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Jasminkumar Kheni
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Rukam Singh Tomar
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Mita Shihora
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Shital Padhiyar
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Hiralben Desai
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Tushar Antala
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

BA Golakiya
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Corresponding Author:
Jasminkumar Kheni
Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

Protein profiling of chickpea (*Cicer arietinum* L.) during wilt disease (*Fusarium oxysporum* f. sp. *ciceri*)

Jasminkumar Kheni, Rukam Singh Tomar, Mita Shihora, Shital Padhiyar, Hiralben Desai, Tushar Antala and BA Golakiya

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Abstract
The proteomic study was carried out in 2DE gel electrophoresis to check differentially expressed proteins during chickpea- wilt interaction in resistance (WR315) and susceptible (JG62) chickpea genotypes. The result of this study showed that total 1070 and 1215 spots were detected in JG-62 and WR-315, respectively**.** Out of 1070 spots, 480 spots were present in control group while 590 spots were present in wilt inoculated susceptible plants. In resistant group 550 spots were found in control while 665 spots were found in wilt inoculated chickpea. The molecular masses of selected spots were identified with the range of 22.5 KDa to 104.4 KDa with pH range 4.00 to 6.76. Many spots were shown the significant level of differential expression in wilt resistant and susceptible chickpea plants. These spots were taken from match area of susceptible variety. Among the % vol. of each spot were identified and match with each other. Among the 12 spots matches during wilt infection majority shows down regulated as they occupy very low spot area.

Keywords: Wilt disease, proteomics, plant-pathogen interaction

Introduction
Chickpea is known as Bengal gram or garbanzo bean. It complies diploid (2n = 2x 6) genome size of 738 Mb [1]. This genome size is comparatively higher than that of the model legume crops such as soybean, peanut, garden pea, alfalfa, and lentil [2]. This genus belongs to the family Leguminoseae and sub-family Papilionaceae. Those are composed of 34 perennial wild species and 9 annual species. Including 9 annual species, chickpea is the only cultivated species [3]. Chickpea helps to improve soil fertility in dry lands and fix atmospheric nitrogen. It is one of the major constituents of the Mediterranean diet and a basic food in Asiatic countries [4]. Due to its high nutritional value, its grain international market is very active. The Chickpea is a good and cheap diet source with high protein quantity for people of developing countries; those are largely vegetarian. There are deluge of breeding efforts have contributed towards improvement of chickpea yield, however the lack of stable production is a major concern for the crop adoption farmers [5]. Due biotic and abiotic stresses that drastically affect grain yield, especially fungal infectious diseases like: Fusarium wilt, Ascochyta blight and drought or cold stresses.
In this present studies we revealed proteomics associated with chickpea-wilt interaction through two dimensional electrophoresis (2DE). This technique is efficient to identify differentially expressed proteins involved in wilt-chickpea interaction [6]. Most of studies of proteomics are performed based on model plant species such as *Arabidopsis thaliana* [7]. However, research based on model organisms requires experimental authentications. Furthermore, some features and processes are differing in commercial crops. Hence, it is apparent that it is difficult to approach via model plant in totality [8].
This study aims to conclude proteomic basis of wilt disease susceptibility and resistance by two controversial genotypes named JG62 and WR315, respectively. There are many proteins differentially regulated in both resistant and susceptible genotype of chickpea at before and after inoculation with foc.

Materials and Methods

Plant growth and fungal treatment

Chickpea (*Cicer arietinum* L.) genotypes JG62 (wilt susceptible) and WR315 (wilt resistant), obtained from Pulse research station of Junagadh Agricultural University (JAU), Junagadh, India were used for experimental analysis. Seeds of both genotypes were grown in a soil sand mixture with conditions of 22 to 28 °C, 35 to 40% relative humidity and 16 h:8 h photoperiod of day and night, respectively [9]. Plants of both genotypes were grown on normal soil without infection served as control. Both control and infected plants were kept under same growth conditions. Root samples from control and infected plants at 2 day after inoculation (dai) were harvested, instantly frozen in liquid nitrogen and stored at -80 °C for further experiments. Proteins were extracted from root colar tissues of each sample.

Protein Extraction and 2D electrophoresis

Protein precipitation was based on the TCA method with some modification [10]. As well GE health care guideline was used when ever needed. Brief, Chickpea root collar tissue was finely powdered in liquid nitrogen with a pre-cooled ceramic mortar and pestle. The resulting powder was suspended in 500 µl cool rehydration buffer (8M Urea, 2% CHAPS, 7 mg of DTT/2.5ml buffer) Once it is finely homogenized, the volume is made up to 1.5 ml. The mixture was incubated at Room temperature for 10 minute. Centrifuge the mixture at 12000rpm for 30 minute. Take supernatant 500 µl and add 10% TCA in acetone allow precipitation of protein at -20 °C for overnight. Next day centrifugation at 12000 rpm for 15 min at 4°C, the protein pellet was washed four-five times with chilled 90% acetone. After centrifugation at 12000 rpm for 15 min between rinses, the supernatant was discarded and the pellet was subjected to air dry. The dried powder was solubilised in lysis buffer (8M urea, 2% CHAPS, 2% ampholyte pH 4–7(GE Healthcare Bio-Science, Little Chalfont, UK), 0.2% DTT, 10 µl/ml Protease inhibitor mix). This is then stored overnight at 4 °C for protein extraction. Then protein was loaded onto isoelectrofocusing (IEF) polyacrylamide gels (IGP Strip) with rehydration or by cup loading method. IEF was carried out on GE healthcare instruments.

The analysis was carried out on 2D protocol was followed as per GE health care standard method with some manual changes [11, 12]. The 24 cm IGP strips were rehydrated in rehydration buffer (8M Urea, 2% CHAPS, 1% Bromophenol Blue, 0.5% IGP buffer (pH 4-7), 7 mg DTT per 2.5 ml rehydration solution at time of use) for at least 10-20 h in 400 µl rehydration buffer based on length of strips. The sample was loaded using cup loading method and sealed with mineral oil. The separation of protein in this method is based on the size of the protein molecules [13]. The SDS PAGE separation was followed by IEF completion. The gel was stained in Coomassie brilliant blue G 250 and distained using methanol, acetic acid and distilled water in ration 40:10:50.

Spot identification and analysis:

When the tracking dye reached the end of the running gel after complete separation of molecules, power supply was turned off. The gel was gently removed from the space between the plates, immersed in staining solution contained in a tray. After sufficient incubation period, the gel was distained by adding the detaining solution followed by Scanning of gel by Typhoon FLA Scanner. Differentially expressed spots were calculated and identified using Platinum Master software (GE healthcare). The graphical representation 2DE protocol is given in Figure 1.

Results and Discussion

Protein Profiling by 2D Gel Electrophoresis

Wilt resistant and wilt susceptible chickpea (*Cicer arietinum* L.) genotypes JG-62 and WR-315 was selected for the study of protein profiling. These both genotypes were selected for wilt inoculation. The samples were collected at 2 dai of *Fusarium* inoculation to plants and same genotypes with un-inoculated. The root collar tissues were selected for protein extraction and 2D-gel electrophoresis.

The Samples were collected after 2 days after inoculation from wilt resistant and wilt susceptible chickpea plant variety. The protein was extracted for 2D gel electrophoresis analysis from fresh root collar of control and wilt inoculated resistant (WR-315) and susceptible (GJ-62) genotypes. Proteins were separated on the basis of their isoelectric point (pI) on the IPG strips (pH 4-7, 110 cm Non Linear) and in second dimension. SDS-PAGE was stained with CBB G-250 and stained gel was shown in Figure 2. The result of study showed that total 1070 and 1215 spots were detected in JG-62 and WR-315, respectively. The gel was analyzed using 2D gel analysis software**.** Out of 1070 spots, 480 spots were present in control group while 590 spots were present in wilt inoculated susceptible plants. In contrast, resistant group 550 spots were found in control and 665 spots were found in wilt inoculated chickpeas. As compared to resistant control group, there were 115 more spots were preset in wilt inoculated chickpeas. Total 60 spots were matched in susceptible genotypes; and in resistant total 112 spots were matched. The match ID was given from 0 to 112 and 0 to 60. From which total 45 spots were shown which contain higher area. The match criteria were selected as per volume (%), PI, CV and molecular masses. The details of spots are given in Table 1 for susceptible group and in Table 2 for resistant group. The different levels of protein expression were showed by the histogram of both resistant and susceptible genotypes Figure 3 and 4. The molecular masses of selected spots were identified with the range of 22.5 KDa to 104. 4 KDa; with pH ranged 4.00 to 6.76. Among Total matched spots, 12 spots mentioned table 1. They were significantly differentiated with expression level between two treatment groups. The volume of each spot was identified and match with each other. Total 12 spots matched during wilt infection majority shows down regulated as they are occupy very low volume while in wilt resistant chickpea they show higher and up regulation among the 12 selected spots. The green area is matched area and it's significantly increase in infected WR-315 plants. It indicates that major up-regulation of proteins related to defense and signaling molecules. The similar study of protein expression in 2D gel electrophoresis founds in chickpea and soybean and further expressed protein were identified for disease resistant [14, 15]. A study also revealed that up regulated proteins involved in disease resistance while down regulated proteins involved in amino acid metabolism and photosynthesis[16]. Another study carried out on DIGE analysis indicates that 47 differentially expressed proteins involved in of salt- and drought mechanisms [17]. Alternatively, Rollins *et al.* (2013) performed leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.) **[18]**. Proteomics of salinity stressed in wheat chloroplasts revealed that antioxidant enzyme regulation protects cells from hydrogen paroxide [19]. A comparison of first dimension IPG and

NEPHGE techniques in two-dimensional gel electrophoresis experiment with cytosolic unfolded protein response in *Saccharomyces cerevisiae* [20]. In this study they compared IPG- based and NEPHGE based 2DE techniques by using the similar types of samples and they concluded that NEPHGE based method is most preferable over other methods [20]. Nevertheless, the narrow range (pH 4-7) IPG technique is ideal for acidic proteins analysis. Another study of plant parasite *Orobanche crenata* and *Pisum sativum* revealed that metabolic and stress-related proteins play an important role during penetration and connection to the vascular system of the parasite [21]. However, due to low reproducibility 2-DE is criticised when comparative analysis of two different gels is performed [22, 23]. There are, however, also some advance genomics techniques available for identification of pathogenesis related proteins from pathogen. The similar study of *Athelia rolfsii* genome indicates that there are several genes responsible for pathogenicity [24]. Nevertheless, advancements in plant-pathogen interactions through model plants become recent trend to apply similar approach in crop plants and help to improve agricultural practices with elite fungicide development in particular crop [25]. The proteomics study of chickpea may provide putative information about protein level regulation during chickpea and wilt disease interaction. Further, functional characterization will help to improve crop variety and other disease resistant traits.

Conclusion

The wilt disease in chickpea showed considerable proteomic changes during chickpea-wilt interaction. There are significant differences among wilt resistant and susceptible chickpea. There are also some up and down regulated proteins with reference to volume and coefficient of variation. This shows importance of proteomics techniques to determine proteins regulation during host pathogen interaction which helps to identify some putative proteins to identify some disease resistance proteins involved during infection.

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The authors are extremely grateful to the Department of Biotechnology, JAU, Junagadh, India for the support and facilitation for carrying out the research work and Pulse research station, JAU, Junagadh, India for providing chickpea genotypes.

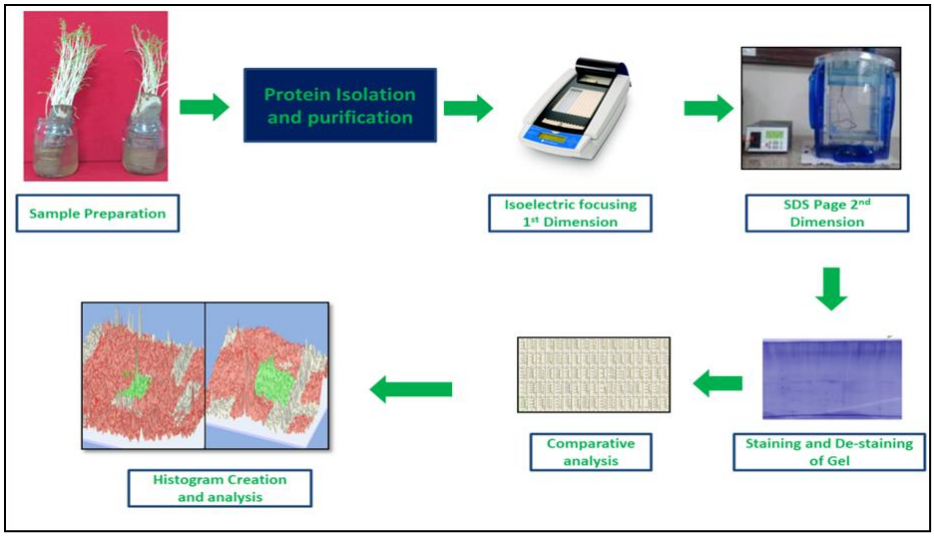

Fig 1: Graphical representation of chickpea-wilt protein profiling using two dimensional gel electrophoresis

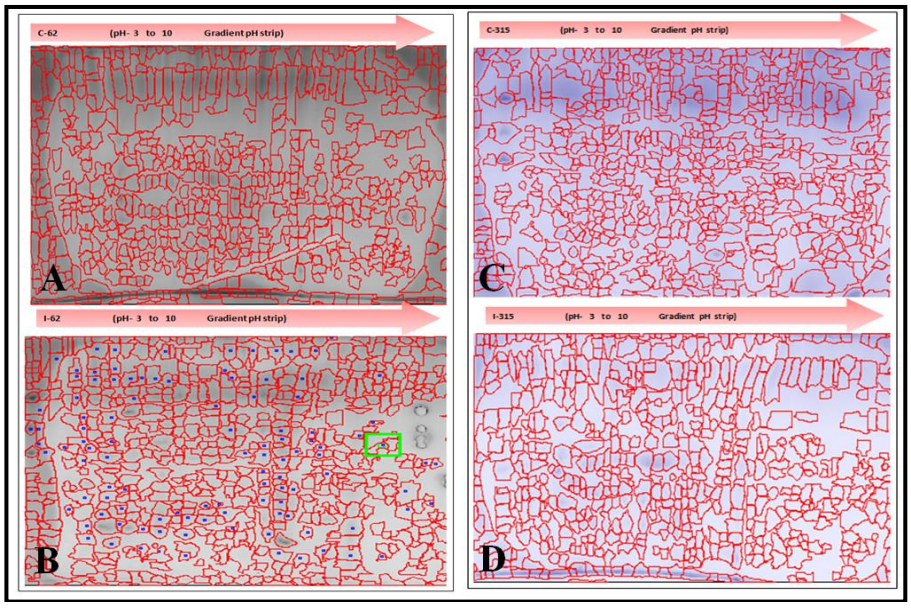

Fig 2: Root proteome profiling of control and *F. oxysporum* f.sp. *ciceri* (foc) infected chickpea genotypes JG62 (A, B); WR315 (C, D); (C= Control; I=Infected)

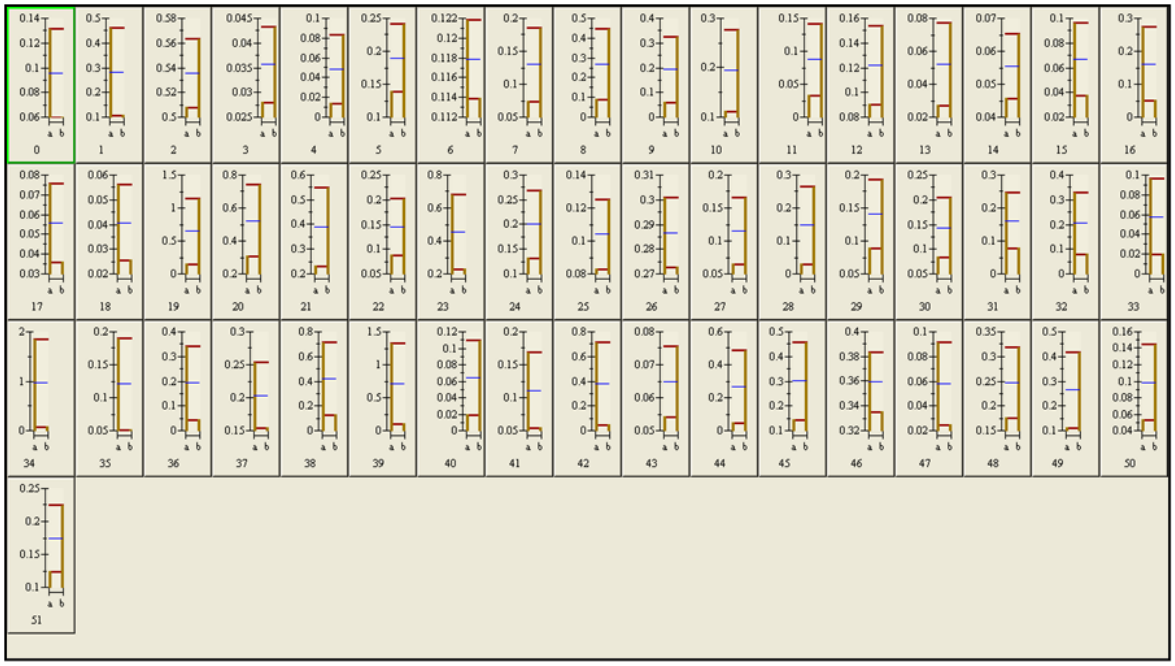

Fig 3: Comparative Histogram of Match ID spots, (a) C-62 (b)I-62 wilt susceptible chickpea genotypes

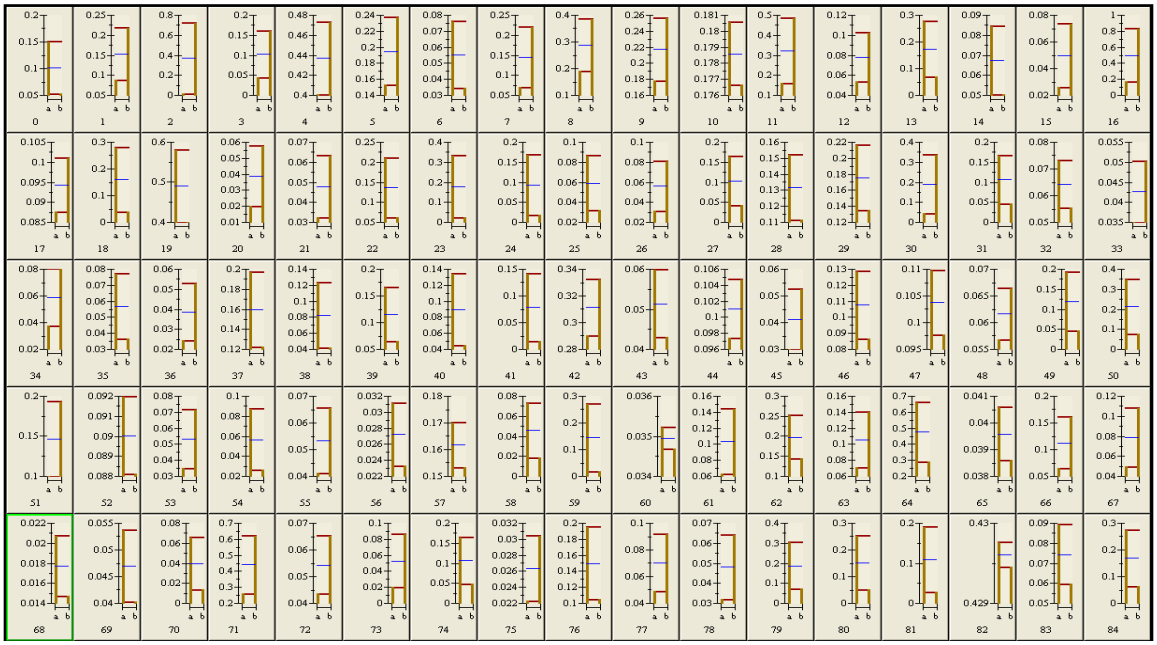

Fig 4: Comparative Histogram of Match ID spots, (a) C-315 (b) I-315 wilt resistant chickpea genotypes

Table 1: Match ID of spots found in 2DE-Gel analysis of Chickpea genotype JG62

Sr. No.	Spot ID	Coef. Variation	Control			Infection		
			% volume	M.W. (K.Dal)	PI	% volume	M.W. (K.Dal)	PI
1	0	0.47478	0.053674	29.0	5.58	0.15071	29.0	6.18
2	1	0.42302	0.218546	29.0	5.14	0.08861	29.0	5.68
3	2	0.95093	0.719361	29.0	5.38	0.01809	29.0	6.04
4	3	0.57310	0.160510	29.0	5.22	0.04356	29.0	5.84
5	4	0.08204	0.472728	29.0	5.39	0.40104	29.0	6.00
6	5	0.21662	0.237257	29.0	5.50	0.15277	29.0	6.06
7	6	0.37623	0.034524	29.0	5.27	0.07617	29.0	5.81
8	7	0.51219	0.219470	29.0	4.71	0.07080	29.0	5.22
9	8	0.34001	0.386180	29.0	4.32	0.19021	29.0	4.88
10	9	0.17991	0.256343	29.0	4.96	0.17817	29.0	5.46
11	10	0.01083	0.176666	29.0	4.73	0.18053	29.0	5.33
12	11	0.50666	0.485666	29.0	4.19	0.15903	29.0	4.78
13	12	0.31347	0.102228	29.0	5.13	0.05343	29.0	5.64
14	13	0.59158	0.071014	29.0	4.68	0.27674	29.0	5.21
15	14	0.25071	0.050599	29.0	5.25	0.08446	29.0	5.75
16	15	0.47144	0.073231	29.0	5.41	0.02631	29.0	5.96

17	16	0.66162	0.826583	29.0	4.18	0.16833	29.0	4.73
18	17	0.07048	0.101004	29.0	4.56	0.08770	29.0	5.08
19	18	0.75705	0.038746	29.0	4.81	0.28021	29.0	5.31
20	19	0.18314	0.400494	29.0	5.15	0.58008	29.0	5.64
21	20	0.48635	0.057683	29.0	5.55	0.01993	29.0	5.98
22	21	0.32072	0.063101	29.0	5.50	0.03245	29.0	5.94
23	22	0.54636	0.061782	29.0	4.64	0.21060	29.0	5.18
24	23	0.86071	0.024796	29.0	5.26	0.33123	29.0	5.67
25	24	0.80414	0.018271	29.0	5.33	0.16830	29.0	5.78
26	25	0.45917	0.031981	29.0	5.39	0.08628	29.0	5.89
27	26	0.43549	0.080577	29.0	5.06	0.03169	29.0	5.51
28	27	0.59467	0.041509	29.0	5.97	0.16331	25.3	6.46
29	28	0.15275	0.111509	29.0	4.74	0.15172	29.0	5.23
30	29	0.23071	0.135293	29.0	6.17	0.21644	30.0	6.66
31	30	0.77108	0.336100	29.0	4.89	0.04344	29.0	5.34
32	31	0.55964	0.047030	29.0	4.56	0.16657	29.0	5.05
33	32	0.13690	0.055556	29.0	4.19	0.07318	29.0	4.68
34	33	0.17751	0.050219	29.0	4.83	0.03508	29.0	5.31
35	34	0.35935	0.079911	29.0	4.96	0.03766	29.0	5.42
36	35	0.35470	0.036677	29.0	4.20	0.07700	29.0	4.66
37	36	0.36337	0.052555	29.0	4.30	0.02454	29.0	4.74
38	37	0.23234	0.122552	29.0	4.76	0.19674	29.0	5.23
39	38	0.49251	0.041916	29.0	5.90	0.12328	29.0	6.34
40	39	0.43074	0.065700	29.0	5.99	0.16512	31.7	6.40
41	40	0.49589	0.045293	29.0	5.77	0.13440	26.9	6.22
42	41	0.80545	0.015216	29.0	5.70	0.14121	29.0	6.16
43	42	0.06721	0.332204	29.0	4.33	0.29036	29.0	4.73
44	43	0.16202	0.043058	29.0	5.78	0.05971	30.9	6.21
45	44	0.03587	0.104652	29.0	5.90	0.09740	35.5	6.30

Table 2: Match ID of spots found in 2DE-Gel analysis of Chickpea genotype WR315

Sr. No.	Spot ID	Coef. Variation	Control			Infection		
			% volume	M.W. (K.Dal)	PI	% volume	M.W. (K.Dal)	PI
1	0	0.36990	0.060251	29.0	5.58	0.130991	29.0	6.18
2	1	0.61800	0.108413	29.0	5.14	0.459193	29.0	5.68
3	2	0.05093	0.562904	29.0	5.38	0.508341	29.0	6.04
4	3	0.21412	0.043253	29.0	5.22	0.027997	29.0	5.84
5	4	0.70492	0.082800	29.0	5.39	0.014331	29.0	6.00
6	5	0.26604	0.240689	29.0	5.50	0.139534	29.0	6.06
7	6	0.03345	0.113958	29.0	5.27	0.121846	29.0	5.81
8	7	0.42300	0.184536	29.0	4.71	0.074826	29.0	5.22
9	8	0.65299	0.442258	29.0	4.32	0.092844	29.0	4.88
10	9	0.68374	0.324130	29.0	4.96	0.060881	29.0	5.46
11	10	0.42106	0.275641	29.0	4.73	0.112296	29.0	5.33
12	11	0.61999	0.141521	29.0	4.19	0.033198	29.0	4.78
13	12	0.25936	0.153779	29.0	5.13	0.090438	29.0	5.64
14	13	0.47939	0.076814	29.0	4.68	0.027032	29.0	5.21
15	14	0.17496	0.065171	29.0	5.25	0.045762	29.0	5.75
16	15	0.43807	0.037500	29.0	5.41	0.095969	29.0	5.96
17	16	0.68652	0.050736	29.0	4.18	0.272957	29.0	4.73
18	17	0.34963	0.036240	29.0	4.56	0.075204	29.0	5.08
19	18	0.37201	0.025579	29.0	4.81	0.055883	29.0	5.31
20	19	0.76292	1.137340	29.0	5.15	0.152949	29.0	5.64
21	20	0.40976	0.736722	29.0	5.55	0.30845	29.0	5.98
22	21	0.40655	0.547662	29.0	5.50	0.231071	29.0	5.94
23	22	0.38922	0.201341	29.0	4.64	0.08852	29.0	5.18
24	23	0.49420	0.230144	29.0	5.26	0.679875	29.0	5.67
25	24	0.33780	0.268553	29.0	5.33	0.132932	29.0	5.78
26	25	0.20154	0.125027	29.0	5.39	0.083083	29.0	5.89
27	26	0.04889	0.272672	29.0	5.06	0.300703	29.0	5.51
28	27	0.43330	0.165268	29.0	5.97	0.065344	25.3	6.46
29	28	0.79276	0.264169	29.0	4.74	0.030537	29.0	5.23
30	29	0.36310	0.191689	29.0	6.17	0.089566	30.0	6.66
31	30	0.41712	0.203171	29.0	4.89	0.083567	29.0	5.34
32	31	0.51602	0.078153	29.0	4.56	0.244805	29.0	5.05
33	32	0.60583	0.080758	29.0	4.19	0.329008	29.0	4.68
34	33	0.65868	0.019760	29.0	4.83	0.096024	29.0	5.31
35	34	0.91138	0.085629	29.0	4.96	1.84695	29.0	5.42

36	35	0.57471	0.051299	29.0	4.20	0.189942	29.0	4.66
37	36	0.77212	0.043900	29.0	4.30	0.341398	29.0	4.74
38	37	0.23880	0.155052	29.0	4.76	0.252338	29.0	5.23
39	38	0.69637	0.127547	29.0	5.90	0.712587	29.0	6.34
40	39	0.85112	0.106321	29.0	5.99	1.32197	31.7	6.40

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