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Research Paper

Metabolic responses of endophytic *Nicotiana benthamiana* plants experiencing water stress

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ABSTRACT

Endophytic fungal colonization may influence how plants respond to environmental stress. Two promising fungal isolates, one resembling *Cladosporium cladosporioides* and another unidentified ascomycetous fungus, isolated from wild *N. benthamiana* plants in northern Australia were inoculated to plants of the research accession of *N. benthamiana* (RA-4). Inoculated seedlings were grown under adequate or water deficit conditions. We examined leaf metabolites using gas chromatography-mass spectrometry (GC–MS) to compare levels of sugars, sugar alcohols, amino acids and other metabolites at various stages of plant growth and stress application. Ninety-three metabolites were detected in leaves, including 20 sugars, 13 sugar alcohols, 21 amino acids, 29 organic and fatty acids and ten other compounds. Endophyte colonization caused significantly differential accumulation of 17–21 metabolites when the plants were grown under well-watered condition. The presence of endophytes under water stress conditions caused differential accumulation of cytosine, diethylene glycol, galactinol, glycerol, heptadecanoate, mannose, oleic acid, proline, rhamnose, succinate, and urea. Accumulation of these metabolites suggests that fungal endophytes influence plants to accumulate certain metabolites under water-stress. Further, plants colonised by the two different endophytes tested, showed some differences in the metabolites they accumulated. Colonization with endophytic fungi significantly increased root dry mass and relative water content in plants under severe water stress condition, suggestive of a symbiotic relationship between these fungi and *N. benthamiana* plants, a species adapted to the hot and unpredictable soil moisture conditions of northern Australia. We reveal that endophyte colonization triggers reprogramming of host metabolism and indices changes in host development. This study sheds lights on the mechanisms underlying increased tolerance to water stress in plants conferred by fungal endophytes. Fungal endophytes have the potentials for application to increase the inherent water stress tolerance of crops.

1. Introduction

In nature, plants are exposed to various environmental stresses that may have significant impacts on size, lifespan and fecundity. Water deficit (commonly referred to as drought) is one of the most widespread abiotic stresses limiting plant growth in many parts of the world (Chaves et al., 2003; Lawlor, 2012). Current climate change models predict that soil water availability in some regions will be significantly reduced (Stocker, 2014). Plant strategies to cope with water stress can broadly be divided into tolerance and avoidance (Claeys and Inzé, 2013). Plant drought tolerance involves detoxification of reactive oxygen species (ROS) and the accumulation of solutes called osmolytes such as sugars, the amino acid proline, and other compounds that maintain the cellular turgor pressure required for cell expansion under stress conditions (Chen and Jiang, 2010; Claeys and Inzé, 2013; Hoekstra et al., 2001; Morgan, 1984; Rodriguez and Redman, 2005). Also, microbial symbionts may play a role in plant adaptation to stress (Coleman-Derr and Tringe, 2014; Rodriguez et al., 2009).

Fungal endophytes live in association with plants while inducing no visible symptoms of pathogenicity. In some cases, fungal endophytes confer benefits to plants exposed to water scarcity (Upson et al., 2009). Possibly the most well-known example endophyte-mediated plant water stress tolerance is the mutualism of tall fescue and perennial ryegrass with the grass endophyte (Class 1, *sensu*: Rodriguez et al. 2009)

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Epichloë coenophiala (Kane, 2011). Non-grass fungal endophytes (Class 2, *sensu*: Rodriguez et al. 2009) have also been reported to improve plant tolerance under water deficit stress (Khan et al., 2013, 2015; Redman et al., 2011; Waqas et al., 2012). Although the role of fungal endophytes to mediate plant water stress tolerance has been described in several studies, the key mechanism(s) are incompletely understood. Endophyte colonization causes (a) increased growth and development (Khan et al., 2013; Redman et al., 2011) (b) enhanced osmotic adjustment (Grover et al., 2001), (c) increased gas exchange and water use efficiency (Bae et al., 2009; Elmi and West, 1995) and (d) improved defence against oxidative stress in host plants (Azad and Kaminskyj, 2016). Metabolomic studies in *Festuca arundinacea* (tall fescue) colonised with *E. coenophiala* have reported a significant impact of the endophyte on primary and secondary metabolism under water deficit conditions (Nagabhyru et al., 2013; Rasmussen et al., 2008). Stress-induced accumulation of sugars, sugar alcohols, amino acids, and mineral ions has been documented in plants (Chen and Jiang, 2010; Hanson and Smeekens, 2009; Loescher, 1987). Class 1 endophytes have a significant effect on the accumulation of simple sugars in the plant under water stress. Effects of the endophyte on accumulations of amino acids, organic acids other metabolites in plants under water stress have not been well characterised.

To the best of our best knowledge, no study has yet been undertaken to investigate the role of Class 2 endophytes on water stress tolerance of hosts at the metabolic level. Our preliminary (Dastogeer et al., 2017b) study identified two ascomycetous fungal endophytes isolated from indigenous Australian *Nicotiana* plants growing in arid conditions that provided significantly improved water stress tolerance in *Nicotiana benthamiana* plants tested in both *in vitro* and greenhouse trials. Analysing these endophyte effects on host plants at the metabolite level using high-throughput metabolite profiling is an approach to elucidate the mechanisms of endophyte-enhanced plant growth and survival under water deficit conditions. The objectives of this study were to investigate and compare the metabolic responses of endophytic and non-endophytic *N. benthamiana* plants grown under well-watered, moderately water stressed and severely water stressed conditions.

2. Materials and methods

2.1. Fungal isolates

Two fungal endophytes, named E-162 (GenBank ID: KU059880) and E-284, (GenBank ID: KU059897) were selected from our previous experiments (Dastogeer et al., 2017b) which indicated that they increased water stress tolerance of *N. benthamiana* plants both *in vitro* and in greenhouse conditions. These endophytes were originally isolated from the roots of wild *Nicotiana benthamiana* plants species collected in northern Western Australia. The strains were isolated from surface-sterilized plant tissue on 0.1 × potato dextrose agar (PDA) medium from symptomless root tissue (Dastogeer et al., 2017a). Combined morphological (colony appearance, mycelial texture, hyphae and conidial structure, etc.) and molecular techniques were used to help in identification of the fungi studied. Molecular identification was done by Sanger sequencing of PCR amplified products of the ITS regions rDNA of the fungi using the universal primers ITS1 or ITS1F with ITS4 (Gardes and Bruns, 1993; White et al., 1990). Based on the closest match (> 98% similarity) from a Blastn search of the NCBI nucleotide UNITE (Abarenkov et al., 2010) database, isolate E-162 was identified as closely resembling isolates of *Cladosporium cladosporioides,* whereas E-284 was not identified below the subkingdom level of Ascomycota. The closest matching sequences (≥95%) to isolate E-284 were all labeled as unidentified Ascomycota (Supplementary Fig. 1). Since the isolate did not sporulate on PDA media, we went no further in describing E-284 in greater detail.

Fungal cultures were stored at −80 °C in potato dextrose broth (PDB) containing 15% (v/v) glycerol. Fungi were sub-cultured from the frozen stock to potato dextrose agar (PDA) and incubated at 25 °C in the dark, prior to use in experiments.

2.2. Inoculum preparation

For inoculum preparation, the fungi were seeded in 250 ml Erlenmeyer flasks containing 0.1 × potato dextrose broth (PDB), with continuous shaking at 100 rpm at 25 °C. Seven-day-old mycelial pellets were harvested and macerated in a liquid homogenizer-mixer into uniform fragments. The suspension was filtered through sterile absorbent cotton wool plugs to remove large hyphal fragments. The inoculum concentration was determined by using a haemocytometer with a compound microscope and was adjusted to 5×10^4 fragments mL^{-1} through sterile dilution. This inoculum concentration was chosen based on our previous findings (Dastogeer et al., 2017b). To assess the viability of the fragments, a germination test was carried out on PDA after incubation for 48 h at 25 °C.

2.3. Stress treatment

Seeds of the research accession of *N. benthamiana* (research accession 4 (RA-4)) (Goodin et al., 2008; Wylie et al., 2015) were surface-sterilised by submerging them in 3% sodium hypochlorite for 3 min and then in 75% ethanol for 2 min, then rinsed with sterile water three times. The seeds were sown in steam-treated sand for germination. Steam sterilization of soil was done at 99 °C for 4 h twice with a gap of 48 h in between steaming sessions to eliminate other fungi. Three-week-old seedlings were removed from the soil, and the roots were washed to remove adhering soil. For inoculation, the seedling roots were submerged in the inoculum suspension for 5 h before planting. The control seedlings were immersed in sterile distilled water for 5 h. Treated seedlings were transplanted in steam-treated sand in pots (10 cm in diameter and 12 cm deep). We used sterilised media to see the effect only these strains in absence of other microbes. In non-sterilised soil the effect of these fungal stains could have been modified by the interaction with indigenous microbial communities and we would not be able to measure the sole effect of these strains. To improve the efficacy of inoculation, the inoculum suspension was also applied to the root-zone of the seedling at the time of planting. The root-zone application was done by spraying of the inoculum suspension into depressions in the sand where the seedlings were to be planted. The plants were maintained in a greenhouse at 22–24 °C, 60 ± 5% RH, and with natural photoperiod. Seedlings were watered to drip point for three weeks before stress treatment was imposed to allow acclimation of plants after transplanting and to allow sufficient growth of plants. The pots were arranged randomly; the position of pots was rearranged every week to minimise environmental variation within the greenhouse, until the imposition of stress. Plant infection status was checked at the end of the experiments by culturing surface-sterilized roots and identifying isolated fungi by microscopy as well as by sequencing of the ITS region as described above.

2.4. Sampling

The first sampling was done at 21 days post-inoculation (dpi), referred to as harvest one (H1, 0 days post-stress). In the following days, half of the pots from each inoculated and non-inoculated group received stress by withholding watering and other half continued to receive watering as before. There were four plants per pot, five pots per replication and four replications in each endophyte or non-endophyte treatment under well-watered condition. The same number of plants was treated with water stress. When the non-inoculated groups started showing stress symptoms as manifested by wilting of one or two leaves from the bottom at 4 days post-stress (at 25 dpi), we considered it as moderate water stress and harvested the second sample (H2). As the stress continued, the non-inoculated group showed shoot tip wilting.