STUDIES ON THE HYPERTROPHIC DISEASES CAUSED BY TAPHRINA SPECIES I. NUTRITIONAL BEHAVIORS OF PATHOGENS

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I. NUTRITIONAL BEHAVIORS OF PATHOGENS

By

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Introduction

The hypertrophic diseases caused by *Taphrina* species have been studied by the authors and the anatomical changes of the leaf curl of peach which was observed periodically after infection, had been reported (4).

From the observation on the process in the tumour formation, it was supposed that the tumour was formed as a result of the action or stimulation of excreted metabolites by the pathogen. Therefore, it was necessary to culture the pathogens and test the excreted metabolites (5), but except for Mix's reports there is scarcely any report concerning to the culture of *Taphrina* species.

Consequently, at first some cultural behaviors of three fungi (*Taphrina deformans* Tul., *T. pruni* Tul. and *T. cerasi* Sadeb.) were investigated.

The outline of these experimental results had already been reported (3) and in this paper the details will be presented.

Materials and methods

The three fungi used in the experiments, were isolated as described below and the mono-sporous isolates preserved.

Ascospores on the diseased leaf, were sweeped gently by a wet soft brush and suspended in 1 per cent cupper sulfate solution. And then after five minutes, one drop of the suspension was spotted on the potato dextrose agar by using the loop or pipette. At three or five days after incubation (24°C), the mycelia grew to about 3 cm in diameter, so the tip of the mycelia were transplanted to another medium.

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These fungi grew at first like yeast and then form a white or pinkish mycelial mat. Single conidium was picked up aseptically from the above described culture.

The fungi were cultured on the potato dextrose agar for twenty days at 18°C. And the produced conidia were used as the inoculum. Conidia were washed twice by sterilized dist. water and suspended in sterilized dist. water. The spore concentration of the inoculum was counted by the Thoma haematometer and always adjusted to about 9×10^6 spores per ml. Every nutrient solution was inoculated with one loop of the spore suspension. From preliminary experiments, we knew that these fungi grew well in the aerobic condition. Therefore, the culture was shaked horizontally with a reciprocating motion during the incubation at 24 ± 1°C (20 ± 1°C for the experiment of vitamin requirement). Shaking was done intermittently every quarter minutes (width 6.7 cm, 100 cycles/min). Culture vessels for shaking were T type test tube 50 ml in volume and always three vessels were used for one plot in every experiment.

After the incubation for a given period, the fungal bodies in the nutrient solution were separated by centrifugation and then washed by dist. water and were dried at 80°C until attaining a constant weight. The growth rate in each of the nutrient solution was compared by the dry weight of fungal bodies.

Fundamental constituents of tested nutrient solutions and its concentration are as follows: KH_2PO_4 0.1 g, $MgSO_4$ 0.05 g and $FeCl_3$ trace in dist. water (100 ml).

Results

1) pH and growth

Experiments to determine the optimum pH for the growth were done as

Table 1. pH and growth

Initial pH	*T. deformans*		*T. pruni*		*T. cerasi*	
	Final pH	F.B. wt.	Final pH	F.B. wt.	Final pH	F.B. wt.
1.05	1.1	0 mg	1.1	0 mg	1.3	0 mg
1.30	1.3	0	1.4	0	1.4	0
1.85	2.0	4.4	2.0	9.1	2.0	3.6
2.30	4.2	30.1	3.8	31.7	4.0	36.1
2.60	4.4	53.5	5.1	39.8	6.2	49.0
4.00	4.5	43.8	4.6	32.3	6.4	53.0
4.45	4.3	51.3	4.7	30.3	6.2	56.9
5.80	4.7	42.5	4.6	33.1	6.4	49.8
6.45	4.9	36.6	5.2	22.8	6.6	48.5
L.S.D.(0.05)		8.4		5.8		12.8

Note : Initial pH were measured after sterilization.
Sterilization was done at 100°C for 10 min.
The period of culture was 7 days.
F.B. : Fungal bodies.

illustrated below. $NaNO_3$ (0.3 g) and glucose (5 g) were added to the fundamental liquid medium of 100 ml and each media were adjusted to different pH by adding 1N HCl or NaOH, and then autoclaved. Initial pH and final pH of the media were measured by the glass-electrode method.

As shown in Table 1, these three fungi could grow in the medium of the initial pH 1.85. Optimum pH value was in the acidic side and its range was comparatively wide i.e., *T. deformans* 2.6~6.5, *T. pruni* 2.3~5.8, *T. cerasi* 2.6 ~6.5.

Accordingly, it was evident that these fungi required an acidic environment for the best growth.

2) Nitrogen source and growth

a) *The utilization of inorganic nitrogen and growth*

The inorganic nitrogen compounds were respectively added to the fundamental medium, as the sole nitrogen, so as to contain the same nitrogen content of $NaNO_3$ (0.3 g). The effects of various compounds upon the growth of the fungi were investigated. In this experiment, glucose (5 g/*dl*) was used as the carbon source.

The results obtained are indicated in Table 2. These fungi were able to grow on every medium, but the growth of *T. pruni* was not so much as the

Table 2. The utilization of inorganic nitrogen and growth

Nitrogen source	*T. deformans*		*T. pruni*		*T. cerasi*	
	Final pH	F.B. wt.	Final pH	F.B. wt.	Final pH	F.B. wt.
NH_4Cl	1.5	103.6mg	1.8	69.3mg	1.8	80.9mg
$(NH_4)_2C_2O_4$	2.5	61.3	2.8	37.5	3.2	78.8
$(NH_4)_2SO_4$	1.8	143.3	1.6	102.7	1.8	115.3
NH_4NO_3	2.4	67.8	2.6	60.7	2.6	75.9
$Ca(NO_3)_2$	4.0	23.0	4.0	29.6	5.4	131.0
KNO_3	4.2	38.9	4.2	38.0	5.2	79.0
$NaNO_3$	4.2	37.5	4.2	36.3	5.6	73.2
$NaNO_2$	5.8	0	5.8	0	5.8	0
KNO_2	5.8	0	5.8	0	5.8	0
check	4.8	0	4.8	0	4.8	0
L.S.D.(0.05)		0.5		10.1		22.2

Note : Initial pH were adjusted so as to become pH 4-5 after sterilization.
Sterilization was done at 110°C for 10 min.
The check does not contain any nitrogen source.
The period of culture was 10 days.

others. The most vigorous growth was obtained on the media containing NH_4-N. Above all, $(NH_4)_2SO_4$ was the most excellent as the nitrogen source, and also NH_4Cl was relatively better. But *T. cerasi* utilized NO_3-N as well

as NH_4-N, and $Ca(NO_3)_2$ was the best nitrogen source among NO_3 salts. So the nature of *T. cerasi* was different from the others.

These fungi could not utilize nitrite nitrogen.

As generally reported, when NH_4-N was added the final pH of the media became acidic and neutral in the case of NO_3-N.

In all culture, it was observed that alcohol fermentation had occured.

b) *The utilization of organic nitrogen and growth*

Organic nitrogen compounds as shown in Table 3 were used. Nitrogen content in the culture medium were regulated the same as $NaNO_3$ (0.3g). Carbon source was glucose (5g/*dl*).

Table 3. The utilization of organic nitrogen and growth.

Nitrogen source	*T. deformans*		*T. pruni*		*T. cerasi*	
	Final pH	F.B. wt.	Final pH	F.B. wt.	Final pH	F.B. wt.
glycine	3.6	35.4 mg	2.8	40.0 mg	4.0	88.6 mg
β-alanine	4.2	29.6	4.2	34.1	4.2	34.5
α-alanine	3.6	33.3	4.0	36.6	4.0	41.1
dl-valine	2.6	34.8	3.4	31.6	3.4	39.2
l-leucine	3.4	41.7	2.8	32.6	3.4	32.5
l-isoleucine	3.4	52.4	3.4	29.9	3.4	7.3
dl-threonine	4.0	4.9	3.8	11.2	4.2	24.8
dl-serine	4.0	9.2	3.6	23.0	4.2	53.5
l-proline	3.8	45.4	4.0	43.3	4.0	240.3
l-tryptophan	4.0	18.7	3.6	19.5	4.0	17.0
l-methionine	3.2	45.6	3.0	47.8	3.0	48.7
l-cysteine	4.2	15.4	2.6	24.2	2.6	25.5
l-aspartic acid	3.9	33.2	3.8	32.8	4.0	121.0
l-glutamic acid	3.8	38.9	4.2	51.5	4.1	134.0
l-lysine	2.5	36.7	2.4	45.1	2.4	70.5
l-arginine	2.6	66.0	2.6	95.8	2.8	87.5
l-histidine	3.6	11.6	2.6	32.0	3.2	31.3
asparagine	3.6	49.9	4.1	50.0	4.0	56.5
urea	4.0	37.0	4.8	0	4.4	71.5
check	4.8	0	4.8	0	4.8	0
L. S. D. (0.05)		1.1		1.4		2.8

Note : See the note of Table 2.

In general, the growth of the fungi on the medium containing amino acid or amide, was less than on the medium with NH_4-N.

But among the compounds, *l*-arginine was commonly a favorable nitrogen source for these fungi and utilized as well as inorganic sources.

T. cerasi indicated different response to amino acids from others. L-proline, *l*-glutamic acid, *l*-aspartic acid and glycocol were utilized quite well. Particularly

l-proline was the most easily utilizable nitrogen source among all kinds of nitrogen sources.

Also, urea was utilized by *T. deformans* and *T. cerasi*, but never by *T. pruni*. Final pH of the media were influenced differently by each fungi and kind of the amino acid. *T. deformens* more acidified respectively the medium with the amino acid such as *l*-arginine, *l*-valine and *l*-lysine.

Identical phenomena were investigated in the culture of *T. pruni* and *T. cerasi*.

In the culture of *T. pruni*, this fungus acidified the media containing *l*-arginine, *l*-cysteine, *l*-leucine or *l*-histidine respectively and also *l*-arginine, *l*-cysteine, *l*-lysine and *l*-methionine gave the same effect on pH of the culture of *T. cerasi*.

But, in the media with organic nitrogen the change of pH was not so severe as with inorganic nitrogen.

3) Carbon source and growth

The relation between carbon sources and the growth observed. Carbon sorces were respectively added to 100 *ml* of the fundamental medium so as to contain the same content of carbon in 5 g of glucose, and $NaNO_3$ (0.3 g) was added as the nitrogen source.

As indicated in Table 4, the favorable carbon soucrce was different in each of the fungi. Starch and fructose for *T. deformans*, starch, sucrose and maltose for *T. cerasi* and raffinose for *T. pruni* were most favorable to their growth.

Table 4. Carbon source and growth

Carbon source	*T. deformans*		*T. pruni*		*T. cerasi*	
	Final pH	F.B. wt.	Final pH	F.B. wt.	Final pH	F.B. wt.
raffinose	6.2	88.1 mg	7.6	78.4 mg	7.2	73.2 mg
xylose	6.2	65.9	6.2	47.3	5.8	19.1
galactose	7.0	26.3	7.0	37.6	6.8	28.5
fructose	6.2	95.7	5.8	54.5	6.6	74.6
glucose	4.2	40.2	4.4	29.0	5.0	75.0
sucrose	4.6	41.2	4.2	27.0	7.8	134.3
maltose	4.2	55.9	4.4	33.2	5.4	118.7
lactose	5.8	3.5	6.0	6.4	5.6	5.5
starch	5.0	104.3	4.8	30.6	7.2	136.2
glycerol	5.8	3.8	5.8	4.4	5.4	4.4
mannitol	7.4	59.8	7.6	60.7	7.6	48.5
check	4.8	0	4.8	0	4.8	0
L.S.D.(0.05)		5.3		2.5		8.9

Note : See the note of Table 2.
The check does not contain any carbon source.

And every fungi could not utilize glycerol and lactose.

Therefore, on the utilization of sugars these fungi had behaviors different from each other.

4) Vitamin requirement and growth

Generally speaking, fungi like other organisms require growth factors for their growth. But it is obscure whether these fungi require an external supply of vitamins. So, their requirement for vitamin was examined.

NH_4Cl (0.189 g), as a nitrogen source, was added to 100 *ml* of the fundamental medium from which the contaminants were eliminated by active carbon. And then, 100 γ of one or more kinds of vitamin were added respectively to the medium (100 *ml*).

The results obtained are indicated in the Table 5 and Table 6. In *T. deformans* promotive effect to the growth with use of almost all vitamins, except biotin, was recognized even after ten days.

Table 5. Vitamin requirement and growth.

Vitamin	*T.deformans*		*T. pruni*		*T. cerasi*	
	Final pH	F.B. wt.	Final pH	F.B. wt.	Final pH	F.B. wt.
		mg		mg		mg
B_1	2.1	62.7	1.8	60.7	2.1	69.9
B_2	2.0	67.1	1.7	60.6	2.1	74.4
B_6	2.1	56.0	1.8	56.9	2.1	74.0
Pan	2.1	74.2	1.9	53.2	2.0	76.9
N	2.1	65.2	1.8	53.4	2.2	75.9
P	2.1	55.2	1.7	61.5	2.0	81.1
Bi	2.4	42.5	1.9	55.3	2.1	74.9
B_1+Bi	2.1	66.1	1.8	58.8	2.1	84.5
B_1+Bi+B_2	2.1	66.2	1.7	60.7	2.0	90.8
B_1+Bi+P	2.1	56.2	1.9	54.5	2.1	88.7
B_1+Bi+P+N	2.1	56.7	1.8	61.8	2.1	84.4
B_1+Bi+P+N+Pan	2.1	51.7	1.8	61.0	2.1	92.5
B_1+Bi+P+N+Pan+B_6	2.1	55.0	1.9	54.1	1.9	86.3
check (1)	2.2	52.2	1.9	54.9	2.2	61.3
check (2)	2.0	56.9	1.8	52.1	2.1	61.7
L. S. D. (0.05)		1.8		3.8		2.1

Note : Initial pH was 4.0.
Sterilized at 100°C for 10 min.
Cultured for 7 days.
(1) Treated with active carbon. Vitamin free medium.
(2) Untreated. Vitamin free medium.
Abbreviation
N : Niacin Pan : Pantothenic acid Bi : Biotin P : PABA

Table 6. Vitamin requirement and growth.

Vitamin	*T. deformans*		*T. pruni*		*T. cerasi*	
	Final pH	F.B. wt.	Final pH	F.B. wt.	Final pH	F.B. wt.
		mg		mg		mg
B_1	2.2	63.8	1.8	64.2	2.0	94.7
B_2	2.1	47.1	1.8	76.8	2.0	94.8
B_6	2.1	63.2	1.8	68.3	2.1	75.9
Pan	2.0	61.8	1.9	64.6	2.1	83.7
N	2.0	65.8	1.9	66.4	2.1	83.1
P	1.9	61.3	1.9	65.0	2.0	96.0
Bi	2.0	63.3	2.0	65.0	2.0	85.3
B_1+Bi	2.0	56.3	1.8	58.0	2.0	98.4
B_1+Bi+B_2	2.1	73.4	1.9	68.9	2.0	106.7
B_1+Bi+P	2.1	56.1	1.9	73.8	2.0	94.5
B_1+Bi+P+N	2.0	61.7	1.9	65.6	2.0	113.7
B_1+Bi+P+N+Pan	2.0	64.1	1.8	64.1	2.1	107.7
B_1+Bi+P+N+Pan+B_6	2.0	60.1	1.9	55.2	2.0	104.9
check (1)	2.1	46.5	1.9	75.7	2.1	97.3
check (2)	2.1	56.3	1.8	71.9	2.0	79.2
L. S. D. (0.05)		5.7		1.1		6.4

Note : Cultured for 10 days.

In *T. pruni*, the effects of vitamins were scarcely observed and in *T. cerasi*, the promotive effect was detected only after seven days with all of the vitamins tested. A mixture of two or more kinds of vitamins did not promote the effect so much as one.

Accordingly, the vitamins tested were not seemed to be indispensable to the growth.

5) Carbon and nitrogen concentration and growth

The effects of the concentration of carbon and nitrogen upon the growth were investigated. Glucose was used as the carbon source and $NaNO_3$ as the nitrogen source. Glucose and $NaNO_3$ were added to the fundamental medium (100 *ml*) in the concentration as follows :

Glucose : $NaNO_3$ 1 : 0.3
1 : 0.6
5 : 0.3 unit : g/*dl*

As shown in the Figs. 1, 3 and 5, the growth of three fungi was the most vigorous on the medium containing 5 g glucose and 0.3 g $NaNO_3$. Especially, this tendency was clear in *T. cerasi* and it was observable to grow vigorously after seven days. However, there were no differences between 1 : 0.3 and 1 : 0.6.

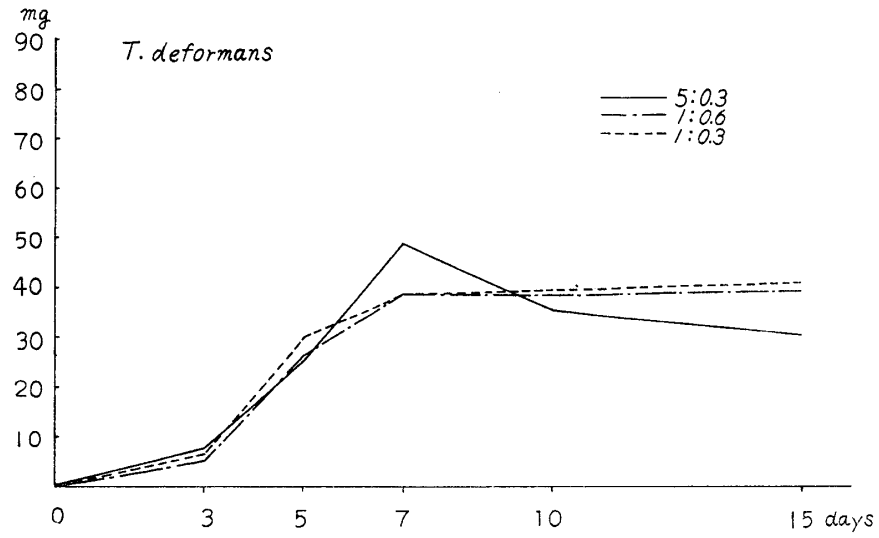


Fig. 1. Carbon and nitrogen concentration and growth.

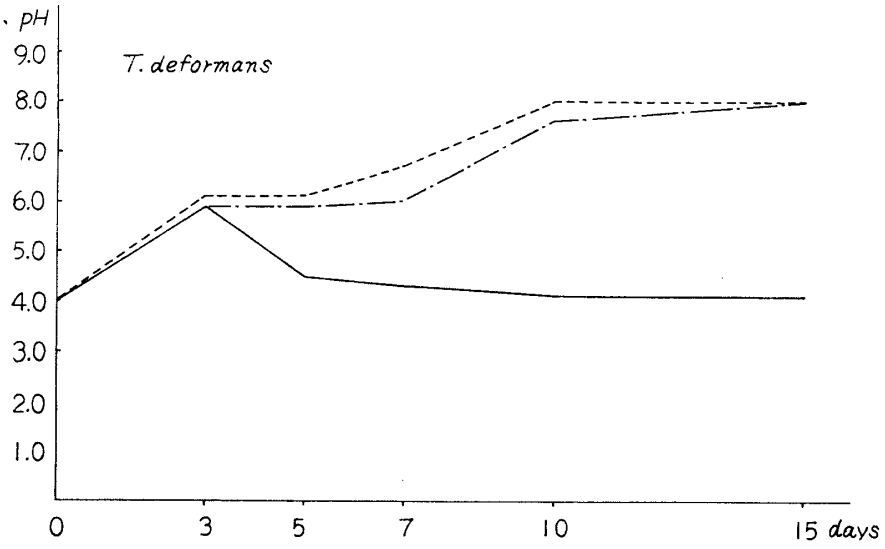


Fig. 2. Change of pH value. Note : Initial pH was 4.0.

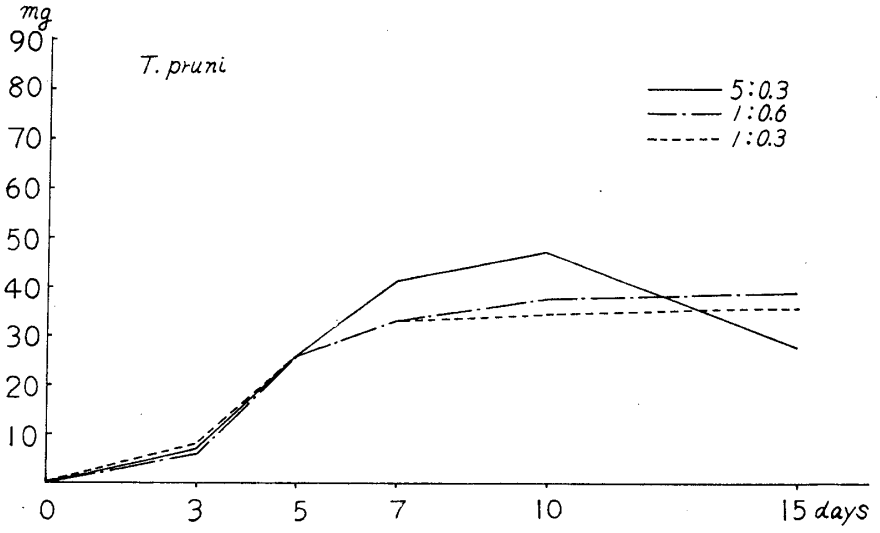


Fig. 3. Carbon and nitrogen concentration and growth.

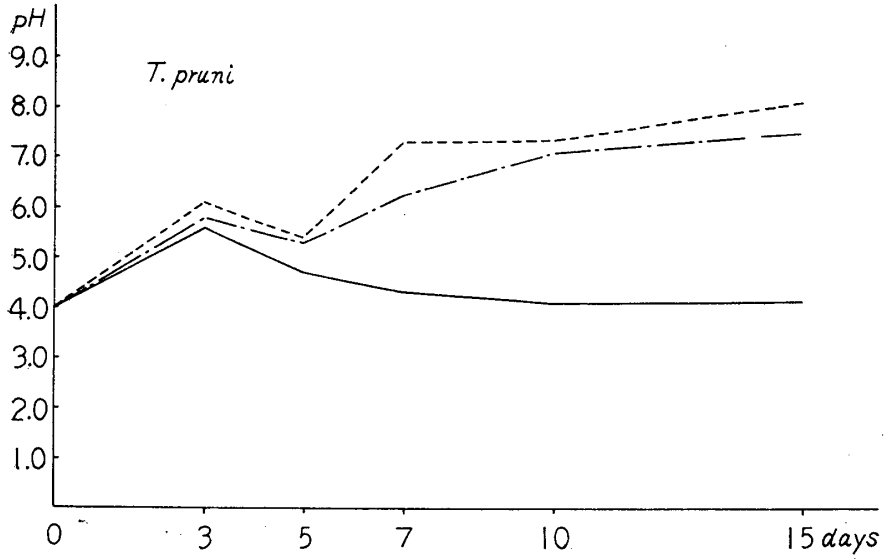


Fig. 4. Change of pH value.

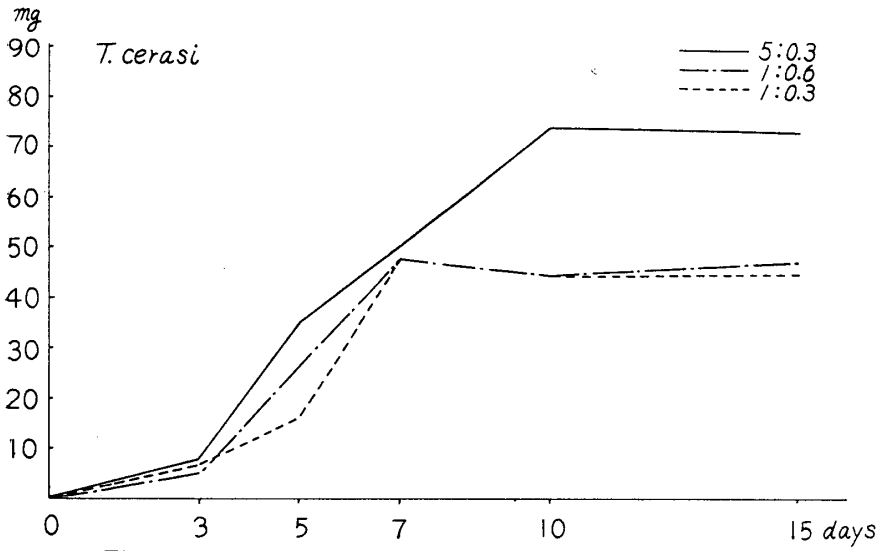


Fig. 5. Carbon and nitrogen concentration and growth.

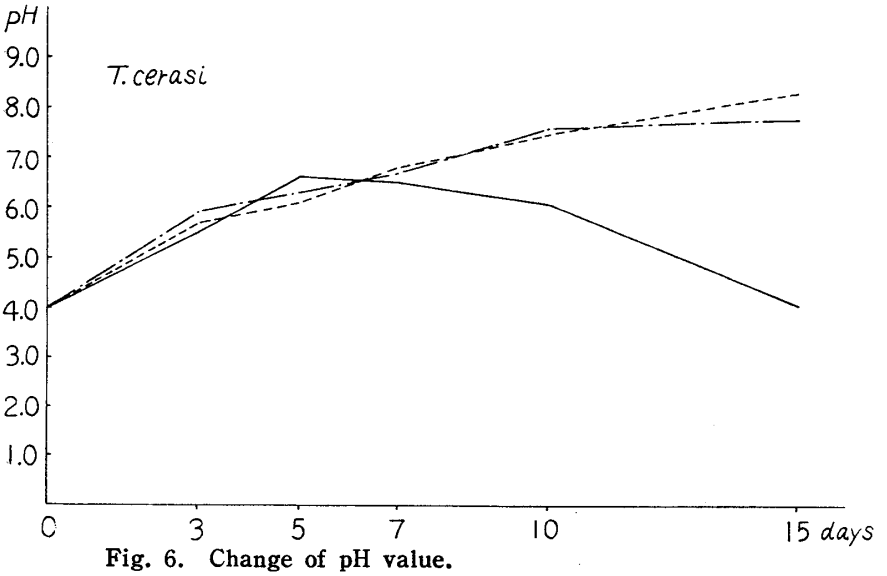


Fig. 6. Change of pH value.

It seemed that the growth of *Taphrina* species depends upon the quantity of sugar. In those experiments, the change of pH of the media was influenced by the growth rate of the fungi. In the media which contains relatively less carbon such as 1 : 0.3 and 1 : 0.6, its pH value changed to the neutral side with increasing growth. But in the medium which contains much carbon as 5 : 0.3, its pH value temporary ascended and then descended. In *T. deformans* and *T. pruni*, this phenomenon was typical and the turning point of pH was observed at three days after inoculation. But this phenomenon occured only when glucose was used as the carbon source and not in the case of galactose.

Discussions and Conclusions

Mix (6) reported that *T. deformans* did not grow below pH 3.3, but it was observed that these three fungi, containing *T. deformans*, were able to grow in pH 1.85 which is lower than 3.3. The reason why such difference occured may be that in Mix's experiment the culture medium was not synthetic and the method of measurement of pH was probably imperfect, Though, the optimum pH 4-5 was similar with our experiment.

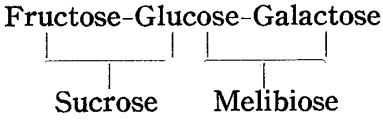
Although NH_4-N is the best nitrogen source for these fungi, *T. cerasi* also utilized NO_3-N as well as NH_4-N. Robbins (10) classified the fungi into four groups by the kind of utilized inorganic nitrogen. According to his classification, these three fungi seem to belong to his second group.

Itoi (2) reported that $(NH_4)_2C_2O_4$ was a good nitrogen source for *Stagonospora carpathica*, because this compound had oxalic acid besides NH_4-N. In our experiment, such phenomenon was not investigated.

These three fungi utilized amino acids and amides, but their growth was not so vigorous as in NH_4-N except *T. cerasi*.

In the yeast and other fungi, it is said that there is an intimate relationship between the utilization of amino acids and vitamin requirement. But we did not observe this relation with these three fungi.

Raffinose was utilized well by the three fungi as a carbon source. Raffinose has the following structure and is decomposed to fructose and melibiose by the invertase. If the fungi excrete the invertase, it should utilize one-third of raffinose i.e., only the portion of fructose, and consequently unutilized melibiose might remain.



Whether the fungi have the melibiase decomposing melibiose to glucose and galactose is not clear. But it was observed that these three fungi grew well on the medium with raffinose so much as one-third of the growth on the medium with fructose, therefore it seemed that fungi were able to utilize

the melibiose.

As stated above, these fungi produce alcohol in the medium, and the degree of fermentation varies according to the kind of sugar. Price *et al.* has already reported this phenomnon (by Mix's report (7)). Injurious effects of ethyl alcohol to the plant had been studied by Nishimura (9), Sheffer & Walker (11) and Nashed & Girton (8). Therefore, alcohol fermentation of these fungi should be studied from the phytopathologic point of view.

These three fungi were able to grow on the vitamin free medium and there were scarcely any differences on the growth between the vitamin free and vitamin added media. These results make us imagine that the tested fungi are autotrophic to vitamins.

In general, the final pH of the medium becomes basic by the selective absorption of NO_3, when $NaNO_3$ was used as the sole nitrogen source of the medium. From that the final pH became acidic on the medium (Composition: glucose 5 g, $NaNO_3$ 0.3 g) as indicated in Figs. 2, 4 and 6, it was considered that these three fungi produced some acidic substances. It was described above that in *T. deformans* and *T. pruni* the pH of the medium ascended temporarily and then descended. This phenomenon seems to be related to the period of production of the acidic substance.

From the experiment reported above, it was concluded that the favorable synthetic media for the culture of *Taphrina* species are as follows.

$(NH_4)_2SO_4$	0.23 g
KH_2PO_4	0.1 g
$MgSO_4$	0.05 g
$FeCl_3$	trace
Carbon source	5 g
Dist. water	100 *ml*
Initial pH	4-5

For the carbon source, the following sugars are suitable. Starch or fructose in *T. deformans*, raffinose in *T. pruni* and starch, sucrose or maltose in *T. cerasi*.

These three fungi grew well under aerobic conditions. So, it is desirable to culture by shaking or air bubbling.

By using this culture medium the fungi of *Taphrina* sp. were cultured. And the plant growth substance was extracted from the culture filtrate and crystallized in 1961 (5). The details of those experiments will be presented in the next report.

Summary

The requirement of nutrients of *Taphrina* species (*Taphrina deformans, T. pruni, T. cerasi*) were experimented.

These fungi were isolated from diseased plants and mono-sporous isolates were used for the experiment. Every culture was done by the shaking method.

1) These three fungi grew well on synthetic medium. Among them, the growth of *T. cerasi* was the most vigorous.

2) The range of the optimum pH for the growth was 2.6-6.5 in *T. deformans*, 2.3-5.8 in *T. pruni* and 2.6-6.5 in *T. creasi.*

3) For the nitrogen source, NH_4-salts were better than NO_3-salts. Especially, $(NH_4)_2SO_4$ was most favorable.

Amino acids and amides were also used well by these fungi. Especially, *l*-arginine was suitable for these three fungi. Further-more, *l*-proline, *l*-glutamic acid, *l*-aspartic acid and glycocol were utilized quite well in *T. cerasi.*

4) For the carbon source, starch, fructose and raffinose were utilized fairly well by *T. deformans*, raffinose by *T. pruni* and starch, maltose and sucrose by *T. cerasi.*

5) The growth of these fungi depended upon the quantity of sugar.

6) These fungi were autotrophic to tested vitamins.

7) When glucose (5g/*dl*) was used for the carbon source, the pH value of the culture medium became acidic at 15 days after inoculation. And this phenomenon was not observed with galactose.

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