Dibutyryl c-AMP as an inducer of sporidia formation: Biochemical and antigenic changes during morphological differentiation of Karnal bunt (*Tilletia indica*) pathogen in axenic culture

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Effect of dibutyryl adenosine 3′,5′-cyclic monophosphate (dbc-AMP), an analogue of c-AMP, was investigated on growth and morphological differentiation of *Tilletia indica*. Exponential growth was observed up to 21 days in both presence and absence of dbc-AMP; however, increasing concentration of dbc-AMP was deleterious to mycelial growth in liquid culture. A slow increase of mycelial biomass up to 21 days and decline at 30 days in the presence of 2·5 mM dbc-AMP was observed, therefore, this concentration was chosen in subsequent investigations. The inhibitory influence of dbc-AMP was further substantiated by decrease in soluble protein. The fungus on exposure to dbc-AMP experienced morphological differentiation from vegetative mycelial phase to sporogenous mycelial phase, and was induced to produce filiform sporidia. Use of quantitative ELISA further suggested that sporidia formation took more than 21 days in the presence of dbc-AMP. Variations of proteins during different stages of *T. indica* grown in the presence and absence of dbc-AMP suggested the expression of stage-specific proteins or differential expression of proteins induced by dbc-AMP. The changes in expression of cell surface antigens as evidenced from decrease and increase binding of anti-mycelial and anti-sporidial antibodies in dbc-AMP treated culture by ELISA was further interpreted on the basis of morphological differentiation from mycelial to sporidial phase.

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1. Introduction

Karnal bunt (KB) caused by *Tilletia indica* Mitra syn. *Neovossia indica* (Mitra) Mundukar is an economically important disease of wheat in the northwestern regions of India and other parts of the world. It is a floret-infecting disease that partially infects seed of wheat and hence also called partial bunt (Mitra 1931). Many aspects concerning disease development are yet to be answered. The proper understanding of KB disease at cellular and molecular level would help to devise strategies for successful management of disease. Researchers interested in the study of the molecular biology of bunt fungi recognized that KB can be made excellent model system because of the yeast-like habit of bunt fungi in pure culture, and availability of both haploid (n) and diploid (2n) cells, it occurs in three morphologically distinct forms in the life cycle (Dhaliwal *et al* 1983; Kumar *et al* 2000). The forms are: (i) haploid sporidial form; (ii) filamentous dikaryon mycelial form; and (iii) a diploid teliospore form. The primary sporidia

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Abbreviations used: dbc-AMP, Dibutyryl adenosine 3′,5′-cyclic monophosphate; KB, Karnal bunt; PDB, potato dextrose broth.

germinate to give rise to inter-convertible filiform and allantoid secondary sporidia. The filiform secondary sporidia form the reproductive entity, while allantoid secondary sporidia form the banana-shaped infectious entity (Dhaliwal and Singh 1988). The disease spread occurs by the production of sporidia that cause infection of individual florets at boot leaf stage (Goates 1988). Therefore, KB has become the bunt fungus of choice for studies on molecular genetics, mating type, pathogenicity, growth habit and other aspects of fungal biology.

Mating is an essential step in the life cycle of most homobasidiomycetes. No special cells are required for mating; fusion between vegetative mycelial/sporidial cells is sufficient to trigger a well-characterized developmental sequence that converts monokaryon into dikaryon. However, evidence for mating interaction to establish the infectious cell type during morphological differentiation of KB is not clearly demonstrated (Kumar *et al* 2000). The molecular signals required for establishing and maintaining the infectious dikaryons would have to be identified by investigating the role of c-AMP in fungal pathogenesis. Studies on mating in lower eukaryotes, including yeast and filamentous fungi, have revealed role of c-AMP in various aspects of fungal biology, including both growth and cell cyclic progression (Pasquale and Good Enough 1987; Good Enough 1992; Bencina *et al* 1997). Moreover, c-AMP has been demonstrated a key role in the virulence of phytopathogenic fungi such as *Ustilago maydis* (Gold *et al* 1994) and *Cryphonectria parasitica* (Chen *et al* 1996). In addition, in the phytopathogens, *Magnaporthe grisea* and *Colletotrichum trifolii*, c-AMP has been demonstrated to play an important role during appressorial differentiation (Lee and Dean 1994; Yang and Dickman 1997). However, whether c-AMP plays any such role in the regulation of morphological differentiation of *T. indica* during pathogenesis and host-parasite interaction or not, is yet to be investigated.

In vitro culture of fungus provides a useful system for generating good deal of information about molecular and cellular mechanisms underlying the transition of vegetative to sporidial phase. Analysis of sporidiation and biomolecules involved in their production offers the opportunity to explore mechanisms of intra cellular and extra cellular signalling, biochemical basis of recognition, fusion and development of sexual pathway and role of adenylate cyclase/c-AMP cascade (Hall *et al* 1999). This cascade is involved in sensing nutrient levels. Mutations in this pathway have pleiotropic effects on differentiation including cell division, mating and sporulation. The pathogenic mechanism to cause KB disease can be traced out by understanding of cAMP-adenylate cyclase system in fungus, *T. indica*. The conversion of vegetative mycelial to sporidial phase in this pathogen may also be centered near this molecular signalling system. However, the molecular events governing sporidiation, formation of infectious dikaryotic mycelial form has not been extensively studied in KB.

Distinct morphological and biochemical changes are invariably associated with morphological differentiation and/or transition of haploid mycelial to sporidial to diploid teliospore phase. During development, *T. indica* appears a phenotypic mixture of genetically identical (haploid) cells but morphologically differentiated cells like haploid mycelial and allantoid/filiform sporidial form or genetically different but mixture of haploid and diploid fungal populations. The process of sporidiation during developmental cycle of fungus accompanied the change in morphology and in turn to appearance and/or disappearance of developmentally related markers on fungal population. Number of these markers is small compared to common structural proteins of different fungal populations. It is therefore, difficult to pinpoint these markers in fungal populations by conventional biochemical methods. Characterization of these changes in relation to growth and morphological differentiation has become difficult without suitable probe. Due to high specificity and sensitivity of antigen antibody reaction, immunological approach is probably the only method to study these markers related to development and differentiation of *T. indica*. In the present study attempts were made to study the influence of dbc-AMP on induction of sporidia formation and biochemical and antigenic changes associated during sporidiation.

2. Materials and methods

2.1 *Culture of* T. indica

The monoteliosporic culture of *T. indica* was prepared as described earlier (Rai *et al* 2000). Cultures of *T. indica* grown on potato dextrose broth (PDB) were used for *in vitro* studies of sexual development of *T. indica*.

2.2 *Dibutyryl cyclic-AMP treated fungal growth in liquid culture*

The fungal mycelia in equal proportion were inoculated in 24 well plate/petri-dishes (containing 2·0 ml PDB per well or 5·0 ml PDB per petri-dish) and allowed to grow for 2 days. At the end of 48 h, different concentration of dbc-AMP in final concentration of 1·25, 2·50 and 5·00 mM was added in different wells and allowed to grow in BOD incubator at 22 ± 1°C. The fungal mycelia growing either in the presence or absence of dbc-AMP were harvested at intervals of 3, 7, 14, 21 and 30 days of growth. Wet weight of mycelial biomass was plotted as a function of growth vs. time. Exponential and stationary phases were determined from the line of best fit.

2.3 *Morphological studies*

T. indica was grown in media containing 1·25 mM of dbc-AMP and fungus growth was observed at intervals of 7, 14, 21 and 30 days of inoculation using aniline blue. The fungal mycelia were teased to separate interwoven mycelial network with the help of needle on slide itself. After teasing, staining was done using aniline blue and mounted on lacto-phenol. To determine the effect of dbc-AMP on morphogenetic development, the stained mycelia or other structures from at least three different regions were examined by light microscopy and scored as morphologically differentiated if they possessed sporidia or chlomydospores.

2.4 *Preparation of partially purified antigen*

The potentially purified antigen (PA) was prepared by fractionating the crude extract of *T. indica* by ammonium sulphate precipitation. Saturated $(NH_4)_2SO_4$ was added to the crude extract to a final concentration of 30%. The 30S precipitate was redissolved in a small volume of PBS and dialyzed against three changes of phosphate buffer without NaCl at 4°C. Ammonium sulphate was added to the resulting supernatant to a final concentration of 70% and the pellet designated as 70S was resuspended in PBS and dialyzed for 24 h at 4°C against three changes of phosphate buffer. The total protein content of pooled 30S and 70S fractions was determined by Bradford method and both fractions were pooled (not sufficient individually for immunological work) and used as fractionated (purified) mycelial antigen (Varshney 2003).

2.4a *Harvesting of sporidia*: The sporidia were harvested from 21 days grown fungal cultures on solid medium. The sterilized distilled water (10 ml) was placed in petriplates and by pressing fungal mycelium with the help of glass rod, the sporidia were harvested by centrifugation and counted by Hemocytometer.

2.5 *Production of polyclonal antibodies*

New Zealand white albino rabbits were used for production of antisera against partially purified mycelial antigens and intact sporidia. The partially purified antigen and intact sporidia were taken separately and emulsified with an equal volume of Freund's complete adjuvant before injection. The antigenic preparations were injected into two female rabbits. The emulsified antigen(s) (0·5 ml/rabbit) containing either 500 µg of mycelial antigen or 2×10^5 sporidia were administered through subcutaneous mode, intra-peritonial mode and footpad at multiple sites. The antigen emulsified in equal volume of Freund's incomplete adjuvant was used for booster injections and given at intervals of 10, 20 and 30 days after primary immunization. One week after the last injection, the rabbits were bled from a marginal ear vein. The clear sera were decanted from the clotted blood into test tubes, centrifuged at 5000 rpm for 5 min to get rid off any remaining cells. The antisera generated against mycelial and sporidial antigens were finally frozen at – 20°C.

2.6 *Preparation of soluble protein extract*

Each frozen sample of mycelial mass was ground in mortar and pestle using acid washed glass beads in extraction buffer (0·05 M, Tris-HCl, pH 7·6 containing ascorbic acid, polyvinyl pyrrolidone and protease inhibitor cocktail) for about 25–30 min under cold conditions. The crushed sample was decanted and centrifuged at 10,000 rpm for 20 min. The supernatant for each sample was collected and stored at – 20°C for electrophoresis of proteins. The total protein in each sample was estimated by Bradford method.

2.7 *Separation of proteins by SDS-PAGE electrophoresis*

Electrophoresis of soluble fungal proteins was carried out on a vertical-slab gel electrophoresis system using 80 µg of protein per lane. Electrophoresis was performed for 5–6 h at 150 mV. Protein patterns were visualized by Coomassie brilliant blue staining.

2.8 *Development of indirect ELISA*

The indirect microtitre ELISAs for detection and quantitation of mycelial and sporidial were developed as per Engvall and Perlmann (1971) with slight modification. After coating the ninety-six well flat bottom microtitre plates with crude fungal extract (25 µg of protein per ml: 100 µl per well) and non-specific blocking with 3% skimmed milk solution in PBS, the plates were incubated for 2 h at 37°C or overnight at 4°C, washed three times in PBST, and incubated for 1 h at 37°C with the pooled rabbit anti-mycelial (1 : 5000) or anti-sporidial (1 : 2000). After three additional washes in PBST, 100 µl of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) *g* chain antibodies (Banglore Genei, Banglore) diluted 1 : 2,500 in PBS was added to each well. The plates were incubated for 1 h at 37°C and washed three times in PBST. p-nitrophenyl phosphate [1 mg/ml in 1 M diethanolamine buffer (pH 9·8)] was used as chromogen. The reaction was stopped after 30 min incubation at room temperature by the addition of 3 N NaOH and the absorbance at 405 nm was determined on a ELISA reader (ECIL, Hyderabad). All tests were performed in triplicate. The controls were uncoated wells and the incubation of coated wells with PBS instead of the immune or culture supernatants.

3. Results and discussion

Fungi employ c-AMP significantly in a variety of processes including the control of differentiation, sexual development and virulence in addition to the monitoring of nutritional status and stress. Furthermore, the c-AMP pathway influences transcription and cell cycle progression (Herskowitz 1988). Recent forays in to filamentous fungi including pathogens of plants and animals have provided revelations about c-AMP signalling in morphological development and virulence. Information accumulated on the role of c-AMP on morphogenesis and stress in these fungi indicates that similar components will be induced and play important role in phytopathogenic fungi like *T. indica*. In the present study it was observed that dbc-AMP showed inhibitory growth response to *T. indica* and induced sporidia formation. The fungal mycelium divided by intercalinary division exponentially (logarthmic growth phase) followed by a decrease in the rate of multiplication (stationary growth phase), both in the presence and absence of dbc-AMP. A close analysis of growth curve (wet weight of mycelial biomass vs time) revealed exponential growth up to 21 days in both presence and absence of dbc-AMP. The mycelial biomass decreased at 30 days of growth in dbc-AMP treated cultures at 2·5 mM and 5·0 mM concentration (figure 1). High concentrations of dbc-AMP were deleterious to both logarithmic phase cells as well as stationary phase cells. The mycelial biomass decreased with increasing concentrations of dbc-AMP.

The growth pattern was affected due to the presence of inducer in the medium. Not only fungal mycelia grow at a slower pace in the presence of dbc-AMP but also the mycelial biomass was reduced by nearly 25% when cultures were treated by 2·5 mM dbc-AMP and hence chosen as optimal inducer concentration in subsequent study. Inhibitory influence of dbc-AMP on fungal growth was substantiated by the estimation of soluble protein concentration (μg/well) of fungal growth in liquid culture (figure 2). The amount of protein increased exponentially till 21 days after which the protein concentration slightly decreased in case of higher dbc-AMP treated cultures whereas in control and lower concentrations of dbc-AMP treated cultures, protein concentration kept increasing till 30 days. The dbc-AMP caused inhibition of mycelial growth of fungus, *T. indica*, though not fully understood. One plausible explanation for growth retarding property of dbc-AMP is due to arrest in the cell cycle progression or it induces the conversion of sizable population of cells from mycelial growth to sporidial phase.

In order to determine whether c-AMP acts as an inducer of sporidia formation, light microscopic examination of lactophenol cotton blue stained fungal cultures revealed that following 14 days exposure of fungal cells to dbc-AMP, the fungal population appeared to undergo morphogenetic changes which showed condensation of mycelia are randomly distributed as flattened morphology and subsequently induced in the form of filiform sporidia (figure 3). The extent of sporidiation continu-

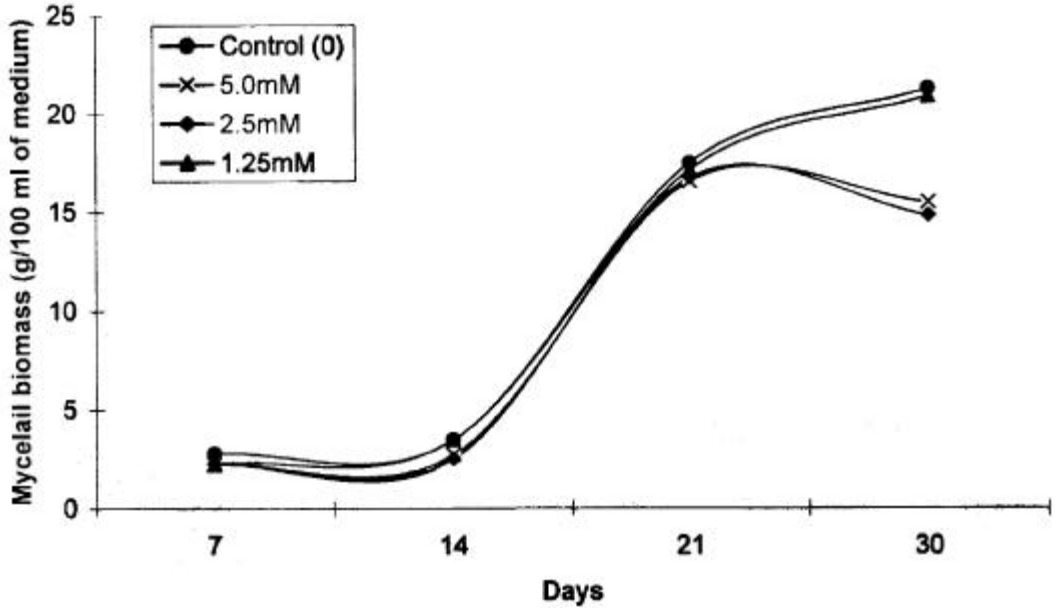

Figure 1. Effect of different concentration of dbc-AMP on growth in terms of mycelial biomass (g/100 ml) of *T. indica*.

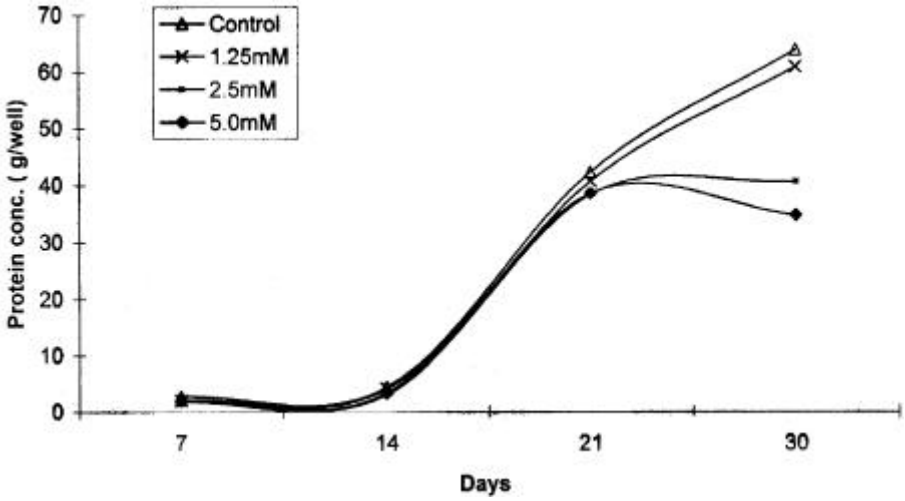

Figure 2. Growth kinetics in terms of soluble protein concentration of control and dbc-AMP treated cultures of *T. indica*.

ously increased following subsequent days of incubation. Fungal cultures grown in the absence of dbc-AMP supported the mycelination in the form of network of fungal hyphae. While fungal culture grown in the presence of dbc-AMP stimulated the process of sporidiation. The controlled culture stimulated the sporidiation process only after 21 days of incubation. Although, morphological differentiation from mycelial to sporidial phase occurred spontaneously *in vitro* cultures, the process was enhanced by addition of dbc-AMP. The chemical agent could therefore, prove extremely useful in inducing sporidia formation and subsequent development as well as altering the virulence of pathogen.

Molecular switch for conversion of sporidia, mating type functions and pathogenesis in *T. indica* may be centered on near c-AMP adenylate cyclase system. Cyclic-AMP appears to control entry into parasitic development pathway by an effect on the mating type locus. The mating type locus acts as a 'master developmental switch' responding to the environmental agents and controlling entry into conjugative, sporulative or vegetative morphogenetic pathways. The results of ELISA for quantification of sporidia further supported the role of dbc-AMP as inducer of sporidiation. It was observed that sporidia formation was enhanced in the presence of dbc-AMP; although it also occurs spontaneously. To observe the formation of sporidia in liquid culture, indirect quantitative ELISA was performed. The fungal cultures were harvested at 3, 7, 14, 21, 30 and 45 days interval. The fungal extracts (100 µl) were coated in ELISA plate in duplicate. The indirect ELISA was performed using anti-sporidial antibody and reactivity of antibody was extrapolated from standard curve of absorbance at 405 nm vs sporidial number (data not given). Indirect ELISA was performed using anti-sporidial antibody and reactivity of anti-sporidial to fungal protein extracts was considered 100% reactivity with sporidial protein and not mycelial protein as cross reactivity of these antibodies with mycellial protein was negligible.

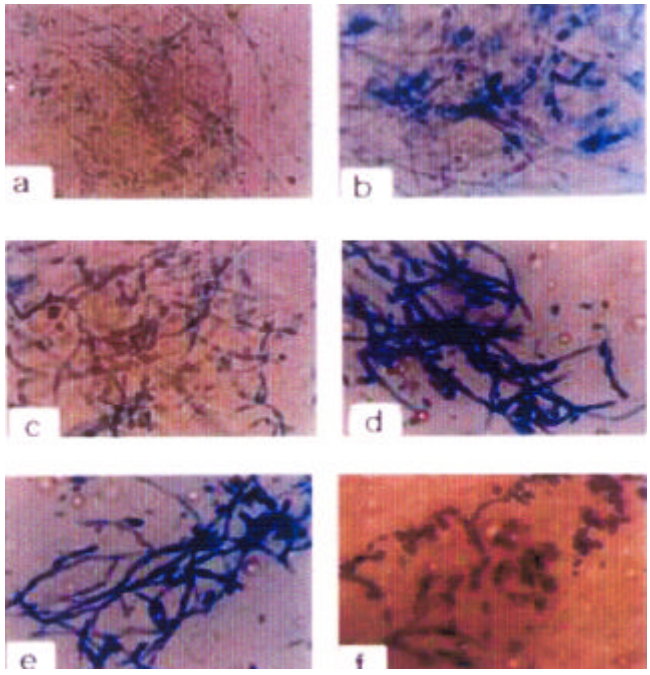

Figure 3. Effect of dbc-AMP (2·5 mM) on morphogenetic development of fungal pathogen (*T. indica*) during different time intervals of growths in axenic culture. (**a**, **c**, **e**) Control (without dbc-AMP). (**a**) 7 days grown culture. (**c**) 14 days grown culture. (**e**) 21 days grown culture. (**b**, **d**, **f**) In the presence of dbc-AMP. (**b**) 7 days grown culture. (**d**) 14 days grown culture. (**f**) 21 days grown culture.

The absorbance at 405 nm increased at 7 to 21 days from 0·12 to 0·460 and after that it was decreased at 30 days. By extrapolating from standard curve (absorbance at 405 nm vs sporidial number), 3×10^3, 4×10^3, $2{\cdot}4 \times 10^6$, and $2{\cdot}1 \times 10^6$ sporidia were formed at 7, 14, 21, and 30 days grown control cultures (figure 4). Maximum reactivity was observed at 21 days, indicating maximum induction of sporidia at this period of growth in liquid culture, i.e. $2{\cdot}4 \times 10^6$ sporidia per 10 ml of culture. However, the number of sporidia was more than doubled i.e. $6{\cdot}8 \times 10^6$ in 21 days grown culture treated with dbc-AMP (figure 5). The study further provides evidence that c-AMP acts as inducer of sporidiation at distinct period during differentiation and c-AMP signalling pathway not remain active throughout the differentiation period of *T. indica* in axenic culture.

Egel (1989) suggested that c-AMP is thought to play a general role in signal transductional pathway that monitors nutritional status of the cell. In earlier experiments also it has been shown that starvation is required for inducing mating in *Saccharomyces pombe*. In case of *Saccharomyces cerevisiae* c-AMP level is regulated via adenylyl cyclase. Two G-proteins, Ras2p and Gpa2p, are thought

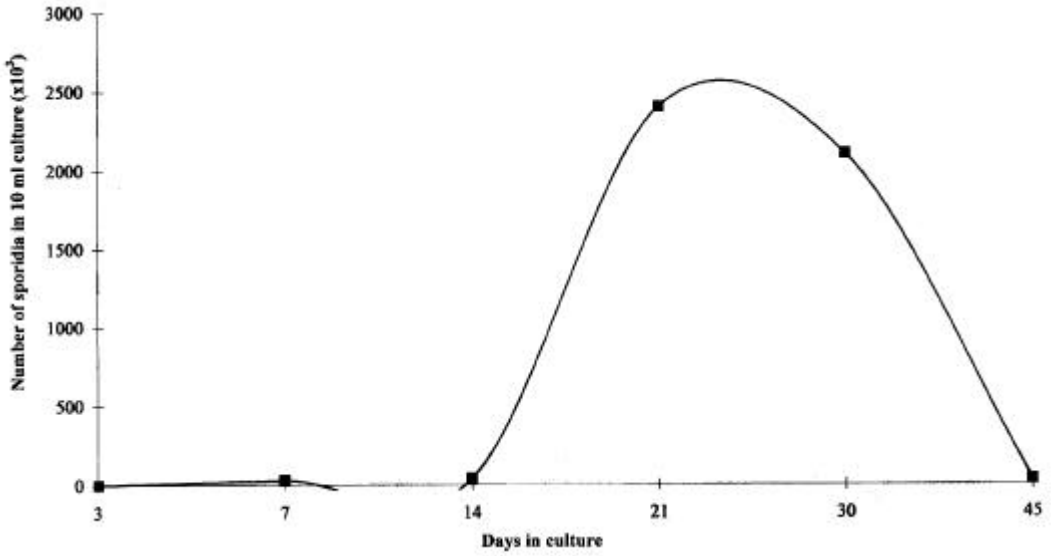

Figure 4. ELISA for determination of induction of sporidiation during growth cycle of *T. indica* in liquid culture.

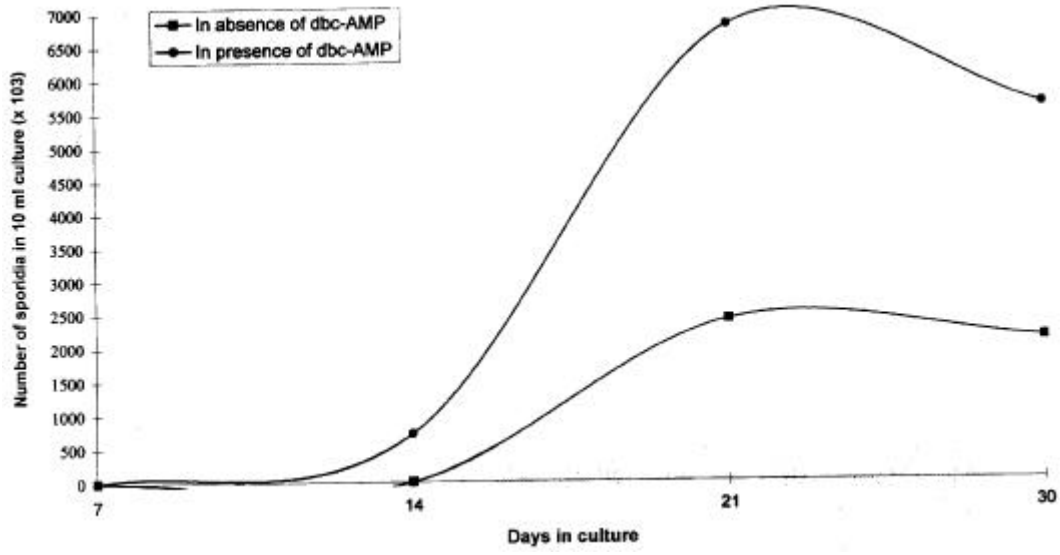

Figure 5. Influence of dbc-AMP on induction of sporidia during growth cycle of *T. indica* in liquid culture by quantitative ELISA using anti sporidial antibodies.

to influence c-AMP levels via regulation of adnylyl cyclase in *S. cerevisiae*. Mutants defective in both genes (*Ras2p* and *Gpa2p*) exhibits a very slow growth phenotype (forming less mycelination) on rich medium (Kubler *et al* 1997; Xue *et al* 1998). This effect can be suppressed by exogenous supply of c-AMP or mutation in *PDE-2* gene encoding c-AMP phosphodiesterase (Kubler *et al* 1997; Lorenz and Heitman 1997; Xue *et al* 1998). The signalling and sensing mechanisms that are involved may provide insight for understanding the transition from filamentous growth in smut fungi and construction of haploid sporidia to the infectious dikaryon. It is clear that general topic of nutritional status and signalling in phytopathogenic fungi requires considerable attention. Hence, these useful paradigms can also be applied in KB (*T. indica*) for understanding the transition, formation of specialized cell types and also in understanding the biology of pathogen. It provides an obvious entry point to learn more about aspects of cell-to-cell signalling, differentiation (e.g. mating structures, sporidia formation) and regulation of gene expression and alteration in surface properties in fungi. In the present investigation attempt has been made to study the influence of dbc-AMP on the differential expression of proteins through comparison of the protein profiles of *T. indica* cultures at different stages of growth cycle and examining the utility of electrophoretic procedures i.e. SDS-PAGE as an aid in assessing the variations during different stages of morphological differentiation of fungi under *in vitro* culture. The protein banding patterns obtained suggests marked changes that have been associated with the transition from vegetative mycelial phase to the sporidial phase.

Such variations may be due to differential expressions of proteins at two stages of development or due to transition from vegetative mycelial to sporidial phase. The commonly used measure of variation is the calculation of similarity coefficient, which is generally employed for comparison of isolates of the same species or sub groups within a species (Tohme *et al* 1996). However, in present study, similarity coefficient has been used for the comparison of fungal population at different stages of growth cycle (table 1). The number of bands present were 14, 12, and 7 in the absence of dbc-AMP while 20, 19 and 9 bands were observed in the presence of dbc-AMP at 14, 21 and 30 days interval, respectively. The similarity coefficient between 14–14, 14–21, 14–30 days are1, 0·46 and 0·24 in the presence of dbc-AMP, while in the absence of dbc-AMP these values are 1, 0·61 and 0·50, respectively. The continuous decrease in similarity coefficient suggests the transition of fungal population from one morphological phase to another. The population which predominates at initial period of growth is the vegetative mycelial phase. However, at later stages of growth, sporidia/sporogenous mycelium gets accumulated/associated with the decrease

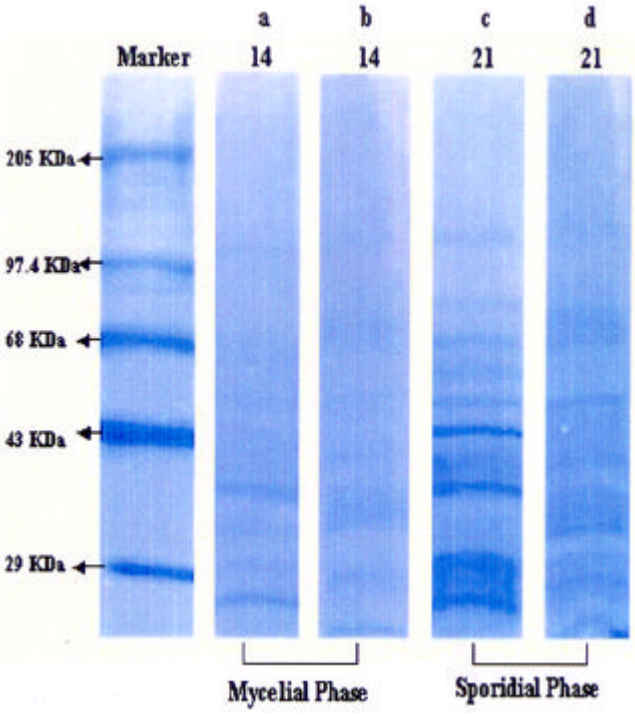

Figure 6. Effect of dbc-AMP on changes in protein profiles of fungal protein of *T. indica* at 14 and 21 days growth in culture. (**a**, **c**) In the absence of dbc-AMP. (**b**, **d**) In the presence of dbc-AMP.

Table 1. Effect of dbc-AMP on changes in protein profiles and similarity coefficient of *T. indica* culture grown at different time intervals.

	In the presence of dbc-AMP				In the absence of dbc-AMP			
	Number of bands				Number of bands			
Days of culture	Similar bands	Dissimilar bands	Total number of bands	Similarity coefficient	Similar bands	Dissimilar bands	Total number of bands	Similarity coefficient
14	6	2	20	1	6	1	14	1
21	6	1	19	0·615	6	1	12	0·462
30	6	–	9	0·500	6	–	6	0·241

in the soluble protein content. There are certain bands, which are present only during the initial stages of growth of the fungus. It has been supported by morphological examination at experimental phase of the growth of either mycelial or sporidial rich fungal population. The fungus is in vegetative phase; hence, these bands are vegetative phase-specific. The bands that are present throughout the stages of the cultures may be those of the structural proteins, which are required throughout the growth cycle of *T. indica*. Hence, they are continuously expressed (figure 6). Some bands were present in all the stages of growth cycle of the fungus but the intensity of the bands greatly varied. This variation may be due to some kind of post-transcriptional and post-translational changes, which may affect the mobility of the band slightly from their original position. There are certain bands, which are associated with only a specific stage. These represent unique kind of protein whose presence marks specifically the actual stage of the culture, since they are expressed only at that stage of growth of fungus. The characterization of this stage-specific protein will enable to provide a useful marker for the study of morphological differentiation of *T. indica*.

In the present study, polyclonal antibodies generated in New Zealand white rabbit against partially purified antigen devoid of high molecular glycoproteins of fungal mycelium and intact sporidia of *T. indica* were used to monitor the changes associated with antigenic properties of fungal population in the presence of dbc-AMP during

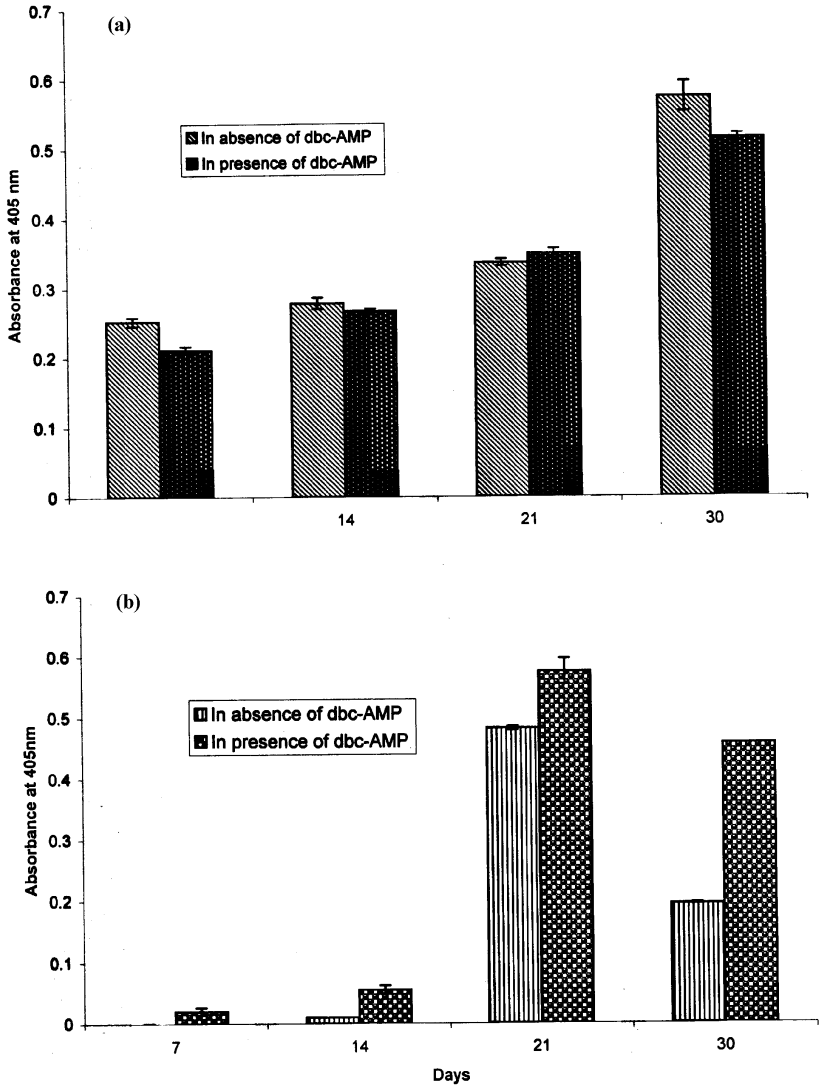

Figure 7. Alteration of expression of all surface antigens during different time intervals of growth as detected by ELISA techniques using polyclonal antibodies raised against partially purified mycelial proteins and intact sporidia. (**a**) Using anti mycelial antibodies. (**b**) Using anti sporidial antibodies.

morphogenetic development. The reactivity of polyclonal antibodies was determined with fungal population growing in the presence of dbc-AMP using microtitre ELISA as described above. Alteration of several cell surface antigen(s) accompanies the change in morphology produced by the influence of signalling molecules/differentiation inducers, viz. dbc-AMP. The reactivity of antisera raised against mycelial and sporidial in the present study was checked with fungal populations grown in the presence and absence of dbc-AMP by microtitre ELISA (Varshney 2003). It was found by the antigen concentration kinetic and antibody dilution studies using ELISA revealed that 250 ng antigen and antibody titre of 1 : 2000 was found to be optimum. It was seen that treatment of fungal cells with dbc-AMP resulted in decrease in binding of anti-mycelial antibodies to fungal proteins when compared to control (without dbc-AMP) at later stages of growth. However, dbc-AMP treatment caused the increased binding of anti-sporidial antibodies during growth progression (figure 7). The reactivity patterns and differential binding of the anti-mycelial and anti-sporidial antibodies to fungal population during growth cycle of *T. indica* further suggested the changes in the antigenic properties of fungus *T. indica* during growth cycle in the presence and absence of dbc-AMP.

Altered distribution of mycelial antigens in the presence of dbc-AMP is associated with morphogentic development of fungus, *T. indica*. The decreased and increased binding of polyclonal antibodies in the presence of dbc-AMP was intimately linked with the switching over from mycelial phase to sporidial phase. The molecular understanding of such changes in the developmental/differentiation marker(s) will provide an insight about KB pathogenesis, sexual development and virulence.

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