J. Microbiol. Biotechnol. (2014), 24(3), 354–358
http://dx.doi.org/10.4014/jmb.1310.10063

jmb

# Lycorine: A Potential Broad-Spectrum Agent Against Crop Pathogenic Fungi

**Jin-Wen Shen[1†], Yuan Ruan[2†], Wei Ren[2], Bing-Ji Ma[2*], Xiao-Long Wang[2], and Chun-Feng Zheng[2]**

[1]*College of Life Sciences, Henan Agricultural University, Zhengzhou 450002, China*
[2]*College of Agronomy, Henan Agricultural University, Zhengzhou 450002, China*

*Received: October 17, 2013*
*Revised: December 10, 2013*
*Accepted: December 13, 2013*

First published online
December 17, 2013

*Corresponding author
Phone: +86-371-63504463;
Fax: +86-371-63558122;
E-mail: mbj123@sina.com

†Thess authors contributed equally to this work.

pISSN 1017-7825, eISSN 1738-8872



A screening test showed that lycorine exhibited significant antifungal activity against 24 pathogenic crop fungi at concentrations of 500 μg/ml and 100 μg/ml, respectively. *Fusarium graminearum* was selected for antifungal mechanism studies by observing its mycelial morphology and investigating the variations in its conductivity. In addition, the substance absorption and metabolism of *F. graminearum* were explored. The mechanism was revealed as being one by which lycorine destroyed the cellular membrane and further influenced substance absorption and cell metabolism.

**Keywords:** Lycorine, antifungus activity, antifungal mechanism, *Fusarium graminearum*

Lycorine is a pyrrolo[*de*]phenanthridine ring-type alkaloid extracted from different Amaryllidaceae genera, whose structure was first determined by Nagakawa *et al.* in 1956 [13]. Lycorine was reported as possessing various biological activities, including anticancer [8, 9, 11], antimalarial [16], antiviral [10, 17], and antifeedant properties for the larvae of the yellow butterfly [14]. In addition, lycorine also showed other biological properties, such as the inhibition of vitamin C biosynthesis [1], inhibition of growth and cell division in higher plants, algae, and yeasts [5], and prevention of cyanide-insensitive respiration [2]. These facets have made lycorine a valuable compound for researchers investigating such drugs and a number of other important physiological processes.

Research during the last decade has also convincingly shown that some natural products, when isolated, play an important role in pharmacology and agriculture as a rich source of bioactive components for crop protection [12, 18]. Although lycorine has been sufficiently investigated by pharmacologists, research into this compound and its screening in crop protection trials/applications is rare. In a continuation of our studies on bioactive secondary metabolites for antifungal agents, lycorine was firstly found to exhibit significant antifungal activity against various pathogenic crop fungi at different concentrations. We herein report the antifungal spectrum of lycorine and its specific mechanism using the strain of *Fusarium graminearum* as a mode fungus.

The antifungal activity of lycorine was determined using a poisoned food technique. The technique entailed the following steps: lycorine at different concentrations (500 and 100 μg/ml) was prepared on potato dextrose agar (PDA) plates. Then the pre-tested fungi that were cultured in the agar disc were inoculated into the center of the Petri dish and then incubated at 28°C. Each fungus was replicated over a further five plates. After incubation for 4 to 7 days when the fungi in the control dishes were almost fully grown, the mycelia growth of fungi (mm) in both treated (T) and control (C) Petri dishes was measured across different diameters. The percentage of growth inhibition (I) was calculated using Eq. (1) below [6]:

$$I\ (\%) = [(C - T)/C] \times 100\% \quad (1)$$

The antifungal activities of lycorine against 24 crop pathogenic fungi are shown in Table 1. Overall, lycorine

**Table 1.** The inhibition rates of lycorine against 24 crop pathogenic fungi *in vitro*.

| Plant pathogetic fungi | Inhibition ratio (%) | |
|---|---|---|
| | 500 μg/ml | 100 μg/ml |
| *Alternaria oleracea* M. Ibrath | 78.1 | 61.9 |
| *Fusarium graminearum* | 74.7 | 63.7 |
| *Colletotrichum gloeosporioides* Penz *et* Sacc | 73.9 | 57.2 |
| *Alternaria solani* (Ell et Mart) Jones *et* Grout | 73.9 | 48.5 |
| *Fusarium graminearum* Schw | 62.3 | 21.8 |
| *Pleospora lycopersici* | 61.3 | 52.6 |
| *Fusarium oxysporum* f. sp. *vasinfectum* | 60.3 | 70.1 |
| *Pseudocercospora variicola* (Wint.) Guo *et* Liu | 58.8 | 46.1 |
| *Cercospora arachidicola* Hori | 54.6 | 45.5 |
| *Fusarium oxysporum* | 53.4 | 42.9 |
| *Exserohilum turcicum* | 51.4 | 42.6 |
| *Colletotrichum ophiopogonis* | 51.2 | 63.2 |
| *Cercospora personata* Berk *et* Curt | 47.1 | 28.2 |
| *Rhizoctonia cereali* | 45.4 | 51.1 |
| *Corynespora cassiicola* | 44.1 | 35.1 |
| *Alternaria alternata* | 43.8 | 13.2 |
| *Gloeosporium loeosporium* sp. | 42.6 | 30.9 |
| *Bipolaris carbonum* Wilson | 36.1 | 36.1 |
| *Phytophthora vignae* Purss | 30.8 | 15.9 |
| *Cladosporium ladosporium* sp. | 28.2 | 29.3 |
| *Alternaria pluriseptata* | 23.3 | 16.9 |
| *Fusarium pointed* watermelon | 9.9 | 4.8 |
| *Trichoderma atrovoride* | 15.5 | 12.2 |
| *Fusarium oxysporum* Schl. f. sp. *vasinfectum* | 3.6 | 0 |

The 24 strains of test pathogenic crop fungi were all cultured on PDA medium at 28°C in the authors' laboratory.

presented a significant, widespread, and intensive strong antifungal activity. The lycorine concentration was positivity correlated with the rate of inhibition on most of the pathogenic fungi tested. With decreasing lycorine concentration, the inhibition rate was correspondingly reduced. Lycorine at 500 μg/ml showed a strong inhibition (inhibitory rate more than 60%) for 7 crop pathogenic fungi (*F. oxysporum* f. sp. *vasinfectum*, *F. graminearum*, *F. graminearum* Schw, *P. lycopersici*, *A. oleracea* M. Ibrath, *A. Solani* (Ell *et* Mart) Jones *et* Grout, and *C. gloeosporioides* Penz *et* Sacc), a mid-strong inhibition (inhibitory rate more than 30%) for 12 crop pathogenic fungi (*P. vignae* Purss, *C. ophiopogonis*, *G. loeosporium*, *E. turcicum*, *C. cassiicola*, *P. variicola* (Wint.) Guo *et* Liu, *C. arachidicola* Hori, *B. carbonum* Wilson, *F. oxysporum*, *C. personata* Berk *et* Curt, *R. cereali*, and *A. alternata*), and a weak inhibition (inhibitory rate less than 30%) for 5 crop pathogenic fungi (*F. oxysporum* Schl. f. sp. *vasinfectum*, *F. pointed* watermelon, *T. atrovoride*, *C. ladosporium* sp., and *A. pluriseptata*).

Lycorine at 100 μg/ml displayed a strong inhibition for 4 crop pathogenic fungi (*F. oxysporum* f. sp. *vasinfectum*, *F. graminearum*, *A. oleracea* M. Ibrath, and *C. ophiopogonis*), a mid-strong inhibition for 11 crop pathogenic fungi (*G. loeosporium* sp., *E. turcicum*, *P. lycopersici*, *A. solani* (Ell *et* Mart) Jones *et* Grout, *C. cassiicola*, *P. variicola* (Wint.) Guo *et* Liu, *C. arachidicola* Hori, *B. carbonum* Wilson, *F. oxysporum*, *C. gloeosporioides* Penz *et* Sacc, and *R. cereali*), and a weak inhibition for 9 crop pathogenic fungi (*F. graminearum* Schw, *F. oxysporum* Schl. f. sp. *vasinfectum*, *P. vignae* Purss, *F. pointed* watermelon, *T. atrovoride*, *C. ladosporium* sp., *C. personata* Berk. et Curt, *A. alternate*, and *A. pluriseptata*). Except in the cases of four crop pathogenic fungi (*F. oxysporum* f. sp. *vasinfectum*, *C. ophiopogonis*, *C. ladosporium* sp., and *R. cereali*), the concentration of lycorine presented a positive correlation with the inhibitory ability on most of the crop pathogenic fungi examined.

*F. graminearum* was selected for the investigation of the antifungal mechanism because the mycelium of this fungus is red, making it easy to judge its growth stages and observe its microstructure. Moreover, the mycelia of *F. graminearum* grow to form clusters, which is convenient to be extracted for the further experiments. The strain was inoculated at the center of the disc of the PDA medium and incubated at 28°C for 5 days. Then the fungus cake (9 mm in diameter) in the disc was inoculated into 100 ml of potato dextrose broth (PDB) medium and spun at 180 rpm and 28°C in a shaking incubator. The strain was firstly cultivated for 24 h in the medium and the culture broth was then divided into two equal parts. The cultivation of one 50 ml culture broth sample was continued for a further 24 h as the treated group after lycorine was added (to 50 μg/ml final concentration). The other 50 ml culture broth sample also continued to be cultivated for a further 24 h and constituted the blank control group.

A 50% inhibitory concentration ($IC_{50}$) test of lycorine against *F. graminearum* was conducted as follows: lycorine PDA plates at concentrations of 500, 100, 50, 25, 12.5, and 6.25 μg/ml were prepared. Then the fungus *F. graminearum* was inoculated into the center of each Petri dish and incubated at 28°C. Each concentration was replicated over five plates. After incubation for 4 days when the fungi in the control dish were almost fully grown, the mycelia growth of fungi in both treated and control Petri dishes were measured across three different diameters. The

percentage growth inhibitions of each of these six concentrations were obtained using Eq. (1). The $IC_{50}$ value of lycorine against *F. graminearum* was approximately 50 μg/ml, which was calculated according to the method reported by Jiang *et al.* [7]. Hence, 50 μg/ml of lycorine was selected as the sensitive concentration for further studies of its antifungal mechanism against *F. graminearum*.

Morphological observation of *F. graminearum* was performed using a Nikon Eclipse 50i microscope (Nikon, Kawasaki, Japan). *F. graminearum* mycelia from the 50 μg/ml lycorine treated and blank control groups were collected and observed using 10× and 40× magnification optical microscopy, respectively. The results are shown in Fig. 1. As expected, the mycelium structure was different between the control and the treated groups. At low power (Figs. 1a and 1b), the control group showed a greater density and a greater number of branches, whereas the treated group showed the opposite. At high power (Figs. 1c and 1d), the control group exhibited a coarse, full mycelium with significant branches, whereas the treated group exhibited the opposite. Overall, lycorine had a significant toxic effect on the morphology of *F. graminearum*.

The cellular permeability of *F. graminearum* was measured using a DDS-11A conductivity meter (INESA Scientific Instrument Co., Ltd, Shanghai, China). The cellular permeability of the mycelium was assayed by measuring the conductivity at pre-set times (0, 2, 4, 6, 8, and 10 h). Each experiment was performed three times. *F. graminearum* was first cultivated for 48 h in a PDB medium and then the mycelia were collected and washed three times using 20 ml of distilled water to remove the culture medium. A blank control group was prepared by mixing 1 g of mycelium with 50 ml of sterilized distilled water. The treated group was made by mixing 1 g of mycelia with 50 ml of lycorine solution at a concentration of 50 μg/ml. The results are shown in Fig. 2. The conductivities of the control and treated groups gradually increased as measured at pre-set times. Moreover, the conductivity difference between the treated group and the control group increased gradually over time. The conductivity of the treated group showed larger variations than the control group. This outcome should be attributed to the fact that lycorine destroyed the structure of the cytomembrane of the mycelium, which in turn led to the exosmosis of intracellular materials.

For the succinate dehydrogenase activity (SDA) assay, the mycelia of the 50 μg/ml lycorine treated group and blank control group were collected and weighed. After the culture media were fully removed, 3 g of mycelia was taken from samples from both groups and ground in liquid nitrogen. The ground mycelia were then dissolved in 0.05 mol/ml Tris-HCl. After freezing and centrifugation at 1,000 ×*g* for 10 min, the supernatants were collected and recentrifuged at 10,000 ×*g* for 15 min. The precipitate obtained was mitochondria. Measurement of SDA in the mitochondria was strictly in accordance with the kit protocol. The process was indicated as follows: colors were compared at 600 nm using a 1 cm light-path-length cuvette. The light absorption values at 5 and 65 sec were recorded with the light absorption decreasing by 0.01 in 1 min as an enzyme activity unit. As a result, owing to the stress induced by the lycorine, the SDA of the treated group

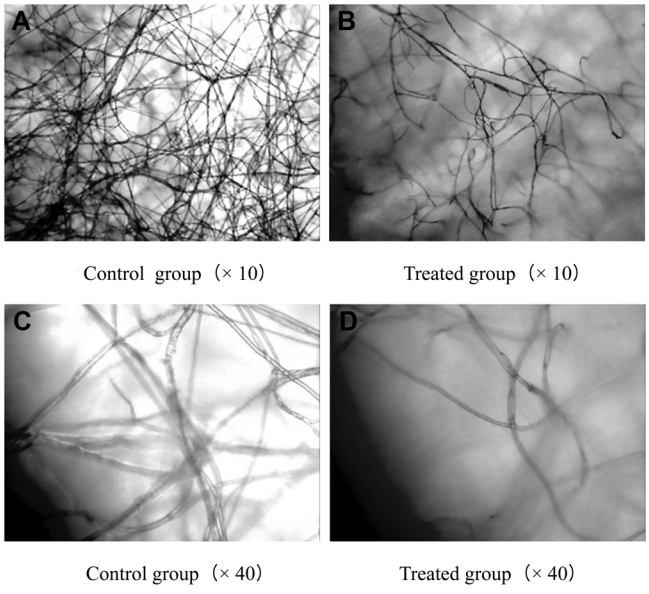


**Fig. 1.** Effects of lycorine on the morphology and structure of *F. graminearum*.

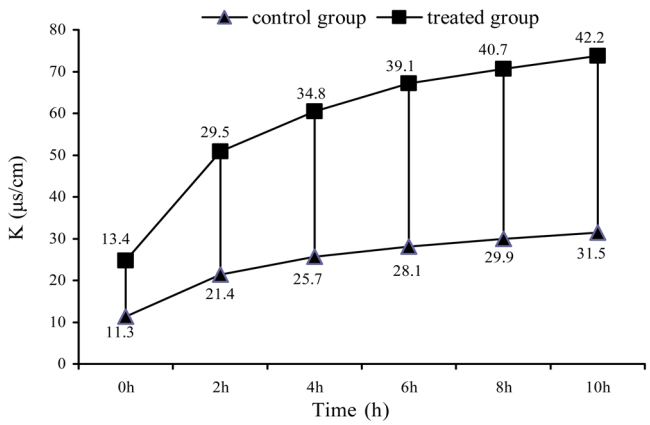


**Fig. 2.** The conductivity curve of lycorine.

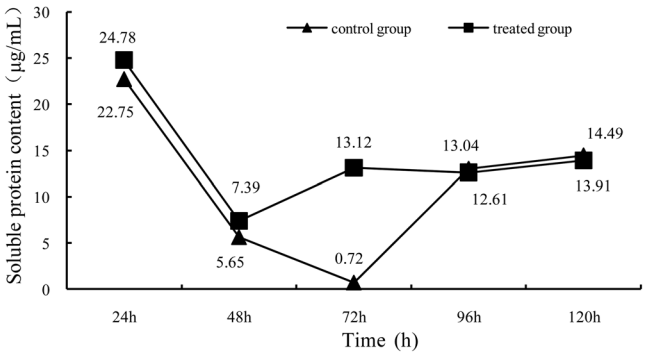


**Fig. 3.** Variations of soluble protein content.

(11.92 U/mg·min·pro) was greatly lower than that of the control group (33.79 U/mg·min·pro). It is conjectured that the energy metabolism of mycelium was interfered with and the growth of mycelium thus inhibited.

For the soluble protein content assay, 1 ml of liquid medium of both the lycorine-treated group and blank control group was collected from each group every 24 h. The liquid medium collected was then centrifuged at 4,000 ×*g* for 10 min and the supernatant was collected. Then 0.5 ml of the supernatant was drawn off and dissolved to 0.5 ml with distilled water. Both the treated group and the control group were dosed with 5 ml of Coomassie brilliant blue G-250 solution. The absorbency was determined at 595 nm and the protein concentration was calculated according to the protein standard curve. The results are shown in Fig. 3, and implied that the soluble protein contents of both groups of *F. graminearum* decreased gradually from the beginning. However, at 48 h, the soluble protein content of the treated group began to increase. This increase could possibly have been attributed to the exosmosis of intracellular substances developed from lycorine-induced cell membrane destruction; after 72 h, the protein content of the control group increased as a result of mycelia shrinkage and cell death in significant quantities.

The semipermeable cell membrane of a pathogenic fungus is a cellular biological barrier. Under adverse growth or inhibition conditions, the fungal cell membrane will be damaged, followed by a loss of semipermeability, low liquidity, and finally the breach of its fungal protection ability. Furthermore, a large number of internal electrolytes (such as $K^+$) then have access to the culture medium, which results in the increase of the conductivity therein. Hence, the changes in medium conductivity reflect the permeability variation of the fungal cell membrane [3]. Our experimental results suggested that the treated group of *F. graminearum* presented a significantly higher conductivity, a larger magnitude of change, and more significant variations than the control group. This outcome revealed that, since lycorine damaged the cellular membrane and changed the permeability of *F. graminearum*, the intracellular substances leaked out on one hand; on the other hand, lycorine exerted influences on mitochondria and thereby changed the cellular energy metabolism within, and across the fungal cell wall.

Succinate dehydrogenase (SD) locates on the membrane of mitochondria [15] and SDA reflects the intensity of cellular metabolism [4]. In this study, the SDA of the treated group was significantly lower than the control group, suggesting that lycorine significantly influenced SDA. The SDA reduction ultimately slowed the metabolism and growth rate of *F. graminearum*.

In conclusion, lycorine is a broad-spectrum agent against crop pathogenic fungi. In this study, the changed morphology of pathogenic fungi was observed using optical microscopy. The cellular permeability of *F. graminearum* was measured to investigate the conductivity variations in pathogenic fungi. Moreover, the substance absorption and metabolism of pathogenic fungi were explored using different methods. Additionally, the related inhibitory mechanism of lycorine to *F. graminearum* was studied. The mechanism is concluded as being such that lycorine destroyed the cellular membrane and further influenced substance absorption and cell metabolism. The inhibition was mainly derived from the influence of lycorine on the cellular membrane structure and on the function of *F. graminearum*. In addition, such influences also affected the metabolism of pathogenic fungi to some extent. As a natural medicinal chemical, lycorine can be developed and utilized as a biological fungicide, which may provide some ideas for the fruitful development of new biological fungicides.

## Acknowledgments

This work was supported by special funds for the construction of modern agricultural technology systems in China. None of the authors have any conflicts of interests to declare.

## References

1. Arrigoni O, Arrigoni LR, Calabrese G. 1975. Lycorine as an inhibitor of ascorbic acid biosynthesis. *Nature* **256:** 513-514.
2. Arrigoni O, Arrigoni LR, Calabrese G. 1976. Ascorbic acid as a factor controlling the development of cyanide-insensitive respiration. *Science* **194:** 332-333.

3. Bi QY, Ma ZQ, Zhang XF, Wang WQ, Han XY, Tian YP. 2009. The influence of cell membrane permeability and leakage of contents with the synergistic and antagonistic mixtures of the partners of thiram and tebuconazole to *Fusarium graminearum*. *Chin. Agri. Sci. Bull.* **20:** 222-227.
4. Cooke D, O'Kennedy R. 1999. Comparison of the tetrazolium salt assay for succinate dehydrogenase with the cytosensor microphysiometer in the assessment of compound toxicities. *Anal. Biochem.* **2:** 188-194.
5. De LP, Dalessandro G, De SA, Arrigoni O. 1973. Inhibitory effect of lycorine on cell division and cell elongation. *Plant Cell Physiol*. **14:** 487-496.
6. Fang ZD. 1998. *Plant Pathogen Method.* 3rd Ed., pp. 68-70. China Agriculture Press, Beijing.
7. Jiang HH, Dong HZ, Yang JL, Gao X. 2013. Study on inhibitory effects of nano-$Cu_2O$ on *Alternaria solani* of tomato. *North. Hort.* **8:** 125-126.
8. Jokhadze M, Eristavi L, Kutchukhidze J, Chariot A, Angenot L, Tits M, *et al.* 2007. *In vitro* cytotoxicity of some medicinal plants from Georgian Amaryllidaceae. *Phytother. Res.* **21:** 622-624.
9. Lamoral-Theys D, Andolfi A, Van Goietsenoven G, Cimmino A, Calvé BL. 2009. Lycorine, the main phenanthridine Amaryllidaceae alkaloid, exhibits significant antitumor activity in cancer cells that display resistance to proapoptotic stimuli: an investigation of structure-activity relationship and mechanistic insight. *J. Med. Chem.* **52:** 6244-6256.
10. Li SY, Chen C, Zhang HQ, Guo HY, Wang H, Wang L, *et al.* 2005. Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Res.* **67:** 18-23.
11. Liu XS, Jiang JK, Jiao XY, Wu YE, Lin JH. 2009. Lycorine induces apoptosis and down-regulation of Mcl-1 in human leukemia cells. *Cancer Lett.* **274:** 16-24.
12. Ma BJ, Wu TT, Ruan Y, Shen JW, Zhou H, Yu HY, *et al.* 2010. Antibacterial and antifungal activity of scabronine G and H *in vitro*. *Mycology* **1:** 200-203.
13. Nakagawa Y, Uyeo S, Yayima H. 1956. The double bond in lycorine. *Chem. Ind.* 1238-1239.
14. Numata A, Takemura T, Ohbayashi H, Katsuno T, Yamamoto K, Sato K, *et al*. 1983. Antifeedants for the larvae of the yellow butterfly, *Eurema hecabe mandarina*, in *Lycods radiate*. *Chem. Pharm. Bull.* **31:** 2146-2149.
15. See RM, Foy CL. 1982. Effect of butanedioic acid mono (2,2-dimethylhydrazide) on the activity of membrane-bound succinate dehydrogenase. *Plant Physiol.* **70:** 350-352.
16. Sener B, Orhan I, Satayavivad J. 2003. Antimalarial activity screening of some alkaloids and the plant extracts from Amarylldaceae. *Phytother. Res.* **17:** 1220-1223.
17. Szlavik L, Gyuris Á, Minárovits J, Forgo P, Molnár J, Hohmann J. 2004. Alkaloids from *Leucojum vernum* and antiretroviral activity of Amaryllidaceae alkaloids. *Plants Med.* **70:** 871-873.
18. Wink M. 1993. Production and application of phytochemicals from an agricultural perspective, pp. 171-213. *In* van Beek TA, Breteler H (eds.). *Phytochemistry and Agricuture*. Clarendon Press, Oxford.