STRAIN IMPROVEMENT OF ENTOMOPATHOGENIC FUNGAL SPECIES *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* BY PROTOPLAST FUSION

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ABSTRACT: Protease hyper producing recombinant strains were produced by inter-generic protoplast fusion of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. β-glucuronidase and KCl were used as lysing enzyme and osmotic stabilizer. Along with inter-generic fusion, intra-strain and inter-strain fusions were also carried out using polyethylene glycol (PEG) as fusogen. When the fused protoplasts were regenerated on Czapekdox agar medium, they exhibited fast mycelial growth and abundant sporulation when compared to non-fusants. Pr1 and Pr2 specific activities were found to be increased by two- fold in recombinant strains than the non-fusants and the parental strains.

Key Words: Entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, Protoplast isolation, Protoplast fusion.

INTRODUCTION

Entomopathogenic fungi exist as ubiquitous saprophyte in soil and often cause widespread epizootics, wiping out insect pest populations on agricultural crops. Conidia are the infective propagules and it is generally assumed that high humidity and optimum temperature are required for the fungus to germinate. Conidia upon contact adhere to insect cuticle under favourable conditions and germinate. The germ tubes secrete extracellular enzymes which breach the insect cuticle made of 75-80 % protein (Pekrul and Grula 1979). Proteinaceous outer integument of insect forms an effective barrier and proteases are the virulent factors that play a key role in degradation (Clarkson and Charnley 1996). Proteases from *B.bassiana* and *M.anisopliae* are capable of degrading wide variety of cuticle proteins. Serine proteases Pr1 and Pr2 have been identified in *B.bassiana* and *M.anisopliae* (St.Leger 1987). Pr1 belong to subtilisin like serine protease and Pr2 is a trypsin like serine protease (St.Leger 1995). Like many fungi *B.bassiana* and *M.anisopliae* lack conventional sexual cycle as they are asexual forms and rely entirely on parasexual recombination (Paccola and Azevedo 1991). As in meiosis, segregation and crossing over of chromosomes occur in parasexual recombination but independent of one another.

Fungal protoplast fusion has been established as a means to transfer genetic material and provides an effective method for genetic manipulation and strain improvement (Stasz 1998). High virulent hybrid strains of entomopathogenic fungi were obtained by certain researchers (Couteaudier et.al 1997). Successful fusion of protoplasts in *Trichoderma* and *Aspergillus has* been well demonstrated (Stasz et.al 1988). Present study aims at strain improvement in entomopathogenic fungi which involves isolation and fusion of protoplasts from two different genera and performing inter-strain, intra-strain and inter-generic fusions through parasexual recombination.

MATERIALS AND METHODS

Fungal cultures

Beauveria bassiana strains UB1 (ARSEF 1788) isolated from *Helicoverpa virescens* in Spain is low extracellular protease producer and UB9 (ARSEF 2033) isolated from *Cocinella* spp. in U.S.A is high extracellular protease producer, *Metarhizium anisopliae* strains UM6 (ARSEF 2596) isolated from *Pyrausta machaeralis* in India is high extracellular protease producer and UM10 (ARSEF 3295) isolated from *Anticarsia gemmatalis* in Mexico is a low extracellular protease producing these strains were grown in Sabouraud's Dextrose [SD] broth (Dextrose-4 gm, Yeast extract-1 gm, Peptone-1 gm, distilled water-100 ml) and incubated in a shaker for 40 hour at 28^0C.

Isolation of protoplasts

After 48 hour of incubation mycelium was harvested and centrifuged for 10 min at 8000 g and 4ºC. The mycelial pellet was washed twice with wash buffer and 1ml of lytic enzyme for 50 mg of mycelium was added, incubated in a shaker at 28ºC and 180 rpm for 3 hour. The protoplasts were separated from mycelial debris by filtration through sterile 30 µm nylon muslin and finally protoplasts were collected by centrifugation at 4000 g for 10 min and the pellet was dissolved in 200 µl of 0.7 M KCl.

Fusion of protoplasts

Intra-strain, inter-strain and inter-generic protoplast fusions were carried out by mixing $1x10^7$/ml protoplasts of each strain from *B. bassiana* (UB1 & UB9) and *M. anisopliae* (UM6 & UM10) by centrifugation. The pellet was incubated in 1 ml of 20% PEG 6000 containing 0.01 M $CaCl_2$ and 0.05 M Glycine (pH 7.5) for 30 min at 28°C. Parental protoplasts were subjected to fusion separately to same PEG treatment.

Regeneration of fusants

The PEG in the fusion mixture was washed away twice using wash buffer. Fused protoplasts each strain by centrifugation at 5000 g for 10 min and were suspended in 0.6 M potassium phosphate buffer.

Protoplasts were plated on Czapekdox agar (5 gm Glucose, 0.1 gm KH_2PO_4, 0.2 gm $NaNO_3$, 0.025 gm $MgSO_4.7H_2O$, 0.001 gm $FeSO_4.7H_2O$, 5.22 gm KCl, 2 gm Agar, 100 ml DW) for regeneration process. Agar plates were incubated for 3-4 days at 28^0C. 100 µl of the suspension was taken for microscopic observation and photographs were taken using trinocular microscope. After 3-4 days of incubation appearance of colonies was observed and regeneration frequency was determined by using the formula:

$$\text{Regeneration frequency} = \frac{\text{Number of colonies regenerated}}{\text{Number of protoplasts inoculated}} \times 100$$

Sub culturing of regenerated protoplasts

Fast growing colonies from agar plates were selected randomly and sub cultured by streaking on Sabouraud's Dextrose [SD] agar slants (4 gm Dextrose, 1 gm Yeast extract, 1 gm Peptone, 2 gm Agar, 100 ml DW) and incubated at 28^0C for 2-3 days under sterile conditions. Fully grown colonies were used for the production of protease and tested for its enzymatic activity.

Cultivation of entomopathogenic fungi for protease production

The fungal colonies from regenerated slants were inoculated into MM amended with casein and incubated for 72 hour at 28^0C. The culture was centrifuged for 10 min at 4^0C and 8000 g. Supernatant was taken as crude enzyme and stored at 4^0C for further assay.

Proteolytic assay

Casein substrate (400 µl) was added to 200 µl of crude enzyme. Later 200 µl of 0.01 M Tris HCl (pH 8.0) was added to the mixture, incubated at 37^0C for 10 min and 100 µl of 1.2 M TCA was added to stop the reaction. Blank was prepared by adding TCA just before the addition of enzyme and absorbance of all the solutions were measured at 280 nm.

Assay of Pr1 & Pr2

Two synthetic substrates mainly N-Succinyl – Alanine prophepanilide and N-α- Benzoyl-DL- Arginine p – nitroanilide were used for the assay of Pr1 & Pr2. The above mentioned synthetic substrates (0.05 ml) were added to 0.85 ml of 15 mM Tris HCl (pH 8.5) and 0.1 ml of crude enzyme from each strain, mixed well and incubated for 1 hour at 28^0C.

Reaction was terminated by adding 250 µl of 30% acetic acid and allowing it to stand for 15 min on ice and centrifugation at 1250 g for 5 min at 4^0C. The absorbance of the supernatant was measured at 410 nm.

RESULTS

Isolation and fusion of protoplasts

Incubation of *Beauveria bassiana* and *Metarhizium anisopliae* mycelia with lysing enzyme resulted in lysis of cell wall and release of protoplasts. Swelling and rounding up of cell contents were observed initially and subsequently the mycelium started lysing after 2 hours. Almost complete digestion of mycelia and release of protoplast occurred prominently after 3hours of incubation (Figure. 1). The protoplasts just released out of mycelium were smaller in size but later they slowly enlarged to a spherical structure. The concentration of released protoplasts in all the fusions is shown in Table 1.

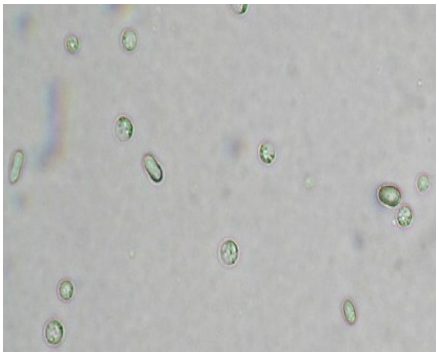

Figure. 1 Protoplast released after 3 hours enzymatic treatment.

Table 1 Concentration of protoplasts and regeneration frequencies in intra-specific and inter-specific fusions of *Metarhizium anisopliae* and *B. bassiana* isolates and inter-generic fusion of *M. anisopliae* and *B. bassiana*

Fusion strains	Concentration of protoplasts	Regeneration Without PEG	Frequency With PEG
M. anisopliae			
UM10 x UM10	1.80 X 10^5 / ml	1.13 %	1.00 %
UM6 x UM6	2.80 X 10^5 / ml	0.92 %	0.89 %
UM10 x UM6	2.00 X 10^5 / ml	0.77%	0.98 %
B. bassiana			
UB1 x UB1	1.35 X 10^5 / ml	1.06 %	1.02 %
UB9 x UB9	1.15 X 10^5 / ml	0.98 %	0.79 %
UB1 x UB9	1.50 X 10^5 / ml	0.85 %	0.94 %
Intergeneric fusion between *M. anisopliae* and *B. bassiana*			
UM6 x UB9	2.00 X 10^5 / ml	0.92 %	1.08 %

Fusion of protoplasts

When the protoplasts were mixed with PEG solution, they stuck together and pairs of protoplasts could be observed. Later the plasma membranes at place of contact in both the protoplasts dissolved and fusion of protoplasmic contents took place (Figure. 2). Subsequently the nuclei of the pairing protoplasts fused together (karyogamy) in many cases and in some cases, dikaryotic stage without nuclear fusion was observed. Finally, the fused protoplasts became single, larger and round or oval shaped structures.

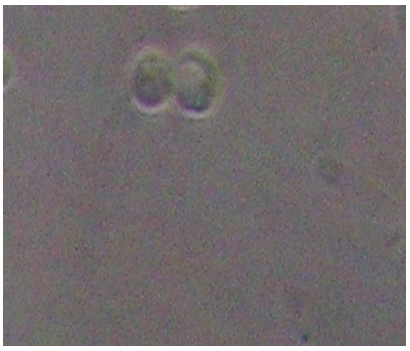

Figure. 2 Protoplast fusion after treatment with PEG for 30 min

Regeneration of fused and non-fused protoplasts

All the fusants of *Beauveria bassiana* and *Metarhizium anisopliae* exhibited luxuriant mycelial growth and profuse sporulation than the non-fusants (Figure. 3). Growth of fusants was observed within 2-3 days but for non-fusants it took up to 4-5 days. Prominent characteristic morphological features were observed among the fusants and non-fusants. For *Beauveria bassiana* white coloured mycelium and spores were observed (Figure. 4). While *Metarhizium anisopliae* initially showed white mycelium and up on sporulation green colouration was observed (Figure. 5). The regeneration frequency was high for inter-generic (1.08%) than inter-strain (0.94%) and intra-generic (0.84%) protoplast fusion. (Table -1).

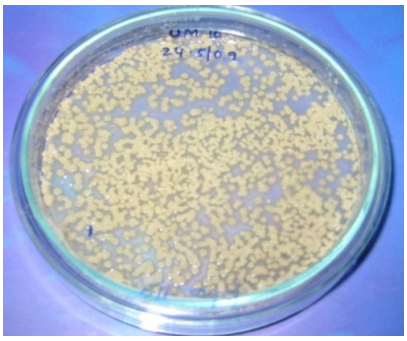

Figure. 3 Colonies of regenerated protoplasts showing white sporulation (UM10)

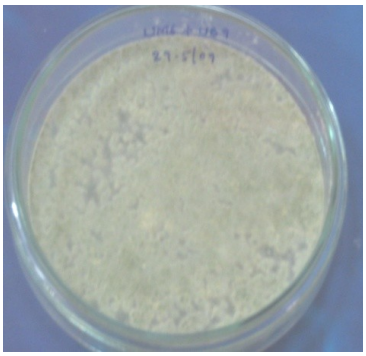

Figure. 4 Colonies of regenerated protoplasts showing greenish white sporulation (UM6 x UB9)

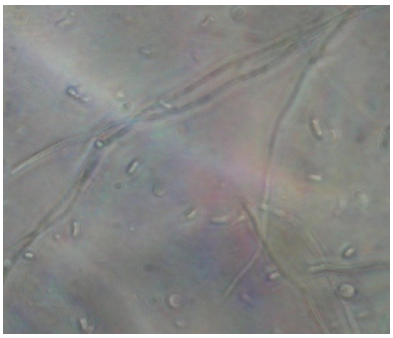

Figure. 5 Microscopic view of mycelia from regenerated protoplasts

Subculturing

On subculturing, the fusants exhibited fast mycelial growth than the non fusants and parental strains. For the fused strains of UB1 x UB9 white mycelium and upon sporulation white spores were observed where as for fused strains of UM6 x UM10 white mycelium and upon sporulation green coloured spores were observed. But for inter-generic fusion (UM6 x UB9) white mycelium and upon sporulation greenish white coloured spores were observed.

The subcultured fusants exhibited fast mycelial growth than non fusants and parental strains. The fusants of UB1 x UB9 exhibited white mycelial growth as well as sporulation where as fusants of UM6 x UM10 showed white mycelium and green sporulation. On the other hand white mycelial growth and greenish as well as whitish sporulation was observed in inter generic fusants of UM6 x UB9.

Enzymatic activity

Pr1 activity in intra-specific fusions of UM10, UM6, UB1, UB9 were 0.752 3.861, 3.231 and 3.434 units/ ml respectively. Pr2 activity in for intra-specific fusion of UM10, UM6, UB1, and UB9 were 0.486, 1.981, 0.362, and 0.422 units/ ml respectively. Pr1 activity in inter-strain fusions of UM10 x UM6, and UB1 x UB9 were 3.983, 3.719 units/ ml respectively. Pr2 activities in inter-strain fusion of UM10 x UM6, UB x UB9 were 2.045, 0.465 units/ ml. Pr1 and Pr2 activities in inter generic fusions of UM6 x UB9 were 5.965, 5.206 units/ ml (Table 2). Similarly the specific activity for Pr1 in intra-specific fusions of UM10, UM6, UB1, and UB9 were 2.622, 0.072, 0.023 and 0.034 mg/ ml respectively. Pr2 activity for intra-specific fusion of UM10, UM6, UB1, and UB9 were 2.081, 0.043, 2.073 and 3.750 mg/ ml respectively. Pr1 activity for inter-specific fusion of UM10 x UM6, UB1 x UB9 were 0.082 and 0.029 mg/ ml. Pr2 activities for inter-specific fusion in UM10 x UM6, UB1 x UB9 were 0.065 and 2.948 mg/ ml respectively. Pr1 and Pr2 activity for inter generic fusions in UM6 x UB9 were 3.445 and 2.817 mg /ml respectively (Table - 2). From the above data it can be inferred that the protease activity and its specific activity of the inter-generic fusion has increased by two-fold.

Table - 2 Enzymatic assay in intra-specific and inter-specific fusions in *Metarhizium anisopliae* and *B. bassiana* isolates and inter-generic fusion in *M. anisopliae* and *B. bassiana*

Strains	Pr1 activity units/ml	Specific Activity units/mg	Pr2 activity units/ml	Specific Activity units/mg
M. anisopliae				
UM10 (Wild-type)	0.60 ± 0.01	3.20 ± 0.03	0.42 ± 0.01	1.80 ± 0.01
UM10 x UM10	0.75 ± 0.07	2.62 ± 0.02	0.48 ± 0.06	2.08 ± 0.09
UM6 (Wild-type)	1.66 ± 0.02	0.03 ± 0.08	1.64 ± 0.01	0.03 ± 0.03
UM6 x UM6	3.86 ± 0.01	0.07 ± 0.06	1.91 ± 0.01	0.04 ± 0.07
UM10 x UM6	3.98 ± 0.05	0.08 ± 0.02	2.04 ± 0.07	0.06 ± 0.02
B. bassiana				
UB1 (Wild-type)	3.15 ± 0.01	0.02 ± 0.00	0.26 ± 0.02	1.92 ± .003
UB1 x UB1	3.23 ± 0.06	0.02 ± 0.08	0.36 ± 0.05	2.07 ± 0.06
UB9 (Wild-type)	3.33 ± 0.01	0.03±0.03	0.38 ± 0.01	2.58 ± 0.03
UB9 x UB9	3.43 ±0.01	0.03 ± 0.03	0.42 ± 0.04	3.75 ± 0.01
UB1 x UB9	3.71 ± 0.04	0.02 ± 0.05	0.46 ± 0.07	2.94 ± 0.09
Inter-generic fusion between *M .anisopliae* and *B. bassiana*				
UM6 x UB9	5.96 ± 0.01	3.44 ± 0.05	5.20 ± 0.05	1.81 ± 0.03

DISCUSSION

Protoplast fusion is an effective tool for bringing genetic recombination and developing superior hybrid strains in filamentous fungi (Stasz 1988). Protoplasts are typically released from germ tubes or hyphal cells with the aid of cell wall, degrading enzymes in the presence of osmoticants. Different factors for efficient protoplast release in filamentous fungi are: culture media, age of the mycelium, incubation time, different enzymatic combinations and osmotic stabilizers. Commercially available multienzyme preparation Novozym 234 has been used successfully with many fungi (Stanz 1989). Mycelium harvested after 40 hours incubation in Sabouraud's dextrose broth showed the best protoplast yield. An incubation time of 3 hours and lysing enzyme (1% w/v β-Glucuronidase) at a concentration of 10 mg/ ml was the best among different enzymatic concentrations which yielded a better protoplast release and the most suitable osmotic stabilizer for efficient protoplast release was 0.7 M KCl. In *Trichoderma,* some strains required treatment of growing hyphae with glucose analog 2-deoxyglucose (Zonneveld 1973) for adequate production of protoplasts with Novozym 234 (Stasz 1989). In the present study intra-specific, inter-specific and inter-generic fusion of protoplast from the strains UM6, UM10 and UB1, UB9, resulted in enhanced production of protease. The release of protoplast was significantly affected by the concentrations of lysing enzymes. At low concentration lysis of fungal mycelium took place only at the tip portion resulting in minimum release of protoplast whereas at high enzyme concentration protoplast burst immediately after release and disintegrated. Among the different concentrations tested, 5mg /ml with 0.7M KCl as osmotic stabilizer released higher number of protoplasts. However, Pe'er and Chet (1990) obtained higher number of protoplasts from *Trichoderma harzianum* using Novozym 234 at 10mg /ml with 0.6 M KCl while Tschen and Li (1997) employed 15mg/ml of Novozym with 0.6 M sucrose to isolate maximum number of protoplasts from *T. harzianium* and *T. koningii.* Fusion of protoplasts was readily accomplished by the addition of PEG and intra-specific, inter-specific, inter-generic protoplast fusions were achieved by using 20% PEG (6000) in 0.01 M $CaCl_2$ and 0.05 M Glycine and pH 7.5. However, Pe'er and Chet (1990) used 33% PEG for inter-specific protoplast fusion in *T.harzianium* and Anne and Peberdy used PEG in the presence of 10-50 mM calcium which induced the formation of small to large aggregates from 2 - 100 protoplasts. The concentration of PEG plays a critical in effective fusion of isolated protoplasts. Higher concentrations of PEG caused shrinking and bursting of protoplasts (Lalithakumari 2000) and the concentration between 40-60% was suitable for protoplast fusion in different strains of fungi (Mrinalini et al. 1998). Upon plating, the colonies of fusants exhibited fast mycelial growth within 3 days. Non fusion protoplast did not germinate develop into colonies even after three days on selective media and took more than 4 days to develop into colonies and their growth rates were slow when compared to fused protoplasts. The colonies appeared white in colour for fusants of UB1 & UB9 and green color in UM6 & UM10. Protease activity for inter-generic fusants increased two-folds when compared to inter-specific and intra-strain fusants.

In conclusion present study clearly demonstrated the scope and significance of protoplast fusion technology for developing superior mycopesticidal strains. Most importantly the intra-specific, inter-specific protoplast fusion in *Beauveria bassiana and Metarhizium anisopliae* resulted in considerable increase of protease activity in most of the fusion strains and more than two fold increase in enzyme activity. Inter-generic (UM6 x UB9) protoplast fusion revealed the potential of strain improvement in *B.bassiana* and *M.anisopliae* which is evident from quantitative assays. Hence, this technique can successfully be used to develop superior hybrid strains of entomopathogenic fungi that lack inherent sexual reproduction.

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