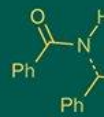


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(2): 191-196
www.biochemjournal.com
Received: 01-09-2025
Accepted: 04-11-2025

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Elucidation of defense-related biochemical mechanisms in clusterbean against *Agrothelia rolfsii*-incited stem rot

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i2c.7420>

Abstract

Cluster bean, a vital legume for arid regions, faces significant yield constraints from stem rot disease caused by the soil-borne fungus *Agrothelia rolfsii*. While the pathogen is known, a comprehensive understanding of the biochemical defense mechanisms it triggers in cluster bean remains limited. This study investigated the impact of *A. rolfsii* infection on key biochemical components—sugars, soluble proteins, and phenols—across three cluster bean varieties (RGC-936, RGC-1003, and RGC-1033). Biochemical analyses conducted 45 days after sowing revealed a significant shift in host metabolism induced by the pathogen. A consistent and significant depletion of carbohydrate reserves was observed, with total, reducing, and non-reducing sugars decreasing most markedly in the susceptible variety RGC-936. Conversely, the infection elicited a robust defense response, characterized by a significant accumulation of soluble proteins and phenolic compounds. The variety RGC-1033 exhibited the most pronounced induction of these defense biomarkers, with increases of 1.88 mg g⁻¹ FW in soluble protein and 0.10 mg g⁻¹ FW in total phenols. The results demonstrate that *A. rolfsii* infection disrupts primary carbohydrate metabolism while simultaneously activating the synthesis of defense-related proteins and phenolics. The varietal differences in the magnitude of these responses suggest a link between biochemical resilience and potential tolerance, providing crucial insights for developing stem rot-resistant cluster bean cultivars.

Keywords: Cluster bean, stem rot, *Agrothelia rolfsii*, biochemical defense, phenols, sugars

1. Introduction

Cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.), a member of the Fabaceae family, is a drought-tolerant annual legume cultivated primarily as a rainfed Kharif crop. Its versatility makes it a crucial commodity for grain, fodder, vegetable, green manure, and seed production, particularly in arid and semi-arid regions (Swamy and Naveena, 2015) [19]. India is the global leader in cluster bean (guar) cultivation, accounting for approximately 80% of world production and 75% of international trade, with an estimated area of 9.6 million hectares in its north-western states, including Rajasthan, Haryana, Gujarat, Punjab, and parts of Uttar Pradesh and Madhya Pradesh (Anon, 2014) [2]. Rajasthan alone contributes significantly, with cultivation spanning approximately 2 million hectares (Anon, 2014) [2]. Despite the development of agronomically stable, high-yielding varieties, the productivity of cluster bean is severely constrained by a complex of phytopathogenic diseases (Anon, 1999) [1]. The crop is susceptible to bacterial blight (*Xanthomonas axonopodis* pv. *cyamopsidis*), Alternaria leaf spot (*Alternaria cucumerina* var. *cyamopsidis*), anthracnose (*Colletotrichum capsici* f. sp. *cyamopsicola*), and charcoal rot (*Macrophomina phaseolina*), among others. Among these, stem rot incited by the soil-borne fungus *Agrothelia rolfsii* (formerly *Sclerotium rolfsii*) poses a significant threat, inducing substantial biotic stress that disrupts key physiological and biochemical processes.

While it is known that *A. rolfsii* infection alters physiological parameters such as chlorophyll and proline content, a comprehensive analysis of its impact on critical biochemical defense components in cluster bean remains limited. Elucidating these pathogen-induced biochemical changes is essential for understanding host-pathogen interactions and developing resilient cultivars. Therefore, this study was designed to investigate the effect of *A. rolfsii* infection on the levels of key biochemical markers—specifically, phenols, sugars, and soluble proteins—across three distinct varieties of cluster bean.

2. Materials and Methods

2.1 Biochemical Analysis

2.1.1 Estimation of Total Sugar Content

The total sugar content was determined using the colorimetric anthrone method (Dubois *et al.*, 1956) [3]. Briefly, 100 mg of fresh plant tissue was hydrolysed with 5 mL of 2.5 N HCl in a boiling water bath for 3 hours. The hydrolysate was cooled, neutralized with solid sodium carbonate, and made up to a final volume of 100 mL with distilled water. After centrifugation, the supernatant was collected. Aliquots (0.5 and 1.0 mL) of the supernatant were used for analysis.

A standard curve was prepared using a working glucose standard solution (100 µg/mL), with concentrations ranging from 0 to 100 µg. Both sample aliquots and standards were brought to a volume of 1 mL with distilled water. Then, 4 mL of anthrone reagent (2 mg/mL in concentrated sulfuric acid) (appendix) was added to each tube. The tubes were heated in a boiling water bath for 8 minutes, rapidly cooled, and the absorbance of the resulting green colour was measured at 630 nm. The total sugar content was calculated from the standard curve and expressed as mg per gram of fresh tissue weight (mg g⁻¹ FW).

2.1.2 Estimation of Reducing Sugars

Reducing sugars were quantified by Nelson's modification of Somogyi's method (Somogyi, 1952) [18]. A 100 mg plant sample was extracted twice with hot 80% ethanol. The combined supernatants were evaporated to dryness, and the residue was dissolved in 10 mL of distilled water.

Aliquots (0.1-0.2 mL) of this alcohol-free extract were pipetted into test tubes. A standard series was prepared using the working glucose standard (0, 20, 40, 60, 80, and 100 µg). The volume in all tubes, including samples and standards, was adjusted to 2 mL with distilled water. One millilitre of alkaline copper tartrate reagent (prepared by mixing 24 mL of Copper Reagent A and 1 mL of Copper Reagent B) (appendix) was added to each tube. The tubes were heated in a boiling water bath for 10 minutes, cooled, and then 1 mL of arseno-molybdate reagent (appendix) was added. The volume in each tube was made up to 10 mL with distilled water, and the absorbance was measured at 620 nm. The reducing sugar content was determined from the standard curve and expressed as a percentage.

2.1.3 Estimation of Non-Reducing Sugars

The non-reducing sugar content was calculated indirectly by subtracting the reducing sugar content from the total sugar content and multiplying the difference by a factor of 0.95.

2.1.4 Estimation of Total Phenol Content

The total phenolic content was estimated according to the method of Thimmaiah (1999) [20]. One gram of fresh shoot tissue was homogenized in 10 mL of 80% ethanol. The homogenate was centrifuged at 10,000 × g for 20 minutes. The supernatant was collected, and the residue was re-extracted with 80% ethanol. The combined supernatants were evaporated to dryness, and the residue was dissolved in 5 mL of distilled water.

An aliquot (0.2 mL) of this extract was mixed with 3 mL of distilled water, followed by the addition of 0.5 mL of Folin-Ciocalteu reagent (appendix). After 3 minutes, 2 mL of 20% sodium carbonate solution was added, and the mixture was vortexed. The tubes were placed in a boiling water bath for

1 minute. After cooling, the absorbance was measured at 650 nm against a reagent blank. A standard curve was prepared using catechol, and the total phenol content was expressed as mg catechol equivalents per gram of fresh weight (mg g⁻¹ FW).

2.1.5 Estimation of Soluble Protein Content

The soluble protein content was determined using the method of Lowry *et al.* (1951) [10]. One gram of fresh shoot tissue was homogenized in 5 mL of 0.1 M sodium phosphate buffer (pH 7.0) (appendix). The homogenate was centrifuged at 16,000 × g for 20 minutes, and the supernatant was used for the assay.

An aliquot (0.1 mL) of the supernatant was mixed with 1 mL of distilled water. Then, 5 mL of Alkaline Copper Solution (Solution C), prepared fresh by mixing 50 parts of Solution A (2% sodium carbonate in 0.1 N NaOH) with 1 part of Solution B (0.5% copper sulfate in 1% sodium potassium tartrate), was added. The mixture was incubated at room temperature for 10 minutes. Subsequently, 0.5 mL of 1N Folin-Ciocalteu reagent (appendix) was added, mixed thoroughly, and incubated in the dark for 30 minutes. The absorbance was measured at 660 nm. The protein concentration was calculated from a standard curve prepared using bovine serum albumin (BSA) and expressed as parts per million (ppm).

3. Results

3.1 Alterations in Sugar Metabolism in Response to Pathogen Challenge

Infection by *A. rolfii* significantly altered the carbohydrate profile across all three cluster bean varieties, manifesting as a consistent reduction in total, reducing, and non-reducing sugar content compared to healthy controls.

Total sugars were highest in healthy plants of RGC-1033 (9.01 mg g⁻¹ FW), followed by RGC-1003 (8.29 mg g⁻¹ FW) and RGC-936 (6.17 mg g⁻¹ FW) (Fig. 1). A similar varietal trend was maintained in diseased plants, but with significantly lower concentrations: RGC-1033 (8.33 mg g⁻¹ FW), RGC-1003 (7.05 mg g⁻¹ FW), and RGC-936 (4.74 mg g⁻¹ FW) (Fig. 1). The magnitude of pathogen-induced depletion was most pronounced in RGC-936 (1.43 mg g⁻¹ FW reduction), followed by RGC-1003 (1.24 mg g⁻¹ FW) and RGC-1033 (0.68 mg g⁻¹ FW) (Fig. 1).

A parallel decline was observed in reducing sugars. Healthy plants of RGC-1033 contained the highest level (5.26 mg g⁻¹ FW), which decreased to 4.79 mg g⁻¹ FW upon infection (Fig. 2). The greatest absolute reduction in reducing sugars occurred in RGC-1003 (0.67 mg g⁻¹ FW), compared to 0.47 mg g⁻¹ FW in RGC-1033 and 0.31 mg g⁻¹ FW in RGC-936 (Fig. 2).

The most substantial relative decrease was noted in non-reducing sugars. The variety RGC-936 exhibited the most severe depletion, with a reduction of 1.21 mg g⁻¹ FW (from 2.20 mg g⁻¹ FW to 0.99 mg g⁻¹ FW) (Fig. 3). The reductions in RGC-1003 and RGC-1033 were comparatively lower, at 0.51 mg g⁻¹ FW and 0.21 mg g⁻¹ FW, respectively (Fig. 3).

3.2 Induction of Defense-Associated Biomarkers

In contrast to the observed decline in sugars, infection by *A. rolfii* triggered a marked increase in soluble protein and phenolic compounds, key indicators of a plant defense response.

The total phenol content was significantly elevated in infected plants. The highest phenolic levels, both in healthy and diseased states, were found in RGC-1033 (0.47 and 0.57 mg g⁻¹ FW, respectively) (Fig. 4). All infected varieties showed a consistent increase in phenolics: RGC-1033 by 0.10 mg g⁻¹ FW, RGC-1003 by 0.08 mg g⁻¹ FW, and RGC-936 by 0.07 mg g⁻¹ FW, suggesting a systemic activation of the phenylpropanoid pathway (Fig. 4).

Similarly, Soluble protein content increased significantly in diseased plants of all varieties (Fig. 5). The most substantial induction was observed in RGC-1033, with an increase of 1.88 mg g⁻¹ FW (from 5.04 to 6.92 mg g⁻¹ FW). Smaller, yet consistent, increases were recorded in RGC-1003 (0.24 mg g⁻¹ FW) and RGC-936 (0.19 mg g⁻¹ FW) (Fig. 5).

4. Discussion

The biochemical constituents of a plant, including sugars, proteins, and phenols, are critically involved in the defense response against pathogen invasion. This study demonstrates that infection with the stem rot pathogen elicits significant alterations in the profiles of these compounds in cluster bean, reflecting the plant's attempt to mount a defense and the pathogen's success in exploiting host resources.

A marked depletion in sugar content was observed in the stems of infected plants across all tested varieties. Total sugars, as well as reducing and non-reducing sugars, were significantly lower in diseased tissues compared to their healthy counterparts. The most pronounced reduction in total sugars was recorded in the susceptible variety RGC-936 (1.43 mg g⁻¹), followed by RGC-1003 (1.24 mg g⁻¹) and RGC-1033 (0.68 mg g⁻¹) (Fig. 1). This decline is consistent with previous findings that pathogenic infection disrupts fundamental physiological processes like photosynthesis and respiration, leading to substantial fluctuations in carbohydrate pools (Saraswathi and Reddy, 2012) [16]. The reduced sugar content may be attributed to the pathogen's direct utilization of sugars for energy, and potentially to the role of sugars in inhibiting key pathogenicity enzymes, such

as pectolytic and cellulolytic enzymes (Jyosthna *et al.*, 2004; Jagtap *et al.*, 2011) [7, 6]. This aligns with reports of decreased sugar content in other host-pathogen systems, including soil-borne fungal infections (Keshgond and Lingaraju, 2016; Reddy and Sireesha, 2013) [9, 15].

In contrast to sugars, the soluble protein content exhibited a significant increase in the infected stems. The variety RGC-1033 showed the highest accumulation (1.88 mg g⁻¹), followed by RGC-1003 (0.20 mg g⁻¹) and RGC-936 (0.19 mg g⁻¹) (Fig. 4). This enhanced protein synthesis is likely of host origin (Singh, 2000) [17] and represents a critical component of the defense machinery. Similar increases in protein content have been documented in groundnut infected with *Cercospora personatum* (Kour and Dhillon, 1990) [8] and in *Vigna mungo* infected with leaf crinkle virus (Malik *et al.*, 2002). This upregulation is presumably due to the activation of enzymes essential for the synthesis of various defense-related compounds (Vidyasekaran, 2001) [21].

Furthermore, a significant induction of phenolic compounds was observed in the diseased stems 45 days after sowing. Phenols, being universally present in vascular plants, are well-established as key players in both passive and active defense mechanisms, ultimately restricting pathogen development (Nicholson and Hammerschmidt, 1992). In this study, the maximum phenolic content was recorded in RGC-1033 (0.10 mg g⁻¹), followed by RGC-1003 (0.08 mg g⁻¹) and RGC-936 (0.06 mg g⁻¹) (Fig. 4). The higher phenolic accumulation in certain genotypes may be linked to the availability of sugars, which act as precursors for the shikimic acid pathway responsible for phenol synthesis. This finding is consistent with the work of Ekbote and Mayee (1983) [4] and supports the general observation that resistant varieties often exhibit a more robust and rapid accumulation of phenolics upon infection (Ganguly, 1995; Singh, 2000) [5, 17]. The concurrent increase in both phenols and proteins, as also reported by Nandi *et al.* (2013) [12] in cowpea, underscores a coordinated biochemical defense response against pathogen challenge.

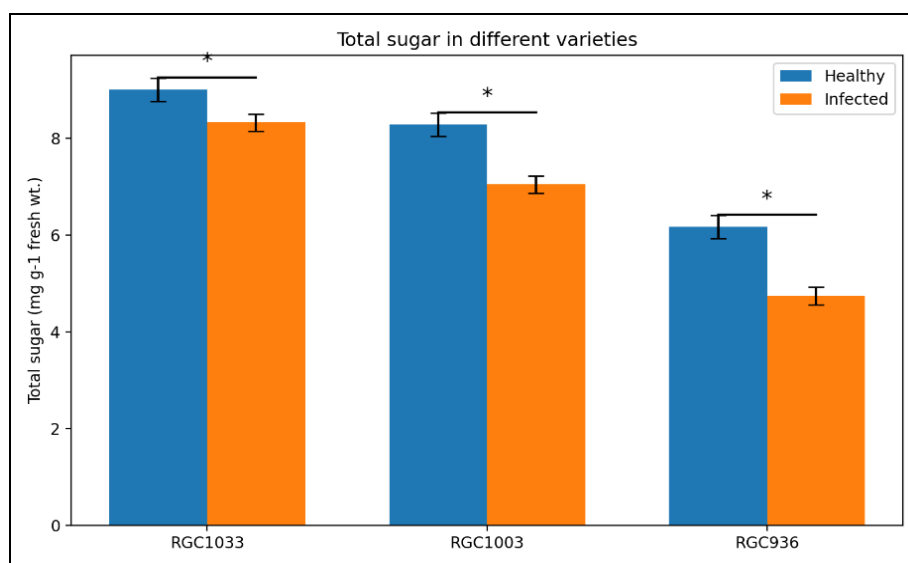


Fig 1: Total sugar content in stems of healthy and stem rot-infected cluster bean plants. Bars represent the mean $\pm$ standard deviation. Different letters indicate statistically significant differences ($p < 0.05$) between healthy and infected plants within each variety.

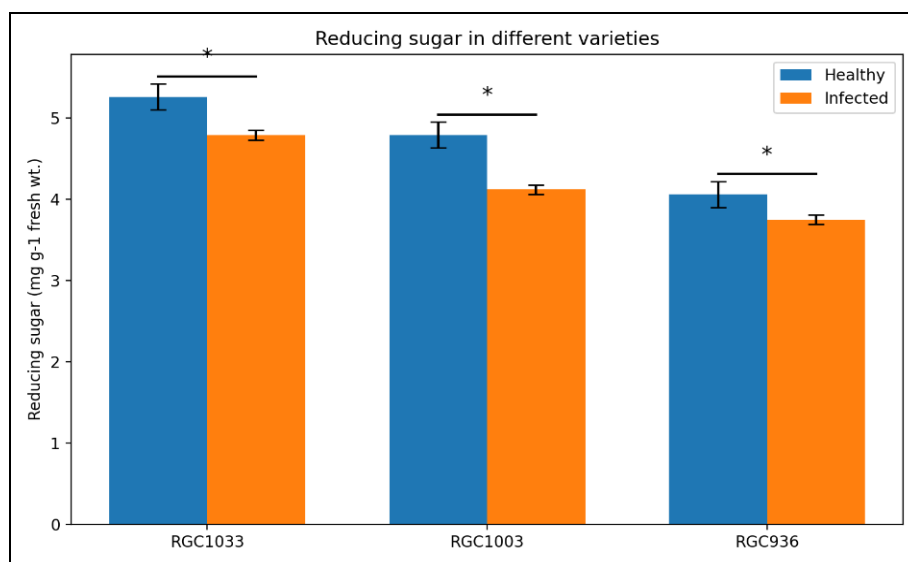


Fig 2: Total reducing sugar content in stems of healthy and stem rot-infected clusterbean plants. Bars represent the mean $\pm$ standard deviation. Different letters indicate statistically significant differences ($p < 0.05$) between healthy and infected plants within each variety.

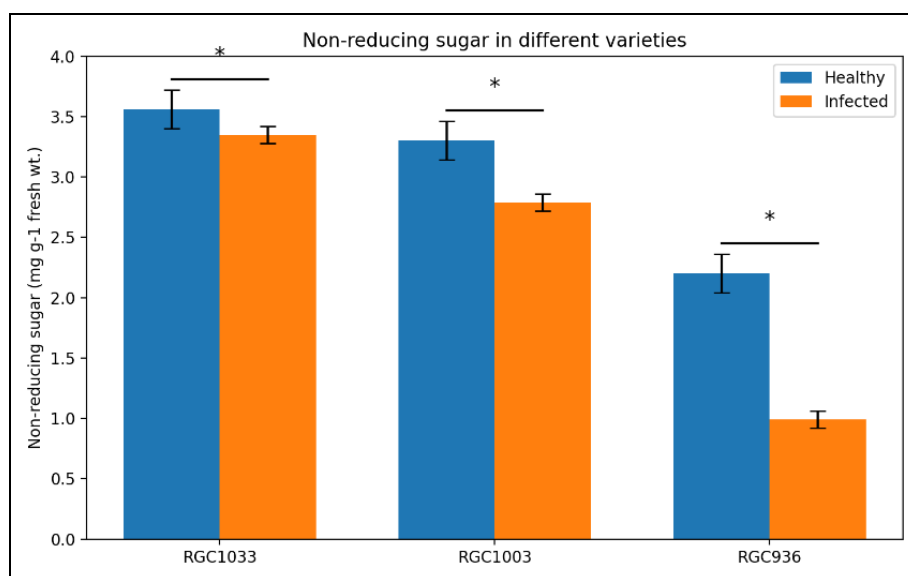


Fig 3: Total non-reducing sugar content in stems of healthy and stem rot-infected clusterbean plants. Bars represent the mean $\pm$ standard deviation. Different letters indicate statistically significant differences ($p < 0.05$) between healthy and infected plants within each variety.

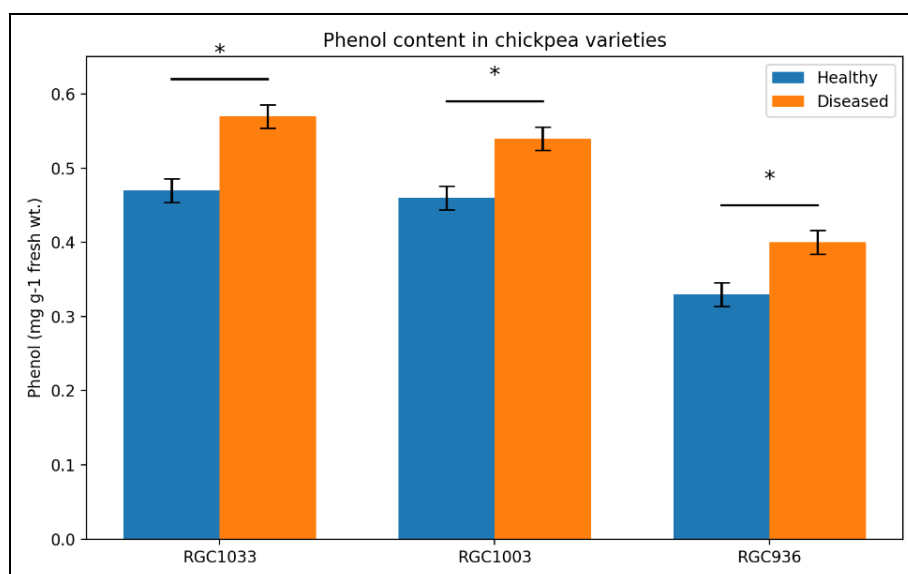


Fig 4: Total phenol content in stems of healthy and stem rot-infected cluster bean plants. Values are presented as the mean $\pm$ standard deviation. Different letters above the bars indicate statistically significant differences between treatments within a variety ($p < 0.05$).

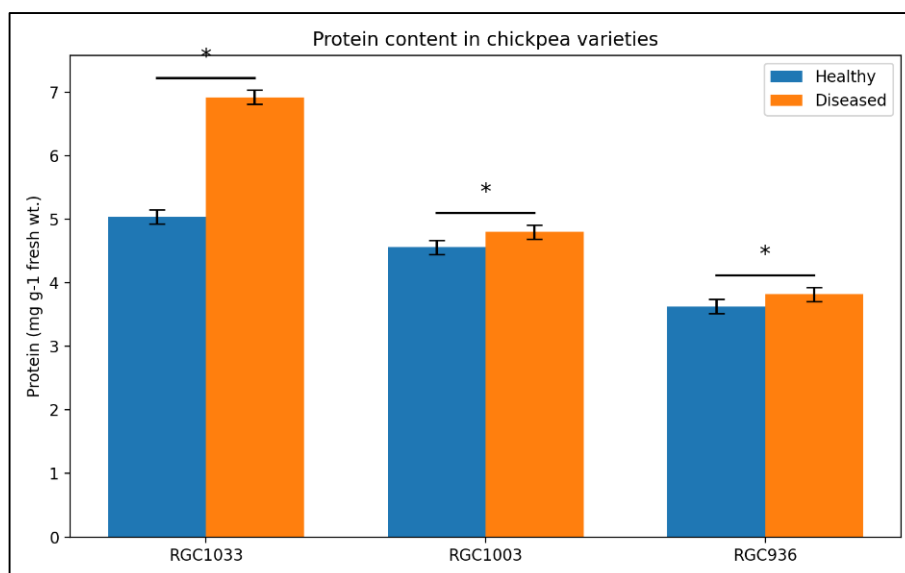


Fig 5: Total soluble protein content in stems of healthy and stem rot-infected cluster bean plants. Values are presented as the mean $\pm$ standard deviation. Different letters above the bars indicate statistically significant differences between treatments within a variety ($p < 0.05$).

5. Conclusion

The findings of this study demonstrate that stem rot infestation in cluster bean precipitates significant biochemical alterations. A consistent and significant decrease in carbohydrate reserves—including total, reducing, and non-reducing sugars—was observed across all infected samples. Conversely, the infection triggered a substantial increase in defense-related compounds, marked by the significant accumulation of soluble proteins and phenolic content in diseased plants compared to healthy controls. These results confirm that stem rot pathogenesis disrupts primary metabolism while simultaneously inducing a defense response in the host plant.

6. Acknowledgements

The author expresses sincere gratitude to the Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agriculture University (SKRAU), Bikaner, for providing the necessary facilities and support that enabled the successful execution of this research work.

Appendix

Reagents

Anthrone Reagent: 2 mg/mL in concentrated sulfuric acid.

Glucose Standard Stock: 1 mg/mL in distilled water.

Working Glucose Standard: 100 μ g/mL, prepared by diluting the stock solution.

Copper Reagent A: Contained 2.5% (w/v) anhydrous sodium carbonate, 2.5% (w/v) potassium sodium tartrate, 2.0% (w/v) sodium bicarbonate, and 20.0% (w/v) sodium sulfate.

Copper Reagent B: 15% (w/v) copper sulfate with one drop of concentrated sulfuric acid.

Arseno-Molybdate Reagent: Contained 2.5 g ammonium molybdate in 2.5 mL concentrated sulfuric acid, mixed with 0.3 g disodium hydrogen arsenate in distilled water, and made up to 70 mL.

Folin-Ciocalteu Reagent: Commercially obtained, diluted to 1N for the protein assay.

Sodium Phosphate Buffer: 0.1 M, pH 7.0.

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