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Biochemical analysis in clusterbean conferring resistance against powdery mildew disease caused by *Leveillula taurica* (Lev.) Arn.

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Abstract
Powdery mildew, caused by *Leveillula taurica* (Lev.) Arn., is a major crop disease of clusterbean in Karnataka and causes substantial economic losses. It reduces the effective photosynthetic leaf area, resulting in significant yield loss. Among the available management strategies, host plant resistance is the most suitable one. So, under greenhouse conditions, nine clusterbean genotypes were screened to identify resistant sources against the disease and understand the bio-chemicals conferring resistance. The role of biochemical parameters such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) activities, total phenol (TP), and chlorophyll content in imparting resistance against powdery mildew in clusterbean was investigated to know the relationship of enzymes in the host with pathogen. None of the genotypes exhibited resistance to the powdery mildew pathogen. Moderately resistant varieties had higher enzymatic activities. Except for chlorophyll, all biochemical parameters increased significantly after inoculation in resistant varieties compared to susceptible varieties. Pathogen infection resulted in an increased PO, PPO and PAL activity, as well as higher phenol, which enhanced the mechanical strength of host cell wall and may also inhibit the fungus growth, as phenolics are fungitoxic in nature. The findings of the study indicate that the evaluated biochemical parameters could be used as reliable biochemical markers for early selection of powdery mildew resistant genotypes. The moderately resistant genotypes identified in this study could also be used as donor parents in breeding programs designed to improve powdery mildew disease resistance in clusterbean.

Keywords: Powdery mildew of clusterbean, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) activities, total phenol (TP), chlorophyll

Introduction
Clusterbean is an important vegetable legume crop belongs to family Fabaceae. It is a drought tolerant crop suitable for cultivation under rainfed conditions in arid and semiarid regions of India. It is rich in vitamins A, B and K, in addition to minerals like calcium, iron, folate and potassium with cis-linoleic acid as the major fatty acid and seeds have 4.53 per cent ash, 3.32 per cent oil, 11.06 per cent fibre, 10 per cent moisture and 33.25 per cent protein (Badr *et al*., 2014) [2]. Green pods and gum are used in the treatment of diabetes and seeds for small pox, leaves in the treatment of moon blindness (Bhosle and Kothekar, 2010) [3]. Because of its richness in nutrients and commercial importance, it has become a cash crop, however its cultivation is hindered by many diseases *viz*. root rot, wilt, blight, powdery mildew and anthracnose diseases. Among these, powdery mildew caused by *Leveillula taurica* is an economically important disease of clusterbean, which can infect more than 710 host species from 59 plant families (Hirata, 1968) [11]. The pathogen causes a yield loss of 50-55 per cent when the weather conditions are most congenial for outbreak of powdery mildew (December and January). The pathogen mainly infects on leaves and pods, severely affected plants are defoliated and weakened by premature drying which leads to death of infected leaves under favorable conditions, the mildew cause considerable defoliation. This disease has become major detrimental factor in hindering the cultivation of clusterbean and its related earnings of foreign exchange. Development of resistant cultivars is the best option for management of this disease. These resistant cultivars are identified by screening germplasms against the pathogen under artificial or natural epiphytotic conditions. Understanding the host biochemical response to pathogen infection is another aspect critically assessed.

Biochemical constituents like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), total phenols (TP) play a key role in inducing host resistance to the crop and in present experiment these were assessed and their relation with host resistance and their biosynthesis before and after pathogen infection in resistant as well as susceptible host plant varieties was aimed to understand.

Materials and Methods

Sample collection: Clusterbean leaves were artificially inoculated 30 days after sowing (DAS) and at 60 DAS when disease was at its peak level, leaves were collected from eight varieties and one hybrid grown in pot for the assessment of activity of defence enzymes *viz*., peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), total phenols (TP) and chlorophyll content.

Enzyme extraction: The leaf tissues collected from plants were immediately homogenized with CTAB buffer. One gram of powdered sample was extracted with 2 ml sodium phosphate buffer, 0.1M (pH 7.0) at 40 ℃. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extract prepared from leaves was used for estimation of PO, PPO, PAL.

Estimation of peroxidase: Peroxidase (PO) activity was assayed spectrophotometrically (Hartee, 1955) [10]. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol (or Gallic acid using standard procedure), 0.5 ml of enzyme extract and 0.5 ml of 1 per cent H_2O_2. The reaction mixture was incubated at room temperature (25 ± 1 ℃). Changes in absorbance at 420 nm were recorded at 30s intervals for 3 minutes and the boiled enzyme preparation served as a blank. Enzyme activity was expressed as the change in the absorbance of the reaction mixture min^{-1} g^{-1} on a fresh weight basis (Hammerschmidt *et al*., 1982) [9].

Estimation of polyphenol oxidase: Polyphenol oxidase **(**PPO) activity was determined as per the procedure given by Mayer *et al*. (1965) [14]. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm in min^{-1} g^{-1} fresh weight of tissue.

Estimation of phenylalanine ammonia lyase: The PAL assay was assessed as per the method described by Ross and Sederoff (1992) [19]. The assay mixture containing 100 µl of enzyme, 500 µl of 50 mM Tris HCl (pH 8.8) and 600 µl of 1 mM L-phenylalanine was incubated for 60 min. The reaction was arrested by adding 2 N HCl. Later, 1.5 ml of toluene is added, vortexed for 30s, centrifuged (1000 rpm for 5 min) and the toluene fraction containing trans-cinnamic acid is separated. The toluene phase was measured at 290 nm against the blank of toluene. The standard curve was drawn with graded amounts of cinnamic acid in toluene as described above. Enzyme activity was expressed as n moles of cinnamic acid min^{-1} g^{-1} fresh tissue.

Estimation of total phenols: The total phenols present in the leaves was estimated following the procedure described by Bray and Thorpe (1954) [4]. Fresh leaf samples (0.5 g each) were blended with 10 ml of 80 per cent ethanol and boiled at 50 ºC for 30 min. The extracts were filtered through cheese cloth and then with Whatman No. 41 filter paper and centrifuged. The volume was made up to 10 ml with ethanol. An aliquot of one ml each was placed in a series of boiling tubes and made up to 3 ml with distilled water. To this, one ml of Folin Ciocalteu reagent and two ml of 20 per cent sodium carbonate were added. The tubes were heated for one min in a boiling water bath and cooled in running water. The solution was diluted to 10 ml with distilled water and the intensity of the blue colour was measured at 660 nm in a spectrophotometer against a blank (a blank was maintained with three ml of distilled water instead of the extract and the colour was developed as described above) for which three replications were maintained. Catechol was used to prepare the standard graph from which the amount of phenol in a given sample was calculated. The content of total phenols was expressed as catechol equivalents in mg 100 g^{-1} fresh weight.

Estimation of chlorophyll content: Total chlorophyll, chlorophyll a and chlorophyll b contents were determined by following the method of Arnon (1949) [1]. Tissues from healthy and powdery mildew infected plants was brought from field. About 100 mg of fresh leaf was weighed from each sample and homogenized with acetone. The extract was filtered through Whatman No.41 filter paper and washed 2-3 times with 80 per cent acetone. The final volume of extract was made up to 25 ml. The absorbance of extract was read at 645, 652 and 663 nm in spectrophotometer and for blank 80 per cent acetone was used. The chlorophyll content was estimated using the formula.

$$\text{Total chlorophyll} = (20.2 \times A_{645} + 8.02 \times A_{663}) \times \frac{V}{1000} \times \frac{1}{\text{Fresh weight (mg/g)}}$$

$$\text{Chlorophyll 'a'} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times \frac{V}{1000} \times \frac{1}{\text{Fresh weight (mg/g)}}$$

$$\text{Chlorophyll 'b'} = (22.9 \times A_{645} - 14.5 \times A_{663}) \times \frac{V}{1000} \times \frac{1}{\text{Fresh weight (mg/g)}}$$

Where,
A_{645} = Absorbance of extract at 645 nm.
A_{663} = Absorbance of extract at 663 nm.
V = Volume of the extract (25 ml).
W = Fresh weight of the sample (0.1g).

Results and Discussion

Biochemical assessment: The clusterbean varieties and hybrids subjected for biochemical analysis to know their defence mechanism against pathogen and the results recorded are presented in the Table 1, Fig. 1.

Assessment of Peroxidase (PO): Hybrid Kamaal (1.138 $min^{-1}g^{-1}$) showed higher content of peroxidase in diseased leaves, as compared to healthy leaves (0.787 $min^{-1}g^{-1}$), followed by variety Rani which also recorded more Peroxidase (PO)

activity in infected leaves (1.051 $min^{-1}g^{-1}$) compared to healthy leaves of same variety (0.644 $min^{-1}g^{-1}$) and Ajeet variety showed maximum PO activity upon infection (0.966 $min^{-1}g^{-1}$) as compared to healthy once (0.494 $min^{-1}g^{-1}$). Whereas in highly susceptible Kushtagi local variety also, the activity of PO was observed more in infected leaves (0.345 $min^{-1}g^{-1}$) compared to healthy leaves (0.113 $min^{-1}g^{-1}$). Gurjar *et al.* (2015) [8] observed higher PO activities in both healthy and diseased leaves of resistant cultivar against grape powdery mildew. Studies of Cherif *et al*. (2007) [7] showed increased PO activity in resistant lines and susceptible lines after pathogen infection.

Assessment of Polyphenol Oxidase (PPO): Higher activity of polyphenol oxidase (PPO) was recorded in diseased leaves of moderately susceptible Kamaal (0.856 $min^{-1}g^{-1}$) genotype compared to healthy leaves (0.634 $min^{-1}g^{-1}$) of the same variety, followed by Rani which also showed more PPO activity in infected leaves (0.835 $min^{-1}g^{-1}$) as compared to healthy leaves (0.597 $min^{-1}g^{-1}$). In Ajeet variety also higher PPO (0.785 $min^{-1}g^{-1}$) was recorded upon infection of powdery mildew as compared to healthy leaves (0.457 $min^{-1}g^{-1}$) of same variety. The least peroxidase activity was observed in highly susceptible variety Kushtagi local (in healthy 0.152 $min^{-1}g^{-1}$ and infected leaves 0.228 $min^{-1}g^{-1}$). These observations dictate that individual variety has its differential ability to produce PPO in response to pathogen infection and varies among the varieties tested. Thus, resistant varieties play an important role in defending against the powdery mildew pathogens in clusterbean. The enzymatic activities of PPO in immune variety against fenugreek powdery mildew were found higher before and after disease incidence (Navanath, 2017) [15].

Assessment of Phenyl ammonium lyase (PAL): Higher concentration of phenyl ammonium lyase was recorded in infected leaves of moderately susceptible genotype Kamaal (0.562 $min^{-1}g^{-1}$) as compared to healthy leaves (0.885 $min^{-1}g^{-1}$), followed by Ajeet which also showed maximum PAL activity upon infection (0.506 $min^{-1}g^{-1}$) compared to healthy leaves (0.674 $min^{-1}g^{-1}$). The infected leaf of Rani also showed more PAL (0.494 $min^{-1}g^{-1}$) than healthy leaves (0.628 $min^{-1}g^{-1}$). The least activity of PAL in highly susceptible Kushtagi local was also higher (0.437 $min^{-1}g^{-1}$) in inoculated leaves compared to healthy leaves (0.209 $min^{-1}g^{-1}$). The observations are in concurrence with earlier two enzymes PO and PPO which were also higher in infected leaves than healthy but differed among the cultivars depending upon their defence ability against the powdery mildew pathogen. Gurjar *et al.* (2015) [8] observed higher PAL activities in resistant cultivar than susceptible cultivar against grape powdery mildew. PAL activity can be induced by plant pathogens interactions and by fungal elicitor treatment (Ramanathan *et al*., 2000) [17]. Analysed data from the present study revealed that, PAL activity was higher in infected plants then the healthy plants.

Assessment of total phenols: Higher content of phenols was recorded in diseased leaves 12.17 mg/g, as compared to healthy leaves (8.504 mg/g) of moderately susceptible genotype Kamaal, followed by Rani which also showed more phenols in infected leaves 10.96 mg/g as compared to its healthy leaves (8.287 mg/g). In Ajeet variety also powdery mildew infected leaves (10.76 mg/g) showed more phenols than its healthy leaves (6.485 mg/g). The least phenols activity was observed in infected plants of highly susceptible variety Kushtagi local, here also infected leaves had higher (3.390) phenols than its healthy leaves (1.456 mg/g). Phenolic compounds act as preformed resistance factors and are generally considered as most responsible parameters for diseases resistance (Sathiyanathan, 1981) [20]. Also, phenolics accumulation is generally higher in resistant genotypes than in susceptible ones (Parashar and Sindhan, 1986; Kalia and Sharma 1988; Chander, 1989; Rathi *et al*., 1998) [16, 12, 5, 18] as observed in the cultivars evaluated in the present investigation.

Assessment of chlorophyll: Infection of powdery mildew on leaves results in destruction of chloroplast and intern it reduces photosynthesis, photophosphorylation and CO_2 assimilation. Hence, experiment was conducted to know the chlorophyll loss in clusterbean as influenced by the development of powdery mildew. The amount of chlorophyll-a, chlorophyll-b and total chlorophyll in healthy and powdery mildew infected leaves (mg/g fresh leaf weight) of eight genotypes was estimated as described in "Material and Methods" and data pertaining to results are presented in the Table 2, Fig. 2.

Highest activity of chlorophyll ‘a’ was observed in moderately susceptible Kamaal (3.059 mg/g) genotype followed by Rani (2.342 mg/g) and Ajeet (2.164 mg/g), which decreased drastically with powdery mildew infection in these varieties by 1.345 mg/g, 1.320 mg/g and 1.254 mg/g respectively. Whereas highest chlorophyll ‘b’ was recorded in moderately susceptible Kamaal genotype (2.035 mg/g) followed by Ajeet (1.599 mg/g) and Rani (1.366 mg/g) which got reduced to 1.326 mg/g, 0.919 mg/g and 0.836 mg/g respectively upon infection by the powdery mildew pathogen. Total chlorophyll was found highest in healthy leaves of moderately susceptible Kamaal (5.093 mg/g) genotype followed by Ajeet (3.723 mg/g) and was found on par with Rani (3.708 mg/g), later with powdery mildew infection chlorophyll content reduced to 2.671 mg/g in Kamaal, 2.239 mg/g in Rani and 2.090 mg/g in Ajeet variety respectively.

Similar studies conducted by Channamma (2015) [6] on clusterbean powdery mildew showed moderately resistant varieties GAUG-13 and RGC-1031 showed more chlorophyll than moderately susceptible and susceptible. Reduction in total chlorophyll contents in disease affected leaves was noted in comparison to healthy leaves in different varieties. This finding agrees with the earlier report of Lobato and Goncalves (2009) [13], where they have reported 15.2 per cent decrease in chlorophyll content in susceptible cultivars infected by *C. lindemuthianum* causing anthracnose of bean.

Table 1: Effect of powdery mildew on enzyme activity in different clusterbean varieties

Varieties	Peroxidase		Poly phenol oxidase		Phenyl ammonium-lyase		Total phenols (mg/g)	
	Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
PNB	0.249^{e*}	0.655^{d}	0.342^{e}	0.563^{e}	0.390^{e}	0.565^{d}	5.960^{e}	8.929^{e}
Darsha	0.206^{f}	0.477^{e}	0.308^{g}	0.538^{f}	0.431^{d}	0.622^{c}	4.333^{f}	7.330^{f}
Anjali	0.209^{f}	0.446^{e}	0.327^{f}	0.506^{g}	0.435^{d}	0.506^{e}	3.794^{g}	6.795^{g}
Aruna	0.124^{g}	0.395^{f}	0.258^{h}	0.478^{h}	0.300^{f}	0.464^{f}	3.369^{h}	5.614^{h}
Varsha	0.383^{d}	0.674^{d}	0.368^{d}	0.695^{d}	0.484^{c}	0.559^{d}	6.053^{d}	10.64^{d}
Kamaal	0.787^{a}	1.139^{a}	0.634^{a}	0.856^{a}	0.562^{a}	0.885^{a}	8.540^{a}	12.17^{a}
Rani	0.644^{b}	1.051^{b}	0.597^{b}	0.835^{b}	0.494^{bc}	0.628^{c}	8.287^{b}	10.96^{b}
Ajeet	0.494^{c}	0.966^{c}	0.457^{c}	0.785^{c}	0.506^{b}	0.674^{b}	6.485^{c}	10.76^{c}
Kushtagi Local	0.113^{h}	0.345^{g}	0.152^{i}	0.228^{i}	0.209^{g}	0.437^{g}	1.456^{i}	3.390^{i}

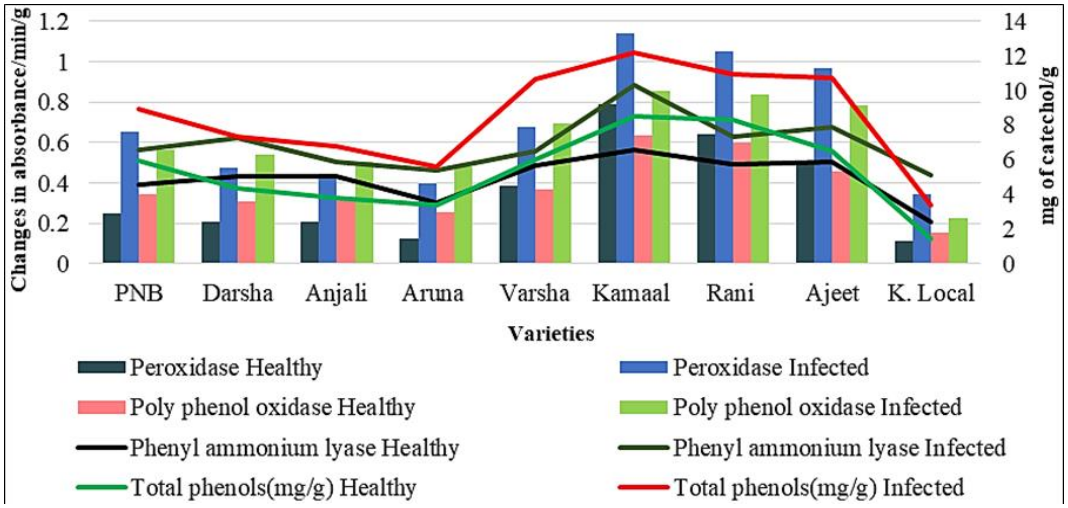

Fig 1: Influence of powdery mildew on PO, PPO, PAL and Phenol activity in clusterbean varieties

Table 2: Effect of powdery mildew on chlorophyll content in clusterbean varieties

Varieties	Chlorophyll 'a' (mg/g)		Chlorophyll 'b' (mg/g)		Total Chlorophyll (mg/g)	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
PNB	1.161^{de*}	0.849^{d}	0.772^{e}	0.570^{cd}	1.932^{d}	1.419^{cd}
Darsha	1.588^{c}	0.874^{d}	1.138^{d}	0.735^{bcd}	2.726^{c}	1.609^{c}
Anjali	1.383^{cd}	0.686^{e}	0.716^{f}	0.510^{cd}	2.098^{d}	1.196^{de}
Aruna	0.895^{ef}	0.636^{e}	0.614^{g}	0.413^{d}	1.509^{e}	1.049^{e}
Varsha	2.026^{b}	0.997^{c}	0.771^{e}	0.501^{d}	2.797^{c}	1.498^{cd}
Kamaal	3.059^{a}	1.345^{a}	2.035^{a}	1.326^{a}	5.093^{a}	2.671^{a}
Rani	2.342^{b}	1.320^{a}	1.366^{c}	0.919^{b}	3.708^{b}	2.239^{b}
Ajeet	2.164^{b}	1.254^{b}	1.599^{b}	0.836^{bc}	3.723^{b}	2.090^{b}
Kushtagi Local	0.737^{f}	0.509^{f}	0.643^{g}	0.465^{d}	1.374^{e}	0.974^{e}

*****Mean of three replications and values in the column followed by common letters are non- significant at p=0.01 as per DMRT.

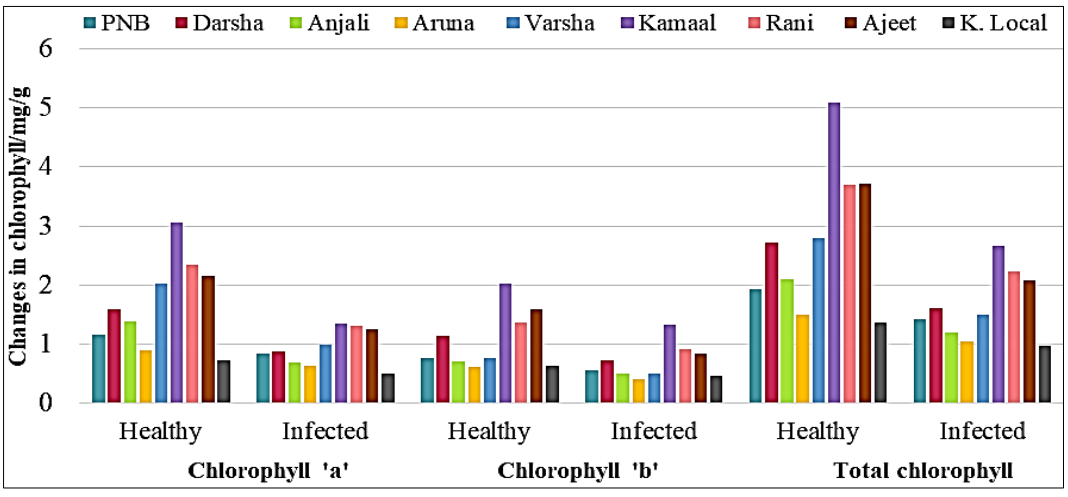

Fig 2: Influence of powdery mildew on Chlorophyll content in clusterbean varieties

Conclusion

The results revealed that biochemical studies on these varieties showed the increase in defensive enzyme peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and total phenolics production with the pathogen invasion, whereas there was sudden decrease in chlorophyll content upon pathogen infection.

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