



## Original paper

# Differential physiological and genetic responses of five European Scots pine provenances to induced water stress



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## ABSTRACT

*Pinus sylvestris* L. (Scots pine) is the conifer with widest natural distribution area. Portugal constitutes its westernmost limit of distribution. Most of the Portuguese populations were planted but two autochthonous populations were recently ascribed to ‘Serra do Gerês’ (NW Portugal), and seem to be well adapted to the temperate climate. However, the ongoing climate changes may compromise their survival. With this study we intend to evaluate the anatomic-physiological and genetic responses of Scots pine from five European provenances (‘Gerês’, ‘Puebla de Lillo’, ‘Montes Universales’, Germany and Sweden) to three water availability regimes, in order to determine which one(s) present higher resistance to drought. Individuals from ‘Gerês’ presented the highest stability in photosynthetic reactions as well as the better photochemical and metabolic behaviours under drought (T3). Hence, the relative expression ratio of three water stress-responsive genes during drought was lower and gradual in ‘Gerês’, compared to all other provenances, followed by Germany. The results achieved in ‘Gerês’ and Germany provenances are very interesting since they reflected that the native populations of ‘Gerês’ along with the Portuguese Scots pine planted populations with a probable German provenance, have ability and high adaptive potential to respond to situations of water deficit. Moreover, the present genetic and physiological data demonstrated the urgent demand for the conservation of Portuguese Scots pine genetic resources as well as its use in plantation/afforestation of areas where the warming and drought has been affecting the survival of this species.

## 1. Introduction

*Pinus sylvestris* L. (Scots pine) is the conifer with largest natural distribution (Matías and Jump, 2012). The Holocene postglacial history of Scots pine resulted in many possible refuges and colonization routes throughout Europe that strongly influenced its genetic diversity and local adaptation (Matías and Jump, 2012; Matías and Jump, 2012). Portugal constitutes the outermost occidental limit of the species distribution. Most of the Portuguese Scots pine populations were planted during the last century and seem to have a German or Central Europe provenance (Cipriano et al., 2013). Two native Scots pine populations were recently ascribed to ‘Serra do Gerês’ (NW Portugal) (Pavia et al., 2014a). These two native populations seem to present high genomic

plasticity and adaptive potential since previous studies revealed chromosomal irregularities proper of peripheral populations that face environmental constraints (Pavia et al., 2014b); high intra- and inter-population genetic diversity (Pavia et al., 2014a); as well as an abundant natural regeneration (Fernandes et al., 2015). The genomic and phenotypic plasticity of Scots pine have been widely reported also for other provenances. Some authors pointed out that the resilience of some Scots pine provenances to future drought situations is mainly due to the wide distribution area of the species, its genetic diversity and adaptation potential (Seidel and Menzel, 2016).

The increasing concentration of gases with greenhouse effect in the atmosphere causes significant climate changes at the global scale, and a strong impact in the environment and in the socio-economical sector.

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An increase of the average temperature and a reduction of water availability in soils are expected for the South of Europe (Meehl et al., 2007; Kang et al., 2009; Kirtman et al., 2013). Such climate changes will mainly affect the growth and distribution of tree species currently adapted to local conditions, and a considerable loss of *P. sylvestris* habitats are predicted (Taeger et al., 2015). Although the apparent adaptation of the native Portuguese Scots pine populations to the Mediterranean climate, they were never characterized relative to the water stress response, which constitutes one of the major causes of seedling mortality in pine forests (Lorenz et al., 2005; Seidel et al., 2016; Seidel and Menzel, 2016). The reduction of water availability in the soil of pine forests prevents seed germination and hence, the ability of natural regeneration in both natural populations and planted stands. Besides, since the Portuguese Scots pine populations are peripheral regarding its distribution area, the climate changes may affect their survival and the susceptibility of carbon metabolism to biotic and abiotic stresses. Thus, the determination of provenances with high anatomical and physiological resistance to drought could be useful for the definition of strategies of forest management, conservation of genetic resources and improvement. Moreover, and based on logical assumptions, we are confident that pine trees more resistant to abiotic stress would be less susceptible to pests and diseases and would produce high-quality forest products such as wood.

The understanding of the physiological mechanisms and/or genetic basis of resistance to drought in forest species has been pursued by plant biologists (see Newton et al., 1991). The plantlets under this stress may be more susceptible to photoinhibition (Bolhar-Nordenkamp et al., 1989), leading to the overproduction of reactive oxygen species (ROS), which can damage cellular components, including DNA, photosynthetic pigments, proteins and membranes (Mittler, 2002).

Genomics and transcriptomics approaches have been developed in order to unravel drought-responsive genes in conifers (Chang et al., 1996; Heath et al., 2002; Chaves et al., 2003; Dubos and Plomion 2003; Dubos et al., 2003; Watkinson et al., 2003). Despite the complexity of the molecular basis of dehydration tolerance in trees, the expressional studies have allowed the identification of several expressed sequence tags (ESTs) from wood-forming and root tissues of unrelated genotypes of *Pinus* sp. subjected to different water availability regimes. Some of those drought-induced candidate genes belong to the small *lp* multigene family, with at least four members which present a distinct pattern of expression during water deficit stress (Padmanabhan et al., 1997; González-Martínez et al., 2006). The *lp3* candidate gene is expressed predominantly in roots but a constitutive basal level of expression could be detected in stems and needles (Padmanabhan et al., 1997). Later on it was isolated a *lp3* clone in *P. taeda* that was similar to a small family of abscisic acid (ABA)-inducible genes and ripening genes identified in other plant species (Padmanabhan et al., 1997).

Endogenous ABA is rapidly produced during drought causing a triggering a cascade of physiological responses which includes stomatal closure due to its transport to the stomata guard cells through passive diffusion (Osakabe et al., 2014). Since the *abaH* gene codes for abscisic acid and water-stress induced protein, a high expression of this transcript is expected in plants under a water deficit regime.

*PAL1* gene codes for phenylalanine ammonia-lyase (PAL) that catalyses the first step of the phenylpropanoids pathway, as well as the synthesis of several products based on the phenylpropane skeleton, such as hydroxycinnamic acid, stilbenes, and flavonoids, which fulfil many essential roles in higher plants (MacDonald and D'Cunha, 2007). PAL is also one of the most extensively studied enzymes with respect to plant responses to biotic and abiotic stress (Kim and Hwang, 2014).

Other major expressional candidate genes for drought-stress response identified in trees encode cysteine proteases (Tranbarger and Misra, 1996), iron storage proteins (Li et al., 1998), antioxidants (Li et al., 1998; Karpinska et al., 2001), pathogenesis related proteins (Dubos and Plomion, 2001; Dubos et al., 2003) and protein kinases (Dubos and Plomion, 2003; Dubos et al., 2003).

A large number of pine genes respond to water stress but its expression is influenced by both water deficit and genotypic variation (Lorenz et al., 2005; Seidel et al., 2016; Seidel and Menzel, 2016).

With this study, we intend to evaluate the drought resistance of Scots pine individuals from five European provenances: 'Serra do Gerês' (NW Portugal); Sweden; Germany; and Spain ('Puebla de Lillo' and 'Montes Universales') after being subjected to three water availability regimes, based on anatomic-physiological performance and expression profiling of the water stress-responsive genes, *abaH* (abscisic acid and water-stress induced protein), *lp3* (water-deficit-induced protein) and *PAL1* (phenylalanine ammonia-lyase 1), using quantitative real-time PCR (qRT-PCR).

## 2. Materials and methods

### 2.1. Plant material and water availability regimes

Three hundreds seeds of each Scots pine provenance: 'Serra do Gerês' (NW Portugal); Sweden; Germany (Nuthe – Nieplitz, NE Germany); 'Puebla de Lillo' (León, NW Spain) and 'Montes Universales' (CE Spain) were allowed to germinate in Petri dishes containing filter paper moistened with distilled water in the dark at 25 °C for two weeks. However, due to variable germination rates among provenances, only 125 seedlings of each provenance were distributed through five plastic trays containing peat and holes in the bottom, and used for water stress induction. The trays were randomly displayed and maintained in natural daylight in a greenhouse with controlled temperature (25 °C) and relative humidity (50% during the day and 70% at night) at University of Tras-os-Montes and Alto Douro (UTAD). Every week, the trays position was randomly changed.

Three water availability regimes were applied to 20-months-old Scots pine seedlings from the five European provenances. The control seedlings (T1) were hand-irrigated to the field capacity of soil every day. The water availability regime T2 consisted in the daily hand-irrigation with half volume of the field capacity (determined in T1 seedlings), consisting in a moderated water stress. The water availability regime T3 was the most severe treatment since the seedlings were not irrigated (induced drought). The seedlings were daily accompanied in order to detect morphological symptoms of water stress such as chlorotic needles. The water stress experiments ended with the death of half of the T3 seedlings which occurred after 37 d.

During the period of water stress induction, three harvests of needle tissue (consisting in a pool of needles from three to five seedlings of the same provenance) from all treatments were performed. The first harvest (A) occurred in the beginning of the water stress treatments; the second one (harvest B) was after the first detection of 1/3 of the seedlings with water stress signals, and the third harvest (C) was done at the 37th d (end of the water stress experiments). In each harvest, the needle samples were immediately frozen in liquid nitrogen and maintained at –80 °C till the isolation of total RNA.

### 2.2. Physiological and anatomical evaluations

Chlorophyll *a* fluorescence features were measured *in situ* with a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England) at the 37th d (end of water stress induction). Maximum quantum efficiency of photosystem II (PSII) was calculated as  $F_v/F_m = (F_m - F_0)/F_m$  by measuring the fluorescence signal from a dark-adapted pine needles when all reaction centres are open using a low intensity pulsed measuring light source ( $F_0$ ) and during a pulse saturating light (0.7 s pulse of 15,000  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  of white light) when all reactions centres are closed ( $F_m$ ). Pine needles were dark-adapted for 30 min using dark-adapting leaf-clips for these measurements. Following  $F_v/F_m$  estimation, after a 20 s exposure to actinic light (1500  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), light-adapted steady-state fluorescence yield ( $F_s$ ) was averaged over 2.5 s, followed by exposure to

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