STUDIES ON THE MICROFLORA OF STORED PEPPER

BY

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THESIS

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1982

DECLARATION

I hereby declare that this thesis entitled "STUDIES ON THE MICROFLORA OF STORED PEPPER" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

Vellanikkara, — 1982.

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Certified that this thesis entitled "STUDIES ON THE MICROFLORA OF STORED PEPPER" is a record of research work done independently by Smt. Estelitta, S, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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CONTENTS

Chapter No.	Title	Page No.
I.	INTRODUCTION	.. 1
II.	REVIEW OF LITERATURE	.. 4
III.	MATERIALS AND METHODS	.. 22
IV.	RESULTS	.. 32
V.	DISCUSSION	.. 81
VI.	SUMMARY	.. 94
	REFERENCES	.. i - viii
	ABSTRACT	

LIST OF TABLES

Table No.	Title		Page
1.	Weather data during the period of study.	..	33
2.	Moisture content of different grades of stored black pepper in different seasons.	..	36
3.	Oleoresin content of different grades of stored black pepper in different periods of the year 1979.	..	41
4.	Piperine content of different grades of stored black pepper in different periods of the year 1979.	..	42
5.	Starch content of different grades of stored black pepper in different periods of the year 1979.	..	43
6.	Mean fungal population in different grades of stored black pepper in different seasons of the year 1979.	..	46
7.	Mean bacterial population in different grades of stored black pepper in different seasons of the year 1979.	..	52

Table No.	Title		Page
8.	Status of infection of different microflora at different levels of humidity.	..	65
9.	Deterioration of the quality in terms of oleoresin content of black pepper due to the attack of different micro-organisms at different humidity levels after 15 and 30 days of incubation.	..	68
10.	Relationship between relative humidity and oleoresin content of stored black pepper inoculated with different micro-organisms.	..	69
11.	Deterioration of the quality in terms of piperine content of black pepper due to the attack of different micro-organisms at different humidity levels after 15 and 30 days of incubation.	..	72
12.	Relationship between relative humidity and piperine content of stored black pepper inoculated with different micro-organisms.	..	73
13.	Deterioration of the quality in terms of starch content of black pepper due to the attach of different micro-organisms at different humidity levels after 15 and 30 days of incubation.	..	76

Table No.	Title	Page
14.	Relationship between relative humidity and starch content of stored black pepper inoculated with different micro-organisms. ..	77
15.	Moisture content of black pepper at different humidity levels after 15 and 30 days of incubation. ..	80

LIST OF FIGURES

Figure No.	Title		Between pages
1.	Weather data during the period of study.	..	82-83
2.	Mean moisture content of different grades of stored black pepper in different seasons.	..	84-85
3.	Oleoresin content of black pepper inoculated with micro-organisms at different humidity levels after 15 days of incubation.	..	89-90
4.	Oleoresin content of black pepper inoculated with micro-organisms at different humidity levels after 30 days of incubation.	..	89-90
5.	Piperine content of black pepper inoculated with micro-organisms at different levels of humidity after 15 days incubation.	..	90-91
6.	Piperine content of black pepper inoculated with micro-organisms at different levels of humidity after 30 days of incubation.	..	90-91

Figure No.	Title		Between pages
7.	Starch content of black pepper inoculated with micro-organisms at different humidity levels after 15 days of incubation.	..	91-92
8.	Starch content of black pepper inoculated with micro-organisms at different humidity levels after 30 days of incubation.	..	91-92

Introduction

INTRODUCTION

The State of Kerala is well known for its spices even from time immemorial. Pepper (*Piper nigrum* L.), known as the 'King of spices', was the prime attractant of the foreign traders to this state. Pepper occupies the most important place in world's spice trade and covers more than 55 per cent of India's trade of spices, accounts for nearly Rs. 35 - 40 crores of foreign exchange annually (Mathew and Sankarikutty, 1977).

The annual production of black pepper, otherwise known as 'black gold', in India is around 35,000 tonnes and the State of Kerala alone accounts for 96 per cent of the production (Anonymous, 1981). Kerala almost holds the monopoly in India for the production and marketing of pepper in different grades and forms. Pepper is mainly produced in homestead gardens as an intercrop and also as a mono-culture. After drying for four to five days in bright sun, the produce goes to the village markets and from there to the assembling markets in bulk or as small lots. From the assembling markets, the black pepper is carried to the exporting centres at Cochin, Calicut and Alleppey and got assembled for effecting sales by the commission agents. Then the produce goes to the wholesale traders and exporters.

In India, pepper is harvested during December-January. The trade of pepper is a continuous process throughout the year, even though the harvest is done only during a short period. So, it involves the process of storage for a long time. The traditional belief is that if pepper is properly dried and stored, it can be kept for years together. But this belief is true only to a certain extent. Pepper, like any other agricultural products, is affected by micro-organisms before and after harvest. Pepper comes under the category of semi-perishable agricultural commodities, of which the quality deterioration due to micro-organisms is very slow. Quality deterioration of stored pepper is also taking place due to storage microflora.

One of the oldest preservation methods is drying and storing, and this has a direct consequence of removal or binding of moisture, without which the micro-organisms cannot grow. For nearly half of the year, highly humid condition is existing in this part of the country and hence there is every chance for the black pepper stored in godowns to absorb moisture from atmosphere and facilitate microbial activity. Very little attention has been given so far to the quality deterioration of stored black pepper. Keeping all these aspects in view, the present study was designed with the following objectives.

1. To find out the moisture status of different grades of black pepper stored in export market during different seasons of the year.
2. To study the periodical variations in the occurrence of microflora associated with the stored black pepper.
3. To isolate the micro-organisms associated with the stored black pepper and to identify the same; and
4. To estimate the deterioration of the quality of the stored black pepper in terms of its oleoresin, piperine and starch contents and to assess the role of each micro-organism in changing the quality of the product at various levels of humidity.

Review Of Literature

REVIEW OF LITERATURE

Agricultural commodities are subjected to considerable spoilage by micro-organisms. The damage to perishable commodities are very well visualised since the perishable nature of the commodities is attributed to the association of micro-organisms. In the case of semi-perishable commodities also micro-organisms are causing damage, but in a relatively slow manner. The qualitative deterioration is taking place in storage due to infestation by micro-organisms under storage conditions (Jay, 1970). The stored black pepper which is of a semi-perishable nature is prond to quantitative and qualitative deterioration in storage due to the attack of microflora.

Christensen and Kaufmann (1965) reported two sources of microflora occurring in stored agricultural commodities, namely, those which are carried from field to storage and those microflora which are usually present in the warehouses. The studies on microflora associated with black pepper have been carried out by very few workers. Moreno-Martinez (1970) studied the fungal flora of black and white pepper as well as the factors affecting their development by examining 71 samples of black pepper. The number of fungal colonies per gramme of ground black and white pepper with moisture contents of 14 to 19 per cent varied from 200 to 6 lakhs and these consisted of seven different species. In general, the ground products had higher microbial counts than unground products, due to the additional steps in processing and due to the greater surface area provided by the particles in the ground product.

Krishnaswamy _et al._ (1971) gave an account of the populations of micro-organisms in spices and spice mixtures. According to them, the total microbial load in spices and spice mixtures ranged from 20×10^3/g to 55×10^6/g. Coliforms were also detected in black pepper. Yeast and mould infestation were present in all types of spices but in black pepper the infestation was heavy (9,800/g). Incidence of mesophilic putrifactives ranged from 26 to 920/g. Non-coagulase type staphylococci was present in some of the spices, sparingly. _Clostridium perfringens_ was present in black pepper while sulphide stinkers and _Salmonella_ sp. were completely absent.

Martines and Christensen (1973) assessed the fungalflora of black and white pepper. They obtained fungal colonies from 42 samples of black and white, whole and ground peppers which averaged 53,000/g the range being 500 to 610,000/g. The major fungi present were *Aspergillus flavus*, *A. glaucus*, *A. ochraceus*, *A. restrictus*, *A. tamarii*, *A. versicolor* and *Penicillium* sp. The moisture content of commercial samples ranged from 8.2 to 16.2 per cent on wet weight basis. They also indicated that if the moisture content of black pepper is kept below 13 per cent, perhaps infestation by fungi could be warded off.

Krishnaswamy *et al.* (1974) determined the microbiological quality of certain spices including black pepper and have stated that fumigation with a mixture of ethylene-oxide and methyl-formate resulted in considerable reduction of total microbial load and complete destruction of coliforms, yeasts and moulds of black pepper.

Pospisil and Ljesevic (1975) observed that the spores of anaerobic bacteria were present in white and black pepper and that *Escherichia coli* and *Proteus* spp. were absent in all samples examined. Masson (1978) studied the hygienic quality of 50 commercial samples of produce including black and white pepper by analysing their microbiological quality. The organisms obtained were coliforms, enterococci staphylococci, *Clostridium perfringens*, yeasts, mildew and moulds.

Nagel *et al.* (1979) determined the aerobic and anaerobic bacterial spore forms occuring in ground black pepper. Bacterial growth was not detected after culturing at 62°C. The maximum counts of bacteria were obtained from the unheated ground black pepper which amounted to about 10^{-4}/g. After heat treatment at 100°C for ten minutes, the counts were as low as 10^{-1}/g.

Von Lorch *et al.* (1979) studied the microbiological aspects of 20 types of herbs and spices including black pepper. The total mesophilic count, yeast + mould count and counts of coliforms, *Escherichia coli*, *Staphylococcus aureus*, sulphite-reducing clostridia, *Clostridium perfringens* in different spices were determined.

Seenappa and Kempton (1980) studied the bacteriological quality of both whole and ground forms of black pepper which were offered for sale in eight retail stores in a Canadian city. Coliforms 1,100/g and enterococci ranging from 5.3×10^4 to 21.5×10^4/g were detected in some of the samples. *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus* and *Streptococcus faecalis* were isolated and biochemically characterised. The aerobic bacteria in the industrial samples ranged from 6.1×10^6 to 73×10^6 in non-ethylene treated and 2.0×10^2 to 41×10^3 in ethylene treated samples.

Moreno-Martinez (1970) reported that eight isolates of the major fungi which they obtained from black and white pepper were lethal to rats to the extent of 64 per cent. On this basis it was concluded that pepper might be a suitable substrate for aflatoxin production. Mislivec *et al.* (1972) determined mycotoxic moulds in samples of imported white and black pepper. Seventy three per cent of non-disinfected black pepper samples contained mycotoxic moulds while only 12.3 per cent of disinfected samples contained moulds, indicating surface contamination of the moulds. *Aspergillus glaucus* predominated both before and after disinfection. All samples of white pepper both before and after disinfection contained mould, indicating internal contamination. *Aspergillus glaucus* was rare in white pepper samples. Isolates of *Aspergillus flavus*, *A. ochraceus*, *A. versicolor* and *P. citrinum* produced mycotoxin on sterile rice.

Pal and Kundu (1972) determined aflatoxin production by *Aspergillus* spp. from various Indian spices ranged from 0.1 to 10.1, 0.1 to 2.5 and 0.0 to 7.5 mg/ml respectively. *Piper nigrum* was found to be the best medium for aflatoxin producing fungi. Julseth and Deibel (1974) reported that none of the organisms detected in stored spices was of any potential public health hazards. Guergue and Ramirez (1977) warned about potential contamination of sausages through aflatoxin contained in red and black pepper used in the preparations. They isolated 525 strains of moulds from samples of red pepper (*Capsicum annum*) and black pepper (*Piper nigrum*) commonly used for spanish sausages. Of these, 100 isolates belonged to *Aspergillus flavus* group out of which 31 were from black pepper among which, 8 strains produced aflatoxin B1.

Most of the spices used in food products are contaminated to varying extent with mould spores, yeasts, bacteria and insects. There are few reports that when the black pepper and white pepper are used for the preparation of food stuffs, the food items get contaminated with micro-organisms.

Volkova (1971) studied about *Bacillus cereus* contamination of foods and environment at institutional feeding centres. Among the food samples and additives analysed it was found that spices and condiments, particularly black pepper were heavily contaminated. Dragoni (1978) reported the role of fungal contamination of black pepper in mould growth on dry sausages. He examined granular, coarse crushed and powdered form of black pepper samples incubated at 25 or 35°C on malt agar with two or three per cent salt to determine the extent of their contamination with moulds. He detected ten different species in the grains and the percentage of samples contaminated with *Penicillium lanosocoeruleum* was the highest being 20 to 70 per cent. *Aspergillus glaucus* contaminated 20 to 50 per cent while *A. candidus* infested 10 to 20 per cent of the samples. In the crushed pepper six species were observed, *A. glaucus* and *P. lanosocoeruleum* (both 1000 to 10,000/g) being the major sources of contamination and in powder, ten species were found, the major contaminants being *A. glaucus* (10,000/g), *A. candidus* and an unidentified species of *Penicillium* (both 1,000 to 10,000/g). In the case of crushed pepper salt mixture four species were found, of which *A. glaucus* and *A. niger* (both 100 to 1,000/g) were the major contaminants.

Moreau and Moreau (1978) conducted mycological analysis of spice samples imported to France and have reported that some of the contaminations spoiled the appearance of the food products thereby rendering the products unsaleable. Some of the other contaminants were toxigenic and were, therefore, dangerous.

Scharner and Schiefer (1978) reported that high loads of bacteria in spices may be responsible for spoilage of spice-containing meat products and may thus cause health hazards to consumers. Approximately 30 samples of spices and spice blends were tested for moulds (*Penicillium* and *Aspergillus* spp.), *Salmonella*, *Bacillus* species, coliforms and sulphite reducing clostridia. Black pepper showed very high levels of contamination (total bacterial counts approximately 10^7/g). They have recommended sterilization of spices by fumigation with ethylene-oxide to reduce the moulds.

Moisture content is well recognised as an imported factor that govern the deterioration of black pepper. For each species of storage fungi, there is a minimum level moisture content below which cannot grow in stored products.

Moreno-Martines (1970) gave results on the moisture content of pepper dried according to three methods of oven drying, namely, 103°C to constant weight, 130°C for one hour and at 98°C under vacuum for five hours, as ranging from

0.8 to 3.2 per cent higher than those determined by the toluene distillation process. Martinez and Christensen (1973) found that moisture content of the commercial samples of black pepper ranged from 8.2 go 16.2 per cent on wet weight basis and indicated that a moisture content of 13 per cent or below afforded protection to stored pepper from invasion by storage fungi.

Jose (1978) reported that under inadequate drying or when black pepper is stored under situations characterised by high ambient moisture content, storage fungi appeared on the product thereby influencing its appearance. Sumathykutty *et al*. (1979) found that moisture content in various commercial grades of garbled and ungarbled materials varied from pinhead to ripening stage at specific intervals in a particular variety. They also reported that the average weight of berries showed increase while the moisture content showed decrease upto the maturity stage. The moisture content is an important factor that influences growth of storage micro-organisms in other agricultural commodities. Fishlock (1929) found *Aspergillus niger* and *A. flavus* growing on insufficiently dried copra. Ward (1937) reported that *A. glaucus* group and *Penicillium glaucum* preferred a moisture content of about seven per cent, while the *A. tamarii*, *A. wentii* and *A. ochraceus* preferred 12 per cent moisture content, while *A. niger*, *Rhizopus nigricans* and *Ceratostomella adiposa* seemed to prefer a moisture content between 15 and 20 per cent. He further stated that sclerotial formation of *A. flavus* tolerated a moisture content ranging from 7 to 15 per cent, with an optimum between 12 and 15 per cent. Dry copra deteriorated rapidly under combined action of bacteria and penetrating moulds when its moisture content was over 12 per cent and when the ambient relative humidity was 80 per cent, the temperature being 28 to 30°C. Below 12 per cent moisture, *Aspergillus flavus* grew superficially but the penetration of the tissues were limited to isolated areas where the bacteria could reach to establish themselves prior to the fungal invasion. Bacteria causing deterioration of copra remained dormant indefinitely on copra having six to eight per cent moisture under ordinary conditions of temperature and humidity.

There is extensive literature about the deterioration of oil seeds such as groundnut, corn and soybean. Pettit *et al.* (1968) observed the occurrence of *Bacillus subtilis* from freshly harvested or dried groundnut kernels, particularly the samples with high moisture content.

Christensen and Lopez (1963) established that the following low limits of moisture content, (based on per cent wet weight) permitted invasion of starchy cereal seeds such as wheat, barley and maize which were kept at 20 to 25°C. *Aspergillus halophilicus* and *A. restrictus* 13.2 per cent, *A. candidus* 15.0 per cent, *A. ochraceus* 15.2 per cent and *A. flavus* 18.0 per cent. The colonies of *A. restrictus* from wheat kept for 100 days at 20 to 25°C at moisture content of 14.4 per cent numbered 7,50,000 while at 14.6 per cent the colony count was 2,710,000 and at 14.9 per cent this was 4,910,000. An increase of 0.2 per cent in the moisture content thus led to five-fold increase of storage microflora.

Christensen and Kaufmann (1968) observed that *Aspergillus* sp. and *Penicillium* sp. could proliferate on rice grains with a moisture content of 12 to 16 per cent, whereas *Rhizopus* sp. required a moisture content of 16 to 22 per cent. Majumder and Venugopal (1969) reported the effect of moisture content on the production of perithecial bodies. Perithecia of *A. repens*, *A. amstelodami*, *A. chevalieri* and *A. ruber*, will be produced on cereal grains if the moisture content is atleast 15.0 to 15.5 per cent. There is a positive correlation between moisture content and growth of storage fungi on sorghum (Burrough and Saucer, 1971); Rice (Christensen and Mirocha, 1976); Sunflower (Christensen, 1972); Wheat (Domenigg *et al.* 1951); Pea (Field and King, 1962); Cotton (Halloin, 1975); Soybean (Milner and Geddes, 1946) and in Ragi (Oblisami and Srinivasa, 1973). Christensen (1972) reported that the moisture content of sunflower seeds and acticity of *Aspergillus glaucus* and *A. restrictus* were positively correlated.

The development of mould depends more on the relative humidity of the storage temperature than the moisture content of the grains (Gosh, 1951). He also concluded that *Aspergillus glaucus* and *A. candidus* can grow on rice with a humidity of 70 per cent or below whereas *Mucor* and *Fusarium* sp. required about 85 per cent relative humidity for their successful growth. Majumder *et al.* (1965) reported that 85 per cent relative humidity could cause visible mould growth in rice, wheat, sorghum, bengal gram, green gram, horse gram, groundnut, cumin and coriander.

Due to the infection by storage microflora, the qualitative deterioration of stored semi-perishable agricultural products is a common phenomenon. Reports on the qualitative spoilage of black pepper due to invasion by storage microflora are scanty.

Studies on the quality deterioration of other semi-perishable agricultural products are numerous. Considerable changes in the quality of coconut oil due to prolonged storage of copra have been reported. Stock (1928) observed that changes in the odour of the oil content of copra could be attributed to the presence of methyl amyll methyl heptyl or methyl nonyl ketones. The fungi

Aspergillus niger, A. flavus, A. ustus, Rhizopus sp., Penicillium sp., Diplodia natalensis, Trichoderma viridae, Cunninghamella verticillata and Syncephalastrum racemosum which occur on copra were found to be capable of utilising coconut oil as a source of carbon for their growth leading to reduction in oil content (Eyre, 1932; Sreemulanathan and Nair, 1971). Varghese (1952) studied on the food value of coconut and reported that the quality of coconut oil depended to a large extent on the quality of copra from which it was extracted. Imperfectly dried mouldy copra or copra obtained from immature nuts yield rancid and cloudy oil with bad taste and smell. Changes in the odour of the oil in copra samples may be due to the presence of odoriferous aldehydes or ketones produced as a result of oxidation due to the infestation by storage microflora (Thorpe, 1960).

Paul et al. (1980) studied the qualitative and quantitative changes of coconut oil on inoculation with Aspergillus niger, A. flavus, A. ustus, Rhizopus sp., Penicillium sp., Diplodia natalensis, Trichoderma viridae, Cunninghamella verticillata, Syncephalastrum *racemosum* and *Bacillus subtilis* singly and in combination, showed that there was a progressive reduction in oil content and rancidity. Maximum reduction in oil was recorded in copra samples inoculated with *A. niger* and *A. flavus* individually.

Ward and Diener (1961) reported several biochemical changes in stored groundnut due to the attack of *Aspergillus tamarii*, four species of *A. glaucus* group and *Penicillium citrinum*. Lalithakumari *et al.* (1971) recorded the reduction in oil content due to storage fungal lipase activity in groundnut and castor.

Deterioration of grain was accompanied by an increase in fatty acids and the value denoted as Fat Acidity Value. Mogee and Christensen (1970) studied the fatty acid content of some cereals after invasion by some seed borne fungi. Individual major fatty acids or total fat acidity increased with increasing levels of invasion by storage fungi. The lipase activity of *Aspergillus candidus* and *Penicillium* sp. was reported in wheat (Dirks, 1954; Domenigg *et al.* 1951) with a moisture level of 15 - 31 per cent. The lipase enzyme activity was studied by Goodman and Christensen (1952) on corn, Mayne Ruth (1956) on cotton, Vidyasekaran and Govindasamy (1966) on paddy and Oblisami and Srinivasa (1973) on ragi. The species of *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Alternaria* and *Helminthosporium* generally increase the free fatty acid. According to Goodman and Christensen (1952), the different storage moulds convert fats into fatty acids at different rates and consumed them as the main source of carbon. Vidhyasekaran and Govindasamy (1968) observed free fatty acid conversion in paddy seeds infected by *Chaetomium bostryctides*. The reduction of starch in terms of quantity will be very low in most of the seeds (Barton, 1961). Considerable reduction in dry weight, starch content and reducing sugars were noticed in paddy seeds infected with seed borne fungi (Vidhyasekharan and Govindasamy, 1968). The reduction in dry weight was found to be due to depletion of starch. Due to the high production of energy, from the depletion of starch, there is an increasing amount of free fatty acids.

Kochu Babu (1976) indicated that carbohydrate, protein, fat, reducing sugars and total nitrogen content were reduced due to mould infection and it was understood that the carbohydrate metabolism is altered by pathogenic invasion.

Cherry *et al.* (1974 and 1975) reported changes in protein and amino acids in peanuts infected with *Aspergillus parasiticus* and also observed that the proteins were hydrolysed first to small polypeptides or insoluble components and then to free amino acids due to the infection.

Materials and Methods

MATERIALS AND METHODS

Location of the study

The experiments connected with this study were conducted at the College of Horticulture, Vellanikkara.

1. Sample collection

Pepper samples used for the study were collected from South India Produce Company, Jew Town, Cochin, one of the leading pepper export company in Kerala. Four different grades of pepper, namely, garbled, ungarbled, light and pinhead were used.

Ungarbled pepper

The black pepper collected from the cultivators (assembling market) containing fractions and grades like dust, stalk, pinhead, hollows, immature and berries of different sizes.

Garbled pepper

The black pepper graded according to the size after cleaning, washing and drying.

winnowing and sieving to remove light and pinhead grades.

Light pepper

The black pepper consisting mainly immature berries which are very light in weight and are seperated by winnowing.

Pinhead pepper

The black pepper, consisting of under-developed berries due to infertilisation or any other physiological disorder and which are very small in size and seperated by sieving.

The samples were collected during four different months namely, January, April, July and October of 1979-80. Care was taken to exclude lots which received pesticidal treatments.

2. Quality determination

The quality aspects of stored black pepper were determined in terms of oleoresin, piperine, starch and moisture.

2.1 Oleoresin content was estimated by the counter current extraction using soxhlet apparatus (A.S.T.A., 1960).

2.2 For piperine, the total nitrogen in the non-volatile ether extract fraction was multiplied by the factor 20.36 (EOA, 1965).

2.3 Starch content was estimated by the method of Barton (1961).

2.4 Moisture content was determined by oven dry method. Samples ~~of~~ weighed were kept in crucibles in a hot air oven at 80°C until constant weights were obtained. Moisture thus determined was expressed in percentage dry weight basis.

3. Estimation of microflora

The quantitative assay of microflora was carried out by serial dilution technique (Pramer and Schmidt, 1966). The microflora on whole and ground pepper berries were estimated by this technique. Grinding was done with the help of a mortar and pestle and the ground pepper was passed through a 20 mesh sieve. Ten grammes each of whole and ground pepper berries were transferred to 100 ml sterile distilled water and these were shaken well in a rotary shaker for one hour. From these, dilutions upto 10^{-7} dilutions were prepared in sterile distilled water. The best dilutions for each group of organisms were previously determined and those dilutions were used for further studies.

3.1 Fungus

One ml of the solution from 10^{-5} dilution was pipetted into sterile petri dishes to which melted and cooled 15 to 20 ml of Martin's rose bengal streptomycin agar (Peptone - 5.0 g; glucose - 10.0 g; agar 20.0 g; potassium dihydrogen phosphate - 1.0 g; magnesium sulphate - 0.5 g; rose bengal solution - 1:300 dilution; streptomycin - 30.0 mg and distilled water - 1,000 ml) was poured. Five petri dishes were used for each sample. The petri dishes with the media were swirled thoroughly to get a uniform distribution. After solidification, the dishes were incubated at room temperature for four days. The fungal colonies developed at the end of four days were counted using dark field colony counter and values were expressed as number of colonies per gramme of dried black pepper.

3.2 Bacteria

For estimating bacteria population, one ml of the solution from 10^{-7} dilution was pipetted into each of the sterile petri dishes and 15-20 ml of nutrient agar (Beef extract - 1.0 g; peptone - 5.0 g; yeast extract - 2.0 g; agar - 20.0g and distilled water - 1,000 ml) was poured. The petri dishes were swirled gently for uniform distribution of media in the dish. The dishes were incubated for 48 hours at room temperature in an inverted position. The bacterial colonies developed were counted with the help of a dark field colony counter and expressed as number of colonies per gramme of black pepper on moisture free basis.

3.3 Actinomycetes

One ml of the solution from 10^{-6} dilution was pipetted into sterile petri dishes. Melted and cooled 15 to 20 ml Kuster's agar (Glycerol - 10.0 g; casein - 2.0 g; magnesium sulphate - 0.5 g;

sodium chloride - 2.0 g; calcium carbonate - 0.2 g; ferrous sulphate - 0.01 g; agar - 20.0 g and distilled water - 1,000 ml) was poured in each petri dish. The dishes were incubated for seven days at room temperature and the actinomycetes colonies were counted. These were expressed as number of colonies per gramme of stored black pepper on moisture free basis.

4. Identification of microflora

All the microflora developed in dilution plate method were studied.

4.1 Morphological studies of fungal flora

The young colonies developed in the dilution plate method were isolated and cultured in potato-dextrose-agar. Single spore isolation/ single hyphal tip isolation was done for maintaining pure culture.

Morphological characters of the fungal flora were studied growing fungi in petri dishes and by slide culture technique. All the cultures were incubated in room temperature. Czapek's agar medium for *Aspergillus* spp. and

Penicillium spp and Potato-dextrose-agar medium for other fungi were used (Ainsworth 1971).

Major morphological characters of the fungi were observed by using Olympus research microscope. The measurements were obtained by taking the means of fifty observations of each characters.

4.2 Identification of bacteria

Bacterial colonies developed in the dilution plate method were streaked in nutrient agar and single colony isolation was made.

From the single colony isolation, Grams' method (1874) was employed for the identification of bacteria.

5. Inoculation studies

Black pepper from the Pepper Research Centre, Vellanikkara, was used for all the *in vitro* studies. Laboratory studies were carried out with the Panniyur-1 variety pepper obtained from the Pepper Research Centre, Vellanikkara. The pepper was brought to a moisture content of eleven per cent and was then surface sterilized with ethanol. The inoculation of different micro-organisms was done by smearing the berries with the following fungi, viz.,Aspergillus niger, A. candidus, A. nidulans, A. versicolor, Curvularia lunata, Penicillium citrinum, Fusarium moniliforme, Rhizopus nigricans and Bacteria gram -ve.

6. Status of infection of different microflora

The growth of different microflora, kept at different levels of relative humidity was observed after incubation for 15 and 30 days and infections were graded as follows:

0 - No growth

1 - Sparse growth

2 - Medium growth

3 - Profused growth

7. Effect of relative humidity

One of the objectives of the study was to find out the effect of different levels of relative humidity on the growth of micro-organisms on pepper and on the consequent qualitative deterioration.

Different levels of relative humidity were maintained inside the desiccator (16-20-5-10 cm) by keeping various concentrations of sulfuric acid as follows:

Sulphuric acid per cent		Relative humidity per cent at 25°C
0	-	100.0
5	-	98.5
10	-	96.1
15	-	92.9
20	-	88.5
25	-	82.9
30	-	75.6
35	-	66.8
40	-	56.8

Ten grammes each of surface sterilized pepper in five mm petri dish was inoculated with the test organisms individually and kept at various levels of relative humidity. There were five petri dishes for each sample. Observations were taken after 15 and 30 days of incubation to find out the effect of relative humidity on the growth of micro-organisms as well as on the quantitative and qualitative deterioration due to specific organisms.

8. Statistical analysis

All the data obtained were analysed statistically by complete randomised design. Data on microbial population were analysed after logarithmic transformation. Co-efficients of correlation between relative humidity and each of the chemical constituents under study were also worked out.

Results

RESULTS

1. Period of sample collection

Samples of five grades of black pepper viz., garbled, ungarbled, light pepper, pinhead and upcountry grade were drawn during four seasons of the year (1979) namely cool dry (January), warm dry (April), South West monsoon (July) and North East monsoon (October). Average rainy days, temperature and relative humidity during the month in which pepper samples were drawn are given in Table 1.

From the above table, it is clear that during January there was no rainfall and the relative humidity was the least (max. 87.75 per cent and mini. 65.25 per cent) compared to other different seasons of the year. The average maximum and minimum temperatures during the month were 31.5°C and 22.4°C respectively. Thus, this period could be classified as cool dry period of the year.

During April, there were four rainy days resulting in a total of 57.1 mm rain, which in turn has resulted in an increase in relative humidity (maximum 91.35 per cent and minimum 68.85 per cent).

Table 1. Weather data (monthly average) during the period of study. (1979)

Period of sampling	Number of rainy days	Total rainfall in mm	Temperature in °C		Relative humidity in %	
			Maximum	Minimum	Maximum	Minimum
January	0	0	31.50	22.40	87.75	65.25
April	4	57.10	37.20	29.60	91.35	68.85
July	26	895.90	28.50	22.00	99.20	93.25
October	19	185.20	32.00	23.50	97.00	89.25

The average maximum and minimum temperatures during this summer month were 37.2°C and 29.6°C respectively. Thus, the month could be considered as the warm dry period of the year.

Out of a total of 31 days, there were 26 rainy days resulting in 895.90 mm rain in July and this was the peak rainy month of the South West monsoon season. As a result of continuous rain, there was an increase in relative humidity (maximum 99.2 per cent and minimum 93.25 per cent) and a decrease in average temperature (maximum 28.5°C and minimum 22.0°C). Thus this month could be considered as the cool moist period of the year.

North East monsoon was not very severe in October. In this month there was only 19 rainy days with 185.20 mm rain. As a result of intermittent rains there was a fluctuation in relative humidity (maximum 97.0 per cent, minimum 89.25 per cent) and temperature (maximum 32°C and minimum 23.5°C). The average temperature during this month was less than that during April and more than that in July. Similarly the rainfall during this month was less then that in July and more than in April. Thus this period could be considered as warm moist period of the year.

2. Moisture status of black pepper in different seasons

Different grades of black pepper were drawn during the four different seasons namely, cool dry period (January), warm dry period (April), cool moist period (July) and warm moist period (October). Moisture contents of the samples were determined and the results are presented in Table 2.

The results reveal that the moisture content of different grades in different seasons vary. The minimum moisture was observed during the warm dry period of the year (April) in all the grades and maximum during the cool moist period of the year (July).

During the month of January, the mean moisture content of the garbled pepper was 10.75 per cent and there was a slight decrease during April, ie., 10.19 per cent. But in July, during the peak period of South West monsoon, there was an increase in the moisture content of garbled pepper and the

Table 2. Moisture content* (per cent) of different grades of stored black pepper during different seasons.

Seasons	Grades of black pepper			
	Garbled	Ungarbled	Light pepper	Pinhead
January	10.75	11.08	11.67	10.97
April	10.19	10.76	10.50	10.50
July	12.08	12.26	12.15	12.87
October	11.08	11.74	12.17	12.23

* Means of four replications

CD (0.05) for season

Garbled pepper	=	0.26
Ungarbled pepper	=	0.39
Light pepper	=	0.56
Pinhead pepper	=	0.56

mean moisture content was 12.08 per cent. Later on by October, the moisture content decreased by one per cent.

The variation of moisture content in four different seasons of the year was found significantly different in the case of garbled stored black pepper. The maximum moisture content was observed during the cool moist period (July) followed by warm moist period (October), but it was on par with the cool dry period (January) and the least was during the warm dry period (April).

The initial mean moisture content of the ungarbled pepper during January was higher (11.08 per cent) than that of garbled pepper (10.75 per cent). The decrease in the moisture content during April was to the tune of 0.32 per cent. But the decrease during the cool moist period was 1.50 per cent. Later, it decreased to 0.52 per cent during the warm moist period (October).

In the case of ungarbled stored black pepper, the moisture status during different periods was found to be significantly different. The maximum mean moisture content was observed during the cool moist period, July (12.26 per cent) followed by warm moist period, October (11.74) per cent). The minimum moisture per cent was observed during the warm dry period, April (10.76 per cent) but it was found to be on par with the cool dry period, January (11.08 per cent).

In January, the moisture content of light pepper was 11.67 per cent and a slight decrease was noticed during April (10.50 per cent). But in July, moisture content increased to a maximum of 12.59 per cent and thereafter decreased in October by 0.42 per cent.

There was significant difference in the mean moisture content of light pepper during different seasons of the year. The maximum moisture content was observed during the cool moist period of the year (July) but it was on par with the warm moist period (October). The mean moisture content during the cool dry period (January) was found to be on par with warm moist period (October). The least moisture content was observed during the warm dry period which differed significantly from the other three seasons.

Pinhead pepper showed a moisture content of 10.97 per cent in January with a slight decrease of 0.47 per cent in April, the warm dry period of the year. In July, a moisture content of 12.87 per cent was noticed, which was 0.64 per cent more than that of the warm moist period (October).

The mean moisture content of pinhead pepper during different seasons of the year was found to be significantly different and the maximum moisture content was observed during the cool moist period (July) followed by warm moist period (October). The least moisture content was observed during the warm dry period (April) but it was on par with the cool dry period (January).

The above data indicate that the moisture status of all the grades of stored black pepper fluctuates according to the season. The minimum moisture content was observed during the warm dry period of the year, April and maximum moisture content was noticed during the cool moist period, July. It is also clear that the temperature and humidity levels in the atmosphere highly influenced the moisture content of dried stored black pepper of different grades in different seasons.

3. Major chemical constituents of black pepper in different seasons

Tables 3,4 and 5 give the composition of four different grades of black pepper viz., garbled, ungarbled, light pepper and pinhead grade in different seasons.

It is evident from the data that the fluctuating moisture status during different seasons led to slight decline in major constituents like oleoresin, piperine and starch in different grades of black pepper. In all the grades of stored black pepper the oleoresin, piperine and starch contents were maximum during the cool dry period of the year, January and the minimum during the warm moist period of the year, October. The decrease in the content of various constituents in garbled, ungarbled, light pepper and pinhead grade from January to October was 1.72 per cent, 1.02 per cent, 1.60 per cent and 0.59 per cent respectively in the case of oleoresin. The corresponding values for piperine were 0.65 per cent, 0.83 per cent, 0.39 per cent and 0.16 per cent respectively. The reduction in the content of starch from January to October was 2.77 per cent,

Table 2. Oleoresin content* (per cent) of different grades of stored black pepper in different periods of the year 1979.

Seasons	Grades of black pepper			
	Garbled	Ungarbled	Light pepper	Pinhead
January	10.92	9.93	12.10	5.12
April	10.86	9.81	12.02	5.00
July	9.26	9.02	10.63	4.67
October	9.20	8.91	10.50	4.53

* means of four replications

CD (0.05) for season
- Garbled pepper = 0.411
- Ungarbled pepper = 0.312
- Light pepper = 0.58
- Pinhead pepper = 0.35

Table 4. Piperine content* (per cent) of different grades of stored black pepper in different periods of the year 1979.

Seasons	Grades of black pepper			
	Garbled	Ungarbled	Light pepper	Pinhead
January	5.41	5.06	4.91	1.16
April	5.08	4.75	4.69	1.08
July	4.95	4.36	4.61	1.03
October	4.76	4.23	4.52	1.00

* Means of four replications

CD (0.05) for seasons

- Garbled pepper = 0.36
- Ungarblad pepper = 0.42
- Light pepper = 0.20
- Pinhead pepper = 0.05

Table 5. Starch content* (per cent) of different grades of stored black pepper in different periods of the year 1979.

Seasons	Grades of black pepper			
	Garbled	Ungarbled	Light pepper	Pinhead
January	34.63	33.33	22.17	14.36
April	34.22	32.67	21.84	14.17
July	32.26	31.87	21.39	13.71
October	31.86	31.73	20.81	13.51

* Means of four replications

CD (0.05) for seasons		
	Garbled pepper	= 1.23
	Ungarbled pepper	= 0.874
	Light pepper	= 0.80
	Pinhead pepper	= 0.44

1.6 per cent, 1.36 per cent and 0.85 per cent in the case of garbled, ungarbled, light pepper and pinhead respectively.

4. Estimation of microflora in stored black pepper

Microbial population of ground and whole samples of four different grades of stored black pepper viz., garbled, ungarbled, light and pinhead were estimated in four different seasons namely, cool dry period, warm dry period, cool moist period and warm moist period of the year 1979. Isolation of different micro-organisms, namely fungi, bacteria and actinomycetes was done with the specific media for particular group of micro-organisms as described in the materials and methods.

Bacterial and fungal populations were present in all the grades of stored black pepper in all the seasons while actinomycetes were absent. There was no variation in the type of microflora associated with the stored black pepper in different seasons.

The results of the population estimates of fungi and bacteria in whole and ground samples of different grades of stored black pepper in different seasons are given in Tables 6 and 7.

4.1 Fungal population in different seasons

The results reveal that ground samples of all the grades yielded more number of fungal colonies when compared to whole samples of all the grades in all the seasons (Table 6). While considering the average mean fungal population of the year, the ground sample of garbled grade yielded 41888 colonies/g whereas whole sample of the same yielded only 26163 colonies/g. In the case of ungarbled grade, the whole sample yielded only 35488 colonies/g while that of the ground pepper yielded a population of 48870 colonies/g. The fungal populations in whole and ground samples of light pepper and pinhead grade were not much pronounced when compared to that of garbled and ungarbled grades. In light pepper, the average yield of fungal colonies/g of whole sample was 42608, but it was only 51463 colonies/g for the ground sample. Similarly the pinhead grade the whole sample yielded a fungal population of 28488 colonies/g and ground sample, 38285 colonies/g.

The results indicate that in the whole samples of all grades of stored black pepper, the minimum

Table 6. Mean fungal population ('000) in different grades of stored black pepper in different seasons of the year 1979.

Seasons	Garbled		Ungarbled		Light pepper		Pinhead	
	Whole	Ground	Whole	Ground	Whole	Ground	Whole	Ground
January	27.00 (4.4314)	38.25 (4.5827)	31.15 (4.4935)	44.50 (4.6484)	37.83 (4.5778)	48.95 (4.6897)	29.25 (4.4661)	36.00 (4.5563)
April	13.50 (4.1303)	29.25 (4.4661)	22.50 (4.3522)	33.75 (4.5282)	27.00 (4.4314)	31.50 (4.4983)	15.75 (4.1973)	22.50 (4.3522)
July	31.15 (4.4935)	55.00 (4.7404)	50.60 (4.7042)	61.60 (4.7896)	57.20 (4.7575)	70.40 (4.8476)	37.40 (4.5729)	50.60 (4.7042)
October	33.00 (4.5185)	45.05 (4.6537)	37.70 (4.5763)	55.63 (4.7483)	48.40 (4.6848)	55.00 (4.7404)	31.50 (4.4983)	44.00 (4.6435)
~~Total~~ Mean	26.163 (4.4176)	41.888 (4.6221)	35.488 (4.5501)	48.870 (4.6890)	42.608 (4.6295)	51.463 (4.7115)	28.488 (4.4547)	38.285 (4.5031)

Figures given in parantheses are logarithmically transformed values.

CD (0.05) for season		Whole	Ground
	Garbled pepper -	0.2327	0.18
	Ungarbled pepper -	0.27	0.21
	Light pepper -	0.253	0.20
	Pinhead pepper -	0.17	0.17

fungal population observed was in garbled grade (26163 colonies/g) followed by pinhead (28488 colonies/g), ungarbled (35488 colonies/g) and light pepper (42608 colonies/g) in that order. In the case of ground sample the minimum count was observed in pinhead (38285/g) followed by the garbled grade (41888/g) and the maximum was for the light pepper (51463/g).

The results reveal that there was much fluctuation in fungal population in all grades of stored black pepper in different seasons of the year. In all the grades, the minimum fungal population was observed for both whole and ground samples during the warm dry period of the year, April. During this period the minimum fungal population in the whole samples was observed in garbled grade (13500 colonies/g) followed by pinhead (15750 colonies/g), ungarbled (22500 colonies/g) and light pepper (27000 colonies/g). For the ground samples the pinhead grade yielded a minimum fungal population of 22500 colonies/g followed by 29250 colonies/g for garbled grade. But there was no appreciable difference in population count between the ground samples of light pepper and ungarbled grades, with a fungal population of

31500 colonies/g for light pepper and 33750 colonies/g for the ungarbled grade during the warm dry period.

The maximum fungal population in all the grades of stored black pepper for all the samples was observed during the cool moist period of the year, July. During this period the maximum fungal population in all the whole samples was obtained in the case of light pepper grade, 57200 colonies/g followed by 50600 colonies/g for ungarbled pepper. The garbled grade yielded a minimum fungal population of 31150 colonies/g during that period followed by 37400 colonies/g in pinhead grade. Maximum fungal population for ground samples during that period was observed in light pepper grade (70400 colonies/g), followed by ungarbled grade (61600 colonies/g) and the minimum fungal colonies for the ground samples was obtained in the pinhead grade (50600 colonies/g), followed by the garbled grade with a fungal population of 55000 colonies/g during the cool moist period.

During the cool dry period and warm moist period, the fungal populations were found to be less than the cool moist period and more than the warm dry period, but it was higher during the warm moist period when compared to the cool dry period. The whole sample of garbled grade yielded a minimum fungal population of 27000 colonies/g during the cool dry period followed by pinhead grade with a population of 29250 colonies/g. The whole samples of ungarbled grade yielded 31150 colonies/g and the same sample of light pepper grade yielded 37830 colonies/g.

Maximum fungal population was obtained during the warm moist period in ground samples of ungarbled grade (55630 colonies/g), and there was not much difference between ground samples of light pepper grade, (55000 colonies/g) and ungarbled grade. There was no appreciable difference between ground samples of garbled grade and pinhead grades which yielded a fungal population of 45050 colonies/g and 44000 colonies/g respectively.

The population variation of different grades of stored black pepper in different seasons was analysed. In the case of whole samples of garbled grade, the maximum fungal population was during the cool moist period but it was on par with the warm moist and cool dry periods and values during these periods differed significantly from that of the warm dry period. The ground samples of garbled grade also yielded maximum fungal population during the cool moist period and that was on par with those of warm moist and cool dry period, but the cool dry period was found to be on par with the warm dry period.

Similarly the whole samples of ungarbled grade yielded maximum fungal population during the cool moist period and was on par with warm moist and cool dry period. The cool dry period was found to be on par with warm dry period. The same trend was observed in ground samples of ungarbled grade also.

In the case of light pepper, like other two grades the maximum fungal population was observed during the cool moist period and was on par with warm moist and cool dry periods, but the cool dry period was found to be on par with the warm dry period. Maximum fungal population in ground samples of light pepper grade was observed during the cool moist period and it was on par with warm moist period and cool dry period and the cool dry period differed significantly from that of warm dry period.

The pinhead grades of both whole and ground samples showed a similar trend as far as the different seasons were concerned. Maximum fungal population was obtained during the cool moist period and it was on par with the warm moist and cool dry period and the warm dry period differed significantly from that of cool dry period.

4.2 Bacterial population

The results show that bacterial colonies were more in ground samples of each grade in different seasons when compared to whole samples of all grades of stored black pepper. The bacterial population ranged from 7427500 colonies/g in the garbled grade to 10721500 colonies/g in pinhead grade. The whole sample of the ungarbled grade yielded a mean average population of 8703000 colonies/g and for the light pepper it was 10472500 colonies/g. The ground sample of garbled grade yielded an average mean population of 8765000 colonies/g and that of the ungarbled

Table 7. Mean bacterial population ('00000) in different grades of stored black pepper in different seasons of the year 1979.

Seasons	Grades of black pepper							
	Garbled		Ungarbled		Light pepper		Pinhead	
	Whole	Ground	Whole	Ground	Whole	Ground	Whole	Ground
January	66.25 (6.8212)	72.00 (6.8573)	68.98 (6.8387)	84.55 (6.9272)	96.10 (6.9827)	102.35 (7.0103)	98.50 (6.9934)	103.50 (7.0170)
April	49.50 (6.6946)	60.75 (6.7836)	62.50 (6.7959)	76.50 (6.8838)	79.00 (6.8976)	87.75 (6.9432)	81.60 (6.9117)	92.25 (6.9652)
July	96.80 (6.9859)	114.40 (7.0584)	113.20 (7.0539)	134.20 (7.1277)	127.80 (7.1066)	143.00 (7.1553)	129.50 (7.1123)	138.60 (7.1418)
October	84.55 (6.9272)	103.45 (7.0149)	103.45 (7.0149)	124.60 (7.0955)	116.00 (7.0645)	127.60 (7.1059)	119.26 (7.0766)	122.00 (7.0864)
~~Total~~ Mean	74.275 (6.8709)	87.650 (6.9427)	87.030 (6.9396)	104.963 (7.0212)	104.725 (7.0199)	115.183 (7.0615)	107.215 (7.0302)	114.088 (7.0573)

Figures given in parantheses are logarithmically transformed values.

CD (0.05) for season			Whole	Ground
	Garbled pepper	-	0.04	0.13
	Ungarbled pepper	-	0.04	0.09
	Light pepper	-	0.014	0.079
	Pinhead pepper	-	0.014	0.06

grade yielded a population of 10496300 colonies/g. The ground samples of light pepper and pinhead grades yielded maximum number of bacterial colonies (11518300 colonies/g and 11408800 colonies/g respectively (Table 7).

While considering the bacterial colonies in different grades of stored black pepper, the whole and ground samples of garbled grade yielded less number of bacterial colonies (7427500 colonies/g and 8765000 colonies/g respectively) then light and pinhead grade (8703000 colonies/g and 10496300 colonies/g respectively). There was not much difference between light and pinhead grades which yielded a population of 10472500 colonies/g and 11518300 colonies/g respectively.

The variation in the occurrence of bacterial population in different grades of stored black pepper in different seasons of the year was much pronounced. The minimum population for both whole and ground samples was obtained in all the grades during the warm dry period of the year, April. The whole samples of all the grades yielded minimum number of bacterial colonies during this period and the least number of bacterial population occurred in the case of garbled grade, 4950000 colonies/g. The whole samples of ungarbled grade yielded 6250000 bacterial colonies/g, which was less than that of garbled grade. The difference in bacterial colonies was slight in the case of whole samples of light and pinhead grades (7900000 colonies/g and 8160000 colonies/g respectively). Almost a similar trend was observed in the case of ground samples also, where the minimum bacterial population was obtained in the garbled grade, (6075000 colonies/g, which was followed by ungarbled grade (7650000 colonies/g). The ground samples of light and pinhead grades yielded 8775000 colonies/g and 9225000 colonies/g, respectively.

The whole and ground samples of all the grades yielded maximum number of bacterial population during the cool moist period of the year, July. During this period maximum population was obtained in the case of ground samples of light pepper grade, (14300000 colonies/g) followed by ground samples of pinhead grade, (13860000 colonies/g). The ground sample of ungarbled grade yielded

13420000 colonies/g. Minimum number of bacterial colonies was observed in the ground samples of garbled grade, (11440000 colonies/g). Among the whole samples, during the cool moist period, maximum population was observed in pinhead grade, (12950000 colonies/g) followed by light pepper grade, (12780000 colonies/g). In the case of whole samples of ungarbled grade, 11320000 bacterial colonies were observed and for garbled grade it was only 9680000 colonies/g.

While considering the warm moist period, October, the bacterial populations of whole and ground samples in different grades were less. During this period the minimum population was observed in the case of garbled grade (8455000 colonies and 10345000 colonies/g for the whole and ground samples respectively). The ground samples of light pepper grade yielded maximum population of 12760000 colonies/g during this period, whereas the whole samples yielded only 11600000 colonies/g. But in the case of pinhead grade, the whole sample yielded more number of bacterial colonies than the whole samples of light pepper grade which was 11926000 colonies/g while the ground samples yielded less number of colonies than the ground samples of light pepper grade, 12200000 colonies/g.

During the cool dry period, January, the bacterial population was less than that of cool moist period and warm moist period, but it was higher than that of warm dry period. Here also, the maximum population was obtained in the case of ground samples of all the grades where the maximum population was noticed in the ground samples of pinhead grade, 10350000 colonies/g and there was no appreciable difference between this sample and ground sample of light pepper grade with a bacterial population of 10235000 colonies/g. The ground sample of ungarbled grade yielded 8455000 colonies/g during this period and minimum population was noticed in the ground sample of garbled grade (7200000 colonies/g). The minimum population was obtained in the whole sample of garbled grade, (6625000 colonies/g) followed by ungarbled grade (6898000 colonies/g). No appreciable difference was noticed between the whole samples of light pepper and pinhead grade (with a population of 9610000 colonies/g and 9850000 colonies/g, respectively) during this period. But it was much higher than that of the whole samples of garbled and ungarbled grades.

Statistically, the variation of bacterial population in the different seasons differed significantly in all grades, both for whole and ground samples.

In the whole samples of garbled pepper, the bacterial population differed significantly from one season to another and the maximum occurred during the cool moist period, followed by warm moist period, cool dry period and the least in warm dry period. But in the case of ground samples of garbled grade, the maximum population was observed during the cool moist period, eventhough being on par with the warm moist period. It differed significantly from that of cool dry period and warm dry period, while the latter two were on par. The whole and ground samples of all other three grades viz., ungarbled, light pepper and pinhead showed exactly the same trend as that of the garbled grade.

5. Morphological characters of microflora

All the grades of stored black pepper examined in different seasons yielded *Aspergillus* spp. (four species), *Penicillium* sp., *Curvularia* sp., *Fusarium* sp., and *Rhizopus* sp. and one type of bacteria. The morphological characters of these fungi were studied and identified as described in the Materials and Methods.

Only one type of bacterium was observed and it was identified as gram -ve bacterium.

5.1 Morphological characters of the fungi were as follows:

(i) *Aspergillus niger* Van Tieghem.

Ann. Sci. nat. Bot. Ser. 5. 8: 240, 1867.

Saccardo, P.A. 1886. *Syll. fung.* 4: 75.

Colonies of Czapek's agar, were found to be fast growing with abundant submerged mycelium; hyaline, aerial hyphae scanty in the early stage, but abundant in old cultures. Conidiophores arising directly from the subtratum, hyaline or slightly yellow to brown near the vesicle only, with thick walls, smooth, non-septate of variable length and width, 320 to 400 x 7 to 10 µ. Conidial heads fuscous, blackish brown to black, conidia in chains, globose or subglobose, thick-walled, 20-50 µ in diameter, hyaline to yellowish brown, fertile over the whole surface. Phialides borne directly on the vesicle, 6-10 x 2-3 µ. Conidia globose, smooth at first, later rough or spinulose.

(ii) Aspergillus candidus Link ex Fries

Syst. Mycol. 3: 385, 1832.

Saccardo, P.A. 1886. Syll. fung. 4: 66.

Colonies on Czapek's solution agar, white becoming yellowish cream with age, often thin with vegetative mycelium mostly submerged, surface growth consisting of conidiophores and heads. Heads white, globose, radiate, varying in the same culture from large, globose masses 200 to 300 µ in diameter to small heads with less than 100 µ in diameter. Conidiophores 500 to 625 u longer or upto 1000 µ, 10 to 20 µ in diameter; with thick walls, smooth, colourless or slightly yellowed below the vesicle. Vesicles typically globose, ranging from 40 µ in diameter and typically fertile over the entire surface. Phialides borne on metulae, both usually colourless. Metulae 15 to 20 µ long. Phialides usually uniform in all heads, 5-8 x 2-3 µ. Conidia hyaline, globose or sub-globose, rarely elliptical, thin walled, smooth, 2.5 to 3.5 μ in diameter.

(iii) Aspergillus nidulans (Eidam) Winter in Rabenhorst's _Kryptogamenft_. 1, _2_: 62, 1884. Saccardo, P.A. 1892. _Syll. fung._ _10_: 524.

Colonies on Czapek's solution agar plain, spreading broadly, dark cress - green due to the abundant conidial heads, reverse of colony in purplish red shades, becoming dark with age. Conidiophores smooth-walled, slightly brown in colour, 70 to 130 μ long, 2.5 to 3 μ in diameter near the foot, 3.5 to 5 μ wide below the vesicle. Conidial heads short, columnar, 60 to 70 μ x 30 to 35 μ. Vesicles hemispherical, 8 to 10 μ in diameter. Phialides borne on metulae, 5 to 6 x 2 to 2.5 μ, metulae 5 to 6 x 2 to 3 μ. Conidia globose, 3 to 3.5 μ in diameter, green in mass.

(iv) _Aspergillus versicolor_ (Vuillemin) Tiraboschi _Ann. Bot._ _7_: 9, 1908. Saccardo, P.A. 1913. _Syll. fung._ _22_: 1261.

Colonies on Czapek's agar initially white, later yellow to varying colour, reverse occasionally colourless or slightly coloured. Colonies slow-growing, compact, velvety and consisting of closely crowded conidiophoses arising from the substratum. Conidiophores colourless, smooth, 350 to 500 µ long, 5 to 10 µ wide near the vesicle. Conidial heads hemispherical, radiate, 100 to 125 µ in diameter. Vesicles 12 to 20 µ in diameter, with fertile area hemispherical. Phialides borne on metulae. Metulae 8 to 10 x 2.5 to 3 µ; Phialides 5 to 10 x 2 to 2.5 µ. Conidia globose, usually delicately echinulate, 2.5 to 3 µ diameter, usually borne in loosely radiating chains.

(v) Penicillium citrinum Thom

Bull. U S. Dep. Agric. Bur. Anim. Ind. 118: 61-3. F. 22, 1910. Emended by Thom, 1930. The Penicillia, PP. 256-7, f.34. Not Penicillium citrinum.

Sopp., 1912. Monograph. PP.166-7, pl.12: f. 120, pl. 18: f. 126, pl. 23: f. 21.

Saccardo, P.A. 1913. Syll. fung. 22: 1260.

Colonies, on Czapek's solution agar furrowed in a radial pattern, close-textured and almost leathery; conidial areas in blue-green shades, becoming dark green to olive-grey in age. Conidiophores arising mostly from the substratum, rarely on aerial hyphae, mostly 75 to 175 µ long and 2 to 3 µ in diameter, usually unbranched, rarely branched and smooth-walled. Penicilli consisting of a terminal group of three, four or occasionally more, divergent metulae. Metulae 10 to 20 x 2 to 3 µ with 6 to 10 in clusters with parallel phialides. Phialides 8 to 10 x 2 to 3 µ, bearing conidia in chains. Conidia globose to subglobose, single celled, usually 2.5 to 3 µ but ranging from 2 to 3.2 µ in diameter and smooth-walled.

(vi) Curvularia lunata (Wakker) Boedijn

Bull. Jard. bot. Buitenz. Ser. 3, 13: 127, 4.2, 3 (11,12), 4(3), 1933.

Saccardo, P.A. 1899. Syll. fung. 14: 1089.

Mycelium septate, profusely branched, hyphae sub-hyaline to light brown; hyphae 2 to 5 µ wide. Conidiophores dark brown, unbranched, septate towards the tip, twisted or straight upto 250 µ long, 2 to 5 µ wide. Conidia boat-shaped, brown, 3 septate, the third cell from the base bulged and conupicuously larger, broader and darker than the other cells, curved or sometimes straight, apical cells sub-hyaline, rounded, basal cell sub-hyaline, some what obconical, bear a scar indicating point of attachment to the conidiophore, 17 to 30 µ long, 10 to 15 µ wide.

(vii) _Fusarium moniliforme_ Sheldon

Ann. Rep. Nebraska agric. Exp. Sta. **17**: 23-32, 1904.

Saccardo, P.A. 1913. _Syll. fung._ **22**: 1485.

Mycelium hyaline, septate, branched. Conidial masses typically formed in sporodochia. Micro-conidia produced in simple, unbranched chains and remaining joined, or formed in false heads, later appearing scattered over the yellowish to rosy-white aerial mycelium, hyaline, single celled, fusiform or egg-shaped, 5 to 8 x 2 to 3 µ. Macroconidia delicate, some-what falcate or straight, tapering towards either end, sometimes slightly hooked at the ends, scattered or grouped in sporodochia, bright coloured in mass, being pink to brown, 3 to 5 septate, 40 to 60 x 2.5 to 4 µ. Chlamydospores absent.

(viii) _Rhizopus nigricans_ Ehrenberg

Saccardo, P.A. 1888. _Syll. fung._ **7**: 212.

Mycelium abundant, soft, initially white cottony growth, branched without septa. Stolon creeping, recurving to the substrate in the form of arachnoid hyphae, strongly raised and distant from the substrate and implanted at each node by means of rhizoids. The internodes often attained a length of 1 to 3 cm and the hyphae more or less branched. Sporangiophores rarely single, united in groups 3 to 5, 0.5 to 4 mm in height x 24 to 42 µ in diameter. Apophyses broad and wedge shaped. Sporangia hemispheric, 100 to 350 µ. Columellae broad, hemispheric, depressed, an average of 70 µ in diameter and 90 µ in height. Spores unequal, irregular round or oval, angular, striate, 9 to 12 µ long x 7.5 to 8 µ in diameter of grey-blue. Zygospores not observed.

6. Status of infection of different microflora

In order to assess the influence of humidity on the growth of micro-organisms, black pepper samples of Panniyur-1 variety collected from Pepper Research Scheme, Vellanikkara were incubated for periods of 15 and 30 days after inoculating with different fungi and bacteria. The micro-organisms used for inoculation isolated from different grades of pepper collected from Cochin export market, were

Table 8. Status of infection of different microflora at different relative humidity levels (Period of incubation 15 and 30 days).

Micro-organisms	Period of incubation (days)	Relative humidity (per cent)								
		56.8	66.8	75.6	82.9	88.5	92.9	96.1	98.5	100
Aspergillus niger	15	0	0	0	1	1	3	3	3	3
	30	0	0	1	2	2	3	3	3	3
Aspergillus candidus	15	0	0	0	1	1	2	3	3	3
	30	0	0	1	2	2	2	3	3	3
Aspergillus nidulans	15	0	0	0	1	1	2	3	3	3
	30	0	0	1	2	2	2	3	3	3
Aspergillus versicolor	15	0	0	0	1	1	1	1	3	3
	30	0	0	1	1	2	2	2	3	3
Curvularia lunata	15	0	0	0	0	1	1	2	3	3
	30	0	0	0	0	1	2	3	3	3
Penicillium citrinum	15	0	0	0	1	1	2	3	3	3
	30	0	0	1	2	2	2	3	3	3
Fusarium moniliforme	15	0	0	0	0	1	1	1	2	2
	30	0	0	0	1	1	2	2	3	3
Rhizopus nigricans	15	0	0	0	0	1	3	3	3	3
	30	0	0	0	0	1	3	3	3	3
Bacteria (Gram -ve)	15	0	0	0	0	1	2	2	2	2
	30	0	0	0	0	1	2	3	3	3

Grade 0 - No growth　　Grade 2 - Medium growth
Grade 1 - Sparse growth　　Grade 3 - Profused growth

Aspergillus niger, A. candidus, A. nidulans, A. versicolor, Curvularia lunata, Penicillium citrinum, Fusarium moniliforme, Rhizopus nigricans and bacterium (gram -ve).

The growth of the micro-organisms under various levels of humidity at 15 and 30 days of incubation was observed and graded as described in Chapter III.

From the Table 8, it can be observed that there was no growth for the micro-organisms after two incubation periods viz., 15 days and 30 days at lower levels of humidity (56.8 to 75.6 per cent), except in the case of all species of Aspergillus and Penicillium citrinum, which showed sparse growth at 75.6 per cent relative humidity after 15 days of incubation. However, the growth behaviour of all micro-organisms varied considerably from 82.9 to 96.1 per cent relative humidity.

At saturated humidity levels (98.5 to 100 per cent), growth of F. moniliforme and bacterium (gram -ve) were medium whereas profused growth was noticed for all other micro-organisms after 15 days of incubation. But when the incubation period was longer ie., 30 days, all micro-organisms showed

Table 9. Deterioration of the quality in terms of oleoresin content of black pepper due to the attack of different micro-organisms at different humidity levels after 15 and 30 days of incubation (on moisture free basis).

Micro-organisms	Period of incubation (days)	Relative humidity (in percentage)								
		56.8	66.8	75.6	82.9	88.5	92.9	96.1	98.5	100
Aspergillus niger	15	9.28	9.62	9.53	8.75	8.42	7.92	7.60	6.44	6.29
	30	9.68	9.23	7.62	6.40	5.54	5.49	5.23	4.86	4.59
Aspergillus candidus	15	9.89	9.63	9.62	9.17	8.79	8.49	8.05	6.61	6.37
	30	9.27	8.76	7.52	6.78	5.47	5.61	5.25	4.96	4.42
Aspergillus nidulans	15	9.86	9.81	9.76	9.35	8.90	8.48	8.03	7.01	6.88
	30	9.36	9.57	8.13	7.18	6.72	5.53	4.89	4.62	4.13
Aspergillus versicolor	15	10.02	9.68	9.92	9.20	9.11	8.68	8.36	7.39	7.09
	30	9.43	9.54	3.98	7.86	7.17	6.06	5.73	5.36	5.01
Curvularia lunata	15	9.87	9.62	9.85	9.53	9.27	8.88	8.74	8.41	7.91
	30	10.18	9.42	9.57	9.26	9.01	8.27	7.08	6.34	5.43
Penicillium citrinum	15	10.06	9.98	9.62	9.51	9.08	8.97	8.72	8.46	8.36
	30	9.68	8.36	7.82	6.37	5.84	5.70	5.02	4.81	4.79
Fusarium moniliforme	15	10.06	9.92	9.72	9.67	9.11	9.02	8.78	8.52	8.33
	30	9.98	10.02	9.54	9.20	9.06	9.04	8.79	7.50	7.36
Rhizopus nigricans	15	10.21	10.02	9.84	9.53	9.21	8.91	8.72	8.68	8.62
	30	10.02	10.08	9.59	9.38	8.85	8.58	8.03	6.91	6.18
Bacteria (gram -ve)	15	9.92	10.02	9.80	9.61	9.03	9.03	8.86	8.78	8.49
	30	10.16	10.21	9.69	9.42	9.42	9.26	8.27	8.06	7.04

<u>Aspergillus niger</u>, <u>A. candidus</u>, <u>A. nidulans</u>, <u>A. versicolor</u>, <u>Curvularia lunata</u>, <u>Penicillium citrinum</u>, <u>Fusarium moniliforme</u>, <u>Rhizopus nigricans</u> and bacterium (gram -ve).

The growth of the micro-organisms under various levels of humidity at 15 and 30 days of incubation was observed and graded as described in Chapter III.

From the Table 8, it can be observed that there was no growth for the micro-organisms after two incubation periods viz., 15 days and 30 days at lower levels of humidity (56.8 to 75.6 per cent), except in the case of all species of <u>Aspergillus</u> and <u>Penicillium citrinum</u>, which showed sparse growth at 75.6 per cent relative humidity after 15 days of incubation. However, the growth behaviour of all micro-organisms varied considerably from 82.9 to 96.1 per cent relative humidity.

At saturated humidity levels (98.5 to 100 per cent), growth of <u>F. moniliforme</u> and bacterium (gram -ve) were medium whereas profused growth was noticed for all other micro-organisms after 15 days of incubation. But when the incubation period was longer ie., 30 days, all micro-organisms showed profused growth.

7. Deterioration in quality of black pepper as influenced by humidity, period of incubation and type of micro-organism

The results presented in Tables 9 to 14 clearly show that the quality of stored black pepper was deteriorated due to the influence of relative humidity, period of incubation and the type of micro-organism used for inoculation.

7.2 Oleoresin

The oleoresin content of stored black pepper was found decreasing corresponding to the changes in levels of humidity (56.8 per cent to 100 per cent), when inoculated with different micro-organisms (Table 9). The ranges were 9.28 to 6.29 per cent (Aspergillus niger), 9.89 to 6.37 per cent. (A. candidus), 9.86 to 6.88 per cent (A. nidulans), 10.02 to 7.09 per cent (A. versicolor), 9.87 to 7.91 per cent (Curvularia lunata), 10.06 to 8.36 per cent (Penicillium citrinum), 10.06 to 8.33 per cent (Fusarium moniliformae), 10.21 to 8.62 per cent (Rhizopus nigricans) and 9.92 to 8.49 per cent (Gram -ve bacteria) after 15 days of incubation. But when the samples were incubated for 30 days, the

Table 10. Relationship between relative humidity and oleoresin content of stored black pepper inoculated with different micro-organisms.

Micro-organisms	Period of incubation			
	15 days		30 days	
	Correlation co-efficient (r)*	Regression equation (Y = A + Bx)	Correlation co-efficient (r)*	Regression equation (Y = A + Bx)
Aspergillus niger	-0.8654	Y = 14.2450-0.0717x	-0.9879	Y = 16.9712-0.1241x
Aspergillus candidus	-0.8557	Y = 14.6780-0.0732x	-0.9874	Y = 16.0006-0.1134x
Aspergillus nidulans	-0.8766	Y = 14.3712-0.0676x	-0.9528	Y = 17.8271-0.1313x
Aspergillus versicolor	-0.8733	Y = 13.9690-0.0610x	-0.9546	Y = 16.8525-0.1143x
Curvularia lunata	-0.8683	Y = 12.4214-0.0392x	-0.8530	Y = 16.0760-0.0925x
Penicillium citrinum	-0.9642	Y = 12.5866-0.0403x	-0.9687	Y = 16.9024-0.1289x
Fusarium moniliforme	-0.9425	Y = 12.5525-0.0394x	-0.8573	Y = 13.5156-0.0542x
Rhizopus nigricans	-0.9785	Y = 12.6708-0.0400x	-0.8657	Y = 15.2221-0.0783x
Bacteria (gram -ve)	-0.9348	Y = 12.2084-0.0347x	-0.8501	Y = 14.0768-0.0596x

* All r values are significant at one per cent level.

ranges were 9.68 to 4.59 per cent (*A. niger*), 9.27 to 4.42 per cent (*A. candidus*), 9.36 to 4.13 per cent (*A. nidulans*), 9.43 to 5.01 per cent (*A. versicolor*), 10.18 to 5.43 per cent (*C. lunata*), 9.68 to 4.79 per cent (*P. citrinum*), 9.98 to 7.36 per cent (*F. moniliformae*), 10.02 to 6.18 per cent (*R. nigricans*) and 10.16 to 7.04 per cent (gram -ve bacterium).

The co-efficients of correlation between humidity and oleoresin content for 15 days and 30 days of incubation were -0.8654 and -0.9879 (*A. niger*), 0.8557 and -0.9874 (*A. candidus*), -0.8766 and -0.9528 (*A. nidulans*), -0.8733 and -0.9546 (*A. versicolor*), -0.8683 and -0.8530 (*C. lunata*), -0.9642 and -0.9687 (*P. citrinum*), -0.9425 and -0.8573 (*F. moniliformae*), -0.9788 and -0.8657 (*R. nigricans*) and -0.9348 and -0.8501 (gram -ve bacterium) respectively. All these values were significant at one per cent level (Table 10).

The regression equations indicated that a unit increase in the relative humidity per cent resulted in the reduction of oleoresin content by 0.07 and 0.12 per cent (*A. niger*), 0.07 and 0.11 per cent (*A. candidus*), 0.06 and 0.13 per cent

(*A. nidulans*), 0.06 and 0.11 per cent (*A. versicolor*), 0.04 and 0.09 per cent (*C. lunata*), 0.04 and 0.13 per cent (*P. citrinum*), 0.04 and 0.05 per cent (*F. moniliformae*), 0.4 and 0.08 per cent (*R. nigricans*) and 0.03 and 0.06 per cent (gram -ve bacterium) after 15 days and 30 days of incubation respectively. However, it was found that in all cases, the effect of humidity was conspicuous at longer periods of incubation.

7.2 Piperine

The piperine content in stored black pepper inoculated with different micro-organisms was found to be lowering corresponding to the increase in levels of humidity (56.8 per cent to 100 per cent)(Table 11). The ranges were 4.97 to 2.01 per cent (*A. niger*), 4.97 to 2.16 per cent (*A. candidus*) 4.98 to 2.28 per cent (*A. nidulans*), 4.98 to 2.45 per cent (*A. versicolor*), 4.98 to 2.32 per cent (*P. citrinum*), 5.06 to 3.41 per cent (*C. lunata*), 4.98 to 3.34 per cent (*F. moniliformae*), 5.02 to 3.96 per cent (*R. nigricans*) and 5.02 to 4.02 per cent (gram -ve bacterium) after 15 days of incubation. But when the samples were incubated for 30 days, the ranges were 4.98 to 0.50 per cent (*A. ni*

Table 11. Deterioration of the quality in terms of piperine content of black pepper due to the attack of different micro-organisms at different humidity levels after 15 and 30 days of incubation (on moisture free basis).

Micro-organisms	Period of incubation (days)	Relative humidity (in percentage)								
		56.8	66.8	75.6	82.9	88.5	95.9	96.1	98.5	100
Aspergillus niger	15	4.97	4.68	4.28	3.87	3.56	3.18	2.56	2.34	2.01
	30	4.98	4.96	3.26	2.49	1.94	1.52	0.83	0.75	0.50
Aspergillus candidus	15	4.97	5.01	4.86	4.08	3.89	3.50	2.97	2.58	2.16
	30	4.98	3.39	2.63	2.08	1.98	1.63	1.41	1.07	0.88
Aspergillus nidulans	15	4.98	4.98	4.87	4.29	4.16	3.87	3.52	2.76	2.28
	30	4.97	4.82	3.93	3.56	3.10	2.78	2.07	1.20	1.05
Aspergillus versicolor	15	4.98	4.98	4.57	4.53	4.32	3.98	3.66	2.89	2.45
	30	4.87	4.24	3.98	[illegible]	3.24	2.82	2.21	2.07	1.24
Penicillium citrinum	15	4.98	4.92	4.87	4.53	4.18	4.02	3.68	2.93	2.32
	30	4.86	4.84	4.24	3.89	3.26	2.98	2.43	1.86	1.32
Curvularia lunata	15	5.06	5.07	5.02	4.97	4.68	4.25	4.01	3.52	3.41
	30	4.96	4.98	4.46	3.96	3.78	3.02	2.92	2.14	2.08
Fusarium moniliforme	15	4.98	5.02	4.98	4.84	4.67	4.28	4.06	3.54	3.34
	30	4.92	4.87	4.56	3.83	3.74	3.07	2.97	3.18	2.16
Rhizopus nigricans	15	5.02	4.98	5.02	4.97	4.81	4.28	4.11	3.98	3.96
	30	5.06	4.92	4.84	4.67	4.62	4.02	3.96	3.45	3.25
Bacteria (gram -ve)	15	5.02	5.02	4.98	4.84	4.81	4.46	4.28	4.17	4.02
	30	4.96	4.96	4.92	4.87	4.64	4.18	4.11	4.01	3.96

Table 12. Relationship between relative humidity and piperine content of stored black pepper inoculated with different micro-organisms.

Micro-organisms	Period of incubation: 15 days — Correlation co-efficient (r)*	15 days — Regression equation (Y = A + Bx)	30 days — Correlation co-efficient (r)*	30 days — Regression equation (Y = A + Bx)
Aspergillus niger	-0.9601	Y= 9.1363-0.0670x	-0.9855	Y= 11.8425-0.1122x
Aspergillus candidus	-0.9227	Y= 9.2461-0.0649x	-0.9801	Y= 9.3009-0.0840x
Aspergillus nidulans	-0.8790	Y= 8.7287-0.0565x	-0.9491	Y= 10.6238-0.0899x
Aspergillus versicolor	-0.8609	Y= 8.3025-0.0507x	-0.9454	Y= 9.3150-0.0732x
Penicillium citrinum	-0.8530	Y= 8.4815-0.0526x	-0.9376	Y= 9.9461-0.0789x
Curvularia lunata	-0.8520	Y= 7.6290-0.0378x	-0.9421	Y= 9.4417-0.0695x
Fusarium moniliforme	-0.8390	Y= 7.4293-0.0358x	-0.9426	Y= 9.2095-0.0667x
Rhizopus nigricans	-0.8529	Y= 6.8341-0.0269x	-0.8833	Y= 7.5787-0.0388x
Bacteria (gram -ve)	-0.8817	Y= 6.5596-0.0230x	-0.8890	Y= 6.6917-0.0259x

* All r values are significant at one per cent level.

4.98 to 0.88 per cent (*A. candidus*), 4.92 to 1.05 per cent (*A. nidulans*), 4.87 to 1.24 per cent (*A. versicolor*), 4.86 to 1.32 per cent (*P. citrinum*), 4.96 to 2.08 per cent (*C. lunata*), 4.92 to 2.16 per cent (*F. moniliformae*), 5.06 to 3.25 per cent (*R. nigricans*) and 4.96 to 3.96 per cent (gram -ve bacterium).

The co-efficients of correlation between humidity and piperine content for 15 days and 30 days of incubation were -0.9601 and -0.9855 (*A. niger*), -0.9227 and -0.9801 (*A. candidus*), -0.8790 and -0.9491 (*A. nidulans*), -0.8609 and -0.9454 (*A. versicolor*), -0.8530 and -0.0376 (P. citrinum), -0.8530 and -0.9376 (*P. citrinum*), -0.8520 and -0.9421 (*C. lunata*), -0.8390 and -0.9426 (*F. moniliformae*), -0.8529 and -0.8833 (*R. nigricans*) and -0.8817 and -0.8890 (gram -ve bacterium) respectively, all being significant at one per cent level (Table 12).

From the regression equations, it could be seen that a unit increase in the relative humidity (per cent) caused a reduction of piperine content by 0.07 and 0.11 per cent (*A. niger*), 0.06 and 0.08 per cent (*A. candidus*), 0.06 and 0.09 per cent (*A. nidulans*), 0.05 and 0.07 per cent (*A. versicolor*), 0.05 and 0.08 per cent (P. citrinum), 0.04 and 0.07 per cent (C. lunata), 0.04 and 0.07 per cent (F. moniliformae), 0.03 and 0.04 per cent (R. nigricans) and 0.02 and 0.03 per cent (gram -ve bacterium) after 15 days and 30 days of incubation. However, the results shown that in all cases the effect of humidity was higher at longer periods of incubation.

7.3 Starch

In the samples of stored black pepper inoculated with different micro-organisms, the starch content was found to be reducing with the increase in levels of humidity (56.8 per cent to 100 per cent) (Table 13). The ranges were 32.16 to 21.8 per cent (A. niger), 33.38 to 23.13 per cent (A. candidus), 34.16 to 23.27 per cent (A. nidulans), 34.58 to 24.37 per cent (A. versicolor), 33.92 to 26.97 per cent (P. citrinum), 33.08 to 26.47 per cent (C. lunata), 33.25 to 26.43 per cent (F. moniliformae), 32.67 to 29.13 per cent (R. nigricans) and 32.82 to 30.92 per cent (gram -ve bacterium) after 15 days of incubation. When the incubation period was 30 days, the ranges of starch content were 32.27 to 17.6 per cent (A. niger), 32.68 to 18.23 per cent

Table 13. Deterioration of the quality in terms of starch content of black pepper due to the attack of different micro-organisms at different humidity levels after 15 and 30 days of incubation (on moisture free basis).

Micro-organisms	Period of incubation (days)	Relative humidity (in percentage)								
		56.8	66.8	75.6	82.9	88.5	92.9	96.1	98.5	100
Aspergillus niger	15	32.16	32.69	32.62	28.13	28.67	27.72	26.55	23.94	21.80
	30	32.27	29.84	39.78	25.60	25.35	23.21	21.76	19.02	17.60
Aspergillus candidus	15	33.38	33.45	32.62	32.26	30.25	28.40	26.78	24.47	23.13
	30	32.68	32.02	27.79	27.41	26.47	25.34	21.60	19.76	18.23
Aspergillus nidulans	15	34.16	33.08	32.54	32.58	30.38	27.07	25.63	24.07	23.27
	30	32.48	31.62	29.45	28.43	26.72	24.85	24.00	21.53	19.61
Aspergillus versicolor	15	34.58	34.56	33.18	32.26	29.86	26.93	25.77	25.93	24.37
	30	31.26	30.42	30.18	27.56	27.49	25.45	22.29	21.57	20.36
Penicillium citrinum	15	33.92	33.48	32.07	31.94	29.36	26.17	25.93	25.63	24.97
	30	33.08	31.67	31.48	31.28	28.73	26.32	24.15	24.09	21.56
Curvularia lunata	15	33.08	32.46	32.18	32.09	31.32	28.00	27.58	27.07	26.47
	30	33.08	31.47	30.46	30.18	29.06	27.01	26.17	26.02	25.42
Fusarium moniliforme	15	33.25	33.16	32.27	32.18	32.09	29.89	27.33	26.69	26.43
	30	32.46	32.38	32.26	30.02	29.17	25.42	25.03	24.96	24.45
Rhizopus nigricans	15	32.67	32.63	32.64	32.52	31.98	31.57	30.66	29.69	29.13
	30	31.68	31.42	31.18	30.46	30.02	29.14	29.06	28.72	28.53
Bacteria (gram -ve)	15	32.52	32.76	32.72	32.68	32.59	32.41	31.82	30.96	30.92
	30	33.17	32.49	32.47	31.38	31.13	29.68	28.92	28.67	28.51

Table 14. Relationship between relative humidity and starch content of stored black pepper inoculated with different micro-organisms.

Micro-organisms	Period of incubation			
	15 days		30 days	
	Correlation co-efficient (r)*	Regression equation (Y = A + Bx)	Correlation co-efficient (r)*	Regression equation (Y = A + Bx)
Aspergillus niger	-0.8828	Y = 47.1551-0.2244x	-0.9531	Y = 51.8096-0.3190x
Aspergillus candidus	-0.8810	Y = 48.7122-0.2291x	-0.9393	Y = 52.2328-0.3150x
Aspergillus nidulans	-0.9029	Y = 50.4764-0.2526x	-0.9472	Y = 49.8552-0.2770x
Aspergillus versicolor	-0.9389	Y = 51.0086-0.2528x	-0.9194	Y = 47.3131-0.2496x
Penicillium citrinum	-0.9404	Y = 48.4698-0.2279x	-0.8811	Y = 47.5950-0.2335x
Curvularia lunata	-0.8886	Y = 43.3112-0.1577x	-0.9692	Y = 43.5208-0.1752x
Fusarium moniliforme	-0.8585	Y = 43.9785-0.1616x	-0.9236	Y = 46.5080-0.2142x
Rhizopus nigricans	-0.8131	Y = 37.6807-0.0734x	-0.9640	Y = 36.5804-0.0778x
Bacteria (gram -ve)	-0.7646	Y = 35.4650-0.0389x	-0.9470	Y = 40.2880-0.1137x

* All r values are significant at one per cent level, except that of Bacteria (gram - ve) after 15 days of incubation.

(A. *candidus*), 32.48 to 19.61 per cent (A. *nidulans*), 31.26 to 20.36 per cent (A. *versicolor*), 32.08 to 21.56 per cent (P. *citrinum*), 33.08 to 25.42 per cent (C. *lunata*), 32.46 to 24.45 per cent (F. *moniliformae*), 31.68 to 28.53 per cent (R. *nigricans*) and 33.17 to 28.51 per cent (gram -ve bacterium).

The co-efficients of correlation between humidity and starch content for 15 days and 30 days of incubation were -0.8828 and -0.9531 (A. *niger*), -0.8810 and -0.9393 (A. *candidus*), -0.9029 and -0.9472 (A. *nidulans*), -0.9389 and -0.9194 (A. *versicolor*), -0.9404 and -0.8811 (P. *citrinum*), -0.8886 and -0.9692 (C. *lunata*), -0.8585 and -0.9236 (F. *moniliformae*), -0.8131 and -0.9640 (R. *nigricans*) and -0.7646 and -0.9470 (gram -ve bacterium) respectively. All these values were significant at one per cent level except in the case of gram -ve bacterium for 15 days of incubation (Table 14).

The regression equations revealed that for each unit increase in the relative humidity (per cent), the starch content was reduced by 0.22 and 0.32 per cent (A. *niger*), 0.23 and 0.32 per cent (A. *candidus*), 0.25 and 0.28 per cent (A. nidulans), 0.25 per cent each (A. versicolor), 0.23 per cent each (P. citrinum), 0.16 and 0.18 per cent (C. lunata), 0.16 and 0.21 per cent (F. moniliformae), 0.07 and 0.08 per cent (R. nigricans) and 0.04 and 0.11 per cent (gram -ve bacterium) for 15 days and 30 days of incubation. These results indicated that the effect of humidity was higher at longer periods of incubation in all the cases except the samples inoculated with A. versicolor and P. citrinum.

7.4 Moisture content of black pepper at different humidity levels

As evident from Table 15 the moisture content of stored black pepper varied according to the changes in humidity levels. The moisture content of black pepper, inoculated with different micro-organisms, was minimum at the lowest level of humidity (56.8 per cent) and maximum at the highest level of humidity (100 per cent). It could also be observed that the moisture content increased when the incubation period of the black pepper inoculated with micro-organisms was prolonged.

Table 15. Moisture content (per cent) of black pepper at different humidity levels after 15 and 30 days of incubation.

Micro-organisms	Period of incubation (days)	Relative humidity (in percentage)								
		56.8	66.8	75.6	82.9	88.5	92.9	96.1	98.5	100
Aspergillus niger	15	11.07	11.48	11.57	12.35	13.60	14.83	14.87	15.23	15.80
	30	11.27	11.48	11.60	12.87	14.30	15.33	18.07	18.50	22.03
Aspergillus candidus	15	11.26	11.42	11.83	12.82	13.51	14.06	14.73	14.90	15.40
	30	11.18	11.67	11.80	13.53	13.70	14.30	15.70	17.50	18.50
Aspergillus nidulans	15	11.13	11.28	11.47	12.59	13.22	13.84	14.33	14.88	15.03
	30	11.24	11.82	12.53	14.70	15.50	16.27	18.07	19.30	20.37
Aspergillus versicolor	15	11.08	11.46	12.82	13.26	13.85	14.33	14.87	15.27	15.57
	30	11.07	11.26	11.93	13.70	15.07	16.43	18.20	18.77	19.27
Curvularia lunata	15	11.17	11.82	12.03	13.12	13.96	14.82	14.96	15.36	15.90
	30	11.24	11.23	11.97	13.73	14.67	16.43	17.33	17.47	19.38
Penicillium citrinum	15	11.26	11.53	12.08	12.68	14.02	14.63	14.59	14.97	15.28
	30	10.98	11.16	11.80	13.93	15.02	15.60	17.12	17.96	19.63
Fusarium moniliforme	15	11.10	11.32	11.82	12.67	13.23	13.96	14.98	15.43	15.52
	30	11.26	11.23	12.90	14.20	14.53	15.80	16.13	17.03	18.77
Rhizopus nigricans	15	11.16	11.53	11.72	12.56	14.26	14.98	15.02	15.38	16.06
	30	11.32	11.98	12.01	12.70	15.97	17.43	19.53	21.50	22.80
Bacteria (gram -ve)	15	11.23	11.42	11.53	12.34	13.43	14.68	15.26	15.72	15.83
	30	11.28	11.97	12.16	14.42	16.28	17.27	18.08	18.10	20.27

Discussion

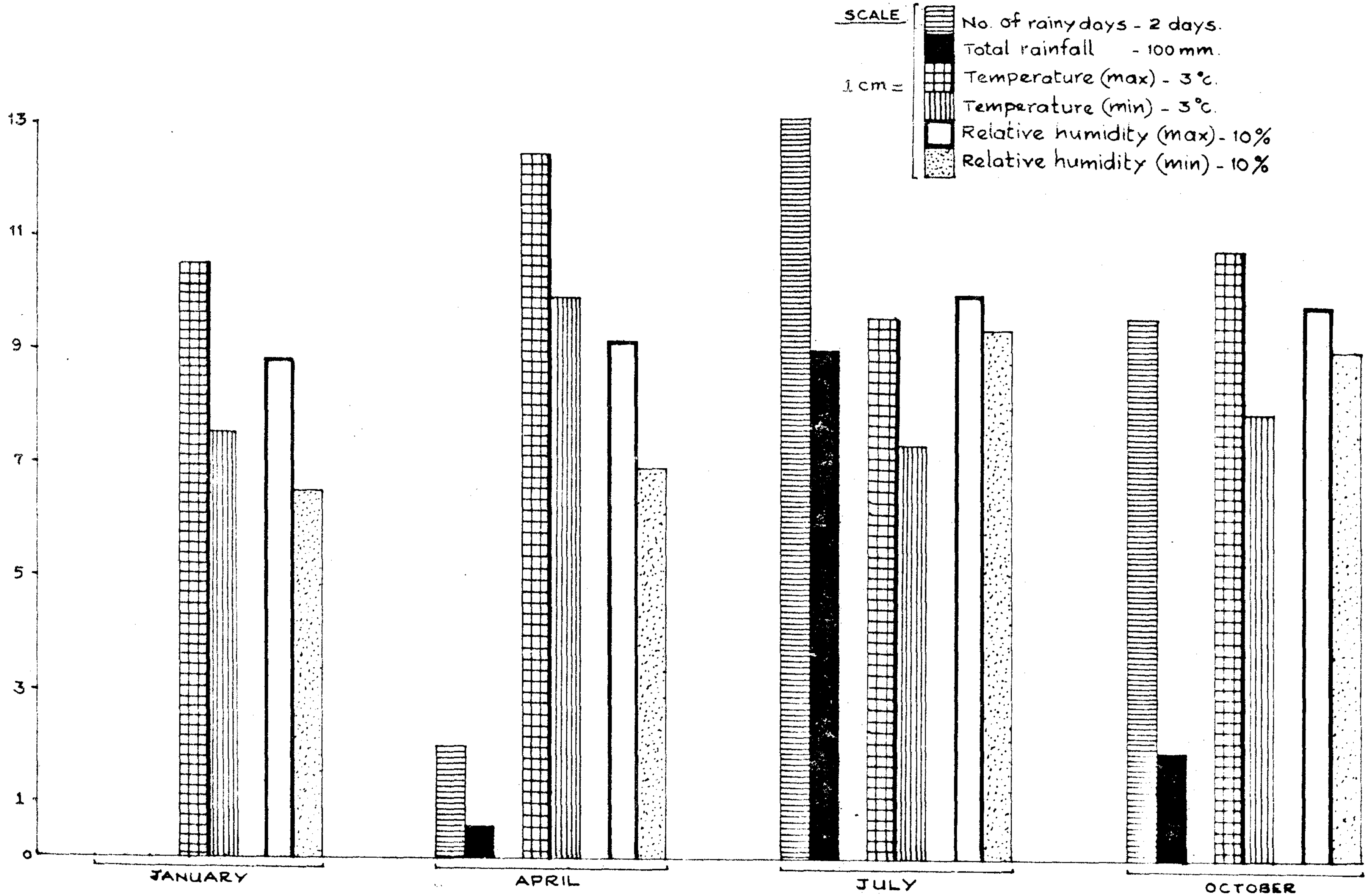
DISCUSSION

All perishable and semi-perishable agricultural commodities are usually subjected to considerable spoilage by microflora which leads to the deterioration of the quality of the products. The deterioration of the quality of perishable commodities is very quick when compared to the semi-perishable ones. Usually, the quality deterioration of semi-perishable products due to the infection by micro-organisms in storage is a slow process. The black pepper, which is semi-perishable in nature, is also subjected to quality deterioration in storage due to the attack of different micro-organisms and this aspect has not been studied in detail so far. In the present investigation, an attempt has been made to study the storage microflora of black pepper and the resultant deterioration in quality.

It is a well recognised fact that the moisture content of any commodity has great influence on the growth of microflora leading to its spoilage. In this study, different grades of stored black pepper samples were drawn during different periods of the year. It is so done because the climatic factors especially relative humidity and temperature in Kerala are varying from season to season, and are important as far as storage of any dried product is concerned.

Among the periods, January is considered to be the cool dry period of the year. As evident from Table 1, the main features of this period are very low relative humidity and comparatively lower temperature during day and night (Fig. 1). The results of moisture estimation of different grades of stored black pepper (Table 2) revealed that the moisture content ranged from 10.75 per cent (garbled pepper) to 11.67 per cent (light pepper) during this period (Fig. 2). But the samples drawn during April have shown slight decrease in moisture content irrespective of the grades and the range was from 10.19 per cent (garbled pepper) to 10.76 per cent (ungarbled pepper). The obvious reason for this lower moisture content is that this period turned to be the hottest part of the year eventhough there was a slight incfease in the relative humidity. The day and

Fig. 1 - WEATHER DATA (MONTHLY AVERAGES) DURING THE PERIOD OF STUDY.



night temperature of April was recorded to be the maximum and consequently the moisture content of the stored black pepper was also reduced.

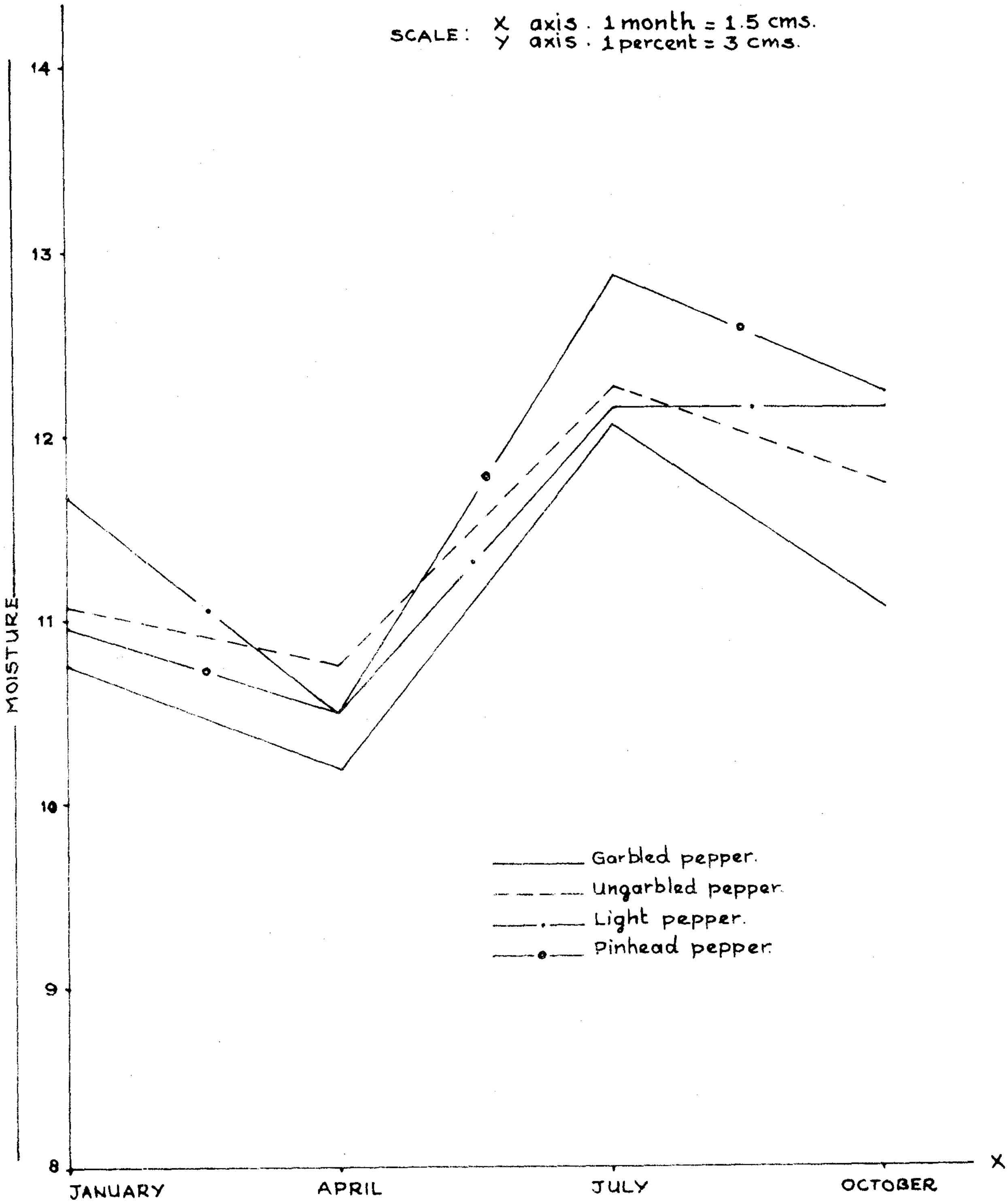
The month of July is often coincides with the peak of South West monsoon and resulting in continuous down pour. During the year of study also, the day and night temperatures came down and the relative humidity reached its saturation levels in July. When compared to other periods of the year, this period could be designated as the cool moist period, and the moisture status of the stored black pepper during this period ranged from 12.08 per cent (garbled pepper) to 12.87 per cent (pinhead pepper), evidently showing much increase. The maximum moisture content of the stored black pepper was observed during this period.

During October, the north east monsoon is active and gives very heavy showers occasionally. Normally, continuous rainfall is not obtained during this period. During the period of study also, the relative humidity showed a wide range, with an increased temperature during day and night in October and it was considered as the warm moist period of the year. During this period, the moisture content of stored black pepper ranged from 11.8 per cent (garbled pepper) to 12.23 per cent (ungarbled pepper) showing a decrease when compared to July. However, this moisture status was higher than that of cool dry period (January) and warm dry period (April).

From these observations, it is clearly understood that the moisture status of stored black pepper was well influenced by the temperature and the level of relative humidity of the atmosphere.

A perusal of Tables 3,4 and 5 reveal the fact that there was a slight decline in the major quality constituents of black pepper viz., oleoresin, piperine and starch, during different seasons studied. The possible reason that could be attached to this finding is the influence of fluctuating moisture status on the chemical constituents. In all the grades analysed, oleoresin, piperine and starch contents were found to be the maximum during the cool dry period of the year (January). Over and above the lower moisture content that helped to keep up the quality constituents, the fresh nature of black pepper during the period might have also contributed

Fig. 2 - MEAN MOISTURE CONTENT (PERCENTAGE) OF DIFFERENT GRADES OF STORED BLACK PEPPER DURING DIFFERENT SEASONS.



to such a result because this agricultural produce generally reaches the market in January, after its harvest and drying during December-January.

The major quality constituents were found to be the minimum during the warm dry period (October). From the data, it was very much evident that the length of storage period coupled with the fluctuations in moisture status contributed to the reduction in the important chemical constituents of the stored black pepper.

A cursory observation of Tables 6 and 7 reveals that the ground samples of all grades of black pepper in all seasons yielded more microbial growth when compared to the whole samples. This might be due to the increased surface area of the black pepper when it was ground. This finding is in conformity with the earlier works of Moreno Martinez (1970) and Martinez and Christensen (1973).

There was much variation in the population of storage microflora in different grades of black pepper. But no difference existed in the type of microflora yielded during different seasons in different grades of the product. The maximum population was observed in light pepper both for whole and ground samples and the minimum was in the garbled pepper for whole sample and in the pinhead pepper for ground sample. The garbled pepper, being more bold in size and having less moisture per cent, which is prepared after washing and drying to remove external moulds, has naturally yielded less microbial growth. But in pinhead grade, as the size is very small, the increase in suface area through grinding was lesser than other grades and the microbial growth was less in the ground sample.

The moisture content of the stored commodities has another important role of influencing the growth of storage microflora leading to the deterioration of the quality of the produce. For each species of the storage microflora, a minimum level of moisture content is required below which the micro-organisms cannot grow and when the moisture content is high, the microbial attack will also be increased.

These results also reveal that there was much fluctuation in the microbial population over different seasons of the year. The maximum population was found during the cool moist period (July) which had maximum moisture content and the minimum population during warm dry period (April) which recorded the minimum moisture content. These findings on the fluctuation of the population of storage microflora in different seasons thus confirm the effect of moisture content on the growth of microflora in stored products. The studies by Moreno-Martinez (1970), Martinez and Christensen (1973) and Jose (1978) on moisture content of stored black pepper have also supported this finding. Similar effect was reported in several other agricultural products by Burrough and Saucer (1971) and Christensen and Mirocha (1976).

It is a well known fact that various micro-organisms thrive well in different humidity levels. Certain micro-organisms, especially fungi, can grow well in lower humidity levels and others in higher humidity levels. This fact has been stressed in the studies of Gosh (1951), Majumdar *et al.* (1965) and Ainsworth and Sussman (1968). In the present study as shown in Table 8, under very low humidity levels ie., below 75 per cent, no fungus growth was observed except in the case of *Aspergillus* spp. However, at 75.6 per cent of relative humidity, these fungi showed a sparse growth and the growth behaviour of all other organisms varied considerably from 82.9 per cent to 96.1 per cent relative humidity. Similar results were also reported by Gosh (1951) after studying the microflora in stored rice and Majumdar *et al.* (1965) after a study on stored dry agricultural products. It was also found in the present study that *Fusarium moniliforme* and bacterium (gram -ve) required almost saturated humidity levels for their better growth behaviour. The results also indicated that if the storage period was prolonged under higher humidity levels, the growth of the micro-organism became profuse. Hence, it could be concluded from the findings that prolonged storage coupled with high humidity played an important role for the growth of micro-organisms in stored black pepper and the findings of Christensen and Mirocha (1976) support this conclusion.

In several occasions, the storage microflora have been reported to cause considerable changes in the stored agricultural produce viz., decrease in germination percentage, discolouration or damage to the embryos, whole kernals or seeds, various biochemical changes including toxin production etc., resulting in drastic reduction in quality or complete spoilage of the produce.

In this study also, the storage microflora were found to be responsible for quality deterioration of the stored black pepper, the deterioration being varied according to the changes in humidity levels and length of incubation period. All the eight fungi isolated, belonged to five genera, and the bacterium (gram -ve) were found to cause the deterioration of the quality when they were inoculated seperately. But the extent of this deterioration varied according to the species of micro-organism inoculated, the level of humidity and the period of incubation. The rate of reduction of important chemical constituents which determine the quality of black pepper viz., oleoresin, piperine and starch was also varying when the samples were inoculated with different micro-organisms.

The oleoresin content of the stored black pepper samples was found to be decreasing due to the

Fig. 3 - OLEORESIN CONTENT OF BLACK PEPPER INOCULATED WITH MICRO-ORGANISMS AT DIFFERENT HUMIDITY LEVELS AFTER 15 DAYS OF INCUBATION

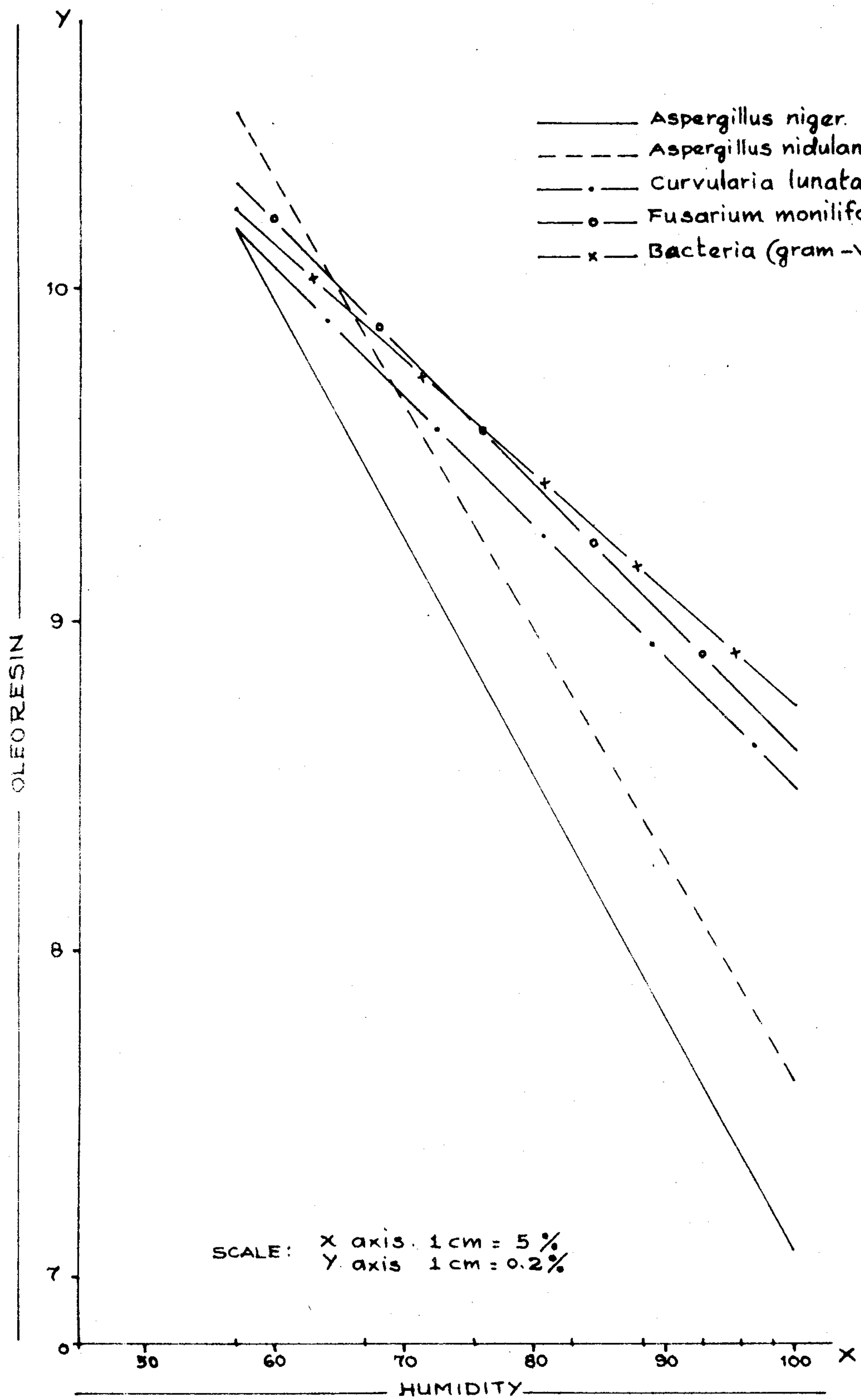
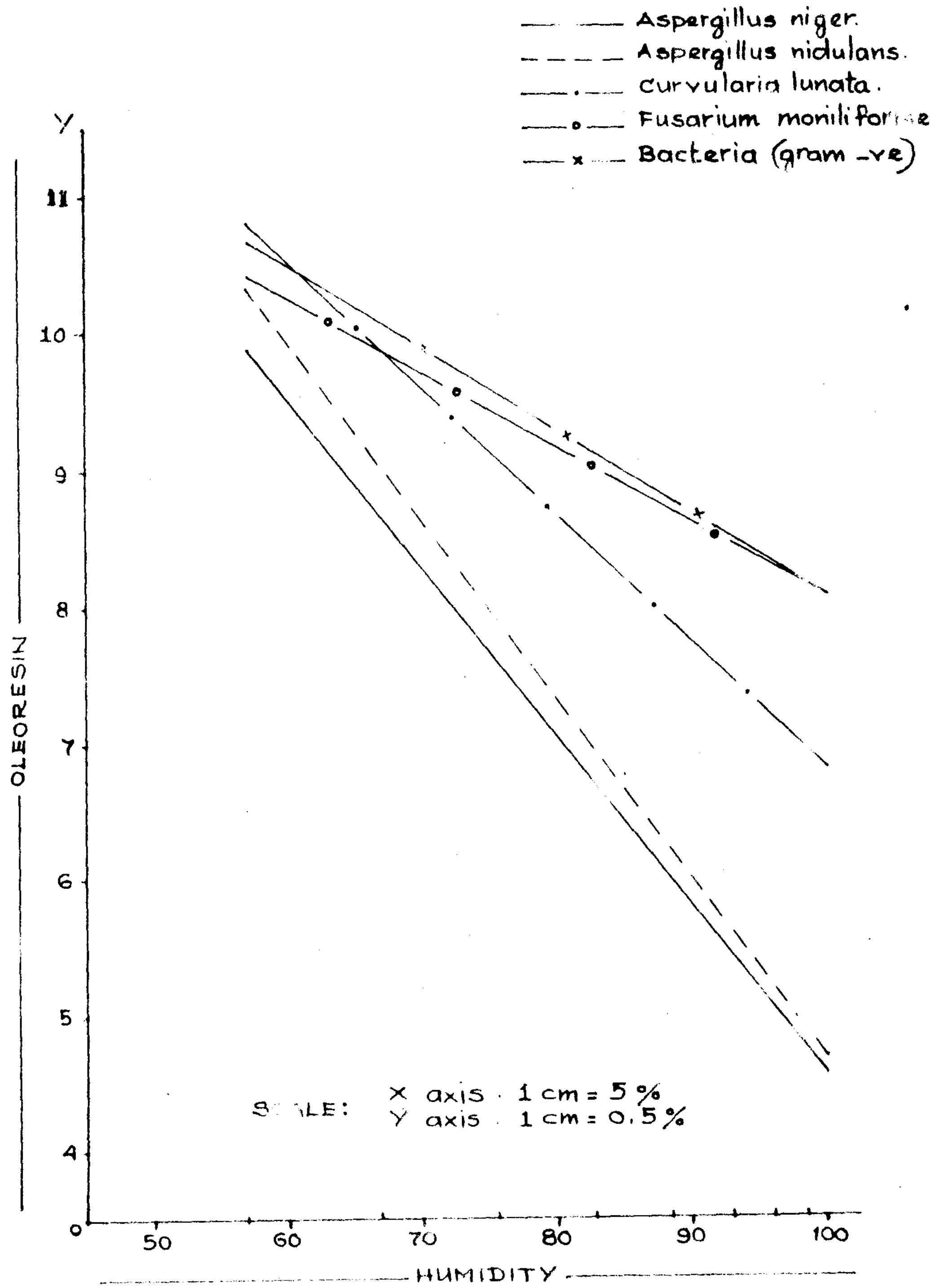


Fig. 4 - OLEORESIN CONTENT OF BLACK PEPPER INOCULATED WITH MICRO-ORGANISMS AT DIFFERENT HUMIDITY LEVELS AFTER 30 DAYS OF INCUBATION



microbial infection (Table 9). It is clear from Fig. 3 and 4 that the reduction of oleoresin content was high at higher humidity levels and it varied according to the growth behaviour of the micro-organism inoculated, showing a negative correlation between relative humidity and the oleoresin content (Table 10). The length of incubation period was also found to be influencing the reduction of oleoresin content.

Previous studies related to the reduction of oleoresin content in black pepper due to microbial attack are scanty. However, there are certain reports on the microbial influence in the reduction of oil content of copra by Eyre (1932) and Sreemulanathan and Nair (1971). Studies on groundnut and castor by Dierner and Davis (1966) and Lalithakumari <u>et al.</u> (1971) have also shown thesame trend. These observations, by and large, support the present finding that the microbial infection led to the reduction of a major quality constituent of the stored produce.

As in the case of oleoresin, the piperine content was also reduced considerably due to the microbial infection (Table 11). It is clear from Fig. 5 and 6 that the reduction rate was more pronounced at

Fig. 5 - PIPERINE CONTENT OF BLACK PEPPER INOCULATED WITH MICRO-ORGANISMS AT DIFFERENT LEVELS OF HUMIDITY AFTER 15 DAYS OF INCUBATION

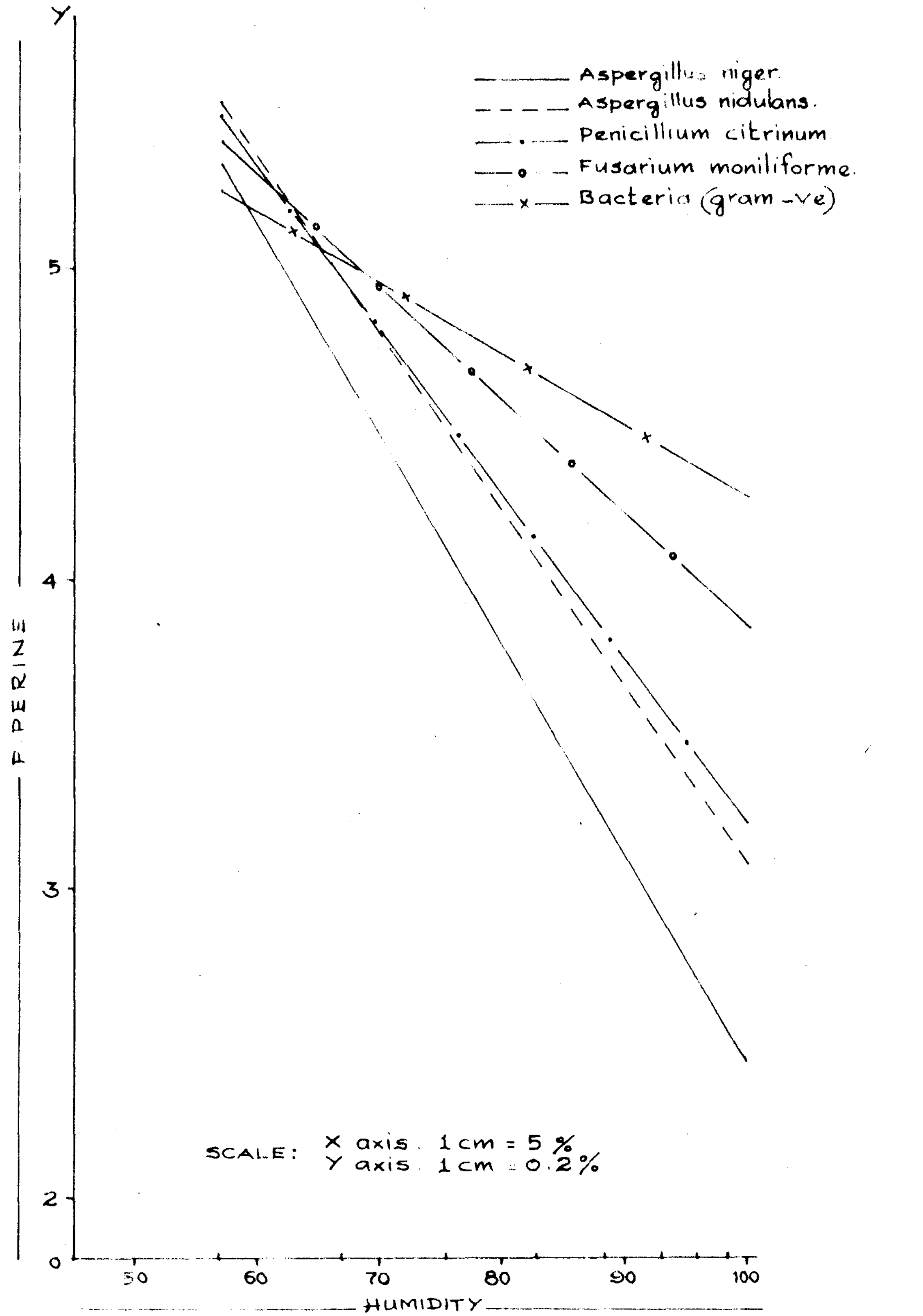
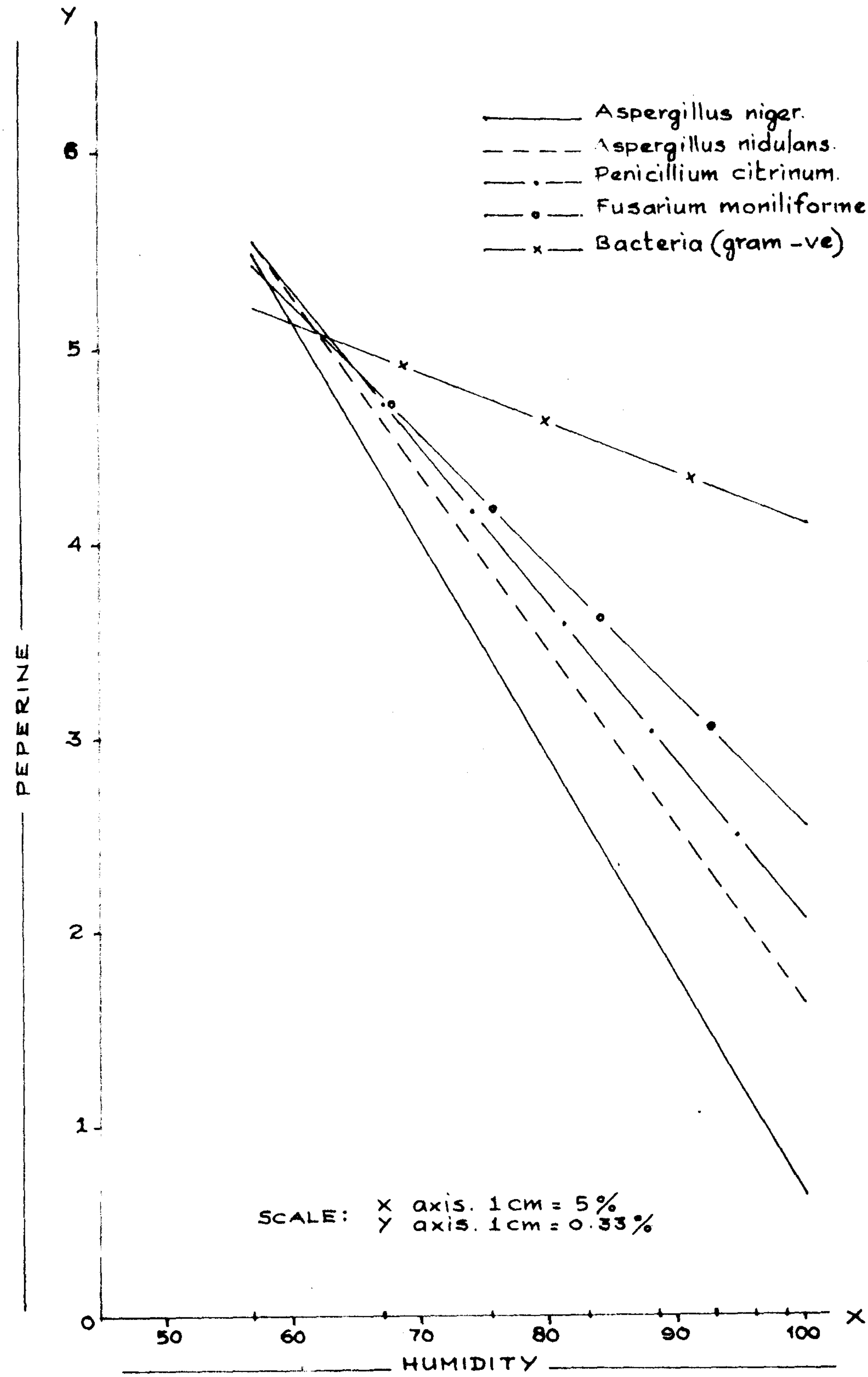


Fig. 6 - PIPERINE CONTENT OF BLACK PEPPER INOCULATED WITH MICRO-ORGANISMS AT DIFFERENT HUMIDITY LEVELS AFTER 30 DAYS OF INCUBATION



higher levels of humidity, when the microbial growth was profused. Here also, a negative correlation between relative humidity and piperine content was observed (Table 12) and the influence of the length of incubation on the reduction of piperine content was seen.

No study has been reported on the microbial infection reducing the piperine content of black pepper, as in the case of oleoresin. Piperine is an amide of piperic acid and piperidene in which nitrogen is the major constituent element. Some workers like Kiraly and Farkas (1989) and Shaw (1961) have reported that the microbial infection could alter the nitrogen content in stored products, which indirectly support the present finding.

The starch content of stored black pepper was also decreasing when the samples were inoculated with different micro-organisms (Table 13). The growth behaviour of the micro-organisms has also shown the same trend as depicted in Fig. 7 and 8, with profused growth at higher humidity levels resulting in the depletion of the starch content. Here also, a negative correlation was found between

Fig 7. STARCH CONTENT OF BLACK PEPPER INOCULATED WITH MICRO-ORGANISMS AT DIFFERENT HUMIDITY LEVELS AFTER 15 DAYS OF INCUBATION

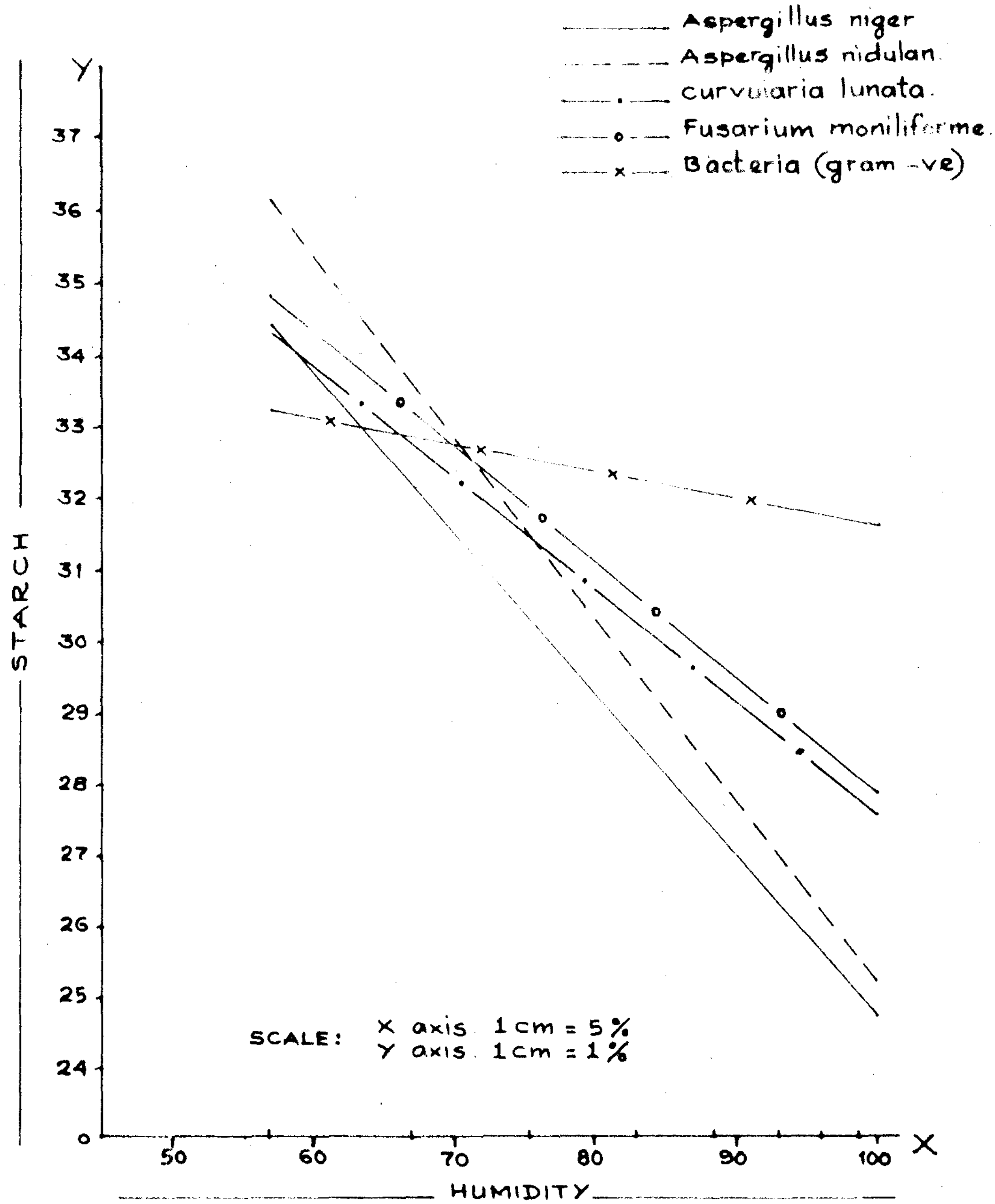
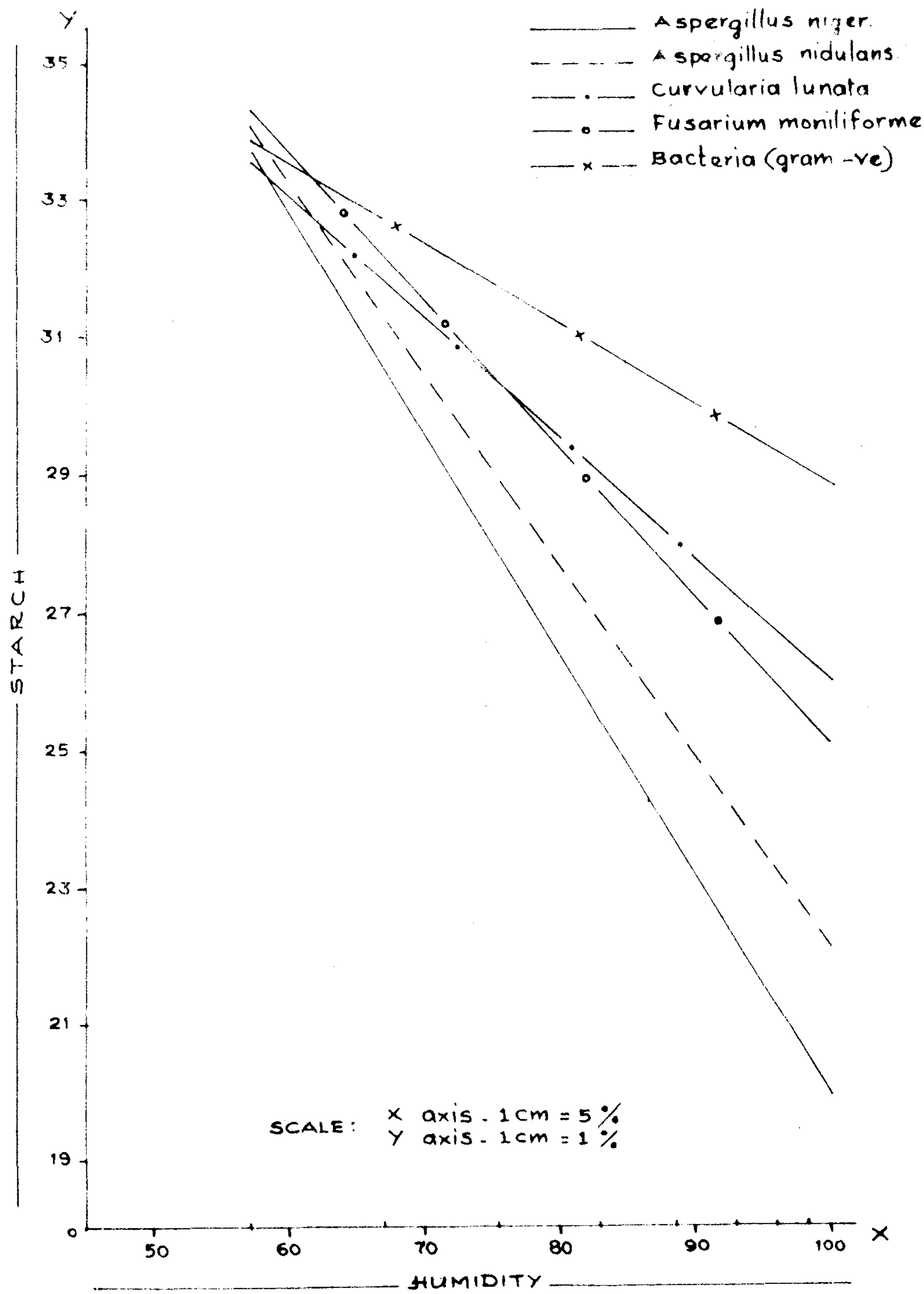


Fig 8 - STARCH CONTENT OF BLACK PEPPER INOCULATED WITH MICRO-ORGANISMS AT DIFFERENT HUMIDITY LEVELS AFTER 30 DAYS OF INCUBATION



the relative humidity and the starch content of black pepper (Table 14).

More reduction of starch was seen in the case of fungal flora than the bacterial infection and there was a direct influence of the length of incubation on the reduction of starch content.

No previous study could be located on the reduction of starch content of black pepper due to microbial infection. However, there are certain reports on the reduction of starch content by microbial attack in some other agricultural produce. Inman (1965) in his study on barley and Kochubabu (1976) in his study on sorghum found this type of starch depletion due to microbial infection, and the findings of the present study has been supported with those reports.

It is logical to expect that the reduction of all those chemical constituents viz., oleoresin, piperine and starch might have caused by the activity of enzymes produced by the storage microflora. Goodman and Christensen (1952) and Dirks (1954) reported on the lipase activity of storage fungi on wheat grains. A similar study by

Vidyasekaran *et al.* (1966) revealed the production of cellulase, pectin methyl esterase, polygalacturonase and protopectinase in the culture filtrates of *Fusarium moniliforme* on paddy seeds. Aujla *et al.* (1976) reported the production of polygalacturonase by *Aspergillus flavus* and pointed out the quality deterioration by the activity of enzyme. As shown in these studies, here also, the enzymatic activity might have resulted in the reduction of the chemical constituents, deteriorating the quality of stored black pepper.

The present study clearly indicate that the dried black pepper should be stored in a cool dry place. The black pepper can absorb moisture from high humidity levels in the atmosphere, which leads to the growth of storage microflora and in turn in the reduction of the quality of black pepper.

Summary

SUMMARY

An investigation on the microflora of stored pepper was conducted at the College of Horticulture, Vellanikkara during 1979-80. The observations and conclusions of this investigation are summarised as follows:

1. There are four distinct seasons for the year in Kerala, which can be categorised as cool dry period (January), warm dry period (April), cool moist period (July) and warm moist period (October).

2. The moisture content of stored black pepper varied in different seasons and the maximum moisture content (12.08 to 12.87 per cent) was observed during the cool moist period (July) and the minimum (10.19 to 10.76 per cent) during the warm dry period (April) for the different grades of black pepper collected for the study.

3. The major chemical constituents of stored black pepper namely, oleoresin, piperine and starch were found to have slight variations in different seasons.

4. Among the different pepper grades, light pepper yielded maximum oleoresin content (12.10 per cent) and the minimum was in pinhead pepper (4.53 per cent). The highest oleoresin content in garbled pepper was 10.92 per cent whereas it was only 9.93 per cent in ungarbled pepper.

5. In all the grades of black pepper, maximum oleoresin content was found in cool dry period (January) and the minimum in warm moist period (October).

6. The maximum piperine content (5.41 per cent) was observed in the garbled grade and the minimum (1.00 per cent) was in pinhead pepper. The ungarbled and light pepper grades yielded 5.06 per cent and 4.23 per cent piperine content respectively.

7. The lowest piperine content in all the grades was observed in warm moist period (October) and the maximum in cool dry period (January).

8. The garbled grade of black pepper contained maximum starch (35.63 per cent) whereas the pinhead grade had the minimum (13.51 per cent) starch content.

9. During the cool dry period (January), the starch content was found to be the maximum in all the grades

and during warm moist period (October) to be the minimum.

10. In all the seasons, association of microflora was observed with different grades of stored black pepper and there was no change in the type of micro-organisms. Only the population of each micro-organism was found to be varying in different grades in different seasons.

11. The ground pepper samples showed more microbial population than the whole samples of different grades of black pepper.

12. The microbial population was minimum in whole samples of garbled pepper and it was maximum in ground samples of light pepper.

13. It was found that eight species of fungi, belonging to five genera, and one gram -ve bacterium were associated with all the grades of black pepper in all the seasons of the year. The fungi isolated and identified were four species of Aspergillus namely, Aspergillus niger, A. candidus, A. nidulans and A. versicolor and Curvularia lunata, Penicillium citrinum, Fusarium moniliforme and Rhizopus nigricans.

14. It was found that all the species of _Aspergillus_ and _Penicillium citrinum_ could grow even at a lower humidity level (75.6 per cent) whereas all other micro-organisms were growing well only at higher humidity levels. There was no growth of microflora upto 66.8 per cent relative humidity.

15. When the micro-organisms were inoculated individually at different humidity levels, the quality deterioration of black pepper was very conspicuous.

16. Oleoresin, piperine and starch content of black pepper reduced considerably when the samples of different grades were inoculated with different micro-organisms, at various humidity levels.

17. Relative humidity was found to be negatively correlated with oleoresin, piperine and starch content of black pepper, when the samples were inoculated with different micro-organisms. Similar relationship was observed between the period of incubation and the quality aspects also.

18. When the dried black pepper was exposed to higher humidity levels, it absorbed the moisture which in turn enhanced the microbial activity.

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STUDIES ON THE MICROFLORA OF STORED PEPPER

BY

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ABSTRACT OF THE THESIS

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ABSTRACT

With a view to study the microflora in stored black pepper, a research project was carried out at the College of Horticulture, Vellanikkara. It was also aimed at estimating the deterioration of the quality of stored black pepper in terms of its oleoresin, piperine and starch contents due to microbial infection and assessing the role of each micro-organisms in changing the quality of the product.

The study revealed that the major chemical constituents of stored black pepper, namely, oleoresin, piperine and starch varied in different grades of black pepper. Slight variations in these quality constituents were observed according to the seasons of storage also.

In all the seasons, association of microflora with all grades of black pepper was observed. The species of micro-organisms were not changed during seasons, but the population varied according to grade of black pepper and season of storage. The micro-organisms found were *Aspergillus niger*, *A. candidus*, *A. nidulans*, *A. versicolor*,

Curvularia lunata, *Penicillium citrinum*, *Fusarium moniliforme*, *Rhizopus nigricans* and Bacterium (gram -ve).

There was no growth of microflora in stored black pepper upto 66.8 per cent relative humidity, whereas profused growth was observed at saturation levels of humidity. Only *Aspergillus* spp. and *Penicillium citrinum* could come up at a lower HUMIDITY LEVEL (75.6 per cent).

In three quality constituents of black pepper viz., oleoresin, piperine and starch reduced considerably when the samples were inoculated with different micro-organisms at different levels of humidity. Reduction in the quality constituents was found corresponding to the increase in level of humidity as well as length of incubation period.