INVESTIGATIONS ON
Morchella esculenta Fr.

THESIS

By

PIARO DEVI

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PALAMPUR - 176062 (H.P.) INDIA

IN

Partial fulfilment of the requirements for the degree

OF

MASTER OF SCIENCE IN AGRICULTURE
(PLANT PATHOLOGY)

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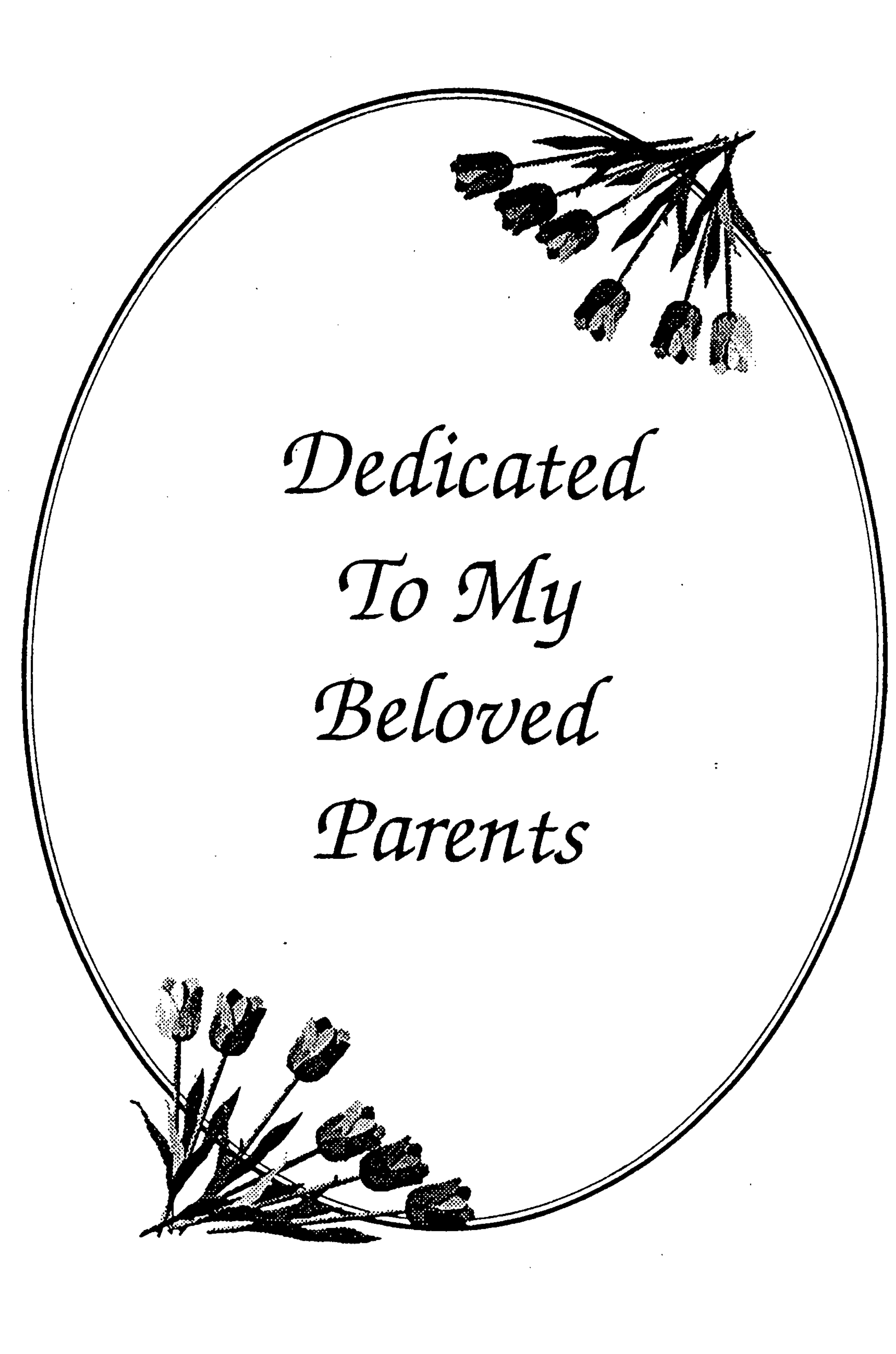

Dedicated
To My
Beloved
Parents

Dedicated
To My
Beloved
Parents

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CERTIFICATE I

This is to certify that the thesis entitled, **"Investigations on *Morchella esculenta* Fr."**, submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Plant Pathology** of Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a *bonafide* research work carried out by **Ms. Piaro Devi** daughter of Shri Prem Kumar under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

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

(B.M. Sharma)
Chairman,
Advisory Committee

Place : Palampur
Dated : the November 9 , 1998.

CERTIFICATE II

This is to certify that the thesis entitled, **"Investigations on *Morchella esculenta* Fr."**, submitted by **Ms. Piaro Devi** daughter of Shri Prem Kumar to the Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Plant Pathology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

Chairman,
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I owe entire responsibility for all errors and omissions.

(PIARO DEVI)

Place: Palampur
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INTRODUCTION

CHAPTER-I

INTRODUCTION

Edible mushrooms are known for their excellent culinary characteristics and gastronomical properties. They have been a part of dietary habits, from the earliest days of mankind. They are considered as a delicacy in food and form choicest table dishes due to which they have come to occupy an important position in human diet.

Nearly 2,000 species of mushrooms have been reported to be edible. Of these, about a dozen species have been reported to be cultivated under controlled conditions. The production of cultivated mushrooms is being dominated by the common white button mushroom (*Agaricus bisporus*) which accounts for approximately three-fourth of the world's total production (Chang and Miles, 1989). Other commercially cultivated species are *Pleurotus* spp., *Auricularia* spp. (Wood ear), *Lentinus edodes* (Shiitake), *Volvariella volvacea* (straw mushroom), *Flammulina velutipes* (winter mushroom) and *Tremella fuciformis* (white jelly fungus).

There is an increasing interest for mushrooms other than the white button mushroom and trend is towards the species with more flavour. Among such mushrooms, the morels possess the most desirable and delicate flavour. The morel (*Morchella esculenta* Fr. and related

species) has been the most prized edible mushroom for many centuries. It is perhaps the best known edible mushroom and is easily identified even by those who have little or no knowledge of mycology. Morels are collected from the wild and are considered as delicacy by the connoisseurs of food.

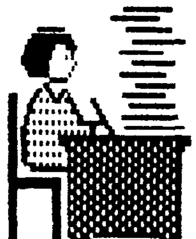
Morels occur in sufficient quantities in the forests of Himachal Pradesh (Waraitch, 1976). More than 2800 quintals of dried morel specimens were taken out from the forests of Himachal Pradesh during 1991-92 (Anonymous, 1993). Its continuous collection is leading to fast depletion of its population in the natural habitat (Sharma *et al*, 1995). The dried specimens of morels fetch a fabulous price of Rs. 4000-5000 per kg in cities.

The obvious commercial application of morels has been attracting the researcher's attention since the beginning of the present century. The morels, however, have defied all the attempts at its consistent indoor cultivation. Recently some break through has reportedly been achieved in cultivating morels (Ower *et al.*, 1986, 1988) in the U.S.A. Systematic studies on various cultivation aspects of this mushroom are being carried out in this laboratory since 1992. Sud (1995) made exhaustive studies on its collection, distribution, natural soil analysis, optimal conditions and requirement of carbon and nitrogen compounds for mycelial growth. Two strains of *M. esculenta* viz.

`Pre-winter' and `Post-winter' have been reported from Himachal Pradesh (Sharma *et al.*, 1997). A process for large scale production of sclerotia has been developed based upon some results obtained from the studies on the nutrition of vegetative hyphae (Sharma *et al.*, 1997).

Present investigations constitute an extension of the research work conducted earlier in this laboratory on various aspects with the aim of achieving the objectives listed below.

1. Collection of specimens and sclerotia of *M. esculenta* from nature.
2. Cultural studies in relation of vitamin and mineral requirements for vegetative growth vis-a-vis sclerotial production.
3. Comparative biochemical analysis of sclerotia collected from nature and those produced in the laboratory.
4. Determination of precise conditions for triggering *in vitro* carpogenic germination of sclerotia.



REVIEW
OF
LITERATURE

CHAPTER-II

REVIEW OF LITERATURE

The species of *Morchella* are commonly referred as morels. Morels grow in a variety of places such as hard wood forests, old orchards and burnt over woods from mid March to May and September to October. Morels are common in the North-Western Himalayan region. The occurrence of morels has been reported by various workers from different parts of world, from time to time. Despite difficulty in fruit body production, the vegetative mycelium of mushroom is easy to grow on wide variety of media. The information available on various aspects of morel's biology and some of the cultivated mushrooms has been reviewed under following headings.

2.1 OCCURRENCE

Morchella esculenta is known to be widely distributed species and has been reported from different parts of the world, in the past.

In India, this is a common species in North-Western Himalayan region and has been reported from various localities by Waraitch (1976). Kaul *et al.* (1981) in their studies at two morel bearing sites in Kashmir Valley found that morel fruiting occurs when soil moisture

is above 20 per cent, relative humidity 58 per cent and soil temperature in the range of 10-23°C above ground and 8.5-20°C below ground. Batra (1983) gave brief account of its ecology, time of fruiting, collection and mode of consumption etc. Guzik *et al.* (1988) and Chen (1989) studied its edibility and medicinal properties. Sharma *et al.* (1995) monitored ascocarp development of *M. esculenta* under natural conditions and reported that fruit body develops at the expense of subterranean sclerotium. The appearance of fruit bodies of *M. esculenta* appeared to be directly related to the prevailing temperature. Sharma *et al.* (1997) also studied physico-chemical characteristics of soils supporting the growth of morel mushroom and reported that soil moisture level of 40-45 per cent, pH around 6 and rich nutrient supply (N, P and K) appear to have a direct relationship with morel's growth. Sharma *et al.* (1997) further reported the occurrence of two strains of *M. esculenta* on the basis of ecological, morphological and cultural characteristics which were designated as `Pre-winter' and `Post-winter'.

2.2 NUTRITIONAL REQUIREMENTS

Sud (1995) while working on physiological studies of the strain of *M. esculenta* on the temperature of 20°C, pH 6.5 and Coon's medium were optimum for mycelial growth. Starch and potassium nitrate were found to be best carbon and nitrogen sources, respectively.

2.2.1 Vitamin nutrition

Madelin (1956) found that a higher concentration of thiamine was needed for sporulation than for growth of *Coprinus lagopus*. Block *et al.* (1959) reported definite requirement of thiamine for good growth of *Pleurotus ostreatus*. However, different workers have recorded different concentrations (0.1 to 0.5 ppm) of thiamine to support optimum mycelial growth of *Pleurotus ostreatus* than any other vitamin (Sugimori *et al.*, 1971; Hashimoto and Takahashi, 1976; Hong, 1978; Bukhalo and Solomko, 1979). Better growth of *Pleurotus sajor-caju* has been recorded with thiamine (0.05 ppm) by Jandaik and Kapoor (1976). Partial deficiency of thiamine in case of *Macrolepiota procera* has been recorded by Thianga and Jandaik (1979). They further observed that optimum concentration for accelerated growth was found to be 50 µg/l. Sharma (1984) while investigating vitamin nutrition of two isolates of *Pleurotus eryngii*, recorded good mycelial growth on biotin (20 ppm). Gupta (1990) reported better growth of four species of *Morchella* with the addition of riboflavin and cyanocobalamine, while addition of para-amino benzoic acid, nicotinic acid, biotin and folic acid resulted in less growth as compared to control.

2.2.2 Mineral Nutrition

Robbins and Hervey (1959) studied the effect of mineral nutrition on *Morchella* showing that wood and tomato extract have a

beneficial effect on growth. Robbins and Hervey (1965) showed that this beneficial effect with wood was due to minerals in its ash, especially manganese and calcium, explaining the common association of morel fruiting with areas that was burned in the previous year. Jandaik (1976) recorded increased growth of *Pleurotus sajor-caju* with 10 ppm of ferric chloride. Increased growth of *P. ostreatus* was also observed by Hong (1978) with magnesium sulphate and potassium dihydrogen phosphate. Iron was found to support maximum growth of *P. eryngii* (Soni, 1979) while synergistic effect of zinc and iron on the growth of *P. cornucopiae* was observed by Delmas and Mamoun (1981). Kurtzman and Zadrazil (1982) recorded stimulatory growth of *Pleurotus* species with manganese sulphate. Sharma (1984) showed that 1 ppm zinc supported maximum growth of Spain isolate of *P. eryngii* while Kabul isolate of the same species responded best to 0.5 ppm manganese. Ghosh and Majumdar (1986) studied the effect of some minerals on mycelial growth of *M. esculenta* and reported that calcium ion when added as $CaCl_2.2H_2O$ has stimulatory effect on the growth of mushroom mycelia.

2.3 NUTRITIVE VALUE

Varying opinions have been expressed regarding the true nutritive value of edible mushrooms. Interest in the nutritive value of edible fungi (mushrooms) has increased in recent years, although its importance was also realized by earlier workers. The nutritive value of

edible mushroom has been reported by several workers (Singer, 1961; Atkinson, 1961; Bano *et al.*, 1964, 1971; Jandaik and Kapoor, 1975; Li and Chang, 1982; Bano and Rajarathnam, 1982; Abou *et al.*, 1987).

Mushrooms are a good source of protein and amino acids apart from vitamins and minerals. Their protein content varies between 19-40 per cent on dry weight basis (Kurtzman, 1975). Purkayastha and Chandra (1976) found 14-27 per cent protein on dry weight basis in *Agaricus compestris, Lentinus subnudus, Calocybe indica* and *Volvariella volvacea.* Haddad and Hayes (1978) indicated that protein in *Agaricus bisporus* mycelium ranged approximately between 32 to 42 per cent of the dry weight. Samajpati (1978) found 30.16 per cent, 28.16 per cent, 34.7 per cent and 29.16 per cent protein in dried mycelium of *Agaricus compestris, Agaricus arvensis, Morchella esculenta* and *Morchella deliciosa*, respectively. The fruit bodies of *Leucocoprinus meleagris* contained 18.80 per cent of protein on dry weight basis (Tapati and Purkayastha, 1983). Abou *et al.* (1987) found 46.5 per cent protein on dry weight basis in *A. bisporus.* Sharma *et al.* (1988) reported 14.71 to 17.34 per cent and 15.20 to 18.87 per cent protein in fruit bodies of *Lactarius deliciosus* and *L. sanguiflus*, respectively. Kanwar *et al.* (1990) has reported a fat content of 11.52 per cent in fruit bodies of *Amanita ceasarea* on dry weight basis. Gupta and Singh (1991) reported 41.4 per cent essential amino acids content in sporophores of *Podaxis pistillaris.*

Mushrooms contain considerable amount of potassium, phosphorus, copper and iron as compared to calcium. Kaul (1978) reported that *M. esculenta* contains Ca (0.5776 mg), P (3.313 mg), Fe (1.213 mg) and K (3.831 mg) per 100 gm dry weight. Varo *et al.* (1980) reported that *Agaricus bisporus* contains N (6.9g), K (6.2g), Ca (0.04g), Mg (0.16g), P (0.75g), Fe (7.8mg), Cu (9.4mg), Mn (0.83mg) and Zn (8.6mg) per kg fresh weight. Abou *et al.* (1987) found that content of potassium and sodium in *Agaricus bisporus* was 300 ppm and 28.2 ppm, respectively.

2.4 CULTIVATION STUDIES

In early 1950's fermentation research showed the possibility to produce morel flavour for use in food preparation without actually producing fruit bodies. It resulted in issuance of patent regarding submerged culture of *Morchella* by Szuecs (1959). The use of fermenter-grown *Morchella* mycelium as a food flavouring material quickly lost much of its appeal when, in a landmark paper, Ower (1982) published the first photographs of the production of morel fruiting bodies in laboratory culture. Ower used sclerotia as a nutrient sink, under defined conditions to observe the first occurrence of the controlled fruiting of *M. esculenta*.

Despite some scattered reports (Baker and Matkin, 1959; Volk and Leonard, 1990) of outdoor cultivation, attempts to control fruiting

indoors met with the success only in 1982. Later, in association with Neogen Company, Ower did some additional work to develop a commercial process and a U.S. patent was issued (Ower *et al.*, 1986). The patent described a process for production of sclerotia of *Morchella* and the subsequent formation of fruiting bodies from sclerotia. The description of process in the patent are, however, vague and attempts at duplicating the process by researchers and mushroom growers outside the Neogen Company have failed. Apparently something of importance has been left out of patent. An additional patent (Ower *et al*, 1988) was later granted that provided minor additions to same technical issues.

Volk and Leonard (1989a) found that rye grain supplemented with peptone, yeast extract, trace element and casamino acid overlaid with perforated aluminium foil and covered with a layer of nutrient poor soil (2 parts potting soil mixture, 1 part peat soil and 2 parts distilled water) gave the best result for sclerotia production. Addition of asparagine or aspartic acid as nitrogen source to the rye also had a beneficial effect on sclerotia formation, while addition of carbon source had no such effect.

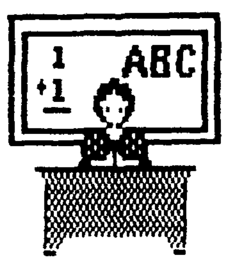
Much of the morel cultivation process was perhaps developed without scientific knowledge of morel life cycle. Only recently a heterokaryon phase has been verified to be a part of life cycle (Volk and Leonard, 1989b) showing that fruiting is not limited to primary mycelium. It was suggested that when hyphae of two genetically different mycelia

interact, heterokaryotic hyphae may arise and if conditions become unfavourable for growth, these may form a heterokaryotic sclerotium. The sclerotium may germinate myceliogenically which regenerates a secondary mycelium or carpogenically which leads to fruiting body production. A comprehensive life cycle of morel has been published by Volk and Leonard (1990). Amir *et al.* (1992, 1993, 1994, 1995a, b) carried out extensive studies on morphology and physiology of *Morchella esculenta* during sclerotial formation. Buscot (1993) studied mycelial differentiation of *M. esculenta* on two nutrient media at different temperatures. Amir *et al.* (1994, 1995a) made measurements of glucose translocation during growth and sclerotial formation and observed the existence of source-sink relationship between mycelium and sclerotia. These studies suggested that the mechanism of translocation consists of turgor driven mass flow.

Royse (1995) reported that commercial cultivation involves mainly the production of sclerotia, an early over-wintering stage of mushroom. Briefly `nutrient primed' sclerotia are produced in soil placed on a layer of sterilized wheat. The production of sclerotia requires about 18 to 24 days under ideal conditions. At maturity the sclerotia are harvested, soaked in clean water for 24 hours and distributed into a thin layer of pasturised `nutrient poor' soil mix. The sclerotia germinate via the production of mycelium. After the mycelium has spread throughout the soil, a continuous (24 hour duration) fine mist of clean water is provided to stimulate the formation of ascocarps.

Sharma *et al.* (1997) studied sclerotial production of *M. esculenta* following Jar method and reported that a 20°C temperature, complete darkness, forest soil and wheat grains gave good sclerotial production. The sclerotia were of large size (upto 4 cm), compact and yellowish brown to brown in colour.

Sud (1995) studied sclerotial germination behaviour and observed that manipulation of incubating temperature results in induction of carpogenic germination and inhibition of myceliogenic germination.



MATERIALS AND METHODS

CHAPTER-III

MATERIALS AND METHODS

3.1 COLLECTION

The fresh specimens and remains of sclerotia of *M. esculenta* were collected during March-May and September-October from various locations in the state and preserved after sun drying.

3.1.1 Isolation and maintenance of pure culture

Isolation from the fresh specimens collected from wild were made following standard tissue culture technique. Fresh specimens were first washed with a jet of sterile water and then cut across the pileal region with the help of sterilized sharp blade and bits (1-2 mm) of tissue were taken using sterilized forceps and dipped in 0.1 per cent mercuric chloride solution for 10-20 seconds. These were repeatedly washed with sterile water (5 washing) and placed on sterilized filter paper to remove excess moisture. The bits were then transferred on to Coon's medium slants, aseptically, with the help of sterilized inoculating needle and incubated at 20 ± 1°C. Stock culture was maintained in the refrigerator at 4°C. Culture was revived after a period of 7-10 days on fresh slants.

3.2 GLASSWARE

In all the experiments, Borosil glassware was used. In the beginning of each experiment, glassware was first cleaned with washing reagent "Teepol" or "Vim" and then washed with running tap water. The glassware was rinsed with boiling chromic acid solution (Potassium chromate 50 gm, concentrated sulphuric acid 50 ml and distilled water 300 ml) and left overnight, washed next day with tap water and then rinsed 2-3 times with distilled water.

3.2.1 Sterilization

Glassware was sterilized in hot air oven at 180°C for 2 hours. The media were sterilized at 15 p.s.i for 20 minutes. Soil sterilization was done at 22 p.s.i for 2 hours. Rectified spirit was used for cleaning homogenizer, forceps, inoculation needle, hands and inoculation chamber.

3.2.2 Preparation of inoculum

Inoculum used in various experiments was obtained from the margin of actively growing colonies (8-10 days old) on Coon's medium slants/plates. For cultural studies on solid media, 6 mm diameter discs of mycelial mat were cut with the help of pre-sterilized cork borer and sufficient care was taken to avoid agar block along with the mycelial discs.

In experiments on liquid media, the inoculum was prepared by thoroughly homogenizing the mycelial bits in sterilized glass homogenizer. A uniform mycelial suspension (12-16 bits per low field of the compound microscope) was used throughout the studies.

3.3 NUTRITIONAL REQUIREMENTS

The nutritional requirement studies were carried out by using Coon's liquid basal medium. Twenty five ml liquid medium was poured into 100 ml `Erlenmeyer' flasks, plugged, sterilized and inoculated with 1 ml of standardized mycelial suspension. At the end of growth period (15 days), the mycelium was filtered through previously dried and weighed filter paper (Whatman No.42) with the help of suction pump.

3.3.1 Vitamin requirement

The basal medium was supplemented with eight vitamins at two concentrations of 25 ppm and 50 ppm as well as their mixture for studying their effect on the mycelial growth.

The basal medium for vitamin studies was purified by heating with 5 g/litre of activated charcoal and then filtered through Whatman No.1 filter paper before sterilization (Mathur *et al.*, 1950). Stock solutions of all vitamins were prepared in double glass distilled water except for biotin which was initially dissolved in 5 ml of 50 per cent ethanol and then the volume was made up with double glass distilled water.

3.3.2 Mineral requirement

The basal medium was supplemented with eight minerals at two concentrations of 5 ppm and 10 ppm for studying their effect on mycelial growth.

The basal medium for mineral studies was purified by addition of 15 gm of $CaCO_3$/litre, autoclaved at 10 p.s.i pressure for 10 minutes and filtered while hot through Whatman No.1. filter paper. 2.5 ml and 5 ml of 50 ml strength of mineral was added to 22.5 ml and 20 ml of the basal medium in 100ml conical flask and sterilized at 13 p.s.i. for 10 minutes (Steinberg, 1935). The pH of the medium was adjusted to 6.5 before sterilization.

3.4 ANALYTICAL METHODS

3.4.1 Determination of moisture, dry matter and ash

The moisture content of the samples (fruit bodies and sclerotia) was determined by taking their fresh weights and drying them at 60±1°C to a constant weight. The difference in the weight was used for calculating the moisture percentage in the samples.

Dry matter content was calculated by drying a known weight of fresh samples in an electrical oven at 70±1°C (A.O.A.C., 1975).

The ash content in fruit bodies and sclerotia was estimated by ashing the dry samples in a muffle furnace at 420±1°C until constant weight (A.O.A.C., 1975).

3.4.2 Fat determination

A weighed powdered (0.5 g) sample was extracted with chloroform-methanol (2:1) in a pestle-mortar. The extract was filtered through filter paper. The extract containing fat and chloroform-methanol was evaporated in water bath and dried in an oven at low temperature (30-35°C) and weighed (Folch *et al.*, 1957).

3.4.3 Protein estimation

Protein estimation was done by Lowry's method (Lowry *et al.*, 1951) as detailed below:

Extraction: 500 mg of the sample was weighed and ground well with a pestle and mortar in 10 ml of phosphate buffer. The extract was filtered through whatman No.1 filter paper.

Reagents:

(A) 2% sodium carbonate in 0.1 N sodium hydroxide

(B) 1% copper sulphate

(C) 2% sodium potassium tartrate

(D) Prepared by mixing the reagent A, B and C in the ratio of 100:1:1, respectively.

(E) Folin-phenol (FP) (50% of the commercial folin-ciocalteu's phenol reagent).

Estimation: To 0.1ml of the extract, 0.9 ml of buffer (0.05M, pH 7.0) was added. 5 ml of `Reagent-D' was added to it and thoroughly shaken. After 10 minutes, 0.5 ml FP reagent was added and again shaken vigorously. The solution was then kept at room temperature (20-25°C) in the dark for 30 minutes which resulted in the development of blue colour and was read at 710 nm. The protein content was calculated with the help of a standard curve prepared by using bovine serum albumin (20-200 μg/ml) and was expressed as g/100 gm dry weight.

3.4.4 Sample preparation for the estimation of major mineral elements

For determining various minerals, the samples previously dried at 65°C for 12 hr were digested on a hot plate in the tri acid (HNO_3; H_2SO_4; $HClO_4$) mixed in a 9:2:1 (V/V/V) ratio. After completion of the digestion, the digest was cooled and diluted with double distilled water to make the volume 100 ml.

3.4.4.1 Determination of Magnesium, Iron, Copper, Calcium and potassium

Three elements viz. magnesium, Iron and Copper were determined on Varian AA-175, Atomic Absorption Spectrophotometer (Lindsay and Norvel, 1978).

Potassium and calcium in the silica free extract were determined with the help of flame photometer by using different filters (Jackson, 1967).

3.5 PRODUCTION OF SCLEROTIA

Sclerotia have been identified as the structures in the morel's life cycle which are competent of producing fruit bodies. Sclerotial formation of *M. esculenta* was studied by Jar method (Ower *et al*, 1986, 1988; Sharma *et al.*, 1997). The jars were nearly half filled with boiled wheat grains (100 g) and separated from upper layer of moist soil (80 g forest soil with 35-40% moisture content) by a perforated aluminium foil. Jars were sealed at top and sterilized at 22 p. s.i for 2 hr and kept overnight. The upper soil layer was then inoculated with 5-6 mycelial bits and jars were incubated at 20±1°C. The sclerotia were harvested after 35-40 days and observations pertaining to fresh weight of sclerotia, final weight of wheat grains and soil were recorded.

Effect of supplementation of Potassium nitrate, starch, vitamins, minerals in the wheat grains and soil amendment with peat moss and vermiculite was studied for the sclerotial production.

3.5.1 Effect of supplementation of potassium nitrate and starch, singly, and in combination, in wheat grains on the sclerotial production of *M. esculenta*

Potassium nitrate and starch have been reported to support good mycelial growth of *M. esculenta* (Sud, 1995). Their effect on sclerotial production was studied by supplementing potassium nitrate (@ 5

g/kg of dried wheat grains) and starch (@ 20 g/kg of dried wheat grains) in the boiled wheat grains singly as well as in combination. Jars were filled, sterilized, inoculated and incubated as described under 3.5. Sclerotia were harvested after 35-40 days of incubation and observations on the fresh weight of sclerotia were recorded as usual.

3.5.2 Effect of supplementation of vitamins in the wheat grains on the sclerotial production of *M. esculenta*

Four vitamins which supported fairly good mycelial growth viz. folic acid, ascorbic acid, nicotinic acid and biotin at two concentrations 25 ppm and 50 ppm were added to boiled wheat grains already supplemented with potassium nitrate (@ 5 g/kg of dried wheat grains). Jars were filled, sterilized, inoculated and incubated as already described.

3.5.3 Effect of supplementation of minerals in the wheat grains on the sclerotial production of *M. esculenta*

Four minerals which supported good mycelial growth viz. iron, zinc, calcium and boron at two concentrations (5 ppm and 10 ppm) were added to boiled wheat grains already supplemented with potassium nitrate. Jars were filled, sterilized, inoculated and incubated as described under 3.5.

3.5.4 Soil amendments

3.5.5 Effect of soil amendment with peat moss and vermiculite on sclerotial production

Different concentrations of peat moss (2, 3, 5, and 7%) and vermiculite (2, 3, 5, 7, 9 and 11 %) in the soil zone were evaluated for the production of sclerotia. Jars were filled, sterilized, inoculated and incubated as usual and observationswere recorded after 35-40 days of incubation.

3.5.6 Mass multiplication of sclerotia

Mass production of sclerotia for further studies on their germination behaviour were carried out by incorporating best treatments of soil amendments and wheat grain supplements following jar method as already explained.

3.5.7 Determination of biological efficiency

The biological efficiency (BE) was calculated as a percentage of the fresh weight of sclerotia to the dry weight of nutrient-rich substrate i.e. wheat grains.

3.6 GERMINATION OF SCLEROTIA

3.6.1 Effect of temperature

Effect of different temperatures viz. 5, 10, 15, 20 and 25°C was evaluated on sclerotial germination. The experiment was conducted in glass petriplates (90 mm dia) filled with forest soil (up to 1 cm thick) as substrate. The soil moisture level was brought to 35 to 40 per cent and petriplates were sterilized at 22 p.s.i for 2 hr. and kept overnight. Petriplates were seeded with 4 to 5 sclerotia stored in the refrigerator for a period of one month, after testing their viability on Coon's medium. Three replications for each treatment were taken. These petriplates were incubated at different temperatures. The experiment was conducted at natural soil pH. Observations were recorded after 5 days of incubation on the basis of growth characteristics.

3.6.2 Effect of initial soil moisture levels

Different initial soil moisture levels viz., 10, 20, 30, 40, 50 and 60 per cent were evaluated for studying the sclerotial germination behaviour. Moisture levels were prepared by adding the required quantity (percentage) of water in the dried forest soil. Preparation of substrate and seeding of sclerotia was done as explained under 3.6.1.

3.6.3 Effect of light

Effect of duration of light viz. 0, 6, 12, 18 and 24 hrs on the germination of sclerotia was studied on forest soil in glass petriplates in the same way as already explained under 3.6.1.

3.6.4 Effect of growth hormones

Stock solution of test growth hormones, except GA_3 were prepared in double distilled water and stored at 5°C in a refrigerator. GA_3 was first dissolved in 10ml acetone and then the volume was made up with double distilled water. Three concentrations of each growth hormones, (5ppm, 10ppm, and 20 ppm) were evaluated for sclerotial germination. Sclerotia stored in the refrigerator for one month were dipped in different concentrations of growth hormones solutions for varying time intervals i.e. 1, 3 and 5 hours. Preparation of substrate and seeding of sclerotia was done as described under 3.6.1.

3.6.5 Effect of different concentrations of starch and potassium nitrate

Coon's medium containing 0.5, 0.75, 1.0, 1.5 and 2.0 per cent starch and potassium nitrate was prepared separately, solidified by using 2.0 per cent agar and pH was adjusted to 6.5. 100ml of the medium of each concentrations of starch and potassium nitrate were taken in 150

ml Erlenmeyer flasks and sterilized at 15 p.s.i for 20 minutes. Flasks were seeded centrally with sclerotia stored in the refrigerator for a period of one month after testing their viability. Three replications for each treatment were taken. Flasks were incubated at 20±1°C for 12 days.

3.6.6 *In situ* sclerotial germination

Mass production of sclerotia was carried out by employing the combination of best treatments (supplementation of potassium nitrate, nicotinic acid and calcium in the wheat grains and soil amendments with vermiculite) found in the experiments on sclerotial production. For studying *in situ* sclerotial germination behaviour of *M. esculenta*, the basal nutrient rich zone of wheat grains was removed and soil zone containing mature sclerotia was left as such in jars. The base of each jar was provided with 10-15 fine holes for water percolations. A set each of five such jars was incubated at temperatures of 5°C, 15°C, 20°C and 25°C. The jars were regularly hydrated so that water percolated through the substratum.

3.7 STATISTICAL ANALYSIS

The data obtained during the course of present investigation were subjected to appropriate statistical analysis wherever necessary using standard procedure given by Cochran and Cox (1970). The significance of differences was tested at 5 per cent level of probability.

EXPERIMENTAL RESULTS

CHAPTER-IV

EXPERIMENTAL RESULTS

4.1 DISTRIBUTION AND COLLECTION OF *M. esculenta*

4.1.1 Distribution

Morchella esculenta is a well established species and is widely distributed. It is fairly common in North-Western Himalayan region, particularly during March-May. The species occurs in sufficient quantity in the forests of Himachal Pradesh and is collected by the local people for their consumption and to be sold in the market. Due to its continuous collection, the specimen population is depleting fast from its natural habitat.

4.1.2 Collection of fresh specimens and sclerotia of M. esculenta

The fresh specimens of *M. esculenta* were collected during September-October and March-May from various locations in the districts of Mandi, Kangra and Kinnaur of Himachal Pradesh. Five collections, each comprising 4-6 specimens were made and designated as indicated in Table 1 (Fig. 1). Collections I and IV were made during September-October (Pre-winter period) and remaining collections were made during April (Post-Winter Period). The sclerotia of *M. esculenta* could not be located from any of the locations. However, sclerotial remains were found and collected along with the specimens only at one locality, Palampur. The samples were sun-dried, moisture content determined and preserved.

Table 1: Collection of *M. esculenta* from different locations of Himachal Pradesh

Collections	Place	Period
I	Chauntra (Mandi)	September-October, 1997
II	Thangi (Kinnaur)	April, 1997
III	Jhatingri (Mandi)	April, 1997
IV	Palampur (Kangra)	September-October, 1997
V	Tiken (Mandi)	April, 1998

4.2 ISOLATION AND MAINTENANCE OF PURE CULTURE

Pure culture of *M. esculenta* was established on Coon's medium following tissue culture method. Repeated and large number of isolations from pileal region of the fruit bodies yielded the same culture type. The mycelial culture made from the specimens collected during 'Pre-winter' period was used for further studies on the various aspects.

4.3 NUTRITIONAL STUDIES

4.3.1 Effect of Vitamins

Vitamins are required in small quantities for the growth of fungi. Fleshy fungi, though rich in vitamins but some of them have been found to respond to exogenous supply as well. Keeping this in mind, eight vitamins, each in two concentrations and their mixture were evaluated to ascertain their effect on the growth of *M. esculenta* as per procedure already explained under Materials and Methods. The results recorded are presented in Table 2.

Table 2: Effect of vitamins on the mycelial growth of *M. esculenta*

Vitamin	Mycelial dry weight (mg)*	
	25 ppm	50 ppm
Folic acid	95.66	75.50
Thiamine hydrochloride	83.00	61.00
Biotin	115.00	18.00
Inositol	5.00	42.00
Pyridoxin hydrochloride	56.00	49.00
Calcium pentothenate	96.00	69.66
Ascorbic acid	99.33	74.33
Nicotinic acid	108.00	66.00
Mixture	94.00	72.66
Control (no vitamin)	117.00	
CD (P=0.05)	37.34	20.38

* Average of three replications

It is evident from the data that vitamins did not show any significant effect on the mycelial growth, in comparison to control. In fact, addition of vitamins reduced the growth at both the concentrations.

4.3.2 Effect of Minerals

It is well established that minerals are constituents of the fungal mycelium. The mineral requirements for vegetative growth even on dry weight basis is not easily demonstrated because these are required in very minute quantities. In the present study, eight minerals with two

concentrations were tested to see their effect on the mycelial growth of *M. esculenta*. Purification of medium, inoculation and incubation were done as described under Materials and Methods. The data were recorded in terms of average dry mycelial weight after 15 days of incubation at 20±1°C and presented in Table 3.

Table 3: Effect of minerals on the mycelial growth of *M. esculenta*

Mineral	Source	Mycelial dry weight (mg)*	
		5 ppm	10 ppm
Iron	Iron sulphate ($FeSO_4.7H_2O$)	80.30	76.30
Zinc	Zinc sulphate ($ZnSO_4.7H_2O$)	72.00	78.60
Copper	Copper sulphate ($CuSO_4.5H_2O$)	76.30	78.00
Manganese	Manganese sulphate ($MnSO_4.H_2O$)	76.30	75.60
Cobalt	Cobalt chloride ($CoCl_2.6H_2O$)	75.00	76.00
Calcium	Calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$]	84.33	84.00
Boron	Boric acid (H_3BO_3)	75.30	88.60
Molybdenum	Ammonium molybdate [$(NH4)_6Mo_7O_{24}.4H_2O$]	71.30	71.30
Control	No mineral	76.30	
CD (P=0.05)		6.67	6.87

* Average of three replications

As is evident from the data, the tested minerals did not support significant mycelial growth as compared to control. However, boron at 10 ppm and calcium at both concentrations showed positive effect on growth. Molybdenum gave poor growth at both the concentrations.

4.4 SCLEROTIAL PRODUCTION

Sclerotia have been identified as the structures in the life cycle of *M. esculenta* which are competent to give rise to fruit body. The various parameters affecting the production of sclerotia were studied employing `Jar method' (Ower *et al.*, 1986, Sharma *et al.*, 1997) as explained under Materials and Methods.

4.4.1 Effect of supplementation of Potassium nitrate and starch, singly, and in combination, in wheat grains on the sclerotial production of *M. esculenta*

Potassium nitrate and starch have been reported to support good mycelial growth of *M. esculenta* (Sud, 1995). Their effect on sclerotial production was studied by supplementing potassium nitrate (@ 5g/kg of dried wheat grains) and starch (@ 20g/kg of dried wheat grains) in the boiled wheat grains singly as well as in combination. Jars were filled, sterilized, inoculated and incubated as already explained under Materials and Methods. Mature sclerotia were harvested after 35-40 days. Observations have been recorded in Table 4 (Fig. 2).

Table 4: Effect of supplementation of potassium nitrate and starch, singly, and in combination, in wheat grains on the sclerotial production of *M. esculenta* *

Treatment	Weight of boiled grain (g)	Initial weight of moist soil (g)	Final weight of soil (g)	Fresh weight of harvested sclerotia (g)	Weight of soil after harvesting sclerotia (g)	Biological efficiency (%)
KNO_3	100.00	80.00	99.00	27.75	71.00	55.50
Starch	100.00	80.00	79.25	9.75	69.50	19.50
KNO_3+Starch	100.00	80.00	95.25	25.00	70.00	50.00
Control (wheat grain)	100.00	80.00	81.00	12.50	68.50	25.00
CD (P=0.05)				2.61		

* Average of four replications

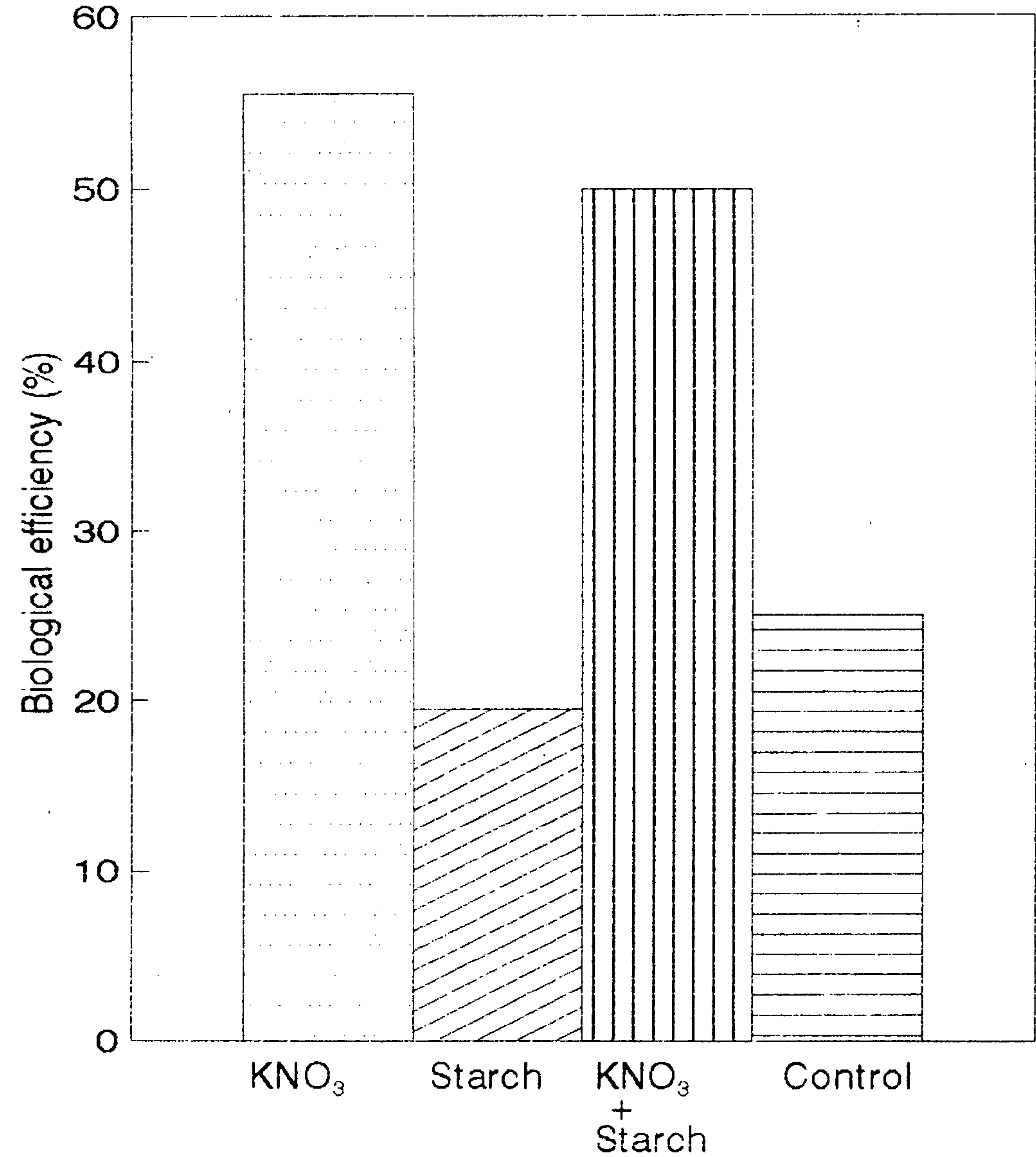


Figure 1: Effect of supplementation of potassium nitrate and starch, singly, and in combination, in wheat grains on the sclerotial production of *M. esculenta*

The results show that starch inhibited the production of sclerotia as compared to control. However, supplementation of potassium nitrate doubled the sclerotial yield. The combination of both enhanced the sclerotial production, significantly. Per cent biological efficiency (BE) i.e. sclerotia (in soil zone) to substrate (grain) ratio was determined by dividing weight of fresh sclerotia with dry weight of grain substrate as given in Table 4. Wheat grains supplemented with potassium nitrate served as control for further experiments on sclerotial production.

4.4.2 Effect of supplementation of vitamins in the wheat grains on the sclerotial production of *M. esculenta*

Four vitamins which were comparatively well utilized for mycelial growth viz. folic acid, ascorbic acid, nicotinic acid and biotin at 25 and 50 ppm concentrations were evaluated for sclerotial production by supplementing in wheat grain substrate Jars were filled, sterilized, inoculated and incubated as described under `Materials and Methods. Data in terms of fresh weight of harvested sclerotia and biological efficiency are recorded in Table 5 (Fig. 3 & 4).

It is evident from the data that all the vitamins tested were utilized by the fungus for sclerotial production as compared to control. Nicotinic acid at 25 ppm was found to be the best vitamin for sclerotial production followed by ascorbic acid at both the concentrations. Two

Table 5: Effect of vitamins on the sclerotial production of *M. esculenta**

Vitamin	Concentration (ppm)	Weight of boiled grain (g)	Initial weight of moist soil (g)	Final weight of soil (g)	Fresh weight of harvested sclerotia (g)	Weight of soil after harvesting sclerotia (g)	Biological efficiency (%)
Folic acid	25	100.00	80.00	90.00	28.66	61.33	57.32
	50	100.00	80.00	91.00	29.33	61.66	58.66
Bictin	25	100.00	80.00	90.66	28.00	62.66	56.00
	50	100.00	80.00	91.66	27.66	64.00	55.32
Ascorbic acid	25	100.00	80.00	98.00	30.66	67.33	61.32
	50	100.00	80.00	94.00	31.33	62.66	62.66
Nicotinic acid	25	100.00	80.00	97.33	32.33	65.00	64.66
	50	100.00	80.00	89.33	28.66	60.66	57.32
#Control		100.00	80.00	97.33	24.00	73.00	48.00
CD (P=0.05)	25				3.45		
	50				3.84		

* Average of three replications;

Supplemented with KNO_3

Plate - I (Fig. 1-4)

1. Fruit bodies of *M. esculenta* (Pre-winter strain)

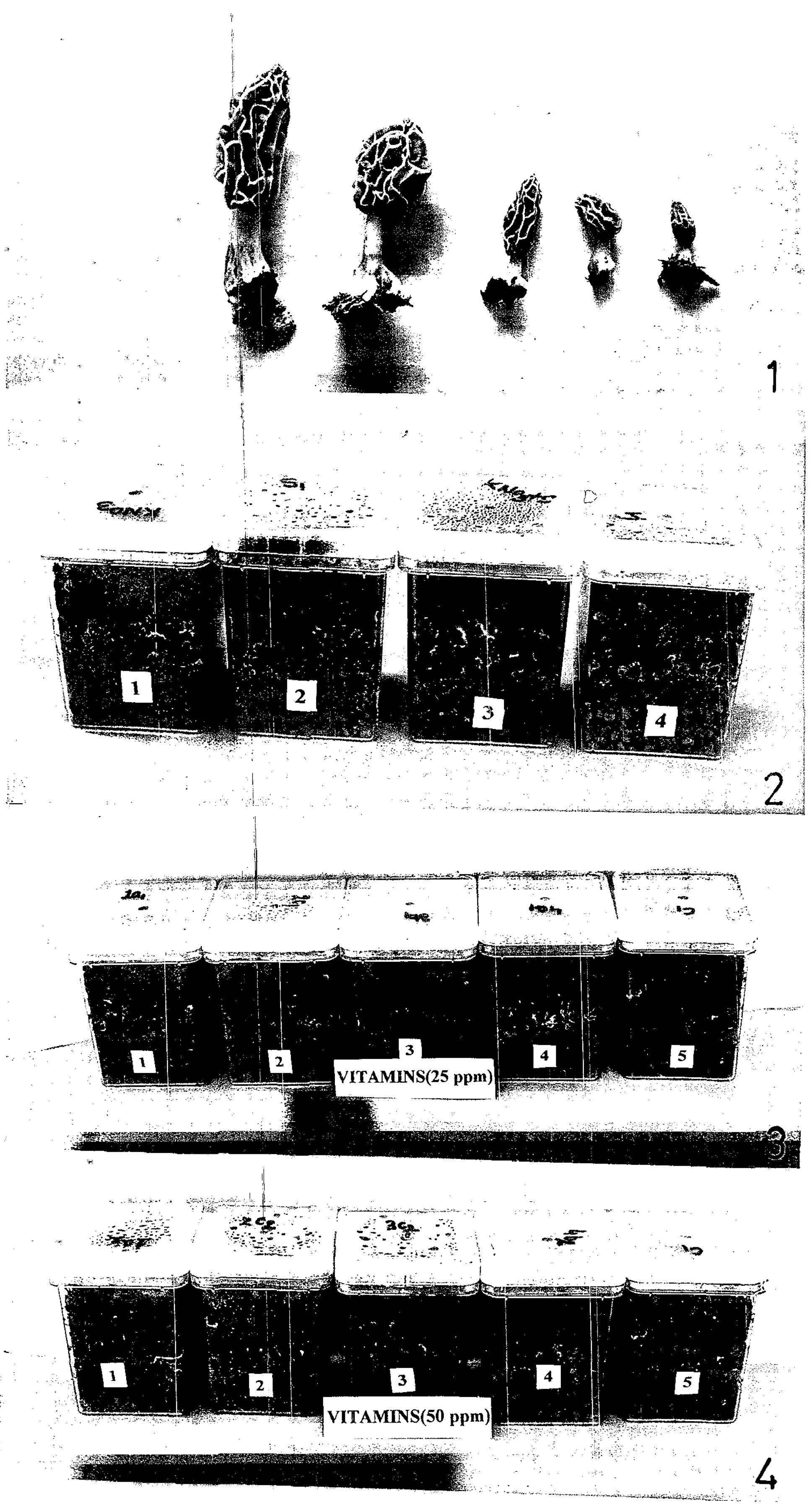
2. Effect of supplementation of potassium nitrate and starch, singly, and in combination, in wheat grains on the sclerotial production of *M. esculenta* (after 35 days)

1. KNO_3, 2. Starch, 3. KNO_3 + Starch, 4. Control

3-4. Effect of vitamins on sclerotial production of *M. esculenta* (after 35 days)

1. Folic acid, 2. Biotin, 3. Ascorbic acid, 4. Nicotinic acid, 5. Control

Plate – 1



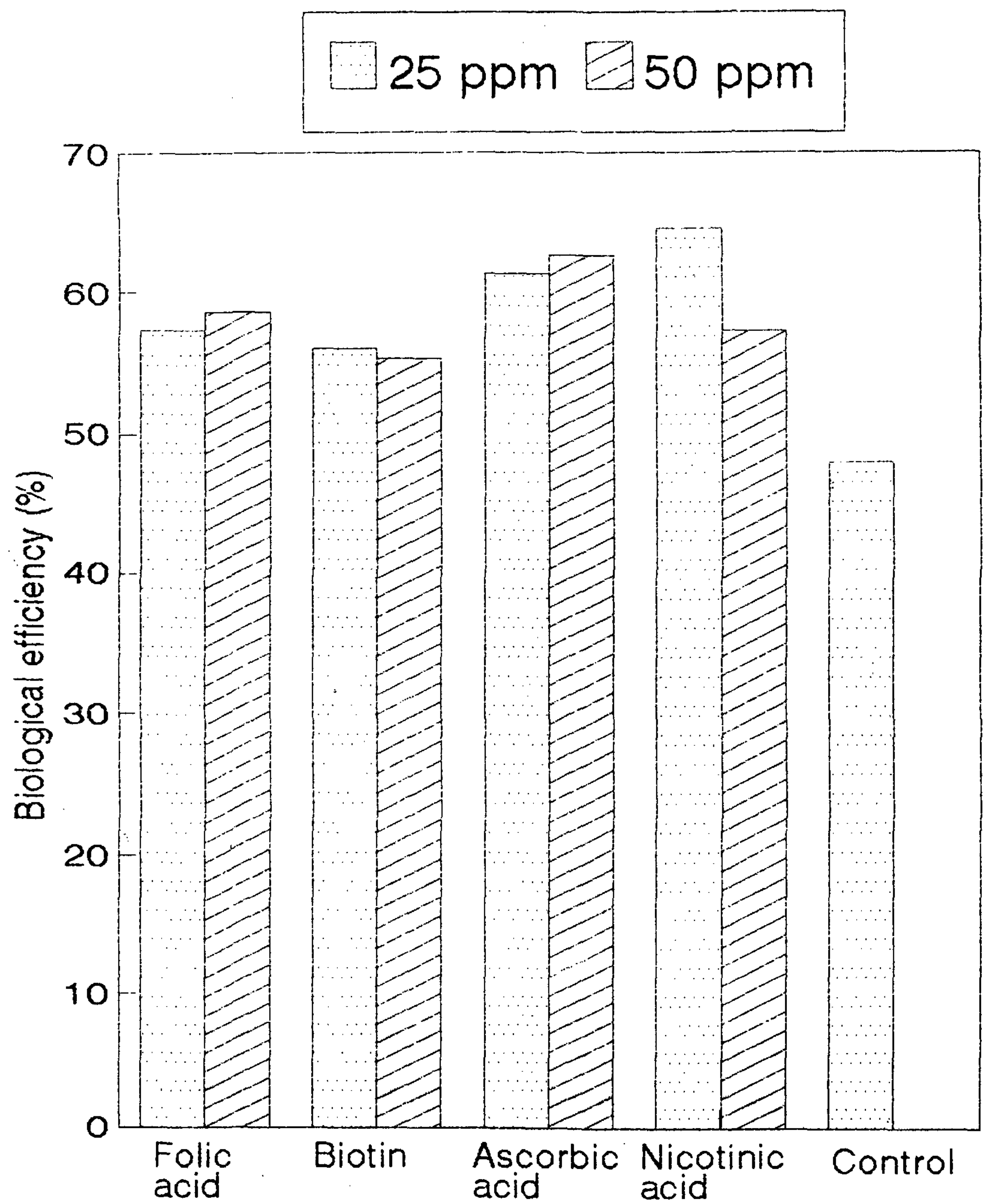


Figure 2: Effect of vitamins on the sclerotia production of *M. esculenta*

vitamins significantly enhanced the biological efficiency of the mushroom. Sclerotial production and biological efficiency in folic acid and biotin was statistically at par with each other.

4.4.3 Effect of supplementation of minerals in the wheat grains on the sclerotial production of *M. esculenta*

Two concentrations (5ppm and 10ppm) of four minerals viz. iron, zinc, calcium and boron were tested for production of sclerotia. Jars were prepared and incubated as usual. Mature sclerotia were harvested and data were recorded in terms of fresh sclerotial weight and biological efficiency. The results are presented in Table 6 (Fig. 5 & 6).

As is evident from the data that calcium, zinc and boron at 10ppm concentration significantly enhanced the sclerotial production in comparison to control. Calcium gave maximum biological efficiency followed by zinc and boron. Sclerotial yield with allthe minerals at 5 ppm concentration was statistically at par with control.

4.4.4 Effect of soil amendment with peat moss on sclerotial production of *M. esculenta*

For improving soil texture, soil medium was amended with different concentrations (2, 3, 5, and 7 per cent) of peat moss to evaluate the effect on sclerotial production. Moisture level of the soil was maintained 35 to

Table 6: Effect of minerals on the sclerotial production of *M. esculenta**

Mineral	Concentration (ppm)	Weight of boiled grain (g)	Initial weight of moist soil (g)	Final weight of soil (g)	Fresh weight of harvested sclerotia (g)	Weight of soil after harvesting sclerotia (g)	Biological efficiency (%)
Iron	5	100.00	80.00	85.33	27.00	56.66	54.00
	10	100.00	80.00	92.00	27.33	64.66	54.66
Zinc	5	100.00	80.00	85.33	25.66	59.66	51.32
	10	100.00	80.00	93.00	33.66	59.33	67.32
Calcium	5	100.00	80.00	87.00	25.33	61.00	50.66
	10	100.00	80.00	90.66	35.66	54.50	71.32
Boron	5	100.00	80.00	86.66	26.66	60.00	53.32
	10	100.00	80.00	90.33	32.33	58.00	64.66
#Control		100.00	80.00	97.33	24.00	73.33	48.00
CD (P=0.05)	5				NS		
	10				3.38		

* Average of three replications;
# Supplemented with KNO_3

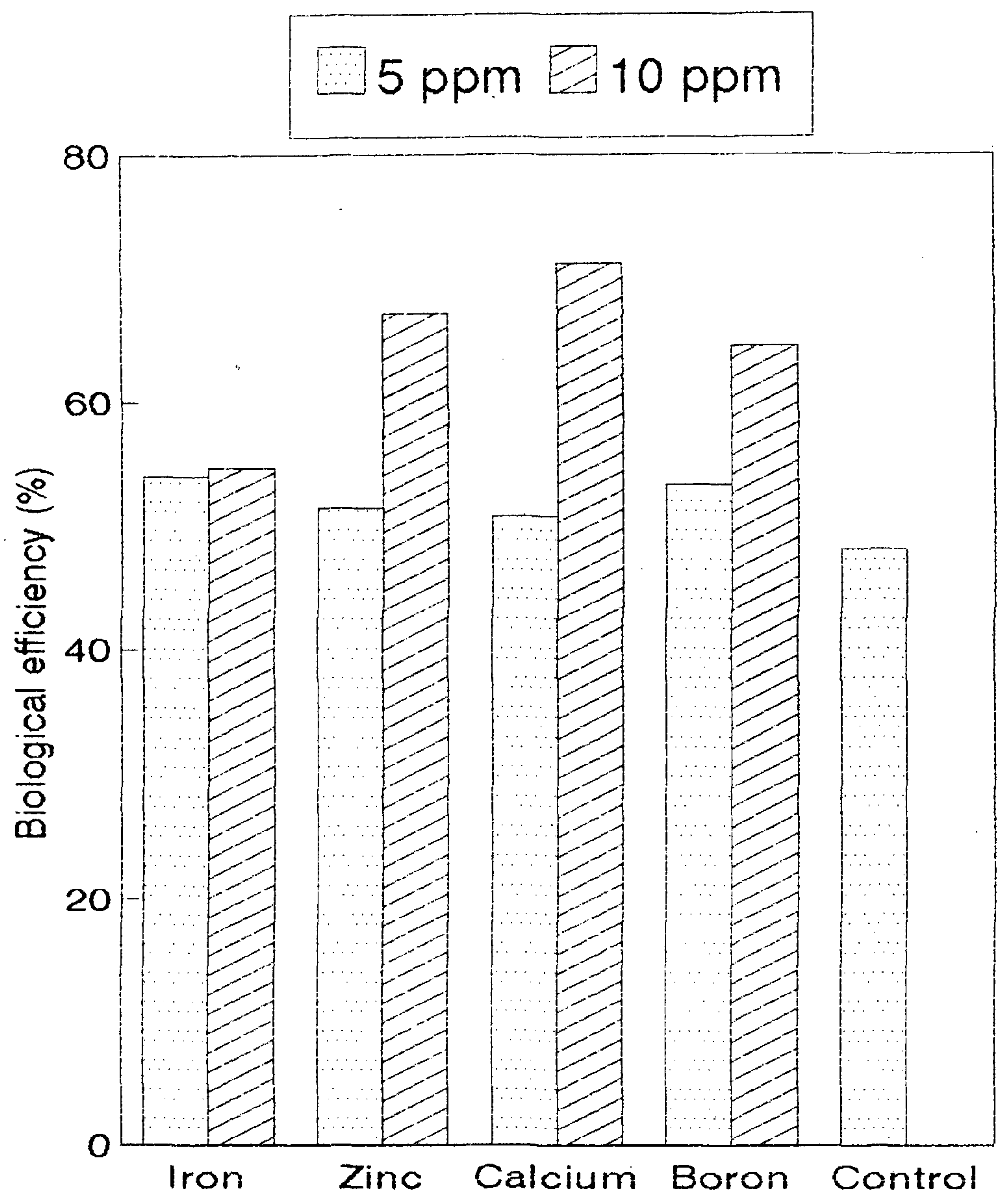


Figure 3: Effect of minerals on the sclerotia production of *M. esculenta*

40 per cent. Inoculation and incubation were carried out as already explained under Materials and Methods. It is evident from the data Table 7 (Fig. 7) that all the concentrations of peat moss did not show any improvement in the sclerotia production in comparison to control. In fact addition of peat moss retarded the production of sclerotia.

4.4.5 Effect of soil amendment with vermiculite on sclerotial production of *M. esculenta*

Soil medium was amended with six different concentrations (2, 3, 5, 7, 9 and 11 per cent) of vermiculite to evaluate the effect on sclerotial production. The data are presented in Table 8 (Fig. 8).

As is evident from the table that sclerotial yield and biological efficiency was maximum at 7 per cent followed by 5 per cent of vermiculite as compared to control. Soil amendment with 7 per cent vermiculite gave maximum sclerotial yield and biological efficiency. Sclerotia harvested from this treatment were large (up to 5 cm) in size, compact and dark brown as compared to all other treatments. The biological efficiency and fresh weight of the harvested sclerotia was at par with other treatments.

Table 7: Effect of soil amendment with peat moss on sclerotial production of *M. esculenta**

Peat moss (%)	Weight of boiled grain (g)	Initial weight of moist soil (g)	Final weight of soil (g)	Fresh weight of harvested sclerotia (g)	Weight of soil after harvesting sclerotia (g)	Biological efficiency (%)
2	100.00	80.00	94.66	23.00	71.33	46.00
3	100.00	80.00	99.00	21.66	77.00	43.32
5	100.00	80.00	94.66	21.00	73.00	42.00
7	100.00	80.00	99.33	20.33	79.00	40.66
#Control	100.00	80.00	97.33	24.00	73.33	48.00
CD (P=0.05)				NS		

* Average of three replications;

Only forest soil

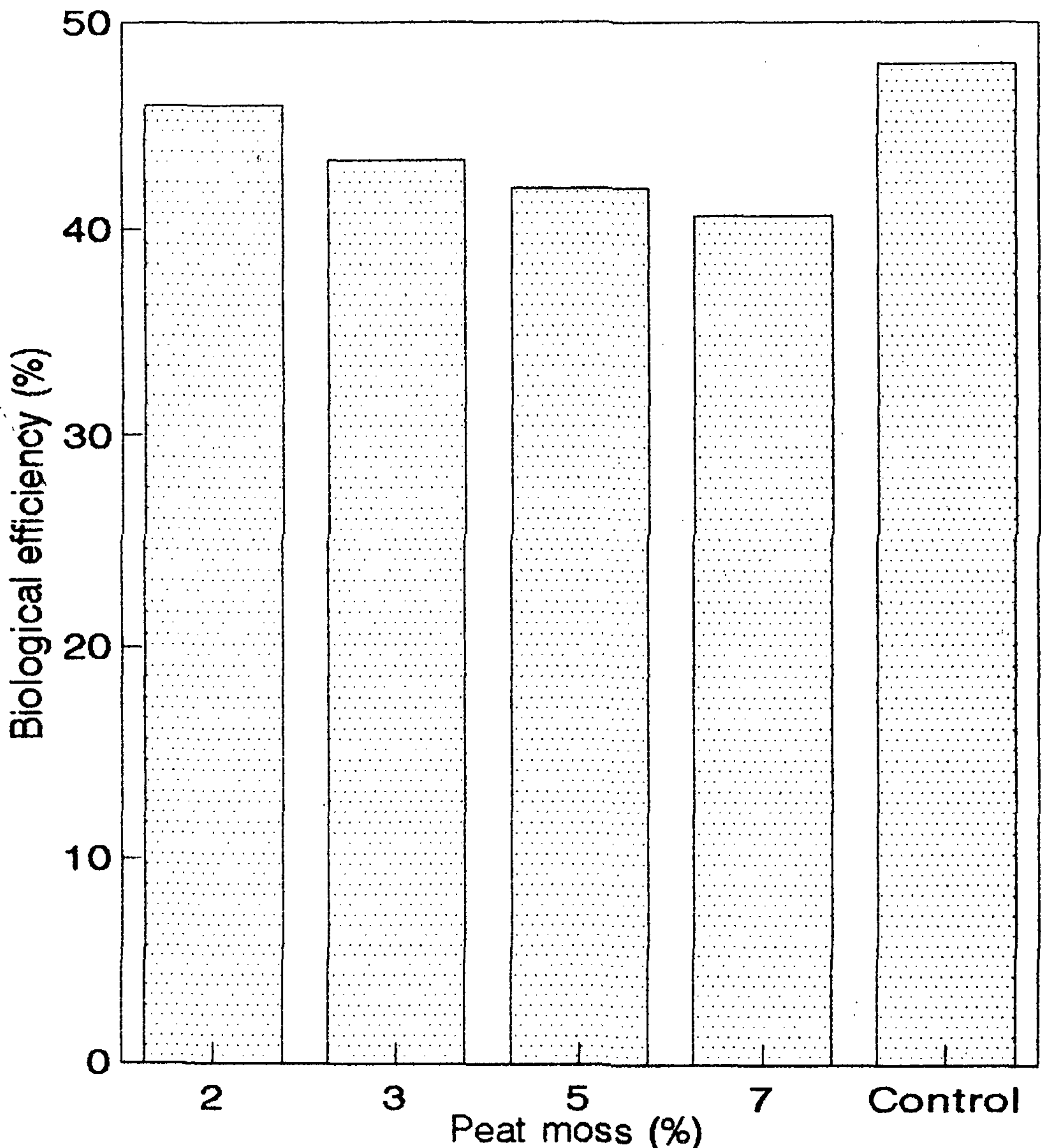


Figure 4: Effect of soil amendment with peat moss on the sclerotia production of *M. esculenta*

Table 8: Effect of soil amendment with vermiculite on sclerotial production of *M. esculenta**

Vermiculite (%)	Weight of boiled grain (g)	Initial weight of moist soil (g)	Final weight of soil (g)	Fresh weight of harvested sclerotia (g)	Weight of soil after harvesting sclerotia (g)	Biological efficiency (%)
2	100.00	80.00	100.00	26.66	73.33	53.32
3	100.00	80.00	101.66	28.33	73.00	56.66
5	100.00	80.00	101.66	33.66	68.00	67.32
7	100.00	80.00	110.00	37.60	72.33	75.20
9	100.00	80.00	101.66	26.33	75.33	52.66
11	100.00	80.00	97.00	25.66	71.00	51.32
#Control	100.00	80.00	97.00	24.00	73.33	48.00
CD (P=0.05)				7.31		

* Average of three replications
# Only forest soil

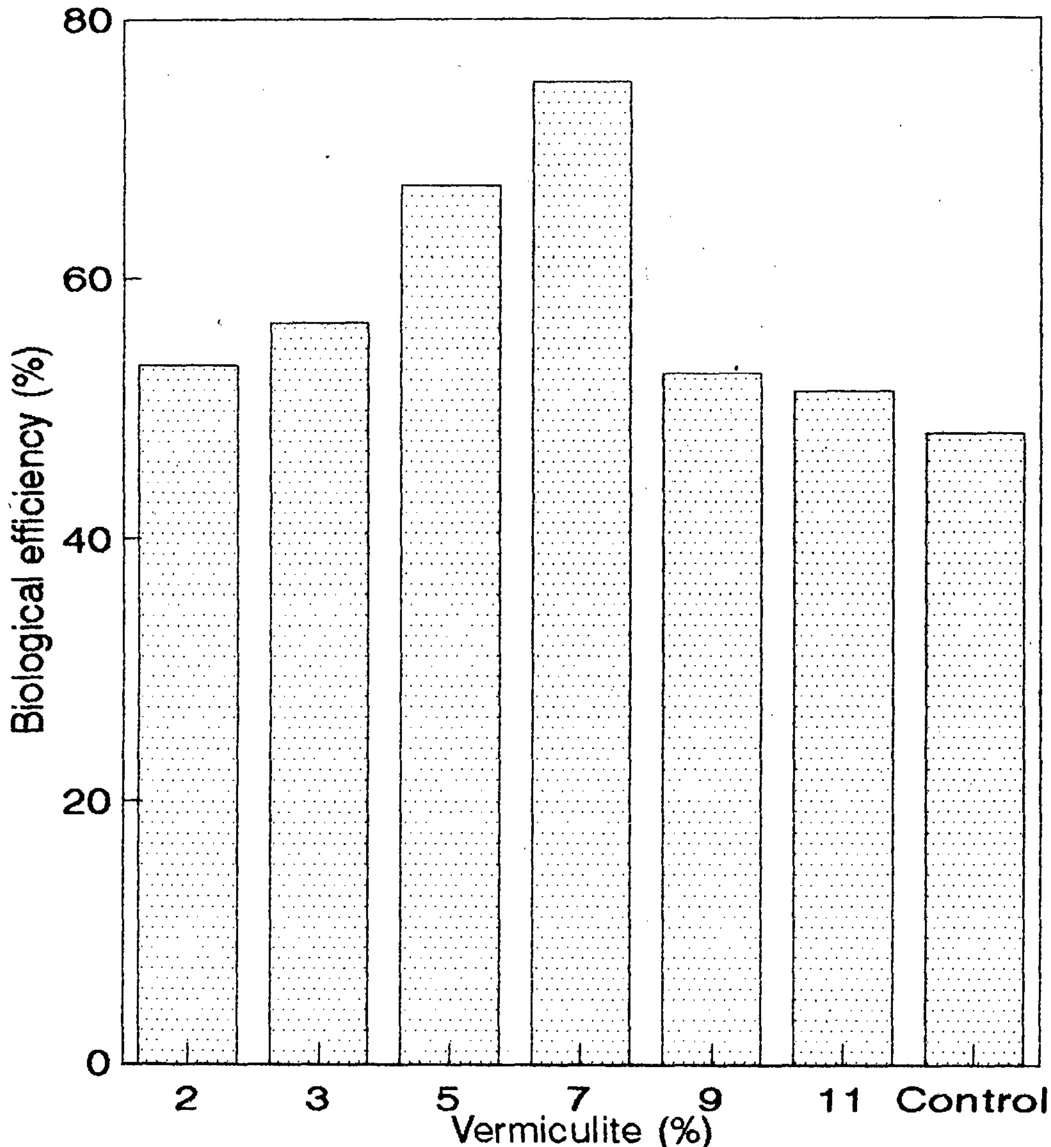


Figure 5: Effect of soil amendment with vermiculite on the sclerotia production of *M. esculenta*

Plate - II (Fig. 5-8)

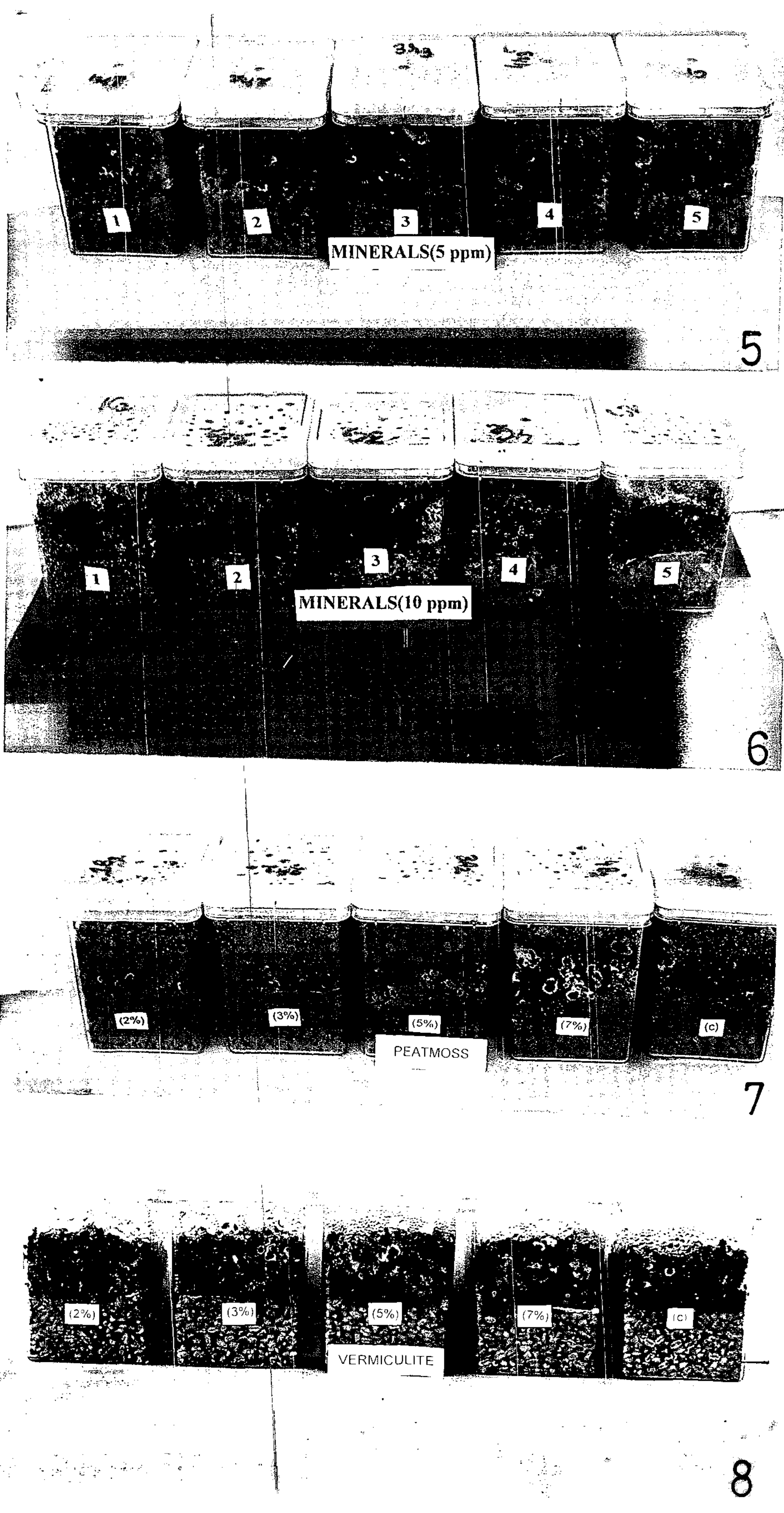
5-6. Effect of minerals on the sclerotial production of *M. esculenta* (after 35 and 15 days, respectively)

1. Iron, 2. Zinc, 3. Calcium, 4. Boron, 5. Control

7. Effect of soil amendment with peat moss on sclerotial production of *M. esculenta* (after 35 days)

8. Effect of soil amendment with vermiculite on sclerotial production of *M. esculenta* (after 15 days)

Plate – II



4.5 CHEMICAL COMPOSITION OF FRUIT-BODIES AND SCLEROTIA

The fruit bodies and sclerotial remanents, of *M. esculenta* collected from the nature and sclerotia produced in the laboratory were analysed for their moisture, protein, fat, dry matter, ash and major mineral elements as per the procedure described in Materials and methods. The results obtained are presented in Table 9.

Table 9: Comparative composition of main constituents (%) and minerals (mg/g) in dry sample of the fruit bodies and sclerotia

Constituents	Sclerotia produced in the laboratory	Fruit-bodies	Sclerotial remanents collected from nature
Moisture	50.00	84.50	42.85
Dry matter	50.00	15.50	57.15
Fat*	16.78	5.84	1.50
Ash*	20.00	4.00	76.00
Protein*	39.60	46.00	11.32
Calcium	0.0125	0.0325	0.0125
Potassium	0.0125	0.030	0.0075
Magnesium	1.30	0.37	0.85
Iron	1.80	0.025	2.43
Copper	0.0036	0.0006	0.0029

* Dry weight basis

The data showed that the moisture content in fruit-bodies were comparatively more than in the sclerotia. However, the reverse was true in case of dry matter content. Protein content in the fruit bodies was comparatively more than in the sclerotia. Sclerotia possessed significantly higher fat contents as compared to fruit bodies. Mineral contents of sclerotia were comparable to that of fruit-bodies. Potassium, however, was quite low in sclerotial remnents as compared to the fruit bodies.

4.6 SCLEROTIAL GERMINATION BEHAVIOUR OF *M. esculenta*

4.6.1 Effect of temperature on sclerotial germination behaviour of *M. esculenta*

To find out optimum temperature for the sclerotial germination behaviour of *M. esculenta*, five temperatures in the range of 5-25°C were evaluated. The observations are presented in the Table 10 (Fig. 10).

Table 10: Effect of temperature on sclerotial germination behaviour of *M. esculenta* after 5 days of incubation

Temp-erature (°C)	Growth characteristics	Colony diameter (mm)*
5	No germination	-
10	Mycelial growth limited only on and around sclerotia	-
15	Extensive mycelial growth, colony fluffy, light brown	47.50
20	Mycelial growth poor, colony fluffy, light brown	15.00
25	Mycelial growth poor, colony fluffy, light brown	15.00

* Average of three replications

There was no sclerotial germination at 5°C. Maximum mycelial growth was recorded at 15°C whereas higher temperatures inhibited the myceliogenic germination.

4.6.2 Effect of initial soil moisture on sclerotial germination behaviour of *M. esculenta*

Six initial soil moisture levels in the range of 10-60 per cent were evaluated for studying the sclerotial germination behaviour of *M. esculenta*. The observations were recorded in terms of growth characteristics presented in Table 11 (Fig. 11).

As is evident from the data, moisture levels of 40 and 50 per cent supported moderate mycelial growth. Lower and higher moisture levels inhibited myceliogenic germination of sclerotia.

4.6.3 Effect of light durations on sclerotial germination behaviour of *M. esculenta*

Different light durations were tested to evaluate their effect on sclerotial germination behaviour of *M. esculenta*. The observations are presented in Table 12 (Fig. 12). The results show that, continuous dark conditions supported extensive mycelial growth. There was inhibition of mycelial growth at 12 hr light duration. Other light durations viz., 18, 24 and 6 hrs supported moderate mycelial growth of sclerotia.

Table 11: Effect of initial soil moisture on sclerotial germination behaviour of *M. esculenta* after 5 days of incubation

Initial moisture level (%)	Growth characteristics	Colony diameter (mm)*
10	Mycelial growth only on sclerotia	-
20	Mycelial growth limited only on and around sclerotia	15.00
30	Mycelial growth poor, fluffy, light brown	20.00
40	Mycelial growth moderate, fluffy, light brown	25.00
50	Mycelial growth moderate, fluffy, light brown	25.00
60	Mycelial growth poor, fluffy, light brown	17.50

* Average of three replications

Table 12: Effect of light durations on sclerotial germination behaviour of *M. esculenta* after 5 days of incubation

Light duration (hrs)	Growth characteristics	Colony diameter (mm)*
0	Extensive mycelial growth, colony fluffy, light brown	50.00
6	Mycelial growth moderate, colony fluffy, light brown	25.00
12	Mycelial growth poor, colony fluffy, light brown	20.00
18	Mycelial grwoth moderate, colont fluffy, light brown	30.00
24	Mycelial growth moderate, colony fluffy, light brown	30.00

* Average of three replications

Plate - III (Fig. 9-12)

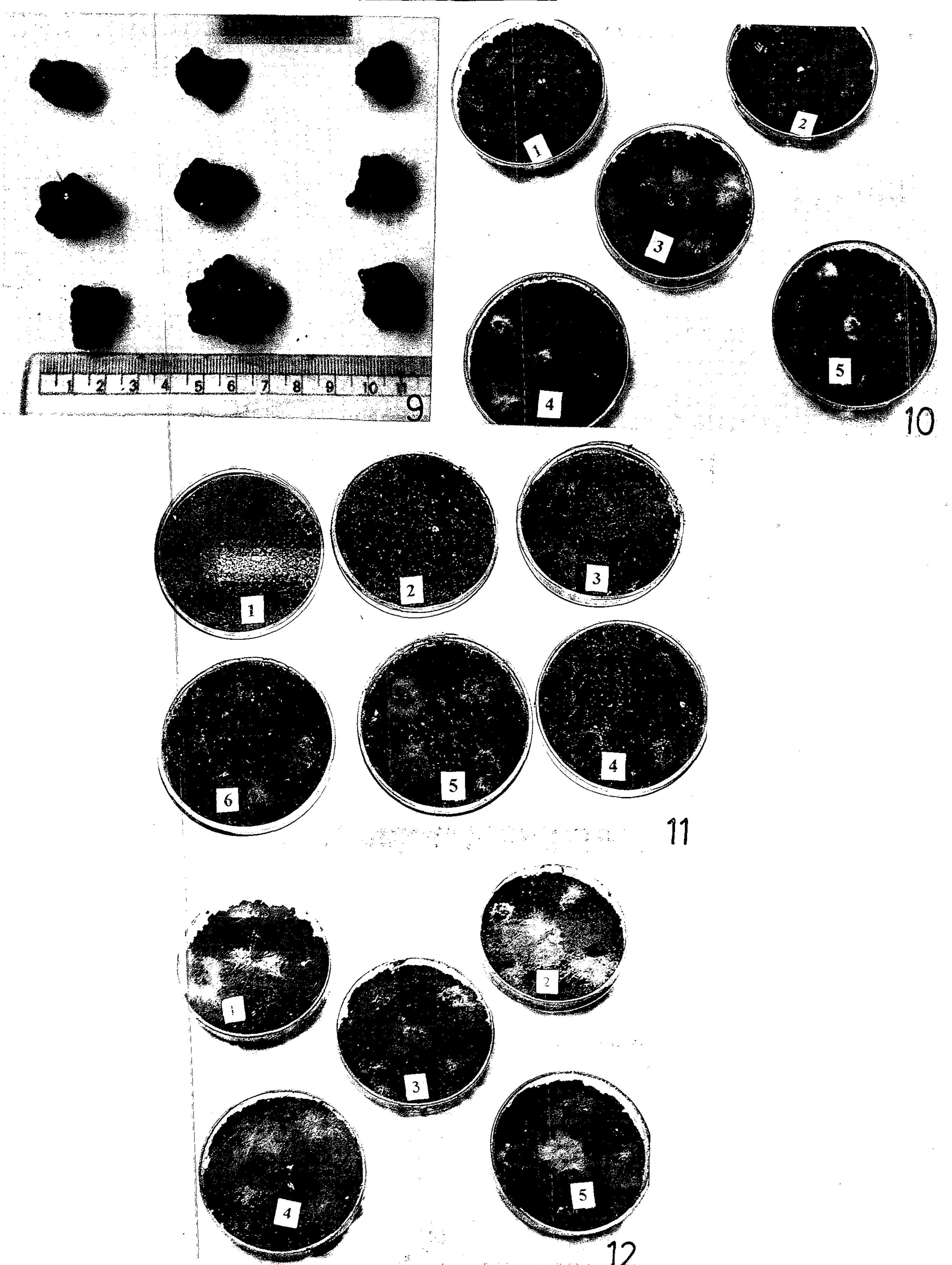
9. Mature harvested sclerotia

10. Effect of temperature (5-25℃) on sclerotial germination behaviour of *M. esculenta*

11. Effect of initial soil moisture (10-60%) on sclerotial germination behaviour of *M. esculenta*

12. Effect of light durations (0-24 hrs) on sclerotial germination behaviour of *M. esculenta*

Plate - III



4.6.4 Effect of growth hormones on sclerotial germination behaviour of *M. esculenta*

Three concentrations of growth hormones viz., Indole acetic acid, indole butyric acid, naphthalene acetic acid, gibbrellic acid, kinetin and benzyl amino purine were tested to evaluate their effect on sclerotial germination behaviour of *M. esculenta*. The observations are recorded in Table 13.

Table 13: Effect of growth hormones on sclerotial germination behaviour of *M. esculenta*

Growth hormones	Soaking time of sclerotia (h)	Growth characteristics after 5 days of incubation		
		5 ppm	10 ppm	20 ppm
IAA	1	+++	+++	+++
	3	+++	+++	+++
	5	-	-	-
IBA	1	-	+++	+++
	3	+++	+++	+++
	5	+++	+++	+++
NAA	1	+++	+++	+++
	3	-	+++	+++
	5	-	+++	+++
GA_3	1	+++	+++	+++
	3	+++	+++	+++
	5	+++	+++	+++
Kinetin	1	+++	+++	+++
	3	-	+++	+++
	5	-	-	+++
BAP	1	+++	+++	+++
	3	-	+++	+++
	5	-	+++	+++

+++ = Extensive mycelial growth, colony fluffy and light brown
- = Poor mycelial growth

The results indicate that all the growth hormones at all concentrations supported the myceliogenic germination of sclerotia. Indole acetic acid at all the concentration, however gave poor mycelial growth of sclerotia, when seeded after dipping for 5 hrs.

4.6.5 Effect of different concentrations of starch on sclerotial germination behaviour of *M esculenta*

Five concentrations of starch were tested to evaluate their effect on sclerotial germination. The observations have been summarised in Table 14 (Fig. 13).

The data (Table 14) reveal that sclerotial germination behaviour of *M. esculenta* was related to the concentration of starch in the medium. Concentration of 0.5, 0.75 and 1.0 per cent supported good mycelial growth of sclerotia. Higher concentrations of starch 1.5 and 2.0 per cent inhibited the mycelial growth and induced the formation of mycelial knots on the sclerotia.

4.6.6 Effect of different concentrations of Potassium nitrate on sclerotial germination behaviour of *M. esculenta*

Five concentrations of potassium nitrate were tested to evaluate their effect on sclerotial germination. The observations have been presented in Table 15.

Table 14: Effect of different concentrations of starch on sclerotial germination behaviour of *M. esculenta*

Concentration of starch (% w/v)	Characteristics	
	After 5 days	After 12 days
0.50	Extensive mycelial growth, colony fluffy, raised, light brown	Extensive mycelial growth, colony fluffy, raised, brown
0.75	Extensive mycelial growth, colony fluffy, raised, light brown	Extensive mycelial growth, colony fluffy, raised, brown
1.00	Extensive mycelial growth, colony fluffy, raised, light brown	Extensive mycelial growth, colony fluffy, raised, brown
1.50	Mycelial growth limited only on and around sclerotia, sparse	Growth appressed around sclerotia, whitish, **knot** formed on sclerotia
2.00	Mycelial growth only on sclerotia, very sparse	Mycelial growth very poor on sclerotia, **knot** formed on sclerotia

Table 15: Effect of different concentrations of potassium nitrate on sclerotial germination behaviour of *M. esculenta*

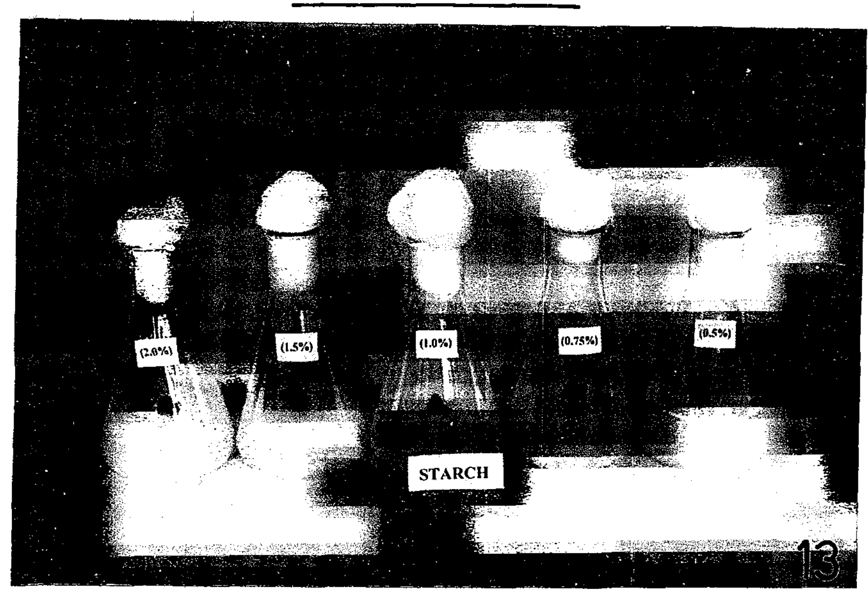
Concentration of KNO_3 (%w/v)	Characteristics	
	After 5 days	After 12 days
0.50	Mycelial growth extensive, light brown	Mycelial growth compact, brown
0.75	Mycelial growth extensive, light brown	Mycelial growth fluffy, creamish brown
1.00	Mycelial growth extensive, light brown	Mycelial growth fluffy, creamish brown
1.50	Mycelial growth extensive, fluffy, light brown	Mycelial growth fluffy, creamish brown
2.00	Mycelial growth only on sclerotia	Mycelial growth restricted, creamish

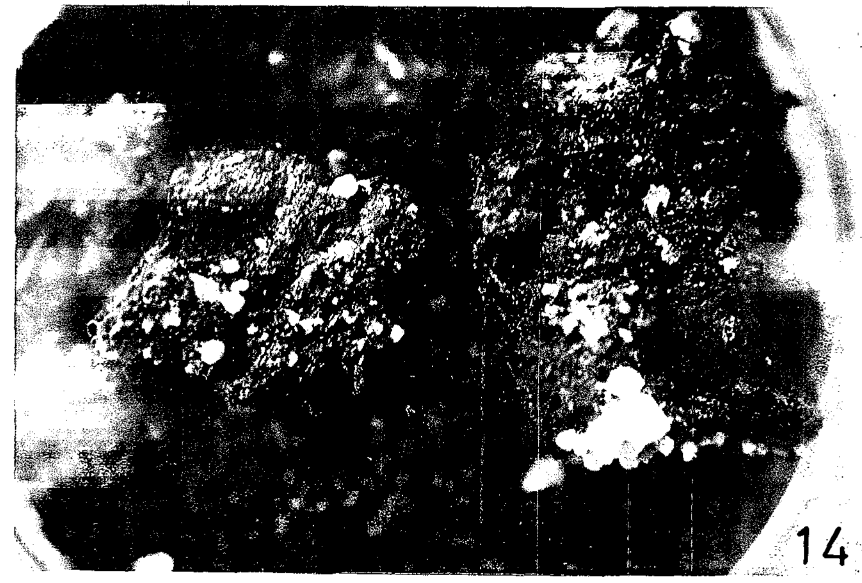
Plate - IV (Fig. 13-14)

13. Effect of different concentrations of starch on sclerotial germination behaviour of *M. esculenta*

14. *In situ* sclerotial germination behaviour of *M. esculenta*

Plate - IV





The data show that sclerotial germination behaviour of *M. esculenta* was not influenced much by varying concentration of potassium nitrate in the medium. Concentrations of 0.5, 0.75, 1.0 and 1.5 per cent supported extensive myceliogenic germination of sclerotia except at 2 per cent where the growth was limited. No knot formation was observed.

4.6.7 *In situ* sclerotial germination behaviour of *M. esculenta*

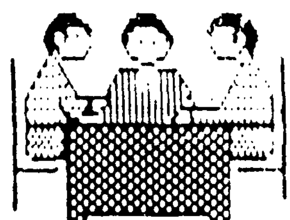
For studying *in situ* sclerotial germination behaviour of *M. esculenta*, the basal nutrient rich zone of wheat grains was removed and soil zone containing mature sclerotia was left as such in jars. The base of each jar was provided with 10-15 fine holes for water percolations. A set each of five such jars was incubated at temperatures of 5°C, 15°C, 20°C and 25^{oC}. The jars were regularly hydrated so that water percolated through the substratum. The data were recorded at an interval of 5 days and are presented in Table 16 (Fig. 14).

Table 16: *In situ* sclerotial germination behaviour of *M. esculenta*

Temperature (°C)	Sclerotial response (days)			
	5	10	15	20
5	-	-	-	-
15	-	-	-	-
20	Mycelial growth only on sclerotia	Whitish pin heads on sclerotia, 2.5 mm in size	Pin heads yellowish brown, 3mm in size	Pinheads brown, 3mm in size
25	Poor mycelial growth around sclerotia	Poor mycelial growth around sclerotia	Poor mycelial growth around sclerotia	Poor mycelial growth around sclerotia

- = No germination

The data reveal that pinhead formation was observed at 20°C temperature. Pinheads which were initially whitish, changed to yellowish brown to brown after a period 10-15 days and grew in size upto 3 mm.



DISCUSSION

CHAPTER-V

DISCUSSION

The experimental results obtained during the present investigations have been discussed under the following headings.

5.1 Distribution and collection

5.2 Establishment of Pure Culture

5.3 Nutritional Requirement

5.3.1 Vitamin nutrition

5.3.2 Mineral nutrition

5.4 Sclerotial production

5.5 Chemical composition

5.6 Sclerotial germination

5.1 DISTRIBUTION AND COLLECTION

Morchella esculenta is fairly common in North-Western Himalayan region during March to May. It occurs in sufficient quantities in the forests of Himachal Pradesh. It can also be collected during September-October but in very meagre quantities. The species is collected from its natural habitat by the local people and dried specimens are sold in the markets at a fabulous price of Rs. 4000-5000 per kg. More

than 2800 quintals of dried morel specimens were taken out from the forests of Himachal Pradesh during 1991-92 (Anonymous, 1993). This continuous collection of morels has resulted in gradual decline of specimens population in nature.

Specimens of *M. esculenta* were collected during September-October and March-May representing `Pre-Winter' and `Post-Winter' strains, respectively (Sharma *et al.*, 1997). Despite repeated efforts, sclerotia could not be collected from nature both during `Pre-winter' and `Post-winter' periods. However, sclerotial remains of *M. esculenta* were collected only at one location (Palampur) during Pre-winter period.

5.2 ESTABLISHMENT OF PURE CULTURE

Pure culture of `Pre-winter' strain was established through tissue culture method and maintained on Coon's medium. Sharma *et al.* (1997) have reported the occurrence of `Pre-winter' and `Post-winter' strains of *M. esculenta* on the basis of ecological, morphological and cultural characteristics. They also reported that `Pre-winter' strain produced sclerotia more efficiently as compared to `Post-winter' strain and suggested that the former may turn out to be potential fruiter. Likewise, the pure culture of `Pre-winter' strain has been used during the present study.

5.3 NUTRITIONAL REQUIREMENT

5.3.1 Vitamins

Vitamins did not support mycelial growth of present strain of *M. esculenta* as compared to control. This is either due to the fact that *M. esculenta* has vitamins in sufficient quantity or due to toxic effect. Higher concentration of vitamins are known to produce inhibitory effect (Lilly and Barnett, 1951). Similarly, higher concentrations of vitamins, also inhibited the mycelial growth of the present strain. Similar finding have been reported for *Morchella* species by Gupta (1990).

5.3.2 Minerals

Mineral elements play a vital role in the life cycle of fungi (Arnon, 1954; Nicholas, 1963). However, in the present study, minerals in general, were poorly utilized by *M. esculenta* for its mycelial growth as compared to control. Boric acid and calcium nitrate tetrahydrate supported comparatively better mycelial growth of the mushroom. Calcium has been reported to support good mycelial growth of *M. esculenta*. Robbins and Hervey (1959) while studying the effects of mineral nutrition of *Morchella*, showed that wood and tomato extracts had a beneficial effect on mycelial growth. They in 1965 concluded that beneficial effect with wood was due to minerals in its ash, especially manganese and calcium. Litchfield *et al.* (1963) recorded stimulatory effect for the growth of *M. esculenta* with calcium chloride. Ghosh and

Majumdar (1986) also reported similar findings with calcium chloride on the growth of *M. esculenta*. Boron gave maximum mycelial growth as compared to other test minerals.

5.4 SCLEROTIAL PRODUCTION

Ower (1982) reported first fruiting of *M. esculenta* from nutrient sink sclerotia. He established that sclerotia are the structures in the life cycle of morels which are competent of giving rise to fruit bodies. His studies on the cultivation of morels led to issuance of two US patents (Ower *et al.*, 1986, 1989). The patents suggest that most important aspect in the cultivation of *M. esculenta* is the production of large sized sclerotia. Similar observations have been made by Volk and Leonard (1989b).

In the present studies, emphasis was given for the production of large sized sclerotia capable of forming fruit-bodies employing jar method (Ower *et al.*, 1986, Sharma *et al.*, 1997). In this method, sclerotia are formed in the upper nutrient poor soil zone. The lower zone comprises a nutrient rich substrate from which the nutrients are translocated back through the mycelia towards the nutrient poor substrate where sclerotial initials are formed. Wheat grains and forest soil have been reported as most suitable nutrient rich and nutrient poor substrates, respectively, for the production of sclerotia at a temperature of 20°C under complete dark conditions by Sharma *et al.* (1997).

For further enhancing the sclerotial production efficiency of *M. esculenta*, different supplementation of nutrient rich medium (Wheat grain) and amendment of nutrient poor zone (forest soil) were tested.

Potassium nitrate as a nitrogen source and starch as a carbon source have been reported to support good mycelial growth of *M. esculenta* (Sud, 1995). Supplementation of nutrient rich medium (wheat grains) with substances known to support vegetative growth might be expected to enhance sclerotial formation. Like wise, addition of potassium nitrate in the grain material significantly improved sclerotial production and biological efficiency. Significantly greater biological efficiency has also been reported by Leonard and Volk (1992) with the addition of nitrogen sources (asparagine and aspartic acid) in the rye grains for *M. crassipes*. Starch, however, did not enhance sclerotial production indicating thereby that carbon availability is not a limiting factors in wheat grains. Similar observations have been made by Leonard and Volk (1992) in relation to exogenous supply of carbon in rye medium for sclerotial production of *M. crassipes*. Supplementation of both potassium nitrate and starch, in combination, gave biological efficiency comparatively lower than with potassium nitrate. However, potassium nitrate alone was supplemented in the wheat grains for further experimentation.

Four vitamins viz. ascorbic acid, folic acid, biotin and nicotinic acid which supported fairly good vegetative growth were also added singly into wheat grains already supplemented with potassium nitrate. Addition of nicotinic acid at 25 ppm and ascorbic acid at 50 ppm in the grain material significantly enhanced the sclerotial production and biological efficiency. These two additives, however, has not shown marked effect on the mycelial growth of the mushroom but influenced the sclerotial production, significantly, indicating their possible role in the ascocarp development. Folic acid and biotin did not show any stimulating effect on sclerotial production as compared to the control.

Four minerals viz. iron, zinc, calcium and boron which were comparatively well utilized by the mushroom for its vegetative growth were also tested to evaluate their effect on the sclerotial production by supplying in the wheat grains already supplemented with potassium nitrate. Among these minerals, supplementation of calcium at 10 ppm concentration remarkably improved sclerotial production as compared to control. The sclerotia harvested from this treatment were comparatively hard and larger in size (upto 3-4 cm) as compared to control and other previous treatments. Better utilization of calcium for sclerotial production provide a clue to explain the common association of morel fruiting with areas that burned the previous year. Supplementation of boron and zinc

at 10 ppm concentration in the grain material also supported significantly better sclerotial production in comparison to control. These findings are in accordance with those of Leonard and Volk (1992) who also observed the positive effect of trace elements when used in the combination with rye, peptone, yeast extract and casamino acids for sclerotial production of *M. crassipes*.

With the aim to further improve the soil texture for sclerotial production the soil was amended with the varying amount of peat moss and vermiculite. All the concentrations of peat moss showed negative effect on the production of sclerotia in comparison to control. It may be due to the fact that peat moss possesses very high water holding capacity of around 300-400% which might have affected the soil texture and aeration etc. resulting into poor development of sclerotia.

Sclerotial yield and biological efficiency was found to be maximum when soil was amended with 7 per cent vermiculite. Also, sclerotia formed in this treatment were comparatively compact, dark and very large in size (up to 4-5 cm) as compared to all other treatments. This can be attributed to higher cation exchange capacity of vermiculite resulting into faster absorption of nutrients. Also addition of vermiculite crystals in the soil might have enhanced aeration, thereby resulting into improved soil texture. It may be mentioned here that the studies relating to the soil amendments have been carried out for the first time.

5.5 CHEMICAL COMPOSITION

Chemical composition of fruit bodies of *M. esculenta* has been well documented in the past. For evaluating nutrient status (Chemical composition) of sclerotia produced in the laboratory, a comparative analysis of chemical composition of specimens of *M. esculenta*, sclerotia produced in the laboratory and sclerotial remnants collected in nature was carried out. The composition of constituents of sclerotia produced in the laboratory was found to be more or less comparable to those of specimens. The dry matter content, however, was more in sclerotia than in the specimens. The crude protein content was almost similar in sclerotia and specimens. Sclerotia possessed significantly higher fat contents as compared to even total fat contents of specimens and sclerotial remains. Fat contents probably get utilized during fruit body development and maturation.

Sclerotia and sclerotial remains contain considerable amount of minerals, particularly magnesium and iron. Contents of different elements, however are variable. Thus, sclerotia produced in laboratory are nutrient sink structures, competent of developing into fruit bodies.

5.6 SCLEROTIAL GERMINATION

Experiments on germination behaviour of sclerotia of *M. esculenta* were carried out using forest soil as the substrates. Harvested

sclerotia were kept in moist soil and stored in refrigerator for a period of one month to simulate winter conditions. Sclerotia germinate either myceliogenically or carpogenically depending upon the prevailing conditions. During the present studies various parameters affecting the sclerotial germination were evaluated with the aim to identify the factors inhibiting myceliogenic germination and inducing carpogenic germination.

Sclerotia of *M. esculenta* did not germinate below 10°C. Mycelial growth was observed to be maximum at 15°C. Higher temperature (20 to 25°C) inhibited the myceliogenic germination of sclerotia though a temperature range of 20-22°C has been found to be optimum for the vegetative growth of the mushroom (Sud, 1995).

Six different soil moisture levels were evaluated to study the sclerotial germination behaviour of *M. esculenta*. Soil moisture level of 40 to 50 per cent supported rich myceliogenic germination of sclerotia. Lower and higher moisture level inhibited the myceliogenic germination. In nature, soil moisture level varies during different stages of development. Soil moisture content above 20 per cent and soil temperature between 10-23°C have been reported conducive for the development of ascocarps of *M. esculenta* (Kaul *et al.*, 1981).

Sclerotial germination behaviour of *M. esculenta* was greatly influenced by light duration. Complete darkness supported extensive myceliogenic germination of sclerotia. However, 12 hour duration of light

significantly inhibited myceliogenic germination which may be conducive for carpogenic germination of sclerotia. In nature also, particularly in the woods, developing ascocarps receive only diffused sun light during most part of the day. Increasing duration of light beyond 12 hours favoured myceliogenic germination. Thus, light plays an important role in determining the germination pattern of sclerotia.

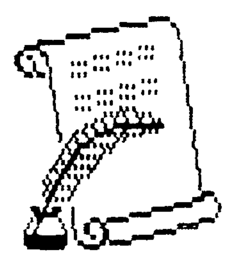
With the aim of inducing carpogenic germination, sclerotia were seeded in the forest soil after dipping in various growth hormones at different concentrations for varying intervals of time. Almost all the growth hormones promoted myceliogenic germination. Indole-acetic acid at all the concentrations, however, gave poor mycelial growth of sclerotia. It appears that plant growth hormones have no role in inducing carpogenic germination of sclerotia.

Different concentrations of starch and potassium nitrate in coon's medium were separately evaluated for their effect on sclerotial germination. Lower concentration of starch (0.5-1.0%) supported extensive myceliogenic germination of sclerotia where as higher concentrations (1.5 and 2.0%) inhibited the myceliogenic germination and induced the carpogenic germination of sclerotia resulting in the formation knots. It is interesting to note that starch was very poorly utilized for sclerotial production and even inhibited the process as it probably was not a limiting factor in wheat grains. However, different concentrations of

starch significantly influenced the germination behaviour of sclerotia. Dextrose concentration of 1.5 per cent in potato dextrose agar medium has been reported to be optimum for primordia formation in *Leucocoprinus* sp. by Sharma *et al.*, 1993.

Potassium nitrate supported extensive myceliogenic germination of sclerotia at all the concentrations except at 2.0 per cent where the growth was limited. No knot formation was observed. Potassium nitrate, however, was well utilized for sclerotia production and enhanced the process significantly.

In situ germination behaviour of sclerotia at different temperature revealed that pinhead formation precedes mycelial growth on sclerotia after a period of 6-7 days at a temperature of 20°C. Pin heads which were initially whitish, changed to yellowish brown to brown after a period of 10-12 days and grew in size also (upto 3mm) but no differentiation took place probably due to lack of appropriate environmental and other conditions of growth which need to be worked out.



SUMMARY

CHAPTER-VI

SUMMARY

Morchella esculenta is the most prized and highly flavoured mushroom. It is common in the North-Western Himalayan region during March-May. Present investigations were undertaken on its collection, vitamin and mineral requirements for its mycelial growth and production of sclerotia, comparative chemical composition of sclerotia and ascocarps and germination behaviour of sclerotia under different set of conditions. The results obtained are summarized below.

The fresh specimens of *M. esculenta* were collected during March-May and September-october. Sclerotial remanents of `Pre-winter' strain of *M. esculenta* were also collected. All the vitamins tested, resulted in the inhibition of mycelial growth of *M. esculenta* in comparison to control. Among the minerals, boron and calcium at 10 ppm concentration supported good mycelial growth of the mushroom.

Supplementation of potassium nitrate in the wheat grains enhanced the sclerotial production, significantly. Starch however, inhibited the sclerotial formation as compared to control. Addition of vitamins in the wheat grains, already supplemented with potassium nitrate, in general, further enhanced the sclerotial yield in comparison to control. Nicotinic acid at 25 ppm gave significantly higher yield.

Calcium (10 ppm) also gave a significant increase in sclerotial production. Sclerotial yield with other minerals at both the concentrations was, however, at par with the control.

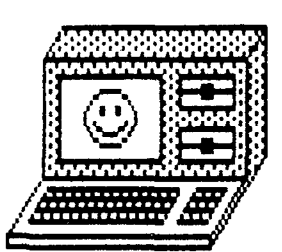
Soil amendment with peat moss showed adverse effect on the sclerotial production. Addition of 7 per cent vermiculite in the soil gave maximum sclerotial yield and biological efficiency. Sclerotia harvested from this treatment were large (upto 5cm) in size, compact and dark brown in colour.

Comparative analysis of composition of the specimens and sclerotia produced in the laboratory revealed higher moisture content in the fruit-bodies. The total protein content of fruit bodies (46%) and sclerotia produced in the laboratory (39.6%) was comparable. Sclerotia possessed significantly higher fat contents as compared to fruit bodies. Minerals concentration (Ca, Mg, Fe, Cu and K) varied in sclerotia and fruit bodies but not significantly.

Experiments on sclerotial germination were conducted under controlled conditions. Forest soil was used as substrate for the germination of sclerotia. A temperature range of 20-25°C, high moisture level of soil (about 40-50%) and 12 hours duration of light inhibited the myceliogenic germination of sclerotia. Growth hormones, in general, stimulated the myceliogenic germination of sclerotia.

Higher concentrations of starch (1.5% and 2.0%) in the Coon's medium induced carpogenic germination in the form of knots on the sclerotia after 7-8 days.

In situ sclerotial germination behaviour of *M. esculenta* at 20°C, showed the formation of knots after 6-7 days which developed into distinct pin heads after 10-12 days. The pin heads did not develop further and aborted after a period of about one month.



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