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Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Prophylactic and Therapeutic Efficacy of a Glycoconjugate Vaccine Against Bovine Brucellosis

F. Mukherjee[1a], K. Nagmani[2a], A. Prasad[1], V.S. Bahekar[1], B. Ramalakshmi[3], T. Mythili[4], L. Rajendra[5], S.K. Rana[1], G.K. Sharma[6] and V.A. Srinivasan[7]*

[1]National Dairy Development Board, R&D Laboratory, Hyderabad, 500032, Telangana, India
[2]Department of Bio-Technology, Jawaharlal Nehru Technological University; Research and Development Centre, Indian Immunological Limited, Gachibowli, Hyderabad 500032, Telangana, India
[3]Research and Development, Indian Immunologicals Limited, Hyderabad, 500032, Telangana, India
[4]38 Callander Street, Thomson East Geelong, Victoria, Australia-3219
[5]Plot No. 68. Flat No. 4A, Challa Pushpa Doyen Dacha Apartments, Sri Nagar Colony, Hyderabad 500073
[6]National Dairy Development Board, Anand, 388001, Gujarat, India
[7]National Dairy Development Board, Gachibowli, Hyderabad 500032, Telangana, India
*For correspondence - srinivasanva1948@gmail.com
[a]*These authors contributed equally to this work*

Abstract

Bovine brucellosis is a zoonotic disease. It impacts dairy industry since it is a major cause of abortion. The disease is caused by bacteria belonging to the genus *Brucella*. The pathogen is excreted in milk, semen and aborted materials. Bovine brucellosis is currently controlled by calf-hood vaccination of females using live attenuated *B. abortus* strains (S19/RB51), however it does not provide 100% protection, and its use is restricted by age and gender. The vaccine strains are infectious to humans. Review of literature indicated that use of glyco-conjugate (GC) vaccines could address the above problems. Therefore, the objective of study was to assess the protective efficacy and therapeutic potential of GC vaccine prepared from *Brucella abortus* S19 (S19GC) in cattle. Immunization of *Brucella* free animals with two doses of 50µg each at an interval of 90 days by sub-cutaneous route resulted in pronounced Th1 mediated interferon gamma (IFN-γ) response (P=0.0061) as observed in ELISPOT assay compared to unvaccinated controls till 90 days post vaccination (DPV). The ability of S19GC to arrest shedding of *Brucella* was studied in brucellosis positive animals, vaccination of infected animals each with 50µg of S19GC by subcutaneous route resulted in arresting of shedding as evidenced by negative culture results, reduction (2 log folds) in genome copy number as observed by real-time PCR (qPCR). The prime-boost strategy of immunization (50 µg dose/ cattle) of a group of cattle infected with brucellosis (> 53%) was only able to arrest bacterial shedding transiently (7-60 DPV) in 66.67% immunized cattle, and after booster bacterial shedding was not recorded by culture in 83.34% of the animal till 250 PI. Also, *Brucella* genome was not detected transiently (7-150 DPV) in samples; but was detected thereafter till 250 DPV. The most promising effect of immunization with S19GC was observed in a group of cattle with a mixed population of brucellosis negative and positive animals of the above farm. Immunization of this group of animals with 100 µg of S19GC resulted in complete stoppage of shedding as indicated by culture and qPCR. The post immunization anti-LPS and anti-OMP antibody IgG1 and IgG2 response differed (P<0.01) at 30 DPV compared to pre immunization, suggesting the involvement of both Th2 and Th1 cells in the immunity conferred by S19GC vaccine.

Key words: *Brucella abortus*, cattle, glycoconjugate vaccine, lipo-polysaccharide, outer membrane protein.

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Introduction

The genus *Brucella* is a gram-negative, non-motile and facultative intracellular pathogen; and is comprised of ten recognized species (1, 2, 3, 4). *B.abortus* is the most widespread (5) and affects cattle as well as other mammalian species (6). World Health Organization (WHO) ranks brucellosis among top seven "neglected zoonosis", a threat to human health and the cause of poverty (7, 8). The disease is a major cause of concern for dairy industry as it causes reduction in milk yield, abortion and infertility in female cattle, poor quality of semen and orchitis in males. There are two major type of vaccines used for control of brucellosis - live attenuated *B.abortus* smooth S19 strain and live attenuated *B.abortus* rough RB51 strain. The major drawback of these conventional vaccines is their ability to shed the live organism and cause disease in associated cattle (9) and humans (10). Live attenuated vaccines have been used in many countries for a long time for control of brucellosis. However, these vaccines are indicated for female calves of 4 months to 11 of age which is a serious limitation for effective control of brucellosis in many endemic countries where slaughter of infected animals cannot be advocated. The S19 vaccine also induces O-lipo-polysaccharide specific antibodies which interfere with serodiagnostic tests (6). Because of these drawbacks, efforts are currently directed towards the development of improved vaccines, which includes formulations of subunit (11) and recombinant sub-cellular vaccines against brucellosis (12, 13).

Subunit vaccines are promising vaccine candidates since they are non-virulent, non-infectious, and therefore do not pose any biohazard, and are well defined antigens that can be adopted for immunization. The use of GC vaccines for prophylactic immunization in humans has been cited earlier. They have been used against human pathogens *Haemophilus influenzae* (HiB), *Streptococcus pneumoniae* and *Niesseria meningitis* (14, 15, 16). In case of brucellosis earlier reports had indicated the use of lipopolysaccharide (LPS) from *B.melitensis* covalently conjugated to BSA (3) and *B.melitensis* LPS non-covalently conjugated to *Neisseria meningitides* OMP (17). More recently, the immune response of a GC vaccine formulation prepared from LPS and OMP extracted from *B.abortus* S19 strain was studied in mice and cattle calves (18, 19). It was shown that BALB/C mice immunized with S19GC vaccine by sub-cutaneous route were protected upon challenge with *B. abortus* 544 virulent strain (19), and also the GC formulation was able to elicit appropriate cell mediated immune response in mice and cattle calves (18, 19). In the present study the prophylactic and therapeutic potential of S19GC vaccine in adult cattle comprising of brucellosis negative and naturally infected animals raised as closed free mixing herd was explored.

Material and Methods

Preparation of Brucella S19GC Glycoconjugate vaccine: The extraction and purification of lipopolysacharide (LPS) and outer membrane protein (OMP) from *B.abortus* S19 strain and further conjugation of LPS and OMP for the preparation of *B. abortus* glycoconjugate vaccine was done in house as described by Mythili *et al.,* 2010 (18). The *B. abortus* S19 vaccine USDA strain used for the preparation of glycoconjugate vaccine was obtained from Animal Disease Research Laboratory (ADRL), National Dairy Development Board, Anand, India.

Pre-vaccine screening of animals: The prevaccination status of brucellosis in animals of three different farms (Farm I, Farm II and Farm III) was determined by cultural isolation, serology, and qPCR. Cultural isolation was done by using modified *Brucella* selective media (MBS) (20) and serology by indirect ELISA using a commercial kit BRUCELISA (VLA, UK). The qPCR was done as per the method developed in house (unpublished data).

Vaccination: Based on the pre-vaccine screening results a total of nine experimental groups were formed (Table 1). The prophylactic

and therapeutic efficacy of GC vaccine was studied post vaccination by the assessment of humoral and cell mediated immune response and shedding pattern of *Brucella* in these groups. Experiments in three groups of brucellosis negative cattle (Group 1, 2 and 3) approved by the Institutional Ethical Committee (IAEC) and the Committee for the Purpose of Control Experiment of Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India (Approval number: IIL-R&D-LA-53/2010 dated 08.09.2010) were conducted according to the standard operating procedures (SOP) and guidelines of IAEC/ CPCSEA. Immunization studies in rest of the 6 groups of animals naturally infected with brucellosis were conducted upon receipt of request by the proprietors of the farms and after obtaining consent from the farm authorities.

Experimental groups in Farm I: The purpose of the study in Farm I was to measure the cell mediated immune response in immunized cattle by assessing the secretion of interferon gamma (IFN-γ). Animals were housed in separate sheds and were not allowed to mix with each other. A total of three groups (Group 1, 2 and 3) of 6 cattle per group were maintained in the Holding Farm, Indian Immunologicals Limited (IIL), Hyderabad. Cattle of Group 1 and 2 were administered with $2x10^{10}$ cfu/dose of live *B. abortus* S19 vaccine (Bruvax, IIL) and 50 µg/Ml/dose of S19GC formulations, respectively, by sub-cutaneous route. Animals in Group 2 were given a booster dose of 50 µg/Ml/dose of S19GC each at day 90 post primary immunization. Animals in the Group 3 were inoculated sub-cutaneous with 1ml of phosphate buffered saline (PBS) served as placebo controls. Heparinized whole blood samples were collected at 0, 7, 14, 21, 30, 60, 90, 105 and 120 DPV (Table 1). Peripheral blood lymphocytes harvested from blood samples were used to study the cell mediated immune (CMI) response by ELISPOT (Table 1).

Experimental groups in Farm II: The purpose of the study was to assess the prophylactic and therapeutic efficacy of the S19GC vaccine in an infected farm. The farm housed approximately 1000 animals. The animals were maintained by a trust under animal charity. The animals were left free for grazing together during the daytime and were tied in different sheds at night as routine. Milking animals however, were housed in separate sheds and were stall fed. There was a separate shed for the calves born of these milking animals. Separate sheds were maintained for male and female animals. Hence the farm housed a mixed population of animals that were either negative or positive for brucellosis and were in contact with each other. A total of four groups of adult female cattle were formed namely Group 4, 5, 6 and 7. In Group 4 (n=8) and 7 (n=8) all the animals were negative by serology, isolation and qPCR. In Group 5 (n=8) all the animals were positive by serology but negative by isolation and qPCR. In Group 6 (n=7) all the animals were positive by serology and qPCR with one of the animal positive by culture. All the animals from these three groups were vaccinated with 50µg/ml/dose of the vaccine except group 7 which was kept as unvaccinated negative control group and were inoculated with 1ml of PBS. Nasal swabs, vaginal swabs, milk, serum and heparinized blood samples were collected on day 0, 7, 14, 21, 30 and 60 after vaccination. The samples were used for isolation by culture, antibody isotype subtypes ELISA and qPCR (Table 1).

Experimental groups in Farm III Phase 1: All cattle constituting the Group 8 (n=6) in Farm III were positive for brucellosis by serology and qPCR, of which two were also positive by culture. The purpose of study was to evaluate the effects of booster and the nature of protection offered by S19GC in infected animals over an extended period of observation (250 days). The farm housed 70 animals that mixed freely while grazing. Hence all animals were always in contact with each other. Animals were primed with 50µg/ml/dose of S19GC by sub-cutaneous route and boosted with same dose 90 days after primary immunization. Samples were collected on 0, 7,

14, 21, 30, 60, 90, 105, 120, 150, 210 and 250 days after primary immunization (Table 1). Samples were subjected to isolation by culture, antibody isotype subtypes ELISA, qPCR, whole blood IFN-γ ELISA (IGRA) and IFN-γ ELISPOT assay (Table 1).

Experimental groups in Farm III Phase 2: Animals in Group 9 (n=9) constituted of 7 cattle that were negative for brucellosis by isolation, serology and qPCR, and 2 were positive only by qPCR. Animals in Group 9 were vaccinated sub-cutaneously with 100µg/ml/dose of S19GC

Table 1: Experimental design to study the prophylactic and therapeutic efficacy of *Brucella* S19GC vaccine.

Farm ID	Group ID / No. of animals	Brucellosis status of the animal	Type, route date and dose of vaccination	Booster dose	Sample collection days	Tests done
Farm I	Group 1, n=6	Isolation -ve / Serology -ve / qPCR -ve	S19 live sub-cutaneously 10.12.10 2 x 10^{10}cfu	No booster	0,7,14,21,30,60, 90,105,120 days	IFN-γ estimation by ELISPOT
Farm I	Group 2, n=6	Isolation -ve / Serology -ve / qPCR -ve	S19GC sub-cutaneously 10.12.10 50 µg/ml	50 µg/ml at day 90	0,7,14,21,30,60, 90,105,120 days	IFN-γ estimation by ELISPOT
Farm I	Group 3, n=6	Isolation -ve / Serology -ve / qPCR -ve	PBS sub-cutaneously 10.12.10 1ml	No Booster	0,7,14,21,30,60, 90,105,120 days	IFN-γ estimation by ELISPOT
Farm II	Group 4, n=8	Isolation -ve / Serology -ve / qPCR -ve	S19GC sub-cutaneously 16.11.11 50 µg/ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm II	Group 5, n=8	Isolation -ve / Serology +ve / qPCR +ve	S19GC sub-cutaneously 16.11.11 50 µg/ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm II	Group 6, n=7	Isolation +ve or Isolation -ve / Serology +ve / qPCR +ve	S19GC sub-cutaneously 16.11.11 50 µg/ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm II	Group 7, n=8	Isolation -ve / Serology -ve / qPCR -ve	PBS sub-cutaneously 16.11.11 1ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm III	Group 8, n=6	Isolation +ve or Isolation -ve / Serology +ve / qPCR +ve	S19GC sub-cutaneously 05.09.12 50 µg/ml	50 µg/ml at day 90	0,7,14,21,30,60, 90,105,120,150, 210,250 days	Isolation, antibody Isotype ELISA, qPCR, IFN-γ estimation by ELISA and ELISPOT
Farm III	Group 9, n=9	Isolation -ve / Serology -ve / qPCR -ve or qPCR +ve	S19GC sub-cutaneously 04.06.13 100 µg/ml	No Booster	0,14,30,60, 90,120 days	Isolation, antibody Isotype ELISA, qPCR, IFN-γ estimation by ELISA and ELISPOT

Table 2. Status of brucellosis by isolation of *Brucella* by culture: Farm-II, Group 4, 5, 6 and 7.

Farm II	Animal ID Group 4	Status of animal before vaccination			Type of vaccine	Date of vaccination	*Brucella* isolation status post vaccination (days)					
		Isolation	Serology	qPCR			0	7	14	21	30	60
1	4a	N	N	N	S19GC 50µg	16.11.2011	N	N	N	N	N	N
2	4b	N	N	N			N	N	N	N	N	N
3	4c	N	N	N			N	N	N	N	N	N
4	4d	N	N	N			N	N	N	N	N	N
5	4e	N	N	N			N	N	N	N	N	N
6	4f	N	N	N			N	N	N	N	N	N
7	4g	N	N	N			N	N	N	N	N	N
8	4h	N	N	N			N	N	N	N	N	N
Farm II	**Group 5**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**0**	**7**	**14**	**21**	**30**	**60**
1	5a	N	P	N	S19GC 50 µg	16.11.2011	N	N	N	N	N	N
2	5b	N	P	N			N	N	N	N	N	N
3	5c	N	P	N			N	N	N	N	N	N
4	5d	N	P	N			N	N	N	N	N	N
5	5e	N	P	N			N	N	N	N	N	N
6	5f	N	P	N			N	N	N	N	N	N
7	5g	N	P	N			N	N	N	N	N	N
8	5h	N	P	N			N	N	N	N	N	N
Farm II	**Group 6**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**0**	**7**	**14**	**21**	**30**	**60**
1	6a	N	P	P	S19GC 50 µg	16.11.2011	N	N	N	N	N	N
2	6b	N	P	P			N	N	N	N	N	N
3	6c	N	P	P			N	N	N	N	N	N
4	6d	P	P	P			P	N	N	N	N	N
5	6e	N	P	P			N	N	N	N	N	N
6	6f	N	P	P			N	N	N	N	N	N
7	6g	N	P	P			N	N	N	N	N	N
Farm II	**Group 7**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**0**	**7**	**14**	**21**	**30**	**60**
1	7a	N	N	N	Phosphate buffer saline 1ml	16.11.2011	N	N	N	N	N	N
2	7b	N	N	N			N	N	N	N	N	N
3	7c	N	N	N			N	N	N	N	N	N
4	7d	N	N	N			N	N	N	N	N	N
5	7e	N	N	N			N	P	P	P	P	P
6	7f	N	N	N			N	N	N	N	N	N
7	7g	N	N	N			N	P	P	P	P	P
8	7h	N	N	N			N	N	N	N	N	N

without booster. Animals of Group 9 were housed in the same cattle shed occupied by cattle in Group 8. The purpose of this study was to assess the effect of increased dose of S19GC on cattle. Samples collected on 0, 7, 14, 21, 30, 60, 90, 105, 120 days post-immunization were subjected to same assays conducted on samples obtained from animals in Group 8 as described above (Table 1).

Assessment of post vaccine response of cattle:

Cultural Isolation: Modified *Brucella* selective medium (MBS) with 1X concentration of antibiotics was prepared as described by Her *et al*., 2009 (20). Two hundred microliters of samples (milk, nasal/vaginal swabs) in tryptic soya broth with 1X concentration of *Brucella* selective supplement (Hi Media, India) consisting of a cocktail of antibiotics comprising of Polymyxin B sulphate, Bacitracin, Nystatin, Cycloheximide, Nalidixic acid, Vancomycin (transport medium) were plated on MBS media plates in a biosafety level 3 (BSL-3) cabinet, and incubated at 37°C with 5%CO_2 for 4 to 5 days. Small pink shiny smooth colonies representative of *Brucella* species was further confirmed by staining with modified Ziehl-Nelson staining and qPCR.

Antibody Isotype IgG1 and IgG2 specific ELISA: The antibody isotype IgG1 and IgG2 titers against LPS and OMP were determined using an indirect ELISA (17). The checker board titration was done using purified LPS and OMP, purified in house as described by Mythili et al., 2010 (18); and known positive and negative cattle serum. The results were used to arrive for optimal concentration of antigen and serum dilution. The optimal concentration of the antigen fixed was 100ng/well and the dilution of serum was from 1:25 till the endpoint of titration. The log2 reciprocal of end point dilution showing optical density value close to or above the cut-off value was taken as the serum antibody titer for IgG1/IgG2. The cut-off value was determined by frequency distribution of the mean value of day zero titers of all negative group of animals and calculated as Mean±3 S.D. The cut off value for IgG1 against LPS and OMP was determined as 0.142 and 0.217 respectively. The cut off value for IgG2 against LPS and OMP was 0.275 and 0.285 respectively.

The LPS and OMP components of *Brucella* of concentration 100ng/well in carbonate bicarbonate buffer (coating buffer, pH 9.0) were coated on 96 well plates (Nunc Maxisorp™, The Netherlands) and incubated overnight at 4°C. Plates were washed with wash buffer, 0.05% Tween-20 in phosphate buffer saline (PBST, pH-7). Further the plates were blocked with 200µl/well of blocking buffer (3% skimmed milk in PBST). The plates were incubated at 37°C for 1 hour. After incubation the plates were washed and the test sera diluted 1:25 in blocking buffer was added and diluted serially and incubated at 37°C for 1 hour. After the incubation the plates were washed and antibovine IgG1/IgG2 (AbD Serotec, Germany) diluted 1:500 was added 100µl/well and incubated at 37°C for 1 hour. After incubation the plates were washed and recombinant protein A/G conjugated with horse radish peroxidase diluted 1:20000 in blocking buffer was added 100µl/well to all the plates and incubated at 37°C for 1 hour. After incubation the plates were washed and 100µl of chromogen or substrate solution (Tetra methyl benzidine, TMB with hydrogen peroxide, Sigma, USA) was added to the plates. The plates were incubated in dark for 10 minutes at room temperature. The reaction was stopped by adding 1.25M sulphuric acid (Merck, Germany) 100µl/well. Absorbance was measured at 450 nm in the ELISA reader (Multiscan®Titertek™, Finland).

Real time PCR: Quantitative real time PCR (qPCR) targeting *Brucella bcsp31* gene encoding for cell salt extractable outer membrane 31kDa protein, using TaqMan chemistry was developed and standardized (unpublished data). The assay had an analytical sensitivity of 30 fg and detected up to one copy number of the positive control plasmid construct, and $1x10^4$ *Brucella* cells/reaction from spiked bovine tissue matrices like blood, milk, nasal and vaginal swabs. The

diagnostic sensitivity and specificity of the qPCR was 100% and 86.55%. The qPCR was reliable, reproducible and could be completed in 72 minutes. This assay was used in the present study to monitor *Brucella* shedding in vaccinated animals. Briefly, the details of the assay (*viz.,* primers, probe sequence, thermal profile) are furnished below:

The sequence of the primers and probe is as follows:
bcsp31 forward primer :
5' CTCGGTTGCCAATATCAATG 3';
bcsp31 reverse primer :
5' ATATGGATCGTTTCCGGGTA 3';
bcsp31 probe :
FAM 5'CCGGTGCCGTTATAGGCCCA 3' TAMRA.

The selected primers were expected to generate an amplicon of 165 bp in the qPCR. The reaction was performed in 0.2 ml PCR strip-tubes (Qiagen, Germany) with a total reaction volume of 25µl which contained 12.5µl of master mix (Qiagen, Germany) and 10 pico-moles of each primer, 10 pico-moles of probe and 5µl of the template. Reaction conditions were set as follows: Hold at 95°C for 5 minutes, cycling at 95°C for 5 seconds and 60°C for 30 seconds consisting of 40 cycles. Reaction was carried out in Rotor Gene Q qPCR cycler (Qiagen, Germany). Plasmid constructs (pCR™2.1-TOPO®-Bru-bcsp31) harboring the 165bp fragment of *bcsp31* was developed in house and used as internal amplification positive control standard. The plasmid was serially diluted from 10^{10} to 1 copy number and real time reaction was performed for each dilution of the standard, positive, negative controls and the test samples (blood, nasal/vaginal swabs and milk). Reaction threshold (C_q) values of the standards were plotted on a graph against the initial copy numbers of the plasmid. The sample C_q values were placed on the standard graph to know their copy numbers. C_q value of 38 was set as the cut off for positive and negative samples. Samples showing a C_q value below 38 were decalred as positive and the samples showing C_q value above 38 were declared as negative for brucellosis.

For conducting the qPCR the DNA from the test samples (blood, milk, nasal and vaginal) of vaccinated animals was extracted by 'blood and body fluid protocol' of Qiagen Blood mini kit, Germany with slight modifications. Prior to DNA extraction all the clinical samples were spiked with known amount of unrelated linearized plasmid DNA containing E6 gene of human papilloma virus (HPV). This exercise was done to normalize the values of *Brucella* copy number obtained. Normalization was necessary to minimize the variations in qPCR results due to sampling, handling, and other technical errors. After the DNA extraction the samples were assayed by both the qPCRs targeting *bcsp31gene* and HPV E6 gene , using the same thermal profile and PCR parameters as described above, to determine the respective copy numbers of them in each sample. The sequence of primers and probe of HPV qPCR used is as follows:

HPV forward: 5'-
TGGAGACCATCCGATAACAC-3';
HPV reverse: 5'-
GGATGTCTTGTTTGTTTCCG-3';
HPV probe: 5'-FAM/TCT GTG TTC ACC ACC CGG GC/36-TAMS/-3'.

The normalized copy number of *Brucella* genome was calculated as bcsp31: HPVE6 copy numbers for a particular sample. When the copy numbers of the bcsp31 gene were compared without normalization, the variation in copy numbers was up to 10^3 folds (though the samples were spiked with equal amount of *Brucella* organisms). However, the variations were reduced to less than 10 folds, when the copy numbers were normalized with the copy numbers of exogenous control. This indicated that normalization with extraneous DNA was essential.

Whole blood Interferon gamma by ELISA (IGRA): The whole blood IFN-γ stimulation assay was performed as described by Wood and Jones

(21). Firstly the IFN-γ was expressed by stimulating heparinized whole blood of the vaccinated and control group of animals on different days of pre and post vaccination. Secondly a capture ELISA was performed to measure the IFN-γ expression. The IFN-γ was expressed as a Stimulation Index (S.I.) value calculated as (Test sample O.D – Blank) / (PBS O.D – Blank). The cut-off S.I. value was determined by frequency distribution of mean of day zero S.I. values of all negative animals and calculated as Mean±3 S.D. 1ml blood of each animal was stimulated with 25µg of LPS and OMP, concanavalin A (8µg/well) as positive control and PBS as negative control in 24 well tissue culture plates. The plates were incubated in CO_2 incubator at 37°C with 5% CO_2 for 24 hours. After 24 hours the plates were centrifuged at 1000 rpm for 10 minutes. The plasma from each well was collected and stored at -20°C till the testing is done. The capture ELISA was performed using IFN-γ specific antibody obtained from AbD serotec (UK). Suitable standards were also kept for data analysis. Standard curve obtained from the standards was used to estimate the IFN-γ quantity.

Interferon gamma estimation by ELISPOT: Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood collected from the individual animal within 24 hours. PBMCs were isolated by density gradient centrifugation using Lymphoprep™ solution (Axie-Shield PoC AS, RodelØkka, Oslo, Norway). The cell count was taken by trypan blue exclusion technique and the cells were stored in horse serum with 10% DMSO under liquid nitrogen until use. ELISPOT assay was done as per the manufacturer's instructions of the kit ELISpot^{PLUS} for Bovine/Ovine/Equine IFN-γ (MABTECH AB, Sweden) with slight modifications. The assay was standardized and done against *Brucella* OMP component. The OMP concentration was optimized at 25µg/well after testing with various dilutions. The 96 well ELISPOT plate was prewet with 70% ethanol (100µl/well) for exactly two minutes. The plate was then washed with sterile distilled water. The coating antibody (bIFN-γ-I) was added 100µl/well with a concentration 7.5µg/ml to the plate. Next morning the plate was washed with sterile PBS. Later 200µl/well of RPMI with 10% horse serum (cRPMI) was added to the plate and incubated at 37°C for ≥ 30 minutes. While the plate was under incubation the PBMCs were revived and washed two times with RPMI media (Hyclone, U.S.A) +10% Fetal bovine serum (cRPMI) by spinning them at 1500 rpm for 8 minutes. After washing the cells were resuspended in cRPMI and were counted. The cells were added $1x10^6$ per well. OMP antigen and concanavalin A (8µg/ml) was added to the cells in triplicates. Three of the wells were left as blank. Hence total 9 wells were used to test one sample or animal. The plate was incubated at 37°C for 24 hours. After 24 hours the plate was washed with sterile PBS. The detection antibody (PAN-Biotin) of concentration 0.25µg/ml in PBS with 0.5% calf serum (PBS-0.5% FCS) was added 100µl/well to the plate. The plate was incubated for 2 hours at room temperature. After the incubation the plate was washed with sterile PBS and 100µl/well streptavidin-ALP 1:1000 dilution was added. The plate was incubated for one hour at room temperature. After the incubation the plate was washed with PBS and 0.45 micron filtered substrate solution was added 100µl/well and incubated at room temperature for 9 minutes. The reaction was stopped and washed by adding 200µl/well of purified sterile distilled water. The plate was then allowed to dry. The dried ELISPOT plate was scanned to measure the number of spots developed using Immunospot® Series 5 UV Reader (CTL, USA). The results were analysed on the same machine using its automated software features Immunocapture® and Immunospot® for user independent setting of counting parameters (Smartcount®) and the gates (Autogate®).

Statistical Analysis: The anti-*Brucella* specific isotype antibodies were expressed as the reciprocal of log2 end point dilution ± Standard Deviation. The significance of differences between groups and days for both anti-*Brucella*

specific antibodies and T cell population producing IFN-γ were analyzed by employing ANOVA followed by Tukey's HSD. For ANOVA and Tukey's HSD, P value of <0.05 were considered statistically significant (22, 23, 24).

Results

Safety of S19GC vaccine: In all the animals after vaccination no local reaction at the site of inoculation except a pea size swelling was observed that persisted for 72 hours post vaccination. All vaccinated animals were healthy and did not exhibit any unwanted systemic reaction during the observation period of 21 days.

Farm I: In this farm for both the Groups 1 and 2, the mean spot forming units (SFU), wherein each spot indicated a IFN-γ secreting cell against outer membrane protein of *B.abortus* S19 strain (S19OMP) were found to be significantly different when compared with unvaccinated controls (P = 0.0061) (Fig 1A, Fig 1B). The mean SFU values in Group 1 and 2 also differed on different days (P <0.0001) post vaccination (7, 14, 21, 30, 60, 90 DPV) but not at post-booster (PB) (P =0.3715) (Fig 2). Also, the IFN-γ response in placebo controls did not differ statistically between different days of observation.

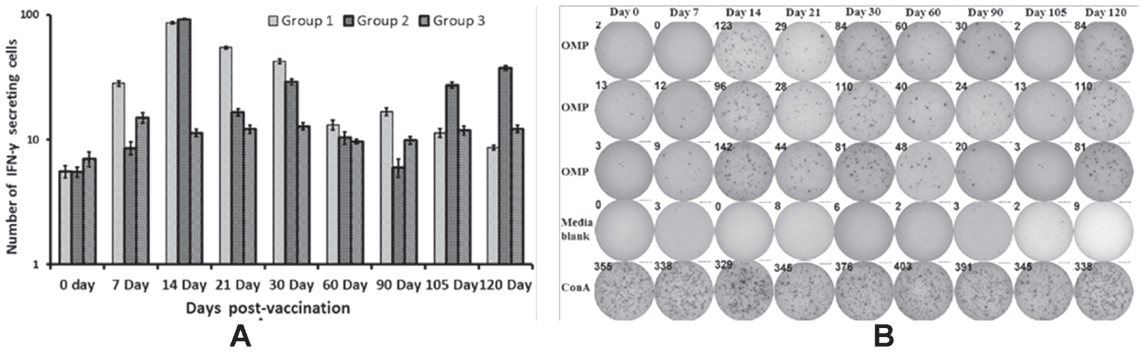

Fig.1. (A) CMI response by ELISPOT: Farm-I, Group 1, 2 and 3 (B) CMI response by ELISPOT-picture: One of the animals of Farm-I, Group 2.

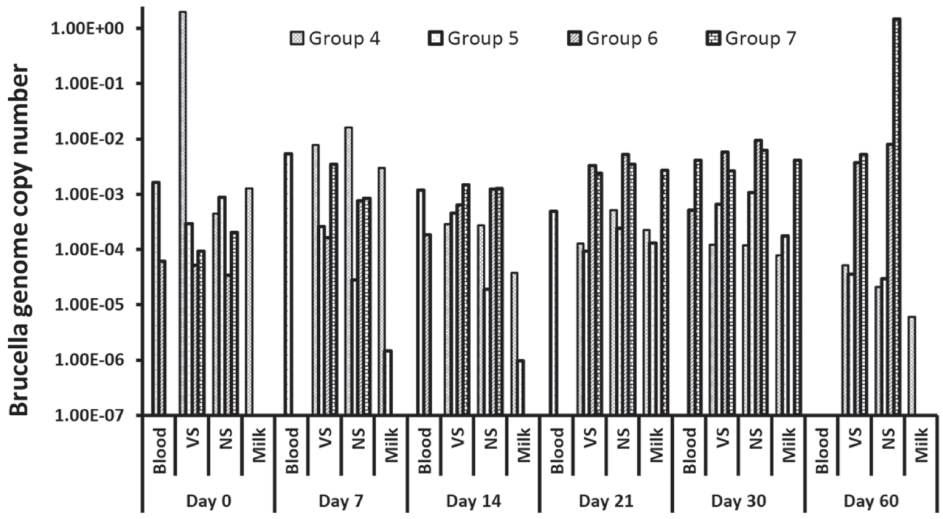

Fig. 2. *Brucella* shedding in blood, nasal, vaginal swabs and milk by bcsp31 qPCR: Farm-II, Group 4, 5, 6 and 7.

Farm II: In this farm one of the animals from Group 6 which was positive by cultural isolation stopped shedding from 7 DPV till 60 DPV. Similarly, in case of qPCR positive and serology positive animals from Group 5 and 6 no bacterial shedding was noticed till 60DPV. Also the vaccinated *Brucella* negative animals in Group 4 remained negative till 60 DPV. Whereas in the unvaccinated control (Group 7) two animals showed shedding from 7 DPV till 60DPV (Table 2). The qPCR results of animals of Group 4, 5 and 6 showed on an average 2 log reduction of *Brucella* genome in case of blood and vaginal secretions on 7 DPV; and also in milk and nasal secretions on 21 DPV. Whereas in unvaccinated animals in Group 7 the *Brucella* genome copy number showed 3 log folds increase in case of nasal secretion and 1 log fold increase in vaginal secretions on 60DPV (Fig 2). The isotype antibody ELISA results of group 4, 5, and 6 indicated that the IgG1 and IgG2 response elicited in terms of log2 end point titers against LPS and OMP components of S19GC vaccine were significantly different from day 0 pre-vaccination when compared with 60 DPV ($P<0.05$) (Figs. 3, 4).

Farm III Phase 1: Two animals in Group 8 positive by culture on day 0, continued to shed *Brucella* till 7DPV. From 14 DPV the shedding was stopped till 30 DPV. On 60 DPV one of the animals out of the two resumed shedding; and in addition, another animal of the group started shedding. The shedding continued till day 90 PI. After the administration of booster dose on day 90 PI, both the animals stopped shedding again, till day 120 PI. From day 150 PI, one animal in the group which was positive by culture from day 0 resumed shedding (Table 3). The *Brucella* genome was not detectable from 7 to 150 DPV. The mean isotype antibody titers in post-vaccinated samples were non-significant when compared to pre-vaccine titers. Similarly, pre-immunization SFU values of IFN-γ secreting cells observed by ELISPOT were not significant when data was compared for all sampling time points after primary immunization and PB. Similarly, the IGRA-ELISA values were non-significant till 90DPV; and analysis of data of samples collected PB showed inconclusive results.

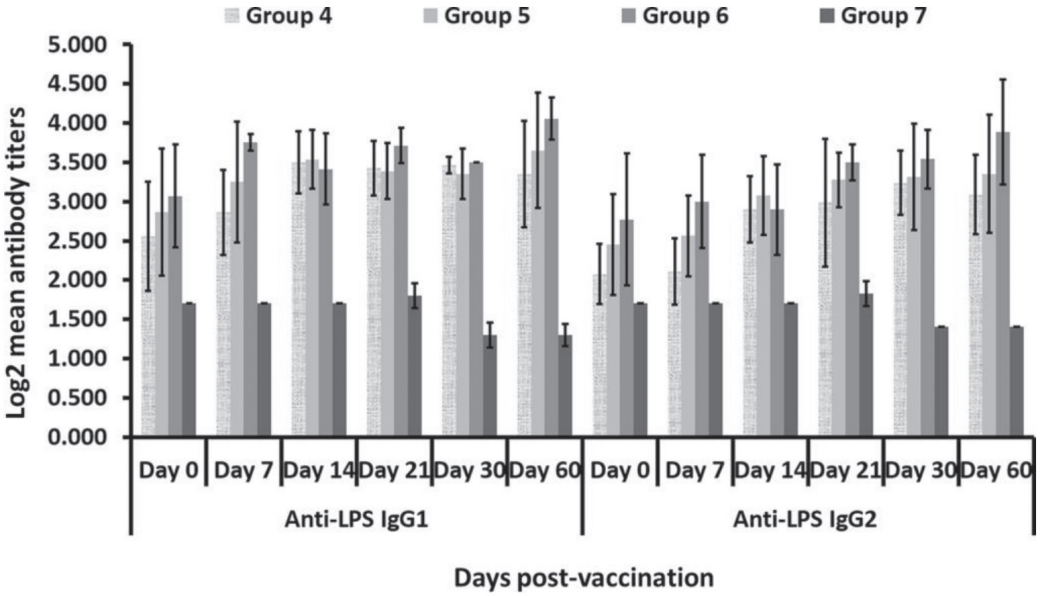

Fig.3. Anti LPS IgG1 and IgG2 response by ELISA: Farm-II, Group 4, 5, 6, and 7.

Farm III Phase 2: Samples from this group of animals were negative by culture (Table 4). The qPCR also did not detect any *Brucella* genome till 120DPV. Comparison of isotype antibody ELISA results between the days showed that the IgG1 and IgG2 titers against LPS and OMP of day 0 pre-vaccination was significantly different from those observed at 30 DPV ($P<0.05$) (Fig 5). However, the IFN-γ response against LPS and OMP as determined by IGRA-ELISA and ELISPOT displayed inconclusive results.

Discussion

Vaccination is a critical component in control and eradication program of bovine brucellosis. In the U.S.A, Australia and in most of the EU countries the disease has been eradicated after large investments and many cycles of culling and vaccinations. As discussed

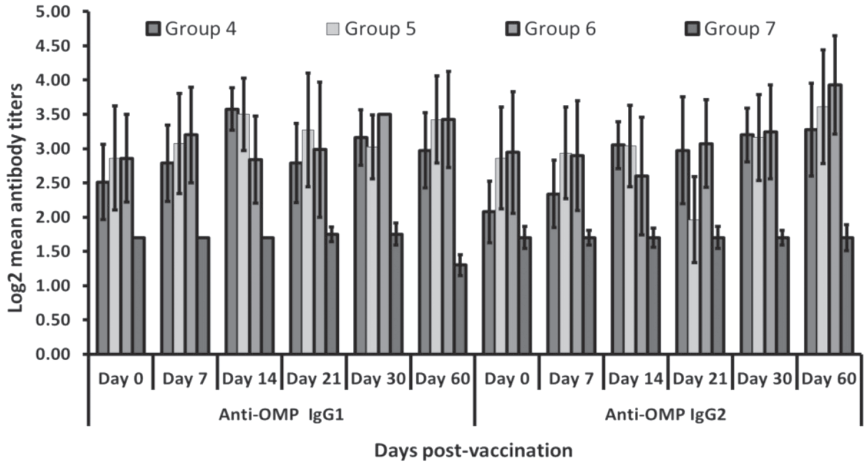

Fig.4. Anti OMP IgG1 and IgG2 response by ELISA: Farm-II, Group 4, 5, 6, and 7.

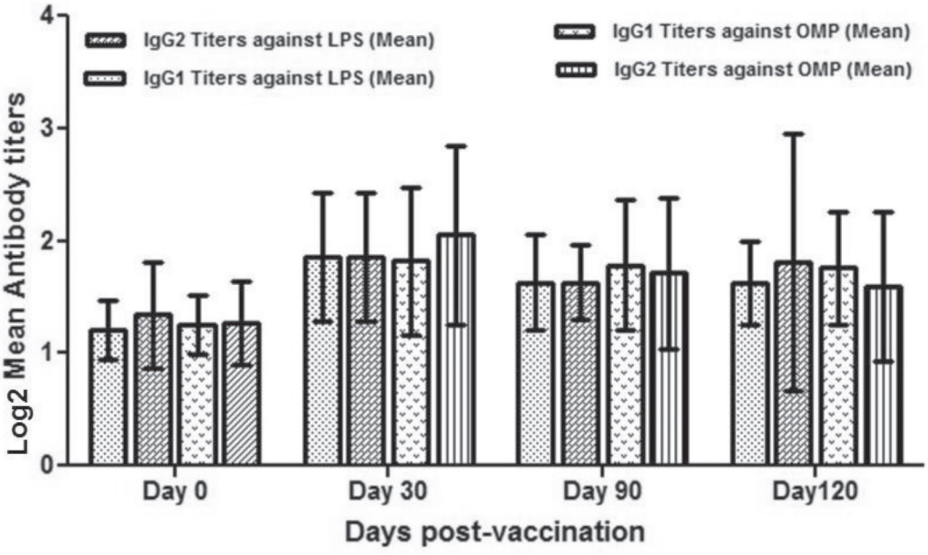

Fig. 5: Anti LPS, anti OMP IgG1 and IgG2 response by ELISA: Farm-III-Phase-2, Group 9.

Table 3. Status of brucellosis by isolation of *Brucella* by culture: Farm-III-Phase-1, Group 8.

Farm III	Animal ID Group 8	Status of animal before vaccination			Type of vaccine	Date of vaccination	*Brucella* isolation status post vaccination (days)					
		Isolation	Serology	qPCR			0	7	14	21	30	60
1	8a	P	P	P	S19GC 50ug	5.09.12	P	P	N	N	N	N
2	8b	P	P	P			P	P	N	N	N	P
3	8c	N	P	P			N	N	N	N	N	N
4	8d	N	P	P			N	N	N	N	N	P
5	8e	N	P	P			N	N	N	N	N	N
6	8f	N	P	P			N	N	N	N	N	N
Farm III	**Group 8**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**90**	**105**	**120**	**150**	**210**	**250**
1	8a	P	P	P	S19GC 50ug	5.09.12	N	N	N	N	N	N
2	8b	P	P	P			P	N	N	P	P	P
3	8c	N	P	P			N	N	N	N	N	N
4	8d	N	P	P			P	N	N	N	N	N
5	8e	N	P	P			N	N	N	N	N	N
6	8f	N	P	P			N	N	N	N	N	N

Table 4. Status of brucellosis by isolation of *Brucella* by culture: Farm-III-Phase-2, Group 9.

Farm III	Animal ID Group 9	Status of animal before vaccination			Type of vaccine	Date of vaccination	*Brucella* isolation status post vaccination (days)					
		Isolation	Serology	qPCR			0	14	30	60	90	120
1	9a	N	N	P	S19GC 100µg	4.06.13	N	N	N	N	N	N
2	9b	N	N	N			N	N	N	N	N	N
3	9c	N	N	P			N	N	N	N	N	N
4	9d	N	N	N			N	N	N	N	N	N
5	9e	N	N	N			N	N	N	N	N	N
6	9f	N	N	N			N	N	N	N	N	N
7	9g	N	N	N			N	N	N	N	N	N
8	9h	N	N	N			N	N	N	N	N	N
9	9i	N	N	N			N	N	N	N	N	N

the present vaccines (S19 and RB51) do not provide 100% protection and are virulent to the animals and humans. Also these vaccines cannot be given to all the age groups and genders. Hence development of subunit vaccines is an attractive alternate approach to immunization since these are expected not to have a set of drawbacks as mentioned above.

It has been shown earlier that immunization with LPS from *B. melitensis* non-covalently linked

to *Neisseria meningitidis* OMP was able to offer protection in mice (17). It has been also shown that S19GC vaccine was able to protect mice against the virulent challenge with *B. abortus* strain 544 and cure splenic infection in 91% mice (19). In addition, the S19GC has been also shown to elicit appropriate and specific antibody and cell mediated immune response in cattle calves immunized by subcutaneous route (18). Recently, Cherwonogrodsky *et al*., 2014 (25) has shown that mice immunized sub-cutaneously with a GC formulation using 90% of polysaccharides (PS) and 1-3% of protein from *B.suis* 145 strain are protected upon challenge with virulent strains of *B. abortus*, *B. melitensis* and *B.suis*.

GC vaccine derived from *B. abortus* S19 (S19GC) was tested in cattle for assessing protective efficacy and therapeutic potential. The assessment of S19GC to elicit appropriate immune response compared to a live *B.abortus* S19 vaccine was studied in a brucellosis free farm (Farm-I) by IFN-γ response employing ELISPOT assay. The 50 µg sub-cutaneous dose of S19GC vaccine was found safe for cattle. The ELISPOT assays demonstrated the ability of S19GC to elicit specific IFN-γ response after stimulation with S19 OMP that indicated a Th1 mediated immune response event. The cattle immunized with S19GC differed significantly from controls and at different time points of observation during the pre-booster stage. A similar IFN-γ response has been shown in cattle calves in whole blood stimulation assays (18). The IFN-γ response plays a significant role in killing of *Brucella* by activation of the macrophages (26). Protective immunity to the intracellular pathogen *Brucella* is mostly cell mediated.

The ability of S19GC to stop shedding was assessed in a brucellosis infected farm (Farm-II) maintained as a closed free mixing herd, in 4 groups of animals (Group 4, 5, 6 and 7) at weekly interval till 60 days post immunization. During the pre-vaccine stage 48.3% (15/31) of the animals screened from the four groups were positive for brucellosis. Following immunization shedding of *Brucella* was arrested in cattle from Group 6 beginning 7 DPV till the end of the study period (60 DPV) as animals remained negative by culture; and the qPCR indicated a 2 log fold reduction in the genome copy number. Immunization of animals in Groups 4 and 5 with S19GC prevented them from acquiring fresh infection by natural challenge up to 60 DPV. However 2 out of 7 animals from the brucellosis free unvaccinated controls (Group 7) continued shedding *Brucella* till 60 DPV. The nasal and vaginal secretions collected from these animals on different days during the period of the study (0, 7, 14, 21, 30, 60 DPV) showed progressive increase in the number of bacteria as estimated by qPCR as illustrated in the Figure 2. Overall they showed a 1 to 3 log folds of increase in the genome copy number. The antibody isotype IgG1 and IgG2 titers against LPS and OMP were significantly different when compared to pre-vaccination status in vaccinated group of animals (Group 4, 5 and 6). Similar response has been observed in cattle calves by Mythili *et al*., 2010 (18), and in mice after immunization with subunit vaccines of *B. melitensis* (17). The antibody isotype subtypes (IgG1 and IgG2) response reported in the current study indicated a Th1 as well as Th2 type of response (27) induced by the S19GC vaccine.

The effect of booster and dose of S19GC in cattle was studied in two phases, in another farm (Farm-III) infected with brucellosis, in two groups of cattle (Group 8 and 9). At pre-immunization 53.33% (9/15) animals included in the study had brucellosis as determined by isolation/serology/qPCR. The maintenance of the herd was closed and free-mixing. The shedding in 66.67% (4/6) cows from Group 8, vaccinated sub-cutaneously with 50 µg dose at Day 0 and 90 DPV, was temporarily arrested for 53 days (from 7 to 60 DPV). The effect of booster was clearly observed since only 1/6 animals resumed shedding 60 days post booster, and in the remaining cows (83.34%) shedding was absent till the end of the observation period (250 DPV). Further, the *Brucella* genome was undetectable in nasal/ vaginal secretions from 7 to 150 DPV, but not thereafter, suggesting that the

immunization regimen adopted was successful in arresting the shedding transiently in an endemic situation. Finally, 9 cows in Group 9 from Farm-III, who were in contact with animals of Group 8, from which 2 were positive by qPCR, upon immunization with 100 µg S19GC by subcutaneous route without booster showed complete stoppage of shedding as evidenced by culture and qPCR till the end of study (120 DPV). The antibody isotype ELISA showed a significant difference of IgG1 and IgG2 response against LPS and OMP components of S19GC between pre-immunization and 30DPV.

The overall results indicated that 100µg dose of S19GC vaccine when administered subcutaneously to adult female cattle is able to induce therapeutic as well as prophylactic effect up to 120 DPV. The vaccine was found to be safe and could trigger both humoral and cell mediated immune response. The involvement of Th1 and Th2 cells was marked by pronounced antibody response which in turn was able to stop shedding in case of infected animals and prevented brucellosis free animals from acquiring fresh infection from in contact animals carrying *Brucella* infection.

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Stacking of Host-induced Gene Silencing Mediated Resistance to Banana Bunchy Top Virus and Fusarium Wilt disease in Transgenic Banana Plants

Siddhesh B Ghag[§], Upendra K S Shekhawat[§], Ashok B Hadapad and Thumballi R Ganapathi*

Nuclear Agriculture & Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400 085, India.
[§]Contributed equally to this work
*For Correspondence - trgana@barc.gov.in

Abstract

Pest and diseases are the main cause of loss in productivity of major food security crops. The objective to increase food production feeding the increasing world population, at most times is seriously compromised by these biotic constraints. Introducing resistant traits in plants against multiple pathogens is the need of the hour. New methods such as RNA interference can be used to develop efficient resistance against several deleterious phytopathogens. RNA interference has previously been applied to control banana bunchy top virus (BBTV) and Fusarium wilt disease in banana. In this study, we introduced both these ihpRNA (intron hairpin RNA) constructs in the single transgenic banana plant by co-transformation so as to stack resistance to both the diseases. Thirty eight transgenic lines were regenerated, out of which 5 lines were found to be co-transformed. To test for resistance to BBTV and Fusarium wilt disease, the four out of these five co-transformed lines were challenged with viruliferous aphids and Fusarium mass culture. In these bioassays, two transgenic lines (VR19 and VR20) showed high level of resistance to BBTV and Fusarium wilt disease. The two resistant lines expressing both the silencing cassettes were characterized at molecular level by PCR using respective T-DNA specific primers. Further, based on these results, we propose that generation of transgenic plants transformed with multiple silencing constructs targeted towards vital pathogen genes could turn out be most appropriate approach to develop effective resistance against a broad array of viral and fungal pathogens.

Keywords : Banana; co-transformation; intron-hairpin-RNA; banana bunchy top disease; Fusarium wilt

Introduction

Agricultural production of the important food security crops is under constant threat of damaging pests lowering the yield substantially (1). An estimated 50% of the crop productivity is lost due to destructive insects and pests (2). Banana is the fourth most important food crop after rice, wheat and maize and it is grown in over 120 countries. Banana is affected by several diseases caused by fungal, viral and bacterial pathogens that result in huge losses globally. Fungal pathogens like *Fusarium oxysporum* f. sp. *cubense* (Foc) and *Mycosphaerella* species and viral pathogens such as banana bunchy top virus are the most destructive banana pathogens and also especially difficult to control via conventional methods (3). Banana bunchy top disease (BBTD) is caused by the member of the Nanoviridae group, banana bunchy top virus (BBTV). Typical symptoms include 'bunchy top' appearance of the infected plant wherein the

plant top becomes choked with a rosette of narrow, short, erect and brittle leaves (4). BBTV is transmitted by banana aphid, *Pentalonia nigronervosa* Coquerel that can feed on all forms of vegetative planting material like suckers, corms and tissue cultured plants (5). Another disease of banana that is of great concern is the Fusarium wilt disease (also know as Panama wilt). Panama wilt caused enormous destruction of the banana plantations in prime banana growing regions of the world in the nineteenth century. Foc, a soil pathogen enters the healthy banana plant via roots and thrives in the corm tissue of the infected plant. The fungus grows further by overcoming the plant defenses and plugs the xylem vessels thereby causing wilting of the plant. The infected plant shows progressive yellowing of the leaves, discoloration of the pseudostem and finally wilts (6). There are four races identified as race1-4 that infect different banana genotypes of which the race 1 and race 4 are the most significant on a global perspective. Race 1 of Foc infects the Silk and Pome varieties, whereas race 4 (tropical race 4, TR4) infects all the varieties of banana including the race 1 resistant Cavendish varieties which dominate the world market. There are no known effective control methods available to manage this dreadful disease of banana.

Both the diseases of banana *viz*. Fusarium wilt and BBTD can reduce the yield from a banana plantation by 50 - 100% which can have significant impact on the economic and social conditions of small and marginal farmers in developing countries especially where this crop also serves as a cash crop (7, 8). Development of resistant cultivars is an alternative strategy to manage these diseases. Banana breeding is complicated due the triploid nature of the elite edible cultivars and unavailability of natural resistance in the available gene pool. Thus, stacking of novel genes imparting resistance to multiple pathogens is considered as the best approach to effectively control several deleterious plant diseases in a single line (9-13). In the last couple of years, RNA interference, mediated by small interfering RNA (siRNA), has been adopted as the strategy of choice for control of several plant pathogens (14). Several proof-of-principle studies have lately been published which demonstrated development of disease resistance in plants by achieving silencing of important pathogen genes (15-18, 5). This concept known as "host induced gene silencing" (HIGS) is based on the recently unearthed evidence that there is a free flow exchange of RNA between the host and the pathogen in early stages of disease development (19). We have conducted two such studies wherein host coded hairpin RNA expression cassettes were used *in planta* to generate siRNAs specifically targeted towards vital pathogen genes thereby leading to inhibition of pathogen growth in case of simulated infection (bioassay). In one of these studies, the transgenic banana plants expressing the intron hairpin RNA (ihpRNA) construct designed to silence the viral master replication initiation protein (Rep) (20) showed resistant to BBTV infection more than 6 months post-inoculation with viruliferous aphid (5). In another study, transgenic banana plants expressing ihpRNA construct targeted against the velvet protein gene of Foc imparted high level of resistance to Fusarium wilt disease of banana (18). Velvet protein (21) used in the above study was a suitable candidate for silencing because it is considered to be one of the most important general pathogenecity proteins involved in all aspects of pathogenesis and disease development (22).

In the present study, an attempt has been made to introduce T-DNAs from both these constructs (ihpRNA-Rep and ihpRNA-VEL) in a single banana plant by co-transformation to impart effective resistance against both BBTV and Fusarium wilt disease (Fig. 1). Embryogenic cell suspension cultures of banana were co-transformed with two ihpRNA constructs targeted against viral *Rep* gene and Foc velvet protein gene. Further, transgenic banana plants having both the T-DNAs were identified and subjected to BBTV and Foc bioassays.

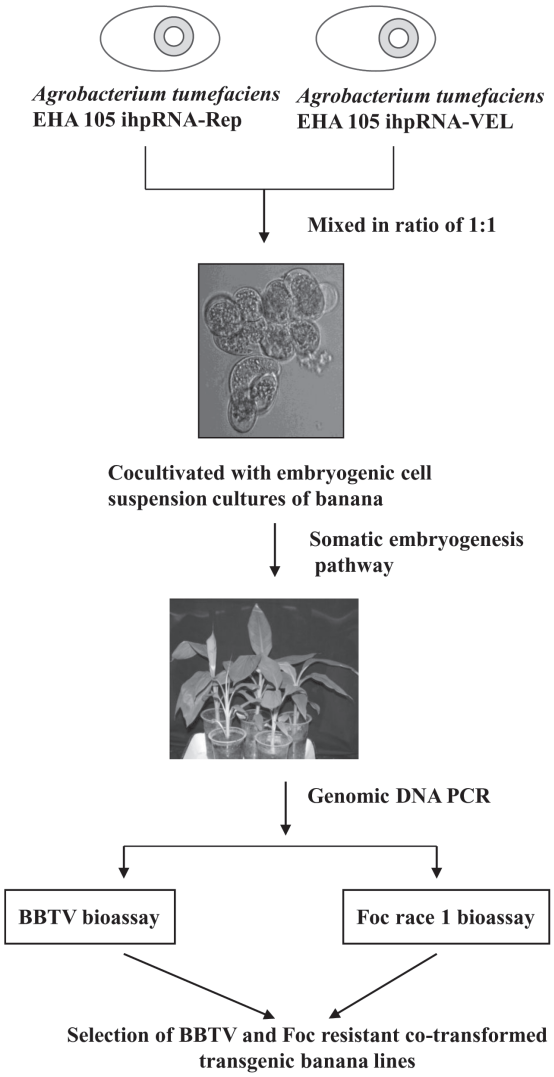

Fig. 1. Schematic representation of generation of co-transformed multiple disease resistant transgenic banana plants.

Materials and methods

Plant material and binary vectors : Previously established embryogenic cell suspension (ECS) cultures of banana cv. *Rasthali* were used for transformation experiments. This ECS culture was initiated from the shoot-tip cultures of cv. *Rasthali* as described before (23). The ECS cultures were subcultured after every eight-days using fresh medium and sieved through an 85µm sieve before being used for *Agrobacterium*-mediated genetic transformation. ihpRNA-Rep (5) and ihpRNA-VEL (18) binary vector constructs were mobilized separately into *Agrobacterium tumefaciens* EHA 105 by electroporation.

Co-transformation : A single *Agrobacterium* colony of each construct (ihpRNA-Rep and ihpRNA-VEL) was inoculated in liquid medium containing 0.75% w/v yeast extract and 0.8% w/v nutrient broth supplemented with 50 mgL^{-1} kanamycin. The bacterial cultures were incubated overnight at 27ºC with an orbital shaking of 180 rpm. Both the overnight grown *Agrobacterium* cultures were resuspended in the same liquid medium supplemented with kanamycin and grown for further 4-5 hours under the same conditions until an OD_{600} nm of ~ 0.6-0.8 was reached. The *Agrobacterium* suspension cultures were centrifuged at 6,500 *g* for 10 minutes and resuspended in M2 medium (24) added with 100 mM acetosyringone (ACS) as described previously (25). Further, the two *Agrobacterium* cultures were mixed in the ratio of 1:1 and vortexed thoroughly to obtain a uniform suspension culture. The *Agrobacterium* suspension culture so obtained was used for cocultivation with banana ECS cultures. The cocultivated cells were aspirated onto sterile glass filter discs and were then transferred onto semi-solid M2 medium supplemented with 100 mM ACS as described previously (23). The plates containing the cocultivated cells were incubated in dark for three days at 23±2 ºC followed by transfer on same medium supplemented with cefotaxime (400 mgL^{-1}). Further, the cells were transferred onto embryo induction medium supplemented with cefotaxime (400 mgL^{-1}) and hygromycin (5 mgL^{-1}). The embryos growing on the selection medium were subcultured for three rounds after every three-week. The embryos were germinated and shoots developed were multiplied to obtain clonal copies of the same transgenic line followed by rooting and hardening in green house as described previously (25).

Genomic DNA PCR : The transgenic banana plants regenerated on hygromycin selection medium were sorted out based on preliminary GUS staining results and further analyzed using genomic DNA PCR using primers designed to amplify hygromycin phosphotrasferase (*hpt*) gene to check for the presence of at least one T-DNA in the banana genome. Genomic DNA was isolated from young banana leaves using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). Subsequently, another PCR set was carried out using primers specific for *VSP* (soybean vegetative storage protein) 3' UTR and *nos* (nopaline synthase) 3' UTR (Supplementary table S1) of the ihpRNA-Rep and ihpRNA-VEL constructs respectively to identify the co-transformed transgenic lines.

BBTV bioassay : The BBTV infected banana plants were maintained under contained controlled conditions. The aphid colonies obtained from the banana growing fields in India were reared in a controlled growth chamber at 25 ± 0.5°C and 12 h light/dark photoperiod. Mature aphids were fed on BBTV infected banana leaves for 24 hours and then transferred onto the transformed banana plants *in vitro* at 25 ± 0.5°C and 12 h light/dark photoperiod (5). After the inoculation-access periods the *in vitro* maintained plants were transferred to soil and subsequently all plants were sprayed with insecticide confidor (Bayer Crop Science). All the inoculated banana plantlets were grown in an insect-proof growth chamber maintained at 25 ± 0.5°C and 12 h light/dark photoperiod. The BBTD symptoms were monitored after 3 and 6 months from inoculation of viruliferous aphids.

Foc bioassay : Foc race 1 was isolated from the infected corm tissue of banana obtained from the banana growing regions of India. This Foc race 1 culture was grown in potato dextrose broth (PDB) for 5 days at 30ºC with an orbital shaking of 160-180 rpm. After 5-days, the spores were separated using cheesecloth and spore count was adjusted to 8 X 10^5 ml^{-1} using haemocytometry. The spores were inoculated in an autoclaved mixture of sand and maize bran in the ratio of 19:1 and incubated at room temperature for 4-weeks (25). Green-house hardened transgenic and untransformed control banana plants were replanted in the mixture of soil and mass culture (1:1). The plants were screened for Fusarium wilt symptoms six-week post-infection (18). For untransformed controls and each transgenic line, a minimum of four replicates were screened for Foc infection twice and representative plants were photographed.

Results

Regeneration of co-transformed banana plants : Embryogenic cell suspension cultures of banana cv. *Rasthali* co-cultivated with 1:1 mixture of two different *Agrobacterium* cultures harboring binary vectors ihpRNA-Rep and ihpRNA-VEL developed into whitish embryos on embryo induction medium supplemented with the antibiotic hygromycin at a concentration of 5 mgL^{-1} (Fig. 2a). The untransformed cells necrosed in presence of the antibiotic. After repeated subculture on the embryo induction medium added with antibiotic, the putatively transformed embryos matured and subsequently germinated on the medium containing 0.5 mgL^{-1} 6-benzylaminopurine (BAP). The shoots so obtained were transferred to shoot multiplication medium containing BAP (2 mgL^{-1}) to get multiple copies of the same transformation event (Fig. 2b). Each shoot was separately rooted in the 1-naphthaleneacetic acid [NAA] based medium for 4-weeks (Fig. 2c) and used for inoculation-access of BBTV followed by hardening in green house under controlled conditions or the rooted plants were first hardened in green house for 2 months (Fig. 2d) followed by challenging them with Foc mass culture. Totally, 38 independent transformed lines were regenerated from the co-transformation experiment. All the transformed plants obtained looked phenotypically similar to the untransformed control plants. The transgenic character was determined by GUS assay and PCR amplification. Out of 38 putatively transformed lines, 36 lines tested positive for GUS assay but all the 38 lines were PCR positive.

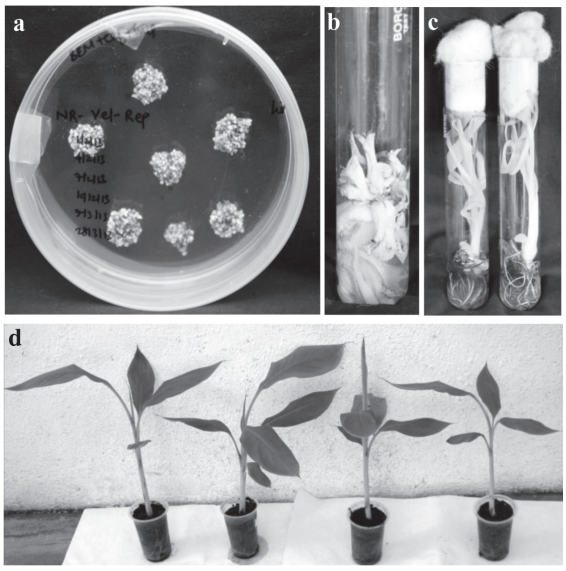

Fig. 2/ Generation of transformed banana plants. Embryogenic cell suspension cultures of banana cv. *Rasthali* were co-cultivated with 1:1 ratio of *Agrobacterium* cultures harboring ihpRNA-Rep and ihpRNA-VEL binary vectors. The putative transformed cells developed into embryos on the hygromycin supplemented medium (a). The plantlets developed by somatic embryogenesis pathway were multiplied in medium containing BAP (b). The shoots were separated and rooted in NAA based medium (c) and hardened in green house for 2 months (d).

A single band of 788 bp matching the coding sequence of *hpt* gene was amplified in all the transgenic lines whereas it is absent in the untransformed controls.

To select the co-transformed plants, *hpt* PCR positive lines were tested for second round of PCR using specific primers to detect the presence of both the T-DNAs in the transgenic lines. The PCR products checked on 1% w/v TAE-agarose gels showed the presence of 497 bp and 316 bp bands corresponding to the *VSP* 3' UTR and *nos* 3' UTR of the ihpRNA-Rep (Fig. 3a) and ihpRNA-VEL (Fig. 3b) constructs respectively. Out of the 38 PCR positive lines, 5 lines showed presence of both the T-DNAs in the transgenic banana plants. Among these, four co-transformed lines (VR18, VR19, VR20 and VR23) were selected for bioassay studies.

Enhanced resistance to BBTV infection : The selected co-transformed transgenic rooted banana plants possessing both the T-DNAs were each inoculated with 10 viruliferous aphids after 24 hours of acquisition-access to BBTV infected banana leaf. Untransformed control plants at the same growth stage were also inoculated with the viruliferous aphids in similar manner. These plants were then maintained in contained greenhouse conditions and observed for BBTD symptoms. After 3 months of BBTV assay, the untransformed control plants showed BBTV infection symptoms such as stunted growth, short narrow leaves and rosette pattern whereas none of the 4 co-transformed lines (VR18, VR19, VR20 and VR23) showed BBTD symptoms 3-months post-inoculation (Fig. 4a). The plants were maintained further under same conditions for 6 months without any visible BBTD symptoms indicating that the ihpRNA-Rep construct was effectively transcribed and processed into respective siRNAs that imparted resistance to BBTV and these did not have any deleterious effects in the transgenic plants.

Improved resistance to Fusarium wilt disease : Four selected co-transformed lines were subjected to Foc bioassay following the acclimatization of plants in green-house for 2-months. The untransformed control plants showed typical Fusarium wilt disease symptom development 4-weeks post-infection. There was yellowing of the older leaves, cracking and discoloration of the pseudostem. Out of the 4 co-transformed lines, VR18 behaved similar to the untransformed control wherein there was yellowing of the leaves followed by discoloration and cracking of pseudostem and eventually wilting. VR20 and VR23 showed mild to moderate symptoms but no wilting. Line VR19 was healthy and did not show any symptoms of Fusarium wilt disease (Fig. 4b). VR19 and VR20 co-transformed lines were further maintained for 6-

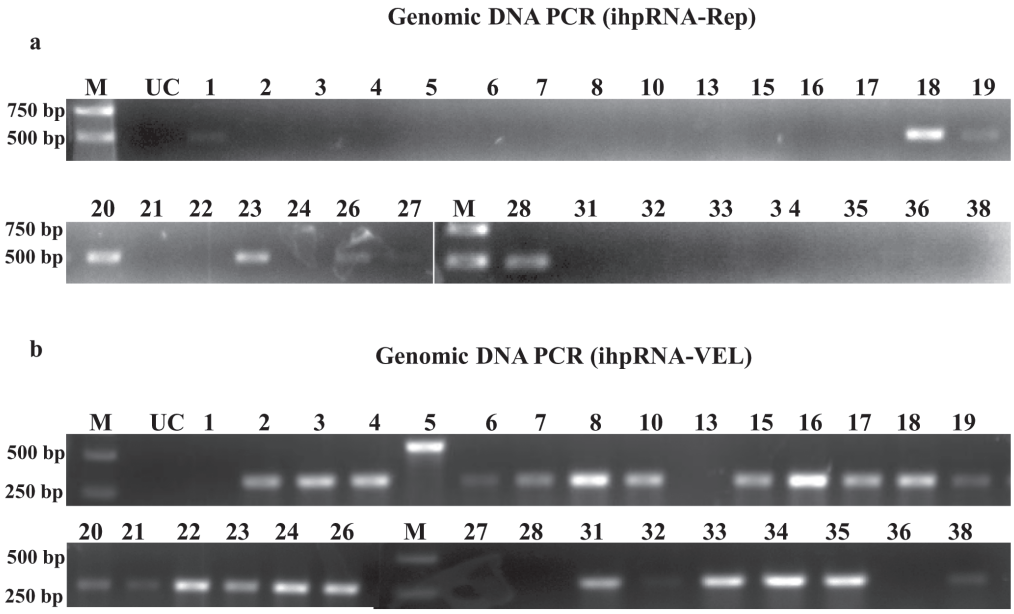

Fig. 3. Genomic DNA PCR for determination of transgenic nature and co-transformation event. All the 38 lines regenerated were tested for the co-transformation event by performing genomic DNA PCR using primers specific for the T-DNA regions of each ihpRNA-Rep (a) and ihpRNA-VEL (b) constructs. The PCR products obtained using genomic DNA from the transgenic lines (VR1-38) were checked on 1% TAE-agarose gel along with the 1 kb DNA marker (M).

Table S1. Primers used in the present study

PCR Reaction		Primer Sequence (5'-3')
Amplification of *hygromycin phosphotransferase* gene	Fw Rv	GTCCTGCGGGTAAATAGCTG ATTTGTGTACGCCCGACAGT
Amplification of soybean *VSP* 3' UTR	Fw Rv	GCACTATTCAAATAGGAGCATTAGC CTTCAAGACGTGCTCAAATCACTAT
Amplification of *nos* 3' UTR (reverse primer binds in vector backbone)	Fw Rv	CTACCGAGCTCGAATTTCCCCGATCGTT TTTCACACAGGAAACAGCTATGA

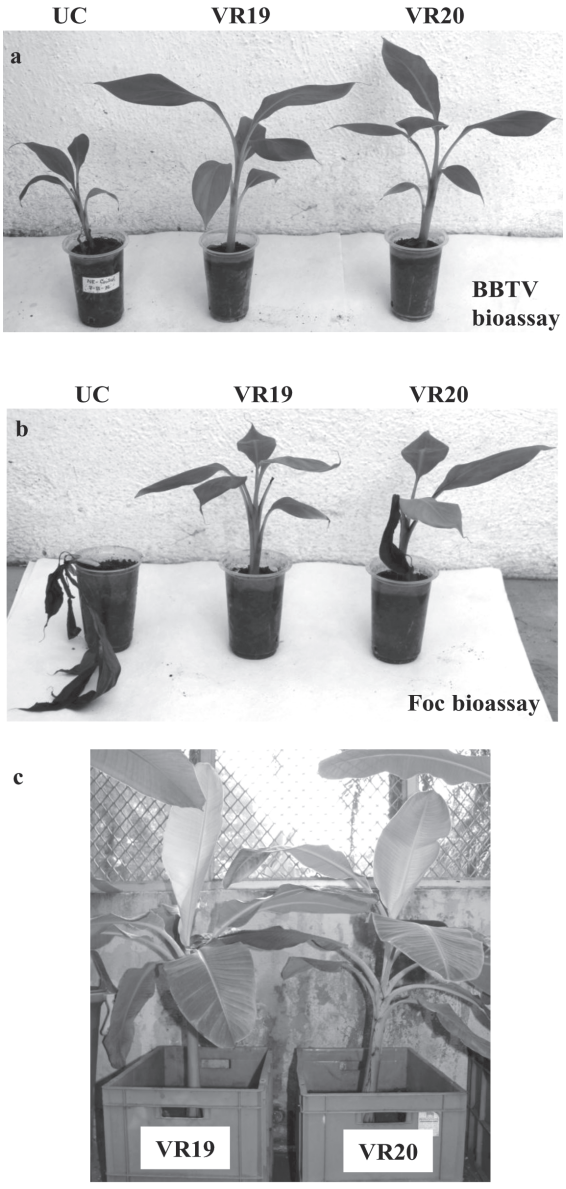

Fig. 4 BBTV and Foc bioassay. Four co-transformed lines were subjected to BBTV and fungal bioassay wherein all the four lines displayed BBTV resistance whereas two lines out of four showed resistance to Fusarium wilt. The representative photograph of the two lines (VR-19 and VR20) displaying resistance to BBTV (a) and Fusarium wilt (b) are shown along with the untransformed control (UC). VR19 and VR20 transgenic banana lines showing no wilt symptoms 6 months post infection (c).

months to observe for any Fusarium wilt disease symptoms. These plants remained free of any Fusarium wilt symptom 6-months post-infection indicating that ihpRNA-VEL overexpressing lines effectively controlled Foc infection (Fig. 4c).

Discussion

Multigene manipulation is an important tool to incorporate two or more important agronomic traits in plants (26). Transgenic approaches for developing crops resistant to a particular phytopathogen have already proved effective in several crops against wide array of pathogens (27). Today, the emphasis is towards developing transgenic crops with multiple qualitative traits so as to enable the plant to resist several pathogens together. In the present study, transgenic banana plants were developed by introducing resistance to two important diseases of banana, BBTD and Fusarium wilt disease by co-transforming these plants with two different ihpRNA constructs to silence the expression of BBTV *Rep* gene and Foc velvet protein gene.

Banana is an important food security crop and it is grown as monocultures throughout the world (3). As a result, the plantations are always under the threat of a disease epidemic. Banana production is severely affected by two economically important diseases namely BBTD and Fusarium wilt disease (28). Management of these diseases is warranted as both these diseases spread easily and there are no known natural resistant edible cultivars. Genetic resistance is the most effective and sustainable method for controlling these diseases. Genetic transformation of elite edible banana varieties has gained importance due to difficult breeding strategies and involvement of huge expenditure (29). In this study, two different T-DNAs containing the silencing cassettes targeted against the BBTV *Rep* gene and Foc velvet protein gene were introduced in the super-virulent strain of *Agrobacterium tumefaciens*. The *Agrobacterium* cultures harboring the respective T-DNAs were mixed in the ratio of 1:1 so as to obtain co-transformed banana plants. Banana

being a monocot, *Agrobacterium*-mediated genetic transformation is a difficult task even in case of single gene transformations. In our co-transformation experiments, we were able to regenerate four co-transformed lines out of the total of 38 lines. The four co-transformed lines were subjected to BBTV and Foc bioassay under greenhouse conditions. The *in vitro* developed plantlets were inoculated with the active aphid cultures which were previously allowed to feed on the BBTV infected banana leaf. The control plants started showing BBTV symptoms such as stunted growth, shortening and narrowing of the leaf lamina and choking of the leaves on the top 4-weeks post-inoculation. Even after the period of 3-months all the four co-transformed lines (VR18, VR19, VR20 and VR23) did not show any symptoms of BBTV infection indicating that the transgenic banana plants were able to suppress the expression of BBTV *Rep* gene which resulted in effective resistance.

In another set of experiments, the co-transformed plants were challenged with Foc mass culture and observed for disease development. Six-week post-infection, VR19 line was healthy and showed no signs of Foc infection. The other plants showed some level of Foc disease symptom development. The two best performing co-transformed lines (VR19 and VR20) obtained from the Foc bioassay studies were maintained under greenhouse conditions for further 6-months and they did not show any BBTV or Fusarium wilt symptoms. These two lines (VR19 and VR20) effectively resisted both the pathogens because they completely silenced the BBTV *Rep* gene and Foc velvet protein genes at the transcriptional level. Further, even though we used two T-DNAs having same marker and reporter cassettes, we did not observe any growth deformities in the regenerated transgenic banana plants.

Conclusions

Stacking of genes in economically important crops vulnerable to serious pathogens offers potential alternative for imparting resistance to multiple pathogens in the single crop plant. In this study, we extended gene pyramiding strategy in combination with RNA interference technology to curb the two most important diseases of banana. The plant showed resistance to both BBTV and Fusarium wilt disease when challenged under greenhouse conditions. These plants also resisted infection symptoms for 6-months after which the experiment was terminated. Thus, in this study we revalidated the potential of using genes and sequences from target pathogens for imparting effective resistance against multiple pathogens in a single transgenic line by simply co-transforming suitable binary vectors.

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Conflict of interest : The authors declare that they have no conflict of interest

Supplementary material : Supplementary table S1: Primers used in the present study

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Phenotypic and Genotypic Characterization of Indigenous Lactobacillus Species from Diverse Niches of India

Kiran Thakur[1*], Narendra Kumar[1], Dhiraj Kumar Nanda[1], and Sudhir Kumar Tomar[1]
National Collection of Dairy Cultures, [1]Dairy Microbiology Division, National Dairy Research Institute, Karnal,Haryana,India-132001
*For Correspondence - sudhirndri@gmail.com

Abstract

Lactobacilli are well reckoned for their technological and probiotic significance and their exercise in dairy products for trade and industry. The present study was carried out to isolate and characterize Lactobacilli from various dairy and non dairy sources of Indian origin. The isolates were subjected to phenotypic and genotypic characterizations followed by partial 16S rDNAsequencing . As many as 40 isolates were identified as Lactobacilli and among them 14 were identified as *L.plantarum*, 5 as *L. fermentum*, 5 as *L. delbrueckii subsp. bulgaricus*, 4 *L. brevis* , 2 *L. pentosus* and 1 isolate as *L. mucosae.* The great diversity was found with the place as well as source of collection.

Key Words: Lactobacilli, human feces, curd and bamboo shoots

Introduction

Belonging to the genus Lactic Acid Bacteria (LAB), lactobacilli are characterized by the formation of lactic acid as the main end product of carbohydrate metabolism and find their habitats in plants or material of plant origin, silage, fermented food as well as in the oral cavities, gastrointestinal tracts (GIT), and vaginas of humans and animals. Also Known as the power houses of food industry (1) they are the most vital groups of acid producing bacteria in the food industry for their use as starter cultures for dairy product sas well as they are gaining increasing attention in the area of probiotics due to their potential health benefits to consumer (2). Being the most dominant microflora of fecal samples, LAB may exist as commensal organisms, which are adapted to intestinal environment (3). Since the composition of the indigenous lactoflora varies from individual to individual, the study of new strains not only provides presumptive knowledge of beneficial microbiota from different parts of the country, but it would also leads to development of novel probiotic strains (4). The health attributes related to probiotics are known to be strain specific and are affected by the nutritional and environmental conditions of the host. In India, there is a large diversity in the food habits of the people. Among all the fermented foods consumed by ethnic people living in the sub-Himalayan regions of India, it is bamboo shoots which are consumed most (5,6). In southern part of India it is Dosa (a fermented crepe or pancake made from rice batter and black lentils) which is is widely popular. In the present study, we have targeted Lactobacillus species from dairy and non dairy sources for their phenotypic and genotypic characterization for their possible future applications as novel functional strains.

Materials and Methods

Cultivation Conditions : Human faeces , sour dough , *(dosa)* batter , fermented bamboo shoots and samples of household curd(dahi) were collected from diverse niches (as mentioned in Table 1) of India .The samples were enriched in MRS broth (Himedia Laboratories Pvt. Ltd, Mumbai, India) followed by streaking and pour plating on MRS agar under anaerobic conditions.

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Isolates were phenotypically assigned to the genus level on the basis of : cell morphology, Gram staining and catalase reaction.

Phenotypic and genotypic characterization : The putative lactobacilli isolates were further subjected to molecular typing methods. Genomic DNA from each isolate was prepared essentially following the method of Pospiech and Neumann (7). *Lactobacillus* genus-speciûc and species specific primer (Table.2) (8-12) were used for the conûrmation of putative Lactobacillus isolates. Polymerase chain reaction (PCR) was performed on an EppendorfMastercycler (Hamburg, Germany) and was veriûed by electrophoresis on 2% (w/v) agarose gel using a 100 bp ladder (Bangalore Genei, Bangalore,India) as a molecular weight marker. 16S rDNA gene of some representative isolates was ampliûed using primer 7F and primer S-G-Lab-0677-R (Table 2). The purified ampliûed PCR products (20 ìL) were sent for sequencing using primer 7F (12, 13) to obtain partial sequence of the 16S rDNA followed by analysis using the Chromas software (version 1.45, http:/ /www.technelysium. com.au/chromas.html). Basic Local Alignment Search Tool (BLAST) analysis was performed for similarity check with the already available database for species determination. The phylogenetic dendrogram was constructed by using unweighted pair-group method with arithmetic averages (UPGMA) of MEGA4.0 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Arizona State University, Phoenix, AZ, USA).

Results

Phenotypic and Genotypic characterization : Initially screened on the basis of microscopic examination and catalase test, 40 isolates appeared to belong to the *Lactobacillus* genus and were further characterized by biochemical and molecular methods. All these isolates conformed to the general phenotypic characteristics of genus Lactobacillus by gram staining methods. These isolates were observed to be invariably rod shaped cells (Figure 1) , gram-positive, catalase-negative, non motile, facultative anaerobic bacteria and were able to grow at 15°C, 2%, and 4% NaCl. The generic status was further conûrmed by PCR as all the isolates showed ampliûcation product of expected size (250 bp) using genus-speciûc primers (Fig. 2) (2.1). Based on the phenotypic characteristics , the isolates were tentatively designated as *L. plantarum*(14), *L. fermentum*(5), *L.delbrueckii subsp. Bulgaricus (5)*, *L. brevis* (4) , *L.pentosus* (2) and *L. mucosae* (1) (Figure 1). For species conûrmation species specific primers were used (Fig 2) (2.2, 2.3, 2.4, 2.5 & 2.6) . Further, partial sequencing of 16S rDNA was performed for representative isolates. The sequences obtained from the isolates were compared to those of reference strains held in GenBank. Sequence data was generated for selected isolates on the basis of their origin, probiotic and technological properties (data not shown) were submitted to the GenBank under the following Accession No.(Table 4). The phylogenetic analysis (Figure 3.0) together with

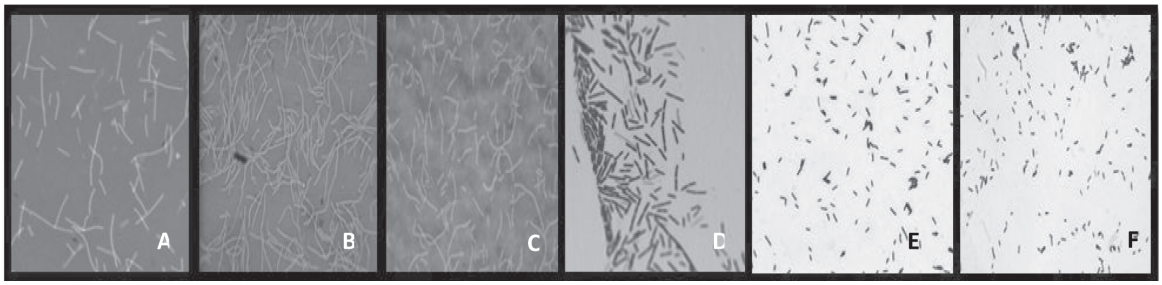

Fig.1. Negative Staining and Gram staining showing the rod shapes of lactobacilli :A. *L.acidophilus,* B. *L.del. bugaricus* C. *L. casei*, D. *L. plantarum ,* E. *L.fermentum* and F.*L. mucosae*

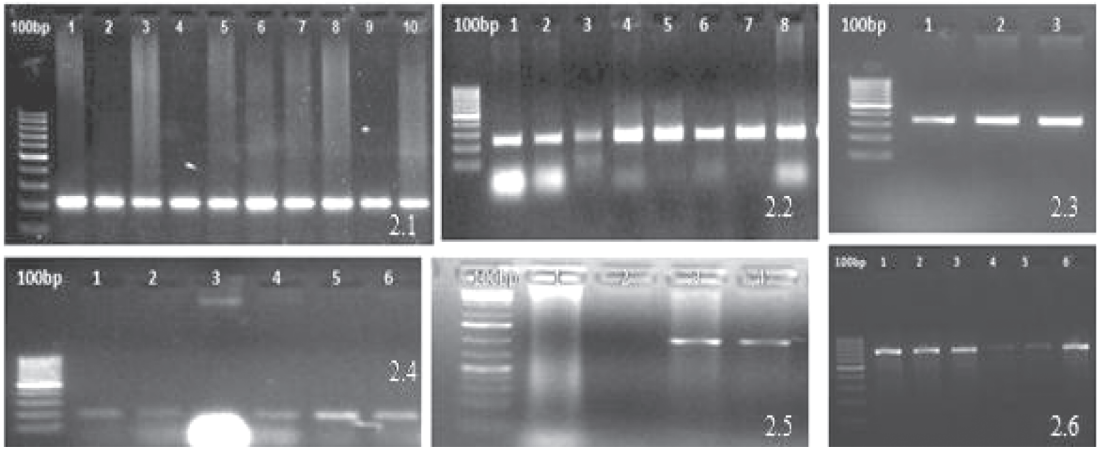

Fig.2 . Genus-speciûc PCR of Lactobacillus , 100 bp ladder, Lane 1-10 are Isolates, 2.2 Species specific PCR for *L. plantarum* (220bp) , 100 bp ladder , 2.3 Species specific PCR for *L. fermentum* (337), 2.4 Species specific PCR for *L.pentosus* (220bp), 2.5 Species specific PCR for *L.delbrueckii* (1065bp)1Kb ladder, 2.6 (7F, *S-G-Lab-0677-R* Isolates selected for 16s rDNA partial sequencing

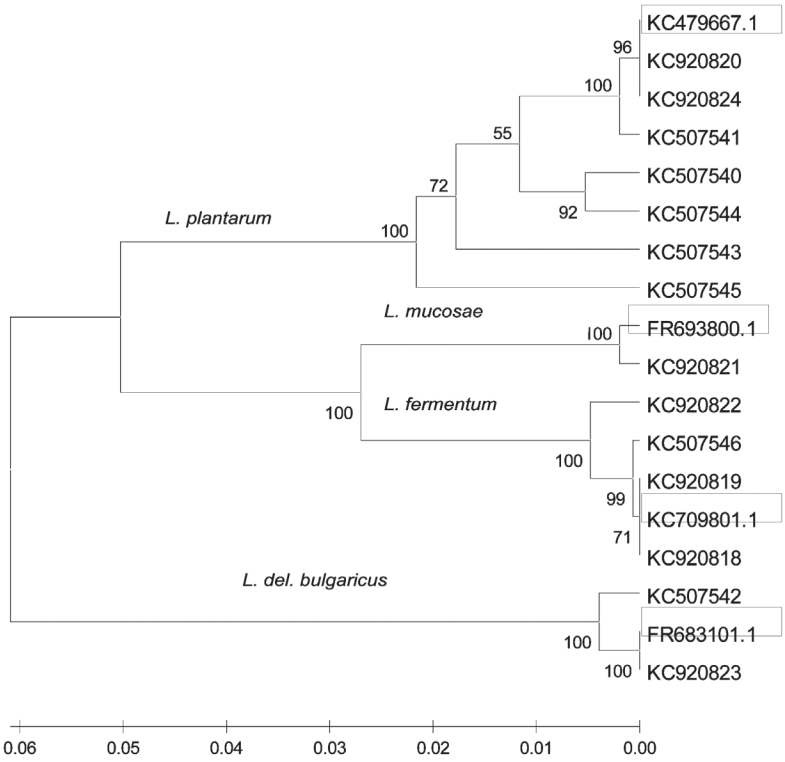

Fig. 3. Phylogenetic analysis of all isolates with reference strains of Lactobacillus using 16S r DNA sequences along with standard sequences obtained from database constructed using the UPGMA method by Mega 4.0 software.The boxed accession numbers are taken as a reference from NCBI genbank Database for similarity check.

Table 1. Collection of samples from different parts of India

1	Human faeces (30)	Haryana and Manipur
2	Sour dough(3)	Himachal Pradesh
3	FermentedBamboo shoots(5)	Manipur
4	Household curd(10)	Puducherry, Kerala
5	Dosa batter(3)	Delhi

Table.2 List of primers used in this study for Lactobacillus identiûcation and their properties

Target	Primer sequence 5 2 – 3 2	Annealing temp. (°C)	Product size (bp)	References
Lactobacillus genus	**CTCAAAACTAAACAAAGTTTC CTTGTACACACCGCCCGTCA**	**55**	**250**	(8)
L. plantarum	**GCTGGATCACCTCCTTTC ATGAGGTATTCAACTTATG**	**53**	**220**	(9)
L. fermentum	***AATACCGCATTACAACTTTG GGTTAAATACCGTCAACGTA***	***50***	***337***	(10)
L. pentosus	**GCTGGATCACCTCCTTTCGTA TTCAACTTATTAGAACG**	**53**	***220***	(9)
L. delbrueckii subsp. Bulgaricus	**AAGTCTGTCCTCTGG CTGGAAAA ATGAAGTTGTTTAAAGTAGGTA**	**58**	**1065**	(11)
16s rDNA (7F, S-G-Lab-0677-R)	***AGAGTTTGATCATGGCTCAG CACCGCTACACATGGAG***	***57***	**700**	(12,13)

species specific PCR profile has showed the diversity in *Lactobacillus* species with respect to their source of origin. Among all the isolates obtained from various sources, *L. plantarum* was the predominant flora.

Discussion

In this study, 40 isolates of Lactobacillus isolated from diversified niches were identified by a combination of conventional and molecular techniques. The great diversity has been found with respect to source of sample and within the sample. The microflora of curd from two different regions of india , Human feces (individual to individual), fermented bamboo shoots (house to house) varied significantly on the species level.*Lactobacillus* isolates were identified by PCR amplification and partial sequencing of 16S rDNA. According to genotypic features, *L. delbrueckii subsp. bulgaricus* and *L. brevis* was most dominant in the household curd from Puducherry, a place in South India. The isolates from curd possessedhomofermentative and heterofermentative characteristics as reported previously by different authors (14, 15). *L. pentosus* and *L. plantarum* were prevalent in Bamboo shoot and dosa batter. There are few reports on isolation of *L. plantarum, L. pentosus, L. brevis*and *L. fermentum,* from fermented bambooshoot products (16, 17). It was reported the isolation of lactobacilli from raw and fermented products like milk, curd, idli batter and pickle from Bangalore, South Indian region. According to earlier reports, occurance of *L.plantarum* as a predominant species in sourdough is in an agreement with our findings as shown in (Table 3) (18). The phylogenetic analysis has shown the close relationship

Table.3. Identiûcation of isolates from four different samples and their Genbank Accesion Numbers

Sr. No.	Source	Organism	Accesion no.
1	Human faeces	*L.fermentum*	KC920818
2	Human faeces	*L.fermentum*	KC920819
3	Bamboo shoot	*L. plantarum*	KC920820
4	Human faeces	*L.mucosae*	KC920821
5	Human faeces	*L.fermentum*	KC920822
6	Human faeces	*L. del.subsp.bulgaricus*	KC920823
7	Sourh dough	*L.plantraum*	KC920824
8	Human faeces	*L.plantraum*	KC507540
9	Bamboo shoot	*L.plantraum*	KC507541
10	Human faeces	*L. del.subsp.bulgaricus*	KC507542
11	Human faeces	*L.plantraum*	KC507543
12	Sourh dough	*L.plantraum*	KC507544
13	Dosa batter	*L.plantraum*	KC507545
14	Human faeces	*L.fermentum*	KC507546

between *L. mucosae* and *L. fermentum*. They can be regarded as closely related species on the basis of similarity index also reported by (19).There are few reports on analysis of composition of lactobacilli in human feces specifically in India. Studying complex environmental samples such as faeces, which generally contain different bacterial species is very interesting.Themicroflora varies in different stages of the host's life. Furthermore, this microflora is also influenced by geographical conditions (20). Among the species characterized from Human feces, *L. plantarum* was dominant species followed by *L. fermentum*. Later has been one of predominant lactobacilli in human intestine and vaginal tract as previously described (21).

Conclusions

This study would pave the way to analyze the composition of unexplored microbiota of Indian population as well dairy and plant sources. The 16s ribosomal DNA partial sequences have been submitted to NCBI. This study has culminated into identification and characterization of a number of indigenous Lactobacilli strains which can be further explored for functional and technological properties and can also be adopted for commercial food applications.

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Live micro-encapsulated *Brucella abortus* vaccine strains offer enhanced protection and sustained immune response in BALB/c mice

F. Mukherjee[1], A. Prasad[2**], L. Rajendra[3], V.S. Bahekar[1], B. Ramalakhsmi[4], K. Nagmani[4], S.K.Rana[1], G.K.Sharma[5] and V. A. Srinivasan[6*].**

[1]Research and Development Laboratory, National Dairy Development Board, Gachibowli, Hyderabad 500032 Telangana, India

[2]Department of Bio-Technology, Jawaharlal Nehru Technological University; Research and Development Centre, Indian Immunological Limited, Gachibowli, Hyderabad 500032, Telangana, India

[3]Plot No . 68. Flat No. 4A, Challa Pushpa Doyen Dacha Apartments, Shri Nagar Colony, Hyderabad 500073

[4]Research and Development Centre, Indian Immunological Limited, Gachibowli, Hyderabad 500032, Telangana, India

[5]National Dairy Development Board, Anand 388001, Gujarat, India

[6]National Dairy Development Board, 33, Telecom Nagar, Gachibowli, Hyderabad 500032, Telangana, India

*For Correspondence - srinivasanva1948@gmail.com

*** Authors contributed equally*

Abstract

Two sustained release vaccine delivery formulations, micro-encapsulated live *Brucella abortus* vaccine strains S19 and RB51 in alginate microspheres, were analyzed for entrapment efficacy and *in-vitro* release kinetic studies.

The level of protection offered to female BALB/c mice after sub-cutaneous (S/C) immunization with both encapsulated vaccine formulations 15 days after intra-peritoneal (I/P) challenge on 30th days post immunization (DPI) with wild type *B. abortus* 544 strain was significantly higher (P<0.01) compared to non-encapsulated live versions. In addition, the protection offered by the encapsulated RB51 formulation was superior (P<0.01) compared to encapsulated S19.The mean number of colony forming units (log_{10} CFU) persisting in spleen in all four experimental groups of immunized mice at 15 day post challenge (DPC) did not differ significantly.

Subtle differences in the antibody isotype and cytokine response pattern were observed during the pre and the post challenge stage in different groups of mice immunized with encapsulated S19, encapsulated RB51, non-encapsulated S19 and RB51. Compared to non-encapsulated version and saline inoculated controls, the enhanced protection exhibited by micro-encapsulated vaccines was reflected in significantly different (P <0.01) IgG1, IgG2b, IgG3 titers in mice immunized with encapsulated RB51, but not with encapsulated S19 at 15 DPC; however, encapsulated S19 immunized group showed significantly different (P<0.01) IgG2a titers. Although, both the mice groups immunized with encapsulated S19 and RB51 elicited significantly higher (P<0.01) IFN-γ response compared to S19 and RB51 non-encapsulates and controls at 15 DPC, the difference (P<0.05) in IL-2 response could be observed in encapsulated RB51 immunized group but not in encapsulated S19. The salient features of pre-challenge immune response in mice immunized with encapsulated S19 smooth strain were characterized by significantly elevated IgG1, IgG2a, IgG2b, IgG3 titers (P<0.05; P<0.05; P<0.01; P<0.01 respectively) at 30 DPI, also, IgG2a (P<0.05) and IgG2b (P<0.01) titers differed

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significantly as early as 7 DPI, accompanied by elevated IL-2 ($P<0.05$) at 21 DPI; while the encapsulated RB51 rough strain elicited enhanced IgG1 ($P<0.05$, $P<0.05$ and $P<0.01$) at 14,21 and 30 DPI and IgG2b ($P<0.01$) response at 30 DPI.

Overall comparison indicated that RB51 micro-encapsulated vaccine formulation is probably a potential candidate as it offered the best level of protection upon challenge and elicited most appropriate immune response.

Keywords: *Brucella abortus,* microencapsulation, vaccine, cytokines, antibody.

Introduction

Bovine brucellosis is mostly caused by a Gram negative intra-cellular pathogen *Brucella abortus* and is one of the major causes of infertility and abortion in cattle and buffaloes (1, 2). The disease causes significant economic loss to the dairy industry, and it has been estimated that the losses accounted for cattle and buffaloes in India was approximately US$ 3.25 billion (3, 4). The pathogen is excreted in milk, uterine, vaginal discharge and semen from infected animals, and humans can acquire infection by aerosol, direct contact through skin abrasions or consumption of unpasteurized milk (5, 6).

Bovine brucellosis is generally controlled by test and slaughter strategy or by mass calf-hood vaccination of females, restriction in the movement of animals and adoption of biosecurity measures (7). Two live attenuated *B. abortus* vaccines S19 and RB51 has been successfully used in mass immunization program in different countries for control of bovine brucellosis (8,9,10). However, the S19 vaccine produces residual virulence in some proportion of immunized animals, and excretes the vaccine strain in the environment, infecting humans (11). The efficacy of protection due to immunization with S19 vaccine varies from 65-70%, but the duration of protection beyond 7 years has not been documented (12,13). Vaccination using S19 strain interferes with standard diagnostic serological tests (14) since anti O-lipopolysaccharide (O-LPS) antibodies are detected in case of natural infection and also due to immunization. This interference is absent following immunization with RB51 vaccine since it lacks the O-LPS. However, RB51 is a rifampicin resistant strain, and exposure of humans to this strain from vaccinated animals or during vaccination has been cited; this observation is significant as it complicates therapeutic regimen with antibiotics in exposed humans (15). In order to address and improve the current limits of immunogenicity, duration and safety offered by the two *B. abortus* S19 and RB51 vaccines, sustained-release delivery vehicles carrying transposon and deletion marked attenuated mutants of *vbjR Brucella meltensis* (16) and *B. abortus* (17) has been used for testing the efficacy of these candidates in mice. These studies in mouse models had showed enhanced efficacy of immunogenicity, protection and safety. The present study was aimed at testing whether the protective and immunogenic efficacy of *Brucella abortus* S19 and RB51 vaccine strains cross linked by Poly-L-lysine and encapsulated in alginate microspheres were superior to non-encapsulated controls in a BALB/c mouse model.

Materials and Methods

Mice : 6 to 8 week old female BALB/c mice were obtained from Small Animal Testing (SAT) unit, Indian Immunologicals Limited, Hyderabad and acclimatized for 2 weeks before start of the experiment. All experimental procedure and animals care were done as per the guideline of Institutional Animal Ethical (IAEC) Committee (Approval No. IIL-R & D SA06/2010).

Bacterial Strain : Freeze dried vials of *Brucella abortus* strain S19 obtained from USDA and RB51 from Virginia Tech, USA were respectively grown on Potato Infusion Agar (BD, USA) and Tryptic Soya Agar slant (Difco,USA) with 5% serum and dextrose (20% w/v) with Rifampicin at a concentration of 20 µg/ml for 4-5 days .Bacteria were harvested from the surface into phosphate buffer saline (PBS) with pH 6.4 .The cultures were pelleted by centrifugation at 4000

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rpm for 20 minutes , re-suspended in PBS 6.4 and washed thrice before getting a final suspension .The viable count of organisms was determined by serial dilution plating method. The culture was finally re-suspended at a final concentration of 6.3 x 10^7 CFU / ml for S19 and 4.7 x 10^7 CFU / ml for RB51.

Preparation and characterization of B.abortus S19 and RB51 microsphere: Microspheres of *B.abortus* S19 and RB51 loaded with 6.3 x 10^7 CFU / ml for S19 and 4.7 x 10^7 CFU / ml respectively were prepared by method previously described (17) with minor modification where the encapsulates were prepared by employing Homogenizer (Polytron) at 2000 rpm for 10 minutes .The culture was permanently cross-linked with Poly-L-lysine and final covering of alginate matrix. Prior to permanent cross-linking with Poly-L-lysine, 1 ml of microspheres suspensions were treated with depolymerizing solution (Tri sodium-citrate 50mM, 0.455mM NaCl and 10mM 3-Morpholinopropane-1-sulfonic acid (MOPS). After treatment, cultures were re-suspended in 1 ml of Brucella broth (BD, USA) and incubated at 37 ° C for 24 hours with 5% CO_2 for RB51 and without CO_2 for S19. After incubation, serially diluted culture was plated on specified plate for S19 and RB51 to determine the post-encapsulation viable count of both organisms. The entrapment efficacy of both the strains were calculated by dividing post-encapsulation count of bacteria by pre-encapsulation count and expressed in per cent. The presence of bacteria encapsulated, morphology and size were determined by direct microscopy (OLYMPUS, Model No. BX 50, Japan) at 100X magnification under oil immersion. Three optical fields were observed and mean diameter of microsphere was determined.

In-vitro bacterial release from the microencapsulate : One ml. of encapsulated culture containing 1.1 x 10^9 CFU/ml for S19 and 2.3 X 10^9 CFU/ml of RB51 were re-suspended in 9 ml. Brucella broth and incubated at 37 ºC for 24 hours. After 24 hours, the culture is spinned at low rpm (1500 -2000) for 5 minutes and the microencapsulates were re-suspended in 10 ml of fresh Brucella broth. One ml of broth culture post spinning was taken for each strain and plated onto Potato infusion agar (PIA) plate for S19 and in 5% Serum dextrose agar (SDA) with Rifampicin for RB51 respectively. Plates were incubated at 37 ºC with and without CO_2 for 3-4 days for RB51 and S19 respectively.. The colonies of the bacteria were counted from each plate and expressed as CFU/ml in terms of number of release of bacteria versus time. The above procedure was repeated till no further release was seen.

Antigen preparation : *B.abortus* S19 and RB51 were grown on PIA and SDA slant with rifampicin respectively and incubated for 4-5 days at 37 ° C .Confluent growth of bacterial lawn were harvested in PBS (pH 6.4) from each slant separately and checked for purity of the culture. The cultures were pelleted by centrifugation at 2000 rpm for 10 minutes in centrifuge tube. The pelleted culture was washed thrice with PBS buffer .The culture was finally re-suspended in PBS (pH 6.4) at fixed volume and equal amount of acetone (50 % v/v) were added to culture. The acetone mixed culture was kept for stirring for 24 hours to kill the bacteria. After 24 hours, 100 µl of killed culture were plated and checked for the viability. The killed culture was confirmed by negative growth on the plate. The protein content of acetone killed antigen were assessed by Bichinconic Acid Method (BCA Method) and used for evaluation of anti-Brucella specific antibody in mice (18).

Immunization of mice : Thirty 6-to 8 weeks' old female BALB/c mice were randomly divided in 5 groups of 6 mice each. Two groups of mice were immunized with a single dose of 0.1ml microencapsulated *B.abortus* S19 and RB51 containing a final count of 1.3 x 10^5 CFU and 2.7 X 10^5 CFU by sub-cutaneous route respectively as per the OIE protocol (19) . Two groups of positive control mice received non-encapsulated live *B.abortus* S19 1.1 x 10^5 CFU and 1.9 x 10^5

CFU for RB51. One group of negative control mice was injected with MOPS buffer.

Bleeding and challenge of mice : Mice were bled by inserting capillary tube in the infra-orbital sinus on day 0 and on 7,14,21,30 and 45 days post-immunization. All mice irrespective of treatment were intra-peritoneally challenged on day 30 with 2.2 x 10^5 CFU of wild type *B.abortus* strain 544.

Protection efficacy : Mice in each group were euthanized by CO_2 asphyxiation on 15 days post-challenge (DPC). Spleen from each individual mouse was collected aseptically. The size and weight of each spleen was determined to observe the presence or absence of spleenomegaly. Spleen was homogenized using sterile mortar and pestle and re-suspended in PBS with volume in ml equal to 10 times the weight of spleen. Ten fold serial dilution of spleen were prepared and 200 µl of suspension in duplicate were plated on PIA plate for S19 and SDA with rifampicin plate for RB51.The suspension was simultaneously plated on Tryptic Soya Agar (TSA) plate in duplicate to determine the bacterial load of *B.abortus* 544 strain in spleen. SDA and TSA plate were incubated at 37 ° C for 4-5 days at 5% CO_2 and PIA plate without CO_2 .The colonies of each Brucella strain were counted and the values were expressed after logarithmic transformation. The efficacy of micro-encapsulated with non-encapsulated live *B.abortus* vaccine and unvaccinated control group were determined by comparing the $\log_{10}$ CFU protection value of treatment group with non-vaccinated group (19).

Determine of cytokine response in -vivo : Mice were bled and serum were obtained from individual mouse of each group on day 0,7,14,21 and day 30 post-immunization and 15 day post-challenge for determination of cytokine level. Quantification of different cytokine (IL-2, IL-6, and IFN-γ) was determined by ELISA (eBioscience, Germany) as per the manufacturer's instruction.

Determination of anti-Brucella specific antibody : To determine the anti-Brucella specific antibody response, mice were bled and serum was collected on day 0, 7,14,21,30 days post immunization (DPI) and on 15 DPC. Sera were used for measuring IgG isotypes. Acetone killed whole cell *B.abortus* S19 and RB51 antigen were used to coat 96 well ELISA plate (Nunc, Denmark) at a concentration of 100ng /100µl / well. Optimal concentration of antigen and serum dilution were determined by checker board titration employing pre immunized and vaccinated sera and the method was qualified for inter-personal and inter-day variability in triplicate by Bland-Altman Plot using MedCalc Software (Data not shown). After overnight incubation at 4 °C, plates were washed 4 to 5 times with PBS and 0.1% Tween-20 (Sigma, Germany) and blocked with 2 % skimmed milk (BD, USA) and kept for 1 hour at 37 ° C. After wash, serum was added at initial dilution of 1:50 in same buffer and serially diluted further two-fold to determine the end-point titer. After addition of serum, plates were incubated at 37 ° C for 1 hour. After washing with PBS-T to remove unbound antibody, mouse monoclonal IgG isotype antibody (SIGMA, Germany) were added at 1: 1000 dilution for each plate in 2% skimmed milk and incubated at 37 ° C for 1 hour. 100 µl of purified recombinant protein A/G peroxidase (ThermoScientific, USA) at 1:20,000 dilutions in 2% skimmed milk was added after washing with PBS-T. After incubation and washing, added 100µl of peroxidase substrate (1 Tablet of Tetramethyl Benzidine dissolved in 10ml of citrate buffer with 3µl of H_2O_2) was added in each well and kept for 10 minutes at room temperature in dark. The reaction was stopped by 100µl of 1.25 M H_2SO_4 and read the absorbance value at 450nm (Synergy ST, BioTek). The Mean OD value $\pm$3 Standard Deviation of pre-vaccinated mouse sera at 1:50 dilution were taken as cut off value for each isotype.

Statistical analysis : The *in vitro* release of bacteria from micro-encapsule was expressed as $\log_{10}$ CFU plotted on Y axis versus time in days on X axis. The difference in spleen weight, intensity of infection and protective efficacy of

vaccination (bacterial clearance from spleen) of each group of 6 mice at 15 DPC was expressed as mean $\log_{10}$ CFU± Standard Deviation was analyzed by Student's *t* test, and the significance of differences between the groups were determined by analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) post-test comparing all groups to one another. The cytokine production *in vivo* was expressed as mean cytokine concentration ±Standard Deviation of each group of 6 mice. The anti-Brucella specific isotype antibodies were expressed as the reciprocal of $\log_2$ end point dilution ±Standard Deviation. The significance of differences between groups for both anti-*Brucella* specific antibodies and cytokines were analyzed by employing ANOVA followed by Tukey's HSD. For ANOVA and Tukey's HSD, P value of <0.05 were considered statistically significant (17, 20).

Results

Microsphere preparation and characterization : Microscopic studies revealed that the preparations from live *B. abortus* S19 and RB51 strains derived from cultures of Batch I and II were of uniform spherical shape ranging from 100 to 200 µm in size, with dotted centers suggestive of proper encapsulation (Fig. 1a and 1b). The viability of micro-encapsulated S19 and RB51 preparations after dissolution of capsules did not show significant batch to batch variation (Table 1). The *in vitro* release kinetics study on microsphere preparations incorporating *B.abortus* S19 and RB51 indicated the initial burst and continued sustained release till 24 days and 27 days for S19 and RB51, respectively (Fig. 2).

Protection efficacy : At 15 DPC (45 DPI) mice from all immunized groups demonstrated a statistically significant decrease in bacterial load in spleen as compared to MOPS inoculated naive mice control, with a 1.40-log reduction (P<0.01) from non-encapsulated S19, 1.99-log reduction (p<0.01) from encapsulated S19, a 3.22-log reduction (p<0.01) from non-encapsulated RB51 and a 3.89-log reduction (p<0.01) from encapsulated RB51 relative to naïve mice (Table 2). Both groups of mice immunized with encapsulated S19 and RB51 showed significantly higher level of protection upon challenge compared to non-encapsulated S19 and RB51 (P<0.01) (Table 2). Moreover, the level of protection offered by encapsulated RB51 (higher by 1.9-log) was significantly higher than that

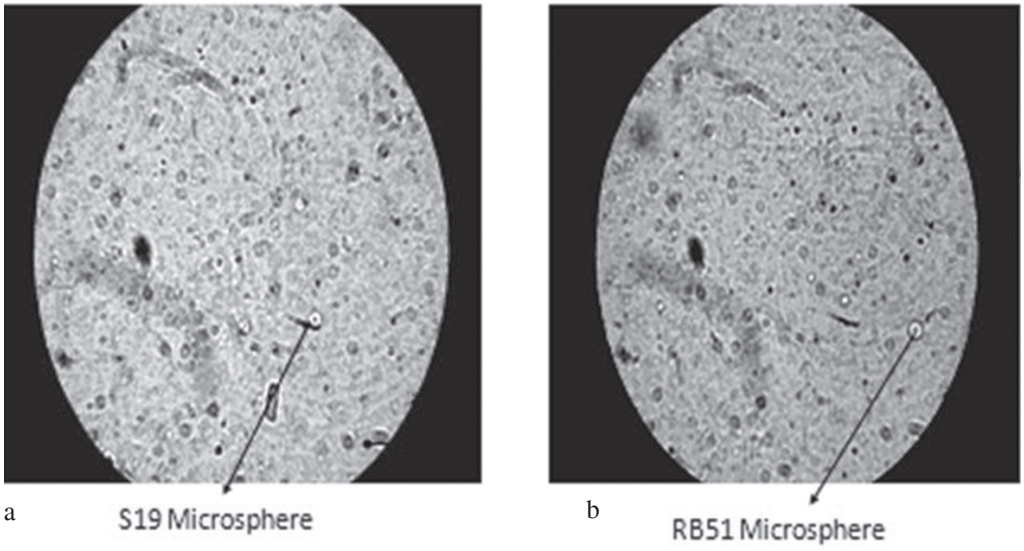

Fig. 1. Microscopic evidence of microencapsulated *B.abortus* S19 (1a) and RB51 (1b) after permanent cross-linking with Poly-L-lysine (100 x).

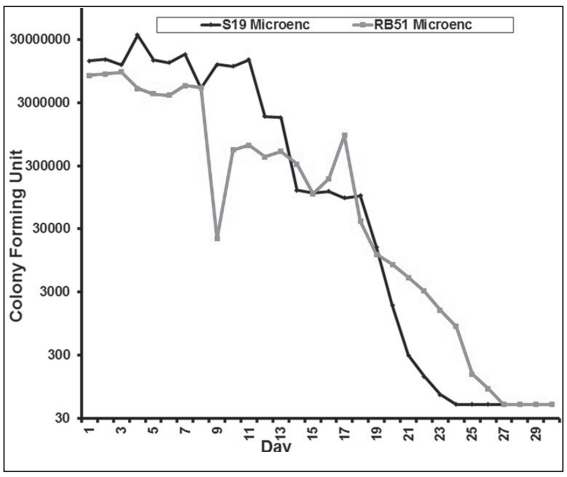

Fig. 2. Kinetics of live bacteria released from microsphere. 1ml of microencapsulates were suspended in 9 ml of Brucella broth and incubated at 37 ° C for 30 days. 1 ml of aliquot was withdrawn from each strain and plated on specific media plate to determine the release of bacteria from microspheres on each day.

offered by encapsulated S19 (P<0.01) (Table 2). However, the mean log10 CFU in spleen of vaccine strains in all groups of immunized mice at 15 DPC did not differ significantly (Table 2). Statistical difference was not observed in the mean spleen weight in all groups of immunized mice at 15 DPC (data not shown).

Comparison of immune response in mice after immunization and challenge: ***Encapsulated live B. abortus S19 and RB51 versus non-encapsulated live S19 and RB51 :*** The IgG1 isotype response in mice immunized with non-encapsulated live RB51 differed (P< 0.01) from MOPS inoculated controls at 7 DPI but in those immunized with encapsulated RB51 the levels differed (P< 0.01) at 7, 14, 21 and 30 DPI. The IgG1 response of mice immunized with encapsulated RB51 and non-encapsulated formats differed at 7 DPI (P< 0.05) and at 14, 21, 30 DPI (P<0.01). Mice groups immunized with non-encapsulated and encapsulated S19 showed IgG1 levels that differed (P<0.01) from MOPS inoculated controls at 30 DPI (Fig.3a). In addition IgG1 response in mice immunized with encapsulated S19 differed (P<0.05) from those inoculated non-encapsulated form at 30 DPI. The levels of IgG1 elicited in mice immunized with RB51 encapsulated form also differed from those immunized with S19 encapsulated form (P<0.05) at 7 and 14 DPI. It was observed that mice immunized with live non-encapsulated S19 and RB51 had significantly different (P<0.01) IgG1 levels with respect to the MOPS inoculated controls at 15 DPC. Further, at 15 DPC the IgG1 levels of mice immunized with encapsulated RB51 were significantly different (P<0.01) from non-encapsulated live RB51.

The IgG2a levels were significantly higher in mice immunized with non-encapsulated RB51 as well as encapsulated RB51 compared to MOPS inoculated controls (P<0.01) at 7,14,21,30

Table 1. Entrapment efficacy of Brucella *abortus* live vaccine with alginate matrix in two different set of preparations.

Batch I : *In –Vitro* Kinetics				
	Pre-Count (X)	**Post –Count (Y)**	**Y/ X x 100**	**%**
S19 RB51	1.9 X 10^9 CFU / ml 3.7 X 10^9 CFU / ml	1.1 X 10^9 CFU / ml 2.3 x10^9 CFU / ml	0.5789 0.6216	57.89 62.16
Batch II : Vaccine Batch For Mice Immunization				
S19 RB51	6.3 x 10^7 CFU / ml 4.7 x10^7 CFU / ml	4.1 x10^7 CFU / ml 2.6 x10^7 CFU / ml	0.6507 0.5531	65.07 55.31

Table 2. Enhanced protection in female BALB/c mice immunized sub-cutaneously with encapsulated *B.abortus* S19 and RB51 compared to non-encapsulated and MOPS control groups followed by intra-peritoneal challenge with 544 strain. Difference in log protection values were compared between experimental and control group(e). Mice immunized with S19 micro-encapsulated form (a); RB51 micro-encapsulated form (b); S19 non-encapsulated form (c); RB51 non-encapsulated form (d) and MPOS buffer inoculated control groupe; S** significant P<0.01 by Student 't' test.

Vaccine Group	Recovery of challenge strain *B.abortus* 544 (Log 10 CFU)	Recovery of vaccine strain *B.abortus* (Log 10 CFU)	Protection indices
Microencapsulated Live RB51	1.87	2.68	3.89**a,b,e
Non-encapsulated Live RB51	2.54	1.95	3.22**b,d,e
Microencapsulated Live S19	3.77	3.83	1.99**a,e
Non-encapsulated Live S19	4.36	3.23	1.40**a,c,e
Unvaccinated MOPS Buffer control	5.76	-	

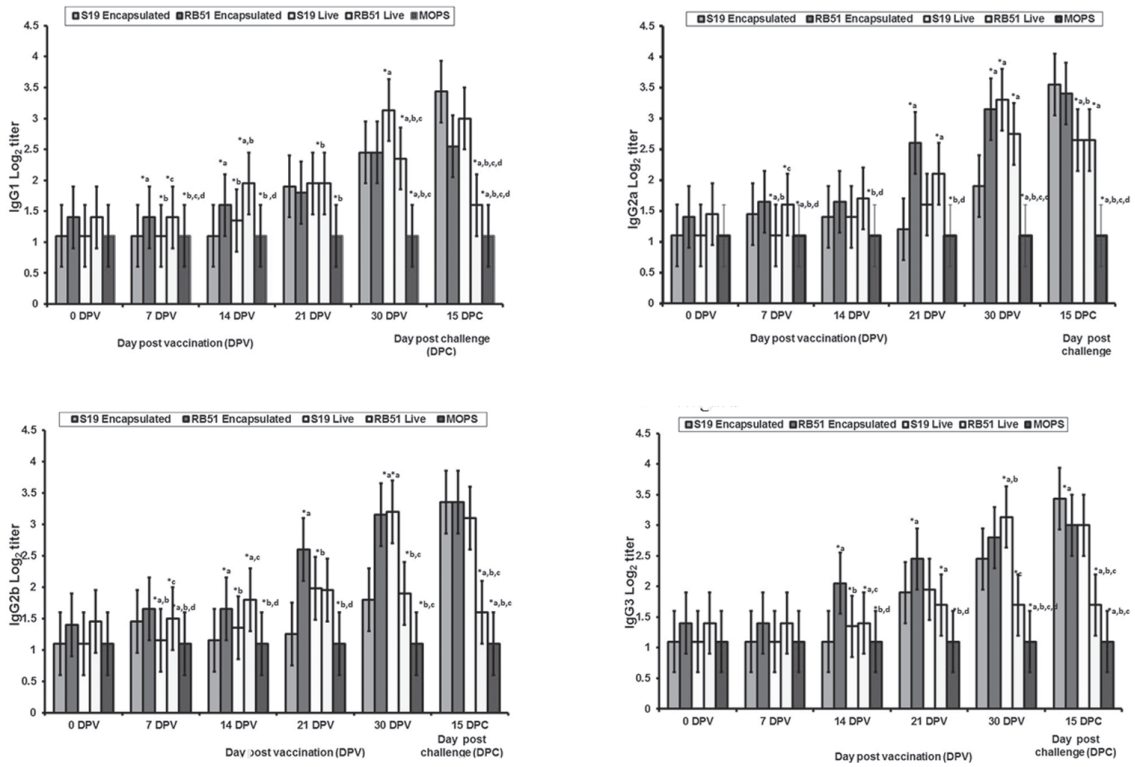

Fig.3. Anti-brucella isotype specific antibody response in BALB / c mice immunized with live microencapsulated *B.abortus* S19(a) and RB51(b) and live non-encapsulated S19(c) and RB51(d) with MOPS control group (e) on days 0,7,14, 21,30 and 15 day post-challenge ; Fig 3a , 3b ,3c and 3d : Response of IgG1 , IgG2a ,IgG2b and IgG3 differ significantly between group , days and day and group ($p < 0.001$)

DPI and also at 15 DPC (Fig.3b). Similarly, in mice groups immunized with non-encapsulated S19 the IgG2a differed from MOPs inoculated controls at 7 DPI ($P<0.05$) and 30 DPI ($P<0.01$), whereas in mice immunized with encapsulated S19 differed significantly from MOPS control at 7 DPI ($P<0.05$) and 30 DPI ($P<0.01$) respectively. The IgG2a levels were higher in mice immunized with S19 encapsulated format compared non-encapsulated group at 7DPI ($P<0.05$), 30 DPI ($P<0.01$) and at 15 DPC ($P<0.01$). The IgG2a were significantly elevated ($P<0.01$) in mice immunized with S19 encapsulated version compared to those immunized with RB51 encapsulated group at 30 DPI.

The IgG2b levels were significantly elevated in mice immunized with non-encapsulated S19 ($P<0.05$ at 30 DPI and 15 DPC), non-encapsulated RB51 ($P<0.05$ at 7, 14 and 21 DPI), encapsulated S19 ($P<0.01$ at 30 DPI and 15 DPC) and encapsulated RB51 ($P<0.01$ at 7, 14, 21 DPI and 15 DPC) compared to MOPs inoculated controls (Fig.3c). Significantly different IgG2b levels were observed in mice immunized with S19 encapsulated version compared to live non-encapsulated S19 ($P<0.01$) at 7 and 30 DPI, whereas in groups of mice immunized with encapsulated RB51 the levels differed ($P<0.01$) at 21 DPI and 15 DPC. Further mice immunized with encapsulated RB51 had significantly different IgG2b levels ($P<0.01$) compared to the group immunized with encapsulated S19 at 14, 21 and 30 DPI.

The IgG3 levels were significantly elevated in mice immunized with non-encapsulated S19 ($P<0.01$ at 15 DPC), encapsulated S19 ($P<0.01$ at 15 DPC), non-encapsulated RB51 ($P<0.01$ at 14, 21 and 30 DPI) and encapsulated RB51 ($P<0.01$ at 14 and 30 DPI, and at 15 DPC, $P<0.05$ at 21 DPI) compared to MOPs controls. Compared to non-encapsulated S19 and RB51, IgG3 was significantly elevated ($P<0.01$) in mice immunized with encapsulated S19 at 30 DPI and those immunized with encapsulated RB51 at 15 DPC. Moreover, IgG3 found to differ significantly in mice immunized with encapsulated S19 compared to those immunized with RB51 at 14 DPI ($P<0.01$) and 21 DPI ($P<0.05$) (Fig.3d).

The IL-2 levels were significantly different ($P<0.05$) in mice immunized with encapsulated S19 compared groups of mice immunized with non-encapsulated S19 at 21 DPI, and MOPS controls at 21 DPI ($P<0.05$) and at 15 DPC ($P<0.01$). In addition the IL-2 level were statistically different in mice immunized with non-encapsulated S19 and RB51 compared to MOPS at 15 DPC ($P<0.01$). Also, IL-2 was significantly different ($P<0.05$) in mice immunized with encapsulated RB51 from non-encapsulated RB51 and MOPs control mice groups at 15 DPC (Fig.4a). The IFN-γ levels in mice immunized with non-encapsulated S19 differed ($P<0.01$) from their MOPS control at 15 DPC. Both groups of mice immunized with S19 and RB51 encapsulated forms showed significantly elevated IFN-γ levels ($P<0.01$) compared to non-encapsulated S19 and RB51 as well as to the MOPS control at 15 DPC (Fig.4b). However the IL-6 levels did not differ significantly among groups after immunization or post-challenge (Fig.4c).

Discussion

Microspheres prepared from live *B. abortus* S19 and RB51 strains ranged from 100-200 µm. Particle size is an important factor for the effective uptake of the immunogen by antigen processing cells/ macrophages (21). Natural and synthetic micro-particles of similar size ranging from 10 to 300 µm has been used to encapsulate Influenza A, HBsAg , *B. abortus* and *B. melitensis* antigens (14, 21, 22), and while the micro-encapsulated Influenza A and HBsAg immunogens were reported to elicit appropriate immune response (21), the micro-encapsulated *B. abortus* and *B. melitensis* exhibited improved protection (16,17). Although the bacterial viability post-encapsulation did not differ statistically between strains and batches in the current study, the values were lower (mean 60%) as compared to previous report (17). The reason for lower viability may probably because of the employment of a

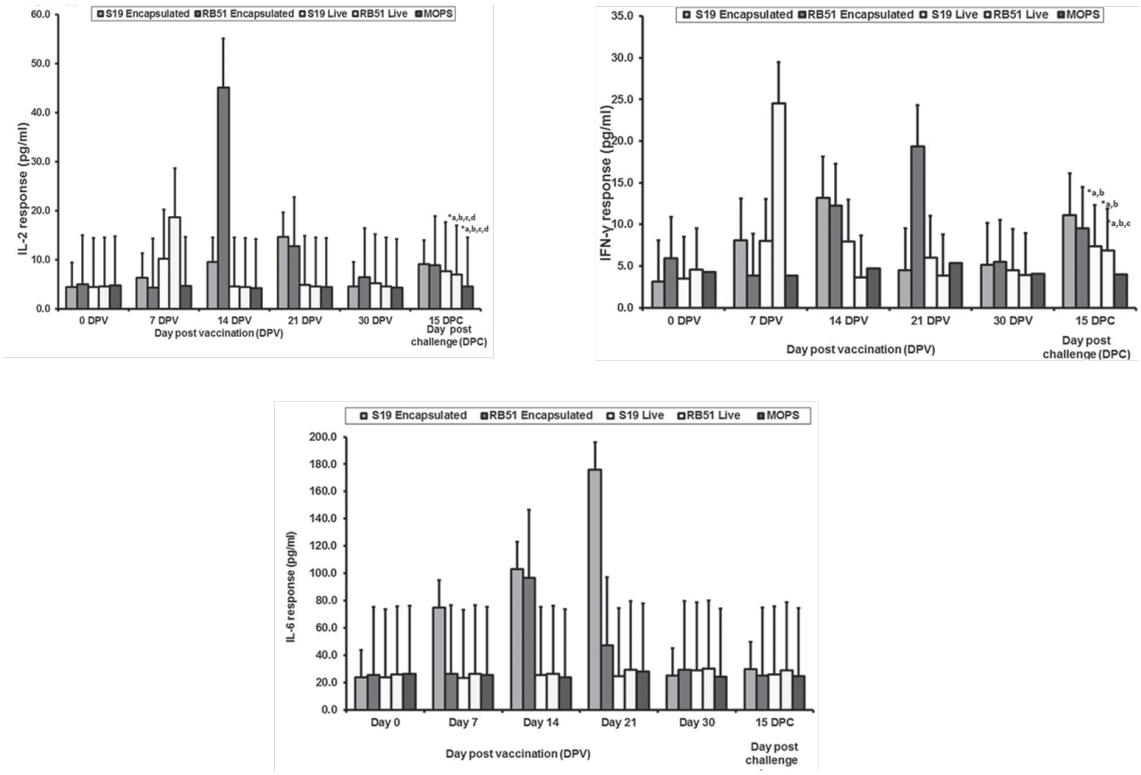

Fig. 4. Comparative quantification of cytokine in serum samples of female BALB / c immunized with live encapsulated *B.abortus* S19(a) and RB51(b) vaccines with non-encapsulated live S19(c) and RB51(d) and MOPS control group(e) on day 0 , 7,14,21, 30 and 15 day post-challenge with *B.abortus* 544 ; Fig 4a and 4b : Response of IL-2 and IFN-γ differ significantly between day and group by ANOVA ($p<0.001$ and $p< 0.015$ respectively).IL-6 does not differ significantly between days and group (4c)

homogenizer for carrying out the encapsulation instead of a specific encapsulator as cited in a previous study (17). Homogenizers generate high stress on cells due to shear force, the encapsulator on the other hand produce minimal stress that helps maintain viability of preparations. Similarly, the release of S19 and RB51 from microencapsulated preparations as observed by *in vitro* release kinetic studies lasted for 24 and 27 days, respectively, compared to a previous report of 36 days in case of a microencapsulated mutant *B. melitensis* (17). Various physicochemical factors may affect the release, including the concentration, surface charge, ligand composition and hydrophobicity (21, 22).

Micro-encapsulated live S19 and RB51 provided enhanced protection in mice by order of magnitude of 0.59-log and 0.67-log respectively compared to non-encapsulated forms when a protocol of sub-cutaneous immunization, challenge at 30 DPI and recovery of challenge strain *B. abortus* 544 at 15 DPC was followed. The efficacy of protection in the current study was similar to a study reported previously (17) where mice immunized intra-peritoneally with live *B. abortus* S19 vjbR::kan mutant encapsulated in alginate microsphere exhibited

superior efficacy (0.8-log) that was statistically different ($P<0.05$) than its non-encapsulated format, following intra-peritoneal challenge with *B. abortus* 2308 at 32 weeks post-immunization and evaluation of the load of *B. abortus* 2308 in spleen at 1 week post-challenge. While in a separate previous study mice immunized intra-peritoneally with encapsulated *B. melitensis* 16M vjbR::Tn5 mutant and challenged intra-peritoneally at 9 weeks post-immunization with *B. melitensis* 16M strain showed much higher level of enhanced protection of the order of 1.84-log compared to non-encapsulates controls (16). Improved efficacy of vaccines by microencapsulation of antigens employing biodegradable polymers have been reported (14, 23, and 24). The degree of efficacy may be affected by the choice of microspheres (natural/ synthetic), physiochemical character of the antigen such as the composition of the outer membrane (25), the nature of lipo-polysaccharide (smooth and rough) (26) as well as the immunization and challenge protocol adopted for the model for testing the efficacy. The most salient finding in the current study was the exhibition of superior protection offered by the encapsulated live *B. abortus* RB51 rough strain by an order of magnitude of 1.9-log compared to encapsulated S19. Probable reasons for enhanced efficacy may be ascribed to the facts such as structural and functional differences in outer membrane in rough *Brucella* strains (25). Further it has been shown that the nature and degree of attachment, mode of entry into a cell (in non-opsonized conditions) and intra-cellular trafficking (in human monocytes, vero cells and macrophages) of rough and smooth strains of *Brucella* are quite distinct (26, 27, 28), the rough strains also induced higher amounts of pro and anti-inflammatory cytokines from monocytes (28).

The characteristics of antibody isotype and cell mediated immune response appeared to be influenced by the choice of the vaccine strain and whether it was used as an encapsulated or a non-encapsulated version for immunization. The probable mechanisms that could have conferred the group of mice a protective advantage by immunization with micro-encapsulated S19 and RB51 compared to the un-encapsulated versions are summarized (Table 3). Similarly, the probable mechanism that conferred the mice group immunized with micro-encapsulated RB51 over those immunized with micro-encapsulated S19 is summarized (Table 4). Thus it appears that antibody isotype levels differs between the encapsulated versions in the post-immunization stage alone, however, no such differences in IL-2 and IFN-γ were observed between the two encapsulated groups either at the post-immunization and post challenge stage.

The significantly different levels of IgG2a, IL-2 and IFN-γ in mice immunized with encapsulated S19 compared to the non-encapsulated group at the DPC stage was probably related to better protection by an order of magnitude of 0.59 logs. While better protection by an order of magnitude of 0.67 logs provided by immunization of mice with encapsulated RB51 version compared to non-encapsulated group may have been due to significantly different levels IgG1, IgG2b, IgG3, IL-2 and IFN-γ at the DPC stage. Therefore it appears that production of significantly higher levels of IgG2a, IL-2 and IFN-γ (S19) or significantly higher levels of IgG1, IgG2b, IL-2 and IFN-γ (RB51) in encapsulated groups compared to the non-encapsulated versions at the DPC stage was due to more efficient recall and recruitment of these specific immune memory cells. This observation probably conferred the protective advantage to mice groups immunized with encapsulated versions over those immunized with live *B. abortus* vaccines. Improved protection observed in mice immunized with encapsulated S19 versions is probably also linked to significantly different levels of IgG2a, IgG2b, IgG3 and IL-2 in the DPI stage. Since together in tandem, they may have been contributory factors that could have possibly facilitated more efficient priming of the immune system, leading to more efficient recruitment of memory cells, later at the DPC stage. But unlike

Table 3. NS: Statistically not significant; S*: Significant P< 0.05; and S** significant P<0.01 by Tukey's Honesty Significant Difference. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated vaccine a protective advantage over the group immunized with non-encapsulated form. Probable mechanisms: Protective advantage to mice conferred by immunization with micro-encapsulated S19 compared to the non-encapsulated form could probably be due to significantly different response of IgG2a, IL-2 and IFN-γ at the DPC stage and that of IgG2a, IgG2b, IgG3 and IL-2 at the DPI stages. Together in tandem this characteristic immune response at the DPI stages probably facilitated more efficient priming of immune cells leading to efficient recruitment immune memory cells later at the DPC stage. Similarly, protective advantage to mice conferred by immunization with micro-encapsulated RB51 compared to the non-encapsulated form could probably be due to significantly different response of IgG1, IgG2b, IgG3, IL-2 and IFN-γ at the DPC stage and that of IgG1 and IgG2a at the DPI stages.

A. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated S19 vaccine a protective advantage

Immune response	**DPI stages**	**15 DPC stage**
Antibody isotype		
IgG1	NS	S*
IgG2a	(7 DPI S*) (30 DPI S**)	S**
IgG2b	(7 and 30 DPI) S**	NS
IgG3	(30 DPI) S**	NS
Cytokine response		
IL-2	(21 DPI)S*	S*
IFN-γ	NS	S**

B. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated RB51 vaccine a protective advantage

Immune response	**DPI stages**	**15 DPC stage**
Antibody response		
IgG1	(14 and 21 DPI)S* (30 DPI S**)	S*
IgG2a	NS	NS
IgG2b	(21 DPI) S* (30 DPI)S**	S**
IgG3	NS	S**
Cytokine response		
IL-2	NS	S*
IFN-γ	NS	S**

the above observation, significantly elevated levels of IgG1 and IgG2a in case of mice immunized with encapsulated version of rough strain *B. abortus* RB51, at the DPI stage, were sufficient enough to render improved priming; that in turn, could have contributed to better recall of memory T cells at the DPC stage.

Similarly, the superior protective efficacy of encapsulated versions of RB51 compared to S19 could probably be linked to significantly higher levels of IgG1 (early stage - 7 DPI) and IgG2b (middle and late stage - 14, 21, 30 DPI) at the DPI stage in mice immunized with encapsulated RB51, but not significantly enhanced IgG2a (late stage -21 and 30 DPI) and IgG3 (mid and late

Table 4. NS: Statistically not significant; S*: Significant P< 0.05; and S**: Significant P<0.01 Tukeys Honesty Significant Difference. Suggested immune response mechanisms that empowered mice immunized with micro-encapsulated RB51 vaccine a protective advantage over the group immunized with S19 encapsulated form. Probable mechanisms: Protective advantage to mice conferred by immunization with micro-encapsulated RB51 compared to the micro-encapsulated S19 form could probably be due to significantly different response of IgG1 at 7 DPI and IgG2b at 14, 21 and 30 DPI, but not significantly elevated IgG2a at 21 and 30 DPI and IgG3 at 14 and 21 DPI as noticed in mice immunized with micro-encapsulated S19.

Micro-encapsulated vaccine form / Immune response	**DPI stages**	**15 DPC stage**
Antibody response		
IgG1		
S19	NS	NS
RB51	(7 and 14 DPI) S*	NS
IgG2a		
S19	(21 and 30 DPI) S*	NS
RB51	NS	NS
IgG2b		
S19	NS	NS
RB51	(14, 21 and 30 DPI) S*	NS
IgG3		
S19	(14 and 21 DPI) S*	NS
RB51	NS	NS
Cytokine response		
IL-2		
S19	NS	NS
RB51	NS	NS
IFN-γ		
S19	NS	NS
RB51	NS	NS

stage -14 and 21 DPI) levels; as noticed in mice immunized with encapsulated S19 compared to encapsulated RB51. This evidence is supported by the observation that the levels of antibody isotypes were statistically non-significant when both encapsulated versions were compared at the DPC stage. Neither, the levels of IL-2 and IFN-γ differed statistically between the groups of mice immunized with encapsulated S19 and RB51 either at the DPI or the DPC stage. These observations emphasize that most probably priming of the immune cell subsets secreting IgG1 and IgG2b at the DPI stage confers the encapsulated rough *B. abortus* RB51 version with a significant protective advantage (>1.9 log) over the encapsulated smooth *B. abortus* S19 version.

The above repertoire of immune response events described above while comparing encapsulated with non-encapsulated groups probably suggested that for both encapsulated versions, in the DPC stage, the cellular immunity was due to both the Th1 and the Th2 types, and these responses were significantly more

pronounced than the non-encapsulated versions, leading to better protection in encapsulated groups. However, during the DPI stages, in the S19 encapsulated group the Th1 driven immune response was more pronounced than controls. This was distinct from that noticed in the RB51 encapsulated group where cells of both Th1 and Th2 lineage were recruited for eliciting a more pronounced immune response than the controls. The most important observation that emerged from this study was that the protective advantage offered by immunization of BALB/c mice with encapsulated RB51 over those with encapsulated S19 seemed to be linked to cellular events that were distinct in character and was noticed in the DPI stage alone. The Th1 driven response alone was more pronounced in the S19 encapsulated group than in the RB51 encapsulated group, as evidenced by recruitment of B cell subsets secreting significantly elevated levels IgG2a and IgG3, in contrast, in RB51 encapsulated mice both the Th1 and Th2 driven responses were prominent compared to the S19 encapsulated group, as evidenced by recruitment of a different subset of B cells secreting significantly elevated levels of IgG2b and IgG1. This observation may have been responsible for conferring the mice immunized with rough encapsulated RB51 the protective advantage over those immunized with smooth encapsulated S19, since no other significantly different Th1 or Th2 driven activity was noticed among the two groups in terms of antibody isotype or cytokine response (IL-2, IFN-γ) either at DPI or DPC stages. The essential involvement Th1 and Th2 cells in the immunity to *Brucella* have been previously described (14, 17, 29, 30, 31 32,). Our current observations are in agreement to these previous reports.

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Enhanced Toxicity of Purified *Bacillus thuringiensis* Cry1Ac δ-endotoxin

Vivek Kumar Singh[1,4] Vikrant Nain[2], Priyanka Sharma[1], K R S Sambasiva Rao[4], Ananta Birah[3], G P Gupta[3] and P. A. Kumar[1,5]*

[1]National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi -110012, India
[2]Gautam Buddha University, Greater Noida, India
[3]Division of Entomology, Indian Agricultural Research Institute, New Delhi -110012, India
[4]Acharya Nagarjuna University, Guntur, India
[5]Indian Institute of Rice research, Rajendra Nagar Hyderabad- 500030, India
*For Correspondence - polumetla@hotmail.com

Abstract

Use of Cry1Ac δ-endotoxin of *Bacillus thuringiensis* (*Bt*) has revolutionized crop protection especially in cotton. Large quantities of Cry1Ac protein are needed for several basic studies. Heterologous expression in *E. coli* facilitates production *of* large quantities of recombinant Cry1Ac protein. However, earlier studies reported that *E. coli* expressed recombinant protein leads to large variations in LC_{50} values. Here we report a method of active Cry1Ac δ-endotoxin purification that is simple, robust and exhibits higher toxicity with consistent results in insect bioassays. In this protocol proteolysis separates completely folded Cry1Ac δ-endotoxin molecules (active toxin) from non-native protein folding forms and other host proteins, followed by extraction of active Cry1Ac toxin by micro filtration. Insect bioassay of purified Cry1Ac toxin with *Helicoverpa armigera* showed 125 times enhanced toxicity (LC_{50} 40 pg/cm^2) as compared to the earlier reports (LC_{50} ng/cm^2) 4.5 to 3500 ng/cm^2. These results provide a new prospective in determination of baseline susceptibility, monitoring resistance development and utilization of its potential.

Key words: δ-endotoxin, *Helicoverpa armegera,* CHAPS, PVDF membrane, PAGE, protein purification, microfiltration.

Abbreviations: CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio.-1-propanesulfonate

Introduction

Insect pest management in agriculture by expressing insecticidal proteins of *Bacillus thuringiensis (Bt)* has been adopted worldwide (1). The high specificity of *Bt* insecticidal crystal proteins towards target insects, which arises due to the need of high alkaline pH (>10) for solubilization, presence of serine proteases in the insect midgut for processing and most importantly the presence of highly specific receptors in the insect midgut epithelial cells for binding of the toxin, narrow down the host range (2). This high margin of safety recommends the use of *Bt* genes in food crops or in other sensitive sites where chemical pesticides may cause adverse effects (3, 4).

Since 1995 when the first Bt transgenic crop was commercialized, *cry1Ac* genes have been most frequently used, either alone or in combination of other genes (5). *Bt* toxins have narrow but overlapping host ranges. Therefore it is imperative to screen insecticidal proteins for their effect on insect pests of economic importance. In addition, the base line susceptibility data is required to monitor the resistance development, that remains a concern after the commercial release of large number of

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Bt transgenic crops world wide. For such studies, we need large quantities of purified protein for diet incorporation or leaf overlay insect feeding assays.

Bt cry genes have been over-expressed in *E. coli* using various bacterial expression vectors (6, 7). However, the major limitation arises with structural similarities Bt proteins (particularly Ist domain) with the membrane proteins (8). Membrane proteins are difficult to recover in active from by heterologus expression, hence the Cry1Ac protein too (9). Cry1Ac toxins when over-expressed in *E. coli* form inclusion bodies. Inclusion bodies are known to be composed of aggregates of unfolded and partially folded protein molecules (10). These inclusion bodies are subsequently *in vitro* solubilized at high alkaline pH (> 10) for recovery and refolding of protein (11). During this *in vitro* refolding process some protein molecules get correct folding while others form misfolded and aggregated protein forms (10, 12). As the fraction of completely folded molecules (active toxin) in different protein preparations always vary, it leads to large variations in the toxicity data (Table1). These protein folding variations manifested in the toxicity data become a cause of concern when determining base line susceptibility or monitoring the resistance development in the insects exposed to *Bt* diet. It warrants development of a protocol that can separate completely folded Cry protein (active toxin) not only from other host proteins but also misfolded and aggregated protein forms.

Common methods for Cry toxin purification are selective precipitation (13), ion exchange chromatography (14), Histidine tag with Ni NTA column chromatography (15), antibody affinity chromatography (16), FPLC Sepharose column (17) and gradient centrifugation (18). All these methods have certain limitations and fail to separate completely folded protein molecules from misfolded or aggregated toxin protein molecules.

Here we report a simple and robust method for the purification of active Cry1Ac δ-endotoxin with enhanced toxicity. *Bt* δ-endotoxins are resistant to proteolytic degradation (19) and this intrinsic property of protease resistance present in all *Bt* toxins is used in the current method of purification. A trypsin and chymotrypsin protease mix digest out all non native protein folding forms while active Cry1Ac molecules having native like structure remains protease resistant. Active Cry1Ac toxin is further purified by extraction from the polyacrylamide gel using PVDF membrane in a centrifugal device.

Material and Methods:

Expression of Cry1Ac Protein in E. coli: Native Cry1Ac gene was provided by Dr. Donald H Dean, Ohio State University and cloned in pKK223-3 vector (Clonetech) (Figure. 1) and mobilized to *E. coli* expression host JM105. The

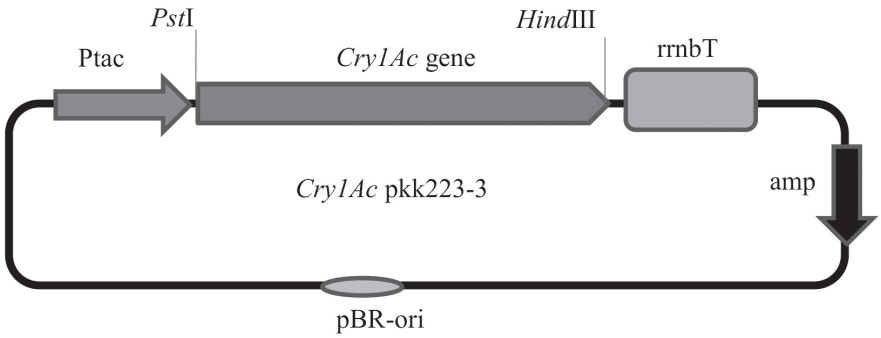

Fig. 1 Scheme of plasmid vector Cry1Ac-pkk223-3 for expression of Cry1Ac protein expression. Cry1Ac gene cloned in pkk223-3 vector using the PstI and HindIII restriction site combination. The scheme shows the genetic elements of pkk223-3vector: P-tac promoter, transcriptional terminator (rrnb T), Ampicilin resistance gene (amp), origin of replication (pBR-ori).

transformed colonies were grown in 500 ml Luria Broth at 37 °C for 12 h. The protein expression was induced by 1mM Isopropyl-â-D-thiogalactopyranoside (IPTG) and cells were grown at 37 °C for another 6 h. Cells were harvested by centrifugation at 6000 x g for 10 min at 4 °C and resuspended in sonication buffer containing 50 mM Tris-Cl (pH 8.0), 5 mM EDTA. Sonication was carried out at a power output of 100W three times for 1 min each. The lysate was centrifuged at 14000 x *g* for 20 minutes at 4 °C. The pellet was processed for the extraction of Cry1Ac protein.

The pellet obtained after the centrifugation of lysate was washed thrice with wash buffer I (0.5 M NaCl, 2% Triton X 100), 5 times with wash buffer II (0.5 M NaCl) and thrice with sterile distilled water. Each washing step was followed by centrifugation at 16000 x *g* for 10 min at 4 ºC. The pellet was lyophilized and stored at -20 ºC until use.

Solubilization of Inclusion bodies: Inclusion bodies were solubilized with carbonate buffer (pH 10.5) for 4 h at 37^{0}C and 120 RPM. The solubilized protein was centrifuged at 11000 RPM for 15 min and the supernatant was taken as solubilized Cry1Ac endotoxin and purified further. Total protein from *E. coli* cells expressing *cry1Ac* gene was resolved on 10% SDS PAGE using Laemmli procedure (20) an total protein concentration was estimated using the Bradford method (21).

Purification of Cry1Ac protein: Protease mix solution was prepared with trypsin 30 mg/ml (Amresco) and chymotrypsin 0.4 mg/ml (Amresco). For removal of other proteins from active Cry1Ac, protease mix was added to the total solubilized protein in a ratio of 1:3 (v/v) in a reaction volume of 100 µL in a 1.5 ml microfuge tube and incubated for 1 h at 37 ^{0}C. Once the reaction was over, the total reaction mixture was resolved on 7.5% native PAGE. To locate the Cry1Ac protein band on the gel one lane of gel was cut with a surgical blade and placed adjacent to the remaining gel after CBB staining. A slice of gel containing Cry1Ac protein was cut with a surgical blade and placed in the gel extraction buffer (50mM carbonate buffer, pH 10.5 and 0.1% CHAPS). Gel slice was crushed to a fine suspension using a sterile polycarbonate pestle in 1.5 ml centrifuge tube and incubated for 4 h at 37 ^{0}C.

Cry1Ac protein was recovered from the gel suspension by filtration with 0.45 ìm, low protein binding Durapore PVDF membrane placed in a centrifugal device (Ultrafree®-DA, Millipore). Gel suspension was placed in the centrifugal device, centrifuged for 15 min at 4000 x g and the flow through was collected. The detergent (CHAPS) was removed from the recovered protein by micro filtration through regenerated cellulose filter having 10kda MW cut-off pore size (Amicon ultra 4 10 kDa, Millipore). To remove traces of remaining CHAPS in the protein solution, it was diluted to 4 ml and re-centrifuged for 30 min and the process was repeated thrice. The concentrated protein was recovered by the swirling of the membrane.

Protein Toxicity Assays: The toxicity of the purified Cry1Ac protein was tested against the first instar larvae of *Helicoverpa armigera*. Different concentrations of the toxin were incorporated in the *H. armigera* diet. The diet of *H. armigera* was prepared by the method described by Singh and Rembold (23). The diet was poured in the 24-well culture plate (1 ml/well) (Cellstar, Greiner Labortechnik, Germany) and allowed to solidify. One first instar larva was released in each well. A set of 30 first instar larvae were tested for each concentration at one time. The plates were covered with Saran wrap and kept in the insect culture room at 28 ± 2 ºC, 60 ± 5 % humidity and 14L: 10D photoperiod. Mortality was recorded after 3 days and LC_{50} values calculated by Probit analysis (24). The experiment was replicated three times.

Results and Discussion

Bt Cry toxins when expressed in *E. coli* form inclusion bodies. As the Cry toxins are present in the form of aggregates of partially folded protein molecules (10), the inclusion

bodies are solubilized at higher pH (>10), for recovery and refolding of the toxins (11). On solubilization of the inclusion bodies some of the protein molecules get properly folded while others either get misfolded or form protein aggregates (10, 12). The proportion of these three protein forms viz., folded, misfolded and aggregated may be influenced by various preparation and purification methods followed in different laboratories. Because of the variation in the content of the active toxin in the protein preparations different levels of toxicity are manifested in the insect bioassays. This discrepancy in the toxicity data is summarized in Table 1, which shows that the LC_{50} values for Cry1Ac against *Helicoverpa armigera* range from 4.5 to 3500 ng/cm^2.

Protease digestion: *Bt* toxins binds to the specific receptors present on insect midgut and kill the insect by pore formation in the gut epithelium cells (3, 4). In order to remain active in the insect gut the *Bt* toxin must protect itself from gut proteases. Hence, *Bt* toxins have acquired protease resistance by embedding the protease sensitive sites within the folded protein structure (17). In the Cry protein molecules that have not folded properly, the protease sensitive sites will get exposed, subsequently leading to proteolytic degradation, while properly folded proteins will remain protease resistant.

In the present study, this intrinsic property of protease resistance among Cry toxins was used to study the *in vitro* folding of Cry1Ac protein. Cry1Ac toxin recovered after *in vitro* solubilization of inclusion bodies (Figure 2A1, 2A2, 2A3) was digested with Trypsin, Chymotrypsin and a mixture of both (Figure 2B1). These results suggest that the quantity of Chymotrypsin resistant core is 37 percent of Trypsin resistant core. In the case of digestion

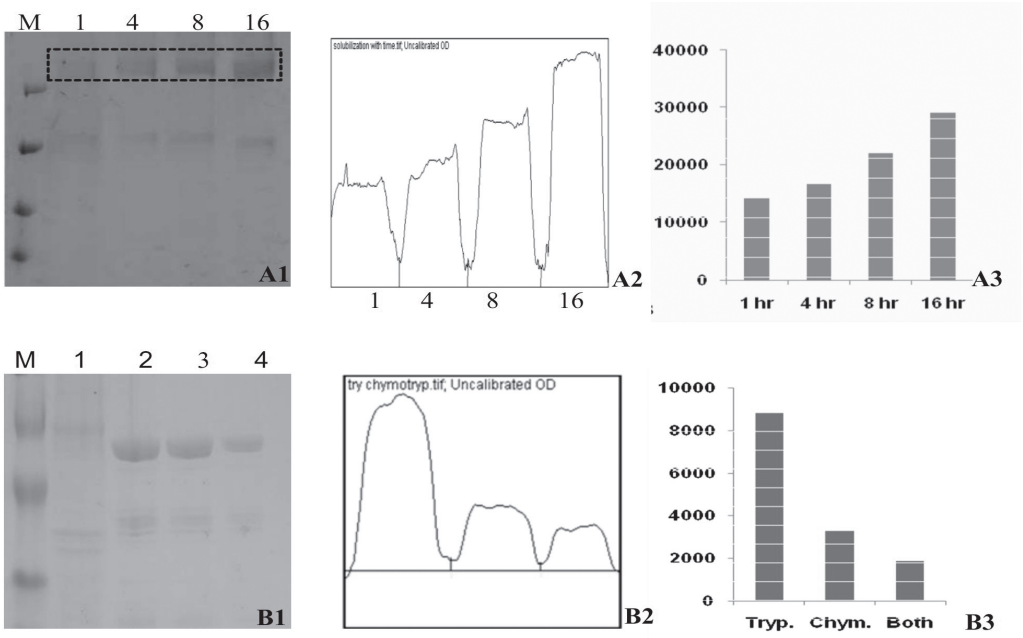

Fig 2. Solubilization and refolding of Cry1Ac protein expressed as IBs in E. coli. A.Solubilization of IBs for different time intervals; 1, 4, 8 and 16 hrs respectively, B.Degree of protein folding estimated in terms of protease resistant core left after trypsin and chymotrypsin treatment. Lane 1. Protoxin, Lane 2-4, Trypsin, Chymotrypsin and both protease treatment respectively. 1. SDS-PAGE, 2-3. OD of required bands represented graphically and quantitatively.

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with both the toxins together the quantity of protease resistant core is approximately 50 and 20 percent of trypsin and chymotrypsin resistant core respectively (Figure 2B2, 2B3). It indicates that some protein molecules resistant to trypsin were sensitive to chymotrypsin digestion and *vice versa*. The possible reason of this quantitative difference in the recovery of protease resistant core may be that the Cry1Ac toxin molecules have 48 trypsin recognition sites and 100 chymotrypsin recognition sites as predicted by peptide cutter (25). Thus in the case of misfolded (or partially folded) protein molecules the chymotrypsin sites will get more frequently exposed in comparison to the trypsin sites, leading to the degradation of more number to molecules. All those molecules having either trypsin or chymotrypsin sites accessible to the respective protease will get digested when treated with the mixture of both the proteases. Presence of protein molecules that are resistant to one but cleavable by another protease suggests the presence of protein folding intermediates, which have all the sites of one protease embedded in the folded protein structure but with the sites for the other protease exposed.

Purification of Cry1Ac protein: In this work the intrinsic property of protease resistance was further utilized to separate the folded (active) *Bt* Cry1Ac toxin from host proteins and other unfolded, misfolded or aggregated Cry1Ac protein forms developed by *in vitro* folding. As trypsin and chymotrypsin mixture removes maximum number of *in vitro* folding intermediates, it was tested for the stability of Cry1Ac toxin over digestion and time required to eliminate the host proteins as well.

In vitro solubilized Cry1Ac protein was mixed with the protease mixture and incubated at 37^{0}C for different intervals, ranging from 15 min to 4 hrs. Results of protease digestion analysis show that most of proteins are digested out within 1 h of incubation (Figure 3). Incubation for more than 1 hr does not degrade the Cry1Ac protein further, indicating the stability of protease resistant Cry1Ac core to over digestion. One hour incubation was found sufficient for the removal of *E. coli* protein from the reaction mix also.

Table1. Comparison of LC_{50} values for *H. armigera* neonate larvae bioassay with Cry1Ac toxin obtained from *E. coli* inclusion bodies.

Type of Assay	LC_{50} (LCL-UCL) ng/cm^2	References
Diet incorporation	20 (12-36)	Chakrabarti, *et al.* 1998
Diet surface	4.7 (2.3-9.4)	Padidam, *et al.* 1992
Diet incorporation	240 (180-310)	Babu, *et al.* 2002
Diet surface	115 (82-159)	Liao, *et al.* 2002
Diet incorporation	3500 (2700-4500)	Avilla, *et al.* 2005
Diet surface	0.1	Kumar *et al.* 2005
Diet surface	40.3 (27.3 -78.2)	Present study with trypsinized toxin
Diet surface	0.04 (0.02-0.05)	Present study with purified toxin

Table 2: Cry1Ac dose response on *H. armigera* mortality

	LC_{50}	LC_{95}	Slope	p value	Chi square value
Purified Cry1Ac	0.04±0.01	0.33±0.13	7.72±1.501	0.94	0.04002
Trypsinized Cry1Ac	40±11.34	91.03±18.98	7.43±1.52	0.93	0.8294

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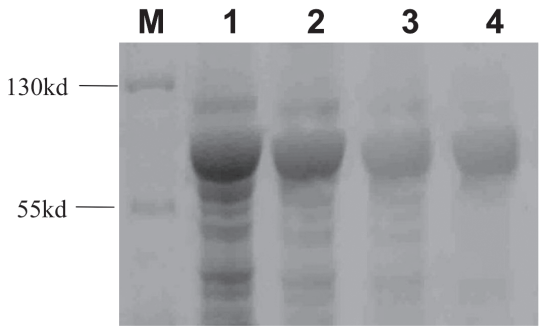

Fig. 3 Purification of the Cry1Ac protein with the protease digestion. M , Marker, lane 1 marker , lane 1 protease digestion for 15min. , lane 2 protease digestion for 30 min, lane 3 protease digestion for 1hour, lane 4 protease digestion for 4hours

Protease digestion of Cry1Ac toxins shows the presence of small peptides of molecular mass less than 40 kDa, as visualized on CBB stained SDS-PAGE gels. These peptides may be fragments generated by cleavage of large protein molecules. These small peptides (<40kDa) and the remaining protease were separated from the Cry1Ac protein (65 kDA) by extraction from the native polyacrylamide gel.

Extraction of Cry1Ac from polyacrylamide gel: Polyacrylamide gel strip (50 mg) containing Cry1Ac toxin was crushed to make a fine suspension in 1 ml gel extraction buffercarbonate buffer (pH 10) with 0.1% CHAPS.. Cry proteins are soluble at alkaline pH (>10.5) and hence the carbonate buffer (pH 10) was used for the protein extraction from polyacrylamide gel. To facilitate the removal of protein from the gel, CHAPS at a concentration of 0.1% was used. CHAPS was selected because of its Ziwtterionic nature, low aggregation number (10) and high critical micelle concentration (6 mM). These properties make CHAPS suitable for removing active protein without affecting the protein structure and its easy removal from the protein solution. To recover the protein it was centrifuged at 4000 x g for 10 min in columns having hydrophilic PVDF membrane with a pore size 0.45µ (Ultrafree®-DA,Millipore). The flow through contains active Cry1Ac δ-endotoxin and CHAPS.

Regenerated cellulose (10kda MW) (Amicon ultra 4 Millipore) was used to remove CHAPS from the Cry protein. Protein solution containing CHAPS was loaded in the column and centrifuged in swing bucket centrifuge at 4000 x g for 15 min. This process removes most of the CHAPS solution. To remove the traces of detergent the concentrated protein solution was diluted with carbonate buffer (pH 10) and recentrifuged under the same conditions for 20 min. The protein sample was recovered from the columns by side by side swirling and resolved on SDS PAGE for analysis and quantification (Figure 4a).

Insect Bioassay: Biological activity of the purified Cry1Ac toxin was tested by insect feeding assay with neonate larvae of *H. armigera*. The effect of purified Cry1Ac toxin with percent mortality of neonate larvae is summarized (Table 1, Table 2). Total mortality was observed with 0.9 ng /cm2 concentration of purified Cry1Ac toxin while no mortality was observed in the control experiment. The data obtained was subjected to probit analysis and the lethal concentration was calculated. The LC_{50} and LC_{95} value for Cry1Ac protein were found to be 0.04 ng/cm^2 and 0.33 ng/cm^2, respectively, with 95 percent fiducial limit. The experiment was repeated five times and reproducible LC_{50} value was obtained. Because all the *Bt* Cry toxins are protease resistant and their size ranges from 60-65 kDa this method may be utilized to purify other *Bt* Cry toxins without any gene specific standardization. This method of purification gives a highly active Cry1Ac toxin. It may be because of the high level of purity achieved using this method. The hydrophilic PVDF membrane used in this method are low protein binding. The non-protein binding property of hydrophilic PVDF membranes has been successfully used in the removal of virus particles from the antibodies (26, 27, 28). The method of protein extraction from the polyacrylamide gel can be used for the

purification of any other protein in general.

Protease digestion of solubilized protein with trypsin and chymotrypsin confirmed that fraction of active protein (properly folded) in the recovered protein is very less. Insect bioassay with purified and properly folded Cry1Ac protein was performed on neonate larvae of H. armigera. There was about 125 times reduction in LC50 value (0.04 ng/cm2), compared with the earlier published best reports.

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Factors affecting Callus Induction in Mothbean [*Vigna aconitifolia* (Jacq.)

Hande V. P[1]., Chauhan R. M.[1], Dapke J. S.[*2] and Vanave P. B.[2]
[1]Department of Genetics & Plant Breeding, C. P. College of Agriculture SDAU, Sk Nagar, India
[2]Department of Genetics & Plant Breeding, N. M. College of Agriculture, NAU Navsari, India
[*]For Correspondence - jitudapke@gmail.com

Abstract

Mothbean [*Vigna aconitifolia* (Jacq.) Marechal] belongs to fabaceae family is an important food grain legume crop that is cultivated over the entire world. Five varieties of mothbean viz., GMO 1, GMO 2, CZM 2, Jwala and RMO 40 were investigated for callus induction. Cent per cent callus induction was obtained in both the explants of five varieties on different medium combinations tested.

Significant differences were observed among the varieties, explants, medium combinations and their two as well as three way interactions for both the days taken for callus initiation and callus fresh weight except for variety × explant × medium interaction in case of days taken for callus initiation. Among the different medium combinations tried, MS + 2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP recorded significantly minimum number of days (6.356) for callus initiation and produced the highest callus fresh weight (2.457 g). Of the different varieties tested, Jwala took minimum number of days to callus initiation (6.944) and produced the highest callus fresh weight (2.056 g). Among the explant types, the hypocotyl explants recorded the least number of days to callus initiation (7.660) and leaf explants produced the highest callus fresh weight (1.932 g). Considering the interaction effects, MS + 2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP for days to callus initiation and callus fresh weight were identified as the most suitable media for callus induction. MS medium supplemented with 1.0 mg l^{-1} 2, 4-D promoted the growth of friable and organized callus in all the varieties and hence, this medium was identified as the most suitable medium for callus maintenance.

Key words: In vitro, mothbean, callus culture

Introduction

Mothbean [*Vigna aconitifolia* (Jacq.) Marechal] (2n = 22) belongs to fabaceae family is an important food grain legume crop that is cultivated over the entire world. It is regarded as a quality pulse crop in India for its excellent protein quality, high digestibility and freedom from flatulent effects associated with other pulses. The seeds of *Vigna aconitifolia* are a rich source of protein and minerals, including calcium, magnesium, iron, zinc and manganese (9) and exhibit high levels of crude lipid. The crop is unable to fulfill increasing demand because of low yield potential, which thought to be result of biotic and abiotic factors as well as low genetic variability.

One of the major steps in any yield improvement program is the creation of a broad based gene pool. Interspecific hybridization has proved impossible due to cross-incompatibility and hybrid sterility. To date, a reproducible and reliable transformation system that would enable genes of interest to be inserted into mothbean lines is not available in existing genotypes. Consequently, genetic transformation combined with traditional breeding methods may prove helpful in improving both the quality and yield of mothbean.

Plant tissue culture offer new strategies for improvement of agricultural crops. Plant cell culture has provided a new and an exciting option for obtaining increased genetic variability relatively rapid and without much sophisticated technology (6). This has led the plant breeders to explore the feasibility of using alternative tissue culture techniques for the improvement of mothbean.

Materials and Methods

Experimental materials: The experimental materials include five genotypes of mothbean *viz*., GMO 1, GMO 2, CZM 2, Jwala and RMO 40. The most commonly grown varieties in a stressed ecosystem were used for the investigation. The seeds harvested from pureline were grown aseptically and hypocotyl and leaf were used as explants.

Experimental conditions*:* The culture room maintained at 25±1 ^{0}C temperature and about 50 per cent relative humidity. Uniform light was provided by cool white fluorescent tubes over a light/dark cycle of 16/8 hours. All the aseptic manipulations were performed carefully under the laminar airflow cabinet.

Culture medium: The Murashige and Skoog, (7) medium (MS) commonly used by several workers was used throughout the period of investigation. Modifications were made to this medium by supplementing with different growth regulators at different concentration depending upon the purpose of the individual experiment.

Preparation of explants: Healthy and uniform seeds were agitated thoroughly in dilute Teepol solution for 5 minutes, seeds were then rinsed under running tap water and then surface-sterilized with 0.1 per cent aqueous mercuric chloride solution for five minutes, followed by 4 to 5 rinses of two minutes duration in sterilized distilled water. Seeds were germinated aseptically on a medium containing salts and vitamins according to Murashige and Skoog (MS), 3 per cent sucrose and 0.8 per cent agar in magenta jars. Different explants were excised from five to seven day old seedling and used for the tissue culture studies.

Hypocotyl explants: The hypocotyl explants were taken from aseptically grown seven day old seedling during the course of investigation. The pieces of hypocotyls were excised from seedlings by removing shoots and roots using scalpel. One explant was placed horizontally on medium into each culture tube.

The leaf explants were prepared from seven day old *in vitro* grown seedlings. The leaves were cut into the uniform pieces of 5 mm^2 with the help of sharp scalpel. Each explants piece was inoculated per test tube in such a way that the lower surface of the explants remain in contact with medium

All the cultures were incubated in a culture room at 26±2ºC and 16/8 hours of light and dark regime. Observations on the induction of callus and other types of responses were recorded periodically. Fresh weight of callus was recorded after 30 days of inoculation. For this calli devoid of original explant tissues were taken out from individual test tubes and weighed in gram.

Callus maintenance: After recording observations from different explants and from different concentrations and combinations of 2, 4-D and K, actively growing calli were maintained routinely in the MS medium containing 1 mg l^{-1} 2, 4-D. The subculturing was done after every three weeks period.

Results and Discussion

The effect of medium, explant, genotype and their interactions on days taken for callus initiation: The mean comparison for days taken for callus initiation on the different media presented in Table 1. Among the different medium combinations tried, MS + 2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP recorded minimum number of days (6.356) for callus initiation. While, MS medium containing 1.0 mg l^{-1} 2, 4-D took significantly the maximum number of days (9.408) for callus initiation. It has been observed that an increase in 2, 4-D levels resulted in the

significant decrease in number of days taken for callus initiation. Similar results also obtained in mothbean (1).

Irrespective of different media and explants used (Table 2), the minimum number of days taken for callus initiation was recorded by the variety Jwala (6.944). The highest number of days for callus initiation was recorded by the variety CZM 2 (8.239). The distinct differences in callus induction amongst different genotypes of mothbean reported by (2).

Among the two explants (Table 3), hypocotyl explants recorded significantly the least number of days for callus initiation (7.660), while the maximum number of days was taken by the leaf explants (8.007). Similar also reported soft, friable and light cream yellow callus from hypocotyl explants of mothbean (1).

The results of variety × explant interaction as given in Table 4 showed that the hypocotyl explants of variety Jwala took the minimum number of days (6.700) for callus initiation. The

Table 1. The influence of different media on days taken for callus initiation

Sr. No.	Media (MS + mg l^{-1})	Mean
1	MS + 1.0 2, 4-D	9.408
2	MS + 2.0 2, 4-D	9.150
3	MS + 1.0 2, 4-D + 1.0 BAP	8.226
4	MS + 1.0 2, 4-D + 2.0 BAP	7.216
5	MS + 2.0 2, 4-D + 1.0 BAP	6.356
6	MS + 2.0 2, 4-D + 2.0 BAP	6.664
S.Em = 0.094	C.D. = 0.263	C.V. % = 8.210

Table 2. The influence of different varieties on days taken for callus initiation

Varieties	GMO 1	GMO 2	CZM 2	Jwala	RMO 40
Mean	8.070	8.027	8.239	6.944	8.035

S.Em = 0.086 C.D. = 0.240 C.V. % = 8.210

Table 3. The influence of various explants on days taken for callus initiation

Explants	Hypocotyl	Leaf
Mean	7.660	8.007

S.Em = 0.054 C.D. = 0.152 C.V. % = 8.210

Table 4. The influence of variety × explant interaction effect on days taken for callus initiation

Sr. No.	Explants	Varieties				
		GMO 1	GMO 2	CZM 2	Jwala	RMO 40
1.	Hypocotyl	7.587	8.157	8.168	6.700	7.952
2.	Leaf	8.533	7.897	8.310	7.187	8.117

S. Em = 0.122 C.D. = 0.340 C.V. % = 8.210

highest number of days (8.533) taken for callus initiation were recorded by the leaf explants of variety GMO 1.

The variety × medium interaction (Table 5) showed that the variety Jwala took significantly the least number of days (5.340) for callus initiation on MS medium supplemented with 2.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} BAP, which was at par with the medium MS + 1.0 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} BAP (5.590) and MS + 2.0 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} BAP (5.640) of the same variety. The maximum number of days (10.190) taken for callus initiation was observed for the variety GMO 1 on MS + 1.0 mg l^{-1} 2, 4-D.

The result of explants × medium interaction revealed that the hypocotyl explants took significantly the least number of days (6.160) for callus induction on MS + 2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP. On the other hand, maximum number of days (9.476) taken for callus induction was recorded by the leaf explants on MS medium containing 2.0 mg l^{-1} 2, 4-D (Table 6).

The results of variety × explant × medium interaction effect showed non-significant differences (Table 7). The results indicate that minimum number of days (5.300) taken for callus initiation was recorded with the hypocotyl explants of variety Jwala on MS + 2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP, followed by the variety GMO 1(6.160) with the same explant and medium combination. Further the days taken for callus initiation by the leaf explants of variety Jwala on MS +2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP took the least number of days (5.380) for callus initiation. The significant interaction effects of explant and media, variety and media indicates that all these factors are important in early response to callus induction.

Callus fresh weight (g): Among the different media tried for callus induction, MS medium supplemented with 2.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} BAP yielded significantly the highest callus fresh weight (2.457 g). The lowest callus fresh weight (1.531 g) was recorded on MS medium containing 1.0 mg l^{-1} 2, 4-D which was at par with the MS medium supplemented with 2.0 mg l^{-1} 2, 4-D (1.553 g) (Table 8). Similar results were reported by (8) in green gram.

Across different media and explants used, the variety Jwala produced significantly higher callus fresh weight (2.056 g) as compared to other four varieties. Significantly the lowest callus fresh weight (1.692 g) was recorded for the variety GMO 1 (Table 9). Among the two explants tried (Table 10), leaf explants recorded significantly the highest callus fresh weight (1.932 g) while the hypocotyl explants produced the least callus fresh weight (1.843 g). The differences in callus fresh weight due to different explants (8).

The variety × explant interaction effects (Table 11) showed that the leaf explants of variety Jwala produced significantly the highest callus fresh weight (2.102 g). On the other hand, the hypocotyl explants of variety GMO 1 recorded significantly the lowest callus fresh weight (1.663 g).

The results of variety × medium as given in Table 12 revealed that significantly the highest callus fresh weight (2.669 g) was produced by the variety Jwala on MS medium containing 2.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} BAP. Significantly the lowest callus fresh weight (1.416 g) was recorded by the variety CZM 2 on MS + 1.0 mg l^{-1} 2, 4-D and it was at par with the callus fresh weight (1.440 g) produced by the varieties GMO 1 on the same medium and the callus fresh weight (1.447 g) produced by the variety CZM 2 on the MS medium containing 2.0 mg l^{-1} 2, 4-D.

The results of explant × medium interaction are presented in Table 13. The maximum callus fresh weight (2.542 g) was produced by the leaf expalnts on MS medium supplemented with 2.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} BAP. Significantly the lowest callus fresh weight (1.444) was recorded by the hypocotyl explants on MS + 1.0 mg l^{-1} 2, 4-D.

The three way interaction effects (Table 14) indicated that the leaf explants of variety Jwala

Table 5. The influence of variety × medium interaction effect on days taken for callus initiation

Sr. No.	Media (MS + mg l^{-1})	GMO 1	GMO 2	CZM 2	Jwala	RMO 40
1	MS + 1.0 2, 4-D	10.190	9.380	9.140	9.220	9.110
2	MS + 2.0 2, 4-D	9.090	9.340	9.510	8.720	9.090
3	MS + 1.0 2, 4-D + 1.0 BAP	8.700	8.360	8.850	7.150	8.470
4	MS + 1.0 2, 4-D + 2.0 BAP	6.990	8.150	7.780	5.590	7.490
5	MS + 2.0 2, 4-D + 1.0 BAP	6.570	6.090	6.830	5.340	6.950
6	MS + 2.0 2, 4-D + 2.0 BAP	6.880	6.840	7.440	5.640	6.520

S.Em = 0.211 C.D. = 0.588 C.V. % = 8.210

Table 6. The influence of explant × medium interaction effect on days taken for callus initiation

Sr. No.	Media (MS + mg l^{-1})	Hypocotyl	Leaf
1	MS + 1.0 2, 4-D	9.424	9.392
2	MS + 2.0 2, 4-D	8.824	9.476
3	MS + 1.0 2, 4-D + 1.0 BAP	7.868	8.584
4	MS + 1.0 2, 4-D + 2.0 BAP	7.096	7.304
5	MS + 2.0 2, 4-D + 1.0 BAP	6.160	6.552
6	MS + 2.0 2, 4-D + 2.0 BAP	6.592	6.736

S.Em = 0.133 C.D. =0.372 C.V. % = 8.210

Table 8. The influence of different media on callus fresh weight (g)

Sr. No.	Media (MS + mg l^{-1})	Mean
1	MS + 1.0 2, 4-D	1.531
2	MS + 2.0 2, 4-D	1.533
3	MS + 1.0 2, 4-D + 1.0 BAP	1.674
4	MS + 1.0 2, 4-D + 2.0 BAP	1.926
5	MS + 2.0 2, 4-D + 1.0 BAP	2.457
6	MS + 2.0 2, 4-D + 2.0 BAP	2.204

S.Em = 0.010 C.D. = 0.027 C.V. % = 4.876

Table 9. The influence of different varieties on callus fresh weight (g)

Varieties	GMO 1	GMO 2	CZM 2	Jwala	RMO 40
Mean	1.692	1.840	1.854	2.056	1.991

S.Em = 0.009 C.D. =0.025 C.V. % = 4.876

Table 10. The influence of various explants on callus fresh weight (g)

Explants	Hypocotyl	Leaf
Mean	1.843	1.932

S.Em = 0.006 C.D. = 0.016 C.V. % = 4.876

Table 11. The influence of variety × explant interaction effect on callus fresh weight (g)

Sr. No.	Explants	Varieties				
		GMO 1	GMO 2	CZM 2	Jwala	RMO 40
1	Hypocotyl	1.663	1.763	1.801	2.010	1.978
2	Leaf	1.722	1.916	1.916	2.102	2.004

S.Em = 0.012 C.D. = 0.035 C.V. % = 4.876

Table 12. The influence of variety × medium interaction effect on callus fresh weight (g)

No.	Media (MS + mg l^{-1})	GMO 1	GMO 2	CZM 2	Jwala	RMO 40
1	MS + 1.0 2, 4-D	1.440	1.632	1.416	1.648	1.518
2	MS + 2.0 2, 4-D	1.504	1.484	1.447	1.642	1.590
3	MS + 1.0 2, 4-D + 1.0 BAP	1.509	1.550	1.732	1.819	1.760
4	MS + 1.0 2, 4-D + 2.0 BAP	1.693	1.838	1.793	2.155	2.149
5	MS + 2.0 2, 4-D + 1.0 BAP	2.059	2.441	2.520	2.669	2.596
6	MS + 2.0 2, 4-D + 2.0 BAP	1.949	2.093	2.242	2.402	2.335

S.Em = 0.022 C.D. = 0.060 C.V. % = 4.876

Table 13. The influence of explant × medium interaction effect on callus fresh weight (g)

Sr. No.	Media (MS + mg l^{-1})	Hypocotyl	Leaf
1	MS + 1.0 2, 4-D	1.444	1.618
2	MS + 2.0 2, 4-D	1.556	1.511
3	MS + 1.0 2, 4-D + 1.0 BAP	1.721	1.627
4	MS + 1.0 2, 4-D + 2.0 BAP	1.908	1.943
5	MS + 2.0 2, 4-D + 1.0 BAP	2.372	2.542
6	MS + 2.0 2, 4-D + 2.0 BAP	2.058	2.350

S.Em = 0.014 C.D. = 0.038 C.V. % = 4.876

yielded significantly the highest callus fresh weight (2.778 g) on MS medium containing 2.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} BAP. The lowest callus fresh weight was recorded by the hypocotyl explants of variety GMO2 on MS medium with 1.0 mg l^{-1} 2, 4-D however, it was at par with the results obtained for the varieties GMO 1 (1.418 g) and CZM 2 (1.436 g) with the same explants and on the same medium. The significant interaction between explant, variety and medium suggest that all these factors are paramount important to have maximum callus fresh weight in mothbean.

Callus maintenance: The callus culture on MS medium containing 1.0 mg l^{-1} 2, 4-D was greenish and light greenish in both hypocotyl and leaf explants of all the varieties, respectively. This medium was identified as suitable medium for the maintenance of callus. Thus, after recording observations from different explants and different concentrations and combinations of growth regulators, actively growing calli were maintained routinely after every three weeks on fresh MS medium supplemented with 1.0 mg l^{-1} 2, 4-D. The calli maintained on above said medium did not show any appreciable changes in morphology. The importance of lower levels of 2, 4-D for good

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Table 7. The influence of variety × explant × medium interaction effect on days taken for callus initiation

Sr. No.	Media (MS + mg l^{-1})	Explants									
		Hypocotyl					Leaf				
		Varieties					Varieties				
		GMO 1	GMO 2	CZM 2	Jwala	RMO 40	GMO 1	GMO 2	CZM 2	Jwala	RMO 40
1	MS + 1.0 2, 4-D	9.920	9.420	9.080	9.280	9.420	10.460	9.340	9.200	9.160	8.800
2	MS + 2.0 2, 4-D	8.580	9.060	9.540	8.240	8.700	9.600	9.620	9.480	9.200	9.480
3	MS + 1.0 2, 4-D + 1.0 BAP	7.980	8.080	8.480	6.480	8.320	9.420	8.640	8.420	7.820	8.620
4	MS + 1.0 2, 4-D + 2.0 BAP	6.660	8.600	7.420	5.420	7.380	7.320	7.700	8.140	5.760	7.600
5	MS + 2.0 2, 4-D + 1.0 BAP	6.160	6.300	6.440	5.300	6.600	6.980	5.880	7.220	5.380	7.300
6	MS + 2.0 2, 4-D + 2.0 BAP	6.220	7.480	7.480	5.480	6.300	7.540	6.200	7.400	5.800	6.740

S.Em = 0.298 C.D. = NS C.V. % = 8.210

Table 14. The influence of variety × explant × medium interaction effect on callus fresh weight (g)

Sr. No.	Media (MS + mg l^{-1})	Explants									
		Hypocotyl					Leaf				
		Varieties					Varieties				
		GMO 1	GMO 2	CZM 2	Jwala	RMO 40	GMO 1	GMO 2	CZM 2	Jwala	RMO 40
1	MS + 1.0 2, 4-D	1.418	1.386	1.436	1.490	1.490	1.462	1.878	1.396	1.806	1.546
2	MS + 2.0 2, 4-D	1.500	1.466	1.528	1.620	1.666	1.508	1.502	1.366	1.664	1.514
3	MS + 1.0 2, 4-D + 1.0 BAP	1.466	1.528	1.598	2.098	1.916	1.552	1.572	1.866	1.540	1.604
4	MS + 1.0 2, 4-D + 2.0 BAP	1.678	1.826	1.772	2.144	2.120	1.708	1.850	1.814	2.166	2.178
5	MS + 2.0 2, 4-D + 1.0 BAP	1.968	2.424	2.364	2.560	2.542	2.150	2.458	2.676	2.778	2.650
6	MS + 2.0 2, 4-D + 2.0 BAP	1.948	1.950	2.108	2.148	2.136	1.950	2.236	2.376	2.656	2.534

S.Em = 0.031 C.D. = 0.085 C.V. % = 4.876

proliferation of callus and as efficient promoter of friable callus has been recognized in mothbean (1, 3, 4 and 5).

Conclusion

From the present study, it concluded that the interaction effects, MS + 2.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} BAP for days to callus initiation and callus fresh weight identified as the most suitable media for callus induction. MS medium supplemented with 1.0 mg l^{-1} 2, 4-D promoted the growth of friable and organized callus in all the varieties and hence, this medium identified as the most suitable medium for callus maintenance.

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Bio-ethanol Production from Lignocellulosic Banana Waste Using Co-Culture Techniques

Rakesh Kumar Meena*, Rajni Sahu, Prashant Shukla and Sanket Thakur
Aditya Biotech Lab & Research Pvt Ltd., Raipur, Chhattisgarh, India
*For Correspondence - dbt.rakesh@gmail.com

Abstract

Ethanol is the next energy molecule and its production from various biomasses is becoming the need of the hour. Plant based biomass is sustainable and available in large quantities for ethanol production. However, separation of cellulose from lignin is important for the production of ethanol. Banana pseudostem is one such source available in large quantities as cellulosic biomass. In this experiment, we have isolated three different strains of yeast which were found to be better for ethanol production. This study investigates the influence of biological pre-treatment method on sugar conversion and ethanol production from banana wastes rich in lignocelluloses that are thrown away. The two different cellulose degrading bacteria (A2 and A3) were used as biological pre-treatment for 24h, 48h and 72h. The three wastes i.e. banana peel (BP), banana dry pseudostem (DS) and banana wet pseudostem (WS), were taken and fermented with activated *S. cerevisiae* separately and taken in separate YPDA (yeast, peptone, dextrose, agar) slants as an adapted organism and labelled as *S. cerevisiae* (D), *S. cerevisiae* (P) and *S. cerevisiae* (W). It was found that the A2 strain efficiently degraded the banana wastes in to its monomer in 72h. The total yield of ethanol was estimated by titration method. Among the three adapted organism, *S. cerevisiae* (D) was found as a good strain for ethanol production. The maximum yield by using dry pseudostem was 0.288 g/g of waste, while by using BP and WS produced 0.19 g/g and 0.2 g/g ethanol respectively.

Key words: Pseudostem, Cellulose, Co-culture, Biological pre-treatment, *S. cerevisiae,* cellulose degrading bacteria, ethanol, lignocellulose,

Introduction

Bioethanol is the most dominant biofuel, considered as a good alternative for liquid transportation fuels with powerful economic, environmental and strategic attributes. Although the usable energy produced by burning ethanol is 68% lower than that of petroleum fuel, the combustion of ethanol is clean (because it contains oxygen). Worldwide production capacity of ethanol in 2005 and 2006 was about 45 and 49 billion litres per year respectively and the total projected demand in 2015 is over 115 billion litres (1). Liquid biofuels are being researched mainly to replace the conventional liquid fuels, such as diesel and petrol. The advantage of the second generation biofuels is the fact that they do not compete directly with the food market. It is possible to use entire above-ground biomass of a plant, thus enabling better efficiency and land use. Downside of the second generation biofuel production is the need for large investments and sophisticated processing equipment, compared to the first generation. In the future, the production of ethanol is expected to include both, traditional grain/sugar crops and lignocellulosic materials. Production of ethanol from lignocellulosic raw material and utilizing it as a substitute for petrol could help promote rural development, reduce greenhouse gases, and achieve independence from outside energy providers (2). Banana pseudostem can be used as raw material for the

production of bioethanol. Banana pseudostem is abundantly available agriculture residues in subtropical and tropical regions. India is the largest producer of banana, contributing to 27% of world's banana production. After harvesting, 60 – 80 t/ha of banana pseudostem is generated in the field. In India, presently after extraction of fibre from pseudostem the resulting biomass is dumped on roadside or burnt or left *in situ* causing detrimental impact on environment. High concentration of holocellulose (72%) with low lignin content (10%) and its easy availability makes banana pseudostem as a potential source of lignocellulosic biomass which could be used for the production of bioethanol. Innovation in the study is that use of co-culture bacteria and yeast for the production of ethanol from banana pseudostem. Large amount of lignocellulosic wastes are generated through forestry and agricultural practices, from sugar industry, pulp and paper industries, timber industries and many agro-industries, bagasse, rice straw, wheat straw, cotton straw, corn stover, groundnut shells, wood, grasses, paper pulp and many others (3).

Materials and Methods

The harvested banana plants were collected from the fields of Aditya Biotech Lab & Research Pvt. Ltd, Chandandih, Nandanvan Road, Raipur, Chhattisgarh, India and Bakery yeast was procured from local market.

Sample preparation: After extraction of fibre from banana pseudostem, the rest of the biomass was categorised as follows. The pseudostem was taken as first pseudostem with juice i.e., wet biomass, second pseudostem without juice i.e., dried biomass and banana peel as third pseudostem.

Inoculum preparation: The bakery yeast was activated by suspending it in slightly warm autoclaved water under laminar air flow. Then activated yeast was taken on YPDA (yeast extract 10 g; peptone 20 g; dextrose 20 g and agar 15 g in per litre) slants by using inoculating loop. The activated yeast was inoculated in fermentation broth containing banana peel, banana dry pseudostem and banana wet pseudostem separately and incubated at 30°C for 48h at 100 rpm. Then the adapted yeast was taken in separate YPDA slants from these fermented broths for further production of ethanol from banana wastes.

Biological pre-treatment and fermentation: After collection of samples and inoculum preparation, the fermentation process was carried out. The batch cultures were carried out in 150 mL Erlenmeyer flasks containing dry pseudostem, wet pseudostem and peel in 50 mL distilled water separately. They were then inoculated with cellulose degrading bacteria (identified in our Laboratory) in each flask, after 24h, 48h and 72h incubation of broth at 30°C at 100 rpm separately then inoculated activated bakery yeast and incubated at 30°C at 100 rpm. Then the production of ethanol was analysed after 24h and 48h by using potassium dichromate method.

Quantitative analysis of bioethanol by potassium dichromate method: This method uses a redox titration to find the concentration of ethanol in an aqueous solution. The ethanol is oxidised to ethanoic acid by reacting it with an excess of potassium dichromate in acid.

$$2\ Cr^{2}O72" + 16\ H^{++} + 3\ C_2H_5OH\ '! \ 4\ Cr^{3++} + 11\ H2O + 3\ CH_3COOH$$

The amount of unreacted dichromate is then determined by adding potassium iodide solution which is also oxidised by the potassium dichromate forming iodine.

$$Cr^{2}O72" + 14\ H^{+} + 6\ I"'! \ 2\ Cr^{3++} + 3\ I2 + 7\ H_2O$$

The iodine is then titrated with a standard solution of sodium thiosulfate and the titration results are used to calculate the ethanol content of the original solution.

$$2\ S_2O_{32}" + I2\ '! \ S_4O_{62}" + 2\ I"$$

Procedure: Five mL of fermented broth was taken in 150 mL conical flask and made up the volume up to 125 mL. Then 20-20 mL of above aliquot was taken in three different 150 mL

conical flasks and 20 mL of 0.04 M potassium dichromate solution was added. Then ten mL of 40 % sulphuric acid was added, the conical flasks were sealed with aluminium foil. They were heated for 10 min in a boiling water bath at 50^0C. The flasks were removed and 2 g of potassium iodide was added in each flask and titrated with burette filled with 0.1 M sodium thiosulfate till ting green colour appeared. One to two mL of 1% starch indicator was added and started titration till equivalence point appeared. The same process was repeated with references (distilled water was taken in the place of aliquot).

Calculations: Average volume of sodium thiosulfate used was determined for sample titration. Average volume of sodium thiosulfate used was also determined for the blank titration (reference). The volume of the sodium thiosulfate solution used for the sample titration was subtracted from the volume used for the blank titration. This volume of the sodium thiosulfate solution was used to determine the alcohol concentration. The number of moles of sodium thiosulfate in this volume was calculated. Using the equations, the relationship between the moles of sodium thiosulfate and the moles of ethanol was determined as 1 mol of S_2O_{32}- is equivalent to 6 mol of Cr^2O72- and 2 mol of Cr^2O72- is equivalent to 3 mol of C_2H_5OH. Then 1 mol of S_2O_{32} - has been found equivalent to 0.25 mol of C_2H_5OH. We used this ratio to calculate the moles of alcohol in the sample solution. Finally the dilution factor was multiplied with it.

Results

The comparative analysis of bioethanol production from three different banana wastes by using two different cellulose degrading bacteria (A2 and A3) for pre-treatment method and fermented with adapted *Saccharomyces cerevisiae* (D, P and W) separately are given below

In the case of comparative study of bioethanol production by using adapted yeast with A2 cellulose degrading bacteria, maximum production was found in 72h pre-treated dry pseudostem (DS). The yield of ethanol was 0.288 g per g of waste after 24h of incubation with yeast (Fig. 1) but it gradually decreased after 48 h where the yield was 0.280 g/g of ethanol. Good yield by using banana peel (BP) and wet pseudostem (WS) was found to be 0.19 g/g of ethanol and 0.2 g/g of ethanol respectively. Similarly, high production was found in dry waste fermented with yeast (P) (Fig. 2). The yield was 0.223 g/g of ethanol but it decreased after 48 h.

Saccharomyces cerevisiae (W) produced 0.161 g/g of ethanol in 24h, but after 48h of fermentation, it yielded 0.173 g/g of ethanol. By using other banana wastes, the yield of ethanol was reduced (Fig. 3). The production of bioethanol by using A3 cellulose degrading bacteria (Fig. 4) was increased after 48 h of incubation. The total yield was found to be 0.135 g/g of ethanol in dry waste while the maximum production was 0.14 g/g of ethanol in banana peel after 24 h incubation with yeast. Maximum production of ethanol was found to be 0.196 g/g from wet pseudostem in 24 h while the production was reduced after 48 h (Fig. 5). Maximum production of ethanol was noticed by using BP and DS and it was found to be 0.186 g/g and 0.196 g/g ethanol respectively. The yield of ethanol by using yeast (W) was found to be 0.156 g/g after 48 h fermentation of dry waste. But by using other wastes, the yield of ethanol was reduced (Fig. 5).

Discussion

In this study, banana wastes were taken as a feedstock for the production of bioethanol. These wastes were banana peel (BP), dried pseudostem (DS) and wet pseudostem (WS). The wastes were pretreated separately with two different cellulose degrading bacteria (A2 and A3) for 24 h, 48h and 72h separately. After pretreatment, these wastes were fermented with adapted yeast. Higher yield was found in dry pseudostem with A2 cellulose degrading bacteria when compared with the other two wastes. Itelima (4) have used co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* for ethanol

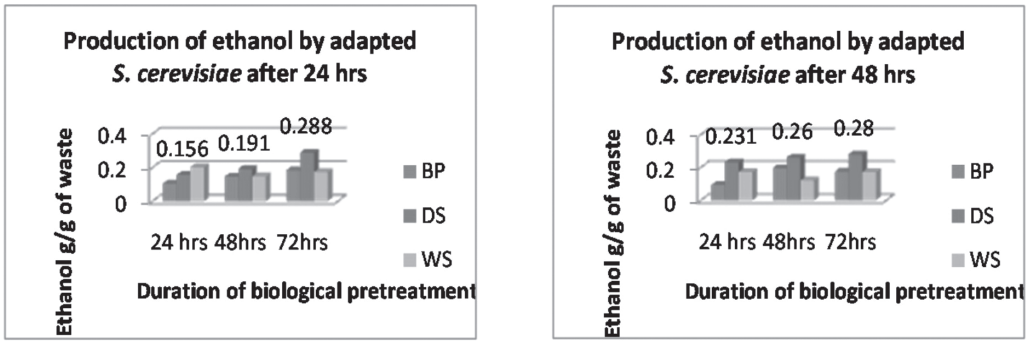


Fig. 1. Production of bioethanol by using A2 cellulose degrading bacteria with adapted *Saccharomyces cerevisiae* (Dry Mass)

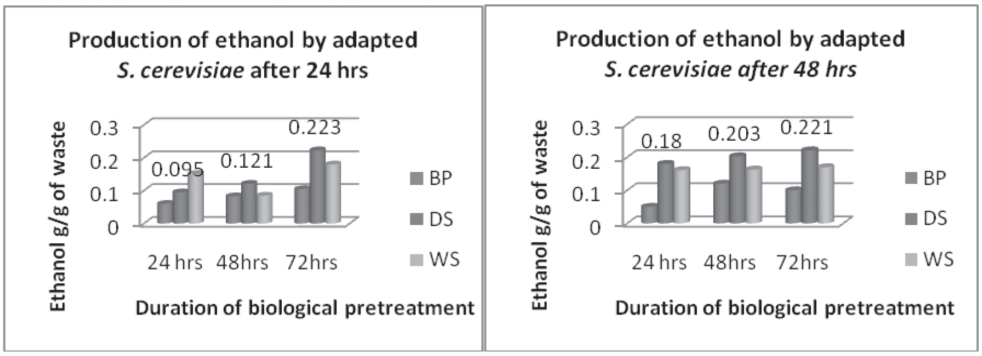


Fig. 2. Production of bioethanol by using A2 cellulose degrading bacteria with adapted *Saccharomyces cerevisiae* (Pulp)

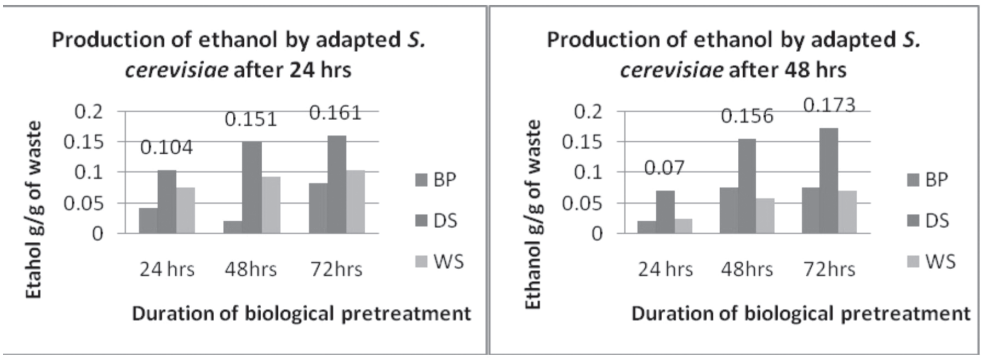


Fig. 3. Production of bioethanol by using A2 cellulose degrading bacteria with adapted *Saccharomyces cerevisiae* (Wet mass)

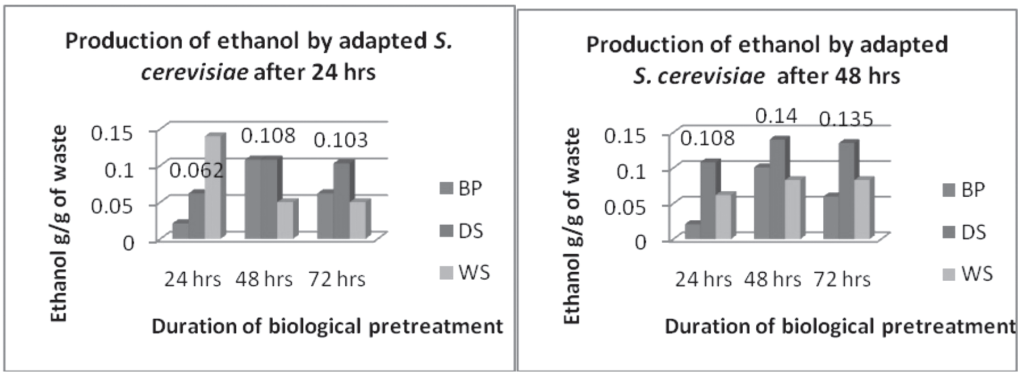


Fig. 4. Production of bioethanol by using A3 cellulose degrading bacteria with adapted *Saccharomyces cerevisiae* (Dry Mass)

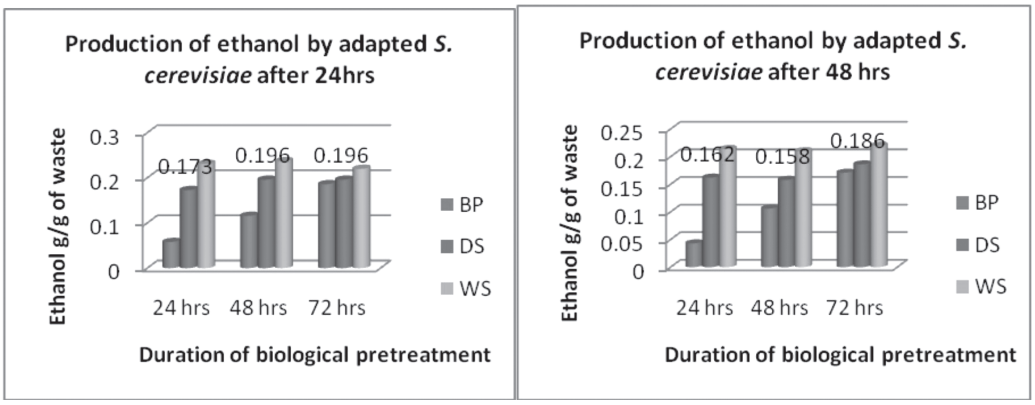
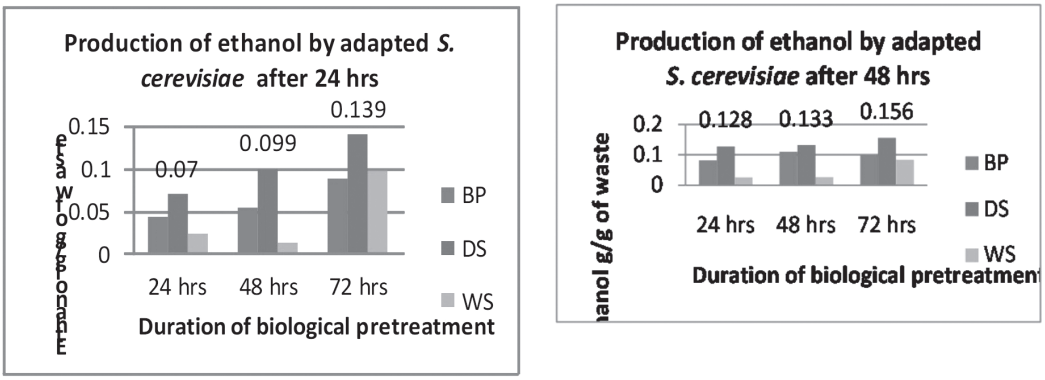


Fig. 5. Production of bioethanol by using A3 cellulose degrading bacteria with adapted *Saccharomyces cerevisiae* (Pulp)



production from corn cobs. Svetlitchnyi (5) have successfully developed a technique for consolidated bioprocessing for converting lignocellulosic biomass into ethanol using thermophilic consortia of bacteria. *Similarly, Park et al. (6) have described Acremonium cellulolyticus and Saccharomyces cerevisiae co-culture for ethanol production. Pichia stipites and Zymomonas mobilis co-culture was used for ethanol production by Laplace et al. (7). Lynd et al. (8) have described the use of consolidated bioprocessing of cellulosic biomass for ethanol production. Awan et al. (9) have described the effect of co-culture of Xanthomonas axonopodispv.* It has been observed that *Citri, Saccharomyces cerevisiae* and *Candida parapsilosis* have reduced the fermentation time significantly. A comparative study of ethanol production by using banana fruit, banana peel and banana pseudostem was reported earlier. In their study, they evaluated the acid hydrolysis of banana residues by using Aspen HYSYS(R) software for simulating the heating, hydrolysis, neutralization and cooling process. In the simulation of the hydrolysis of the pulp, peel, and pseudostem wastes, the energy consumed was found approximately 300.6 kJ/h for the pulp, 309.1 kJ/h for the peel, and 310.4 kJ/h for the pseudostem. The pseudostem was the most efficient glucose producer, producing approximately 0.87 g/h. For the analysis of the hydroxymethylfurfural formed, it was found that the highest production was for the pulp (mass fraction of 2.86 x 10-4), indicating that there is inhibition of production by this compound. The ratio of energy consumption to quantity sugars and glucose formed showed better results for the pseudostem, and this is the ideal waste product suited for hydrolysis because the highest quantity of sugar is formed in relation to the energy consumed (10).

Conclusions :

It can be concluded that the pseudostem is a good feedstock and A2 cellulose degrading bacteria efficiently degrade the dried pseudostem than the other pseudostem wastes in to its monomers, which were utilized by *Saccharomyces cerevisiae* for the production of ethanol. This is a good alternative technology for the production of ethanol from banana pseudostem waste because the farmers dispose off the banana waste after harvesting the crop and these waste can be utilized by this process. It will also help in controlling soil pollution.

Acknowledgement

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Comparison of the Efficacies of Different ELISA formats in Sero-detection of Antibodies to HPV 16 E7 Oncoprotein

Ravi Ranjan Verma [a], Rajan Sriraman [b], Samir Kumar Rana [c], NM Ponnanna [c], Shikha Srivastava[d], J K Roy [d], Sadhana Gupta [e] and V A Srinivasan [c *].

[a] Department of Biotechnology, Jawaharlal Nehru Technological University Hyderabad, Hyderabad, Telangana, and R&D Centre, Indian Immunologicals Ltd., Gachibowli P.O., Hyderabad 500032, India.
[b] B13/166 Sonarpura, Varanasi 221001, India
[c] National Dairy Development Board, R&D Laboratory, Gachibowli P.O., Hyderabad 500032, India.
[d]Department of Zoology, Banaras Hindu University, Varanasi 221005, India.
[e]Department of Obstetrics & Gynaecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India.
*For Correspondence - srinivasanva1948@gmail.com

Abstract

Different formats- the indirect ELISAs with either peptide or the E7 whole protein as antigens and the more recent GST-E7 capture ELISA have been employed in sero-epidemiological studies for the determination of HPV infection. Further, several studies have attempted to correlate sero-prevalence with prognosis and/or early diagnosis of cancer. This study compares the efficacy of ELISA in sero-detection when different forms of the E7 antigen *viz.,* E7 peptides, E7 whole-antigen, and GST-E7 fusion protein are used. The sera samples collected from hospital-visiting women were grouped into two categories- "suspect" negative (n=20; from women 18 years of age and negative for HPV 16 L1 antibodies determined by the VLP ELISA) and "suspect" positive (n=40; from women whose corresponding Pap smear samples showed presence of HPV 16 DNA and serum showed sero-positivity for HPV 16 L1 antibodies). The suspect negative sera showed absorbance readings below the respective cut-off points in all formats of the ELISA. However the suspect positive samples showed varied results with ~4%, ~20% and ~73% of samples showing absorbance readings above the cut-off in the E7 Peptide, E7 whole-antigen and GST-E7 capture ELISAs respectively. The results indicate that the GST-E7 based capture ELISA might be best suited for use in the sero - detection of antibodies in individuals with unknown disease or infection status.

Key words: HPV16 E7, E7 Peptide ELISA, E7 whole-antigen ELISA, GST-E7 Capture ELISA

Introduction

The Human Papillomavirus genotype 16 (HPV 16) is the predominant high-risk HPV type associated with squamous cell carcinoma (Cervical cancer, Head and neck cancer etc.). The hallmark of an HPV associated cancer is the long-latency from infection to full-blown cancer. Therefore, early prognosis is quintessential to the effective management of the disease. Mandatory screening through Pap smears, prompt follow-up and treatment is proven to effectively check disease progression and mortality in developed nations (1). But in lesser-developed regions the skill and resources needed to emulate the management strategy are scarce. Hence there has been intense interest in adopting tests such as ELISA for prognosis and early diagnosis (2).

The HPV E6 and E7 onco-proteins have been suggested as potential serological markers of HPV associated cancer since tumorigenic cells show constitutive expression of these viral proteins (3). ELISAs reported for the sero-detection of E7 specific antibodies employ the E7 antigen as peptides (4), recombinant whole-antigen (5) or as fusion antigens, most notably tagged with Glutathione-S-Transferase (GST-E7) (6).

This article presents the evaluation of the ELISA for detection of E7 antibodies with respect to the use of different forms of the E7 antigen in the assay.

Materials and Methods

Serum samples: Serum samples analyzed in this study were collected from consenting women who visited the Sir Sundarlal Hospital, Banaras Hindu University. Sera for comparison were classified into "suspect positive" and "suspect negative" based on the criteria detailed below.

Suspect positive sera (n=45): a. Sera (n= 45) from donors who also tested positive for HPV 16 DNA in the DNA hybridization assay using the commercial Linear Array HPV Genotyping Test Kit from Roche Molecular system, USA in the corresponding cervical smear samples as described elsewhere (7).

b. The above sera that also showed positivity for HPV 16 major capsid protein L1 in the VLP based ELISA as described elsewhere (8)

Suspected Negative sera (n=20): Sera obtained from girls below the age of 18 years and showing no reactivity in the VLP-based ELISAs

Genes, vectors and bacterial hosts: Codon-optimized HPV16 E7 gene for expression in E. coli was obtained from GeneArt, Germany as a plasmid borne synthetic construct. Plasmid propagation and sub-cloning were performed in the host strain, E. coli Top 10 cells (Life Technologies™, USA). The E7 gene was sub-cloned in the commercial expression vector pGEX4T1 (GE Healthcare, USA) between *Eco* RI and *Not* I sites. Expression of the GST-E7 fusion protein was performed in the in *E. coli* BL21 cells (GE Healthcare, USA).

Expression and Purification of GST-E7**:** Sub-cloning was performed following standard procedures (9). Briefly, The E7 gene from Geneart™ was digested with the *Eco* RI and *Not* I restriction enzymes (New England Biolabs Inc., USA) and inserted into a pGEX4T1. The insert: vector ligations were performed using the Rapid Ligation Kit™ from Roche, USA. pGEX4T1/E7 clones were propagated in E. coli Top 10 cells after transformation of the chemically competent bacterial cells. Further, pGEX4T1/E7 plasmids were obtained from overnight bacterial cultures of transforming E. coli Top 10 cells using the Qiagen MiniPrep Kits™ as per the manufacturer's instructions (Qiagen, USA).

Recombinant E7 expression was carried out in the E. coli BL21 strain cells (GE Healthcare, USA) by induction with 1mM IPTG (Genie, Bangalore) at an OD600 of 0.6. After an induction period of 4 h at 37°C the cells were lysed using a sonicator (Vibra-Cell™ VCX-700, Sonics & Materials Inc., USA) in Potassium phosphate buffer (pH 7.2) containing 0.2M NaCl, 5mM DTT and 2mM EDTA. The GST-E7 from the cell-lysates was then purified by affinity chromatography using the Glutathione Sepaharose 4B matrix (GE Healthcare, USA) according to manufacturer's instructions. Determination of expression and analysis of purification was performed by protein electrophoresis (PAGE) following standard methodologies (9).

E7 protein and E7 peptides**:** HPV16 E7 was obtained by the proteolytic digestion of the affinity-purified preparation of GST-E7 protein using the thrombin cleavage kit from Novagen® Darmstadt, Germany. After digestion, the mixture was subjected to Glutathione Sepharose 4B affinity chromatography for removal of GST. The residual thrombin (biotinylated) was removed by affinity chromatography using Streptavidin-Agarose matrix.

Synthetic overlapping peptides (20mer with 13mer overlaps) of the full-length E7 protein were obtained from JPT Peptide Technologies, GmbH, Germany.

Indirect peptide and E7 ELISA: Maxisorp™ 96-well plates (Nunc; Thermo Scientific, USA) were coated overnight at 4°C with either HPV 16 E7 (for E7 ELISA) or with the Synthetic E7 peptides (Peptide ELISA) at the concentration of 200 ng / well/100µl in 0.05M carbonate buffer (pH 9.6). Blocking was done by incubation (1.0h) with 2% casein in phosphate buffer saline contain 0.05% Tween 20 (PBST) Sera for analysis (suspected positive and suspected negative) were added in triplicates at a dilution of 1:25. To facilitate the antigen-antibody binding, plates were incubated for 1h at 37°C. Bound antibodies were then probed with 1:5000 dilution of goat anti-human IgG peroxidase™ (ABD Serotec, UK). Between the ELISA steps plates were washed with PBST using a Microtitre plate Washer (BioRad, USA). The assay was developed with 3, 3, 5, 5-Tetramethylbenzidine Liquid Substrate system (Sigma-Aldrich, USA). Post development for 10 min, the reaction was stopped by the addition of 1.25N H_2SO_4. The absorbance at 450nm was measured in the Microtitre Plate absorbance reader (Beckman Coulter DTX 880 Multimode detector USA). Appropriate experimental controls, *viz.,* wells treated with influenza peptides (for peptide ELISA) and GST protein (for indirect E7 whole antigen ELISA) was incorporated in the assays. Commercial monoclonal antibody to HPV16 E7 (Santa Cruz, USA) served as the positive antibody control in the assays.

Glutathione capture ELISA: Maxisorp™ 96-well Microtitre plate wells were coated with 200ng/ well/of casein- glutathione in 0.05M carbonate buffer, pH9.6 and were incubated overnight at 4°C. Wells were blocked with 2% casein in PBST for 1.0h. Binding antigens, either GST-E7 or only GST at 200ng/well were added and incubated at 37°C for 1h. Sera for evaluation were added in triplicates at 1:25 dilution. The rest of the ELISA steps *viz.,* developing, stopping and recording absorbance was done as detailed above for the E7 protein and peptide ELISAs.

Determination of Cut-off value: As reported elsewhere (10), the cut-off value was decided as the average plus three times standard deviation of the absorbance values obtained for experimental negative sample in triplicates in the ELISAs. For the peptide ELISA wells coated in triplicates with long-peptides of influenza formed the experimental negative samples. For the E7 whole antigen indirect ELISA and GST-E7 capture ELISA wells coated in triplicates with recombinant GST alone represented the experimental negative samples.

Comparison of the ELISA results: The absorbance values in the ELISA were presented in a Box-and-Whisker plot using the Origin pro software.

Results

Purified proteins, GST-E7 and E7: The recombinant GST-E7 expressed and purified from the *E. coli* BL21 showed a 37.0 kDa product in SDS-PAGE. Recombinant E7, obtained as a thrombin cleavage product from the fusion GST-E7, subscribed to the size of ~12kDa (Figure 1).

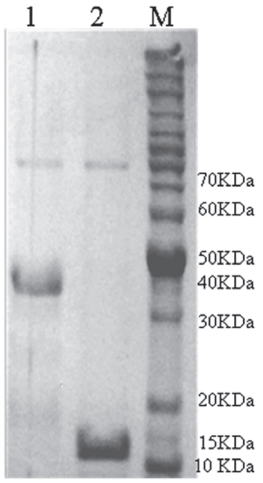

Fig. 1. SDS-PAGE profile Lane 1- Affinity purified HPV16 GST E7 protein; Lane 2: HPV16 E7 protein after thrombin cleavage; Lane M: Protein molecular-

Cut-off points: The cut-off point for the E7 peptide was A_{450} of 0.146 while the cut-off point for E7 whole-antigen and GST-E7 capture ELISAs was A_{450}= 0.148.

Comparison of the three ELISA formats: Out of the 45 suspect positive sera 2 (~4%), 9 (20%), and 33 (73%) samples showed positivity in the peptide, whole-antigen (E7) and capture (GST-E7) ELISAs respectively. None of the suspect-negative sera (20 samples) showed absorbance values higher than the cut-off in any of the ELISAs viz., Peptide, whole-antigen E7 or the capture ELISA.

Discussion

Approach of the comparative study: Different ELISA protocols have been reported (4, 5 and 6) for the detection of antibodies to HPV. Early studies involving the detection of antibodies to E7 have mostly been performed with indirect ELISAs using synthetic E7 peptides (13, 14). The ease of availability of synthetic peptides rather than the considerable effort involved in the generation of recombinant protein may have been the reason for the choice of antigen. E7 peptide ELISAs, even among cancer patients have usually reported seroprevalence of only about 30% of the evaluated cases (14).

Capture ELISA using recombinant GST-E7 generated from E. coli has been a more recent advent for serological detection of antibodies to E7 and other HPV proteins (6). Recent reports pitch for the GST- E7 capture ELISA as an economical yet specific assay for detection of antibodies against HPV proteins, including E7 owing to the presence of the GST tag (6). A recent article reports nearly 66% of the cervical cancer patients showing seroprevalence of antibodies to HPV proteins E6 and/or E7 (13). One of the reasons that may be accorded to the increased sensitivity of the GST fusion protein based capture ELISA is the antigen in holistic form *vis-a-vis* peptides.

Here, we have attempted to compare the effectiveness of the peptide ELISA, whole-antigen ELISA and GST-E7 based capture ELISA in sero-detection of antibodies. Available literature points at a distinct, positive correlation between seroprevalence of antibodies to HPV proteins and progression towards cervical cancer (15, 16). Further, it has been reported that persons with persistent infection often show antibodies to HPV proteins L1, E6 and E7. On this premise, the serum samples were grouped into suspect positive. And therefore these serum samples were from individuals whose cervical cytological samples showed the presence of HPV 16 DNA while also the sera contained antibodies to HPV 16 L1, the outer capsid protein.

Sexual activity has been known to facilitate HPV infection and transmission (11). Therefore the suspect negative samples constituted sera from young unmarried women (=also below 18 years) which also did not show L1 antibodies. With this grouping of serum samples used in the comparative study, we reasoned a call on the effectiveness of the each of the different formats of the assay could be made with better certainty.

The results of the ELISA as represented in the Box-and-Whisker plot (Figure 2) indicate that the capture ELISA is the best format for the detection of the sero prevalence. Nearly 73% of the suspect positive samples showed absorbance values higher than the cut off point for the ELISA. While the E7 whole antigen ELISA fared better than the peptide ELISA (20% vs 4%) the results are more in line with the previously reported studies (17). The high percentage of positivity for antibodies to onco-proteins E6 and E7 with individuals most likely harbouring HPV has been reported recently using the GST capture ELISA (15).

The large difference between peptide ELISA and the GST-E7capture ELISA in detecting antibodies may be attributed to the profound difference in the physical conformation of the antigens (peptide vs GST-E7). Linear peptides can be expected to discount largely or even completely the conformation specific antibodies. GST-E7 on the other hand can expectedly display E7 protein in a tertiary

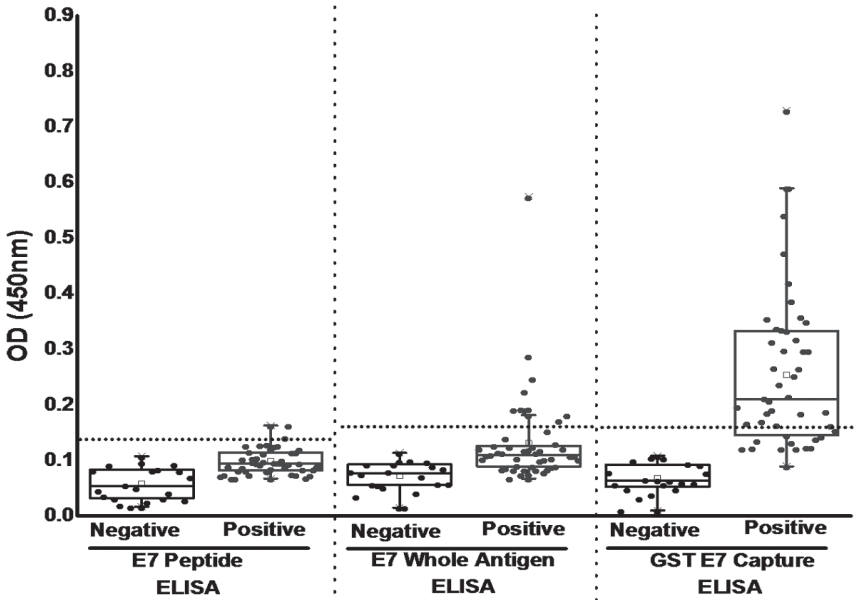

Fig. 2. Comparison of three ELISA formats for the determination of anti-HPV16 E7 antibodies in sera represented in a Box-and-Whisker plot. The graph depicts the spread of samples ordered based on the absorbance values. Whiskers and boxes represent interquartile regions, with the lower boundary of each box representing the 25th percentile and the upper boundary, the 75th percentile. The line inside the boxes represents the median value. The whiskers represent the 5th and 95th percentile. Points beyond the whiskers are samples that are outliers.

configuration and ensures fully accessibility for antibody binding in the capture ELISA. The low number positive samples in the E7 whole antigen ELISA relative to the capture ELISA need further investigation. However, it may be mentioned that the reported propensity of E7 to aggregate at near neutral pH (18) may have some bearing on antigenic form influencing the detection. Besides, it is also not uncommon for the distortion of antigen on binding to microtitre plate wells (19), especially with high-binding surfaces (MaxiSorp™).

Conclusion:

The results indicate that the GST-E7 based capture ELISA might be best suited for use in sero-detection of antibodies in individuals with unknown disease or infection status. Additionally the GST tag confers significant advantage in protein purification. Hence the GST-E7 based capture format of ELISA might find use in sero-epidemiological studies and probably a role in prognosis and/or early- diagnosis of cancer.

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Immobilization of Laccase Produced by *Pycnoporus sanguineus* for Reuse in the Remediation of Catechol

L. F. Garcia, G. S. Lobón, M. F. Santiago and E. de S. Gil*
Department a Faculty of Pharmacy, Federal University of Goiás,
AV. Universitária S/N, 74605-220 Goiânia, GO, Brazil.
*For correspondence - ericsgil@gmail.com

Abstract

The catechol (1,2-dihydroxybenzene) can be oxidized by enzymatic action, and then turned into quinone. Laccase (EC 1.10.3.2) is an enzyme produced mainly by fungi of white decomposition, which contains copper, and catalyzes the oxidation of phenolic substrates by coupling the reduction of O_2 to water. The aim of this study is to evaluate the reutilization of Alginate Chitosan Laccase (ACL) beads for remediation of the catechol. In this study, the laccase was produced by the fungus *Pycnoporus sanguineus*. For the immobilization of the enzyme, 2% calcium alginate and the culture supernatant of the fungus cultivation were used. The mixture was dropped into a solution containing 2% $CaCl_2$, for formation of beads. To ensure resistance enabling the reuse the beads, they were immersed in 0.3% chitosan solution. Enzymatic activity, optimum pH and optimum temperature were determined for the ACL beads and free enzyme. For reuse assay, 13 enzymatic cycles were realized, under agitation. As a result, we obtained optimum pH of 5.0 for free enzyme and ACL beads, optimum temperature of 40 °C for free enzyme and 50 °C for ACL beads. In the reuse test, it was shown that after 13 cycles of enzymatic activity, *i.e.* oxidation of catechol, it was stable until the 7º cycle. It can be concluded that the present study was effective for immobilization of laccase enzyme and oxidation of catechol.

Key words : Bioremediation, micropollutants, phenols, laccase.

Introduction

Every day, a huge amount of untreated sewage is dumped into the water resources, thus having a global impact over the aquatic ecology. The phenolic compounds figure as one of the most widespread and dangerous pollutants to the environment. Thus, since such pollutants are very typical in industrial activities, their removal and or transformation in safer compounds are imperative. Indeed, the safety limits of phenolic compounds are established by legislation, and CONAMA 357 is the related resolution in Brazil (1).

The removal efficiency of pollutants depends on the treatment suitability, according to the complexity of aquatic system. For instance, physico-chemical (eg. filtration, adsorption, decanting, settling, flocculation and distillation), chemical (eg. incineration, photocatalysis, ozonation, anodization) and biological (eg. bioremediation, phytoremediation) methods of treatment, as well as their combinations have been used to achieve this task. The physico-chemical processes, generally referred to as primary treatments, consist basically in the separation, transfer or transition of phases, in which the contaminant is concentrated in a reduced phase, remaining unchanged. Hence, it is necessary to use additional techniques that are able to destroy the pollutant, namely a secondary treatment process (2, 3).

In this context, biological processes based on the use of fungi and / or bacteria, have been

used to convert the pollutant into less toxic products, *i.e.* CO_2 and H_2O in aerobic fermentation or CO_2 and CH_4 anaerobic fermentation (2, 4). Therefore, concerning to the toxicity of generated compounds, such alternatives of treatment are more attractive than chemical processes, *i.e.* dioxins and furans coming from incineration and organochlorines from chlorine injection (2, 3).

The phenolic compounds can be biochemically oxidized into their quinones by many enzymes, namely the oxidoreductases or phenoloxidase (5, 6). Thus, laccase, manganese peroxidase, lignin peroxidase are few examples of oxidoreductases that have been studied for biological treatment of xenobiotics (7, 8).

The laccases, benzenediol oxygen oxidoreductases, are metalloenzymes presenting four copper atoms at the catalytic site. They are able to use oxygen to oxidize a great variety of organic compounds that act as hydrogen donors (9). In fact, over a hundred compounds were identified as substrates for fungal laccases. Hence, owing to their broad activity, these enzymes have been the target of many researchers, especially ones focusing on environmental biotechnology, *i.e.* bioremediation, biosensors for environmental monitoring and green chemistry fields (10).

Despite the low specificity of laccases, their activities, as well as the stability vary regarding the experimental conditions, mainly the immobilization procedure, pH and temperature. As consequence, the applicability of such enzymes can be modulated (11).

The immobilizing procedure reinforces the structural stability avoiding conformational changes and chemical inactivation, resulting in more pure products and allowing the reuse (12).

Among the different methods of immobilization, the entrapment in biopolymeric matrices, such as alginates offers many advantages, including: biocompatibility, lower occurrence of denaturation, low cost and eco-friendly (12, 13).

On the other hand, beads of calcium alginate have showed to present low mechanical stability and high porous dimension, leading high lixiviation rate of entrapped enzymes (13, 14). Therefore, many researchers have been looking for new polymeric materials. In turn, the ionic environment of the biopolymer chitosan has been explored in order to enhance the electrostatic properties of alginate. The resulting alginate-chitosan polymeric membrane exhibited higher mechanical stability and low porosity. Furthermore, the strengthening of electrostatic interactions between proteins and the polymeric system hampered the lixiviation process, increasing the life cycle (15).

Therefore, the aim of this work was the development and optimization of immobilization procedures of laccase (L) (*Pycnoporus sanguineus*) by entrapment in beads of alginate (A) - chitosan (C) and applie the best system on the bioremediation of phenols.

Materials and Methods

Organism and culture conditions: *Pycnoporus sanguineus* (CCT-4518) was obtained from Fundação André Tosello, Campinas, São Paulo, Brazil. The fungus was grown on Petri dish with Potato Dextrose Agar (PDA) at 37 ºC, for 7 days. Five fungal discs, measuring 5 mm in diameter taken from the borders of PDA cultures, were transferred to Erlenmeyer flasks (250mL) with 50mL of liquid medium, containing 1.25% (w/v) of malt extract, 0.0005% (w/v) of $CuSO_4.H_2O$, and 50 $mg.L^{-1}$ of 2,5-xylidine (16). Laccase activity was monitored at intervals 24 hours.

Enzymatic activity assay: Laccase activity was determined using: 0.1 M catechol ($å_{390\,nm}$ = 1417 $M.cm^{-1}$) (17). The reaction mixture contained: substrate, 50 mM sodium acetate buffer (pH 5.0), and the culture supernatant (free enzyme) or immobilized enzyme. The oxidation of the substrates was measured after 5 min in spectrophotometer. An enzyme unit U was defined as the amount of enzyme required to oxidize 1ìM of substrate per minute. The result is expressed in $U.mL^{-1}$.

Preparing the Alginate: Chitosan: Laccase (ACL) beads: For immobilization, varied concentrations of sodium alginate (0.5, 1, 1.5 and 2% w/v), calcium chloride (0.5, 1, 1.5 and 2% w/v) and chitosan (0.1, 0.2 and 0.3% w/v, prepared by dissolving chitosan in 0.1 M acetic acid solution), were used to achieve the best immobilization and yield (14, 15).

In assay tubes were added the culture supernatant with laccase (0.5, 1, 1.5 and 2 mL) and an alginate solution volume to 4 mL complete. The ACL beads were formed by dripping the mixture into 10 mL of $CaCl_2$ solution, the process was stirred at 150 rpm for 1 hour.

The formed ACL beads were immersed for 30 minutes in 5 mL of the solution of chitosan. Then, they were harvested and washed with 0.5% $CaCl_2$ (w/v) to remove the chitosan solution which was not incorporated. Each experiment was run in triplicate.

Enzyme activity in $CaCl_2$ solution, used in the formation of ACL beads, in solution of chitosan and in 0.5% $CaCl_2$ washings, called residual activity, were determined. The percentages of immobilization and yield were defined as:

$$\%\,Immobilization = \frac{Activity\ of\ free\ enzyme - Residual\ activity}{Activity\ of\ free\ enzyme} x100$$

$$\%\,Yield = \frac{Activity\ of\ immobilized\ enzyme}{Activity\ of\ free\ enzyme} x100$$

Stability tests: The optimum pHs of free laccase and ACL beads were compared and studied by incubating the samples in 0.2 M sodium acetate buffer ranging from pH 3.5-5.5 and 0.2 M sodium phosphate buffer pH 6.0. The enzymatic activity was estimated for all the different pH ranges as described in the Section "Enzymatic activity assay".

The optimum temperatures of free laccase and ACL beads were determined in the range of 20-80 °C. Both were incubated in 50 mM sodium acetate buffer (pH 5.0) for 5 minutes, for each temperature. After this period, the catechol substrate was added, and incubated for another 5 minutes, according to Section "Enzymatic activity assay".

Thermal stability was assayed by incubating the free enzyme and ACL beads simultaneously at 50 °C for 60 minutes. The activity was measured as described in the Section "Enzymatic activity assay".

Storage stability assay was performed to determine the stabilities of free and immobilized laccase. For storage stability measurements, ACL beads were kept at 4 ºC in distilled water. The activity of ACL beads was followed for 7 days and determined by the laccase activity assay procedure reported in the Section "Enzymatic activity assay".

Reuse assay of ACL beads: 13 tests for reuse with the same batch of ACL beads with catechol substrate were performed. Each cycle lasted 5 minutes. The beads were removed from the solution and washed with 50 mM sodium acetate buffer (pH 5.0) at least 5 times.

Results and Discussion

Laccase production: The laccase was produced by using strains of the fungus, *Pycnoporus sanguineus* CCT-4518, according to methodology well established in our lab team (11). The enzymatic activity was checked each 24 h, and the peak production was achieved in the third day (Figure 01). The efficiency of production is similar those obtained in previous works (7).

ACL beads*:* Besides the optimum proportion between, enzyme and biopolymers, the optimization of beads of alginate-chitosan was performed by evaluating three main parameters, pH, temperature and of ion calcium (18). Whereas, the porosity of such polymeric system is the main indicator to reach the ideal conditions (15). According to literature data, the best concentration of alginate and calcium is of 2% for each reagent (14, 15) and the same results

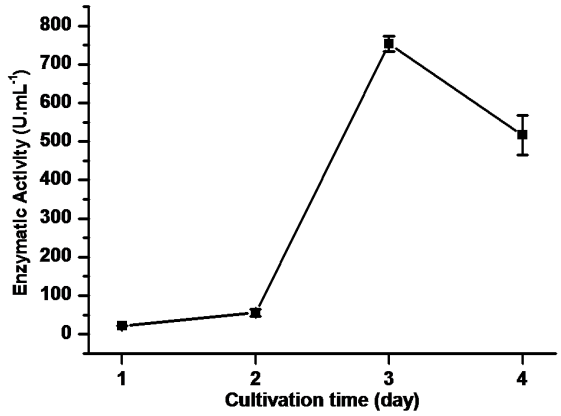

Fig. 1. Enzymatic Production of *Pycnoporus sanguineus* according to the cultivation time. Enzymatic activity determined with the catechol substrate.

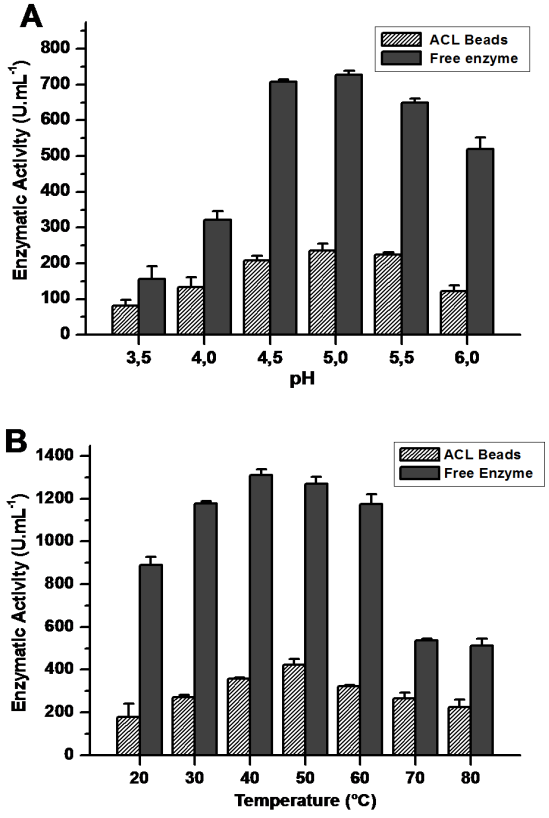

Fig. 2. Enzymatic activity of laccase (*P. sanguineus*) on the catechol oxidation for different pH (A) and

were herein obtained. Yet the best concentration chitosan was of 0.3%. The Table 01, presents the optimum conditions for the preparation of beads.

The final proportion between enzyme and biopolymer system was of 0.5/3.5 (v/v). At this condition, the beads presented diameter of 3.5 0.5 mm and have exhibited higher catalytic performance. The final enzymatic activity of c.a 40.42% (± 0.50) is close to the results obtained in similar systems (Table-2).

Stability tests: The influence of pH and temperature on the laccase activity was evaluated. Moreover, in order to make valuable comparisons, the free and immobilized enzyme and two different substrates, seryngaldazine and catechol were used in these experiments. The results obtained for catechol are presented in Fig. 2.

The Fig. 2-A show that the higher enzymatic activity of ACL beads against catechol was achieved at pH 5.0.

In turn, the optimum temperature for all substrates was of 50 ºC. Akin to the ACL beads, for the free enzyme the optimum pH was from 5.0 and temperature from to 40 ºC. Such results are in agreement with previous works (11, 19).

Catapane et al. (20), for the laccase from *Trametes versicolor* immobilized on polyacrylonitrile (PAN) and Geethanjali and Subash (21) for protease immobilized in calcium alginate beads, found that the optimum pH was not changed and optimum temperature increased by 10 °C for the enzyme immobilized, similar to the data found. The improvement in the resistance against temperature might be due to enzyme immobilization at polymeric matrix. In fact, the polymer support provides stability allowing enzyme-substrate complex formation (22).

The thermal stability of the free and ACL beads was investigated at 50 ºC in 60 minutes (Figure 03). The laccase of *P. sanguineus* is quite resistant to temperature 50 ºC, which is the optimum temperature (11). Was obtained 27.28%

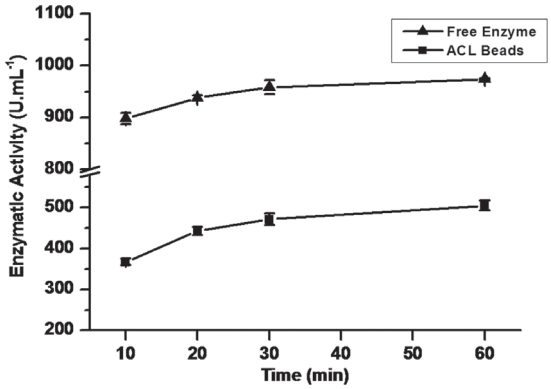

Figure 03: Thermo stability of free laccase and ACL beads at 50 ºC.

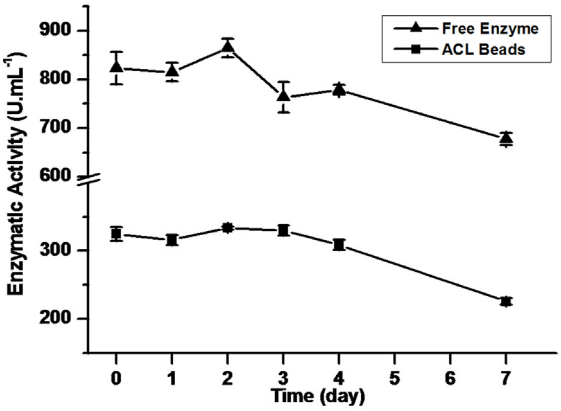

Fig. 4 Time stability of free laccase and ACL beads.

Table-1. Comparative studies involving alginate beads for enzyme immobilization against different substracts.

Polymeric System	Substract	% Yield	Reference
Alginate:Chitosan:Laccase	Syringaldazine	17.93	Our work
Alginate:Chitosan:Laccase	ABTS	34.22	Our work
Alginate:Chitosan:Laccase	Guiacol	15.33	Our work
Alginate:Clay:Alpha-amylase, Glucoamylase and Cellulase	Cassava roots	52.14	(13)
Alginate:Chitosan:Laccase	ABTS	46.93	(15)
Alginate:Laccase	ABTS	93.3	(14)
Alginate:Laccase	Grey dye Lanaset	49.0	(14)
Alginate:Chitosan:Catechol 1,2-Dioxigenase	Catechol	56.0	(12)

increase in enzymatic activity for ACL beads and only 7.75% for the free enzyme. Lu, Zhao and Wang (15) and Daâssi et al (14) also have obtained higher activity for immobilized enzyme.

The ACL beads were stored at 4 °C in distilled water to determine the storage stability. After 7 days of storage, the residual ACL beads activity was retained at 80.43%, and free laccase was retained at 82.26% (Figure 04), which demonstrates good storage stability.

The stability of ACL beads was testified after repetitive cycles of catechol oxidation

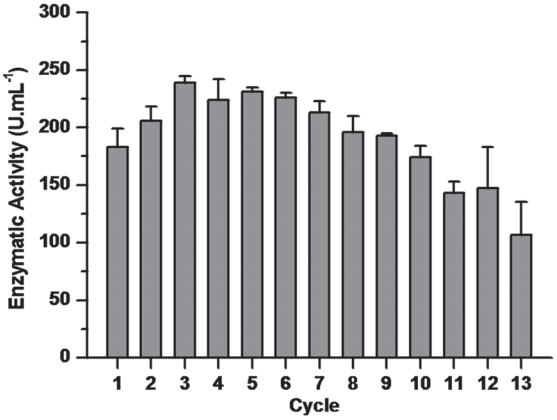

Fig. 5. Reuse of ACL beads for catechol oxidatiom. Each cycle of oxidation occurring within 5 min, pH 5.0 and room temperature.

(Figure 05). It was observed that the ACL beads activity remained almost stable till the seventh cycle of five minutes of enzymatic oxidation. Then the activity started falling as consequence of enzyme lixiviation due to ACL washing procedures.

The remaining activity of 70% until the twelfth cycle, is similar to the results obtained by Yanto, Tachibana and Itho (23), but higher than the results obtained by Lu, Zhao and Wang (15), in which was obtained only 35% of initial performance.

Conclusion

The immobilization in beads of alginate and chitosan, showed to be a good alternative to increase the life cycle and applicability of laccase from *Pycnoporus sanguineus*. The enzymatic

Table 2. Experimental parameters evaluated on the development of ACL beads

Experimental Condition	Range evaluated	% Immobilization	% Yield	Optimum Performance
Alginate (%) w/v	1	49.48 (± 0.37)	22.26 (± 2.53)	2
	1.5	61.46 (± 2.68)	27.97 (± 0.71)	
	2	80.34 (± 0.40)	40.42 (± 0.50)	
	2.5	58.85 (± 1.05)	29.07 (± 3.11)	
Calcium Chloride (%) w/v	1	67.71 (± 1.52)	29.44 (± 3.01)	2
	1.5	72.22 (±1.01)	29.62 (± 1.68)	
	2	80.34 (± 0.40)	40.42 (± 0.50)	
	2.5	73.26 (± 0.51)	28.70 (± 1.42)	
Chitosan (%) w/v	0.0	76.92 (± 2.90)	20.03 (± 1.47)	0.3
	0.1	78.63 (± 2.47)	20.91 (± 1.46)	
	0.2	81.20 (± 1.60)	21.44 (± 2.52)	
	0.3	80.34 (± 0.40)	40.42 (± 0.50)	
Laccase Extract/Alginate v/v	0.5/3.5	80.34 (± 0.40)	40.42 (± 0.50)	0.5/3.5
	1/3	50.72 (± 0.16)	17.73 (±0.15)	
	1.5/2.5	46.24 (± 1.21)	11.70 (±0.59)	
	2/2	45.52 (± 0.17)	10.01 (±0.20)	

activity of ACL remained quite good, being observed no change in the optimum pH, whereas the slight increase of optimum temperature.

The ACL beads kept high catalytic efficiency for catechol oxidation even after repetitive reuse with the same batch of immobilized enzymes.

Since laccases have broad activity over many polluting compounds, the methodology of immobilization can be extended to other processes.

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Biotechnological Approaches to Evolve Sorghum (*Sorghum bicolor* (L.) Moench) for Drought Stress Tolerance and Shoot fly Resistance

K N S Usha Kiranmayee[1,2],C Tom Hash[3], Santosh P Deshpande[1], K V G K Varaprasadand P B Kavi Kishor[2]

[1]International Crops Research Institute for the Semi-Arid Tropics, Hyderabad 502324, India
[2]Department of Genetics, Osmania University, Hyderabad 500007, India
[3]International Crop Research Institute for the Semi-Arid Tropics, BP12404, Niamey, Niger
*For Correspondence - s.deshpande@cgiar.org

Abstract

Sorghum is a model tropical grass that uses C_4 photosynthetic activity.But its yield is affected by many abiotic stresses likeheat, drought, cold,salt and also biotic stresses such as shoot fly, midges, and stem borerfromseedling stages to maturity. This article summarizes the terminal drought stress tolerance mechanism with stay-green phenotype expression during post-flowering and also mechanisms of early shoot fly resistance during seedling stages of crop growth. The trait stay-green is extensively studied and its correlation to yield makes the stay-green trait more special for research and in marker assisted back cross programs. Under terminal drought stress conditions, stay-green trait is expressed with a complex mechanism involving many transcription factors, chlorophyll retention and nitrogen remobilization from leaves to maintain longer photosynthetic activity. Shoot fly resistance on the other hand, involves manyphysico-chemical, biologicaland morphological traits. Out of the many morphological traits, seedling leaf blade glossiness and trichome density are well characterized at genetic level and can assist as shoot fly resistance sources in marker-assisted breeding programs as they are highly negatively correlated with shoot fly dead heart formation. However, quantitative trait loci (QTL) mapping studies and candidate genes identified for the stay-green and shoot fly component traits need to be further validated with fine mapping, gene cloning and expression level studies. Pyramiding these two traits into a high yielding sorghum variety may lead to multiple stress resistance which could ultimately benefit the marginal farmers in India.

Keywords: Sorghum, shoot fly resistance, stay-green, drought tolerance, QTL, marker-assisted selection

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a cultivated tropical crop plant that belongs to the family Poaceae, tribe Adropoganeae and genus *Sorghum*. Sorghum is largely self-pollinated diploid crop (2n=2õ=20) with fully sequenced genome length of ~730Mb (1). It is the fifth most important cereal crop globally (2) providing food, feed, fiber, fuel, and chemical/ biofuels feed-stocks across a range of environments and production systems.USA, India, México, Nigeria, Sudan and Ethiopia are the major producers of sorghum. Other sorghum producing countries include Australia, Brazil, Argentina, China, Burkina Faso, Mali, Egypt, Niger, Tanzania, Chad and Cameroon. Grain is mostly used as food (55%), in the form of breads and porridges in Asia and Africa, and as feed (33%) in the Americas. Its stover is an increasingly important source of dry season

fodder for livestock, especially in Asia (http://www.icrisat.org/crop-sorghum.htm). Its remarkable ability to produce yields under adverse conditions like arid and semi-arid regions, where water limited conditions exists alongside heat stress. This makes sorghum an important 'fail-safe' source of food, feed, fiber, and fuel in the global agro-ecosystem. Sorghum is a representative of tropical grasses that use C_4 photosynthesis, which results from complex biochemical and morphological specializations that improve carbon assimilation at high temperatures. While the world's average annual yield for sorghum was 1.08 tonnes per hectare in the year 2012, total production from all sorghum producing countries was 57 million tonnes. FAO reported the United States of America as the top sorghum producer with a harvest of 1.22 million tonnes followed by India, Nigeria, Mexico and Sudan (3). In India, with its large population and fragile balance in the production and demand equation for food grains, sorghum plays a crucial role in national food security. Attempts to increase the production of sorghum with the introduction of new high-yielding varieties and hybrids since 1966, was largely unsuccessful because of the susceptibility of the improved cultivars to various abiotic (drought)and biotic(shoot fly) (4,5,6,7)stresses. But therate of loss due to biotic and abiotic stresses in sorghumyear by year is increasing.

Drought stress and stay-green trait : Abiotic stresses are the most harmful constraints concerning the growth and productivity of crops worldwide. After soil nutrient deficiency, drought stress is the most important abiotic constraint for sorghum production globally(8) Sorghum is well adapted to semi-arid environments and regarded as model crop for studying drought stress tolerance among grass species. So, breeders mostly have focused on improving drought stress tolerant varieties of sorghum (9). If plants withstand drought spell occurring at grain filling stage, it is defined as terminal drought tolerance. Drought stress during and after flowering typically causes premature leaf senescence which in turn lead to stalk lodging, stalk rot disease, reduced grain filling, and significant grain and stover yield losses.Plant characters best associated with post-flowering drought tolerance, may be due to delay in leaf senescence or non-senescence or "stay-green" trait(9,10,11,12,13,14,15,16). Therefore, the "stay-green" trait is more than the ability of the plant to maintain functional green leaf area (GLA), to improve quality of residues (17), to support the continuation of carbon fixation and supply of starch to the grain filling site (18), to prevent premature death and stalk lodging (19) andto sustain grain-filling under water stress to improve yield (14,20).Stay-green is of three types. Type A stay-green phenotypes have a delayed onset and a normal rate of senescence following its onset. Type B stay-green phenotypes initiate leaf senescence normally but the rate of senescence is comparatively slower. Type C stay-green phenotypes retain chlorophyll despite the normal onset and progression through senescence(21). Many crop plants other than sorghum like rice, wheat, maize, barley, cotton, tobacco have been reported till date with stay-green character.

Mechanism of drought tolerance/stay-greenand factors associated with stay-green : Molecular mechanisms underlying delay in senescence which extend the duration of active photosynthesis in sorghum have not been elucidated completely. Rosenow et al. (20) observed positive impact of delayed leaf senescence on crop performance of plants under water limited conditions during grain filling. Presence of stay-green phenotype is a result of balance between nitrogen (N) demand by grain and nitrogen captured by vegetative parts of plants like increasing the supply of water by modified root architecture which increases water extraction from soil or reducing water demand by reducing transpiration loss. Nitrogen remobilization from leaves maintain longer photosynthetic activity and supply adequate carbohydrates to developing grains (10,22,23). It appears that carbon, nitrogen ratios and ABA levels affect senescence. Besides them,

cytokinins also play a role in leaf senescence and increased production of cytokinins lead to delayed leaf senescence (24).Stay-green was influenced by genetic factors, environmental factors like high temperature, soil-water holding capacity, soil moisture content at planting, vapor pressure depict, rain fall during cropping and management factors like population size and planting time (14). Leaf chlorophyll content was also significantly correlated with stay-green scores under drought conditions as pointed out by Xu et al. (25).

Nodal root angle depends on vertical and horizontal distribution of rootsin soil. Theirprofile is relevant to drought adaptation and is co-localized with stay-green genomic regions which show that roots and their growth are related tostay-green phenotype expression (12,26,27).Stay-green is highly negatively correlated with flowering time and stover yield (9). These correlation studies indicate early flowering is associated with green leaf area. But, stay-green shows positive association with grain yield (9,11,14). Stay-green is negatively correlated with flowering time, canopy size, size of upper leaf,tillering. Under drought conditions stay-green enhances grain yield, by altering the canopy development and modifyingthe size of the leaf (leaf anatomy), root growth (nodal root angle) and water uptake mechanisms (11,12,28). Reduction in leaf size leads to transfer of photosynthetic nutrients to grains without undergoing the drought stress.

Identification of genetic factors involved in stay-green : Genomic regions responsible for stay-green trait were detected with the help of molecular markers and the phenotyping data of the stay-green lines locate the variation in the genomic regions which are important for drought tolerance breeding programs. Quantitative trait loci(QTLs) for stay-green have much importance in improving the productivity under drought stress conditions (23).Many QTL mapping studies contributing to stay-green expression under drought stress conditions have been evaluated in mapping populations (8,15,29, 30,31,32,33, 34,35,36,37,38) introgression lines (9) and near isogenic lines (29,30,31,33,34,35,15). Several stay-green sources have been field evaluated and used for crosses (39,40). Best stay-green sources are B35,E36-1,and SC56 that are involved in different marker assisted breeding programs. Cross B35 (stay-green) × R16 (senescent) was developed (9) and their introgression lines were field evaluated.B35 (stay-green) × Tx7000 (senescent) was also extensively studied and their introgression lines were used for fine mapping of different stay-green QTLs (15,33,35). B35 × Tx430 (32), SC56 × Tx7000 (36), N13× E36-1, IS9830 × E36-1 (8),M35-1× B35 (16) crosses were made and different stay-green QTLs were identified.Stay-green was extensively studied in crops other than sorghum like in maize (41), wheat (42), barley(43), rice (44),and *Arabidopsis*(45).It appears therefore that stay-green genotypes need to be utilized in sorghum breeding programs aimed at developing drought tolerant plants.

Marker-assisted breeding for stay-green : Drought stress may be alleviated by developing crops that are well adapted to dry-land environments with marker assisted breeding crop improvement programs.Increasing marker density and identifying QTLs and narrow downing the QTLs to smaller regions will improve marker assisted breeding.Different types of stay-green QTLs are influenced by different backgrounds (28) and many crossing programs introgressed stay-green into senescent breeding lines. Therefore, marker assisted breeding programs help us develop drought tolerant lines in sorghum.

Stay-green candidate genes : An alteration in the chlorophyll break down mechanism influenced by many key factors like plant hormones, transcriptional factors and genes lead to delayed degradation of chlorophyll.Cytokinins are plant hormones involved in regulating senescence process, and thecytokinin receptor (AHK3), the type-B response regulator (ARR2) and the recently identified cytokinin response factor (CRF6) are involved in senescence signal responses (46). No apical meristem (NAC/NAM)

transcriptional factor is a developmental regulator and accelerates senescence and increases nutrient remobilization from leaves to developing grains (47).In *Arabidopsis*,*At*NAP encodes NAC transcription factor which is closely associated with senescence (48).OsNAP is a NAC transcriptional activator identified in rice involved in senescence pathway. Reduced OsNAP expression lead to improved grain filling and seed setting and subsequently increased grain yield (49).Senescence associated genes (SAGs) were up- and downregulated under stress conditions (50). Chlorophyll catabolic enzymes and STAYGREEN1 (SGR1), STAYGREEN2 are regulators of chlorophyll degradation and their mutants (sgr) exhibit stay-green phenotype which is a desired phenotype for drought tolerance (45).WRKY family transcriptional factors are also involved in senescence pathwayand over expression of WRKY transcriptional factors lead to improved drought tolerance (51). Thus, the above candidate genes appear to be crucial for imparting drought stress tolerance. Their overexpression in sorghum can certainly lead to transgenic sorghum lines that can withstand water limited conditions.

Shoot fly resistance : Apart from abiotic stresses, many biotic stresses are caused by plant pathogens and insect pests.Nearly, 150 species of insect pests damage sorghum, of which sorghum shoot fly *Antherigonia soccata* (Rondani), is the major insect pest in Africa, Asia and Mediterrian Europe (6). Shoot fly belongs to the family Muscidae and is a devastating pest in sorghum. It mostly attacks tropical grass species like wheat, barley and sorghum.Female shoot fly lays white, elongated,cigar shaped eggs singly on abaxial (lower) surface of leaf, parallel to mid-rib. Eggs hatch in 1-2 days of incubation and larvae crawl into central leaf whorl and cuts the growing tip resultingin typical wilting and drying of the central whorl leaf known as 'dead heart'. As a result of dead heart formation, the young seedlings may be killed outright or they may produce axial tillers, which are rarely productive. The axial tillers serve as a mechanism of recovery resistance if they remain undamaged, but if shoot fly infestation continues, the seedling may die or present a rosette appearance and fail to produce any grain (52). Larvae feed on the decaying tissuewhich may lead to seedling mortality and the crop gets damaged within 1-4 weeks after seedling emergence.

Mechanisms of shoot fly resistance : Agronomic practices (timely sowing), natural and synthetic insecticides, natural enemies and host plant resistance (HPR), are all components of integrated pest management practices used to minimize sorghum losses due to shoot fly infestation. Early sowing during rainy season can also be one of the resistance mechanisms (53); but HPR and timely sowing remains most preferred as they are cost-effective, eco-friendly and easily adapted by farmers. Mechanism of resistance to shoot fly is complex and depends on interplay of many component characters of plant, insect and environmental factors (54).Improvement in resistance will increase ecological fitness, reduces pesticide use, and facilitates creation of a sustainable production system with increased efficiency, profitability and enhances grain quality traits. Antixenosis for oviposition is the primary mechanism of resistance for shoot fly resistance in sorghum (55,56). Antibiosis and tolerance also plays important shoot fly resistance mechanism (52,57).Of many important morphological components of sorghum HPR identified, seedling leaf blade glossiness (58), seedling leaf blade trichome density (59), seedling vigor, and leaf sheath pigmentation are all positively associated with Shoot Fly Resistance (SFR). Leaf glossiness reflects the flies from the host and increased trichome density inhibits the larval movement on leaf surface and acts as barrier between the leaf and fly to prevent egg laying (antixenosis)(60). Rapid growth of seedling due to seedling vigor inhibits the larvae movement to reach the central leaf whorland this reduces the frequency of dead hearts(60). Cytoplasmic male sterilityalso influences the expression of shoot fly resistance mechanism (61,62). Chlorophyll content and leaf

surface wetness, and waxy bloomhave been reported to be associated with shoot fly susceptibility (63). Increased secondary metabolites also take path in shoot fly resistance mechanism (64). Shoot fly resistant genotypes were used in the breeding programs as a source forresistance. Genotypes such as IS2122, IS18551, IS2146, IS1054, IS2312, SFCR151, ICSV705, SFCR125 were used in many crossing programs as resistant donors for shoot fly resistance (65,66,67). However, many of these resistance mechanismsstill need to be evaluated clearly at the molecular level. Genes associated with these mechanisms and their cloning and overexpression studies are also needed for validation.

Factors associated with shoot fly resistance : Resistance to shoot fly is mediated by many physico-chemical, morphological,biological, environmental, biochemical, cytoplasmic and genetic factors.Chemicals and pesticides were used to control shoot flies in the field. Fipronil and imidacloprid were successfully evaluated for shoot fly control (68).As the chemicals and pesticides are not affordable by poor farmers and can cause serious environmental hazards,it is necessary to develop cultivars with shoot fly resistance with the help of marker assisted back cross (MABC) methods (64). Morphological traits like seedling leaf blade glossiness, trichome density in lower and upper leaf portions,leaf sheath pigmentation, seedling vigor are negatively correlated with percent shoot fly 'dead heart' and positively associated with shoot fly resistance.Significant correlation was observed between shoot fly dead hearts and yield(53). Morphological components like glossiness and trichome density are negatively correlated to shoot fly dead heart percentage and are significantly associated to shoot fly resistance. Combined effects of trichome density on abaxial (lower), adaxial (upper) and leaf glossiness have been shown to reduce dead heart percentage and high shoot fly resistance (66).These observations point out that glossiness and trichome density are vital for shoot fly resistance in sorghum. Environment is a major factor associated with shoot fly resistance as the rainy(Rabi) season is most suitable for shoot fly infestation when compared to the post rainy (Kharif) season.Biochemical factors like p-hydroxy benzaldehyde, cinnamic acid, luteolin, apigenin, and some unidentified compounds from damaged and undamaged seedlings of sorghum were associated with expression of resistance for shoot fly as pointed out by Chamarthi et al. (69). QTLs associated with shoot fly resistance have beenidentified in many populations and different crosses responsible. However, candidate genes need to be identified and validated in sorghum. SFR component traits have been mapped and the putative QTLs identified for individual traits and subsequently validated by marker-assisted backcross (MABC)-introgression into genetic backgrounds highly susceptible to shoot fly. The cross BTx623 × IS18551(70, 71, 72, 73) mapped the shoot fly resistance (SFR) QTLs on SBI-01, SBI-05, SBI-07, and SBI-10. Similarly, using crosses 296B (susceptible) × IS18551 (resistant) (60,74) and cross 27B (susceptible) × IS2122 (resistant) (75)mapped the SFR. In a reciprocal cross IS18551 × 296B, Apotikar et al.(76) found SFR QTLs on SBI-01 and SBI-03. Five putative QTLs for SFR component traits from IS18551 were then validated by MABC-introgression into the genetic backgrounds of elite shoot fly-susceptible hybrid seed parent maintainer lines 296B and BTx623 (77). Thus, these studies point out that it is possible to transfer shoot fly resistance through classical breeding programs.

MABC for shoot fly resistance : Many crossing programs at the National and International Research Centers like Directorate of Sorghum Research and ICRISAT, Patancheru, India, resulted in the development of introgression lines for shoot fly resistance which can be used in further breeding programs. Jyothi(77) introgressed SFR QTLs into BTx623 (fully sequenced) (1) and into 296B backgrounds. 296B× IS18551 and BTx623× IS18551 (60,70,71,72,73,74,77) (crosses were

extensively studied and their introgression lines were field evaluated for the introgressed trait validation. Utilizing these introgression lines in future molecular breeding programs may help in increasing the shoot fly resistance in different genetic backgrounds and can be pyramided along with other preferred traits to attain multiple resistances to the sorghum plants.Gene pyramiding is a breeding strategy that serves to combine favorable alleles at multiple genetic loci into a single plant genotype. This process of stacking of genes/QTL into a single elite cultivar background can now be efficiently performed by marker-assisted selection (MAS), using backcrossing or pedigree approaches. This approach expedites the varietal development process by providing the opportunity to select for all desirable genes/QTLs simultaneously, as well as eliminating the time-consuming process of inoculation for different races or isolates at different time intervals (78). Pyramiding of multiple genes or common major QTLs for biotic and abiotic stresses are important approaches for genetical improvement of any sorghum genotype. Fine mapping can be achieved by large scale population with more markers showing more recombination events. In early generation populations like F_2, F_3populations many recombination events can be utilized but, heterozygosity segregation distortion, dominance and epistasis need to be overcome to fine map the interested regions. Advance molecular toolsincrease the precision of crop improvement. A genome-wide association study (GWAS) is a further advanced method to understand the marker trait associations based on linkage disequilibrium and can identify the SNP associated with the candidate genes (79).

Candidate genes responsible for shoot fly resistance : Candidate genes underlying the target QTLs like seedling leaf blade glossines and trichome density have been reported by Satish et al. (60,74) and Aruna et al. (75). Data derived fromsorghum genome database and studies on trichome density and glossiness in different crops are consistent with the identified QTLs. Identification of genes, pathways and mechanism involved in sorghum seedling leaf blade glossiness and trichome density have not yet been clearly studied nor cloned in sorghum. Majority of the studies were carried out in model crop plants like *Arabidopsis* and maize. But studies on sorghum are very few. Wax deficient mutant loci in*Zea mays* (maize), *Brassica napus* and sorghum are defined as '*glossy*' loci whereas in *Arabidopsis thaliana* and *Hordeum vulgare* (barley), they were named as '*ceriferum*' (cer) mutant loci (80). In *Arabidopsis*,shine (*shn*) mutants were reported. Ithas been found that the *shn* gene encodes for APETALA (AP2)/ethylene response element binding protein (EREBP) transcriptional factors that act in up- and downregulation of lipid biosynthesis (81). More than 30 '*glossy*' loci have been identified and a few were cloned (*gl1, gl2*, *gl3*, *gl4*, *gl8*, *gl13* and *gl15*) in maize (82) and their functional role in glossiness has been reported. Similarly,many studies reported that WRKY, MYB transcription factors play important roles (83,84,85,86)for developmental regulation of trichomes and trichome morphology can also play important roles in SFR (60). Further, *Mir*1 gene encodes for cysteine proteasewhich can reduce the growth of larvae as reported by (60). Transparent Testa Glabra1 (TTG1), Glabrous 2 (Gl2) and Glabrous 3 (Gl3) are involved in trichome initiation and TTG2 is also involved in trichomes throughout their development (83,87). Thus, these data appear that genes associated with both glossiness and trichome density have been identified and can be used in genetic engineering techniques for generating transgenics with better resistance.

Conclusions

Recent advances in genomics, molecular breeding and next generation sequencing and re-sequencing methodologies can be utilized in future to decipher stay-green and morphological traits of shoot fly resistance in sorghum. We need to further fine map the mapped QTL genomic regions and look for the maker trait associations with the help of genome wide association studies

(GWAS) in sorghum. Genes responsible for stay-green, leaf blade glossiness and trichome density need to be cloned and their introgression and expression level studies should be made in sorghum in order to enhance the genetic architecture. In future, both these studies need to be targeted with MABC and it could be possible to pyramid the stay-green trait alongside shoot fly component traits inorder to achieve a multiple resistant variety for improved sorghum productivity.

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Secondary Metabolites of Plants and their Role: Overview

Saurabh Pagare[1*], Manila Bhatia[1], Niraj Tripathi[2], Sonal Pagare[3] and Y.K. Bansal[1]
[1]Department of Biological Science, Rani Durgavati Vishwavidyalaya,
Jabalpur (M.P.)-482001, India
[2]Directorate of Weed Science Research, Jabalpur (M.P.)-482004, India
[3]NTPC Hospital,Korba (C.G.)-495450, India
*For Correspondence - saurabhhind@gmail.com

Abstract

Secondary metabolites (SM) are compounds that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment. These compounds are often involved in plants protection against biotic or abiotic stresses. Secondary metabolites are from different metabolites families that can be highly inducible in response to stresses. Primary metabolites perform essential metabolic roles by participating in nutrition and reproduction. A few SMs are used as especially chemical such as drugs, flavours, fragrances, insecticides, and dyes and thus have a great economic value. These new technologies will serve to extend and enhance the continued usefulness of the higher plants as renewal sources of chemicals, especially medicinal compounds. A continuation and intensification efforts in this field is expected to lead to successful biotechnological production of specific, valuable and as yet unknown plant chemicals.

Keywords: Secondary metabolites, drugs, flavours, fragrances, biotechnology

Introduction

Plants possess capacity to synthesize different organic molecules called secondary metabolites. Unique carbon skeleton structures are basic properties of plant secondary metabolites. Secondary metabolites are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its surroundings, ensuring the continued existence of the organism in its ecosystems. Formation of SMs is generally organ, tissue and cell specific and these are low molecular weight compounds. These compounds often differ between individuals from the same population of plants in respect of their amount and types. They protect plants against stresses, both biotic (bacteria, fungi, nematodes, insects or grazing by animals) and abiotic (higher temperature and moisture, shading, injury or presence of heavy metals). SMs are used as especially chemical such as drugs, flavours, fragrances, insecticides, and dyes by human because of a great economic value.

In plants, SMs can be separated into three groups (Terpenoids, Polyketides and Phenypropanoids) based on their biosynthesis origin (1). Alkaloids are additional class of SMs, which are nitrogenous organic molecules biosynthesized mainly from amino-acids, e.g., tryptophan, tyrosine, phenylalanine, lysine and arginine using many unique enzymes (2). Many of the most important therapeutic agents are alkaloids. The sites of biosynthesis are compartmentalised at cellular or sub-cellular level. However SMs can be transported long distances and accumulate from their location of synthesis.

Primary Vs Secondary Metabolites : Primary metabolites are found in all plants and execute vital metabolic responsibilities, by participating in

nutrition and reproduction (2). Sometimes it is hard to discriminate primary and secondary metabolites. For example, both primary and secondary metabolites are found among the terpenoids and the same compound may have both primary and secondary roles. Secondary metabolites are broad range of compounds from different metabolite families that can be highly inducible in stress conditions. Carotenoids and flavonoids are also involved in cell pigmentation in flower and seed, which attract pollinators and seed dispersers. Therefore, they are also involved in plant reproduction (3). Plant primary products refer to the compounds of nucleic acids, proteins, carbohydrates, fats and lipids and are related to structure, physiology and genetics, which imply their crucial role in plant development. In contrast, secondary metabolites usually take place as minor compounds in low concentrations. Primary metabolism refers to the processes producing the carboxylic acids of the Krebs cycle. Secondary metabolites, on the other hand, are non-essential to life but contribute to the species' fitness for survival. In fact, the specific constituents in a certain species have been used to help with systematic determination, groups of secondary metabolites being used as markers for botanical classification (chemotaxonomy). Plants secondary metabolites can be divided into three chemically distinct groups viz: Terpenes, Phenolics, N (Nitrogen) and S (sulphur) containing compounds.

I)Terpenes : Terpenes comprise the biggest group of secondary metabolites and are free by their common biosynthetic origin from acetyl-coA or glycolytic intermediates. An immense bulk of the diverse terpenes structures produced by plants as secondary metabolites that are supposed to be concerned in defense as toxins and feeding deterrents to a large number of plant feeding insects and mammals. Terpenes are divided into monoterpenes, sesquiterpenes, diterpene, Triterpenes and polyterpenes. The pyrethroid (monoterpenes esters) occur in the leaves and flowers of Chrysanthemum species show strong insecticidal responses to insects like beetle, wasps, moths, bees, etc and a popular ingredient in commercial insecticides because of low persistence in the environment and low mammalian *toxicity.* In Gymnoperms (conifers) á-pinene, â-pinene, limonene and myrecene are found. A number of sesquiterpenes have been till now reported for their role in plant defense such as costunolides are antiherbivore agents of family composite characterized by a five member lactone rings (a cyclic ester) and have strong feeding repellence to many herbivorous, insects and mammals. ABA is also a sesquiterpene plays primarily regulatory roles in the initation and maintenance of seed and bud dormancy and plants response to water stress by modifying the membrane properties and act as a transcriptional activator (4). Abietic acid is a diterpene found in pines and leguminous tress. It is present in or along with resins in resin canals of the tree trunk . Another compound phorbol (Diterpene ester), found in plants of euphorbiaceae and work as skin irritants and internal toxins to mammals. The milkweeds produce several better tasting glucosides (sterols) that protect them against herbivors by most insects and even cattle. Several high molecular weight polyterpenes occur in plants. The principal tetraterpenes are carotenoids family of pigments.

(*II) Phenolic compounds* **:** Plants produce a large variety of secondary products that contain a phenol group, a hydroxyl functional group on an aromatic ring called Phenol, a chemically heterogeneous group also. They could be an important part of the plants defence system against pests and disease including root parasitic nematodes (5). Elevated ozone (mean 32.4ppb) increased the total phenolic content of leaves and had minor effects on the concentration of individual compounds (6).Coumarin are simple phenolic compounds widespread in vascular plants and appear to function in different capacities in various plant defense mechanisms against insect herbivores and fungi. They derived from the shikimic acid pathway, common in bacteria, fungi and plants but absent in animals

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(7). Some coumarin derivatives have higher anti-fungal activity against a range of soil borne plant pathogenic fungi and exhibit more stability as compared to the original coumarin compounds alone (7). Furano is Also a type of coumarin with special interest of phytotoxicity, abundant in members of the family umbelliferae including celery parsnip and parsley. Psoraline, basic linear furacoumarin, known for its use in the treatment of fungal defence and found very rarely in SO_2 treated plants (8). Ligin is a highly branched polymer of phenyl- propanoid groups, formed from three different alcohols viz., coniferyl, coumaryl and synapyl which oxidized to free radical (ROS) by a ubiquitous plant enzyme-peroxidises, reacts simultaneously and randomly to form lignin. Its physical toughness deters feeding by herbivorous animals and its chemical durability makes it relatively indigestible to herbivorous and insects pathogens. Lignifications block the growth of pathogen and are a frequent response to infection or wounding. Flavanoids perform very different functions in plant system including pigmentation and defence. Two other major groups of flavanoids found in flowers are flavanones and flavanols function to protect cell from UV-B radiation because they accumulate in epidermal layers of leaves and stems and absorb light strongly in the UV-B region while letting visible (PAR) wavelengths throughout uninterrupted (9). In addition exposure of plants to increased UV-B light has been demonstrated to increase the synthesis of flavanones and flavanols suggesting that flavanoids may offer measures of protection by screening out harmful UV-B radiation (6). Isoflavanoids are derived from a flavanones intermediate, naringenin, ubiquitously present in plants and a play a critical role in plant developmental and defence response. They secreted by the legumes and play an important role in promoting the formation of nitrogen fixing nodules by symbiotic rhizobia (10). Moreover, it seems that synthesis of these flavanoids is an effective strategy against reactive oxygen species (ROS). The analysis of activity of antioxidant enzymes like SOD, CAT, POX, APX, GPX and GR suggested that peroxidases were the most active enzymes in red cabbage seedlings exposed to Cu^{++} stress (11).Tannins included in the second category of plant phenolic polymers with defensive properties. Tannins are general toxins that significantly reduce the growth and survivorship of many herbivores, and also act as feeding repellents to a great diversity of animals.

(III) Sulphur containing secondary metabolites: They include GSH, GSL, Phytoalexins, Thionins, defensins and allinin which have been linked directly or indirectly with the defence of plants against microbial pathogens (12,13,14). GSH is the one of the major form of organic sulphur in the soluble fraction of plants and has an important role as a mobile tool of reduced sulphur in the regulation of plant growth and development and as a cellular antioxidants in stress responses (15), reported as a signal of plant sulphur sufficiency that down regulates sulphur assimilation and sulphur uptake by roots.

GSL is a group of low molecular mass N (nitrogen) and S (sulphur) containing plant glucosides that produced by higher plants in order to increase their resistance against the unfavourable effects of predators, competitors and parasites because their break down products are release as volatiles defensive substances exhibiting toxic or repelient effects for example, mustard oil glucosides in cruciferae and allyl cys sulfoxides in alllum (16). They are metabolised and absorbed as isothiocyanates that can affect the activity of enzymes involved both in the antioxidant defence system and in the detoxification from zenobiotics abd significantly affect GST activity and cell protection against DNA damage (17) whereas toxicity of glucosinolatic products is well documented but their mode of action has not yet been elucidated and results from experiments with Brassica plants modified in GSL content generated doubts about their contribution to plant defences.

Phytoalexins are synthesized in response to bacterial or fungal infection or other forms of stress that help in limiting the spread of the

invading pathogens by accumulating around the site of infection, appears to a common mechanism of resistance to pathogenic microbes in a wide range of plants. Many of these changes are linked to a rapid apoptotic response, resulting in death of one or a few invaded plant cells, known as the hypersensitive response (HR). Most plant families produce organic phytolexins of diverse chemistry; these groups are often associated with a family, for example sesquiterpenoids of Solanaceae, isoflavonoids of Leguminosae, while phytoalexins from Brassica have an indole or related ring system and one S atom as common structural features. Crucifereae appears to be the only plant family producing these S metabolites, which are clearly different from the other well- known GSL.Cruciferous crops are cultivated worldwide because they are extremely valuable and for the last decades, various research groups have investigated cruciferous phytoalexins as well as their biological activity.Typically, there are multiple responses involving several related derivatives such as up to nine wyerone (Furano-acetylenic derivatives) forms in Vicia fava and several forms of phaseollin in Phaseolus vulgaris and glyceollin in Glycine max, postin in Pisum sativum pods, Ipomearone in sweet pototo, orchinol in orchid tubers, trifolirhizin in red clover. Defensins, thionins and lectins are S-rich non-storage plant proteins synthesize and accumulate after microbial attack and such related situations. They inhibit growth of a broad range of fungi. Additionally defensins genes are partly pathogen-inducible and others that are involved in resistance can be expressed constitutively. Some plant species produce lectins as defensive proteins that bind to carbohydrates or carbohydres containing proteins.

(IV) Nitrogen containing secondary metabolites: They include alkaloids, cyanogenic glucosides, and non-proteins amino-acids. Most of them are biosynthesized from common amino-acids. Alkaloids found in approximately 20% of the species of vascular plants, most frequently in the herbaceous dicot and relatively a few in monocots and gymnosperms. Generally, most of them, including the pyrrolizidine alkaloids (PAs) are toxic to some degree and appear to serve primarily in defense against microbial infection and herivoral attack. Cyanogenic glucosides constitue a group of N-containing protective compounds other than alkaloids, release the poison HCN and usually occur in members of families viz., *Graminae, Roosaceae and leguminosesae.* They are not themselves toxic but are readily broken down to give off volatile poisonous substance like HCN and volatile H_2S when the plant crushed; their presence deters feeding by insects and other herbivorous such as snails and slugs. Amygdalin, the common cynogenic glucoside found in the seeds of almonds, apricot, cherries and peaches while Dhurrin, found in Sorghum bicolar.

Many plants also contain unusual amino acids called non-protein amino-acids that incorporated into proteins but are present as free forms and act as protective defensive substance. For examples, canavanine and azetidine-2 carboxylic acid are close analogs of arginine and proline respectively. They exert their toxicity in various ways. Some block the synthesis of or uptake of protein amino acid while others can be mistakenly incorporated into proteins. Plants that synthesized non-protein amino acid are not susceptible to the toxicity of these compounds but gain defence to herbivorous animals, insects and pathogenic microbes.

Transport, Storage and Turnover: SMs can be water soluble (hydrophilic) compounds or lipophilic (needs organic solvents), therefore needs different cellular mechanism for their transport, storage and turnover. Most substances are synthesized in the cytoplasm, the ER or in the organelles. Hydrophilic SMs are usually stored in the vacuole after their formation in cytoplasm, whereas lipophilic substances are sequestered in resin ducts, laticifers, glandular hairs, trichomes, thylakoid membranes or on the cuticle. Hydrophilic SMs have to pass the tonoplast, which is impermeable to many of the polar secondary metabolites. For some alkaloids

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and flavanoids, a specific transporter has been described, which pumps the compounds into the vacuole. In order to avoid autotoxicity, plants cannot store these compounds in the vacuole but usually sequester them on the cuticle, in dead resin ducts or cells which are lined by a biomembrane but an impermeable solid barrier. In many instances, the site of biosynthesis is restricted to a single organ such as roots, leaves or fruits, but an accumulation of the corresponding products can be detected in several other plant tissues. Long distance transport must take place in these instances. The xylem or phloems are likely transport routes but an apoplastic transport can also be involved. Storage can also be tissue and cell- specific, depending upon the protection providing to the plants.In a number of plants, specific idioblasts have been detected that contain tannins, alkaloids or glucosinolates. More often, SMs are concentrated in trichomes or glandular hairs (many terpenoids in Labiatae, Asteraceae), stinging hairs (many amines in urticaceae) or the epidermis itself (many alkaloids, flavanoids, anthocyanins, cynogenic glycosides, coumarins, etc.) flowers, fruits and seeds are usually rich in SMs, especially in annual plants. In perennial species, high amounts of SMs found in bulbs, roots, rhizomes and the bark of roots and stems. It is well-established that profiles of SMs vary with time, space and developmental stage. Since related plant species often show similarities in the profiles of their SMs, they have been used as taxonomic tool in plant systematic. However, profiles of closely- related plants quite often differ substantially or those of unrelated plant group show strong similarities; this clearly shows that SM patterns are not unambiguous systematic markers but that convergent evolution and selective gene expression are common themes.

Extraction of Secondary Metabolites from Plant : Plant secondary metabolites are currently the subject of much research interest, but their extraction as part of phytochemical or biological investigations presents specific challenges that must be addressed throughout the solvent extraction process. Successful extraction begins with careful selection and preparation of plant samples. During the extraction of plant material, it is important to minimize interference from compounds that may coextract with the target compounds, and to avoid contamination of the extract, as well as to prevent decomposition of important metabolites or artifact formation as a result of extraction conditions or solvent impurities. Researchers from a variety of scientific disciplines are confronted with the challenge of extracting plant material with solvents, often as a first step toward isolating and identifying the speciûc compounds responsible for biological activities associated with a plant or a plant extract. The impetus for this research arises largely because plants form the foundation of traditional pharmacopeias, and because many of our currently important pharmaceutical drugs are obtained from plants. Further interest arises from the growing awareness that many of the secondary metabolites of organisms, including plants, serve important biological and ecological roles, mainly as chemical messengers and defensive compounds. Investigators engaged in the isolation of secondary metabolites from plants soon discover the need for considerable laboratory finesse in the apparently routine “sample preparation” steps that convert crude plant material into an extract suitable for chemical analysis, biological testing, or chromatographic separation.

Major Secondary Metabolite Pathways : In plants particularly three pathways are the source of most secondary metabolites: The shikimate pathway, the isoprenoid pathway and the polyketide pathway. After the formation of the major basic skeletons, further modifications result in plant species specific compounds. The shikimate pathway is the major source of aromatic compounds. It is found in microorganisms and plants, but not in mammals, making it an interesting target for herbicides and antibiotics, as these compounds are expected not to have any effect on the mammalian system. Glyphosate is a well known example. The

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enzymes channeling chorismate into the aromatic amino acids pathways are chorismate mutase and anthranilate synthase. Although, in several plant species for both chorismate mutase and anthranilate synthase more than one gene has been cloned, only in case of chorismate mutase a plastidial and a cytosolic enzyme have been found. The phenylpropanoid pathway is one of the most important metabolic pathways in plants in terms of carbon flux. In a cell more than 20% of the total metabolism can go through this pathway, the enzyme chorismate mutase is an important regulatory point. The importance of this pathway is due to the fact that it leads to among others lignin, lignans, flavonoids, and anthocyanins. Key to these products is the enzyme phenylalanine ammonia lyase (PAL), which converts phenylalanine into trans-cinnamic acid by a non-oxidative deamination. This enzyme can be found in all plants, in some plants a single enzyme is found, whereas others may have several iso-enzymes. The other important pathway in plants is that of the terpenoids, also known as isoprenoid pathway. Terpenoids include more than one third of all known secondary metabolites. Moreover, the C_5-building block is also incorporated in many other skeletons, e.g. in anthraquinones, naphtoquinones, cannabinoids, furanocoumarines, and terpenoid indole alkaloids. In the "decoration" type of reactions in various types of secondary metabolites C_5-units are attached to the basic skeleton, e.g. hop bitter acids, flavonoids and isoflavonoids.

Functions of Secondary Metabolites : Many secondary compounds have signalling functions influence the activities of other cells, control their metabolic activities and co-ordinates the development of the whole plant. Other substances such as flower colours serve to communicate with pollinators or protect the plants from feeding by animals or infections by producing specific phytoalexines after fungi infections that inhibit the spreading of the fungi mycelia within the plant (18). Plants use secondary metabolites (such as volatile essential oils and colored flavonoids or tetraterpenes) also to attract insects for pollination or other animals for seed dispersion, in this case secondary metabolites serve as signal compounds. Compounds belonging to the terpenoids, alkaloids and flavonoids are currently used as drugs or as dietary supplements to cure or prevent various diseases (19) and in particular some of these compounds seem to be efficient in preventing and inhibiting various types of cancer (20, 21). It has been estimated that 14-28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno-medicinal use of the plants (22). Secondary metabolites are a metabolic intermediates or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism and biosynthetized from one or more general metabolites by wider variety of pathways than is available in general metabolism.

Presence of volatile monoterpenes or essential oils in the plants provides an important defense strategy to the plants, particularly against herbivorous insect pests and pathogenic fungi. These volatile terpenoids also play a vital role in plant-plant interactions and serve as attractants for pollinators (23). They act as signalling molecules and depict evolutionary relationship with their functional roles. Soluble secondary compounds such as cyanogenic glycosides isoflavoids and alkaloids can also be toxic to animals.

Biotechnology and Secondary Metabolites : Since SM have evolved as compounds that are important for the ûtness of the organisms producing them, many of them interfere with the pharmacological targets, which make them interesting for several biotechnological applications. Controlled clinical studies have shown the efficacy of several, for example extracts from *Ginkgo biloba*, *Hypericum perforatum*, *Piper methysticum*, *Chamomilla recutita*, *Crataegus monogyna*, *Silibum*

marianum, *Melissa ofûcinalis*, *Mentha piperita*, *Valeriana ofûcinalis*.

The use of stimulants (such as caffeine, nicotine, ephedrine), fragrances (several essential oils), ûavours (essential oils, capsaicin, piperine, etc.), natural dyes, poisons (strychnine) and hallucinogens (morphine, heroin, cocaine, tetrahydro cannabinol) is based on SM. Since many SM are insecticidal, fungicidal and phytotoxic, they may be used in agriculture as natural plant protectants. Before the advent of synthetic pesticides about 60 years ago, plant-derived insecticides (including nicotine, rotenone, quassin, ryanodine, pyrethrins and azadirachtins) were a common theme. Applications unequivocally showed that these natural insecticides worked. One ecological advantage is that SM are readily degraded in plants and in soil, is also their disadvantage and synthetic pesticides are more resistant and persistent. Moreover, modern pesticides are usually more potent than biopesticides. On the other hand, plants are easy to grow and biopesticides could be a sustainable source of plant protectants for farmers in countries that do not have access to Western synthetic pesticides. Unfortunately, legislation does not favour mixtures of compounds to be used as pesticides; therefore, the development of biorational pesticides has to face many obstacles. Nevertheless, natural compounds do provide an underexplored alternative. As a consequence of these various applications, a world market for plant extracts and isolated SM exists, which exceeds 10 billion US dollars annually. Therefore, it is a challenge for biotechnologists to find ways to produce these compounds in sufûcient quantity and quality.

The main and traditional way is to grow the respective plants in the ûeld or in greenhouses and to extract the products from them. For several species, new varieties have been selected with improved yields and quality. In this context, cell and organ culture are important techniques for in vitro propagation. In a few instances, genetic engineering of secondary metabolism has already had a direct inûuence for example, when *Atropa belladonna* plants were transformed with the gene that encodes the enzymes converting L-hyoscyamine into L-scopolamine, new plants were generated which produced scopolamine as the major product. More often, ûavonoid metabolism has been altered genetically, producing plants with different ûower colours. It is a challenge for future research to isolate the genes of biosynthetic pathways and to express them either in transgenic plants or in microbes.

If successful, recombinant bacteria or yeasts might be grown someday, which will produce valuable plant SM. Combinatorial biosynthesis might then be an open field. Using genes encoding enzymes for the biosynthesis of antibiotics, this strategy has already been successful. It has also brought about renewed interest in the regulation of SM synthesis and in the location and means of sequestration of these substances within the plant. In recent years, attempts have been made to express the genes of alkaloid biosynthesis in microorganisms. Ultimately, it might be possible to produce valuable alkaloids from recombinant bacteria or yeast. If the corresponding SM (both from plant or microbial origin) confers resistance to insects or pathogens, genetic transformation of susceptible crop plants could be another valuable avenue for Exploitation. For more than two decades, scientists around the world have tried to produce valuable SM in cell or organ cultures. Whereas undifferentiated cell cultures have often failed to produce such a compound in reasonable yields, differentiated organ cultures (e.g. transformed root cultures) are often as active as the intact plant .Cell- and tissue-speciûc gene expression appears to control these processes. In addresses the production of SM in vitro (Table 1).

It is possible that genetic engineering may help to improve plant cell cultures as biotechnological production systems in the future.

Table1. Secondary Metabolites from Plant Cell, Tissue and Organs Cultures

Plant Name	Active Ingredient	Culture Type
Adhatoda vasica	Vasine	Shoot culture(24)
Agastache rugosa	Rosmarinic acid	Hairy root(25)
Ammi majus	Umbelliferone	Shootlet(26)
	Triterpenoid	Suspension(27)
Angelica gigas	Deoursin	Hairy root(28)
Arachis hypogaea	Resveratol	Hairy root(29)
Artemisia annua	Artemisinin	Callus(30)
Aspidosperma ramiflorum	Ramiflorin	Callus(31)
Azadirachta indica	Azadirachtin	Suspension(32)
Brucea javanica	Cathin	Suspension(33)
Bupleurum falcatum	Saikosaponins	Root(34)
Camellia chinensis	Flavones	Callus(35)
Capsicum annum	Capsiacin	Callus(36)
Cassia acutifolia	Anthraquinones	Suspension(37)
C. senna	Anthraquinone	Hairy root(38)
Catharanthus roseus	Indole alkaloids	Suspension(39)
	Vincristine	Suspension(40)
	Catharathine	Suspension(41)
Cayratia trifoliata	Stilbenes	Suspension(42)
Centella asiatica	Asiaticoside	Hairy root(43)
		Callus(44)
Drosera rotundifolia	7-Methyljuglone	Shoot culture(45)
Eleutherococcus senticosus	Eleuthrosides	Suspension(46)
Eriobotrya japonica	Triterpenes	Callus(46)
Fabiana imbricata	Rutin	Callus and Suspenson(47)
Fagopyrum esculentum	Rutin	Hairy root(48)
Fritillaria unibracteata	Alkaloids	Multiple shoot(49)
Gentiana macrophylla	Glucoside	Hairy root(50)
Gentianella austriaca	Xanthone	Multiple shoot(51)
Glycyrrhiza glabra	Glycyrrhizin	Hairy root(52)
Gymnema sylvestre	Gymnemic acid	Callus(53)
Hemidesmus indicus	Lupeol, Rutin	Shoot culture(54)
Hypericum perforatum	Hypericin	Multiple shoot(55)
Mentha arvensis	Terpenoid	Shoot(56)
Momordica charantia	Flavonoid	Callus(57)

Conclusion

This review has dealt with a small selection of plant secondary- metabolites and their potential roles in defence mechanisms and ecological adaptation, in addition to the topics we have covered. there is an enormous range of other compounds present in the plant kingdom, with a very varied distribution. Plant secondary metabolism produces products that aid in the growth and development of plants but are not required for the plant to survive. Secondary metabolites have important ecological functions in plants: They protect plants against being eaten by herbivores and against being infected by microbial pathogens. They serve as attractants (odor, color, taste) for pollinators and seed-dispersing animals. They function as agents of plant-plant competition and plant-microbe symbioses. The ability of plants to compete and survive is therefore profoundly affected by the ecological functions of their secondary metabolites. Biotechnological approaches are also involved in production of secondary metabolites through genetic engineering process. Plant tissue culture may also play a major role for the same.

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NEWS ITEM

Dr. APJ ABDUL KALAM PASSES AWAY

Shillong: India's 11th President APJ Abdul Kalam died on Monday evening at a hospital in Meghalaya, where he had gone to deliver a lecture at Indian Institute of Management, Shillong . He was 83years old.

Dr.Abdul Kalam is known as the *Missile Man of India* for his work on the development of ballistic missile and launch vehicle technology. He also played a pivotal organizational, technical and political role in India's *Pokhran-II* nuclear tests in 1998, he was the project director of India's first Satellite Launch Vehicle (SLV-III) which successfully deployed the *Rohini* satellite in near-earth orbit in July 1980. He has received several prestigious awards, including the Bharat Ratna, India's highest civilian honour. The former President had collapsed during the lecture at the Indian Institute of Management, Shillong, around 6.30 pm. He was taken to the Bethany hospital. Doctors said he had suffered from a massive cardiac arrest.

SCIENTIFIC FINDINGS

New species of frog, fish found in Western Ghats : Researchers have discovered a new fish species and three types of tadpoles in Western Ghats of India. Newly discovered fish species: Pethiastriata- it was found along the streams of the Tunga in the Kudremukh National Park, Karnataka. Pethiastriata is described as a small fish, with a length of around 4 cm. The male is reddish incolour and the female,greyish.The species thrive in shallow pools of gently flowing water and are found in small groups of around four.The fish differ from existing species on seven characteristics, including dark outer edges of scales that give them a distinct striped pattern. Species of tadpoles: They belong to the species of Nyctibatrachus ('night frog') - *N. kumbara* ('potter frog'), *N. kempholeyensis* (named after the Kempuhole stream) and *N. jog* (named after the waterfall). These species of tadpoles were found for the first timein the narrow streams of the Sharavati in Karnataka.

2. First malaria vaccine likely by October : The RTS,S/AS01 vaccine was developed for use in sub-Saharan Africa where malaria still kills around 1300 children every day. The first malaria vaccine candidate to reach phase-3 of clinical testing was found to partially protect children against the disease up to four years after vaccination and may be available by as early as October this year, scientists reported. The results suggest that the vaccine candidate RTS,S/AS01 could prevent a substantial number of cases of clinical malaria, especially in areas of high transmission. "The European Medicines Agency (EMA) will assess the quality, safety, and efficacy of the vaccine based on these final data," said corresponding author Brian Greenwood, Professor at the London School of Hygiene & Tropical Medicine in Britain."If the EMA gives a favourable opinion, WHO (World Health Organization) could recommend the use of RTS,S/AS01 as early as October this year. If licensed, RTS,S/AS01 would be the first licensed human vaccine against a parasitic disease," Greenwood added. The findings revealed that vaccine efficacy against clinical and severe malaria was better in children than in young infants, but waned over time in both groups.However, protection was prolonged by a booster dose, increasing the average number of cases prevented in both children and young infants.There is currently no licensed vaccine against malaria anywhere in the world. The findings were published in the journal The Lancet.

At 603 kmph, Japan's maglev train breaks its own speed record : A Japanese maglev, which is the fastest passenger train in the world, has broken its own speed record. Operator JR Central said the train reached 603 kmph in a test run surpassing its previous record of 581 kmph set in 2003. The train travelled for about 2 km at a

speed exceeding 600 kmph. Japan's high-speed rail services are among the most advanced in the world, with hundreds of trains running each day with minimal delays. The Maglev Test Line, near Mount Fuji about 80 km west of Tokyo, is developing technology for use on a future link between Tokyo and Osaka. The magnetic levitation trains hover above rails, suspended by powerful magnets.

Earthquakes unveil deep rock structure under East Asia : Scientists have found a towering rock structure deep under East Asia by using 3-D supercomputer simulations of data from 227 earthquakes that hit the region during 2007-2011. The finding could throw light on the fate of the subducted continental plates beneath the Tibetan Plateau, known as 'the roof of the world,' which rises about 5km above sea level. The work may also help find hidden hydrocarbon resources, and more broadly it could help explore the Earth under East Asia and the rest of the world, researchers said."We are combining different kinds of seismic waves to render a more coherent image of the Earth," said principal investigator and lead author Min Chen from the Rice University. Researchers combined seismic records from thousands of stations for each earthquake to produce scientifically accurate, high-resolution 3-D tomographic images of the subsurface beneath immense geological formations. Like a thrown pebble generates ripples in a pond, earthquakes make waves that can travel thousands of miles through the Earth. The research was published in the Journal of Geophysical Research, Solid Earth.

Scientists document 'virgin births' of endangered sawfish in Florida : Scientists have documented in Florida a series of "virgin births," reproduction without mating, in acritically endangered sawfish species pushed to the brink of extinction by over-fishing and habitat destruction. Scientists say that for the first time the phenomenon called parthenogenesis has been seen ina vertebrate in the wild. They also say that some females may be resorting to asexual reproduction because small tooth sawfish numbers are so low that mating opportunities may not exist

CSIR succeeds in Whole Genome Sequencing of Holy basil (Tulsi) : CSIR-Central Institute of Medicinal & Aromatic Plants (CSIR-CIMAP), Lucknow, has published wholegenome sequence of Ocimum sanctum, the wonder plant 'Holy basil' or 'Tulsi'. This is the first report of complete genome sequence of a traditional and most respected medicinal plant of India, using a composite next generation sequencing technologies.Whole genome sequencing is a laboratory process that determines the complete DNA sequence ofan organism's genome at a single time. Benefits of Whole genome sequencing: Considering the metabolic and therapeutic potential of this revered plant, the availability of whole genome sequence is the first step to understand and unravel the secrets of this 'mother of all herbs' and to provide scientific validity to the traditional claims of its utility in diverse medicinal usage. The availability of the genome sequence now opens the possibility to identify genes involved inproducing therapeutic molecules and to produce them in vitro.This will also facilitate identification of not yet identified genes involved in the synthesis of important secondary metabolites in this plant.

Rat brain cells power a computer : A rat trained to control a robot is an interesting 'Pavlovian' experiment by itself. But, independently-cultured rat brain cells aiding a robot navigate through an obstacle seems straight out of science fiction. Having cultured brain cells on a glass plate and kept in sterilized conditions, a group of researchers at Indian Institute of Science (IISc) demonstrated that this tissue culture can read signals from an infra-red enabled robot, process the problem of obstacles, and give an appropriate, accurate solution The results of the 2.5-year-long experiment were described by the Scientists — from the Center for Nanoscience and Engineering (CeNSE) and Electrical Communications Engineering at IISc — took the

rat brain cells (hippocampus of just-born rat pups) and cultured it on a specialized glass plate that is covered with multiple electrodes that can detect the most minute spiking in voltages generated by the cells. The cultured cells start to grow dendrites —The cells form a network that shows spontaneous electrical activity through tiny voltage spikes. Interpretation of these spikes is done through an electronics platform that can detect as well as send electrical impulses (of just 500mV amplitude) to the cultured tissue through the embedded electrodes. These impulses are fed through the computer to the cells, which process the information, and the resulting voltage spikes — for commands of front, back, left and right — are translated into codes for the robot. Run over 10 minutes, with obstacles moved around in random, the robot was able to navigate successfully nearly 98 per cent of the time. Though still nascent, the "Neuro-electronic hybrid systems" experiment allows researchers to develop electronic systems that use the learning and processing abilities inherent in brain cells. Jude Baby George from CeNSE believes the system has the potential to build a computing system with "wetware" — a combination of hardware, software and neural functions of organic matter — capable of adapting and solving real-world problems.

MDR-TB spreads less within households

There are greater chances of controlling the spread of MDR-TB due to its lower fitness. : Unlike people with drug-susceptible TB, those with multi drug-resistant TB (MDR-TB) are less likely to transmit disease to others living in the same household (also known as household contacts), a study published on June 23 in the journal PLOS Medicine found.Although it may not be right to extrapolate the findings to the community level, within households, MDR-TB surely has "relatively low fitness [be less capable of spreading] compared with drug-susceptible TB."The study results agree with those of previous animal and laboratory studies, as well as molecular epidemiology studies that had estimated that the fitness level of drug-susceptible TB bacteria to spread within populations was higher than MDR-TB bacteria. But animal and lab studies do not take into account the clinical, environmental and socio-economic factors that influence infection. Hence, the latest study has great significance.The study carried out in South Lima and Callao, Peru followed up people living in the same household as the index patient for three long years (2010-2013). The study tracked 213 MDR-TB index patients and 1,055 of their household contacts. In the case of drug-susceptible TB patients, the study followed 487 index patients and their 2,362 household contacts.While only 35 of 213 of MDR-TB contacts developed MDR-TB disease, 114 of 2,362 drug-susceptible TB contacts developed disease. The hazard ratio for TB disease for household contacts of MDR-TB index cases was "half" that of the household contacts of those with drug-susceptible TB. As a rule, people who are in close contact for extended periods of time with a person who has TB disease — either drug-susceptible TB or MDR-TB — are at heightened risk of getting infected and suffering from TB The study found that male household contacts and those who slept in the same room as the index patient had higher incidence of active TB than those who did not share a sleeping room. Also, household contacts who had diabetes or HIV or who had previous history of active TB had higher incidence of active TB.While contacts from the lower socio-economic strata were more likely to suffer from active TB, crowding (large number of people in a household) was "not significantly" associated with higher incidence of TB disease. The results of the study indicate that there are greater chances of controlling the spread of MDR-TB due to its lower fitness. But there can be no room for complacency as the MDR-TB can become fitter with time and be equally transmissible as drug-susceptible TB.

Bombay Blood: how the rare blood type was discovered : A heart-warming piece of news was reported about two weeks ago regarding a life-saving instance of blood donation. An infant,

Sandesh Kumar from Gorakhpur, U.P., was found to have an inborn heart condition that needed surgery to set it right. This required blood donation. But the youngster has a very rare blood type. His blood is not O, A, B, or AB, but a special type called (hh)- a rare one first discovered in Bombay in 1952, and hence christened as Bombay Blood. People who carry this rare blood type, about 1 in 10, 000 Indians, can accept blood only from another Bombay Blood type individual, and not from anyone who is O, A, B or AB type. Why is it called Bombay Blood, and how was it discovered?. Dr Durgadas Kasbekar of CDFD Hyderabad has written a detailed and lucid article about it in the forthcoming issue of the journal Indian Journal of History of Science, What is the biology behind this exceptional blood type? To understand this, let us first look at what each blood group type contains. Blood contains red blood cells (and other cells that are not relevant for us here, floating in a fluid called plasma. Red blood cells carry on their surface a set of markers with which the plasma interacts. This compatibility or cross-talk between the cell and the plasma is what makes each blood type special. The markers on the cell are determined by a master type called H, out of which are generated types A, B, AB and AO. Bombay doctors found that the hh type (Bombay type people) can accept only from other hh type, and also can receive only from the hh types. This makes the Bombay Blood types a very special and rare category of people. How did this happen and why are these people so rare? It is largely because of extensive inbreeding within the same lineage or close-community marriages, often consanguineous, such that the 'blood type' or the gene pool is greatly restricted. Such intra-community marriages have happened in small isolated communities such as the gypsies, Russian Jewish or Parsi communities. It is thus likely that the Bombay Blood types have common ancestral origins.

Phase I clinical trials for Ebola virus concluded : At this time, there are no vaccines to protect against EVD licensed for use in humans. Clinical trials for several candidate vaccines are in various phases and a safe and effective vaccine is hoped for by the end of 2015.Phase I clinical trials for two vaccine candidates – ChAd3-ZEBOV, developed by GlaxoSmithKline (GSK), in collaboration with the US National Institute of Allergy and Infectious Diseases (NIAID) and rVSV-ZEBOV, developed by NewLink Genetics and Merck Vaccines USA, in collaboration with the Public Health Agency of Canada – were concluded in January. Both have been shown to be safe and well tolerated in humans in Phase I clinical trials. The results from the trials for rVSV-ZEBOV were recently published in the New England Journal of Medicine.

POPULAR SCIENCE NEWS

Kasturirangan report to be implemented by year-end : Union Minister of State for Environment and Forests Prakash Javadekar recently said that the recommendations made by the Kasturirangan Committee report on eco-sensitive zones in the Western Ghats would be implemented by this year-end.He said that the Union government had asked the State governments concerned to hold consultation with the local population and submit their comments.While Kerala has submitted its comments, Karnataka is yet to do so. Tamil Nadu, Goa, Maharashtra, and Gujarat are expected to submit their reports shortly. The Kasturirangan panel was set up to study the Gadgil committee report on the Western Ghats. The Gadgil panel report had faced unanimous opposition from state governments for recommending that almost three-fourth of the hills, including plantations, cultivated lands and large habitations, be turned into a restricted development zone with an over-arching authority to regulate the region supersedingthe elected authorities' role. Recommendations made by the Kasturirangan panel:Around 60,000 sq km of Western Ghats, spread across six states, should be turned into a no-goarea for commercial activities like mining, thermal power plants, polluting industries and largehousing plans.It has suggested that 90% of

the natural forests left in the Western Ghats complex – adding upto60,000 sq km and constituting 37% of the entire hilly belt — be conserved under theEcologically Sensitive Area (ESA) provisions of the green law. The forest area falling within theESA would also cover 4,156 villages across the six states. The villages falling under ESA will beinvolved in decision making on the future projects. All projects will require prior-informed consentand no-objection from the gram sabha (village council) of the village.The panel has recommended that there should be a complete ban on mining activity in this zoneand current mining activities should be phased out within five years, or at the time of expiry of the mining lease.

'Over 70% of Everest glacier may be lost by 2100' : A paper published in "The Cryosphere", a journal of the European Geosciences Union, is a first approximation to how the Himalayan glaciers will react to increasing temperatures in the region. Over 70 per cent of the glacier volume in the Mount Everest region in the Himalayas could be lost in 85 years if greenhouse gas emissions continue to rise, a new paper suggests. It also indicates more flood risk in the future in the Kosi river downstream from Nepal to India. The paper, "Modelling glacier change in the Everest region, Nepal Himalaya", published on Wednesday in The Cryosphere, a journal of the European Geosciences Union (EGU), said the glacier volume could be reduced between 70 and 99 per cent by 2100. paper stresses that "the signal of future glacier change in the region is clear and compelling" and that decreases in ice thickness and extent are expected for "even the most conservative climate change scenario".

Large Hadron Collider resumes atom smashing after two-year pause : Scientists say they have successfully restarted the world's biggest particle collider after a two-year shutdown and upgrade and it's now producing almost double the collision energy of its first run. The European Organisation for Nuclear Research, or CERN, said on Wednesday the Large Hadron Collider will now run around the clock for the next three years. The collider underwent a $150 million-upgrade after its first run, which produced results that helped confirm the existence of an elusive subatomic particle, the Higgs boson. CERN's director, Rolf Heuer, says physicists hope the new run might lead to discoveries that could help "explain remaining mysteries such as dark matter". The LHC, located in a 27-km tunnel beneath the Swiss-French border, is now smashing together protons at 13 trillion electronvolts.

SCIENTIFIC INNOVATION

Smartphone technology enables blind to 'see' : Scientists are developing new adaptive mobile technology that could enable visually-impaired people to 'see' through their smartphone or tablet. Specialists in computer vision and machine learning based at the University of Lincoln, UK, funded by a Google Faculty Research Award, are aiming to embed a smart vision system in mobile devices to help people with sight problems navigate unfamiliar indoor environments.Based on preliminary work on assistive technologies done by the Lincoln Centre for Autonomous Systems, the team plans to use colour and depth sensor technology inside new smartphones and tablets to enable 3D mapping and localization, navigation and object recognition.The team will then develop the best interface to relay that to users - whether that is vibrations, sounds or the spoken word.

OPPORTUNITIES

Postdoctoral fellowships: Research Associate (Postdoc) at Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research Bengaluru. Job description: The applicant should have a Ph.D. The applicant should be well versed in mammalian cell culture, fluorescent microscopy, and basic molecular biology techniques. Proficiency in handling and carrying out mouse work would be a plus. Should have good oral and written communication skills and must be able to work in a team. http://www.jncasr.ac.in/ravim/index.html. Application

Deadline : 31/08/2015
Contact-Ravi Manjithaya-ravim@jncasr.acin

Employer -WT/DBT India Alliance. Location-Road No. 12, Banjara Hills, Hyderabad, Andhra Pradesh, India. Discipline -Life Sciences, Health Sciences. Position Type -Full Time

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The National Centre for Biological Sciences (NCBS) and University of Cambridge: The National Centre for Biological Sciences (NCBS), Bangalore, the Institute for Stem Cell Biology and Regenerative Medicine (inStem), Bangalore, and the University of Cambridge are pleased to announce the NCBS-inStem-Cambridge Postdoctoral Fellowship.The Fellowship allows for the joint-appointment of up to 4 Postdoctoral Fellows per year to work in Cambridge for half the period of a 4 year term, with the other half of the tenure to be completed in a partnering research group based in NCBS or inStem. **Further information:** Further information on NCBS and in Stem is available through their respective annual reports here and here. Please contact Dr Mani Shankar Narayanan at the University of Cambridge's International Strategy Office (msn28@cam.ac.uk), or Dr Rashi Tewari at the NCBS Academic Office (rashi@instem.res.in) for any additional information.

ICGEB (International Centre for genetic Engineering and Biotechnoogy) offers competitive Postdoctoral Fellowships in the Life Sciences to highly motivated scientists wishing to pursue postdoctoral research in a world-class scientific environment. The Fellowships consist of a very competitive package including stipend, health insurance and additional benefits. The most successful fellows will also be eligible to apply for ICGEB Early Career Research Grants to support their own research programmes as young PIs upon return to an ICGEB Member State. Closing Dates for Applications: 31 March and 30 September 2015.

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Prof. K. SRINIVASULU
KL University, Vaddeswaram, Guntur Dt., Andhra Pradesh, India
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