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Alternaria Alternata Infection in Tomato Adversely Affects the Nutritional Parameters of Tomato

Arti Tiwari* and Ram Sanmukh Upadhyay

Laboratory of Mycopathology and Microbial Technology, Center of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005.

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The study aims the determination of nutritional components in tomato fruits after infection by *Alternaria alternata*. The tomato (*Solanum lycopersicum)* a foremost vegetable crop, possesses significant number of nutritional components which provide prevention and cure for various important diseases. Tomato plants are sternly affected by the fungus *A. alternata* which causes stem canker, leaf spot, and fruit rot diseases. The objective of the present study was to investigate the lycopene, β-carotene, total carotenoides and proline content in tomato fruits infected by *A. alternata*. The lycopene content was observed maximum at 0-hours after treatment with *A. alternata* and was found to be declined up to 96-hours. The highest percent decrease in lycopene content of 85.12% was calculated in 96 hours after treatment. A similar trend was also observed in the case of β-carotene and total carotenoids content. The highest decrease of 30.82% in β-carotene and 73.92% in total carotenoides was observed in 96 hours after treatment with *A. alternata*. An increment in proline content was found in every 24 hours and the maximum content was estimated at 96-hours after treatment of *A. alternata*. The results indicated that *A. alternata* has a colossal effect on all the studied components of tomato fruits.

Keywords: *Alternaria alternata,* carotenoids, lycopene, proline, tomato.

Introduction

Tomato (*Solanum lycopersicum* L.) is second most extensively consumed vegetable after the potato (Ilić *et al.*, 2014) and known as one of the world's most imperative vegetable crops. From a nutritional point of view, the tomato is popularly known as "The Poor Man's Apple", which is one of the chief vegetable crops in India. The French people named it as 'The Apple of Love' whereas the Germans as 'The Apple of Paradise' (Rick 1978; Vishnu and Patil, 2014).

Tomato is consumed in various forms directly in the form of salads, as ingredients in many recipes, processed products, and juices and in the form of soups. Tomato is one of the low-calorie vegetable, holding just 18 calories per

***Corresponding author**: Arti Tiwari; **E-mail**: aarti0222@gmail.com

100 grams. Tomato fruits are very low in fat contents and have zero cholesterol level. Nevertheless, they are excellent sources of antioxidants, dietary fiber, carbohydrates, flavor compounds, minerals, protein, fats and glycoalkaloids amino acids, minerals, and vitamins (Stahl and Sies, 1996; Clinton, 1998; Rao and Agarwal, 1999; Davies and Hobson, 1981; Gundersen *et al.,* 2001). In addition to the vitamins, antioxidants and other compounds present, the tomato fruit also include several flavonoid compounds namely quercetin, kaempferol, rutin, myricetin and naringenin, as well as phenolic acids including chlorogenic acid, gallic acid (Hallman, 2012), rutin, plastoquinones, tocopherol, and xanthophylls (Beecher, 1998; Leonardi *et al.,* 2000). The presence of majorly of nutritive compounds leads nutritionists and dieticians to recommend tomatoes in a diet for weight loss and cholesterol control programs. The antioxidants are found to be defensive against cancers, including colon, prostate, breast, endometrial, lung, pancreatic tumors and several cardiovascular diseases (Giovannucci *et al.,* 1995; Gerster, 1997; Clinton, 1998; Giovannucci, 1999; Paiva and Russell, 1999). Tomato contains chlorogenic acid and coumaric acid too, that protect the body from carcinogens which are produced by cigarette smoke. Tomatoes help in improving the functioning of the digestive system and liver, and also prevent constipation.

Lycopene, a flavonoid antioxidant, is a unique phytochemical compound present in the tomatoes. It imparts red fruit color and also acts as a dietary antioxidant. Lycopene along with carotenoids may help protect cells and other structures in the human body from harmful free radicals and singlet oxygen (Di Mascio *et al.,* 1989; Stahl and Sies, 2003). Various researches showed that lycopene protects the skin from ultra-violet (UV) rays and thus offers some defense against skin cancer (Johnson, 2002; Sies and Stahl, 2003). β-carotene is well known as a pro-vitamin A carotenoid and is an essential nutrient in the human diet due to retinoid activity (Tee, 1992; Omenn *et al.,* 1994). It has been known that intake of high β-carotene containing fruit and vegetables can be associated with a reduced risk of heart disease and certain cancers (Ziegler, 1989; Doll, 1990; Block *et al.,* 1992; Omenn *et al.,* 1994). β-carotene has been identified for performing the role in various vital activities such as immunity booster, antioxidants, cell communication promotion, cancer prevention, eyesight improvement and protection of skin from oxidative damage. Carotenoids are water-insoluble pigments possessing 40 carbon rings. Carotenoids have a major role in plants i.e. photosynthesis (Gross *et al.,* 1991), provitamin and antioxidants (Kritchevsky, 1999).

Proline is one of the proteogenic amino acids which contains α-amino group as a secondary amine. Proline plays a major role in plant-pathogen interaction and cell death as it protects protein structures from denaturation and

detoxification of hydroxyl radicals (Fabro *et al.*, 2004; Verslues and Sharma, 2010). It is also involved in redox buffering and energy transfer. Several types of plant stresses cause proline to accumulate to high levels in many plant species ((Hare *et al.*, 1999; Ruiz *et al.*, 2002; Rivero *et al.*, 2004; Claussen, 2005).

In recent years numerous research articles have been published in the area of food, health and nutrition but still there are hardly few articles reciting the effects of pathogens (fungi) on nutritional components of tomato fruits. The effect of the pathogen on the physiological and biochemical properties of different plants by fungi, bacteria and viruses are well known by various research studies but still the effect on the dietary parameters are unknown. The aim of the present study was to investigate the effect of *Alternaria alternata* on some nutritionally important dietary components in tomato fruits.

Materials and methods

Isolation and Identification of Alternaria alternate

Infected tomato fruits showing typical disease symptoms (Fig. 1) were collected from different vegetable farms of Banaras Hindu University (BHU) and brought into the laboratory. The diseased fruits were immediately given a surface wash with sterile distilled water to remove all the dirt and dust adhered (care was taken not to dislodge much of the black sooty mass having spores and mycelia of the pathogen). The infected portions of the fruits were cut into small pieces of approximately 2 cm diameter. These tissues were then placed on potato dextrose agar (PDA) medium and incubated at 25-27℃ for seven days and were observed daily for the fungal growth. A mycelial plug was cut from the margins of the growing colony with the help of a cork borer and this plug was aseptically placed on fresh PDA plates for subculture. The pure isolate was obtained by subsequent transfer of the culture obtained on a regular basis on PDA plates (Figure 2a and 2c). Once the pure isolates were obtained, their microscopic examination was carried out for identification. These slides were observed under 10X and 40X magnification of Dewinter light microscope (Fig. 2b and 2d). The conidial morphology of each fungal isolate was carefully noted and compared with those given in the standard manuals for its confirmation. The identification of the cultured pathogen as *Alternaria alternata* was also carried out based on colony morphology and conidial size (Ellis, 1971; Shakir *et al.*, 1997). The pathogenic cultures were maintained on PDA slant at 25℃. The isolates were sub cultured on the regular basis in a customary time interval.

Fig. 1 Tomato disease symptoms

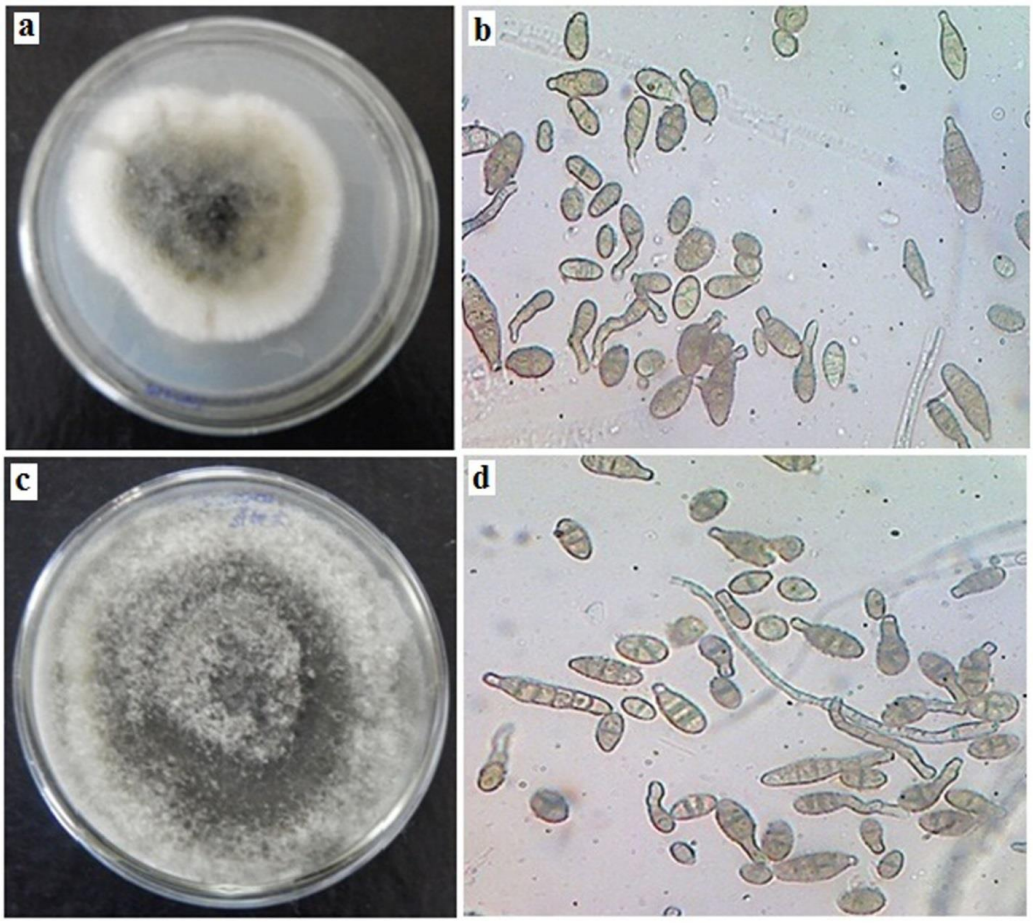

Fig. 2 Isolats on PDA and spores

Pathogenicity test

Pathogenicity test for all isolates of *A. alternata* was done by following Koch's Postulates method. The pathogenicity test was carried out by inoculating uninjured 15-20 cm long plant with the spore suspension of *A. alternata* containing $4X10^5$ spores/ml followed by 48-hours of incubation in a humid chamber and then plants were placed in the green house bench. Isolation of the pathogen was done again from the artificially infected tomato plants. Spores and culture morphology were compared with the isolates from naturally infected plants. Greenhouse temperature ranged from 25 °-29 ℃ with a 14-hours light and 10-hours dark cycle.

Preparation of samples

The seeds of tomato (cv. Punjab chhuhara susceptible to *Alternaria alternata* were obtained from Indian Institute of Vegetable Research (IIVR), Varanasi and were sown in the field for the experiments. The less ripened tomato fruits were collected and washed properly. The tomato fruits were teased with the help of sand paper and spore suspension containing $4X10^5$ spores/ml of *A. alternata* was inoculated to these tomato fruit samples. Treated tomato fruits were kept in a plastic tray containing moist blotting paper and the tray was covered with transparent cling film to attain moisture appropriately (Fig. 3).

Fig. 3 Tomato fruits in moisture appropriately

Lycopene content

Lycopene content was estimated according to Concepcion and Gruissem (1999) with slight modifications. Tomato fruits (3 gm) were cut into halves, the seeds were removed and the pericarp and mesocarp were ground in liquid nitrogen and extracted with 10 ml of acetone: hexane (2:1) solution. The suspension was centrifuged at 5,000g for 10 min in 50 ml centrifuge tubes. The upper hexane layer was removed and 1:10 dilution of this extract was determined in the spectrophotometer at 453, 505 and 663 nm using hexane as blank. The experiments were done in triplicates.

β-carotene content

Beta-carotene content was estimated according to the Nagata and Yamashita (1992) and Azeez *et al.* (2012) with slight modifications. Samples (3 gm) were ground in liquid N_2 and extracted with 10 ml of acetone: hexane (4:6) mixture and filtered through Whatman No. 4 filter paper. Absorbance was measured at different wavelength (663, 505, and 453 nm). This assay was carried out in triplicates. β-carotene was measured by using the following formula,

$$\beta\text{-carotene (mg/100ml)} = 0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}.$$

The values were expressed in mg/100 ml.

Total carotenoids content

Total carotenoids were estimated by Zakaria *et al.* (1979). The experiment was carried out in the dark to avoid photolysis of carotenoids once the saponification was complete. The sample (3 gm) was homogenized and saponified with 2.5ml of 12% alcoholic potassium hydroxide in a water bath at 60℃ for 30 minutes. The saponified extract was transferred to a separating funnel containing 10-15 ml of petroleum ether and mixed well. The lower aqueous layer was then transferred to another separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colorless. The absorbance of the yellow color was observed in a spectrophotometer at 450nm and 503 nm using petroleum ether as blank.

Proline Content

Proline extraction and colorimetric determination using acidic ninhydrin reagent (2.5 g ninhydrin/100 mL of a solution containing glacial acetic acid, distilled water and ortho-phosphoric acid 85% at a ratio of 6:3:1) was carried out as Chinard (1952) and Bates *et al.* (1973). The tomato fruits (3 gm) were ground in a mortar following the addition of 10 ml of 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was filtered through two layers of Whatman No. 2 filter paper, and the clear filtrate was then used in the assay. Glacial acetic acid and ninhydrin reagent (1 ml each) were added to 1 ml of the filtrate. The closed test tubes with the reaction mixture were kept in a boiling water bath for 1 h, and the reaction was terminated in an ice bath for 5 min. The reaction mixture was extracted with 4 ml toluene, mixed robustly with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance determined at 520 nm using toluene as a blank. The proline concentration was calculated from a standard curve and calculated on a fresh weight basis (µM / g FW).

The standard proline having the purity of 95% obtained from the M/S Sigma-Aldrich, Bombay and all the chemicals used for analysis were of HPLC grade. The stock solution of standard proline was prepared by different concentration from 10 µM to 100 µM and was plotted against absorbance at 520 nm.

Data Analysis

All the experiments were repeated twice and the results of two experiments were in close agreement. The data from two experiments were combined. All data were analyzed by one-way analysis of variance (ANOVA) in the statistical software SPSS 16.0. Mean separations were performed by Duncan's multiple range tests (DMRT). The significance was determined at $p<0.05$.

Results and discussions

In order to study the interaction of any pathogen with its host plant, the prime and most important effort is to isolate and characterize that pathogen. To accomplish this purpose, a piece of tissue obtained from the diseased tomato plants was placed on PDA media. A small-spored long-chained pathogen grew from 95% in the tissues and in most of the isolations this pathogen was

obtained. The cultures on PDA under fluorescent lights were at first fluffy and off-white but become dusky neutral gray with an off-white border within 48 hours (Fig. 2a). The colony then extended over the entire plate, sporulation was profuse and the colony became appressed and almost black. The conidiophores were brown, straight, bearing light brown conidia present in long chains and were obclavate and muriform, surface smooth to verruculose, with a short conical or cylindrical, pale beak, less than one-third of the length of the conidium. The conidia had 3-7 transverse septa and typically numerous longitudinal or oblique septa (Fig. 2b and 2d).

Artificially infected tomato plants showed disease symptoms in the pathogenicity test. Re-isolation of the pathogen from artificially infected tomato plants on PDA media showed similar growth and morphology on the plates as isolated from the naturally infected tomato plants. Results showed pathogenicity of *Alternaria* isolates after 14 days. Pathogenicity test confirmed *Alternaria* as *Alternaria alternata*. The most virulent pathogen was used for further experiments on the basis of disease severity caused in unnaturally infected tomato plants in the laboratory.

In the study it was found that lycopene content decreased steadily from 0-hour to 96-hours after *A. alternata* treatment as comparison to untreated control fruits (Fig. 4). Lycopene content was observed maximum at 0-hour post treatment and which was minimum at 96-hours post treatment. The percent decrease in lycopene content was calculated as 25.62% in 24-hours, 58.40% in 48-hours, 77.68% in 72 and the highest decrease of 85.12% was observed at 96-hours after *A. alternata* treatment. The result showed that *Alternaria* influenced enormously the lycopene content of tomato fruits and diminished the lycopene along with other components. Numerous studies of lycopene assay have been already conducted using tomatoes (Maruyama *et al.,* 2015; Vinha *et al.,* 2014; Kumcuoglu *et al.,* 2014; Ilahy *et al.,* 2011; Beerh and Sidappa, 1959; Adsule and Dan, 1979; Sadler *et al.,* 1990; D'Souza *et al.,* 1992; Arias *et al.,* 2000) but still there are very fewer studies on interaction with fungi.

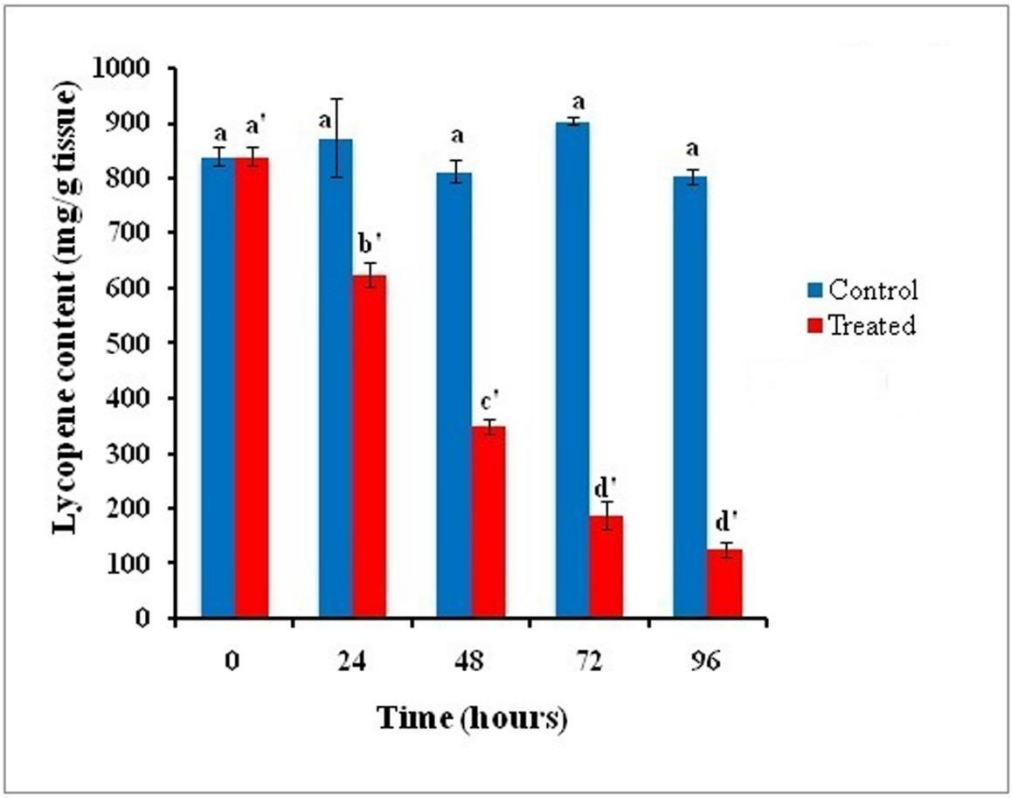

Fig. 4

β-carotene is well-recognized disease preventing, health promoting and vitamin A active carotenoid found in tomato fruits. From the results, it can be concluded that *Alternaria* had a lesser effect on the β-carotene content. β-carotene content was also found highest at 0-hour post treatment and then it was decreased slowly up to 96-hours. A significant decrease in the amount of beta-carotene was observed at 96-hours after treatment (Fig. 5). The percent decrease in beta carotene content was investigated as 4.51%, 12.03%, 21.80% and 30.82% respectively at 24, 48, 72 and 96 hours from 0 hours. Abushita (1997) reported that β-carotene was gradually increased in tomatoes as ripening progressed and was most intense at the highest level of fruit maturity in floriset cultivar which was not in accordance with the observation of Biacs *et al.* (1987), who found that β-carotene approached its maximum level in yellow colored fruits of Ventura cultivar and then declined. This variation is possibly due to the effect of some varietal factors involved in carotenogenesis in tomato fruit.

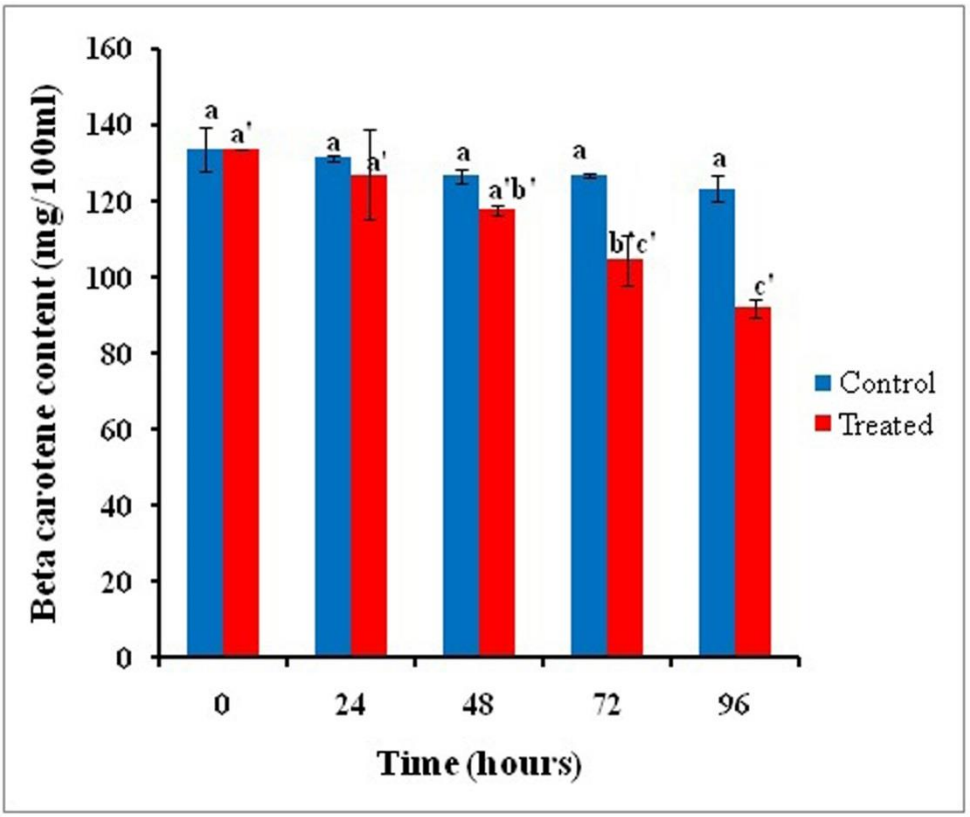

Fig. 5

Total carotenoids constitute all the carotenoids existing in the tomato fruits including lycopene, α and β-carotenes, xanthophylls, leutins etc which are one of the most crucial components of tomatoes. In the present study it was found that total carotenoids decreased from 0-hour to 96-hour after treatment of *A. alternata*. Total carotenoids were found to be highest at 0-hour and then decreased progressively up to the lowest level at 96-hours (Fig. 6). The content of total carotenoids declined swiftly by 56.28% after 24-hour post treatment and then decreased slowly up to 96-hours. The percent decrease observed were 60.68%, 69.11% and 73.92% after 48, 72 and 96-hours respectively. Total carotenoids content was affected significantly during first 24-hours after treatment and then very little decrease was found. This shows that *Alternaria* seriously affects total carotenoids in the first twenty-four hours.

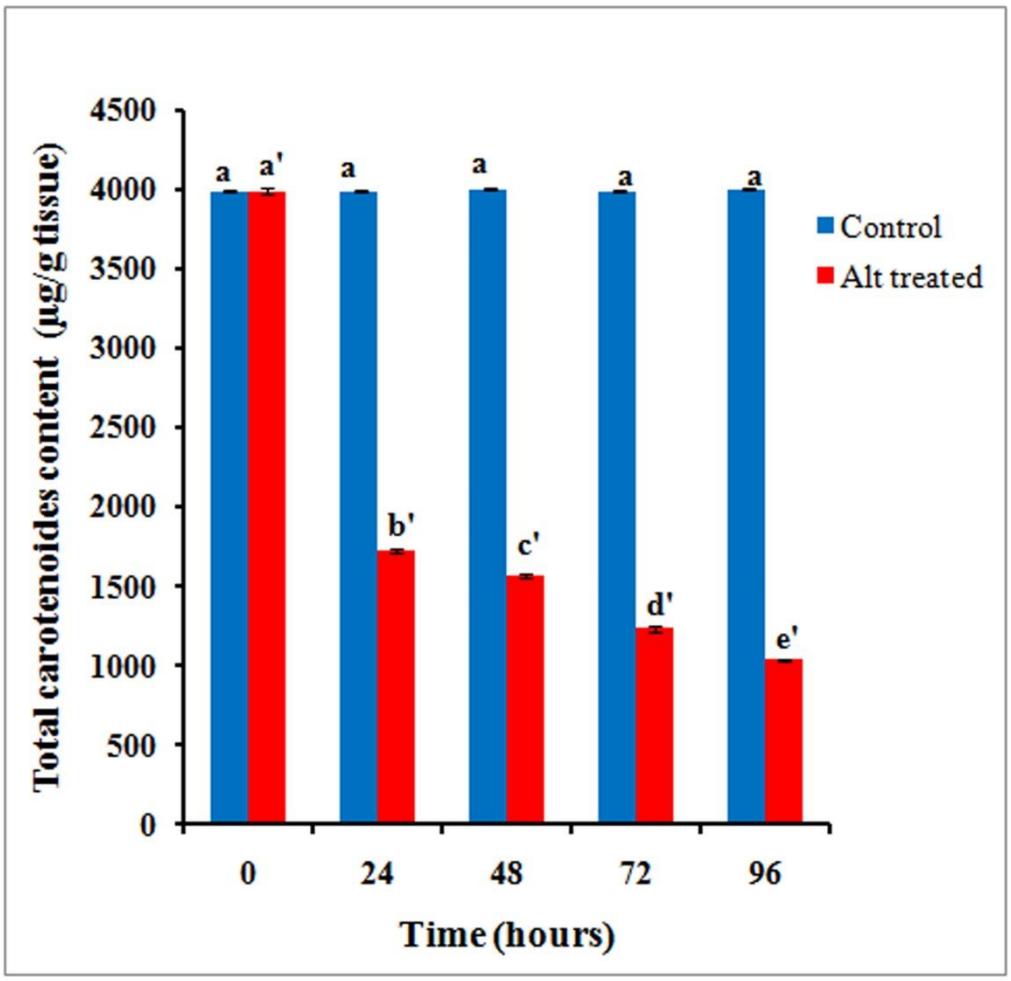

Fig. 6

The proline content was found to be slowly augmented from 0-hour to 48-hours, but fast increase in its content was found after 48-hours and the maximum amount of proline was accumulated at 96-hours (Fig. 7) after treatment with *A. alternata*. Rosales *et al.* (2007) found an increase in proline content in cherry tomato fruits. The reason for proline accumulation in stressed plants may be due to protein breakdown (Becker and Fock, 1986), inhibition of protein synthesis (Dhindsa and Cleland, 1975) and inhibition of leaf development (Davies and Van Volkenburg, 1983). Proline has various roles to play in plants. For example it play role in regulation of cell pH, protein stability, and increased protection from the cold and adjusts the redox potential; the increased enzyme leads to greater cell compromise with stress and protects the cytosol and the structures of the cell. Proline is accumulated predominantly in

the cytoplasm to balance the osmotic potential (Arnon 1949). Many plants synthesize proline as non-toxic protective at salinity conditions (Slama *et al.*, 2014). During stress, proline synthesis is induced and its amount becomes large because proline is a key amino acid in adjusting osmotic potential (Ashoori *et al.*, 2015). Thus during infection *A. alternata* in tomato remoulds the enzymatic processes leading to the formation of increased proline.

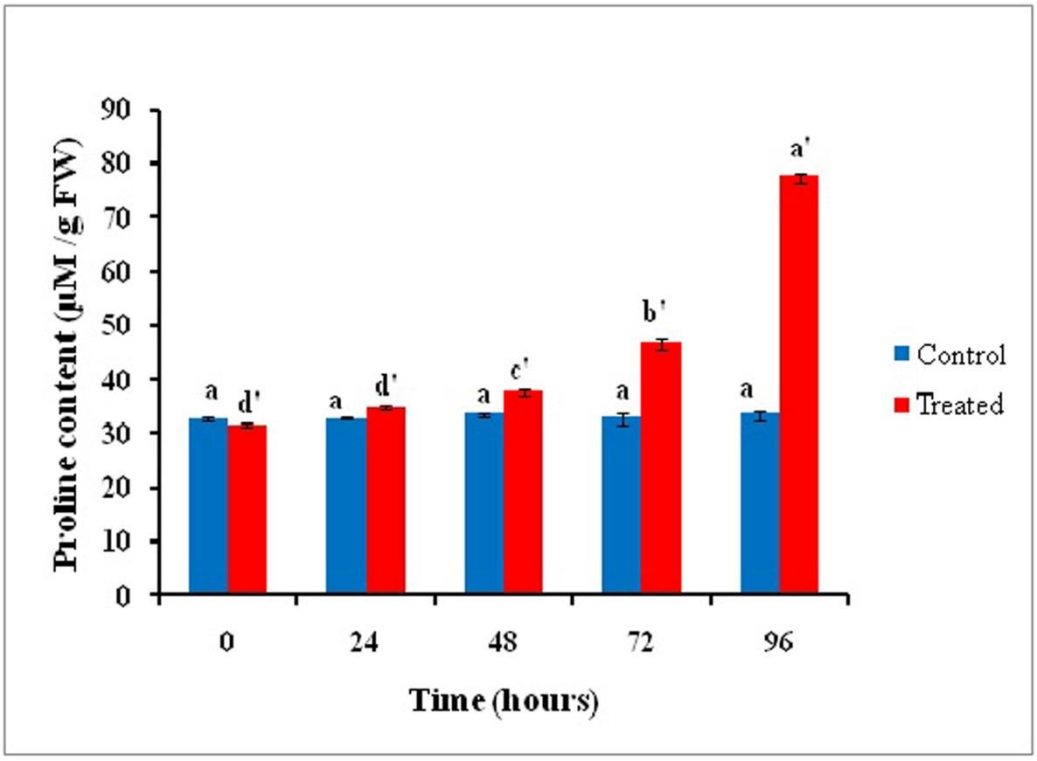

Fig. 7

Conclusion

The maximum content of lycopene was observed at 0-hours after treatment in *A. alternata* treated tomato fruit samples and lowest content was investigated at 96 hours post treatment. A similar trend was obtained in the β-carotene and total carotenoids content. The highest amount was accumulated at 0-hours and the lowest at 96-hours post treatments. It clearly shows that the *Alternaria* is inhibiting the formation and diminishing the level of these components in tomato fruit samples in various trends. Proline showed the different phenomenon from other components after infection of *Alternaria*. The proline content was found to be increased slowly from 0-hours to 96-hours and maximum content was calculated at 96-hours post treatment with 148 %

increase (highest) in comparison to the content at 0 hours. *Alternaria* majorly affects the enzymes involved in the synthesis and maintenance of the lycopene content, β-carotene content, total carotenoids and the proline content. Based on the obtained results, it can be concluded that while observing all the samples, a significant decrease in the lycopene, β-carotene and total carotenoids content was recorded. A noteworthy amount of increase was observed for proline content.

Through this study, further the mechanism of action of *Alternaria* on these tomato components can be achieved and the site at which *Alternaria alternata* is functioning and diminishing or gaining the components can be further studied in detail. This study could be an effective strategy to unlock the sites and enzymes involved in the synthesis of essential compounds, being affected by this fungus.

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