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Fungal endophytes promote the accumulation of Amaryllidaceae alkaloids in *Lycoris radiata*

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Summary

***Lycoris radiata* is a main source of Amaryllidaceae alkaloids; however, the low content of these alkaloids *in planta* remains a limit to their pharmaceutical development and utilization. The accumulation of secondary metabolites can be enhanced in plants inoculated with fungal endophytes. In this study, we analysed the diversity of culturable fungal endophytes in different organs of *L. radiata*. Then, by analysing the correlation between the detectable rate of each fungal species and the content of each tested alkaloid, we proposed several fungal candidates implicated in the increase of alkaloid accumulation. This was verified by inoculating these candidates to *L. radiata* plants. Based on the results of two independent experiments conducted in May 2018 and October 2019, the individual inoculation of nine fungal endophytes significantly increased the total content of the tested alkaloids in the entire *L. radiata* plants. This is the first study in *L. radiata* to show that fungal endophytes are able to improve the accumulation of various alkaloids. Therefore, our results provide insights into a better understanding of interactions between plants and fungal endophytes and suggest an effective strategy for enhancing the alkaloid content in the cultivation of *L. radiata*.**

Introduction

Amaryllidaceae alkaloids are known for their various medicinal activities (Ding *et al*., 2017). For example, galanthamine is a cholinesterase inhibitor and has been licensed for the clinical treatment of Alzheimer's disease; lycorine has anti-neoplastic, anti-malarial, anti-viral, antimicrobial and anti-inflammatory properties; and narciclasine has anti-neoplastic property. However, the chemosynthesis of Amaryllidaceae alkaloids is challenging because of the high cost and low yield, resulting from its complex procedures and mixed stereoisomers (Wang *et al*., 2014). Therefore, being unique producers of Amaryllidaceae alkaloids in nature (Takos and Rook, 2013), plants of Amaryllidaceae remain a major source of these alkaloids for the pharmaceutical industry (Berkov *et al*., 2011; Singh and Desgagné-Penix, 2014).

Among Amaryllidaceae, *Lycoris* is native to the subtropical regions of eastern Asia, from southwestern China to southern Korea and Japan (Liu *et al*., 2012); *Lycoris radiata* is the most widespread species (Hsu, 1994). It has been documented that *L. radiata* contains a greater variety of alkaloids than other *Lycoris* species do (Tian *et al*., 2015) and it is widely used in Asia as a source for Amaryllidaceae alkaloids (Berkov *et al*., 2011). However, the present utilization of Amaryllidaceae alkaloids is limited because of their low contents *in planta* (Singh and Desgagné-Penix, 2014). In this study, the concentration of galanthamine in *L. radiata* plants is less than 0.1% of dry weight. Because of the various medicinal properties of Amaryllidaceae alkaloids, their further clinical development and utilization should not be restricted by their limited availability (Takos and Rook, 2013). Therefore, the development of cultivation methods that might enhance the accumulation of alkaloids in *L. radiata* is of interest.

Ecologically, the accumulation of alkaloids is an important chemical defensive strategy used by plants to adapt to environmental stresses, such as endophytes, pathogens, herbivores and others (Joosten and van Veen, 2011; Matsuura and Fett-Neto, 2017). Fungal endophytes are ubiquitous to nearly all the plants and are characterized by the feature of not causing harm to the host plants (Schulz and Boyle, 2005). The interactions between host plants and fungal endophytes can be explained by the hypothesis of 'balanced antagonism' (Schulz and Boyle, 2005), and plant secondary metabolites play key roles in maintaining this delicate balance (Bednarek, 2012). Actually, plant defensive responses are aroused by perceiving fungal endophytes (Camehl *et al*., 2010; Yuan *et al*., 2019). Then,

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the activated signalling elements, such as reactive oxygen species and phytohormones, trigger the downstream synthetic pathways of secondary metabolites *in planta* (Yuan *et al*., 2016b; Zhai *et al*., 2017). These bioactive secondary metabolites can regulate the growth of fungal endophytes, which help symbiotic interactions to be maintained (Liu and Reinscheid, 2004; Schulz and Boyle, 2005). Therefore, fungal endophytes may play a role as a class of stimuli holding immense potential to enhance the accumulation of valuable secondary metabolites in host plants, such as vindoline in *Catharanthus roseus* (Pandey *et al*., 2016), dragon's blood in *Dracaena cochinchinensis* (Wang *et al*., 2011), and sesquiterpenoids in *Atractylodes lancea* (Yuan *et al*., 2016a). Taken together, it is suggested that fungal endophytes may be exploited and developed to enhance the accumulation of alkaloids in *L. radiata*. However, to the best of our knowledge, no studies have applied fungal endophytes to improve the accumulation of medicinal alkaloids in *L. radiata*; so far, there has been one report to mention a fungal endophyte, *Trichoderma atroviride*, isolated from the bulb of *L. radiata* (Zhou *et al*., 2017). As yet, the diversity of fungal endophytes in *L. radiata* remains unclear, although fungal endophytes are phylogenetically diverse and represent one of the most ubiquitous symbionts on earth.

In this study, our objective was to systemically explore the diversity of culturable fungal endophytes in *L. radiata*, thereby searching for effective fungal endophytes that have positive influences on the accumulation of Amaryllidaceae alkaloids. Also, we propose a strategy for improving the yield of alkaloids in *L. radiata* during the cultivation.

Results

Potential influence of environmental factors on the accumulation of alkaloids in L. radiata

In the biosynthesis of Amaryllidaceae alkaloids, their common precursor is 4′-*O*-methylnorbelladine, and its cyclization via three ways of *C-C* phenol coupling will lead to the formation of three types of alkaloids, including lycorine-type, galanthamine-type and narciclasine-type (Fig. S1; Kilgore and Kutchan, 2016). Therefore, we chose to test five alkaloids (including narciclasine, lycorine, galanthamine, lycoramine and tazettine) that would represent these three types of alkaloids, for profiling the alkaloid distribution in *L. radiata*. As shown in the chromatogram produced by liquid chromatography (LC) (Fig. 1), five peaks in methanol extracts of *L. radiata* had the same retention times as those of corresponding alkaloid standards. Meanwhile, the LC extracts of interest were subjected to mass spectrometry (MS), and each of the five candidate alkaloids was verified by comparing it with a corresponding standard alkaloid. Finally, according to the confirmed data from the LC–MS analysis (Fig. 1), we can conclude that these five Amaryllidaceae alkaloids of interest are present in *L. radiata*.

Because the accumulation of alkaloids is an essential defensive response *in planta* (Joosten and van Veen, 2011), the stimulation caused by microbial invasion and colonization could be involved in this process. Therefore, we tested the potential influences of environmental factors, including microbes from soil and/or air, on the accumulation of the five alkaloids in *L. radiata*. Under our experimental conditions (Fig. 2A), after 14 days of exposure to external environmental factors, the total content of the five alkaloids in the treated *L. radiata* tissue culture plantlets was 1.5-fold higher ($P < 0.01$) than that in the sterile mock counterparts (Fig. 2B). The results indicate that LC analysis is sensitive enough to detect the changes in the content of alkaloids in the plantlets. Compared with other environmental factors, such as herbivores or pathogens, endophytes are intimately associated with host plants. Some reports have described the role of fungal endophytes in the accumulation of secondary metabolites in their host plants (Wang *et al*., 2011; Pandey *et al*., 2016; Yuan *et al*., 2016a), and therefore, the fungal colonization might be one of the key factors promoting alkaloid accumulation in *L. radiata*. In this study, we were interested in and focused on the fungal endophytes in *L. radiata* and further testing their potential for enhancing the accumulation of Amaryllidaceae alkaloids.

Composition of the isolated fungal endophytes in L. radiata

A total of 108 fungal strains were isolated from different organs of *L. radiata* plants growing in Nanjing Botanical Garden Mem. Sun Yat-Sen using the culture-dependent method (Saunders and Kohn, 2009). Of these fungal endophytes, there were 22 strains named as Lr**S**F1-22 obtained from **S**capes, 16 strains as Lr**L**F1-16 from **L**eaves, 55 strains as Lr**B**F1-55 from **B**ulbs and 15 strains as Lr**R**F1-15 from **R**oots. However, we were not able to isolate fungal endophytes from flowers, which include the flower stalks, receptacles, sepals, petals, pistils and stamens. Because we were able to isolate bacterial endophytes from the flower of *L. radiata* in other tests (data not shown), the sterilization process used in this study was applicable and reliable. Hence, flowers were excluded in our subsequent experiments. In addition, there were three fungal strains LrSF19, LrLF2 and LrRF5 that we were not able to culture after isolation; therefore, these strains were also excluded.

To identify each fungal strain, we analysed its genetic background, based on the fungal genomic DNA Basic Local Alignment Search Tool (BLAST) search. All the strains were determined as fungal species since the

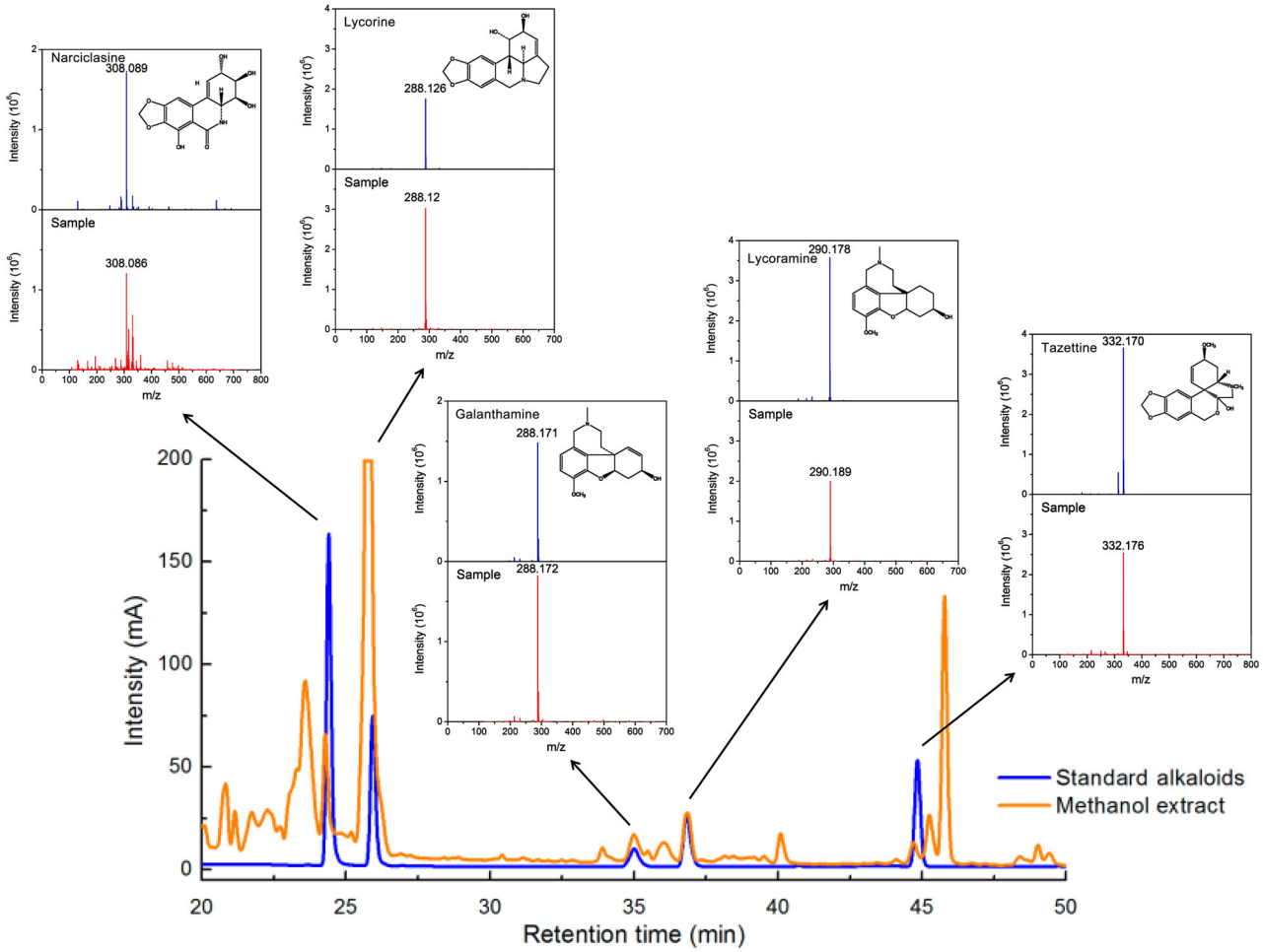

Fig. 1. Liquid chromatogram of the methanol extract of *L. radiata* and the standard Amaryllidaceae alkaloid mixture containing narciclasine, lycorine, galanthamine, lycoramine and tazettine. Embedded graphs showing the individual mass spectrum of the five Amaryllidaceae alkaloids in the methanol extract and their corresponding standard. [Color figure can be viewed at wileyonlinelibrary.com]

sequences of the internal transcribed spacer (ITS) region had a high degree of similarity with corresponding sequences deposited in the GenBank database (Table S1). The similarity of ITS sequences from the fungal endophytes ranged from 85% to 100%. In addition, the fungal endophytes were further identified according to their morphological characteristics, by observing fungal colonies, mycelia and spores and comparing these characteristics with those of reported fungal species (Table S1). Then, the fungal endophytes isolated from *L. radiata* were confirmed using both molecular and morphological identification. Therefore, the endophytes were classified into 27 species (Fig. 3).

As shown in Fig. 3, the fungal species of *Gibberella intermedia* and *Fusarium proliferatum* were predominant, occupying 19.05% and 18.10% of all the fungal endophytes respectively. Another 13 fungal species were isolated at least twice in *L. radiata*. Twelve species were isolated only once (Fig. 3). Ten fungal species were verified from scapes, nine were from leaves, 12 were from bulbs and 9 were from roots (Fig. 4C). However, we did not find any of the obtained fungal species appearing in all organs of *L. radiata*. Our observations showed that fungal endophytes in *L. radiata* were phylogenetically diverse and their distribution was uneven in different organs.

Diversity of the isolated fungal endophytes in different organs

In total, 27 species of fungal endophytes were identified (Fig. 3) and these belonged to 14 genera (Fig. 4A); the fungal composition in different organs of *L. radiata* was further compared at the genus level (Fig. 4A). As shown in Fig. 4B, the fungal endophytes were distributed unevenly across the different plant organs. Only *Fusarium* and *Gibberella* were observed in all organs of *L. radiata*. Half of the identified fungal genera were only

observed from one specific organ. *Sarocladium* was exclusively found from the scape; *Stagonosporopsis* and *Glomerella* were found from the leaf; *Phoma* was found from the bulb; and *Galactomyces*, *Metacordyceps* and *Diaporthe* were found from the root (Fig. 4A). The results for *L. radiata* are consistent with the perspective that the composition of fungal endophytes in different organs of most plants is quite different (Kumar and Hyde, 2004; Mishra *et al*., 2012; Wearn *et al*., 2012; Clay *et al*., 2016; Potshangbam *et al*., 2017). It has already been explained that some defensive factors *in planta*, such as plant secondary metabolites, can regulate the colonization and growth of fungal endophytes in different plant organs (Saunders and Kohn, 2009).

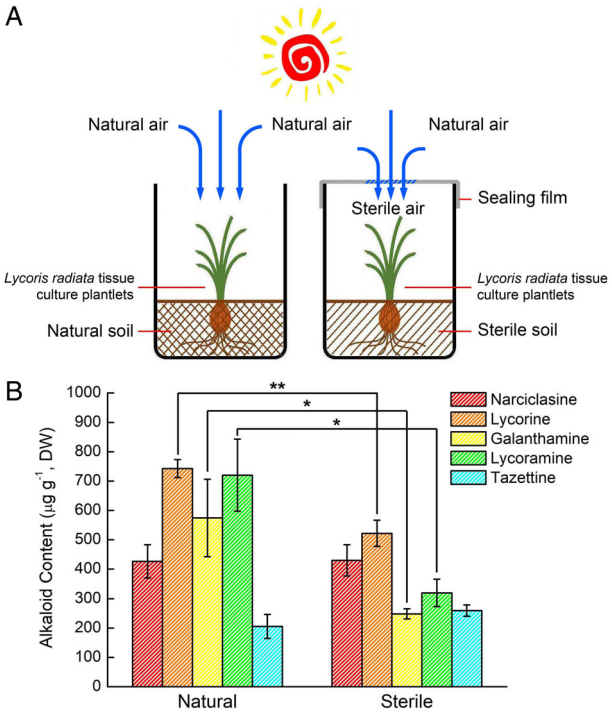

Fig. 2. Schema showing the experimental design for testing the influence of environmental factors on the accumulation of alkaloids in *L. radiata* (A) and the corresponding results (B). Results are means from three biological replicates. Error bars indicate standard deviations. Double asterisks indicate a significant difference at $P < 0.01$. Single asterisk indicates a significant difference at $P < 0.05$. The quantification of alkaloids in *L. radiata* was conducted with HPLC. [Color figure can be viewed at wileyonlinelibrary.com] [Color figure can be viewed at wileyonlinelibrary.com]

We isolated 55 strains from bulbs, which harboured substantially more fungal endophytes than other organs did. In fact, our data indicated that these 55 strains represented 12 fungal species and the frequency of appearance in terms of fungal species in bulbs was very similar to those in other organs (Fig. 4C). Additionally, the fungal diversity in different organs was compared using alpha diversity indices as statistical assessments (Yang *et al*., 2016). Such alpha diversity indices, including the Shannon–Wiener, Margalef and Pielou Indices, were calculated based on the isolating numbers of fungal strains as well as species in each organ. Our data demonstrated that there were no obvious differences in alpha diversity in different organs (Fig. 4D). Taken together, it was suggested that with respect to the detectable rates of the fungal species and strains, the fungal diversity in different organs of *L. radiata* was similar, although the compositions were quite different.

Correlations between fungal endophytes and alkaloids

We measured the contents of narciclasine, lycorine, galanthamine, lycoramine and tazettine in different organs of wild *L. radiata*. All five alkaloids of interest were measurable in the tested organs (scape, leaf, bulb and root) (Fig. 5). Moreover, it was clear that the content of different alkaloids varied considerably in different organs; this has also been observed in other Amaryllidaceae plants (Lubbe *et al*., 2013; Reis *et al*., 2019). In *L. radiata*, leaves and roots contained significantly more alkaloids of interest than scapes and bulbs. As shown in Fig. 4B, leaves and roots contained more exclusive fungal genera than scapes and bulbs. Thus, we postulated that the existence of some special fungal endophytes

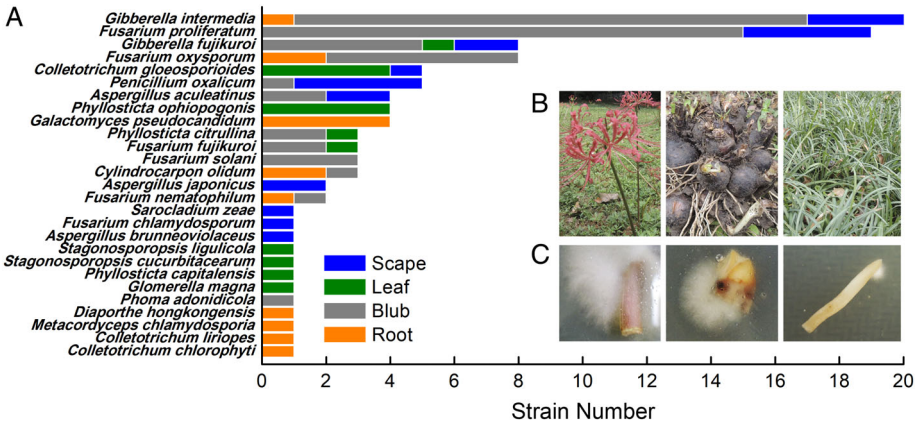

Fig. 3. Fungal species isolated from *L. radiata*. A. Distribution of fungal species in different organs. B. Different organs of *L. radiata* collected for fungal isolation. C. Fungal colony grown from the cuts of plant tissues. [Color figure can be viewed at wileyonlinelibrary.com]

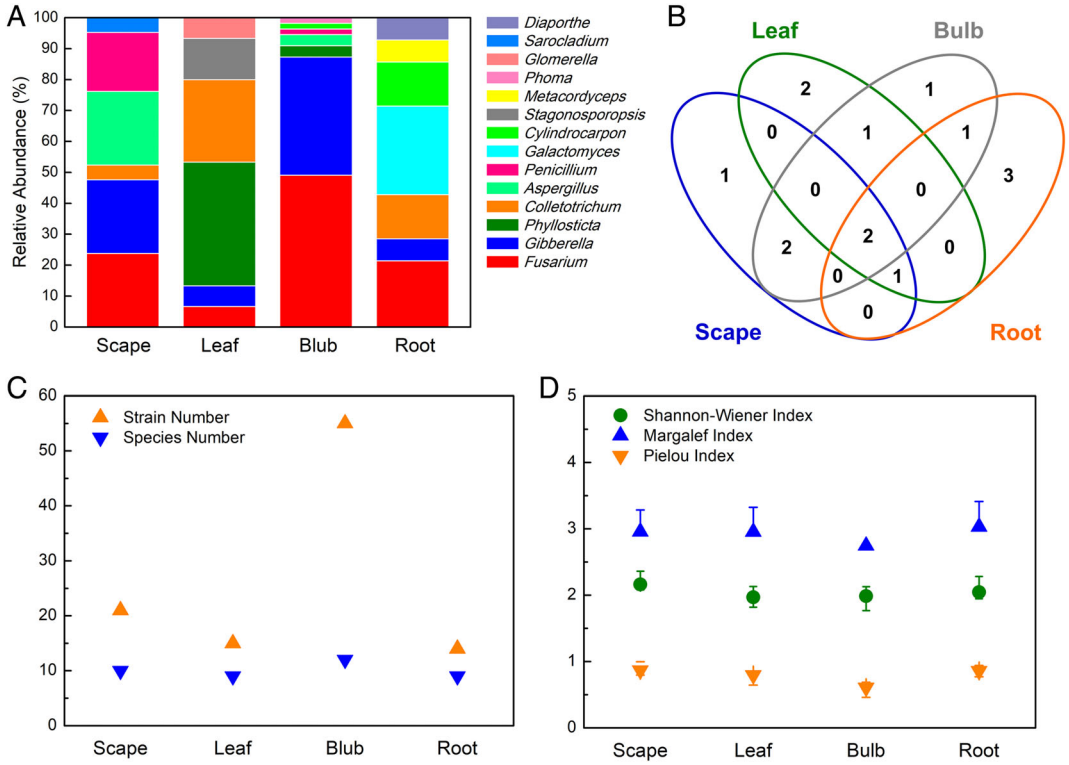

Fig. 4. Distribution pattern and alpha diversity of fungal endophytes in different organs of *L. radiata*. A. The relative abundance of different fungal genera in scape, leaf, bulb and root of *L. radiata*. B. Venn diagram of fungal genera in different organs, showing the number of shared and exclusive genera in organs. C. The number of fungal strains and species in different organs. D. Shannon–Wiener diversity index, Margalef richness index and Pielou evenness index calculated based on the number of fungal strain and species in each organ. [Color figure can be viewed at wileyonlinelibrary.com]

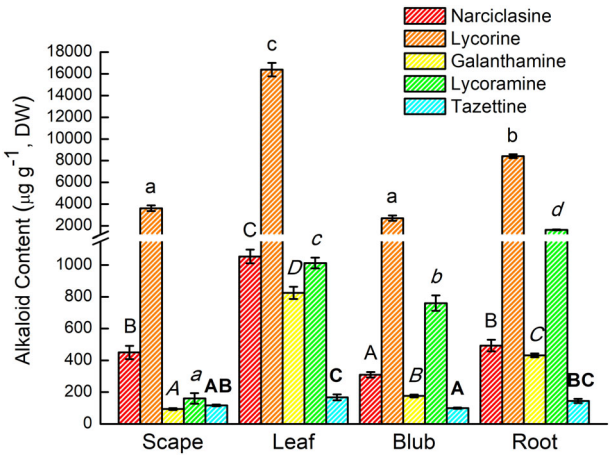

Fig. 5. Contents of five Amaryllidaceae alkaloids in different organs of *L. radiata* plants collected from Nanjing Botanical Garden Mem. Sun Yat-Sen. The quantification of alkaloids in *L. radiata* was conducted with HPLC. Results are means from three biological replicates. Error bars indicate standard deviations. Significant differences ($P < 0.05$) among different alkaloids across plant organs are indicated with different types of letters. [Color figure can be viewed at wileyonlinelibrary.com]

might be one of the environmental factors that induce the accumulation of alkaloids in these plant organs.

Furthermore, given that the distribution patterns and isolation rates of different fungal species, as well as the contents of different alkaloids were variable in different organs of *L. radiata* (Figs 3 and 5), we wondered whether the existence of some specific fungal endophytes would enhance the accumulation of alkaloids in *L. radiata*. Correlation analysis has been applied to reveal interactions between hosts and their microbiome (Yang *et al*., 2016; Valles-Colomer *et al*., 2019); we, therefore, analysed the correlations of the distribution patterns between fungal endophytes and alkaloids in different organs of *L. radiata*, and then searched for fungal endophytes that could be involved in beneficial influences on the accumulation of Amaryllidaceae alkaloids.

As shown in Fig. 6, there was a positive correlation between the presence of some fungal endophytes and alkaloid accumulation. The correlation coefficients are presented in Table S2. Briefly, we found that six fungal species, *Glomerella magna*, *Phyllosticta capitalensis*, *Stagonosporopsis cucurbitacearum*, *Stagonosporopsis ligulicola*, *Phyllosticta ophiopogonis* and *Colletotrichum gloeosporioides*, showed significantly positive correlations with four of the five tested alkaloids (narciclasine, lycorine, galanthamine and tazettine), and another six fungal species, *Colletotrichum liriopes*, *Colletotrichum chlorophyti*, *Metacordyceps chlamydosporia*, *Diaporthe hongkongensis*, *Galactomyces pseudocandidum* and

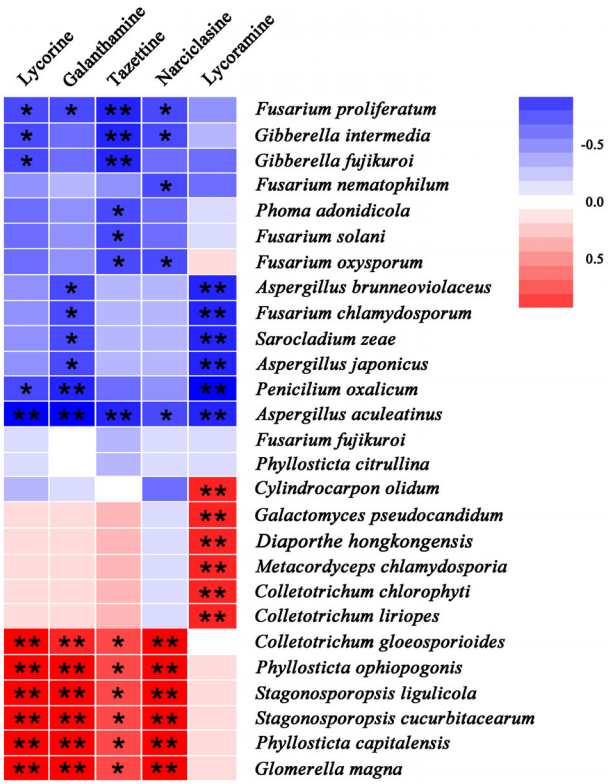

Fig. 6. Bivariate correlations between fungal species and Amaryllidaceae alkaloids. The colour intensity for each block represents the correlation between each of the fungal species and the content of each alkaloid. [Color figure can be viewed at wileyonlinelibrary.com]

Cylindrocarpon olidum, showed significantly positive correlations with lycoramine. In general, these observations suggested that some fungal endophytes have the potential to promote the accumulation of different alkaloids in *L. radiata*.

Promotion of fungal endophytes to the accumulation of alkaloids in L. radiata *plants*

To further verify whether the fungal endophytes showing significantly positive correlations with Amaryllidaceae alkaloids could have beneficial influences on the accumulation of alkaloids *in planta*, bulbs of *L. radiata* of similar size were collected in May 2018 and October 2019, and were cultivated in flowerpots for 2 months; then, individual fungal species of interest were inoculated to the *L. radiata* plants under controlled conditions (Fig. S2). At 14 days post-inoculation, we measured the influence of each fungal species of interest on the growth of *L. radiata* plants. For the pot experiment in May 2018, there were negative influence of inoculation with *C. gloeosporioides* LrLF6, *P. ophiopogonis* LrLF16, *D. hongkongensis* LrRF11 or *C. chlorophyti* LrRF15 on the dry weight of *L. radiata* plants; however, for the pot experiment in October 2019, the negative influences of these fungal endophytes were not observed and each of fungal strains in test led to little change of plant biomass (Fig. S3). It was speculated that the negative influence of some fungal endophytes on plant growth may be a random phenomenon, which may be influenced by the growth and physiological status of different plants; thus, when the number of biological replicates was increased from three (May 2018) to five (October 2019), it was no longer observed.

Later, we observed that fungal endophytes showing significantly positive correlations with Amaryllidaceae alkaloids enhanced the accumulation of alkaloids in different organs of the inoculated *L. radiata* plants in both pot experiments conducted in May 2018 and October 2019 (Table S3). Moreover, it was observed that, in this condition, some fungal endophytes could enhance the alkaloid accumulation, not only in the organ where they had been originally isolated but also in other organs (Fig. 3 and Table S3). It might be explained from three aspects: first, fungal endophytes could translocate among organs *in planta*, so they could function in organs other than the organ where they had been isolated (Hiruma *et al*., 2016); then, the fungal colonization could activate long-distance signalling cascades, such as reactive oxygen species or hormones, which could transmit to other organs and might target genes involved in the Amaryllidaceae alkaloid biosynthesis pathways (Pandey *et al*., 2016; Yuan *et al*., 2016b); finally, the newly synthesized alkaloids as the response of fungal colonization in one plant organ may be transported to other organs *in planta* (Shitan *et al*., 2003).

To address relations between the inoculated fungal species of interest and the accumulation of alkaloids, we analysed the results with respect to the alkaloid content of whole plants. As shown in Fig. 7A, combined results obtained from the two independent experiments conducted in May 2018 and October 2019, nine fungal strains, including *C. olidum* LrBF4, *P. capitalensis* LrLF1, *S. cucurbitacearum* LrLF4, *C. gloeosporioides* LrLF6, *G. magna* LrLF12, *S. ligulicola* LrLF13, *P. ophiopogonis* LrLF16, *M. chlamydosporia* LrRF8 and *G. pseudocandidum* LrRF9, were able to significantly increase the total content of the tested alkaloids. Compared with non-inoculated plants, the total content of the tested alkaloids of the plants inoculated with these strains was 18.6%, 23.4%, 28.3%, 9.2%, 25.8%, 16.9%, 10.2%, 11.9% and 15.1% greater, respectively. These results indicated that most of the fungal species positively correlated with the alkaloid accumulation (Fig. 6) were able indeed to increase the content of alkaloids in the *L. radiata* plants (Fig. 7A). Actually, as shown in Fig. 7B–F, fungal strains highlighted by five-pointed stars were species that had significantly positive

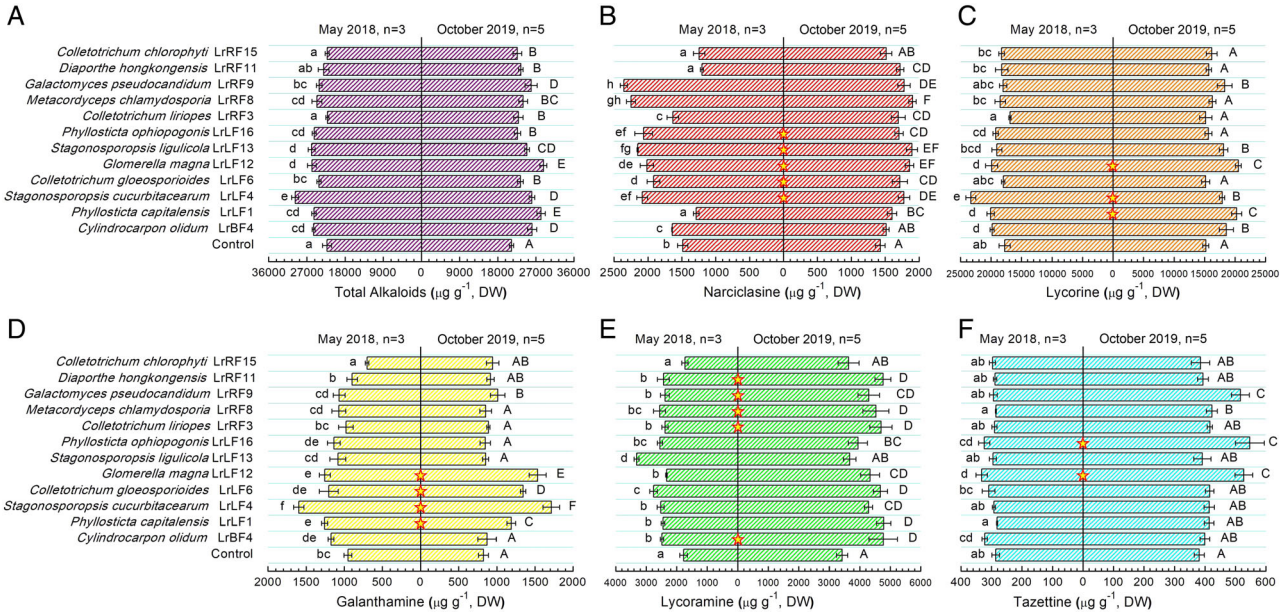

Fig. 7. Influence of different fungal endophytes on the accumulation of alkaloids in *L. radiata* plants. A. The total content of the five alkaloids of interest. B. Content of narciclasine. C. Content of lycorine. D. Content of galanthamine. E. Content of lycoramine. F. Content of tazettine. Results are the means from three biological replicates for the May 2018 experiment and five replicates for the October 2019 experiment. Error bars indicate standard deviations. Significant differences ($P < 0.05$) in different fungal inoculations are indicated by different lowercase letters (May 2018) or different uppercase letters (October 2019). Five-pointed stars highlight fungal species that had significantly positive correlations with corresponding alkaloids and significantly increased the content of these alkaloids. The quantification of alkaloids in *L. radiata* was conducted with HPLC. [Color figure can be viewed at wileyonlinelibrary.com]

correlations ($P < 0.05$) with corresponding alkaloids and then significantly increased the content of these alkaloids ($P < 0.05$) in both pot experiments conducted in May 2018 and October 2019. Importantly, we found that all these patterns were about closely in match with the result of correlation analysis (Fig. 6). In detail, five of the six inoculants (*S. cucurbitacearum*, *C. gloeosporioides*, *G. magna*, *S. ligulicola* and *P. ophiopogonis*) demonstrated to have a significantly positive correlation with narciclasine, could significantly increase the corresponding narciclasine content in *L. radiata* plants compared with the control (Fig. 7B); three of the six inoculants (*P. capitalensis*, *S. cucurbitacearum*, and *G. magna*) positively correlated with lycorine, could significantly increase the lycorine content in plants (Fig. 7C); four of the six inoculants (*P. capitalensis*, *S. cucurbitacearum*, *C. gloeosporioides* and *G. magna*) positively correlated with galanthamine, could significantly increase the galanthamine content (Fig. 7D); five of the six inoculants (*C. olidum*, *C. liriopes*, *M. chlamydosporia*, *G. pseudocandidum* and *D. hongkongensis*) positively correlated with lycoramine, could significantly increase the lycoramine content (Fig. 7E); and two of the six inoculants (*G. magna* and *P. ophiopogonis*) positively correlated with tazettine, could significantly increase the tazettine content *in planta* (Fig. 7F). Thus, it may be suggested that analysing the correlations between fungal endophytes and alkaloid contents could be a feasible method in searching for candidate fungal endophytes able to enhance the accumulation of alkaloids in *L. radiata*.

Furthermore, both experiments in May 2018 and October 2019 showed that the influences of different fungal endophytes on the accumulation of alkaloids could be various, implying various mechanisms implicated in the alkaloid accumulation induced by fungal endophytes, which deserves further explorations.

Colonization of fungal endophytes in L. radiata

To figure out whether the beneficial influences of fungal endophytes on the Amaryllidaceae alkaloid accumulation were due to their endophytic colonization or just their physical contacts to plant cells, we re-isolated the corresponding fungal endophyte from the sterile tissue culture plantlets of *L. radiata* inoculated with the specific strain. By doing so, we could rule out the obstacle caused by the original microbiome in *L. radiata*. The tested fungal strains included *C. olidum* LrBF4, *P. capitalensis* LrLF1, *S. cucurbitacearum* LrLF4, *C. gloeosporioides* LrLF6, *G. magna* LrLF12, *S. ligulicola* LrLF13, *P. ophiopogonis* LrLF16, *C. liriopes* LrRF3, *M. chlamydosporia* LrRF8, *G. pseudocandidum* LrRF9, *D. hongkongensis* LrRF11 and *C. chlorophyti* LrRF15. All the tested fungal strains were successfully re-isolated from the inoculated *L. radiata* plantlets and were identified by comparing their

morphological characteristics with each of the original strains (Fig. S4). All the tested fungal endophytes could colonize the bulbs of the plantlets. Since the fungal endophytes were inoculated besides bulbs, fungal mycelia would preferentially penetrate the epidermis of bulbs, then translocate into other organs *in planta*. It was also observed in a study by Hiruma *et al*. (2016).

Discussion

Lycoris radiata is one of the unique producers and a major source of Amaryllidaceae alkaloids, which have various medicinal activities (Ding *et al*., 2017). However, the availability of Amaryllidaceae alkaloids is limited because of their very low content *in planta*. Previously, Wang *et al*. (2010) extracted only 60 g of alkaloids from 15 kg of dried bulbs of *L. radiata*, which was about equal to the alkaloid content detected in this study (~4.056 mg g^{-1} in the dried bulbs of wild *L. radiata*). As one essential defensive response, the accumulation of plant secondary metabolites can be enhanced by abiotic or biotic stimuli, including the influence of fungal endophytes (Wang *et al*., 2011; Pandey *et al*., 2016; Yuan *et al*., 2016a). Thus, inoculation with fungal endophytes may be a strategy that can be used to promote the accumulation of Amaryllidaceae alkaloids. As a perennial herb, *L. radiata* possesses many opportunities to harbour abundant fungal endophytes. In this study, we investigated the occurrence of fungal endophytes on wild *L. radiata* (Figs 3 and 4), and its correlation with the accumulation pattern of Amaryllidaceae alkaloids in different organs (Fig. 6); then, we inoculated fungal endophytes of interest to *L. radiata* plants (Fig. S2) and demonstrated that several fungal endophytes were able to promote the accumulation of the alkaloids of interest (Fig. 7).

In this study, a total of 105 fungal strains belonging to 14 of genera have been confirmed from the scape, leaf, bulb and root of healthy *L. radiata* (Figs 3 and 4). Among these fungal strains, members in the genera *Fusarium*, *Gibberella*, *Phyllosticta*, *Colletotrichum*, *Aspergillus*, *Penicillium*, *Phoma*, *Glomerella* and *Diaporthe* are widely distributed in nature and have a high spore production (Mishra *et al*., 2012), which give them more chances to colonize a wide range of plants (Schulz and Boyle, 2005; Aly *et al*., 2011; Zheng *et al*., 2016). Moreover, species in *Galactomyces* (Clay *et al*., 2016), *Cylindrocarpon* (Wearn *et al*., 2012), *Stagonosporopsis* (Cui *et al*., 2015) and *Sarocladium* (Potshangbam *et al*., 2017) have been observed as fungal endophytes in specific plants. In particular, *Penicillium* sp. reported to be isolated from the bulb of *Lycoris aurea* (Luo *et al*., 2011) was also observed in this study (Fig. 3). Whereas, other fungal endophytes reported previously, including *Trichoderma* sp. from the bulb of *L. radiata* (Zhou *et al*., 2017) and *Mucor* sp. from the bulb of *L. aurea* (Yang *et al*., 2010), were not repeatable in our observation, indicating that fungal assemblages *in planta* may be influenced by comprehensive factors, such as infection pattern, plant age and environmental conditions (Aly *et al*., 2011). Notably, *Metacordyceps* sp., which has been reported to exist in the rhizosphere (Shi *et al*., 2019) is reported as a plant–fungal endophyte for the first time in this study. It may exist in the rhizosphere of *L. radiata* and colonize the plant occasionally.

The interaction between plants and endophytes has been described as a balanced symbiotic continuum ranging from mutualism through commensalism to latent pathogenicity (Schulz and Boyle, 2005). Fungal lifestyles could change in response to host internal signals or environmental factors (Rai and Agarkar, 2014). As expected, some fungal endophytes isolated in *L. radiata* have been reported as latent pathogens in other plants, such as members in the genera *Fusarium*, *Phyllosticta*, *Colletotrichum*, *Phoma* and *Diaporthe* (Biggs, 1995; Rodriguez and Redman, 2008; Wikee *et al*., 2013; Demers *et al*., 2015; Sessa *et al*., 2018). We assumed that these latent pathogens could exhibit an endophytic lifestyle in *L. radiata*, because of the existence of alkaloids having antimicrobial activity *in planta* (Liu and Reinscheid, 2004; Bednarek, 2012; Pusztahelyi *et al*., 2015). Amaryllidaceae alkaloids might play a key role in maintaining a delicate balance between *L. radiata* and fungal endophytes.

The preference of fungal endophytes to stay in different organs was observed in *L. radiata* (Fig. 4); similar results have been described in many other plants (Kumar and Hyde, 2004; Mishra *et al*., 2012; Wearn *et al*., 2012; Clay *et al*., 2016; Potshangbam *et al*., 2017). This may be because the diverse physiological status of different organs leads to different conditions for fungal endophytes (Aly *et al*., 2011). For example, plant roots spread in soil that is an important reservoir for fungal endophytes (Griffin and Carson, 2018); thus, in this study, there were more fungal genera found exclusively in the roots than in other organs. Moreover, the colonization rates of fungal endophytes were similar in the scape, leaf and root but were much higher in the bulb (Fig. 4C). Possibly, this could be explained by the fact that the scape and leaf are annual, whereas the bulb is perennial (Zheng *et al*., 2016). Moreover, the bulb provides more space and carbohydrates for fungal colonization (Iqbal *et al*., 2006).

As one response to biotic stimuli, some studies have reported enhanced secondary metabolism after inoculation with fungal endophytes; such as *vindoline* in *C. roseus* inoculated with *Curvularia* sp. and *Choanephora infundibulifera* (Pandey *et al*., 2016), dragon's blood in *D. cochinchinensis* with *F. proliferatum*

(Wang *et al*., 2011), and sesquiterpenoids in *A. lancea* with *Gilmaniella* sp. (Yuan *et al*., 2016a). In this study, both the fungal composition (Fig. 4A) and the alkaloid content (Fig. 5) varied in different organs of *L. radiata*. Moreover, the leaf and root, which harboured more exclusive fungal species than other organs (Fig. 4B), had a much higher content of alkaloids (Fig. 5). Thus, the existence of some special fungal species might be one key factor that induced the accumulation of alkaloids in *L. radiata*. Therefore, correlation analysis was employed to assess the potential influence of each fungal species on the alkaloid accumulation (Fig. 6). We found that 12 fungal species had significant positive correlations with at least one of the alkaloids tested. Furthermore, most of these fungal endophytes could significantly promote the accumulation of the corresponding alkaloids in the *L. radiata* plants (Fig. 7), that is to say, most patterns were about closely in match with the result of correlation analysis (Fig. 6). These results indicate that the correlation analysis is a feasible and convenient method to identify fungal endophytes to promote the accumulation of alkaloids in *L. radiata*. Of course, it should not be overlooked that rather than being the cause of the accumulation, some fungal endophytes that were positively correlated with one or more of the alkaloids tested, rather are adapted to and coexist with the secondary metabolites used as defences by host plants (Liu and Reinscheid, 2004; Huang *et al*., 2008).

In this study, the effects of different fungal endophytes on the accumulation of alkaloids in *L. radiata* were quite different (Fig. 7); this may be because the mechanisms underlying the accumulation of secondary metabolites induced by fungal endophytes could depend on the endophyte and/or plant species (Zhai *et al*., 2017). Diverse fungal elicitors could be recognized by pattern recognition receptors on plant cell membranes (Chen *et al*., 2016) and then different signalling cascades would be activated, which will further enhance secondary metabolite synthesis (Hao *et al*., 2010; Yuan *et al*., 2016b; Zhai *et al*., 2017). Since we observed that some fungal endophytes promoted the accumulation of alkaloids, not only in the organ where they had been originally isolated but also in other organs of *L. radiata* (Table S3), the results indicate that the fungal colonization was involved in initiating certain long-distance signalling cascades, which later regulated the alkaloid synthesis in other locations. Moreover, we observed that in the pot experiment in May 2018, the inoculation of *P. ophiopogonis* LrLF16 significantly decreased the dry weight of *L. radiata* plants and enhanced the accumulation of all the tested alkaloids (Fig. 7 and Fig. S3), which hints that the reduced biomass may condense the alkaloids *in planta* in some cases. Except for direct influences of fungal endophytes on the accumulation of alkaloids in *L. radiata* plants, the colonization of fungal endophytes may influence the composition and then the function of the original microbiome *in planta*, which may further influence host plants (Zhang *et al*., 2019). Generally, the mechanisms underlying the accumulation of alkaloids in *L. radiata* induced by fungal endophytes are complex and require further study.

The synthesis of secondary metabolites has ecological importance, as a strategy applied by plants to resist pathogenic invasion (Pusztahelyi *et al*., 2015) or regulate endophytic growth (Liu and Reinscheid, 2004; Huang *et al*., 2008; Saunders and Kohn, 2009). For instance, inoculation with endophytic *Gilmaniella* sp. enhanced the production of phenylpropanoids and terpenoids but downregulated the expression of defence-related genes in its host plant *A. lancea*, so the accumulation of plant secondary metabolites would function as a defensive strategy applied by the host plant to compensate for the weakening of plant immunity caused by fungal colonization (Yuan *et al*., 2019). In this study, most fungal endophytes enhanced the accumulation of alkaloids in *L. radiata* but had little influence on the plant growth (Fig. 7 and Fig. S3). This may be because, as a defence response, the accumulation of secondary metabolites induced by endophytes may be enhanced at the expense of plant growth (Karasov *et al*., 2017; Zhou *et al*., 2018).

In summary, we isolated fungal endophytes in *L. radiata* and described their distribution pattern in different organs. Combined with the distribution pattern of Amaryllidaceae alkaloids of interest in *L. radiata*, the correlations between the detectable rate of each endophytic fungal species and the content of each alkaloid were analysed. Therefore, we identified candidates of fungal endophytes that are associated with the accumulation of alkaloids *in planta*. Furthermore, the ability of these candidate endophytes to enhance alkaloid accumulation was verified by inoculating the *L. radiata* plants with each fungal strain of interest. To the best of our knowledge, the study is the first to indicate that inoculation with fungal endophytes can facilitate the accumulation of various alkaloids in *L. radiata*. We, therefore, propose this as a strategy for increasing the content of certain medicinal alkaloids during plant cultivation. This study also broadens our view of the interaction between this medicinal plant and its fungal endophytes.

Experimental procedures

Plant sampling

The sampling site was located in a *L. radiata* plot at Nanjing Botanical Garden Mem. Sun Yat-Sen (118°83′E; 32°05′N), Jiangsu Province, East China. *Lycoris radiata* was sampled in the autumn of 2016. In total, 20 healthy plants (about 10 years old) were sampled and each sampled plant was located at least 5 metres apart from

other sample plants. Different organs of the sampled plants were separated using a sterilized knife and the same organs were stored in a clean plastic bag. The subsequent processes were carried out as soon as possible in the laboratory.

Isolation of fungal endophytes from different organs

Plant materials were washed under running tap water to remove soil and then all procedures were conducted aseptically as described by Saunders and Kohn (2009) with some modifications. Briefly, plant materials were surface sterilized by immersing in 75% (v/v) ethanol for 1 min, soaking in 1% (w/v) mercury chloride for 4 min (flowers were soaked for 2 min), immersing in 75% (v/v) ethanol for another 1 min, and thoroughly rinsing five times in sterilized distilled water. Then, plant tissues were dried with sterilized absorbent paper. To validate the effect of surface sterilization, the tissues from different plant organs were selected randomly and pressed onto potato dextrose agar (PDA) as described by Yang *et al*. (2016). Subsequently, 100 1-cm long segments from each of the following organs (flower stalks, pistils and stamens, scapes and roots), and 100 1-cm^2 square segments from each of the following organs (flower petals, receptacles and sepals, leaves and bulbs) were cut and placed on PDA plates. Plates were incubated at 28°C for 2 weeks and emergent fungal colonies from the cuts of plant tissues were transferred to fresh PDA plates. Each fungal colony was separated, purified and preserved as routinely processed in the laboratory.

Fungal DNA extraction, amplification and sequencing

Total DNA was extracted from the fresh mycelia of each fungal endophyte using the Multisource Genomic DNA Miniprep Kit (Axygen Bioscience, Tewksbury, MA, USA) according to the manufacturer's protocol. The ITS region, consisting of two ITSs and the 5.8S gene in the eukaryotic rRNA cistron, has the highest resolution to discriminate closely related fungal species (Schoch *et al*., 2012). Thus, primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS region of each fungal endophyte isolated in this study (Mishra *et al*., 2012). The amplified products were purified and sequenced by Sangon Biotech (Shanghai, China). To identify the fungal endophytes at the molecular level, the ITS sequence of each fungal strain was compared with corresponding sequences deposited in the GenBank database using the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/).

Morphological identification of fungal endophytes

All the isolated fungi were further identified to the level of species according to their colonial and microscopic morphology. Colonial morphology included the shape and colour of the fungal colony. To observe the microscopic morphology of fungi, a sterilized coverslip was inserted into PDA in the plate and a mycelial disc (d = 7 mm) of the individual fungal endophyte was placed near the coverslip. Fungal mycelia that grew onto the coverslip were stained using Lactophenol Cotton Blue solution and then the stained mycelia were observed through a microscope using a 100× oil objective. In this study, the microscopic observation mainly included whether the fungal mycelia were septate, whether the fungus produced spores, and the size and shape of the fungal spores. Morphological characteristics of individual fungal strain were compared with those of reported strains having the same taxonomic status according to their molecular identification (Table S1). Consequently, fungal endophytes isolated from *L. radiata* were identified to the level of species according to both molecular and morphological characteristics.

Extraction, identification and quantification of alkaloids

The content of five main alkaloids (narciclasine, lycorine, galanthamine, lycoramine and tazettine) was examined in this study. The authentic standards of narciclasine, lycorine, galanthamine and lycoramine were purchased from Shanghai Yuanye Biotechnology (Shanghai, China) and their purity was more than 99%. The authentic standard of tazettine was purchased from Shenzhen Xianjin Technology (hk) (Shenzhen, China) and its purity was more than 95%.

To detect the content of these five alkaloids in different organs of wild *L. radiata*, three individual plants were harvested and dried at 65°C to constant weight. The total alkaloids from different organs of the individual plant were extracted as described by Mu *et al*. (2010). Briefly, 1 g of dried plant materials was ground and extracted in 5 ml of methanol for 24 h at 4°C with sonicating for three times (30 min each) at regular intervals. After centrifugation at 4000*g* for 20 min, the methanol extracts were filtered through a 0.22 μm sterile filter before the analysis of LC–MS or HPLC.

LC–MS analysis was performed on an Agilent 1260 UPLC-DAD-6530 ESI-Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) according to the method of Sun *et al*. (2018) with some modifications. The separation of different alkaloids was performed on an Agilent Zorbax Eclipse XDB-C_{18} column (100 mm × 4.6 mm × 1.8 μm) with 0.1% formic acid in water (A) and methanol (B) as the mobile phase. The elution time was 0–60 min, with 5%–50% of methanol. The flow rate was

0.3 ml min^{-1} and the column was maintained at 30°C. The injection volume was 10 μl. Samples and standard alkaloids were detected by positive ionization mode with a capillary voltage of 4000 V and a fragmentor voltage of 170 V. The mass spectra were recorded from *m/z* 100 to 1000. Then, the mass spectrum of individual peaks in the total ion chromatography of the methanol extracts of *L. radiata* was compared with the mass spectrum of authentic standards of narciclasine, lycorine, galanthamine, lycoramine and tazettine to verify the existence of these alkaloids.

HPLC analysis was performed on a Shimadzu LC-20AT HPLC equipped with a Diode Array Detector (Shimadzu Corporation, Tokyo, Japan). The separation of different alkaloids was on a reverse-phase column (InertSustain C_{18}, 250 mm × 4.6 mm × 5 μm) using the same program as described above for the LC–MS analysis. The detection was performed at 290 nm, at which the five alkaloid standards showed the maximum absorption peaks. Qualitative analysis was performed according to the retention times of authentic standards. Quantitative measurement was conducted by comparing the detection results to standard curves that had been constructed according to concentrations and peak areas of alkaloid standards in the chromatograms.

Lycoris radiata *tissue culture plantlet adaptation and transplanting*

Lycoris radiata aseptic tissue culture plantlets were established using the following method. The bulb of wild *L. radiata* was cut into quarters and washed under running tap water to remove mucilage, after which all procedures were conducted aseptically. The cut bulbs were surface sterilized by immersing in 75% (v/v) ethanol for 1 min, soaking in 1% (w/v) mercury chloride for 10 min, immersing in 75% (v/v) ethanol for another 1 min, and thoroughly rinsing five times in sterilized distilled water. Then, the cut bulbs were transferred to 50 ml of MS agar supplemented with 6.0 mg L^{-1} 6-benzyladenine (6-BA) and 0.5 mg L^{-1} naphthaleneacetic acid (NAA) in 300 ml tissue culture vessels to induce the formation of new buds. When a sufficient number of buds had germinated, they were separated and transplanted to 50 ml of MS Agar supplemented with 0.2 mg L^{-1} NAA in 300 ml tissue culture vessels to develop into complete plantlets. All aseptic tissue culture plantlets were kept in a growth chamber with a photoperiod of 12 h, a light density of 3400 lm m^{-2} and a temperature cycle of 25/18°C day/night.

Four-week-old rooting plantlets were randomly divided into two groups. The plantlets in the treatment group were adapted to the natural environment for 1 week by opening the tissue culture vessels, and the plantlets in the control group were still grown in the sealed tissue culture vessels for this week. Then, the plantlets in the treatment group were transplanted into natural soil in open tissue culture vessels for 2 weeks; meanwhile, plantlets in the control group were transplanted into sterilized soil in sterilized tissue culture vessels sealed with a sealing film, which allowed air into the vessels and filtered the air to keep it sterile. Thus, the composition of the sterile air in sterilized tissue culture vessels in the control group was the same as the air outside. The culture vessels containing the plantlets from the treatment group and the control group were randomly placed under the same environmental conditions. All plantlets were harvested 2 weeks after transplanting and dried to a constant weight to test the influences of environmental factors on the accumulation of alkaloids in *L. radiata*. At the same time, several *L. radiata* plantlets in the control group were randomly selected. Their leaf, bulb and root were cut into small pieces and placed on PDA plates, which were incubated at 28°C for 2 weeks. No fungal colonies grew from the cuts of plant tissues, indicating the absence of fungal endophytes in the plantlets in the control group (Fig. S5).

Fungal inoculation of potted plants

Similar-sized bulbs of *L. radiata* were collected from the Nanjing Botanical Garden Mem. Sun Yat-Sen. Each bulb was washed using running tap water to remove soil and cultured in individual flowerpots containing 100 g sterilized vermiculite; the pots were watered with 100 ml of one-fourth MS solution every week. All bulbs were kept in a growth chamber with a photoperiod of 12 h, a light density of 3400 lm m^{-2} and a temperature cycle of 25/18°C day/night, which mimicked the environmental conditions that could induce *L. radiata* to leaf. After 2 months, potted plants that had germinated and had similar leaf numbers were chosen and used for the inoculation of fungal endophytes.

Potted plants were inoculated with particular fungal endophytes in independently conducted experiments in May 2018 and October 2019, according to the following steps (Fig. S2). Individual fungal endophytes were grown in potato dextrose broth at 28°C, 180 rpm for 5 days. Then 1 g of fungal mycelia was collected by filtering the fungal fermentation through sterilized gauze; the mycelia were washed with sterilized double-distilled water three times, and then re-suspended in 10 ml sterilized double-distilled water. Each of the *L. radiata* plants was inoculated with 1 ml of the fungal suspension of one of the fungal endophytes by application to the bulbs and roots. The inoculation of each fungal endophyte was carried out for three plants in the May 2018 experiment and five plants in the October 2019 experiment. Control plants were treated with 1 ml of sterilized double-distilled water.

All inoculated and control plants were randomly placed in the growth chamber. *Lycoris radiata* plants were harvested and dried to constant weight 2 weeks after treatments. The dried plants were stored for the analysis of the content of different alkaloids.

Re-isolation of fungal endophytes from L. radiata

To confirm the colonization of fungal endophyte in *L. radiata*, individual strains were inoculated to *L. radiata* aseptic tissue culture plantlets. One week after inoculation, the plantlets were collected and surface sterilized as described above. Then, the leaf, bulb and root were separated, cut into small pieces, and placed on PDA plates. Plates were incubated at 28°C for 1 week and emergent fungal colonies from the cuts of plant tissues were compared with the original fungal colonies.

Statistical analyses

Alpha diversity indices (Shannon–Wiener, Margalef and Pielou) of fungal endophytes were calculated based on the number of fungal strains and species in each organ of *L. radiata* using PAST 3.06 and the bootstrap value was set as 99% (Hammer *et al*., 2001). The ratio of the fungal number in each genus to the total fungal number in one organ was defined as the relative abundance of each fungal genus in this organ. A Venn diagram was plotted based on the number of fungal genera in each organ using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html; Oliveros, 2007).

Bivariate correlation between the number of each endophytic fungal species (Fig. 3) and the content of each tested alkaloid (Fig. 5) in each organ of *L. radiata* was analysed using SPSS Statistics 20.0 (SPSS, Chicago, USA). The Pearson correlation coefficient was calculated and the significant correlation is shown in Table S2. Then, a heatmap of the Pearson correlation coefficients between different endophytic fungal species and alkaloids was produced using the heatmap function from the Vegan package in R 2.15.3.

In the May 2018 experiment (three plants) and the October 2019 experiment (five plants), each plant represented one biological replicate. After each measurement, the average value and standard deviations (SD) of three/five plants were calculated and shown. The means and SD were calculated using SPSS Statistics 20.0 and the final experimental data were represented as mean ± SD. When an analysis consisted of only a control and an experimental group, an independent *t*-test was performed using SPSS Statistics 20.0, and when three or more groups were compared, a one-way analysis of variance was performed, followed by Tukey's multiple-comparison test ($P < 0.05$) (Gleeson *et al*., 2006; Zhou *et al*., 2016).

Accession number

ITS sequences of all 108 fungal strains isolated from *L. radiata* were submitted and deposited in the GenBank under the accession numbers MG543681-MG543788.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 The molecular and morphological identifications of fungal endophytes isolated form *L. radiata*.

Table S2 Exact Pearson correlation coefficients between the number of each endophytic fungal species and the content of each tested alkaloid.

Table S3 Influence of fungal endophytes on the accumulation of alkaloids in different organs of *L. radiata* plants. Results are means from three biological replicates for the May 2018 experiment and five replicates for the October 2019 experiment, which are represented as mean $\pm_{SD}$. Significant differences ($P < 0.05$) in influence of different fungal inoculations on the content of individual alkaloid in each plant organ are indicated by different lowercase letters. The quantification of alkaloids in *L. radiata* was conducted with HPLC.

Figure S1 The cyclization of 4′-*O*-methylnorbelladine via three ways of *C-C* phenol coupling leading to the formation of lycorine-type, galanthamine-type and narciclasine-type alkaloids.

Fig. S2 Some *L. radiata* plants inoculated with different fungal endophytes having significantly positive correlations with the accumulation of Amaryllidaceae alkaloids.

Fig. S3 Influence of fungal endophytes on the biomass of *L. radiata* plants. Results are means from three biological replicates in May 2018 and five replicates in October 2019. Error bars indicate standard deviations. Significant differences ($P < 0.05$) in different fungal inoculations are indicated by different lowercase letters (May 2018) or different uppercase letters (October 2019).

Fig. S4 Re-isolation of fungal endophytes from the tissue culture plantlets of *L. radiata* inoculated with corresponding fungal strains. In each embedded graph, the original fungal colony is on the left and the corresponding re-isolated colony is on the right.

Fig. S5 Confirmation of the absence of fungal endophytes in the axenic *L. radiata* plants. No fungal colonies were obtained from the cuts of plant tissues after being incubated at 28°C for two weeks.