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PRODUCTION OF SINGLE CHAIN FRAGMENT VARIABLE (scFv) MONOCLONAL ANTIBODIES AGAINST *SCLEROTIUM ROLFSII* LECTIN (SRL) USING PHAGE DISPLAY TECHNOLOGY

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ABSTRACT
In this study we tried to produce monoclonal antibodies using Tomlinson libray from obtained from (MRC cambridge), screening was performed using *Sclerotium rolfsii* fungal pathogen, against lectin was obtained from sclerotial bodies, four rounds of biopanning were conducted using lectin as antigen, at the end of this 45 clones were randomly selected and further screened for higher binding affinity against SRL, there is wide range from lower to higher 0.072 to 1.426 spectrophotometric ELISA reading at 415nm at 60 minutes, finally there are two clones which showed high affinity to 1.426, and 1.142 spectrophotometric ELISA reading at 415nm at 60 minutes target antigen(SRL) respective clone are ScFvSRL-20 and ScFvSRL-34, these two selected clones may be utilized for detection of *Sclerotium rolfsii* fungal pathogen .

INTRODUCTION

Sclerotium rolfsii is a necrotrophic, soil borne phytopathogen, its impact can be observed on numerous agricultural and horticultural crops throughout worldwide loss about millions of dollars annually (Mulrean *et al*., 1984). Have broad host range at least 400 species in 100 families are badly affected, major crops are legumes, crucifers and cucurbits, oilseeds, sometimes peanuts, potatoes, and soybeans, yield loss can be estimated to 100% (Bowen *et al*., 1992; Cintas and Webster, 2001). *Sclerotium rolfsii* capable to produce huge amount sclerotia hardy survival structure in soil for several years (Singh *et al*., 2003). Management of this soil borne pathogen using chemical fungicides is not so very effective and difficult, as it requires large quantity of pesticides reduce its population, also leads to soil and environmental pollution and may leads to fungicide resistance in the pathogen (Singh *et al*., 2014). Botanicals and antagonists are ecofriendly and economically feasible and best way to control soil borne pathogens (Jabbar *et al*., 2014). Because of large diversity in fungal kingdom many of fungi genera's are available in nature capable to produce different sized sclerotial may cause difficulties in proper identification and detection of particular pathogen (Smith *et al*., 2014). Due to advances in recent molecular and immunological techiniques several tools for detecting pathogens such ELISA, PCR. (McCartney *et al*., 2003; Ward *et al*., 2004). These techniques have numerous advantages when compared to traditional methods in that they are easy, faster, more reproducible and not depends on the personal judgment of visual observations. To avoid crop loss early, rapid, and specific detection and identification of plant pathogens is essential for effective plant disease management. Without specific disease diagnosis, proper control measures cannot be used at the appropriate time (McCartney *et al*., 2003)

Phage display antibodies are a unique type of phage-display construct in which the displayed peptide is an antibody molecule or, different formats of antibodies structure, a domain of the antibody molecule that includes the site that binds antigen. A phage-antibody library real sense it contains a billions of clones, capable of displaying billions of antibodies with different antigen specificities obtained through screening of specific antigens by the process biopanning**.** Phage expressing a specific antibody against a given antigen is then selected and enriched by biopanning. It has broad application in field of Biomedical, Immunology, Pathology, Agriculture (Kushwaha *et al*., 2013), *etc*. phage display library was usedfor detection of numerous different antigens of pathogens such as bacteria (Morton *et al*., 2013), fungi (Hu *et al*., 2015), glycoproteins (Montserrat *et al*., 2015), oligosaccharide (Stewart *et al*., 2012), lipoproteins (Negi *et al*., 2016), toxins (Wang *et al*., 2012), allergens (Cruz *et al*., 2015), inhibitors (Zou *et al*., 2016), enzyme (Hekim *et al*., 2006), Biomarker (Abou *et al*., 2016), biological threat agents (Petrenkoa *et al*., 2005) and plant lectin (Agdour *et al*., 2007). Based on these information it has been planned to produce antibodies to detect soil borne pathogen *Sclerotium rolfsii* using Tomlinson libraries from the Medical Research Council (MRC), Winter's laboratory, Cambridge, England.

MATERIALS AND METHODS

Phage display library and strains

The Tomlinson (I+J) human synthetic naive phage display single chain antibody fragment libraries (in phagemid/scFv format- which is fused to the pIIIcoat protein of M13 bacteriophage), helper phage KM1307, E. coli strains TG1, were obtained from The Medical Research Council (MRC), Winter's laboratory, Cambridge,

England (Lee et al. 2007). The size of the library I is 1.47×10^8phagemid clones in E.coli TG1 cells, and has a high proportion of functional antibody fragments with approximately 96% of clones containing inserts. The library J size contains1.37×10^8 with approximately 88% of clones containing inserts.

Selection of monoclonal antibodies against SRL

The libraries stock I and J and stock helper phage KM1307 were expanded in order to obtain enough quantities for future use of several rounds of selections. Purification of phage populationwas performed using the Poly Ethylene Glycol (PEG) precipitation method (Lee *et al*., 2007; Marks and Hoogenboom, 1991). The selection procedure was described previously in detail (Harrison *et al*., 1996; Chames *et al.,* 2002), with some modifications.The phages obtained from the 4th round of biopanning bound with much higher affinity than those from 1st, 2nd and 3rdrounds of biopanning. Therefore, these phages were used for production of MAbs by the procedure given below. Five ml 2XTY-ampicillin (2μg/ml) medium was inoculated with *E. coli* TG1 cells and incubated with shaking at 37ºC until OD_{600} reached 0.6. The cells were infected with phages obtained from 4thbiopanning in the ratio of 1:20 and incubated without shaking in a 37ºC water bath for 30 min. Infected cells were spun at 3,300g for 10 min at 4ºC to pellet the cells. The cells were resuspended in 0.5 ml of 2XTY and plated on TYE plates containing ampicillin (2μg/ml) and 1 per cent glucose. Plates were incubated overnight 37ºC. Single bacterial colonies were picked from the plate and each of them inoculated into 3 ml of the above medium and shaken at 37ºC until OD_{600} was 0.6. One ml of this culture was infected with M13K07 helper phage and incubated without shaking in 37ºC water bath for 30min. Infected cells were spun at 3,300 g per 10 min and gently resuspended in 100 μl 2XTY. Each such culture was further inoculated into 10 ml of 2XTY containing kanamycin 25 μg/ml and ampicillin 2 μg/ml. Tubes containing different colonies were incubated over night at 37ºC.

Screening of SRL using monoclonal ELISA

In this experiment, phages obtained from different clones were used and the best ones were identified as follows. The ELISAs were performed essentially as described (Lee *et al*., 2007) with few modification. Each ELISA plate well was coated with 60 μg of SRL (antigen) in 200 μl of carbonate buffer along with appropriate controls the incubated for overnight at room temperature. The wells were rinsed thrice with 1 x PBS and excess buffer was removed by flipping over the ELISA plate. All the wells were blocked with 200 μl of 3 per cent BSA in 1x PBS for 90 min at room temperature. Then wells were then rinsed thrice with 1x PBS. To each well 200 μl of $1x10^{12}$ScFv's phage raised against SRL obtained from different clones stored in 3 per cent BSA-1x PBS were added. Plate was incubated for 90 min at room temperature. The wells were washed thrice with 1xPBS-0.05 per cent Tween-20 and then three times with 1x PBS. Anti M13-HRP conjugate was diluted 1:5000 times in 3 per cent BSA-1x PBS and 200 μl was added to each well. ELISA plate was incubated for 90 min at room temperature. Wells were washed thrice with 1x PBS. Then substrate (200 μl) was added to each well and incubated in dark for 30 min and A_{415} was measured.

RESULTS AND DISCUSSION

Generation of monoclonal antibodies against SRL

After 4 rounds of panning, 50-90percent bound specifically to antigen. Lectin (antigen) from *S. rolfsii,* after phage rescue, the expression of soluble V_H fragments was induced from 45 (each for SRL) randomly picked bacterial colonies and these were screened by ELISA (Table 1). there is wide smaller to higher range were found in spectrometric readings at 415nm at 60 min. 45 clones shows both low affinity and high affinity clones but most random selected clone comes under low and medium range affinity finally we selected two clones. The specificity of the isolated ScFv phages was shown in the monoclonal ScFv phage ELISA for SRL depicted. The result obtained from monoclonal ELISA show that the absorbance values were greater than the control values (Note: the control values have been subtracted from values obtained for each test monoclonal ScFv phages). It evident that the ELISA values varied from one ScFv to another but only few ScFv that showed more binding affinity to the antigen even though same concentration of phages (*i.e.* $1E+10^{12}$ phage/well) was used for ELISA. Hence, the monoclonal ScFv phages had the value above (1 Standard deviation + Mean) *i.e.* 1.093 SRL was used for further experiment. Right monoclonal antibody selected at the end of fourth biopanning clone ScFvSRL-20 and ScFvSRL-34(Fig.1). Laura *et al*. (2009) reported that the lectin from *Helix pomatia* (HPA) binds O-linked *á*-GalNAc but also recognizes Gal (*â*1–3) GalNAc and *á*-GlcNAc interactions of HPA A 12-mer phage display library was screened with a GalNAc-specific lectin to identify an amino acid sequence that binds to the lectin. Phage particles that were eluted from the lectin with free GalNAc which considered to a GalNAc-binding site. Peptides were synthesized with the selected sequence as a quadravalent structure to facilitate receptor cross linking. Quadravalent peptide has biological activity with a degree of specificity which effects occurred at concentrations in the nanomolar range in contrast to free sugars that generally bind to proteins in the micro to milimolar range. Similar reports obtained from many researcher (Wang *et al*., 2012) used Tomlinson I and J libraries screened against Cry1C, four rounds of biopanning performed, Approximately 10^{11} phage particles used at each round of biopanning, finally 192 phage clones randomly selected for monoclonal ELISA, 24 clones were showed positive binding for Cry1C, among them three clones showing higher binding ability to antigen above 0.9 absorbance in ELISA assay. (Cruz *et al.,* 2016) performed three round of biopanning were performed against almond protein using phage display libraries, finally monoclonal ELISA assay results reveals that, 32 clones which show positive binding to

Table 1: Spectrometric quantification of monoclonal phages and ELISA

ScFv phages	Absorbance 269 nm	320 nm	No. of phages/ μl	Phage volume required in μl	Volume required for2replications	Volume of PBSto makeup to400 μl	Readings after 60 min at 415 nm.
1	0.012	0.007	2.54E+10	39	78	322	0.132
2	0.015	0.006	2.96E+10	34	68	332	0.271
3	0.003	0.011	1.69E+10	59	118	272	0.162
4	0.098	0.040	7.91E+10	13	26	374	0.306
5	0.076	0.012	8.75E+10	11	22	378	0.251
6	0.001	0.015	2.12E+10	47	94	306	0.192
7	0.002	0.013	2.12E+10	47	94	306	0.968
8	0.009	0.015	3.39E+10	39	78	322	0.954
9	0.004	0.012	2.45E+10	37	74	326	0.194
10	0.066	0.012	7.62E+10	13	26	374	0.562
11	0.01	0.011	2.96E+10	34	68	332	0.431
12	0.021	0.005	3.80E+10	26	52	348	0.159
13	0.147	0.038	1.52E+10	7	14	386	0.072
14	0.044	0.007	5.21E+10	19	38	362	0.321
15	0.023	0.001	3.12E+10	32	64	336	0.406
16	0.005	0.014	2.81E+10	35	70	330	0.094
17	0.008	0.016	3.37E+10	30	60	340	0.101
18	0.016	0.012	3.65E+10	27	54	346	0.603
19	0.04	0.006	6.32E+10	16	32	368	0.735
20	0.012	0.017	4.62E+10	23	46	356	1.426
21	0.003	0.014	2.68E+10	37	74	326	0.826
22	0.03	-0.005	4.94E+10	20	40	360	0.762
23	0.008	-0.003	2.95E+10	34	68	332	0.521
24	0.035	0.007	3.82E+10	26	52	348	0.432
25	0.125	-0.04	2.32E+10	4	8	392	1.092
26	0.013	-0.011	3.29E+10	30	60	340	0.364
27	0.001	-0.015	2.45E+10	42	84	316	0.244
28	0.007	-0.012	2.83E+10	35	70	330	0.364
29	0.021	-0.06	1.75E+10	9	18	382	0.448
30	0.005	-0.12	2.86E+10	37	74	376	0.786
31	0.011	-0.11	3.26E+10	30	60	340	0.102
32	0.014	-0.004	3.73E+10	37	74	376	0.192
33	0.004	-0.018	3.24E+10	47	94	306	0.249
34	0.005	-0.007	2.21E+10	29	58	342	1.142
35	0.026	-0.003	3.49E+10	42	84	316	0.264
36	0.003	-0.013	2.19E+10	12	24	376	0.342
37	0.021	-0.009	1.02E+10	15	30	370	0.562
38	0.082	-0.022	6.25E+10	31	62	338	0.786
39	0.061	-0.016	3.14E+10	8	16	384	0.512
40	0.001	-0.013	1.89E+10	35	70	330	0.461
41	0.009	-0.006	2.86E+10	18	36	364	0.321
42	0.039	-0.001	5.32E+10	26	52	348	0.461
43	0.014	-0.014	3.92E+10	33	66	334	0.392
44	0.003	-0.017	3.12E+10	19	38	362	0.623
45	0.062	-0.024	3.63E+10	23	46	354	0.738

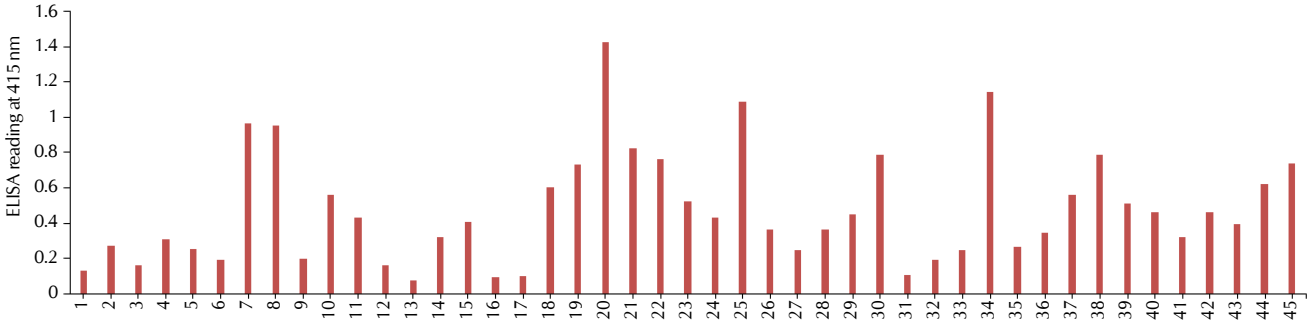

Figure 1: Spectrometric quantification of monoclonal phages and ELISA reading for lectin

almond extract, these clones selected were further used for analysis. (Zou *et al* ., 2016) in his studies used phage display library of 12-mer peptides used to isolate monoclonal antibodies penicillinase, after three round of biopanning screening, finally 16 clones was selected as monoclonal antibodies. (Abou *et al*., 2016) also used The Tomlinson I+J human single fold synthetic naïve phage displaysingle chain antibody fragment libraries, performed separate screening from each I and J library screened against L-Carnitine which is a biomarker. The absorbance difference for the library I-selected phage for L-Carnitinewas 0.06–0.07 and for Library J-selected phage was 0.07–0.08 comparing to Ubiquitine absorbance which was 0.003 for both library I and J, respectively.

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