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Peanut priming induced by biocontrol agents

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ABSTRACT

Selected strains of rhizobacteria induce systemic resistance in plant (ISR), enhancing the capacity to mobilize infection-induced cellular defense responses (priming). *Bacillus* sp. CHEP5 and *Pseudomonas* sp BREN6 strains reduced root and stem wilt disease severity caused by *Sclerotium rolfsii* in *Arachis hypogaea* L. Strains inoculation increased the activity of phenylalanine ammonia-lyase and peroxidase, after pathogen-challenge, indicating priming. CHEP5 primes plants to produce more ethylene upon stimulation with ACC. *A. hypogaea* L is an ISR-positive plant and BREN6 and CHEP5 strains enhance the plant's defense capacity by priming for potentiated activity of defense proteins and ACC-converting capacity.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an economically important crop throughout the world. It is susceptible to many pathogens, with most damage being caused by fungi. A soil borne fungal disease that adversely affects peanut health and productivity all over the world growing areas is root and stem wilt caused by *Sclerotium rolfsii*. Depending on severity of field infestation, yield losses due to such soil borne disease may be as high as 50% [1]. Effective control of root and stem wilt disease can be reached by supplying a recommended fungicide. However, this strategy could contribute to environmental pollution and to greater production cost. Biological control of fungal diseases of plants is eco-friendly and a potential component of integrated disease management.

Rhizobacteria are used as inoculants to enhance crop yield and for biological control of fungal pathogens. Certain strains of rhizosphere bacteria stimulate plant growth and are, therefore, called plant growth-promoting rhizobacteria (PGPR). Some of them promote plant growth by suppressing soil-borne pathogens. This biological control activity can be the result of competition for nutrients, siderophore-mediated competition for iron, or antibiosis [2]. In addition, selected strains of non-pathogenic rhizobacteria can reduce disease in above-ground plant parts through the induction of a defense state that is commonly referred to as rhizobacteria-induced systemic resistance (ISR) [3] and is mediated by jasmonic acid and/or ethylene. PGPR that colonize root systems and protect plants against foliar diseases include *Pseudomonas fluorescens*, *Pseudomonas putida*, *Bacillus pumilus*, and *Serratia marcescens* [4,5].

A common feature of induced resistance caused by beneficial microorganisms is "priming". Inoculation of plants with some rhizobacteria shows that ISR-positive plants are primed as they display faster and/or stronger activation of the various cellular defense responses that are induced following attack by pathogens [6]. The enhanced plant's defense capacity by priming is correlated with a potentiated expression of defense genes and *de novo* synthesis of antimicrobial compounds such as pathogenesis-related proteins, which are expressed in uninfected tissue in response to a first infection [7]. Although the phenomenon has been known for decades, most progress in our understanding of priming has been made over the past few years. The molecular mechanisms underlying priming and its importance in the overall plant resistance still remain to be investigated.

Pathogenesis-related proteins are generally used as ISR markers [8] but increased activities and accumulation of these proteins depend mainly on the inducing agent as well as plant genotype, physiological condition, and pathogen [9]. Some of these proteins have chitinase or β-1,3-glucanase activity that degrade the fungal cell wall and cause lysis of fungal cell [3]. Enzymes related to phytoalexin biosynthesis (phenylalanine ammonia lyase (PAL)) or

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that could act in cell wall reinforcement by catalyzing lignifications such as the oxidative enzyme peroxidase (PO), have also been associated with ISR [10]. Furthermore, another phenotypic trait of rhizobacteria-mediated ISR reported in *Arabidopsis* is the plant enhanced capacity for conversion of ACC (1-amino-cyclopropane-1-carboxylic acid) to ethylene, providing a greater capacity for producing this hormone after pathogen challenge [11].

ISR phenotype has been demonstrated in many plant species, e.g., carnation, bean, radish, tobacco, tomato, and has been reported to be effective against a broad spectrum of plant pathogens, including viruses, bacteria and fungi [3]. However, there are scarce publications reporting attempts to use PGPR strains as elicitors of ISR in peanut and their results are controversial. Kishore et al. [12] reported that *S. marcescens* GPS5 and *Pseudomonas aeruginosa* GSE18 activated defense-related enzymes in peanut leaves inoculated with *Phaeoisariopsis personata*, the causal agent of late leaf spot disease. Both strains reduced the lesion frequency and enhanced the activities of peanut defense-related enzymes chitinase, β-1,3-glucanase, PO and PAL. Madhaiyan et al. [9] reported induced systemic resistance activity in peanut against rot pathogens inoculated in leaves, in response to methylotrophic bacteria as the activities of PAL, β-1,3-glucanase and PO were significantly higher in treatments with *Methylobacterium* challenge-inoculated with *Aspergillus niger* or *S. rolfsii*. Zhang et al. [13] tested the induction in peanut of systemic resistance to late leaf spot disease (caused by *Cercosporidium personatum*) by PGPR that elicited ISR in other plants and by the addition of chemical elicitors. Neither the 19 PGPR strains including the genera *Paenibacillus* and *Bacillus*, nor the chemical inducers evaluated showed consistent results, indicating plant protection from the fungal pathogen attack. Then, the authors proposed that peanut is not a systemically inducible plant.

We have previously selected and characterized endophytic and epiphytic bacteria from peanut plants for their potential use in the biocontrol of fungal pathogens [14]. The bacterial collection obtained includes the isolates *Bacillus* sp. CHEP5 and *Pseudomonas* sp. BREN6, able to inhibit the growth of *S. rolfsii*. To gain a better understanding about ISR induction in peanut, the objectives of this research were a) to determine whether *Bacillus* sp. CHEP5 and *Pseudomonas* sp. BREN6 induces systemic protection against root and stem wilt caused by *S. rolfsii*, b) to know whether peanut plants are primed by determining if inoculated plants display stronger activation of the enzymes β-1,3-glucanase, phenylalanine ammonia lyase (PAL) and peroxidase (PO) following *S. rolfsii* challenge, and c) to know if the rhizobacteria-mediated ISR is associated with peanut enhanced capacity for conversion of ACC to ethylene.

2. Material and methods

2.1. Bacterial strains, pathogens and culture conditions

Pseudomonas sp. BREN6 and *Bacillus* sp. CHEP5 isolated from roots and leaves, respectively, of peanut plants growing at field, and able to inhibit the growth of *S. rolfsii* [14] were used in this study. Both of them showed antibiosis against *S. rolfsii* and BREN6 is also a siderophore producer strain. They were cultured in TSA (trypticase soy agar) medium. The fungal pathogen *S. rolfsii* was obtained from infected peanut plants and grew on potato dextrose agar at 25 °C for 7 days.

2.2. Plant material and growth conditions

Peanut cultivar Tegua, which is susceptible to root and stem wilt, was provided by J. Soave, criadero "El Carmen". Seeds were surface sterilized by soaking in ethanol for 30 s followed by 20% H_2O_2 for 15 min, and then washed six times with sterile distilled water. Seeds were germinated at 28 °C in sterilized Petri dishes with one layer of Whatman N° 1 filter paper and moist cotton, until the radicle reached approximately 2 cm. Seedlings were sown in previously sterilized plastic cups filled with quartz sand, watered regularly and supplied once a week with Hoagland medium [15]. Plants were grown under controlled environment (light intensity of 200 μmol m^{-2} s^{-1}, 16-h day/8-h night cycle, at a constant temperature of 28 °C and a relative humidity of 50%).

2.3. Bioassays for induced systemic resistance

Root system of 15 days old plants growing in pots with quartz sand was separated (without cutting) using the split method described by Fuchs et al. [16]. Each root part was put in 100 ml glass tubes containing agarized Hoagland medium (0.6%) and one of the root parts was inoculated with the bacterial biocontrol agent (3 ml of a TSB culture containing 10^9 CFU ml^{-1}). A week later, the other root part was challenged with the pathogen by adding, per tube, one wheat seed infested with *S. rolfsii* mycelium (20 mg). Non-pathogenized and non-bacterized control plants were also included. At 13 days post-pathogen challenge, disease symptoms were recorded and plants were harvested to determine their shoot and root dry weights and lengths. At this time, the activity of enzymes involved in systemic resistance was also evaluated in leaves from these plants. The experiment was repeated three times with six replicates for each treatment.

2.4. Total chlorophyll determination

The amount of total chlorophyll in plants inoculated with *Bacillus* sp. CHEP5 or *Pseudomonas* sp. BREN6 before and after *S. rolfsii* challenge was determined by the method described by Arnon [17]. Briefly, approximately 0.1 g of fresh weight peanut leaves was placed into a mortar and the tissue was grinded to fine pulp after the addition of 80% acetone. The resulting extract was transferred to a Buchner funnel containing a pad of Whatman filter paper. While filtering the extract, the gridding of the leaves pulp was repeated to adjust the final volume of the filtrate to 10 ml. The optical density of the chlorophyll extract was read with a spectrophotometer set at 650 and 665 nm. The amount of total chlorophyll present in the extract was calculated on the basis of μg of chlorophyll per gram of fresh leaf tissue, according to the following equation [18]:

$$\text{Total chlorophyll} = 6.45\ (\text{Abs}\ 665) + 17.72\ (\text{Abs}\ 650)$$

2.5. Determination of enzyme activities

Peanut leaves in measured quantities were washed under running tap water and homogenized with liquid nitrogen using a mortar and pestle containing appropriate buffer solution (50 mM potassium phosphate and 1 mM EDTA, pH 7.8) and 1% PVP (polyvinylpyrrolidone) and then filtered through a 0.20 mm nylon filter into a centrifuge tube. The tissue extract was centrifuged at 12,000 g for 40 min at 4 °C. The supernatant to be used for the enzymatic activities determination was stored at −20 °C. A colorimetric assay for enzymatic activity was performed with a GENESYS2 spectrophotometer. The reaction rates were linear and proportional to the enzyme or protein concentration added. The protein concentration was determined by the method described by Bradford [19].

To assay PO, the method described by Frick [20] was used. Leaf extract (100 μl) was added to 790 μl of buffer solution (50 mM potassium phosphate, 0.1 mM EDTA pH 7) and mixed with 100 μl guaiacol (10 mM in the mixture). The reaction was started by the