Variability studies in *Mycogone perniciosa* causing wet bubble of *Agaricus bisporus*

By

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CERTIFICATE-I

This is to certify that this thesis entitled, **"Variability studies in *Mycogone perniciosa* causing wet bubble of *Agaricus bisporus*"** submitted for the degree of **Doctor of Philosophy** in the subject of **Plant Pathology** to **Chaudhary Charan Singh Haryana Agricultural University, Hisar** is a bonafide research work carried out by **Mr. Man Mohan, Admn. No. 2012A45D,** under my supervision and that no part of the thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been duly acknowledged.

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ABBREVIATIONS

et al.	:	*et alia* = and others
i.e.	:	id est = that is
pH	:	Negative logarithm of hydrogen ion concentration
viz.	:	Namely
µm	:	Micro meter
%	:	Per cent
bp	:	Base pairs
CPT	:	Candidate plus treez
CTAB	:	Cetyl trimethyl amonium bromide
DNA	:	Deoxyribo Nucleic Acid
dNTP	:	Deoxyribonulceotide triphosphate
EDTA	:	Ethylene diamine tetra acetic acid
Lab	:	Laboratory
NTSYS-PC	:	Numerical taxonomy and multivariate analysis system
PCR	:	Polymerase chain reaction
RNase	:	Ribonuclease
RAPD	:	Random amplified polymorphic DNA
RNA	:	Ribo nucleic acid
Taq	:	*Thermus aquaticus*
TBE	:	Tris-Borate EDTA
TE	:	Tris-EDTA
Tris	:	2-amino-2 (hydroxymethyl)- 1,3-propandiol
UPGMA	:	Un-weighted pair group method with arithmetic mean
UV	:	Ultra-violet

CHAPTER-I

INTRODUCTION

Diversification in any farming system imparts sustainability and mushroom cultivation is one such component that imparts diversification and helps in addressing the problems of quality food, health and environmental sustainability. Mushroom farming is today being practiced in more than one hundred countries. Presently, three geographical regions *viz*, Europe, America and East Asia contribute about ninety six per cent of world mushroom production. The world mushroom production is continuously increasing from 0.30 to 3.41 million tonnes over period of last 50 years from 1961-2010 as per FAO statistics. The three major mushroom producing countries are Chine, USA and Netherland which account for more than sixty per cent of world production. The other leading countries which contribute much for mushroom production are Poland, Spain, France, Italy, Ireland, Canada and United Kingdom. The current Mushroom production in India is 1.2 lakh metric tons. In seventies and eighties button mushroom was grown as seasonal crop in hills, but with the development of the technologies for environmental control and increased understanding of the cropping system, mushroom production shot up from 5000 tonnes in 1970 to over 1,20,000 tonnes in 2011. In Haryana the annual production of white button mushroom is 10,000 tons (Prakasam, 2012).

Button mushroom comprise a good nutritious diet for all ages groups of people and under all conditions of health. They are rich source of proteins especially amino acids like lysine and tryptophan that are deficient in cereals. The carbohydrate content ranges from 4.5 to 5.0 per cent but are in the form of glycogen, chitin and hemicellulose instead of starch. The fat is as low as 0.3 per cent but is rich in linoleic acid, which is an essential fatty acid. Cholesterol is absent which is replaced by ergo-sterol and its later gets converted to vitamin D in the human body. Button mushroom is a good source of vitamin C and vitamin B-complex, particularly thiamine, riboflavin, niacin, biotin and pantothenic acid (Singh *et al*., 2011). Folic acid and vitamin B_{12}, which are generally absent in most vegetables, are present in high amount in this mushroom. Such vitamins also supply a range of valuable minerals especially potassium and iron (Kouser and Shah, 2013).

Mushroom cultivation is affected by a large number of biotic and abiotic factors. Fungi, bacteria, viruses, nematodes, insects and mites are different biotic factors that damage the mushroom crop directly or indirectly. Among the fungal pathogens, *Mycogone perniciosa* (wet bubble), *Verticillium fungicola* var. *fungicola* (dry bubble), *Trichoderma* spp. (green mould) and *Dactylium dendroides* (cobweb) are the prominent mycopathogens inflicting considerable yield losses (Sharma *et al*., 2011).

Mycogone perniciosa (Magnus) Delaroix is the causal organism of wet bubble disease, which is also known as La Mole of *Agaricus bisporus* (Lange Imbach). This disease is very contagious resulting in severe crop loss (Umar *et al.,* 2000). This disease has been known for many years worldwide wherever, *A. bisporus* is grown (Fletcher *et al*., 1995). It has been reported a serious disease in major mushroom growing countries viz., China, USA, United Kingdom, Taiwan, South Africa, Brazil, Hungry, Australia and Poland from time to time. In India, this disease was reported for the first time in 1978 from some mushroom farms in Jammu and Kashmir (Kaul *et al.,* 1978). Later, this disease has been reported from the state of Himachal Pradesh, Haryana and Maharashtra (Sharma and Kumar, 2000, Bhatt and Singh, 2000). A considerable loss in yield due to this disease has been reported which varied from 15.7 to 80.1 per cent whereas, artificial inoculation can result in yield loss of upto 100 per cent (Bhatt and Singh, 2000). Recently, seasonal mushroom grower of Haryana has been regularly facing the incidence and the quality and quantity loss due to this disease in mushroom farms.

Mycogone perniciosa infects *Agaricus bisporus* at various stages of its development. The results from infection at pinhead-stage is tumorous masses, later infection leads to malformation of the mushroom, while in severe infection it causes the appearance of white fluffy growth and distortion of stalk generally. The disease leads to drastic change in the shape of mushroom as well as malformation, white, fluffy mycelial growth and amber brown droplets have been investigated as the morphological and phenotypic changes (Fletcher *et al.,* 1994). The symptoms studies by Fletcher *et al*., (1995) revealed that the colonies have either an even or uneven edge and the mycelial growth v,
.0aries from aerial, dense and sparse, while the colour of colonies varies from yellow-brown, dark brown, pale brown to white.

Gray and Morgan-Jones (1980) reported that *M. perniciosa* produces small thin-walled conidia (referred to as phialospores) on *Verticillium*-like conidiophores together with much larger bicellular conidia (commonly referred to as either aleuriospores or chlamydospores). Holland and Cooke (1991) described additional spore forms of *M. perniciosa*, including lateral smooth conidia, intercalary chlamydospores and arthroconidia. The primary source of pathogen is contaminated casing soil. When contaminated casing soil act as primary source of mushroom infection, the symptoms appear within 10 to 14 days. The water-borne dissemination of the spores is the most important means of dispersal, whereas, the dry spores of *M. perniciosa* can also be spread by wind (Cross and Jacobs, 1969). Spores may survive on the surface of buildings or may be carried in crop debris and in this way can contaminate crops. The mushroom pickers may be the active disseminator of the pathogen by their hands, tools, boxes (Fletcher *et al*., 1994).

Variability in colony morphology and physiology has been observed among the isolates of *Mycogone perniciosa.* Lapierre *et al*. (1971) found that some pathogenic strains, which are slow growing on agar, were highly pigmented and produced numerous aleuriospores. Other isolates were weakly pathogenic, producing more vegetative growth with little pigmentation. Kim (1987) found that the strains of *M. perniciosa* were more virulent on cream coloured strains of *A. bisporus* than normal strain of this pathogen. The virulent strains had a similar optimum growth temperature to the normal strains but differed in optimum range of pH, thermal death point and the fungicidal sensitivity (Lambert, 1930).

Molecular variability studies of *M. pernociosa* was done for the isolates from different regions by using different markers *viz*., ITS and RAPD. Multilocus genotyping of ten isolates of *M. perniciosa* collected from different locations/regions were identical at intra-species levels by the RAPD profiling of isolates by five RAPD primers (Sharma *et al*., 2011). PCR amplification of ITS region of rRNA gene yielded an ITS fragment of approximately 600 bp length in all the ten isolates. No inter or intra species ITS length diversity was detected. The DNA nucleotide sequence of all ten isolates of *Mycogone perniciosa* were identical. No intra-species variation in internal transcribed spacer (ITS) regions of 5.8s gene was found and the identical RAPD profile suggests that all ten isolates belongs to a single strain of *M. perniciosa.* This strain is widely distributed across different geographical locations and results showed the variability among isolates (Sharma *et al*., 2011). Fletcher *et al*., (1995) reported that polymorphism in two species of *Mycogone* clearly distinguished *M. perniciosa* and *Mycogone rosae* by RAPD primers. Eight isolates of *Mycogone perniciosa* showed homogeneity in rDNA, except two isolate from China had variation by RAPD primers.

Till now, meager information is available on *Mycogone perniciosa* in India, especially regarding variability in this pathogen. In this context present study was carried out to find out the variability among the isolates of *Mycogone perniciosa* infecting *Agaricus bisporus* in relation to cultural, morphological, physiological and molecular variability. Keeping this in view, the present study has been conducted to ascertain the following objectives:

1. To study the morphological, physiological and chemosensitive variability of different isolates of *Mycogone perniciosa.*
2. Pathogenic and molecular variability among selected isolates of *Mycogone perniciosa*

CHAPTER-II

REVIEW OF LITERATURE

Agaricus bisporus (Lange) Imbach, the button mushroom is the most commonly cultivated mushroom worldwide. The Cultivation of button mushroom is a highly specialized process which is commercially grown under strictly controlled environmental conditions. In Northern India, its cultivation is seasonal under poor hygiene and sanitation which leads to the build-up of pathogens. The success of mushroom production is dependent upon many interacting factors, of which disorders/diseases caused by pests and pathogens play a significant role. Fungi are most important group of pathogens and their effect is most common on mushroom fruiting bodies rather than on mushroom mycelium (Pieterse, 2005). Recently, the disease has engaged as a serious problem causing considerable yield losses in various parts of India. It also leads to losses in quality and quantity parameters. Therefore, present review of literature pertaining to variability in *Mycogone perniciosa* causing wet bubble of *Agaricus bisporus* has been presented hereunder.

2.1 History of mushroom cultivation

The term mushroom was initially used to describe only the edible fungi originating from the order Agaricales, while poisonous varieties were referred to as toadstools (Eicker, 1990). The first book describing cultivation of mushrooms was written by De Tournefort in 1707. The history of mushroom cultivation goes back to the ages of "VEDA" wherein the mention was made in the classical religious scriptures like "Rig Veda" and "Atherva Veda" about the use of juice from fly agaric mushroom (*Amanita muscaria*) as an intoxicating drink named as Soma. *A. bisporus* was first cultivated in seventeenth century when Louis XIV encouraged people to grow mushrooms in their outdoor gardens (Pacioni, 1985). However, the systemic research on mushroom domestication was not aimed at that time for quite a long period (Prakasam, 2012).

2.2 Diseases of *Agaricus bisporus*

Mushrooms are affected adversely by a large number of biotic and abiotic factors. Among the biotic agents, fungi, bacteria, viruses, nematodes, insects and mites cause damage to mushrooms directly or indirectly. A number of harmful fungi were encountered in compost and casing soil during the cultivation of white button mushroom. Many of these act as competitor moulds, thereby adversely affecting spawn run, whereas, others attack the fruiting bodies at various stages of crop growth producing distinct disease symptoms. There is complete crop failure depending upon the stage of infection, quality of compost and environmental conditions (Sharma *et al.,* 2007). The identification of fungal pathogens and competitor moulds associated with cultivated mushrooms has shown that the fungi involved

are *Mycogone perniciosa, Cladobotryum dendroids, Verticillium fungicola,* while the moulds were *Arthrobotrys oligospora, Botryotrichum piluliferum, Botrytis cinerea, Chaetomium olivaceum, Chromelosporium fulvum, Chrysosporium merdarium, Geotrichum candidum, Merium* spp. *Mortierella ramanniana, Papulospora byssina, Scopulariopsis brevicaulis, Sepedonium niveum, Sporotrichum roseum, Trichoderma harzianum, Trichoderma viridea* and *Trichoderma roseum.* These fungi are more common under Indian conditions, whereas, significant proportion of the total mushroom production is affected by the use of unpasteurized compost prepared by long method, by seasonal growers, which harbors several parasitic and weed moulds (Elicker *et al.,* 1989). Weed moulds compete with mushroom mycelium for space, water and nutrients.

2.3 Wet bubble disease

Wet bubble in white button mushroom incited by *Mycogone perniciosa* has been reported as one of the serious diseases from all the major mushroom growing countries of the world. Wet bubbles or La Mole (*M. perniciosa*), first described from Paris in 1888, and stated to be responsible for the heaviest losses in mushroom beds in France, England and the United States (Nielson, 1932). The disease has also been reported to assume serious problem in other major mushroom growing countries of the world such as the United Kingdom, the Netherlands, USA, China, Taiwan, South Africa, Brazil, Hungary, Australia and Poland from time to time.

In India, this disease was reported for the first time in 1978 from some mushroom farms in Jammu and Kashmir (Kaul *et al*., 1978). Later, this disease has been reported from the states of Himachal Pradesh, Haryana and Maharashtra (Sharma, 1994, Sharma and Kumar, 2000, Bhatt and Singh, 2000).

2.3.1 History

Mycogone disease of button mushrooms also called wet bubble disease and La Molé, is caused by *Mycogones perniciosa*. The disease has been known for many years, and is reported worldwide, wherever, *A. bisporus* is grown (Fletcher *et al*., 1995). *M. perniciosa* causes distortion of affected mushrooms and the characteristic undifferentiated lumps of tissue, which, is also called as sclerodermoid mushrooms.

According to Smith (1924) the earliest scientific records of *Mycogone* disease of mushrooms are those of Magnus (1888) and Cooke (1889). Magnus was unable to identify the fungus and although the ascus stage was not seen, he named the teleomorph stage *Hypomyces perniciosa* by analogy with *Hypomyce schryosospermus* (Smith, 1924 and Eicker *et al*., 1989). Cooke stated that this fungus he found resembled both *Mycogone rosea* and *Mycogone alba* but differed in having amber coloured chlamydospores (Smith, 1924). Constantin and Dufour (according to Smith, 1924) made an exhaustive study of the disease and after growing the fungus side by side with *Mycogone rosea* decided that it was a distinct

species and named it *Mycogone perniciosa*. Smith, (1924) stated that wet bubble caused heaviest losses among all diseases in mushroom beds in France, England and United States.

In USA, *M. perniciosa* was isolated from 3.7 per cent samples collected from various mushroom farms. Forer and his associates (1974) while estimating the qualitative and quantitative losses caused by wet bubble and dry bubble in Pennsylvania (USA), reported that these two diseases induced 2.2 million lbs as qualitative and 19.7 million lbs as quantitative loss of mushrooms. Nair (1976) conducted a survey of 24 mushroom farms in New South Wales during 1975-76 and observed that the most economically important disease in these farms was wet bubble. Sharma and Kumar (2000) reported that the natural incidence of wet bubble disease of button mushroom ranged from 1 to 100 per cent in northern India. Loss in yield in *A. bisporus* due to this disease under artificial inoculation conditions has been reported to vary from 15.72 to 80.13 per cent. Bhatt and Singh (2000) have reported the yield loss up to 100 per cent as a result of artificial inoculation of *M. perniciosa*.

2.3.2 Host range

Mycogone perniciosa, though a major pathogen of *Agaricus bisporus*, is also capable of infecting other mushroom species. It was reported that *Mycogone perniciosa* can infect *Agaricus campestris*. It was reported that *Pleurotus eryngii* and *Plerrotus nebrodensis* were also susceptible to *Mycogone perniciosa* (Sharma *et al*., 2007). Sharma and Kumar (2000) reported that the different strains of *Agaricus bisporus viz*., U-3, S-11, S-791, S-910 and *Agaricus bitorqus* is *viz*., NCB-6, NCB-13 were susceptible to *Mycogone perniciosa* under *in vivo* conditions.

2.3.3 Epidemiology

Spread of *Mycogone perniciosa* occurs primarily through casing soil but the introduction of pathogen through other agencies, like spent compost and infected trash. The infection can be air-borne, water-borne or may be mechanically carried by mites and flies (Garcha, 1978). Hsu and Han (1981) reported water splash as an important factor for wet bubble spread on the beds. Bech *et al*. (1981) reported that spread of the pathogen occurred through contact during watering and especially during harvesting. They also observed that contaminated containers can be an active source of spread over a long distanc. Contrary to other reports it was also shown that spores of *Mycogone perniciosa* were also spread by air current (Tu and Liao, 1989).

Kumar and Sharma, (1998) reported that the transmission of *Mycogone perniciosa* under *in vitro* conditions by sciarid and phorid flies was 100 per cent on malt extract agar (MEA) medium and 4-12 per cent on compost. Chlamydospores have been reported to survive for a long period of time (upto 3 years) in casing soil which served as the primary source of inoculum. The aleuriospores produced on the surface of monstrous structures are probably responsible for their secondary infection.

2.3.4 Symptomatology

Many workers have described symptoms of wet bubble at different stages of mushroom development. Smith (1924) recognized two types of symptoms in the infected sporophores and the sclerodermoid masses, which he considered as the result of infection by *M. perniciosa* at different stages in the development of sporophores. Thus, when disease infection took place before the differentiation of stipe and pileus the selerodermoid forms, whereas, infection after differentiation resulted in the production of thick stipe with deformation of the gills (Fletcher and Ganney, 1968).

These symptoms appeared in severe form in places with high humidity. The early infection badly affected fruiting bodies, turning them into shapeless mass covered with the pathogens white fluffy mycelium and deformed carpophores became brown and started to decay. Internal tissues were also discolored, watery and showed holes resulting from decay. Symptoms were accompanied by an unpleasant odour. Under dry conditions, tissues remain dry as in the case of infections caused by *Verticilium* and if the stipe is affected, it decayed and turned red brown. In addition to distortion *Mycogone perniciosa* may produce small fluffy white patches of mycelium on the surface of casing, following the infection of a developing mushroom below the casing surface. White mycelium growth was usually very distinct from mushroom mycelium and turn brown in later stage (Sabharwal and Kapoor, 2015).

Garcha, (1978) reported the irregular, nodular and tumorous fungal masses were formed and no differentiation or organogenesis of the cell mass takes place. This Mycopathogen grew on the surface as fluffy mycelium but was absent in deeper lesions. Transmission EM revealed two kinds of cell wall reactions, either focal swelling like cushion at the site of adhesion of *M. perniciosa* or focal lytic changes with swollen mitochondria.

2.4 Cultural and morphological variability

The wet bubble disease is caused by *Mycogone perniciosa* and its perfect stage is *Hypomyces perniciosa.* Mycelium of the pathogen was compact white but it was brown or yellow in some cases. Hyphae were branched interwoven, septate, hyaline, 3.5µm in diameter. The conidiophore were short , slender, branched, hyaline measuring 200x3-5µm and having sub-verticillate to verticillate branches which bear thin walled, one-celled conidia measuring 5-10x4-5 µm in size with two celled chlamydospores where upper cell was warty, thick walled, globose, bright colored measuring 15-30x10-20 µm, whereas, the lower cell was hyaline, smooth, 5-10x4-5 µm thicker (Sharma *et al.,* 2007).

Variations in colony morphology and physiology have been observed among the isolates of *Mycogone perniciosa*. Lapierre *et al.*(1971) found that some pathogenic strains were slow growing on agar, were highly pigmented and produced numerous aleuriospores. The other isolates were weakly pathogenic, producing higher vegetative growth with little pigmentation. The colonies were regular in growth with either dense or aerial, while some isolates were irregular in growth. Colony colour, which to some extent indicates the

production of aleuriospores, varied from white to dark brown (Glaoclija *et al.*, 2007). *Mycogone perniciosa* produces a copious flocculent mycelium on most substrata (Smith, 1924). In the early stages this mycelium was white which later changes to a light amber brown. Fletcher *et al.* (1995) found that colonies had either an even or uneven edge and the amount of aerial mycelium varied from dense to sparse, while the colour of colonies were yellow brown, dark brown, pale brown or white. *Mycogone perniciosa* and *Mycogone rosae* can be differentiated from each other by the pale color of the chlamydospores, colony and by the *Verticilium* state (Brady and Gibson, 1976).

2.5 Spore characteristics

Smith (1924), reported that *Mycogone perniciosa* exhibited two type of spores aleuropspores and phialospores on *Agaricus bisporus.* Gray and Morgan-Jones (1980) reported that *M. perniciosa* produced small thin-walled conidia called as phialospores on *Verticillium*-like conidiophores together with much larger bicellular conidia called as aleurospores. The phialoconidia were the first spores to appear on an infected mushroom and these were found at the edges of the *M. perniciosa* mycelium. These were also the first spores produced *in vitro* when the nutrient levels was still high, whereas, the conidia were produced by the formation of a constriction near the apex (Smith, 1924). The portion beyond the constriction enlarges until a long cylindrical spore with pointed ends was cut - off. When the formation of the conidium was practically complete a median septum appeared. The mature conidium was septate with equal sized cells (Smith, 1924), whereas, phialospore size varied within the range of 13.6-17.5 x 3.7-6.1µm (Fletcher *et al.*, 1995). The upright vertically branched conidiophores that bear the conidia (Smith, 1924), varied from range 27.0-58.2 x 3.0-3.7µm in size (Fletcher *et al.*, 1995).

The Chlamydospores were formed later on the short branches at the base of conidiophores and these were much more resistant than thin-walled conidia (Smith, 1924). The upper cell of the chlamydospores swelled and its wall thickened more quickly than the lower cell (Smith, 1924). Thus, a bicellular spore was formed, the upper cell of which was almost spherical with a thick stratified wall (Holland and Cooke, 1991). Chlamydospore size varied from 18.3-23.7 x 19.9-26.1 mm for the upper cell and 10.4-14.5 x 12.5-17.3 mm for the lower cell (Fletcher *et al.*, 1995). Brady and Gibson (1976) also found the chlamydospore size fall within this range (Pieterse, 2005).

2.6 Effect of Temperature and pH

The growth of *M. perniciosa* at different pH and temperatures were investigated. The mycelia growth of *M. perniciosa* isolates was best at a temperature of 25°C and only slightly worse at the temperature of 20°C. On the other hand, the mycelial growth reduced significantly at temperature of 15 °C (Siwulski *et al.*, 2011). The optimum temperature for their mycelia growth was 24 °C (Lambert, 1930). Light and darkness seemed to have little or no effect but higher temperature *viz.,* 25 °C accelerated their optimum growth (Smith, 1924).

Studies on the cultural characteristic of different *M. perniciosa* isolates was conducted and 25°C was found to be optimum for their development (Glamoclija *et al.*, 2008). A slight above temperature was optimum for the growth of *M. perniciosa* mycelium (Tan *et al*., 1994). An incubation temperature of 30°C was found to considerably reduce mycelium development of all the examined *M. perniciosa* isolates. In France, the situation of mushroom cultivation in underground bunkers, where the temperature amounted to 15°C, disease caused by *M. perniciosa* occurred less than in traditional cultivation at the temperature 16-19°C (Bech *et al*., 1989). *M. perniciosa* chlamydospores can survive at 105°C for ten minutes in the dry state (Bech and Kovacs, 1981).

M. perniciosa, mycelium developed best at pH 5.5, irrespective of the examined isolate. Within the medium pH values, the mycelia of all isolates developed at similar rates (Siwulski *et al.,* 2011). The optimal pH for the development of *M. perniciosa* mycelium ranged from 5.0 to 5.6 (Tan *et al.,* 1994). Radial mycelial growth of the pathogen in the pH range from 4-8 varied from 54.4 to 78.18mm, maximum being at pH 5.0 (Sharma and Kumar 2000). This was slightly less than the optimum pH of 6.2 (Hsu and Han 1981). No growth of the pathogen was recorded at pH 3.5 and 9.0 (Sharma and Kumar, 2000).

2.7 Chemosensitivity and interaction.

Bhatt and Singh, (2002) reported that *M. pernociosa* was completely inhibited by Bavistin (25 µg/ml), Captaf (30 µg/ml), Ridomil MZ (300 µg/ml) and Bavistin+Formalin (12+12 µg/µl/ml). Lower concentrations of these fungicides also affected the growth of pathogen significantly. However, Bavistin (20 µg/ml), Sporgon (15 µg/ml), Ridomil MZ (25 µg/ml), Bavistin+Formalin (10+10 µg/µl/ml) inhibited the growth of this pathogen more than 90 per cent after 7 days of inoculation.

The role of carbendazim, iprodione, prochloraz-Mn, thiabendazole and thiophanate-methyl were tested *in vitro* and *in vivo* for their effect on *Mycogone perniciosa*, the pathogen that causes wet bubble disease of white button mushroom. *In vitro* experiments showed that prochloraz-Mn (ED_{50}=0.006-0.064µg/ml) and carbendazim (ED_{50}=0.031-0.097µg/ml) were the most effective fungicides for inhibiting the mycelial growth of *M. perniciosa*, while iprodione (ED_{50}=1.90-3.80µg/ml) was the least effective (Francisco *et al*., 2010).

The efficacy of five selected fungicides were tested at different concentrations against *H. rosellus,* Bavistin (10 µg/ml), Sporgon (10 µg/ml), Captaf (20 µg/ml) and Bavistin+Formalin (4+4 µg/ml) inhibited 100 per cent growth of pathogen after 24 hrs of incubation. Bavistin (7 µg/ml) showed 100 per cent growth inhibition at 24 to 48 hrs and was found effective in inhibiting the growth of pathogen by 92.95 per cent in 68 h (Bhatt and Singh, 2002). Formalin have also been reported to inhibit the survival of *M. perniciosa*. The inhibition of *M. perniciosa* was 62.5 to 100 per cent when inoculation discs were drenched in 0.5-2 per cent formalin solution for 5 seconds. Exposure of *M. perniciosa* culture to vapours of 1-4 per cent formalin for 6-24 hrs also resulted on 100 per cent inhibition of fungal growth (Sharma, 1999).

Infection on *A. bisporus* by *M. perniciosa* and subsequent development of disease has to take place due to interaction between the host and the pathogen, as in the case with other mycoparasitism reactions *e.g. Verticillium fungicola*, causing dry bubble disease of *A. bisporus* (Dragt *et al*., 1996). Cooke and Rayner (1984) described three possible outcomes when two mycelial systems interact: (1) neutral intermingling of hyphae, (2) deadlock, in which neither individual mycelium enters territory occupied by the other and (3) invasion of domain, with one mycelium partially or completely replacing the other. It is not documented in which of these categories interaction of *M. perniciosa* and *A. bisporus* fits best.

2.8 Molecular variability

Wet bubble disease was first described from Paris in 1888. Later in 1892 dry bubble disease was first reported by Constantin and Dufour, who described all bubble disease then known, and referred to them as *la mole* disease. The word *Mole* was presumably derived from the Latin word *mole* for mass. Constantin and Dufour suggested that all bubble disease were caused by one fungus, *Hypomyces perniciosae,* which could appear in different forms: one bearing two types of spore, a chlamydospore and big *Verticillium-* like conidia, and a second form bearing only small *Verticillium-* like conidia (Pieterse, 2005).

Dry bubble and wet bubble disease were distinguished from each other and described two different fungi as causal agent. The use of DNA polymorphism as a trait has added a new dimension to the recognition and classification of genetic variability in fungi. Classical taxonomy, based upon morphological characteristic and pathogenicity were used for identification of different *Mycogone perniciosa* isolates (Wendyand Lynne, 1998; Liu Weicheng *et al.,* 2003; Gupta *et al.,* 2009; Darvishnia, 2013).

The variability among the *Mycogone perniciosa* isolates at the genetic level have been found which showed their genetic relatedness or genetic distance from each other. On the basis of RAPD, it was concluded that isolates collected from different countries and areas of *M. perniciosa* might be very different from others. Isolates from Fijian, China and other countries showed 70 per cent or more similarity (Tan qi *et al.,*1994). RAPD marker analyses showed a high variability among the twenty three isolates of *M. perniciosa*. Twelve primers detected 105 scoreable products. All of these scorable bands were used to analyses kin relationship among isolates by UPGMA (Unweighted pair group method using arithmetic mean) program. The UPGMA analysis revealed the similarity among eight isolates of *M. perniciosa* from Shanghai and Jiangsu was over 90 per cent (Tan qi *et al.,* 1994).

Sharma *et al.* (2011) reported that etiology and molecular variability of wet bubble disease caused by fungus *Mycogone perniciosa* . Disease samples were collected from various parts of mushroom growing areas. These isolates showed distinct molecular identities varied from 90-91 per cent with other mushroom pathogen.

CHAPTER-III

MATERIALS AND METHODS

The present investigation entitled "Variability studies in *Mycogone perniciosa* causing wet bubble of *Agaricus bisporus"* was carried out at Mushroom Technology Laboratory, Department of Plant Pathology in collaboration of Department of Molecular Biology and Biotechnology, CCS Haryana Agricultural University, Hisar, during winter 2013-2014 and 2015-2016 seasons. Hisar is situated at a latitude of 29°10'N, longitude 75°46'E and an altitude 215.2 m above mean sea level and fall in semi-tropical regions of Western Zone of India. Details of the materials used and methodology adopted during the course of this investigation are given below.

3.1 Materials used in research work

3.2 Collection, isolation, purification and pathogenicity test

3.3 Morphological variability of *M. perniciosa* isolates

3.4 Physiological variability of *M. perniciosa* isolates

3.5 Chemosensitve variability of *M. perniciosa* isolates

3.6 Interaction of *M. perniciosa* isolates with different strains of *A. bisporus*

3.7 Molecular variability in different isolates of *M. perniciosa*

3.1 Materials used in research work

3.1.1 Glasswares

The glasswares *viz*., Petri plates, conical flasks, beakers, funnels, measuring cylinder and culture tubes, etc. used were of the borosilicate quality. The glasswares were kept in the cleaning solution containing 60 g potassium dichromate and 60 ml of concentrated sulphuric acid (H_2SO_4) in one litre of water for 24 hours. Afterwards, the glasswares were cleaned thoroughly with liquid detergent solution and washed in running tap water, rinsed with distilled water and dried in hot air oven.

3.1.2 Equipments

Equipments used during the course of investigation included microscope, autoclave, hot air oven, BOD incubator, laminar air flow, refrigerator, microwave oven, weighing balance, mixer & grinder, pH meter, haemocytometer, bacterial proof filters, electronic camera, LPG gas burner, centrifuge, spectrophotometer, shaker, water bath, vortex, hot plate, mortar & pestle, Eppendof tubes, PCR tubes, PCR tube stand, floaters, micropipettes, thermo cycler, gel-electrophoresis unit, Gel-Doc system *etc.* Small instruments like inoculation needle, glass slides, scalpel, cork borer, razor, glass rods, Oakridge tubes, spatula, syringe, filter papers, plastic tray, muslin cloths, *etc.* were also used.

3.1.3 Sterilization

All types of glasswares including Petri plates, test tubes, conical flasks and pipettes used in the present investigation were sterilized in a hot air oven at 180°C for 1 hr. Inoculation needle, scalpels, forceps, *etc.* were flame sterilized prior to use. Nutrient media were sterilized at 1.1 kg/cm^2 (121.6°C) for 15 minutes in autoclave.

3.1.4 Chemicals, buffers and solutions

Chemicals used for DNA extraction and PCR amplification were of molecular biology grade and were obtained from Life Technologies (India) Pvt. Ltd. All other chemicals used in the present investigation were of molecular/analytical grade. The above chemicals/reagents used for preparing buffers and solutions were sterilized as per need

Table 3.1: Composition and concentration of important solutions and buffers

Reagent	Composition	Concentration
CTAB extraction buffer	Tris base (pH 8.0)	1 M
	EDTA* (disodium, pH 8.0)	0.5 M
	Sodium chloride	5 M
	CTAB	2.0 %
	-mercaptoethanol	2.0 %
Wash 1 solution	Ethanol	70%
	Sodium acetate	0.2 M
Wash 11 solution	Ethanol	70%
	Ammonium acetate	10 mM
RNase A (10 mg/ml)	Tris (pH 8.0)	10 mM
	Sodium chloride	15 mM
	RNase A	10 mg
TE buffer	Tris base (pH 8.0)	10 mM
	EDTA* (disodium. pH 8.0)	1 mM
1X TBE buffer	Tris base (pH 8.0)	0.9 M
	EDTA* (disodium. pH 8.0)	0.02 M
	Boric acid	0.9 M

* Ethylene diaminetetra acetic acid

3.2 Collection, isolation, purification and pathogenicity test

In the present study, fifteen isolates of *M. perniciosa* were collected from different districts of Haryana, Punjab and Himachal Pradesh (Table 3.2). The samples were washed thoroughly with tap water, dried and then kept in paper bags for further isolation of pathogen. The fungi was cultured on fresh potato dextrose agar (PDA) medium for isolation, purification and pathogenicity test. The pathogen was purified and maintained by repeated sub-culturing of each isolates after every month and kept in a refrigerator at 4°C for the further studies.

Table 3.2: Collection of samples of *Mycogone perniciosa* from different areas

S. No.	Isolates	Place of Collection
1	LDP	Punjab Agricultural University, Ludhiana, Punjab
2	SHP	Directorate of Mushroom Research, Solan, Himachal Pradesh
3	NAS	Nauni, Solan, Himachal Pradesh
4	TPN	Taharpur, Panipat, Haryana
5	HSN	HAIC R&D, Murthal, Sonipat, Haryana
6	RHT	Rohtak, Haryana
7	BFT	Bhodia, Bhattu road, Fatehabad, Haryana
8	SKK	Sudha mushroom farm, Kurukshetra, Haryana
9	RSN	Rohat, Sonipat, Haryana
10	BSN1	Bhagana, Sonipat, Haryana
11	KSN	Kharkhoda, Sonipat, Haryana
12	AFT	Akanwali, Fatehabad, Haryana
13	BKK	Bhorsayeda, Kurukshetra, Haryana
14	BSN2	Bainyapur, Sonipat, Haryana
15	SHS	Satrod, Hisar, Haryana

3.2.1 Isolation of pathogen

Isolation of the *Mycogone perniciosa* was made from the infected fruiting bodies, showing typical symptoms of wet bubble disease. The diseased sporophores/sclerodermoid masses were first examined for the associated pathogen by teasing the diseased portion with the help of a needle and observed under microscope. For isolation of the causal fungus, 5 mm small disc segments were cut from the infected sporophore with the help of sterilized cork borer, surface sterilized with 0.1% mercuric chloride for 30 seconds followed by rinsing thrice with sterilized distilled water, blotter dried and inoculated under aseptic conditions on PDA medium in sterilized Petri dishes and incubated the plates at 25 ±1°C.

3.2.2 Purification and maintenance of pathogen

The culture was purified by hyphal tip culture method (Pathak, 1972). The pure culture was obtained and maintained by repeated sub-culturing at monthly intervals. The stock culture in PDA slants was stored at 4°C in a refrigerator. The repeated sub-culturing was done for further studies to avoid the possible loss of pathogenic behavior of the fungus.

3.2.3 Pathogenicity test

In order to prove the pathogenicity of *Mycogone perniciosa* and development of infection on the sporophores, two sets of experiments were carried out. In the first set plastic baskets of 5 kg compost capacity. The baskets were disinfected with 2 per cent formalin and rinsed thrice with sterilized distilled water. The baskets were filled with pasteurized compost, spawned and kept in spawn run room at 24 ± 2°C. In the first set of experiment, spore

suspension ($2\times10^{6}ml^{-1}$) were inoculated in sterilized casing mixture at the time of casing. However, in the second set of experiment, the isolated pathogen was inoculated on healthy pinheads and the fruit bodies with aleuriospore suspension ($2\times10^{6}ml^{-1}$) and also with the mycelial discs from culture plate of the pathogen to observe the development of wet bubble symptom. After inoculation, the baskets were incubated in an isolated room at a temperature 25 ±1°C with relative humidity of more than 85 per cent. An un-inoculated basket was also maintained under similar conditions as check in isolation apart to avoid any contamination. Both the sets of pathogenicity tests were closely monitored for the development of symptoms. Re-isolation of the micro-organism was done from the artificially inoculated sporophores/ sclerodermoid masses of the diseased pinheads to prove the Koch's postulates.

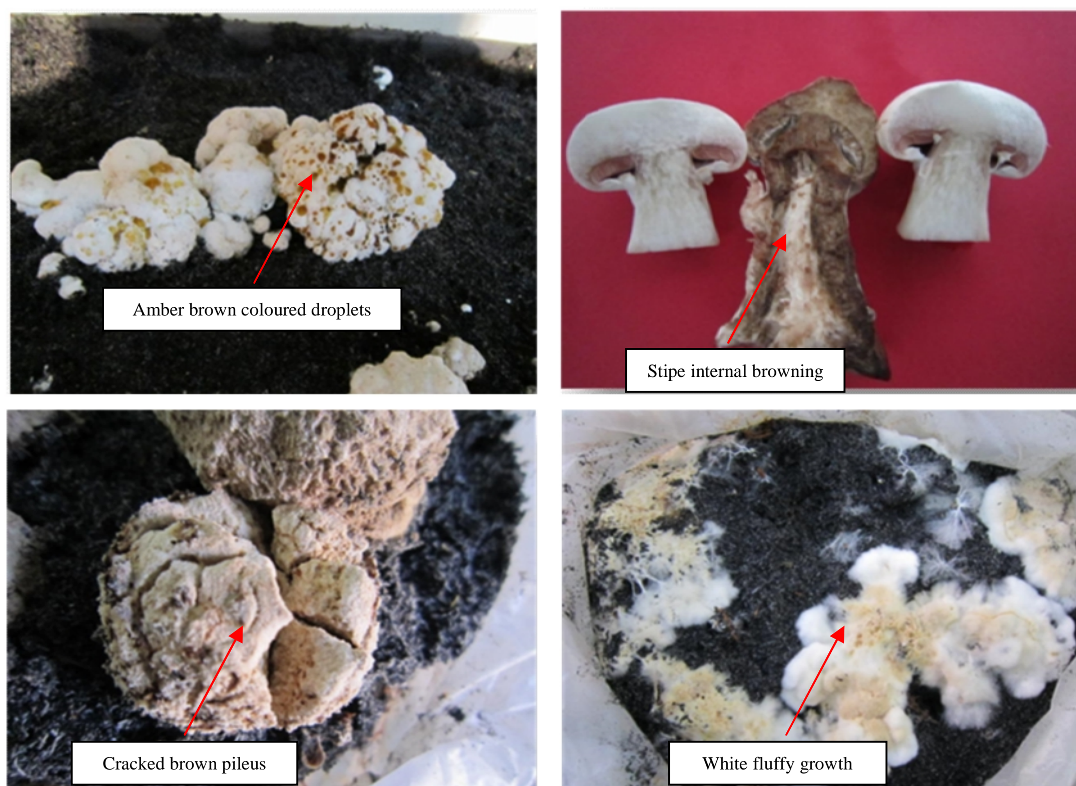


Plate 3.1: Mushroom infected with wet bubble, (A) Amber brown coloured droplets (B) Stipe internal browning (C) Cracked brown pileus (D) White fluffy growth

3.3 Morphological variability of *Mycogone perniciosa* isolates

The morphological studies were performed on the potato dextrose agar (PDA) medium in the present investigation. The medium was sterilized at 1.1 kg/cm^2 for 15 minutes in an autoclave. Twenty five ml of sterilized PDA was poured aseptically in each sterilized Petri plates. On solidification of the medium Petri plates were inoculated with 5 mm mycelial disc from the periphery of actively growing colony of seven days old cultures of fifteen different isolates and then incubated at 25±1°C. The colony growth, mycelium, colony colour and spore size were recorded after 48 hrs intervals up to ten days of incubation. Three replications were maintained for each isolate in completely randomized design (CRD).

3.3.1 Measurement of spore size

Size of the spore was measured using ocular and stage micrometer. The number of divisions of stage micrometer coinciding with division of the ocular micrometer were noted, the calibration factor ocular index (μ) was calculated by the formula as follows:

$$\text{Ocular index } (\mu) = \frac{\text{Number of division of stage micrometer coinciding with division in ocular}}{\text{Number of divisions of ocular micrometer coinciding with division in stage}} \times 10$$

3.4 Physiological variability in isolates of *Mycogone perniciosa*

3.4.1 Effect of temperature on the growth in isolates of *M. perniciosa*

To study the effect of temperature on the radial growth of isolates of *Mycogone perniciosa* on PDA medium under *in vitro* conditions. The sterile Petri plates (90mm) containing 25 ml of medium with three replication were inoculated with 5 mm mycelial disc of each isolate taken from periphery of seven days old actively growing colony on PDA plates. The inoculated Petri plates were kept in BOD incubator at 15, 20, 25 and 30°C temperature. The colony diameters of the isolates were recorded at two days interval upto ten days of incubation. The average diametric growth of each isolate was measured.

3.4.2 Effect of pH on the growth of isolates of *M. perniciosa*

The effect of pH on growth of *M. perniciosa* isolates was studied on PDA medium under *in vitro* conditions. The pH of medium was adjusted to 6.0, 7.0, 8.0 and 9.0 using 0.1 HCl or 0.1 NaOH and buffered with citrate phosphate buffer (Dhingra and Sinclair, 1986). Sterile Petri plates (90mm) containing 25 ml of medium were inoculated with 5 mm mycelial disc of each isolate taken from periphery of actively growing colony of seven days old on PDA. The Petri plates were kept in BOD incubator at 25±1°C. The colony diameter of the isolates was recorded at two days interval up to ten days after incubation. The average diametric growth of each isolate was measured.

3.5 Chemosensitve variability of isolates of *Mycogone perniciosa*.

The sensitivity of fungicides against the *Mycogone perniciosa* isolates were determined by poisoned food technique (Grover and Moore, 1962). Two fungicides *viz.,* carbendazim (25µg/ml) and Metalaxyl MZ (300µg/ml) were used. Stock solution of these fungicides were prepared by dissolving required quantity in sterilized distilled water. Autoclaved potato dextrose agar (PDA) medium was amended with different stock solutions to obtain the desired concentration of fungicides before being poured into Petri plates. The Petri plate with unamended PDA served as check. Four Petri plates for each concentration of the fungicides were inoculated with each isolate of *M. perniciosa* by placing 5 mm actively growing mycelial disc of seven days old culture. The radial growth was recorded after two days of incubation in BOD incubator at 25±1°C up to eight days. The growth inhibition of the

pathogen at various concentrations of fungicides were calculated over the check by using Vincent's formula (1947).

$$I = \frac{(C - T)}{C} \times 100$$

Where,

I = Per cent inhibition

C = Radial growth in control

T = Radial growth in treatment

3.6 Interaction of isolates of *M. perniciosa* with different strains of *A. bisporus*

A. bisporus strains *viz*., ABL-1, ABL-2, ABL-3, ABL-4, ABL-5, ABL-6, ABL-7 and U-3 were sub-cultured on PDA medium at 25°C for three weeks and maintained on PDA slants. The interaction studies were done by inoculating the pathogen and host were grown in dual culture method on PDA in Petri plates. The isolates of *M. perniciosa* and strains of *A. bisporus* were inoculated by growing five mm mycelial disk cut from actively growing colonies of seven days old culture of each fungus were placed 6 cm apart on the surface of PDA in Petri plates. The Petri plates were incubated at 25±1 ^{o}C in BOD incubator for ten days. The mycelial inhibition was measured over control at 48 hrs intervals by using Vincent's formula.

3.7 Molecular variability in different isolates of *M. perniciosa*

3.7.1 Isolation of genomic DNA

The total genomic DNA of the isolates of *Mycogone perniciosa* was isolated from mycelia using CTAB method of Saghai-Maroof *et al.* (1984) with slight modifications.

1. Isolates were inoculated on 50 ml potato dextrose broth (200 g peeled potato, 20 dextrose in one liter distilled water and autoclaved at 121.6 ^{o}C for 15 min) and incubated at 25±1 ^{o}C for seven days.
2. Mycelia were harvested by filtration through double layers of filter paper, dried and stored at -20° C.
3. Five gram of dried mycelium was ground to fine powder in pre-chilled pestle and mortar using liquid nitrogen and transferred in 50 ml centrifuge tube containing 10 ml of 2% CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer.
4. The contents were mixed by gentle shaking and incubated for 1 h at 65°C with occasional mixing. To this, same volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. The mixture was centrifuged at 10,000 rpm for 20 min. the aqueous upper phase was transferred to fresh tubes.
5. The above step was repeated 2-3 times and the aqueous layer was taken in another fresh polypropylene tube,

6. Added equal volume of chilled isopropanol and kept in deep freezer for 1h for precipitation of the DNA.
7. The samples were centrifuged at 8000 rpm for 10 min at $4^{o}C$ and the pellet in each was washed with 70% ethanol.
8. The DNA sample were dried and dissolved in TBE buffer (pH 8.0) then stored in freeze at $4^{o}C$ for further use.

3.7.2 RNase treatment

To remove RNA contamination, DNA samples were treated with lµl of RNase-A solution (10 mg/ml) per 50 µl of DNA sample and incubated in water bath at 37°C for 3 hrs.

3.7.3 Agarose gel electrophoresis

Agarose gel electrophoresis (0.8%) was used to check quality and quantity of genomic DNA. Gel casting plate was washed, air-dried and its ends were sealed with tape. Agarose (Agrose-LE, MB grade, Affymetrix) was melted in 1X TBE buffer and ethidium bromide (5µg/ml) (Sigma, USA) was added. Gel solution was then poured into gel casting plate, an appropriate comb was inserted to get a 0.5 cm thick gel. After setting of gel, sealing tapes were removed from both the ends. Gel plate was placed in the electrophoresis chamber and submerged using 1X TBE buffer and comb was removed gently. Samples were prepared by adding 2.0 µl of 6X loading dye (Sucrose 4 gm, bromophenol blue 0.025g, xylene cyanol 0.025g, final volume 10 ml). Samples (10µl) were loaded in the wells and electrophoresis was carried out at constant voltage (80V) for 3 hrs. in 1X TBE buffer. The gel was visualized in a UV trans-illuminator. The quality and quantity of DNA was analyzed by spectrophotometrically using spectrophotometer (Thermo-scientific Ltd.) estimated as good (if sharp and discrete band appeared), sheared (if smear was there), improper dissolution (if thin lane appeared) and the presence of alcohol was indicated if cup shaped band was present.

3.7.4 Polymerase Chain Reaction (PCR) Amplification

PCR reaction was carried out for obtaining the best amplification of DNA of the isolates of *M. perniciosa* by using RAPD primers. The PCR reaction was performed in 25 µl of reaction mixture containing 1.0 µl DNA (50ng/µl), 2.5 µl of 10X PCR buffer, 0.5 µl dNTP's (2.5mM of each dATP, dCTP, dGTT, dTTP), 0.5 µl of each primer (0.4µM), 0.2 µl Taq DNA polymerase (2.5U) and 19.8 µl double distilled autoclaved water. The DNA amplification was performed by using MastercyclerepTM gradient thermal cycler (Eppendorf AG, Germany).

1. Initial Denaturation 95°C for 30 sec.
2. Denaturation 95°C for 30 sec.
3. Annealing 50°C-56°C for 1 m.
4. Extension 72°C for 1 m.
5. Final Extension 72°C for 5 m.

Amplified products were stored at -20°C till further use.

3.7.5 Scoring of the bands/amplifications

DNA bands for RAPD analysis was scored based on presence (taken as 1) or absence (taken as 0) of bands (Ghosh *et al.,* 1997). The 0/1 matrix was used to calculate similarity or genetic distance using 'simqual' sub-program of software NTSYS-PC version 2.02 (Numerical Taxonomy and Multivariate Analysis System Programme) (Rohlf, 1997). The resultant distance matrix was employed to construct dendrogram by the un-weighted pair-group method with arithmetic average (UPGMA) sub-programme of NTSYS-PC.

3.7.6 Data analysis

The analysis of data was done by using statistical package of program OPSTAT (Sheoran, 2006). The data was changed to per cent for angular transformation for the statistical analysis.

CHAPTER-IV

EXPERIMENTAL RESULTS

Button mushroom (*Agaricus bisporus*) is an important edible mushroom cultivated in India and abroad, its production is affected by different fungi, bacteria and viruses which affect not only its quality but yields also. Wet bubble disease caused by *Mycogone perniciosa* results in huge losses by reducing the yield and sometimes leading to crop failures, depending upon the severity and stage of infection. The present investigations were undertaken to study the variability among *Mycogone perniciosa* isolates collected from predominant button mushroom cultivating states *viz*., Haryana, Punjab and Himachal Pradesh of India. So an attempt was made to find the cultural, morphological, physiological, chemosensitivity and molecular variability in *Mycogone perniciosa*. The results of present work are described in the subsequent paragraphs under following headings:

4.1 Symptomatological variability
4.2 Morphological variability
4.3 Physiological variability
4.4 Chemosensitive variability
4.5 Interaction between *Mycogone perniciosa* isolates and *Agaricus bisporus* strains.
4.6 Molecular variability

4.1 Symptomatological variability

Mycogone perniciosa is a mycoparasite that infects *Agaricus bisporus* at various stages of its development. The infection at pin head stage causes the appearance of tuberous masses and later infection lead to malformation of the fruiting bodies. The symptoms produced by different isolates of *Mycogone perniciosa* varied from thick stipe to sclerodermoid having brown coloured droplets to internal stipe browning. These symptoms were categorized on the basis of appearance of brown amber coloured droplets, gill infection and foul smell. Three isolates *viz*., LDP, RSN and BSN showed dark brown to light brown coloured symptoms on pileus, whereas, isolates *viz.,* SHP, SKK, KSN and BSN2 exhibited brown amber coloured droplets with no gills infection (Table 4.1; Plate 4.1).

A perusal of data revealed that the isolates BFT, SKK, KSN and BSN2, showed white fluffy growth on casing surface, while the isolate LDP exhibited small sclerodermoid with no internal stipe browning (Plate 4.1). However, isolate TPN resulted in a thick stipe with gills infection, while isolate NAS showed deep cracking on fruiting bodies along with foul smell (Table 4.1).

However, six *Mycogone perniciosa* isolates *viz*., LDP, TPN, RHT, RSN, BSN1 and AFT were more damaging in nature and these depicted gill infection in all the mushrooms inoculated and fruiting bodies and (Plate 4.1). Four isolates produced brown amber coloured droplets that emited foul smell. However, six isolates *viz*., SHP, RHT, SKK, BSN1, KSN and BSN2 showed the similarity regarding appearance of amber coloured droplets, gill infection and foul smell, while, two isolates *viz*., HSN and BKK did not show the presence of brown amber coloured droplets, gills infections with foul smell (Table 4.1; Plate 4.1).

Among the fifteen isolates, ten isolates *viz*., SHP, NAS, RHT, BFT, SKK, RSN, BSN1, KSN, BSN2 and SHS upon infection emitted foul smell but remaining five isolates did not give the foul smell. Among these fifteen isolates, four was *viz*., SHP, SKK, KSN and BSN2 isolates also produced the brown amber coloured droplets as well as foul smell whereas, eleven isolates did not show these symptoms.

Table 4.1: Symptoms produced by isolates of *Mycogone perniciosa* on white button mushroom

Isolates	Distinguishing symptoms	Amber droplets	Gill infection	Foul smell
LDP	Sclerodermoid with white pileus	-	+	-
SHP	Sclerodermoid with brown amber droplets	+	-	+
NAS	Sclerodermoid with cracked brown pileus	-	-	+
TPN	Thick stipe	-	+	-
HSN	Sclerodermoid with dry pileus	-	-	-
RHT	Stipe internal browning	-	+	+
BFT	Mixed infection with white fluffy growth with dry Sclerodermoids	-	-	+
SKK	White fluffy cottony growth on fruiting pileus	+	-	+
RSN	Sclerodermoid with dark brown pileus	-	+	+
BSN1	Sclerodermoid with brown pileus	-	+	+
KSN	White fluffy growth on casing material	+	-	+
AFT	Sclerodermoid with large dark brown spots on	-	+	-
BKK	Sclerodermoid with small brown spots	-	-	-
BSN2	White fluffy growth with brown mycelium	+	-	+
SHS	Mixed infection with brown sclerodermoid	-	-	+

-: Absent, +: Present

Thus, the fifteen isolates were categorized on the basis of symptoms induced into four categories *viz*., highly virulent, virulent, moderately virulent and less virulent. Among these four isolates *viz*., SHP, SKK, KSN and BSN2 were highly virulent releasing brown amber coloured droplets with foul smell. Three isolates *viz*., RHT, RSN and BSN1 were virulent exhibiting gills infection with foul smell. However, three isolates *viz*., LDP, TPN and AFT were found moderately virulent, whereas, rest of the three isolates *viz*., NAS, BFT and SHS were categorized less virulent. It is evident from data that all the isolates evaluated were virulent in nature however degree of virulence varied. All the fifteen isolates into present

studies were virulent which produces the distinguished symptoms on the button mushroom (Plate 4.1: Plate 4.1).

4.2 Morphological variability

To study the variability in *Mycogone perniciosa* isolates were cultured on PDA medium and incubated at 25°C for ten days to study the various morphological characteristics. The isolates exhibited variation among themselves regarding colony growth, mycelial growth, colony colour and spore size (Table 4.2; Plate 4.2). Three isolates LDP, HSN and RSN showed uneven colony growth, while, the rest of isolates had even colony growth (Plate 4.3). The feathery growth was observed in seven isolates *viz*., HSN, BFT, SKK, RSN, KSN, BKK and BSN2, while, rest of the isolates did not show this character.

The mycelium varied from aerial, dense, sparse to fluffy among isolates. The seven isolates *viz*., LDP, SHP, NAS, RHT, RSN, BKK and BSN2 was aerial whereas, isolate TPN showed the dense mycelium. The four isolates *viz*., HSN, BFT, BSN1 and AFT had sparse mycelium however, three isolates *viz*., SKK, KSN and BSN2 had fluffy mycelial growth but it was even and very feathery in nature (Plate 4.2). The colony colour among the isolates of *Mycogone perniciosa* varied from brown, white to lemon colour and five isolates *viz*., TPN, RHT, SKK, KSN and BSN2 showed white colony colour whereas, isolate HSN showed lemon colour (Plate 4.2).

Plate 4.1: Symptoms induced by isolates of *Mycogone perniciosa* on white button mushroom

The microscopic examination *Mycogone perniciosa* spores revealed that the spore length ranged from 18.6-21.8 while the maximum breadth was 16.9 µm and the minimum was 11.2 µm. The two isolates *viz.*, KSN and BSN2 had maximum spore size, 21.8x16.9, 21.6x16.7 µm respectively, whereas, the minimum sized spore (18.6x11.1 µm) was recorded in RHT isolate (Table 4.2).

Table 4.2: Morphological variability of *Mycogone perniciosa* isolates collected from white button mushroom

Isolates	Colony growth	Mycelium	Colony colour	Spore size (µm)
LDP	Uneven, feathery	Aerial	Brown	21.1x11.2
SHP	Even, feathery	Aerial	Brown	21.3x11.4
NAS	Even, feathery	Aerial	Brown	21.4x15.6
TPN	Even, slightly feathery	Dense	White	20.4x12.2
HSN	Uneven, very feathery	Sparse	Lemon	20.8x14.4
RHT	Even, feathery	Aerial	White	18.6x11.1
BFT	Even, very feathery	Sparse	Brown	20.2x12.2
SKK	Even, very feathery	Fluffy	White	20.4x12.7
RSN	Uneven, very feathery	Aerial	Brown	20.2x15.2
BSN1	Even	Sparse	White	21.4x14.1
KSN	Even, very feathery	Fluffy	White	21.8x16.9
AFT	Even	Sparse	White	20.2x16.4
BKK	Even, very feathery	Aerial	Brown	20.3x14.7
BSN2	Even, very feathery	Fluffy	White	21.6x16.7
SHS	Even, feathery	Aerial	Brown	20.7x13.3

4.3 Physiological variability

4.3.1 Effect of temperature on *Mycogone perniciosa* isolates

The effect of different temperature regimes was studied on growth behavior of *Mycogone perniciosa* isolates cultured on PDA medium under in vitro conditions at 15, 20, 25 and 30° C. The radial growth was recorded at two days interval up to ten days. The perusal of data revealed that all isolates evaluated had mycelial growth from 15-30°C results have been presented in (Table 4.3a and 4.3b).

The perusal of results indicated that *Mycogone perniciosa* isolates growth was observed in the temperature range 15-30°C but its growth reduces with the deviation in temperature increase or decrease from 25°C. At 15°C, the maximum mean radial growth (55.8 mm) was in case of isolate BSN2 followed by isolate KSN (51.6 mm) while the minimum mean radial growth (9.9 mm) was observed in LDP isolate after ten days of incubation (Table 4.3a & 4.3b). Whereas, at 20° C different trend was obtained and maximum growth was obtained in isolates KSN (69.6 mm) followed by in isolate BSN2 (69.2 mm)

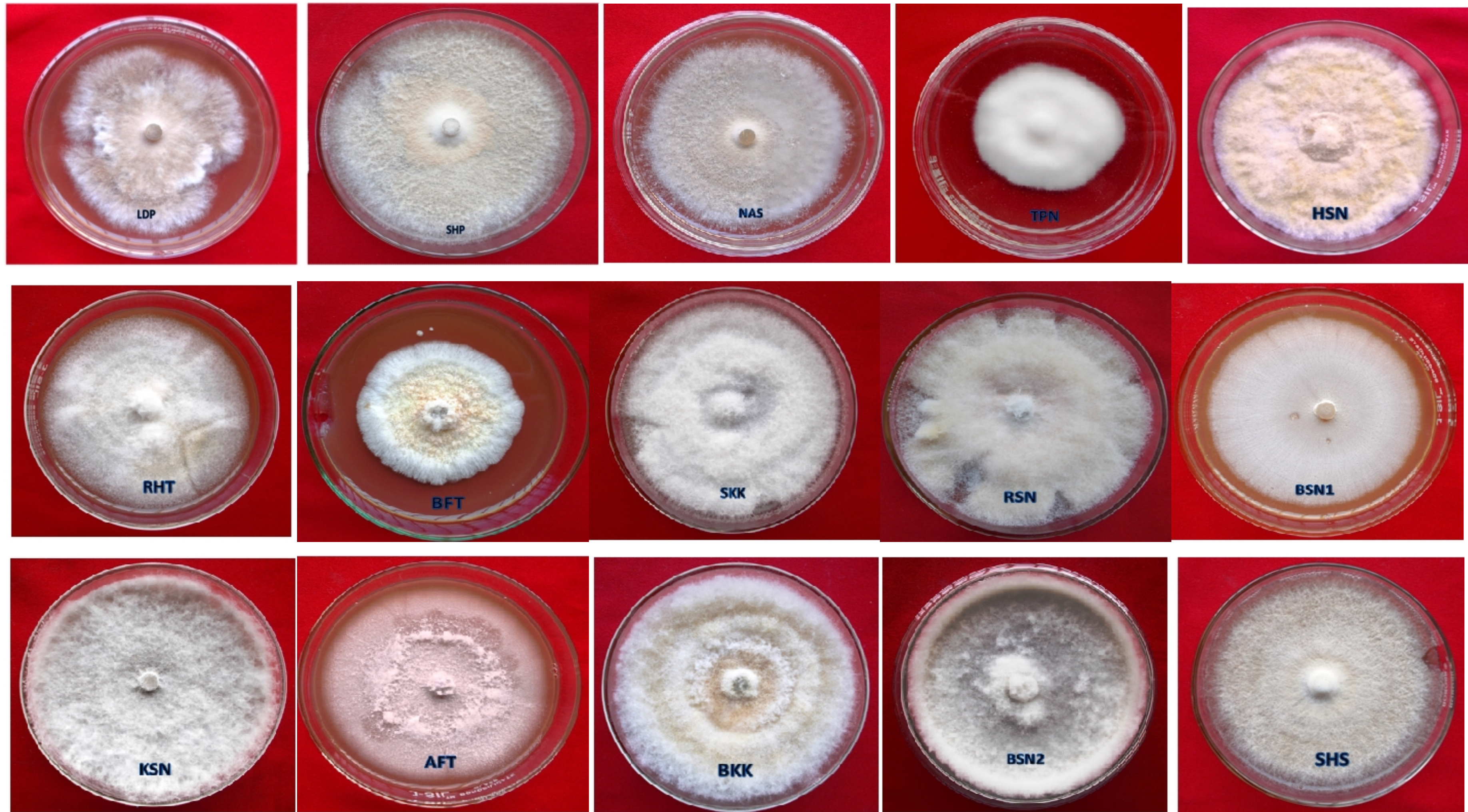


Plate 4.2: Morphological variability among isolates of *Mycogone perniciosa* collected from white button mushroom

On the other hand, the minimum growth was recorded in isolates in NAS (19.9 mm) isolate after ten days of incubation.

However, at 25°C the growth as higher in all the isolates studied on ranged to 15, 20, 25 and 30°C. The maximum radial growth (76.0 mm) was exhibited by isolates BSN2, while it was minimum in case of isolate SHS. (Table 4.3b). At 30°C the maximum radial growth (56.6 mm) was recorded in isolate BSN2 followed by KSN (55.2 mm), while, minimum mean radial growth was recorded in isolate SHP (14.9 mm) after ten days of incubation (Table 4.3b). On the contrary maximum mean radial growth was found at 25°C (88.7 mm) followed by 20°C (54.8 mm) 30°C (41.2 mm) and the least growth was observed at 15°C (30.8 mm). Thus, 25°C was most suitable temperature for mycelial growth of *Mycogone perniciosa* (Table 4.3a & 3b: Plate 4.3).

Further these isolates were categorized three different groups *viz*., fast growing, moderately growing and slow growing in nature at 25°C. Of fifteen isolates two isolates KSN and BSN2 were fast growing (>71 mm) whereas, six isolates *viz*., LDP, SKK, RSN, BSN1, AFT and BKK were moderately growing (50-70mm) and rest of isolates *viz*., SHP, NAS, TPN, HSN, RHT, BFT and SHS were slow growing (20-50mm).

Table 4.3a: Effect of temperature on growth of different isolates of *Mycogone perniciosa*

Radial growth (mm)												
	Temperature (15°C)						**Temperature (20°C)**					
Isolates	**2 DAI**	**4 DAI**	**6 DAI**	**8 DAI**	**10 DAI**	**Mean**	**2 DAI**	**4 DAI**	**6 DAI**	**8 DAI**	**10 DAI**	**Mean**
LDP	7.2	9.8	10.0	11.2	11.2	9.9	9.3	13.8	21.0	25.5	33.2	20.6
SHP	8.2	10.8	11.7	12.8	13.8	11.5	8.8	15.0	26.5	31.0	41.7	24.6
NAS	7.7	11.0	11.3	11.8	13.2	11.0	9.2	12.0	22.0	25.3	31.0	19.9
TPN	10.2	14.2	14.2	16.8	17.8	14.6	12.7	15.7	23.3	26.0	31.0	21.7
HSN	13.0	16.0	17.7	22.5	23.8	18.6	21.8	30.2	39.5	47.2	57.3	39.2
RHT	10.3	16.8	17.5	18.8	20.8	16.9	15.7	26.8	41.7	46.5	52.5	36.6
BFT	10.2	15.2	17.3	17.8	19.0	15.9	12.8	18.0	25.2	27.7	30.0	22.7
SKK	7.7	10.5	13.0	16.3	18.3	13.2	12.7	22.8	42.8	45.3	55.0	35.7
RSN	10.8	18.7	28.8	33.7	35.2	25.4	18.8	31.0	53.3	59.2	64.5	45.4
BSN1	15.0	21.8	32.8	34.7	43.0	29.5	23.0	35.5	55.3	60.7	70.0	48.9
KSN	22.0	40.0	48.7	57.3	90.0	51.6	38.0	58.0	72.0	90.0	90.0	69.6
AFT	10.0	13.5	14.7	16.2	18.2	14.5	15.3	23.0	43.0	47.5	61.2	38.0
BKK	11.5	19.7	27.2	30.2	35.8	24.9	21.7	35.8	61.7	66.8	83.0	53.8
BSN2	24.3	47.5	53.0	64.2	90.0	55.8	43.7	51.8	70.7	90.0	90.0	69.2
SHS	7.5	9.3	10.5	10.5	12.2	10.0	9.0	15.0	25.2	28.7	31.0	21.8
Mean	11.7	18.3	21.9	25.0	30.8		18.2	27.0	41.5	47.8	54.8	

DAI: Days after incubation

Factors	CD at 5%	Factors	CD at 5%
A= Isolates	1.0	A= Isolates	1.2
B= Days	0.6	B= Days	0.7
AxB (Interaction)	2.3	AxB (Interaction)	2.7

Table 4.3b: Effect of temperature on growth of different isolates of *Mycogone perniciosa*

Radial growth (mm)												
	Temperature (25°C)						Temperature (30°C)					
Isolates	**2 DAI**	**4 DAI**	**6 DAI**	**8 DAI**	**10 DAI**	**Mean**	**2 DAI**	**4 DAI**	**6 DAI**	**8 DAI**	**10 DAI**	**Mean**
LDP	13.3	35.2	59.5	85.3	90.0	56.7	5.8	16.3	19.3	23.7	25.2	18.1
SHP	9.3	26.5	45.8	71.7	90.0	48.7	5.0	14.0	15.3	19.0	21.3	14.9
NAS	9.8	25.0	46.7	71.7	90.0	48.6	5.0	17.3	17.8	19.3	19.3	15.8
TPN	14.2	23.7	46.8	69.0	71.2	45.0	10.5	14.8	16.5	17.0	17.7	15.3
HSN	12.3	22.5	46.5	71.2	90.0	48.5	11.2	30.8	40.8	51.2	57.8	38.4
RHT	15.8	21.5	26.8	58.0	90.0	28.7	13.0	16.0	21.0	26.3	31.2	21.5
BFT	15.2	18.8	28.2	62.0	90.0	29.0	10.3	17.3	20.8	21.5	22.8	18.6
SKK	26.8	36.5	61.2	90.0	90.0	60.9	9.3	25.5	30.7	34.7	38.0	27.6
RSN	17.7	34.2	54.0	82.7	90.0	55.7	12.3	16.8	23.7	30.5	40.7	24.8
BSN1	26.0	45.7	65.7	90.0	90.0	63.5	18.0	34.2	54.0	60.5	61.2	45.6
KSN	37.0	57.7	84.3	88.2	90.0	71.4	33.7	43.0	54.8	65.8	78.8	55.2
AFT	18.5	32.8	55.3	82.2	90.0	55.8	16.3	24.8	44.3	55.7	57.5	39.7
BKK	30.3	56.5	80.8	90.0	90.0	69.5	12.5	25.5	38.3	40.0	41.0	31.5
BSN2	48.5	61.5	90.0	90.0	90.0	76.0	27.8	46.2	59.2	67.7	82.2	56.6
SHS	16.5	30.8	54.7	70.3	90.0	28.1	5.7	17.8	21.8	23.0	23.7	18.4
Mean	20.1	34.2	54.4	73.0	88.7		13.1	24.0	31.9	37.1	41.2	

DAI: Days after incubation

Factors	CD at 5%	Factors	CD at 5%
A= Isolates	1.1	A= Isolates	1.7
B= Days	0.6	B= Days	1.0
AxB (Interaction)	2.5	AxB (Interaction)	3.7

4.3.2 Effect of pH on *Mycogone perniciosa* isolates

The effect of different pH regime was studies on growth behavior of *M. perniciosa* isolates cultured on PDA medium under *in vitro* conditions at pH 6.0, 7.0, 8.0 and 9.0. The observations regarding influence of pH on mycelial growth of *Mycogone perniciosa* isolates were recorded after ten days of inoculation and results thus obtained has been resented in the Table 4.4.

The analysis of data indicated that the growth of *Mycogone perniciosa* isolates was better in the pH range 5.0 to 8.0, however, it attained the maximum growth at pH 7.0. Most of the isolates had maximum growth (90 mm) at pH 7.0 except one isolate TPN which attained (40.7 mm) radial growth after ten days of incubation and the growth of isolates decreased significantly with any deviation from a pH level 7.0 and the reduction was highest at pH 9.0 followed by pH 6.0 and pH 8.0 irrespectively of *M. perniciosa* isolates evaluated.(Table 4.4)

Plate 4.3: Effect of temperature on growth of different isolates of *Mycogone perniciosa*

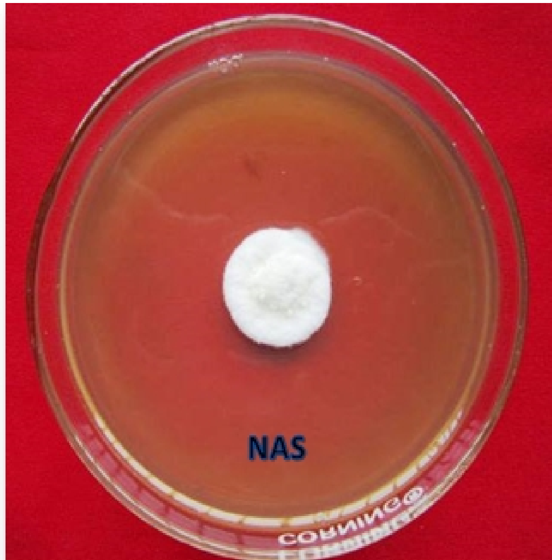
The perusal of results present in table 4.4 indicated that fourteen *M. perniciosa* isolates attained maximum growth (90 mm) at pH 7.0 except isolate TPN (40.7 mm). The pH level of 7.0 was found optimum for mycelial growth of the isolates studied (Table 4.4; Plate 4.5)

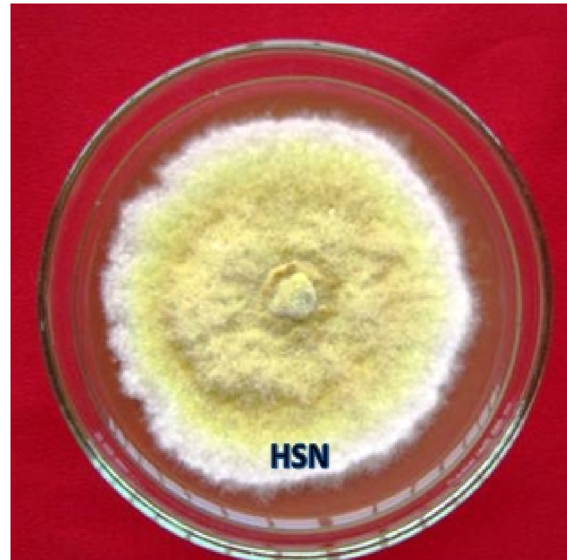
At pH 6.0, seven isolates *viz*., BFT, SKK, RSN, BSN1, AFT, BKK and BSN2 attained the maximum radial growth 90 mm, while rest of isolates of *M. perniciosa* did not attained the 90 mm radial growth after ten days of incubation (Table 4.4). Four isolates RHT, AFT, BKK and SHS was highly sensitive to higher pH and has no growth when pH was more than 7.0.

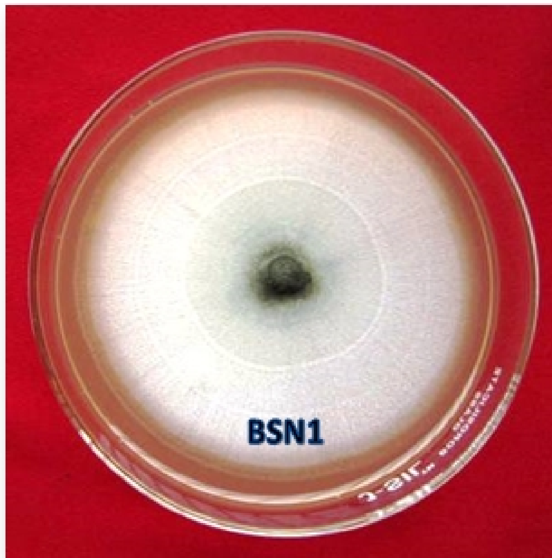
Table 4.4: Effect of different pH on growth of *Mycogone perniciosa* isolates

	Radial growth (mm) after 10 Days				
Isolates	**pH 6.0**	**pH 7.0**	**pH 8.0**	**pH 9.0**	**Mean**
LDP	23.5	90.0	23.3	8.0	36.2
SHP	17.7	90.0	19.3	6.0	33.3
NAS	33.0	90.0	20.0	7.3	37.6
TPN	72.3	40.7	31.3	14.0	51.9
HSN	72.8	90.0	32.0	0.0	48.7
RHT	19.3	90.0	0.0	0.0	15.0
BFT	90.0	90.0	40.0	12.7	58.2
SKK	90.0	90.0	42.0	14.0	59.0
RSN	90.0	90.0	46.0	9.3	58.8
BSN1	90.0	90.0	44.7	17.3	60.5
KSN	88.3	90.0	77.3	22.7	69.6
AFT	90.0	90.0	0.0	0.0	45.0
BKK	90.0	90.0	0.0	0.0	45.0
BSN2	90.0	90.0	80.7	26.7	71.8
SHS	85.0	90.0	0.0	0.0	43.8
Mean	69.5	86.7	30.4	9.2	

Factors	CD at 5%
A= Isolates	1.3
B= pH	0.6
AxB (Interaction)	2.6







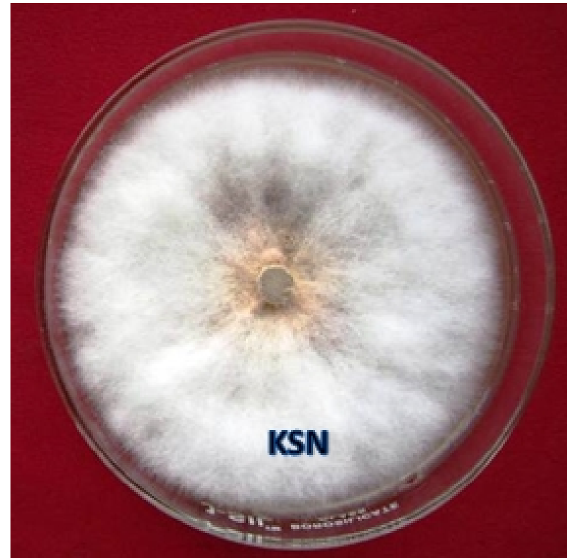


Plate 4.4: Effect of pH 6.0 on growth of different isolates of *Mycogone perniciosa*

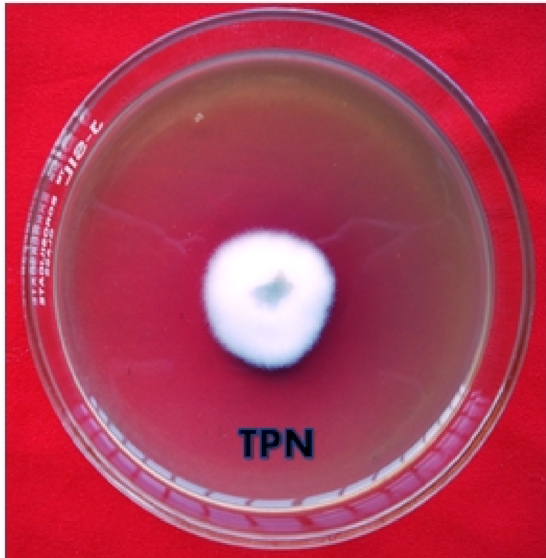


Plate 4.5: Effect of pH 7.0 on growth of different isolates of *Mycogone perniciosa*

4.4 Chemosensitive variability

Two fungicides *viz*., carbendazim and metalaxyl MZ were evaluated for their chemosensitivity towards *M. perniciosa* isolates at different concentration using poisoned food technique. In case of nine isolates, their growth was completely checked by carbendazim at 25 µg/ml, while in others the inhibition varied among the remaning isolates. The perusal of data presented in Table 4.10 revealed that in majority of the *M perniciosa* isolates mycelial growth was inhibited at 25 ppm under carbendazim was used except isolates *viz*., NAS, SKK, BSN1, AFT, BSN2 and SHS which exhibited resistance (Table 4.5).

On the basis of chemosensitivity to carbendazim (25 µg/ml) and metalaxyl MZ (300 µg/ml) the fifteen isolates of *Mycgone perniciosa* were categorized into three groups *viz*., highly sensitive (HS, 81-100% inhibition), sensitive (S, 41-80% inhibition) and less sensitive (R, 0-40% inhibition). Out of these isolates, nine isolates *viz*., LDP, SHP, TPN, HSN, RHT, BFT, RSN, KSN and BKK were highly sensitive, two isolates *viz*., NAS and BSN2 were sensitive, rest of isolates *viz*., SKK, BSN1, AFT and SHS were less sensitive to the carbendazim (Table 4.9).

Four isolates SKK, BSN1, AFT and SHS exhibited the 50 per cent inhibition due to carbendazim while no isolates exhibited the 50 per cent or less than 50 per cent inhibition due to metalaxyl MZ (Table 4.5). Among these isolates *viz*., LDP, SHP, NAS, TPN, HSN, RHT, BFT, RSN, KSN and BKK were found highly sensitive to carbendazim and metalaxyl MZ. In the present study significant variation was observed in sensitivity of isolates to both the fungicides evaluated. The results revealed that *M. perniciosa* isolates were sensitive towards both the chemicals used and the degree of sensitivity varied with the fungicides and it also taken for the present work.

Table 4.5: Effect of carbendazim and metalaxyl MZ on growth of isolates of *Mycogone perniciosa*

	carbendazim (25µg/ml)					metalaxyl MZ (300µg/ml)				
	Inhibition (%) over control					Inhibition (%) over control				
Isolates	**2 DAI**	**4 DAI**	**6 DAI**	**8 DAI**	**Mean**	**2 DAI**	**4 DAI**	**6 DAI**	**8 DAI**	**Mean**
LDP	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
SHP	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
NAS	100.0	68.7	63.8	65.1	74.4	100.0	100.0	100.0	100.0	100.0
TPN	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
HSN	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
RHT	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
BFT	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
SKK	51.7	21.8	12.4	16.8	25.6	100.0	100.0	75.1	75.1	87.6
RSN	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
BSN1	36.7	27.5	21.7	44.5	32.6	100.0	100.0	52.3	52.3	76.2
KSN	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
AFT	36.7	16.3	23.6	45.3	30.5	100.0	100.0	51.2	51.2	75.6
BKK	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
BSN2	100.0	61.9	73.4	68.4	75.9	100.0	100.0	100.0	100.0	100.0
SHS	30.0	19.0	21.8	49.2	30.0	100.0	100.0	56.1	56.1	78.0
Mean	83.7	74.3	74.4	79.3		100.0	100.0	89.0	89.0	

DAI: Days after incubation

Factors	CD at 5%
A= Isolates	0.9
B= Days after incubation	0.5
AxB (Interaction)	1.8

Factors	CD at 5%
A= Isolates	0.4
B= Days after incubation	0.2
AxB (Interaction)	0.7

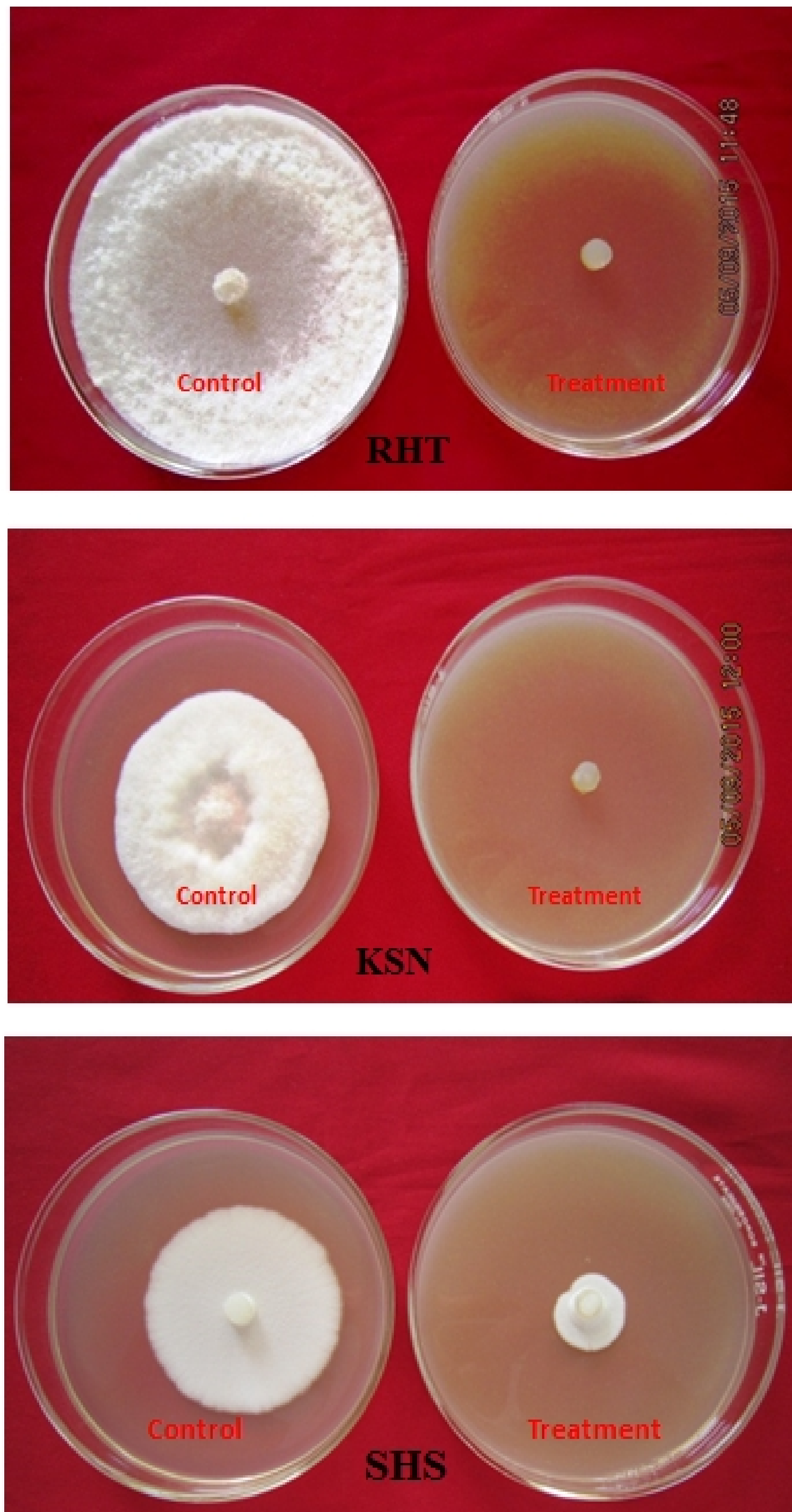


Plate 4.6: Effect of carbendazim on the growth of different isolates of *Mycogone perniciosa*

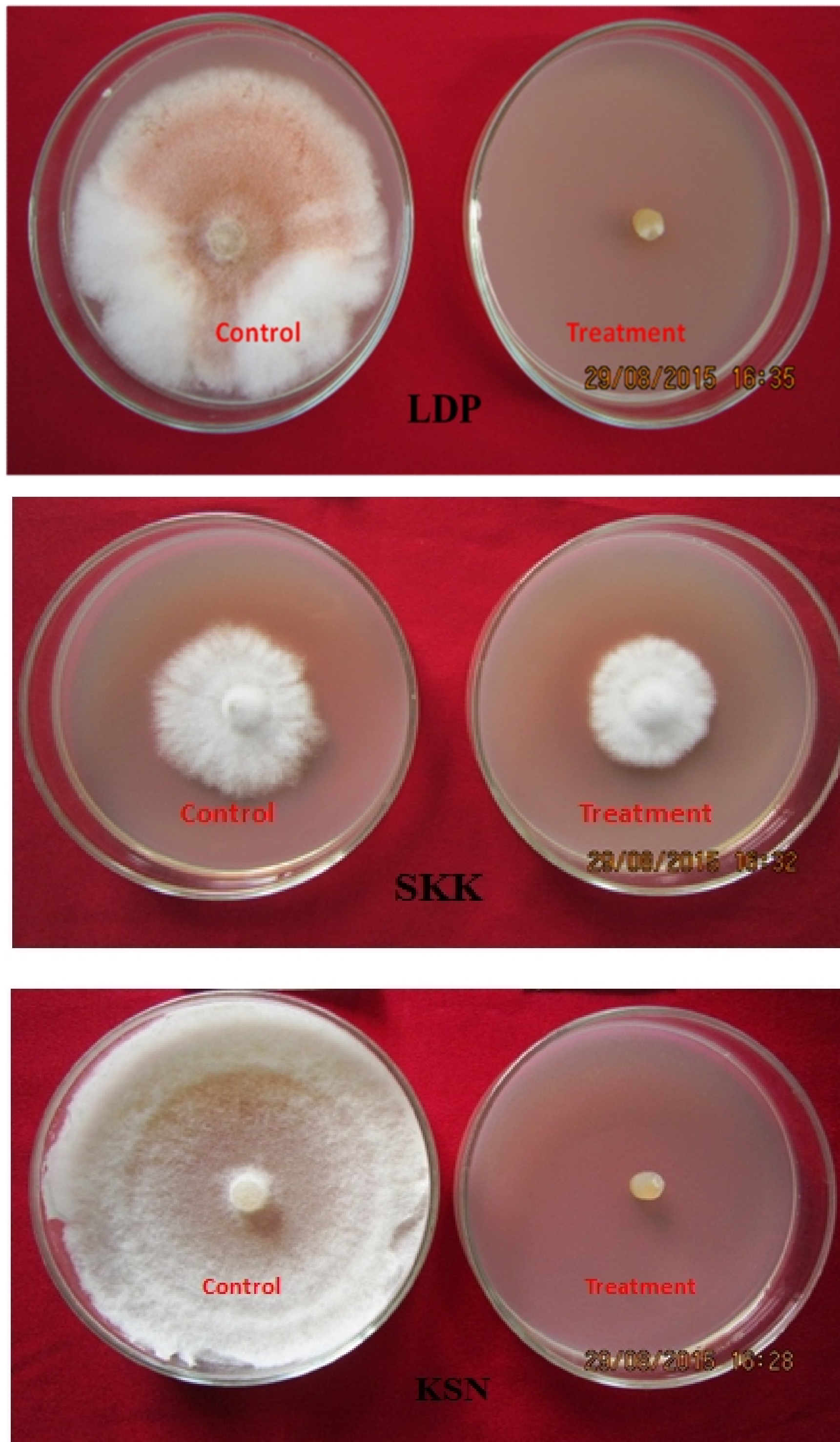


Plate 4.7: Effect of metalaxyl MZ on the growth of different isolates of *Mycogone perniciosa*

4.5 Interaction of *Mycogone perniciosa* isolates and *Agaricus bisporus* strains.

The variability within the *Mycogone perniciosa* isolates was determined on the basis of *A. bisporus* mycelial inhibition due to *M. perniciosa*. Eight different strains of *A. bisporus viz.*, ABL-1, ABL-2, ABL-3, ABL-4, ABL-5, ABL-6, ABL-7 and U-3 were screened against *Mycogone perniciosa* and mycelial inhibition was calculated. Two strains U-3 and ABL-5 of *A. bisporus* showed maximum sensitivity to all the *M. perniciosa* isolates, whereas, four *Mycogone perniciosa* isolates *viz.,* RSN, BSN1, KSN and BSN2 were pathogenic against all the *A. bisporus* strain (Table 4.6).

All the isolates of *M. perniciosa* significantly differed regarding mycelial growth of the *A. bisporus* strains screened. TPN, HSN, BFT and SHS strains of *M. perniciosa* were at par in per cent inhibition of *A. bisporus* strains (Table 4.6).

4.6 Molecular variability

The molecular variability among the isolates is considered to represent its evolutionary potential. The molecular variability is helpful in understanding of intrinsic factors and variation at species level.

Molecular variability of fifteen isolates of *Mycogone perniciosa* collected from Haryana, Punjab and Himachal Pradesh of India was studied by using a set of 36 random primers to amplify DNA fragments from the genomic DNA of fifteen isolates of *Mycogone perniciosa.*

Genomic DNA isolated from seven days old mycelium was resolved on agarose gel. PCR reactions for RAPD analyses in *M. perniciosa* isolates were optimized with respect to DNA concentration, dNTPs concentration and number of cycles amplification. Polymerase chain reaction based DNA amplification by commercially available arbitrary decamer random primers were used to generate RAPD profiles among the fifteen isolates of *M. perniciosa.*

4.6.1 Polymorphism analysis

Out of thirty six random primers screened, only twenty nine primers responded by producing detectable bands. In RAPD profiling, total of 275 scorable bands were produced with an average of 9.48 bands per primer, of which 266 bands were polymorphic and nine bands were monomorphic in nature (Table 4.7). The number of bands produced per primer ranged from 5 to 17. Twenty nine polymorphic primers were out of twenty two primers were completely polymorphic and remaining seven exhibited variable range of polymorphism. Average number of polymorphic and monomorphic bands per assay was 9.17 and 0.31 respectively (Table 4.7).

4.6.2 Genetic similarity and UPGMA based cluster analysis

Jaccard's similarity coefficient calculated from data matrix showed that the genetic relatedness among the isolates ranged from 0.42-0.92 with an average similarity of 0.67. The highest similarity coefficient (0.92) was obtained between isolates RSN and BSN1, whereas, the lowest similarity coefficient (0.42) was obtained between isolates KSN and BSN1.

Table 4.6: Interaction of *Mycogone perniciosa* isolates with different strains of *Agaricus bisporus*

Inhibition (%) after 6 days of inoculation									
Isolates	**ABL 1**	**ABL 2**	**ABL 3**	**ABL 4**	**ABL 5**	**ABL 6**	**ABL 7**	**U 3**	**Mean**
LDP	14.0 (21.9)	16.0 (22.9)	12.0 (20.8)	14.0 (18.3)	26.0 (30.4)	8.0 (16.7)	14.0 (22.2)	38.0 (39.8)	17.7 (24.1)
SHP	14.0 (21.4)	14.0 (21.9)	12.0 (21.3)	16.0 (23.5)	20.0 (25.8)	10.4 (19.3)	16.0 (23.8)	30.0 (33.4)	16.7 (23.8)
NAS	12.0 (20.5)	10.0 (19.0)	10.0 (18.7)	14.0 (21.1)	26.0 (30.4)	16.5 (23.8)	16.0 (23.5)	40.0 (40.8)	18.4 (24.7)
TPN	14.0 (21.6)	16.0 (23.8)	14.0 (21.9)	12.0 (20.1)	30.0 (34.4)	16.0 (24.6)	12.5 (20.4)	40.0 (39.2)	19.7 (25.8)
HSN	12.4 (20.5)	18.0 (25.3)	14.0 (20.8)	18.0 (26.4)	28.0 (33.1)	10.0 (18.7)	10.0 (18.8)	42.0 (42.2)	19.9 (25.7)
RHT	16.0 (24.8)	14.0 (22.5)	16.0 (23.5)	17.0 (24.5)	34.0 (35.6)	18.0 (25.8)	8.0 (16.4)	42.0 (40.3)	21.1 (26.7)
BFT	16.0 (23.5)	16.0 (24.8)	14.0 (22.4)	14.5 (22.2)	32.0 (32.9)	12.0 (21.7)	9.5 (17.6)	44.0 (42.3)	20.1 (25.9)
SKK	20.0 (27.0)	10.0 (18.7)	22.0 (30.2)	21.0 (29.3)	36.0 (37.2)	14.0 (22.2)	10.0 (20.2)	44.0 (39.8)	23.1 (28.0)
RSN	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)
BSN1	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)	0.0 (4.0)
KSN	10.0 (19.0)	14.0 (18.3)	16.0 (22.9)	10.0 (19.0)	12.0 (20.8)	13.0 (21.9)	18.0 (25.8)	19.0 (26.0)	14.0 (21.7)
AFT	14.0 (22.2)	14.0 (17.4)	8.0 (16.3)	15.4 (22.0)	32.0 (33.8)	12.0 (19.9)	10.0 (19.0)	40.0 (39.4)	17.4 (23.8)
BKK	14.0 (22.2)	12.0 (21.0)	12.0 (21.9)	14.0 (21.1)	28.0 (32.5)	16.0 (25.0)	18.0 (6.3)	32.0 (34.8)	19.2 (25.6)
BSN2	10.0 (19.0)	14.0 (18.3)	10.0 (19.0)	12.0 (20.8)	16.0 (22.9)	18.0 (25.8)	20.0 (27.0)	38.0 (39.8)	17.2 (24.0)
SHS	10.0 (18.40)	14.0 (22.9)	12.0 (19.6)	12.0 (21.3)	27.0 (32.0)	10.0 (19.3)	20.0 (25.3)	38.0 (39.4)	18.5 (24.8)
Mean	**10.7 (17.3)**	**10.4 (17.1)**	**10.3 (16.9)**	**11.3 (17.8)**	**21.5 (24.9)**	**10.2 (16.9)**	**9.9 (16.6)**	**29.5 (29.8)**	

*: *Agaricus bisporus* strain
Figures in parenthesis are angular transformed values

Factors	CD at 5%
A= Isolates	0.8
B= strains	0.6
AxB (Interaction)	2.1

The dendrogram constructed by using similarity matrix generated from the data of fifteen isolates, revealed distinct genetic relatedness among the isolates. The cluster analysis using combined data of all the RAPD primers depicted wide range of genetic relatedness, which ranged from 0.42-0.92, *i.e*., 42-92 per cent with an average similarity of 67 per cent among the isolates of *Mycogone perniciosa*. However, the clustering pattern (dendrogram/ phylogenetic tree) based on unweighted pair group method with arithmetic means (UPGMA) analysis in NTSYS-PC Version 2.02h software (numerical taxonomy and multivariate analysis system programme) at lowest similarity coefficient of 0.42, separated the isolates into two major clusters *viz*., cluster A and cluster B, respectively (Fig. 4.5).

The unweighted pair group method with arithmetic means (UPGMA) analysis differentiated the fifteen *Mycogone perniciosa* isolates into two major clusters A and B at similarity coefficient of 0.67. All the *Mycogone perniciosa* isolates in the major cluster A was further clustered into sub-cluster A_1 and sub-cluster A_2 at similarity coefficient of 0.54. Sub-cluster A_2 was a large group having nine isolates *viz*., SHP, NAS, HSN, BFT, RHT, RSN, BSN1, AFT and BKK showing closeness to each other, whereas, sub-cluster A_1 was constituted by only three isolate *viz*., LDP, TPN and SKK having wide distance from other isolates. Within sub-cluster A_2 the maximum genetic similarity (92%) was detected between isolates RSN and BSN1, whereas, the isolates *viz*., SHS and SHP were distantly related with the lowest genetic similarity. KSN and BSN2 exhibited 84 per cent genetic similarity. Overall, the maximum genetic similarity (92%) among *Mycogone perniciosa* isolates was detected between isolates BSN1and KSN, whereas, the minimum genetic similarity (42%) was shown by isolates *viz*., BSN1 and KSN with a wide genetic distance.

However, cluster B was constituted by only two isolates *viz*., KSN and BSN2having wide distance from other isolates. In the present study, the RAPD analyses successfully differentiated the isolates of *Mycogone perniciosa* which showed high level of polymorphism (Table 4.7, Fig. 4.5).

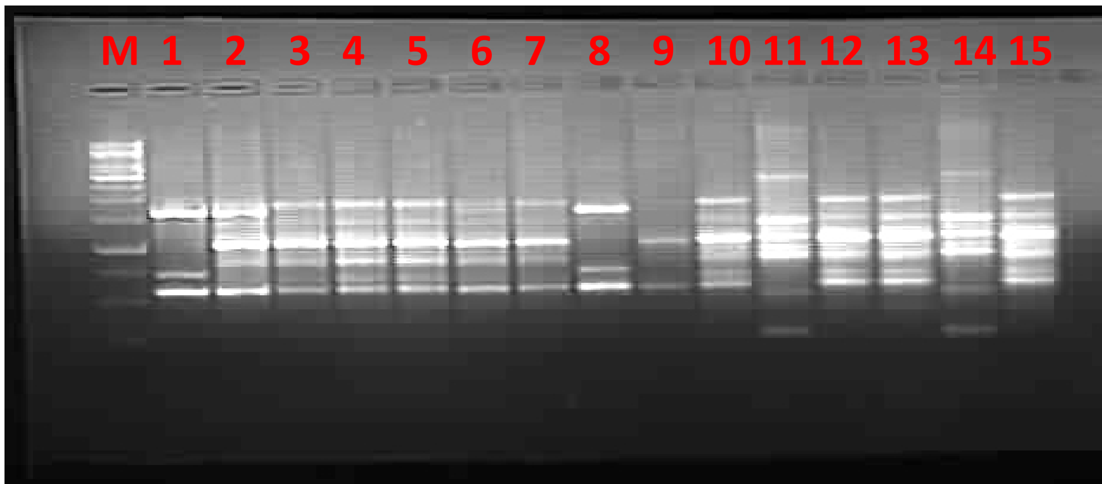


Fig. 4.1: DNA polymorphism in fifteen isolates *Mycogone perniciosa* using RAPD primer OPD-09, M; Marker, Line 1 = LDP, Line 2 = SHP, Line 3 = NAS, Line 4 = TPN, Line 5 = HSN, Line 6 = RHT, Line 7 = BFT, Line 8 = SKK, Line 9 = RSN, Line 10 = BSN1, Line 11 = KSN, Line 12 = AFT, Line 13 = BKK, Line 14 = BSN2, Line 15 = SHS.

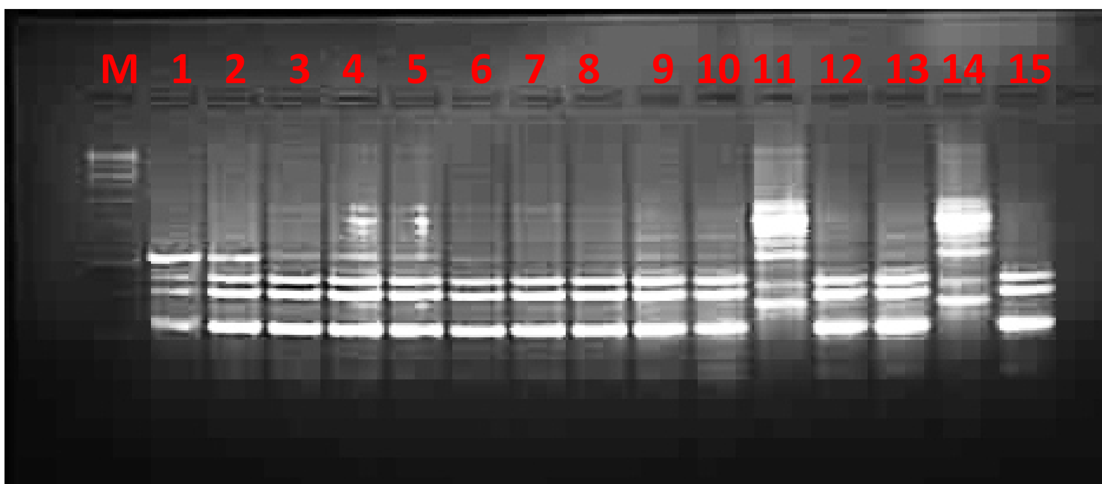


Fig. 4.2: DNA polymorphism in fifteen isolates *Mycogone perniciosa* using RAPD primer OPE-01, M; Marker, Line 1 = LDP, Line 2 = SHP, Line 3 = NAS, Line 4 = TPN, Line 5 = HSN, Line 6 = RHT, Line 7 = BFT, Line 8 = SKK, Line 9 = RSN, Line 10 = BSN1, Line 11 = KSN, Line 12 = AFT, Line 13 = BKK, Line 14 = BSN2, Line 15 = SHS.

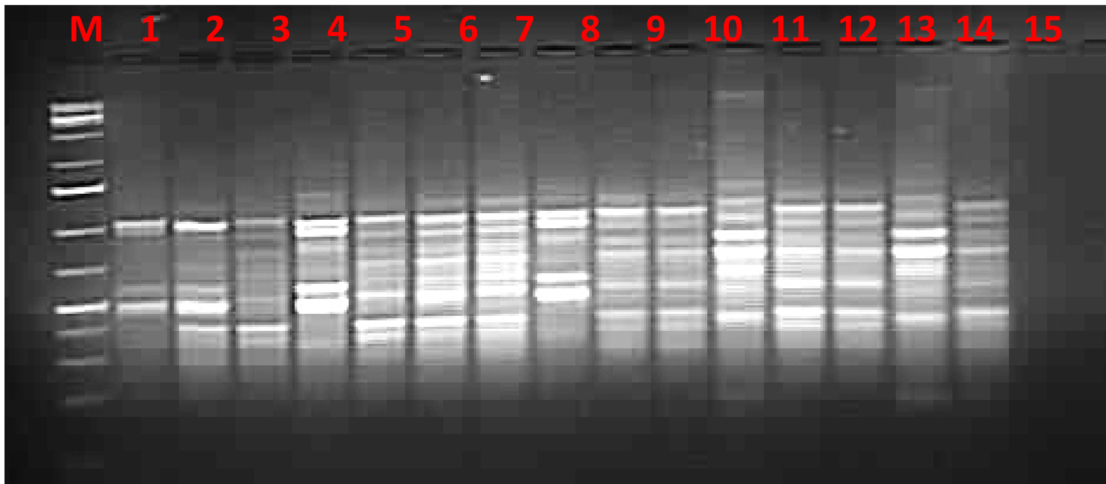


Fig. 4.3: DNA polymorphism in fifteen isolates *Mycogone perniciosa* using RAPD primer OPL-02, M; Marker, Line 1 = LDP, Line 2 = SHP, Line 3 = NAS, Line 4 = TPN, Line 5 = HSN, Line 6 = RHT, Line 7 = BFT, Line 8 = SKK, Line 9 = RSN, Line 10 = BSN1, Line 11 = KSN, Line 12 = AFT, Line 13 = BKK, Line 14 = BSN2, Line 15 = SHS.

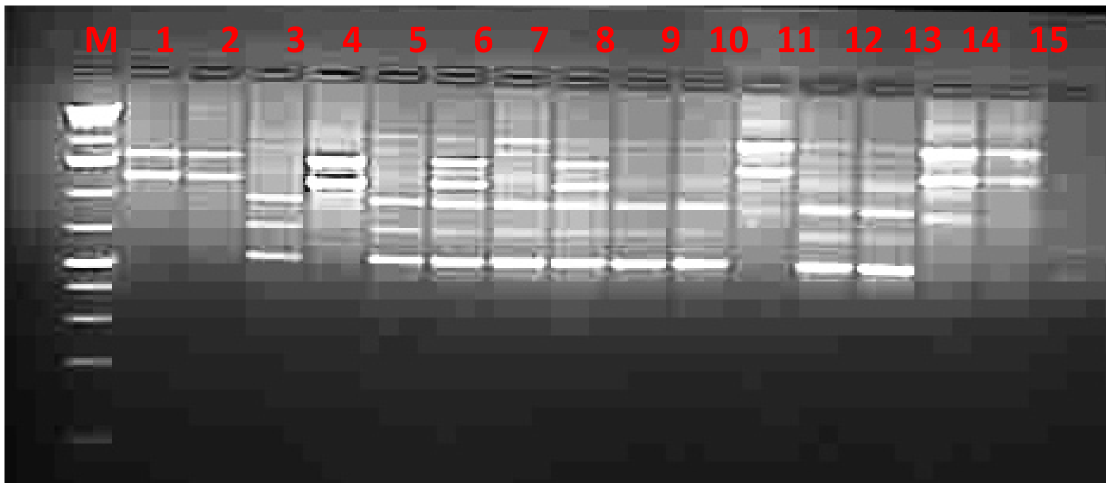


Fig. 4.4: DNA polymorphism in fifteen isolates of *Mycogone perniciosa* using RAPD primer OPE-10, M; Marker, Line 1 = LDP, Line 2 = SHP, Line 3 = NAS, Line 4 = TPN, Line 5 = HSN, Line 6 = RHT, Line 7 = BFT, Line 8 = SKK, Line 9 = RSN, Line 10 = BSN1, Line 11 = KSN, Line 12 = AFT, Line 13 = BKK, Line 14 = BSN2, Line 15 = SHS.

Table 4.7: Random primers showing polymorphism among *Mycogone perniciosa* isolates

Primer	Primer Sequence (5´-3´)	Total No. of bands	Monomorphic bands	Polymorphic bands	Percentage Polymorphism
OPC-01	GGTCCCTGAC	8	0	8	100.00
OPC-02	GAAACGGGTG	8	0	8	100.00
OPC-03	GTGACGTAGG	5	0	5	100.00
OPC-04	GTGATCGCAG	6	1	5	83.33
OPC-05	CAATCGCCGT	7	2	5	71.42
OPC-07	CAGCACCCAC	5	1	4	80.00
OPC-08	TCTGTGCTGG	7	0	7	100.00
OPC-09	TTCCGAACCC	7	1	6	85.71
OPC-10	TGATCCCTGG	8	0	8	100.00
OPD-01	CATCCCCCTG	7	1	6	85.71
OPD-02	GGACTGGAGT	8	0	8	100.00
OPD-04	TGCTCTGCCC	6	0	6	100.00
OPD-05	GTCCACACGG	6	1	5	83.33
OPD-06	TGGGGGACTC	6	2	4	66.67
OPD-08	CTGCTGGGAC	7	0	7	100.00
OPD-09	AAAGCTGCGG	16	0	16	100.00
OPD-10	TGTCATCCCC	8	0	8	100.00
OPE-01	AAGCCTCGTC	13	0	13	100.00
OPE-02	TGCGTGCTTG	12	0	12	100.00
OPE-03	GACGGATCAG	12	0	12	100.00
OPE-05	ACCGCGAAGG	10	0	10	100.00
OPE-06	GGACCCAACC	9	0	9	100.00
OPE-07	GTCGCCGTCA	11	0	11	100.00
OPE-09	TGAGCGGACA	13	0	13	100.00
OPE-10	ACTCCTGCGA	15	0	15	100.00
OPL-01	GTCCCGTGGT	10	0	10	100.00
OPL-02	TGGGCGTCAA	17	0	17	100.00
OPL-04	TGTAGCAGGG	13	0	13	100.00
OPL-05	GACTGCACAC	15	0	15	100.00
Total		**275**	**9**	**266**	**95.04**
Average		**9.48**	**0.31**	**9.17**	**-**

Table 4.8: Matrix of Jaccard´s genetic similarity co-efficient among *Mycogone perniciosa* isolates using RAPD data.

Isolates	LDP	SHP	NAS	TPN	HSN	RHT	BFT	SKK	RSN	BSN1	KSN	AFT	BKK	BSN2	SHS
LDP	1.00														
SHP	0.73	1.00													
NAS	0.45	0.70	1.00												
TPN	0.75	0.65	0.51	1.00											
HSN	0.43	0.68	0.91	0.53	1.00										
RHT	0.51	0.73	0.83	0.59	0.89	1.00									
BFT	0.44	0.68	0.84	0.55	0.90	0.87	1.00								
SKK	0.71	0.57	0.49	0.74	0.52	0.58	0.53	1.00							
RSN	0.46	0.63	0.82	0.49	0.84	0.80	0.87	0.53	1.00						
BSN1	0.46	0.64	0.82	0.51	0.84	0.79	0.86	0.52	0.92	1.00					
KSN	0.47	0.48	0.44	0.53	0.47	0.49	0.45	0.47	0.45	0.42	1.00				
AFT	0.47	0.65	0.81	0.53	0.81	0.76	0.83	0.54	0.83	0.85	0.45	1.00			
BKK	0.46	0.64	0.80	0.53	0.81	0.78	0.82	0.55	0.81	0.87	0.44	0.90	1.00		
BSN2	0.47	0.47	0.46	0.55	0.49	0.52	0.47	0.48	0.48	0.46	0.85	0.45	0.49	1.00	
SHS	0.54	0.67	0.75	0.56	0.75	0.75	0.75	0.52	0.76	0.78	0.55	0.81	0.79	0.55	1.00

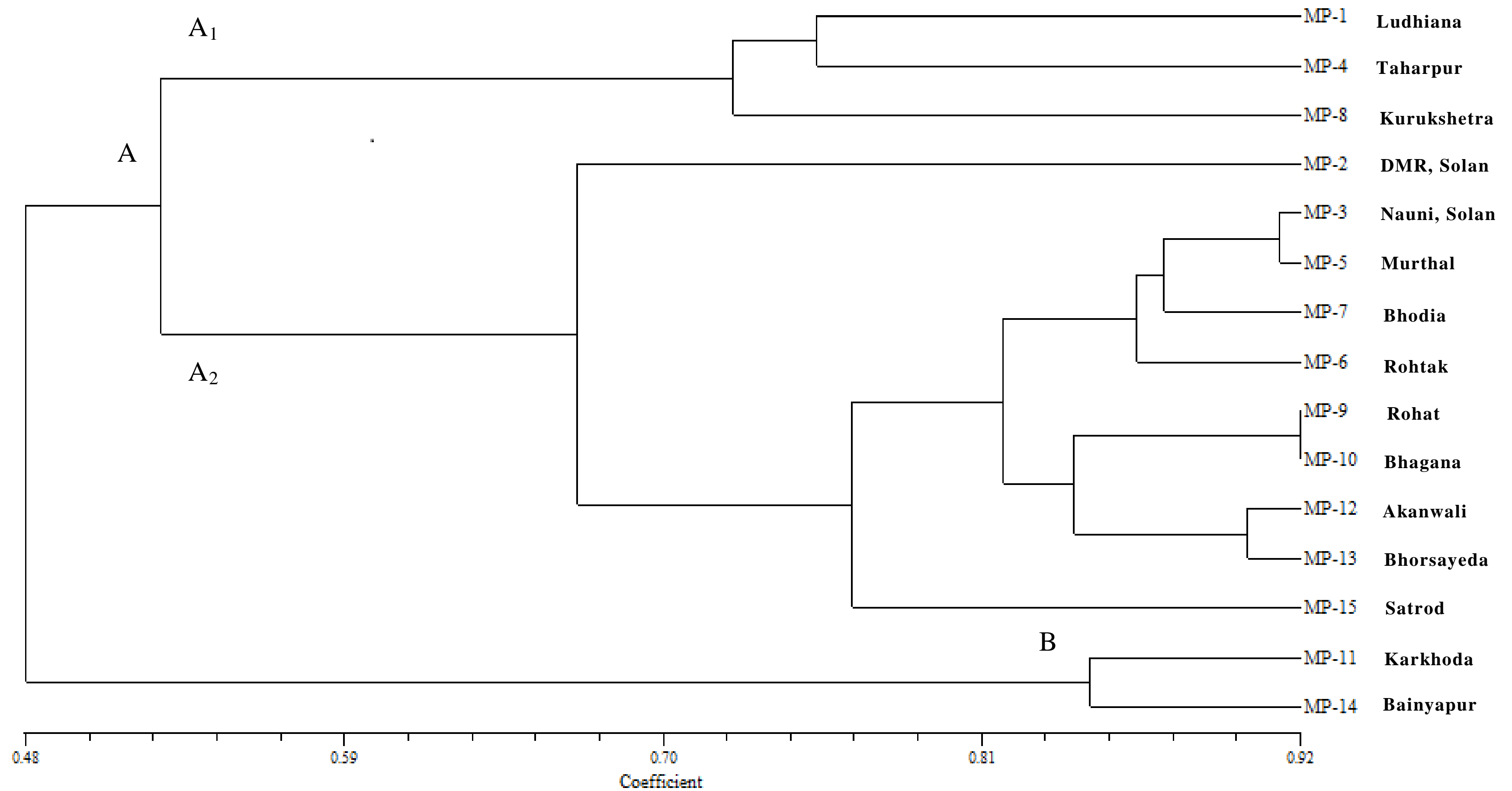


Fig. 4.5: UPGMA dendrogram showing relationship among fifteen isolates of *Mycogone perniciosa* based on RAPD primers, MP-1 = LDP, MP-2 = SHP, MP-3 = NAS, MP-4 = TPN, MP-5 = HSN, MP-6 = RHT, MP-7 = BFT, MP-8 = SKK, MP-9 = RSN, MP-10 = BSN1, MP-11 = KSN, MP-12 = AFT, MP-13 = BKK, MP-14 = BSN2, MP-15 = SHS.

CHAPTER-V

DISCUSSION

Wet bubble of button mushroom caused by *Mycogone perniciosa* (Magnus) earlier considered to be a minor disease, has emerged as destructive in recent years causing considerable yield losses in button mushroom growing areas. In view of economic importance of the disease, limited information is available on variability of this pathogen. Existence of variability is well known phenomenon in all organism including pathogenic fungi. Study of variability helps to know the evolutionary pattern of new strains or races of a pathogen in different geographical regions. To date scanty information is available on existence and distribution of variants of *Mycogone perniciosa* infecting button mushroom. Hence, the results obtained on symptomatological, morphological, physiological, chemosensitivity and genetic variability among the fifteen isolates of this pathogen is discussed here.

The symptomatic variability studies of the fifteen isolates of *Mycogone perniciosa* exhibited marked difference in the symptoms. The symptoms produced by the *Agaricus bisporus* strains by infection of different isolates of *Mycogone perniciosa* varied from thick stipe to sclerodermoid having brown coloured droplets to internal stipe browning. These symptoms were categorized on the basis of appearance of brown amber coloured droplets, gill infection and foul smell. Three isolates *viz*., LDP, RSN and BSN showed dark brown to light brown coloured symptoms on the pileus, whereas, four isolates *viz.,* SHP, SKK, KSN and BSN2 exhibited brown amber coloured droplets with no gills infection.

The results obtained in present investigations are in agreement with Fletcher *et al.* (1995) was showed varuation in symptoms produced on *Agaricus bisporus* strains which varied from amber droplets, gills infection, internal discoloration of stipe and cap spotting. Umar (2000) observed that pathological changes occured as a result of *M. perniciosa* infection which included dedifferentiation, tissue necrosis and an excessive, nodular proliferation of hyphae. Cytopathic changes are characterized by ribosomal condensation, rupture and peri-mitochondrial aggregations of the ribosomes, hydropic degeneration of many segments, hypertrophy or occlusion of dolipore septa by coagulated materials, cell wall thickening or lysis. The hyphae of the mycopathogen coiled around the host hyphae with firm adhesions causing their death.

Sharma and Kumar (2000) also observed the infected areas on the mushroom become creamish-brown in colour due to the formation of dark coloured chlamydospores. Exudation of accumulated extracellular fluid resulted in a teardrop phenomenon and clear brown coloured drops are exuded as a result of the activity of the putrefying bacteria in that infected parts of the mushroom.

In the present study four isolates *viz*., BFT, SKK, KSN and BSN2 showed white fluffy growth on casing surface, while the isolate LDP exhibited small sclerodermoid with no internal stipe browning. However, isolate TPN resulted in a thick stipe with gills infection, while isolate NAS showed deep cracking on fruiting bodies along with foul smell. Ghazzawi and Beig (2011) also observed similar results the early infection badly affected the fruiting bodies, turning them into shapeless masses covered with white fluffy mycelium and the fruiting bodies became brown and started to decay. The internal tissues were also discoloured, watery with the appearance of holes resulting in decay with foul smell. When fruiting bodies became dry, it started to decay and turned red brown.

The morphological variability studies of fifteen isolates of *M. perniciosa* exhibited variation in the colony growth, mycelium, colony colour and spore size. On the basis of colony growth three isolates *viz*., LDP, HSN and RSN showed uneven colony growth, while, the rest of isolates showed even colony growth. Feathery growth was observed in seven isolates *viz*., HSN, BFT, SKK, RSN, KSN, BKK and BSN2, while, rest of the isolates did not show this character.

In the present study mycelium varied from aerial, dense, sparse to fluffy among *Mycogone perniciosa* isolates. Amongst the isolates *viz*., LDP, SHP, NAS, RHT, RSN, BKK and BSN2 was aerial whereas, isolate TPN showed dense mycelium. Four isolates *viz*., HSN, BFT, BSN1 and AFT had sparse mycelium, however, three isolates *viz*., SKK, KSN and BSN2 had fluffy mycelial growth but it was even and very feathery in nature. The colony colour among the isolates of *Mycogone perniciosa* varied from brown, white to lemon colour and five isolates *viz*., TPN, RHT, SKK, KSN and BSN2 showed white colony colour whereas, isolate HSN showed lemon colour.

The present studies are in accordance with Fletcher *et al*. (1995) was showed that the colonies of *Mycogone perniciosa* had either an even or uneven edge and the mycelium varied from dense to sparse, while the colour of colonies were yellow brown, dark brown, pale brown and white. Similarly, Glamoclija *et al*. (2007) also observed that colony characteristic in different isolates were variable. The colonies growth of some isolates were dense, sparse, fluffy, aerial and irregular in shape. According to Basha and Chatterjee (2007) variations in type of mycelial growth of isolates of of *Mycogone perniciosa* was observed as the colonies of seventeen isolates were fluffy, whereas, three, showed dense mycelial growth.

The spores size in present studies was measured, the length ranged from 18.6-21.8 µm while the maximum breadth was 16.9 µm and the minimum was 11.2 µm. Among the isolates, two isolates *viz*., KSN and BSN2 had maximum spore size, 21.8x16.9 and 21.6x16.7 µm, respectively, whereas, the minimum sized spore (18.6x11.1 µm) was

recorded in isolates RHT. The present investigation is in accordance with earlier workers (Ghazzanoi and Being, 2011; Sabharwal and Kapoor, 2015)

The radial growth of fifteen isolates studied at four temperature level revealed variability. The maximum growth of the isolates was observed at 25°C, while it was minimum at 20°C, and 30°C, whereas, the least mycelia growth was recorded at 15°C. On the basis of their relative growth rate these isolates were categorized into three different groups *viz*., fast growing, moderately growing and slow growing in nature at 25°C. Of the fifteen isolates two isolates KSN and BSN2 were fast growing (>71 mm), whereas, six isolates *viz*., LDP, SKK, RSN, BSN1, AFT and BKK were moderately growing (50-70mm) and rest of the seven isolates *viz*., SHP, NAS, TPN, HSN, RHT, BFT and SHS were slow growing (20-50mm). The present results are in conformity with the observations made by Sabharwal and Kapoor (2015) who observed that the optimum temperature for the mycelial growth of this pathogen was 25°C, while poor growth was recorded at 15°C, 20°C and the worst growth was examined at 30°C.

The variability within the *Mycogone perniciosa* isolates were recorded on the basis of different growth rate of the mycelia of these isolates at different pH levels *viz.,* 6.0, 7.0, 8.0 and 9.0. The results in present study indicated that *Mycogone perniciosa* grow at the range from 6.0 to 9.0. However, it attains the maximum growth at pH 7.0. Most of the isolates of *Mycogone perniciosa* exhibited higher (90 mm) growth except one isolates TPN that attained 40.7 mm radial growth after ten days of incubation. The radial growth of *Mycogone perniciosa* decreased with increase or decrease in pH from 7.0, while the reduction was higher at pH 9.0, followed by pH 6.0 and 8.0 respectively. It was interesting that four isolates *viz*., RHT, AFT, BKK and SHS were highly sensitive to higher values of pH and had no growth when pH was more than 7.0.

The present findings are in accordance with the findings of Sabharwal (2015) *Mycogone perniciosa* mycelium developed best at pH 7.0 in solid medium. The radial growth of *Mycogone perniciosa* isolates decreased as pH increased or decrease from pH 7.0, while reduction was higher at pH 9.0. Similar results have been observed by earlier workers (Tan *et al*., 1994; Glamoclija *et al*.; 2008 and Siwulski *et al*., 2011)

The efficacy of two fungicides was studied for mycelial growth inhibition at different concentrations using poisoned food technique under *in vitro* conditions. The results showed the different isolates of *Mycogone perniciosa* showed variability in their sensitivity to carbendazim and metalaxyl MZ. metalaxyl was more toxic to all the isolates as compared to carbendazim. On the basis of chemosensitivity to carbendazim (25 µg/ml) and metalaxyl MZ (300 µg/ml) the fifteen isolates of *Mycgone perniciosa* were categorized into three groups *viz*., highly sensitive (HS, 81-100% inhibition), sensitive (S, 41-80% inhibition) and less sensitive (LS 0-40% inhibition). Out of these isolates, nine

isolates *viz*., LDP, SHP, TPN, HSN, RHT, BFT, RSN, KSN and BKK were highly sensitive, two isolates viz., NAS and BSN2 were sensitive, rest of the four isolates *viz*., SKK, BSN1, AFT and SHS were less sensitive to the carbendazim. Out of these fifteen isolates, eleven isolates *viz*., LDP, SHP, NAS, TPN, HSN, RHT, BFT, RSN, KSN, BKK and BSN2 were highly sensitive, four isolates *viz*., SKK, BSN1, AFT and SHS were sensitive, while none of the isolate were under less sensitive to the *Mycogone perniciosa* isolates.

The results of this study are in agreement with Bhatt and Singh, (2002) was observed that the mycelial growth of *M. pernociosa* isolates was completely inhibited by Bavistin (25 µg/ml) and Ridomil MZ (300 µg/ml). Lower concentrations of these fungicides also affected the growth of pathogen significantly. However, Bavistin (20 µg/ml) and Ridomil MZ (25 µg/ml) inhibited the growth of this pathogen more than 90 per cent.

Similarly, Francisco *et al*. (2010) under *in vitro* experiments showed that carbendazim and prochoraz-Mn were most effective fungicides by inhibiting the mycelial growth of *Mycogone perniciosa*, while, iprodione was least effective. The fungitoxic effect of carbendazim and metalaxyl MZ is provided by interfering with a number of cellular processes such as mitosis, meiosis, intracellular transport of molecules and the maintenance of cell shape (Orbach *et al.*, 1986; Peterbauer *et al.*, 1992; Tikhomirova and Inge-Vechtomov, 1996)

In present investigation, the interaction between *Mycogone perniciosa* isolates and *Agaricus bisporus* strains were evaluated under *in vitro* conditions for the inhibition of mycelial growth of *Agaricus bisporus*. During this interaction four isolates *viz*., NAS, HSN, RHT and BFT showed maximum inhibition of strain U3 (40.3 to 42.3% inhibition), which showed *Agaricus bisporus* strain U-3 was most sensitive to these four isolates, whereas, the *Agaricus bisporus* strain ABL-7 was least sensitive during the interaction. The present findings are in agreement with the report given by Pietrese (2005) the interaction of *Mycogone perniciosa* with *Agaricus bisporus* strains was found significant inhibition of mycelial growth of *Agaricus bisporus* by *Mycogone perniciosa* was more pronounced.

Calonje *et al*. (1997) reported that the *Verticilium fungicola* induced to produce large number of hydrolytic enzymes like chitanase, protease, glucanases, xylanases and glucosidases in presence of inducers like cell wall and different carbon source on encountering with host *Agaricus bisporus*. Similar results has been reported by Basher and Chakma (2014) in case of two non-related fungi the highest inhibition of mycelial growth was noticed in *T. harzianum* (45.9%), followed by *T. viride* (31.9%), proved effective in mycelial growth inhibition of test fungi. However, Srideepthi and Krishan

(2015) reported the efficacy of *T. viride* and *T. harzianum* against pathogen, which showed 68.4 and 34.2 per cent of mycelial growth respectively. This variation in effectiveness might be due to difference in nature, quality and quantity of the inhibitory substances produced by pathogens.

Molecular markers have been used widely to characterize fungal plant pathogen populations, in particular for the assessment of genetic diversity, phylogenetic relationships and the characterization of pathotypes (Brown, 1996; Michelmore and Hulbert, 1987). Recent developments in the field of DNA technology provides exciting avenues for identification of host genes, pathogens diversity and phylogenetic relationship within and outside the pathogen population (Bielikova *et al.*; 2002; Xu *et al.* 2003; Zamani *et al.*, 2004; Gouveia *et al.*, 2005). Several molecular markers have been used in diversity analyses of *Mycogone perniciosa* isolates, among these random amplified polymorphic DNA (RAPD) is a novel marker with potential use in taxonomic, diversity and phylogenic analyses especially at inter- and intra-species levels, where it allows generating discrete DNA fragments that are species or strain-specific (Sharma *et al.*, 2011).

Out of thirty six random primers screened, only twenty nine primers responded by producing detectable bands. In RAPD profiling, total of 275 scorable bands were produced with an average of 9.48 bands per primer, of which 266 bands were polymorphic and nine bands were monomorphic in nature the number of bands produced per primer ranged from 5 to 17. Twenty nine primers were polymorphic out of which twenty two primers were completely polymorphic and remaining seven exhibited variable range of polymorphism. Average number of polymorphic and monomorphic bands per assay was 9.17 and 0.31 respectively. RAPD based pairwise genetic similarity of fifteen isolates of *Mycogone perniciosa* illustrated that highest genetic similarity value (0.92) was recorded between RSN and BSN1, while the lowest similarity value (0.42) was between KSN and BSN1.

The unweighted pair group method with arithmetic means (UPGMA) analysis differentiated the fifteen *Mycogone perniciosa* isolates into two major clusters A and B at similarity coefficient of 0.67. All the *Mycogone perniciosa* isolates in the major cluster A was further clustered into sub-cluster A_1 and sub-cluster A_2 at similarity coefficient of 0.54. Sub-cluster A_2 was a large group having nine isolates *viz.*, SHP, NAS, HSN, BFT, RHT, RSN, BSN1, AFT and BKK showing closeness to each other, whereas, sub-cluster A_1 was constituted by only three isolate *viz.*, LDP, TPN and SKK having wide distance from other isolates. Within sub-cluster A_2 the maximum genetic similarity (92%) was detected between isolates RSN and BSN1, whereas, the isolates *viz.*, SHS and SHP were distantly related with the lowest genetic similarity. KSN and BSN2 exhibited 84 per cent genetic similarity. Overall, the maximum genetic similarity (92%) among *Mycogone perniciosa* isolates was detected between isolates BSN1 and KSN, whereas, the minimum genetic similarity (42%)

was shown by isolates *viz*., BSN1 and KSN with a wide genetic distance. However, cluster B was constituted by only two isolates *viz*., KSN and BSN2 having wide distance from other isolates. In the present study, the RAPD analyses successfully differentiated the isolates of *Mycogone perniciosa* which showed high level of polymorphism.

The present study are in conformity with Sharma *et al.* (2011) who observed that ten isolates of *Mycogone perniciosa* collected from different mushroom growing farms and different regions showed genetic variability by using five different RAPD primers. Yadav *et al.* (2003) reported that the genetic relationship of nine isolates *Agaricus bitorquis* based on RAPD markers was depicted by dendrogram and exhibited two major phylogenetic clusters. First cluster comprise of six strains showing intra cluster variation and second has three genetically distinguish strains. The average genetic distance between these two groups was 91.5 per cent indicating high level of polymorphism.

Largeteau *et al*., (2006), reported that DNA polymorphism was observed in different isolates of *Verticillium* var. *fungicola* cluster analysis of pairwise dissimilarity between isolates of var. *fungicola* isolates into a distinct cluster, remote from the var. *aleophilum* isolates. All primers distinguished between VX02, VTAW, and the American isolates, shown to be remote on the dendrogram, but V01 and V35 grouped together. Fifteen of the eighteen European var. *fungicola* isolates did not show any RAPD polymorphism and can be considered clonal. VCF, VV1 and VCTC showed polymorphism with one (OPH20), two (OPA12 and OPH20) and nine primers (OPA09, OPA11, OPA12, OPA13, OPA17, OPA18, OPD04, OPH19 and UBC30)out of 24, respectively. Variability was characterised by the presence or absence of a single band. Analysis of the AFLP banding pattern yielded 198 bands of which 71 were informative. All seven strains screened could be identified from each other but genetic distances were very large.

Collopy *et al*., (2002) reported that genetic variability within isolates of *V. fungicola* investigated and used a random amplified polymorphic DNA (RAPD) analysis to examine the within-species variation of this organism. Sixty-six isolates collected over a 45-year period (1950-1995) were compared with respect to colony morphology, response to fungicides, virulence, geographic origin and RAPD grouping. When all 66 isolates were analyzed together, no correlation was found between RAPD grouping, colony morphology and virulence. However, all recently collected isolates (1993-1995) were similar in their fungicide responses, virulence, colony morphologies, geographic origins, and RAPD groupings. This high level of genetic dissimilarity within recent isolates of *V. fungicola* was obtained.

CHAPTER-VI

SUMMARY AND CONCLUSION

In present studies an efforts has been made to differentiate the variability among the isolates of *Mycogone perniciosa* collected from different geographical areas of Haryana, Punjab and Himachal Pradesh. Wet bubble disease caused by *Mycogone perniciosa* is one of the devastating disease of *Agaricus bisporus* in the mushroom growing areas. Hence, the studies were carried out on symptomatological, cultural, morphological, physiological, chemosensitive and genetic variabilities in the isolates of *Mycogone perniciosa.* The outcome of present investigation about variability in *Mycogone perniciosa* are summarized here.

The symptomatological studies revealed the variability among the isolates of *Mycogone perniciosa* varied from thick stipe to sclerodermoid having brown coloured droplets to internal stipe browning. These symptoms were categorized on the basis of appearance of brown amber coloured droplets, gill infection and foul smell. Three isolates *viz*., LDP, RSN and BSN showed dark brown to light brown coloured symptoms on the pileus, whereas, isolates *viz*., SHP, SKK, KSN and BSN2 exhibited brown amber coloured droplets with no gills infection. These fifteen isolates were categorized on the basis of symptoms induced into four different categories *viz*., highly virulent, virulent, moderately virulent and less virulent. Among these four isolates *viz*., SHP, SKK, KSN and BSN2 were highly virulent, releasing brown amber coloured droplets with foul smell. Three isolates *viz*., RHT, RSN and BSN1 were virulent exhibiting gills infection with foul smell. However, three isolates *viz*., LDP, TPN and AFT were moderately virulent, whereas, rest of the three isolates *viz*., NAS, BFT and SHS were categorized less virulent. It is evident from data that all the isolates under studies were virulent in nature. The strain of *Agaricus bisporus* showing variation in the degree of virulence of these isolates.

In case of morphological variability studies of *Mycogone perniciosa* isolates showed that the isolates exhibited variation among themselves regarding colony growth, mycelial growth, colony colour and spore size. Three isolates LDP, HSN and RSN showed uneven colony growth, while, rest of the isolates showed even colony growth. The feathery growth was observed in seven isolates *viz*., HSN, BFT, SKK, RSN, KSN, BKK and BSN2, while, rest of the isolates did not show this character. The mycelium varied from aerial, dense, sparse to fluffy among isolates. The seven isolates *viz*., LDP, SHP, NAS, RHT, RSN, BKK and BSN2 was aerial whereas, isolate TPN showed the dense mycelium. The four isolates *viz*., HSN, BFT, BSN1 and AFT had sparse mycelium however, three isolates *viz*., SKK, KSN and BSN2 had fluffy mycelial growth but it was even and very feathery in nature. The colony colour among the isolates of *Mycogone perniciosa* varied from brown, white to lemon colour

among these five isolates *viz*., TPN, RHT, SKK, KSN and BSN2 showed white colony colour whereas, isolate HSN showed lemon coloured.

In case of physiological variability, isolates grow at 15°C, 20°C, 25°C and 30°C to assess their growth behavior. The maximum radial growth (76.0 mm) was exhibited by isolates BSN2, while it was minimum in case of isolate SHS. At 30°C the maximum radial growth (56.6 mm) was four in isolate BSN2 followed by KSN (55.2 mm), while, minimum mean radial growth was recorded in isolate SHP (14.9 mm) after ten days of incubation. On the contrary maximum mean radial growth was found at 25°C (88.7 mm) followed by 20$_{o}$C (54.8 mm) 30°C (41.2 mm) and the least growth was observed at 15°C (30.8 mm). The temperature of 25°C was most suitable temperature for mycelial growth of *Mycogone perniciosa* .

On the basis of mycelial growth at different pH levels of *Mycogone perniciosa* isolates the growth was better in the pH range 5.0 to 8.0, however, it attained the maximum growth at pH 7.0. Most of the isolates had maximum growth (90 mm) at pH 7.0 except one isolate TPN which attained (40.7 mm) radial growth after ten days of incubation and the growth of isolates decreased significantly with any deviation from a pH level 7.0 and the reduction was highest at pH 9.0 followed by pH 6.0 and pH 8.0 irrespectively of *M. perniciosa* isolates evaluated under the studies. The pH level of 7.0 was found optimum for mycelial growth of the isolates of *Mycogone perniciosa.*.

On the basis of chemosensitivity to carbendazim and Metalaxyl MZ these fifteen isolates of *Mycogone perniciosa* were categorized into three groups *viz*., highly sensitive, sensitive and less sensitive (LS, 0-40% inhibition).The chemosensitivity studies revealed the isolates of *Mycogone perniciosa* were more sensiyive to metalaxyl MZ. On the basis of chemosensitivity of these fifteen isolates, eleven isolates were highly sensitive, four isolates *viz*., SKK, BSN1, AFT and SHS were found sensitive while none of isolate found less sensitive to *Mycogone perniciosa*.

The variability within the *Mycogone perniciosa* isolates was determined on the basis of mycelial inhibition *A. bisporus* due to *M. perniciosa* during the interaction to studies. Eight different strains of *A. bisporus* were screened against *Mycogone perniciosa*.and mycelial inhibition was calculated. Two strains U3 and ABL 5 of *A. bisporus* showed maximum sensitivity to all the *M. perniciosa* isolates, whereas, four *Mycogone perniciosa* isolates *viz.,* RSN, BSN1, KSN and BSN2 were pathogenic against all the *A. bisporus* strain.

The investigation on molecular variability of fifteen isolates of *Mycogone perniciosa* collected from Haryana, Punjab and Himachal Pradesh of India showed the variation at the molecular level. A set of 36 random primers to amplify DNA fragments from the genomic DNA of fifteen isolates of *Mycogone perniciosa.* Out of thirty six random primers screened, only twenty nine primers responded by producing detectable bands. In RAPD profiling, total

of 275 scorable bands were produced with an average of 9.48 bands per primer, of which 266 bands were polymorphic and nine bands were monomorphic in nature The number of bands produced per primer ranged from 5 to 17. Twenty nine primers were polymorphic out of which twenty two primers were complete polymorphism and remaining seven exhibited variable range of polymorphism. Average number of polymorphic and monomorphic bands per assay was 9.17 and 0.31 respectively.

RAPD based pairwise genetic similarity of fifteen isolates of *Mycogone perniciosa* illustrated that highest genetic similarity value (0.92) was recorded between RSN and BSN1, while the lowest similarity coefficient value (0.42) between KSN and BSN1. The association amongst isolates was presented in the form of UPGMA generated dendrogram. It clearly segregated all the fifteen isolates, into two major clusters *viz*., A and B at similarity coefficient of 0.48, *i.e*, 48 per cent. The major cluster A was further divided into two sub-clusters *viz*., A_1 and A_2. First subcluster (A_1) of cluster A, comprising three isolates *viz.,* LDP, TPN and SKK belonging to Ludhiana, Taharpur, Panipat and Kurukshetra, respectively showing closeness to each other. Similarly, major cluster B comprising two isolates and in this the maximum genetic similarity 85 per cent was observed between isolates KSN and BSN2 belonging to Kharkhoda (Sonipat) and Bainyapur (Sonipat) of Haryana. These two isolates *viz*., KSN and BSN2 were highly diversed from rest of isolates of the *Mycogone perniciosa* showing wide genetic distance.

Furthermore, no comprehensive study in India has been done on variability in this pathogen infecting *Agaricus bisporus*. It is opined that significant differences in symptomatology, morphological, physiological, chemosensitivity and genetic variability were observed among different isolates collected from different locations.

The while symptoms variation observed among the isolates of *Mycogone perniciosa* along with morphological, physiological showed the isolates, KSN and BSN2 were highly variable and fast in growth nature. Which showed that these isolates were highly diversed to all. The genetic distance of these two isolates *viz.,* KSH and BSN2 was 48 per cent to rest of isolates. In future it is likely these two isolates of *Mycogone perniciosa* can be used in the screening programme of strains of *Agaricus bisporus.*

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ABSTRACT

Title of Thesis	:	**Variability studies in *Mycogone perniciosa* causing wet bubble of *Agaricus bisporus***
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Key words: *Agaricus bisporus*, *Mycogone perniciosa*, morphology, physiology, Variability

(An abstract of the thesis submitted to the CCS Haryana Agricultural University, Hisar in the partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology)

Wet bubble disease caused by *Mycogone perniciosa* is one of the most devastating fungal disease and causes considerable yield loses in *Agaricus bisporus.* The present investigation on symptomatology, morphology, physiology, chemosensitivity and genetic variability was carried out under *in vitro* with fifteen isolates of *M. perniciosa* collected from major mushroom growing areas. The symptoms induced by different isolates of *M. perniciosa* varied from thick stipe to sclerodermoid having brown amber coloured droplets to internal browning. These were categorized on the basis of brown amber colour droplets, gills infection and foul smell occurrence. Isolates *viz.,* LDP, RSN and BSN1 showed the symptoms on pileus like dark brown to light brown colour, while some isolates exhibited the brown amber droplets. A wide variation in symptoms was also detected when isolates were compared with isolates from widely reported origin of geographical regions in Haryana, Punjab and Himachal Pradesh. Isolate TPN showed thick stipe, slow mycelial growth with slightly feathery, dense mycelium and lemon colony colour. While symptoms variation observed among the isolates of *M. perniciosa* were compared with morphological, physiological and genetic variability, it was observed that the isolates, KSN and BSN2 produced fluffy, even, very feathery and brown in colony colour was recorded fastest growing. In results 25°C was found most suitable temperature for mycelial growth of *Mycogone perniciosa*. While, the pH level 7.0 was found optimum for mycelial growth of the isolates studied. These two isolates *viz*., KSN and BSN2 were highly diversed from rest of isolates of the *M. perniciosa* showing wide genetic distance. On the bsasis of chemosensitivity to metalaxyl MZ among these fifteen isolates, eleven isolates of pathogen were highely sensitive but none of isolate were under less sensitive group to *Mycogone perniciosa* isolates. Interaction studies between *Mycogone perniciosa* isolates and *Agaricus bisporus* strains showed mycelial inhibition. The highest pair wise genetic similarity i.e., 92.0 per cent was observed between sub cluster isolates RSN and BSN1 followed by 85.0 per cent genetic similarity between isolates KSN and BSN2 of major cluster B. In future it is likely these two isolates of *Mycogone perniciosa* can be used in the screening programme of strains of *Agaricus bisporus.*

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- **1st position in Quiz contest** and got **1st position in camp activities** in 10 days annual camp NSS, 2007.

(m) **Participation in seminar/conference/symposium** :

- **Participated in 1st National Brassica Conference on Production Barriers & Technological Options in Oilseed Brassica** at CCS Haryana Agricultural University, Hisar, March, 2-3, 2012.
- **Participated in National Seminar on Sustainable Agriculture & Food Security: Challenges in Changing Climate** "SAFS-2012" at CCS,HAU, Hisar, March, 27-28, 2012.
- Participated and **presented a paper** in DRDO Sponsored National Conference on **GREEN TECHNOLOGIES FOR GREEN ENVIRONMENT**, at G.V.M. Girls College, Sonepat, February, 18-19, 2013.
- **Participated in 35th Annual Conference and Symposium of ISMPP on "Innovative and Eco-friendly Research Approaches for Plant Diseases Management"** at Dr. PDKV, Akola, Maharashtra, January, 8-10, 2014.
- **Participated and presented Poster in National Symposium of IPS and ISMPP on "Holistic Approach for Plant Health Management"** at Dr YSPUH&F, Nauni, Solan, Himachal Pradesh, India. September, 28-29, 2015.

List of Publications

- **Manmohan**; 2013. Influence of sowing time and spacings on the severity of important diseases of rapeseed-mustard. *Forage Res.,* **38** (4): 221-224.
- Ved Prakash, Ramniwas, M.L.Khichar, Dinesh Sharma, **Manmohan** and Baljit Singh. 2015. Agrometeorological indices and intercepted photosynthetically active radiation in cotton crop under different growing environments.**29** (2).
- **Manmohan,** Surjeet Singh, Ashwani Kumar and Jagdeep Singh. Variability studies of *Mycogone perniciosa* isolates causing wet bubble disease of *Agaricus bisporous* based on physiological parameters. In annual meeting of IPS and ISMPP on "Holistic approaches for plant health management" at Dr YSP University of horticulture & forestry, Nauni, Solan, Himachal Pradesh, India, September, 28-29, 2015, p. 164.
- **Manmohan,** Ashwani Kumar, Surjeet Singh and Jagdeep Singh. Cultural and morphological variability studies on *Mycogone perniciosa* isolates causing wet bubble disease of button mushroom. In annual meeting of IPS and ISMPP on "Holistic approaches for plant health management" at Dr YSP University of horticulture & forestry, Nauni, Solan, Himachal Pradesh, India, September, 28-29, 2015, p. 165.

(Related to thesis work only)

Manmohan

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I also undertake that, patent, if any, arising out of the research work conducted during the programme shall be filed by me only with due permission of the competent authority of CCS Haryana Agricultural University, Hisar.

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