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Tables: 2, Figs: 3

# SHORT COMMUNICATION

# Purification, identification and bioactivity of phytotoxic compounds from the fungus *Exserohilum monoceras*

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## ABSTRACT

Barnyardgrass (*Echinochloa crus-galli* L.) is a problematic weed worldwide that competes with crops and reduces their yields. The fungus *Exserohilium monoseras* (Drechsler) Leonard and Suggs is a potential biocontrol agent for barnyardgrass. This study aimed to isolate and identify the phytotoxic compounds from *E. monoceras* strain X27. The fungus was cultured in potato dextrose (PD) media. A crude extract of X27 fermentation filtrate (5.0 g/L) drastically inhibited the barnyardgrass shoot growth by 94.9% and root growth by 79.0%. The crude toxins in the fermentation filtrate were isolated using thin-layer chromatography (TLC). Four compounds were isolated and identified by gas chromatography-mass spectrometry (GC-MS) as oleic acid amide ($C_{18}H_{35}NO$), dibutyl phthalate (DBP; $C_{16}H_{22}O_4$), stearic acid ($C_{16}H_{32}O_2$) and octadecanoic acid ($C_{18}H_{36}O_2$). Of the 4-compounds in the mixture, DBP showed the greatest suppression of plant growth, decreasing root growth by 84% and shoot growth by 76% at 1 g/L concentration.

**Key words:** Barnyardgrass, *Echinochloa crus-galli*, *Exserohilum monoceras*, identification, purification

## INTRODUCTION

Barnyardgrass (*Echinochloa crus-galli* L. Beauv.), an annual weedy grass, causes yield reduction in 36 crops in 61 countries and in 36 crops (11). It is a major weed in paddy fields, where it competes with rice (*Oryza sativa* L.) and causes reductions in yield. Competition from 25 barnyardgrass plants·$m^{-2}$ causes 50% reduction in rice yields (7). The wide use of herbicides to control barnyardgrass (i) contributes to the environmental pollution and (ii) loss of biodiversity (5,6). Use of some herbicides has resulted in emergence of herbicide resistant biotypes of *E. crus-galli* (15,17). Biological control, which uses living organisms to control or reduce the population of undesirable weed species, is an alternative to herbicides (14). The use of exotic plant pathogens to control weeds was first reported in the early 1970s (13).

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*Exserohilum monoceras* (Drechsler) Leonard and Suggs is a phytopathogenic fungus in Southeast Asia, North America and Australia (16,17). It causes leaf blight in *Echinochloa* species and is being evaluated as a potential bioherbicide for *Echinochloa* control (16,17). Monocerin, the first chemical isolated from the *E. monoceras* culture, was not characterized as a phytotoxin but rather as an antibiotic to protect wheat (*Triticum aestivum* L.) against powdery mildew (*Erysiphe graminis* D.C. ex Mrat) (12). Subsequently, monocerin isolated from the *Exserohilum turcicum* (Pass.) proved phytotoxic against johnsongrass (*Sorghum halepense* (L.) Pers.) and Canada thistle (*Cirsium arvense* (L.) Scop.) (12). It has received much attention, as it causes leaf blight in *Echinochloa* species (1) and is considered for commercialization as a biocontrol product for *Echinochloa* species (10). However, the identification of the phytotoxic compounds from *E. monoceras* and their efficiency in controlling barnyardgrass has not been reported.

This study aimed to isolate and identify the phytotoxic substances from fungal pathogen *E. monoceras* strain X27 and examine their bioactivities against barnyardgrass.

## METHODS AND MATERIALS

*Exserohilum monoceras* (Drechsler) Leonard and Suggs strain X27 (X27; isolated by our Laboratory) was cultured in potato dextrose (PD) media in 500 mL Erlenmeyer flasks at 28°C for 5 d with shaking at 180 rpm and then filtered through filter paper (5). The fermented broth (250 mL) was extracted with ethyl acetate (3 × broth volume). The ethyl acetate extract was evaporated to dryness under reduced pressure at < 40°C. The crude extract mixture was dissolved with distilled water containing 0.4% Tween-20 to prepare toxin solutions of 5.0 g/L concentration. For the control treatment, culture medium was prepared in the same way as the fermented broth but without fungal inoculation.

Seeds of barnyardgrass (*Echinochloa crus-galli* L. Beauv.) harvested in 2006 from fields of ~~our~~ Zengcheng Experimental Station were surface-sterilized with 1 g/L mercuric chloride ($HgCl_2$) for 10 min, then with 750 g/L ethanol ($C_2H_5OH$) for 10 s and finally rinsed with sterile water three times (5). Sterilized seeds were dried with autoclaved filter paper and kept at 4°C for subsequent use.

**Broth Bioassay**

The fermented broth was diluted 1:1 with sterile distilled water (5). Fifty germinated seeds and one piece of filter paper were placed in each beaker (50 mL). Five mL of different concentrations of broth were added to each beaker. Incubation conditions and data collection methods were the same as described above. Experiments were repeated in triplicate.

**Extracts of broth and mycelia**

The fermented broth (250 mL) was extracted with ethyl acetate (3 × broth volume), dichloromethane and petroleum ether, separately to determine the best method to extract the phytotoxins (5). The solvent was evaporated to dryness under reduced pressure at 55 to 60°C. The residue in the evaporation flask was dried on a jet stream of

$N_2$ gas and then dissolved in 5 mL methanol. Filter paper in beaker (50 mL) was treated with 0.1 mL of methanol extracts of broth. To avoid the toxic effects of solvents, filter paper was placed in a fume hood for 1 h to allow complete solvent evaporation. Subsequently, 5.0 mL water and 50 germinated seeds were added to each beaker. The control beaker received 0.1 mL methanol. Incubation conditions and data collection methods were the same as described above. Experiments were repeated in triplicate.

**TLC Isolation**

Thin-layer chromatography (TLC) followed the method of Chen and Sung (3). Silica gel and silica GF 254 were purchased from Qingdao Haiyang Chemical Ltd., Qingdao, China. All solvents were of analytical grade. After evaporation to dryness, the residue was dissolved in distilled water, adjusted to 50 mg/L and tested for its effects on germination and growth of barnyardgrass. Spots that inhibited the lettuce emergence were collected, dissolved in acetone and used for gas chromatography-mass spectrometry (GC-MS) analysis (5).

**GC-MS Analysis**

The fraction with the highest activity was analyzed using gas chromatography-mass spectroscopy (GC-MS TRACE 2000) following the method of Chen *et al.* (5,6).

**Phytotoxicity of pure compounds**

The phytotocity of four pure compounds with 5-concentrations (50, 100, 200, 500 and 1000 mg/L) was determined using barnyardgrass as test species (5,6). The compounds were dissolved in ethanol and the solution was transferred to Petri dishes (10 cm dia) with filter paper. After the ethanol volatilized, distilled water (5 mL) containing 0.4% Tween-20 was added to each Petri dish. Petri dishes for the control (CK) received only ethyl alcohol, distilled water and Tween-20. Germinating barnyardgrass seeds were sown in the Petri dishes, which were then placed in a growth chamber at 28°C. The root and shoot length of the seedlings were measured after 7 d. Experiments were repeated in triplicate.

**Statistical Analysis**

All treatments were arranged in a completely randomized design with four replications. Bioassays were replicated five times. Data were analyzed with a one-way ANOVA statistical test (SPSS 10.0, SPSS Inc. Chicago, USA) using analysis of variance and least significant difference at the 0.05 probability level.

## RESULTS

**Phytotoxicity of crude extract**

The crude extract of *Exserohilium monoceras* strain X27 fermentation broth was phytotoxic to barnyardgrass (Table 1). The crude ethyl acetate extract drastically reduced the growth of roots (85.8%) and shoots (75.6%). While the dichloromethane crude extract mixture reduced the root and shoot growth by 10.1 and 9.0%, respectively.

The petroleum ether crude extract reduced the roots and shoots growth by only 1.5% and 3.8%, respectively. Therefore, ethyl acetate was the best solvent to extract the allelochemicals produced by X27 fungal strain.

Table 1. Effects of different extractions of fermented broth of *Exserohilum monoceras* strain X27 on the seedling growth of barnyardgrass (concentration = 5 g/L)

| Solvent extraction | Root growth (% inhibition) | Shoot growth (% inhibition) |
|---|---|---|
| Ethyl acetate | 85.8 ± 3.4 a | 75.6 ± 4.0 a |
| Dichloromethane | 10.1 ± 2.7 b | 9.0 ± 1.4 b |
| Petroleum ether | 1.5 ± 0.7 c | 3.8 ± 1.0 c |

The data are means ± SE (n = 4). Different letters within columns indicate a significant difference.

The 2.0, 3.0, 4.0 and 5.0 g/L concentrations of ethyl acetate crude extract decreased the germination of barnyardgrass by 15.0, 55.0, 69.7 and 77.5%, respectively (Fig. 1). Similarly, the ethyl acetate crude extract mixture at 2.0, 3.0, 4.0 and 5.0 g/L decrease the barnyardgrass shoot growth by 9.3, 69.1, 89.2 and 94.9% respectively. The ethyl acetate crude extract at 2.0, 3.0, 4.0 and 5.0 g/L decreased the barnyardgrass roots by 2.2, 42.0, 62.0 and 79.0% respectively. The ethyl acetate crude extract mixture was more inhibitory to shoot growth than root growth of barnyard grass.

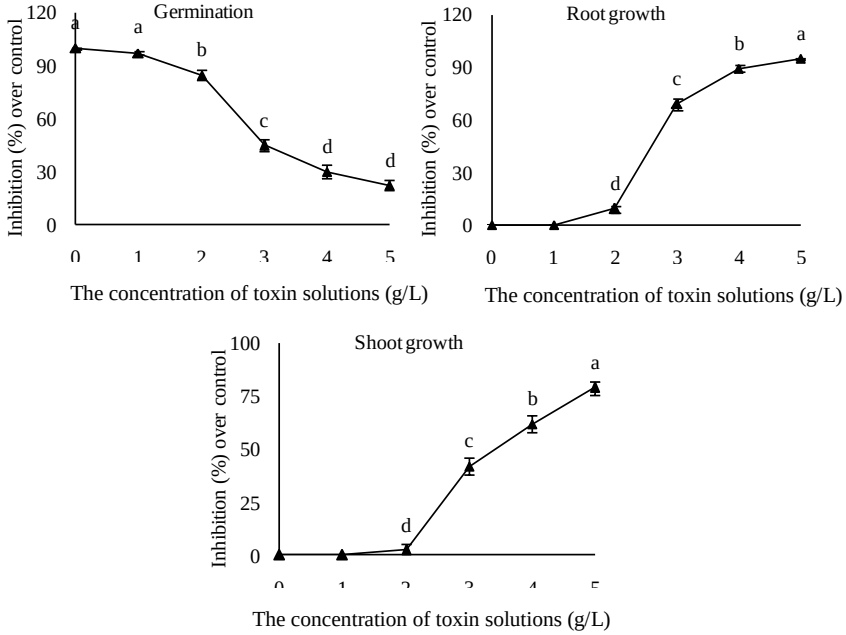



Figure 1. Phytotoxicity of fungal extract on the germination rate and roots and shoots growth of barnyardgrass.

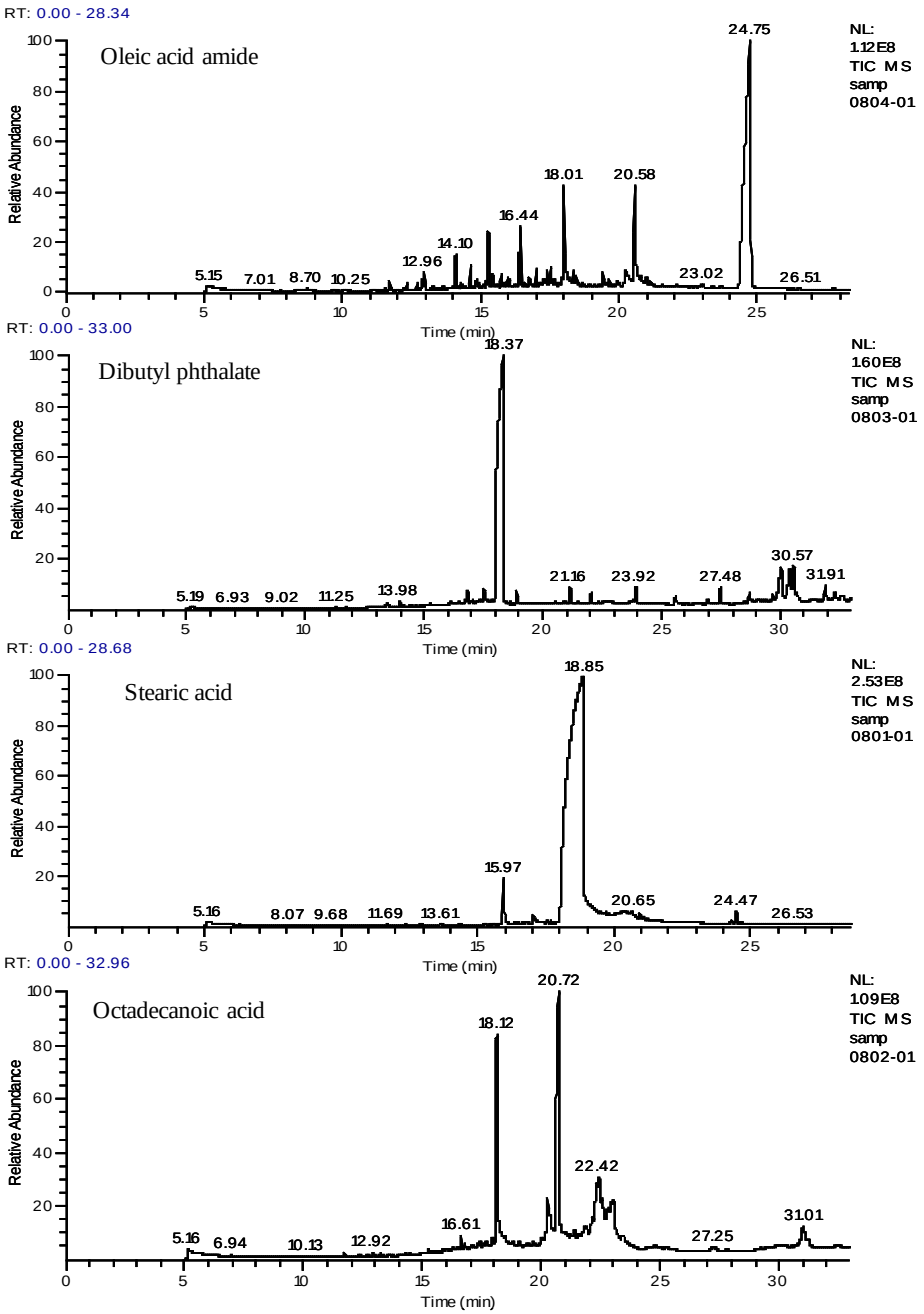
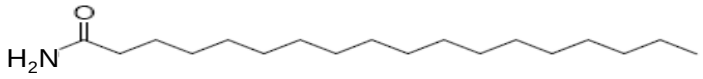


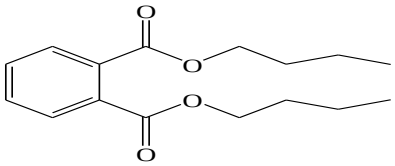

Figure 2. Four compound fraction was analyzed by gas chromatography-mass spectrometry.

**Phytotoxicity of pure compounds**

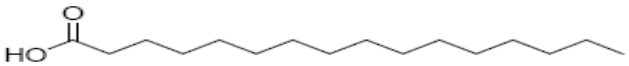
Four compounds detected by GC (Fig. 2) were identified by MS as oleic acid amide, dibutyl phthalate (DBP), stearic acid and octadecanoic acid. Their chemical structures are described in Fig. 3. The phytotoxicity of these 4-compounds isolated from X27 was assayed (Fig. 2 and 3). All isolated compounds inhibited the shoot growth of barnyardgrass (Table 2). The oleic acid amide, stearic acid and octadecanoic acid at 1 g/L concentration inhibited the root growth of barnyardgrass by 41.6, 34.9 and 33.5%, respectively (Table 2) and shoot growth of barnyardgrass by 27.9, 25.3 and 27.4%, respectively.



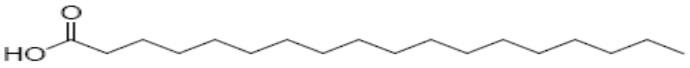
$C_{18}H_{35}NO$
Mol. Wt.: 281.48
Oleic acid amide
301-02-0



$C_{18}H_{22}O_4$
Mol. Wt.: 278.34
Dibutyl phthalate
87-74-2



$C_{16}H_{32}O_2$
Mol. Wt.: 256.42
Stearic acid
57-10-3



$C_{18}H_{36}O_2$
Mol. Wt.: 284.48
Octadecanoic acid
57-11-4

Figure 3. Chemical structures of compounds involved in inhibitory activity of *Exserohilum monoceras* strain X27.

DBP was most inhibitory to plant growth, even at lower concentrations than other three compounds. DBP at 50 mg/L and 1, 000 $mg \cdot L^{-1}$ drastically decreased the barnyard grass root growth by 73.9 and 84.5% and shoot growth by 59.4 and 76.2%, respectively (Table 2).

Table 2. Effects of 4-compounds on the growth of barnyardgrass roots and shoots

| Compounds | Concentration (mg/L) | Root growth (% inhibition) | Shoot growth (% inhibition) |
|---|---|---|---|
| Oleic acid amide | 50 | 26.0 ± 3.0a | 15.9 ± 0.9b |
| | 100 | 30.4 ± 3.8a | 21.8 ± 4.1ab |
| | 200 | 30.0 ± 5.3a | 22.4 ± 2.5ab |
| | 500 | 31.4 ± 5.4a | 24.5 ± 2.4ab |
| | 1000 | 33.5 ± 3.5a | 27.4 ± 3.7a |
| | Mean | 30.26 | 22.40 |
| DBP (Dibutyl phthalate) | 50 | 73.9 ± 2.2b | 59.4 ± 2.1b |
| | 100 | 81.6 ± 4.0ab | 71.8 ± 1.9a |
| | 200 | 82.2 ± 2.6ab | 74.8 ± 0.8a |
| | 500 | 84.0 ± 2.5a | 73.3 ± 1.6a |
| | 1000 | 84.5 ± 1.3a | 76.2 ± 1.7a |
| | Mean | 81.24 | 71.10 |
| Stearic acid | 50 | 30.2 ± 1.8b | 23.9 ± 1.3a |
| | 100 | 36.0 ± 3.1ab | 27.4 ± 0.7a |
| | 200 | 36.7 ± 1.8ab | 27.1 ± 1.0a |
| | 500 | 37.7 ± 2.1a | 25.1 ± 2.2a |
| | 1000 | 41.6 ± 2.0a | 27.9 ± 3.1a |
| | Mean | 36.44 | 26.28 |
| Octadecanoic acid | 50 | 26.1 ± 1.9a | 20.1 ± 2.0b |
| | 100 | 29.0 ± 1.5a | 22.7 ± 1.3ab |
| | 200 | 31.5 ± 5.9a | 23.4 ± 3.0ab |
| | 500 | 30.2 ± 1.8a | 29.2 ± 3.6a |
| | 1000 | 34.9 ± 6.2a | 25.3 ± 3.1ab |
| | Mean | 30.34 | 24.14 |

The data are means ± SE (n = 4). Different letters within columns indicate a significant difference.

## DISCUSSION

Among the tested solvents, the ethyl acetate crude extract was most phytotoxic. The ethyl acetate crude extract at 5.0 g/L decreased the barnyard grass germination shoot and root growth by 77.5, 94.9 and 79.0% respectively. Many studies (5,17) reported that *E. monoceras* inhibited the barnyardgrass (*Echinochloa crus-galli* L.) growth. In this study, we isolated and identified the *E. monoceras* (Drechsler) strain that possessed phytotoxicity and inhibited the growth of barnyardgrass.

Monocerin was the first chemical substance isolated from the *Exserohilum turcicum* (Pass.) to be phytotoxic against johnsongrass (*Sorghum halepense* (L.) Pers.) and Canada thistle (*Cirsium arvense* (L.) Scop.) (12). In 1990, *Exserohilum monoceras* was identified to cause the leaf blight in *Echinochloa* species. DBP exhibited the highest activity and accounted for 9.24% of the total peak area (5), suggesting that DBP may be one of the primary active compounds produced by *E. monoceras*. In this study, four allelochemicals were isolated and identified from *E. monoceras* fermentation broth. Bioassays demonstrated that all four compounds had antimicrobial activity against barnyardgrass. The two fatty acids (palmitic acid and stearic acid) were mildly phytotoxic. While these lipids appear to contribute little to the over activity of the extract,

other fatty acid with shorter hydrocarbon chains (e.g., pelargonic acid) have sufficiently good herbicidal properties to be commercialized as contact herbicides (8). Of the four isolated compounds, dibutyl phthalate (DBP) had the strongest activity against barnyardgrass. DBP isolated from *Streptomyces nasri*-H35 and *S. melanofaciens* had antimicrobial activity (9) and the dibutyl phthalate isolated by us from the X27 was strongly herbicidal against barnyardgrass. Little is known about the mechanism of action of DBP, but it is structurally related to the herbicide endothall and its natural analog cantharidin (2). These compounds are known inhibitors of serine/threonine protein phosphatates in plants and it is possible that DBP may have a similar molecular target site.

In conclusion, allelochemicals produced by *Exserohilum monoceras* strain X27 could be extracted using ethyl acetate and DBP was the main chemical compound responsible for the inhibition of barnyardgrass growth.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Ash, G.J., Chung, Y.R., Mckenzie, C. and Cother, E.J. (2008). A phylogenetic and pathogenic comparison of potential biocontrol agents for weeds in the family Alismataceae from Australia and Korea. *Australasian Plant Pathology* **37:** 402-405.
2. Bajsa, J., Pan, Z., Dayan, F.E., Owens, D.K. and Duke, S.O. (2011). Validation of serine-threonine protein phosphatase as the herbicide target site of endothall. *Pesticide Biochemistry and Physiology* **102:** 38-44.
3. Chen, W.L. and Sung, H.H. (2005). The toxic effects of phthalate esters on immune responses of giant freshwater prawn (*Macrobrachium rosenbergii*) via oral treatment. *Aquatic Toxicology* **74:** 160-171.
4. Chen, M., Zhou, J.R., Li, C.Y., Song, Y.Y., Xie, L.J., Chen, S. and Zeng, R.S. (2009). Isolation, identification and bioactivity of allelochemicals of *Streptomyces sp.* strain 6803. *Allelopathy Journal* **23:** 411-424.
5. Chen, Y., Zheng, W., Wang, L.M., Cui, H.L., Li, G.X., Liu, X.G., Han, C.C. and Zeng, R.S. (2009). Effect of toxins isolated from *Exserohilum monoceras* (Drechsler) Leonard and Suggs on *Echinochloa crus-galli* (L.) Beauv. *Agricultural Sciences in China* **8:** 972-978.
6. Chen, Z.Y., Min, H., Wu, W.X., Chen, M.C., Zhang, F. D. and Zhao, J.Q. (2003). Effects of pesticide-contamination on population size and denitrification activity of denitrifying bacteria in paddy soils. *Chinese Journal Applied Ecology* **14:** 1765-1769. (In Chinese)
7. Chin, D.V. (2001). Biological management of barnyardgrass, red sprangletop and weedy rice. *Weed Biology and Management* **1:** 37-41.
8. Dayan, F.E., Cantrell, C.L. and Duke, S.O. (2009). Natural products in crop protection. *Bioorganic and Medicinal Chemistry* **17:** 4022-4034.
9. EI-Naggar, M.Y.M. (1997). Dibutyl phthalate and the antitumor agent F5A1, two metabolites produced by *Streptomyces nasri* submutant H35. *Biotechnology Letters* **55:** 125-131.
10. Hirase, K., Nishida, M. and Shinmi, T. (2006). Effect of δ-aminolevulinic acid on the herbicidal efficacy of foliar-applied MTB-951, a mycoherbicide to control *Echinochloa crus-galli* L. *Weed Biology and Management* **6:** 44-49.

11. Holm, G.L., Plucknett, D.L., Pancho, J.V. and Herber, J.P. (1991). *The World's Worst Weeds-Distribution and Ecology*. Krieger Publishing Company, Malabar, FL, USA. pp. 32, 341, 609.
12. Robeson, D.J. and Strobel, G.A. (1982). Monocerin, a phytotoxin from *Exserohilum turcicum* (*Drechslera turcica*). *Agricultural and Biological Chemistry* **46:** 2681-2683.
13. Tuat, N.V., Hanh, T.H., Hetherington, S.D., Chin, D.V. and Auld, B.A. (1999). Potential use of *Exserohilum monoceras* as a biological control agent for *Echinochloa spp*. (barnyardgrass) in Vietnam. *Plant Protection Quarterly* **14:** 136-138.
14. Xuan, T.D., Chung, III M., Khanh, T.D. and Tawata, S. (2006). Identification of phytotoxic substances from early growth of barnyardgrass (*Echinochloa crus-galli*) root exudates. *Journal of Chemical Ecology* **32:** 895-906.
15. Vidotto, F., Tesio, F., Tabacchi, M. and Ferrero, A. (2007). Herbicide sensitivity of *Echinochloa* spp. accessions in Italian rice fields. *Crop Protection* **26:** 285-293.
16. Zhang, W.M. and Watson, A.K. (1997). Efficacy of *Exserohilum monoceras* for the control of *Echinochloa* species in rice (*Oryza sativa* L.). *Weed Science* **45:** 144-150.
17. Zhang, W. M., Moody, K. and Watson, A. K. (1996). Response of *Echinochloa* species and rice (*Oryza sativa* L.) to indigenous pathogenic fungi. *Plant Disease* **80:** 1053-1058.