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A study on physico-biochemical efficiency of LBG 17 a Blackgram (*Vigna mungo* L. Hepper) genotype against powdery mildew

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Abstract: *LBG 17 is a resistant inbred of Blackgram developed from susceptible parents Nethiminumu and chikkuduminumu to defend the damage due to powdery mildew. In present study the physiological efficiency of LBG 17, Nethiminumu and chikkuduminumu over powdery mildew was studied in terms of chlorophyll, phenols, soluble proteins, soluble carbohydrates, total carbohydrates, peroxidase, superoxide dismutase and polyphenol oxidase. Powdery mildew effect on yield efficiency was also studied in LBG 17, Nethiminumu and chikkuduminumu in terms of seeds per pod, 100 seed weight, number of pods and yield per acre and correlated with the respected physiological and biochemical parameters. The chlorophyll content (mg/g), soluble proteins (µg/mg), total carbohydrates (µg/mg) were highest in resistant variety on fungal infection whereas these characters recorded less in susceptible cultivars. The activity of phenols (µg/mg), peroxidises (µg/mg), super oxide disumutase (µg/mg) and polyphenol oxidase (µg/mg) were significantly increased in tolerant genotype compared to its susceptible parents. Significant variation in the protein molecular mass of LBG 17 was also observed at 66 KD over its parents. The regression analysis studies revealed that the increased physico, biochemical activities in LBG 17 showed a positive correlation with the increased yield which in turn confirm the inherent ability of this genotype over its parents against powdery mildew.*

Keywords: *Blackgram, Powdery mildew, Hybrid vigour, LBG 17, Biochemical, Superoxide dismutase*

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I. Introduction

The reactive oxygen species (ROS) such as superoxide radicals (O_2-), hydrogen peroxide (H_2O_2), OH, are chemically active molecules which were produced by the plants during pathogen attack[1]. Formation of these ROS indicates that these are initial cellular responses exhibited by plants upon successful pathogen recognition. However, the enhanced ROS inside the cells results in cellular damage and destroy macromolecules like proteins, pigments, lipids and nucleic acids [2]. In order to overcome the damage caused by ROS, plants accommodate with antioxidant enzymes like POX, PPO, SOD and CAT [3]. On the other side phenolic compounds can assist plants in protecting cells from free radicals formed due to reactive oxygen species [4, 5]. Yield loss in pulses is increasing year by year due to these unwanted biotic factors [6].

Blackgram (*Vigna mungo* L. Hepper) is one of the most ancient and important pulse crop of Asia, found to be originated in India. It is having more nutritional quality and suitable to all types of cropping systems. It is commonly known as "**poor man's meat**" as it is dietary protein for the large section of vegetarian population of Indian Sub Continent with a seed protein content of 24% [7]. In India, Andhra Pradesh is one of the major blackgram cultivating states with an yield of 13.74 lakh tonnes from an area of 32.99 lakh ha with a productivity of 417 kg/ha. In spite of cultivating in large area, the productivity was decreasing year by year; this decrease in productivity is may be due to the low genetic yield potentiality, different biotic and abiotic stresses. Among the biotic stresses, diseases due to fungal or bacterial are responsible for an estimated yield loss of about 20 to 30% [8]. Among the foliar fungal diseases of blackgram powdery mildew found to be significant caused by *Cercospora* results in major yield losses. The powdery mildew occurs throughout the year under favourable conditions and it is more severe in late sown *kharif* crop. Hence, the use of fungicides has become inevitable in controlling the foliar diseases in the absence of suitable resistant cultivars. On the other hand development of powdery mildew tolerant varieties found to be a good remedy to stabilize the blackgram yields. Regional Agricultural Research Station (RARS), Lam, Guntur had developed a variety of blackgram LBG 17 resistant against powdery mildew by crossing Nethiminumu and Chikkuduminumu. In present study we aimed at to determine and compare the physiological, biochemical and yield alterations during powdery mildew infection in tolerant LBG 17 and recessive parents Nethiminumu and Chikkuduminumu.

II. Materials And Methods

The present work has been carried out by using Blackgram varieties viz., Nethiminumu and Chikkuduminumu and their cross LBG 17 at Botanical Garden of Acharya Nagarjuna University. The seeds were sown in the field in eight replications in complete randomized block design. The seeds were sown in 5 rows of 5m length with plant to plant and row to row spacing of 10 cm and 30 cm respectively. The plants were inoculated with spore suspension (10^6 conidia/ml) of *Erisiphae polygoni* DC on healthy leaves after 30 days of sowing. Disease scoring was performed through visual observation of the powdery spots, which appeared on the leaves and compared with a 0 to 9 scale. Observations on morphological, physiological and yield attributes were recorded by taking 5 randomly selected plants in each replication.

2.1 Estimation of Total chlorophyll

Chlorophyll content of experimental material was estimated by the method of [9]. Finely cut leaf samples of 50 mg were dissolved in 10 ml of Dimethyl Sulphoxide (DMSO) and the tubes were incubated overnight in the dark. The extracted chlorophyll pigment containing solution was measured spectrophotometrically at 645 nm and 663 nm.

$$\text{Total chlorophyll} = (20.2 \times \text{O.D at 645 nm} + 8.02 \times \text{O.D at 663 nm}) \times \frac{V}{10} \times W$$

2.2 Estimation of Phenols

The amount of phenols present in the leaf samples was estimated by Folin-ciocalteau reagent method [10]. Hundred milligrams of leaf sample was macerated with 1 ml of 80% ethanol and centrifuged at 15000 rpm for 15 minutes. To the supernatant 2 ml of petroleum ether was added vortexed for 3 minutes. Later petroleum ether was removed by keeping in water bath. After drying 5 ml of phenol stock was prepared by adding distilled water. To the 100 µl stock solution 3 ml of distilled water was added followed by 0.5 ml of Folin-ciocalteau reagent to this 2 ml of 20 percent sodium carbonate solution was added after 3 minutes. The contents were boiled for about 8 minutes and cooled. Change in colour was measured at 650 nm.

2.3 Estimation of Soluble Proteins

Soluble protein was estimated according Lowry *et al*. [11]. One gram of leaf sample was ground into fine mixture using prechilled pestle and mortar by adding 1.5 ml of 50 mM pH 7.5 phosphate buffer. The homogenate was centrifuged at 15000 rpm for 15 minutes. To the 1 ml of supernatant containing protein sample 5 ml of alkaline copper solution was added and incubated for ½ hr later the samples were allowed to react with follins reagent and copper sulphate to get a blue colour complex. After 30 minutes the absorbance i.e., blue colour was measured at 660 nm in a spectrophotometer.

2.4 Estimation of Soluble Carbohydrates

The amount of soluble carbohydrates present in the leaf sample was estimated by Anthrone method [12]. A gram fresh leaf material was ground into fine mixture using 1.5 ml of 50 mM pH 7.0 phosphate buffer containing β- Mercaptoethanol. Later the homogenate was centrifuged at 15000 rpm for 15 minutes at 4°C. To the 0.1 ml of supernatant 4 ml of Anthrone reagent was added and the test tubes were placed in a boiling water bath for 8 minutes and cooled rapidly in ice. The absorbance of the green color solution was measured at 630 nm. The amount of sugars present in the extract was calculated using a standard curve prepared from glucose.

2.5 Estimation of Total Carbohydrates

The amount of total carbohydrates present in the leaf sample were estimated by Anthrone method [13]. 100 mg of fresh leaf sample was taken into a boiling tube and the tube was kept in boiling water bath for 3 hours with 5 ml of 2.5 N HCl. The sample was cooled to room temperature and then neutralized with solid sodium carbonate until the effervescences ceases. The final volume of the sample was made to 25 ml with distilled water in volumetric flask. 0.1 ml of supernatant was taken in a test tube and volume made to 1 ml with distilled water and 4 ml of Anthrone reagent was added. The test tube was placed in a boiling water bath for 8 minutes and cooled rapidly in ice. A reagent blank was treated similarly by taking 4 ml of Anthrone reagent only. The absorbance of the green color solution was measured at 630 nm. The amount of sugars present in the extract was calculated using a standard curve prepared from glucose.

The activities of peroxidase and polyphenol oxidase were assayed according to Prathibha Devi [14]. 500 mg of plant material was grounded using pre chilled pestle and mortar by adding 30 - 40 ml phosphate buffer (0.02 M). The contents were filtered through cheese cloth and centrifuged at 2000 rpm for 10 min. The extract was made up to 100 ml by adding phosphate buffer and preserved for further biochemical analysis.

2.6 Peroxidase activity

Reaction mixture was prepared by adding 3 ml of pyrogallol phosphate buffer and 0.1 ml of enzyme extract into a cuvette. To the reaction mixture 0.5 ml of H_2O_2 was added and gently shaken. The absorbance was measured after 3 min at 420 nm. The same procedure was continued to know the control value by using boiled enzyme extract. The enzyme activity was measured by subtracting the absorbance value of the blank from the sample and expressed the enzyme activity as absorbing units per 1 g fresh weight per 3 minutes.

2.7 Polyphenol oxidase

The reaction mixture was prepared by adding 2 ml of buffer, 1ml of enzyme extract. The mixture was incubated for 5 min and the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. Optical density was measured at 420 nm against a blank containing 1 ml of H_2SO_4, 2 ml of buffer, 1 ml of pyrogallol and 1 ml of boiled enzyme extract. Enzyme activity was calculated by subtracting the absorbance value of blank from the sample and expressed the enzyme activity as absorbing units per 1 g fresh weight per 5 min.

2.8 Superoxide dismutase

Leaf samples of 500 mg were homogenized in ice cold 50 mM potassium phosphate buffer (pH 7.8) with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and centrifuged at 4 °C in cooling micro centrifuge (Eppendorf – 5415 R) at 10, 000 rpm. The supernatant was used for enzyme activity assay [15] within 12 h of extraction. SOD activity was estimated by recording the decrease in absorbance of superoxide nitro blue tetrazolium complex by the enzyme [16].

A reaction cocktail of 33 ml was prepared by mixing the reagents in the following ratio (60 µl 50 mM Phosphate buffer; 390 µl 13 mM Methionine; 0.6 µl 02 µM Riboflavin; 60 µl 0.1 mM EDTA; 300 µl 75 mM NBT and 50 µl Enzyme extract). A blank was set without enzyme and NBT to calibrate the spectrophotometer. Another control was set having NBT but no enzyme as reference control. All the tubes were exposed to 400 W bulbs (4×100 W bulbs) for 15 min. The absorbance was measured at 560 nm immediately and calculated the percentage inhibition of the reaction between riboflavin and NBT in the presence of methionine which is taken as 1 unit of SOD activity.

2.9 Separation of proteins from black gram varieties by SDS-page method

The proteins from different black gram cultivars viz., Nethiminumu, Chikkuduminumu and LBG17 were separated by SDS-PAGE method and analyzed further for the difference in protein pattern. Seedlings of three blackgram varieties were taken for the separation of proteins by SDS-PAGE method.

The single seedling was transferred into a fresh 1.5 ml tube, to this 500 µl extraction buffer was added and grinded with a sterile pestle. The samples were intermittently vortexed for homogenization. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4 ºC. The supernatant from each sample was transferred into a fresh eppendorf (1.5 ml) tubes, to this equal volume of 2x samples loading buffer was added and mixed well. The entire setup was incubated at 95 ºC for five minutes. It was used for SDS-PAGE analysis.

2.10 Yield attributes

Yield parameters like number of clusters per plant, number of pods per plant, pod length (cm), number of seeds per pod, 100 seed weight (g), seed yield per plant (g) were analysed by using standard protocols.

III. Results And Discussion

The physiological and biochemical analysis were carried out with respect to total chlorophyll (mg/g) soluble carbohydrates (mg/g), total carbohydrates (mg/g), soluble proteins (mg/g) and phenols (mg/g) recorded in three blackgram varieties are presented in Table 1.

Total chlorophyll content in the leaves of three varieties was recorded and it was found to be lower in susceptible parents. The highest total chlorophyll content was recorded in LBG 17 (2.1 mg/g) followed by Chikkuduminumu (2.0 mg/g) and Nethiminumu (1.6 mg/g) (Table 1). Similar results were observed in grapes [17] and lucerne [18].

The phenol content in leaves of different test varieties of blackgram was studied (Table 1). Highest phenol content was observed in LBG 17 (3.68 mg/g) followed by Chikkuduminumu (2.77 mg/g) and Nethiminumu (2.68 mg/g). Phenolic compounds were considered to be the most responsible parameters for diseases resistance [19]. Similar trend of results were observed in cucumber [20], pea [21] and groundnut [22].

The soluble protein content in leaves of different blackgram cultivars was estimated. Highest soluble protein was recorded in LBG 17 (4.18 mg/g) followed by Chikkuduminumu (2.68 mg/g) and Nethiminumu (2.59 mg/g). Variation in soluble protein content among the three varieties was statistically significant (Table 1). The soluble carbohydrate content was estimated in the leaves of blackgram varieties. The highest soluble carbohydrate content was observed in LBG17 (42.62 mg/g) whereas it is medium in Chikkuduminumu (32.39

mg/g) and least in Nethiminumu (20.14 mg/g). Statistically this parameter showed very significant variation among the varieties studied (Table 1). The total carbohydrate content of leaves was estimated in all the three varieties (Table 1). The highest total carbohydrate was found in LBG 17 (45.59 mg/g) followed by Chikkuduminumu (36.10 mg/g) and Nethiminumu (23.73 mg/g). Present findings were in agreement with results obtained in peach [23], okra [24] and greengram [25].

Table 1 Physico-biochemical performance of LBG 17 over parents Nethiminumu and Chikkudu minumu

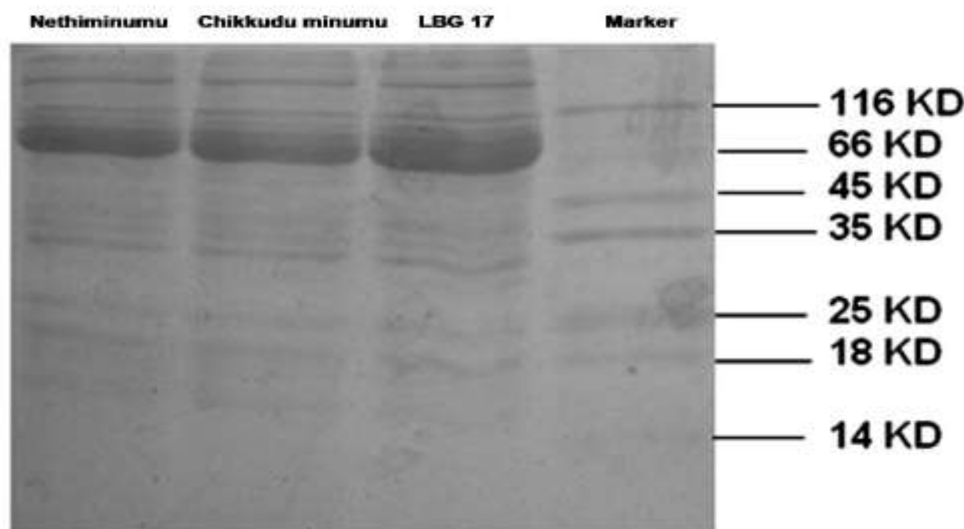
Variety→ Character↓	Blackgram			Mean	SEM	CD
	Nethi minumu	Chikkudu minumu	LBG 17			
Total Chlorophyll (mg/g)	1.8	2.0	2.3*	2.03	0.10	0.05
Phenols (mg/g)	2.68	2.77	3.68*	03.04	0.112	0.34
Soluble proteins (mg/g)	2.59	2.68	4.18*	03.77	0.17	0.68
Soluble carbohydrates (mg/g)	20.14	32.39	42.62	32.66	0.24	0.94
Total carbohydrates (mg/g)	23.73	36.10	45.59	36.43	0.32	1.29
Peroxidase (u/g)	0.13	0.14	0.16	0.143	0.120	0.03
Polyphenol oxidase (u/g)	0.12	0.14	0.18	0.146	0.100	0.54
Superoxide dismutase (u/g)	22.32	25.10	28.90	25.44	1.12	01.45
Number of clusters	10.00	10.50	14.00	11.30	0.88	NS
Pods per plant	40.00	41.30	65.30	48.80	2.02	07.94
Pod length (cm)	05.30	05.00	06.00	05.40	0.77	NS
Seeds per plant	06.60	06.80	08.60	07.20	0.33	01.30
100 seed weight (g)	05.10	05.80	06.90	05.93	0.09	00.35
Seed yield per plant (g)	13.40	10.60	19.30	14.40	0.21	00.82

The activity of ROS enzymes also showed significant changes between the susceptible and tolerant genotypes. The maximum peroxidase activity of 0.16 u/g was recorded in tolerant inbred LBG 17 whereas the lower activity 0.13 u/g and 0.14 u/g was observed in susceptible parents Nethiminumu and Chikkudu minumu. Peroxidases (POXs) catalyze the oxidation of several substrates at the expense of H_2O_2, playing a key role in the detoxification of H_2O_2 during the H_2O_2 -dependent polymerization of hydroxycinnamyl alcohols involved in the lignification process (lignin biosynthesis). Several studies have shown POX induction associated with the defence against biotic stress in Arabidopsis [26], in *Phytophthora coloccasiae* [27] and in cucurbita [28].

A highest polyphenol oxidase activity was observed in tolerant hybrid LBG 17 (0.18 u/g) followed by Chikkudu minumu (0.14 u/g) and Nethiminumu (0.12 u/g). The increased activity of poly phenol oxidase was reported due to either solubilization of polyphenolases from cellular compartments or activation of latent polyphenol oxidase [29]. Similar increase in both polyphenol oxidase and peroxidase enzymes following infection has also been reported in *Beta vulgaris* [30] and *Zea mays* [31].

The superoxide dismutase activity was found to be more in LBG 17 (28.90 µu/g). The SOD activity was found to be less in Chikkudu minumu and Nethiminumu i.e 25.10 µu/g, 22.62 µu/g respectively. Superoxide dismutase plays a vital role in removal of the excess H_2O_2 generated spontaneously [3] and similar trend of increase in SOD on fungal infection was reported in tomato [32], in strawberry [33] and in rice [34].

The protein profiles of resistant versus susceptible cultivars carried out through electrophoresis using standard marker. Size of the protein bands generated by SDS-PAGE (measured by Unstained Protein Molecular Weight Marker ranging from 14.4 to 116 kDa) ranged from 13.5 to 100 kDa. All the three varieties contain similar bands. But at 66 kDa larger band width was observed in resistant variety LBG 17 (Fig. 1). Variability in intensity was viewed in protein bands of LBG 17 showed the amount of protein peptides mounting up at a specific molecular weight confirming that production of more proteins in LBG 17 in the antioxidant enzymes form which further supporting its efficiency against powdery mildew.



Yield attributes also revealed that LBG 17 found to be more productive than the susceptible parents chikkuduminumu and nethiminumum. Maximum number of clusters (14.00), pods/plant (65.30), pod length (6.00 cm), seeds per pod (8.60), 100 seed weight (6.90 g) and seed yield per plant (19.30 g) were recorded with LBG 17 and these values were comparatively more against the parents (Table 1). Increased resistance to fungal infection and maintaining of normal yields in tolerant varieties was previously reported in pea [35] and in fenugreek [36].

IV. Conclusion

The variety LBG 17 was showed varied and significant differences over the parents Nethiminumu and Chikkudu minumu upon infection with *Erisiphae polygoni* DC. Basing on these results it could be confirmed that increased physiological and biochemical activities in LBG 17 on infection with powdery mildew may be the reason for its potential tolerance against the disease and it should be recommended as potential blackgram variety against powdery mildew disease caused by *Erisiphae polygoni* DC.

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