The role of oxidative burst and cell wall in tomato interaction with various taxonomic groups of Rhizoctonia spp

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Abstract

In this research, interaction of multinucleate *Rhizoctonia solani* and a binucleate isolate of *Rhizoctonia* sp. (BNR) was investigated with using tomato as a susceptible host. The highest levels of O_2^-, H_2O_2, callose accumulation and superoxide dismutase activity were observed for the isolate of *R. solani* AG 3, which caused lower disease severity compared to highly pathogenic *R. solani* isolates belonging to AG 4 HG-I and AG 4 HG-II. Xanthine/xanthine oxidase treatment, which induced O_2^- production, increased the disease symptoms. Whereas, glucose/glucose oxidase, which increased H_2O_2, decreased the disease symptoms. Ascorbate, as an inhibitor of H_2O_2 accumulation, reduced callose deposition and increased the disease severity. Analyzing activity of cell wall degrading enzymes (pectinase and cellulase) produced by different fungi showed higher activity of these enzymes for the isolates with higher pathogenicity. Therefore, defense components of host plant and pathogenicity factors of fungi are both involved in the outcome of plant-fungi interaction.

Key Message

Reactive oxygen species, antioxidants and callose are involved in tomato defense responses against various taxonomic groups of *Rhizoctonia*, as a destructive necrotrophic fungal pathogen

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most important vegetables, which plays a crucial role in human nutrition and health. The fungal genus *Rhizoctonia* is a destructive plant pathogenic fungus with a wide host range normally found in soils all over the world. The fungus causes diseases in many monocotyledons (monocots) and dicotyledon (dicots) such as crops, ornamental plants, vegetables and forest species in more than 530 genera of higher plants (Gonzalez et al, 2006). *Rhizoctonia solani* is the most important species of *Rhizoctonic*, which is widely recognized and studied. To date, multinucleate isolates of *R. solani* are placed into 13 anastomosis groups from AG1 to AG13 and binucleate isolates are classified into 16 AGs (Carling et al, 2002; Sharon et al, 2008). *Rhizoctonia solani* is the causal agent of crown and root rot of tomato, which leads to high yield losses worldwide (Daroodi et al. 2021, Pourmahdi and Taheri 2014).

Management of *Rhizoctonia* diseases is very difficult due to the high level of genetic diversity in the pathogen populations, long-term survival of the sclerotia of this soil-borne phytopathogen in the soil, and its wide host range (Taheri et al. 2007). Using partially resistant or tolerant cultivars together with application of biological and natural strategies for plant protection are amongst the most effective methods for controlling different diseases caused by *Rhizoctonia* spp. on various plants (Kheyri and Taheri 2021, Kheyri et al. 2022)

During plant-pathogen interaction, a variety of defense mechanisms are activated in the host plant to block the penetration, progress and proliferation of potential pathogens. A successful plant defense

strategy depends on quick recognition of the pathogen and activation of various biochemical and structural defense mechanisms (Taheri 2022; Thordal-Christensen, 2003). Plants have several successful defense mechanisms against different pathogens. Plant immunity system creates a variety of protection strategies, including hypersensitivity response, production of antimicrobial compounds, and biosynthesis of enzymes that can break down pathogen's cell wall. In addition to these specialized defense mechanisms, plant cell wall manifests the first line of defense. In other words, it is actively reinforced through creation and deposition of papillae in the sites where reaction with microbial pathogens happen. Strengthening the plant cell wall by papillae deposition, known as callose, is one of the most important plant defense mechanisms against pathogens with different lifestyles (Usak et al. 2023). Callose can be formed in the penetration sites as a powerful physical barrier, which reduces pathogen development in the host tissues (Miya, 2007; Bednark and Osbourn, 2009). Papilla is a complex structure that is formed between plasma membrane and the inner wall of plant cells. Papillae biochemical composition can vary in different plant species. Some common compositions found in the papilla are phenolics, reactive oxygen species (ROS), cell wall proteins and polymers. Among these polymers, β-1,3-glucan or callose is one of the most abundant compounds. Improved performance of defensive compositions is directly related to strengthening of cell wall or an antimicrobial effect. Priming of defense strategies such as papillae formation is a key factor for successful plant defense (Li et al. 2023; Voigt, 2014).

Various types of ROS can play critical roles in changing the cell wall structure, defensive signals and hypersensitivity reactions, or can directly kill different pathogens (Asselbergh et al, 2007). Rapid and unstable accumulation of ROS can activate oxidative burst as a defense-related reaction, which is regulated by enzymatic and non-enzymatic antioxidants (Melilo et al, 2011, Taheri 2022). In order to protect the cells under stress conditions and maintain the level of ROS, plant tissues produce several antioxidants, such as catalase (CAT) and peroxidase (POX), which regulate the amounts of ROS. Hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are among the most important types of ROS, which are involved in cell wall strengthening via increasing the connections or cross-linking between proteins and phenolics in plant cell wall as an effective defense mechanism against various pathogens with different lifestyles (Nikraftar et al. 2013; Taheri & Kakooee 2017).

Fungal pathogens evolved various strategies for overcoming physical and biochemical defense responses in host plants. The ability of producing various cell wall degrading enzymes (CWDEs) is necessary for phytopathogens to have successful penetration through cell wall and plasma membrane, as the first layers of plant defense. Increased production and activity of CWDEs secreted by the pathogens is directly associated with increased disease progress in the host tissues (Anitha et al, 2011; Khaledi et al. 2017; Pannecoucque and Höfte 2009).

This study aims to investigate the interaction of tomato plants (cultivar Mobil) with three multinucleate isolates and one binucleate isolate of the necrotrophic fungus *Rhizoctonia*. The main purposes of this study were to investigate (i) virulence of the four *Rhizoctonia* isolates on tomato seedlings and leaf discs, (ii) infection process of each isolate at different time points after inoculation and determine its association with pathogenicity of each fungal isolate, (iii) the amount of callose deposition, (iv)

production of ROS in plant cells as a defense mechanism against the pathogen, and (v) the levels of antioxidant activities and expression of corresponding genes. Furthermore, activity of cell wall degrading enzymes (including pectinase and cellulose) produced by different fungal isolates and its correlation with virulence of each isolate and the disease progress were evaluated.

2. Materials and methods

2.1. Tomato plant growth conditions in greenhouse

Tomato cultivar Mobil, which has good growth and high yield in the climatic conditions of Iran, was used in this study. The seeds were surface sterilized with 1% sodium hypochlorite for 1 min, rinsed 3 times with sterile distilled water and incubated for 5 days on a wet sterile filter paper in Petri dishes at 28°C. Germinated seeds were each sown in the 15 cm-diameter plastic pots filled with a commercial potting soil, which had been autoclaved at 121°C for 60 min grown in greenhouse (with conditions of 28 ± 4°C and 16/8 h light/dark photoperiod). The seeds of this cultivar were obtained from Agricultural Research Center of Khorassan-Razavi province. The tomato seedlings were transferred to pots containing autoclaved soil and reared in favorable greenhouse conditions (16 hours of light and 8 hours of darkness, temperature 26–28°C). Four weeks after germination, the seedlings were used for inoculation with the fungal isolates.

2.2. Preparation of fungal inoculum

In this study, isolates of *R. solani*, as a plant pathogenic fungus belonging to the anastomosis groups AG 3, AG 4 HG-I, AG 4 HG-II which were previously isolated from tomato in Iran (Pourmahdi and Taheri, 2014), and an isolate of binucleate *Rhizoctonia* sp. were obtained from the fungal collection of Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad in Iran. To prepare the inoculum, 2 kg of wheat seeds are wetted with 500 ml distilled water and then sterilized in the autoclave 3 times with 24 h intervals at 5.1 atmosphere pressure for 20 min. Then, 12 g of sterilized seeds were distributed on the surface of each Petri dish containing Potato Dextrose Agar (PDA) medium. Afterwards, they were colonized by adding 3 mycelial plugs with 0.5 cm diameter to each Petri dish and incubating for 7 days at 28°C (Misawa and Kuninaga, 2010). The wheat seeds distributed on the PDA medium without the fungus were used as a negative control in the pathogenicity tests.

2.3. Pathogenicity tests on tomato seedlings and leaf discs

For inoculating each seedling, 3 g of wheat seeds colonized by each fungal isolate were used. This inoculum was placed next to the crown of tomato seedling at 4 weeks growth stage. Then, the pots were covered with plastic bag to keep moisture and avoid evaporation. One week after fungal inoculation, development of the disease symptoms was evaluated and the disease severity was investigated by determining the lesion length on tomato seedlings.

To perform the leaf disc assay, circular discs (2 cm diameter) were cut out from apical leaflet of the first outer leaves of 4-week-old tomato plants using a cork borer (Nikraftar et al. 2013). Each of these discs

were placed on a glass slide inside a Petri dish containing two wet filter papers. One mycelial plug of *Rhizoctonia* (5 mm diameter) was placed in the center of each leaf disc. Petri dishes were kept in laboratory conditions (25°C; 12/12 h of light/dark photoperiod). The symptoms were assessed 5 days after inoculation (Taheri and Tarighi, 2011) and the disease index (DI) on each leaf disc was estimated using the formula described by Taheri and Tarighi (2010).

2.4. Evaluation of infection structures formation by the fungal isolates

To investigate formation of infection structures, microscopic analysis of the infected leaf discs was carried out using the method of Smith et al. (2004), in which the fungal hyphae were stained by aniline blue. For this purpose, the leaf discs were placed in the bleach solution for 24 h. To keep bleaching and softening leaf tissues, the discs were kept in lactophenol solution for 24 h at 37°C. Finally fungal hyphae were stained using 1% aniline blue and investigated using a light microscope (Olympus, BH2, Tokyo, Japan).

2.5. Histochemical detection of hydrogen peroxide in tomato leaves

Accumulation of H_2O_2 at various time points after inoculation with each fungal isolate was investigated via histochemical detection method using a color reaction with 3,3'-diaminobenzidine (DAB), as described by Thordal-Christensen et al. (1997). Tomato leaf discs infected with *Rhizoctonia* isolates were kept in a 0.1 mg/ml DAB- HCl solution overnight. Polymerization of DAB at the spots where peroxidase and H_2O_2 are accumulated creates a brown color, which is visible under a microscope (Asselbergh et al, 2007). The DAB staining intensities were quantified using Image J software (http://rsb.info.nih.gov/ij/index.html).

2.6. Superoxide anion detection

Reaction between nitro blue tetrazolium (NBT) and superoxide (O_2^-) changes the color of the spot where O_2^- is accumulated and turns it into blue, which can be easily examined under a microscope. The color change was surveyed using the methods described by Adam et al (1989) and Schrauder et al (1998). The NBT staining intensities were quantified using Image J software.

2.7. Evaluation of callose deposition

For determining the role of callose in tomato-*Rhizoctonia* spp. interaction, the leaf discs were inoculated by different fungal isolates. Then, callose deposition was estimated at several time points after the fungal inoculation. Chlorophyll was removed from the leaf tissues using ethanol and the samples were immersed in 1% aniline blue for 1 h for callose detection. Then, the samples were placed in 60% glycerin to make them more softened and improve the microscopic analysis. Callose deposition was investigated using Olympus BX41 microscope and Olympus BX51 Fluorescence microscope. Intensity of callose formation was quantified by determining the number of pixels per million pixels using image J software.

2.8. Manipulating ROS levels by glucose/glucose oxidase and xanthine/xanthine oxidase systems

To clarify the role of ROS in tomato interaction with *Rhizoctonia* isolates, the experiment was performed using xanthine/xanthine oxidase (X/XO) and glucose/glucose oxidase (G/GO) reaction systems, which produce O_2^- and H_2O_2 in plants, respectively. Tomato leaf discs were treated with either X/XO (0.5 mM/0.05 unitss/ml) or G/GO (2.5 mM/25 units/ml) for 3 h. The solutions were prepared using 10 mM sodium phosphate buffer (pH 7). Tomato leaf discs were immersed in each solution for 3 h and then inoculated by *Rhizoctonia* isolates. After 5 days, disease symptoms were investigated and the DI was estimated (Taheri and Tarighi, 2010).

2.9. Evaluating effect of ascorbate on callose production

Tomato leaf discs were placed in 5 mM ascorbate solution, as a potent H_2O_2 scavenger, for 2 h to determine the role of this treatment on callose deposition during tomato-*Rhizoctonia* interaction. The treated leaf discs were inoculated by different isolates of *Rhizoctonia*. Chlorophyll was removed using ethanol at different time points after the fungal inoculation and the samples were stained in 1% aniline blue for 1 h to detect callose deposition in the leaf tissues. Finally, the samples were immersed in 60% glycerin and microscopically investigated for detecting callose deposition as previously described.

2.10. Protein extraction from the plant samples and antioxidant enzymes assays

Total protein was extracted from the plant samples with different treatments at 0, 12, 24, 36 and 48 hours post-inoculation (hpi) with the fungal isolates. The first leaves of tomato plants (500 mg) were ground in liquid nitrogen and homogenized in 3 mL of 100 mM potassium phosphate buffer (pH 6.8). The homogenate was centrifuged at 14,000 g for 20 min at 4 °C and the supernatant was obtained and used to investigate activity of the antioxidant enzymes (Kar and Mishra, 1976). Protein content in the extract was estimated using bovine serum albumin as a standard (Bradford, 1976).

Superoxide dismutase (SOD) activity was determined via measuring reduction of NBT at 560 nm (Sadasivam and Manickam 1996). The reaction mixture (3 mL) contained potassium phosphate buffer (50 mM, pH 7.8), methionine (13 mM), riboflavin (2 µM), EDTA (0.1 mM), NBT (75 µM) and enzyme extract (100 µL).

For investigating peroxidase (POX) activity, the protein extracts (containing 30 mg of total protein) were added to 30 ml of 200 mM guaiacol (as an electron donor substrate) and 25 mM citrate phosphate (pH 5.4). For each sample, 30 ml of 30% H_2O_2 was added, and the absorbance was determined at 470 nm by spectrophotometry. The POX activity was calculated using the extinction coefficient of 26.6 mM − 1 cm^{-1} for guaiacol (Djebali et al., 2011).

Finally, the activity of POX was expressed as μmol min^{-1} mg^{-1} protein and the SOD activity was expressed as U SOD mg^{-1} protein.

2.11. RNA extraction and gene expression analyses

Transcription analyses of the antioxidant genes, including SOD and POX, in tomato plants were performed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method. Inoculation of tomato plants was performed using with the BNR and *R. solani* isolates and the leaf samples were collected at different time point after the fungal inoculation. Total RNA was extracted from tomato leaves using TRIzol reagent according to instructions of the manufacturer (Invitrogen GmbH, Karlsruhe, Germany). Each RNA sample was treated with RNase-free DNase (TURBO DNase, Ambion, USA) to remove DNA. Quantity of each RNA sample was checked via spectrophotometry. First-strand cDNAs were synthesized using oligodT-(18) primer and SuperScript reverse transcriptase (Invitrogen) following the manufacturer's instruction. Primer sequences specific for amplifying the cDNA fragment of POX (designed in this study using Primer 3 software), SOD (Rai et al. 2018), and *Actin* (Zheng 2013) were used in qRT-PCR (Table 1). The qRT-PCR amplifications were performed by using Quantitect SYBR green PCR kit (Qiagen), according to the descriptions of the manufacturer using the ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR conditions performed consisted of an initial denaturation step at 95°C for 15 min, followed by 35 to 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The PCR efficiency for each target cDNA was checked by the slope of a standard curve. Transcription levels of the antioxidant genes were presented as the fold increase in expression level compared to expression of the *Actin* gene as a calibrator and presented using the means (± SE) of three replicates in an experiment with three independent repetitions.

Table 1
Evaluation of infection structures formation by *Rhizoctonia* isolates on tomato leaves.

Rhizoctonia isolates	Structures first observation (hpi)			Number of structure		
	Lopat Appresorrium	Infection Cushions	Penetration of hyphae through stomata	Lopat Appresorrium	Infection Cushions	Penetration of hyphae through stomata
AG 4 HG-II	12	18	24	8	6	4
AG 4 HG-I	18	24	24	5	3	2
AG 3	24	36	48	3	2	1
BN	48	60	-	2	1	-

2.12. Activity of pectinase and cellulase secreted by the fungal isolates

The method described by Khairy et al (1964) was used to investigate the activity level of pectinase and cellulase enzymes secreted by various taxonomic groups of *Rhizoctonia* under laboratory conditions and

determine their association with pathogenicity of the fungal isolates on tomato.

Pectinase activity was measured based on the amount of sugars reduction (D-galacturonic acid) released in the supernatant liquid of the medium. To determine the amount of D-galacturonic acid, dinitrosalicylic acid colorimetric method was employed and the absorbance was measured at 540 nm wavelength. According to the standard curve, the unit of enzyme activity is 1 mM galacturonic acid per minute. Standard curves were created based on absorbance at various concentrations (micrograms per milliliter) of D-galacturonic acid. To measure the cellulase activity, the method described by Wood and Baht (1988) was used. Absorbance was measured at 550 nm wavelength and the amount of dropped glucose released was calculated through standard curve of glucose. One unit of cellulase activity is defined as the amount of enzyme that hydrolyzes 1 micromole of glucose per minute during the reaction.

2.13. Statistical analysis

The spss 21 software was used to analyze the data obtained from different experiments and the mean values calculated were compared with each other using Duncan's multiple range test (MRT) ($p \leq 0.05$). Each experiment was repeated at least three times with three repetitions for each treatment in all assays.

3. Results

3.1. Pathogenicity tests on tomato leaf discs and seedlings

Pathogenicity levels of the fungal isolates on tomato seedlings and leaf discs were evaluated (Fig. 1). The results of pathogenicity tests on tomato leaf discs indicated that the highest and lowest disease index values were observed for the isolates of AG 4 HG-II isolate (88.25 ± 2.17) and the binucleate *Rhizoctonia* (BNR) isolate (13 ± 0.81), respectively. Among multinucleate isolates, the lowest virulence level was observed for the isolate of AG 3 (57 ± 2.48) (Fig. 1).

According to the results obtained via investigating the pathogenicity of different *Rhizoctonia* isolates on tomato seedlings, the highest and lowest virulence index values were observed for the AG 4 HG-II isolate (74.75 ± 0.85) and the BNR as a hypovirulent isolate (11.75 ± 0.94). Among three multinucleate *R. solani* isolates tested, the lowest disease severity was observed for the AG 3 isolate (41.25 ± 1.37) (Fig. 1).

3.2. Investigating infection structures formation by various taxonomic groups of the pathogens

After inoculation of *Rhizoctonia* isolates on tomato leaf discs, microscopic analysis was performed to investigate infection structures formed by the fungal isolates at different time points after inoculation (Fig. 2). *Rhizoctonia* hyphae spread on the leaf surface and penetrate the leaf tissue directly or indirectly via stomata. At the first day after inoculation, the isolates only grew on the surface of tomato leaves. From the second day onwards, infection structures began to form, followed by disease symptom appearance in the third day after the inoculation. According to the results of examining infection structures formation at different time points after inoculation, maximum number of lobate appressorium

(LA) and infection cushion (IC) was observed at 48 h after inoculation for the isolate of *R. solani* AG 4 HG-II. The hypovirulent isolate of Binucleate *Rhizoctonia* (BNR) produced the minimum number of these infection structures on tomato leaves (Table 1).

3.3. Histochemical detection of hydrogen peroxide and superoxide

Accumulation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) at different time points after inoculating tomato leaves with *Rhizoctonia* isolates was investigated. The DAB compound is quickly polymerized after exposure to H_2O_2 in the presence of peroxidase and forms a brown deposit where hydrogen peroxide is accumulated (Tordal-Christensen et al, 1997). Histochemical detection of H_2O_2 revealed that its highest level was observed in the leaves inoculated with the R. solani AG 3, whereas the lowest levels of H_2O_2 were detected in the interaction of tomato with the BNR isolate at various time points tested (Fig. 3). Accumulation of H_2O_2 increased until 24 hpi and decreased afterwards.

Histochemical detection of O_2^- using the NBT staining revealed that accumulation of this anion in the leaves infected by AG 3 was higher compared to the leaf discs inoculate with AG 4 HG-I, AG 4 HG-II, and the binucleate isolate. According to the microscopic observations, accumulation of O_2^- increased until 24 hpi in the interaction of tomato leaves with *R. solani* isolates (AG 3, AG 4 HG-I, and AG 4 HG-II), and then clearly decreased (Fig. 4).

3.4. Callose deposition

Investigating callose deposition in tomato leaves infected with different isolates of *Rhizoctonia*, observed as blue areas in microscopic images, revealed that the highest amount of callose was produced in the leaf discs infected with *R. solani* AG 3 at various time points tested. On the other hand, callose deposited in the leaves interacted with the binucleate isolate of *Rhizoctonia* was lower than those of the leaf discs infected with *R. solani* isolates. Callose accumulation showed increasing trend until 72 hpi (Figs. 5 and 6).

3.5. Effect of ROS-generating systems on the disease progress and callose

Statistical analysis revealed the presence of significant differences among the disease progress on the leaves treated with G/GO, X/XO and controls. Comparing the data showed that the disease index decreased significantly on the leaf discs treated with G/GO, which caused H_2O_2 production in the leaf tissues, compared to the controls (Fig. 7A). However, in the leaf discs treated with X/XO (which produced both H_2O_2 and O_2^-) significant increase in disease progress was observed. The X/XO treatment clearly decreased callose deposition in the leaf discs infected with different *Rhizoctonia* isolates (Fig. 7B).

3.6. Effect of treating the leaves with ascorbate on disease progress and callose accumulation

Treating the leaves with ascorbate significantly increased the disease progress on tomato leaves infected with various isolates of the pathogen (Fig. 8A). In addition, decreased levels of callose deposition were observed in tomato leaves treated with ascorbate and inoculated with various taxonomic groups of *Rhizoctonia*, compared to the controls (Fig. 8B)

3.7. Antioxidant enzymes activities

Effects of inoculating tomato plants with each isolate of *Rhizoctonia* on the activity of antioxidant enzymes, such as SOD and POX, were investigated at 0, 12, 24, and 48 and 72 hpi (Fig. 9). Investigating the SOD activity revealed that the maximum activity level of this antioxidant was observed in the plants inoculated with the hypovirulent BNR isolate at most of the time points tested. Whereas, the minimum level of SOD activity was observed for tomato plants inoculated with *R. solani* AG 4 HG-II, which had the highest pathogenicity among the isolates tested. The SOD activity reached a peak at 12 hpi, followed by a decreasing trend for all *Rhizoctonia* isolates inoculated on tomato plants (Fig. 9A).

For the POX activity, the highest level of this antioxidant enzyme activity was observed for the plants inoculated with *R. solani* AG 4 HG-I. The maximum level of POX activity was observed at 12 hpi for all treatments tested, which followed by a decreasing trend until 72 hpi.

3.8. Transcription analysis of the antioxidant genes

Enzyme activity assays revealed the priming of SOD activity in tomato plants, whereas lower level of this antioxidant enzyme activity was observed for the plants inoculated with BNR compared to the other treatments at 12 hpi. Therefore, it was interesting to investigate the correlation between activity of the antioxidant enzymes and transcript levels of the corresponding genes. The data obtained via qRT-PCR revealed that maximum level of the *SOD* upregulation was observed at 12 hpi in tomato plants with the BNR inoculation, without significant difference with that of *R. solani* AG 3 treatment. Whereas lower upregulation of the SOD gene was observed for the plants inoculated with *R. solani* AG 4 HG-II, compared to the other treatments tested (Fig. 10A).

The highest levels of *POX* transcript accumulations were observed for all treatments at 12 hpi and decreased afterward, as observed for the *SOD* gene expression. Tomato plants treated with *R. solani* AG 4 HG-I showed higher upregulation of the POX gene at 12 and 48 hpi, compared to the other treatments tested. Whereas at 24 hpi, significant differences were not observed among the *POX* expression levels for various treatments. At 72 hpi, the BNR treatment showed higher level of the *POX* upregulation compared to the other treatments tested (Fig. 10B).

3.9. Activity of pectinase and cellulase enzymes secreted by the pathogens

Differences were observed in the activity of pectinase and cellulase, as the main cell wall degrading enzymes secreted by various taxonomic groups of *Rhizoctonia* (Fig. 12). Under laboratory conditions, maximum level of pectinase activity was observed for the isolate of *R. solani* AG 4 HG-II (6121/6 μ $g.ml^{-}$

^{1}) at 168 h after culturing the fungus in broth medium, and it declined afterward. Lowest level of pectinase activity level was observed for the binucleate isolate (Fig. 11A). Maximum activity of cellulase was observed for AG 4 HG-I (1254/9 μ g.ml^{-1}) at 192 h after culturing the fungus in broth medium. Lowest level of cellulase activity was detected for the binucleate isolate (Fig. 11B).

4. Discussion

Plants use advanced strategies to defend themselves against biotic and abiotic stresses. Such basic resistance protects plants when facing pathogen attacks and helps them to reduce destructive effects of diseases via application of various biochemical, cellular and molecular defense mechanisms (Nikraftar et al, 2011). This means that plant simply doesn't provide the suitable environment for pathogens to attack.

In this study, the highest level of disease progress was observed on tomato seedlings and leaf discs infected with *R. solani* AG 4 HG-II. Infection caused by the binucleate isolate was less than multinucleate *R. solani* isolates. Generally, binucleate isolates of *Rhizoctonia* are reported as hypovirulent pathogens on various host plants, which in some cases have potential capability of controlling highly virulent multinucleate isolates of *R. solani* (Keshavarz-Tohid and Taheri, 2015; Sharon, et al., 2011). Therefore, it is interesting to find out if the hypovirulent binucleate isolate of *Rhizoctonia* used in this study is capable of inducing defense responses and protecting tomato plants against the highly virulent *R. solani* isolates, which can be the subject of future researches in this pathosystem.

Performing microscopic investigations of infection process in tomato-*Rhizoctonia* spp. interaction revealed the existence of direct correlation between the number of infection structures formed by the fungal isolates and disease progress on the leaves. The highest number of lobate appressoria and infection cushions was produced by AG 4 HG-II isolate, which caused the highest level of disease progress on tomato seedlings and leaf discs. Whereas, the lowest number of these infection structures was produced by the hypovirulent isolate of binucleate *Rhizoctonia*. These findings are in agreement with a previous report comparing the infection structures formed by *R. solani* AG 3 on a partially resistant (Falat) compared to a susceptible tomato cultivar (Mobile) for determining the correlation of infection structures formed by the pathogen with disease progress (Nikraftar et al. 2013). Infection cushions produced by *R. solani* AG 3 in Mobil cultivar at 48 hpi were more than of those of Falat cultivar (Nikraftar et al. 2013). Production of this infection structures in turn lead to the emergence of disease symptoms at the junction spot on the plants. Pannecoucque and Höfte (2009) stated that there is a difference between invasion activity and production of infection structures by various taxonomic groups of *Rhizoctonia* on cauliflower, which is in accordance with the findings of present study in tomato-*Rhizoctonia* pathostystem. Formation of infection structures also depends on plant cultivar and the site of pathogen attack. Verma (1996) explains that infection cushions are formed on hypocotyl of both resistant and susceptible plants but they spread faster and grow deeper in susceptible host plants. Bassi et al (1978) investigated resistant and one susceptible tomato cultivars to *Rhizoctonia* and observed that the pathogen spreads in the resistant cultivar much slower than in the susceptible one. Thus, inability of

Rhizoctonia to form a lot of infection cushions in the partially resistant cultivar could be associated with lower level of disease development on it.

One of the most important types of ROS is H_2O_2, which is known to be involved in tomato resistance against biotrophic fungal pathogens such as *Cladosporium fulvum* (Borden and Higgins, 2002), *Oidium neolycopersici* (Mlikova et al, 2004), and hemibiotrophics such as *Colletotrichum coccodes* (Mellersh et al, 2002). In addition, several studies revealed the importance of H_2O_2 cumulated in tomato as a defense response against necrotrophic fungi such as *Botrytis cinerea* (Asselbergh, 2007), *Fusarium oxysporum* f.sp. *lycopersici* (Mandal et al, 2008)d *solani* (Nikraftar et al, 2013). The results of this study revealed that the highest amounts of not only H_2O_2, but also O_2^- were produced in the leaf tissues of tomato plants infected with *R. solani* AG 3, while the lowest levels of these molecules were produced in plant cells interaction with the hypovirulent isolate of binucleate *Rhizoctonia*. Therefore, it can be concluded that oxidative burst is one of host defense responses against aggressive isolates of *R. solani.* Accumulation of higher amount of ROS is correlated with lower levels of disease progress and higher resistance of the host plant in tomato-*R. solani* interaction.

Callose is a 1,3- β glucan polymer found in plants, which is produced in response to lesions, biotic or abiotic stresses (Usak et al. 2023; Li et al. 2023). In the present research, it was observed that inoculating tomato plants with highly virulent *R. solani* isolates (belonging to AG 4 HG-I and AG 4 HG-II) led to accumulation of lower levels of callose compared to the amount of callose accumulated in plants infected with the isolate of AG 3, with lower level of virulence. Therefore, virulence of *R. solani* isolates was inversely related to callose deposition in the host cells. In other words, higher level of tomato resistance to *R. solani* AG 3 compared to AG 4 isolates was correlated with higher amounts of callose deposition. These data suggest that callose deposition can be considered as a resistance marker in tomato-*R. solani* pathosystem. This finding is in agreement with the report of Gindro et al (2006) in grape-*Plasmopara viticola* interaction, which stated that the percentage of infected stoma representing callose deposition can be used as a marker to examine resistance of various grape varieties to downy mildew. Researchers found that callose is deposited with more delay in mesophyll of susceptible grape varieties compared to the resistant ones. By forming papillae, callose deposition prevents penetration of haustoria formed by phytopathogenic fungi into the epidermal cells of host plants (Heinz et al, 1990), therefore it can be a powerful physical barrier for penetration of phytopathogens into the plant cells. Romero et al. (2008) demonstrated that oxidative burst and strengthening of plant cell wall through accumulation of callose and lignin in melon varieties resistant to powdery mildew, caused by *Podosphaera fusca*, is more than those of susceptible varieties, which is in accordance with our findings.

Employment of X/XO leads to production of O_2^- and H_2O_2, resulting in increased disease progress by *R. solani*, similar to the findings of other researchers for necrotophic pathogens such as *Botrytis cinerea* (Govrin and Levin, 2000) and *Cochliobolus miyabeanus* (De Vleesschauwer, 2009). On the other hand, G/GO treatment increases H_2O_2 accumulation in plant tissues (Bennett et al, 2005; Mur et al, 2005) that can be associated with activation of defense mechanisms leading to decrease disease symptoms.

Analysis of defense genes induced by lesions in tomatoes revealed that H_2O_2 functions as a secondary messenger and plays an important role in induction of defense genes (Orozco-Cárdenas et al, 2001). The results of this study also indicated that increased amount of H_2O_2 by G/GO results in a significant reduction of disease severity. It is assumed that H_2O_2 produced over the interaction between plant and the necrotophic fungus R. *solani* functions as a secondary messenger that induces defense genes just like in lesions. According to the result of evaluating callose deposition in the leaf discs treated with G/GO and X/XO and inoculated with different taxonomic groups of the pathogen, callose accumulation in the G/GO treated leaves was higher compared to X/XO treated leaves. Therefore, it can be concluded that lower levels of disease progress in G/GO treated leaved is correlated with higher amount of callose deposition via this treatment.

Ascorbic acid (ascorbate) is a potent antioxidant, which is involved in removing H_2O_2 from living cells. The findings of this study revealed that accumulation of H_2O_2 is of great importance in basal resistance of tomato to different taxonomic groups of *Rhizoctonia*, as susceptibility of the host plant to these fungi increased via treating leaf discs with ascorbate. Asselbergh et al (2007) also reported that tomato leaf discs treated with ascorbate showed increased susceptibility to the necrotrophic fungus *B. cinerea.* Ascorbate treatment, on the other hand, leads to unwanted changes in the natural metabolism of plant cells. The reason is that ascorbate is used as an electron donor in reduction reactions of biological processes (Taheri 2022; Noctor, 2006).

Investigating activity of the antioxidant enzymes, such as the SOD and POX, which are involved in regulating ROS levels, revealed higher level of SOD activity for the plants with the hypovirulent BNR and *R. solani* AG-3 treatments. This observation was in agreement with the data obtained in transcription analyses of the corresponding gene. Considering the function of SOD, which converts O_2^- into H_2O_2 and increase H_2O_2 accumulation, like the G/GO treatment which produces H_2O_2 and leads to decreased disease progress, the data obtained in enzyme activity, gene expression and ROS manipulations assay are in accordance. As we observed that the *R. solani* AG 3 isolate, which caused lower levels of the disease symptoms compared to other isolates of *R. solani* tested, showed the highest SOD activity and higher amounts of H_2O_2 accumulation at the early time point investigated (12 hpi). H_2O_2 is known as a second messenger involved in plant defense against *R. solani* (Kheyri et al. 2022, Nikraftar et al. 2014) and also may be involved in production of callose and lignin as the main physical barriers which prevent progress of the fungal pathogen in the plant tissues (Taheri 2022).

The POX activity and upregulation of the corresponding gene, which was higher in the early time point 12 hpi in the plants inoculated with *R. solani* AG 4 HG-I and AG 4 HG-II, might be related to the role of this antioxidant in degrading H_2O_2 as a defense related signaling molecule, which lead to higher level of the disease progress on the plants inoculated with these taxonomic groups of *R. solani*. These findings are in accordance with the results obtained by Kheyri et al. (2022) about detecting lower levels of H_2O_2 in the plants without the protectant treatment that had higher disease progress, compared to the plants with resistance inducer treatments which showed higher H_2O_2 accumulation at some early time points.

Plant cell wall is composed of various polysaccharides and several proteins. In all cases, polysaccharides (such as cellulose) constitute a major part of plant cell walls and pectin can be found in middle lamella. The cell polysaccharides are classified into cellulose, hemicellulose and pectin and these three can be found in almost all plant cell walls in different proportions (Harholt et al, 2010; Harholt et al, 2006). To destroy the cell wall, exocellular proteins such as cellulolytic, hemicellulolytic, pectolytic and proteolytic enzymes are produced by phytopathogens, which are able to attack cell wall components (Viler, 1975). Therefore, pathogens can produce and secret a wide range of enzymes capable of degrading plant cell wall ingredients, which are necessary for successful penetration into the host cells (Jayasinghe et al. 2004; Khaledi et al. 2015). Findings of this study clarified presence of a direct correlation between pathogenicity of *Rhizoctonia* isolates and activity of cellulase and pectinase enzymes produced by them. However, it seems necessary to make further investigations on other taxonomic groups of this fungus and use several isolates of each taxonomic group to survey the relationship between activity of cell wall degrading enzymes secreted by pathogens and their pathogenicity on the host plant.

In accordance with our data, Pannecoucque and Höfte (2009) demonstrated that during the pathogenic interactions between *Rhizoctonia* isolates and cauliflower, pectin degrading enzymes are important and diffused ahead of the fungus and pathogen ingress is coupled with host cell deformation and pectin lysis at locations not in direct contact with fungal hyphae. For several phytopathogenic fungi, including *Rhizoctonia*, the role of cellulase and pectin degrading enzymes in penetrating plant tissues, virulence and aggressiveness of the pathogen is demonstrated (Jayasinghe et al. 2004; Xue et al. 2018). Bateman (1964) reported that *R. solani* produces cellulase which may assist penetration of the fungus into host cells. Pectinase activity was regarded to be the most important predictor for virulence of *R. solani* (Weinhold and Bowman 1974; Mandal et al. 2013). Degradation of pectin in middle lamella and cellulose contents of the cell wall plays a considerable arole in development of pathogen in plant tissue and providing nutriments for it. Therefore, cellulase and pectinase are potentially important for aggressiveness and virulence of various phytopathogens. Investigating their activity together with the activity of other CWDEs for several isolates from each taxonomic group may be used as helpful biochemical markers for estimating pathogenicity of numerous fungal isolates, without performing time-consuming greenhouse trials.

Declarations

Acknowledgment

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The authors declare that they have no conflict of interest.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Parissa Taheri, Fatemeh Hosseini-Zahani, and Saeed Tarighi

The manuscript was written by Parissa Taheri and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

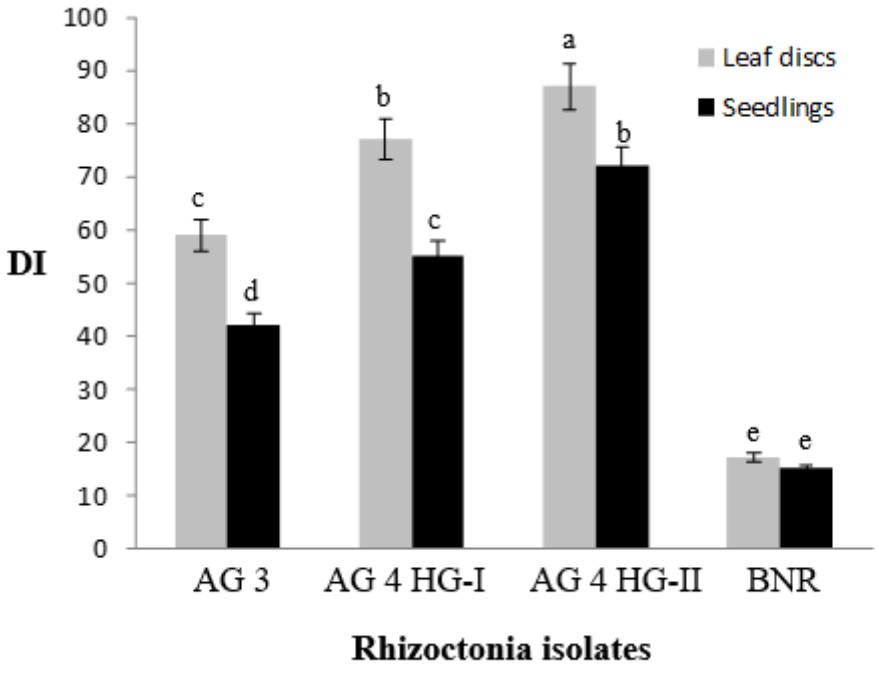

Figure 1

Disease index (DI) caused by various isolates of *Rhizoctonia* on tomato leaf discs and seedlings at 5 days after inoculation. Lines on the columns represent standards error (±SE). Treatments depicted with different letters are significantly different from each other at p≤ 0.05. BNR: Binucleate *Rhizoctonia* sp. AG-Bb.

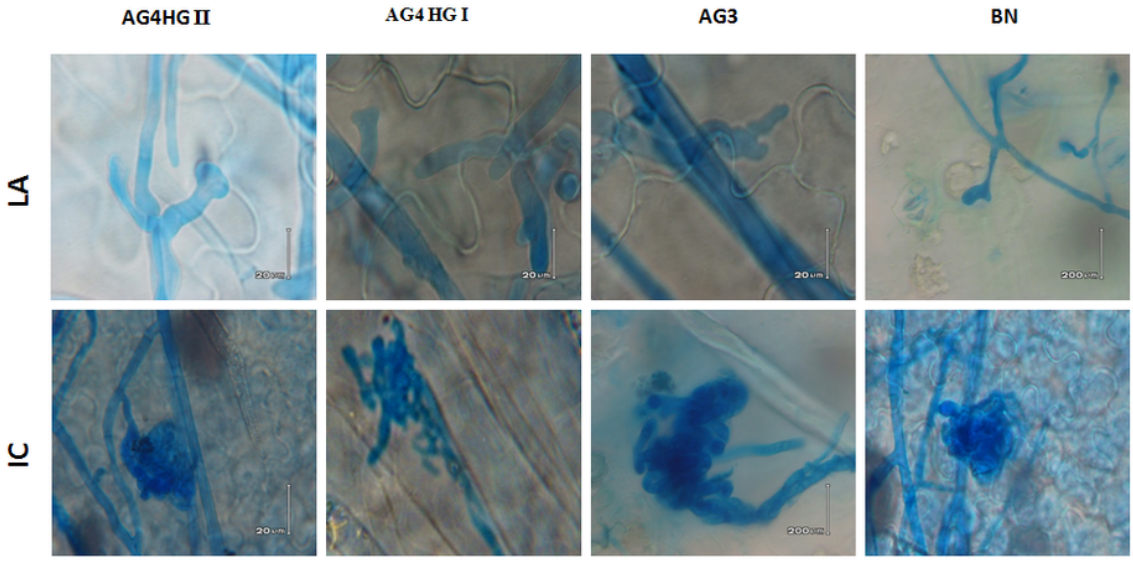

Figure 2

Infection structures of *Rhizoctonia*isolates on the leaves of tomato cultivar Mobil at 48 h after inoculation.Scale bars =40µm. LA: Lobate appresorium; IC: Infection cushion. BNR: Binucleate *Rhizoctonia*sp. AG-Bb.

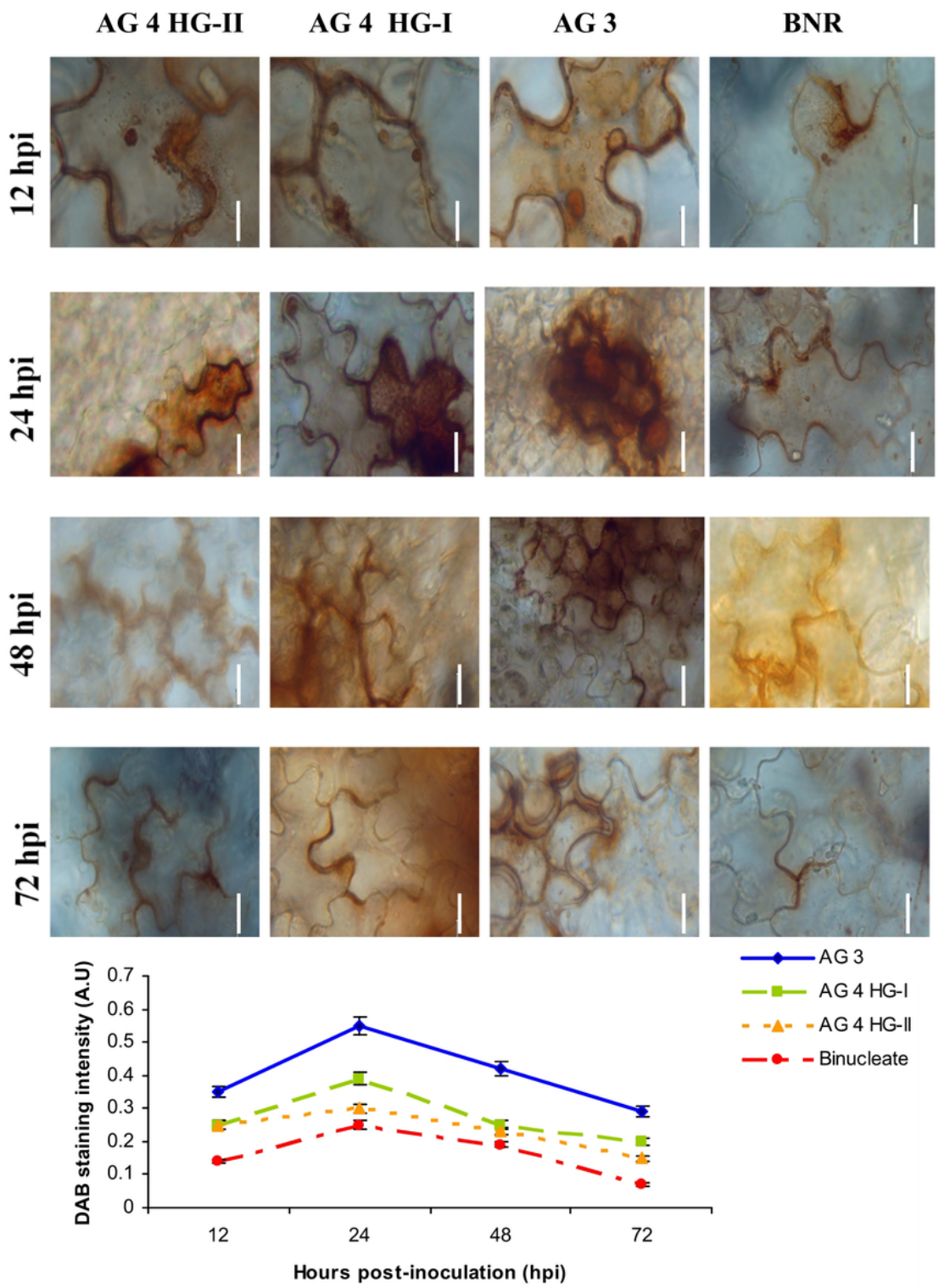

Figure 3

Histochemical detection of hydrogen peroxide in tomato leaves at various time points after inoculation with *Rhizoctonia* isolates. hpi: hours post-inoculation, Scale bars= 40 µm. BNR: Binucleate *Rhizoctonia* sp. AG-Bb.

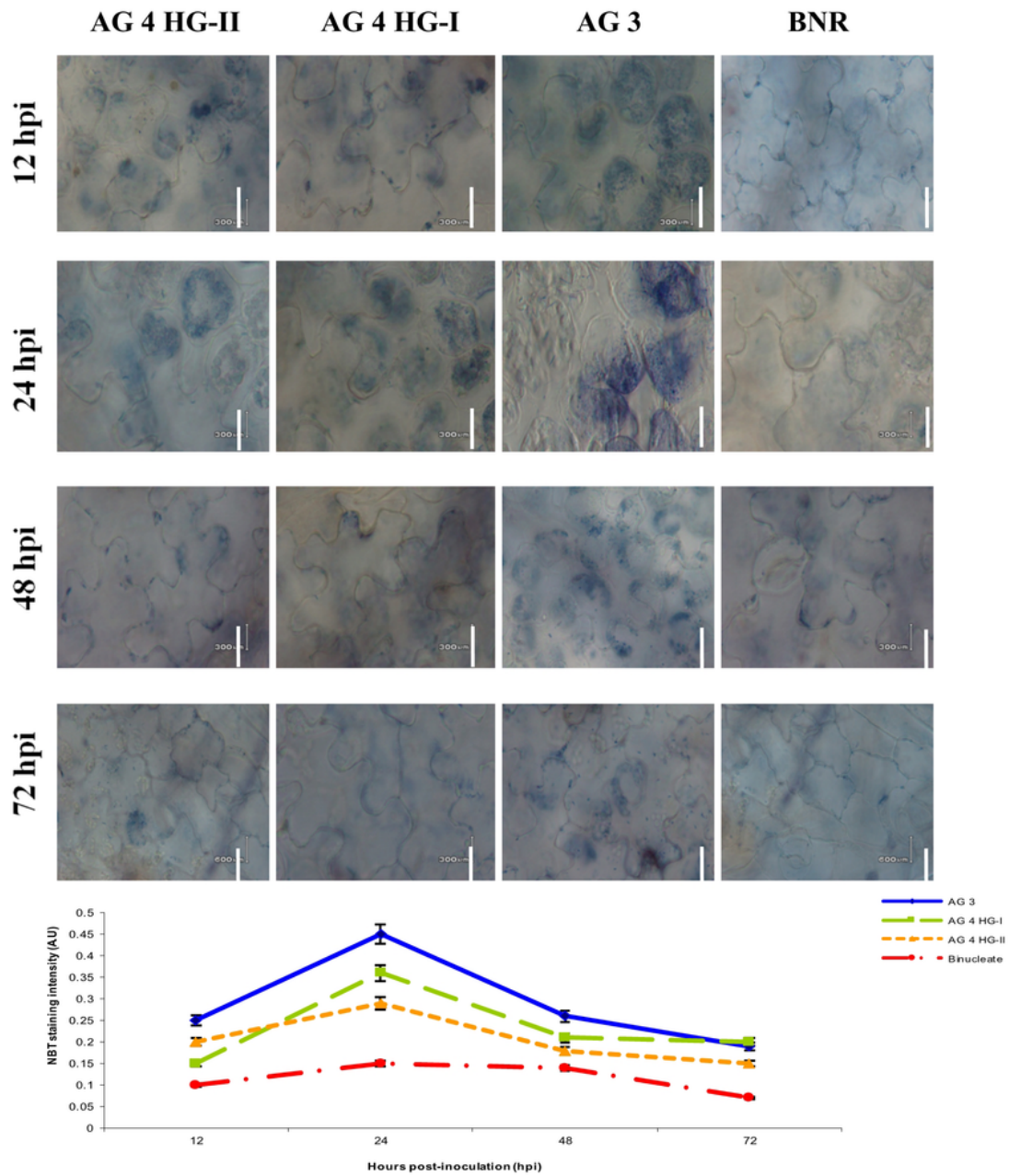

Figure 4

Histochemical detection of superoxide in tomato leaves at various time points after inoculation with *Rhizoctonia* isolates. hpi: hours post-inoculation, Scale bars= 40 µm. BNR: Binucleate *Rhizoctonia* sp. AG-Bb.

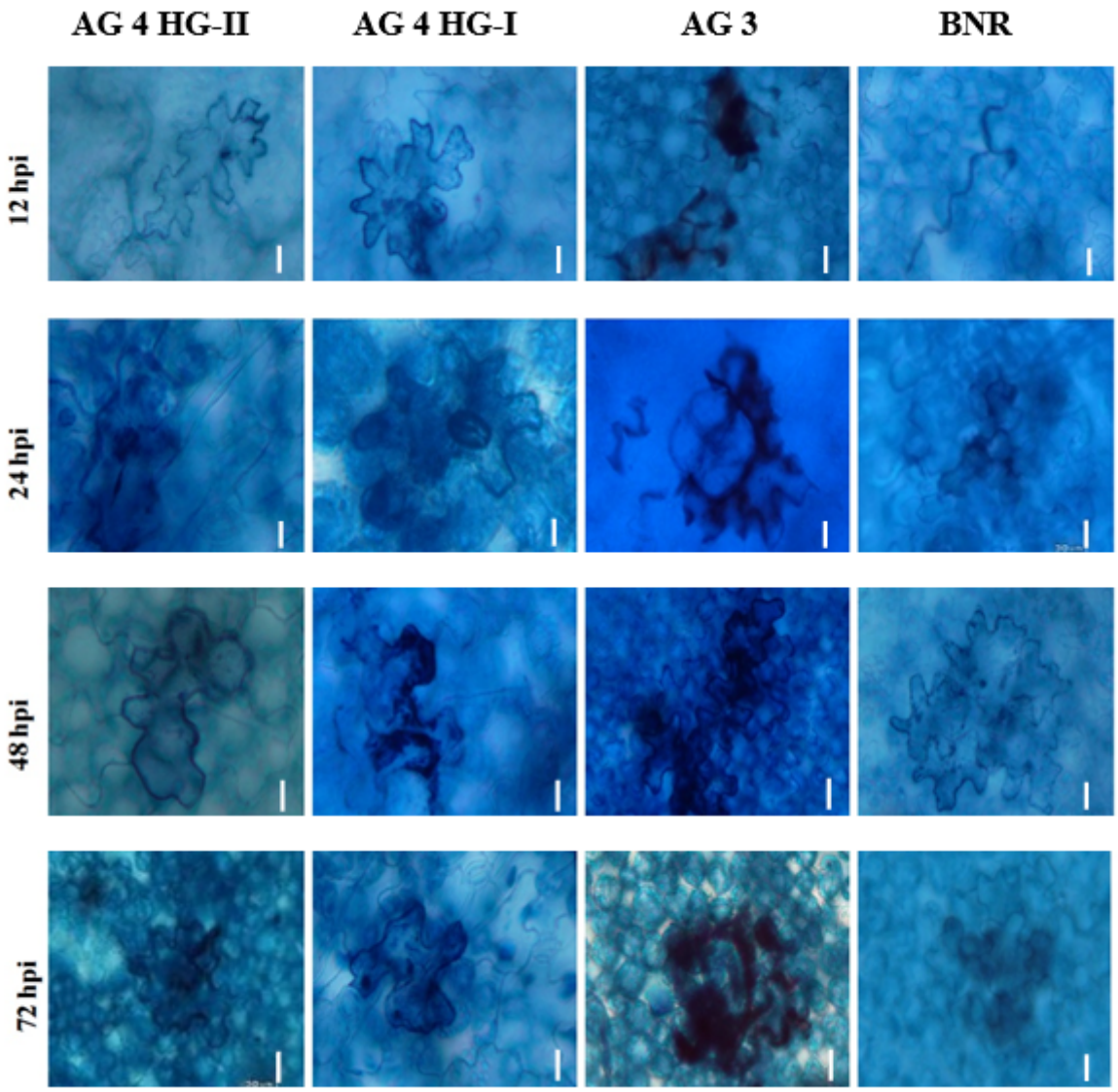

Figure 5

Callose deposition in tomato leaves at various time points after inoculation with *Rhizoctonia* isolates. hpi: hours post-inoculation, Scale bars= 40 µm. BNR: Binucleate *Rhizoctonia*sp. AG-Bb.

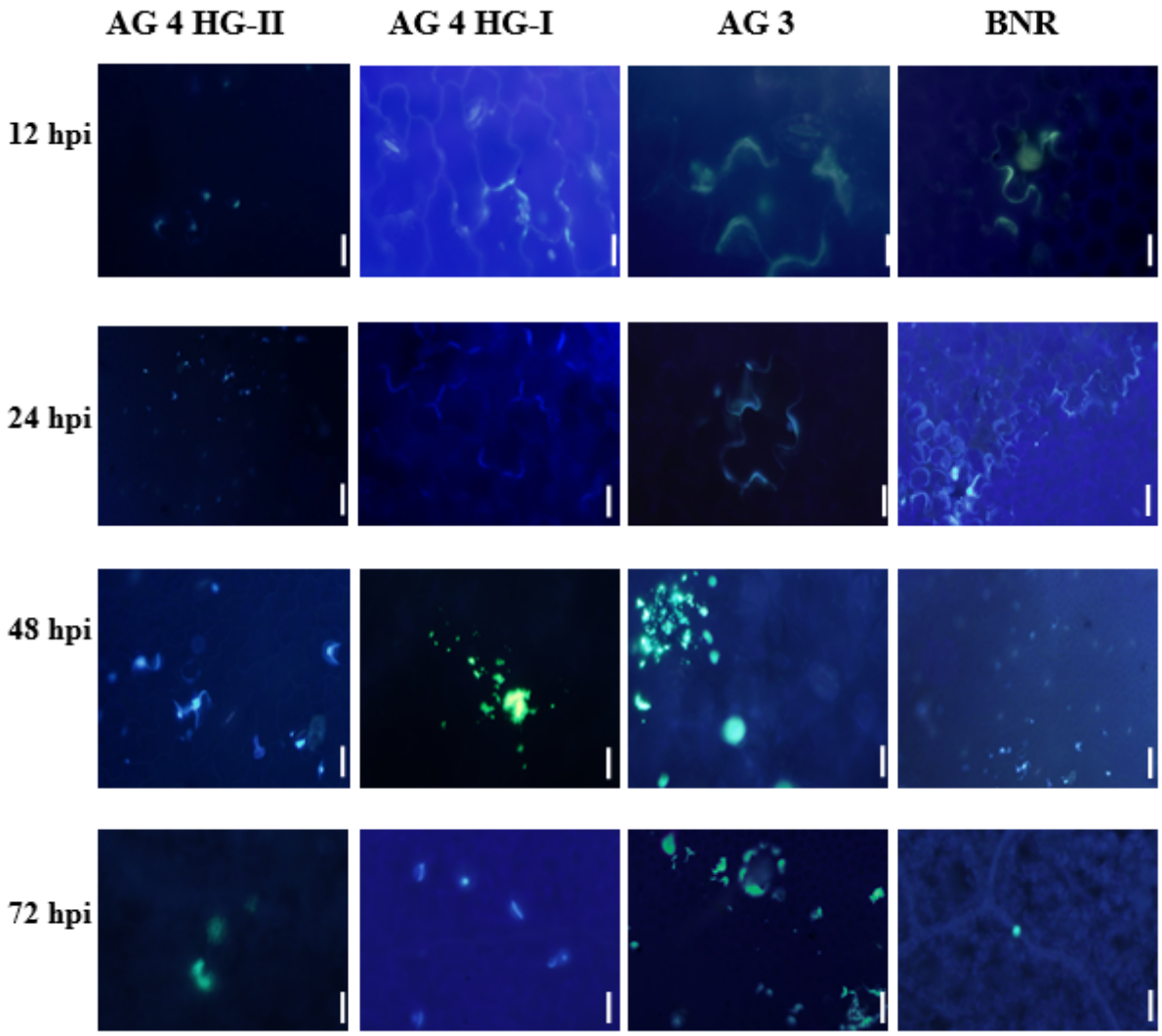

Figure 6

Fluorescent images of callose deposition in tomato leaves at various time points after inoculation with *Rhizoctonia* isolates. hpi: hours post-inoculation, Scale bars= 40 µm. BNR: Binucleate *Rhizoctonia* sp. AG-Bb.

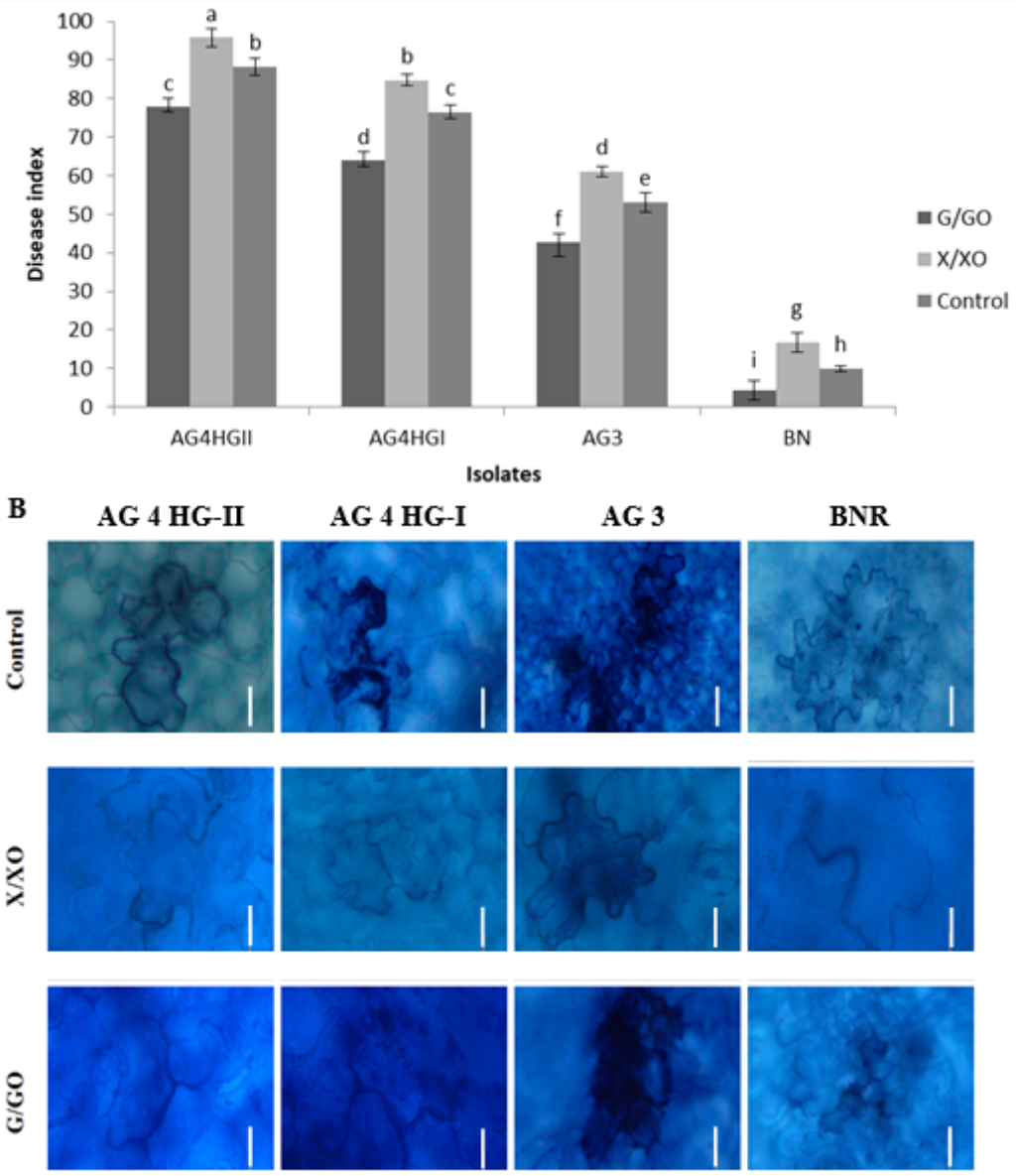

Figure 7

Effect of treating tomato leaf discs with G/GO and X/XO on progress of the disease caused by *Rhizoctonia* isolates at 5 days after inoculation (A) and on callose deposition (B). Lines on the bars represent standards error (±SE). Different letters on the bars are indicating significant differences. Scale bars =40µm. BNR: Binucleate *Rhizoctonia* sp. AG-Bb.

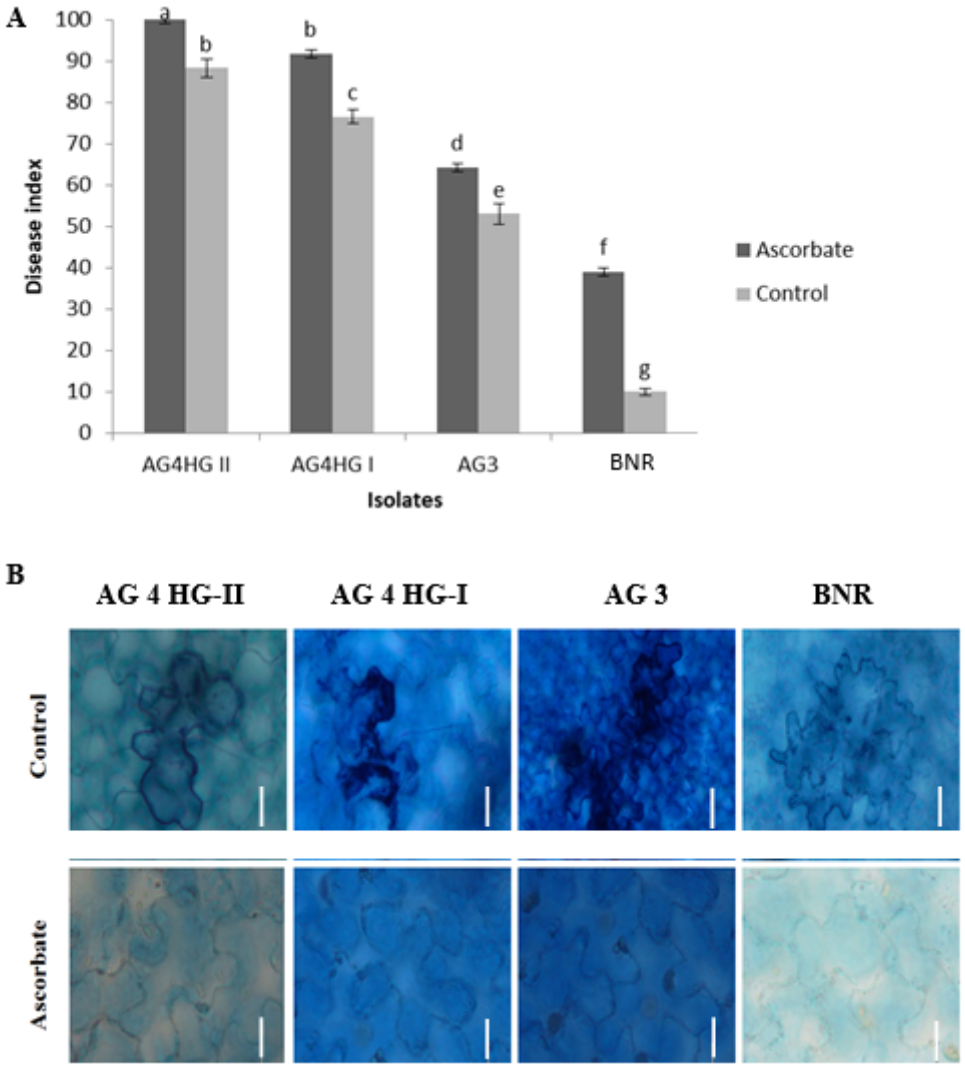

Figure 8

Effect of treating tomato leaf discs with ascorbate on progress of the disease caused by *Rhizoctonia* isolates at 5 days after inoculation (A) and on callose deposition (B). Lines on the bars represent standards error (±SE). Different letters on the bars are indicating significant differences. Scale bars =40µm. BNR: Binucleate *Rhizoctonia*sp. AG-Bb.

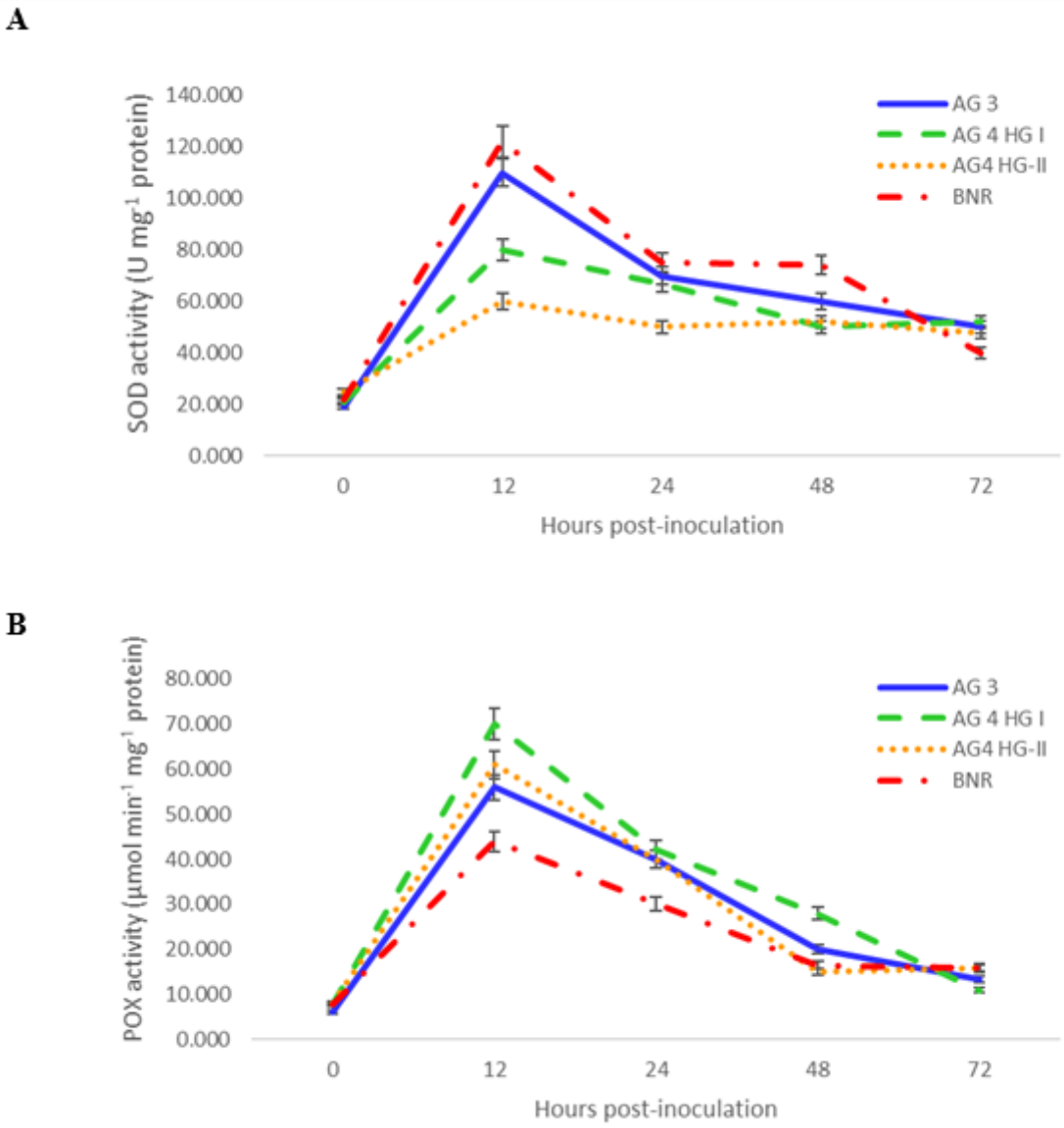

Figure 9

Superoxide dismutase (SOD; A) and peroxidase (POX; B) activity in tomato plants at various time points after inoculation with *Rhizoctonia* isolates. Data are means (± standard error) of three replicates of a representative experiment. Each replicate consisted of one sample pooled from six individual plants. The experiment was repeated three times with similar results. BNR: Binucleate*Rhizoctonia* sp. AG-Bb.

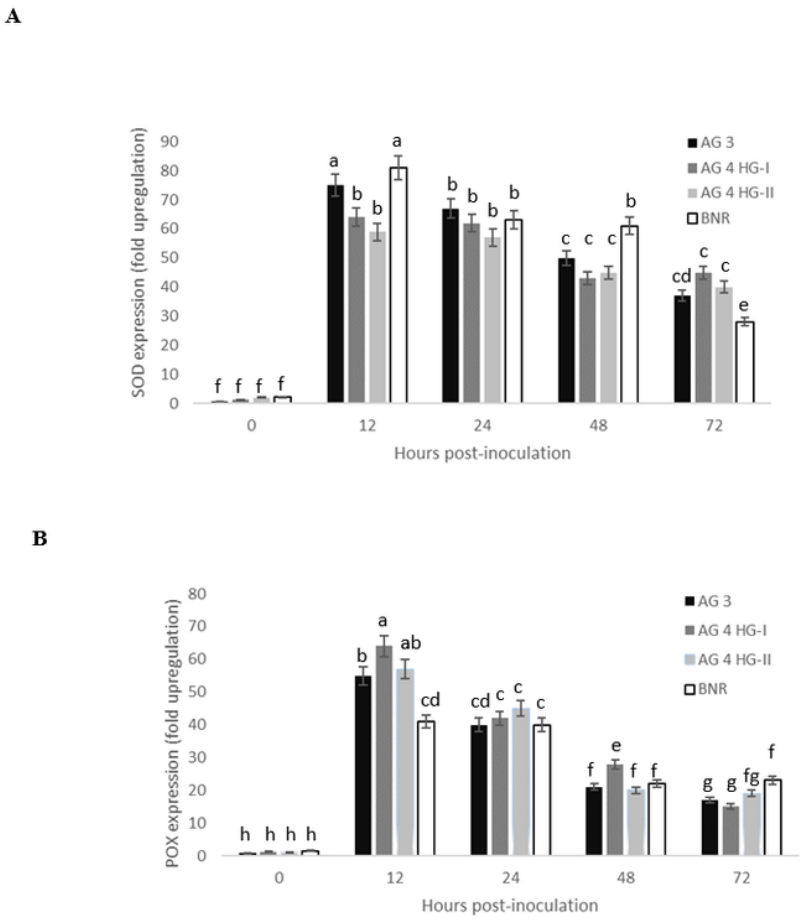

Figure 10

Gene expression analyses the *SOD* (A) and *POX* (B) genes at various time points after inoculation of tomato plants with *Rhizoctonia* spp, isolates. Expression levels of the gene transcripts were determined using qRT-PCR and expressed as fold upregulation compared to the transcript levels of the *Actin*gene, as an internal control. Values are the means ± standard error (SE) of three replicates of an experiment with three independent repetitions. Means with different letters at each time point statistically significant difference according to the Kruskall-Wallis tests completed by Mann-Whitney analysis at P≤ 0.05 in the SPSS software. BNR: Binucleate *Rhizoctonia* sp. AG-Bb.

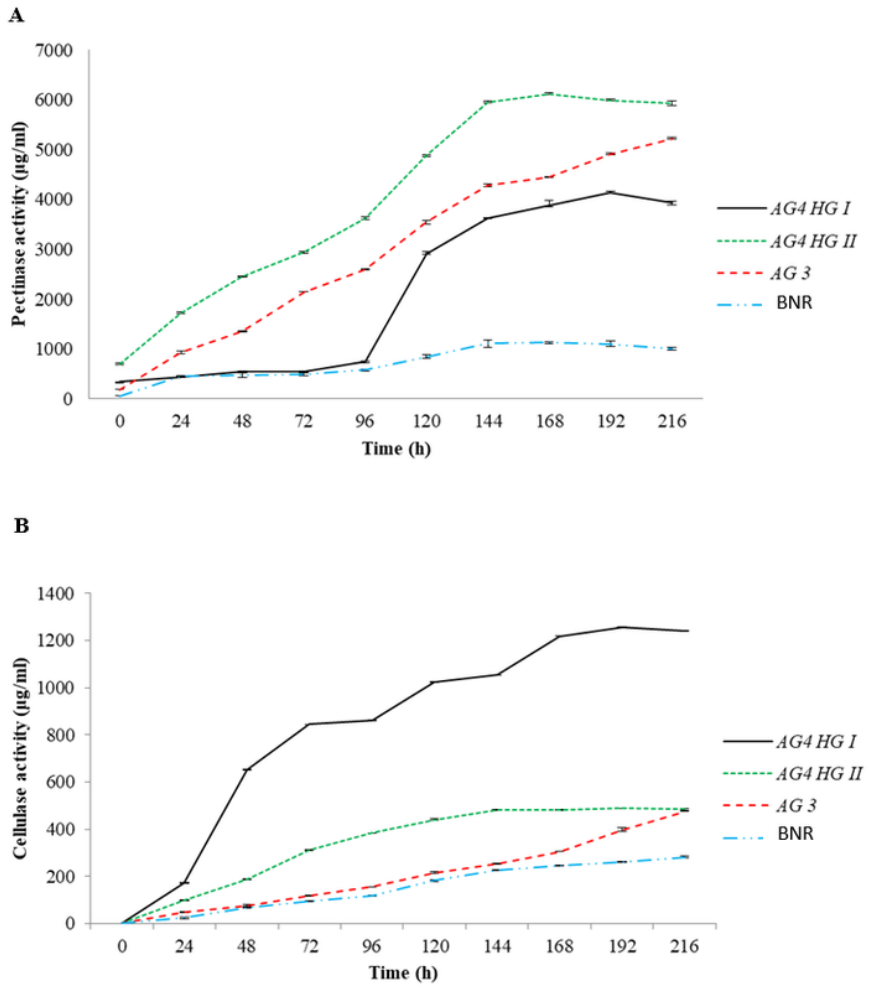

Figure 11

Activity of pectinase (A) and cellulose (B) secreted by different isolates of *Rhizoctonia* at various time points after culturing them in broth media. BNR: Binucleate*Rhizoctonia* sp. AG-Bb.