HIGHER EDUCATION COMMISSION

H-9, Islamabad (Pakistan)

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Proposal Identification Number

RESEARCH GRANT APPLICATION FORM

COVER SHEET FOR PROPOSAL

A. TITLE OF PROPOSED PROJECT: **Isolation, cloning and expression of novel antimicrobial genes from medicinal plants**

B. WHETHER PROPOSED RESEARCH IS BASIC ✔ OR APPLIED ☐

C1. RESEARCH DOMAIN

✔ Sciences ☐ Engineering & Technology ☐ Social Sciences ☐ Humanities

C2. STATE FIELD OF RESEARCH AND SPECIALIZATION (For example; Major: Chemistry, Specialization: Organic)

Major **BIOCHEMISTRY** Specialization **MOLECULAR BIOLOGY**

D. PROJECT DIGEST. Describe the proposed research using (about 250) words geared to the non-specialist reader.

Fungal and bacterial infections have been increased tremendously during the past few years. One major reason is the development of resistance in pathogenic microorganisms against the available antimicrobial compounds and drugs. This may lead to fatal infections in many cases. This situation is not only a growing threat to humans, but also for plants. Therefore, there is a dire need to investigate natural sources for new antimicrobial compounds. Antifungal and antibacterial proteins and peptides have been found in many plants. We also have isolated and purified such proteins from some of the plants. However, for application of such antimicrobial compounds, large scale production is necessary. One such method is to isolate the genes corresponding to antimicrobial compounds and hyperexpress in simple systems that can give high yields. In this project we will isolate novel antimicrobial genes from some medicinal plants that are known to exhibit antimicrobial proteins/peptides. The genes will be isolated using Differential Display (DD)-PCR which is relatively a new technique for detection of novel genes under certain stress conditions. We have developed expertise and facilities for the technique in our lab. The seeds of the potential medicinal plants will be induced for the antimicrobial genes and subjected to DD-PCR for isolation of the genes. The antimicrobial genes will be expressed in *E. coli* and the corresponding proteins will be tested for antimicrobial activity. The outcome of the project will be highly beneficial for the country as it will provide an opportunity to explore novel antimicrobial proteins from our natural resources. This will also contribute towards scientific knowledge for fighting against antimicrobial infections.

E1. PRINCIPAL INVESTIGATOR NAME (full with no initials)	E2. HIGHEST DEGREE	E3. POSITION/TITLE
Dr AMER JAMIL	**PhD**	**Associate Professor**

E4. DEPARTMENT/SECTION	E5. UNIVERSITY/INSTITUTION	E6. MAILING ADDRESS
Chemistry and Biochemistry	**University of Agriculture, Faisalabad**	**Molecular Biochemistry Lab., Dept. of Chemistry & Biochemistry, University of Agriculture, Faisalabad-38040**

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F: CO-PRINCIPAL INVESTIGATOR

Name & Position: **Dr Muhammad Shahid, Assistant Professor**

Professional Address: **Protein Molecular Biology Lab., Dept of Chemistry & Biochemistry, University of Agriculture, Faisalabad**

G1. PROPOSED DURATION OF PROJECT: (*in months*)	G2. PROPOSED STARTING DATE	H. TOTAL FUNDS REQUESTED
24 months	**July 1, 2009**	**Rs. 3.965209 million**

CERTIFICATES

1) **Certified that the PI is a full time Faculty Member/Foreign Professor/ Eminent Scholar/Eminent Researcher of the University / Degree awarding institutes.**
2) **Certified that the equipment(s) demanded for the subject project is / are not available in the University / Institute**.
3) **Certified that the project under reference has not been submitted to any other funding agency including HEC.**
4) **Certified that No portion of the project has been funded by any other funding agency including HEC in the past.**

SIGNATURE OF PRINCIPAL INVESTIGATOR

SIGNATURE THE HEAD OF INSTITUTION
(Vice-chancellor/Rector of University, Director of Degree-awarding Institutions)

SIGNATURE OF PRINCIPAL INVESTIGATOR

Date: **March 20, 2009**

SINATURE OF CO-PRINCIPAL INVESTIGATOR

Date **March 20, 2009**

ENDOSEMENT OF THE HEAD OF INSTITUTION (Vice-chancellor/Rector of University, Director of Degree-awarding Institutions)

Signature & Date

Name **Prof. Dr. Iqrar A Khan** Title: **Vice Chancellor**

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PROJECT DETAILS

1. PROJECT SUMMARY

Describe the proposed research using (about 250) words.

A remarkable increase in resistance in fungal and bacterial strains against the antimicrobial compounds has been found during the last few decades. This has lead to a very serious situation in many cases especially in immunosuppressive individuals. Economic losses are also observed due to fungal infections in crops and other plants. Therefore, there is a strong need to explore new antimicrobial compounds from different sources, including medicinal plants. We have isolated and purified some antifungal and antibacterial proteins from some plants. Heterologous expression of the genes related to such proteins is an efficient way to meet the ever increasing demand of the antimicrobial compounds. Differential Display (DD)-PCR has been emerged as a very impressive and reliable technique for isolation of novel genes under different conditions from a variety of sources including plants. We have optimized this technique in our lab. The present project focuses on isolation of novel antimicrobial genes from different medicinal plants, followed by their expression in heterologous hosts. The seeds of the medicinal plants will be induced with fungal infection for expression of the antimicrobial genes. RNA from induced and non-induced samples will be isolated. First strand of cDNA will be made and subjected to PCR. The differentially expressed genes will be isolated from the gel, cloned and sequenced. The potential genes that code for antimicrobial proteins will be cloned in suitable expression vectors and expressed in *E. coli*. The expressed proteins will be tested for antimicrobial activity and molecular mass determination. The project will contribute towards new scientific knowledge for fighting against antimicrobial infections. It will also be beneficial for the country as will help explore our natural resources for isolation and expression of novel antimicrobial compounds.

2. PROPOSED GOALS/OBJECTIVES (please identify quantifiable goals)

i. If the proposed research is basic, please identify or postulate scientific hypothesis on which your proposed goal is based.
ii. If the proposed research is applied, please clearly identify the output in the form of a product or process, need or relationship to industry and also identify the end-user of your output/ product. P.I. is encouraged to make preliminary inquiries with the proposed end user and attach any certificate/ document in support of the proposed research.

HYPOTHESIS/BASIS OF RESEARCH (if basic research)

Medicinal plants express antimicrobial proteins/peptides, therefore, novel antimicrobial genes may be isolated from medicinal plants and expressed in heterologous hosts.

GOALS/OBJECTIVES (please quantify your objectives in case of Applied research)

1. **Isolation of differentially expressed genes under fungal induced conditions.**

2. **Cloning, sequencing and analysis of the genes for identification of the genes related to antimicrobial compounds.**

3. **Expression of the antimicrobial genes in heterologous hosts.**

4. **Characterization of the recombinant antimicrobial proteins.**

IDENTIFY END USER/ BENEFICIARY INDUSTRY (if applied research)

N/A

3. INTRODUCTION (not to exceed one page)

The introduction should consist of three paragraphs; the first paragraph should indicate the scientific hypothesis/commercial basis on which the project is based. The second paragraph should introduce the precise nature of the project, and the final paragraph should indicate the proposed objectives in the light of the first two paragraphs and explain clearly what the reader will see in the main body of the proposal.

Fungal and bacterial infections have been increased dramatically during the last few years mainly due to increased use of antibiotics, success in organ transplantation, immunosuppressive therapy, international travels, exploitation of new habitats etc. On the other hand resistance of fungal and bacterial strains against implemented antimicrobial compounds has also increased tremendously. This can lead to serious and fatal infections especially in immunosuppressive individuals. Such situation has created a great threat not only to humans, but also to crops as well. Different fungi can cause serious diseases in plants and animals. They can degrade wood, leading to economic losses; therefore, it is of growing interest to detect antifungal compounds to control the development of plant-destroying fungi (Blanchette, 1994). In this regard the researchers have directed their research focus during the last few years towards the exploration of natural sources (Yadev et al., 2007). As many of the antibiotics and other synthetic drugs have shown sensitization reactions, main thrust of research has been towards the extraction of aini-infectional compounds including antimicrobial peptides/proteins from plants, animals and microorganisms (Selitrennikoff, 2001).

Medicinal plants are highly efficient to cure diseases, and occupy a significant place in modern medicine (Bhattacharjee, 2001). These also cater the needs of people who reside in villages and remote areas. Besides the demands made by these systems as their raw material, the demands for medicinal plants made by the modern pharmaceutical industries has increased manifold (Gupta *et al.*, 1999; de Lucca *et al*., 2005). Antimicrobial compounds have also been isolated and reported from plants (Theis and Stahl, 2004). Antifungal proteins from plants are organized into five major groups based on sequence analysis (van Loon, 1985) and termed Pathogenesis-related proteins: PR-1 (cystein-rich and small proteins of ~15-17 kDa), PR-2 (β-glucanases), PR-3 (chitinases), PR-4 (chitin-binding proteins), PR-5 (thaumatin-like proteins). We have investigated many plants (*Hygrophila auriculata*, *Abrus precatorius*, *Moringa oleifera*, *Croton tiglium*, *Withania somnifera*, *Solanum nigrum* and *Psoralae corylifolia*) for antimicrobial activities (Jamil *et al*., 2007). We also have isolated antifungal proteins from some plants (Jamil, 2008; Shahid *et al*., 2008). The present project has been developed based on outcome of the previous research projects completed in our lab. (Jamil, 2008; Jamil, 2009).

Novel genes expressing antimicrobial compounds need to be isolated and hyperexpressed for practical applications. In this project we will concentrate on isolation, cloning and expression of novel antimicrobial genes from plants. We will isolate the genes by differential display PCR technique that has already been optimized in our lab. The genes will be cloned in plasmids and expressed in *E. coli*. Hyperexpression of the proteins will be achieved by using strong promoter systems in the expression vectors. The recombinant antimicrobial proteins will be tested for antimicrobial activity. Apart from expression of the antifungal genes, analysis of the differentially expressed genes would also help understand the nature of host-fungal interaction as only a little information is available in this area (Sturtevant, 2000). This may lead to the development of novel antifungal drug targets. The project will contribute towards new scientific knowledge for fighting against antimicrobial infections.

4A. BACKGROUND OF THE RESEARCH PROBLEMS TO BE ADDRESSED (Not to exceed two pages)

i. In case of basic research, a comprehensive and up-to-date literature survey clearly highlighting the existing gaps and what new information will be added to the existing pool of knowledge.
ii. In case of applied research, please also identify the industry in Pakistan, which should benefit from the process/product. Please justify how the proposed research will contribute to the national economy/social sector. Please justify your claim by giving figures of import/export, present market, future trends etc. The principal Investigator is encouraged to discuss the proposed research with the proposed beneficiary and attach supporting documentation.

Several plants have been shown to exhibit antimicrobial activities (Jamil *et al*., 2007). Alkoloidal extracts of *Zanthoxylum chiloperone* var. angustifolium have been found to exhibit antifungal activity against *Candida albicans and Asperigillus fumigatus* (Thouvenel *et al*., 2003). Similarly, *Zingiber officinale* (ginger) and *Juglans cinerea* (butternut) had pronounced antifungal activity against a variety of human pathogenic fungi (Christine *et al*., 2002). A defensin-like antifungal peptide has been reported from French bean seeds (Miyakawa *et al*., 2007; Leung *et al*., 2008). *Ginkgo biloba* seeds exhibited antifungal activity against some fungi (Sawano *et al*., 2007). Chinese cabbage (*Brassica campestris* L.) also possessed antifungal activity (Lee *et al*., 2007; Park *et al*., 2007). Antifungal activity has also been shown in the seeds of *Pouteria torta* (Boleti, 2007). Plant chitinases have also been shown to possess antifungal activities against many fungi (Kirubakaran and Sakthivel, 2007; Ho and Ng, 2007; Onaga and Taira, 2008). Athikomkulchai *et al.* (2006) reported two compounds, 3'-(4''-hydroxy-3'',5''-dimethoxyphenyl)-propyl benzoate (1) and 3'-(4''-hydroxyphenyl)-propyl benzoate isolated from the branches of *Croton hutchinsonianus*. The phenylpropyl benzoates were found to exhibit antifungal activity against *Candida albicans*. Oil from the species *Croton cajucara*

essential oil inhibited the growth of reference samples of *Candida albicans*. Nihei *et al.* (2005) reported different compounds from a methanol extract of *Croton jatrophoides.* Furthermore, phorbol diesters isolated from a methanolic extract of the seeds of *Croton tiglium* have been found to inhibit the growth of some microbes (Mekkawy *et al*., 2000).

Many proteins or peptides with antibacterial or antifungal activity have been isolated in recent years from various plants. Huynh *et al*. (2001) purified a protein of molecular mass of 30 kDa possessing potent and broad-spectrum antifungal activity from the leaf extracts of *Engelmannia pinnatifida.* Similarly, Cheong *et al*. (1997) purified an antifungal pathogenesis related (PR) group of 5 proteins (BFIP) with a molecular mass of 27 kDa from the floral buds of *Brassica compestris*. In another study, Casadoa *et al*. (2000) purified a 23 kDa thaumatin like protein termed as CsTL1 from mature chestnut (*Castanea sativa*) cotyledons. The purified protein had an antifungal activity against *Trichoderma viride* and *Fusarium oxysporum*. Similarly, Tonon *et al*. (2002) isolated an antifungal protein, β-1,3-glucanase (GLU-39) having a molecular mass of 39 kDa, from potato cultivar (*Solanum tuberosom* L.). A 45 kDa antifungal protein has been reported from blast fungus (*Magnaporthe grisea*)-treated rice leaves (Lee *et al*., 2007). Another 53 kDa homodimeric protein was purified from American ginseng (*Panax quinquefolium*) roots exhibiting antifungal, ribonuclease and anti-HIV-1 reverse transcriptase activities, designated as quinqueginsin (Wang and Ng, 2000). A protein with molecular mass of 30 kDa possessing strong and broad-spectrum antifungal activity is reported from the leaf extracts of *Engelmannia pinnatifida* (Huynh *et al*., 1996). Similarly, 30 kDa ribosome-inactivating protein possessed antifungal potential, and a 28 kDa antifungal protein, were isolated from dehusked barley grains (Leah *et al*., 1991). Esmeraldino *et al.* (2005) and Suarez *et al*. (2003) found antimicrobial activity in aqueous extracts from *Croton urucurana and Croton malambo*, respectively. Tang *et al.* (1994) purified croton I and II from *Croton tiglium,* having Mr 40 kDa and 19 kDa, respectively. Terras *et al.* (1992) isolated an antifungal protein from the seeds of 4 Brassicaceae species. Dahot *et al.* (1997) separated 7 and 14 peptides on Sephadex G-25 from acetone and ethanol prepared samples of *Moringa oleifera.* Ye and Ng (2002) isolated a variety of antifungal proteins from leguminous plants. A 13 kDa protein with antifungal activity against *Mycosphaerella arachidicola* was isolated from *Allium sativum* (garlic) by Xia and Ng (2005). The protein was purified on gel filtration (Sephadex 75) and ion-exchange columns. Mincoff *et al*. (2006) isolated a 30 kDa antifungal protein from seeds of *Sorghum bicolor* using different chromatographic techniques including reverse-phase HPLC. Wang and Ng (2006) have reported a 6 kDa antifungal peptide from baby lima bean. The peptide was isolated with ion-exchange chromatography on DEAE-cellulose and CM-cellulose columns, gel filtration on Superdex 75 (FPLC), and affinity chromatography on Affi-gel blue. The peptide had inhibitory activity against different fungi. Similar methodology was adopted for isolation of an antifungal peptide (Mr 9412) from seed of *Brassica campestris* (Lin *et al*., 2007). Antifungal peptides from French bean seeds (Leung *et al*., 2008), red lentil seeds (Wang and Ng, 2007b), *Phaseolus vulgaris* (Wang and Ng, 2007a) and *Amaranthus hypochondriacus* (Rivillas-Acevedo and Soriano-Garcia, 2007) have also been isolated by different chromatographic procedures. A novel protein from *Croton tiglium* with antifungal and antibacterial activities has been reported by Shahid *et al*. (2008). The protein was isolated with the help of gel filtration followed by ion-exchange chromatography.

Relatively little reports are found on antifungal genes. Some workers have isolated antifungal genes from plants with the help of cDNA. Hanselle *et al.* (2001) isolated a pathogenesis related protein induced by infection with *Ascochyta rabiei* from *Cicer arietinum L*. chickpea leaves. The cDNA clone for this protein was isolated from the chickpea cDNA library. Cho *et al*. (2004) constructed a cDNA library using mRNA extracted from rice leaves infected with *Xanthomonas oryzae pv. oryzae*. Screening of cDNA library led to the isolation of many induced genes including pathogenesis related protein. Park *et al*. (2007) isolated a protein identical to human FK 506-binding protein from cDNA library of Chinese cabbage leaf. The protein was expressed in *E. coli* and found to inhibit pathogenic fungal strains.

PCR-based techniques are being now applied due to ease of operation, fast and reliability. Among these, differential display-PCR technique has emerged as less labor-intensive with much better results (Sturtevant, 2000). DD-PCR has been used in identification and isolation of differentially expressed genes from plants (Song and Allen, 1997; Chen *et al*., 2003; Chotikacharoensuk *et al*., 2006; Torres *et al*., 2006; Bozkurt, 2008; Caruso *et al*., 2008). Differential expression of fungal genes after exposure to the host has also been demonstrated in some studies. Colonna-romano *et al*. (1998) isolated differentially expressed genes by *Histoplasma capsulatum* during macrophage infection. Differentially expressed genes in *Candida albicans* were identified by DD-PCR after its exposure to the host cells (Muhlschlegel *et al*., 1998). Genes were also identified from *C. albicans* after stress induction with a toxic heavy metal Cadmium (Hong *et al*., 1998). Genes corresponding to antimicrobial compounds from plants may also be isolated after induction of fungal stress. Asiegbu *et al.* (2003) inoculated seeds of *Pinus sylvestris* with root rot fungus *Heterobasidion annosum* and isolated a novel antimicrobial peptide gene (Sp-AMP) homologue. Novel antimicrobial genes may be identified with DD-PCR technique from plants after fungal induction.

4B. RESEARCH PLAN: SCHEDULE/PHASING (Not to exceed one page)

The studies will be completed in two years:
Year I: Induction of antimicrobial genes in the selected medicinal plants with fungal stress followed by isolation and cloning of differentially expressed genes by DD-PCR technique

Year 2: Sequencing, characterization and expression of the isolated and cloned antimicrobial genes

Brief Methodology:

i. **Medicinal plants that potentially contain antimicrobial proteins/peptides will be explored for isolation of the genes.** For example, *Nigella sativa* (blackseed), *Foeniculum vulgare* (fennel), *Ricinus communis* (castor oil plant), *Cichorium intybus* (common chicory), *Capsicum frutescens* (chili pepper), *Ammi majus* (lace flower), *Trachyspermum copticum* (carom seeds), *Linum usitatissimum* (common flax), *Carthamus tinctorius* (safflower)

ii. Gene induction with fungal stress: Plant seeds, after washing, will be placed separately on Whatman filter paper in a Petri plate and incubated at 25 ˚C (Bachem, 1996). The seedlings will be inoculated with a fungus *Fusarium solani* in order to induce antifungal genes (Lee and Hwang, 2006).

iii. DD-PCR: Total RNA will be isolated by using Qiagene RNeasy plant mini (or equivalent) kit according to the manufacturer's instructions at different time intervals. DNA will be removed by DNase treatment. The integrity of the isolated sample will be checked by ethidium bromide staining through agarose gel electrophoresis (Sambrook and Russell, 2001). First strand of cDNA will be synthesized by Hminus-MMLV-reverse transcriptase kit (Fermentas) using primers anchored to oligo-dT. It will be subjected directly to PCR by using the same anchored primers and arbitrary upstream primers (Torres et al., 2006). The amplified products will be fractionated by denaturing polyacrylamide gel electrophoresis and visualized by silver staining (Deng et al., 1999).

iv. Cloning and sequencing: The gel bands of differentially expressed genes will be excised and the genes will be isolated and re-amplified by using the same set of primers as used above (Deng et al., 1999). The re-amplified products will be ligated in appropriate vector using cloneJet PCR cloning kit (Fermentas). Sequencing of the expressed genes cloned in vectors will be done from DNA sequencing facility (such as Macrogen, Korea).

v. Bioinformatics tools will be employed to find out the novel genes after sequencing.

vi. Expression of the genes in *E. coli*: The genes with potential antimicrobial sequences will be cloned in expression vector (e.g., pET) and transformed in *E. coli* (Sambrook and Russell, 2001). In order to get full-length genes 3' and 5' RACE will be performed. The cloned genes will be induced under IPTG induction and the expressed proteins will be isolated and purified using different chromatographic procedures (Deutcher, 1990). Concentration of protein will be determined by Bradford method (Bradford, 1976). SDS-PAGE will be run to confirm the protein purification. The proteins will be subjected to antimicrobial assays.

vii. Antifungal assays: Fungal strains (e.g., *Aspergillus niger, Fusarium solani, Trichoderma harzianum, Mucor mucedo, Alterneria alterneria*) will be grown on Sabouraud's glucose agar medium (Cruickshank *et al*., 1975). For antifungal assay, the sterilized growth medium will be transferred to autoclaved petri plates. Chloramphenicol solution (40 mg/L) will be added to growth medium to make it selective for fungal growth (Emmons *et* al., 1970). The petri plates will be incubated at appropriate temperature for 48 h, for the growth of fungus. Filter paper discs will be laid flat on the growth medium having fungal growth, 100 uL of extract will be applied on each disc and again incubated for 48 h. The extracts having antifungal activity will exhibit clear zones around the disks showing growth inhibition. Discs containing the standard antimicrobial compounds will be run in parallel as control. The zones of inhibition will be measured in millimeters using zone reader (Haung *et al*., 2000; Huynh *et al*., 2001; Rehman *et al.,* 2001).

4C. REFERENCES (cited in 3, 4A & 4B; not to exceed two pages)

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5. IMPACT (of proposed research on teaching/training of manpower, institutional capability building and on local industry)

The proposed project will have very positive and significant impact on different aspects of national development. It will definitely enhance the capabilities of researchers and students in the area of gene expression which is a leading field of life sciences in the developed world. We would be able to produce highly skilled manpower in this area of research of global importance. The project proposal will also be beneficial for institution of facilities and advanced techniques in the department. We will be in a better position to deliver the practical knowledge to our students. The trained graduates will be able to serve as leaders in our future research endeavors. The project will have sound impact on the economy of the country as our natural resources will be exploited for isolation and expression of novel antimicrobial genes and proteins. Any novel compound with potent antimicrobial activity will lead us to the production of the compound at large scale, catering our indigenous needs. It may have therapeutic implications after undergoing clinical trials. Furthermore, foreign exchange may be earned by patenting such compounds with international agencies. However, this would require further studies after completion of the current research project.

The project will be helpful in achieving the goal of Pakistan Govt. to improve the quality, relevance, or capacity of education and research at Pakistani Institutions of higher education in science and technical fields.

6. COLLABORATING LABS

In case of collaboration with national/international research group or local industry, please identify clearly the parts of research that will be carried out in the participating laboratories and please identify complimentarity and/or justify the need for collaboration) P.I.s are encouraged to find collaborating partners within Pakistan, particularly in less developed areas. Include a letter from Collaborating agency expressing willingness to collaborate.

NA

7. FACILITIES AND FUNDING

7A. Facilities: equipment available for the research project IN THE HOST UNIVERSITY/INSTITUTION

Electrophoresis, centrifuge machines, autoclave, orbital shakers, refrigerators, liquid nitrogen containers, weighing balances, pH meter, gel documentation and analysis system, ovens, water bath, laminar air flow cabinet.

7B. Scientific Personnel

a. Available: PI and CoPI

b. Required*: One Research Associate with minimum qualification as MPhil Biochemistry (will have to enroll, preferably, PhD biochemistry program in the Dept.). Preference will be given to the candidates having research experience on DDPCR from plants.

*Involvement of research students is encouraged.

7C. Other funding available for the proposed studies (if any) **Nil**

8. PRINCIPAL INVESTIGATOR

A brief resume of research accomplished in the last 05 years. Please specify title of the research proposal(s), duration, funding source(s) and award amount(s).

The research area of the PI is gene expression. Regarding the current research as proposed in this proposal, he initiated work on purification, characterization and expression of antifungal proteins and peptides from medicinal plants. Such work has been presented in HUPO conference held in China (Oct., 2004), IUBMB symposium Japan (Nov. 2005), International Symposium on Medicinal Chemistry Turkey (Aug. 2006), 55th international congress and annual meeting of the society for medicinal plant research Austria (2007) and many national conferences, and highly appreciated by the fellow scientists. Dr. Jamil has published good quality research publications. He is also author of two chapters on gene expression and biotechnology, in foreign books. Based on his work, Dr. Jamil was awarded with TWAS (Third World Academy of Sciences) prize for Young Scientists in the South in the field of Biology for the Year 2002 and PAS (Pakistan Academy of Sciences) Gold Medal in Biochemistry for the year 2007.

He also has experience of running research projects related to gene expression work as follows:

Title of research proposal	Duration	Funding Source	Award Amount
Purification and characterization of antifungal peptides/proteins from potential medicinal plants and construction of cDNA libraries for hyperexpression.	Three years (completed)	Higher Education Commission, Govt. of Pakistan.	Rs. 1.60 million
Pilot scale production, purification and characterization of xylanase from hyperexpressed mutant of *Chaetomium thermophile*	Three years (Development Project) (completed)	Higher Education Commission, Govt. of Pakistan.	Rs. 11.73 million
Purfication, characterization and hyperexpression of antifungal proteins/peptides from potential medicinal plants (supportive grant to the project No. 1 above).	Two years (completed)	International Foundation for Science	US $ 6000
Hyperexpression of lysine and transfer of cellulase genes in *Brevibacterium flavum* for recycling of agro-industrial wastes.	Three years (completed)	Pakistan Science Foundation	Rs. 0.754878 million
Studies on poly(A) site strength and interaction of 3'-end processing of mRNA to transcription for understanding the mechanism of gene regulation in eukaryotic systems	Three years (in progress)	Higher Education Commission, Govt. of Pakistan.	Rs. 6.034800 million

8. PRINCIPAL INVESTIGATOR: continued

1.	Please attach C.V.	**CV is attached**		
2.	Number of Publications during the last five years & page numbers on the C.V. where these publications are listed	National: **11** International: **14**	Please see pages **6-8** of CV Please see pages : **6-7** of CV	
3.	Number of research projects completed & page number where this information appears	Basic: **06** Applied: _____	Please see pages **2-3** of CV Please see pages_____ of CV	

9A. ESTIMATED BUDGET FOR THE PROPOSED RESEARCH PERIOD

DESCRIPTION	% of time devoted to Project	YEAR 1	YEAR 2	YEAR 3	Amount (in million Rs.)
A. Salaries and Honorarium					
PI: One month/year of basic pay @	40	0.031	0.031		0.062
Co-PI: One month basic pay for the entire duration @	20	0.025			0.025
Research Associate @ Rs. 13000/month	100	0.156	0.156		0.312
Lab Attendant @ Rs. 6000/month	100	0.072	0.072		0.144
Office Assistant (honorarium to existing employee)	50	0.018	0.018		0.036
Subtotal:		0.302	0.277		0.579

B. Permanent Equipment (Please attach invoice/quotation and expected delivery date for items costing over Rs. 0.1 million.)				
-80°C freezer with card locking system and CO_2 backup	0.79565			
Micropipettes one set of four pipettes, Gilson	0.08			
Subtotal:	0.87565			0.87565

C. Expendable supplies *(year wise quantity with full justification)*				
List attached as Annexure-I	1.418689	0.381002		1.799691
Subtotal:	1.418689	0.381002		1.799691

9A. ESTIMATED BUDGET FOR THE PROPOSED RESEARCH PERIOD (continued)

DESCRIPTION	YEAR 1	YEAR 2	YEAR 3	Amount (in million Rs.)
D. Others D1. Literature, documentation, information, online literature search, contingencies, postage, etc.				
	0.01	0.01		0.02
Subtotal:	0.01	0.01		0.02

D2. Local Travel (Destination and purpose with full justification)				
POL/TADA	0.01			0.01
Subtotal:	0.01			0.01

D3. Miscellaneous				
Audit Fee (Max. Rs 10,000)		0.01		0.01
Accountant Fee (Max. Rs. 10,000)		0.01		0.01
Subtotal:		0.02		0.02
Subtotal (D1 + D2 + D3):	0.02	0.03		0.05
E. Indirect cost (University overheads) 20 % of Total direct cost to meet office support, utilities, etc.	0.523268	0.1376		0.660868
Grand Total (A + B + C + D+E):	3.139607	0.825602		**3.965209**

9B. JUSTIFICATION (Please justify your request in a background of the existing facilities available at the host Institute.)

A. **Salaries & Allowances** (All positions, other than PI and Co-PI, must be fully justified. Please give qualifications/requirements of each of the new full-time positions requested for in the Proposal.)

1. **Research Associate** will be the person mainly responsible for the conduct of research work in the project. A full time researcher who can devote 100 percent time to the project work is absolutely necessary for achieving the targets. Minimum qualification of the researcher would be MPhil Biochemistry preferably with research experience on DDPCR from plant samples.
2. **Lab Attendant** is highly necessary for the project. The person should be well aware of general lab. equipment and procedures such as autoclave, water bath, glassware cleaning, storage of chemicals etc. He will also assist in sampling of plants from the filed. Moreover, he will facilitate the purchases and other requirements of the project. No such person is available from the department/university. Execution of the project is not possible without such person.
3. **Office Assistant/typist** is demanded on part time basis for preparation and submission of project bills and maintaining the project record. Already employed persons from the dept. will be engaged in the project and honorarium will be given to such person.

B. **Permanent Equipment** (Please identify major items (over Rs. 25,000). Major pieces of equipment costing over Rs. 0.1 million must be fully justified. Minor items (under Rs. 25,000) may be lumped into one.)

1. **-80^{o}C freezer with card locking system and CO_2 backup** is needed to preserve the plant and RNA samples. RNA is degraded very rapidly at high temperatures. The best recommended temperature is **-80^{o}C**. Time course experiments have to be conducted in the project, therefore, a large number of samples will have to be preserved. The samples will be placed in liquid nitrogen followed by their storage in the freezer. Moreover, bacterial competent cells and strains also demand this temperature. The long term storage of fungal spores is also done at low temperatures. The freezer with **CO_2 backup** is especially demanded due to the electricity problem in the country. The backup system will help in keeping the temperature to -80 ^{o}C even if electricity fails for more than 24 hours. It is also needed due to intermittent power failure throughout the day. No such facility is available in the department. Success of the project is very much dependent upon this equipment, otherwise there are chances of sample and strain losses.

2. **Micropipettes one set of four pipettes** is demanded as dedicated pipettes are needed for RNA work due to its rapid degradation. The project work involves extensive work on RNA, therefore, a set of four micropipettes covering the whole range is requested.

C. **Expendable supplies** (With full justification and details of **quantity required** for the project)

1. Glassware/disposables: Nuclease-free glassware and plastiware is required for molecular biology work proposed in the project, which is highly expensive.
2. The chemicals and kits needed for molecular biology work are exclusive and very expensive.

The year wise list of expendable supplies with their potential use is given as Annexure-I.

D. **Other Costs.** (Travel must be justified.)

A very small amount for POL and TA/DA is demanded in the proposal as seed and fungal samples have to be collected. Moreover, the PI and researcher have to travel for meetings regarding the project.

Annexure-I List of Expendable Supplies

The items mentioned below are essentially required for the conduct of research work in the proposed project. The estimates are based on 09 plants with 09 time intervals for each plant and 02 replicates (minimum total number of samples = 162 (without any repetition in case a sample is lost due to mishandling). The prices mentioned in the table are based on current market situation, and may vary in both directions at the time of purchase.

(Price Pak. Rs.)

Items	Catalog #	Company	Packing	Unit Price	Qty Year 1	Price	Qty Year2	Price	Total Price	Use
Top vision LEGQ agarose	R0491	Fermentas	1x100g	18500	0	0	1	18500	18500	DNA separation
RiboRular RNA Ladder	SM1823	Fermentas	5x40 uL	22500	1	22500	0	0	22500	RNA analysis
2X RNA loading dye solutuion	R0641	Fermentas	1 mL	4800	3	14400	0	0	14400	RNA loading on gel
RevertAid First Strand cDNA synthesis kit	K1621	Fermentas	1x100 reac	34000	2	68000	0	0	68000	First strand synthesis of RNA has to be made from RNA.
2 mM dNTPmix	R0242	Fermentas	5x1 mL	12500	1	12500	0	0	12500	PCR
TBE buffer	B52	Fermentas	1x1 L	5600	4	22400	0	0	22400	
Rapid DNA Ligation & Transformation Kit	K1432	Fermentas	30 react	16500	0	0	1	16500	16500	The plasmids will be cloned and expressed in *E. coli*
RiboLock™ RNase Inhibitor	EO0382	Fermentas	4x2500 U	28500	1	28500	0	0	28500	RNA isolation. RNA is rapidly degraded without the inhibitor.
RNase A/T1 Mix	EN0551	Fermentas	1x1ml	11300	0	0	1	11300	11300	RNA degradation during plasmid DNA isolation.
GeneJET™ Plasmid Miniprep Kit	K0503	Fermentas	1x250 prep	34000	1	34000	0	0	34000	Plasmid isolation.
GeneJET™ Gel Extraction Kit	K0692	Fermentas	1x250 prep	36700	1	36700	0	0	36700	The expressed genes will be isolated from gel and cloned.
PageSilver™ Silver Staining Kit	K0681	Fermentas	1x25 app	17500	4	70000	0	0	70000	The DD-PCR products have to be analyzed by silver staining
IPTG Solution	R1171	Fermentas	10x1.5mL	9600	0	0	1	9600	9600	Expression of genes
X-Gal Solution	R0941	Fermentas	1x10 mL	14600	0	0	1	14600	14600	Expression of genes
Alkaline Phosphatase	EF0511	Fermentas	1x500 U	13600	0	0	1	13600	13600	Gene cloning
Klenow Fragment	EP0051	Fermentas	1x300 U	9600	0	0	1	9600	9600	Gene cloning
Taq DNA Polymerase	EP0402	Fermentas	5x500 U	20000	1	20000	1	20000	40000	Gene amplification

CloneJet PCR cloning kit	K1232	Fermentas	1x40 react	32500	4	130000	0	0	130000	Cloning of PCR products
Bacterial Transformation kit	K2711	Fermentas	40 transfor	9700	4	38800	0	0	38800	Transformation of PCR ligated products
PageRuler™ Prestained Protein Ladder	SM0671	Fermentas	2x250 uL	14500	0	0	1	14500	14500	Protein detection by SDS-PAGE for checking gene expression
RNeasy plant minikit		Qaigen	50 prep	53000	4	212000	0	0	212000	Isolation of RNA
pET expression system with competent cells		Novagen		70000	0	0	1	70000	70000	Expression of genes
Primers			per base	44	800	35200	0	0	35200	For amplification of the genes
DNA sequencing			per run	700	0	0	150	105000	105000	Sequencing of the genes will be done
Sodium acetate	T-6066	Sigma/ Merck	1 kg	6500	1	6500	0	0	6500	Buffer preparation
Glacial acetic acid	S-150501	RdH/ Merck	2.5 L	1650	0	0	1	1650	1650	Buffer preparation and staining of protein gels
NaH_2PO_4	S-281100	RdH/ Merck	1 kg	1600	1	1600	0	0	1600	Buffer preparation
Na_2HPO_4	2402394	Merck/ Merck	1 kg	1850	1	1850	0	0	1850	Buffer preparation
EDTA	E-5134	Sigma/ Merck	250 g	8500	1	8500	0	0	8500	Buffer preparation
NaOH pellets	1.06462	Merck/Sigma	1 kg	1800	1	1800	0	0	1800	Preparation of EDTA
KCl	A 1364	AppliChem/ Merck	1 kg	1600	1	1600	0	0	1600	Buffer preparation
Polyvinylpolypyrrolidone	PVP10-100G	Sigma/ Merck	100 g	8400	2	16800	0	0	16800	RNA isolation
Isoprapanol	I-9516	Sigma/ Merck	500 mL	9200	2	18400	0	0	18400	RNA precipitation
Ethanol molecular biology grade	K26225745	Merck/Sigma	1 L	9000	4	36000	0	0	36000	RNA/DNA precipitation
Bradford reagent	B-6916	Sigma/ Merck	500 mL	13400	0	0	1	13400	13400	Protein estimation after expression
Streptomycine sulfate	S-9137	Sigma/ Merck	25 g	6900	1	6900	0	0	6900	Inhibitor of bacteria during growth of fungi
Chloramphenicol	190321	ICN/ Merck	5 g	4000	2	8000	0	0	8000	Inhibitor of bacteria during growth of fungi
Antimicrobial discs		Oxoid	pkt	1000	0	0	10	10000	10000	Antimicrobial assays
Nutrient agar	CM0003	Oxoid	500 g	4200	2	8400	0	0	8400	Bacterial growth
Sabroud liquid medium	CM0147	Oxoid	500 g	6000	2	12000	0	0	12000	Fungal growth

Potato dextrose agar	CM0139	Oxoid	500 g	7500	2	15000	0	0	15000	Fungal growth
$(NH_4)_2SO_4$	A 4418	Sigma/ Merck	1 kg	16500	1	16500	0	0	16500	Buffer preparation
Sodium sulfide	350328-31	Uni Chem/ Merck	500 g	1800	1	1800	0	0	1800	Protein isolation
H_2SO_4		LabScan/ Merck	2.5 L	6500	1	6500	0	0	6500	Preparation of dialyzing membranes
Acrylamide	A-3553	Sigma/ Merck	100 g	7200	4	28800	0	0	28800	PAGE for RNA and proteins
N,N,-bis-methylene-acrylamide	M-2022	Sigma/ Merck	100 g	42000	1	42000	0	0	42000	PAGE for RNA and proteins
Conc. HCL		LabScan/ Merck	2.5 L	6500	1	6500	0	0	6500	Buffer preparation (pH maintenance)
Tris base	T-6066	Sigma/ Merck	1 kg	21900	2	43800	0	0	43800	Buffer preparation
Ammonium persulfate	A-9164	Sigma/ Merck	100 g	6800	1	6800	0	0	6800	PAGE
TEMED	T- 7024	Sigma/ Merck	100 mL	19400	1	19400	0	0	19400	PAGE
Sodium dodecyl sulfate	102918	ICN/ Merck	1 kg	5200	1	5200	0	0	5200	SDS-PAGE
Sample buffer, Laemmli Electrophoresis reagent	S-3401	Sigma/ Merck	1 vial	7200	0	0	2	14400	14400	Protein loading after gene expression
Methanol (HPLC grade)	M/4056/17	Fisher/ Merck	2.5 L	1100	5	5500	0	0	5500	Staining of protein gels
Glycine	DR0235	Sigma/ Merck	2.5 kg	16500	1	16500	0	0	16500	SDS-PAGE and other buffers
Glycerol		Acros/ Merck	1 L	2000	1	2000	0	0	2000	Storage of cultures
2-mercaptoethanol	63700	Fluka/ Merck	250 mL	5200	1	5200	0	0	5200	Protein isolation
Brilliant Blue R		Acros/ Merck	100 g	9800	1	9800	0	0	9800	Staining of protein gels
water RNase free	D0121	Biobasic/ Merck	500 mL	4500	4	18000	0	0	18000	RNA handling needs RNase free water
Formamide	151160	ICN/ Merck	100 mL	6600	1	6600	0	0	6600	RNA gels
Restriction enzymes (various)		Fermentas	1 vial	7500	3	22500	2	15000	37500	Cloning of genes in plasmids
Ampicillin			5 g	7000	2	14000	2	14000	28000	Selective growth of *E. coli*
Pestal mortor				190	3	570	0	0	570	Grinding of samples
Liquid nitrogen			1 L	100	500	50000	0	0	50000	Sample preparation for RNA isolation, and isolation of RNA
Eppendorf tubes RNase/DNase free	3445		1x1000	2400	15	36000	0	0	36000	All manipulations

1.5 mL									
Gloves disposable latex		1 pkt	700	15	10500	0	0	10500	All manipulations
Tips, blue DNase/RNase free	2160	1x1000	1850	14	25900	0	0	25900	All manipulations
Tips, yellow DNase/RNase free	2100.C	1x1000	1450	14	20300	0	0	20300	All manipulations
Tips, white DNase/RNase free	2040	1x1000	1450	10	14500	0	0	14500	All manipulations
Plastic (Saran) wrap		1 pkt	200	5	1000	5	1000	2000	Covering the gels
Aluninium foil		1 roll	198	24	4752	24	4752	9504	Sample storage, autoclaving
Beakers pyrex 100 mL		1 pc	150	6	900	0	0	900	Solution preparation
Beakers pyrex 250 mL		1 pc	195	12	2340	0	0	2340	Solution preparation
Beakers pyrex 500 mL		1 pc	225	6	1350	0	0	1350	Solution preparation
Beakers pyrex 1000 mL		1 pc	350	3	1050	0	0	1050	Solution preparation
Flasks volumetric pyrex 25 mL		1 pc	320	3	960	0	0	960	Solution preparation
Flasks volumetric pyrex 50 mL		1 pc	325	3	975	0	0	975	Solution preparation
Flasks volumetric pyrex 100 mL		1 pc	325	3	975	0	0	975	Solution preparation
Flasks volumetric pyrex 250 mL		1 pc	590	3	1770	0	0	1770	Solution preparation
Flasks volumetric pyrex 500 mL		1 pc	800	3	2400	0	0	2400	Solution preparation
Flasks volumetric pyrex 1000 mL		1 pc	1040	3	3120	0	0	3120	Solution preparation
Flasks Erlenmeyer pyrex 250 mL		1 pc	220	12	2640	0	0	2640	Growth of fungi and bacteria
Flasks Erlenmeyer pyrex 500 mL		1 pc	320	12	3840	0	0	3840	Growth of fungi and bacteria
Flasks Erlenmeyer pyrex 1000 mL		1 pc	435	6	2610	0	0	2610	Growth of fungi and bacteria
Storage bottles 50 mL autoclavable		1 pc	275	6	1650	0	0	1650	Storage of samples, media, buffers
Storage bottles 100 mL autoclavable		1 pc	290	12	3480	0	0	3480	Storage of samples, media, buffers
Storage bottles 250 mL autoclavable		1 pc	348	12	4176	0	0	4176	Storage of samples, media, buffers
Storage bottles 500 mL autoclavable		1 pc	460	12	5520	0	0	5520	Storage of samples, media, buffers
Storage bottles 1 L autoclavable		1 pc	670	6	4020	0	0	4020	Storage of samples, media, buffers

Storage bottles 2 L autoclavable	1 pc	1400	3	4200	0	0	4200	Storage of samples, media, buffers
Petri plates disposable	1 pair	9	400	3600	400	3600	7200	Bacterial growth
Perti plates (glass, large)	1 pair	234	24	5616	0	0	5616	Fungal growth
Forceps	1 pc	45	3	135	0	0	135	Sample handling
Spatula	1 pc	120	6	720	0	0	720	Sample weighing
Parafilm	1 roll	2450	1	2450	0	0	2450	Storage of Petri plates
Beckman Paper wicks	1 pkt	3800	2	7600	0	0	7600	Antifungal assays
Labelling tapes	1 pkt	9000	1	9000	0	0	9000	Labeling of samples for storage at -80 ^{o}C
Weighing boats	50 pcs	10000	1	10000	0	0	10000	Sample weighing
Labeling permanent marker	1 pc	60	12	720	0	0	720	Labeling of samples for storage at -80 ^{o}C
Filter paper round Whatmann	1 pkt	1600	1	1600	0	0	1600	Filtration of different solutions
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