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Pre-treatment with calcium enhanced defense-related genes' expression in the soybean's isoflavones pathway in response to *Sclerotinia sclerotiorum*

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary is a fungal plant that causes serious losses in important crops. This study examined the expression of selected defense-related genes in the isoflavonoids pathway after soybean inoculation with two *Sclerotinia* isolates possessing different levels of aggressiveness (SSPetri, highly aggressive and SS18, weakly aggressive) and the effects of pre-treatment with calcium on disease symptoms. *PAL1* displayed an early expression after inoculation regardless of the isolate used. Expression of *IFS2* and *IFR2* were more pronounced in leaves inoculated with the weakly aggressive isolate SS18 than with its highly aggressive counterpart. Oppositely, *CHS3*, *CHS5* and *CHI1B1–CHI1B2* had higher transcript abundance in presence of the highly aggressive isolate SSPetri. Pre-treatment with calcium enhanced the expression of *CHS3*, *CHS5*, *CHI1B1–CHI1B2 IFS2* and *IFR2* in the leaves inoculated with SSPetri. An increase in isoflavone aglycones and their malonyl and acetyl conjugates was recorded in the inoculated leaves, with higher contents in response to SSPetri. Pre-treatment with calcium improved the accumulation of daidzein, genistein and acetyl glycitin, especially in the leaves inoculated with SSPetri. *De novo* synthesis of glyceollins occurred in response to inoculation, especially in response to the highly aggressive isolate SSPetri.

1. Introduction

The stem rot (SSR) caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is a major disease in many field crops including soybeans [19]. Strategies to control this disease are limited due to tighter rotations with susceptible hosts and the lack of effective management of crop residues. Current control strategies in most areas growing soybeans rely on the use of partially resistant cultivars [23], along with chemical fungicides. The efficacy of fungicides has not been steady in achieving full protection, making the return on investment challenging for growers who use them [31,34]. Induced resistance could make a good contribution to the fight against plant pathogens [15,16,38], but research on resistance inducers is still lacking in many pathosystems involving *S. sclerotiorum*. In this context, key inorganic nutrients may be used to trigger plant defense responses and/or inhibit fungal growth. For instance, calcium is a secondary nutrient known to regulate various cell functions, from nutrient uptake to changes in cell status, and can be used in helping plants cope with both biotic and abiotic stresses [21,44]. Since cell wall Ca^{2+} achieves its effects through interaction with pectates [41], it is often referred to as the plant's first line of defense. Some pathogens, including *S. sclerotiorum*, secrete oxalic acid, which sequesters calcium from the cell walls to form calcium oxalate [13], thereby shifting this element away from its structural and signaling functions. Increasing calcium levels in tissues can greatly decrease the pathogen's ability to invade the leaf [9,10,35,42]. For example, the use of calcium chloride significantly reduced the incidence and severity of potato early blight under artificial conditions [17]. Used as a plant nutrient, calcium has also been reported to enhance potato resistance to soft and dry rots [33].

Soybeans accumulate isoflavone phytoalexins in response to biotic and abiotic stress, with the main compounds being genistein, daidzein and glycitein, which are synthesized by a branch of the phenylpropanoid pathway [18]. This extended metabolic route leads to the synthesis of other important secondary metabolites

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such as tannins, lignins, lignans, anthocyanins, flavones, flavonols, and glyceollins, previously shown to possess antimicrobial activity [47]. Glyceollins are derived from the precursor daidzein via isoflavone reductase (IFR), the last and crucial enzyme in glyceollin biosynthesis [28]. However, the first reaction of this biosynthetic pathway is the diamination of L-phenylalanine to cinnamic acid by phenylalanine ammonia lyase (PAL). Chalcone synthase (CHS), a multigene family (8 members) in soybean, constitutes the first critical enzyme for flavonoid synthesis [12]. Other important enzymes in the pathway for isoflavone synthesis are chalcone isomerase (CHI), which converts chalcones to flavanones, and chalcone reductase (CHR), required for daidzein and glycitein formation. However, the enzyme that specifically sets apart the isoflavone-producing plant species from the others is isoflavone synthase (IFS) [3,22,39]. In soybean genome, two gene copies of IFS, IFS1 and IFS2 have been identified [22]. Both convert naringenin and liquiritigenin to genistein and daidzein, respectively. However, only IFS2 was detected in soybean hypocotyls and roots in response to infection [12]. Many studies reported on the importance of soybean phytoalexin accumulation upon pathogen attacks, especially glyceollins [25]. However, the kinetics of isoflavone production, i.e., genistein, daidzein, glycitein, and their conjugates in soybeans in response to pathogens is lacking. The objectives of this study were to assess the effects of a foliar calcium-based formulation on (i) the development of weakly- and highly-aggressive isolates of *S. sclerotiorum* in soybeans, (ii) the differential expression of key genes in the phenylpropanoid pathway, and (iii) the accumulation of their respective isoflavones during the early stages of infection.

2. Material and methods

2.1. Fungal growth

Two *S. sclerotiorum* isolates, SSPetri (highly aggressive, HA) and SS18 (weakly aggressive, WA) were selected for this study. SSPetri was isolated from a sclerotium collected from a diseased soybean plant from a field in Manitoba, Canada and SS18 was isolated from a diseased lentil plant in Saskatchewan, Canada. The mycelium was grown on Potato Dextrose Agar (PDA) and stored at 4 °C until needed. The inoculum was prepared as a subculture on fresh PDA incubated at room temperature. Plugs were prepared by cutting five-mm discs from the edge of two-day-old cultures.

2.2. Plant material

Seeds of a commercial soybean cultivar Thunder 27005RR2 (Thunder Seed, Canada) were selected for this study. Seedlings were grown in 15-cm-diameter pots filled with a 50–50 soil and sand mixture, for 21 days in a growth chamber maintained at 20 °C with 60–80% relative humidity and a photoperiod of 16 h. Six seedlings were used per pot as an experimental unit. Two days before inoculation, the seedlings were divided into two groups of 63 pots. One group was sprayed with a calcium-based formulation (P3; OMEX Agriculture Inc., Canada) using a hand atomizer while the other was left untreated. The whole experiment was conducted three times independently. All experiments were conducted according to a randomized complete block design.

The plants were then inoculated with *S. sclerotiorum*, at the V2 leaf stage characterized by the presence of two sets of unfolded trifoliate leaves. Plugs with inoculum were placed side down in a center position on each leaf. After inoculation, all treated and control plants were misted with water and covered with plastic bags to increase the relative humidity for two consecutive days. Leaf samples were harvested at 6, 24, 48, 72, 96 and 120 h post inoculation (hpi), immediately immersed in liquid nitrogen, and kept at −80 °C until extracted for secondary metabolites and RNA analyses. The following treatments were considered: (i) Control: water control; (ii) Control + Ca–F: treated with the calcium formulation; (iii) SSPetri: inoculated with the highly aggressive isolate SSPetri; (iv) SSPetri + Ca–F: treated with calcium and inoculated with the highly aggressive isolate SSPetri; (v) SS18: inoculated with the weakly aggressive isolate SS18, and (vi) SS18 + Ca–F: treated with calcium and inoculated with the weakly aggressive isolate SS18.

2.3. Disease assessment

Eighteen plants from each experimental unit were visually rated for disease at 6, 24, 48, 72, 96 and 120 hpi. The leaves were also photographed to obtain digital images from which a percentage of diseased area was calculated using the lesion assay software ASSESS 2.2 [24]. Three replicates per experimental unit were included for each treatment and experiment. The whole experiment was repeated independently three times.

2.4. Primer design

Primer pairs for the tested genes (*PAL1*, *CHS3*, *CHS5*, *CHI1B1*, *IFS2* and *IFR2*) were designed based on available soybean mRNA sequences using GenScript Real-time PCR Primer Design Software (http://www.genscript.com/cgi-bin/tools/primer_genscript.cgi). The constitutively expressed gene *actin* was used to normalize the quantification of expression of each target gene (Table 1).

2.5. RNA extraction

Total RNA was extracted from the leaf tissues using the RNeasy plant mini kit (QIAGEN) and treated with RNase-free DNase I (Ambion) according to the manufacturer's recommendations. Briefly, 100 mg of tissues reduced into a fine powder using liquid nitrogen were suspended in 450 µl of Buffer RLT. The homogenate was mixed using a vortex and incubated at 56 °C for 3 min then transferred to a QIA shredder spin column and centrifuged for 2 min at 15,000 rpm. To the supernatant of the flow-through, 0.5 v/v of 100% ethanol was added and mixed by pipetting then transferred to an RNeasy spin column. The column was centrifuged for 15 s at 9000 rpm. The sample was then washed once with 700 µl of Buffer RW1 and twice with 500 µl of Buffer RPE. The samples were eluted to a final volume of 40 µl. The RNA concentration was determined at 260 nm using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and its quality checked on a 1% agarose gel.

2.6. RT-qPCR

The RNA extracted from soybean leaf tissues was used to synthesize the first-strand cDNA using a Promega reverse transcription kit (Promega, Madison, WI, USA) following the manufacturer's recommendations. Briefly, one µg of RNA was used in a 50 µl solution containing 5 ng/µl of oligo dT, 500 µM of each dNTP, 0.4X First-Strand buffer (20 mM Tris–HCl (pH 8.3), 30 mM KCl, 1.2 mM $MgCl_2$), 4 mM DTT, 40 U RNaseOUT and 200 U MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase. The first-strand cDNA was synthesized at 37 °C for 2 h and the reaction was inactivated by heating the mixture to 70 °C for 15 min.

qPCR was performed for each target gene as well as the reference gene *actin*, using CFX96TM Real Time System (C1000™ Thermal Cycler, Biorad) and SsoFast EvaGreen Supermix, QPCR master mix (Biorad, Hercules, CA, USA) following the manufacturer's recommendations. Three biological replicates were used per