STUDIES ON THE HYPERTROPHIC DISEASE CAUSED BY TAPHRINA SPECIES VI. THE SECRETION OF NUCLEIC ACID RELATING SUBSTANCE

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VI. THE SECRETION OF NUCLEIC ACID RELATING SUBSTANCE

By

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Introduction

As reported previousely, in the leaf curl disease the remarkable cell division of infected tissue occurs (10). And in witches' broom disease, the abnormal cell division following abnormal genesis of twigs occurs. In the infected tissue of the leaf curl disease, the DNA content of nucleus increased (14). It was clarified by recent reports that the DNA and RNA synthesis, nuclear division, cell division and morphogensis were controlled by co-operative action of native auxin and native kinin. And moreover it was indicated that when one of them did not exist, the cell division did not occur and when auxin or kinin was supplied externally, cell division was promoted.

Taphrina fungi excrete various auxins and hemiauxins (13). However these substances promoted the DNA and RNA synthesis, but do not initiate singly the nuclear division and cellular division (2, 3, 10, 21). From the above it is assumed that the phytokinin secreted by *Taphrina* fungi is one of the important agents in the cell division of the hypertrophic tissue. Therefore the secretion of kinin by these fungi was exmained. Results of these experiments will be reported in this paper.

Material and Method

I. Isolation from fungal bodies

In the preliminary test, we extracted the active substance, which promoted the callus growth from fungal bodies by chloroform-gel method. The extract was autoclaved at 120°C for 60 min., and then the active substance was extracted with

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basic ether. But this method contains the process of heat treatment. So this substance is likely an artifact as the kinetin. Therefore, the phenol method was employed as a mild method for the extraction of nucleic acid relating substances.

Taphrina wiesneri was cultured in the synthetic medium reported previously (II) at 25°C for 10 days with aeration. The fungal bodies of stationary growth phase were used as the sample. The sample was ground with a quarz sand by adding 2% sodium dodecyl sulfate solution of one-tenth volume of fungal bodies under low temperature. Five times volume of 0.05 M tris buffer (pH 7.4) containing 0.05 M $MgCl_2$ was added into the homogenate and the mixture was poured into the separation funnel, and then shaked with the equal volume of 90% phenol for 5–10 min., and centrifuged at 5,000×g for 20 min. The deproteinization process was repeated and then ether was added to eliminate phenol which dissolved slightly in the aqueous solution. White color substance which may be a mixture of DNA and RNA sedimented. This nucleic acid relating substance was removed and dried. This substance was dissolved in tris buffer stated above, and filtered by Seiz filter and added aseptically to the following tissue culture medium in the various concentrations.

The stem of tobacco plant (*Nicotiana tabacum* var. White barley) was used for the callus formation. The stem was cut to 5 mm long and then sectioned vertically.

The outside tissue of the stem was shaved out and sterilized for a short time with lime chloride solution (10 g of bleaching powder added to water and filtered). This piece was transplanted on the agar medium containing Murashige and Skoog's composition (16). Twenty days after culture at 25°C, the piece of generated callus was removed to the new made medium. The transplantation was repeated and from this precultured callus tissue the small piece of the same size was cut out and transplanted to the various media examined in this experiment. Five pieces of the callus were used for one 200 ml flask. The constituents of various culture media examined :

A	:	Basal medium (Murashige and Skoog's solution from which IAA and kinetin were eliminated)			
B	:	〃	〃	+	IAA (10 γ)
C_1	:	〃	〃	+	Extract (0.2 mg)
C_2	:	〃	〃	+	〃 (2.0 mg)
C_3	:	〃	〃	+	〃 (20.0 mg)
D_1	:	〃	〃	+	〃 (0.2 mg) + IAA (10 γ)
D_2	:	〃	〃	+	〃 (2.0 mg) + IAA (10 γ)
D_3	:	〃	〃	+	〃 (20.0 mg) + IAA (10 γ)
		Total	50 ml		

Forty-two days after culture, dry weight and fresh weight without roots were measured and compared with each others.

2. Secretion into buffer solution

It was assumed that this substance may be excreted out of the fungal bodies. So to investigate whether this substance is excreted, secretion from fungal bodies was examined. *Taphrina deformans*, *T. pruni* and *T. wiesneri* were used. Each fungus was inoculated to $(NH_4)_2SO_3$ added medium respectively and cultured at 24°C for 5 days. Developed fungal bodies were collected by centrifugation at 3,000 rpm for 3 min. Obtained fungal bodies were washed twice by sterilized water and 300 mg (dry wt.) of fungal bodies was suspended into 100 ml of sterilized 0.08 M citric acid buffer (pH 6.0). Five ml of this suspension was incubated at 25°C for 6 hrs. After the incubation the fungal bodies were removed by centrifugation. By orchin- and diphenylamine reaction, the existence of nucleic acid relating substance was examined and the secreted amount was measured by the optical density at 260 mμ.

To fractionate the excreted substance, ion exchange chromatography by Berghirst's modified method (6) was used. The resin is Dowex IX, 10, 200–400 mesh and formic acid type. From the sample, contaminated ion was eliminated by active carbon (6, 17) and alcohol solution of sample was used for the chromatography. The elution was done with stepwise concentration by the following eluent. Each 10 ml of the fractionated eluate was sampled by a fraction collector and the optical density at 260 mμ was estimated by a spectrophotometer.

The constitutents and concentrations of the solution which was used for the elution:

1.	H_2O					100 ml
2.	0.005 N	HCOOH				200 ml
3.	0.02 N	〃				200 ml
4.	0.1 N	〃				400 ml
5.	0.05 N	HCOONa	in	0.1 N	HCOOH	400 ml
6.	0.1 N	〃	〃	〃	N	600 ml
7.	0.3 N	〃	〃	〃	N	200 ml
8.	0.6 N	〃	〃	〃	N	300 ml

The commercial yeast RNA was hydrolyzed at 37°C for 8 hrs. with 1 N KOH and absorbed to the column and fractionated. This pattern of elution was used as the control. The identification of eluates was done by the position of elution and RF value of paper chromatogram. The development of the paper chromatogram was carried on by water saturated n-butanol solution at 20–22°C for 15 hrs.

3. Synergistic action of secreted substances

In order to examine the synergistic action among succinic acid, auxin and kinin, this experiment was done. The auxin used in this experiment was IAA, and kinetin was used as a kinin. Murashige and Skoog's medium was used again here along with tobacco callus.

Results

Nucleic acid relating substance extracted from fungal bodies promoted the tobacco callus growth. Results were shown in Fig. 1, 2. Namely in D-medium containing IAA and the extract, the dry wt. and fresh wt. of the callus increased much more than these of B-medium containing IAA alone. The increase of fresh wt.

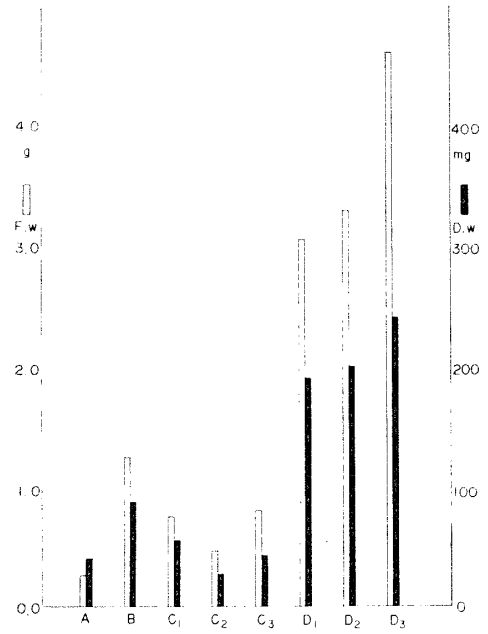


Fig. 1 The effect of the nucleic acid fraction which was extracted from the fungal bodies

Note: A: Check, B: IAA, C_1: Extract (0.2 mg), C_2: Extract (2.0 mg), C_3: Extract (20 mg), D_1: Extract (0.2 mg)+IAA, D_2: Extract (2.0 mg)+IAA, D_3: Extract (20 mg)+IAA.
White line : Fresh weight, Black line : Dry weight

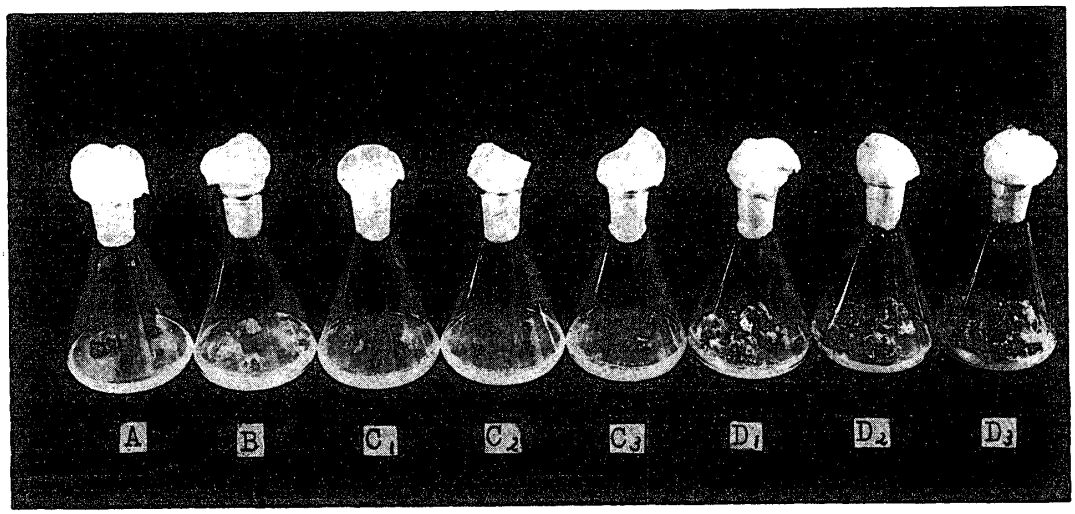


Fig. 2 The effect of active substance which was extracted from the fungal bodies

Note: A, B, C_1, C_2, C_3, D_1, D_2, D_3, was shown in Fig. 1

was especially, notable. On the contrary, cell division and cell enlargement did not occur in the C-medium which contained the extract alone.

The orchin- and diphenylamine reaction of this extract was positive. And also the substance had a peak of UV spectrum at 260 mμ. Thus the existence of DNA and RNA was ascertained.

By the same methods, it was observed that these fungi secreted the orchin reaction positive substance into the same buffer solution. The amount of this substance secreted by the three fungi was much more in order of *T. pruni*>*T. wiesneri*>*T. deformans* as indicated in Fig. 3.

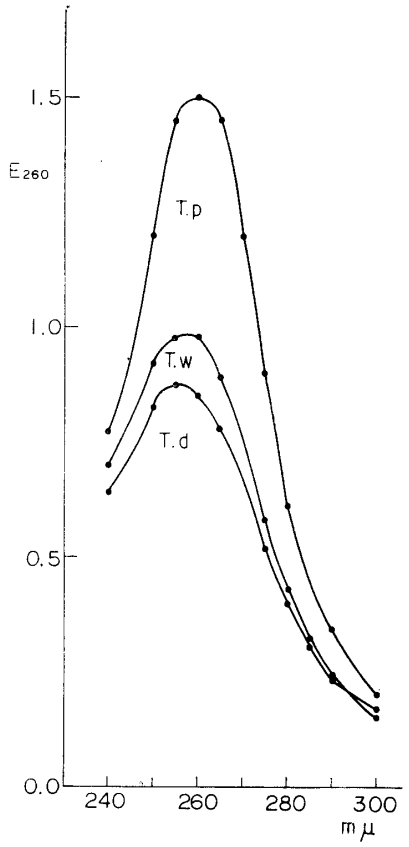
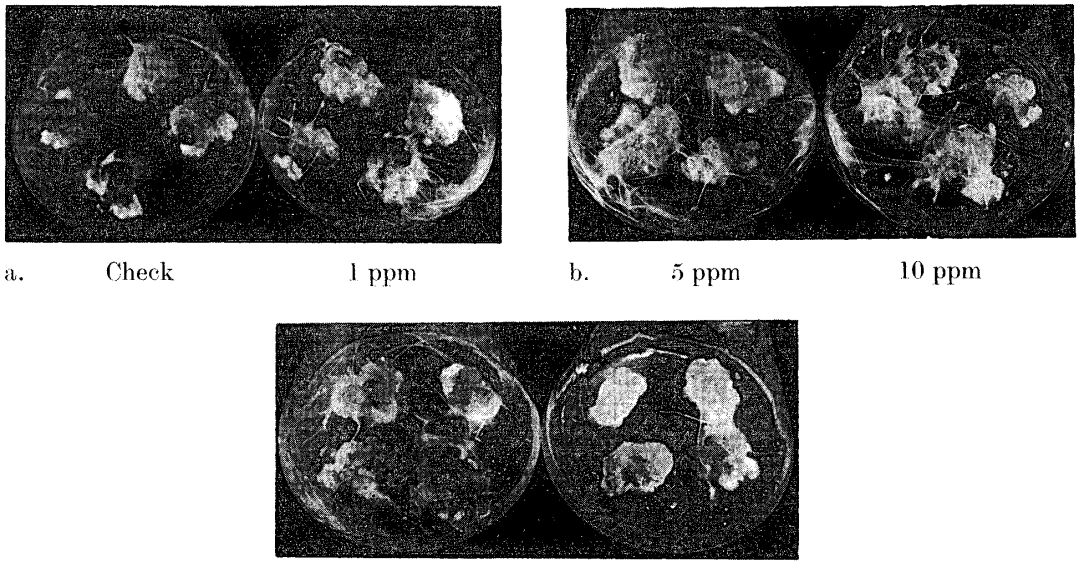


Fig. 3 The amount of the secretion of nucleic acid relating substance into the buffer solution by the three species
Note: Abscissa: Optical density
Ordinate: Wave length
T.p=*Taphrina pruni*, T.w.=*T. wiesneri*,
T.d=*T. deformans*

In the ion exchange column chromatography for this material, about 50 % of absorbed substance was eluted by 0.005 N formic acid. The orchin reaction of this substance was negative, but had a peak at 260 mμ. By the paper chromatography for 0.005 N formic acid eluted fraction, a substance of Rf 0.38 in this fraction was identified as the hypoxanthine. The residual 50 % of the absorbed substance which orchin reaction was positive, was not eluted by the formic acid solution used in this experiment. It is assumed that these substances may be oligonucleotides or more polymelized substances.

The results of synergistic action of excreted substances are shown in Fig. 4, 5, 6, 7. Succinic acid increased the callus growth. Promoting action of succinic



a. Check 1 ppm b. 5 ppm 10 ppm

c. 50 ppm 100 ppm

Fig. 4 a,b,c The effect of succinic acid. The root was formed notably at 5–50 ppm.
Note: IAA–0.2 ppm, Kinetin—0.2 ppm

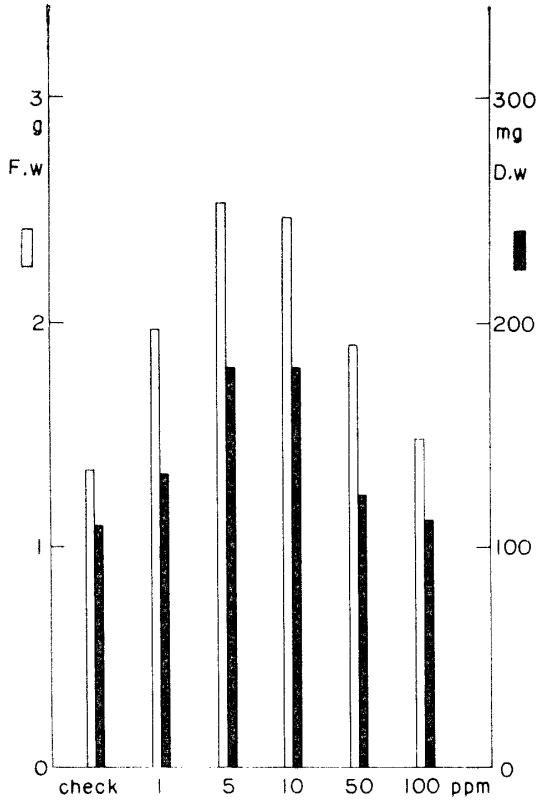
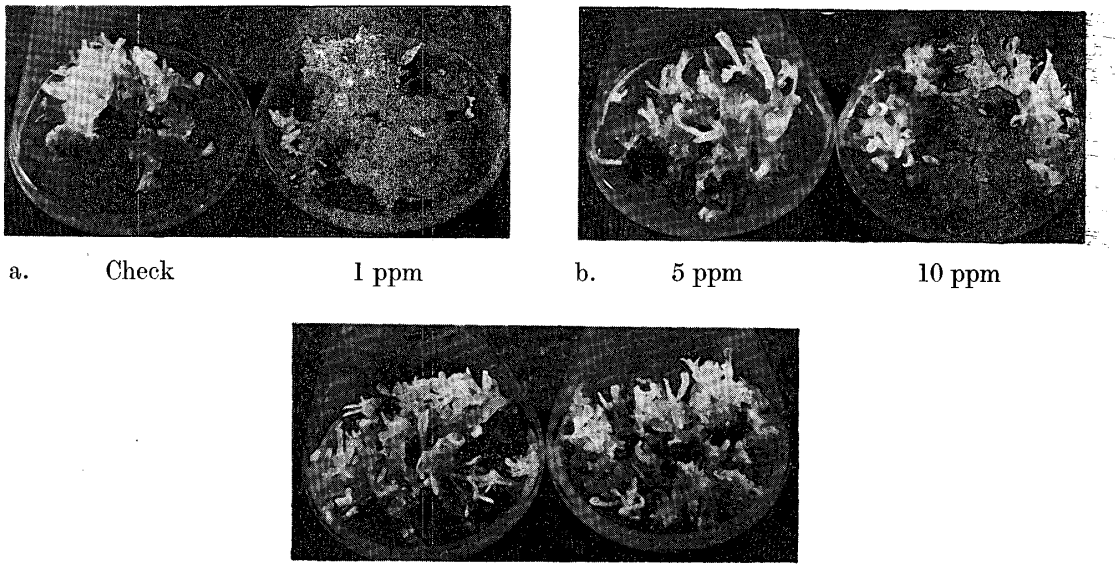


Fig. 5 The effect of succinic acid.
Note: Abscissa indicates the concentration of succinic acid.
White line : Fresh weight, Black line : Dry weight,
IAA — 0.2 ppm, Kinetin — 0.2 ppm



a. Check 1 ppm b. 5 ppm 10 ppm

c. 50 ppm 100 ppm

Fig. 6 a,b,c The effect of succinic acid. The bud formation was stimulated.
Note: IAA—0.03 ppm, Kinetin—1.0 ppm

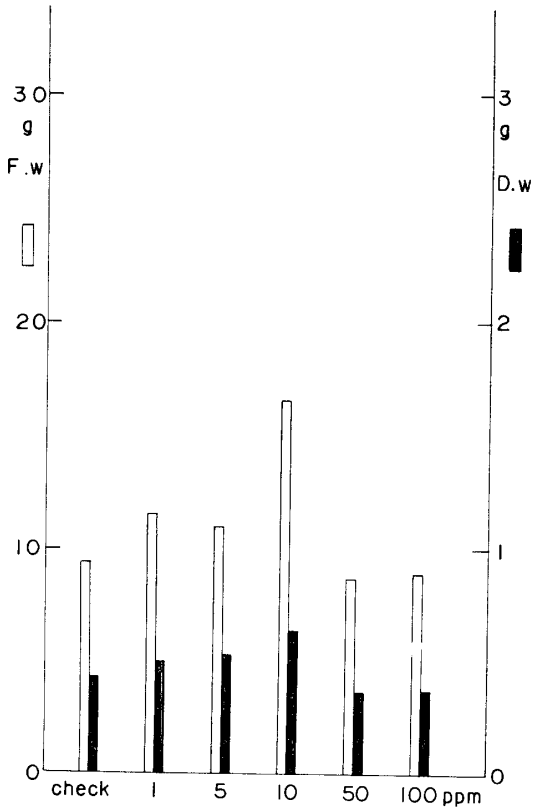


Fig. 7 The stimulation of bud formation. The optimum concentration is 10 ppm.
Note: Abscissa indicates the concentration of succinic acid IAA—0.03 ppm, Kinetin—1.0 ppm.

acid was excellent in the concentration of 10 ppm. When succinic acid was added into the medium containing 0.2 ppm of IAA and 0.2 ppm of kinetin, the root genesis and its elongation were induced. The optimal concentration of succinic acid was 5–10 ppm. And when 0.03 ppm of IAA and 1 ppm of kinetin were used, the bud formation occured, but the bud formation was slightly promoted by succinic acid (Fig. 6, 7).

Discussion

Auxins, hemiauxins and succinic acid promoted the cell enlargement, but not the cell division directly. Recent reports stated that the cell must be at the special age, and auxin and kinin co-existence were needed. So the cell division of the diseased tissue caused by *Taphrina* spp. seems to be a result of the action of auxin and kinin excreted by the fungi. In general, it is assumed that the kinin is a nucleic acid relating substance. Therefore, it is presumed that the active substance which promoted the cell division existed in the nucleic acid fraction we extracted. There is the risk of making an artifact when the extracting method which contains the heat treatment is used, but it is supposed that the phenol method is mild and the extracted substance may be intact. Therefore, the physiological action to the plant tissue cell was examined for the fraction which was extracted by the phenol method. The result showed that this fraction had a promotive action to the growth of tobacco callus. Hildebrandt (7), Satarova (20) and Manil et al (4) reported that the foreign nucleic acid promoted the plant cell division. And Horn, Katsuka and Chivremont & Brachet (4) reported similar results to animal tissue. In the case of diseases caused by *Taphrina* spp., the cell division of infected host tissue may be promoted by the foreign nucleic acid which is secreted by the causal fungus. It has been reported that the active nucleic acid which stimulates the cell division must be extremely polymerized (4). And also it was reported that the remarkably high molecular nucleic acid could permeate the cell membrane (4). Therefore, a considerably polymerized one may be effective in nucleic acid relating substances extracted from the fungal bodies.

Recently Marigault (9) extracted DNA from the fungal bodies of *Agrobacterium tumefaciens* and succeeded in the genesis of crown gall aseptically by using this DNA. This report is of special interest when the results of our experiment are taken into consideration.

Taphrina fungi excrete the hypoxanthine into the buffer solution. Steinert (4) reported that the frog egg contained free hypoxanthine abundantly and it acted in the DNA synthesis. And there are reports that hypoxanthine, adenine and etc. are necessary for the growth of the young leaf tissue and callus growth in a minute amounts (7, 1). In this case it is not clear, whether the nucleic acid-bases are used merely as substrates of nucleic acid synthesis or stimulate the cell divis-

ion as the activator. It is necessary to investigate further the relationship between the gall formation and various nucleic acid bases.

Thus, the various substances which were excreted by *Taphrina* spp. were examined, but there was not the tendency that the different specific stimulative substances were excreted among species of *Taphrina* fungi. Heinis (5) reported that the plum-pocket symptom occured when *T. pruni-subcorditae* infected *Prunus subcordatae* Benth, but when Italian or French prunus was infected, the witches' broom and leaf curl occured. The same phenomenon was reported by Mix (15). Therefore it is supposed that the difference of the symptoms caused by the various *Taphrina* species does not merely depend on the difference of the stimulative substances. And it is assumed that the effective substances excreted by every examined species of *Taphrina* fungi did not act singularly, but acted synergistically in intimate relations to each other.

As stated previously, Patau (18) reported that the cell division was controlled by the balance of auxin and kinin. And IAA does not only elongate the cell wall, but also occupies the important role on the DNA and RNA synthesis (2, 3, 19, 21). Therefore, it is assumed that the concentration balance of the active substances is an important factor for the tumour formation. And in order to ascertain this hypothesis the experiment described above was carried out. Succinic acid greatly promoted callus formation, root genesis and elongation, and bud initiation. It was also clarified that the concentration balance of IAA and kinetin was concerned intimately with the morphogenesis.

Loewenberg (8) reported that citric acid promoted excellently the bud formation of the tobacco callus. So the organic acids of the TCA cycle may be one of the causal agent of witch's broom symptom.

Thus it is supposed that each characteristic symptom caused by *Taphrina* spp. is induced by the synergistic action of auxin, hemiauxin, kinin like substance and succinic acid excreted by these fungi. And also it is presumed that the genesis of each characteristic symptom depends on the concentration balance of the various stimulative substances, and on the species of the host and the infected position of the host. In addition, physiological factor such as the age of the infected tissue or host may be an important factor for the genesis of the hypertrophic symptom.

Summary

The nucleic acid fraction which was extracted from the fungal bodies of *Taphrina pruni, T. wiesneri* and *T. deformans* promoted the growth of tobacco callus tissue. This fraction was only effective when IAA was simultaneously added. The increase of fresh wt. was especially notable. The orchin and diphenylamine reaction of this fraction were positive and had a peak of UV absorption spectrum at 260 mμ.

These fungi secreted the orchin reaction positive substance into the buffer solution. The amount of secreted substance was much more in order of *T. pruni*> *T. wiesneri*>*T. deformans*.

This fraction contains an orchin reaction negative substance and this substance was identified as hypoxanthine. The relationship between these nucleic acid relating substances, succinic acid or auxins, and hypertrophic symptoms caused by *Taphrina* species was discussed.

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