

***In silico* identification of agriculturally important molecule(s) for defense induction against bacterial blight disease in Soybean (*Glycine max*)**

Saril Mamgain, Shalini Dhiman, Rajesh Kumar Pathak, Mamta Baunthiyal*

Department of Biotechnology, Govind Ballabh Pant Institute of Engineering & Technology, Pauri Garhwal-246194, Uttarakhand, India

*Corresponding author: mamtabaunthiyal@yahoo.co.in

Abstract

The productivity of *Glycine max* (Soybean), one of the economically important crops of India, is seriously affected by bacterial blight disease which is mainly caused by *Pseudomonas syringae*. The disease results in significant yield losses in Soybean crops. Since no proven resistant source is available against bacterial blight, the only option remaining is to utilize biotechnological strategies which could lead to inhibition of pathogenic proteins of the *Pseudomonas syringae* responsible for disease progression. Phytoalexins are well known to inhibit bacterial growth and trigger defense response against diseases in crop plants. The present study was conducted to identify the molecules which could inhibit the growth and development of bacteria. A few proteins were selected from literature analysis viz., Ornithine carbamoyl transferase 2, phaseolotoxin-insensitive, avirulence protein AvrRpt2, HarpinHrpZ, Sensor protein GacS, and Translation initiation factor IF-3 of *Pseudomonas syringae* as possible molecular target of phytoalexins. The molecular modeling of these proteins were done by using their amino acid sequence on Phyre2 and I-TASSER tool followed by model validation through energy minimization and Ramachandran plot analysis. Subsequently molecular docking was performed using some selected phytoalexins produced by members of *Brassicaceae*, *Fabaceae*, *Solanaceae*, *Vitaceae* and *Poaceae* family with each modeled protein structure by AutoDock vina. Based on the molecular docking study, we identified efficient defense molecules, which can be used for the development of agrochemicals for protection of *G. max* against infection of *Pseudomonas syringae*.

Key words: *Glycine max*; *Pseudomonas syringae*; Bacterial blight; Molecular docking; Agriculturally important molecule.

Abbreviations: *G. Max*_ *Glycine max*; *P. syringae*_ *Pseudomonas syringae*; BLAST_Basic local alignment search tool; PDB_Protein data bank; SPDB_Swiss PDB Viewer; ProSA_Protein Structure Analysis; ProQ_Protein model quality prediction.

Introduction

Soybean (*Glycine max*) is one of the most important crops in the World. It is a source of dietary proteins and oil. Soybeans are beneficial for health since it contains α -Linolenic acid, Iso-flavones, Lecithins, Lectins and Linoleic acid *etc* (Ahuja et al., 2012). The crop is greatly challenged by bacterial pathogen, *Pseudomonas syringae* that is a gram-negative rod-shaped bacterium, which infects a variety of plant species. In Soybean it causes bacterial blight disease on leaves as well as on stems, petioles, and pods (Park, 1986; Block et al., 2010). It is an early season disease, which appears in winters in the field on Soybean. Primarily the disease appears when wind or splashing water droplets spread *Pseudomonas* cells from crop plant on the soil surface to leaves. The *Pseudomonas* enters the Soybean leaves through stomata subsequently producing a toxin that is responsible for breakdown of Chlorophyll (Chen et al., 2000). From literature studies, some proteins were identified for their role in pathogenesis viz., Ornithine carbamoyl transferase 2, phaseolotoxin-insensitive avirulence protein AvrRpt2, HarpinHrpZ, Sensor protein GacS, and Translation initiation factor IF-3 (Hrabak et al.,

1992; He et al., 1993; Dixit et al., 2011; Echeverri et al., 2012; González et al., 2012; Jeandet, 2015). Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive plays a vital role in the continued existence and pathogenicity of *P. syringae*; Cysteine protease avirulence protein AvrRpt2 is required for the degradation of plant cell RIN4 and consequent activation of RPS2 during bacterial infection by thiol protease activity. The activation of RPS2 is enough for the initiation of hypersensitive response (HR) and resistance in plants. The cleavage of RIN4 by AvrRpt2 also interferes with RPM1-mediated resistance activated by either AvrRpm1 or AvrB. It inhibits PAMP (pathogen-associated molecular patterns) and blocks the plant callose deposition. The molecular mechanism of virulence is not known as yet, but this activity is independent of ethylene and salicylic acid response pathways as well as independent of RIN4 disappearance. Sensor protein GacS may be involved in lesion production, swarming and in the formation of extracellular protease, syringomycin as well as N-acyl-L-homoserine lactone (acyl-HSL) and is required for pathogenicity on bean plants; Harpin HrpZ are proteins

capable to elicit hypersensitive response (HR) in non-host plants and are required for pathogenesis in host plants. HrpZ forms types of ion-conducting pores permeable for cations. Such pore-forming activity may allow nutrient release and are responsible for delivery of virulence factors during bacterial colonization of host plants.

In comparison with animal immune systems, plants lacks adaptive immune systems, they produces several short-lived molecules (generally within 72-96 hours) to protect themselves from the attack of abiotic and biotic stresses (Jeandet et al., 2014). These molecules have been known as phytoalexins, but as the intensity of these stresses increases, the defense responses rapidly decrease in time dependant manner (Kitten and Willis, 1996; Kelley et al., 2015; Pathak et al., 2013). Studies have demonstrated that the exogenous application of phytoalexins is able to induce defense in a concentration-dependent fashion (Mukherjee et al., 2011; Lamberth et al., 2013; Jeandet et al., 2014; Kumar et al., 2015). Computational approaches may facilitate understanding of the interactions of phytoalexins with selected target pathogenic proteins. Such knowledge may allow designing and development of novel agrochemicals against bacterial blight disease caused by *Pseudomonas syringae*. It will also allow better understanding of the plant-pathogen interaction at the molecular level (Kumar et al., 2015; Pathak et al., 2017a).

Results and Discussion

Primary sequence analysis and prediction of secondary structure

The amino acid sequence of the target proteins, viz., Ornithine carbamoyl transferase 2 phaseolotoxin-insensitive, avirulence protein AvrRpt2, HarpinHrpZ, Sensor protein GacS, Translation initiation factor IF-3 from *Pseudomonas syringae*, which are probable molecular target against BB disease of *Glycine max*, were downloaded from Uniprot database (<http://www.uniprot.org/>) (Table 1). These sequences were subjected to BLASTp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) program for identification of suitable template structure and method that is used for modeling of its three dimensional structure. The SOPMA online tools were employed for identification of amino acid residues involved in the formation of protein secondary structure i.e., helix, sheet, coil and turn, this information can be also utilized for the modeling of the protein structure (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

3D structure modeling, model quality assessment and validation

Advances in computational biology techniques to predict protein structure and function reduces the experimental cost and time. On average, 50–70% of a genome can be structurally modeled using computational techniques (Whalen et al., 1991). Widely used method for protein structure prediction involved comparing a sequence of interest with available database sequences, to build an evolutionary or statistical profile of that sequence and then

scans this profile against a database profiles for known structures. This results in an alignment between two sequences, one of unknown structure and one of known structure at sequence level. On the basis of such comparison, a model of sequence using the information of known structure(s) sequence is built. When the similarity of sequence between the protein of interest and the protein(s) structure available in database is low, then detection of the relationship and the subsequent alignment may be enhanced if structural information is included to augment the analysis of sequence.

In the present study, Pyre2 and I-TASSER were employed to model 3D structure of target protein sequences (Yang et al., 2015). SPDB Viewer was used for stabilizing its stereochemical properties through energy minimization. The stability of the target protein model was validated by RAMPAGE server. The Ramachandran plot statistics of target protein models in range of 96.7 to 77.3% (favored regions), 2.2 to 15.9% (allowed regions) and 0.8 to 6.6 (outer regions) are shown in Table 2. The results of the RAMPAGE analysis revealed that the relatively low percentage of residues have phi/psi angles in the outer regions suggesting the acceptability of Ramachandran plots for target proteins. The stereochemical quality of the predicted models found to be satisfactory were taken for molecular docking studies (Pathak et al., 2013; 2016).

Ligand designing and molecular docking

The structure of selected phytoalexins was constructed using ChemSketch <http://www.acdlabs.com/resources/freeware/chemsketch/>. Each phytoalexins structure and target protein structures were imported to AutoDock tool for preparation of ligand.pdbqt and protein.pdbqt file. PDBQT is a type of file format that stores atomic coordinates, partial charges and all other information of macromolecules required for molecular docking in AutoDock vina. The each target protein model was entirely covered under the grid box and made configuration file for molecular docking with each phytoalexins. For each compound, out of the many docking poses, only those having the highest docking score and relatively good hydrogen bond interaction were selected for further analysis. Arachidin2 were docked with Ornithine carbamoyltransferase2, phaseolotoxin-insensitive with binding free energy -6.4 kcal/mol; Cysteine protease avirulence protein AvrRpt2 with binding free energy -8.1 Kcal/mol; Sensor protein GacS with binding free energy -7.5 Kcal/mol; Harpin HrpZ with binding free energy -8.5 Kcal/mol and Translation initiation factor IF-3 with binding free energy -6.8 Kcal/mol respectively. Arachidine 3 were docked with all proteins with binding free energy -6.8, -8.0, -7.7, -8.4 and -6.7 Kcal/mol; Arachidin 1 were docked with all proteins with free binding energy -7.3, -8.2, -7.5, -8.5 and -6.8 Kcal/mol; Avenanthramide were docked with all proteins with binding free energy -6.7, -8.0, -7.3, -8.5 and -7.5 Kcal/mol; Brassilexin were docked with all proteins with binding free energy -5.2, -6.8, -5.5, -6.6 and -6.9 Kcal/mol; Camalexin were docked with all proteins with binding free energy -5.5, -6.8, -5.7, -6.7, -6.9 Kcal/mol; Capsidiol were docked with all proteins with binding free energy -5.5, -6.3, -5.6, -6.7, -5.5 Kcal/mol; E_viniferin were docked with all proteins with binding free energy -8.1, -8.9,

Table 1. Detail information regarding pathogenic protein sequences of *P. syringae*.

S.N.	Name of protein	Length	UniProtKB ID
1	Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive	327	P68746
2	Cysteine protease avirulence protein AvrRpt2	255	Q6LAD6
3	Sensor protein GacS	907	P48027
4	Harpin HrpZ	341	P35674
5	Translation initiation factor IF-3	183	P0A133

Table 2. Ramachandran plot statistics of top *P. syringae* proteins model.

Protein name	Favored region (%)	Allowed region (%)	Outlier region (%)
Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive	92.5	5.7	1.6
Cysteine protease avirulence protein AvrRpt2	92.5	3.7	3.7
Sensor protein GacS	95.9	3.3	0.8
Harpin HrpZ	77.3	15.9	6.8
Translation initiation factor IF-3	96.7	2.2	1.1

Table 3. Molecular docking studies of selected phytoalexins with pathogenic proteins: illustrates minimum binding free energy (Kcal/mol).

Receptors	Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive	Cysteine protease avirulence protein AvrRpt2	Sensor protein GacS	Harpin HrpZ	Translation initiation factor IF-3
Ligands					
Arachidin2	-6.4	-8.1	-7.5	-8.5	-6.8
Arachidine3	-6.8	-8.0	-7.7	-8.4	-6.7
Arachidin1	-7.3	-8.2	-7.5	-8.5	-6.8
Avenanthramide	-6.7	-8.0	-7.3	-8.5	-7.5
Brassilexin	-5.2	-6.8	-5.5	-6.6	-6.9
Camelexin	-5.5	-6.8	-5.7	-6.7	-6.9
Capsidiol	-5.5	-6.3	-5.6	-6.7	-5.5
ϵ -viniferin	-8.1	-8.9	-8.2	-9.7	-5.9
Glyceollin I	-8.1	-7.7	-8.3	-9.3	-6.3
Glyceollin II	-7.6	-8.5	-7.7	-9.1	-6.5
Kauralexin A1	-7.3	-7.2	-7.8	-8.5	-5.5
KauralexinB1	-7.2	-7.2	-7.2	-8.6	-5.6
Luteolin	-7.5	-8.1	-7.5	-8.8	-7.2
Medicarpin	-6.8	-7.8	-7.0	-8.4	-7.1
Monilactone A	-8.5	-8.0	-6.9	-8.1	-5.5
Phytocassane A	-7.2	-7.5	-7.3	-8.9	-6.2
Pistin	-7.5	-8.2	-7.4	-8.2	-5.7
Resveratrol	-6.2	-7.4	-6.9	-7.6	-6.4
Rutalexin	-6.1	-7.1	-6.4	-7.5	-5.8
Sakuranetin	-6.9	-7.9	-6.9	-8.3	-7.0
Scopoletin	-6.0	-6.7	-6.2	-6.8	-6.7
Spirobrassin	-5.6	-6.3	-6.2	-7.3	-5.5
Wighteone	-7.0	-9.0	-7.3	-9.1	-7.5
Zealexin A1	-6.2	-7.7	-6.6	-7.7	-7.2
Zealexin B1	-6.1	-7.9	-6.9	-7.6	-7.9

Table 4. Amino acids residues of pathogenic proteins involved in interactions with top identified phytoalexins viz., E_viniferin, Glyceollin I and Monilactone A, minimum binding free energy corresponding to each phytoalexins are given.

S.N.	Proteins	E_viniferin		Glyceollin I		Monilactone A	
		Binding free energy (Kcal/mol)	Amino acid residues involved in protein-ligand interaction	Binding free energy (Kcal/mol)	Amino acid residues involved in protein-ligand interaction	Binding free energy (Kcal/mol)	Amino acid residues involved in protein-ligand interaction
1	Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive	-8.1	Lys56, Arg109, Gln139, Asn168, Arg313,	-8.1	Thr61, Arg313	-8.5	Ser58, Arg60, Thr61, His136
2	Cysteine protease avirulence protein AvrRpt2	-8.9	Val173, Asp177, Ser206	-7.7	No H bond interactions	-8.0	Ser133
3	Sensor protein GacS	-8.2	Gly295, His298, Gln301	-8.3	Ile360	-6.9	Lys275
4	Harpin HrpZ	-9.7	Ser122, Asp125, Asn337	-9.3	Ser240, Thr305, Ala304, Asp308	-8.1	Leu177
5	Translation initiation factor IF-3	-5.9	Glu104, Gln108, Asp150, Asp151	-6.3	Asp151	-5.5	Gln108

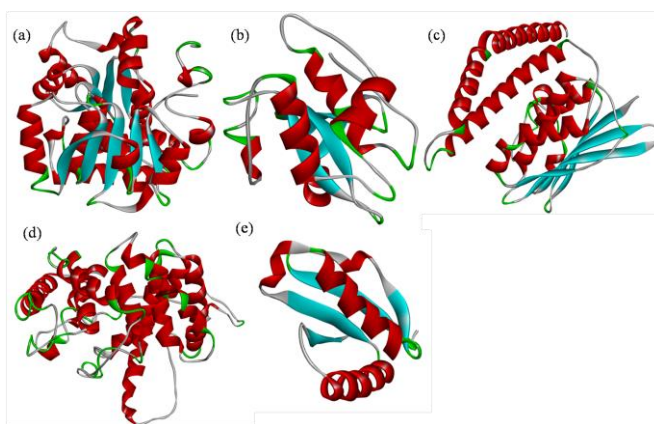


Fig 1. 3D Structure of pathogenic proteins of *Pseudomonas syringae* (a) Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive, (b) Cysteine protease avirulence protein AvrRpt2, (c) Sensor protein GacS, (d) Harpin HrpZ, and (e) Translation initiation factor IF-3 modeled through computational methods using amino acid sequence.

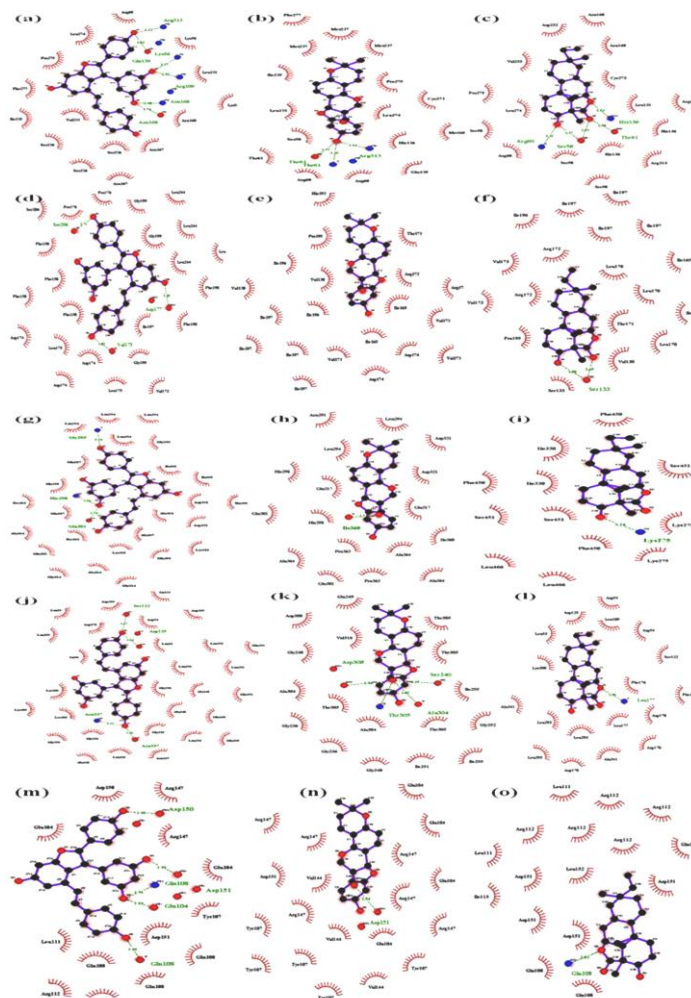


Fig 2. 2D interaction diagram of the docked structures of top identified ligands with pathogenic proteins generated by Ligplot (a) Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive docked with E_viniferin; (b) Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive docked with Glyceollin I; (c) Ornithine carbamoyltransferase 2, phaseolotoxin-insensitive docked with Monilactone A; (d) Cysteine protease avirulence protein AvrRpt2 docked with E_viniferin; (e) Cysteine protease avirulence protein AvrRpt2 docked with Glyceollin I; (f) Cysteine protease avirulence protein AvrRpt2 docked with Monilactone A; (g) Sensor protein GacS docked with E_viniferin; (h) Sensor protein GacS docked with Glyceollin I; (i) Sensor protein GacS docked with Monilactone A; (j) Harpin HrpZ docked with E_viniferin; (k) Harpin HrpZ docked with Glyceollin I; (l) Harpin HrpZ docked with Monilactone A; (m) Translation initiation factor IF-3 docked with E_viniferin; (n) Translation initiation factor IF-3 docked with Glyceollin I; (o) Translation initiation factor IF-3 docked with Monilactone A; showing H-bond_(green) and Hydrophobic_(red) interactions.

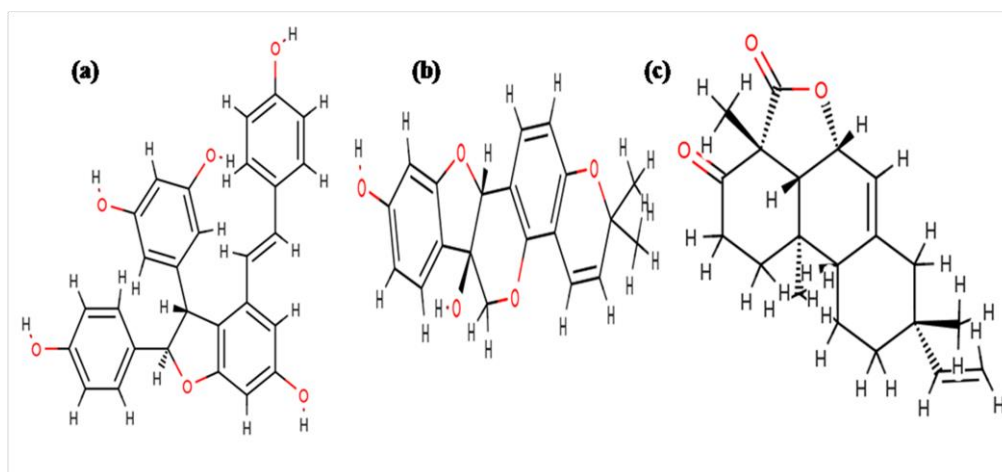


Fig 3. Structure of (a) ϵ -viniferin (b) Glyceollin I (c) Monilactone A: Agriculturally important molecules identified through molecular docking studies.

-8.2, -9.7 and -5.9 Kcal/mol; Glyceollin I were docked with all proteins with binding free energy -8.1, -7.7, -8.3, -9.3, -6.3 Kcal/mol; Glyceollin II were docked with all proteins with binding free energy -7.6, -8.5, -7.7, -9.1 and -6.5 Kcal/mol; Kauralexin A1 were docked with all proteins with binding free energy -7.3, -7.2, -7.8, -8.5 and -5.5 Kcal/mol; Kauralexin B1 were docked with all proteins with binding free energy -7.2, -7.2, -7.2, -8.6 and -5.6 Kcal/mol; Luteolin were docked with all proteins with binding free energy -7.5, -8.1, -7.5, -8.8 and -7.2 Kcal/mol; Medicarpin were docked with all proteins with binding free energy -6.8, -7.8, -7.0, -8.4 and -7.1 Kcal/mol; Monilactone A were docked with all proteins with binding free energy -8.5, -8.0, -6.9, -8.1 and -5.5 Kcal/mol; Phytocassane A were docked with all proteins with binding free energy -7.2, -7.5, -7.3, -8.9 and -6.2 Kcal/mol; Pistin were docked with all proteins with binding free energy -7.5, -8.2, -7.4, -8.2 and -5.7 Kcal/mol; Resveratrol were docked with all proteins with binding free energy -6.2, -7.4, -6.9, -7.6 and 6.4 Kcal/mol; Rutalexin were docked with all proteins with binding free energy -6.1, -7.1, -6.4, -7.5 and -5.8 Kcal/mol; Sakuranetin were docked with all proteins with binding free energy -6.9, -7.9, -6.9, -8.3 and -7.0 Kcal/mol; Scopoletin were docked with all proteins with binding free energy -6.0, -6.7, -6.2, -6.8 and -6.7 Kcal/mol; Spirobrassinin were docked with all proteins with binding free energy -5.6, -6.3, -6.2, -7.3 and -5.5 Kcal/mol; Wighteone were docked with all proteins with binding free energy -7.0, -9.0, -7.3, -9.1 and -7.5 Kcal/mol; Zealexin A1 were docked with all proteins with binding free energy -6.2, -7.7, -6.6, -7.7 and -7.2 Kcal/mol; Zealexin B1 were docked with all proteins with binding free energy -6.1, -7.9, -6.9, -7.6 and -7.9 Kcal/mol (Fig. 2; Table 3).

The world's population is increasing rapidly and could reach to 9 billion people in year 2050. Agriculture is a major driver for securing food and nutritional security but crop plants are challenged with several pathogenic microorganisms that directly attack on crops, reducing agricultural productivity. Bioinformatics has immense potential to identify novel agrochemicals that can protect crop plants and thus increase agricultural production. Bioinformatics based discovery and designing is relatively new in agrochemical industries. The development of scytalone dehydratase inhibitors for rice blast as fungicides is one of the most detailed examples (Walter, 2002). Bioinformatics-based identification of agriculturally important molecules may reduce use of agrochemicals (Wiederstein et al., 2007; Pathak et al., 2017b).

Identification of Agriculturally important lead molecule(s)

A response to several biotic and abiotic stresses phytoalexins E_{viniferin} is produced by the members of Vitaceae family; Glyceollin I is produced by the members of Fabaceae family and Monilactone A is produced by members of Poaceae family (Ahuja et al., 2012), These phytoalexins demonstrated greatest affinity towards pathogenic proteins of *Pseudomonas syringae* as compared to other phytoalexins selected in present study during docking simulation. E_{viniferin} showed H-bond interactions with Ornithine Carbamoyltransferase 2, phaseolotoxin-insensitive amino acid residue Lys56, Arg109, Gln139, Asn168 and Arg313 with binding free energy -8.1 Kcal/mol; Cystein protease avirulence protein AvrRpt2 amino acid residue Val173,

Asp177, Ser206 with binding free energy -8.9 Kcal/mol; Sensor protein GacS amino acid residue Gly295, His298 and Gln301 binding free energy -8.2 Kcal/mol; Harpin HrpZ amino acid residue Ser122, Asp125, and Asn337 with binding free energy -9.7 Kcal/mol and Translation initiation factor IF-3 amino acid residue, Glu104, Gln108, Asp150 and Asp151 with binding free energy -5.9 Kcal/mol. Glyceollin I showed H-bond interaction with Ornithine carbamoyl transferase 2, phaseolotoxin insensitive amino acid residue Thr61, Arg313 with binding free energy -8.1 Kcal/mol; Cysteine protease avirulence protein AvrRpt2 with binding free energy -7.7 Kcal/mol, however no hydrogen bond interaction were detected during Ligplot analysis; Sensor protein GacS amino acid residue Ile360 with binding free energy -8.3 Kcal/mol; Harpin HrpZ amino acid residues Ser240, Thr305, Ala304, Asp308 with binding free energy -9.3 Kcal/mol and Translation initiation factor IF-3 amino acid residue Asp151 with binding free energy -6.3 Kcal/mol. Monilactone A showed H-bond interaction with Ornithine carbamoyltransferase 2, phaseolotoxin insensitive amino acid residue Ser58, Arg60, Thr61 and His136 with binding free energy -8.5 Kcal/mol; Cysteine protease avirulence protein AvrRpt2 amino acid residue Ser133 with binding free energy -8.0 Kcal/mol; Sensor protein GacS amino acid residue Lys275 with binding free energy -8.2 Kcal/mol; Harpin HrpZ amino acid residue Leu177 with binding free energy -9.7 Kcal/mol and Translation initiation factor IF-3 amino acid residue Gln108 with binding free energy -5.5 Kcal/mol (Fig. 3; Table 4). The results of present study suggest that the phytoalexin ϵ -viniferin, Glyceollin I and Monilactone A may decrease the effect of pathogen on Soybean crop against Bacterial Blight disease.

Materials and Methods

Retrieval of target sequences

The amino acid sequence of the target pathogenic proteins of *P. syringae* viz., Ornithin Carbomoyltransferase 2, phaseolotoxin-insensitive; Cysteine protease avirulence protein AvrRpt2; Sensor protein GacS; Harpin HrpZ; and Translation initiation factor IF-3 were retrieved from UniProt database (<http://www.uniprot.org/>) (Table 1).

Secondary structure prediction

Amino acid sequence of target proteins were subjected to SOPMA server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) for identification of amino acid residue involved in the formation of secondary structure i.e. Helix, Sheet, Turn and Coil.

3D structure prediction and visualization

Target protein sequence of *P. syringae* was subjected to NCBI BLASTp (Altschul et al., 1990) against PDB database (<http://www.rcsb.org/pdb/home/home.do> for identification of suitable template as well as determined method for modeling of 3D model. In addition to BLASTp search, Pyre2 (Pathak et al. 2013) and I-TASSER were employed to model 3D structure of target protein sequences (Fig. 1). Discovery studio visualizer was used for the visualization of 3D models

(<http://accelrys.com/products/collaborative-science/biovia-discovery-studio/>).

Quality assessment and evaluation of predicted model

The structural refinements through energy minimization of each predicted models were performed by SPDB viewer (<http://spdbv.vital-it.ch/refs.html>). The overall quality of each models were done by ProSA (Protein Structure Analysis) (Sawada et al., 1997) and ProQ (Protein model quality prediction) (Trott and Olson, 2010). RAMPAGE server was used to analyze the Ramachandran plot of the predicted protein models (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) (Table 2).

Ligand designing and molecular docking

The structure of each selected phytoalexins was constructed using ChemSketch (<http://www.acdlabs.com/resources/freeware/chemsketch/>). Molecular docking was performed using phytoalexins with pathogenic proteins by AutoDock vina (Wallner and Elofsson, 2003), which requires the three dimensional structure of ligands and receptors and uses a sophisticated gradient optimization method for molecular docking.

Conclusion

The present computational study provides an insight about the interactions between phytoalexins and pathogenic protein of *Pseudomonas syringae*. It explains the mode of inhibition of bacterial activity. The phytoalexins ϵ -viniferin, Glyceollin I and Monilactone A may be utilized for further studies to validate its role for curtailing the incidence of bacterial blight disease of Soybean and other agriculturally important diseases of crop plants. This information may be beneficial for scientific community to develop novel molecules using phytoalexins for sustainable agriculture that directly replace the use of hazardous bactericides, thus ensuring food and nutrition security of the rapidly growing world population.

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Compliance with Ethical Standards

The authors declare that they have no competing financial interests.

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