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STUDIES ON THE QUALITATIVE CHANGES IN THE AMINO ACID, SUGAR AND ORGANIC ACID CONTENTS OF TOMATO FRUIT DURING PATHOGENESIS BY *ALTERNARIA SOLANI*

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ABSTRACT

Qualitative changes in the amino acid, sugar and organic acid contents of the tomato fruit during pathogenesis causaed by *Alternaria solani* were investigated chromatographically. Marked differences in the amino acid, sugar and organic acid contents of the healthy and diseased tomato fruits were noted. Total in all only eight different amino acids, viz., Lysine, Arginine, Histidine, Aspartic acid, Glutamic acid, Alanine, Methionine and one Unidentified amino acid (Rf. 0.36) were detected in the extracts of healthy tomato fruits while thirteen amino acids namely as Cysteine, Lysine, Arginine, Histidine, Aspartic acid, Glutamic acid, Alanine, Tyrosin, Methionine, Valine, Leucine, Asparagine and one Unidentified amino acid having Rf value 0.38, were successfully detected in the extracts of diseased tomato fruits. These results indicated that five new amino acids, i.e., Cystine, Tyrosine, Valine, Leucine and Asparagine were synthesized in the diseased tissues after infection of *Alternaria solani*. The concentration of two amino acids namely Glutamic acid and Methionine have been found to be increased in diseased tissues in comparison to healthy tissues, as observed visually by seeing the intensity of colour of spots on the developed chromatograms. The chromatographic analysis of extract of healthy tissues showed the presence of four different sugars, i.e., Sucrose, Glucose, Maltose and Fructose in healthy tomato fruits. When extract of diseased tissues of inoculated (infected) tomato fruits were analyzed then it was found that only two sugars namely Fructose and Sucrose have been detected and remaining two susgars, i.e., Maltose and Glucose have not been detected even upto 12 days incubation periods. These results indicated that these two sugars may utilized during pathogenesis by *Alternaria solani*. The different length of given incubation period (4 days to 12 days) have no pronounced effect on the sugar levels, i.e., their concentration and composition. In the healthy tomato fruits (uninoculated) only five organic acids namely Fumaric acid, Malonic acid, Malic acid, Citric acid and Tartaric acid have been detected, in all the given incubation periods. When the inoculated diseased tomato fruits of 4, 8 and 12 days incubation, were analyzed for organic acids detection then it was found that total six organic acids present. Besides, five organic acids which were initially present in healthy tissues, only one new organic acid, i.e., Succinic acid was produced in diseased tissues. These results indicates that Succinic acid was synthesized after infection during the disease development. There was no remarkable changes in the concentration and compositin of detected organic acids in diseased and healthy tissues during various incubation periods.

INTRODUCTION

It is well known that various pathogenic fungi not only blemish, disfigure or cause rot to various fruits of economic importance but these pathogenic fungi also bring the biochemical changes which altered the nutritive value of fruits during their pathogenesis. These biochemical changes in the fruits may affect on the composition of many biochemical substances like amino acids, carbohydrates, organic acids, phenols etc. such biochemical changes would, however be, depend on the severity of disease, virulence of pathogen, proneness of the fruit to diseases and environmental conditions. Once the susceptible fruit infected and the pathogen is well established within the host tissues, the further

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spread of pathogen or in other words further progress of the disease will depend on the supply of required nutrients received by pathogen at the infection site. In certain cases the pathogen may be more advance from the infection site for drawing the required nutrients from the fruit tissues. After successful establishment of pathogen in the host tissues during pathogenesis in many cases the biochemical changes in the biochemical status of many substances may takes place in the fruits, depending upon the nature of pathogen and host pathogen relationships. The study of such biochemical changes within diseased fruit tissues certainly would helps in understanding the damage caused by pathogen. Therefore, a number of workers, have attempted to analyse the concentration and composition of various amino acids, sugars, organic acids, phenols etc. in healthy as well as in diseased tissues infected by various fungal pathogen (Chandra and Tandon, 1963; Mac Comb and Winsted, 1964; Ghosh *et al*., 1964; Stretch and Capellini, 1965; Tandon, 1967; Tandon and Mishra, 1969; Kapoor and Tandon, 1969; Srivastava and Tandon, 1970; Kirpal *et al*., 1970; Bisen and Agarwal, 1972; Chahal and Grover, 1972; Nema, 1983; Reddy *et al*., 1984; Madhukar and Reddy, 1991; Rajeswari *et al*., 1997). Amino acids are one of the most important metabolic product of healthy tomato fruits.

Due to pathogenic activity of pathogen, during disease development, the level of amino acids in diseased fruits may changed, some of them may be disappeared or new one may be produced or their concentration may changed. (Singh, 1968; Kapoor and Tondon, 1969; 1970 a; 1970 b; Grewal and Grover, 1974; Madhukar and Reddy, 1991; Rajeswari *et al*., 1997). The nutritive value of tomato fruits largely dependent on various sugars (carbohydrates) which are present in the healthy tissues. Like amino acids, composition of various sugars may change during the development of disease. (Ghosh *et al*., 1964; Mac Comb and Winsted, 1964; Singh and Tandon, 1970; Grewal and Grover, 1974). Organic acids are also another important metabolite of healthy fruit of tomato which may play the important role in defense. After penetration of pathogen, during development of disease the composition and concentration of organic acids may also effected (Srivastava, 1966; Singh, 1968; Kapoor and Tandon, 1969; Singh and Tandon, 1970). Keeping in mind the above facts, in the present study the various metabolites like amino acids, sugars and organic acids were analyzed chromatographically in healthy as well as in disease tissues of tomato fruits.

MATERIALS AND METHODS

Organism

The *Alternaria solani* was isolated from diseased tomato fruits (Chaurasia and Chaurasia, 2010; Chaurasia et.al., 2013b). Single spore culture was obtained and maintained on potato dextrose agar medium.

Preparation of tissue extract

Healthy semi ripe tomato fruits, after plucking were surface sterilized by 0.01% mercuric chloride solution and then inoculated with *Alternaria solani* by cavity method (Granger and Horne, 1924; Chaurasia *et al*., 2009; Chaurasia and Chaurasia, 2010). The inoculated fruits were placed in sterilized moist chamber and incubated at 28ºC for 4, 8 and 12 days. Healthy uninoculated surface sterilized semi-ripe tomato fruits were kept as control. After 4, 8 and 12 days of incubation, two gram of tissue from each sample of healthy and diseased tissue was grinded in a Waring blender with 60 ml of 80 % ethanol for 5 minutes. The resultant decoction was squeezed through a muslin cloth and later filtered through Whatman No. 42 filter paper. The filtrate was centrifuged at 10,000 rpm for 20 minutes. The clear supernatant liquid was decanted and was used for analysis.

Analysis of fruit extracts

Extracts from the healthy tomato fruit and from the tissue infected with *Alternaria solani* were analyzed for amino acids, sugars and organic acids by descending paper chromatographic techniques.

Paper Chromatography

The whole sheet (57 X 46.5 cm) of whatman No. 1 chromatographic paper was used. It was suspended carefully in the chromatographic chamber for saturation in the solvent system. After an overnight saturation the paper was taken out and dried at room temperature. The chromatographic paper was used as such for the screening of various organic substances. In case of organic acid, the paper was activated for 30 minutes at 60ºC before use.

Spotting of the extracts

The spots of the extracts of different samples were put on the Whatman No. 1 chromatographic papers, 2.5 cm above the base and the 3.0 cm apart from each other with the help of lambda pipettes (Drummond Scientific Company, U.S.A.). These spots were concentrated by repeated applications of the extract till 100 lambdas (µ l) of the extract sample was applied on each spot.

Development of the chromatogram

The spotted papers were developed in different solvent systems in chromatographic chambers. Paper was allowed to run overnight till the solvent traveled about 15 cm. The paper was then removed and air-dried. Spots were detected by spraying suitable reagents on the paper. After spraying, papers were heated if required in chromatographic oven adjusted at a suitable temperature for development of the coloured spots. Colour, shape, size, density (Visually) and Rf values were noted for each spot.

Solvent systems and detecting reagents

The following solvent system and detecting reagents were used for the separation and detection of amino acids, sugars and organic acids.

Detection of amino acids

Solvent System: n-Butanol : Acetic acid : Water (3:1:1 v/v)

Detecting Reagent: The reagent used was ninhydrin. This was prepared by dissolving 300 mg ninhydrin in 100 ml acetone and 3 ml of glacial acetic acid. After spraying, the

chromatogram was treated for 7 to 10 minutes at 105ºC temperature for the development of colour.

Detection of sugars

Solvent System: n-Butanol : Acetic acid : Water (4:1:5 v/v)

Detecting Reagent: The reagent used was diphenylamine. this was prepared by the following manner; 2% w/v diphenyl-amine in acetone : 2% w/v aniline in acetone 85% H_3PO_4 (Phosphoric acid) = 5:5:1 v/v prepared fresh. After spraying, the chromatogram was air-dried and then heated at 100ºC for 5 to 10 minutes. Sugars appered as coloured spots.

Detection of organic acids

Solvent System: Ethanol: NH_4OH : Water (80:5:15 v/v)

Detecting Reagent: The reagent used was 2, 6 - Dichlorophenolindophenol. This was prepared by dissolving 100 mg 2, 6 - dichiorophenolindophenol in 100 ml ethanol. After spraying, spots appeared as pink on blue background. Bromocresol green was also used as a spraying reagent. This was prepared by dissolving 0.04 g bromocresol green in 100 ml ethanol. 0.1 N NaOH was then added to this solution till a blue colour develops and the solution reached to pH 10.0. The developed paper was heated at 60ºC for 30 minutes and was cooled to room temperature for overnight. After spraying with bromocresol green the organic acids appeared as bright yellow spots against bluish green background.

Identification of the spots

The spots of various samples were identified by comparing these with the known standards of the same compounds which were on chromatographed simultaneously. Identification was based upon the similarity of the colour and the Rf value of the unknown compound with that of the known standard. The Rf value was calculated by the following formula:

$$Rf = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

The intensity of the spots were noted and categorized into low (1^+), medium (2^+) and high (3^+).

RESULTS AND DISCUSSION

To understand the biochemical alteration in the metabolic post – infection changes, the extracts of healthy and diseased tomato fruits were analyzed chromatographically and various amino acids, sugars and organic acids were detected and estimated qualitatively at different incubation periods.

Detection of various amino acids in healthy and diseased tomato fruits

The result of detected amino acids in the extracts of healthy and diseased tomato fruits after different incubation periods are presented in Table 1. From the data it is clear that in the extract of healthy tomato fruits, only eight amino acids namely, Lysine, Arginine, Histidine, Aspartic acid, Glutamic acid, Alanine, Methionine and one Unidentified amino acid (Rf. 0.36) were detected. When the extracts of artificially infected tomato fruits were analyzed chromatographically, it showed the presence of thirteen amino acids namely, Cysteine, Lysine, Arginine, Histidine, Aspartic acid, Glutamic acid, Alanine, Tyrosine, Methionine, Valine, Leucine, Asparagine and one Unidentified amino acid having Rf value 0.36, after the various incubation periods of 4, 8 and 12 days. From the above results, it is clear that the free amino acid pool was considerably altered in the diseased tomato fruits during the pathogenesis by *Alternaria solani*. It was found that in diseased tomato fruits, five new amino acids i.e., Cystine, Tyrosine, Valine, Leucine and Asparagine showed their presence which were actually absent in the healthy tomato fruits. The appearance of above said five amino acids in the extract of diseased tomato fruits confirmed the post infection metabolic changes during pathogenesis. Kapoor and Tandon (1969) have been also reported that two amino acids i.e., Valine and Tyrosine were absent in the healthy tomato fruits were synthesized and detected in diseased tomato fruits, infected by *Drechslera australiense*.

Table 1. Detection of various amino acids in healthy and diseased tomato furits

Amino Acids	Healthy Tissue			Diseased Tissue		
	Days after incubation			Days after incubation		
	4	8	12	4	8	12
Cysteine	-	-	-	1^+	1^+	1^+
Lysine	1^+	1^+	1^+	1^+	1^+	1^+
Arginine	1^+	1^+	1^+	1^+	1^+	1^+
Histidine	1^+	1^+	1^+	1^+	1^+	1^+
Aspartic acid	1^+	1^+	1^+	1^+	1^+	1^+
Glutamic acid	1^+	1^+	1^+	2^+	3^+	3^+
Alanine	1^+	1^+	1^+	1^+	1^+	1^+
Tyrosine	-	-	-	1^+	1^+	1^+
Methionine	1^+	1^+	1^+	2^+	3^+	3^+
Valine	-	-	-	1^+	1^+	1^+
Leucine	-	-	-	1^+	1^+	1^+
Asparagine	-	-	-	1^+	1^+	1^+
Unidentified (Rf 0.36)	1^+	1^+	1^+	1^+	1^+	1^+

- = Absent 3^+, 2^+ and 1^+ = Relative concentrations of the substances as determined by the intensity of the spots.

From the presented data it was also observed that the concentration of two amino acids, i.e., Glutamic acid and Methionine were increased in the diseased tomato fruits in comparison to healthy tomato fruits. These results are also agreement with the observations of Kapoor and Tandon (1969) who have also reported the increase in the amount of Glutamic acid, as a result of post infection changes brought about by *Drechslera australiense*. Reddy *et al*. (1984) have been also reported, the increase in level of Glutamic acid in diseased tomato fruit during the pathogenesis of *Phoma exigua*. The composition of various free and bound amino acids in healthy and diseased fruits has also studied by many workers like Singh (1968), Kapoor and Tandon (1969, 1970 b), and Mehta (1973). These workers have reported the increase in the amount of some amino acids in the diseased fruits during pathogenesis, probably due to either by host parasite interaction or by secretion of proteolytic enzymes by pathogen. several other workers have reported the increase in amino acids my be due to proteolysis of the host protein catalysed by normal host or pathogens enzymes (Kiraly and Farkas, 1959; Rubin and Artsikhorskaya, 1963) or may be due to the de - novo Synthesis by the host (Rohringer, 1957; Pagg Sequaira, 1968) or Synthesis by the pathogen itself

(Rohringer, 1957; Ven andel, 1966). The increse may also be attributed to the activation of enzymes involved in amino acid and amide synthesis (Goodman *et al*., 1967) or transamination or activated hydrolysis of certain proteins of the host pathogen complex (Mehta *et al*., 1975). Transportation of amino acids to the infected portion from the healthy tissue may also cause increase in some of the amino acids (Hare, 1966; Van Andel, 1966; Goodman *et al*., 1967). On the whole it is concluded that the composition of free amino acids pool in the healthy tomato fruits is considerably influenced and altered during the pathogenesis of *Alternaria solani*. Total in all only eight different amino acids were detected in the extracts of healthy tomato fruits while thirteen amino acids were successfully detected in the extracts of 4, 8 and 12 days old infected fruits. From these observations it is concluded that five new amino acids, namely Cysteine, Tyrosine, Valine, Leucine and Asparagine were synthesized in the diseased tissues after infection. It is also concluded from the visual observations of developed chromatograms that concentration of Glutamic acid and Methionine amino acids considerably increased in the diseased tissues infected by *Alternaria solani*.

Detection of various sugars in healthy and diseased tomato fruits

Data regarding the detection of various sugars in the healthy and diseased tomato fruits by chromatographic technique are presented in Table 2. The analysis of extract of healthy tissues showed the presence of four different sugars namely Sucrose, Glucose, Maltose and Fructose in the healthy tomato fruits. When the extracts of diseased tissues of various incubation periods were analyzed then it was found that only two sugars, i.e., Fructose and Sucrose showed their presence in diseased tomato fruits. The above results clearly indicated that the two sugars, i.e., Glucose and Maltose which were present in healthy tomato fruits completely disappeared in diseased tissues as a result of post-infection changes by pathogen *Alternaria solani*. Kapoor and Tandon (1970 a) and Mehta (1973) have also reported the loss of Glucose and Maltose in diseased tomato fruits during pathogenesis of *Dreshslera australiense* and *Alternaria solani* respectively. Rai (1971) has also came to conclusion that rapid reduction and ultimate loss of Glucose occurs in Chillies fruits, as a result of *Colletotichum capsici* infection. A number of other reports also indicate a well marked disappearance or reduction of sugars during pathogenesis caused by various fungal pathogens (Mc Combs and Winsteed, 1964; Dayal and Joshi, 1968; Chopra and Jhooty, 1974). Show and colotalo (1961) discussed that this decrease in sugar contents during pathogenesis is due to increased respiration of infected host tissue and subsequently the quick utilization of substrate.

Table 2. Detection of various sugars in healthy and diseased tomato fruits

Sugars	Healthy Tissue			Diseased Tissue		
	Days after incubation			Days after incubation		
	4	8	12	4	8	12
Sucrose	1^+	1^+	1^+	1^+	1^+	1^+
Glucose	1^+	1^+	1^+	-	-	-
Maltose	1^+	1^+	1^+	-	-	-
Fructose	1^+	1^+	1^+	1^+	1^+	1^+

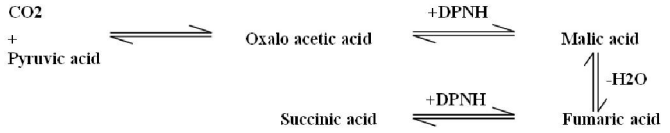
- = Absent
1^+ = Present

From present study, it is concluded that incubation period did not show any effect on the level (concentration) of sugars in diseased tissues. From results, it is also concluded that after 4 days incubation, further increase in the incubation period upto 12 days, has no effect on level (concentration) of Sucrose and Fructose and were found to be the same in diseased tissues (as chromatogram observed visually). These findings are also in agreement with observation of Mehta (1973). On the whole, it can be concluded that only four various sugars, i.e., Sucrose, Glucose, Maltose and Fructose have been detected in the healthy tomato fruits. In diseased tissues of artificially infected tomato fruits, only two sugars, i.e., Sucrose and Fructose have showed their presence and remaining two sugars, i.e., Maltose and Glucose were found to be disappeared. These results clearly indicated that Maltose and Glucose were successfully utilized by pathogen *Alternaria solani* during pathogenesis. Utilization of maltose and glucose by many fungi in the culture medium has been also reported by Hasija (1968) and Reddy (1969). According to Cochrane (1958), the utilization of these substances is dependent on enzymatic system which are constitutive in same organisms and inducible in others.

Detection of various organic acids in healthy and diseased tomato fruits

Results regarding the detection of organic acids in healthy and diseased tomato fruits are presented in Table 3. The various organic acids, present in healthy and in diseased tomato fruits were successfully analyzed by paper chromatography. From the results, it is revealed that only five organic acids were present in the extract of healthy tomato fruits. The organic acids which detected in healthy tomato fruits were Fumaric acid, Malonic acid, Malic acid, Citric acid and Tartaric acid. When the extract of diseased tomato fruits was analyzed after 4, 8 and 12 days incubation periods, total six organic acids namely Fumaric acid, Succinic acid, Malonic acid, Malic acid, Citric acid and Tartaric acid were detected. Out of these six organic acids, only Succinic acid showed its presence in the diseased fruits, which was initially absent in healthy tomato fruits. From these results it is concluded that only Succinic acid was synthesized during pathogenic activity of *Alternaria solani*. There was no remarkable changes in the concentrations and composition of detected organic acid in the diseased and healthy tissues, during the various incubation periods.

The presence of Succinic acid in the diseased tomato fruits, during the pathogenesis of *Drechslera australiense*, was reported by Kapoor and Tandon (1969). Several workers like Raistrick and Smith (1935), Nord and Sciarini (1946) and Cantino (1949) have also reported the appearance of Succinic acid in the diseased tissues during the pathogenic activity of various pathogens which caused the fruit rots. Tandon (1970) reported that the sudden appearance of a particular organic acid may be the result of interaction between the host and the pathogen. Singh (1968) and Kapoor and Tandon (1969) also reported production of succinic acid during pathogenesis. It has been reported by many workers including Cantino (1964), Nord and Sciarini (1946) that a number of microorganisms synthesize this acid from carbohydrates. McElory (1961) suggested the following scheme for the process:



On the whole, it is concluded that the five organic acids, namely Fumaric acid, Malonic acid, Malic acid, Citric acid and Tartaric acid showed their presence in the healthy as well in the diseased tomato fruits, during the given incubation period, i.e., 4, 8 and 12 days incubation. Besides, the above five organic acids, Succinic acid was only the organic acid which was detected only in the diseased tomato fruit. The presence of Succinic acid in the diseased tissues, suggested that it was synthesized after infection by pathogen, i.e., during the pathogenesis of *Alternaria solani*. There was no other remarkable changes in the concentration of detected organic acids in healthy as well as in diseased tomato fruits, during various given incubation periods, as observed visually.

Table 3. Detection of various organic acids in healthy and diseased tomato fruits

Organic acids	Healthy Tissue			Diseased Tissue		
	Days after incubation			Days after incubation		
	4	8	12	4	8	12
Fumaric acid	1^+	1^+	1^+	1^+	1^+	1^+
Succinic acid	-	-	-	1^+	1^+	1^+
Malonic acid	1^+	1^+	1^+	1^+	1^+	1^+
Malic acid	1^+	1^+	1^+	1^+	1^+	1^+
Citric acid	1^+	1^+	1^+	1^+	1^+	1^+
Tartaric acid	1^+	1^+	1^+	1^+	1^+	1^+

- = Absent
1^+ = Present

Absence of succinic acid in the healthy tissues and its presence in the diseased ones may be regarded as a fundamental evidence for the active role of the polygalacturonase (PG), polymethylgalacturonase (PMG) and cellulase (Cx) enzymes in causing the rots. Presence of all the three enzymes in the diseased tissues of the tomato fruits in appreciable amounts and degradation of pectic and cellulose substances originally present in the tomato fruits were additional evidences (Chaurasia *et al*., 2013a; Chaurasia *et al*., 2013c).

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