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XXXI CICLO DEL DOTTORATO DI RICERCA IN AMBIENTE E VITA

FUNCTIONAL TRAITS AS A TOOL TO PREDICT INVASIVE POTENTIAL OF ALIEN SPECIES IN NATIVE COMMUNITIES

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DOTTORANDO
FRANCESCO PETRUZZELLIS

COORDINATORE
PROF. GIORGIO ALBERTI

SUPERVISORE DI TESI
PROF. GIOVANNI BACARO

CO-SUPERVISORE DI TESI
PROF. ANDREA NARDINI

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Table of contents

Abstract	II
Riassunto	IV
Preface	VI
General introduction	1
Chapter 1	
<i>Study 1</i> - Sampling intraspecific variability in leaf functional traits: practical suggestions to maximize collected information	15
<i>Study 2</i> - A simplified framework for fast and reliable measurement of leaf turgor loss point	44
Chapter 2	
<i>Study 3</i> - Less safety for more efficiency: water relations and hydraulics of the invasive tree <i>Ailanthus altissima</i> (Mill.) Swingle compared with native <i>Fraxinus ornus</i> L.	65
<i>Study 4</i> - Plasticity of functional traits of tree of heaven is higher in exotic than in native habitats	98
Chapter 3	
<i>Study 5</i> - Invasive potential of alien plant species explained by mechanistic traits: a step forward in understanding alien invasion mechanisms	124
General conclusions	149
Publication list	153

Abstract

The spread of alien invasive species (IAS) is occurring at unprecedented rates, causing strong impacts on ecosystems. Indeed, IAS are deemed one of the major causes of loss of biodiversity and habitat simplification worldwide. In this light, finding determinants of invasiveness has become an uprising research topic in plant ecology. Such features are generally described in terms of ‘functional traits’, i.e. morphological, physiological, or phenological characteristics which are measurable at the individual level from the cell to the whole-organism. The research activities described in this Thesis, aimed at highlighting which traits and associated functions might be at the basis on the invasion process, by measuring common functional traits as well as mechanistic traits (with clearer linkage to a physiological function) on a large number of species.

Despite the large use of traits, there are still some critical issues to be solved in trait-based ecology. In the first part of this Thesis, I provided an analysis of the intraspecific variability of two model traits and I suggested a minimum and optimal sampling size to measure functional traits with a desired level of precision and accuracy (Study 1). Mechanistic traits have been seldom included in trait-based studies, because of difficult and time-expensive procedures for their measurement. In this light, in Study 2 I proposed a simplified framework for the measurement of leaf turgor loss point, a key mechanistic trait related to drought resistance.

After addressing these methodological issues, in the second part of this Thesis I focused my research activities on *Ailanthus altissima* (Aa), which is one of the most invasive species in Europe. In Study 3, I compared functional and mechanistic traits of Aa *Fraxinus ornus* (competing Aa in the same areas). The superior fitness of Aa might be related to its lower resistance to drought stress, which was counterbalanced by higher water transport efficiency. I also detected larger phenotypic plasticity of Aa in response to light availability and I wondered whether this feature was also present in individuals growing in its native range (China). The comparison between individuals of Aa growing in its exotic vs native range (Study 4) suggested that novel features related to turgor loss adjustment and the higher plasticity in traits related to leaf-construction costs could increase Aa fitness in response to different environmental conditions in its invasive range.

To test whether the features described for Aa were shared by other IAS, I analysed several functional and mechanistic traits related to resource acquisition, with special reference to water-use strategies, on a large number of herbaceous and woody species (78 native and 15 invasive species) (Study 5). Results showed that IAS were characterised by traits that favour fast-growth, independently of growth form or habitats features. IAS consistently shared lower drought resistance

and denser venation network, which are mechanistic traits directly influencing leaf construction costs and photosynthetic and growth rates. The coordination between construction costs, drought resistance and photosynthesis-related traits, suggested that IAS outperform native species due to higher potential for carbon gain and biomass accumulation, while sharing similar or higher levels of water-use efficiency.

In conclusion, mechanistic traits could provide novel insight in the mechanisms underlying plant invasion. Novel techniques recently developed could allow the measurement of such traits on a wide number of species with reduced cost. Widening the scale of the analyses provided in this Thesis might help in the definition of the ‘Spectrum of functions associated to plant invasion’.

Riassunto

Le specie aliene invasive (IAS) si stanno diffondendo a velocità sempre più elevata, con effetti molto negative a livello degli ecosistemi. Infatti, le IAS sono considerate una delle maggiori cause della perdita di biodiversità e semplificazione degli habitat a livello globale. Di conseguenza, uno dei più grandi obiettivi in campo ecologico è quello di evidenziare le caratteristiche che ne favoriscono la diffusione. Quest'ultime sono generalmente descritte dai 'tratti funzionali', definiti come caratteristiche morfologiche, fisiologiche o fenologiche, misurabili in un individuo dal livello cellulare sino a quello organismico. Lo scopo principale di questa Tesi è di evidenziare i tratti funzionali e meccanicistici (cioè tratti chiaramente associati a specifiche funzioni fisiologiche) alla base del processo di invasione.

Nonostante il largo uso dei tratti funzionali, esistono ancora delle questioni parzialmente risolte riguardanti i metodi di misura. Nella prima parte di questa Tesi, ho quantificato la variabilità intraspecifica di due tratti modello, nell'ottica di definire un minimo e adeguato numero di repliche per misurare i tratti funzionali con una certa accuratezza e precisione (Studio 1). I tratti meccanicistici sono stati scarsamente utilizzati in campo ecologico, probabilmente perché la loro misura spesso richiede procedure difficili e molto lunghe. In questo senso, nello Studio 2 ho proposto un metodo semplificato per misurare il punto di perdita del turgore cellulare, un tratto meccanicistico direttamente associato alla resistenza all'aridità.

Dopo aver cercato di trovare una risposta a domande di natura metodologica, ho concentrato le attività di ricerca su una delle più diffuse specie invasive, *Ailanthus altissima* (Aa). Nello Studio 3, ho confrontato tratti funzionali e meccanicistici di Aa e *Fraxinus ornus* (Fo) (che competono nelle stesse aree). La maggiore fitness associata ad Aa può essere legata alla sua bassa resistenza all'aridità, che è però controbilanciata da una alta efficienza di trasporto dell'acqua. In più, Aa ha mostrato una maggiore plasticità fenotipica di Fo in risposta a differenti condizioni di irraggiamento e nello studio 4 ho testato se questa sia una caratteristica presente anche nell'areale nativo di Aa (Cina). Tale studio dimostra che diverse modalità di aggiustamento del punto di perdita di turgore e una maggiore plasticità dei tratti legati ai costi di produzione delle foglie, possono essere alla base della maggiore fitness di Aa nel suo areale invasivo.

Al fine di testare se i tratti descritti in Aa fossero condivisi da altre IAS, ho esteso l'analisi di tratti funzionali e meccanicistici su un più alto numero di specie (78 native e 15 IAS) sia erbacee che legnose (Studio 5). I risultati dimostrano che le IAS possiedono tratti che favoriscono una rapida crescita, indipendentemente dalla forma di crescita (erbacea o legnosa). Nello specifico, le IAS

sembrano condividere una bassa resistenza all'aridità e una maggiore densità di venature fogliari, che influenzano i costi di produzione delle foglie e i tassi di crescita e di fotosintesi. Il coordinamento tra costi di produzione, livello di resistenza all'aridità e tratti legati alla fotosintesi può suggerire che la superiorità delle IAS possa dipendere da un maggiore capacità di accumulare biomassa pur con una simile o maggiore efficienza dell'uso dell'acqua.

In conclusione, i tratti meccanicistici possono fornire nuove informazioni sui meccanismi alla base del processo di invasione delle IAS. Le nuove tecniche sviluppate per la loro misura potrebbero permettere di aumentare il numero di specie su cui misurare tratti meccanicistici riducendone i costi associati. La possibilità di generalizzare i pattern visti in questa Tesi ad una scala più ampia potrebbe infine portare alla definizione di un generale 'Spettro di funzioni associate al processo di invasione'.

Preface

This thesis provides an analysis of functional and mechanistic traits associated to invasiveness of alien plant species, from the definition of protocols for sampling and measuring functional traits to the analysis of the differences between native and invasive species.

In the introduction, I briefly discussed what functional traits are and the issues still partially unresolved in trait-based ecology. Moreover, I discussed about the definition of invasive species and provided a brief description of the causes and effect of their spread in exotic areas. In the last paragraph of this section I provided a summary of advances on the determination of functional traits associated to invasiveness of alien species.

In the first thesis chapter, I provided an analysis of the intraspecific variability of two model traits and I suggested a minimum and optimal sampling size to measure functional traits with a desired level of precision and accuracy (Study 1). In the second part of this chapter, I provided a simplified framework for the measurement of leaf turgor loss point, a key mechanistic trait related to drought resistance of plant species (Study 2).

In the second chapter, I focused on the differences of functional and mechanistic traits between one invasive species (*Ailanthus altissima*) and one native species (*Fraxinus ornus*) (Study 3). Then, I tested whether functional and mechanistic traits and their associated plasticity were different in *A. altissima* in its native vs exotic range (Study 4).

In the third chapter, I analysed several functional and mechanistic traits related to resource acquisition, with special reference to water-use strategies, on a large number of species (78 native and 15 invasive species), with the aim to highlight novel features underlying the invasive nature of alien species in natural habitats (Study 5).

The conclusions chapter provides a general overview and synthesis on the key findings across the five studies of the Thesis, suggesting further analysis for future research.

General Introduction

The concept of functional traits in plants

The concept of trait dates back since Darwin (1859), who defined traits as proxies of organisms' performance. Since then, the improved research activities in community (Grime, 1974; Petchey & Gaston, 2002; McGill *et al.*, 2006) and ecosystem (Chapin *et al.*, 1993; Lavorel & Garnier, 2002) ecology moved the concept of trait beyond the original definition, and trait-based approaches are now used in studies ranging from organisms to ecosystems level. Despite the large use of traits in ecology, their definition was not always clear and unambiguous. For instance, Petchey *et al.* (2004) measured several so-called "plant traits" ranging from plant level (i.e. stature of a plant, seed mass) to plot level (i.e. vegetation cover, canopy height). In the same year, Eviner (2004) quantified ecosystem properties on the basis of "plant traits" as plant standing biomass, soil conditions and microbial phosphorus. As pointed by Violle *et al.* (2007), using the term "trait" to designate such different aspects of a community could lead to an unproductive confusion in the identification of mechanisms underlying community structure and ecosystem functioning. They proposed to define traits as "any morphological, physiological or phenological feature measurable at the individual level, from the cell to the whole-organism level, without reference to the environment or any other level of organization".

Based on this definition, different categories according to questions addressed have been proposed to classify plant traits (see Tab. 1 for an example of trait categories).

<i>Trait category</i>	
Functional traits	- Physiological process (e.g. photosynthesis, respiration)
	- Life-history process (e.g. germination, growth, reproduction)
	- Individual fitness
	- Performance measures
Performance traits	- Fitness components (growth reproduction, survival)
Response traits	- Response of a plant to environmental changes
Effect traits	- Effect of a plant on ecosystem functioning

Tab. 1. Trait categories proposed in Violle *et al.*, 2007.

In particular, “functional traits”, defined as morpho-physio-phenological traits which impact plant fitness indirectly via their effects on growth, reproduction and survival (Violle *et al.*, 2007), have been increasingly used in trait-based studies. The term “functional” refers to the linkage between functional traits and plant functions. For instance, specific leaf area (SLA), the ratio between leaf area and its oven dry mass, is one of the most used functional trait and it is well-related to plant functions as relative growth rate and photosynthetic rate (Poorter & Remkes, 1990). Moreover, plants adapted to arid and poor-nutrients habitats usually show thicker and smaller leaves with higher lifespan and lower values of SLA (Pérez-Harguindeguy *et al.*, 2013). The high success of functional traits was mainly determined by the existence of coordination between some traits, which in turn allowed the detection of important trade-offs among different physiological functions. Among these, one of the most important is represented by the Leaf Economic Spectrum (LES, (Wright *et al.*, 2004), which describes the coordination between leaf costs and photosynthetic traits. The use of functional traits in a large number of studies allowed to address a wide range of ecological issues, such as the determination of ecosystems level processes (Díaz & Cabido, 2001; Escudero & Valladares, 2016), in modelling vegetation changes under different environmental pressure (Noble & Gitay, 1996; Hobbs, 1997) or in managing ecosystem services (Lavorel & Garnier, 2002). In the last years, global datasets (e.g. TRY database, Kattge *et al.*, 2011) have been developed favouring the determination of patterns of functional traits variation on a global scale. In their seminal paper, Díaz *et al.* (2016) described the global spectrum of plant form and function analysing coordination and trade-offs between six functional traits. More recently, Butler *et al.* (2017) mapped the global distribution and variability of three functional traits related to photosynthesis and foliar respiration. These are just few examples of the variety of analyses made possible from the advent of global databases, and the inclusion of novel traits in such tools holds promises to furtherly deepen our knowledge on plants-environment interaction at global scale.

Unresolved issues in trait-based ecology

Despite the large use of functional traits, there are still some critical issues to be solved in trait-based ecology. The first is related to the cost-benefits of different sampling strategies to capture the variability of functional traits between and within communities. The estimation of the functional structure of a given community relies on accurate measurements of the trait values of the individuals that compose it (Carmona *et al.*, 2015). The best choices would be to sample all the individuals present in the studied community, but clearly this is not possible. The common practice is to sample

a reduced number of individuals and average traits values measured on this reduced sample. However, the choice of the best sampling strategy to measure functional traits is still an actual debate in trait-based ecology. Sampling a reduced number of individuals from a community could in fact lead to underestimation of trait's variability, thus potentially introducing bias in the analysis. To overcome this problem, standardized protocols have been recently introduced providing useful advice on how to measure traits, thus allowing comparison of the variability of different studies and to perform general inferences on ecological mechanisms (Cornelissen *et al.*, 2003; Pérez-Harguindeguy *et al.*, 2013). Moreover, these protocols support decisions for the selection of an appropriate sample size depending on the purpose of the study and on the desired precision level. Before protocols application, however, optimal sampling parameters should be assessed on the basis of the extent of intraspecific variability (ITV) in the study area (Cornelissen *et al.*, 2003). Indeed, recent papers claimed that intraspecific variability (ITV) should be taken into account when measuring functional traits (Albert *et al.*, 2010; Albert, 2015), at least for SLA, leaf dry matter content (LDMC) and chemical traits (Siefert *et al.*, 2015). According to Albert *et al.* (2011), ITV could be divided in: (1) between population level variability (ITV_{POP}); (2) between individual variability (ITV_{BI}); (3) within individual variability (ITV_{WI}). ITV_{WI} is defined as the feature of traits that vary within individuals (Albert *et al.*, 2011) and could arise due to genotypic, phenotypic, or ontogenetic processes (Messier *et al.*, 2010; Valladares *et al.*, 2014). Large attention had been dedicated to the first two levels, while the latter had been scarcely investigated in a rigorous way.

Another issue still partially unresolved regards the choice of traits that must be included to functionally characterize a species or a community. Recently, Brodribb (2017) suggested the dichotomy between “mechanistic” traits, which comprehends plant's features clearly associated to a physiological process, and general functional traits (such as SLA or its reciprocal leaf mass per unit area, LMA), which rather represent “syndromes” that could be driven by several different physiological functions and associated trade-offs. Among mechanistic traits, water relation parameters and hydraulics (e.g. Ψ_{tp} , the water potential at turgor loss point, or Ψ_{50} , the water potential inducing 50% loss of hydraulic conductivity) have been used to model plant species distribution (Costa-Saura *et al.*, 2016; Larter *et al.*, 2017), and they were shown to correlate with growth rate and risk of mortality under drought (Fan *et al.*, 2012; Choat *et al.*, 2012). Despite the advantages promised by mechanistic traits, they have been seldom included in trait-based studies, probably because of difficult and time-consuming procedures that often requires expensive instrumentation for their measurement. However, in the last years, novel techniques have been developed to simplify procedures for some mechanistic traits measurements (Bartlett *et al.*, 2012; Brodribb *et al.*, 2016), thus favouring the inclusion of these traits in future studies.

Invasive species around the globe

In the last decades, a novel branch called “Invasion Ecology” raised in plant ecology to study determinants, patterns and consequences of biological invasion (Pyšek & Richardson, 2006). Indeed, a growing number of species spread in areas where they usually were not present. These species are the so-called “alien species”, which are defined as any taxa intentionally or accidentally introduced by humans in a given area (Richardson *et al.*, 2000; Pyšek & Richardson, 2006). However, most introduced species fail to establish away from sites where they are introduced but need continuous human-mediated introduction to persist in their non-native areas (“casual alien species”) (Richardson *et al.*, 2000). On the contrary, “Invasive alien species” (IAS) are alien species producing large number of propagules able to form self-sustaining populations far away from their parents plants with the potential ability to alter ecosystem features (Richardson *et al.*, 2000). The introduction of alien species dates back to the start of human activity, but the recent improvement of trades and transport network after worldwide globalization exponentially increased the number of introduction pathways, and thus the rate of invasion (Hulme, 2009). Many studies reported that the number of IAS occurring in a given area is positively correlated to the degree of urbanization and the magnitude of human activities (Tordoni *et al.*, 2017). Human involvement is a crucial aspect to describe the invasion process for two main reasons. First, propagule pressure, that is the number of individuals (or viable propagules) released into a region where they are not native (Lockwood *et al.*, 2005), is higher in urban areas. Secondly, human-disturbed areas are usually nutrient enriched, thus favouring colonization and persistence of IAS (Davis *et al.*, 2000). At global scale, islands are on average more invaded than mainland (Lonsdale, 1999); undisturbed tropical forests are less invaded than temperate mainland regions (Rejmánek *et al.*, 2013) and mesic environments seem to be more susceptible to invasion than extreme ones (with some exceptions) (Sax, 2001).

The colonization by alien species have strong impacts, both on ecosystem and socio-economic levels. Regarding natural habitats, IAS are considered a major threat to biodiversity, as their presence is often associated to modifications of natural ecosystems and loss of species richness of invaded communities (Hejda *et al.*, 2009). The strong impacts on economics are mainly associated to reduced crops and forestry yields (Pimentel *et al.*, 2001), along with costs associated to the management of invaded areas. Direct and indirect effects of human health have been also reported, as some IAS could cause allergic reactions or could be vectors for disease.

All these factors contributed to aim the research activities in Invasion Ecology towards a fully comprehension of the mechanisms responsible of the invasion by alien species, and the

functional approach have been claimed as the most proper to achieve this goal. In this light, finding determinants of invasiveness of alien species is the major aim of my PhD thesis.

A functional trait approach to study plant invasion

The number of studies on plant invasion including functional traits steeply increased after late 1990s (Pyšek & Richardson, 2007). The most used approach to highlight determinants of invasiveness consists on multispecies comparisons between native and/or non-invasive alien species vs IAS (Daehler, 2003; van Kleunen *et al.*, 2010; Funk & Zachary, 2010). The first approach (native vs IAS) aims at highlighting traits enhancing the ability of IAS to increase in abundance over native species, while the second one (non-invasive alien vs IAS) would emphasise traits promoting IAS invasive potential (Pyšek & Richardson, 2007). Studies comparing functional traits of species with different growth forms and biogeography have revealed consistent patterns in traits' differences between IAS and native/non-invasive alien species, with some exceptions. In their review, Pyšek & Richardson (2007) reported that IAS consistently grow taller and faster than native/non-invasive alien species; they had higher photosynthetic rates, higher SLA and higher efficiency in seed dispersal. In a more recent meta-analysis including more studies and traits than the previous, van Kleunen *et al.*, (2010) showed that IAS have higher values of functional traits related to leaf area or leaf biomass allocation, shoot biomass allocation, growth rate and size, thus supporting previous results. These results suggested that IAS occupy a position within the leaf economic spectrum (Wright *et al.*, 2004) that favours fast growth (Leishman *et al.*, 2014). However, despite the great number of studies and substantial meta-analytic efforts, a unique set of traits responsible for the invasive potential of some plant species has not been identified to date. For instance, Daehler (2003) found that growth rates, competitive ability, or fecundity did not differ between IAS and native species. They concluded that differences between the two groups of species often depend on growing conditions. These discrepancies suggest that invasion is a context dependent process and that multiple suites of traits could promote invasiveness in different environments (van Kleunen *et al.*, 2015; Funk *et al.*, 2016). In this light, different hypotheses have been proposed to explain the success of invasive plant species. The “try harder” hypothesis (Tecco *et al.*, 2010) suggests that successful aliens deal better with the local conditions than resident species, expressing a set of functional traits different from those of native species. On the other hand, the “join the locals” hypothesis (Tecco *et al.*, 2010) predicts similarities among alien invasive and native species.

Another feature that might be associated to invasiveness of alien species is phenotypic plasticity (Heberling *et al.*, 2016), defined as the ability of an organism to develop different phenotypes under different environmental conditions (Nicotra *et al.*, 2010). High phenotypic plasticity has been suggested to favour colonization and rapid spread of alien species over large and heterogeneous areas (Godoy *et al.*, 2011). However, only few studies reported higher phenotypic plasticity in IAS, while in others no differences between IAS and native species were detected (Davidson *et al.*, 2011; Godoy *et al.*, 2011). More recently, Heberling *et al.* (2016) have proposed comparing functional traits of alien invasive species in their native vs exotic habitat as a tool to investigate their invasive potential. Up to date, only few studies have investigated alien invasion on the basis of this approach (van Kleunen *et al.*, 2011; Leishman *et al.*, 2014; Taylor *et al.*, 2016; Heberling *et al.*, 2016). Most of them reported that IAS had higher specific leaf area (SLA), better water content regulation and higher relative growth rate (RGR) in their exotic vs native range. Further comparisons are still needed to test the consistency of this pattern and to assess whether these range-level differences have a genotypic or phenotypic basis.

Mechanistic traits have been seldom included in studies comparing IAS and native species, and results are still somehow contrasting. In few studies, IAS had higher resistance to drought than native species, as water potential inducing 50% loss of conductivity (Ψ_{50}) was lower (Yazaki *et al.*, 2010; Crous *et al.*, 2012). On the contrary, Zeballos *et al.* (2014) showed that wood density (a proxy of Ψ_{50}) of invasive tree species was on average lower than that of native trees, thus suggesting lower drought stress resistance in IAS. It is unlikely that hydraulic or other single mechanistic traits explain alone invasiveness of alien species, but the coordination between mechanistic, cost-associated and performance related traits could be at the basis of the differences between IAS and native species.

Aims

Due to the rapid spread of IAS worldwide, finding the determinants of invasiveness is fundamental to predict which species will likely become invasive and to plan adequate management actions. Hence, the major aim of this Thesis is to highlight which functional and mechanistic traits are associated with the invasive potential of alien species.

The choice of the sampling strategy that should be adopted when measuring functional traits is still an opening issue in trait-based ecology. Moreover, the need to include mechanistic traits in trait-based analyses requires the development of novel and simpler techniques for their measurements. In this light, in the first part (Chapter 1) of this Thesis I aimed at:

- a) assessing the minimum and optimal sampling size in order to take into account the intraspecific variability of two model traits (one functional and one mechanistic) (Study 1);
- b) proposing a simplified framework to estimate the leaf water potential at turgor loss point, a key mechanistic trait related to drought resistance (Study 2).

The second aim of this thesis (Chapter 2) was to highlight functional and mechanistic traits associated to the invasiveness of *A. altissima* (Aa), which is one of the most invasive species in Europe. Specifically, I aimed at:

- a) testing for inter-specific differences between Aa and *Fraxinus ornus*, which competes with Aa in the same areas, in terms of performance in resource use and acquisition, and plasticity of functional and mechanistic traits in response to light availability (Study 3);
- b) testing whether functional traits values and their associated plasticity in Aa are higher in exotic rather than native habitats (China) (Study 4).

In the last chapter (Chapter 3, Study 5), I analysed several functional and mechanistic traits on a large assemblage of woody and herbaceous native and invasive species (93 species in total, 78 natives and 15 IAS), co-occurring in three different habitats with contrasting ecological conditions. Specifically, I tested whether:

- a) IAS generally share an acquisitive strategy irrespectively of growth form or habitat features;
- b) mechanistic traits related to drought tolerance and leaf venation were higher in IAS than in native species;
- c) mechanistic and functional traits are coordinated in trade-offs.

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Chapter 1

Study 1

Sampling intraspecific variability in leaf functional traits: practical suggestions to maximize collected information

Francesco Petruzzellis^{1*}, Chiara Palandrani^{1,2}, Tadeja Savi¹, Roberto Alberti¹, Andrea Nardini¹ and Giovanni Bacaro¹

¹University of Trieste, Department of Life Sciences, Via L. Giorgieri 10, 34127 Trieste, Italy

²University of Udine, Department of Agricultural, Food, Environmental and Animal Sciences, via delle Scienze 206, 33100, Udine, Italy

*Corresponding author

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Summary

1. The choice of the best sampling strategy to capture mean values of functional traits for a species/population, while maintaining information about traits' variability and minimizing the sampling size and effort, is an open issue in functional trait ecology.
2. Intraspecific variability (ITV) of functional traits strongly influences sampling size and effort. However, while adequate information is available about intraspecific variability between individuals (ITV_{BI}) and among populations (ITV_{POP}), relatively few studies have analysed intraspecific variability within individuals (ITV_{WI}).
3. Here, we provide an analysis of ITV_{WI} of two foliar traits, namely specific leaf area (SLA) and osmotic potential (or π), in a population of *Quercus ilex* L. We assessed the baseline ITV_{WI} level of variation between the two traits and provided the minimum and optimal sampling size in order to take into account ITV_{WI}, comparing sampling optimization outputs with those previously proposed in the literature.
4. Different factors accounted for different amount of variance of the two traits. SLA variance was mostly spread within individuals (43.4% of the total variance), while π variance was mainly spread between individuals (43.2%).
5. Strategies that did not account for all the canopy strata produced mean values not representative of the sampled population. The minimum size to adequately capture the studied functional traits corresponded to 5 leaves taken randomly from 5 individuals, while the most accurate and feasible sampling size was 4 leaves taken randomly from 10 individuals.
6. We demonstrate that the spatial structure of the canopy could significantly affect traits variability. Moreover, different strategies for different traits could be implemented during sampling surveys.
7. We partially confirm sampling sizes previously proposed in the recent literature, and encourage future analysis involving different traits.

Keywords

Osmotic potential; PERMANOVA; Precision; *Quercus ilex*; SLA; Variance partitioning

Abbreviations

BTV	Interspecific variability (or between species variability)
CV	Coefficient of variation
FD	Functional diversity
ITV	Intraspecific variability
ITV _{BI}	Intraspecific variability between individuals
ITV _{POP}	Intraspecific variability among populations
ITV _{WI}	Intraspecific variability within individuals
LDMC	Leaf dry matter content
SE	Standard error
SLA	Specific leaf area
π	Osmotic potential

Introduction

Plant traits are defined as any morphological, physiological or phenological feature measurable at the individual level, from the cell to the whole-organism level (Violle *et al.*, 2007). In the last decades, plant functional traits have been widely included in trait-based studies. They are defined as any trait which impacts fitness indirectly via its effects on growth, reproduction and survival (Violle *et al.*, 2007) and reflect the trade-offs among different physiological and ecological functions (Lavorel *et al.*, 2007, Díaz & Cabido, 2001; McIntyre *et al.*, 1999). A wide range of ecological issues can be conveniently addressed using a functional trait approach. For example, functional diversity helps addressing questions about determination of ecosystems level processes (Díaz & Cabido, 2001; Escudero & Valladares, 2016) or to disentangle processes underlying invasions by alien species as well as invasion resistance (Drenovsky *et al.*, 2012; Funk *et al.*, 2008). Furthermore, assessment of species traits can be used in modelling vegetation changes under different environmental pressure (Hobbs, 1997; Noble & Gitay, 1996) and in managing ecosystem services (Lavorel & Garnier, 2002).

The number of studies based on the analysis of functional traits is steadily increasing in recent years. Yet, there are still some critical issues to be solved, related to the cost-benefits of different sampling strategies to capture the variability of functional traits between and within communities. A very actual debate in “trait-based ecology” is focused on the importance and relative magnitude of interspecific (BTV, B stands for between species) and intraspecific variability (ITV) (Albert *et al.*, 2010). While sources and effects of BTV on functional-trait based studies have been widely investigated (Díaz *et al.*, 2004; Wright *et al.*, 2005), the contribution of ITV to the total variability of a trait has been underestimated (Violle *et al.*, 2012). ITV is defined as the overall variability of trait values and trait syndromes (set of trait values including trait trade-offs) expressed by individuals within a species (Albert *et al.*, 2011). The commonly accepted paradigm is that the BTV is much larger than ITV (Albert *et al.*, 2011), leading to the so-called $ITV < BTV$ assumption (Garnier *et al.*, 2001; Wilson *et al.*, 1999). Therefore, ITV has been often considered negligible. Recently, a growing number of studies has shown that this assumption is not always correct (Albert, 2015; Albert *et al.*, 2010; Siefert *et al.*, 2015) and provided frameworks and suggestions on procedures to account for ITV. For example, Siefert *et al.* (2015) demonstrated that different traits have different ITV magnitude and that ITV must be taken into account when specific leaf area (SLA), leaf dry matter content (LDMC) or leaf chemical traits are included in functional traits based studies. Albert *et al.* (2011) also proposed a framework to assess when and how ITV should be taken into account.

According to Albert *et al.* (2011), ITV can be decomposed into three levels: i) between population level variability (ITV_{POP}); ii) between individual variability (ITV_{BI}); iii) within individual variability (ITV_{WI}). Large attention had been dedicated to the first two levels, while the latter had been scarcely investigated in a rigorous way. ITV_{WI} is defined as the feature of traits to vary within individuals (Albert *et al.*, 2011) and could arise due to genotypic, phenotypic or ontogenetic processes (Messier *et al.*, 2010; Valladares *et al.*, 2014). In particular, significant micro-environmental gradients can occur even within the canopy of single trees (Niinemets, 2016), thus affecting leaf traits values magnitude and distribution within a single individual.

Leaves display a series of attributes that are linked to specific functions (functional leaf traits) and/or show responses to biotic and abiotic stress factors (stress response traits), which can be subdivided into: (a) morphological traits; (b) chemical traits; (c) physiological traits; (d) syndromes. The analysis of functional leaf traits is a useful tool for tree species and provenance phenotyping, due to the adaptation of trees to environmental stress (Gratani *et al.*, 2003). Additionally, functional leaf traits can be used as response factor in long term and large spatial scales surveys of forest and crops conditions (Apgaua *et al.*, 2017; Martin *et al.*, 2017). Indeed, leaf sampling and analysis is a tool applied in research projects and monitoring programs (Rautio *et al.*, 2010), but sampling adequate numbers of leaves can be a difficult, time-consuming and costly task, because of horizontal structure of forest making samples difficult to access.

Under this perspective, the choice of traits and the number of replicates to be measured to capture the mean value (and the associated variability) of leaf traits parameters for target populations remains an issue only partially solved in trait-based ecology. In this perspective, standardized protocols are mandatory to compare the variability of different studies and to perform general inferences on ecological mechanisms. In the last 10 years, multiple handbooks of protocols (e.g. Cornelissen *et al.*, 2003; Pérez-Harguindeguy *et al.*, 2013) have listed the most used plant traits and proposed sampling standards for each of them (i.e., how to measure traits, the minimum and preferred number of replicates, etc). Moreover, these protocols support decisions for the selection of an appropriate sample size depending on the purpose of the study and on the desired precision level. Before protocols application, however, optimal sampling parameters should be assessed on the basis of the extent of ITV in the study area (Cornelissen *et al.*, 2003). Here, we provide an analysis of ITV_{WI} of two foliar traits, one “functional” (SLA) and one “mechanistic” (osmotic potential or π), in a population of *Quercus ilex* L. (Holm oak). According to Brodribb (2017), mechanistic traits are characterized by a clear association with a specific physiological function, while general functional traits (such as SLA) rather represent a ‘syndrome’ that can be associated to different physiological functions and associated trade-offs. Despite the deeper physiological

insights provided by mechanistic traits than by functional ones, they are more difficult to measure (in terms of costs and time) and, therefore, are scarcely included in trait-based studies. Consequently, very little is known about mechanistic traits variability, while several studies investigated variation of soft traits at different ecological scales (Messier *et al.*, 2010). Anyway, recent studies have proposed new techniques for time- and cost-effective estimation of different mechanistic traits (e.g. Bartlett *et al.*, (2012a) for osmotic potential, or Skelton *et al.* (2017) for vulnerability to xylem embolism).

Based on an intensive spatially explicit sampling of the two described foliar traits, this study is aimed at: *i)* assessing the baseline ITV_{WI} level of variation between the two traits; *ii)* assessing the minimum and optimal sampling size in order to take into account ITV_{WI} ; *iii)* comparing sampling optimization outputs with those previously proposed in the literature. *iv)* proposing practical advises and sampling scenarios useful for ecologists and biologists to plan traits sampling campaigns.

Materials and methods

Study area and sampling design

This study was performed in the Cernizza woodland (45°46'37.4" N, 13°45'21.2" E), an area located in the Karst region (NE Italy) at 40 m a.s.l. The climate is humid-temperate with higher precipitation in October-November and a relatively dry spell in August-September (Furlanetto, 2003). The woodland hosts typical Mediterranean evergreen species, including *Quercus ilex*, *Phyllirea latifolia*, *Ostrya alba*, *Smilax aspera*, *Ruscus aculeatus* and *Rubia peregrina* (Del Favero & Poldini, 1998).



Fig. 1 Individual of *Quercus ilex* in the study area.

Q. ilex (Fig. 1) is the dominant woody species and occurs in different environmental conditions due to the heterogeneous substrate of the study area (Nardini *et al.*, 2016). For these reasons, we choose *Q. ilex* as the study species. We first identified three areas (0.6 ha each), approximately 100 m apart from each other and characterized by the highest density of *Q. ilex* according to a recent map by Furlanetto (2003) (Fig. S1). We extrapolated the centroid of each area using the software Quantum Gis (v. 2.12.0 – Lyon. QGIS Development Team, 2015) and the *Q. ilex* individual closest to the centroid represented the centre of a 10x10 m quadrat. We sampled

every individual of *Q. ilex* with trunk diameter at breast height (DBH) ≥ 5 cm within each quadrat. In total, we sampled 34 individuals from three quadrats.

The sampling procedure was designed in order to collect as the highest possible (given time and cost constraints) number of leaves within all the individuals in each quadrat. Clearly, a complete census even of a single crown is unrealistic. Hence, we realized an intensive sampling effort consisting in 12 pairs of leaves for each individual. Specifically, we sampled 12 twigs from each individual and one leaf pair was selected from each twig. We sampled leaf pairs because the measurement of the selected traits (see below) imply samples destruction. With the aim to capture as much variability as possible within each considered individual, each tree was divided in two height classes (“a” from base to 2.5 m, “b” from 2.5 m to the top) and in two vertical strata (external = E, internal = I). A total of 6 leaf pairs were selected for each height class; 3 pairs of them were external leaves (E) while the other 3 were internal leaves (I). The exposure (north, south, east, west) of the leaf pairs along the canopy was randomly assessed according to the following scheme. We generated a random series of number from 0 to 360. When this number was between 45 and 135 ($45 < x < 135$) sampled leaf pairs should be exposed to east; when $135 < x < 225$ to south; $225 < x < 315$ to west and $315 < x < 45$ to north. Such stratification was designed to assess the contribution of different leaf position in the canopy to the total variance of the traits, as micro-environmental gradients within the canopy could affect leaf traits values (Niinemets, 2016). Twigs bearing leaf pairs were detached, wrapped in cling film, put in humid sealed plastic bags and stored in coolbags until measurements in the laboratory. A total of 408 leaves from 34 different individuals were sampled from all the 3 quadrats in 3 different days, two in December 2015 and one in January 2016. The number of individuals per sampling unit differed, as expected, depending on the number of different trees occurring in each quadrat (5 individuals in the first quadrat; 17 individuals in the second quadrat; 12 individuals in the third quadrat). All data are deposited in Supporting Information.

Measurements of leaf traits

As mentioned above, SLA and π were measured for each leaf pair.

SLA was calculated as the ratio between fresh leaf area and its dry weight and it is expressed in $\text{mm}^2 \text{mg}^{-1}$.

$$\text{SLA} = (\text{Leaf Area})/(\text{Leaf Dry Weight}) [\text{mm}^2 \text{mg}^{-1}]$$

SLA is generally considered a “soft” structural trait, well-related with relative growth rate and photosynthetic rate (Poorter & Remkes, 1990). Plants adapted to arid and poor-nutrients habitats usually show thicker and smaller leaves with higher lifespan and lower values of SLA

(Pérez-Harguindeguy *et al.*, 2013). Fresh leaves were scanned using a scanner and leaf area was measured using the software ImageJ (Schneider *et al.*, 2012). Leaves were then put in the oven for 48 h at 72°C and then leaf dry weight was measured using an analytical balance.

The osmotic potential (π) is considered a mechanistic trait, due to the time-consuming procedures and to the specific instruments required for its measurements (Cornelissen *et al.*, 2003). The standard method for the measurement of π is based on the elaboration of leaf water potential isotherms (or pressure-volume curves) as described by Tyree & Hammel (1972). Recently, Bartlett *et al.* (2012a) proposed an alternative procedure to measure π using vapour-pressure osmometry of freeze-thawed leaf discs. We used this method, with some modifications from the protocol proposed by Bartlett *et al.* (2012a). Fresh leaves (without the petiole) were roughly crumbled and sealed in cling film. Then they were immersed in liquid nitrogen (LN₂) for 2 min. Leaves (still sealed in cling film) were then ground to the smallest possible size and stored in sealed plastic bottles at -20°C until measurements. Finally, π was measured using a dew point potentiometer (Model WP4, Decagon Devices Inc., Pullman, Washington, USA) within two weeks after samples preparation.

Partitioning of spatial variability

The first goal of our analysis aimed at assessing the spatial variation of ITV of the two measured traits. Hence, we performed a partitioning of spatial variability of SLA and π . Specifically, we assessed the variation in the two traits across 4 hierarchical organizational levels, namely quadrat (3 levels, fixed factor), individual (34 levels, fixed nested within quadrat), height class (2 levels, h_class, fixed) and position within the canopy (2 levels, E/I, fixed) through a multivariate analysis of variance by permutation (PERMANOVA, Anderson, 2001; McArdle & Anderson, 2001). The following interaction terms were tested: quadrat*h_class, quadrat*E/I, h_class*E/I, individual (quadrat)*h_class, individual (quadrat)*E/I, quadrat*h_class*E/I and individual(quadrat)*h_class*E/I. Euclidean distance was used as multivariate distance in this analysis. The percentage of variance explained by each factor was obtained by dividing the sum of squares (SS) calculated on the differences of distances from centroid of each factor by the total sum of squares.

The components of multivariate variance were tested for statistical significance with respect to 999 permutations of residuals under a reduced model (Anderson, 2001), using an a priori chosen significance level of $\alpha = 0.05$.

The analysis was computed using software PRIMER (Clarke & Gorley, 2006) including the add-on package PERMANOVA+ (Anderson *et al.*, 2008).

Selection of most adequate sampling strategy and minimum and optimal sampling size

The second goal of our study was to provide the most adequate sampling strategy to measure SLA and π . We aimed at selecting the sampling strategy that minimize the number of leaves and individuals (from here sampling size) needed to estimate traits values with desired precision and accuracy.

First, we calculated the minimum precision required to estimate the two traits adequately. Hence, we randomly resampled an increasing number of leaves (from 1 to 408) from the dataset, each time calculating the standard error (SE) as a precision measurement. The higher the SE was, the lower the precision was. We run the simulation 4999 times for each number of leaves considered and, at the end, we were able to construct the relationship between SE and number of leaves considered. We assumed that the minimum desired precision corresponded to the SE value at the flex point (SE_{min}) of this relationship. In fact, after this point, the relationship became linear and the slope slightly changed. The flex point was calculated using segmented function from package “SEGMENTED” (Muggeo, 2008) for R software (R Core Team, 2015).

Secondly, we formulated 15 different sampling strategies adopting different selection criteria for the choice of leaves and individuals, constrained on the different levels of spatial organization (Fig. 2, Table S1). RANDOM strategy was the less complex strategy, as leaves and individuals were sampled randomly, discarding all the spatial levels. In Q_fixed, an intermediate complex strategy, leaves were sampled randomly from an equal number of individuals within each quadrat. Finally, in stQ_FIXED, the most complex strategy, a fixed number of leaves were sampled from each canopy stratum (h_class and E/I) and from a fixed number of individuals per quadrat. All the strategies are resumed in Tab. S1. For each sampling strategy, we resampled an increasing number of leaves and individuals, starting from 1 leaves from 1 individual (“STRATEGY”_1_1) to

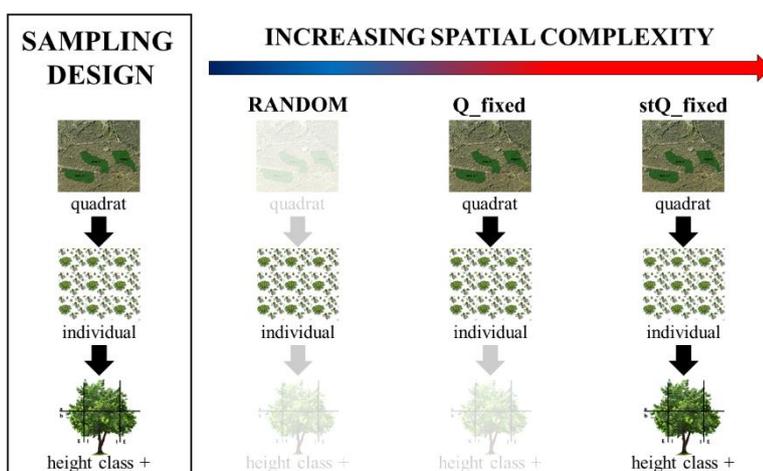


Fig. 2 Illustration of sampling hierarchy (left boxes) and three examples of sampling strategies with different spatial complexity tested in this study.

12 leaves from 34 individuals (“STRATEGY”_34_12). This analysis was conducted using mstage function from SAMPLING package (Tille & Matei 2008) for R software. In this way, we were able to test not only different sampling strategies, but also to select the minimum and optimal sampling size (e.g. number of individuals and leaves from each individual) to

estimate SLA and π values. We simulate each possible combination of sampling size in each sampling strategy 4999 times, each time calculating SLA and π mean values, standard error (SE) and coefficient of variation (CV).

We then organized the selection of the most adequate sampling strategy and minimum and optimal sampling size in 2 steps. In the first step, we discarded all the strategies not meeting the following criteria:

- a) Sampling strategy should produce a mean value of the two traits ($SLA_{\text{strategy}}, \pi_{\text{strategy}}$) that lies within the C.I. of the mean value calculated on the whole dataset ($SLA_{\text{whole_data}}, \pi_{\text{whole_data}}$);
- b) Sampling strategy should have a mean SE equal or lower SE_{min} , otherwise it was considered an unprecise strategy.

In the second step, we assessed the minimum and optimal sampling size of the sampling strategy selected as described above. More in detail, we measure the accuracy of each sampling size calculating the standardized deviations of the traits values estimated with each sampling size from $SLA_{\text{whole_data}}$ and $\pi_{\text{whole_data}}$:

$$S = |X_m - \check{X}_{\text{whole_data}}| / SD(\check{X}_{\text{whole_data}})$$

Where:

S= standardized deviation from mean value; $X_m = \pi$ or SLA mean value of the corresponding sampling size; $\check{X}_{\text{whole_data}} = \pi$ or SLA mean value of the original dataset; $SD(\check{X}_{\text{whole_data}}) = \pi$ or SLA standard deviation of the mean value. Sampling size that minimized the standardized deviation of the two traits were considered the most accurate.

At the end of this selection process, we were able to select the most adequate sampling strategy and minimum and optimal sampling size measuring their precision and accuracy.

Results

Factors expressing the spatial arrangement of leaves in the sampled areas, namely quadrat, individual, height class (h_class) and external/internal leaves (E/I) significantly affected variability of SLA and π (Table 1), and explained ~64% of the total variance in SLA measurements and ~60% of the total variance in π (Fig. 3). Factors related to the spatial structure of the canopy (h_class, E/I and their interactions with other variables) accounted for 43.4% of the total variance of SLA, while they explained ~23% of the total variance of π . On the other hand, factors related to the distribution of individuals (quadrat, individual and their interactions with other variables) accounted for ~20% of the variance of SLA, while they explained 43.2% of the total variance of π .

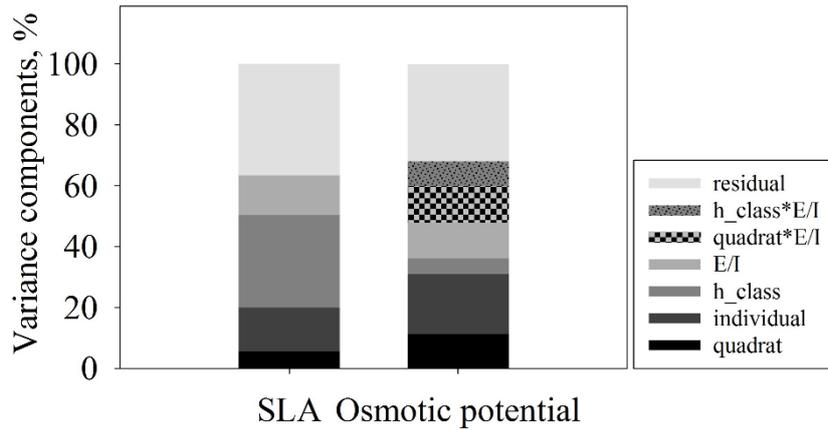


Fig. 3 Estimated components of variance (expressed as percentages) in specific leaf area (SLA, mm²/mg) and osmotic potential (π , -MPa) values calculated for each factor

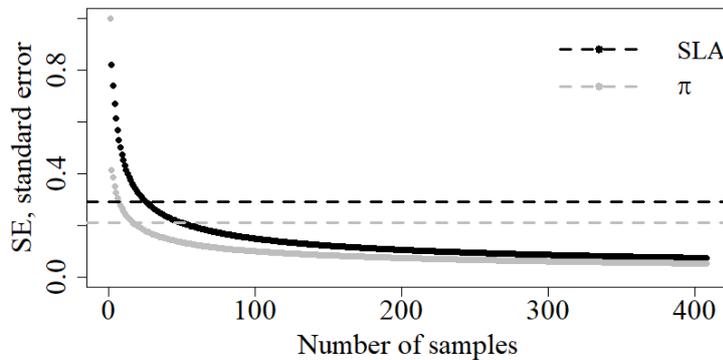


Fig. 4 Relationship between number of samples considered and associated standard error (SE) of SLA (black points) and π (gray points)

Fig. 4 shows the relationship between the standard error, as a precision measurement, calculated on the 2 traits taking into account an increasing number of leaves. Fig. 5 summarize the mean standard error and mean values of the two traits obtained resampling data adopting the tested sampling strategy. Strategies were not considered representative of the sampled population if the mean values of SLA and π were out of C.I. range (Table 2) and/or SE higher than SE_{min} . Only 4 of the 15 tested resampling strategies satisfied these criteria: RANDOM, Q_fixed, stRANDOM and stQ_fixed.

RANDOM had the lowest deviation from the mean values of the two traits and were consequently considered the most accurate strategies to take into account trait's variability with respect to the whole set of sampled leaves. Fig. 6 summarizes the statistics related to different sampling sizes within RANDOM family sampling strategies. The minimum size corresponded to 5 leaves taken randomly from 5 individuals (25 leaves in total, equal to the 6% of the whole set of

sampled leaves), while the most accurate and feasible sampling size was 4 leaves taken randomly from 10 individuals (40 leaves in total, equal to the 10% of the whole set of sampled leaves) (Fig. 6).

	<i>Source</i>	<i>Df</i>	<i>SS</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>
SLA	quadrat	2	4.67	2.33	3.72	0.033
	h_class	1	69.77	69.77	111.14	0.001
	E/I	1	9.94	9.94	15.84	0.001
	individual (quadrat)	31	56.68	1.83	2.91	0.001
	quadrat*h_class	2	0.21	0.11	0.17	0.842
	quadrat*E/I	2	1.01	0.50	0.80	0.454
	h_class*E/I	1	0.38	0.38	0.61	0.440
	individual (quadrat)*h_class_	31	21.84	0.70	1.12	0.263
	individual (quadrat)*E/I	31	22.84	0.74	1.17	0.260
	quadrat*h_class*E/I	2	.49	1.74	2.78	0.065
	individual(quadrat)*h_class*E/I	31	18.19	0.59	0.93	0.590
	residual	269	168.88	0.63		
π	quadrat	2	16.21	8.10	10.42	0.001
	h_class	1	5.93	5.93	7.61	0.006
	E/I	1	10.66	10.66	13.71	0.002
	individual(quadrat)	31	91.99	2.98	3.81	0.001
	quadrat*h_class	2	3.93	1.97	2.53	0.084
	quadrat*E/I	2	9.94	4.97	6.39	0.005
	h_class*E/I	1	4.27	4.27	5.49	0.018
	individual(quadrat)*h_class	31	15.32	0.49	0.63	0.938
	individual(quadrat)*E/I	31	26.90	0.87	1.11	0.327
	quadrat*h_class*E/I	2	2.72	1.36	1.75	0.163
	individual(quadrat)*h_class*E/I	31	17.44	0.56	0.72	0.855
	Residual	269	209.26	0.78		

Tab. 1 Results of PERMANOVA analysis of canopy structure variability at each spatial scale (quadrat, individuals within quadrats, height classes, external/internal leaves). Df: degrees of freedom; SS: sum of squares, MS: mean squares; Pseudo-F: pseudo-F statistics. Bold text indicates p-values < 0.05.

Discussion

The major goal of our analysis was to assess the sampling strategy that can adequately capture mean values of functional traits for a species/population, while maintaining information about traits' variability and minimizing the sampling size and effort. In this perspective, we provided an analysis of the contribution of different factors, both spatial and biological, to the observed variability of two foliar functional traits, SLA and π . The factors tested in this study accounted for different proportion of the variance of the two traits, suggesting a different response of the traits to the different micro-environmental conditions occurring through the canopy and within the study area. Several studies have reported that sources of traits' variation depend on the vertical (height), horizontal (outer or inner branches) and azimuthal (aspect) position of the leaves and branches within the crown (Niinemets *et al.*, 2004). Considering the canopy's structure, light availability decreases moving from the top to the base and from external to internal portion of the crown (Niinemets *et al.*, 2015). SLA respond to this gradient, as it generally increases as light availability decreases (Poorter *et al.*, 2009). A higher SLA could arise because of an increase in leaf area and/or a reduction in leaf biomass. Larger leaves improve the ability of the plant to intercept sun light and, consequently, the rate of photosynthesis. Alternatively, the reduction in biomass could allow plants to reduce the investment costs of shaded leaves (Nardini *et al.*, 2012). These mechanisms could explain why h_{class} and E/I resulted the main factors contributing to the variance of SLA (43.4% of the total variance of SLA).

Factors related to the canopy structure accounted for ~23% of the total variance of π . As mentioned above, light availability changes accordingly to canopy structure. Consequently, higher and external leaves intercept a higher amount of light radiation, inducing stomatal aperture. Moreover, the wind speed is higher in the outer canopy, reducing the boundary layer resistance to water vapour diffusion from leaves to the atmosphere. All these factors lead to decrease water potential of leaves in the outer canopy, and the decrease of leaves π might help leaves to maintain turgor despite larger water losses. This could explain why factors h_{class} and E/I and their interaction significantly affected π variance. Interestingly, factors related to the spatial structure of the study area (quadrat, individual and their interaction with other factors) accounted for the highest amount of variance of π (43.2 %). This suggest that ITV of π is mostly spread among individuals rather than within individuals, contrary to SLA variance. The reason for this pattern may be related to the fact that the water status of a plant strongly depends on soil water availability (Binks *et al.*, 2016). Different quadrats could have different soil water availability, because of heterogeneous soil structure or different competition for water with co-occurring species. In the last years, different

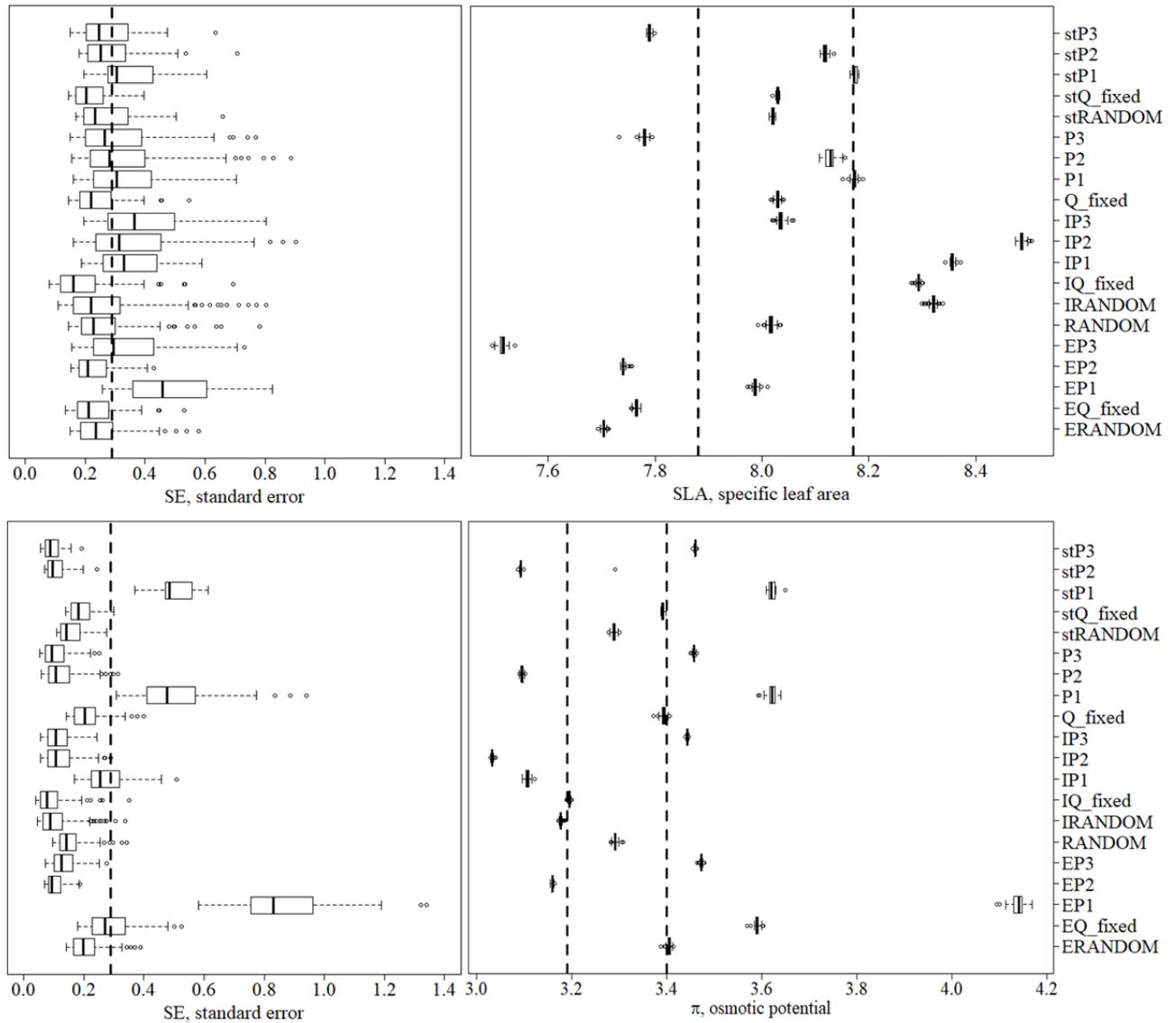


Fig. 5 Median values, 25th and 75th percentiles of standard error (SE, left boxes) and median values, 25th and 75th percentiles of specific leaf area (SLA, mm²/mg, upper right box) and osmotic potential (π , -MPa, lower right box) calculated for each resampling strategy tested in this study. Dotted line in SE boxes indicates breakpoint values of SE of the two traits (0.29 for SLA and 0.22 for π), while dotted lines in upper and lower right boxes indicates 95% CI calculated for SLA and π

studies investigated traits variability and, in particular, Messier *et al.* (2010) assessed variability at different ecological scales of leaf dry matter content (LDMC) and leaf mass per area (LMA), the inverse of SLA. Patterns in ITV of LDMC and LMA were similar to those founded by us in SLA but not for π : In fact, plot (which corresponds to quadrat in our study) accounted for the less amount of variance of SLA and trees (individuals in our study) accounted for ~ 20% of the total variability. Otherwise, as described above, the pattern of ITV of π was the opposite, as the main drivers of its variability were factors related to the study area spatial structure (e.g. quadrat and individuals). This difference reported between the soft traits (SLA, LMA and LDMC) and the mechanistic trait (π)

opens interesting questioning about the patterns of ITV, encouraging future analyses including more mechanistic traits and more species.

The unequal contribution of the tested factors to the total variability of the two functional traits may imply that different traits should be sampled following different strategies. Over the last years, one of the most debated issues in trait-based ecology has in fact regarded the choice of appropriate sampling strategy (Baraloto *et al.*, 2010; Carmona *et al.*, 2015; Paine *et al.*, 2015). Trees that belong to the same species and inhabit the same population of natural origin can show strong genetic and phenotypic differences (Messier *et al.*, 2010). The expression of the phenotype is in turn heavily influenced by the macro- and micro-environmental conditions and by the relationships with the neighbouring trees (competition and/or co-operation) (Abakumova *et al.*, 2016). To the best of our knowledge, no study has investigated the ITV_{WI} and only few reports have provided information on sampling strategies based on measurements of precision (Cornelissen *et al.*, 2003; Pérez-Harguindeguy *et al.*, 2013). RANDOM, stRANDOM, Q_fixed and stQ_fixed resulted the strategies that better estimated the mean values of the traits with an adequate precision (SE lower than SE profiles flex points, Fig. 2) and dispersion of probability distribution (CV higher than CV profiles flex points, Fig. S2). Of these 4 strategies, the most accurate was RANDOM strategy, as it minimized deviations from traits mean value.

	SLA, mm ² mg ⁻¹	π , -MPa
<i>Mean value</i>	8.02	3.29
<i>Upper C.I. (95%)</i>	8.17	3.40
<i>Lower C.I. (95%)</i>	7.88	3.19

Tab. 2 Mean values and upper and lower C.I. at 95% of specific leaf area (SLA, mm² mg⁻¹) and osmotic potential (π , -MPa) calculated on the entire population.

The minimum size required to accurately and precisely estimate the two traits was represented by RANDOM_5_5 (sampling 5 random individuals from the 3 quadrats and 5 leaves per individual). Increasing sampling size resulted in a decrease of SE (increase of precision), but this trend was not consistent for S (Fig. 6). This means that an increase of the sampling effort not always translates in better accuracy. The most significant drop of trait S and sum of S was at RANDOM_10_4 (4 leaves from 10 random individuals), which could be considered as a good compromise between sampling effort and precision of the measurements of the two traits rather than RANDOM_32_2, RANDOM_26_1, RANDOM_27_1 and RANDOM_28_1.

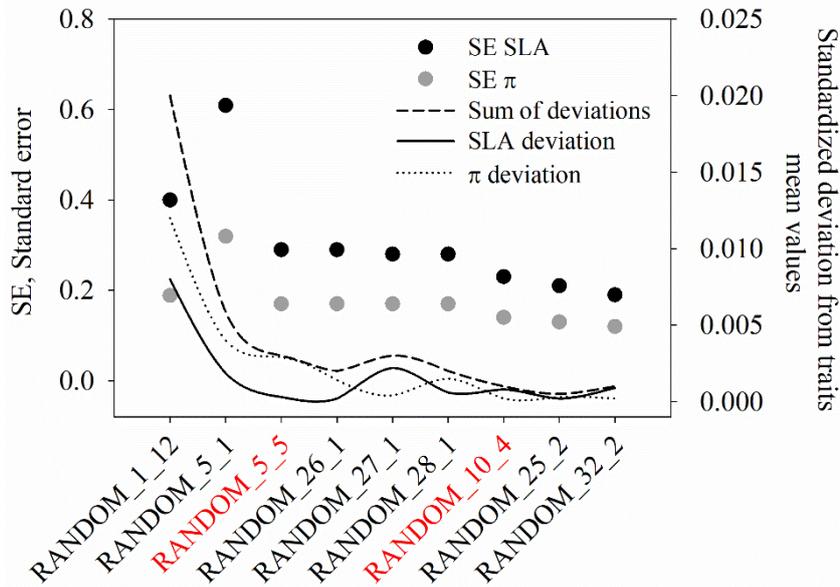


Fig. 6 Standard error (SE) and deviations from mean values of specific leaf area (SLA, mm²/mg) and for osmotic potential (π , -MPa) in different sampling size of RANDOM sampling strategy. Sampling sizes in red represent the minimum (RANDOM_5_5) and optimal (RANDOM_10_4) sampling sizes to estimate SLA and π with desired precision and accuracy

Our findings partially confirm precedent results from Baraloto *et al.* (2010) and Carmona *et al.* (2015), as they reported that strategy equivalent to the RANDOM strategy was the most precise and accurate to estimate species mean trait values and Community weighted mean (CWM). They also concluded that sampling at least one individual per species could be a good compromise to describe species mean traits value. On the contrary, in our study, sampling all possible leaves of one individual (RANDOM_1_12) produced traits estimates far under the desired level of precision and accuracy (Fig. 6). This difference could be due to the different spatial and ecological scales of our analysis vs Baraloto *et al.* (2010) and Carmona *et al.* (2015) analyses. Our analysis was conducted at local scale, where an accurate estimate of the variance of trait values is vital rather than at broader scale (Baraloto *et al.*, 2010). Hence, we suggest including the minimum and preferred sampling size provided here in studies aimed at highlighting differences in species/communities at local scale, while the approach proposed by Baraloto *et al.* (2010) and Carmona *et al.* (2015) could be adopted in analyses at broader scales. We also compared our results with those included in standard protocol proposed by Pérez-Harguindeguy *et al.* (2013). They provide a minimum and a preferred number of individuals and leaves per individual based on the calculation of CV of each trait considered in different studies and on common practice. For SLA, they recommend sampling 5 leaves from 5

individuals (minimum) or 4 leaves from 10 individuals (preferred). However, they also suggested sampling “sun leaves” (leaves positioned in the outer canopy stratum) for the measurements of SLA. We interpreted this suggestion in two possible ways. Outer canopy could be represented by leaves in the external stratum of the canopy at any height (see *cor*, *cor_min*, *per*, *per_min* strategies, Table S2) or by leaves in the upper and external stratum of the canopy (see *cor_b*, *cor_min_b*, *per_b*, *per_min_b* strategies, Table S2). The mean values of the two traits calculated for each resampling strategy listed above (Table S2) lies outside the 95% C.I. range (Table 2) calculated on the whole dataset. Hence, SLA values measured following standardized protocols results significantly different from SLA mean values measured on the entire dataset. Sampling “sun leaves” was proposed in the past to control variation of leaf traits to avoid shading bias (Messier *et al.*, 2010). However, our data suggest that the exclusion of other canopy strata could produce underestimation not only of mean traits values, but also of traits variability, as CV measured only on external leaves was lower than minimum CV (Table S3, Fig. S3). Such underestimation can lead to underestimations of physiological leaf processes that change as function of leaf surface such as transpiration rate, gas exchanges and photosynthesis rate (Poorter *et al.*, 2009). Moreover, Keenan & Niinemets (2016) demonstrated that most of the measurements of SLA in current available databases are strongly biased by shading effect. In fact, leaves experience strong changes in terms of light availability during their development (i.e. light gradients within a canopy or across gap-understory continua). Consequently, the dichotomy between “sun” and “shade” leaves could be considered ambiguous. As previously proposed by Messier *et al.* (2010), it would be preferable to distinguish between measurements of SLA only considering sun leaves (SLA_{max} or SLA_{sun}) from measurements considering the whole canopy (SLA_{tree}). In the light of the above, we recommend sampling leaves considering all the strata composing the canopy to estimate correctly the studied traits values, especially when interested in assessing traits variability at species level.

Conclusion

Our analysis confirms the role of ITV in determining leaf functional traits variability. Moreover, we demonstrate that the spatial structure of the canopy could significantly affect traits variability. Interestingly, different factors accounted for different proportion of the variability of the two traits, suggesting that different strategies for different traits could be implemented during sampling surveys.

We also provided practical advices to optimize sampling procedures of functional traits: a minimum (5 leaves from 5 individuals) and optimal (4 leaves from 10 individuals) sample size based on

measurements of precision, partially confirming sampling sizes previously proposed by literature. The results presented here should encourage future analysis involving different traits, in order to get global insights into ITV as based on multiple trait analysis.

Authors' Contributions

FP, AN and GB conceived and designed the experiment; FP, CP, TS, RA and GB collected the data; FP, CP and GB analysed the data; FP, AN and GB wrote the manuscript, with the contribution of all authors.

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Data accessibility

The complete dataset has been deposited in Dryad: [doi:10.5061/dryad.6362p](https://doi.org/10.5061/dryad.6362p)

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Supplementary material

<i>Resampling strategies</i>	<i>Description</i>
RANDOM	. “i” individuals from all the 34 sampled are chosen randomly, then “f” leaves per individual are randomly sampled)
Q_fixed	a fixed number of individuals per quadrat (“f” leaves per individual are randomly sampled)
P1	only individuals from quadrat n°1 were randomly chosen
P2	only individuals from quadrat n°2 were randomly chosen
P3	only individuals from quadrat n°3 were randomly chosen
stRANDOM	Hierarchical stratified = the choice of individuals is the same of previous resampling strategies, but leaves were systematically chosen from the canopy stratum defined by height class (“a” or “b”) and external (E) or internal (I) foliage.
stQ_fixed	
stP1	
stP2	
stP3	
ERANDOM	The choice of individuals is the same of first 5 resampling strategies, but leaves were randomly selected only from external (E) foliage.
EQ_fixed	
EP1	
EP2	
EP3	
IRANDOM	The choice of individuals is the same of first 5 resampling strategies, but leaves were randomly selected only from internal (I) foliage.
IQ_fixed	
IP1	
IP2	
IP3	

Tab. S1 List of virtual resampling strategies tested.

<i>Resampling strategies</i>	<i>Description</i>	
Cornelissen et al. (2003)	cor	Preferred number of leaves taken randomly from external canopy (E).
	cor_b	Preferred number of leaves taken randomly from external (E) and upper canopy (b).
	cor_min	Minimal number of leaves taken randomly from external canopy (E).
	cor_min_b	Minimal number of leaves taken randomly from external (E) and upper canopy (b).
Pérez-Harguindeguy et al. (2013)	per	
	per_b	The same as the previous sampling strategies but referred to preferred and minimal number of leaves
	per_min	to be sampled by Pérez-Harguindeguy et al. (2013)
	per_min_b	

Tab. S2 Resampling strategies following standardized protocol suggested criteria (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013).

<i>Resampling strategy</i>	SLA, mm² mg⁻¹			π, -MPa		
	<i>Mean</i>	<i>SE</i>	<i>CV</i>	<i>Mean</i>	<i>SE</i>	<i>CV</i>
RANDOM	8.02	0.27	0.18	3.29	0.16	0.25
Q_fixed	8.03	0.25	0.17	3.39	0.21	0.35
P1	8.17	0.33	0.14	3.62	0.48	0.47
P2	8.12	0.30	0.19	3.10	0.12	0.19
P3	7.78	0.28	0.17	3.46	0.09	0.13
stRANDOM	8.02	0.28	0.18	3.29	0.16	0.25
stQ fixed	8.03	0.22	0.17	3.39	0.19	0.35
stP1	8.17	0.34	0.14	3.62	0.50	0.46
stP2	8.12	0.30	0.19	3.10	0.11	0.19
stP3	7.79	0.28	0.17	3.46	0.10	0.13
ERANDOM	7.70	0.29	0.17	3.41	0.22	0.31
EQ_fixed	7.76	0.24	0.17	3.60	0.29	0.45
EP1	7.99	0.49	0.15	4.14	0.88	0.52
EP2	7.74	0.33	0.17	3.16	0.14	0.19
EP3	7.51	0.34	0.15	3.47	0.14	0.14
IRANDOM	8.32	0.26	0.18	3.18	0.10	0.18
IQ_fixed	8.29	0.19	0.17	3.19	0.09	0.21
IP1	8.36	0.36	0.11	3.11	0.28	0.23
IP2	8.49	0.36	0.18	3.03	0.12	0.17
IP3	8.04	0.40	0.17	3.44	0.12	0.11
cor	7.69	0.30	0.17	3.40	0.24	0.30
cor_b	6.98	0.21	0.13	3.23	0.15	0.21
cor_min	7.71	0.35	0.17	3.40	0.25	0.25
cor_min_b	6.98	0.31	0.12	3.23	0.21	0.18
per	7.70	0.22	0.18	3.40	0.19	0.33
per_b	6.99	0.17	0.13	3.23	0.13	0.21
per_min	7.70	0.27	0.17	3.41	0.22	0.30
per_min_b	7.00	0.24	0.13	3.23	0.17	0.20

Tab. S3 Mean, SE and CV values of specific leaf area (SLA, mm² mg⁻¹) and osmotic potential (π , -MPa) calculated for each resampling strategies listed in Table 1 and Table 2. Bold text indicates the most precise resampling strategies within the entire pool of strategies tested.

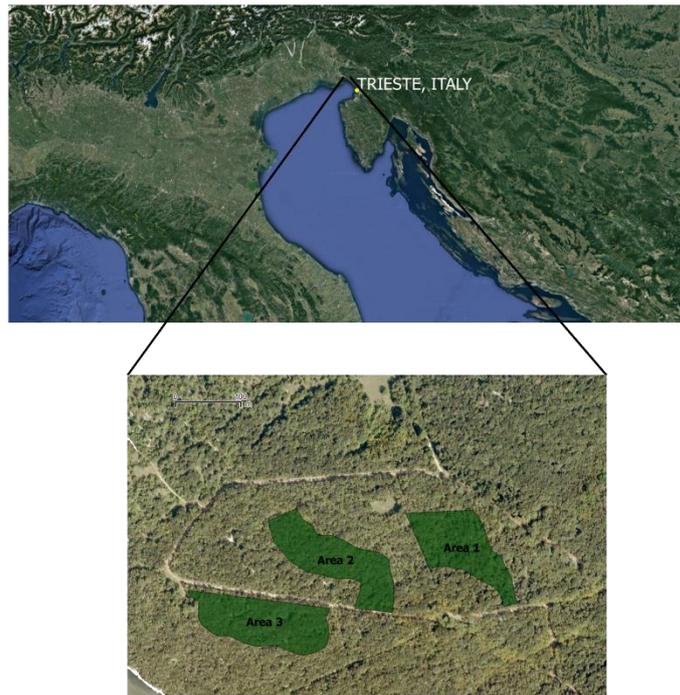


Fig. S1 Map of the three study areas with the highest density of *Q. ilex*, where samples were collected.

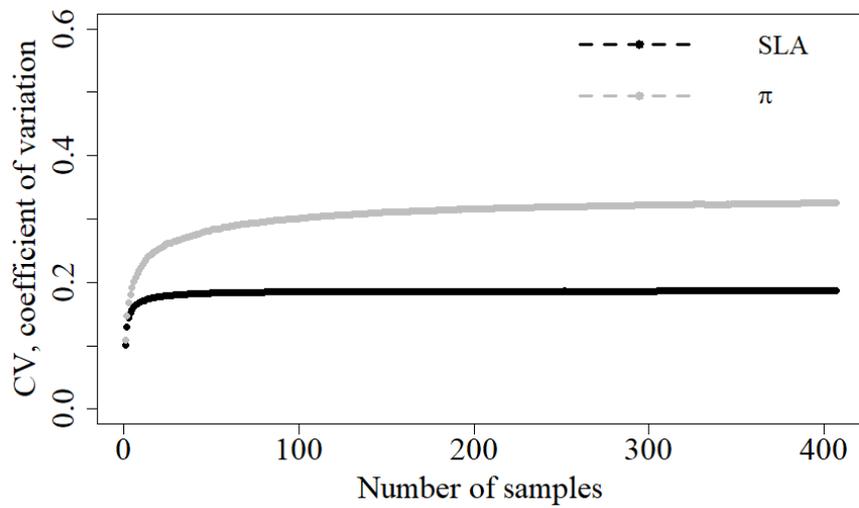


Fig. S2 Relationship between number of samples considered and associated coefficient of variation (CV) of SLA (black points) and π (grey points).

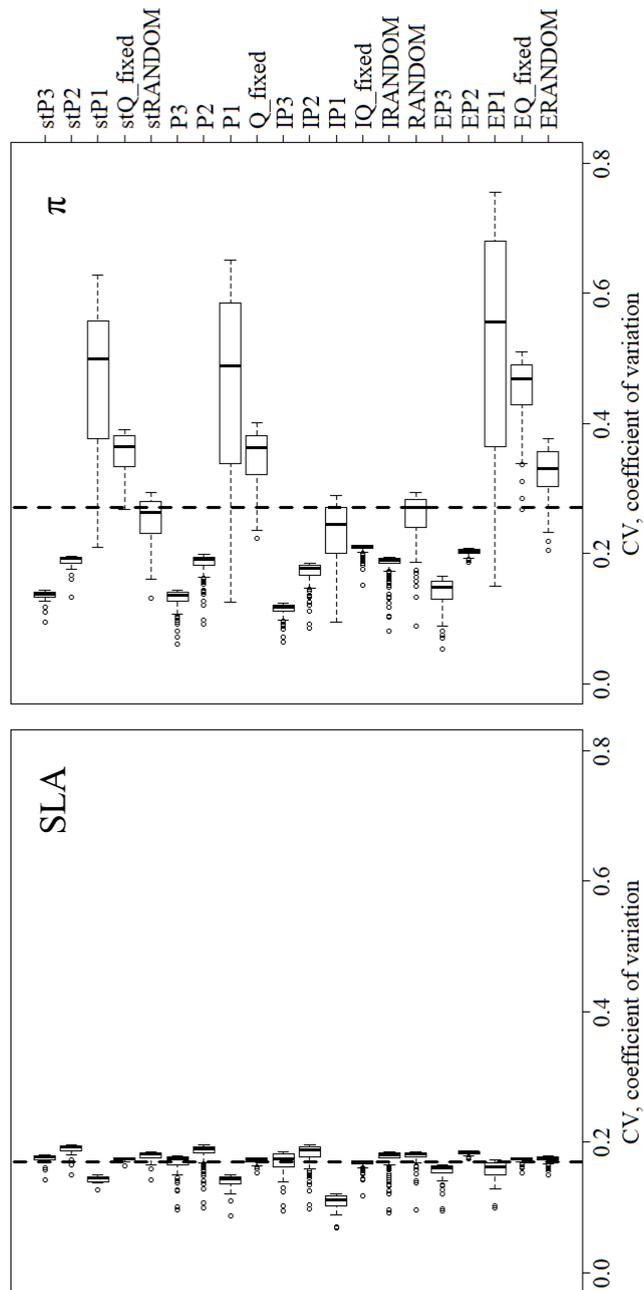


Fig. S3 Median values, 25th and 75th percentiles of coefficient of variation (CV) of specific leaf area (SLA, mm² mg⁻¹) and osmotic potential (π , -MPa) calculated for each resampling strategy tested in this study. Dotted lines indicate maximum CV of the two traits calculated on the whole dataset.

Study 2

A simplified framework for fast and reliable measurement of leaf turgor loss point

Francesco Petruzzellis^{1*}, Tadeja Savi², Giovanni Bacaro¹, Andrea Nardini¹

¹Dipartimento di Scienze della Vita, Università degli Studi di Trieste, via L. Giorgieri 10, 34127, Trieste, Italia

²University of Natural Resources and Life Sciences, Vienna, Department of Crop Sciences, Division of Viticulture and Pomology, Konrad Lorenz Straße 24, A-3430 Tulln, Austria

*Corresponding author

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Abstract

Drought tolerance shapes the distribution of plant species, and it is mainly determined by the osmotic potential at full turgor (π_0) and the water potential at turgor loss point (Ψ_{tlp}). We provide a simplified framework for π_0 and Ψ_{tlp} measurements based on osmometer determination of π_0 (π_{0_osm}). Specifically, we ran regression models to i) improve the predictive power of the estimation of π_0 from π_{0_osm} and morpho-anatomical traits; ii) obtain the most accurate model to predict Ψ_{tlp} on the basis of the global relationship between π_0 and Ψ_{tlp} . The inclusion of the leaf dry matter content (LDMC), an easy-to-measure trait, in the regression model improved the predictive power of the estimation of π_0 from π_{0_osm} . When π_{0_osm} was used as a simple predictor of Ψ_{tlp} , discrepancies arose in comparison with global relationship between π_0 and Ψ_{tlp} . Ψ_{tlp} values calculated as a function of the π_0 derived from π_{0_osm} and LDMC (π_{0_fit}) were consistent with the global relationship between π_0 and Ψ_{tlp} . The simplified framework provided here could encourage the inclusion of mechanistically sound drought tolerance traits in ecological studies.

Keywords

dewpoint hygrometer; mechanistic traits; osmotic potential, water availability; water potential

Introduction

Plant functional traits are defined as morphological, physiological, or phenological features measurable at the individual level, from the cell to the whole-organism (Violle *et al.*, 2007). Recently, Brodribb (2017) suggested to distinguish “mechanistic” traits, which comprehends plant’s features clearly associated to a physiological process, from general functional traits (such as leaf mass per unit area), which rather represent “syndromes” that could be driven by several different physiological functions and associated trade-offs. Mechanistic traits have been increasingly included in trait-based studies and provided novel insights into several ecological processes, ranging from species assembly rules (Blackman *et al.*, 2012; Brodribb *et al.*, 2014), invasion of alien plant species (Petruzzellis *et al.*, 2018), and vegetation dynamics under ongoing climate changes (Anderegg, 2015). As an example, hydraulic traits (e.g. Ψ_{50} , the water potential inducing 50% loss of hydraulic conductivity, or K_s , the stem specific hydraulic conductivity) have been used to model plant species distribution (Costa-Saura *et al.*, 2016; Larter *et al.*, 2017), and they were shown to correlate with growth rate and risk of mortality under drought (Fan *et al.*, 2012; Choat *et al.*, 2012; Anderegg *et al.*, 2015).

Leaf water relation parameters have been recently proposed as predictors of the position of a species along the “fast-slow” whole plant economic spectrum (Blackman, 2018; Zhu *et al.*, 2018), as they correlate to both leaf hydraulic and economic traits (Nardini & Luglio, 2014; Trifiló *et al.*, 2016). Specifically, leaf osmotic potential at full turgor (π_0) and the leaf water potential at turgor loss point (Ψ_{tlp}) are strongly linked to species-specific ability to tolerate leaf dehydration (Bartlett *et al.*, 2012b) and consequently to sustain stomatal conductance, photosynthesis and growth even under water shortage conditions (Tognetti *et al.*, 2000; Bartlett *et al.*, 2016). In particular, Ψ_{tlp} indicates the water potential inducing loss of cell turgor pressure (Bartlett *et al.*, 2012b), which is critical to maintain gas exchange and growth (Brodribb *et al.* 2003). In their recent analysis, Zhu *et al.* (2018) have reported that Ψ_{tlp} is correlated with leaf carbon investment, as species with lower Ψ_{tlp} tend to have higher leaf density (d_{leaf}) and leaf mass per unit area (LMA). Turgor loss point also correlates with habitat moisture, as species living in arid environments usually have lower values of Ψ_{tlp} than species living under higher water availability (Lenz *et al.*, 2006; Bartlett *et al.*, 2012b; Zhu *et al.*, 2018). Given the correlation between Ψ_{tlp} , hydraulic and economic traits and environmental features, the inclusion of the turgor loss point in ecological studies holds promises to provide important insights on ecological and evolutionary patterns in plants.

Ψ_{tlp} has been traditionally estimated from water potential isotherms (or pv-curves, Tyree and Hammel 1972), i.e. by measuring the progressive decrease of the water potential and of the water

content during leaf dehydration. This procedure is time-consuming, and this probably limited the inclusion of Ψ_{tlp} in studies involving large numbers of species/individuals and/or study sites. Recently, Bartlett *et al.* (2012b) have reported that the variation of Ψ_{tlp} both between and within species is mainly driven by changes in π_0 , which reflects solutes concentration in cells at full turgor. These two traits resulted highly correlated to each other, as species with lower Ψ_{tlp} also have lower values of π_0 (Bartlett *et al.*, 2012b). Consequently, both traits could be considered as useful parameters to predict species drought tolerance, and π_0 could be used as a proxy of turgor loss point. Alternative methods have been proposed to obtain π_0 , e.g by directly measuring the osmotic potential of sap extracted from leaf tissues using a thermocouple psychrometer. In particular, rapid freeze and thaw of leaf samples, that induces cell disruption and the release of symplastic contents, is considered the most accurate procedure to measure π_0 with an osmometer (π_{0_osm}). Recently, Bartlett *et al.* (2012a) proposed a framework to predict both π_{0_pv} (osmotic potential at full turgor derived from pv-curves) and Ψ_{tlp} from π_{0_osm} measurements. In their analysis, the authors tested various models including different morpho-anatomical leaf traits, and they reported that models including bulk modulus of elasticity (ϵ) and d_{leaf} significantly improved the ability to predict π_{0_pv} from π_{0_osm} measurements. However, ϵ is generally derived from pv-curves, so that including this parameter in the derivation of π_{0_pv} does not represent a major advantage. Also, d_{leaf} measurements can be laborious and prone to errors as far as volume estimates are concerned. Hence, a simplified framework for estimation of π_0 would be useful for ecological studies.

In this study, we measured several functional traits as well as water relation parameters derived from pv-curves in 27 species, with the aim to provide a simple framework to estimate π_{0_pv} and Ψ_{tlp} from measurements of π_{0_osm} obtained with a dewpoint hygrometer. The specific aims were to i) obtain a model to predict π_{0_pv} on the basis of π_{0_osm} and easy-to-measure functional traits (like LMA or leaf dry matter content); ii) obtain the most accurate model to predict Ψ_{tlp} on the basis of the global relationship between π_0 and Ψ_{tlp} .

Materials and methods

Leaf traits measurements

To model the estimation of the osmotic potential at full turgor (π_0) and of the water potential at turgor loss point (Ψ_{tlp}) from π_0 values obtained with a dewpoint hygrometer (π_{0_osm} ; see below), we selected 27 temperate and Mediterranean woody species (Table S1) with different levels of drought resistance. Species were sampled in natural habitats in the Karst region (NE Italy), or in the

Botanical Garden of University of Trieste. Additional data were obtained from previous studies performed in our laboratory (see Table S1 for references).

Three leaves were sampled from different individuals of each species to measure π_{0_osm} . Twigs were detached from branches and were rehydrated overnight. One leaf per twig was roughly crumbled and sealed in cling film. Then, it was immersed in liquid nitrogen (LN₂) for 2 min. The leaf (still sealed in cling film) was then carefully ground and stored in sealed plastic bottles at -20°C. Before measurements, samples were thawed at room temperature for 5 min while still sealed in cling film and in plastic bottles, to avoid evaporation effect on measurements (Bartlett *et al.*, 2012a). Finally, π_{0_osm} was measured with a dewpoint hygrometer (Model WP4, Decagon Devices Inc., Pullman, Washington, USA).

Water potential isotherms (pv-curves) were measured to obtain reference values for Ψ_{tip} and π_{0_pv} . Fresh leaves were rehydrated for 12 h with their petioles immersed in pure water and pv-curves were measured using the bench dehydration technique, by repeatedly measuring water loss and water potential with a balance and a pressure chamber (model 1505D, PMS Instruments, Albany, OR, USA), respectively, during progressive sample dehydration. Water potential (Ψ_{leaf}) and cumulative weight loss (WL) of leaves were measured until the relationship between $1/\Psi$ and WL became strictly linear, indicating the loss of cell turgor. Pv-curves were then elaborated according to Salleo (1983) to calculate the osmotic potential at full turgor (π_{0_pv}), the water potential at turgor loss point (Ψ_{tip_pv}) and the modulus of elasticity (ϵ).

For each species, leaf morpho-anatomical parameters were measured on 6 leaves from the same individuals sampled for the measurements of π_{0_osm} and pv-curves. Specifically, we measured leaf thickness (Th, μm), leaf dry matter content (LDMC, mg g^{-1}), leaf mass per unit area (LMA, mg cm^{-2}) and leaf density (d_{leaf} , g cm^{-3}).

Th was measured after rehydrating leaves for 3h using a digital calliper on three portions of the leaf (top, middle, bottom). Values were then averaged for each leaf.

LDMC and LMA were calculated as:

$$\text{LDMC} = \text{Leaf dry weight} / \text{Leaf turgid weight} \quad (\text{eqn 1})$$

$$\text{LMA} = \text{Leaf dry weight} / \text{Leaf area} \quad (\text{eqn 2})$$

Fresh leaves were first rehydrated for 3h and leaf turgid weight was measured with an analytical balance. Leaves were scanned and leaf area was measured using the software Image J (Schneider *et al.*, 2012). Leaves were finally oven-dried for 48 h at 72°C and leaf dry weight was measured.

d_{leaf} was calculated as:

$$d_{leaf} = \text{Leaf dry weight} / \text{Leaf fresh volume} \quad (\text{eqn 3})$$

Leaf fresh volume was measured using a water displacement method (Hughes 2005), while leaf dry weight was measured as described above.

Statistical analysis

The first aim of this study was to find an easy and fast method to measure the leaf osmotic potential at full turgor, based on the work of Bartlett *et al.* (2012a). To improve of the predictive power of the estimation of π_{0_pv} from π_{0_osm} measurements, we ran a multiple linear regression model to predict π_{0_pv} (response variable) as a function of π_{0_osm} and the leaf traits described above. A Minimum Adequate Model (MAM) was obtained using package “glmulti” (Calcagno 2013) via minimization of the corrected Akaike informative criterion (AICc) plus a backward procedure to avoid multicollinearity among selected explanatory variables. To compare results obtained by Bartlett *et al.* (2012a), we evaluated two other linear regression models, setting π_{0_pv} as the response variable. In a first model, only π_{0_osm} was set as the predictive variable, while in the second we considered both π_{0_osm} and d_{leaf} as predictive variables. Models were then compared on the basis of their predictive power (adjusted R^2 , R^2_{adj}) and, in order to take in account the number of predictors included in the model, the AICc and their mean absolute error (MAE).

The second aim of this study was to test the ability to predict $\Psi_{t_{lp_pv}}$ both from π_{0_osm} measurements and the fitted values of the MAM (π_{0_fit}) and compare models' output with the global relationship between $\Psi_{t_{lp}}$ and π_0 described in Bartlett *et al.* (2012b). We first fitted two separated simple linear regression models on the data measured in the present study, considering $\Psi_{t_{lp_pv}}$ (response variable) as a function of π_{0_osm} or π_{0_fit} , respectively. For each model, we calculated coefficient estimates and associated 95% confidence intervals (95% C.I.), R^2_{adj} , AICc and MAE.

Because the number of species analysed in Bartlett *et al.* (2012b) differed from the present study (248 and 27 respectively), we set up a bootstrap procedure (999 replicates) to obtain comparable values of estimated coefficients. π_0 and $\Psi_{t_{lp}}$ values of 27 randomly selected species from the dataset in Bartlett *et al.* (2012b) were chosen. From this selection, we fitted a simple linear regression model calculating coefficient estimates, 95% C.I., R^2_{adj} , AICc and MAE. At the end of this bootstrap procedure, averaged values were calculated. Differences between $\Psi_{t_{lp_pv}}$ prediction from π_{0_osm} , π_{0_fit} and the one derived from Bartlett *et al.* (2012b) were determined comparing 95% C.I. of the coefficient estimates and AICc. Specifically, predictions were assumed to differ if 95% C.I. of coefficient estimates did not overlap and if the difference between AICc values were > 2 (Burnham and Anderson 2004). All statistical analyses were performed using R 3.4.1 (R Foundation for Statistical Computing, Vienna, AT).

Results

Species scientific name, abbreviation, and associated mean values of leaf traits are summarized in Tab. S1. Species that sustained higher leaf construction costs (higher LMA, LDMC and Th) also had higher drought resistance (lower $\Psi_{\text{tlp}_{\text{pv}}}$), as shown by correlation analysis reported in Tab. S2.

Although $\pi_{0_{\text{osm}}}$ resulted a significant predictor $\pi_{0_{\text{pv}}}$ (Tab. 1), it underestimated $\pi_{0_{\text{pv}}}$ at less negative values and overestimated it at more negative values (Fig. 1). The best model to predict $\pi_{0_{\text{pv}}}$ included $\pi_{0_{\text{osm}}}$ and LDMC as predictive variables (Tab. 1). The inclusion of LDMC significantly improved the predictive power of the model, as the R^2_{adj} was higher and AICc were lower than those calculated on the model including only $\pi_{0_{\text{osm}}}$ or the one including both $\pi_{0_{\text{osm}}}$ and ε (Tab. 1).

$\pi_{0_{\text{osm}}}$ and $\pi_{0_{\text{fit}}}$ were tested as predictors of $\Psi_{\text{tlp}_{\text{pv}}}$. The parameters of the two derived models were compared with those derived from the relationship between $\Psi_{\text{tlp}_{\text{pv}}}$ and $\pi_{0_{\text{pv}}}$ reported in Bartlett *et al.* (2012b) and from the iterative procedure described above. The average a and intercept estimates calculated on a reduced number of species were not statistically different from those calculated including the whole dataset provided by Bartlett *et al.* (2012b) and on the models including $\pi_{0_{\text{osm}}}$ and $\pi_{0_{\text{fit}}}$ as predictive variables, as 95% C.I. overlapped each other (Tab. 2). However, the model including $\pi_{0_{\text{fit}}}$ had higher predictive ability than the one including $\pi_{0_{\text{osm}}}$ (Tab.1), as the R^2_{adj} was higher and AICc and MAE were lower (Tab. 2). In addition, the model including $\pi_{0_{\text{osm}}}$ led to overestimation of $\Psi_{\text{tlp}_{\text{pv}}}$ for values < -2 MPa. As shown in Fig. 1 and Fig. 2, mean values of slope (a) and intercept calculated on the model including $\pi_{0_{\text{fit}}}$ as predictive variable were much closer to those calculated on 27 randomly selected species from Bartlett *et al.* (2012b).

π_{0_pv} estimation	Estimate	Std. error	p-value	R^2_{adj}	AICc	MAE
$\beta * \pi_{0_osm} + \text{intercept}$				0.46	17.8	0.18
β	0.681	0.143	<0.001			
intercept	-0.434	0.240	0.08			
$\beta * \pi_{0_osm} + \beta_1 * \text{LDMC} + \text{intercept}$				0.58	11.4	0.12
β	0.506	0.138	0.001			
β_1	-0.002	0.001	0.007			
intercept	0.013	0.258	0.96			
$\beta * \pi_{0_osm} + \beta_1 * \epsilon + \text{intercept}$				0.53	16.1	0.17
β	0.654	0.226	0.17			
β_1	-0.013	0.132	<0.001			
intercept	-0.319	0.006	0.03			

Tab. 1 Summary of the models predicting the osmotic potential at full turgor measured through pv-curves (π_{0_pv}) from osmotic potential at full turgor obtained with a dewpoint hygrometer (π_{0_osm}) alone, including leaf dry matter content (LDMC) or the modulus of elasticity of cell walls (ϵ). R^2_{adj} = adjusted r^2 . AICc = Akaike informative criterion corrected for low number of observations. MAE = mean absolute error of the model

$\Psi_{\text{tlp_pv}}$ estimation	Estimate	2.5%	97.5%	R^2_{adj}	AICc	MAE
$\beta^* \pi_{0_osm} + \text{intercept}$				0.47	31.4	0.31
β	0.899	0.530	1.268			
intercept	-0.581	-1.197	0.034			
$\beta^* \pi_{0_fit} + \text{intercept}$				0.61	23.9	0.25
β	1.313	0.902	1.725			
intercept	-0.032	-0.673	0.609			
$\beta^* \pi_{0_pv} + \text{intercept}$ (27 species)				0.87	6.82	0.18
β	1.211	1.027	1.394			
intercept	-0.057	-0.391	0.276			
$\beta^* \pi_{0_pv} + \text{intercept}$ (Bartlett <i>et al.</i>, 2012b)				0.87	50.1	0.18
β	1.205	1.146	1.263			
intercept	-0.062	-0.170	0.044			

Tab. 2 Summary of the models predicting the water potential at turgor loss point measured through pv-curves ($\Psi_{\text{tlp_pv}}$) from osmotic potential at full turgor obtained with a dewpoint hygrometer (π_{0_osm}) alone, fitted values of the MAM (π_{0_fit}) and from π_0 values provided by Bartlett *et al.* (2012b). 2.5% and 97.5% represent 95% confidence interval limits. R^2_{adj} = adjusted R^2 . AICc = Akaike informative criterion corrected for low number of observations. MAE = mean absolute error of the model.

Discussion

As reported by Bartlett *et al.* (2012a), π_{0_osm} significantly correlated with π_{0_pv} (Fig. 1), but the regression line was different from the desired 1:1 relationship. A reason for this discrepancy is that osmometer-based measurements of π_0 could be biased by errors due to sample preparation. In fact, the disruption of cell walls could cause the dissolution of cell wall solutes that could lead to more negative π_0 values. On the other hand, symplastic fluids could be diluted by apoplastic water, leading to higher π_0 values. In this light, testing whether this prediction could be improved is fundamental to provide a solid framework for fast and reliable π_0 estimation. In the present study, we measured several leaf morpho-anatomical traits in order to enhance the predictive power of π_{0_pv} from measurements done with an hygrometer (π_{0_osm}) on the basis of the framework proposed by Bartlett *et al.* (2012a).

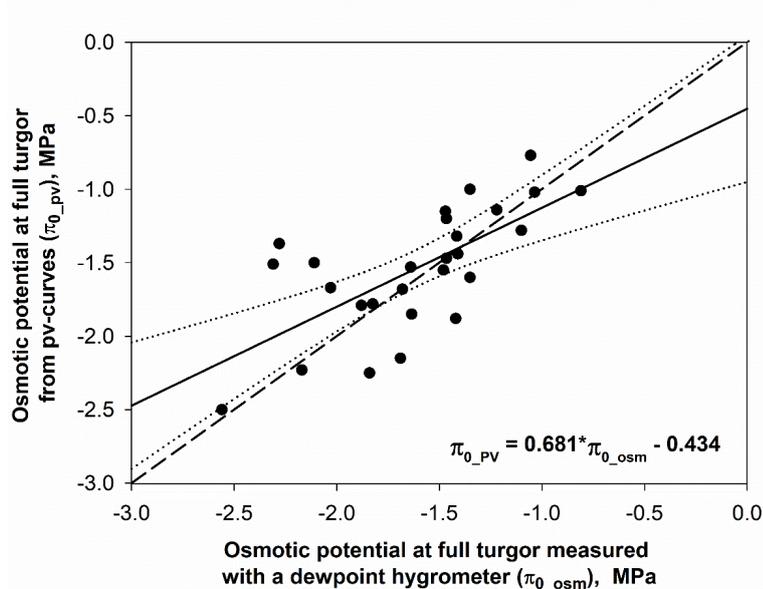


Fig. 1 Relationship between osmotic potential at full turgor measured with a dewpoint hygrometer (π_{0_osm}) and osmotic potential at full turgor measured through pv-curves (π_{0_pv}). Dotted lines represent confidence intervals of the regression line (solid line). Dashed line represents the 1:1 line.

The best model to predict π_{0_pv} included π_{0_osm} and LDMC, enhancing the predictive power of the model including only π_{0_osm} , as R^2_{adj} was higher and AICc and MAE were lower (Tab. 1). As previously suggested by Bartlett *et al.* (2012a), the inclusion of LDMC in the predictive model could account for both errors associated to osmometer measurements. Higher values of LDMC are associated to greater cell wall investment, which in turn could improve the maintenance of relatively high water content, thus accounting for apoplastic dilution. On the other hand, higher LDMC could

also reflect thicker cell walls or leaf with smaller but more numerous cells, thus accounting for solutes concentration enrichment derived from cell walls disruption.

In their analysis, Bartlett *et al.* (2012a) found that the best models to predict π_{0_pv} included d_{leaf} , π_{0_osm} and their interaction, or just ϵ and π_{0_osm} . In our analysis, d_{leaf} was discarded during model selection and the model including ϵ had less predictive power than the model including LDMC. These discrepancies could be due to multiple factors. Both studies included a limited number of species (30 in Bartlett *et al.* (2012a) and 27 in the present study), adapted to different environments. Most of the species in the present study are typical of Mediterranean biomes, while most of the species in Bartlett *et al.* (2012a) originate from temperate and tropical biomes. Consequently, drought tolerance and turgor loss point could be driven by different morpho-anatomical features in the two sets of species. Moreover, whereas minimum and maximum π_{0_pv} values were nearly the same between the two datasets, the distribution of density probability of π_0 was more skewed in Bartlett *et al.* (2012a) (Fig. S1), indicating a higher density of observations in a narrower range of π_0 values. However, a sort of consensus approach could be derived from these analyses. In both studies, the best model to predict π_{0_pv} included traits which reflects leaves carbon investment (d_{leaf} , LDMC and ϵ), suggesting that species that sustain higher leaf construction costs (denser and/or thicker leaves), and thus occupy the “slow-growing” space of leaf economic spectrum (Wright *et al.*, 2004), also have higher drought resistance. The framework provided by Bartlett *et al.* (2012a) could allow to estimate π_0 and $\Psi_{t_{lp}}$ on a large number of species and samples strongly reducing the time needed for its measurement using pv-curves. The framework provided in this study further simplifies the model proposed by Bartlett *et al.* (2012a), as the measurement of LDMC is faster and simpler than the procedure for d_{leaf} measurement.

π_0 and $\Psi_{t_{lp}}$ are strongly correlated each other (Bartlett *et al.*, 2012b) and thus, it is possible to estimate $\Psi_{t_{lp}}$ from measurements of π_0 . A significative linear relationship between π_0 and $\Psi_{t_{lp}}$ was found in the regression models ran on the data provided by the authors (Tab. 2). We used parameters estimates of this model as a reference to compare regression models with π_{0_osm} or π_{0_fit} as predictive variable. The model including π_{0_osm} resulted very similar to the one obtained by Bartlett *et al.* (2012a):

$$\Psi_{t_{lp_pv}} = 0.832 \pi_{0_osm} - 0.631 \quad (\text{eqn 4, from Bartlett } et al. 2012a)$$

$$\Psi_{t_{lp_pv}} = 0.899 \pi_{0_osm} - 0.581 \quad (\text{eqn 5})$$

However, the regression model in eqn 5 had a lower predictive power and parameters' estimates were slightly different than those calculated from the model ran on data from Bartlett *et al.* (2012b) (Tab. 2). As shown in Fig. 2, we detected a discrepancy between the regression model considering π_{0_osm} as the predictive variable and the one calculated on data from Bartlett *et al.* (2012b).

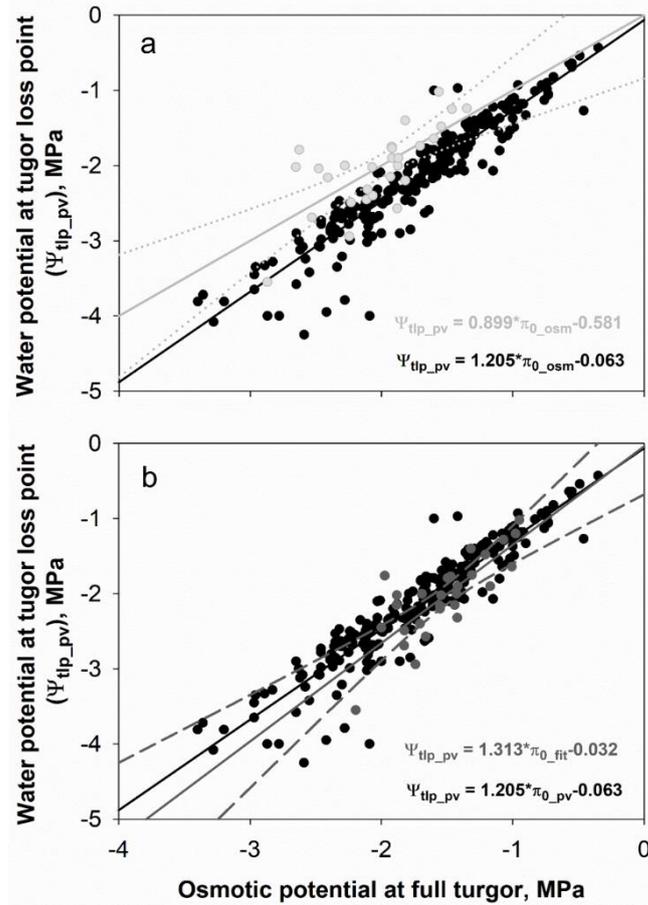


Fig. 2 Relationship between water potential at turgor loss point measured through pv-curves (Ψ_{tlp_pv}) and the osmotic potential at full turgor measured with a dewpoint hygrometer (π_{0_osm} , light grey circles, a), the fitted values of the MAM (π_{0_fit} , dark grey circles, b) and the osmotic potential at full turgor from Bartlett *et al.* (2012b, black circles). Dotted light green line represents confidence intervals of the regression line calculated considering π_{0_osm} (solid light grey line, a). Dashed dark grey line represents confidence intervals of the regression line calculated considering π_{0_fit} (solid dark grey line, b). Solid black lines represent regression lines calculated on data from Bartlett *et al.* (2012b).

In particular, Ψ_{tlp} values < -2 MPa tended to be overestimated by eqn 2. On the contrary, the model considering π_{0_fit} as explanatory variable produced parameters' estimates much closer to those obtained from data provided in Bartlett *et al.* (2012b) (Fig. 2) and no discrepancy was detected. The number of studies including π_0 and Ψ_{tlp} estimation from osmometer/hygrometer measurement of π_0 rapidly increased in the last years (Maréchaux *et al.*, 2015; Savi *et al.*, 2016; Petruzzellis *et al.*, 2017, 2018), and it is likely that the number of species with associated Ψ_{tlp} values will increase as well. In this light, the standardization and the simplification of the framework for Ψ_{tlp} estimation is crucial to build a solid global dataset. To improve the predictive power of the estimation of Ψ_{tlp} , we suggest measuring LDMC as well as π_{0_osm} from leaves attached to the same twig or at least

belonging to the same individual. To estimate π_0 and Ψ_{tlp} we then suggest applying the following equations:

$$\pi_{0_fit} = 0.506\pi_{0_osm} - 0.002LDMC \text{ (expressed in mg g}^{-1}\text{)} \quad (\text{eqn 6})$$

$$\Psi_{tlp_pv} = 1.313\pi_{0_fit} - 0.032 \quad (\text{eqn 7})$$

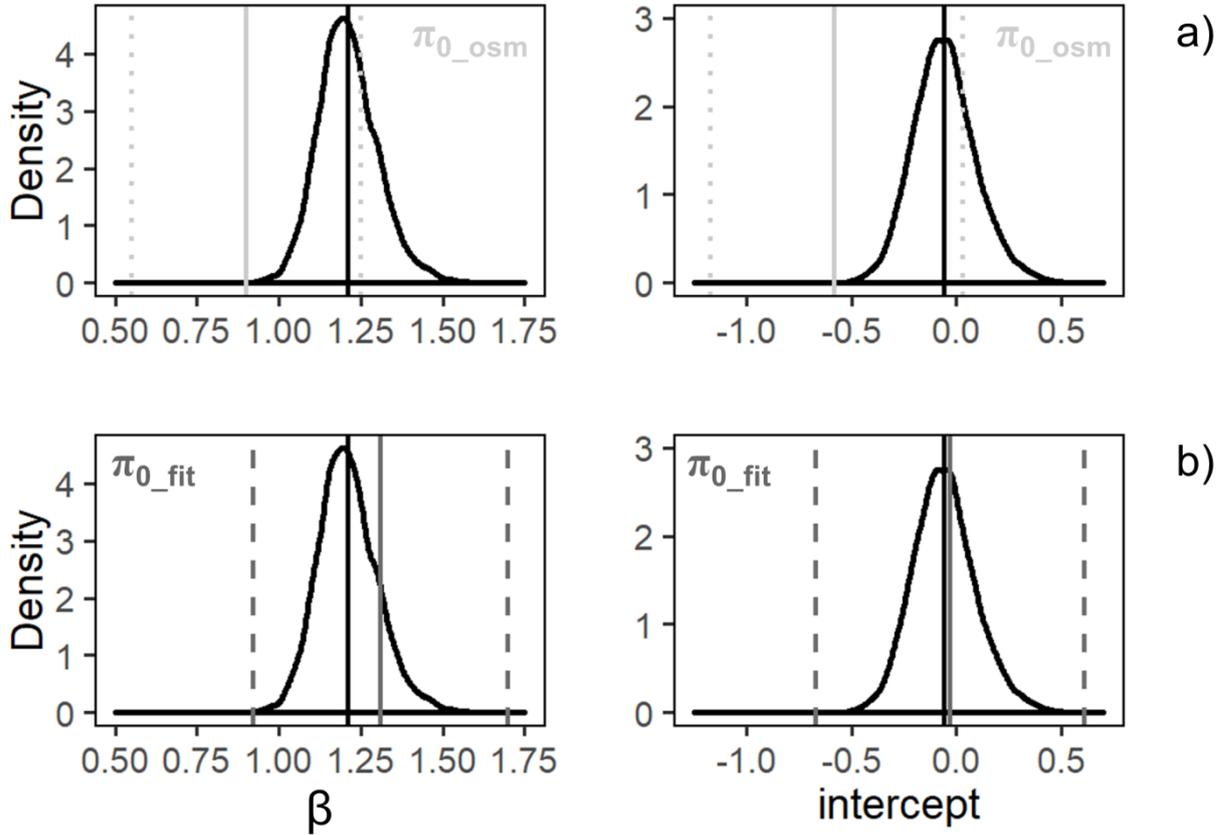


Fig. 3 Density probability and associated mean values (black solid lines) of coefficient β and intercept of the model predicting Ψ_{tlp_pv} calculated on a reduced subset of data from Bartlett *et al.* (2012b). Solid and dotted light grey lines represent mean values and 95% C.I. of coefficient β and intercept calculated on the model including π_{0_osm} as predictive variable (a). Solid and dashed dark grey lines represent mean values and 95% C.I. of coefficient β and intercept calculated on the model including π_{0_fit} as predictive variable (b).

Clearly, the inclusion of more species and π_0 values in this type of analysis is needed to furtherly optimize the framework for Ψ_{tlp} estimation.

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Supplementary information

Tab. S1. Species name (and abbreviation) and associated mean values \pm standard deviation of morpho-anatomical trait, osmotic potential measured with a dewpoint hygrometer (π_{0_osm}) and pv-curves derived water relation parameters. Th = leaf thickness. LDMC = leaf dry matter content. LMA = leaf mass per unit area. d_{leaf} = leaf density.

Species	Th, μm	LDMC, mg g^{-1}	LMA, mg cm^{-2}	d_{cell} , g cm^{-3}	$\pi_{0,\text{osm}}$, -MPa	$\pi_{0,\text{pv}}$, -MPa	$\Psi_{\text{tp,pv}}$, -MPa	ϵ , MPa	Source of water relation parameters
<i>Ailanthus altissima</i> (Mill.) Swingle	46.3±10.8	235.5±4.2	3.6±0.1	0.8±0.2	-1.2±0.1	-1.1±0.2	-1.3±0.4	32.4±8.0	(Savi <i>et al.</i> 2016b)
<i>Acer campestre</i> L.	71.8±23.9	362.4±0.9	4.9±0.5	0.7±0.2	-1.5±0.01	-1.2±1.1	-1.9±0.1	5.2±1.3	(Nardini <i>et al.</i> 2012)
<i>Acer monspessulanum</i>	111.2±6.4	474.0±4.9	7.0±0.2	0.6±0.05	-1.4±0.03	-1.6±0.2	-2.2±0.3	9.6±3.2	(Nardini <i>et al.</i> 2012)
<i>Acer pseudoplatanus</i>	109.8±22.1	340.1±0.6	5.8±1.2	0.5±0.05	-1.4±0.01	-1.0±0.1	-1.4±0.1	7.0±3.2	(Nardini <i>et al.</i> 2012)
<i>Arbutus unedo</i> L.	211.0±4.4	381.8±26.2	11.4±1.4	0.5±0.05	-1.6±0.01	-1.5±0.4	-2.0±0.5	8.7±4.7	(Savi <i>et al.</i> 2017)
<i>Corylus corygyria</i>	102.8±2.5	379.9±15.7	6.1±0.5	0.6±0.03	-1.4±0.3	-1.3±0.3	-2.0±0.4	6.6±2.3	(Savi <i>et al.</i> 2016c)
<i>Cistus scabifolius</i> L.	136.0±37.9	244.4±31.3	6.4±2.6	0.5±0.03	-1.1±0.2	-1.3±0.1	-1.6±0.2	5.5±1.4	(Savi <i>et al.</i> 2016c)
<i>Emerus major</i> Mill.	42.4±6.6	238.9±11.2	4.1±0.1	1.0±0.1	-1.4±0.03	-1.4±0.3	-1.9±0.3	9.4±5.0	(Savi <i>et al.</i> 2016c)
<i>Hedera helix</i> L.	165.7±36.0	373.4±13.9	8.9±2.0	0.5±0.1	-1.0±0.1	-1.0±0.1	-1.5±0.1	5.1±1.7	(Nardini <i>et al.</i> 2012)
<i>Ilex aquifolium</i> L.	319.5±3.4	393.6±1.6	17.2±0.5	0.5±0.05	-1.5±0.1	-1.5±0.05	-1.8±0.01	18.0±2.7	(Nardini <i>et al.</i> 1996)
<i>Launus nobilis</i> L.	175.0±7.4	576.5±74.0	11.3±0.6	0.7±0.05	-1.8±0.02	-2.2±0.02	-2.5±0.31	40.7±1.5	(Nardini <i>et al.</i> 1996)
<i>Ligustrum vulgare</i> L.	113.3±12.2	303.3±13.4	6.3±0.9	0.6±0.01	-1.5±0.4	-1.2±0.2	-1.8±0.2	2.9±2.8	(Savi <i>et al.</i> 2016c)
<i>Malus punila</i> Mill.	118.3±9.8	426.8±4.8	9.5±0.6	0.8±0.01	-2.1±0.04	-1.5±0.01	-2.2±0.2	2.0±0.02	Present study
<i>Olea oleaster</i> Hoffmanns. & Link	439.1±7.8	470.0±4.3	23.6±1.3	0.5±0.02	-2.6±0.3	-2.5±0.1	-3.6±0.1	19.3±1.6	(Lo Gullo <i>et al.</i> 1998)
<i>Phyllirea angustifolia</i>	216.9±71.6	481.2±42.9	13.9±4.7	0.6±0.05	-1.8±0.01	-1.8±0.3	-2.5±0.03	9.4±12.6	(Savi <i>et al.</i> 2016c)
<i>Phyllirea latifolia</i> L.	210.1±33.3	466.4±7.8	12.0±2.3	0.6±0.02	-1.6±0.1	-1.9±0.3	-2.4±0.1	2.3±0.1	Present study
<i>Pistacia lentiscus</i> L.	142.1±52.6	376.2±86.0	9.0±4.9	0.6±0.1	-2.2±0.1	-2.2±0.1	-2.7±0.3	16.1±5.8	(Savi <i>et al.</i> 2016c)
<i>Prunus mahaleb</i> L.	76.0±18.2	366.6±14.9	6.4±0.6	0.9±0.1	-1.5±0.4	-1.6±0.2	-2.2±0.2	8.8±3.6	(Savi <i>et al.</i> 2016c)
<i>Palurus spina-christi</i> Mill.	48.2±17.0	366.1±67.0	4.2±1.7	0.9±0.1	-2.3±0.2	-1.5±0.05	-2.0±0.2	5.5±3.0	(Savi <i>et al.</i> 2016c)
<i>Prunus spinosa</i> L.	65.7±11.9	272.8±44.8	3.4±0.7	0.5±0.03	-2.0±0.1	-1.7±0.2	-2.2±0.1	10.1±1.5	(Savi <i>et al.</i> 2017)
<i>Pyrus pyrastet</i> (L.) Burgsd.	185.8±1.7	300.3±14.0	8.2±0.7	0.4±0.04	-1.7±0.07	-1.7±0.5	-2.3±0.5	5.6±6.6	(Savi <i>et al.</i> 2017)
<i>Quercus ilex</i> L.	201.3±26.4	507.0±2.7	12.3±1.7	0.6±0.05	-1.4±0.1	-1.9±0.2	-2.6±0.2	16.9±5.0	(Nardini <i>et al.</i> 2012)
<i>Quercus pubescens</i> ^{W:11.4}	125.0±17.2	432.5±3.1	7.3±0.7	0.6±0.01	-1.8±0.1	-2.3±0.1	-2.9±0.2	11.8±1.8	(Savi <i>et al.</i> 2016b)
<i>Rhamnus alaternus</i> L.	186.7±2.0	427.0±25.6	11.9±1.0	0.6±0.05	-2.3±0.1	-1.4±0.4	-1.8±0.3	6.1±7.9	(Savi <i>et al.</i> 2017)
<i>Robinia pseudacacia</i>	52.2±7.0	314.3±13.6	4.5±0.3	0.9±0.05	-0.8±0.1	-1.0±0.2	-1.2±0.04	14.6±8.6	(Savi <i>et al.</i> 2016b)
<i>Sambucus nigra</i> L.	58.5±1.9	221.9±30.3	4.0±0.9	0.7±0.1	-1.1±0.1	-0.8±0.03	-1.0±0.05	7.4±0.4	(Nardini <i>et al.</i> 2012)
<i>iburnum tinus</i> L.	154.2±8.1	385.2±4.6	8.3±0.2	0.5±0.02	-1.9±0.1	-1.8±0.1	-2.0±0.1	35.0±7.2	(Nardini <i>et al.</i> 2012)

	Th, μm	LMA, g cm^{-2}	dleaf, g cm^{-3}	LDMC, mg g^{-1}	π_{0_osm} , MPa	π_{0_pv} , MPa	Ψ_{tip_pv} , MPa
LMA, g cm^{-2}	0.95						
dleaf, g cm^{-3}	-0.53	-0.29					
LDMC, mg g^{-1}	0.69	0.81	-0.02				
π_{0_osm} , MPa	-0.36	-0.40	0.06	-0.43			
π_{0_pv} , MPa	-0.54	-0.57	0.17	-0.66	0.68		
Ψ_{tip_pv} , MPa	-0.48	-0.54	0.11	-0.67	0.66	0.95	
ϵ , MPa	0.11	0.13	0.04	0.25	-0.10	-0.43	-0.27

Tab. S2 Correlation matrix (Sperman's rank coefficient) of the traits included in the present study. In bold the significant coefficients ($P < 0.05$)

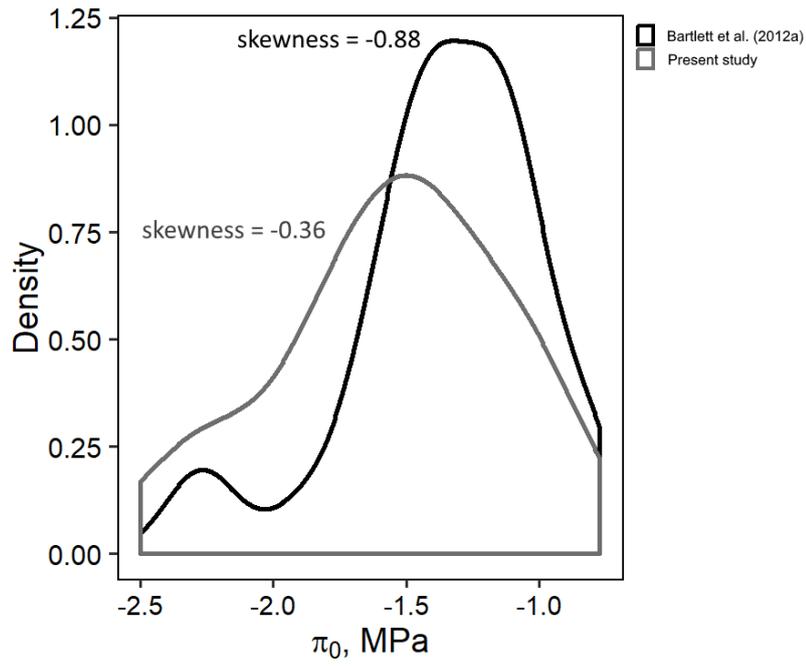


Fig. S1 Density probability distribution of π_0 values in the present study (dark grey) and in the dataset from Bartlett *et al.* (2012a, black).

Chapter 2

Study 3

Less safety for more efficiency: water relations and hydraulics of the invasive tree *Ailanthus altissima* (Mill.) Swingle compared with native *Fraxinus ornus* L.

Francesco Petruzzellis^{1*}, Andrea Nardini^{1*}, Tadeja Savi², Vanessa Tonet¹, Miris Castello¹ and Giovanni Bacaro¹, Andrea Nardini¹

¹Dipartimento di Scienze della Vita, Università degli Studi di Trieste, via L. Giorgieri 10, 34127, Trieste, Italia

²University of Natural Resources and Life Sciences, Vienna, Department of Crop Sciences, Division of Viticulture and Pomology, Konrad Lorenz Straße 24, A-3430 Tulln, Austria

*Corresponding authors

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Abstract

Invasion of natural habitats by alien trees is a threat to forest conservation. Our understanding of fundamental ecophysiological mechanisms promoting plant invasions is still limited, and hydraulic and water relation traits have been only seldom included in studies comparing native and invasive trees. We compared several leaf and wood functional and mechanistic traits in co-occurring *Ailanthus altissima* (Mill.) Swingle (Aa) and *Fraxinus ornus* L. (Fo). Aa is one of the most invasive woody species in Europe and North America, currently outcompeting several native trees including Fo. We aimed at quantifying inter-specific differences in terms of: (i) performance in resource use and acquisition; (ii) hydraulic efficiency and safety; (iii) carbon costs associated to leaf and wood construction; and (iv) plasticity of functional and mechanistic traits in response to light availability. Traits related to leaf and wood construction and drought resistance significantly differed between the two species. Fo sustained higher structural costs than Aa, but was more resistant to drought. The lower resistance to drought stress of Aa was counterbalanced by higher water transport efficiency, but possibly required mechanisms of resilience to drought-induced hydraulic damage. Larger phenotypic plasticity of Aa in response to light availability could also promote the invasive potential of the species.

Keywords

functional traits, gas exchange, hydraulic conductance, invasive plant, plasticity, turgor loss point.

Introduction

The invasion of natural habitats by alien plants is one of the most important threats to biodiversity conservation and ecosystem stability at regional and global scales (Pejchar & Mooney, 2009; Pyšek *et al.*, 2012). Invasion by alien trees is of particular concern because forests represent the largest carbon stock in terrestrial ecosystems and are also at the base of several biogeochemical and hydrological processes of fundamental importance (Bonan, 2008). Hence, alterations in tree species' composition and abundance following invasion by alien trees is predicted to produce large and possibly irreversible impacts (Moser *et al.*, 2009). This is especially worrying in the context of ongoing climate change, leading to higher frequency and intensity of droughts that are apparently increasing the dieback and mortality rates of forest trees (Hember *et al.*, 2017; Neumann *et al.*, 2017). Invasion by alien trees and resulting competition for water might accelerate these processes (Cavaleri & Sack, 2010; Schachtschneider & February, 2013), increasing the risk of native forest decline in the coming decades. Our understanding of the fundamental ecophysiological mechanisms promoting invasion by alien tree species is still limited. Studies comparing functional traits of species with different growth forms and biogeography have revealed consistent patterns in traits' differences between alien and native species (van Kleunen *et al.*, 2010). In particular, invasive trees have been frequently reported to display higher values for traits related to resource acquisition compared with native tree species. These traits include high photosynthetic capacity, high specific leaf area and extensive root systems, all leading to increased biomass production in invasive species (Stratton & Goldstein, 2001; Lamarque *et al.*; 2011, Zeballos *et al.*, 2014; Luo *et al.*, 2016). In fact, invasive and native species typically occupy opposite ends in the leaf economic spectrum (Wright *et al.*, 2004; Zeballos *et al.*, 2014; Funk *et al.*, 2016). Phenotypic plasticity, defined as the ability of a genotype to express different phenotypes in different environments (Nicolson *et al.*, 2010), has been also proposed as an important trait favouring the invasive potential of alien species (Luo *et al.*, 2016; Oliveira *et al.*, 2017). Richards *et al.* (2006) proposed that plasticity of functional traits may allow invasive species to better acclimate to different environmental conditions than natives, thus broadening their ecological spatial amplitude or extending the period of active photosynthesis and biomass production on a seasonal scale (Stratton & Goldstein 2001; Nardini *et al.*, 2003; Caplan & Yeakley, 2013; Luo *et al.*, 2016).

Despite large efforts devoted to identify functional traits related to invasiveness of alien woody species, most studies dealing with this topic have focused on traits relatively easy to measure, but often without clear mechanistic linkages with plant physiological performance. As an example, specific leaf area is one of the most used parameters in trait-based ecology, although it

actually represents a ‘syndrome trait’ resulting from the interaction of several different processes including mass-based light-saturated photosynthetic rate, leaf lifespan and nutrient concentration, all finally influencing the plant-level growth rate (Poorter *et al.*, 2009). However, ‘mechanistic’ traits (sensu Brodribb, 2017) clearly associated with physiological processes hold better promise to provide meaningful information on fitness and performance of invasive plants compared with native ones. Among these, traits related to plant water relations and hydraulics are very interesting for studies focused on invasive trees, because the efficiency of root-to-leaf water transport is one of the factors most closely correlated to leaf gas exchange rates, maximum net photosynthesis, competition for water and relative growth rates (Nardini *et al.*, 1999; Nardini, 2002; Tyree, 2003; Brodribb, 2009). On the other hand, hydraulic safety expressed in terms of critical water potential values inducing xylem embolism (Urli *et al.*, 2013; Zhu *et al.*, 2017) or cell turgor loss (Binks *et al.*, 2016; Savi *et al.*, 2017) is the most critical factor for tree resistance to drought and persistence under water-limited conditions (Nardini *et al.*, 2014a). Most importantly, it has been suggested that a trade-off exists between tree hydraulic safety and efficiency (Nardini & Luglio, 2014; Gleason *et al.*, 2016), and this trade-off might be important to explain interaction and competition processes during the invasion process.

Hydraulic and water relation traits have been only seldom included in studies comparing native and invasive trees, and results are somehow contrasting. As an example, Crous *et al.* (2012) reported that the invasive *Acacia mearnsii* had higher resistance to xylem embolism, and hence tolerance to drought, compared with two native species in fynbos riparian ecotones. A similar finding was reported by Yazaki *et al.*, (2010) showing that saplings of the invasive *Psidium cattleianum* were more resistant to xylem embolism than native *Trema orientalis*. Opposite findings were reported by Zeballos *et al.* (2014), showing that wood density of invasive tree species was on average lower than that of native trees. This would suggest higher vulnerability to xylem embolism in invasive trees, considering the frequently reported correlation between wood density and xylem resistance to embolism (Markestejn *et al.*, 2011; Nardini *et al.*, 2013; Barotto *et al.*, 2018). By comparing five pairs of cooccurring native and invasive trees, Pratt & Black (2006) concluded that hydraulic traits do not explain alone the invasive nature of tree species, although in two out of the five pairs considered, the invasive species turned out to be more vulnerable to embolism than the native ones.

Ailanthus altissima (Mill.) Swingle (hereafter referred to as Aa) is one of the most invasive woody species in Europe and North America, where it was introduced for ornamental and economical purposes from East Asia (Sladonja *et al.*, 2015). The genus *Ailanthus* belongs to the family of Simaroubaceae. Members of this family have a primarily pantropical distribution (Clayton

et al., 2007). However, the genera *Leitneria*, *Castela*, *Holacantha*, *Ailanthus*, *Picrasma* and *Brucea* include subtropical and temperate members. Although *Ailanthus* has an extensive fossil record dating from the early Eocene across the entire Northern Hemisphere (Corbett & Manchester, 2004), *Aa* is at present the only species of the family represented in Europe, where it is considered alien and invasive. In its invasive range, the species is common in urban or disturbed areas, but in the Mediterranean region it also occurs in some natural habitats with increasing negative impacts on native outcompeted species (Gómez-Aparicio & Canham, 2008; Constan-Nava *et al.*, 2010). In the area selected for this study (Classical Karst, NE Italy), *Aa* is currently outcompeting the native tree *Fraxinus ornus* L. (hereafter referred to as *Fo*) in post-disturbance vegetation successions as well as in natural forest regeneration processes, where *Fo* acts as a pioneer tree well adapted to exploit warm, sunny and water-limited habitats (Kalapos & Csontos, 2003; Chiatante *et al.*, 2006; Gortan *et al.*, 2009). *Fo* is a member of the Oleaceae. The genus is represented in Europe by other species like *Fraxinus excelsior* or *Fraxinus angustifolia*, but these have quite different ecological requirements as they generally occur in mature forests with higher water availability.

The distribution ranges of *Aa* and *Fo* in Europe are largely overlapping (Caudullo *et al.*, 2017), and the two species show similar ecological requirements and habitat preferences (<http://forest.jrc.ec.europa.eu/european-atlas-of-forest-tree-species>). *Aa* and *Fo* share several common features, including small-to-medium size, presence of compound leaves, high seed production, fast growth, insect pollination, winged fruits (samaras) dispersed by wind, and the tendency to colonize and spread in open habitats. By measuring several leaf and wood functional and mechanistic traits in co-occurring *Aa* and *Fo*, we specifically aimed at testing inter-specific differences in terms of: (i) performance in resource use and acquisition; (ii) hydraulic efficiency and safety; (iii) carbon costs associated to leaf and wood construction; and (iv) plasticity of functional and mechanistic traits in response to light availability.

Materials and methods

Study area

The study area was located near the village of Gropada (North-Eastern Italy, 45.667390N, 13.846307E) in the Classical Karst, a limestone plateau extending by ~500 km² and characterized by a sub-Mediterranean climate. Summers are warm (average temperature 24.2 °C) with a short dry period occurring in July, while winters are mild (average temperature 7.5 °C), although frost events are not rare. Mean annual rainfall averages 900 mm (data from ARPA-OSMER, <http://www.osmer.fvg.it>, reference period 1996–2016). The natural vegetation is dominated by

deciduous thermophilous mixed oak woodland, which alternates with *Pinus nigra* J.F. Arnold subsp. *nigra* plantations.

Two sites, each with an extension of $\sim 1500 \text{ m}^2$ and characterized by contrasting light conditions (hereafter, L = site with high light availability and S = shaded site) were selected in the study area. The L site was located in an open grassland area undergoing progressive encroachment, where Aa was dominating over the native Fo. Here, individuals of both species had heights ranging from 1.5 to 5 m. The S site was located within a nearby pinewood where the dominant species in the canopy was *P. nigra* with a mean plant height in the range of 10–15 m and several individuals of Aa and Fo were present in the understory, with average heights of 1–3 m. Air temperature (T, °C), relative humidity (RH, %) and incident photosynthetic photon flux density (PPFD, $\mu\text{mol m}^{-2} \text{ s}^{-1}$) were measured in each site at the same dates and times selected for field physiological measurements, using a thermo-hygro-anemometer (model 45160, Extech Instruments, Nashua, NH, USA) and a portable light metre (model HD 9021, Delta OHM srl, Padova, Italy), respectively. Five individuals of Aa and five of Fo were randomly selected in each site, and the functional traits listed in Table 1 were measured. All field measurements and sample collection for laboratory analyses were performed during 10 sunny days between the end of June and early July 2016.

Plant water status and gas exchange

Leaf conductance to water vapour (g_L), minimum leaf water potential (Ψ_{\min}) and pre-dawn leaf water potential (Ψ_{pd}) were measured on two leaves per individual selected from the outer part of the canopy. Both g_L and Ψ_{\min} were measured between 12:30 and 15:00 h, using a steady-state porometer (SC-1 Decagon Devices Inc., Pullman, WA, USA) and a pressure chamber (mod. 1505D, PMS Instrument Company, Albany, OR, USA), respectively. Ψ_{pd} was measured between 4:00 and 5:00 h. For Ψ_{pd} and Ψ_{\min} , leaves were detached from branches, wrapped in cling film and put in plastic bags with a piece of wet paper inside. Samples were stored in cool bags until measurements in the laboratory within 1–2 h from collection.

Estimating leaf transpiration rate and whole-plant hydraulic conductance

Leaf transpiration rate (E_L) was calculated as: $E_L = (\text{VPD}/p_{\text{atm}}) \times g_L$, where VPD is the leaf-to-air vapour pressure deficit (kPa), p_{atm} is the atmospheric pressure (kPa) and g_L is the leaf conductance to water vapour ($\text{mmol m}^{-2} \text{ s}^{-1}$). The VPD is given by $\text{VPD} = \text{VP}_{\text{leaf}} - \text{VP}_{\text{air}}$, where VP_{leaf} is the saturated water vapour pressure in the sub-stomatal cavity and VP_{air} is the partial pressure of water vapour in the air. VP_{air} and VP_{leaf} were calculated as $\text{VP}_x = \text{VP}_0 \times (1 - \text{RH})$, where VP_0 is the vapour

pressure at saturation and RH is the relative humidity recorded at the time when g_L was measured (see above). Relative humidity was assumed to be 100% in the leaf interior spaces.

On the basis of E_L values as well as Ψ_{\min} and Ψ_{pd} (taken as a proxy for soil water potential measurements), whole-plant hydraulic conductance (k_{plant}) was calculated as $k_{\text{plant}} = E_L / (\Psi_{pd} - \Psi_{\min})$.

<i>Functional trait</i>	<i>abbreviation</i>	<i>unit</i>
Leaf conductance to water vapour	g_L	$\text{mmol m}^{-2} \text{s}^{-1}$
Leaf transpiration rate	E_L	$\text{mmol m}^{-2} \text{s}^{-1}$
Pre-dawn leaf water potential	Ψ_{pd}	MPa
Minimum leaf water potential	Ψ_{\min}	MPa
Leaf osmotic potential at full turgor	π_0	MPa
Leaf water potential at turgor loss point	Ψ_{tlp}	MPa
Stem specific hydraulic conductivity	K_s	$\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$
Whole-plant hydraulic conductance	k_{plant}	$\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$
Water potential at 50% loss of conductivity	Ψ_{50}	MPa
Safety margin	SM	MPa
Wood density	WD	g cm^{-3}
Wood capacitance	C_{wood}	$\text{kg MPa}^{-1} \text{m}^{-3}$
Leaf dry matter content	LDMC	mg g^{-1}
Specific leaf area	SLA	$\text{mm}^2 \text{mg}^{-1}$
Major vein length per unit area	VLA_{maj}	mm mm^{-2}
Minor vein length per unit area	VLA_{min}	mm mm^{-2}
Leaf C to N ratio	C:N	/
Leaf C content	C	%
Leaf N content	N	%
Leaf ^{13}C isotopic composition	$\delta^{13}\text{C}$	‰

Tab 1. List of functional and mechanistic traits with their respective abbreviation and unit measured in *A. altissima* and in *F. ornus*.

Water relation parameters

Osmotic potential at full turgor (π_0) and water potential at turgor loss point (Ψ_{tp}) were measured on five leaves from five individuals, sampled on the basis of a hierarchically stratified random design (Petruzzellis *et al.*, 2017). Leaves were detached from branches, wrapped in cling film and put in plastic bags with a piece of wet paper inside to avoid dehydration. Samples were stored in cool bags until processing in the laboratory. Measurements were done according to Bartlett *et al.* (2012) with some modifications. Fresh leaf laminas were first rehydrated overnight and were then roughly crumbled and sealed in cling film. Then, they were immersed in liquid nitrogen for 2 min. Leaves (still sealed in cling film) were then carefully ground and stored in sealed plastic bottles at $-20\text{ }^{\circ}\text{C}$ until measurements, when samples were thawed at room temperature for 5 min while still sealed in cling film and in plastic bottles. Measurements of π_0 were done with a dew point hygrometer (Model WP4, Decagon Devices Inc.). Measurements of π_0 using the method described above could be influenced by sample dilution due to apoplastic water. To overcome this limitation, π_0 were corrected for different leaf dry matter content (LDMC; see below) using the following equation (Petruzzellis *et al.*, submitted, see Study 2): $\pi_0 = (0.5303 \times \pi_{\text{WP4}}) + (0.0019 \times \text{LDMC}) - 0.001306$. Ψ_{tp} was finally calculated as: $\Psi_{\text{tp}} = (1.31 \times \pi_0) - 0.03$.

Leaf morpho-anatomical traits

Five leaves from five individuals in each site and for each species were sampled as described for water relation parameters and leaf morpho-anatomical traits were measured. Leaf dry matter content and specific leaf area were calculated as:

$$\text{LDMC} = \text{Leaf dry weight} / \text{Leaf turgid weight}.$$

$$\text{SLA} = (\text{Leaf area}) / (\text{Leaf dry weight}).$$

Fresh leaves were first rehydrated overnight, and leaf turgid weight was measured with an analytical balance. Leaves were scanned using a scanner, and leaf area was measured using the software ImageJ. Leaves were finally oven-dried for 48 h at $72\text{ }^{\circ}\text{C}$ and leaf dry weight was measured.

The lengths of major and minor veins per unit surface area (VLA_{maj} and VLA_{min} , respectively) were measured as: $\text{VLA} = \text{Vein Length} / \text{Leaf sample area}$. To measure VLA_{maj} , fresh leaves were scanned and the ratio between vein length and sample area were measured using PhenoVein software (Bühler *et al.*, 2015). For VLA_{min} , fresh leaves were cleared in 1 M NaOH solution for 48–72 h at room temperature, replacing solution when it turned dark coloured. Then, small portion of leaves (1 cm^2) were cut and bleached in 5% NaClO for 1–2 min. Samples were treated in a sequence of ethanol solutions at increasing concentration (25%, 50%, 75% and 100%) and then immersed in an alcoholic solution of toluidine blue (3%) overnight. Finally, samples were

processed in a series of ethanol solutions at decreasing concentration and microscopic slides were prepared. Images of small portions (~5 mm²) of leaves were captured with an optical microscope at 4× magnification equipped with a digital camera and VLA_{min} was measured using PhenoVein software.

Nutrient concentration and stable isotopes analysis

Nitrogen content (N %), carbon content (C %), C:N ratio and carbon isotopic composition ($\delta^{13}C$) were measured on one dried pulverized leaf randomly sampled from each individual of each species in L and S sites. Leaves were sampled as described above, oven-dried and then pulverized in a mortar. Samples were analysed for C and N contents (% dry weight), and stable isotope ratios were assessed via elemental analyser/continuous flow isotope ratio mass spectrometry using a CHNOS Elemental Analyser (vario ISOTOPE cube, Elementar, Hanau, Germany) coupled with an IsoPrime 100 mass spectrometer (Isoprime Ltd, Cheadle, UK). All isotope analyses were conducted at the Centre for Stable Isotope Biogeochemistry at the University of California, Berkeley. Long-term external precision based on reference material 'NIST SMR 1577b' (bovine liver) is 0.10‰ and 0.15‰ for C and N isotope analyses, respectively.

Wood density, wood capacitance and hydraulic conductivity measurements

Wood density (WD) and wood capacitance (C_{wood}) were measured on 2-year-old segments from one stem per each individual (10 stems per species, 5 per each site).

Wood density was calculated as:

$$WD = \text{Wood dry weight} / \text{Wood dry volume}$$

Bark was removed from 3 cm long segments before oven-drying the samples at 70 °C for 24 h. Samples were then weighed, and their dry volume was measured using a water displacement method (Hughes, 2005).

To measure wood capacitance (C_{wood}), 3 cm long segments were longitudinally split in two parts. Bark was removed, and samples were soaked in water overnight. Fresh volume was measured as described for WD. Sequential measurements of sample weight and water potential (using the WP4 hygrometer, see above) were performed during bench dehydration. Measurements were performed in the Ψ range between 0 and -2.0 MPa. C_{wood} was calculated as the slope of the cumulative water loss vs Ψ linear relationship, normalized by fresh volume (Savi *et al.*, 2017).

To estimate the vulnerability of the study species to drought induced embolism formation, vulnerability curves were measured with classical hydraulic techniques coupled with bench dehydration. Embolism-induced loss of stem hydraulic conductance was measured on stem

segments sampled from 2-year-old branches, randomly collected from all individuals included in the study (see above). Stems were detached from branches and the cut section was immediately put in water. Additional cuts were made under water to remove any eventual embolism induced by the initial cutting (Trifilò *et al.*, 2014; Beikircher & Mayr, 2016) and stems were immediately transported to the laboratory, where they were rehydrated overnight. Stems were left dehydrating in the laboratory for a minimum of 1 h up to a maximum of 4 days. At different time intervals, two apical leaves were wrapped in cling film and the stem was enclosed in a black plastic bag containing wet paper tissue. After 30 min of equilibration, the xylem water potential (Ψ_{xyl}) was measured. Stem segments were obtained from branches and progressively re-cut under water to the desired length of 5–10 cm. About 1 cm of bark was removed at both ends, and samples were connected to an hydraulic apparatus (Xyl'Em; Bronkhorts France S.A.S., Montigny-Les-Cormeilles, France). The measurement solution was a 10 mM KCl solution (filtered at 0.2 μm) in degassed mineral water (Nardini *et al.*, 2007). Native stem hydraulic conductance (k_i) was initially measured under low water pressure ($P = 7$ kPa). Samples were then flushed at $P = 0.2$ MPa for 10 min to remove embolism and k was re-measured at low pressure to get maximum stem hydraulic conductance (k_{max}). The percent loss of stem hydraulic conductivity (PLC) was calculated as:

$$\text{PLC} = 100 \times [1 - (k_i/k_{\text{max}})]$$

One vulnerability curve (VC, sigmoidal with four parameters) for each species and in each site was generated by plotting all PLC values vs the corresponding Ψ_{xyl} , and the reference value Ψ_{50} (xylem water potential at 50% loss of conductance) was calculated.

Values of k_{max} were also used to calculate stem specific hydraulic conductivity (K_s) for each species and site as:

$$K_s = (k_{\text{max}} \times l)/A_x$$

where l is sample length and A_x is the transverse xylem area as calculated on the basis of xylem diameter measured using a digital calliper immediately after hydraulic measurements.

Finally, the safety margin (SM) experienced by the two species in the different sites was calculated as $\text{SM} = \Psi_{\text{min}} - \Psi_{50}$.

Statistical analysis

After checking for data normality and homogeneity of variances, pairwise comparisons between means of each functional trait measured in the two study species were performed using a two-tailed Student's t-test where, for each couple of normally distributed populations, the null hypothesis that the means are equal was verified. This analysis was run through 't.test' function in 'stats' package

for R software. Each functional trait was treated as a response variable, while species were treated as explanatory variables.

Two-way parametric ANOVA analysis was run to test differences between functional traits values of the study species through ‘aov’ function, in ‘stats’ package for R software. Each functional trait was treated as a response variable, while species and site were treated as explanatory variables. After checking for data normality and homogeneity of variances, post-hoc Tukey’s Honestly Significant Differences comparisons were run through ‘TukeyHSD’ function in ‘stats’ package for R software when the main terms and their interaction in the ANOVA model resulted significant. Vulnerability curves were fitted through ‘fitplcs’ function in ‘fit-PLC’ package for R software and Ψ_{50} values and confidence intervals were calculated for each vulnerability curve. The Ψ_{50} value of a single vulnerability curve was considered different from the others if its confidence intervals did not overlap with confidence intervals calculated from the other vulnerability curves.

To summarize and visually describe the differences among traits of individuals of the two species in the two study sites, a Principal Component Analysis (PCA, R mode) was run through ‘princomp’ function in ‘stats’ package for R software. Before processing data, traits values were standardized (mean = 0; standard deviation = 1).

Results

Air temperature at the time of measurements ranged between 25.7 ± 0.5 and 29.4 ± 1.2 °C in the S and L site, respectively. Relative humidity was similar in the two sites ($41.9 \pm 2.1\%$ in L and $41.5 \pm 3.1\%$ in S), while marked differences were recorded in terms of PPFD, ranging from 21 ± 6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in S to 1386 ± 249 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in L.

The mean values for all traits measured in Aa and Fo in the two study sites, and the relative standard deviations are summarized in Table 2 (data aggregated by species and site) and in Table S1 available as Supplementary Data at Tree Physiology Online (data aggregated by species). Individuals of both species had higher g_L and E_L in L site compared with the S one, with no differences between species (Figure 1). In particular, g_L was almost double in leaves under high irradiance compared with those in the shade, and these differences translated into an even larger increase of E_L (Figure 1) due to higher air temperatures in the L site than in S one.

Despite similar leaf-level rates of water loss, the water status of the two species was different. In fact, Ψ_{min} values measured in L site were significantly higher (less negative) in Aa than in Fo, while no inter-specific difference was recorded in S site (Figure 2). Ψ_{pd} values varied within a very narrow range and averaged -0.4 MPa (Figure 2). The water potential at turgor loss point

(Ψ_{tip}) changed accordingly to different light regimes (Figure 2), with significant lower values in L site compared with S site in both species. However, in both sites Fo had lower Ψ_{tip} values (−2.4 MPa in L site and −1.9 MPa in S site) compared with Aa (−1.8 MPa in L site and −1.5 MPa in S site). These differences in Ψ_{tip} were similar to those recorded for osmotic potential that in both light regimes was lower in Fo than in Aa (Figure 2).

Values of both K_s and k_{plant} were higher in Aa compared with Fo, especially in L site where K_s and k_{plant} were respectively fourfold and twofold higher in Aa than in Fo (Figure 3). Values of Ψ_{50} calculated from vulnerability curves of each species (Figure 4) did not differ between L and S sites but were different in the two species. Fo had higher resistance to drought stress than Aa, with Ψ_{50} averaging −3.2 MPa and −1.2 MPa, respectively. The safety margin against embolism formation experienced at midday was significantly lower in Aa where it was close to 0, than in Fo where it was very large and ranged between 1.5 and 1.9 MPa (Table 2, Figure 5).

The cost associated with the production of tissues devoted to water transport was lower in Aa than in Fo, as WD was $\sim 0.5 \text{ g cm}^{-3}$ in the former species compared with 0.7 g cm^{-3} in the latter (Figure 5). Differences between species in terms of WD apparently translated into differences in C_{wood} , which was higher in Aa than in Fo, with no significant differences between sites (Figure 5). Also leaf structural costs were different between species and sites. In fact, Aa had higher SLA values in the S site than in the L one, while Fo had similar values in both sites. Notably, in the S site the SLA of Aa was about double that of Fo. VLA_{maj} did not change significantly between species and sites, while VLA_{min} was significantly higher in Aa compared with Fo, again with no difference between sites (Figure 6).

Nutrient concentration varied according to the sites. Specifically, in both species C:N was higher in the L site than in the S site (Figure 7). Moreover, C:N was higher in Fo than in Aa. These differences mostly derived from variation in N content, as C content was rather constant at 47% across species and sites, while N was higher in Aa than Fo, and generally higher in S site compared with L site. Leaf carbon isotopic composition was similar in the two species, but values were significantly different in the two sites (Figure 7). In particular, $\delta^{13}\text{C}$ was significantly higher in the S site compared with the L site in both Aa and Fo. The projection of individuals on axis 1 and 2 of the PCA (see Figure S1 available as Supplementary Data at Tree Physiology Online) confirmed a separation between the two species (axis 1, 47% of total explained variance) and between the two study sites with contrasting light availability (axis 2, 24% of total explained variance).

	<i>A. altissima</i>		<i>F. ornus</i>	
	L	S	L	S
g_L , mmol m ⁻² s ⁻¹	443.4±76.6 a	219.3±98.6 b	514.5±177.3 a	234.1±38.9 b
E_L , mmol m ⁻² s ⁻¹	8.1±1.9 a	2.7±1.0 b	10.5±4.4 a	3.0±0.7 b
Ψ_{pd} , MPa	-0.5±0.1 a	-0.5±0.1 a	-0.4±0.1 a	-0.3±0.03 b
Ψ_{min} , MPa	-1.1±0.1 a	-1.0±0.2 a	-1.8±0.3 b	-1.3±0.2 a
π_0 , MPa	-1.5±0.3 a	-1.3±0.3 a	-1.9±0.2 b	-1.4±0.8 a
Ψ_{tlp} , MPa	-1.8±0.1 a	-1.5±0.1 b	-2.4±0.1 c	-1.9±0.4 a
K_s , kg m ⁻¹ s ⁻¹ MPa ⁻¹	8.0±2.4 a	8.0±3.0 a	1.6±0.9 b	1.6±0.6 b
k_{plant} , mmol m ⁻² s ⁻¹ MPa ⁻¹	15.4±6.0 a	5.5±1.4 b	7.5±2.1 b	3.2±0.7 b
Ψ_{50} , MPa	-1.1	-1.3	-3.3	-3.0
SM, MPa	-0.03±0.1 a	0.3±0.2 a	1.5±0.3 b	1.9±0.2 b
WD, g cm ⁻³	0.5±0.05 a	0.4±0.1 a	0.7±0.1 b	0.6±0.1 b
C_{wood} , kg MPa ⁻¹ m ⁻³	66.1±9.3 a	58.3±8.8 a	21.5±5.5 b	31.9±12.5 b
LDMC, mg g ⁻¹	411.9±23.1 a	230.6±5.2 b	408.8±31.6 a	395.6±12.7 a
SLA, mm ² mg ⁻¹	14.0±3.3 a	37.0±7.0 b	11.0±2.4 a	16.8±4.1 a
VLA _{maj} , mm ² mm ⁻¹	0.3±0.02 a	0.3±0.1 a	0.3±0.1 a	0.2±0.1 a
VLA _{min} , mm ² mm ⁻¹	12.5±0.9 a	12.2±1.9 a	8.8±0.8 b	6.8±1.2 b
C:N	23.8±3.2 a	13.8±3.2 b	29.7±1.3 c	22.1±1.3 a
C, %	47.2±1.0 a	46.9±0.7 a	47.8±0.7 a	47.3±0.9 a
N, %	2.0±0.3 a	3.5±0.8 b	1.6±0.1 a	2.1±0.1 a
$\delta^{13}C$, ‰	-27.7±0.5 a	-29.4±1.1 b	-27.6±0.8 a	-29.1±1.2 ab

Tab. 2. Mean values ± standard deviation of functional traits measured in *A. altissima* and *F. ornus* in the site with high irradiance (L) and in shaded site (S). Different letters indicate significant differences between groups ($p < 0.05$)

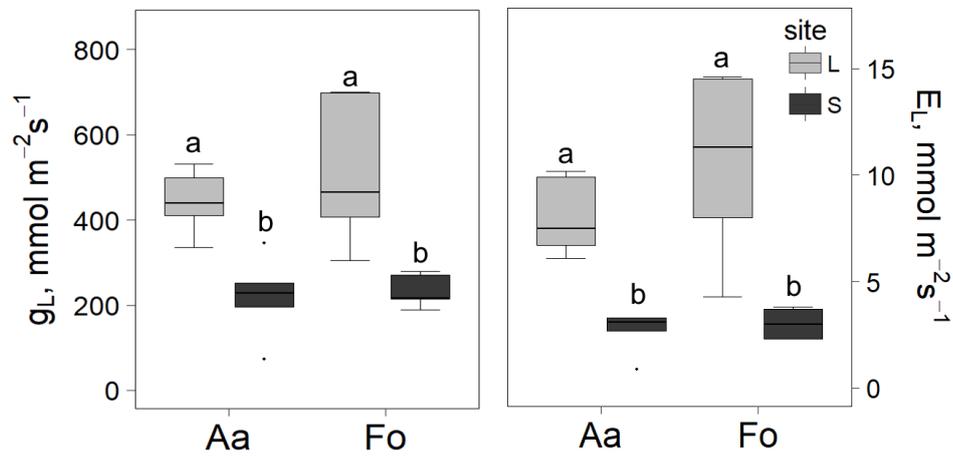


Fig 1 Median values, 25th and 75th percentiles of leaf conductance to water vapour (g_L) and leaf transpiration rates (E_L) measured in *A. altissima* (Aa) and in *F. ornus* (Fo) in L site (grey boxes) and in S site (dark grey boxes). Different letters indicate statistically significant differences among groups ($P < 0.05$).

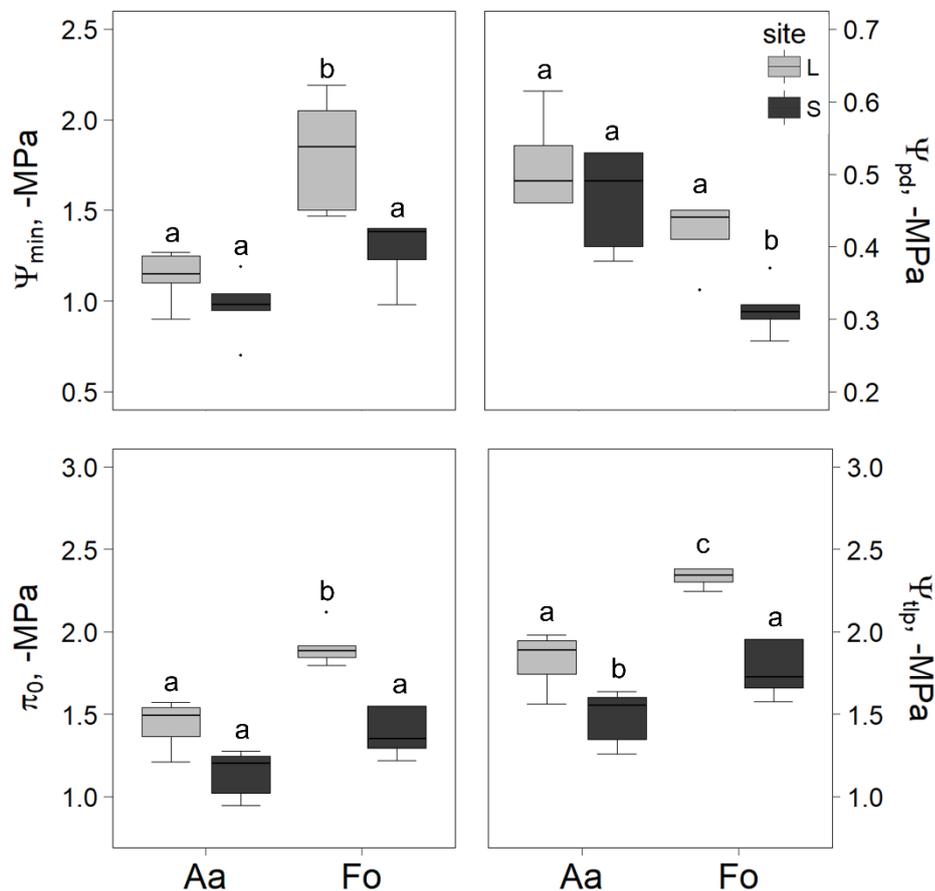


Fig. 2 Median values, 25th and 75th percentiles of minimum leaf water potential (Ψ_{\min}), pre-dawn leaf water potential (Ψ_{pd}), osmotic potential at full turgor (π_0) and water potential at turgor loss point (Ψ_{tlp}) measured in *A. altissima* (Aa) and in *F. ornus* (Fo) in L site (grey boxes) and in S site (dark grey boxes). Different letters indicate statistically significant differences among groups ($P < 0.05$).

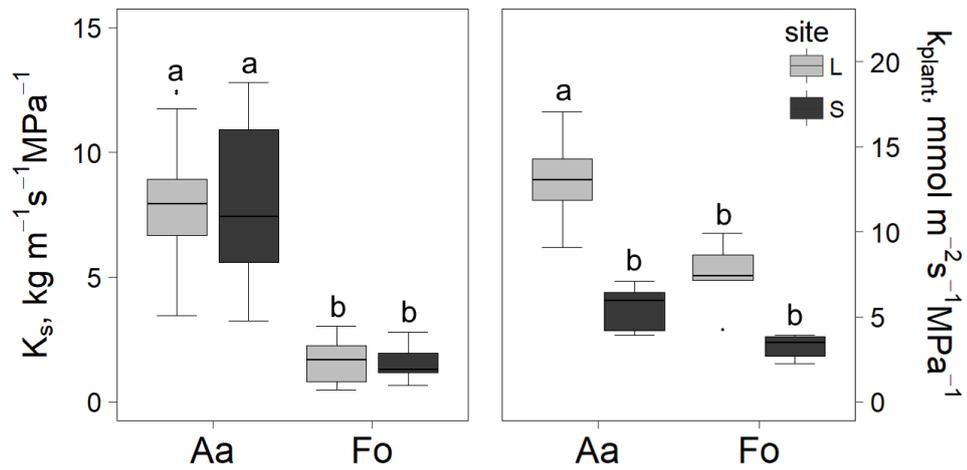


Fig 3 Median values, 25th and 75th percentiles of stem specific hydraulic conductivity (K_s), and whole-plant hydraulic conductance (k_{plant}) measured in *A. altissima* (Aa) and in *F. ornus* (Fo) in L site (grey boxes) and in S site (dark grey boxes). Different letters indicate statistically significant differences among groups ($P < 0.05$).

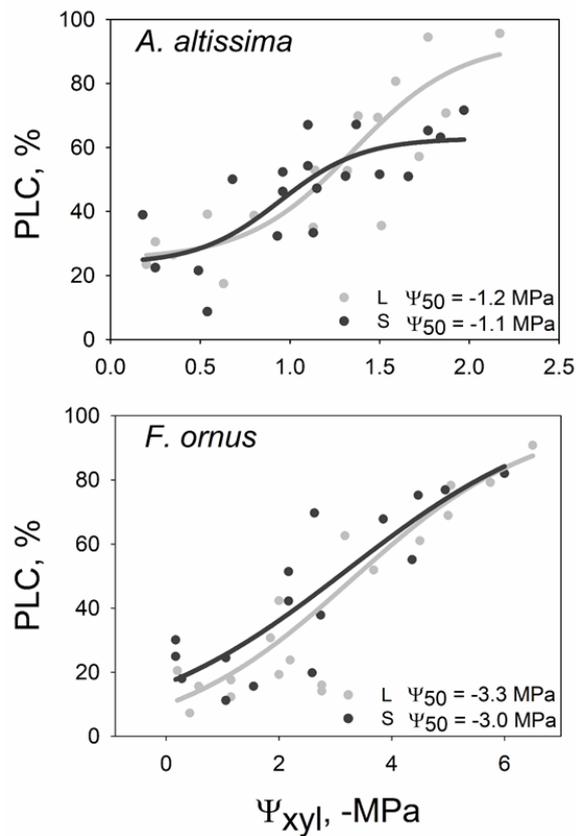


Fig 4 Vulnerability curves reporting the relationship between percent loss of hydraulic conductivity (PLC) and xylem water potential (Ψ_{xyl}) measured in *A. altissima* and in *F. ornus* in L site (grey point and lines) and in S site (dark grey points and lines).

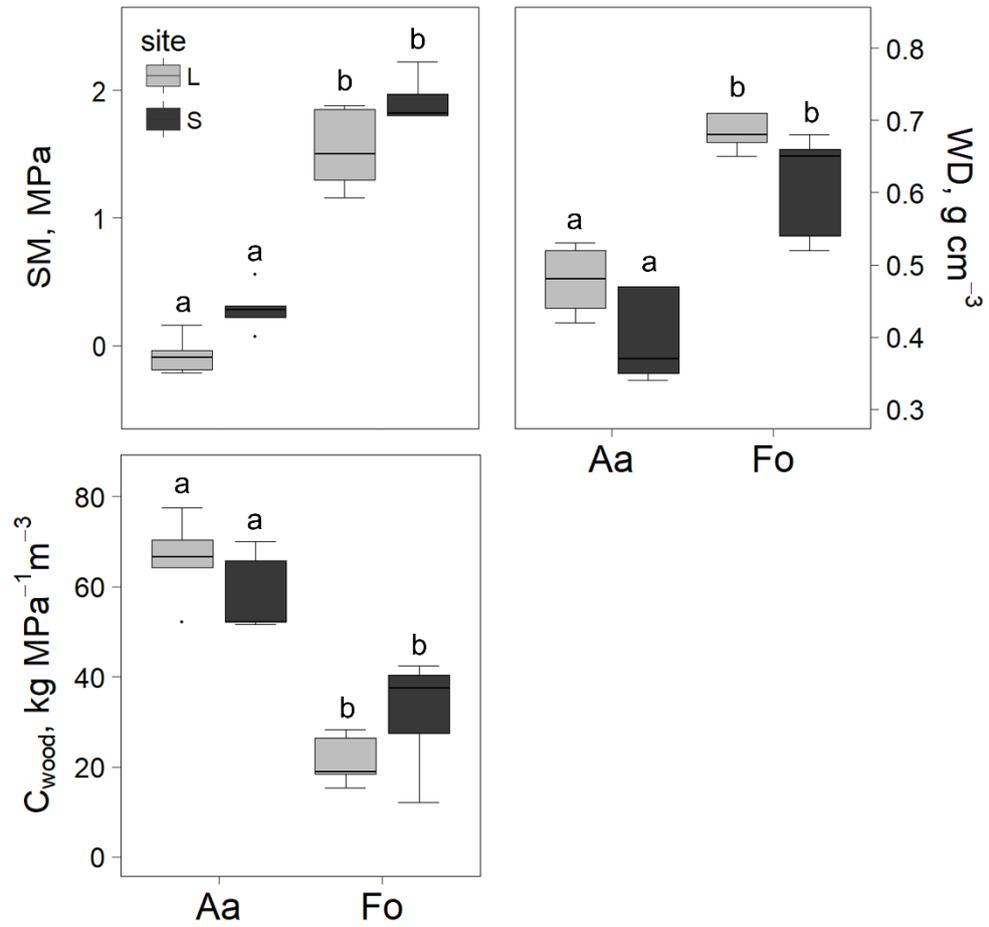


Fig. 5 Median values, 25th and 75th percentiles of safety margin (SM), wood density (WD), and wood capacitance (C_{wood}) measured in *A. altissima* (Aa) and in *F. ornus* (Fo) in L site (grey boxes) and in S site (dark grey boxes). Different letters indicate statistically significant differences among groups (P < 0.05).

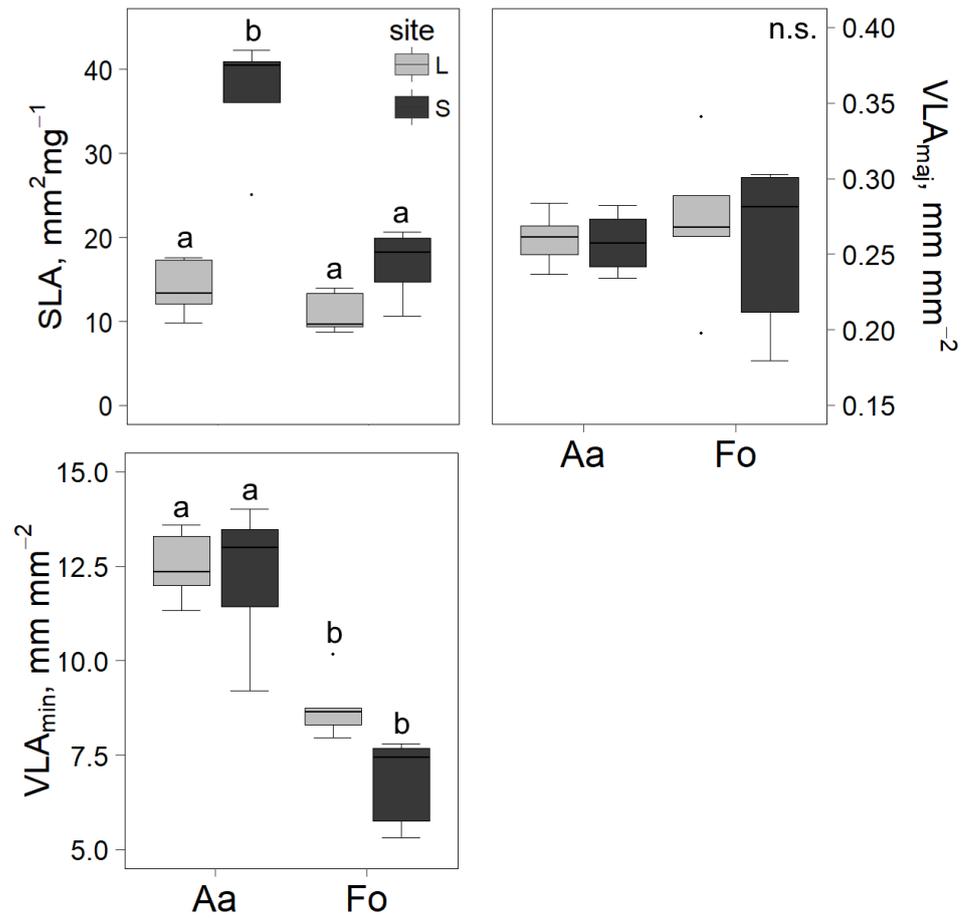


Fig. 6 Median values, 25th and 75th percentiles of specific leaf area (SLA), major vein length per unit area (VLA_{maj}) and minor vein length per unit area (VLA_{min}) measured in *A. altissima* (Aa) and in *F. ornus* (Fo) in L site (grey boxes) and in S site (dark grey boxes). Different letters indicate statistically significant differences among groups ($P < 0.05$), while n.s. indicates no significant differences ($P > 0.05$).

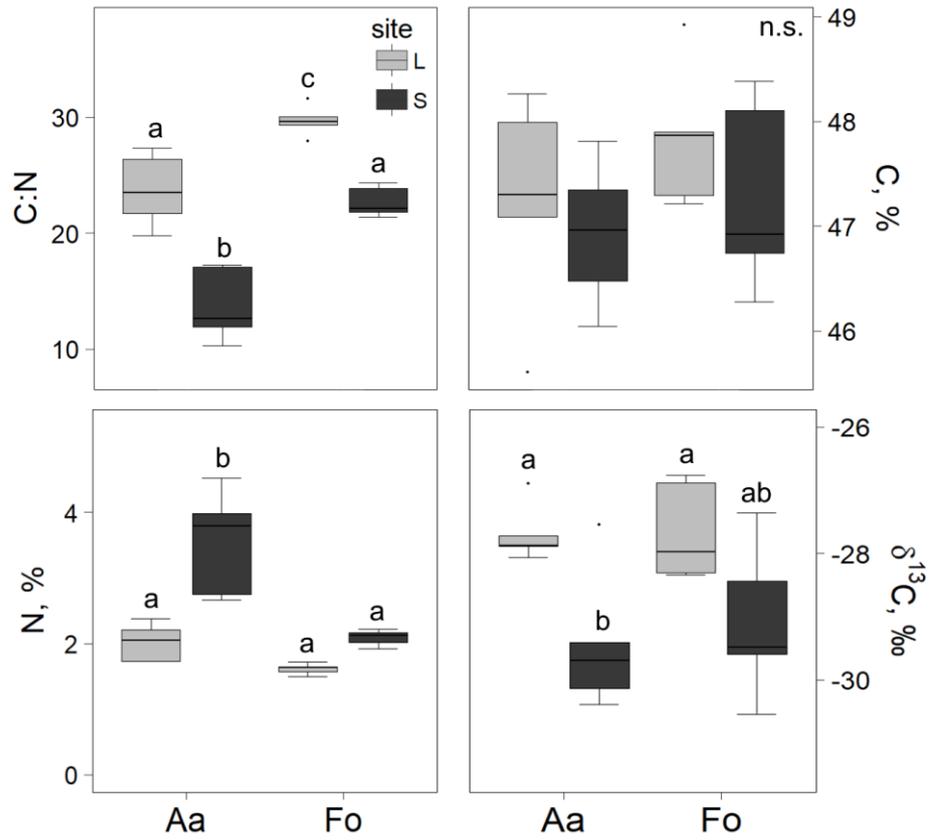


Fig. 7 Median values, 25th and 75th percentiles of leaf C to N ratio (C:N), leaf C content (C), leaf N content (N) and leaf ¹³C isotopic composition ($\delta^{13}C$) measured in *A. altissima* (Aa) and in *F. ornus* (Fo) in L site (grey boxes) and in S site (dark grey boxes). Different letters indicate statistically significant differences among groups ($P < 0.05$), while n.s. indicates no significant differences ($P > 0.05$).

Discussion

Understanding which functional/mechanistic traits promote invasiveness of alien species is fundamental to understanding the mechanisms underlying plant invasions in different native vegetation types, and eventually predict the vulnerability of forests to alien tree invasions. In this study, key functional traits related to resource use and acquisition, with special reference to water use strategies, were compared in an alien species and a cooccurring native tree, growing in two sites with different light availability. We observed significant differences in some physiological and morphological traits, overall suggesting that the invasive potential of Aa is driven by a combination of reduced carbon costs for leaf and stem construction and high efficiency of water transport, translating into high productivity even though at the expense of hydraulic safety.

Similar gas exchange rates but more favourable water status in invasive A. altissima compared with native F. ornus

The different incident light radiation in the two study sites had significant effects on gas exchange rates, as both species had lower g_L and E_L in the S site compared with the L one, but no differences were detected between the two species in this respect, as also confirmed by similar $\delta^{13}C$ values that represent a proxy for stomatal aperture integrated over longer time intervals than those typical of gas exchange measurements. Interestingly, despite similar leaf-level transpiration rates, plant water status was different in the two species. Aa had higher Ψ_{min} than Fo, with lower values in the L site for both species, in agreement with higher evaporative water losses under high irradiance. In general, Ψ_{min} is considered a proxy for the maximum water stress level experienced by an individual on a daily or seasonal scale. Different daily Ψ_{min} values in co-occurring species experiencing similar rates of water loss can be explained by three nonexclusive factors: (i) access to different water sources characterized by different soil water potential (Ehleringer & Dawson, 1992); (ii) different hydraulic resistance in the soil-to-leaf water transport pathway (Sperry *et al.*, 1998); and (iii) different hydraulic capacitance of stem and leaf tissues possibly buffering the water potential drop (McCulloh *et al.*, 2012).

We did not estimate rooting depth of the study species. To the best of our knowledge, there are no data available about maximum rooting depth in Aa, although previous studies have reported that lateral roots can spread to distances of ~27 m from the base of the plant, and down to 2 m below the soil surface (Kowarik & Säumel, 2007). On the other hand, Fo was reported to undergo marked seasonal drop in pre-dawn water potential (Nardini *et al.*, 2003), possibly suggesting prevalence of relatively shallow roots in this species. Thus, it is possible that Aa had access to more abundant

water sources than Fo, and future analysis of xylem sap isotopic composition could provide useful information on rooting depth in these species (Nardini *et al.*, 2016). However, it has to be noted that Ψ_{pd} , a commonly used proxy to estimate the water potential of soil volumes explored and exploited by the root system (Sellin, 1999), was similar and quite high in both species. This would suggest that, regardless of eventual differences in rooting depth, the two species had no limitations in terms of water access and availability when measurements were performed.

Calculations of whole-plant hydraulic conductance (k_{plant}), as based on transpiration rates and soil-to-leaf water potential drop, revealed that Aa was about twofold more efficient in terms of root to- leaf water transport capacity than Fo. This indirect estimate of k_{plant} was consistent with hydraulic measurements of stem specific hydraulic conductivity (K_s), which was fourfold higher in Aa than in Fo. Also, k_{plant} values were in agreement with recorded differences in terms of minor vein density (VLA_{min}), which was ~60% higher in Aa than in Fo. Vein density has been reported to correlate to leaf hydraulic conductance (Nardini *et al.*, 2014b; Scoffoni *et al.*, 2016). In turn, leaf hydraulic conductance is an important determinant of whole-plant hydraulic efficiency (Nardini, 2001; Sack & Holbrook, 2006). Hence, high vein density and high K_s of Aa probably contributed to an important extent to decrease resistances associated with long distance water transport, leading to moderate drop of leaf water potential even at relatively high transpiration, while similar water loss rates induced a much larger water potential drop in Fo. Finally, high wood capacitance recorded for Aa (~60 kgMPa⁻¹ m⁻³ vs only 20–30 kgMPa⁻¹ m⁻³ in Fo) was consistent with its lower wood density compared with Fo, and possibly contributed to buffer the water potential drop on a daily basis as previously suggested for other woody species (McCulloh *et al.*, 2012; De Guzman *et al.*, 2017; Epila *et al.*, 2017). The larger daily leaf water potential drop recorded in Fo compared with Aa did not necessarily translate into a major reduction of turgor pressure in the native tree, as also suggested by high midday values of g_L . This was apparently due to the fact that Fo had more negative values of π_0 and Ψ_{tlp} than Aa. Indeed, values of $\Psi_{tlp} - \Psi_{min}$ were similar in the two species (~-0.6 MPa), suggesting that they experienced similar water stress levels during daytime. However, this was achieved by Fo only thanks to osmoregulation, which likely required important energetic inputs to accumulate and or synthesize solutes in mesophyll cells. Hence, a secondary advantage of high k_{plant} and C_{wood} of Aa was reduced costs for osmoregulation and turgor maintenance, so that it can be hypothesized that more carbohydrates remained available for growth and biomass accumulation in this species than in the native one (Attia *et al.*, 2015).

The safety–efficiency trade-off in invasive A. altissima compared with native F. ornus

Previous studies on different species' assemblages have suggested the existence of a trade-off between hydraulic efficiency and hydraulic safety (Tyree *et al.*, 1994; Gleason *et al.*, 2016). The current paradigm is that high hydraulic conductance derives from anatomical features, like wide xylem conduits and/or thin and permeable inter-conduit pit membranes, which increase the vulnerability of xylem to drought-induced embolism formation. We could detect this trend in our study species, as the high k_{plant} and K_s of Aa was coupled to high vulnerability to xylem embolism. Values of Ψ_{50} in Aa were ~ -1.2 and -1.1 MPa in L and S site, respectively. Fo was significantly more resistant to embolism, with Ψ_{50} of ~ -3.2 MPa consistent with low k_{plant} and K_s in this species. These differences were also in agreement with the higher wood density of Fo compared with Aa, as this trait has been reported to be correlated to embolism resistance (Nardini *et al.*, 2013). Values of Ψ_{50} represent the intrinsic vulnerability of xylem to embolism. However, the actual risk of hydraulic failure to which different species are exposed does not depend simply on Ψ_{50} , but rather on the difference between Ψ_{50} and the actual Ψ_{min} reached by the species during the day or the whole growing season, i.e., on the so-called 'safety margin' (Choat *et al.*, 2012). In Aa, the safety margin was close or slightly above in L and S site, respectively. On the other hand, Fo maintained a very large safety margin of ~ 1.5 – 2.0 MPa. These findings suggest that Fo was not experiencing a significant risk of embolism development, consistent with a safe hydraulic construction, but likely requiring substantial carbon investment into wood construction as revealed by high WD. On the other hand, low WD in Aa probably significantly reduced carbon costs invested in wood formation, translating into high hydraulic efficiency per unit carbon invested, but also into a risky hydraulic strategy implying likelihood of embolism formation on a daily or seasonal scale. Interestingly, Aa has been reported to promptly close stomata under severe drought (Trifilò *et al.*, 2004) and to rapidly recover from embolism following post-drought irrigation (Savi *et al.*, 2016). In general terms, species with low WD and high C_{wood} have been reported to be more capable of embolism reversal than those with high WD and low C_{wood} (Trifilò *et al.* 2015). Hence, Aa and Fo might be an example of two species lying along the recently proposed trade-off between water use strategy, wood capacitance and embolism reversal ability (Klein *et al.* 2018, Nardini *et al.* 2018).

The wood and leaf economic spectra of A. altissima and F. ornus

Recently, Eller *et al.* (2018) depicted the possible trade-off between safety margin and tree growth rate, emerging from coordination between WD and Ψ_{50} . It was suggested that low construction costs (low wood density) in fast-growing species with narrow safety margins could allow these plants to achieve higher volumetric xylem production rates. As a consequence, the higher xylem volume

available could allow fast-growing species like Aa to transport larger water volumes thus favouring expansion growth. Our results are overall consistent with this structural/ physiological safety–efficiency framework, where higher efficiency in water transport in the invasive Aa implies higher hydraulic vulnerability but at lower wood construction costs, with potential benefits for carbon assimilation rates, growth and competition with native tree species. Hence, Aa and Fo would lie at the opposite extremes of this ‘wood economic spectrum’.

Recent studies and meta-analyses (Leishman *et al.*, 2007, van Kleunen *et al.*, 2010) have reported that invasive species generally occupy a position along the global leaf economic spectrum that favours fast growth, with higher SLA, nitrogen content and photosynthetic capacity per unit leaf mass compared with native species. In this study, SLA was higher in Aa than in Fo, especially in the shaded site. This would suggest that Aa undergoes less carbon investment per unit leaf area construction, despite higher VLA_{\min} (Nardini *et al.*, 2012) and similar carbon content on a mass basis. Also, Aa had higher nitrogen concentration and lower C:N ratio than Fo in both L and S sites, suggesting high investments in the photosynthetic machinery in Aa (Gulías *et al.*, 2003). However, these inter-specific differences did not translate into differences in terms of $\delta^{13}C$, consistent with similar levels of stomatal aperture in both Aa and Fo.

Functional plasticity and shade-tolerance of A. altissima and F. ornus

The magnitude of changes in different functional traits in response to light availability was overall larger in the invasive Aa compared with native Fo. In particular, Aa showed larger variations in terms of SLA, k_{plant} , C:N and N content between L and S sites, when compared with Fo. This would suggest that the functional plasticity of Aa is larger than that of Fo.

Interestingly, Aa is generally described as a shade-intolerant species, but the species has been recently reported to invade closed forest stands dominated by *Castanea sativa* L. in Southern Switzerland (Knüsel *et al.*, 2017). The presence of large invasion spots in shaded habitats in our study area is in agreement with this report, and apparently consistent with the large functional plasticity of Aa. In particular, k_{plant} was much higher in the L site compared with S one, and this allowed Aa to exploit high light availability to maintain fully open stomata while buffering the diurnal water potential drop. Because K_s was similar in S and L sites, this increase in k_{plant} was likely due to changes at the root or leaf level, most likely in the extra-vascular water pathways that are known to be very dynamic and responsive to light conditions (Tyree *et al.*, 2005). The large increase of SLA recorded in Aa individuals growing in S site compared with L one is a typical response of shade-tolerant plants, where it helps to minimize leaf carbon construction costs while maximizing photosynthetic carbon gain per unit surface. The twofold increase of SLA in response

to shade in Aa is in striking contrast to that of Fo, where SLA increased only by ~50%, suggesting that the native tree species is competitively disadvantaged even under low light conditions. Large phenotypic plasticity has been proposed as an important factor promoting invasiveness of alien plant species (Davidson *et al.*, 2011) and this pattern is apparently confirmed by our data.

Conclusions

The comparison of several key functional and mechanistic traits of Aa, one of the most invasive species in Europe, and the native and outcompeted Fo, have revealed interesting patterns and overall suggest that a safety–efficiency trade-off provides a mechanistic framework to explain the invasive success of the alien tree. Traits related to leaf and wood construction costs and drought resistance significantly differed between the two species, with Fo sustaining higher structural costs than Aa. The lower resistance to drought stress of Aa was apparently counterbalanced by higher water transport efficiency, possibly requiring mechanisms to assure resilience to drought-induced hydraulic damage. Large phenotypic plasticity of Aa in response to light availability could also play a role in determining the invasive potential of this species. While we note that caution should be used when inferring species-specific drought resistance on the basis of physiological measurements performed over limited time intervals during the growing season, our data show that the measurement of mechanistic traits related to resource acquisition, with special reference to water-use strategies, could provide important novel insight into the mechanisms underlying the invasive nature of alien tree species in natural forest habitats.

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Supplementary information

	<i>A. altissima</i>	<i>F. ornus</i>	t value	p value
g_L , mmol m ⁻² s ⁻¹	331.3±144.5	374.3±191.0	-0.57	0.578
E_L , mmol m ⁻² s ⁻¹	5.4±3.2	6.8±5.0	-0.76	0.461
Ψ_{pd} , MPa	-0.5±0.1	-0.4±0.1	2.86	0.111
Ψ_{min} , MPa	-1.0±0.2	-1.5±0.4	-3.76	0.002
π_0 , MPa	-1.3±0.2	-1.7±0.3	-7.74	<0.000
Ψ_{tip} , MPa	-1.6±0.2	-2.1±0.4	-7.74	<0.000
K_s , kg m ⁻¹ s ⁻¹ MPa ⁻¹	8.0±2.6	1.6±0.7	13.76	<0.000
k_{plant} , mmol m ⁻² s ⁻¹ MPa ⁻¹	10.5±6.7	5.3±2.7	2.26	0.043
Ψ_{50} , MPa	-1.2	-3.1	/	/
SM, MPa	0.1±0.2	1.7±0.3	12.75	<0.000
WD, g cm ⁻³	0.4±0.1	0.7±0.1	-6.50	<0.000
C_{wood} , kg MPa ⁻¹ m ⁻³	62.2	26.7	7.86	<0.000
LDMC, mg g ⁻¹	291.0±94.3	400.9±92.3	-2.54	0.032
SLA, mm ² mg ⁻¹	25.5±13.2	13.9±4.4	4.61	<0.000
VLA_{maj} , mm ² mm ⁻¹	0.3±0.1	0.3±0.1	0.37	0.712
VLA_{min} , mm ² mm ⁻¹	12.3±1.4	7.8±1.4	14.79	<0.000
C:N	18.8±6.0	26.2±3.9	-3.27	0.005
C, %	47.1±0.8	47.6±0.8	2.89	0.016
N, %	2.8±1.0	1.8±0.3	-1.27	0.219
$\delta^{13}C$, ‰	-28.6±1.2	-28.4±1.2	-0.35	0.729

Tab. S2 Mean values ± standard deviation of functional traits measured in *A. altissima* and in *F. ornus*. The output of two-tailed Student's t-test (t and p values) is also reported. α level = 0.05.

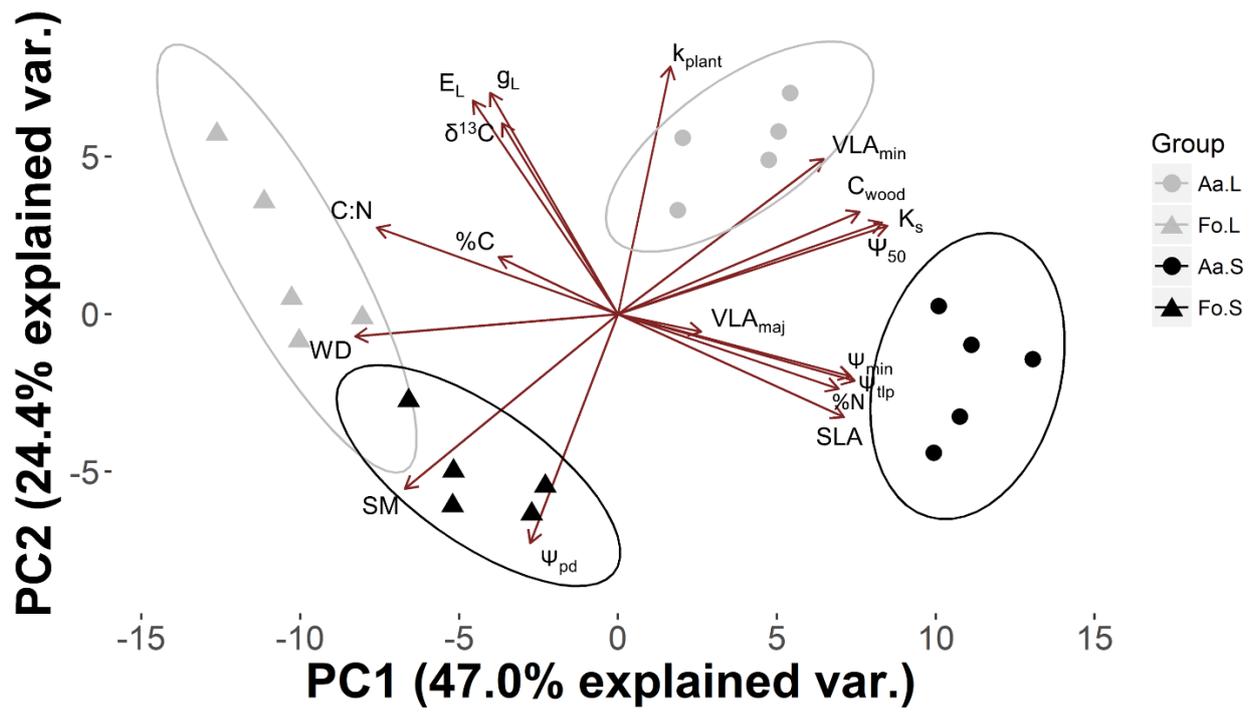


Fig S1 Principal Component Analysis (PCA) of standardized and centered functional traits measured in *A. altissima* and *F. ornus* in the site with high irradiance (L) and in the shaded site (S).

Study 4

Plasticity of functional traits of tree of heaven is higher in exotic than in native habitats

Francesco Petruzzellis*¹, Guoquan Peng², Melvin T. Tyree², Vanessa Tonet¹, Tadeja Savi³,
Valentina Torboli¹, Alberto Pallavicini¹, Giovanni Bacaro¹ and Andrea Nardini¹

¹University of Trieste, Department of Life Sciences, via L. Giorgieri 10, 34127 Trieste

²Zhejiang Normal University, College of Chemistry and Life Sciences, Jinhua, Zhejiang, China

³University of Natural Resources and Life Sciences, Division of Viticulture and Pomology,
Department of Crop Sciences, Konrad Lorenz Straße 24, A-3430 Tulln, Wien, Austria

*Corresponding author

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Abstract

Phenotypic plasticity has been recently proposed to play an important role in invasion process. More precisely, phenotypic plasticity of alien invasive species could be higher in their exotic rather than native habitat, probably because of the release from biotic or abiotic constraints. In this paper, several plant functional traits were analysed on *Ailanthus altissima* (Mill.) Swingle, a highly invasive species in Europe, aiming at providing a comparison among key functional traits measured on individuals growing in their native habitat and to test if values of functional traits and their related plasticity are higher in exotic habitats. Our analysis pinpointed that variability of functional traits in the native habitat was mainly driven by different irradiance, temperature and evaporative demand of the studied sites, in accordance to the traits trade-offs in the Leaf Economic Spectrum. Physiological traits related to drought tolerance were different between native and exotic habitats. In the native one, *A. altissima* relied on osmoregulation processes to adjust leaf turgor loss point (Ψ_{tp}), as the osmotic potential at full turgor (π_0) were lower in drier sites. In the exotic habitat, individuals in drier sites had similar π_0 but lower wall elasticity (ϵ), suggesting that leaves had thinner cell walls. Moreover, plasticity in specific leaf area and ϵ were higher in the exotic habitat. The novel features related to Ψ_{tp} adjustment and the higher plasticity in traits related to leaf-construction costs could increase *A. altissima* fitness in response to different environmental conditions in its invasive range.

Keywords

Ailanthus altissima; DNA barcoding; drought tolerance; turgor loss point; variability

Key message

Novel features related to turgor loss adjustment and higher ability to modulate leaf construction costs could improve *A. altissima* fitness in its invaded range.

Introduction

Invasion by alien plant species is occurring at unprecedented rates on a global scale, portending important impacts on natural ecosystems and human health (Vilà *et al.*, 2011). Over recent decades, an increasing number of studies have analysed patterns of biological invasions to disentangle the underlying ecophysiological processes. Under this perspective, the recent focus on the functional traits or the syndrome of traits enhancing the invasive potential of alien invasive plant species, holds promise to develop predictions on which species are more likely to become successful invaders in different habitats (van Kleunen *et al.*, 2010; Drenovsky *et al.*, 2012). Functional trait ecology represents a relatively novel approach to investigate this phenomenon. Functional traits provide tools for measuring functional diversity (FD), as they impact plant fitness via effects on growth, reproduction and survival (Violle *et al.*, 2007), and reflect the trade-offs among different physiological and ecological functions (McIntyre *et al.*, 1999; Díaz & Cabido, 2001; Lavorel *et al.*, 2007). Most of the previous studies in this field have focused on comparative analysis of functional traits between native and invasive species, or between invasive and non-invasive alien species (Daehler, 2003a; Pyšek & Richardson, 2008; van Kleunen *et al.*, 2010; Funk & Zachary, 2010). The former comparison might highlight traits enhancing the ability of alien species to increase in abundance over native species, while the latter would emphasise traits promoting their invasive potential (Pyšek & Richardson, 2008). Despite the great number of studies and substantial meta-analytic efforts, a unique set of traits responsible for the invasive potential of some plant species has not been identified to date. This probably arises because the character of the invasion process is context dependent and thus, it is unlikely that a unique set of traits could successfully relate to the performance of alien invasive species worldwide, as multiple suites of traits will be more or less favourable in different environments (Tecco *et al.*, 2010; van Kleunen *et al.*, 2015). As an example, (Pyšek & Richardson, 2008) have reported that the invasive potential is higher in fast-growing alien species, but this pattern was not observed by (Daehler, 2003b). In their meta-analysis, (van Kleunen *et al.*, 2010b) showed that invasive species have higher values of functional traits related to leaf area or leaf biomass allocation, shoot biomass allocation, growth rate and size, supporting the view that invasive plants occupy a position within the leaf economic spectrum (Wright *et al.*, 2004) that favours fast growth (Leishman *et al.*, 2014).

Different hypotheses have been proposed to explain the success of invasive plant species. The “try harder” hypothesis (Tecco *et al.*, 2010) suggests that successful aliens deal better with the local conditions than resident species, expressing a set of functional traits different from those of native species. On the other hand, the “join the locals” hypothesis (Tecco *et al.*, 2010) predicts

similarities among alien invasive and native species. More recently, phenotypic plasticity has been proposed to play an important role in invasion process (Heberling *et al.*, 2016). This is defined as the ability of an organism to develop different phenotypes under different environmental conditions (Valladares *et al.*, 2000). Hence, high phenotypic plasticity is predicted to favour colonization and rapid spread of alien species over large and heterogeneous areas (Godoy *et al.*, 2011). However, a global correlation between high phenotypic plasticity and invasiveness has not been demonstrated (Davidson *et al.*, 2011; Godoy *et al.*, 2011). A possible explanation for this lack is that many studies have been based on multispecies comparison, enabling more robust ecological conclusion but generally limiting the analysis to a restricted number of traits, thus possibly jeopardizing the analysis of important insights into underlying physiological processes (Godoy *et al.*, 2011).

More recently, Heberling *et al.* (2016) have proposed comparing functional traits of alien invasive species in their native vs exotic habitat as a tool to investigate their invasive potential, suggesting three possible outcomes. Under the first scenario, functional traits values and plasticity of alien species do not differ in the two ranges, suggesting that successful invaders are pre-adapted to the environmental conditions of the new habitat. Under the second possible scenario, invasive species would have higher ability to exploit resources and, in this case, trait mean values would be similar, but plasticity would be higher in the invaded range. Under the third scenario, traits values might differ in the two ranges, thus suggesting that novel traits are developed in the invaded habitat. Up to date, only few studies have investigated alien invasion on the basis of this approach (Caño *et al.*, 2008; van Kleunen *et al.*, 2011; Leishman *et al.*, 2014; Taylor *et al.*, 2016; Heberling *et al.*, 2016). In most of them, the performance of invasive species improved when moving from native to invaded ranges, mainly related to higher specific leaf area (SLA), better water content regulation and higher relative growth rate (RGR). Caño *et al.* (2008) and Heberling *et al.* (2016) also concluded that invasive species display larger traits' plasticity in the invaded range than in the native one, suggesting a better resource use ability. Nonetheless, Leishman *et al.* (2014) suggested that all these modifications could be associated with factors linked with the introduction to the novel environment, such as release from leaf herbivory and other biotic or abiotic constraints, rather than with particular environmental conditions.

Ailanthus altissima (Mill.) Swingle is one of the most invasive woody species in Europe, where it was introduced for ornamental and economical purposes (Sladonja *et al.*, 2015). It is also reported to be one of the fastest growing invasive trees in North America (Knapp & Canham, 2000) and U.K. (Mabberley, 1997), with a great ability to colonize different habitats (Sladonja *et al.*, 2015). It can propagate both from seeds and clonally from root suckers. It has a wide temperature tolerance, although it is somehow vulnerable to cold stress (Sladonja *et al.*, 2015). It is drought

resistant, featuring effective early stomatal closure to save water and limit xylem embolism formation (Trifilò *et al.*, 2004), as well as prompt recovery of hydraulic efficiency upon drought relief via embolism reversal (Savi *et al.*, 2016). The species is also known to produce bioactive molecules (e.g. aianthone) assuring resistance to herbivores, and inducing allelopathic effect on other plant species (Sladonja *et al.*, 2015). In invaded areas, its primary occurrences are cities and other disturbed sites like agricultural lands and transportation corridors, but it is increasingly reported to occur even in natural environments (Sladonja *et al.*, 2015). All these features make *A. altissima* an ideal species to analyse the occurrence and magnitude of functional traits plasticity as a determinant of the invasion processes.

Aiming to disentangle this conundrum, we measured leaf and shoot morpho-anatomical and physiological traits on individuals of *Ailanthus altissima* (Mill.) Swingle in their native range (China) and one exotic range (Italy). Specifically, the aims of the study were to: *i*) provide a reference value and variability of key functional traits related to resource acquisition and drought tolerance of individuals of *A. altissima* growing in their natural habitat; *ii*) test whether functional traits values and their associated plasticity are higher in exotic rather than native habitats.

Materials and methods

Study areas

The study areas in Italy were located in the Karst region (NE Italy, 45°40'–45°42'N and 13°50'–13°43'E), a limestone plateau covering a surface of about 500 km². This area is characterized by a transitional climate between the oceanic-mediterranean type and the continental one (Poldini, 1989; Poldini *et al.*, 1992). The regional climate is discontinuous because of the morphological diversity of the area, causing the formation of complex and fragmented microclimate, influencing plant and animal communities. Classical Karst hosts natural woods, generally dominated by thermophilous *Quercus* species (*Q. ilex*, *Q. petraea*, and *Q. pubescens*), *Fraxinus ornus*, *Acer campestre*, are mixed with *Pinus nigra* plantations (Poldini *et al.*, 1992).

The study areas in China were in the Qinling Mountains (32°30'N–34°45'N and 104°30'E–112°45'E), a major east-west mountain range in southern Shaanxi Province (People's Republic of China). The climate of the Qinling Mountains consists of long, wet summers and cold winters. Low-elevation foothills are dominated by northern temperate deciduous trees like oak (*Quercus acutissima*, *Q. variabilis*), elm (*Ulmus* spp.), walnut (*Juglans regia*), maple (*Acer* spp.), ash (*Fraxinus* spp.) and *Celtis* spp. Evergreen species include both broadleaf trees (*Castanopsis sclerophylla*, *Cyclobalanopsis glauca*) and conifers like *Pinus massoniana*. Although many of these

trees are congeneric with species that are important in European forests, the taxonomic diversity within most of these genera is significantly higher in Central China than Europe (Mackinnon, 1996). At middle elevations, conifers like *Pinus armandii* co-occur with *Betula* spp., *Quercus* spp. and *Carpinus* spp. *A. altissima* is widely distributed in all regions of China except Hainan, Heilongjiang, Jilin, Ningxia, Qinghai (<http://www.efloras.org>). The study areas in China are located in the centre of the *A. altissima*'s distribution range, according to (Fang *et al.*, 2011).

Climate data of the study areas are summarized in Tab. 1. On average, the study sites in China experienced more arid conditions during the growing season (April to September), as Aridity Index (AI), calculated as the ratio between the sum of mean monthly precipitation (MMP, mm) and sum of mean monthly evapotranspiration (MET, mm), was lower and mean temperature was higher. Data were obtained from the WorldClim database (<http://worldclim.org/current>) (Fick & Hijmans, 2017).

	ITALY				CHINA			
	<i>MMP,</i> <i>mm</i>	<i>MET,</i> <i>mm</i>	<i>Mean T,</i> <i>°C</i>	<i>Min T,</i> <i>°C</i>	<i>MMP,</i> <i>mm</i>	<i>MET,</i> <i>mm</i>	<i>Mean T,</i> <i>°C</i>	<i>Min T,</i> <i>°C</i>
April	103.0	73.0	10.2	6.2	58.4	97.4	12.1	7.0
May	97.0	107.0	14.5	10.3	74.6	133.4	17.2	11.9
June	110.0	123.0	18.2	14.0	75.4	152.0	21.7	16.4
July	89.0	137.0	20.9	16.4	127.8	149.4	23.8	19.3
August	107.0	117.0	20.6	16.2	123.0	132.0	22.5	18.1
September	125.0	78.0	17.2	13.2	135.2	90.2	17.2	13.2
AI	0.99				AI 0.79			

Tab. 1 Mean monthly precipitation (MMP), mean monthly evapotranspiration (MET), mean monthly temperature (Mean T), minimum monthly temperature (Min T) and Aridity Index (AI) of the vegetative period in the two study areas. Data were downloaded from database online (<http://worldclim.org/current>) (Fick & Hijmans, 2017)

Sampling design

Two sites characterized by contrasting light habitats were selected in the study areas in both countries (Fig. S1 and Fig. S2). Air temperature (T, °C), relative humidity (RH, %), incident photosynthetic photon flux density (PPFD, $\mu\text{mol m}^{-2} \text{s}^{-1}$) at the time of sample collection were measured using a digital thermometer and a portable light meter (model LI-250A, LI-COR), respectively (Table 2). Shaded sites in Italy were located within a pinewood where the dominant species was *Pinus nigra* with a mean plant height in the range of 15-20 m. Shaded sites in China were in stands where the dominant species were *Juglans regia* (mean height of 20-25 m), species of *Betula* (mean height of 10-15 m), *Carpinus turczaninowii* (mean height of 10-15 m), *Fraxinus chinensis* (mean height of 5-10 m). Individuals of *A. altissima* had a similar plant height in the two ranges (about 3-6 m).

In Italy, 5 individuals were randomly selected in each site (20x20 m, see Petruzzellis *et al.* 2018). Five leaves were collected for each individual and for each trait measured, following sampling procedure suggested in Petruzzellis *et al.* (2017). In total, 50 leaves were sampled from 10 individuals (5 in the site with high irradiance and 5 in the shaded site). Additionally, one branch per individual was sampled and wood density (WD) was measured on the last year stem growth.

In China, we applied a more intensive sampling strategy to provide a reference value and variability of key functional traits related to resource acquisition and drought tolerance (see below) of individuals growing in their natural habitat. Specifically, 3 plots of 20x20 m were selected in each site, and 5 to 6 individuals were sampled in each plot. For sampling purposes, each tree was divided in two height classes (h_class): a lower class ("l", $0 < x < 2.5$ m) and an upper class ("u", $x > 2.5$ m). This stratification was designed to assess the contribution of different leaf position in the canopy to the total variance of the traits, as micro-environmental gradients within the canopy could affect leaf traits values (Niinemets, 2016; Petruzzellis *et al.*, 2017). Eight leaves were collected from each individual for each trait measured, 4 leaves from each height class. In some cases, individuals had leaves only in one of the two height classes. In total, 176 leaves were sampled from 31 individuals (16 in the site with high irradiance and 15 in the shaded site). Leaves were detached and immediately wrapped in cling film, placed in humid sealed plastic bags and stored in cool bags until measurements in the laboratory (about 3 hours later). Diameter at breast height (DBH, cm) of each sampled tree was recorded and individuals were classified in 3 categories according to diameter length ('A' $0 < d \leq 5$ cm, 'B' $5 < d \leq 10$ cm and 'C' $10 < d \leq 15$ cm). WD was measured as described above.

	ITALY		CHINA	
	<i>L</i>	<i>S</i>	<i>L</i>	<i>S</i>
T (°C)	29.4±1.2	25.7±0.5	32.0±5.0	24.6±3.5
RH (%)	41.9±2.1	41.5±3.1	70.7±14.6	74.3±10.8
PPFD ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	1386.4±249.4	21.4±6.4	1216.5±278.1	35.64±2.2
Altitude (m)	406	406	800<alt<700	1000<alt<800

Tab. 2 Mean values \pm standard deviation of air temperature (T), air relative humidity (RH) and photosynthetic photon flux density (PPFD) measured in sites with high light availability (L) and in shaded sites (S) in the two

Traits measured

The following traits were measured on samples collected in the field: specific leaf area (SLA, $\text{mm}^2 \text{mg}^{-1}$), minor and major vein length per unit area (VLA_{min} and VLA_{maj} , mm mm^{-2}), osmotic potential at full turgor (π_0 , -MPa), water potential at turgor loss point (Ψ_{tlp} , -MPa), modulus of elasticity (ϵ , MPa), wood density (g cm^{-3}).

SLA was calculated as:

$$\text{SLA} = \text{Leaf Area} / \text{Leaf Dry Weight} [\text{mm}^2 \text{mg}^{-1}] \quad (1)$$

SLA is generally considered a “soft” structural trait, which correlates to relative growth rate, nutrient status and photosynthetic rate (Poorter & Remkes, 1990). Images of fresh leaves were obtained with a scanner and leaf area (A_{leaf}) was measured using the software ImageJ (Schneider *et al.*, 2012). Leaves were then oven-dried for 48 h at 72°C and leaf dry weight was measured using an analytical balance.

Leaf venation architecture comprehends several structural features influencing plant performance in terms of photosynthetic capacity and drought tolerance (Sack & Scoffoni, 2013). We specifically measured vein length to area of major and minor veins (VLA_{maj} and VLA_{min}) as:

$$\text{VLA} = \text{Vein Length} / \text{Leaf sample area} [\text{mm mm}^{-2}] \quad (2)$$

For VLA_{maj} , fresh leaves were scanned and the ratio between vein length and sample area were measured using PhenoVein software (Lobet *et al.*, 2013). To measure VLA_{min} , fresh leaves were treated in 1M NaOH solution for 48-72 h at room temperature, replacing solution when it turned from transparent to dark coloured. After initial clearance, small portion of leaves of about 1 cm^2 were cut and bleached in 5% NaClO for 1-2 min. Then, samples were treated in a sequence of

ethanol solutions at increasing concentration (25%, 50%, 75%, 100%) and then immersed in an alcoholic solution of toluidine blue (3%) overnight. Finally, samples were processed in a series of ethanol solutions at decreasing concentration and microscopic slides were prepared. Images of smaller portions ($\sim 5 \text{ mm}^2$) of leaves were captured with an optical microscope at 4x magnification equipped with a digital camera (model Syrio-2, Pbinternational) and VLA_{\min} was measured using PhenoVein software.

Water relation parameters, namely π_0 , Ψ_{tip} and ε were derived from the elaboration of water potential isotherms (or PV-curves) (Tyree & Hammel, 1972). π_0 , Ψ_{tip} and ε are physiological traits providing information on drought tolerance levels of species/individuals (Savi *et al.*, 2016). Plants adapted or acclimated to drought-prone environments generally display more negative π_0 and Ψ_{tip} values (Bartlett *et al.*, 2012). PV-curves were measured using the bench dehydration technique, by repeatedly measuring water potential with a pressure chamber (model 1505D, PMS Instruments, Albany, OR, USA) during progressive sample dehydration. Fresh leaves were rehydrated for 12 h with their petioles immersed in distilled water. Water potential (Ψ_{leaf}) and cumulative weight loss (WL) of leaves were measured until the relationship between $1/\Psi$ and WL became strictly linear indicating the loss of cell turgor. PV-curves were then elaborated according to (Salleo, 1983) in order to calculate the water relation parameters.

WD is a stem structural trait and was calculated as:

$$\text{WD} = \text{wood dry weight/Wood dry volume [g cm}^{-3}\text{]} \quad (3)$$

WD has been suggested to be a good and simple proxy of species-specific drought resistance, as it relates to xylem implosion resistance and to the resistance to xylem embolism (Hacke *et al.*, 2001; Nardini *et al.*, 2013). A stem segment about 5 cm long was cut from the current year branch from each individual, and bark was removed before putting the sample in oven at 70°C for 24h. Samples were then weighted, and dry volume was measured following Archimede's principle (Williamson & Wiemann, 2010; Pérez-Harguindeguy *et al.*, 2013).

DNA barcoding

DNA barcoding was used to check for species identity of individuals sampled in China and Italy. DNA was extracted from young leaves of 16 individuals (8 from Italy and 8 from China). The complete coding sequence for ribulose-1,5-carboxylase/oxygenase (rubisco) large subunit chloroplast gene (rbcL) was amplified by the PCR method for each sample, using rbcLA (Kress and Erickson 2007) as primer set. *A. altissima* rbcL novel sequences were then checked for quality and ambiguous nucleotides resolved in MEGA7 software suite (Kumar *et al.*, 2016). The sequences were then used to carry out a GeneBank nucleotide BLAST.

Statistical analysis

To summarise and visually describe the differences among traits of individuals growing in the two habitats, a Principal Component Analysis (PCA) was run through “princomp” function in “stats” package for R software (R Core Team, 2015). Before processing data, traits values were standardized dividing each trait value by standard deviation of that trait.

Generalized linear models (GLMs) and generalized linear mixed effect models (GLMMs) were used to model traits variation in China and to highlight differences between traits values measured in the two countries.

GLMMs were fitted through “glmer” function in “lme4” package (Bates *et al.*, 2015) for R software, using SLA, VLA_{maj} and VLA_{min} as dependent variables to model traits’ variation in China. The fixed effects tested were Site (‘S’, shaded sites and ‘L’, high irradiance sites), height class (h_class, ‘l’ leaves in the lower portion of the canopy and ‘u’ leaves occurring in the upper canopy), diameter class (diam_class, ‘A’ $0 < d \leq 5$ cm, ‘B’ $5 < d \leq 10$ cm and ‘C’ $10 < d \leq 15$ cm) and photosynthetic photon flux density (PPFD). The random variable was Individual. For the other traits, namely π_0 , Ψ_{tp} , ε and WD, we evaluated GLMs through “glm” function in “stats” package for R, testing the same fixed effect listed before and without the random effect, because only one measure for each individual was available.

When comparing functional traits in the two countries, we fitted GLMMs using A_{leaf} , SLA, VLA_{maj} and VLA_{min} as dependent variables. In this case, the fixed effects were Site and Country (‘I’, Italy and ‘C’, China) and the random effect was Individual nested in Site. For π_0 , Ψ_{tp} , ε and WD, we evaluated GLMs testing Site and Country as fixed effects. For any of the tests that were statistically significant, we performed a multiple comparison using function “glht” within package “multcomp”.

Aiming at testing whether plasticity was different in the two countries, we applied a multivariate approach. First, we calculated multivariate centroids of each trait for each country given by the average value of individuals belonging to that country. Then, we calculated the distances from centroids of each trait in each country using the following equation:

$$D_j = |y_j - c| / c$$

Where:

D_j is the distance of observation j ; y_j is the mean trait value of individual j and c is the trait centroid. We then tested statically significant differences of distances from centroids of different traits measured in Italy and China using Wilcoxon test.

Results

Variability in the native range

Traits variation in China was related to different factors, as highlighted by model outputs reported in Tab. 3. Generally, the main driver of variability was light availability in the study sites. More precisely, SLA was higher in shaded sites and in younger individuals (we consider as young individuals those belonging to diam_class “A”). Furthermore, different micro-environmental conditions occurring through the canopy significantly affected SLA, as h_class resulted significant (Tab. 3). Water relation parameters also varied accordingly to light availability, with higher π_0 and Ψ_{tlp} values in shaded sites. The variation of turgor loss point was mainly due to osmoregulation processes, as ε did not change significantly. Wood density (WD) was higher in individuals growing in sites with higher light availability.

	<i>site</i>	<i>h_class</i>	<i>diam_class</i>	<i>site*h_class</i>
SLA	4.76*	19.77***	6.99*	5.11*
VLA_{min}	1.11 ^{n.s.}	0.79 ^{n.s.}	0.69 ^{n.s.}	0.42 ^{n.s.}
VLA_{maj}	0.09 ^{n.s.}	0.01 ^{n.s.}	2.08 ^{n.s.}	0.01 ^{n.s.}
π_0	8.65***	/	5.54 ^{n.s.}	/
Ψ_{tlp}	9.61**	/	2.74 ^{n.s.}	/
ε	0.62 ^{n.s.}	/	2.20 ^{n.s.}	/
WD	0.47 ^{n.s.}	/	3.93 ^{n.s.}	/

Tab. 3 GLMMs and GLMs results summary for each trait measured in China. χ and significance level are reported for site, h_class, diam_class and interaction between site*h_class. * = p value < 0.05; ** = p value < 0.01; *** = p value < 0.0001; n.s. = p > 0.05

Comparison of traits in the native vs exotic habitats

DNA barcoding was used to check for species identity. The first set of 100 Blast hits gave 100-percentage similarity for the rbcL gene between the extracted sequences (see Supplementary Material) and the respective GeneBank sequences of *A. altissima*.

The projection of individuals on axis 1 and 2 of the PCA (Fig. S3) clearly suggests a separation between individuals growing in the exotic vs native habitat, suggesting possible

differences in functional traits between the two groups. π_0 and Ψ_{tip} (associated to axis 1), ε and VLA_{min} (associated to axis 2) are the traits that contributed most to the identification of two groups of individuals, belonging to native (China) and exotic (Italy) groups.

Functional traits mean values and their related standard deviation measured in each site in each country are summarized in Tab. S1. SLA was not different in China vs Italy, but it changed significantly according to site exposure (higher in shaded sites) (Fig. 1). Ψ_{tip} was different in the two countries and changed accordingly to light availability in both countries (Fig. 1). Acclimation processes to likely higher water stress in sites characterized by higher light availability were different in the two countries. Significant variation of π_0 in response to light availability occurred only in China, but not in Italy (Fig. 1). On the other hand, individuals in Italy had more elastic cell walls in high-irradiance sites, and generally ε was lower in the invaded range (Fig. 1). WD changed accordingly to light availability only in the native range (Fig. 1), and it was also higher in China. VLA_{maj} was higher in Italy, while VLA_{min} was higher in China, and both parameters did not change in response to light availability.

Plasticity of functional traits related to leaf-construction costs was different in the two habitats. In particular, individuals in the exotic habitat had higher SLA and ε plasticity (Fig. 2).

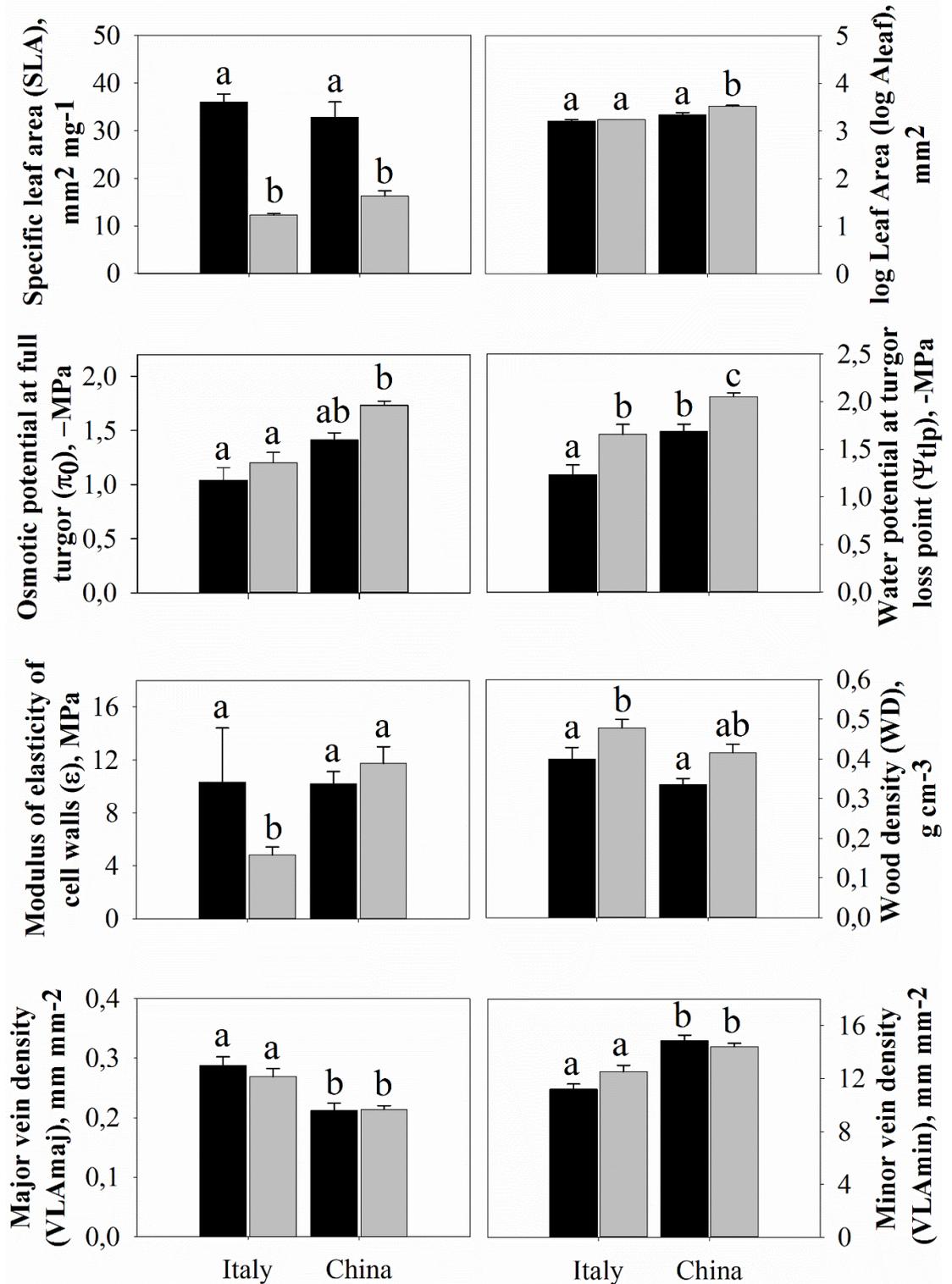


Fig. 1. Mean values \pm SEM of measured functional traits in Italy and China. Black histograms represent data measured on individuals growing in shaded sites, while grey histograms represent data measured on individuals growing in sites with higher light availability. Different letters indicate statistical significant differences among groups ($p < 0.05$).

Discussion

The first goal of the study was to describe the variability of the functional traits measured on *A. altissima* in its native range (China). The different light regimes in the study sites was the main driver of traits variability, rather than canopy spatial structure and/or individual age (diam_class), suggesting that trait-variability mainly occurred among individuals rather than within individuals. In the native area, surveyed individuals growing in sites with higher irradiance invested more of their biomass into structural features (like leaves with low SLA and high-density wood) and more energy in adjustment of physiological parameters related to drought tolerance (lower π_0 and Ψ_{tp}). This syndrome of traits could represent an acclimation to higher temperature and evaporative demand under high irradiance, in accordance to the traits trade-offs of the Leaf Economic Spectrum (Bartlett *et al.*, 2012; Scoffoni *et al.*, 2015; Costa-Saura *et al.*, 2016; Savi *et al.*, 2017).

The competitive pressure on *A. altissima* in its native range could be much higher than in the invaded ranges, and this could limit *A. altissima* spread. Native co-occurring species may be better competitors than species already present in invaded range for resource acquisition, or could be better adapted to tolerate allelopathic compounds released by *A. altissima* roots. On the other hand, *A. altissima* could be more able to colonize a larger number of environments when introduced in exotic ranges, and this could be possible if novel traits arise in the invaded ranges or if individuals have higher trait-plasticity (phenotypic plasticity), probably because of reduced competition with co-occurring species in exotic habitats. In support to the latter hypothesis, phenotypic plasticity has been recently suggested to play a role in plant invasions (Davidson *et al.*, 2011) but, surprisingly, few studies compared functional traits and plasticity of invasive species in their native vs invaded range. To address this point, we compared trait-values and plasticity measured in the native and invaded ranges (China vs Italy) in response to different light availability. Ψ_{tp} decreased from shaded to sunny sites, probably in response to higher temperature and drought stress (Fig. 1). The maximum difference recorded in the two ranges was 0.45 MPa, a value previously suggested as the maximum possible Ψ_{tp} adjustment for different species on a seasonal scale (Bartlett *et al.*, 2014). Consequently, even if Ψ_{tp} was significantly lower in China, this difference might be interpreted as a seasonal adjustment due to the different measurement time (late June 2016 in Italy, middle August 2016 in China) and to the higher aridity in the native range. Nonetheless, observed differences could suggest that *A. altissima* is one of the most plastic species in drought tolerance traits potentially enhancing its ability to colonize environments with different light availability. Interestingly, the drop of Ψ_{tp} was driven by different factors in the two countries. In the native range, π_0 was significantly lower in sites with high light availability, suggesting that osmoregulation processes

underlies acclimation to higher drought stress condition. On the contrary, in the invaded range, no significant differences were detected in π_0 , while cell walls were more elastic in sites with higher light availability (i.e. lower ϵ values).

Mean values of ϵ were higher in China than in Italy (Fig. 1). This implies that cell walls were more rigid and stiffer (Taiz & Zeiger, 2010) in the native range. On the contrary, the lower values of ϵ in the exotic range could represent an advantage for *A. altissima*, as these might allow maintaining cell turgor even for large water losses (Schulte, 1992). Moreover, stiffness of cell walls has been directly correlated to mesophyll CO₂ conductance (Peguero-Pina *et al.*, 2017; Onoda *et al.*, 2017), accounting for 25-50 % of the total mesophyll resistance to CO₂ diffusion (Evans *et al.*, 2009). In particular, more rigid and thicker cell walls (high ϵ) likely limit mesophyll CO₂ conductance and this could in turn reduce the photosynthetic rates (Reich & Flores-Moreno, 2017; Peguero-Pina *et al.*, 2017; Onoda *et al.*, 2017) and whole-plant performances. Thus, we suggest that *A. altissima* relies on osmoregulation processes and on the construction of leaves with thicker cell walls to cope with drought stress in the native range, with consequent limitations for productivity. In contrast, in the invaded range, the climatic conditions are apparently more favourable and *A. altissima* could invest less energy in the synthesis of solutes and the construction of leaves with thinner cell walls that could benefit photosynthesis and, thus, enhance productivity. In support to this hypothesis, individuals growing in Italy had higher VLA_{maj}, a trait often associated with higher biomass productivity and negatively correlated with mortality rate (Brodribb *et al.*, 2010; Iida *et al.*, 2016). All these factors could promote *A. altissima* invasive potential and explain why it has been widely reported as a fast growing species, especially in the exotic ranges.

Previous studies also reported that invasive plants shift their traits to favour faster growth strategy in the invaded range by increasing their SLA and decreasing root/shoot biomass (van Kleunen *et al.*, 2011;

Leishman *et al.*, 2014) and their photosynthetic performance (Caño *et al.*, 2008). In this study, SLA did not differ significantly in the two countries but its plasticity was higher in Italy (Fig. 2). This difference could be a result of the higher

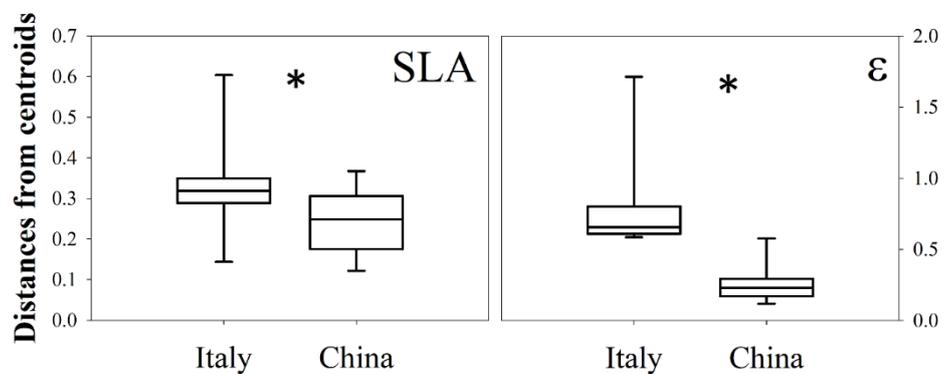


Fig. 2. Median distances from centroids \pm standard deviation measured in specific leaf area (SLA) and modulus of elasticity of cell walls (ϵ) in each country. * indicate statistical significant differences among groups ($p < 0.05$)

plasticity in ϵ in the invaded range, supporting the above hypothesis whereby *A. altissima* could acclimate to different light regimes by modulating thickness of cell walls, with implication on photosynthetic rates and productivity. However, we still do not directly know all the possible sources contributing to trait-values and plasticity in the exotic vs native range. As previously suggested by (Heberling *et al.*, 2016), environmental conditions might be important but could not be the only factor affecting traits assemblage in the two ranges. Exotic ranges may lack of biotic filtering occurring in the native ranges, i.e. competition with co-occurring species or herbivory, with implications for plant performance. Moreover, genetic variation may also be important, as *A. altissima* is reported to be widely distributed both in the native and the exotic range (Liao *et al.*, 2014; Sladonja *et al.*, 2015). Although moderate genetic diversity was identified in *A. altissima* in China, the haplotype relationships investigated in (Liao *et al.*, 2014) suggested that there were multiple refugia during the Quaternary glacial and that three dispersal routes existed over the evolutionary history for some common haplotypes. The DNA barcoding analysis ran in the present study confirmed that individuals sampled in Italy and in China belonged to the same species, but future analysis could be useful to assess wheter a particular genotype has been introduced in the exotic ranges (Neophytou *et al.*, 2018). Moreover, a functional vs genetic comparison on multiple populations in the native and exotic ranges will assess if these range-level differences have a genotypic or phenotypic basis.

In conclusion, both the arise of novel features related to Ψ_{tp} adjustment and the higher plasticity in traits related to leaf-construction costs could increase *A. altissima* fitness in response to different environmental conditions in its invaded range. Clearly, future studies including more traits and more populations are required to disentangle processes underlying *A. altissima* invasion at a wider spatial scale.

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Supplementary material

	Italy		China	
	<i>L</i>	<i>S</i>	<i>L</i>	<i>S</i>
SLA, mm ² mg ⁻¹	12.3±0.6	36.06±3.7	16.29±3.8	32.8±11.9
A _{leaf} , mm ²	1607.4±496.8	1630.2±634.2	3240.0±1466.8	2242.5±1089.8
π ₀ , -MPa	1.2±0.2	1.04±0.3	1.7±0.1	1.4±0.3
Ψ _{tip} , -MPa	1.7±0.2	1.2±0.2	2.1±0.1	1.7±0.3
ε, MPa	4.8±1.3	10.32±5.0	11.74±4.4	10.19±3.7
WD, g cm ⁻³	0.5±0.1	0.4±0.1	0.5±0.1	0.3±0.1
VLA _{maj} , mm mm ⁻²	0.27±0.03	0.29±0.03	0.21±0.05	0.21±0.02
VLA _{min} , mm mm ⁻²	12.5±1.1	11.2±0.9	14.4±0.9	14.9±1.7

Tab. S1 Mean values ± standard deviation of specific leaf area (SLA), leaf area (A_{leaf}), osmotic potential at full turgor (π₀), water potential at turgor loss point (Ψ_{tip}), modulus of elasticity (ε), wood density (WD), major vein length per unit area (VLA_{maj}) and minor vein length per unit area (VLA_{min}) measured in sites with high irradiance (L) and in shaded sites (S) in the native (China) and exotic (Italy) habitats.

	<i>Group (site*country)</i>
SLA	45.787***
A_{leaf}	26.40***
π₀	43.46***
Ψ_{tip}	45.28**
ε	7.7*
WD	23.39***
VLA_{min}	40.60***
VLA_{maj}	22.01***

Tab. S2 GLMMs and GLMs results summary of the comparison of each trait measured in China and Italy. χ and significance level are reported for each Group (site*country). * = p value < 0.05; ** = p value < 0.01; *** = p value < 0.0001.



Fig. S1. Map of the study area in Italy.

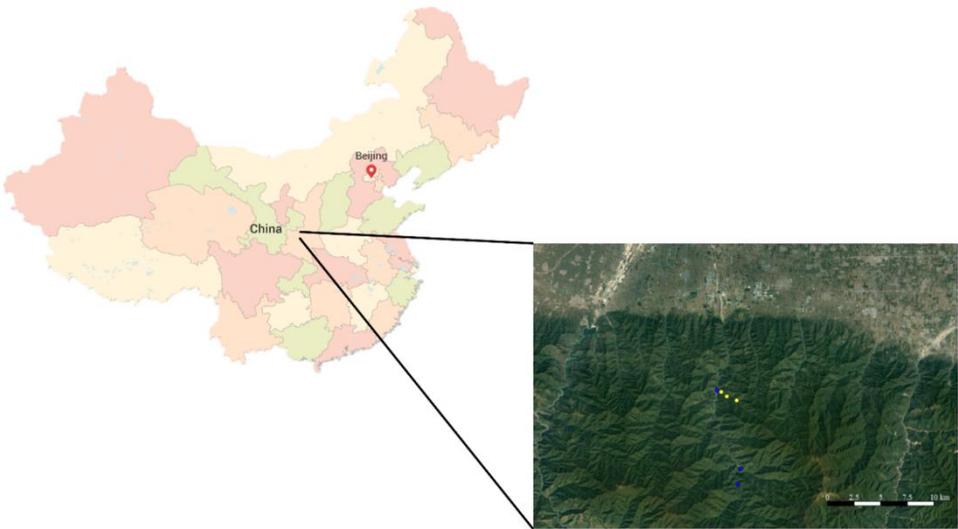


Fig. S2. Map of the study area in China.

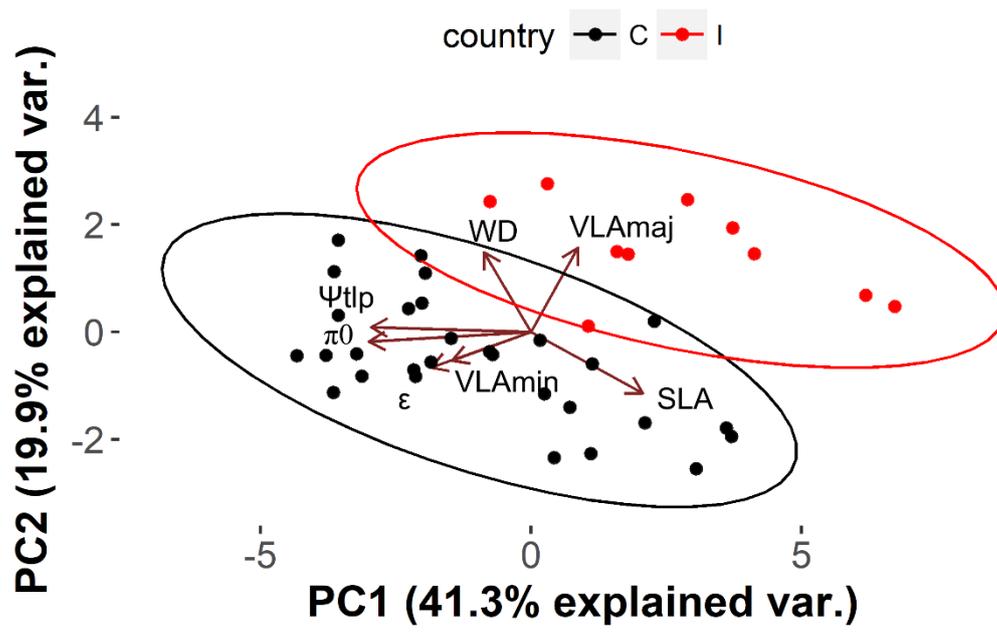


Fig. S3. Principal Component Analysis (PCA) of standardized functional traits measured in individuals of *A. altissima* growing in the native (China, C) and exotic (Italy, I) habitat.

Chapter 3

Study 5

Invasive potential of alien plant species explained by mechanistic traits: a step forward in understanding alien invasion mechanisms

Francesco Petruzzellis¹, Enrico Tordoni¹, Vanessa Tonet¹, Tadeja Savi², Martina Tomasella¹, Chiara Palandrani³, Miris Castello¹, Andrea Nardini¹ and Giovanni Bacaro¹

¹University of Trieste, Department of Life Sciences, Via L. Giorgieri 10, 34127 Trieste, Italy

²University of Natural Resources and Life Sciences, Division of Viticulture and Pomology, Department of Crop Sciences, Konrad Lorenz Straße 24, A-3430 Tulln, Wien, Austria

³University of Udine, Department of Agricultural, Food, Environmental and Animal Sciences, via delle Scienze 206, 33100, Udine, Italy

Abstract

Invasive alien species are a serious threat to biodiversity. Several studies and meta-analyses have been focused on the comparison between invasive (IAS) and native species but, to date, a unique set of traits responsible for the invasive potential of alien species at a global scale has not been clearly identified. Most previous studies analysed few species and traits simple to measure or were based on reviews or meta-analyses. In this study, we present a comparison on several woody and herbaceous IAS (15 species) and native species (78), based on functional and mechanistic traits measured in areas along a water availability gradient. We tested whether i) IAS generally share an acquisitive strategy independent of growth form or habitat features; ii) mechanistic traits related to drought tolerance and leaf venation were higher in IAS than in native species; iii) mechanistic and functional traits are coordinated in trade-offs. Our results show that IAS are characterised by traits that favour fast-growth, independently of growth form or habitats features. IAS consistently shared lower drought resistance and denser venation network, which are mechanistic traits directly influencing leaf construction costs and photosynthetic and growth rates. The coordination between construction costs, drought resistance and photosynthesis-related traits, suggested that IAS outperform native species due to higher potential for carbon gain and biomass accumulation, while sharing similar or higher levels of water-use efficiency.

Introduction

Invasive alien species (IAS) are defined as plants naturalized outside their native range, producing reproductive offspring, often in very large numbers and at considerable distances from the parent plants, and potentially spreading over a large area (Pyšek *et al.*, 2004). IAS spread has dramatically increased in the last decades (Seebens *et al.*, 2017), and they are currently considered a serious threat to biodiversity and ecosystem function on a global scale (Simberloff *et al.*, 2013). Hence, the identification of the biological and ecological mechanisms promoting IAS ecological success is an uprising research topic in plant ecology. Such ‘features’ are generally described in terms of ‘functional traits’, i.e. morphological, physiological, or phenological characteristics which are measurable at the individual level from the cell to the whole-organism (Violle *et al.*, 2007).

Including functional traits in the analysis of ecological invasion has provided helpful insights in basic processes underlying this phenomenon. Comparative analyses of functional traits between native and invasive species have revealed that IAS tend to share traits of the leaf economic spectrum (Wright *et al.*, 2004a) that favours fast growth (the so-called ‘fast-return strategy’) (Pyšek & Richardson, 2008; Leishman *et al.*, 2014). A recent meta-analysis has suggested that IAS usually have higher values of functional traits associated to performance (i.e. photosynthetic rate, growth rate, size, ecc.) and lower values associated to carbon-costs of important plant structures (i.e. specific leaf area, wood density, ecc) (van Kleunen *et al.*, 2010). Nevertheless, a unique set of traits responsible for the invasive potential of alien species at a global scale has not been clearly identified, as multiple suites of traits might promote IAS spread in different environments (van Kleunen *et al.*, 2015; Funk *et al.*, 2016), suggesting that invasion is a context-dependent process (Funk & Vitousek, 2007). According to the “environmental filtering” or “join-the-local” hypothesis (Tecco *et al.*, 2010; Kraft *et al.*, 2015), IAS are functionally similar to native species occurring in the same habitat, because only a specific suite of traits could enable the establishment and the persistence in a certain habitat. On the other hand, the “limiting similarity” or “try-harder” hypothesis (MacArthur & Levins, 1967; Tecco *et al.*, 2010) suggests that IAS species display different traits from natives, to avoid niche overlap and competition with native species or to outcompete them.

The scarce of evidence for common traits associated to invasiveness of alien species possibly derives from the fact that most previous studies were based on the analysis of traits simple to measure (van Kleunen *et al.*, 2010), but often without a clear linkage with plant physiology. For instance, specific leaf area (SLA) is commonly included in such studies, although it represents a ‘syndrome trait’ resulting from the interaction of several different processes (i.e. photosynthetic

rate, leaf lifespan, nutrient concentration) all finally somehow influencing the plant-level growth rate (Poorter *et al.*, 2009). The use of “mechanistic” traits, i.e. plant features clearly associated to a physiological function (Brodribb, 2017), could help disentangling the fundamental basis of the invasion process. Despite the advantages offered by mechanistic traits, they have been seldom included in studies on plant invasion, probably because of the difficult and time-consuming procedures needed to measure them. However, in the last years novel and simple techniques have been developed to simplify their measurements (Bartlett *et al.*, 2012a; Brodribb *et al.*, 2016) and, as a result, mechanistic traits such as hydraulics and water relation parameters have been increasingly included in ecological studies, providing novel mechanistic insights into plant-environment interaction (Blackman *et al.*, 2012; Fan *et al.*, 2012; Choat *et al.*, 2012; Brodribb *et al.*, 2014; Anderegg, 2015). In particular, the water potential inducing loss of cell turgor (Ψ_{tlp}) is a direct measurement of species drought tolerance (Bartlett *et al.*, 2012b), and species living in xeric environments typically have lower values of Ψ_{tlp} than species living in mesic habitats (Lenz *et al.*, 2006; Bartlett *et al.*, 2012b; Zhu *et al.*, 2018). From a physiological point of view, the maintenance of cell turgor is critical to maintain gas exchange and growth (Brodribb *et al.*, 2003), and recent analyses have reported that Ψ_{tlp} is correlated with other traits of the Leaf Economic Spectrum (LES) (Wright *et al.*, 2004), e.g. maximum CO₂ assimilation rate (A_{max}) and leaf mass per unit area (LMA) (Zhu *et al.*, 2018). These relationships suggest that the inclusion of mechanistic traits associated to water use (as Ψ_{tlp} , leaf carbon isotopic composition, vein length per unit area) in coordination with cost-related and performance-related traits might be important to explain interaction and competition processes during plant invasion (Petruzzellis *et al.*, 2018).

Most of the previous studies on plant invasions were based on meta-analysis, or included comparisons between a relatively low number of native and invasive species with different growth forms and/or traits (van Kleunen *et al.*, 2010). Here we present an analysis of the differences of several functional and mechanistic traits measured on a large assemblage of woody and herbaceous native and invasive species (93 species in total, 78 natives and 15 IAS), co-occurring in different areas along a water availability gradient. Specifically, we tested whether *i*) IAS share an acquisitive strategy independent of growth form or habitat features; *ii*) mechanistic traits related to drought tolerance and leaf venation were higher in IAS than in native species; *iii*) mechanistic and functional traits are coordinated along trade-offs.

Methods

Study areas and sampling design

Six sampling sites (three for woody and three for herbaceous species) were selected in NE Italy, ranging from coastal dune to wet areas and arid grasslands to woodlands. The climate of the study area is transitional between the Mediterranean and continental type (Poldini *et al.*, 1992), thus favouring the colonization of a wide variety of species occupying different ecological niches. We grouped sampling sites according to the gradient of water availability (high, mild- and low-water stress).

Two sampling sites (one for woody and one for herbaceous species) with high water stress were characterized by pronounced xericity, and they were represented by areas dominated by typical Mediterranean evergreen species (e.g. *Quercus ilex*) and by dune habitats, hosting natural vegetation typical of the northern Adriatic coastline (e.g. *Ammophila arenaria*).

The larger portion of the study area is occupied by Karst, a limestone plateau settled between the Adriatic Sea and the Alps, extending for over 500 km². Here, we located two sampling sites with mild water stress, which vegetation is dominated by thermophilus *Quercus* species (e.g. *Q. pubescens*), intermixed with *Fraxinus ornus*, *Ostrya carpinifolia* and *Prunus mahaleb* (Poldini, 1989). Herbaceous communities are generally represented by species of arid meadows, like *Chrysopogon gryllus* and *Satureja montana*.

Two sampling sites characterized by low water stress were placed in areas close to small lakes and rivers which neighbours the Karst plateau, hosting herbaceous species adapted to high water availability (e.g. *Carex* species, *Agrostis stolonifera*, ecc.) and woody species typical of riparian zones, such as *Populus nigra* and *Salix* sp. (e.g. *Salix alba*, *Salix eleagnos*) (Poldini, 1989).

For woody species, we selected 9 of the most representative native species in the study area (Poldini, 1989) and 3 invasive species (see Tab. S1). Five individuals from each of the most abundant native and invasive species present in each site were randomly sampled following Petruzzellis *et al.* (2017), and mechanistic and functional traits listed below were measured as detailed below.

For herbaceous species, 22 to 30 squared plots of 16 m² were randomly selected in each site, and one individual for each of the herbaceous species present in each plot was randomly sampled. In total, we sampled 81 species (69 native and 12 invasive species, Tab. S1). The status of a species (native or invasive) was assessed according to the updated checklist of the Italian alien vascular flora (Galasso *et al.*, 2018). Fieldwork was carried out in Spring-Summer during the period 2016 - 2018.

Measurements of mechanistic and functional traits

The complete list of functional and mechanistic traits, their associated physiological functions and relative number of leaves and individuals sampled are reported in Tab. 1.

		Trait category	N leaves (individuals)	
			<i>Woody</i>	<i>Herbaceous</i>
LMA	mg cm ⁻²	Costs	5 (5)	1 (2 to 7)
LDMC	mg g ⁻¹		5 (5)	1 (2 to 7)
C:N	/		1 (5)	1 (2 to 7)
C_{mass}	%		1 (5)	1 (2 to 7)
π₀	MPa	Vulnerability to drought	5 (5)	1 (2 to 7)
Ψ_{tip}	MPa		5 (5)	1 (2 to 7)
VLA_{min}	mm mm ⁻²	Productivity	5 (5)	1 (2 to 7)
N_{mass}	%		1 (5)	1 (2 to 7)
δ¹³C	‰	/	1 (5)	1 (2 to 7)

Tab. 1. List of functional and mechanistic traits with associated unit, trait categories and number of leaves per individuals (in parenthesis) measured in this study.

Leaf and wood morpho-anatomical traits. The following traits were measured on five leaves, randomly sampled from five individuals for each woody species in each sampling site, while for herbaceous species one leaf from each individual was randomly sampled in each sampling site (see above).

Leaf dry matter content (LDMC) and leaf mass per unit area (LMA) were calculated as LDMC = Leaf dry weight/Leaf turgid weight and LMA = Leaf dry weight/Leaf area. Fresh leaves were first rehydrated overnight, and leaf turgid weight was measured with an analytical balance. After that, leaves were scanned and leaf area was measured using the software ImageJ (Schneider *et al.*, 2012). Then, leaves were oven-dried for 24h at 70°C and dry weight was measured.

The length of minor veins (3rd and higher orders) per unit surface area (VLA_{min}) was measured as VLA = Vein Length/Leaf sample area. Fresh leaves were treated in 1 M NaOH solution for 48-72 h. Then, a portion of 1 cm² was cut from the central portion of each leaf (carefully avoiding the

midrib) and cleared in 1% NaClO for 5 min. Samples were then dehydrated in a sequence of ethanol solutions at increasing concentrations (25%, 50%, 75% and 100%) and then immersed in an alcoholic solution of toluidine blue (3%) overnight. Samples were then treated in a series of ethanol solutions at decreasing concentration and microscopic slides were prepared. Images of leaf portions of ~5 mm² were captured with an optical microscope at 4× magnification equipped with a digital camera and VLA_{min} was measured using PhenoVein software (Bühler *et al.*, 2015).

Water relation parameters. Leaves for the measurement of osmotic potential at full turgor (π_0) and water potential at turgor loss point (Ψ_{tp}) were sampled as described above. Twigs were detached, wrapped in cling film and put in plastic bags with a piece of wet paper inside to avoid dehydration. Samples were stored in cool bags until processing in the laboratory. π_0 and Ψ_{tp} were determined according to a recently developed framework (Bartlett *et al.*, 2012a) with some modifications. Twigs bearing leaves were first rehydrated overnight and then one leaf per twig were roughly crumbled and sealed in cling film. Then, it was immersed in liquid nitrogen for 2 min. Leaves (still sealed in cling film) were then carefully ground and stored in sealed plastic bottles at -20 °C until measurements. Samples were thawed at room temperature for 5 min while still sealed in cling film and in plastic bottles. Measurements of π_0 were done with a dew point hygrometer (π_{0_osm}) (Model WP4, Decagon Devices Inc.), and to overcome possible bias due to apoplastic dilution or cell wall enrichment of symplastic fluids (Bartlett *et al.*, 2012a), π_0 was estimated with the following equation (Petruzzellis *et al. submitted*, see Chapter 1, Study 2): $\pi_0 = 0.506\pi_{0_osm} - 0.002LDMC$ (expressed in mg g⁻¹). Ψ_{tp} was finally calculated as: $\Psi_{\text{tp}} = (1.31 \times \pi_0) - 0.03$.

Nutrient concentration and stable isotopes analysis. Nitrogen and carbon content (N_{mass} and C_{mass} , respectively), C:N ratio and carbon isotopic composition ($\delta^{13}\text{C}$) were measured on the same leaves sampled for LMA and LDMC. Leaves were oven dried (70 °C for 48h) and then pulverized in a mortar. Elemental composition and $\delta^{13}\text{C}$ were measured by an elemental analyzer/continuous flow isotope ratio mass spectrometry using a CHNOS Elemental Analyzer (vario ISOTOPE cube, Elementar, Hanau, Germany) coupled with an IsoPrime 100 mass spectrometer (IsoPrime Ltd, Cheshire, UK). All isotope and elemental composition analyses were performed by the Center for Stable Isotope Biogeochemistry (University of California, Berkeley). Long-term external precision based on reference material “NIST SMR 1577b” (bovine liver) is 0.10‰ and 0.15‰, respectively for C and N isotope analyses.

Statistical Analysis

Spectrum of traits associated with invasiveness. To summarize and visually describe the differences among traits between native and IAS, we first performed a Principal Component Analysis (PCA) using the R function ‘princomp’. All values were standardized (zero mean, unit variance) and centered before the analysis, and the number of significant PCA axes to be retained without loss of significance was then calculated (Dray, 2008). To estimate the occurrence probability of IAS and native species in specific regions of the two-dimensional space defined by PC axes 1 and 2, we used two-dimensional density kernel estimation, following the procedure reported in DÍaz *et al.* (2016). Traits were then further classified in categories reflecting their functional meaning. π_0 and Ψ_{tp} , were considered associated to vulnerability to drought; VLA_{min} and N_{mass} to productivity and LMA, C:N and LDMC to leaf construction costs. In order to reliably assess the magnitude of the difference of functional strategies between native and invasive species, we calculated the effect size of the difference of each trait category (Borenstein *et al.*, 2010) using R package “metafor” (Viechtbauer, 2010). Specifically, for each trait, we calculated the effect size (standardized mean difference or Hedges’ g) of the difference between IAS and native species as follows:

$$g = \frac{\bar{X}_{invasive} - \bar{X}_{native}}{S} J$$

where \bar{X} is the mean trait value of each species group, S is the pooled standard deviation and J is a weighting factor correcting for its slight positive bias for small sample size (Hedges & Olkin, 1985). Positive values of Hedges’ g indicated that invasive species had a larger value compared to native species in terms of units of standard deviation. Unbiased estimates of Hedges’ g sampling variance were also calculated as:

$$v_g = \frac{\bar{X}_{invasive} + \bar{X}_{native}}{X_{invasive} X_{native}} + \left(1 - \left(\frac{\bar{X}_{invasive} + \bar{X}_{native} - 2}{(\bar{X}_{invasive} + \bar{X}_{native}) J^2} \right) \right) g^2$$

Testing single-trait differences between native species and IAS. Single-trait differences between native species and IAS was assessed using a bootstrapped t-test procedure. This procedure was necessary because of the unbalanced number of species in the two groups (78 natives and 15 IAS), both in woody (9 natives and 3 IAS) and in herbaceous species (69 natives and 12 IAS). In order to provide comparable dimension of the same number of native species with respect to the number of sampled IAS, we applied a restricted resampling procedure as follow. From each site, 4 native herbaceous species from each site and 1 native woody species, thus obtaining a subset of 15 species (12 herbaceous and 3 woody). Then, via classic t-test (two tail, $\alpha = 0.05$), statistical differences

between the mean trait values of the randomly selected set of 15 native species and those of the 15 sampled IAS, were tested for each trait. Values of the t-statistic, p-value ($\alpha = 0.05$) and effect size r , calculated as:

$$r = \sqrt{\frac{t \text{ statistic}^2}{(t \text{ statistic}^2 + \text{degrees of freedom})}}$$

were averaged along 9999 randomization and their 95% confidence intervals calculated.

All the analyses were performed using R software version 3.5.1.

Trade-offs between traits. Spearman's rho (ρ) correlation between traits was calculated independently of growth form. For $\delta^{13}\text{C}$ we calculated the correlation only on C3 species. The analysis was carried out using 'rcorr' function for R software.

Results

The projection of species on the first two axes of the PCA (62.6 % of the total variance explained by PC1 and PC2) confirmed a separation between IAS and native species independently of growth form and habitat type (Fig. 1). In general, IAS occupy the extreme region of the functional space associated to acquisitive traits (i.e. high values of Ψ_{tlp} , N_{mass} and VLA_{min} and low values of LMA and C:N). Herbaceous IAS separated from herbaceous natives along PC1, while woody IAS tended to differ from woody natives along PC2 (Fig. 2a). In addition, when considering different habitats, most IAS shared the functional space associated to natives occurring in mesic habitats (Fig. 2b).

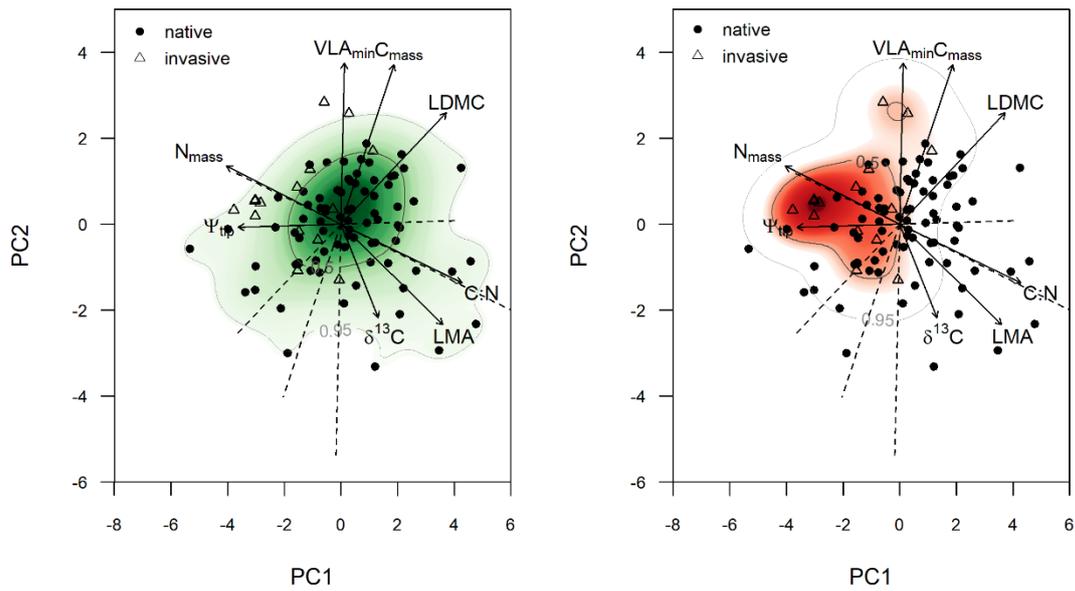


Fig. 1. Principal Component Analysis (PCA) of standardized and centered functional and mechanistic traits measured in IAS (triangles) and native species (solid circles). Solid arrows indicate direction and weighing of vectors representing the traits considered. The colour gradient indicates regions of highest occurrence probability of native species (green) and IAS (red) in the trait space defined by PC1 and PC2, with contour lines indicating 0.5 and 0.95 quantiles.

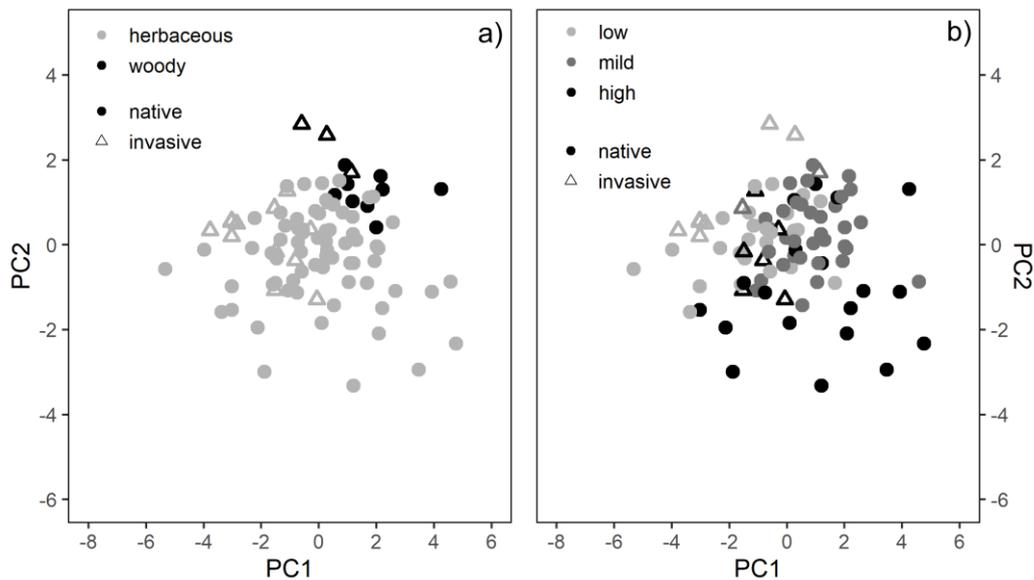


Fig. 2. Principal Component Analysis (PCA) of standardized and centered functional and mechanistic traits measured in IAS (triangles) and native species (solid circles). Panel a reported the location of different growth forms, while panel b reported the location of different sites. Light grey indicates species occurring in habitats with low water stress, dark grey indicates those occurring in habitats with mild water stress and black indicates species occurring in habitats with high water stress.

The separation of the two groups of species along the first two axes of the PCA, suggested the presence of difference trait assemblages between IAS and native species. For comparison of trait categories between IAS and native species, mean effect sizes were significantly larger than zero for traits associated to vulnerability to drought ('Vulnerability' category, i.e. π_0 , Ψ_{tlp}) and productivity ('Productivity' category, i.e. VLA_{min} and N_{mass}), whereas mean effect sizes were significantly lower than zero for cost-associated traits ('Costs' category, i.e. LMA, C:N and LDMC) (Fig. 3).

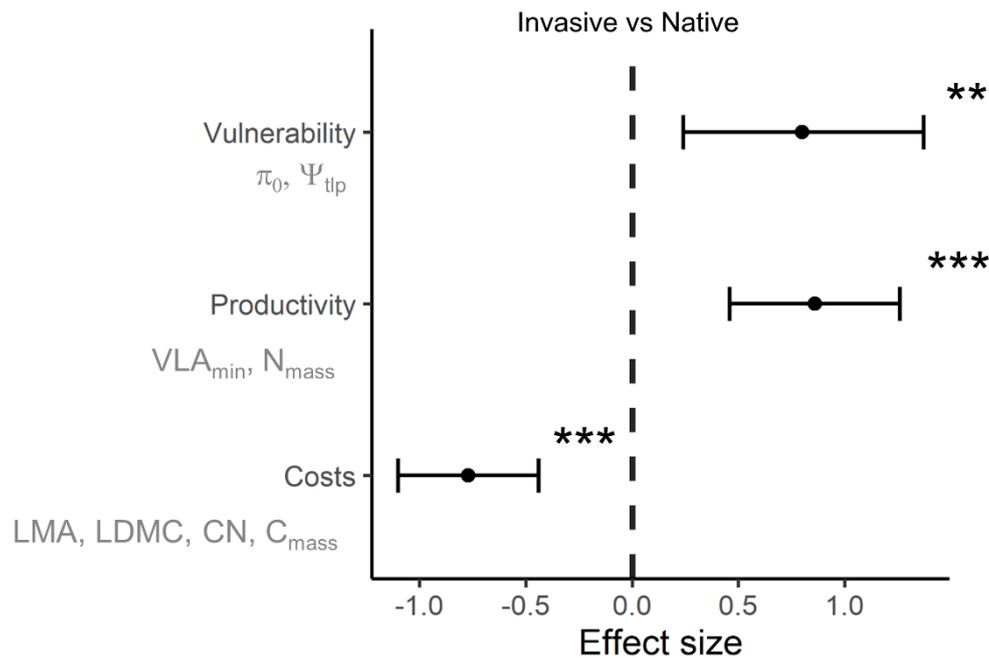


Fig. 3. Mean effect sizes (Hedges' g) and associated confidence intervals (95%, bars) of differences between IAS and native species the three trait categories (Table 1). A mean effect size was considered significantly different from zero when its 95%-confidence interval does not include zero. ** = p -value < 0.01, *** = p -value < 0.001.

We also tested for differences at single-trait level to highlight which trait was consistently shared by IAS (Tab. 2). IAS had significantly higher π_0 ($p < 0.01$, $r = 0.48$), Ψ_{tlp} ($p < 0.01$, $r = 0.49$), VLA_{min} ($p = 0.05$, $r = 0.36$) and N_{mass} ($p < 0.05$, $r = 0.42$), whereas C:N ($p < 0.05$, $r = 0.43$) and LMA ($p < 0.05$, $r = 0.39$) were significantly lower.

Traits	<i>IAS</i>	<i>native</i>	<i>Mean t-value</i>	<i>C.I. 2.5%</i>	<i>C.I. 97.5 %</i>	<i>p</i>	<i>r</i>
LMA	5.0±1.9	7.7±3.4	-2.65	-3.73	-1.75	0.02	0.44
LDMC	254.1±77.6	327.4±104.7	-2.22	-3.83	-0.91	0.04	0.38
C:N	16.7±5.7	24.3±8.9	-2.77	-3.91	-1.69	0.01	0.46
C_{mass}	44.1±2.8	44.6±3.9	-0.45	-1.89	0.70	0.65	0.12
π_0	-1.0±0.3	-1.4±0.4	2.81	1.70	4.10	0.01	0.46
Ψ_{tip}	-1.4±0.4	-1.9±0.5	2.80	1.67	4.10	0.01	0.46
VLA_{min}	9.5±2.8	7.7±2.4	1.80	0.74	2.82	0.05	0.32
N_{mass}	3.0±0.9	2.1±0.8	2.78	1.32	4.10	0.01	0.46
$\delta^{13}\text{C}$	-28.6±4.9	-27.6±4.4	-0.5	-1.35	0.46	0.62	0.11

Tab. 2. Mean values \pm standard deviation of the functional and mechanistic traits measured on IAS and native species. Mean t-value, 95 % confidence intervals (C.I. 2.5% and C.I. 97.5%), p-value (p) and effect size (r) calculated after bootstrap t-test procedure.

Traits' correlation matrix is summarized in Tab. S2. We found that LMA was positively correlated with $\delta^{13}\text{C}$, but negatively with Ψ_{tip} and N_{mass} (Fig. 4). Moreover, Ψ_{tip} was negatively correlated with $\delta^{13}\text{C}$ and positively with N_{mass} (Fig. 5a and 5b). Also, lower $\delta^{13}\text{C}$ values were associated with higher N_{mass} (Fig. 5c).

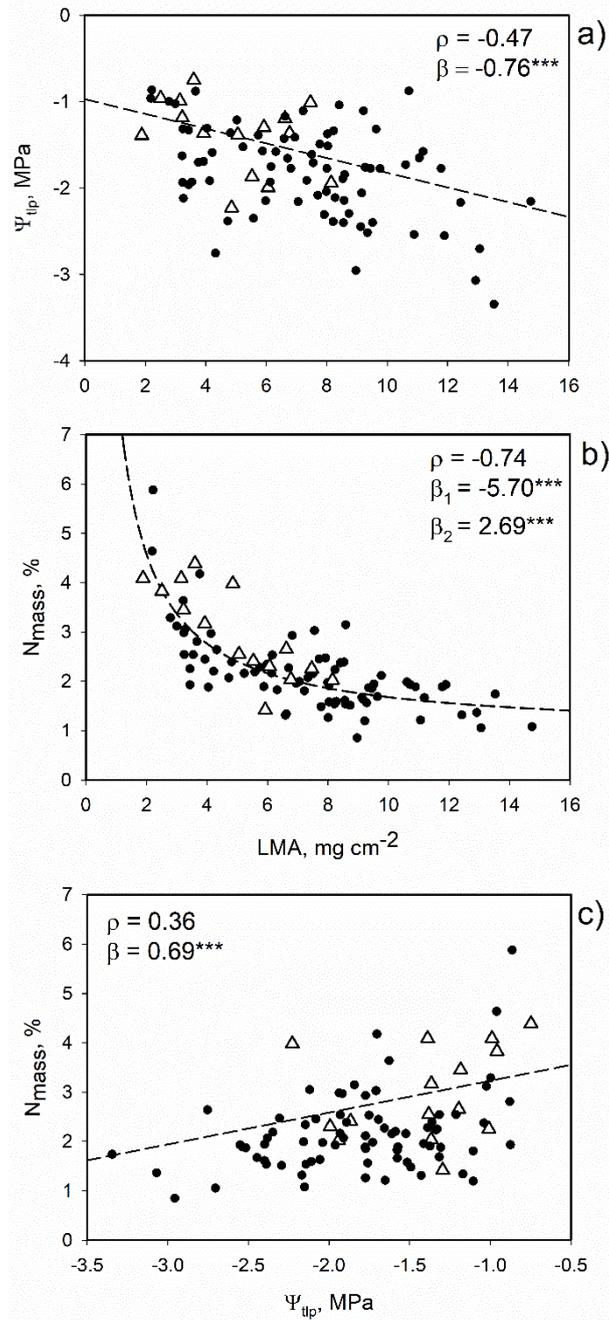


Fig. 4. Relationship between Ψ_{tlp} (a), $\delta^{13}C$ (b) and N_{mass} (c) vs LMA. Spearman's rho (ρ) and β coefficients are also reported. *** = p-value < 0.001. Triangles indicates IAS, while solid circles represent native species.

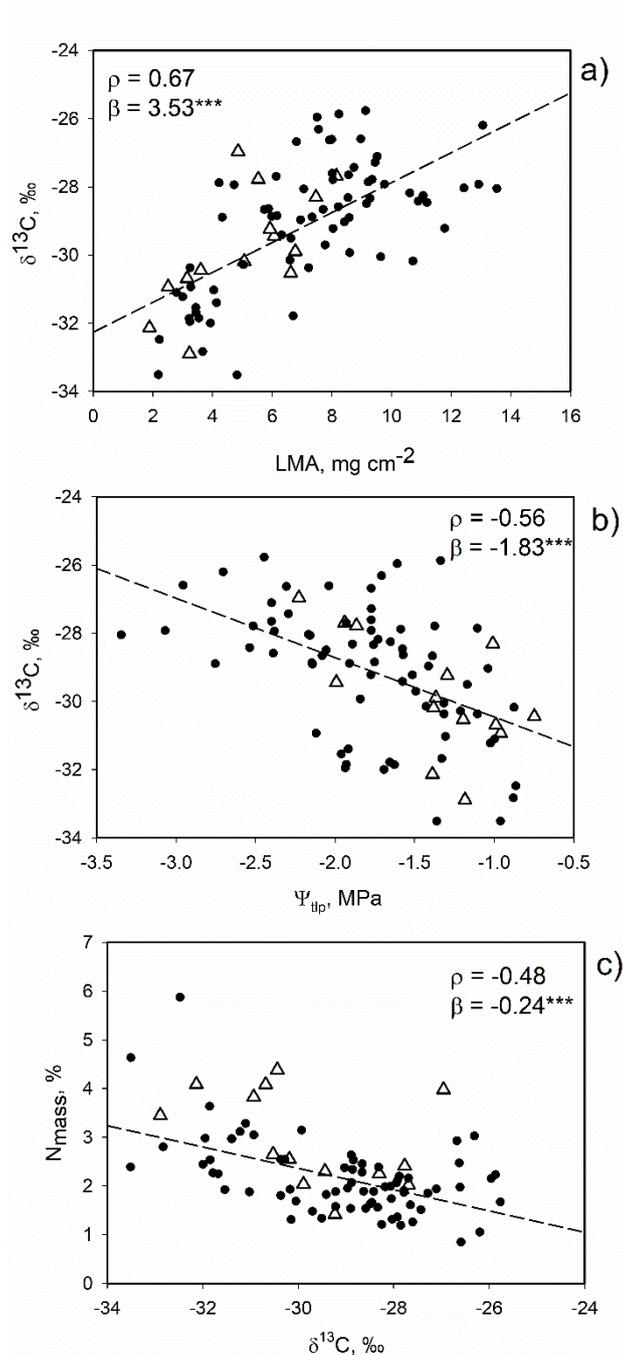


Fig. 5. Relationship between $\delta^{13}\text{C}$ (a) and N_{mass} (b) vs Ψ_{tlp} , and between N_{mass} vs $\delta^{13}\text{C}$ (c). Spearman's rho (ρ) and β coefficients are also reported. *** = p-value < 0.001. Triangles indicates IAS, while solid circles represent native species.

Discussion

The identification of eventual traits promoting alien plant invasiveness across bio-geographical and ecological scales is a still unresolved issue. Our study is one of the few examples of analysis based on direct measurements performed on a large number of alien and native species growing in contrasting environmental conditions (van Kleunen *et al.*, 2010; Funk *et al.*, 2016). Moreover, this is one of the very few studies reporting measurements of mechanistic traits, coupled to more classical functional traits.

Despite the scepticism on the possible existence of traits generally associated to invasiveness of alien species, recent meta-analyses and reviews showed that indeed some traits might promote invasiveness under many different circumstances. In general, IAS were suggested to adopt an acquisitive strategy, as shown by traits of the LES that might favour fast growth (Pyšek & Richardson, 2008; van Kleunen *et al.*, 2010). Our results confirmed this pattern, as IAS generally separated from natives along PC1, occupying the region of the functional space associated with low LMA and C:N and high N_{mass} (Fig. 1). Most importantly, our data showed that the consistency of this pattern is furtherly confirmed when accounting for growth form and site. Woody species and herbaceous species were divided along PC2 (Fig. 2a), and again PC1 determined the separation between woody IAS and woody natives. When accounting for sites, IAS tended to occupy the same region of the functional space occupied by species growing in low-stress and resource-rich habitats (Fig. 2b), which typically have an acquisitive strategy. This suggest that on average IAS, even when occurring in habitats with contrasting environmental conditions, share a common assemblage of traits reflecting functions that might be generally associated to their invasive potential of alien species.

We hypothesized that higher vulnerability to drought could be a novel feature associated to invasiveness of alien species, in coordination with lower construction costs and higher productivity-related traits. The output of effect-size and single trait bootstrap t-test analyses confirmed our hypothesis. Specifically, IAS tended to have higher values of traits associated to ‘Vulnerability’ and ‘Productivity’ categories, whereas traits associated to ‘Costs’ were lower when compared to native species (Fig. 3). Lower π_0 and Ψ_{tp} reflect the ability of a species to maintain cell turgor during drought stress (Bartlett *et al.*, 2012b; Zhu *et al.*, 2018), but both were consistently higher in IAS than in natives (Tab. 2). Both traits were negatively correlated with leaf carbon costs (LMA) (Fig. 4a), which was higher in native species (Tab. 2). We also found a coordination between Ψ_{tp} , LMA and N_{mass} (Fig. 4) which was higher in IAS, reflecting higher benefits of photosynthetic potential on a cost basis (Díaz *et al.*, 2016).

The trade-off between drought vulnerability, lower construction costs and higher photosynthetic rates has been previously reported in other studies (Villagra *et al.*, 2013; Zhu *et al.*, 2018) and might be related to structural properties that affect water transport and carbon gain within leaves. In particular, leaf venation traits (as VLA_{\min}) are mechanistically linked with gas exchange, photosynthetic and relative growth rates (Sack *et al.*, 2013; Iida *et al.*, 2016), and might play a role in the different strategy adopted by IAS and native species. In support to this hypothesis, we detected higher VLA_{\min} in IAS ($p = 0.05$, Tab. 2). Generally, higher VLA_{\min} values are associated with higher leaf hydraulic conductance (Sack *et al.*, 2013; Scoffoni *et al.*, 2016), which reflects the efficiency of water transport within leaves. A denser leaf vein network shortens the extra-vascular water pathway from xylem to stomata (McKown *et al.*, 2010), thus significantly affecting leaf hydraulic conductance, stomatal aperture and photosynthetic rates. According to the recently proposed trade-off between leaf hydraulic safety (as resistance to embolism formation in xylem) and efficiency (Nardini *et al.*, 2012; Nardini & Luglio, 2014), IAS might be positioned in the low safety-high efficiency region of this relationship. Recently developed techniques (Brodribb *et al.*, 2016) could be used to include embolism quantification in future studies to test whether an increased hydraulic efficiency and lower hydraulic safety might promote invasiveness.

We hypothesized that denser leaf venation network might ensure higher gas exchange rates and carbon gain in IAS. However, $\delta^{13}C$, which is an integrated measurement of stomatal conductance (Dawson *et al.*, 2002) and intrinsic water-use efficiency (Prieto *et al.*, 2017), was not different between the two species groups (Tab. 2). However, we detected a coordination between Ψ_{tlp} , $\delta^{13}C$ and N_{mass} (Fig. 5), where species with higher Ψ_{tlp} have lower (more negative) $\delta^{13}C$ (Fig. 5a) and higher N_{mass} (Fig. 5b). IAS were mostly positioned in the region associated with lower drought resistance and higher N_{mass} of these trade-offs, suggesting that they could have higher photosynthetic rates than natives at any given stomatal conductance, with similar or higher levels of water-use efficiency.

Overall, our data showed that IAS tend to share an acquisitive strategy independently of growth form and habitat type, showing traits of the LES that favour fast-growth. Mechanistic traits such as Ψ_{tlp} and VLA_{\min} might play a central role in determining invasive potential of IAS, by providing stronger mechanistic linkages between construction costs, and photosynthetic and growth rates. Future studies including mechanistic traits related to water transport efficiency as well as root traits will help in detecting clear physiological function underlying the invasion process.

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Supplementary Material

	LMA	LDMC	C:N	C _{mass}	π_0	Ψ_{tlp}	VLA _{min}	N _{mass}
LDMC	0.45***							
C:N	0.75***	0.56***						
C_{mass}	0.38***	0.72***	0.43***					
π_0	-0.48***	-0.73***	-0.42***	-0.43***				
Ψ_{tlp}	-0.47***	-0.73***	-0.41***	-0.43***	0.99***			
VLA_{min}	0.06 ^{n.s.}	0.12 ^{n.s.}	0.04 ^{n.s.}	0.25**	-0.06 ^{n.s.}	-0.06 ^{n.s.}		
N_{mass}	-0.74***	-0.45***	-0.97***	-0.27**	0.36***	0.36***	-0.01 ^{n.s.}	
$\delta^{13}\text{C}$	0.67***	0.56***	0.53***	0.51***	-0.56***	-0.56***	0.08 ^{n.s.}	-0.48***

Tab. S2. Spearman's rho coefficient (ρ) calculated on functional and mechanistic traits measured in this study. *** = p-value < 0.001, ** = p-value < 0.01, n.s. = p > 0.05.

<i>Species</i>	<i>abbreviation</i>	<i>growth form</i>	<i>Status</i>	<i>Site (stress level)</i>
<i>Ailanthus altissima</i>	Aa	woody	invasive	mild
<i>Amorpha fruticosa</i>	Af	woody	invasive	low
<i>Acer monspessulanum</i>	Am	woody	native	high
<i>Fraxinus ornus</i>	Fo	woody	native	mild
<i>Ostrya carpinifolia</i>	Oc	woody	native	mild
<i>Prunus mahaleb</i>	Pm	woody	native	mild
<i>Populus nigra</i>	Pn	woody	native	low
<i>Pistacia terebinthus</i>	Pt	woody	native	mild
<i>Quercus ilex</i>	Qi	woody	native	high
<i>Quercus pubescens</i>	Qp	woody	native	mild
<i>Robinia pseudoacacia</i>	Rp	woody	invasive	low
<i>Salix eleagnos</i>	Se	woody	native	low
<i>Agrostis stolonifera</i>	Agrostol	herb	native	low
<i>Allium sphaerocephalon</i>	Allispha	herb	native	mild
<i>Ambrosia artemisiifolia</i>	Ambrarte	herb	invasive	low
<i>Ambrosia psilostachya</i>	Ambrpsil	herb	invasive	high
<i>Ammophila arenaria</i>	Ammoaren	herb	native	high
<i>Anthyllis vulneraria</i>	Anthvuln	herb	native	mild
<i>Apocynum venetum</i>	Apocvene	herb	native	high
<i>Aristolochia clematitis</i>	Ariscllem	herb	native	low
<i>Atriplex portulacoides</i>	Atriport	herb	native	high
<i>Atriplex prostrata</i>	Atripros	herb	native	high
<i>Bidens frondosa</i>	Bidefron	herb	invasive	low
<i>Bidens vulgata</i>	Bidevulg	herb	invasive	low
<i>Bromus erectus</i>	Bromerec	herb	native	mild
<i>Cakile maritima</i>	Cakimari	herb	native	high
<i>Carex elata</i>	Careelat	herb	native	low
<i>Carex extensa</i>	Careexte	herb	native	high
<i>Carex vesicaria</i>	Carevesi	herb	native	low
<i>Cenchrus longispinus</i>	Cenclong	herb	invasive	high
<i>Centaurea rupestris</i>	Centrupe	herb	native	mild
<i>Chrysopogon gryllus</i>	Chrygryl	herb	native	mild
<i>Crepis neglecta</i>	Crepnegl	herb	native	mild
<i>Cynodon dactylon</i>	Cynodact	herb	native	high
<i>Cyperus capitatus</i>	Cypecapi	herb	native	high
<i>Cytisus procumbens</i>	Cytiproc	herb	native	mild
<i>Dictamnus albus</i>	Dictalbu	herb	native	mild
<i>Elymus pungens</i>	Elympung	herb	native	high
<i>Erigeron annuus</i>	Erigannu	herb	invasive	high
<i>Erigeron canadensis</i>	Erigcana	herb	invasive	high
<i>Eryngium amethystinum</i>	Erynamet	herb	native	mild
<i>Euphorbia nicaeensis</i>	Euphnica	herb	native	mild
<i>Festuca sp.</i>	Festsp	herb	native	mild
<i>Galium verum</i>	Galiveru	herb	native	mild
<i>Gratiola officinalis</i>	Gratoffi	herb	native	low

<i>Hainardia cylindrica</i>	Haincyli	herb	native	high
<i>Hedera helix</i>	Hedeheli	herb	native	low
<i>Hippocrepis comosa</i>	Hippcomo	herb	native	mild
<i>Iris pseudacorus</i>	Irispseu	herb	native	low
<i>Jurinea mollis</i>	Jurimoll	herb	native	mild
<i>Koeleria pyramidata</i>	Koelpyra	herb	native	mild
<i>Leersia oryzoides</i>	Leeroryz	herb	native	low
<i>Limbarda crithmoides</i>	Limbcrit	herb	native	high
<i>Limonium narbonense</i>	Limonarb	herb	native	high
<i>Lotus germanicus</i>	Lotugerm	herb	native	mild
<i>Lysimachia vulgaris</i>	Lysivulg	herb	native	low
<i>Lythrum salicaria</i>	Lythsali	herb	native	low
<i>Mentha arvensis</i>	Mentarve	herb	native	low
<i>Oenothera stucchii</i>	Oenostuc	herb	invasive	high
<i>Oxalis dillenii</i>	Oxaldill	herb	invasive	low
<i>Persicaria dubia</i>	Persdubi	herb	native	low
<i>Persicaria hydropiper</i>	Pershydr	herb	native	low
<i>Phragmites australis</i>	Phraaust	herb	native	low
<i>Physalis alkekengi</i>	Physalke	herb	native	low
<i>Plantago major</i>	Planmajo	herb	native	low
<i>Plantago subulata</i>	Plansubu	herb	native	mild
<i>Potentilla reptans</i>	Poterept	herb	native	low
<i>Potentilla tommasiniana</i>	Potetomm	herb	native	mild
<i>Ranunculus repens</i>	Ranurepe	herb	native	low
<i>Rorippa sylvestris</i>	Rorisylv	herb	native	low
<i>Rubus caesius</i>	Rubucaes	herb	native	low
<i>Salvia pratensis</i>	Salvprat	herb	native	mild
<i>Sanguisorba minor</i>	Sangmino	herb	native	high
<i>Satureja montana</i>	Satumont	herb	native	mild
<i>Scabiosa triandra</i>	Scabtria	herb	native	high
<i>Scorzonera villosa</i>	Scorvill	herb	native	mild
<i>Senecio inaequidens</i>	Seneinae	herb	invasive	mild
<i>Silene vulgaris</i>	Silevulg	herb	native	high
<i>Spartina versicolor</i>	Sparvers	herb	native	high
<i>Stachys officinalis</i>	Stacoffi	herb	native	mild
<i>Stipa pennata</i>	Stippenn	herb	native	mild
<i>Teucrium chamaedrys</i>	Teuccham	herb	native	mild
<i>Teucrium montanum</i>	Teucmont	herb	native	mild
<i>Teucrium scordium</i>	Teucscor	herb	native	low
<i>Thalictrum flavum</i>	Thalflav	herb	native	low
<i>Thalictrum minus</i>	Thalminu	herb	native	mild
<i>Thymus pulegioides</i>	Thympule	herb	native	mild
<i>Trifolium rubens</i>	Trifrube	herb	native	mild
<i>Verbascum densiflorum</i>	Verbdens	herb	native	high
<i>Vincetoxicum hirundinaria</i>	Vinchiru	herb	native	low
<i>Xanthium orientale</i>	Xantorie	herb	invasive	high

General conclusions

Advances on methodological issues

The first chapter of this Thesis aimed at addressing methodological issues related to the sampling strategy that should be adopted when measuring functional traits (Study 1) and to the development of a simplified framework to measure leaf turgor loss point (Study 2).

Recent studies highlighted that intraspecific variability (ITV) should be considered when sampling functional traits, as it affects leaf traits variability (Albert *et al.*, 2015). An underestimation of ITV could in fact introduce bias in trait-based analyses. In study 1 we demonstrated that the spatial structure of the canopy could significantly affect traits variability, as previously suggested by other studies. Indeed, different spatial factors accounted for different proportion of the variability of the two tested traits, suggesting that different sampling strategies for different traits could be implemented during sampling surveys. We provided a minimum and optimal sample size, partially in accordance with those previously suggested in widely-used standardize protocols. The minimum size to adequately capture the studied functional traits corresponded to 5 leaves taken randomly from 5 individuals, while the most accurate and feasible sampling size was 4 leaves taken randomly from 10 individuals. We also suggested to sample leaves considering all the strata composing the canopy to estimate correctly the studied traits values, especially when interested in assessing traits variability at species level. As suggested by Messier *et al.* (2010), the dichotomy between “sun” and “shade” leaves is ambiguous, and current databases reporting data measured on “sun” leaves are biased by a shaded effect (Keenan & Niinemets, 2016).

Another issue still partially unresolved in trait-based ecology regards the choice of traits that must be included to functionally characterize a species or a community. The recently proposed mechanistic traits reflect functions that can be clearly physiologically defined, and their inclusion in trait-based studies might deepen our knowledge on ecological and evolutionary processes. Leaf turgor loss point (Ψ_{tlp}) is a mechanistic trait directly associated to drought resistance, and it is related to hydraulic and economic traits as well as environmental features. In study 2, I provided a simplified framework for Ψ_{tlp} measurements based on osmometer determination of the osmotic potential at full turgor (π_{0_osm}). Due to their relationship, it is possible to estimate Ψ_{tlp} from π_0 . To improve the predictive power of the estimation of Ψ_{tlp} , we suggest measuring leaf dry matter content (LDMC) as well as π_{0_osm} from leaves attached to the same twig or at least belonging to the same

individual. I provided two simple equations to estimate π_0 and Ψ_{tp} , encouraging the inclusion of these mechanistically sound drought tolerance traits in ecological studies.

Key findings on traits associated to invasiveness of alien species

The major goal of this Thesis was to highlight which functional and mechanistic traits enhance the invasive potential of alien species. The results gained in Chapter 1 allowed me to measure functional and mechanistic traits with an optimal accuracy and precision, and I was able to include Ψ_{tp} on several native and IAS, which constitute a novelty in this type of comparisons. I found clear differences of both functional and mechanistic traits between IAS and native species, and traits trade-offs provided useful information on mechanisms underlying the invasion process.

The comparison between *A. altissima* (Aa), one of the most invasive species in Europe, and the native and outcompeted *F. ornus* (Fo) (Study 3), have revealed that a safety–efficiency trade-off might be at the basis of the invasive success of alien trees. The lower resistance to drought stress of Aa was apparently counterbalanced by higher water transport efficiency and determined lower structural costs than Fo. Large phenotypic plasticity of Aa in response to light availability could also play a role in determining the invasive potential of this species. Moreover, it has been suggested that phenotypic plasticity of IAS could be higher in their exotic rather than native habitat. In study 4, I performed a comparison of functional and mechanistic traits of Aa measured in its exotic and native (China) ranges. Novel features related to Ψ_{tp} adjustment arose in the exotic range along with higher plasticity in traits related to leaf-construction costs, possibly determining an increased fitness of Aa in response to different environmental conditions in its invaded range.

The results obtained in Chapter 2 suggested that measurement of mechanistic traits related to resource acquisition, with special reference to water-use strategies, could provide important novel insight into the mechanisms underlying the invasive nature of alien species in natural habitats. In this light, in Chapter 3 I extended the measurement of these traits on a larger number of species (93, 78 natives and 15 IAS). Our results show that IAS possess traits that favour fast-growth, independently of growth form or habitats features. Moreover, IAS consistently shared lower drought resistance and denser venation network, which are mechanistic traits directly influencing leaf construction costs and photosynthetic and growth rates. The coordination between construction costs, drought resistance and photosynthesis-related traits, suggest that IAS outcompete native species due to higher potential for carbon gain and biomass accumulation, while sharing similar water-use efficiency.

Towards the ‘Spectrum of functions associated to plant invasion’

The consistent differences between IAS and native species strongly suggest that mechanistic traits do play a role in the invasion process. In this Thesis I mainly focused on leaf traits, but the results of Study 3 showed that also traits at stem level might contribute to define functions associated with plant invasion. Recent developed techniques might contribute to measure other mechanistic traits on a large number of samples and on different plant organs. Among these, the ‘optical method’ recently proposed by Brodribb *et al.* (2016) has been used to estimate vulnerability to embolism formation in different organs (from leaves to roots), significantly reducing time and costs associated with this kind of measurement with standard techniques (e.g. bench-dehydration hydraulics). Moreover, root traits have been seldom included in trait comparisons between native and IAS. The differences in drought resistance levels between native and IAS reported in this Thesis might suggest that IAS could access to different water sources. Including measurement of sap oxygen stable isotopes ($\delta^{18}\text{O}$) could provide useful information about root depth and to test whether IAS occupy a different hydrological niche than native species. The chance to measure mechanistic traits on a larger set of species would allow widening the scale of the analyses provided in this Thesis, to test whether IAS consistently share the assemblage of traits described here.

In conclusion, I suggest that the inclusion of leaf, stem and root functional and mechanistic traits in the study of plant invasion might finally converge in the definition of the ‘Spectrum of functions associated to plant invasion’.

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