Antifungal Effects of Lycorine on Botrytis Cinerea and Possible Mechanisms

Shixue Zhao
Hainan University of China Tropical Agriculture: Hainan University
Yanhua Guo
Hainan University of China Tropical Agriculture: Hainan University
Qiannan Wang
Hainan University of China Tropical Agriculture: Hainan University
Bang An (anbang@hainanu.edu.cn)
Hainan University https://orcid.org/0000-0003-4200-5777

Research Article

Keywords: Lycorine, antifungal activity, Botrytis cinerea, postharvest

Posted Date: March 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-251600/v1

Version of Record: A version of this preprint was published at Biotechnology Letters on April 15th, 2021.
See the published version at https://doi.org/10.1007/s10529-021-03128-8.

Abstract

Botrytis cinerea cause postharvest diseases on fruit and lead economic losses. Application of environment-friendly natural compounds is an alternative for synthetic fungicides to control postharvest disease. Lycorine is an indolizidine alkaloid which is widely used for human drug design, however, application of lycorine in controlling postharvest disease and the underlying mechanisms have not been reported. In this study, the effects of lycorine on mycelium growth, spore germination, disease development in apple fruit, cell viability, cell membrane integrity, cell wall deposition, and expression of mitogen-activated protein kinase (MAPK) and GTPase of *B. cinerea* were investigated. Our results showed that lycorine was effective in controlling postharvest gray mold caused by *B. cinerea* on apple fruit. In the *in vitro* tests, lycorine strongly inhibited spore germination and mycelium spreading in culture medium. Investigation via fluorescein diacetate and propidium iodide staining suggested that lycorine could damage the membrane integrity and impair cell viability of *B. cinerea*. Furthermore, the expression levels of several MAPK and GTPase coding genes were reduced upon the lycorine treatment. Taken together, lycorine is an effective and promising way to control postharvest disease caused by *B. cinerea*.

Introduction

Fungal pathogens cause severe postharvest diseases on fruit and lead to considerable economic losses during transportation and storage (Tian et al., 2016). *Botrytis cinerea*, the causal agent of gray mold, could infect over two hundred plant species, including a number of common fruits (Dean et al., 2012). Synthetic fungicides have been widely used to control both pre- and postharvest diseases of fruit. However, indiscriminate use of synthetic fungicides has caused widespread concerns over food security and environmental contamination (Droby et al., 2009), which encourages researchers to identify and develop environment-friendly antifungal compounds for controlling postharvest diseases. A great number of natural chemicals have been found to be effective in controlling fungal disease in postharvest fruit till now. Treatment with phytohormones such salicylic acid and methyl jasmonate could induce the plant immunity and decrease postharvest decay in fruit (Yao et al., 2005; Cao et al., 2013; Wang et al., 2014). Plant essential oils and some isolated constituents could directly inhibit the growth of fungal pathogen in postharvest fruit (Mendel et al., 2002; Ji et al., 2018). Natural compounds such as chitosan and boron showed strong antimicrobial effect on fungal pathogens (Meng et al., 2010; Liu et al., 2007; Qin et al., 2010). Lycorine, an indolizidine alkaloid, is identified in *Amaryllidaceae* plants. In recent years, researchers discovered that lycorine and their derivatives have excellent properties including anti-tumor (Yui et al., 1998), anti-inflammatory (Çitoğlu et al., 2012), anti-malarial (Kogure et al., 2008), anti-virus (Liu et al., 2011), and other functions. In addition, lycorine is safe for most people when taken by mouth in low amounts. Thus, lycorine and their derivatives have been widely used for drug design. However, to our knowledge, application of lycorine in controlling postharvest disease and the underlying mechanisms have not been reported.

The objectives of this research were to i) study the inhibitory activity of lycorine on *B. cinerea* both *in vitro* and *in vivo*; ii) evaluate mechanisms by which lycorine inhibit growth of *B. cinerea* via analyzing cell

viability, cell wall structure, and cell membrane integrity.

Materials And Methods

Chemicals

Lycorine (CAS. No. 476-28-8) was purchased from Solarbio Science & Technology (Beijing). Lycorine was resolved in DMSO to make the stock solution at 0.696 mol L^{-1} before use.

Pathogens

Botrytis cinerea was isolated from infected tomato fruit and maintained on potato dextrose agar (PDA). For germination and inoculation tests, spores were obtained from 10-day old PDA cultures and suspended to 10^5 CFU mL^{-1} with ddH_2O containing 0.05% tween 80.

Fruit

Healthy apple fruit (*Malus pumila* Mill cv. *Fuji*) at commercial maturity were purchased from market. Fruit without physical injuries and infections were selected based on size uniformity. Before treatments, fruit surfaces were disinfected with 2% sodium hypochlorite for 3 min, then rinsed with tap water, and air-dried.

In vitro antifungal activity assay

To evaluate the effects of lycorine on mycelium growth of *B. cinerea*, aliquots of lycorine stock solution were added to potato dextrose agar (PDA) medium to generate final concentrations of 0, 1, 2, and 5 mmol L^{-1} respectively; in addition, extra DMSO were added into the medium to make DMSO at same concentration. The PDA media were poured into sterilized Petri dishes (90mm in diameter). 2 mm of mycelium together with agar were cut form the growing edge of 3 d-old cultures of *B. cinerea*, and transferred into the center of each Petri dish. Then the plates were cultured at 22°C and the diameter of colony was determined. Each treatment contained three replicates. To evaluate the effects of lycorine on spore germination and germ tube elongation of *B. cinerea*, spores were incubated in potato dextrose broth (PDB) containing lycorine at various concentrations. At least 100 spores were counted to calculate the germination rate. Measurement of germ tube length were performed on triplicates of 50 spores using ImageJ.

In vivo antifungal activity assay

The *in vivo* assay was carried out according to the method described previously (Ji et al., 2018). Apple fruit were wounded (3 mm wide and 4 mm deep) at the equator using a sterilized scalpel. Droplets of 5 µL of spore suspension at 10^5 spore mL^{-1} were inoculated into the wounds and air-dried for 1 h. Then 5 µL of lycorine solution diluted in ddH_2O at 0, 5, 10, and 30 mmol L^{-1} were added into the wounds. Fruit treated with ddH_2O was used as the control. Then the treated fruit were kept in moisture environment and stored at room temperature, and lesion diameter were recorded at 2, 3, and 4 day. Each treatment contained three replicates with 10 fruit per replicate, and the experiment was performed twice.

Fluorescence microscopy

B. cinerea spore were incubated in PDB containing lycorine as mentioned above. Fluorescent dyes fluorescein diacetate (FDA; Sigma) was used to determine cell viability; propidium iodide (PI; Sigma) was used to determine membrane integrity; Calcofluor white M2R (Sigma) was used to stain cell wall. After staining, *B. cinerea* spores were examined using a fluorescence microscope (LEICA DM4000M). At least 100 spores were counted to calculate the percentage of staining.

RNA extraction and qRT-PCR

B. cinerea were grown on cellophane paper plated on the PDA containing different concentration of lycorine. Then mycelium was collected and disrupted in liquid nitrogen by grinding in a mortar with a pestle, and RNA was extracted using TRIzol Reagent (Invitrogen). Reverse transcription was conducted with Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher) according to the manufacturer's instructions. The Quantitative real-time PCR was then performed using FastStart Universal SYBR Green Master (Roche, Switzerland) with a LightCycler 96 System (Roche, Switzerland) instrument. Relative expression levels of three mitogen-activated protein kinase (MAPK), including BcMPS1 (BCIN_02g08170), pathogenicity MAP kinase 1 (BcPmk1, BCIN_09g02390), and protein kinase A (BcPKA, BCIN_16g01130), and three GTPase, including Bccdc42 (BcBCIN_13g00090), BcRac (BCIN_01g02000), BcRas1 (BcIN_12g05760), were estimated using the $2^{-\Delta\Delta Ct}$ method. The *B. cinerea* actinA coding gene (BCIN_16g02020) was used as an endogenous control for normalization. The primers used for quantitative RT-PCR are listed in Table S1.

Statistical analysis

Statistical analysis was performed with SPSS version 10.0 (SPSS Inc., Chicago, USA). Data analyzed by one-way ANOVA, and mean separations were determined using Duncan's multiple range test. Differences at $P < 0.05$ were considered significant.

Results

Lycorine inhibited mycelial growth and spore germination in vitro

The *in vitro* assay showed that lycorine markedly inhibited mycelial growth of *B. cinerea* with a dose-dependent manner (Fig. 1). The colony diameter of the control sample was about 50 mm after inoculation for 3 day; whereas that of the sample treated with lycorine at 1 mmol L^{-1} was smaller than half of the control. Furthermore, the mycelial growth was completely inhibited when the lycorine concentration increased to 5 mmol L^{-1} (Fig. 1B). Germination is the first step for *B. cinerea* spore to infect plant host. Thus, the effect of lycorine on spore germination was examined. As shown in Fig. 2, the inhibitory effect of lycorine on germination also showed a dose-dependent manner. After incubation for 10 h, about 95% of spore in the control sample was germinated; in comparison, the germination rate was

only 17% in the sample treated with 5 mmol L^{-1} lycorine (Fig. 2A and 2B). In addition, the germ tube elongation was completely inhibited by 5 mmol L^{-1} lycorine (Fig. 2C).

Effect of lycorine in controlling gray mold in apple fruit

Consistent with the results of the *in vitro* test, lycorine was also effectively in controlling gray mold in apple fruit. As shown in Fig. 3, after inoculation for 2 day, the disease incidence was only 50% in the sample treated with 30 mmol L^{-1} lycorine, whereas the control sample showed disease symptoms in all wounds (Fig. 3B). The lesion diameters also decreased as the lycorine dose increased. Lycorine at concentration of 30 mmol L^{-1} significantly alleviated fruit decay in apple fruit with the lesion diameters of 6, 8, 13 mm at 2, 3, 4-day post inoculation; in comparison, the lesion diameters of control fruit were 8, 16, 27 mm, respectively.

Lycorine impaired membrane integrity and decreased cell viability of B. cinerea

After incubation for 8 h, *B. cinerea* spore were collected, stained with FDA and PI respectively, and observed under a fluorescence microscope. The results showed that about 95% of spores of control were stained with FDA in high fluorescence intensity; whereas only a few spores were stained in the lycorine treated sample (Fig. 4A and 4C). Meanwhile, less spores were stained with PI in the control sample compared with the lycorine treated sample (Fig. 4B and 4D). These results suggested lycorine could damage the cell membrane integrity and decrease cell viability of *B. cinerea*.

Polarized cell wall deposition was impaired by lycorine in B. cinerea

Calcofluor white M2R could be used for staining of microbe cell walls. As shown in Fig. 5, there is an obvious polarized deposition of cell wall compounds in the germ tube of *B. cinerea*, with intense calcofluor white fluorescence observed in the apical region. Whereas lycorine treated spore showed uniformly distributed deposition of cell wall and lack polarity.

Lycorine down-regulated MAPK and GTPase in B. cinerea

We also carried out a qRT-PCR assay to assess the effect of lycorine on transcription of MAPK and GTPase coding genes, which are involved in cell growth and virulence of *B. cinerea*. The results (Fig. 6) suggested that lycorine showed inhibitory effect on expression of GTPase *BcMPS1*, *Bccdc42*, *BcRac*, and *BcRas1* with a dose-dependent manner; and lycorine at 2 mmol L^{-1} significantly depressed expression of the genes compared with the control sample. Meanwhile, treatment with lycorine at 1 mmol L^{-1} up-regulated the transcription of *BcPmk1* and *BcPKA*; but when the concentration of lycorine come up to 2 mmol L^{-1}, the transcription of *BcPKA* was depressed significantly.

Discussion

Lycorine is identified as the most common alkaloid within the Amaryllidaceae family (Zhong et al., 2005). Due to its pharmacological potential, the alkaloid has been widely used in medicine design. In the present study, we found that lycorine shows efficient antifungal activity against *B. cinerea* both *in vitro* and *in vivo*. In the *in vitro* assays, the inhibitory effect of lycorine on *B. cinerea* was with a dose-dependent manner: the mycelium growth and the spore germ tube elongation were completely inhibited by lycorine at 5 mmol L^{-1} (Fig. 1, Fig. 2). In the *in vivo* test, lycorine also showed a dose-dependent manner on controlling disease severity in apple fruit (Fig. 3). However, the effect concentration of *in vivo* tests is relatively higher than that of *in vitro* tests, which might be explained by the complex environment in the wound of fruit, including carbon sources, pH value, physical barrier of host, which influence the antifungal effect of lycorine (Meng et al., 2010; Ma et al., 2019).

Cell viability play a vital role in spore germination and expansion of mycelium in fungal pathogen. To investigate whether lycorine showing antifungal activity by impair cell viability of *B. cinerea*, the FDA and PI staining assays were conducted. FDA is a cell-permeant esterase substrate that can serve as a viability probe. PI is a fluorescent intercalating agent that binds to DNA but cannot passively traverse into cells that possess an intact plasma membrane. The results showed that, compared with the control, fewer spores were stained with FDA (Fig. 4C) and more spore were stained with PI (Fig. 4D) upon the lycorine treatment. These results indicate that lycorine could damage the plasma membrane integrity and decrease the cell viability, which lead to the inhibition of spore germination and decrease in mycelium growth. Similar results were found in the previous reports that lycorine treatment inhibited cellular viability and induced cell death in multiple myeloma cell lines and primary myeloma cells (Jin et al., 2016). Polarized growth of germ tube and fungal hyphae is important for pathogenicity of *B. cinerea* to plant host (An et al., 2016), and this kind of polarized growth requires sustained deposition of cell wall components to the apical region (Fischer-Parton et al., 2000). In the present study, treatment of lycorine damage the polarized deposition of cell wall in germinated spore (Fig. 5); and this kind of impair effect may be attributed to injuries to cell viability.

Mitogen-activated protein kinase (MAPK) signaling pathway regulates various developmental and infection processes in filamentous fungal pathogens (Jiang et al., 2018); and many fungicides kill pathogens by direct targeting at MAPK signaling components. To investigate effect of lycorine on MAPK signaling, the transcription level of three kinases, *BcMPS1*, *BcPKA*, and *BcPmk1* were measured. The kinase BcMPS1 is conserved in filamentous fungi to regulate cell wall integrity and pathogenesis (Li et al., 2012; Bashi et al., 2016). In the present study, the transcript level of *BcMPS1* was significantly down-regulated upon the lycorine treatment (Fig. 6). And the result could also partially explain the impair effect of lycorine on cell wall deposition (Fig. 5). BcPmk1 and BcPKA are important for spore germination, appressorium formation, stress response, and virulence of *B. cinerea* and other fungal pathogens (Schumacher et al., 2008; Guo et al., 2016 Turra et al., 2014). As showed in Fig. 6, lycorine at concentration of 1 mmol L^{-1} up-regulated the transcription of *BcPmk1* and *BcPKA*, suggesting that the two genes are involved in response to lycorine stress. BcCdc42, BcRas1 and BcRac belong to small GTPases of the Ras superfamily, which participate in various cellular processes; furthermore, the three

GTPases are important for polarized growth of *B. cinerea* (Schumacher et al., 2008; Marschall et al., 2016). Here we found that lycorine significantly depressed the expression of *Bccdc42*, *BcRas1* and *BcRac* (Fig. 6), which might lead to inhibition of spore germination and hyphae growth. Taken together, these results suggested that lycorine showing anti-fungal activity by interfering with the MAPK and GTPase signaling.

Conclusions

Lycorine could inhibit pathogenicity of *B. cinerea* by disrupting cell membrane integrity, decreasing cell viability, impairing polarized growth, and interfering with MAPK and GTPase signaling pathways. lycorine and their derivatives can be used for controlling postharvest gray mold caused by *B. cinerea*, offering an environmental-friendly alternative for fungicide.

Declarations

Disclosure statement

The authors declare that there are no conflicts of interest.

Funding

This work was supported by the National Natural Science Foundation of China (31760037 and 31772117).

References

1. An B, Li B, Li H, Zhang Z, Qin G, Tian S (2016) Aquaporin8 regulates cellular development and reactive oxygen species production, a critical component of virulence in botrytis cinerea. New Phytol 209(4):668-1680
2. Bashi ZD, Gyawali S, Bekkaoui D, Coutu C, Lee L, Poon J, Rimmer SR, Khachatourians GG, Hegedus DD (2016) The Sclerotinia sclerotiorum Slt2 mitogen-activated protein kinase ortholog, SMK3, is required for infection initiation but not lesion expansion. Can J Microbiol 62:836-850
3. Cao J, Yan J, Zhao Y, Jiang W (2013) Effects of postharvest salicylic acid dipping on Alternaria rot and disease resistance of jujube fruit during storage. J Sci Food Agr 93:3252–3258
4. Çitoğlu GS, Ö Bahadır Acıkara Yılmaz BS, Özbek H (2012) Evaluation of analgesic, anti-inflammatory and hepatoprotective effects of Lycorine from sternbergia fisheriana (herbert) rupr. Fitoterapia 83(1):81-87
5. Dean R, Kan JALV, Pretorius ZA, Hammond-Kosack KE, Pietro AD, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, et al. (2012) The top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol 13(7):414-430

6. Droby S, Wisniewski M, Macarisin D, Wilson C (2009) Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest Biol Technol 52:137–145
7. Fischer-Parton S, Parton R.M, Hickey P.C, Dijksterhuis J, Atkinson HA, Read ND (2000) Confocal microscopy of FM4-64 as a tool for analysing endocytosis and vesicle trafficking in living fungal hyphae. J Microsc 198:246–259
8. Guo L, Breakspear A, Zhao G, Gao L, Kistler HC, Xu JR, Ma LJ (2016) Conservation and divergence of the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway in two plant-pathogenic fungi: Fusarium graminearum and F. verticillioides. Mol Plant Pathol 17:196-209
9. Ji D, Chen T, Ma D, Liu J, Xu Y, Tian SP (2018) Inhibitory effects of methyl thujate on mycelial growth of, botrytis cinerea, and possible mechanisms. Postharvest Biol Technol 142:46-54
10. Jiang C, Zhang X, Liu H, Xu JR (2018) Mitogen-activated protein kinase signaling in plant pathogenic fungi. PLoS Pathog 14(3):e1006875
11. Jin Z, Zhou S, Zhang Y, Ye H, Jiang S, Yu K, Ma Y (2016) Lycorine induces cell death in mm by suppressing janus kinase/signal transducer and activator of transcription via inducing the expression of socs1. Biomed Pharmacother 84:1645-1653
12. Kogure N (2008) New Lycorine-type alkaloid from lycoris traubii and evaluation of antitrypanosomal and antimalarial activities of Lycorine derivatives. Bioorg Med Chem 16(24):10182-10189
13. Li G.; Zhou X.; Xu JR (2012) Genetic control of infection-related development in Magnaporthe oryzae. Curr Opin Microbiol 15:678-684
14. Liu J, Tian SP, Meng XH, Xu Y (2007) Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. Postharvest Biol Technol 44(3):300-306
15. Liu J, Yang Y, Xu Y, Ma C, Qin C, Zhang L (2011) Lycorine reduces mortality of human enterovirus 71-infected mice by inhibiting virus replication. Virol J 8(1):483-483
16. Ma D, Ji D, Zhang Z, Li B, Qin G, Xu Y, Chen T, Tian S (2019) Efficacy of rapamycin in modulating autophagic activity of Botrytis cinerea for controlling gray mold Postharvest Biol Technol 150:158-165
17. Marschall R, Siegmund U, Burbank J, Tudzynski P (2016) Update on nox function, site of action and regulation in Botrytis cinerea. Fungal Biol. Biotechnol 7:8.
18. Mendel F, Henika PR, Mandrell RE (2002) Bactericidal activities of plant essential oils and some of their isolated constituents against campylobacter jejuni, escherichia coli, listeria monocytogenes, and salmonella enterica. J Food Prot 65(10):1545-1560
19. Meng XH, Yang LY, Kennedy JF, Tian SP (2010) Effects of chitosan and oligochitosan on growth of two fungal pathogens and physiological properties in pear fruit. Carbohyd Polym 8:70–75
20. Qin GZ, Zong YY, Chen QL, Hua DL, Tian SP (2010) Inhibitory effect of boron against Botrytis cinerea on table grapes and its possible mechanisms of action. Int J Food Microbiol 138:145–150
21. Schumacher J, Kokkelink L, Huesmann C, Jimenez-Teja D, Collado IG, Barakat R, Tudzynski P, Tudzynski B (2008) The camp-dependent signaling pathway and its role in conidial germination,

growth, and virulence of the gray mold Botrytis cinerea. Mol Plant Microbe Interact 21(11):1443-1459

22. Tian SP, Torres R, Ballester AR, Li BQ, Vilanova L (2016) González-Candelas, L. Molecular aspects in pathogen-fruit interactions: virulence and resistance. Postharvest Biol Technol 122:11–21
23. Turra D, Segorbe D, Di Pietro (2014) A Protein kinases in plant-pathogenic fungi: conserved regulators of infection. Annu Rev Phytopathol 52:267-288
24. Wang K, Jin P, Han L, Shang HT, Tang SS, Rui HJ, Duan YF, Kong FY, Xu K, Zheng YH (2014) Methyl jasmonate induces resistance against penicillium citrinum in chinese bayberry by priming of defense responses. Postharvest Biol Technol 98:90–97
25. Yao H, Tian SP (2005) Effects of pre- and post-harvest application of salicylic acid or methyl jasmonate on inducing disease resistance of sweet cherry fruit in storage. Postharvest Biol Technol 35:253–262
26. Yui S, Mikami M, Kitahara M, Yamazaki M (1998) The inhibitory effect of Lycorine on tumor cell apoptosis induced by polymorphonuclear leukocyte-derived calprotectin. Immunopharmacology 40(2):151-16
27. Zhong J (2005) Amaryllidaceae and Sceletium alkaloids. Nat Prod Rep 22:111–126

Supplemental

Table S1 is not available in this version of the manuscript.

Figures

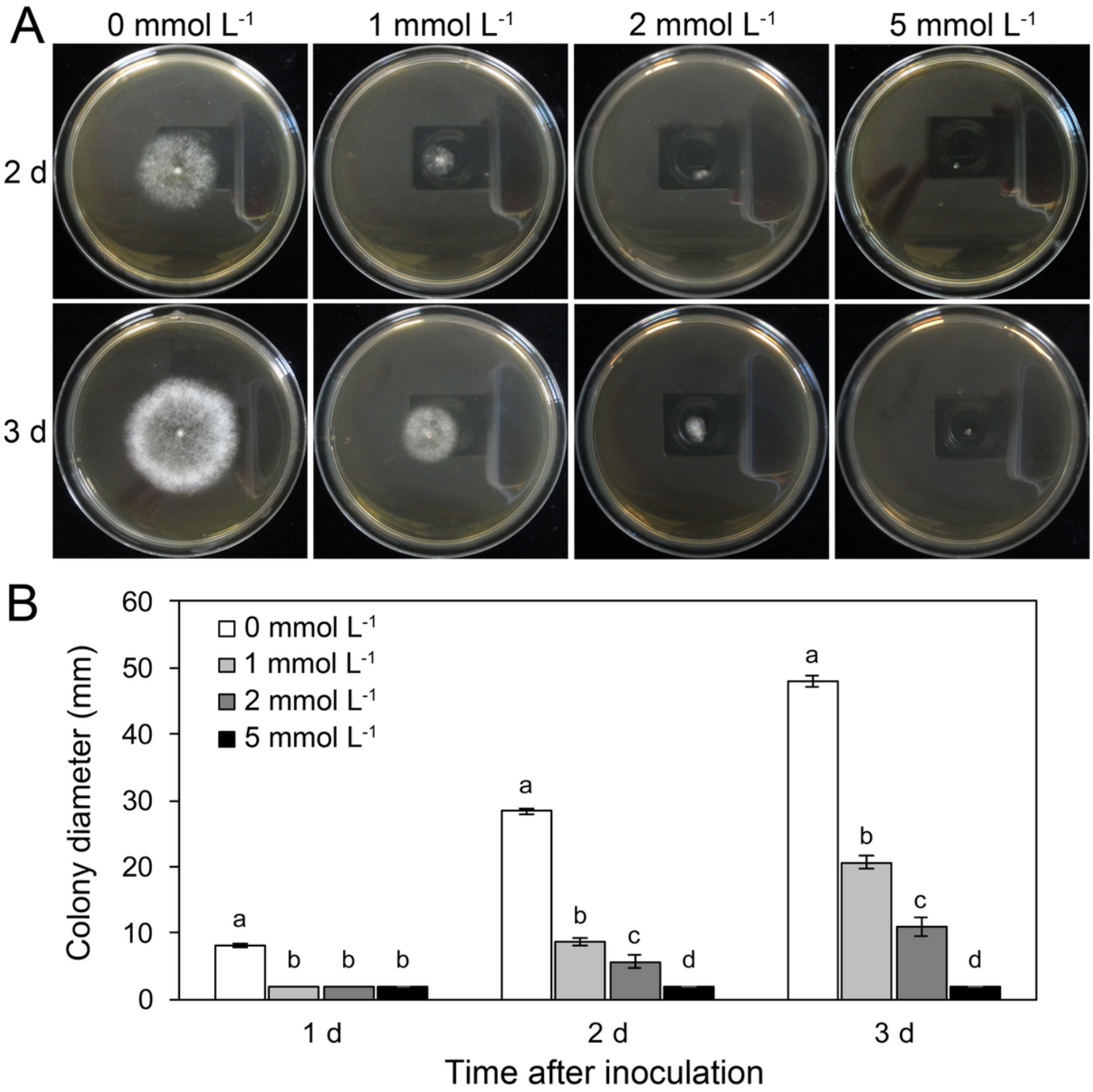

Figure 1

Effect of lycorine on mycelial growth of B. cinerea. (A) In vitro assay for inhibitory effects of lycorine on colony spreading of B. cinerea. (B) Statistical analysis of colony diameter. Data are means ± standard deviations from three replicates. Columns with different letters represent significant difference at each time point according to Duncan's multiple range test at $P < 0.05$.

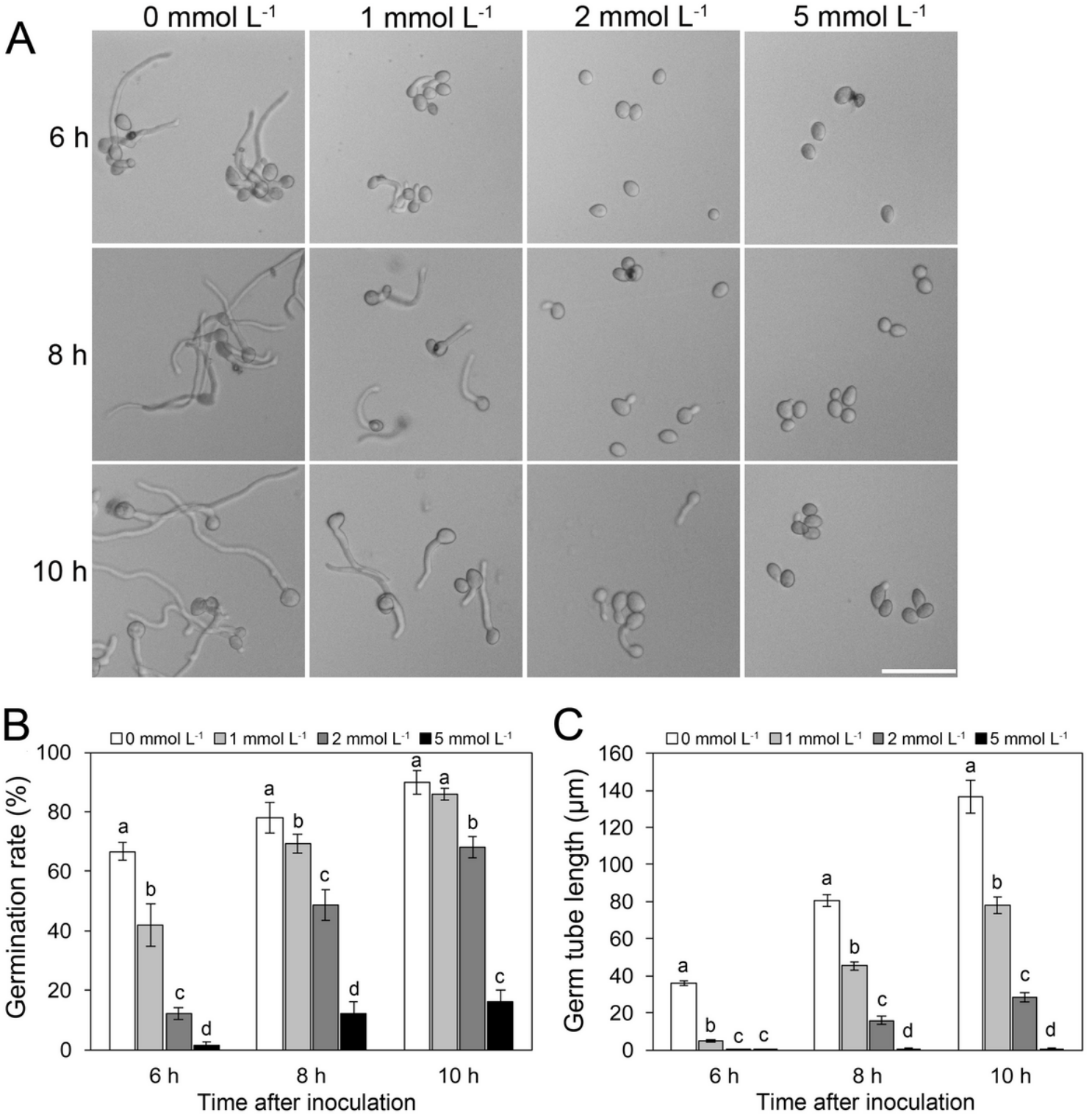

Figure 2

Effect of lycorine on spore germination and germ tube elongation of B. cinerea in potato dextrose broth. (A) Relative photographs of the germinating spore of B. cinerea. (B) Germination rate of B. cinerea under lycorine treatment; (C) Germ tube length of B. cinerea after lycorine treatment. A total of 100 spore were counted to calculate the germination rate and germ tube length. The statistical analysis was performed on triplicates of 100 spore and data are means ± standard deviations from three replicates. Columns with

different letters represent significant difference at each time point according to Duncan's multiple range test at $P < 0.05$. Bar = 50 μm.

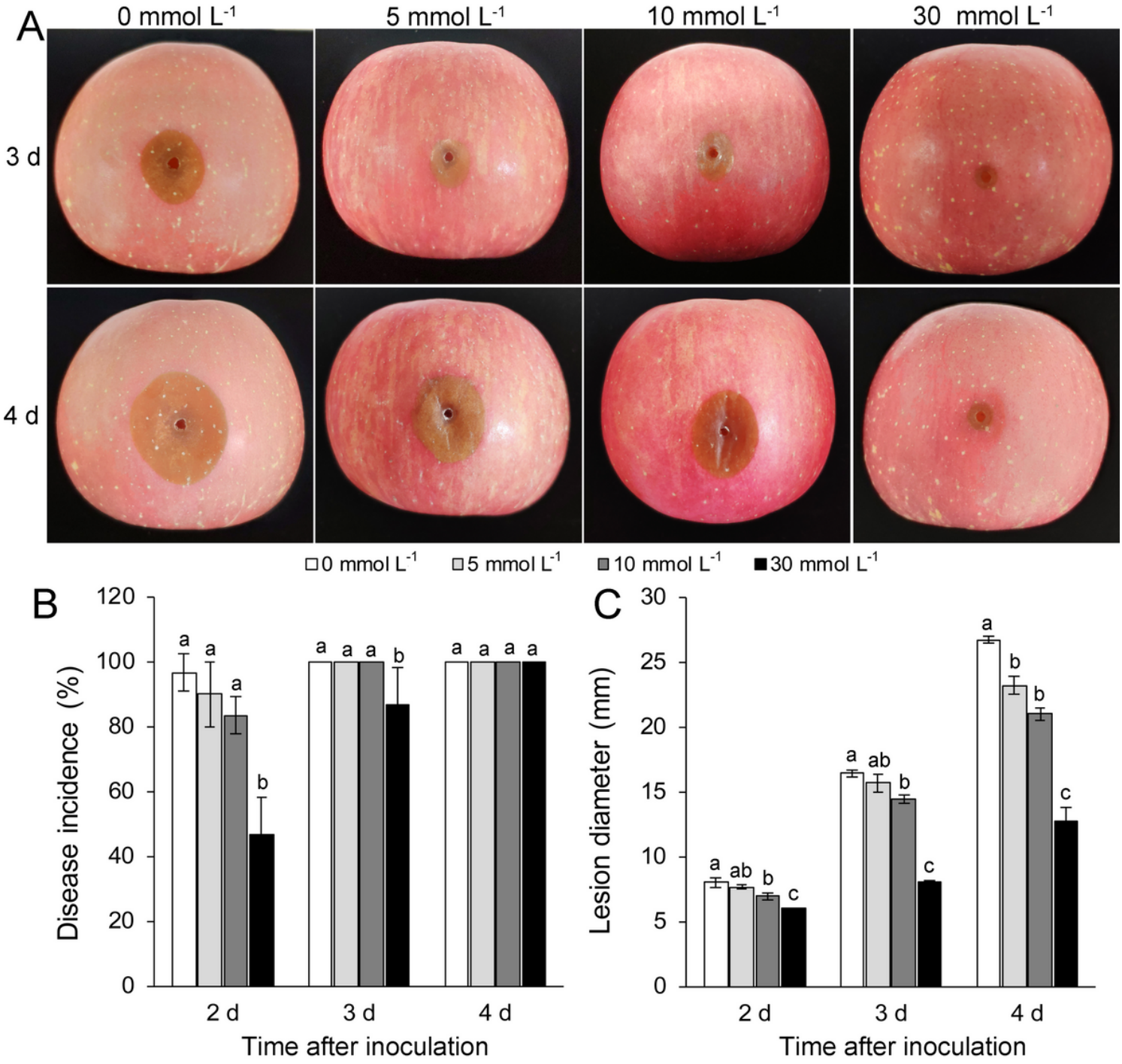

Figure 3

Efficacy of lycorine on disease severity of gray mold caused by B. cinerea on apple fruit. (A) Disease symptom of representative samples of apple fruit on 2, 3- and 4- day post inoculation. (B) Statistical analysis of disease incidence. Data are means ± standard deviations from three group of replicates. Columns with different letters represent significant difference at each time point according to Duncan's multiple range test at $P < 0.05$. (C) Statistical analysis of lesion diameter. Data are means ± standard

errors from three group of replicates. Columns with different letters represent significant difference at each time point according to Duncan's multiple range test at $P < 0.05$.

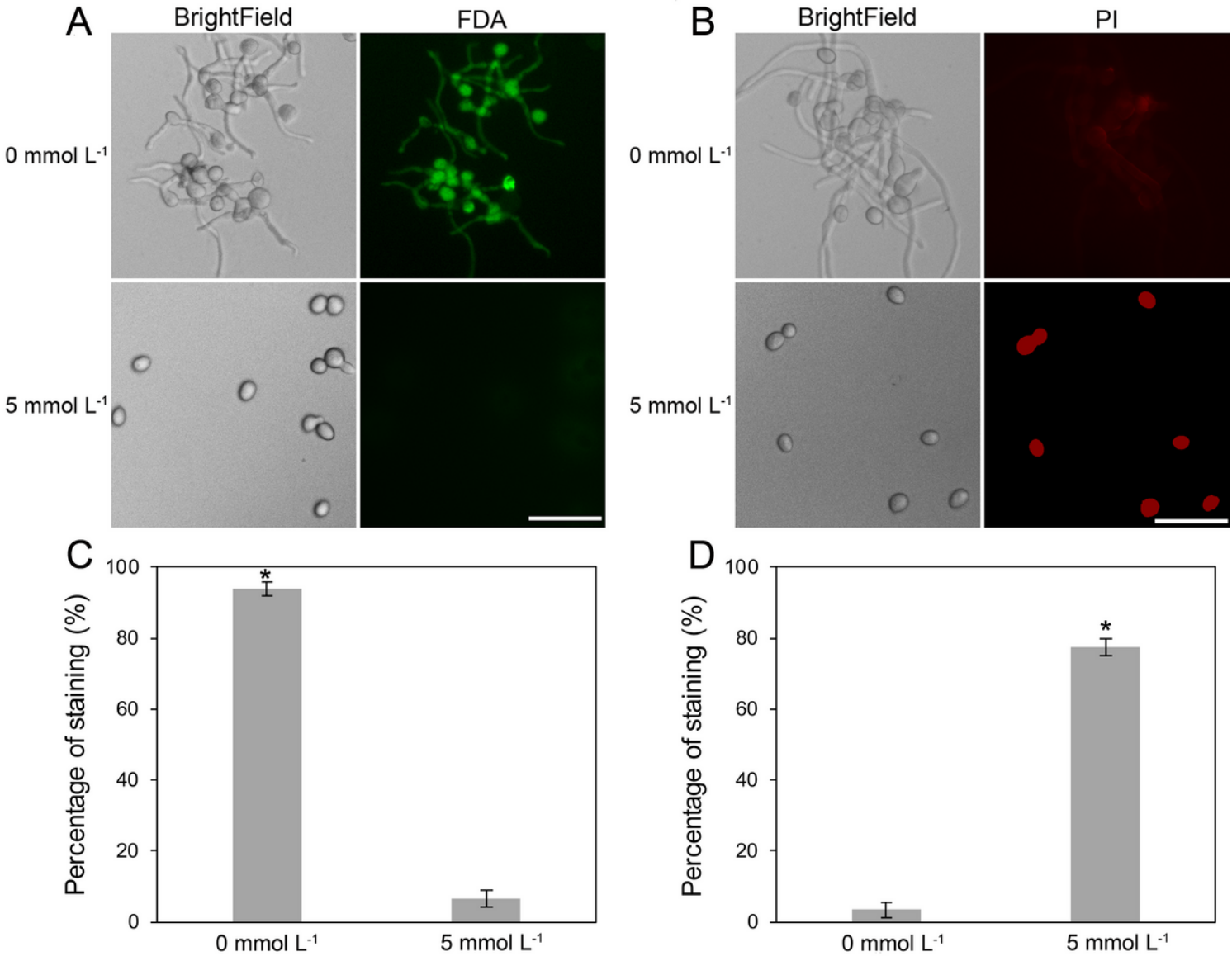

Figure 4

Effect of lycorine on cell viability and plasma membrane integrity of B. cinerea after incubation for 8 h in potato dextrose broth. (A) FDA staining assay. (B) PI staining assay. (C) FDA staining percentage. (D) PI staining percentage. The statistical analysis was performed on triplicates of 100 spore and data are means ± standard deviations from three replicates. Columns with Asterisk symbols (*) represent significant difference according to Duncan's multiple range test at $P < 0.05$. Bar = 50 μm.

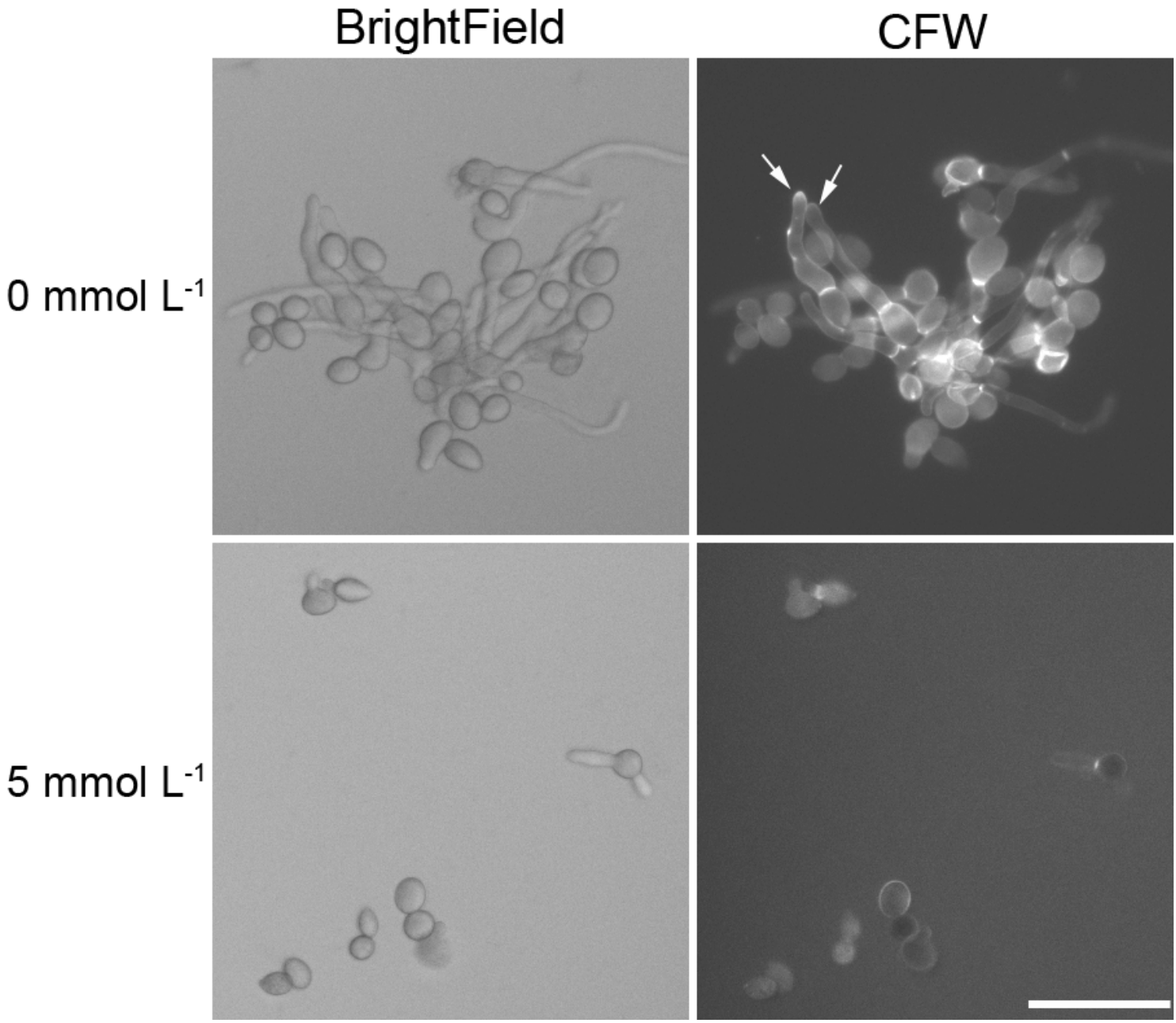

Figure 5

Effect of lycorine on cell wall depositions of B. cinerea after incubation for 8 h in potato dextrose broth. Arrows represent significant enhanced deposition at the apex of germ tube. Bar = 50 μm.

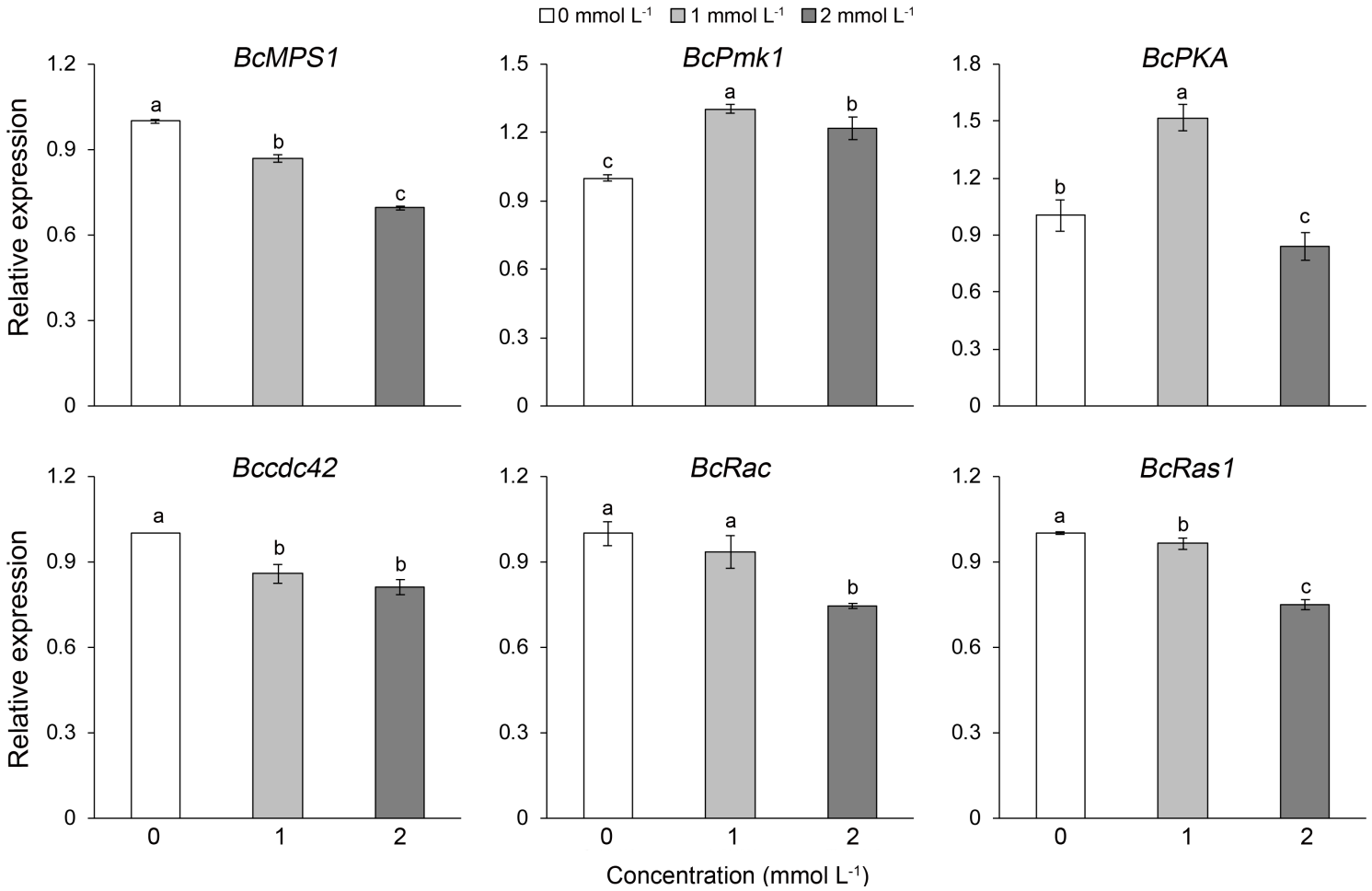

Figure 6

Effect of lycorine on transcription of six MAPK and GTPase coding genes of B. cinerea. Data are means ± standard deviations from three biological replicates. Columns with different letters represent significant difference according to Duncan's multiple range test at $P < 0.05$.