STUDIA FORESTALIA SUECICA

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Enzymatic Splitting of Sucrose by some Strains of *Valsa nivea* Fr.

Enzymatisk spjälkning av sackaros med några stammar av Valsa nivea Fr.

by

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Enzymatic Splitting of Sucrose by some Strains of *Valsa nivea* Fr.

In an earlier paper (Persson 1955) a parasitic crown-mycose on hybrid aspen, *Populus tremula* × *Populus tremuloides*, caused by the ascomycete *Valsa nivea* Fr. was reported. This fungus appears as a saprophyte on the native aspen *Populus tremula* L. (Ursing 1949) and parasitic only on strongly weakened individuals of *Populus tremuloides* Michx. within the transatlantic distribution range of the species (Schreiner 1931). Among the field trials in the south of Sweden, where the disease was first discovered, two progenies of hybrid aspen were included besides the control parcels of native aspen. The pandemic character of the attack was recognized only in the parcels of hybrid aspen, whereas *Populus tremula* was left unattacked. Experiments in vitro cultivating the fungus on agar plates including ground pieces of bark from the parent species and from the hybrid aspen indicated great differences in growth rate.

On the bark-agar plates with *Populus tremula* the growth was sparse and after some time inhibited; on the plates with *Populus tremuloides* the growth was markedly luxuriant, and on the bark-agar of hybrid aspen the fungus grew outstandingly with a dense mycelium. These results may be interpreted as the occurrence of factors stimulating the growth of *Valsa nivea* on hybrid aspen. The opposite conditions may be valid for the parent species.

The problem

With regard to the experiences gained from the bark-agar experiments, where the different species of aspen yielded distinctly different growth, these variations were made subject of an examination regarding some of the biologically active substances in bark of aspen possibly affecting the growth of the fungus.

Salicin is reported to be specific in the genera *Salix* and *Populus* (Jowett and Potter 1902). It is a glucoside giving by hydrolysis besides glucose ortho-hydroxy-benzyl alcohol. This substance was firstly investigated. Secondly, the free sugars attracted the interest and became dominating, as will be accounted for on the following pages.

Finally the correlation between the sucrase activity and the growth of the fungus in vitro was discussed. Incidentally the attention was drawn to a strain of *Valsa nivea* represented in these experiments, which was shown apatogenic in experiments in vivo (Kern 1957).

Material and methods

Bark extraction

The epidermis, periderm, phloem and cambium, here simply named bark, were collected from 5 to 10 stems of hybrid aspen (10 progenies), of *Populus tremula* (3 progenies), and from *Populus tremuloides* (2 progenies) in September at the Swedish Match Co. Research Station, Mykinge. The bark was stored during transport in plastic sacs. It was rapidly ground in a turmix and extracted with 70 % alcohol in a Soxhlet extractor to isolate the free amino acids and the sugars according to PAECH and TRACEY (1955).

The extraction for salicin was performed with boiling water, lead acetate was added, and the precipitate was extracted in a Soxhlet with 90 % alcohol, evaporated and taken up in distilled water (JOWETT and POTTER 1902).

Nutrient solution

KHG-nutrient solution (KERN 1957) was prepared and modified as follows:

	Standard	Modified I	Modified II
$Ca(NO_3)_2 \cdot 4\ H_2O$	1.00 g	1.00 g	1.00 g
$MgSO_4$	0.25 g	0.25 g	0.25 g
KH_2PO_4	0.25 g	0.25 g	0.25 g
KCl	0.25 g	0.25 g	0.25 g
$FeCl_3 \cdot 6H_2O$	0.01 g	0.01 g	0.01 g
Yeast extract Difco	5.00 g	0.50 g	—
Sucrose	20.00 g	20.00 g	20.00 g
Distilled water	1000 ml	1000 ml	1000 ml
Aneurine			50 γ
Biotine			5 γ
Inosite			0.1 g

The modification to Mod. I is made in order to keep the level of other carbons than that of sucrose very low. In the nutrient solution KHG, Mod. II, there is a total lack of yeast extract which is therefore substituted by aneurine, biotine and inosite used in growth substance experiments with *Valsa* species (FRIES 1938).

Enzyme preparation

13 strains of *Valsa nivea* were grown at 25° C in KHG standard nutrient solution for 20 days, at the end of which luxuriant growth was obtained. The mycelial mats were removed by centrifuging and filtering

at + 3° C. The mycelium was washed with a citrate phosphate buffer solution of pH 4.62 (v. EULER et al. 1924), dried in vacuo at — 13° C and homogenized in a turmix. The mycelial sucrase treated in this way and stored at — 20° C was found excellently active still 14 days after preparation.

Determination of mycelial dry weights

Mycelial mats were filtered off on papers dried to known constant weights in 100° C and cooled in an exsiccator. At the filtering, the mycelium was washed repeatedly with distilled water. After the mycelium and paper had been dried at 100° C over night, cooled in an exsiccator and weighed, the mycelial weights were calculated.

Paper partition chromatography

The identification of sugars and salicin was made on Whatman No. 1 chromatography paper. For the sugars a three-phase system with ethylacetate—acetic acid—water (64—17—15.5) was used (AUGUSTINSSON 1952) in an ascending run. The spots were developed at 105° during 5

Strains of *Valsa nivea*

Fungus strain	Isolated from	Collected	Pathogenic for
V 1	Hybrid aspen, 1943	Ekebo, Sweden	Hybrid aspen
V 2	" " 1943	" "	" "
V 4	*Populus tremula* L.	Bogesund, Sweden	Saprophytic
V 5	" *tremuloides* Michx.	Mykinge, "	"
V 6	Hybrid aspen, 1944	Ekebo, "	Hybrid aspen
V 7	" " 1943	Mykinge, "	" "
V 8	" " 1943	" "	" "
V 9	" " 1952	" "	" "
V 620	*Populus alba* L.	Ann Arbor, USA	*Populus alba* L., *P. androscoggin*, *Prunus laurocerasus* L.
V 643	" *tremula* L.	Zürich, Switzerland	*Populus tremula* L., *Prunus laurocerasus* L.
V 663	" *tremuloides* Michx.	Michigan, USA	*Populus alba* L., *P. tremula* L., *P. androscoggin*, *Prunus laurocerasus* L., *Prunus lusitanica*, *Fraxinus excelsior* L.,
V 690	" *alba* L.	Kaiserstuhl, Germany	*Populus alba* L., *P. androscoggin*, *Prunus laurocerasus* L., *Prunus lusitanica*, *Fraxinus excelsior* L.
V 701	" *tremula* L.	St Véran, France	None

minutes with benzidin—trichloroacetic acid—ethanol as a spraying reagens (LINSKENS 1959).

The salicin of the bark was identified on the chromatogram, run in a butanol—acetic acid—water solvent (4—1—5) and developed at 75°—80°C by spraying the paper with 2 N sulphuric acid (PERSSON 1958).

Semi-quantitative determination of salicin

STANGE (in LINSKENS 1959) describes a method of determining carbohydrates photometrically. The density of the spots of salicin has been determined by an EEL densitometer (PERSSON 1958).

Salicin

The crude salicin extracted from equivalent quantities of bark including the parent species and the hybrid aspen was solved in water, plotted on a chromatography paper and developed. As a reference substance salicin p.a., solved in water, was used.

The absorption values are collected in Table 1, and should be read horizontally. The vertical differences probably depend on different papers and different times of heating.

Table 1. **Absorbance of the spots of salicin from hybrid aspen and the parent species.**

Control	Absorption adjusted to the blank			
2 % salicin	*Populus tremula*	*Hybrid aspen*		*P. tremuloides*
0.16	0.12	0.19	0.27	0.22
0.12	0.18	0.16	—	0.17
4 % salicin				
0.05	0.06	0.10	—	0.05

No distinct trends concerning the contents of salicin in bark extracts can be read from this table, and for the moment this object was abandoned for the sugars.

Sugars

The qualitative determinations of carbohydrates in hybrid aspen and the parent species are graphed in Figure 1. For every 5 mm distance from the starting point of the chromatogram the absorption values have

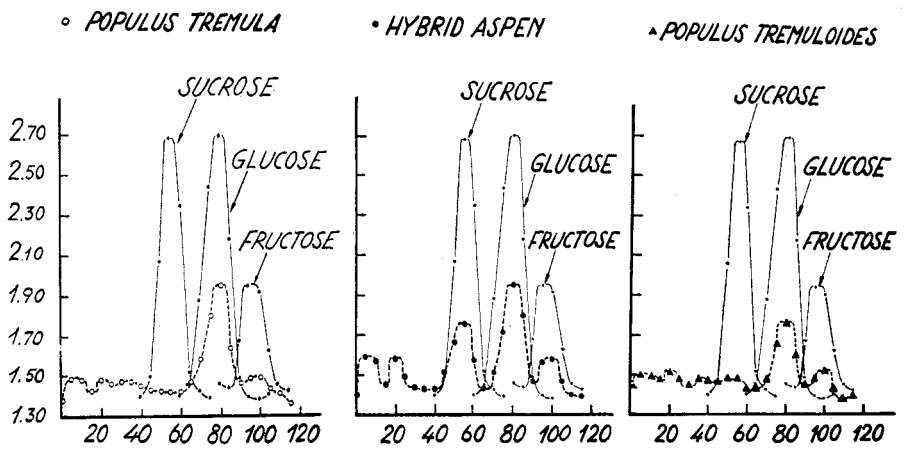


Fig. 1. The sugars of the hybrid aspen and the parents are represented by dotted lines. Reference sugars are graphed full drawn.

been read and plotted along the y-axis, reference substances being 2 % water solutions of sucrose, fructose and glucose.

For the actual time of sampling distinct differences can be noted concerning the carbohydrates. The contents of glucose and fructose present small variations in the species investigated. The sucrose is only lacking in the parent species but quite observable in the extract from hybrid aspen. Further, the hybrid aspen more emphasizedly possesses two unidentified sugars with low R_f-values than the parent trees do.

It is a wellknown fact that glucose and fructose are very common in plant tissues. The sucrose is found abundantly in trees and is considered to be present in the vacuolar sap of all plants (KRAMER and KOZLOWSKI 1960).

Studies made on the carbohydrates of the inner living bark of *Robinia pseudoacacia* L. state optimum production of total carbohydrate in September—October decreasing to a minimum in June. For sucrose the seasonal cycle is roughly the same (SIMINOVITCH et al. 1953). In *Acer saccarum* MARSH. the sucrose is reported to reach a slight optimum in December and a minimum in June. The curve of the total carbohydrate is analogue but more strongly pronounced (JONES and BRADLEE in KOZLOWSKI 1960). In *Pinus echinata* MILL. a comparison was made on the reserve carbohydrate of stem wood and stem bark for a year. The bark contained 7—10 % carbohydrates with a slight optimum in April, the wood 1.5—3 % with an optimum in June (HEPTING 1945). The contents of total carbohydrates in *Fagus silvatica* L. was found to be

larger in stems than in roots (GÄUMANN 1935), and in *Liquidambar styraciflua* L. the concentration in bark was higher than in wood (WENGER 1953).

From this experience it seemed to be of interest to investigate the capacity of utilizing the sucrose of 13 strains of *Valsa nivea*. This was accomplished in two different ways, i.e., considering the splitting of sucrose by enzyme preparations as well as the consumption of the sugar by the fungus in growth experiments in vitro.

Enzymatic experiments

pH spectrum

The importance of pH on the sucrase activity of fungi is known from 1889 in *Aspergillus niger* v. TIEGHEM. Later (KANITZ 1903; v. EULER et al. 1924; HOFMANN 1934; NEUBERG et al. 1950) the sucrase activity from different ascomycetes was studied. von EULER et al. pointed out a pH optimum for *Penicillium glaucum* LINK between 4—6, but a more narrow one for yeast sucrase, 3.5—5. The optimum in preparations from *Aspergillus niger* and *A. oryzae* (AHLB.) COHN, i.e., taka invertase, was determined to 4.9 and 5 respectively (HOFMANN 1934). On yeast invertase NEUBERG (in SUMMER and MYRBÄCK 1950) summarizes: "yeast invertase is most active at slightly acid pH and most stable at pH 4—5. Careful studies of the optimum conditions led to the generally accepted value of pH 4.5, but the divergence at pH 3.5 or 5.5 is small, i.e., the optimum range is rather wide. Invertases from *Aspergillus oryzae* have a higher optimum pH of 6—8. In alkaline solution purified preparations rapidly lose activity."

20 mg enzyme preparation from each strain of *Valsa nivea* was mixed in Ellerman tubes with 1 ml 2 % solution of sucrose buffered with Mc Ilvaine's citrate—phosphate buffer solution. The following pH values, 4.6, 5.6, 6.6, and 7.6 respectively, were represented in these experiments. The tubes were incubated at 37° C, a temperature commonly used to obtain rapid enzymatic decomposition (NEUBERG and MANDL 1950). Samples were removed after 2, 4, 8, 24 and in some instances 72 hours and plotted on Whatman No. 1 chromatography papers. These were run and developed as described earlier.

Disregarding the rate differences in splitting sucrose by the present 13 strains of the fungus, the pH-activity within the chosen range does not offer any distinct optimum. Exceeding the neutral point in alkaline direction, the enzymatic splitting is strongly decreased if not quite inhibited. Contrarily, the enzymatic activity is significantly reestabilished

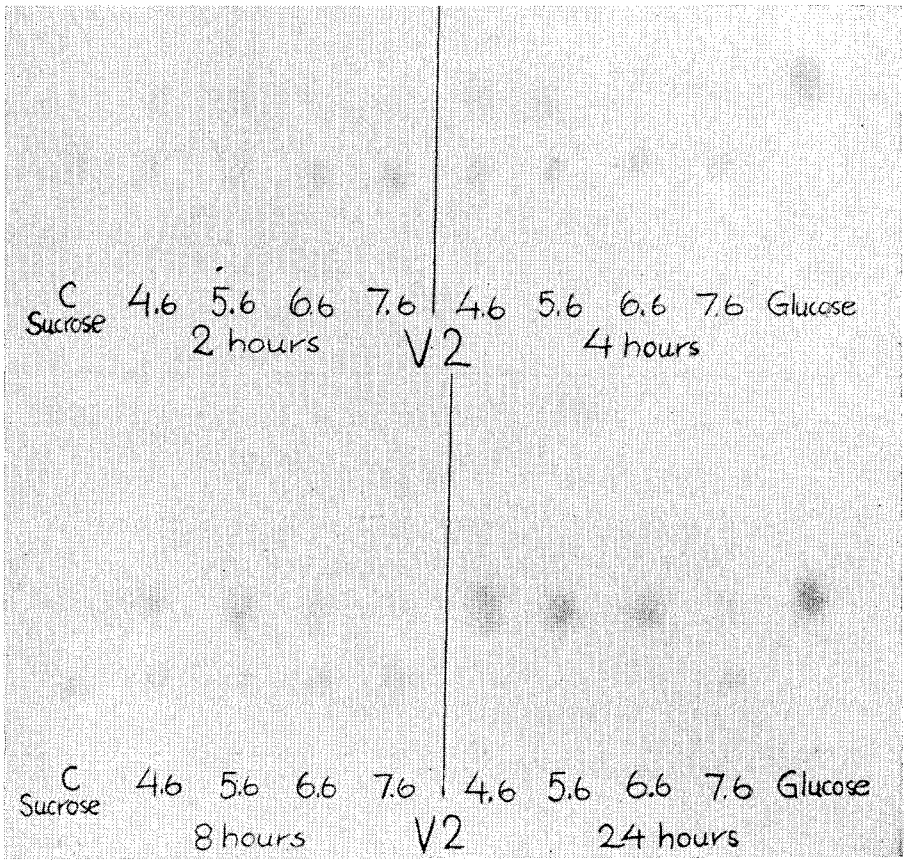


Fig. 2. The influence of pH on the splitting of sucrose by one strain of *Valsa nivea*.

already at the pH value 6.6. Generally the sucrose tends to disappear with increasing time. All the strains, except two, are capable of decomposing the sucrose molecule within the times chosen for this experiment. Strains No. V 8 and V 701 are obviously lacking sucrase.

The following part of the investigation includes a closer study by paper chromatography of sucrase activity as a function of time, and was accomplished with 2 % solutions of sucrose buffered to pH 4.6, which was found a stable range of sucrase activity as proved in the preceding experiment. The remaining conditions were in accordance with the pH-experiment above. Samples were plotted on the paper after 0.5, 1, 2, 3, 4, 8 and 24 hours of incubation. 2 % solutions of sucrose, glucose, and fructose served as reference substances. The sucrose reference solution was buffered to pH 4.6 and incubated for 24 hours at 37° C as a control.

Table 2. **The influence of pH on the splitting of sucrose by strains of *Valsa nivea* at different time.**

Strain No.	Sucrose split to glucose and fructose after																Remarks
	2 hours				4 hours				8 hours				24 hours				
	pH				pH				pH				pH				
	4.6	5.6	6.6	7.6	4.6	5.6	6.6	7.6	4.6	5.6	6.6	7.6	4.6	5.6	6.6	7.6	
V 4	○	○	○	—	○	○	○	—	○	○	○	○	+	+	+	○	+ + + +
V 2	○	○	○	—	○	○	○	—	○	○	○	○	+	+	+	○	(72 hours)
V 1	○	○	○	—	○	○	○	—					+	+	○	○	
V 663	○	○	○	—	○	○	○	—					+	+	+	○	
V 5	○	○	○	—	○	○	○	—					+	+	+	○	+ + + +
V 6	○	○		—	○	○		—	+	+		○	+	+		+	(72 hours)
V 7	○	○	+	○	+	+	+	○	+	+	+	○	+	+	+	+	
V 9	○	○	○	○	○	○	○	○	+	+	○	○	+	+	+	○	
V 620	○	○	○	○	○	○	+	○	+	+	+	+	+	+	+	+	
V 643	○	○	○	—	○	○	○	○	+	+	+	○	+	+	+	○	
V 690	○	○	○	○	+	+	+	○	+	+	+	○	+	+	+	+	
V 8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
V 701	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	○ — — (+) (102 hours)

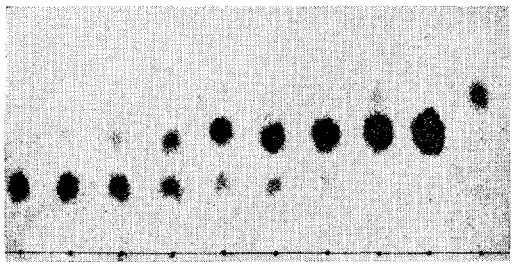
Sucrose split: + completely
○ partly
— none

The figures indicate a great variation in enzymatic splitting of the sucrose to a glucose and a fructose molecule within the 13 strains of *Valsa nivea*. The fotocopies only very faintly reproduce the spots of fructose which on the original Whatman paper appear with light-yellow colour. The copying was unfortunately performed with lamps including much yellow light.

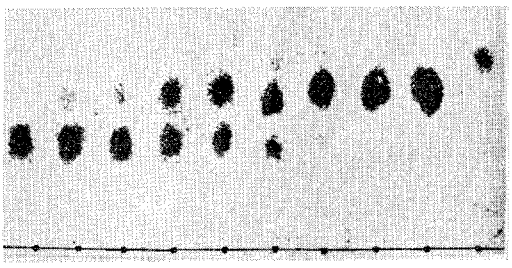
Taking into account the time used for complete splitting off the sucrose molecule, a rough classification of the sucrase activity is presented in the table below.

The inverted ratio of time may serve as a measure of the sucrase activity in the equivalent preparations with the fungus. In the pile diagram below the activity is graphed.

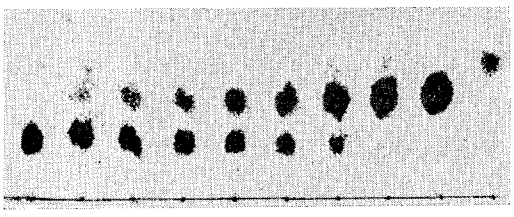
The inverting action of yeast was discovered by PERSOZ in 1833 and in 1860, BERTHELOT (in SUMNER and MYRBÄCK 1950) isolated the enzyme and named it "ferment inversif". From this term "invertase" and "invertin" have occurred. "Sucrase" and "saccharase" are synonyms. According to NEUBERG and MARX (1907) and HOFMANN (1934) there are two sucrases acting on the sucrose molecule. Depending on which end of the molecule they attack, the enzymes are termed β-fruc-



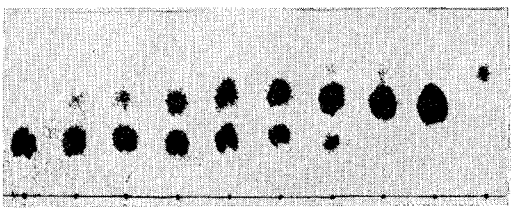
C 0.5 1 2 3 4 8 24 Gluc Fruc
Suc V 663



C 0.5 1 2 3 4 8 24 Cluc Fruc
Suc V 2

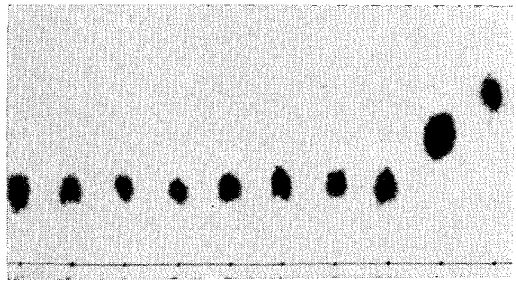


C 0.5 1 2 3 4 8 24 Gluc Fruc
Suc V 4

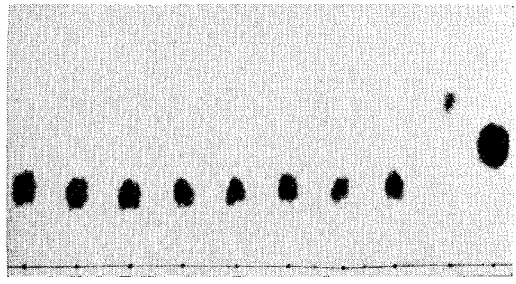


C 0.5 1 2 3 4 8 24 Gluc Fruc
Suc V 5

Fig. 3. Photocopies of chromatograms showing sucrase activity of four strains of *Valsa nivea*. Abbreviations: Suc=sucrose, Gluc=glucose, Fruc=fructose, C=control.



C 0.5 1 2 3 4 8 24 Gluc Fruc
Suc V 701



C 0.5 1 2 3 4 8 24 Fruc Gluc
Suc V 8

Fig. 4. No splitting of sucrose by the strains V 701 and V 8 still after 24 hours of treatment.

tosidase and α-glucosidase. In sucrose both ends of the molecule are open to attack, but in the trisaccharide raffinose the glucose end is blocked by galactose linkage. The α-glucosidase present is inactive on raffinose. Melibiose is a disaccharide of galactose and glucose.

Schematically:

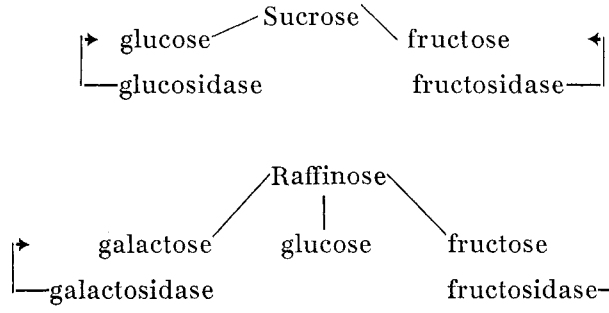


Table 3. **The rate of splitting of sucrose in vitro by 13 strains of *Valsa nivea*.**

	Enzymatic splitting of sucrose					none
	completely					
	3 hours	4 hours	8 hours	24 hours	48 hours	48 hours
Fungus strain No.	V 7 V 643	V 9 V 690	V 6 V 620	V 2 V 4 V 5 V 663	V 1	V 8 V 701

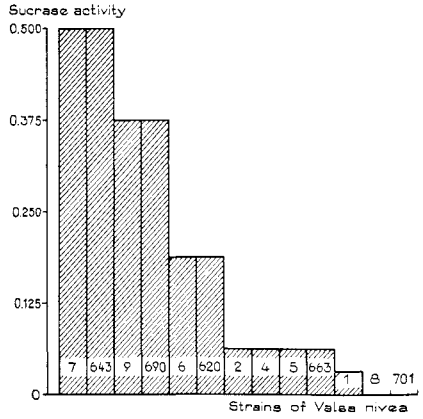


Fig. 5. The relative sucrase activity of 13 strains of *Valsa nivea*.

Enzyme preparations from three strains of *Valsa nivea* were left acting on 2 % raffinose in a buffered (pH 4.6) solution. Samples were plotted on Whatman No. 1 paper, run and developed as above.

The spots on the chromatogram after 2 hours manifest fructose and melibiose produced, which excludes the presence of α-glucosidase in the enzyme preparations from the fungi and indicates β-fructosidase as the acting enzyme on sucrose.

Growth experiments

In the last part of this investigation some growth experiments all including sucrose are reported. In an earlier paper (KERN 1957) 5 of the strains handled in the present experiments were investigated concerning the growth responses to Difco yeast extract in the KHG nutrient solution. With 5 % yeast extract KERN obtains optimum growth at 14 to 21 days of culture at 26—27° C. The strains No V 701 and V 690 were totally yeast-heterotrophic. No V 643 and V 663 displayed sparse

growth, and the strain No. V 620 grew more abundantly without yeast extract.

In a rough test all the strains of *Valsa nivea* were grown with each 4 Erlenmeyer flasks containing 40 ml KHG standard nutrient solution for 18 days in + 25° C. They were harvested as described earlier.

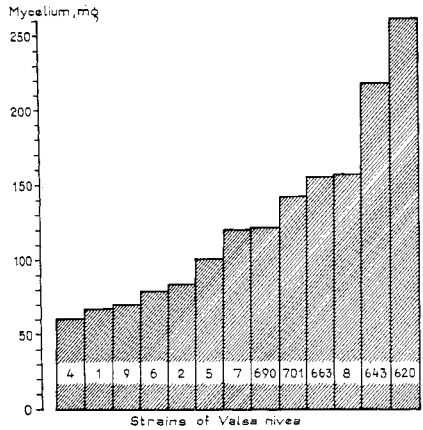


Fig. 6. Mycelial weights from an 18 days' culture of *Valsa nivea* in the KHG standard nutrient solution.

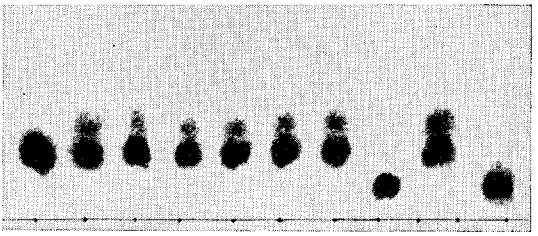
The pile diagram above offers a support of the dependence of yeast extract in those strains of *Valsa nivea* investigated by KERN (1957). The Swedish strains are all yielding relatively high dry weights, and especially V 8 is abundantly growing. This strain, however, was lacking sucrase as shown in the enzyme experiments.

During the time of growth samples were collected from the nutrient solutions of the flasks after 3, 6, 9, 14 and 18 days, plotted on a Whatman paper, and developed in order to analyse the variations concerning the contents of sucrose.

In Figure 7 the copies of chromatograms indicate the rate of metabolizing the split sucrose components glucose and fructose of different strains. After growing for 14 days, the following strains of *Valsa nivea*, V 2, V 4, V 5, V 9 and V 643, have completely utilized the saccharides, which by the strains V 1, V 6, V 7, and V 690 has been achieved after growing for 18 days. The strains V 620 and V 663 only sparsely utilized the glucose-fructose from the split sucrose molecule. V 8 and V 701 persist in leaving the sucrose undecomposed still 18 days after inoculation.

V1 V2 V4 V5 V6 V7 V8 V9 Suc V663 V620

3 days

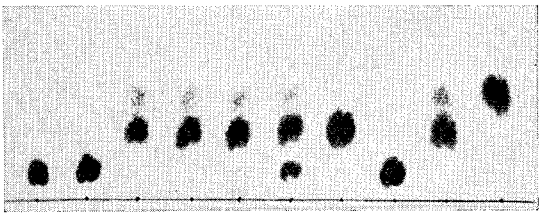


Gluc V1 V2 V4 V5 V6 V7 V8 V9 Suc

6 days

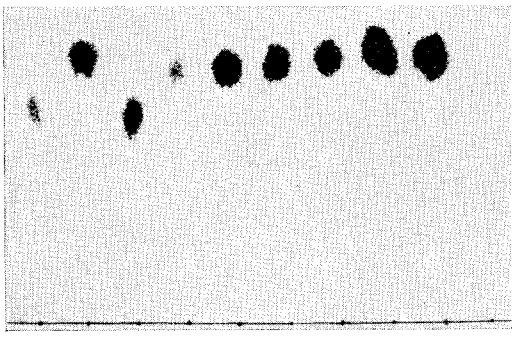
Suc V690 V643 Suc V701 V1

3 days



Suc V701 V643 V690 V620 V663 Gluc V8 V1 Fruc

6 days

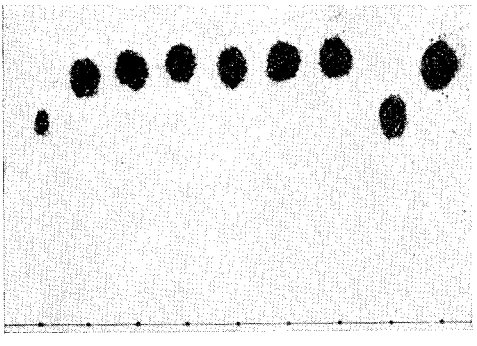


Suc V1 V2 V4 V5 V6 V7 V8 Gluc Fruc

9 days

Suc V1 V2 V4 V5 V6 V7 V8 Gluc Fruc

18 days



Suc V9 V701 V643 V1 V690 V663 Gluc Fruc

9 days

Suc V9 V701 V643 V690 V620 V663 V1 luc Fruc

18 days

Fig. 7. Chromatograms on the utilization of sucrose by the strains of *Valsa nivea* at successive times of harvesting. The fungi have grown in KHG standard nutrient solution.

There are no direct correlations observed between the disappearance of saccharides and the mycelial growth. This unusual condition may be explained by the occurrence of yeast extract in the semisynthetic KHG-solution, which according to KERN 1957 (p. 167) contains a.o. aspartic acid, glutamic acid, glycocolle, isoleucine, lysine, phenylalanine, threonine and valine as sources of carbon. The evident growth of V 8 and V 701, unaffected in spite of the disaccharide, may depend on their capacity of utilizing other sources of carbon than sucrose.

Growth in KHG, Mod. I, nutrient solution

In the KHG standard nutrient solution there are included 5 g Difco yeast extract pro liter constituting a source of carbon beside sucrose. In order to reduce this carbon to a nimimum, but still utilizing the effect of yeast extract as a growth substance, the concentration was diluted to 0.05 %. The fungi were grown under conditions reported earlier. Two flasks were harvested from each strain of the fungus after 6, 12, 18, 24, and 36 days of culture. The mycelial weights are graphed in the figures below.

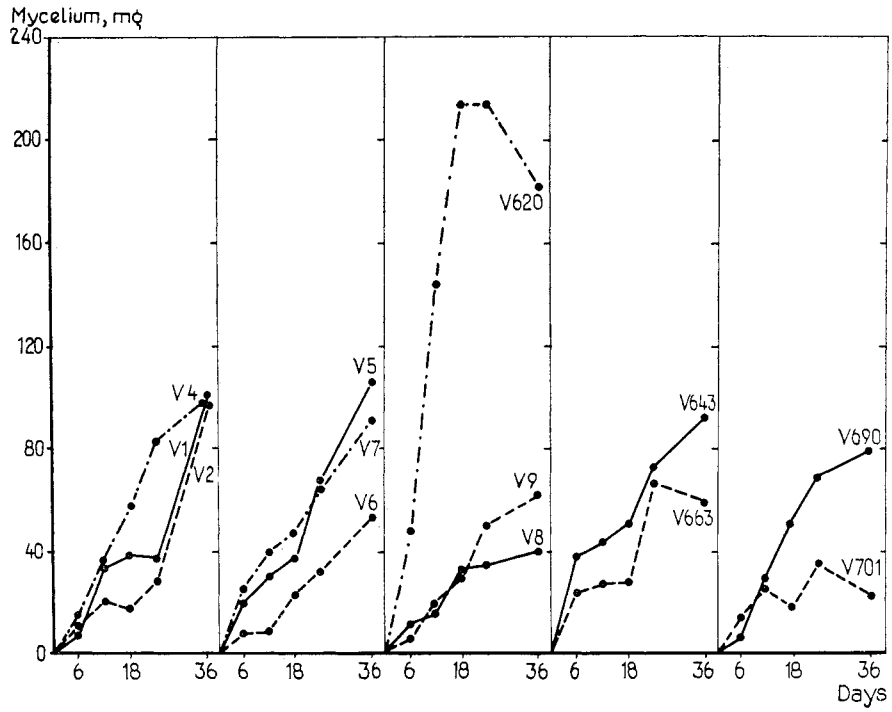


Fig. 8. Growth of 13 strains of the fungus in KHG Mod. I nutrient solution.

Suc KHG V1 V2 V4 V5 V6 V7 Gluc Fruc

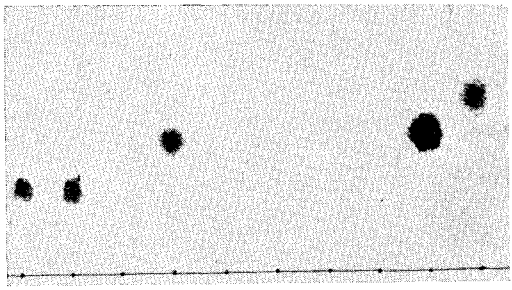
Suc KHG V8 V9 V620 V643 V663 V690 V701 KHG

6 days

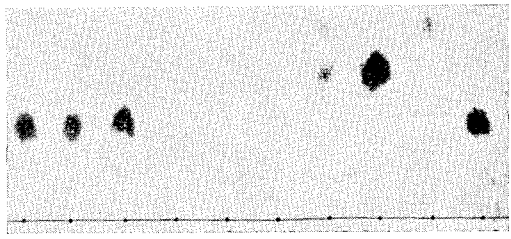
Fig. 9 Photocopies of chromatograms on the utilization of sucrose after 6 days of growth in KHG Mod. I nutrient solution.

By comparing the mycelial weights after 18 days in KHG standard and KHG Mod. I nutrient solutions, see fig. 6 and fig. 8, some distinct trends may be noticed. Thus the strain V 620 of *Valsa nivea* retains its rapid rhythm of growth even with a lower concentration of yeast extract, which was only to be expected as it has been reported to grow luxuriantly without yeast extract (KERN 1957). The mycelial yield of the strain V 643 decreased from 218 mg to 50 mg with the lower concentration of yeast extract, and the values of V 663 decreased from 155 to 27 mg dry mycelium. There was also a pronounced decline of V 690 and V 701 with the lower concentration of yeast extract giving a decrease from 122 to 50 mg and 140 to 17 mg mycelium respectively. The weights will not be quite comparable because the flasks with KHG standard nutrient solution contained 40 ml while those with KHG Mod. I only contained 30 ml per flask.

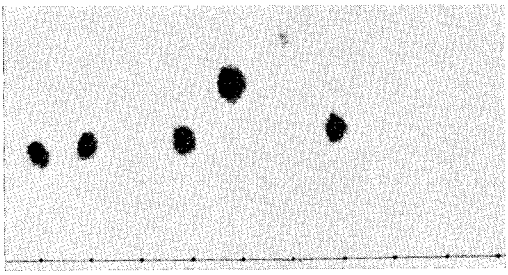
Within the time used for this experiment including KHG Mod. I, the strains V 8 and V 701 grew sparsely giving the lowest yield of all



KHG Suc V1 V2 V4 V5 V6 V7 Gluc Fruc



KHG Suc V8 V9 V620 V643 V663 Gluc Fruc KHG



KHG Suc V690 V701 Gluc Fruc KHG

24 days

Fig. 9 b. Photocopies of chromatograms on the utilization of sucrose after 24 days of growth in KHG Mod I nutrient solution.

strains investigated. These two strains are characterized by lacking sucrase.

The chromatograms from this experiment principally repeat the experiences reported in Fig. 7. The sucrose molecule is still after 36 days of growth left unattacked by the strains V 8 and V 701 as can be seen from the figure below. No quantitative determinations are made on the sucrose of the nutrient solutions harvested, and the chromatograms only give the qualitative picture of the sugars.

In another attempt to cultivate the fungus, a pure synthetic nutrient solution was used where the yeast extract was substituted for aneurine, biotine and inosite as has been reported favourable for the growth of

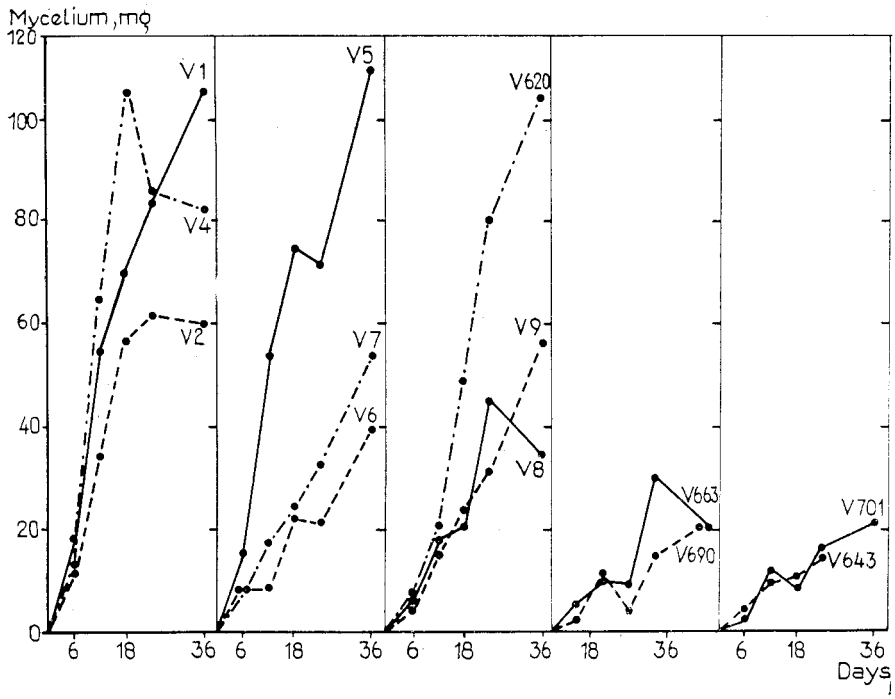


Fig. 10. Growth of 13 strains of the fungus in KHG Mod. II nutrient solution.

Valsa pini ALB. et SCHWEIN. and *Valsa ceratophora* TUL. (FRIES 1938). The dominant source of energy was 2 % sucrose. The mycelia were harvested after 6, 12, 18, 24 and 36 days of growth and the weights determined from 4 flasks of every strain as described earlier.

A comparison of the mycelial yields from growth in KHG standard, from KHG mod. I and from KHG Mod. II drawn from Table 4 clearly shows the strong stimulating effect of yeast extract on all the strains of *Valsa nivea*.

By comparing the growth in KHG Mod. I and KHG Mod. II, a more differentiated picture is obtained, and in this range of concentration the yeast extract works as a growth substance like the aneurine, biotine and inosite of KHG Mod. II. This apprehension was more precisely studied in the following experiment which included one series with sucrose and one lacking it. The yeast extract was added in the following concentrations 0; 0.01; 0.25 and 2.50 %. The concentration of sucrose in the former series amounted 5 %, which was found to yield an optimal mycelial growth of four strains tested. Two strains of special interest were included in this experiment, i.e. V 663, strongly pathogenic in

Tabell 4. **Mycelial growth in 3 different nutrient solutions and the relative sucrase activity of 13 strains of *Valsa nivea*.**

Strain No.	Growth mg after 18 days			Sucrase activity
	KHG standard	KHG mod. I	KHG mod. II	
V 620	276.1	213.0 (213.5)	48.2 (102.8)	0.18
V 643	217.8	50.4 (91.2)	10.7 (15.7)	0.50
V 8	156.8	31.9 (39.7)	20.1 (44.3)	0
V 663	155.6	27.9 (66.5)	9.1 (30.2)	0.06
V 701	142.0	17.4 (34.6)	8.8 (30.2)	0
V 690	120.9	50.1 (79.1)	4.0 (21.3)	0.38
V 7	120.8	46.2 (90.3)	24.4 (54.5)	0.50
V 5	100.4	36.1 (105.2)	74.4 (108.7)	0.06
V 2	82.9	17.5 (98.3)	57.0 (61.8)	0.06
V 6	79.7	22.3 (52.1)	22.4 (39.3)	0.18
V 9	69.9	30.9 (71.4)	23.2 (55.9)	0.38
V 1	66.8	37.3 (100.2)	69.7 (104.5)	0.03
V 4	61.8	57.6 (97.8)	103.8 (103.8)	0.06

( ) = The maximum mycelial yield

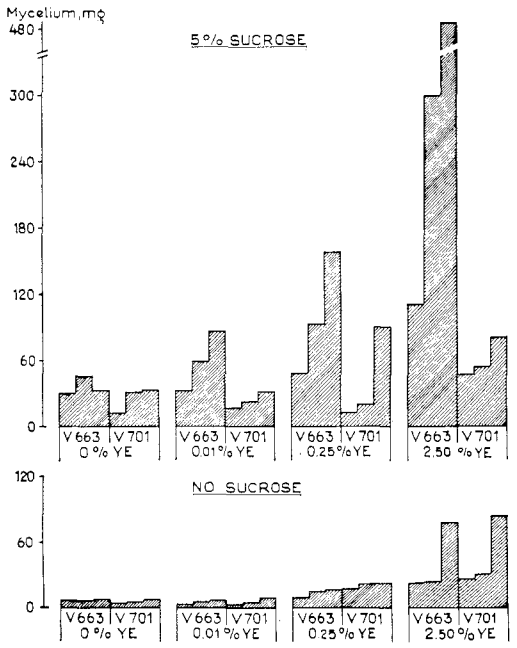


Fig. 11. Growth of two strains of *Valsa nivea* at different concentrations of yeast extract (Y E). Harvests are performed after 6, 18 and 36 days of growth.

field tests, and V 701 lacking sucrase and only at random causing symtoms on hybrid aspen.

From the pile diagram in fig. 11 it appears that the utilization of sucrose is quite different between the strains. V 663 pays rapid responses to increasing concentrations of yeast extract depending on an increasing utilization of sucrose. In this respect the strain is superior to V 701, which dry mycelium after 36 days of growth in 2.5 % yeast extract is only 16 % of that of V 663.

By comparing the mycelial weights above with those representing no sucrose of the solutions, two distinct differences are observable. Firstly the absolute yields of both strains are markedly less and secondly the great superiority of V 663 is changed in favour of V 701. The differences are however small.

From these experiments the importance of yeast extract in combination with sucrose as a source of energy is demonstrated. Without sucrose in the solutions the mycelial yields increase with increasing concentration of yeast extract. With sucrose included, yeast extract stimulates the utilization of sucrose in relation to concentration. This stimulation is however very small concerning the strain V 701.

In order to investigate the growth of four characteristic strains of *Valsa nivea* V 1, V 6, V 620, and V 701 were separately cultivated in nutrient solutions containing the main saccharides of hybrid aspen, i.e. glucose, sucrose and fructose at a concentration of 5 %.

The diagrams above are rather uniform indicating that all the sugars serve as sources of energy. Less attractive is fructose. The individual differences between the monosaccharide glucose and the disaccharide

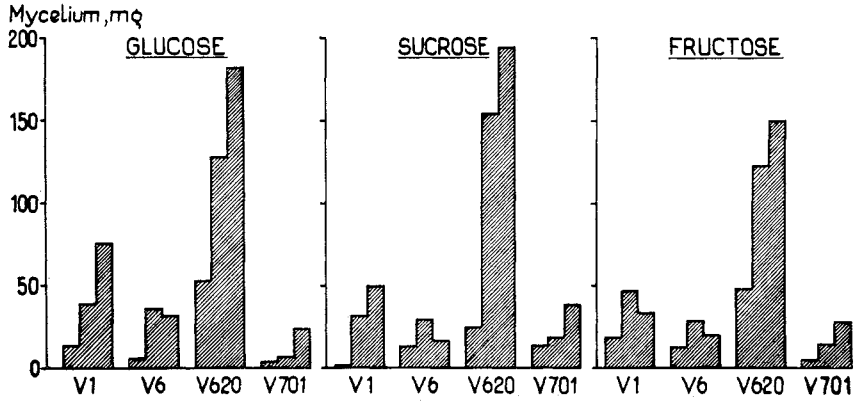


Fig. 12. Growth of four strains of the fungus at a concentration of 5 % of the sugars respectively, harvested after 6, 18 and 36 days.

sucrose are small measured as dry mycelial weights. The strain V 620 being high-yielding with all sugars in contrast to strain V 701, which is growing sparsely on the three different sources of energy.

From the qualitative chromatograms of extracted bark of hybrid aspen and the parent species three sugars were identified. In the following experiment these sugars are mixed in the KHG-solution up to the concentration of 2.4 %. With only glucose in the solution the concentration of it thus amounts 2.4 %. If glucose and fructose are mixed each is added in a concentration of 1.2 %, and if all three sugars are included they amount 0.8 % each. Harvests were performed after 12, 24 and 36 days of growth. The results are summarized below.

Of all combinations present two may be comparable qualitatively with the sugars of hybrid aspen and the parent species, i.e. glucose + fructose + sucrose (hybrid aspen) and glucose + fructose (the parent species). The comparison between the actual combinations was however little elucidating. The combination including all the three sugars yielded, with the strains chosen, inferior dry weights compared with those containing glucose + fructose, though the differences were slight. Also in this experiment the characteristic "sugar strain" V 620 grew vigorously and V 701 sparsely.

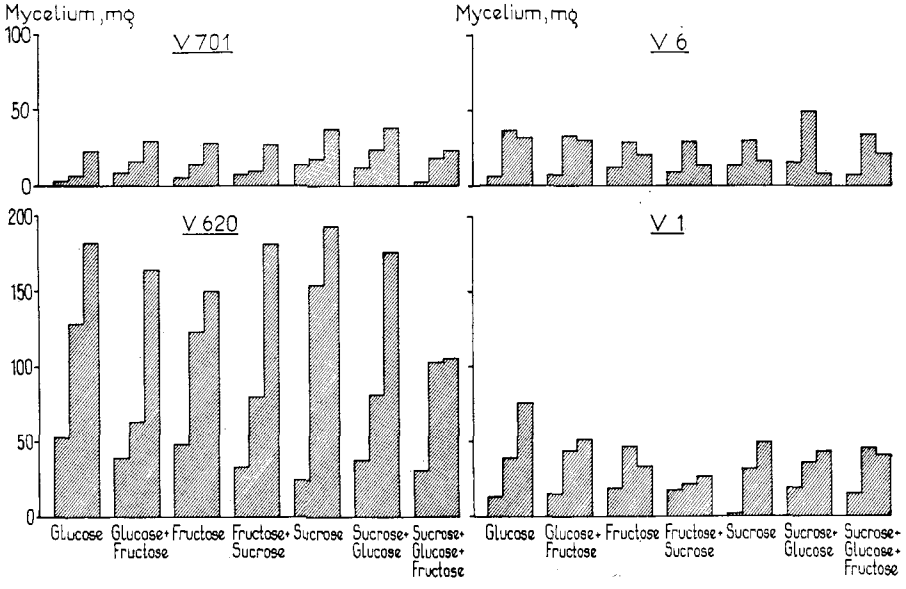


Fig. 13. Growth of four strains of *Valsa nivea* in KHG nutrient solution with glucose, glucose + fructose and glucose + fructose + sucrose as carbon sources. The mycelia are harvested after 6, 18 and 36 days of culture.

Discussion

In the present investigation mainly the sucrase activity of some strains of *Valsa nivea* has been studied.

The reason for these experiments was the fact that a qualitative difference of the saccharides was found in extracts from periderm, phloem and cambium of *Populus tremula, P. tremuloides* and the hybrid aspen. In the present samples sucrose is identified in hybrid aspen, but is lacking in the parent species. As the hybrid aspen is found seriously attacked by *Valsa nivea*, whereas *Populus tremula* is established quite resistent (PERSSON 1955), and only weakened individuals of *P. tremuloides* are attacked (SCHREINER 1931), both the opinion of *Valsa nivea* (syn. *Leucostoma nivea*) as a perthophyte causing apoplexie in *Prunus* species (GÄUMANN 1946), and that describing the fungus as a saprophyte on dead twigs of *Populus tremula* (URSING 1949), may be correct.

Among the multitude of factors in the host which may influence an attack, one metabolic substance has been chosen, and the ability of the fungus to utilize this one is investigated. In an earlier paper (PERSSON 1955) the size of the lenticels in *Populus tremula* and in hybrid aspen is determined. The considerably larger lenticels of the hybrid aspen are presumed to facilitate the establishment of an attack. Growth experiments on bark agar plates, however, suggested that the fungus is developing rapidly on material from both *Populus tremuloides* and hybrid aspen. The growth on *P. tremula* bark agar was sparse and after some time ceasing.

Principally there are at least two opposite ways to discuss the conception of susceptibility—resistance. The susceptibility of the host can decisively depend on substances stimulating or necessary for the growth of the parasite. Generally nitrogen has been found to enhance and potassium to reduce the development of obligate parasites (LAST 1953, ALLEN 1954).

The effects of growth substances have not given uniform results. Thus *Sclerotinia* species grew most luxuriantly on press juice from beets containing the highest concentration of biotine, which was in agreement with the field resistance possessed by the most biotine-deficient beet species (SUCHORUKOW, GERBER, BARABANOWA, BORODULINA 1933). On floating leaves an increase of powdery mildew is reported compared with that of the controls, when thiamine was sprayed on the leaves (PRYOR 1942). On the contrary, there was no correlation between thiamine, riboflavine, nicotinic acid, ascorbic acid or indole

acetic acid and the resistance of the host against *Puccinia* species (GOTTLIEB and HART 1943).

The effect of the carbohydrate content on the host's susceptibility lacks the controversiary aspect of the growth substances. On floating leaves in sucrose media there is a positive correlation between development of mildew and concentration of sucrose. Among the saccharides investigated, the mildew on wheat leaves yields a more luxuriant growth when sucrose is supplied and less vigorous growth with glucose and glycerine supply (TRELEASE and TRELEASE 1929), *Puccinia sorghi* ERIKSS. developed on seedlings of maize furnished with starch, sucrose, maltose or glucose and the number of plants infected in the sucrose series were superior to those of the glucose series (MAINS 1917). Between the susceptibility of potatoes to *Synchytrium endobioticum* SCHILB. and the content of mono- and disaccharides a positive correlation exists. This correlation is stronger with the disaccharides (SUCHORUKOW 1958).

The reverse of susceptibility, the resistance, may thus be explained either as a lack of substances promoting the development of the parasite inside the host, or as a presence of fungistatic or fungicide substances. Among the latter a couple of polyphenolic substances in the heartwood of many needle trees has been isolated and investigated for fungicidal action on some importanct wood rotting fungi (RENNERFELT 1943). Pinosylvin and pinosylvin-monomethylether isolated from *Pinus silvestris* L. have an inhibiting effect on the growth of *Fomes annosus* FR. and several other fungi. Thujaplicin has proved fungicidal on blue stain and decay fungi (RENNERFELT 1948).

Tannin, a phenolic substance included in *Castanea mollissima* BL. and *C. dentata* BORKH. is responsible for the resistance of these two species against attacks of *Endothia parasitica* (MURR.) P. J. et H. W. AND. *C. sativa* MILL. possesses less tannin and is severely attacked by the fungus (NIENSTAEDT 1953). In experiments with the esterase and polyphenolase activity of *Endothia parasitica*, *Herpotrichia nigra* HART. and *Fomes annosus*, however, the fungi were found to decompose the substances responsible for resistance in vivo, by enzymes (BAZZIGHER 1957).

Salicin is a glucoside exclusively appearing in the genera *Salix* and *Populus*. It has been isolated and determined chemically from samples collected at different times during a year (JOWETT and POTTER 1902). A maximum yield of salicin is reported from extractions made in July. In author's own experiments on determining salicin (PERSSON 1958) of hybrid aspen and its parents, the differences between the species were of no significance.

Summary

The results of the present investigation may be summarized and interpreted as follows:

1. Bark extracts from hybrid aspen and its parent species contained different sugars. The sucrose was lacking in the extracts from the parent species. If this is occasional, depending on the time of sampling, or constitutional is still obscure. It is, however, often observed that the annual growth of the hybrid aspen is finished several weeks later than that of the native aspen. These differences may be accounted for as being responsible for the great qualitative variations regarding the sugars. With a genetically based shorter time of seasonal growth the native aspen will earlier transform the free sugars into storage products, i.e. starch, than does the hybrid aspen.

2. If the fungus will, on the whole, establish and develop an attack inside the host, the capacity of utilizing the present sources of energy may be decisive. In the parent species there were attacks reported on the Canadian father only under extreme conditions (SCHREINER 1931). Sucrose was a lacking substance of the parent species in the present investigation, and therefore, the interest was focused on this substance. To get a first information of the behaviour of the different strains of the fungus regarding sucrose, an enzymatic examination was performed. Two strains of thirteen in all were unable to decompose the sucrose molecule enzymatically. The rest could be ranked according to the time used for splitting the sucrose. One of the sucrose-lacking strains, V 701, has been tested in vivo against hosts from the genera *Populus*, *Prunus* and *Fraxinus* (KERN 1957). It was found totally apathogenic. In resistant field trials at Mykinge Research Station author's inoculations of 126 trees with the sucrase-lacking strain V 8 all gave negative results. Another two strains were tested simultaneously giving characteristic symtoms. The routine resistance tests of new progenies of hybrid aspen in the field are all performed with the strain V 663, which in the sucrase experiments has given the low value of enzymatic activity, 0.06. The strain was chosen because it has given a most reliable effect in experiments in vivo under field conditions.

3. In experiments in vitro the effect of sucrose as a source of energy has been studied. In accordance to KERN (1957) the growth stimulating yeast extract was added initially to a concentration that served as a source of energy. This could possibly explain, that on the chromatograms no observable decrease in the content of sucrose appeared

concerning the strains V 8 and V 701, though their growth was roughly equal to that of the rest.

In a second experiment the concentration of yeast extract was decreased to a tenth of the first. The mycelial yield was markedly decreasing, though now the effect of yeast extract could hardly be considered a source of energy. In a third experiment the yeast extract was substituted for a complex synthetic growth substance yielding still smaller dry mycelial weights.

In another experiment concerning the sucrase lacking strain V 701 and the field pathogenic V 663 in media with sucrose and without, the effect of yeast extract was studied. The strain V 701 yielded about the same dry weights with as without sucrose. In contrast the strain V 663 was extremely stimulated by the presence of sucrose in cooperation with yeast extract.

The experiences of the last experiments performed in vitro concerning the role of sucrose are principally supported by the investigation made on the host hybrid aspen. The pathogenity of different strains of *Valsa nivea* is related to their ability to utilize the sucrose present in hybrid aspen, but lacking in the parent species. The experiments indicate, however, that other substances of growth substance type may influence the vitality of the strains of *Valsa nivea*.

From these experiments two distinct facts may be read. a) All the strains of *Valsa nivea* investigated are able to grow in a nutrient solution containing sucrose as the only source of energy. The undecomposed sucrose molecule on the chromatograms is only determined qualitatively, part of the sucrose molecule probably being transformed and utilized in an unknown manner and not manifested on the chromatograms.

b) The different strains behave quite differently to growth substances. V 1, V 4, V 5, V 8 and V 701 make no distinction between the yeast extract and the synthetic growth substances. V 2, V 6, V 7 and V 9 are more sensitive and V 620, V 643, V 663 and V 690 grow outstandingly in nutrient solutions containing yeast extract, but poorly in solutions with synthetic growth substances.

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Sammanfattning

Enzymatisk spjälkning av sackaros med några stammar av *Valsa nivea* Fr.

Föreliggande undersökning är en fortsättning på arbeten redovisade i en tidigare publikation (Persson 1955) rörande uppträdandet av *Valsa nivea* som parasit på hybriden mellan *Populus tremula* × *P. tremuloides*. Problemets ekologiska sida har emellertid ej berörts i denna senare uppsats utan här har i stället några stammar av svampen undersökts med avseende på enzymaktivitet samt behov av kolkälla i relation till tillväxtsubstanser. De huvudsakliga resultaten kan sammanfattas sålunda.

1. Barkextrakt från hybridasp och de båda föräldraarterna har analyserats i första hand med avseende på energikällor tjänliga för *Valsa nivea*. Denna ascomycet uppträder som parasit i hybridaspens krona under det att den på föräldraarterna är känd som en harmlös saprofyt. Medelst papperskromatografisk analysmetod identifierades hos föräldraarterna och hybridasp glykos och fruktos. Hos hybridasp tillkommer emellertid en betydande fraktion sackaros, vilken saknas hos svensk asp och nordamerikansk asp, d. v. s. föräldraarterna.

2. Med utgångspunkt från denna markanta metaboliska olikhet underkastades 13 stammar av *Valsa nivea* en granskning omfattande den enzymatiska aktiviteten med avseende på sackaros in vitro. 2 % sackaroslösning tillfördes frystorkat mycel och prover uttogs med bestämda tidsintervall och överfördes till kromatogram. Den hastighet med vilken sackarosmolekylen spjälkades till glykos och fruktos tjänade som mått på sackarasaktiviteten. En distinkt uppdelning av svampstammarna kunde göras. Två av dem saknade helt sackaras; dessa båda visade sig i resistenstest i fält vara nära nog apatogena. Den stam, V 663, som i detta sammanhang huvudsakligen används för att undersöka resistens hos hybridaspavkommor har emellertid en låg sackarasaktivitet. Det föreligger således inget enkelt samband mellan sackarasproducerande förmåga och patogenitet.

3. Tillväxtförsök med de aktuella svampstammarna i KHG-näringslösning innehållande sackaros som energikälla visade något överraskande att även de båda stammar som in vitro ej kan spjälka sackaros ändå växte bra i nämnda näringslösning. I denna ingår emellertid jästextrakt innehållande aminosyror och vitala tillväxtämnen. I en försöksserie

varierades kvantiteten jästextrakt under det att sackaroskoncentrationen hölls konstant på två nivåer: 0 % och 5 %. I det förra fallet växte den starkt patogena stammen V 663 sämre än den apatogena V 701; i det senare avsevärt mycket bättre, och detta i relation till koncentrationen av jästextrakt.

Om ett samband föreligger mellan patogenitet och tillväxt kan man sammanfattningsvis påstå att förekomsten av sackaros hos värdväxten ej ensam kan förklara den selektiva angreppstyp, som *Valsa nivea* framkallar hos hybridasp. Substanser av tillväxtämneskaraktär synes verka stimulerande på patogenens förmåga att utnyttja värdväxtens kolhydrater.