Mycopath (2014) 12(2): 67-75 **Research Article**

Alteration in β-1, 3-glucanases enzyme in *Sesamum indicum* L. infected with *Macrophomina phaseolina*

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Abstract

Interaction between plant and a phytopathogens causes the induction of defense system in plant against that pathogen which is regulated by a complex network of different signals, as a result of which they produce a large amount of secondary metabolites i.e., phenolic compounds, PR proteins and other enzymes. Plant β-1,3-glucanases are the major PR proteins and belong to the PR-2 family, which are believed to play an important role in plant defence against the phytopathogens. So this study was carried out to analyze the modification in β-1,3-glucanases specific activity in Sesame (*Sesame indicum* L.) plant after the infection with the pathogen [*Macrophomina phaseolina* (Tassi) Goid.]. For the experiment, 7 days and 14 days old *Sesame* plants were used. The specific activity of β-1,3-glucanases enzyme in infected plants was considerably exceeded in contrast to control plants. The obtained results give important information concerning the plant-pathogen interactions, in the defense response for *Sesame* improvement programs seeking the adaptation to diverse range of fungal attack along with adverse environmental factors.
Keywords: β-1, 3-glucanases, defense response, *Macrophomina phaseolina, Sesamum indicum.*

Introduction

Plants evoke a series of general defense reactions, including the production of phytoalexins and antimicrobial proteins after pathogen attack. Plants are defended against pathogens by two types of barriers: constitutive and inducible barriers. Induced resistances can be of two types: localized acquired resistance and systemic acquired resistances. The localized acquired resistance is expressed locally at the site of infection. The metabolic alterations in localized acquired resistances includes: production of phytoalexins (Agrios, 2005), the fast production of reactive oxygen species (ROS) (De Gara *et al.,* 2003), synthesis of PR proteins, or activation of programmed cell death, also called the hypersensitive reactions (HR) along with the accumulation of broad range of defense related proteins and peptides (Castro and Fontes, 2005). PR proteins are acid-soluble, protease resistant proteins of low molecular weight that accumulate in plant cells during incompatible interactions with viruses, bacteria or fungi. The synthesis and accumulation of these PR-proteins have long been thought to play an important role in the plant defense response. Among all the PR-proteins, β-1,3-glucanases (EC 3.2.1.39) are able to partially degrade fungal cell walls by catalysing the hydrolysis of β -1,3-d-glucosidic linkages in β -d-glucans, which is the major cell wall component of most pathogenic fungi (Adams, 2004). Sesame (*Sesameum indicum* L.) is an annual plant belongs to family Pedaliaceae and it is grown for oil having high antioxidant activity. Sesame is produced in the warm regions of the world, mainly in India, but it has great loss of its yield because it is mainly affected by the soil borne fungus *M. phaseolina* causes charcoal or root rot disease in this plant. This phytopathogen plays a crucial role in losses of 500 plant species but plant has endowed a mechanism to defend them against the phytopathogen, they have the capability to resist themselves by activating a signalling cascade and producing phytoalaxins at the infected and uninfected site. So, to investigate the role of β-1, 3-glucanase in sesame defence against the fungus *M. phaseolina*, *in vivo* experiments are carried out. For the experiments 7 and 14 days old sesame cultivars inoculated with fungal spore suspension and without inoculation as control were used. The experiment was conducted at different time interval, for example, at short time interval starting from 0 hour up to 10 hour and at long time interval starting from 24 hour up to 168 hour of inoculation.

Material and Methods

Plant material and growth conditions

Seeds of different cultivars of *S. indicum* were procured from Rajasthan, Tamil Nadu and

Uttar Pradesh (India). Seeds were sterilized with 0.1% $HgCl_2$ and grown under controlled conditions in green house at temperature 30±2 °C and 77% humidity. For investigation, 7 days and 14 days old plants were selected for *in vivo* systems at two time intervals i.e. 0, 2, 4, 6, 8 up to 10 h and 24, 48, 72, 96, 120, 144 up to 168 h. Spore suspension of *M. phaseolina* (10^5 spores mL^{-1}) (MTCC 166) was sprayed on sesame plants.

Determination of β-1,3-glucanase activity

The following reagent / buffers were used:
1 M potassium acetate buffer (pH 7.5)
0.05 M potassium acetate buffer (pH 5)
2% solution of laminarin (w/v)
The β-1,3-glucanase was assayed using the method of Abeles *et al*. (1970).

Extraction of β-1, 3-glucanase

The control and fungal pathogen inoculated tissue i.e. entire leaf of *Sesamum* plants were taken at the different time intervals of fungal spore inoculation and was homogenized in a pre-chilled mortar pestle in 4 mL of 0.05 M of potassium acetate buffer, pH 5. The homogenate was filtered through pre-moistened four layered muslin cloth and filtrate was centrifuged at 10,000 rpm in a cooling centrifuge for 10 min at 4 °C. The supernatant was collected and further used for the enzyme estimation.

Determination of β 1, 3 glucanases and chitinases:

Determination of β 1, 3 glucanases was done by using the method of Abeles *et al.* (1970).

Assay of β-1, 3-glucanase

The extract from both, control and inoculated plants were taken for the assay. One milliliter reaction mixture was prepared by mixing of 50 µL sample, 450 µL buffer (0.05 M potassium acetate, pH 5) and 500 µL of 2% laminarin as substrate. This reaction mixture was incubated for 1 h at 40 °C. After incubation the released glucose was further assayed using the methods of Nelson (1944), and Somogyi (1952).

Calculation

The β-1, 3-glucanase activity was calculated in µkatal g^{-1} fresh weight using the standard value obtained from the standard curve of glucose. The specific activity was calculated by determining the protein content using Lowry *et al.* (1951).

Characterization of proteins by SDS-PAGE

Protein profile in the normal and infected plant samples were analyzed by using SDS-PAGE following the method of Laemmli (1970).

Statistical analysis

All data of healthy and diseased plants were subjected to one-way analysis of variance (ANOVA) (Petkovšek *et al.,* 2008). Differences between healthy and infected plants were tested with least significant differences (LSD) with the level of significance of $p<0.05$. Data were analyzed by using the SPSS 16.0 program (Table 1 and 2).

Results and Discussion

Plants have developed a number of strategies to defend themselves against pathogens and environmental stress. Over the years, many studies have been performed to analyze plant-pathogen interaction. Many plant defense genes are triggered in response to infection by pathogen. Expression of these proteins correlates with the development of systemic acquired resistance in plants (Ryals *et al.,* 1996). β-1, 3- glucanase known as laminarinases enhanced fungal resistance in plants (Kirubakaran and Sakthivel, 2007). In the present study, in order to determine the maximum activity of β-1,3- glucanase enzyme in pathogen inoculated and control plants after short time interval (0 to 10 hours) and long time interval (24 hours up to 168 hours), the β-1,3-glucanase enzyme activity was determined in 7 days old and 14 days old cultivars.

Change in β 1, 3- glucanase activity in sesame after foliar spray of spore suspension of *M. phaseolina* at short time interval

In order to investigate an early change in enzyme activity under *in vivo* conditions, the leaves were analyzed at the difference of 2 hours up to 10 hours after pathogen inoculation. It was observed from that after 7 days maximum production of β 1,3- glucanase occurs at 6 hours after inoculation of fungal spore suspension and the values were found higher at every stage of infection progression. The values noticed in all the four infected experimental cultivars i.e. RT-46, GT-2, T-12 and TMV-3 were 10.72±0.07, 7.35±0.02, 4.95±0.03 and 2.91±0.14 µkatal mg^{-1} protein at 6 hpi while these values in control of the same cultivars at 6 hour are 9.56±0.01, 6.10±0.21, 4.09±0.05 and 2.32±0.01 µkatal mg^{-1} protein. From the above data it was clear that in all the varieties, infected cultivars showing higher values in comparison with healthy tissues. Same results

have also been observed after 14 days in all the four sesame cultivars. In both the cases i.e. 7 and 14 days old plants RT-46 showed the best results and the percentage increase in RT-46 is 2%, 8%, 9%, 9%, 3% and 3% in comparison of control ones (Table 3, Fig. 1). These results are in support with Radhajeyalakshmi *et al.* (2009), who observed the higher glucanase activity in suspension cultured cells and leaves of tomato plants after inoculation of *A. solani* and its elicitor. Previously, it was shown by Forslund *et al*. (2000) that β 1, 3-glucanases were induced in barley upon attack by *R. padi*.

Change in β-1, 3-glucanase activity in sesame after foliar spray of spore suspension of *M. phaseolina* at long time interval

In the present study on β-1,3-glucanase produced in *S. indicum* after exposure of fungal spore suspension, the results showed that β -1,3-glucanase activity started increasing rapidly after 24 hpi and found maximum at 96 hour of infection and after that declined steadily in 7 days old plants. The values were measured in µkatal mg^{-1} protein at the difference of 24 hours up to 168 hours after infestation. Among all the four investigational cultivars RT-46 showed the best results as in all the above parameters. The percentage increase in RT-46 was recorded as 4%, 6%, 9%, 25%, 19%, 16% and 10% at 24, 48, 72, 96, 120, 144 and 168 hours of infection in comparison of control ones and had maximum value (15.50±0.05 µkatal mg^{-1} protein) at 96 hpi which was higher when compared to control one (12.37±0.16 µkatal mg^{-1} protein). Rest three investigational cultivars show the aligning results, that is, these were also showing the maximum activity at 96 hpi as in RT-46 and always found higher values in comparison to control tissues. In the same way, 14 days old plants in all the four investigational cultivars showed maximum activity at 96 hours after infection of fungal pathogen and same pattern of percentage increase was found as in 7 days old sesame cultivars which was noticed as 3%, 11%, 11% 30%, 24%, 15% and 14% at 24, 48, 72, 96, 120, 144 and 168 hour of infection (Table 4). In 14 days old plants, among all the four experimental sesame cultivars RT-46 showed the best results as in 7 days old cultivars and in all the cases infected cultivars were showing higher enzyme activity in comparison of control tissues at every stage of fungal pathogen infection. Fourteen days old cultivars showed higher amount of enzyme production when compared to 7 days old plants and the comparison is shown in Fig. 2.

Saikia *et al*. (2005) reported three β-1,3-glucanase isozymes in *Fusarium oxysporum* induced chickpea plants. Maximum level in the activity of β-1,3-glucanase was reached to maximum level after three days of inoculation which was more than four fold in comparison of control ones, subsequently it decreased progressively. The latest pronouncement regarding glucanase activity after fungal pathogen (*Alternaria brascicoli*) attack in *Eruca sativa* plant was observed by Gupta *et al.* (2012). They observed that in 10 days old RTM-202 (resistant) cultivar, the maximum activity was at 48 hpi which was 2.4 fold higher in comparison of healthy tissues while in T-27 (susceptible) cultivar the maximum activity was at 72 hpi which was 1.8 fold higher in comparison of healthy tissues and in one month old cultivars the maximum activity in RTM-202 was at 24 hpi which was 1.5 fold higher while in T-27 maximum activity was at 48 hpi and 1.4 fold higher when compared to control ones.

Qualitative analysis of β-1, 3-glucanase enzyme using SDS-PAGE

The purified β-1,3-glucanase enzyme of healthy and inoculated four sesame cultivars were qualitatively analysed by SDS-PAGE at different time intervals. Purified samples of all the four sesame cultivars were characterized by 12.5% resolving gel. Medium range molecular weight protein marker was used to analyse the molecular weight of obtained protein bands. For the analysis of 14 days old healthy and inoculated sesame cultivars were taken by using *in vivo* system; the reason being sufficient time for fungal invasion and the changes thereafter. From the data, it is clear that number of protein bands were found more when compared to control ones. Fig. 3 shows the number of bands in 14 days old plants in all the four cultivars. Number of bands were found higher in cultivar RT-46 in comparison of the rest three investigational cultivars. For example, numbers of bands observed were 6 in RT-46 control and 9 in RT-46 inoculated, whereas in GT-2C these were 5 and 7 in GT-2 I. Similarly, total number of peaks in T-12C were 5 while in inoculated T-12 number of bands were 7. On the other hand these numbers of bands in TMV-3C were noticed to 5 while in inoculated TMV-3, these were 6.

Analysis of β 1, 3- glucanase enzyme gel using the scion image program

Analysis of SDS-PAGE gel of β1,3-glucanase enzyme was done by using scion image program. For the analysis of 14 days old plants in all the four investigational cultivars extract was

taken at 96 hpi because according to Table 4 and Fig. 2, maximum calculated amount and peak value were obtained at 96 hour after pathogen infestation in all the four experimental cultivars.

Variation in the total area of individual peaks in the inoculated plants in comparison of control plants was observed. The area occupied by the peak in the inoculated plants was observed more than that of healthy ones. For example, in RT-46 maximum peak area was 825 in inoculated plants which was more than that of 712 in control at 96 hours of infection while this area was noticed 675 in GT-2C and 786 in inoculated plants. On the other hand, peak area value in T-12C was 469 and 541 in T-12 inoculated plants. TMV-3C showed the area peak value in range of 195 which was always lower than inoculated plants i.e. 257. Among all the four cultivars, RT-46 showed the best results having maximum peak and peak area values (Fig. 4). Similar results were also found in 7 days old plants but concentration of protein content was low when compared to 14 days old plants. Bands were light and peak area values attained by the bands of 7 days old plants protein extract were found lower than 14 days old ones. But in both the cases i.e. 7 and 14 days old cultivars values were found higher in inoculated cultivars when compared to control ones. Similar results were reported by Kim and Hwang (1997), who isolated a 34-kDa β-1,3-glucanase from a pepper stem which inhibit the hyphal growth of *P. capsici*.

Table 1: Descriptive analysis of glucanase (µkatal g^{-1} protein) at short time and long time interval in *Sesamum indicum* plants in control and after infection with *Macrophomina phaseolina* using *in vivo* system

Variety	Groups	N	Mean	SD	SE
RT- 46	Control	33	13.1940	3.40252	0.59230
	Infected	33	14.1046	3.89903	0.67873
	Total	66	13.6493	3.65982	0.45049
GT-2	Control	33	7.7759	1.00083	0.17422
	Infected	33	8.5499	1.23625	0.21520
	Total	66	8.1629	1.18221	0.14552
T-12	Control	33	4.8206	0.54324	0.09457
	Infected	33	6.2498	0.61169	0.10648
	Total	66	5.5352	0.92084	0.11335
TMV-3	Control	33	4.3586	0.58549	0.10192
	Infected	33	5.1522	0.87659	0.15259
	Total	66	4.7554	0.84078	0.10349

Table 2: One way analysis of variance (ANOVA) for Glucanase at short time and long time interval in *Sesamum indicum* plants in control and after infection with *Macrophomina phaseolina* using *in vivo* system.

Variety	Source of Variance	Sum of Squares	Degree of freedom	Mean Square	F value	Sig.
RT- 46	Between Groups	13.682	1	13.682	1.022	0.316
	Within Groups	856.946	64	13.390		
		870.628	65			
GT-2	Between Groups	9.886	1	9.886	7.815	0.007
	Within Groups	80.959	64	1.265		
		90.845	65			
T-12	Between Groups	33.700	1	33.700	100.708	0.000
	Within Groups	21.417	64	0.335		
		55.117	65			
TMV-3	Between Groups	10.391	1	10.391	18.702	0.000
	Within Groups	35.559	64	0.556		
		45.950	65			

Table 3: Specific activity of β 1,3- glucanase (µkatal mg^{-1} protein) at short time interval in *Sesamum indicum* plants after inoculation with *Macrophomina phaseolina* using *in vivo* system.

7 days Old Plants								
Hours	RT 46		GT 2		T 12		TMV 3	
	C	I	C	I	C	I	C	I
0	9.39±0.35	9.53±0.04	6.01±0.03	6.20±0.01	3.98±0.01	4.04±0.32	2.27±0.02	2.54±0.15
2	9.39±0.35	9.63±0.07	6.03±0.03	6.21±0.02	4.15±0.14	4.26±0.13	2.32±0.06	2.58±0.04
4	9.49±0.03	9.88±0.04	6.12±0.03	6.22±0.02	4.04±0.02	4.66±0.03	2.38±0.06	2.62±0.12
6	9.56±0.01	10.72±0.07	6.10±0.21	7.35±0.02	4.09±0.05	4.95±0.03	2.32±0.01	2.91±0.14
8	9.53±0.25	10.14±0.17	6.29±0.03	7.06±0.06	4.17±0.01	4.86±0.03	2.49±0.06	2.59±0.07
10	9.54±0.04	9.98±0.07	6.09±0.01	6.91±0.03	4.18±0.05	4.75±0.03	2.52±0.01	2.58±0.08
14 Days Old Plants								
Hours	RT 46		GT 2		T 12		TMV 3	
	C	I	C	I	C	I	C	I
0	10.83±0.24	11.03±0.05	8.01±0.09	8.31±0.06	4.77±0.01	5.42±0.03	3.27±0.03	3.39±0.12
2	10.91±0.41	11.76±0.00	8.15±0.30	8.49±0.01	4.86±0.02	5.52±0.01	3.28±0.24	3.45±0.27
4	10.95±0.02	11.94±0.09	8.19±0.02	9.67±0.16	5.03±0.04	5.63±0.01	3.31±0.19	3.46±0.25
6	11.07±0.10	12.02±0.29	8.28±0.02	9.76±0.07	5.39±0.03	5.84±0.18	3.33±0.02	4.06±0.05
8	11.29±0.12	11.65±0.08	8.28±0.23	9.68±0.26	5.30±0.01	5.72±0.01	3.34±0.11	3.90±0.25
10	11.24±0.01	11.53±0.02	8.37±0.04	8.93±0.07	5.50±0.03	5.64±0.01	3.40±0.08	3.91±0.23

Table 4: Specific activity of β 1, 3- glucanase (µkatal mg^{-1} protein) at long time interval in *Sesamum indicum* plants after inoculation with *Macrophomina phaseolina* using *in vivo* system

7 days Old Plants								
Hours	RT 46		GT 2		T 12		TMV 3	
	C	I	C	I	C	I	C	I
24	12.46±0.05	12.96±0.21	6.49±0.02	6.82±0.01	4.80±0.03	5.20±0.07	2.65±0.14	3.77±0.29
48	12.46±0.05	13.17±0.14	6.50±0.02	7.09±0.05	4.87±0.01	5.28±0.04	2.62±0.02	3.96±0.17
72	12.38±0.34	13.55±0.19	6.60±0.01	7.92±0.03	4.88±0.01	5.80±0.10	2.74±0.18	4.36±0.01
96	12.37±0.16	15.50±0.05	6.64±0.03	8.38±0.04	4.90±0.19	7.39±0.27	2.80±0.05	4.99±0.39
120	12.31±0.25	14.59±0.25	6.57±0.04	8.09±0.02	5.16±0.01	6.94±0.03	2.83±0.15	4.75±0.02
144	12.44±0.05	14.42±0.05	6.90±0.22	7.99±0.05	5.24±0.12	6.33±0.27	2.90±0.11	3.85±0.05
168	12.40±0.10	13.58±0.05	7.00±0.16	7.33±0.03	5.36±0.34	5.95±0.24	2.86±0.18	3.69±0.11
14 Days Old Plants								
Hours	RT 46		GT 2		T 12		TMV 3	
	C	I	C	I	C	I	C	I
24	15.13±0.05	15.64±0.17	8.00±0.02	9.35±0.18	6.32±0.02	7.65±0.02	3.45±0.03	4.17±0.01
48	15.14±0.34	16.76±0.28	8.58±0.02	9.98±0.04	6.50±0.01	8.49±0.04	3.55±0.02	4.45±0.01
72	15.17±0.10	16.85±0.02	8.87±0.32	10.47±0.03	6.61±0.03	8.76±0.01	3.50±0.11	4.95±0.02
96	15.18±0.09	19.71±0.23	9.09±0.34	11.24±0.01	6.81±0.01	10.44±0.13	3.56±0.04	5.79±0.02
120	15.19±0.08	18.86±0.46	9.12±0.36	10.78±0.1	7.1±0.07	9.94±0.2	3.56±0.04	5.57±0.03
144	15.12±0.11	17.39±0.10	9.14±0.05	10.68±0.01	7.14±0.10	9.8±0.04	3.53±0.02	5.05±0.02
168	15.11±0.12	17.18±0.08	9.27±0.11	9.90±0.31	7.32±0.01	8.79±0.13	3.59±0.27	4.76±0.01

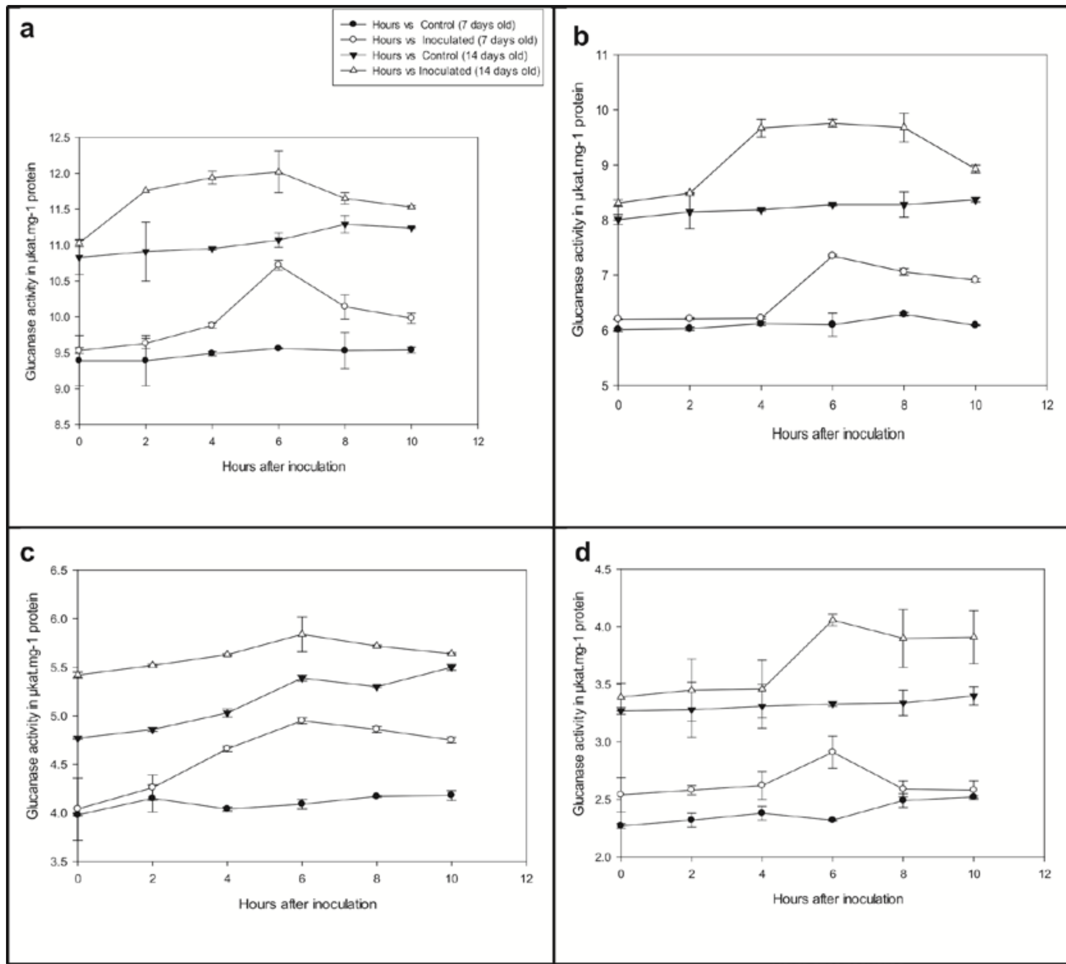
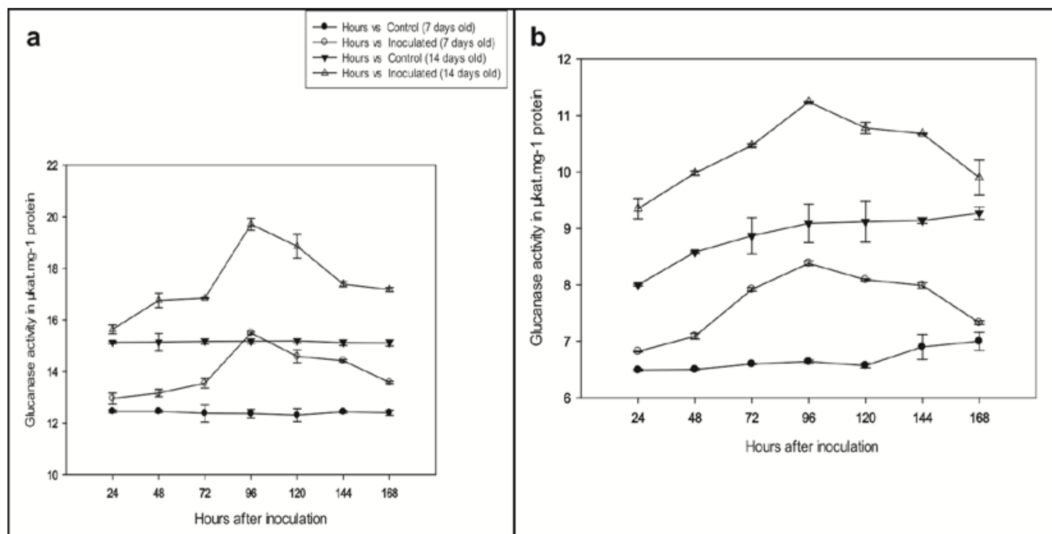


Fig. 1a-d: Specific activity of β 1,3- glucanase ((µkatal mg^{-1} protein) after short time interval in control and inoculated **(a):** RT-46 cultivar; **(b):** GT-2 cultivar; **(c):** T-12 cultivar; **(d):** TMV-3 cultivar with foliar sprays of spore suspension of *M. phaseolina* using *in vivo* system.



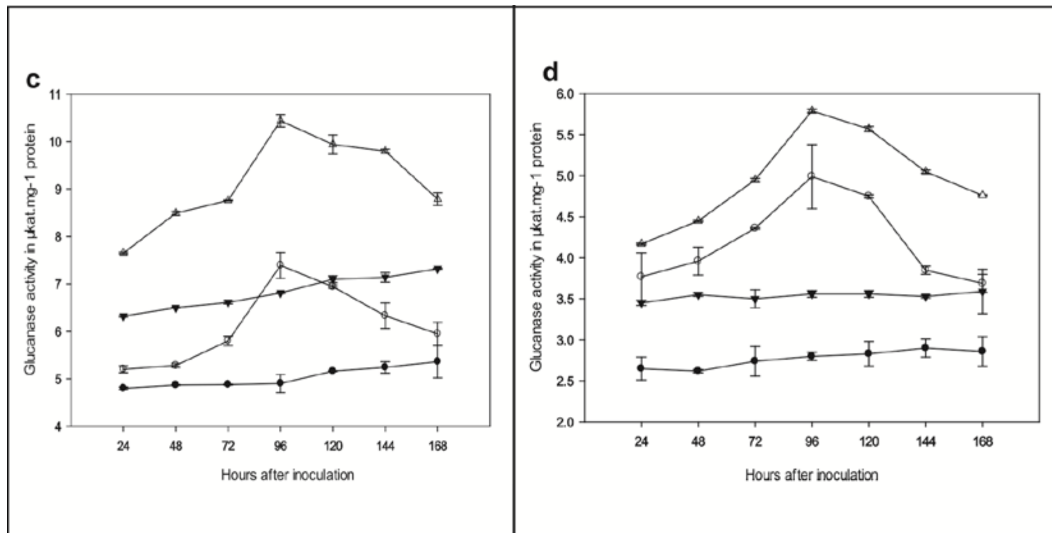


Fig. 2a-d: Specific activity of β 1,3- glucanase (µkatal mg^{-1} protein) after long time interval in control and inoculated **(a):** RT-46 cultivar; **(b):** GT-2 cultivar; **(c):** T-12 cultivar; **(d):** TMV-3 cultivar with foliar sprays of spore suspension of *M. phaseolina* using *in vivo* system.

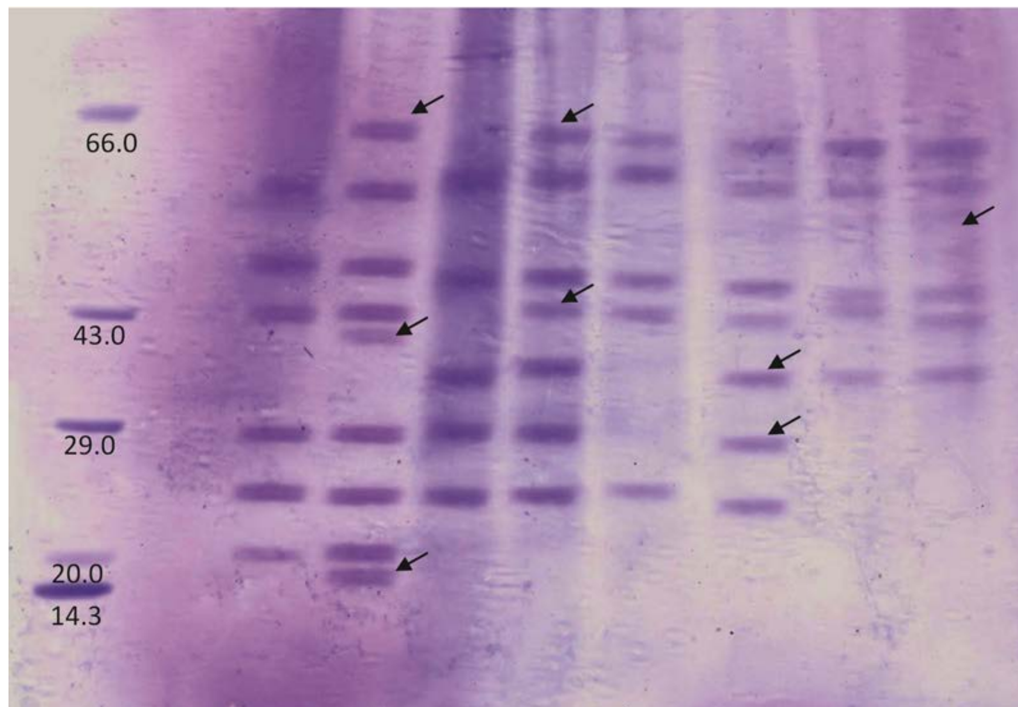


Fig. 3: SDS-PAGE profile of purified β 1, 3- glucanase enzyme of control and inoculated 14 days old four sesame cultivars (RT-46, GT-2, T-12 and TMV-3) after inoculation of 96 hours.

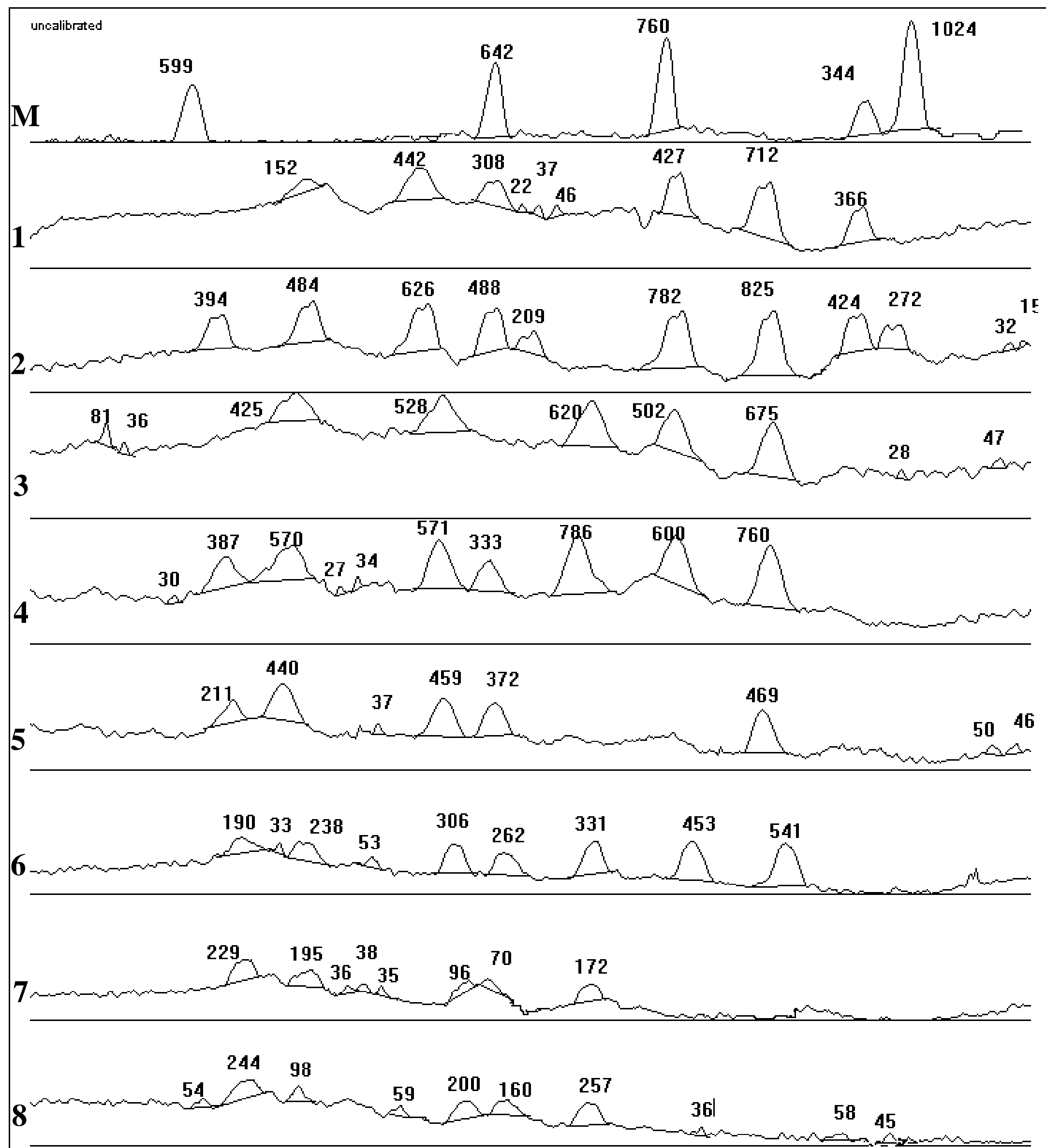


Fig. 4: Plot profile of β 1, 3- glucanase enzyme of 14 days old four cultivars of sesame (RT-46, GT-2, T-12 and TMV-3) at 96 hours after inoculation using the scion image programme (M: Medium range molecular weight marker; 1: Protein profile of control RT-46 cultivar; 2: Protein profile of infected RT-46 cultivar; 3: Protein profile of control GT-2 cultivar; 4: Protein profile of infected GT-2 cultivar; 5: Protein profile of control T-12 cultivar; 6: Protein profile of infected T-12 cultivar; 7:Protein profile of control TMV-3 cultivar; 8: Protein profile of infected TMV-3 cultivar).

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