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3 **Defense Gene Expression of *Vigna radiata* (L.) Wilczek., against *Cercospora* leaf spots**  
4 **(CLS)**

5 **Running Title:** Defense Gene Expression of *Vigna radiata* (L.) Wilczek.....  
6

7 **ABSTRACT**

8 Most cultivars of *Vigna radiata* (L.) Wilczek grown in Indian subcontinent are susceptible to various biotic and  
9 abiotic stresses. *Cercospora* leaf spot (CLS) is a major biotic stress resulting in poor yield of this crop.  
10 Therefore, it is essential to investigate resistance status of different cultivars to CLS and develop effective  
11 strategy. Present investigation was focused on the role of biochemical compounds in resistance response of this  
12 crop to CLS in naturally grown population and after artificial induction with pathogen derived elicitor. The  
13 defense responses *in vivo* and *in vitro* were analyzed in the form of phytoalexin genestein, PAL and PR- proteins  
14 in their leaves. PR-proteins, PAL and genestein were assayed employing established protocols. In naturally  
15 grown population, four cultivars- Kopergaon, TARM-1, TARM-2 and TARM-18 showed lesser accumulation of  
16 genestein and lower level of PAL and PR- proteins. However, Pant M-3, ML 1037 and ML-936 showed  
17 resistant interaction with very high accumulation of genestein, PAL and PR proteins. Similar trends of  
18 accumulation of these biochemicals were observed in *in vitro* condition after elicitation with pathogen derived  
19 elicitor. The correlation study showed that the cultivars with lower defense related expression showed high  
20 disease incidence (51-61%) and with higher defense related expression were with less than 5% CLS incidence.  
21 It could be stated that PR-proteins, PAL and genestein has prominent role in defense mechanism of mungbean  
22 against CLS as biochemical markers and further their utility in early screening for disease resistance of crop  
23 plants could be explored.

24 **Key Words:** Defense, *Cercospora* leaf spots, Phytoalexins, Resistant interaction.

25 **1. INTRODUCTION**

26 *Vigna radiata* (L.) Wilczek. (mungbean) is a major pulse crop of India and popular as cheapest source of plant  
27 protein worldwide, especially in developing countries. It is a short duration legume crop, cultivated worldwide  
28 for its dry seeds. Annual mungbean production worldwide is around 2.5 to 3.0 million tonnes, harvested from  
29 about 5.0 million ha [1]. India is the largest producer of mungbean contributing more than 50% of total world  
30 mungbean production [2]. It is used as pulse in the preparation of various Indian food items as a main source of  
31 plant protein for the vegetarian diet.

32 The genus *Vigna* includes about 150 species, of which 22 are native to India. Most of the cultivars of  
33 mungbean, cultivated worldwide and especially in Indian subcontinent, are susceptible to diverse pathogens that  
34 include Fungi, Bacteria, Viruses and Nematodes. Most severe of these are *Cercospora* leaf spot (CLS) caused  
35 by *Cercospora canescens* Ellis & Martin., leading to huge loss in grain productivity [3]. Therefore, to induce or  
36 enhance resistance or to develop the cultivar with resistance to this pathogen is a major breeding objective.  
37 Some biochemical compounds synthesized by host plant possesses antimicrobial property and play vital role in  
38 plant defense [4]. Induced resistance involves multiple mechanisms that include increased level of PR proteins,  
39 Phenylalanine ammonia lyase (PAL) and Peroxidases (PO) [5, 6].

40 Present study is focused on assessing the role of PR- proteins, Phenylalanine ammonia lyase (PAL) and  
41 phytoalexin genestein in defense array of mungbean against CLS incidence. Considering this, analysis of these  
42 biochemicals in leaves of naturally infected populations of mungbean cultivars was done. And cotyledons and  
43 seedling parts of these cultivars were elicited by *Cercospora* cell wall elicitor to induce the defense in vitro and  
44 role of these biomolecules in defense mechanism was revealed.

## 45 2. MATERIALS AND METHODS

### 46 2.1 Germplasm Collection

47 The germplasm of mungbean was procured from BARC Mumbai (TARM-1, TARM-2, TARM-18) and Punjab  
48 Agriculture University Ludhiana (Pant M-3, ML- 1037, ML- 936); and cultivar Kopergaon was taken as local  
49 reference. Seeds of all these cultivars were sown in the field for multiplication and the status of disease  
50 resistance was assessed under field conditions in both, kharif and rabi seasons. The resistant status was  
51 analyzed by measuring percent infection per leaflet as disease incidence.

### 52 2.2 Preparation of *Cercospora* cell wall elicitor (CCWE)

53 *Cercospora* cell wall elicitor was prepared and elicitation dose was standardized as per the method developed by  
54 Koche and Choudhary [7].

### 55 2.3. Analysis of defense related biochemicals

56 The level of phytoalexin- genestein and expression of PAL and chitinase and  $\beta$ ,1-3 glucanase genes were  
57 analyzed in the field grown plants. As the mungbean plants starts developing symptoms of leaf spot disease, by  
58 35- 40 days onwards, leaves of each cultivar were harvested after every 15 day from the day of germination to  
59 the age of 60 days. The harvested leaves were frozen in liquid nitrogen and then stored at  $-20^{\circ}\text{C}$ , until use.

60 The *in vitro* defense response to *Cercospora* cell wall elicitor (CCWE) of each cultivar was analyzed in  
61 cotyledons, roots, hypocotyl and epicotyl.  $\beta$ -1, 3- glucanase, chitinase and PAL were analyzed by employing  
62 the established methods. The glucanase and chitinase assays were performed according to procedure set by  
63 Kauffmann et al. and Reissig et al [8, 9]. Enzyme PAL was assayed according to the procedure given by Lamb  
64 et al. [10]. Protein concentration was measured according to Bradford [11] and for analysis of phytoalexin  
65 genestein method of Edward and Strange [12] was adopted.

66

## 67 3. RESULTS

68 To determine the role of different biochemical like phytoalexin genestein, PAL and PR- proteins as markers in  
69 assigning the resistance to a particular cultivar, their accumulation was analyzed in leaves of seven mungbean  
70 cultivars, naturally infected with *Cercospora canescens*. The *in vitro* analysis of these defense related  
71 biomolecules was also done in cotyledons and different seedling parts elicited by CCWE.

### 72 3.1 Analysis of defense related biomolecules in field grown plants

73 Genestein accumulation was analyzed periodically in the leaf tissues naturally grown cultivars. The  
74 leaves of each cultivar were harvested periodically from 15<sup>th</sup> day of germination to the age of 60 days. In the  
75 field, the disease symptoms start to appear from about 40<sup>th</sup> day of germination and around 60<sup>th</sup> day it reaches to  
76 its maximum severity. At the time of peak severity (period of pod setting and maturation), in susceptible  
77 cultivars, the infected leaf area ranged between 35% in (TARM-2) and 61% (in Kopergaon), while in ML-1037,  
78 ML-936 and Pant M-3 cultivars, percent infected leaf area was always found to remain below 5% and very few  
79 spots could be seen on the ageing leaves of these resistant cultivars (Table- 1).

80 In this investigation the correlation between the level of glucanase, chitinase and genestein in the leaves of  
81 mungbean cultivars and their resistant status was observed. Their accumulation in resistant cultivars, ML-1037,  
82 ML-936 and Pant M-3 was observed to be considerably more than in susceptible cultivars. The steady increase  
83 in their accumulation was also noticed in resistant cultivars after the germination till maturity. On the contrary,  
84 their level in susceptible cultivars was very low during this period. The peak activities of PR- proteins and  
85 genestein content 60 days after germination of each cultivar were presented in table-1. This clearly indicates the  
86 difference of PR proteins and genestein accumulation defining their resistant status.

### 87 3.2 In vitro Analysis of defense related biomolecules

88 Defense response to CLS was also analyzed in cotyledons and different parts of seedlings after elicitation with  
89 CCWE. After elicitation, the samples were fixed after every 5 hrs and proceed for the analysis of PR- proteins.  
90 The peak values of these biomolecules was observed 35 hrs after elicitation.

#### 91 3.2.1. PR Proteins

92 The peak  $\beta$ -1,3 glucanase level in cotyledons and different seedling parts after elicited by CCWE is given in  
93 fig1. It reveals that, the level of  $\beta$ -1,3 glucanase in resistant cultivars (ML-1037, ML-936, Pant M-3) is quite  
94 higher than rest of the susceptible cultivars. The highest glucanase activity was found in cotyledons of cultivar  
95 ML-1037 (8.18 ( $\mu$ g/g protein)). Further, it is observed that, the level of  $\beta$ -1,3 glucanase is highest in cotyledons  
96 followed by hypocotyls of each cultivars and least is root parts (Fig. 1).

97 Peak chitinase expression ( $\mu$ g/g protein) in cotyledons and different parts of seedlings elicited by CCWE is  
98 mentioned fig. 2. It showed that chitinase activity in resistant cultivars was 1.5 to 2 fold more than of susceptible  
99 cultivars. Both resistant and susceptible cultivars cotyledons and seedling parts showed increase in chitinase  
100 levels and their peak activities are 2 fold high in susceptible while about 4 fold more than control in resistant  
101 cultivars (Fig. 2).

#### 102 3.2.2. PAL (Phenylalanine ammonia lyase)

103 Peak PAL content ( $\mu$ Kats/Kg protein) in cotyledons and different seedling parts of mungbean cultivars  
104 elicited with CCWE is noted in fig.3. The peak activity of PAL in all cultivars was noted 4hrs after elicitation. It  
105 was observed that PAL level in all seedling parts of resistant cultivars was significantly higher than in elicited

106 susceptible cultivars. Among resistant cultivars, Pant M-3 showed highest PAL activity (57.64 -65.93  $\mu$ Kats/ Kg  
107 protein). In all cases, PAL activity in hypocotyls region of all cultivars was found to be highest followed by in  
108 epicotyls and least in roots (Fig. 3).

### 109 3.2.3. Phytoalexin Genestein

110 Phytoalexin genestein start accumulating in cotyledons and seedlings immediately after elicitation with CCWE.  
111 The highest level of genestein accumulation was observed 45hrs after elicitation. The data on peak genestein  
112 accumulation in cotyledons and different seedling parts of mungbean cultivars is given in table-2. Overall it was  
113 seen that elicited parts of resistant cultivars showed more than 20 to 40 fold increase of genestein content over  
114 control while this increase was about 3 to 6 times of control level in susceptible cultivars (Table-2).  
115 Comparative higher level of genestein accumulation was observed in elicited cotyledons of all cultivars  
116 followed by hypocotyls region.

## 117 4. DISCUSSION

118 The CLS defense response was investigated in three resistant and four susceptible cultivars of mungbean by  
119 analyzing the level of phytoalexin genestein (a potent antimicrobial compound) and studying the biochemical  
120 expression of defense related genes such as PAL (a key enzyme involved in phytoalexin biosynthesis) and two  
121 PR proteins i. e.  $\beta$ -1,3 glucanase and chitinase (both singly or in combination shows anti-fungal activity). The  
122 expressions of these genes were studied in terms of their extractable enzyme content.

123 Traditional screening for stress tolerance or disease resistance at field level was based on the necrotic scores and  
124 reduction in biomass on stress exposure. It was a laborious and time consuming, environment dependent  
125 destructive method. Since last two decades biologists are working on its non-destructive, easy and effective  
126 method to assess the resistance status of particular crop in early stages.

127 During present study it was noted that genestein accumulated rapidly and at higher level in the resistant cultivars  
128 (ML-1037, ML-936 and Pant M-3) as compared to the susceptible cultivars. In the leaves of naturally infected  
129 resistant cultivars the level of genestein was in between 78.84 to 111.30  $\mu$ g/ gm fresh tissue weight, whereas in  
130 the leaves of susceptible cultivars it was 2.28- 4.81  $\mu$ g/ gm fresh tissue weight. The level of  $\beta$ -1,3 glucanase and  
131 chitinase was also found to be higher in the leaves of naturally infected field grown resistant cultivars as  
132 compared to the susceptible cultivars. The similar trend of accumulation of these biochemicals after elicitation  
133 with CCWE was observed during *in vitro* experimentation.

134 PR proteins are constitutively expressed in plants at low levels, but the expression of most of the PR  
135 proteins is turned on in response to pathogen attack. Induction of PR proteins is a consequence of the activation  
136 of plant defensive pathways, which limit the entry or further spread of the pathogen [13, 14]. Originally, PR  
137 proteins were detected and defined as being absent in healthy plants but accumulating in large amounts after  
138 infection or induction [15]. Our study supports the hypothesis that lower level of PR protein results in high  
139 disease incidence and vice versa conferring the respective resistance status to cultivars.

140 The similar observations were made by Strange et al. [16] in lettuce- *B. cinerea* interaction and Paiva et  
141 al. [17] in alfalfa- *Phoma* interaction. These reports were earlier supported by different workers indicating that  
142 induction of PR- proteins and other defense related biochemical using pathogen derived elicitors [18-20].

143 Jyotsna et al. [21] reported that biochemical and morphological markers are useful to investigate  
144 *Phaeoisariosps*- Groundnut pathosystem. Role of biochemical markers such as chitinase, glucanase and PAL  
145 was also demonstrated by other workers [22-24]. Few workers also reviewed the utility of these biochemical  
146 markers in plant breeding [25, 26]. Further, this screening made available a solid platform for the molecular  
147 screening for disease resistance in crop plants [27]

## 148 5. CONCLUSION

149 On the basis of observations, made during this study, it is concluded that, defense responses induced by  
150 *Cercospora* cell wall elicitor (CCWE) in different plant parts and at different developmental level are different.  
151 Generally, the pathogen derived biotic elicitors induced defense genes more rapidly and at higher level. The  
152 effectiveness of the elicitor also varies from cultivar to cultivar. The seedling study indicated that each part  
153 exhibit different response to single elicitor indicating tissue specific expression of defense genes. Cotyledons  
154 and hypodotyls followed by epicotyl were found to be more responsive than roots with respect to defense  
155 induced by CCWE.

156 Present study clearly define that phytoalexin genestein, PAL and PR proteins are playing the vital roles,  
157 in resistant interaction between *Vigna radiata* – *Cercospora* pathosystem. The level and accumulation of these  
158 biochemicals has positive correlation with the resistant status or defense behavior of the plants in field. This also  
159 supports that early detection of resistant status using these biochemical markers in cotyledons could be a fruitful  
160 idea for further agricultural practices.

## 161 Conflict of Interest

162 We declare here that there is no conflict of interest in our research.

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227 markers in identification and characterization of resistance to rice blast in India. PLoS ONE 12(04): e0176236,  
228 2017.
- 229 **Table-1:** Analysis of % infected leaf area,  $\beta$ -1,3 glucanase, chitinase and genestein in naturally infected plants  
230 of seven mungbean cultivars.

Cultivar	Resistant status	% infected leaf area	Peak chitinase content	Peak glucanase content	Genestein content in leaves
ML-1037	Resistant	2.53	3708.62	1877.39	111.30
ML- 936	Resistant	4.59	1872.30	1158.77	78.84
Pant M-3	Resistant	3.30	3226.84	1580.16	82.68
TARM- 1	Susceptible	51.57	1063.07	490.10	4.49
TARM- 2	Susceptible	35.90	856.05	527.59	3.24

<b>TARM- 18</b>	Susceptible	54.99	890.52	697.08	4.81
<b>Kopergaon</b>	Susceptible	61.03	1245.34	885.88	2.28

231 **Note:** The analysis was done in leaves of naturally grown mungbean population 60 days after germination. The  
 232 peak values of glucanase and chitinase are in  $\mu\text{g/g}$  protein and genestein content in  $\mu\text{g/g}$  of fresh leaf tissue.

233

234

235 **Table- 2:** Peak Genestein accumulation ( $\mu\text{g/g}$  fresh tissue weight) in cotyledons and different seedling parts of  
 236 mungbean cultivars elicited by CCWE.

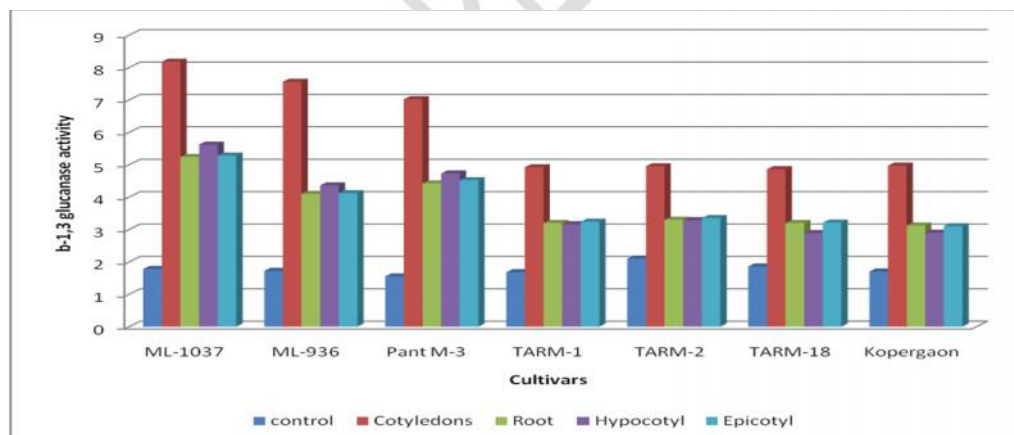
Cultivars	O hr Control	Cotyledons		Root		Hypocotyl		Epicotyls	
		Con	Eli	Con	Eli	Con	Eli	Con	Eli
<b>ML- 1037</b>	0.161	16.60	312.83	9.80	196.80	18.96	249.3	9.50	190.50
<b>ML- 936</b>	0.356	10.00	267.87	11.66	241.55	19.33	216.0	10.65	230.50
<b>Pant M- 3</b>	0.882	7.60	271.22	12.57	294.92	18.22	243.0	10.55	214.90
<b>TARM- 1</b>	0.436	7.63	50.63	1.68	31.91	4.16	38.00	8.65	30.68
<b>TARM- 2</b>	0.601	6.81	36.05	2.83	28.30	6.39	45.00	5.85	25.38
<b>TARM- 18</b>	0.420	9.57	43.14	1.22	27.65	12.46	49.27	7.22	25.60
<b>Kopergaon</b>	0.253	9.35	26.94	2.60	24.85	23.58	45.50	9.65	26.20

237 **Note:** Con = Control, Eli = Elicited; The peak genestein activity was noted 45 hrs after elicitation.

238

239

240 **Figure 1:** Peak glucanase content ( $\mu\text{g/g}$  protein) in different seedling parts and cotyledons of mungbean  
 241 cultivars elicited with CCWE determined 35 hrs after elicitation.

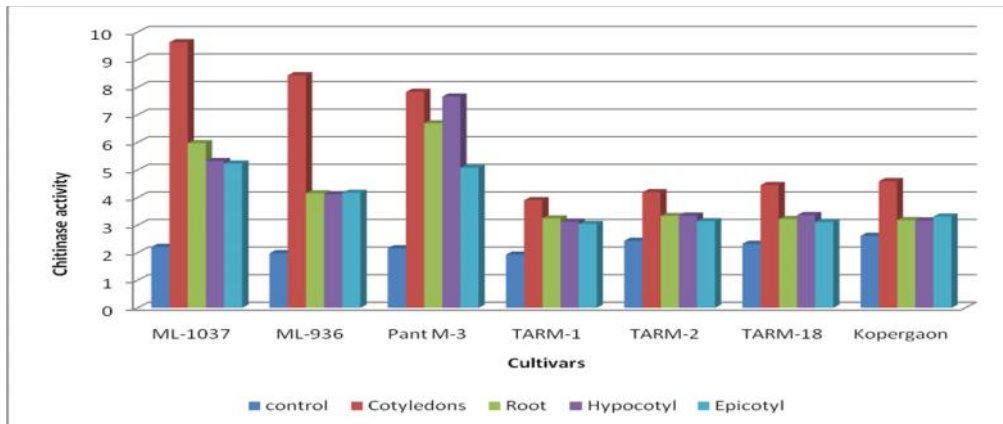


242

243 **Note:** content/ level is expressed in terms of activity in fig.

244 **Figure 2:** Peak Chitinase level ( $\mu\text{g/g}$  protein) in different seedling parts and cotyledons of mungbean cultivars  
 245 elicited with CCWE determined 35 hrs after elicitation.





246

247 Note: content/ level is expressed in terms of activity in fig.

248

249

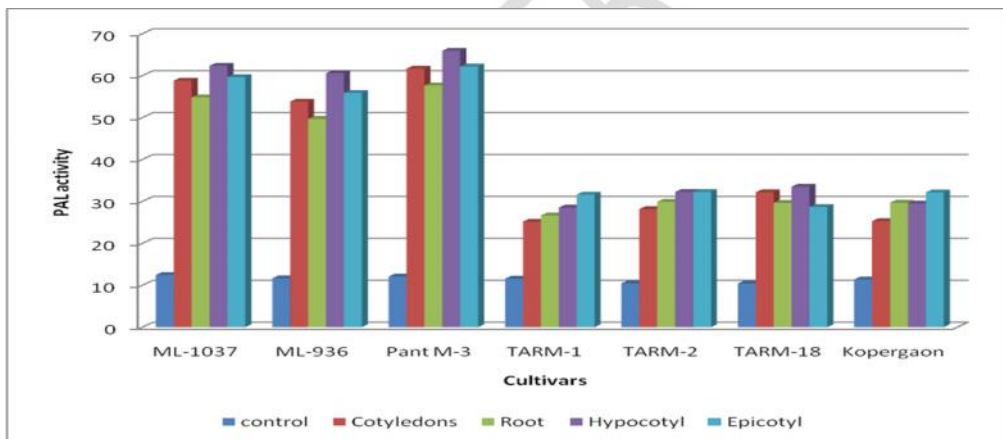
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254 **Figure 3:** Peak PAL content ( $\mu$ Kats/Kg fresh weight of tissue) in different seedling parts and cotyledons of  
 255 mungbean cultivars elicited with CCWE, determined 4 hrs after elicitation.



256

257 Note: content/ level is expressed in terms of activity in fig.

258

259