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Comparative efficacy of three different fungal antagonists (*Gonatobotrys simplex*, *Trichoderma harzianum* and *Penicillium chrysogenum*) against *Alternaria alternata* causing black rot of tomato fruits by modulating innate antioxidant expression of tomato fruits

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ABSTRACT

In view of the huge post harvest losses coupled with undesirable effects of the synthetic fungicides against the fungal rots, use of plant based secondary metabolites has received much attention in recent years as one of the alternatives to chemicals that are not only harmless to human beings but are also safe for the ecosystem. Realizing these facts, in the present investigation, therefore, the use of non-chemical strategies both under in vitro and in vivo conditions via modulating antioxidant system of host were undertaken to manage A. alternata rot of tomato fruits. Three different fungal antagonists viz., Gonatobotrys simplex, Trichoderma harzianum and Penicillium chrysogenum were used to post harvest pathogen of tomato fruits via modulating the innate antioxidant expression of tomato fruits. All the antagonists were able to inhibit the growth of the pathogen both under in vitro as well as in vivo conditions depicted by the decrease in mycelial growth and rot development respectively. There was a significant enhancement in lipid peroxidation during pathogen stress which significantly got reduced after application of fungal antagonists. Application of antagonists also enhanced the antioxidant expression of tomato fruits (various enzymes like superoxide dismutase, guaiacol peroxidase, catalase, ascorbate peroxidase and non-enzymatic antioxidants like ascorbic acid, total phenols, proline and glutathione). The above investigation collectively comes with recommendation of an efficient and environmental safe approach, which can be used to control various fungal pathogens.

I INTRODUCTION

Fresh fruits and vegetables contain approximately 65 to 95% water, abundant nutrients such as carbohydrates, proteins and lipids and growth factors such as vitamins and minerals which make them a suitable substrate for the development of various micro-organisms which are widespread in the air, soil and water. As fruits and vegetables continue to become more popular with consumers, the role of fungi in spoilage and safety of these products have increased in importance. The economic loss incurred through storage diseases might exceed that caused by field diseases because of the large investments on the product from harvest until it reaches the consumer. It reduces market supply, increases retail price and drastically impedes the export potential (Pelczar *et al.,* 1993; Bukar *et al.,* 2009). Among the various fruit vegetable crops, tomato (*Solanum lycopersicum* L.; family- Solanaceae) is one of the most important crops cultivated all over the world and is placed sixth in terms

of total production out of 15 vegetables listed by Food and Agricultural Organization (Tanwar *et al.,* 2010). Globally, tomato occupies an area of 4.55 million hectares with a production of 122.9 million tonnes (FAO, 2001). In India, tomato is mainly grown in the states like Andhra Pradesh, Orissa, Karnataka, West Bengal, Bihar, Gujarat, Maharashtra, Chhattisgarh, Jharkhand, Madhya Pradesh and Uttar Pradesh, with Bihar being the leading producer followed by Karnataka, Punjab, West Bengal and Assam with an average production of around 48,00 metric tonnes per year (Guuntekin *et al.*, 2009; Pila *et al.*, 2010).

Post harvest decays of fruits and vegetables account for significant levels of post harvest losses. It is estimated that about 20-25% of the harvested fruits and vegetables are decayed by pathogens during post-harvest handling even in developed countries (Droby *et al.*, 2009; Abano and San-Amaoh, 2012). In developing countries, post harvest losses are often more severe (more than 30%) due to inadequate post harvest handling, packaging, transportation and storage which may result in decay and production by micro-organisms which become activated because of the changing physiological state of the fruit (Singh and Sharma, 2007). Fruits due to their low pH, high moisture content and nutrient composition are very susceptible to attack by pathogenic fungi, which in addition to causing rots, may also make them unfit for consumption by producing mycotoxins (Moss, 2002).

Tomato fruits are also infected by a number of fungal micro-organisms (Wani, 2011). Among different pathogens, *Alternaria alternata* has been known to cause 80% loss in tomato fruits (Waller *et al.*, 2002). The most important method of protecting the fruits and vegetables against the fungal attack is the use of fungicides (Kanetis *et al.,* 2007). However, many fungicidal agents available in the market are toxic and have undesirable effects on other organisms present in the environment. Some synthetic fungicides are non-biodegradable and hence can accumulate in the soil, plants and water and consequently, affect the humans through the food chain. Moreover, the development of resistance of pathogenic fungi towards the synthetic fungicides is of great concern (Tapwal *et al.*, 2011). Therefore, the health and environmental concerns associated with the continuous use of synthetic fungicides have alarmed legal enforcers and consumers to demand green technology and quality products from the food industry as well as the scientific community. This called for a new paradigm shift from the use of synthetic fungicides to a safer and environmentally friendly alternative for reducing the post harvest decay in fruits and vegetables (Mari *et al.,* 2007).

Among the replete of bio-control approaches, the use of microbial antagonists is quite promising and gaining popularity (Sharma *et al.,* 2009). The antagonists micro-organisms that are naturally present on the surfaces of fruits and vegetables, which when after isolation are used for the control of post harvest diseases are called naturally occurring antagonists (Sobiczewski *et al.,* 1996). Droby *et al.* (2009) has well documented the commercial antagonistic micro-organisms available in the global market for the post harvest control of decay in fruits and vegetables. The antagonists are sprayed directly onto the surfaces or are applied by dipping (Sharma *et al.,* 2009). Moreover, from the available literature, it appears that post harvest application of microbial antagonists is a better, practical and useful method for controlling post harvest diseases of fruits and vegetables (Irtwange, 2006).

Extensive survey of literature showed a plethora of information on the use of various non-chemical methods against post harvest diseases of fruits and vegetables. However, a little information is available on the use of

post harvest management of tomato fruits by using fungal antagonists especially under *in vivo* conditions via modulating antioxidant system of host. Therefore, our endeavour was to manage *A. alternata* rot of tomato fruits by using *Trichoderma harzianum*, *Gonatobotrys simplex* and *Penicillium chrysogenum*.

II MATERIAL AND METHODS

2.1 Survey of vegetable markets and isolation of *A. alternata* from diseased tomato fruits

The various vegetable markets were surveyed for the collection of visibly cracked and bruised tomato fruits in pre-sterilized polythene bags. *A. alternata* was isolated from visibly infected fruits after incubating them at 28 ± 2°C for three days. The purified cultures were maintained in duplicates on sterilized potato dextrose agar (PDA). The Koch's postulates were performed for testing the pathogenicity of *A. alternata*.

2.2 Preparation of the spore suspension of selected fungal antagonists (FA)

Different fungal antagonists were recovered from tomato fruits (healthy) itself. Five day old cultures of *Gonatobotrys simplex*, *Penicillium chrysogenum* and *Trichoderma harzianum* were flooded with 10 ml of sterilized distilled water and the spores were rubbed from the surface of the Petri plate with a glass rod. The spore density was calculated by counting the spores in a haemocytometer.

The resulting spore suspension was considerd as stock solution. Three different test concentrations were made from the stock solution *viz.*, $1x10^{8}$, $1x10^{10}$, and $1x10^{12}$ cfu/ml.

2.3 Antifungal efficacy of selected FA

2.3.1 *In vitro* antifungal efficacy of FA

Five-day-old cultures of *A. alternata* were flooded with 10 mL of sterilized distilled water. The spores were rubbed from the surface of Petri dish and spore density was calculated by using haemocytometer to obtain a uniform suspension of $1x10^{5}$ spores mL^{-1}. The antifungal efficacy of different FA was performed against *A. alternata* by the poisoned food technique (Perucci *et al.* 1994). Different concentrations of FA were poured in Petri dishes followed by addition of 9.5ml PDA. Likewise, control sets were prepared using equal amount of distilled water replacing various treatments. The prepared plates were then inoculated aseptically with discs of test fungus and incubated at 28±2°C for 7 days. The percentage mycelial inhibition was calculated by using the formula:

$$\text{Percentage mycelial inhibition} = \frac{dc-dt}{dc} \times 100$$

Where dc is the mean mycelial growth diameter of colony of control sets

dt is the mean mycelial growth diameter of colony of treatment sets

2.3.2 *In vivo* antifungal efficacy of FA

To analyse the efficacy of various treatments by pre inoculation (PRI) treatment, healthy fruits were weighed and surface sterilized with 1% sodium hypochlorite solution and then dried under aseptic conditions and rinsed with 70% alcohol. Pre-weighed tomato fruits prior to inoculation were dipped in the respective solutions for 30 mins and thereafter inoculated with 5µl of spore suspension of *A. alternata* ($1x10^5$spores/ml). In case of post inoculation (POI), pre-weighed tomato fruits prior to treatment were inoculated with 5µl of spore suspension and thereafter treated with respective solutions. Inoculated tomato fruits were then incubated in sterilized polythene bags for 3 days 28±2°C, R.H. 90%.

2.3.3 Calculation of percent rot development and percent rot control

After 3 days of incubation percentage rot was calculated by using the formula:

$$\text{Percentage rot} = \frac{(W-w)}{W} \times 100$$

Where, W= Weight of the fruit before inoculation
w= Weight of the fruit after removal of rotten tissue

Similarly, percentage rot control was evaluated by using the formula:

$$\text{Percentage control} = \frac{\%\text{ decay in untreated fruit} - \%\text{ decay in treated fruit}}{\%\text{ decay in untreated fruit}} \times 100$$

2.4 Biochemical estimation of various physiological parameters

2.4.1 Lipid peroxidation

The peroxidation of lipids was estimated according to the method of Heath and Packer (1968). Briefly, tomato fruits [0.5 g fresh weight (FW)] were homogenized in 3 mL of 0.1% trichloroacetic acid (TCA) as previously described in Choudhary *et al.* (2011).

2.4.2 Protein content and antioxidant enzymes

2.4.2.1 Preparation of fruit extract

The fruit tissue (0.5 g FW) was homogenized in 3 mL of 0.1 M potassium phosphate buffer using pre-chilled pestle and mortar, then centrifuged at 15,000 rpm for 20 min. Supernatants obtained were used for the estimation of protein contents and activities of the antioxidant enzymes.

2.4.2.2 Protein content

The protein content was estimated according to method of Lowry et al. (1974).

2.4.2.3 Estimation of enzymatic activities

The activities of superoxide dismutase, guaiacol peroxidase, catalase, ascorbate peroxidase and glutathione reductase were estimated by following the methods of Kono (1978), Putter (1974), Aebi (1983), Nakano and Asada (1981), and Nordhoff et al. (1993) respectively.

2.5 Non-enzymatic antioxidants

The estimation of glutathione, total phenols, ascorbic acid and proline contents was done by following the methods of Sedlak and Lindsay (1968), Ragazzi and Veronese (1973) and Cakmak and Marschner (1992) and Bates et al. (1973) respectively.

2.6 Statistical analysis

All the experiments were performed in triplicate. Data shown are the means of three replicates along with standard error (n = 3). Student's t-test was carried out and data were presented at $p \leq 0.05$. All the statistical calculations were performed using IBM SPSS 20.0 software.

III RESULTS AND DISCUSSION

3.1 Isolation of *A. alternata* from diseased tomato fruits

Different wholesale and retail markets of Jammu district were surveyed regularly for collection of visibly cracked and bruised tomato fruits. The infected fruits were incubated at 28±2ºC for three days for isolation of *A. alternata*. Twenty two different isolates were recovered from bruised tomato fruits (Fig. 1). After identification, different isolates of *A. alternata* were inoculated in healthy tomato fruits to check their infection causing potential. The experiment was performed in triplicates with three fruits. Out of all, A19 (CHA) isolate was the destructive in terms of loss in biomass. This particular isolate was used for further experiments.

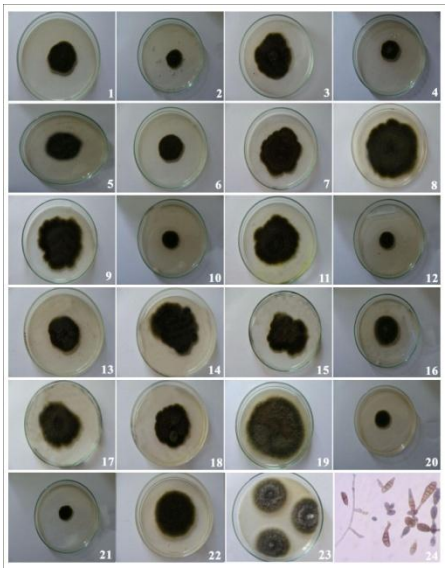

Figure-1 Different isolates of *A. alternata* isolated from diseased tomato fruits.

3.2 Symptoms of tomato fruits inoculated with *A. alternata*

A. alternata (A19 CHA) was inoculated in healthy tomato fruits to study its effect on loss in terms of fruit biomass and various biochemical parameters. Rot development in tomato fruits after inoculation of *A. alternata*

has been shown in Fig. 2 (a-f). Symptoms were observed after three days of inoculation and then the observations were made after every two days interval upto a total of 13 days.

Initially, 72 hours after inoculation (72hi), the rot appeared as a dark, brown to black lesions which were upto 8-8.7 mm in diameter with white mycelium around the boundary. After 5 days, the lesions became more dark and broadened in size (12.5-13.2 mm) with marked depression in the centre and elevated portion around the diameter. The lesions became slightly sunken after 7 days, which were of firm texture and 24.0-25.4 mm in diameter. After 9-11 days, the lesions became more sunken and subsequently, got covered by grayish black mass giving it a fluffy appearance. Concentric rings of mycelium were formed after 13 days followed by secondary infections. The rot extended deep into the fruit rendering the tissues black, soft and watery.

Alternaria rot has been considered as the most common disease of tomato fruits and causes heavy losses in quality of the fruits, thus rendering large quantity of tomato fruits unfit for consumption (personal observation). It is generally considered to be a weak pathogen requiring injured or weakened tissue to germinate and develop. Thus, the fungus tends to develop in tomatoes affected with mechanical and chilling injury, sunscald, growth cracks etc. At times over 80% of the overall decay in the stored tomatoes has been caused by this particular fungus (Snowdon, 1991). Our results of isolation of *A. alternata* as post harvest pathogen of tomato fruits are consistent with the previous reports of Spalding (1980) and Hasan (1995), who isolated *A. alternata* from tomato fruits and when inoculated in healthy tomato fruits could significantly initiate rot development. Abdel-Mallek *et al.* (1995) also reported that *A. alternata* was one of the most commonly isolated pathogens from healthy or diseased tomato fruits. Recently, El-Katatny and Emam (2012) and Pane *et al.* (2012) found *A. alternata* responsible for the post harvest spoilage of tomato fruits. Similarly, Feng and Zheng (2007) observed more than 50% loss of tomato fruit biomass caused by *A. alternata*.

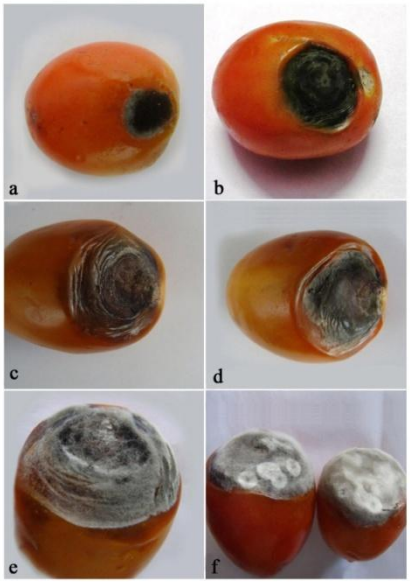

Figure-2 Different stages of tomato fruits inoculated with *A. alternata*.

a, b, c, d, e and f: after 3, 5, 7, 9, 11 and 13 days respectively.

3.3 Effect of *A. alternata* on biochemical constituents of tomato fruits

Lipid peroxidation (Malondialdehyde, MDA)

The extent of cell damage caused by production of reactive oxygen species (ROS) and oxidative stress related to plant's response to pathogenic stress can be estimated by determining the products of membrane lipids peroxidation. Lipid peroxidation, measured in terms of malondialdehyde (MDA) content, a thiobarbituric reactive substance, was found to increase (16.02-fold) in IN tomatoes as compared to healthy CN at $P \leq 0.05$ as well as $P \leq 0.01$ as shown in Table 1

Table 1: Effect of *A. alternata* on different biochemical constituents of tomato fruits.

S.No	Parameter	Control (Healthy tomato fruits)	Inoculated (tomato fruits with *A. alternata*)
1	Malondialdehyde (µmol g^{-1} (F.W.)	6.23±1.02	16.2±1.2^{*+}
2	Protein (mg g^{-1} (F.W.)	11.6±1.0	6.45±1.0^{*+}
3	Superoxide dismutase UA mg^{-1} Prot g^{-1} (F.W.)	1.01±0.2	1.98±0.2*
4	Guaiacol peroxidase UA mg^{-1} Prot g^{-1} (F.W.)	0.5±0.12	0.24±0.1^{*+}
5	Catalase UA mg^{-1} Prot g^{-1} (F.W.)	2.39±0.45	5.38±1.2^{*+}
6	Ascorbate peroxidase UA mg^{-1} Prot g^{-1} (F.W.)	4.32±1.1	5.67±1.2*
7	Glutathione reductase UA mg^{-1} Prot g^{-1} (F.W.)	0.98±0.12	1.23±0.2*
8	Ascorbic acid (mg g^{-1} (F.W.)	3.7±0.98	7.57±0.5^{*+}
9	Total phenol (mg g^{-1} (F.W.)	8.29±1.24	13.2±1.2^{*+}
10	Proline (mg g^{-1} (F.W.)	3.2±0.45	5.47±0.4*
11	Glutathione (mg g^{-1} (F.W.)	8.9±1.21	5.9±0.73*

Values represent means of replicates with standard error (SE)
* Significant difference from control at $P \leq 0.05$ and $^{+}$ Significant difference from control at $P \leq 0.01$

The peroxidation of unsaturated lipids of biological membranes is the most prominent symptom of oxidative stress in living organisms that is triggered by the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) which are known to attack lipids leading to formation of malondialdehyde (Mittler, 2002; Gobel *et al.*, 2003; Campo *et al.*, 2004). Intensive lipid peroxidation here in this study is an indication of compatible nature of the host-pathogen combination as also reported earlier by Mandal *et al.* (2008) who observed an enhancement in MDA content in *S. lycopersicum* inoculated with *Fusarium oxysporum*.

Protein (PR)

A significant decrease in PR content was observed in tomato fruits inoculated with *A. alternata* which was 4.43-fold less as compared to control after 72hi at P≤0.05 as well as P≤0.01. Perusal of literature, however, did not reveal any such report on changes of PR content in tomato fruits inoculated with *A. alternata*. Therefore, the present study constitutes the first report of change in PR content in tomato fruits.

Antioxidant enzyme activities

The results relating to antioxidant enzymes are presented in Table 1. The stimulating effect (4.87-fold) of inoculation of tomato fruits with *A. alternata* on SOD activity was revealed. On the other hand, there was a significant decrease in unit activity of GPOX which was 5.2-fold lower as compared to CN. An increase in CAT activity (12.5-fold) was observed as a result of interaction of tomato fruits and *A. alternata*. APOX and GR in tomato fruits responded to the pathogen attack by their increased activity respectively (3.12- and 2.55-fold) and the changes were significant when compared with the activity of other enzymes at P≤0.05. At P≤0.01, out of all antioxidant enzymes, changes were significant only for CAT and GPOX as compared to CN.

In plant cells, enzymes and redox metabolites that are produced under pathogen attack act in synergy to carry out ROS scavenging (Gara *et al.,* 2003). SOD catalyses the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), H_2O_2 is in turn dismutated into oxygen and water by CAT and APOX by utilizing ASA as a specific electron donor (Mittler *et al.,* 2002). These are considered as the main enzymatic components responsible for protection of cells against oxidative damage (Tommasi *et al.,* 2001). In the present study, unit activity of SOD, CAT, APOX and GR significantly got enhanced after 72hi, but GPOX showed a reduction. Results of our experiments are consistent with previous findings as evidenced by a significant enhancement in defense related enzymes (SOD, CAT, PPO and PAL) in jujube fruits infected with *P. expansum* (Zhu *et al.,* 2010). However, our study presents the first incident of increase of antioxidant enzymes under pathogenic stress of *A. alternata*.

Non-enzymatic antioxidant molecules

Various non-enzymatic antioxidants (ASA, PL and TPC) evaluated in tomato fruits inoculated with *A. alternata* also exhibited a rising trend except GSH, which showed a decrease in IN fruits. In our study, ASA content increased sharply (10.4-fold) after 72hi in IN tomato fruits as compared to control. An enhancement was observed in TPC and PL content as a result of interaction of host and pathogen with maximum increase of 6.15- and 5.12-fold respectively. At P≤0.01, only TPC and PL were significantly different from CN as depicted in Table 1.

Presumably, accumulation of non-enzymatic antioxidants helped in decimating the negative effects of oxidative stress as previously observed in the studies of Noctor and Foyer (1998). Amongst non-enzymatic antioxidants, phenols are widely distributed in plants and constitute an important component of defense mechanism (Friedman and Levin, 2009). Rapid production of phenols at infection site depicts initial activation of defense mechanism in plants, thereby, restricting or slowing down pathogen growth (Matern and Kneusel, 1988). In

addition, phenols restrict the growth of invading pathogens by binding with hydrolytic enzymes, released by fungal pathogens during cell division (Dai *et al.* 1995). Similar to our study, Ruelas *et al.* (2006) noticed an enhancement in various phenols in tomato fruits infected with *A. alternata*. Alternatively, in the study of Chatage and Bhale (2012) on ivy gourd fruits inoculated with *A. pluriseptata*, a reduction in phenolic content was noticed.

Furthermore, ASA and PL content in tomato fruits inoculated with *A. alternata* indicated a rising trend as compared to CN fruits. Ascorbic acid (ASA) is a water soluble antioxidant that helps in scavenging ROS (Smirnoff, 1996). It reacts rapidly with $O_2^{\cdot-}$, singlet oxygen (O_2^1), ozone (O_3) and H_2O_2 and thus, helps in removal of ROS (Asada, 1992). Similar to our study, increased level of ASA was found in *Avena sativa* after infection with virulent *Drechslera* species (Gonner and Schlosser, 1993). Opposite trends were observed in the study of El-Zahaby and Gullner, (1995), where decreased level of ASA was observed in barley infected with *Erysiphe graminis*. An enhancement in PL content might be helping in decimating ROS by acting as a compatible osmolyte as noted by Verbuggen and Herman (2008).

Interestingly, in the present study a reduction in GSH content in inoculated tomato fruits was observed, which could be attributed to the accumulation of phytoalexins, that play an important role during pathogen stress as indicated in the studies of Guo *et al.* (1993). Wintage *et al.* (1988) proposed that GSH is involved in signal transduction during the initiation of phytoalexin response by fungal elicitors. Kuzniak and Sklodowska (2001) also found a decrease in GSH content in tomato leaves infected with *Botrytis cineria*.

Overall schematic representation of production of ROS and their detoxification mechanism have been given in Fig. 3 and 4.

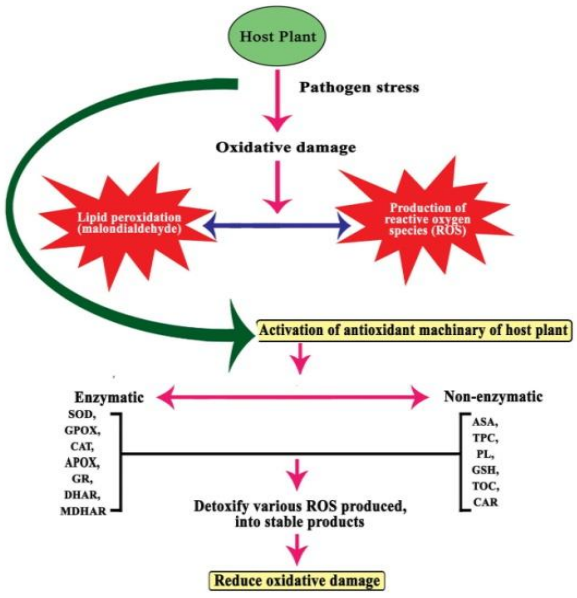

Figure-3 Overall schematic representation of antioxidant machinery in a plant system during pathogen attack

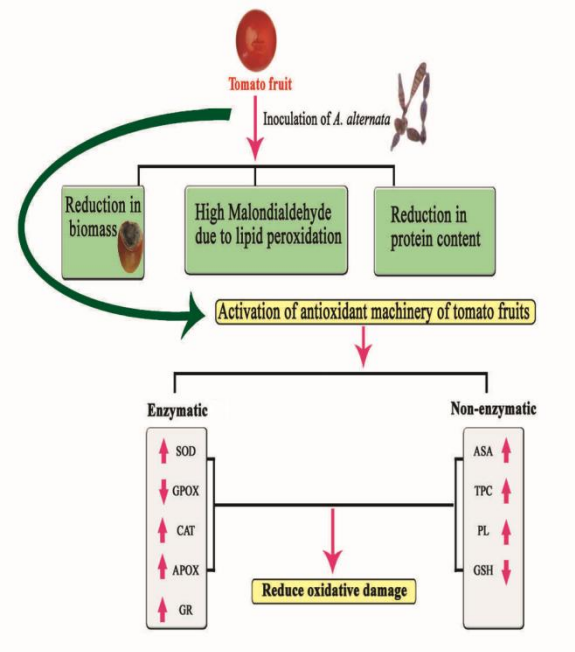

Figure 4 Overall schematic representation of antioxidant machinery in tomato fruits after inoculation of *A. alternata*.

3.4 *In vitro* and *in vivo* antifungal efficacy of various fungal antagonists against *A. alternata*

3.4.1 *In vitro* antifungal efficacy

Percent mycelial inhibition and percent reduction in conidial count

The results related to percent mycelial inhibition and percent reduction in conidial count are given in Table 2. All the antagonists inhibited mycelial growth of *A. alternata* (Fig. 5, 6 and 7. All three antagonistic fungi caused significant inhibition of test pathogen at higher concentrations. Maximum reduction of mycelial growth was recorded for *T. harzianum* (83.29%) followed by *P. chrysogenum* (69.26%) and *G. simplex* (59.23%) at 10^{12} cfu/ml over control. Percent mycelial inhibition and conidial count reduction at 10^{8} cfu/ml was insignificant over control. *T. harzianum* was superior over all the antagonists used. All these bioagents also had a negative effect on conidial count of the pathogen, but *T. harzianum* was most effective and caused maximum inhibition of 89.26%, whereas, it was least for *G. simplex* (68.26%) at 10^{12} cfu/ml. Since, *T. harzianum* was recovered from tomato fruit surface itself, the study suggests that the naturally occuring microbes on the fruit surface can be effectively recruited as effective competitors of various rot causing pathogens of the fruit itself. *T. harzianum* could also inhibit mycelial growth of *A. alternata* as denoted earlier by Pandey (2010). After *T. harzianum, P. chrysogenum* was second best antagonist in reducing the mycelial growth of *A. alternata*. In the study of Soytong *et al.* (2005). *P. chrysogenum* was used to control *C. gloeosporioides* causing anthracnose of grapes. Results indicated antifungal activity of *P. chrysogenum* against mycelial growth of test pathogen more than 50% and reported inhibition of spore production. A number of *Penicillium* species have been reported as antagonists of plant pathogens with a mechanism of action based on the induction of resistance (Madi and Katan, 1998), the

production of antibiotic compounds (Nicoletti *et al.,* 2004; Yang *et al.,* 2011) and the establishment of mycoparasitic interactions (Sempere and Santamarina, 2010).

G. simplex also showed satisfactory results regarding mycelial growth and reduction in conidial count. As per survey of literature, there are no reports on use of this fungus to control any pathogens so far. Thus, this study is the first report on use of *G. simplex* to control *A. alternata* causing tomato fruit rot. However, in the reseacrh conducted by Whaley and Barnett (1963), a biotrophic mycoparasite relationship between *G. simplex* (as parasite) and *A. alternata* (as host) has been described that was later on confirmed by Hoch (1976) through scanning electron microscopy.

Table 2: Effect of different fungal antagonists on mycelial inhibition and reduction in conidial count of *A. alternata.*

Name of the antagonist	Concentrations (cfu/ml)	Percent mycelial inhibition (%)	Percent reduction in conidial count (%)
Gonatobotrys simplex	10^8	22.53±1.26[a]	21.16±1.16[a]
	10^{10}	40.16±1.60[b]	50.13±2.36[b]
	10^{12}	59.23±1.29[c]	68.26±1.23[c]
Penicillium chrysogenum	10^8	26.52±3.26[a]	23.12±1.09[a]
	10^{10}	43.26±2.29[b]	54.90±3.20[b]
	10^{12}	69.26±3.19[c]	73.13±5.26[c]
Trichoderma harzianum	10^8	35.11±1.28[a]	42.16±3.42[a]
	10^{10}	52.16±1.36[b]	66.29±3.26[b]
	10^{12}	83.29±4.23[c]	89.26±5.19[c]

Values represent means of replicates with SE
Different letters are significantly different between the treatments at P≤0.05

3.4.2 *In vivo* antifungal efficacy

Percent rot development and percent rot control

T. harzianum was most effective in controlling decay of tomato fruits incited by *A. alternata*, followed by *G. simplex* and *P. chrysogenum* at 10^{12} cfu/ml at PRI after 72hi (Table 4). In POI, maximum rot control was recorded for *G. simplex* followed by *T. harzianum* and *P. chrysogenum* at 10^{12} cfu/ml as compared to IN fruits (Table 3). In general, the antagonists were found to be more effective when applied as pre-inoculation than post inoculation treatment which might be due to the fact that they get sufficient time to establish themselves in the host against the pathogen. Such results are consistent with the observations of Fan and Tian (2001) and Wang *et al.* (2010).

Post harvest application of *T. harizianum*, *T. viride*, *Gliocladium roseum* and *Paecilomyces variotii* resulted to control *Botrytis* rot in strawberries and *Alternaria* rot in lemons via a decrease in rot development (Pratella and Mari, 1993). Similarly, Sharma *et al.* (2012) used *Trichoderma hamatum*, *T. harzianum*, *T. viride* against *P. digitaum* causing rot in kinnow fruits and found all antagonists to be effective against test pathogen with

maximum rot control shown by *T. harzianum* followed by *T. viride*. Likewise, *P. chrysogenum* and its antifungal extracts could significantly reduce spore germination of *Botrytis fabae* and decreased disease incidence of beans (Jackson *et al.*, 1993). Soytong *et al.* (2005) also found *P. chrysogenum* to be effective against *Colletotrichum gloeosporioides* fruit rot of grapes. Intersetingly, *G. simplex*, was also able to curb *A. alternata* infection under *in vivo* conditions. Prior to this study, literature shows no use of *G. simplex* to control any rot of the fruits and vegetables, in general and *Alternaria* rot of tomato, in particular. Hence, this study reports the first sucessful application of *G. simplex* as an antagonist.

Lipid peroxidation [Malondialdehyde (MDA]

Inoculation of *A. alternata* in healthy tomato fruits led to an enhancement in MDA content as compared to CN fruits while as application of fungal antagonists in tomato fruits reduced MDA content with maximum reduction noticed for *T. harzianum* at 10^{10} cfu/ml in PRI after 72hi as compared to IN fruits (Table 3). Similarly, in POI, maximum reduction in MDA content was recorded in tomato fruits treated with *T. harzianum* followed by *P. chrysogenum* and *G. simplex* at 10^{12} cfu/ml in comparison to IN fruits (Table 3). No such reports have been reported in inhibiting MDA content in host plant by application of fungal antagonists.

Protein (PR)

Data related to PR content of IN and treated fruits are given in Table 3 and 4. *P. chrysogenum* was the most effective fungal antagonist in enhancing PR content at 10^{12} cfu/ml in PRI after 72hi followed by *T. harzianum* and *G. simplex* as compared to IN fruits. However, in POI application, significant changes were noticed only for *T. harzianum* at 10^{10} cfu/ml. Perusal of literature did not report any such use of antagonists in controlling disease incidence of fruits by modulation of PR content.

Antioxidant enzyme activities

A significant increase in SOD activity was noticed only for *T. harzianum* treated fruits and that too only at 10^{10} cfu/ml as compared to IN fruits. On the other hand, application of *G. simplex* and *P. chrysogenum*, brought down SOD activity, with maximum decrease noticed for *G. simplex* application. The effects were similar for POI as that of PRI with significant increase noticed only for *T. harzianum* at 10^{10} cfu/ml. Out of PRI and POI, maximum increase was recorded for POI (Table 5 and 6). Similarly, amongst the three antagonists, *T. harzianum* was the most effective in enhancing GPOX unit activity at all applied concentrations with maximum increase at 10^{12} cfu/ml at PRI. It was followed by *P. chrysogenum* and *G. simplex*. In POI also, maximum unit activity of GPOX was recorded for *T. harzianum* at 10^{10} cfu/ml and 10^{12} cfu/ml, followed by *P. chrysogenum* at 10^{12} cfu/ml over IN fruits. Out of PRI and POI, maximum increase was noticed in *T. harzianum* in PRI application at 10^{12} cfu/ml.

Pre-inoculation of *T. harzianum* showed maximum enhancement in CAT activity at 10^{10} cfu/ml, followed by *G. simplex* at 10^{12} cfu/ml and *P. chrysogenum* at 10^{12} cfu/ml, as compared to IN fruits. *T. harzianum* again showed maximum enhancement in CAT after POI at 10^{10} cfu/ml, followed by *P. chrysogenum* and *G. simplex* at 10^{10} cfu/ml only when compared to IN fruits. In PRI, maximum increase in APOX activity was noticed after

application of *P. chrysogenum* at 10^{10} cfu/ml. *T. harzianum* was the next most effective antagonist at 10^{10} cfu/ml followed by *G. simplex* at 10^{10} cfu/ml over IN fruits. In post-inoculated application, maximum unit activity was noticed for *P. chrysogenum* treated fruits at 10^{12} cfu/ml followed by *T. harzianum* at 10^{10} cfu/ml over IN fruits. Out of PRI and POI, highest increase in unit activity of APOX was noticed in PRI application of *P. chrysogenum* at 10^{10} cfu/ml.

T. harzianum showed maximum enhancement in GR activity at 10^{12} cfu/ml in PRI after 72hi followed by *P. chrysogenum* at 10^{10} cfu/ml and *G. simplex* at 10^{10} cfu/ml as compared to IN fruits (Table 5 and 6). In POI, unit activity of GR was maximum in tomato fruits treated with *P. chrysogenum* at 10^{12} cfu/ml only over IN fruits. Changes were insignificant after application of other two fungal antagonists.

Overall, it was observed that there was an enhancement in unit activity of antioxidant enzymes of tomato fruits treated with *T. harzianum*. Ozaby and Newmann (2004) stated specific strains of genus *Trichoderma* colonize and penetrate host plant and initiate a series of morphological and biochemical changes considered to be a part of the plant defense responses. Recently, Prasad and Ganesh (2014) treated black grams with *T. harzianum* and inoculated with *A. alternata* which showed an improvement in SOD and CAT activity over inoculated seeds.

In pear fruits treated with *Cryptococcus laurentii* to control decay caused by *A. alternata*, significant enhancement in enzyme activities such as peroxidase, phenyl ammonia lyase, polyphenol oxidase and β-1,3-glucanase resulted in decrease of decay caused by *A. alternata* (Tian *et al.*, 2006). Recently, innate antifungal resistance in tomato (inoculated with *A. alternata*) was studied by using *Penicillium oxalicum* as a biocontrol agent, which significantly decreased disease incidence of *A. alternata*. During quantification of assays, more or less 2-fold increase was recorded in these enzymes (Ahmad *et al.*, 2014). These studies proved that the enzymatic system of host might have got triggered by the use of various antagonists as has been evidenced in the present investigation.

Although, a plethora of information on activity of some enzymes in different fruits and vegetables treated with different antagonists is available but reports on the modulation of enzymatic system (SOD, CAT, GPOX, APOX and GR) of tomato fruits inoculated with *A. alternata* and treated with fungal antagonists is strikingly novel and significant approach.

Table 3 Effect of different fungal antagonists on percent rot development, percent rot control, lipid peroxidation and protein content in tomato fruits pre-inoculated (PRI) with *A. alternata*.

Parameter	Inoculated fruits	*Gonatobotrys simplex* (cfu/ml)			*Penicillium chrysogenum* (cfu/ml)			*Trichoderma harzianum* (cfu/ml)		
		1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}
Percent rot development (%)	40.3±2.7^{a}	25.6±1.2^{b}	19.23±1.32^{c}	13.45±0.98^{d}	33.32±2.21^{a}	21.54±1.09^{b}	15.97±0.54^{c}	28.09±0.78^{b}	25.67±1.5^{b}	12.34±1.03^{c}
Percent rot control (%)	-	36.3±1.4^{a}	52.30±3.32^{b}	66.64±2.43^{c}	17.36±1.08^{a}	46.57±1.78^{b}	60.59±1.21^{c}	30.33±4.56^{a}	36.33±1.90^{a}	69.39±2.21^{b}
Malondialdehyde (µmol g^{-1} (F.W.)	16.2±1.2^{a}	14.7±1.1^{a}	10.96±1.02^{b}	12.3±0.97^{b}	14.21±1.23^{a}	13.98±1.19^{a}	10.4±0.67^{b}	10.39±0.56^{b}	9.98±0.64^{b}	9.79±1.02^{b}
Protein (mg g^{-1} (F.W.)	6.45±1.0^{a}	6.54±1.2^{a}	9.83±0.98^{b}	7.32±0.92^{a}	10.3±0.5^{b}	15.23±0.7^{c}	16.59±0.21^{c}	7.89±0.9^{a}	12.32±0.5^{b}	9.8±0.49^{b}

Table 4 Effect of different fungal antagonists on percent rot development, percent rot control, lipid peroxidation and protein content in tomato fruits post-inoculated (POI) with *A. alternata*.

Parameter	Inoculated fruits	*Gonatobotrys simplex* (cfu/ml)			*Penicillium chrysogenum* (cfu/ml)			*Trichoderma harzianum* (cfu/ml)		
		1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}
Percent rot development (%)	40.3±2.7^{a}	35.4±2.3^{a}	25.43±1.2^{b}	15.43±1.02^{c}	35.5±2.21^{a}	26.67±0.98^{b}	22.34±1.34^{b}	33.32±1.56^{a}	21.34±1.65^{b}	15.67±1.21^{c}
Percent rot control (%)	-	12.0±0.9^{a}	36.93±2.1^{b}	61.73±2.54^{c}	11.85±0.67^{a}	33.84±1.86^{b}	44.59±1.49^{c}	17.36±1.02^{a}	47.07±2.21^{b}	61.13±1.23^{c}
Malondialdehyde (µmol g^{-1} (F.W.)	16.2±1.2^{a}	15.2±1.4^{a}	12.98±0.89^{b}	13.21±2.03^{b}	12.31±1.13^{b}	10.21±1.09^{b}	9.79±0.34^{b}	15.75±2.12^{a}	13.31±0.56^{b}	12.32±1.12^{b}
Protein (mg g^{-1} (F.W.)	6.45±1.0^{a}	6.9±1.12^{a}	6.75±0.98^{a}	6.45±0.78^{a}	7.67±0.67^{a}	7.15±0.59^{a}	7.89±0.45^{a}	6.89±0.78^{a}	11.08±0.7^{b}	8.76±0.69^{b}

Table 5: Effect of different fungal antagonists on unit activity of different antioxidant enzymes in tomato fruits pre-inoculated (PRI) with *A. alternata*.

Antioxidant enzymes UA mg^{-1} Prot g^{-1} (F.W.)	Inoculated fruits	*Gonatobotrys simplex* (cfu/ml)			*Penicillium chrysogenum* (cfu/ml)			*Trichoderma harzianum* (cfu/ml)		
		1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}
Superoxide dismutase	1.98±0.2^{a}	0.9±0.03^{b}	1.16±0.07^{a}	1.34±0.035^{c}	1.33±0.2^{a}	2.13±0.42^{b}	1.14±0.46^{a}	11.3±0.2^{a}	2.37±0.04^{b}	1.89±0.04^{a}
Guaiacol peroxidase	0.24±0.1^{a}	0.36±0.1^{b}	0.54±0.12^{c}	0.98±0.23b	0.92±0.2^{b}	1.02±0.87^{b}	0.96±0.078^{b}	0.9±0.1^{b}	1.03±0.13^{b}	1.09±0.1^{b}
Catalase	5.38±1.2^{a}	6.23±1.2	6.29±0.92	6.53±0.78	5.89±0.8	6.03±0.9	6.12±0.67	5.19±0.8	7.23±0.67	5.28±0.77
Ascorbate peroxidase	5.67±1.2^{a}	4.8±1.09^{a}	7.54±1.23^{b}	6.98±0.99^{b}	6.84±1.2^{b}	8.34±1.12^{c}	6.12±1.54^{b}	5.4±1.02^{a}	7.98±1.12^{b}	5.89±0.99^{a}
Glutathione reductase	1.23±0.2^{a}	1.56±0.1^{b}	2.01±0.13^{b}	1.65±0.15^{b}	1.63±0.7^{b}	2.01±0.12^{c}	1.76±0.56^{b}	0.76±0.1^{b}	1.55±0.13^{c}	2.13±0.17^{d}

Values represent means of replicates with SE; Different letters are significantly different between the treatments at P≤0.05.

Table 6: Effect of different fungal antagonists on unit activity of different antioxidant enzymes in tomato fruits post-inoculated (POI) with *A. alternata*.

Antioxidant enzymes UA mg^{-1} Prot g^{-1} (F.W.)	Inoculated fruits	*Gonatobotrys simplex* (cfu/ml)			*Penicillium chrysogenum* (cfu/ml)			*Trichoderma harzianum* (cfu/ml)		
		1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}	1 x10^{8}	1 x10^{10}	1 x10^{12}
Superoxide dismutase	1.98±0.2^{a}	1.02±0.3^{a}	1.23±0.45^{a}	1.43±0.29^{b}	1.39±0.23^{b}	1.28±0.19^{b}	1.67±0.14^{a}	2.01±0.12^{a}	2.97±0.1^{b}	1.78±0.19^{a}
Guaiacol peroxidase	0.24±0.1^{a}	0.21±0.09^{a}	0.28±0.08^{a}	0.24±0.06^{a}	0.34±0.04^{b}	0.39±0.08^{b}	0.43±0.03^{b}	0.53±0.06^{b}	0.84±0.05^{c}	0.59±0.071^{b}
Catalase	5.38±1.2^{a}	5.69±0.98^{a}	6.7±0.78^{b}	5.63±0.68^{a}	6.78±1.09^{b}	6.89±0.9^{b}	6.75±0.79^{b}	6.05±0.98^{b}	7.67±0.78^{b}	5.45±0.45^{a}
Ascorbate peroxidase	5.67±1.2^{a}	4.54±1.07^{a}	5.12±0.98a	5.23±0.96^{a}	6.06±0.97^{a}	7.89±0.56^{b}	6.65±0.56^{b}	4.53±1.09^{a}	7.02±1.02^{b}	5.34±0.97^{a}
Glutathione reductase	1.23±0.2^{a}	1.12±0.1^{a}	1.31±0.08^{b}	1.25±0.079^{a}	1.12±0.09^{a}	1.45±0.08^{b}	1.53±0.05^{c}	1.08±0.2^{a}	1.27±0.09^{a}	2.34±0.07^{b}

Non-enzymatic antioxidant molecules

Effect of application of three different antagonists on ASA are given in Table 7 and 8. In PRI application of antagonists, there was maximum increase in ASA content after application of *T. harzianum* followed by *P. chrysogenum* and *G. simplex* at 10^{12} cfu/ml as compared to IN fruits inoculated with *A. alternata* alone. On the other hand, differential results were noticed after POI such that application of *T. harzianum* and *G. simplex* caused a significant reduction in ASA content over IN fruits and fruits treated with *P. chrysogenum* showed insignificant changes in ASA content as compared to IN fruits. TPC of PRI as well as POI treated fruits with fungal antagonists are also detailed in Table 8 and 9. Maximum enhancement in TPC occurred in tomato fruits treated with *P. chrysogenum* at 10^{12} cfu/ml followed by *T. harzianum* and *G. simplex* at 10^{10} cfu/ml as compared to IN fruits. In POI application, significant changes were noticed only for *T. harzianum* followed by *P. chrysogenum* at 10^{10} cfu/ml over IN fruits. Maximum enhancement was noticed for POI tomato fruits treated with *T. harzianum* at 10^{10} cfu/ml.

G. simplex was the most effective antagonist in increasing PL content followed by *T. harzianum* at 10^{10} cfu/ml. In POI, maximum enhancement in PL content was noticed for *T. harzianum* treated fruits followed by *G. simplex* at 10^{10} cfu/ml as compared to IN fruits. Changes were insignificant for *P. chrysogenum* at both PRI as well as POI. Out of PRI and POI highest change in PL was recorded for *G. simplex* treated fruits at 10^{10} cfu/ml (Table 7 and 8).

GSH content of challenged tomatoes with *A. alternata* and exposed to various concentrations of fungal antagonists are given in Table 7 and 8. In PRI application of fungal antagonists, maximum GSH content was noticed for *T. harzianum* at 10^{12} cfu/ml as compared to IN fruits. *G. simplex* was the next most effective antagonist with maximum enhancement of GSH at 10^{10} cfu/ml followed by *P. chrysogenum* at 10^{12} cfu/ml. Differential response was noticed after POI application. There was a significant decrease in GSH content after application of antagonists used except *T. harzianum* in which GSH content showed significant enhancement.

Overall, *T. harzianum* was superior in enhancing different antioxidants of tomato fruits inoculated with *A. alternata*.

These findings go well with the study of Ahmad *et al.* (2014), who noticed an enhancement in phenolics in tomatoes inoculated with *A. alternata* and treated with *P. oxalicum* as an antagonist. Overall, among different fungal antagonists used, spore suspension of *T. harzianum* was able to cause maximum inhibition of mycelial growth of *A. alternata* while *G. simplex* was able to reduce rot development under *in vivo* conditions. Overall, the application of *T. harzianum* could up-regulate maximum modulation of different biochemical parameters. The comparison among different antagonists is given Fig: 5.

Table 7: Effect of different fungal antagonists on concentration of different non-enzymatic antioxidants in tomato fruits pre-inoculated (PRI) with *A. alternata.*

Non-enzymatic antioxidants (mg g^{-1} (F.W.)	Inoculated fruits	*Gonatobotrys simplex* (cfu/ml)			*Penicillium chrysogenum* (cfu/ml)			*Trichoderma harzianum* (cfu/ml)		
		1 x10^8	1 x10^{10}	1 x10^{12}	1 x10^8	1 x10^{10}	1 x10^{12}	1 x10^8	1 x10^{10}	1 x10^{12}
Ascorbic acid	7.57±0.5^a	1.66±0.9^b	3.32±0.52^c	8.34±0.32^a	6.33±1.2^a	9.23±1.87^b	8.98±1.09^a	3.56±0.9^b	9.32±0.16^b	8.56±0.18^b
Total phenol	13.2±1.2^a	11.9±0.8^b	14.52±1.1^a	11±0.65^b	11.32±0.3^b	7.5±0.21^b	17.5±0.19^c	12.5±0.7^b	17.43±0.65^c	14.32±0.9^d
Proline	5.47±0.4^a	5.39±0.9^a	9.83±0.89^b	7.32±0.61^b	3.26±0.8^b	5.93±0.56^a	6.12±0.67^a	4.36±0.3^a	9.02±0.33^b	5.99±0.25^a
Glutathione	5.9±0.73^a	4.43±0.9^a	7.51±0.52^b	6.83±0.32^a	6.63±0.9^a	3.25±0.25^b	6.99±0.57^a	6.56±0.9^a	8.53±0.82^b	10.91±0.52^c

Table 8: Effect of different fungal antagonists on concentration of different non-enzymatic antioxidants in tomato fruits post-inoculated (POI) with *A. alternata*.

Non-enzymatic antioxidants (mg g^{-1} (F.W.)	Inoculated fruits	*Gonatobotrys simplex* (cfu/ml)			*Penicillium chrysogenum* (cfu/ml)			*Trichoderma harzianum* (cfu/ml)		
		1 x10^8	1 x10^{10}	1 x10^{12}	1 x10^8	1 x10^{10}	1 x10^{12}	1 x10^8	1 x10^{10}	1 x10^{12}
Ascorbic acid	7.57±0.5^a	2.34±0.89^b	4.56±0.79^c	4.32±0.24^c	7.09±0.49^a	7.65±0.74^a	7.23±0.81^a	4.56±0.45^b	4.43±0.39^b	5.34±0.43^b
Total phenol	13.2±1.2^a	10.23±1.2^b	12.23±.1.4^a	11.32±1.98^a	13.42±1.2^a	14.89±1.1^a	13.45±1.1^a	11.09±1.9^a	15.67±1.1^b	13.42±1.43^a
Proline	5.47±0.4^a	5.23±0.14^a	7.82±0.24^b	6.54±0.34^a	5.31±0.13^a	5.98±0.19^a	6.01±0.89^a	5.12±0.19^a	8.09±0.29^b	5.83±0.43^a
Glutathione	5.9±0.73^a	4.32±0.29^a	4.59±0.31^a	5.12±0.39^a	5.12±0.19^a	5.87±0.24^a	5.89±0.43	5.31±0.18^a	9.02±0.28^b	11.23±0.67^c

Values represent means of replicates with SE; Different letters are significantly different between the treatments at P≤0.05.

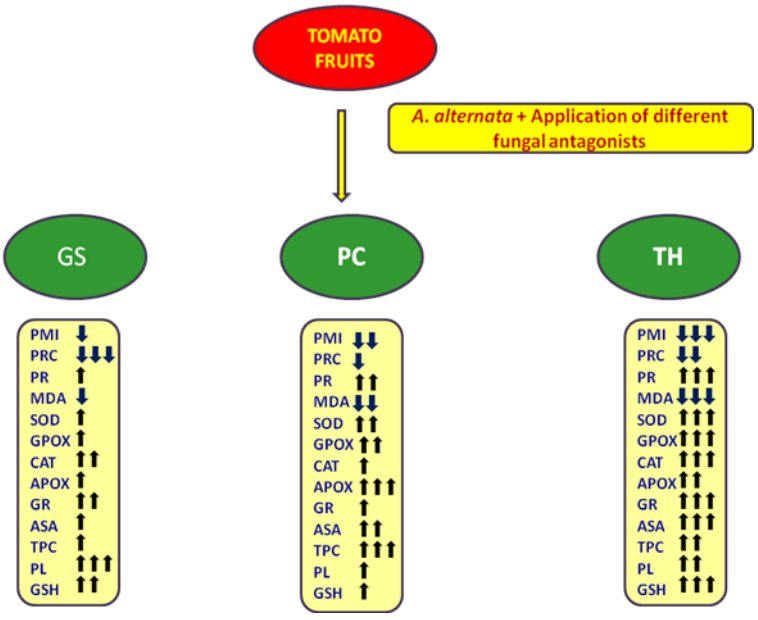

Figure 5 Overall comparison between different fungal antagonists.

IV CONCLUSIONS

In conclusion, the study unveiled that the treatment of inoculated tomato fruits with various fungal antagonists improved the tolerance of substrate (tomato fruit) against pathogenic stress incited by *A. alternata*. This is elucidated by the observation that despite the negative effect of pathogen on lipid peroxidation and protein content, the treated fruits gave better results and possessed more biomass as that of untreated control. The reduction in pathogenic stress by application of different treatments resulted in: a) better fruit biomass, b) prevented membrane damage as revealed by a reduction in MDA content, c) augmentation of innate antioxidant enzymes as well as non-enzymatic anti-oxidant system of tomato fruits. Better antioxidant capacity facilitates proficient scavenging of reactive oxygen species, thereby preventing tomato fruits from oxidative damage.

The reduction of MDA content by application of different treatments can be co-related with the enhancement in total phenolic content of tomato fruits. Phenols have the strong ability to donate electrons or hydrogen atoms thus neutralizing various ROS which are involved in the lipid peroxidation. Moreover, they also modify membrane lipid packing order and decrease fluidity of membranes. These changes could strictly hinder diffusion of free radicals and thus reduce peroxidative reactions. Moreover, other enzymatic antioxidants such as SOD, GPOX, APOX, CAT and GR and non-enzymatic antioxidants like ASA, TPC, PL and GSH are also involved in the detoxification of various ROS that are responsible for peroxidation of lipids. Thus, the enhancement of various enzymatic as well as non-enzymatic antioxidants by application of various treatments is responsible for the amelioration of the pathogen stress.

The mitigation of pathogenic stress in tomatoes by these non-chemical methods entails a myriad of antioxidant enzymes as well as non-enzymatic antioxidants that interact with one another to resist the effect of invading pathogen. It is, therefore, recommended that this non-chemical, eco-friendly integrated module of plant extracts, fungal antagonists and 24-epibrassinolide be used for effective management of the *Alternaria* rot of tomato fruits.

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