

Biochemical Changes in Carbendazim Sensitive and Resistant Isolate of *Alternaria Alternata* Causing Root Rot to Fenugreek (*TRIGONELLA FOENUM GRAECUM L.*)

Nilkanth K. Khandare

Department of Botany, Krantisinh Nana Patil College, Walwa, 416 313, Dist- Sangli, Maharashtra, India

Abstract: Fenugreek (*Trigonella foenum graecum L.*) is an important plant grown for its vegetable, fodder and medicinal, was infected by *Alternaria alternata* (Fr.) Keissler, causing root rot disease to fenugreek. Carbendazim was used for management of the disease. The carbendazim sensitive and resistant isolates show biochemical variation when assessed against untreated healthy ones. Biochemical constituents like Carbohydrates, starch, reducing sugar, DNA, RNA as well as Iron, Zinc, Copper, Manganese, and Magnesium contents were seen to be reduced due to infection of *Alternaria alternata* in sensitive and resistant isolates as compared to healthy plant, while Calcium, total ash and polyphenol contents were increased in both of the isolates.

Keywords: Fenugreek, *Alternaria alternata*, carbendazim sensitive and resistant, biochemical constituents

1. Introduction

Fenugreek (*Trigonella foenum graecum L.*) is an important plant grown for its vegetable, fodder and medicinal value. It is attacked by many fungal pathogens like *Rhizoctonia solani*, *Fusarium oxysporum*, *Curvularia lunata* etc. *Alternaria alternata* (Fr.) Keissler, causing root rot disease to fenugreek is severe in all pathogens infecting Fenugreek (Khandare and Kamble, 2013). The sensitivity of pathogen was calculated in both sensitive (Aa-2) and resistant isolates (Aa-8) of *Alternaria alternata* (Khandare and Kamble, 2013). The root rot causes infection to plant and reduces the quality of plant. The plant is rich in carbohydrate, starch and other elements which make the plant valuable for use for various purposes. The biochemical analysis of the plant in healthy and infected plant shows lot of variations during biochemical analysis. The aim of present investigation is to analyse the biochemical constituents in healthy and infected fenugreek.

2. Material and Methods

2.1. Material

Fenugreek (*Trigonella foenum graecum L.*), healthy and infected by *Alternaria alternata*. This was studied by inoculating the *Trigonella foenum graecum L.* plants with spore suspension of resistant and sensitive isolates of *Alternaria alternata*. After 10 days of inoculation plants were dried at 40°C in hot air oven and powder was obtained in grinder. Healthy and inoculated samples were extracted in ethanol and were analyzed for all the biochemical estimations.

2.2. Preparation of Reagents and Solutions

2.2.1. Alkaline Copper tartarate

Solution A

Anhydrous sodium carbonate 2.5 gm, Potassium sodium tartarate 2.5 gm, sodium sulphate (anhydrous) 20 gm were dissolved in distilled water and final volume was made up to 100 ml with distilled water, filtered and stored at room temperature.

Solution B

Copper sulphate 15 gm were dissolved in a small volume of distilled water and one drop of sulphuric acid was added and final volume was made up to 100 ml with distilled water. 4 ml of solution B and 96 ml of solution A were mixed before use.

2.2.2. Arsenomolybdate Reagent:

2.5 gm ammonium molybdate was dissolved in 45 ml distilled water. To this 2.5 ml sulphuric acid was mixed well. Then 0.3 gm disodium hydrogen arsenate dissolved in 25 ml distilled water was added and incubated at 37°C for 24 to 28 hours.

2.2.3. Standard Glucose Solution

Stock

100 mg of Glucose was dissolved in 100 ml distilled water.

Working Standard

10 l of stock diluted to 100 ml with distilled water. (0.1mg/ml)

2.2.4. Folin –Ciocolteu reagent-

Commercially prepared reagent (2N) was mixed with equal quantity of distilled water and stored in amber color bottle at 2 °C.

2.2.5. DNA Standard solution

30 mg deoxyribonucleic acid (DNA) salt from Herring sperm free acid. Hi Media Laboratory, Pvt. Ltd. Mumbai was dissolved in 100 ml 0.3 N KOH solution to give a solution containing 30 mg DNA per ml of KOH.

2.2.6. Diphenylamine Reagent

1 gm diphenylamine was dissolved in 96 ml glacial acetic acid to which 4 ml concentrated H₂SO₄ added and stored at 20°C.

2.2.7. RNA Standard solution

50 mg of RNA from yeast, pure (BDH) chemicals Ltd. Poole (England) was dissolved in 100 ml 0.3 N KOH and stored at 20°C. Each ml of this solution contained 500 mg RNA. This was diluted with KOH to obtain various concentrations of RNA.

2.2.8. Orcinol Reagent

2 ml of 10% solution of ferric chloride 6H₂O was added to 400 ml of concentrated HCl.

2.3 Biochemical Estimation**2.3.1. Estimation of carbohydrates:**

Carbohydrates were estimated according to the method described by Nelson (1944). 0.5 gm oven dried plant material was homogenized in mortar and pestle and extracted with 80% alcohol. It was filtered through Buckner's funnel using Whatman no. 1 filter paper. The residue on filter paper was washed with 80% alcohol repeatedly. All the washing and filtrate were mixed together. The residue was used for starch estimation.

2.3.2. Estimation of Reducing Sugar

The filtrate was condensed on the water bath to about 2-3 ml and to it added lead acetate and potassium oxalate (1:1) to decolorize the extract. It was mixed together with the help of glass rod with addition of some distilled water. It was again filtered and washed with distilled water, collecting the washings in the same filtrate. The final volume of filtrate was made to 50 ml with distilled water. The filtrate was used for estimation of reducing sugars.

2.3.3. Estimation of Starch

The residue on the filter paper used for estimation of starch was transferred to a conical flask with 50 ml of distilled water and 2-3 ml of concentrated HCl, autoclaved at 15 lbs pressure for 30 minutes. The autoclaved filtrate was cooled and neutralized with Na₂CO₃ and filtrated. This filtrate contained reducing sugars produced as a result of hydrolysis of starch. Aliquots of 2ml (from reducing sugar and starch) were taken separately in 10ml marked test tubes. 0.5, 1.0, 1.5, 2.0, 2.5 ml of working standard glucose solution were taken in a series of test tubes. The Alkaline copper tartarate reagents 1 ml was added to each test tube. Test tubes were then kept in boiling water bath for 10 minutes. After cooling the tubes, 1 ml Arsenomolybdate reagent was added to all the tubes. The final volume was made 10 ml with distilled water. A blank was prepared as stated above with working standard glucose solution. After adjusting the spectrophotometer with blank after 10 minutes, the absorbance was read at 560 nm on double beam spectrophotometer (Shimadzu, UVVIS-190). A standard curve of glucose (0.1 mg/ml) was prepared and the sugar content was calculated.

2.3.4. Estimation of Polyphenol –

The polyphenols were estimated following the method by Folin and Denis (1951). 1 gm oven dried root infected

powder of *Trigonella foenum graecum* was homogenized in mortar and pestle and extracted with 80% acetone. It was filtered through Buckner' funnel, using Whatman No.1, filter paper. The total volume was made 25ml with 80% acetone. This filtrate was used for estimation of polyphenols. From the acetone extract, 2ml were taken separately in 10 ml marked test tubes. 1.00, 2.00, 3.00, 4.00 ml of the standard tannic acid solutions were taken in a series of test tubes. To this mixture 10 ml 20% Na₂CO₃ was added in each test tube. In the above mixture 2 ml of Folin- Denis reagent was added mixed well and diluted to 50 ml with distilled water. The standard tannic acid solution (0.1 mg/ml) was used for the preparation of standard polyphenol curve. A blank was prepared without standard tannic acid. After development of colour the absorbance was read at 660 nm on Shimadzu, UV-VIS, and 190 double beam, spectrophotometer.

2.3.5. Estimation of nucleic acids-

The nucleic acids were separated from the roots of fenugreek by using method of Cherry (1962). Oven dried root infected powder (200 mg) of *Trigonella foenum graecum* roots were homogenized in 2 ml cold methanol. Insoluble pellet was washed with 2 ml methanol and centrifuged at 500 rpm for 15 minutes. Aqueous state discarded. Pellet was again washed with 4 ml cold 0.2 N perchloric acid and centrifuged at 5000 rpm for 15 minutes at 40°C aqueous state discarded. Pellet was suspended in 5 ml ethanol. (other mixture (2:1) and kept at 50°C for 15 minutes, centrifuged in cooling centrifuge at 5000 rpm for 15 minutes. Pellet was discarded. Supernatant was used for estimation of DNA with a Diphenylamine and RNA with Orcinol.

2.3.6. Estimation of DNA-

1 ml of supernatant was added to 2.5 ml freshly prepared diphenyl amine reagent. The contents were mixed and heated for 5 minutes in boiling water bath. These samples were cooled and absorbance read at 540 nm in a spectrophotometer (Schimadzu-UV-VIS-190). A similarly treated blank was used for zero setting. The quantity of DNA per test was determined from the standard curve by using standard DNA solution.

2.3.7 Estimation of RNA-

1ml distilled water and 9 ml freshly prepared Orcinol reagent was added to 0.2 ml of extract. The contents were mixed and heated for 20 minutes in a boiling water bath. The solution was cooled and absorbance read at 260 nm (Schimadzu-UV-VIS- 190). A blank was prepared by using distilled water for zero setting. Standard curve for RNA was prepared by using standard RNA solution.

2.3.8 Estimation of Total ash-

The residue after incineration of the sample at 550-660 °C was known as ash. For this purpose the sample was subjected to a high temperature up to 600 °C and then ash content was determined. During ignition to such a high temperature all organic compounds decomposed and pass off in the form of gases, while the mineral elements remained in the form of ash.

2.4 Procedure

1 gm oven dried powder of sample was taken in a previously weighed vitrosil silica crucible. It was heated on a hot plate for about 30 minutes, till the sample was sufficiently charred and turns black. The lid of crucible was replaced and kept it in a muffle furnace. The temperature was allowed to raise up to 550 °C and kept constant for 4 hrs. The crucible was removed on cooling and transferred directly to desiccators and weighed immediately. The weight of ash obtained was determined per 1 gm of sample and calculated the ash content as percent dry matter.

3. Result and Discussion

In the experiment it was investigated that by inoculating the *Trigonella foenum graecum* plants with spore suspension of resistant isolate and sensitive isolate of *Alternaria alternata* and compared with healthy plant, (Table 1) indicate that, carbohydrates, starch, reducing sugar, DNA, RNA as well as Iron, Zinc, Copper, Manganese, and Magnesium content reduced due to infection of sensitive and resistant isolates of

Alternaria alternata as compared to healthy plant, while Calcium, total ash and polyphenol content was increased due to the infection of both the isolates of *Alternaria alternata*. The biochemical comparison among sensitive and resistant isolates show highest reduction in biochemical constituents like Carbohydrates, starch, reducing sugar, DNA, RNA, Iron, Zinc, Copper, Manganese, and Magnesium contents, while Calcium, total ash and polyphenol contents were highly increased.

Rana and Sengupta (1976), reported more production of amino acid in carbendazim resistant strain of *Macrophomina phaseolina* than the sensitive one. Jagtap *et al.*, (2011), studied biochemical changes in turmeric rhizome infected with *Sclerotium rolfsii*. There are similar results observed by other workers and the results are in aggregation of earlier workers who have studied biochemical characteristics of fungal pathogens Nagaraja, T.G. (2007), Sindhan and Parshar (1996), Ushamalani, *et al* (1998) and Choudhari and Kareppa (2013).

Table 1: Biochemical constituents of carbendazim sensitive, resistant and healthy (untreated) Fenugreek

Sr. no	Biochemical constituents	Healthy Fenugreek	Fenugreek infected with sensitive isolate (Aa-2)	Fenugreek infected with resistant isolate (Aa-8)
1	Carbohydrate gm/100gm	9.4	8.8	8.2
2	Reducing sugar gm/100gm	1.88	1.20	1.0
3	Starch gm/100gm	6.0	4.70	4.0
4	Polyphenols mg/100g	900	920	940
5	DNA mg/g	1.600	750	0.825
6	RNA mg/g	2.20	2.05	1.08
7	Element Analysis (mg/100g)			
a.	Iron	450	6 430	419.9
B	Zinc	15.95	4.50	2.32
C	Copper	11.13	7.25	5.77
d	Calcium	1.28	2.10	2.56
e	Manganese	0.73	0.30	0.10
f	Magnesium	11.84	9.20	8.27

4. Conclusion

The sensitive and resistant isolates after biochemical comparison with healthy one show reduction in biochemical constituents like Carbohydrates, starch, reducing sugar, DNA, RNA as well as Iron, Zinc, Copper, Manganese, and Magnesium contents, while Calcium, total ash and polyphenol contents were increased in both of the isolates. The resistant isolate show highest reduction in biochemical constituents like Carbohydrates, starch, reducing sugar, DNA, RNA, Iron, Zinc, Copper, Manganese, and Magnesium contents, while Calcium, total ash and polyphenol contents were highly increased.

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Author Profile



Nilkanth K. Khandare is a postgraduate in Botany and awarded Ph.D in plant pathology, from Shivaji University Kolhapur, (Maharashtra). He is assistant Professor of Botany and has 18 years teaching experience at Krantisinh Nana Patil College, Walwa, Dist- Sangli, (Maharashtra), India.