Original Research Article

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

Running Title: Defense Gene Expression of Vigna radiata (L.) Wilczek…..

ABSTRACT

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

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

1. INTRODUCTION

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

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

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

2. MATERIALS AND METHODS

2.1 Germplasm Collection

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

2.2 Preparation of Cercospora cell wall elicitor (CCWE)

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

2.3. Analysis of defense related biochemicals

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

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

3. RESULTS

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

3.1 Analysis of defense related biomolecules in field grown plants

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

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

3.2 In vitro Analysis of defense related biomolecules

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

3.2.1. PR Proteins

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

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

3.2.2. PAL (Phenylalanine ammonia lyase)

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

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

3.2.3. Phytoalexin Genestein

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

4. DISCUSSION

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

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

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

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

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

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

5. CONCLUSION

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

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

Conflict of Interest

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

REFERENCES

1. Poehlman JM. The Mungbean. Oxford and IBH publishing corp., New Delhi, India,1991.

2. Heuze V, Trang G, Bastianelli D, Lebas F. Mungbean (*Vigna radiata*). Feedipedia, a programme by INRA, CIRAD, AFZ and FAO. http://www.feedipedia.org/node/235, 2015.

3. Iqbal SM, Zubair MH. Resistance in Mungbean to Cercospora leaf spot disease. International J. Agric. Biol. 2004; 6(5): 792-793

4. Latha P, Anand T, Ragupathi N, Prakasam V, Samiyappan R. Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by mixtures of PGPR strains and Zimmu leaf extract against *Alternaria solani*. Biol. Control. 2009; 50: 85-93.

5. Maurhofer M, Hase C, Maurwly D, Metraux JP, Defago G. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root colonizing *Pseudomonas fluorescens* strain CHA0: influence of the gac A gene and of pyoverdine production. Phytopathology. 1994; 84:136 -140.

6. Xue L, Charest PM., Jabaji-Hare SH. Systemic induction of peroxidases, β-1,3glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. Phytopathology. 1998; 88:359- 365

7. Koche DK, Choudhary AD. Elicitor induced gene expression of β-1.3 glucanase and chitinase genes in *Vigna radiata* (L) Wilczek. Bionature. 2005; 25 (1&2): 69-74.

8. Kauffmann S, Legrand M, Geoffroy P, Fritig B. Biological function of pathogenesis- related proteins: Four PR- proteins of tobacco have 1,3 glucanase activity. EMBO J. 1987; 6: 3209-3212.

9. Reissig JL, Strominger JL, Leloir LF. A modified calorimetric method for the estimation of N-acetyl amino sugars. J. Biol. Chem. 1955; 217: 959-966

10. Lamb CJ, Lawton MA, Taylor SJ, Dixon RA. Phenylalanine ammonia lyase: Regulation of its induction and its role in plant development. Phytochemisrty. 1980; 23(7): 1349-1359.

11. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein –dye biding. Anal Biochem. 1976; 72: 248- 254

12. Edward C, Strange RN. Separation and identification of phytoalexins from leaves of groundnut (*Arachis hypogaea*) and development of method for their determination by reversed phase high performance liquid chromatography. J. Chromatography. 1991; 547(1): 185- 193.

13. Baker B, Zamsryski P, Staskaweiz B, Dinesh-Kumar SP. Signaling in plant-microbe interactions. Science. 1997; 272:726-733

14. Conrath U, Pieterse MJ, Mauch-Mani B. Priming in plant-pathogen interactions. Trends Plant Sci. 2002; 5:210-16

15. Sticher L, Mauch-Mani B, Métraux JP. Systemic acquired resistance. Annual Review Phytopath. 1997; 35:235-270.

16. Strange RN, Ingham JL, Cole DL, Cavill ME, Edwards C, Cooksey CJ, Garatt PJ. Isolation of phytoalexin medicarpin from leaflets of *Arachis hypogaea* and related species of tribe Aeschynomeneae. Z Nature. 1985; 40 C: 313-316.

17. Paiva NL, Oommen A, Harrison MJ, Dixon R. Regulation of isoflavonoid metabolism in alfalfa. Plant Cell Tissue Organ Cult. 1994; 38: 213- 220.

18. Crammer CL, Bell JN, Ryder TB, Bailey JA, Schuch W, Bolwell GP, Robbins MP, Dixon RA, Lamb CJ. Coordinated synthesis of phytoalexin biosynthetic enzymes in biologically stressed cells of bean (*Phaseolus vulgaris* L.). EMBO J. 1985; 5: 285- 289

19. Dixon RA. The phytoalexin response: Elicitation, signaling and Control of host gene expression. Biol. Rev. 1980; 61: 239- 91

20. Yamada T, Hayashi M, Nakatsuka S, Muraya K, Kato H, Shiraishi, T. Suppression of pisatin and phenylalanine ammonia- lyase m-RNA in a compatible reaction between *Pisum sativum* L. cv. midoriusui and *Pseudomonas syringae* pv pisi. Annals Phytopath. Soc Japan 1994; 60(1): 66- 73.

21. Jyotsana MK, Eswara Reddy NP, Chalam TV, Reddy GLK. Morphological and biochemical characterization of *Phaeoisariopsis personata* resistant and susceptible cultivars in Groundnut (*Arachis hypogaea*). Plant Path. Bull. 2004; 13: 243-250.

22. Kavino M, Kumar N, Damodaran T, Harish S, Saravankumar D. Biochemical markers as useful tool for the early identification of *Fusarium oxysporium* f. sp. cubene, race 1 resistance in banana clones. Arch. Phytopath. Plant Prot. 2009; 42(11): 1069-1078.

23. Selvamathiazhagan N, Kannan R, Rajamanickam C, Suyambulingam, AK, Subbiah, SN, Michael JS, Sengottayan S. Effect of plant compounds on induced activities of defense-related enzymes and pathogenesis related protein in bacterial blight disease susceptible rice plant. Physiol. Mole. Plant Path. 2012; 80: 1- 9.

24. Krishna VV, Girish Kumar K, Pradeepa K, Santosh Kumar S., Shashi Kumar R. Biochemical marker assisted screening of *Fusarium* wilt resistance in *Musa paradisiacal* (L.) cv. Puttable micropropagated clones. Indian J. Exp. Biol. 2013; 51: 531-542.

25. Narshimhulu R, Naidu NV, Shanthi PM, Gowardhan G, Rupes KR, Hariprashad RK. Marker assisted selection in disease resistance breeding. J. Plant Breed. Genet. 2013; 1(2): 90-109

26. Mantri N, Patade V, Pang E. Recent advances in Rapid and sensitive screening for abiotic stress tolerance; In : Improvement of crops in era of climate change (Ed: P. Ahmad et. al.) @ Springer Science- Business Media, New York, 2014.

27. Yadav MK, Aravindan S, Ngangkham U, Subudhi HN, Bag MK, Tolan A, Jena M. et al. Use of molecular markers in identification and characterization of resistance to rice blast in India. PLos ONE 12(04): e0176236, 2017.

Table-1: Analysis of % infected leaf area, β-1,3 glucanase, chitinase and genestein in naturally infected plants 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

TARM- 18	Susceptible	54.99	890.52	697.08	4.81
Kopergaon	Susceptible	61.03	1245.34	885.88	2.28

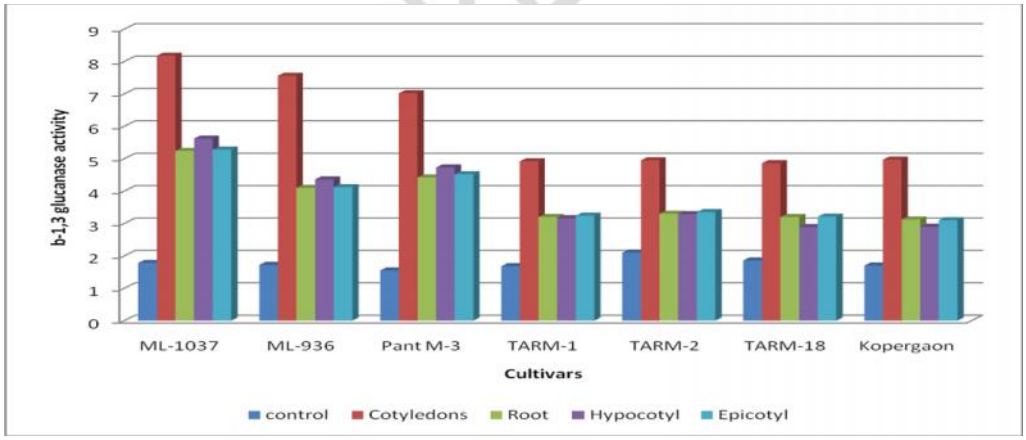
Note: The analysis was done in leaves of naturally grown mungbean population 60 days after germination. The peak values of glucanase and chitinase are in µg/g protein and genestein content in µg/ g of fresh leaf tissue.

Table- 2: Peak Genestein accumulation (µg/g fresh tissue weight) in cotyledons and different seedling parts of mungbean cultivars elicited by CCWE.

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

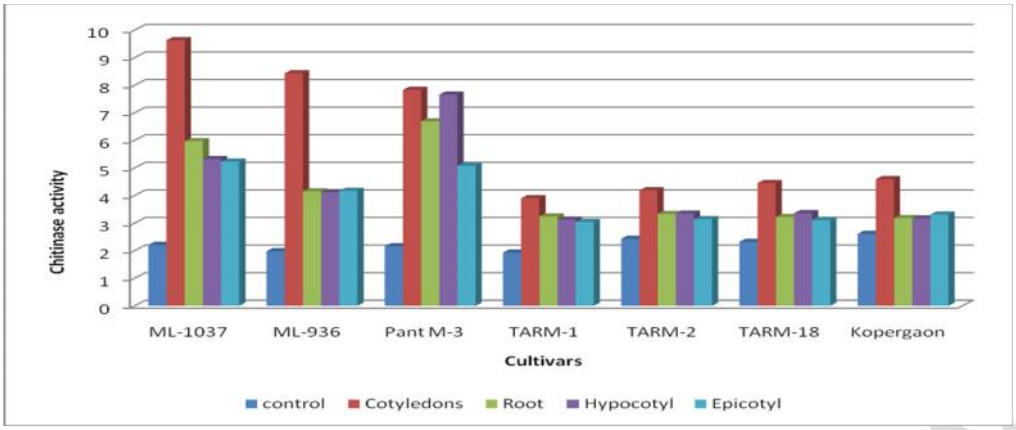
Note: Con = Control, Eli = Elicited; The peak genestein activity was noted 45 hrs after elicitation.

Figure 1: Peak glucanase content (µg/g protein) in different seedling parts and cotyledons of mungbean cultivars elicited with CCWE determined 35 hrs after elicitation.



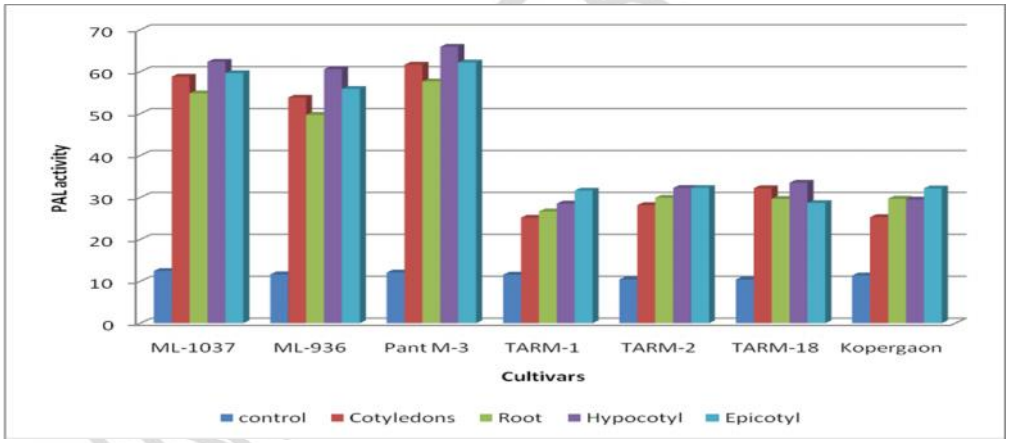
Note: content/ level is expressed in terms of activity in fig.

Figure 2: Peak Chitinase level (µg/g protein) in different seedling parts and cotyledons of mungbean cultivars elicited with CCWE determined 35 hrs after elicitation.



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

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



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