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FINAL MASTER THESIS

Pathogenicity tests of *Phytophthora alni* and *Phytophthora plurivora* in *Fraxinus excelsior* and *Alnus glutinosa* seedlings

Author:
Susana Durães

Advisor:
Dr. Julio Javier Diez Casero

Co-advisor:
Dr. Jorge Martín-García

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INDEX

LIST OF FIGURES

ABSTRACT

Durães, SM. 2015. Pathogenicity tests of *Phytophthora alni* and *Phytophthora plurivora* in *Fraxinus excelsior* and *Alnus glutinosa* seedlings.

Phytophthora alni and *P. plurivora* have been associated to damage and death of *Alnus glutinosa* in Europe in recent decades. Recently, *Fraxinus excelsior* mortality was also associated to *Phytophthora plurivora*. In Spain, *A. glutinosa* appears with *F. excelsior*, creating riparian forests with a high ecological importance. The main objective of the study was to describe the susceptibility growth of Common alder and European ash from natural riparian populations in Northern Spain, using *P. alni* and *P. plurivora* isolates recovered also in Northern Spain. This study was divided into two independent experiments; Experiment I aimed at knowing the effect of soil infestation and stem inoculation with *P. plurivora* and *P. alni* on European Ash 1-year-old seedlings, and Experiment II aimed at evaluating *P. plurivora* infestations on different 3-month-old A. glutinosa seedlings from 5 different provenances.

On Experiment I, *Phytophthora plurivora* and *P. alni* Spanish isolates didn't prove to be pathogenic towards *Fraxinus excelsior*. All seedlings persisted until the end of the experiment on both treatments (Soil infestation and Stem inoculation), with no significant plant yield differences between isolates and control. On Soil infestation, the two *P. plurivora* isolates were successfully recovered from soil samples, while *P. alni* and control didn't appear any necrosis on vegetal baiting. Likewise on Stem inoculation, the two *P. plurivora* isolates were also isolated from necrotized stem pieces; however the appeared necrosis were only superficial. This determine that *P. plurivora* is able to penetrate inside *F. excelsior*, but this species is likely able to stop the progress of this pathogen.

On Experiment II, most of the *A. glutinosa* seedlings from different provenances survived until the end of the experiment, showing that soil inoculation method it was not properly effective in a short term. Nevertheless, it was possible to re-isolate the two isolates from roots and soil samples. Observing relative height growth, root length and aerial dried weight results, all control values were smaller and with significant differences comparing with both *P. plurivora* isolates, while on root dried weight, control values were higher and with significant difference comparing only with one *P. plurivora* isolate.

In both experiments, the two *P. plurivora* isolations didn't show any aggressiveness significance between each other.

To conclude, *P. plurivora* proved its ability to persist in soil, which enhances the spread of *P. plurivora* around the world via nursery trade of contaminated plant material, even not showing typical *Phytophthora* symptoms on the trading plants.

KEYWORDS: Common alder, European ash, pathogenicity, provenances, *Phytophthora plurivora*.

RESUMEN

Durães, SM. 2015. Pruebas de patogenicidad de *Phytophthora plurivora* y *Phytophthora alni* en *Fraxinus excelsior* y *Alnus glutinosa* plántulas.

Phytophthora alni y *P. plurivora* han sido asociados al decaimiento y muerte de *Alnus glutinosa* en Europa en las últimas décadas. Del mismo modo, más recientemente la mortalidad de *Fraxinus excelsior* también ha sido asociada a *Phytophthora plurivora*. En España, alisos y fresnos son especies ecológicamente muy importantes al formar parte de la orla vegetal de los bosques de ribera. El objetivo principal de este estudio fue determinar la susceptibilidad de las poblaciones de aliso y fresno del norte de España frente a *P. alni* y *P. plurivora*. Este estudio consistió de dos experimentos independientes; Experimento I cuyo objetivo era determinar la susceptibilidad de plántulas de fresno común de 1 año de edad frente a ambos patógenos mediante ensayos de inoculación en suelo y tallo, y el Experimento II cuyo objetivo fue evaluar la susceptibilidad de distintas regiones de procedencia de aliso común del norte de España frente a *P. plurivora*, para lo que se inocularon plántulas de 3 meses de edad mediante la aplicación directa de zoosporas en el substrato.

En el experimento I, Las cepas españolas de *Phytophthora plurivora* y *P. alni* no se han comportado como patógenas frente a *Fraxinus excelsior*. Ninguna plántula murió en ninguno de los métodos de inoculación utilizados. Sorprendentemente, los dos aislamientos de *P. plurivora* fueron reaislados tanto desde el tallo en la zona necrótica como desde el suelo mediante la técnica de trampas vegetales. Sin embargo, *P. alni* no fue reaislado ni en tallo ni en el suelo. Esto determina que *P. plurivora* es capaz de penetrar en el interior *F. excelsior*, pero esta especie probablemente es capaz de detener el avance de este patógeno.

En el Experimento II, la mayoría de las plántulas de aliso de las distintas procedencias permanecieron asintomáticas, no observándose ningún daño ocasionado por *P. plurivora*. Sin embargo, el patógeno fue reaislado tanto desde las raíces como de muestras de suelo. El crecimiento relativo en altura, longitud de la raíz y el peso seco de la parte aérea de los controles fueron menores que de las plántulas inoculadas, mientras que el peso seco de la parte radicular de los controles fue mayor que de las plántulas inoculadas con un aislamiento de *P. plurivora*.

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Para concluir, *P. plurivora* ha mostrado su capacidad para persistir en el suelo, lo que podría favorecer su dispersión alrededor del mundo a través del mercado globalizado de plántulas infestadas asintomáticas.

PALABRAS CLAVE: aliso común, fresno común, patogenicidad, procedencias, *Phytophthora plurivora*.

INTRODUCTION

Genus Phytophthora

Phytophthora is a genus of microorganisms in the kingdom Chromista, phylum Oomycota, class Oomycetes, order Pythiales, family Pythiaceae (Hawksworth *et al.*, 1996). Recently, Phytophthora had been grouped into the kingdom Chromalveolata, which also includes brown algae and other protists (Adl *et al.*, 2005). Also *Phytophthora* has been placed in order Pythiales with *Pythium* and related genera, but more recent phylogenetic analysis has indicated a closer affiliation with downy mildews (i.e *Peronospora farinose*) and white rusts (i.e. *Albugo candida*) in the Peronosporales (Beakes & Sekimoto, 2008). However, additional multi-gene analysis with a larger number of downy mildew species is needed to better characterize this relationship and the proper placement of *Phytophthora spp.* (Blair *et al.*, 2008).

Phytophthora species resemble true fungi because they grow by means of fine filaments (hyphae), and produce spores. However, *Phytophthora* and other oomycetes have different characteristics from fungi. Unlike true fungi, their cell walls contain cellulose instead of chitin, and have diploid phase in their life cycle, rather than the haploid phase (Erwin & Ribeiro, 1996). Another important feature of *Phytophthora* is produces swimming spores, called zoospores, during one phase of its life cycle (Brasier, 1992).

Most of the *Phytophthora* species are soil borne oomycetes that cause destruction in the roots system and can survive a long period in soil without host. Some species are specific, just attacking one or two plant species, but others have a wide range of plant hosts (Erwin & Ribeiro, 1996).

Usually, *Phytophthora* is in soil as a form of survival structures, the chlamydospores (asexual spores) and also oospores (sexual spores). When conditions are appropriate, particularly warm temperature and presence of water in the soil, these structures germinate to produce sporangia. When sporangia get maturity, they release biflagellate zoospores. The zoospores are the most important structures of infection; although they are short-lived, they can produce several generations for a short time if environmental conditions remain appropriate (Erwin & Ribeiro, 1996). They may move actively in the water, through the movement of their flagella, or be swept away by rainwater to potential infection sites (Duniway, 1976; Zentmyer, 1980). Normally they are chemically attracted to root exudates of potential hosts where they settle and encyst (positive chemotaxis) (Carlile, 1983; Hardham, 2001). In addition, the zoospores contain information about the chemical environment and may also include information about the

electrical environment, which allows zoospores distinguish living roots from the dead roots and contribute to the selection of infection site on the root surface (Morris & Gow, 1993).

When zoospores find a potential root surface, they lose their flagella and with enzymes capable of causing destruction of root's cell walls, they germinate to produce hyphae, which allow the pathogen to grow into plant cells (inter- and intracellular) to obtain nutrients (Hardham, 2001).

After the invasion of the host tissues, the pathogen can sporulate in cortical cells, if the conditions are not the most favorable; or sporangia on the root surface, if the conditions remain favorable. Sporangia turn to release zoospores and the cycle restarts. These continue to infect the root or adjacent roots. The chlamydospores, when stimulated by the presence of a host plant and good environmental conditions, germinate giving life to new infectious hyphae (Hardham, 2001).

In Figure 1 is shown briefly the life cycle of *Phytophthora spp*.

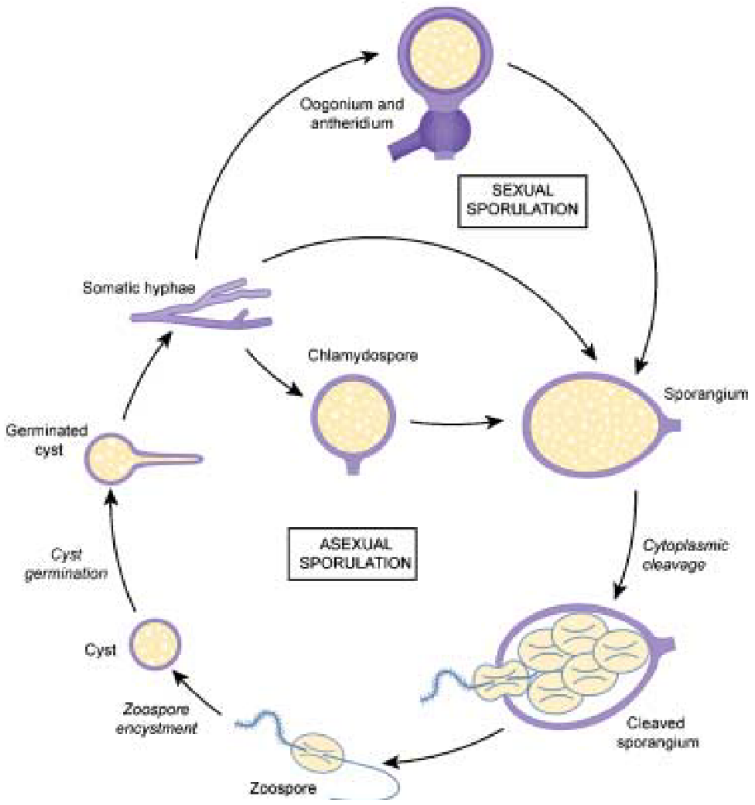


Figure 1 - Generalized life cycle of *Phytophthora* (adapted from Hardham, 2005).

Endemic *Phytophthoras* in their native ecosystems have co-evolved with their hosts, and co-exist while not usually causing severe diseases. Disease problems most often arise when the pathogen is introduced to a new habitat. They can cause major problems to plants in nurseries

and natural ecosystems where plants have little resistance against the foreigner pathogen and where it has few or any natural enemies (Rytkönen, 2011).

In the past years, multiple new species of *Phytophthora* have been described associated with nursery trade or natural ecosystems. Many of these may be recent invasive, due to the increasing international movement of plants leading to export and spread of exotic *Phytophthora spp.* (Brasier, 2007).

Alnus glutinosa disease and its pathogen: *Phytophthora alni* & *Phytophthora plurivora*

In 1993, Gibbs recorded for the first time an unknown deadly disease of *A. glutinosa* in southern Britain (Gibbs, 1995). Investigations revealed that the diseased trees were spread mainly along riverbanks, but also in forest plantations, some of which were away from watercourses (Cech, 1998; Gibbs *et al.*, 1999). Initially it was thought to be caused by *Phytophthora cambivora,* because of its similarities. However *P. cambivora*, a fungus well-known as a pathogen of broadleaved trees (i.e. *Castanea*, *Fagus* and *Malus*) was not previously reported from alder. So, it quickly became clear that the pathogen was an entirely new species (Gibbs *et al.*, 1999).

Affected trees presented typical symptoms of *Phytophthora* root and collar rot: unusually small, sparse and often yellowish foliage, a dieback of the crown, rusty colored exudates on the lower part of the stem, and early and often excessive fructification with abnormally small cones (Cech, 1998; Gibbs *et al.*, 1999; Webber *et al.*, 2004; Černý & Strnadová, 2010) (see Figure 2).

Brasier *et al.* (1999) stated the causal organism was an interspecific hybrid of heterothallic *P. cambivora* and an unidentified *Phytophthora* associated to homothallic *P. fragariae*. Neither of these organisms is a known pathogen of alder. Eventually, the alder *Phytophthora* was formally designated by Brasier *et al.* (2004) as a new species, *Phytophthora alni* Brasier & S.A. Kirk.

Phytophthora alni had three variants which vary in their virulence and pathogenicity: *P. alni* ssp. *alni* (PAA), corresponding to former 'standard' type; *P. alni ssp. uniformis* (PAU), corresponding to Swedish variant; and *P. alni ssp. multiformis* (PAM), corresponding to Dutch, German and UK variant. Later, was discovered PAU and PAM are parental species of PAA (Ioos *et al.*, 2006; Aguayo *et al*., 2013), and its hybridization maybe occurred in European nurseries (Brasier *et al.*, 1999, Brasier & Jung, 2003; Jung & Blaschke, 2004). Therefore, Husson *et al*. (2015) proposed to raise PAA, PAU and PAA to species status and to rename them to *Phytophthora* x *alni*, *Phytophthora uniformis* and *Phytophthora* x *multiformis*, respectively.

Most records of alder *Phytophthora* have come from the common alder (*A. glutinosa*), but the oomycete has also been detected in grey alder (*A. incana*), and Italian alder, (*A. cordata*) (Gibbs, 1995; Cech, 1998; Santini *et al*., 2003; Adams *et al.*, 2009). Brasier & Kirk (2001)

compared the relative susceptibility of the three species indicates that *A. glutinosa* is most susceptible to the disease, while *A. incana* is the most resistant.

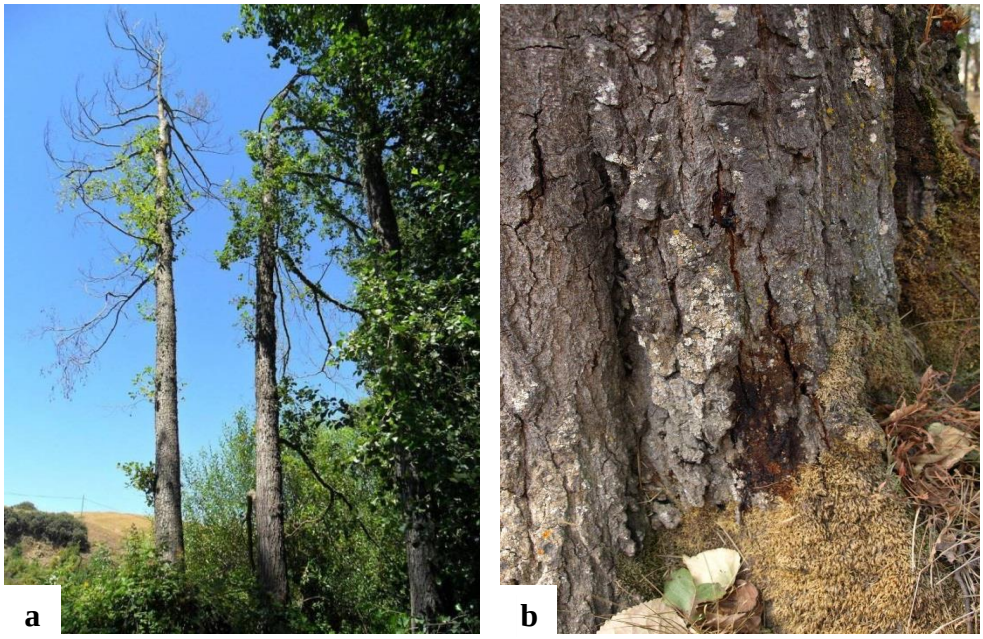


Figure 2 – Typical symptoms of *Phytophthora* root and collar rot in *Alnus glutinosa*. **a**. dieback of the crown; **b**. rusty colored exudates on the lower part of the stem (Courtesy of Masum Haque).

Concerning pathogen infection locations, Alder trees along rivers are mainly infected via lenticels and adventitious roots during temporary flooding events, while roots are the primary infection site in nursery-grown planted alder seedlings on non-flooded sites (Jung & Blaschke, 2004).

Indeed, environmental factors play an important role in the occurrence of the disease, and several risk factors have been identified including low water flow speed, seasonal flooding by contaminated water, fine soil texture and high water temperatures (Jung & Blaschke, 2004; Thoirain *et al.*, 2007; Elegbede *et al.*, 2010; Strnadová *et al.*, 2010; Aguayo *et al*., 2014).

A limiting factor and lead to tree recovery could be poor pathogen overwintering in soil. Aguayo *et al.* (2014) showed a clear annual pattern of *P. alni* viable inocolum in infected trees with limited survival after cold winters. It has already been shown that *P. alni* does not survive severe frosts (Černy *et al.*, 2012; Černý & Strnadová, 2012). In fact, *P. alni* does not produce chlamydospores, and because it is a triploid homoploid hybrid, being unable to complete meiosis, produce non-viable oospores (Delcán & Brasier, 2001; Brasier *et al.*, 2004). As a

consequence of this lack of resistant spores, *P. alni* has been demonstrated to persist in soil for only few months in the absence of host (Jung & Blaschke, 2004; Elegbede *et al.*, 2010).

However, global warming and associated climate change patterns are likely to impact on plant-pathogen interaction, and several researchers have focus on this problematic (e.g. Anderson *et al.*, 2004; Bergot *et al.*, 2004; Garrett *et al.*, 2006; Desprez-Loustau *et al.*, 2007; La Porta *et al.*, 2008; Pautasso *et al.*, 2010). The impact of many diseases is likely to increase with a temperature rise in the winter, resulting in more successful pathogen survival (Bergot *et al.*, 2004; Černý & Strnadová, 2012).

The respective disease and damage of alder stands has become a crucial problem in many European countries (i.e. U.K., France, Germany, Italy, the Netherlands, Czech Republic, Hungary, Spain) and North America countries (Gibbs, 1995; Santini *et al.*, 2001; Streito *et al.*, 2002; Nagy *et al.*, 2003; Santini *et al.*, 2003; Jung & Blaschke, 2004; Érsek & Nagy 2008; Adams *et al.*, 2009; Černý & Strnadová, 2010; Solla *et al.*, 2010; Varela *et al.*, 2010; Aguayo *et al*., 2014).

Alder decline has recently become an important problem in Spain because of the rapid spreading of the pathogen. The riparian alder decline and mortality in northern Spain was firstly observed by Tuset *et al.* (2006), and later was associated to *P. alni ssp. alni* (*Phytophthora xalni*) as the main pathogen agent (Solla *et al.*, 2010; Varela *et al.*, 2010; Haque & Diez, 2012).

The spreading way of alder *Phytophthora* may be related to its introduction with the planting materials, which may become infected in nurseries where the cross infection between different hosts is frequent due to asymptomatic infections (Santini *et al*., 2003; Jung & Blaschke, 2004).

Alders could become infected in the nursery either by watering with contaminated river water or through contact with already infected material. According to this, alder population may be in danger as they co-exist with hardwoods and ornamental woody species contaminated by *Phytophthora* in the same nurseries. Therefore, container grown seedlings in nurseries may act as a major carrier facilitating further dispersion of *Phytophthora* to the natural ecosystems (Jung & Blaschke, 2004; Webber *et al.*, 2004; Adams *et al.*, 2009; Haque & Diez, 2012).

Regarding physiological aspects, *Phytophthora xalni* produces non-caducous and non-papillate sporangia. Sporangial shape varies from ovoid to ellipsoid. Furthermore, it produces ornamented oogonia and elongated amphigynous antheridia (see Figure 3) (Brasier *et al.*, 2004; Haque *et al.*, 2015).

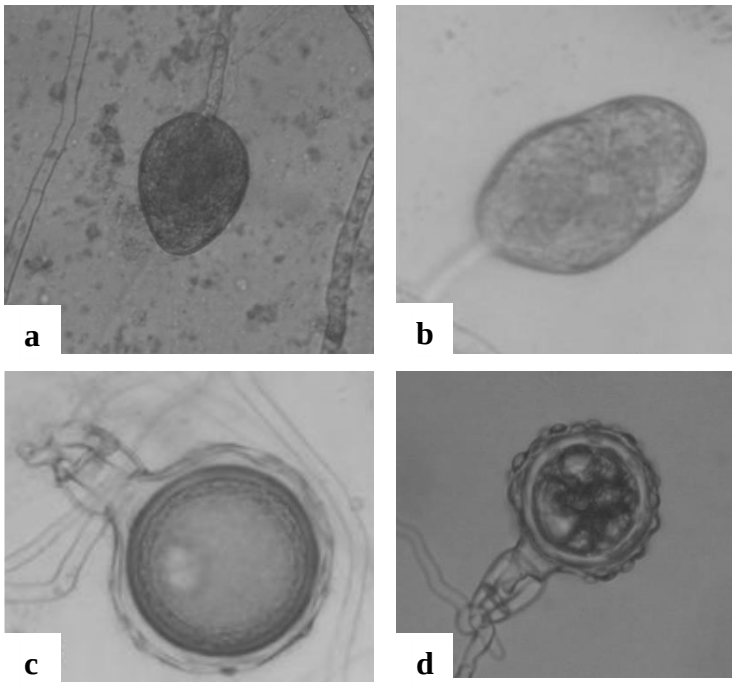


Figure 3 – Morphological sexual structures of *P. alni ssp. Alni*. **a**. ovoid shape non-papillate sporangium. **b.** Ellipsoid, non-papillate sporangium. **c.** ornamented oogonium with two-celled amphigynous antheridia. **d.** ornamented ooginium with aborted oospores (Courtesy of Masum Haque).

Besides *P. alni ssp. alni*, another *Phytophthora* was recovered recently from necrotic bark at lower stems of *A. glutinosa* in Spain. The ITS region of rDNA was amplified, sequenced and compared with the reference sequence of *P. plurivora* showing 100% identity with it. Pathogenicity test were performed using *P. plurivora* isolates inoculated on seedlings of *A. glutinosa* and after three months of inoculation, all inoculated plants wilted and died (Haque *et al.*, 2014).

Phytophthora plurivora is a hemibiotrophic root pathogen with worldwide distribution, attacking mainly woody plant species, as *Fraxinus excelsior* (explain in detail in next section) and others (i.e *Fagus sylvatica, Quercus robur)* (Mrázková *et al*., 2013; Jankowiak *et al*., 2014). *P. plurivora* shows homothallic, paragynous, antheridia and usually globose oogonia. Sporangia are semi-papillate, non-caducous, less frequently bi- or tripapillate. They show more frequently an ovoid or limoniform shape (see Figure 4) (Jung & Burgess, 2009; Dalio, 2013). Like *P. alni*, *P. plurivora* does not produce chlamydospores (Jung & Burgess, 2009). Schoebel *et al.* (2014) concluded in

their study that *P. plurivora* was most likely introduced from Europe (Belgium and the Netherlands) to the US. In Europe, *P. plurivora* may have primarily been spread from German nurseries. Moreover, the lack of geographic structuring in *P. plurivora* sampled in this study reflected the importance of plant international trade for the dispersal of this pathogen.

P. plurivora has been reported to cause several severe plant diseases such: collar rots, bark cankers, extensive fine root losses or dieback of crowns on young and mature of hundreds of tree and shrub species (Jung *et al*., 2005; Orlikowski *et al*., 2011). Jung & Burgess (2009) described that some *P. citricola* isolates from different plant hosts around the world, represented morphologically similar but genetically distinct species. Therefore, many of these strains have been classified as belonging to a new species, *Phytophthora plurivora* Jung & Burgess.

Due to its wide host range, its high aggressiveness to major native tree species and the involvement in several widespread, devastating tree declines, *P. plurivora* (possibly together with *Phytophthora cambivora* and *Phytophthora cinnamomi*), is presently the most threatening *Phytophthora* species (Jung & Burgess, 2009).

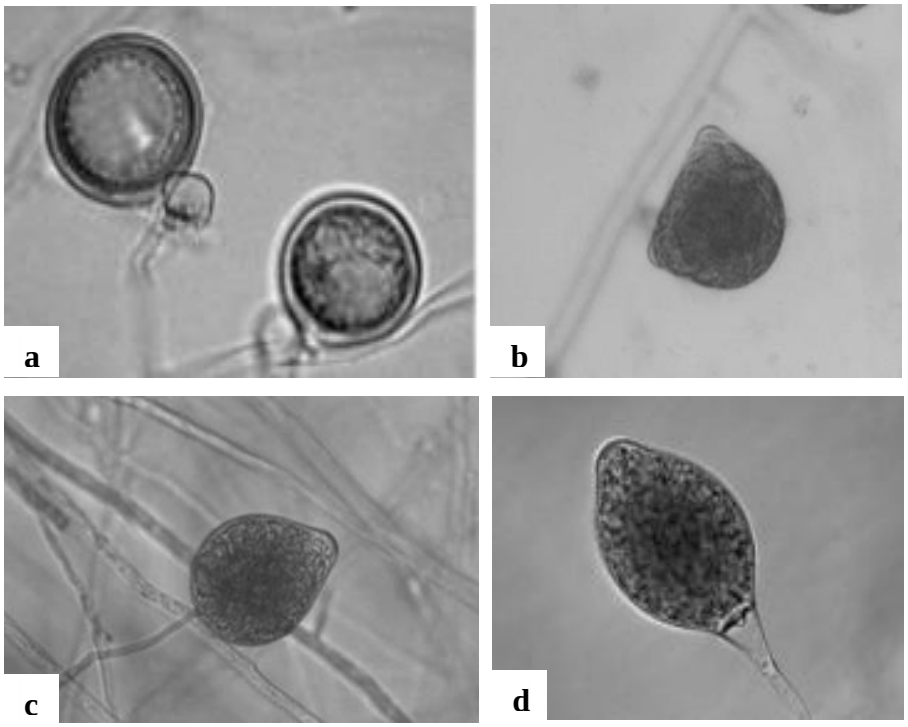


Figure 4 – Reproductive structure (asexual and sexual) of *P. plurivora*. **a.** Mature oogonia with slightly aplerotic oospore and paragynous antheridium (Source: Jung & Burgess, 2009). **b.** Sporangium non-caducous, semipapillate, bipapillate (Courtesy of Masum Heque). **c.** Sporangium non-caducous, semipapillate, ovoid (Courtesy of Masum Heque). **d.** Sporangium non-caducous, semipapillate, limoniform (Source: Jung & Burgess, 2009).

Susceptibility of *Fraxinus excelsior* to *Phytophthora plurivora*

Until now there have been only few reports on the occurrence of *Phytophthora* spp. on *F. excelsior* (Przybyl, 2002). Plus it has usually been considered as relatively resistant towards *Phytophthora* species (Jung & Nechwatal, 2008). However, *F. excelsior* seedlings were observed in forest nurseries in Poland (Orlikowski *et al*., 2004). From the necrotic tissues, a *Phytophthora* species was isolated being firstly identified as *Phytophthora citricola* (Orlikowski *et al*., 2011) and further as *P. plurivora* (Jung & Burgess, 2009). Pathogenicity of *P. plurivora* to young ash trees was demonstrated (Orlikowski *et al*., 2004; Orlikowski *et al*., 2011). However, in Jung & Nechwatal (2008) study showed opposite results, which may indicate significant levels of within-species variation in pathogenicity or host specificity of different *P. plurivora* isolates that was supported by Orlikowski *et al*. (2011) study.

Isolates of *Phytophthora* spp. in necrotic tissue and rhizosphere of declining ash trees in several stands in Poland and Denmark was also been obtained by Orlikowski *et al*. (2011), suggesting an involvement of these pathogens in ash decline in these stands.

It is probable that roots and collar infections by *Phytohpthora* species weaken trees by driving them to allocate resources into the replacement of lost fine roots and defence reactions against bark infections. Therefore, affected trees most probably have reduced resources for defence against *Chalara fraxinea* infections (a fungal pathogen of *F. excelsior*), and this might contribute to the rapid mortality of ash trees observed in eastern and central Europe (Orlikowski *et al*., 2011). In oak decline in Europe, a similar interaction with defoliating insects has been suggested (Jung *et al*., 2000).

Genus *Alnus*

Genus *Alnus* belongs to the birch family (Betulaceae) and in Europe there are four native species: *Alnus glutinosa* (common alder or black alder); *Alnus incana* (grey alder); *Alnus cordata* (Italian alder), native from Southern Italy and Corsica; and *Alnus viridis* (green alder), a shrub species found in mountainous to subalpine altitudes. In general, species of the genus *Alnus* are able to colonize bare areas (pioneer species), not very tolerant to shade; it grows on wet and clay soils, settling in riparian or marshy areas. The roots have specialized nodules which fix atmospheric nitrogen as a result of a symbiotic association with Actinomycete *Frankia*. Consequently these species can grow on poor soils with low nutrients status (Cech, 1998; Jung & Blaschke, 2004; Webber *et al.*, 2004; Adams *et al.*, 2009).

Common alder and grey alder in particular have considerable landscape value and are widely used in watersides reforestation; they play a vital role in habitat restoration, as shading and

cooling streams, thereby improving fish habitat and their root system helps to stabilize riverbanks. More rarely, they can be found in pure stands, which in good conditions can grows as fast as ash (*Fraxinus spp.*), maple (*Acer spp.*) or cherry (*Prunus spp.*) and its wood is equally with high quality (Cech, 1998; Jung & Blaschke, 2004; Webber *et al.*, 2004; Thoirain *et al.*, 2007; Adams *et al.*, 2009; Claessens *et al.*, 2010).

A. glutinosa is native to Eurasia and North Africa. More specifically, common alder occurs throughout most of Europe and across Russia to Siberia. Its range also includes the Caucasus, Iran, Turkey, and in North Africa is native to Tunisia, Algeria and Morocco (Featherstone, 2014). In Iberian Peninsula, its presence is very common when the climate is sufficiently humid. This climatic condition and its affinity to substrates with low carbonates explain the preferred distribution in Iberian Peninsula: western peninsula and transition areas Mediterranean-Atlantic (see Figure 5) (Blanco *et al*., 2005).

It can grow up to 20-30 meters high, and on the best sites can reach 35 meters. *A. glutinosa* is fast growing species and can grow in a wide range of soils, either acidic or basic, but preferring wet and clay soils with pH ranging 4.2 to 7.5; this trait increases its invasiveness in susceptible environments. However, it has a relatively short life span up to 160 years.

Leaves are simple and alternate. Common alder is deciduous, and the new leaves open out in April. Flowers appear before the emergence of leaves in early spring. *A. glutinosa* is monoecious. Male catkins are dark yellow-brown in color, while female flowers are brown cone-like catkins. Fruit form in autumn, as ovoid catkins. The fruits grow in clusters of up to 4 at the end of twigs. These ripen by October and release many small nutlets. The seeds have no wings; therefore, despite their small size they are usually not spread more than 30 to 60 m by the wind. The seeds contain an air bladder and float in water, and McVean (1955) holds that rather than wind, running water and wind drift over standing water are the major agents of dispersal. *A. glutinosa* is virtually self-sterile. The root system is nodulated and dense, and can be fairly shallow (McVean, 1955; Claessens *et al.*, 2010; Featherstone, 2014).

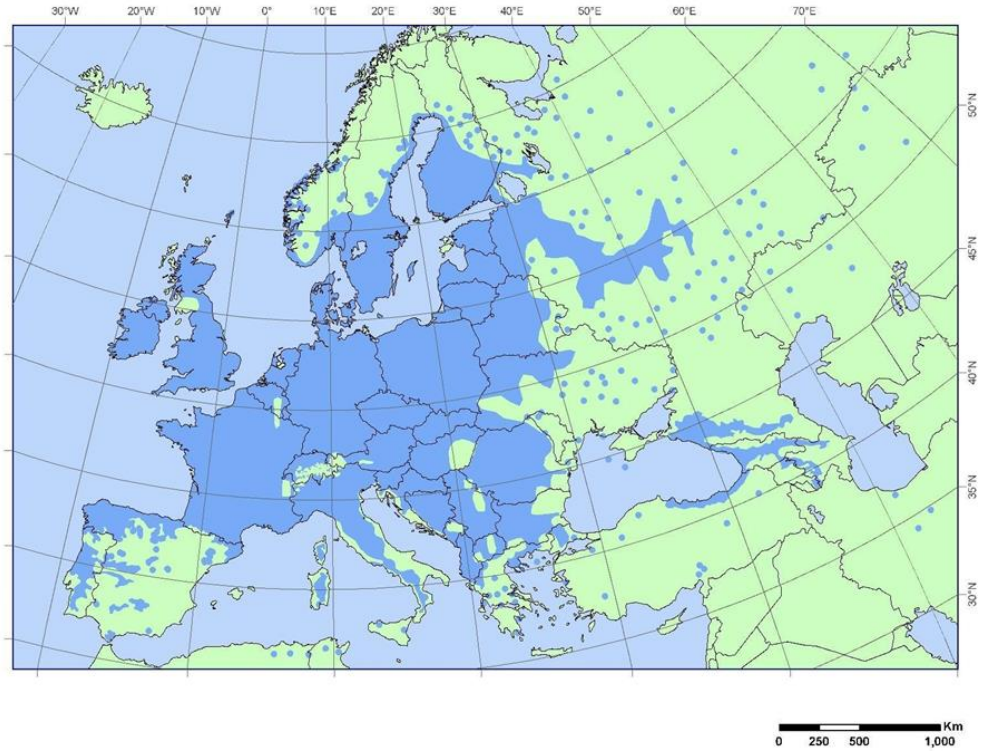


Figure 5 - Native distribution of *Alnus glutinosa* (Source: EUFORGEN, 2009).

Genus Fraxinus

Genus *Fraxinus* belongs to Oleaceae family and in Europe there are 3 native species: *Fraxinus excelsior* (common ash or European ash); *Fraxinus angustifolia* (narrow-leaved ash); and *Fraxinus ornus* (manna ash). *F. excelsior* and *F. angustifolia* are both large wind-pollinated trees, whereas *F. ornus*, with its white inflorescences, attract insect pollinators. Manna ash is more closely related to Asian species of *Fraxinus* than to the other European species (Wallander, 2001; FRAXIGEN, 2005). Common ash and narrow-leaved ash are very closely related, and have a big similarity that sometimes they are difficult to distinguish, especially when they are in mixed stands (FRAXIGEN, 2005; Wallander, 2008). Also there are cases of hybridization in areas where they occur together (i.e. Balkans, Italy, and southern France) (Fernandez-Manjarres, *et al*., 2006; Heuertz *et al*., 2006).

Until now, common ash and narrow-leaved ash have been widely planted for timber production throughout Europe, because its fast-growth and high quality timber when in optimal conditions. However, there has been an increased recognition of the importance of managing forests for a

long-term sustainability, conservation of biodiversity and ecological restoration (FRAXIGEN, 2005; Pautasso *et al*., 2013).

F. excelsior is common throughout Europe except for central and southern parts of the Iberian Peninsula, south-east Turkey, northern Scandinavia and Iceland (see Figure 6). In the northern and western parts of its natural range, European ash grows in lowland forests, while in central and southern Europe, it occurs in mountainous areas at altitudes of up to 1600–1800 m and in northern Iran up to 2200 m (FRAXIGEN, 2005). It plays an important role in both primary and secondary succession. In Europe, It often occurs in mixed broadleaved forest or as a component of forests dominated by *Fagus sylvatica* (European beech), *Quercus petraea* (sessile oak), *Quercus robur* (pedunculate oak), *Acer pseudoplatanus* (sycamore), and *Alnus glutinosa* (black alder) or *Alnus incana* (grey alder) (Dobrowolska *et al*., 2011).

European ash grows on a wide range of site types, except on acidic soils, but dominates mainly on sites that are unfavorable for beech, oak and, in some cases, alder (Dobrowolska *et al*., 2011). It has been suggested as a very adaptable hardwood, being able to grow well due to a high tolerance to water and nutrient supply (Kerr & Cahalan, 2004). In eastern parts of its natural range, ash may become a dominant species in marshy sites and on moist clay-loam lowland areas (Dufour & Piegay, 2008). On the contrary, European ash may also be a dominant species in relatively dry sites, typically on stony mountain slopes (Weber-Blaschke *et al.*, 2008). In central parts of Europe and at the northern limit of its natural range, ash may dominate on relatively dry calcareous sites (Dobrowolska *et al*., 2011). For optimal conditions, European ash grow better in calcareous or sedimentary parent material soils, rich in clay or silt (pH 5-8), moist and well drained, having high contents of available nitrogen and phosphorus (FRAXIGEN, 2005).

Specifically in Spain, *F. excelsior* appears with *A. glutinosa* and other tree species (i.e *Salix* spp., *Populus tremula*, *Betula alba*, *Acer pseudoplatanus*), in altitude higher than 1200 meters, creating riparian forests with a high ecological importance (Blanco *et al*., 2005). In fact, in EU Council Directive 92/43/EEC on the conservation of natural habitats and wild fauna and flora, riparian forests, and particularly Alder forests with European ash, are classified as priority habitat (*i.e* Alno-Padion, Alnion incanae, Salicion albae).

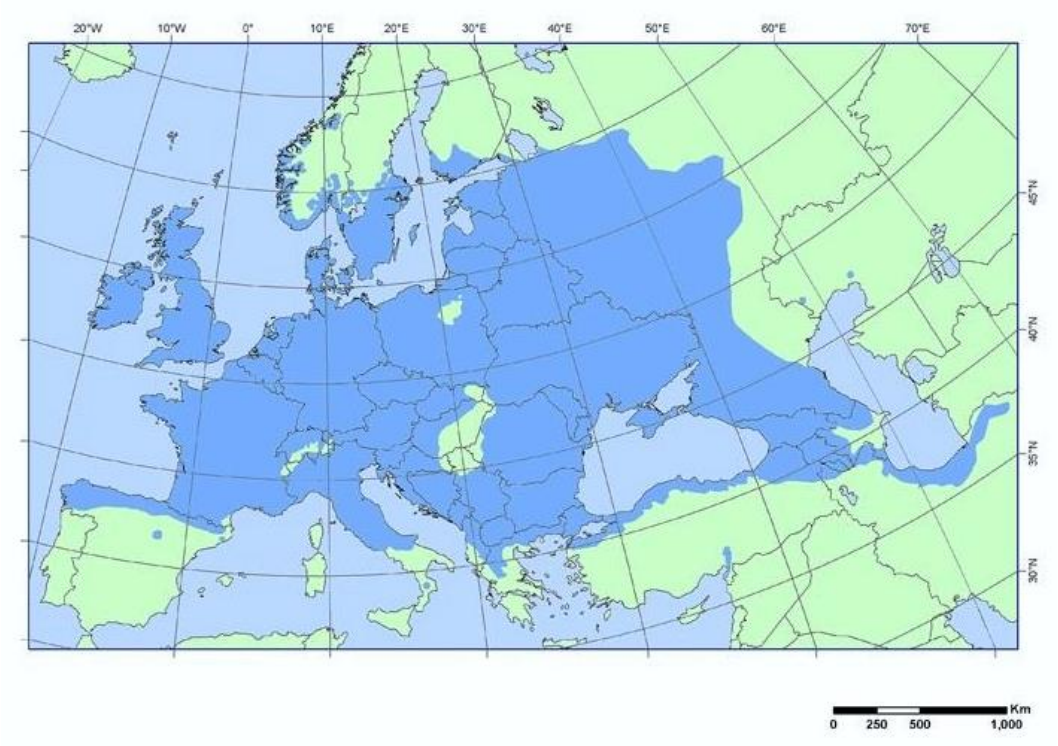


Figure 6 - Natural distribution of *Fraxinus excelsior* (Source: EUFORGEN, 2009).

F. excelsior it is a large tree, growing up to 20-30 meters high rarely exceeding 250 years old (Wallander, 2001; Pautasso *et al*., 2013). Leaves are pinnate compound, with 7-13 leaflets (Leugnerová, 2008). It is a wind-pollinated tree with small, simple flowers lacking nectar, scent and petals. The flowers may be: male, female, or hermaphrodite. Female and hermaphrodite flowers can together be called pistillate flowers because they both have a functional pistil containing an ovary. The ovary contains four ovules but only one of these is usually fertilized, resulting in a one-seeded fruit. The fruit (samara) has a flattened wing facilitating dispersal by wind and watercourses. Many pistillate flowers occur together in inflorescences; containing around 100 flowers while male inflorescences contain at least twice as many pistillate flowers, on average 200-400 (see Figure 4). Both male and female flowers can occur on the same tree; however it is more common to find pure male and pure female individuals. Moreover a tree that is male one year can produce female flowers the next one, and vice versa. The inflorescences emerge March to April from lateral buds on the shoot from the previous year. Leafing (or flushing) occurs after flowering has finished, on the shoots that emerge from the terminal buds.

The pistillate flowers frequently have male parts that are reduced in size and function. Pistillate flowers with functional anthers can potentially father seeds by self-fertile or outcrossing to other trees (FRAXIGEN, 2005; Dacasa-Rüdinger *et al*., 2008; Wallander, 2008).
Compared with riparian trees such as *Populus spp*. (poplars), *Salix* spp. (willows) and *Alnus* spp. (alders), young ash seedlings have a superficial root system. In adult trees, the root system is quite regular with deeply penetrating vertical roots originating from the developed horizontal roots (Dobrowolska *et al*., 2011).

Disease Management

Despite of the *Phytophthora* species, it is not recommended to eliminate the oomycete from a site through the felling or winching out of the affected trees. On riparian sites, the disturbance created by this activity, including bringing machinery on site, may even spread the disease by allowing infective spores and fragments of the oomycete from diseased trees or soil to come into contact with healthy trees further downstream (Webber *et al.*, 2004).
Unfortunately *Phytophthora* pathogens cannot be suppressed with well-known fungicides, because as oomycetes they do not synthesize chitin and ergosterol. However, many investigations have shown that different salts of phosphoric acid, the phosphites are effective to control growth of *Phytophthora* pathogens (Jackson *et al*., 2000; Hardy *et al.*, 2001; Dalio *et al.*, 2014). Besides interfering with defence pathways of host plants (Eshraghi *et al.*, 2011), phosphite was shown to interact directly with *Phytophthora* pathogens (Jackson *et al.*, 2000). However, Wilkinson *et al.* (2001) showed that phosphite treatment reduced infection but did not prevent the production of viable zoospores on infected trees. So they concluded that phosphite application lower the amount of infection by *Phytophthoras,* but may not remove the risk of *Phytophthora* spreading from already infected trees.
In order to reduce the spread of *Phytophthora* diseases in plantations and nurseries, the following measures could be adapted (Jung & Blaschke, 2004; Webber *et al*., 2004; Haque & Diez, 2014): a) careful selection of plant material free of diseases; b) avoidance of frequent seedling transportation with soils from nurseries to planting sites; c) assessment of plant health growing season in nurseries at a regular interval; d) inspection and testing of nursery soil and water reservoir used to irrigate nurseries; e) Routine disinfection of nursery tools before performing silvicultural treatments; f) planting susceptible trees in stands where nursery plants have not been planted for a long period of time; and g) development of molecular based detection protocol for a rapid and effective identification of *Phytophthora* species.

Coppicing encourages the regeneration of new growth, especially if the tree has a diseased root system that can no longer support the entire crown. It also prevents diseased trees from becoming unstable and causing damage to the riverbank. Studies on the potential for *Phytophthora* disease management through the coppicing of affected alder trees were showed by Gibbs (2003). Natural riparian alder stands in the UK appeared with high sprouting rates and low re-infections after coppicing. Besides these results, coppicing was not recommended for plantations infected with *P. alni* by Jung & Blashcke (2004). However, these authors state that coppicing infected trees and shoots can be a short-term control measurement, but in the long term, a variety of resistant genotype is needed in order to sustain riparian alder stands.

So there is another possible approach of managing the disease and is the selection of native tree populations for naturally occurring resistance, using a series of artificial infection experiments, and the subsequent breeding and broad use of these resistant genotypes (Štochlová *et al*., 2012). This thought was disproved by Gibbs (2003), who carried out field and laboratory experiments with young alder plants from different European provenances. However, healthy mature alder trees can be found in the same stand as both diseased and dead trees (Streito *et al.*, 2002; Jung & Blaschke, 2004; Štochlová *et al*., 2012). Consequently, a programme was started in Germany to select and breed for resistant alders (Jung & Blaschke, 2004). Positive results of a selection breeding programme was recently demonstrated for *Eucalyptus marginata* and *Phytophthora cinnamomi* (Hüberli *et al.*, 2003), and for *Chamaecyparis lawsoniana* and *Phytophthora lateralis* (Sniezko & Hansen, 2000; Sniezko *et al*., 2003; Oh *et al.*, 2006).

OBJECTIVES

The main aim of study was to describe the susceptibility growth of Common alder and European ash from natural riparian populations in Northern Spain, using *P. alni ssp. alni* and *P. plurivora* isolates recovered also from Northern Spain.

The Experiment I aimed at determining the pathogenicity of *Phytophthora* species in European ash, in order to evaluate the risk that these pathogens may affect natural riparian ecosystems, since European ash and Common alder occur together in Northern Spain, so that cross-infection in riparian populations and nurseries may be avoided.

More specifically, the objectives were:

- To know the effect of soil infestation with *P. plurivora* and *P. alni* on European Ash;
- To know the effect of stem inoculation with *P. plurivora* and *P. alni* on European Ash;

The Experiment II aimed at testing the hypothesis that Common alders from different provenances/populations have different resistances towards *Phytophthora plurivora* and in future could be an alternative approach to manage the disease in Northern Spain.

More specifically, the objective was:

- To evaluate the effect of soil infestation with *P. plurivora* on different Common alder provenances;

MATERIAL & METHODS

Experiment I

Plant Material

Fraxinus excelsior seedlings with 1-year-old were produced in Central Nursery of the Government of Castilla y León (Spain). These seedlings are originally from Castilla y León region, Spain.

Phytophthora isolates and inoculum production

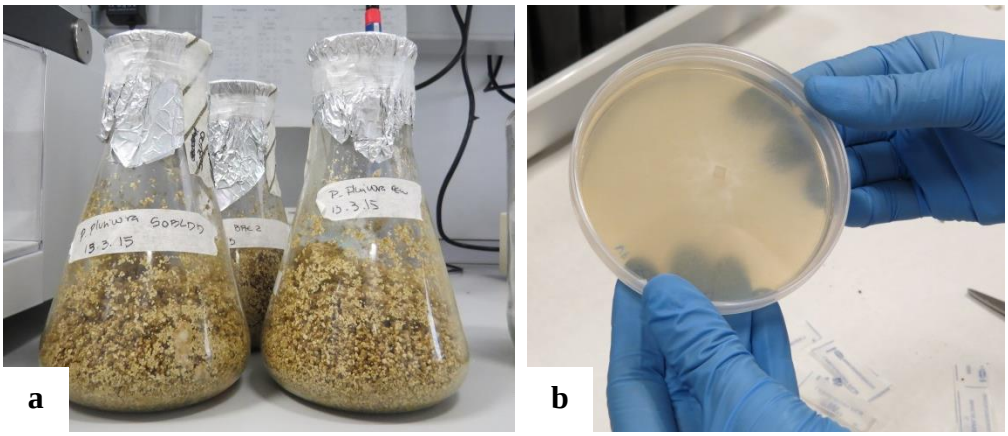
Two isolates of *P. plurivora* and one isolate of *P. alni ssp. alni* recovered in 2012 by Laboratorio de Plagas y Enfermedades Forestales, Campus de Palencia, Universidad de Valladolid [Laboratory of Forestry Pests and Diseases, Campus Palencia, University of Valladolid] (Palencia, Spain) from diseased *A. glutinosa* trees growing on banks of river Tera, Tormes and Esla (Spain), respectively, were used on soil pathogenicity tests (see Table 2 and 3). For soil infestation essay, individual isolates of *Phytophthora* spp. were transferred to grow at 20ºC in 500 ml Erlenmeyer flasks on an autoclaved mixture of 250 ml of fine vermiculite and 20 ml of whole oat-grains with 175 ml of Vegetable Juice broth (200 ml/l Vegetable Juice Hipercos products, 800 ml/l distilled water, 3 g/l $CaCO_3$) for 4 to 6 weeks (Jung *et al*., 1996) (see Figure 7a). For stem inoculation essay, colonies of *P. plurivora* and *P. alni ssp. alni* were sub-cultured for 1 week at 20ºC in the dark onto 90 mm Petri dishes containing sterilized V8 agar (V8 agar: clarified 100 ml/l Vegetable Juice Hipercos products, 2 g/l $CaCO_3$, 16 g/l Agar Technical DIFCO, Detroit, MI, USA) (see Figure 7b).

Table 1 - *Phytophthora spp.* used in pathogenicity tests.

Specie	Code	Recovery Year	Host	Country	GenBank Accession Nº
Phytophthora alni alni	ZABRE02	2012	*Alnus glutinosa*	Spain	-
Phytophthora plurivora	SORLDD14	2012	*Alnus glutinosa*	Spain	KF413075
Phytophthora plurivora	LAMASA1	2012	*Alnus glutinosa*	Spain	KF413074

Table 2 - Specific locations of *Phytophthora spp.* recovered in Spain.

Code	Location	River	Latitude	Longitude
ZABRE02	Betrocino, Zamora	Esla	41°54'48"N	5°44'45.6"W
SORLDD14	Langa de Duero, Soria	Tera	41°36′34″N	3°25′10″W
LAMASA1	La Maya, Salamanca	Tormes	40°41′42″N	5°35′36″W



a **b**

Figure 7 – Different *Phytophthora* spp. inoculum production. **a.** *Phytophthora* spp. growing on a mixture of fine vermiculite and whole oat-grains with a Vegetable Juice broth, used in soil infestation essay. **b.** *Phytophthora* spp. growing on a petri dish with V8 agar, used in stem inoculation essay.

Experimental design

The essays were conducted in forestry containers with individual cells. Each cell had the capacity of 250cm^3 with disease free peat. These containers were placed on trays to storage infested drained water. Both essays took place in April 2015 and kept in room temperature with natural light.

Soil Infestation essay: Inocula broth consisting of 5 weeks old were added on the soil surface (2% of the total cell volume) and then covered with sterilized peat (see Figure 8). 13 seedlings of *F. excelsior* were infested by each *Phytophthora* isolate. Sterile vegetable juice broth was added to 11 cells containing the control plants. To stimulate zoospore release and disease development, the forestry containers were flooded up to 1 cm above soil level for 48 h once every 2 weeks (Jung *et al*., 1996; Santini *et al.,* 2003). After each flooding treatment, the water was removed and decontaminated. The flooding process was repeated four times.

Stem inoculation essay: 13 seedlings of *F. excelsior* were inoculated by each *Phytophthora* isolate, except for *Phytophthora alni* that were used 15 replicates. An U-shaped wound was cut in the bark at the collar level using a sterile scalpel. A mycelial plug from the margin of a growing colony of *Phytophthora* was introduced into the wound, which was then sealed with Parafilm® (American National Can Co., Neenah, USA) (see Figure 9). Controls (13 seedlings of *F. excelsior*) received only sterile V8 agar plugs (Santini *et al.*, 2003). Plants were watered twice per week.

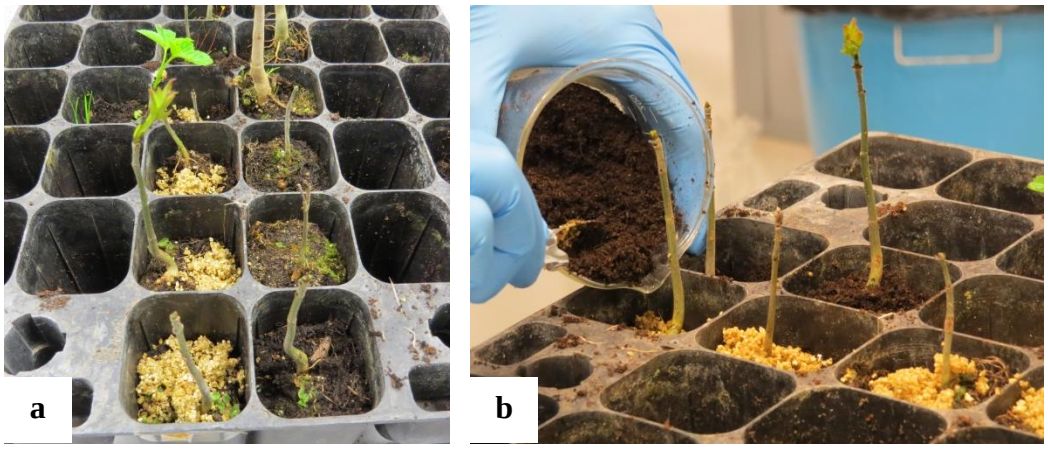


Figure 8 – Soil infestation process. **a.** Inocula broth added into the soil surface. **b.** Afterwards it was covered with peat.

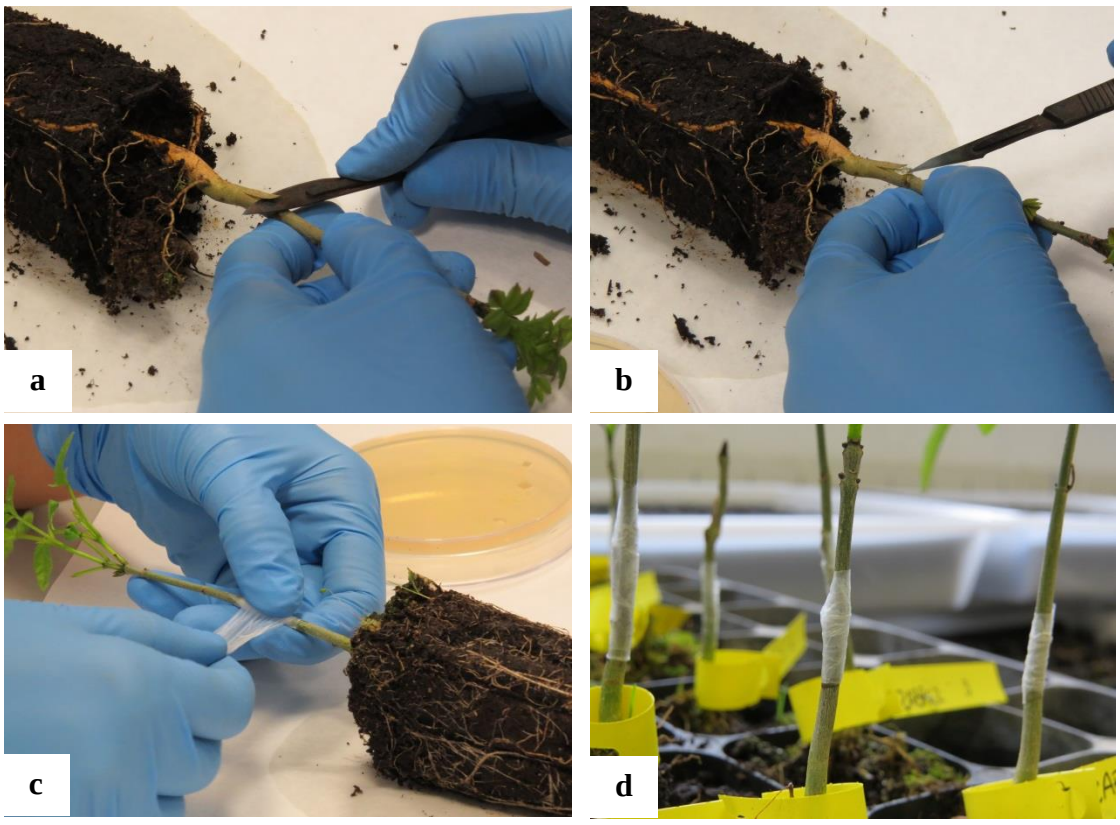


Figure 9 – Stem inoculation essay showing all the steps. **a.** A U-shaped cut in the bark at the collar level using a sterile scalpel. **b**. A mycelial plug from the margin of a growing colony of *Phytophthora* was introduced into the wound. **c.** Afterwards it was sealed with Parafilm®. **d.** An image showing the final result.

Damage assessment

The development of symptoms was recorded weekly during the entire test period (from 21 April to 23 June) of both essays. The seedling conditions were summarized in a damage index followed by Santini *et al.* (2006), that was based mainly on foliar symptoms scored with a 6-point scoring system in which: 0 = healthy seedling; 1 = seedling with 10–25% yellowing/wilting; 2 = 25–50% yellowing/wilting; 3 = 50–75%; 4 = 75–100%; and 5 = dead seedling. Also the seedling growth was taken into account, recording weekly the height of each replicate. Consequently, the relative height growth was obtained using the following formula:

$$Relative\ height\ growth = \frac{Xf - Xi}{Xi}$$

Where: Xi: Initial height; Xf: Final height.

For stem inoculation essay, the damage of each plant was also assessed by measuring the length of the stem lesion (Santini *et al.,* 2006). Moreover, to obtain all seedlings dried weight, they were dried at 75ºC for 24h (dried weight was constant at this point). The weight was divided as: aerial part, radical root and secondary roots.

Finally, the re-isolation of the pathogen was performed. For stem inoculation, it was cultivated affected bark in selective media V8-PARPH (V8-PARPH: clarified 100 ml/l Vegetable Juice Hipercos products; 2 g/l CaCO3; 16 g/l Agar Technical AppliChem GmbH, Darmstadt, Germany; 0.4 ml/l Pimaricin; 0.25 g/L Ampicillin; 0.01 g/ml Rifamycin; 0.07 ml/l Hymexazol; 5 ml/l of alcohol diluted with 0.5% of PCNB) and incubated at 20ºC in the dark. For soil infestation, it was performed the re-isolation of the pathogen through soil using vegetal baiting. It was used 400 ml of each soil sample, and then flooded with distilled water. The leaflets used as baiting were from *Quercus robur*, *Quercus ilex* and *Prunus avium*. Leaflets showing necrosis were cut and plated onto selective media V8-PARPH and incubated as well at 20ºC in the dark (see Figure 10). Then, it was possible to identify the pathogen, observing its sexual structures.

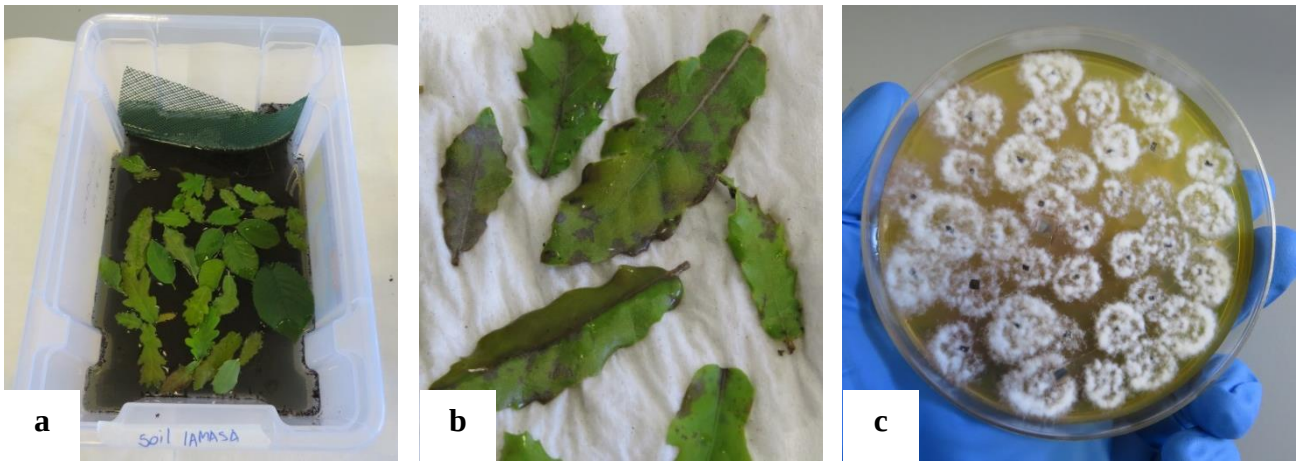


Figure 10 – Re-isolation of *Phytophthora* spp. through soil using vegetal baiting. **a.** This method consisted in using plastic boxes (one for each soil sample), previously disinfected with 70% alcohol, containing 400 ml of soil sample. Then, it was flooded with distilled water until reaches 3cm high. After 2 hours, it was placed a fine grid to join the water impurities. Subsequently it was collected susceptible leaflets and placed on water surface with the leaves underside turned down, covering the entire water surface. **b.** After 3 days, the leaflets that show typical *Phytophthora* necrosis are collected. **c.** Leaflets showing necrosis were cut into small pieces and plated onto selective media V8-PARPH. The image shows *Phytophthora plurivora* mycelial growth from the leaflets with 5 days old.

Statistical analysis

Before performing any statistical analysis, dried weight data was standardized, without this procedure the variables measured at different scales would not have contributed equally to the analysis. Because the initial height was different between replicates, dried weight was standardized using this formula:

$$Standardized\ dried\ weight = \frac{Wf}{Xi}$$

Where: Wf: Final dried weight; Xi: Initial height.

Normality was tested in all variables. The non-normal data was transformed to normal using Tukeys ladder of transformations, giving several common transformations to correct upward and downward skew (i.e. log(x); $\sqrt{x}$; x^2; x^3; antilog(x)). Then, it was tested variance homogeneity, carrying out Levene's test. For relative height growth and standardized dried weight were performed Analysis of variance (ANOVAs) and multiple comparison procedures to test the effects of *P. plurivora* and *P. alni* inoculations on height growth and weight. These procedures were carried out in IBM SPSS Statistics 20 program.

For necrosis length, analysis of variance (ANOVAs) and multiple comparison procedures were performed to test the effects of *P. plurivora* and *P. alni* inoculations on necrosis length. As the data violated two of the ANOVA assumptions (normality and homogeneity of variances) and this particular variable presented many zeros, robust statistical methods were applied. In particular, heteroscedastic one-way ANOVA was performed using the generalized Welch procedure and a 0.1 trimmed mean transformation. This ANOVA was carried out using the "Wilcox' Robust Statistics (WRS)" package (Wilcox & Schönbrodt, 2014) implemented in the R software environment.

Experiment II

Plant Material

Common alder free of disease seeds, from different provenances (see Table 3), were supplied by the Central Nursery of the Government of Castilla y León (Spain) in order to be used in the experiments. Before use, seeds were cleaned as following: firstly they were dipped into hydrogen peroxide (3%) for 30 minutes; then washed twice with sterilized distilled water to remove excess of hydrogen peroxide and finally they were dried, rested and stored at 4ºC until starting with the pre-chill procedure.

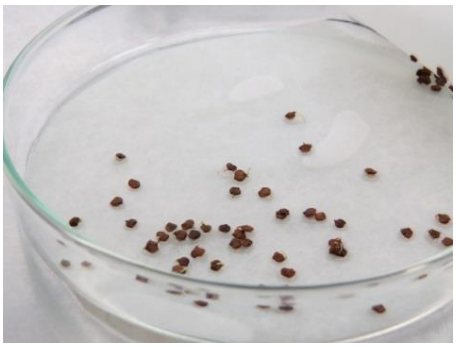


Figure 11 – Alder seeds showing radical root after 3 days in growth chamber.

Seeds from the six provenances were placed on top of moist sterilized filter paper (ANOIA Barcelona, 73 grade) in sterilized glass petri dishes, sealed with Parafilm® (American National Can Co., Neenah, USA), to avoid major contaminations, and pre-chilled for 9 to 19 days at 4ºC, before transfer to germination conditions. Afterwards, all petri dishes were kept into growth chamber at 25ºC and photoperiod (day/night 16h/8h) to promote germination (see Figure 11) (adapted from Gosling *et al*., 2009). Some petri dishes were transferred directly to the growth chamber with the same conditions mentioned above.

Table 3 - Seed source of *Alnus glutinosa* used in the experiment.

Code	Specie	Provenance
C467	*Alnus glutinosa*	Merindad de Valdeporres, Burgos
C683	*Alnus glutinosa*	Rebollar, Salamanca
C737	*Alnus glutinosa*	Gallegos del Río/Vegalatrave, Zamora
C773	*Alnus glutinosa*	Arenas de San Pedro, Ávila
C846	*Alnus glutinosa*	Vegas del Condado, Léon

Phytophthora isolates and inoculum production

Two isolates of *P. plurivora* recovered in 2012 by Laboratorio de Plagas y Enfermedades Forestales, Campus de Palencia, Universidad de Valladolid [Laboratory of Forestry Pests and Diseases, Campus Palencia, University of Valladolid] (Palencia, Spain) from diseased *A. glutinosa* trees growing on banks of river Tera and Tormes (Spain), respectively, were used on soil pathogenicity test (see Table 1 and 2). Colonies of *P. plurivora* were sub-cultured for 1 weeks at 20ºC in the dark onto 90 mm Petri dishes containing sterilized V8 agar (V8 agar: clarified 100 ml/l Vegetable Juice Hipercos products; 2 g/l $CaCO_3$; 16 g/l Agar Technical AppliChem GmbH, Darmstadt, Germany).

Sporangia were obtained by placing 7-10 plugs of young actively-growing *P. plurivora* into 90 mm petri dish and flooded with distilled water (15 ml per petri dish). Petri dishes were kept for 2-3 days into a growth chamber at 25ºC and photoperiod (day/night 16h/8h). The distilled water was renewed after 48hours. Once sporangia have formed, petri dishes were placed in a refrigerator at 4ºC for 1h, and then returned to growth chamber (25ºC) during another 1-2h to promote zoospore releasing. Zoospore concentration was determined by using a haemocytometer (adapted from Jeffers, 2006).

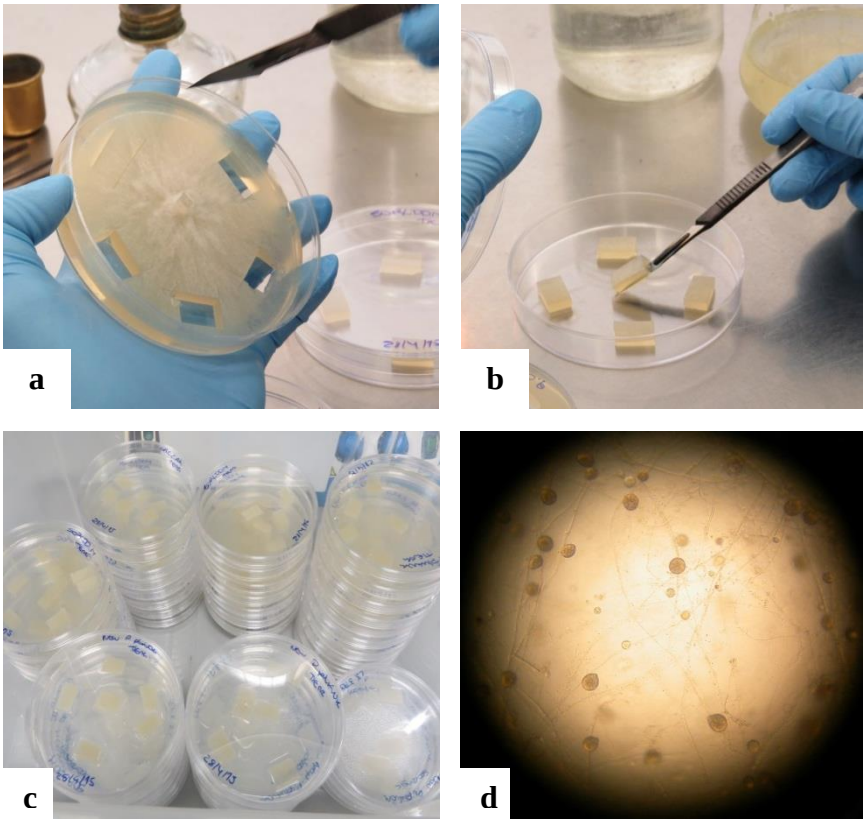


Figure 12 – *P. plurivora* sporagia production. 7-10 plugs of young actively-growing *P. plurivora* (**a**) are placed into 90 mm petri dish (**b**) and flooded with distilled water (**c**). After 2-3 days, it is possible to observe from the microscope mature sporangia (100x) (**d**).

Soil pathogenicity test

The experiment was conducted in plastic containers with individual cells. Each cell had the capacity of 43 cm^3. These containers were placed on trays to storage infested drained water. Germinated seeds, showing a healthy radical root, were sowed in individual cells containing non-infested, autoclaved soil mixture (1:2, v/v) of sand and peat (see Figure 13a). Alder replicates, with 3 months old, were used in the experiment (see Table 4 and Figure 13b). The experiment took place in June 2015 and kept in a growth chamber at 25ºC and photoperiod (day/night 16h/8h). In the beginning of the pathogenicity test (day 0), replicates were infested with 10^3 zoospores/ ml, after 10 days (day 10) were infested with 10^4 zoospores/ml, and finally after another 10 days (day 20) were infested with 10^5 and 10^4 zoospores/ml, LAMASA1 and SORLDD14 respectively. This last zoospore concentration added was different between isolates

due the insufficient zoospores produced in SORLDD14. This particular isolate was more difficult in stimulating zoospore release comparing with LAMASA1. 1 ml of distilled water was added in control replicates (see Figure 13c). This method had the purpose to observe foliar symptoms gradually and not brusquely during all the experiment period.

Table 4 - Alder replicates used to be infested by each *Phytophthora plurivora* isolate.

Code	Provenance	LAMASA1 replicates	SORLDD14 replicates	CONTROL replicates
C467	Merindad de Valdeporres, Burgos	21	18	22
C683	Rebollar, Salamanca	41	17	23
C737	Gallegos del Río/Vegalatrave, Zamora	31	17	16
C773	Arenas de San Pedro, Ávila	22	24	43
C846	Vegas del Condado, Léon	21	22	37

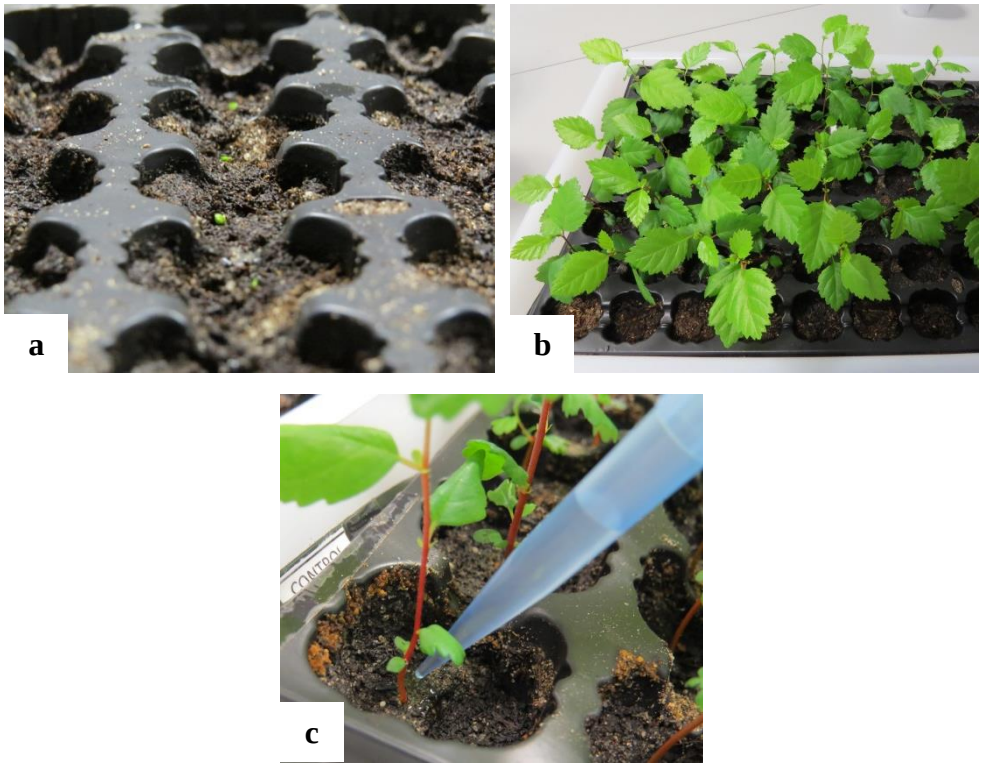


Figure 13 – Soil pathogenicity test using *P. plurivora* zoospores. **a.** Germinated alder seeds, showing a healthy radical root, were sowed in individual cells containing non-infested, autoclaved soil mixture (1:2, v/v) of sand and peat. **b.** Alder replicates, with 2 to 3 months old, were used in the experiment. **c.** Replicates were infested with different zoospores concentration in three different times (0, 10, and 20).

Damage assessment

The development of symptoms was recorded 2-3 times per week during the entire test period (from 6 June to 15 July). The plant conditions were summarized in a damage index adapted from Santini *et al*. (2006), which was based on foliar symptoms scored with a 5-point scoring system in which: 0 = healthy seedling; 1 = seedling with 25–50% yellowing/wilting; 2 = 50–75% yellowing/wilting; 3 = 75-100%; 4 = dead seedling. From these data was possible to calculate AUDPC (Area Under the Disease Progress Curve) of each seedling using the following formula:

$$AUDPC = \sum_{i}^{n-1} [\left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)]$$

Where: yi: Initial score; yi_{+1}: Final score; t_{i+1}: Final time; ti: Initial time

Also the plant height were taken into account, recording also 2-3 times per week. Therefore, the relative height growth was obtained using the following formula:

$$Relative\ height\ growth = \frac{Xf - Xi}{Xi}$$

Where: Xi: Initial height; Xf: Final height.

Finishing the pathogenicity test, the roots lengths were measured and it was also obtained the seedlings dried weight, they were dried at 60ºC for 48h (dried weight was constant at this point). The weight was divided as: aerial part and roots. Moreover, it was performed the re-isolation of the pathogen through roots (plated directly into selective media V8-PARPH) and soil using vegetal baiting as explained in Experiment I.

Statistical analyses

Before performing any statistical analysis, dried weight and root length data were standardized, without this procedure the variables measured at different scales would not have contributed equally to the analysis. Because the initial height was different between replicates, dried weight was standardized using this formula:

$$Standardized\ dried\ weight = \frac{Wf}{Xi}$$

Where: Wf: Final dried weight; Xi: Initial height.

Normality was tested in all variables. The non-normal data was transformed to achieve the normality using Tukeys ladder of transformations, giving several common transformations to correct upward and downward skew (i.e. log(x); $\sqrt{x}$; x^2; x^3; antilog(x)). Then, it was tested variance homogeneity, carrying out Levene's test. For relative height growth, standardized dried weight and root length were performed Analysis of variance (ANOVAs) and multiple comparison procedures to test the effects of *P. plurivora* inoculations on height growth, weight and root length. These procedures were carried out in IBM SPSS Statistics 20 program.

For AUDPC, no damage was recorded in most of the seedlings (only 3,68%) and therefore statistical analyses was not necessary.

RESULTS

Experiment I

Relative Height Growth

In the end of the experiment, analysis of variance (Two-way ANOVA) showed some differences in relative height growth with a confidence level of 95%. In Figure 14 shows the mean relative height growth of all isolations and control on the different treatments used in the study (Soil infestation and Stem inoculation). Regarding only stem inoculation, there were no significant differences between isolations and Control. While in soil infestation, there was only significant differences between isolation ZABRE and Control (F=5,498; P=0,028).

However, when observing the significance between treatments, there is a high significance between each other (F=32,217; P<0.001). In fact, Soil infestation showed higher values with mean relative growth of 1,211 ± 0,086 cm, while Stem inoculation only showed mean relative growth of 0,461 ± 0,083 cm (see Figure 15).

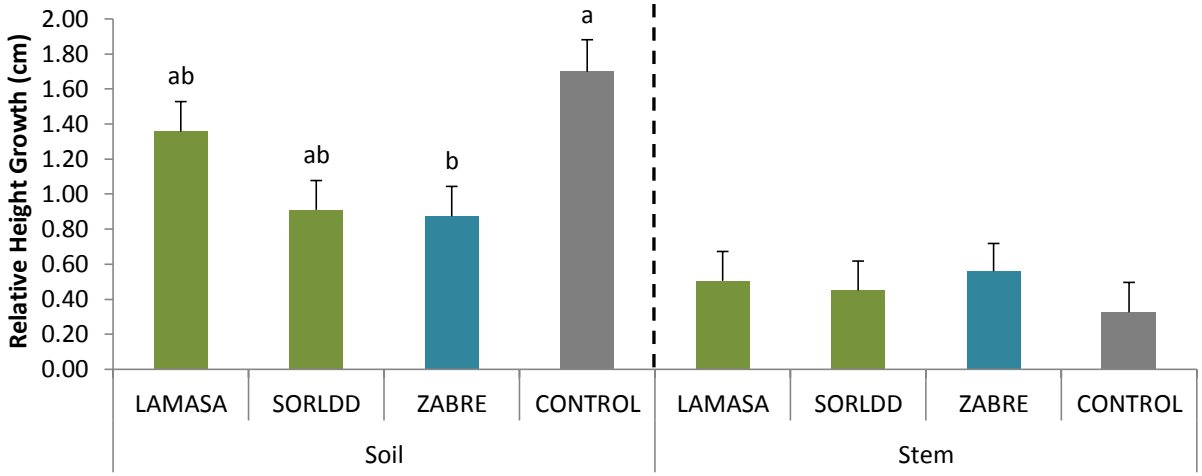


Figure 14 - Comparison of the mean relative height growth of *F. excelsior* inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD) and one isolate of *P. alni* ssp. *alni* (ZABRE) in the different treatments (Soil infestation and Stem inoculation). Green: *P. plurivora* isolates; Blue: *P. alni ssp. alni* isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences (p < 0.05).

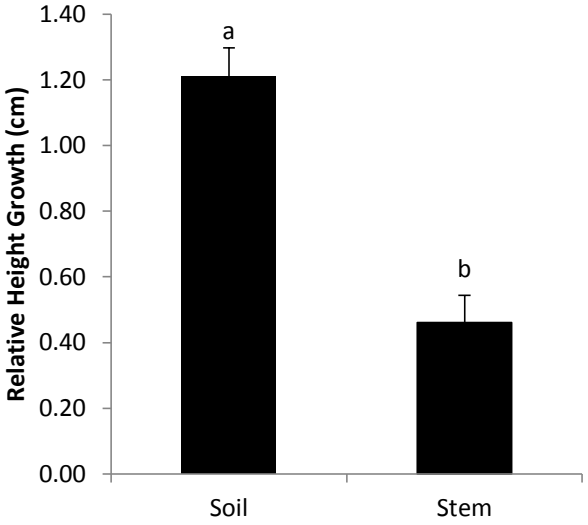


Figure 15 - Comparison of the mean relative height growth of *F. excelsior* between the different treatments; Soil infestation and Stem inoculation. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

Dried Weight

Beginning with standardized aerial dried weight, there was not showed significant significances between isolations and control, in both treatments (see Figure 16). Although, between treatments there was a high significance between each other (F=22,843; P<0,001). Likewise, Soil infestation showed higher values with mean standardized aerial dried weight of 0,089 ± 0,005, while Stem inoculation showed values of 0,057 ± 0,005 (see Figure 17).

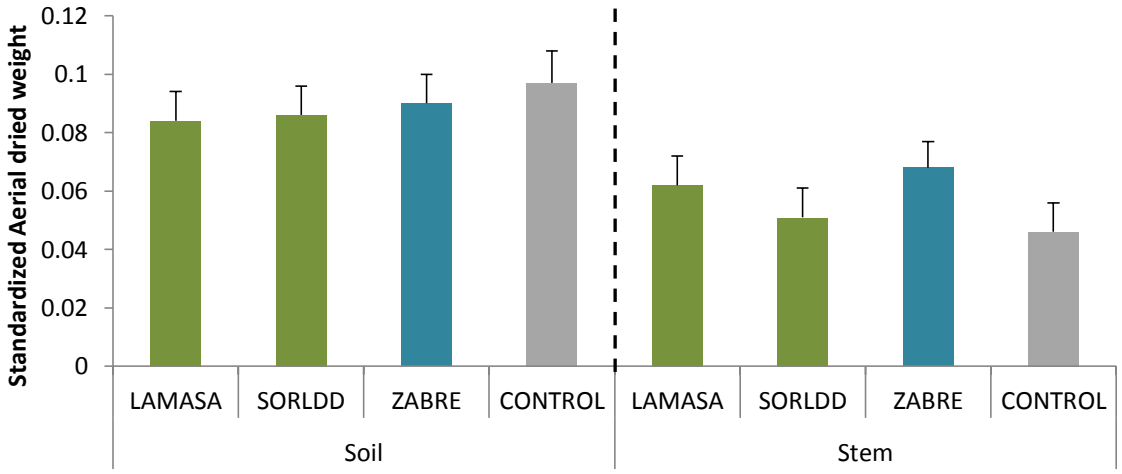


Figure 16 - Comparison of the mean standardized aerial dried weight of *F. excelsior* inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD) and one isolate of *P. alni* ssp. *alni* (ZABRE) in the different treatments (Soil infestation and Stem inoculation). Green: *P. plurivora* isolates; Blue: *P. alni ssp. alni* isolate; Grey: Control. Note: Error bars show the standard error.

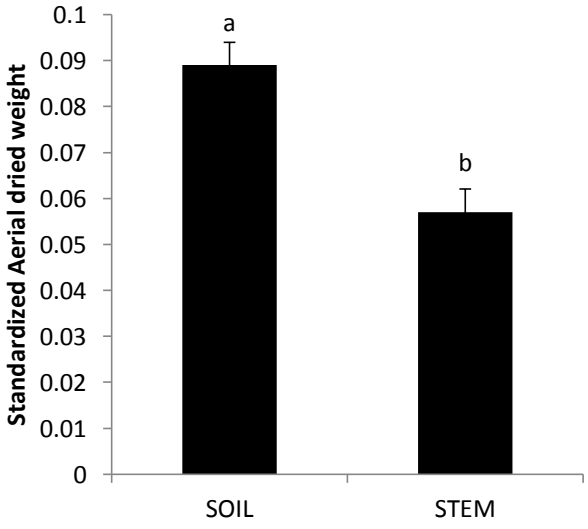


Figure 17 - Comparison of the mean standardized aerial dried weight of *F. excelsior* between the different treatments; Soil infestation and Stem inoculation. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

Concerning Standardized radical root dried weight, only significant differences between ZABRE and Control (F=6,102; P=0,020) in stem inoculation treatment were found (see Figure 18). Between treatments, in this case, there was no significant difference between each other.

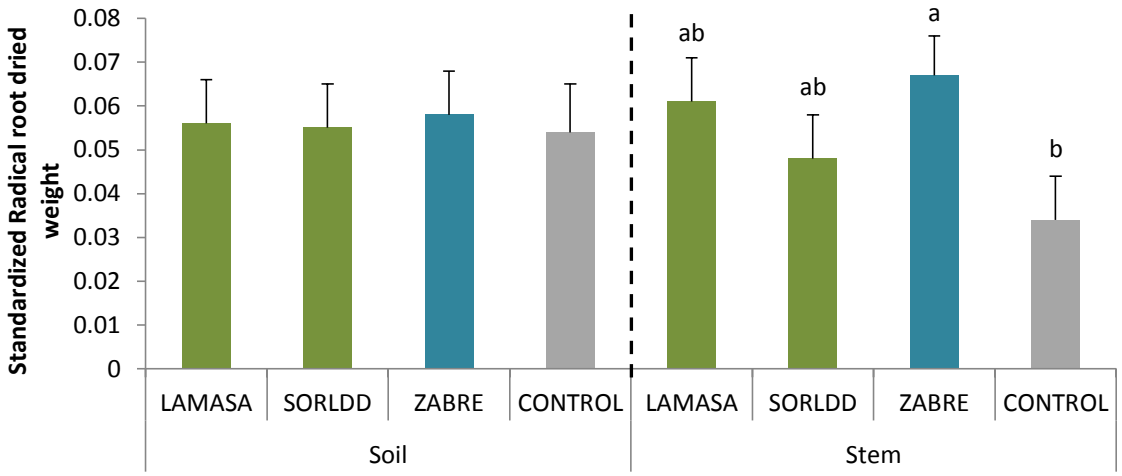


Figure 18 - Comparison of the mean standardized radical root dried weight of *F. excelsior* inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD) and one isolate of *P. alni* ssp. *alni* (ZABRE) in the different treatments (Soil infestation and Stem inoculation). Green: *P. plurivora* isolates; Blue: *P. alni ssp. alni* isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

Lastly, the Figure 19 shows the mean standardized secondary roots dried weight of all isolations and control on the different treatments used in the study (Soil infestation and Stem inoculation). In this case, there were no significant differences between treatments or between isolations in each treatment.

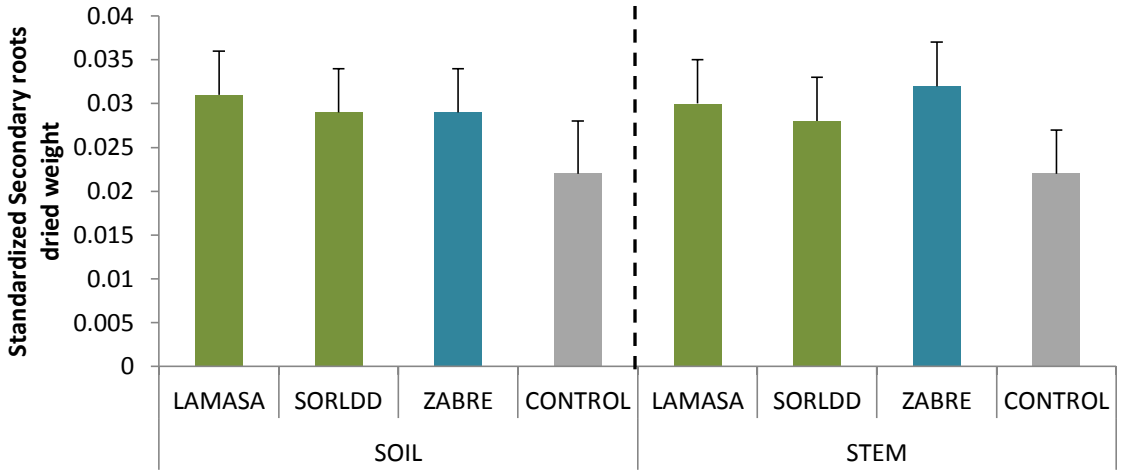


Figure 19 - Comparison of the mean standardized secondary roots dried weight of *F. excelsior* inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD) and one isolate of *P. alni* ssp. *alni* (ZABRE) in the different treatments (Soil infestation and Stem inoculation). Green: *P. plurivora* isolates; Blue: *P. alni ssp. alni* isolate; Grey: Control. Note: Error bars show the standard error.

Necrosis Length

Stem Inoculation treatment, described above in Material & Methods section, it was carried out by placing continuously growing mycelial plug on a wound previously made in the plant tissue. It was only in the end of the experiment that necrosis length was measured. In Figure 20 shows the mean Necrosis length in all isolations and control on Stem inoculation treatment. The analysis of variance indicated significant differences between isolates with a confidence level of 95% (Fwe=4,071; P=0,021). Only LAMASA and SORLDD showed with necroses with no significant difference between each other, however not in all replicates appeared necroses, 62% and 31% of total replicates showed necrosis, LAMASA and SORLDD respectively. In other hand, ZABRE and Control didn't show with any necrosis (see Figure 21). LAMASA showed mean values of 0,369 ± 0,096 and SORLDD showed mean values of 0,200 ± 0,094.

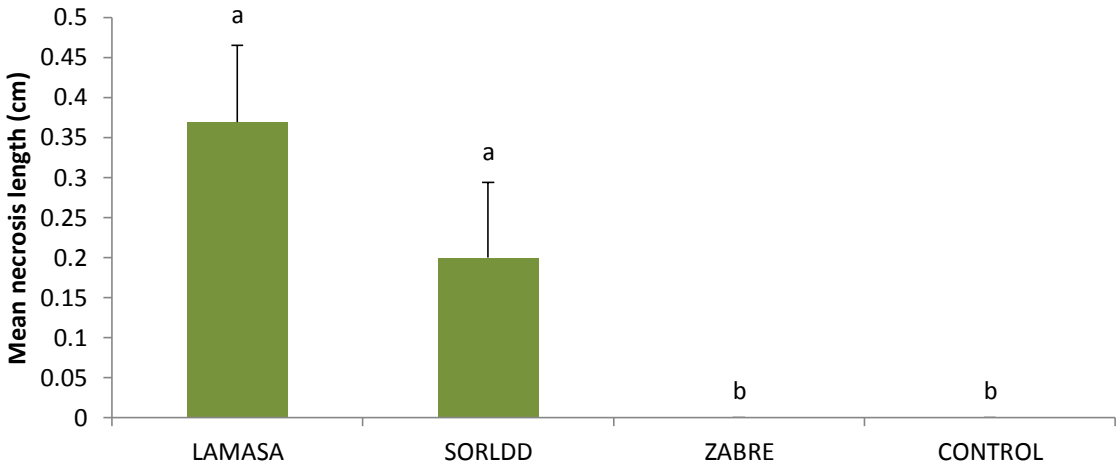


Figure 20 - Comparison of the mean necrosis length of *F. excelsior* inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD) and one isolate of *P. alni* ssp. *alni* (ZABRE) in Stem inoculation treatment. Green: *P. plurivora* isolates. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

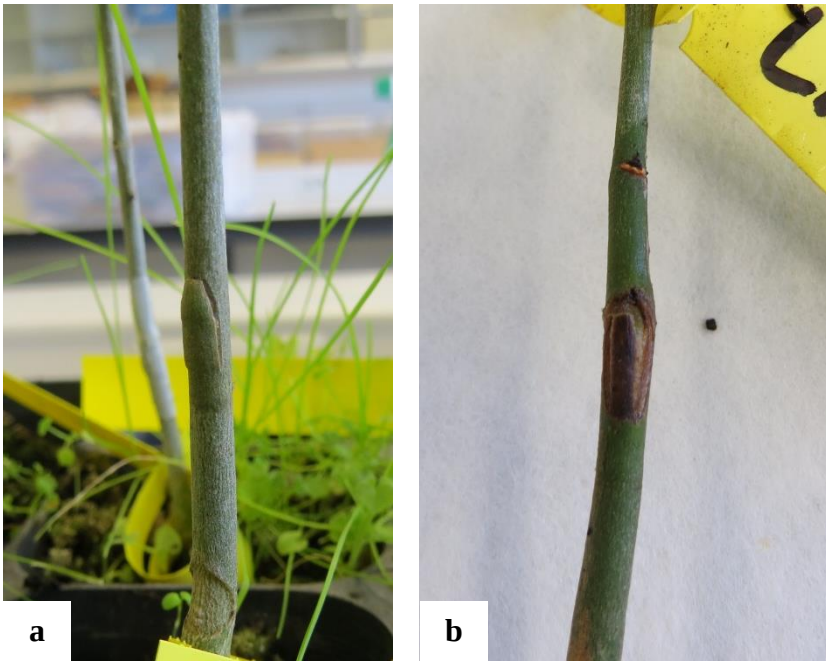


Figure 21 – Necrosis length comparison after 62 days. **a**. Control seedling showing no necrosis symptoms. **b**. Inoculated stem by *P. plurivora* showing necrosis on the wound location.

Pathogen Re-isolation

After the experiment was complete, it was performed the oomycete re-isolation. In Soil infestation treatment, it was positively re-isolated the two *P. plurivora* isolations from the soil samples using vegetal baiting method (results not showed). For stem inoculation, not from all the affected bark samples was possible to re-isolate the pathogen. The Figure 22 shows the percentage of re-isolated pathogen through affected necrosis on Stem inoculation treatment. From the total replicates with necrosis, it was possible to re-isolate 38% and 25% of the pathogen, LAMASA and SORLDD respectively.

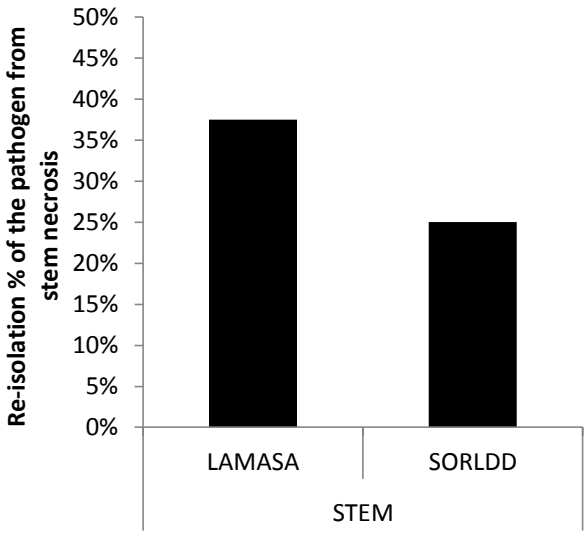


Figure 22 – Percentage of the pathogen re-isolation of affected bark on *F. excelsior* replicates in Stem inoculation treatment.

Experiment II

Relative Height Growth

In the end of the experiment, analysis of variance (Two-way ANOVA) showed many significant differences in relative height growth with a confidence level of 95%. To get a good view in these results, it was made 3 graphics; a graphic comparing *P. plurivora* isolations and control relative height growth in each provenance, graphic showing relative height growth differences between *P. plurivora* isolations and control, and finally graphic showing relative height growth differences between provenances. Firstly, the Figure 23 shows the mean relative height growth of all isolations and control on the different provenances used in the study (C467, C683, C737, C773 and C846). Provenances C773 and C846 didn't have significant differences between isolations and Control, while in the remaining provenances (C467, C683 and C737) there were significant differences between isolations and control. In these 3 situations, *P. plurivora* isolations had higher relative height growth comparing with Control. In provenance C467, LAMASA and SORLDD were significant between each other (F=7,159; P=0,011), and both significant to Control (F=82,339; P<0,001 and F=41,749; P<0,001, respectively). In provenance C683, LAMASA and SORLDD didn't have significant differences between each other, but were both significant to Control (F=15,186; P<0,001; F=10,219; P=0,003, respectively). Lastly, in provenance C737, LAMASA and SORLDD didn't have significant differences between each other, but were both significant to Control (F=32,490; P<0,001; F=17,194; P<0,001, respectively).

In Figure 24 shows the mean relative height growth between *P. plurivora* isolations and control, independent of the provenances. In this graphic there is a clear significant difference between *P. plurivora* isolations and Control (LAMASA: F=61,254; P<0,001; SORLDD: F=48,481; P<0,001), while between isolations there were no significant differences. Moreover, LAMASA and SORLDD had higher values comparing with Control; 2,567 ± 0,178, 2,055 ± 0,188 and 1,286 ± 0,105, respectively. In Figure 25 shows the mean relative height growth between provenances independent of isolations. Here, it is visible the differences between provenances (F=47,629; P<0,001). C737 was the provenance with the highest mean relative growth, showing 3,868 ± 0,321, while C846 showed the lowest value with 1,211 ± 0,158.

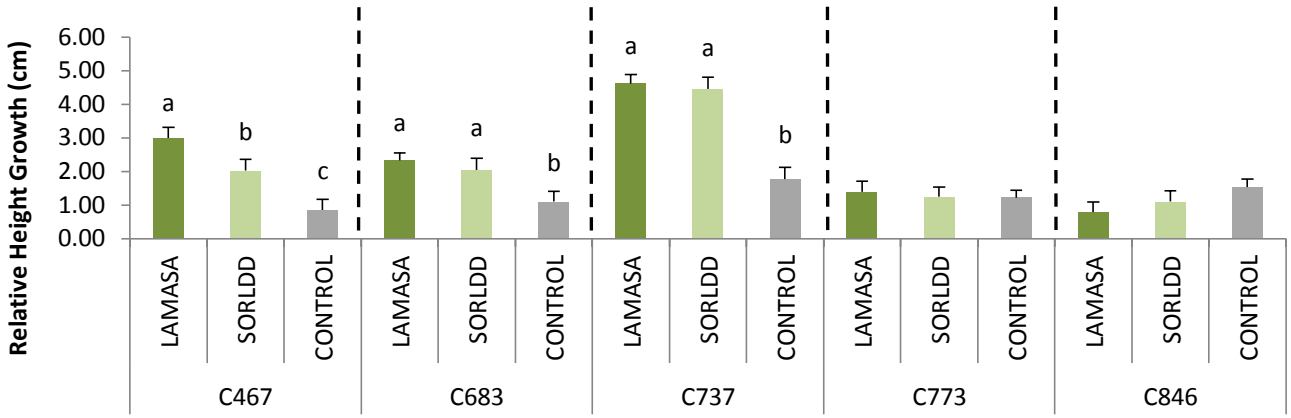


Figure 23 - Comparison of the mean relative height growth of different *A. glutinosa* provenances inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD). Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

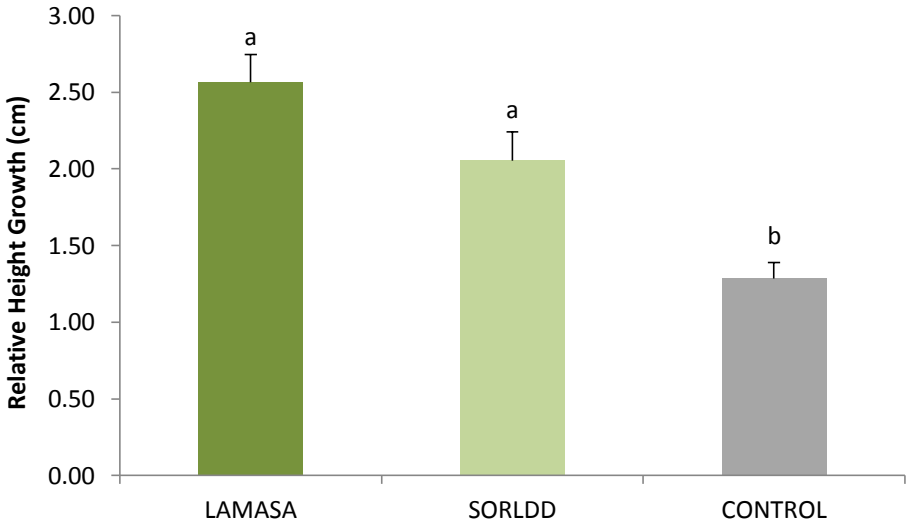


Figure 24 - Comparison of the mean relative height growth of *A. glutinosa* between the different isolations and Control. Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

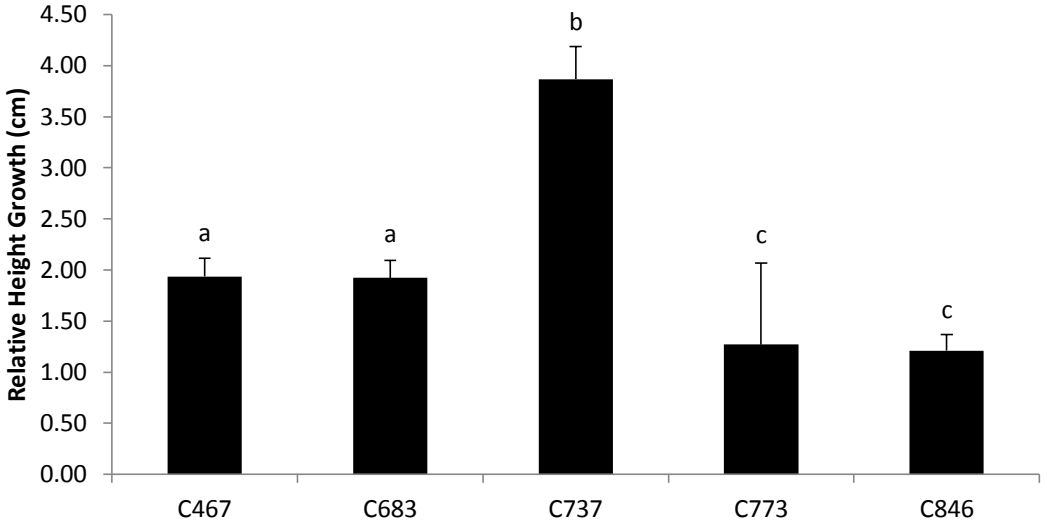


Figure 25 - Comparison of the mean relative height growth of *A. glutinosa* between the different provenances. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

Root Length

Finishing the pathogenicity test, the roots lengths were measured and converted to standardized root lengths. Analysis of variance (Two-way ANOVA) showed many significant differences in mean standardized root length with a confidence level of 95%. Likewise mean relative height growth, it was also made 3 graphics; a graphic comparing *P. plurivora* isolations and control standardized root length in each provenance, graphic showing standardized root length differences between *P. plurivora* isolations and Control, and finally graphic showing standardized root length differences between provenances. Firstly, in Figure 26 shows the mean standardized root length of all isolations and control on the different provenances used in the study (C467, C683, C737, C773 and C846). Provenance C773 didn't have significant differences between isolations and Control, while in the remaining provenances (C467, C683, C737 and C846) there were significant differences between isolations and control. In these 4 situations, *P. plurivora* isolations had higher or the same standardized root length comparing with Control. In provenance C467, LAMASA and SORLDD were not significant between each other, and both significant to Control (F=9,472; P=0,004 and F=30,287; P<0,001, respectively). In provenance C683, LAMASA and SORLDD again didn't have significant differences between

each other, but were both significant to Control (F=15,044; P<0,001; F=4,553; P=0,040, respectively). In provenance C737 the same situation appeared, LAMASA and SORLDD didn't have significant differences between each other, but were both significant to Control (F=10,468; P=0,002; F=11,833; P=0,002, respectively). Lastly, in provenance C846, SORLDD didn't show significant differences between Control, while LAMASA had significant differences with Control (F=18,640; P<0,001).

In Figure 27 shows the mean standardized root length between *P. plurivora* isolations and Control, independent of the provenances. In this graphic there were significant differences between *P. plurivora* isolations and Control (LAMASA: F=18,183; P<0,001; SORLDD: F=21,604; P<0,001), while between isolations there were no significant differences. Additionally, LAMASA and SORLDD had higher values comparing with Control; 2,222 ± 0,105; 2,334 ± 0,120 and 1,781 ± 0,106, respectively. In Figure 28 shows the mean standardized root length between provenances independent of isolations. Here, it is visible the differences between provenances (F=12,464; P<0,001). C737 was the provenance with the highest mean standardized root length, showing 2,921 ± 0,154 while C846 showed the lowest value, 1,699 ± 0,135.

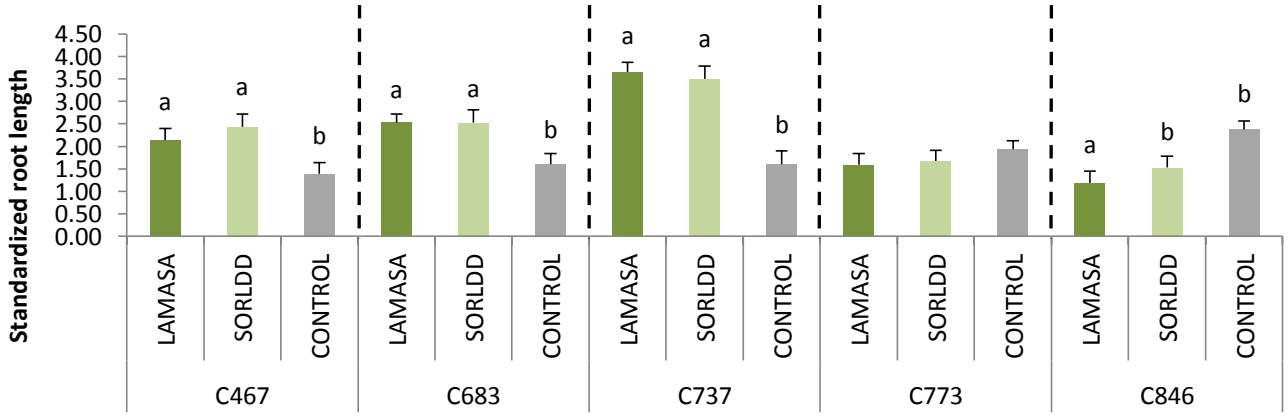


Figure 26 - Comparison of the mean standardized root length of different *A. glutinosa* provenances inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD). Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

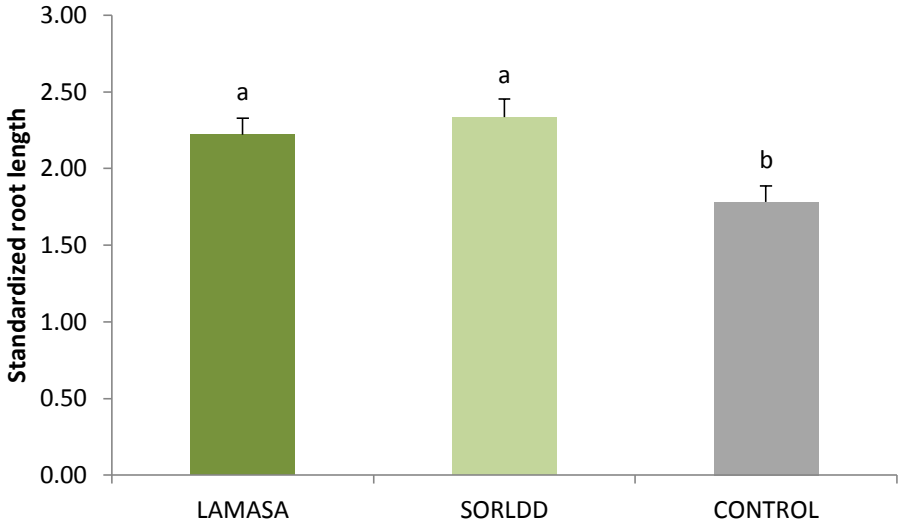


Figure 27 - Comparison of the mean standardized root length of *A. glutinosa* between the different isolations and Control. Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

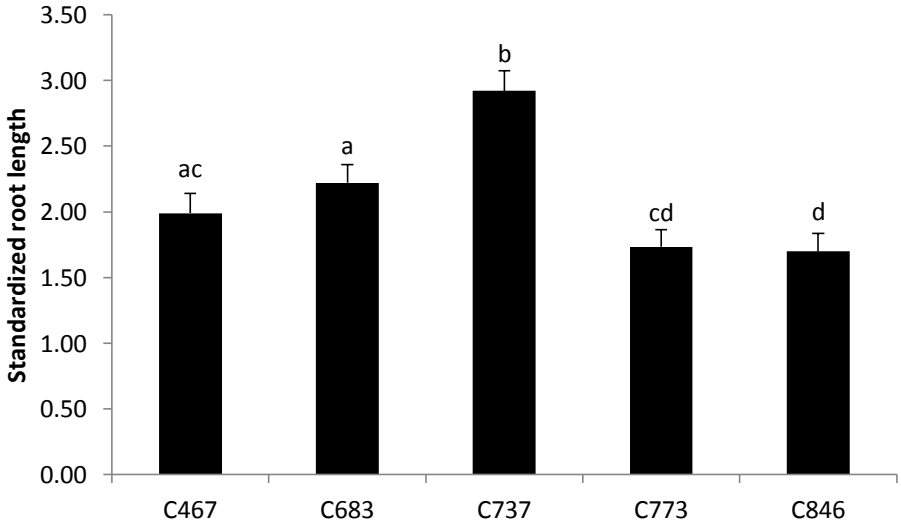


Figure 28 - Comparison of the mean standardized root length of *A. glutinosa* between the different provenances. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

Dried Weight

Beginning with standardized aerial dried weight, it was created the same type of graphics as in the previous variables. In this case, only two provenances showed significant differences between *P. plurivora* isolations and Control (C467 and C737). The Figure 29 shows the mean standardized aerial dried weight of all isolations and Control on the different provenances used in the study (C467, C683, C737, C773 and C846). In provenance C467, LAMASA and SORLDD were not significant between each other, and both significant to Control (F=35,076; P<0,001 and F=30,660; P<0,001, respectively). In provenance C737, only SORLDD showed significance to Control (F=8,182; P=0,008). The Figure 30 shows the mean standardized aerial dried weight between *P. plurivora* isolations and Control, independent of the provenances. In this graphic there were significant differences between *P. plurivora* isolations and Control (LAMASA: F=16,672; P<0,001; SORLDD: F=9,450; P=0,002), while between isolations there were no significant differences. Additionally, LAMASA and SORLDD had higher values comparing with Control; 0,042 ± 0,002; 0,042 ± 0,002 and 0,033 ± 0,002, respectively. In Figure 31 shows the mean standardized aerial dried weight between provenances independent of isolations. Here, it is visible the differences between provenances (F=30,682; P<0,001). C737 was the provenance with the highest mean standardized aerial dried weight, showing 0,064 ± 0,003 while C773 showed the lowest value, 0,024 ± 0,002.

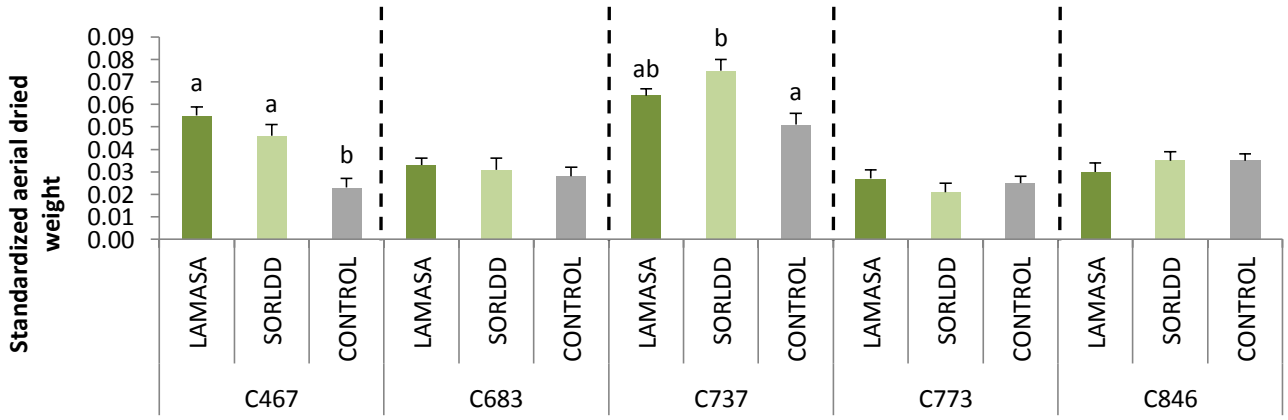


Figure 29 - Comparison of the mean standardized aerial dried weight of different *A. glutinosa* provenances inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD). Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

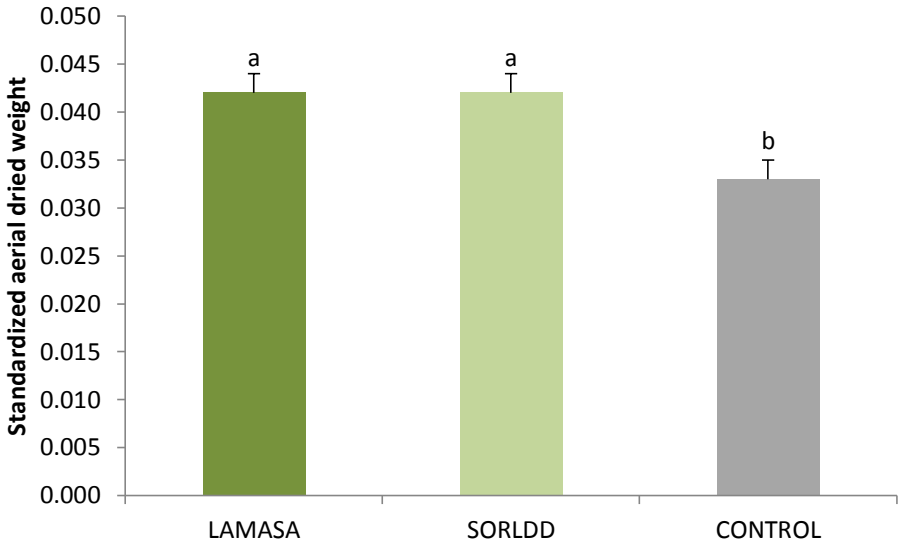


Figure 30 - Comparison of the mean standardized aerial dried weight of *A. glutinosa* between the different isolations and Control. Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

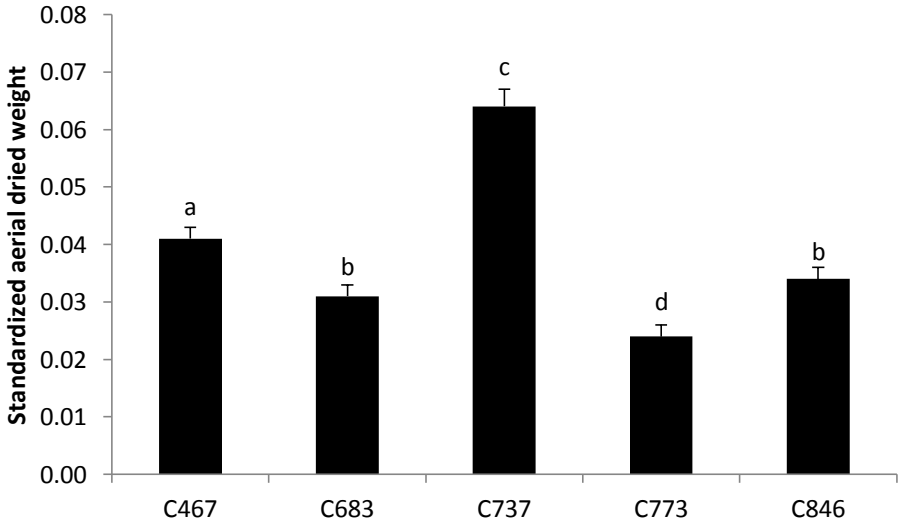


Figure 31 - Comparison of the mean standardized aerial dried weight of *A. glutinosa* between the different provenances. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

The Figure 32 shows the mean standardized roots dried weight of all isolations and Control on the different provenances used in the study (C467, C683, C737, C773 and C846). In this case, there were no significant differences between *P. plurivora* isolations and Control in provenances C683 and C737. In provenance C467, SORLDD was not significant to Control, in other hand LAMASA was significant to Control, as SORLDD and Control with higher values (F=14,277; P=0,001). In provenance C773, LAMASA and SORLDD didn't have significant differences between each other, however were both significant to Control (F=27,364; P<0,001; F=20,226; P<0,001, respectively). In provenance C846, only LAMASA showed significance to Control (F=10,950; P=0,002). The Figure 33 shows the mean standardized roots dried weight between *P. plurivora* isolations and Control, independent of the provenances. In this graphic there were significant differences between LAMASA and Control (F=25,098; P<0,001), while between SORLDD and Control there were no significant differences. In this particular situation, SORLDD and Control had higher values comparing with LAMASA; 0,119 ± 0,010; 0,113 ± 0,008 and 0,084 ± 0,008, respectively. Last but not least, the Figure 34 shows the mean standardized roots dried weight between provenances independent of isolations. Here, it is visible the differences between provenances (F=8,971; P<0,001). Again, C737 was the provenance with the highest mean standardized roots dried weight, showing 0,161 ± 0,012 while C773 showed the lowest value, 0,060 ± 0,010.

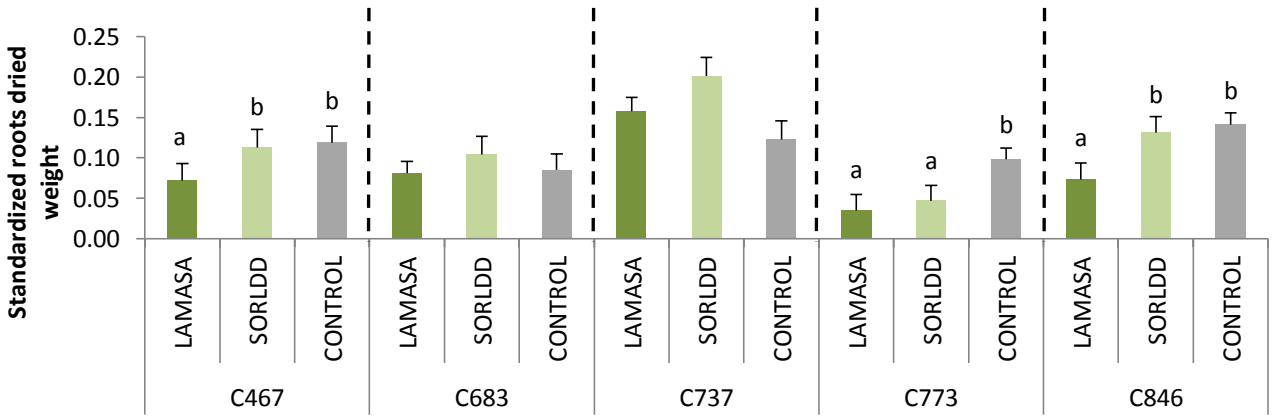


Figure 32 - Comparison of the mean standardized roots dried weight of different *A. glutinosa* provenances inoculated with two isolates of *P. plurivora* (LAMASA and SORLDD). Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

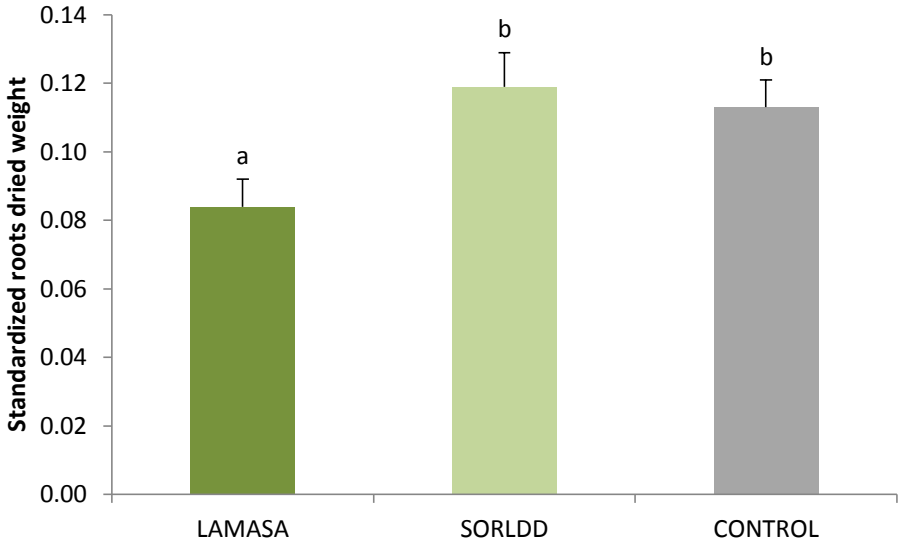


Figure 33 - Comparison of the mean standardized roots dried weight of *A. glutinosa* between the different isolations and Control. Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

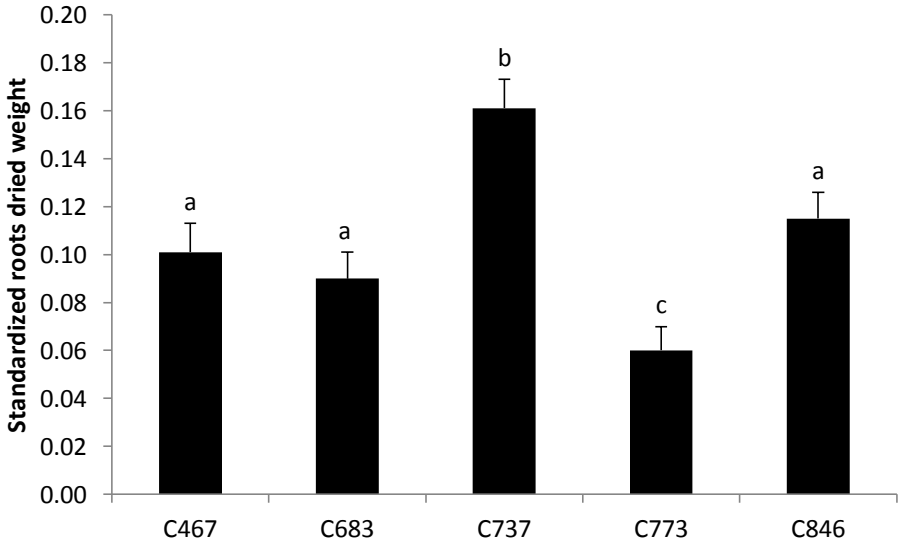


Figure 34 - Comparison of the mean standardized roots dried weight of *A. glutinosa* between the different provenances. Note: Error bars show the standard error. Different letters indicate significant differences ($p < 0.05$).

AUDPC

The development of symptoms of *A. glutinosa* replicates was recorded 2-3 times per week during the entire experiment period. From these data was possible to calculate AUDPC (Area Under the Disease Progress Curve) of each seedling. However only 3,68% of total replicates appeared wilted and after 40 days died. The Figure 35 shows the mean of AUDPC between *P. plurivora* isolations and Control, independent of the provenances. In this graphic is possible to see only replicates from LAMASA got foliar symptoms, however because only few seedlings got damage, statistical analyses was not necessary. Consequently, there was not any significance between isolations and Control. When the provenances were separated, it was possible to see that only provenance C683 didn't appear with any foliar symptoms (Figure 36).

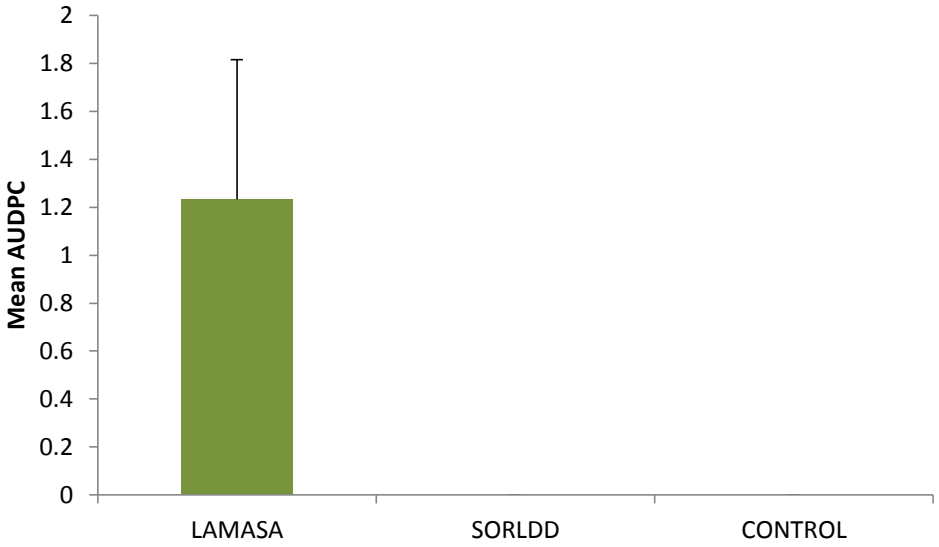


Figure 35 - Comparison of AUDPC of *A. glutinosa* between the different isolations and Control. Dark Green: LAMASA isolate; Light Green: SORLDD isolate; Grey: Control. Note: Error bars show the standard error.

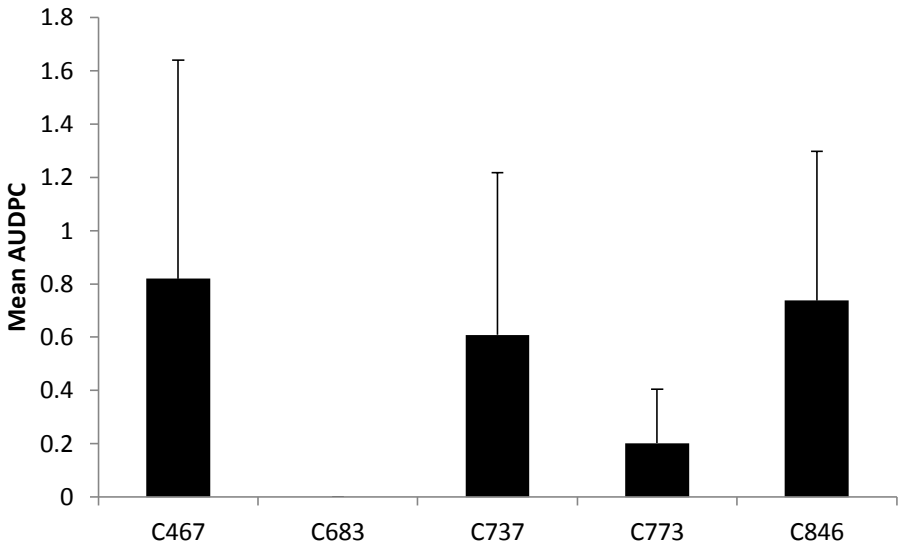


Figure 36 - Comparison of mean AUDPC of *A. glutinosa* between the different provenances. Note: Error bars show the standard error.

Pathogen Re-isolation

After the experiment was complete, it was performed the oomycete re-isolation from soil and from roots. Random roots from each provenance were plated directly into selective media V8-PARPH. From roots, it was possible to re-isolate *P. plurivora* LAMASA of all provenances, except for C846, however *P. plurivora* SORLDD was only possible to re-isolate from provenances C737 and C846. Concerning soil *Phytophthora* contamination, it was used vegetal baiting to "catch" *P. plurivora*. In this case it was possible to re-isolate the two *P. plurivora* isolations from soil (results not showed).

DISCUSSION

Experiment I

The pathogenicity of *Phytophthora plurivora* and *P. alni* was tested in laboratory in European ash, using two different inoculation methods. This was the first study to test pathogenicity of *P. alni* and *P. plurivora* in *F. excelsior* in Spain, using different methods simultaneously. Concerning survival, all the seedlings replicates persisted until the end of the experiment on both treatments, so there was no need to perform a survival test. Contrary, on Zamora-Ballesteros (2014) study, who performed pathogenicity tests with the same *P. plurivora* and *P. alni* Spanish isolates in 1-year-old alder seedlings through stem inoculation, obtained mortality on contaminated replicates, concluded host susceptibility. A variable that was not taken into account either in this experiment was the development of symptoms summarized in a damage index, this happened because replicate's foliar parts from stem inoculation were severed attacked by insects from Curculionidae family. This negative consequence affected the foliar symptoms evaluation, so it was not considered in the final results. Although using an insecticide, it didn't kill all the insect individuals. Since it was used flooding on soil infestation replicates, the damage was much less comparing with the ones from stem inoculation, this because the insects came with the replicates from the nursery in a larvae state. This problem could also affect relative height growth and aerial dried weight, as showed significant differences between treatments, which soil infestation had higher values comparing with stem inoculation. Santini *et al* (2003) also performed pathogenicity tests using stem inoculation and soil infestation. In this study, inoculation tests were carried out on seedlings of several hardwoods with 1-year-old (*Alnus cordata*, *A. glutinosa*, *Castanea sativa*, *Juglans regia* and *Quercus robur*) to determine their susceptibility to *Phytophthora alni*. In their results no statistically significant differences were found between these two methods. So it is possible that our results in this experiment could be interfered by the insects attack.

As the natural penetration of most *Phytophthora* sp. into the host takes place through the fine roots (Erwin & Ribeiro, 1996), susceptible hosts start to get damaged roots, and that reflect in root dried weight. In this experiment, it was not the case. *Fraxinus excelsior* didn't not show any significant differences between control and inoculated replicates, even between treatments no significant differences were found. In Santini *et al* (2003) study was found necrotized rootlets in contaminated seedlings, contrary in these experiment control rootlets had the same appearance as contaminated ones. One study that contradicts these findings is Orlikowski *et al* (2011) study. In this experiment it was performed soil infestation tests towards fine and feeder roots of 1-year-

old European ash seedlings. The results showed an average root mortality of approximately 42% using Polish *P. plurivora* isolates, concluding host susceptibility. All these findings suggests that, apart from different levels of resistance in host individuals, this may indicate different degree of within-species variation in pathogenicity or host specificity of different isolates of *P. plurivora*. So it is possible to suggest future experiments, using different *P. plurivora* isolates to test their aggressiveness on *F. excelsior*.

Regarding necrosis length in stem inoculation replicates, it was found significant differences between *P. plurivora* isolates and Control. Both Control and *P. alni* replicates didn't appear with any necrosis. However, necrosis length showed mean values of 0,369 ± 0,096 and 0,200 ± 0,094, LAMASA and SORLDD respectively. Comparing these results with other studies, Zamora-Ballesteros (2014) got similar values using the same Spanish isolates in *Alnus glutinosa*, where ZABRE isolate and SORLDD isolate necrosis length showed the same mean value approximately 0,380 cm, while LAMASA had value approximately 0,200 cm. Though, Zamora-Ballesteros's necrosis length values showed no much difference with these results; in relation to replicates survival it was completely different; in this study, replicates with necrosis didn't die or showed significant differences between Controls and in addition, the necrosis appeared just superficial, while in Zamora-Ballesteros's study, the replicates showed decaying comparing with control replicates. These may indicate that lesion length may not be the most suitable indicator for assessing damage caused by *Phytophthora* sp.

In the end of the experiment, it was performed the re-isolation of the pathogen. In stem inoculation, from the total replicates it was possible to re-isolate 38% and 25% of the pathogen, LAMASA and SORLDD respectively. These results don't mean the other necrosis didn't have the pathogen, as some plates got contaminated with bacteria and *Pythium* sp. and was not possible to confirm pathogen presence. Moreover, *P. plurivora* proved to be able to penetrate inside *F. excelsior* seedlings lesions, but the species might have any kind of induced defence to stop the advance of the pathogen. For soil infestation, it was performed the re-isolation of the pathogen through soil using vegetal baiting. Also, it was positively re-isolated the two *P. plurivora* isolations from the soil samples using this method, while *P. alni* and control didn't appear any necrosis on vegetal baiting. These findings, confirms that *P. alni* doesn't persist too much months in soil without the host (Jung & Blaschke, 2004; Elegbede *et al*., 2010). However, *P. alni* is considered only damaging to *Alnus glutinosa*, Santini *et al*. (2003) showed that this pathogen also produces symptoms in other species, including chestnut and walnut species. Nevertheless it was not the case with European ash. Actually the significant differences showed in this experiment were between control and ZABRE (i.e. in relative height growth and radical

root dried weight). These differences should not taken into account as *P. alni* was not re-isolated in either treatment, concluding that *P. alni* was not present in the end of the experiment. On contrary, *P. plurivora* continued actively in the soil and in lesions, even it was not damaging the replicates. Moreover, roots were directly inoculated into selective media, only obtaining bacteria and *Phytium* sp., not possible to confirm the pathogen in the roots.

This confirms that *P. plurivora*, more than *P. alni*, has a greater ability to persist in soil (Jung & Blaschke, 2004). This feature enhances the spread of *P. plurivora* around the world via nursery trade of contaminated plant material, even not showing typical *Phytophthora* symptoms on the trading plants.

Experiment II

The pathogenicity of *Phytophthora plurivora* was tested in laboratory on Common alder on different provenances from Northern Spain, using zoospores infestation. This was the first study to test pathogenicity of *P. plurivora* on different provenances of *A. glutinosa* in Spain. Concerning survival, in this experiment only a real small percentage of seedlings didn't persist until the end of the experiment (3,68%), so there was no need to perform a survival test. Contrary, on Zamora-Ballesteros (2014) study, who performed pathogenicity tests with the same *P. plurivora* Spanish isolates in 1-year-old alder seedlings through stem inoculation, obtained mortality on contaminated replicates, concluded host susceptibility. In this experiment, was really a surprise how alder seedlings survived until 40 days, while in Zamora-Ballesteros (2014) study after one month of inoculation it was already visible foliar symptoms. Moreover, in this experiment it was used younger seedlings (3-month-old) and it was stated that ontogeny of plants is known to influence their response towards *Phytophthora* spp., and susceptibility usually decreases with age (Oßwald *et al*., 2014), and in this case happened the opposite. So, probably the difference of these two experiments is in the different inoculation method. Here it was used zoospore infestation directly on soil, while Zamora-Ballesteros (2014) study was used a more invasive method; it was made a wound and placed directly *Phytophthora* sp. mycelial on the lesion. On natural infections, *P. plurivora* can initiate infection directly on the trunk, and in some cases can also start on the roots (Jung & Blaschke, 2004; Oßwald *et al*., 2014). In fact, stem inoculation may not be representative of natural infections that occur throughout European riparian forests as *P. plurivora* mainly progress towards the stem from the roots. In addition, 3-month-old seedlings doesn't have a strong stem to make stem inoculation method, so it was performed zoospore infestation into to the soil to try to "copy" the most possible way natural infections. Also, Jung & Blaschke (2004) stated that alder trees along rivers are mainly infected

via lenticels and adventitious roots during temporary flooding events, while roots are the primary infection site in nursery-grown planted alder seedlings on non-flooded sites. So in this experiment tested in laboratory conditions, we can consider the second situation. Navarro (2014, 2015) performed a similar zoospore inoculation method and demonstrated that *P. plurivora* caused root damage on red alders with 1 to 2 years old. A difference between Navarro study (2014, 2015) zoospore method and our experiment was that zoospores were induced to encyst by vortexing before soil inoculation. Moreover, Navarro seedlings were flooded with water every two weeks, while in this experiment seedlings were watered abundantly but not fully flooded. Navarro's study took the same period as our experiment, however used different alder species and used *P. plurivora* American isolates. Furthermore, inter-study comparison of isolates aggressiveness should be considered with caution because pathogenicity varies depending on the growing season in which inoculation was carried out (Brasier & Kirk, 2001; Navarro, 2015) as well as the provenance or age of inoculated seedlings (Jung & Blaschke, 2006). Hence, all these factors may play an important role on disease efficacy. One recent study (Štochlová *et al*., 2015) that proves variation in natural susceptibility of *A. glutinosa* was studied *in vitro* using branch inoculation tests. In this essay, different *A. glutinosa* genotypes were used, and inoculated with two types of *P. alni*. Host susceptibility varied significantly.

In the end of experiment, re-isolation of the pathogen was performed from soils and roots. These results were surprising, because pathogen was found on roots and on soil. One suggestion to improve this experiment is to extend the experiment period, to flood the seedlings to get early disease damage or even change the soil inoculation method (i.e. freshly inocula broth).

Observing relative height growth, root length and aerial dried weight results, all control values were smaller and with significant differences comparing with LAMASA and SORLDD isolations, while on root dried weight, control values were higher and with significant difference comparing only with LAMASA isolation. In fact, Fleischmann *et al*. (2004) regarded a pattern of spread after soil inoculation: in particular, they found that belowground biomass of beech seedlings infected with *P. plurivora* (referred to as *P. citricola*) was lower than in control plants, although aboveground biomass was the same. Considering LAMASA and SORLDD aggressiveness variation it was not shown significant differences between each other. In Zamora-Ballesteros (2014) study also didn't found any significance between these two isolations on plant yield either.

Considering seedlings provenance yield differences, one provenance really stood out from the others; C737 had always higher values with significant differences from the other provenances.

CONCLUSION

1. *Phytophthora plurivora* and *P. alni* Spanish isolates didn't prove to be pathogenic towards *Fraxinus excelsior*. All seedlings persisted until the end of the experiment on both treatments (Soil infestation and Stem inoculation).
2. Most of the *A. glutinosa* seedlings from different provenances survived after 40 days of experiment, showing that soil inoculation method it was not properly effective in a short term.
3. Remarkably, the two *P. plurivora* isolations were successfully recovered from soil samples of both experiments, using vegetal baiting, while *P. alni* and control didn't appear any necrosis on vegetal baiting. These findings, confirmed that *P. alni* doesn't persist too much months in soil without host.
4. To conclude, *P. plurivora* proved its ability to persist in soil, which enhances the spread of *P. plurivora* around the world via nursery trade of contaminated plant material, even not showing typical *Phytophthora* symptoms on the trading plants. Nursery trading measurements play a great role of protecting natural ecosystems from *Phytophthora* sp. diseases.

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