

## PLANT EXTRACTS INDUCING SYSTEMIC RESISTANCE IN *SOLANUM LYCOPERSICUM* (TOMATO) SEEDLINGS AGAINST *RHIZOCTONIA SOLANI*: MODULATING ANTIOXIDANT ENZYMES AND PR-PROTEINS EXPRESSION

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### Abstract

Intensive and indiscriminate use of fungicides is responsible for environmental pollution effecting the living organisms. Natural plant extracts emerge to be potential candidates having competitive abilities to overcome the plant pathogens without effecting human health and environment. *Rhizoctonia solani*, a root rot causing agent is responsible to cause huge economic losses with limited controlling agents available. The effect/potential of two plant extracts neem (*Azadirachta indica*) and eucalyptus (*Eucalyptus globulus*) was studied against *R. solani* on tomato seedlings. The altered biochemical activities and stress induced markers were monitored in diseased and treated conditions for tomato seedlings at different growth intervals. Results revealed that stress induced markers such as accumulation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Malondialdehyde (MDA) indicated significant increase as compared to the control. Antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) enzymes were also significantly increased in diseased plants treated with plant extracts. Moreover, during protein expression analysis, presence of low molecular proteins PR-2 (33-37kDa) were evident in diseased plants treated with neem and eucalyptus extracts as compared to control. Thus, it is concluded that application of plant extracts triggered systemic resistance by the expression of pathogenesis related proteins (PR) and alteration of antioxidant enzyme activities in tomato seedlings.

**Key words:** Antioxidant enzymes, Pathogenesis related proteins, Plant extracts, *Rhizoctonia solani* and Systemic resistance.

### Introduction

*Rhizoctonia solani* is a well-known pathogenic fungus causing severe damage to young seedlings by morphological and biochemical alterations (Long *et al.*, 2019). So far, several disease management options have been employed such as moderately resistant varieties, cultural control, biological control and synthetic fungicides. Among these, excessive use of fungicides may lead to resistant fungal strains along with adverse effects on environment that calls an alternative input for sustainable agriculture. Thus, to overcome this dilemma, alternative strategies using plant extracts are being investigated since last decade (Choudhury *et al.*, 2018). These botanicals contain wide range of bioactive compounds, well known for antimicrobial activity and activate natural defense mechanism. Additionally, these extracts also contain different substances such as plant hormones, nutrients, antioxidants and osmoprotectants that efficiently strengthen the defense systems (antioxidant enzymes) of plants against environmental stresses and play a vital role in growth promotion (Desoky *et al.*, 2019). These extracts may ultimately hinder disease development by activating defense responses regarded as induction of systemic Resistance (Rashad *et al.*, 2018) possess defensive enzymes and trigger Pathogenesis related proteins (PR proteins) (Prasannath, 2017).

Plants counteract pathogen attack by inducible responses (Malo *et al.*, 2017) like generation of active oxygen species (ROS) such as superoxide ion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and causing oxidative damage by lipid peroxidation, membrane damage, inactivation of proteins and genetic (DNA) mutations which can be controlled by the stable expression of antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD)

and catalase (CAT) (Das & Roychoudhury, 2014). Mostly, the innate immunity of plants fail to maintain healthy growth of plants, therefore, exogenous support such as plant extracts is required to increase plant tolerance to the stress (Desoky *et al.*, 2018; Ur Rehman *et al.*, 2018).

*Azadirachta melia* (Neem) leaves have higher total phenolic content (Naseer *et al.*, 2014) while *Eucalyptus globulus* (eucalyptus) has monoterpene citronellal. Their extracts are responsible to treat various fungal diseases of plants through activating defense mechanisms (Goel *et al.*, 2016; Shafique *et al.*, 2019) and stimulate immunity of plant. The implications of plant extracts are considered safe compared to synthetic chemicals and are effective against different plant diseases. Consequently, the present work was planned to explore the systemic acquired resistance in response to the aqueous extract of neem and eucalyptus against the attack of *Rhizoctonia solani* by activating defense system.

### Materials and Methods

**Isolation and inoculum preparation:** Tomato (*Solanum lycopersicum*) seedlings having root rot symptoms were collected. After washing, small root pieces (1 cm) were cut from diseased plants and surface sterilized with 2% sodium hypochlorite solution for 2 minutes followed by washing with autoclaved water. The roots were transferred to potato dextrose agar (PDA) at 25-30°C for a week to observe the growth of *Rhizoctonia solani*. 95 g garden soil with 5 g flour was mixed and autoclaved after the addition of 10 ml of water for preparation of soil inoculum. After that, fungal disc was introduced aseptically with incubation period of about 15 days. Flasks were agitated daily to avoid mycelial compaction.

**Preparation of leaves extracts:** For the preparation of plant extracts 50 g of powder of neem and eucalyptus was added in 100 ml distilled water, kept overnight and then filtered through muslin cloth. The extracts were poured in bottles and heated at 100°C for 20 minutes to avoid contamination.

**Green house experiment:** The 20 days old healthy seedling of tomato varieties were divided into six groups of each variety. Three groups of each variety were transferred to pots containing natural-pathogen-free soil where the first group was grown in untreated soil that served as control; the second group was grown in soil treated with neem extract, whereas, the third group was grown in soil treated with eucalyptus extract. The remaining three groups of each variety were grown in a comparable set of treatments but with soil artificially infested with 5% inoculum of *R. solani* w/w. The plants were uprooted at three different intervals (5, 15 and 30 days) after the application of plant extracts to determine the percentage colonization of roots by *R. solani*. The roots were washed to remove soil, cut into 1 cm long pieces and placed on PDA plates after surface sterilization with 2% sodium hypochlorite solution for 2 minutes. Root colonization was recorded after incubation for 5 days by using the following formula:

$$\text{Colonization (\%)} = \frac{\text{Number of root pieces colonized by the fungus} \times 100}{\text{Total number of root pieces}}$$

**Hydrogen peroxide determination:** Hydrogen peroxide was measured according to the method of Jessup (Jessup *et al.*, 1994). Fresh leaf tissues (500mg) were extracted in 5 ml of tri-chloro acetic acid (0.1%) and supernatant was separated after centrifugation at 12,000 rpm for 15 minutes. Reaction mixture was prepared by adding 500 µl of plant supernatant, 500 µl of K- phosphate buffer (pH 7.0) and 1 ml of potassium iodide. The absorbance was measured at 390 nm.

**Malondialdehyde (MDA) determination:** The malondialdehyde (MDA) content was estimated by the method of (Heath & Packer, 1968). For this, 1 g fresh leaves was mixed in 2 ml of trichloroacetic acid (5%) and supernatant was collected after centrifugation at 12,000 rpm for 15 minutes. 0.5% TBA and 500 µl of the supernatant was mixed and boiled for 30 minutes at 95°C then placed at room temperature for cooling. The mixture was again centrifuged at 7500 rpm for 5 minutes at 4°C. Absorbance of supernatant was taken at 532 nm and 600 nm by spectrophotometer.

**Proline assay:** Proline was estimated by the method of Bates (Bates *et al.*, 1973). For this, 500 mg tissue (leaf) were extracted using sulfosalicylic acid and then centrifuged at 12000 rpm for 15 minutes. Reaction mixture containing 1 ml of supernatant, 1 ml of acid ninhydrin solution and glacial acetic acid was boiled for 1 hour. Reaction was stopped and toluene (4 ml) was added

and vortexed; the resulting mixture was placed for 30 minutes at room temperature. Layer formation appeared in the solution from which absorbance was taken at 520 nm. For blank only toluene was used.

**Antioxidant enzymes assays:** Fresh leaves of tomatoes were used to extract antioxidant enzymes (Elavarthi *et al.*, 2010). 0.1 g of leaf samples were homogenized in 1.2 ml potassium phosphate buffer (0.2M, pH-7.8). The homogenized mixture was centrifuged at 12000 rpm at 4°C for 15 minutes. Supernatant was used for enzyme quantification. For superoxide oxide dismutase quantification, assay mixture was prepared by adding 3 ml of K.P buffer (EDTA, l-methionine, 50 µM NBT and Triton-X100 (0.025%)), riboflavin (20 µl) and plant supernatant (20 µl). The reaction started when the tubes were exposed by fluorescent tube light for 15 minutes then absorbance was measured at 560 nm (Beauchamp & Fridovich, 1971). For catalase quantification, the reaction mixture consisted of K.P buffer (50Mm, pH-7.8), 15mM H<sub>2</sub>O<sub>2</sub> and 100 ul of leaf extract. OD was observed at 240 nm after every 30 second (Aebi, 1984). For peroxidase quantification, the reaction mixture was prepared by adding Guaiacol 2.7 mM, K.P buffer (50 mM, pH-7.0) and Hydrogen peroxide (2 mM). 100 µl of enzyme extract was mixed with reaction mixture and OD was taken at 470 nm (Polle *et al.*, 1994).

**Qualitative detection of Pathogenesis related proteins (PR-2) through western blotting:** Total soluble protein is extracted using Phosphate Buffer Saline (PBS). For this 100 mg tissues are homogenized in 1ml PBS and then centrifuged at 12000 rpm for 20 minutes at 4°C. Concentration of total soluble protein was determined through Bradford assay (Bradford, 1976). About 30-40 µg protein samples from each treatment at seedling stage was electrophoresed on 12% gel at 100 V along with broad range protein ladder (16-270kDa) (Laemmli, 1970). The proteins separated by SDS-PAGE were electrophoretically transferred to PVDF membrane (0.45 µm) by using (Towbin *et al.*, 1979) method. The membrane was probed with primary antibody of PR-2 protein for 2-4 h with concentration of 1:1000 followed by rabbit polyclonal antibody (2°Ab) conjugated with alkaline phosphatase (AP). The blot was developed with alkaline phosphatase buffer containing BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium) till the bands became visible. The blot then rinsed with distilled water, dried and scanned.

### Statistical analysis

The above experiment was performed three times. The statistical analyses of all treatments in the experiment were made in the groups and then results were evaluated. Two way (ANOVA) variance analysis was applied for the evaluation of the data. The difference between the applications was determined using the Least Significant Design (LSD) at the  $p < 0.05$  probability level.

## Results

In tomato seedlings the induced resistance through aqueous extract of neem and eucalyptus were analyzed in root rot diseased seedlings of tomato at three different intervals.

**Root colonization percentage:** No root colonization was observed in plants grown in soil not infested with *R. solani* inoculum. Neem extract proved more effective than eucalyptus extract by exhibiting low percentage of colonization in both the varieties. The root colonization percentage reduced from 33% in tomato plant growing in untreated infested soil to 20% in neem and eucalyptus extract treated soil after 15 days in variety Rio grande. While in variety Jumbo, it declined from 80% to 40% and 20%, respectively (Fig. 1). After 30 days of application of neem extract, the reduction in root colonization percentage was from 46.6% in untreated plants to 26.6% in variety Rio grande and from 60% to 26.6% in Jumbo while eucalyptus extract reduced the colonization to 33.3% in both the varieties.

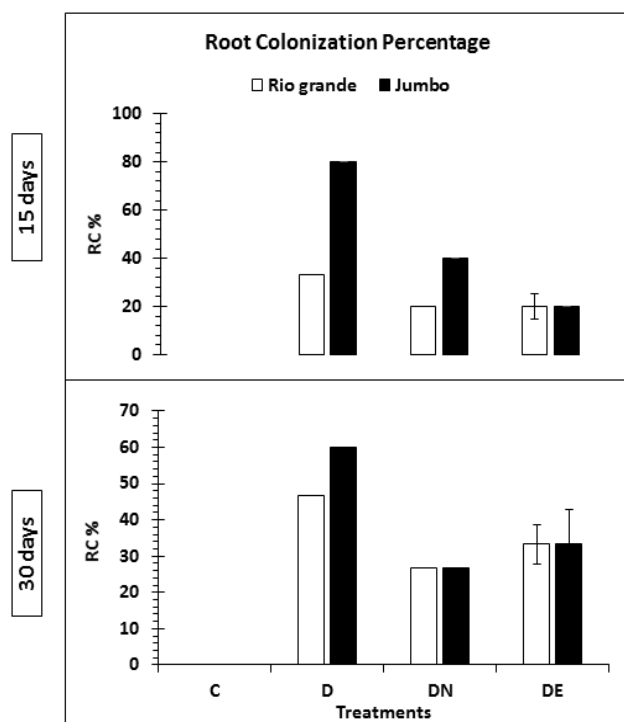


Fig. 1. Root colonization percentage of two tomato varieties (Rio grande and Jumbo) at seedling stage after 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE). The figure showed data from a representative experiment that was repeated three times.

**Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>):** Statistical analysis revealed that hydrogen peroxide content was not significantly different ( $p>0.05$ ) after 5- and 15-days intervals among the varieties while after 30 days, varieties showed significant difference ( $p<0.05$ ) in hydrogen peroxide levels. At the three different intervals, treatments (T) and their interactions with varieties ( $V \times T$ ) showed significant

differences ( $p<0.05$ ). After 5- and 15-days intervals, highest hydrogen peroxide content was found in Rio grande as compared to jumbo in diseased plants (Fig. 2a). While H<sub>2</sub>O<sub>2</sub> content was remarkably decreased in Rio grande and Jumbo only after neem extract application.

**Malendialdehyde (MDA):** Statistical analysis revealed that after 5, 15 and 30 days of plant extract application, varieties (V), treatments (T) and their interaction ( $V \times T$ ) showed significant differences ( $p<0.05$ ) for this parameter. The concentration of MDA was significantly higher in diseased plants of both the varieties at 5, 15- and 30-days intervals (Fig. 2b). At 5 days interval, MDA content was slightly increased in Rio grande while remarkably decreased in Jumbo in *Azadirachta indica* (neem) treated seedlings. Eucalyptus application declined MDA content in both the varieties. At 15- and 30-days interval, lipid peroxidation declined in both varieties where neem extract was applied.

**Proline:** Statistical analysis of proline content revealed that varieties (V), treatments (T) and their interaction ( $V \times T$ ) showed significant differences ( $p<0.05$ ) at 5, 15- and 30-days intervals after plant extract application. In diseased seedlings, proline content was decreased in both the varieties at all the intervals (Fig. 3a). The accumulation of proline increased remarkably after application of both plant extracts at 5, 15- and 30-days interval in both the varieties.

### Antioxidant enzyme analysis

**Superoxide dismutase:** Regarding superoxide dismutase enzyme showed significant difference ( $p<0.05$ ) among varieties (V), treatments (T) and their interaction ( $V \times T$ ) at three different intervals. Under diseased conditions, both varieties exhibited minimum activity (Fig. 4a). The activity of SOD was rapidly increased in Rio grande after application of plant extract after 5 and 30 days and in Jumbo after 15 days. Whereas Jumbo exhibited maximum SOD activity.

**Catalase:** Statistical analysis of catalase enzyme revealed that varieties (V), treatments (T) and their interaction ( $V \times T$ ) showed significant differences ( $p<0.05$ ) after 5, 15 and 30 days of plant extract application. At 5- and 15-days intervals, in diseased condition higher catalase activity was found in Rio grande as compared to Jumbo but opposite results were found after 30 days (Fig. 4b). At 30-days interval, expression of CAT was evident only in variety Jumbo in both the extracts treatments.

**Peroxidase:** Statistical analysis revealed that varieties (V), treatments (T) and their interaction ( $V \times T$ ) showed significant differences ( $p<0.05$ ) at the three different intervals. Under diseased conditions, at both 5- and 30-days intervals, both neem and eucalyptus extracts application showed slightly increased peroxidase activity in both the varieties where higher peroxidase activity was found in Rio grande compared to Jumbo (Fig. 5).

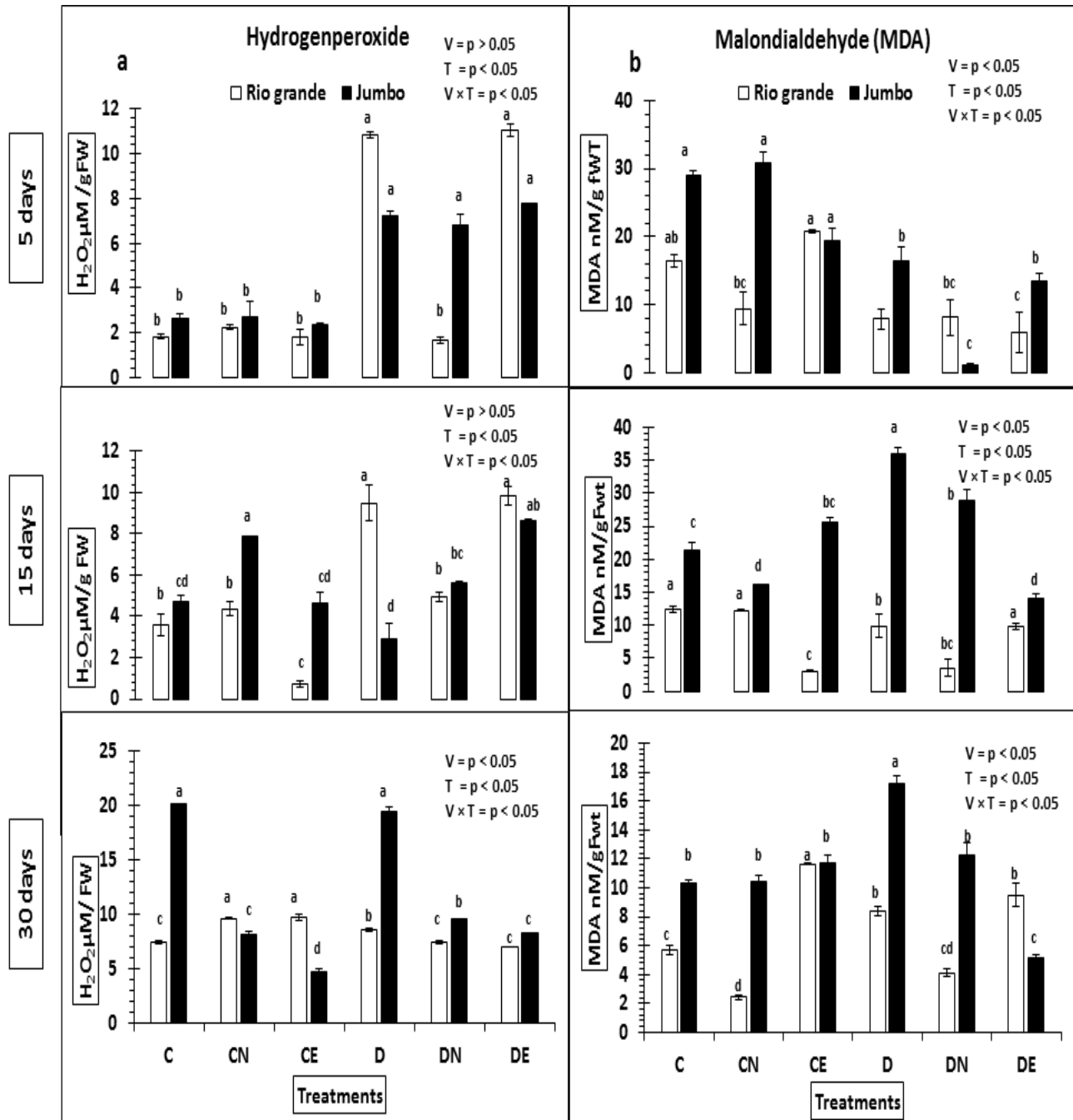


Fig. 2. (a) Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (b) Malendialdehyde (MDA) content of two tomato varieties (Rio grande and Jumbo) at seedling stage after 5, 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE). The figure showed data from a representative experiment that was repeated three time and were significantly different at  $p \leq 0.05$  according to LSD.

**Qualitative detection of Pathogenesis related proteins (PR-2) through western blotting:** Statistical analysis of total soluble protein showed that after 5, 15 and 30 days of plant extract application, varieties (V), treatments (T) and their interaction (V × T) exhibited significant differences ( $p < 0.05$ ). At all the three intervals, maximum amount of protein was observed in Jumbo in both control and diseased conditions compared to Rio Grande (Fig. 3b). Total protein concentration gradually increased in both the varieties after neem and eucalyptus extracts application in control as well as in diseased stress plants. Protein profiling of the control, diseased and plant extract

treated groups showed six visible different molecular weight protein bands 16kDa, 30kDa 33kDa, 37kDa, 52kDa and 66kDa. Under disease treatments, two types of molecular weight protein bands (33 and 37kDa) were evident (Figs. 6 and 7). In both the varieties, polypeptides with the molecular weight of 33kDa appeared in diseased seedlings. However, after plant extract treatment, decrease in expression of 33kDa protein but increase in the expression of 35kDa 37kDa was observed (Fig. 8). Both the varieties expressed the pathogenesis induced protein (PR-2) with higher intensity in neem compared to eucalyptus extract application.

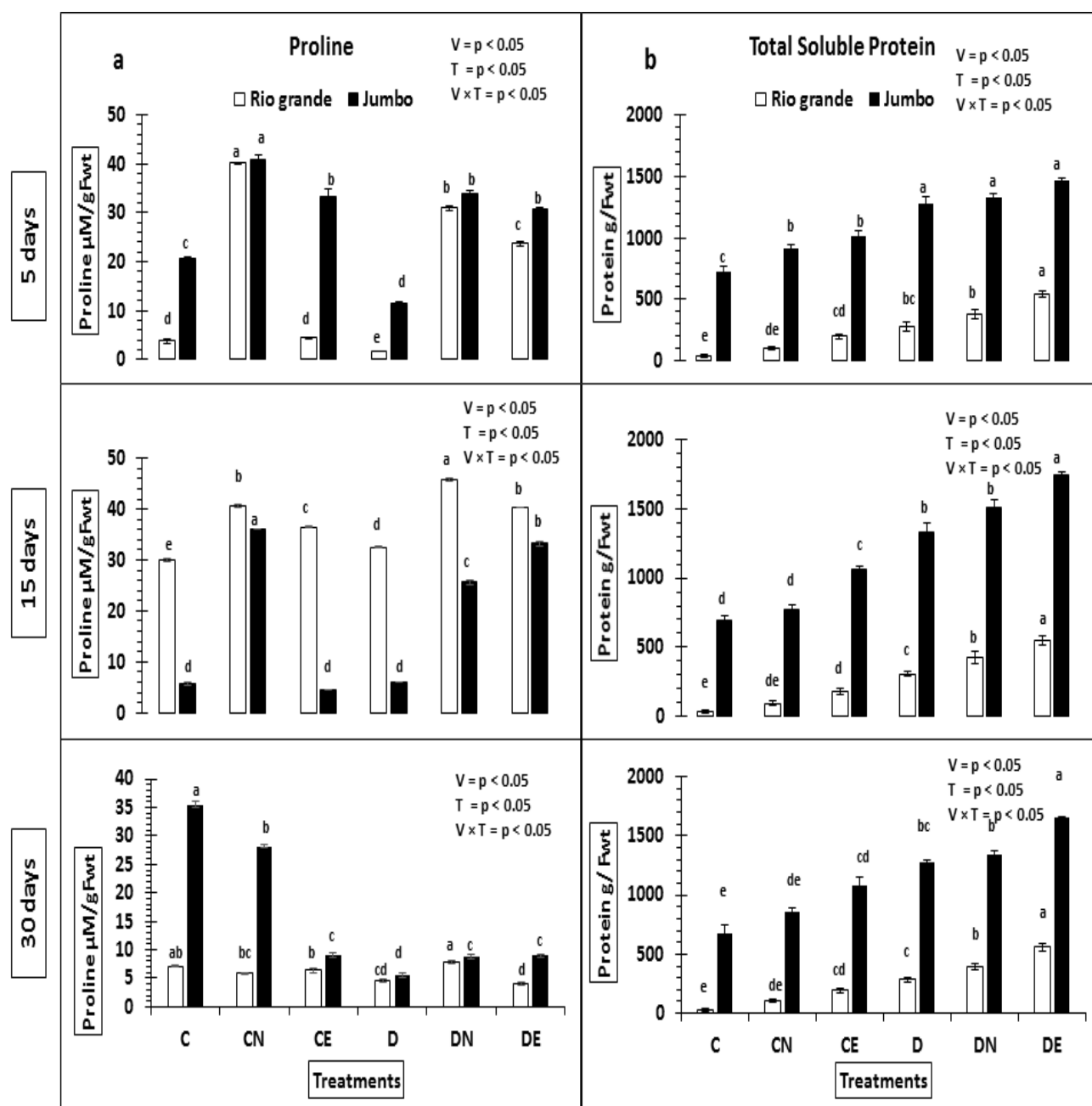


Fig. 3. (a) Proline content (b) Total soluble protein of two tomato varieties (Rio grande and Jumbo) at seedling stage after 5, 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE). The figure showed data from a representative experiment that was repeated three time and were significantly different at  $p \leq 0.05$  according to LSD.

## Discussion

This study has explained the use of neem and eucalyptus extracts as potential botanical agents against the *Rhizoctonia* root rot of tomato. Plant extracts exhibit potential antifungal activities against pathogenic fungi of plants (Abed-Ashtiani *et al.*, 2018). The results revealed that in both the varieties root colonization percentage was reduced significantly by application of aqueous extract of neem. Neem showed more effectiveness compared to eucalyptus extract due to broad range of valuable bioactive compounds such as quercetin, salannin, azadirachtin, nimbin, nimbidin, nimbin and nimbidol (Rodrigues, 2019) having antimicrobial properties.

Stress tolerance indicators such as lipid peroxidation (MDA) and  $H_2O_2$  content in diseased plants indicated more damage due to increased oxidative stress in response to pathogen induced biochemical, cellular and molecular alterations (Taheri *et al.*, 2014) such as accumulation of reactive oxygen species. It is an earliest response of plant against biotic stress which inhibits the pathogen growth. ROS act as antimicrobial molecules as well as trigger immune responses (Camejo *et al.*, 2016). Farmer & Mueller, 2013 reported that lipid peroxidation (measured as MDA) was an indicator of ROS proliferation (Moldes *et al.*, 2016) while  $H_2O_2$  content served as an elicitor in plant pathogen interaction (Zhai *et al.*, 2017) and could improve resistance against *Fusarium* (Sorahinobar *et al.*, 2015). The

high content of both  $H_2O_2$  and MDA were also reported in *Vicia faba* leaves infected by bean yellow mosaic virus (Radwan *et al.*, 2010; Spanic *et al.*, 2017).

In this study, lipid peroxidation and hydrogen peroxide content were reduced after application of neem and eucalyptus extracts. Among both the extracts, the maximum reduction in stress tolerance markers (MDA and  $H_2O_2$ ) was found in neem extract treated seedlings, suggesting role in mitigating the root rot disease development by reduction in oxidative stress. This reduced level of lipid peroxidation may be due to a mechanism that activates the antioxidant defensive enzymes. It is reported that extract of *Azadirachta indica* has compounds that are rich source of antioxidants due to which it plays an important role as free radical scavenger (Alzohairy, 2016).

Plants have different defense mechanisms against pathogens. The best-known mechanisms are antioxidant enzymes that constantly modulate against number of reactive oxygen species which act as first line of defense and systemic acquired resistance which include the production and accumulation of PR proteins (Oliveira *et al.*, 2016). Plants possess fundamental mechanism of antioxidant enzymes against stress oxidative damage (Wu *et al.*, 2014). In this study, antioxidant enzymes activity

such as superoxide dismutase showed minimal activity in diseased condition but after the applications of both the plant extracts, SOD activity was increased in Rio grande. This increase in SOD levels caused more accumulation of  $H_2O_2$  and it is suggested that it is an essential element involve in resistance against pathogen diseases (Sharma *et al.*, 2012; Li *et al.*, 2015). Catalase and Peroxidase activity was also increased significantly in diseased leaves of both the varieties compared to control. Treatment of seedlings with neem and eucalyptus extracts lead to increased enzyme activity that resulted in suppression of the disease due to the antifungal activity and phenolic compounds that are associated with their antioxidant activities. Moreover, the balance between  $H_2O_2$  scavenging enzymes and SOD are crucial in determining the state level of  $O_2$  and  $H_2O_2$ . (Saravanakumar *et al.*, 2011). The peroxidase enzymes are reported to have a dual role as an important scavenger of  $H_2O_2$  as well as helper in the oxidation of phenolic compounds against pathogens (Li *et al.*, 2015). It shows that phenolic compounds are associated with antioxidants exhibiting redox properties, due to which acting as reducing agent hydrogen donors and singlet quenchers (Asif, 2015) as well as induce systemic resistance by the accumulation of PR-Proteins.

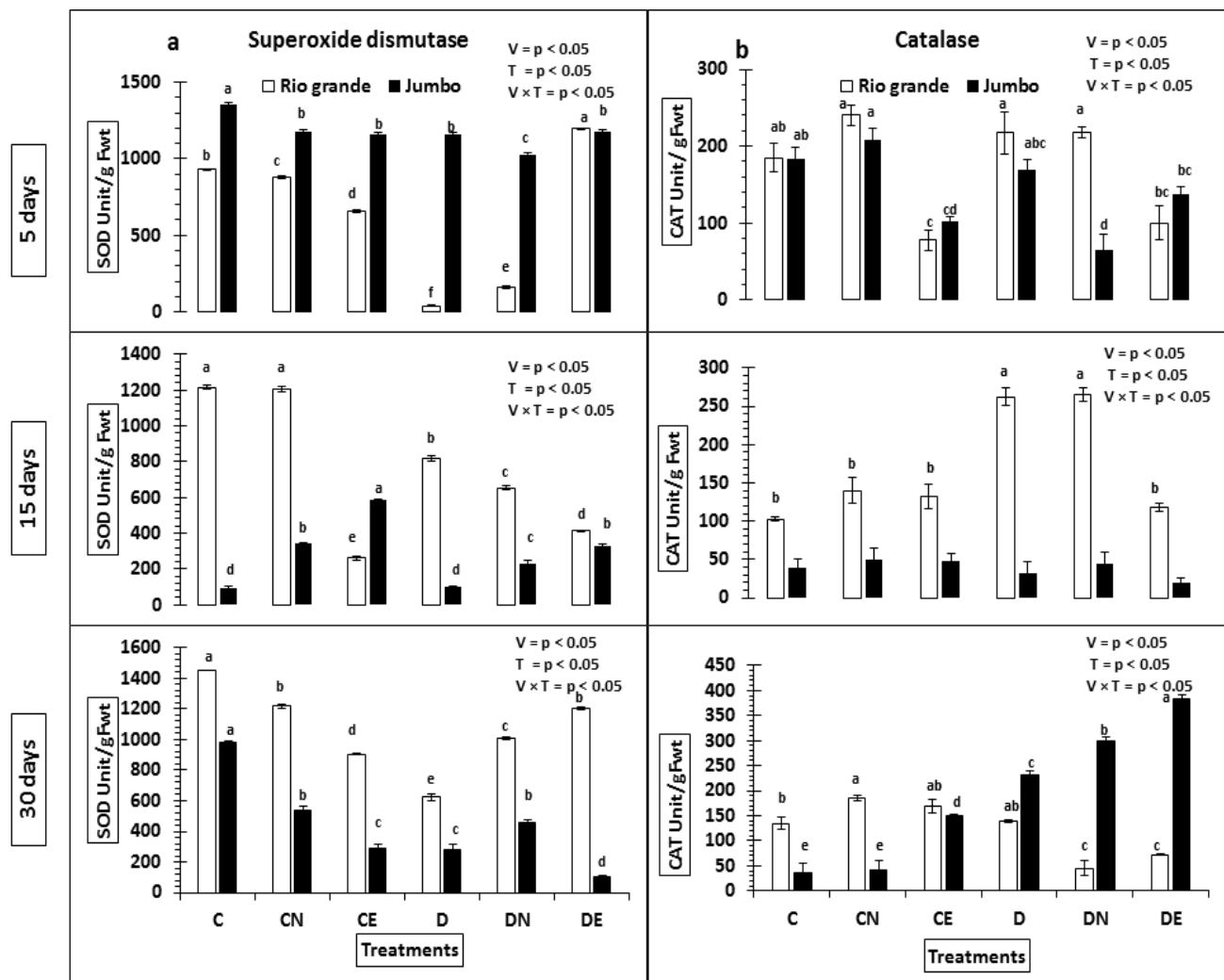


Fig. 4. (a) Superoxide dismutase (SOD) enzyme (b) Catalase enzyme activity of two tomato varieties (Rio grande and Jumbo) at seedling stage after 5, 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE). The figure showed data from a representative experiment that was repeated three time and were significantly different at  $p \leq 0.05$  according to LSD.

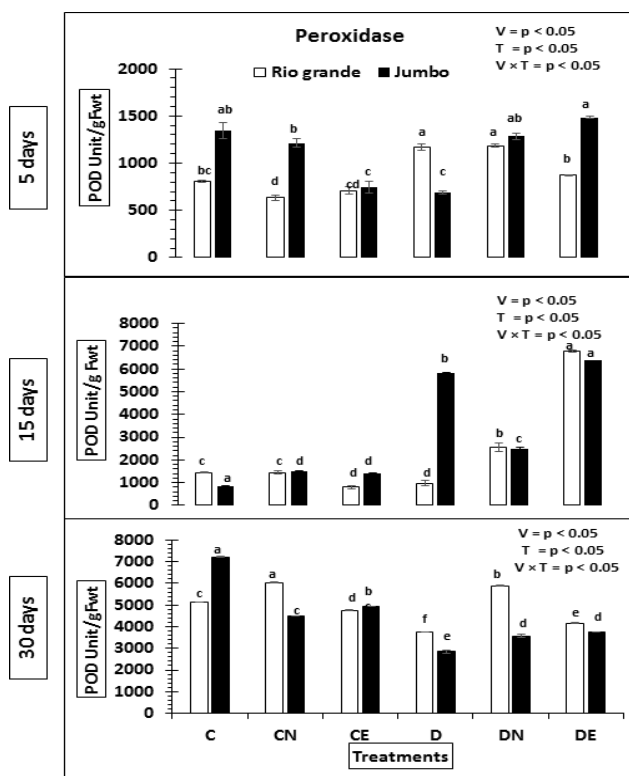


Fig. 5. Peroxidase (POD) enzyme activity of two tomato varieties (Rio grande and Jumbo) at seedling stage after 5, 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE). The figure showed data from a representative experiment that was repeated three time and were significantly different at  $p \leq 0.05$  according to LSD.

As reported in previous studies, these plant extracts had direct antifungal activity against fungal pathogens; indirectly they might trigger the host defense by successful induction of immunity system of plant against pathogens (Akladios *et al.*, 2015; Abkhoo & Jahani, 2017) and protecting the plant by enhancing expression of defense related proteins (PR proteins). SDS- PAGE analysis revealed that different protein bands were apparently induced under disease condition and 33 and 37 kDa also appear after plant extracts treatments. This study revealed that number of proteins are induced to enhance the defense system of tomato against root rot disease. Therefore, high expression of proteins bands of molecular weight 33 and 37kDa was found in extract-treated plants which showed their importance in plant resistance and they may be PR proteins. In both the varieties, PR-2 proteins (33-37kDa) were found in neem treated seedlings. These PR proteins generate the long lasting and broad range of resistance against pathogens in different crop varieties (Ali *et al.*, 2017; Ali *et al.*, 2018b). There are 17 different families of PR-proteins, among them PR-2 proteins are potential antifungal proteins in plants (Ali *et al.*, 2018a) which target the fungal cell wall or hydrolyze them and cause cell death (Agarwal *et al.*, 2014). It is hence concluded that plant extracts application is an ecofriendly approach to provide possibilities for induction of systemic resistance against different diseases to trigger antioxidant enzyme activity and accumulate pathogenesis related proteins. Moreover, plant extracts as SAR inducers reduce the adverse effect on environment as well as give good and equal effective protection as fungicides.

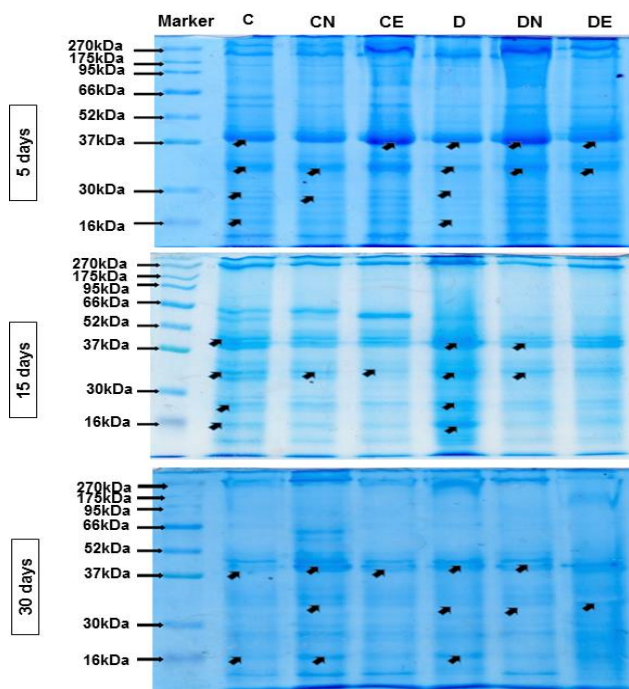


Fig. 6. SDS-PAGE protein profiling of variety Rio grande at seedling stage after 5, 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE).

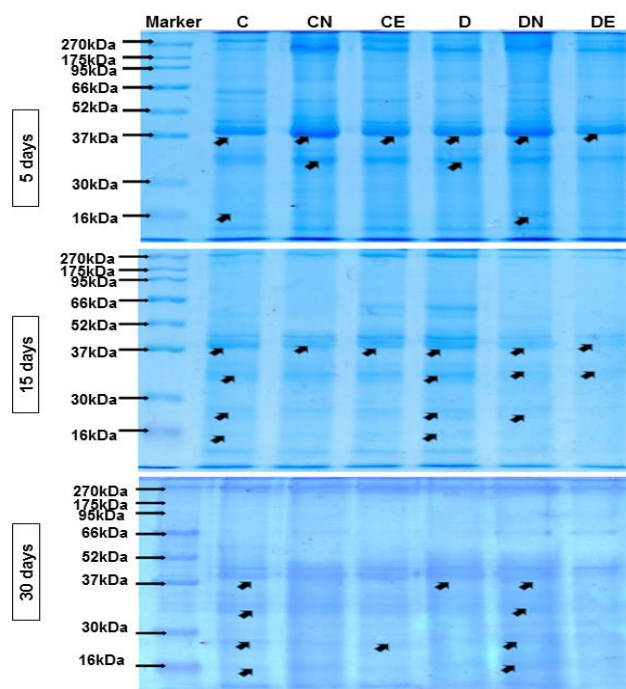


Fig. 7. SDS-PAGE protein profiling of variety Jumbo at seedling stage after 5, 15 and 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE).

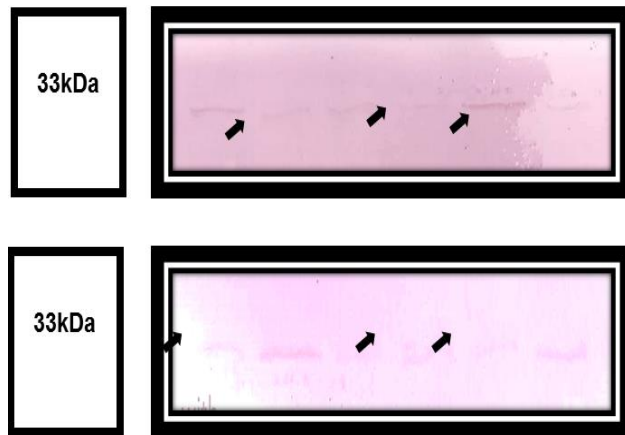


Fig. 8. Immunoblotting of two tomato varieties Rio grange and Jumbo at seedling stage after 30 days of *Azadirachta indica* and *Eucalyptus globulus* extract application. Control (C), Control Neem (CN), Control Eucalyptus (CE), Diseased (D), Diseased Neem (DN) and Diseased Eucalyptus (DE).

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