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Carbon starvation by light deprivation does not constrain the ability of young pines to produce induced chemical defences in response to a bark-chewing herbivore

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ABSTRACT

Pine tree chemical defence is based on constitutive and inducible carbon-rich substances (mainly oleoresin and phenolic compounds) present in pine tissues in conspicuously high concentrations. Resistance at early stages is essential for future fitness, and allocation of carbon resources to chemical defences may signify a relevant carbon sink for juvenile pines. However there is still poor knowledge about the extent to which allocation priorities of recently assimilated and stored C pools to chemical defences are plastic in response to biotic stress. We aimed to determine the extent to which the production of induced chemical defences in pine trees in response to herbivory depends on the availability of current photosynthates or if they can be built upon stored C pools. Two years-old trees of three pine species (*Pinus pinaster*, *Pinus sylvestris* and *Pinus radiata*) were exposed to real herbivory by the pine weevil *Hylobius abietis*, a bark-chewing insect, during one week under conditions of either natural sunlight or complete light deprivation. Weevil damage and the concentration of chemical defences in the whole stem were measured in a time-course design with destructive sampling. We analysed, using an ANCOVA approach, whether the ability to increase the concentration of chemical defences in response to weevil damage was altered by light deprivation. We hypothesised that if induced defences are built mainly upon current photoassimilates, then carbon starvation by light deprivation should strongly hamper the production of new defences. For all pine species, concentration of non-volatile resin significantly increased in response to weevil damage, which was more intense under dark conditions. ANCOVA analysis showed that light deprivation did not constrain the ability of young pines to respond to weevil damage by increasing non-volatile resin content in the stems. No significant covariation was found for total polyphenolics. By evaluating chemical defences in the whole stem, and restricting C assimilation by complete light deprivation, we can infer that young pines were able to use other carbon sources than current photosynthates, likely stored carbon pools, for *de novo* synthesis of induced chemical defences in response to insect herbivory. Disentangling the plasticity in the use of C sources for the synthesis of induced defences is crucial for understanding the ability of pine trees to respond to multiple stressors in a changing environment. Please refer to the video summary (Appendix F) for a quick overview.

1. Introduction

Pine chemical defence is founded in the production and accumulation of a mixture of carbon-rich compounds, mainly terpenes and phenolics, that serve as wound-sealant, antifeedants or deterrents, providing resistance against insect herbivores (Eyles et al., 2010; Franceschi et al., 2005; Moreira et al., 2014). Pests and diseases can cause severe damage and extremely high mortality rates at the juvenile stage (Nordlander et al., 2011; Zas et al., 2011), and therefore defence against biotic threats at early stages is essential for pine fitness (Wainhouse et al., 2005; Zas et al., 2011). Terpenoid and phenolic compounds are present in all pine tissues in conspicuously high concentrations, in the order of 10–100 mg of secondary chemicals per gram of d.w. tissues (Moreira et al., 2014; Sampedro et al., 2011a). The production of both types of defences therefore signifies a relevant carbon sink for juvenile pines (Eyles et al., 2010; Gershenzon, 1994).

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Pines have both constitutive defences, which are present in absence of attack, and induced defences, which are triggered after biotic challenge (Franceschi et al., 2005; Mumm and Hilker, 2006). In pines, herbivore-induced responses involve a rapid increase in the activity of terpene synthases (e.g. Funk et al., 1994; Lewinsohn et al., 1991; Steele et al., 1995) and in the concentration of major chemical defences (Zas et al., 2014; Zhao et al., 2011), qualitative changes (Moreira et al., 2013; Zhao et al., 2011) and even anatomical modifications such as the genesis of traumatic resin ducts (Franceschi et al., 2005; Moreira et al., 2015). An increasing number of studies have shown the critical relevance of those induced defences for effective protection against particular insect herbivores (Schiebe et al., 2012; Zas et al., 2011; Zhao et al., 2011).

Induced defences are considered a cost saving strategy because they are produced only when needed (Karban and Baldwin, 1997). However, once triggered, the production of induced defences is also costly and requires large amounts of carbohydrates (Cipollini and Heil, 2010; Sampedro et al., 2011a). Due to resource limiting conditions imposed by water, light and nutrient availability in nature, young pine trees have to optimize the allocation of resources to constitutive defences across heterogeneous environments (Moreira et al., 2014). Resource availability may also affect the allocation to induced defences, but evidence in this case is inconclusive, especially in long-lived trees (Eyles et al., 2010). For instance, some studies found greater inducibility of defences under growth-limiting conditions (Najar et al., 2014; Sampedro et al., 2011a), while others found the opposite (Lombardero et al., 2000). In order to understand this apparent inconsistency, and given that investment in defensive chemistry directly depends on carbon availability, the origin of the carbon-resources used for producing induced defences is worth considering, i.e. to what extent do carbon-resources used in induced defences come from current photosynthates, and to what extent can they be built upon stored carbohydrates (Ruan et al., 2013). Recently assimilated carbon is known to be a major source for terpenoid biosynthesis (Gershenzon, 1994). There is a close link between CO_2 uptake and biosynthesis of terpenoids in conifer foliage, where ^{13}C-labeled monoterpenes quickly appear after spiking young pines with $^{13}CO_2$ (Ghirardo et al., 2010). Up to 58% of the monoterpene emission was *de novo* synthesized from current assimilated carbon in *Pinus sylvestris* saplings (Ghirardo et al., 2010 and references therein), although alternative carbon sources (stored non-structural carbohydrates and recycled carbon) may also contribute (Ghirardo et al., 2011). Interestingly, starch grains and other carbon storage molecules found in phloem parenchyma may be available for induced synthesis of new phenolic compounds and also for the synthesis of resin in the epithelial cells of the resin canals (Krekling et al., 2000).

Furthermore, even when the carbon for chemical defences comes primarily from photosynthetic assimilation, carbon sources for oleoresin and phenolics could differ as the initial substrates required for the synthesis of those compounds differ (Schultz et al., 2013). Both types of compounds are synthesized through well differentiated pathways. Phenolic compounds are synthesized through the phenylpropanoid pathway from precursors of aromatic amino acids (Eyles et al., 2010; Moore et al., 2014), whereas terpenoids derive from isopentyl pyrophosphate and dimethylallyl pyrophosphate synthesized via the mevalonate and methylerythritol pathways (Bohlmann et al., 1998; Eyles et al., 2010; Moore et al., 2014).

The relative contribution of current photosynthates and stored carbon pools to plant respiration, biomass allocation, soil transfer processes and whole tree carbon balance have received significant attention in recent years (e.g. Epron et al., 2012; Högberg et al., 2008; Kuptz et al., 2011). It is also known that C allocation patterns and within-plant mobilization of C resources may change immediately following biotic damage signalling in *Arabidopsis* (Ferrieri et al., 2013), tomato (Gómez et al., 2010; Hanik et al., 2010), tobacco (Machado et al., 2013) and poplar (Arnold et al., 2004). There is also evidence that jasmonates, a family of plant hormones deeply involved in defensive responses, could be also regulators of sugar catabolism in tobacco, thus interfering both with the allocation of carbon resources and with tissue quality and palatability (Ferrieri et al., 2015; Machado et al., 2015). All these processes may contribute to the drastic changes in biomass allocation found in young pine trees in response to herbivory signalling (Moreira et al., 2012b). However there is still poor knowledge about the extent to which allocation priorities of stored and recently-fixed carbon to chemical defences are plastic in response to biotic stress. Particularly in long-lived woody plants, large non-structural carbohydrate pools in roots and stems may be crucial for physiological processes under stressful situations (Appel et al., 2012; Najar et al., 2014).

The aim of the present study is to determine the extent to which the production of induced chemical defences in pine trees in response to herbivory depends on the availability of current photosynthates or stored C. To this end, we exposed 2 years-old pine trees of three pine species to real herbivory by a harmful bark-chewer insect, under conditions of either natural sunlight or complete light deprivation. We measured in a time-course design the concentration of the main chemical defences (non-volatile resin and total polyphenolics) and explored whether light deprivation conditioned the increase of these defences in response to weevil damage. If induced defences are built mainly upon current photoassimilates, then light deprivation should strongly hamper the production of new defences because of carbon starvation. However, if stored carbon is the main source for the synthesis of new induced defences, the effect of light limitation on their production would be less relevant. Besides, induced synthesis of both terpenoids and phenolics could be differentially affected as their carbon sources divert from different biosynthesis pathways.

2. Material and methods

2.1. Experimental design

Maritime pine (*Pinus pinaster* Ait.), Scots pine (*P. sylvestris* L.) and Monterey pine (*P. radiata* D. Don) were subjected to experimental herbivory by the pine weevil (*Hylobius abietis* L.) under two light treatments (complete light deprivation and natural sunlight as a control) during a one-week time-course experiment with destructive sampling. The experiment followed a randomized split-plot design replicated in 8 blocks, with light deprivation (two levels: light deprivation, 'dark' and natural sunlight, 'light') as the whole factor, and time exposure to the pine weevil (Time, four levels: 0 (constitutive), 1.5, 3.5 and 7 days) as the split factor. In total, we used 189 plants, corresponding to 8 blocks × 2 light treatments × 4 times of exposure to the pine weevil = 64 plants per pine species (61 in the case of Maritime pine due to lack of enough plant material). Additionally, a 'dark' treatment without weevils was applied to 8 extra plants of each species in order to check for potential side effects of the dark treatment on defensive allocation.

2.2. Plant material, insect rearing and plant culture conditions

Seed sources for Maritime pine, Monterey pine and Scots pine were Massif Landais (France), Astur-Cantabro provenance (Coastal North Spain) and Sierra de Guadarrama provenance (Central Spain Mountains), respectively. Maritime pine is naturally present in southern Europe and northern Africa around the Mediterranean basin (Richardson and Rundel, 1998). Scots pine is the most