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Biochemical Pathway for the Formation of Abnormal Sclerotia of *Sclerotinia sclerotiorum*

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ABSTRACT

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Biochemical differences between normal (white medulla) and abnormal (brown medulla) sclerotia of *Sclerotinia sclerotiorum* were compared using samples collected from diseased heads of sunflower grown in commercial fields in Manitoba in 1977 and 1979 and in Alberta in 1985 and 1986. Of the 21 free amino acids detected in medullary tissues, only tryptophan (Trp) was significantly ($P<0.05$) reduced in abnormal sclerotia, compared to normal ones. Further analyses of medullary tissues revealed that 5-hydroxytryptamine (serotonin) (5-HT) was present in large amounts in normal sclerotia, but was in small quantity (1977 sample) or absent (1979, 1985 and 1986 samples) in abnormal sclerotia, whereas 5-hydroxyindole acetic acid (5-HIAA) was present only in small quantity in normal sclerotia but was present in large amounts in abnormal sclerotia. When the chemicals 5-HIAA, monoamine oxidase inhibitor (MAOI), Trp, 5-hydroxytryptophan (5-HTP) and 5-HT were tested at 1000 ppm, only the sclerotia from cultures grown on potato dextrose agar amended with 5-HIAA had a high frequency (88.7%) of abnormal sclerotia. The evidence of depleting the neurotransmitter or endocrine modulator of 5-HT and its precursor Trp to its metabolite 5-HIAA in abnormal sclerotia suggests that the serotoninergic pathway is involved in the formation of abnormal sclerotia of *S. sclerotiorum*.

Key words: *Sclerotinia sclerotiorum*, abnormal sclerotia, serotonin, neurotransmitter, serotoninergic pathway

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan species of plant pathogen which has a host range of 408 species in 75 families [(4)], including sunflower (*Helianthus annuus* L.). In Canada and the United States, *S. sclerotiorum* can cause two types of diseases on sunflower, wilt and head rot [(14)]. Wilt is caused by infection of roots by mycelia from myceliogenic germination of sclerotia [(16)] and head rot is caused by infection of sunflower heads by ascospores produced from carpogenic germination of sclerotia [(20)]. Generally, wilt is more predominant than head rot in the major sunflower production areas of North America including North Dakota, South Dakota and Minnesota, the United States [(9)] and Manitoba, Canada [(11)] but in 1986, there was a severe outbreak of Sclerotinia head rot of sunflower in the eastern region of North Dakota where the head rot occurred in 98% of the surveyed fields [(10)].

Sclerotia are the primary survival structure of *S. sclerotiorum* [(5,19)]. Mycelia in stems of diseased sunflower plants survived poorly under the Canadian prairie winter conditions [(18)]. Three types of sclerotia of *S. sclerotiorum* namely normal, abnormal and tan sclerotia, were reported to occur under natural conditions. Normal sclerotia have smooth surface, black rind and white medulla, whereas abnormal sclerotia have wrinkled surface, black rind and brown medulla [(13)]. Compared to normal sclerotia, abnormal sclerotia are structurally deformed causing severe leakage of nutrients [(15)] and reducing longevity [(19)]. Huang [(13)] reported that the formation of abnormal sclerotia is due to physiological factors, not genetic factors. Tan sclerotia are produced by aberrant strains of *S. sclerotiorum* collected from diseased sunflower [(12)] and lettuce [(7)] which produced brown sclerotia and albino apothecia.

Abnormal sclerotia of *S. sclerotiorum* were found in samples collected from diseased sunflower heads in Manitoba and Alberta [(13,19)]. The frequency of abnormal sclerotia varied with samples ranging from 0 to 30% [(13,19)]. Little is known

about the etiology and biochemical aspects of formation of abnormal sclerotia. A preliminary analysis on chemical components of normal and abnormal sclerotia revealed that there were no significant differences between these two types of sclerotia in the content of oil, protein, alcohol-soluble substances and free fatty acids including palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid [15]. The objective of this study was to further investigate the possible biochemical pathway involved in the formation of abnormal sclerotia of *S. sclerotiorum*.

MATERIALS AND METHODS

Sclerotial samples

Sclerotia of *S. sclerotiorum* collected from diseased sunflower heads in 1977 and 1979 in Manitoba [13] and in 1985 and 1986 in Alberta [19] were used in this study. They were stored in paper bags at room temperature (20 ±2℃) for samples from Manitoba and at -4℃ for samples from Alberta. To collect medullary tissues for chemical analyses, normal and abnormal sclerotia were selected from each field sample, soaked in water for 2 hr, trimmed with a sharp razor blade to remove the melanized rind (Fig. 1), air dried and stored at 4℃ for use.

Sample preparation for chemical analyses

Samples of normal and abnormal sclerotia of *S. sclerotiorum* collected from Manitoba fields in 1977 and 1979 and Alberta fields in 1985 and 1986 were subjected to GC analysis for amino acids and HPLC analysis for determinations of biogenic amines, including 5-HT (5-hydroxytryptamine), 5-HIAA (5-hydroxyindole-3-acetic acid) and 5-HTP (5-hydroxytryptophan). Samples for analysis of amino acids were prepared according to the method described by Yeung *et al.* [24] and samples for the bioactive amines analysis were prepared according to the method described by Yeung and Friedman [23]. Since sclerotia were hard and dry, pre-soakings to soften the tissue were necessary. Briefly, a sample of medullary tissues (14-15 mg) was soaked in cold phosphate buffered saline (1 ml, order No. P3813, Sigma, St. Louis, Missouri) for 2 hr. The medullary tissues were then manually cut up into small pieces using a pair of forceps and a scalpel, homogenized for 10 sec and centrifuged for 5 min (48,000 g, 4℃). The supernatant was collected for GC (gas chromatography) and HPLC (high performance liquid chromatography) analyses. The entire sample preparation procedure was done on ice or in a cold room.

All the GC and HPLC analyses were conducted at the Neurochemical Research Unit, Department of Psychiatry, University of Alberta, Edmonton, Alberta, Canada. Data were analyzed statistically using *t*-test for the amino acids in normal and abnormal sclerotia and Pearson analysis for the biogenic amines.

Formation of abnormal sclerotia in culture

Five chemicals including 5-HIAA (order No. H8876, Sigma, St. Louis, Missouri), 5-HT (order No. H7752, Sigma, St. Louis, Missouri), pargyline (monoamine oxidase inhibitor, MAOI) (order No. P8013, Sigma, St. Louis, Missouri), 5-HTP (order No. H9772, Sigma, St. Louis, Missouri), and L-tryptophan (Trp) (order No. H8659, Sigma, St. Louis, Missouri), were used to test the effect of each chemical on discolouration of medullary tissues of sclerotia of *S. sclerotiorum*. Stock solutions were prepared by dissolving 0.2 g of each chemical in 1 ml of 95% ethanol and then added with sterile water to 10 ml. The stock solutions were incorporated to the potato dextrose agar (PDA) medium, at 5 ml per 100 ml medium for the concentration of 1000 ppm after the agar medium was autoclaved and cooled to 47℃. PDA media containing the same amount of 95% ethanol used in the chemical media were used as control. The media were then poured into Petri dishes (5.5 cm in diameter) at 10 ml/dish.

A single ascospore culture of *S. sclerotiorum*, isolate SS 9, collected from a diseased sunflower plant in Altona, Manitoba [17] was used in this study. Agar blocks (5-mm diameter) containing mycelial mat were removed from the colony of 1-wk-old, PDA culture, inoculated on agar media (PDA only or PDA with test chemicals) in Petri dishes, 1 block per dish. After incubation at 20℃ for 3 wk, sclerotia produced in each dish were removed, cut open and examined for colour of medullary tissues. The experiment was repeated once and for each experiment; there were five replicates (dishes) per treatment.

RESULTS

Free amino acids of normal and abnormal sclerotia

Twenty-one free amino acids were detected in normal and abnormal sclerotia of *S. sclerotiorum* collected from diseased sunflower fields (Table 1). With the exception of tryptophan (Trp), there were no significant differences ($P>0.05$) for the amount of free amino acids between normal and abnormal sclerotia. In the abnormal sclerotia, the amount of Trp was only 62% of that in the normal ones (Table 1). In the chromatogram of amino acid analysis, a major additional peak eluted at 24.5 min of retention time was detected in samples of abnormal sclerotia but this peak was absent in samples of normal sclerotia. However, attempts to identify this unknown component by mass spectrometry were unsuccessful.

5-HT and 5-HIAA in normal and abnormal sclerotia

In order to find out if the reduction of Trp in abnormal sclerotia has any physiological significance, further examinations of the signal transduction of serotonin pathway were carried out using medullary tissues of normal and abnormal sclerotia collected from diseased sunflower heads in fields. Results of biochemical analysis of medullary tissues

Table 1. Free amino acids in medullary tissues of normal and abnormal sclerotia of *Sclerotinia sclerotiorum* collected from diseased sunflower heads

Amino acid	Sclerotia [1]	
	Normal [2]	Abnormal [2]
Alanine (Ala)	0.582	0.614
Glycine (Gly)	0.165	0.235
β-Alanine (β-Ala)	0.898	0.926
Valine (Val)	3.176	3.085
Leucine (Leu)	0.835	0.834
Isoleucine (Ile)	3.055	3.056
Threonine (Thr)	6.896	6.823
γamino-n-butyric acid (GABA)	0.101	0.080
Asparagine (Asn)	3.447	3.475
Methionine (Met)	7.160	7.160
Asparctic acid (Asp)	0.990	1.051
Phenylalanine (Phe)	0.940	1.010
Glutamic acid (Glu)	0.670	0.667
Serine (Ser)	1.235	1.057
Cysteine (Cys)	1.690	1.690
Citruline (Cit)	2.650	2.650
Histadine (His)	2.530	2.560
Ornithine (Orn)	1.490	1.450
Tryptophan (Trp)	1.280	0.790 [3]
Lysine (Lys)	3.110	3.130
Tyrosine (Tyr)	1.120	1.100

[1] Sclerotia were collected from diseased sunflower heads in Manitoba in 1977 and 1979 and in Alberta in 1985 and 1986. They were stored in paper bags at 20±2℃ for the 1977 and 1979 samples and at -4C for the 1985 and 1986 samples.
[2] All values of amino acids are μmol/g.
[3] The difference between normal and abnormal sclerotia is significant at $P<0.05$ (*t*-test).

showed that normal sclerotia had high concentrations of 5-HT (43.8 ng/g in the 1979 samples and 89.0 ng/g in the 1986 samples), and low (22.5 ng/g in the 1985 samples and 43.0 ng/g in the 1986 samples) to undetectable levels of 5-HIAA (Table 2). Compared to normal sclerotial samples, abnormal sclerotia had less 5-HT and much higher concentrations of 5-HIAA. Thus, the effective conversion of neurotransmitter 5-HT to its metabolite 5-HIAA is only evident in abnormal sclerotia. The ratio of 5-HT/5-HIAA was less than 1 for abnormal sclerotia but was larger than 1 for normal sclerotia (Table 2).

Results of 5-HIAA analysis from the normal sclerotia collected in 1977 and 1979 showed a similar trend as those obtained in 1985 and 1986. However, in the 1977 and 1979 sclerotial samples, no detectable levels of 5-HIAA were observed in normal sclerotia, rather than low, but detectable, levels of 5-HIAA in normal sclerotia of 1985 and 1986 samples (Table 2).

Formation of abnormal sclerotia in culture

Sclerotia of *S. sclerotiorum* from the controls (PDA cultures without any of the test chemicals) or PDA cultures treated with 1000 ppm of 5-HTP, Trp, MAOI, or 5-HT had numerous small faint-coloured specks distributed in the white medullary tissues (Figs. 2, 3), whereas sclerotia from cultures grown on PDA containing 1000 ppm of 5-HIAA had large brown to dark brown spots (Fig. 2) or patches (Fig. 3) in the medullary tissues. Among the sclerotia formed on PDA (control) or PDA amended with 5-HIAA, MAOI, Trp, 5-HTP or 5-HT at 1000 ppm, only the sample from 5-HIAA cultures had a significantly ($P<0.05$) higher number of sclerotia with brown medullary tissues. The frequency of sclerotia with brown medulla was 88.7%, 3.1%, 11.1%, 10.5%, 5.2% and 12.5% for the treatments of 5-HIAA, MAOI, Trp, 5-HTP, 5-HT, and PDA, respectively. The brown medullary tissues from sclerotia produced on 5-HIAA treated cultures were more sensitive to air-dry treatment than the white medullary tissues from sclerotia produced on untreated control (Figs. 3a and 3b) or on other chemical treated cultures. A rapid deformation was evident in the brown medullary tissues of sclerotia from the cultures treated with 5-HIAA (Figs. 3a and 3b).

DISCUSSION

GC analysis of amino acids of normal and abnormal sclerotia of *S. sclerotiorum* reveals that only Trp was significantly reduced in abnormal sclerotia. In order to find

Table 2. Amount of 5-hydroxytryptamine (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA), in medullary tissues of sclerotia of *S. sclerotiorum*

Sclerotia [1]	5-HT [2]		5-HIAA [2]		Ratio (5-HT/5-HIAA) [2]	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
1977	80.3±59.1	27.1±31.6	nd	60.3±18.3	-±- [3]	0.5±0.6
1979	43.8±50.9	nd [4]	nd	8.3±9.6	-±-	-±-
1985	83.3±24.9	nd	22.5±8.7	69.2±16.7	3.7±1.3	-±-
1986	89.0±28.3	nd	43.0±5.5	98.3±43.7	2.1±0.7	-±-

[1] Sclerotia were collected from diseased sunflower heads in Manitoba in 1977 and 1979 and in Alberta in 1985 and 1986. They were stored in paper bags at 20±2℃ for the 1977 and 1979 samples and at -4C for the 1985 and 1986 samples.
[2] All values are mean in ng/g±S. D. (n=4)
[3] Undefined values are signified by "—"
[4] nd = not detected

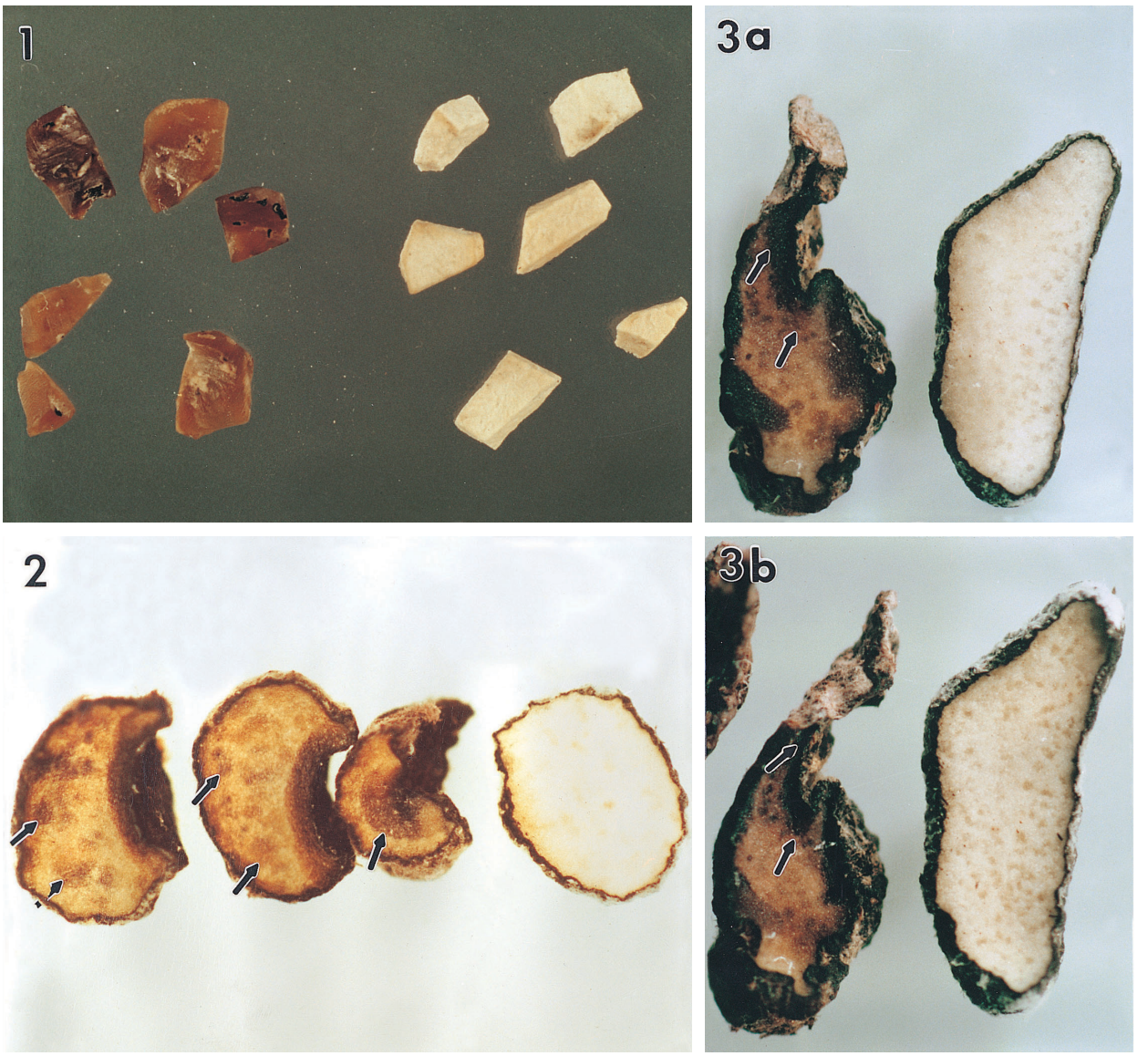

Fig. 1. Field samples of normal (right) and abnormal (left) sclerotia of *Sclerotinia sclerotiorum* collected from diseased sunflower heads showing white medullary tissues in normal sclerotia and brown medullary tissues in abnormal ones.

Fig. 2. Cross section of sclerotia of *Sclerotinia sclerotiorum* produced on PDA cultures (the sclerotium to the right) and on PDA containing 5-HIAA at 1000 ppm (three sclerotia to the left). Note white medullary tissues in the normal sclerotium and brown patches (arrows) in the medullary tissues of 5HIAA-treated sclerotia.

Figs. 3a, b. Cross section of a normal sclerotium from PDA culture (right) and an abnormal sclerotium from PDA containing 5-HIAA at 1000 ppm (left) after air-drying for 3 hrs (3a) and 8 hrs (3b). Note brown patches (arrows) in the alonormal sclerotium and the differences between the normal sclerotium (right) and the abnormal sclerotium (left) in tissue shrinkages after air-dried for 3 hrs (3a) and 8 hrs (3b).

out if the reduction of Trp has any physiological function in the formation of abnormal sclerotia, we examined the serotonin pathway in the sclerotia. The biochemical analyses of field samples reveals that normal sclerotia have high concentrations of 5-HT and low concentrations of 5-HIAA, while abnormal sclerotia have low to undetectable amount of 5-HT and high concentrations of 5-HIAA (Table 2). The observed shift or depletion of 5-HT and its precursor Trp to its metabolite 5-HIAA in the abnormal sclerotia indicates that serotonin may play a critical role in the development and formation of abnormal sclerotia. This increased turnover of 5-HT to 5-HIAA may involve any one or more enzymes like tryptophan hydroxylase (TH), aromatic amino acid decarboxylase (AAAD) and monoamine oxidase (MAO) according to the following pathway.

$$\text{Trp} \xrightarrow{\text{TH}} \text{5-HTP} \xrightarrow{\text{AAAD}} \text{5-HT} \xrightarrow{\text{MAO}} \text{5-HIAA}$$

Consequently, any of these enzymes, either alone or in combination, might have been activated or upregulated causing the depletion of 5-HT. However, in any biological systems, there are other physiological adaptations trying to maintain homeostasis. Therefore, it is also possible the increased 5-HIAA might have a negative feedback inhibitory effect on one or multiple of these enzymes, but unable to offset the imbalance, and the sclerotia became abnormal [3,8]. The involvement of the serotoninergic pathway is further confirmed in the observation of culture studies that abnormal sclerotia are formed in high frequency when *S. sclerotiorum* is grown on PDA amended with 5-HIAA.

This is the first report on presence of serotonin in sclerotia of *S. sclerotiorum*. Homeostatic regulations of physiological functions by hormones and neurotransmitter, such as serotonin (5-HT), represent a vital mechanism for survival in both animals and plants [2,6,8,21,22,23]. Serotonin has been known for decades to influence cellular proliferation, mitosis, physiological functions, and to participate in neurotransmission. Whether the mitogenic effect is initiated through cellular receptors, organelles, or serotonin transporters depends on the cell type, and is currently unresolved [3,6,8]. The serotonin signal transduction is a complex process, and has recently been reviewed [3,21]. Our data demonstrated biochemical changes in abnormal sclerotia. The observed changes in serotoninergic cascade might characterize the inability of the sclerotia to preserve such balance, or due to impaired physiological adaptation.

Results of analysis from the older sclerotia collected in 1977 and 1979 showed a similar trend as those obtained in 1985 and 1986. However, in the 1977 and 1979 sclerotial samples, no detectable levels of 5-HIAA were observed in normal sclerotia, rather than low, but detectable, levels of 5-HIAA in 1985 and 1986 normal samples (Table 2). It is not clear whether the difference in 5-HIAA contents between normal sclerotia of old (1977 and 1979) samples and new (1985 and 1986) samples is due to storage conditions, such as duration, temperature, and/or viability of the samples. Meanwhile, this study indicates that the ratio of 5-HT/5-HIAA in abnormal sclerotia is always less than1 (Table 2) and it may serve as a useful biochemical marker for the neurotransmitter turnover, or an index for 5-HT metabolism or utilization, to differentiate normal and abnormal sclerotia.

Previous reports indicate that abnormal sclerotia of *S. sclerotiorum* are mummified [13] with fractured rind and sparse filamentous hyphae in an amorphous martrix of the brown medullary tissues [15]. Hyphae in the brown medullary region are non-viable as most of the viable hyphae are confined in the white medullary region [15]. The tissue impairment in abnormal sclerotia is further confirmed by the observation of present study that air-drying treatment causing drastic desiccation and deformation of the brown medullary tissues of sclerotia of *S. sclerotiorum* which are formed on 5-HIAA amended cultures (Fig. 3).

Abnormal sclerotia of *S. sclerotiorum* have been found to occur on diseased sunflower heads under natural conditions [13,19]. It has not been reported in any other host plants of *S. sclerotiorum*. Despite the head rot of sunflower [14] and the white mold of bean [1] are both caused by infection of ascospores of *S. slerotiorum*, a survey of sclerotial samples collected from diseased bean plants in Alberta failed to detect the existence of abnormal sclerotia in the samples (H. C. Huang, unpublished). This suggests that sunflower head tissues may be important in triggering formation of abnormal sclerotia. Since the formation of abnormal sclerotia is a physiological nature, not genetical [13] and is related to the conversion of Trp to 5-HIAA, further studies on factors affecting formation of abnormal sclerotia in sunflower heads and other hosts are warranted.

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摘 要

黃鴻章[1,3]、Yeung J. M.[2] 2002. 菌核病菌產生異常菌核的生化途徑. 植病會刊 11:1-6. ([1] 加拿大農業部 Lehbridge 研究中心；[2] 1350 I Street NW, Washington, DC 20005, 美國華盛頓 國家食品加工協會 (National Food Processors Association)；[3] 聯絡作者，電子郵件：huangh@em.agr.ca；傳眞機：(403) 382-3156)

在 1977-1986 年間，從加拿大Manitoba 及 Alberta 兩省的向日菌核病 (由 *Sclerotinia sclerotiorum* 引起) 的樣本中發現兩種不同菌核存在，即有白色中髓的正常菌核 (normal sclerotia) 與中髓褐變的異常菌核 (abnormal sclerotia)。初步生化分析田間樣本，結果顯示正常菌核與異常菌核均含有 21 種游離氨基酸 (free amino acids)，而且其中只有色氨酸 (Tryptophan) 含量在正常菌核中有顯著降低的情形。進一步分析結果證明，正常菌核的白色中髓含有大量的 5-Hydroxytryptamin (5-HT) (即神經傳導物質、或稱 Serotonin) 及少量的 5-Hydroxyindole acetic acid (5-HIAA)。相反地，異常菌核的褐色中髓內的 5-HT 含量甚低而 5-HIAA 的含量甚高。將菌核病菌培養於馬鈴薯瓊脂培養基「分別添加 1000 ppm 的 5- HT，5-HIAA，Monoamine oxidase inhibitor (MAOI)，Tryptophan，或 5-Hydroxytryptophan (5-HTP)」上所形成的菌核切開檢查，發現只有添加 5-HIAA 的培養基能促使大量菌核 (88.7%) 的中髓褐變。這種色氨酸及 Serotonin (5-HT) 降低與 5-HIAA 增加的特性，證明異常菌核的形成是與色氨酸的代謝過程 (即 Serotoninergic Pathway) 有關。

關鍵詞：菌核病菌、異常菌核、Serotonin Serotoninergic Pathway