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Nitika Kumari
Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India

AK Meena
Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India

Vinay Kumar Kardam
Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India

Suresh Kumawat
Department of Soil Science and Agricultural Chemistry, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India

Vijay Shree Gahlot
Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India

Corresponding Author:
Nitika Kumari
Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India

Biochemical analysis of cowpea against charcoal rot incited by *Macrophomina phaseolina* (Tassi.) Goid

Nitika Kumari, AK Meena, Vinay Kumar Kardam, Suresh Kumawat and Vijay Shree Gahlot

Abstract
Cowpea (*Vigna unguiculata* (L.) Walp) also known as 'vegetable meat' is an important pulse crop. It is affected by several fungal, bacterial and viral diseases but charcoal rot of cowpea incited by *Macrophomina phaseolina* (Tassi) Goid. is emerging as a severe problem in arid regions of Rajasthan (India). Various biochemical changes in host plants are observed before and after the incidence of disease. The total sugar, reducing sugar, non reducing sugar and soluble protein were higher in healthy roots as compared to diseased roots in all the tested varieties *i.e.*, RCP-27, C-152 and RC-19. Maximum reduction in total sugar and non reducing sugar was found in RCP-27 followed by C-152, while maximum reduction in reducing sugars was found in C-152 and soluble proteins in RC-19. Total phenol content was higher in diseased roots as compared to healthy tissues of all the tested varieties. Maximum increase in total phenol was observed in diseased roots of RC-19 followed by C-152.

Keywords: Charcoal rot, Macrophomina phaseolina, biochemical changes, souluble proteins, phenols

1. Introduction
Cowpea (*Vigna unguiculata* (L.) Walp.) is a crucial legume crop of arid, semi-arid, tropical and sub-tropical regions around the world (Paino D'urzo *et al.*, 1990) [13]. It belongs to the family Fabaceae (Cobley, 1956) [5]. Due to its high nutritional value and early maturity, it is often referred to as the "poor man's meat" (Boukar *et al.*, 2019) [4]. It is also a rich source of carbohydrates (56.24%), lipids (3.99%) and gross energy (1.51%) (Owolabi *et al.*, 2012) [12]. The crop is affected by many fungal, bacterial, viral, phytoplasmal and nematodal diseases (Emechebe and Lagoke, 1979) [7]. Among all the fungal diseases of cowpea, the one of the most destructive is charcoal rot of cowpea caused by *Macrophomina phaseolina* causing potential yield losses. A charcoal like appearance can be observed at the collar region of affected host plants; hence the disease is named charcoal rot. In advance stage scattered sclerotial bodies may be seen on the affected tissues (Singh and Srivastava, 1988) [15]. *M. phaseolina* induce biotic stress and affects various physiological functions like chlorophyll, proline and sugar content (Kuldeep *et al.*, 2012) [8]. So, the aim of this study was to examine the effect of *M. phaseolina* on phenols, sugars and soluble proteins of three varieties of cowpea.

2. Materials and Methods
2.1 Estimation of total sugar
2.1.1 Reagents
1. Anthrone reagent (2mg/ml conc, sulphuric acid)
2. Standard glucose solution (1mg/ ml): dissolved 100 mg glucose in 100 ml distilled water.
3. Working standard solution (100 mg/ml) Dilute 10 ml standard solution to 100 ml with distilled water
4. 5N HCl

Total sugar content was determined by colorimetric method using anthrone reagent. In this method, 100 mg of sample was taken in a boiling tube and hydrolyzed in boiling water bath for 3h with 5ml of 2.5N HCl and cooled to room temperature. It was neutralized with solid sodium carbonate until the effervescence ceased and the volume was made to 100ml and centrifuged. The supernatant was collected. 0.5 and 1 ml aliquots were taken for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard and the volume was made to 1 ml in all the tubes including the sample tubes by adding distilled water.

After that, 4ml of anthrone reagent was added, heated for 8 min in a boiling water bath, cooled rapidly and the green to dark green colour was read at 630 nm. The amount of sugars in the sample was plotted against standard curve prepared from glucose. The sugar content in plant samples was expressed as mg g^{-1} fresh tissue (Dubois *et al*, 1956) [6].

2.2 Estimation of reducing sugar

2.2.1 Reagents

2.2.2 Copper reagent "A"

Sodium carbonate (anhydrous)	2.5 g
Potassium sodium tartrate	2.5 g
Sodium bicarbonate	2.0 g
Sodium sulphate	20.0 g
Distilled water	80.0 ml
Volume	100 ml

2.2.3 Copper reagent "B"

Copper sulphate	15 g
Conc. Sulphuric acid	1 drop
Volume	100 ml

2.2.4 Alkaline copper tartrate

Copper reagent "A"	24 ml
Copper reagent "B"	1 ml

2.2.5 Arseno-molybdate reagent

Ammonium molybdate	2.5 g
Conc. Sulphuric acid	2.5ml
Disodium hydrogen arsenate	0.3 g
Volume	70 ml
Standard glucose solution (1 mg/ ml)	Dissolve 100 mg glucose in 100 ml distilled water
Working standard solution (100mg/ml)	Dilute 10 ml standard solution to 100 ml with distilled water

Reducing sugar content was measured following "Nelson's modification of somogyi's method" (Somogyi, 1952) [16] using arseno-molybdate colour forming reagent and two copper reagent "A" and "B". In this 100 mg of sample was taken and the sugar was extracted with hot 80% alcohol twice. The supernatant was collected and evaporated on water bath; 10 ml water was added for dissolving the sugars. Aliquots of 0.1 or 0.2 ml of alcohol-free extract was pipetted to separate test tubes. Then 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution was taken in a series of test tubes. The volume in both samples and standard tubes was made to 2 ml with distilled water. 2 ml distilled water was taken into a separate tube to serve as a blank. 1 ml of alkaline copper tartarate reagent was added to each tube. The tubes were placed in boiling water for 10 min, cooled and 1 ml of arsenomolybdate reagent was added to all the tubes. The volume in each tube was made to 10 ml with water. Absorbance was measured at 620 nm on Spectrophotometer. The value was plotted against a standard curve prepared from glucose. The figures were expressed on percentage basis.

2.3 Estimation of non- reducing sugar

The amount of non-reducing sugar was obtained by subtracting reducing sugar from the amount of total sugar and multiplying the resultant with a constant factor 0.95.

2.4 Estimation of total phenol content

The total phenol content was estimated by the method described by Thimmaiah (1999) [18]. One gram root or shoot sample was grinded in mortar and pestle with 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was filtered and the residue was re-extracted with five-time volume of 80 per cent ethanol. Supernatant was cooled and evaporated to dryness in water bath. The residue was dissolved in 5 ml of distilled water. An aliquot of 0.2 ml was transferred in test tube and volume was made to 3 ml with distilled water. Folin-ciocalteau reagent (0.5ml) was added in each test tube. After three minutes, 2 ml of 20 per cent sodium carbonate was added in each tube and mixed thoroughly. The tubes were then placed in boiling water for one minute. After cooling, the absorbance was recorded at 650 nm against a reagent blank. The standard curve was prepared by taking different concentrations of catechol. The phenol content was express as mg g^{-1} fresh tissue.

2.5 Estimation of soluble protein content

The soluble protein content of the samples was assayed by using the method of Lowry *et al.* (1951) [9]. One gram of root or shoot was macerated in mortar with 5 ml 0.1 M sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 16,000 rpm for 20 minutes. The supernatant was used for estimation of soluble protein content. For this purpose, two per cent sodium carbonate (anhydrous) in 0.1 N NaOH (Solution A) was prepared. Similarly, 0.5 per cent copper sulphate ($CuSO_4.5H_2O$) in 1 per cent sodium potassium tartarate (freshly made) was prepared (solution B). From these two reagents, solution C (alkaline copper sulphate) was prepared by mixing 50 ml of solution A with 1 ml of solution B just before use. An aliquot of 0.1 ml supernatant was taken in test tube and the volume was made to 1 ml with distilled water followed by addition of 5 ml solution C mixed well and incubated at room temperature for ten minutes. 0.5 mL of folin ciocalteu reagent was diluted to 1N, mixed well and incubated at room temperature in dark for 30 minutes. The absorbance was recorded at 660 nm against blank. The amount of protein in sample was computed from the standard curve prepared by using different concentrations of bovine serum albumin. It was expressed as part per million (ppm).

3. Results

3.1 Effect on total sugar

Data presented in table 1 and fig 1 indicates that all the diseased samples have significant reduction in total sugar, reducing and non-reducing sugar contents in all the three tested varieties. The total sugar content were observed low in diseased plants of test varieties. The total sugar content in healthy plants were found maximum in RCP-27 (7.60 mg g^{-1} fr.wt.) followed by C-152 (6.72 mg g^{-1} fr.wt.) and RC-19 (5.75 mg g^{-1} fr.wt.). The maximum total sugar content was observed in diseased samples of RCP-27 (6.28 mg g^{-1} fr.wt.) followed by C-152 (5.78 mg g^{-1} fr.wt.) and RC-19 (4.75 mg g^{-1} fr.wt.). The highest reduction in total sugar in diseased samples was observed in RCP-27 (1.32 mg g^{-1} fr.wt) followed by RC-19 (1 mg g^{-1} fr.wt) and C-152 (0.94 mg g^{-1} fr.wt).

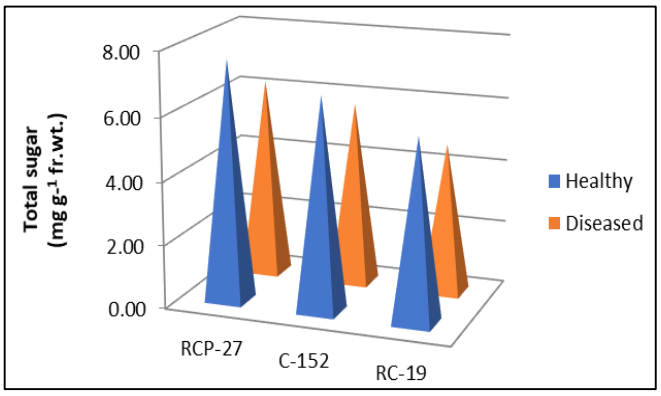

Fig 1: Biochemical changes in total sugar in cowpea plants

3.2 Effect on reducing sugar

The data in table 1 and fig 2 showes that reducing sugar content was maximum in healthy samples of variety RCP-27 (5.56 mg g^{-1} fr.wt.) followed by C-152 (4.85 mg g^{-1} fr.wt.) and RC-19 (3.68 mg g^{-1} fr.wt.). A similar trend was observed in diseased samples showing maximum reducing sugar content in RCP-27 (4.50 mg g^{-1} fr.wt.) followed by C-152 (3.68 mg g^{-1} fr.wt.) and RC-19 (3.16 mg g^{-1} fr.wt.). It clearly exhibited that reducing sugar were observed minimum in diseased samples as compared to healthy ones. The highest reduction in reducing sugar in diseased samples was observed in C-152 (1.17 mg g^{-1} fr.wt) followed by RCP-27 (1.06 mg g^{-1} fr.wt) and RC-19 (0.52 mg g^{-1} fr.wt).

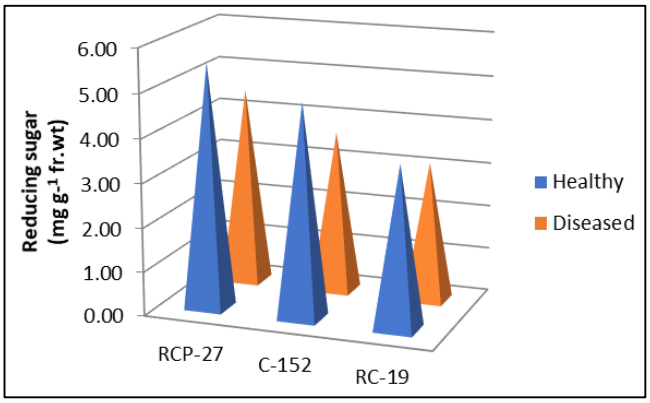

Fig 2: Biochemical changes in reducing sugar in cowpea plants

3.3 Effect on non-reducing sugar

The data pertaining to effect of charcoal rot on non-reducing sugar content are presented in table 1 and fig 3. Non-reducing sugar were found decreased in all the tested diseased samples as compared to healthy samples. The maximum non-reducing sugar was observed in healthy samples of RCP-27 (2.59 mg g^{-1} fr.wt.) closely followed by C-152 (2.31 mg g^{-1} fr.wt.) and RC-19 (2.29 mg g^{-1} fr.wt.). Similarly in diseased samples, RCP-27 showed highest value of 1.81 mg g^{-1} fr.wt, followed by C-152 (1.79 mg g^{-1} fr.wt.) and RC-19 (1.70 mg g^{-1} fr.wt.). The maximum reduction in non-reducing sugar content was observed in diseased samples of variety RCP-27 (0.78 mg g^{-1} fr.wt) followed by RC-19 (0.59 mg g^{-1} fr.wt) and C-152 (0.52 mg g^{-1} fr.wt).

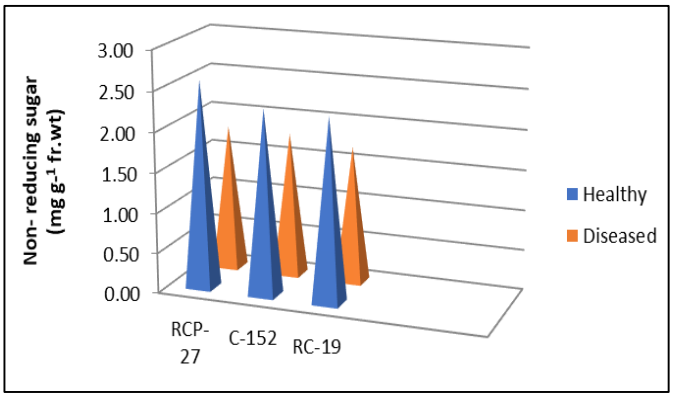

Fig 3: Biochemical changes in non-reducing sugar in cowpea plants

3.4 Effect on soluble protein

There was a significant decrease in soluble protein content in diseased plant samples as compared to healthy ones in all the tested varieties. The table 2 and fig 4 showed maximum soluble protein in healthy samples of variety RCP-27 (14.95 mg g^{-1} fr.wt) followed by C-152 (13.86 mg g^{-1} fr.wt) and RC-19 (12.84 mg g^{-1} fr.wt). Similarly in diseased samples of RCP-27 showed highest protein content of 13.74 mg g^{-1} fr.wt followed by C-152 (11.91 mg g^{-1} fr.wt) and RC-19 (9.68 mg g^{-1} fr.wt). The soluble protein content reduction was observed in diseased samples of all tested varieties *viz.*, RC-19 (3.16 mg g^{-1} fr.wt), C-152 (1.95 mg g^{-1} fr.wt) and RCP-27 (1.21 mg g^{-1} fr.wt).

Table 1: Biochemical changes in total sugar, reducing and non-reducing sugar in cowpea plants

Variety	Total sugar (Mg g^{-1} fr.wt.)		Reducing sugar (Mg g^{-1} fr.wt.)		Non-reducing sugar (Mg g^{-1} fr.wt.)	
	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
RCP-27	7.60 (15.99)*	6.28 (14.51)	5.56 (13.63)	4.50 (12.24)	2.59 (9.25)	1.81 (7.73)
C-152	6.72 (15.02)	5.78 (13.91)	4.85 (12.71)	3.68 (11.05)	2.31 (8.73)	1.79 (7.69)
RC-19	5.75 (13.87)	4.75 (12.59)	3.68 (11.05)	3.16 (10.24)	2.29 (8.69)	1.70 (7.49)
S.Em±	0.12	0.15	0.11	0.12	0.08	0.03
CD (P=0.05)	0.37	0.47	0.34	0.37	0.24	0.08

*Figure in parenthesis are angular transformed values

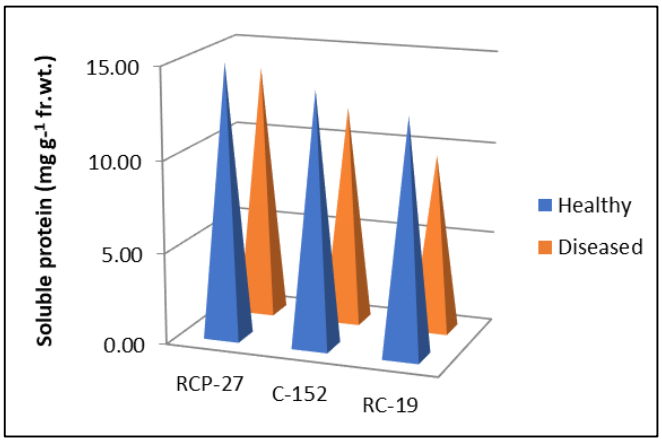

Fig 4: Biochemical changes in soluble protein in cowpea plants

3.5 Effect on total phenol

As presented in table 2 and fig 5 there was a significant increase in phenol content of cowpea plants due to charcoal rot as compared to healthy ones. Maximum phenol content was found in healthy samples of RCP-27 (1.69 mg g^{-1} fr.wt) followed by C-152 (1.54 mg g^{-1} fr.wt) and RC-19 (1.37 mg g^{-1} fr.wt). Maximum phenolic content was observed in diseased samples of RC-19 (2.53 mg g^{-1} fr.wt) followed by C-152 (2.13 mg g^{-1} fr.wt) and RCP-27 (1.77 mg g^{-1} fr.wt). The total phenol increase was observed in diseased samples of all tested varieties *viz*., RC-19 (1.16 mg g^{-1} fr.wt), C-152 (0.59 mg g^{-1} fr.wt) and RCP-27 (0.08 mg g^{-1} fr.wt).

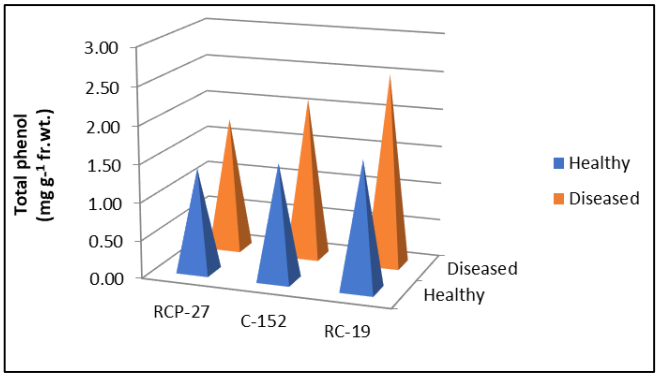

Fig 5: Biochemical changes in total phenol in cowpea plants

Table 2: Biochemical changes in soluble protein and total phenol in cowpea plants

Variety	Soluble protein (mg g^{-1} fr.wt.)		Total phenol (mg g^{-1} fr.wt.)	
	Healthy	Diseased	Healthy	Diseased
RCP- 27	14.95 (22.74)*	13.74 (21.75)	1.69 (7.46)	1.77 (7.63)
C-152	13.86 (21.85)	11.91 (20.18)	1.54 (7.12)	2.13 (8.38)
RC-19	12.84 (20.99)	9.68 (18.11)	1.37 (6.70)	2.53 (9.16)
S.Em±	0.12	0.14	0.06	0.06
CD (P=0.05)	0.37	0.43	0.18	0.18

*Figure in parenthesis is angular transformed values

4. Discussion

Biochemical changes in total, reducing and non- reducing sugar as well as soluble protein and total phenol from charcoal rot infected plants were compared with healthy plants and estimated in the laboratory.

Charcoal rot infestation resulted in significant reduction in total sugar, reducing and non-reducing sugar content of cowpea. Data of table 1 revealed that total sugar content was observed low in diseased plant tissues as compared to healthy ones of all the test varieties. The maximum total sugar content was observed in diseased samples of RCP-27 (6.28 mg g^{-1} fr.wt.) followed by C-152 (5.78 mg g^{-1} fr.wt.) and RC-19 (4.75 mg g^{-1} fr.wt.). The similar trend was also found in reducing and non-reducing sugar. In the present studies, total, reducing and non-reducing sugar was observed to be low in disease infected roots as compared to healthy roots of plant. The reduction in sugars content after infection may be due to rapid hydrolysis of sugars during pathogenesis through enzymes (hydrolases) secreted by pathogens and subsequent utilization by pathogen for their development.

There was a significant decrease in soluble protein content in diseased tissues as compared to healthy tissues in all the tested varieties. Diseased samples of RCP-27 showed highest protein content of 13.74 mg g^{-1} fr.wt followed by C-152 (11.91 mg g^{-1} fr.wt) and RC-19 (9.68 mg g^{-1} fr.wt). The data indicate that the soluble protein was observed more in healthy roots compared to diseased root. The results concluded by Arya *et al.,* (2016) [2] reported the reduction in the contents of total sugar, reducing and non-reducing sugar in the dry root rot infected tissues in groundnut. They also found that soluble protein content was significantly decreased in diseased roots of groundnut. Similar pattern was reported by Sultana *et al.,* (1998) [17]. These findings are very much similar with our findings. The results are in agreement with the findings of Ushamalini *et al.,* (1998) [19] and Pancham *et al.,* (2016) [14]. They studied the effect of seed-borne fungi of cowpea and concluded that *Fusarium oxysporum* f.sp. *trachelphilum* caused maximum reduction in sugars as compared to other fungi, while in *Macrophomina phaseolina* infection, protein content reduced drastically with respect to control. The same findings are reported by Anila *et al.* (2016) [1] who observed decreased protein and sugar content in diseased variety in comparison to healthy plants.

There was a significant increase in phenol content of infected cowpea plants due to charcoal rot as compared healthy ones. Maximum phenolic content was observed in diseased samples of RC-19 (2.53 mg g^{-1} fr.wt) followed by C-152 (2.13 mg g^{-1} fr.wt) and RCP-27 (1.77 mg g^{-1} fr.wt). Findings revealed that total phenols in all the varieties were found to be higher due to infection. Accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development owing to its toxicity to pathogens. Phenolic compounds are fungitoxic in nature, thus the accumulation of phenolic compound increase the physical and mechanical strength of host cell wall resulting in the inhibition of fungal invasion (Benhamou *et al.,* 2000) [3].The phenolic compounds act as adaptive mechanism in the host plant against the fungal infection. Phenolic substances are known to participate in a number of biochemical process such a oxidation reduction reaction and stimulation as well as inhibition of auxin activity (Misaghi, 1982) [11]. Phenolic compounds were shown to inhibit the production of cell wall degrading enzymes by the pathogen (Mandavia *et al.,* 1997) [10].

5. Conclusion

Biochemical contents such as total sugar, reducing, non-reducing sugar and soluble proteins were significantly reduced in all the samples of charcoal rot infested cowpea plants. While there was a significant increase in phenol

content of cowpea plants due to charcoal rot as compared to healthy ones.

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