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STUDIES ON MANNITOL PRODUCTION DURING INFECTION OF *LYCOPERSICUM ESCULENTUM* WITH *ALTERNARIA ALTERNATA*

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ABSTRACT
The present work was carried out with an objective to characterize the production of Mannitol during *Alternaria alternata* and tomato plant interaction leading to pathogenesis. Mannitol, as a quencher of ROS takes an important role in host pathogen interaction by allowing the fungus to suppress ROS mediated plant response. To examine this, naturally occurring infected tomato plant was identified and characterized as *A. alternata* using culture techniques as well as molecular techniques using PCR ampliphication. The production of mannitol in plant tissues infected with host pathogen was observed and conformed by using TLC and HPLC that mannitol was being produced in plant infected tissues but not in healthy plant tissues. From the result it is proved that mannitol is somewhere required by *A. alternata* for its pathogenicity during interaction with tomato and is important for normal disease development.

KEYWORDS: PCR ampliphication, infected tomato, mannitol, pathogenicity.

INTRODUCTION

Tomato (*Lycopersicum esculentum*, Family-Solanaceae*)* is extensively used as a model plant for genetic studies related to fruit quality, stress tolerance (biotic and abiotic) and other physiological traits. Current advance in biotechnology, including structural and functional genomics, can provide important tools for tomato improvement in developed and developing countries (Mutsukura *et al*., 2008). During the last two decades the use of molecular marker has facilitated identification, mapping and transfer of many disease resistance genes into tomato (Foolad 2007; Labate *et al*., 2007). Tomato fruits have good resources of vitamin A, C and minerals, recent studies have shown the importance of lycopene a major component of red tomatoes, which has antioxidant properties and may help to protect against diseases, such as cancer and heart disease. It also causes upper respiratory tract infections in AIDS patient asthma in people with sensitivity (Droby *et al*., 1984). Tomato plant shows susceptibility to the pathogen *Alternaria alternata f. sp. lycopersici*. This pathogen causes leaf spot disease of tomato plants (*Lycopersicum esculentum*) which leads to reduction in photosynthetic yields. *Alternaria alternata* a weak pathogen, attacking already stressed plants, but may also be pathogenic on healthy plants, grows in long chains, with dark brown conidiophores and conidia (Ellis and Ellis, 1985). The fungal plant interplay depends on mutual recognition, signaling, and the expression of pathogenicity and virulence factors, from the fungal side, and the existence of passive, preformed, or inducible defense mechanisms in the plant, resulting in compatible (susceptibility) or incompatible (nonhost, basal or host specific resistance) interactions. The interaction of both gene products leads to the activation of host defense responses, such as the hypersensitive response, that arrests the growth of fungi. *Alternaria* species have different lifestyles, ranging from saprophytes to endophytes to pathogens (Thomma, 2003). One reason for the success of these pathogens may be attributed to their production of diverse phytotoxins (Nishimura and Kohmoto 1983, Montemurro and Visconti 1992). The host-selective toxins (HSTs) produced by many members of the genus *Alternata* have unique modes of action and toxicity to their respective host plants. Production of HST is critical for successful pathogenesis because HST-deficient mutants are incapable of attacking their host plants (Hatta *et al*., 2002, Harimoto *et al*., 2007). It has been shown that poyhydroxy alcohol mannitol ($C_6H_8(OH)_6$) production and secretion by a fungal pathogen of tomato, *Alternaria alternata*, is massively induced by host extracts (Jenning *et al*., 1998). A variety of different physiological functions have been attributed to these polyols, among them carbohydrate storage, translocation, osmoprotection, and coenzyme regulation and storage of reducing power (Jennings, 1984). Polyols are frequently found in fungal spores and are thought to act as storage compounds. In imperfect fungi the mannitol cycle is made up of the following enzymes: mannitol 1-phosphate dehydrogenase, mannitol 1-phosphatase, mannitol dehydrogenase, and hexokinase. It is hypothesized that polyols produced by fungi "quench" oxidants generated by the oxidative burst, and therefore, the ability to synthesize and accumulate these polyols is necessary for the fungi to be able to suppress or resist this host defense (Chaturvedi *et al*., 1997). Because reactive oxygen serves such a central role in plant responses to pathogen attack, successful pathogens must be able to evade or suppress ROS-mediated defenses. In view of the above facts the present work was taken up with an objective to characterize mannitol production during tomato pathogenesis by *Alternaria alternata*.

MATERIALS & METHODS

Isolation and Identification of Fungal culture

The isolate of *Alternaria alternata* was isolated from a field infected tomato plant with typical stem canker symptoms. The leaves of infected tomato plants were collected and were given a surface wash with sterile distilled water followed by cutting into small circular pieces of approximately 2cm diameter. These discs were then placed on Potato Dextrose Agar (PDA) medium and incubated at 25-27°C under 12 hour light and 12 hour dark period for 5 days. Pure isolate was obtained by subsequent transfer of the culture obtained on a regular basis on PDA plates. The identification of the cultured pathogen as *Alternaria alternata* f. sp. *lycopersici* was carried out based on colony morphology and conidial size and its pathogenicity was confirmed by following Koch's postulates. The pathogenic culture was maintained on PDA slant at 25°C under 12 hour light and 12 hour dark period. The isolate was sub cultured after every 2 weeks.

Preparation of media for fungus growth

PDA and PDB media was used for growth of *Alternaria alternata*.

Plant material and growth

Seeds of tomato cv. Co-3 were grown in suitable thermocol pots filled with sterilized soil and kept under a 14 h light and 10 h dark cycle at 28±0.5°C and the plants were allowed to attain a height of 15-20 cm before testing for sensitivity to the pathogens and for other experiments.

Pathogenicity Test

Pathogenicity test was carried out by spraying uninjured 15-20 cm long plants to runoff with spore suspension, containing 3×10^5 spores/ml followed by 48 hours of incubation in moist chamber after which the plants were returned to the greenhouse bench. Greenhouse temperature ranged from 25-29°C with a 14 hour light and 10 hour dark cycle. The leaf tissue was scored for necrotic lesions after every 24 hours for 10 days. A control plant without pathogen infection was kept.

DNA extraction from the fungal isolate and PCR amplification

For extraction of fungal genomic DNA the fungal isolate was grown in potato dextrose broth for 15 days. After 15 days of growth the fungal mycelia was collected by filtration through a Buchner funnel and the resulting mycelium was collected and lyophilized. Approximately 0.5 g of lyophilized mycellar pad was grinded in a mortar and pestle and was transfered to a 50 ml tube. To this was added 10 ml of CTAB extraction buffer (0.1 M Tris-HCl pH 7.5, 1% CTAB, 0.7 M NaCl, 10 mM EDTA and 1% β-mercaptoethanol) and was gently mixed to wet all powdered pad. This was then incubated in a 65°C water bath for 30 min Following incubation it was cooled and an equal volume of chloroform/isoamyl alcohol (24:1) was added. The contents of the tube were mixed and centrifuged at 2000 *g* for 10 min at room temperature. The aqueous supernatant was transferred to a new tube and an equal volume of isopropanol was added. The resulting precipitate was rinsed with 70% ethanol. After rinsing the precipitate was air dried and 2 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 20 µg/ml RNAase A was added and placed in 37°C water bath for 1 h to resuspend the samples. Following this the sample was kept in water bath at 65°C for 10 min to deactivate the RNAase A. To check for the integrity of the extracted DNA 5 µl of it was electrophoresed on 1% agarose gel prepared in 0.5x TBE buffer at 100 V. Ethidium bromide to a final concentration of 0.5µg/ml was used in the gel. The presence of DNA in the gel was observed by a UV illuminated gel documentation system.

Following genomic DNA isolation from the fungal isolate PCR amplification from the DNA was carried out for identification of *Alternaria alternata*. Amplification reactions were performed in 25 µl reaction mixture containing 2.5 µl 10x reaction buffer (100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 0.01% (w/v) gelatin), 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1.5 µM of each primer, 1.5 units of TaqDNA-polymerase and 10-100 ng DNA. PCR cycling conditions consisted of denaturation at 94°C for 2 min, annealing at 70°C for 40 s, extension at 72°C for 1 min for 35 cycles and a final extension at 72°C for 10 min, followed by cooling at 4°C until recovery of the samples. To check for the specific amplicon in the amplified product 5 µl of the amplification product was separated on a 2% agarose gel by electrophoresis in 0.5x TBE buffer at 100 V. Ethidium bromide to a final concentration of 0.5µg/ml was used in the gel. The presence of DNA in the gel was observed by a UV illuminated gel documentation system. The PCR primers used for identification of *A. alternata* were as follows;

AAF2 5′ TGCAATCAGCGTCAGTAACAAAT 3′
(Forward primer)
AAR3 5′ ATGGATGCTAGACCTTTGCTGAT 3′
(Reverse primer)

Mannitol extraction and TLC separation method -

Mannitol was extracted from the healthy and pathogen infected tissues by killing the tissue samples in boiling 80% ethanol. This sample was left to stand overnight. Following overnight stand the ethanol fraction was collected. After that a further extraction for 5 min was given with boiling ethanol and again the ethanol fraction was collected. These two fractions collected were pooled. These extracts together form soluble extract containing mannitol.

Thin Layer Chromatography based separation and identification of mannitol in the prepared samples was carried out on silica gel 60 plates. Standards (2%) which consists of glucose, glycerol and mannitol are spotted along with cell extracts onto silica gel 60 plates and developed with acetonitrile : ethyl acetate: propanol: water (17:4:4:3) as the mobile phase. The spots are developed by spraying with 0.5% $KMnO_4$ in 1N NaOH.

Mannitol analysis by HPLC

HPLC System

Samples were analyzed by HPLC detector (Photodiode Array Detector - PDA Detector 2998) at 254 nm. The separation of mannitol was done by (C-18 Waters Spherisorb®, ODS2, 5.0µm, 4.6mm × 250mm column at 25 °C). The mobile phase consisted of 80% acetonitrile and 20% of double distilled water. The flow rate was 0.70 mL/min and run time was 20 minutes. The injection volume was 10µL.

RESULTS

Isolation of pathogen and performance of pathogenicity test

Pieces of tissue obtained from the diseased tomato plants were plated on PDA. A small-spored long-chained pathogen grew from 95% of the tissues and in most isolation this was the only fungus obtained. 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. 1A). After the colony extends over the entire plate sporulation is abundant and the colony becomes appressed and nearly black. The fungus *Alternaria alternata* was consistently isolated and identified based on morphological characters. Conidiophores were brown, straight, bearing light brown conidia formed in long chains and were obclavate and muriform, often with a short conical or cylindrical, pale beak, less than one third of the length of the conidium. Conidia had 3-7 transverse septa and usually several longitudinal or oblique septa. (Fig. 1B).

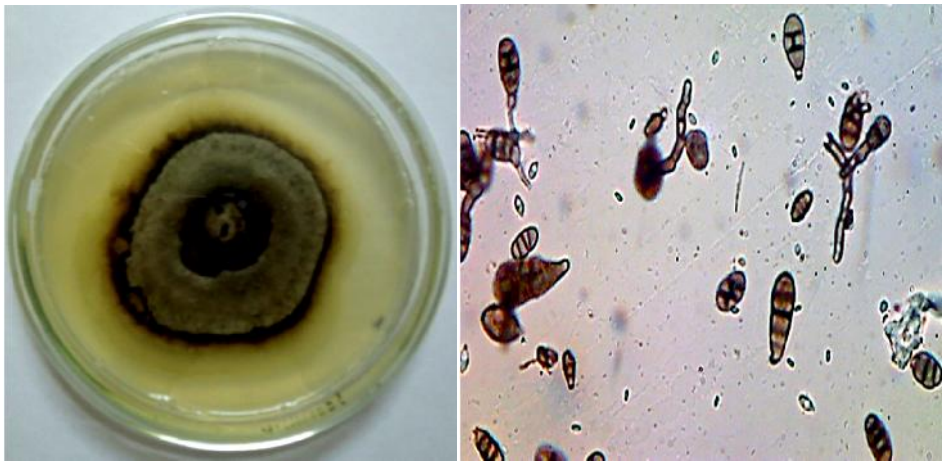

FIGURE 1: Colony morphology of *Alternaria alternata* grown on PDA (A); Conidia as seen under the light microscope (B)

The pathogen isolated was obtained in pure form by a series of regular subculture, carried out on the Potato Dextrose Agar medium. Once the pure isolate was obtained based on its colony morphology and conidial structure it was identified as *Alternaria alternata*.

Upon infection the isolate induced symptoms of sunken necrotic lesions on tomato stems in 3-4 days. Infection on leaves produced symptoms consisting of brown to black sunken necrotic lesions with typical concentric rings, which increased regularly and covered almost 3/4 of the leaf area within 10 days. The isolated pathogen from these infected portions produced similar kind of colony growth and conidial morphology and exhibited pathogenicity for tomato establishing the Koch's postulates. Isolation from diseased plants yielded cultures of *Alternaria* sp. similar in all respects to isolate used for inoculation. These results confirmed the isolated pathogen as *Alternaria alternata*.

Molecular identification of the isolated pathogen

Many *Alternaria* spp. can infect tomato plant and produce disease on it. In order to confirm the isolated pathogen as *Alternaria alternata* the identification of the isolated pathogen was also carried out by PCR amplification of a specific region within the ITS sequences of *A. alternata*. For this specific primer pair AAF2 and AAR3 were used. These primers are specific for *A. alternata* and a specific 341bp amplification product was obtained from the total genomic DNA extracted from the isolated pathogen (Fig. 2). This result further confirmed the isolated pathogen as *A. alternata*.

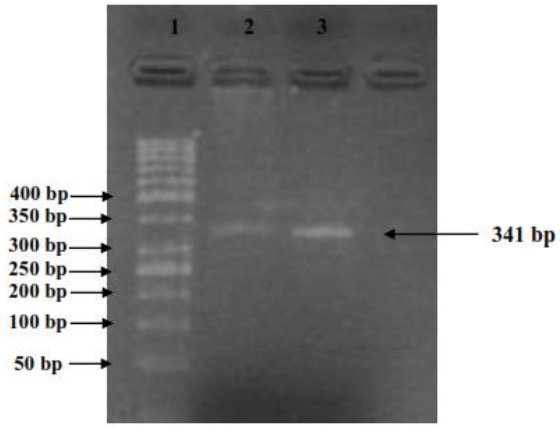

FIGURE 2. Gel electrophoresis of PCR product with primers AAF2 and AAR3 from the isolated pathogen. Lane 1- DNA marker; Lane 2- PCR amplified product from the isolated pathogen; Lane 3- PCR amplified product from the pathogen reisolated after pathogenicity test.

TLC analysis of Mannitol

To characterize the production of mannitol during pathogenicity of *Alternaria alternata* against tomato mannitol extraction was carried out from infected tomato plants as well uninfected tomato plants serving as control. It was found that a large amount of mannitol was produced in tomato plants infected with *Alternaria alternata* while no mannitol production was found in uninfected healthy tissue of tomato (Fig. 3). Mannitol production was also checked in the mycelia of *Alternaria alternata* growing in PDB but no mannitol production was observed.

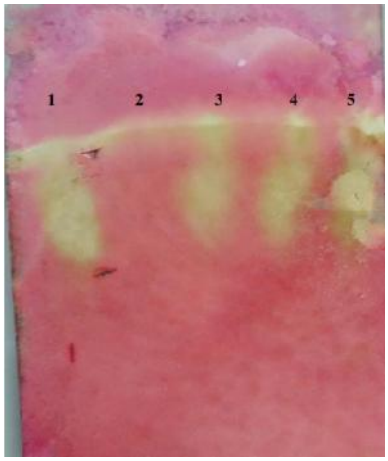

FIGURE 3. TLC seperation of the mannitol; Lane 1-Pure mannitol; Lane2- uninfected healthy leaf tissue; Lane 3 to 5- pathogen infected leaf tissue samples.

HPLC analysis of Mannitol

Once the production of mannitol was characterized during pathogenesis of *Alternaria alternata* on tomato using TLC separation, the production was further characterized by HPLC system. The results obtained from HPLC chromatogram confirmed the production of mannitol during *Alternaria alternata* – tomatao interaction (Fig. 4). A very significant amount of mannitol was observed in the infected tissue samples and it was in the range of 80-110 µg/g tissue samples.

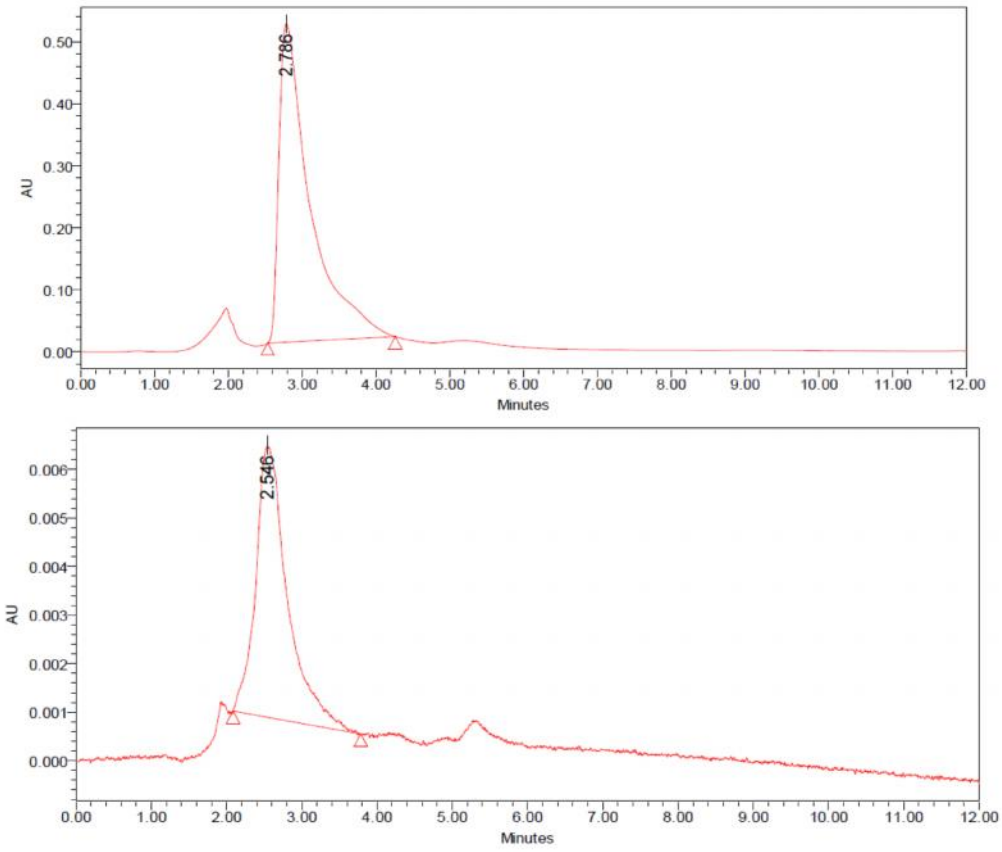

FIGURE 4. HPLC chromatogram of the mannitol (A) pure mannitol; and (B) pathogen infected sample containing mannitol.

DISCUSSION

Mannitol plays a role in fungi as a storage or translocated carbohydrate, and is important in spore germination and utilization under starvation conditions (Horikoshi *et al*., 1965; Dijkema *et al*., 1985; Witteveen & Visser, 1995). Mannitol also quenches reactive oxygen species (ROS) (Smirnoff & Cumbes, 1989; Chaturvedi *et al*., 1997; Voegele *et al*., 2005), leading to the hypothesis that it can play an antioxidant role in host–pathogen interactions. For example, mannitol-deficient mutants of *Cryptococcus neoformans* are less virulent than wild type, presumably due to mannitol protecting against oxidative killing by phagocytic cells (Chaturvedi *et al*., 1996a, b). In 1998, Jennings et al., 1998 discovered that tobacco, which does not synthesize mannitol, has an endogenous mannitol dehydrogenase (MTD; EC 1.1.1.255; 1-oxidoreductase which converts mannitol to mannose) that is induced by fungal colonization and chemical elicitors. They concluded that this enzyme was induced to degrade pathogen produced mannitol, allowing for ROS-mediated plant defenses to be effective against the fungus. To test this hypothesis, they transformed tobacco to constitutively express a celery MTD gene (Jennings et al., 2002). The transgenic plants expressing the MTD enzyme had enhanced resistance to *Alternaria alternata* infection; supporting the hypothesis that mannitol is required for infection by this pathogen. In *A. alternata*, mannitol was proposed to be produced through a mannitol cycle (Hult & Gatenbeck, 1978). In this cycle, fructose 6-phosphate is converted to mannitol 1-phosphate by mannitol 1-phosphate 5-dehydrogenase (MPDH; EC 1.1.1.17). The phosphate group is removed by a phosphatase (EC 3.1.3.22) (Yamada *et al*., 1961; Ramstedt *et al*., 1986), producing inorganic phosphate and mannitol. Mannitol is then utilized by the enzyme mannitol dehydrogenase (MtDH; EC 1.1.1.138), a 2-oxidoreductase that catalyzes a reversible conversion of mannitol to fructose. To complete the cycle, fructose is phosphorylated to fructose 6-phosphate by a hexokinase (EC 2.7.1.1). The polyol mannitol has been shown to quench ROS both in vitro and in vivo (Smirnoff & Cumbes, 1989; Chaturvedi *et al*., 1997; Voegele *et al*., 2005), and a role for mannitol in antioxidant defense in fungi have been documented in several fungi (Chaturvedi *et al*., 1997; Jennings *et al*., 1998; Voegele *et al*., 2005). Studies have also suggested that mannitol may be important in pathogenesis to counteract antioxidant defenses induced by both plant and animal hosts (Chaturvedi *et al*., 1996a, b). The strongest data in support of a role for mannitol in plant pathogenesis come from Jennings *et al*. (1998), who hypothesized that fungal pathogens secrete mannitol to quench ROS during infection of tobacco plants, because tobacco (a nonproducer of mannitol) expresses a mannitol-degrading enzyme (mannitol dehydrogenase) when challenged with fungal elicitors and inducers of plant defense responses. The enzyme was hypothesized to convert the pathogen-produced mannitol to mannose, allowing the ROS-mediated plant defense response to be effective against the fungus. In support of this hypothesis, transgenic tobacco plants constitutively expressing a mannitol dehydrogenase from celery were created; these transgenic plants were shown to have enhanced resistance to *A. alternata* (Jennings *et al.,* 2002). To evaluate the production of mannitol by *A. alternata* during tomato infection this work was taken. It was very clear from the results obtained in the present work that mannitol is definitely produced during interaction of *A. alternata* with tomato.

The observation that an ROS-quencher such as mannitol is required for normal disease development by *A. alternate* on tobacco suggests that plant-generated ROS are not used by this pathogen for disease development. Necrotrophic pathogens like *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Exserohilum turcicum* take advantage of the ROS from the plant to facilitate their infection (Govrin & Levine, 2000; Keissar *et al*., 2002). For these pathogens, protective mechanisms against plant-generated ROS would be predicted to inhibit infection. The mechanisms used by *A. alternata* to infect tobacco are not completely known. Toxins such as alternariol, altenuene, and tenuazonic acid have been implicated in disease development. Also *A. alternata* produces cellulase, polygalacturonase, and pectinmethylesterase in vitro, and pectin lyase has been recovered from brown spot lesions of naturally infected tobacco leaves. These results suggest that this pathogen utiltizes toxins and enzymes to kill cells, allowing for colonization of the tissues.

CONCLUSION

The present work was carried out with an objective to characterize the production of mannitol during *A. alternata* – tomato interaction leading to pathogenesis. To accomplish this isolate of *A. alternata* was isolated from a naturally infected tomato plants growing in field. The isolated was identified and characterized as *A. alternate* using culture techniques revealing its colony morphology and spore characteristics; as well as by molecular method using PCR amplification of a specific gene fragment of 341 bp characteristic for *A. alternata*. The pathogenicity of the isolated *A. alternata* for tomato was confirmed by validating Koch's postulates. Production of mannitol was checked in the plant tissues infected with *A. alternata*. It was observed that mannitol was being produced in all the infected samples and this was confirmed by TLC and HPLC analysis. A very significant amount of mannitol was observed to be present in the infected plant tissues, while the healthy plant tissues of tomato were completely lacking the presence of mannitol. From the results obtained in the present work it can be concluded that mannitol is somewhere required by *A. alternata* for its pathogenicity during its interaction with tomato and plays a very pivotal role in the process of disease development on tomato.

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