatum* and *Sclerotinia minor*, for which there are no data available with respect to strigolactone mimics effect on growth and colony morphology, to the best of our knowledge.

MATERIALS AND METHODS

Materials

The following culture media and chemicals were used: Potato Dextrose Agar (PDA, Scharlau, Barcelona, Spain), dimethylformamide (DMF), 2-hydroxy-benzo[de]isoquinoline-1,3-dione (Across Organic, Thermo Fisher, Waltham, MA, USA), potassium carbonate and 5-bromo-3-methyl-5H-furan-2-one (Sigma-Aldrich, Merck Group, Darmstad, Germany), GR24, racemic, (StrigoLab, Turin, Italy).

Synthesis of SL mimic 5

SL mimic 5 was synthesised as previously described (Oancea et al., 2017). Briefly, SL mimic 5, 3-Methyl-5-(benzo[de]isoquinoline-1,3-dione-2-yloxy)-5H-furan-2-one, bearing a naphthalimide core linked by an ether group to the furan-2-one ring, was obtained in DMF solvent and in the presence of K_2CO_3, by the coupling reaction, at room temperature for 24 hours, of 2-hydroxy-benzo[de]isoquinoline-1,3-dione with 5-bromo-3-methyl-5H-furan-2-one.

Fungal strains and cultivation methods

The used fungal strains were *C. acutatum* CBS 112980 (Culture collection of fungi and yeasts, Westerdijk Fungal Biodiversity Institute, Utrecht, Netherland) and *S. minor* DSM 63016 (DSMZ - German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany).

The PDA medium was prepared following the standard protocol and it was sterilized in an autoclave (MLS - 3751L, PHCBI, Tokyo, Japan) at 121ºC for 15 minutes. The PDA medium was poured in sterilized 90 mm Petri dishes, 20 ml per dish. Fresh cultures of *C. acutatum* and *S. minor* were prepared by inoculating mycelial disks excised from the edge of old cultures into the centre of Petri dish with PDA medium. The inoculated Petri dishes with the phytophatogenic fungi were kept in an incubator (MIR-154-PE, PHCBI, Tokyo, Japan), at 28ºC, for 5 days.

Preparation of test solutions

For each tested SL, a stock solution with 10 mM concentration was prepared by dissolving 1.5 mg GR24, and respectively 1.54 mg SL mimic 5 in 500 µl acetone. Working solutions of or responding concentrations were prepared by diluting the stock solution in acetone. The working solutions were used to obtain the final concentrations (5 x 10^{-6}, 10^{-5}, 5 x 10^{-5} M) of each synthetic SL by dissolving into warm, sterile water agar 1.8% agar concentration. The final concentration of acetone was 0.5% (v/v).

Experimental design

The warm water agar with GR24 or SL mimic 5 was spread onto 45 mm Petri dishes, 10 ml per dish. Water agar 1.8% and water agar supplemented with 0.5% (v/v) acetone were used as controls. GR24 was used as a reference chemical structure. After cooling to room temperature, the Petri dishes were each inoculated with a piece of mycelium-agar taken from the edge of the 5-day-old phytopathogenic fungal colony using a flame sterilized cork borer (5 mm diameter). The inoculated Petri dishes were incubated at 28ºC for 3 days (incubator MIR-154-PE, PHCBI, Tokyo). After 3 days, the developed fungal colonies were macroscopically observed, the colony diameter was measured and the Petri dishes were examined under a Leica DM 1000 LED microscope (Leica Microsystems, Wetzlar, Germany) provided with a digital camera ICC50W. The images of the fungal colonies were captured on the 3rd day.

The number of hyphal branches of different orders, from second till, the maximum observed in each case was recorded on each primary branch, moving back from the end of

the youngest hyphal tip. In the case of *S. minor* the hyphae were counted along a distance of 1000 µm. In the case of *C. acutatum*, the hyphae were counted along two distances, 1000 and 2000 µm, because the maximum hyphae order was observed at longer distance for some experimental treatment. In the case of crooked primary branch, the distance was drawn step-wise, from linear fragments. Each experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics, version 26. Ten hyphae from three replicates for each experimental treatment were used for the statistical analysis. The experiments were performed twice, and the presented images are from one of them. One-way ANOVA, Welch's ANOVA and Brown-Forsythe method were used to determine whether or not significant differences exist between the tested groups.

RESULTS AND DISCUSSIONS

The effects of test solutions on branching activity of phytopathogenic fungi

Figures 1 and 2 show typical hyphae development of *C. acutatum* and *S. minor*, respectively, in our experiment treatments.

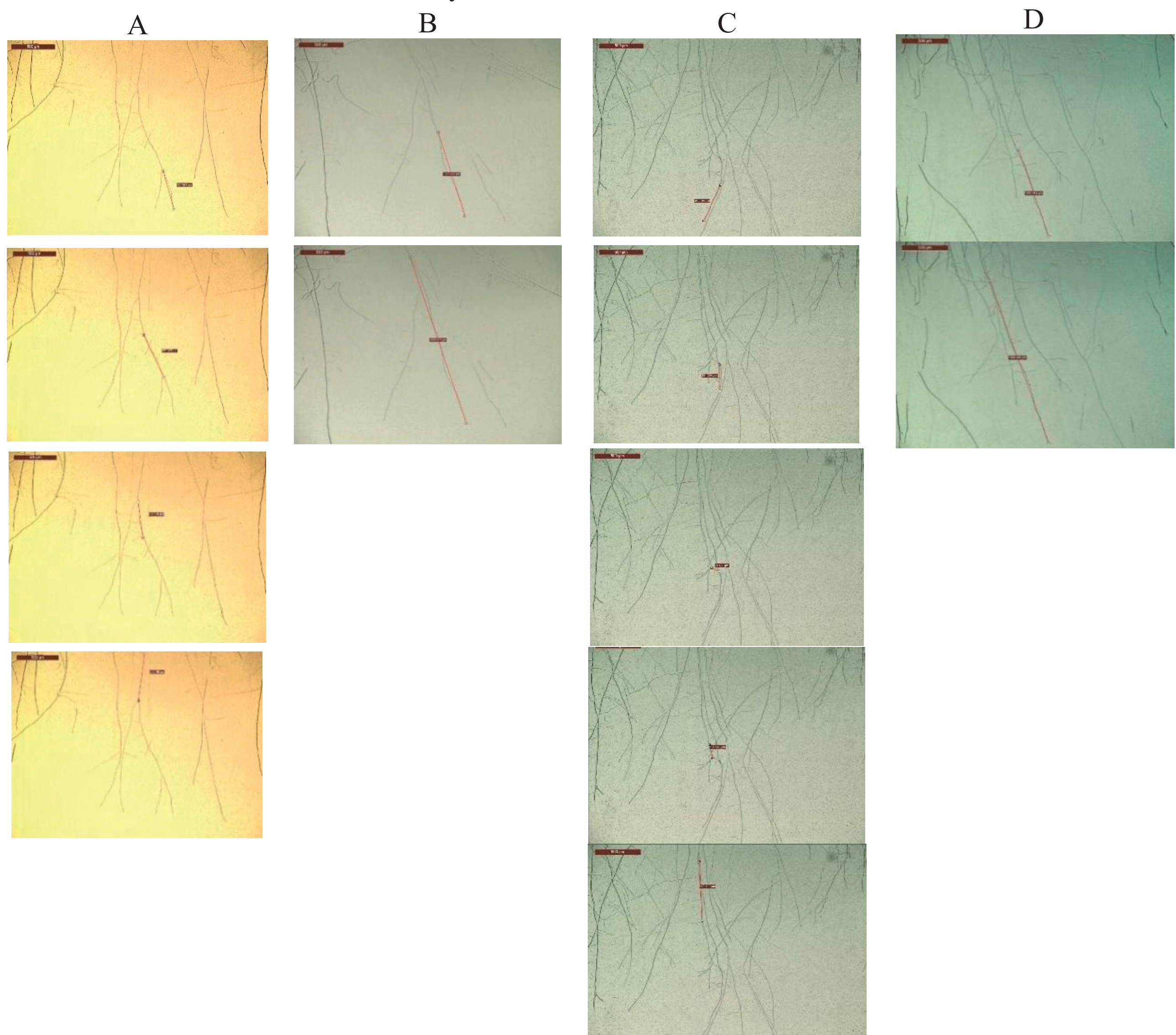


Figure 1. The aspect of hyphal branching of *C. acutatum* in control water agar (A), acetone (B), GR24 C2 = 10^{-5} M (C), SL mimic 5 C1 = 5 x 10^{-6} M (D), recorded on two total lengths of 1000 and 2000 µm, beginning from the end of the youngest hyphal edge. In the case of A and C, because the primary hyphae were not straight, the distances were built from several linear fragments

In the case of *C. acutatum*, the number of hyphae was recorded on two total lengths of 1000 and, respectively, 2000 µm, beginning from the end of the youngest hyphal edge,

because the maximum hyphae order was observed at distances longer than 1000 µm. Because many primary hyphae were not straight, the total distance consisted of several shorter fragments along linear hyphae length in these cases. Already the qualitative analysis shows that GR24 and SL mimic 5 had an effect on the branching of *C. acutatum* (Figure 1, C and D versus A and B). The fungal network of *C. acutatum* in treated samples is denser, with visibly more 2nd and 3rd order branches. Less evident effects are observed for *S. minor* (Figure 2, C and D versus A and B), also because of the denser hyphal network of controls.

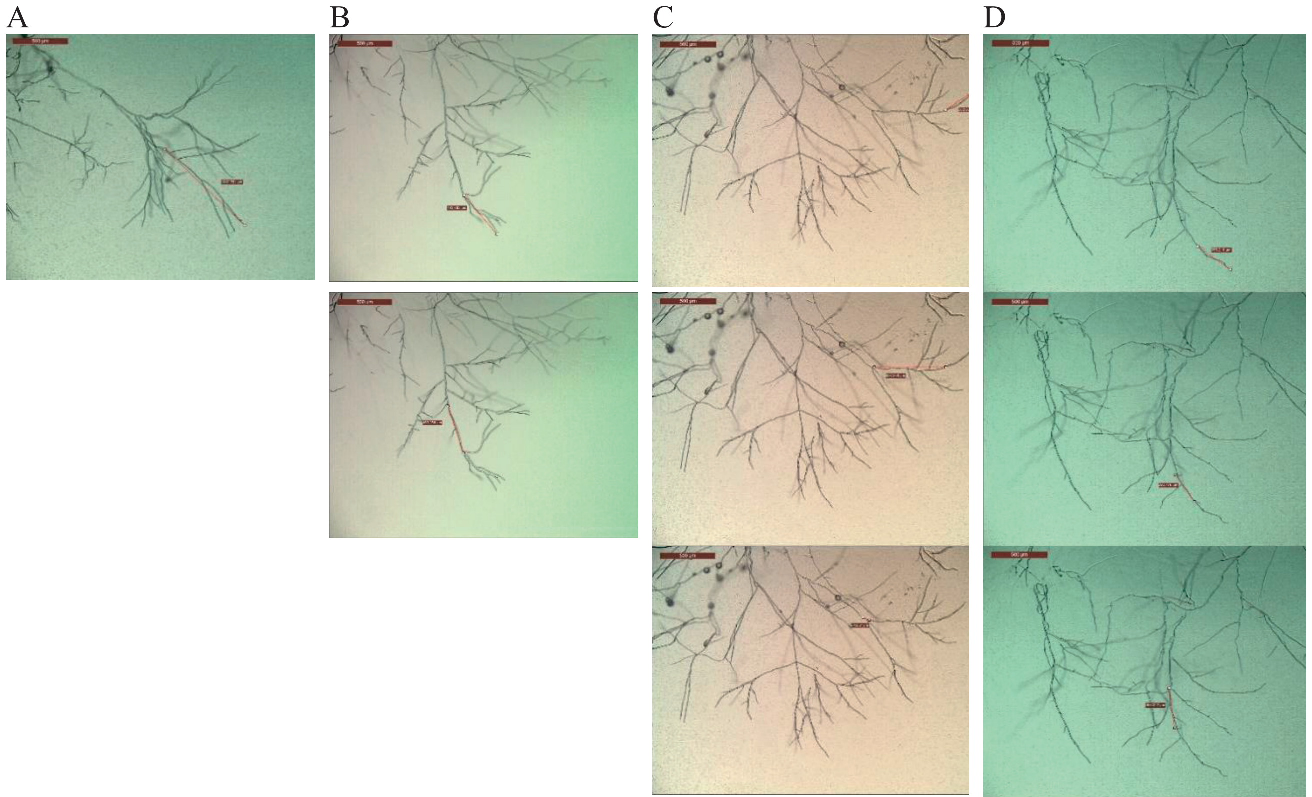


Figure 2. The aspect of hyphal branching of *S. minor* in control water agar (A), acetone (B), GR24 C2 = 10^{-5} M (C), SL mimic 5 C1 = 5 x 10^{-6} M (D), recorded on total length of 1000 µm, beginning from the end of the youngest hyphal edge. In the case of B, C, and D, because the primary hyphae were not straight, the distances were built from several linear fragments

The 2nd, 3rd and 4th order hyphae were counted for each replicate in each treatment representing the dependent variables which were evaluated in this experiment. Each group of data corresponding to each dependent variable under each of the 8 treatments was analyzed by the commonly used normality tests: Shapiro-Wilk and Kolmogorov-Smirnov (with Liliefors Significance correction). In most cases, the obtained p-values were well over 0.05 which suggested that the null hypothesis of these tests could not be rejected. The two normality tests which were used assume that the data sets follow the normal distribution. Since the null hypothesis could not be rejected, the data sets were considered to be normally distributed confirming the normality assumption for one-way ANOVA.

For *C. acutatum* 1000 µm and *C. acutatum* 2000 µm, based on the Levene's test of homogeneity of variance, the calculated p-value was < 0.05 which indicated that the null hypothesis needs to be rejected. This meant that the treatment groups were not homoscedastic between themselves. Since this is an important assumption for one-way ANOVA, the alternative Welch and Brown-Forsythe were used, which are not sensible to differences of variance in the compared groups.

For *S. minor* 1000 µm, the assumption of homoscedastic behaviour was not violated, which meant that one-way ANOVA could be applied. The choices made in each situation

with respect to the 2nd and 3rd order hyphae counts are based on the values represented in Table 1 which depicts the p-value of Levene's test and the corresponding analysis of variance test considered (highlighted with grey).

One-way ANOVA, Welch's ANOVA and Brown-Forsythe method are used to determine whether or not significant differences exist between the tested groups. Post-hoc tests are necessary to evaluate pairwise differences between the groups.

When the ANOVA alternatives were used, Games-Howell test was applied to evaluate the heteroscedastic groups (*C. acutatum* 1000 µm and *C. acutatum* 2000 µm) and Tukey honest significance test to evaluate the homoscedastic groups (*S. minor* 1000 µm).

Table 1. Statistical tests for interpreting differences between the groups using ANOVA, Welch's ANOVA and Brown-Forsythe

	2nd order				3rd order			
	Levene	ANOVA	Welch's ANOVA	Brown-Forsythe	Levene	ANOVA	Welch's ANOVA	Brown-Forsythe
***C. acutatum* 1000 µm**	0.033	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	0.001
***C. acutatum* 2000 µm**	0.012	<0.001	<0.001	<0.001	0.091	<0.001	0.001	<0.001
***S. minor* 1000 µm**	0.250	0.073	0.005	0.081	0.647	0.045	0.045	0.047

The groups in each case were organized in homogeneous subsets denoted by letter labels as seen in Figure 3, Figure 4 and Figure 5. These results are shown with error bars representing the standard error, which were added to the columns in each of the three figures.

Figure 3 treats the 2nd and 3rd order hyphae counted in the case of *C. acutatum* 1000 µm. For the 2nd order, three homogeneous subsets were detected. All treatments except GR24 C3 were significantly different than the two control groups presenting a higher count for the 2nd order hyphae branches. GR24 C2 presented the highest mean being significantly different than all other groups except GR24 C1 and SL mimic 5 C1. A p-value of 0.1 was set as the threshold to include both significant and marginally significant differences. In the case of 3rd order branches, only one pair of groups presented significance of p-value < 0.1, namely GR24 C1 and the water agar control group. Compared with acetone, only GR24 C1 had a marginally significant effect on the 3rd order branches, the effects of the other variants being not significant, probably also because of the relatively low number of branches, therefore we decided to consider also 2000 µm (see below). The homogeneous subsets observed for the case of *C. acutatum* 1000 µm were not entirely consistent with the ordered means of the groups because of the heteroscedastic nature of the groups. However, as a general tendency, synthetic SLs presence, both analogues and mimics, determined a significant increase of plant pathogen branching, with a bimodal response in the case of GR24 (in the case of 2nd order branching) and a linear response in the case of SL mimic 5.

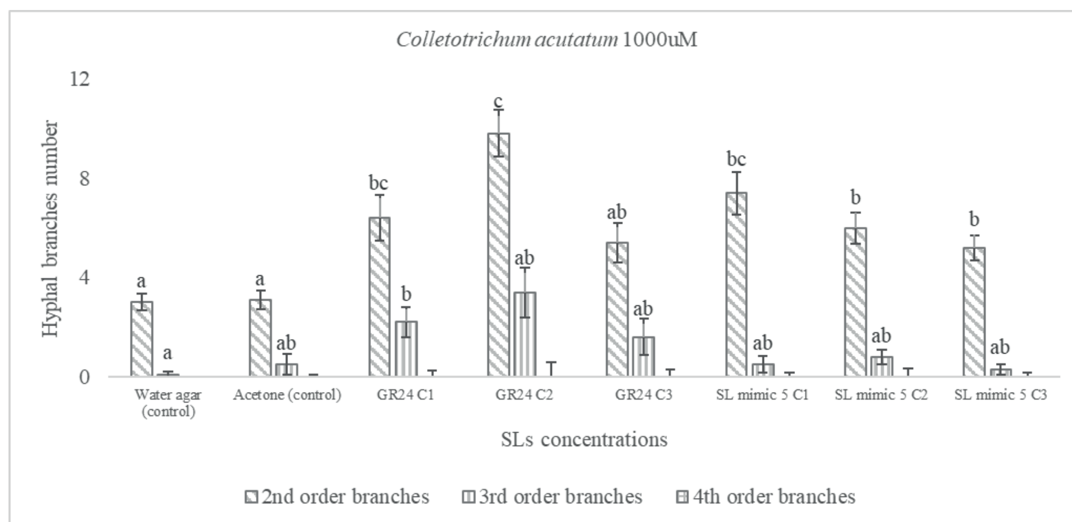


Figure 3. The influence of SLs on fungal hyphal branching for *C. acutatum*. The total number was recorded on a total length of 1000 µm.C1, C2, C3 are 5 x 10^{-6}, 10^{-5}, and 5 x 10^{-5}M concentration, respectively. The bars represent the standard error. Significant differences are shown by using different letters at $p < 0.05$

For the *C. acutatum* 2000 µm experiment, the 2nd order hyphae branches found under the 8 different treatments fell under 3 homogeneous subsets as represented in Figure 4. Subset "a" included the control groups together with treatments SL mimic 5 C2 and SL mimic 5 C3. Subset "b" included the 6 middle groups, almost all of the analyzed groups, except the acetone control group and GR24 C2. Subset "c" containing groups GR24 C1, GR24 C3, SL mimic 5 C1 and GR 24 C2 is significantly different than the acetone control.

Not enough 4th order hyphae branches were found in order to establish any statistical relevant difference between groups, but within 2000 µm, almost no 4th order hyphae was present in the acetone control. Also the apparent tendency is to increase the number of 4th order hyphae, especially at concentration C2 of strigolactone (Figure 4).

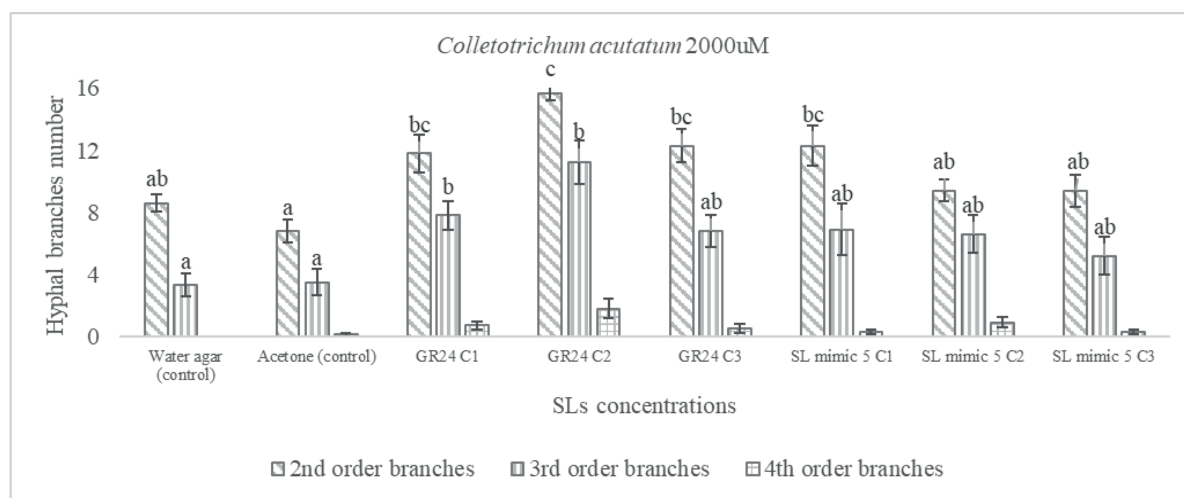


Figure 4.The influence of SLs on fungal hyphal branching for *C. acutatum*. The total number was recorded on a total length of 2000 µm.C1, C2, C3 are 5 x 10^{-6}, 10^{-5}, and 5 x 10^{-5}M concentration, respectively. The bars represent the standard error. Significant differences are shown by using different letters at $p < 0.05$

Figure 5 shows the compared means for *S. minor* 1000 µm. In this case, Levene's homogeneity of variance test showed that the groups tested were homoscedastic. In this case, ANOVA was used to detect overall significant differences. Pairwise comparison was done using Tukey's HSD post-hoc test. In terms of the number of 2nd order hyphae branches, no significant difference was found between any of the pairs; all groups were part of the same homogeneous subset. The only significant number of 3rd order branches was found for SL

mimic 5 C2 which is significantly different than the water control, but not the acetone control. All the other groups are part of homogeneous subsets with both the water agar control group (group a) and the SL mimic 5 C2 group (group b), so no significant difference can be reported with each other or with either the water agar control group or the SL mimic 5 C2 group. The numbers of the 4th order branches were not enough to establish any difference between groups, but we noticed their absence in both controls, few 4th order hyphae appearing only in the presence of strigolactones, both GR24 and SL mimic 5.
However, it is possible to conclude that synthetic SLs stimulate plant pathogenic branching. The response of *C. acutatum* CBS 112980 type strain to GR24 is similar to the response of an isolate from diseased strawberry, identified as being from the same species, and tested for response to GR24 (Dor et al., 2011). *C. acutatum* is a species complex, which includes phytopathogens producing a plant disease called (generally) antracnose. This species complex shows a wide diversity of important traits, such as mode of reproduction, host infection strategy, host range and host preference (Baroncelli et al., 2017) . The similar response of different strains from this species complex to GR24 SLs analogue suggests the existence of a response pathway to SLs in these plant pathogenic fungi.

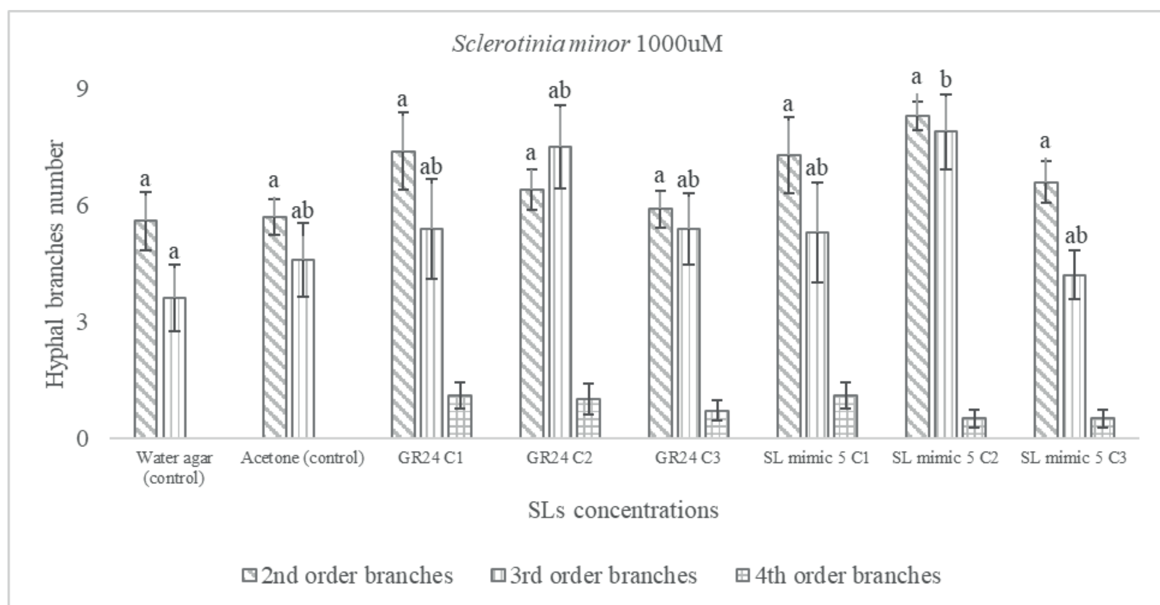


Figure 5. The influence of SLs on fungal hyphal branching for *S. minor*. The total number was recorded on a total length of 1000 µm.C1, C2, C3 are 5 x 10^{-6}, 10^{-5}, and 5 x 10^{-5}M concentration, respectively. The bars represent the standard error. Significant differences are shown by using different letters at $p < 0.05$

The influence of SLs on radial fungal growth

The experimental results showed that both GR24 and SL mimic 5 inhibited the growth of the two tested phytopathogenic fungi, the effect being in general detectable from the lowest concentration applied, 5 × 10^{-6} M (Figures 6, 7). Both compounds inhibited the strains in a dose-dependent manner. In the case of *C. acutatum*, SL mimic 5 seems to have stronger effect on the colony diameter than GR24 (Figure 6). In order to assess the statistical differences in the data, ANOVA analysis was used, based on Tukey–Kramer Honestly Significant Difference test. Except GR24 C1, all the other variants were statistically significant compared with the control.

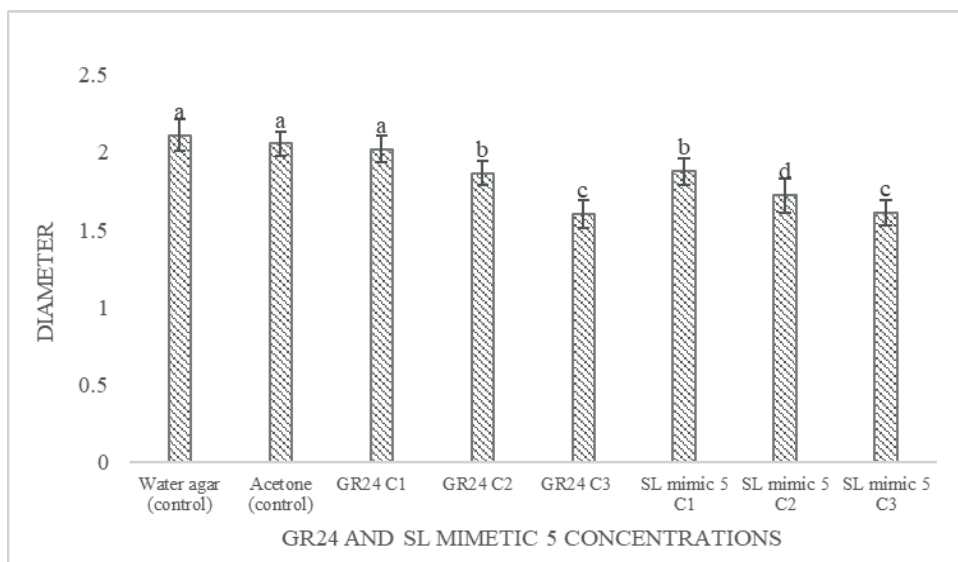


Figure 6. Diameter of *C. acutatum* colonies in the absence and presence of strigolactones (C1, C2, C3 are 5 x 10^{-6}, 10^{-5}, and 5 x 10^{-5}M concentration, respectively). The bars represent the standard error. Significant differences are shown by using different letters at $p < 0.05$

For *S. minor*, GR 24 was slightly more effective inhibitor than SL mimic 5 (Figure 7). In order to assess the statistical differences in the data, Welch's ANOVA analysis was performed, based on Games-Howell post hoc test. All treatments with strigolactones were significantly different from the control. The effect of both compounds was more pronounced in the case of *S. minor*. All in all, SL mimic 5 and GR24 show similar response on *C. acutatum* and *S. minor* in general. The presence of these compounds in the culture media appears to inhibit the growth of both fungal plant pathogens.

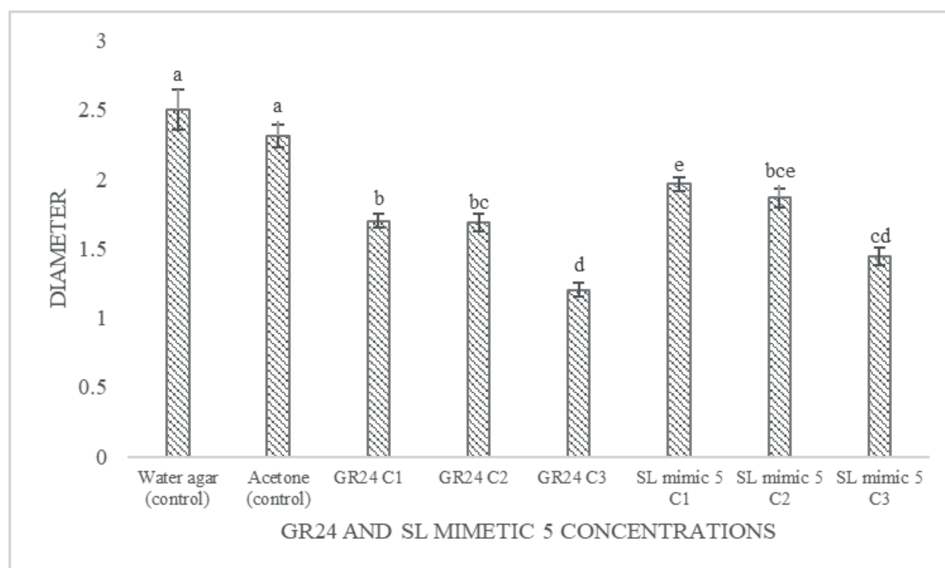


Figure 7. Diameter of *S. minor* colonies in the absence and presence of strigolactones (C1, C2, C3 are 5 x 10^{-6}, 10^{-5}, and 5 x 10^{-5}M concentration, respectively). The bars represent the standard error. Significant differences are shown by using different letters at $p < 0.05$

The *in vitro* response of the plant pathogenic fungi to synthetic SLs largely depends on the application method. When GR24 was applied in a hole made in the water agar, no influence of this synthetic SL on different fungi, ectomycorrhizal fungi, *Trichoderma* and *Piriformospora indica*, foliar pathogens *B. cinerea* and *Cladosporium* sp. or soil-borne pathogens was observed (Steinkellner et al., 2007). Other studies, wherein small fiberglass discs soaked with different concentrations of GR24 were placed on the surface of the agar

media, reported no effect of fungi different from AMF (Torres-Vera et al., 2014). Also, spreading GR24 from an acetone solution on the surface of the agar media produced no influence of SLs on *Fusarium oxysporum* hyphal growth (Foo et al., 2016).

Our experimental method, of mixing GR24 with agar medium, determined different behavior regarding the fungal plant pathogen response (Oancea et al., 2017).

Similar results were reported by the studies of Belmondo et al. (2017) and Dor et al. (2011), which evidenced an inhibition of radial fungal growth when GR24 was embedded into water agar media.

Most probably the AMF fungi respond to lower concentration than phytopahogenic fungi, and for that reason there are differences between the 2 tested methods. In our study, the used GR24 concentrations were in the range 10^{-5} and 10^{-6} M, being similar with other reported studies (Matusova et al., 2005; Joel et al., 2011; Dorr et al., 2011). Here we should also consider the extremely low solubility of these hydrophobic SLs in aqueous media.

The biological significance of the response of plant pathogenic fungi is not yet clear. Our results show quite similar response for SLs analogue GR24 and a SLs mimic, SL mimic 5, both in term of hyphal branching and inhibition of radial growth. The inhibition of radial fungal growth can be a result of a stress response (Sabbagh et al., 2012; Asante et al., 2008; Rodríguez-Urra et al., 2009).

Most probably, the secretion of SLs has not only a role in recruitment of AMF in phospohorus deficit conditions (Lopez-Raez et al, 2011). The strigolactone “crying-for-help” in root exudate is probably an adaptive mechanisms, by which plant modulate their rhizospheremicrobiome (Aquino et al., 2021; Liu et al., 2020; Rolfe et al., 2019).

Our results demonstrated similar results for a SLs analogue and a SLs mimic, in terms of plant pathogens response. Both synthetic SLs share a common D-ring. Karrikins, plant germination stimulant compounds derived from smoke, which also share similarities with D-ring of natural strigolactones (Scaffidi et al., 2012), most probably support plant response to biotic and abiotic stress by shaping the plant rhizomicrobiome (Nasir et al., 2020).

SLs mimics are less expensive than SLs analogues because are easier to synthetize. Present results support the idea of using SL mimic 5 as a new type of plant biostimulant, enhancing plant tolerance to biotic and abiotic stress by rhizomicrobiome shaping. Such mode of action is analogue to prebiotic effect on probiotic microflora in animal digestive systems, and represents a new intervention mean in supporting cultivated plants to adapt to the biotic and abiotic stress enhanced by climatic changes. Further studies are necessary in this direction.

CONCLUSIONS

In this study we presented several tests regarding the influence of synthetic SLs, GR24 analogue and SL mimic 5, on two phytophatogenic fungi, *C. acutatum* and *S. minor*. Our results suggest that the mimic compound has the same effect as GR24, inducing hyphal branching stress response and inhibiting phytopathogen growth. The synthesized SL mimic can be further used, for in depth investigation of SLs - plants and SLs microorganisms interactions, for both fundamental studies (role and receptor of SLs D-ring) and practical applications - shaping plant rhizomicrobiome.

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