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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

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Design, Synthesis and Antibacterial Activity Evaluation of 9-phenyl-10-(2-phenylalkyl) acridinium bromide: A Novel Acridine Based Antibacterial Agent

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Abstract

The present study was carried out to develop an effective antibacterial agent from a new derivative of acridine. Acridine had been known as an active antibacterial agent with low toxicity since past. The novel acridine derivative, 9-phenyl-10-(2-phenylalkyl)acridinium bromide was synthesized and tested for antibacterial activity against Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli*. The combination reaction between 9-phenylacridine and alpha-(bromo) methylstyrenein THF medium was carried out to synthesize 9-phenyl-10-(2-phenylalkyl) acridinium bromide. Synthesis of 9-phenyl-10-(2-phenylalkyl) acridiniumbromide was confirmed by ^{1}H-NMR spectroscopy. Antibacterial screening was carried out using disc diffusion and broth dilution methods. The activity was compared with protonated and unprotonated forms of 9-phenylacridine, which were the core starting materials of the novel derivative. The novel compound exerted an effective antibacterial activity against both *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 with MICs (Minimum Inhibitory Concentration) of 7.48 µg/ml and 4.41 µg/ml, also with MBCs (Minimum Bactericidal Concentration)of 375 µg/ml and 6000 µg/ml respectively. The activity against *E. coli* was higher than that against *S. aureus*. Thus, 9-phenyl-10-(2-phenylalkyl) acridinium bromide can plausibly be developed as an effective antibacterial agent with a broad spectrum of activity.

Keywords: Acridine derivatives, 9-phenyl-10-(2-phenylalkyl)acridinium bromide, Antibacterial activity,Minimum Inhibitory Concentration.

Introduction

The extensive development of drug resistance in bacteria, lack of new antibacterial agents in use, emergence of both new and old diseases and emergence of new virulent strains from commensals prompt to design and synthesize a novel antibacterial agent (1). Most of the synthetic antibacterial agents are heterocyclic dye molecules and an alkylamino side chain can be seen frequently among them (2). Acrdine is an unsaturated heterocyclic dye molecule (1). Many acridine derivatives were previously studied and were found to be effective as antibacterial agents (3, 4).

Although penicillin is superior to acridine, researchers' focuses have been drawn to acridine with the extensive increase of drug-resistance in bacteria (2). Capability for cationic ionization, high levels of ionization at neutral pH and a planar molecular surface area make acridine an excellent candidate for an antibacterial agent (3). It was found that acridine exerts its action by intercalating with bacterial nucleic acid (5). The novel acridine derivative, 9-phenyl-10-(2-phenylalkyl)acridinium bromide, is structurally very much analogous to 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide which was identified as a vesicular monoamine transporter inhibitor and a bacterialcell wall synthesis inhibitor (4, 6). Therefore, it prompts a clue that 9-phenyl-

10-(2-phenylalkyl) acridinium bromide might have an alternative mechanism of action on bacterial cell wall other than DNA intercalation. Furthermore, 9-phenyl-10-(2-phenylalkyl) acridinium bromide is an ionic liquid (7, 8). Its absence of volatility offers a much lower toxicity as compared to low-boiling-point solvents (7, 9).

Materials and Methods

Chemicals and Equipments: All chemicals used were of analytical grade. All antibiotics used in the experiment were purchased from State Pharmaceutical Co. Ltd, Sri Lanka. The ^{1}H (300 MHz) spectra were recorded on a Bruker 300 NMR spectrometer (with TMS for ^{1}H as internal references).

Culture media and Microorganisms used: Two standard bacterial species: *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 obtained from the Faculty of Medicine, University of Colombo, were employed for the study. The bacterial stock cultures were maintained on nutrient agar slants at 4 ^{0}C. Each working culture was reactivated prior to susceptibility testing by inoculating into a separate test tube containing nutrient broth and incubated overnight at 37 ^{0}C. Then streak plates were prepared from working cultures using Mueller Hinton agar medium and incubated at 37 ^{0}C for 24 hours. The bacterial colonies were suspended in 0.85% sterile saline solution and the turbidity was adjusted to that of 0.5 McFarland standard. This suspension was used to prepare the spread plates for the subsequent experimentation. All the dehydrated media were purchased from HiMedia, Mumbai, India and prepared according to the manufacturer's instructions.

Synthesis of novel acridine derivatives:

(a) Synthesis of 9-phenylacridine (Fig. 1a), the first acridine derivative, was done via microwave enhanced Bernthsen reaction (10). Melting point was checked after recrystalization with ethanol water mixture, to confirm the presence of the compound.

(b) Then to produce second derivative, 9-phenylacridinium ion (Fig. 1b), protonation of 9-phenylacridine was done by adding concentrated Hydrochloric acid until the reaction medium was acidic.

(c) To synthesize the novel acridine derivative, initially, alpha-(bromo)methylstyrene (Fig. 2c) was synthesized by allylicbromination of

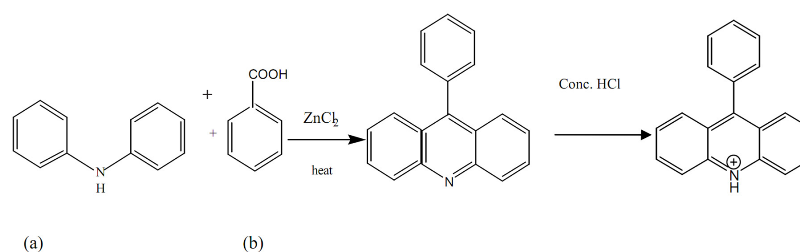

Fig. 1. Bernthsen 9-Phenylacridine synthesis

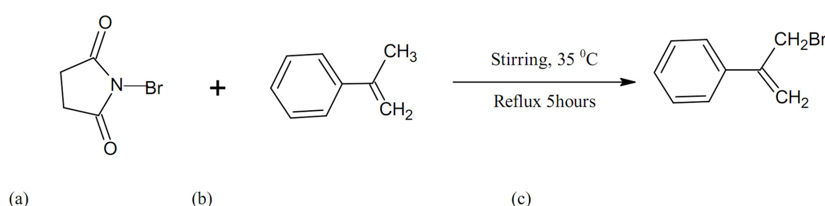

Fig. 2. Synthesis of α (-bromo) methlystyrene

alpha-methylstyrene (Fig. 2b)using N-bromosuccinamide (Fig. 2a)(11). The product was purified by running it in a silica column with hexane and TLC was carried out for the eluted fractions using alpha-methylstyrene as the reference. Formation of alpha-(bromo) methylstyrene was confirmed using ^{1}H-NMR analysis (300 MHz). Then the combination reaction between previously synthesized 9-phenylacridine and α-(bromo)methylstyrene in THF medium with overnight stirring was carried out to synthesize the novel acridine derivative, 9-phenyl-10-(2-phenylalkyl) acridinium bromide (Fig. 3a) (11, 12, 13). The temperature was maintained at 0 ^{0}C during initial 2 hours of the reaction. This compound was purified by running it in a silica column with hexane: ethyl acetate 9:1 mixture and TLC was done for the eluted fractions, running reactants on the same chromatogram. Formation of 9-phenyl-10-(2-phenylalkyl) acridinium bromide was confirmed using ^{1}H-NMR analysis (300 MHz).

Detection of antibacterial activity: Antibacterial activity was tested by using Kirby-Bauer method (14) for the compounds 9-phenylacridine, 9-phenylacridinium ion and 9-Phenyl-10-(2-phenylalkyl)acridinium bromide. Bacterial suspensions having turbidities similar to that of 0.5 McFarland standard (100µl per plate) were aseptically (15) spread on separate Mueller-Hinton agar plates and allowed to stand for 30 min. To determine MIC, a separate dilution series from each compound was prepared: from 9-phenylacridine and 9-phenylacridinium ion: 5 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.2 mg/ml, 0.1 mg/ml; 9-Phenyl-10-(2-phenylalkyl)acridinium bromide: 2.4 µg/ml, 0.6 µg/ml, 0.36 µg/ml, 0.12 µg/ml, 0.06 µg/ml. Sterilized (15) paper discs having 6 mm diameter were aseptically impregnated (10 µl per disc) from each dilution of the series and from positive control (gentamycin 3 mg/ml) and were air dried. The limits of the concentration gradient for this study were determined through a preliminary study. Sterile double distilled water was the negative control. The discs were placed equidistantly from each other on the plates and were incubated for 24 h at 35 ^{0}C. The test was done in triplicates. After incubation, the presence of antibacterial activity was assessed based on the appearance of clear halos, the inhibition zones, around the paper disks. The inhibition zone diameters were measured along two perpendicular axes and recorded.

Statistical analysis: Analysis of Variance (ANOVA) was carried out to determine the significant differences (P<0.05) between the means using Minitab 16. The significant means

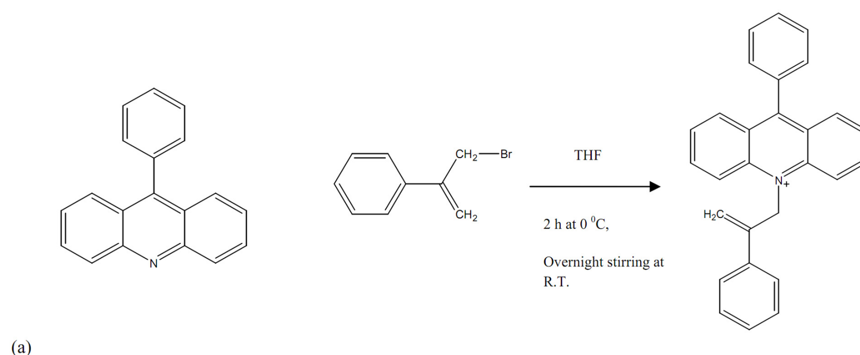

Fig. 3. Combination reaction of 9-phynylacridine and α-(bromo) methlystyrene

were compared using the Least Significant Difference (LSD) analysis.

Determination of minimum inhibitory concentration (MIC): Two models: Free diffusion model and the dissipative diffusion model were fitted to above data as the development of an inhibition zone depends on the nature of the diffusion of respective compound through agar(16).

In the free diffusion model, MIC was determined by plotting the squared value of the inhibition zones (x) against the natural logarithm of the antimicrobialcompound concentration (C)(17).

$$\ln(MIC) = \ln(C) - \frac{x^2}{4Dt}$$

Diffusion coefficient is represented by D and t indicates the time of antimicrobial compound diffusion.

The equation shown below expresses the dissipative diffusion model (17).

$$\ln(MIC) = \ln(C) - 2D^{-1}\left(V \pm \sqrt{V^2 - 4D}\right)x$$

Coefficient characterizing the dissipation rate, antibiotic concentration and inhibition zone diameter are denoted by V, C and x respectively. According to this model, the MIC of the compound can be calculated by dividing the intercept of the plot of inhibition zone diameter (x) against natural logarithm of the antimicrobial compound concentration (C) from its gradient (17).

Minimum Bactericidal Concentration (MBC):Two series of dilutions were prepared separately for the two test organisms from the novel compound. 9-Phenyl-10-(2-phenylalkyl) acridinium bromide was incorporated into sterilized Mueller Hinton broth (total volume of 100 µl), ranging from 600 µg/ml to 0.75 µg/ml for *S. aureus* and from 12000 µg/ml to 1200 µg/ml for *E. coli* was prepared and labeled. A volume of 100 µl from the bacterial suspension in 0.5% NaCl which was similar in turbidity to McFarland turbidity standard (3×10^8cfu/ml) were added into each of these tubes and were incubated at 37 ^{0}C. After 24 hours, streak plates were prepared from each sample and were incubated at 37^{0}C. After 24 hours, the plates were observed for the presence of bacterial growth (1).

Results and Discussion

Microwave enhanced Bernthsen reaction for synthesis of 9-phenylacridine (Fig. 1a) was given a yield of 80%. According to *in vitro* biological evaluation, this first acridine derivative, unprotonated 9-phenylacrdine (Fig. 1a, melting point 184-185^{0}C) was not effective against *S.aureus*, but there was an activity against *E.coli* (Table 1). Presence of both

Table 1. Mean diameters of inhibition zone for different concentrations of acridine derivatives against *S. aureus* and *E. coli*

Mean Inhibition zone diameter (mm) ±SD										
Compound	1 µg/disc		2 µg/disc		5 µg/disc		10 µg/disc		50 µg/disc	
	Ec	Sa	Ec	Sa	Ec	Sa	Ec	S.a	Ec	Sa
1a	ND	ND	ND	ND	4.12 ± 0.71	ND	5.35 ± 0.30	ND	9.32 ± 1	ND
1b	ND	ND	ND	5.64 ± 1.10*	5.25 ± 0.90	7.54 ± 0.55	7.31 ± 0.22	9.81 ± 0.10	1.32 ± 1 1.12	14.84 ± 1.55*

Note: Ec- *E. coli*; Sa- *S. aureus*; ND- not detected
* Significant at $p<0.05$ level

lipopolysaccharide surface and the outer membrane barriers are vital factors that explain the greater resistance of Gram negative organisms to antimicrobials than Gram positive organisms (18). Hence the activity against *E. coli* makes the compound clinically important and it can be suggested that the drug molecule might be transported in to Gram negative cell either via pores in the membrane or by dissolving in lipid fractions of the outer membrane (4).

However, the establishment of positive charge increased the spectrum of antibacterial activity of 9-phenylacridine (Table 1) affecting both *S. aureus* and *E. coli.* 9-phenylacridinium ion (Fig.1b) exhibited an inhibition zone diameter of 14.84 ± 1.55 mm against *S. aureus* and that of 11.32 ± 1.12 mm against *E. coli* (11.00 ± 1.11 mm) for the disc strength at 50 µg/disc. Because the positive charge on 9-phenylacridinium ion is unstable, 9-phenyl-10-(2-phenylalkyl)acridinium bromide (Fig. 3a) was synthesized to increase the stability of the molecule by establishing a permanent positive charge. The combination reaction between the core molecule, 9-phenylacridine (Fig. 1a)and α-(bromo) methylstyrene(Fig. 2c) (^{1}H-NMR signals (acetone, δ, ppm) at chemical shifts 4.8 (s,2H, $=CH_2$), 6.2 (s,2H, CH_2X), 7.11-7.47 (m,5H, ArH)) (19) were resulted a yield of 68% of 9-phenyl-10-(2-phenylalkyl) acridinium bromide (Fig. 3a). Its formation was confirmed by the presence of ^{1}H-NMR signals (acetone, δ, ppm) at chemical shifts 3.8 (s,2H, CH_2), 5.0 (s,2H, $=CH_2$), 7.24-7.47 (m,8H, ArH), 7.8-7.4 (m,5H, ArH) and 8.0-7.8 (m,5H, ArH) (19). 9-phenyl-10-(2-phenylalkyl) acridinium bromide is an ionic liquid with a density of 1.2 g/ml. It is a liquid at room temperature and turns in to solid state at reduced temperature.

The novel compound showed further enhanced activity against both test organisms at a very low concentration of 2.4×10^{-2} µg/disc and it was given an inhibition zone of 12.84 ± 0.81 mm against *S. aureus* and an inhibition zone of 11.32 ± 0.58 mm against *E. coli* (Table 1 and 2, $p<0.05$). The positive control, gentamycin gave an inhibition zone of 24.50 mm for the disc strength at 30 µg/disc and no inhibition was given for the negative control with distilled water.

Table 2. Mean diameters of inhibition zone for different concentrations 9-phenyl-10-(2-phenylalkyl)acridinium bromide against *S. aureus* and *E. coli*

Mean Inhibition zone diameter (mm) ±SD					
	6×10^{-4} µg/disc	1.2×10^{-3} µg/disc	3.6×10^{-3} µg/disc	6×10^{-3} µg/disc	2.4×10^{-2} µg/disc
E. coli	-	-	7.24 ± 0.47	8.39 ± 0.12	11.32 ± 0.58
S. aureus	7.11 ± 0.67*	8.35 ± 0.35*	9.56 ± 0.04*	10.72 ± 0.25*	12.84 ± 0.81

* Significant at $p<0.05$ level

Table 3. Comparison of MIC and MBC values of 9-phenyl-10-(2-phenylalkyl)acridinium bromide and other acridine derivatives for *S. aureus* and *E. coli*

Acridine derivative	MIC		MBC	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
9-phenylacridine	ND	2.42 mg/ml	-	-
9-phenylacridinium ion	3.42 mg/ml	2.74 mg/ml	-	-
9-phenyl-10-(2-phenylalkyl) acridinium bromide	7.48 µg/ml	4.41 µg/ml	375 µg/ml	6000 µg/ml

Though both the dissipative diffusion model and free diffusion model indicated linearity, the coefficient of determination (R^2) of linear regression was higher for the dissipative diffusion model (Fig. 4 and 5, Fig. 6 and 7, Fig. 8 and 9). Thus, the drug diffuses through the agar medium via a dissipative diffusion process and therefore, dissipative diffusion model was used to determine MIC values of these drugs. MIC of 9-phenyl-10-(2-phenylalkyl)acridinium bromide (Fig. 5) for *S. aureus* (7.48 µg/ml) and for *E. coli* (4.41 µg/ml) were lower than MIC of 9-phenylacridinium ion

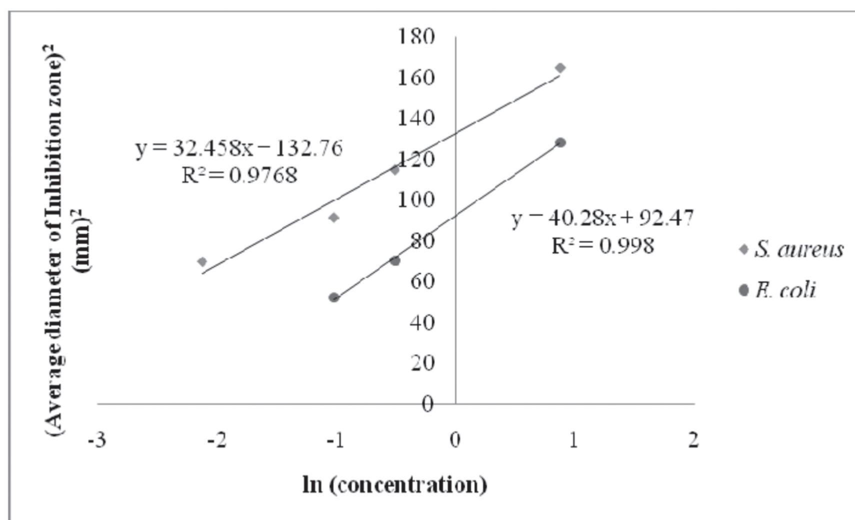

Fig. 4. Free diffusion model for9-phenyl-10-(2-phenylalkyl)acridinium bromide against *S.aureus* and *E. coli*

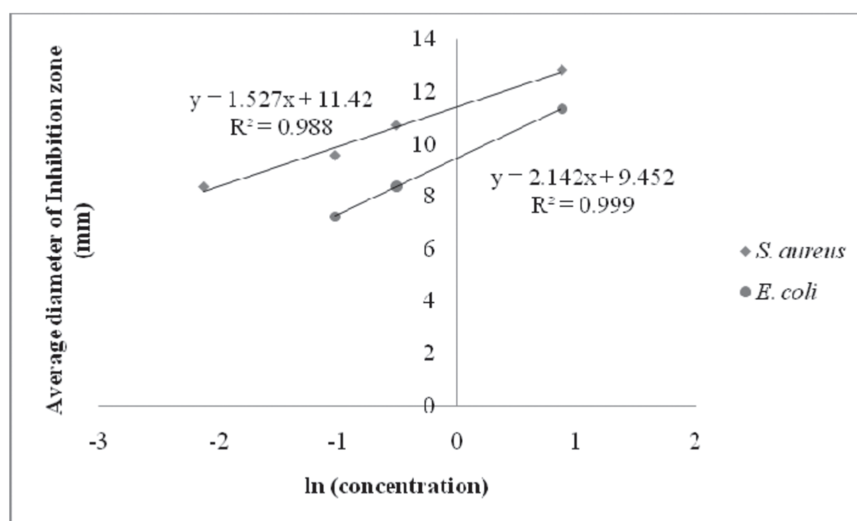

Fig. 5. Dissipative diffusion model for 9-phenyl-10-(2-phenylalkyl)acridinium bromide against *S. aureus*and*E.coli*

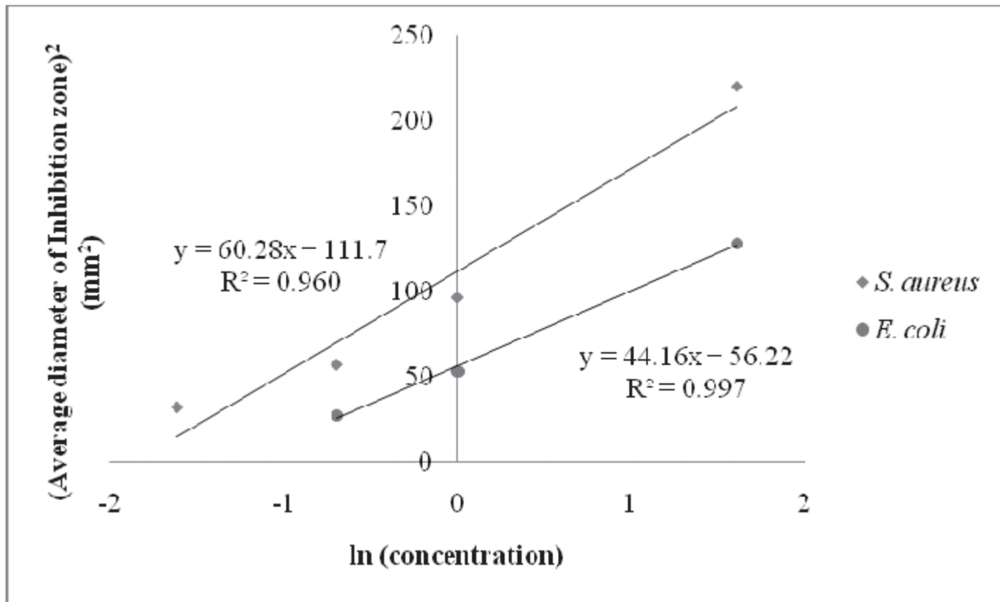

Fig. 6. Free diffusion model for 9-phenylacridinium ion against *S.aureus* and *E. coli*

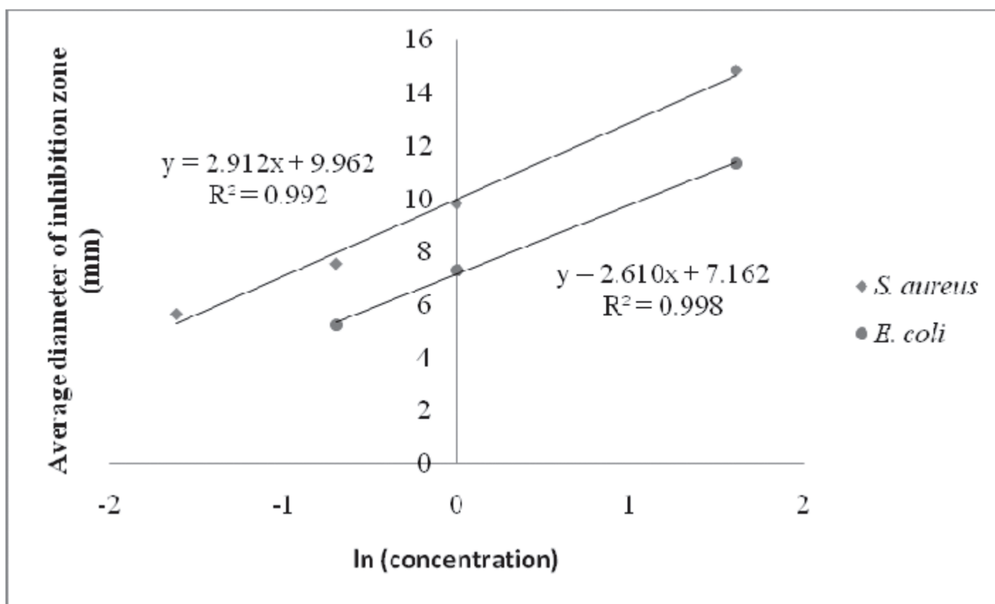

Fig. 7. Dissipative diffusion model for 9-phenylacridinium ion against *S. aureus* and *E.coli*

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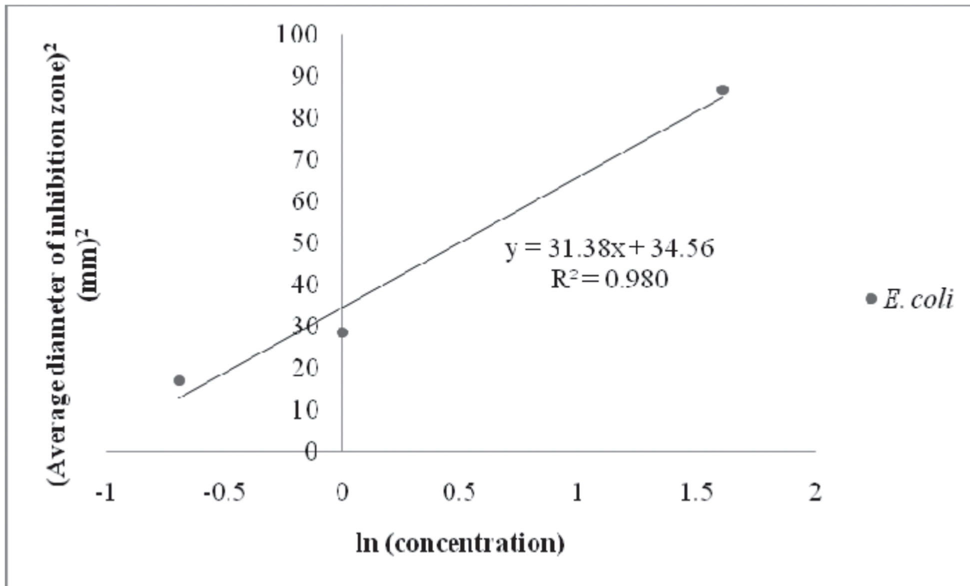

Fig. 8. Free diffusion model for 9-phenylacridine against *E. coli*

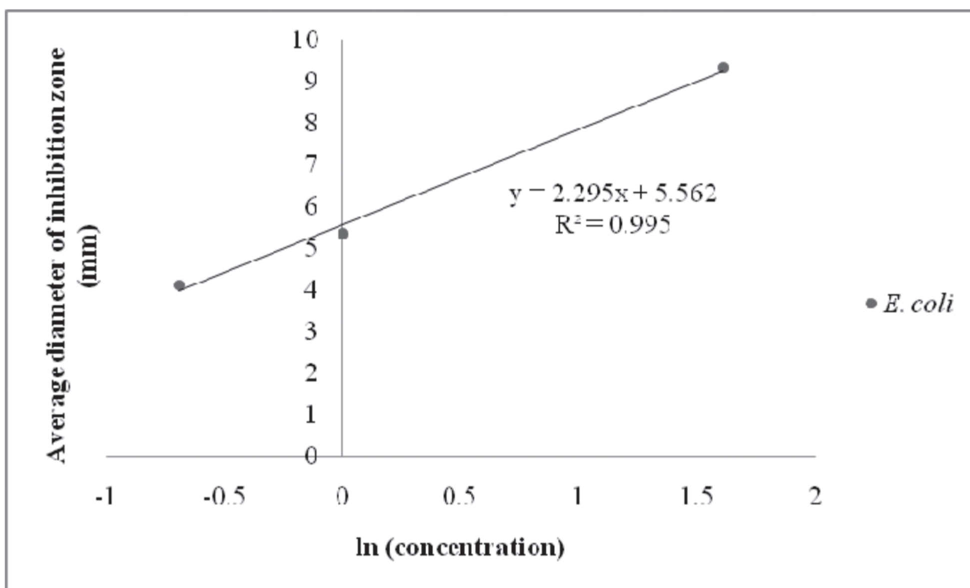

Fig. 9. Dissipative diffusion model for 9-phenylacridine against *E.coli*

(Fig. 7) for *S. aureus* (3.42 mg/ml) and for *E. coli* (2.74 mg/ml). MIC of structurally modified 9-phenylacridine, 9-phenyl-10-(2-phenylalkyl) acridinium bromide, was smaller than the MIC of 9-phenylacridine itself (Fig. 9), showing its enhanced antibacterial activity against both *S. aureus* and *E. coli* (Table 3).

According to literature the MIC for positive control, gentamycin, against *S. aureus* is 64 µg/ml and against *E. coli* it is 32 µg/ml (20, 21). MIC of the novel antibacterial agent, 9-phenyl-10-(2-phenylalkyl)acridinium bromide, was smaller than the MIC of the existing antibiotic gentamycin, indicating even higher and broader antibacterial activity against both *S. aureus* and *E. coli*. Also the MIC for *E. coli* is lower than that for *S. aureus* indicating higher sensitivity towards *E. coli*.This increased spectrum and efficiency of antibacterial activity of 9-phenyl-10-(2-phenylalkyl)acridinium bromide was resulted by the establishment of permanent positive charge on ring nitrogen. Heterocyclic ring system in 9-phenyl-10-(2-phenylalkyl)acridinium bromide gives it the lipophilicity and the permanent positive charge on the ring nitrogen improves its hydrophilicity. This amphipathic nature is a prime pharmacokinetic property which aids drug molecule to cross cell wall and cell membrane barriers (4).Apart from permanent positive charge, bulkiness of 9-phenyl-10-(2-phenylalkyl)acridinium bromide summed up by the methyl styryl group, fore-grounded its amphipathic nature which resulted in better penetration and effective DNA intercalation, thus a high antibacterial activity. Also, before protonation, 9-phenylacridine was assumed to be soluble in lipid fraction of the membrane due to its increased nonpolarity. Then the increase in water solubility after protonation may have had an effect on increased sensitivity of 9-phenyl-10-(2-phenylalkyl)acridinium bromide, as ion is more soluble in water.

Furthermore, according to MBC analysis of 9-phenyl-10-(2-phenylalkyl)acridinium bromide, MBC values for both *S. aureus* (375 µg/ml) and *E. coli* (6000 µl/ml) were significantly higher than its MICs (Table 3). The reason for the discrepancy between MIC and MBC might be the mechanism of action which the drug exerts the bactericidal effect. MIC and MBC are similar in bactericidal compounds such as lytic substances and membrane disorganizers. But protein synthesis inhibitors and substances affecting DNA do not cause immediate death and need a higher concentration to kill (22). Therefore, MBC of such compounds are higher than MIC.

Conclusion

In summary, the novel acridine derivative was shown to possess an *in vitro* antibacterial activity against both Gram-positive and Gram-negative bacteria. Compared to the MIC of the positive control Gentamycin, 9-phenyl-10-(2-phenylalkyl)acridinium bromide was shown to be effective at a lower concentration with MIC values of 4.41 µg/ml and 7.48 µg/ml for *E. coli* and *S. aureus*, respectively. Future work can be extended to develop an effective antibacterial agent against both *S. aureus* and *E coli* using 9-phenyl-10-(2-phenylalkyl) acridinium bromide. Particularly, such extension would be worthwhile considering continually developing drug resistance.

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References

1. Popiolek, L., Kosikowska, U., Mazur, L., Dobosz, M. and Malm, A. (2013). Synthesis and antimicrobial evaluation of some novel 1,2,4-triazole and 1,3,4-thiadiazole derivatives, 22:3134-3147.
2. Wainwright, M. (2001).Acridine-a neglected antibacterial chromophore, 47:1-13.
3. Shaikh, B. M., Konda, S. G., Mehare, A. V., Mandawad, G. G., Chobe, S. S. and Dawane, B. S. (2010). One-pot multicomponent synthesis and antibacterial evaluation of some novel acridine derivatives, 2:25-29.

4. Jayatissa, R. N., Perera, R. P., Hettiarachchi, C. M. and Weerawarna, P. M. (2012). In vitro antibacterial activity of 4-phentl-1-(2-phenyl-allyl) pyridinium bromide: A novel class of pyridinium based antibacterial compounds, 52: 83-87.

5. Albert, A., Rubbo S. D., Goldacre, R. J., Davey, M. E. and Stone, J.D. (1945). Steps in the correlation of structure with biological activity, 26:160–92.

6. Anderson, G. G. and O'Toole, G. A. (2008). Innate and Induced Resistance Mechanisms of Bacterial Biofilms,322: 85-105.

7. Huddleston, J. G., Visser, A. E., Reichert, W. M., Willauer, H. D., Broker, G. A. and Rogers, R. D. (2001).Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazoliumcation, 3: 156-164.

8. Li, J. J. (2011). Reactions in Heterocyclic Chemistry II, John Wiley and Sons, pp.161.

9. Welton, T. (1999). Room-temperature ionic liquids. Solvents for synthesis and catalysis, 99: 2071-2083.

10. Seijas, J. A., Vazquez-Tato, M. P., Martinez, M. M. and Rodriguez-Parga, J. (2002). Microwave enhanced synthesis of acridines- A new aspect in the Bernthsen reaction, 4:390-391.

11. Wimalasena, D. S., Perera, R. P., Heyen, B. J., Balasooriya, I. S. and Wimalasena, K. J. (2008). Vesicular monoamine transporter substrate/inhibitor activity of MPTP/MPP+ derivatives: A structure-activity study, 51: 760-768.

12. Ma, Z., Day, C. S. and Bierbach, U. (2007). Unexpected reactivity of the 9-Aminoacridine chromophore in guanidylation reactions,72: 5387-5390.

13. Worsfold, D. J. and Bywater, S. (2003). Anionic polymerization of á-methylstyrene, 26: 299-304.

14. Clinical and Laboratory Standards Institute M02-A10 (2009). Performance standards for antimicrobial disk susceptibility tests; approved standard(10th edition), Clinical and Laboratory Standards Institute, Wayne.

15. Gillespie, S. H. (1994). Medical Microbiology-Illustrated, Butterworth Heinemann Ltd., United Kingdom, pp. 234–47.

16. Hawkey, P. M. and Lewis, D. A. (1994). Medical bacteriology: A practical approach, Oxford University press, United Kingdom, pp. 181–94.

17. Bonev, B., Hooper, J. and Parisot, J. (2008). Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method, 61: 1295-1301.

18. Singleton, P. (2004). Bacteria in Biology, Biotechnology, Medicine(6th edition), Wiley-Blackwell, pp. 57.

19. Wade, L. G. and JR (2003). Organic Chemistry (5th edition), USA, pp. 575.

20. Betriu, C., Rodriguez-Avial, I., Sanchez, B. A., Gomez, M., Alvarez, J. and Picazo, J. J. (2002). In vitro activities of Tigecycline (GAR-936) against recently isolated clinical bacteria in Spain, 46(3):892-895.

21. Wise, R., Andrews, J. M. and Edwards, L. J. (1983). In vitro activity of Bay 09867, a new quinoline derivative compared with those of other antimicrobial agents, 23(4):559.

22. Lorian, M. D. V. (1975). Some effects of subinhibitory concentrations of antibiotics on bacteria, 51:1046-1055.

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Phenotypic Assessment of Bt *Cry2A* Transgenic Tomato Resistant to Neonate larva of *Helicoverpa armigera*

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Abstract

Tomato (*Solanum lycopersicon*) is the most imperative vegetable crop cultivated across the world. In India, the tomato productivity is stumpy and one of the major limiting factors is the damage caused by tomato fruit borer *Helicoverpa armigera* (Lepidoptera: Noctuidae). At present there is no source of genetic resistance existing in tomato germplasm against this pest. The conventional methods for management of this particular pest are futile. Bt transgenic technology provides a secure and consistent means for management of this pest. With this analysis, the *cry2A* Bt transgenic tomato cv. Arka Vikas was developed for resistance to fruit borer through *Agrobacterium* mediated transformation. The *Agrobacterium* strain EHA 105 harboring pBINBT *cry2A* containing CaMv35S promoter, OCS terminator, the synthetic *cry2A* gene coding region and neomycin phosphotransferase II (*nptII*) selectable cassette was used for transformation. Transformants were obtained through efficient regeneration and transformation protocol developed in our lab. Using PCR and qualitative ELISA, confirmation of transgene integration and expression respectively were respectively were examined. The detached leaf insect bioassays were performed with *in vitro* reared freshly hatched first instar neonate of *Helicoverpa armigera* larvae. Transgenic plants showed extensive resistance to *Helicoverpa armigera* and we found a significant mortality (95 %) of *H. armigera* within 24 hr of bioassays and outcome of this examination on Bt tomato lines revealed that these transgenic lines were effective in management of *H. armigera*.

Keywords: *cry2A, Helicoverpa armigera, Agrobacterium,* Tomato.

Introduction

A new epoch has been reputable in area of plant biotechnology addressing the major problems faced in related area making beneficial progress in agriculture to increase the productivity by minimizing the losses occurred. Tomato is second most (FAOSTAT Database 2010) important solanaceous vegetable crop cultivated in Indian sub continent for human consumption. In view of economy, it is a short duration high yielding dietary vegetable crop consumed for nutrition. The major limiting factor in tomato production is *Helicoverpa armigera*, commonly called as tomato fruit borer (1) a polyphagus pes (2) affecting many crops and is largely distributed in its population. Application of chemical insecticides is the common practice followed for control of insect pests. Insect pests have almost developed resistance to all major chemical insecticides and its repeated application led to perilous effect on humans (3). An alternative strategy is required for the control of these insect pests. Bt transgenic technology provides a safer

and reliable means for the control of insect pests and reported earlier in tomato (4).

Bt proteins are very much transient in nature, as the toxicity of Bt proteins is specific to insect pests (5). Cry toxins significantly proved active against lepidopteran (6) and other insect pests in case of transgenic crops (7). Bt crops shows a promising way for insect control that are highly insect specific (8). All Bt crops are environmentally safe and globally 58 million hectares Bt crops were cultivated (9) and can majorly reduce amounts of chemical pesticides entering the environment. However, in few cases there is report of resistance developed to this Bt crops which demands finding of other alternative cry genes. In this regard, *cry2A* gene was opted since earlier studies on cotton plants harboring this gene showed increased resistance to *Helicoverpa armigera* (10, 11). Plant tissue culture is being the major catalyst in genetic transformation and using *Agrobacterium tumefaciens* (12) the transformation and regeneration of tomato using *cry2A* gene was introduced into local cultivar Arka Vikas (13) using MS media along with other growth regulators (BAP, IAA). *Agrobacterium* mediated gene transformation was performed by co cultivation for production of Bt transgenic tomato plants, since *Agrobacterium* mediated gene transfer have an added advantage over other biolistic gene transfer methods being genotype independent (14). *In vitro* regeneration of cultivated tomato has been a subject of research (15) and Bt transgenic tomato against Tomato fruit borer *Helicoverpa armigera* is being done due the commercial value of tomato crop and its amenability for further improvement via genetic manipulation.

Materials and Methods

The experiment was conducted at the Biopesticides laboratory, Division of Biotechnology, Indian Institute of Horticultural Research Bangalore. *H. armigera* larva were reared in a controlled environment (25 ±2°C, 65±5% R.H.,) and freshly hatched neonate larvae were used for challenging and observation. The transgenic plants raised by the means of *Agrobacterium* mediated transformation. This transformation was mainly done using hypocotyls part as explants. Shoot regeneration were achieved in the transformed explants by different hormonal combination like (BAP, IAA), then the root were induced by the hormone IBA followed by hardening in glasshouse. The transformed explants were isolated from the non-transformants in the kanamycin selection media. The presence of transgene was confirmed by polymerase chain reaction using *npt*II (for kanamycin resistance) and *cry2A* gene specific primers. The protein expression was confirmed qualitatively by lateral flow immunodiagnostic assay method (Bt strip method, Desigen™ Jalna). The molecularly confirmed transformants were challenged with *in vitro* reared freshly hatched neonate larvae of *Helicoverpa armigera* Hubner. The bioassays performed *in vitro* and results of this testing were discussed.

***Plasmid and bacterial strains used*:** Plasmids pBin *cry2A* were transformed into *Agrobacterium* strain EHA- 105 along with the gene *cry2A*, selectable marker *npt*II – (neomycin phosphotransferase II) which was used for selection of transformed plants (Fig.1).

***Plant transformation*:** Tomato seedlings were grown aseptically on half-strength Murashige and Skoog (MS) medium. Hypocotyls from 8-10 day old seedlings were used for co-cultivation with *A. tumefaciens*(16). Overnight grown *A. tumefaciens* containing gene in modified M_9 medium for 24 h at 28°C and diluted 20-fold before use. Explants were co-cultivated with diluted *A. tumefaciens* containing *cry2A* gene. The explants were incubated in Petri dishes on regeneration/selection medium containing MS salts, 3% sucrose, 100 mg/l kanamycin, 250 mg/l Cefotaxime and 0.25% Gelrite (pH 5.7-5.8). The culture conditions were maintained at 25°C, 16 h photoperiod. The explants were regularly sub-cultured every two weeks. The regenerated shoots were grown on shoot elongation media and rooting were done on root induction medium containing MS salts, 3% sucrose, 50 mg/l

kanamycin. The rooted plants were transplanted into soilrite for hardening. After establishment, the plants were shifted to pots into the net house.

Conformation of cry2A transgene by Polymerase chain reaction (PCR): The presence of the transgene was investigated in the T_1 generation obtained from the selfing of the T_0 plants, by analyzing for the presence of *cry2A* transgene by PCR. Genomic DNA was isolated from the leaves of all putative transformants. PCR analysis was carried out by using the primers specific to *npt*II gene.

Forward primer: 5'-AGAAGAACTC GTCAAGA AGGC-3'.

Reverse primer: 5'- GAACAAGATG GATTGCA CGCA-3'.

PCR was carried out using standard reaction mixture and thermal cycling was performed for 30 cycles using following parameters: 94°C-10 min; 54°C -90 sec and 72°C, 45sec.

Insect bioassays: *Helicoverpa armigera* was artificially reared in the laboratory on modified semi-synthetic diet(17) with slight modifications. The leaves of transformed plants hardened in glasshouse were collected and placed on moisture white filter paper (Fig. 3). The freshly hatched neonate larvae starved for 2-3 hr and released on leaves of control and transgenic leaves. Insect mortality data was recorded with 24 hr intervals. The experiment was repeated thrice for each plant and observation was taken along with confirmation of alive and dead larva after treatment under stereo microscope. All laboratory experiments conducted for bioassay were kept at 25±2°C, 65±5% RH.

Assessment of transgenic plants in the glasshouse for insect-resistance was also performed with challenge inoculation of tomato fruit borer *H. armigera* at glasshouse conditions and this test includes *cry2A* transgenic lines (T_1 generation), the cultivar cv. Arka Vikas as susceptible control for comparison. Using fine camel hairbrush two larvae were released on both fruit in plant and detached fruit simultaneously for bioassay. In detached leaf bioassay three larvae were released individually and observations were recorded.

Results and Discussion

The development of familiar execution procedure is necessary and reports in case of cotton indicate that Bt genes are tremendous against lepidopteran insects ensuing in elevated yields (18) and hence *cry* genes in case of tomato to local cultivars was employed. Earlier many researchers proved *cry1Ab* protein was efficient in control of tobacco hornworm but higher proteins was essential for tomato fruit worm, *Helicoverpa* species (19) and lack of resistant varieties in germplasm strongly motivates for new approach of control measures like adaptation of synthetic genes like *cry2A* that are highly specific against lepidopteran pests. This study includes *Agrobacterium* mediated transformation in tomato and molecular characterization of transgene and insect phenotyping in putative transformants. Diverse essential crops like cotton, brinjal, maize are transformed with synthetic Bt genes under the control of constitutive promoters such as CaMV 35S exhibited a wide range of levels of foreign protein expression in whole plant benefiting the farmers with less application of chemical pesticides. Many researchers in developing transgenic plants relay heavily on molecular characterization of transformants and concentrate finally on phenotypic characterization like bioassay protocols. It becomes essential that phenotypic screening should be done first so that molecular characterization can be cross checked in bioassay positive plants reducing the input in molecular screening.

In the present study, *Agrobacterium* transformation in tomato and phenotypic assessment of CRY2A protein on *Helicoverpa armigera* was performed by insect bioassays for selection of high insect resistant lines. Four transgenic lines were developed and characterized(20) and PCR was performed for both gene specific and *npt* II (Fig. 2) primers and

positive lines were selected. The insect bioassay results indicate that CRY2A protein confers superior levels of resistance towards neonate larva of *Helicoverpa armigera* on expression of high levels of Bt toxin (Fig. 3). The concentrations of Bt protein expression varies from crop to crop (21) and among individual lines with similar Bt construct(22) and for stable expression throughout the plant 35SCaMV promoter was used for enhancing the production of Bt protein in transgenic plants. Previous reports have shown that use of 35SCaMV promoter resulted in a constitutive and enhanced production of transgene derived protein (23). From *in vitro* larval insect bioassays and resistance phenotyping (24) using 1st instar larvae of *H. armigera* on the transgenic plants (T_1 generation) proved that Bt protein expression is lethal to neonate larva. In this study, we have maintained two groups of larva which among one group is allowed to feed on control tomato leaves, whereas other group of larva were allowed to feed on Bt cry2A transgenic plant leaves. Here we observed mortality only in larva group which fed on transgenic leaves (Fig.4). This indicates that the observed mortality perhaps due to consumption of cry protein. Over expression of CRY2A Bt protein may have a negative impact on growth, development of *H. armigera* (25). Larval mortality in challenged neonate larva was 90 to 95% on Bt plants except those expressed less protein and in control plants the larva entered into next instar stages. A significant difference was observed regarding mortality between control and treated larval groups and a critical difference (CD) at 5% showed a range from 5-9% during 1st day to 3rd day of post-treatment in case of transgenic leaves fed larval groups compared to control (Table 1). Plants which showed relatively high levels of CRY2A protein expression gave the better response for causing larval mortality. This study is a preliminary analysis in order to understand whether these Bt transgenics were having any effect on larvae or not and we found that these transgenic are effective in inducing mortality of *H. armigera*. Further studies are required in order to fully characterize a transgenic plant which is essential in releasing these transgenic plants for commercialization.

Conclusions

Tomato (*Solanum lycopersicum*) being an agronomically significant solanaceae crop grown primarily for its nutritional benefits and commercial values. Tomato is prone to many diseases and pests causing serious crop loss economically. Numerous chemical insecticides have been applied for controlling the propagation of harmful insects affecting food chains. The insect populations are resistant to many chemical insecticides leading to opt the most efficiently preferred toxins released by *Bacillus thuringiensis.* However analysis showed consistently high expression of *cry2A* gene in the transgenic plants exhibited total protection against the target pest in neonatal stages. Insect bioassay clearly indicates, *cry2A* transgenic tomato gave total 95% mortality of neonate larva of *Helicoverpa armigera*. Some lines transgenic tomato having *cry2A* gene developed during this experiment showed high mortality rates of *H. armigera* larvae proving their potential in insect controlling strategies and this also may help in delaying the resistance development to Bt toxins. This phenotyping screening based challenge inoculation in other crop species can be utilizes for genetic improvement as well as for diverse investigations in the field of transgenic screening. This study is an example in demonstrating the effectiveness of *cry2A* gene in controlling of *H. armigera* and this demonstration could be precedent for Bt transgenic mediated management of other pests in other crops.

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References

1. Mitter, C., Poole, R.W. and Matthews, M. (1993). Biosystematics of the Heliothinae (Lepidoptera: Noctuidae). Annual Review of Entomology. 38: 207-255.

2. Fitt, G. (1989).The ecology of Heliothis species in relation to agro ecosystems. Annual Review of Entomology. 34: 17-52.

3. Ferre, J., Van Rie, J. and MacIntosh, S.C. (2008). Insecticidal genetically modified crops and insect resistance management (IRM). In: Romeis J, Shelton AM, Kennedy GG (eds) Integration of insect-resistant genetically modified crops within IPM programs. Springer, Berlin, Heidelberg, pp 41–85.

4. Rhim Seong-Lyul, Cho Hwa-Jin, Kim Byung-Dong, Schnetter Wolfgang and Geider Klaus (1995). Development of insect resistance in tomato plants expressing the ä-endotoxin gene of *Bacillus thuringiensis* subsp. *tenebrionis*. Molecular Breeding. 1 (3) pp. 229–236.

5. Crickmore, N., D. R. Zeigler, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, A. Bravo and D. H. Dean. (2012). Bacillus thuringiensis Toxin nomenclature. (http://www.lifesci. sussex.ac.uk/Home/Neil _ Crickmore/Bt/).

6. Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and D. Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins.- Microbiology and Molecular Biology Reviews. 62: 775-806.

7. Mandal, C.C., Gayen, S., Basu, A., Ghosh, K.S., Dasgupta, S., Maiti, M.K., and Sen, S.K. (2007). Prediction-based protein engineering of domain I of Cry2A entomocidal toxin of Bacillus thuringiensis for the enhancement of toxicity against lepidopteran insects. Protein Eng Des. Sel. 20: 599– 606.

8. Bravo, A., Likitvivatanavong, S., Gill S. and Soberon, M. (2011). *Bacillus thuringiensis*: a story of a successful bio insecticide. *Insect Biochemistry and Molecular Biology.* 41:423–231.

9. James, C. (2010). Global Status of Commercialized Biotech/ GM Crops: ISAAA Brief No. 42. ISAAA: Ithaca, NY.

10. Alejandra Bravo, Isabel Gomez, Helena Porta, Blanca Ines Garcia-Gomez, Claudia Rodriguez-Almazan, Liliana Pardo and Mario Soberon (2013). Evolution of Bacillus thuringiensis Cry toxins insecticidal activity. Microbial Biotechnology, Jan 6(1):17–26. doi: 10.1111/j.1751-7915.2012.00342.x

11. Wu, K. M., Lu, Y.H., Feng, H.Q., Jiang, Y.Y. and Zhao, J.Z. (2008). Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin-containing cotton. Science. 321: 1676-1678.

12. Poonam Bhatia, Nanjappa Ashwath, Tissa Senaratna and David Midmore (2004). Tissue culture studies of tomato (*Lycopersicon esculentum*) Plant Cell, Tissue and Organ Culture. 78: 1–21.

13. Vageeshbabu S. Hanur, Harendra Modak, P. M., Shamseer K. Boopal, Srividya, K.N., Purushothama, A. and Asokan, R. (2009). Development of Bt transgenic eggplant (*Solanum melongena* L.) and tomato (*Solanum lycopersicon*) for resistance to the fruit borers. International Conference on Horticulture, Bangalore, 9-12, November, Oral Presentation.

14. Christou, P. (1995). Strategies for variety independent genetic transformation of important cereals, legumes and woody species using particle bombardment, Euphytica. 85:13-27.

15. Evans, D.A. and Sharp, W.R. (1983). Single gene mutations in tomato plants regenerated from tissue culture. Science. 221: 949–951.

16. Suresh Kumar, Amaresh Chandra and Pandey, K.C. (2008). *Bacillus thuringiensis* (Bt) transgenic crop: An environment friendly insect-pest management strategy.

Journal of Environmental Biology September: 29(5): p 641-653.

17. Hamed, M. and Nadeem, S. (2008). Rearing of *Helicoverpa armigera* (Hubner) on Artificial Diets in Laboratory. Pakistan Journal of Zoology. 40(6): 447-450.

18. Artim, L. (2003). Application for determination of non-regulated status for lepidopteran insect protected VIP3A cotton transformation event COT102. Submitted by Syngenta Seeds, Inc., Research Triangle Park, NC 27709-2257 to the Biotechnology Regulatory Services, Riverdale, MD.

19. Suresh Kumar, Amaresh Chandra and Pandey, K.C. (2008) *Bacillus thuringiensis* (Bt) transgenic crop: An environment friendly insect-pest management strategy. Journal of Environmental Biology September: 29(5): p 641-653.

20. Vageeshbabu, S., Hanur K. Boopal, Srividya, K.N., Poovarasan, S. Shraddha Bhalewar, Saraswathi, M. S, Vijeth V. Arya and Rami Reddy, P.V. (2011). "Development of *Cry2A* Bt transgenic tomato for resistance to the tomato fruit borer, *Helicoverpa armigera*". In Tumkur University & CSIR sponsored National Level Conference on "Dissecting the Complexities of Plant Biotechnology in the Post- Genomic Era" on 21st September, # PP- 16, P32.

21. Cohen, M.B., Gould, F. and Bentur, J. S. (2000). Bt rice: practical steps to sustainable use. International Rice Research Notes. 25:4–10.

22. Ramesh, S., Nagadhara, D., Pasalu, I.C., Kumari, A.P., Sarma, N.P., Reddy, V.D. and Rao, K.V. (2004). Development of stem borer resistant transgenic parental lines involved in the production of hybrid rice. Journal of Biotech. 111:131–141.

23. Mohammad Razi Anuar, Ismanizan Ismail and Zamri Zainal (2011). Expression analysis of the 35S CaMV promoter and its derivatives in transgenic hairy root cultures of cucumber (*Cucumis sativus*) generated by *Agrobacterium rhizogenes* infection African Journal of Biotechnology Vol. 10(42), pp. 8236-8244, 8 August, Doi: 10.5897/AJB11.130 ISSN 1684–5315.

24. Vageeshbabu S. Hanur, Boopal, K., Srividya, K.N., Poovarasan, S. Shraddha Bhalewar, Saraswathi, M. S., Vijeth V. Arya and Rami Reddy, P.V. (2012) "Resistance phenotyping of Bt Transgenic eggplant and tomato" at International conference on Plant Biotechnology for Food Security held on February 21-24.

25. Maqbool, S.B., Husnain, T., Riazuddin, S., Masson, L. and Christou, P. (1998). Effective control of yellow stem borer and rice leaf folder in transgenic rice indica varieties Basmati 370 andM7 using the novel d-endotoxin cryIIA Bacillus thuringiensis gene. Molecular Breeding 6:1–7.

Statistical Optimization of Anti-leukemic Enzyme L-Asparaginase Production by *Penicillium cyclopium*

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Abstract

A sequential optimization strategy, based on statistical experimental designs is employed to enhance L-Asparaginase production. First, a 2-level Plackett-Burman design was applied for bioprocess parameters screen that significantly influence the enzyme production. Seven culture ingredients and fermentation period were examined for their significance on enzyme production and specific activity using Plackett–Burman factorial design. $MgSO_4.7H_2O$, KCl and $(NH_4)_2SO_4$ were the most significant factors improving enzyme production process. The second optimization step was performed using fractional factorial design in order to optimize the amounts of highest positive variables had significant effect on enzyme activity. The second optimization step was to identify optimal values of the three factors that bringing about maximum L-asparaginase activity, using central composite designed experiments. The different media components were initially optimized using the conventional one-factor-at-a time method. L-asparaginase activity in the optimized medium was 160 IU mL^{-1} which increased 1.54-fold over that of the conventional one-factor-at-a time method basal medium (104.5±1.6 IU mL^{-1}).

Keywords: L-asparaginase, Plackett-Burman design, Response surface methodology, central composite design, *Penicillium cyclopium.*

Introduction

L-asparaginase (L-asparagine amido hydrolase, E.C, 3.5.1.1) belongs to an amidase group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonia. L-asparaginase is an important enzyme as therapeutic agent used in the treatment of acute lymphocytic leukemia, Hodgkin disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosacroma (1-3). Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine (4) and (5). Hence, they are not capable of producing L-asparagine and mainly depend on the L-asparagine from circulating plasma pools (3). Clinical trials indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man (6). L-asparaginase have been produced by bacteria such as *E. coli* (7,8), *Pseudomonas aerginosa* (9), *Erwinia chrysanthemi* (10), *Zymononas mobilis* (11), *Staphylococcus aureus* (12) and *Enterobacter aerogenes* (13). However L-asparginase produced by bacteria leads to adverse effects in human trials. Therefore, there is an extensive search for other sources of L-asparaginase production. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera such as *Aspergillus, Penicillium* and *Fusarium* are commonly reported

to produce asparaginase with less adverse effects (14) and (15). Screening and selection of the optimum concentration of the medium component are very important to determine the overall economic feasibility of the production process, (3). Optimization studies enveloping a-one-factor- at – a – time approach is tedious and tend to overtook the effects of interacting factors but might lead to misinterpretations of results, (16). In contrast, statistically planned experiments effectively tackle the problem which involves the specific design of experiments that minimizes the error in determining the effect of variables and the results achieved in an economical manner, (17). Statistical experimental designs can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (18-21).

Our objective was to elaborate the best conditions for production of extracellular L-asparginase by *Penicillium cyclopium* through a stepwise optimization strategy including: firstly, elucidation of medium and environmental components that affect enzyme production significantly using a two-level screening design (22) and (23). Secondly,optimization of the most significant components by central composite design. Creating a mathematical model expressing the relationship between optimized factors level and L-asparaginase production and verification of the model and monitoring the production pattern.

Materials and Methods

Microorganism: *Penicillium cyclopium* was kindly obtained from department of natural and microbial product, National Research Centre (NRC), Cairo, Egypt. The fungal strain was routinely grown on potato-dextrose agar (PDA) medium at 30°C and preserved at 80ºC in 50% (v/v) glycerol.

Cultivation conditions and crude enzyme extraction: The fungal strain was grown for 3 days at 30 °C in the fermentation medium which has the following composition (g/l) : sucrose, 2; L-aspargine 10.0, ; $NH_4(SO_4)_2$, 8.77 , KH_2PO_4 1.52; KCl , 0.52; $MgSO_4.7H_2O$, 0.52; and $Cu(NO_3)_2.3H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ as trace elements. The pH of the medium was initially adjusted at pH 6.2, the flasks were autoclaved for 15min at a pressure of 1.5 Ib/inch^2 to raise the temperature to 121°C. 250 ml Erlenmeyer flasks each containing 100ml of the fermentation medium were then inoculated with 18 ml of the fungal suspension previously prepared as standard inocula (6ml contain 13 x 10^6 spores/ml) then incubated at 30°C on a reciprocal shaker (200 rpm) for 3 days . Extracellular enzyme was prepared by centrifugation at 5000 rpm for 20 min, The supernatant obtained from the culture broth was used for the determination of the L-asparaginase activity.

Protein determination:The protein content was determined according to Lowry method (24).

Enzyme assay : L-asparaginase was assayed colorimetrically (25). A standard curve was prepared with ammonium sulfate. L-asparaginase activity was expressed in terms of International unit (IU). One unit (IU) of L-asparaginase is defined as the amount of enzyme which liberates 1 μ mole of ammonia /ml/min at 37°C.

Medium Optimization Using One-Factor-at-a-Time: The production of L-asparaginase was optimized based on varying the carbon sources, nitrogen sources, incubation period, inoculum size, inoculum age, various initial pH as part of the culture conditions. The influence of various carbon sources namely glucose, maltase, galactose, lactose, sorbitol, fructose, sucrose and dextrin was studied for the enzyme production by *P. cyclopium*. The effect of different nitrogen sources organic or inorganic nitrogen sources on the L-asparaginase production was also examined.

Statistical designs

Plackett-Burman design: For screening purpose, various medium components as well

as incubation time were evaluated. The different factors were prepared in two levels, (-1) for low level and (+1) for high levels, based on a Plackett-Burman statistical design (23). This design is recommend when more than five factors are under investigation (26). This design is practical especially when the investigator is faced with large number of factors and is unsure which settings are likely to produce optimal or near optimum responses (27). The actual values of the variables at low levels (-1) and high levels (+1) is given in Table (2) while Table (3) illustrates the factors under investigation as well as levels of each factor used in the experimental design. Plackett-Burman experimental design is based on the first order model, each row represents a trial run and each column represents an independent variable concentrations.

$$Y= B_0 + \Sigma B_i X_i \qquad \text{Eq. 1}$$

Where Y is the response (L-asparaginase activity) , B_0 is the model intercept and B_i is the variables estimates. The effect of each variable was determined by following equation:

$$E(X_i) = 2(\Sigma M_i^{+} - M_i^{-})/N \qquad \text{Eq. 2}$$

Where $E(X_i)$ is the effect of the tested variable. M_i^{+} *and* M_i^{-} represent L-asparaginase activity from the trials where the variable (X_i) measured was present at high and low concentrations, respectively and N is the number of trials in Eq. 2. The standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (*p*-value) of each concentration effect was determined using student's *t*-test

$$t(X_i) = E(X_i)/SE \qquad \text{Eq. 3}$$

Where $E(X_i)$ is the effect of variable X_i

This model describes interaction among factors and it is used to screen and evaluate the important factors that influence asparaginase production and L-asparaginase activity. Seven independent variables were screened in eight combinations organized. All trials were performed in duplicates and the average of L-asparaginase yield observations were treated as responses.

Central composite design: After the identification of components affecting the production by Plackett–Burman design three variables ($MgSO_4 . 7H_2O$, KCl, $(NH_4)_2SO_4$ concentrations) were selected for response surface methodology of central composite design (CCD), (28) and (29). CCD proposed by (30-32) ,was selected for this study, a 2^3 factorial design with six star points and six replicates at the central points were employed to fit the second-order polynomial model, the experimental design consisted of 20 runs and the independent variables were studied at five different levels. The experimental design used for the study is shown in Table 3. All the experiments were done in triplicate and the average of L-asparaginase production obtained was taken as the dependent variable or response (*Y*). The second-order polynomial coefficients were calculated and analyzed using the 'SPSS' software (Version 16.0) Second degree polynomials, Eq.(4), which includes all interaction terms, were used to calculate the predicted response:

$$Y_{Activity} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \qquad \text{Eq. 4}$$

Where $Y_{Activity}$ is the predicted activity of L-asparaginase (U/ml), and X_1, X_2 and X_3 are the independent variables corresponding to the concentration of $MgSO_4 . 7H_2O$ and KCl, $(NH_4)_2 SO_4$ respectively; β_0 is the intercept, β_1, β_2 , β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are quadratic coefficients, β_{12}, β_{13}, β_{23} are cross product coefficients. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination for each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6).

Results and Discussion

Optimization of L-asparaginase production by P. cyclopium using the conventional one-factor-at-a time method (22): Some factors affecting L-asparaginase production were studied aiming at optimization of antileukemic enzyme activity Table 1. Incubation period played as important role in enzyme productivity as maximum enzyme activity was obtained from 48-72h reaching (68 U/ml) after 72h only after incubation on a rotary shaker (200 rpm) at 30°C. Similar results have been reported by Lapmak *et al* (33) where the highest activity of 6.3U/ml for 72h using *Bioplaris sp BR 438*. At longer incubation periods, the enzyme activity decreased which might be due to the depletion of nutrients, accumulation of toxic end products and the change in pH of the medium or loss of moisture (34).

Table 1. Effects of different compounds on L-asparaginase production by *Pencillium cyclopium*

Optimization condition Different inoculum sizes(ml)	L-asparaginase activity(U/ml)
3	26.5±2.2
6*	68±1.5
9	68.5±3.7
12	69±4
15	69.4±4.7
18	70±3.6
21	30±3.5
Different inoculum ages (day)	
1	79.5±3
2	70 ±3.6
3	17.5 ±1.8
Different pH Values	
3	25±1.5
4	30±2.3
5	40±2.5
6.2*	79.5±3
7	17.5±2
Carbon sources	
Glucose*	3±79.5
Maltose	35±4.6
Glactose	46±1.5
Lactose	53±1.8
Sorbitol	34.3±1.8
Fructose	-
Sucrose	90±1.2
N-sources	
control*	90±1.2
Ammonium sulfate	104.5±1.6
Yeast extract	35.5±5
Urea	15±2.3
Soybean meal	10.6±2
Beef extract	2.6±35.5
Peptone	15±1.5
Ammonium oxalat	26.5±1.5
Amino acid	
Tyrosine	47.7±2.3
Tryptophan	19.4±0.7
L-lysine	27±1
L-arginine	29.5±1.7
D-alanine	30.5±1.8
L-asparagine*	104.5±1.6
Glutamic acid	20.5±2

Inoculum size in terms of spores count using the Haemocytometer had a positive effect on enzyme activity up to 18ml compared to the control which had 6ml inoculum size ($13x10^6$ spores/ml); higher inoculum size resulted in leveling off the activity. Prakasham *et al.* (22) abstracted that incubation temperature, inoculum level and medium pH among all fermentation factors were major influential parameters at their individual level and contributed to more than 60% of total L-asparaginase production. Nearly similar results were obtained by Hosamani and Kaliwal (34). They found that L-asparaginase production by *Fusarium equiseti* increased readily with increase in the inoculum level and maximum enzyme activity was obtained at 20% (V/W) and least activity was obtained at 50% (V/W) inoculum level. Chanakya *et al.* (35) have reported the maximum L-asparaginase

Table 2. Actual values of the process variables

Process variables (g/l)	Sucrose X_1	L-asparagine X_2	KH_2PO_4 X_3	$MgSO_4$ $7H_2O$ X_4	KCl X_5	$(NH_4)_2SO_4$ X_6	Time X_7
Low level (-1)	1	0.5	0.75	0.32	0.32	5.7	3
High level (+1)	3	1.5	1.75	0.72	0.72	11.7	5

Table 3. Plackett-Burman experimental design for evaluation of factors (Coded levels and real values) affecting asparaginase activity.

	Tested variables									
Trial no	Sucrose	L-Asparagine	KH_2PO_4	$MgSO_4.7H_2O$	KCl	$(NH_4)_2SO_4$	Time	L-asparaginase activity (U/ml)	Protein (mg/ml)	D.W (g/100ml)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7			
1	-1(1)	-1(0.5)	-1(0.75)	+1(0.72)	+1(0.72)	+1(11.7)	-1(3)	121.333±2.0	0.23	0.30
2	+1(3)	-1(0.5)	-1(0.75)	-1(0.32)	-1(0.32)	+1(11.7)	+1(5)	21.96667±1.7	0.19	0.13
3	-1(1)	+1(1.5)	-1(0.75)	-1(0.32)	+1(0.72)	-1(5.7)	+1(5)	26±1.03	0.20	0.18
4	+1(3)	+1(1.5)	-1(0.75)	+1(0.72)	-1(0.32)	-1(5.7)	-1(3)	46.16667±2.56	0.24	0.19
5	-1(1)	-1(0.5)	+1(1.75)	+1(0.72)	-1(0.32)	-1(5.7)	+1(5)	16.33333±1.52	0.25	0.08
6	+1(3)	-1(0.5)	+1(1.75)	-1(0.32)	+1(0.72)	-1(5.7)	-1(3)	34.66667±1.52	0.23	0.25
7	-1(1)	+1(1.5)	+1(1.75)	-1(0.32)	-1(0.32)	+1(11.7)	-1(3)	62.63333±2.48	0.26	0.26
8	+1(3)	+1(1.5)	+1(1.75)	+1(0.72)	+1(0.72)	+1(11.7)	+1(5)	14.23333±1.70	0.27	0.10

production with inoculum level of 1.5ml of *Fusarium oxysporum*. The reason may be because of higher inoculum density is inhibitory to the enzyme production as too much biomass can deplete the substrate nutrients or accumulates of some non-volatile self inhibiting substances that inhibit the product formation (36). On the other hand, Mudgetti (37) reported that lower density may give insufficient biomass causing induced product formation whereas higher inoculum may produce too much biomass which is inhibitory to the product formation.

Regarding the inoculum age, the enzyme activity of *P.cyclopium* was maximum after 24h (79.5 U/ml and specific activity 397.5 U/mg protein). Further increase in the age, resulted in the decrease in enzyme production which may be due to the occurrence of microbial death phase (38). The L-asparaginase activity at various initial pH (3-7) of the fermentation media showed that maximum enzyme activity of 79 U/ml was obtained at the initial pH of 6.2. As the initial pH was increased from 3-5 the enzyme activity was found to increase and it was found

to maximum for initial pH of 6.2. The activity was found to decrease for further increase in initial pH beyond 6. Similarly, Gulati *et al*. (39) reported pH 6.2 was optimum for L-asparaginase production by *A. terreus* . Also, Sarquis *et al*. [14] and Mohsin *et al*. (40) found that L-asparaginase activity using *A. terreus* and *Penicillium sp* respectively increased with the increase of the pH of the medium up to 6. On the other hand, Ali *et al*. (41) reported that pH 7.0 was optimum for maximum production of fungal L-asparginase under submerged fermentation. This variation in pH optima may be due to the type of organism used, chemical composition of substrate, fermentation system and finally the condition in which the fermentation took place.

The influence of various carbon sources namely glucose, maltase, galactose, lactose, sorbitol, fructose, sucrose and dextrin was studied for the enzyme production by *P. cyclopium*. It was reported that the microbial synthesis of L-asparaginase is under catabolic repression and requires less amount of carbon source (42- 44). Hence, the concentration of carbon sources was fixed at 0.2% it was observed that sucrose was the best carbon source. The maximum L-asparaginase activity observed was 104.5 U/ml for sucrose followed by glucose with 79 U/ml. On the contrary, Hosamani and Kaliwal (34) reported that maximum enzyme activity was observed on using glucose as carbon source and the least activity was with sucrose.

The effect of different nitrogen sources on the L-asparaginase production was examined. Therefore, equivalent amounts to nitrogen content of the basal nitrogen source (L-asparaginase 1%) of the fermentation medium. The results showed that the most suitable nitrogen sources for L-asparaginase production was L-asparagine (104 U/ml) when it was the sole nitrogen source. Other organic or inorganic nitrogen sources repressed L-asparaginase production. Sarquis *et al*. (14) reported that ammonium sulphate enhanced both growth and enzyme activity of *A. terreus*.

Narayan *et al*. (45) have reported 2% of yeast extract as the best nitrogen source for enzyme production by *Streptomycin albidoflavus*. Amena *et al*. (46) have reported 0.25% ammonium sulphate can be used as best inorganic nitrogen source by *S.gulbargensis*. Thus, most microorganisms utilize nitrogen sources either inorganic or organic or sometimes both.

Evaluation of factors affecting L-asparaginase activity using Plackett-Burman Experimental Design: The data on L-asparaginase activity adopting the screening Plackett-Burman experiments Table 2. showed a wide variation from 14.233 to 121.333 IU. This variation reflects the importance of medium optimization to attain higher activity. The main effects of examined factors on asparaginase activity were calculated and are presented graphically in (Fig. 1). On analysis of regression coefficients, calculated t-test and *P*-values of the tested 7 ingredients Table 3., those which showed a positive effect for asparaginase activity were $(NH_4)_2SO_4$, $MgSO_4$. $7H_2O$ and KCl , while sucrose, L-asparagine, KH_2PO_4 .$7H_2O$ and incubation time showed negative effect. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Some investigators find that the confidence levels greater than 70% are acceptable (32). The first order linear model describing the correlation between the seven

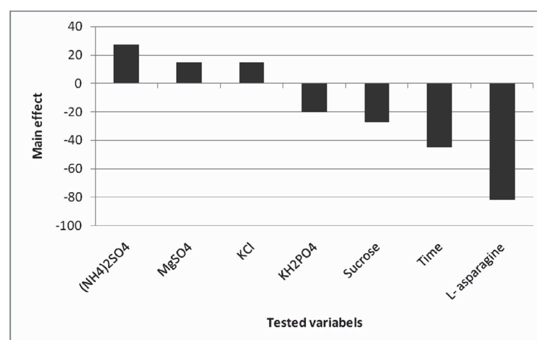

Fig. 1. Effect of culture conditions and medium composition on L-asparaginase (U/ml) produced by Penicillium cyclopium.

Table 4. Statistical analysis of Plackett-Burman design showing coefficient values, t and P-values for each variable.

Variable	Coefficients	t-statistics	P-value	Confidence level %
Sucrose	-13.65	-1.97	0.2105	0.7895
L-asparagine	-5.65	05.988	0.0003	0.9997
$KH_2 PO_4$	-10.95	01.46	0.0937	0.9063
$MgSO_4 . 7H_2O$	6.60	1.09	0.1548	0.8452
KCl	6.14	1.07	0.1587	0.8413
$(NH_4)_2SO_4$	12.12	1.99	0.0434	0.9566
Time	-23.28	-3.28	0.0067	0.9933

Table 5. Experimental plan for optimization for L-asparaginase production using central composite design.

Run	$MgSO_4 . 7H_2O$ X_1		KCl X_2		$(NH_4)_2 SO_4$ X_3		L-asparaginase activity (U/ml)	
	Coded	Actual g/l	Coded	Actual g/l	Coded	Actual g/l	Experimental	Predicted
1[a]	-1	0.75	-1	0.75	-1	9.73	83.6	88.8
2[a]	+1	1.25	-1	0.75	-1	9.73	79.9	78.9
3[a]	-1	0.75	+1	1.25	-1	9.73	105.0	128.6
4[a]	+1	1.25	+1	1.25	-1	9.73	73.0	74.8
5[a]	-1	0.75	-1	0.75	+1	13.73	74.5	70.4
6[a]	+1	1.25	-1	0.75	+1	13.73	80.0	80.7
7[a]	+1	0.75	+1	1.25	+1	13.73	132.0	137.3
8[a]	-1	1.25	+1	1.25	+1	13.73	102.0	104.1
9[a]	+1	0.5	0	1.0	0	11.73	108.0	98.6
10[b]	+1	1.5	0	1.0	0	11.73	40.0	44.5
11[b]	-2	1.0	2	0.5	0	11.73	30.0	22.6
12[b]	+2	1.0	2	1.2	0	11.73	94.5	85.9
13[b]	0	1.0	0	1.0	-2	7.73	160.0	152.4
14[b]	0	1.0	0	1.0	+2	15.73	118.5	114.3
15[c]	0	1.0	0	1.0	0	11.73	117.0	114.3
16[c]	0	1.0	0	1.0	0	11.73	117.0	114.3
17[c]	0	1.0	0	1.0	0	11.73	116.5	114.3
18[c]	0	1.0	0	1.0	0	11.73	117.0	114.3
19[c]	0	1.0	0	1.0	0	11.73	118.4	114.3
20[c]	0	1.0	0	1.0	0	11.73	119.0	113.1

a: Factorial 20 factorial designb: Star points c: Central points

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factors and L-asparaginase activity could be presented as:

$Y_{activity} = -13.65x_1 - 5.65X_2 - 10.95X_3 + 6.60 X_4 + 6.14 X_5 + 12.12 X_6 - 23.23X_7$ .... (Eq5)

Where X_1, X_2, X_3, X_4, X_5, X_6 and X_7 are sucrose, L-asparagines, KH_2 PO_4, $MgSO_4$. $7H_2O$, KCl, $(NH_4)_2$ SO_4 and time respectively.

On the basis of the calculated t-test and P-values Table 4. it was evident that the medium components MgSO4. 7H2O, KCl and $(NH_4)_2$ SO_4 were found to be the most positively significant variables affecting L-asparaginase activity. Other variables with less significant effect were not included in the next optimization experiment, but instead were used in all trials at their level (-1). According to these results, a medium of the following composition (g/l), sucrose, 1; L-asparagine , 0.5; KH_2 $PO_4.7H_2O$, 0.75 and $MgSO_4$. $7H_2O$, 0.72; KCl, 0.72; $(NH_4)_2$ SO_4, 11.7 and time 3 days were used as a plane medium for further investigations.

Central Composite Design and Response Surface Methodology: Based on Plackett-Burman design, $MgSO_4$. $7H_2O$, KCl and $(NH_4)_2$ SO_4 were selected for further optimization using response surface methodology. To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of -2, 0 & 2 in relation to production of L-asparaginase Table 5.

The results obtained from the central composite design were fitted to a second order polynomial equation to explain the dependence of L-asparaginase production on these three components.

$Y_{activity} = 33.431 + 398.054 X_1 + 558.814X_2 - 70.812 X_3 - 193.162 X \quad -240.424 X_2^2 + 2.051 X_3^2 - 175.600 X_1 X_2 + 10.300 X_1 X_3 + 13.710 X_2 X_3$ ….. (Eq6)

Where $Y_{activity}$ is the predicted response of L-asparaginase activity, X_1, X_2, and X_3 are the coded values of $MgSO_4$. $7H_2O$, KCl and $(NH_4)_2SO_4$ respectively. Presenting the experimental results in the form of surface plots (Fig. 2, 3, 4) clearly showed the response surface and contour plots of (KCl & MgSO4. 7H2O) , ($MgSO_4$. $7H_2O$ & $(NH_4)_2$ SO_4) and (KCl & $(NH_4)_2$ SO_4) on L-asparaginase production respectively, keeping the other components at the fixed (-1) level. The analysis of variance of the quadratic regression model suggested that the model is very significant as was the evident from the Fisher's F-test Table 6. The model goodness to

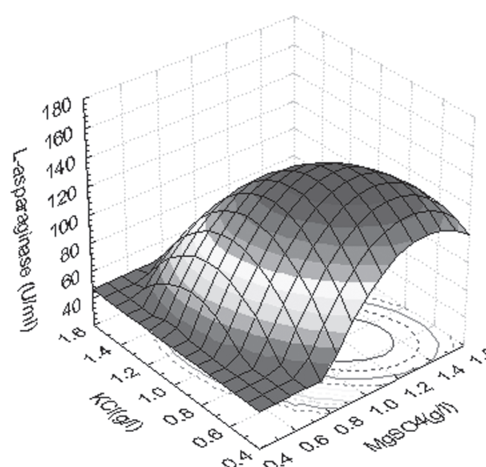

Fig. 2. Response surface plot and its contour plot of L-asparaginase production by Penicillium cyclopium showing the Interactive effects of different concentrations of KCl and MgSO4. 7H2O at X3= 0

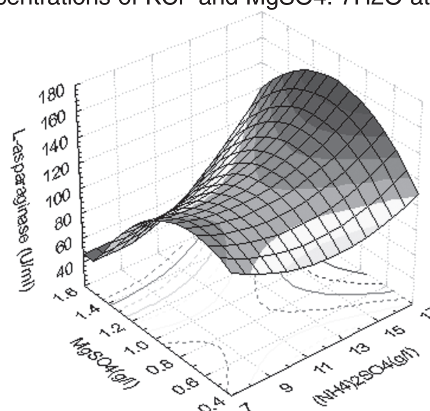

Fig. 3. Response surface plot and its contour plot of L-asparaginase production by Penicillium cyclopium showing the Interactive effects of different concentrations of MgSO4. 7H2O and (NH4)2 SO4 at X2= 0

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Table 6. Analysis of variance for L-asparaginase production by *Pencillium cyclopium* **.**

Source	Regression Coefficient	Standard error	T-test	P-value
Intercept	33.431	2369.8	0.141	0.981
X_1	398.054	161.819	2.46	0.34
X_2	558.814	179.077	3.121	0.011
X_3	-70.812	27.101	-2.613	0.26
X_1^2	-193.16	42.795	-4.514	0.001
X_2^2	-240.42	56.929	-4.223	0.002
X_3^2	2.051	0.883	2.323	0.043
X_1X_2	-175.6	76.997	2.281	0.046
X_1X_3	10.3	9.625	1.07	0.31
X_2X_3	13.71	8.004	1.713	0.117

F value =10.895; P>F=.0001; R2 =0 .907; R = 0. 953; Adjusted R2= 0 .824; SE= 13.611

fit was checked by determination coefficient (R^2). In this case , the value of R^2 (0.824) closer to 1 denotes better correlation between the observed and predicted responses . The P-values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interaction between the variables. The optimal value of L-asparaginase production increased 1.5 fold the basal conditions, which reflects the necessary and value of optimization process.

Our result show graphically, the relationship and the interaction between the independent variables ($MgSO_4$. $7H_2O$, KCl, $(NH_4)_2$ SO_4 and response (L-asparaginase activity. These factors are the most important factors and needs fine regulation for effective L-asparaginase production. However at intercalative terms, the effect of KCl vs. $MgSO_4$. $7H_2O$ interaction (-175.6) was higher than other factors interaction effects, when $(NH_4)_2SO_4$ is maintained at zero level. The results identify that these two factors are critical in the production of L-asparagines, while increasing KCl and $MgSO_4$. $7H_2O$ up to 1.4&1.4 g/ l respectively, L-asparaginase production increased steadily and, thereafter, declined sharply.

Results identify that KCl is the key factor for L-asparaginase activity this monitored by its higher regression coefficient 558.814 and t-test value 3.121 and lower p-value 0.011. Results obtained in this study are in accordance with other findings which reported that $MgSO_4$. $7H_2O$ and KCl had positive effect on the production of L-asparaginase (16). However ammonium sulphate was reported also for its importance for L-asparaginase activity **(**14).

Verification experiment: The optimized medium (of trial 1 formula) recorded maximum L-asparaginase activity of 121.3 U/ml, which is more than that of basal control medium (104 U/ml) after 3 days of fermentation. In order to verify the optimization results a verification experiment performed under the predicted optimal condition was practically compared with the basal culture medium of Plackett- Burman design in triplicates.

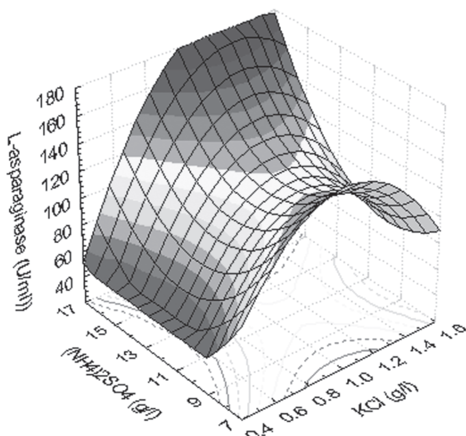

Fig. 4. Response surface plot and its contour plot of L-asparaginase production by Penicillium cyclopium showing the Interactive effects of different concentrations of KCl and (NH4)2 SO4 at X1= 0

The results showed an actual maximum L-asparaginase production 116.15%, this indicates the effectiveness of Plackett–Burman design as a tool for elucidating the most important variables affecting the response. Hoping that the present results could be considered as a contribution of description of the best fermentation conditions leading to the formation of L-asparaginase from the local isolate *Penicillium cyclopium.*

Herein, we investigated the characterizations and applications of crude L-asparaginase enzyme. The optimum temperature and optimum pH were 40 ^{0}C and 8, respectively. Activation energy 3.47 (Kcal/mol). Deactivation rate constant (min^{-1}) at 70 ^{0}C 3.7 x $10^{-3.}$ L-asparaginase enzyme production by *Penicillium cyclopium* has been successfully tested for its cytotoxic effect. The crude-enzyme extract has antiproliferative activity in different cell lines growth, however, the highest antitumor activity was recorded towards PC3 (Prostate carcinoma cell line) with (IC_{50}=11.9). Considering these properties, *Penicillium cyclopium* is a promising producer of microbial antileukemic L-asparaginase.

Conclusion

In this report, traditional optimization methods change one independent variable, while keeping the other variables fixed at a certain level. Some factors affecting L-asparaginase production were studied aiming at optimization of antileukemic enzyme activity. However, this single-dimensional search is laborious, time-consuming, and incapable of reaching a true optimum owing to the interactions among the variables. Therefore, this study applied RSM to optimize the medium conditions for L-asparaginase, we used a sequential optimization stratigy based on statistical experimental design in the production of asparaginase enzyme from *Penicillium cyclopium .* First, a- 2 -level Placket Burman design was applied to screen the bioprocess parameters that significantly influence the asparaginase production. Second, optimization step was performed using Central Composite design in order to optimize the amount of variables having the highest positive significant effect on asparaginase production. A high similarity was observed between the predicted and experimental results which reflected the accuracy and applicability of Response Surface methodology to optimize the process for asparaginase production

References

1. Stecher, A.L. , De Deus, P.M., Polikarpov, I. and Abrahão- Neto, J. (1999). Stability of L-asparaginase , an enzyme used in leukemia treatment. Pharmaceutica Acta Helvetiae. 74 : 1-9.

2. Verma, N., Kumar, K., Kaur, G. and Anand, S. (2007) . *Escherichia coli* K-12 asparaginase-based asparagine biosensor for leukemia. Artif Cells Blood Substit Immobil Biotechnol . 35: 449-456.

3. Venil, C. and Lakshmanaperumalsamy, P. (2009) .Solid state fermentation for production of L-asparaginase in rice bran by *Serratia marcescens* SB08. Inter J Microbiol . 7 : 10-18.

4. Manna, S., Sinha, A., Sadhukhan, R. and Chakrabarty, S.L. (1995). Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri MB-* 405. Curr Microbiol . 30: 291-298.

5. Swain, A.L., Jaskólski, M., Housset, D., Rao, J.K. and Wlodawer, A. (1993).Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. .Proc Natl Acad Sci USA. 90: 1474- 1478.

6. Oettgen, H.F., Old, L.J., Boyse, E.A., Campbell, H.A., Bayard, D., Clarkson, B.D., Tallal, L., Leeper ,R.D. and Schwartz, M.K. (1967) .Inhibition of leukemias in man by L-asparaginase. Cancer Res. 27: 2619-2631.

7. Derst, C., Wehner, A., Specht, V. and Rohm, K.H. (1994). States and functions of tyrosine residues in *Escherichia coli* asparaginase. Eur J Biochem. 224: 533-540.

8. Khushoo, A., Pal, Y., Singh, B. and Mukherjee, K. (2004). Extracellular expression and single step purification of recombinant *Escherichia coli* L-as paraginase [22]. Prot Exp Purif. 38 : 29-36.

9. El-Bessoumy, A.A., Sarhan, M. and Mansour, J. (2003) .Production, isolation and purification of l-asparaginase from *Pseudomonas Aeruginosa* 50071 using solid-state fermentation. J Biochem Mol Biology. 37: 387- 393.

10. Kotzia, G.A. and Labrou , N.E . (2007). L-asparaginase from *Erwinia Chrysanthemi* 3937 Cloning, expression and characterization. J Biotechnol . 127 : 657-669.

11. Pinheiro, I.O., Araujo, J.M., Ximenes, E.C.P.A., Pinto, J.C.S. and Alves, T.L.M. (2001). Production of L-asparaginase by *Zymomonas mobilis* strain CP4. Biomater Diagnos .6: 243-244.

12. Muley, R.G., Sarker, S., Ambedkar, S.and Nail, S.R. (1998). Influence of alkali treated corn steep liquor containing medium on protein production by *Staphylococcus aureus*. Folia Microbiol. 43: 31-34.

13. Mukherjeem, J., Majumdar, S.and Scheper, T. (2000). Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. Appl Microbiol Biotechnol. 53: 180-184.

14. Sarquis, M.I.D.M., Oliveira, E.M.M., Santos, A.S. and da Costa, G.L. (2004). Production of L-asparaginase by filamentous fungi. Mem. Inst. Oswaldo Cruz. 99: 489-492.

15. Baskar, G. and RenganathanIndian, S. (2009). Application of latin square design for the evaluation and screening of supplementary nitrogen source for L asparaginase production by *Aspergillus terreus* MTCC 1782. J Sci Technol. 2: 50-54.

16. Abdel-Fattah,Y.R. and Olama, Z.A. (2002). L-asparaginase production by *Pseudomonas aeruginosa* in solid-state culture, evaluation and optimization of culture conditions using factorial designs. Proce Biochem. 38: 115-122.

17. Balakrishnan, K. and Pandey, A.(1996). Production of biologically active secondary metabolites in solid state fermentation. *J Sci Ind Res* . 55: 365-372.

18. Kim, H.O., Lim, J.M., Joo, J.H., Kim, S.W., Hwang, H.J.,Choi, J.W. and Yun, J.W. (2005). Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. Bioresour Technol. 96:1175-1182.

19. Nawani , N.N. and Kapadnis, B.P.(2005). Optimization of chitinase production using statistics based experimental designs. Proce Biochem. 40: 651-660.

20. Wang, Y.X. and Lu ,Z. (2005).Optimization of processing parameters for the mycelial growth and extracellular polysaccharide production by *Boletus spp.* ACCC 50328. Proce Biochem. 40 :1043-1051.

21. Kotra, S.R., Prudvi, N., Kra, S.S., Mannava, K.K., Peravali, J.B., Anmol, K., Krs, S.R. and Pulicherla, K.K. (2013). Cost effective process for the production of fungal L-asparaginases from *pencillium sps.* isolated from local soil sample . j Pharm Med Sci. 2: 45-50.

22. Prakasham, R.S., Rao, C.S., Rao, R.S., Lakshmi, G.S. and Sarma, P.N. (2007). L-asparaginase production by isolated *Staphylococcus sp*.-6A: design of experiment considering interaction effect for process parameter optimization. J Appl Microb. 102: 1382-1391.

23. Plackett , R.L. and Bunnan, J.P. (1946). The design of multifactorial experiments. Biometrika . 33: 305-325.

24. Lowry , O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with folin phenol reagent. J Biol Chem. 193: 265-275.

25. Imada, A., Nakahama, K., Igarasi, S. and Isono, M.(1973). Asparaginase and glutaminase activities of microorganisms. J Gen Microbiol. 76: 85-99.

26. Yu, X. , Hallett , S.G. , Sheppard, J . and Watson, A.K. (1997) .Application of the Placett- Burman experimental design to evaluate nutritional requirements for the production of *Colletetrichum coccodes* spores. Appl Microbiol Biotechnol. 47 :301-305.

27. Strobel, R. and Sullivan, G. (1999). Experimental design for improvement of fermentations. In: Demain AL, Davies JE, eds. *Manual of Industrial Microbiology and Biotechnology*. Washington: ASM Press., 80–93.

28. Box ,G. E. P. and Draper, N. R. (1987). Empirical model building and response surface , John Wiley & Sons , New York.

29. Mason, R.L., Gunst, R.F. and Hers, J.L. (1989). *Statistical Design and Analysis of Experiments with Application to Engineering and Science.* New York: John Wiley & Sons. ISBN 0–471–85364-X.

30. Box, G. E. P., Hunter, W.G.and Hunter, J.S. (1978). Statistics for experimenters :an introduction to design , data analysis, and model building , John Wiley & Sons , New York.

31. Adinarayana, K., Ellaiah, P. , Srinivasulu, B., Bhavani, R. and Adinarayana, G. (2003). Response surface methodological approach to optimize the nutritional parameters for neomycin production by *Streptomyces marinensis* under solid-state fermentation. Proce Biochem. 38 : 1565-1572.

32. Stowe, A.R. and Mayer, R.P. (1966). Efficient screening of process variables. Ind Eng Chem . 58:36-40.

33. Lapmak, K., Lumyong, S., Thongkuntha, S., Wongputtisin. P. and Sardsud, U. (2010). L-asparaginase production by *Bipolaris sp*. BR438 isolated from brown rice in Thailand. Chiang Mai J Sci.37:160-164.

34. Hosamani, R. and Kaliwal, B. (2011). L-asparaginase – an antitumor agent production by *Fusarium equiseti* under solid state fermentation. Int J Drug Discovery .3:88-99.

35. Chanakya, P., Nagarjun, V. and Srikanth, M. (2011). Production of a tumor inhibitory enzyme, L-asparaginase through solid state fermentation using *Fusarium oxysporum*. Inter J Pharm Sci Rev Res. 7:189-192.

36. Bilgrami, K.S. and Verma, R.N. (1981). Physiology of fungi, 2nd edition, Vikas Publishing, Pvt. Ltd:pp.313-315.

37. Mudgetti, R.E. (1986). Solid-state fermentations. In: Microbiolog In Manual of Industrial , Demain Biotechnology Ed, A.L , Solmen , editors. N.A. 66–83. Washington DC: American Society for Microbiology.

38. Suresh, J.V. and Raju, J. K. (2013). Studies on the Production of L-asparaginase by Aspergillus terreus MTCC 1782 using agro-residues under mixed substrate solid state fermentation. J Chem Bio Phy Sci Sec. 4: 314–325.

39. Gulati, R., Saxena, R.K.and Gupta, R. (1997). A rapid plate assay for screening L-asparaginase producing microorganisms. Lett Appl Microbiol. 24:23-26.

40. Mohsin, S.M., Sunil, D.P., Siddalingeshwara, K.G., Karthic, J., Jayaramu, M., Naveen, M., Vishwanatha, T. and Prathibha, K.S. (2012). Optimization of fermentation conditions for the biosynthesis of L-asparaginase by *Penicillium sp*. J Acad Indus Res. 1:180-182.

41. Ali, S.S., Rai, V., Soni, K., Kulshrestha , P. and Lai, S.K. (1994). A fungal L-asparaginase with potential antitumor activity. Ind J Microbiol .34: 73-76.

42. Rózalska, M. and Mikucki, J. (1992). *Staphylococcal* L-asparaginase: catabolic repression of synthesis. Acta Microbiol. 41:145-50.

43. Geckil, H. and Gencer, S.(2004). Production of L-asparaginase in *Enterobacter aerogenes* expressing Vitreoscilla hemoglobin for efficient oxygen uptake .Appl Microbiol Biotechnol. 63: 691- 697.

44. Baskar, G. and Renganathan, S. (2011). Screening of supplementary nitrogen source for fungal L-asparaginase production from soya bean meal flour using latin square design . Inter J Res. Biotech Biochem. 1 :1-7.

45. Narayana, K.J.P., Kumar, K.G. and Vijayalakshmi, M. (2007). L-asparaginase production by *Streptomyces albidoflavus.* Ind J Microbiol . 48: 331-336.

46. Amena, S., Vishalakshi, N., Prabhakar, M., Dayanand, A. and Lingappa, K. (2010). Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis* . Braz J Microbiol. 41: 173-178.

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Effect of Growth Regulators on *In Vitro* Organogenesis and Long Term Storage of *Plectranthus barbatus* Andr. (Syn.: *Coleus forskohlii* (Wild.) Briq.)

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Abstract

This report describes a large scale micropropagation protocol for *Plectranthus barbatus* Andr. (Syn.: *Coleus forskohlii* (Wild.) Briq.) an important medicinal herb belonging to the family Lamiaceae. Multiple shoots were obtained from nodal explants on MS medium fortified with varying concentrations of different cytokinins in combination and with auxins. Profound proliferation of multiple shoots was noticed on the medium with cytokinins used in combination. The medium supplemented with BAP (0.25mg/l) and KN (0.25mg/l) resulted with an average of 41 multiple shoots. The shoots were very healthy with broad leaves. Individual shoots were excised and rooted *in vitro* on half strength MS medium without growth regulators. The rooted shoots were hardened in growth chamber with 90% humidity and later transferred to the field with 95% of survival rate. *In vitro* slow growth for the conservation of the plant is maintained by elimination of cytokinin for two to three passages for long-term maintenance. The root biomass of *in vitro* raised plants was compared with vegetatively propagated plants, which were raised from the same selected mother plants. The regenerated plantlets did not show detectable phenotypic variation. The protocol can be successfully applied for the production of plants suitable for field cultivation and can be conserved from over exploitation of the natural resources of the species.

Key words: Cytokinins, auxins, nodal explants, multiple shoots, conservation, long term cultures.

Abbreviations: BAP-6-benzyl amino purine; KN-kinetin; IAA – indole-3-acetic acid; IBA – indole-3- butyric acid; NAA - naphthalene acetic acid; AC - activated charcoal; $HgCl_2$ - mercuric chloride; MS -Murashige and Skoog medium.

Introduction

The use of medicinal plants is increasing worldwide. According to the World Health Organization (WHO), approximately 80% of the world's population currently uses herbal medicines in one form or another (1). In view of the medicinal, economic and commercial utility of plants and with their limitation in propagation, it becomes imperative to establish micropropagation procedures. The method provides new means of conserving and rapid propagation methods for valuable, rare and endangered medicinal plants. Excellent *in vitro* methods for mass propagation were successfully developed in certain important medicinal plants viz., *Phyllanthus urinaria* (2), *Plumbago zeylanica* (3) and *Chlorophytum* (4).

Plectranthus barbatus (syn.: *Coleus forskohlii*) is an important medicinal herb belonging to the Lamiaceae was selected for the present study. It has become a hot plant with lots of commercial value in recent years. Forskolin, a labdane diterpene possesses cardiotonic,

hypotensive and anti-inflammatory properties (5). It stimulates the production of cAMP by activating adenylate cyclase (6). The wild plants, which are the only source of forskolin have now got exhausted and pharmacy industry is today dependent on the cultivation of this plant. Hence, this plant is domesticated very recently and many scientists including pharmacy sector focused their attention on various aspects of research in this plant. *In vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (7). Plant tissue culture approach seems to be a profitable alternative among the different approaches in conservation and sustainable management of this species. It fills the gap between demand and supply of its tuberous roots for commercial gains and to provide cost-effective planting material for conventional propagation methods at a rapid rate in a short span of time. Based on these observations *in vitro* studies were carried out in *Plectranthus barbatus* by many researchers and reported a maximum of 12 multiple shoots per explant (8, 9, 10, 11, 12, 13). The principle of elimination of cytokinin for two to three passages is followed for long-term maintenance. In conclusion, the protocol standardized through this study demonstrates a rapid and cost effective method of *in vitro* regeneration and conservation of *Plectranthus barbatus.*

Materials and Methods

Plant Material and explants preparation: Germ plasm of *Plectranthus barbatus* was collected from various geographical locations of Southern India viz. Attur, Reddiarpalyam, Thiruvanamalai, Kallakurchi in Tamil Nadu, Hyderabad in Andhra Pradesh, Bangalore, Sindgi, Belgaum in Karnataka and Kolhapur in Maharashtra. Germplasm was planted in medicinal plants garden maintained by M/s Rishi Herbal Technologies, Bangalore. The nodal explants from identified genotype with high yielding forskolin were used as source of explants. The nodal segments were excised and cultured on MS medium supplemented with 0.5 mg/l BAP in order to select most suitable explant for shoot multiplication and to establish clean mother cultures. Number of shoots regenerated, nature and percent frequency of response were recorded at the end of 15 days.

Aseptic technique: The explants were washed under running tap water for 30 minutes, with Teepol for 5 minutes and ethanol 70% for 15 minutes before taking them to laminar air flow chamber. They were surface sterilized in 0.1% $HgCl_2$ (7 min) followed by 0.05% $HgCl_2$ (4 min) and again rinsed thoroughly with sterile Double Distilled Water. The nodal explants of about 1cm length were excised and inoculated on to the MS medium supplemented with 0.5 mg/l BAP for bud break and further for shoot bud regeneration on different media supplemented with combination of cytokinins, and with auxins.

Medium and cultural conditions: MS medium was used for the cultures. Excised shoot buds were then transferred on MS medium with BAP and KN and various combinations of auxin and cytokinins. The pH of the medium was adjusted to 5.6 using 0.1N NaOH or 0.1N HCl. Subcultures to the same fresh medium was carried out every 15 days initially and there after 20 days. Individual shoots were excised and rooted *in vitro* on half strength MS medium without growth regulators. They were acclimatized in growth chamber with high humidity and later in the soil with 95% of survival.

For acclimatization a mixture of soil, vermiculate and sand (2:1:1) was used in portrays and healthy roots appeared after two weeks. The survival rate of the propagation of clones was about 95%. The culture room was maintained at a temperature of 25±2°C. The cultures were kept under the light intensity of 2,000 Lux at the level of culture tubes using white fluorescent lamps. Photoperiod of 12 hrs per day was maintained. The relative humidity of the room was maintained at 90 percent. Ten/twenty replicates were kept for treatment and this experiment was repeated at least thrice to confirm the results.

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Analysis of forskolin: After harvesting roots from plants, they were dried and finely powdered. The powdered roots were extracted with benzene at 70^0 C for two hours. The benzene extracts were filtered and concentrated in vacuo. The residue was then assayed for its content of forskolin by HPLC (14).

Mobile phase and chromatographic parameters: Hexane, ethylacetate and methylene chloride were mixed in the proportion of 70:20:10 (v/v) and degassed before use. The column was equilibrated for half an hour. The mobile phase was pumped at the rate of 0.8 ml/ min with a back pressure of 200 psig. The injector and the detector were flushed with the mobile phase. The refractive index detector was set at 4x and the potentiometer chart speed was set at 0.5 cm/min. The injection volume was 10 ml and 20 ml in the analysis work. The parameters were changed for semi preparative analysis of the plant extract. They were as follows: flow rate 0.5ml/ min, pressure 100 psig, chart speed 2cm/min, detector attention 32x, injection volume 100ml.

Method: Samples of forskolin (25 mg) was dissolved in chloroform (0.5ml) and diluted to 10ml by mobile phase. The internal standard solution was prepared by dissolving in chloroform (0.5 mg) and mobile phase (10ml). Forskolin prepared in triplicates to flow the stock solutions to contain 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2, 2.25, 2.5 mg/ml in which the concentration of internal standard was always maintained at 3 mg/ ml. Each solution (20ml) was injected into the column and results were noted.

Statistical Analysis: Each experiment was repeated three times and each treatment had 20 replicates. The number of explants exhibiting regeneration was identified and the number of the shoots and roots were determined. The data of regenerated shoots and roots per explant were analyzed using ANOVA.

Results and discussion

Effect of cytokinins in different combination on shoot multiplication: The rate of multiplication depends upon usage of the correct combination of growth regulators and suitable medium for that specific organ or tissue. Subsequently the *in vitro* grown shoots from nodal explants (Fig. 1A) were used to assess optimal growth regulator requirements for maximum multiplication of shoot buds further by fortifying the medium in combination with different concentrations of BAP and KN. It is the first report that cytokinins in different combinations were studied and the results are given in Table 1. Profound proliferation of multiple shoots was noticed on the medium with cytokinins used in combination. The medium supplemented with BAP (0.25mg/l) and KN (0.25mg/l) resulted in average 41 of multiple shoots (Fig. 1B). Callus from the cut ends was observed as the concentrations of growth regulators increased. Proliferation drastically improved after 2nd and 3rd subcultures. Many cultures regenerated more than 150 shoot buds on this medium by the subsequent subculturing.

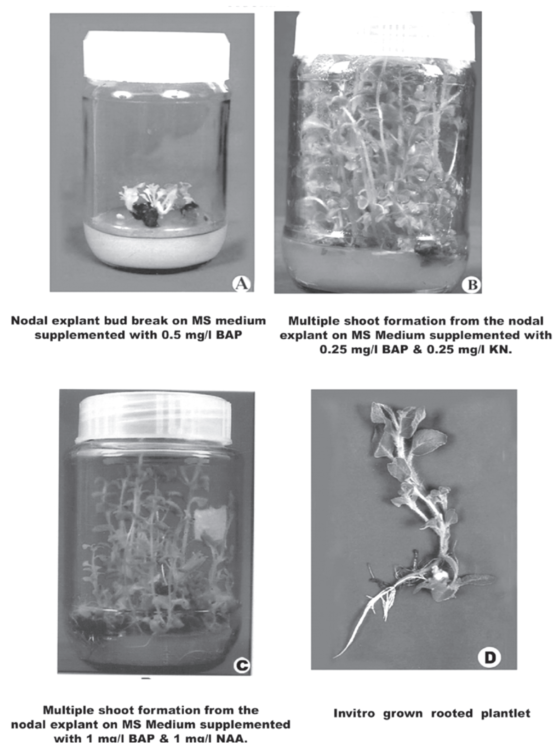

Fig. 1. *Invitro* propagation of *plectranthus barbatus*

The data subjected for statistical F-test indicate a significant difference in cultures subjected to various treatments in terms of average number of shoots (F= 1930.09**) and percentage of explants showing callus (F= 916.40**). The results indicate that the requirement of hormones for the differentiation of shoots, or roots or both is specific and well defined and a slight modification in the required level alters the growth pattern of the cultured explants (15). The result is in agreement with Malathy and Pai (16) in *Hemidesmus indicus,* Komalavalli and Rao (17) in *Gymnema sylvestre*. Callus formation from the cut ends was pronounced in the higher concentrations and might have covered shoot formation sites, besides inhibiting the already formed shoots and a similar opinion has been presented by Sreekumar *et al*. (18) in *Hemidesmus indicus.*

Effect of cytokinins and auxins in different combinations on shoot multiplication: The growth stimulating effect on MS medium supplemented with various combinations of auxin and cytokinins on multiple shoot regeneration was also observed in this study (Table: 2). Shoot emergence was observed (up to 14.67 shoots) within 10 days of sub culture in the medium BAP(1mg/l) and NAA (1mg/l) (Fig 1C). However, in this case the addition of either NAA or IAA to the MS medium supplemented with different concentrations of BAP significantly reduced the number of shoots per explant, but did not affect shoot length. Only 12 shoots per explant were developed with supplementation of IAA and KN (11) in *Coleus forskohlii.* Whereas, Sen and Sharma (19) in contrast reported 20 number of shoots in the medium fortified with BAP and IAA (2.0 and 0.05mg/l).

The data subjected for statistical analysis indicate the significant difference in cultures subjected to various treatments with respect to the percent frequency of cultures responding (F= 5.54**), average number of shoots (F= 185.27**) and percentage of explants showing root formation (F=1921.90**).The combination of cytokinins and auxins stimulate the *in vitro* multiplication and the growth of shoots of several plant species (20). However, in our case the addition of NAA or IAA to the multiplication medium significantly reduced the number of shoots per explant and the result is in accordance with the earlier report shown in *Cunila galioides* (21). In contrast the effective role of NAA/IAA in combination with BAP for shoot induction of

Table 1. Effect of combination of cytokinins for multiple shoot bud induction in *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Hormone treatment (mg/l)		Average no. of Multiple shoots/ Explant	Percentage of explants showing Callus
BAP	KN		
0.25	0.25	41.00	26.65
0.50	0.50	10.33	10.00
0.25	0.50	30.33	33.35
0.50	0.25	19.67	75.00
F – Value		1930.09**	916.40**
SEm ±		0.54	1.60
CD at 5% level		1.25	3.69

** Significant at 1% level

Data represents average of 3 experiments with 20 replications

Table 2. Effect of Cytokinins and Auxins on Multiplication of Shoots in nodal Explants of *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Growth Regulator (mg/l)		Percentage of explants showing multiple shoot formation	Average no. of shoots regenerated / explant	Percentage of explants showing root formation
BAP	NAA			
0.5	0.5	56.7	1.00	13.33
1.0	1.0	63.3	6.33	36.67
1.0	1.0	83.3	14.67	43.33
2.0	2.0	73.3	8.33	66.67
2.0	2.0	66.7	1.00	86.67
BAP	IAA			
0.5	0.5	53.3	1.00	23.33
1.0	0.5	56.7	4.33	30.00
1.0	1.0	76.7	12.33	30.00
2.0	1.0	73.3	6.33	56.67
2.0		66.7	1.00	83.33
F – Value		5.54**	185.27**	1921.90**
SEm ±		4.71	0.52	5.58
CD at 5% level		9.83	1.08	11.64

** Significant at 1% level

Data represents average of 3 experiments with 10 replications

multiple shoots has been reported in *Rauwolfia* (22).

Maintenance and multiplication from long - term cultures: In the present study, the vigour of regeneration capacity of the initial explants through different passages was studied. Maintenance of long-term cultures play a significant role in germplasm conservation. Particularly the present study which is dealing with selection of high yielding lines is very much needed as *Plectranthus barbatus* is prone to variations and abnormalities and after 3-4 sub-cultures on multiplication medium tend to show abnormalities in leaf morphology. Similar observations were reported by Sen and Sharma (19) and Sharma *et al*. (8). To avoid such abnormalities Bhattacharya and Bhattacharya (11) proposed single passage cultures. Growth retardants such as mannitol and sorbitol were added to reduce the growth and maintained germplasm conservation by least subculture technique. The principle of reduced growth storage is based on manipulation of culture condition by adding growth retardants to allow the culture to remain viable by negligible growth which could be targeted for ex-situ conservation (23).

However in the present investigation an efficient protocol was identified and optimized for maintenance of long-term cultures without use of these growth retardants. Initially cultures were initiated on a very low concentrations of cytokinin i.e. BAP (0.25mg/l). After obtaining clean cultures the axillary buds and terminal buds were transferred on to multiplication medium for a single sub-culture with duration of only 10 days.

Later these multiplied shoot buds were transferred on to basal medium. Sub-cultures were performed at 25 days interval regularly. Whenever the multiplication is needed again axillary and terminal buds from these cultures were excised and transferred to multiplication medium. By adopting this technique cultures were maintained for 3 years without any abnormalities. Rate of recovery and retainment of caulogenic potentiality was also satisfactory. Thus the elimination of cytokinin for two to three passages is essential for long-term maintenance otherwise callusing at the cut edges and other abnormalities results in. This type of response may be due to increase in endogenous hormone levels which may finally lead to the callusing and other responses generally appears with the increased levels of hormones. This shows that high cytokinin levels are necessary to initiate shoot proliferation, but once induction started the concentration has to be reduced and this result agrees with Stimart (24). In general, explants derived from micropropagated shoots have an early and greater capacity for morphogenesis than tissues excised from field plants (25) which is attributed to absence of lag period between explanting and adaptation of explants to *in vitro* conditions and presence of smaller yet active meristamatic centers of the micro plants compared to relatively larger but quiescent meristems of the shoots of mature plants (25). *In vitro* slow growth method is a better option for conserving germplasm of plants (26). Hence, it is always advisable to maintain long-term cultures than fresh initiation phases.

Rooting of shoots: The regenerated micro shoots of 7 – 8 cm length derived from nodal culture were transferred to different rooting media (Table 3). From the basal cut portion of the shoots, root regeneration was observed within 10 days of inoculation. Interestingly best root regeneration response was observed from shoots implanted on ½ MS basal medium indicating growth regulator independent rhizogenic response of shoots (Fig. 1D). All the growth regulators are effective in root induction with varied percent frequencies. However, the most critical observation is callus proliferation at the cut edges of the shoots. The percent frequency and callus mass produced significantly enhanced with increase in concentration of growth regulators. Shoots cultured on basal medium showed little or no callus at the cut edges. During rooting stage, proliferation of callus at the cut edges is not advisable as it may hamper acclimatization. This might be due to the rhizogenesis from callus instead of base of the stems. Hence, out of all the rooting media tried, basal medium is most suitable with high percent frequency (86.67%) of healthy roots. The percentage of shoots showing callus on these media is very less i.e., 8.35% and even this mass of callus in these shoots is also negligible. In the presence of NAA and IBA roots were stunted with callus on the surface. However, IAA (0.5mg/l) showed second better response (76.65%) than NAA (0.5mg/l) with 51.65% and IBA (0.5mg/l) with 61.65% in rhizogenic experiments. Reddy *et al.* (12) in their study in *Plectranthus barbatus* also confirmed the advantage of using basal media for rooting. However, Sharma *et al.* (8) and

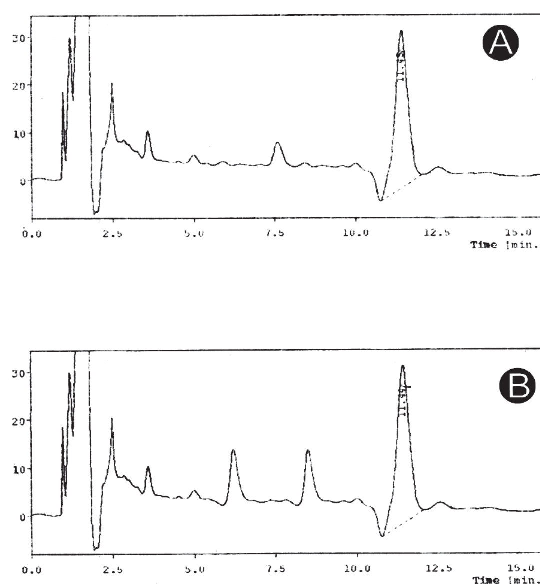

FIGURE - 2. 2A. Chromatogram (HPLC) for forskolin in invitro grown Plant with 1.9%
2B. Chromatogram (HPLC) for forskolin in vegetatively propagated Plant with 1.2%

Tabel 3. Effect of different hormone treatments (without charcoal) for *in vitro* rooting in *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Media + Hormone (mg/l)	Percentage of explants showing rooting	Percentage of explants showing callus	Average No. of roots/ Explant	Average Root Length (cm)
1/2MS + 0.50 NAA	51.65	48.35	13.33	10.00
1/2MS + 1.00 NAA	26.65	73.35	10.67	9.33
1/2MS + 1.50 NAA	8.35	91.65	9.33	8.67
1/2MS + 2.00 NAA	0.00	100.00	4.33	8.67
1/2MS + 0.50 IAA	76.65	23.35	17.67	11.67
1/2MS + 1.00 IAA	46.65	53.35	14.67	10.67
1/2MS + 1.50 IAA	26.65	73.35	10.67	8.67
1/2MS + 2.00 IAA	6.65	93.35	4.67	7.67
1/2MS + 0.50 IBA	61.65	38.35	11.67	9.67
1/2MS + 1.00 IBA	41.65	26.67	9.67	9.33
1/2MS + 1.50IBA	20.00	80.00	8.67	7.33
1/2MS + 2.00 IBA	6.65	93.35	3.67	6.67
1/2MS control	86.67	8.35	17.33	11.33
F – Value	11.20**	394.22**	133.38**	9.57**
SEm $\pm$	2.38	2.05	0.55	0.66
CD at 5% level	4.91	4.23	1.13	1.36

**Significant at 1%level

Data represents average of 3 experiments with 20 replications

Table 4. Comparative analysis of *in vitro* grown plants obtained in a period of 6months raised from each selected mother plant of *Plectranthus barbatus* (syn.: *Coleus forskohlii*)

Content of forskolin in mother plant	Vegetative Progagation			Propagation through tissue culture		
	No.of Plants obtained	Root Biomass (on dry weight basis/Kg)	Forskolin Content (on dry weight)	No.of Plants obtained	Root Biomass (on dry weight basis/Kg)	Forskolin Content (on dry weight)
1.9	493	0.082	1.2	1592	0.098	1.9

Bhattacharya and Bhattacharya (11) reported better rooting response in *Coleus forskohlii* on media containing IAA. In the present study, IAA induced callus indicating unsuitability of usage of IAA in rhizogenesis experiments and confirmed the advantage of using basal media for rhizogenesis. Regenerated plants were transferred to non-aseptic conditions for acclimatization and then to conditions of progressively lower humidity levels. Completely adapted plants were transferred to field with 100% survival rate.

For acclimatization a mixture of soil, vermiculate and sand (2:1:1) was used in portrays and healthy roots appeared after two weeks. The survival rate of the propagation of clones was 95%. The plants were hardened for 10-15 days before being transferred to the green house. In conclusion, the protocol standardized through this study demonstrates a rapid and effective method of *in vitro* shoots multiplication and plant regeneration in *Plectranthus barbatus.*

Evaluation of plants developed through vegetative propagation and tissue culture:The potential of shoot cultures and plantlets *in vitro* as a source of forskolin has been demonstrated. More or less differentiated axenic cultures, namely transformed and untransformed root cultures (5), shoot cultures (10, 11, 19) and shoot – forming callus cultures (12, 13) were reported to produce forskolin. However there is no available literature on screening of high yielding lines and their *in vitro* multiplication. In the present investigation a schematic and systematic approach was presented which resulted in production of large number of planting stocks with high yield of forskolin (1.9%).

In vitro raised plants were much healthier with broader leaves. The root number and biomass is relatively higher (Table 4). The root biomass of *in vitro* raised plants was compared with vegetatively propagated plants, which were raised from the same selected mother plants possessing forskolin 1.9% (Fig. 2A) . The forskolin content of these vegetatively propagated plants is 1.2% (Fig. 2B). The regenerated plantlets did not show detectable phenotypic variation. This might be attributed to the presence of growth regulators and continuous supply of balanced nutrition. This clearly suggests the advantage of micropropagation over conventional propagation.

References

1. Julsing, K. Matthys, Quax, J. Wim and Kayser Oliver (2007). The Engineering of medicinal plants: Prospects and limitations of medicinal plant biotechnology. In Oliver Kayser and Wim J. Quax (eds.) Medicinal Plant Biotechnology. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 978-3-527-31443-0

2. Kalidass, C. and Mohan, V.R. (2009). *In vitro* rapid clonal propagation of *Phyllanthus urinaria* Linn. (Euphorbiaceae). A medicinal plant. Researcher 1(4): 56-61.

3. Kalidass, C., Daniel Arjunan and Mohan, V.R. (2010). Rapid Micropropagation of *Plumbago zeylanica* L. An important mdicinal Plant. Journal of American Science. 6 (10): 1027-1031

4. Sautrik Basu and Baran Jha, T. (2011). *In vitro* propagation of *Chlorophytum nepale-nse*. J. Tropical Med. Plants.12:33-40

5. Mukherjee, R., Ghosh, B. and Jha, S. (1996). Forskolin synthesis in *in vitro* cultures of *Coleus forskohlii* Briq. transformed with *Agrobacterium tumefaciens*. Plant Cell rep. 15: 691 – 694.

6. Daly, J.W., Padgett, W., Seamon, K.B. (1982). Activation of cyclic AMP genetic systems in brain membranes and slices by the diterpene forskolin: Augmentation of receptor mediated responses. J Neurochem 38: 532-544.

7. Murch, S.J., Krishna Raj, S. and Saxena, P.K. (2000). Tryptophan is a precursor for melatonin and serotonin biosynthesis in in-vitro regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. Plant Cell Rep. 19: 698-704.

8. Sharma, N., Chandel, K.P.S., Srivastava, V.K. (1991). *In vitro* propagation of *Coleus forskohlii* Briq., a threatened medicinal plant. Plant Cell Rep. 10: 67 – 70.

9. Sen, J., Sharma, A.K., Sahu, N.P., Mahato, S.B. (1992). Production of forskolin in *in vitro* cultures of *Coleus forskohlii*. Planta Med. 58: 324 – 327.

10. Suryanarayan, M. and Pai, J.S. (1998). Studies in micropropagation of *Coleus forskohlii*. J. Med. Aromatic plant Sci. 20: 379 – 382.

11. Bhattacharya, R. and Bhattacharya, S. (2001). *In vitro* multiplication of *Coleus forskohlii* Briq.: An approach towards shortening the protocol. *In vitro* Cell Dev. Biol. 37: 572 – 575.

12. Reddy, P.S., Rodrigues, R. and Rajasekharan, R. (2001). Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. Plant Cell, Tissue Organ Cult. 66: 183 –188.

13. Balasubramanya, S., Rajanna, L. and Anuradha, M. (2012). Effect of plant growth regulators on morphogenesis and forskolin production in *Plectranthus barbatus* Andrews. In vitro cell Dev. Biol.-plant 48:208-215.

14. Inamdar, P.K., Kanitkar, P.V., Reden, J., De Souza, N.J. (1984). Quantative determination of forskolin by TLC and HPLC. Planta Med. 51:3 – 34.

15. Ali, G., Purohit, M., Mughal, M.H., Iqbal, M., Srivastava, P.S. (1996). Arapid protocol for micropropagation of *Bacopa monnieri* (L.) Wettst. – an important medicinal plant. Plant tissue culture and Biotechnology. 2: 208 – 211.

16. Malathy, S. and Pai, J.S. (1998). *In vitro* propagation of *Hemidesmus indicus.* Fitoterapia. 69: 533 – 536.

17. Komalavalli, N. and Rao, M.V. (2000) *In vitro* micropropagation of *Gymnema sylvestre* – A medicinal plant. Plant Cell, Tissue Organ Cult. 61: 97 – 105.

18. Sreekumar, S., Seeni, S. and Pushpangadan, P. (2000). Micropropagation of *Hemidesmus indicus* for cultivation and production of 2-hydroxy 4-methoxy benzaldehyde. Plant Cell, Tissue Organ Cult. 62: 211- 218.

19. Sen, J. and Sharma, A.K. (1991b). *In vitro* propagation of *Coleus forskohlii* Briq., a threatened medicinal plant. Plant Cell Rep. 9: 696 – 698.

20. George, E.F. (1993). Plant propagation by tissue culture. Part 1. The Technology. Exegetics Ltd., Edington. UK.

21. Fracaro, F. and Echeverrigaray, S. (2001). Micropropagation of *Cunila galiodes,* a popular medicinal plant of south Brazil. Plant Cell, Tissue Organ Cult. 64: 1 – 4.

22. Ghosh, K.C., Bhattacharya, G.N. and Benarjee, N. (2001). *In vitro* production of genetically stable clones of *Rauwolfia tetraphylla* Linn. Perspectives in cytology and genetics. 10: 725 – 729.

23. Asamenew, T. and Narayanaswamy, M.P. (2004). *In vitro* shoot multiplication and rooting in *Coleus forskohlii* Briq., AGRIS Rec.4:126-134.

24. Stimart, D.P. 1986. Commercial micropropagation of florist flower crops. In; Tissue culture as a plant production system for horticultural crops. (eds.) R.H. Zimmerman, R.J.Griesbach, F.A. Hammerschlag and R.H.Lawson. Dordrecht: Martinus Nijhoff Pub. Pp: 301-315.

25. Amin, M.N. and Jaiswal, V.S. (1987). Rapid clonal propagation of guava through *in vitro* shoot proliferation on nodal explants of mature trees. Plant Cell, Tissue Organ Cult. 9: 235 – 243.

26. Uyoh, E.A., Nkang, A.E. and Eneobong, E.E. (2003). Biotechnolgy, genetic conservation and sustainable uses of bioresources. African J Biotechnolgy.2:704-709.

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In Vitro Callus Induction and Plantlet Regeneration Studies through Anther Culture in Two *Indica* Rice (*Oryza Sativa* L.) Varieties

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Abstract

Effect of organic adjuvants, synthetic plant growth regulators and diverse carbon sources in influencing anther culture response in two *indica* rice genotypes (GR 11 and Gurjari) were assessed due to genotypic difference. Androgenic callus induction as well as green plantlet regeneration was more when callus was induced on N6 fortified with 100 mgl^{-1} yeast extract (YE). However, casein hydrolysate (CH) at 50, 250 and 500 mgl^{-1} concentrations encouraged green plantlet regeneration. High callus induction was observed with synthetic growth regulators in comparison with the control set. However, green plantlet regeneration was observed when callus induction medium (CIM) was supplemented with 2, 4-D, NAA and Kn in both the varieties. Between two varieties, Gurjari showed better callus induction on N6 medium supplemented with 6% sucrose. Maximum callus induction was observed in Gurjari when 6% sucrose was used as carbon source. Plantlet regeneration in the present study was found to be very low. This study elucidates that the genotypic differences to *in vitro* response and the probability of the exploitation of androclonal variation in *indica* rice.

Keywords: Anther culture, doubled haploids, 2,4-D, NAA, Yeast extract, Casein hydrolysate, *Oryza sativa*.

Introduction

In the recent past anther culture in rice has been improved substantially. However, detailed study on various factors governing culture response of anthers under *in vitro* conditions especially in *indica* rice is extremely limited. Generally green plant regeneration from androgenic calli is very low irrespective of the race (1). Low anther culture response, high percent of albino plantlet regeneration and abundance of haploids are the principal constraints in establishing successful anther culture in rice.

Plantlets developed directly from microspores provide ample scope in developing homozygous doubled haploids (DH) without interference of heterozygosity. Anther culture provides an easy to handle *in vitro* selection for genetic improvisation of characters (2) for superior performance. Androclonal variation seems to have immense prospect albeit it was quantified in a very few cases in rice (3). DH lines produced from microspore culture and/or anther culture were proved to be highly potential in genetic improvement by broadening the genetic diversity through production of homozygous lines within short time (4,5), in contrast to numerous cycles of inbreeding or back crossing as conventionally practiced *en route* plant breeding. Owing to the importance of anther culture in speeding up the breeding

process, plants with special agronomic characters such as development of earliness, increased grain weight, superior grain quality, disease resistance (6,7), dwarf plant type and abiotic stress tolerances have been developed (8). Unlike somaclonal variation, utilization of androclonal variation (AV) in rice genetic improvement is reported to be exceedingly less. Upon culturing anthers from an inbred line or from F_1 recombinants, one might generate androclones with altered characters different from the parent due to culture induced AV. Thus, anther culture prospects amply in rice genetic improvement. Considering the above aspects of anther culture, the present study was aimed at increasing the green plantlet regeneration by optimizing plant growth regulators (PGR), organic adjuvants and carbon sources and to asses the range and magnitude of variation in pollen derived plants of traditional cultivars, GR 11 and Gurjari which were widely grown in the state of Gujarat and are popular for its high demand in domestic and international markets owing to high consumer preference.

Materials and methods

Plant materials and callus induction: The varieties, GR 11 and Gurjari were employed for this study. The panicles with boot leaf sheath were wiped with 70% ethanol. They were covered with moist tissue paper, kept in polyethylene bag and cold shocked at 8ºC for eight days in a refrigerator prior to anther plating. On the day of culture, selected spikelets were surface sterilized in Erlenmeyer flask with 0.1% freshly prepared $HgCl_2$ solution for 10 min. The $HgCl_2$ was drained off and the panicles were washed three times in sterile distilled water. Fifty to sixty spikelets were cut at a time on sterile petri dishes under laminar airflow (LAF) bench. Individual spikelets were cut at the base to free the anthers from the filaments. They were plated aseptically onto autoclaved Erlenmeyer flasks (100 ml capacity, Borosilicate glass) containing callus induction medium (CIM) - N6 (9) supplemented with different concentrations of plant growth regulators (2,4-D at 1.0 and 2.0 mgl^{-1}; NAA at 1.0 and 2.0 mgl^{-1} and Kinetin at 0.5 and 1.0 mgl^{-1}), organic adjuvants (Yeast extract at 100, 200 and 500 mgl^{-1} and casein hydrolysate at 50, 250 and 500 mgl^{-1}) and carbon sources (sucrose, maltose and glucose at 4, 6 and 8% singly) in liquid form. The cultures were kept in dark at 25 $\pm$ 2ºC. The flasks were examined periodically at weekly intervals to observe the progress in respect of callus formation. Embryogenic calli of at least ~2 mm diameter were transferred to 25 x 150 mm culture tubes (Borosil) containing 14 ml regeneration medium consisting of MS (10) supplemented with different concentrations (2.0, 3.0 and 4.0 mgl^{-l}) of Kinetin, (1.0 and 2.0 mgl^{-l}) GA_3, 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 with 0.1N HCl or 0.1N NaOH before adding agar and autoclaved at 121ºC, 15 lb pressure for 20 minutes. The culture tubes were plugged with non-absorbent cotton wrapped in one layer of cheesecloth. Inoculated cultures were kept for four weeks under 16/8 h light (~130 $\mu E\ m^{-2}\ s^{-1}$) /dark at 25 $\pm$ 2ºC.

Results and Discussion

Effect of plant growth regulators: Higher order interaction of mean values for the effect of 2,4-D, NAA, Kn and variety on liquid medium for callus induction per cent and days to callus induction (Table 1) showed non-significant values. Analysis of variance revealed that there was significant differences for 2,4-D, NAA, Kn, variety, 2,4-D and NAA interaction, NAA and variety interaction, Kn and variety interaction and 2,4-D, NAA and Kn interaction, 2,4-D, NAA and variety interaction and NAA, Kn and variety interaction.

Present investigation showed that 2.0 mgl^{-1} 2,4-D incorporated in N6 media gave high callus induction per cent (2.07%). Overall, N6 medium supplied with 1.0 mgl^{-1} concentration of NAA showed significantly high callus induction per cent (2.13%) among NAA levels. At the same time when N6 medium was supplemented with 0.5 mgl^{-1} concentration of Kn showed overall best callus induction per cent (2.33%) as well as early callus induction (46.50 days) when only Kn levels

Table 1. Effect of 2,4-D, NAA, Kn and variety on mean values for percentage of callus initiation.

Treatment		Variety (D)			2,4-D	NAA	Kn	Variety	A x B	B x C	C x D	AxBxC	AxBxD	BxCxD
2,4-D (A)	NAA (B)	Kn (C)	GR 11	Gurjari	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
1.0	1.0	0.5	0.77	2.71	1.77	2.13	2.33	GR 11 1.70	1.65	2.42	1.84	1.74	1.16	1.74
		1.0	1.55	1.55								1.55	2.13	3.10
	2.0	0.5	2.33	2.71					1.89	1.84	2.81	2.52	1.84	2.13
		1.0	1.35	1.16								1.26	1.94	1.55
2.0	1.0	0.5	2.71	3.48	2.07	1.70	1.51	Gurjari 2.13	2.62	2.23	1.57	3.10	2.71	1.94
		1.0	2.71	1.55								2.13	2.52	2.52
	2.0	0.5	1.55	2.33					1.52	1.18	1.45	1.94	1.10	1.00
		1.0	0.65	1.55								1.10	1.94	1.36
			A x B x C x D											
S. Em. ±			0.22		0.08				0.11			0.16		
C. D. 0.05			NS		0.23				0.32			0.45		
C. V. %			20.13											

S.Em.: Standard error of mean, C.D. 0.05: Critical difference at 5.00% probability, C.V. %: Coefficient of variance per cent

Table 2. Effect of organic adjuvants and their concentrations on callus initiation per cent[A].

Treatment		Variety (C)		Average of organic adjuvant	Average of concentration	A x B Average
Organic Adjuvant (A)	Concentration mgl^{-1}(B)	GR 11	Gurjari			
Yeast Extract	100 (Low)	1.54(2.37)	1.54(2.38)	1.25 (1.57)	1.34 (1.79)	1.54 (2.37)
	200 (Medium)	1.31(1.73)	1.31(1.73)			1.31 (1.72)
	500 (High)	0.92(0.87)	0.91(0.85)		1.31 (1.72)	0.91 (0.84)
Casein Hydrolysate	50 (Low)	1.14(1.30)	1.14(1.30)	1.12 (1.26)		1.14 (1.29)
	250 (Medium)	1.31(1.73)	1.31(1.73)		0.91 (0.84)	1.31 (1.72)
	500 (High)	0.92(0.87)	0.91(0.85)			0.91 (0.84)
S. Em. ±	0.0887	0.0362	0.0444	0.0627		
C. D. 0.05	NS	0.11	0.13	0.18		
C. V. %	12.94					

[A] Square root transformation, re-transformed values in parenthesis. S.Em.: Standard error of mean, C.D. 0.05: Critical difference at 5.00% probability, C.V. %: Coefficient of variance per cent

were compared and these values were found significant. Between two varieties, Gurjari showed significantly high callus induction per cent (2.13%) than variety GR 11.

Medium N6 supplemented with growth regulator combination of 2.0 mgl^{-1} 2,4-D+ 1.0 mgl^{-1} NAA showed significantly high callus induction per cent (2.62%) than any other medium over variety and concentrations of Kn. Liquid N6 medium supplemented with 1.0 mgl^{-1} NAA + 1.0 mgl^{-1} Kn showed highest callus induction per cent (2.42%) and the value was at par with 2.0 mgl^{-1} NAA + 1.0 mgl^{-1} Kn medium. Variety Gurjari showed highest callus induction per cent on liquid N6 medium supplied with 1.0 mgl^{-1} Kn (2.81%). Liquid medium N6 supplied with 2.0mgl^{-1} 2,4-D + 1.0 mgl^{-1} NAA + 0.5 mgl^{-1} Kn showed high callus induction per cent (3.10%) which was found significant. Variety GR 11 showed highest callus induction per cent (2.71%) on medium N6 supplied with 2.0 mgl^{-1} 2,4-D + 1.0 mgl^{-1} NAA and at the same concentration of growth regulator on variety Gurjari the value was at par in the experiment. Variety Gurjari showed significantly high callus induction per cent (3.10%) when anthers were cultured on liquid N6 medium supplemented with 1.0 mgl^{-1} NAA + 0.5 mgl^{-1} Kn.

Compact embryogenic calli of ~2 mm diameter were transferred to regeneration medium (RM) for further plant regeneration. Albino plantlets were also obtained in both the varieties.

Effect of organic adjuvants: Two organic adjuvants *viz*., Yeast Extract and Casein Hydrolysate at three different concentrations were studied. Mean values for organic adjuvants, their concentration and variety interaction (Table 2) showed non significant differences for callus induction per cent and days to callus induction. Analysis of variance showed significant differences for type of organic adjuvants, concentration of organic adjuvants and interaction between organic adjuvant and its concentration for callus induction per cent. Variety showed non-significant effect for callus induction per cent as well as days to callus induction. Days to callus induction showed non-significant differences for organic adjuvants, its concentration and interaction effects.

Both the varieties produced androgenic calli in N6 supplemented with three different concentrations (100, 200 and 500 mgl^{-1}) of YE and (50, 250 and 500 mgl^{-1}) CH each. High callus induction (1.79%) was observed at low concentration (100 mgl^{-1}) of YE in both anther culture responsive varieties. This showed that lower concentration of YE facilitated androgenic callus initiation. The results also indicate substantial variation in callus induction percent for type of organic adjuvants as well as among different concentrations.

Between two organic adjuvants tried, yeast extract gave significantly higher callus induction per cent (1.25) than casein hydrolysate (1.12). Low concentration of organic adjuvant showed significant difference for callus induction per cent (1.34) and was at par with medium concentration (1.31) for the same.

When N6 medium was supplemented with yeast extract at low concentration (100 mgl^{-1}) showed significantly high callus induction per cent (1.54). At the same time medium concentration of casein hydrolysate (250 mgl^{-1}) showed better callus induction per cent among the different concentrations of casein hydrolysate.

Effect of carbon sources: Diverse carbon sources *viz*. sucrose, glucose and maltose were used to pinpoint the appropriate compound for prolific callus induction and facile green plantlet regeneration in anther culture system. Maximum callus induction (3.34%) was observed in Gurjari on N6 with 6% sucrose. Callus induction was extremely low in GR 11. However, callus induction was observed in all the treatments for both the varieties.

Mean values for type of sugars, their concentrations and variety showed non-significant interactions (Table 3). Analysis of

Table 3. Effect of sugars and their concentrations on callus initiation per cent.

Treatments		Variety (C)		Average of type of sugar	Average of concentra-tion	Average of variety	A x B	B x C
Sugar (A)	Concentration (B)	GR 11	Gurjari				Interaction	Interaction
Sucrose	4% (Low)	1.61	1.81	2.20	1.94	GR 112.05	1.71	1.88
	6% (Medium)	3.01	3.34				3.18	2.01
	8% (High)	1.61	1.81				1.71	
Glucose	4% (Low)	1.81	1.81	1.95	2.79		1.81	2.68
	6% (Medium)	1.81	2.34			Gurjari2.39	2.08	2.90
	8% (High)	1.61	2.34				1.98	
Maltose	4% (Low)	2.21	2.41	2.51	1.93		2.31	1.61
	6% (Medium)	3.21	3.01				3.11	2.25
	8% (High)	1.61	2.61				2.11	
S. Em. ±		0.17		0.0703		0.0574	0.12	0.0994
C. D. 0.05		NS		0.20		0.16	0.35	0.16
C. V. %		13.42						

S.Em.: Standard error of mean, C.D. 0.05: Critical difference at 5.00% probability, C.V. %: Coefficient of variance per cent

Table 4. Effect of Kn and GA_3 on percentage of plantlet regeneration for variety GR 11 and Gurjari

Treatments	Green Plantlet % [A]		Albino Plantlet % [A]		Total Plantlet % [B]	
Kn GA_3	GR 11	Gurjari	GR 11	Gurjari	GR 11	Gurjari
2.0 1.0	4.21 (17.24)	2.99 (8.45)	2.73 (6.97)	2.49 (5.68)	30.12 (25.18)	24.34 (16.98)
2.0	4.16 (16.82)	2.99 (8.45)	2.73 (6.97)	2.23 (4.46)	31.45 (27.22)	23.01 (15.28)
3.0 1.0	4.01 (15.60)	3.50 (11.74)	3.76 (13.60)	2.23 (4.46)	33.07 (29.78)	24.63 (17.37)
2.0	4.67 (21.29)	2.99 (8.45)	2.99 (8.45)	2.73 (6.97)	34.40 (31.92)	24.63 (17.37)
4.0 1.0	4.01 (15.60)	3.25 (9.42)	3.24 (10.00)	2.23 (4.46)	30.54 (25.82)	24.34 (16.98)
2.0	3.95 (15.13)	3.76 (13.60)	3.50 (11.74)	2.73 (6.97)	31.74 (27.68)	25.96 (19.16)
Variety Average	4.17 (16.89)	3.25 (10.04)	3.16 (9.48)	2.44 (5.45)	31.89 (27.90)	24.48 (17.18)
S. Em. ±	0.22		0.37		1.18	
C. D. 0.05	0.62		0.16		3.35	
Kn x GA_3 x Variety interaction						
S. Em. ±	0.53		0.53		2.89	
C. D. 0.05	NS		NS		NS	
C. V. %	32.07		42.01		22.92	

[A] Square root X + 0.5 Transformation, [B] ARC sin transformation, Re-transformed value in parenthesis
S.Em.: Standard error of mean, C.D. 0.05: Critical difference at 5.00% probability, C.V. %: Coefficient of variance per cent

variance showed significant difference for the mean values of type of sugar, concentration of sugar, variety, interaction between sugars and its concentration and interaction between sugar concentration and variety for callus induction per cent. Variety showed significant difference for days to callus induction. Interaction between sugar, its concentration and variety did not show significant difference for callus induction and days to callus induction.

In this experiment N6 medium supplemented with maltose showed significantly higher callus induction per cent (2.51%) followed by sucrose (2.20). Over all, medium concentration of sugar (6%) showed significantly higher callus induction per cent (2.79%) than other concentrations of sugar. Variety Gurjari showed significantly higher callus induction per cent (2.39%) but induced later callusing (50 days) than variety GR 11 (2.05% and 48 days) in the experiment.

Interaction of sugar and its concentrations on callus induction per cent showed significant differences. Sucrose at medium concentration (6%) showed highest callus induction per cent (3.18%) and was at par with the same concentration of maltose (3.11%). Gurjari variety showed significantly higher callus induction per cent (2.90) at medium concentration (6%) of sugar.

The embryogenic calli were transferred to RM for regeneration of plantlets. High percent of albino plantlets were observed from all the *in*

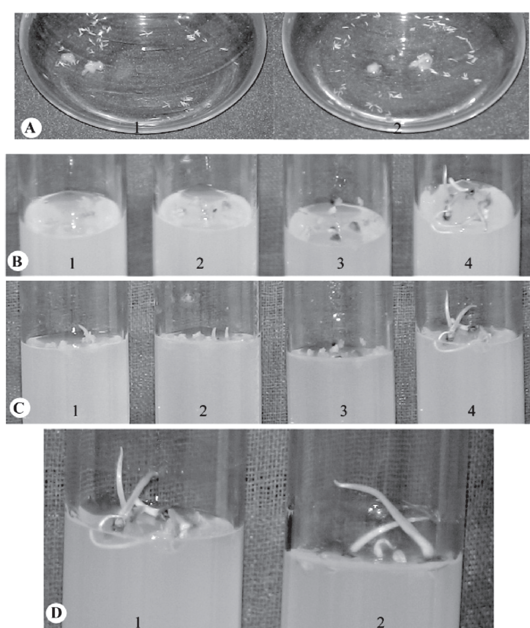

Plate 1. A callus initiation of Liquid N6 + 2.0 mgl-1 2,4 - D+1.0 mgl-1 NAA+0.5 mgl-1 kn for variety 1) GR 11 and 2) Gujari,
B1 - 4 Embryo development of variety GR 11
C1 - 4 Embryo development of variety Gurjari
D Embryo germination on semi-sold medium
1) MS+3.0 mgl-1Kn+2.0 mgl-1 GA, +3% Sucrose+0.8% Agar-agar, variety GR II
2) MS+4.0mgl-1 Kn+2.0 mgl-1 GA3 + 3% sucrose+0.8 Agar-agar, variety gurjari

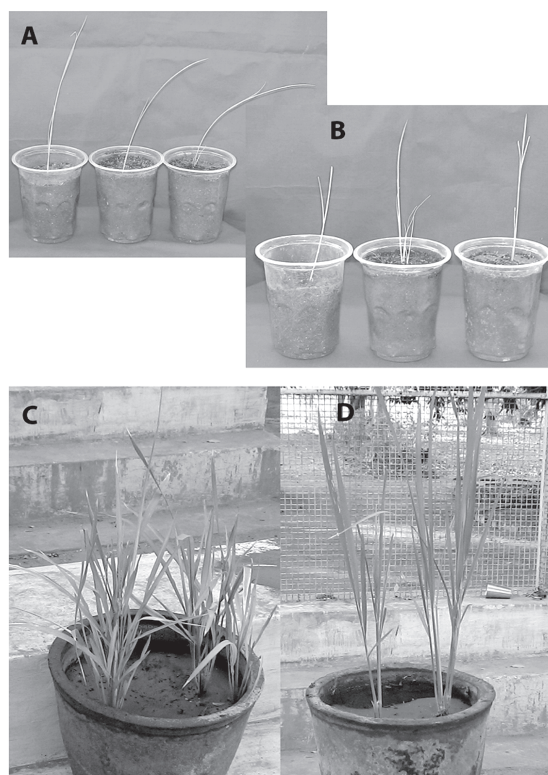

Plate II : Primary Hardened Plantlets variety a) GR II Gurjari Hardened plants established in earthen pots variety C) GR II D) Gurjari

vitro culture responsive cultivars. Mean values of green, albino and total plantlet regeneration for the effect of Kn, GA_3 and variety interaction (Table 4) showed non-significance differences. Analysis of variance showed that variety had significant effect for the number of green, albino and total plantlet regeneration. Kn and GA_3 did not show any effect on plantlet regeneration individually as well as interaction.

Variety GR 11 showed significantly high mean values for number of green (4.17), albino (3.16) and total (31.89) plantlet regeneration than variety Gurjari.

Manipulation of chemical and physical culture environments is essential to provoke microspore to switch on to an embyogenic rather than a gametophytic pattern of development (11). The effect of different treatments and varieties play an important role for callus induction and plantlet regeneration. It was also found that YE (100 mgl^{-1}), 2,4-D (2 mgl^{-1}) and 6% glucose in CIM promoted green plantlet regeneration. N6 supplemented with organic adjuvants like YE and CH showed enhanced androgenic callus induction in *indica* rice to a considerable extent. Null relationship was discernable between callus induction/plantlet regeneration and different concentrations of medium/organic adjuvants, plant growth regulators, carbon sources and genotypes. This indicates that the callus induction and subsequent green plantlet regeneration were strictly genotype specific. However, application of 6% maltose as carbon source in the medium inflated plantlet regeneration substantially. Green plantlet regeneration was found to be very low. It is mentionable that the success of plantlet regeneration under *in vitro* culture system depends on the type of medium used in each phase of culture starting from callus induction, proliferation to plantlet regeneration and the type and dose of different growth regulators especially auxins, and cytokinins used in cereal anther culture. Among the auxins, 2,4-D was found to be useful for callus induction and subsequently in green plantlet regeneration. Interestingly, number of responding calli was high in GR 11. Very low culture response was observed in Gurjari. Among the treatments, more culture response was observed when medium was added with organic adjuvants like CH and YE, plant growth regulators (2, 4-D, NAA and Kn) and maltose as carbon source. Both the varieties showed green plantlet regeneration (Plate: 1 and 2). The regenerated plantlets were rooted in the same medium. After two weeks on RM, profuse tillers were also observed. The rooted plantlets were shifted to experimental glass house for hardening. After two weeks of hardening, plants were finally transferred to earthen pots. The androgenic plantlets obtained from GR 11 showed flowering on time, however, produced cent percent spikelet sterility owing to their haploid nature. The plants developed in Gurjari also flowered and produced filled grains and with high sterility perhaps due to their genesis and development on relatively harsh *in vitro* environment.

References

1. Roy, B. and Mandal, A.B. (2005). Anther culture response in *indica* rice and variations in major agronomic characters among the androclones of a scented cultivar, Karnal local. Afr. J. Biotechnol. 4: 235-240.

2. Janhe, A., Hazze, P.A. and Lorz, H. (1991). Regeneration of fertile plants from protoplast derived from embryogenic suspension of barley (*Hordeum vulgare* L.). Plant Cells Rep. 10: 1-6.

3. Mandal, A.B., Sheeja, T.E. and Roy, B. (2000). Assessment of androclonal variation in *indica* rice PTB28. Indian J. Exp. Bio. 38: 1054 -1057.

4. Morrison, R.A. and Evans, D. A. (1988). Haploid plants from tissue culture: new plant varieties in shortened time frame. Biotechnol. 6: 684-690.

5. Foroughi-Wehr, B. and Wenzal, G. 1989. Androgenic haploid production. IAPTC News Lett. 58: 11-18.

6. Zhang, Z.H. (1989). In: Mujeeb Kazi A. and Sitch L. A. (Eds.) Review of Plant Biotechnology 1985-88: The practicability of anther culture breeding in rice. CIMMYT, Mexico and IRRI, Philippines p. 31-42

7. Zhan, Z., Zheng, Z., Goa, Y. and Cao, H. (1984). In: Cassell, T. and Cassell, P.L.C. (Eds.) Genetic manipulation of crops: Breeding and utilization of anther cultured varieties 'Xinxion' and 'HuaZao' in rice (*Oriza sativa* L.). Published for IRRI, Philippines and Academic Sinica, China, London, UK.

8. Alejar, M.S., Zapata, D., Khush, G.S. and Datta, S.K. (1995). In: M. Terzi *et al*. (Eds.) Current Issues in Plant Molecular and Cellular Biol.: Utilization of anther culture as a breeding tool in rice improvement. Kluwer Academic Pub. Netherlands, pp. 137-142.

9. Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C., Chu, C.Y. and Bi, F.Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Scientia Sinica. 5: 659-668.

10. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plantarum. 15: 473-497.

11. Srivastava, P. and Johri, B. (1988). Pollen embryogenesis. J. Palynol. 23: 83-99.

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Impact of Heavy Metals (Cr, Pb and Sn) on *In Vitro* Seed Germination and Seedling Growth of Green Gram (*Vigna radiata* (L.) R.Wilczek)

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Abstract

Heavy metal toxicity has been known for more than a century and focus on the effects of heavy metals particularly on plant growth and development has only recently received more attention due to increasing pollution day by day. The effects of chromium (Cr), lead (Pb) and tin (Sn) on green gram [*Vigna radiata* (L.) Wilczek] were studied in the present investigation under *in vitro* growth conditions. The doses of 1, 10, 20, 50 and 100 ppm were used in the present experiments. The percentage of seed germination and early seedling growth were affected by Cr, Pb and Sn at higher concentrations. It has been observed that the effect of chromium is very significant and a drastic decrease in seed germination was noticed as well as early seedling growth with increasing concentrations especially at 50 and 100 ppm when compared to other heavy metals. Both lead and tin exhibited gradual/moderate reduction in seed germination and shoot length with increasing concentrations. In contrast, an abnormal root growth was noticed in tin treatments. The content of total chlorophyll decreased with increasing chromium concentrations. Similarly, abnormal total chlorophyll content was noticed in both lead and tin treatments. This investigation may be useful to study plant growth patterns particularly in areas polluted with heavy metals.

Keywords: Green gram, *In vitro*, Tin, Toxicity, Seed germination, Seedling growth

Introduction

Heavy metals usually exist as cations under biological conditions and they affect the crop plants in many ways. They play vital role in plant metabolism. On one hand some heavy metals at low doses are essential micro nutrients for plants. On the other hand, they are toxic when they exceed their critical levels. They often lead to various metabolic disorders and inhibition of growth and development in plants (1,2). Moreover, modern civilization undoubtedly polluted the environment of our planet. Due to anthropogenic activities natural resources like air, water including soils are polluted to critical levels and have become a major threat in the recent times. Industrial wastes are the major sources of soil pollution that originate from chemical, mining and metal processing industries (3). Industrial effluents and sewage sludge are affecting our plant life (4). Moreover, these heavy metals make their way into food chains and in turn prove harmful specifically to human beings.

As mentioned above currently the agriculture land is being contaminated by heavy metals like Pb, Cd, Cu, Ni, Zn, Cr etc. There are many evidences to show that these heavy metal concentration in plants increases and they adversely affect several biological processes.

Symptoms of phytotoxicity include inhibition of seed germination or early seedling development, reduction of shoot and root growth and biochemically they affect structural and permeability properties of membranes, enzymatic activities, cause nutrient imbalances, decreased rate of photosynthesis and transpiration (5, 6, 7). However, plants have evolved several effective mechanisms to deal with the excess of heavy metals in the soil. The uptake, translocation and accumulation of heavy metals in plants are mediated by an integrated network of physiological, biochemical and molecular mechanisms. Overall, all these networks are not necessarily be always same to all the crop species. Different plants may have different tolerance mechanisms. So, it is difficult to assess the growth pattern of each crop in any heavy metal polluted areas.

Green gram is one of the most important pulse crops, native to the Indian subcontinent and widely cultivated throughout India, China, other parts of Southeast Asia and different parts of the world. Seeds of green gram are small, ovoid in shape and green in colour. These seeds are protein rich, edible and the seedlings are rich with vitamins and amino acids and are used directly without cooking (8). Apart from this, the post harvested crop is widely used as forage.

However, the productivity and quality of the grain is severely reduced due to salinity and heavy metal toxicity in many parts of the country. In the present investigation, the ability of green gram seed germination and early seedling growth under the influence of different doses of Cr, Pb and Sn were observed. There were few reports on Cr and Pb toxicity on green gram by using pot based experiments (8, 9, 10), but we used *in vitro* (under control) cultural conditions. This work may be useful to know the effect of heavy metal on the crop growth and developmental pattern in green gram.

Materials and Methods

Local seed varieties of *Vigna radiata* were used as source material for the present research investigation. The media used in the present study were without any nutrients and growth regulators. The media employed are shown in Table 1. Percentage of seed germination and the length of the shoots and roots were recorded for each metal treatment. A minimum of three replicates were taken for each experiment and all experiments were repeated thrice. The mean and excel programming techniques were used in personal computer for different parameters. Seeds were sterilized with 70% ethanol for 1 min and the ethanol was removed immediately and the material was washed once with distilled water. Seeds were then sterilized with a solution of 0.1%. $HgCl_2$ [(w/v) Fisher (P) Ltd., Mumbai, India] for 8-10 min. Surface sterilization was followed by 5-6 rinses using sterile distilled water.

All the experiments for seed germination were carried out using tap water, distilled water and finally 1, 10, 20, 50 and 100 ppm of heavy metals in identical conditions. All the cultures were incubated in a culture room at 25 ± 2°C with a relative humidity of 50-60% and 16 h photo period at a photo flux density of 15-20 Em^2S^{-1} of white fluorescent tubes. Total chlorophyll estimation was carried out using young leaves by modified methods of Arnon (11) and Witham *et al*. (12).

Media concentrations and combinations: The chromium (as potassium dichromate), lead (as lead nitrate) and tin (as stannous chloride) as sources of chemicals and entire list of media are given in table 1.

Results and Discussion

In the present study, an attempt has been made to know the effect of different heavy metals on green gram seed germination and their early growth. All the cultures along with controls (tap water and distilled water media) were studied under identical culture conditions and a comparative account of the cultures in terms of percentage of germination, shoot and root lengths were documented.

There was a reduction in percentage of seed germination with increasing concentrations of chromium. Significant reduction in 50 and 100

Table 1. Different media used in the present study

No.	Combinations	Cr (PPM)	Pb (PPM)	Sn (PPM)
1	Tap water + 0.8% Agar + pH5.7-5.8			
2	Distilled water + 0.8% Agar + pH5.7-5.8			
3	Distilled water + 0.8% Agar + pH5.7-5.8	1	1	1
4	Distilled water+ 0.8% Agar + pH5.7-5.8	10	10	10
5	Distilled water+0.8% Agar + pH5.7-5.8	20	20	20
6	Distilled water+0.8% Agar + pH5.7-5.8	50	50	50
7	Distilled water+0.8% Agar + pH5.7-5.8	100	100	100

ppm was observed when compared to control (both in tap water and distilled water media). Moreover, only 5% of seed germination at 100 ppm was noticed (Fig. 2). Such a growth inhibition was reported in green gram by Mumthas et al. (9) but we found complete inhibition at 100 ppm. Peralta and coworkers (13) also noticed suppression of seed germination in alfalfa with chromium treatments. Chromium suppressed the shoot length/growth at 20, 50 and 100 ppm. Similarly, root growth and total chlorophyll content also gradually decreased with increasing Cr concentrations (Table 2 and Fig. 1A). Fozia *et al*. (14) observed similar decrease in shoot and root lengths in sunflower with increased Cr concentrations. Chromium is widely used in industries and is toxic in its valence states both in Cr (IV) and Cr (III) and high concentration of chromium in plants show symptoms like inhibition of seed germination, reduced shoot and root growth (10).

Reduction in percentage of seed germination with increasing lead concentrations was observed in the present study (Fig. 3). Similar reduction in seed germination was observed at 75 ppm in *Leucaena leucocephola* as noticed by Shafiq et al. (15) and in wheat and lens (16). In our experiments 60% germination was recorded at 100ppm of Pb which supports the works of Singh et al. (8). Lead is one of the major heavy metal pollutants in the soil and though it does not hold any biological significance, its accumulation in plant cells and tissues may lead

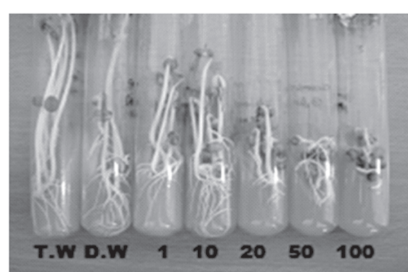

Fig-A

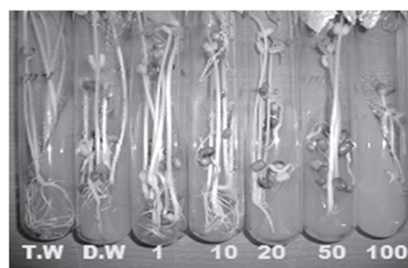

Fig-B

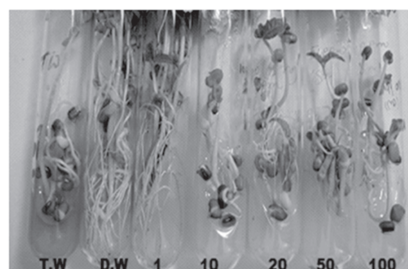

Fig-C

Fig. 1. A, B and C: Effect of chromium (Fig-A), lead (Fig-B) and tin (Fig-C) on seed germination and seedling growth
T.W- Tap water sample
D.W-Distilled water sample
1, 10, 20, 50, 100 PPM samples

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to decreased seed germination as noticied by Yang et al. in wheat (17). On the other hand, a gradual decrease of shoot and root lengths with increasing concentrations of Pb. Such a decline in shoot and root lengths was also observed by Pourrat et al. (18) especially at 100ppm (Table 3 and Fig. 1B).

Tin (Sn) is also an important heavy metal to investigate along with other heavy metals (19, 20). The effect of tin (Sn) on different aspects of early seedling germination and morphology did not vary much as noticed in lead treatments. Gradual/moderate reduction but no inhibition of percentage of seed germination was observed with increasing tin concentrations (Fig. 4). Similarly shoot length decreased with increasing Sn. But interestingly, increase in root length at 20 and 100 ppm of Sn concentrations was

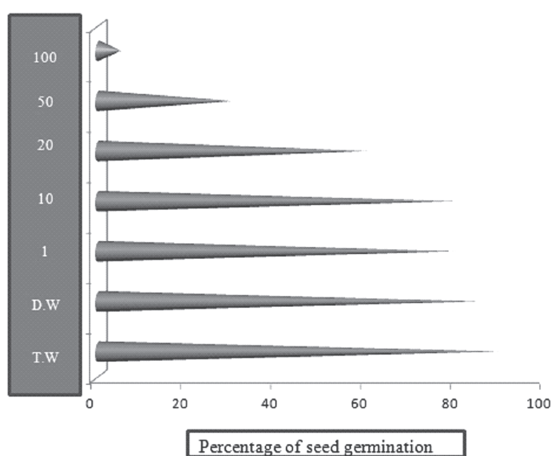

Fig. 2. Effect of chromium on percentage of seed germination

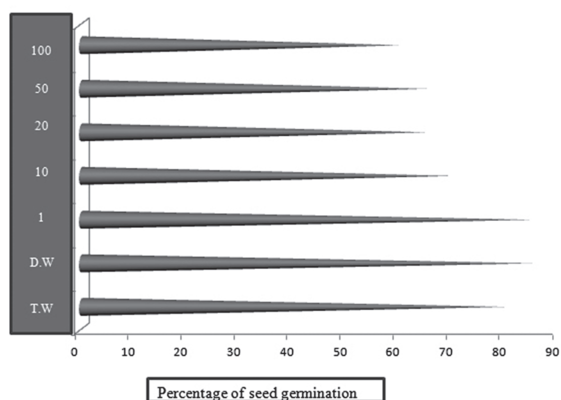

Fig.3. Effect of lead on percentage of seed germination

Table 2. Effect of chromium on early seedling growth

Conc. (Cr)	*Vigna radiata* Seedling Morphology		
	Shoot length (cm)	Root length (cm)	Total chlorophyllin mg/g.tissue
T.W	9.4	4.9	0.46
D.W	9.2	4.8	0.39
1ppm	6.2	4.4	0.35
10ppm	6.2	3.8	0.31
20ppm	3.6	1.4	0.28
50ppm	2.1	0.8	0.27
100ppm	0.5	0.5	0.24

The above data were collected from 2-3 week-old seedlings Values above represented are mean of 3 replicates

Table 3. Effect of lead on early seedling growth

Conc. (Pb)	*Vigna radiata* Seedling Morphology		
	Shoot length (cm)	Root length (cm)	Total chlorophyll in mg/g.tissue
T.W	9.6	4.1	0.38
D.W	9.6	3.9	0.32
1ppm	9.5	4.0	0.31
10ppm	9.3	3.8	0.29
20ppm	9.3	3.1	0.28
50ppm	8.9	1.1	0.29
100ppm	3.5	0.8	0.24

The above data were collected from 2-3 week-old seedlings Values above represented are mean of 3 replicates

observed (Table 4 and Fig. 1C). An unexpected increase in total chlorophyll content was noticed with 50ppm of Pb and 20 ppm of Sn treatments (Tables 3 and 4).

Table 4. Effect of tin on early seedling growth

Conc. (Sn)	*Vigna radiata* Seedling Morphology		
	Shoot length (cm)	Root length (cm)	Total chlorophyll in mg/g.tissue
T.W	8.9	4.1	0.55
D.W	8.8	7.1	0.48
1ppm	8.1	7.2	0.41
10ppm	6.3	3.4	0.37
20ppm	5.0	4.8	0.39
50ppm	4.8	2.1	0.36
100ppm	4.7	2.7	0.36

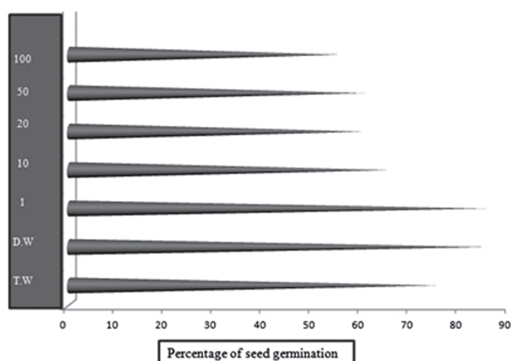

Fig.4. Effect of tin on percentage of seed germination

Conclusions

In conclusion, significant decrease in seed germination as well as seedling growth was noticed with increasing chromium concentrations. Both lead and tin exhibited moderate reduction in percentage of seed germination. Shoot length was also affected moderately with both lead and tin at higher concentrations. In contrast, abnormal root growth in different Sn treatments was observed.

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References

1. Moffat, A.S. (1995). Plants proving their worth in toxic metal cleanup, Science. 269: 302-303

2. Abbassi, S.S., Abbassi, N. and Soni, R. (1998). Heavy metals in the environment, Mittal Publication, New Delhi, India, pp314

3. Raskin, I. and Ensley, B.D. (2000). Phytoremediation of toxic metals: using plants to clean up the environment. John Wiley and Sons, New York, pp303

4. Reeves, R.D. and Baker, A.J.M. (2000). Metal accumulating plants, In: I. Raskin and B.D. Ensley (Ed.) Phytoremediation of toxic metals: using plants to clean up the environment, John Wiley and Sons, Inc, Toronto, Canada, pp303

5. Bishnoi, N.R., Chugh, L.K. and Sawhney, S.K. (1993). Effect of chromium on photosynthesis, respiration and nitrogen fixation in peas (*Pisam sativam* L.) seedlings. J Plant Physiol 142: 25-35

6. Bisht, S.S., Sharma, C.P. and Kumar, A. (1976). A Plant response to excess concentration of heavy metals. Geophytol, 6: 296-307

7. Burhan, N., Shaukat, S.S. and Tahira, A. (2001). Effect of zinc and cobalt on germination and seedling growth of *Pennisetum americanum* (L.) Schumann and *Parkinsonia aculeata* L. Pakistan J. Biol. Sci. 4: 575-580

8. Singh, R.P., Tripathi, R.D., Dabas, S., Rizvi, S.M., Ali, M.B., Sinha, S.K., Gupta, D.K., Mishra, S and Rai, U.N. (2003). Effect of lead on growth and nitrate assimilation of *Vigna radiata* (L.) Wilczek seedlings in a

salt affected environment. Chemosphere. 52: 1245-50

9. Mumthas, S., Chidambaram, A.L.A., Sundaramoorthy, P. and Sankar Ganesh, K. (2010). Impact of heavy metals on growth and metabolism of *Vigna radiata*. Int. J.Curr. Res. 2: 52-56

10. Abbasi., Hisamuddin., Robab, M.I., Akhtar, A. and Sharf, R. (2012). Chromium toxicity in mung bean, *Vigna radiata* and bioremediation by *Pseudomonas fluorescens.* Int. J. of Plant, Animal and Envi. Sci. 2: 99-101

11. Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. Plant Physiol. 24: 1-15.

12. Witham, F.H., Blaydes, B.F. and Devlin, R.M. (1971). Experiments in plant physiology, Van Nostrand Reinhold, New York, USA, pp167-200

13. Peralta, J.R., Gardea-Torresdey, J.L., Tiemann, K.J., Gomez, E.A.S. and Parsons, J.G. (2000). Study of the effects of heavy metals on seed germination and plant growth on alfalfa plant (*Medicago sativa*) grown in solid media. Proceedings of the Conference on Hazardous Waste Research, 135-140.

14. Fozia, A., Muhammad, A.Z., Muhammad. A and Zafar, M.K. (2008). Effect of chromium on growth attributes in sunflower (*Helianthus annuus* L.). J. Environ. Sci. (China). 20: 1457-80

15. Shafiq, M., Zafar, M.I. and Muhammad, A. (2008). Effects of Lead and Cadmium on germination and seedling growth of *Leucaena leucocephala*. J.Appl.Sci. Environ. Manage 12: 61-66

16. Mesmar, M.N. and Jaber, K. (1991). The toxic effect of lead on seed germination, growth, chlorophyll and protein contents of wheat and lens. Acta Biol Hung., 42: 331-44.

17. Yang, Y., Wei, X., Lu, J., You, J., Wang, W. and Shi, R. (2010). Lead-induced phytotoxicity mechanism involved in seed germination and seedling growth of wheat (*Triticum aestivum* L.). Ecotoxicol. Environ. Saf. 73: 1982-7

18. Pourrat, B., Shahid, M., Dumat, C., Winterton, P. and Pinelli, E. (2011). Lead uptake, toxicity, and detoxification in plants. Rev. Environ. Contam. Toxicol. 213: 113-36.

19. Van Assche, F. and Clijsters, H. (1990). Effects of metals on enzyme activity in plants. Plant Cell Environ. 13:195-206

20. Schafer, S.G. and Femfert, U. (1984). Tin—a toxic heavy metal? A review of the literature. Regul. Toxicol. Pharmacol. 4: 57-69.

A High-Throughput DNA Extraction Protocol and its Utilization in Molecular Characterization of Noni (*Morinda citrifolia* L.) Genotypes.

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Abstract

This study describes the standardization of DNA isolation protocol and DNA based molecular characterization of Noni, potentially designated as *Morinda citrifolia* L. Total Genomic DNA was isolated from fresh and young leaves of *M.citrifolia* and *M.tomentosa* following CTAB method with three indispensable modifications which includes methanol and PVP treatment to remove phenolics with PCI and long incubation in ethanol for enhancement of quantity of DNA. Upon gel documentation of isolated DNA by modified method evinced single discrete band of genomic DNA and yielded significantly superior, 441.20 ng/ìl average concentration of DNA over thirteen different genotypes tested with absorbance ratio of DNA at A260/A280 with a mean value of 1.81 as compared to 1.66 by conventional method. Molecular characterization of 13 Noni genotype was done with 40 RAPD and 15 SSR molecular markers. Among 40 RAPD markers, only 20 showed polymorphism among the 13 genotype of Noni. RAPD dendrogram showed two major clusters with co-efficient value 0.39. The genotypes of *M. tomentosa* were found in cluster (A) and (B) whereas, *M. citrifolia* was observed in cluster (A). Genotypes CHESN11 and CHESN12 had highest similarity with maximum co-efficient value (0.699) which falling under same cluster A while CHESN8 and CHESN1 with least similarity and noted in different Sub cluster A2 and A1.Out of the 15 SSR primers, 5 detected polymorphic with 25 scorable bands among 13 accessions of *M. citrifolia* and *M. tomentosa*. The sizes of amplified products ranged from 66 to 5229 bp. Dendogram for SSR based on Jaccard's similarity co-efficients showed two major clusters with co-efficient value 0.53. The genotypes of *M. tomentosa* were found in cluster (A) and cluster (B), while *M. citrifolia* was observed only in cluster (A). Genotypes CHESN11 and CHESN12 had highest similarity with maximum co-efficient value (0.857) which falling under same cluster A even as CHESN12 and CHESN3 with least similarity observed in two different clusters A and B.

Keywords: DNA isolation, *M. citrifolia*, *M. tomentosa,* RAPD, SSR

Introduction

Among medicinal plants discovered by the ancestors of Polynesians, Noni is one of the important traditional folk medicinal plants that have been used for over 2000 years in Polynesia (1). It is botanically designated as *Morinda citrifolia* L. belongs to sub-family *Rubioideae* of family *Rubiaceae*. *Morinda citrifolia* L. is native to Malaysia, Australia and Polynesia (2). The

genus *Morinda* is distributed world over and the presence of as many as 80 different species have been reported (3).

Noni is a small evergreen tree or shrub of 3-10 m in height at maturity. The plant sometimes supports itself on other plants as a liana. There is much variation in overall plant form, fruit size, leaf size and morphology, palatability, odour of the ripe fruit and number of seeds per fruit. Leaves, fruits, stems, and roots are used in various medicinal preparations throughout the pacific region (4). Green fruits are crushed and the juice extracted as a remedy for lesions or sores. Root and bark is used to treat inflammation and infections. Fevers, skin diseases, respiratory problems, gastrointestinal issues, menstrual and urinary problems, diabetes and venereal diseases are just a few of the ailments that this fruit is said to cure. Modern day proponents claim that the Noni fruit and its juice can be used to treat cancer, diabetes, heart disease, cholesterol, high blood pressure, AIDS, rheumatism, psoriasis, allergies, infection, and inflammation. Some believe that the fruit can relieve sinus infections, menstrual cramps, arthritis, ulcers, sprains, injuries, depression, senility, poor digestion, atherosclerosis, addiction, colds, flu, and headaches. It is further claimed that the juice can heal scratches on the cornea of the eye (5-11). Today one of the most widespread uses of Noni is as a fruit juice. The fruit is edible although its rather unpleasant smell and bitter taste are off-putting and for this reason the plant is not cultivated widely in gardens. It is however, useful for coastal erosion control (12).

The genetic diversity analysis can be carried out by the use of morphological, biochemical and molecular markers. The study of morphological variability is still the only approach used in many species to assess genetic diversity. PCR-based DNA markers, such as RAPD, and SSR (simple sequence repeat) have been widely used to investigate the genetic structure of a population (13-14). The selections of RAPD and SSR were based on their relative technical simplicity, level of polymorphism they detect, cost effectiveness, easily applicable to any plant species and target those sequences which are abundant throughout the eukaryotic genome and are rapidly evolved (15-16). Molecular markers have been applied in the analysis of specific genes, as well as to increase understanding of gene action, generate genetic maps and assist in the development of gene transfer technologies. Molecular markers have also critical roles in studies of phylogeny and species evolution, and have been applied to increase our understanding of the distribution and extent of genetic variation within and between species. To test genetic resources for their productivity, quality parameters and stress tolerance, field trials are usually time consuming, therefore, molecular markers and DNA technology are used to differentiate the germplasm and to assess diversity in the gene pool to identify genes of interest and to develop a set of markers for screening elite genotypes (17). Since, the DNA isolation protocol has not been standardizes in Noni and only few reports are available on characterization of genetic diversity of *Morinda* cultivars through molecular marker. Hence, this study was aims to standardize the protocol for DNA isolation and to assess the genetic diversity in *Morinda* spp. using RAPD and SSR markers for identifying elite genotype for its improvement.

Material and Methods

Plant Material: Experimental material consisted 2-3 days young leaves of twelve genotypes of *Morinda tomentosa* and one genotype of *Morinda citrifolia* (Table 1).

Genomic DNA Extraction: The plant genomic DNA isolation was carried out by following the modified CTAB method as described by Doyle and Doyle (18) with different modifications.

Reagents: DNA extraction buffer [2.5% CTAB, 1.5M NaCl, 100mM Tris –Cl (pH 8), 25mM EDTA (pH 8.0)], Phenol: Chloroform: Isoamylalcohol (25:24:1), 0.2% β- mercaptoethanol, 1% Polyvinylpyrrolidone, Methanol and High salt TE.

Table 1. List of genotype under study with its source

S.N.	Genotype	Spp.	Source
1	CHESN1	*Morinda tomentosa*	Central Horticultural Experiment Station, Vejalpur, Panchmahal (Godhra)
2	CHESN2	*Morinda tomentosa*	
3	CHESN3	*Morinda tomentosa*	
4	CHESN4	*Morinda tomentosa*	
5	CHESN5	*Morinda tomentosa*	
6	CHESN6	*Morinda tomentosa*	
7	CHESN7	*Morinda tomentosa*	
8	CHESN8	*Morinda tomentosa*	
9	CHESN9	*Morinda tomentosa*	
10	CHESN10	*Morinda tomentosa*	
11	CHESN11	*Morinda tomentosa*	
12	CHESN12	*Morinda tomentosa*	
13	MCIT13	*Morinda citrifolia*	Botanical garden, C.P.C.A. , S.D.A.U, SKNagar, Dantiwada

***Method for DNA extraction*:** About 0.5 gm of leaf tissue was chopped into small pieces by removing mid rib and treated with 100 ml methanol by stirring about 60 minutes at 30 rpm. The methanol treated chopped leaf tissue was transfer into 1% PVP solution and stirred for 60 minutes at 30 rpm. Subsequently, the tissues were gently grind in the presence of pre-warmed CTAB buffer and 0.2 % â- merceptoethanol. The solution was mixed well by inverting the tubes several times and the homogenate was incubated at 65°C for 60 min in water bath with intermittent shaking. Tubes were cooled down to room temperature and equal volume of Phenol: Chloroform: Isomyl-alcohol (25:24: 1) was added and mixed gently for 1.5 min. The tubes were centrifuged at 12000 rpm for 10 min at 25°C. 100% ethanol was added to supernatant and mixed gently for 2 min. Thick, bright white pellets of DNA were visible. Tubes were kept at -20°C for 20 min for better precipitation of DNA. DNA so pelleted was centrifuged at 12000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet was washed thrice by 70% ethanol. Pellets were air dried and dissolved in 100 µl of Tris-EDTA (TE) buffer. Purity and quantification of DNA was carried out on Nanodrop and 0.8% agarose gel electrophoresis.

Analysis of Random Amplification of Polymorphic DNA (RAPD) and SSR markers: RAPD analysis was performed by a method described by Mathew et al. (19). Amplified fragments were separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide in 1X TBE buffer. PCR for SSR analysis was standardized by composing 25µl reaction volume containing 10X Taq Buffer B followed by 25mM MgCl2, 2.5mM each dNTPs (Bangalore GeNei Pvt. Ltd), 1U Taq DNA polymerase (Bangalore GeNei Pvt. Ltd), Millipore sterilized water and 50 ng template DNA. Amplification condition were maintained at 94°C for 4 min, 40 cycles at 94°C for 1 min (denaturation), 56°C for 1 min (annealing), 72°C for 2 min (elongation) followed by final extension at 72°C for 7 min. The PCR products were separated by electrophoresis in a 2.0 % agarose gel containing ethidium bromide (0.5 µg/ml) using 1X TBE (Tris base, Boric acid, EDTA) buffer (pH 8.0) and visualized under UV light. Data was scored for amplified PCR product were '1' for the presence and '0' for absence of

band. Phylogenetic analyses for all data were performed using online analysis tool DendroUPGMA (http://genomes.urv.cat/UPGMA) by jacard coefficient and bootstrap analysis. Dendrogram were constructed with TREX (Tree and reticulogram Reconstruction).

Result and Discussion

Standardization of Genomic DNA extraction protocol: Total Genomic DNA was isolated from fresh and young leaves of *M.citrifolia* and *M.tomentosa* following CTAB method by Doyle and Doyle (18) with incorporating three important modifications which included methanol and PVP treatment with PCI and long incubation in 100% ethanol yielded high concentration DNA (441.20 ng/µl) over Doyle and Doyle method (245.10 ng/µl), respectively. The purity of total genomic DNA was confirmed by gel documented picture, showing clear, distinct and dense bands (Fig.1) which is further substantiated by ratio of A260/280 (1.81). The standardized protocol yielded maximum genomic DNA (820 ng/µl) in genotype CHESN 3 (Table 2). Noni is one of the important traditional folk medicinal plants and chemical constituents of medicinal plants interfere in DNA isolation (20) and isolated DNA that contains impurities is not suitable for PCR amplification (21). Due to maximum concentra-tion of polyphenols and polysaccharides, DNA is not extracted easily by using the protocol of Doyle & Doyle (18) in Noni. Owing to unavailability of standardized protocol for DNA isolation in Noni, study was conducted with incorporating three important modifications in Doyle and Doyle method. The modification included PVP, removes the phenolics while maintaining a higher yield of DNA (22). The second modification comprised of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added during centrifugation which functions as protein precipitating solution (23). The precipitation of DNA increases by third modification gleaning 100% ethanol as compare to isopropanol (24). In plant molecular biology extraction of high quality of DNA from plant material is an important for genome characterization (25-27).

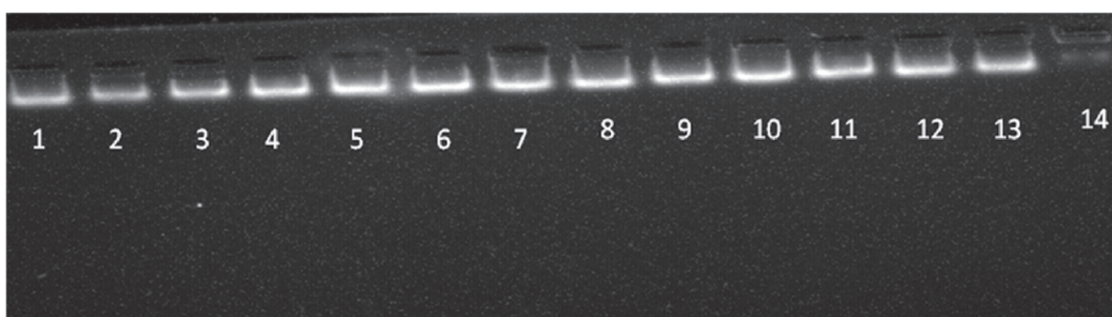

Fig.1. Genomic DNA isolated by modified method in 13 genotypes of Noni.

Lane: **1**=MCIT, **2**=CHESN 1, **3**=CHESN 2, **4**=CHESN 3, **5**=CHESN 4, **6**=CHESN 5, **7**=CHESN 6, **8**= CHENS 7, **9**=CHENS 8, **10**= CHESN 9, **11** =CHESN 10, **12**= CHESN 11, **13**= CHESN 12, **14**= Lambda DNA

RAPD and SSR Analysis

The RAPD analyses of genomic DNA were carried out using 40 RAPD primers in 13 different genotypes of Noni. 20 of the 40 RAPD primers produced clear and unambiguous banding pattern among genotypes. A total of 206 bands were amplified, out of which 200 were polymorphic. This evinced on an average 10.00 polymorphic bands/primer; though the average number of amplified bands per primer were 10.33. The number of amplified loci varied from five in OPH 15 to 28 in OPH 1. The size of amplified bands ranged from 36 bp to 11425 bp. The PIC value ranged from 0.734 (OPH15) to 0.956 (OPH1) with a mean of 0.862(Table 3). Similarity indices were estimated on the basis of 20 RAPD primers ranged from 0.189 (between CHESN8 and CHESN1) to 0.699 (between CHESN11 and CHESN12). UPGMA-based dendrogram clustered all the 13 genotypes into 2 main group viz., A and B. The genotypes of *M. tomentosa* were found in cluster (A) and (B) and *M. citrifolia* was observed in cluster (A). The cluster A contained 7 genotypes viz. MCIT13, CHESN12, CHESN11, CHESN10, CHESN9, CHESN8 and CHESN7.The cluster B contained 6 genotypes viz. CHESN6, CHESN5, CHESN4, CHESN3 CHESN2, CHESN1. Genotypes CHESN10 and CHESN12 had highest similarity with maximum co-efficient value (0.699) which falling under same cluster A while, CHESN8 and CHESN1 with least similar and fallen in different cluster Fig. 2.

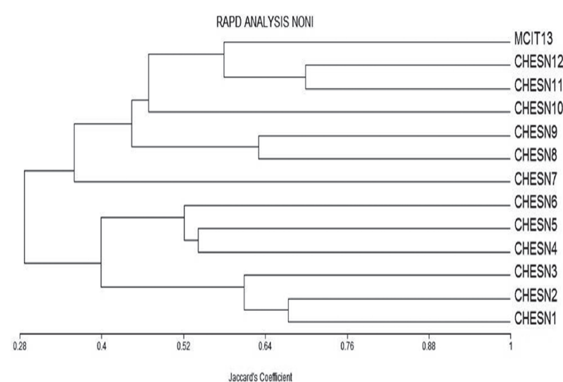

Fig. 2. RAPD based Dendrogram showing clustering of different genotypes of Noni

Table 2. Quantity and Quality of genomic DNA extracted from different genotypes of Noni using modified CTAB method

Sr. No.	Genotype	Conventional Method		Modified Method	
		DNA(ng/µl)	O.D. (260 /280)	DNA (ng/µl)	O.D. (260 /280)
1	*MCIT*	125.20	2.20	227.40	1.93
2	CHESN 1	285.00	1.58	432.70	1.84
3	CHESN 2	476.00	1.87	748.80	1.82
4	CHESN 3	377.00	1.94	820.30	1.82
5	CHESN 4	430.20	1.70	670.70	1.81
6	CHESN 5	250.90	1.66	501.10	1.81
7	CHESN 6	200.30	1.50	302.80	1.77
8	CHESN 7	60.30	1.47	109.70	1.78
9	CHESN 8	150.50	2.22	204.10	1.85
10	CHESN 9	100.70	1.98	209.90	1.78
11	CHESN 10	350.20	1.98	780.40	1.77
12	CHESN 11	300.30	1.94	568.60	1.78
13	CHESN 12	80.30	1.89	159.00	1.79
	Mean	**245.10**	**1.84**	**1.84**	**1.81**
	Range	60.30-476	1.47-2.22	1.47-2.22	1.77-1.93

Table 3. Polymorphism obtained with different RAPD primers generated from Noni genotypes

Sr no.	Primer	Range of band size (bp)	Total polymorphic band	Total monomorphic band	Total band	Polymorphism %	PIC
1	OPH 1	36-731	28	0	28	100.0	0.956
2	OPH 2	235-5699	11	1	12	90.90	0.859
3	OPH 3	178-1493	8	1	9	87.50	0.868
4	OPH 4	105-1182	9	1	10	88.88	0.849
5	OPH 5	88-696	8	0	8	100.0	0.845
6	OPH 6	103-1300	8	1	9	87.50	0.856
7	OPH 7	65-814	9	0	9	100.0	0.860
8	OPH 8	84-804	8	0	8	100.0	0.857
9	OPH 9	113-772	8	0	8	100.0	0.838
10	OPH 10	117-907	9	0	9	100.0	0.864
11	OPH 11	411-4931	7	1	8	85.71	0.847
12	OPH 13	345-11425	11	0	11	100.0	0.884
13	OPH 14	255-7695	12	0	12	100.0	0.894
14	OPH 15	326-3269	4	1	5	75.00	0.734
15	OPH 17	217-3894	11	0	11	100.0	0.878
16	OPH 19	91-3726	12	0	12	100.0	0.898
17	OPH 20	126-1293	10	0	10	100.0	0.877
18	OPH 21	144-1076	9	0	9	100.0	0.877
19	OPH 31	126-6368	9	0	9	100.0	0.843
20	OPH 33	295-3832	9	0	9	100.0	0.858

Among the 15 SSR primers, 5 were found polymorphic in different genotypes of Noni. The PIC value for all five SSRs ranged from 0.355 to 0.806 (Table 4). The CM 3 showed the highest number of bands among the 13 genotypes of Noni while only two bands were produced by CM 8. The product size was found between 66bp to 5229 bp, respectively (Table 4). Dendrogram (Fig.3) showed two major clusters with co-efficient value 0.53. The genotypes of *M. tomentosa* were found in cluster (A) and cluster (B) while genotype of *M. citrifolia* was observed only in cluster (A). The cluster A was further grouped in two clusters A1 and A2. The cluster A1 contained 3 genotypes viz. CHESN12, CHESN11, and MCIT 13. The cluster A2 included 3 genotypes i.e. CHESN9, CHESN10 and CHESN8. The cluster B was further grouped in two clusters B1 and B2. The cluster B1 contained 4 genotypes i.e. CHESN7, CHESN6, CHESN5 and CHESN3, while the cluster B2 comprised CHESN4, CHESN2 and CHESN1. Genotypes CHESN11 and CHESN12 had highest similarity with maximum co-efficient value (0.857) which falling under same cluster B while CHESN12 and CHESN3 with least similar in two different cluster A1 and B1.

Table 4. Polymorphism obtained with different SSR primers generated from Noni genotype

Sr.No. Name	Seq.	Range of Band Size(bp)	Total Polymorphic Bands	Total Monomorphic Bands	Total Bands	% Polymor-phism	PIC
1	CM2	133-247	4	0	4	100	0.355
2	CM3	136-334	8	0	8	100	0.738
3	CM5	66-5229	7	0	7	100	0.806
4	CM6	133-261	4	0	4	100	0.698
5	CM8	66-212	2	0	2	100	0.506

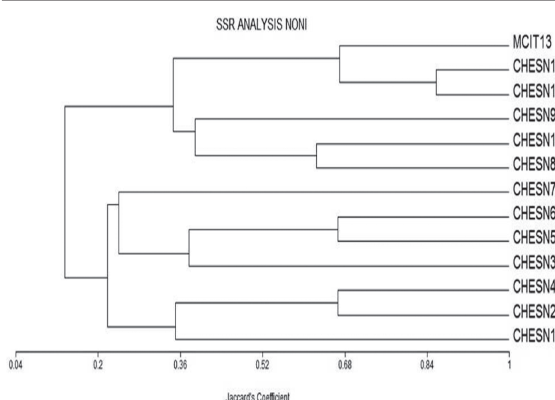

Fig. 3. SSR based Dendrogram showing clustering of different genotypes of Noni

Molecular markers have been used in diversity analysis for classification of germplasm accessions based on genetic distance. In present study RAPD and SSR marker were used, which generated good diversity for present set Noni's different genotypes. In this study RAPD (70% polymorphism) were found to be more efficient then SSR markers. Similar results have been obtained for coffee (28).

Comparatively, both SSR and RAPD analysis is depicting that, both *M. citrifolia* and *M. tomentosa* has evolved from moderately distal genetic lineage as both having maximum genetic distance (0.791) and also good similarity as per Jaccard's similarity coefficient (0.623). Our study, also match with previous study done by singh et al., (29) and singh et al., (30) who showed that a close genetic similarity in some of the cultivars analysed also shown by high values of similarity index. Based on similarity index using simple matching coefficient, they recorded similarity values between all the *Morinda* spp. from 38-95% for RAPD, such trend was also observed in present investigation ranging from 20% to 70%.

Conclusion

The newly developed protocol for the DNA isolation is user friendly and extremely helpful as no standard DNA isolation protocol is available for Noni. Hence, this protocol was optimized to isolate the genomic DNA from the leaves of noni and study provide evidence that RAPD and SSR polymorphism could be used as efficient tools to assess genetic diversity in Noni, which can help for selection of genotypes and to make cross combination for further improvement.

Acknowledgement

We are highly thankful to Central Horticultural Experiment Station, Vejalpur, Panchmahal (Godhra) for providing the genotypes of *M. tomentosa*.

Reference:

1. Mathivanan, N., Surendiran, G., Srinivasan, K., Sagadevan, E., and Malarvizhi, K. (2005). Review on the current scenario of Noni research: Taxonomy, distribution, chemistry, medicinal and therapeutic values of *Morinda citrifolia*. International Journal of Noni Research 1(1): 1-17.

2. Brown, W. H. (1946). Useful plants of the Philippines. Vol. 3 (Tech. Bull. 10) Phil.

Department of Agriculture and Commerce, Manila.

3. Morton, J.F. (1992). The ocean-going Noni, or Indian mulberry (*Morinda citrifolia*, *Rubiaceae*) and some of its "colourful" relatives. Ecological Botony 46, 241–256.

4. Nelson, S.C. (2001). Noni cultivation in Hawaii. University of Hawaii CTAHR–Cooperative Extension Service PD–19.

5. Duke, J., Bogenschutz, M. and Duke, P. Handbook of medicinal plants, 2nd edn (CRC Press, Boca Raton, FL, USA) 2002,529.

6. McClatchey, W. (2002). From Polynesian healers to health food stores: Chnaging perspectives of *Morinda citifolioa* (*Rubiaceae*). Integrated Cancer Theory, 1:110-120.

7. Wang, M. Y. and Su, c. (2001). Cancer preventive effect of *Morinda citrifolia* (Noni), Annals of the New York Academy of Sciences, 952: 161-80.

8. Liu, G., Bode, A., Ma, W.Y., Sang, S. and Ho C-T. (2001). Two novel glycosides from the fruits of *Morinda citrifolia* (noni) inhibit AP-1 transactivation and cell transformation in the mouse epidermal JB6 cell line. Cancer Research, 61:5749-56.

9. Chan-Blanco, Y., Vaillant, F., Perez, A.M., Reynes, M., Brillouet J-M and Brat, P. (2006). The Noni fruit (*Morinda citrifolia* L.): A review of agricultural research, nutritional and therapeutic properties. Journal of Food Composition and Analysis, 19:645-654.

10. Sudhakar, R., Reddy, K.N., Murthy, E.N. and Raju, V.S. (2009). Traditional medicinal plants in Seshachalam hills, Andhra Pradesh, India. Journal of Medicinal Plants Research, 3:408-412.

11. Rout, S.D., Panda, T. and Mishra, N. (2009). Ethno-medicinal plants used to cure different diseases by tribals of Mayurbhanj District of North Orissa, Ethno-Medicine, 3:27-32.

12. Abraham, Z., Senthilkumar, R. and Joseph, J.K. (2008). Collection of plant genetic resources from Andaman and Nicobar Islands. Genetic Resources and Crop Evolution, 55: 1279-1289.

13. Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B. and Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142:169–196.

14. Charcosset, A. and Moreau, L. (2004). Use of molecular markers for the development of new cultivars and the evaluation of genetic diversity. Euphytica 137:81–94.

15. Brennan, J. P., Rehman, A., Raman, H., Milgate, A. W., Pleming,D. and Martin,P. J. (2005). Aneconomic assessment of the value of molecular markers in plant breeding programs.In 49th Annual Conf. of the Australian Agricultural and Resource Economics Society, Coffs Harbour, Australia, 9-11 February.

16. Sheikh, W., Acharya, S. and Patel J.B. (2012). Molecular markers in plant genome analysis: A review. AGRES- An International e-Journal, 1(2): 107-125.

17. Agarwal, M., Shrivastva, N. and Padh, H. (2008). Advances in molecular marker techniques and their applications in plant sciences. Plant cell Reports, 27:617-631.

18. Doyle, J. J. and Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus, 12: 13-15.

19. Mathews, M.D., Srinivasachary, Sujatha, R., JeVrey, L.B., Mike, D., Gale and Katrien, M.D. (2007).The genetic map of Finger millet Eleusinecoracana. Theoretical Applied Genetics, 114:321-332.

20. Puchooa, D. and Venkatasamy, K. (2005). A protocol for isolation of DNA from Trocetia

boutoniana. International Journal of Agriculture and Biology, 7: 82-85.

21. Bansal, K.C., Lenka, S.K. and Mondal, T.K. (2014). Genomics resources for breeding crops with enchanced abiotic stress tolerance. Plant breeding, 133: 1-11.

22. Patel, K.A., Acharya, S., Vagehla, K.O., Patel, J.B., Patel, B.T., Kanbi, V.H. and Sheikh, W.A. (2010). Efficient modified CTAB DNA isolation protocol for cultivated and wild backgrounds of pigeonpea. GAU Research Journal, 35(2):75-79.

23. Collard,B.C.Y. and Mackill, D.J.(2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philosophical Transactions of the Royal Society of London A, 363:557-572.

24. Barker, D., Hurley, D. and Thien L. (1998). Plant DNA isolation: a method to efficiently remove polyphenolics, polysac-charides and RNA. Taxon, 44: 379-386.

25. Barzegari, A., Vahed, S.Z., Atashpaz, S., Khani, S. and Omidi, Y. (2010). Rapid and simple methodology for isolation of high quality genomic DNA from coniferous tissues (Taxus baccata). Molecular Biology Reports, In press

26. Li, J.F., Li, L. and Sheen, J. (2010). Protocol: a rapid and economical procedure for purification of plasmid or plant DNA with diverse applications in plant biology. Plant Methods, 61.

27. Smyth, R.P. (2010). Reducing chimera formation during PCR amplification to ensure accurate genotyping. Genetics, 469: 45-51.

28. Mishra, M., Narayana, S., Bhat, A., Nayani, S., Saya S., Kumar, A. and Jayarama. (2011). Genetic molecular analysis of Coffea arabica (Rubiaceae) hybrids using SRAP markers. International Journal of Tropical Biology, 59 (2): 607-617.

29. Singh, D. R., Singh, S., Minj, D., Anbananthan, V., Salim, K. M., Kumari, C., and Varghese, A. (2012). Diversity of Morinda citrifolia L. in Andaman and Nicobar Islands (India) assessed through morphological and DNA markers. African Journal of Biotechnology, 11(86): 15214-15225.

30. Singh, D. R., Srivastava, A.K., Srivastava, A. and Srivastava, R.C. (2010). Genetic diversity among three Morinda species using RAPD and ISSR markers. Indian journal of biotechnology, 10:285-293.

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Detection of Some useful Phytochemicals in Prop Root Extracts of *Ficus benghalensis*

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Abstract

The various plant parts (stem, leaf, bark, fruit and sap) extract of *Ficus benghalensis* had been known for their anathematic, analgesic, anti-inflammatory, antioxidants, antidiabetic, immuno-modulatory and antimicrobial activity for ancient time. These plant part extracts are used in various ayurvedic medicines to treat diarrhea, dysentery and piles, teeth disorders, rheumatism, skin disorders like sores and to boost immune system. The present study was focused on the prop root extracts (hydro & ethanolic) of *F. benghalensis* against the pure bacterial strains of human oral cavity which inhibits gingival and supragingival diseases and was also analyzed for the bioactive phytochemicals compounds. The qualitative analysis of the *F. benghalensis* prop roots crude hydroextract revealed the presence of flavonoids, saponins, steroids and reducing sugars while ethanolic extracts showed the presence of alkaloids, tannins, and glycosides additionally. The Liquid Chromatography Mass Spectroscopy (LCMS) showed presence of α-ethyl-α-phenyl glutarimide, 1, 2- Dihexanoyl-*sn*-Glycero-3-Phosphocholine, Antimony trisulfide, 2, 2, 2-trichloroethanol, Antimony trioxide at retention time of 1.586 min., 12.377 min.,13.646 min., 14.376 min., and 22.185 min respectively in crude hydroextract of prop roots of *F. benghalensis*. The Thin Layer Chromatography (TLC) product as bioactive compound was identified as 2-chloroethyl phosphonic acid. The antibacterial activity of crude hydroextract were also determined and found the encouraging results against bacterial strain *Streptococcus* sp. (8 mm zone of inhibition), *Lactobacillus* sp. (9 mm), *Actinobacillus* sp. (7 mm), *Fusobacterium* sp. (9 mm) but TLC separated product (2-chloroethyl phosphonic acid) showed antibacterial activity only against *Lactobacillus* sp. as 17 mm zone of inhibition.

Key words: *Ficus benghalensis,* TLC, prop roots, immuno-modulatory, phytochemicals, LCMS.

Introduction

More than 700 species of bacteria inhabit the oral cavity and many of these are associated with oral diseases (1). The development of dental caries are due to the acidogenic and aciduric Gram-positive bacteria (*Streptococcus mutans* and *S. sobrinus*), *lactobacilli* and *actinomycetes*, that metabolize sucrose to organic acids mainly lactic acid which dissolve the calcium phosphate in teeth, causing decalcification and eventual decay. Dental caries is therefore a supragingival stage (2) and periodontal diseases are subjected as subgingival stage that is linked to anaerobic Gram-negative bacteria (*Porphyromonas gingivalis*, *Actinobacillus* sp., *Prevotella* sp. and *Fusobacterium* sp.) (1, 3). Various parts of *F. benghalensis* is used as ayurvedic medicine against various microbial diseases. These plant

parts are acrid, astringent, anodyne, sweet, refrigerant, depurative, vulnerary, anti-inflammatory, antiarthritic, antidiarrhoeal, antiemetic, ophthalmic, stypic, diaphoretic and tonic traditionally (4). Mukherjee *et al*, 1998 (5); and Husain *et al*., 1992 (6) used some plant parts of *F. benghalensis* to treat diarrhea, dysentery and piles, Warrier *et al*, 1993 (7) used as rheumatism, skin disorders like sores and to boost immune system (8), as a hypoglycemic in ayurveda (9, 10, 11, 12). It is found to show antibacterial activity against dental caries causing bacteria *Actinomyces vicosus* (13).

F. benghalensis is the world's largest tree in terms of its spread and some of old trees covers over an acre of ground. It is also the national tree of India. Its name banyan is named for the merchants who set up their shop under the spreading trees. *F. benghalensis* is an epiphyte, in the beginning of its life, growing on another tree where some fig-eating bird deposited a seed. As it grows, it produces prop roots from its horizontal branches which then take root where they touch the ground. These prop roots create a forest on their own. This tree can get 100 feet tall and with its massive limbs supported by prop roots can spread over an area of several acres. It has large and thick leathery leaves (14). Plant-derived substances have become of great interest due to their availability, cellular totipotency, and various natural applications. These medicinal plants are the richest bioresource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (15). Natural constituents of plants can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components (16). The chemical constituents in plants may be therapeutically active or inactive. The bioactive compounds of plants are alkaloids, flavonoids, tannins, glycosides, steroids, reducing sugars, saponins etc.

The phytochemicals research based on the use of prop roots extract of *F. benghalensis* as an herbal remedy could be considered an effective and novel approach in the discovery of new anti-infective agents. The knowledge of the prop root chemical constituents of *F. benghalensis* could open the doors, not only for the discovery of economic materials (tannins, oils, gums, precursors for the synthesis of complex chemical substances) but also may be valuable in disclosing new sources of therapeutic agents such as anathematic, analgesic, anti-inflammatory, antioxidants, antidiabetic, immuno-modulatory and antimicrobial activity and could be use cure various diseases like subgingival and supragingival of tooth disorders.

Material and Methods

The sample of prop roots of *F. benghalensis* was collected from Surajpur, Baddi, Himachal Pradesh, India-13205 in the month of February when the atmospheric temperature was 18^0C. The specific locations were $30^0$53'15.44"N, $76^0$48'29.26"E and 1935 feet elevation. Sample was washed under running tap water to remove surface impurities and then was cut into small pieces of 2-3 cm, dried under reduced pressure, grinds to prepare a fine powder and stored in polypropylene container at 4^0C for future use.

***Aqueous Extraction*:** The 10% w/v aqueous prop root extract was subjected to extraction in a Soxhlet apparatus at 100^0C for 48 hours in distilled water. Ten grams of the prop roots powder were loaded in the thimble of Soxhlet apparatus. It was fitted with appropriate size round bottom flask with 100 ml distilled water, and upper part was fitted with condenser. Constant heat was provided by heater for recycling of the solvent. After complete extraction, extracted material was evaporated to 20 ml at 50^0C under reduced pressure. Furthermore, the extract in round bottom flask was transferred into clean boiling tubes and stored at 4^0C.

***Ethanolic Extraction*:** The 10% w/v ethanolic prop root extract was subjected to extraction in

a Soxhlet apparatus at 78.3^{0}C for 48 hours in ethanol. After complete extraction, extracted material was evaporated to 20 ml at 35^{0}C under reduced pressure. Furthermore, the extract in round bottom flask was transferred into clean boiling tubes and stored at 4^{0}C. The percentage yield of extraction was calculated as:

Percentage yield of extract = mass of extract (mg) / mass of sample taken (mg) X 100

***Bioactive compound analysis*:** The qualitative experiments were performed on both of the extracts to confirm the presence of the bioactive chemical constituents such as alkaloids, flavonoids, tannins, saponins, glycosides, steroids and reducing sugar by standard procedures. As for alkaloid detection, 10 ml of extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent (Dissolve 1.358 g of $HgCl_2$ in 60 ml of H_2O and pour into a solution of 5g of KI in 10 ml of H_2O, final volume was made to 100 ml with distilled water). The sample was then observed for the presence of turbidity or yellow precipitation (17). For flavonoids, 1 ml of 10% NaOH was added to 3 ml of the extract. The sample was then observed for the formation of yellow color (17). For tannins, 4 ml of 5% $FeCl_3$ was added to 2 ml of extract. Then the sample was observed for the development of a dark green precipitate. In the second test, 4ml of freshly prepared 10% KOH was added to 4 ml of extract. It was then observed for the formation of a dirty white precipitate (18). For saponins, Frothing Test: 2 ml of extract was taken in a test tube and vigorously shaken for 2 minutes. The sample was then observed for the presence of froth at least 1 cm in height. Emulsion Test: Five drops of olive oil were added to 3 ml of concentration extract in a test tube and the mixture was vigorously shaken. Formation of stable emulsion was then observed in the sample (19). For glycosides estimation, 20 ml of 50% H_2SO_4 was added to 2 ml of conc. extract in a test tube. The mixture was heated in a water bath for 15 minutes. 10 ml of Fehling's solution was then added and the mixture was boiled. The sample was then observed for the development of brick red precipitate. For steroids, 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. Concentrated H_2SO_4 was carefully added to the filtrate to form lower layer and observed for the formation of reddish brown color at the interface (17). And for the confirmation of reducing sugar, 0.5 ml of extract, 1 ml of water and 5-8 drops of Fehling's solution was added at hot. It was observed for development of brick red precipitate (20).

***Preparative TLC Separation of bioactive compounds*:** The TLC plates were cleaned and dried in hot air oven. Slurry was prepared by mixing silica gel G with distilled water in clean beaker with continuous stirring. Then slurry was poured over the glass plates with the help of TLC plate spreader to make film of 2.0 mm thick. Then the plate was air dried and activated by heating in a hot air oven at 120^{0}C for 1 hour. After the plate was allowed to cool at room temperature then marked about 2.0 cm from the bottom as the baseline. 10 µl aqueous extract was loaded at the center of the baseline of the plate.

***Development of Chromatogram*:** The development tank was saturated with solvent system of chloroform, methanol and water (10:10:3) for the analysis of bioactive compounds. The plate was kept in the tank without touching baseline by solvent and left for development. After running the solvent about I hour, the final solvent front was marked and the plate was air dried. Few pieces of iodine crystals were kept in the tank. The tank was covered with glass plate to saturate the tank with iodine vapor. Then the plate was kept in iodine vapor saturated tank and left for few minutes till the spot appear onto the TLC plate. R_f value was calculated by measuring the distance traveled by the each bioactive compound to the total distance traveled by solvent in cm and each compound was also compaired with respective controls.

***Collection of the TLC products*:** Spots on the

preparative TLC silica gel G plates were scratched with the help of clean and dry spatula and collected in a beaker containing 10 ml of methanol and left overnight. Then the content in the beaker was stirred and filtered through Whatman no. 42 filter paper. The filtrates of each spot were collected in clean and dry test tubes. The filtrates were then concentrated. The filtrates containing bioactive compound were used for the LCMS identification (20, 21).

***Antibacterial activity determination*:** Four different pure bacterial strains were isolated from the oral cavity of human mouth. These strains were identified as *Streptococcus* sp., *Lactobacillus* sp., *Actinobacillus* sp., and *Fusobacterium* sp. Discs were made out of absorbent paper. The sterilized discs were then soaked in 1 ml of bioactive compound of preparative TLC product, crude aqueous extract, and crude ethanolic extract. These discs were left overnight in the respective solutions of natural compounds. Next day, nutrient agar (peptone 5.0 g, beef extract 3.0 g, sodium chloride 5.0 g, pH 7.0, agar 15 g in one liter distilled water) plates were prepared. The 100µl inoculum of each bacterial strain was spreaded on to the agar plates. The discs of appropriate natural compounds were then placed on the agar plates after that the plates were incubated at 37^0C for 24 hours. Then plates were observed for any inhibition of bacterial growth as indicated by a clear zone around the discs. The size of each the zone of inhibition was measured and expressed in mm.

***LCMS identification of the compounds involved*:** The prop roots hydro-extract and TLC products of *F. benghalensis* were concentrated under reduced pressure with the help of rotatory evaporator and dried to powdered extracts. Dried extracts were dissolved in 10 ml of methanol and filtered through Whatman filter paper no. 42. Then these samples were passed through Na_2SO_3 column, collected and concentrated to 1ml. Liquid chromatography/mass spectrometry (LC/MS) with a quadruple ion trap MS was done. The column used was Symmetry (Walters) C18 column (250 × 4.6 mm). A 25µl sample volume was injected using the system's autosampler. Solvent A contained 5% formic acid in water and solvent B consisted of HPLC-grade methanol. The flow coming from the LC was split 1:28 and introduced to the ESI (electrospray ionization) turbo spray. A scan rate of 4.000 amu/s was performed under negative ionization in the enhanced scan mode. The UV response during LC/MS was monitored at 360 nm. The LC/MS was operated in the positive- ion mode using Diode array detector and electrospray ionization (ESI) source: Ion spray voltage: -4.500 V, Declustering potential: -50 V, Entrance potential: -10 V, Collision energy: -90 to 10 V, Curtain gas: 40 psi, Nebulizer gas: 45 psi, Turbo gas: 80 psi. Finally compounds identification was corroborated based on the relative retention time and mass fragmentation pattern spectrums with those of standards and the NIST147 library database of the LCMS system (22).

Results and Discussion

During detection of phytochemicals in sample extracts showed the variation in type of phytochemicals present in different solvents (hydro and ethanol). The analysis for the phytochemicals of the *F. benghalensis* prop roots hydroextract revealed the presence of flavonoids, saponins, steroids and reducing sugars as well as ethanolic extract confirmed the detection of alkaloids, tannins and glycosides. Kawo (23); Yusha'u *et al*. (24); and Kawo *et al*. (25), supports the present study after concluding the result that these phytochemicals having the ability to dissolve into specific type of solvents. For rest of the nondetectable phytochemicals in both (hydro & ethanol) extracts of the prop root, Adoum *et al*. (26) and World Health Organization (WHO 2003) report explained the seasonal variations and the geographical location of a plant can affect the presence active constituents and chemical composition of the plants which may be induced the biological activity by many factors like climate, soil, and propagation method. Odugbemi (27), well explained the time of collection of plant sample for these detections also affects its efficiency.

The preparative TLC, Liquid chromatography and mass spectroscopy were performed for the analysis of bioactive chemical compounds present in hydroextract of the sample. As the Fig. 1.0 showed the TLC product at R_f value of separated bioactive compound found as 0.7005. However this compound was confirmed as 2-chloroethyl phosphonic acid by compaired with control compound R_f value and Liquid chromatography and mass spectroscopy techniques (Fig. 3.0 to Fig. 3.1). The Liquid Chromatography Mass Spectroscopy (LCMS) also showed presence of α-ethyl-α-phenyl glutarimide (Doriden), 1, 2- Dihexanoyl-*sn*-

Fig. 1.0. TLC chromatogram of the bioactive compounds shown by arrow mark.

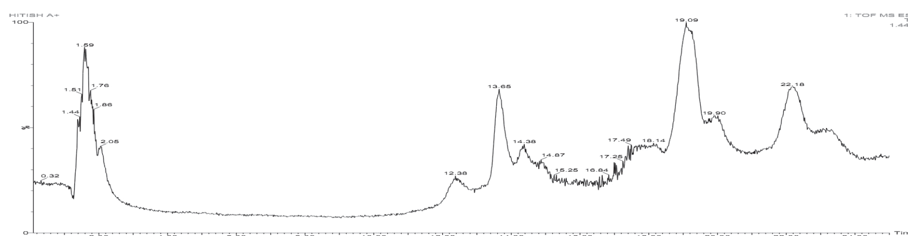

Fig. 2.0. LC chromatograph of prop root crude hydroextract.

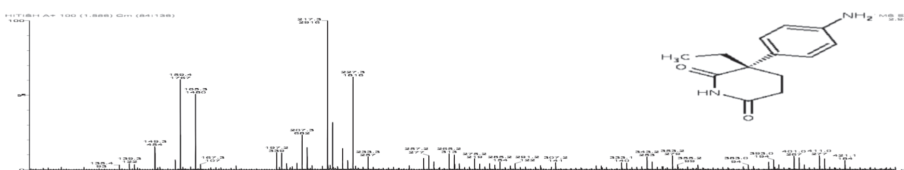

Fig. 2.1. MS of LC peak having retention time 1.586. α-ethyl-α-phenyl glutarimide ($C_{13}H_{15}NO_2$) having molecular weight 217.3 was identified.

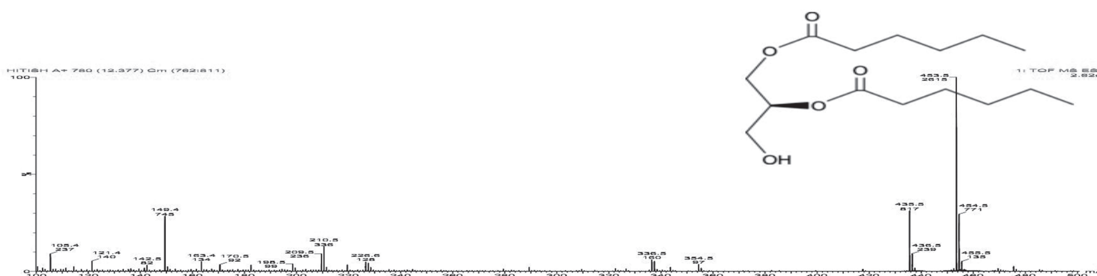

Fig. 2.2. MS of LC peak having retention time 12.377, 1, 2- Dihexanoyl-sn-Glycero-3-Phosphocholine ($C_{20}H_{40}NO_8P$) having molecular weight 453.5 was identified.

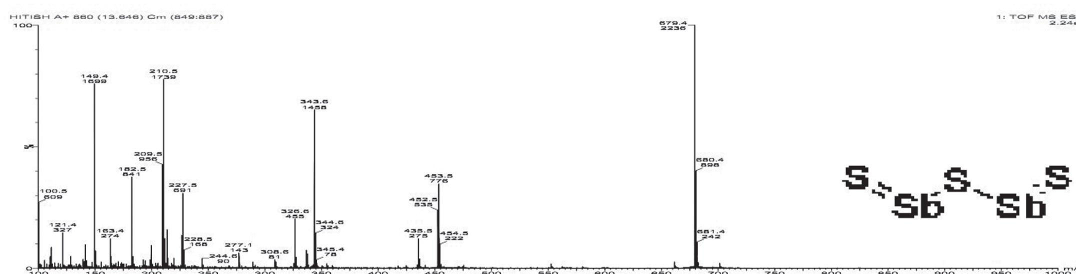

Fig. 2.3. MS of LC peak having retention time 13.646, Antimony trisulfide (S_6Sb_4) having molecular weight 679.4 was identified.

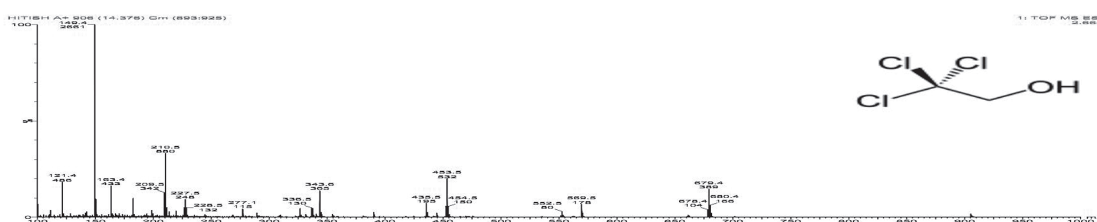

Fig. 2.4. MS of LC peak having retention time 14.376, 2, 2, 2-trichloroethanol ($C_2H_3OCl_3$) having molecular weight 149.4 was identified.

Glycero-3-Phosphocholine, Antimony trisulfide, 2, 2, 2-trichloroethanol, Antimony trioxide at retention time of 1.586 min., 12.377 min.,13.646 min., 14.376 min., and 22.185 min respectively in crude hydroextract of prop roots of *F. benghalensis* (Fig. 2.0 to 2.5). The LC pattern of active compounds separated after TLC is shown in Fig. 3.0.

As so many researchers isolated and purified many plant part extracts of variety of plants for the confirmed the presence of various phytochemicals for the exploiting of the pharmacological importance such as analgesic, antidiabetic, antibacterial, and antibacterial properties. Jothy *et al*. (22) carried out to isolate active compounds in four steps using multiple extractions, fractionation using column chromatography and purification using preparative thin-layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS) from *Cassia fistula* seed extract. HPLC and LC/MS tests on this distinct peak showed the presence major bioactive compound with anti yeast activity in the active fraction isolated from crude methanolic extract of *C. fistula* seeds.

However, in case of *F. benghalensis* 18 compounds were identified by various scientists from the methanol fractions. The plant samples revealed the synthesis of Phenol, 1,5-Heptadiene-3,4-diol,2-Cyclopenten-1-one, 2,3,4,5-tetramethyl, Piperazine, 1,2-Benzenediol,1-Propanone, 2-amino-1-phenyl, Phenol, 2,6-dimethoxy, Phenol, 2-methoxy-3-2-propenyl, Benzaldehyde, 3-hydroxy-4-methoxy, Phenol, 2- (trimethylsilyl), Dodecanoic acid, Oxalic acid, 2-ethylhexyl ethyl ester, Androstan-7-one, 3- (acetyloxy) -, (3α,5α), 9H-Pyrido[3,4-b]indole, 8-hydroxy-1-methyl, 4-Decenoic acid, 3-methyl, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, Hexadecanoic acid, methyl ester, Phenol and 2- (2-penten-4-yl)-4-methyl.

Bastin and Boominathan (28) identified the major components of extracts of *Allium cepa* and *Ficus benghalensis* by gas chromatography coupled with mass spectrometry (GC/MS) analysis. In this case nearly 40 compounds were identified in the methanol fractions. Patel *et al*. (29) carried out the phytochemical investigation of *Dendrophthoe* by using HPTLC and LC-MS/MS analysis. LC-MS/MS analysis of ethyl acetate fraction of *D. trigona* established the presence of two compounds. The MS-MS fragmentation pattern of these compounds confirmed the presence of quercetin, (-) - epicatechin and/or (+) - catechin in ethyl acetate fraction of *D. trigona*.

In the present work, the bioactive compound (2-chloroethyl phosphoric acid) separated by TLC was investigated for their antibacterial activity. The antimicrobial activity was found maximum against *Lactobacillus* sp. as 17 mm zone of inhibition at 0.15 mg/ml concentration of compound. The antibacterial activity of crude hydroextract were also determined and found against bacterial strain *Streptococcus* sp. (8 mm zone of inhibition), *Lactobacillus* sp. (9 mm), *Actinobacillus* sp. (7 mm), *Fusobacterium* sp. (9 mm). The mass spectrum pattern of active compounds separated after TLC is shown in Fig. 3.1.

The antibacterial activity of *F. benghalensis* Linn bark was reported by Bhangale *et al*. (13) on *Actinomyces viscosus*. The hydro alcoholic extract of plant was used and found to be effective against *Actinomyces viscosus.* The maximum zone of inhibition was found to be 15.2 mm at 0.10 mg/ml concentration of extract. Study concluded the seven bioactive phytochemicals (flavonoids, saponins, steroids, reducing sugars, alkaloids, tannins, and glycosides) were detected during qualitative analysis of both crude (ethanolic and hydro) extracts of *F. benghalensis.* Crude hydroextract was having varying degree of antibacterial activity against pure oral dental bacterial (*Streptococcus* sp., *Lactobacillus* sp., *Actinobacillus* sp., and *Fusobacterium* sp.) strains. Furthermore efforts were made for the identification chemical compounds of crude hydroextract of *Fusobacterium* sp. 2-chloroethyl phosphonic acid was purified on TLC followed by LCMS. The compounds α-ethyl-α-phenyl

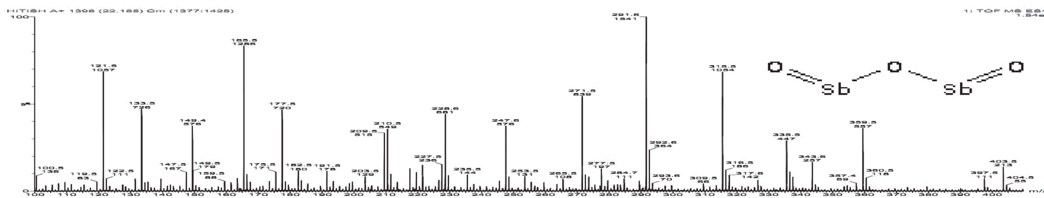

Fig. 2.5. MS of LC peak having retention time 22.185, Antimony trioxide (Sb2O3) having molecular weight 291.6 was identified.

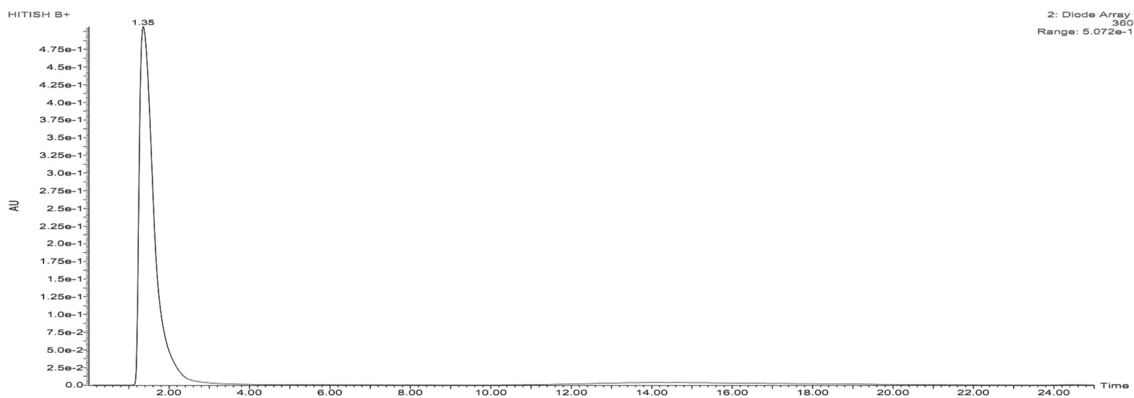

Fig. 3.0. LC chromatograph of a preparative TLC product.

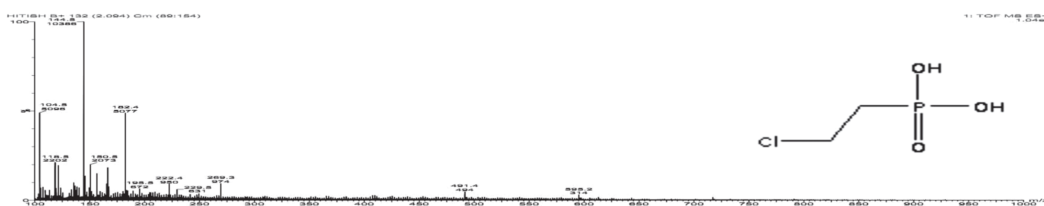

Fig. 3.1. MS of LC peak having retention time of 2.049, 2-chloroethyl phosphonic acid (C2H6ClO3P) having molecular weight 144.5 was identified.

glutarimide, 1, 2- Dihexanoyl-*sn*-Glycero-3-Phosphocholine, Antimony trisulfide, 2, 2, 2-trichloroethanol, and Antimony trioxide were also identified by LCMS. These pure chemicals could be use as an ayurvedic medicine for the treatment of various teeth disorders (gingival, subgingival and supragingival stages) and to boost immune system.

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References

1. Jenkinson, H.F. and Lamont, R.J. (2005). Oral microbial communities in sickness and in health. *Trends in Microbiology*, 13, (12): 589–595.
2. Loesche, W. (2007). Dental caries and periodontitis: contrasting two infections that have medical implications. Infectious Disease Clinics of North America, 21(2): 471–502.
3. Tichy, J. and Novak, J. (1998). Extraction, assay, and analysis of antimicrobials from plants with activity against dental pathogens (*Streptococcus sp.*). *Journal of* Alternative and Complementary Medicine, 4(1): 39–45.
4. Sala, A.V. (1997). Indian Medicinal Plants-A compendium of 500 species. Orient Longman Ltd., Madras, 3: 20-26.
5. Mukherjee, P.K., Saha, K., Murugesan, T., Mandal, S.C., Pal, M. and Saha, B.P. (1998). India. Screening of antidiarrhoeal profile of some plant extracts of a specific region of West Bengal, India. Journal of Ethnopharmacology, 60: 85-89.
6. Husain, A., Virmani, O.P., Popli, S.P., Misra, L.N., Gupta, M.M., Srivastava, G.N., Abraham, Z . and Singh, AK. (1992). Dictionary of Indian Medicinal Plants, CIMAP, Lucknow, India, 546.
7. Warrier, P.K., Nambiar, V.P. and Ramanbutty, C. (1993). Indian Medicinal Plants. Orient Longman Ltd., Madras, 1-5.
8. Gabhe, S.Y., Tatke, P.A. and Khan, T.A. (2006). Evaluation of the immuno-modulatory activity of the methanol extracts of *Ficus benghalensis* roots in rats. Indian Journal of Pharmacology, 38, (4): 271-275.
9. Shroti, D.S. and Aiman, R. (1960). The relationship of the post-operative state to the hypoglycemic action studies on *Ficus benghalensis* and *Ficus glomerata*. Indian Journal of Medical Research, 48: 162-168.
10. Deshmukh, V.K., Shroti, D.S. and Aiman, R. (1960). Isolation of a hypoglycemic principle from the bark of *Ficus benghalensis*. Indian Journal of Physiology and Pharmacology, 4: 182-185.
11. Augusti, KT. (1975). Hypoglycemic action of bengalenoside: A glucoside isolated from *Ficus benghalensis* Linn, in normal and Alloxan diabetic rabbits. *Indian Journal of Physiology and Pharmacology*, 19: 218-20.
12. Augusti, K.T., Daniel, R.S., Cherian, S., Sheela, C.G. and Nair, C.R. (1994). Effect of Leucopelargonin derivative from *Ficus bengalensis* Linn on diabetic dogs. Indian Journal of Medical Research, 99: 82-86.
13. Bhangale, S.C., Patil, V.V. and Patil, V.R. (2010). Antibacterial activity of *Ficus Bengalensis* Linn. bark on *Actinomyces viscosus*. International Journal of Pharmaceutical Sciences, 2, (1): 39-43.
14. Chew, W.L. (1989). Flora of Australia. Australian Government Printing Service, Canberra, 3: 15-68.
15. Ncube, N.S., Afolayan, A.J. and Okoh, A.I. (2008). Assessment techniques of antimicrobial properties of natural

compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, 7(12): 1797-1806.

16. Parekh, J., Karathia, N. and Chanda, S. (2006). Evaluation of antibacterial activity and phytochemical analysis of *Bauhinia variegata* L. bark. African Journal of Biomedical Research, 9: 53-56.

17. Trease, G.E. and Evans, W.C. (1978). A textbook of pharmacognosy. Eleventh Edition, Bailliere Tindall, London, 530.

18. El-Olemyl, M.M., Al-Muhtadi, F.J. and Afifi, AA. (1994). AQ laboratory manual, College of Pharmacy, King Saud University. King Saud University Press, 1-134.

19. Harborne, J.B. (1973). Phytochemical methods - A guide to modern techniques of plant analysis, Third edition, Chanpman and Hall publishers, London, 7-279.

20. Joshi, B., Sah, G.P., Basnet, B.B., Bhatt, M.R., Sharma, D., Subedi, K., Pandey, J. and Malla, R. (2011). Phytochemical extraction and antimicrobial properties of different medicinal plants*: Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem). Journal of Microbiology and Antimicrobials, 3(1): 1-7.

21. Mehrotra, V., Mehrotra, S., Kirar, V., Shyam, R., Misra, K., Srivastava, AK. and Nandi, SP. (2011). Journal of Microbiology and Biotechnology Research, 1 (1): 40-45.

22. Jothy, S.L., Zakaria, Z., Yeng, Y., Chen, YL Lau, Latha, L.Y., Shin, LN. and Sasidharan, S. (2011). Bioassay-directed isolation of active compounds with antiyeast activity from a *Cassia fistula* seed extract. Molecules, 16: 7583-7592.

23. Kawo, A.H. (2007). Water purification potentials and *in-vivo* toxicity evaluation of the aqueous and petroleum-ether extracts of *Calotropis procera* (Ait.F.) Ait.F. latex and *Moringa oleifera* LAM seed powder. *PhD thesis*. Microbiology Unit, Department of Biological Sciences, Bayero University, Kano, Nigeria, 184.

24. Yusha, U.M., Bukar, A. and Balarabe, A.I. (2008). Prevalence and sensitivity of enterobacterial isolates from patients with urinary tracts infections to *Acalypha wilkisenia* extracts. Biological and Environmental Sciences Journal for the Tropics, 5(3): 72–76.

25. Kawo, A.H., Mustapha, A., Abdullahi, B.A., Rogo, L.D., Gaiya, Z.A.and Kumurya, A.S. (2009). Phytochemical properties and antibacterial activities of the leaf and latex extracts of *Calotropis procera* (Ait.F.) Ait.F. Bayero Journal of Pure and Applied Sciences, 2(1): 34-40.

26. Adoum, O.A., Akinniyi, J.A. and Omar, T. (1997). The effect of geographical location on the antimicrobial activities and trace element concentrations in the root of *Calotropis procera* (Ait.) R. Br. Annals of Borno, 13(14): 199-207.

27. Odugbemi, T. (2008). A textbook of medicinal plants from Nigeria. University of Lagos Press, Nigeria, 20-323.

28. Bastin, T.M. and Boominathan, M. (2011). Comparative studies on the antimicrobial activity of *Allium cepa* and *Ficus bengalensis* fractions against *Streptococcus pneumoniae*. International Journal of Universal Pharmacy and Life Sciences, 1(2): 2249-6793.

29. Patel, B.H., Joshi, A.A. and Parmar, R.R. (2012). Phytochemical investigation of *Dendrophthoe trigona (Wt. and Arn.) Danser*. Current Pharma Research, 2(3): 580-583.

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Glucose Regulated Protein 78 (*GRP* 78) as a Cytoprotection against Apoptosis in Small Cell Lung Carcinoma

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Abstract

In a previous study, has been found that mushroom crude extract (*Cantharellus sp)* is able to induce apoptosis in human cervical cancer cell line (HeLa) and the resistance to Small Cell Lung Carcinoma (H157). To clarify the mechanism, we carried out comparative proteomics analysis between H157 and HeLa cell lines. Differentially expressed proteins were separated electrophoretically and identified by Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI-TOF-MS). Peptide tandem mass spectra were searched against a comprehensive data base containing known proteins derived from data bases. There were profound changes in 14 proteins related to mitochondrial function and oxidative stress. The results also showed up regulation of GRP78 in treated H157 cell line. Up regulation of GRP78 was further confirmed by western blotting analysis and it was suggested that GRP78 is responsible for apoptosis resistance of H157 cell line when treated with *Cantharellus* extract at low concentration.

Keywords: Glucose Regulated protein 78, Apoptosis, *Cantharellus*, Small Cell Lung Carcinoma cell line.

Introduction

In a stress conditions, cells have to undergo a defense mechanism one of which is the activation of unfolded protein response (UPR) (1). The function of UPR is the induction of 78-kDa glucose regulated protein (GRP78) also known as immunoglobulin binding protein (BiP) that belong to the Hsp70 family (2). GRP78 composed of three domains; the peptide binding domain, ATPase domain and a C-terminus domain (3).

GRP78 is a multifunction endoplasmic reticulum (ER) protein involved in many cellular processes. As a molecular chaperone, it prevents the formation of malfolded proteins by binding to unfolded polypeptides using energy derived from ATP hydrolysis to promote proper folding and prevent aggregation (2). It is targeting misfolded protein for proteosome degradation, translocating newly synthesized polypeptides across the ER membrane, it possesses the capacity to bind Ca^{2+} which helps to immobilize Ca^{2+} and maintain ER calcium homeostasis, serving as a sensor of ER stress and also it plays a role in antibody synthesis therefore it may be crucial for B-cell maturation (4).

The expression of GRP78 can be enhanced by various drugs that are capable of generating unfolded protein load, by means of interruption of glucose metabolism and disruption of protein glycosylation (5). Over expression of GRP78

results in cytoprotection in a wide variety of tumors and protects tumor cells against apoptosis caused not only by disturbance of ER homeostasis but also by various cancer therapeutic agents (6). The potential mechanisms responsible for this protection include preventing protein misfolding, binding of ER Ca^{2+} and blocking activation of caspase and proapoptotic proteins associated with the ER (7).

In recent years, proteomic technologies have started to be used for investigating cell function and disease mechanisms, as they offer opportunity to answer biological questions that were never thought possible (8). Among many different strategies for proteomic analysis, two-dimensional electrophoresis (2-DE) is a powerful and widely used method for the complex protein mixtures extracted from cells, tissues or other biological samples (9). Through proteomics technologies, various level of proteins that are involved in the signaling pathways targeted by the drugs have been identified, (4, 10, 3).

In a previous study, has been found that crude extract of *Cantharellus strain* is able to induce apoptosis in human cervical cancer cell line (HeLa) and the resistance to Small Cell Lung Carcinoma (H157) (11). In this study, we carried out comparative proteome analysis of HeLa and H157 cells treated with crude extract of *Cantharellus* with the aim of identify and functionally characterize crucial proteins that lie upstream of the actual event of *Cantharellus* induced apoptosis. This paper reports the cytoprotection effects of glucose regulated protein 78 upon apoptosis induced by *Cantharellus* extract.

Materials and Methods

***Preparation of crude extract*:** Fruiting bodies of *Cantharellus sp* were collected from miombo woodlands located in Tabora region, Western Tanzania. The collection was done by hand and the fungi were put into small basket and brought to the laboratory for identification by a mushroom taxonomist. Thereafter, specimens were cut into small pieces and sun dried for period of three days. The dried materials were further pulverized to powder using a national super mixer grinder (MX-119) (Emerging planet India Ltd., Coimbatore 641011, India). About 200g of the powder materials were extracted by soaking in ethyl acetate. The ethyl acetate crude extracts were concentrated in vacuo using a rotary evaporator (HEIDOLPH® Ltd) Essex Scientific laboratory suppliers Ltd with the bath temperature maintained at 40°C. The organic extracts obtained were kept at 4°C until further use.

***Preparation of protein and protein separation*:** H157 and HeLa cell lines obtained from cell lines collection at the Biochemistry laboratory, Department of Biotechnology, University of the Western Cape, Cape Town, South Africa. Culture media composed of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and penicillin (1 µg/ml). Cells were seeded at a density of $2.5x10^4$ cells per ml in 24-well culture plates and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 hours. When reached 80% confluences, were treated with 2mg/ml of *Cantharellus* extract and incubated for 24 hr. Other flasks were not treated, act as control. After incubation, proteins were extracted from the cells using 9M urea solution, the obtained protein pellet were re-suspended in urea solution stored at -20 ÚC for further use. This was done in four different experiments. Protein concentration was estimated by the Bradford assay (BIO-RAD, Alfred Nobel Drive, Hercules, CA, USA) (12). Protein sample (20 µg) were run in 12% resolving and 5% stacking one-dimensional Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (1D SDS-PAGE) using the Mini-Proteam 3 Electrophoresis cell system (BIO-RAD) and visualised using Coomassie Brilliant Blue (CBB) staining solution. Protein samples (150 µg) from both cell lines treated and untreated were run in two-dimensional Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (2D SDS-PAGE). The proteins were focused using the 7 cm ReadyStrip™ IPG strips at a pH range of 4 – 7. .

***PDQuest™ Basic software image analysis and MALDI-TOF Mass Spectrometry*:** After separation of proteins spots by 2D-PAGE and staining with CBB stain. The gels were scanned using a PharosFX™ Plus Molecular Imager scanner (BIO-RAD) using QuantityOne software. The scanned gel images were analysed using PDQuest Basic version 8.0 for differential protein expression profiles. The spot viewer was then used to identify the differentially expressed spots between the control and experimental triplicate groups in the analysis set (either quantitative or *T*-test). The quantitative test was used with an upper limit of 1.5 and lower limit of 0.5 fold difference and a *T*-test with the significance level of 95%. A total of 107 and 87 spots from HeLa and H157 untreated and 95 and 112 spots from HeLa and H157 treated were analyzed.

***Western blot analysis*:** Proteins isolated from both HeLa and H157 were separated by 12% SDS-PAGE and then electro blotted to polyvinylidene difluoride (PVDF) transfer membranes. The membranes were washed with TBS tween (TBST) 0.1 % (v/v) Tween-20, 1x TBS and placed in 5 % blocking solution fat free milk prepared in TBS tween-TBST. GRP78 (E-4) primary antibodies (1° Ab) Santa Cruz biotechnology was diluted at 1:500 in 3 % bovine serum albumin (BSA) and added onto the membranes then incubated overnight at 4°C on with shaking to detect GRP78. Then membrane was washed with TBST and incubated with goat anti-mouse IgG-HRP: sc-2031 as secondary antibody on a shaker for 30 minutes at room temperature. The SuperSignal West Pico (Thermo Fisher Scientific, USA) chemiluminescence detection substrate was added onto the surface of the blot containing proteins followed by incubation in a dark place for 5 minutes. After incubation excess detection solution was drained off and detection was done using autoradiography. The blots were exposed to X-ray film for 20 minutes in a dark room. After exposure the film was developed using an x-ray processor (13).

Results

***Preparation of protein and protein separation*:** The standard approach for resolving proteins involves two dimensional gel electrophoresis (14). Protein spots from untreated and treated H157 and HeLa cells separated on narrow 4-7 pH range 7 cm IPG strips in 12% SDS-PAGE and visualized by CBB staining solution (Fig. 1). Spots indicated by arrows were selected for MALD-TOF MS analysis.

***PDQuest™ Basic software image analysis and MALDI-TOF Mass Spectrometry*:** The analysis set of differentially expressed proteins in HeLa and H157 cell lines present in the *T*-test and Quantitative test obtained from 2D SDS-PAGE (Table 1). Total of 14 spots from both cell lines were selected and excised automatically using ExQuestTM Spot Cutter. Out of the 14 selected protein spots, 6 spots were up regulated, while 8 were down regulated (Table 2). This was followed by protein identification using MALDI-TOF MS in combination with database searching or peptide mass fingerprinting.

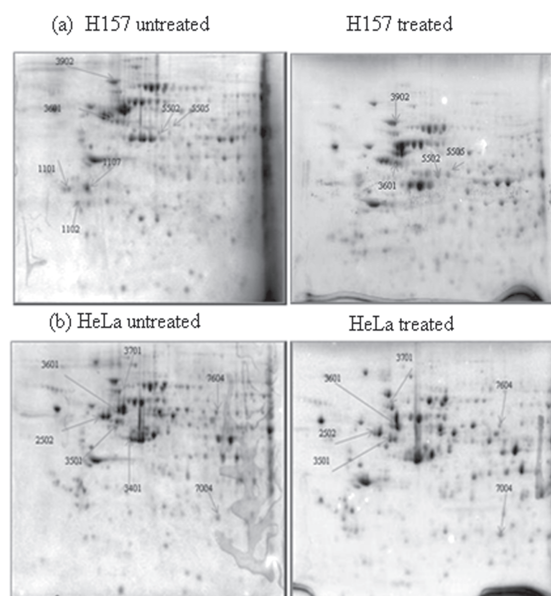

Fig.1. 12 % 2D SDS-PAGE showing proteomes of (a) untreated and treated H157 cells and (b) untreated and treated HeLa cells

Protein identification by MALDI-TOF MS: Peptide masses obtained from MALDI-TOF MS were correlated with protein sequence from a protein database technique known as peptide mass fingerprinting (PMF). The peptide masses obtained from MALDI-TOF MS were then submitted to MASCOT and searched using National Center for Biotechnology Information (NCBInr) database search. Out of the 14 protein spots selected for MS analysis 5 spots were positively identified (Table 3).

Western blot analysis: The GRP78 polyclonal antibody was used to recognize the inducible expression of GRP78 in time dependent manner upon treated H157 cell lines. The expression levels were increased with treatment time (Fig. 2) whereas the GRP78 expression was not observed in HeLa.

Discussion

Our earlier studies on the investigation on pro-apoptotic effects of *Cantharellus* extract showed that H157 cell line is resistant to apoptosis induced by the extract. This study employed comparative proteomic analysis to evaluate the responsible protein. The standard approach for resolving proteins involves two dimensional gel electrophoresis (15). This report describes the evaluation using 2-De MALDI-TOF-MS to profile differential protein expression in H157 and HeLa treated and untreated using *cantharellus* extract induced cell death. There were profound changes in 14 protein spots, out of 14 spots; six were up regulated while eight were down regulated. Using database searches with high confidence based upon high scores and sequences coverage, we identified 5 proteins related to mitochondrial function, oxidative stress and cell motility. The results indicated that mitochondrial dysfunction

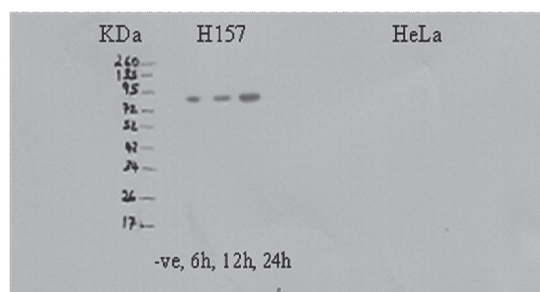

Fig. 2. Expression of Grp78 in H157 cell line in three different time points incubation with *Cantharellus* extract.

Table 1. Number of different expressed proteins in HeLa and H157 cell lines

Cell line	Total number of spots		*T*-test	Quantitative test
	Untreated	Treated		
HeLa	107	95	40	13
H157	87	112	50	17

Table 2. The expression levels of protein spots identified by PDQuest

HeLa cell line		H157 cell line	
Spot number	Expression level	Spot number	Expression level
2502	downregulated	1101	downregulated
3402	down regulated	1102	downregulated
3501	upregulated	1107	downregulated
3601	downregulated	3601	upregulated
3701	upregulated	3902	upregulated
7004	upregulated	5502	downregulated
7604	downregulated	5505	upregulated

might play a role in HeLa induced apoptosis. This was also confirmed by decrease in the mitochondrial transmembrane potential and increase reactive oxygen species (ROS) generation in HeLa treated cells (data not shown).

Results identified spot S 3902 (Fig. 1 and Table 3) as GRP78_Human, 78kDa glucose-regulated protein and was found overexpressed in H157 and nothing observed in HeLa treated cell lines. GRP78 overexpression is linked with the resistance of H157 cell upon treatment with *Cantharellus* extract. This observation supports the observation reported elsewhere that overexpression of GRP78 renders cell more resistance to a wide variety of severe insults including apoptosis inducing chemical substances through their chaperoning and anti-apoptotic effects (16). Further the observation was confirmed on transformed HeLa cell with GRP78, the results revealed 50 % protection on the transformed cell (data not shown)

GRP78 as inhibitors of apoptosis, it tends to form complex with BCL-2 interacting killer (BIK). BIK is a proapoptotic tumor suppressor in several human tissues and has been used as a therapeutic target for anti-cancer drugs and mediates apoptosis through the mitochondrial pathway (17). It has been shown that, GRP78 over expression blocked BIK-induced apoptosis, suppressed estrogen starvation-induced BAX activation and mitochondrial permeability transition (18, 4). GRP78 is able to suppress both BIK and NOXA – mediated apoptosis either individually or in combination. Thus overexpression of GRP78 could negate apoptosis resulting from BIK induction even if it is assisted by another BH3-only protein such as NOXA. Recently it was observed that BCL2 is strongly implicated in the survival pathway activated by GRP78 overexpression (19, 4).

GRP78 over expression binds and inhibits the activation of caspase -7 activated by both ER stress and genotoxic drugs. As executor caspases, caspases-3, -6 and -7 can act in a concert to facilitate the apoptotic process. Therefore, another mechanism for the protective

Table 3. Spots identified by MASCOT search engine

Cell line	Spot number	Database search results
H157	3902	SwissProt 2010_11 (522019 sequences; 184241293 GRP78_HUMAN Mass: 72288 Score: 113 Expect: 1e-07 Matches: 1378 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2
	3601	NCBInr 20101113 (12273660 sequences; 4194265988gil89574029 Mass: 48083 Score: 72 Expect: 0.014 Matches: 8, mitochondrial ATP synthase, H+ transporting F1 complex beta subunit [Homo sapiens]
HeLa	7004	NCBInr 20101113 (12273660 sequences; 4194265988gil4504517 Mass: 22768 Score: 124 Expect: 9.2e-08 Matches: 8heat shock protein beta-1 [Homo sapiens]
	3501	NCBInr 20101113 (12273660 sequences; 4194265988gil89574029 Mass: 48083 Score: 116 Expect: 5.8e-07 Matches: 11mitochondrial ATP synthase, H+ transporting F1 complex beta subunit [Homo sapiens]
	3402	NCBInr 20101113 (12273660 sequences; 4194265988gil297702014 Mass: 39774 Score: 154 Expect: 9.2e-11 Matches: 13PREDICTED: actin, cytoplasmic 2-like isoform 3 [Pongo abelii]

effect of GRP78 is the suppression of the full activation of the multiple caspase-mediated cell death pathways in drug-treated cells contributing to the development of drug resistance (20). It has been shown that GRP78 blocks caspase -7 activation by facilitating the formation of an inhibitory complex suppressing its activation. Recent reports have shown that the ATPase domain of GRP78 binds to procaspase -7, blocking its activation and decrease cellular apoptosis (20, 21). It can also form more inhibitory complexes as well as preserving the integrity of the existing inhibitory complex. GRP78 resembles the X-linked inhibitor of apoptosis (XIAP) such that both genes contain an internal ribosome entry site element that allows efficient translation under physiological stress conditions (1). The results reported here indicated that, 78kDa glucose-regulated protein might responsible for H157 resistance to apoptosis when H157 treated with *Cantharellus* extract. Western blot analysis was used for validation of GRP78 protein overexpression at three different time points in H157 and HeLa cell lines after and before treatment with *Cantharellus* extracts. The results showed that GRP78 overexpressed with time (FIg. 2). It has been observed from previous studies that overexpression of GRP78 promotes tumor proliferation and metastasis, that reducing GRP78 expression resulted in an increase in tumor cell apoptosis (17, 6). This implies that drugs that targeted GRP78 activity might complement conventional cancer therapy

Human cancer cells are derived from dissimilar tissues therefore they can use different diverse signaling and defense mechanisms to acquire resistance to specific drugs. Thus, the cytoprotection of GRP78 to variety of drug is likely to vary among cancer cells. More number of cell lines is needed to be evaluated in order to establish the mechanism of action among cancer cells for the development of possible drug.

Conclusion

The results suggested that, overexpression of GRP78 protein in H157 cell is responsible for the apoptosis resistance of H157 upon *Cantharellus* treatment. This was also confirmed by western blotting analysis. Therefore, successful suppression of GRP78 protein activity can offer a new therapeutic approach to H157

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References

1. Reddy, R.K., Mao, C., Baumester, P., Austin, R.C., Kaufman, R.J. and Lees, A.S. (2003). Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7activation. J. Biol. Chem, 278(23): 20915-20924
2. Lee, A.S. (2005). The Er chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods, 35: 373-381
3. Luo, S., Mao, C., Lee, B. and Lee, S.A. (2006). GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early Mouse embryonic development. Mol cellular Biol 26 (15): 5688-5697
4. Zhang, Y., Liu, R., Ni, M., Gill, P. and Lee, A.S. (2011). Cell surface relocalization of the endoplasmic reticulum chaperone and unfolded protein response regulator GRP78/BiP. J. Biol Chem 285: 5065-15078
5. Zhang, L. and Zhang, X. (2010). Roles of GRP 78 in physiology and Cancer. AMJ Transl Res 2(1): 65-74.

6. Virrey, J.J., Dong, D., Stiles, C., Patterson, J.B., Pen, L., Ni, M., Schonthal, A.H., Chen, T.C., Hofman, F.M. and Lee, A.S. (2008). Stress chaperone GRP78/Bip confers chemoresistance to tumor-associated endotheliar cells. Mol Cancer Res 6: 1268-1275

7. Zhou, H., Zhang, Y., Fu, Yong, Chan Lauren, and Lee S.A. (2011). A novel mechanism of anti-apoptotic function of 78kDa Glucose regulated protein (GRP 78), an endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BcL-2) from BcL-2 interacting killer (BIK). J biol Chem, 10: 1074-1080

8. Yim, E., Bae, J., Lee, S., Lee, K., Kim, C., Namkoong, S., Um, S. and Park, J. (2004). Proteome Analysis of differential Protein Expression in Cervical Cancer Cells after Paclitaxel Treatment. Canc Res Treatment 36(6): 395-399

9. Henzel, W.J., Billeci, T.M., Stults, J.T., Wong, S.C., Grimley, C. and Watanabe, C. (1993). Identifying proteins from two-dimensional gels by molecular mass searching peptide fragments sequences database. Pro Natl Acad Sci, 90(11): 5011-5015

10. Pyrko, P., Schonthal, A.H., Hofman, M.F., Chen, C.T. and Lee, A.S. (2007). The unfolded protein response regulator GRP78/BiP as novel target for increasing chemosensitivity in malignant Gliomas, Cancer Res 1567: 9809

11. Masalu, R., Hosea, K.M.M., Meyer, M., Lyantagaye, S. and Kanyanda, S. (2010). Induction of early apoptosis and reactive oxygen species (ROS) production by Tanzanian basidiomycete (Cantharellus miomboensis). Intern J. Biol Chem Sciences, 4 (4): 825-833

12. Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254

13. Simon, H.U., Yehia, A.H. and Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis, 5(5): 415-8

14. Alexander, H., Stegner, A.L., Wagner-Mann, C., Du Bois, G.C., Alexander, S., Sauter, E.R. Cancer Res, 10: 7500-7510

15. Martin, S., Lamb, H.K., Brady, B., Lefkove, B., Bonner, M.Y., Thomson, P., Lovat, P.E., Arbiser, J.L., Hawkins, A.R. and Redfern, C.P.F. (2013). Inducing apoptosis of cancer cells using small-molecule plant compounds that binds to GRP78. BJC, 109: 433-443.

16. Suzuki, M.T. and Giovannomi, S.J. (2007). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol, 62: 625-630

17. Ni, M., Zhang, Y. and Lee, S.A. (2011). Beyond the endoplasmic reticulum: a typical GRP78 in cell viability, signaling and therapeutic targeting. Biochem. J, 434: 181-188

18. Min, N.I., Zhang, Y.I. and Lee, A.S. (2011). Beyond the endoplasmic reticulum: atypical GRP78 in cell viability signaling and therapeutic targeting. Biochem J, 434: 181-188

19. Morris, J.A., Domer, A.J., Edwards, C.A., Handershort, L.M. and Kaufman, R.J. (1997). Immunoglobulin binding protein (Bip) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective protein. J. Biol. Chem, 272 (7): 4327 – 4334

20. Sugawara, S., Takeda, K., Lee, A. and Dennert, G. (1993). Suppression of stress protein GRP 78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. Cancer Res, 53: 6001-6005

21. Lee, S.W.and Tian, L. (2010). X–linked inhibitor of apoptosis protein as therapeutic target in metastatic melanoma. J. Cell Biochem. 23(5): 675 – 682.

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Phytochemical Analysis and Antimicrobial Potential of *Abutilon indicum* Stem Extracts against GUTI Pathogens

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Abstract

Abutilon indicum is a well-known traditional medicinal shrub and used as an agent of anthelminthic, hepatoprotective and hypoglycemic. However limited studies are available on its potential against Genitourinary tract infections. The present investigation deals with the analysis of possible phytochemicals exist in the stem of *Abutilon indicum* followed by evaluating its antimicrobial potential against various pathogenic microbes involved in Genitourinary tract infections. The phytochemicals analysis showed the existence of flavonoids, steroids, tannins, phenols etc upon subjecting to sequential extraction process employing n-hexane, ethyl acetate, methanol and aqueous solvent systems. Evaluation of extracts against a panel of GUTI-pathogens revealed that against all the tested extracts, the ethyl acetate extract showed considerable inhibitory effect against all the pathogens. The extract was separated by TLC and silica gel column chromatography to fractionate the bioactive constituents. Evaluation of chemical nature of ethyl acetate fractions revealed that the presence of alkaloids, steroids, phenolic compounds, tannins, tripenoids and saponins. The isolated compounds were further screened for antimicrobial activity against GUTI. Bioactive alkaloid fraction showed 95.10% purity by HPTLC was subjected to FTIR analysis.

Keywords: *Abutilon indicum*; Ethyl acetate, Bioactive, GUTI.

Introduction

Bacteria are abundant life form on Earth with wide genetic diversity resulting ever increasing adverse medical conditions leading to morbidity and mortality associated with microbial infectious diseases (1, 2). To treat such communicable diseases and microbial infections several drugs and synthetic medicines were developed. However, during the passage of time the microbes developed resistance to synthetic drugs and to survive in multiple environmental habitats and harsh conditions (3). Frequent use of antibacterial, cytotoxic and immunosuppressive drugs increased microbial infections (1, 4) due to increased resistance.

In oral cavity, skin, gastrointestinal tract and genitourinary tract system diverse bacterial assemblage colonizes. Pathogenic invasion of genitourinary tract includes kidneys, periurethra, urethra, bladder and cervix leading to inflammatory response refers to genitourinary tract infections (GUTI). GUTI affects more than 300 million women and female children per year worldwide. These infections are caused by *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumonia, Enterococcus foecalis, Candida albicans, Gardenerellavaginalis etc* (5).

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In the past Bacterial Vaginalis (BV), one of the most GUTI not considered as pathogenic but recently found to be associated with obstetric infections (6). *Gardenerellavaginalis*one of organisms involved in Bacterial vaginalis causes vaginal discharge, pruritis and odor. Metronidazole or clindamycin are common antibiotics used for infections whereas recurrent infections occurred due to frequent use and their adaptability and survival of microbial flora (7). Resurgence of infectious disease increased the impact of immunocompromised population over last three decades due to antibiotic resistant microorganisms. In addition, synthetic drugs are costly and are out of range to a lay person lead to a growing interest to unlock the secrets of therapeutic components present in plants and their pharmacological evaluation. On the contrary, the therapeutic value of plant extracts has higher potential, less side effects, well tolerated remedies and safe for human beings economically. Bioactive plant extracts serves as a guide post for new antibacterial drug discovery and is ecofriendly (3).

India is enriched with flora and fauna, since ancient times most of the plants have been using for human ailments (8). *Abutilon indicum* (Malvaceae family) commonly known as *Atibala* (Sanskrit); an erect, pubescent, perennial shrub native to subtropical and tropical regions. Seeds, bark, roots, leaves, flowers in fact whole plant is used in traditional medicine as a laxative, diuretic, demulcent, pulmonary, aphrodisiac, expectorant, sedative, anti-inflammatory and antihelminthic to treat gonorrhoea, headache, bladder infections and ulcers (9-12). However survey of literature afforded limited systematic approach, made to study antimicrobial activity of stem extract of *A.indicum* invitro. The present investigation objective is to evaluate phytochemical analysis, antimicrobial activity, composition, determination, standardization and chemical characterization of *Abutilon indicum* stem ethyl extract to provide scientific evidence for its antimicrobial potential against GUTI.

Materials and Methods

Chemicals: Muller hinton agar, mullerh inton broth, ethyl acetate, methanol, n-hexane were purchased from Hi media, Mumbai, India. The standard antibiotics ciprofloxin, metronidazole, flucanozole were obtained from Sigma-Aldrich. Silica gel for column chromatography was from Finar chemicals India Pvt. Ltd.

Collection of plant material: Fresh stems of *Abutilon Indicum* were procured from the regions of Kanigiri, Prakasam (Dt), India. Dr.K. Guravareddy Scientist of Regional Agriculture Research Centre (RARC) identified and authenticated the plants in the regions of Guntur, Andhra pradesh, India. The plant material was washed thoroughly with distilled water and air dried at room temperature. These dried samples were pulverized into fine powder and stored at 4°C in zip-lock bags (13).

Preparation of solvent extracts:The processed stem material was subjected to fractionation in order of increasing polarity (n-Hexane > Ethyl acetate > Methanol > Water). Extraction was carried out using Serial Exhaustive Extraction process at room temperature (mass to volume ratio 1:10). The solvents were evaporated using Rotary evaporator at 40°C.

Phytochemical screening*:* Using standard qualitative method, the stem extract of *A.indicum* was subjected to phytochemical screening for constituents including carbohydrates, steroids, phenols, tannins, flavonoids, cardiac glycosides, saponins and starch.

Microbial cultures: Pure cultures of *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus foecalis, Candida albicans, Gardenerellavaginalis* were provided by Mrs. L. Jayalakshmi, Department of Microbiology of Osmania Medical College, Hyderabad, India. Thereafter, the MH agar slants of all the strains were prepared and stored at 4°C.

Antimicrobial screening of solvent extracts: All the solvent free extracts of *A.indicum* stem were evaluated for antimicrobial activity by Kirby Bauer method (agar well diffusion method) (14). Microbial strains were subcultured into fresh medium and incubated at 37°C for 18h, then by pour plate method inoculated aseptically to Muller Hinton Agar (MHA) medium. 100µl of each extract with different concentrations (100 - 400mg/ml) was added to the wells punched on agar medium (6mm). Ciproflaxin (5µg/ml), Metronidazole (5µg/ml), Flucanazole (20 µg/ml) were taken as standard drugs.

Minimum inhibitory (MI) and bactericidal (MB) concentrations: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the stem extracts were determined by broth dilution methods (15). A serial two fold dilution ranging from 10 – 0.15 mg/ml was used for determining MICs and MBCs. The lowest concentration where no growth is visible is considered as the minimum inhibitory concentration and where no growth in drug free media is minimum bactericidal concentration.

Separation and purification of bioactive compounds:The crude extracts were subjected to column chromatography to separate and purify the bioactive compounds. In brief, 1 gm of dried bioactive plant extract was dissolved in suitable solvent (Ethyl acetate) and ground with 3 gm of silica gel to eliminate the formation of air bubbles. Then the trodden material was loaded on the top of silica gel packed in a glass column. Initially, 100 ml of a non-polar solvent such as n-Hexane was passed in order to elute residual non-polar compounds. Later, the polarity of eluting solvent was increased with ethyl acetate. Each fraction was collected and stored in separate screw cap container. Thin layer chromatography was performed for each fraction and then the fractions were evaluated for antibacterial activities.

Purity of bioactive fractions:The samples were spotted on pre-coated silica gel plates 60 F 254 (2cm x 2cm with 0.2mm thickness (E.Merck, KGaA64271 Darmstadt, Germany) using a CAMAG automatic sample spotter (CAMAG TLC Scanner 3). Initially the TLC plates were washed with ethyl acetate and activated at 60°C for 5 minutes prior to performing. The sample was spotted just 1cm above from the bottom and 1cm from the left edge of the TLC plate. The loaded plates were placed in a solvent system containing hexane-ethyl acetate 4:1 ratio in glass TLC chamber (20±10cm) previously saturated with the solvent for 30min (temperature 25±2°C) till the solvent front reached to 5.9cm for the separation of components. TLC plates were air dried and scanning was performed on a CAMAG TLC scanner 3 at absorbance of 254nm fitted with win CATS planar chromatography manager software V1.4.4 version.

Characterization of bioactive compound:The fourth fraction obtained from column chromatography of ethyl acetate extraction of stem was analysed by Fourier- transform infra red spectroscopy (FT-IR). One drop of sample was placed between two cells of sodium chloride to form a thin film. Then the spectrum was recorded.

Results

***Phytochemical analysis*:** The preliminary phytochemical studies showed the presence of flavonoids, phenols, steroids, saponins, tannins and glycosides, starch and terpenoids in n-Hexane extract of *A.indicum* stem. In ethyl acetate extract, phenols, steroids, carbohydrates, saponins, glycosides, cardiac glycosides, starch and terpenoids were observed. Methanol extracts revealed the presence of phenols, steroids, carbohydrates, tannins, cardiac glycosides, starch and terpenoids. Whereas, the aqueous extract exhibited presence of flavonoids, phenols, steroids, carbohydrates, tannins, glycosides and starch (Table 1).

Antimicrobial screenings

***Zone of inhibitions*:** The antimicrobial activity of n-hexane, ethyl acetate, aqueous and methanol extracts of *A.indicum* stem was tested against various pathogens involved in genitourinary tract infections such as *Escherichia coli, Staphylococcus aureus, Staphylococcus*

epidermidis, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus foecalis, Candida albicans, Gardenerellavaginalis (Tables 2-5). The inhibition pattern was observed to be varied with the sort of microorganism employed, solvent type and their concentration of extract. The zone of inhibition increased with the concentration of extract used. The inhibition zones were observed to be in the ranges from 06±0.1 to 18±0.1mm for hexane extract, 07±0.2 to 21±0.2mm for ethyl acetate extract, 07±0.3 to 19±0.1mm for methanol and 06±0.2 to 15±0.1mm aqueous extracts whereas the zone of inhibition for standard drug ranges from 18±0.1 to 35±0.4. Among all solvent extracts, maximum zone inhibition was found for ethyl acetate extract (21±0.2mm) at 40mg/ml concentration (Table 3). The antifungal activity against *Pseudomonas aeuriginosa* (concentration 20mg/ml) showed 21±0.2mm zone of inhibition while, 16±0.2mm

Table 1. Phytochemical constituents present in the stem of*Abutilon indicum*.

Phytochemical compounds	Extracts			
	Hexane	Ethyl acetate	Methanol	Water
Alkaloids	-	-	-	-
Flavonoids	+	-	-	+
Phenols	+	+	+	+
Steroids	+	+	+	+
Carbohydrates	-	+	+	+
Saponins	+	+	-	-
Tannins	+	-	+	+
Glycosides	+	+	-	+
Cardiac glycosides	-	+	+	-
Starch	+	+	+	+
Terpenoides	+	+	+	-

Note:(-) refers as absent and (+) refers as present.

Table 2. Antimicrobial activity of hexane extract of stem

Microorganisms	Diameter of zone of inhibition (mm) at different concentration levels Hexane extract				
	100mg/ml	200mg/ml	300mg/ml	400mg/ml	Standard drug (µg/ml)
E.coli	08±0.2	09±0.3	10±0.1	15±0.2	32±0.2
E.foecalis	06±0.1	06±0.2	07±0.4	07±0.1	28±0.1
P.aeruginosa	14±0.4	17±0.2	18±0.1	18±0.1	35±0.4
S.aureus	07±0.2	07±0.1	07±0.1	08±0.4	35±0.1
S.epidermidis	07±0.3	08±0.2	08±0.2	08±0.1	09±0.4
K.pneumonia	06±0.1	08±0.1	10±0.3	12±0.2	32±0.3
G. vaginalis	08±0.2	11±0.1	12±0.3	13±0.2	18±0.3
C. albicans	08±0.1	11±0.3	13±0.2	16±0.1	30±0.1

Note: Standard drug used: ciproflaxcin for G (+Ve) and G(-Ve) bacteria of concentration (5µg/ml); Flucanozole for *Candidaalbicans* (5µg/ml), Metronidazole 20µg/ml for Gardenerellavaginalis. Data are means (n=3) ± standard deviation of three replicates.

zone of inhibition formed for ethyl acetate extract against *Gardenerellavaginalis.* The antimicrobial activity of n-hexane, methanol, aqueous extracts were lower when compared to extract of ethyl acetate *Abutilon indicum* (Table 2, 4 and 5). The ethyl acetate extract contributed highest zone of inhibition 21±0.2mm for *P.aeruginosa* however the same extract inhibited *S.aeureus* poorly (0.7±0.2mm).

***Minimum Inhibitory and bactericidal concentrations*:** The MICs of the *Abutilon indicum* stem extracts observed against all the pathogenic strains and they were found to be in

Table 3. Antimicrobial activity of ethyl acetate extract of stem

Microorganisms	Diameter of zone of inhibition (mm) at different concentration levels				
	Ethyl acetate extract				Standard
	100mg/ml	200 mg/ml	300mg/ml	400mg/ml	drug (µg/ml)
E. coli	08±0.4	11±0.2	13±0.2	20±0.1	32±0.2
E. foecalis	12±0.2	14±0.1	18±0.3	19±0.1	28±0.1
P. aeruginosa	11±0.3	13±0.2	16±0.1	21±0.2	35±0.4
S. aureus	07±0.2	09±0.1	11±0.3	13±0.1	35±0.1
S. epidermidis	10±0.1	12±0.4	14±0.2	16±0.3	35±0.4
K. pneumonia	10±0.2	11±0.1	12±0.1	15±0.2	32±0.3
G. vaginalis	07±0.3	08±0.2	13±0.1	16±0.2	18±0.3
C. albicans	10±0.2	11±0.3	14±0.1	19±0.1	30±0.1

Note: Standard drug used: ciproflaxcin G(+Ve) and G(-Ve) bacteria of concentration (5µg/ml);Flucanozole for Candida albicans (5µg/ml), metronidazole 20 µg/ml for Gardenerellavaginalis. Data are means (n=3) ± standard deviation of three replicates.

Table 4. Antimicrobial activity of methanol extract of stem

Microorganisms	Diameter of zone of inhibition (mm) at different concentration levels				
	Methanol extract				Standard
	100mg/ml	200mg/ml	300mg/ml	400mg/ml	drug (µg/ml)
E. coli	07±0.3	08±0.2	11±0.1	12±0.2	32±0.2
E. foecalis	08±0.1	09±0.1	10±0.2	10±0.4	28±0.1
P. aeruginosa	13±0.2	15±0.2	17±0.1	18±0.1	35±0.4
S. aureus	11±0.2	12±0.1	12±0.3	15±0.2	35±0.1
S. epidermidis	10±0.4	13±0.2	16±0.1	19±0.1	35±0.4
K. pneumonia	09±0.1	09±0.3	11±0.2	12±0.2	32±0.3
G. vaginalis	08±0.2	08±0.1	10±0.3	13±0.1	18±0.3
C. albicans	11±0.1	11±0.2	13±0.2	14±0.1	30±0.1

Note: Standard drug used: Ciproflaxcin for G(+Ve) and G(-Ve) bacteria of concentration (5µg/ml); Flucanozole for *Candida albicans* (5µg/ml), Metronidazole 20µg/well for Gardenerellavaginalis. Data are means (n=3) ± standard deviation of three replicates

range from 0.62mg/ml to 5mg/ml for bacterial strains and for *Candida albicans*. In case of bacterial strains, ethyl acetate extract showed potent activity against the *E.coli* and *P.aeruginosa* having MICs 0.62mg/ml and 0.31mg/ml, respectively (Table 6). Similarly, the ethyl acetate extracts of *Abutilon indicum* showed the lowest MICs against C*andida albicans* when compared to other extracts. The MBCs observed were in the range of 0.31 to 10 mg/ml for bacterial strains and *Candida albicans*. Similarly the ethyl acetate extract showed the lowest MBCs against bacterial

Table 5. Antimicrobial activity of aqueous extract of stem

Microorganisms	Diameter of zone of inhibition (mm) at different concentration levels				
	Aqueous extract				Standard
	100mg/ml	200 mg/ml	300mg/ml	400mg/ml	drug (µg/ml)
E. coli	06±0.2	07±0.2	09±0.1	11±0.3	32±0.2
E. foecalis	11±0.3	14±0.1	14±0.2	15±0.1	28±0.1
P. aeruginosa	11±0.1	12±0.2	13±0.1	13±0.4	35±0.4
S. aureus	10±0.2	11±0.1	14±0.2	14±0.3	35±0.1
S. epidermidis	13±0.1	13±0.3	15±0.2	15±0.1	35±0.4
K. pneumonia	06±0.2	08±0.4	08±0.1	11±0.3	32±0.3
G. vaginalis	0.6±0.2	10±0.1	12±0.3	15±0.1	18±0.3
C. albicans	0.6±0.1	0.9±0.2	11±0.2	12±0.1	30±0.1

Note: Standard drug used: Ciproflaxcin for G (+Ve) and G(-Ve) bacteria of concentration (5 µg/ml); Flucanozole for Candida.albicans (5µg/ml), Metronidazole 20 µg/ml for Gardenerellavaginalis. Data are means (n=3) ± standard deviation of three replicates.

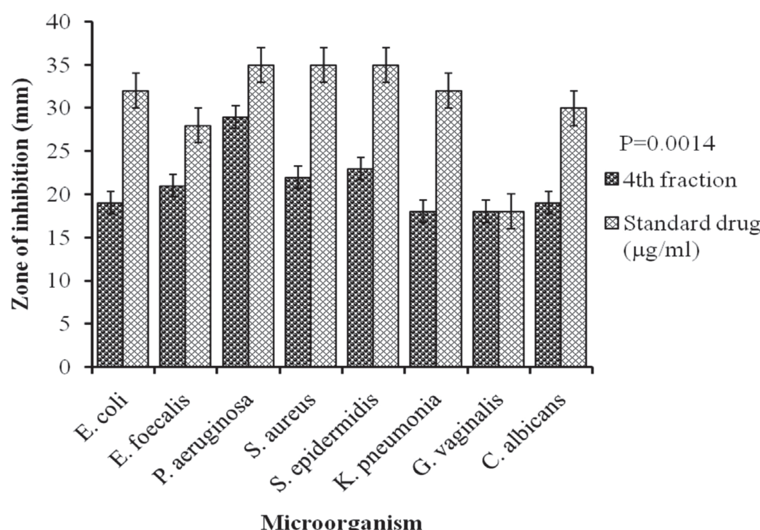

Fig. 1. Antibacterial effect of purified bioactive compound of (4th fraction) ethyl acetate stem extract in comparison with standard drugs (a positive control antibiotics) (**Note:** F- Fractions (1-7), NI- No zone of Inhibition.Standard drug used: Ciproflaxcin for G (+Ve) and G(-Ve) bacteria of concentration (5 µg/ml); Flucanozole for *Candida albicans* (5µg/ml), Metronidazole 20 µg/ml for *Gardenerellavaginalis*. Data are means (n=3) ± standard deviation of three replicates. P=0.01<0.05 means significant)

strains and *Candida albicans* when compared to the other extracts.

***Purification and antimicrobial property of bioactive fraction*:** The crude ethyl acetate extract of *A.indicum* stem was subjected to purification by column chromatography and among the obtained fractions, the 4th fraction showed good inhibitory activity ranging from 18±0.4mm to 29±0.4 mm. Whereas, the standard positive control drugs showed inhibition zones ranging from 18±0.3mm to 35±0.4mm. It was observed that, from table 8 and Fig. 1, the highest inhibitory effect was observed on *P.aeruginosa* (29±0.4) and whereas the lowest effect on *G.vaginalis* (18±0.4). The purified fractions of ethyl acetate extracts were also screened for MICs and MBCs determination against selected microbial strains and noticed that the fourth fraction revealed the highest zone of inhibition (table 9). Observed MICs for microbial strains were in the range from 0.077 to 0.310 and MBCs range from 0.150 to 0.620 and whereas for the standard drug the range was 0.052 to 0.372. The MIC for *P.aeruginosa* (0.077) showed lowest concentration and *S.aureus* (0.310) showed highest concentration. Similarly MBC observed for *P.aeruginosa* (0.150) has minimum concentration and *S.aureus*, *Candida albicans* (0.620) has maximum concentration. Furthermore, the phytochemical analysis of ethyl acetate extract showed the presence of steroids, phenolic compounds, tannins and trace of tripenoids (Table 7).

HPTLCanalysis: The 4th fraction (Fig. 2a) obtained from ethyl acetate extract of stem was subjected to HPTLC analysis for the generation of HPTLC finger printing profile which is represented as a chromatogram. The solvent system used in the investigation was found to have compact spots for extracts at different Rf values and there was no overlap with any other component in the analyzed sample at 254nm. The solvent system used in the investigation was found to give a strong uphill peak with purity of about 95.10% for the fourth fraction on with Rf values between 0.77-0.86. No

Table 6. MIC and MBC of different solvent extracts of Stem

Name of the organism	Solvent extracts								Standard drug (µg/ml)
	Hexane		Ethyl acetate		Methanol		Aqueous		
	MICmg/ml	MBCmg/ml	MICmg/ml	MBCmg/ml	MICmg/ml	MBCmg/ml	MICmg/ml	MBCmg/ml	
E.coli	2.50	5.00	0.31	0.62	1.25	2.50	5.00	10.0	0.058
E.foecalis	5.00	10.0	0.62	0.31	2.50	5.00	2.50	5.00	0.026
P.aeruginosa 0.0	0.31	0.62	2.50	5.00	5.00	10.0	0.035		
S.epidermidis	1.25	2.50	0.62	0.31	1.25	2.50	1.25	2.50	0.043
K.pneumonia	2.50	5.00	0.62	0.31	1.25	2.50	2.50	5.00	0.124
Candida albicans	5.00	10.0	0.62	0.31	5.00	10.0	5.00	10.0	0.028
Gardenerellavaginalis	5.00	1.25	0.31	0.62	2.50	5.00	2.50	5.00	0.062

Standard drug used: Ciprofloxacin for G (+Ve) and G(-Ve) bacteria and Fluconazole for *Candida albicans*, Metronidazole for *Gardenerellavaginalis*
MIC: Minimum Inhibition Concentration, MBC: Minimum Bacterial Concentration

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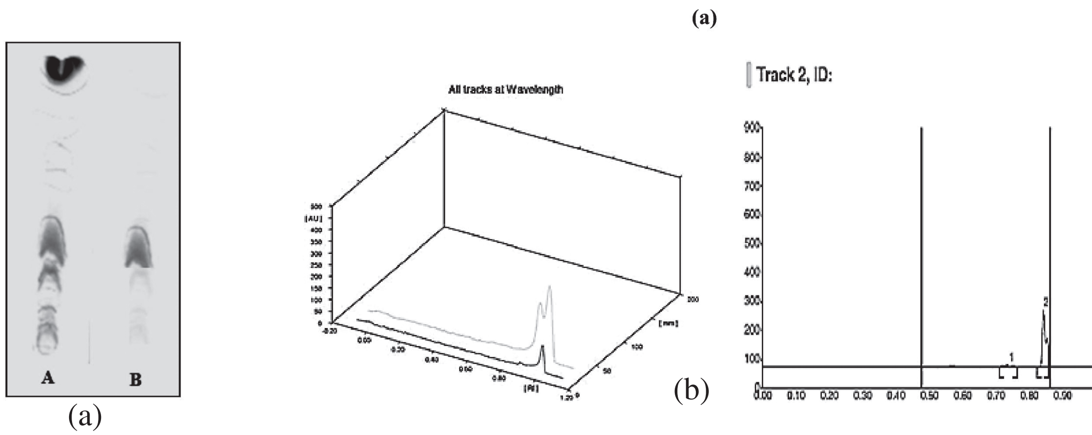

Fig.2. (a) TLC chromatogram of EA extract (A) and purified fraction (B);(b) HPTLC analysis showing finger printing of the bioactive compound (4th fraction) of ethyl acetatestemextractof *Abutilon indicum*.

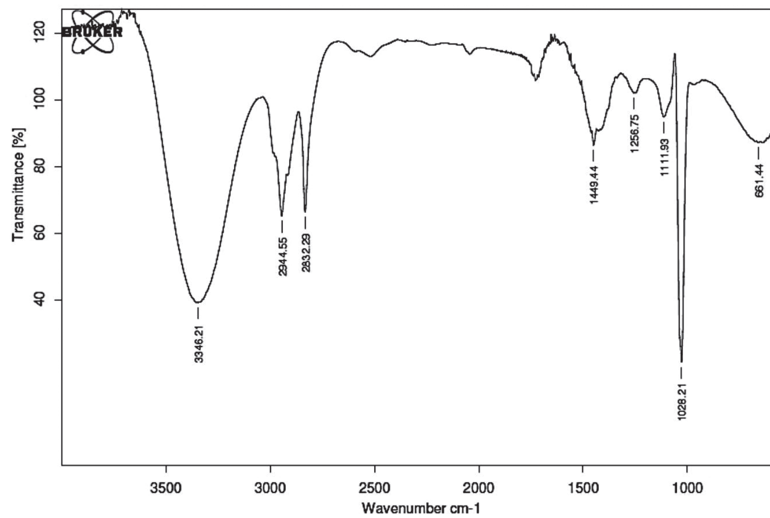

Fig. 3. FTIRspectrapeakvaluesofthebioactive fraction-(4)(ethyl acetate stem extracts) of Abutilon indicum.

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overlap with any other peaks was observed in the analyzed sample; this shows the purity of a bioactive fraction. The results and observations are presented in table 10 and Fig. 2b.

FT-IR analysis: Spectroscopic analysis was performed for the TLC fractions that showed antimicrobial activity. The FT-IR spectrum obtained with that of the reference chart revealed the presence of functional groups such as alcohols, alkyl halides, aliphatic amines, amines, alkanes and alkenes in the stem extract. The fourth fraction of ethyl acetate extract of stem was analysed by FT-IR for determining the functional groups of the bioactive component based on its peak ratio and electron transition of compounds. The peak values observed within the IR range (4000-400cm-1) include 3346.21 (alcohol) and 2944.55, 2832.29 and 1449.24 (alkanes) and 1256.75 (alkyl halides), 1028.21 (amines), 661.44 (alkenes) and 1111.95 (aliphatic amines). More alkane functional groups in the active fraction are found (Table 11 and Fig.4).

Discussion

Table 7. Phytochemical components present in purified *Abutilon indicum*ethylacetatedried stem extract.

Bioactivecompounds	Result
Alkaloids	-
Steroids	+
Tripenoids	±
Phenolic Compounds	+
Tannins	+
Saponins	-
Glycosides	-
Triterpenes	-

Note: "+" = Present; "-" = negative, "**±**" = Trace.

Plants comprise of numerous bioactive phyto-compounds which are imperative for the development of antimicrobial agents. The plants belonging to Malvaceace family have emperical applications in the treatment of skin diseases, wounds and also show antimicrobial activity (16). The phytochemical components of *Abutilon indicum* have been established in previous studies and these include majorly tropane alkaloids such as â-sitosterol, D-amylorin, eugenol which could be responsible for its hepatoprotective, anti-inflammatory, antimicrobial activity (12). Successive isolation of a bioactive compound from any herbal plant is mainly based on the type of solvent employed in the extraction procedures (17). In the present investigation, different polar solvents such as n-hexane, ethyl acetate, methanol and aqueous solvents were employed for extracting the bioactive constituents from stem of *Abutilon indicum*. The ethyl acetate extracts of stem showed good inhibitory effect against antibiotic resistant strains isolated from GUTI (Table 2 to 5). Even the aqueous stem extract of *A.indicum* screened against human pathogenic bacteria showed similar type of results as reported by (18). While as shown in the present study, differential activities were observed between a less polar (n-hexane ethyl acetate) solvent and a high (methanol) polar solvent extracts of stem. Ethyl acetate extract of stem has shown moderate activity. Though several compounds are known to present in *Abutilon indicum* and only some will show the bioactivity. This is further evidenced in the present study too where only one fraction of the ethyl acetate extract of stem revealed moderate bioactivity. Further analysis of active fraction by HPTLC and FT-IR (Fig.4) depicted 95.10% purity. This is confirmed based on the fact that testing of crude ethyl acetate extract fraction and purified fraction of stem showed that the pure fraction was more effective against GUTI pathogens. In view of the above and present data on the demonstration of antimicrobial activity of *Abutilon indicum* against GUTI provide an immense scope for developing synergetic compounds to enhance the magnitude of synthetic antibiotics.

Conclusion

In view of the continuous rise of antibiotic resistant microbial strains, the present study gains

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Table 8. Antimicrobial effect of column chromatography purified fraction of dried stems of ethyl acetate extract of *Abutilon indicum*

Diameter of zone inhibition (mm) Dried stems of Ethyl acetate Fractions (1mg/ml)								
Test organisms	**1 F**	**2 F**	**3F**	**4F**	**5F**	**6F**	**7F**	**Standard drug (µg/ml)**
E. coli	NI	NI	NI	19±0.2	NI	NI	NI	32±0.2
E. foecalis	NI	NI	NI	21±0.3	NI	NI	NI	28±0.1
P. aeruginosa	NI	NI	NI	29±0.4	NI	NI	NI	35±0.4
S. aureus	NI	NI	NI	22±0.1	NI	NI	NI	35±0.1
S. epidermidis	NI	NI	NI	23±0.4	NI	NI	NI	35±0.4
K. pneumonia	NI	NI	NI	18±0.3	NI	NI	NI	32±0.3
G. vaginalis	NI	NI	NI	18±0.4	NI	NI	NI	18±0.3
C. albicans	NI	NI	NI	19±0.1	NI	NI	NI	30±0.1

Note: F- Fractions (1-7), NI- No zone of Inhibition.Standard drug used: Ciproflaxcin for G (+Ve) and G(-Ve) bacteria of concentration (5 µg/ml); Flucanozole for Candida.albicans (5µg/ml), Metronidazole 20 µg/ml for Gardenerellavaginalis. Data are means (n=3) ± standard deviation of three replicates.

Table 9. Minimum inhibitory and bactericidal concentrations of the 4th fraction (purified) of dried stems of *Abutilon indicum*ethyl acetate extract.

Test organisms	**4th fraction (1mg/ml)**		**standard(1mg/ml)**	
	MIC	**MBC**	**MIC**	**MBC**
E. coli	0.150	0.310	0.058	0.174
E. foecalis	0.310	0.620	0.026	0.052
P. aeruginosa	0.077	0.150	0.078	0.234
S. aureus	0.310	0.620	0.035	0.105
S. epidermidis	0.077	0.150	0.043	0.086
K. pneumonia	0.150	0.310	0.124	0.372
G. vaginalis	0.150	0.310	0.028	0.056
C. albicans	0.150	0.620	0.062	0.186

Note: Standard drug used: Ciproflaxcin for G (+Ve) and G(-Ve) bacteria of concentration (1mg/ml); Flucanozole for Candida.albicans (1mg/ml), Metronidazole (1mg/ml) for Gardenerellavaginalis. Data are means (n=3) ± standard deviation of three replicates.

Table 10. HPTLC analysis for purity of bioactive compound of the 4th fraction (dried) stem ethyl acetate extract *Abutilon indicum*

Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.71	1.1	0.74	10.3	4.90	0.77	0.4	188.1	10.99
2	0.82	0.0	0.84	200.0	95.10	0.86	65.9	1524.0	89.01

Table 11. FTIR peak values of the 4th fraction of ethyl acetate stem extracts of *Abutilon indicum.*

S.No.	Peak valves Absorption (cm^{-1})	Functional Group Names
1	3346.21	Alcohol
2	2944.55	Alkanes
3	2832.29	Alkanes
4	1449.44	Alkanes
5	1256.75	Alkyl Halides
6	1111.95	Aliphatic Amines
7	1028.21	Amines
8	661.44	Alkenes

importance to exploit the synergitic antibacterial activity of the extracts of *A. indicum* and the findings clearly indicate that *A.indicum* has profound antimicrobial activity against the GUTI strains. These results are supportive to the traditional use of *A.indicum*in phyto-medicine. Further research should be focused for the structural elucidation of active fraction by LC-MS and NMR studies.

References

1. Murat E. Marcela, Christopher, Z., Taylor, M., Scot E. Dowd, David H.Martin and Michael J. Ferris (2011). Exploring the Diversity of *Gardnerellavaginalis* in the Genitourinary Tract Microbiota of Monogamous Couples through Subtle Nucleotide Variation; www.plosone.org, 6:(10)e26732.
2. Shokeen, P., Bala, M. and Tondon (2009). Evaluation of the activity of 16 medicinal plants antibacterial potential against UTI isolates against *Neisseria gonorrhoeae*.Int. J. Antimicrob. 33: 86-91.
3. Subramaniyan V. Sellan, C. and Srinivasan, P. (2013). Screening of ethnomedicinal plants for antibacterial activity. International Journal of Medicine and Pharmaceutical Sciences (IJMPS), 3(2): 11-18 .
4. Joanne, L., May Anna, K., Christine, A. and Warren (1997). Fluconazole disc diffusion testing for the routine laboratory. Journal of Antimicrobial Chemotherapy. 40: 511–516.
5. Gregor, R. (2001).Probiotic agents to protect the urogenital tract against infection. The American journal of clinical nutrition,73: (437S–43S).
6. Jihan, M., Al-Muk and Hassan, J., Hasony (2001). Isolation of *GardnerellaVaginalis* from Pregnant Women with Bacterial Vaginosis in Basrah, Iraq; Bahrain Medical Bulletin, Vol.23, No.3, September 2001.
7. Nagaraja, P. (2008). Antibiotic resistance of *Gardenerellavaginalis*in recurrent bacterial vaginolis. Indian journal of Medical Microbiology.26:(2): 155-7.
8. Ganga Suresha, P., Ganesana, R., Dharmalingama, M., Baskara, S., Senthil, P. and Kumar, B. (2011). Evaluation of wound healing activity of “*Abutilon Indicum*” linn in wister albino rats. Int J Biol Med Res. 2(4): 908 – 911.
9. Karthikeyan, R., Venkatesh, P. and Chandrasekhar, N. (2012). Morpho anatomical studies of leaves of *Abutilon indicum* (linn.). Sweet Asian Pacific Journal of Tropical Biomedicine. 464-469.
10. Chandan, D., Sujit, D., Durga Charan, S., Arnabaditya, M. and Abhiram, R. (2012). Pharmacognostical characterization and standardization of *Abutilon indicum*bark, Linn. Asian Journal of Plant Science and Research. 2 (2):143-150.
11. Ahmed, I. and Beg, A.Z. (2001). The antibacterial phytochemical studies on 45 Indian medicinal plants potential of these plants against UTI causing pathogens against multi-drug resistant human pathogens. J. Ethnopharmacol. 74: 113-123.

12. Guno Sindhu Chakraborthy and Prashant M. Ghorpade (2009). Pharmacognostical and Phytochemical Evaluation of stem of *Abutilon indicum*(Linn.); International Journal of Pharmaceutical Sciences and Drug Research. 1(3): 188-190

13. Lam, K.S. (2007). New aspects of natural products in drug discovery.Trends Microbiology. 15: 279-289

14. Isao, K., Hisae, M. and Aya, K. (1994). Naturally occurringantiacne agents. Journal of Natural Products, 57: 9-17.

15. Harborne, J.B. (1992). *Phytochernical Methods,* Chapman and Hall publication, London. 7-8.

16. Ayesha, M., Suresh, P.V.K. and Parwez, A (2011). Evaluation of Antibacterial activity of *Cuscuta reflexa* and *Abutilon indicum*. International Journal of Pharma and Bio Sciences. 2(4): 355-561.

17. Pandey, D.P., Rather, M.A., Nautiyal, D.P. and Bachheti, R.K. (2011). Phytochemical analysis of *Abutilon indicum*; International Journal of ChemTech Research . 3(2): 642-645.

18. Anjana, S., Rani, V and Padmini, R. (2009). Antibacterial Activity of Some Medicinal Plants UsedbyTribals Against Uti Causing Pathogens.World Applied Sciences Journal 7 (3): 332-339.

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Transferability of Rice SSR Marker in Wheat (*Triticum aestivum*)

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Abstract

Simple sequence repeat (SSR) markers are short, tandem repeat DNA sequences of few base pairs (1-6 bp). They provide high level of polymorphism and information in crop genomic research. Development of SSR markers is extremely expensive and time consuming because of the requirement of prior sequence knowledge for design of locus specific primers. Transferability of 25 rice microsatellites was studied using six wheat cultivars. 60% of the primers were found to amplify in wheat. Primers RM 351 RM 217, RM 341, RM 3589, RM 188, RM 179, RM 108, RM 239, RM 224 and RM 177 could produce multiple alleles in all the varieties of wheat whereas; RM 212, RM 209, RM 269 and RM 5863 produced single alleles. Therefore microsatellite markers developed for one species could be very valuable in related species.

Introduction

Common wheat *Triticum aestivum* (2n = 6x = 42), belonging to the family poaceae is the second most abundant staple crop grown worldwide. It provides about one-half of human food calories and nutrient requirements. The major wheat producing countries are China, India, USA, France, Russia, Canada, Australia, Pakistan, Turkey, UK, Argentina, Iran and Italy. In India, wheat is mainly grown during the winter season, planted during October–November and harvested during March–April. The gradual increase in population demands substantial in-crease in productivity of wheat. Wheat has always been subjected to extensive and aggressive genetic research to maximize grain production and productivity. DNA based markers provide suitable tools for detailed genetic analysis, gene mapping and estimation of genetic diversity. Microsatellites, also known as Simple Sequence Repeats (SSRs), are short, tandem DNA repeats of few base pairs (1-6 bp). They are abundant in 5'-UTRs, 3'-UTRs, exons and introns. SSRs are used in pedigree analysis to determine kinship among individuals, fingerprinting, forensics, genetic mapping and phylogenetic analysis (1). Microsatellite markers have been developed in many crop species such as soybean (2), wheat (3), maize (4), barley (5), rice (6) potato (7) and many others. In 1998, the first SSR map in bread wheat of 279 loci was reported.

However, development of microsatellite markers is extremely expensive and time-consuming. Only about 30% of all primer pairs developed from microsatellite sequences is functional and suitable for genetic analysis. The high cost of developing microsatellite markers is the main factor limiting their widespread use. A good alternative would be the use of a set of primers across cross-species to avert species barrier. Comparative mapping studies clearly reveal ~~the~~ presence of synteny within the genomes of closely related species of Poaceae

family, such as wheat, barley, rye, triticale, rice and maize. Sufficient homology exists among several crop genomes in the sequences flanking the SSR loci. Thus, primer pairs of a species can be used in related species called "transferability". This similarity and genomic relationship has allowed exchange of SSR primers between crop species. These characteristics are particularly interesting for rice and wheat researchers since a huge number of SSR markers are available in rice and based on its high information content, it can provide good estimate of genetic variability and extensive genomic analysis in other important crops of grass family. Keeping all these in preview, objectives were set in the present study to study the transferability rate of rice genomic SSR markers in wheat and to estimate the polymorphism level of cross-genus or cross-species SSR markers.

Materials and Methods

Six varieties of wheat were taken for the present study (Table 1). The plant material was collected from the germplasm maintained at Directorate of Seed Research, Mau, Uttar Pradesh. Pure seeds were germinated under appropriate conditions and leaves from two weeks old plantlets were collected for the purpose of isolation of DNA. Total genomic DNA was isolated from the fresh leaf using modified method of Guillemant and Laurence (8). About 3g of tender leaf was ground using a pre-cooled mortar and pestle. The leaf powder obtained was transferred to 50ml polypropylene tube and 15ml of the extraction buffer containing 3M sodium acetate, 4M NaCl, 0.5 MEDTA (pH 8.0), 4M Tris HCl (pH 8.0), 3% SDS and 6% PVP was added. The mixture was incubated at 65^{0}C for 30 minutes with 10M ammonium acetate, followed by chloroform-isoamyl alcohol extraction method. After ethanol precipitation, DNA was resuspended in double distilled autoclaved water and incubated for 30 minutes at 37 ^{0}C. To remove the RNA, RNAase 10µg/ml was added into the DNA solution. DNA was further precipitated by following the steps of phenol-chloroform-isoamyl alcohol (25:24:1), chloroform and finally with ethanol. Quantification of the DNA was done by electrophoresis on 0.8% agarose gel (1XTAE) stained with ethidium bromide (0.5 µg/ml). Purified DNA samples were estimated by taking OD value by spectrophotometer at 260 and 280 nm. After quantification, PCR amplification of DNA to check the transferability of twenty five rice genomic SSR markers chosen from www.gramene.org (Table 2) was carried out in 0.2 ml thin wall PCR tubes using a thermal cycler. PCR mixture of 25µl contained 25ng of genomic DNA template, 0.5 U of Taq DNA polymerase,1mM of oligonucleotide primer, 2.5 µl of 10X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5mM $MgCl_2$) and 0.25ìl of pooled dNTPs (100mM each of dATP, dCTP, dGTP and dTTP). The PCR mixture was properly vortexed in mini centrifuge. The PCR method was completed in 4 steps of 35 cycles. The temperature and time (initial denaturing step at

Table 1. Wheat varieties used in SSR analysis

S. No.	Cultivar name	Pedigree	Year of release/ Notification
1.	DBW 17	*CMH 79A.95/3* CNO 79//Raj 3777*	2006
2.	CBW 38	CNDO/R 143/ENTE/ EXI_2/3/Ae.SQUARROSA (TAUS)/4/WEAVER/5/2*PASTOR	2008
3.	Kundan (DL 153-2)	Tanori 71/NP 890	1981
4.	Pusa Gold(WR 544)	Kalyan Sona/HD 1999//HD 2204/DW 38	2003
5.	Raj 4037	DL 788-2/RAJ 3717	2003
6.	HD 2733	ATTILA/3/TUI/CARC//CHEN/CHTO/4/ATTILA	2001

94^{0}C for 3 minute followed by 34 cycles of 94^{0}C for 1 minute, 55^{0}C for 1 minute and 72^{0}C for 2 minutes, last cycle, primer extension at 72^{0}C for 7 minutes) set up was followed to complete PCR reaction. Amplified products were separated on 3% metaphor agarose gel containing ethidium bromide with 1XTBE buffer. A constant voltage of 80V was provided for 3 hours. DNA fragments were visualized under UV light. The banding patterns were photographed using gel documentation system and saved as digital pictures. To evaluate transferability of rice SSR markers to wheat, percentage of primers showing transferability and quality of banding pattern was recorded. For quality the microsatellite bands were classified into three classes based on the band intensity and ease of scoring: (++) for strong and easily scorable bands; (+) for weak and difficult to score bands; and (-) for no band (9).

Results and Discussion

The Poaceae family includes important crops, such as wheat, barley, rice and maize. Among them, rice has the smallest genome (415Mb), the maize genome is about 6 times larger than rice, barley is about 12 times and hexaploid wheat genome is about 3 times the size of barley compare to rice only. The advent of DNA marker technology has facilitated the rapid generation of many high density genetic and physical maps permitting genetic map comparison using common markers between closely, less commonly and distantly related species. Despite the fact that the genome size of cereals varies upto 40 folds, comparative genetic mapping among several species of Poaceae has revealed extensive genome colinearity (10; 11). In this context, 30 linkage blocks from the rice genetic map are sufficient to reconstitute the seven Triticeae (*e.g.*, wheat, rye and barley), 12 rice and 10 maize chromosomes (10). Microsatellites have many advantages in molecular genetic studies. DNA sequences flanking the SSR loci are highly conserved among closely related species. This homology among several crop genomes allow utilization of primer pairs of one species in related species and is called "transferability" (12). Transferability has been used in many species like wheat, rye and triticale (12), wheat and rye (13), wheat, barley and rye (14).

Of the three microsatellite generating approaches namely generating, mining and transferability, the first approach is very costly, laborious and time-consuming, the second approach is cost-effective, but does not always necessarily produce microsatellites for a species of interest due to lack of genomic information and the third approach costs minimum and is time saving. Clearly, in case of SSR marker information in a related species, the transfer approach is advantageous (15). The availability of molecular markers that are transferable among species allows the identification of allelic variability, QTL mapping, QTL-allele tagging, comparative QTL mapping, gene-flow studies and direct search for new allelic variations within germplasm collections (16). Transferable markers from comparable regions of related genomes can be used where there is shortage of polymorphic marker in specific regions of the genome. This approach would be a lot easier and cheaper than developing new SSR primers (17).

Twenty five rice SSR markers were tested for transferability in wheat in this study. Fifteen rice markers showed amplification with clear scorable bands in wheat. Of the 25 rice SSR markers tested 15 (60%) (RM 351, RM 212, RM 209, RM 224, RM 239, RM177, RM 217, RM 341, RM 269, RM 179, RM 108, RM 188, RM 3252, RM 3589 and RM5862) amplified sequences in wheat (Table 2). However, rest of the ten markers (RM 32, RM144, RM 169, RM174, RM176,RM 178, RM 264, RM 274, RM 336 and RM 330AB) did not amplify in wheat genome suggesting their non- transferability. Thirteen of these markers (RM 88, RM 209, RM 269, RM 3252, RM 351, RM 217, RM 341, RM 3589, RM 179, RM 108, RM 239, RM 224 and RM 177) produced multiple alleles among the test varieties of wheat whereas RM 212 and RM 5862 amplified a single allele in all the varieties tested. Primers RM 209 and RM

3252 produced polymorphic alleles. Among transferable 15 primers, primer RM 188 showed amplification in only one variety whereas the other primers RM 351, RM 212, RM 209, RM 224, RM 239, RM 177, RM 5862, RM 269, RM 3252, RM 3589, RM 341, RM 179, RM 108 and RM 217 showed amplification in all six wheat varieties. Among the cultivars of six wheat variety analyzed, RM 209 can be also used for identification of genetic purity in variety HD2733 (Fig. 1). As described in Table 2, 15 (60%) of the rice SSR primers tested gave positive amplification in wheat (scored as + and ++). Among these, 10 (66.7%) primers showed clear and easily scorable bands scoring ++. Amplified products were not specific with none of them showing band alike in allele size to that of the rice. There is no fixed size for rice and it depends on the genotype in use. Transferability of rice SSRs to other cereal species in the present study is comparable and higher than those as noted in earlier studies (14; 19; 23; 28; 30). Higher transferability rates could be the result of the fact that the SSR markers used in the present study were selected among good quality SSR markers, *i.e.*, single copy and clear amplicon making in the original genus (17).

For transferable SSR marker development, researchers require high rate of transfer and high level of polymorphism. Considerable difference in degree of transferability is observed in related species. Contradictory reports in the literatures are available to compare the levels of polymorphism among transferable genomic SSR markers and genic (EST) SSR markers. Several papers reported that the level of polymorphism was higher in genomic SSR markers than genic SSR markers (1;18;19). However, certain studies reported that the level of polymorphism was higher from genic SSR markers than genomic SSR markers (20; 21). Certain SSR transfer studies among cereals indicate low levels of SSR polymorphisms due to the fact that they used EST-origin SSRs and amplified highly conserved regions, which have high level of transferability but low level of polymorphism (1; 22; 23). According to Gupta et al. (1), Thiel et al. (24) and Cordeiro et al. (25) EST derived SSRs showed significant transferability in contrast to genomic SSRs (14; 26; 27). Varshney et al. (28) and Roder et al. (29) detected only 20 -30% homoeoloci of the genomic SSRs in an amphiploid species like bread wheat with three related genomes. Higher levels of transferability of EST-derived SSRs as compared to genomic DNA-derived SSRs reflect the conserved nature of coding sequences compared to non-coding genomic DNA.

The level of polymorphism revealed by transferrable SSR markers depends on the phylogenetic distance among species and the

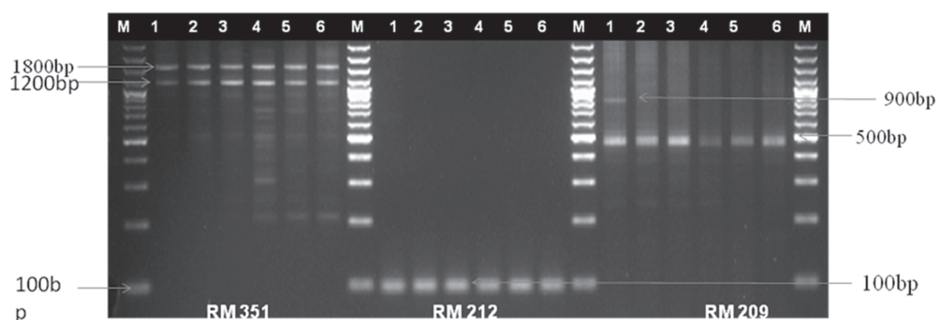

Fig. 1. DNA amplification pattern using microsatellite primer RM209 in major wheat varieties; Lane 1: HD 2733; Lane 2: CBW38; Lane 3: WR 544; Lane 4: DL 1532; Lane 5: DBW 17; Lane 6: Raj 4037; M: 100 base pair ladder

Table 2. Transferability of rice SSR markers to wheat

S. No.	Primer Designation	Chromosome number of rice	Forward and reverse sequence	Observed allele size in wheat (base pair)	Expected specific allele size in rice	Number of alleles	Quality
1.	RM 32	8	AGTCTACGTGGTGTACACGTGG TGCGGCCTGCCGTTTGTGAG	No Amplification	168	-	-
2.	RM 88	8	ACTCATCAGCATGGCCTTGCC TAATGCTCCACCTTCACCAC	300-1200	180	3	+
3.	RM 108	9	TCTCTTGCGCGCACACTGGCAC CGTGCACCACCACCACCACCAC	600-1500	80	3	++
4.	RM 144	11	TGCCCTGGCGCAAATTTGATCC GCTAGAGGAGATCAGATGGTAGTCATG	No Amplification	237	-	-
5.	RM 169	5	TGGCTGGCTCCGTGGGTAGCTG TCCCGTTGCCGTTCATCCCTCC	No Amplification	167	-	-
6.	RM 174	2	AGCGACGCCAAGACAAGTCGGG TCCACGTCGATCGACACGACGG	No Amplification	208	-	-
7.	RM 176	3	CGGCTCCCGCTACGACGTCTCC AGCGATGCGCTGGAAGAGGTGC	No Amplification	134	-	-
8.	RM 177	4	CCCTCTTAGACAGAGGCCAGAGGG GTAGCCGAAGATGAGGCCGCCG	700-3000	195	4	++
9.	RM 178	5	TCGCGTGAAAGATAAGCGGCGC GATCACCGTTCCCTCCGCCTG	No Amplification	177	-	-
10.	RM 179	12	CCCCATTAGTCCACTCCACCACC CCAATCAGCCTCATGCCTCCCC	450-800	190	3	+
11.	RM 209	11	ATATGAGTTGCTGTCGTGCG CAACTTGCATCCTCCCCTCC	500-900	134	2	++
12.	RM 212	1	CCACTTTCAGCTACTACCAG CACCCATTTGTCTCTCATTATG	100	136	1	++
13.	RM 217	6	ATCGCAGCAATGCCTCGT GGGTGTGAACAAAGACAC	160-1350	133	5	+
14.	RM 224	11	ATCGATCGATCTTCACGAGG TGCTATAAAAGGCATTCGGG	340-1500	157	3	+
15.	RM-239	10	TACAAAATGCTGGGTACCCC ACATATGGGACCCACCTGTC	900-3800	144	6	++
16.	RM 264	8	GTTGCGTCCTACTGCTACTTC GATCCGTGTCGATGATTAGC	No Amplification	178	-	-
17.	RM 269	10	GAAAGCGATCGAACCAGC GCAAATGCGCCTCGTGTC	280-600bp	182	4	++

Transferable SSR markers

18.	RM-274	5	CCTCGCTTATGAGAGCTTCG CTTCTCCATCACTCCCATGG	No Amplification	160	-	-
19.	RM 336	7	CTTACAGAGAAACGGCATCG GCTGGTTTGTTTCAGGTTCG	No Amplification	154	-	-
20.	RM 341	2	CAAGAAACCTCAATCCGAGC CTCCTCCCGATCCCAATC	550-3000	172	9	++
21.	RM 351	7	CCATCCTCCACCGCCTCTCG TGTAGGAAGGAAAGGGGACG	210-1800	134	13	++
22.	RM 330	10	CAATGAAGTGGATCTCGGAG CATCAATCAGCGAAGGTCC	No Amplification	177	-	-
23.	RM 3252	1	GGTAACTTTGTTCCCATGCC GGTCAATCATGCATGCAAGC	50-180	172	2	+
24.	RM 3589	7	TGAAGTTTTCGAGTCCACCG AGATCAGGGTAGAGGGCTGC	500-1200	108	6	++
25.	RM 5862	2	TTAGTACCTCATCATAGCTG CTCTAATCTTCTCTCATTATCA	150	223	1	++

genetic distance within the species and not the types of transferable SSR markers. This may partly explain the contradictory published reports. The polymorphism detected may not be related to the transfer rate of SSR markers. High transfer rate markers were not necessarily revealing high level of polymorphism. In terms of the degree of outcrossing of the species, SSR markers may not be easily transferred to out-crossing species because their sequence divergence may occur rapidly compared with other species (15). Successful amplification was not always correlated with conservation of SSR motif structure between related species (27: 30). The level of polymorphism detected across species or genera depends on the genetic divergence of species tested and primers (31). PCR stringency is also one of the important dependable that reported factors which largely determine the rate of transferability hence this has to be monitored strictly while doing such experiments. Wang et al. (32) reported that mis-priming is an important factor in cross species and genera amplifications.

Comparative genomics between grasses has provided evidence for remarkable conservation of marker and gene order, and revealed extensive interspecies genome co-linearity (10). On the other hand, similarity between wheat and rice (35%) or maize (44%) verified the opinion of Gaut (33) that grass genomes were less conserved than previously thought. Our findings confirm that rice SSRs is transferable to other cereal species. SSRs can also be transferred from major cereals such as wheat, rice, maize and sorghum to minor grass species such as finger millet with more than 50% success rate depending upon the genetic relatedness between genera (23).

Yet another result showed that RM209 SSR primer produced unique clear amplification in wheat variety HD2733 indicating that rice SSR markers could be transferred among wheat and can be used in genetic purity analysis which is an important requirement of quality seed sector. Admixtures cause loss of 20 to 25% in wheat production. This loss can be recovered by using genetically pure quality seed. Quality seed is seed that is genetically uniform, highly viable and free from seed borne pathogens.

Conclusion

The transferability of rice SSR primers in wheat was studied and found to be 60%

transferable with clear scorable bands. Obviously, this transferability approach would be a lot easier and cheaper than developing new SSR primers. Specificity of primer depends on several factors such as choice of primers (genic or genomic), genus proximity and PCR conditions. Transferable primers can also turn to be distinctive marker for assessing genetic purity of seed lot. In our case, the primer RM209 was unique in wheat variety HD2733. Genetic purity is an essential requirement in quality seed production.

References

1. Gupta, P.K., Rustgi, S., Sharma, S., Singh, R., Kumar, N. and Balyan, H.S. (2003). Transferable EST-SSR markers for the study of polymorphism and genetic diversity in bread wheat. Mol. Gen. Genome, 270: 315–323.

2. Akkaya, M., Bhagwat, A. and P. Cregan. (1992). Length polymorphisms of simple sequence repeat DNA in soybean. Genetics, 132: 1131-1139.

3. Korzun, V., Röder, M., Worland, A.J. and Börner, A. (1997). Intrachromosomal mapping of genes for dwarfing (*Rht12*) and vernalization response (*Vrn1*) in wheat by using RFLP and microsatellite markers. Plant Breeding, 116: 227-232.

4. Senior, M.L. and Heun, M. (1993). Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. Genome, 36: 884-889.

5. Varshney, R.K., Marcel, T.C., Ramsay, L., Russell, J., Röder, M.S., Stein, N., Waugh, R., Langridge, P., Niks, R.E. and Graner, A. (2007). A high density barley microsatellite consensus map with 775 SSR loci. Theor. Appl. Genet., 114: 1091-1103.

6. Wu, K.S. and Tanksley, S.D. (1993). Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol. Gen. Genet., 241: 225–235.

7. Provan, J., Powell, W. and Waugh, R. (1996). Microsatellite analysis of relationships within cultivated potato (*Solanum tuberosum*). Theor. Appl. Genet., 92: 1078-1084.

8. Guillemant, P. and Laurence, M.D. (1992). Isolation of Plant DNA: A fast, inexpensive, and reliable method. Plant Mol. Biol. Rep., 10: 60-65

9. García, J.M.M., Leonardo Velasco José, M., Fernández-Martínez and Begoña Pérez-Vich. (2008). Transferability of sunflower microsatellite markers to safflower, Seventh International Safflower Conference, Wagga wagga, Australia

10. Devos, K.M. and Gale, M. D. (2000). Genome relationships: the grass model in current research. Plant Cell, 12: 637–646.

11. Paterson A.H., Bowers, J.E. Burow, M.D. Draye, X. Elsik, C.G. Jiang, C.X., Katsar, C.S., Lan, T.H., Lin, Y.R., Ming, R. and Wright, R.J. (2000). Comparative genomics of plant chromosomes. Plant Cell, 12: 1523–1540.

12. Kuleung, C. Baenziger, P.S. and Dweikat, I. (2003). Transferability of SSR markers among wheat, rye and triticale. Transferability of SSR markers among wheat, rye, and triticale. Theor. Appl. Genet., 108: 1147-1150.

13. Caudrado, A. and Schwarzacher, T. (1998). The chromosomal organization of simple repeats in wheat and rye genomes. Chromosoma, 107: 587-594.

14. Roder, M.S., Plaschke, J., Konig, S.U., Bo¨rner, A., Sorrells, M.E., Tanksley, S.D. and Ganal, M.W. (1995). Abundance, variability and chromosomal location of microsatellites in wheat. Mol. Gen. Genet., 246: 327–333.

15. Wang, M.L., Barkley, N.A. and Jenkins, T.M. (2009). Microsatellite Markers in Plants and

Insects. Part I: Applications of Biotechnology Genes. Genomes and Genomics, 3: 1523–1539.

16. Brondani, R.P.V., Brondani, C., Tarchini, R. and Grattapaglia, D. (1998). Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *Eucalyptus urophylla*. Theor. Appl. Genet., 97: 816–827.

17. Yildirim, A., Kandemir, N., Ateþ Sönmezoðlu, O.T. and Eserkaya Güleç, T. (2009). Transferability of microsatellite markers among cool season cereals. Biotechnol. Biotechnol. Eq., 23: 1299-1302

18. Cho, Y.G., Ishii, T., Temnykh, S., Chen, X., Lipovich, L., McCouch, S.R., Park, W.D., Ayres, N.S. and Cartinhours, S. (2000). Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). Theor. Appl. Genet., 100: 713-722

19. Eujay, I., Sorrells, M., Baum, M., Wolters, P. and Powell, W. (2001). Assessment of genotypic variation among cultivated durum wheat based on EST-SSRs and genomic SSRs. Euphytica, 119: 39-43

20. Liewlaksaneeyanawin, C., Ritland, C.E., El-Kassaby, Y.A. and Ritland, K. (2004). Single-copy, species-transferable microsatellite markers developed from loblolly pine ESTs. Theor. Appl. Genet., 109: 361-369

21. Varshney, R.K., Graner, A. and Sorrells, M.E. (2005). Genic microsatellite markers in plants: features and applications. Trends in Biotechnol., 23: 48-55

22. Palop, M., Palacios, C. and Gonzales-Candelas, F. (2000). Development and across species transferability of microsatellite markers in the genus *Limonium* (Plumbaginaceae). Conservation Genet., 1: 177-179.

23. Wang, M.L., Barkley, N.L., Yu, J., Dean, R., Newman, M.L., Sorrells, M. and G.A.Pederson, G.A. (2005). Transfer of simple sequence repeat (SSR) markers from major cereal crops to minor grass species for germplasm characterization and evaluation. Plant Genet. Res., 3: 45-57.

24. Thiel, T., Michalek, W., Varshney, R.K. and Graner, A. (2003). Exploiting EST databases for the development of cDNA derived microsatellite markers in barley (*Hordeum vulgare* L.). Theor. Appl. Genet., 106: 411–422.

25. Cordeiro, G.M., Casu, R., McIntyre, C.L., Manners, J.M. and Henry, R.J. (2001). Microsatellite markers from sugarcane (*Saccharum* spp.) ESTs cross transferable to Erianthus and Sorghum. Plant Sci., 160: 1115–1123.

26. Sourdille, P., Tavaud, M., Charmet, G. and Bernard, M. (2001). Transferability of wheat microsatellites to diploid Triticeae species carrying the A, B and D genomes. Theor. Appl. Genet., 103: 346–352.

27. Peakall, R., Gilmore, S., Keys, W., Morgante, M. and Rafalski, A. (1998). Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. Mol. Biol. Evol., 15: 1275–1287.

28. Varshney, R.K., Kumar, A., Balyan, H.S., Roy, J.K., Prasad, M. and Gupta, P.K. (2000). Characterization of microsatellites and development of chromosome specific STMS markers in bread wheat. Plant Mol. Biol. Rep., 18: 1–12.

29. Roder, M.S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M.H., Leroy, P. and Ganal, M.W. (1998). A microsatellite map of wheat. Genetics, 149: 2007–2023.

30. Chen, X., Cho, Y.G. and McCouch, S.R. (2002). Sequence divergence of rice microsatellites in *Oryza* and other plant species. Mol. Gen. Genom., 268: 331–343.

31. Reddy, N.R., Madhusudhana, R., Prasanthi, M., Srinivas, G., Murali Mohan, S., Satish, K. and Seetharama (2011). Assessment of transferability of sorghum (*Sorghum bicolor*) EST-SSR markers among its wild species and other members of Gramineae family. Indian J.Agric. Sci., 81: 1063–1067

32. Wang, M.L., Gillaspie, A.G., Newman, M.L., Dean, R.E., Pittman, R.N., Morris, J.B. and Pederson. G.A. (2004). Transfer of simple sequence repeat (SSR) markers across the legume family for germplasm characterization and evaluation. Plant Genet. Res., 2: 107-119

33. Gaut, B.S. (2002). Evolutionary dynamics of grass genomes, New Phytol., 154: 15–28.

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Can we Achieve Salt Stress Tolerance in Crop Plants by Genetic Engineering Methods?

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Abstract

Nearly 20% of the world's irrigated land is affected by soil salinity. In fact, salt stress is one of the major constraints for crop productivity throughout the world and can result in losses to the tune of billions of US dollars on a global scale. But, to maintain the global food production rates in the future to meet the food demands, plant breeding and genetic engineering technologies need to be complemented so as to alter the salt stress tolerance of crop plants. Recent progress in molecular genetics has helped us in identifying the mechanisms and several of the quantitative trait loci (QTLs) and validation of individual genes that have been identified with salt stress tolerance. Many of these genes have also been tested in different plants as a proof of concept. In this review, we like to highlight the importance of genetic engineering technologies to generate transgenic crops that can help to mitigate the salt stress in crop plants.

Keywords: Salt stress, sodium exclusion, ion sequestration, osmolytes, reactive oxygen species.

Introduction

To match the growing population, food production needs to be increased by 50% during the next 40 years or so (1). But, both drought and soil salinity are the major constraints for crop productivity. Approximately, 800 million hectares of land is affected by soil salinity and this accounts for 7% of the total land area in the world (2, 3). Soil salinity is characterized by a high concentration of soluble salts. The presence of both salt tolerant halophytes and salt susceptible glycophytes leads us to believe that there is a genetic basis for salt stress in plants (4). Salt stress is a multigenic trait and many efforts over the years has resulted in the identification of quantitative trait loci in crop plants like rice, wheat, barley, tomato and others (5, 6). Further, several individual genes associated with salt stress tolerance have been identified and isolated. The incorporation of these genes into crop plants has resulted in the generation of transgenics which exhibited salt stress (7, 8). In this review, we will outline the mechanisms of salt stress tolerance in higher plants and cite examples of transgenic crop approaches to overcome the salinity stress.

Mechanisms of salinity tolerance : Plant growth is severely affected with the imposition of salt stress. Salt stress causes not only ionic but also osmotic stress. Higher concentration of Na^+ ions in the soil drives water out of the cell. But water immediately moves from the vacuole into the cytosol to compensate the loss of water. This results in the reduction of turgor and plant growth (9). The loss of cell volume and turgor however, is transient (10). But, plants adjust themselves to this osmotic condition very soon. Further, Na^+ ions are also toxic to the cells since these ions compete with K^+ for major binding sites in key metabolic processes like protein synthesis,

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enzymatic reactions and ribosome functions (11). If salt stress prolongs for a long time, inhibition of lateral shoot development, death of older leaves, early flowering, reduced floret number or total inhibition of reproductive growth occurs in many plants (12). Over the years, plants have developed several mechanisms to cope up with this situation. One such mechanism is to restrict the entry of Na^+ and exclusion of Na^+ back into the soil using the plasma membrane bound salt overly sensitive (SOS1) Na^+/H^+ antiporter proteins (13). Such an exclusion of toxic ions and tolerance to osmotic stress is vital to the survival of plants. Na^+/H^+ antiporter activity is driven by the electrochemical gradient of protons (H^+) generated by the H^+-ATPase located at the plasma membrane (14, 15). The second mechanism is tissue tolerance to Na^+ or Cl^- ions, which requires compartmentalization of excess amounts of Na^+ into the vacuoles *via* the vacuolar membrane bound Na^+/H^+ antiporter (16) as shown in the Fig. 1. Compartmentalization of ions at the cellular and intracellular level thus avoids Na^+ and Cl^- accumulation in the cytoplasm. The sequestration of Na^+ into the vacuole is mediated by the vacuolar membrane bound Na^+/H^+ antiporter (*AtNHX1*) in *Arabidopsis* and others (17, 18). Contrary to this, a third mechanism appears to operate in halophytes, where salts are usually excluded through the salt glands or bladders located at the leaf margins (15). Under salt stress, cell internal K^+ concentration decreases significantly (19). As the Na^+ levels increase in the cytoplasm, loss of K^+ from the cells also increases through K^+ outward-rectifying (KOR) channels (20, 21) causing severe damage and death to the cells (22). Over and above, cellular K^+ also leaks out of the cells *via* non-selective cation channels (NSCC) stimulated by reactive oxygen species (ROS) generation (22). It appears therefore, prevention of K^+ loss through the KOR and NSCC channels when plants are exposed to salts is important in order to maintain ion homeostasis. It has been reported that compatible solutes and polyamines can prevent NaCl induced K^+ leakage and improve plant productivity (23, 24). Stomatal opening and closure is mediated by changes in guard cell K^+ contents involving various channels

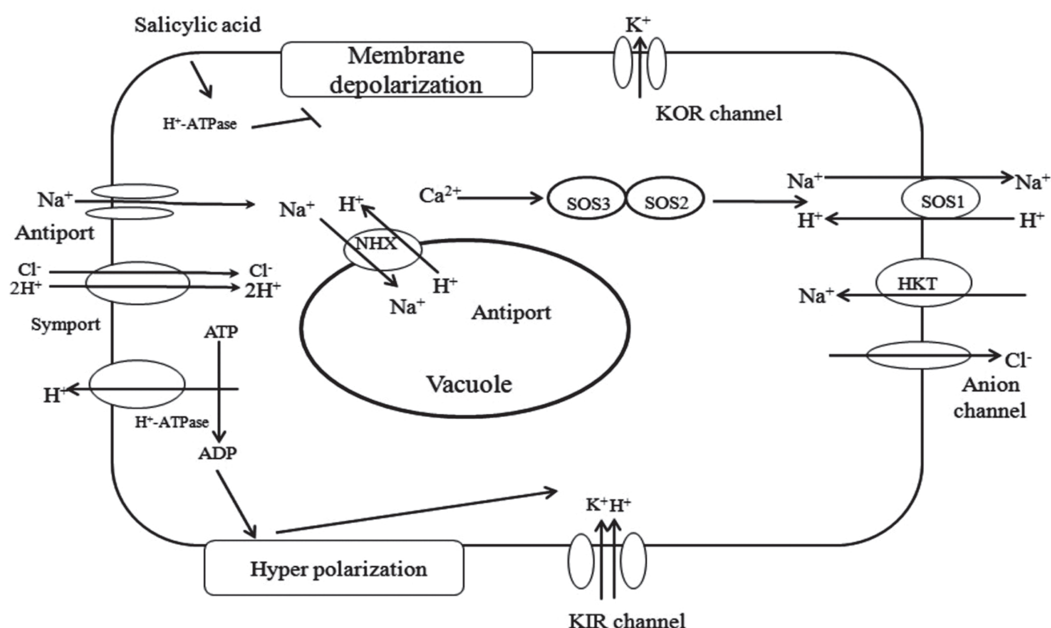

Fig.1. Cellular mechanisms involved in the exclusion and compartmentalization of Na^+ ions. Chloride is transported through a symporter into cells and excluded by an anion channel.

working in a coordinated way in the plasma and vacuolar membranes (tonoplast). Two types of K^+ -permeable voltage-gated channels (inwardly and outwardly rectifying) in the plasma membrane have been characterized. Out of the two, the inwardly rectifying KIR channels activate on membrane hyperpolarization and involved in the influx of K^+ into the cells. On the other hand, the outward rectifying KOR channels activate on membrane depolarization, at positive membrane potentials than the K^+ -equilibrium potential and thus allow K^+ release (25, 26). These channels are encoded by *Shaker* type of family genes which comprises nine members in *Arabidopsis* (27). Among them, a voltage-gated guard cell outward rectifying K^+ channel (GORK), is important and activated by salt stress. It has been speculated that salicylic acid upregulates the plasma membrane H^+-ATPase activity and thus helps in ion homeostasis. It is demonstrated that salicylic acid alleviates salt stress due to enhanced K^+ retention in the cells resulting from enhanced H^+-ATPase activity and decreased K^+ loss *via* a GORK channel. Further, it has been found recently that arbuscularmycorrhizal fungi (AMF) enhance maize salt tolerance through improved ion homeostasis (28). Maize plants inoculated with three AMF displayed increased K^+ and reduced Na^+ accumulation when compared to the control plants. Further, such an enhanced K^+/Na^+ ratio has been correlated with *ZmAKT2*, *ZmSOS1* and *ZmSKOR* gene expression levels in the roots of maize exposed to salt stress. Thus, AMF also contribute to K^+ and Na^+ homeostasis in plants colonized by native AMF. On the whole, plant's ability to withstand salinity may be achieved by reduced Na^+ delivery into the shoots, Na^+ recirculation in the phloem and compartmentation of Na^+ ions into such structures like senescing leaves, leaf sheath or epidermis. Recirculation of Na^+ ions from the leaves into the roots may be mediated by high affinity K^+ transporters (HKT) in many plants (29, 30).

Transgenic approaches for salt stress tolerance: Several transcription factors associated with both salt and drought stresses have been identified and tested (31). Therefore, this will not be dealt in this review. Rather, transgenic crops that have been developed so far for salt stress tolerance using (1) Na^+ and K^+ transporter genes, (2) osmoprotectant biosynthetic pathway genes, (3) detoxifying genes and (4) late embryogenesis abundant (LEA) genes will be discussed.

Na^+ and K^+ transporter genes: As mentioned earlier, Na^+ exclusion takes place at the plasma membrane level through the SOS pathways. Genes that encode salt exclusion at the plasma membrane as well as inclusion into the vacuoles (Na^+/H^+antiporters) have been identified in crop plants like rice, sorghum and others besides the model plants. The SOS pathway is highly complex, but conserved and consists of three proteins, namely SOS3, a calcium sensor protein (32); SOS2, a serine/threonine protein kinase (33); and SOS1, a protein that helps in the exclusion of Na^+ out of the cells (34). SOS3 protein interacts and activates SOS2 (35) to recruit SOS2 to the plasma membrane where it interacts again with SOS1 protein (36). Transgenic cotton plants expressing *AtNHX1* were developed by He *et al.* (37) which show more biomass and fibers under salt stress conditions. It has been suggested that enhanced fiber yield was due to better photosynthetic performance and nitrogen assimilation rates in the transgenics. Expression of a plasma membrane Na^+/H^+ antiporter *SOD2* from *Schizosaccharomyces pombe* in rice exhibits salt tolerance (38). Transgenic plants accumulate more K^+, Ca^{2+} and Mg^{2+} and less Na^+ in the shoots compared to untransformed controls. Such plants also exhibit higher levels of photosynthesis and root proton exportation. Chen *et al.* (39) expressed *OsNHX1* gene in maize that confers salt tolerance and promotes plant growth in the field. Osmotic potential and proline levels in transgenic leaves were lower than in wild type plants. Likewise, from *Salicornia brachiata*, an extreme halophyte, *SOS1* gene was isolated and overexpressed in tobacco. It has been shown that *SbSOS1* enhances Na^+ loading in xylem and confers salt tolerance in

tobacco (40). A list of transgenics (not a comprehensive one) generated through the overexpression of *SOS*, *NHX* and potassium transporter genes is shown in Table 1.

Rus et al. (41) expressed halotolerance gene, *HAL1*, isolated from yeast in transgenic tomato plants. This gene helps in K^+ acquisition and lowers Na^+ levels in yeast under salt stress. Transgenic tomato plants expressing *HAL1* gene displayed better fruit growth under salt stress. Ellul *et al.* (42) also developed *HAL1*-expressing watermelon plants that conferred salt tolerance. Obata et al. (43) isolated a potassium channel *OsKAT1* from a Japonica rice variety Nipponbare that helped to tolerate yeast and rice cells to the salt stress. This channel is suggested to be associated with cytosolic cation homeostasis during salt stress and thus protects cells from Na^+ toxicity (Table 1). However, several of the sodium-proton antiporters (NHX) and K^+ transporters have not been cloned and their functions have not yet been demonstrated.

Osmoprotectants: Under salt stress conditions, plants accumulate different types of organic osmolytes that are compatible with cell milieu. Among them, glycine betaine, proline, mannitol, pinitol, trehalose, and polyamines have been found to be very important. Genes associated with the glycine betaine biosynthetic pathway have been cloned from higher plants as well as from bacteria and inserted into crop plants. Mohanty et al. (44) showed that transgenic rice plants accumulated glycine betaine content with the incorporation of *codA* gene and displayed salt stress tolerance. Also, enhanced glycine betaine synthesis with the engineering of *BADH* gene in maize plants was noticed (45). These transgenics survived well in the presence of salt stress but not the controls, demonstrating that glycine betaine is a good osmoprotectant. The proteogenic amino acid proline, has been recognized as a versatile molecule with multifarious functions. The key gene in proline biosynthetic pathway, *pyrroline 5-carboxylic acid synthetase* (*P5CS*) and also its catabolic pathway genes *proline dehydrogenase* (*ProDH*) and *pyrroline 5-carboxylate dehydrogenase* (*P5CDH*) have been up and down-regulated in several crop plants (46). Hmida-Sayari *et al.* (47) demonstrated that the yield of transgenic potato tubers is better under salt stress when compared to the plants grown under normal tap water. Similarly, transgenic sorghum seeds (engineered with *Vigna aconitifolia P5CS* gene) displayed better germination and growth in presence of NaCl and PEG when compared to wild type plants (48). Transgenic plants engineered with proline metabolic pathway genes like *P5CS* and its mutated version *P5CSF129A*, *ProDH*, and *ornithine δ-aminotransferase* (*OAT*) and their salt stress tolerance is shown in Table 2.

Among the sugar alcohols, mannitol has been shown to be associated with salt stress. Sickler et al. (49) cloned *mannose-6-phosphate reductase* (*M6PR*) gene from celery and incorporated it in *Arabidopsis*. While the transgenics exhibited higher photosystem II (PSII) yield in the presence of salt stress, wild type plants showed reduction in PSII. Thus, *mannitol dehydrogenase* (isolated from bacteria) and *M6PR* genes have potential to be used in other crop plants for imparting salinity tolerance. Trehalose is an important disaccharide sugar distributed in fungi and higher plants. Glucose acts as the precursor for the synthesis of trehalose. Trehalose acts as a compatible solute and protects membranes and proteins against denaturation under abiotic stress conditions. *Trehalose phosphate phosphatase* (TPP) gene involved in the biosynthetic pathway was cloned from rice and overexpressed it in rice (50). Transgenic rice plants displayed more vigorous growth and viability over the controls. In addition to sugars, polyamines like spermidine, spermine, putrescine are involved in tolerance to salt, hyperosmosis, hypoxia, low and high temperature stresses (51). Polyamines are aliphatic amines widely distributed in plants. They are also known to be associated with several abiotic stresses. The biosynthetic pathway for the synthesis of polyamines is known and hence the genes that encode the enzymes have been cloned. Over-

Table 1. Transgenic plants developed using *sodium-proton antiporter* (*NHX*) genes (located at the plasma and vacuolar membranes) for salt stress tolerance (not a comprehensive list)

Gene	Isolated from	Validated in	Phenotypic effects of transgenic plants
AtNHX1	*Arabidopsis thaliana*	Cotton	Enhanced capability to grow on high saline soils, increased fiber yield
PgNHX1	*Pennisetum glaucum*	Tomato	More tolerance to salts and retained more chlorophyll
KcNHX1	*Karelinia caspica*	*Karelinia caspica*	Gene silencing reduced tolerance to high NaCl
OsNHX1	*Oryza sativa*	*Zea mays*	Confers salt tolerance
TaNHX1	*Triticum aestivum*	*Arabidopsis*	Improves salt stress
AtNHX1	*Arabidopsis*	*Festuca arundinacea*	Enhanced salt tolerance
PtNHX2	*Populu stomentosa*	Tobacco	Maintains ion homeostasis and shows salt tolerance
LeNHX2	*Lycopersicon esculentum*	*Lycopersicon esculentum* and Yeast	Improves K^+ homeostasis and displays salt stress tolerance
KcNHX2	*Karelinia caspica*	*Karelinia caspica*	Showed tolerance towards salinity
HvNHX3	*Hordeum vulgare*	*Hordeum vulgare*	Regulates pH for cell integrity and shows salt stress tolerance
McNHX3	*Mesembryanthemum crystallinum*	*Mesembryanthemum crystallinum*	Showed salt tolerance
AtNHX5	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Expressed in roots, leaves, stems, flowers and siliques. Detected at low levels in roots and shoots, exhibits salt stress
AtNHX6	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Expressed in roots, leaves, stems, flowers and siliques. Detected at low levels in roots and shoots, shows salt stress
PeNHX6	*Populus euphratica*	*Populus euphratica*	Transcript levels were upregulated in roots, shows salt sress tolerance
AtNHX7	*Arabidopsis thaliana*	*Arabidopsis thaliana*	More expressed in roots than shoots
AtNHX8	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Expressed more in leaves than roots and flowers, exhibits salt stress
MdSOS1	*Malus*	*Malus* species	Shows more tolerance to salinity
PpSOS1	*Physcomitrella patens*	*Physcomitrella patens*	Showed Na^+ efflux, and salt stress tolerance
SbSOS1	*Salicornia brachiata*	*Nicotiana tobacum*	Confers salt tolerance
AtSOS2	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Transcripts lelvels were upregulated, plants exhibit salt tolerance
OsSOS2	*Oryza sativa*	*Oryza sativa*	Regulates ion homeostasis, and shows salt tolerance
SlSOS2	*Solanum lycopersicum*	*Solanum lycopersicum*	Confers salt tolerance
SOS3	*Brassica napus*	*Wheat*	Regulates ion homeostasis, and exhibits salt tolerance
AtSOS3	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Maintains intracellular ion homeostasis, and salt tolerance
HvSOS3	*Hordeum vulgare*	*Hordeum vulgare*	Controls sodium exclusion and ameliorates salt stress
AtSOS4	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Required for root hair development, and plants show salt tolerance
TaSOS4	*Triticum aestivum*	*Aegilops crassa*	Expression was up regulated in roots, and transgenics show salt tolerance
OsKAT1	*Oryza sativa*	*Oryza sativa*	Confers salt stress
HAL1	*Sachharomyces*	*Lycopersicon esculentum*	Improved salt tolerance

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Table 2. Transgenics developed from proline biosynthetic pathway genes that conferred salt/abiotic stress tolerance

Gene	Isolated from	Validated in	Phenotypic effects of transgenic plants
P5CS	*Vigna aconitifolia*	Rice	Salt stress tolerance
P5CS	*Vigna aconitifolia*	Rice	Increased biomass production under drought and salinity stress
P5CS	*Vigna aconitifolia*	Rice	Salt stress (200 mM NaCl) tolerance
P5CS	*Vigna aconitifolia*	Rice	Stress inducible promoter increases biomass under salt stress
P5CS	*Vigna aconitifolia*	Wheat	Enhanced proline levels and salt tolerance
P5CS	*Vigna aconitifolia*	Wheat	Water stress tolerance
P5CS	*Vigna aconitifolia*	Sugarcane	Transgenic sugarcane plants displayed antioxidative defense
P5CS	*Vigna aconitifolia*	Carrot	Tolerance to salt stress
P5CS	Not known	*Olea europaea* (Olive)	Enhanced tolerance to salinity
P5CS	*Vigna aconitifolia*	*Larix leptoeuropaea*	Transgenics tolerant to cold, salt, and freezing stresses
P5CS	*Vigna aconitifolia*	*Medicago*	Proline essential for the maintenance of nitrogen-fixing activity under osmotic stress
P5CS	*Vigna aconitifolia*	*Cicer* (Chickpea)	Enhanced proline and salt tolerance
P5CS	*Vigna aconitifolia*	Wheat	Drought tolerance
P5CS	*Arabidopsis*	Potato	Enhanced salt tolerance
P5CS	Not known	*Festuca*	Not known
P5CS2	*Oryza sativa*	Rice	Enhanced salt and cold stress tolerance
P5CS2	*Phaseolus vulgaris*	Tobacco	Proline accumulation, improved drought tolerance in transgenic tobacco
P5CSF129A	*Vigna aconitifolia* *Poncirus trifoliata*	*Citrus sinensis×* (Carrizo citrange)	Drought tolerance and increased antioxidative capacity due to increased proline
P5CSF129A	*Vigna aconitifolia*	*Citrus paradisi×* *Citrus trifoliata* (Swingle citrumelo)	Drought tolerance and antioxidant enzymatic activities
P5CSF129A	*Vigna aconitifolia*	*Lactuca* (Lettuce)	Enhanced tolerance to freezing
P5CSF129A	*Vigna aconitifolia*	*Cicer* (Chickpea)	Enhanced proline and drought stress tolerance
P5CSF129A	*Vigna aconitifolia*	*Sorghum bicolor*	Enhanced proline, salt and drought stress tolerance
P5CSF129A	*Vigna aconitifolia*	*Festuca arundinaceae*	Transgenics tolerant to drought stress
P5CSF129A	*Vigna aconitifolia*	Rice	Enhanced proline, better biomass and growth performance under salt stress
P5CSF129A	*Vigna aconitifolia*	*Eucalyptus*	Four-fold increase in proline levels
P5CSF129A	*Vigna aconitifolia*	Tobacco	Mild but distinct positive effect on abiotic stress
P5CSF129A	*Vigna aconitifolia*	*Cajanus cajan*	Salt stress tolerance
ProDH	*Brassica oleraceae italica*	Broccoli	Antisense ProDH resulted in low proline level
ProDH	*Arabidopsis*	Rice	Antisense transgenics accumulated higher proline than wild type plants
OAT	*Arabidopsis*	Rice	Increased proline and improved yield under salt and drought stress conditions
OAT	*Oryza sativa*	Rice	*Drought and oxidative stress tolerance*

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expression of the genes like *arginine decarboxylase* (*ADC*), *ornithine decarboxylase* (*ODC*), *S-adenosylmethionine decarboxylase* (*SAMDC*), or *spermidine synthase* (*SPDS*) showed improved tolerance to abiotic stresses in plants (Liu et al. 2007). Apple plants engineered with *SPDS* gene exhibited higher antioxidant enzyme activities compared to untransformed controls (52). Thus, besides carbohydrates, polyamines also play vital roles in alleviating the salt stress.

Detoxifying genes: Salt stress produces reactive oxygen species (ROS) such as singlet oxygen, superoxide radical, hydroxyl radical and hydrogen peroxide. Oxidative damage can be alleviated by both enzymatic and non-enzymatic methods. Several enzymes like glutathione peroxidase (GPX), glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD) help in the detoxification of ROS. Over expression of cytosolic (*AcGPX*) and chloroplastic *GPX* (*ApGPX*) in *Arabidopsis* led to enhanced tolerance to oxidative damage caused by H_2O_2 or methyl viologen (53). *SOD* from a mangrove plant *Avicennia marina* was cloned and transferred into rice which exhibited bettertolerance towards methyl viologen mediated oxidative stress compared to controls (54). Similarly, chloroplastic *APX* was isolated from barley (*HvAPX1*) and introduced into *Arabidopsis*. Transgenic lines showed better salt stress tolerance compared to untransformed controls. Salt stress tolerance in the transgenics is suggested to be due to reduction in the oxidative damage (55). *Glutathione reductase* (*GR*) gene from Chinese cabbage (*BcGR1*) was cloned and introduced into tobacco (56). Thus, transgenic plants displayed increased salt stress tolerance compared to wild type plants. It appears therefore that detoxification of the ROS is vital for the plants to protect themselves against abiotic stresses.

Late embryogenesis abundant (LEA) proteins: Late embryogenesis abundant proteins (LEA) are synthesized in response to dehydration stress. Since salt stress also creates dehydration stress, plants exposed to salinity stress also synthesize LEA proteins. They help the plants in water status stabilization, ion sequestration, prevention of membrane leakage, and protection of proteins against denaturation. Based on conserved sequence motifs, they are divided into five different groups besides LEA-like proteins that exist in plants (57, 58). *LEA* genes have been genetically manipulated in many plants. Transgenic lettuce (*Lactuca sativa*) containing *B. napus LEA* gene demonstrated increased growth under salt stress over that of controls (59). A *dehydrin-5* (*Dhn-5*) gene when over expressed in *Arabidopsis* resulted in stronger growth rate under NaCl stress (60). Further, transgenic leaves accumulated more Na^+ and K^+ compared to the controls. A group 3 LEA protein *HVA1* gene was overexpressed recently in mulberry which displayed salt stress tolerance (61). Transgenic mulberry plants also showed better water use efficiency as compared with controls. The authors suggested that HVA1 proteins increased the performance of the transgenic mulberry by protecting the plasma and chloroplast membrane stability under stress conditions.

Conclusion

Salt stress is a complex genetic trait. Salt-tolerant and susceptible plants exhibit wide range of adaptations in nature. Further, halophytes display exclusion of Na^+ and acquisition of K^+ often, along with salt glands that help them in salt exclusion. At the same time, breeders need to utilize interspecific hybridization, marker-aided selection for salt stress tolerance along with QTLs that have been identified so far in major crop plant systems. Identification of individual genes, their isolation and validation can help the breeders in future to target such genes in their breeding programs. Further, most of the transgenics have been evaluated at the pot conditions in the green house. But, they need to be tested under field conditions not only as a function of yield but also to find out gene x environment interaction. Use of constitutive promoters to express the alien genes would result in the yield penalty due to metabolic and energy costs. Therefore, use of

GATEWAY vectors along with salt stress inducible promoters is advisable for multiple gene transfers and to generate crop plants with better salt resistance.

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References

1. Rengasamy, P. (2006). World salinization with emphasis on Australia. J. Exp. Bot. 57: 1017-1023.
2. Munns, R. (2005). Genes and salt tolerance: bringing them together. New Phytol. 167: 645-663.
3. FAO (2009). FAO land and plant nutrition management service. www.fao.org/ag/agl/agll/spush/.
4. Yamaguchi, T. and Blumwald, E. (2005). Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci. 10: 615-620.
5. Flowers, T.J. and Flowers, S.A. (2005). Why does salinity pose such a difficult problem for plant breeders? Agr. Water Manag. 78: 15-24.
6. Jenks, M.A., Hasegawa, I., Mohan, P.M.and Jain, S. (eds) (2007). Advance in molecular breeding toward drought and salt tolerant crops. Springer, Dordrecht, The Netherlands.
7. Sangam, S., Jayasree, D., Reddy, K.J., Chari, P.V.B., Sreenivasulu, N. and KaviKishor, P.B. (2005). Salt tolerance in plants – transgenic approaches. J. Plant Biotech. 7: 1-15.
8. Vij, S. and Tyagi, A.K. (2007). Emerging trends in the functional genomics of the abiotic stress responsein crop plants: review article. Plant Biotechnol J. 5: 361-380.
9. Yeo, A.R., Lee, K.S., Izard, P., Boursier, P.J. and Flowers, T.J. (1991). Short- and long-term effects of salinity on leaf growth in rice (*Oryza sativa* L.). J. Exp. Bot. 42: 881-889.
10. Bartels, D. and Sunkar, R. (2005). Drought and salt tolerance in plants. Crit. Rev. Plant Sci. 24:23-58.
11. Marschner, H. (1995). The mineral nutrition of higher plants.Academic Press, London.
12. Munns, R. and Tester M. (2008). Mechanisms of salt tolerance. Ann. Rev. Plant Biol. 59: 651-681.
13. Zhu, J.K. (2003). Regulation of ion homeostasis under salt stress.Curr.Opin. Plant Biol. 6: 441-445.
14. Chinnusamy, V. and Zhu, J.K. (2003). Plant Salt tolerance. In: Hirt H, and Shinozaki K (eds) Plant responses to abiotic stress. Springer, Berlin, pp. 241-270.
15. Tester, M. and Davenport, R. (2003). Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot. 91: 503-527.
16. Zhang, H.X. and Blumwald, E. (2001). Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat. Biotechnol. 19: 765-768.
17. Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L. and Fink, G.R. (1999). The *Arabidopsis thaliana* proton transporters, *AtNhx1* and *Avp1*, can function in cation detoxification in yeast. Proc. Nat. Acad. Sci. USA, 96: 1480-1485.
18. Shi, L.Y., Li, H.Q., Pan, X.P., Wu, G.J. and Li, M.R. (2008). Improvement of *Toreniafournieri* salinity tolerance by expression of *Arabidopsis AtNHX5*. Funct. Plant Biol. 35: 185-192.
19. Carden, D.E., Walker, D.J., Flowers, T.J.

and Miller, A.J. (2003). Single-cell measurements of the contributions of cytosolic Na^+ and K^+ to salt tolerance. Plant Physiol.131: 676-683.

20. Flowers, T.J. and Hajibagheri, M.A. (2001). Salinity tolerance in *Hordeum vulgare*: ion concentrations in root cells of cultivars differing in salt tolerance. Plant and Soil231: 1-9.

21. Shabala, S. and Cuin, T.A. (2008). Potassium transport and plant salt tolerance. Physiologia Plant.133: 651-669.

22. Demidchik, V., Cuin, T.A., Svistunenko, D., Smith, S.J., Miller, A.J., Shabala, S., Sokolik, A. and Yurin, V. (2010). *Arabidopsis* root K^+-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death. JournalCell Sci.123:1468-1479.

23. Cuin, T. and Shabala, S. (2007). Amino acids regulate salinity-induced potassium efflux in barley root epidermis. Planta. 225:753-761.

24. Jayakannan, M., Bose, J., Babourina, O., Rengel, Z. and Shabala, S. (2013). Salicylic acid improves salinity tolerance in Arabidopsis by restoring membrane potential and preventing salt-induced K^+ loss via a GORK channel. J. Exp. Bot. 64: 2255-2268.

25. Schroeder, J. I., Raschke, K. and Neher, E. (1987). Voltage dependence of K^+ in guard-cell protoplasts.Proc. Natl. Acad. Sci. USA. 84: 4108-4112.

26. Roelfsema, M. R. G. and Prins, H. B. A. (1997). Ion channels in guard cells of *Arabidopsis thaliana* (L.). Planta. 202: 18-27.

27. Very, A.A. and Sentenac, H. (2002). Cation channels in the *Arabidopsis* plasma membrane.Trends Plant Sci.7:168-175.

28. Estrada, B., Aroca, R., Maathuis, F.J.M., Barea, J.M. and Lozano, J.M.R. (2013). *Arbuscular mycorrhizal fungi* native from a Mediterranean saline area enhance maize tolerance to salinity through improved ion homeostasis. Plant Cell Environ. 36: 1771-1782.

29. Rus, A., Lee, B.H., Munoz-Mayor, A., Sharkhuu, A., Miura, K., Zhu, J.K., Bressan, R.A. and Hasegawa, P.M. (2004). *AtHKT1* facilitates Na^+ homeostasis and K^+ nutrition in planta. Plant Physiol. 136: 2500-2511.

30. Davenport, R.J., Munoz-Mayor, A., Jha, D., Essah, P.A., Rus, A. and Tester, M. (2007). The Na^+ transporter *AtHKT1;1* controls retrieval of Na^+ from the xylem in Arabidopsis. Plant Cell Environ. 30: 497-507.

31. Jewell, M.C., Campbell, B.C. and Godwin, I.D. (2010). Transgenic plants for abiotic stress resistance. Kole C (eds). Transgenic Crop Plants, DOI 10.1007/978-3-642-04812-8_2, Springer-Verlag, Berlin, Heidelberg.

32. Munns, R. and Tester, M. (2008). Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59:651-681.

33. Liu, J. and Zhu, J.K.(1998). A calcium sensor homolog required for plant salt tolerance. Science280: 1943-1945.

34. Liu, J.P., Ishitani, M., Halfter, U., Kim, C.S. and Zhu, J.K. (2000).The *Arabidopsis-thaliana* SOS2 geneencodes a protein kinase that is required for salt tolerance. Proc. Natl. Acad. Sci. USA,97: 3730-3734.

35. Shi, H., Ishitani, M., Kim, C. and Zhu, J.K. (2000). The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+antiporter. Proc. Natl. Acad. Sci. USA,97: 6896-6901.

36. Halfter, U., Ishitani, M. and Zhu, J.K. (2000). The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc. Natl. Acad. Sci. USA,97: 3735-3740.

37. Quan, R.D., Lin, H., Mendoza, I., Zhang, Y., Cao, W., Yang, Y., Shang, M., Chen, S., Pardo, J.M. and Guo, Y. (2007). SCABP8/ CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect *Arabidopsis*shoots from salt stress. Plant Cell. 19: 1415-1431.

38. He, C., Yan, J., Shen, G., Fu, L., Holaday, A.S., Auld, D., Blumwald, E. and Zhang, H. (2005). Expression ofan Arabidopsis vacuolar sodium/proton antiporter gene in cotton improves photosynthetic performance under salt conditions and increases fiber yield in the field. Plant Cell Physiol. 46: 1848-1854.

39. Zhao, F., Guo, S., Zhang, H. and Zhao, Y. (2006). Expression of yeast *SOD2* in transgenic rice results inincreased salt tolerance. Plant Sci. 170: 216-224.

40. Chen, M., Chen, Q.J., Niu, X.G., Zhang, R., Lin, H.Q., Xu, C.Y., Wang, X.C., Wang, G.Y. and Chen, J. (2007). Expression of OsNHX1 gene in maize confers salt tolerance and promotes plant growth in the field. Plant Soil Environ 53: 490-498.

41. Yadav, N.S., Shukla, P.S., Jha, A., Agarwal, P.K. and Jha, B. (2012). The *SbSOS1* gene from the extreme halophyte *Salicorni-abrachiata* enhances Na^+ loading in xylem and confers salt tolerance in transgenic tobacco. BMC Plant Biology. 12: 188 doi:10.1186/1471-2229-12-188.

42. Rus, A.M., Estan, M.T., Gisbert, C., Garcia-Sogo, B., Serrano, R., Caro, M., Moreno, V. and Bolarin, M.C. (2001). Expressing the yeast *HAL1* gene in tomato increases fruit yield and enhances K^+/Na^+ selectivity under salt stress. Plant Cell Environ. 24: 875-880.

43. Ellul, P., Rios, G., Atares, A., Roig, L.A., Serrano, R. and Moreno, V. (2003). The expression of the *Saccharomyces cerevisiae HAL1* gene increases salt tolerance in transgenic watermelon [*Citrullus lanatus* (Thunb.) Matsun. & Nakai.]. Theor. Appl. Genet. 107: 462-469.

44. Obata, T., Kitamoto, H.K., Nakamura, A., Fukuda, A. and Tanaka, Y. (2007). Rice shaker potassium channel *OsKAT1* confers tolerance to salinity stress on yeast and rice cells. Plant Physiol. 144: 1978-1985.

45. Mohanty, A., Kathuria, H., Ferjani, A., Sakamoto, A., Mohanty, P., Murata, N. and Tyagi, A.K. (2002). Transgenics of an elite indica rice variety Pusa Basmati 1 harbouring the*codA* gene are highly tolerant to salt stress. Theor. Appl. Genet. 106: 51-57.

46. Wu, W., Su, Q., Xia, X., Wang, Y., Luan, Y. and An, L. (2008). The *Suaeda liao-tungensis* Kitag betaine aldehyde dehydrogenase gene improves salt tolerance of transgenic maize mediated with minimum linear length of DNA fragment. Euphyt. 159: 17-25.

47. KaviKishor, P.B. and Sreenivasulu, N. (2013). Is proline accumulation *per se* correlated with stress tolerance or is proline homeostasis a more critical issue? Plant Cell Environ. 37: 300-11.

48. Hmida-Sayari, A., Gargouri-Bouzid, R., Bidani, A., Jaoua, L., Savoure, A. and Jaoua, S. (2005). Overexpression of D-1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. Plant Sci. 169: 746-752.

49. Reddy, P.S. (2011). Evaluation of transgenic *Sorghum bicolor* (L.)Moench

plants for resistance to abiotic stresses. Ph.D. thesis submitted to the Osmania University, Hyderabad, India.

50. Sickler, C.M., Edwards, G.E., Kiirats, O., Gao, Z. and Loescher, W. (2007). Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress.Funct. Plant Biol. 34: 382-391.

51. Ge, L.F., Chao, D.Y., Shi, M., Zhu, M.Z., Gao, J.P. and Lin, H.X. (2008). Over-expression of the trehalose-6-phosphate phosphatase gene *OsTPP1* confers stress tolerance in rice and results in the activation of stress responsive genes. Planta 228: 191-201.

52. Liu, J.H., Kitashiba, H., Wang, J., Ban, Y. and Moriguchi, T. (2007). Polyamines and their ability to provide environmental stress tolerance to plants. Plant Biotechnol. 24: 117-126.

53. He, L., Ban, Y., Inoue, H., Matsuda, N., Liu, J. and Moriguchi, T. (2008). Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple spermidine synthase in response to salinity and hyperosmosis. Phytochem. 69: 2133-2141.

54. Gaber, A., Yoshimura, K., Yamamoto, T., Yabuta, Y., Takeda, T., Miyasaka, H., Nakano, Y. and Shigeoka, S. (2006). Glutathione peroxidase-like protein of Synechocystis PCC 6803 confers tolerance to oxidative and environmental stresses in transgenic *Arabidopsis*. Physiol. Plant. 128: 251-262.

55. Prashanth, S.R., Sadhasivam, V. and Parida, A. (2008). Over expression of cytosolic copper/zinc superoxidedismutase from a mangrove plant *Avicennia marina* in indica rice variety Pusa Basmati-1 confers abiotic stress tolerance. Transgen Res. 17: 281-291.

56. Xu, W.F., Shi, W.M., Ueda, A. and Takabe, T. (2008). Mechanisms of salt tolerance in transgenic *Arabidopsis thaliana* carrying a peroxisomalascorbate peroxidase gene from barley. Pedosphere. 18: 486-495.

57. Lee, H. and Jo, J. (2004). Increased tolerance to methyl viologen by transgenic tobacco plants that overexpressthe cytosolic glutathione reductase gene from *Brassicacampestris*. J. Plant Biol. 47: 111-116.

58. Wang, Y., Jiang, J., Zhao, X., Liu, G., Yang, C. and Zhan, L. (2006). A novel LEA gene from *Tamarixandrossowii* confers drought tolerance in transgenic tobacco. Plant Sci. 171: 655-662.

59. March, T.J., Able, J.A., Schultz, C.J. and Able, A.J. (2007). A novel late embryo-genesis abundant protein and peroxidase associated with black point in barley grains. Proteomics 7: 3800-3808.

60. Wakui, K. and Takahata, Y. (2002). Isolation and expression of LEA gene in desiccation-tolerant microspore-derived embryos in *Brassica* spp. Physiol. Plant. 116: 223-230.

61. Brini, F., Hanin, M., Lumbreras, V., Amara, I., Khoudi, H., Hassairi, A., Pages, M. and Masmoudi, K. (2007). Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. Plant Cell Rep. 26: 2017-2026.

62. Lal, S., Gulyani, V. and Khurana, P. (2008). Overexpression of *HVA1* gene from barley generates tolerance to salinity and water stress in transgenic mulberry (*Morus indica*). Transgenic Res. 17: 651-663.

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NEWS ITEM

Southeast Asia region including india is declared polio free by WHO

With India having finally eradicated polio, the entire World Health Organisation (WHO) Southeast Asia region was formally certified polio free on March 27, 2014. India is one of the 11 countries in the South East Asian region which have been certified as being free of the wild polio virus.

In January this year India completed three consecutive years without any fresh case of polio being reported the condition for a country to be declared polio free. Afghanistan, Pakistan and Nigeria are the only countries in the world left where the virus remains endemic.

"India has been polio free since January 2011. India embarked on the programme to eradicate polio 19 years ago in 1995, when the disease used to cripple more than 50,000 children in the country every year. This achievement makes the South-East Asia Region, the fourth WHO Region to be certified as polio-free, after the Region of the Americas in 1994, the Western Pacific Region in 2000 and the European Region in 2002." Azad said.

The Health Minister expressed gratitude towards WHO, UNICEF, Rotary International, Bill and Melinda Gates Foundation and others stakeholders including the parents of the children, for their strong technical and operational support to this collective effort in this region.

Dr. Poonam Khetrapal Singh, regional director, WHO southeast Asia said, "while we celebrate this hard won battle today, let us not forget that the war against polio is far from over. We still have two WHO regions to achieve the same goal. And as long as there is polio in any corner of the world, all parts of the world remain at risk of polio returning. Therefore, we cannot let our guards down." She emphasised the need to step up surveillance of acute flaccid paralysis that presents symptoms similar to polio.

Govt approves Rs 650 crore for Nano mission

NEW DELHI: Union Cabinet on Thursday gave its approval for continuation of Nano mission - a mission on Nano Science and Technology - in its second phase in the 12th Plan Period (2012-17) and sanctioned Rs 650 crore for the purpose.

Nano Technology is a knowledge-intensive and "enabling technology" which is expected to influence a wide range of products and processes with far-reaching implications for the national economy and will aslo strengthen activities in nano science and technology by promoting basic research, human resource development, research infrastructure development, international collaborations, orchestration of national dialogues and nano applications and technology development", said an official statement of the government.

The government had launched the Nano mission im May 2007 as an "umbrella capacity-building programme. The nano mission, in this new phase, will also make greater effort to promote application oriented R&D so that some useful products, processes and technologies also emerge. It will be anchored in the Department of Science and Technology and steered by a nano Mission Council chaired by an eminent scientist.

High Court Judges counsel parents of mentally challenged children

Mentally challenged children were god's children and they should not be considered a burden by their families," said G.M. Akbar Ali, Judge, Madurai Bench of Madras High Court. Addressing parents of children with mental disabilities, Mr. Ali said people should never look down upon such children as they also formed part of the society.

Some of the anxious parents of mentally challenged children, who took part in the fourth anniversary of Free Legal Aid Clinic, expressed concern over the future of their children after their death. V. Ramasubramanian, Madras High Court Judge, said there was no scientific answer to the

question of what would happen to the children after the death of their parents.

However, things would change and a time will come when they would have access to good education and a secure future as in the case of other children, he added. Mr. Ramasubramanian observed that there was not much awareness of mental disability and mental illness in the society.

As per a report presented on the occasion, 98 persons received guardianship, 165 received disability certificates, 140 got free treatment, 53 got their property disputes settled, 59 got their parents' pension, nine marital disputes were settled and 41 received counselling.

Earlier, during an interaction with the Judges, parents of mentally challenged children sought guidance on legal issues pertaining to guardianship, family pension benefit, need for institutional care for destitute and so on.

The Free Legal Aid Clinic, with the support of Madurai District Legal Services Authority, functions on the premises of M.S. Chellamuthu Trust and Research Foundation in K.K. Nagar between 2 p.m. and 5 p.m.

MITE gets centre of excellence award

MANGALORE: Vision group on Science & Technology (VGST) of the state department of science and technology has awarded 'Centre of Excellence in Science, Engineering & Medicine (CESEM)' award to the department of Physics of Mangalore Institute of Technology & Engineering (MITE), Moodbidri. The CESEM award comprises of a grant of Rs 20 lakh per year for a period of three years.

Asha Rao, professor and head of the department of Physics is the programme coordinator to set up the Centre of Excellence on "A study on the enhancement of efficiency of solar cells by various modification processes and synthesis / characterization of NLO materials" under the department.

CESEM is the prestigious award of VGST given with an objective of providing infrastructure needed to conduct 'theme based research' only to five selected colleges, University Departments and Research Institutions in the state. Selected institution is eligible to receive a total of grant of Rs 60 lakh for three years, subject to the fulfilment of guidelines, terms and conditions of VGST. The second and third instalment of the award grant will be released to grantee institution ony after the evaluation of progress achieved in the first year grant, stated S Ananth Raj, consultant VGST, in a release.

Other institutions that received the award in the state are SDS Tuberculosis Research Centre & Rajiv Gandhi Institute of Chest Diseases, Bangalore, NET Pharmacy College, Raichur, Alliance College of Engineering and Design, Bangalore and MS Engineering College, Bangalore, he added.

SCIENTIFIC ARTICLES

A New Pill to Delay Ageing Process and Improve Health

A new pill that prevents ageing and keeps people healthier for longer may be a step closer. Activating a protein called siurtuin1 extends lifespan, delays the onset of age-related metabolic diseases, and improves general health in mice, a new study has found. The findings point to a potentially promising strategy for improving health and longevity. Sirtuin1, or SIRT1, is known to play an important role in maintaining metabolic balance in multiple tissues, and studies in various organisms have shown that activating the protein can lead to health benefits.

Powerful HIV Vaccine Comes Closer to Reality

Scientists in National institute of Health, USA have discovered a mechanism that helps HIV evade antibodies and stabilise key proteins. The finding will pave way for more effective vaccine for the deadly virus.

Scientists found the mechanism involved in stabilising key HIV proteins and thereby concealing sites where some of the most powerful HIV neutralising antibodies bind. Numerous spikes just out of the surface of HIV, each containing a set of three identical, bulb-shaped proteins called gp 120 that can be closed together or spread apart like the petals of a flower, researchers sad.

Some of the most important sites targeted by HIV neutralising antibodies are hidden when the three gp 120 trimer remains closed until the virus binds to a cell.

In this process, called sulfation, the amino acids acquire a sulphur atom surroundedby four oxygen atoms. By either blocking or increasing sulfation of these amino acids, the researchers changed the sensitivity of the virus to different neutralising antibodies, indicating that the trimer was being either opened or closed.

A Blood Test will Reveal When You are Going to Die

A novel blood test may predict if you are at the risk of dying in next five years. With nuclear magnetic resonance (NMR) spectroscopy over 200 biomarkers predicting short term mortality have now been discovered .

Leader of the research team, Professor Mika Ala Korpela of the University of Oulu, Finland, claimed, “if a person belongs to a risk group based on these biomarker concentrations, he/she has a multi fold risk of dying in the next five years compared to the general population.”

The study is based on blood samples of over 17,000 Finnish and Estonian People. In the research, mortality was related to four biomarkers in the blood :levels of two proteins (albumin andalpha-1 acidic glycoprotein particles responsible for lipid metabolism in the body) and citric acid concentration. These biomarkers relate to normal metabolism and are present in the blood of all people, but according to the study, their relative amounts are crucial.

The biomarkers were independent of known mortality risk factors such as age, smoking, alcohol use, cholesterol, obesity, and blood pressure.

Mind-Controlled Helicopter Flies on Brainwaves

Researchers of the university of Minnesota have developed a mind-controlled system that allows users to fly a helicopter with just their thoughts. The research team has created a brain-computer interface-a system that allows the brain to communicate directly with an external device- that lets participants to control the path of a flying object, known as a quodcopter by thinking about specific movements.

The interface requires users to wear an electroencephalography (EEG) cap with attached electrodes that pick up signals from the brain.

When participants think about a specific movement up, down, right or left, for instance, neurons in the brain’s motor cortex produce tiny electric signals which are then sent to a computer.

The signals coming from the brain are being picked-up by the sensors and then decoded and sent through a Wi Fi system to control the flying quodcopter.

EDUCATION

Admission To Ph.d. Program-2014 at Csir-institute Of Genomics And Integrative Biology, North campus, Mall Road, Delhi-110007 And South campus, Mathura Road, New Delhi-110025.

The CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB) invites applications from bright young individuals from diverse backgrounds with strong motivation and good academic credentials to pursue research in frontier areas of modern biology leading to a Ph.D degree. The broad interdisciplinary research areas are functional genomics, molecular medicine and disease genomics, bioinformatics and computational biology, systems biology, respiratory disease biology and environmental biotechnology.

Who can apply : Masters’ degree (M.Sc./ M.Tech.) with 60% (or equivalent in CGPA) in any branch of Life Sciences/Biotechnology/Chemistry/ Physics/Mathematics/Statistics/Computer Science or allied subjects with special interest in Biology. B.Tech (in Biological Sciences or other branches with special interest in Biology)/B.Pharm. candidates with 60% (or equivalent in CGPA) may also apply.

How to apply : Links to application form will be available from April 20, 2014 to May 31, 2014 atwww.igib.res.in. Instructions for filling in the application form and last date of application will be given therein. Candidates will have to submit a non-refundable application fee of Rs. 500/- (Rs. 250/- for SC/ST candidates). The instructions for the payment will be available on the website.

Admission to the THSTI-JNU PhD program 2014 in biomedical and clinical research

Applications are invited for the THSTI-JNU PhD programs under two tracks, (i) biomedical research (ii) clinical research.

Eligibility : Candidate must have Master’s degree in any branch of life science (including biomedical, health, pharmaceutical, nutritional science, public health, or nursing), veterinary science, engineering, mathematics, or a medical degree (MBBS, BDS, or equivalent) from a recognized university. Candidate must have scored 60% or higher marks in all examinations and 55% or higher marks in their qualifying examination. Except for candidates with medical or allied background (MBBS, BDS, or equivalent) and those with Master’s degree in public health, nutrition, and nursing, all others must have qualified the junior research fellowship examination of CSIR, UGC, ICMR, NBHM, DST and DBT. Candidates with medical or allied background (MBBS, BDS, or equivalent) or those with Master’s degree in public health, nutrition, and nursing, without a research fellowship will be required to appear in an objective type admission test to be conducted by THSTI in Gurgaon on 15th June 2014.

OPPORTUNITIES

Post-Doctoral Fellowship at Institute of Nano Science And Technology

Advertisement for Postdoctoral Research Fellowships 2014 Institute of Nano Science and Technology invites applications for Postdoctoral Research Associates. Interested candidates who have either a PhD degree or have submitted their PhD thesis can apply for these positions. Applications should be sent by email to postdoc@inst.ac.in. Applications should accompany a cover letter, one page synopsis of PhD thesis, two pages write up of a suitable research proposal and a brief bio data.

ELIGIBILITY: The applicants should either hold a PhD degree in Science or Engineering or should have submitted the PhD thesis with strong research interest in Nano Science and Technology proven research competence corresponding to their equivalents in the best institution of the world and should have a very good academic record. Applicants should be below 40 years of age as on date of interview and have evident capabilities of becoming independent investigators in future.

Details of Fellowship :The fellowship carries a consolidated monthly stipend of Rs.35,000/- besides a contingency grant of Rs.1,00,000/- per annum. The postdoctoral fellowship is for a period of 3 years reviewed annually. Candidates who have submitted the PhD thesis but are yet to be awarded the PhD degree, if selected, will be paid Rs. 25,000/- per month till the award of the degree.

Mode of Selection:The candidates will be shortlisted based on their bio data and evaluation by a shortlisting committee and will be called for interview. Final selection will be done based on the recommendations of a National Selection Committee after the performance in the interview. The interview will be held at the INST campus during the last week of July. Candidates called for interview will be paid II class railway (non-AC)/bus fare by the shortest route from their home town. Shortlisted candidates will be asked to bring their original certificates (B.Sc., M.Sc., M.Tech, PhD) at the time of the interview. The shortlisted candidates should request three referees.

Important Information: Number of Fellowships: 10-15,Last date of online application submission: May 20th 2014,Interview: During July 25th-26th, 2014. Joining Date: Around September 1st, 2014.

Post-Doctoral Fellows Appointment Of Post-Doctoral Fellow at NCBS - Max Planck Lipid Centre, Bangalore

The National Centre for Biological Sciences (NCBS) - Tata Institute of Fundamental Research (TIFR), Bangalore (www.ncbs.res.in) is a premier centre for research and teaching in frontier areas of biological sciences. The NCBS-Max Plank Lipid Centre is a joint initiative of NCBS and the Max Planck Society (MPS). The goal of the Centre is to advance collaborative research activity between scientists at NCBS and the Max Planck Institute for Cell Biology and Genetics Dresden, Germany in understanding the function of lipids, specific chemical components of living cells.

NCBS-Max Planck Lipid Centre is looking for appointment of Post-doctoral fellow to work on cutting edge mass spectrometry based analysis of lipids in biological systems.

Qualifications & Experience: Candidates should be a Ph.D in biological or chemical sciences with strong exposure to biochemistry, analytical chemistry or hands on experience with mass spectrometers and liquid chromatography.

Desirable: Candidates with hands on experience in mass spectrometry based lipidomics including sample preparation, data acquisition and interpretation. A backgroud in lipid chemistry would be an added advantage.

This position is available for a period of three years. Please email your CV along with a summary of your achievements and name & email ID of two professional referees to lipids@ncbs.res.in. Last Date of receipt of applications by email only: 30th April, 2014.

ICRISAT seeks applications from Indian nationals for the position of Scientific Officer (IRRI) to be based at *Patancheru, Andhra Pradesh,* India.

ICRISAT is a non-profit, apolitical, international organization for science-based agricultural development. Established in 1972, it is an Alliance of centers of the Consultative Group on International Agricultural Research (CGIAR), and supported by more than 48 governments, foundations, and development banks. To reduce poverty, enhance crop productivity and food security and to protect the environment, ICRISAT research focuses on farming systems and crop improvement, management and

utilization of sorghum, pearl millet, pigeonpea, chickpea, and groundnut in the semi-arid tropics.

Requirements:
- M.Sc. (Ag) in Plant Breeding and genetics;
- 2 years' experience on germplasm evaluation in breeding trials;
- Knowledge of plant breeding design trials, data analysis, data and report compilation, seed exchange and coordination;
- Preference to candidates with knowledge and skills in the use of MAS for breeding;
- Excellent verbal and written English are necessary

How to Apply: The application should include an up-to-date resume, a recent passport size photograph, names and addresses (including phone & e-mail) of three referees and date of availability. Please send applications stating the job title clearly on the application/envelope, to Human Resources Services, ICRISAT, Patancheru, Andhra Pradesh 502 324, or by E-mail to icrisat.nrsjobs @cgiar.org, latest by 30 April 2014. Only short-listed candidates will be contacted.

Scientist Positions in Rajiv Gandhi Centre For Biotechnology India

Rajiv Gandhi Centre for Biotechnology (RGCB) at Thiruvananthapuram in India is an autonomous research institution of the Government of India, Department of Biotechnology. RGCB invites applications for group leaders in Disease Biology (Cardiovascular and Diabetes Biology, Computational Biology/Bioinformatics and Cancer & Viral Vaccines Program). Complete details are posted on the institute website http://www.rgcb.res.in. Applications that do not fulfill the minimum conditions required will be rejected and no intimation given.

Research Assistant in Case Western Reserve University Comprehensive Cancer Center, Cleveland, OH Bioinformatics

A unique translational cancer research opportunity is available to work with a multi-investigator team consisting of clinicians and basic scientists within the CWRU GI Specialized Program of Research Excellence (SPORE) and other groups at the Case Western Reserve (CWRU) Comprehensive Cancer Center.

The motivated Bioinformatics RA will be a member of the newly established translational bioinformatics effort at CWRU comprehensive cancer center, led by Dr. Vinay Varadan, focusing on the integrative analysis of next generation sequencing and multi-omics data generated within the cancer center.

Requirements:

Master's degree in computer science, engineering, bioinformatics, statistics or biostatistics, with 0-2 years experience in related areas.

Applicants are expected to have fundamental knowledge and experience in one or more of the following areas:

- Next Generation Sequencing Analysis pipelines
- Whole exome or whole genome sequencing based variant/mutation detection
- RNA sequencing based transcript abundance estimation
- Computational biology/Bioinformatics
- Sequence alignment and phylogenetic analysis and clustering algorithms
- Differential gene expression, gene set enrichment
- Protein-protein interaction networks and pathway analysis

Additional experience in any of the following will be considered an advantage:

- Machine learning and/or data mining
- Data visualization and software development

The RA will be involved in the development and maintenance of novel translational bioinformatics pipelines and is therefore expected to be proficient in R, Perl, Python or C/C++ programming along with UNIX scripting.

Contact Information: Vinay Varadan, Assistant Professor, Case Comprehensive Cancer Center, Case Western Reserve University, 2103 Cornell Rd, WRB 3125, Cleveland, OH-44106. Email: vxv89@case.edu

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