



## Does fungal endophyte inoculation affect the responses of aspen seedlings to carbon dioxide enrichment?

Tendry R. Randriamanana<sup>a,\*</sup>, Katri Nissinen<sup>a</sup>, Anu Ovaskainen<sup>a</sup>, Anu Lavola<sup>a</sup>, Heli Peltola<sup>b</sup>, Benedicte Albrechtsen<sup>c</sup>, Riitta Julkunen-Tiitto<sup>a</sup>

<sup>a</sup> Natural Products Research Laboratory, Department of Environmental and Biological Sciences, University of Eastern Finland, P.O. Box 111, FI-80101 Joensuu, Finland

<sup>b</sup> Dynamics and Management of Boreal Forests, School of Forest Sciences, University of Eastern Finland, P.O. Box 111, FI-80101 Joensuu, Finland

<sup>c</sup> Department of Plant Physiology, Umeå University, Umeå Plant Science Centre, SE-901 87, Umeå, Sweden

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### ABSTRACT

Endophytes are microorganisms that live inside plants without causing visible symptoms, at least during some parts of their life cycle. We studied, for the first time, the combined effects of CO<sub>2</sub> enrichment (700 ppm) and fungal endophyte inoculation on the growth, the concentrations of low-molecular weight phenolics, and condensed tannins of aspen (*Populus tremula*) seedlings. As expected, we found that the endophyte strain we inoculated was neutral to plant growth and was able to bypass major plant defences. In addition, CO<sub>2</sub> enrichment alone boosted plant growth, but had only minor effects on plant phenolics. Neither did it affect the plant-endophyte relationship. Based on our findings, we suggest that the successful and asymptomatic colonization of endophytes that we found in aspen might be due to the endophytes' special attributes enabling them to thrive inside plant tissues and to avoid or counteract the plant's chemical defences.

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### 1. Introduction

Microorganisms play eminent roles in shaping plant metabolic, hormonal and defence pathways, conferring novel nutritional capabilities and even providing protection against biotic and abiotic factors in plants (Rosenblueth and Martínez-Romero, 2006; Schulz and Boyle, 2006; Friesen et al., 2011). Fungal and bacterial endophytes are microorganisms that “at some time in their life, can colonize internal plant tissues without causing apparent harm to their host” (Petrini, 1991). They benefit from the spatial structure of the plants and from the protection from environmental stresses and the nutrients they offer (Aly et al., 2011). In return, they may directly promote the growth of plants by facilitating nitrogen acquisition (Doty et al., 2016; Khan et al., 2016), regulating plant phytohormones (Xin et al., 2009a, 2009b), solubilizing inorganic phosphate to improve the plants' phosphorus nutrition (Khan et al.,

2015), and producing siderophores for improved iron uptake of the plants (Santoyo et al., 2016). Endophytes may also indirectly improve plant growth through the release of antibiotics (antibiosis) or lytic enzymes that inhibit plant pathogens, and through induced systemic resistance (Hardoim et al., 2015; Santoyo et al., 2016).

In fact, *in vitro* inoculation of field-sampled endophytes on the leaves on *Theobroma cacao* have protected this woody plant against a *Phytophthora* leaf pathogen, which causes leaf necrosis and premature mortality (Arnold et al., 2003). Likewise, seedlings of *Pinus monticola* pre-inoculated with white pine blister rust not only showed both reduced disease severity and higher survival rate, but also had a longer lifespan than did endophyte-free plants (Ganley et al., 2008). In Salicaceae species (poplar and willow), *in vitro* assays demonstrated that a consortium of widespread endophytes could produce several plant growth-promoting traits and also induced antagonistic activities against numerous and universal plant pathogens (Kandel et al., 2017). In some genotypes of *Populus angustifolia*, endophytes also reduced the severity of symptoms from a necrotrophic leaf pathogen *Drepanopeziza populi* (Busby et al., 2013). However, these beneficial effects depended on the

\* Corresponding author.

E-mail address: [tendry.randriamanana@gmail.com](mailto:tendry.randriamanana@gmail.com) (T.R. Randriamanana).

endophyte species and on the genotypes of the host plants. For instance, *Populus trichocarpa* plants inoculated with endophytes were more resistant to rust infection (Raghavendra and Newcombe, 2013), while other endophyte species from *P. trichocarpa* had no effect on rust severity in the host plant, but even acted as pathogen facilitators (Busby et al., 2016).

Some studies suggest that plants and endophytes may exist in balanced antagonism. Balanced antagonism refers to the interplay or asymptomatic interaction between the two partners, resulting in a momentary equilibrium between endophytes virulence and plant defence. On one hand, plants have several barriers and produce different secondary compounds to repel herbivores and pathogens. On the other hand, fungi and bacteria produce specialized enzymes and secondary metabolites to overcome these barriers and defence and colonize the plant host without developing disease. Endophytic fungi, for example, could synthesize toxic compounds, while the plants produce antifungal metabolites such as condensed tannins (Schulz et al., 1999). Although plant chemistry may have a potential influence on the outcome of the plant-endophyte interaction, only a few studies conducted with woody plants have so far linked their chemistry to endophyte presence. In *T. cacao*, plant chemistry may improve the growth of some endophyte species and potentially influence endophyte species composition (Arnold et al., 2003). In the genus *Populus*, the relationship between phenolic compounds and endophytes is not always straightforward. In twigs of *Populus fremontii*, condensed tannins have inhibited endophyte colonization (Bailey et al., 2005), while in *P. angustifolia*, endophyte abundance did not correlate with concentrations of twigs' condensed tannin (Lamit et al., 2014). In *Populus tremula*, leaf salicylate content and profile influenced fungal endophyte composition and richness (Decker, 2016), but another study found no relationships between phenolic compounds and leaf endophytes (Randriamanana et al., 2015). Further studies are thus needed to advance our knowledge on the largely unexplored impacts of phenolic compounds on the balanced antagonism between woody plants and their associated endophytes.

According to the Earth System Research Laboratory (ESRL), the annual global mean carbon dioxide (CO<sub>2</sub>) concentration in the atmosphere in 2016 was 402.41 ppm (NOAA/ESRL, 2017), which is 44% higher than pre-industrial levels. A multi-model scenario predicts that this value will approach 1000 ppm by the end of the 21st century (IPCC, 2013). So far, few studies have dealt with the effects of CO<sub>2</sub> enrichment on plant-endophyte interactions. This is probably because endophytes live within plant tissues and are thus shielded from the direct effects of abiotic factors. However, endophytes depend largely on plants for nutrients and thus are very likely to be influenced by elevated CO<sub>2</sub> through its indirect effects on plant physiology and chemistry (Grover et al., 2015). CO<sub>2</sub> enrichment is expected to increase the leaf concentrations of carbon-based secondary compounds such as phenolics (Penuelas et al., 1997; Zvereva and Kozlov, 2006). In aspen, it may increase leaf concentrations of phenolic compounds, including condensed tannins, but the magnitude and direction of these changes depend on plant genotype and developmental stage (Couture et al., 2014, 2017). The effects of CO<sub>2</sub> enrichment on the fungal endophytes of several grass species are variable, ranging from no effect to increased fungal endophytes colonization (Compant et al., 2010; Brosi et al., 2011). Most studies with grass endophytes were, however, carried out under optimal environmental conditions, optimizing the cost-benefit balance between plants and endophytes and leading to a mutualistic relationship (Albrectsen and Witzell, 2012). Less favorable environmental conditions may switch the direction of plant-endophyte interaction towards a more antagonistic relationship (Saona et al., 2010), which further emphasizes the need to study the responses of plant-endophyte

relationships under various environmental conditions. In contrast to the positive effects of grass endophytes on their host fitness (Aly et al., 2011; Albrectsen and Witzell, 2012), little is known about the roles of endophytes inhabiting woody plants, which have received less attention (Saikkonen et al., 2010). Furthermore, we are not aware of any study investigating the effects of CO<sub>2</sub> enrichment on the leaf endophytes of woody plants.

In this study, we aimed to investigate whether fungal endophyte inoculation affects the responses of aspen seedlings to enriched CO<sub>2</sub>. We expected two different scenarios: (i) Endophytes would improve the plants' nutrient acquisition, which may increase the positive effects of CO<sub>2</sub> enrichment on aspen growth and phenolic defence. (ii) Alternatively, endophytes would be neutral to plant growth and phenolic defence. This would imply that endophyte colonization would not impose any apparent costs on the growth and chemical defence of the host plant.

## 2. Materials and methods

### 2.1. Experimental design

We conducted the experiment in the growth room facility at Mekrijärvi research station (Eastern Finland) during summer 2013. The study setup was a full factorial design with combination treatments of ambient (400 ppm, C) and elevated CO<sub>2</sub> (730 ppm; CO<sub>2</sub>), and using endophyte-free and endophyte-inoculated (E) aspen seedlings: C, CO<sub>2</sub>, E, E + CO<sub>2</sub>. We replicated these treatment combinations four times in 16 identical greenhouse rooms in total. The growth rooms have been described in detail in the study by Zhou et al. (2012). Briefly, each growth room consisted of a double-layer of toughened glass fixed with stainless steel structures. The glass walls and roofs transmit 75% of the visible light, cutting out part of the infrared radiation and transmitting about 1.7 W/m<sup>2</sup> of UV-B radiation. We provided additional UV-B radiation using two UV-B lamps (Philips, TL 40 W/12RS SLV) wrapped in cellulose diacetate filters (0.95 mm FilmSales Ltd, London, UK). Each room was equipped with inlet and outlet vents with internal controlled fan and filters for fresh air ventilation. CO<sub>2</sub> was supplied from a set of cylinders with pure CO<sub>2</sub> (AGA Oy, Finland) transmitted through pipes at 60 s intervals before being injected into the rooms through an electronically controlled proportioning valve. Each room had its own set of sensors connected to a computer by means of a modulator software UIO32 Programmable Logic Controller (Computec Oy, Joensuu, Finland) and the Citect SCADA automation program (Computec Oy, Joensuu, Finland), which controlled the temperature, gas, humidity and UV-B lamps in the chambers.

### 2.2. Plant material

The seedlings used in the experiment originated from micro-propagated aspen buds that were collected from various locations in eastern and southern Finland (see for more details, Randriamanana et al., 2014). On 11th June 2013 (starting date of the CO<sub>2</sub> treatment), we randomly distributed 12 aspen genotypes in each of the 16 independent growth rooms (192 seedlings in total), so that each growth room contained the same set of 12 replicated genotypes. We kept the seedlings under axenic conditions and planted them in 3 l-pots filled with a sterilized and fertilized mixture of 70% unfertilized sphagnum peat and 30% vermiculite. At the beginning of the experiment, we fertilized the seedlings once with Ingstad's basic nutrient solution (Ingstad, 1962) on 25th June 2013. Thereafter, we watered the seedlings every other day.

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