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ISOLATION OF PROTOPLAST FROM SCLEROSPORA GRAMINICOLA

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ABSTRACT

Sclerospora graminicola is a plant pathogen that causes downy mildew in pearl millet, foxtail millet, and maize worldwide. It affects the yield and quality of crops by disrupting chlorophyll biosynthesis and photosynthesis. Fungal transformation provides an excellent approach to genetic manipulation involving protoplasts. Scientists till date have used protoplasts for transformation studies. Intra and inter specific hybridization of fungal species have been reported for strain improvement and fungicide resistance in many fungal strains. Firstly, we studied the optimization method of protoplast preparation of Sclerospora graminicola and found various factors that may affect the yield during protoplast preparation, such as mycelial stages, pH values, and osmotic stabilizers. Secondly, in the protoplasts isolated, we explored the effects of various osmotic conditioners on the protoplasts viability. The results showed, a protocol has been developed to isolate protoplasts from the infective propagules viz., sporangia, zoospores, oospores and mycelia of S. graminicola using commercially available enzyme NOVOZYME-249 possessing pectinase and proteinase activity. The method standardized for liberation of protoplasts is rapid, reliable and high yielding.

Key Words: Protoplast, Osmotic stabilizers, Sporangia, Oospores, Mycelia.

1. INTRODUCTION

Protoplasts are the organised entity of the living components of plant cells that carryout active metabolism, biosynthesis and energy transfer in contrast to the extra-cellular, essentially metabolically inactive secondary plant matter. They offer an important biological entity for many physiological and biochemical studies as evidenced for instance, by studies on the biochemical properties, isolation of individual components in the cells and regeneration of the cell wall mechanisms. A number of research and review articles are available on various applications of isolated protoplasts in different organisms.

The first and the foremost step in the isolation of fungal protoplast is the removal of cell wall [1]. Removal of cell wall is possible through digestion using different lytic enzymes and also further stabilization of the released protoplasts with the osmotic stabilizers. Protoplasts have been prepared from species representing all major taxonomic groups belonging to different classes of Zygomycotina, Ascomycotina, Basidiomycotina and Deuteromycotina. No single enzyme is effective for the lysis of cell wall due to the fact that the cell wall components of the fungus are resistant to individual enzymes. Combination of enzyme mixture, period of

treatment and concentration are the critical factors and have to be standardized for a particular fungus.

Protoplast release efficiency depends on the chemical composition and rigidity of the cell wall at different stages during the life cycle. The composition of cell walls of fungal species varied at different stages of growth. Due to variation in chemical composition in the different stages like growing mycelium, conidia chlamydospores, sclerotia and other spores, variation within the same fungal cell wall composition during its development was also observed. Of all the stages, hyphal stage was most suitable for protoplast release since more number of protoplasts was released and apart from it, if young mycelia were used they were highly susceptible to the lytic enzymes [2].

Spores would provide the most useful source of protoplast from many filamentous fungi, especially in species where they are uninucleate. Spore protoplasts are more homogenous in their organelle composition and therefore show uniform behavior [3]. In general, the spore wall is more resistant to lysis and an attempt to isolate protoplasts has been successful in only a few instances. Conidial protoplasts show homogeneity in the number of nuclei per protoplasts and thus will be suitable for further manipulation genetically.

Osmotic stabilizers are essential for external protection of the released protoplasts as reviewed [4]. A wide range of osmotic stabilizers viz., inorganic salts, sugars and sugar alcohols have been successfully used. However, no universal stabilizer has been reported for all fungi.

It is advantageous to use protoplasts from single culture and one isolation for genetic studies to maintain uniformity in genetic composition [5]. It is therefore of interest to develop a reliable method of storage for these protoplast preparations. The released protoplasts were generally monitored visually for the isolation of the cell wall and formation of spherical body microscopically. Monitoring the release using florescence brighteners of the cell wall was also employed.

2. MATERIALS AND METHODS

Pathogen used: A virulent pathotype of S. graminicola isolated from and maintained on its susceptible host (cultivar HB3 of pearl millet) under green house conditions at 22± 2°C was used for the study.

The procedure for collection of the samples viz., oopores, sporangia, zoospores and mycelia are described in detail below:

Oospores: Physiologically dried pearl millet leaves showing downy mildew symptoms were used for the study. Leaves were thoroughly grinded with pestle and mortar to obtain fine powder. This powder was passed through muslin cloth several times and the fine powder in the filtrate containing the oospores was used for the study.

Sporangia: Isolation of sporangia was as per the method of Safeeulla [6]. Infected leaves of pearl millet were collected in the evening and the remnants of previous crop of sporangia removed by flushing in running tap water. The leaves were blot dried, cut into 1cm long and incubated for sporulation at 25± 2°C under 60-70% relative humidity. Sporangia were harvested in the following morning into cold sterile distilled water. Precaution was taken not to take sporangiophore and other parts with sporangia.

Zoospores: Zoospores were released from the sporangia collected from the infected leaves of pearl millet following the method of Safeeulla [6]. The leaves were blot dried and then cut to

5-6 cm length pieces and then pinned over onto the blotter which was fixed to the lid of the petri plate. 5-10 ml of sterile distilled water was placed in the lower lid. These petri plates were incubated at 25±2°C in dark at 60-70% relative humidity for 8-10 h. The released sporangia dropped into the water in the lower lid and the zoospores were released.

Mycelia: Dual culture of the pathogen was established by inoculation of the malformed inflorescence axes onto Murashige and Skoog's (MS) basal medium supplemented with 5ppm of 2,4-D. The culture was incubated at 12 h of light under laboratory condition of 25± 2^0C. The mycelium started appearing on the calli within 25 days of inoculation onto the MS medium. For isolation of protoplasts, the mycelium was collected superficially with a pair of sterile forceps taking care not to collect the plant cells.

Isolation of protoplast:

Test samples - oospores, sporangia, zoospores and mycelia at the varying concentrations specified below were pre-incubated with 1% mercaptoethanol for 10 min. at 4°C and pelleted by centrifugation at 10,000 rpm at 4°C. The pellet was resuspended in the reaction mixture consisting of the enzyme at different concentrations and mercaptoethanol (0.1%) in specified osmotic stabilizer and incubated at 31°C at different time intervals as specified. After incubation, the preparation was centrifuged at 8x 10^3 g for 30 min. The pellet consisting of the protoplasts was washed in 0.4 M $CaCl_2$ and stored in the same solution at 4 °C till use. To maximize protoplast yield, protocol for isolation was standardized as follows:

Osmotic stabilizers:

Inorganic salts viz., ammonium chloride (NH_4CI), potassium chloride (KCI) and magnesium sulphate ($MgS0_4$) with molarities ranging from 0.8 to 1.4 were tested. With the exception of $MgS0_4$, the other two salts were dissolved in 0.2 M phosphate buffer. MgS04 was dissolved in 0.02 M phosphate buffer.

Organic compounds viz., sorbitol and mannitol were also tested as osmotic support at molarities of 0.8 to 1.4 after dissolving the compounds in 0.2 M phosphate buffer.

Concentration of samples:

The samples used at different concentrations are listed below:

Sample	Concentration (ml^{-1})
Oospores	7.5x10^4 to 6xl0^5 cells
Sporangia	5x 10^4 to l0x 10^5 cells
Zoospores	5x 10^4 to l0x 10^5 cells
Mycelia	5 to 50 mg

TABLE 1 CONCENTRATION OF THE SAMPLE

Oospore: Oospores were tested at final concentrations of 7.5x10^4 to 6x10^5 cells/ ml in the reaction mixture.

Sporangia: Sporangia were tested at 5x 10^4 to l0x 10^5 cells /ml in the reaction mixture.

Zoospore: Zoospores were tested at 5x 10^4 to l0x 10^5 cells /ml in the reaction mixture.

Mycelium: Mycelia were tested at concentrations of 5, 10, 20, 30, 40, 50 mg/ml.

Incubation period: Effect of incubation time over release of protoplasts was tested at different time intervals ranging from 15 to 120 min at 31°C. Sporangia and zoospores at a

concentration of $8x10^5$ cells ml^{-1}, oospores at $3x10^5$ cells $ml^{-1}$and 10 mg ml^{-1} of mycelium were used individually in a reaction mixture with KCI as stabilizer. Protoplast release was calculated using haemocytometer.

Concentration of lytic enzyme: The lytic enzyme from Aspergillus nidulans NOVOZYME-249 was obtained from NOVOLABS, Denmark. Effect of concentration of enzyme on protoplast release and stability was tested with varying concentrations at a range of 2-20% in the reaction mixture.

Isolation of cell wall from sporangia: $8x10^5$ sporangia/ml were pre-treated with 1 % mercaptoethanol followed by incubation with NOVOZYME and 0% mercaptoethanol in 1M KCI for 75 mins at 31°C. This preparation was centrifuged at 8 x 10^3 rpm and the supernatant used as source of cell wall.

Cryopreservation of isolated protoplasts: Cryoprotectants viz., dimethyl sulphoxide (DMSO), glycerol and polyvinyl pyrolidone (PVP) were tested for their efficiency to cryopreserve the isolated samples at the concentrations ranging from 0 to 25% in calcium chloride ($CaCl_2$). Polyvinyl pyrolidone at a concentration of 15% was used in $CaCl_2$. The isolated protoplasts were diluted to obtain a uniform concentration of $lx10^6$ cells/ml. The suspension was mixed gently and stored at -70°C by step down cooling of the suspension from 4^0c. The samples were collected by slowly thawing the suspension to room temperature. They were tested for viability at different day interval by FDA staining.

$$\text{Percentage of viable protoplasts} = \frac{\text{Number of viable protoplasts x 100}}{\text{Total number of protoplasts}}$$

Yield of protoplasts:

Yield of protoplast from the different infective propagules was calculated by taking haemocytometer counts of the sample after removal of debris. The data was represented in percentage (for oospore, sporangia and zoospore) and as number of protoplasts (mycelial) released /ml of the reaction mixture before and after the treatment with the enzyme. For each count 1000 micro liter of sample was taken in a haemocytometer and the number of cells counted under microscope.

Yield was represented ml^{-1} after calculating the sample using the formula:

Yield =Average number of protoplasts in a square x Total number of squares x 10^4 /ml

The protoplast yield percentage was represented as

$$\% \text{ Yield of protoplast} = \frac{\text{No. of protoplast x 100}}{\text{No. of samples taken}}$$

Protoplast morphology:

The isolated protoplasts were observed for granulation, shape and size. Twenty- five protoplasts were considered for evaluation, from each protoplast preparation, from different infective propagules and the experiment was repeated three times. The size of the released protoplasts was measured using an eye piece micrometer. Sequential pattern of release of protoplasts from different infective propagules was also observed.

Protoplast viability:

Viability of isolated protoplasts was evaluated by flourecein diacetate (FDA) method as described by Widholm [7]. Hundred cells from protoplasts preparation of oospore, sporangia,

zoospore and mycelia were observed by fluorescence microscopy. The cells that fluoresced under UV indicated viable protoplasts, while non-fluorescing ones were considered non viable.

The data obtained were repeated thrice with three replicates and subjected to Fisher's least significant test.

3. RESULTS

Optimization of Isolation of Viable Protoplasts from Different Infective Propagules of Sclerospora graminicola.

Effect of osmotic stabilizers: The zoospores and sporangia were tested for the effect of osmotic stabilizers on the release of protoplasts at a concentration of $8x10^5$/ml with 4% NOVOZYME under incubation for 75 min. The oospore release was tested at $3x\ 10^5$ /ml and mycelia at 10 mg/ml in 12% NOVOZYME with an incubation of 90 and 180 min respectively. All osmotic stabilizers tested, released protoplasts from the different infective propagules of S. graminicola but to varying degrees. Maximum yield was obtained with KCl followed by NH_4CI, $MgS0_4$, mannitol and least in sorbitol (Figures 1-4).

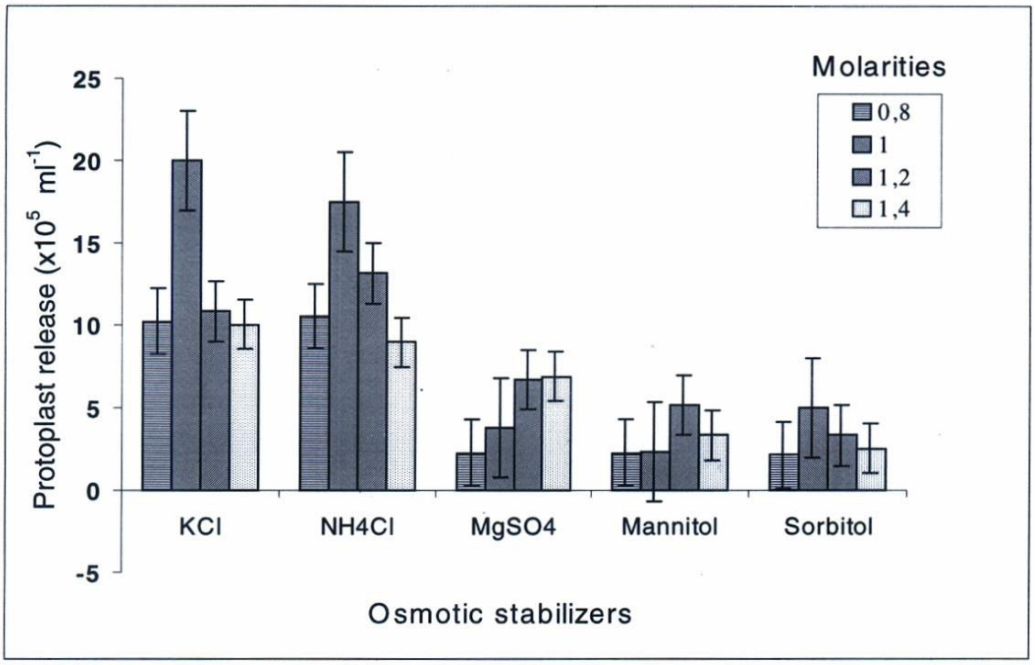

FIG. 1 EFFECT OF OSMOTIC STABILIZER ON RELEASE OF PROTOPLASTS FROM SPORANGIA OF SCLEROSPORA GRAMINICOLA

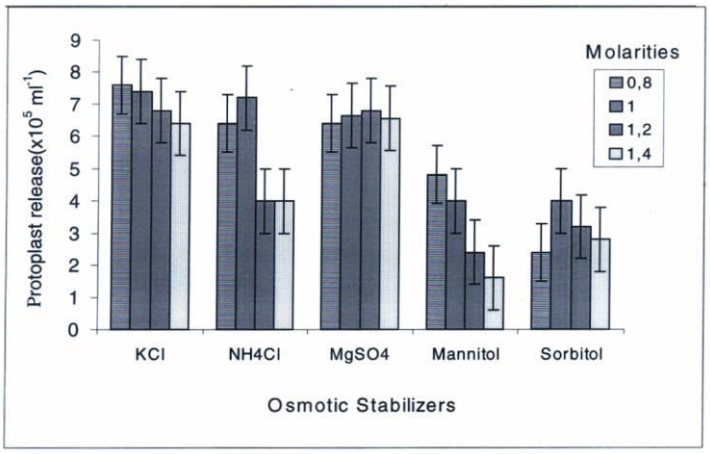

FIG. 2 EFFECT OF OSMOTIC STABILIZER ON RELEASE OF PROTOPLASTS FROM ZOOSPORES OF SCLEROSPORA GRAMINICOLA

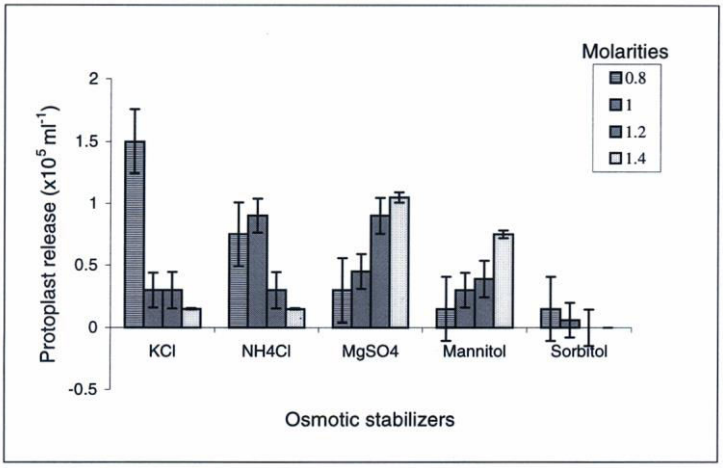

FIG. 3 EFFECT OF OSMOTIC STABILIZER ON RELEASE OF PROTOPLASTS FROM OOSPORES OF SCLEROSPORA GRAMINICOLA

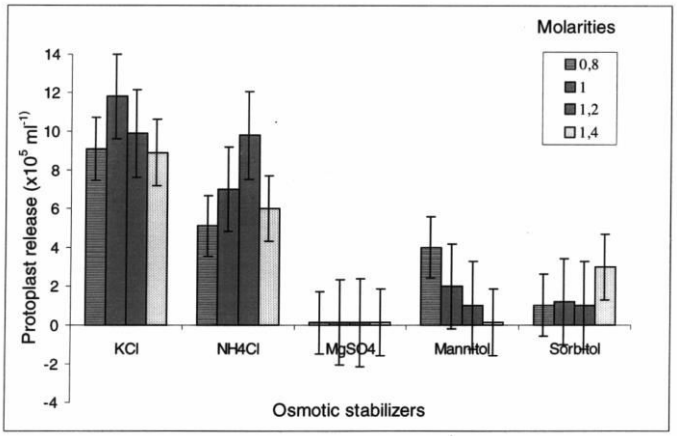

FIG. 4 EFFECT OF OSMOTIC STABILIZER ON RELEASE OF PROTOPLASTS FROM MYCELIA OF SCLEROSPORA GRAMINICOLA

Though KCI released maximum percentage of protoplasts from all the propagules, concentration

dependent yield was observed. 1M concentration released the highest in all propagules (93% in sporangia, 95% in zoospores and $12x10^5$ /ml in mycelia) except oospores (10% release), where 0.8 M was found to be more efficient (50%). Further increase in concentration of stabilizers resulted in lysis of released protoplasts. Good digestion was observed with NH_4CI also with a release of 81, 90, 35% and $9.9x10^5$ /ml of protoplasts from sporangia, zoospores, oospores and mycelia respectively at a concentration of 1 M. Both the organic stabilizers least supported the release from all the infective propagules. They were ineffective in maintaining osmoticum for the released protoplasts eventually causing plasmolysis.

Effect of sample concentration: Effect of sample concentration on protoplast release is represented in Figures (5-6).

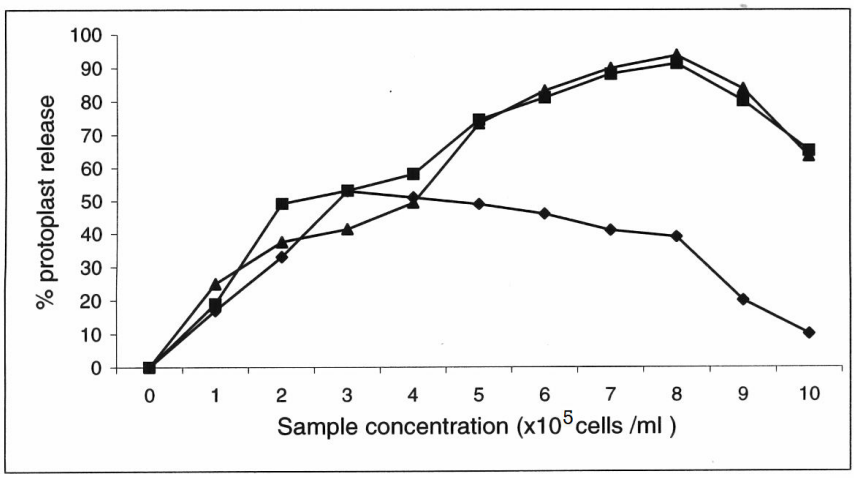

FIG. 5 EFFECT OF SAMPLE CONCENTRATION ON RELEASE OF PROTOPLASTS FROM SPORANGIA(▲), ZOOSPORES(■) AND OOSPORES(◆) OF SCLEROSPORA GRAMINICOLA

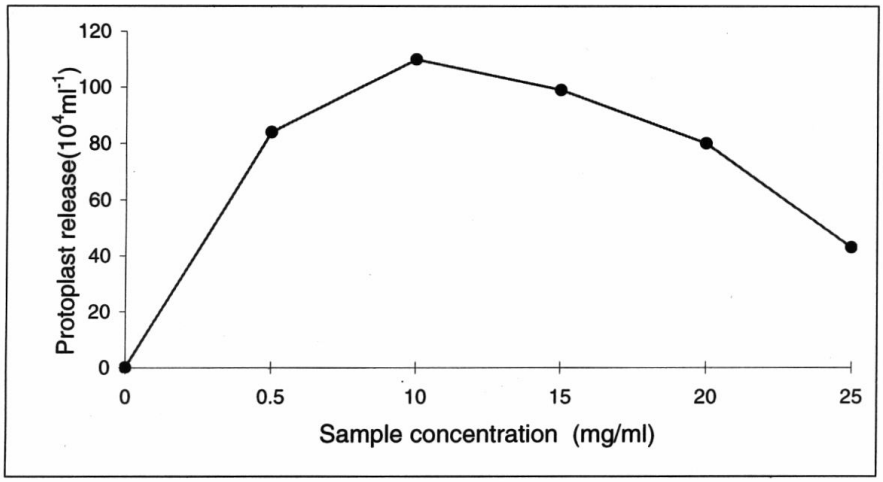

FIG. 6 EFFECT OF SAMPLE CONCENTRATION ON RELEASE OF PROTOPLASTS FROM MYCELIA OF SCLEROSPORA GRAMINICOLA

Extensive release of zoospore (93%) and sporangial (93.75%) protoplasts were obtained when $8x10^5$ spores ml were used in 1 M KCI and 4% NOVOZYME. Maximum digestion of oospores to release 62% of protoplasts was recorded when $3X10^5$ cells /ml was taken in 0.8 M KCI with 12% NOVOZYME and incubated for 90 min in the reaction mixture. In spore concentrations higher and lower to this, protoplast yield decreased. Protoplast release of $8.9X10^5$ /ml from mycelia in 1 M KCl with 12% NOVOZYME was observed at a concentration of 5 mg which increased to 10 $xl0^5$ /ml When 10 mg of mycelia was used. While sample concentrations higher to that reduced protoplast yield lower concentrations led to lysis of the released protoplasts.

Effect of incubation period: Yield of protoplast in KCI at optimal sample concentration was estimated over a period of lytic digestion of 120 min. and the data obtained is represented in Figures (7-8).

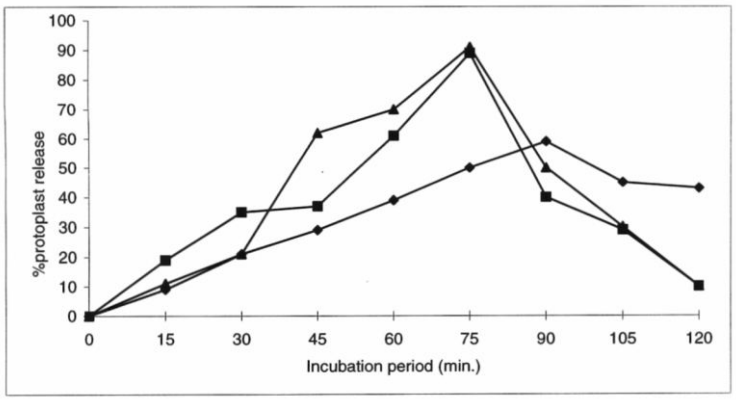

FIG. 7 EFFECT OF INCUBATION PERIOD ON RELEASE OF PROTOPLASTS FROM SPORANGIA(▲), ZOOSPORES(■) AND OOSPORES(◆) OF SCLEROSPORA GRAMINICOLA

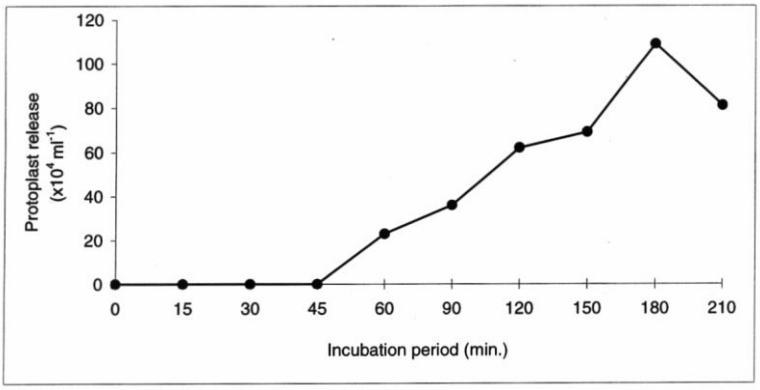

FIG. 8 EFFECT OF INCUBATION PERIOD ON RELEASE OF PROTOPLASTS FROM MYCELIA OF SCLEROSPORA GRAMINICOLA

Release of protoplast was initiated as early as 15 min of incubation from sporangia and zoospores (19 and 15 % respectively) and increased with increase in time and reached the maximum at 75 minute with 92 and 90 % in sporangia and zoospores respectively. Further incubation decreased the release of protoplasts in both the test samples and lysis of released

protoplast was also observed. On the other hand, digestion in the reaction mixture of oospores started only after 45 min of incubation and a maximum of 62% release was observed on 90 min of incubation. Protoplast release in mycelia was initiated after 90 min. of incubation. However, maximal release ($10x10^5$ /ml) was obtained after a period of incubation of 180 min. Effect of enzyme concentration: Varying concentrations of NOVOZYME was tested for optimal release of protoplasts. Observations indicated 2% to be sufficient to initiate the release of 70% in sporangia, 60% in zoospores and 8% of the enzyme concentration in the reaction mixture was required to release 60% protoplast in oospores and $8x10^5$ cells/ ml in mycelia (Figures 9-10).

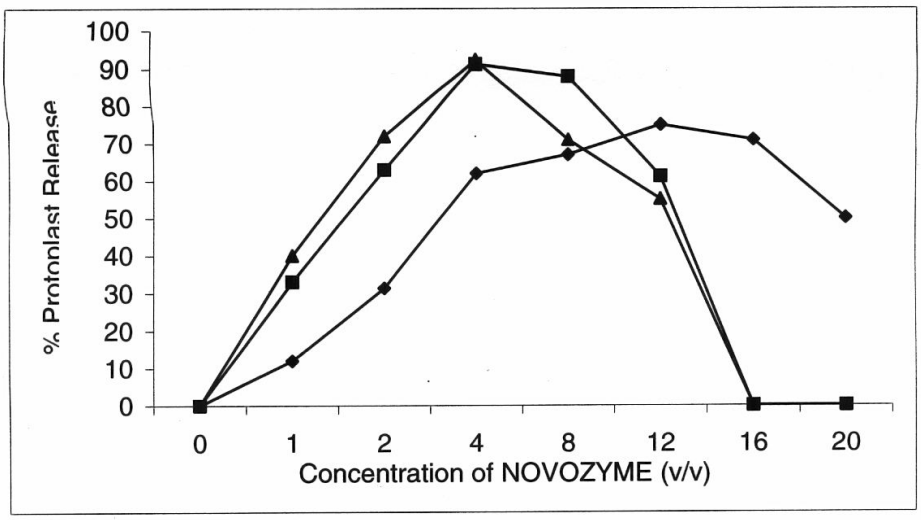

FIG. 9 EFFECT OF INCUBATION PERIOD ON RELEASE OF PROTOPLASTS FROM SPORANGIA(▲), ZOOSPORES(■) AND OOSPORES(◆) OF SCLEROSPORA GRAMINICOLA

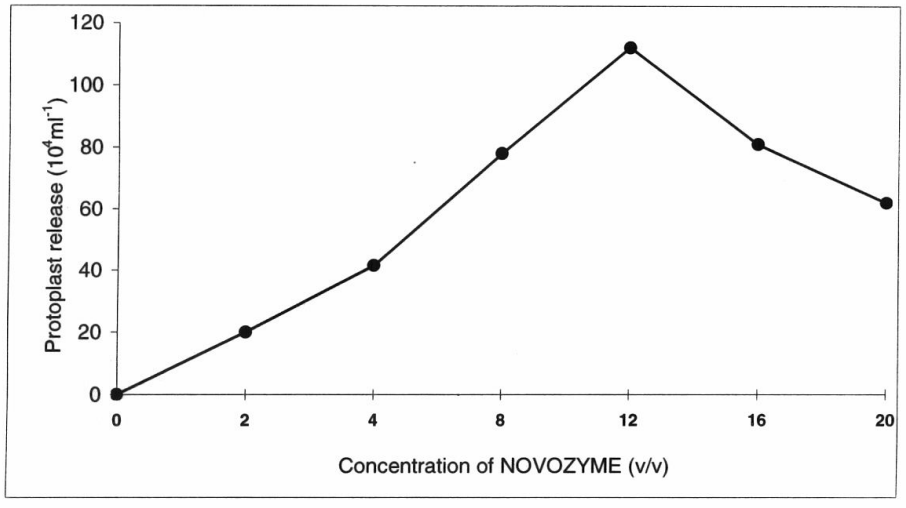

FIG. 10 EFFECT OF NOVOZYME CONCENTRATION ON RELEASE OF PROTOPLASTS FROM MYCELIA OF SCLEROSPORA GRAMINICOLA

However, maximum release of protoplasts in sporangia (90%) and zoospores (90%) required 4% of the enzyme. It was interesting to note that while 12% enzyme concentration was

essential to release a maximum of 62% protoplasts from oospores and 10 x 10^5 ml^{-1} in mycelia, the same concentration was found to be non optimal for sporangia and zoospores as evidenced by lysis of the released protoplasts.

Protoplast viability: Viability of isolated protoplasts was tested by staining with FDA and observed under UV using fluorescence microscope. Out of the hundred cells observed in each protoplast preparation from oospores, sporangia, zoospores and mycelia, 98 % fluoresced brightly, thus indicating viable nature of majority of the isolated protoplasts.

Protoplast morphology: Morphology of the released protoplast varied with the nature of the osmotic

stabilizer used. Of all the stabilizers tested, KCl and NH_4Cl were effective at all the concentrations and released protoplasts from all the infective propagules. Though KCI and NH_4CI gave rise to orderly release of protoplasts, the released ones were shrunken in NH_4CI treatment. KCI released oospores, sporangia and zoospores protoplasts were spherical and granulated. Mycelial protoplasts were also spherical but hyaline. NH_4CI released protoplasts from oospore, sporangia and zoospore were granulated but were plasmolysed at all concentrations tested. Mycelial protoplast released in NH_4Cl were hyaline but plasmolysed. Protoplasts released from the other three osmotic stabilizers ($MgSO_4$, mannitol and sorbitol) were heavily plasmolysed at all concentrations tested. Sporangial and zoospore protoplasts ranged from 8-12 µ and 4-8 µ in diameter respectively. Oospore protoplasts were also of different size ranging from 12-24 µ in diameter. Mycelial protoplasts ranged from 15-23 µ in diameter.

Pattern of protoplast release from the different infective propagules also varied.

Protoplasts from sporangia and zoospores were released through a single site in the cell wall (Fig. 11) whereas release from mycelia was at multiple sites (Fig. 13).

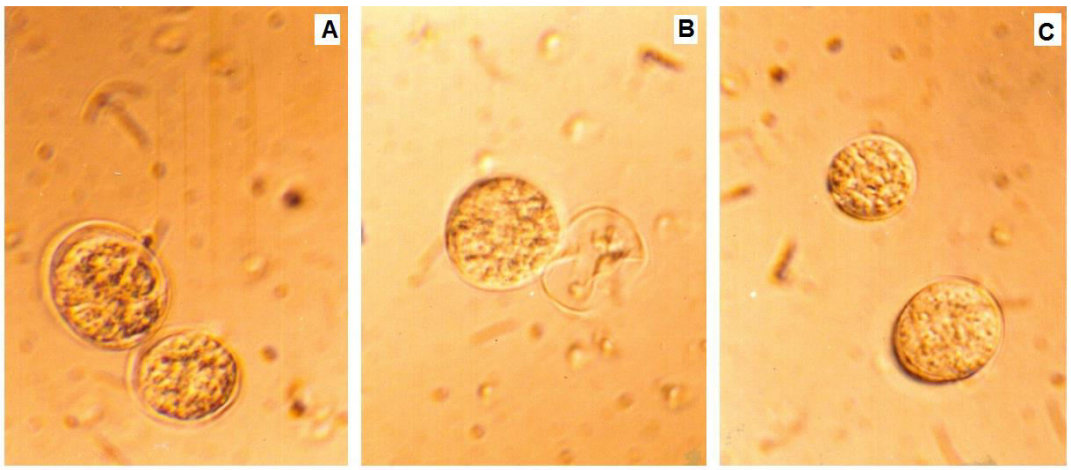

FIG. 11 SEQUENTIAL RELEASE OF PROTOPLAST FROM SPORANGIA OF SCLEROSPORA GRAMINICOLA BY ENZYMATIC DIGESTION

A-Sporangia undigested; B-Release of protoplast at single site; C-Release of protoplast

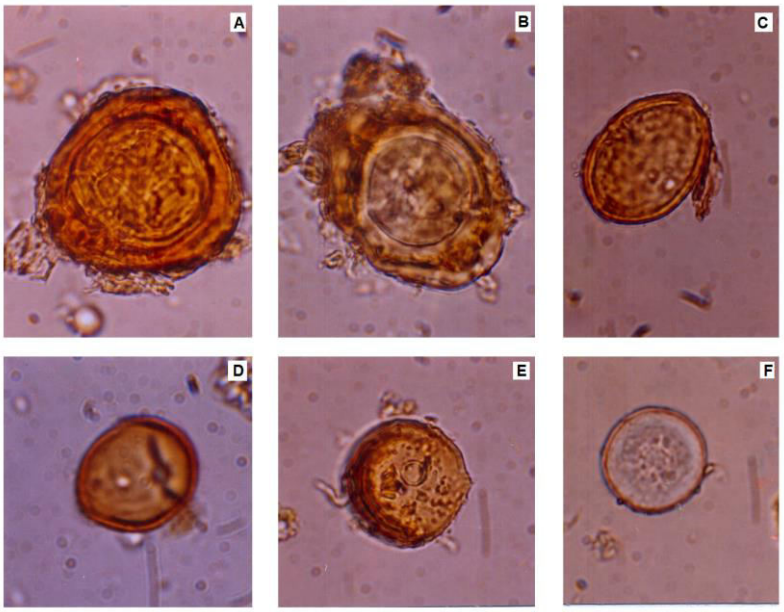

FIG. 12 SEQUENTIAL RELEASE OF PROTOPLAST FROM OOSPORES OF SCLEROSPORA GRAMINICOLA BY ENZYMATIC DIGESTION
A-Sporangia undigested; B, C, D & E-Sequential peeling oospore layer one after another;

F-Released oospore

Release of protoplasts from mycelium was initiated by bulging of mycelial cell wall at several points in the mycelium, migration of protoplasts into these spaces, and subsequently released from that spot. Oospore protoplast release was through sequential peeling of the three wall layers one after the other (Fig. 13), and thus differed from that in the other propagules.

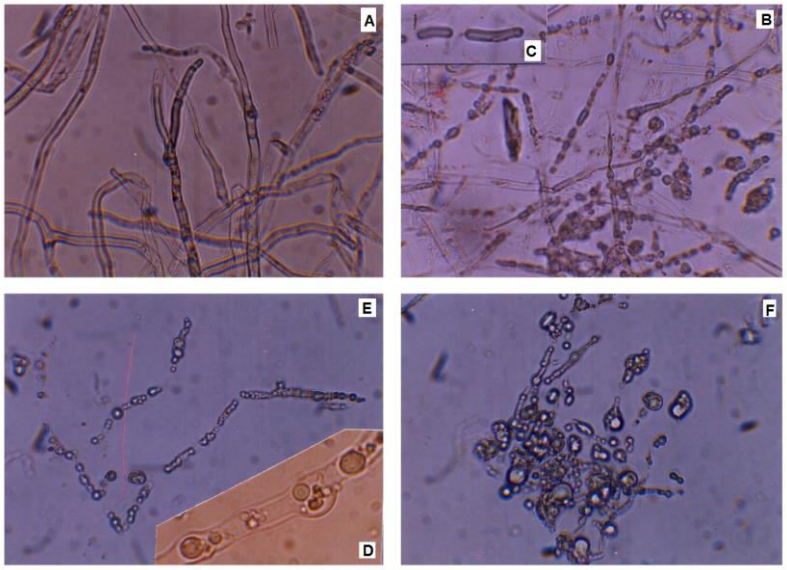

FIG. 13 SEQUENTIAL RELEASE OF PROTOPLAST FROM MYCELIA OF

SCLEROSPORA GRAMINICOLA BY ENZYMATIC DIGESTION

A-Intact undigested hyphae showing coenocytic nature; B & C- Cytoplasm occupying space after bulging of cell wall; D-Magnified view of cytoplasm; E & F-Release of protoplast by budding

Cryopreservation of isolated protoplasts: The three cryoprotectants tested viz., DMSO, glycerol and PVP at concentrations of 1 to 25% for their ability to cryopreserve the isolated protoplasts varied in their efficiency (Fig. 14).

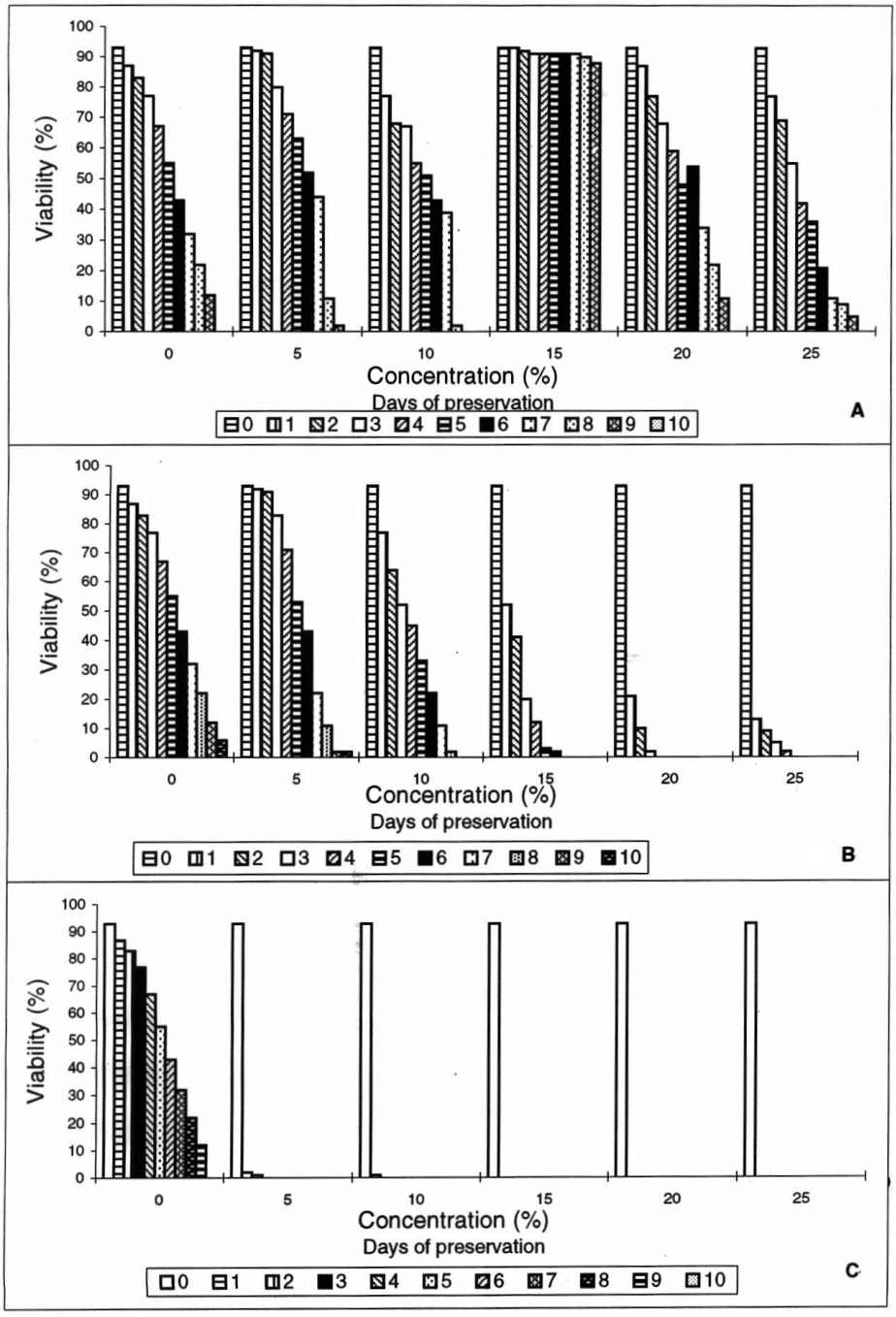

FIG. 14 EFFECT OF CRYOPROTECTANTS ON PROTOPLAST VIABILITY DURING STORAGE

A-Polyvinyl pyrrolidone, B-Dimethyl sulfoxide, C-Glycerol

The three compounds, PVP at 15% concentration showed a maximum of 92% of viable protoplasts at the end of 7th day of preservation, whereas other concentrations of the same chemical showed decreased viability. DMSO was efficient to protect 91% protoplasts till 3 days at 5% concentration in solution. There was drastic decrease in viability thereafter. Samples of protoplasts preserved in glycerol showed 2% viability on 24h of incubation. Thus of the three stabilizers tested, 15% PVP was selected as cryopreservative for preserving the isolated protoplasts. The isolated protoplasts in this solution were viable even on cryopreservation for up to 60 days with a constant viability of 90%. However, further preservation affected the viability with drastic decrease to 70% on seventieth day and further decrease thereafter to 50 and 28% on 75 and 80 day of cryopreservation (Fig. 15).

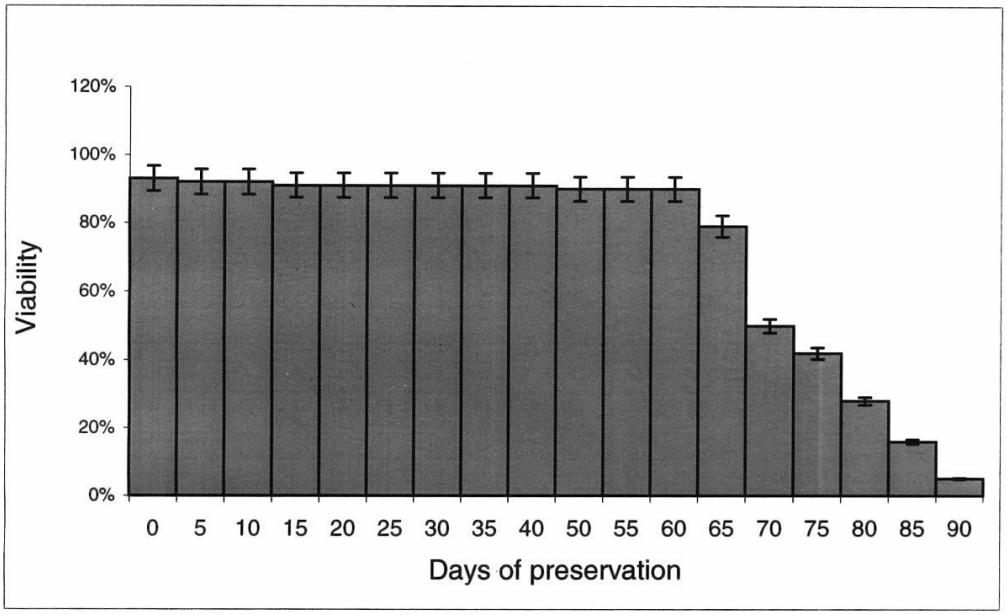

FIG. 15 HISTOGRAM SHOWING THE VIABILITY OF SPORANGIAL PROTOPLASTS ON CRYOPRESERVATION AT -70°C IN 15% POLYVINYL PIRROLIDONE IN CALCIUM CHLORIDE.

4. DISCUSSION

In this study, a protocol has been developed to isolate protoplasts from the infective propagules viz., sporangia, zoospores, oospores and mycelia of S. graminicola using commercially available enzyme NOVOZYME-249 possessing pectinase and proteinase activity. The method standardized for liberation of protoplasts is rapid, reliable and high yielding. The protocol employed for the release of protoplast yielded viable protoplasts as evaluated by FDA staining. Fluorescein diacetate is a non-fluorescing, non-polar compound, which is freely permeable across intact plasmalemma. On coming in contact with esterase present over the plasma membrane, FDA dissociates to produce fluorescein. Fluorescein is a fluorescent polar product and is not freely permeable across intact plasma membrane. Therefore, fluorescein accumulates in viable cells but not in dead cells [8]. Protoplast viability, as evidenced by fluorescence of viable cells, was observed in all the protoplast preparations indicating the valid measurement of the efficiency by the different parameters employed in this study for optimal release of viable protoplasts in the test fungus.

Osmotic stabilizers tested in the present study and their concentrations had differential influence over the yield of protoplasts. While inorganic salts were favorable when added at a suitable molarity with the best result on treatment with KCI, organic stabilizers were ineffective in maintaining osmoticum for the released protoplasts, eventually causing plasmolysis. Probable reason for this variation may be due to the dissociation of the inorganic salts into ions in solutions, which are capable of mobilizing in and out of the cell, thus contributing to maintenance of the stability of the protoplasts. On the other hand, organic compounds are of high molecular weight and hence they themselves can neither enter the cells nor dissociate in solution. Hence, the osmotic environment outside the test sample for release will be high leading to plasmolysis of the cells [9]. Variation in the effective concentration of osmotic stabilizers in releasing protoplasts from different propagules of S. graminicola may be due to internal osmotic potential of the cells or due to the effect of stabilizer on fungal membrane [10]. Microscopic examination of the decreasing release of protoplast with increasing sample concentrations at constant enzyme concentration and reaction volume suggested that enzyme concentration as well as reaction volume were major factors governing protoplast yield. We also observed variation in morphology of the released protoplast.

Such variation in the effect of osmotic stabilizers on the yield of protoplasts has been reported [11], [12], [13], during the study on sphaeroplast forming rate in Saccharomyces cereviseae. Sequential pattern of protoplast release from different infective propagules also varied. Protoplast release in sporangia and zoospore was through a single site (Figures 11 & 12) whereas release from mycelia was at multiple sites and the released protoplasts were of different sizes (Fig. 13). The reason attributed to the variation in the size of protoplasts released from the mycelia may be due to the coenocytic nature of the cytoplasm. It was also observed that a maximum of 62% release of protoplast was achieved from oospores after lytic digestion with 12% enzyme and further increase in the enzyme concentration resulted only in lysing of the released protoplasts rather than increasing the yield suggesting that the enzyme concentration is the major limiting factor for the liberation of protoplast from oospores. Variation in the parameters required for the optimal release of protoplasts from the propagules and their pattern of release suggested the possible variation in composition and distribution among cell wall components that are differentially digested. These observations are consistent with previous findings by Dietz et al., [14], [15] and Struck et al., [16] who reported that the variation in appearance of the protoplasts, requirement of enzyme concentration, incubation period and pattern of release as detected in epidermal and mesophyll protoplasts were due to variation in polypeptide composition and ion compartmentalization on the plasma membrane.

It is advantageous to use protoplasts from single culture and one isolation for genetic studies to maintain uniformity in genetic composition [5]. It is therefore of interest to develop a reliable method of storage for these protoplast preparations. Attempts of freezing protoplasts of S. graminicola demonstrated that the addition of a cryoprotectant was essential. Without the cryoprotectant, the protoplasts lysed either during freezing or thawing. Investigations on the use of DMSO or glycerol as cryoprotectants indicated marked physiological effects on the protoplasts. These changes suggest that these chemicals are osmotically active, can penetrate cell membrane due to low molecular weight and may be toxic to the ells. Compounds of high molecular weight, like PVP (Mol. Wt. 40,000D) do not penetrate the cell membrane.

Observations based on retention of viability of protoplasts revealed negligible physiological effect and are very effective in preventing intracellular freezing. Therefore in our experiments, PVP was chosen as cryoprotectant. To maximize viability of protoplasts and also to minimize negative physiological effects, different concentrations of PVP was tested and 15% (w/v) was found very effective. Protoplasts of S. graminicola were tested for cryopreservation and the observations indicate stable viability for 60 days, which decreased further due to unknown reasons. However, stable viability for 60 days is advantageous for studies on pathological as well as genetic aspects of the biotrophic fungus S. graminicola.

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