Some physiological and biochemical aspects of the action mechanism of fungal parasites during fruit storage.

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During the first phases of infection of apples by *Pezicula malicorticis* the permeability of the tissues is reduced. This results in inadequate nutrition of the parasite. Another important point concerns the organic acids and pH. The buffering capacity decreases with the age of apple tissues (cv. Cox's Orange) especially in storage, while the pH increases, which facilitates fungal invasion. The role of phenolic compounds is not now considered very important. *P. malicorticis* essentially builds up endo-PMG and endo-PG *in-vitro*. The effect of several substances influencing synthesis or the functioning of enzymes is discussed, especially : galactose and polysaccharides, organic acids, esters and alcohols, phenolic compounds, and potassium content. Ripening produces metabolic modifications which enable the parasite to overcome the resistance of the fruit.

MECANISME D'ACTION DES CHAMPIGNONS PARASITES AU COURS DU STOCKAGE DES FRUITS. QUELQUES ASPECTS PHYSIOLOGIQUES ET BIOCHIMIQUES.

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RÉSUMÉ - Lors des premières phases de l'infection des pommes par le *Pezicula malicorticis*, la perméabilité des tissus est réduite. Ce qui entraîne une nutrition insuffisante du parasite. Un autre point important concerne les acides organiques et le pH. La capacité tampon décroît avec l'âge des tissus de pomme (cv. Cox's orange) surtout en stockage, tandis que le pH augmente, ce qui facilite l'invasion fongique. Le rôle des composés phénoliques n'est pas considéré comme véritablement important actuellement. Le *P. malicorticis* produit des endo-PMG et endo-PG *in vitro* d'une manière constitutive. L'effet de plusieurs substances qui agissent sur la synthèse ou le fonctionnement de ces enzymes est discuté, en particulier : galactose et polysaccharides, acides organiques, esters et alcools, composés phénoliques, teneur en potassium. Le mûrissement entraîne des modifications métaboliques qui permettent au parasite de surmonter la résistance du fruit.

Despite intensive and precisely directed plant protection measurements in the fruit growing industry almost everywhere various fruit rots still occur and are responsible for considerable annual losses. Numerous studies strengthen the necessity for investigating the physiological and biochemical interactions in a host-parasite-relationship to elucidate the possible resistance mechanisms.

One of these storage diseases which is particularly important in the northern fruit growing area of Germany is the Gloeosporium fruit rot of apple and pear due to *Pezicula malicorticis* (JACKS.) NANNF. (stat. conid. *Gloeosporium perennans* ZELLER et CHILDS, syn. *Cryptosporiopsis malicorticis* (CORDL.) NANNF. (SCHULZ 1974, 1975). This particular disease gives us the possibility of studying 2 phenomena at once : first the general aspects of pathogenesis in a fruit rot and second, because of the very special life cycle of the fungus, the problem of 'latent infection' which is a special form of resistance. Both phenomena can hardly be separated. Thus I will try to give an integrated picture of the total disease complex covering as well the situation of other fruit rots shortly though there is not very much information available concerning the pathophysiology in fruit diseases.

Before the success of an infection can be seen microscopically by active fungal growth or macroscopically by the appearance of a specific symptom, different variations on the molecular basis must happen within the host, so that the

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development of the parasite will be initiated at all. The nutrients immediately available are exhausted very fast and the parasite needs constant supplement. These substances can only originate from the plant's cytoplasm. This basis is built up by the parasite itself through the production and secretion of toxins and/or membranolytic enzyms. The first reaction can be seen in a change of the permeability of the plasmamembrane. In addition to that the apple fruit itself shows up a characteristic change during ripening which also results in a variation of the permeability.

Normally the permeability of the host tissue is significantly and permanently increased by an microbial infection as numerous examples of bacterioses and mycoses in leaves, stems, roots and tubers demonstrate. There is only one paper of BYRDE, FIELDING, ARCHER and DAVIES (1973) available showing an increase of permeability of the parenchymaceous tissue of apple infected with *Sclerotinia fructigena*.

Under comparable conditions we studied the permeability of apple fruit tissue infected with *P. malicorticis*. The results, however, were different from these obtained with *S. fructigena*. The permeability of the fruit tissue is heavily reduced following natural infection of the apples, as determined by the electrolyte efflux (figure 1). This effect can be measured up to 4 mm from the edge of the rot spot while in the case of *S. fructigena* no change of permeability more than 1 mm in advance of the rot spot can be detected.

In determining the change of the electrolyte permeability during the early phases of pathogenesis artificial inoculations of fruit slices of rips apples have been made. The incubation of this material was made under controlled conditions at 4° C, 10° C and 20°C. The relative change of the conductivity of the test solution after 90 min incubation at 22°C has been determined. Independent of the incubation temperature first an increase of the permeability of ripe diseased apple fruit can be observed ; this is in accordance with the literature, the stimulation, however, never exceeds 30 % over the noninoculated control fruit slices. This change can be seen earlier than any symptom. The duration of the phase with increased permeability, however, is in strong correlation with the incubation temperature, it is the shorter the higher the temperature is. At 20 C incubation the conductivity values of the test medium with the diseased material fall below the control data already after 20 h. Exactly the same pattern can be observed at lower incubation temperatures, the phase of stimulation, however, is extended. The reduction of the permeability will be obtained after 4 days incubation. In every case it is reduced to 50 % of the control. Because of the strict temperature dependence the effect must be assumed to be a toxigenic, actively physiological process. Of much greater interest, in particular in the context of 'latent infection'

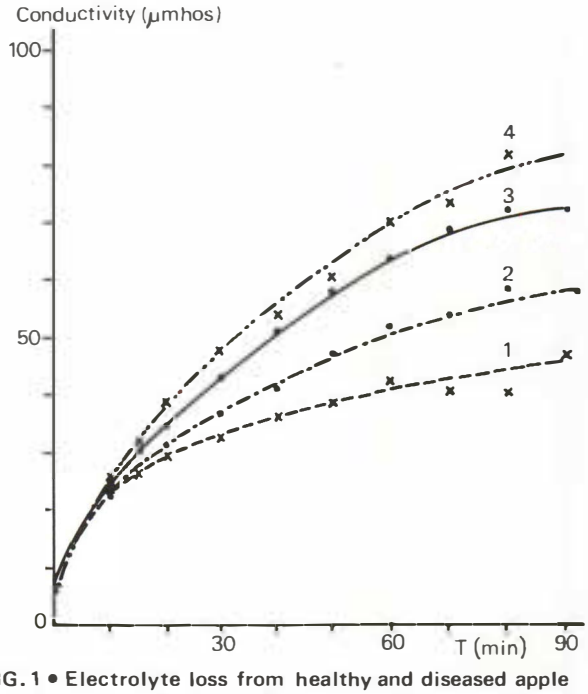

FIG. 1 • Electrolyte loss from healthy and diseased apple fruit tissue (cv. Cox Orange).
1 . Fruit tissue naturally infected by Pezicula malicorticis
2 . 0-2 mm surrounding healthy area
3 . 2-4 mm surrounding healthy area
4 . Healthy tissue of noninfected fruit

are the same experiments using slices of unripe fruits. The results clearly demonstrate that the initial increase in permeability following infection of the apples with *P. malicorticis* does not occur. On the contrary the fruit tissue immediately reacts with the reduction of the electrolyte loss.

Basing on this host reaction, in addition to the weak growth character of *P. malicorticis*, we assume that following a successful infection the deficit of nutrients is responsible for the immediate stop of fungal and disease development during vegetation. This idea can already be read with EDNEY (1956) assuming that the nutrients start diffusing into the lenticel cavities during fruit ripening and not earlier, to reactivate the growth of the parasite.

Following KNEE (1973) one can suppose that the cross binding between the wall polymers are splitted by the parasite as during the normal ripening process. This decomposition, however, occurs because of insufficient conditions within the fruit tissue only slowly and incompletely so that a swelling of the plant's cell wall can be observed resulting in a gellike condition. Thus a passage of substances which are essential for the further development of the parasite is not possible.

This seems a new idea in explaining the phenomenon of 'latent infection' though the aspect of insufficient nutrient

supply often has been mentioned but never has been demonstrated. For the amount of carbohydrates, for instance in an unripe apple, always is in the range for sufficient growth of the fungus. In accordance with numerous data of the literature we found that the amount of reducing sugars as well as total carbohydrates constantly increases during vegetation. During the later storage there is a further slight increase in reducing sugars which possibly will be of importance in the later stages of pathogenesis.

There is still another idea which should be mentioned as a possible explanation for the differential behaviour of *P. malicorticis* on one hand and *Sclerotinia* or *Penicillium* on the other hand. As demonstrated *Pezicula* reduces the membrane permeability while the other parasites increase the permeability ; possibly there exists a correlation to symptom extention. While *Pezicula* reacts with a limited rot spot, both the other fungi cause a total rot of the fruit.

A further point of interest for disease development is the content of the fruit of organic acids in connection with pH of the tissue. The pH shows a typical behaviour independent of the apple variety studied. Immediately after petal fall it is relative high with a value of 5-6 ; then it decreases very fast to values of about or below 3, so that no fungus development can proceed. With increasing physiological age of the fruit the pH increases as well, particularly expressed during storage. Various factors influence this behaviour.

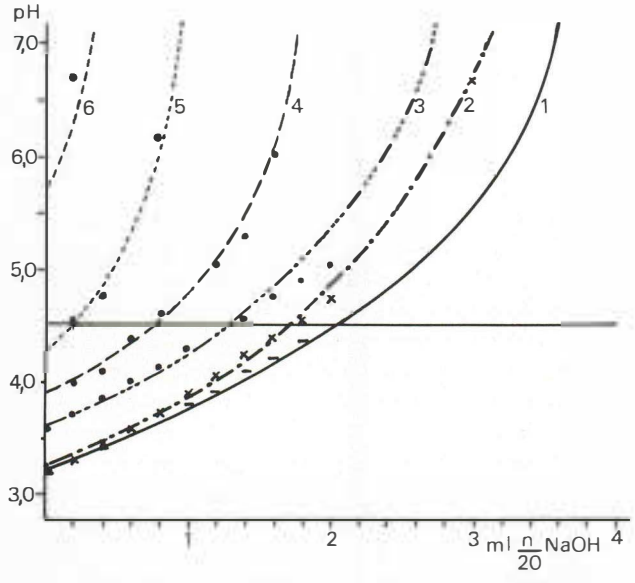

FIG. 2 • Buffer capacity of fruit tissue and callus homogenates (cv. Cox Orange)
Harvest date : 1. September 9, 1974
2. September 17, 1974
3. October 6, 1974
4. October 6, 1974 ; 16 weeks, storage at 4°C
5. October 6, 1974; 16 weeks, storage at 8°C
6. Callus, 6 weeks old.

As a measure of the free acidity SCHOLZ and STEPHAN (1974) regard the buffer capacity which they graphically express by the area below the optimal pH line for the fungus development up to the intersection point with the titration curve for the special case. These studies have been made under various considerations in our laboratory with the Pezicula-apple-relationship (fig. 2). We can observe that the buffer capacity of the fruit tissue (cv. Cox's Orange) decreases with increasing age. This effect can already be recognized with fruits on the tree ; it is even more evident during storage. The storage temperature is of much importance for the dynamic of the pH development.

If one correlates these observations with the pathophysiological behaviour of *Pezicula malicorticis*, other new aspects in the discussion of 'latent infection' and rot development in this disease can be obtained. The pH of the physiologically young fruit is in the optimal range for the development of the parasite ; because of the reduced permeability which is equivalent to the insufficient nutrient supply there is no fungus growth and no symptom expression. With increasing physiological age of the fruit the pH decreases considerably. Simultaneously the fruit has a high buffer capacity so that the parasite is absolutely unable to degrade the fruit parenchyme under field conditions starting the rot symptom. This happens during ripening, when the buffer capacity is reduced and the pH increases.

By applying this hypotheses some phenomena of the disease can be explained. Considering that the buffer capacity of fruits from the same trees varies from year to year (fig. 3), this result parallels exactly with the disease severity in the respective years. Fruits from the 1971 vegetation show a rotting of 2.2 %, while those from the 1974 harvest have a rotting of 26.0 %. Finally a correlation between the buffer capacity and the susceptibility of the apple varieties can be demonstrated (fig. 4). The buffer capacity of the very susceptible variety 'Cox Orange' is much less expressed under comparable conditions than the capacity of the less susceptible variety 'Boskoop'. The importance of pH for pathogenesis and disease development has only been studied to a limited extent and needs further investigation. In this disease complex the pH seems to be the key for various triggering mechanisms.

A further complex which often has been studied in diseased plants and which has been correlated to disease resistance is the phenol metabolism. In particular apple fruits are rich in phenolic compounds which are mainly located in the fruit skin. During vegetation a considerable quantitative und qualitative decrease of the total phenolics in the apple skin can be demonstrated (NEVERMANN 1974). During the following storage no further decrease occurs. In discussing the Gloeosporium fruit rot various reports exist which show only a secondary role of phenolic compounds for disease genesis. In toto the content of the fruit phenolic

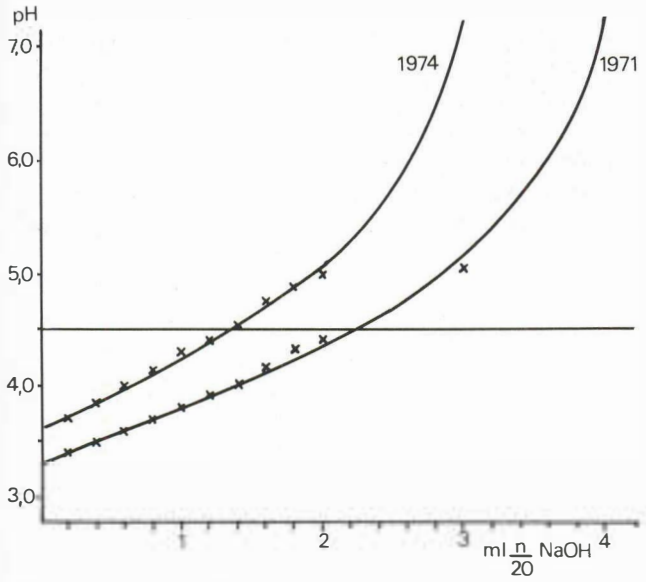

FIG. 3 • Buffer capacity of fruit tissue from different vegetations Harvest date : October 3, 1971 and October 8, 1974.

substance is too low or one must assume a concentration of certain phenolics at definite locations in the fruit skin. Our investigations demonstrate, however, an even distribution of the rot spots all over the fruit surface. EDNEY (1964) working since a long time with this disease in England showed that there is no difference in the spread of a single rot spot between a red and a green part of the fruit surface. JACKSON et al. (1971) even have maintained from their field experiments that fruits colored deeper red from the external part of a fruit tree are more susceptible to the parasites of the Gloeosporium fruit rot. The same result can be

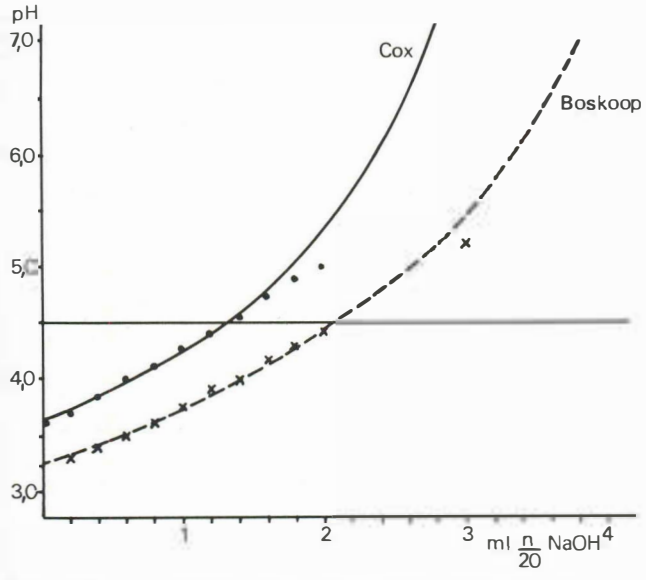

FIG. 4 • Buffer capacity of fruit tissue from differentially susceptible cultivars.

seen from ATKINSON (1974) who described a correlation between the position in the fruit yard and susceptibility of the fruit in that sense that the wider stand of the trees causes better coloration but simultaneously higher susceptibility. Thus the importance of phenolic compounds in situ must at least be regarded as uncertain as VERHOEFF (1974) in this review on the latency in fungi has expressed.

A key position in the genesis of plant diseases is often given to various extracellular enzymes, in particular pectinases, cellulases, proteases. Some of these enzymes have been studied intensively in our department as well as by EDNEY (1964) and BOMPEIX (1972). First of all the pectinases which are responsible for the decomposition of the cell wall constituent pectin and which are subdivided in various single enzymes depending on the complexicity of the substrate, the action point at the polymer and the action mechanism.

A systematic testing of the enzyme spectrum demonstrated that *P. malicorticis* has only a limited pectinolytic activity compared to *Penicillium* or *Sclerotinia* by producing only endo-PMG and endo-PG in our experiments. Both enzymes can be obtained constitutively on a minimal medium in vitro. The enzyme formation is not correlated with the hyphal growth as can be demonstrated by viscosimetric or electrophoretic testing of the culture filtrates. There is also no correlation between the enzyme production and the rot spot development of various isolates of *Pezicula*. These results allow the conclusion that the genetic ability of this parasite for the formation of one or several pectinolytic enzymes alone cannot be used for explaining 'latent infection' and rotting.

The formation of all pectinases strongly depends on the C source as the regulating agent (SCHULZ, 1972). Under the influence of monosaccharides the production of endo-PMG is less expressed than after using polysaccharides. The maximum enzyme formation is obtained after 18 days growth. Surprisingly the endo-PMG production is rather poor by using galactose and sorbitol. The same situation can be seen in the production of endo-PG. Here galactose does not allow any enzyme formation. Galactose therefore must be regarded as a specific enzyme inductor. This fact could be of particular importance in the Gloeosporium fruit rot since galactose represents the most important sugar component of those glycosides in the apple skin which easily can be decomposed by fungal parasites. On the other hand the galactose content decreases autolytically during ripening so that the inhibiting effect on enzyme production slowly is reduced. A similar situation has been described by KEEGSTRA, ENGLISH and ALBERSHEIM (1972) for *Colletotrichum lindemuthianum*. Increasing amounts of monomers in the growth medium demonstrated that *Pezicula* did not react on a fivetimes raised carbohydrate content by stimulating endo-PMG production in contrast to *Glomerella cingulata*,

Sclerotinia or *Penicillium* spp. thus characterizing *Pezicula* as the cause of a low-sugar-disease.

Besides the various sugars other organic substances occurring in the fruit also act regulating on the endo-PMG production of *P. malicorticis* like organic acids, esters, alcohols or phenolic substances. The importance of the latter is disputed. This doubt can still be confirmed by the fact that the formation of endo-PMG is stimulated by chlorogenic acid in the basal medium. Under these conditions in particular the start of enzyme production is accelerated ; simultaneously the hyphal growth is improved. A similar observation has been published by WAKIMOTO et al. (1958) for *Gloeosporium kawakami*.

Finally the inorganic elements show a regulating effect on the production of the pectin degrading enzymes. This seems particularly true for potassium which at least in the early stages of pathogenesis has an evident influence on the endo-PMG formation (SCHULZ, 1976). This result can be regarded conincidental with reports of higher disease incidence and rotting following additional K fertilization. At the moment, however, there is no specific explanation at hand for the increased rotting under these circumstances. It is assumed that the higher rate of rotting depends on the production of larger fruits following the manuring. However, we never found a positive correlation between fruit size and disease incidence of *P. malicorticis* under field conditions.

It is concluded from results presented here that K nutrition acts indirectly on the susceptibility of the apple fruit by altering the carbohydrate and cell wall metabolism. It is known that low K content of a cell or tissue correlates with high sugar content and vice versa. And furthermore, if there is an imbalance between K and Ca because of the additional K manuring, the structure of the cell wall may be different from normal so that the fungus attack is actually facilitated.

Summing up this part we can say that the carbohydrates, in particular the amount of monomers, plays the most important role in the formation of endo-PMG which according to our results is the most essential catabolic enzyme for fruit rotting in this disease complex.

The regulation of the enzyme production by the presence and relation of nutritional substances is directed by further endogenous and exogenous factors. In particular the pH of the fruit changes during its development into a range where enzyme production is initiated (fig. 5). At pH 2.5 which is dominating during fruit growth, no hyphal growth can occur so that no enzyme production is possible. During ripening the pH is changed and enzyme formation immediately starts. The fundamental importance of the pH for the formation and secretion of pectinolytic enzymes as well as pathogenesis is also described by TANI (1967) for *Gloeosporium kaki* which rots kaki fruits.

As the third enzyme of the pectinase group PME must briefly be mentioned. This enzyme is able to precondition the complex pectin for the degradation by PG. The enzyme is produced by all fruit rotting fungi including *P. malicorticis*. We hare seen, however, that the conditions for the formation of PME are very limited. The enzyme could only be produced at pH 3.5 (KOCH, 1975).

The most important exogenous factor influencing enzyme formation in *P. malicorticis* is without doubt the temperature (fig. 6). These results show most interestingly that at 4°C after a long lag phase a constant increase in the production of endo-PMG can be obtained, while the fastest enzyme formation occurs at 12°C. These values surprisingly correlate with the development of the rot spots which is also optimal at 12°C while the hyphal growth is best at 20°C. These data let assume again that the endo-PMG actually plays a key role in the pathogenesis of *P. malicorticis*.

The influence of the incubation temperature on the production of the endo-PG shows a totally different picture. Even after 5 weeks incubation at 4°C there is no enzyme formation measurable. From these results the greater importance of *P. malicorticis* compared to *Glomerella cingulata* as a storage pathogen is evident.

Without discussing the in vivo production of the various catabolic enzymes in detail, figure 7 gives the typical distribution pattern shown as the relative decrease in viscosity for endo-PMG, endo-PG and β-glucanase. In the center of a rot spot as well as in the 5 mm outer margin all 3 enzymes can be detected showing rather high activities. The great importance of endo-PMG depends on the incubation conditions in the cold storage, which means at low temperatures. The most interesting point in this result is that in the healthy area 5 mm in front of the rot spot the pectin as well as the cellulose degrading enzymes can be determined. This completely agrees with the results of BOMPEIX (1972) shown for *P. alba* and *P. malicorticis*. The endo-PMG from the healthy fruit tissue is without any doubt of fungal origin. Our own investigations as well as some other new papers clearly demonstrate that there is no endo-PMG or endo-PG in the healthy fruit tissue. Since the mycelium of *Pezicula* never grows out of the rot spot the distribution pattern of the enzymes can only be explained by the diffusion of the secreted enzymes into the healthy area. The same behaviour could recently be demonstrated for *Sclerotinia fructigena* in peach by HALL (1971) and for *Sclerotinia* in apple by BYRDE and al. (1973). The fruit tissue in front of a rot spot shows a dramatic metabolic change depending on the parasite attack.

In summarizing the results presented here the following point must be mentioned.

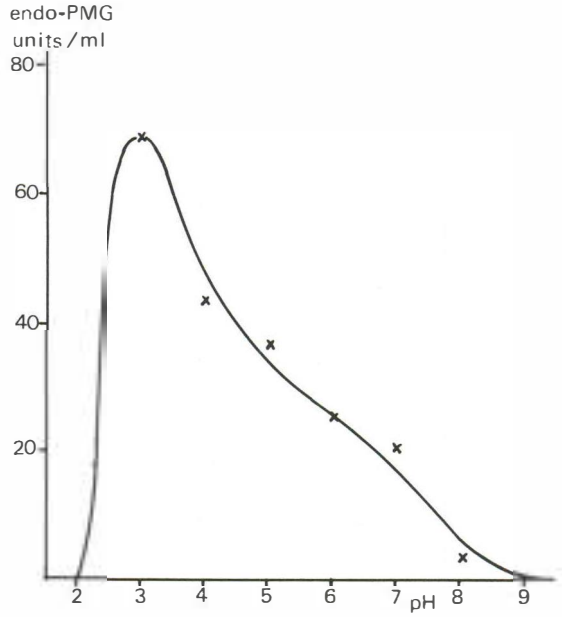

FIG. 5 • Influence of pH of the nutrient solution on the production of endo-polymethylgalacturonase (endo-PMG) by Pezicula malicorticis.

- *Pezicula malicorticis* is able under suitable conditions to produce and secrete a number of enzymes which are of importance for pathogenesis ; mycotoxins with phytotoxic properties sensu stricto are not produced in contrast to *Glomerella, Sclerotinia* and *Penicillium*.

- During vegetation the activity of various enzymes of the fruit is inhibited because of the physiological and the intracellular compartimentation of the fruit ; in addition the formation of the catabolic enzymes of the parasite can be stopped so that no symptom expression occurs.

- Ripening is characterized by a metabolic change of the fruit which is a prerequisite for overcoming the resistance of the fruit against *Pezicula*. This can be seen in the production of PME, the reduction of the buffer capacity and the splitting of monomers from glycosides so that a sugar pool originates.

- Following this change the fungus is able to reactivate and to start enzyme production ; simultaneously the action sites for the enzymes are built up so that the symptom expression can start.

- By synergistic action of the glycosidases from the host and the parasite during storage an oversupply of monomeric sugars can originate so that the production of the

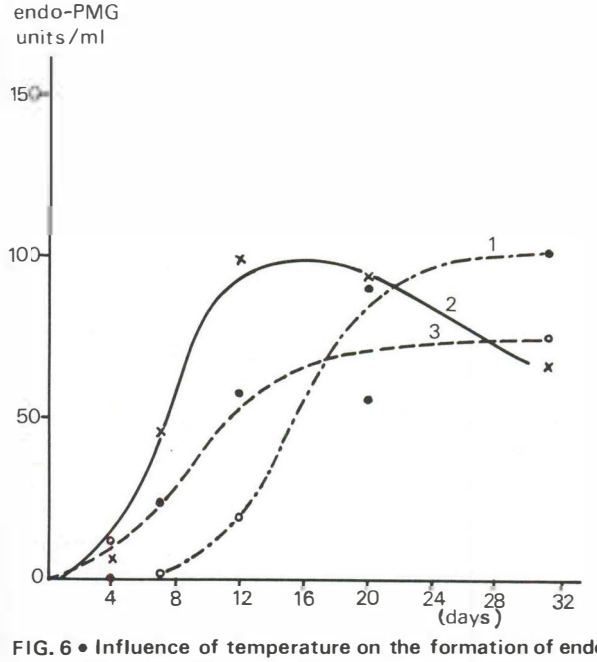

FIG. 6 • Influence of temperature on the formation of endo-polymethylgalacturonase by Pezicula malicorticis.
1. Incubation at 4°C
2. Incubation at 12°C
3. Incubation at 22°C

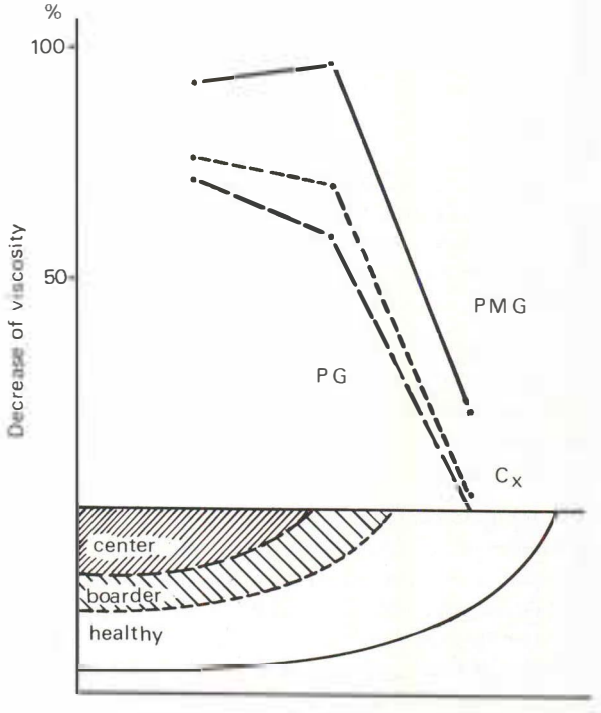

FIG. 7 • Distribution of endo-polymethylgalacturonase (endo-PMG), endo-polygalacturonase (endo-PG) and endo-ß-1,4-glucanase in a rot spot caused by Pezicula malicorticis and the surrounding healthy apple fruit tissue (cv. Cox Orange).

catabolic enzymes in particular endo-PMG is stopped again. This means that the symptom expression comes to an end again.

In toto we can say that the *Pezicula*-apple relationship represents a very complicated pathophysiological situation which primarily consists in the metabolism of the fruit independent of the variety used. This can be the reason for very difficult and almost unsuccessful breeding programs in increasing fruit resistance against *P. malicorticis* and related fungi.

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