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Metabolic Pathway Responsible for the Production of Elemental Sulfur by *Phomopsis viticola*

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Elemental sulfur accumulates in the cirrhus of the fungus *Phomopsis viticola* Sacc., where it acts as self-inhibitor of alpha spores germination. A highly concentrated suspension of alpha spores (more than 10^{12} spores/ml) produces hydrogen sulphide (PEZET, 1975; PEZET and PONT, 1975). Accumulation of sulfur seems to be a general phenomenon for fungi, particularely for microorganisms which produce dormant spores, chlamydospores or sclerotia (PEZET and PONT, 1977). The metabolic pathway responsible for sulfur production in rat liver was studied by KUN and FANSHIER (1959). They showed that an enzyme was able to desulfurate the β-mercaptopyruvate with a concomitant production of pyruvate and sulfur. Nevertheless, *in vivo* no accumulation of sulfur is mentioned. According to these authors, sulfur is transformed into thiosulfate and eliminated by the kidneys. *P. viticola* produces elemental sulfur and accumulates it by the same metabolic process. In this paper, a possible role of sulfur as an energy level regulator during the biological cycle of the fungus is discussed.

Material and methods

Organism and culture media.

The strain of *P. viticola* employed was isolated from a grapevine shoot. A single spore culture was derived from this isolate and grown on malt agar medium. It was designated: *P. vit. VIN 1 (30).* The isolate was cultivated on a synthetic medium of the following composition: KH_2PO_4 (lg), NaCl (0,5 g), $CaCl_2 \cdot 2\ H_2O$ (o,1 g), $FeCl_3$ (5 mg), $MgSO_4$ (1 g), glucose (10 g), maltose (10 g), saccharose (5 g), KNO_3 (5 g), oligoelements (1 ml) (WESTERGAARD and MITCHELL, 1947),

H_2O (1000 ml). The pH was adjusted to 5,5 with NaOH 1 N, before autoclaving. The medium was then poured in 100 ml Erlenmeyer flasks with 50 ml per flask (medium L), or mixed with 25% of microcristalline cellulose (medium C), or mixed with 1,2% of Oxoid agar (medium A). Media A and C were poured into Petri dishes. Medium L was inoculated with 1 ml of a suspension of alpha spores in sterile distilled water (about 10^5 alpha spores / mm^3) and media A and C were inoculated by pouring 2 ml of the same suspension onto the surface of each medium. ^{35}S-$MgSO_4$ was incorporated into medium C (1,1 mCi, 1051 mg of ^{35}S-$MgSO_4$, 0,126 μCi / μmole) and medium A (1,1 mCi, 1005 mg of ^{35}S-$MgSO_4$). Medium A agar contained sulfate (0,6–0,7%) and the initial specific activity for ^{35}S-$MgSO_4$ was 0,102 μCi / μmole. Cultures in medium L were incubated at 22°C (± 2°C) under 12 hours alternated light (Philips TL / 40–55) and with continuous shaking. Cultures on media A and C were incubated at 22°C (± 0,5°C) under 12 hours alternated light (Sylvania Cool White F 14 T12 CW).

Chromatography

Thin-layer chromatography (T LC) was done on commercial plate of silica gel 5 x 20 cm (Merck 60 F_{254}), with the following solvent system:

A: chloroform – methanol – NH_3 8 : 7 : 3 (15 cm)
B: chloroform – methanol – NH_3 4 : 6 : 4 (15 cm)
C: chloroform (5 cm) and after drying, cyclohexane – chloroforme 99 : 1 (10 cm)

Elemental sulfur was revealed by triphenyltetrazolium chloride, standard β-mercaptopyruvate by natrium periodate – benzidine, and cysteine by ninhydrine – copper nitrate (KIRCHNER, 1967). Radioactive products chromatographed on TLC, were detected with a Geiger-Müller counter (Actigraph III, Nuclear-Chicago). These products were scratched off and their radioactivity counted directly by liquid scintillation (diethylene glycol monoethyl ether toluene / PPO – POPOP) without elution (Unilux, Intertechnique). The amount of determinated products were small and the counts obtained with the radioactive assays were approximations. The utilization of ^{35}S was essentially useful in obtaining qualitative results.

Extractions

The compact layers of mycelium and pycnidia formed on medium C by *P. viticola* were washed with distilled water, dried at 40°C, pulverized in a mortar, and extracted with chloroform – methanol (1:1). The resulting extract was chromatographed on a column of silica gel in chloroform (20 cm x 2,5 cm). The eluted fraction (250 ml) was then chromatographed on a silica gel column (20 cm x 2,5 cm) in cyclohexane-chloroform (99:1). One hundred samples of 3,5 ml were collected. Elemental sulfur separated out in the fractions 25 to 35. On medium A it was not possible to separate the mycelium from the medium. Therefore, agar was mixed with the fungus in 30% ethanol. This extract contained elemental sulfur and a compound chromatographically identical with cysteine, which interfered with any further enzymatic reaction (solvent A). The residue was suspended in a mixture of chloroform-methanol (1:1) and extractions were made with continous shaking for 4 hours. The filtrate of the last extraction constituted the C/M extract. This extract contained β-mercaptopyruvic acid and was always kept cool and in darkness to prevent degradation of the compound.

Extraction and enzymatic reaction

An enzyme with the ability to carry out the desulfuration of β-mercaptopyruvate was obtained from rat liver (KUN and FANSHIER, 1959). 2-mercaptoethanol was not used as a stabilizing agent because it reacted with the elemental sulfur produced by the enzymatic reaction. The enzyme used in these reactions was the crude extract designated F_{II} by these last authors. This enzyme was isolated from cultures of *P. viticola* grown on medium L. For each extraction 5 mycelial cultures were combined and the enzyme was extracted using the same procedure. The end products of enzymatic desulfuration of β-mercaptopyruvate are pyruvate

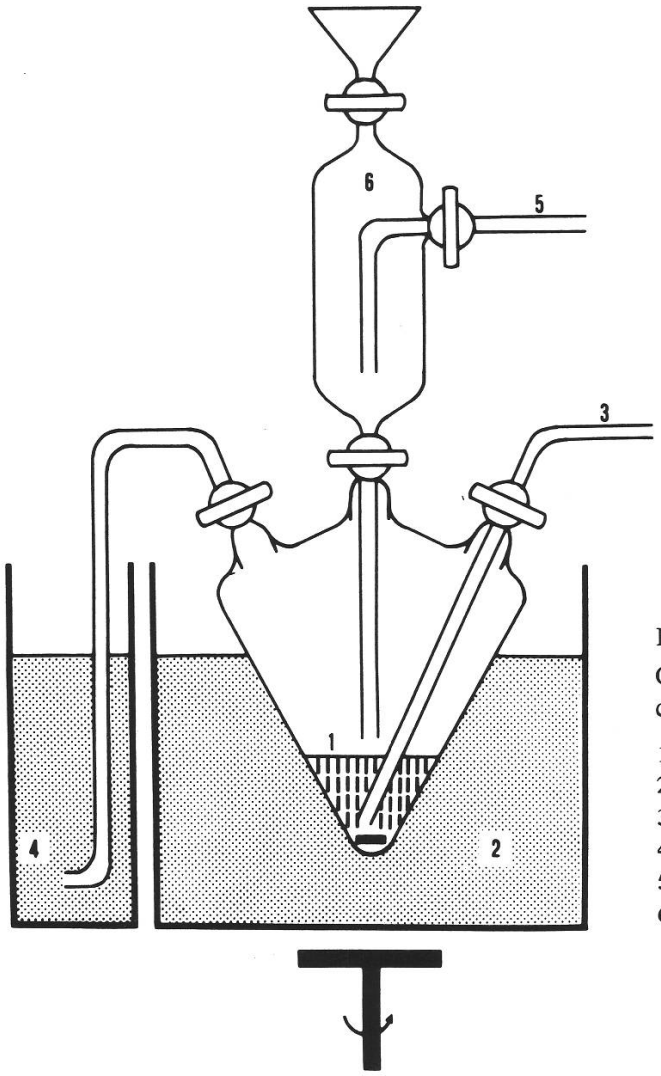

Fig. 1:
Glass-apparatus to analyze quantitatively elemental sulfur (S^o)

1. Solution to be analyzed
2. Thermostatically controlled water-bath
3. Tube for introduction of gas
4. Hydrogen sulfide trap
5. Tube for introduction of gas
6. Reagents introduction chamber

and elemental sulfur. Enzymatic activity was measured by the determination of these products. The method of BERNTSSON (1955) was used to determine the amount of pyruvate. Elemental sulfur precipitates during the enzymatic reaction and forms a sulfur-protein complex if no acceptor, such as cysteine, mercaptoethanol, or reduced glutathion is present (MEISTER et al., 1954). Sulfur can be extracted from the complex by CS_2 or hot methanol. However, in any case, about 40% of the sulfur remains attached to the insoluble portein residue. If this residue is treated by a 0,25 N cysteine solution, a H_2S emission takes place. H_2S was captured in a solution of zinc acetate and the zinc sulfide formed was evaluated by the method of FROMAGEOT (1947). In the case of radioactive material (^{35}S), the ZnS produced by this reaction was measured directly by liquid scintillation. In view of the difficulties of extraction of elemental sulfur from the insoluble protein residue formed after the enzymatic reaction, it is easier and more precise to make the reaction in the presence of cysteine in the apparatus described in Fig. 1, so that a concomitant emission of H_2S occurs. We were able to visualize the reaction by producing it in the apparatus described by PEZET and PONT (1977). This resulted in the formation of black lead sulfide. When the products of the enzymatic reaction are radioactive, the black lead sulfide is also radioactive and can be estimated by fixing the paper disk on a glass plate and counting the radioactivity in an Actigraph III counter against a standard product with known radioactivity. The measurement of pyruvate presented some difficulties even the β-mercaptopyruvate is precipitated with $HgSO_4$ according to the method of MEISTER et al. (1954). However, the results of pyruvate measurement presented in this paper show that this compound is formed during the enzymatic reaction. It is nevertheless more precise to establish the enzymatic activity by measuring free sulfur rather than pyruvate.

Synthesis of ammonium β-mercaptopyruvate

Synthetic ammonium β-mercaptopyruvate, used for certain enzymatic reactions, was prepared according to the method of KUN (1957). Its spectral picture, as shown by IR spectroscopy with KBr, was comparable to that obtained by KUMLER and KUN (1958). Dissolved in phosphate buffer (0.01 M, pH 7,9) this compound in UV spectrophotometry exhibits a band at 290 nm (KUN, 1957). The melting point of the 2, 4-dinitrophenyhydrazone of β-mercaptopyruvate is $159^0 - 161^0$C. KUN (1957) gives $161^0 - 162^0$C for the same product. The IR spectra of this derivative gives: 3080 cm^{-1} (OH), 2580 cm^{-1} (SH), 1680 cm^{-1} (C = O), 1620 cm^{-1} (C= N). Ammonium β-mercaptopyruvate was kept in a cold dark room and used for enzymatic reactions at 10 μmoles/ml.

Determination of desulfuration activity and accumulation of sulfur during the growth of P. viticola.

The fungus was allowed to grow in liquid medium L and five cultures were taken periodically to determine the dry weight and elemental sulfur. These cultures were pounded in chloroform-methanol (1:1) and free sulfur was measured in ethanol by the method described by FROMAGEOT (1947) with the apparatus shown in Fig. 1. The enzyme was extracted as described previously and enzymatic activity determined by measuring the H_2S produced by the action of enzyme (F_{II}) on synthetic ammonium β-mercaptopyruvate in the presence of cysteine.

Results

From cultures of *P. viticola* on medium C + $^{35}S - MgSO_4$ (27 Petri dishes = 1 liter of medium) 3 μg of radioactive sulfur were isolated. Elemental sulfur was produced by *P. viticola* and conforms with previous results (PEZET and PONT, 1975, 1977). The C/M extract obtained from cultures on medium A (27 Petri dishes = 1 liter of medium) contained β-mercaptopyruvate-^{35}S. This acid was detected by co-chromatography with authentic product (system B). It was not very stable and formed decomposition products visible at R_f = 0,69 and 0,71, but no disulfide. β-mercaptopyruvate was located at R_f = 0,67.

This result was confirmed by enzymatic reactions. The radioactivity for cysteine (system A, R_f = 0,43) was not detected. The count of radioactivity in the C/M crude extract was 225.000 dpm, theoretically equal to 195 μg (1,62 μmoles) of β-mercaptopyruvate (desintegration of ^{35}S during 60 days = 0,063 μCi/μmole). The radioactivity of this compound is 6,5% of the C/M crude extract. The difference is explained by two unknown products occuring at R_f = 0,30 and 0,48. For calculations we used the relation which gives the activity N after a period t (in hours): $N = N_O \cdot e^{-0,00033\ t}$. To test the ability of β-mercaptopyruvate to produce pyruvate and sulfur, β-mercaptopyruvate desulfurase extracted from rat liver (F_{II}) was used. The presence of this enzyme in extract F_{II} was shown by the UV-visible spectrum and enzymatic test. The absorption bands at 420 nm and 275 nm (KUN and FANSHIER, 1959; FANSHIER and KUN, 1962) were present. The enzymatic test using synthetic

Table I:

Sulfur produced by enzymatic reactions between F_{II} from rat liver and synthetic β-mercaptopyruvate in the presence of cysteine (0,25 N, pH 6,8).

Reactions	µg S^o produced
β-mercaptopyruvate + F_{II} + cysteine	101
β-mercaptopyruvate + cysteine	2,5
F_{II} + cysteine	0

β-mercaptopyruvate and F_{II} (rat liver) also showed that β-mercaptopyruvate desulfurase was present in F_{II} (Table I). β-mercaptopyruvate is a labile substance (KUN, 1974) and the small amount of sulfur produced without F_{II} is probably due to decomposition of the substrate. The C/M extract from *P. viticola* contained β-mercaptopyruvate but no cysteine and sulfur (determination by TLC). After the action of β-mercaptopyruvate desulfurase (F_{II} from rat liver) on an aliquot of the C/M extract containing 0,32 µmoles of β-mercaptopyruvate, 0,80 µg (8%) of free radioactive sulfur (3500 dpm, 0,063 µCi/µmole) was identified. This result was confirmed by a count of ^{35}S-H_2S produced by a similar reaction from 0,064 µmole of β-mercaptopyruvate (C/M extract) but in the presence of cysteine. The resulting ^{35}S-H_2S was captured on a filter paper disk impregnated with lead acetate and counted with a Geiger-Müller Actigraph III. By this means we were able to identify an amount of H_2S corresponding to 0,15 µg of free sulfur (7%, 700 dpm, 0,069 µCi/µmole after 50 days). On account of the difficulties of the method of analysis (see Materials and methods), the amount of pyruvate determined was much too high (x 400 approximatively). However, the results confirmed that the C/M extract contained β-mercaptopyruvate and that the action of β-mercaptopyruvate desulfurase (F_{II} from rat liver) can produce free sulfur and pyruvate (Table II).

Table II:

Production of ^{35}S-H_2S and pyruvate during the enzymatic reaction between β-mercaptopyruvate (C/M extract form *P. viticola*) and β-mercaptopyruvate desulfurase (from rat liver).

	Reactions of β-mercaptopyruvate[a] (C/M extract) with		
	Cysteine[b]	F_{II}[c]	F_{II}[c] + cysteine[b]
dpm ^{35}S-H_2S produced	0	0	700
mg of pyruvate produced	0	0,19	0,13
H_2S disk-test[d]	neg.	neg.	pos.

a 0,064 µmole
b 0,25 N, pH 6,8
c from rat liver
d neg. = no H_2S produced, H_2S disk-test is white
pos. = H_2S is produced, H_2S disk-test is black

β-mercaptopyruvate desulfurase from rat liver was used previously to identify β-mercaptopyruvate in the C/M extract. Now, in order to show the presence of β-mercaptopyruvate desulfurase in *P. viticola,* this enzyme was extracted from 8 and 13 days old cultures (Medium L, 5 Erlenmeyer flasks). The desulfuration activity was determined by calculating the amount of free sulfur produced from synthetic β-mercaptopyruvate. 11,5 and 42,5 μg of free sulfur were produced respectively. These positive reactions showed that β-mercaptopyruvate desulfurase is present in *P. viticola.* It was of great interest to know the evolution of this desulfuration activity and the accumulation of free sulfur during the life cycle of *P. viticola.* The results (Fig. 2) illustrate that there are two important growth stages. The exponential growth period of the fungus (5–12 days from the inoculation) with a high desulfuration activity, without accumulation of free sulfur, and the differentiation period (formation of the pycnidia) with a low enzymatic activity and an important accumulation of sulfur. This accumulation begins just before the appearance of the first pycnidia.

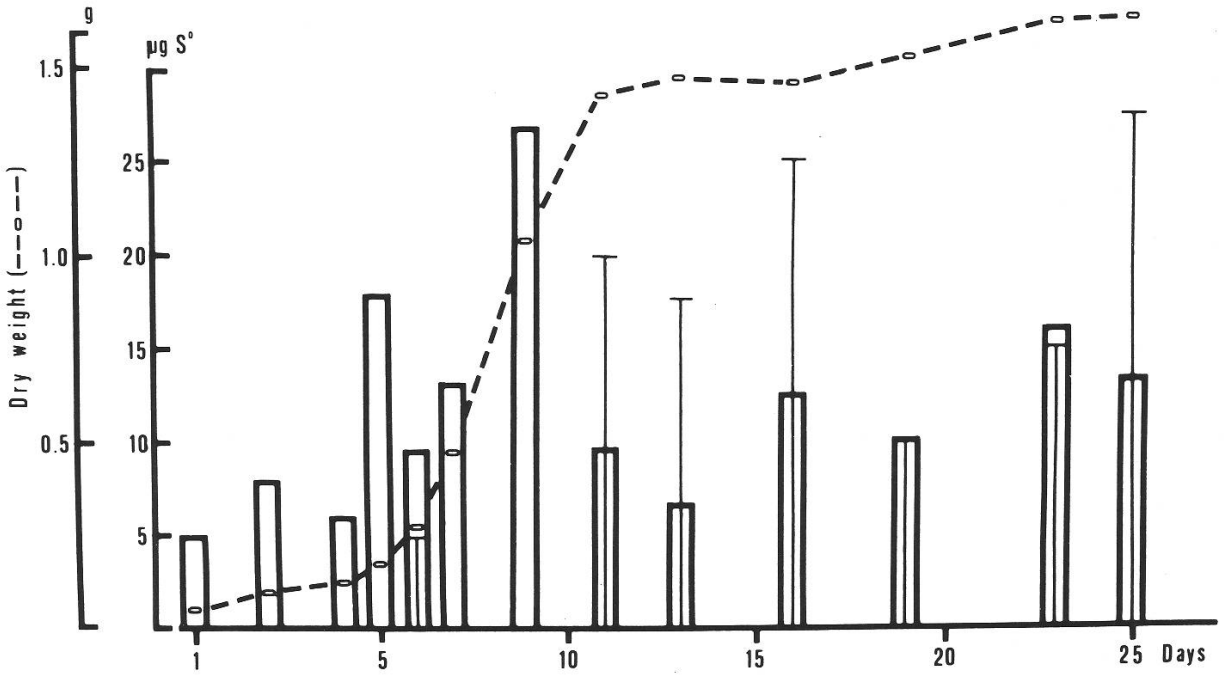

Fig. 2:

Mercaptopyruvate desulfurase activity and production of elemental sulfur during the growth of *Phomopsis viticola.*

o - - - - o:	dry weight (g)
Vertical columns:	β-mercaptopyruvate desulfurase activity (μg of sulfur produced from synthetic β-mercaptopyruvate (10 μmoles/ml) by a β-mercaptopyruvate desulfurase extract (from *P. viticola*)
Vertical lines:	elemental sulfur accumulated (μg)

Discussion

It has been shown that free sulfur is accumulated by *P. viticola* during the desulfuration of β-mercaptopyruvate by the enzyme β-mercaptopyruvate desulfurase. The fungus begins to accumulate elemental sulfur just before the end of the exponential growth period and the formation of the first pycnidia. It seems to bear an important relationship to the enzymatic production of elemental sulfur, accumulation of the element and the two important biological states of the fungus: growth and differentiation. The action of elemental sulfur, as a fungicidal agent, has been studied by MILLER et al. (1953) who showed that conidia of several different fungi produce hydrogen sulfide in the presence of exogenous sulfur. These authors have demonstrated that H_2S was less toxic than S^o. They explained this toxicity by the similarity between O_2 and S^o and by the possibility of transferring hydrogen on S^o instead of O_2 in the terminal electron transport system. A few years later, TWEEDY and TURNER (1966) worked with *Monilia fructicola* and confirmed this hypothesis. They determined that the action of free sulfur (as a fungicidal agent) was located on cytochrome b and c and concluded that free sulfur contributes to decreasing the energy level of the cell by increasing the oxidation of metabolites without conservation of the derived energy in usable form. From this basis, then it is possible to propose the following hypothesis. During the exponential growth period of *P. viticola*, a high desulfuration activity takes place without accumulation of free sulfur. The sulfur may be metabolized and could result in a decrease in the energy level of the cells, with a concomitant decrease in the rate of metabolism. The end of the growth period could be determined, in part, by this phenomenon. The ensuing accumulation of sulfur could help maintain this low level during differentiation. The phenomenon of accumulation of free sulfur seems to be very common in fungi which produce dormant spores, chlamydospores or sclerotia, and could play a role in regulating cell energy, the phenomenon of differentiation, and the maintenance of dormancy (PEZET and PONT, 1977). CHET and HENIS (1975) have published a review about fungal sclerotia where they mentioned that the formation of sclerotia in *Sclerotium rolfsii* is stimulated by iodoacetic acid and inhibited by cysteine. The role of cysteine for the maintenance of growth of the yeast phase cells by *Histoplasma capsulatum* has been shown by BOGUSLAWSKI et al. (1976). In fact, cysteine could compete with cytochromes to capture sulfur and thus enhance maintenance of the growth phase by a high energy level. Iodoacetic acid, by its action on glyceraldehyde phosphate dehydrogenase, could contribute to a decrease in the pool of pyruvate and indirectly stimulate the enzyme β-mercaptopyruvate desulfurase by decreasing the end-product of the reaction. A concomitant production of free sulfur could occur. However, no relations are scientifically established between elemental sulfur, iodoacetic acid, and cysteine, and their effects on growth and differentiation. But it seems to be interested to emit such an hypothesis in a view of a further research.

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Summary

Elemental sulfur is accumulated in the cirrhus matrix of *Phomopsis viticola* Sacc. where it acts as self-inhibitor of germination of alpha spores. The desulfuration of β-mercaptopyruvate by the enzyme β-mercaptopyruvate desulfurase, as described previously by several authors for rat liver, is responsible, in *P. viticola* cultures, for this accumulation of free sulfur with a concomitant production of pyruvate. The determination of the substrate and enzyme in *P. viticola* are made by the use of ^{35}S-$MgSO_4$ incorporation and enzymatic tests. The enzymatic activity of β-mercaptopyruvate desulfurase increases during the growth phase of the fungus without free sulfur accumulating. Sulfur accumulation starts just before the end of growth and before the differentiation of the first pycnidia. The possible role of sulfur on the decrease of the metabolism energy level of the cells, the resulting end of the growth, and the induction of the differentiation is discussed.

Résumé

Du soufre élémentaire s'accumule dans les cirrhes de *Phomopsis viticola* Sacc. où il agit comme auto-inhibiteur de la germination des spores alpha. La désulfuration du β-mercaptopyruvate par l'enzyme β-mercaptopyruvate désulfurase dans le foie du rat, comme cela a été décrit par plusieurs auteurs, est responsable, chez *P. viticola,* de l'accumulation du soufre libre avec production simultanée de pyruvate. La mise en évidence chez ce champignon, du substrat et de l'enzyme cités ci-dessus, a été rendue possible par l'utilisation de ^{35}S-$MgSO_4$ et de tests enzymatiques. L'activité enzymatique de la β-mercaptopyruvate désulfurase augmente pendant la phase de croissance du champignon sans accumulation de soufre libre. Celui-ci commence à s'accumuler juste avant la fin de la croissance et le début de la différenciation des premières pycnides. Le rôle possible du soufre sur une diminution du niveau énergétique du métabolisme cellulaire, sur la fin de la croissance et sur l'induction de la différenciation est discuté.

Zusammenfassung

In der Matrix der Sporenranken von *Phomopsis viticola* Sacc. wird elementarer Schwefel angereichert und wirkt als Hemmstoff gegen die Keimung der Alpha-Sporen. Die Anreicherung von elementarem Schwefel erfolgt unter der Wirkung der β-Mercaptopyruvat-desulfurase aus β-Mercaptopyruvat. Substrat und Enzym wurden in Kulturen von *P. viticola* durch Einbau von ^{35}S-$MgSO_4$ und enzymatische Teste bestimmt. Die Aktivität der β-Mercaptopyruvat-desulfurase nahm während der Wachstumsphase des Pilzes ohne Anreicherung freien Schwefels zu. Die Schwefelanreicherung begann unmittelbar vor dem Ende der Wachstumsphase und vor der Ausbildung der ersten Pyknidien. Die Bedeutung des Schwefels für die Verminderung des Energieniveaus des Zellstoffwechsels, für das Ende der Wachstumsphase und die Induktion der Pyknidienbildung wird diskutiert.

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