INTERGENERIC PROTOPLAST FUSION OF *AGROBACTERIUM TUMEFACIENS* AND *BACILLUS THURINGIENSIS*

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BY

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DEDICATED TO MY PARENTS
AND FAMILY

CONTENTS

CERTIFICATE

Certified that the work incorporated in the thesis "Intergeneric protoplast fusion of *Agrobacterium tumefaciens* and *Bacillus thuringiensis*" submitted by Mrs. Ulka Shrirang Puntambekar was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

P K Ranjekar

Dr. P.K. Ranjekar
Research Guide

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Ulka Puntambekar

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LIST OF ABBREVIATIONS

kD	kilodalton
Kb	Kilo base pairs
°C	degrees centigrade
MW	molecular Weight
rpm	revolutions per minute
h	hour
min	Minute
v/v	volume by volume
µg	microgram
ml	millilitre
mg	milligram
g	gram
l	litre
µl	microlitre
IU	International Units
Ap	ampicillin
Km	kanamycin
Oct	octopine
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol

SYNOPSIS

The use of protoplast fusion technique to generate novel strains and new products unobtainable by conventional methods is an exciting one. This technology has aroused a great deal of interest in breeding intraspecies, interspecies and intergeneric hybrids for improving abilities of industrial microorganisms, as it helps to overcome the noncompatible barriers between mating types, species and also genera (Hopwood, 1981). This technique has been used as a tool for genetic studies (Akamatsu and Sekiguchi, 1987; Illing *et al*,,1989) or for plasmid transfer (Vander Vossen *et al*,,1988), and thus it could add new dimensions to genetics of microbes fortifying conventional genetic techniques.

Agrobacterium tumefaciens and *Bacillus thuringiensis* are the two bacterial genera which play an important role in plant genetic engineering technology. The gene for the insecticidal crystal protein or delta endotoxin has been engineered into plant system via *Agrobacterium* mediated plant transformation so that the transgenic plants are protected from the specific insects (Vaeck *et al*,, 1987). Introduction of Ti plasmid or tumor inducing capacity into taxonomically related bacterial species has been demonstrated, but to transfer the same into more distant bacteria did not lead to tumor induction strain (Hille *et al*,,1983; van

Veen *et al*,,1988). With a view to exploit the newly rising protoplast fusion technology and to develop a new strain which will be an important tool in plant genetic engineering work, an attempt has been made to fuse the protoplasts of *Agrobacterium tumefaciens* and *Bacillus thuringiensis* and to isolate the fusants having tumor inducing capacity alongwith the insecticidal property. A brief outline of these studies is as follows.

Screening of *Agrobacterium tumefaciens* and *Bacillus thuringiensis* strains for protoplast fusion :

For more than a decade *Agrobacterium tumefaciens* has been used as a plant vector for trasnferring desired genes into plants. During infection, the bacterium transfers a portion of its Ti plasmid, the T-DNA segment to the plant genome. Transcription and translation of genes present on the T-DNA causes crown gall tumors in plants. It also contains the genes that encode synthesis of opines, which are modified amino acids and the presence of these compounds in plant extract is unique in case of infected plant tissues.

The first step in the selection of *Agrobacterium tumefaciens* strain having the ability to infect pigeonpea (*Cajanus cajan*) is to develop a suitable transformation method toward this plant. In this work, I have used differ-

ent wild type *Agrobacterium tumefaciens* strains and have determined their relative virulence using various plant transformation methods.

A quick method for *in vitro* tumor formation in pigeonpea was developed. Infections in 72 h old germinating seedlings were able to produce tumors within 10-12 days, whereas tumor formation *in vivo* was observed after 4-6 weeks by infecting the 3-4 weeks old plants. Thus, this method was useful in screening a number of virulent strains within a short period. Opine expression in tumors was varified by analysis of tumor tissue extracts by high voltage paper chromatography.

A gram positive sporulating bacterium *Bacillus thuringiensis* produces a protein (delta endotoxin) which is specifically lethal towards the Lepidoptera, Diptera and Coleoptera larvae. These delta endotoxins are produced during sporulation as parasporal crystals which are toxic when ingested by susceptible larvae. Genetic studies have shown that the structural toxin genes are often located on one or several high molecular weight plasmids. The pigeonpea crop is mainly affected by lepidoptera pest. From the view to obtain a highly active *Bacillus thuringiensis* strain against Lepidopteran class of larvae, various *Bacillus thuringiensis* subspecies were screened by insecticidal

bioassay. *Bacillus thuringiensis* subsp. *kurstaki* (NCIM 2514) at 10^8 spores/ml concentration showed maximum mortality (more than 90%) with the neonate *Spodoptera litura* larvae by using artificial diet and leaf painting methods. These results were confirmed by using the purified crystal protein of this strain.

From this screening work, the highly active strains of *Agrobacterium tumefaciens* (NCIM 2943, Ap^s Km^r $Octopine^+$) and *Bacillus thuringiensis* (NCIM 2514, Ap^r Km^s $Octopine^-$) were selected for isolation and fusion of protoplasts.

Efficient protoplast regeneration of *Agrobacterium tumefaciens* and *Bacillus thuringiensis*

For any genetic work on protoplast fusion, successful regeneration of protoplasts into normal cells is essential in order to give a progeny which could be genetically characterized. Subtle differences in experimental conditions and techniques can have profound effects on isolation and regeneration of protoplasts. I have optimized the conditions for formation and regeneration of *Agrobacterium tumefaciens* and *Bacillus thuringiensis* protoplasts. Treatment of cells in early exponential growth phase (8h) with lysozyme (1mg/ml) resulted in efficient protoplastiza-

tion (more than 90%) and 10-12% protoplast regeneration in both the strains. Interestingly, it was also observed that there was ten fold increase in the protoplast fusion frequencies when the 8h grown cells were used for isolation of protoplasts.

Intergeneric protoplast fusion between *Agrobacterium tumefaciens* and *Bacillus thuringiensis*, isolation of fusants and their general characterization

The protoplasts of *Agrobacterium tumefaciens* and *Bacillus thuringiensis* were fused in presence of PEG and the fusants were isolated on selection medium containing both the antibiotics (Ap^r and Km^r) and octopine as a sole source of C and N which is the unique property of *Agrobacterium tumefaciens*. The stable fusants were tested for a few morphological and biochemical characters. Some of the important features of the fusants are as follows:

[1] Fusants showed mixed phenotypic and genotypic characters of both the parental strains.

[2] Five of the hybrids irrespective of their morphological nature (either gram negative or gram positive rods) showed octopine positive tumor formation in pigeonpea seedlings. This finding confirmed the presence of Ti plasmid in the hybrids and that during transformation the T-DNA was

transferred into plant genome.

[3] All the hybrids were Km^r and Ap^r and it was confirmed by neomycin phosphotransferase II (NPTII) and penicillinase activities respectively.

[4] One of the tumorogenic gram positive hybrid gave the positive signal for immunological test against insecticidal protein specific antibodies. Presence of toxin gene was confirmed by DNA hybridization. This hybrid was further studied in detail for its toxicity.

Comparative toxicity of protoplast fusant and of transgenic *E.coli* containing *bt* gene

To prove the efficiency of protoplast fusion technique, a transgenic *E.coli* containing *bt* gene was constructed. Total plasmids from *Bacillus thuringiensis* (2514) were purified by CsCl (EtBr) gradient and a plasmid DNA library was constructed in vector pUC18. The vector DNA cut with *HindIII* was ligated to the total plasmid DNA prepared from 2514 and cut with *HindIII* and transformed into *E.coli* HB101. Ap^r colonies were selected for β-galactosidase expression by plating on X-gal. The transformants were further screened by colony hybridization and southern hybridization using delta-endotoxin specific probes. One of the

positive transformants pBt15 showed larvicidal activity against *Spodoptera litura*. The insecticidal toxicity bioassay with this pest showed 10% higher activity with the protoplast fusant AB0242 compared to the *bt* gene clone pBt15. The Lc50s and potency in terms of International Units were determined and it was found that the protoplast fusant was more potent than the transgenic construct.

In summary, this work established the applicability of intergeneric protoplast fusion technique in bacterial cell engineering studies and demonstrated that the recombination events could generate different organisms by protoplast fusion between the two distantly related genera. The entire thesis is organized in five chapters :

CHAPTER I : A general review on protoplast fusion.

CHAPTER II : Screening of *Agrobacterium tumefaciens* and *Bacillus thuringiensis* strains for protoplast fusion.

CHAPTER III : Efficient regeneration of protoplasts from *Agrobacterium tumefaciens* & *Bacillus thuringiensis*.

CHAPTER IV : Intergeneric protoplast fusion between *Agrobacterium tumefaciens* & *Bacillus thuringiensis*

CHAPTER V : Comparative toxicity of protoplast fusant and transgenic *E.coli* construct containing *bt* gene.

The overall discussion and concluding remarks on the present work are given at the end of the thesis.

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CHAPTER I

BACTERIAL PROTOPLAST FUSION

A GENERAL REVIEW

SUMMARY

Protoplasts can be isolated from bacterial cells by digestion of the cell wall with the help of lysozyme in presence of osmotic stabilizers. Fusion of protoplasts can be induced by chemical fusogens like polyethylene glycol. The electrofusion technique has been reported in bacteria in which the fusion frequency is much higher than that obtained by PEG induced protoplast fusion. This technology allows recombination to take place not only between related species but also between unrelated genera and has a great potential in the breeding and improvement of industrial strains. This review includes the information and developments on the protoplast fusion in bacteria with special reference to genetic recombination by protoplast fusion between phylogenetically unrelated bacteria.

INTRODUCTION

The traditional approach to the improvement of industrial strains involves mutagenesis of a suitable strain followed by screening of a large number of isolates for the new desired characteristics. This approach has undoubtedly been successful as evident from its applications in advances in basic sciences and technology (Rawlands, 1984a;b). However, random screening is somewhat laborious since a large number of isolates need to be tested to detect strains with desired traits. A variety of other methods and mechanisms by which genetic recombination may be achieved are already well understood and several reports have indicated their application in industrial strain improvement (Calam *et al.*, 1976; Ball, 1982). With the advent of protoplast fusion technology, the situation has changed and rapid breeding techniques based on protoplast fusion are now available for a variety of industrial organisms. Cell engineering through protoplast fusion helps in transfer of multigenic phenotypes across the barrier of genera which is otherwise impossible, even with the help of recombinant DNA technology. Another advantage of protoplast fusion is that it does not require organisms which are deficient in recombination and restriction-modification systems. This latest advance in protoplast technology requires a detailed

study of protoplast systems in relation to their isolation and regeneration.

PRINCIPLES OF PROTOPLAST ISOLATION, REGENERATION AND FUSION

Protoplast Isolation

The term protoplast is normally used to describe a cell which is completely devoid of cell wall residues. Specific conditions for protoplast isolation have been developed in plant cells (Kao *et al.*, 1974; Power *et al.*, 1970; Tanaka *et al.*, 1984), fungi (Gold *et al.*, 1983; Picatoggio *et al.*, 1983) and bacteria (Fodor and Alfoldi, 1976) including aerotolerant anaerobes such as *Clostridia* (Allcock *et al.*, 1982; Knowlton *et al.*, 1984; Minton and Morris, 1983).

Protoplasts are readily obtained from gram positive organisms using lysozyme to digest the cell wall (Ghuysen *et al.*, 1966). Novick *et al.* (1980) have used lysostafin instead of lysozyme for obtaining protoplasts from *Streptomyces*. Protoplast isolation from gram negative organisms is usually more difficult due to the presence of a more complex cell wall (Costerton *et al.*, 1974). The peptidoglycan is shielded from the attack of lysozyme by the outer layer of lipopolysaccharides. The earlier attempts to isolate protoplasts from gram negative organisms have been

unsuccessful, wherein formation of osmotically sensitive bodies, which retained part of the cell wall was observed. These osmotically sensitive bodies were called sphaeroplasts. Weiss (1976) has described a procedure wherein the combination of lysozyme and EDTA converted *E.coli* cells to true protoplasts. The main disadvantage with this method is that, as EDTA being toxic, its prolonged treatment decreases the survival of protoplasts. Another procedure described by Rodicio *et al.* (1978) for isolating true protoplasts has used antibiotic fosfomycin in growth medium which inhibits cell wall synthesis. However, this procedure was not suitable for obtaining good yield of protoplasts from other gram negative organisms like *Serratia marcescens*. A number of other β-lactam antibiotics like carbenicillin and cephalosporins (Cephalotin, cephacitrile, cephamandole, cefoxitime) have also been used for protoplast isolation (Martin, 1983). Some gram negative bacteria were converted to sphaeroplasts by treatment with ceffizoxime in presence of Ca^{2+} without any osmotic stabilizers (Toda *et al.*, 1988). The standard lysozyme EDTA method sometimes does not work with some gram negative bacteria like *Zymomonas mobilis* as shown by Yanase *et al.* (1985). They subcultured *Zymomonas mobilis* cells in a medium containing penicillin G or glycine to generate sphaeroplasts and observed that almost all the cells were converted to protoplasts after 10 hours. A highly

efficient procedure for quantitative conversion of *E.coli* cells to sphaeroplasts has been developed by Marvin and Witholt (1987) in which a much lower concentration of lysozyme was used. The physiological status of microorganisms at the time of protoplasting is the major factor in determining protoplast yield. It has been shown that the culture in the exponential growth phase gave the highest protoplast yield (Shahin, 1972; Okanishi *et al.*, 1974; Peberdy *et al.*, 1976). Temeyer (1987) pointed out that the sensitivity of the cell wall to lysozyme was dependent on the conditions and treatment given to cells during growth and harvest. Physiologically active but non-growing mycelium of *Streptomyces* obtained by two stage culture system was found to be essential for the isolation of protoplasts with higher regeneration frequencies (Chater *et al.*, 1982; Ogawa *et al.*, 1983). The growth medium also has important effect in protoplast formation. Sagara *et al.* (1971) have found that *Streptomyces* mycelium cultured into the medium containing enough glycine was more sensitive to lysozyme than the mycelium grown in absence of glycine. The levels of glycine in the medium vary from 0.8% to 3.5% in different species (Hopwood *et al.*, 1977; Baltz, 1978). Similarly protoplasts of *Micromonospora echinospora* cells were obtained when they were grown

in a medium containing 0.15% glycine (Love *et al.*, 1992). The significance of glycine in increasing the susceptibility of mycelium to lysozyme has not been resolved. In some species of *Streptomyces*, no lysozyme treatment is required to obtain protoplasts from mycelium when grown in presence of glycine (Hopwood, 1981). Rodicio *et al.* (1978) have observed that very young mycelia of some species of *Streptomyces* when grown even in absence of added glycine were converted to protoplasts after treatment with lysozyme. Okanishi *et al.* (1974) have used a mixture of lysozyme and lytic enzyme No.1 from *Cytophaga* and observed more rapid protoplast formation than by lysozyme alone. Other enzymes like lysoamidase (Petrov *et al.*, 1991), protease K, actinase E or α-amylase (Ishii *et al.*, 1990) were used either in combination with lysozyme or individually for the formation of bacterial protoplasts. These observations necessarily suggest that the variations in cell wall composition affect the protoplast formation and differences in response to various enzymes.

Protoplast regeneration

For any genetic work on protoplast fusion or protoplast transformation, successful regeneration of protoplasts into normal cells is essential in order to give a progeny which could be genetically characterized. The

minimum requirement for protoplast regeneration is the presence of osmotic stabilizer in solid media. The osmotic stabilizers generally used are inorganic salts in case of yeast or fungi and 20% sucrose or 16% sorbitol for bacteria. Addition of plasma expanders such as polyvinylpyrolidone, vinylpyrolidone or dextran at 3% concentration to regeneration medium increased the regeneration frequency of *B.subtilis* protoplasts (Akamatsu and Sekiguchi, 1981). However, no such effects of plasma expanders were observed on regeneration of *Z.mobilis* protoplasts (Yanase *et al.*, 1985). In addition to osmotic stabilizer, 25mM each of $CaCl_2$, $MgCl_2$ and low levels of phosphate have been found to be necessary for efficient regeneration of *Streptomyces purvulus* protoplasts (Ochi *et al.*,1979). Regeneration frequency is generally affected by various factors. The temperature at which the plates are incubated for regeneration can have a profound effect on the efficiency of regeneration. Ambient incubation temperatures (29°-30°C) are generally optimum for the regeneration of *Streptomyces* protoplasts (Baltz and Matsushima, 1981). In case of bacilli, the temperature during bacterial growth and lysozyme treatment can affect the regeneration frequency. It has been found by Gabor and Hotchkiss (1979) that a differential between the temperature used for the growth of the cells before

protoplast formation and temperature of the lysozyme treatement increase the extent of regenerating behaviour. With many *Streptomyces* spp., autoinhibition may occur during the regeneration of the protoplasts, that is, protoplasts which regenerate fast, inhibit the regeneration of surrounding protoplasts (Baltz, 1978: Baltz and Matsushima, 1981: Hopwood *et al.*, 1977). Partial dehydration of medium can essentially eliminate autoinhibition, shorten regeneration time and result in increased regeneration frequencies.

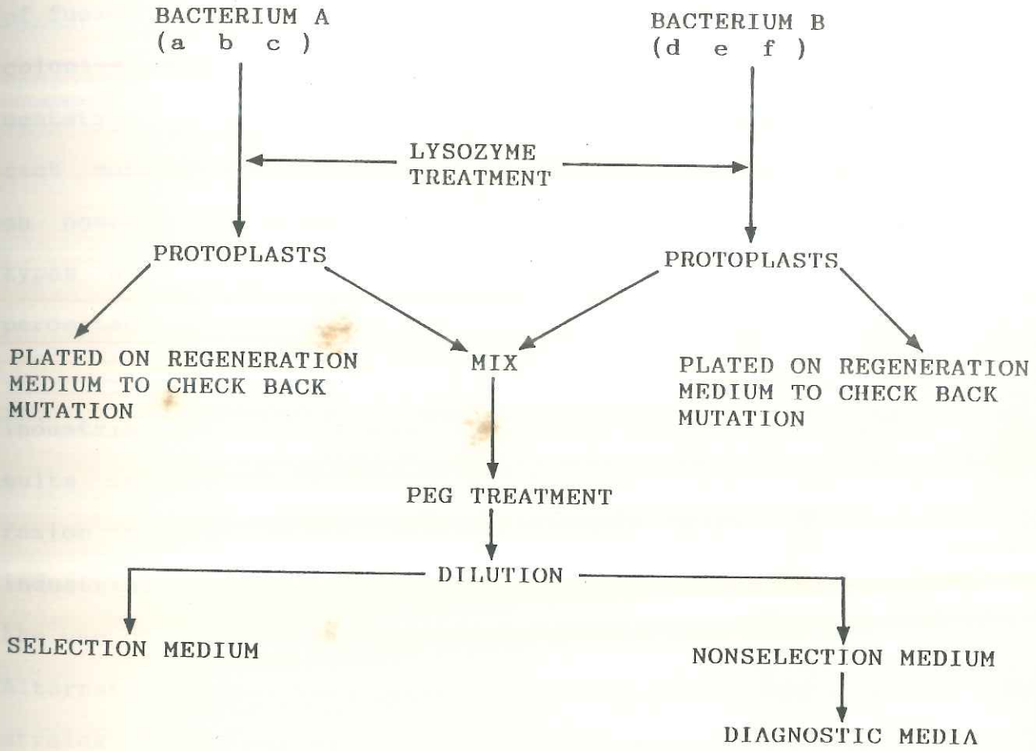
The effects of concentration of penicillin G or glycine added at the time of sphearoplast formation on the regeneration of *Zymomonas mobilis* protoplasts have been investigated by Yanase and coworkers (1985). The protoplasts of *Z.mobilis* isolated by treatment of low concentration of penicillin G or glycine showed higher regeneration frequencies probably by stimulating cell wall synthesis (Yanase *et al.*, 1985). Similar results were obtained in case of *Cellulomonas* protoplasts (Kim and Lee, 1985). The supplementation of BSA during the formation of protoplasts from *Streptomyces clavuligerus* supported the highest viability and thus improved the regeneration. However, incorporation of BSA in the regeneration medium caused significant reduction in regeneration frequency (Illing *et al.* 1989a). In general, one can see from available reports dealing with various conditions for obtaining opti-

num yield of regenerating protoplasts of bacteria, that there are more state of art protocols than general fundamental techniques for bacteria as a whole.

Protoplast Fusion

The schematic representation of protoplast fusion in bacteria is given in Fig 1.1. The generalized procedure for bacterial protoplast fusion involves the use of the parental strains that are genetically marked with complementary auxotrophies or additional selection markers unique to either parent or generated through prior mutagenesis. Protoplasts of both parental strains are mixed in equal numbers and treated with fusogens. Polyethylene glycol (PEG) induces the aggregation of protoplasts and fusion events occur after fusogen is washed or diluted away. The mechanism of the fusion process has been studied in animal cells. Although the precise mechanism is unknown, membrane/membrane contact is established among protoplasts within each aggregate. It is thought that during this contact, localized reorganization of the membrane proteins occurs and small cytoplasmic bridges are formed which enlarge as the two protoplasts fuse (Ferenczy, 1981). The presence of Ca^{2+} and the dilution of the PEG solution are the crucial part of the fusion process (Ferenczy,

FIG. !.!: SCHEMATIC REPRESENTATION OF PROTOPLAST FUSION IN BACTERIA



1981). The PEG treated protoplasts are then plated onto suitable media and the fusion products are recovered by direct or indirect selection methods. In the direct method, the fusion products were recoverd applying strong selection (Ferenczy *et al.*, 1975; Anne and Peberdy, 1976) and a slight relaxation from the strong selection normally allows segregation of the parental types. In this method, the frequency of fusion is assessed by determining the ratio of number of colonies on minimal medium arising from nutritional complementation to number of colonies on complete medium. Indirect method involves the screening of colonies developed on nonselective media for parental and recombinant genotypes and in this case the frequency is determined on the percentage of recombinants obtained.

The introduction of genetic markers in industrial strains is time consuming and frequently results in the loss of productivity. When the protoplast fusion is applied to the production of new genotype of industrial microorganisms, a selection procedure based on the use of auxotrophic strains may be used with reluctance. Alternative approaches other than the use of auxotrophic strains have been employed. Fodor *et al.* (1978) have shown that heat killed protoplasts of *B.megaterium* could be fused with viable protoplasts to give rise to recombinant progeny. Here nonviable protoplasts function as the donor of genetic

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material. Levi *et al.* (1977) have successfully fused streptomycin killed *B.subtilis* protoplasts with streptomycin resistant strains but the fusion frequency was ten fold less compared to that obtained using viable protoplasts.

In all the protoplast fusion experiments, generally PEG is used as a fusogen to induce fusion. This method, however, has more disadvantages than the electrical fusion developed by Zimmerman and Schnettler (1983). The electrofusion technique has been reported in yeasts (Schnettler *et al.*, 1984; Schnettler and Zimmerman, 1985); *A.nidulans* (Lynch *et al.*, 1989) and in *Streptomyces* (Okamura *et al.*, 1988; 1989). With this technique, the fusion frequency is found to be much higher than that obtained by PEG induced protoplast fusion (Okamura *et al.*, 1988).

APPLICATIONS OF PROTOPLAST FUSION TECHNOLOGY

Protoplast fusion as a tool for genetic studies

There are well established mechanisms for genetic transfer in microorganisms leading to recombination. These mechanisms provide a basis for genetic studies. Transformation and transduction are well known techniques for genetic mapping in *B.subtilis* (Young and Wilson, 1972). Transformation is very useful for determination of linkages of markers separated by short distances (Henner and

Hoch, 1980). However, a great many transformation trials are required to map mutations unless additional supplementary information is available. Transduction is used to reveal linkages between markers separated by 6% or 10% of the total chromosomes (Young and Wilson 1972; Henner and Hoch, 1980). It could be hoped that protoplast fusion could add new dimensions to genetics of microbes fortifying the conventional genetic techniques. Fusion of bacterial protoplasts generates the material suitable for the investigation of the gene product interaction in the genetic recombination and biosynthesis. Some of these possibilities and the identification of the fusion products (Schaeffer *et al.*, 1976;) are based on the common expectation that in diploids complementation will normally occur between alleles of the same gene. Protoplast fusion as a technique for chromosome mapping has been reported for *Streptomyces* species (Baltz, 1980), *Staphylococcus aureus* (Stahl and Pattey, 1983); *B.stearothermophilus* (Chen *et al.*, 1986) and *B.subtilis* (Akamatsu and Sekiguchi, 1987). A preliminary position for 11 markers on the *Streptomyces clavuligerus* genetic map is proposed with the use of protoplast fusion (Illing *et al.*, 1989b). Genetic analysis using multiple auxotropic markers in intergeneric hybrids has been carried out in case of protoplast fusion between *Cellulomonas* sp.

and *Bacillus subtilis* (Gokhale *et al.*, 1984).

Generation of novel strains by protoplast fusion

Intra and Interspecies Fusion : The use of protoplast fusion technique to produce novel strains and new products unobtainable by conventional methods is an exciting one. This technology has aroused a great deal of interest in breeding intraspecies, interspecies and intergeneric hybrids for improving abilities of industrial microorganisms. *Streptomyces* and *Bacillus* are the two genera among most important groups of industrial microorganisms. Bacilli produce variety of enzymes and also a few antibiotics of minor commercial significance while *Streptomyces* produce many of the naturally produced antibiotics. Two other genera, *Micromonospora* and *Brevibacterium*, are noted for the production of several antibiotics and amino acids respectively. Not surprisingly, therefore, these organisms have been mainly used in early protoplast fusion experiments. Earlier, intraspecies and interspecies protoplast fusion in *Bacillus* and *Streptomyces* were carried out for genetic studies. Only recently, the trend has been developed to use this technique for generating new strains with higher productivities. *Streptomyces* producing new antibiotics have been generated by PEG induced protoplast fusion (Schlegel and Fleek, 1980; Mazieres *et al.*, 1981;

Gomi *et al.*, 1984; Yamashita *et al.*, 1985). A hybrid strain of *Streptomyces* was obtained by electrofusion which produced totally new antibiotics active on a wide range of gram negative bacteria (Okamura *et al.*, 1989). The production of new antibiotics might be a result of expression of silent genes or the development of the hybrid biosynthetic pathways caused by fusion. Improvement in glucose metabolising activity in *Brevibacterium* (Karasava *et al.*, 1986) and enhancement in lignin degradation activity (Pettey and Crawford, 1984) are the other examples of application of protoplast fusion technique to industrial strain improvement.

Intergeneric Protoplast Fusion : Successful isolation of intergeneric hybrids in bacteria by protoplast fusion between *Cellulomonas* sp. and *Bacillus subtilis*, is first reported by Gokhale *et al.* (1984) wherein the hybrids were generated with altered phenotypic expression and regulation. Chen *et al.* (1987) have carried out protoplast fusion between two unrelated genera, *Fusobacterium varium*, a strictly gram negative rod and *Enterococcus faecium* a facultative anaerobic gram positive coccus, under strictly anaerobic conditions for enhancing degradation of lignin realted compounds. A stable anaerobic recombinant (FE7), obtained by this fusion, was further fused with a cellulo-

lytic anaerobe, *Ruminoccocus albus* and resulted in having a fusant which could simultaneously utilize both cellobiose and dehydradivanillin (Chen *et al.* 1988). Prakash and Cummings (1988) have created a novel actinomycete capable of fixing atmospheric nitrogen by fusion of *Frankia* and *Streptomyces* protoplasts. Recently, hybrids between protoplasts of strain of *B. subtilis*, having an ability to degrade xylan and *Corynbacterium acetoacidophilum*, a lysin producer have been isolated and these are found to inherit the useful traits of both the parents (Deb *et al.* 1990). This suggests that genetic recombination can take place between unrelated genera generating hybrids albeit with low frequencies. Intergeneric recombinations in bacteria also tend to changes the properties of the parental enzymes. Gokhale and Deobagkar (1990) have reported that the hybrid between *Cellulomonas* sp. and *B.subtilis* produced aryl β-glucosidase which was more thermostable than that of *Cellulomonas*. Similarly thermostability of amylase secreted by the hybrid between *Z.mobilis* and *B.subtilis* was increased (Gokhale, 1988). The thermostable forms of enzymes are known to be generated by specific amino acid substitutions, mainly of the hydrophilic to hydrophobic type (Suzuki and Inahori, 1973; Mozhaev and Martinek, 1984). Since fusion products are formed by multiple genomic recombination, introduction

of new amino acids in the primary sequence of the enzymes is a possibility.

Intergeneric Plasmid Transfer

The technique of plasmid transfer by PEG mediated protoplast fusion has been successfully applied to *Streptococcus lactis* (Gasson, 1980; Okamoto *et al.*, 1983). Plasmid transfer is also shown in interspecies crosses between *Streptococcus cremosis* and *Streptococcus lactis* (Okamoto *et al.*, 1985). It is established that plasmids can be transferred from phylogenetically unrelated *Streptococcus lactis* to *Lactobacillus reuteri* by means of protoplast fusion (Cocconcelli *et al.*, 1986). It has been shown that the plasmids could be shuttled between *Bacillus subtilis* and *Streptococcus lactis* in an efficient way (Van der Vossen *et al.*, 1988) This intergeneric protoplast fusion resulted in the same efficiency of the plasmid transfer as in the case of intraspecific plasmid transfer.

Interkingdom genetic exchange by protoplast fusion

Alongwith the plasmid transfer in industrially important microbes, the protoplast fusion technology has also been used in transfer of bacterial DNA into higher organisms. Schaffner (1980) has shown that monkey cells were transformed by *E.coli* harbouring a recombinant plasmid of

simian virus 40, where the infection frequency was greatly increased by fusion of lysozyme-treated bacteria with monkey cells in presence of PEG. Hasezawa *et al* (1981) have tried to develop sphearoplast methods for transformation of plant protoplasts independent of *Agrobacterium* infection. They fused the spheroplasts of *Agrobacterium tumefaciens* harbouring a octopine type Ti plasmids with the *Vinca rosea* protoplasts by treating with polyethylene glycol (PEG) or polyvinyl alcohol (PVA). Transformants producing octopine were obtained after selection on medium containing no hormones which was further confirmed by Southern hybridization from transformed clones (Okada *et al*, 1985). Hain *et al*, (1984) have used spheroplasts of different *Agrobacterium tumefaciens* strains having various selection markers like opine synthesis, antibiotic resistance or hairy root formation and fused each type with *Nicotiana tabacum* protoplasts with PEG and obtained the calli with presence of respective marker from *Agrobacterium tumefaciens* strain. To prove one of the merits of the spheroplast method, Baba *et al* (1986) have tried to transform rice cells by PEG mediated fusion of *Agrobacterium tumefaciens* spheroplasts with rice protoplasts and obtained the nopaline positive clones. Even though these studies could not produce transformed plants, they offer new dimensions towards the use of protoplast

fusion for the development of plant transformation studies.

The variety of reorganization of regulatory sequences or genes or both in the stable intergeneric hybrids thus leads to a method of manipulation of a set of genes such that various new combinations of their interactive expression can emerge, although such manipulation is random and fortuitous. This could be a desirable outcome when an industrially important microbe needs to be complemented with another phenotype from an unrelated bacterium.

Sandhya. 30-4-96

Bs	Ps	
auxotrophic markers	halophilic	MM + NaCl.
	2% NaCl.	
~~amylase~~	intracellular hydantoinase.	

No growth on MM.

(1) Selection of antibiotic markers.

(2) Standardization of Ps. protoplast.

(3) Fusion.

(4) selection & characterization.

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CHAPTER II

SELECTION OF HIGHLY ACTIVE *AGROBACTERIUM TUMEFACIENS* AND *BACILLUS THURINGIENSIS* STRAINS FOR PROTOPLAST FUSION

SUMMARY

Extensive work on screening of *A.tumefaciens* and *B.thuringiensis* strains for their tumor forming capacity in pigeonpea plants and insecticidal property against Lepidopteran pest respectively was carried out to select suitable strains for intergeneric protoplast fusion.

A germinating seed assay was developed to determine the susceptibility of different strains of *A.tumefaciens* towards pigeonpea (*Cajanus cajan*). Tumors, tested for opine synthesis using high voltage paper electrophoresis, showed two types of opines confirming the susceptibility of pigeonpea to infection with *Agrobacterium*. Based on the extent of virulence of *Agrobacterium* strains as judged from the capacity of tumor formation, *A.tumefaciens* (NCIM 2943) which was kanamycin resistant octopine type tumor inducing strain was selected for protoplast fusion work.

Various *B.thuringiensis* subspecies showed differential susceptibility towards the tested pest belonging to the Lepidoptera class. In bioassays, effect of parameters like age of the cells and spore concentration was studied and based on these results *B.thuringiensis* subsp *kurstaki* (NCIM 2514) showing maximum larvicidal activity towards *Spodoptera litura* and potato tuber moth larvae was further taken for protoplast fusion work.

INTRODUCTION

In 1977, Chilton *et al* came forth with an altogether novel system of genetic transformation by *A.tumefaciens* in dicotyledonous plants, which further emerged into the widely used plant transformation vector. A soil bacterium *A.tumefaciens* is a natural plant vector (Ti plasmid), which on infecting at the wounded site of the plant transfers a segment of its DNA (T-DNA) into plant chromosome. Transcription and translation of genes present in the T-DNA cause the formation of crown gall tumors in plants. These tumors are capable of proliferating in the absence of phytohormones (auxins and cytokinins), whereas the normal plant tissue cannot. The T-DNA also contains genes that encode the synthesis of opines (modified amino acids), the presence of which is unique in case of tissues infected with *Agrobacterium* (Tempe and Goldman, 1982). Based on this transformation method, extensive work has been carried out and genes of choice have been transferred to plant cells. (Hooykaas and Schilperoort, 1992).

The first step in the development of a functional *Agrobacterium* mediated gene transfer system in pigeonpea is identification of a suitable strain with tumor inducing capacity. Although *Agrobacterium* sp. are generally considered to have a broad host range including a wide variety of

dicotyledonous and a few monocotyledonous plants (De Cleene and De Ley, 1976) some strains are host specific. Appearance of tumor formation and/or the synthesis of specific opines (Hooykaas-Van Slogteren *et al*, 1984) have been used as evidence of successful infection by *Agrobacterium*. So evaluation of susceptibility of pigeonpea to the different strains of *A.tumefaciens* and their type of synthesis of opines is essential for selecting a strain which will be suitable for further protoplast fusion work and its application for development of *Agrobacterium* mediated transformation system. Factors such as plant cultivar, tissue age, bacterial strain and its concentration also plays an important role in transformation efficiencies of *A.tumefaciens* strains (Armsted and Webb, 1987; and Davis *et al* 1991). Therefore, various assays and parameters have been used to study tumor formation and tried to develop a convenient assay system for determination of virulence of wild type and protoplast fusion hybrid towards pigeonpea plants.

Strains of *B.thuringiensis* produce a crystalline protein inclusions (delta-endotoxins), which are extremely toxic, specifically towards lepidopteran, dipteran or coleopteran pests (Whiteley and Schnepf, 1986). In determining the host range and relative efficiency of the individual *B.thuringiensis* strains towards lepidopteran class of larvae the most important step is the selection of highly active

B.thuringiensis strain for protoplast fusion work. Different strains of *B.thuringinesis* are known to have one or several types of crystal proteins with minor and major differences (Hofte and Whiteley, 1989). Since different proteins can have different host range, the activity of the strains has to be tested against different pests belonging to the same class. The bioassay of microbial insecticides is the evaluation of the response of a group of living microorganisms to the target insect. The bioassay of a microbial insecticides contains concepts which is totally opposite to the bioassay of a chemical insecticide. In case of the chemical, one determines the qulaity (purity) of the active ingredient, whereas in case of microbial insecticides the product is not perfectly homogeneous. Thus the accuracy of the toxicity estimate depends on many things, like the measure of the observations, the number of individual insects in the population being tested, the quality of insect population and the repeatability of the bioassay. Many types of bioassay systems using both artificial diets and plants are available. In the present work, intially the bioassay method was standardized and then various *B.thuringiensis* strains were tested for their insecticidal property towards Lepidopteran insects. The highly active strain selected was further confirmed for the presence of crystal protein and its activ-

ity against the tested pest.

MATERIALS AND METHODS

Chemicals

All the chemicals used throughout the work were of Analytical Reagent (AR) or Guaranteed Reagent (GR) grade and were obtained from Qualigens, British Drug House (BDH), Sarabhai Chemicals or E. Merck India. Bacteriological products like tryptone, peptone, beef extract, yeast extract and agar were obtained from Difco Laboratories, USA. All the fine chemicals were obtained from Sigma Chemical Co., USA like Tris (hydroxymethyl) amino methane, N,N'- methylene bis acrylamide (Bis), N,N,N'N' tetramethyl ethylene diamine (TEMED), β-mercoptoethanol, coomassie briliant blue R- 250, Sodium dodecyl sulfate (SDS), SDS-protein molecular weight markers, lysozyme, octopine, nopaline, arginine, phenanthrenequinone, ethidium bromide, bromophenyl blue, 5 Bromo-4-Chloro-3-Indolyl phosphate (BCIP).

The antibiotics like ampicillin, kanamycin, tetracyclin, spectinomycin, chloramphenicol, rifampicin were also obtained from Sigma Chemicals Co. USA. Antibiotic Cefotaxime or Claforan was from Hoechst India Limited and *Penicillin-G* was obtained from Hindustan Antibiotics Limited, India.

Polyethlene glycol 7500 was obtained from Polyscience Inc. USA.

Hybond-N membranes, radiolabelled α-p-^{32}-dATP or gamma p^{32} dATP, labelling kits for preparation of probe were obtained from Radiochemical Centre, Amersham, UK.

Sephadex G-50 was from Pharmacia Fine Chemicals, Uppsala. Sweden.

Bacteriological Techniques

All *A.tumefaciens* strains (listed in Table 2.1) and *B.thuringiensis* (listed Table 2.3) were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory. The non-oncogenic Ti plasmid pGV3850::1103 and disarmed plasmid pGV3850, were the kind gifts from Prof. J. Schell Max-Planck Institute Germany.

Nutrient media

Maintenance media : *A.tumefaciens* strains were grown on YEB medium which contain g/1 of beef extract 5.0; Bacto Yeast extract 1.0; Bacto peptone 5.0, Sucrose 5.0 and $MgSO_4.7H_2O$ 0.3 at pH 7.0.

B.thuringiensis strains were maintained on LB (Luria Bertani) medium containing g/1 of Tryptone 10.0; yeast extract 5.0 and NaCl 10.0 at pH 7.2. For solidification medium was supplemented with 1.5% agar.

Growth media : For sporulation and isolation of high molecular weight plasmids from *B.thuringiensis* strains, cultures were grown in modified Spizizen's medium (SPY) (Kronstad *et al* 1983) which contains $(NH_4)_2 SO_4$ 2.0; K_2HPO_4 14.0; KH_2PO_4 6.0; Tri-sodium citrate 1.0; $MgSO_4.7H_2O$ 0.2 g/l. This medium was supplemented with 0.1% yeast extract and 0.5% glucose.

MS medium (Murashige and Skoog, 1962) was used for plant growth which contained following components per litre. $CaCl_2.2H_2O$ 0.44g; NH_4NO_3 1.65g; KNO_3 1.90g, KI 0.83mg; $CoCl_2.6H_2O$ 0.025mg; KH_2PO_4 0.17g; H_3BO_3 6.2mg; $Na_2MoO_4.2H_2O$ 0.25mg; $MgSO_4.7H_2O$ 0.37g; $MnSO_4.4H_2O$ 22.0mg; $CuSO_4.5H_2O$ 0.025mg; $ZnSO_4.7H_2O$ 8.6mg, Fe-NaEDTA 36.7mg, glycine 2.0mg; inositol 0.1g; Nicotinic acid 0.5mg; Pyridoxine HCl 0.5mg; Thiamine HCl 0.1mg and sucrose 30g. pH of the medium was adjusted to 5.6. Solid medium was supplemented with 0.8% agar (Difco).

Phytopathological tests

In vivo and *In vitro* tumor formation : *A.tumefaciens* on infecting the wounded tissue develops an undifferentiated growth in the plant. The techniques for infecting plant tissues with *A.tumefaciens* are relatively easy to perform. The plants are injured mechanically with a scalpel and a suspension of agrobacteria is applied to the site of wound and on incubation, tumors are developed in the plants. The

tumor formation is scored by the infection carried out by *in vivo* or *in vitro* methods. In the present work, pigeonpea seeds of T-21 variety were used for all the plant infection experiments with tomato (Pusa red) as control plants.

For *in vivo* infection the pigeonpea plants were grown in separate pots in the garden for 3-4 weeks and were infected with the overnight grown *A.tumefaciens* strains in YEB medium using hypodermic syringe and needle. Five plants were inoculated with each strain at every batch of experiment. The inoculated area was wrapped with a piece of parafilm to protect from drying.

For *in vitro* inoculation the seeds were surface sterilized for 1 minute with 70% ethanol, thoroughly washed with sterile distilled water followed by treatment with 0.05 to 0.1% $HgCl_2$ solution for 8-10 minutes. After a number of washings with sterile water, seeds were soaked overnight in water, decanted and further germinated in dark for 48 hours at 28°C. The germinated seeds were wounded at the embryonic (micropile) region with the sterile needle dipped in overnight grown slope culture of the test organisms. The infected seedlings were placed on MS medium containing 250µg/ml of Claforan in the boiling tubes and incubated at 28°C under 16 h light and 8h dark photoperiod.

Co-cultivation of pigeonpea leaf discs : Co-cultivation or leaf disc method is the most widely used technique for plant transformation which results in better trannsformation frequencies. Co- cultivation is the co-incubation of a part of plant tissue with bacteria. Small parts of leaves are dipped into a bacterial suspension and then cultivated on synthetic medium.

For co-cultivation, leaf pieces (approximately $1cm^2$) or stem segments (about 5mm) were excised from the seven days old germinating seedlings grown *in vitro* on 0.8% agar. The explants were co-cultivated with the overnight grown culture of *A.tumefaciens* pGV3850::1103 in YEB containing 50µg/ml of kanamycin at the bacterial concentration of 10^8cells/ml in the petridish for 48 h. The co-cultivated explants were washed 3 times in MS liquid medium and then placed on MS solid medium containg 1.0mg/1 6-BAP, 0.1 mg/1 NAA, 500µg/ml Claforan and 250µg/ml kanamycin (sigma). The petridishes were incubated at 28°C under 16h light and 8h dark photoperiod.

Opine assay : The tumors producd by *A.tumefaciens* synthesize opines which are absent in the normal plant tissues. Opines are compounds (mostly amino acids linked to sugar moieties such as pyruvate or α-ketoglutarate) which are formed via enzymes present in tumor cells. A given strain of *Agrobacte-*

rium can utilize specific type of opines and based on these specificities the agrobacteria have been classified.

Opines in tumor tissues can be detected either by direct method or by identifying enzymes responsible for opine synthesis. In the present work, opines were directly identified by high voltage paper electrophoresis of the extract of tissues, by the modified method as described by Otten and Schilperoort (1978). 50mg of tumor tissue collected from *in vivo* grown plants was crushed in micro-fuge vial containing 40µl of acidified methanol. For tumors grown *in vitro*, tissue was collected from number of batches, kept in frozen condition and then about 50mg of tissue was taken for extraction. 20µl of the briefly centrifuged clear supernatent was spotted on a Whatman 3MM paper (size about 11.5 cm x 28.0cm). 10µl of stock solution of octo-pine, nopaline and arginine (1mg/ml each) was spotted as standard. The paper was saturated with the electrophoretic buffer (formic acid : acetic acid : water in the ratio of 5:15:80) by carefully pipetting onto the paper. The elec-trophoresis was carried out from positive to negative elec-trode at 400 to 500 V for 1-1.5h. When the electrophoresis was over, the paper was completely dried and sprayed with the phenanthrenequinone reagent for staining the opine spots. The 0.02% (Sigma) phenanthrenequinone was dissolved

in absolute ethanol and mixed with 10% NaOH solution in equal proportion. After drying, the electrophoregram was observed under shortwave UV light source.

Bioinsecticidal assay

Bioassays are developed to measure insecticidal activity of microbial insecticides. The exact procedures in these assays vary with respect to the method used to expose insects to toxin and the period of exposure which ultimately depends upon the test insect and its response to microbial insecticide being assayed.

For these assays, neonate larvae of Tobacco Leaf eating Caterpillar, *Spodoptera litura* (F.) (Lepidoptera : Noctuidae) was used. All the *B.thuringiensis* strains were grown in SPY medium (Kronstad *et al*, 1983) for 72h at 30°C. 10^8 spores were spread evenly on the artificial diet (Nagarkatti and Prakash, 1974) and air dried as described in the method by Ignoffo *et al* (1977). One neonate larva of *S.litura* was placed on the treated surface in each container and kept in an incubator maintained at 28°C and 60% humidity until the end of observation period. Mortality count was made after 7 days of exposure to the treated surface. The bioassay was repeated several times to check reproducibility. Ten larvae/cone with 5-7 replication and

five concentration levels were used. Assays were replicated on 3 separate occasions, and the mean values and standard deviation (SD) were determined. The bioassays were also carried out by feeding the neonate larvae on castor leaf discs applied with test samples as used in case of artificial diet.

For assays with the potato tuber moth (*Phthorimaea operculella*), the neonate larvae were released on the fresh and clean potato surface layered with the known amount of spore suspension. For observations average weight of the larvae was taken into consideration.

Extraction of crystal protein : For routine checking of the presence of crystal protein in *B.thuringiensis* strains, the procedure described by Donovan *et al.* (1988) was followed. The 72h grown cells on the LB agar medium were removed from the surface with a spatula, washed with sterile distilled water and suspended in water at a concentration of 100mg cells (wet wt) per ml. An equal volume of 100mM Tris-HCl (pH 7.0), 20mM EDTA and 100mg/ml of lysozyme was added and the mixture incubated at 37°C for 1 hour. To this mixture, 0.2% (final concentration) of SDS was added for maximum cell lysis, vortexed and centrifuged for 7 minutes at 10,000rpm. The pelleted material consisting of spores and insoluble material from lysed cells including insoluble

crystal proteins was suspended in 0.1% SDS-10mM EDTA solution.

Purification of crystal protein : Insecticidal crystal protein, δ-endotoxin was purified by the method described by Thomas and Ellar (1983) with some modification. *B.thuringiensis* subsp. *kurstaki* (NCIM 2514) was grown in SPY medium for 72 hours at 30°C on a rotary shaker and was harvested by centrifugation at 6000rpm for 5-10 minutes at 4°C. Pelleted cells were washed two - three times with sterile distilled water and three times with 1M NaCl at 0-5°C. The later treatement helped to separate foam (enriched in sporangial debris) from the crystals pellet. After the removal of the foam, the pellet containing crude crystals and spores was resuspended in minimal volume of water with 0.01%, Triton X-100. This crystal spore suspnesion was disaggregated by sonication for 2 minutes discontinuously, diluted and centrifuged at 3000rpm for 10 minutes and the supernatant was collected. This treatment was repeated for 4-5 times for maximum separation of crystals and spores from the debris and the supernatent was pooled together and centrifuged for 10,000rpm for 20 minutes. Pellet enriched with maximum spores and crystals was suspended in minimal volume of distilled water containing 0.01% Triton X-100 to minimize activity of adsorbed proteinases. Spores and crystals were

seperated using differential centrifugation through a discontinuous sorbitol density gradient. 70% sorbitol was diluted to yield discontinous gradients of 60%, 50% and 40% in ditilled water. 8ml of varying percentages of sorbitol (70%-40%) layered carefully in 50cc Sorvall SS-34 tubes and kept on ice. 1ml of aqueous crystal enriched suspension was loaded on top of the gradient and was centrifuged at 15,000rpm for 40 minutes at 4°C in IEC centrifuge. Different interphases were carefully collected with pasteur pipettes, centrifuged at 10,000rpm for 15 minutes and washed with distilled water to remove sorbitol and the purity of the crystals was checked on SDS-PAGE.

Since crystal δ-endotoxin is soluble in alkaline buffer and solubilized toxin retains its activity (Thomas and Ellar, 1983), the crystal protein from *B.thuringiensis* (NCIM 2514) was treated with 0.05M Na_2CO_3 (pH 10.5) buffer at 37°C for 2 hours. Insoluble material was separated by centrifugation at 10,000rpm for 10 minutes and the soluble fraction was collected in supernatent. The pellet was suspended in 0.1M phosphate buffer (pH 7.0). All the soluble and insoluble fractions were checked on SDS-PAGE.

SDS-Polyacrylamide gel electrophoresis : Protein concentration was determined by the method of Lowry *et al.* (1951). The presence of the crystal protein and its purity was

checked on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli and Fovre (1973). The protein samples were dissolved in 0.125 M Tris-HCl buffer (pH 6.8), containing 0.1% SDS, 10% glycerol (w/v) and 0.05% bromophenol blue. The electrophoresis buffer contained 25mM Tris, 192mM glycine and 0.1% SDS. About 50 µg of protein was loaded on polyacrylamide gels and run at 20mA constant current till the tracking dye reached 1cm from the botttom of the gel. The gel was carefully removed from glass plates, rinsed with water and fixed for one hour in 10% TCA in 40% methanol. The gel was stained overnight at room temperature with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 20% (w/v) methanol and 10% (w/v) acetic acid. The gel was destained in a solution containing 20% (w/v) methanol and 10% acetic acid with several changes. Molecular weights were estimated by the relation of log MW to the Rf of SDS/polyacrylamide gel electrophoresis marker proteins.

RESULTS

In vivo and *In vitro* tumor formation and opine analysis

Virulence of different *A.tumefaciens* strains was scored by tumor forming frequency using *in vivo* and *in vitro* infection methods.

For *in vivo* tumor formation studies, total 10 strains of *Agrobacteria* (including 7 wild and 3 constructs)

were used for infecting pigeonpea and tomato (control) plants. To check the reproducibility of infection, five plants were taken for each strain and minimum three such experiments were carried out. Figure 2.1 indicates the tumor formation in pigeonpea 4 weeks after the inoculation of plants grown in the pots and the size of the tumor was 10-12mm in diameter.

Table 2.1 summarizes the data with respect to tumor formation frequency in pigeonpea and tomato plants. Among the wild strains tested, except NCIM 2145, all the other strains were virulent towards both pigeonpea and tomato plants, but the strain 2145 showed virulence only with the tomato plant. The tumor formation frequency relatively varied with *Agrobacterium* strains as well as with the target plant. With tomato plants, for example all the strains showed 80-100% tumor formation. In pigeonpea, the tumor formation varied from 20-60% with the different strains under similar field conditions.

The tumors grown *in vivo* were checked for the type of opine produced. From Table 2.1, it can be seen that the strains 2147 and 2232 are nopaline type and all others are octopine type.

After studying the virulence of different *Agrobacterium* strains towards pigeonpea and determining the

TABLE 2.1 : Analysis of tumor formation and type of opine of *Agrobacterium tumefaciens* strains in pigeonpea and tomato plants *in vivo*

Strains	Opines	Tomato No.of tumors formed/plants	% tumor formation	Pigeonpea No.of tmors formed/plants	% tumor formation
Control	-	0/5	0	0/5	0
2145	Octopine	2/5	40	0/20	0
2146	Octopine	3/5	60	8/20	40
2147	Nopaline	4/5	80	15/25	60
2148	Octopine	4/5	80	8/20	40
2232	Nopaline	5/5	100	5/25	20
2822	Octopine	2/5	40	4/25	16
2939 (pTiB6S3	Octopine	ND	-	5/20	25
2943 (pGV 2215, tms-,Km^r)	Octopine	ND	-	5/25	20
pGV 3850	-	ND	-	0/5	0
pGV 3850:: 1103	-	ND	-	0/5	0

BBRC 184, 692-699, 1992.

Bt. sandiego — layered onto discontinuous sucrose gradient (79%, 72%, 67% w/v) and ultracentrifuged at 80,000 g at 4°C for 3h in swinging bucket rotor (Sorvall AH 627). Crystals formed a distinct band at the interface of the 72% & 79% sucrose.

Bti — spore/crystal mixture (50 mg) layered on top of a 30 ml discontinuous sucrose gradient comprising 10 ml each of 67%, 72% & 79% (W/V) sucrose in 50mM-Tris-HCl (pH 7.5) containing 10mM KCl. (V) Bechman L5-50 ultra centrifuge in SW25.1 rotor operating at 80,000 g for 14h at 4°C suspended in deionized water

Btk — spore/crystal mixture (50 mg) layered on 45%, 67%, 87% (W/v) sucrose in deionized water. Crystals formed a major band at the interface of 67-87% sucrose.

	maximum rpm	g	no. of tubes
SW 65 Ti	65 000	421000	3 x 5 ml
SW 41 Ti	41 000	286000	6 x 13.2 ml

$\frac{80}{5} \times 421000$

80

राष्ट्रीय रासायनिक प्रयोगशाला (वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद) पुणे - 411 008

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Dr. Mrs. Ulka S. Puntambekar
Scientist
Division of Biochemical Sciences

May 18, 1995

Prof. C. Ratledge
Editor-in-Chief
World Journal of Microbiology
& Biotechnology
Department of Applied Biology
University of Hull
Hull HU6 7RX, UK.

Subject : Submission of manuscript

Dear Prof. Ratledge

I am resubmitting herewith the manuscript entitled " Cellulase production by an edible mushroom *Volvariella diplasia* " for the possible publication as a short note in WJMB. The present work is a report of findings of the properties of a well described enzyme cellulase. To my knowledge the production of cellulolytic enzyme by this edible mushroom in submerged culture has not been reported and this study provides an essential information on enzyme production by *V.diplasia* on pure cellulose under defined conditions in submerged culture.

With kind regards

Yours sincerely

Mrs. Puntambekar

FIG 2.1: *In vivo* tumor formation by *A.tumefaciens* (NCIM 2232) in pigeonpea plant.

A : Tumor in pigeonpea plant.

B : Magnified tumor region.

FIG. 2.1

FIG 2.1: *In vivo* tumor formation by *A.tumefaciens* (NCIM 2232) in pigeonpea plant.

A : Tumor in pigeonpea plant.

B : Magnified tumor region.

FIG. 2.1

nature of opines in transfomred pigeonpea plants, an attempt was made to use germinating seedlings (*in vitro*) of pigeonpea for checking the virulence of all the above strains. It was observed that germinating seeds when infected at micropile i.e. embryonic regions showed a high response towards tumor formation. When the infections were done at the other surfaces of seedlings either by injecting seedlings with syringe or by wounding with a needle dipped in agar culture, low frequency of tumor formation was observed. The seedlings with the previously wounded surface, when co-cultivated in suspension culture, showed no response to tumorgenecity. About 10-30 seedlings of different age (2-7 days) were taken for *in vitro* infection by each strain and tumor formation was assessed after 7-10 days of infection. Table 2.2 gives the data on percent tumor formation by infecting the pigeonpea seedlings in 2-7 days after germination with different strains of *Agrobacterium*. The tumor formation in 3 days old seedling is 6- 28%, which is significantly higher as compared to that in 2 days old germinating seedling. In case of 7 days seedling, till the end of observation period majority of plants in test tubes got dried. Although the tumor formation frequency is low in case of *in vitro* method, as compared to *in vivo* method, this method is useful for screening number of strains for their virulence easily and quickly whereas tumor formation in plants grown

TABLE 2.2 : *In vitro* scoring of tumors by *Agrobacterium tumefaciens* strains in pigeonpea

Strains	2 days seedlings No. of tumors/ No. of infected seedlings	% tumors formation	3 days seedlings No. of tumors/ No. of infected seedlings	% tumor formation	7 days seedlings No. of tumors/ No. of infected seedlings	% tumor formation
Control	0/10	0.0	0/10	0.0	0/10	0.0
2145	0/15	0.0	0/10	0.0	0/8	0.0
2146	0/16	0.0	6/24	25.0	1/8	12.5
2147	1/20	5.0	8/28	28.5	1/8	12.5
2148	0/12	0.0	2/20	10.0	0/10	0.0
2232	1/22	4.5	7/32	21.0	1/10	10.0
2822	0/12	0.0	1/16	6.25	0/10	0.00
2939	1/24	4.1	3/24	12.5	ND	-
2943	1/24	4.1	2/24	8.3	ND	-
pGV 3850	ND	-	0/22	0.00	ND	-
pGV 3850:: 1103	ND	-	0/24	0.00	ND	-

in fields is more time consuming.

Co-cultivation of pigeonpea leaf discs

Since normal pigeonpea tissue is sensitive to kanamycin, we used a chimeric gene that conferred kanamycin resistance to transformed plant cells. To transfer and integrate such a resistant gene into pigeonpea chromosomal DNA, the Ti plasmid derived vector pGV3850::1103 was employed. In this vector, all the oncogenic functions have been deleted and replaced by pBR322 sequences and two copies of a chimeric kanamycin resistant gene consisting of nopaline synthase promoter, coding region of amino glycoside phosphotransferase (APT-II) gene of Tn_5 and poly(A) signal region of octopine synthase gene (Czernilofsky *et al*. 1986).

To transform pigeonpea explants by co-cultivation we adopted the leaf disc transformation method developed to Horsch *et al*. (1985) as described in materials and methods. After one month, callus formation was observed on MS medium with kanamycin on the co-cultivated leaf discs, but control leaf discs and stem segments (non-transformed) were not able to grow on antibiotic containing medium. Callus formation was however, observed in the nontransformed leaf discs, on MS medium without antibiotics (Figure 2.2). This confirmed that the normal pigeonpea tissue was unable to regenerate (callus formation) on kanamycin containing

FIG 2.2: Co-cultivation of pigeonpea leaf discs pGV3850::1103

A : Leaf discs without co-cultivation on MS medium

B : Leaf discs without co-cultivation on MS medium and kanamycin (250µg/ml).

C : Leaf discs with co-cultivation on MS medium and kanamycin (250µg/ml).

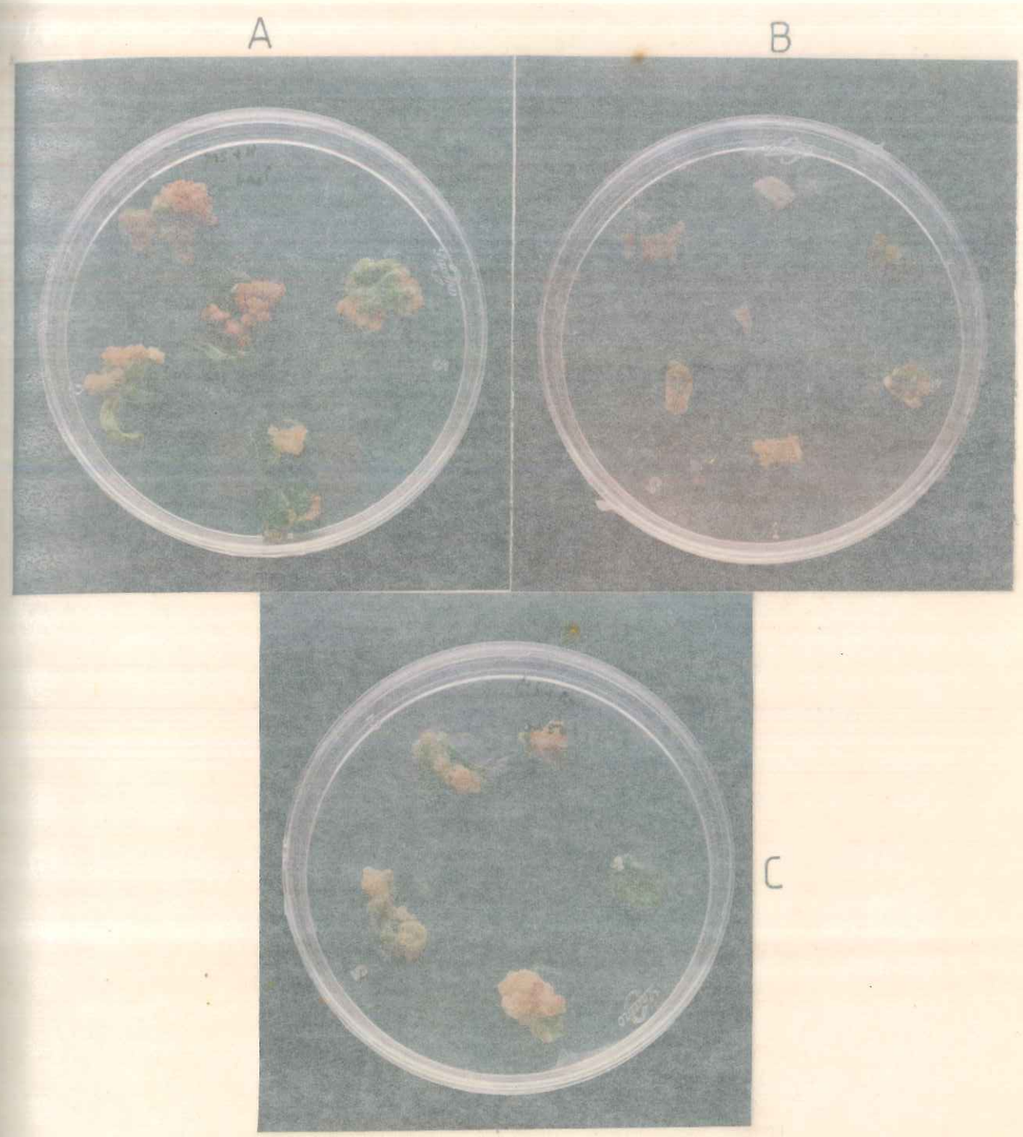

FIG. 2.2

FIG 2.2: Co-cultivation of pigeonpea leaf discs pGV3850::1103

A : Leaf discs without co-cultivation on MS medium

B : Leaf discs without co-cultivation on MS medium and kanamycin (250µg/ml).

C : Leaf discs with co-cultivation on MS medium and kanamycin (250µg/ml).

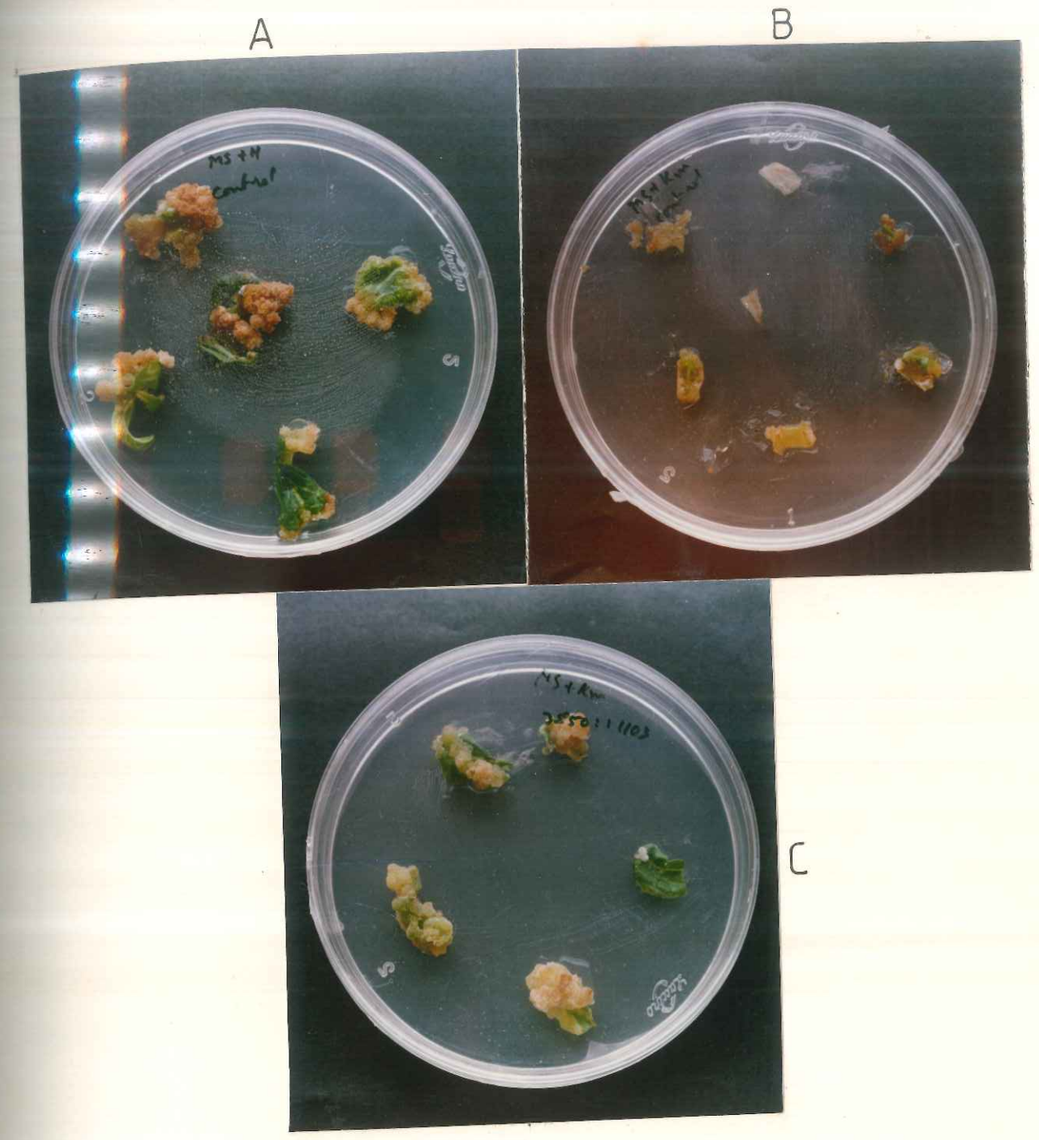

FIG. 2.2

medium. The transformed callus was further transferred to fresh medium with antibiotics for regeneration but after 5th subculture the same tissue failed to regenerate.

Larvicidal bioassays

Different *B.thuringiensis* strains exhibit differential host specificity. Table 2.3 gives the insect mortality data when nine different *B.thuringiensis* strains were tested against *Spodoptera litura* and potato tuber moth. Here HD-73, taken as a standard for comparing activities, is 100% toxic towards *S.litura* and 86% toxic with potato tuber moth. Among all the other strains of different subspecies, *B.thuringiensis* subsp *kurstaki* (NCIM 2514) is 93% and 85% lethal towards *S.litura* and potato tuber moth respectively. Other strains are not toxic to the test larvae as judged by a very low rate of mortality compared to HD-73 and *B.thuringiensis* (NCIM 2514).

Based on these results, *B.thuringiensis* (NCIM 2514) was considered as a candidate for protoplast fusion and was used for protein production and determination of bioassays.

The crude crystal protein was extracted from *B.thuringiensis* (NCIM 2514) as described in material and methods. The protein was dissolved in SDS-PAGE sample buffer by heating in water bath for 5-7 minutes. As seen in Figure

TABLE 2.3 : Larval toxicity test of different *Bacillus thuringiensis* subspecies against Lepidopteran larvae

B.thuringiensis subsp & NCIM No.	*Spodoptera litura* % mortality	Potato tuber moth % mortality
israelensis 2513	27.3	-
kurstaki 2514	93.3	85.5
subtoxicus 2515	13.0	-
subtoxicus 2975	6.6	82.8
kenyae 2976	26.6	54.0
finitimus 2977	26.6	65.5
thompsoni 2978	3.3	72.2
HD-187 2979	20.0	-
HD-73	100.0	86.6
Control	3.3	0.0

FIG 2.3: SDS-polyacrylamide gel electrophoretogram of insecticidal crystal protein from *B.thuringiensis* (NCIM 2514).

LANE 1 : Molecular Weight markers (SDS-6H, Sigma).

LANE 2 : Purified crystal protein.

LANE 3 : Crude protein extract.

LANE 4 : Crystal protein treated with 0.05 M Na_2CO_3 buffer (pH 10.5) : Insoluble fraction.

LANE 5 : Soluble protein in alkaline buffer.

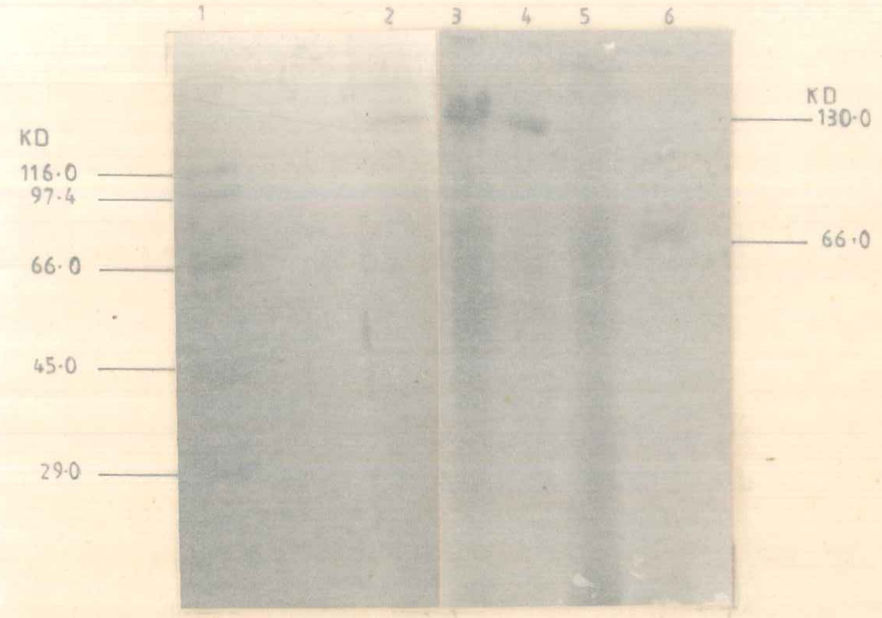

FIG. 2.3

FIG 2.3: SDS-polyacrylamide gel electrophoretogram of insecticidal crystal protein from *B.thuringiensis* (NCIM 2514).

LANE 1 : Molecular Weight markers (SDS-6H, Sigma).

LANE 2 : Purified crystal protein.

LANE 3 : Crude protein extract.

LANE 4 : Crystal protein treated with 0.05 M Na_2CO_3 buffer (pH 10.5) : Insoluble fraction.

LANE 5 : Soluble protein in alkaline buffer.

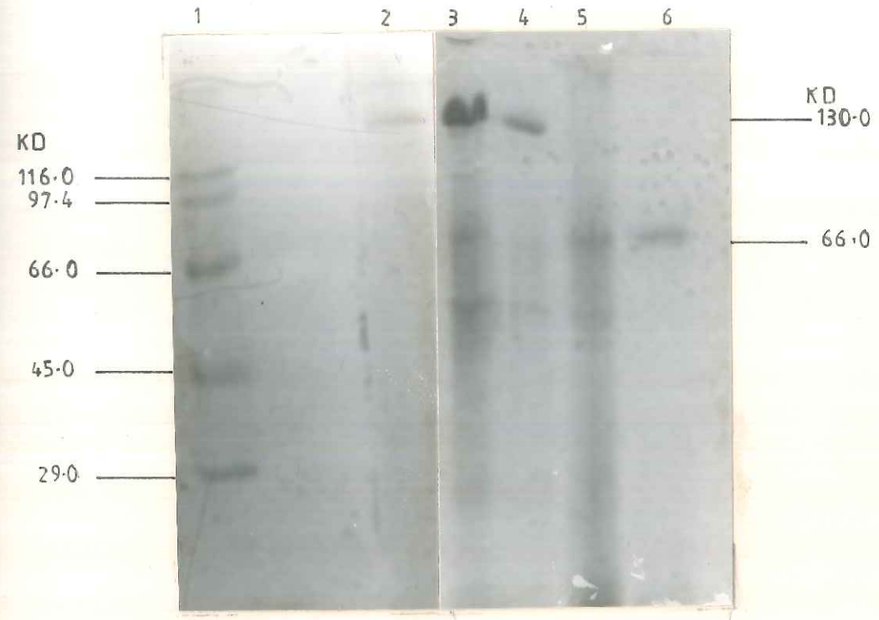

FIG. 2.3

TABLE 2.4 : Larval toxicity test of *Bacillus thuringiensis* subsp kurstaki (NCIM 2514) using artificial diet and castor leaf discs

Sample No.	Artificial diet		Castor leaf discs	
	% mortality $\bar{X} \pm SD$	Larval weight (mg) $\bar{X} \pm SD$	% mortality $\bar{X} \pm SD$	Larval weight (mg) $\bar{X} \pm SD$
Control	3.3 ± 0.2	12.9 ± 1.9	10.0 ± 1.0	15.3 ± 2.1
2514 (10^8 spores)	93.3 ± 11.5	0.7 ± 0.1	85.3 ± 6.2	4.0 ± 0.8
130kD (1.5mg protein)	96.6 ± 12.7	0.3 ± 0.0	90.0 ± 10.0	0.6 ± 0.0
66kD (1.5mg protein)	90.0 ± 10.0	0.4 ± 0.0	73.4 ± 4.2	1.0 ± 0.0

2.3, there is a prominent band of protein corresponding to 130kD molecular weight. To confirm the presence of δ-endotoxin, the crystals were purified by discontinuous sorbitol gradient. On SDS-PAGE, this purified protein shows single band corresponding to 130kD. The yield of this protein was very low where from 2g wet weight of cells 100µg of crystal protein was obtained.

Thomas and Ellar (1983) have shown that the toxin crystals are soluble in alkaline buffer. When the crude crystal protein preparation was solubilized in alkaline buffer (0.05M Na_2CO_3 pH 10.5) at 37°C for 2h, the 130kD band was almost absent and only a band corresponding to 66kD molecular weight was observed on SDS-PAGE. These alkali treated fractions were used for bioassays.

The *B.thuringiensis* (NCIM 2514) crystals spores and protein samples were tested for their toxicity using artificial diet and castor leaf discs. It is observed from Table 2.4 that mortality values are 90-96% with spore suspensions or protein samples when the artificial diet is used for feeding the insects. But the same values are decreased to 73-90% when the larvae are released on the castor leaf. The results from Table 2.4. also indicate that the 66kD protein obtained by solubilization of 130kD δ-endotoxin protein is equally active when tested by bioassays confirming that at alkaline pH the protoxin is solublized and toxin

with low molecular weight is produced.

DISCUSSION

In matured pigeonpea plants, tumors were seen 4-6 weeks after infection with *A.tumefaciens*. However, when seedlings grown *in vitro* were used, tumor formation was more rapid (within 7-10 days) suggesting that an assay for gall formation in pigeonpea could be developed using this system for scoring susceptibility at differnet strains towards this legume. The data also showed a variation in tumor induction *in vivo* and *in vitro* infection and the infectivity of *A.tumefaciens* to the pigeonpea was found to depend on the age of the seedlings and tissue of infection. When the germinating seeds were infected at micropile i.e. embryonic regions, they showed high response towards tumor frequency. Similar observations were seen in case of intact maize seedlings when the meristematic regions were used for T-DNA transfer (Graves and Goldman, 1986).

Since lepidopteran class of insect is the major pest towards pigeonpea *B.thuringiensis* strains were screened for the toxicity against Lepidopteran. The host specificity or differential susceptibility of *B.thuringiensis* strains is suggested to be because of the mode of action of the crystal toxin in the infected larvae. (Slaney *et al.* 1992). These

crystals which are protoxins are referred as insecticidal crystal proteins (ICP) or crystal proteins (27-140kD). When the crystals are ingested by a susceptible larva, they are broken down to toxic peptides of 60-70kD or less. This proteolytically activated toxin after binding to the brush border induces permeability in the epithelium cells (Hoffman *et al.* 1988 a,b). As a result, the cell lysis takes place and the larva stops feeding and eventually dies. In the present work, *S.litura* has been used as a target insect because it has been considered as one of the resistant pest species of field crops of economic importance in India. At present only chemical pesticides are effective in its control. The most commonly used commercial biopesticide *B.thuringiensis* HD-1, is marginally susceptibile (30-50%) to *S.litura* (Amonkar *et al.* 1985, Whitlock *et al.* 1991). In the bioassays carried out, *B.thuringiensis* (NCIM 2514) was found to be more lethal to the test larvae (more than 90% mortality) with significant loss in larval weight. This strain was also active against potato tuber moth larvae (more than 80% mortality). These bioassays were carried out with neonate larvae of *S.litura* and potato tuber moth and the neonate bioassays were shown to be more suitable for accurate determination of potencies of *B.thuringiensis* strains (Navon and Klein; 1990).

It has been reported that the varieties of

B.thuringiensis crystal δ-endotoxin can be solubilized by alkaline buffer (pH > 12) (Bulla *et al.* 1981) or a combination of alkaline buffer (pH 9-10) under reducing conditions (Huber *et al.* 1981; Thomas and Ellar, 1983). When the crystal protein preparation was dissolved in 50mM carbonate buffer (pH 10.5) in absence of reducing agent, δ-endotoxin (130kD) was solubilized, releasing the 66kD toxin. The purified protein and the polypeptide released by alkali solubilization (66kD) from *B.thuringiensis* (NCIM 2514) preparations exhibited the toxicity by bioassays which confirmed that this strain was able to produce δ-endotoxin which was toxic towards lepidoptera larvae.

From the screening procedure followed in this chapter, *A.tumefaciens* (NCIM 2943) and *B.thuringiensis* (NCIM 2514) were selected for protoplast fusion work and in the following chapter the efficient protoplast formation and regeneration of the two strains will be discussed.

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CHAPTER III

EFFICIENT PROTOPLAST REGENERATION OF *AGROBACTERIUM TUMEFACIENS* AND *BACILLUS THURINGIENSIS*

SUMMARY

Conditions for isolation and regeneration of protoplasts of *A.tumefaciens* and *B.thuringiensis* were optimized. Treatment of cells in the early exponential growth phase (8h) with lysozyme (1mg/ml) resulted in efficient protoplastization (90-99%) in both the strains. Of all the osmotic stabilizers used, sucrose (0.6M) and sorbitol (0.8M) were most suitable for regeneration of *B.thuringiensis* protoplasts on both minimal and complete media. However, in case of *A.tumefaciens* protoplasts, only 0.6M sucrose gave the highest regeneration. The plasma expander, bovine serum albumin (BSA), did not enhance the regeneration of the protoplasts. Maximum fusion frequency was obtained when protoplast from 8h grown cells were used for fusion experiments. This is the first report where the protoplast regeneration from *A.tumefaciens* and *B.thuringiensis* has been achieved to the extent of 10-12% on a minimal medium using a minimum concentration of lysozyme for protoplast preparation in the absence of BSA.

INTRODUCTION

The success of the protoplast fusion technology depends on various aspects like protoplast formation and regeneration; protoplasts fusion conditions and the stability of fusants. Some small changes in the experimental conditions during isolation, regeneration and fusion of protoplast can have profound effects on efficient fusion and appropriate phenotypic transfer. From the literature survey as discussed in Chapter I, it has been shown that the ability to form protoplast may vary greatly from organism to organism and efficient formation of protoplast does not ensure its efficient regeneration. Earlier, the spheroplasts of *A.tumefaciens* were fused with plant protoplasts to develop the method for transformation of plant protoplasts independent of *Agrobacterium* infection (Hasezawa *et al.*, 1981; Hain *et al.*, 1984 and Baba *et al.*, 1986). Although these reports have indicated the merits of protoplast fusion technology towards plant transformation, there are no state of art protocols developed for isolation and regeneration of *A. tumefaciens* spheroplasts.

Transformation of *B.thuringiensis* protoplasts has been successfully carried out by various workers (Martin *et al.*, 1981; Alikhanian *et al.*, 1981; Miteva *et al.*, 1981; and Fisher *et al.*, 1984). However, the frequency of transforma-

tion seems to be very low, probably, due to poor protoplastization or inefficient regeneration of protoplasts.

To our knowledge, there have been no systematic studies on optimization of protoplast isolation and regeneration from *A. tumefaciens* and *B. thuringiensis* which are used in the production of insect resistant plants in plant genetic engineering work. This chapter deals with the optimization of conditions for formation and regeneration of protoplasts and their effect on improved regeneration of frequency of fusion in these two strains.

MATERIALS AND METHODS

Bacterial strains

As described in previous chapter *A.tumefaciens* (NCIM 2943) and *B.thuringiensis* (NCIM 2514) were selected for protoplast fusion. For detection of selection markers in these strains, the antibiotic assays were carried out by plate assay methods. The minimal inhibitory concentration for different antibiotics is given in Table 3.1.

To check the ability of these strains to utilize octopine as a sole source of carbon and nitrogen, these cultures were grown on minimal medium (without $(NH_4)_2SO_4$ and glucose) supplemented with 10mg/100ml of filter sterilized octopine.

TABLE 3.1 : Antibiotic resistance of *A.tumefaciens* and *B.thuringiensis*

Antibiotic	Minimum inhibitory concentration µg/ml	
	A.tumefaciens **(NCIM 2943)**	***B.thuringiensis*** **(NCIM 2514)**
Kanamycin (Km)	100	10
Ampicillin (Ap)	20	100
Tetracyclin (Tc)	10	10
Spectinomycin (Sp)	20	20
Chloramphenicol (Cm)	20	20
Claforan or Cefotaxime (Cf)	20	10
Rifampicin (Rf)	5	5

Protoplast isolation

Protoplast formation is the phenomenon mainly dependent on cell wall structure. The physiological status of an organism at the time of protoplasting is the major factor in determining protoplast yield. To remove the cell wall, antimicrobial agents like lysozyme or penicillin G are used.

To check the effect of cell age on protoplast formation, the overnight grown cultures of *A.tumefaciens* and *B.thuringiensis* were inoculated in 50ml of YEB and LB medium respectively and incubated at 30°C on a rotary shaker. Cells at various time intervals were centrifuged and washed twice with 0.01M Tris.HCl buffer (pH 8.0). As the addition of EDTA allows more complete access of lysozyme to the cell wall (Weiss, 1976), the pelleted cells were suspended in TES buffer containing (Tris.HCl 0.01M; Na-EDTA, 0.025M; sucrose, 0.6M at pH 8.0) and kept at 37°C for 30 minutes with gentle shaking. Filter sterilized lysozyme prepared in TS buffer (0.01M Tris.HCl pH 8.0 and 0.6M sucrose) was added at the concentration of 1mg/ml and the EDTA treated cells were further incubated at 37°C with slow shaking to allow the protoplast formation.

Protoplast formation was monitored by treating samples to osmotic shock in distilled water. The protoplasted cells diluted in distilled water were plated on a com-

plete medium and the grown colonies were counted as osmoresistant. After maximum protoplastization, 50mM $MgCl_2$ was added to stabilize the protoplast mixture.

Protoplast regeneration

Following media were used for protoplast regeneration:

Minimal Medium : $MgSO_4.7H_2O$ 0.3; $MnSO_4.2H_2O$ 0.05; $CaCl_2.2H_2O$ 0.08; $ZnSO_4.7H_2O$ 0.005; $CuSO_4.5H_2O$ 0.005; $FeSO_4.7H_2O$ 0.005; K_2HPO_4 0.5, $(NH_4)_2SO_4$, 1.0 and Glucose 5.0g per litre, pH 7.0

Regeneration Medium : The minimum requirement for the regeneration of protoplast is the presence of osmotic stabilizer in the solid medium. Hence the minimal medium or complete medium (LB or YEB) was supplemented with 0.8% agar and 0.6M sucrose.

Selection Medium : For regeneration of fused protoplasts, minimal medium was fortified with ampicillin and kanamycin (50µg/ml each) alongwith 0.8% agar and 0.6M sucrose. For further selection of the protoplast fusants based on the markers of parental strains minimal medium without $(NH_4)_2SO_4$ and glucose was supplemented with 0.01% octopine (Sigma) and antibiotics (Ap and Km), 50µg/ml each.

The suitably dilluted protoplast suspension in TS

buffer was plated on LB or YEB regeneration medium or on a minimal regeneration medium and incubated at 30°C for 48-72h till the colonies appeared. The number of regenerated protoplasts was determined by deducting the number of osmoresistant cells (cells obtained by suspending the protoplast mixture in distilled water) from regenerated cells on the regeneration medium. The frequency of regeneration was expressed as the ratio of number of regenerated protoplasts to the total number of protoplasts.

Protoplast fusion

The generalised procedure for bacterial protoplast fusion is given in Table 1.1. Protoplast of both the parental strains are fused by fusogen like polyethylene glycol, which induces aggregation of protoplasts. In the present work identical number of protoplasts from each strain was mixed gently and fused in presence of 30% polyethylene glycol 7500 (wt/vol) for 3 min and washed with TS buffer to remove PEG, as the fusion events occur after the fusogen is removed or diluted a way. The PEG treated fused protoplasts were mixed with antibiotic containing minimal regeneration medium and poured in plates which were incubated at 30°C for 10-15 days. The fusion frequency was assessed by ratio of total number of colonies appeared on

minimal medium with antibiotics to the total number of protoplasts taken for fusion.

RESULTS

Antibiotic resistance of *A.tumefaciens* and *B.thuringiensis*

For isolation of hybrids after protoplast fusion between two strains, one of the essential factors is to have some selection markers like auxotrophic or antimicrobial resistance in the parent strains to facilitate the selection of such hybrids.

As described in Chapter II, *A.tumefaciens* (NCIM 2943) is a kanamycin resistant shooty mutant of octopine type Ti plasmid $PTiB_6S_3$ and is able to produce octopine when infected to pigeonpea plants. In case of *B.thuringiensis* subsp *kurstaki* (NCIM 2514). it was necessary to find out the selection markers. Table (3.1) shows the minimal inhibitory concentrations of various antibiotics towards both the strains. Since the two strains possesed opposite markers with respect to antibiotic resistance, it helped to formulate a suitable medium to isolate fusants. Thus the hybrids between *A.tumefaciens* (NCIM 2943) (Km^r Ap^s Oct^+) and *B.thuringiensis* subsp *kurstaki* (NCIM 2514) (Km^s Ap^r Oct^-) protoplasts were selected on minimal medium with kanamycin, ampicillin and octopine as a sole source of C and N as

described in detail in chapter IV.

Effect of various factors on protoplast isolation and regeneration

Table 3.2 summarizes the data on protoplast formation and regeneration in *A.tumefaciens* and *B.thuringiensis* as a function of culture age. Cells harvested after 8, 12, 16 and 24h were used for protoplast isolation and their 90-99% protoplast are formed in both the strains and there is no significant effect of culture age on the protoplast formation frequency. The regeneration frequency of protoplast is however affected by culture age. The maximum protoplast regeneration frequency is obtained when 8h grown cells of both the strains are used for protoplast formation and regeneration (10.4% for *A.tumefaciens* and 7.0% for *B.thuringiensis*) on minimal and complete medium. The regeneration frequency is drastically reduced with an increase in culture age and is of the order of 0.55% in *A.tumefaciens* and 0.012% in *B.thuringiensis* with culture age of 24h. Although the number of colonies appeared on minimal medium was relatively low as compared to number of colonies on rich medium, there was no substantial change in the regeneration frequency of protoplasts. Addition of 50mM $MgCl_2$ was strictly necessary for protoplast viability and stability.

In the present study, the 8 hour grown cells were

TABLE 3.2 : Protoplast Formation and Regeneration in *A.tumefaciens* and *B.thuringiensis*

Organism	Culture Age h	Starting no. of cells (Z)	Osmoresistant colonies[b] (Y)	Regenerated colonies[a] (X)	Protoplast formation frequency[c] %	Regeneration frequency[d] %
A.tumefaciens	8	5.8×10^7	9.3×10^4	6.9×10^6	98.55	10.30
	12	7.4×10^9	2.8×10^6	4.2×10^8	99.28	5.63
	16	6.0×10^9	1.2×10^5	1.2×10^8	99.16	1.63
	24	3.8×10^7	3.0×10^6	2.1×10^{10}	99.52	0.55
B.thuringiensis	8	4.0×10^7	2.7×10^5	3.1×10^6	91.20	7.07
	12	2.4×10^8	6.6×10^5	1.4×10^7	95.00	5.54
	16	6.3×10^9	8.4×10^5	2.4×10^7	96.20	0.36
	24	2.5×10^{12}	2.4×10^7	2.8×10^8	91.40	0.12

a. Regenerated colonies include colonies from regenerated cells and osmoresistant cells.
b. Colonies from nonprotoplasts were derived from lysozyme treated cells which resisted lysis upon dilution in water.
c. Protoplast formation frequency = X-Y / X x 100
d. Regeneration frequency = X-Y / Z x 100

used to find out the effect of incubation of different concentration of lysozyme (0.1-15.0mg/ml) at 30°C and 37°C. Maximum regeneration frequencies were obtained from the cells incubated at 37°C and with 1mg/ml lysozyme concentration. Figure 3.1 shows the effect of incubation of cells in presence of lysozyme on protoplast regeneration. In case of *A.tumefaciens*, incubation of protoplast with 1mg/ml lysozyme for 2 h lead to more than 10% protoplast regeneration, whereas in *B.thuringiensis* 7.5% regeneration was obtained after 3 h of incubation with lysozyme. Prolonged treatment with lysozyme resulted in decreased regeneration frequencies in case of both the strains.

To study the effect of different osmotic stablizers on protoplast regeneration, protoplasts of *A.tumefaciens* and *B.thuringiensis* were plated on regeneration medium containing different osmostabilizers as shown in Table 3.3. In case of *B.thuringiensis*, 0.6M sucrose and 0.8M sorbitol were most suitable for regeneration of protoplasts but in case of *A.tumefaciens* only 0.6M sucrose was a favourable osmostabilizer. Addition of plasma expander, BHI (Brain Heart Infusion) increased the regeneration frequency only in case of *A.tumefaciens*, but BSA did not enhance the regeneration in both the strains.

FIG 3.1 : **Effect of incubation of cells in presence of lysozyme on protoplast regeneration of *Agrobacterium tumefacins* and *Bacillus thuringiensis***

8h grown cells were protoplasted with lysozyme (1mg/ml) at 37°C. Protoplasts obtained were plated on regeneration medium.

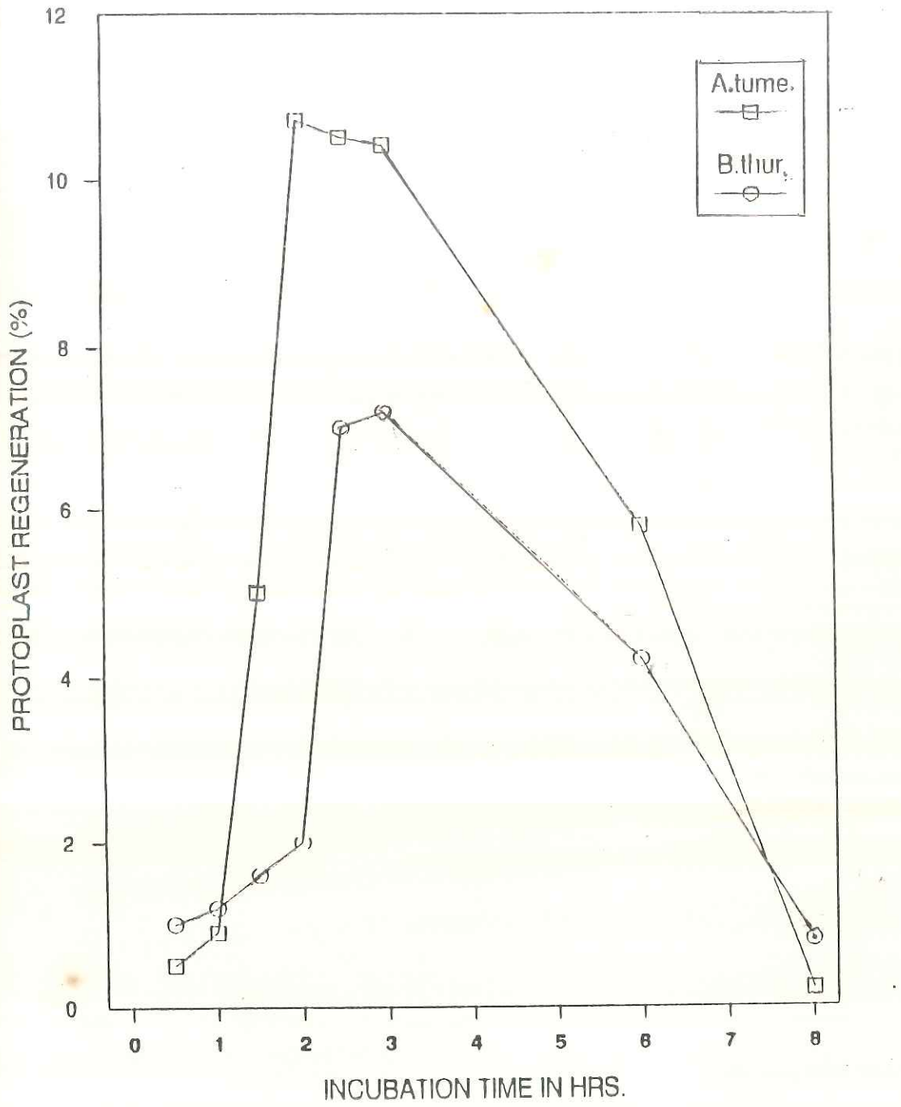

FIG. 3.1

TABLE 3.3 : Effect of different osmotic stabilizers on regeneration of protoplasts.

Stabilizer	Regeneration frequency (%)	
	A.tumefaciens	*B.thuringiensis*
0.6M KCl	0.02	0.17
0.5M NaCl	0.04	0.43
0.5M Na-succinate	0.10	0.22
0.5M Sucrose	8.04	7.00
0.6M Sucrose	10.30	7.20
0.8M Sorbitol	0.05	6.90
0.4M Mannitol	0.06	1.02
2.0% Brain Heart Infusion	14.10	3.02
0.5% Gelatin	0.69	0.01
1.0% BSA	8.50	7.00

Protoplasts obtained as described in text were allowed to regenerate on the regeneration media supplemented with different osmostabilizers.

Effect of cell age on protoplast fusion frequency

To study the effect of cell age on protoplast fusion, identical number of protoplasts isolated from cells of different age were fused in presence of 30% PEG-7500 for 3 minutes. Here it was found that the removal of PEG after protoplast fusion was an essential step in the fusion experiments.

Protoplasts isolated from 8, 16 and 24 h grown cells were used for fusion. In all the cases, 10^8 protoplasts were fused from both the strains and the colonies were regenerated on minimal medium with ampicillin and kanamycin. Table 3.4 summarizes the data on fusion frequencies between the protoplasts of *A.tumefaciens* and *B.thuringiensis* isolated from different age of cells. As seen from Table 3.4, protoplast fusion with the 8 h cells regenerats 2482 colonies, whereas 1782 and 149 colonies regenerats from fused protoplast obtained from 16 h and 24 h cells respectively. With the 8 h protoplasted cells, the fusion frequency is 1.9×10^{-5}. When the 16h and 24 h protoplasted cells are used for fusion, the frequencies are 2.5×10^{-6} and 7.1×10^{-7}. These results show that the fusion frequency is enhanced nearly by ten fold when the protoplasts derived from cells at early exponential phase (8 h) are used in fusion experiments.

TABLE 3.4 : Fusion frequencies between the protoplasts of *A.tumefaciens* (Km^r Ap^s) and *B.thuringiensis* (Km^s Ap^r) isolated from different age of cells

Age of protoplasted cells h	Total No of fused protoplasts	Total No of* regenerated fusants	Fusion frequency %
8	1.3×10^8	2482	1.90×10^{-5}
16	6.9×10^8	1782	2.58×10^{-6}
24	2.1×10^8	149	7.13×10^{-7}

* Regenerated colonies isolated on minimal medium with ampicillin and kanamycin (50µg/ml each).

DISCUSSION

For any genetic work on protoplast fusion or protoplast transformation, successful regeneration of protoplasts into normal cells is essential in order to give a progeny which could be genetically characterized. It is well known that regeneration of *B. thuringiensis* protoplasts is very difficult to achieve (Martin *et al.*, 1981; Alikhanian *et al.*, 1981; Fisher *et al.*, 1984) and this could be attributed to poor protoplastization. It is also known that *B. thuringiensis* is highly resistant to lysozyme due to the chemical structure of its cell wall (Kingan and Ensing, 1968). Miteva *et al.* (1981) have developed an efficient procedure for the isolation of protoplasts from *B. thuringiensis*. The treatment of penicillin to the middle-log phase cells of *B. thuringienesis* allowed them to use less concentration of lysozyme (5mg/ml). In the present study, cells in the early exponential phase (8 h) have been used for isolation of protoplasts, where the cells were subjected to mild osmotic shock by EDTA followed by lysozyme treatment (1mg/ml). This resulted in about 90-95% protoplast formation. Temeyer (1987) has pointed out that the sensitivity of the lysozyme to the cell wall was dependent on the condition and treatment of cells during growth and harvest. With our results, we have found that even though *B.thuringiensis* is a

gram-positive organism, the mild osmotic shock given by EDTA seems to be favourable for lysozyme activity which is most commonly used in case of gram-negative organisms (Weiss, 1976).

Rubinstein and Sanchez-Rivas (1988) used the autoplasts for transformation and fusion to avoid the undesirable effects of lysozyme during protoplastization and regeneration. To achieve the highest regeneration of protoplasts, they formulated various media supplemented with casmino acids and BSA (bovine serum albumin), but the regeneration frequencies obtained were only 0.3% of the original number of protoplasts. Such a regeneration frequency seems to be low for any transformation experiments. It has been reported that the regeneration frequency of protoplast is raised by the addition of artifical expanders to the regeneration medium (Akamatsu and Sekiguchi, 1981; Pulido Vega *et al.*, 1991). However, our results have shown no significant change in the regeneration frequency by the addition of BSA. In case of *B. thuringiensis*, both sucrose and sorbitol were suitable osmostabilizers, but in case of *A. tumefaciens*, only sucrose could act as a suitable osmostabilizer. In case of gram negative organism such as *Zymomonas mobilis*, sorbitol has been found to be suitable for the regeneration of protoplasts (Yanase *et al.*, 1985). It is further observed that

the fusion frequency is enhanced when the protoplasts derived from early exponential phase (8 h grown cells) are used in fusion experiments. This suggests that improvement in fusion frequency necessarily requires higher regeneration of protoplasts.

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CHAPTER IV

INTERGENERIC PROTOPLAST FUSION BETWEEN *AGROBACTERIUM TUMEFACIENS* AND *BACILLUS THURINGIENSIS*

SUMMARY

Intergeneric protoplast fusion between *A.tumefaciens* and *B.thuringiensis* was performed and the hybrids were selected on medium containing antibiotics and octopine as a sole source of carbon and nitrogen. The hybrid nature was initially established by continuous subculturing on selection medium and later they were screened for some of the morphological and biochemical tests exhibited by both the parents. Morphologically, the hybrids either resembled the parent or showed different morphology. The presence of selection markers in the fusants was confirmed by the enzymatic assays related to kanamycin resistance (neomycin phosphotransferase II activity) and opine production. For phytopathogenicity, indirect tests were carried out like formation of 3-keto lactose or ferric ammonium citrate pellicle. The fusants showing positive response to above mentioned tests were further characterized for their ability to form tumors. These successfully isolated hybrids were stably maintained for more than four years and they did not segregate to give rise to parental type even after multiple transfers on rich medium.

INTRODUCTION

The use of protoplast fusion technique to generate novel strains and new products unobtainable by conventional methods is an exciting one. Successful isolation of intergeneric hybrids in distantly related bacteria by protoplast fusion was first reported in our laboratory (Gokhale *et al*, 1984) followed by many other reports (Chen *et al*, 1987; 1988; Prakash and Cummings, 1988; and Deb *et al*, 1990). The new strains generated through protoplast fusion exhibit the morphology and growth characteristics either resembling to the parent strain (Chen *et al*, 1987; 1988) or altogether different morphologies (Gokhale *et al*, 1984). In case of protoplast fusion between *Cellulomonas* and *B. subtilis*, the hybrids showed altered phenotypic expression and regulation (Gokhale *et al*, 1984; Gokhale and Deobagkar, 1989, 1990). Such changed regulation was possibly a result of multiple recombinations between two genomes during protoplast fusion (Gokhale and Deobagkar 1989; Chen *et al*, 1987).

We were interested in undertaking the protoplast fusion work between *A.tumefaciens* and *B.thuringiensis* as both these strains are considered to be very important from plant genetic engineering point of view. The primary objective to carry out such a fusion was to transfer the tumor inducing property into distantly related *Bacillus* strains

which may provide a base for the possibility of using such a protoplast fusion hybrid towards plant genetic engineering work.

In this chapter, the successful intergeneric protoplast fusion between *A.tumefaciens* and *B.thuringienis* strains is described. The stable hybrids selected on the selection medium are further characterized for some of their phenotypic characters.

MATERIALS AND METHODS

Maintenance of stock cultures

A.tumefaciens (NCIM 2943) and *B.thuringiensis* subsp. *kurstaki* (NCIM 2514) were grown on YEB and LB agar slants respectively at 30°C. All the protoplast fusion hybrids were grown on YEB agar medium containing 50µg/ml each of ampicillin and kanamycin. The cultures on slants were preserved at 4°C and were periodically subcultured every two months on freshly prepared medium. The cultures were also preserved in glycerol broth at -70°C. For this purpose, overnight grown cultures in 10ml of liquid medium with antibiotics were pelleted and suspended in 5ml of 30% glycerol, chilled immediately in liquid nitrogen and stored at -70°C. These vials served as master stock cultures.

Isolation and morphological characterization of fusants

Protoplasts were isolated from *A.tumefaciens* and *B.thuringiensis* strains from the 16h grown cells in LB and YEB medium respectively as described in previous chapter III. The colonies were isolated from the selection medium containing antibiotics and octopine as a sole source of carbon and nitrogen. The colonies grown on this medium were continuously subcultured for more than 10 transfers. The single colonies were purified and further characterized to establish their hybrid nature.

Gram staining : All the cultures were grown overnight in YEB at 30°C and were harvested by centrifugation at 3000rpm for 20 minutes. Cells were washed with distilled water, fixed on slide and were further treated for Gram staining. Slides were observed under light microscope.

Scanning electron micrographs : For scanning electron microscopy, bacterial cultures were grown overnight in YEB at 30°C and the cells were harvested by centrifugation at 3000rpm for 20 minutes. Cells were washed with distilled water and fixed in 3% glutaraldehyde at 4°C overnight. Fixed cells were washed with distilled water twice and suspended in minimal volume of distilled water. They were spread on aluminium foil to form a thin film. After gold

sputtering, specimens were observed in JOEL Model 5200 scanning electron microscope and photographed.

Colony morphology : Hybrids alongwith the parent strains were grown on LB and $CaCO_3$ agar slopes, containing yeast extract 10.0; glucose 20.0 and $CaCO_3$ 20.0g/l at pH 7.0.

To check the heat resistance all the hybrids alongwith the parental strains were grown overnight in LB, heated at 70°C for 15 minutes and streaked on LB agar plates.

Biochemical characterization of fusants :

Penicillinase test : Penicillinase activity of the hybrids and parents was determined according to Slavnova *et al.* (1986). Overnight grown cultures were spotted on agar plates containing 5000 units/ml of Penicillin-G and incubated at 30°C, for 16h. The overnight grown cells of *Staphylococcus aureus* (NCIM 2079) were mixed in LB agar (at 50°C) and layered on the overnight grown agar plates and further incubated at 30°C for 24h. Zone of growth of *S. aureus* surrounding the original colonies was measured.

3-keto lactose production : 3-Keto lactose test for crown gall producing bacterium (*Agrobacterium*), was carried out as described by Bernearts and De Ley (1963). All the cultures

were grown for 24-48h on $CaCO_3$ containing (as per the medium for colony morphology) agar slopes and then spotted on the medium containing lactose 10.0; yeast extract 1.0 and agar 15.0g/l. The plates were incubated at 30°C for 24-48h, then flooded with a layer of Benedict reagent and kept at room temperature for about 1h. If 3-Keto lactose was present, a yellow ring of Cu_2O was visible around the cell mass and the diameter was measured.

Ferric ammonium citrate pellicle formation : This test was carried out as described by Kersters *et al.* (1973). All the test organisms were grown in 10ml medium containing (g/l) of yeast extract 10.0; glucose 20.0; ferric ammonium citrate 10.0. After 24-48h, a dark brown pellicle formation at the surface was observed.

Neomycin phosphotransferase (NPTII) activity

The Km^r was estimated by the enzymatic detection of NPTII activity according to the procedure of Roy and Sahasrabudhe (1990).

The protoplast fusants alongwith *B.thuringiensis* (NCIM 2514) as negative control and plasmid pRIP harbouring NPTII activity as a positive control, were used for NPTII assay. Bacterial crude extracts were prepared by centrifugation of 1ml of overnight grown culture in LB medium; the

cell pellet was resuspended in 200µl extraction buffer (25mM Tris-HCl pH 8.0; 10mM EDTA and 50mM glucose containing 1mg/ml lysozyme). The mixture was incubated on ice for 10-15 min. Then 1/5th volume of 10% Triton X100 was added to complete the lysis. The clear lysate was obtained after centrifugation for 10 min at 10000rpm (IEC centrifuge). The supernatant was dialyzed against 100mM Tris-HCl, pH 7.4 and used for NPTII activity determination or preserved at -70°C without loss of activity for three months.

Assay conditions : The reaction mixture contained in 50µl volume, 100mM Tris-HCl pH 7.4; 10mM $MgCl_2$, 20µg kanamycin sulphate and 0.5µCi [^{32}P] ATP (3000 Ci/mmol) with or without 200µM ATP. Sample proteins ranging from 0.1 to 1µg for bacterial extracts were used. The reaction mixture was incubated for 30 min at 18-20°C before applying to the Whatman No 3 chromatographic paper. From the reaction mixture, 10µl aliquots were spotted on the paper using micropipette which was then developed in a solvent system containing n-propanol/ammonia/water (5:4:1 v/v/v, pH 9.5) by ascending paper chromatography.

Once the solvent front had reached the top (ca. 14cm), the paper was air-dried prior to autoradiography. Time of exposure varied from 1 h to 16 h (at -70°C) with an intensifying screen depending upon the specific activity of

[^{32}P] ATP.

RESULTS

Protoplast fusion between *A.tumefaciens* and *B.thuringiensis*

Under the experimental conditions used, almost 99% of the *Agrobacterium* cells and 90% of *Bacillus* cells were converted to protoplasts. The regeneration frequency of these protoplasts was 2% in *A.tumefaciens* and 0.3% in *B.thuringiensis*. The protoplasts from the two strains were fused in presence of PEG as described in material and methods and the fused protoplasts were recovered on the selection medium containing antibiotics. These colonies grown on selection medium were further transferred on minimal medium with octopine as the sole nitrogen and carbon source and were designated as AB 001, AB002 and so on. The hybrid nature of fusants was confirmed since all the hybrids could grow well on the medium containing antibiotics and octopine for more than 10 transfers. Even after plating 10^{10} cells of each parental strain, no colony was observed on the selection medium ruling out the possibility of spontaneous mutation in both the parental strains. Independent complementation frequencies for antibiotic and octopine markers in the fusion products are given in Table 4.1. and the fusion frequencies are 5.3×10^{-6} to 2.0×10^{-7} with

TABLE 4.1 : Fusion frequency of protoplasts on selection medium

Markers	Total number of colonies	Fusion frequency Total number of fused protoplasts 1.5×10^8
Ap^r Km^r	792	5.3×10^{-6}
Ap^r Km^r Oct^+	30	2.0×10^{-7}

antibiotics and octopine markers respectively.

Morphological and colonial features of the hybrids

A.tumefaciens and *B.thuringiensis* are very different in taxonomy, physiology and morphology. *A.tumefaciens* is a gram negative, octopine degrading bacterium whereas *Bacillus thuringiensis* is a gram positive, sporulating bacterium which produces endosporal crystal protein at its sporulation stage. A detailed microscopic observations of the hybrids alongwith the parental strains under light microscope revealed that out of 26 stable hybrids tested, 6 hybrids were gram positive rods like *B.thuringiensis* and the remaining hybrids were gram negative rods as in case of *A. tumefaciens*. The hybrids were further observed under scanning electron microscope. Figure 4.1 shows the scanning electron micrographs of few protoplast fusants alongwith the parental strains. The hybrid AB0242 which is a gram positive strain possesses similar rods as present in *B.thuringiensis* whereas the hybrid AB017 (gram negative) resembles the *A.tumefaciens* parent. The hybrid AB010 which is a gram negative bacterium shows very long rods, which differ in size from both the parents.

The colony morphology of *A.tumefaciens*, *B.thuringiensis* and one of the gram positive hybrids AB0242 on $CaCO_3$ containing medium is shown in Figure 4.2. In case of

FIG 4.1: Scanning electron micrographs

A : *A.tumefaciens* (NCIM 2943)

B : *B.thuringiensis* (NCIM 2514)

C : Protoplast fusant AB0242

D : Protoplast fusant AB017

E : Protoplast fusant AB010

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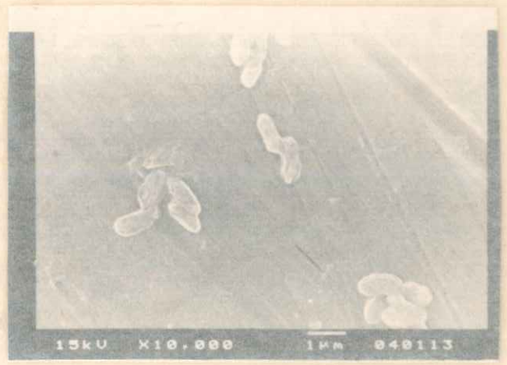

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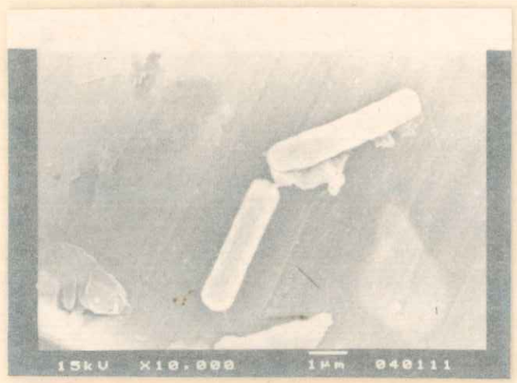


FIG. 4.1

FIG 4.1: Scanning electron micrographs

A : *A.tumefaciens* (NCIM 2943)

B : *B.thuringiensis* (NCIM 2514)

C : Protoplast fusant AB0242

D : Protoplast fusant AB017

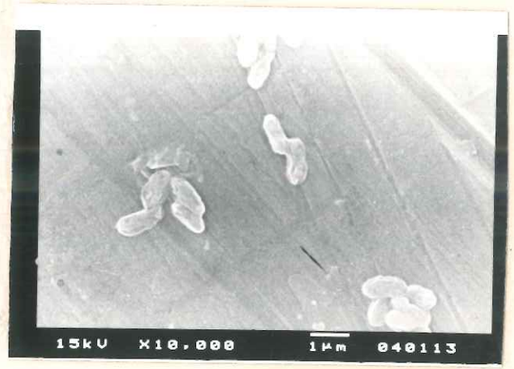
E : Protoplast fusant AB010

A

B

C

D



E

FIG. 4.1

A.tumefaciens, there is a difference in the growth on agar slope of two different media. When grown on LB medium, the colony is colourless and slimy but on the $CaCO_3$ containing medium it is crumpled (as shown in Fig 4.2). In case of *B. thuringiensis*, the growth is white and dry and there is no change in the colony morphology depending on the medium. When the hybrids are grown on both the medium, irrespective of gram reaction all the hybrids resemble with *Agrobacterium* parent except in case of gram positive AB0242, where the colony morphology is similar to *Bacillus* strain.

B.thuringiensis is a gram positive, sporulating organism and spores only can survive at 70°C for 10-15 min. When the overnight grown cells of fusants were heated at 70°C for 15 min and then plated on LB agar plates, 13 hybrids grew well showing heat resistance property from *B.thuringiensis*. From this morphological study and heat resistance character,it is found that hybrid AB0242 resembles more with the parent *B. thuringiensis* strain.

Biochemical characterization of the hybrids

Apart from the morphological and colonial features, the hybrids are characterized by their biochemical tests which are inherited from the parental strains. The biochemical test like penicillinase production is used as an indirect criteria for selection of insecticidal *B.thuringi-*

FIG 4.2: Colony morphology of *A.tumefaciens*, *B.thuringiensis* and protoplast fusant AB0242 on $CaCO_3$ containing medium.

1. AB0242
2. *B. thuringiensis* (NCIM 2514)
3. *A. tumefaciens* (NCIM 2943)

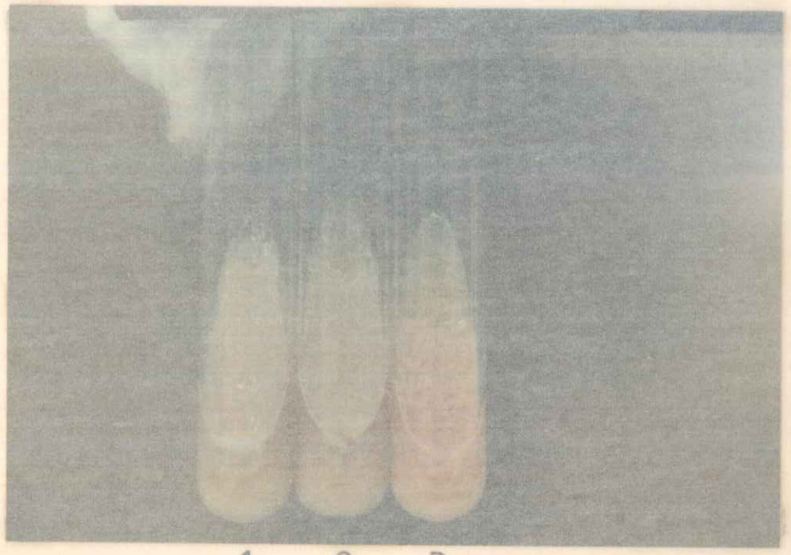
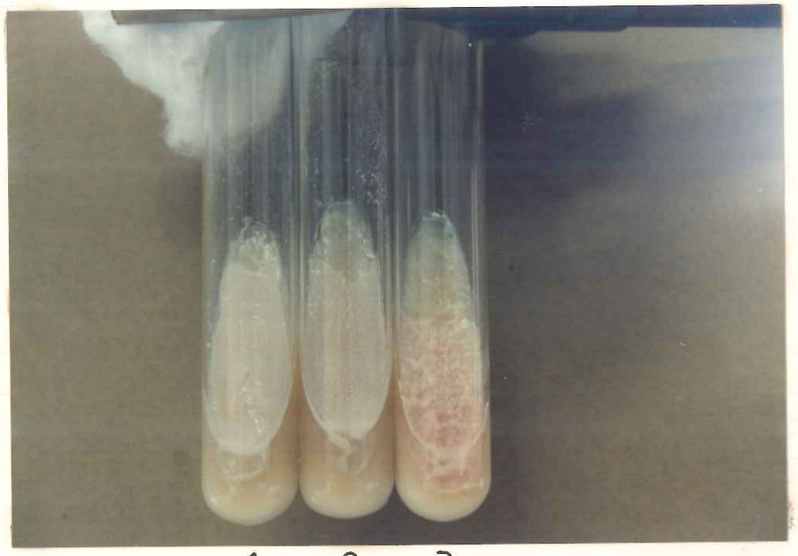


FIG. 4.2

FIG 4.2: Colony morphology of *A.tumefaciens*, *B.thuringiensis* and protoplast fusant AB0242 on $CaCO_3$ containing medium.

1. AB0242
2. *B. thuringiensis* (NCIM 2514)
3. *A. tumefaciens* (NCIM 2943)



1 2 3

FIG. 4.2

ensis strains (Slavnova *et al.*, 1986). In case of *A.tumefaciens*, the 3-keto-lactose positive and ferric ammonium citrate pellicle forming bacterial strains are shown to be phytopathogenic, (Bernaerts and De Ley, 1963; Kersters *et al.*, 1973). Table 4.2 gives the detailed results of these biochemical tests exhibited by the hybrids alongwith the control strains.

Penicillinase activity : In this test, the hybrids are grown on penicillin containing agar plates and are layered with overnight grown *Staphylococcus aureus* culture which is sensitive to penicillin. During incubation, the hybrids which are able to produce penicillinase, deplete the penicillin in the medium and the *S. aureus* is able to grow in that region. Thus the growth of *S. aureus* surrounding the colony in penicillin containing medium is an indication of penicillinase activity.

As seen from Table 4.2, irrespective of gram reaction, out of 23 hybrids 8 hybrids are penicillinase producer and in all these strains more than 0.5cm diameter of *S. aureus* growth zone is observed. With the parental *B.thuringiensis* (NCIM 2514) which is lethal to the lepidopteran larvae (as shown in Chapter II), more than 1.0cm of growth zone is surrounded by the test colony. These results show that among the hybrids tested for this enzyme activity,

TABLE 4.2 : Biochemical characters of the parental strains exhibited by the hybrids

Organisms	3 keto lactose production[a]	Ferric ammonium citrate pellicle[b]	Penicillinase activity[c]
A.tumefaciens (NCIM 2943)	+++	++	-
B.thuringiensis (NCIM 2514)	-	-	+++
Gram positive hybrids			
042,0102	-	-	-
0242,017	-	-	++
0101,0121	++	++	-
0151	+	+	-
Gram negative hybrids			
0171,011	+	++	++
0241	-	-	++
022	++	+	++
010	+	+	-
001,002, 018	++	+++	-
0151,006,001,} 0153 }	+	+	-
019,012,021, } 004 }	++	+	-

a. 3-keto lactose :

+++	1.5 - 1.8cm	diameter
++	1.0 - 1.5cm	diameter
+	0.5 - 1.0cm	diameter

b. Ferric ammonium citrate pellicle :

+++	dark brown (blackish) colour
++	dark brown colour
+	faint brown colour

c. Penicillinase activity :

+++	> 1.0cm	diameter
++	> 0.5cm	diameter
+	slight growth	

only 26% of the hybrids are pencillinase producer but compared to parental strain their activity is low.

3-keto Lactose and Ferric ammonium citrate tests : *A. tumefaciens* is unique in its mechanism because it has the ability to oxidize carbon 3 moiety of disccharides and bionic acids (Bernaerts and De Ley, 1963) like 3-keto lactose (3-keto-galactopyranosyl- glucose), 3-keto maltose, 3-keto sucrose, 3-keto lactobionic acid, 3-keto maltobionic acid. These compounds can easily be detected because they quickly reduce the usual sugar reagents (Fehling, Benedict etc) at room temperature.

According to Kersters *et al.* (1973) most of the 3-keto-lactose positive strains of *A.tumefaciens* are phytopathogenic and produce brown surface pellicle when grown on ferric ammonium citrate. Thus, these are the two easy tests for identification of phytopathogenicity in *A.tumefaciens*.

As shwon in Table 4.2, out of 23 hybrids, 18 hybrids are positive to both the tests. In case of 3-keto-lactose test, the organisms when grown on lactose medium, in positive strains a yellow ring of Cu_2O is observed with Benedicts reagent. The diameter of the yellow ring varies from 0.5-1.8cm.

When the hybrids alongwith the virulent strains

were grown in the citrate medium, a brown colour pellicle was observed in the positive hybrids. When these cultures were grown on agar medium, the slope turned into blackish brown colour. These results showed that out of the hybrids tested for this property, 78% were positive towards this test indicating that majority of the hybrids possessed *A.tumefaciens* property.

Phytopathogenicity of hybrids towards pigeonpea

A.tumefaciens is an unique bacterium which can anabolize and catabolize octopine. As seen earlier these bacteria on infecting the plant tissue produce tumors and synthesize opines which are otherwise absent in normal plant tissue.

Scoring of the susceptibility of the fusants towards legume pigeonpea was carried out by infecting the 72h germinating seedlings at embryonic region as described in Chapter II. Table 4.3 gives the tumor formation frequency by protoplast fusants towards pigeonpea. Twenty three fusants which were able to grow on ampicillin, kanamycin and octopine as a sole source of C and N for more than 10 subcultures were used for screening the susceptibility towards pigeonpea. Out of the twenty three fusants, two gram positive and three gram negative fusants showed tumor formation within ten days of infection. When the three days old germi-

TABLE 4.3 : Scoring of tumor formation by protoplast fusants between *A.tumefaciens* and *B.thuringiensis* towards 3 days old pigeonpea seedlings

Strains	Gram characteristic	No of tumors/ No. of infected seedlings	% Tumor formation
A.tumefaciens (NCIM 2943)	-ve	2/24	8.3
B.thuringiensis (NCIM 2514)	+ve sporulating	0/20	0.0
AB 0102	+ve	1/20	5.0
AB 0242	+ve	2/34	5.0
AB 0171	-ve	1/20	5.0
AB 0241	-ve	2/35	5.7
AB 010	-ve	2/30	6.6

nating seedlings were infected with the fusants, the tumors are formed with 5-6% frequency whereas with parental *A.tumefaciens* strain the frequency is 8.3%. Figure 4.3 shows the photograph of tumor produced by hybrid AB0242 under *in vitro* condition. The opine analysis is carried out as described in Chapter II and it is found that the tumors produced by the hybrids are octopine positive.

Dot blot assay for neomycin phosphotransferase (NPTII) activity

A.tumefaciens (NCIM 2943) contains a chimeric NPTII gene which confers the Km^r in the transformed plant (Zambryski *et al.*, 1984). At the time of selection of protoplast fusants, Km^r was used as one of the selection markers, since *A.tumefaciens* (NCIM 2943) is Km^r, Ap^s, and *B.thuringiensis* (NCIM 2514) is Km^s, Ap^r. The selected fusants are Km^r and Ap^r.

The expression of this dominant selectable marker gene has been monitored in the transformed plant cells in two ways. (1) By the ability of transformed plant tissue to form callus on kanamycin containing medium and (2) by direct assay for neomcyin phosphotransferase.

When the pigeonpea leaf discs were co-cultivated with the hybrids, callus formation was observed on kanamycin containing MS medium but it failed to regenerate after

FIG 4.3: *In vitro* tumor formation by AB0242 in 3 days old germinating seedlings of pigeonpea after 10 days.

FIG. 4.3

subsequent subculturing. For detection of the kanamycin resistance character, dot blot assay method was used as it is quite simple method and large number of samples could be estimated within 2-3 hours (Roy and Sahasrabudhe, 1990). One of the other advantage of this method is that, it is very sensitive and upto 0.00125µg protein/spot could be detected.

Figure 4.4 gives the autoradiogram of paper chromatography of NPTII assay of the cell extracts of the hybrids alongwith the control *B.thuringiensis*. As described in materials and methods, the cell extracts were mixed in the assay mixture containing Γ^{32}P-ATP and chromatographically separated on the paper. The paper was washed with phosphate buffer for 3-5 times as this buffer helped in eliminating background counts. When the exposed X-ray film was developed, the hybrids showed positive signals alongwith the *A.tumefaciens* (NCIM 2943) and plasmid pRIP (Figure 4.4). This confirmed that the Km^r property was positively transfered in two of the hybrids, AB0242 and AB010 which were gram positive and negative respectively.

DISCUSSION

A.tumefaciens and *B.thuringiensis* are very different in taxonomy, physiology and morphology. *A.tumefaciens* is a Gram negative tumorogenic, octopine degarding bacterium while *B.thuringiensis* is a Gram positive, sporulating bacte-

FIG 4.4: **Autoradiogram of papaer chromatography of NPT II assay of the bacterial cell extracts**

LANE 1 : HB101 + pRIP

LANE 2 : *B.thuringiensis* (NCIM 2514)

LANE 3 : *A.tumefaciens* (NCIM 2943)

LANE 4 : AB0242

LANE 5 : AB010

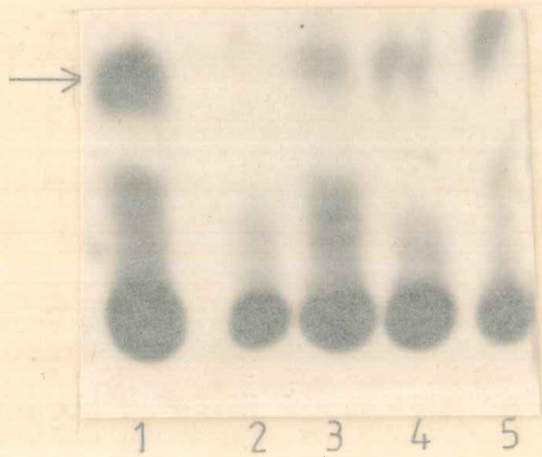


FIG. 4.4

FIG 4.4: **Autoradiogram of papaer chromatography of NPT II assay of the bacterial cell extracts**

LANE 1 : HB101 + pRIP

LANE 2 : *B.thuringiensis* (NCIM 2514)

LANE 3 : *A.tumefaciens* (NCIM 2943)

LANE 4 : AB0242

LANE 5 : AB010

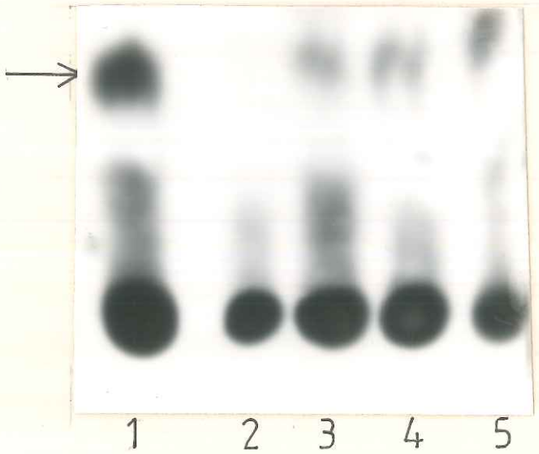


FIG. 4.4

Table 2

In vitro

Tumor formation after infecting the pigeonpea seedlings of different age.

Bacterial strains NCIM No.	~~2 days~~ 2 days			3 days			7 days		
	No. of seeds	No. of tumors	% tumor formation frequency	No. of seeds infected	No. of tumors formed	% tumor formation frequency	No. of seeds	No. of tumors formed	% tumor formation frequency
2146	16	0	0.0	24	6	25.0	8	1	12.5
2147	20	1	5.0	28	8	28.5	8	1	12.5
2148	12	0	0.0	20	2	10.0	10	0	0.0
2232	22	1	4.5	32	7	21.0	10	1	10.0
2822	12	0	0.0	16	1	6.25	10	0	0.0
2145	15	0	0.0	10	0	0.0	8	0	0.0

a. No tumor formation was observed in the 24 h soaked seeds. Most of the infected seedlings were unable to germinate.

b. Tumors were formed after 10 - 15 days of infection.

rium. The hybrids exhibited mixed morphological and biochemical characters of both the parents. Agrobacteria are known to form tumors in plant which synthesize specific opines that are not persent in normal plant tissue and are used by them as a sole source of carbon. Five of the hybrids have induced octopine positive tumors in pigeonpea.

The problem often encountered in the application of protoplast fusion technology is the stability of fusion products. The intraspecies (Ochi *et al.*, 1979; Hotchkiss and Gabor, 1980; Fleischer and Vary, 1985) and interspecies (Okamoto *et al.*, 1985) protoplast fusion resulted in the production of heterokaryons, diploids and true prototrophic recombinants. The heterokaryons are unstable and easily generate parental segregants, whereas the haploid recombinants can maintain genetic stability. The protoplast fusion hybrids between *A.tumefaciens* and *B.thuringiensis* were stably maintained for more than four years and they did not segregate to give rise to parental type evenafter multiple transfer to rich medium. Hybrids generated through protoplast fusion between *Cellulomonas* and *B. subtilis* (Gokhale *et al.*, 1984) or *Cyronbacterium acetoacidophilum* and *B. subtilis* (Deb *et al.*, 1990) were also found to be very stable evenafter growing them for a number of generations without selection pressure.

In case of intergeneric hybrids, the morphologi-

cal and growth characters resemble either the parental strains or possess different morphologies. This observation agrees with the findings made by Gokhale (1988) and Chen *et al.* (1987, 1988). The fusion hybrids between *Cellulomonas* and *B. subtilis* (Gokhale *et al.*, 1984) have shown the altered phenotypic expression and regulation. Such changed regulation was possibly a result of multiple recombinations between two genomes during protoplast fusion (Gokhale and Deobagkar, 1989; Chen *et al.*, 1987). Thus, for the stepwise characterization and finding out the presence of distinctive parental properties and their expression by fusants, biochemical tests indirectly related to the desired phytopathogenicity and insecticidal activity were carried out with the hybrids. For the selection of highly active strains of *B.thuringiensis*, penicillinase test has been employed by Slavnova *et al.* (1986) and it has been shown that virulent clones of *B.thuringiensis* possessed high levels of penicillinase activity. This observation was confirmed by testing this enzyme activity with the different toxic subspecies of *B.thuringiensis*. The 3- keto lactose production and ferric ammonium citrate pellicle formation are the important but easy tests for identification of phyotopathogenicity of *A.tumefaciens* strains into hybrids. When the hybrids were scored on the basis of their biochemical characters, it was

found that more than 70% hybrids possessed *Agrobacterium* characters, whereas 26% of the hybrids possessed the *Bacillus* property.

In these hybridization experiments, the selection of the fused protoplasts was carried out on the medium containing ampicillin, kanamycin and octopine as a sole source of carbon and nitrogen. To rule out the possibility of residual growth of any of the parental strain on these antibiotics, the individual parental strain was plated at higher cell density (10^{10} cells) on selection medium and no growth was observed. The kanamycin resistance in the hybrids was established by neomycin phosphotransferase II (NPTII) activity and two of the hybrids were NPTII positive.

The phytopathogenicity of the hybrids was tested by infecting the 72h germinating seedlings of pigeonpea. The germinating seed assay was very useful and it was possible to screen a large number of fusants for this test within short period, which was otherwise very arduous and time consuming. The appearance of tumor and synthesis of octopine are a good indication for transformation via *A.tumefaciens* i.e. Ti plasmid. As five of the hybrids, irrespective of their gram reaction, were able to produce octopine positive tumors in pigeonpea, it was confirmed that during protoplast fusion Ti plasmid region was transferred into hybrids along-

with the essential genes responsible for plant transformation (T-DNA region).

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CHAPTER V

COMPARATIVE TOXICITY OF PROTOPLAST FUSANT AND OF TRANSGENIC *E.COLI* CONTAINING *BT* GENE TOWARDS *SPODOPTERA LITURA* (F.)

SUMMARY

Five of the octopine positive phytopathogenic hybrids were screened for the presence of δ-endotoxin genes originating from *B.thuringiensis* by DNA-DNA hybridization using toxin specific probes and the expression of *bt* gene was monitored by immunoblot reaction using anti-delta endotoxin antibody. One of the gram positive phytopathogenic hybrids showed presence and expression of *bt* gene and the hybrid was lethal towards *Spodoptera litura* larvae.

To obtain a transgenic *E. coli* containing *bt* gene, a plasmid library from the *B.thuringiensis* (NCIM 2514) parental strain was constructed in pUC18 by digesting the plasmid DNA as well as the vector DNA by *HindIII* followed by ligation and transformation in *E.coli* DH5α. The transformants were screened by colony hybridization using delta endotoxin probe as well as oligoprobe specific for *bt* gene.

The relative toxicity potential of the intergeneric protoplast fusant and the *bt* positive transgenic *E.coli* construct was compared in terms of mortality, LC_{50} and potency in International Units against *Spodoptera litura* larvae. The percent mortality with protoplast fusant was 80% whereas that with *bt* clone was 66% against the test larvae.

INTRODUCTION

Since protoplast fusion technology allows a combination to take place not only between related species but also between unrelated genera, it has a great potential in breeding and improvement of industrial strains. Cell engineering through protoplast fusion helps in transfer of multigenic phenotypes across the barrier of genera which is otherwise impossible even with the help of recombinant DNA technology. With the advent of this technology, the possiblity that novel strains and products could be obtained is very exiciting. Recombination in unrelated bacteria could activate hitherto silent genes and add new characters to the existing phenotypes of either parents leading to new strains with altered phenotypic expression and regulation.

In the previous chapter, a successful protoplast fusion between *A.tumefaciens* and *B.thuringiensis* and identification of stable hybrids has been described. Five of these stable hybrids were found to produce octopine positive tumors in pigeonpea seedling, indicating the presence of Ti coding region transferred into the hybrid from *Agrobacterium* during protoplast fusion. These five fusants were taken as a starting material to confirm the presence of *B.thuringiensis* gene (or delta-endotoxin) by DNA hybri-

dization using *B. thuringiensis* specific probes. The expression of *bt* gene was further assessed by immunological reaction with anti-delta endotoxin antibody. One of the gram positive tumor inducing hybrids showed presence and expression of *B.thuringiensis* gene. Parallel to this work, *bt* gene was cloned in *E.coli* and its toxicity to *Spodoptera litura* was compared with that of the above fusant.

MATERIALS AND METHODS

Bacterial strains and plasmid

The protoplast fusants were isolated by intergeneric protoplast fusion between *A. tumefaciens* (NCIM 2943) and *B. thuringiensis* subsp *kurstaki* (NCIM 2514) as described in chapter IV. The *E.coli* host used for cloning the toxin gene was DH5α. The plasmid vector pUC18 and the enzymes used for cloning were obtained from Bangalore Genei, India.

Immunoblotting for expression of delta endotoxin

Screening of the protoplast fusants for the expression of the delta-endotoxin was done by immunoblotting 0.5µg and 1.0µg of protein, isolated from overnight grown cells in LB on nitrocellulose paper. The reaction was carried out with anti- delta endotoxin antibody, raised in rabbit against polyacrylamide gel purified 130kD protoxin which was a kind gift from Dr. P. Roy, Hindustan Lever

Research Centre, Bombay (Roy, 1992). Goat anti-rabbit IgG-alkaline phosphatase conjugate was used as the second antibody and BCIP (5 Bromo, 4 Chloro, 3 Indolyl phosphate) and NBT (Nitro Blue Tetrazolium) were used as the substrates for alkaline phosphatase.

Plasmid DNA Preparation

Total plasmid preparation of *A.tumefaciensis*, *B.thuringiensis* and protoplast fusant was carried out as described by Kronstad *et al.* (1983). *B.thuringiensis* (2514) total plasmids were further purified by CsCl density gradient centrifugation as described by Sambrook *et al.* (1989). Recombinant clones were analysed by preparing plasmid DNA by the rapid alkali-denaturation method (Birnboim and Doly, 1979).

Delta endotoxin specific probes

The *B.thuringiensis* specific toxin probe was 750bp long *ECoRI* fragments of pRT200 clone which was a kind gift by Dr. R. Tuli (Tuli *et al*, 1989).

The 21-mer oligonucleotide probe with DNA sequence of 5'-GGGACTGCAGGAGTGATAATA -3' was synthesized by Dr. K.N. Ganesh, National Chemical Laboratory, Pune using phosphoramidite chemistry. The probe was designed to be 100%

homologous to part of the coding region of the 6.6kb gene from HD-73 (Adang *et al*, 1985).

Cloning and identification of *Bt* toxin gene

Plasmid DNA of *B. thuringiensis* and pUC18 DNA digested with *HindIII* were used for ligation reaction. The ligated mixture was then used to transfer competent *E.coli*, DH5α cells as described by Sambrook *et al* (1989). The transformed cells were then plated on LB agar plates containing Ap and X-gal (5-Bromo-4-Chloro-3-indolyl β-D-galactopyranoside). The recombinant clones were subjected to colony hybridization using both the delta endotoxin specific probes available with us. The colonies giving positive signals with both the probes when used one at a time under stringent conditions of hybridization and washing, were considered to be the positive transformants.

Larvicidal Bioassays

Larvicidal bioassays were carried out as described in Chapter II. The computer programme used to work out the Regression equation was GRAPHER^R. LC_{50} values were calculated from computer analysis. Different concentrations were used as dose in terms of mg/cm^2 of the diet. For determination of potency, Bactospeine (Duphar B.V. Weesp, Holland) with 16000 IU/mg was taken as the standard.

LC_{50} and potency (International Units) was determined as described by Dulmage (1983)

$$\text{Potency (IU)} = \frac{100 \times LC_{50} \text{ standard}}{LC_{50} \text{ test preparation}}$$

and

$$\text{Potency of sample (IU/mg)} = \frac{LC_{50} \text{ standard} \times \text{potency}}{LC_{50} \text{ test preparation.}}$$

RESULTS

Characterization of protoplast fusants for presence and expression of δ-endotoxin protein

Five of the fusants with phytopathogenic property were used for detection of *B. thuringiensis* specific insecticidal properties by DNA hybridization and immunoblot analysis. For DNA hybridization, total *B.thuringiensis* DNA, toxin probe and oligoprobe specific for *bt* gene were used. Figure 5.1 gives the results of DNA dot blot hybridization using different DNA probes. From Figure 5.1, it can be seen that the hybrid AB0242 has given positive signals with all these probes indicating the presence of *bt* gene in it. Here the 21mer oligoprobe, as demonstrated by Adang *et al.* (1985), was designed to be 100% homologous to the HD-73

FIG 5.1: DNA dot blot hybridization using

A : Total *B.thuringiensis* (NCIM 2514) DNA

B : Toxin probe

(a) 1µg (b) 5µg (c) 10µg total DNA spotted on nytran memberane

LANE 1 : *B.thuringiensis* (NCIM 2514)

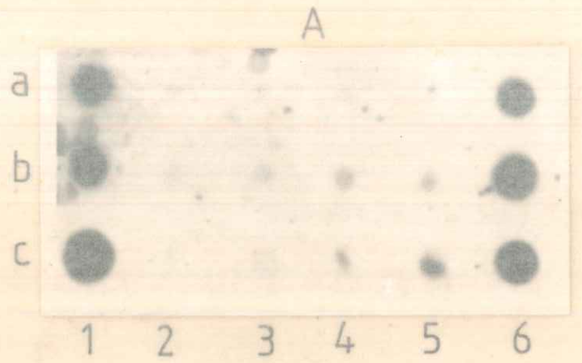
LANE 2 : *A.tumefaciens* (NCIM 2943)

LANE 3 : Protoplast fusant AB010.

LANE 4 : Protoplast fusant AB017.

LANE 5 : Protoplast fusant AB0241.

LANE 6 : Protoplast fusant AB0242



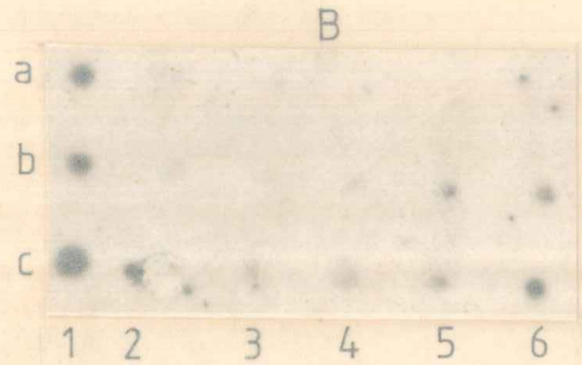


FIG 5.1

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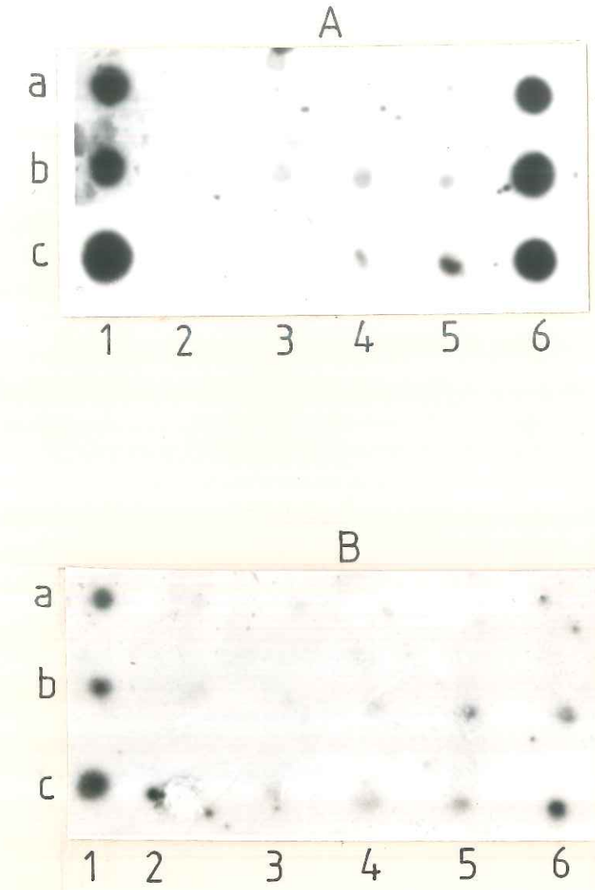

FIG 5.1

6.6kb gene. It has been shown that HD-73 is a single-gene strain, and therefore any hybridization signal coming from it can be attributed to the crystal gene itself. (Figure not shown).

The ability of the hybrids to synthesize the toxin crystal protein was assessed by using the anti-delta endotoxin antibody raised in rabbit against polyacrylamide gel purified 130kD protoxin. Figure 5.2 depicts the immunoblotting of protoplast fusants using delta endotoxin specific antibody. The positive reaction of the hybrid with the antibody suggests that the *bt* gene inherited in the hybrid has been expressed. As seen in Figure 5.2, the hybrid AB0242 is positive in the crystal protein antigen-antibody reaction which shows that the crystal protein is synthesized by this hybrid. The DNA hybridization and immunoblot analysis results thus confirm the presence of insecticidal crystal protein gene as well as its expression in AB0242.

Cloning of *bt* gene in *E.coli*

The cloning of *bt* gene was carried out from the same *B. thuringiensis* strain which was used for protoplast fusion work. Figure 5.3 describes the plasmid pattern of *B.thuringiensis* (NCIM 2514). The crude plasmid preparation was carried out as described by Kronstad *et al*. (1983). The plasmid precipitated by PEG was checked on 0.8% agarose.

FIG 5.2: Immunoblotting of protoplast fusant of *S. endotoxin*

LANE 1 : 0.5µg and

LANE 2 : 1.0µg of protein spotted on nitrocellulose membrane.

The blot was reacted sequentially with anti-δ-endotoxin and anti-rabbit IgG-alkaline phosphatase antibody at 1:500 dilution.

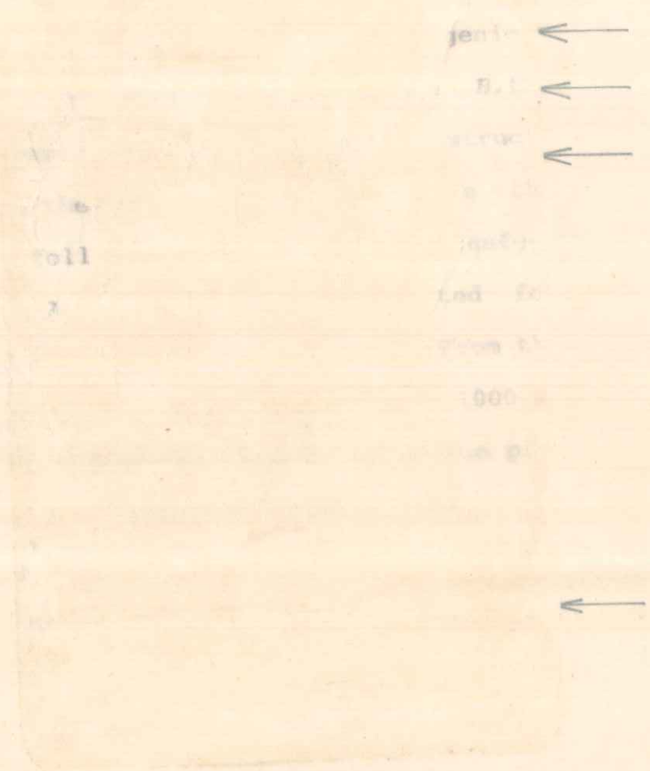

FIG. 5.2

This plasmid was further purified on CsCl-EtBr density gradient. This plasmid profile is show in Figure 5.3 (A), which was further blotted on nytran membrane as described by the procedure of Sambrook *et al.* (1989). The purified plasmid preparation of this strain indicated a number of bands. Southern hybridization of this plasmid with toxin probe revealed the presence of one band in the undigested plasmid preparation (28.8kb) and one band with the *HindIII* digest (7.2kb). To obtain a transgenic *E.coli* containing *bt* gene, a plasmid library from the *B.thuringiensis* (NCIM 2514) parental strain was constructed in pUC18 by digesting the plasmid DNA as well as the vector DNA by *HindIII* followed by ligation and transformation in *E.coli* DH5α. The Amp^r colonies were selected for β-galactosidase expression by plating on X-gal. From the mixture of the blue and white colonies, more than 1000 white colonies were picked up and transferred on LB medium plates containing Amp and X-gal. After 4-5 subcultures, 120 colonies were survived which were further screened by colony hybridization using toxin probe and *B.thuringiensis* specific oligoprobe. Colonies which gave positive signals with both the probes under more stringent conditions (1xSSC) were selected as *bt* gene transformants.

FIG 5.3: Location of sequence homologous to the toxin probe on plasmids of *B.thuringiensis* (NCIM 2514)

A : Photograph of agarose gel stained with EtBr showing plasmid profile in the preparation.

B : Autoradiograph resulting from hybridization of the ^{32}P-labelled toxin probe to blot of 0.8% agarose gel.

LANE 1 : Lambda DNA *HindIII* fragments.

LANE 2 : Undigested plasmid profile.

LANE 3 : Plasmid digested with *HindIII*

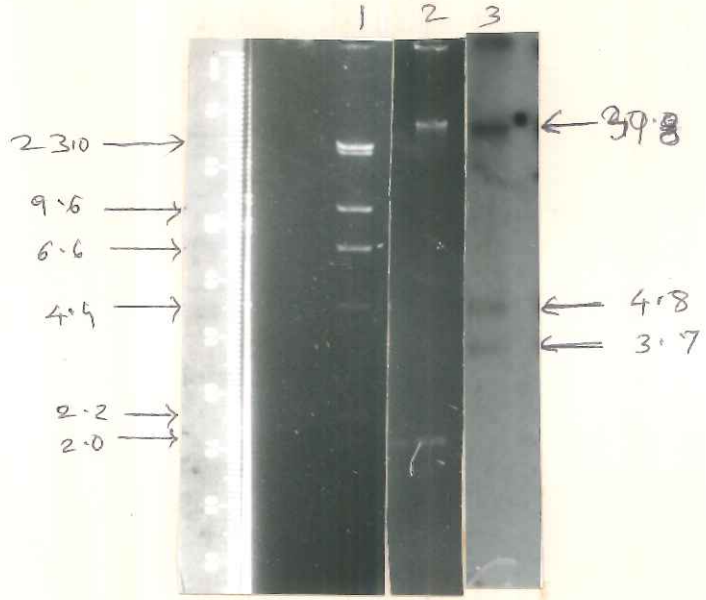
1
2
3
23.0
9.6
6.6
4.4
2.2
2.0
9.8
4.8
3.7

FIG 5.3: Location of sequence homologous to the toxin probe on plasmids of *B.thuringiensis* (NCIM 2514)

A : Photograph of agarose gel stained with EtBr showing plasmid profile in the preparation.

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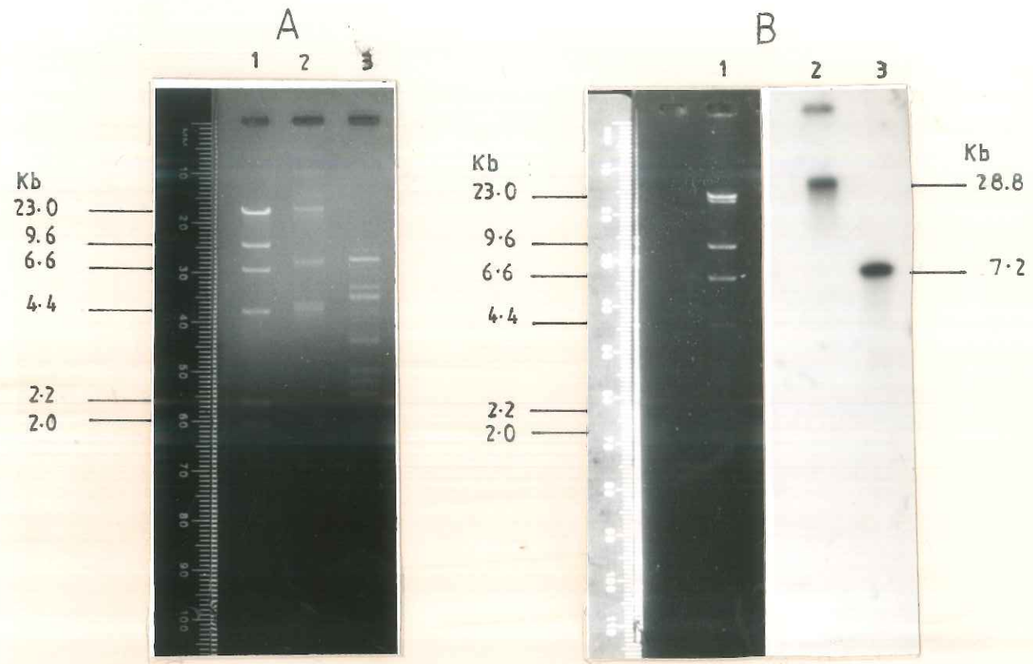

FIG. 5.3

Toxicity of protoplast fusant AB0242 and of transgenic *E.coli* containing *bt* gene pBt15

Our next objective was to compare the expression of *bt* gene in protoplast fusant and of transgenic *E.coli* containing *bt* gene as judged by insecticidal bioassay studies. *S. litura* belonging to the order Lepidoptera was used for these studies. Table 5.1 summarises the comparative data of the percent mortality, LC_{50} and potency in terms of IU against *S. litura* neonate larvae. The mortality results confirm the presence of active *bt* gene in constructs obtained by protoplast fusion as well as by gene cloning. To compare the toxicity levels of both these constructs, it is more appropriate to define the evaluation of death which is determined by LC_{50} values. Statistically, the most accurate measurement of a death response of an insect population is that concentration of toxin which will kill 50% of the insect population. For this determination, a series of concentrations were used and the percent mortality values were calculated. Figure 5.4 gives the graph of dose of the insecitide against the percent mortality. The standard formation of bactospeine with the 0.01-0.05mg/cm^2 dose, resulted in 22.8-82.5% mortality. With the protoplast fusant AB0242 and *bt* clone pBt15, 0.04-0.12mg/cm^2 does revealed 21.0-82.0% mortality.

TABLE 5.1 : Comparative toxicity of protoplast fusant (AB0242) and *bt* clone (pBt15) against neonate larvae of *Spodoptera litura*

Test sample	% Mortality $\bar{X} \pm SD$	LC_{50} µg/cm^2	Potency IU	Potency IU/mg
B.thuringiensis (NCIM 2514)	96.67 ± 12.7	-	-	-
AB0242	80.00 ± 4.00	73.0	383.5	6136
pBt15	66.67 ± 5.33	81.3	345.6	5531
Bactospeine	- -	28.5	-	16000

Then through computer analyses, the LC_{50} values were calculated. Table 5.1 gives the comparative toxicity of protoplast fusant (AB0242) and *bt* clone (pBt 15) against neonate larvae of *S.. litura*. As shown in this Table, the LC_{50} for standard was 28µg/cm^2 and was 73 and 81µg/cm^2 for AB0242 and pBt15 respectively for *S.litura* larvae. To establish the reproducibility and reliability of the bioassay, the potencies were expressed in terms of International Units (IUs). Compared to the standard bioinsecticide potency, AB0242 was 38% wherease pBt15 was 34% potent against *S. litura* larvae. The mortality results were also confirmed by feeding the neonate larvae using artificial diet as well as on castor leaf discs applied with the known concentration of the test samples (Figure 5.5). With the artificial diet as shown in Table 5.1, the percent mortality with protoplast fusant was 80%, whereas that with *bt* clone it was 66% against the test larvae.

DISCUSSION

Different *B.thuringiensis* subspecies and even strain within a single subspecies, exhibit substantial differences in insecticidal activities. For the improvement in efficacies of *B.thuringiensis* insecticides, essentially two strategies have been followed. The first one is the genetic manipulation of toxin gene to improve the

FIG 5.4: Dose of insecticide against the percent mortality values of *Spodoptera litura* by Bactospeine alongwith protoplast fusant AB0242 and clone pBt15

Bactospeine △——△

AB0242 ◇——◇

pBt15 ✳——✳

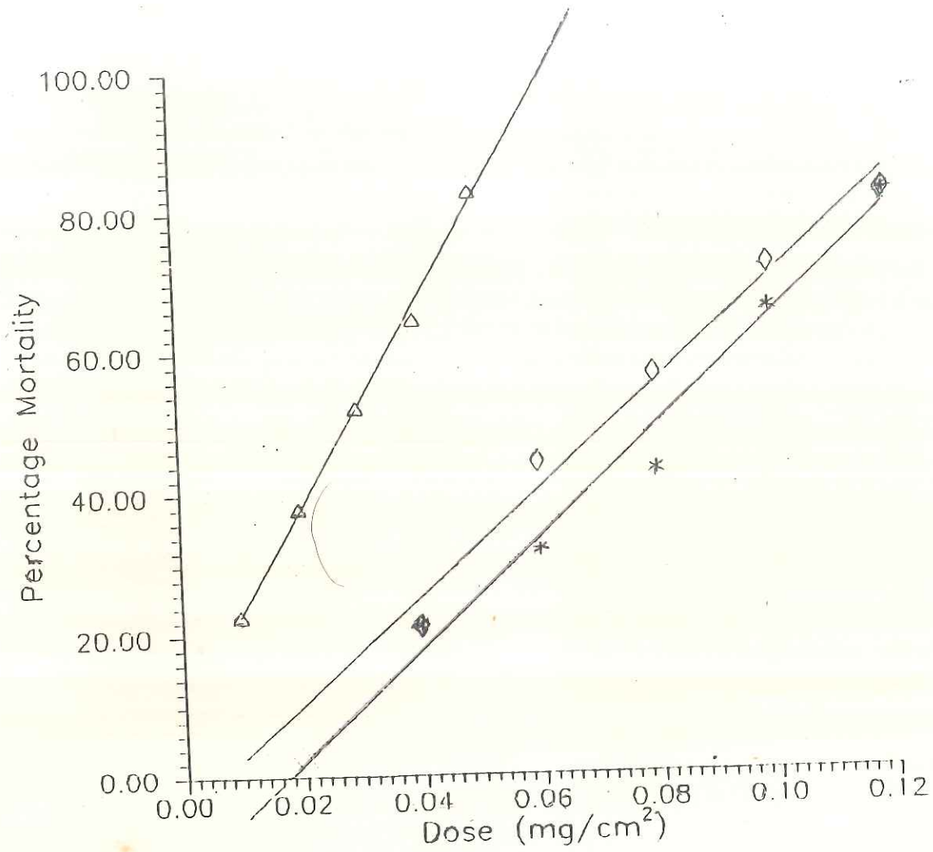

FIG 5·4

FIG 5.5: Larval toxicity test of neonate larvae of *Spodoptera litura* fed on castor leaf discs applied with :

A : Control.

B : *B.thuringiensis* (NCIM 2514).

C : Protoplast fusant AB0242.

D : Delta-endotoxin clone pBt15.

FIG 5.6: Larval toxicity tests of neonate larvae of potato tuber moth fed on clean, fresh potatoes applied with :

A : Control.

B : *B.thuringiensis* (NCIM 2514).

C : Protoplast fusant AB0242.

D : Delta-endotoxin clone pBt15.

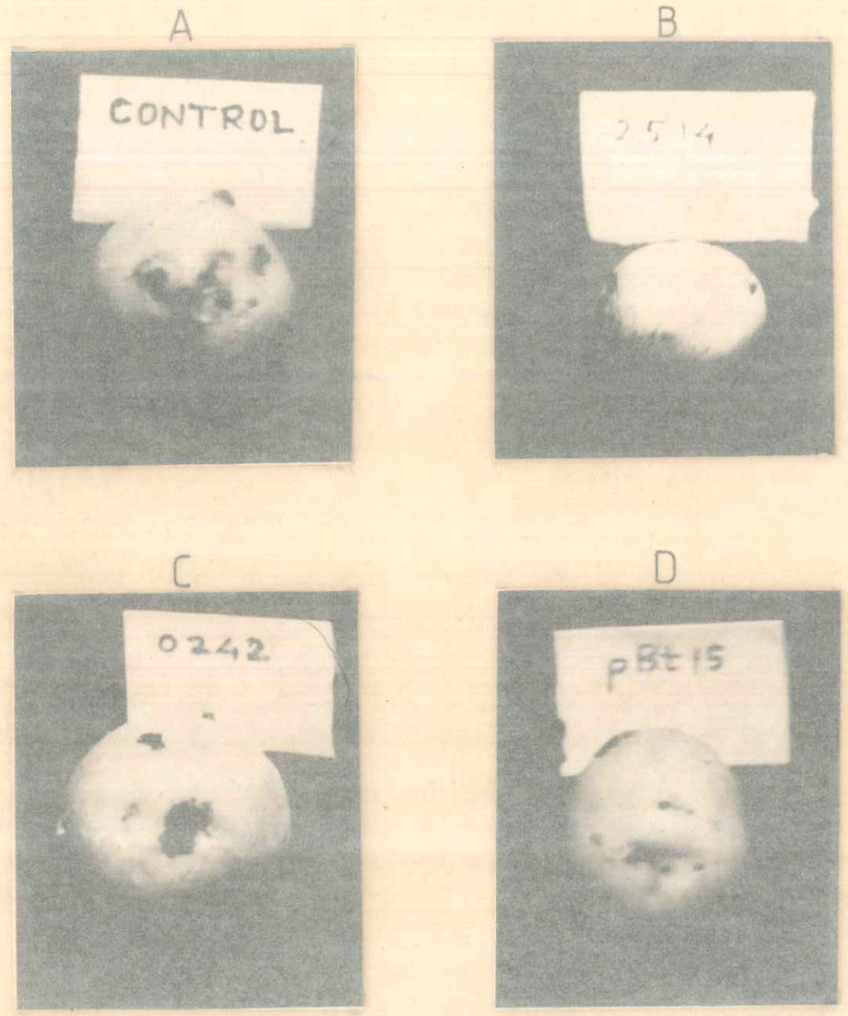

FIG. 5.6

FIG 5.6: Larval toxicity tests of neonate larvae of potato tuber moth fed on clean, fresh potatoes applied with :

A : Control.

B : *B.thuringiensis* (NCIM 2514).

C : Protoplast fusant AB0242.

D : Delta-endotoxin clone pBt15.

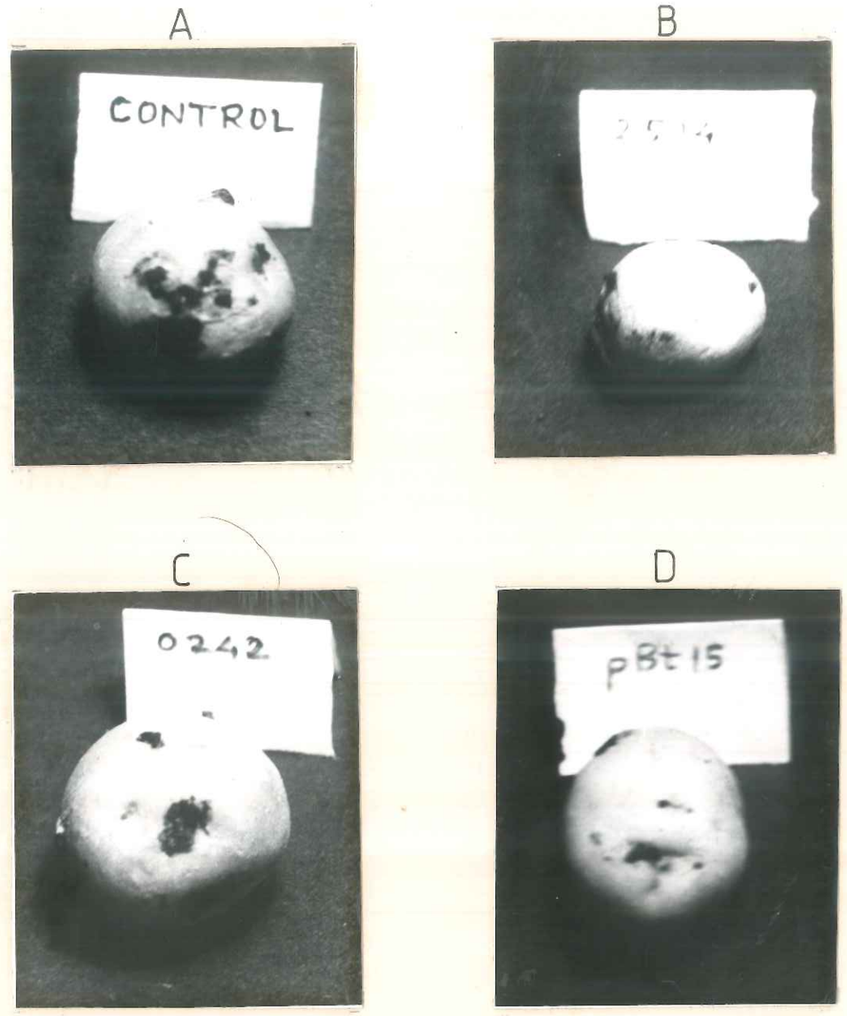

FIG. 5.6

potency and spectrum of insecticidal activity and the other is the development of improved delivery systems like the use of plant associated heterologous hosts (*Pseudomonas fluorescens*; *Clavibacter xyli*). In the present protoplast fusion study, the successful isolation of intergeneric hybrid with the insecticidal property has opened up a new dimension for the development of bioinsecticides, since during recombination some changes may take place resulting in alteration in the gene expression which leads to hyperproduction of specific gene product.

The relative expression levels of the delta-endotoxin genes in the protoplast fusant was studied by comparing its toxicity with that of the *bt* clone. *Spodoptera litura* has been considered as one of the important species of field crops of economic importance in India with indications of resistance towards conventional insecticides. The commercially available HD- 1 preparation elicits 30-50% mortality against this pest (Amonkar *et al*, 1985; and Whitlock *et al*, 1991). The neonate bioassay has been shown to be more suitable for accurate determination of potencies of *B.thuringiensis* (Navon and Klein, 1990). In the present of work, these bioassays with neonate larvae have shown mortality with *B.thuringiensis* (2514) of the order of 80% and 66% with the protoplast fusant and *bt* clone respectively. When these were tested against the

potato tuber moth, there was no drastic change or alteration in the host specificity during gene transfer (Figure 5.6). It may be concluded that the intergeneric protoplast fusion between *A.tumefaciens* and *B.thuringiensis* has resulted in creation of a novel tumor inducing insecticidal strain. It is true that this strain cannot be useful to directly transfer insect resistance into plants, as *bt* gene transfer into plant systems and its expression is a very critical process. But this work definitely serves as a model system for use of intergeneric protoplast fusion technology towards plant genetic engineering. It can also be employed to develop strains with broader spectrum of insecticidal activity by fusion of different subspecies of *B.thuringiensis* strains.

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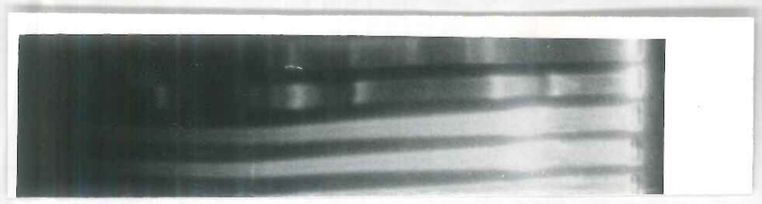

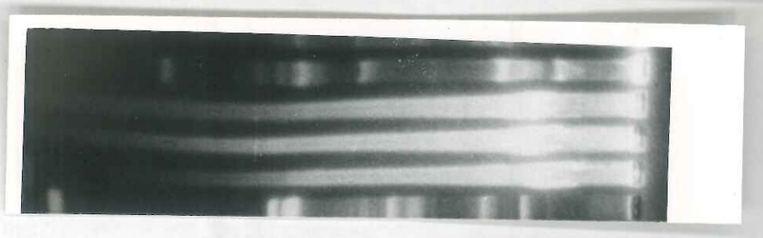

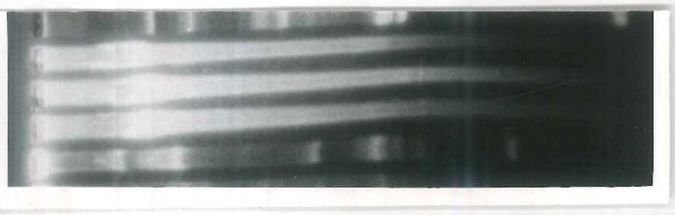

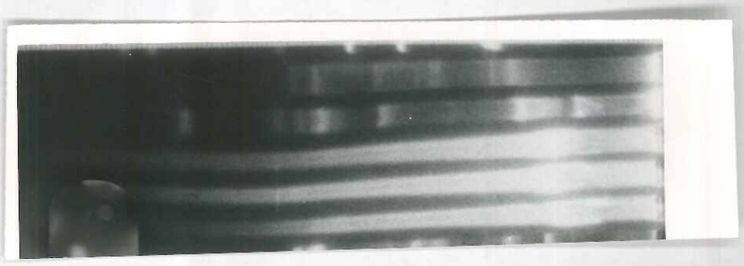

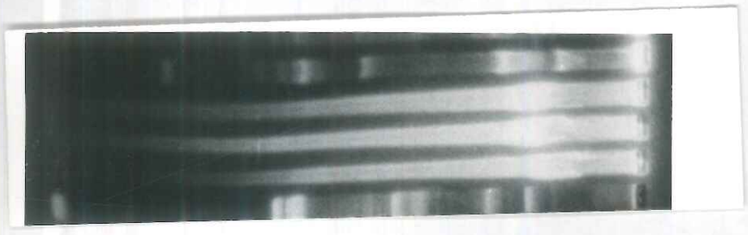

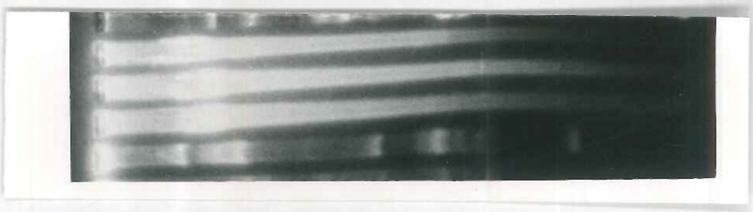

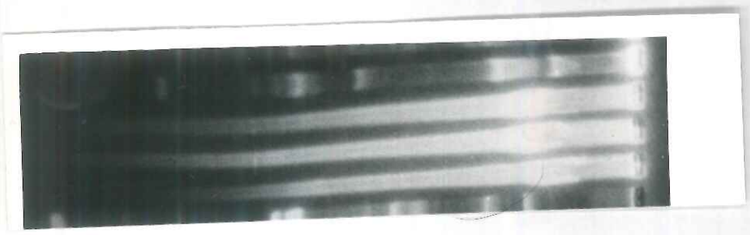

AN OVERVIEW

Successful gene transfer by protoplast fusion depends on protoplast isolation, regeneration and fusion and these conditions vary from organism to organism. Although, *A. tumefaciens* and *B. thuringiensis* are important from plant genetic engineering point of view, there were no state of art protocols developed for protoplast isolation and regeneration from both the strains when the present work was undertaken. Hence for the isolation of protoplast fusion hybrids between these two strains with the desired traits, it was necessary to optimize the different experimental conditions. It was observed that the physiological status of the cell played a pivotal role in maximum protoplast isolation and regeneration. When the cells in early exponential phase were used for protoplastization, maximum protoplasts were obtained from both the strains with increased regeneration frequency. The fusion from these protoplasts resulted in ten fold higher fusion frequencies. Isolation of intergeneric hybrids from the protoplast fusion of *A. tumefaciens* and *B.thuringiensis* suggested that genetic recombination could take place between unrelated bacteria, generating new hybrids possessing parental characters. These hybrids were stably maintained for more than four years and were found to be very stable evenafter growing them for number of generations without selection pressure. In order to establish the

nature of the hybrids, various methodologies analysing inherent phenotypic and genotypic characters of the parents were carried out.

The protoplast fusion hybrids between *A. tumefaciens* and *B. thuringiensis* were isolated at a fusion frequency of 2.0×10^{-7} using antibiotics and octopine as selection markers. Among the 23 stable hybrids, five of the hybrids were virulent towards pigeonpea legume indicating that tumor inducing capacity was transferred and expressed in these hybrids from *Agrobacterium* parent. Two of these five phytopathogenic hybrids exhibited the presence of neomycin phosphotransferase II activity (Km^r) which was again inherited from *Agrobacterium* parent.

The hybrids possessing *Agrobacterium* properties were further tested for the presence of *Bacillus* properties. This was established by detecting the desired insecticidal crystal protein gene in the above hybrids. When the hybrid DNA was probed with parental *B. thuringiensis* DNA alongwith the specific probes developed for detection of delta-endotoxin (*bt*) gene, one of the gram positive and NPTII positive phytopathogenic hybrids showed the inheritance of *bt* gene. The expression of this gene was confirmed by immunoblot reaction and insecticidal bioassays. Thus, this novel protoplast fusant was capable of producing octopine positive tumors in pigeonpea legume alongwith the capacity to produce

insecticidal crystal protein (delta-endotoxin) and it opened new dimensions in protoplast fusion technology.

Tumor formation in plants is a good indication of transfer of T-DNA into them. *Agrobacterium* on infecting the wounded site of plant transfers a segment of its Ti-plasmid (T-DNA) into plant chromosomes and transcription and translation of genes present on T-DNA cause the formation of tumors in plants. The T-DNA also contains genes responsible for opine synthesis, and the presence of these compounds is unique in case of tumorous tissue. Thus, the octopine positive tumor formation by the *A.tumefaciens* and *B.thuringiensis* hybrid is a clear indication of presence of Ti-plasmid in it. Secondly, transfer of tumor inducing characters (Ti) from *A. tumefaciens* by conventional methods was possible in case of taxonomically related *Rhizobium trifolli* (Hooykaas *et al.*, 1977) or *Phyllobacterium myrsinacearum* (van Veen *et al.*, 1988). However, introduction and maintenance of this property into more distantly related bacteria such as *E.coli* or *Pseudomonas aeruginosa* could not result in tumor inducing strain (Hille *et al.*, 1983). Isolation of tumor inducing intergeneric protoplast fusant AB0242 between *A. tumefaciens* and *B. thuringiensis* has shown that the protoplast fusion overcomes the non-compatibility barriers. The ability to produce tumors in pigeonpea by parental *A. tumefaciens*

strain alongwith the protoplast fusant has demonstrated the possibility of transforming the legume pigeonpea (*Cajanus cajan*). Legume species like alfalfa, *Medicago sativa* (Deak *et al.*, 1986; Shahin *et al.*, 1986; and Hill *et al.*, 1991), Soybean, *Glycine max* (Hinchee *et al.*, 1988; and Delzer *et al.*, 1990), and pea, *Pisum sativum* (Puonti-Kaerlas *et al.*, 1990; and De Kathen and Jacobson, 1990) have been successfully transformed using *A. tumefaciens* as a vector. However, very little progress in this regard has been made in Indian legume crop pigeonpea (Suseelan *et al.*, 1987). It is essential to improve this legume in terms of disease and pest resistance and quality of storage proteins through genetic approaches. The present work has provided some evidence that pigeonpea is amenable to transformation by *A.tumefaciens*.

During the screening of various *A. tumefaciens* strains for their transformation ability using different transfer methods, a rapid seed germinating assay was developed (tumor formation within 7-10 days). Hence it was useful for screening number of hybrids for their phytopathogenicity towards pigeonpea within short period. The cocultivation results of the pigeonpea leaf discs with non-oncogenic Ti plasmid derived vector (pGV 3850::1103) revealed the possibility of transforming pigeonpea. Here, although the regeneration of whole plants from transformed callus tissue was unsuccessful, this problem seems to be

typical in regeneration of entire legume plant *in vitro* and to my knowledge there are no reproducible regeneration systems via somatic embryogenesis or organogenesis developed in case of pigeonpea plants. The results obtained by the procedure described in the present work are of importance as they yield nontumorous callus tissue which may be useful for exploiting the possibility of transferring foreign genes into this legume.

While different *B. thuringiensis* strains exhibit differential insecticidal activities, the parental *B. thuringiensis* is more than 90% lethal towards target *Spodoptera litura* (Lepidopteran) insect which has been considered as one of the resistant pest species of the field crop of economic importance in India. At present only chemical pesticides are effective in its control. The protoplast fusant AB0242 has exhibited more than 80% mortality which has indicated that during protoplast fusion no substantial change in the activity has been observed. When the insecticidal activity of the protoplast fusant was compared with the *E.coli* constructs containing the *bt* gene no significant change in the activity or host specificity was observed.

The results and observations made in the course of the present work reported in this thesis have led to the following conclusions :

1. Intergeneric protoplast fusion in bacteria could be used as a tool to obtain stable hybrids with desirable phenotypes.

2. Genes coding for tumor induction in plants and insecticidal crystal protein could be stably transferred and also expressed in *Bacillus* type hybrid.

3. Hybrid status of the organism was confirmed through complementation of the selection markers and biochemical properties of the parental strains as well as from the DNA hybridization experiments.

4. Comparative toxicity data between the protoplast fusant and *bt* gene transformant in *E.coli* proved that protoplast fusion technique could be a substitute to recombinant DNA techniques in cell engineering by introducing desirable phenotypes in unrelated bacteria.

5. Presence of tumor inducing capacity and insecticidal character in the hybrid AB0242 suggested that the parental genomes might have recombined in the hybrids.

The results reported herein have thus opened a new possibility of engineering bacteria which are of agronomical importance and are unrelated taxonomically to complement the complex mechanisms like tumor induction and expression of crystal protein gene. In this protoplast

fusion technique, unlike the recombinant DNA technology, the recipient organism need not require to be completely compatible with the donor bacterium as long as a proper selection system exists to isolate the desirable hybrid organism.

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EFFICIENT PROTOPLAST REGENERATION OF *BACILLUS THURINGIENSIS* AND *AGROBACTERIUM TUMEFACIENS*

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SUMMARY: Treatment of *Bacillus thuringiensis* and *Agrobacterium tumefaciens* taken from the early growth phase (8 h) with lysozyme at 1 mg/ml gave 90-99% protoplast formation and 10-12% protoplast regeneration on the minimal medium in absence of plasma expander (Bovine serum albumin). Enhanced fusion frequency was obtained when protoplasts from 8 h grown cells were used for fusion experiments.

INTRODUCTION: Protoplast fusion is used to generate interspecies or intergeneric hybrids with improved properties. We have reported intergeneric protoplast fusion between *A. tumefaciens* and *B. thuringiensis* (Puntambekar and Ranjekar, 1989), leading to the formation of hybrids possessing the phenotypes from both the organisms. However, the intergeneric hybrids were obtained with low frequencies. The efficiency of fusion and appropriate phenotype transfer depend on efficient protoplast formation and regeneration and small differences in experimental conditions and techniques can have profound effects on isolation and regeneration of protoplasts. To our knowledge there have been no systematic studies on optimizing protoplast isolation and regeneration from *A. tumefaciens* and *B. thuringiensis* which are used in the production of genetically engineered insect-resistant plants and in plant genetic engineering work, respectively. In this report, we have optimized the conditions for formation and regeneration of protoplasts from both these strains and to study the effect of improved regeneration rates on protoplast fusion frequency.

MATERIALS AND METHODS: *B. thuringiensis* subsp. *kurstaki*, NCIM 2514 (Ap^rKm^s) was maintained on LB medium containing (g/l) Tryptone, 10.0; Yeast extract, 5.0 and NaCl, 10.0. *A. tumefaciens* NCIM 2943 (Ap^sKm^r) was maintained on YEB medium consisting of (g/l) Beef extract, 5.0; Yeast extract, 1.0; Peptone, 5.0; $MgSO_4.7H_2O$, 0.2 and Sucrose, 5.0.

Protoplast formation: The overnight grown cultures of these strains were inoculated in 50 ml of LB or YEB medium and incubated at 30°C with shaking. Culture samples were centrifuged and cells washed twice with 0.01 M Tris-HCl buffer (pH 8.0). The pelleted cells were finally suspended in 10 ml buffer containing 0.01 M Tris-HCl, 0.025 M EDTA and 0.6M sucrose, pH 8.0 and kept at 37°C for 30 min with gentle shaking. Filter-sterilized lysozyme (Sigma) prepared in buffer (0.01 M Tris-HCl; 0.6 M sucrose, pH 8.0) was added at 1 mg/ml concentration and the cells were incubated at 37°C with slow shaking. Protoplast formation was monitored by treating samples to osmotic shock in distilled water and counting the osmo-resistant cells. After protoplast formation, 50 mM $MgCl_2$ was added to stabilize the protoplast mixture.

Protoplast Regeneration and Fusion: Protoplast regeneration, fusion and isolation of fusants were carried as described earlier (Puntambekar and Ranjekar, 1989). The number of regenerated cells was determined by deducting the number of osmo-resistant cells from the regenerated cells on the regeneration medium. The frequency of regeneration is expressed as the ratio of number of regenerated cells to the number of protoplasts. The fusion frequency was assessed by ratio of total number of colonies appeared on minimal medium with antibiotics to the total number of fused protoplasts.

RESULTS: The effect of cell age at the time of protoplastization on the regeneration of protoplasts is shown in Table 1. The yield of protoplasts was maximum (>98%) after 90 min in *A. tumefaciens* whereas maximum protoplastization of *B. thuringiensis* cells required 3 h of incubation with lysozyme. The protoplasts isolated from cells of 8 h old cultures regenerated most efficiently on complete medium. Although, the number of colonies appeared on minimal medium was relatively low as compared to number of colonies on rich medium, there was no substantial change in the regeneration frequency of protoplasts.

Using different concentrations of lysozyme (0.1-15.0 mg/ml) on 8 h grown cells at 30°C and 37°C, maximum regeneration frequency was with 1 mg/ml lysozyme at 37°C. Though in case of *A. tumefaciens*, 99% protoplasts were obtained within 90 min, protoplasts isolated after 2 h of incubation with lysozyme gave better regeneration frequency (Fig. 1). With *B. thuringiensis*, maximum regeneration was with protoplasts isolated after 3 h of incubation with lysozyme. Prolonged treatment of lysozyme gave decreased regeneration in case of both the strains (Fig. 1).

Of a number of different osmotic stabilizers, sucrose (0.6 M) and sorbitol (0.8 M) were most suitable for regeneration of *B. thuringiensis* protoplasts. However, *A. tumefaciens* protoplasts regenerated with high frequencies when only 0.6 M sucrose was used as an osmoticum. Interestingly, the addition of plasma expander, BHI (Brain Heart Infusion) increased the regeneration frequency only in case of *A. tumefaciens*, but BSA failed to enhance the regeneration in both the strains (Table 2).

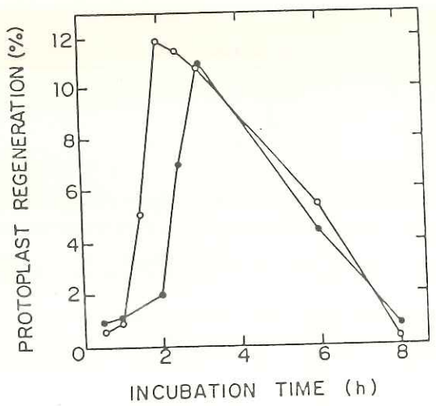

Fig. 1. Regeneration efficiency of protoplasts from A. tumefaciens (o) and B. thuringiensis (•)

8 h grown cells were protoplasted with lysozyme (1 mg/ml) at 37°C. Protoplasts obtained were plated on regeneration media

To examine the effect of the improved regeneration of protoplasts on fusion frequencies, the fusion was carried out using the protoplasts isolated from the cells at different age and the results are given in Table 3. Interestingly, the fusion frequency was enhanced (approximately ten fold) when the protoplasts derived from the 8 h grown cells were used.

DISCUSSION: We have earlier reported the protoplast fusion between *A. tumefaciens* and *B. thuringiensis* (Puntambekar and Ranjekar, 1989) with an objective to obtain intergeneric hybrids possessing the phenotype of both the strains. However, the fusion frequency was low (10^{-6} and 10^{-7}) which could be attributed to the use of protoplasts derived from 16 h grown cells for fusion. Tenmeyer (1987) has pointed out that the sensitivity of the lysozyme to the cell wall was dependent on the condition and treatment of cells during growth and harvest.. With our results, we have found that even though *B. thuringiensis* is a gram-positive organism, the mild osmotic shock given by EDTA seems to be favourable for lysozyme activity which is most commonly used in case of Gram-negative organisms (Weiss, 1976). Addition of 50 mM $MgCl_2$ was strictly necessary for protoplast viability and for enhancing the stability. It is further observed that the fusion frequency is enhanced when the protoplasts derived from early exponential phase (8 h grown cells) are used in fusion experiments. This suggests that improvement in fusion frequency necessarily requires higher regeneration of protoplasts.

Table 1: Protoplast formation and regeneration; *A. tumefaciens* and *B. thuringiensis*

Organism	Cell age h	Starting of cells (Z)	No. Osmoresistant colonies (Y)[b]	Regenerated colonies (X)[a]	Protoplast formation frequency[c] (%)	Regeneration frequency[d] (%)
A. tumefaciens	8	5.8×10^7	9.3×10^4	6.9×10^6	98.5	10.3
	12	7.4×10^9	2.8×10^6	4.2×10^8	99.2	5.6
	16	6.0×10^9	1.2×10^5	1.2×10^8	99.1	1.9
	24	3.8×10^{12}	3.0×10^6	2.1×10^{10}	99.5	0.5
B. thuringiensis	8	4.0×10^7	2.7×10^5	3.1×10^6	91.2	7.0
	12	2.4×10^8	6.6×10^5	1.4×10^7	95.0	5.5
	16	6.3×10^9	8.4×10^5	2.4×10^7	96.2	0.3
	24	2.0×10^{12}	2.4×10^7	2.8×10^8	91.4	0.0

a Regenerated colonies include colonies from regenerated cells and osmoresistant cells.
b Colonies from nonprotoplasts were derived from lysozyme treated cells which resisted lysis upon dilution in water.
c Protoplast formation frequency = X - Y / X x 100.
d Regeneration frequency = X - Y / Z x 100

Table 2 : Effect of different osomotic stabilizers on regeneration of protoplasts

Protoplasts obtained as described in text were allowed to regenerate on the regeneration media supplemented with different osmostabilizers.

Stabilizers	Regeneration Frequency (%)	
	A.tumefaciens	*B.thuringiensis*
0.6 M KCl	0.0	0.1
0.5 M NaCl	0.0	0.4
0.5 succinate	0.1	0.2
0.5 Sucrose	8.0	7.0
0.6 M Sucrose	10.3	7.2
0.8 M Sorbitol	0.0	6.9
0.4 M Mannitol	0.0	1.0
2.0 % Brain Heart Infusion	14.1	3.0
0.5 % Gelatin	0.6	0.0
1.0 % BSA	8.5	7.0

Table 3 : Fusion frequencies between the protoplast of *A.tumefaciens* (Km^r Ap^s) and *B.thuringiensis* (Km^s Ap^r) isolated from different age of cells.

Age of protoplasted cells (h)	Total No of fused protoplasts	Total No of* regenerated fusants	Fusion frequency (%)
8	1.3×10^8	2482	1.9×10^{-5}
16	6.9×10^8	1782	2.5×10^{-6}
24	2.1×10^8	149	7.1×10^{-7}

*Regenerated colonies isolated on minimal medium with ampicillin and kanamycin (50 µg/ml each).

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INTERGENERIC PROTOPLAST FUSION BETWEEN AGROBACTERIUM TUMEFACIENS AND BACILLUS THURINGIENSIS subsp.KURSTAKI

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SUMMARY : Intergeneric protoplast fusion between A. tumefaciens and B. thuringiensis was performed. The fusants exhibited some properties of both the parental strains. One of the Gram positive fusants with most of the Bacillus properties showed tumor inducing capacity in pigeonpea (Cajanas cajan).

INTRODUCTION

In recent years, the protoplast fusion technique has aroused an interest as a means of producing intergeneric hybrids (Prakash and Cummings, 1988). In our laboratory, a successful transfer of cellulase gene from Cellulomonas sp. to Bacillus subtilis has been carried out (Gokhale et al., 1984). Using the same experimental approach, we have fused protoplasts of A. tumefaciens and B. thuringiensis and have characterized a few hybrids in relation to their phenotypic characters and phytopathogenic property towards pigeonpea. The immediate objective was to establish the feasibility of protoplast fusion between a Gram negative A. tumefaciens and a Gram positive B. thuringiensis. Since both the strains are important from the plant genetic engineering point of view, the present work will form a base for further work in this direction.

MATERIALS AND METHODS

A. tumefaciens NCIM 2943 (Km^r Ap^s Octopine type Ti plasmid)

and B. thringiensis subsp. kurstaki NCIM 2514 (Km^S Ap^r) were used. Lysozyme, octopine and kanamycin were from Sigma Chemical Co., U.S.A. All other reagents were of analytical grade.

A. tumefaciens and B. thuringiensis were maintained on YEB and LB slopes respectively. YEB medium contained (g/l) beef extract, 5.0; yeast extract, 1.0; sucrose, 5.0; and $MgSO_4 . 7H_2O$, 0.2. LB medium contained (g/l) Tryptone 10.0; Yeast extract 5.0 and NaCl 10.0. The selection medium for the isolation of hybrids were (i) SM medium containing (g/l) $MgSO_4 . 7H_2O$, 0.3; $MnSO_4 . 2H_2O$, 0.05; $CaCl_2 . 2H_2O$, 0.08; $ZnSO_4 . 7H_2O$, 0.005; $CuSO_4 . 5H_2O$, 0.005; $FeSO_4 . 7H_2O$, 0.005; K_2HPO_4, 0.5 (with 0.1% $(NH_4)_2SO_4$ and 0.5% glucose) and (ii) SMNO medium containing SM salts with 0.01% octopine. Both the media were supplemted with 50 µg/ml of filter sterilized ampicillin (Ap) and kanamycin(Km) after autoclaving.

Protoplast formation, regeneration and fusion

Protoplasts were isolated according to Weiss(1976) with some modification. A. tumefaciens and B. thuringiensis were grown for 16 h in 10 ml of YEB and LB broth respectively. Cells were centrifuged, washed and resuspended in TES buffer(Tris-HCl, 0.01M; Na-EDTA, 0.025M; sucrose, 0.6M at pH 8.0) containing lysozyme(1 mg/ml). The suspension was incubated at 37°C for 3 h with frequent shaking. After adding 0.05M $MgCl_2$, protoplast formation was confirmed by microscopic observation as well as by osmotic shock. Protoplasts of Agrobacterium and Bacillus were regenerated on YEB and LB agar plates respectively containing 0.6M sucrose and 0.8% agar. Identical numbers of protoplasts from each strain were gently mixed and fused in presence of 30% polyethylene glycol 6000(wt/vol) for 3 min and washed with TES buffer. The fused protoplasts were mixed in SM medium with 0.6M sucrose and 0.8% agar, and poured in the plates which were incubated at 30°C for 10 to 15 days.

Phenotypic and phytopathogenic characterization of fusants

The colonies isolated on SM medium were transferred on the same medium containing 1.5% agar and were further subcultured on SMNO medium for more than 10 transfers. The hybrids were

purified and checked for Gram reaction. To check heat resistance(HR), fusants were grown for 24 h in LB broth heated at 70°C for 15 min and then streaked on agar plates. Penicillinase activity (Pen)was determined according to Slavnova et al(1986). Lactose to 3-ketolactose(keto) conversion and ferric ammonium citrate(FAC) pellicle formation tests were carried out as described by Bernaerts and Delay(1963) and Kersters et al(1973) respectively.

Pigeonpea seeds(T-21 variety) were surface sterilized by washing with 70% ethanol and then with 0.01% mercuric chloride solution followed by thorough washings with sterile distilled water. They were soaked overnight in distilled water and then germinated for 72 h at 28°C under dark condition. The germinated seedlings were infected at embryonic region with a sterile needle dipped in the agar culture and then placed in MS agar medium(Murashige and Skoog, 1962) containing Claforan(Hoechst) at a concentration of 500 µg/ml and incubated at 28°C under 16: 8 h photoperiod.

Opine formation was detected according to the method described by Otten and Schilperoort(1978) with some modifications.

RESULTS

Protoplast formation, regeneration and fusion

Under the conditions used, almost 99% of the Agrobacterium cells and 90% of Bacillus cells were converted to protoplasts. Regeneration frequencies of protoplasts of parental strains were 2% for A. tumefaciens and 0.3% for B. thuringiensis. The colonies grown on SM medium were further transferred on SMNO medium containing ampicillin, kanamycin and octopine as a sole carbon and nitrogen source. These fusion products were designated as AB 001, AB 002 and so on. The hybrid nature of fusants was confirmed since all the hybrids grew well on the medium containing antibiotics and octopine for more than 10 transfers. Even after plating 10^{10} cells of each parental strain, no colony was observed on the selection medium ruling out the possibility of spontaneous mutation in both the parental strains. Independent complementation frequencies for

antibiotic and octopine markers in the fusion products are given in Table 1.

Table 1: Fusion frequency on selection medium

Markers	Total number of colonies	Fusion frequency. Total number of fused protoplasts 1.5×10^8
Ap^r Km^r	792	5.3×10^{-6}
Ap^r Km^r Oct^+	30	2.0×10^{-7}

Phenotypic and phytopathogenic characterization of fusants

All the hybrids were tested for a few morphological and biochemical characters of both the parental strains. Table 2 summarizes these properties and it is clear that only one Gram negative hybrid(AB 0171) showed all the tested properties of Bacillus and Agrobacterium. To test tumor inducing capacity, hybrids were inoculated to 72 h germinating seedlings of pigeonpea. Visible tumors of 2-3 mm size were observed after 15-20 days only in case of four hybrids which is a property of Agrobacterium. These tumors were found to be octopine positive(Fig 1). The hybrid AB 0242 appeared to be very interesting, as it was sporulating, had the Bacillus type colony morphology and revealed the capacity to induce octopine positive tumor in pigeonpea seedling.

DISCUSSION

The successful intergeneric protoplast fusion between A. tumefaciens and B. thuringiensis is the first report to our knowledge. Both the strains are very different in taxonomy, physiology and morphology. A. tumefaciens is a Gram negative, tumorogenic, octopine degrading bacterium while B. thuringiensis is a Gram positive, sporulating bacterium with penicillinase activity. In our work, the fusants have shown mixed phenotypic and biochemical characters of both the parents. Agrobacteria are known to form tumors in plants which synthesize specific opines that are not present in

Table 2 : Phenotypic characterization of fusants between A. tumefaciens ($Km^rAP^sOct^+$) and B. thuringiensis ($Km^sAp^rOct^-$)

Fusants	Morphological and Biochemical characters
A. tumefaciens	Gram negative, HR^-, Pen^-, $Keto^+$, FAC^+, $tumor^+$
B. thuringiensis	Gram positive, sporulation, HR^+, Pen^+, $Keto^-$, FAC^-, $tumor^-$
	Gram positive rods
AB042	HR^+, Pen^-, $Keto^-$, FAC^-, $tumor^-$
AB0102	HR^+, Pen^-, $Keto^-$, FAC^-, $tumor^+$
AB0242	sporulation, HR^+, Pen^+, $Keto^-$, FAC^-, $tumor^+$
AB0101, AB0121, AB0151	HR^+, Pen^-, $Keto^+$, FAC^+, $tumor^-$
AB017	HR^+, Pen^+, $Keto^-$, FAC^-, $tumor^-$
	Gram negative rods
AB0171	HR^+, Pen^+, $Keto^+$, FAC^+, $tumor^+$
AB0011	HR^+, Pen^+, $Keto^+$, FAC^+, $tumor^-$
AB0241	HR^+, Pen^+, $Keto^-$, FAC^-, $tumor^+$
AB022	HR^-, Pen^+, $Keto^+$, FAC^+, $tumor^-$
AB010	HR^+, Pen^-, $Keto^-$, FAC^+, $tumor^+$
AB001, AB0151, AB018, AB019	HR^+, Pen^-, $Keto^+$, FAC^+, $tumor^-$
AB002, AB004, AB006, AB011, AB012, AB015, AB021	HR^-, Pen^-, $Keto^+$, FAC^+, $tumor^-$

other plant tissue and are used by them as a sole source of carbon and nitrogen. All the hybrids have been selected on octopine and four of them have induced octopine positive tumors in pigeonpea. The successful isolation of recombinants using these two strains supports the usefulness of protoplast fusion technique in producing intergeneric recombinant bacteria. Work is now in progress in obtaining Agrobacterium strains with insecticidal activity from Bacillus thuringiensis.

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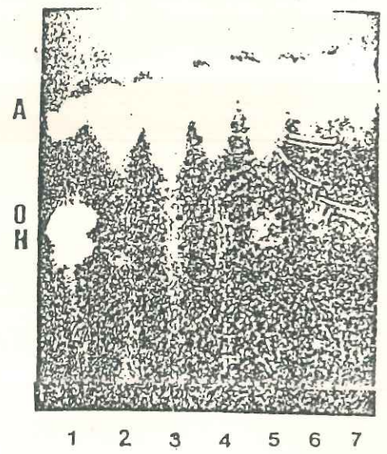

Fig.1. Opine assay of tumors induced by hybrids and A. tumefaciens.
1. Standard : A- Arginine
O- Octopine
N- Nopaline
2. Hybrid AB 010
3. Hybrid AB 0102
4. Control pigeonpea tissue
5. Hybrid AB 0171
6. Hybrid AB 0242
7. A. tumefaciens NCIM 2943