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RESEARCH ARTICLE

DETECTION OF CryI AC GENE EXPRESSION IN *Bt* COTTON THROUGH TWO DIMENSIONAL AGAR GEL PRECIPITATION TEST

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ABSTRACT

Hyper-immune serum was developed in three groups of rabbits. *Bacillus thuringiensis* (CEMB no. 30023 strain no. PR 17.2) was used to produce delta endotoxin, the product of *Cry*1 Ac gene which was used as antigen. Toxoid was prepared having 0 (control), 0.5 and 1.0 mg of delta endotoxin which was injected in rabbits which produced the hyper-immune serum. These antibodies were further used to detect the expression of *Cry*I AC gene. Agarose gel precipitation test was done against standard antigen and antiserum, as well as against antiserum and delta endotoxin from the *Bt* plants. All the Agarose gel precipitation tests, showed positive results which indicated that antibodies were successfully developed and expressed in rabbits against delta endotoxin derived from *B. thuringiensis*.

INTRODUCTION

Bacillus thuringiensis (generally Known as "*Bt*"), a Gram-positive, entomopathogenic bacterium, produces many kinds of toxic proteins during sporulation stage. These toxic proteins are composed of one or various crystal inclusions (also called δ- endotoxins). Few of these proteins have adverse affection with certain insects. They are not toxic to other organisms, including wildlife and beneficial insects (Schnepf *et al.*, 1998; DeMaagd *et al.*, 2001). Formulations based on *Bt* have been used for decades as biological insecticides in agriculture and forestry, as well as vector control against mosquitoes and black flies (Boisvert 2005). There is a growing interest in *Cry* proteins during the last two decades due to their matchless qualities as specificity and environment friendly which are not present in any conventional insecticide (Whalon and Wingerd 2003). The *Cry*I Ac genes encode for lepidopteran specific insecticidal crystal proteins having molecular weight ranging from 130 -140 KDa (Hofte and Whiteley 1989). Most of the cotton crops have been released in to the market in the recent years claiming the *Bt* variety. Although a variety of molecular approaches are reported for the direct identification of the genetically modified organisms (GMOs) through Polymerase Chain Reaction (PCR) followed by agarose gel electrophoresis and random fragment length polymorphism (RFLP), southern blotting and DNA sequencing (Walschus *et al.*, 2002) but the gene expression in certain varieties of plant, including *Bt* cotton, still require verification regarding the expression of *Bt* toxin in the plant. Direct detection of specific gene was evaluated on transgenic potato, tomato, herbicide resistant maize, *Bt*-maize, soybean and processed products (Hupfer *et al.*, 1997). The prime weakness of these technologies is the lack of tendency to provide any information about transgene expression. Gene silencing was reported, in which the transgenes remained in the genome, but were no longer expressed. Moreover, from the bio-safety point of view structural instability is observed. There is a tendency for the recombinant DNA to rearrange or become lost in successive generations. This could change the transgenic line in unpredictable ways and could be health and environmental risk (Mae-Wan-Ho and Cummins 2010). Immunoassay provides an alternative mean for the detection of GMOs based on the determination of specific protein product of the foreign gene. Polyclonal antibodies or antisera are antibodies that are produced from different B-lymphocyte cell resources. They are a combination of immunoglobulin molecules, specifically the immunoglobulin G (IgG), produce against a specific antigen each of them identifying a different epitope. The results of the study provide an easy to apply laboratory approach of two dimensional agarose gel precipitation test for the direct detection of *Bt* toxin expression in the leave extract of cotton plant.

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MATERIALS AND METHODS

Source of *Bacillus thuringiensis* standard suspension *Bacillus thuringiensis* (CEMB no. 30023 strain no. PR 17.2) was obtained from Center of Excellence in Molecular Biology (CEMB), Lahore. *Bacillus thuringiensis* was recultivated to prepare fresh suspension of bacteria.

Cultivation of Bacillus thuringiensis

Bacillus thuringiensis was grown in two different media. *Bt* was inoculate in LB media (Yeast extract 5 g/l, Trypton 10 g/l, NaCl 5 g/l and ager 14 g/l) plates and T_3 broth media (Yeast extract 1.5 g/l, Trypton 3 g/l, Tryptose 2 g/l, $MnCl_2$ 0.005 g/l and Sodium Phosphate

05 M), (Keshavarzi, 2008). Microscopy was done to study the basic characteristics of *B. thuringiensis*. For these purpose slides of *B. thuringiensis* were prepared and stained with Gram's staining method (Gram Stain Protocols 2010).

Separation of Delta endotoxin

The selective T_3 medium containing spores, parasporal crystal bodies and cell debries were separated by the method described by Zouari and Jaoua (1997). Briefly, a volume of 1 ml of suspension from T_3 medium was taken and shifted in 1.5 ml microtube. These tubes were centrifuged for 10 min at 10,000Xg and 24º C. All contents were pelleted at the bottom. The supernatant was discarded; the pellet was dried for one hour. Then one ml of 1 M NaCl was added in each tube and vortexed. When pellets were completely dissolved micro tubes were centrifugated at 10,000Xg and 24º C. All contents were pelleted. The supernatant was discarded and 1 ml of 1 M NaCl was added in each tube and then vortexed again. Followed by the centrifugation at 10,000Xg for 10 minutes and 24º C. The supernatant was discarded and then pellet was dissolved in distilled water and centrifuged for 10 min at 10,000Xg and 24º C. Then the pellet was washed with ice chilled d_3H_2O thrice. In the last the pellets of each micro tube stored at -20ºC. A 50 mM NaOH solution was prepared and adjusted to pH at 12.3. Parasporal bodies were suspended in 50 mM NaOH solution and incubated for 2, 4, 6, 8, 10 and 12 hours at room temperature. Solubilzed delta endotoxin was recovered in the supernatant after centrifugation for 10 min at 10,000Xg and 24º C.

Confirmation of Delta toxin

For the confirmation of delta endotoxin sodium dodycyl sulphat polyacrylamide gel electrophoresis (SDS-PAGE) was applied. SDS PAGE separated proteins on the basis of their molecular weight. The technique was performed in a Bio-Rad Mini Protein Tetra Cell gel apparatus model no. 165-8001EDU. The *Acrylamide* concentration for resolving gel was 10 %; that of stacking gel was 4 %. The delta endotoxins in the sample buffer were run on the gel and stained with the Coomassie stain (0.25 % Coomassie Brilliant Blue, 45 % methanol and 9 % glaciac acetic acid) for 30 minutes at room temperature (Zouari and Jaoua 1997). Formalin inactivated toxoid was mixed with equal ratio of Montanide (Cipic, France) adjuvant and kept in dark at 4° C. Rabbits were used as experimental units. First inoculation was made through interaperitoneal route followed by injections of the antigen (0.25ml) given thigh area through subcutaneous (S/C) route. The rabbits were re-immunized and booster at two days interval for eight injections and the rabbits were maintained for two weeks further without any inoculation. Total blood was obtained and kept in falcon tubes for 3 hour at room temperature. By this process serum was separated from blood contents in new falcons. Serums were pooled separately for each group after centrifugation for 5 minutes so that red blood cells settled down at the bottom. Supernatant were shifted in new falcons. Pure serum was heat inactivated at 56ºC for 20 min in the water bath and stored at -20º C (Protocol for Heat Inactivating Serum, 2010).Two dimensional technique of agar gel precipitation test (AGPT) was conduct with antibodies produced and protein extracted from a *Bt* plant that contained delta endotoxin.

Extraction of Bt cotton leave

For this purpose protein was extracted from *Bt* cotton verity Ali Akbar 703 by the method described by Granier (Granier 1988).

RESULTS

Cultural characters in LB medium

In LB medium and the culture showed the appearance of colonies having creamy white color with raised and smooth surface with entire edges. The colonies were flat to slightly elevated ranging from 2-7 mm in diameter. The plates of inoculated LB agar showed typical cream colored growth when incubated at 50ºC (Fig .1).

Fig. 1. Growth of *Bacillus thuringiensis* on LB medium after 24 hours of incubation at 50ºC

Morphological characteristics

Pure growth of culture showed the cells stained blue which was declared as Gram positive. *B. thuringiensis* also showed spore formation as seen microscopically with typical oval shaped and non-bulging unstained mass within the Grams positive cells located at sub-terminal position as viewed in Fig.2 both phases were differentiated based on their specific characters.

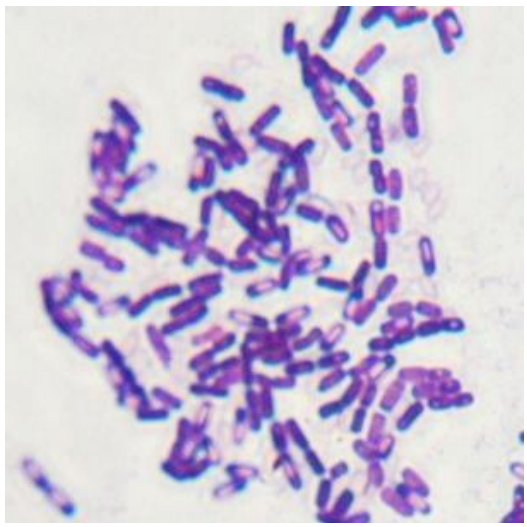

Fig. 2. Microscopic view of the culture growth of *B. thuringiensis grown* inT_3 medium after 48 hours of incubation, showing typical rods with sub terminal spore which are oval and non-bulging illuminated space within the gram positive cells(400X)

After 72 hours of growth the sporulation was completed and checked under microscopic examination through malachite green staining method as light green colored spore.

Isolation of delta endotoxin

Spore suspension was further processed for the separation of Delta endotoxin produced by parasporal bodies during sporulation phase. . *B. thuringiensis* spores were counted after culturing in T_3 medium. The average count was found with 3.1 x 10^7 spores/ml. The purified delta endotoxin was containing as total protein contents of 350µg/ml.

Sodium Dodecyl Sulphate (SDS) PAGE of protein derived from *B. thuringiensis* showed the presence of delta endotoxin in the band. The molecular weight showed by protein ladder (200, 116, 97, 66, 55 kDa) was 130 KDa.

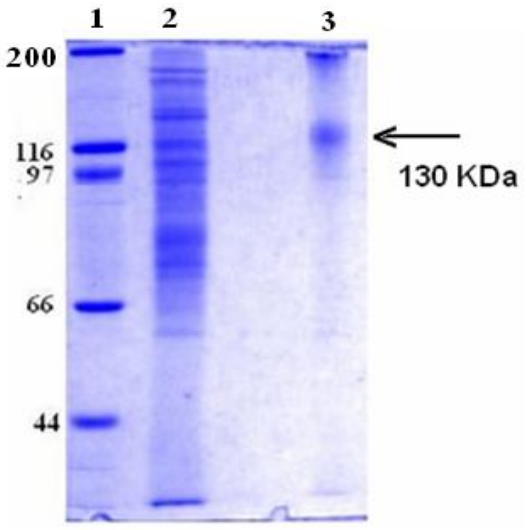

Fig.3. SDS PAGE confirmation. lane 1 protein ladder, lane 2 bacterial culture (crude form) with parasporal bodies which is used to purify the delta endotoxin and in lane 3 purified delta endotoxin (130 KDa)

SDS PAGE run for different incubation period for protein extraction showed that by increasing time, the production of delta endotoxin was also increased as shown in Figure 4.

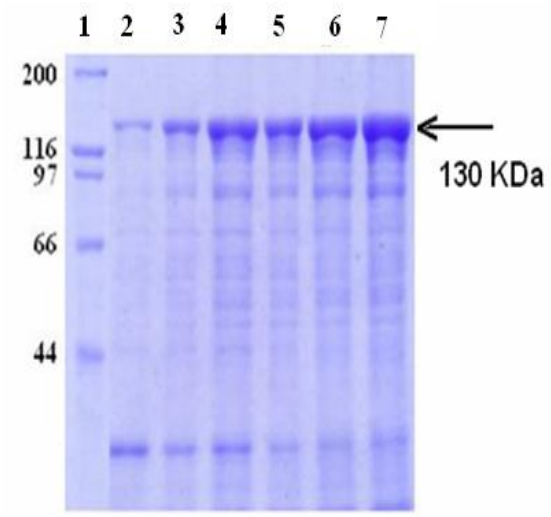

Fig. 4. Expression of Delta toxins through SDS PAGE as observed at different Incubation Periods. In lane 1 protein ladder, and in lane 2, 3, 4, 5, 6 and 7 delta endotoxin derived from 2, 4, 6, 8, 10 and 12 hours of incubation periods, respectively.

Confirmation through agarose gel precipitation test (AGPT)

Antiserum obtained was confirmed through Agarose Gel Precipitation Test (AGPT). AGPT was positive with bands formation which indicated specific antibodies purified from the blood of rabbit. White precipitation band formation indicated specificity of the antigen and antibody system thus proved the positivity. Serum of group A had weak precipitation band (Fig. 5), while serum of the group C was remained negative. Clear visibility of precipitation band was indicated in the serum of group B rabbits.

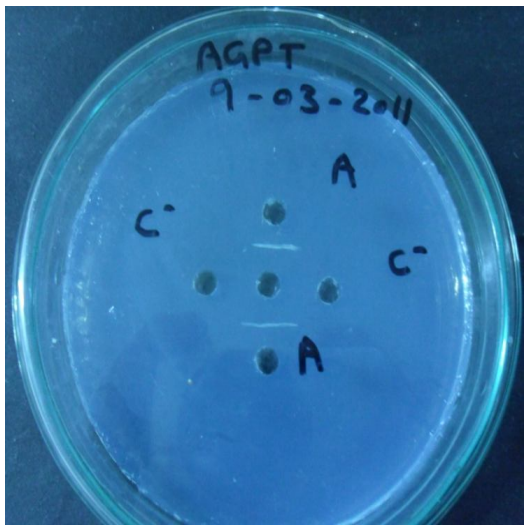

Fig. 5. Agarose Gel Precipitation Test results of group A (standard antigen), Group C (serum from control group), and its antiserum raised in rabbits in central well.

Confirmation of delta endotoxin derived from Bt plant by AGPT

AGPT for the confirmation of delta endotoxin present in plant protein was conducted. AGPT result showed the presence of delta endotoxin in *Bt* cotton variety Ali Akbar 703 with the band formation on the gel as shown in Fig. 6.

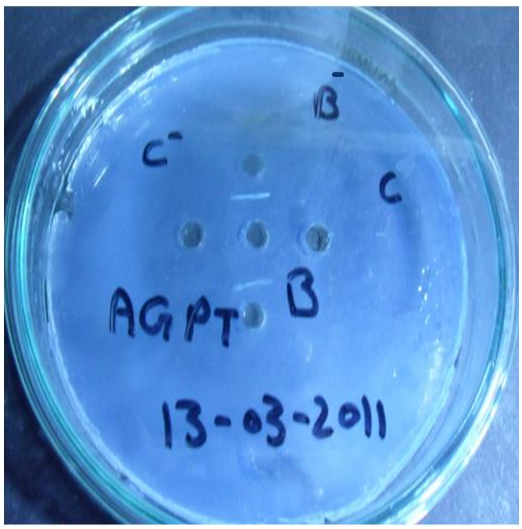

Fig.6 Agarose Gel Precipitation Test result positive results with B (stander antigen), B (standard antigen from plant extract), C (distilled water as antigen) and C (Plant extract from non-bt plant). In well A antibodies and in well B protein from plant

DISCUSSION

Biomonitoring is necessary for *Bt* varieties from time to time in order to determine the current status of the *Bt* cotton variety. Initial confirmation of *B. thuringiensis* as a Gram positive bacteria which showed typical growth in the formation of turbidity in T3 broth with straw colored suspension (Gill *et al.,* 1992; Moraga *et al.,* 2004). The culture growth on LB medium having creamy white color colonies, with entire edges (Peng *et al.,* 2001). LB medium the vegetative growth of culture was enhanced with the suspension appeared as chain of bacilli, In T_3 medium, the culture was more supportive for the development of spores, thus deemed as fit for the propagation of parasporal bodies, which produced insecticidal delta endotoxin (Manasherob *et al.,* 1998). Delta endotoxin was derived from *B.*

thuringiensis confirmed by SDS PAGE which was indicated with the appearance of 130 KDa protein band product of *Cry* I Ac gene. The purification of delta endotoxin purified from the parasporal bodies was evident through SDS-PAGE profile when compared with the crude suspension of bacterial suspension. Appearance of several bands in the culture suspension and the single band of 130 KDa in the Purified delta endotoxin suspension confirmed the successful production of delta endotoxin Another SDS PAGE experiment showed the relation between the time of incubation of parasporal bodies and the production of delta endotoxin. The results showed increased production of delta endotoxin, with increasing incubation time. Hence, the bands were more visible (Jurat-Fuentes and Adang, 2004). This insecticidal delta endotoxin was used as antigen to produce hyperimmune serum which contained antibodies against delta endotoxin. The study showed that AGPT of these standard antiserum and specific antigen showed positive results by forming a band. Similar results were obtained when AGPT was conducted with plant protein containing delta endotoxin by forming bands (King and Meyer, 1963).

Conclusion

A simple technique of two dimensional agar gel precipitation test was optimized for the easy detection of delta endotoxin expression in the *Bt* cotton plant. This technique may be utilized for the assessment of new and old varieties of *Bt* cotton crops for their verification regarding the expression of Cry1 Ac gene.

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