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Antioxidant defense response induced by *Trichoderma viride* against *Aspergillus niger* Van Tieghem causing collar rot in groundnut (*Arachis hypogaea* L.)

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ABSTRACT

The study was conducted to examine the antioxidant enzymes induced by *Trichoderma viride* JAU60 as initial defense response during invasion of rot pathogen *Aspergillus niger* Van Tieghem in five groundnut varieties under pot culture. Seed treatment of *T. viride* JAU60 reduced 51–58% collar rot disease incidence in different groundnut varieties under pathogen infected soil culture. The activities of the antioxidant enzymes, viz., superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (GPX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), elevated in response to pathogen infection, in higher rate by tolerant varieties (J-11 and GG-2) compared with susceptible (GAUG-10, GG-13, GG-20) and further induced by *T. viride* treatment. *Trichoderma* treatment remarkably increased the 2.3 fold SOD, 5 fold GPX and 2.5 fold APX activities during disease development in tolerant varieties and the same was found about 1.2, 1.5 and 2.0 folds, respectively, in susceptible varieties. Overall, *T. viride JAU60* treated seedlings (T_3) witnessed higher activities of SOD (1.5 fold), GPX (3.25 fold) and APX (1.25 fold) than pathogen treatment (T_2) possibly suggest the induction of antioxidant defense response by *Trichoderma* bio-controller to combat oxidative burst produced by invading pathogen.

1. Introduction

Plant cells produce reactive oxygen species (ROS) when interact with phytopathogens. The ROS production like, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), causes oxidative damage, leads to lipid peroxidation with membrane destruction, protein inactivation or DNA mutation [1]. The H_2O_2 is generally thought as the most versatile of the ROS, with a number of different possible functions in a defense strategies for plants. The increase in H_2O_2 level may cause pathogen destruction or is involved as a secondary messenger in the systemic signal network of plant cells [2].

Plants possess a number of enzymatic and non-enzymatic mechanisms of detoxification to efficiently scavenge for either the ROS themselves or their secondary reaction products under conditions of normal healthy growth [3]. Various enzyme systems participate in ROS metabolism during the pathogen attack in plants [4]. Major ROS scavenging enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (GPX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) are produced to avoid cellular disintegration by ROS [5]. The first enzyme in ROS metabolism is SOD which catalyzes dismutation of O_2^- and $H_2O_2^-$ to H_2O_2. The APX belongs to important H_2O_2-scavenging enzymes, removing H_2O_2 through a mechanism known as Halliwell–Asada–Foyer pathway. However, GPX is included in different physiological processes like cross-linking of the cell wall proteins, pectins by diferulic bridges and the oxidation of cinnamyl alcohols prior to their polymerization during lignin and suberin formation. Peroxidases are an important component of an early plant-response system to pathogen attack [6,7]. Natural antioxidants such as a tocopherol, carotenoids, ascorbic acid, uric acid and glutathione is also play an important role to scavenge free radicals or ROS generated by oxidative stress [8,9].

Generally, constitutive and inducible mechanisms are coevolved by plants to cope with pathogen infection [10]. The ROS production has been well established in several plant tissues infected by biotrophs and associated with the expression of a hypersensitive response or systematic acquired resistance [11]. In contrast, very little is known about successful exploitation of bio-control agents

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on disease management through induced systemic resistance (ISR). *Trichoderma* have been used as biological control agents against soil born phytopathogens [12]. All *Trichoderma* strains could not work equally against specific soil born disease. Our previous study [13,14] showed that, among 12 isolates of *Trichoderma*, *Trichoderma viride* JAU60 was found to be best antagonist for *in vitro* growth inhibition of disease causing *Aspergillus niger*.

To our knowledge, there has been no report published on the induction of antioxidative bursts by treatment with *Trichoderma* in groundnut against rot pathogen. In the backdrop of this scenario, the aim of the present study was to investigate expression of antioxidant enzymes induced by *T. viride* JAU60 as initial responses in groundnut (*Arachis hypogaea* L.) against the invading pathogen *A. niger* Van Tieghem, the causal organism of collar rot disease which is one of the most prevalent and damaging diseases of groundnut.

2. Materials and methods

2.1. Preparation of microbial cultures

Groundnut seedlings which showed typical symptoms of collar rot were cut into small bits with the help of sterilized blade and the pure culture of pathogen (*A. niger*) was made by hyphal tip isolation method on the solidified PDA medium in petri plates. *T. viride* JAU60 was originally selected for its specific features regarding biocontrol agent, based on our previous study [13] on *in vitro* antagonism of 12 isolates of *Trichoderma* with test pathogen *A. niger* Van Tieghem. The molecular identification of *T. viride* JAU60 strain was also carried out according to our preceding study. The strain was maintained on PDA media and prepared for mass inoculums using sand maize meal medium (SMMM) as previously described by Sinclair and Dhingra [15]. The *T. viride* JAU60 and *A. niger* Van Tieghem inoculums, thus, obtained were harvested and used for preparation of talc based formulations [16] for bio-control agent and pathogen infection, respectively, in pot culture study.

2.2. Determination of microbes load (cfu)

Serial dilution plate method was used to determine the microbe's load of pathogen *A. niger* or bio-control agent *T. viride* JAU60 in their respective talc based powder mass formulations [17]. Before using in experiment, microbes load as a colony forming unit (*cfu*) measured by serial dilution from both the microbes mass formulations individually.

2.3. Preparation of pathogen – A. niger infested soil

Field soil having the chemical properties of 236 kg ha^{-1} available nitrogen, 12 kg ha^{-1} available P_2O_5, 265 kg ha^{-1} available K_2O, pH 6.5, 0.5 dSm^{-1} electrical conductivity (EC) was passed through a 4-mm sieve and mixed along with farm yard manure (FYM) in a 1:1 ratio and sterilized in autoclave in 1:036 kg/cm^2 for 1 h for three consecutive days. Talc based formulation of pathogen *A. niger* was then added to the soil in the proportion of 1:10 (Talc based Inoculums: Sterilized mixture of soil). The pots were filled with these mixtures @10 kg as a pathogen infested soil per pot. The pots, having pathogen infested soil or healthy soil, were irrigated water to bring the soil at field capacity conditions for three days. Microbial load of pathogen was determined from pathogen infested soil by serial dilution method.

2.4. Pot culture study and collar rot disease incidence

Groundnut seeds were sown in pot culture (earthen pots of 35 cm diameter) with following treatments.

T1 = Groundnut seeds were treated with talc based powder containing carboxymethyl cellulose (CMC) and sown in normal soil pots as a control.
T2 = Groundnut seeds were treated with talc based powder containing CMC and sown in *A. niger* infested soil pots having pathogen load 1.5×10^7 *cfu*.g^{-1} soil.
T3 = Groundnut seeds were treated @4 g kg^{-1} seeds with talc powder based formulation of bio-control agent – *T. viride* JAU60 (microbial load 1.83×10^6 *cfu*.g^{-1} talc powder) and sown in *A. niger* infested soil pots.

Sowing was made for five groundnut varieties – J-11 (bunch variety – V_1), GG-2 (bunch variety – V_2), GAUG-10 (Spreading variety – V_3), GG-13 (Spreading variety – V_4) and GG-20 (Semi spreading – V_5) in three independent experiments. Observations as per cent collar rot disease incidence was recorded at 3 days interval up to 15 days after sowing (DAS) [0 (S_0), 3 (S_1), 6 (S_2), 9 (S_3), 12 (S_4), 15 (S_5) DAS]. The seedlings were harvested on a time course to perform analyses.

2.5. Determination of antioxidant – ascorbic acid

Ascorbic acid was extracted from groundnut seedlings (0.500 g) with 5 ml of meta-phosphoric acid – acetic acid solution (15 g meta – phosphoric acid in 40 ml of glacial acetic acid and made the volume 200 ml with distilled water). The homogenate was filtered through whatman no. 1 filter. The known volume of filtrate was titrated against the indophenol dye [18]. Blank was run with meta-phosphoric acid solution without the sample. The Standard ascorbic acid solution (50 mg) was also titrated with indophenol dye until light pink color persists. Based on standard titer, the amount of ascorbic acid was calculated and expressed as mg.g^{-1} Fr.Wt. tissues.

2.6. Antioxidative enzyme activities in relation to oxidative burst

Groundnut seedlings were homogenized with a pre-chilled mortar and pestle under ice cold condition in 10 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4) with the addition of 1 mM EDTA and 1% (W/V) polyvinylpyrolidone [19]. The homogenates were centrifuged under refrigeration (4 °C) at 15,000 rpm for 20 min and the supernatant was used for the assay of superoxide dismutase, guaiacol peroxidase and ascorbate peroxidase activities. Protein concentration was measured according to Folin lowry method [20].

2.6.1. Superoxide dismutase (SOD) activity (EC 1.15.1.1)

The SOD activity was measured following the method of Van Rossun et al. [21] by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. The reaction mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract (0.1 ml distilled water in case of blank). It was kept under two 15-W fluorescent lamps for 15 min at 25 °C, followed by transferring to dark for 15 min and then the absorbance was read at 560 nm. One unit of the SOD activity was defined as the amount of enzyme required to inhibit reduction of NBT by 50%. Enzyme unit of SOD was calculated according to formula given by Constantine and Stanley [22] and expressed as U.mg^{-1} protein.

2.6.2. Guaiacol peroxidase (GPX) activity (EC 1.11.1.7)

The GPX activity was assayed by measuring increase in absorbance at 470 nm due to oxidation of guaiacol to tetraguaiacol. The reaction mixture (3 ml) consisted of 20 mM guaiacol, 0.1 mM acetate buffer (pH 5.0), 40 mM H_2O_2. The reaction was initiated by