



黄蜻幼虫肠道真菌QTYC-51的鉴定及其除草活性成分

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摘要: 【目的】鉴定黄蜻幼虫肠道中具有除草活性的真菌, 并分析其除草活性成分。【方法】通过形态学观察和分子生物学5.8S rDNA-ITS序列分析, 明确菌株QTYC-51的分类地位。结合培养皿生物分析法测定菌株发酵液和单体化合物的除草活性, 采用色谱方法从该菌乙酸乙酯粗提物中分离活性成分, 并利用质谱和核磁共振谱鉴定活性物质。【结果】菌株QTYC-51被鉴定为拟盾壳霉属菌(*Paraconiothyrium* sp.), 其发酵液对稗草和反枝苋幼根生长有较好的抑制作用, 抑制率分别为76.9%和56.5%。从QTYC-51发酵产物中分离到5个单体化合物, 分别为1,8-dihydroxyanthraquinone, 1-hydroxy-10-methoxy-dibenz[b,e]oxepin-6,11-dione, hydroxyvertixanthone, globosuxanthone和1,3,6,8-tetrahydroxyanthraquinone。在供试浓度为100 μg/mL时, 化合物globosuxanthone对稗草和反枝苋幼根生长有明显的抑制作用, 抑制率分别为94.1%和79.0%, 与阳性对照2,4-二氯苯氧乙酸效果相当; 化合物1-hydroxy-10-methoxy-dibenz[b,e]oxepin-6,11-dione具有较好的除草活性, 对稗草和反枝苋幼根的生长抑制率分别为50.3%和58.6%。【结论】菌株QTYC-51具有开发为微生物源除草剂的潜能。

关键词: 黄蜻幼虫, 拟盾壳霉属, 生物源除草剂, 除草活性

稗草和反枝苋是世界性分布的恶性杂草, 对农业生产有很大的破坏作用^[1-2]。目前人们一般使用化学农药进行杂草防除, 但除草剂的频繁使用已造成杂草种群的演变与环境的严重污染^[3], 人们迫切需要找寻一种新的天然无污染且具有高效选择性的除草剂来代替原始药剂^[4], 生物源除草剂具有资源丰富、毒性小、选择性强、不破坏生

态环境等优点, 已逐步引起人们的重视^[5-6]。当前, 新型除草剂的研究主要集中在土壤放线菌和植物病原菌两个方面, 对于特殊生境微生物的研究则较少^[7]。

昆虫肠道菌是生活在健康昆虫肠道的一类微生物, 昆虫种类的多样性、分布地区的复杂性使其体内微生物在生态、代谢特征和生理活性等方

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面均有一定的特殊性。据报道,有些昆虫肠道菌能够产生一些结构新颖的活性代谢产物^[8],它们可能是新型除草剂的重要来源。本文以1株具有除草活性的黄蜻(*Pantala flavescens*)幼虫肠道真菌QTYC-51为研究对象,并对其活性成分进行分离鉴定,旨在为开发新型微生物源除草剂奠定一定的基础。

1 材料和方法

1.1 材料

1.1.1 菌株来源:将健康黄蜻(*P. flavescens*)幼虫(采自浙江省金华市婺城区北山路与北二环路附近)经肠道解剖、涂布研磨、分离等处理后得到菌株QTYC-51。

1.1.2 供试杂草种子:稗草(*Echinochloa crusgalli*)种子采自浙江师范大学实验稻田,反枝苋(*Amaranthus retroflexus*)种子购于河北省唐山市北方种子基地。

1.1.3 培养基:麦芽浸汁培养基:生麦芽20 g(煮沸30min,滤去残渣),蔗糖20 g,蛋白胨1 g,琼脂15–20 g,蒸馏水补足1 L, pH自然。不加琼脂者为相应的液体培养基(蔗糖、蛋白胨、琼脂粉等购自北京奥博星生物技术有限责任公司)。

1.2 QTYC-51菌株的鉴定

形态学鉴定:接种QTYC-51菌株于MEA固体培养基中,28 °C条件下放入恒温培养箱中培养,每天定时观察菌落生长形态。挑取少量菌落制片,利用显微镜观察菌丝形态和产孢结构等。

分子生物学鉴定:按照生工Ezup柱式真菌基因组DNA抽提试剂盒说明书提取方法,从培养好的QTYC-51新鲜菌体中提取DNA。以QTYC-51菌株的基因组DNA为模板,采用真菌通用引物ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')和ITS4 (5'-TCCTCCGCTTA TTGATATGC-3') 对其进行PCR扩增。其中PCR反应程序为:94 °C 2 min, 94 °C 1 min, 55 °C 1 min, 72 °C 1 min, 进行

35个循环,72 °C 10 min, 4 °C保存。PCR产物纯化后进行测序分析,测序由上海生工公司完成。将QTYC-51菌株的核酸序列与GenBank中相关核酸序列进行BLAST比对,之后利用MEGA 5.05软件构建系统发育树,确定菌株的分类地位。

1.2.1 QTYC-51菌株发酵液除草活性测试:QTYC-51菌株在条件为28 °C、180 r/min的摇床中培养7 d,经纱布过滤后获得发酵液,采用培养皿生物分析法^[9]测试菌株发酵液的除草活性。将杂草种子置于培养箱,28 °C催芽1–2 d,露白后备用。用移液枪取5 mL发酵液,置于铺有滤纸(直径为9 cm)的培养皿中,选取露白的稗草或反枝苋种子放在铺有滤纸的培养皿上,每个培养皿平均放10粒,以蒸馏水为空白对照,2,4-D为阳性对照,重复3次。在温度为28 °C、相对湿度70%、定时光照(白天12 h,黑夜12 h)的人工气候培养箱中进行培养,4 d后测量种子根长。根生长抑制率按照公式(1)计算。

根生长抑制率/%=(对照平均根长-处理平均根长)/对照平均根长×100 公式(1)

1.2.2 QTYC-51菌株活性物质的分离、鉴定和除草活性测试:接种QTYC-51菌株于50 mL ME液体的250 mL锥形瓶中,在28 °C、180 r/min的摇床里培养3 d,得到种子液。取10 mL液体于盛有500 mL ME液体的1000 mL锥形瓶内,同样条件下发酵7 d。发酵所得液体50 L经3层纱布过滤后用等体积乙酸乙酯萃取3次,萃取液在旋转蒸发仪(上海市爱朗仪器有限公司)中进行浓缩,获得粗浸膏。乙酸乙酯粗提物经硅胶柱层析、凝胶柱层析、薄层层析等色谱方法分离、纯化,得到纯化的单体化合物。用Mariner System 5304质谱仪(美国ABI公司)测定单体化合物的分子量,用Bruker AVANCE-600 MHz核磁共振仪(瑞士Bruker公司)对化合物进行¹H-NMR谱和¹³C-NMR谱分析。参考文献[9]的方法测试单体化合物的除草活性。

2 结果和分析

2.1 QTYC-51菌株的鉴定

在MEA培养基上, QTYC-51菌落生长缓慢(28 °C下生长8 d, 菌落直径为1.2–1.6 cm), 绒毡状, 颜色初为白色, 数天后转为浅褐色, 背面褐色(图1)。显微镜观察可见: 菌丝纤细、无色、具隔膜; 分生孢子圆形、单胞、无色。QTYC-51真菌的形态特征与文献[10]中的拟盾壳霉属(*Paraconiothyrium* sp.)的形态特征基本一致, 因此将黄蜻幼虫肠道内生真菌QTYC-51初步鉴定为拟盾壳霉属*Paraconiothyrium* sp..

QTYC-51菌株经过PCR扩增所得5.8S rDNA-ITS基因片段长度为570 bp。测序结果经BLAST比对, 发现QTYC-51的序列与拟盾壳霉属菌

(*Paraconiothyrium* sp.)的相关序列(GenBank登录号: KC510275.1)相似性达98.6%, 两者在系统发育树上处于同一分支(图2)。结合形态特征, 将菌株QTYC-51鉴定为拟盾壳霉属菌株(*Paraconiothyrium* sp.)(GenBank登录号: KX148027)。

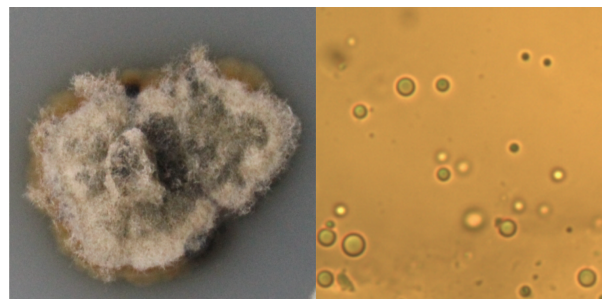


图 1. 菌株QTYC-51的菌落与分生孢子形态(400×)
Figure 1. The colony and spore morphology of strain QTYC-51 (400×).

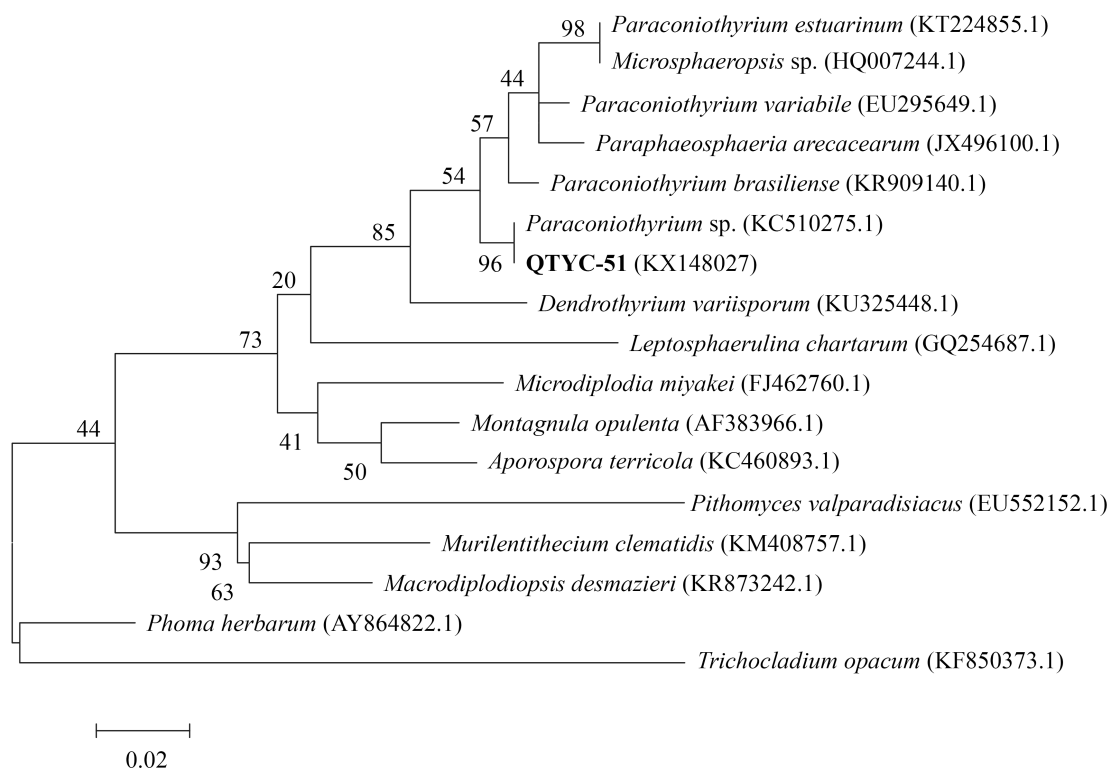


图 2. 基于真菌QTYC-51 5.8S rDNA-ITS基因片段序列构建的系统发育树

Figure 2. Neighbour-joining phylogenetic tree of 5.8S rDNA-ITS sequences of QTYC-51. Numbers in bracket represent the sequences accession number in GenBank. The number at each branch points is the percentage supported by bootstrap. Bar, 0.02 sequence divergence.

2.2 QTYC-51菌株的除草活性

QTYC-51菌株发酵液对稗草和反枝苋幼根生长的抑制结果见表1。发酵液对稗草和反枝苋幼根的生长均有较好的生长抑制作用,抑制率均大于56%,仅比阳性对照2,4-D的抑制效果稍弱。

2.3 QTYC-51菌株发酵产物的分离

50 L QTYC-51菌株发酵液经乙酸乙酯萃取、浓缩后获得粗浸膏24.4 g,经硅胶(200–300目)层析分离,二氯甲烷:甲醇梯度洗脱,得到7个组分(A 100:0、B 100:1、C 100:2、D 100:4、E 100:8、F 100:16、G 100:32)。经过生物活性测试,发现A组分(二氯甲烷:甲醇/100:0)活性最好。对A组分进一步使用硅胶柱层析,用二氯甲烷溶液对其进行洗脱,得到5个小部分(R1–R5),使用重结晶的方法在前3部分分别获得化合物1、化合物5与化合物3,剩下R5部分经过LH-20葡聚糖凝胶(瑞典Pharmacia Biotech公司)柱色谱层析得到化合物4。B–G组分经过TLC检测,合并相似成份,使用葡聚糖凝胶柱层析法反复分离纯化,获得化合物2。采用质谱和核磁共振谱技术对上述5个化合物的结构进行分析,具体波谱数据如下:

化合物1: ESI-MS: m/z 239.1546 [M-H]⁻, ¹H-NMR (CD₃Cl) δ 12.06 (2H, s), 7.83 (2H, dd, J=7.4, 0.8 Hz), 7.68 (2H, m), 7.30 (2H, dd, J=8.4, 0.8 Hz); ¹³C-NMR (CD₃Cl) δ : 193.1, 181.7, 162.6, 137.3, 133.6, 124.6, 120.1, 115.9。上述数据与文献报道的1,8-dihydroxyanthraquinone一致^[11], 确定该化合物

为1,8-dihydroxyanthraquinone。

化合物2: ESI-MS: m/z 293.0407 [M+Na]⁺, 309.0139 [M+K]⁺, ¹H-NMR (CD₃Cl) δ 12.23 (1H, s), 7.77 (1H, dd, J=8.4, 7.4 Hz), 7.61 (1H, t, J=8.3 Hz), 7.55 (1H, m), 7.33 (1H, dd, J=7.3, 0.8 Hz), 6.94 (1H, d, J=8.4 Hz), 6.81 (1H, d, J=8.3 Hz), 4.03 (3H, s)。上述数据与文献报道的1-hydroxy-10-methoxy-dibenz[*b,e*]oxepin-6,11-dione数据基本吻合^[12], 确定该化合物为1-hydroxy-10-methoxy-dibenz[*b,e*]oxepin-6,11-dione。

化合物3: ESI-MS: m/z 287.0533 [M+H]⁺, 309.0351 [M+Na]⁺, ¹H-NMR (DMSO-*d*₆) δ 12.25 (1H, s), 10.49 (1H, s), 7.73 (1H, t, J=8.3 Hz), 7.65 (1H, d, J=9.1 Hz), 7.50 (1H, d, J=9.1 Hz), 7.07 (1H, dd, J=8.4, 0.7 Hz), 6.81 (1H, dd, J=8.2, 0.7 Hz), 3.86 (3H, s)。以上数据与参考文献所述hydroxyvertixanthone相符^[13], 因此, 确定该化合物为hydroxyvertixanthone。

化合物4: ESI-MS: m/z 305.0645 [M+H]⁺, 327.0468 [M+Na]⁺, ¹H-NMR (DMSO-*d*₆) δ 12.45 (1H, s), 7.67 (1H, t, J=8.3 Hz), 7.09 (1H, d, J=8.2 Hz), 6.83 (1H, d, J=8.0 Hz), 6.64 (1H, dd, J=10.0, 4.3 Hz), 6.51 (1H, dd, J=10.0, 0.8 Hz), 5.86 (1H, d, J=8.0 Hz), 4.28 (1H, m), 3.60 (3H, s)。¹³C-NMR (DMSO-*d*₆) δ : 181.1, 172.1, 160.4, 155.3, 141.8, 136.5, 120.2, 115.1, 111.7, 110.7, 107.8, 75.4, 71.9, 52.2。上述数据与文献中globosuxanthone数据基本吻合^[14], 确定化合物4为globosuxanthone。

表1. 菌株QTYC-51发酵液对稗草和反枝苋幼根生长的抑制效果

Table 1. Inhibitory activity of the fermentation broth of QTYC-51 on radicle growth of *E. crusgalli* and *A. retroflexus*

Item	<i>E. crusgalli</i>		<i>A. retroflexus</i>	
	Root length/mm	Inhibiting rate/%	Root length/mm	Inhibiting rate/%
QTYC-51 Fermentation broth	6.1±0.5 b	76.9±1.6 b	8.9±0.5 b	56.5±6.5 b
2,4-D	2.1±0.2 c	92.4±0.6 a	2.4±0.5 c	88.1±4.1 a
CK	26.6±0.5 a	/	20.5±2.2 a	/

The data in the table are averages of 10 replicates; CK means blank control; 2,4-D means positive control; the concentration for the test is 100 μ g/mL; data are mean±SD. Different letters in the same column indicate significant difference by Duncan's new multiple range test ($P<0.05$).

化合物**5**: ESI-MS: m/z 271.1972 $[M-H]^-$, 543.4095 $[2M-H]^-$, 1H -NMR (DMSO- d_6) δ 12.28 (2H, s), 7.27 (3H, d, $J=2.4$ Hz), 6.67 (3H, d, $J=2.4$ Hz)。测得的数据与参考文献中1, 3, 6, 8-tetrahydroxyanthraquinone相同^[11], 确定化合物**5**为1, 3, 6, 8-tetrahydroxyanthraquinone。

2.4 化合物的除草活性测定

5种化合物除草活性测试结果见表2。在供试浓度为100 $\mu\text{g/mL}$ 时, 化合物**4**对稗草和反枝苋幼根生长有明显的抑制作用, 抑制率分别为94.1%

和79.0%, 与阳性对照2,4-D (对2种杂草的抑制率分别为95.5%和89.1%)的抑制效果相当; 化合物**2**具有较好的除草活性, 对稗草和反枝苋幼根的抑制率分别为50.3%和58.6%。其余化合物的除草活性较弱。

进一步测试了化合物**4**的除草活性, 结果见图3和图4。除草活性随着浓度提高而逐渐增强, 比同浓度的阳性对照2,4-D的活性稍弱。计算出化合物**4**对稗草和反枝苋根生长抑制活性的 IC_{50} 值分别为3.2 $\mu\text{g/mL}$ 和10.2 $\mu\text{g/mL}$ 。

表2. 化合物**1-5**对稗草和反枝苋幼根生长的抑制效果

Table 2. Inhibitory activity of compounds **1-5** on radicle growth of *E. crusgalli* and *A. retroflexus*

Metabolites	<i>E. crusgalli</i>		<i>A. retroflexus</i>	
	Root length/mm	Inhibiting rate/%	Root length/mm	Inhibiting rate/%
1	17.8±2.6 b	21.7±6.8 c	13.6±0.2 d	34.8±2. d
2	11.1±1.7 c	50.3±5.7 b	8.5±0.4 e	58.6±5.1 c
3	19.2±1.1 b	15.8±6.9 c	16.7±0.6 c	24.8±3.7 e
4	1.5±0.6 d	94.1±1.9 a	4.5±0.3 f	79.0±3.0 b
5	20.1±1.9 a	11.8±8.8 d	18.2±1.7 b	11.6±3.9 f
2,4-D	0.9±0.4 d	95.5±0.0 a	2.2±0.1 g	89.1±1.9 a
CK	22.7±1.9 a	/	20.8±2.1 a	/

The data in the table are averages of 10 replicates; CK means blank control; 2,4-D means positive control; the concentration for the test is 100 $\mu\text{g/mL}$; the concentration of compounds is 100 $\mu\text{g/mL}$; data are mean±SD. Different letters in the same column indicate significant difference by Duncan's new multiple range test ($P<0.05$).

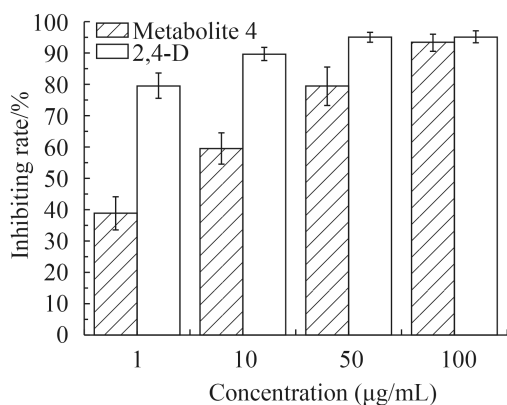


图3. 不同浓度化合物**4**及**2,4-D**对稗草幼根生长的除草活性

Figure 3. Phytotoxic effects of the compound **4** and 2,4-D on radicle growth of *E. crusgalli* at different concentration.

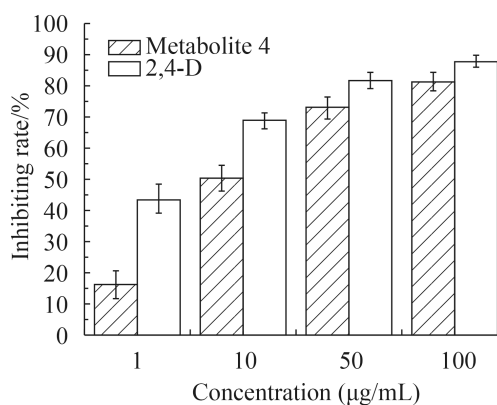


图4. 不同浓度化合物**4**及**2,4-D**对反枝苋根生长的除草活性

Figure 4. Phytotoxic effects of the compound **4** and 2,4-D on radicle growth of *A. retroflexus* at different concentration.

3 讨论

本文的菌株QTYC-51经鉴定属于腔孢纲(Coelomycetes)、球壳孢目(Sphaeropsidales)、球壳孢科(Sphaeropsidaceae)的拟盾壳霉属(*Paraconiothyrium*), 该属是2004年被描述的新属。有文献表明^[15], 拟盾壳霉属菌粗提物具有显著抗氧化与抗恶性肿瘤扩增的特性, 同时经酶活性测试实验^[16]发现, 该属菌能够产生乳糖酸作为受体氧化剂。但对拟盾壳霉属菌活性次级代谢产物的研究较少, 本实验对菌株QTYC-51次级代谢产物进行分离, 得到5个单体化合物, 为了解该属真菌的化学物质多样性奠定了一定的基础。

本研究从蜻蜓幼虫肠道共生菌QTYC-51分离到的化合物**2**、**4**均属于近几年刚发现的天然产物, 两者均可从海绵共生菌*Beauveria bassiana*中分离得到, 化合物**2**(1-hydroxy-10-methoxy-dibenz[b,e]oxepin-6,11-dione)有一个特殊的dibenz[b,e]oxepine结构, 目前已报道的类似衍生物仅有6种^[12]。考虑到化合物**2**独特的结构, 或许通过化学合成生物研究可以得到类似新颖衍生化合物。Wijeratne等于2006年首次从植物共生真菌*Chaetomium globosum*中分离得到化合物**4**^[14]。文献报道化合物**4**具有抑制致病真菌白念珠菌和显著抑制肿瘤生长的特点^[12,14], 但未见其它生物活性的报道。本文首次阐明化合物**2**与**4**可从昆虫肠道菌中分离得到, 且两者具有潜在的除草活性: 在供试浓度为100 $\mu\text{g/mL}$ 时, 化合物**2**对稗草和反枝苋幼根生长的抑制率均大于50%; 化合物**4**对稗草与反枝苋幼根生长的抑制率分别为94.1%和79.0%, 其与阳性对照的除草活性相当。因此, 化合物**2**和**4**是QTYC-51具有除草活性的物质基础, 作为具有除草活性的先导化合物, 值得深入研究。但关于化合物**4**和化合物**2**的除草机制尚不清楚。另外, 菌株QTYC-51的其它活性成分分离鉴定、活性作用机制及大田试验等, 还有待更进一步的研究。

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Identification and phytotoxic activity of fungus QTYC-51 from the gut of *Pantala flavescens* larvae

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Abstract: [Objective] To isolate the fungus with phytotoxic activity from the gut of *Pantala flavescens* larvae. [Methods] Strain QTYC-51 was identified by morphological observation and 5.8S rDNA-ITS sequence analysis. Petri dish bioassay was used to test the phytotoxic activity of fermentation broth and monomer compounds of strain QTYC-51 on *Echinochloa crusgalli* and *Amaranthus retroflexus*. Bioactive components were isolated from ethyl acetate extracts via chromatographic methods, and the structures were determined by mass spectrum and nuclear magnetic resonance analyses. [Results] QTYC-51 was identified as *Paraconiothyrium* sp.. The fermentation broth had good phytotoxic activity on radical growth of *E. crusgalli* and *A. retroflexus* with the inhibition rates of 76.9% and 56.5%, respectively. Five monomer compounds were purified from the fermentation products, including 1,8-dihydroxyanthraquinone, 1-hydroxy-10-methoxy-dibenz[*b, e*]oxepin-6,11-dione, hydroxyvertixanthone, globosuxanthone and 1,3,6,8-tetrahydroxyanthraquinone. At the concentration of 100 µg/mL, compound globosuxanthone was found to possess obvious phytotoxic effects on radical growth of *E. crusgalli* and *A. retroflexus* with the inhibition rates of 94.1% and 79.0%, respectively, which were comparable to that of positive control 2,4-dichlorophenoxyacetic acid. Compound 1-hydroxy-10-methoxy-dibenz [b,e] oxepin-6,11-dione showed potent phytotoxic activity against *E. crusgalli* and *A. retroflexus* with inhibition rates of 50.3% and 58.6%, respectively. [Conclusion] Strain QTYC-51 could be potentially developed as a microbial herbicide.

Keywords: *Pantala flavescens* larvae, *Paraconiothyrium* sp., microbial herbicide, phytotoxic activity

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