Article

Semi-Quantification of Lectins in Rice (*Oryza sativa* L.) Genotypes via Hemagglutination

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Abstract: Lectins are unique glycoproteins that react with specific sugar residues on cell surfaces resulting in agglutination. They offer enormous applications in therapeutics, diagnostics, medicine, and agriculture. Rice lectins are naturally expressed during biotic and abiotic stresses suggesting their importance in stress resistance physiology. The objective of this study was to determine the presence and relative concentration of lectins in different accessions of rice obtained from IABGR/NARC Islamabad mainly originated from Pakistan. About 210 rice accessions including 02 local varieties and 05 transgenic seeds were screened for seed lectins using a hemagglutination (HA) assay with 5% Californian bred rabbits' erythrocytes. A protein concentration of 3–8 mg/100 mg of seed flour was measured for all the rice accessions; the highest was 8.03 mg for accession 7600, while the lowest noted was 3.05 mg for accession 7753. Out of 210 accessions, 106 showed the highest HA activity. These 106 genotypes were further screened for titer analysis and specific activity. The highest titer and specific activity were observed for accession 7271 as 1024 and 236 hemagglutination unit (HAU), respectively. The selected accessions' relative affinity and HA capability were evaluated using blood from four different sources: human, broiler chicken, local rabbit, and Californian-breed rabbit. The highest HA activity was observed with Californian-breed rabbit RBCs. The lectin assay was stable for about 1–2 h. After the required investigations, the accessions with higher lectin concentration and HA capability could be used as a readily available source of lectins for further characterization and utilization in crop improvement programs.

Keywords: rice; *Oryza sativa* L.; lectins; hemagglutination assay; rabbit RBCs; titer values; specific activity

1. Introduction

Lectins are carbohydrate-binding proteins present in every form of life. These are non-immune in origin and can recognize and bind unique sugars present on cell surfaces, resulting in cell agglutination. These are major glycoproteins that react with specific

saccharide residues in the cell membranes offering various applications in therapeutics, diagnostics, the food industry, plant breeding, agriculture, and biotechnology. The potential applications of plant lectins in glycobiology, medicines, and agriculture have been studied and reported. Plant lectins are produced commercially for their use in histochemistry, cytochemistry, blotting, affinity chromatography, biosensors, and microarray technologies [1–7].

Oryza sativa L. is a salt-sensitive plant species with significant genetic variability within the cultivated gene pool that can be exploited to develop salt-resistant varieties. The effectiveness of plant lectins on pathogenic fungi, insects, HIV, and cancer, as observed for numerous legume lectins, also needs to be explored for other plant species [8–11]. More than a hundred lectin molecules have been isolated from plants, viruses, bacteria, invertebrates, and vertebrates, including mammals. About 18 different types of lectins of plants are reported to have immunomodulatory effect in case of infection or disease such as cancer [12]. The role of lectins in abiotic and biotic stress resistance in rice has been highlighted by many researchers [13–21]. Knowing about the genes encoding these lectins and their role in plant defense offers many applications in plant breeding and genetic engineering to improve cultivars. Differences in lectin structure and carbohydrate specificity are related to their different functions. Depending on carbohydrate specificity, major lectins are divided into mannose-binding lectins, galactose, N-acetyl galactosamine binding lectins, N-acetylglucosamine binding lectins, N-acetyl neuraminic acid-binding lectins, and fucose binding lectins. Plant defense is the primary application of plant lectins in response to biotic and abiotic stresses [22–24]. The product of a novel rice gene ‘RAB21’, induced by abscisic acid and water stress, express a protein ‘Rab 21’ [25,26] and organ-specific expression of another rice gene ‘SALT’ in response to salt stress and drought clarifies the activation of specific genes during stress conditions [15–20].

Moreover, the regulation of the salt-responsive gene SALT from rice by hormonal and developmental signals showed the differential expression of these genes. The amino acid sequence of rice protein encoded by three genes (a gene SALT (length 145), SALT, and OsJ_01682) showed 100% similarity [16–19]. Jiang et al. examined the evolutionary history of lectins in rice, Arabidopsis, and soybean and their expression analysis from full-length cDNA, expression sequence tag (EST), microarray, and massively parallel signature sequencing (MPSS) datasets. There are 267 lectin coding genes in rice, of which 254 have been confirmed by expression profiles, which belong to two families, i.e., B-lectin (mannose-binding lectins) and leg-B (legume lectins). Of 267 genes, 91 are related to biotic stresses, 68 to abiotic stresses such as cold, drought, salinity, and 109 genes to biotic and/abiotic stresses. Lectin expression has also been found to be tissue-specific. Data analyses (MPSS database methods) have shown that after infection by the fungus (*Megnaporthe grisea*) and bacterium (*Xanthomonas oryzae*), a total of 58 and 62 lectin genes were differentially expressed, respectively, among which both of the pathogens induced regulation of 29 genes [23]. Various lectin engineering methods are available, by which specificity and stability of a known lectin scaffold could be improved, and non-lectin proteins maybe modified to acquire a sugar-binding function [27]. Hemagglutination (HA) assays are a convenient and quick way of measuring the presence and amount of lectin molecules in a given sample. Lectins of any origin, including multivalent plant lectins, can bind the sialic acid on RBC surfaces and cause blood agglutination. HA is a very convenient method to detect these entities in laboratory settings. Classically, a visual observation method is widely employed in laboratories for routine coagulation assays due to their simplicity. Visual observations in microplates can readily determine hemagglutination titer [28–37]. This study aimed to assess the presence of seed storage lectins in various rice accessions mainly originated from Pakistan without stress to know the inherent capability of rice genotypes expressing phyto-agglutinin.

2. Materials and Methods

The study was carried out in the Institute of Biotechnology and Genetic Engineering (IBGE), Agricultural University Peshawar (AUP), Khyber Pakhtunkhwa, Pakistan.

2.1. Collection of Plant Material

A total of 210 rice accessions (Table 1) were obtained from IABGR/NARC Islamabad and tested for lectins' presence using HA. Two local varieties, viz., Fakhre Malakand and Boti, and five transgenic rice seeds, making a total of 217 genotypes, were also screened for the existence of lectins for the purpose of comparison with the wild. Gum Arabic (Sigma Aldrich, St. Louis, MO, USA) was used as positive control and simple PBS as a blank. Out of 210 accessions of rice seeds provided by IABGR/NARC Islamabad, only four originated from China, 186 from Pakistan, 12 from IRRI, and the source of 8 accessions was not mentioned in the catalog. Out of 186 accessions originating in Pakistan, 172 belong to Khyber Pakhtunkhwa province, 04 from Punjab, 02 from AJK, 01 from Gilgit Baltistan, and the source provinces of 07 were unknown (Table 1).

Table 1. Rice (*Oryza sativa* L.) genotypes used, provided by IABGR/NARC Islamabad.

S. No	Accession No.	Country, Year of Release	Province/ Donor No/Area	District
1–4	7216–7219	China, 1983	C-12, C-13, C-14, C-15 respectively	NA
5	7225	Pakistan, 1983	Punjab	Sheikhupura
6	7234	//	Khyber Pakhtunkhwa	Unknown
7	7235	//	//	Malakand
8–11	7236–7239	//	//	Unknown
12–13	7241–7242	//	//	Unknown
14	7258	Pakistan, 1984	//	Parachinar
15	7260	//	//	Kurram Agency
16–18	7261–7263	//	//	Pachinar
19	7268	//	//	Parachinar
20	7269	//	//	Unknown
21	7271	//	//	Unknown
22	7280	//	//	Unknown
23	7281	Pakistan, 1987	//	Mansehra
24–29	7284–89	Pakistan, 1984	//	Chitral
30–35	7290–7295	//	//	Swat
36–39	7296–99	//	//	Mansehra
40	7396	Pakistan, 1987	//	//
41	7397	//	//	Swat
42	7411	//	Unknown	Unknown
43	7413	//	//	//
44	7414	//	//	//
45	7416	//	//	//
46–47	7418–7419	//	//	//
48	7420	//	Gilgit, Baltistan	NA
49–51	7593–7595	//	Khyber Pakhtunkhwa	Malakand
52–55	7597–7600	//	//	//
56	7601	Pakistan, 1989	//	Dir
57–64	7603–7610	//	//	//
63–84	7611–7630	//	//	Chitral
85–93	7632–7640	//	//	Swat
94	7641	//	//	Unknown
95–104	7648–7657	//	//	Mansehra
105–106	7672–7673	//	Punjab	Gujrat
107–108	7734–7735	//	AJK	NA
109–110	7737–7738	//	Khyber Pakhtunkhwa	Unknown
111–113	7753–7755	//	//	Besham
114	7756	//	//	Unknown
115	7759	//	//	Mansehra
116	7760	//	//	Unknown
117	7762	//	//	//
118	7763	//	Punjab	Gujranwala
119	7765	//	//	Bahawalnagar
120	7766	//	//	Bahawalpur

Table 1. Cont.

S. No	Accession No.	Country, Year of Release	Province/ Donor No/Area	District
121–147	7783–7809	Pakistan, 1991	Khyber Pakhtunkhwa	Chitral
148–155	7811–7818	//	//	//
156–159	7819–7822	//	//	//
160–163	7824–7827	//	//	//
164–166	7828–7830	//	//	Unknown
167–169	7831–7833	//	//	//
170	7836	//	//	//
171	7839	//	//	Unknown
172	7847	Pakistan, 1973	RGP-ARC	NA
173	7850	//	//	//
174	7859	//	//	//
175	7863	//	//	//
176–177	7864–7865	Pakistan, 1972	//	//
178	7869	Pakistan, 1973	//	//
179	7883	//	//	//
180	7886	//	//	//
181	7937	Pakistan, 1991	//	Chitral
182	8083	//	//	//
183	8085	//	//	//
184	17940	//	//	Swat
185–186	17,941–17,942	Pakistan, 2000	//	Nowshera
187–189	17,943–17,945	Pakistan, 1996	//	Chitral
190	17,946	Pakistan, 1997	//	Mansehra
191–202	17,947–17,958	IRRI, 1999	NA	NA
203	18,231	Source not mentioned in the catalog		
204	23,718	//		
205–206	23,727–23,728	//		
207–208	23,731–23,732	//		
209	23,736	//		
210	23,744			
211	Boti	Pakistan	Khyber, Pakhtunkhwa	Swat
212	Fakhre Malakand	//	//	Malakand
213–217	Five Transgenic seeds	NA	NA	NA

2.2. Extraction of Lectins from Rice

The dry, mature seeds were finely grounded in a mortar and pestle. Seed meal was defatted with two volumes of hexane twice by centrifugation, and then dried overnight at room temperature [38]. About 0.2 g of defatted seed flour was added to 1 mL of 1 X phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4), shaken for 4 h on an orbital shaker before centrifugation. The suspension was then cleared by centrifugation at 10,000 × g for 10 min at 4 °C, and the extract was stored at −20 °C [21].

2.3. Preparation of RBCs

About 2 mL of Californian breed rabbit blood was obtained in ethylene diamine tetraacetic acid (EDTA) tubes from animal husbandry of Agriculture University Peshawar Khyber Pakhtunkhwa, Pakistan. It was immediately centrifuged at 2000 rpm for 10 min. The serum was discarded, and the pellet was washed twice with 1 X PBS. Finally, the washed erythrocytes were suspended in 1 X PBS at a concentration of 5% in different flasks and used for HA [33].

2.4. HA Assay

The assay was carried out in a standard U-bottomed microplate. The accessions showing the highest agglutination were serially diluted 2-fold as Bing et al. described [34]. A 50 µL of the clarified lectin extract was mixed with half volume (25 µL) of 5% Californian breed rabbit erythrocytes and incubated for 30 min at room temperature. HA was observed with the naked eye, measured titer, representing reciprocal of highest dilution upon which

full agglutination was achieved. The specific activity of HA is defined as the titer of HA per mg of protein [21,39].

2.5. Total Protein Estimation

A standard but miniaturized photometric Bradford method was used to estimate the protein content in the seed extracts having BSA as standard using a 96 well U-bottomed microplate via reader at 595nm [40,41]. The detailed method is given in Table 2.

Table 2. Method of two-fold serial dilution, corresponding titer values, HA units/mL, and specific activity.

Dilution Number	Ratio and %Age Concentration of the Diluted Sample	Dilution Factor		HA Titer Value (HU/mL)	Specific Activity = Titre/mg of Protein
Original sample	1:1 (100%)	1/2 ⁰	1	1	e.g., Protein Concentration = 6.2 mg/mL and titre = 16 Therefore, Specific activity of the plant/animal lectins showing visible HA will be 16/6.2 = 2.58
1	1:2 (50%)	1/2 ¹	1/2	2	
2	1:4 (25%)	1/2 ²	1/4	4	
3	1:8 (12.5%)	1/2 ³	1/8	8	
4	1:16 (0.063%)	1/2 ⁴	1/16	16	
5	1:32 (0.0313%)	1/2 ⁵	1/32	32	
6	1:64 (0.01563%)	1/2 ⁶	1/64	64	
7	1:128 (0.007813%)	1/2 ⁷	1/128	128	
8	1:256 (0.0039063%)	1/2 ⁸	1/256	256	
9	1:512 (0.00195313%)	1/2 ⁹	1/512	512	
10	1:1024 (0.0009766%)	1/2 ¹⁰	1/1024	1024	
11	1:2048 (0.0004883%)	1/2 ¹¹	1/2048	2048	
12	1:4096 (0.000244141%)	1/2 ¹²	1/4096	4096	

The percentage concentration of the diluted sample is calculated as a ratio multiplied by 100. Reciprocal of highest dilution showing visible agglutination.

2.6. Hemagglutination Inhibition Assay (HIA)

Hemagglutination inhibition assay (HAI or HI) using D-Glucose was performed as described by Adenike and Ereton, (2004) [42]. Serial dilutions of the carbohydrate solution (D-Glucose; initial concentration 200mM) were incubated at room temperature with equal volume of the lectin extract for 30 min. After that 50 µL of 5% rabbit erythrocytes were added, mixed, and incubated for further 30 min.

2.7. Screening Blood Affinity for Rice Lectins

The affinity of different blood types with rice lectins was screened using various percentages (3–5%) of erythrocytes obtained from various sources, including human (blood group B+), broiler chicken, and two strains of rabbit, i.e., local and Californian breed rabbit [20,42].

3. Results

3.1. Rice Lectins Extraction

The results of lectins extraction from rice for the analysis are given in Tables 3 and 4. The procedure of lectin assay was miniaturized as a large number of samples (i.e., 217) of rice (*Oryza sativa* L.) had to be analyzed; also, the plant material available was less in quantity (5–10 seeds). Thus 96-welled microplate was used for HA.

Table 3. Hemagglutination activity (HA) with Californian breed rabbit RBCs and protein content (mg/mL) in the seeds of rice (*Oryza sativa* L.) accessions using Bradford method.

S. No	Accession No.	HA	Protein Conc. mg/mL	S. No	Accession No.	HA	Protein Conc. mg/mL	S. No	Accession No.	HA	Protein Conc. mg/mL	S. No	Accession No.	HA	Protein Conc. mg/mL
1	7216	+	5.815	54	7599	+	5.38	107	7734	+ ³	4.269	160	7822	+ ³	6.425
2	7217	+	5.18	55	7600	+	8.03	108	7735	+ ²	4.808	161	7824	+	4.608
3	7218	+	5.865	56	7601	+	6.33	109	7736	+	6.245	162	7825	+ ²	6.545
4	7219	+	5.77	57	7603	+	5.67	110	7737	+ ²	4.931	163	7826	+	6.785
5	7225	-	7.3	58	7604	+	4.994	111	7738	+	4.998	164	7827	+ ²	4.956

Table 3. Cont.

S. No	Accession No.	HA	Protein Conc. mg/mL	S. No	Accession No.	HA	Protein Conc. mg/mL	S. No	Accession No.	HA	Protein Conc. mg/mL	S. No	Accession No.	HA	Protein Conc. mg/mL
6	7234	+	4.621	59	7605	+	6.65	112	7753	+	3.056	165	7828	+ ²	4.897
7	7235	+ ³	4.996	60	7606	+	5.965	113	7754	+	4.703	166	7829	+ ²	4.672
8	7236	+	3.87	61	7607	+ ²	4.878	114	7755	+ ²	5.42	167	7830	+	5.125
9	7237	+ ²	4.731	62	7608	+ ²	5.2	115	7756	+ ³	5.285	168	7831	+ ³	5.585
10	7238	+ ²	4.344	63	7609	+	6.25	116	7759	+ ³	7.84	169	7832	+	4.28
11	7239	-	4.293	64	7610	+	6.94	117	7760	+ ³	4.770	170	7833	+	6.335
12	7241	+	4.624	65	7611	+ ³	6.115	118	7762	+ ³	5.375	171	7836	+ ³	5.685
13	7242	+ ²	4.871	66	7612	+ ³	5.965	119	7763	+ ²	4.167	172	7839	+ ²	4.354
14	7258	+ ³	5.735	67	7613	+ ²	6.575	120	7765	+	4.463	173	7847	+ ²	4.912
15	7260	+ ³	6.26	68	7614	+ ³	6.995	121	7766	+	5.155	174	7850	+	4.504
16	7261	+ ²	4.98	69	7615	+	6.665	122	7783	+	6.04	175	7859	+	5.79
17	7262	+ ³	4.326	70	7616	+ ³	6.82	123	7784	+	4.884	176	7863	+	5.045
18	7263	+	3.372	71	7617	+	5.16	124	7785	+ ³	6.845	177	7864	+	4.197
19	7268	+ ³	4.365	72	7618	+ ³	6.235	125	7786	+ ³	6.675	178	7865	+	4.398
20	7269	+ ²	4.706	73	7619	+ ²	6.465	126	7787	+	6.825	179	7869	+	4.515
21	7271	+ ³	4.326	74	7620	+ ³	5.3	127	7788	+ ²	7.13	180	7883	+	4.327
22	7280	+ ³	5.105	75	7621	+ ³	5.815	128	7789	+ ²	6.14	181	7886	+ ³	5.665
23	7281	+	5	76	7622	+ ³	6.305	129	7790	+ ³	6.815	182	7937	+	5.94
24	7284	+	6.165	77	7623	+	4.915	130	7791	+ ³	7.585	183	8083	+ ³	6.215
25	7285	+ ³	5.445	78	7624	+ ³	4.421	131	7792	+ ²	6.62	184	8085	+ ³	6.78
26	7286	+ ³	6.95	79	7625	+ ²	5.755	132	7793	+ ²	6.8	185	17940	+ ²	6.765
27	7287	+ ³	6.565	80	7626	+	6.785	133	7794	+ ²	5.76	186	17941	+	5.51
28	7288	+ ³	6.13	81	7627	+	6.98	134	7795	+ ³	6.185	187	17942	+	5.54
29	7289	+ ³	6.995	82	7628	+ ³	5.375	135	7796	+	7.28	188	17943	+	6.055
30	7290	+	5.07	83	7629	+	5.6	136	7797	+ ²	7.56	189	17944	+	4.716
31	7291	+	4.418	84	7630	+	6.855	137	7798	+ ³	7.525	190	17945	-	5.395
32	7292	+ ²	4.037	85	7632	+	6.43	138	7799	+ ³	7.51	191	17946	+ ²	6.915
33	7293	+ ²	3.761	86	7633	+	5.1	139	7800	+ ³	5.765	192	17947	+	5.375
34	7294	+	3.929	87	7634	+ ²	5.315	140	7801	+	3.545	193	17948	-	5.795
35	7295	+	4.376	88	7635	+	5.25	141	7802	+ ³	3.352	194	17949	+	5.515
36	7296	+	7.245	89	7636	+ ³	5.22	142	7803	+	4.465	195	17951	+	5.27
37	7297	+	4.712	90	7637	+ ³	4.695	143	7804	+ ³	7.29	196	17952	+	4.99
38	7298	+ ²	4.560	91	7638	+ ²	4.382	144	7805	+ ³	5.31	197	17953	+	5.755
39	7299	+	3.718	92	7639	+	7.16	145	7806	+ ³	5.505	198	17954	+	4.9185
40	7396	+ ³	7.35	93	7640	+	6.81	146	7807	+ ³	3.845	199	17955	+	6.26
41	7397	+	5.265	94	7641	+ ²	5.11	147	7808	+ ²	4.815	200	17956	+	5.875
42	7411	+ ³	6.26	95	7648	+	4.913	148	7809	+ ³	6.081	201	17957	+	5.38
43	7413	+ ³	5.1	96	7649	+	5.175	149	7811	+ ³	4.56	202	17958	-	5.59
44	7414	+ ³	5.65	97	7650	+	4.617	150	7812	+ ³	5.67	203	18231	+	4.0485
45	7416	+ ³	4.985	98	7651	+ ²	4.105	151	7813	+ ³	6.12	204	23718	+	5.68
46	7418	+ ³	5.4	99	7652	+ ²	4.565	152	7814	+ ³	4.84	205	23727	+	5.575
47	7419	+ ³	6	100	7653	+ ²	5.78	153	7815	+ ³	4.495	206	23728	+ ²	5.66
48	7420	+ ³	5.63	101	7654	+	6.785	154	7816	+ ²	4.315	207	23731	+ ²	3.577
49	7593	+	4.168	102	7655	+	5.63	155	7817	+ ³	6.31	208	23732	+ ³	3.8585
50	7594	+	7.5	103	7656	+	7.335	156	7818	+	4.39	209	23736	+ ³	6.22
51	7595	+	4.522	104	7657	+	6.23	157	7819	+ ³	7.07	210	23744	+	3.6395
52	7597	+	4.579	105	7672	+ ²	4.114	158	7820	+ ³	5.64	211	Boti	+ ²	
53	7598	+	5.29	106	7673	+	4.958	159	7821	+ ²	6.56	212	F.M	+ ²	2.79

213–217 = Five transgenic seeds of rice, where only two showed visible HA (+²) with 3.54 and 3.79 protein content.

The hemagglutination (HA) results of 217rice (*Oryza sativa* L.) genotypes; accessions (210), local varieties (02) and transgenic seeds (05) showing weak (+), high (++/+²), highest (+++/+³) and negative (-), using 5% Californian breed rabbit RBCs provided by IABGR/ NARC Islamabad. Above values of protein concentration are mg/100mg of rice flour. Protein contents were measured via microplate reader at 595 nm using Bradford reagent and the difference of (±0.4) was noted between the values of protein concentration. (... ..) represent (missing value); Boti & F.M (Fakhre Malakand) are local varieties.

Table 4. HA, protein content (mg/mL), titer values, and specific activity in the seeds of 106 rice (*Oryza sativa* L.) accessions.

S. No	Accession No.	HA	Protein Conc. mg/mL	Titre Value	Specific Activity HU/mg	S. No	Accession No.	HA	Protein Conc. mg/mL	Titer Value	Specific Activity HU/mg	S. No	Accession No.	HA	Protein Conc. mg/mL	Titre Value	Specific Activity HU/mg
1	7235	+ ³	4.996	2 ⁷ = 128	25.621	37	7619	+ ²	6.465	2 ³ = 8	1.237	73	7800	+ ³	5.765	2 ² = 4	0.693
2	7236	+ ²	3.87	2 ⁵ = 32	8.268	38	7620	+ ³	5.3	2 ⁵ = 32	6.037	74	7802	+ ³	3.352	2 ³ = 8	2.386
3	7237	+ ²	4.731	2 ⁷ = 128	27.055	39	7622	+ ³	6.305	2 ³ = 8	1.268	75	7804	+ ³	7.29	2 ³ = 8	1.097
4	7238	+ ²	4.34	2 ⁷ = 128	29.49	40	7624	+ ³	4.4205	2 ⁴ = 16	3.6195	76	7805	+ ³	5.31	2 ² = 4	0.753
5	7242	+ ²	4.871	2 ⁷ = 128	26.28	41	7625	+ ²	5.755	2 ² = 4	0.695	77	7806	+ ³	5.505	2 ³ = 8	1.453

Table 4. Cont.

S. No	Accession No.	HA	Protein Conc. mg/mL	Titre Value	Specific Activity HU/mg	S. No	Accession No.	HA	Protein Conc. mg/mL	Titre Value	Specific Activity HU/mg	S. No	Accession No.	HA	Protein Conc. mg/mL	Titre Value	Specific Activity HU/mg
6	7258	⁺ ³	5.735	2 ⁸ = 256	44.63	42	7627	+	6.98	2 ¹ = 2	0.286	78	7807	⁺ ³	3.845	2 ³ = 8	2.081
7	7260	⁺ ³	6.26	2 ² = 4	0.638	43	7628	⁺ ³	5.375	2 ⁹ = 512	95.25	79	7808	⁺ ²	4.815	2 ² = 4	0.8307
8	7261	⁺ ²	4.98	2 ² = 4	0.803	44	7634	⁺ ²	5.315	2 ² = 4	0.752	80	7809	⁺ ³	6.08	2 ¹ = 2	0.3289
9	7262	⁺ ³	2.163	2 ² = 4	1.849	45	7636	⁺ ³	5.22	2 ⁴ = 16	3.065	81	7811	⁺ ³	4.56	2 ³ = 8	1.754
10	7268	⁺ ³	4.365	2 ³ = 8	1.8328	46	7637	⁺ ³	4.695	2 ⁴ = 16	3.407	82	7812	⁺ ³	5.67	2 ³ = 8	1.411
11	7269	⁺ ²	4.706	2 ² = 4	0.8499	47	7641	⁺ ²	5.11	2 ⁴ = 16	3.131	83	7813	⁺ ³	6.12	2 ² = 4	0.654
12	7271	⁺ ³	4.326	2 ¹⁰ = 1024	236.70	48	7651	⁺ ²	4.105	2 ² = 4	0.974	84	7814	⁺ ³	4.84	2 ³ = 8	1.653
13	7280	⁺ ³	5.105	2 ³ = 8	1.5670	49	7652	⁺ ²	4.565	2 ² = 4	0.876	85	7815	⁺ ³	4.495	2 ⁵ = 32	7.119
14	7285	⁺ ³	5.445	2 ³ = 8	1.469	50	7655	+	5.63	2 ⁸ = 256	45.47	86	7816	⁺ ²	4.315	2 ⁰ = 1	0.231
15	7286	⁺ ³	6.95	2 ³ = 8	1.151	51	7672	⁺ ²	4.114	2 ² = 4	0.972	87	7819	⁺ ³	5.07	2 ³ = 8	1.1315
16	7287	⁺ ³	6.565	2 ³ = 8	1.2185	52	7735	⁺ ²	4.8085	2 ⁶ = 64	13.309	88	7820	⁺ ³	7.64	2 ⁴ = 16	2.8368
17	7288	⁺ ³	6.13	2 ³ = 8	1.3050	53	7737	⁺ ²	4.9315	2 ⁷ = 128	25.955	89	7821	⁺ ²	6.56	2 ² = 4	0.609
18	7289	⁺ ³	6.995	2 ³ = 8	1.1436	54	7755	⁺ ²	5.42	2 ⁵ = 32	5.9040	90	7822	⁺ ³	6.425	2 ³ = 8	1.245
19	7292	⁺ ²	4.0375	2 ⁷ = 128	31.702	55	7756	⁺ ³	5.285	2 ² = 4	0.7568	91	7825	⁺ ²	6.545	2 ⁴ = 16	2.444
20	7293	⁺ ²	3.761	2 ² = 4	1.0635	56	7759	⁺ ³	5.2266	2 ³ = 8	1.2374	92	7827	⁺ ²	4.956	2 ⁵ = 32	6.456
21	7298	⁺ ²	4.5605	2 ⁷ = 128	28.067	57	7760	⁺ ³	3.1803	2 ⁵ = 32	10.07	93	7828	⁺ ²	4.897	2 ⁵ = 32	6.5346
22	7396	⁺ ³	7.35	2 ² = 4	0.5442	58	7762	⁺ ³	3.5833	2 ¹ = 2	0.558	94	7829	⁺ ²	4.672	2 ⁶ = 64	13.698
23	7411	⁺ ³	6.26	2 ¹⁰ = 1024	163.57	59	7763	⁺ ³	2.7783	2 ² = 4	1.4397	95	7831	⁺ ³	5.585	2 ¹⁰ = 1024	183.34
24	7413	⁺ ³	5.1	2 ³ = 8	1.5686	60	7785	⁺ ³	4.5633	2 ⁴ = 16	3.506	96	7836	⁺ ³	5.685	2 ² = 4	0.7036
25	7414	⁺ ³	5.65	2 ³ = 8	1.4159	61	7786	⁺ ³	4.45	2 ³ = 8	1.797	97	7839	⁺ ²	4.354	2 ² = 4	0.9188
26	7416	⁺ ³	4.985	2 ³ = 8	1.6048	62	7788	⁺ ²	4.7533	2 ⁸ = 256	53.857	98	7886	⁺ ³	5.665	2 ³ = 8	1.412
27	7418	⁺ ³	5.4	2 ⁴ = 16	2.9629	63	7789	⁺ ²	4.0933	2 ⁵ = 32	7.8176	99	8083	⁺ ³	6.215	2 ³ = 8	1.287
28	7419	⁺ ³	6	2 ¹⁰ = 1024	170.66	64	7790	⁺ ³	4.5433	2 ⁶ = 64	14.086	100	8085	⁺ ³	6.78	2 ⁹ = 512	75.516
29	7607	⁺ ²	4.8785	2 ² = 4	0.8199	65	7791	⁺ ³	5.0566	2 ³ = 8	1.582	101	17940	⁺ ²	6.765	2 ² = 4	0.591
30	7608	⁺ ²	5.2	2 ² = 4	0.7692	66	7792	⁺ ²	4.4133	2 ² = 4	0.906	102	17946	⁺ ²	6.915	2 ¹ = 2	0.289
31	7611	⁺ ³	6.115	2 ³ = 8	1.3082	67	7793	⁺ ²	4.5333	2 ² = 4	0.882	103	23728	⁺ ²	5.66	2 ⁹ = 512	90.45
32	7612	⁺ ³	5.965	2 ³ = 8	1.3411	68	7794	⁺ ²	3.84	2 ² = 4	1.0416	104	23731	⁺ ²	3.577	2 ⁶ = 64	17.89
33	7613	⁺ ²	6.575	2 ³ = 8	1.2167	69	7795	⁺ ³	4.1233	2 ² = 4	0.9700	105	23732	⁺ ³	3.859	2 ⁶ = 64	16.586
34	7614	⁺ ³	6.9995	2 ⁴ = 16	2.2858	70	7797	⁺ ²	5.04	2 ⁰ = 1	0.198	106	23736	⁺ ³	6.22	2 ³ = 8	1.286
35	7616	⁺ ³	6.82	2 ³ = 8	1.1730	71	7798	⁺ ³	5.0166	2 ³ = 8	1.594	107	F.M	⁺ ²	2.79	2 ¹ = 2	0.71
36	7618	⁺ ³	6.235	2 ³ = 8	1.2830	72	7799	⁺ ³	5.0066	2 ³ = 8	1.597	108	109TS	⁺ ²	3.54,3.79	2 ¹ = 2	0.28, 0.26

The 109 rice (*Oryza sativa* L.) genotypes; accessions (106), local varieties (02), transgenic seeds (02), showing the presence of lectins (+) high (⁺²/⁺⁺) or highest (⁺³/⁺⁺⁺) (HA) results using Californian breed 5% Rabbit RBCs, were screened further for titer values and specific activity (titer/mg of protein) F.M; Fakhre Malakand, local variety. TS.; transgenic seeds. The above values of protein concentration are mg/100mg of rice flour. Protein contents were measured via a microplate reader at 595 nm using the Bradford reagent, and the difference of (± 0.4) was noted between the values of protein concentration when multiple readings were taken. Titer values are the reciprocal of the highest two-fold dilution (i.e., $1/2n = 2n$, where $n = 0, 1, 2, 3$) showing visible agglutination. Specific activity was measured by dividing titer values over the protein content (titer/mg of protein).

3.2. HA Assay

Approximately all the accessions showed the presence of lectins via HA out of 217 (210 wild, 2 cultivated and five transgenic) accessions, 109 accessions (106 wild, 1, cultivated, 2 transgenic) gave strong agglutination results (Tables 3 and 4; Figure 1a–g). The accessions (109) exhibiting high HA activity were further serially diluted for titer analysis and specific activity as shown in Table 4. The respective titer values observed for 7816 and 7271 were 4–1024, making a range of 4.315 (lowest)–1024 (highest) HA unit (HAU) and their specific activity as 0.231–236. This micro assay requires only 25 to 100 μ L of extract, whereas the conventional glass slide method requires at least 1000 μ L of plant extract mixed with the same amount of RBC suspension for a single assay [32,33].

HA is used to detect lectins in the crude or clarified extracts of plants. Purified rice seed extracts were serially diluted by a two-fold dilution method till well#10. The last wells (11 and 12) were reserved for positive (gum arabic) and blank (PBS).

Figure 1a–g shows most of the accessions' HA assay for lectins using 5 % Californian breed rabbit erythrocytes. The microplate selected for the HA assay was a U-bottomed with 196 wells distributed in 12 columns (from 1–12) and 08 rows (from A–H). Numbering rows as A, B and C etc. in the figures was for convenience to correctly insert the data in the tables below.

Usually, HA is carried out at room temperature, and an equal volume of lectin extract and RBCs suspension is used [21,32,39]. However, this ratio may vary as described in other studies (Zhang et al., 2000) [9]. In this microassay, precise agglutination results were obtained with 25 μ L of rabbit RBCs mixed with 50 μ L of rice extract [Figure 1a–g]. For

accurate and rapid results during cold weather, the microplate can be kept in an incubator instead of room temperature. A visible lattice of RBCs at the bottom of the well was taken as positive and button formation was recorded as negative. The interpretation of HA on a glass slide is different [32]. One of the rules of selection is that the agglutination should settle fast and non-agglutination should settle slowly. Strong positive, weak positive, and intermediate positive were differentiable with the naked eye. In a U-bottom microplate, strong positive samples showed a lattice or network spread throughout the bottom. Diffused button at the bottom showed partial agglutination and sharp button shows negative agglutination results [32,33]. However, these observations and interpretations can vary from lab to lab, expert to expert, type and concentration of RBCs, incubation time, nature of the sample agglutinin, and method of hemagglutination assay [31]. The lectin assay was stable for about 1–2 h, also reported by Zhang et al. [9].

	1	2	3	4	5	6	7	8	9	10	11	12
A	7737 (++)	7738 (+)	7755 (++)	7760 (++)	7756 (+++)	7762 (+++)	7763 (++)	7766 (+)	7765 (+)	18231 (+)	-VE CONTROL (-)	+VE CONTROL (+++)
B	7624 (+++)	7625 (++)	7627 (+)	7628 (+++)	7636 (+++)	7281 (+)	7831 (+++)	7833 (+)	7859 (+)	7863 (+)	-VE CONTROL (-)	+VE CONTROL (+++)
C	7235 (+++)	7241 (+)	7292 (++)	23731 (++)	23732 (+++)	23744 (++)	23728 (++)	7829 (++)	7850 (+)	7234 (++)	-VE CONTROL (-)	+VE CONTROL (+++)
D	7236 (++)	7237 (++)	7238 (++)	7239 (-)	7242 (++)	7290 (+)	7291 (+)	7294 (+)	7295 (+)	7298 (++)	-VE CONTROL (-)	+VE CONTROL (+++)
E	17940 (++)	17941 (+)	17942 (+)	17943 (+)	17944 (+)	17945 (-)	17946 (++)	17947 (+)	17948 (-)	17949 (+)	-VE CONTROL (-)	+VE CONTROL (+++)
F	17951 (+)	17952 (+)	17953 (+)	17954 (+)	17955 (+)	17956 (+)	17957 (+)	17958 (-)	17951 (+)	8083 (+++)	-VE CONTROL (-)	+VE CONTROL (+++)
G	7296 (+)	7258 (+++)	7286 (+++)	7287 (+++)	7288 (+++)	7284 (+)	7289 (+++)	7260 (++)	7285 (+++)	7397 (+)	-VE CONTROL (-)	+VE CONTROL (+++)
H	7597 (+)	7598 (+)	7595 (+)	7594 (+)	7593 (+)	7599 (+)	7601 (+)	7600 (+)	7604 (+)	7603 (+)	-VE CONTROL (-)	+VE CONTROL (+++)

(a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	7622 (+++)	7614 (+++)	7608 (++)	7617 (+)	7619 (+++)	7615 (+)	7612 (+++)	7611 (+++)	7610 (+)	7623 (+)	-VE CONTROL (-)	+VE CONTROL (+++)
B	7613 (++)	7616 (+++)	7620 (+++)	7652 (++)	7618 (+++)	7606 (+)	7651 (++)	7654 (+)	7605 (+)	7609 (+)	-VE CONTROL (-)	+VE CONTROL (+++)
C	7607 (++)											

(b)

Figure 1. Cont.

(c)

(d)

(e)

Figure 1. Cont.

Figure 1. HA capability of rice (*Oryza sativa* L.) lectins (a–g) against 5% Californian breed rabbit RBCs. The + signs are showing HA activity. The HA activities for different rice genotypes are weak (+), high (++/+2), highest (+++/+3) and negative (-), using 5% Californian breed rabbit RBCs.

3.3. Total Protein Content

Results of the protein content determination in the rice accessions are represented in Tables 3 and 4. For all (i.e., 217) of the tested genotypes, a protein concentration of 3–8 mg/100mg of seed flour was observed, the highest among which was 8.03 mg for accession 7600, while the lowest was 3.05mg for accession 7753 [40]. Sun et al. observed 5–13 g/100g of proteins in rice [41].

3.4. HAI

The rice lectins from all the genotypes were not inhibited by simple monosaccharide (D-glucose) moieties [9]. Other carbohydrates specificity, like mannose and complex polysaccharides needs to be sorted out for these rice accessions. In this study only D-glucose was used, as large number of samples had to be analyzed.

3.5. Affinity Comparison of Various Types of Blood for Rice Lectins

HA capability of the rice lectins against different types of blood RBCs was compared with each other as represented in Figure 1. The blood obtained from humans (B+), rabbits (local and Californian breed), and broiler chickens were screened for comparative agglutination results with rice lectins. Californian breed rabbit erythrocytes showed more precise results than human and broiler erythrocytes. However, we have recognized broiler

chickens as a cheap and readily available source for blood agglutination assays of plant lectins on a large scale (Figure 2). Heard (1955) identified the different blood groups in rabbits and found that some rabbits' red cells are agglutinated more strongly than the red cells of others and to a higher titer. In this study, Californian breed rabbit erythrocytes showed more affinity for rice lectins than local rabbits [33,35].

Figure 2. Comparison of rice lectins capability of HA is shown against 1% rabbit RBCs (A), 3% B⁺ human RBCs (B), 5% Californian-breed rabbits RBCs (C), 5% broiler chicken RBCs (D), and 3% local rabbit RBCs (E).

4. Discussion

Identification of stable and resistant genotypes through evaluation of proteins involved in defense is integral for varietal development and release. Plant defense is the primary application of plant lectins in response to biotic and abiotic stresses [10,11]. The study shows that seed storage lectins are present in nearly all 217 rice genotypes (Table 3). A significant highest lectin activity was observed in 109 (i.e., 106 wild) genotypes out of 217 (210 wild), which were further investigated for titer values and specific activity. Twenty-two accessions showed high titer values (ranges 64–1024) and specific activities (ranges 13–170 HU/mg).

The 106 wild accessions of rice (*Oryza sativa* L.) that exhibited highest agglutination as compared to the rest (210) showed their inherent capability of expressing higher concentration of phytoagglutinins (Table 4). One of the few parameters at molecular level that can easily be used to find the resistant genotypes to biotic and abiotic stresses [16–22]. For semi-quantification of seed lectins in rice a method described in Table 2 was used. The detail of which are given in Table 3. The same method is used for the finding of viral titer [30].

Two local varieties (Fakhre Malakand, Boti) and five transgenic rice varieties were used for the purpose of comparison with the wild relatives. We have found a considerable higher protein concentration, titer values and specific activity in wild accessions of rice as compared to the cultivated and transgenic ones, depicting greater genetic diversity of seed storage lectins [Tables 3 and 4].

HA experiments were carried out at room temperature, i.e., 25 °C. This lectin assay of rice (*Oryza sativa* L.) was miniaturized as only a limited seed sample of each accession was available, and a large number of samples (i.e., 217) had to be analyzed. Thus, 96-welled microplate was used for HA. The ratio of seed flour (g) to protein extraction buffer (mL) was kept the same as used by Bhagyawant et al. [33]. However, this ratio may vary in different studies of plant lectins [2,23].

The U-bottomed microplate was used for HA and the results were recorded through visual observation. In U-shaped wells of microplate strong positive samples appeared as a completely spread lattice while the partially agglutinated showed a diffused button

and sharp button reflected negative agglutination results. This interpretation may vary from lab to lab or according to the type of microplate (i.e., U-bottomed or V-bottomed) or simple glass slide used [32]. Visual reading of the plate also depends on the concentration of erythrocytes used. Usually, granular appearance of the button and resistance to flow was considered positive.

There is a difference between the extent of HA of different blood types with rice lectins, viz. blood of human (B+) and two rabbits (local and Californian breed) and chicken broiler as observed in the study. Californian breed rabbits' 5% red cells agglutinated with rice lectins more strongly than the red cells of the local strain and had higher titer (Figure 1a–g. Heard (1955) identified the same results with different rabbit blood groups [38]. Levine and Landsteiner [36] and Kellner and Hedel [37] encountered the same variation in the agglutinability of rabbit RBCs. The 5% red cells of Californian bred rabbit agglutinated with rice lectins more strongly than the red cells of the local strain, and to a higher titer. Human blood group B+ (3%) and 1% rabbit RBCs did not give precise agglutination results, while 5% broiler chicken RBCs and 3% local rabbit RBCs showed clear and satisfactory results (Figure 2). The titer values and specific activities observed for rice kernel lectins may increase with trypsinized rabbit RBCs. However, we have not used trypsin-treated blood for HA [2,16].

The values observed by Peumans et al. 1998 for untreated and trypsin-treated rabbit red blood cells (0.3 and 0.6 $\mu\text{g/mL}$, respectively) indicate that the treatment has little effect on HA. Trypsin-treated human erythrocytes agglutinated only at higher concentrations of Calsepa (5 $\mu\text{g/mL}$), whereas no clear agglutination was observed with untreated human red blood cells [2]. Hirano et al. [16] and Zhang et al. [20] used 1% trypsinized rabbit RBCs to detect lectins in rice extracts, while Bhagyawant et al. [33] used 3% trypsin treated rabbit erythrocytes for the detection of legume lectins and Gulzar et al. [32] used 2% human RBCs for various plants.

Hemagglutination inhibition assay (HAI) was carried for carbohydrate specificity of rice lectins using D-glucose. No apparent inhibition was noticed in the HAI. Therefore, our samples did not show specificity for glucose. Most of the stress-related lectins found in rice are mannose-specific, although glucose specific lectins have also been found in rice [17–24].

According to the analysis of Zhang et al., [20] mannose-binding rice lectins (MRL) have a specific role in plant defense, as they appear exclusively as a response to salt stress. This may also suggest the role of protein-carbohydrate interaction in plant defense against stress. In the same year 2000, Hirano and his coworkers discovered jacalin-related MRL as a mixture of iso-lectins related to salt inducible 'SALT' gene product with constitutive expression as lectin activity was detected in all parts of the plant at all the stages of growth. Furthermore, they argue that some unintentional stress factors during growth, such as water stress, may have induced the jacalin-related MBL expression in rice [16,20].

In short, lectins are a complex group of proteins with structural diversity and an affinity for several carbohydrates; they are involved in a global response, and respond to biotic and abiotic stresses.

5. Conclusions

A selected range of indigenous (192) and few exotic (25) rice accessions were investigated for lectin concentrations in this study. They exhibited high HA, reflecting a high lectin concentration in the seeds of wild rice. The findings suggest inclusion of the species in plant breeding programs for lectin alleviation in the cultivated varieties. HA variation for investigated lectins can be utilized for mapping lectin encoding genes governing biotic and abiotic stress resistance in rice as lectins are present in every part of the non-stressed rice plant. Thus, lectins as part of the seed storage proteins can be used as a passive-defense mechanism which might reflect their inherent resistance to biotic and abiotic stresses. Further experiments are needed in this connection to validate the present results in present and other cultivars of rice.

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