motor. The weight of this device is approximately 1 kg and dimensions are 220 x 150 x 64 mm.

IV 4.3 Bruisometer Two

The variable force for this device could be applied using 13 block masses each of 15 g. These block masses were located on vertical guides. They could be pinned away from the test-head or positioned on the test-head. The mass variation on the test-head ranged from 15 g (minimum) (0.147 N) and could be increased by 15 g increments (0.147 N) to a maximum of 195 g (1.92 N).

The block masses can be lowered onto the mushroom which is held on a triangular arrangement of spikes on a pivot-bed. The slip-shear treatment is delivered by the weight of the block masses while the mushroom pivots over a range of 50^{o}. The pivot-bed is operated by a battery-driven motor and gear set. This device was fitted with a control circuit to operate the speed and direction of the pivoting action. The weight of this device is approximately 2.6 kg and the dimensions are 115 x 235 x 265 mm.

IV 4.4 Future Use and Development of the Bruisometers

Two bruisometers, of different design, were released to HRI in May and June 1998. Bruisometer Two was demonstrated to the HDC Mushroom Panel at a meeting on 20 May when it was able to distinguish 'bruisability' between first and third flush mushrooms and between mushrooms of different strains. These bruisometers should be viewed as prototypes which will require validation and design improvements before commercialisation or use in experimentation.

Bruising is a big quality problem for the industry. This represents the first opportunity to measure bruisability and therefore to find out which agronomic and environmental factors lead to high bruisability.

HDC have agreed to fund a project on the 'Validation of the Mushroom Bruisometer' (M 19a). Furthermore, Kerry Burton of HRI, will be working with two more design teams from Coventry University, starting October 1998, looking at improvements in both the mechanical and industrial design.

IV.5 Discussion

It has been found that the mushroom stiffness is dependent on the fresh matter as well as on the dry matter. The regression coefficients of each of them show how much impact they have on mushroom stiffness. Although mushrooms are made of 90 % water the imput of water on stiffness is low; its imput is about a seventh of the dry matter. Or in other words, the strength of the tissue is coming from about 85% from the hyphal wall structure and composition.
Although the strength is coming mainly from the dry weight, one could argue that we should take in account only the dry matter content to estimate the stiffness. But the results have shown that if only the dry weight is taken in account, the result is only 50% accurate, furthermore if the fresh weight alone is taken in account the accuracy is 52%. When both, the dry weight and the fresh weight, are considered for the estimated stiffness, the accuracy is 72%.

The analysis of the data collected in farms have shown that there is a great similarity between the firmness evaluated by the operator and the estimated stiffness measured with our equation. In some cases there were some differences in the estimated stiffness and the evaluated stiffness which is probably just a question of reference on the day of assessment. This proves that squeezing mushrooms between your fingers is very subjective and a more reliable method is needed. On the positive side no sample was evaluated firm and was found to be soft.

The purpose of the test was to have a method of measuring firmness, which would be easy to use, reliable and cheap. After a conversation with the growers, they agreed that this test is very feasible on the farm, it doesn't take very long so could be included in the quality assessment of mushrooms. It is very important to know how firm are mushrooms, as this is one of the main quality attributes of which consumers are aware. Growers will be able to evaluate the firmness of their crops and try to

B. PART V

Environment and agronomic effects

V.1 Introduction

There is anecdotal evidence that the same strain when cultivated in different farms can have different textural properties, suggesting that texture can be influenced by growing techniques. As variations in texture can not come from their genotype (considering the same strain), texture might be influenced by external factors like humidity, atmospheric gas, growing conditions and techniques. The importance of these experiments is for the mushroom growers who will be able to repeat the results on their farms. Although the growers try to optimize the growing conditions to produce high quality mushrooms, they can still lose their expected quality. By the design of these experiments, it is hoped to identify how environmental and agronomic effects influence mushroom texture and quality and how growers could adjust the growing conditions during a crop if needed.

The agronomic and environmental effects on mushroom texture and quality were tested in a series of factorial experiments:

- Storage
- Casing type x Humidity level
- Casing depth x Compost depth
- CO_2 level x Strain-cultivars
- Supplements
- Water potential x Casing type

V.1.1 Storage

Mushrooms often arrive a couple of days after harvesting on the supermarket shelves. During this time, the mushrooms have had time to continue their development. The aim of this experiment was to look at the effects of storage on mushroom texture and to see if mushroom skin, from fresh and stored mushrooms, affects mushroom texture.

V.1.2 Casing type x Humidity level

At the HRI mushroom unit, mushrooms are grown using casing based on sugar beet lime and peat. Usually, the choice of casing for growers is based on the availability of the products, often waste from industries or local products are used. For that reason, the formulation of casing varies from areas and countries. The optimum relative humidity of the growing rooms is about 85 %, at high humidity diseases could proliferate (Brown blotch, Trichoderma, verticillium...) and at low humidity the mushroom yield is reduced and mushrooms dry out. Nevertheless, mushrooms were grown at these 3 humidity levels and tested for the quality. The aim of the experiment was to see if casing mixture and the growing room relative humidity could affect mushroom texture and quality.

V.1.3 Casing depth x Compost depth

Mushrooms are grown on compost and casing depth which vary from farm to farm because it depends on which casing and compost are used. The compost and casing amount used for growing mushrooms has an effect on the amount of nutrients available, the water availability and the fruit body induction. Experiments were designed to test how the compost and casing depth can affect mushrooms texture and quality.

V.1.4 CO_2 level x Strain-cultivars

There are many *A. bisporus* strains available from the Spawn Company. Each strain has it own characteristic and mushrooms produced have different quality and textures. The quality properties of four strains were tested in this experiment. Mushrooms usually grow at HRI in rooms were the CO_2 level is around 1000 ppm. A lower level of 800 ppm and a higher level of 1200 ppm were set up in the growing rooms to look at the effect of CO_2 level on mushroom texture and quality.

V.1.5 Supplements

Manufacturers claim that adding supplements during the preparation of compost can increase the mushroom yield. In this experiment three commercial supplements, added at two different rates, were tested to determine their effect on texture and quality.

V.1.6 Water potentials

The major compound in mushrooms is water, which represent 85 to 92 % of the weight. Mushrooms can lose up to 10 % water after one day's storage at 15-21°C (Gormley *et al.*, 1967) and this can affect their texture. Water also has a great influence on discoloration. It is well known that wet mushrooms, when harvested, are more susceptible to discoloration. An experiment was designed to study mushroom texture and discoloration on mushrooms grown on casing of variable water potentials.

V.2 Materials and methods

V.2.1 Growing conditions and agronomic effects

V.2.1.1 Storage

Mushrooms were harvested and stored 1, 2, 3 and 4 days at 18°C under a relative humidity of about 90% before being tested.

V.2.1.2 Casing types

- French style: based on 25% peat and 75% of oolitic limestone from Smiths limestone, Broadway, Gloucester. The limestone is a mixture of 2/3 of larger ground size plus 1/3 of smaller ground size limestone. The final preparation has a yellow-beige colour and has the consistency of moist ready-mix concrete.
- Peat based casing: consists of Irish Spagnum peat supplemented with chalk (1 vol of chalk for 12 vol of peat). It has the consistency of porridge with a light brown colour. This casing is used in Ireland.

- English style: consists of Nooyen ready mix (80% peat, 20% sugar beet lime) which has a dark colour and a consistency of fibrous mud, for more details see II.
- Bulk peat based casing: consists of a mixture of sugar beet lime (25 %) and bulk peat (75 %).
- Mill peat based casing: consists of a mixture of sugar beet lime (25 %) and mill peat (75 %).

V.2.1.3 Humidity of the growing chambers

Mushrooms were grown at a standard relative humidity of 85 %. An experiment was designed to determine the effects of a lower and a higher relative humidity (75 and 95 %) on mushroom texture.

Error! Bookmark not defined.**V.2.1.4 Casing and compost depths**

Mushrooms were grown under standard conditions on compost and casing of three different depths. Trays were filled with 3 different amounts of compost: shallow, medium and deep which represent 4.5, 10.5, 19 kg of compost respectively. The trays were then covered with a sugar beet lime casing of a depth of: 25, 40 or 55 mm.

V.2.1.5 Compost supplements

During the preparation of compost for mushroom cultivation, the supplements Betamyl, Springboard or Promycel were added at a rate of 0.5% and 1% (w/w).

V.2.1.6 Water potential in casing

First, the moisture content of the casing was measured on a weight basis. Then the water potential was determined from a calibration curve for water potential and moisture content. The water potential of each casing is summarised in the table below. The potential is expressed in mm of water. A low water potential is equivalent to a wet casing and a high potential to a dry casing.

Water Potential	P1	P2	P3	P4	P5	P6
Bulk peat	1.17	250	478	569	963	1107
Mill peat	215	263	381	747	1251	1551

V.2.2 Mechanical tests performed on in each growing condition and agronomic effect experiment

V.2.2.1 Storage experiment

Whole mushrooms with the stipe removed were compressed at a displacement of 1 mm. Tests were performed on top and side of the sporophore with the skin retained or removed. Skin was dissected from the cap by peeling from the edge to the centre. The compression tests were performed on freshly harvested mushrooms (day 0) and on mushrooms stored for 1, 2, 3 and 4 days at 18°C.

The experiment had a factorial design with the following number of treatments: 2 positions (tops or sides) x 2 tissues (skin retained or removed) x 5 storage days (day 0, 1, 2, 3 and 4). For each treatment combination, 15 mushrooms were examined. The data were statistically analyzed by analysis of variance.

V.2.2.2 Casing type x humidity experiment

Mushrooms were grown in chambers under 3 different relative humidity: 79%, 85% and 95%. Mushrooms from the first three flushes of each treatment were harvested and cubes of mushrooms were compressed at a displacement of 1 mm. The total energy supplied and the plastic deformation were recorded.

The experiment had a factorial design with the following number of treatments: 3 casing type x 3 humidity. For each treatment combination, 10 mushrooms were tested. The data were statistically analyzed by analysis of variance.

V.2.2.3 Casing depth x compost depth

Stage 2 mushrooms were harvested and cubes were taken of for compression at a displacement of 4 mm. The total energy supplied and the plastic deformation were recorded.

The experiment had a factorial design: 3 compost depth levels (Shallow, Medium, Deep) x 3 casing depth levels (25, 40, 55). For each treatment combination, 10 mushrooms were tested. The data were statistically analyzed by analysis of variance.

V.2.2.4 Mushroom strain x CO_2 Level experiment

Mushrooms, strain A12, U3, S130 and U1 were grown under 3 different levels of $C0_2$: 800, 1000 (usual level) and 1200 ppm. Cubes of mushrooms were taken for compression at a displacement of 4 mm. The total energy supplied and the plastic deformation were recorded.

The experiment had a factorial design: 4 strain types x 3 CO_2 levels. For each treatment combination, 15 mushrooms were tested. The data were statistically analyzed by analysis of variance.

V.2.2.5 Supplements experiment

The stiffness of the mushrooms was measured on cylinders removed from the top in the vertical orientation, 2 mm underneath the surface (see III.).

The experiment had a factorial design: 3 supplement types x 2 rates. For each treatment combination, 10 mushrooms were tested. The data were statistically analyzed by analysis of variance.

V.2.2.6 Water potential x casing type

The stiffness of the mushrooms was measured on cylinders removed from the top in the vertical orientation, 2 mm underneath the surface (see III.).

The experiment had a factorial design: 6 water potential levels x 2 casing types. For each treatment combination, 10 mushrooms were tested. The data were statistically analyzed by analysis of variance.

V.2.3 Bruise area calculation

Cubes of mushrooms were compressed according to the method described in III. The cubes were stored for 2 days at room temperature and then, a slice of about 2 mm thick was cut across the compression. The slices were placed on a U.V. transilluminator (UVP, Inc., San Gabriel, USA) to reveal the bruise area caused by compression, and photographic records were taken with a Mitsubishi video copy processor. The bruise area was then measured by image analysis.

V.2.4 Dry weight

Cylinders taken from the mushroom caps for stiffness measurements were allowed to dry for one week at room temperature.

V.2.5 Tyrosinase activity

The skin of the sporophore was obtained by peeling the mushroom from the side to the centre. The skin was rapidly frozen in liquid nitrogen and stored at -18°C. Samples were ground and homogenised in 7 ml 100 mM sodium phosphate buffer, pH 8.0. The extracts were centrifuged for 20 min at 35,000 g and the supernatant was tested spectrophotometrically for tyrosinase activity in 10 mM catechol, 10 mM proline in 100 mM sodium phosphate buffer pH 6.0. The activity was measured at 525 nm at 30°C by measuring the initial slope of increase in absorbance. The activity was also measured in the presence and absence of SDS in a final concentration of 0.1% (w/v).

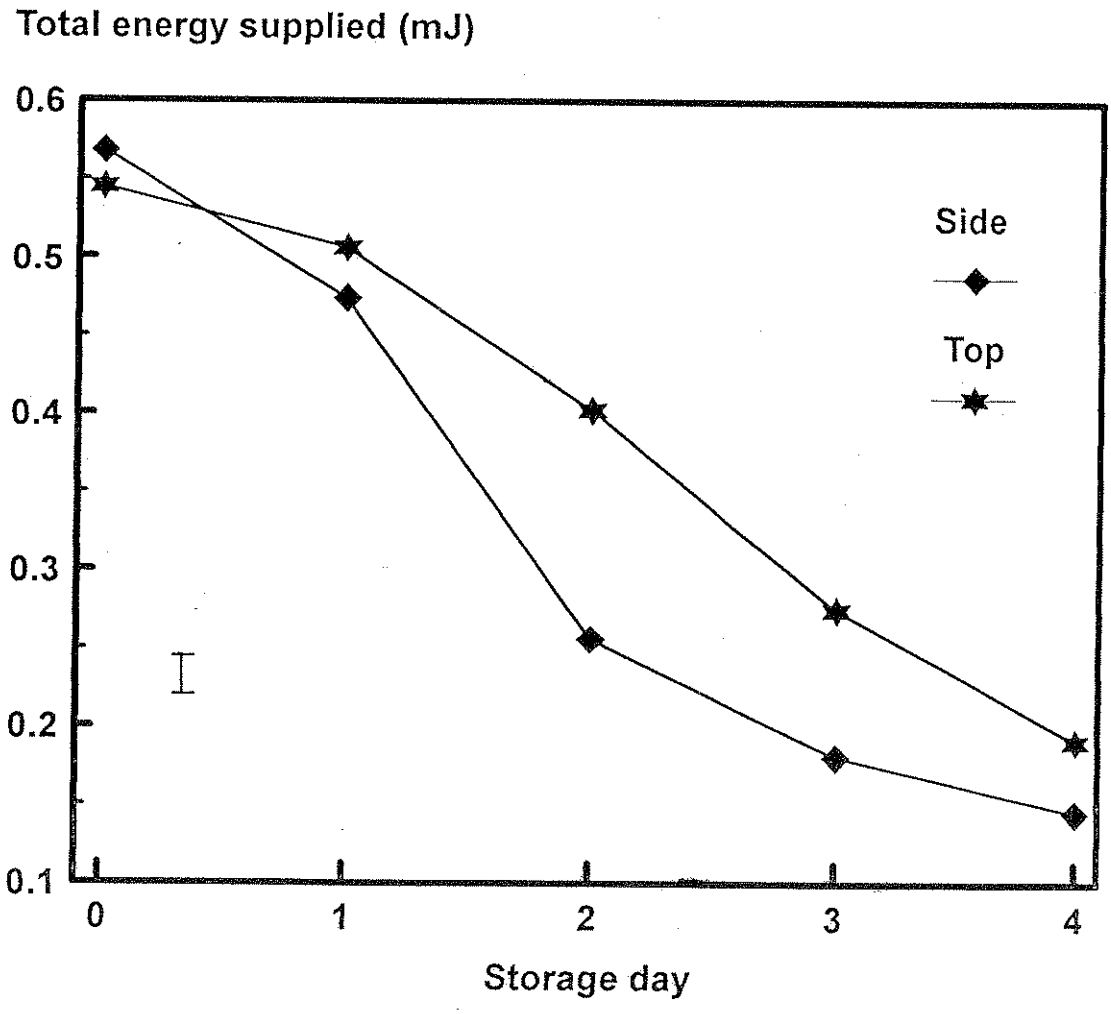
V.3 Results

V.3.1 Storage

The analysis of variance has shown that there is no significant difference in the energy absorbed to compress the mushroom tissue with or without the skin. However a significant difference ($p = 0.001$) was identified between the top and the side of the mushroom under compression (Figure V.1). When the results over the storage period

are combined, the top of the mushroom cap was found to require 18% more energy (i.e. firmer) to be compressed by 1 mm than the sides ($p = 0.001$). However, the analysis of variance reveals that the energy to compress either tops or sides of fresh mushrooms (day 0) is approximately the same. During storage, the energy required decreased for both tissues, but the decline was significantly more rapid in the sides than the tops ($p = 0.001$). A decrease of 55% for the side and 26% for the top after 2 storage days has been found.

Figure V.1: Total energy supplied on fresh and stored mushrooms



V.3.2 Casing x Humidity

Mushrooms from flush 1 were the most plastic (i.e. most liable to permanent deformation) followed by flush 3 and then flush 2 (the mushrooms most resistant to deformation). The sides of the mushrooms were found to have significantly ($p < 0.001$) higher plasticity (i.e. softer) than the tops. When french style casing was used the difference between tops and sides was more pronounced (Figure V.2).

Similarly, the analysis of variance of the energy required for the probe to compress the tissue by 1 mm, found no significant differences between humidity or casing treatments (Figure V.3). Flush 2 mushrooms were found to require significantly ($p < 0.001$) more energy (i.e. therefore firmer) than mushrooms in flush 3 follow by flush 1. Also analysis of the energy requirement showed that sporophore tops required more energy (i.e. firmer) than the sides (Figure V.4).

The analysis of variance showed that there are no significant differences in tyrosinase activity between the humidity treatments and the casing type treatments in presence or absence of SDS.

V.3.3 Casing depth x Compost depth

The analysis of variance of plasticity data showed that the main treatment effect of plasticity come from casing depth ($p<0.001$). The 25 mm casing treatment had the lowest plasticity (most resistant to permanent deformation) followed by 40 mm treatment and then 55 mm treatment (Figure V.5). This trend was more prominent for flush 1 mushrooms than flush 2.

The analysis of variance of the energy requirement to make the compression revealed that both casing depth and compost depth were highly significant ($p < 0.001$), Figure V.6. When examining the overall treatments, deep compost produced the highest energy requirement (i.e. firmest) followed by medium and then shallow compost. The casing treatment followed the opposite trend, the highest energy requirement (firmest) being observed with the 25 mm casing depth. Therefore, of the treatment combinations, the highest energy requirement was with mushrooms grown in deep compost and shallow casing.

Figure V.2: Percentage of plastic deformation on top and side of mushroom caps for the three casing types.

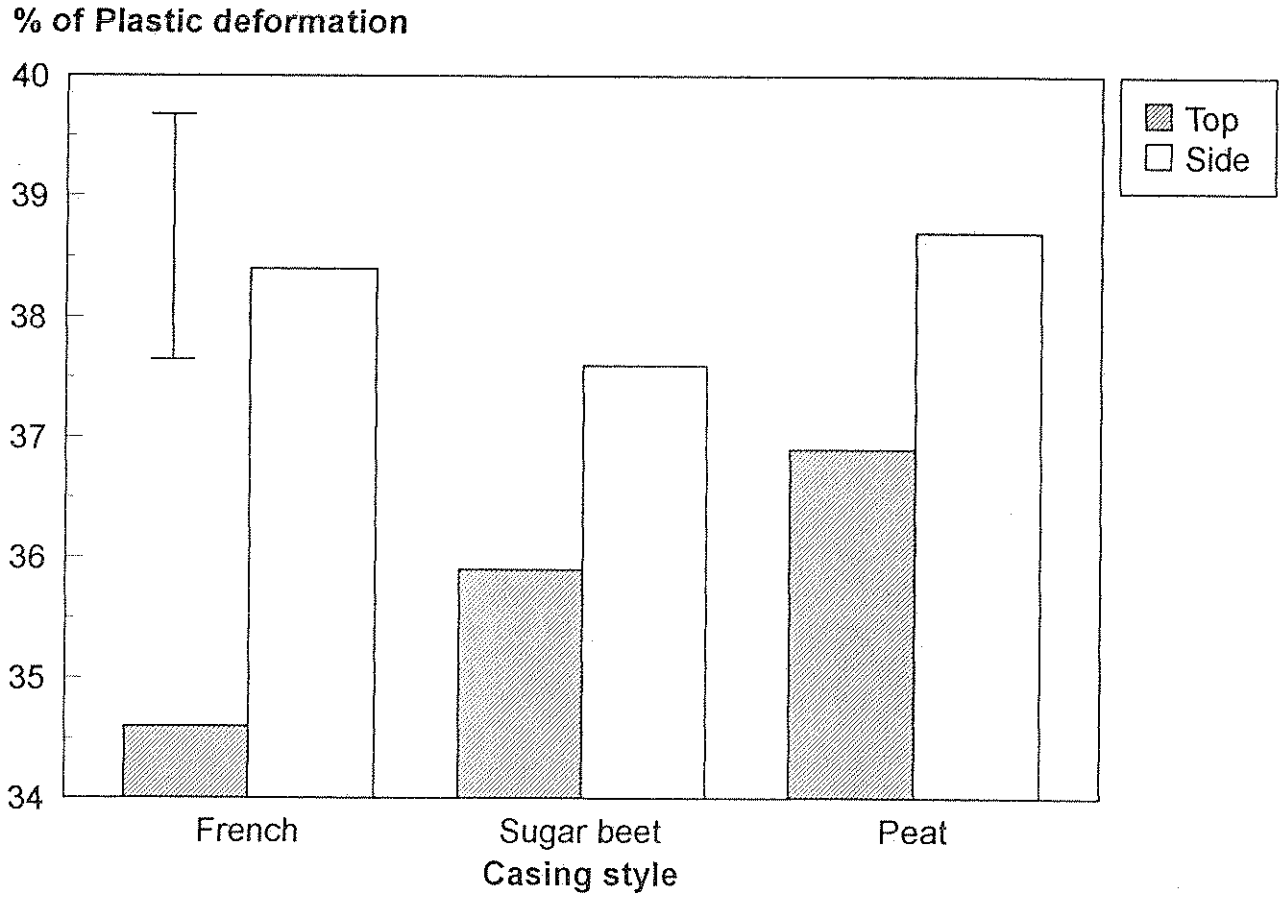


Figure V.3: Total energy supplied on top and side of mushroom caps for the three casing types

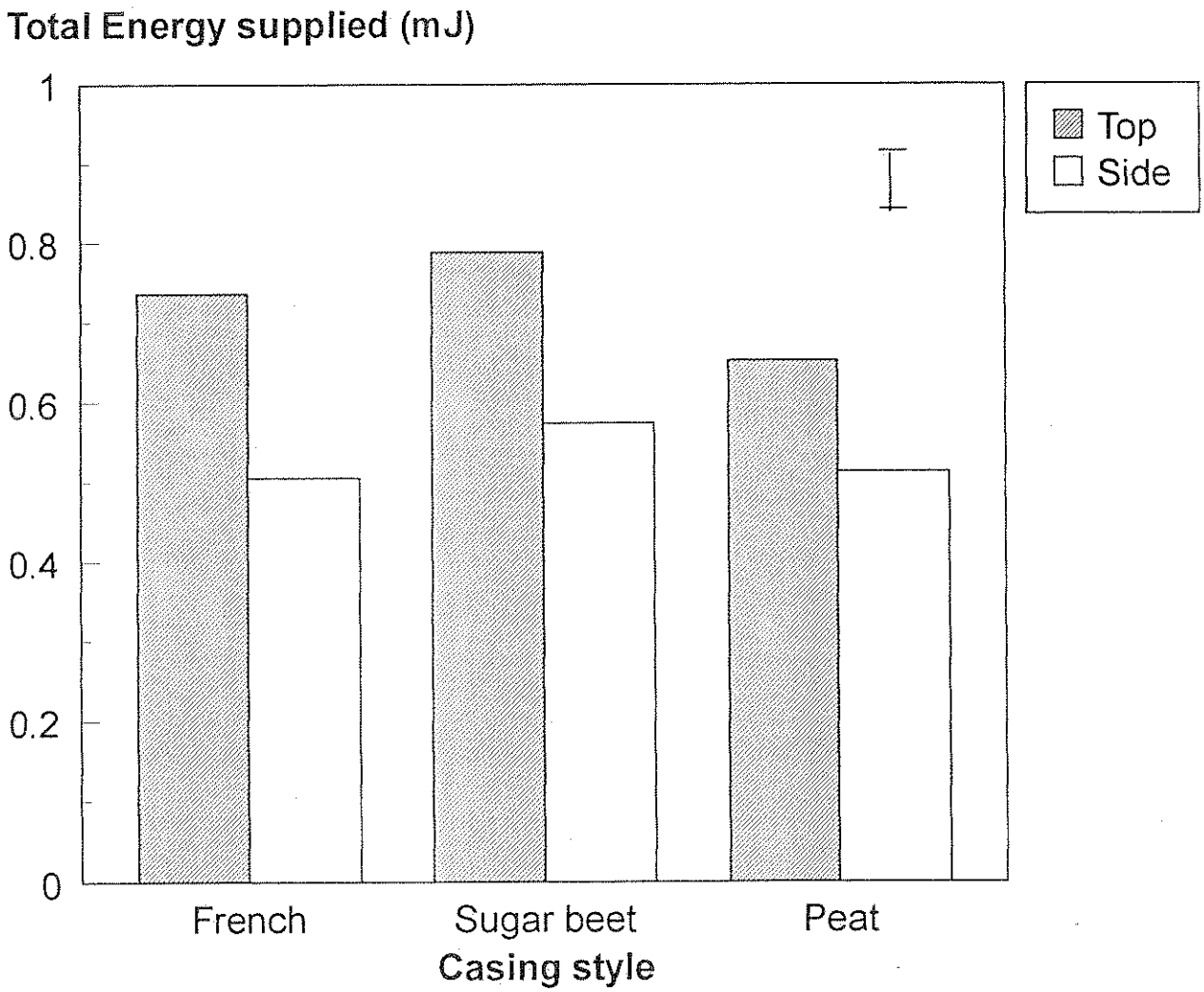
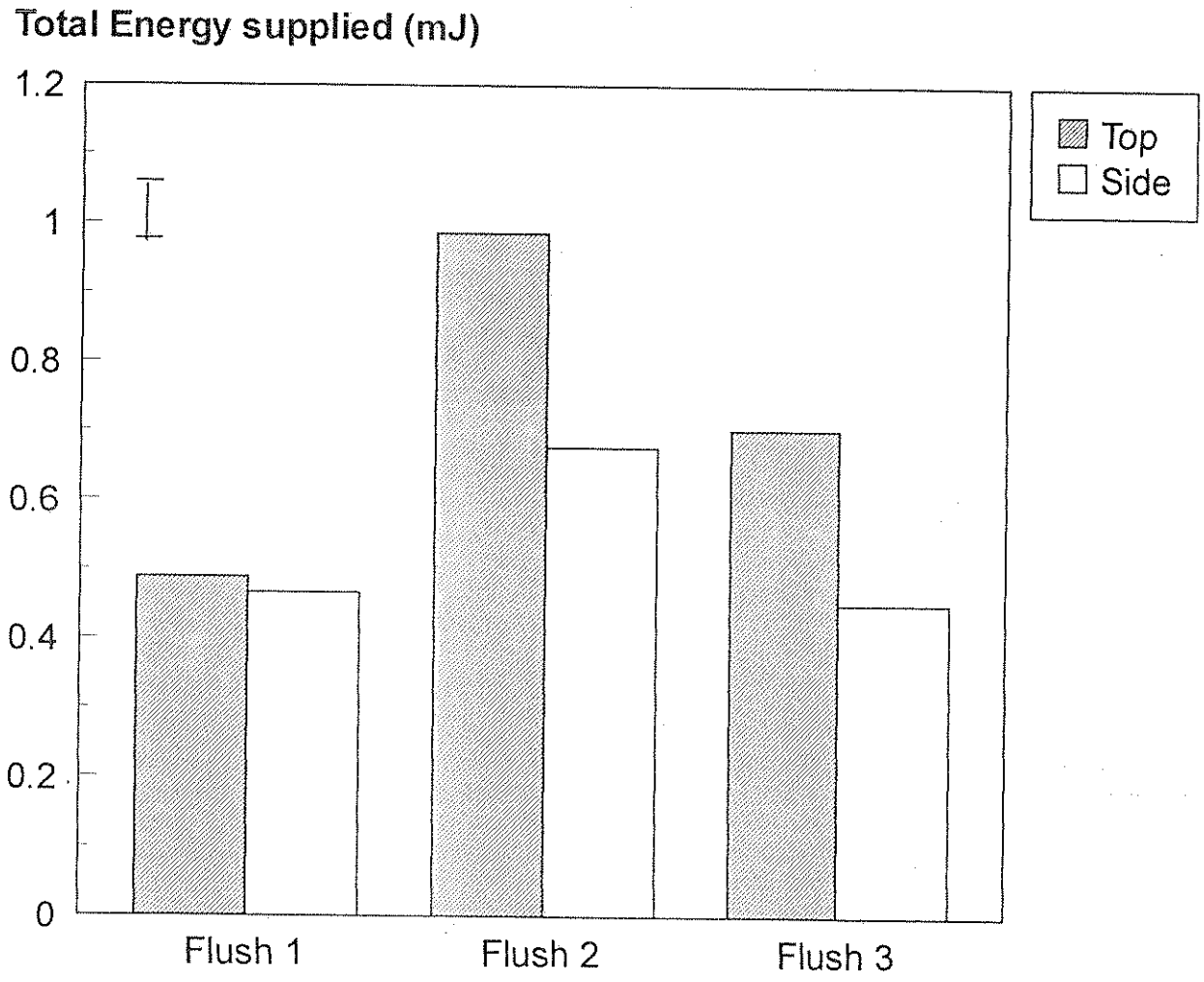


Figure V.4: Total energy supplied on top and side of mushroom caps over the first three flushes.



However, the analysis of the percentage of dry matter content showed that treatment 25D had a higher percentage of dry matter than 40M which in turn had a higher percentage of dry matter than 55S (Figure V.7).

V.3.4 CO_2 level x Mushroom strain

The statistical analysis has shown a significant difference ($p < 0.001$) in energy requirement between the CO_2 level treatment (Figure V.8). There is a trend in firmness for the three CO_2 levels. Mushrooms grown under low CO_2 level (800 ppm) had a higher energy requirement (firmer), than the mushrooms grown under medium CO_2 level (1000 ppm) which in turn were higher than the mushrooms grown at 1200 ppm which produced the softest mushrooms. Measurement of energy in the four strains has revealed a significant difference ($p < 0.1\%$). Strain U3 was found to be much firmer than strain U1, S130 and strain A12 that had approximately similar values (Figure V.10).

The analysis of variance on plasticity revealed that both strains and CO_2 levels are significant ($p < 0.1\%$). There is a trend for CO_2 level, mushrooms produced under a low CO_2 level were more plastic (less resistant to permanent deformation) than mushrooms produced under high or medium CO_2 level (Figure V.9). Strain U3 was found to be the more plastic over the first three flushes than S130, U1 and A12 which were similar to one another (Figure V.11). The statistical analyses appear to be contradictory, as low CO_2 level grown mushrooms required more energy for compression (i.e. firmer) but that compression leads to greater plastic deformation.

Figure V.5: Total energy supplied to compress mushroom caps grown on variable casing and compost depths.

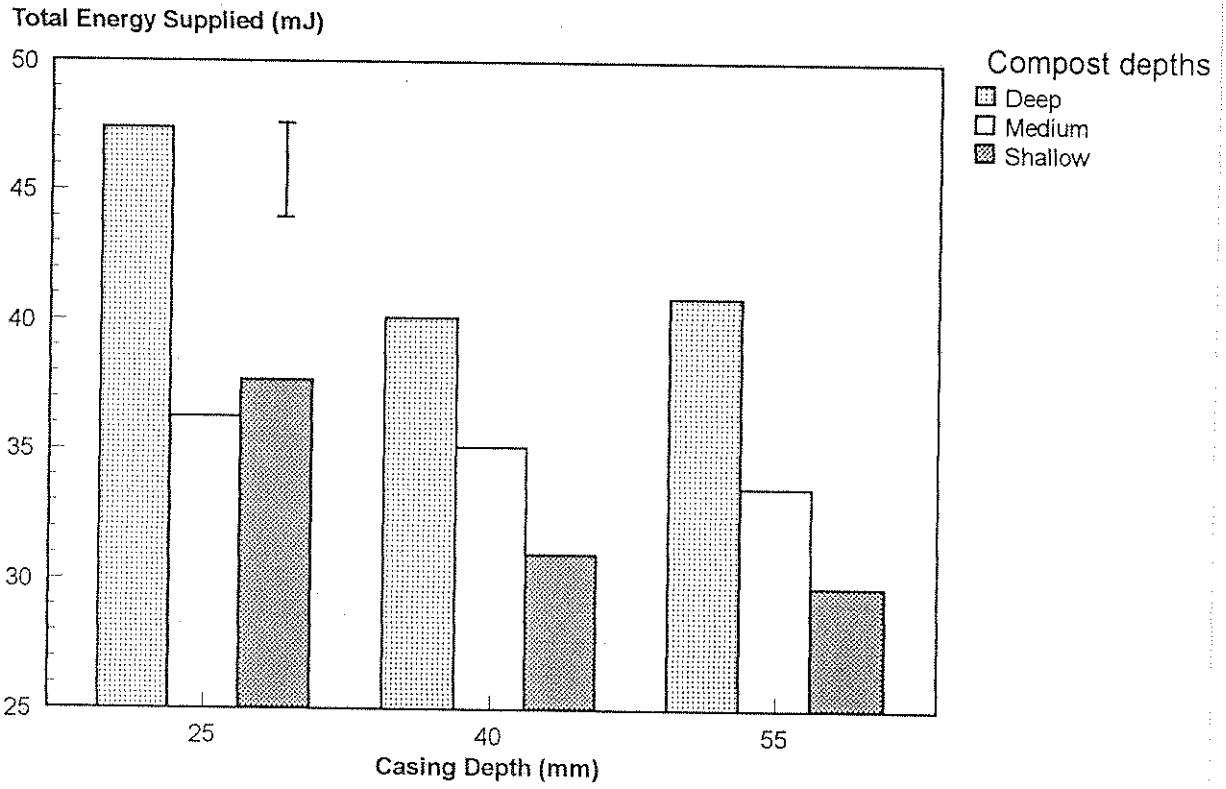


Figure V.6: Percentage of plastic deformation after compressing mushrooms grown on variable casing and compost depths.

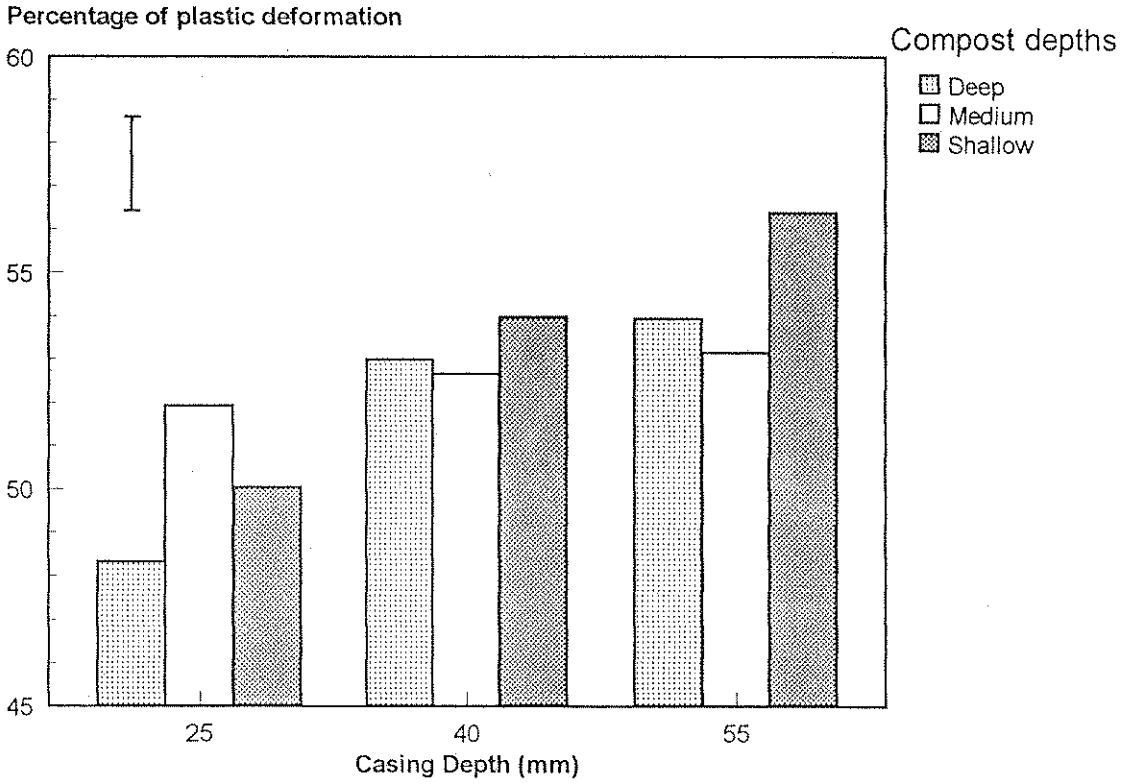


Figure V.7: Percentage of dry matter content of mushrooms grown on variable casing and compost depths.

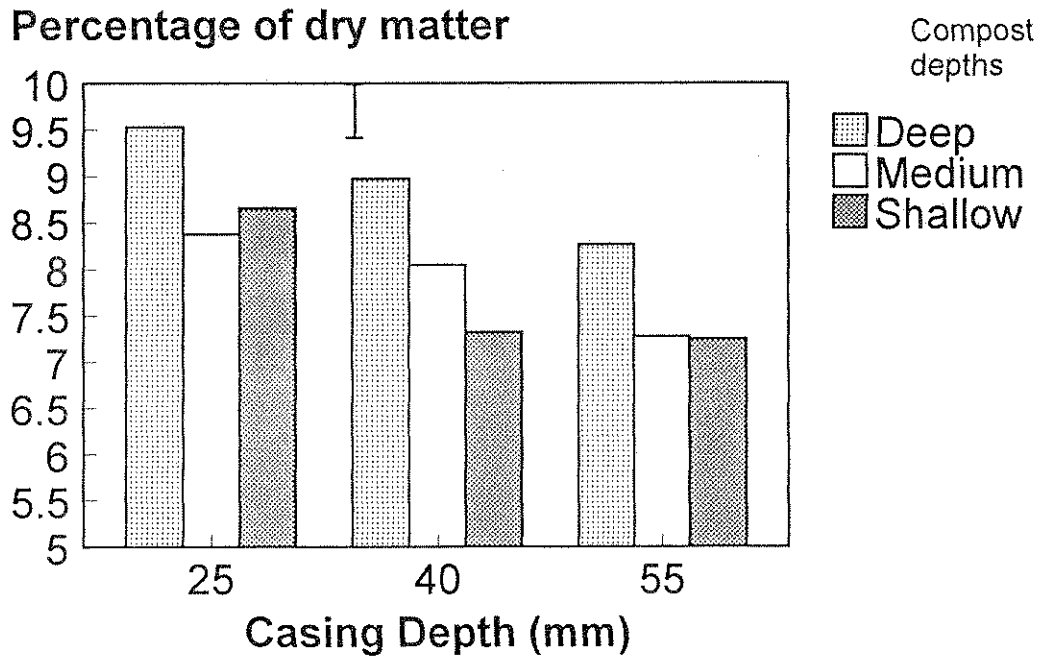


Figure V.8: Total energy supplied to compressed mushroom caps grown at three atmospheric CO_2 levels.

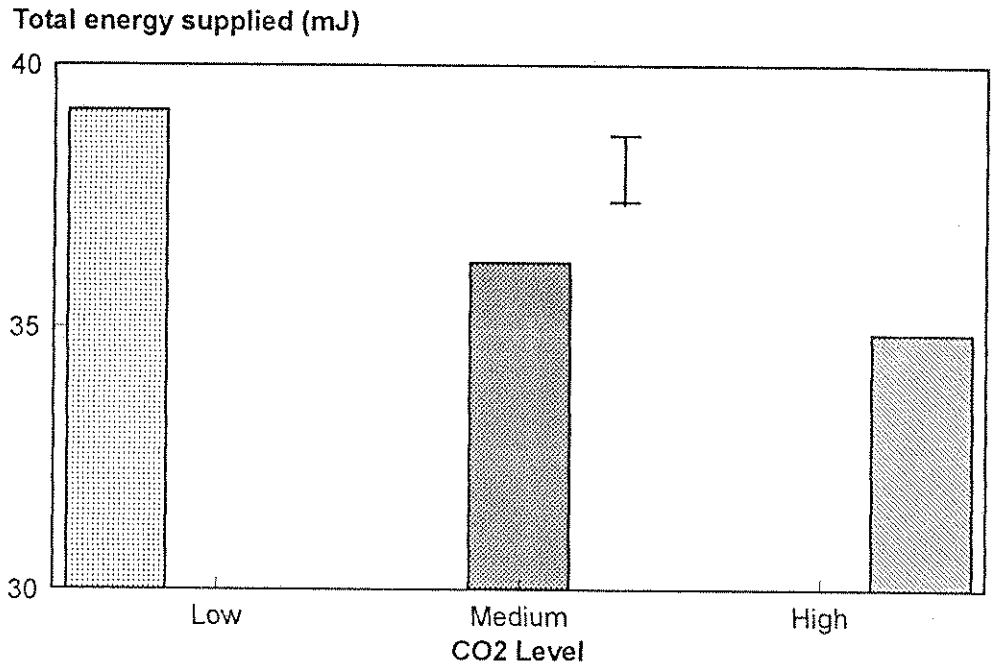


Figure V.9: Percentage of plastic deformation after compression of mushrooms grown at three atmospheric CO_2 levels.

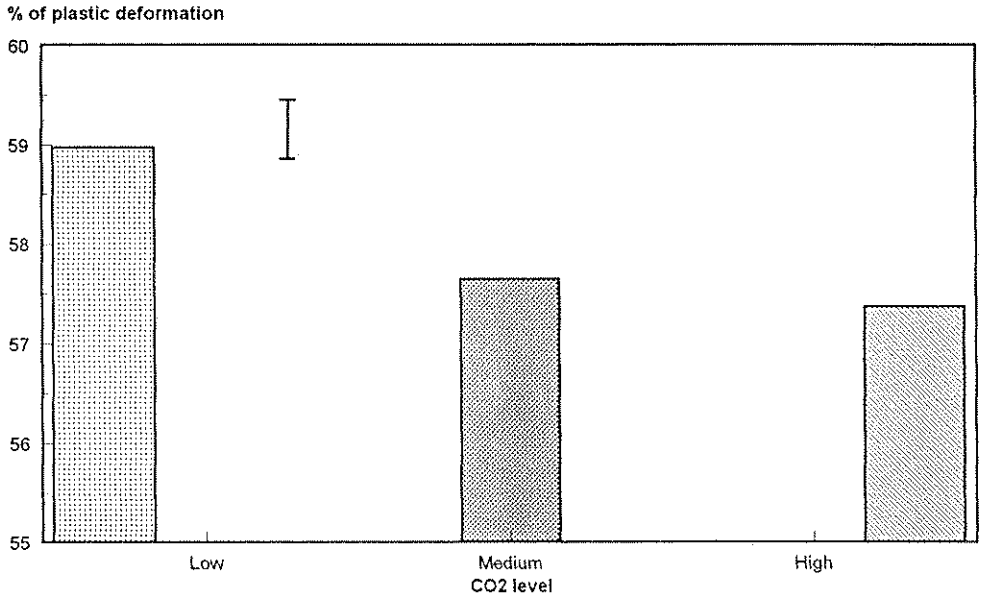


Figure V.10: Total energy supplied to compressed mushroom caps of four commercial mushroom strains

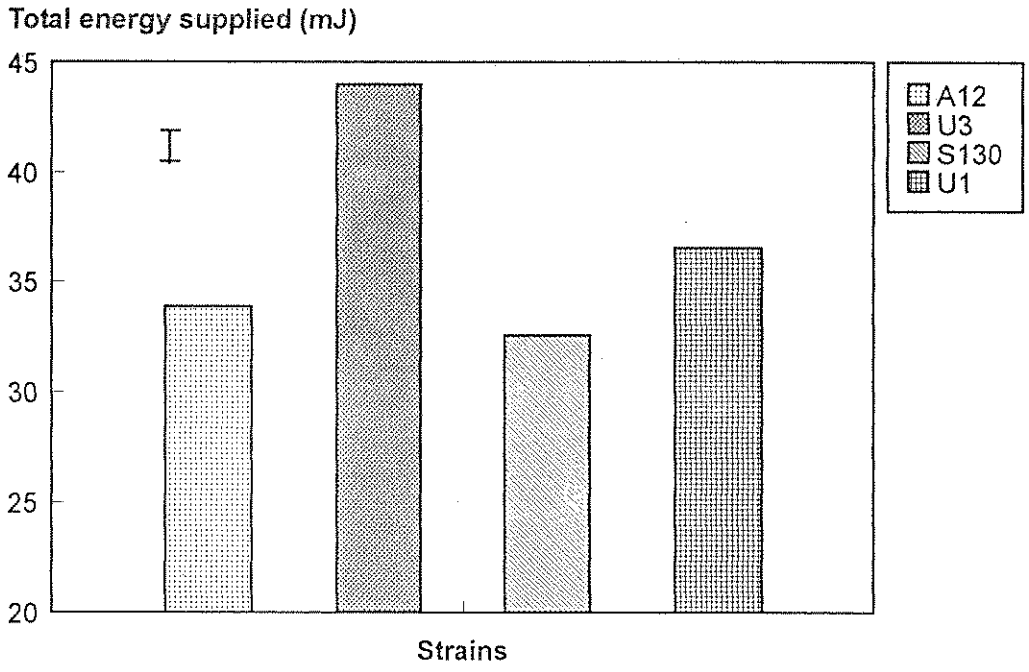
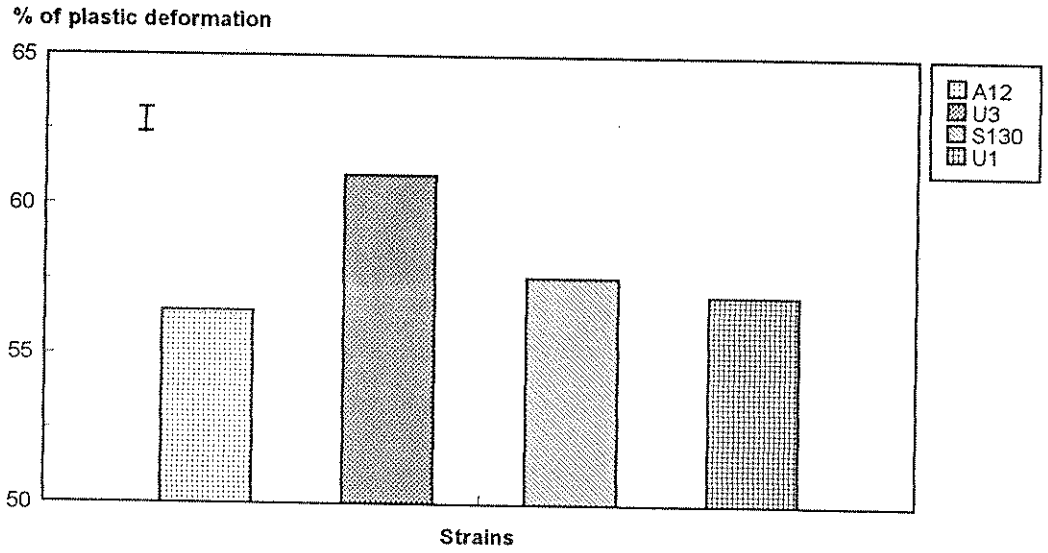


Figure V.11: Percentage of plastic deformation after compression of four commercial mushroom strains



V.3.5 Compost supplements

The analysis of variance has shown that there is no significant difference, on average, in stiffness between mushrooms grown on supplemented compost or not (Figure V.12). A major effect was found in flush 3 which produced significantly less stiff mushrooms ($p<0.001$) for both supplemented and non-supplemented compost.

There is, however, a major effect ($p<0.001$), on average, of the dry weight of mushroom cores between the control (no supplement added to compost) and the supplemented compost (Figure V.13). The control compost had a higher dry weight of mushroom cores. By looking in detail at the supplemented compost, only Betamyl and Promycel added at a rate of 0.5% produced mushrooms with lower dry weight of mushroom cores ($p<0.05$). It was found that, on average, flush 3 mushrooms cores had a lower dry weight ($p<0.001$). During the first flush, there is no significant difference in mushroom core dry weight, between supplemented and non-supplemented compost. Then, during flush 2 and 3, the supplemented compost produced mushrooms with significantly ($p<0.05$) lower mushroom core dry weight.

A positive correlation was found between the dry weight of mushroom cores and their stiffness (Figure V.14). The average regression equation is $y = - 0.2684 + 0.0237\ x$ ($r^2 = 0.95$). The yield produced in this crop is higher than the average yield produced in farms, however, the treatments with 0.5 % Betamyl and 1 % Springboard had a slightly lower yield than the other treatments (Figure V.15).

V.3.6 Water potential x casing type

There is a significant ($p<0.001$) effect of casing on mushroom stiffness. Mushrooms grown on bulk peat were found, on average, stiffer than mushroom grown on mill peat. The water potential of the casing had also, on average, a significant ($p<0.005$) effect on mushroom stiffness. Potential P1 and P2 produced significantly stiffer mushrooms. When the interaction between casing and water potential was analyzed, mushrooms grown on bulk peat were the stiffest mushrooms at potential P1 and mushrooms grown on mill peat were the stiffest at P2 (Figure V.16). At all other potential, mushrooms grown on mill peat were not significantly different in stiffness. Flush 3 mushrooms

Figure V.12: Stiffness of mushrooms grown on supplemented compost

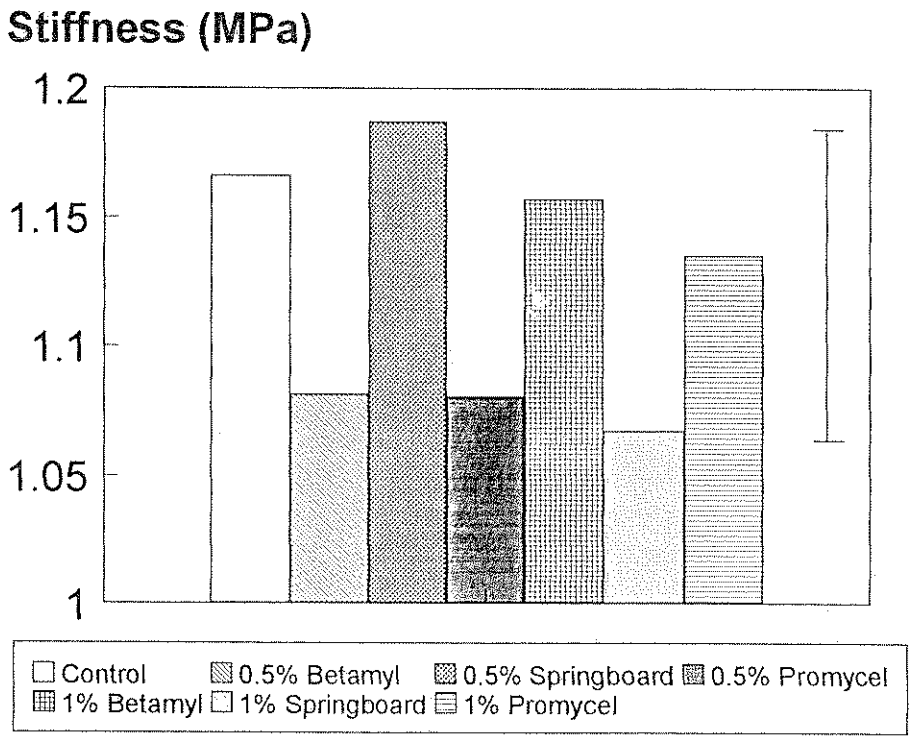


Figure V.13: Dry weight of mushrooms grown on supplemented compost

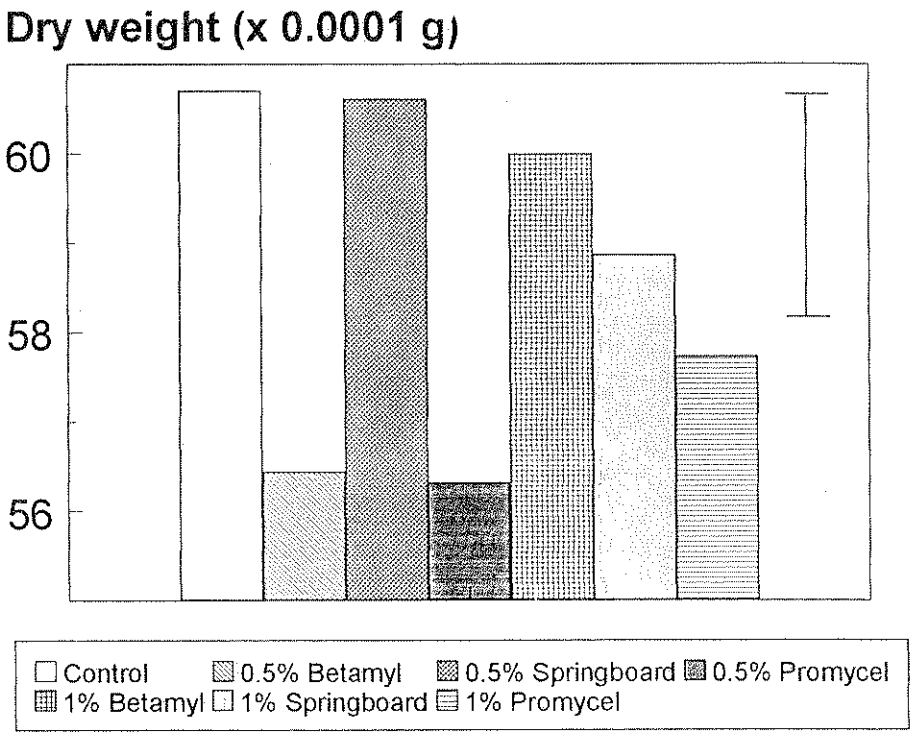


Figure V.14: Relationships between stiffness and dry weight of mushrooms grown on supplemented compost

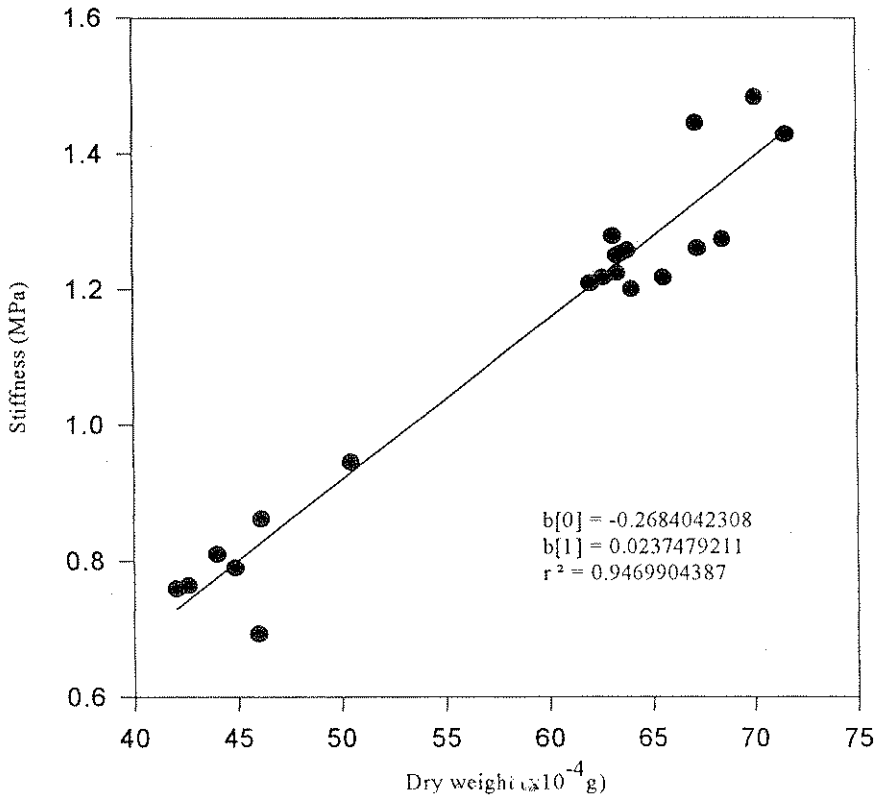
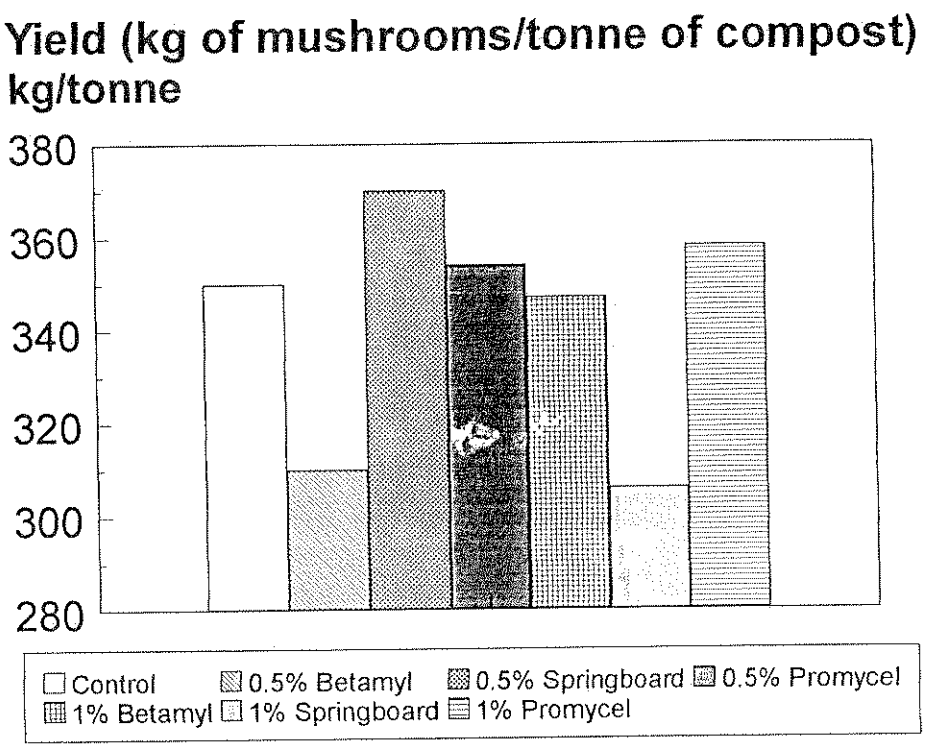


Figure V.15: Yield of mushrooms grown on supplemented compost



were found to be significantly ($p<0.001$) the stiffest of the 3 flushes. Mushrooms grown on bulk peat based casing had significantly ($p<0.001$) higher dry weight of mushroom cores than mushrooms grown on mill peat based casing. There is a significant decreasing trend of the dry weight on mushroom cores between potential P1 and potential P5. This trend is mainly due to mushrooms grown on bulk peat. Mushrooms grown on mill peat had a significant higher dry weight only when grown on water potential P2 (Figure V.17). Flush 3 mushrooms were found to have the highest dry weight of mushroom cores of the 3 flushes.

A positive correlation was found between the dry weight of mushroom cores and their stiffness from mushrooms grown on mill peat based casing (Figure V.18). The average regression equation is $y = - 0.3749 + 0.0263\ x$ ($r^2 = 0.69$). No significant correlation ($r^2=0.20$) was found between the dry weight of mushroom cores and their stiffness from mushrooms grown on bulk peat based casing (Figure V.19).

V.4 Discussion

The agronomic and environmental experiments have shown that firm or soft mushrooms can be produced by variation of growing treatments. A major effect observed was that the second or third flush consistently produced the firmest mushrooms. It was found that the mushroom top was on average firmer than the side. Although these tests were carried out on whole or half mushrooms, it has been found earlier (see chapter IV) by measuring the stiffness of mushroom tissue that the top was firmer than the side.

The skin tissue either at the top or side of sporophore appears to offer no protective function against mechanical damage to the rest of the mushroom. This is in contrast with apples, bananas, kiwis etc., where the skin plays an important role in preventing cracking and damages.

Over a storage period of 5 days, it was found that mushrooms keep their texture during the first storage day, but then, the mushroom firmness decreases.

When mushrooms where grown at high or low relative humidity, no affect on texture

Figure V.16: Stiffness of mushrooms grown on 2 types of casing and at various water potentials.

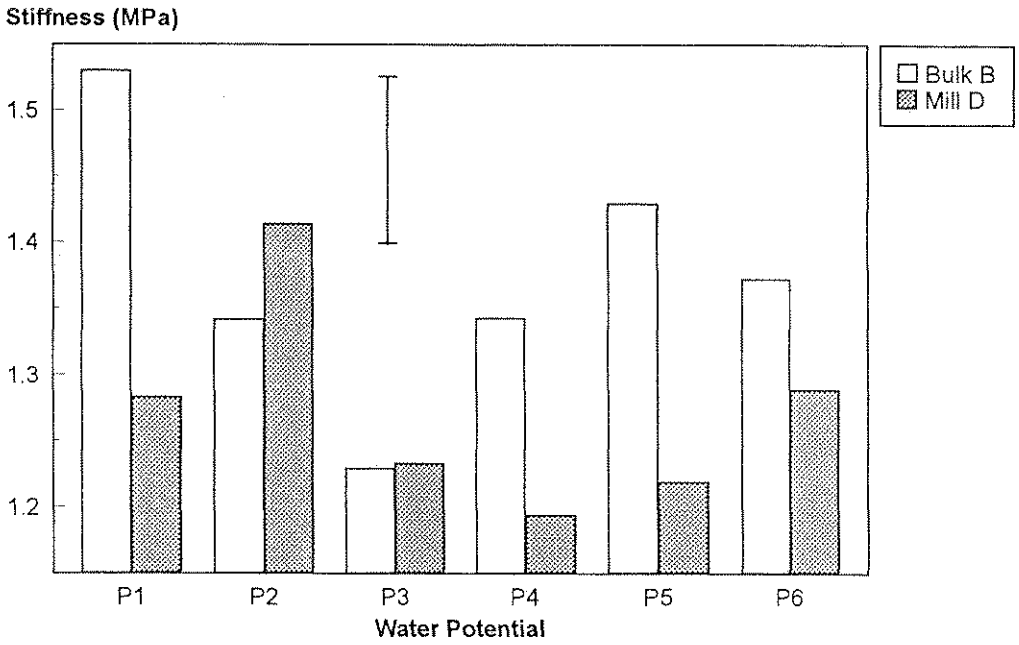


Figure V.17: Dry weight of cores from mushrooms grown on 2 types of casing and at various water potentials.

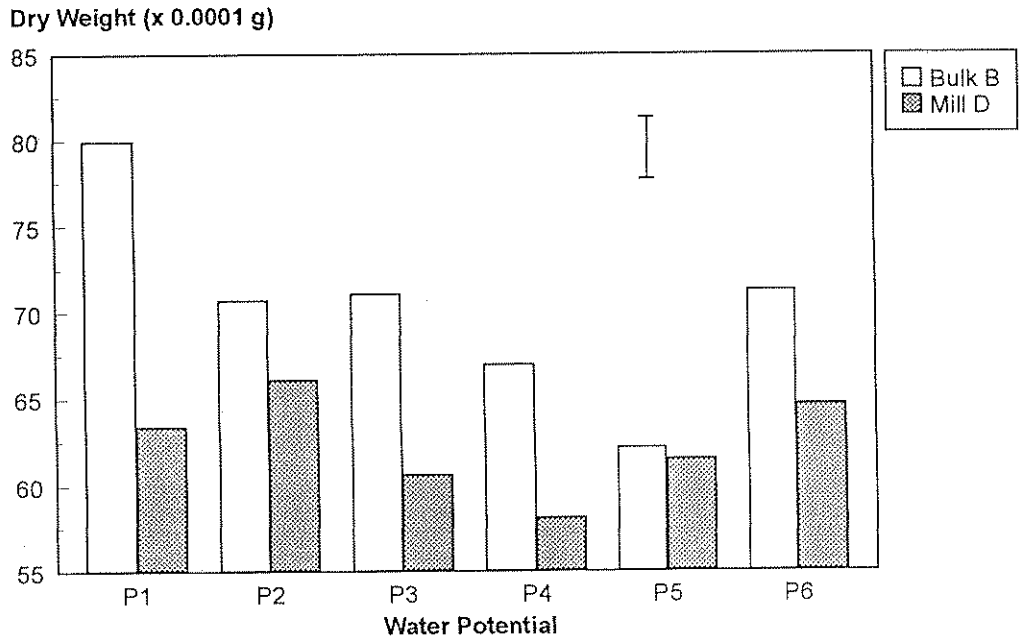


Figure V.18: Relationship between stiffness and dry weight of mushrooms grown on mill peat.

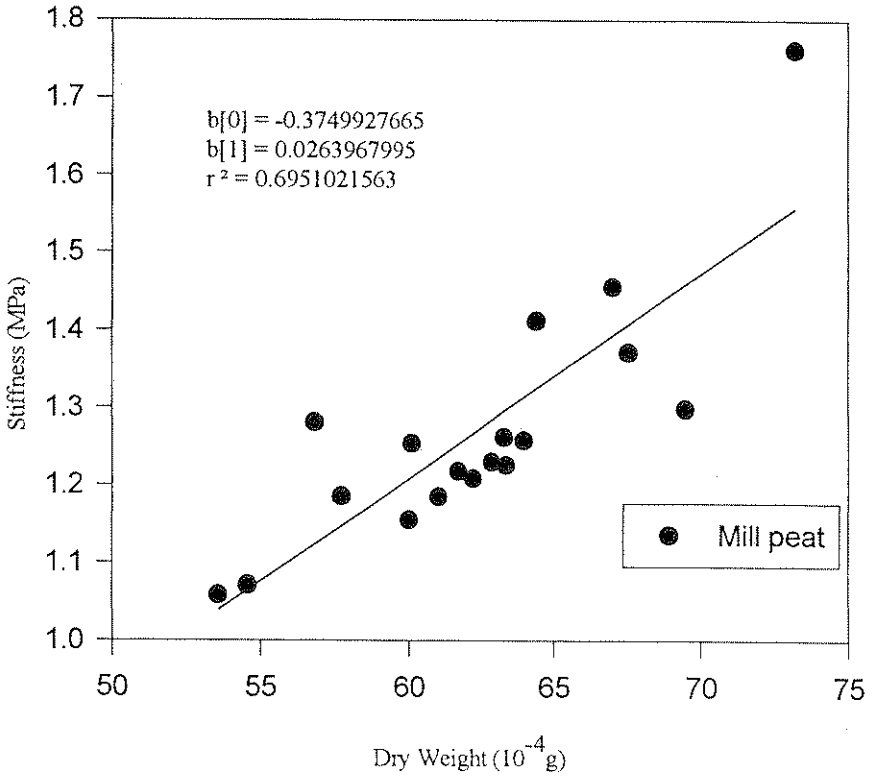
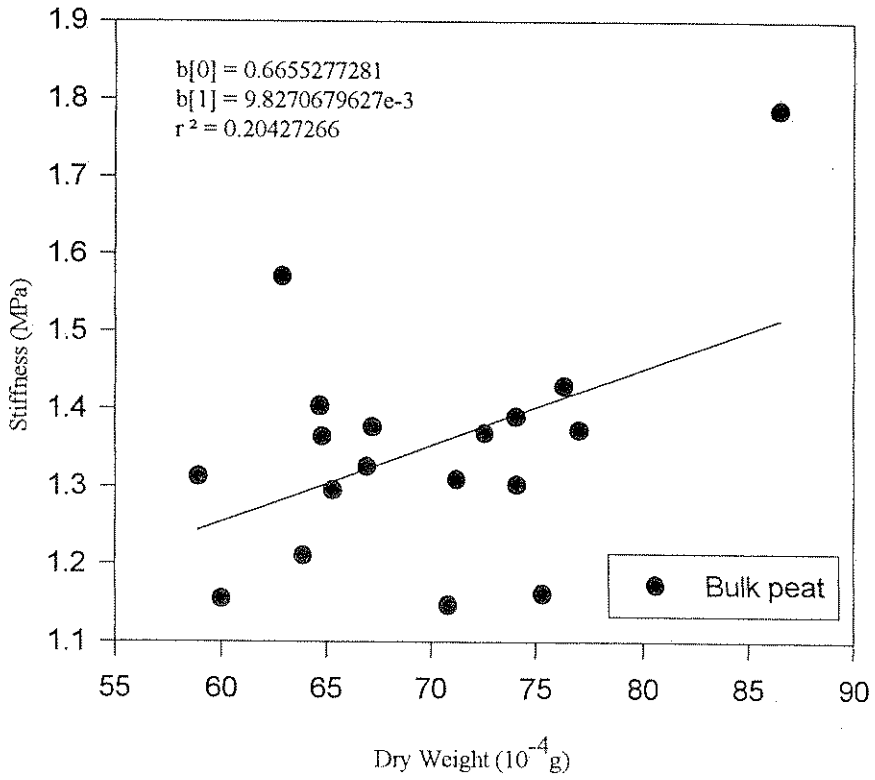


Figure V.19: Relationship between stiffness and dry weight of mushrooms grown on bulk peat.



was observed. However, when mushrooms were grown on casing (wet to dry), a significant difference in stiffness was found. The main source of water for mushrooms is from casing and compost, so it is possible that mushrooms grown at low relative humidity counterbalance the lack of humidity by taking more water form the casing or compost. Mushrooms contained more water when grown on wet casing than on dry casing and were significantly firmer. It seems that mushrooms are more affected by the low water potential of the casing (wet casing) than the high relative humidity.

Texture of mushrooms grown on different casing types were measured either by the total energy supplied or the stiffness of mushroom tissue. No difference was found in firmness between the French style casing, the peat based casing and the English style casing. However, a significant difference was found between the mill peat based casing and the bulk peat based casing. Mushrooms were stiffer when grown on that later casing.

Mushrooms grown at low CO_2 level (800 ppm) were found to be firmer than mushrooms grown at high CO_2 level (1200 ppm). It is, therefore, important to maintain sufficient ventilation in growing rooms to produce good quality mushrooms.

The choice of cultivar depends on which mushroom quality it is expected from them. Some cultivars might be resistant to discoloration, others may behave well under storage, others might be firm, others might have strong flavour and others may produce a good yield. There is no strain available which has all the features cited above. In our study, four mushroom strains, U3, U1, S130 and A12 were tested for their quality regarding firmness. Strain U3 was found to be significantly firmer than the other strains.

Supplemented compost is often used to increase mushroom yield. There was a concern that, the increase in yield might affect (negatively) mushroom quality. No difference in stiffness has been found between mushrooms grown on supplemented and non-supplemented compost. Surprisingly, the mushroom yield with supplemented compost was not higher than the yield with non-supplemented compost but the overall yield of the crop was higher than the average yield found in farm.

A great influence on texture was found in the variation of the casing and the compost depth. Mushrooms grown on deep compost and shallow casing were found to be the firmest (highest energy requirement for compression) and the more plastic. The shallow casing, on average, has also produced mushrooms with the lowest plasticity (more likely to spring back to original position after compression).

B. PART VI

Localisation of the browning reaction

VI.1 Introduction

When mushroom grow in crops, they usually have an immaculate white colour unless it is a brown strain. However their whiteness will eventually turn brown as the natural process of senescence takes place. There is however another mechanism which discolours the cap more than the senescence process. This mechanism is the mechanical damage caused during harvest, packing and transport of mushrooms. The discolouration resulting from the damage is the major cause of browning and is an important factor in the loss of the mushroom quality.

The mechanical damage can be as subtle as just touching the mushroom surface which might leave fingerprints on the cap. The damage observed is a pink patch which will turn brown after a couple of hours. It was also observed in some cases that although there was no discolouration straight after an impact, some discolouration would appear after a couple of hours.

Early research on the discolouration mechanism showed that the enzyme tyrosinase is involved in the process. It was suggested that tyrosinase and its substrate mono- and di-phenols are in 2 different compartments in an intact cell but after an impact, the integrity of the inner membranes are lost which allow tyrosinase to oxidize the phenols. Atkey *et al.* (1983) observed on mushrooms which had been handled, that on the surface, hyphae had coalesced and there was some exudate released from the cells. It was thought that it is this exudate which is responsible for the brown colour on the cap surface. There is however a contradictory claim from Braaksma *et al.* (1994) who observed no loss in the membrane integrity during senescence although the mushroom surface appeared brown. It is then not clear how the discolouration process is taking place. A lot of research has been done on the physiological and enzymological aspect of the tyrosinase but its mode of action is not known yet.

The purpose of this project was to localise the enzyme tyrosinase in the cells and to determine how it is activated after a mechanical damage. It is hoped that localising tyrosinase and understanding its activation could lead to further biotechnological research to preventing discolouration on the cap surface.

VI.2 Materials and Methods

VI.2.1 Hand damaged mushrooms

The cap of freshly harvested mushrooms was roughly rubbed with the back of a fingernail to mimic the bruise caused during harvest, packing and transport. The area of the cap surface, which had turned brown, was removed for further observations under scanning microscopy.

VI.2.2 Compressed mushrooms

A cube of 19x19x10 mm (side x side x height) was taken from a freshly harvested mushroom cap surface. This cube was compressed with the Instron machine to a displacement of 4 mm. The force necessary in such compression is about 0.02 kN which is the equivalent to a finger pushing into the cap with the force produced by the weight of 2 kg. The compressed area was either observed under scanning microscopy or processed for light microscopy or transmission electron microscopy.

VI.2.3 Preparation for scanning electron microscopy

See method described in **II.2.3**

VI.2.4 Preparation for light microscopy

Small cubes (less than 5 mm side) of mushroom tissue were taken from the compressed areas on the cap surface. They were fixed 24 hours in 2.5% glutaraldehyde in cacodylate buffer. After 5 rinses in cacodylate buffer the samples were fixed in 2% osmium tetroxide in cacodylate buffer. They were again rinsed 5 times in cacodylate buffer. The samples were dehydrated one hour in each solution starting with a solution of 25% ethanol followed by 50, 70, 80 90 and twice in 100% ethanol. The samples were kept overnight in 100% ethanol. All the procedure described above was held at 4°C or on ice. An additional change in 100% ethanol

was done prior to resin embedding. The samples were put in 25% Spurrs resin for 7 h, 50% and 75% Spurrs resin for 24h and in 100% Spurrs resin for 48h. The samples were embedded in flat molds in fresh resin for 8 h at 70 °C.

The resin blocks were sectioned with an ultramicrotome (Reichert-Jung Ultracut E, Leyca, UK) at a thickness of 1 μm. Sections were mounted on slides and stained with toluedene blue.

VI.2.5 Preparation for transmission electron microscopy

VI.2.5.1 Compressed mushrooms

The mushroom samples were prepared as for light microscopy (**VI.2.4**). However the resin blocks were sectioned with the ultramicrotome at a thickness of 70 nm. The sections were mounted on grids and stained 40 min in uranyl acetate followed by 10 min in lead citrate. The sections were observed under a transmission electron microscope (Jeol 100CX).

The control samples were taken from an undamaged mushroom cap. The samples were processed the same way as the damaged mushroom.

VI.2.5.2 Cell walls

Two samples of 20 μg of isolated cell walls (see method **VI.2.6**) were incubated 30 min and 24 h in 1ml of 10mM DOPA in 100mM sodium phosphate buffer, pH 6.0. The samples were centrifuged on a bench centrifuge and the surpernatants discarded. About 0.5 ml of 1.5% agarose solution (Sigma agarose, low melting point) was added to each pellet. The low melting point of the agarose was important, as the low temperature would not affect the tyrosinase activity. When the agarose was set, the blocks were cut in small pieces and fixed in 2.5% glutaraldehyde in cacodylate buffer. The following procedure is the same as the one described in preparation for light microscopy (**VI.2.4**).

The control samples were done with 20 μg of isolated walls mixed with 0.5 ml of agarose solution.

VI.2.6 Preparation of cell walls

The mushroom skin was peeled away like an apple with a sharp scalpel. The skin was immediately frozen in liquid nitrogen and grounded. Three grams of the skin powder was homogenized in 6-8 ml of 100 mM sodium phosphate buffer, pH 8.0. The suspension was then sonicated for a few seconds in a sonicateur (Soniprep 150 MSE) and centrifuged for 5 min at 3,000g. The supernatant was discarded and the pellet washed 10 times with the sodium phosphate buffer. Between each wash the pellet was resuspended in the buffer by a treatment of a few seconds in the sonicateur.

VI.2.7 Enzymatic degradation of Cell wall

Two enzyme solutions were prepared to degrade the cell walls: 40mg/ml cellulase and 1mg/ml chitinase in maleate buffer, pH 5.5.
The cell wall preparation was mixed with the cellulase or the chitinase solution at a concentration of 200 mg/ml enzyme solution and the suspensions were incubated in a water bath at 37°C.
The suspensions were centrifuged on a bench centrifuge after 15, 30 and 60 minutes and 2 and 4 hours of incubation to measure any Tyrosinase activity released in the supernatant. The activity was measured with 10 or 15 µl of supernatant mixed in 3ml of DOPA solution.

VI.3 Results

VI.3.1 Hand damaged mushrooms

The brown patches produced on the cap surface as a result of the hand damage were observed under scanning electron microscopy. On the cap surface it was observed that some hyphae were distorted (Photo VII.1) but others looked intact (Photo VII.2). When the surface was damaged by rubbing a fingernail on, it has formed a bundle of hyphae (Photo VII.3). The bundle contained both torn hyphae and intact hyphae bound together by the exudate released (Photo VII.4). The area observed next to the bundle

(Photo VII.5) showed neither torn hyphae nor exudate released from hyphae although it was a brown area.

VI.3.2 Compressed mushroom

VI.3.2.1 Observed under scanning electron microscopy

Under the microscope it was possible to see a crack on the surface, this crack was in the middle of the compressed area (Photo VII.6). The compressed cap surface showed some patches of exudate, it was not clear what happened to the hyphae underneath as the frozen exudate was masking them (Photo VII.6). Other parts of the compressed area showed no apparent damage to the hyphae (Photo VII.7 and VII.8) other than a crack across some hyphae (see arrow). The compressed area was fractured in the preparation chamber of the microscope to observe any damage below the surface. The hyphae observed underneath the surface (Photo VII.9 and VII.10) were not torn apart, they were not compressed (they still had a circular cross section) and there was no exudate released underneath the compressed area.

VI.3.2.2 Observed under light microscopy

The thin sections of the compressed area showed mainly cross sections of hyphae. The outside structure of the hyphae was intact since the cross sections of hyphae looked perfectly round and there was no sign of broken walls (Photo VII.11 and VII.12). In the hyphae near the surface (Photo VII.11), it was noticeable that the inside of some of the cells was full of vesicles (see arrow). In the control mushroom they were no vesicles present inside hyphae (Photo VII.13).

In the area below the compressed surface, there is again no sign of broken hyphae but there are signs of damaged hyphae (Photo VII.12). Some cross sections of hyphae did not look perfectly round, one hyphae looked like it had been flatten by the two surrounding hyphae. Another hyphae, observed in the longitudinal section had its walls indented. The vesicles, which were present inside hyphae at the cap surface, did not seem to be present in hyphae below the surface.

Photo VII.1: Scanning electron micrograph of distorted hyphae on the surface of hand-damaged mushroom cap.

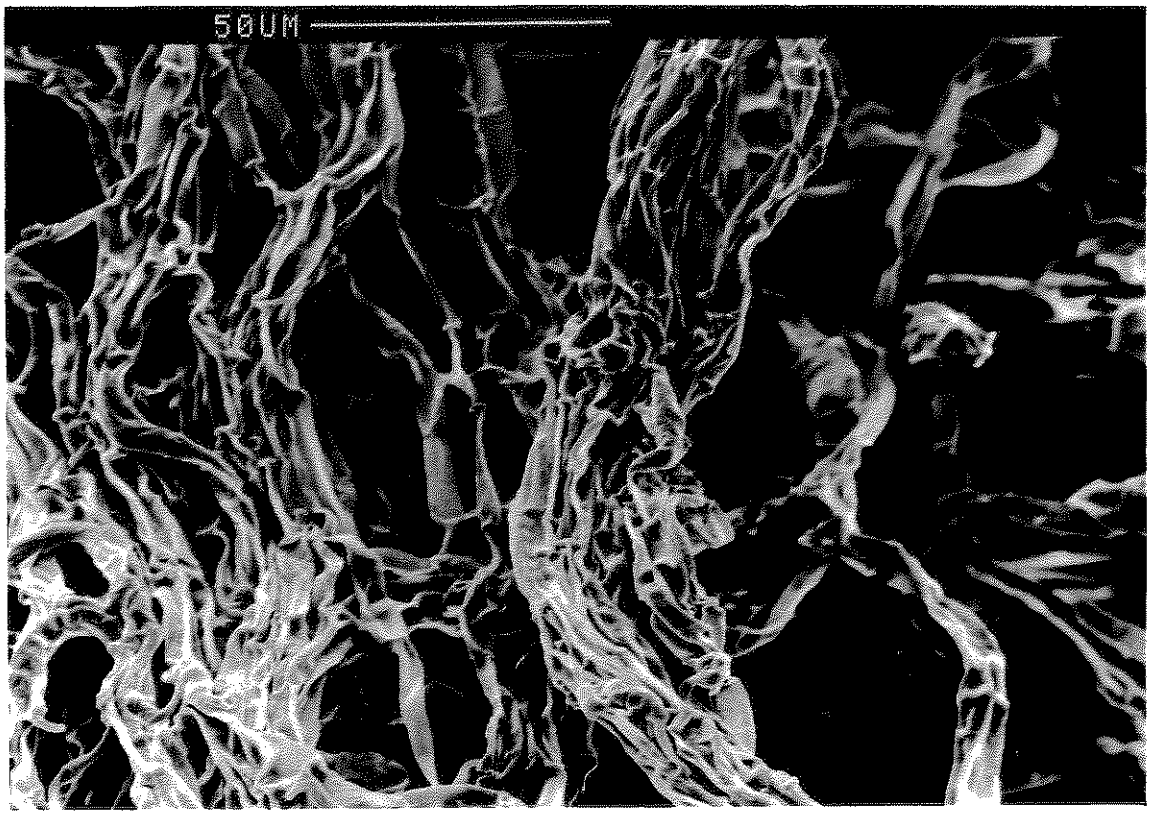


Photo VII.2: Scanning electron micrograph of the surface of hand-damaged mushroom cap.

Photo VII.3: Scanning electron micrograph of hand-damaged cap. A bundle of hyphae has formed when the surface was rub with a fingernail.

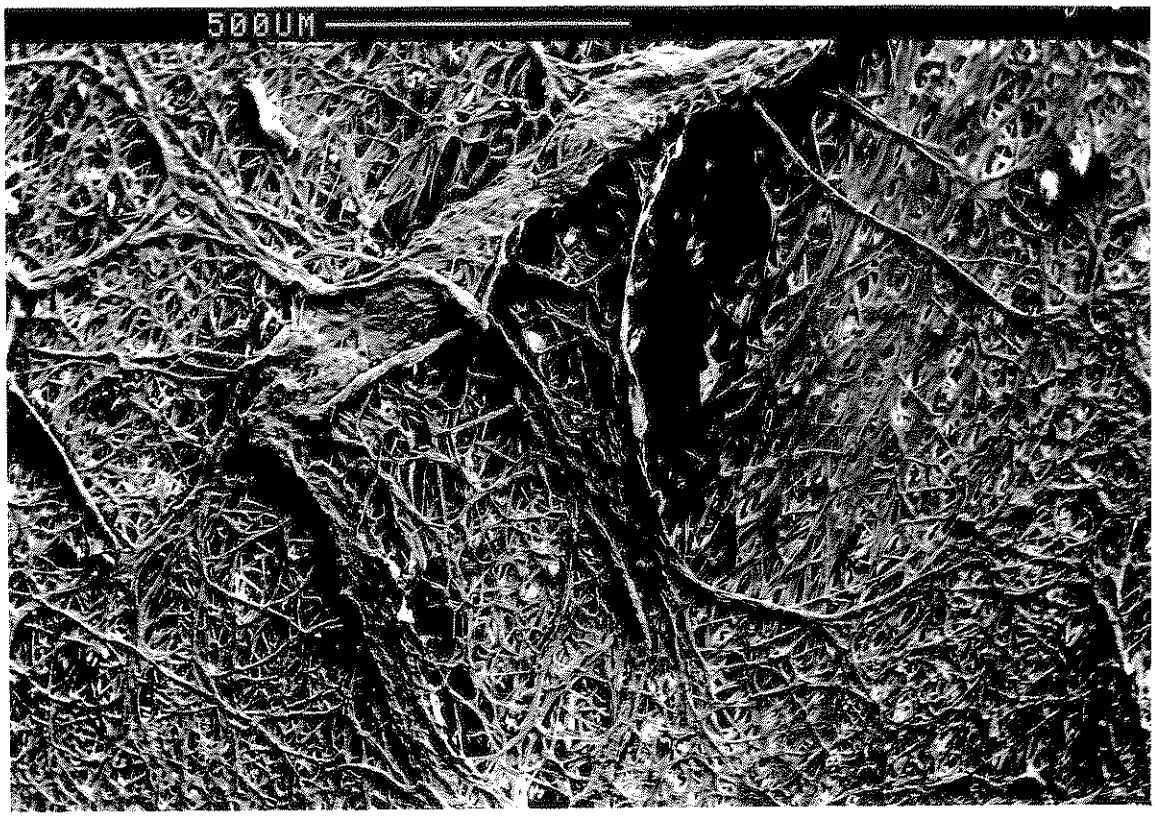


Photo VII.4: Scanning electron micrograph of a close-up of the bundle showing intact and torn hyphae bound together by the exudate released from the cells.

Photo VII.5: Scanning electron micrograph of an area next to the bundle of hyphae showing mainly intact hyphae although the area was brown.

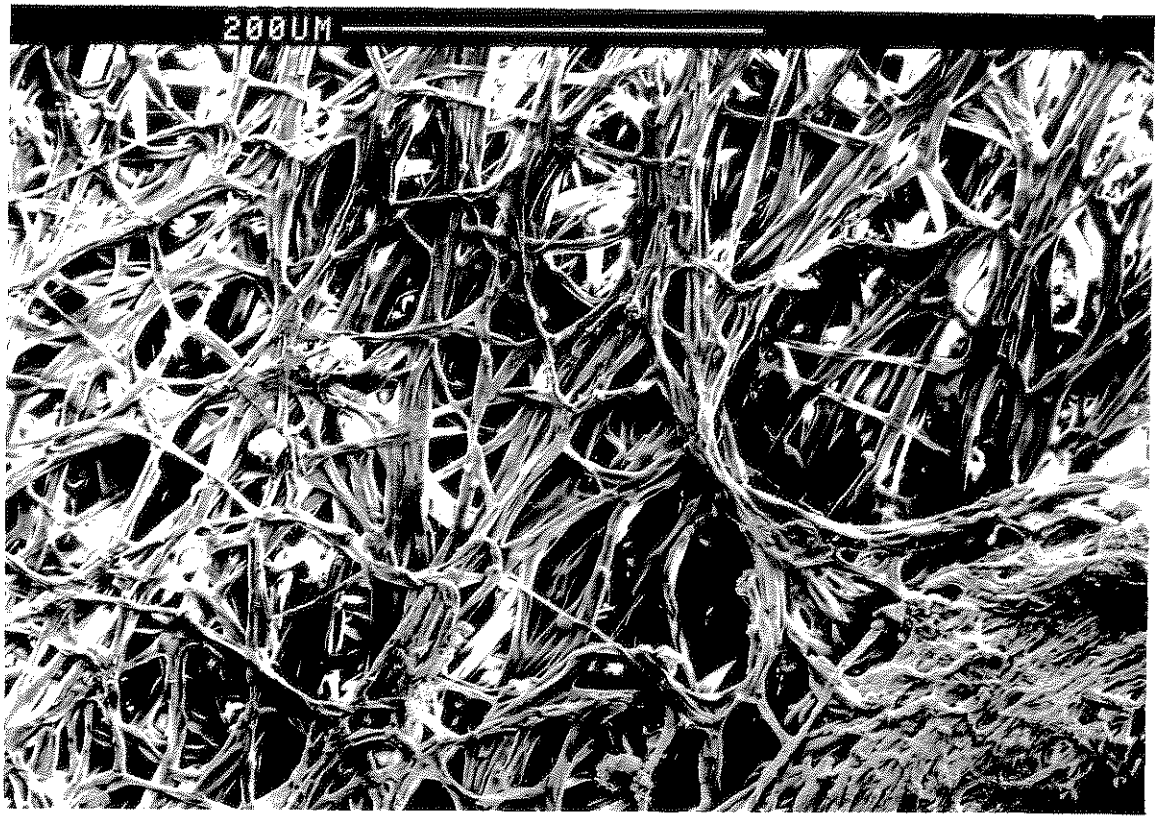


Photo VII.6: Scanning electron micrograph of compressed surface a crack in the middle of the area and patches of exudate (*).

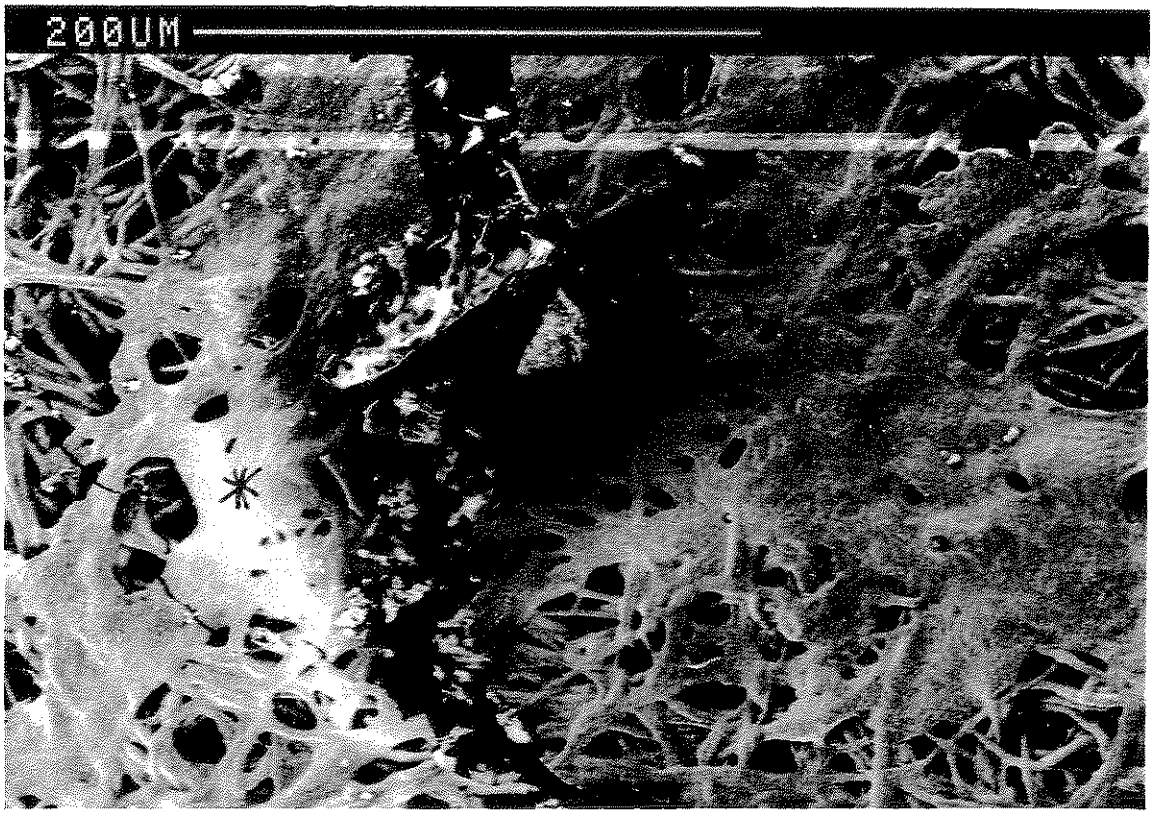


Photo VII.7: Scanning electron micrograph of compressed surface showing intact hyphae and hyphae damaged by a crack (arrow).

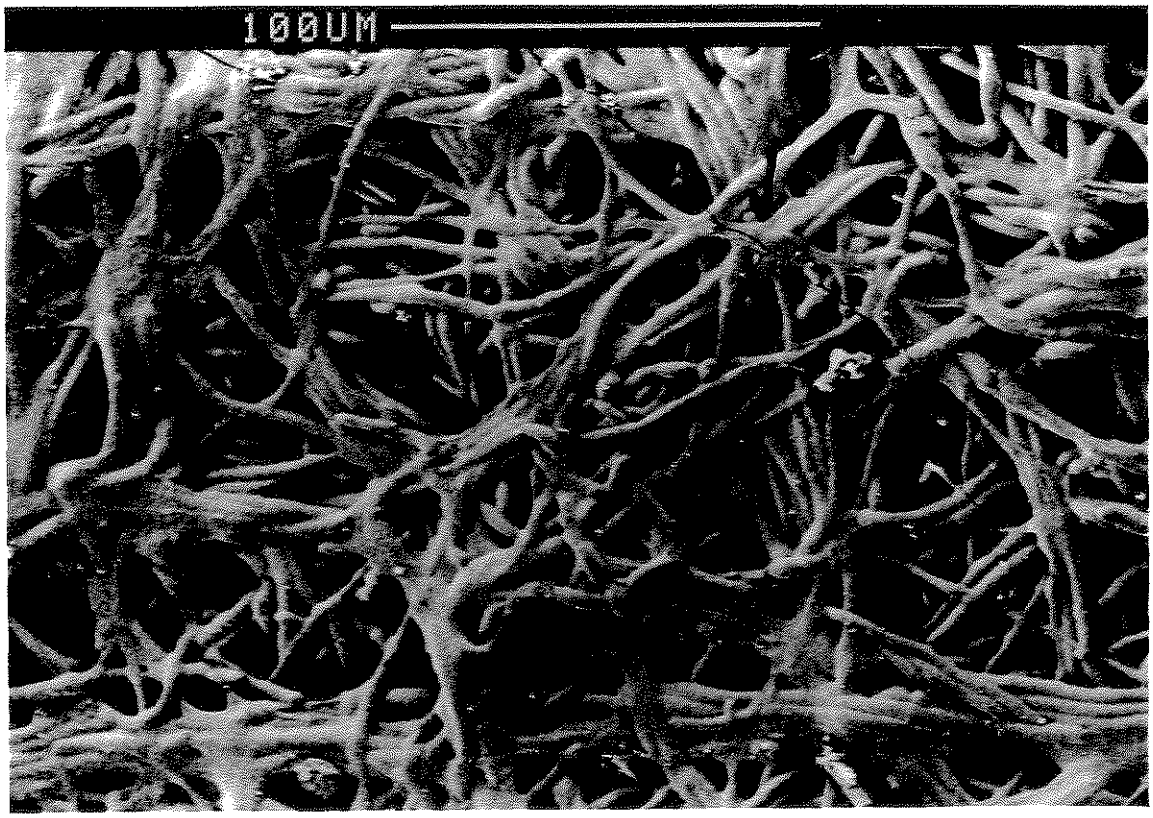


Photo VII.8: Scanning electron micrograph of compressed surface showing intact hyphae next to a patch of exudate.

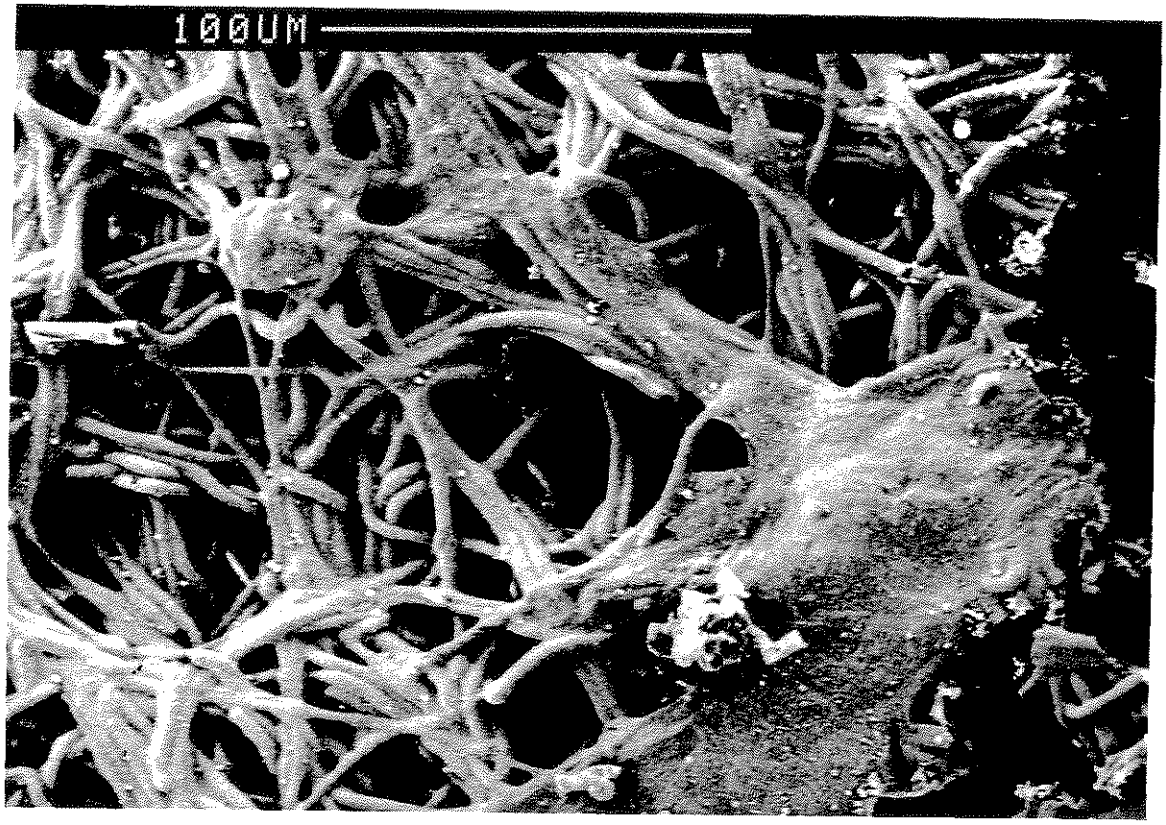


Photo VII.9: Scanning electron micrograph of the area underneath the compressed surface.

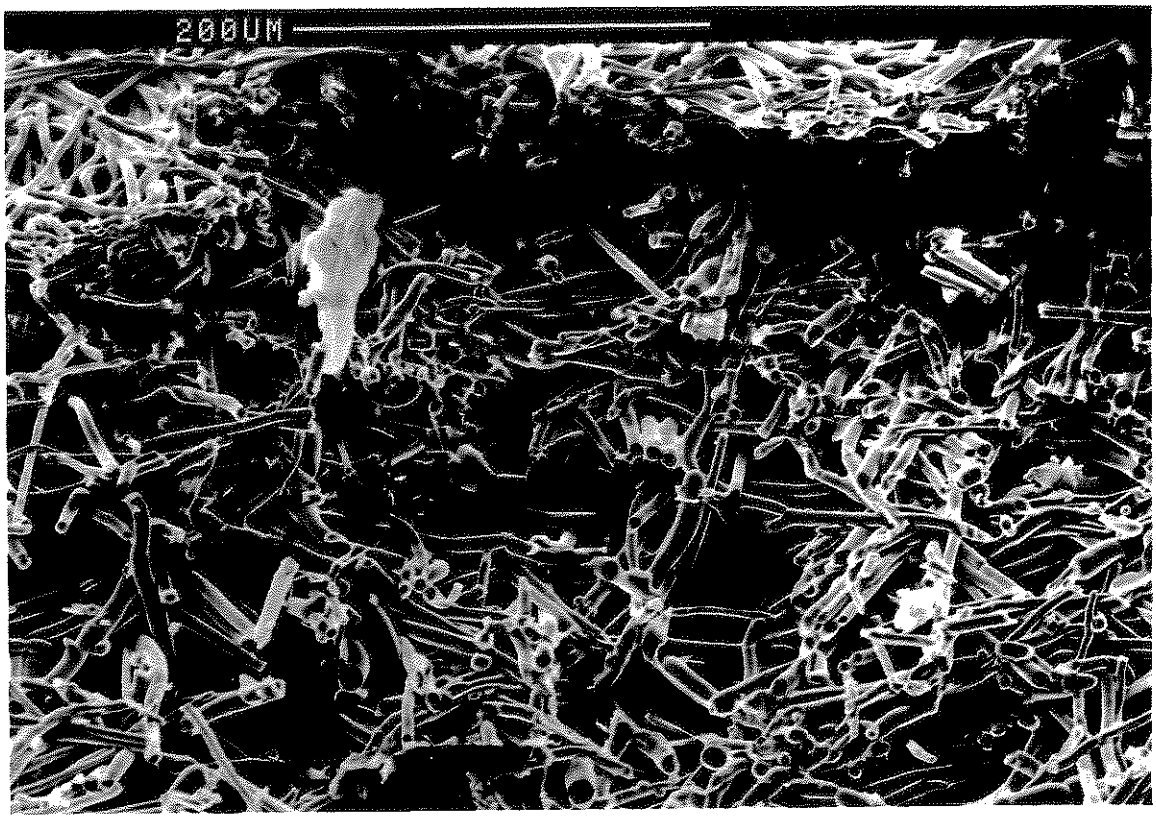


Photo VII.10: Scanning electron micrograph showing intact hyphae underneath the compressed surface.

Photo VII.11: Light micrograph showing cross sections of hyphae on the surface of compressed mushroom. Note the vesicles present inside hyphae (arrow).

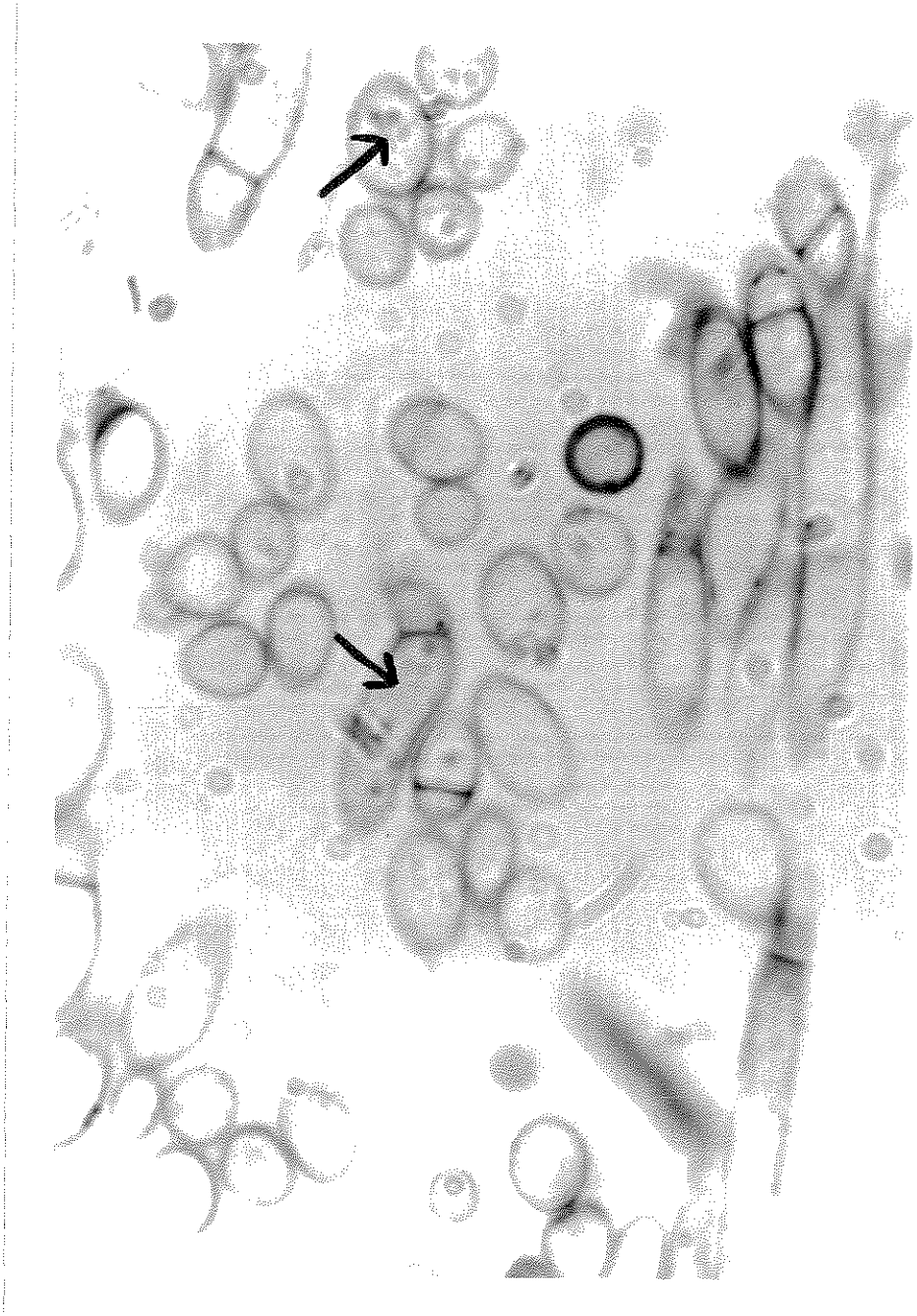


Photo VII.12: Light micrograph showing hyphae below the compressed surface. Note the hyphae squashed between 2 cells (arrow) and the longitudinal section of hyphae with indented wall (*).

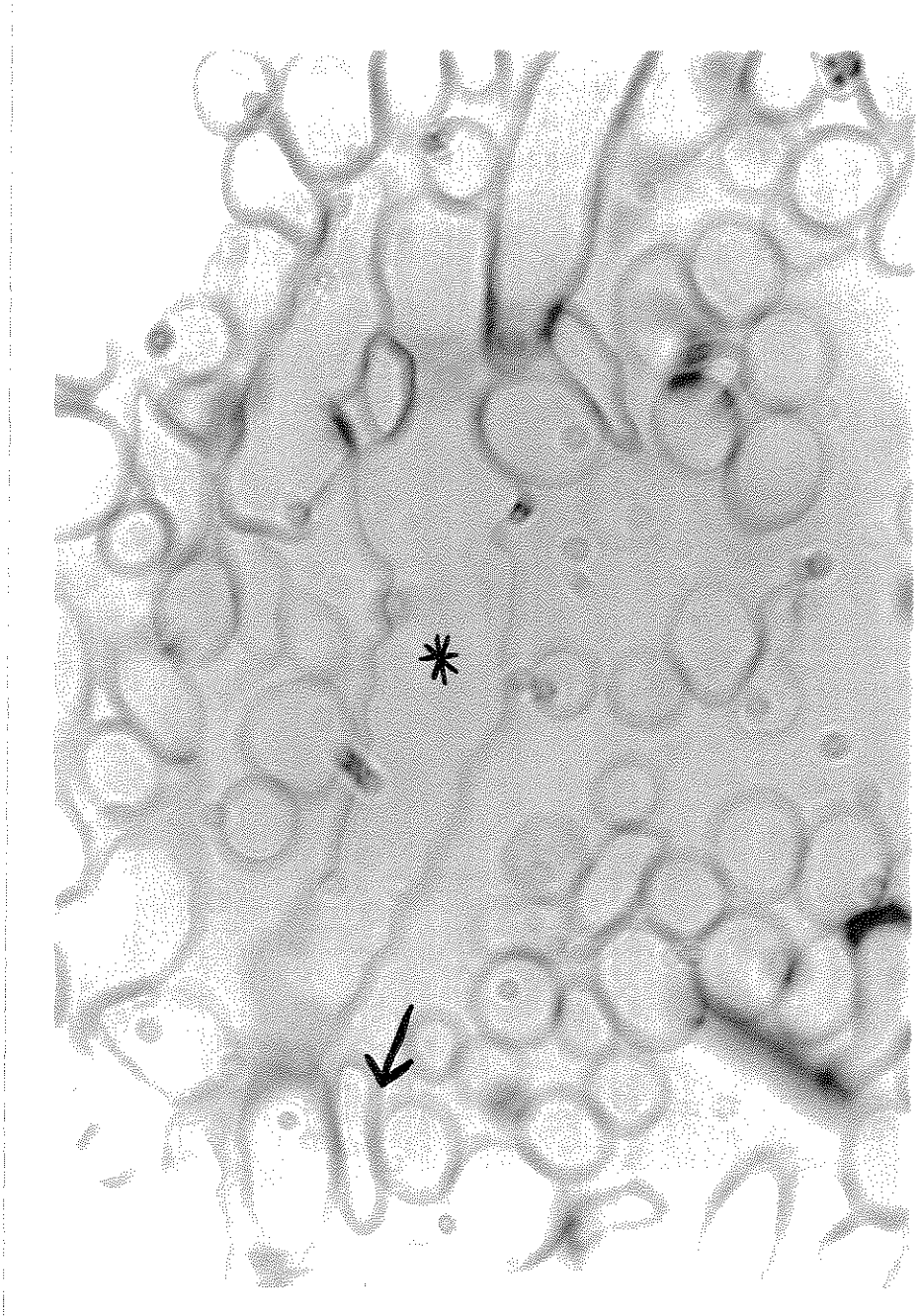
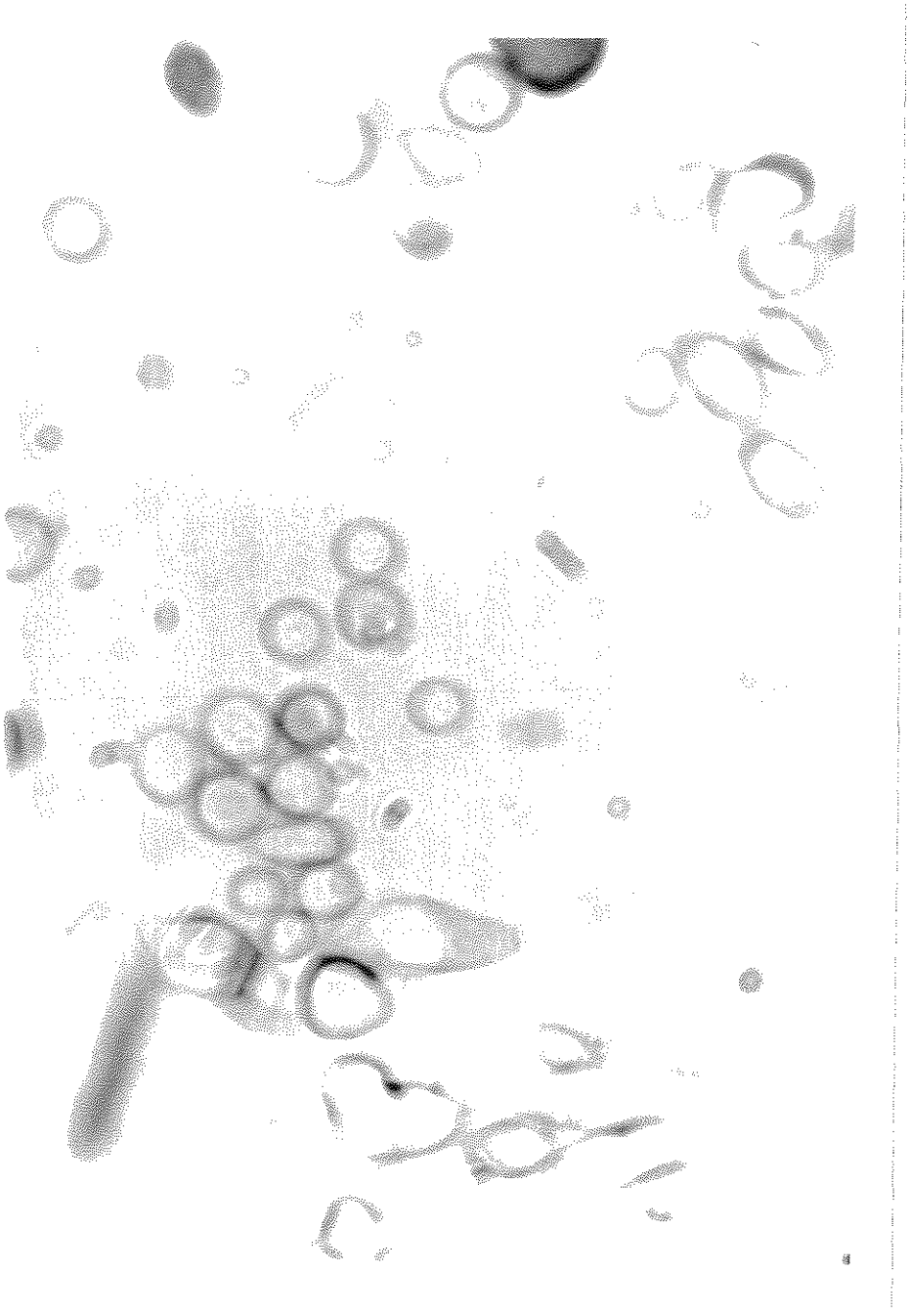


Photo VII.13: Light micrograph showing cross sections of hyphae at the surface of an undamaged mushroom.



VI.3.2.3 Observed under transmission electron microscopy.

The compressed areas observed under transmission electron microscopy have shown that the cell walls of hyphae were not broken during the compression, any debris of broken walls had been found in the sections (Photo VII.14 to VII.17). However some cells were found with a distorted shape. One cell had a triangular shape and by the two cells surrounding it, one can imagine that it is the compression that produced such a shape (Photo VII.14). Other cells (Photo VII.15) had surprising indentations on the walls since hyphal walls are usually straight. In normal hyphae there is usually one vacuole but in these damaged hyphae, although the outside walls of the cells did not appear broken, the inside vacuole had burst into several vesicles (Photo VII.15). In one of the sections, at least 25 vesicles were counted inside one hyphae. In the control mushroom, sometimes the vacuole wasn't on the section or there were 1 to 5 vacuoles present in hyphae (Photo VII.16). However the cells which contained more than one vacuole were not as frequent as in the compressed mushrooms.
When the inside of hyphae was observed at higher magnification, it was noticed in the vesicles that there were some black spots located on the inside membrane of the vesicle (Photo VII.17).

Photo VII.14: Transmission electron micrograph of hyphae at the surface of compressed mushroom. Note the cell with a surprising triangular shape (arrow).

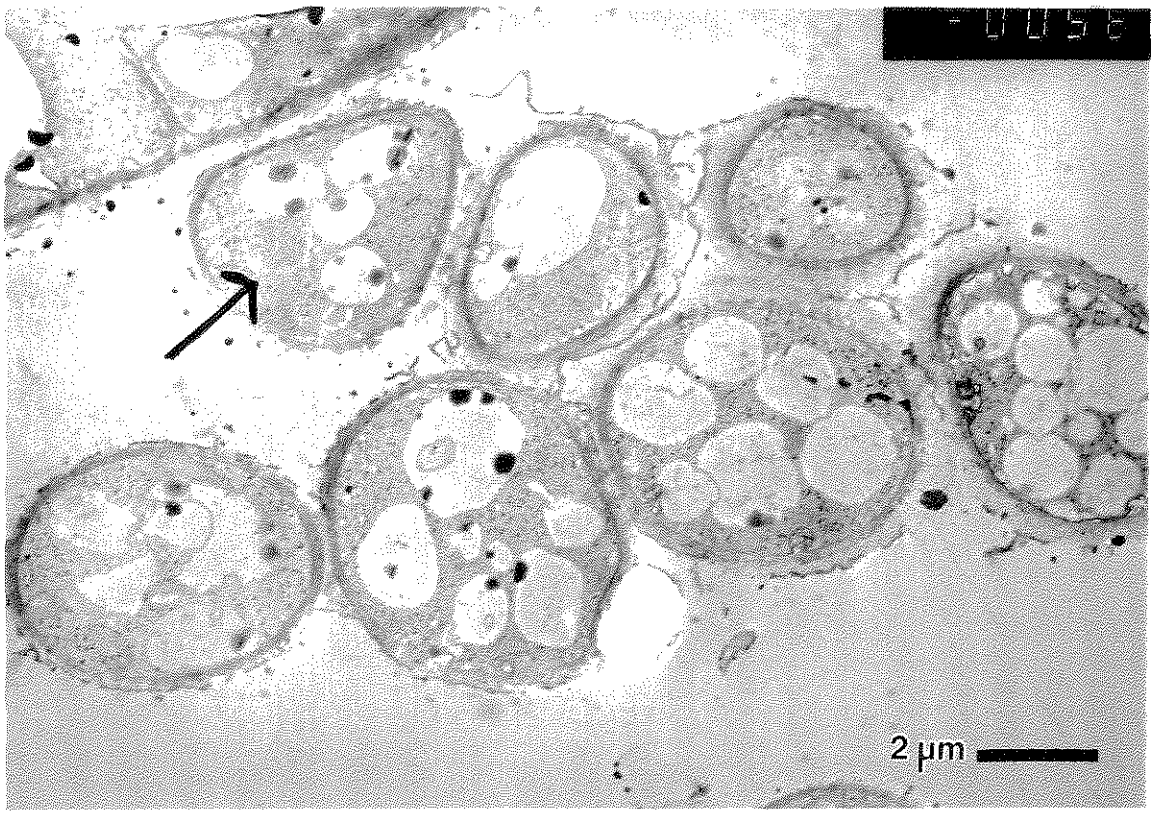


Photo VII.15: Transmission electron micrograph showing hyphae on the compressed surface. Note the indented walls and the number of vesicles inside hyphae.

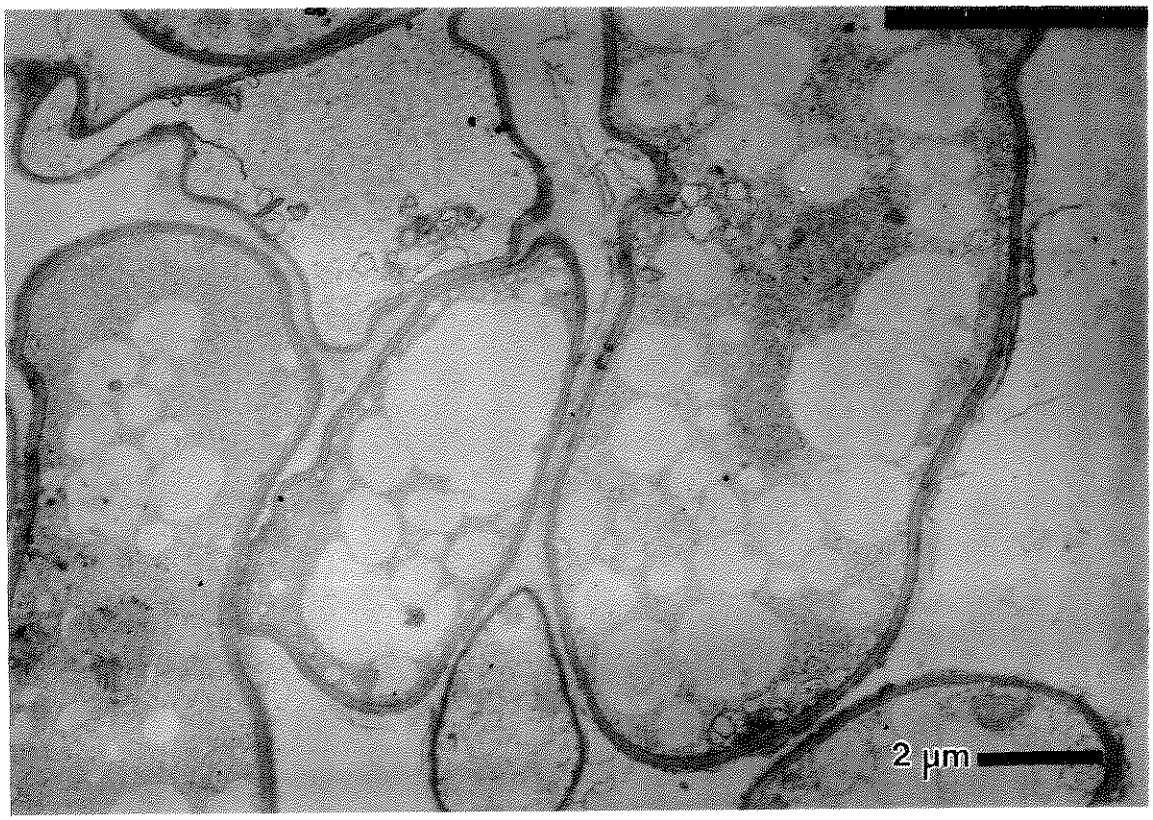


Photo VII.16: Transmission electron micrograph of hyphae from an undamaged mushroom.

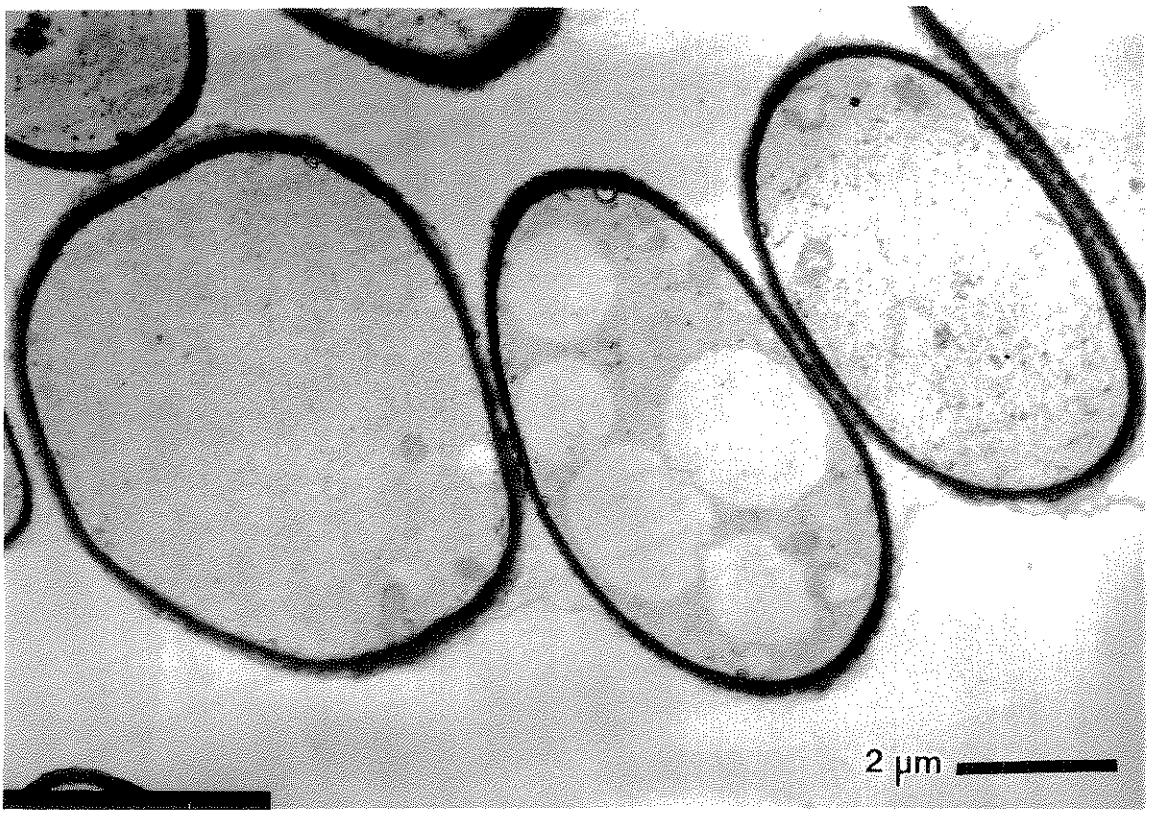
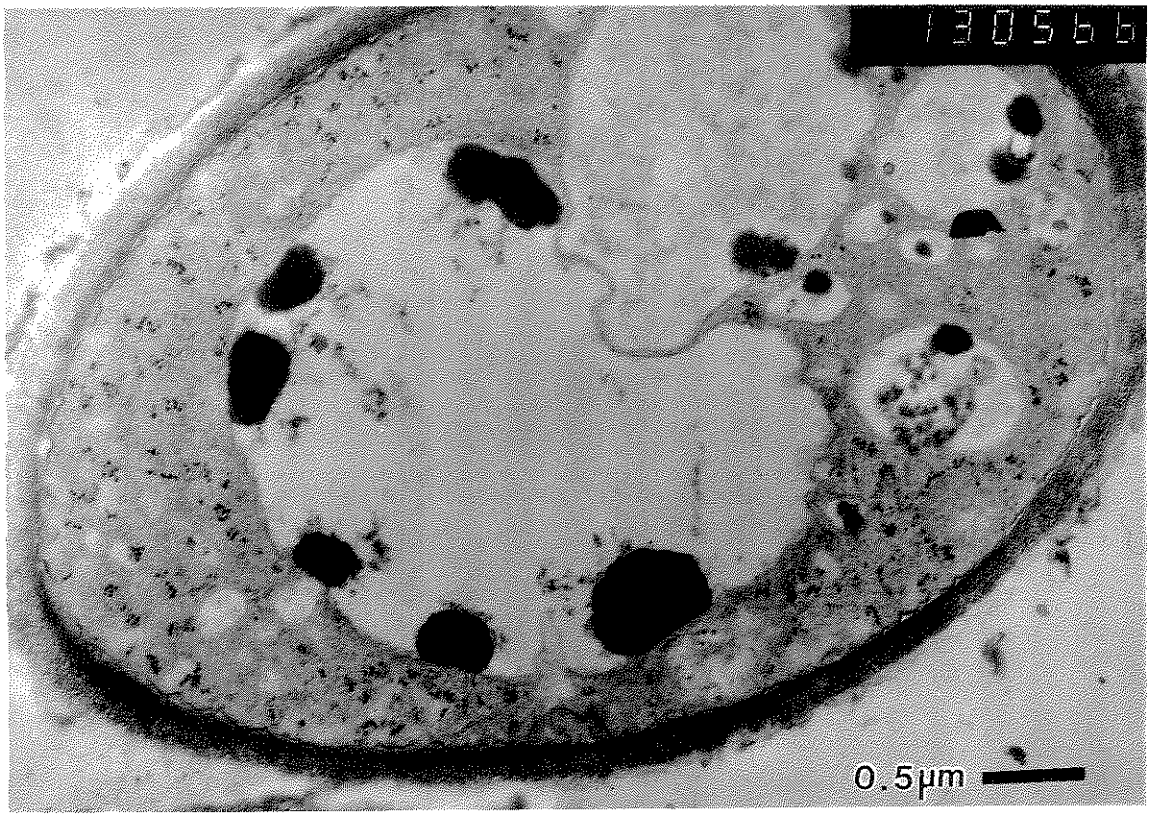


Photo VII.17: Transmission electron micrograph showing the inside of hyphae. Note the dark spots located on the inside membrane of the vacuole.



VI.3.3 Localisation of tyrosinase in the cell walls

VI.3.3.1 Cap surface in presence of DOPA solution

When a piece of the mushroom cap was put into a DOPA solution, both the solution and the mushroom tissue turned pink. Furthermore, after 1 h in the solution the tissue showed distinct layers of different pink shades from the surface to the flesh. This suggested that there might be different specific enzymatic complexes for each of these layers. It also showed that the outermost surface layer has a different enzymatic system from the underneath flesh regarding the discolouration process.
When a drop of DOPA solution was put on the cap surface, the colour of the drop turned from clear to translucent pink in a couple of minutes. After 5 min the colour went darker to a pink-purple colour. Because of both, the very quick reaction and the change in colour of the drop, it was hypothesized that the enzyme Tyrosinase might be located on the outside walls of the hyphae. The substrate (DOPA) would be taken from the drop and the product released in there giving its pink colour to the drop.

VI.3.3.2 Detection of tyrosinase activity in cell wall

For the preparation of cell walls, mushroom cells were smashed and centrifuged so mainly cell walls would be in the pellet. The pellet was washed several times to remove any impurity present in it. During these washes, samples were taken from both the supernatant and the pellet to measure the tyrosinase activity (Figure VI.1). In the first supernatant collected the total relative activity of the soluble tyrosinase was measured and used as reference (total relative activity = 100). In the second wash of the pellet, the total relative activity of tyrosinase was two thirds of the total relative activity present in the first wash. In the third wash the total activity was only a third of the activity detected in the first wash. In subsequent washes, the total relative activity of tyrosinase kept decreasing and in the tenth wash no significant activity was measured.
In parallel to the measurement of the enzyme activity in the supernatant, the total relative activity in the pellet was measured. The total relative activity in the pellet

after the second wash was high and then decreased in the following washes. After ten washes a significant tyrosinase activity was measured in the pellet.
In the preparation of the cell walls, no tyrosinase activity was measured in the supernatant after ten washes, however, a significant enzyme activity was measured in the pellet. This is suggesting that after ten washes all the soluble tyrosinase had gone but an insoluble tyrosinase was present and associated with the cell walls.

To prove that the enzyme tyrosinase was associated with the cell walls, these latter were treated with specific enzymes to degrade them. Tyrosinase activity was then measured in the medium where the walls had been treated.
Some of the cell wall preparation was mixed with the enzyme solutions cellulase and chitinase. After 15, 30, 60 minutes and 2 and 4 hours of incubation the suspensions were centrifuged on a bench centrifuge and the tyrosinase activity was measured from the supernatant.

VI.3.3.2.1 Cell walls treated with cellulase

Significant tyrosinase activity was measured in the supernatant when cell walls were treated with cellulase (Figure VI.1). The activity increased during the first hour of incubation and then decreased. The optimum temperature for cellulase is 37°C but tyrosinase is susceptible to high temperature, so it is probable that the decrease in activity is due to the effect of the temperature on the enzyme.

VI.3.3.2.2 Cell walls treated with chitinase

Significant tyrosinase activity was also measured in the supernatant when cell walls were treated with chitinase (Figure VI.1). The activity increased slowly up to 4 h of incubation. After 30 min of incubation the enzyme activity measured was about 9 times lower than the activity measured when walls were treated with cellulase.

Figure VI.1: A- Tyrosinase relative activity measured in the cell wall preparation. **B-** Tyrosinase relative activity released during cell wall degradation.

A	Supernatant	Pellet
First wash	100	n/m
Second wash	66	128
Third wash	33	n/m
Fourth wash	18	50
Fifth wash	12	n/m
Tenth wash	0	12

B	Cellulase	Chitinase
15 min	100	17
30 min	144	36
60 min	147	n/m
2 h	122	n/m
4 h	86	44

(n/m: not measured)

Chitinase has a very slow enzymatic reaction; this is why little activity was released from the walls.

VI.3.3.2.3 Observation in transmission microscopy of cell walls incubated with DOPA solution

The preparation of cell walls was mixed with DOPA solution. The walls were then processed for observations under transmission electron microscope. On sections of walls incubated 20 min in DOPA, it is possible to see dark spots associated all along the walls (Photo VII.18). On the walls incubated 24 h in DOPA solution, there is a thick dark layer associated or partially associated with the walls (Photo VII.19).

VI.4 Discussion

Discolouration on the mushroom cap surface is responsible to a great extent for the loss of quality. It occurs naturally on the cap surface during senescence however the browning is stronger when the cap surface had been subjected to mechanical damage (i.e. handling and transport). Early research has shown that the enzyme tyrosinase is responsible for the browning; it oxidizes mono- and di-phenols into melanin products. It was hypothesised by Burton (1986) that the enzyme and the substrate are present in two separate compartments, the cytoplasm and the vacuole, and after a mechanical damage the integrity of the inner membranes is lost allowing tyrosinase to react on the phenols. Atkins *et al.* (1983) observed the damaged surface of mushrooms under scanning electron microscopy. He found that some exudate was released on the cap surface from damaged hyphae. This exudate is oxidized leaving brown patches on the surface.

This experiment was repeated in our laboratory using hand-damaged mushrooms and mushrooms damaged by compression. The brown areas which occurred after the damage were observed under scanning electron microscopy. It was found on these areas that there was some exudate released on the surface, there were distorted hyphae together with undamaged hyphae. When the cap surface was subjected to

Photo VII.18: Transmission electron micrograph showing dark spot (arrow) associated with the walls.

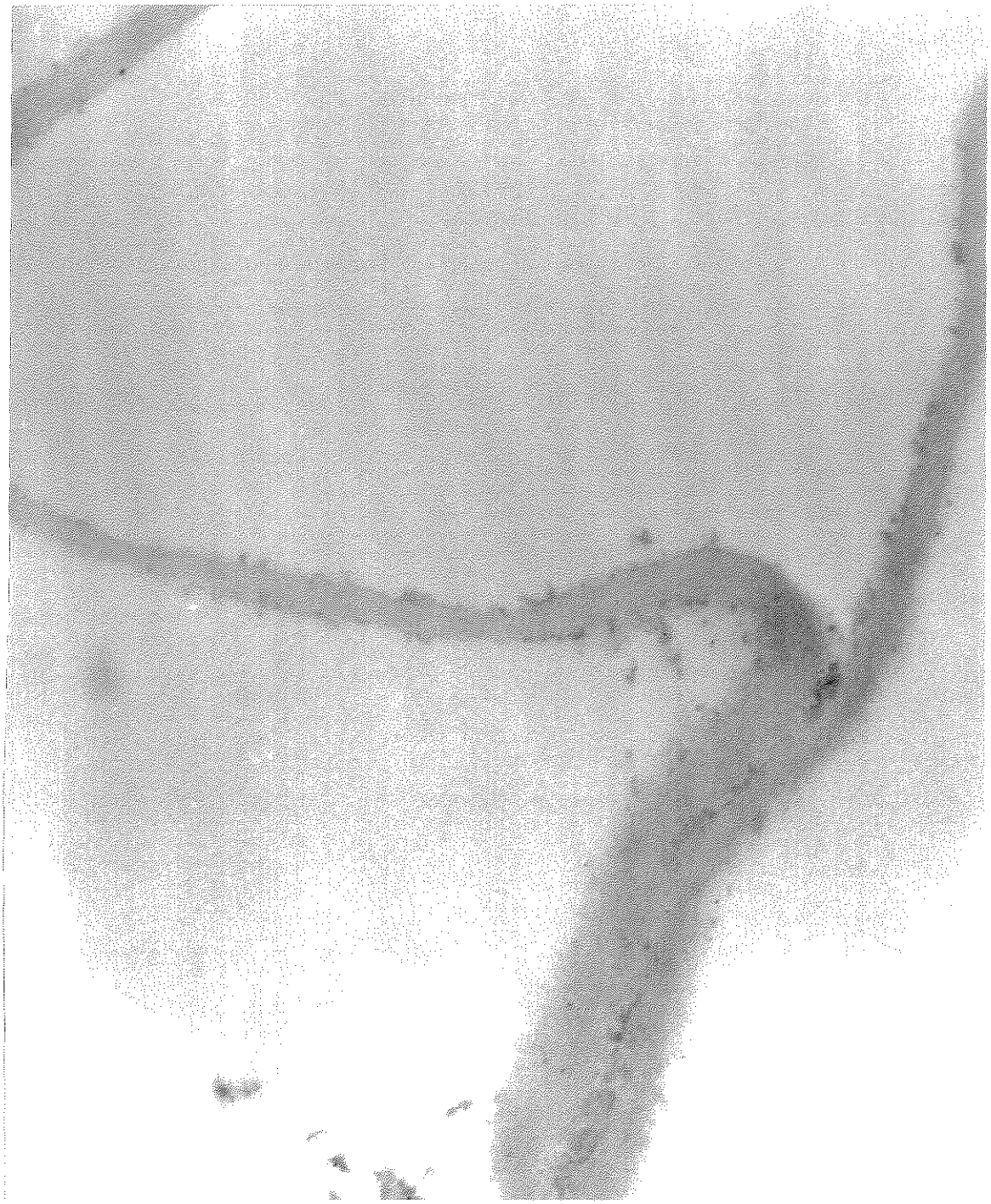
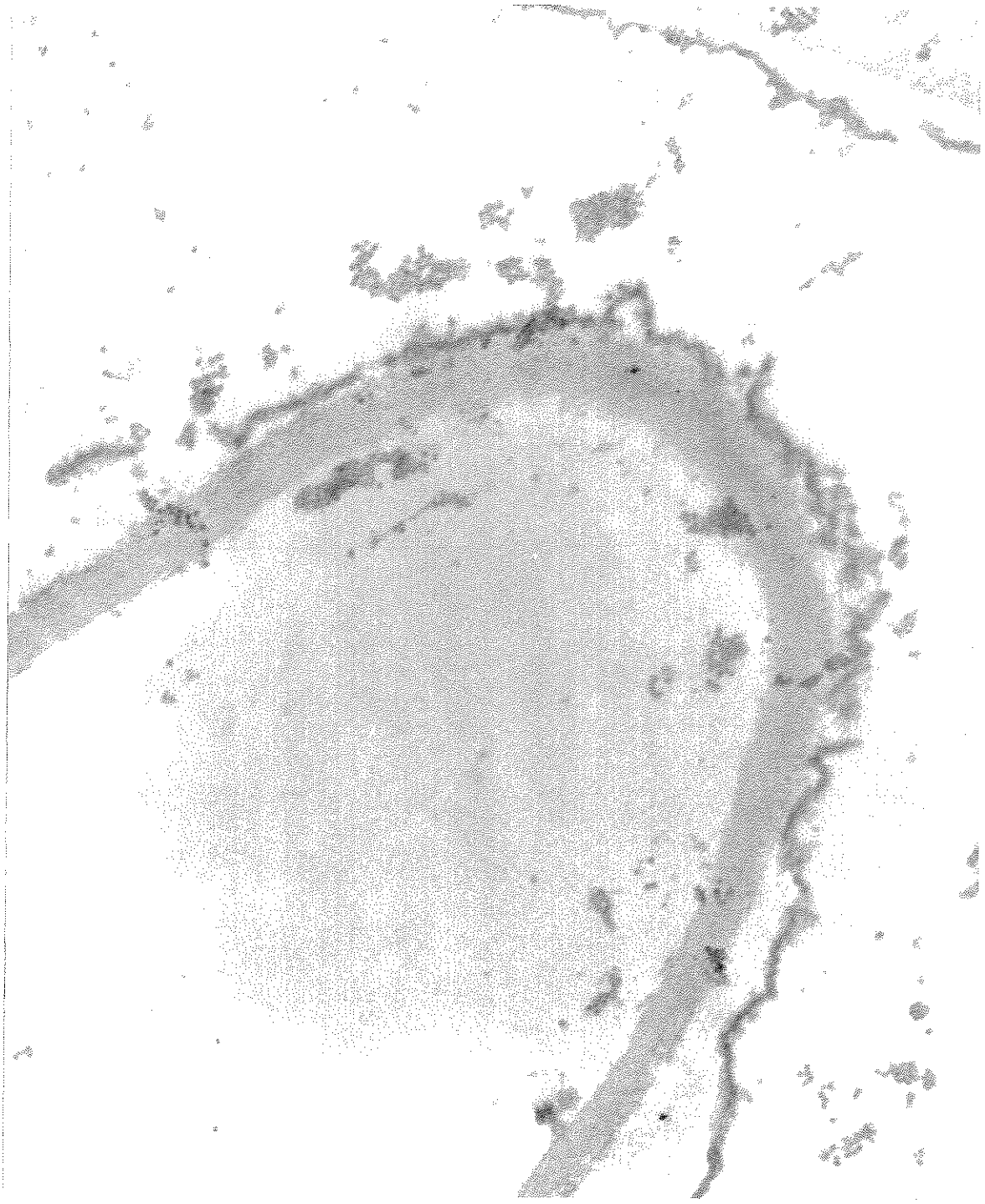


Photo VII.19: Transmission electron micrograph showing a dark layer associated or partially associated with the wall.



compression, it was observed under transmission electron microscopy that the walls of hyphae did not break under the pressure of the compression. The main constituent of cell walls is chitine which probably gives the walls a very strong structure. A compression equivalent to 2kg did not break the cell walls so it is not expected that a slight pressure of fingers on the surface could break the walls.

Thus, mainly intact hyphae were seen on brown surface areas and hyphal walls showed to be difficult to break. There is then a doubt on the hypothesis that discolouration is a result of exudate released from broken hyphae. If this is not the case then discolouration could take place inside the hyphae or could be located on the outside walls of hyphae.

When a drop of DOPA solution (substrate of tyrosinase) was put onto the cap surface, the drop turned pink in a couple of seconds. When the drop was washed away the surface underneath was just slightly pink. This observation and the observations from electron microscopy suggested that the discolouration process could be localized on the cell walls of hyphae.

There is, without doubt, a soluble tyrosinase located inside hyphae (Yamagutchi *et al.*, 1970) but it is probable that this enzyme is not the one involved in the discolouration process on the cap surface.

A preparation of cell walls was carried out in the purpose of isolating the walls and testing them for tyrosinase activity. During the preparation, the pellets containing the cell walls were washed several times and samples were taken in both the washing buffer and in the pellet to be tested for tyrosinase activity. It has been found that the soluble tyrosinase was gradually removed from the pellet; after 10 washes there was no significant tyrosinase activity in the washing buffer. However, a significant amount of tyrosinase activity was detected in the pellet containing the cell walls.

When the isolated walls were put in the presence of DOPA solution, dark aggregates and a dark layer were seen associated or partially associated with the walls.

The conclusion of this work on the discolouration of the cap surface is that there is an insoluble enzyme associated with the walls of hyphae which could be responsible for producing melanin products after a mechanical damage. This is a very important result as it is thought that the enzyme is located inside the cells and all the work carried out on

tyrosinase is done on that enzyme. Further work will be required to isolate and identify this enzyme associated with the walls. There is a hope that in the near future the understanding of the discolouration process will lead to a treatment to prevent the enzyme activation and by consequence the cap discolouration.

B. PART VII

Water uptake in the sporophore

VII.1 Introduction

Waterlogging is an occasional but serious quality problem. At its extreme, waterlogged mushrooms are so soaked with water that water can be seen dripping from the underside of the cap. These mushrooms will have a greyish colour and any form of handling, however gentle, will result in permanent deformation and discolouration. A less extreme form of waterlogging can also sometimes be observed. The symptoms of these mushrooms are small, discrete waterlogged areas with a maximum use of approximately 5 mm across.

A possible cause of waterlogging was water uptake into the mushroom by capillary action *between* the cells. This theory was attractive because (a) the morphology of the stipe (cells orientated in parallel along the length of the stipe) would aid capillary action, and (b) it would explain high volumes of water uptake direct from wet casing. This theory was tested by the use of dyes.

VII.2 Methods and Results

Solutions of dyes were used which did not cross membranes. These solutions were either poured onto casing or mushrooms were carefully harvested with minimum damage and placed into beakers containing dye solution. In either case, dye was not taken up into the sporophore over a 5-day period. When harvested mushrooms were trimmed (lower half of the stipe removed) and placed in the dye, within 20 hours the dye was visible in most parts of the mushrooms.

VII.3 Conclusions

The conclusion of this work then is that it appears that water is not taken up by capillary action between the cells. This infers that water is taken up into mushrooms by the biological route through the cells. The excess water in waterlogged mushrooms must therefore be a consequence of aberrant physiology of the mushroom rather than a direct effect of water in the casing.

C. OVERALL CONCLUSIONS

1. The mechanical properties of the mushroom sporophore have been studied. It is a viscoelastic material. When subjected to a slow compressive force followed by release of the force, the mushroom material shows some elastic behaviour but permanent deformation also takes place. The proportion of elasticity and permanent deformation do not correlate.

2. The morphology of the mushroom sporophore, the packing of the cells, has been studied to provide a basis of explanation for the observed mechanical properties. The mushroom consists of hyphal (tube-like) cells. Four layers of cells have been observed in the first millimetre from the surface of the mushrooms. These layers are distinguished by their orientation, density and intensity of staining by histochemical stains. The outermost layer (the hyphal fringe) is approximately 0.015 mm thick and is largely separated from the rest of the sporophore by extensive air spaces. When the mushroom is subjected to a compressive force, these cells are forced into the air spaces below with little resistance and hence damage is caused. The cells in the layers below the hyphal fringe are radially orientated. When firm mushrooms were compared with soft mushrooms, the firm mushrooms were found to have smaller diameter hyphae, more densely packed and with greater turgor pressure.

3. Mushroom texture is known to vary within and between crops. The effect of agronomic factors on mushroom texture was examined with a view to providing an explanation for the variation in texture and to provide practical information of how agronomy might be used to optimise texture.

   Flush number had a large effect on texture. Second flush mushrooms were the firmest followed by third and then first flush mushrooms. The relative depths of compost and casing were able to affect firmness. Mushrooms grown on deep compost and shallow casing were firm while those grown on shallow compost and deep casing were soft. The carbon dioxide levels in the growing rooms had an inverse effect on texture, ie. the lower the CO_2 level the firmer the mushrooms. The extremes of water potential of casing (ie. very wet or

very dry) led to firmer mushrooms, particularly when using bulk extracted peat.

4. Two methods have been developed to measure the textural properties of mushrooms at the farm. The first involves a procedure of taking the fresh weight and dry weight of a plug of tissue and fitting those data into a formula, which leads to an estimate of the firmness. The second technique is the design of a device to inflict a controlled amount of slip-shear force to cause a bruise.

5. The biochemical basis of the browning reaction involves the oxidation of naturally occurring phenols catalysed by the enzyme, tyrosinase. A new cellular location of tyrosinase has been identified. It is located on the surface of the cell walls.

6. No evidence was found for growing mushrooms to be able to take up water from casing by capillary action between the cells. This theory can therefore be ruled out as a possible cause of waterlogging.

D. RECOMMENDATIONS

1. The major agronomic factors which have been shown to affect texture (compost depth, casing depth, CO_2 level, casing water potential and peat source) should be considered to see if these factors could be altered at the farm level to optimise quality. Obviously these agronomic factors may affect other aspects of the farm's practice and so any beneficial effects on quality would need to be weighed against other possible effects.

2. The formula to estimate mushroom texture using fresh weight and dry weight measurements should be considered as a possible method to include in quality control procedures.

3. The concept of the bruisometer based on controlled slip-shear force looks promising. Further work will be carried out in the HDC funded project M 19a 'Validation of the mushroom bruisometer'.

4. The relationship between agronomy and water uptake should be examined. This will provide information on the mechanism and control of water logging and how to optimise watering practices for quality and yield.

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