

Zeamatin-Like Protein Gene is Associated with Resistance against Bacterial strains in Maize (*Zea mays* L)

Maryam Iqbal¹, Syeda Memoona Zaheer¹, Zunera Hassan², Zunaira Akhtar³, Faiza Meer⁴, Muhammad Farhan Tabassum^{5,*}

¹Department of Optometry and Vision Sciences, Faculty of Allied Health Sciences, University of Lahore, Pakistan.

²Department of Zoology, University of the Punjab, Lahore, Pakistan

³Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

⁴Department of Radiology, Faculty of Allied Health Sciences, University of Lahore, Pakistan

⁵Department of Mathematics, University of Management and Technology, Lahore, Pakistan

*Corresponding Author: Muhammad Farhan Tabassum, E-mail: farhanuet12@gmail.com

Abstract

Maize is the maximum produced cereal crop within side the global and the maximum tailored to distinct ecosystems. Pathogenesis-Related (PR) proteins and Anti-Microbial Peptides (AMPs) are a fixed of several molecules which may be prompted thru phytopathogens further to safety related signalling molecules. They are the vital aspect components of plant innate immune device mainly Systemic Obtained Resistance (SOR), and are appreciably used as diagnostic molecular markers of safety signalling pathways. Zeamatin Like Protein (ZLP) is a member of thaumatin-like proteins from maize with a molecular weight of twenty-two kDa. It is likewise remoted from the seeds of *Zea mays*. Previously there's no statistics to be had at the comparative expression of ZLP transcripts upon contamination through bacterial strains. Structure and expression in their corresponding genes. We now document the isolation of ZLP, it's in all likelihood position in extended resistance (in general protein contents received post-inoculation) towards decided on bacterial strains. The expression of ZLP was compared across different maize lines to determine the corresponding transcriptional responses of ZLP gene and it's infection by Bacterial strains. Purification of the total proteins content from the cells (before and after inoculation) was carried out and analyzed their comparative antibacterial assays in vitro. The results provide valuable information for comprehensive understanding of ZLP's function.

Keywords: Thaumatin Like Proteins, ZLP, PR Proteins, Maize, Antibacterial

Introduction

Maize (*Zea mays* L.) is an essential meals and feed crop of the world. Maize yield is closely related to plant population (Shrestha & Subedi, 2018). Maize manufacturing has elevated the maximum

due to its extra version to distinct ecological structures and sturdy call for bioethanol and animal feed and for the manufacturing of sweeteners and different non-food business products (i.e., biodegradable packaging materials) (Caballero, Finglas, & Toldrá, 2016). Maize is grown in spring and autumn in Pakistan. Since the introduction of spring maize harvesting in Pakistan, there is evenly increase in planting of maize during spring season in the soaked low land areas of Punjab (Khan, 2013).

Wheat (*Aestivum* L.) and maize (*Zea mays* L.) are critical cereal vegetation grown worldwide. In Pakistan, numerous forms of wheat and maize are grown and some of biotic and abiotic elements impact its production. These are particularly used as meals supply and uncooked fabric for animal feed.

Maize is grown in all other provinces of Pakistan, but Punjab and NWFP are the major areas of Pakistan for production of maize. In Pakistan, it is grown on an area of 1.02 million hectares with the annual grain production of 2.96 million tons with average grain yield of 2893 kg/ha-1 (Shahzad et al, 2012). The grain yield production in Pakistan was 1.59 times insignificant than the average grain yield of the world because of high temperature and other abiotic stresses like low temperature, drought and salinity). In a corn seed there are adequate nutrients for its seedling establishment. Under warm, moist and soil conditions the seedling emergence occurs after 6 to 10 days of planting (Naveed et al, 2014).

Corn is a versatile crop, and every part on a corn plant is useable. No part of the corn is emaciated. In making tamales the husk of the corn is used as traditionally. The kernels are ground into food. The stalks become animal food and for medicinal teas the corn silks are used (Sailer, 2012). Plant defences are small cationic, cysteine-wealthy peptides with a stabilized tertiary shape through disulphide- bridges and characterised through a huge variety of organic functions (Amin & Kashgry, 2020).

Worldwide crop production is going through numerous challenges, like climatic change, urbanization, atmospheric pollutants, and excessive utilization of agrochemicals, pesticides, and fertilizers. The remarkable upward thrust in meals call for is exaggerating those problems. Global modifications with inside the agricultural area on the structural degree arise because of the fast improvement of technological innovations. In this connection, traits in technology are made that might have an impact on the fine and amount of the crop and in the end shield the environment (Soliman, 2020).

Plant increase and improvement is regularly challenged with the aid of using numerous abiotic and biotic stresses, which includes drought, cold, salinity, wounding, heavy metals, and pathogen attacks, respectively. A plant responds to those threats with the aid of using activating a cascade of genes, encoding exclusive effectors, receptors, and signalling and defensive molecules. Among all, the induction and accumulation of pathogenesis-related (PR) proteins in flora in reaction to those negative situations could be very important (Deepti Jain Email, Jitendra Paul Khurana 2018). These identified proteins can be grouped into three individual categories (based on their peptide sequences). Category (1) consists of storage protein that are late embryogenesis abundant proteins (LEA3, LEA14), globulin 1 and globulin 2. Category (2) consists, of stress related proteins, that includes glyoxalase I protein (GLX I), aldose reductase (ALD), peroxiredoxin antioxidant (PER1),

heat shock proteins (HSP) and antifungal proteins which include pathogenesis related proteins and trypsin inhibitor. Pathogenesis- Related (PR) proteins and antimicrobial peptides (AMPs) are a set of numerous molecules which can be triggered through phytopathogens in addition to protection associated signalling molecules. They are the important thing additives of plant innate immune gadget particularly systemic received resistance (SAR), and are extensively used as diagnostic molecular markers of protection signalling pathways. ZLP is a member of thaumatin-like proteins from maize with a molecular weight of 22 kDa. It is also isolated from the seeds of *Zea mays* (Wilson et al, 2008).

Materials and Methods

Maize Hybrids: Five maize hybrids were tested for the bacterial attack. These hybrids were MMRI, Tag 1, Ifgol, Yousaf Wala, 3 and Agaiti. All maize hybrids were collected from Maize and Millet Research Institute Yousafwala, Pakistan. Seeds were cultivated during summer season in Sialkot. All seeds were cultivated in rows. About 40-50 seeds per hybrid were sown in each row on the ridges. The sowing date of seeds was March 09, 2014. The sown seeds were monitored for germination and growth whereas the irrigation and fertilizers were applied according to normal agronomical requirements.

Plants were inoculated by the bacterial strains *E.coli*, *Pseudumons aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium* - about 60 to 65 days after sowing. The bacterial strains were collected in the syringes and at least three plants of each variety were inoculated by it. Plants were inoculated at growing regions i.e., at the meristematic region. Each plant was inoculated by the bacterial strain media of about 1-2 ml quantity. After it the plant samples were collected after two weeks for the estimation of the ZLP protein production through comparative gene expression.

Samples were collected after fifteen days of inoculation. The growing or meristematic tissues were collected. These samples were then kept at freezing temperature of about -20°C until the purification of RNA/DNA.

RNA Extraction: For each analytic sample, multiple RNA extractions per selected variety were made by using Purelink RNA and DNA extraction kits (Invitrogen) according to the manufacturer's instructions.

Detection of the gene expression: RNA purified from each of the selected varieties was utilized in qPCR.

Protein Purification/Extraction: 5X Sample Buffer, Reagents: 1.5M Tris-HCl (pH 6.8) 4ml, Glycerol 10ml, 2-Mercaptoethanol 5ml, 10% SDS 2g, 1% bromophenol blue 1ml.

Direction: 1ml of 1% bromophenol blue was added to 4ml of 1.5M Tris-HCl (pH 6.8). Then 10ml of glycerol was added and mixed in it. After mixing, 2g of SDS was added and mixed (SDS takes a few minutes to dissolve). It was followed by the addition of 5ml of β -Mercaptoethanol. The mixture was stored at -20°C. The protein purification from the samples of six maize hybrids was carried out in cold room at 4°C. Approximately 1 gm. sample tissue of each hybrid was weighed and heated at 95°C for 20 minutes. Tissue was ground individually in mortar and pestle

with a pinch of sand till the fine powder was obtained. Each sample was mixed with 1-5 ml 5x sample buffer and stirred to get the semisolid paste like form. The mixture was centrifuged at 14,000rpm for 20 mins. The elute was obtained and stored at -20°C for further processing.

SDS Page: For each gel, one spacer plate, one short plate and one comb were necessary. These all were found with gray rack by the sink. The plates were cleaned with 70% ethanol and dried with the tissue. The combs were washed thoroughly with tap water to remove all dust and small particles, particularly any remains of polyacrylamide.



Figure 1. Gel preparation apparatus

The stacking gel had 5% concentration. A 15% resolving gel was prepared and poured between the plates avoiding bubble formation which could inhibit polymerization. The gel was layered with 70% ethanol on top. The gel was allowed to solidify for at least 20-30 min. After gel solidification, the excess ethanol was carefully removed. The comb was inserted to make wells. The comb was removed after 20-30 mins.

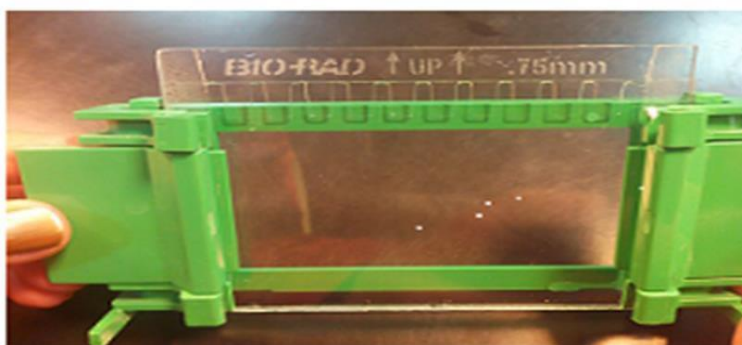


Figure 2. Prepared gel

6x loading dye and protein sample were used in a ratio of 1:2. 5-8ul sample was loaded into each well whereas one well contained ladder (3-5ul). The running module was placed in the gel box which contained 1x TAE running buffer or tank buffer. The electrophoresis took place at initially at 80V and later at 100-120V. The gel was run until dye entered into running buffer. The

gels were placed into the staining solution and shaken for 20 min. The gels were de stained overnight so that bands showed on gel.

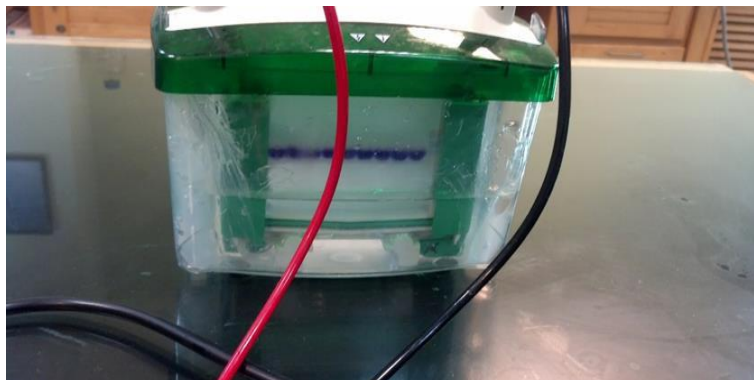


Figure 3. Running Gel

Results and Discussions

Previously there is no information available on the comparative expression of ZLP transcripts upon infection by bacterial strains. Structure and expression of their corresponding genes. We now report the isolation of ZLP protein, its probable role in increased resistance (in total protein contents obtained post-inoculation) against selected bacterial strains.

First of all, conventional PCR was performed on tissue samples to confirm the validity of primers selected for qPCR. As expected, bands of 490bp and 600bp were obtained for gapdh and ZLP respectively.

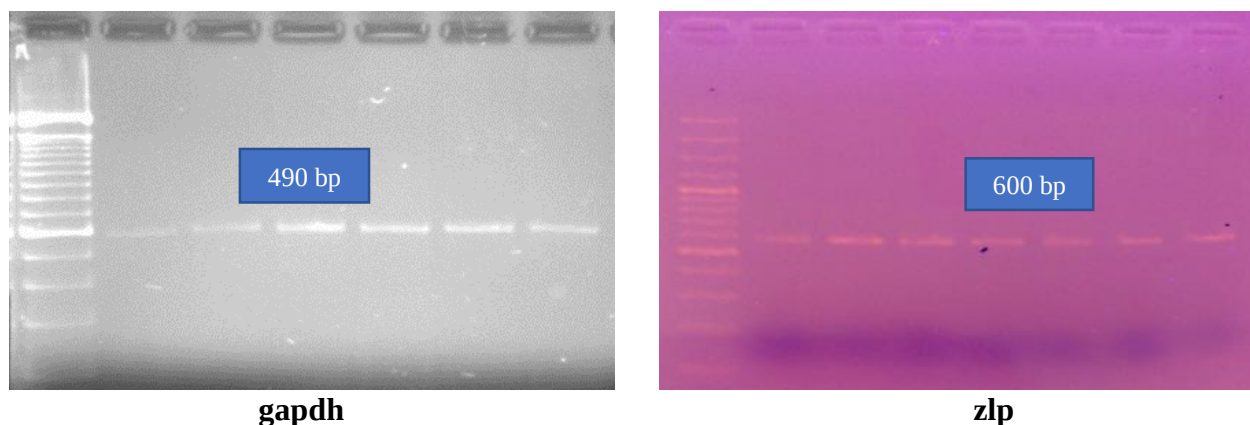


Figure 4. Conventional PCR results

It was followed by protein purification which is performed to separate the desired protein from the complex mixture e.g., cells, tissues, or whole organism. This process is necessary for the analysis of function, structure and interaction of the target protein. The extraction process separates the protein and non-protein parts, and finally the desired protein from the mixture is isolated.

Protein extraction was done from the meristematic tissue samples of six maize hybrids viz: MMRI, Tag 1, IFGOL, Yousaf Wala, 3 and Agaiti. SDS-PAGE separated the desired protein from the mixture. Sodium dodecyl sulphate is an anionic detergent and breaks globular structure of proteins into linear structure, and imparts negative charge on them. SDS binds to the polypeptide chains according to their relative molecular masses. It is also used to find out the relative abundance of proteins and to determine the distribution of proteins among fractions. Here SDS PAGE was performed to find out the molecular mass of zeamatin carrying a molecular mass of 22kDa.

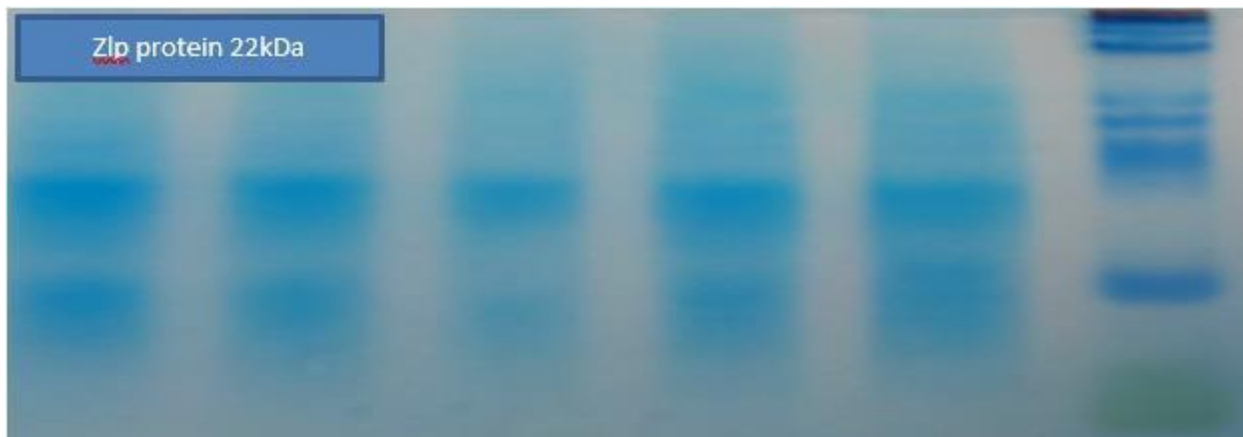


Figure 5. SDS page result

The gene expression analysis for *zlp* was followed by assays for antimicrobial activity of the total protein's purification obtained before and after infection bacterial strains. Some pathogen related proteins have been identified in maize including (name of PRP's). In order to investigate antimicrobial activity of total protein content (MMRI, Tag 1, IFGOL, Yousaf Wala, 3 and Agaiti) of maize were selected and total protein contents of seeds were isolated. The activity of total proteins contents (TPCs) was tested against *E. coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium*. The activity was measured through agar well diffusion assay.

Total protein contents of MMRI, Tag 1, IFGOL, Yousaf Wala, 3 and Agaiti gave antibacterial activity against *E. coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium*. Total protein contents of MMRI were found most active against *E. coli* with a zone of inhibition of 18 mm. while Tag 1, Agaiti and 3 had zones of 15, 10 and 9 mm respectively. IFGOL and Yousaf Wala were inactive against *E. coli*. In the case of *Staphylococcus aureus*, Yousafwala exhibited more antibacterial activity zone 19mm, while 3, IFGOL, Agaiti gave 16mm, 15mm and 8mm inhibitory zones respectively. Tag 1 and MMRI were found inactive. The antibacterial activity of total protein contents of Tag 1 and Agaiti showed the most active inhibitory zone of 15mm against *pseudomonas*, while IFGOL and 3 gave inactive zones. On the other hand, MMRI was found with 10mm inhibitory zone. The total protein contents of Tag 1, Agaiti, and Yousaf Wala showed 15mm inhibitory zones against *Bacillus*,

while IFGOL gave non-reactive zone. MMRI and 3 exhibited 10mm inhibitory zones. The antibacterial activity of total protein contents of Yousaf Wala showed 19mm against *Salmonella typhimurium*, MMRI, 3, Agaiti, Tag 1 gave 16mm, 15mm, 14mm, 12mm inhibitory zones respectively. IFGOL was found inactive. In figure 6 arrows showing antibacterial activity of total protein contents of MMRI, YOUSAF WALA, IFGOL, TAG 1, AGATI and 3 against *Escherichia coli*. In figure 7 arrows showing antibacterial activity of total protein contents of MMRI, YOUSAF WALA, IFGOL, TAG 1, AGATI and 3 against *Staphylococcus aureus*. In figure 8 arrows showing antibacterial activity of total protein contents of MMRI, YOUSAF WALA, IFGOL, TAG 1, AGATI and 3 against *Pseudomonas aeruginosa*. In figure 9 arrows showing antibacterial activity of total protein contents of MMRI, YOUSAF WALA, IFGOL, TAG 1, AGATI and 3 against *Bacillus Cereus*. In figure 10 arrows showing antibacterial activity of total protein contents of MMRI, YOUSAF WALA, IFGOL, TAG 1, AGATI and 3 against *Salmonella typhimurium*.

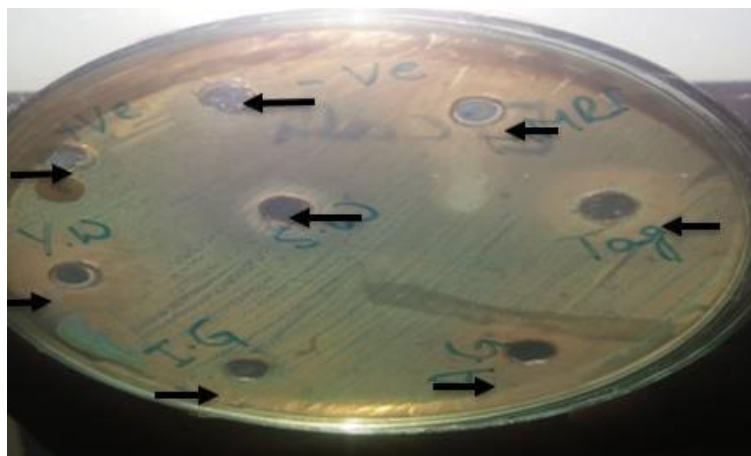


Figure 6. Total protein contents showing antibacterial activity against *Escherichia coli*

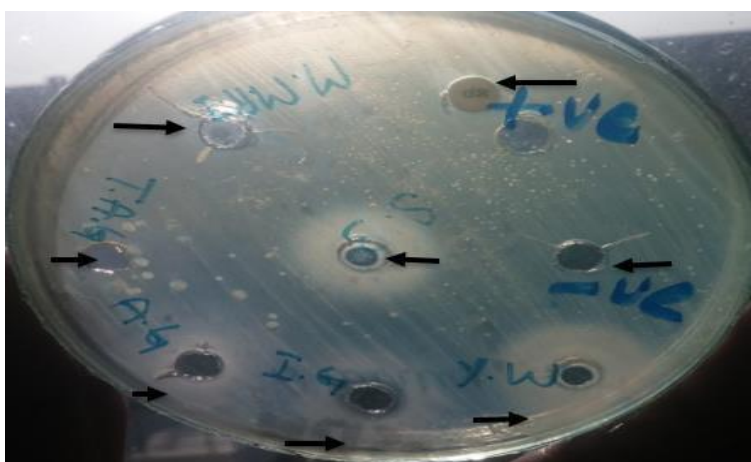


Figure 7. Total protein contents showing antibacterial activity against *Staphylococcus aureus*

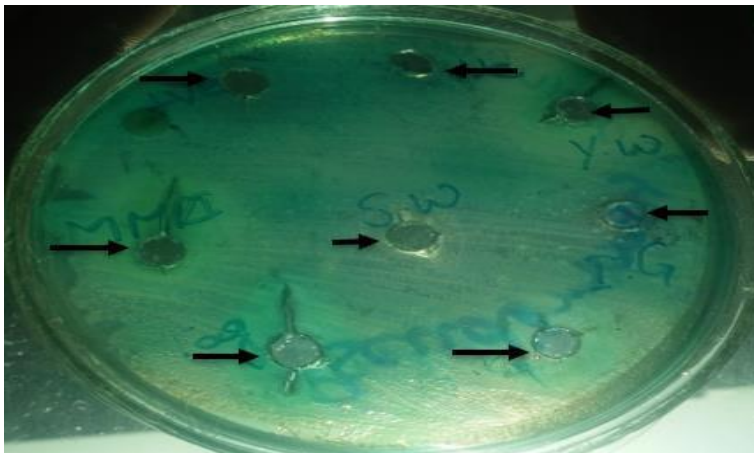


Figure 8. Total protein contents showing antibacterial activity against *Pseudomonas aeruginosa*

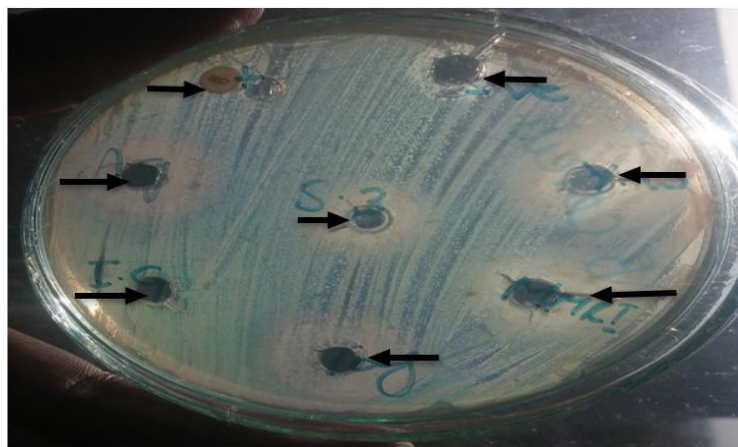


Figure 9. Total protein contents showing antibacterial activity against *Bacillus Cereus*

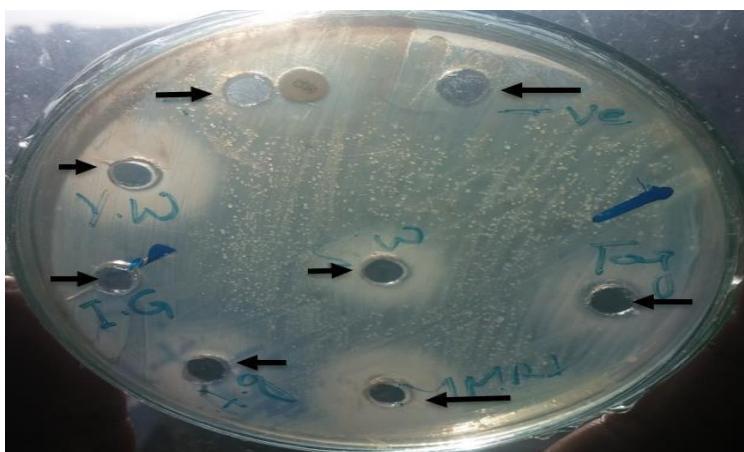


Figure 10. Total protein contents showing antibacterial activity against *Salmonella typhimurium*

Table 1. ATCC Numbers of Bacterial Strains

Bacterial Strains	ATCC #
<i>Escherichia coli</i>	25922
<i>Staphylococcus aureus</i>	25923
<i>Pseudomonas aeruginosa</i>	27853
<i>Bacillus Cereus</i>	11778
<i>Salmonella typhimurium</i>	14028

Table 2. Antibacterial activity (zones of inhibition in mm) total protein contents of various protein samples

Bacterial Strains	Inhibitory Zones of Total protein Contents (mm)						Standard antibiotic	
	MMRI	Tag 1	IFGOL	Yousaf Wala	Agaiti	3	Positive Control Colistin Sulfate	Negative Control 5x sample buffer
<i>Pseudomonas aeruginosa</i>	10	15	0	10	15	0	0	0
<i>Salmonella typhimurium</i>	16	12	0	19	14	15	10	0
<i>Staphylococcus aureus</i>	0	0	15	19	8	16	0	0
<i>Bacillus Cereus</i>	10	15	0	15	15	10	0	0
<i>E.coli</i>	18	15	0	0	10	9	0	0

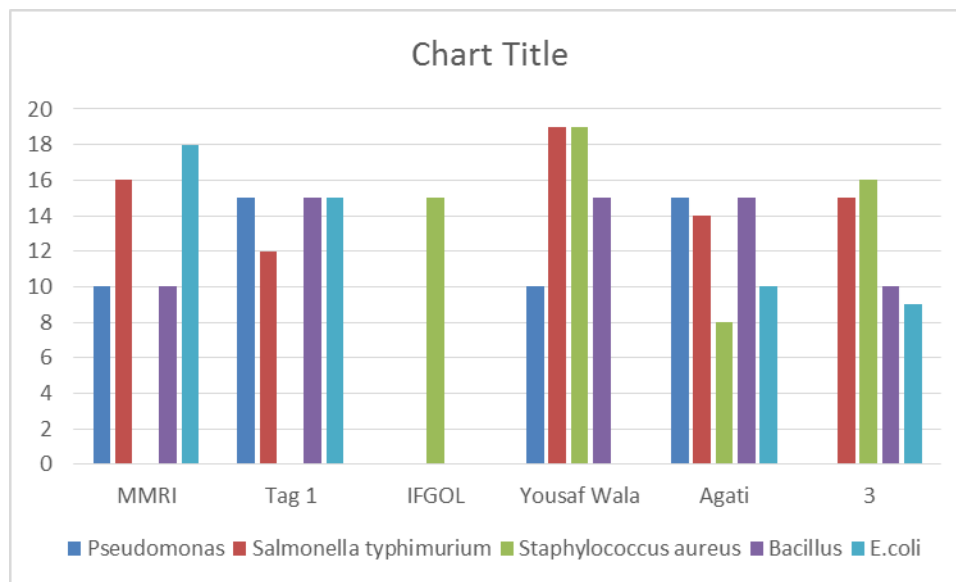


Figure 11. Graphical expression of total protein contents against *E. coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium*

TLPs are generally resistant to proteases and pH- or heat-induced denaturation, and this may be due to the presence of 16 cysteines, which have been shown to be involved in the formation of eight disulfide bonds in thaumatin and ZLP a TLP isolated from maize seeds (16). ZLP, a seed

protein consisting of 206 amino acids with a Mt. of 22,077 from maize, is also a TL protein. It has a 52% sequence homology with that of thaumatin II (Richardson *et al*, 1987). Proteins similar to ZLP have been subsequently detected in the grains of other plants, including barley, flax, oats, sorghum, and wheat (Vigers *et al*, 1992). An optimized and established cell-suspension culture of maize (*Zea mays*) was demonstrated to constitutively secrete a number of pathogenesis-related proteins comprising the antifungal protein ZLP (P33679) with a final yield in the vicinity of 3 mg/l medium (Perri *et al*, 2008).

Conclusion

The present establishes no direct link between the infection of maize with bacteria and subsequent increase in mRNA transcripts or overall enhancement of resistance offered by total proteins contents against the invading pathogen. However, the anti-microbial assays of the post-inoculated proteins samples show an overall enhanced activity against selected bacterial strains.

Limitations and Future Studies

Maize leaves after inoculation must be used within time. Long freezing time for Maize leaves can decrease the antimicrobial activity of Maize. During protein purification, lab measurements should be followed properly. Contamination can change the result.

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