Effects of nitrogen on mineral nutrients and cadmium accumulation in a strain of DSE mycelium under cadmium stress

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Abstract. The dark septate endophytic fungus (DSE) Exophiala pinsciplilia was used to study the effect on growth and mineral nutrients (Ca, Mg, N, P, S) and cadmium (Cd) accumulation of DSE under the different nitrogen concentration and cadmium stress (100 mg·L⁻¹). Under cadmium stress, treatment with low nitrogen significantly increased the pH of the mycelium and culture medium, while high nitrogen significantly reduced the mycelium dry weight, the number of spores, and the pH of the culture medium. The minerals in the mycelium were significantly affected by nitrogen concentration; the Ca and P contents were significantly increased under high nitrogen conditions, while Mg and N content decreased significantly. Under normal nitrogen conditions while under cadmium stress, the nitrogen content of the mycelium was significantly decreased, while higher nitrogen significantly increased the cadmium content of the mycelium. The dry weight of DSE mycelium, the mycelium Cd content, and the mycelium Mg content were significantly negatively correlated, along with the dry weight of P and hyphae. Studies have shown that under cadmium stress, exogenously added high concentrations of nitrogen reduce DSE cadmium tolerance, inhibit growth, and cause the absorption and utilization of minerals; in contrast, low concentrations of nitrogen enhance the growth of the strain.

1. Introduction

Dark septate endophytes (DSE) are a small class soil fungi with unidentified conidia or asexual spores, that colonize plant root tissue cells or intercellular spaces with typical diaphragm and microsclerotial structures; these fungi belong to the ascomycetes or deuteromyces (Jumpponen, 2001; Wagg C et al., 2008). DSE are globally distributed across various habitats. In heavy metal contaminated environments DSE are widely colonized by wild plant roots and display high tolerances to heavy metals (Zhang Y et al., 2013).

This heavy metal tolerance can be influenced by a number of external factors. Nitrogen (N) is a necessary nutrient for fungal life, and plays a very important role in tolerance to heavy metals (Zhang et al., 2018). As well, under cadmium and zinc stress glutamine has been demonstrated to significantly
increase the heavy metal tolerance of a wild strain of *Oidiodendron maius*, as well as increasing the dry weight of mycelium compared to that seen when the fungus was treated with sodium nitrate (Khouja et al., 2014). Rajkumar et al. also found that under conditions of nickel, copper, and zinc stress, fungi can reduce the toxicity of heavy metals and increase biomass by increasing nitrogen absorption as well as the synthesis of proteins and amino acids (Rarkumar and Freitas, 2008). Studies have also shown that medium containing nitrate and ammonia has an inhibitory effect on fungal growth under high concentrations of copper and zinc, but this inhibitory effect varied with nitrogen concentrations (Sazanova et al., 2015). Due to the important role that nitrogen plays in DSE heavy metal tolerance, this interrelationship has attracted the attention of more and more researchers.

Under heavy metal stress, nitrogen also affects the absorption of essential mineral nutrients (such as nitrogen and phosphorus), directly improving mineral nutrition and heavy metal resistance. Heavy metals also inhibit the absorption and conversion of certain mineral nutrients (such as nitrogen) by microorganisms, but this effect is concentration-dependent (Hamsa et al., 2017). Despite the important role that nitrogen plays in DSE heavy metal resistance, there are few reports on the effects of nitrogen nutrition on DSE growth, mineral nutrition, and cadmium tolerance under conditions of cadmium stress.

Here, the DSE *Exophiala pinsciphila* was cultured under cadmium stress (100 mg·L$^{-1}$), and five varying N levels were used to study the growth of DSE mycelium and mineral nutrition (N, P, S, Ca, Mg), as well as the impact that N had on fungal cadmium content.

2. Materials and methods

2.1. Test materials
In this experiment, the DSE fungi *Exophiala pinsciphila* ACCC32496 was cultured using MMN liquid medium (Barros et al., 2006).

2.2. Experimental design
Under stress conditions of 100 mg·L$^{-1}$Cd$^{2+}$ (1.015 g CdCl$_2$·2.5H$_2$O dissolved in 5 L MMN medium), five nitrogen concentrations were tested (0.03, 0.15, 0.3, 1.5, 3.0 g NaNO$_3$ per 900 mL of cadmium-containing MMN medium; 0.06, 0.29, 0.59, 2.94 and 5.88 mmol·L$^{-1}$ final N). Na$^+$ was balanced with NaCl and 900 mL of MMN medium was aliquoted into six 150 mL bottles and a single 250 mL flask. A square of the DSE strain (4-6 cm) was placed in each, and was cultured continuously for 7 days at 28°C, 120 min/r in a constant temperature shaker.

2.3. Indicator determination
The culture solution was filtered and the hyphae were separated from the culture solution. Hyphae were dried at 75°C for 48 h, and the dry weight of the hyphae was determined. The number of spores was determined using the blood cell counting method (Briggs C, 2009), and the pH of the culture solution was measured with a pH meter. The content of Cd, Ca, Mg, and S in mycelium was determined by digest with concentrated HNO$_3$–HClO$_4$ before flame atomic absorption spectrometry was performed. The content of S was also determined by inductively coupled plasma mass spectrometry. The content of N and P in hyphae was determined from a concentrate prepared by H$_2$SO$_4$–H$_2$O$_2$ digestion, using the Nessler method and molybdenum antimony anti-colorimetric determination, respectively.

2.4. Data processing in statistical analysis
Experiments were performed using Microsoft Excel and IBM SPSS Statistics 21 for data collation and statistical analysis, and figures were drawn using Origin 9.1.
3. Analysis of results

3.1. Effects of different nitrogen concentrations on the growth of DSE mycelium and the pH of culture medium under cadmium stress

Under cadmium stress, with increasing nitrogen concentration the dry weight of DSE mycelium, the number of spores, and the pH of the culture medium decreased. With 5.88 mmol·L⁻¹ nitrogen, there was a significant decrease in the dry weight and spore count of mycelium, decreasing by 75.9% and 25.5%, respectively, compared with the values seen with 0.06 mmol·L⁻¹ nitrogen treatment (decreases of 70.3% and 35%, respectively, as compared with 0.59 mmol·L⁻¹ nitrogen treatment) (Table 1). It can be seen that the mycelial growth of DSE was severely inhibited when treated with 5.88 mmol·L⁻¹ nitrogen.

Table 1. Effects of different N concentration on DSE Dry weight of hyphae, Spore number and culture colution pH value under cadmium stress

| N concentration (mmol·L⁻¹) | Dry weight of hyphae (mg) | Spore number×10³ (N/ml) | pH value     |
|---------------------------|---------------------------|--------------------------|--------------|
| 0.06                      | 146.7±24.7a               | 5.5±0.8ab                | 6.14±0.4a    |
| 0.29                      | 96.3±18.5b                | 4.7±0.85ab               | 5.85±0.28ab  |
| 0.59                      | 119.0±23.5ab              | 6.3±1.61a                | 6.06±0.28a   |
| 2.94                      | 88.7±4.7b                 | 4.0±0.95b                | 5.93±0.31a   |
| 5.88                      | 35.3±7.1c                 | 4.1±0.46b                | 5.45±0.08b   |

Note: The different lowercase and uppercase letters in a column indicate significant differences among treatments at P<0.05 and P<0.01 levels, respectively.

3.2. Effects of different nitrogen concentrations on the mineral content in mycelium of DSE grown under cadmium stress

Under cadmium stress, nitrogen concentration significantly affected the mineral content in the DSE mycelium. The hyphae Ca, Mg, N, P, and S concentrations each showed different changes with various nitrogen exposures; treatment with 5.88 mmol·L⁻¹ nitrogen significantly increased mycelial Ca content to 0.45 mg·g⁻¹, 0.59 mmol·L⁻¹ nitrogen significantly increased mycelial Mg content to 0.413g·kg⁻¹, and treatment with both 2.94 and 5.88 mmol·L⁻¹ nitrogen significantly reduced mycelial Mg content to 0.116 and 0.153 g·kg⁻¹(Fig. 1). Treatment with 0.06 mmol·L⁻¹ significantly increased the content of both N and S in the mycelium, and the mycelial P increased significantly when treated with 2.94 mmol·L⁻¹ nitrogen, reaching a concentration of 1.142 mg·kg⁻¹ (Fig. 1).
3.3. **Effects of different nitrogen concentrations on cadmium content in mycelium of DSE grown under cadmium stress**

Different nitrogen concentrations significantly affected the cadmium content of the mycelium in DSE cultured for 7 days under 100 mg·L⁻¹ cadmium stress. When treated with 2.94 mmol·L⁻¹ nitrogen, the mycelium cadmium content was significantly higher than at the other four nitrogen concentration levels, reaching 8.11 g·kg⁻¹ (Fig. 2). The cadmium content of hyphae treated with 0.06, 0.29, 0.59 and 5.88 mmol·L⁻¹ nitrogen decreased by 25.2%, 12.5%, 29.4% and 20%, respectively, when compared to the concentration of cadmium seen when the fungus was grown with 2.94 mmol·L⁻¹ nitrogen. The cadmium content of the DSE mycelium was lowest when grown with 0.59 mmol·L⁻¹ nitrogen, reaching a concentration of 5.73 g·kg⁻¹ (Fig. 2).

![Fig.2 Effect of different nitrogen concentrations in culture medium on cadmium content in DSE mycelium](image)

4. **Correlation analysis**

The correlations between Cd content, dry weight, and N content of the culture medium and the N, P, S, Ca and Mg content of hyphae were analyzed, to determine if any of the correlations were significant ($p<0.05$). The results showed that the mycelial Cd content was only significantly negatively correlated with the Mg content, with a correlation coefficient of 0.67. There was a significant negative correlation between both DSE dry weight and mycelial P and Mg contents (correlation coefficients of 0.62 and 0.65, respectively), while there was no correlation between N content in the culture medium and the mycelial mineral content (Table 2).
Table 2. Correlation analysis between mycelial biomass, Cd content and mycelial mineral content.

| Related indicators | N    | P    | S    | Ca   | Mg    |
|--------------------|------|------|------|------|-------|
| Hyphae Cd content  | -0.448 | 0.383 | 0.179 | 0.052 | -0.656** |
| Dry weight of hyphae | 0.473  | -0.622* | 0.132 | -0.388 | -0.65** |
| Culture medium N content | 0.621  | -0.482 | 0.613 | -0.402 | 0.600 |

Note: * indicates significant correlation, ** indicates extremely significant correlation.

5. Discussion

Under stress, both the growth and number of microorganisms are important indicators of their tolerance to stress conditions (Wang et al., 2018). Here, it was demonstrated that, under cadmium stress, low nitrogen concentrations had no significant effect on DSE biomass and spore count, while treatment with high nitrogen significantly reduced biomass and spore count. DSE tolerance to cadmium was also found to decrease with increasing nitrogen concentrations. This may be because high nitrogen is in itself a type of stress for microorganisms, and this stress is synergistic with cadmium, affecting the normal physiological metabolism and reducing biomass (Zhou et al., 2018). Wang et al. also reported that the absorption of Cd^{2+} by Thalassiosira pseudonana significantly increased under nitrogen-rich nutrient conditions (Wang and Dei, 2001). This may be caused by the high concentrations of cadmium and nitrogen destroying the cell membrane, damaging enzymes, and interfering with metabolic links, making the cells unable to carry out normal metabolic processes and severely inhibiting growth.

Microbial absorption and utilization of mineral nutrients is affected by various factors as well as interactions between the elements themselves (Fellbaum et al., 2012). Here, the high concentration of cadmium and nitrogen the fungus was exposed to significantly increased absorption of mineral elements by the DSE hyphae; the hyphae content of Ca and P increased significantly, however Mg, N, and S content significantly decreased. The decrease in Mg content in the hyphae may be the result of Cd competing with binding sites for Mg (Suksabye et al., 2016), and the increased absorption of Ca and P may be related to the interaction between the mineral elements (Thirkell et al., 2016). However, when a large amount of cadmium enters the cell, the anabolic function of the cell is disturbed, and the synthesis of related ion channels and related recognition proteins (nitrate reductase, etc.) is disrupted, affecting the absorption of N and S; this likely explains the decrease in these elements observed for the hyphae of the fungus grown under stress.

Studying the effects of different nitrogen concentrations on the heavy metal tolerance of DSE can provide a theoretical reference for the future development of DSE as a new technology for heavy metal remediation. However, under the combined conditions of high nitrogen and cadmium, the molecular mechanisms by which the two synergistically act to inhibit microorganisms as well as the factors related to those processes still need further study. In addition, the nitrogen form and ratio could have significantly different impacts on DSE metabolism, and thus these factors must also be examined.

6. Conclusion

Under cadmium stress, high concentrations of nitrogen significantly decreased the dry weight of DSE hyphae, the number of spores, the pH value of the culture medium, and the content of mineral nutrients (Mg, N, and S) in the fungal mycelium; in contrast, the content of Ca, P, and Cd in hyphae was significantly increased. From these results it can be determined that high concentrations of nitrogen are able to reduce the cadmium tolerance of DSE as well as inhibit its normal growth.
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