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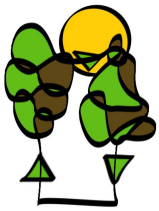
Department of Plant Production and Forest Resources



IDENTIFICATION, CHARACTERIZATION AND PATHOGENICITY OF *Phytophthora* spp. ASSOCIATED WITH THE MORTALITY OF *Alnus glutinosa* IN SPAIN

The present thesis fulfils the necessary requisites to obtain the Doctorate Mention through the University of Valladolid

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TO MY PARENTS

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CONTENTS

CONTENIDOS

ABSTRACT

Haque MM. 2014. Identification, characterization and pathogenicity of *Phytophthora* spp. associated with the mortality of *Alnus glutinosa* in Spain.

Mortality of alders (*Alnus* spp.) has emerged as an important problem in the natural ecosystems of many European countries including Spain. The main objective of the study was to identify and characterize the pathogens involved in riparian alder mortality in Spain. For this purpose, disease affected riparian alder stands in several provinces of the country were surveyed and sampled. The pathogens recovered were characterised by analysing their morphology, physiology and genetic traits. The frequently isolated pathogen was homothallic in nature and had terminal and ornamented oogonia mostly with two-celled amphigynous antheridia. Colony patterns developed on several growth media were usually uniform, radial or irregular with appressed and woolly morphology. rDNA sequences from the internal transcribed spacer region (ITS), comparison with GenBank and subspecies specific primers amplification confirmed identity of the pathogen as *Phytophthora alni* ssp. *alni*, reported earlier in several European countries. In addition to that, another homothallic *Phytophthora* sp. was also isolated from diseased *Alnus glutinosa*. Morphological, physiological and molecular studies identified the pathogen as *P. plurivora*, previously reported on diseased alders in few countries of Europe. Seeds and seedlings of *A. glutinosa* were inoculated with zoospore suspensions and mycelial agar discs of *P. alni* ssp. *alni*, *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora*. The study was done to assess the susceptibility of these two reproductive materials to the *Phytophthora* species tested under controlled environment. Results have suggested that the common alder and its seeds and seedlings are at risk to be infected by them. Pathogenicity of the three subspecies of *P. alni* (*P. alni* ssp. *alni*, *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis*) on detached leaves, twigs and branches of *A. glutinosa* were examined under artificial conditions. Results have demonstrated that wounds and temperatures have significantly influenced virulence of the isolates of three subspecies. This is one important finding concerning pathogenicity of *P. alni* as plant parts of *A. glutinosa* could act as potential sources of inoculums, which may prompt spreading of the pathogen to the natural ecosystems and hamper alder regeneration.

KEYWORDS: Common alder, mortality, decline, susceptibility, pathogenicity, *Phytophthora alni*.

RESUMEN

Haque MM. 2014. Identificación, caracterización y patogenicidad de las especies de *Phytophthora* asociadas a la mortalidad de *Alnus glutinosa* en España.

La mortalidad de los alisos (*Alnus* spp.) es un problema reciente en los bosques de muchos países europeos, incluido España. El principal objetivo de este trabajo fue identificar y caracterizar los patógenos implicados en la mortalidad de los alisos en los bosques de ribera españoles. Para ello, varias riberas con presencia de alisos con claros síntomas de decaimiento de varias provincias españolas fueron muestreadas. Las características morfológicas, fisiológicas y genéticas de los patógenos aislados fueron utilizadas para su caracterización. El patógeno más frecuentemente aislado fue homotálico, mostrando oogonios terminales y ornamentados por lo general con anteridios bicelulares posicionados en forma anfigina. Las colonias desarrolladas en distintos medios de cultivo fueron generalmente uniformes, radiales o irregulares de poco grosor y de aspecto lanoso. La comparación de las secuencias del ADN obtenidas de la región ITS con las secuencias depositadas en el GenBank y la amplificación de los cebadores específicos de cada subespecie confirmaron que el patógeno estudiado era *Phytophthora alni* ssp. *alni*, previamente encontrado en varios países europeos. Por otro lado, otra especie homotálica del género *Phytophthora* fue posteriormente aislada desde otros alisos enfermos. El estudio de sus características morfológicas, fisiológicas y genéticas sirvió para identificar al patógeno como *P. plurivora*, apenas aislada previamente en alisos enfermos de Europa. Semillas y plántulas de *A. glutinosa* fueron inoculadas con una suspensión de esporas y mediante micelio de *P. alni* ssp. *alni*, *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora*. El ensayo fue llevado a cabo para evaluar la susceptibilidad de estos dos materiales forestales de reproducción bajo condiciones ambientales controladas. Los resultados han sugerido que el aliso común, tanto semillas como plántulas, son susceptibles de ser infectadas por las especies inoculadas. A su vez, un estudio de la patogenicidad de las tres subespecies de *P. alni* (*P. alni* ssp. *alni*, *P. alni* ssp. *uniformis* y *P. alni* ssp. *multiformis*) fue llevado a cabo sobre hojas, ramillos y ramas bajo condiciones controladas en laboratorio. Este trabajo ha demostrado que tanto el efecto de la herida como la temperatura influyeron significativamente en la virulencia de los aislamientos de las tres subespecies. Se trata de un hallazgo relevante, en lo referente a la patogenicidad de *P. alni*, ya que partes de

plantas de *A. glutinosa* podrían actuar como fuente potencial de inóculo, el cual podría dispersar rápidamente el patógeno a ecosistemas naturales y dificultar la regeneración de los alisos.

PALABRAS CLAVE: Aliso común, mortalidad, decaimiento, susceptibilidad, patogenicidad, *Phytophthora alni*.

LIST OF ORIGINAL ARTICLES

The thesis is based on the following manuscripts, which in the text will be referred to by their Roman numerals (I-V).

I. Solla A, Pérez-Sierra A, Corcobado T, Haque MM, Diez JJ, Jung T. 2010. *Phytophthora alni* on *Alnus glutinosa* reported for the first time in Spain. Plant Pathology 59, 798.

II. Haque MM, Hidalgo E, Martín-García J, De-Lucas AI, Diez JJ. 2014. Morphological, physiological and molecular characterization of *Phytophthora alni* isolates from western Spain. (Recommended for publication after minor revision in 'European Journal of Plant Pathology', Ref. EJPP-D-14-00378R1).

III. Haque MM, Diez JJ. 2012. Susceptibility of common alder (*Alnus glutinosa*) seeds and seedlings to *Phytophthora alni* and other *Phytophthora* species. Forest Systems 21(2), 313-322.

IV. Haque MM, Martín-García J, Diez JJ. 2014. Variation in pathogenicity among the three subspecies of *Phytophthora alni* on excised leaves, twigs and branches of *Alnus glutinosa*. Preliminary manuscript.

V. Haque MM, Martínez-Álvarez P, Lomba JM, Martín-García J, Diez JJ. 2014. First report of *Phytophthora plurivora* causing collar rot on common alder in Spain. Plant Disease 98(3), 425.

Articles have the kind permission from the co-authors to be use as part of this Doctoral Thesis.

INTRODUCTION

The genus, *Phytophthora*

The word *Phytophthora* derived from Greek words 'Phyton' and 'pthora' literally means 'plant destroyer', is a genus of Oomycetes which mainly differ from fungi by having primarily diploid hyphae and cell walls composed of cellulose and β-glucans instead of chitin (Erwin and Ribeiro 1996). They also differ in terms of zoosporic dispersal and oogamy, characteristic features that are not owned by true fungi (Brasier and Hansen 1992). Earlier *Phytophthora* was considered as fungi because of its morphological and physiological similarities to true fungi. Nevertheless, because of evolutionary phylogeny and structures of zoospores, *Phytophthora* has recently been grouped into the kingdom Chromalveolata, which also includes brown algae and other protists, and is considered as one of the six major groups within the Eukaryota (Adl *et al*. 2005). Most of the *Phytophthora* species are pathogenic to herbaceous and woody plant species and have been usually associated with root and crown rot and stem necroses of woody plants (Erwin and Ribeiro 1996). The number of *Phytophthora* species increased linearly over the first half of the last century, however an exponential increase has been taken place since then, as a result of intensive sampling campaigns and the developments on molecular techniques that have enhanced the differentiation of species genetically close (Figure 1). Until 1999, 55 species of *Phytophthora* have been described (Erwin and Ribeiro 1996; Jung *et al*. 1999). However, since 2000 and onward, more than 50 new *Phytophthora* species (morphologically and molecularly identified but not formally described) have been added to the list (Brasier 2007).

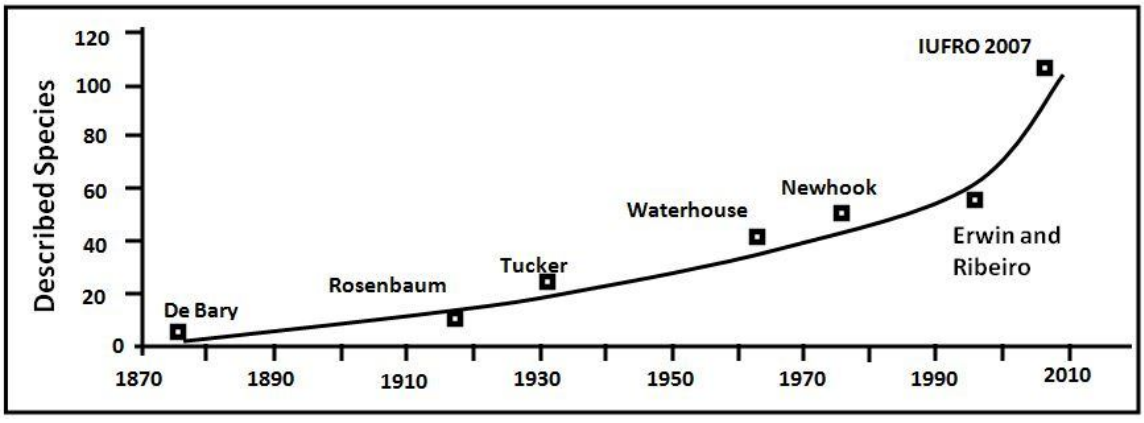

Figure 1. Gradual increase of the number of *Phytophthora* species over time (Brasier 2007)

The infective units of *Phytophthora* species include zoospores, oospores, chlamydospores, sporangia, and hyphal fragments. However, zoospores constitute the dominant infection units (Thomson 1972). In the presence of water, chlamydospores or oospores germinate to produce sporangia. When sporangia get maturity, they release motile biflagellate zoospores which move passively or swim actively in water to potential infection sites (Duniway 1976). Zoospores can swim short distances in water and chemo-tactically attracted to roots of potential hosts where they settle and encyst (Ho and Zentmeyer 1977; Hardham 2001).

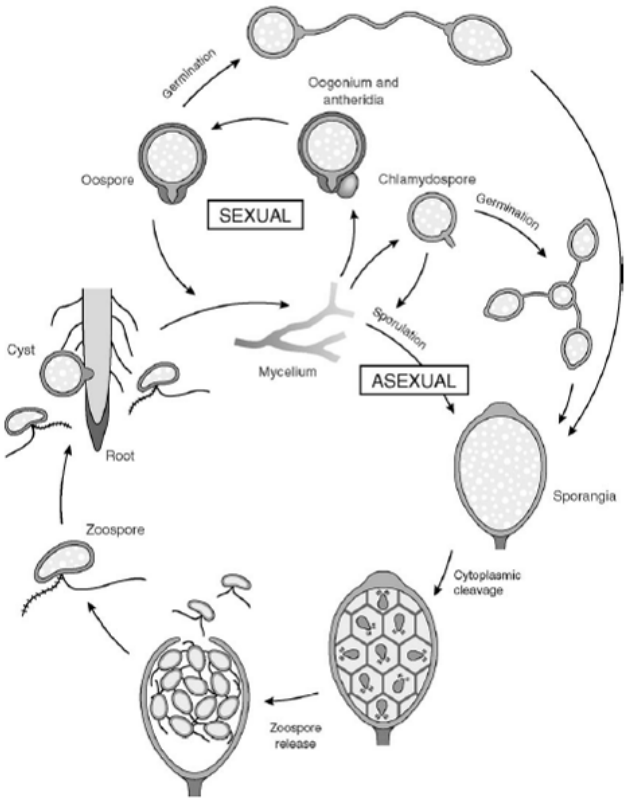

Figure 2. Generalized life cycle of *Phytophthora* (adapted from Adrienne Hardham, The Australian National University, Australia)

Source: http://www.environment.gov.au/system/files/resources/23925ac2-8fda-4036-aa56-5451f5d8b06d/files/appendix1.pdf

The zoospore cyst germinates to form microscopic thread like structures called hyphae which allow the pathogen to grow into plant cells to obtain nutrients (Hardham 2001). Once *Phytophthora* infect plants, it produces chlamydospores in cortical cells and sporangia on the root surface (Ho and Zentmeyer 1977; Hardham 2001). The cytoplasmic contents of sporangia undergo cytokinesis to form uninucleate motile zoospores that are released through an apical pore of the sporangia. The discharged

zoospores move or swim for new infection sites (Deacon and Donaldson 1993). Thus the infection cycle is repeated.

The alder pathogen: *Phytophthora alni*

Origin and taxonomic features of P. alni

Phytophthora alni (Brasier and S.A. Kirk) involved in alder mortality was first detected in southern Britain in 1993 (Gibbs 1995). Since then, it has been reported from several European countries: Austria, Belgium, Czech Republic, France, Germany, Hungary, Ireland, Italy, Lithuania, the Netherlands, Poland, Slovakia, Slovenia, Sweden and Spain (Figure 3) (Gibbs *et al*. 1999; Brasier and Kirk 2001; Santini *et al*. 2001; Brasier 2003; Gibbs *et al*. 2003; Santini *et al*. 2003; Jung and Blaschke 2004; Ioos *et al*. 2005; Cerný and Strnadová 2010; Solla *et al*. 2010; Varela *et al*. 2010, 2012). In Europe, the pathogen has been reported to cause diseases on alders growing mainly along riverbanks, in orchard shelterbelts and forest plantations (Gibbs 1995; Gibbs *et al*. 1999, 2003; Jung and Blaschke 2004).

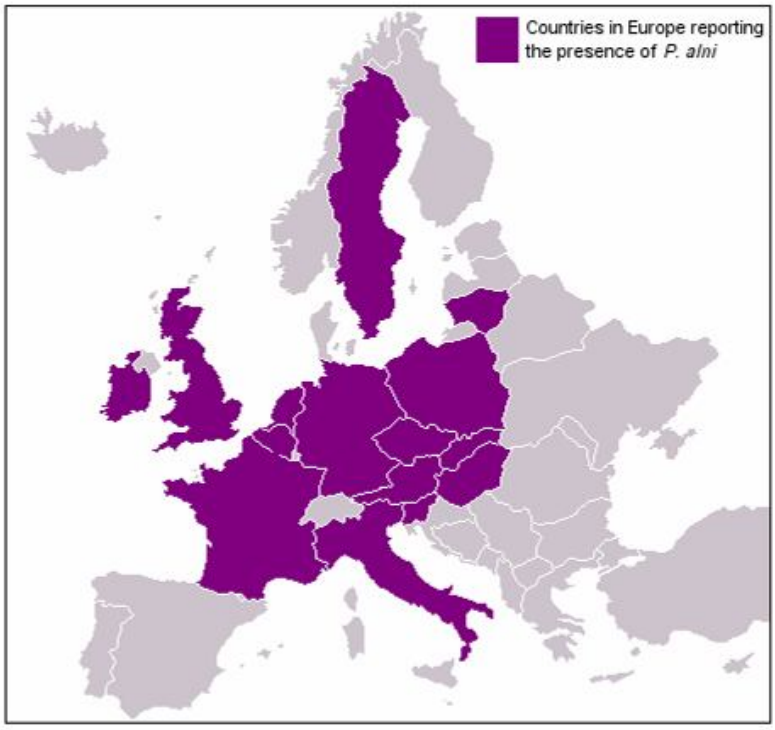

Figure 3. Distribution map of *P. alni* in Europe (the map has been published before 2010. So, presence of *P. alni* in Spain has not been indicated).
Source:http://www.forestry.gov.uk/website/forestresearch.nsf/ByUnique/INFD737J2S

Initially, it was believed that the pathogen responsible for alder mortality was similar to *P. cambivora* (Petri) Buisman in its gametangial morphology while it differed in

homothalism, colony growth patterns, optimum temperature for growth, high level of zygotic abortion and poorly developed oogonia (Brasier *et al*. 1995, 1999). Later, it was hypothesized that the *Phytophthora* engaged in alder mortality evolved as a result of hybridization between heterothallic *P. cambivora* and another unknown taxon of *Phytophthora* closely related to homothallic *P. fragariae* Hickman (Brasier *et al*. 1999). Further investigation by them demonstrated that the alder *Phytophthora* consisted of a variety of heteroploid hybrid species and later they divided it into a 'standard' type and several variants. Analysis of the internal transcribed spacer (ITS) region revealed that the isolates of 'standard' type displayed an unusual ITS polymorphism i.e., dimorphic sites within the ITS sequences while the variants were monomorphic for the same genome region or monomorphic at some sites and dimorphic at other sites (Brasier et al. 1999, 2004). Brasier *et al*. (2004) formally named alder *Phytophthora* as *P. alni* and based on morphological studies, cytological evidences and genetic data, and they divided *P. alni* into three subspecies: *P. alni* ssp. *alni* (Brasier and S.A. Kirk) corresponding to former 'standard' type, *P. alni* ssp. *uniformis* (Brasier and S.A. Kirk) corresponding to Swedish variant and *P. alni* ssp. *multiformis* (Brasier and S.A. Kirk) corresponding to the Dutch, German and UK variants. More recent genetic studies have hypothesized that Paa has occurred from a single or multiple hybridization event between Pau and Pam, although the origin and genetic diversity of these taxa are still under discussion (Ioos *et al*. 2006, 2010). All three sub-species of *P. alni* showed distinctive colony morphologies and different growth-temperature relations on culture media. Sporangia produced by the sub-species were non-caducous and non-papillate and formed conspicuous basal plugs in empty sporangia. Sporangial shape varied from ovoid to ellipsoid with a rounded or occasionally tapered base (Figure 4a, 4b). *P. alni* ssp. *alni* produced ornamented oogonia and elongated two-celled antheridia whereas both *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis* produced unique unusual combinations of oogonial structures and amphigynous or perigynous antheridia (Figure 4c, 4d). Likewise, while *P. alni* ssp. *uniformis* formed oogonia having smooth surface, *P. alni* ssp. *multiformis* produced highly ornamented oogonia. Further morphological studies showed that the isolates of *P. alni* ssp. *alni* produced less viable oospores than remaining two subspecies (Brasier *et al*. 1999, 2004).

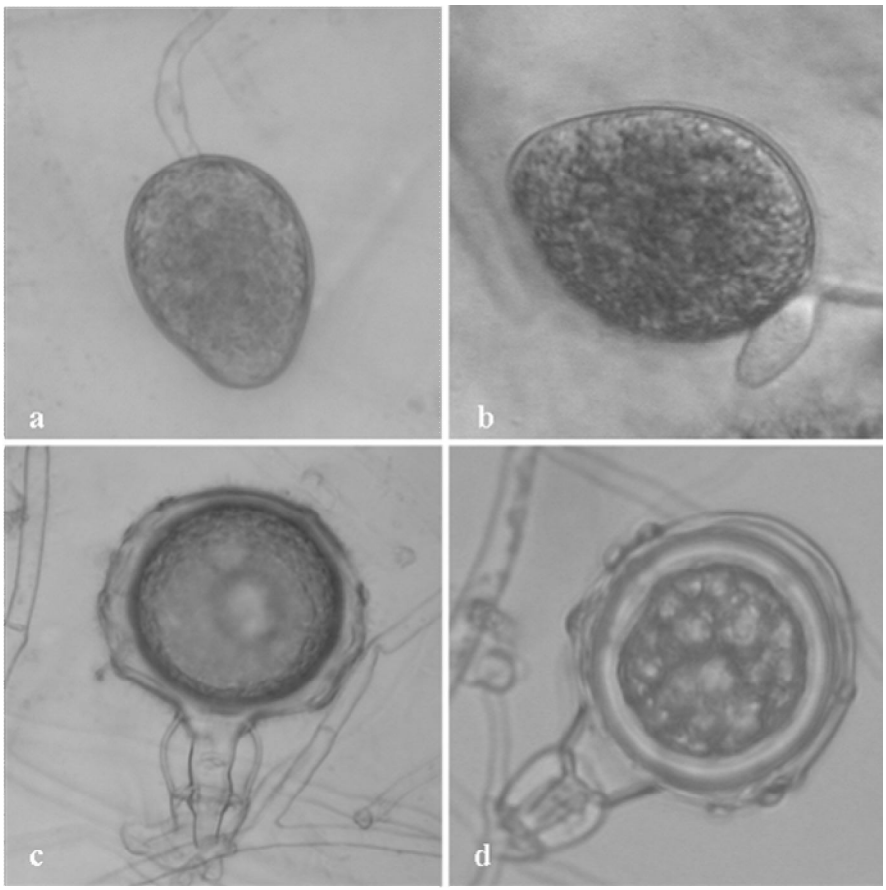

Figure 4. Reproductive structure (asexual and sexual) of *P. alni*. a, b. Non-papillate and ovoid sporangia; **c.** ornamented oogonia with two-celled amphigynous antheridia; **d.** ornamented oogonia with aborted oospore.

The genus '*Alnus*'

'*Alnus*' belongs to family Betulaceae and is characterized by their ability to colonize abandoned land and tolerate high groundwater tables and periodic flooding (Webber *et al*. 2004). There are four species of *Alnus* which are common in Europe. Common alder [*A. glutinosa* (L.) Gaertn.] is the most common species in Europe which grows on wet and clay soils, colonizing riverbanks or swampy areas. It has high conservation value, and is widely used in reforestation and stabilizing riverbanks. It also produces valuable timber. Grey alder [*A. incana* (L.) Moench] is a central to eastern European species and grows mainly in mountain areas. Another species, green alder [*A. viridis*] (Chaix) DC. is a shrub species in the Alps, colonizing steep slopes and banks of mountain streams. Italian alder [*A. cordata* (Loisel.) Duby] is an endemic species in southern Italy and well adapted to both clay and dry soil. It is widely plated for timber production and riverbank stabilization (Santini *et al*. 2003; Jung and Blaschke 2004; Webber *et al*. 2004).

A. glutinosa is the most widespread species occurring spontaneously in almost every region of Spain, except in arid southeast part. This species grows in small groups along riverbanks, streams and in wet places. The distribution map of common alder in Spain is given in Figure 5.

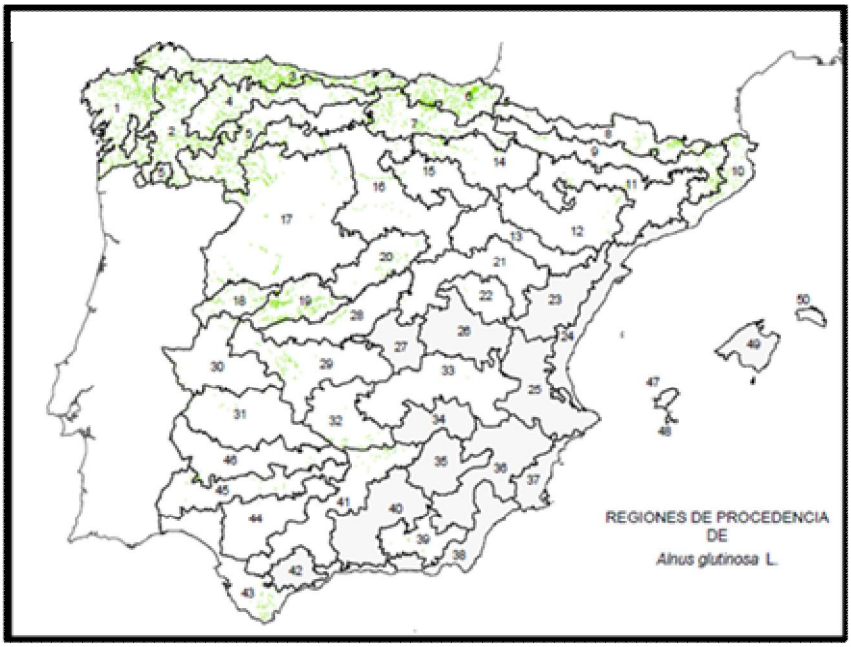

Figure 5. Distribution map (highlighted in green) of *A. glutinosa* in Spain
Source: http://www.inia.es/gcontrec/pub/alnus_glutinosa_publi_1186051443234.pdf

A. glutinosa grows well in moist soils and can reach up to a height of 20-30 meters and exceptionally up to 37 metres with a normal diameter ranged 0.6-0.7 metres. The root system of the tree is shallow, strong and well branched, especially in wet and shallow soils. Young trees have a habit of grow upright with a main axial stem where as the older develop an arched crown with crooked branches. The trunk is straight, cylindrical and overall pretty clean. The bark is smooth, shiny and greenish-brown in the young while in the older trees it is dark grey in colour. The buds are thick, ovate or oblong, hairless, slimy, coated by two or three scales. The tree is characterized by its short-stalked green coloured rounded leaves becoming wedge-shaped at the base and with a slightly toothed margin. The flowers are monoecious. The catkins appear in summer and developed at the end of next winter. The catkins of both sexes appear together at the apex of the twigs of the same year, in clusters of 3-6 (Cela *et al.* 1998).

A. glutinosa is a fast growing species and has a high adaptability towards adverse environmental conditions. It can grow in a place where it is not possible for others to grow, such as in wetlands. It is also an excellent tree species for restore former

forestland or abandoned farmland and other problematic soil that do not support vegetation easily. It has excellent ability to ameliorate soil due to a root symbiosis with actinomycetes (*Frankia*) which are able to fix atmospheric nitrogen. It has traditionally been used in furniture and veneer industry as well as for window and door production. Besides, its leaves and bark have medicinal properties.

The genus, *Alnus* also has high ecological value because of its conservation role (Claessens 2003; Thoirain *et al*. 2007). However, despite its numerous benefits, riparian zones, and in particular alder forests, have been disturbed by human activities over the past century (Kauffman *et al*. 1997; Naiman and Décamps 1997). In fact, virgin vegetation of these riparian zones was almost totally lost when stream flow was regulated by storage reservoir and canalizations in the middle of the 20th century (Schnitzler 1994; González and García 2007).

The disease

Decline of the riparian alder population has recently become an important issue in Europe, and particularly in Spain, because of the rapid spreading of *P. alni*. Establishment of plantations on former agricultural land and riverbanks to stabilize slopes with infested alders and use of water for irrigation from rivers contaminated by diseased alders contributing a lot to the spreading of alder *Phytophthora* to natural ecosystems (Jung and Blaschke 2004). Production of zoospores in the presence of water significantly contributes to the dispersal of *Phytophthora* via irrigation (Yamak *et al*. 2002; Hong *et al*. 2006). Importation of nursery stock for afforestation purposes and use of contaminated river water to irrigate nurseries may have resulted rapid increase of *P. alni* in Europe. Besides, out-planting with *Phytophthora* infected nursery stocks have contributed to the dissemination process of *Phytophthora* to natural ecosystems (Gibbs *et al*. 2003; Jung and Blashchke 2004). Falling of seeds, young shoot or leaves onto the contaminated water or soil and later disseminating far distances through water ways. In Great Britain, spreading of alder *Phytophthora* has occurred through watercourses (Gibbs *et al*. 1999) whereas in Bavaria (Germany) *P. alni* has been introduced into many places either by planting infected nursery stock or by irrigation water (Jung and Blaschke 2004).

P. alni, being a soil-borne pathogen (Elegbede *et al*. 2010) usually infects *Alnus* through fine roots and causes root rot and collar and stem necroses (Figure 6a). Diseased trees characteristically show die-back symptoms (Figure 6b), small, yellowish and sparse

foliage (Figure 6c). Tarry spots (6d) and rusty exudates (6e) on the surface of the bark or in its cracks at collar and lower stem with excessive fructification are the common characteristics. In case of severely affected trees, leaves often fall prematurely, leaving branches bare. Sometimes, the tarry spots can occur up to 2-3 meters from ground level (Figure 4f). Tarry spots indicate the infections and necroses persist in the inner bark and cambium (Gibbs *et al*. 1999, 2003; Jung and Blaschke 2004).

Figure 6. *Phytophthora* decline of common alder. a. Tree stem with bleeding canker; **b.** typical die-back of *A. glutinosa*; **c.** trees with small, sparse and yellowish leaves; **d.** tarry spots at lower stems; **e.** rusty and dark coloured exudates coming out from the point of infection; **f.** necroses moving towards upward direction.

Management of alder disease

Alders play an important role in stabilizing banks of rivers and is considered as the key element of riparian ecosystems in Europe. Spreading of *P. alni*, may lead drastic changes in the riparian ecosystems and may cause serious economic losses at the same time. Attempts to reduce the impact of the disease for short term period by coppicing in the natural riparian alder stands of UK have been found useful by Gibbs (2003) that showed low infection a couple of years after coppicing. However, coppicing was not recommended for plantations infected with *P. alni* by other authors (Jung and Blashchke 2006). Provenance trial could be an option to mitigate alder mortality. However, field and laboratory experiments conducted with alder seedlings collected from different provenances in Europe rejected the idea of resistance of alders against *P. alni* at provenance level (Gibbs 2003). For long term control, another possible strategy could be the genetic selection of a variety of clones in order to maintain healthy alder as it was possible for *Eucalyptus marginata* against *P. cinnamomi* Rands in Australia (Hüberli *et al*. 2003; Jung and Blaschke 2006).

There are currently no known methods to completely eradicate *P. alni* from an infested site. But, there is a need of good integrated management of nursery and irrigation system to stop dissemination of the pathogen. Molecular-based identification system should be initiated for rapid and effective detection. It is also important to ensure safer environment for *A. glutinosa* to grow in nurseries and in plantations. Finally, a common strategy and co-ordinated programme for limiting the transportation of infected plants from country to country is needed in Europe. In order to mitigate spread of *P. alni* in nurseries and plantations including riparian ecosystems, the following measures could be adapted: (a) production of alder seedlings from disease-free areas, (b) avoidance of frequent seedling transportation with soils from nurseries to plantations, (c) examination of seedlings before planting to check if they have any disease symptoms and elimination of symptomatic seedlings, (d) monitoring of health status of plants growing in nurseries periodically, (e) inspection and testing of water sources before watering plants in nurseries, (f) sterilization of nursery tools before and after performing silvicultural practices, (g) planting of *Alnus* in the stands where nursery plants have not been planted for a long period of time, (i) planting of alder with other non-susceptible tree species and (j) maintaining a 3 or 4-year rotation in order to eliminate alder pathogen from sites infected (Jung and Blaschke 2004; Oszako 2010).

AIMS OF THE STUDY

The main objective of the study was to identify and characterize the pathogens involved in common alder mortality in Spain. Furthermore, studies were conducted to obtain information on the potential risks that may pose by the pathogens to *A. glutinosa* and its propagating materials.

More specifically, the aims of the individual study were:

1. To isolate and identify *Phytophthora* species involved in common alder (*Alnus glutinosa*) mortality in Spain (**I & V**)
2. To characterized the *Phytophthora* species by analysis their morphology, physiology and molecular traits (**II**)
3. To examine susceptibility of seeds and seedlings of *A. glutinosa* to *Phytophthora* species (**III**)
4. To examine variation in pathogenicity among the three subspecies of *Phytophthora alni* to *A. glutinosa* (**IV**).

MATERIALS AND METHODS

A brief description of materials and methods is given below, but for detailed information, please refer to the original articles (Roman numerals).

Materials

Sampling sites

- Common alder (*Alnus glutinosa*) growing stands with disease symptoms (**I, II & V**).

Phytophthora isolates

- Cultured Spanish isolates of *Phytophthora alni* (**I, II, III & IV**).
- Cultures French isolates of *P. alni* (**II & IV**).
- Cultured Spanish isolates of *P. cinnamomi* Rands, *P. citrophthora* (R.E. Sm. and E.H. Sm.) Leonian, *P. nicotianae* Breda de Haan (= *Phytophthora parasitica* Dastur) and *P. palmivora* (E.J. Butl.) (**III**).
- Cultured Spanish isolates of *P. plurivora* sp. nov. (**IV**).

Plant materials

- Seeds of *A. glutinosa* were obtained from the National Centre for Forest Breeding, Guadalajara, Spain (**III**).
- Seedlings were obtained from Viveros Fuenteamarga S.L., Valladolid, Spain (**V**).
- Vegetative materials of *A. glutinosa* (leaves, branches and twigs) were collected from the province of Salamanca, Spain (**IV**).

Methods

Sampling of diseased and healthy trees

- Trees selected for sampling having symptoms of die-back, small, sparse and yellowish leaves, tarry spots and rusty exudations at collar and lower stems (**I, II, IV & V**).
- Bark samples were taken with the help of a hammer and chisel, placed in glass jars in distilled water and transported to laboratory under cool conditions (**I, II, V**).

- Fully expanded fresh leaves were collected from mature healthy *A. glutinosa* and kept in poly bags moistened with distilled water and transferred to cool boxes to avoid desiccation (**IV**).
- Branches and twigs were collected from both disease-free and disease affected areas, cut into small segments, wrapped in moist tissue during transport and placed in cool boxes (**IV**).

Isolation method

- Small pieces were cut from the active lesions of inner bark, dried on filter paper and plated on selective media V8-PARPH agar. Isolations were made according to a method previously described (Jung and Blashchke 2004) (**I, II & V**).

Identification method

Morphological study

- Colony morphologies were produced on V8 juice agar (V8A) (**I, II & V**), carrot juice agar (CA) (**II**), corn-meal agar (CMA) (**II**) and potato-dextrose agar (PDA) (**II**).
- Sporangia and oogonia were produced according to protocol as described by Jung *et al*. (1999) (**I, II & V**)

Physiological study

- Cardinal and radial growth rates response to temperatures (**I, II, V**), pHs (**II**) and osmotic potentials according to methodology described by Lira-Méndez and Mayek-Pérez (2006) (**II**) were examined on V8A.

Molecular analyses

- Molecular identification protocol adapted from Vilgalys and Hester (1990) (**II**); White *et al*. (1990) (**II & V**); Gardes and Bruns (1993) (**II**); Vainio *et al*. (1998) (**II**); Cooke *et al*. (2000) (**I & V**); Jung and Burgess 2009 (**V**).
- Identification up to subspecies was done following protocols as described by Ioos *et al*. (2005, 2006) (**II**).

Inoculation

- For inoculation with mycelial agar discs (**III, IV & V)**, colonies of the *Phytophthora* species were sub-cultured at 20ºC in the dark onto Petri dishes containing V8 agar.

- In case of zoospore inoculation (**III & IV**), zoospore suspensions were prepared following the method adapted from Denman *et al*. (2005).

Data recording

- Seed germination and seedling mortality percentage were started to record 7 days after inoculation. Data on germination and mortality were taken at every 5 days interval until 42 days and 67 days respectively (**III**).
- Leaves, branches and twigs were examined for lesions 6 days after inoculation and lesion lengths were measured (**IV**) and seedlings were examined 3 months after inoculation for lesions (**V**).

Reisolation

- To confirm the infections caused by the *Phytophthora* spp., small segments from the inoculated plant materials were surface disinfected and placed directly on V8-PARPH agar in order to re-isolate the pathogens (**IV & V**).

Statistical analyses

- Analysis of variance (ANOVA) and Tukey's HSD post-hoc tests ($\alpha = 0.05$) were used to test the effects of pH and osmotic potential (**II**).
- Repeated measures analysis of variance (ANOVA) were performed according to Tukey multiple range test (**III**).
- Three-way fixed factors effects ANOVAs were performed under non-normality and inequality of variances using the generalized Welch procedure and a 0.2 trimmed mean transformation (**IV**) and Poisson regressions were carried out using Generalized Linear Models (GLMs) to count gridlings (**IV**).

RESULTS AND DISCUSSION

A brief description of results and discussion is given below. But for details, please refer to the original articles (Roman numerals)

Phytophthora species involved in the mortality of *Alnus glutinosa* (I, II & V)

Phytophthora alni ssp. *alni and its characteristic features* (**I & II**)

P. alni was recovered from necrotic bark at collar regions and lower stems of diseased *A. glutinosa* in both studies (**I & II**). In culture media, the isolates produced abundant terminal, spherical oogonia which were ornamented with two-celled amphigynous antheridia. Rarely comma-shaped smaller oogonia and very often oogonia with aborted oospores were also observed. Sporangia were non-caducous and nonpapillate, and shape of the sporangia were ovoid to ellipsoid. The length: breadth ratios of sporangia ranged from 1.3 to 1.6. Sporangiophores were simple or sympodial with terminal sporangia proliferating internally, often nested, and extended internal proliferation and wide exit pores. Colony patterns developed on CA were usually appressed and irregular in growth with very limited aerial mycelium, whereas on V8A, colonies had slightly woolly morphology. Besides, fluffy and considerably woolly growth patterns were observed on CMA and PDA. rDNA sequences from internal transcribed spacer (ITS) were compared with the sequences available in the GenBank showed that the sequences of all isolates were nearly identical. On the basis of the best hits and comparison with GenBank another time, the pathogen was identified as *P. alni* ssp. *alni* (**I & II**). As a whole, morphological, physiological and genetical features of the pathogen described in this study corresponded to those of *P. alni* ssp. *alni*, previously known as the 'standard' type (Brasier *et al*. 2004).

Discriminating among the three subspecies of P. alni (**II**)

Analysis of internal transcribed spacer (ITS) region revealed that isolates of *P. alni* ssp. *alni* (**I & II**) showed an unusual ITS polymorphism i.e., dimorphic sites within the ITS sequences. This type of dimorphism has been described as typical of *P. alni* ssp. *alni*, whereas *P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis* tend to be monomorphic (Ioos *et al*. 2006). Ambiguities that have been related to the recent hybrid condition of *P. alni* ssp. *alni*, believed to be the result of a unique or multiple hybridization events of *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis* (Ioos *et al*. 2007; Aguayo *et al*. 2013).

Isolates of *P. alni* ssp. *alni* from (**II**) showed a unique and common pattern of amplifications with a series of species specific primers previously described (Ioos *et al*. 2005, 2006). Amplification with PA primers indicated that the isolates belonged to *P. alni* complex, but failed to differentiate between *P. alni* ssp. *alni*, *P. alni* ssp. *uniformis* or *P. alni* ssp. *multiformis*; PAM amplification excluded *P. alni* ssp. *uniformis*; and TRP and RAS primers amplifications excluded *P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis*, respectively.

P. plurivora and its characteristic features (**V**)

During conducting surveys (**V**) for alder *Phytophthora*, another homothallic species of *Phytophthora* was isolated from active fresh lesions at collar of declining *A. glutinosa*. On culture media, isolates produced smooth-walled spherical oogonia having paragynous antheridia with both plerotic and aplerotic oospores. Sporagia were non-caducous, semipapillate, mainly ovoid and obpyriform, obovoid to limoniform but sometimes distorted or bilobed. Colonies developed on V8 juice agar (V8A) displayed radiate and slightly chrysanthemum-like growth pattern with limited aerial mycelium at the centre. These morphological and physiological features of the pathogen (**V**) corresponded to the characteristics features of *P. plurivora* as described previously by (Jung and burgess 2009). For molecular identification, the internal transcribed spacer (ITS) region of the rDNA was amplified using the protocol as described by (White *et al*. 1990; Cooke *et al*. 2000). Sequences obtained were compared with the reference sequences which appeared in Jung and Burgess (2009).

Impact of pH, osmotic potential and temperature on mycelial growth of *P. alni* ssp. *alni* (II)

Mycelial growth in response to pH

Mycelial growth of *P. alni* ssp. *alni* isolates (**II**) was observed on a wide range of pH. Although, the isolates grew at higher pH, they demonstrated a tendency of low and slow growth with increasing pH value which indicated that they are less tolerant to higher pH. Optimum growth of the isolates of *P. alni* ssp. *alni* (**II**) was recorded at pH 7, which in turn corresponded to the best pH for growth and sporangia formation of *P. alni* as previously determined (Schumacher *et al*. 2006; Kong *et al*. 2012). *Phytophthora* species are water molds in terms of their zoospore activity. Irrigation reservoirs in which pH and other water quality parameters continuously change could have profound

impacts on the survival of many *Phytophthora* (Kong *et al*. 2009). However, studies are necessary to know more about the survival of the pathogens in different aquatic environment under pH stress. In soil environment, *Phytophthora* species are considered to be tolerant to a wide range of pH and increasing pH generally favours growth (Weste 1983; Andrivon 1994). In this view, our findings are very important as *P. alni* could remain active over a wide pH range, being well adapted to both aquatic and soil environment and pose threat to new areas through dispersal.

Mycelial growth in response to osmotic potential

Most favourable growth of the isolates of *P. alni* ssp. *alni* (**II**) was recorded at higher osmotic potential and declined with decreasing osmotic potential. Studies carried out with other *Phytophthora* species also revealed a similar trend for mycelial growth concerning higher and lower osmotic potentials (Sommers *et al*. 1970; Turco *et al*. 2005). The studies showed the importance of a minimum amount of water in the environment for development of *Phytophthora*. Osmotic potential has been identified as an important factor in the growth and ecology of pathogens. Free water in soils or in the environment is essential to all stages of their life cycle (Davis *et al*. 2000). However, water needs may differ among the pathogens throughout their reproduction time and tolerance to dry conditions. For example, *P. quercina* sp. nov. and *P. citricola* Sawada recovered from dry upland sites in Turkey where they were able to survive over extended dry periods in the absence of suitable host (Balci and Halmschlager 2003).

Mycelial growth in response to temperature

Isolates of *P. alni* ssp. *alni* (**I & II**) showed optimum mycelial growth at between 20 to 25°C. This range of temperature for optimum growth is in accordance with previous studies (Brasier *et al*. 1995, 2004) and is the most ideal temperature range at which many *Phytophthora* were reported to be very active and virulent (Harris and Tobutt 1986; Brasier *et al*. 1995; Hardham 2001). Susceptibility of plant species to invasion by *Phytophthora* is strongly temperature dependent (Matheron and Mateika 1993). Temperature is one of the most important factors that influence the growth and sporulation of *Phytophthora* (Doster and Bostock 1988; Horner and Wilcox 1996; Schumacher *et al*. 2006). It is vital to know the optimum temperatures for growth and sporulation of *Phytophthora* as these two factors could aid in disease management when the risk of sporulation and subsequent disease development is high. In addition to that,

environmental factors such as site temperature along with several other risk factors have been accounted which influence the occurrence of disease by *P. alni* (Jung and Blaschke 2004; Schumacher *et al*. 2006; Elegbede *et al*. 2010). Isolates of *P. alni* ssp. *alni* recovered during the study (**II**) did not show any grow at 2 and 32°C. Alder *Phytophthora* has been reported to have less probability to survive in cold winters or under extreme frosts and poor oospores viability (Schumacher *et al*. 2006; Cerny and Strnadova 2012). On the other hand, an increase in the temperature of river water again increases the probability of disease incidence (Thoirain *et al*. 2007). However, Chandelier *et al*. (2006) reported that the sporangia production of *P. alni* was affected by microbial communities present in the river water together with high temperature. Being a thermophilous pathogen with relatively high optimum growth temperatures (22-25°C), temperature might influence the survival of the pathogen and disease development.

Inoculum effects on seed germination and seedling mortality (III)

Seed germination (**III**)

Isolates of *P. alni* ssp. *alni* (**I & III**), *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* (**III**) reduced significantly seed germination when zoospore suspension was used to inoculate. Forty two days after inoculation, all the isolates of the *Phytophthora* species tested hampered significantly germination regardless of the method used for inoculation. When zoospore suspension was applied at the centre of the plate (method CE), no differences were found among the tested isolates, but when suspension was applied in each seed (method IS), *P. cinnamomi* significantly reduced the germination percentage than that of the others. On average, seed germination percentage when inoculation was made for each seed, was 26.04%; and when zoospore suspension was applied at the centre, the percentage was 36.97%. In controls, it was higher than 80% in all cases. Pathogenic ability of *P. alni* on seeds of *A. glutinosa* has been revealed from a similar laboratory test made by Schumacher *et al*. (2006). *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* have not been reported to infect seeds of alder (*Alnus* spp.) to date, but in our study (**III**), these *Phytophthora* have proved their ability to hamper and reduce seed germination under laboratory conditions. So, it seems to point out that *P. alni* and other *Phytophthora* species tested might also infect alder seeds in natural environment.

Seedling mortality (**III**)

Seedling mortality was significantly influenced by the isolates of *P. alni* ssp. *alni* (**I and III**), *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* (**III**), inoculation methods and time period of the experiment. Inoculated seedlings started to die after 7 days of inoculation with a progressive manner reaching the maximum number after 15-20 days. Such maximum mortality was achieved by both isolates of *P. alni* and *P. citrophthora*. Between inoculation methods, differences were found mainly in the first 2-5 measurements depending of the isolate. In those first records, inoculation with the mycelial agar plugs at each seedling (method IT) caused greater seedling mortality than the inoculation in the centre of the plate (method CE). At the end of the experiment all the isolates caused a seedling mortality rate higher than 90%, regardless of the inoculation method. But, *P. cinnamomi* produced a mortality which was 46.9% when inoculation method (method CE) was applied, whereas it was 78.1% for inoculation method (method IT). Controls did not result any seedling mortality. Ability of *P. alni* and *P. citrophthora* to cause seedling mortality has also been revealed from inoculation studies made by Santini *et al.* (2003, 2006). No information is available so far on the pathogenicity of *P. cinnamomi*, *P. nicotianae and P. palmivora* on seedlings of *Alnus* but, in our present inoculation test (**III**), these pathogen species have revealed their capacity to cause seedlings mortality. So, it can be assumed that container grown seedlings of *Alnus* are vulnerable to the *Phytophthora* species as they may exist with other contaminated plants in the same nurseries. Furthermore, as most infected seedlings may not attain emergence and, therefore, this kind of damage might otherwise be attributed to other causes. These results also suggest that seedlings of common alder are at risk to be infected by them and showed relative non-host-specificity of this genus. However, this experiment does not provide reliable findings on host and pathogen interaction at the natural level.

Influence of damage, temperature and sampling location on the pathogenicity of *P. alni* (IV)

Effect of damage (non-wound vs. wound) on lesion development

Pathogenic ability of *P. alni* ssp. *alni*, *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis* (**II**) on wounded and non-wounded leaves of *A. glutinosa* was evaluated using both mycelial agar plugs and zoospore suspension (**IV**). They failed to produce any lesion on inoculated non-wounded leaves, whereas lesions were developed after 6 days of

incubation on inoculated wounded leaves. This study (**IV**) has proved that wounding was an influential factor to cause infection by the subspecies of *P. alni* and they might have entered into the tissues through wounds. Such wounding effect is also consistence with other previous findings (Erwin and Ribeiro 1996; Thomidis 2003; Kaminski and Wagner 2008), who demonstrated that growth of *Phytophthora* was associated with wounds and wounded plant species were more vulnerable to infection than the non-wounded ones. At the end of experiment, when the isolates of three subspecies of *P. alni* were ranked according to their virulence, two isolates of subspecies *P. alni* ssp. *alni* were the most virulent based on length of lesion, although no significant differences in lengths were noted for remaining isolates of *P. alni* ssp. *alni* and the other two subspecies. This means, all three subspecies were equally pathogenic on the wounded leaves of *A. glutinosa*. Pathogenic ability of *Phytophthora* species on leaves of their woody hosts have been well documented where artificial wounding was necessary in most incidences to cause infections and necroses. *In vitro* leaf inoculation studies on leaves of broad-leaved and coniferous trees using zoospores of *P. ramorum* Werres, DeCook & Man in't Veld by Denman *et al*. (2005) showed that necroses and disease incidence increased significantly when wound inoculations were done. Ability of *P. alni* to cause lesions on leaves following artificial wounding and failure to produce any lesions on non-wounded leaves suggest that the pathogen would unlikely to be a foliar invader for alders.

Effect of temperature and sampling location on lesion and girdling formation

Temperature has been reported to influence on growth, reproduction and pathogenicity of *Phytophthora* species (Sujkowski 1987; Sing and Chauhan 1988; Matheron and Matejka 1992). In the current tests (**IV**), temperature has also been considered as a key factor that significantly influenced the pathogenicity of the three subspecies of *P. alni* tested. Isolates of *P. alni* ssp. *alni* and *P. alni* ssp. *multiformis* have appeared to be more virulent on both inoculated twigs and branches of *A. glutinosa* whereas the isolate of *P. alni* ssp. *uniformis* has found relatively less virulent, particularly on branches at all tested temperatures. Similar observations were found in a previous study done by Brasier and Kirk (2001), where pathogenicity test was carried out on detached logs of *A. glutinosa* using several isolates of three subspecies of *P. alni*. Their results also demonstrated that the isolates of *P. alni* ssp. *alni* and *P. alni* ssp. *multiformis* were highly virulent while *P. alni* ssp. *uniformis* were weakly pathogenic on the inoculated

logs of *A. glutinosa*. Largest lesions occurred on excised twigs and branches at 25ºC followed by 20ºC, in case of all isolates of three subspecies. Temperatures range (20ºC-25ºC), was considered as the most suitable range at which *P. alni*, *P. cactorum* var. *applanata* Chester and *P. cryptogea* Pethybr. and Laff. were reported to be very active and pathogenic (Harris and Tobutt 1986; Hardham 2001; Brasier *et al*. 2004). A similar range of temperature was also reported to be ideal for the occurrence of largest lesions on inoculated branches by *P. cactorum* and *P. citrophthora* (R.E. Smith and E.H. Smith) Leonian (Thomidis 2003). A temperature of 25ºC was demonstrated as the most suitable temperature to form girdling on the inoculated branches. Similarly, at the same temperature, girdlings were formed on the inoculated twigs by all three subspecies including *P. alni* ssp. *uniformis*. Here, it is very important to note that, *P. alni* ssp. *uniformis* could not produce any girdling on inoculated branches. Finally, we have suggested that young common alder sprouts may be more vulnerable to infection by *P. alni* ssp. *uniformis* (considered to be weakly pathogenic; Brasier and Kirk 2001) than mature trees. Effect of sampling locations on the virulence of the isolates of three subspecies was considered as negligible, on both inoculated twigs and branches. Here, it could be pointed out that the current disease-free areas are still under threat to infection by *P. alni*.

CONCLUSIONS

1. *Phytophthora alni*, causal agent of alder (*Alnus* spp.) mortality in Europe, is also involved in the mortality and decline process of common alder (*Alnus glutinosa*) in the north and central-western part of Spain. Although *P. alni* has been reported as the primary agent of riparian common alder mortality in Spain, *P. plurivora* has also been isolated from declining *A. glutinosa*. Further investigations are necessary in order to examine the severity and damage caused by this *Phytophthora* species on *A. glutinosa*. In addition, further surveys are crucial in order to locate the presence of *P. plurivora* in different alder growing areas of Spain.

2. *P. alni* can easily be distinguished from other homothallic *Phytophthora* species by its unique morphological, physiological and genetic traits. Features like two-celled ornamented oogonia with amphigynous antheridia, nonpapillate and non-caducous sporangia, growth patterns on culture media, optimum and maximum temperatures for growth have given the distinctiveness to alder *Phytophthora*. Considering its morphological, physiological features, and ITS sequences, the isolates recovered from common alder clearly belong to *P. alni* spp. *alni* which is considered to be the most aggressive one among the three subspecies of *P. alni*. Additional surveys are essential in order to trace out the existence of remaining two subspecies of *P. alni* (*P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis*) as they might convey equal threat to the existence of riparian alder populations along the rivers and other waterways in Spain.

3. Results show that *P. alni* ssp. *alni* and other *Phytophthora*, such as *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* have proved their ability to infect seed and seedlings of *A. glutinosa*. Susceptibility of such reproductive materials to *P. alni* is certainly a threat as the pathogen can be brought into nurseries and distributed with seeds and seedlings to plantations or riparian sites. In addition to that, as other *Phytophthora* spp. are able to infect alder seeds and seedlings, it is important to apply further controls on nursery management and irrigation system to avoid dissemination of *Phytophthora* to

natural ecosystems. Since plantation establishment with infected alder seedlings is one of the primary pathways by which *P. alni* spread, so careful management and selection of forest reproductive material is important.

4. Pathogenicity tests performed on the detached plant materials (leaves, twigs and branches) of *A. glutinosa* concerning damage (wound vs. non-wound), temperature (15, 20, 25 and 30°C) and sampling location (disease-free areas vs. diseased areas) has confirmed variation in the virulence among the isolates of three subspecies of *P. alni*. Wounds and temperatures have appeared as the most important influential factors in the process of lesion development. Results have showed that wounding has significantly influenced the virulence of the isolates compared to non-wound and control. Seasonal changes effect on the lesion development by *Phytophthora* is dependent on temperature. Results have showed that a temperature range suitable for optimum mycelial growth of *P. alni* at which the tested isolates were more virulent to produce largest lesions on the inoculated twigs and branches than the rest. The results have also demonstrated that sampling locations could not produce any significant effect on the pathogen regarding lesion lengths. Here, it seems to point out that the current disease-free areas for *P. alni* would be susceptible to be infected if the pathogen spreads.

CONCLUSIONES

1. *Phytophthora alni*, causante de la mortalidad de alisos (*Alnus* spp.) en Europa, está también implicado en la mortalidad y decaimiento del aliso común (*Alnus glutinosa*) en el norte y centro occidental de España. Aunque *P. alni* ha sido identificado como el agente principal de la mortalidad de los alisos en España, *P. plurivora* ha sido también aislado en alisos enfermos. Más investigación es necesaria para conocer la distribución de *P. plurivora* en España y examinar su virulencia y daños causados en *A. glutinosa.*

2. *P. alni* puede ser fácilmente distinguible de otras especies homotálicas del género *Phytophthora* por sus características morfológicas, fisiológicas y genéticas. Sus oogonios ornamentados con anteridios bicelulares y posicionados de forma anfigina, sus esporangios no caducos y no papilados, junto con la morfología de sus colonias en medio de cultivo, temperaturas óptimas y máximas de crecimiento diferencian a *P. alni*. A tenor de las características morfológicas, fisiológicas y de las secuencias de la región ITS, los aislamientos obtenidos corresponden claramente a *P. alni* spp. *alni*, la cual es considerada la más agresiva de las tres subespecies de *P. alni*. Otros muestreos de campo serían fundamentales para determinar la existencia de las otras dos subespecies de *P. alni* (*P. alni* ssp. *multiformis* y *P. alni* ssp. *uniformis*) en otros ríos y cursos fluviales de España.

3. Los resultados muestran que *P. alni* ssp. *alni* y otras especies del género *Phytophthora*, tales como *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* son capaces de infectar semillas y plántulas de *A. glutinosa*. La susceptibilidad de estos materiales vegetales de reproducción a *P. alni* es una amenaza porque el patógeno podría ser introducido en viveros y posteriormente diseminado a plantaciones y bosques de ribera. Además, teniendo en cuenta que otras especies del género *Phytophthora* son capaces de infectar semillas y plántulas de aliso, es fundamental llevar a cabo más controles en los viveros y en los sistemas de riego para evitar la propagación de estos patógenos a los ecosistemas naturales. Teniendo en cuenta que plantaciones con plántulas de

aliso infectadas es una de las vías principales para la dispersión de *P. alni*, un correcto manejo de los viveros y una selección adecuada del material forestal de reproducción a utilizar es fundamental.

4. Los ensayos de patogenicidad llevados a cabo en material vegetal (hojas, ramillos y ramas) de *A. glutinosa* en relación al método de inoculación (provocando una herida o sin herida), temperatura (15, 20, 25 y 30°C) y localización (zona con o sin presencia de la enfermedad) han confirmado las diferencias en virulencia entre los aislamientos de las tres subespecies de *P. alni*. Provocar una herida y la temperatura fueron los factores más relevantes en el desarrollo de la enfermedad. En concreto, generar una herida incrementaba el daño causado por todos los aislamientos en las hojas, mientras que en el tratamiento sin daño ningún aislamiento fue capaz de causar infección. Del mismo modo, todos los aislamientos se mostraron más virulentos en los rangos de sus temperaturas óptimas de crecimiento en medio de cultivo, produciendo los mayores daños tanto en ramillos como en ramas. Este estudio también ha demostrado que la localización del material vegetal utilizado (desde zonas infectadas o libres de la enfermedad) no tiene ningún efecto en el daño causado por el patógeno. Lo cual parece indicar que las zonas libres de la enfermedad serían también susceptibles de ser infectadas si el patógeno llegase a dispersarse en estas zonas.

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ARTICLES

ARTICLE I

Phytophthora alni ON *Alnus glutinosa* REPORTED FOR THE FIRST TIME IN SPAIN

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ABSTRACT

Surveys were carried out for alder *Phytophthora* along the river Miño, Galicia, Spain. Bark samples including cambium were taken from the active fresh lesions at collar of diseased *Alnus glutinosa* and isolates were obtained by placing small segments on a selective agar medium. The isolates were homothallic mainly with two-celled amphigynous antheridia, occasionally comma-shaped oogonia having ornamentation. In soil-extract they produced nonpapillate, ellipsoid to ovoid sporangia. Colony developed on V8 agar, had slightly woolly morphology and showed radial growth. DNA of the ITS region was amplified and sequences obtained were compared with the GenBank showed 99% identity with *Phytophthora alni* ssp. *alni*. This is the first report of *P. alni* in Spain.

KEYWORDS: *Alnus glutinosa*, amphigynous, oogonia, sporangia, *Phytophthora alni*.

RESUMEN

Muestreos de campo para la detección de *Phytophthora alni* fueron llevados a cabo en el curso del río Miño (Galicia, España). Muestras de corteza con cambium fueron tomadas del cuello de la raíz y las partes bajas del tronco de alisos enfermos con daños frescos. Los aislamientos fueron obtenidos tras cultivar pequeños fragmentos de dicho material vegetal en un medio de cultivo selectivo. Los aislamientos fueron homotálicos, generalmente con anteridios bicelulares posicionados de forma anfigina y oogonios ornamentados. Produciendo en los extractos de suelo esporangios no papilados, con formas desde elipsoides a ovoideas. Las colonias desarrolladas en el medio de cultivo V8 agar mostraron un aspecto ligeramente lanoso con un crecimiento radial. El ADN de la región ITS fue amplificada y sus secuencias confrontadas con las depositadas previamente en el GenBank obteniéndose una homología del 99% con las identificadas como *P. alni* ssp. *alni*. Este es la primera cita de *P. alni* en España.

PALABRAS CLAVE: *Alnus glutinosa*, anfigina, oogonio, esporangio, *Phytophthora alni*.

Since the mid 2000s extensive mortality of common alder [*A. glutinosa* (L.) Gaertn.] has been observed along many rivers of northern Spain (Tuset e*t al*. 2006). Symptoms include sparse yellowish and small-sized foliage, dieback of branches, increased fruit production and dark-stained necrosis of the bark at the collar and lower stem. These resemble symptoms of the root and collar rot epidemic of alders which is caused by the different subspecies of the host-specific pathogen *Phytophthora alni* (Brasier and S.A. Kirk) and has led to high mortality of riparian alders across 14 countries in Western, Central and Northern Europe (Gibbs *et al*. 2003; Jung and Blaschke 2004).

In September 2009, samples from five symptom-bearing *A. glutinosa* trees were collected along the river Miño (Lugo, Galicia, 42º59′N 7º32′W, 367 m above sea level). Bark samples, including the cambium, were taken from the upper 20 cm of the orange-brown active lesions, placed in distilled water and transported to the laboratory in cool jars. Over 2–3 days the water was replaced four times per day in order to remove excess polyphenols. Small pieces (c. 4 × 4 × 2 mm) were cut from the lesions, blotted dry and plated onto V8-PARPH agar (Jung and Blaschke 2004). After five days incubation at 20ºC, tentative *Phytophthora* isolates were obtained from four trees. The isolates were homothallic with amphigynous antheridia, predominantly two-celled, and produced ornamented, occasionally comma-shaped oogonia ranging from 33 to 50 μm in diameter. In soil-extract they produced nonpapillate, ellipsoid to ovoid sporangia (60 × 49 μm). On V8 agar, colonies had a slightly woolly morphology and showed radial growth rates of 3.6–7.5, 2.2–5.4 and 0.0 mm day^{-1} at 20, 25 and 30 ºC, respectively. These features resemble those of *P. alni* ssp. *alni* (Brasier *et al*. 2004). DNA of the ITS region was amplified as described previously (Cooke *et al*. 2000). The sequences of all isolates were identical (GU175431) and a Blast search at GenBank showed 99% identity with *P. alni* ssp. *alni* (AF139366.1). This is the first report of *P. alni* on *A. glutinosa* in the Iberian Peninsula.

Common alder is the most widespread species of *Alnus* occurring in Spain. It has high ecological value for its conservation role and used for timber production, riverbank and slope stabilization because of its excellent soil amelioration properties. But mortality of *A. glutinosa* due to *P. alni* may lead drastic changes in the riparian ecosystems in coming years, causing economic losses and ecological changes. That's why, it is important to apply control measures to prevent spread of the disease to natural ecosystems. Surveys are needed, which will allow the administration to adopt proper

management strategies. In addition to that, a common strategy and co-ordinated programme for limiting the transportation of infected plants from country to country is needed. Further studies and surveys are necessary to carry out in other alder growing areas of Spain in order to find out whether *P. alni* and other *Phytophthora* are involved in the mortality of *A. glutinosa*.

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ARTICLE II

MORPHOLOGICAL, PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Phytophthora alni* ISOLATES FROM WESTERN SPAIN

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ABSTRACT

During extensive surveys for *Phytophthora* associated with alder mortality in western Spain, isolates were consistently recovered from necrotic bark of the collar and lower stem of *Alnus glutinosa*. Morphological and molecular characteristics of the selected isolates together with their physiology were examined. Morphologically, the isolates were homothallic and characterized by abundant production of terminal oogonia predominantly with two-celled amphigynous antheridia exhibiting ornamentation. Simple sporangiophores were observed bearing terminal, non-papillate, ellipsoid to ovoid sporangia. Colony growth patterns developed on V8 juice agar (V8A), carrot agar (CA), corn-meal agar (CMA) and potato-dextrose agar (PDA) showed uniform, radial or irregular growth patterns with appressed and/or woolly morphology. Temperature (2°C, 5°C, 10°C, 15°C, 20°C, 25°C, 27°C, 30°C and 32°C), pH (5, 7, 9 and 11) and osmotic potential (–0.6, –1.2, –1.8 and –2.4 MPa) significantly influenced radial mycelial growth of the isolates under laboratory conditions. Molecular analyses including ITS DNA region sequencing and subspecies specific primers amplification confirmed identification of the isolates as *Phytophthora alni* ssp. *alni*. Further research is under way to carry out more surveys in order to determine the extent of damage and severity of the disease in different regions of Spain.

KEYWORDS: *Alnus glutinosa*, amplification, decline, oogonia, oomycetes, sporangia.

RESUMEN

Intensos muestreos de campo fueron llevados a cabo para la detección de *Phytophthora* causando mortalidad en alisos del oeste de España, obteniéndose aislamientos a partir de corteza necrótica del cuello de la raíz y las zonas bajas del tronco de *Alnus glutinosa*. Las características morfológicas, fisiológicas y genéticas de los aislamientos seleccionados fueron estudiadas. Morfológicamente, los aislamientos fueron homotálicos y caracterizados por la abundante producción de oogonios terminales, ornamentados y generalmente con anteridios bicelulares posicionados de forma anfigina. Esporangióforos simples fueron observados manteniendo esporangios terminales, no papilados y con formas de elipsoides a ovoideas. Las colonias desarrolladas en zumo V8 agar (V8A), zanahoria agar (CA), maíz agar (CMA) y patata-dextrosa agar (PDA) mostraron un crecimiento uniforme, radial o irregular de poco grosor y de aspecto lanoso. La temperatura (2°C, 5°C, 10°C, 15°C, 20°C, 25°C, 27°C, 30°C y 32°C), pH (5, 7, 9 y 11) y el potencial osmótico (–0.6, –1.2, –1.8 y –2.4 MPa) influyeron significativamente en el crecimiento micelial de los aislamientos bajo condiciones de laboratorio. Análisis moleculares de las secuencias de ADN de la región ITS de los aislamientos amplificados con cebadores específicos confirmaron que los aislamientos correspondían a *Phytophthora alni* ssp. *alni*. Más investigación está siendo llevada a cabo para determinar la extensión y severidad del daño ocasionado por esta enfermedad en otras regiones de España.

PALABRAS CLAVE: *Alnus glutinosa*, amplificación, decaimiento, oogonio, oomicete, esporangio

INTRODUCTION

In Spain, common alder (*Alnus glutinosa* L. Gaertn.) is the most widespread species among alders which is distributed along streams, rivers and in wet woodland ecosystems. It grows well on clay and wet soils in association with tree species like *Fraxinus* spp., *Populus* spp., *Salix* spp. and other. *Alnus* has high ecological value because of its conservation role and is widely planted in programs of riverbank restoration and stabilizing slopes.

Alder decline caused by *Phytophthora alni* (Brasier & S.A. Kirk) was initially observed along the rivers and the horticultural shelterbelts in southern Britain in the early 1990s (Gibbs *et al*. 1994) and, subsequently, in many other European countries (Brasier *et al*. 1995, 2004; Brasier and Kirk 2001; Gibbs *et al*. 1999, 2003; Jung and Blaschke 2004). Affected trees show typical die-back, small sparse and yellowish leaves, excessive fructification, and tarry and rusty exudates on the surface of the bark at collar and lower stem (Gibbs *et al*. 1999). However, mortality of *A. glutinosa* in the Iberian Peninsula was first observed along many rivers of northern Spain by Tuset et al. (2006), the identification of *P. alni* as the causative pathogen of common alder decline has recently been reported (Solla *et al*. 2010; Varela *et al*. 2010, 2012).

At first, it was believed that the *Phytophthora* sp. responsible for alder mortality was similar to *P. cambivora* (Petri) in its gametangial morphology while it differed in homothalism, colony growth patterns, optimum temperature for growth, high level of zygotic abortion and poorly developed oogonia (Brasier *et al*. 1995, 1999). Later, it was hypothesized that the *Phytophthora* engaged in alder mortality evolved as a result of hybridization between heterothallic *P. cambivora* and another unknown taxon of *Phytophthora* closely related to homothallic *P. fragariae* Hickman (Brasier *et al*. 1999). Further investigation by Brasier *et al*. (1999) demonstrated that the alder *Phytophthora* consisted of a variety of heteroploid hybrid species and later they divided it into a ‘standard’ type and several variants. Analysis of the internal transcribed spacer (ITS) region revealed that the isolates of ‘standard’ type displayed an unusual ITS polymorphism i.e., dimorphic sites within the ITS sequences while the variants were monomorphic for the same genome region or monomorphic at some sites and dimorphic at other sites (Brasier *et al*. 1999, 2004). Brasier *et al*. (2004) formally named alder *Phytophthora* as *P. alni* and based on morphological studies, cytological evidences and

genetic data, and further they divided *P. alni* into three subspecies: *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 the Dutch, German and UK variants. More recent genetic studies have hypothesized that Paa has occurred from a single or multiple hybridization event between Pau and Pam, although the origin and genetic diversity of these taxa are still under discussion (Ioos *et al*. 2006, 2010).

All three sub-species of *P. alni* as described (Brasier *et al*. 1999, 2004) showed distinctive colony morphologies and different growth-temperature relations on culture media. Paa produced ornamented oogonia and elongated two-celled antheridia whereas both Pau and Pam produced unique unusual combinations of oogonial structures and amphigynous or perigynous antheridia. Likewise, while Pau formed oogonia having smooth surface, Pam produced highly ornamented oogonia. Further morphological studies showed that the isolates of Paa produced less viable oospores than Pau or Pam.

Although, a few reports have been published on alder mortality caused by *P. alni* in Spain (Solla *et al*. 2010; Varela *et al*. 2010, 2012), no detailed studies concerning characterization of the Spanish isolates of *P. alni* are available to date. Therefore, the present study was undertaken to characterize the isolates of *P. alni* by analysing their morphology, physiology and genetic traits.

MATERIALS AND METHODS

Sampling and Phytophthora isolation

Study sites were focused on several rivers (Table 1), located in the western part of Spain. The sites were characterized by seasonal flooding and distributed in areas where mortality of common alder was previously observed. Samplings were made during July–September 2010 and August–October 2011. Ten trees were selected and sampled from each study site on the basis of external symptoms (i.e., small and yellow leaves, tarry spots and rusty exudations, increased fruit production). Bark samples together with the cambium were taken from active fresh lesions at collar region with the help of a hammer and chisel, placed in glass jars in distilled water and transported to laboratory under cool conditions. Over 48 hours, water was replaced several times per day in order to remove excess of polyphenols. Small pieces were cut from the lesions, dried on filter

paper and plated on selective media V8-PARPH agar [V8 juice agar media amended with 10 µg/mL pimaricin, 200 µg/mL ampicillin, 10 µg/mL rifampicin, 25 µg/mL pentachloronitrobenzene (PCNB), and 50 µg/mL hymexazol]. Petri dishes were incubated at 22°C in the dark and examined regularly for *Phytophthora* colonies. Colonies developed on isolation plates were transferred to fresh V8A plates for initial confirmation.

Table 1. Location of the study sites

Name of the site	Region	River	Latitude	Longitude
Puente de Castraz a Sancti-Spiritus	Castraz	Huebra-Yeltes	40°42'02.8"N	06°20'41.1"W
El Manantio	Sancti-Spiritus	Huebra-Yeltes	40°44'21"N	06°20'41"W
Puente Bogajo a Yecla de Yeltes	Bogajo	Huebra-Yeltes	40°56'51"N	06°32'38.3"W
Aceña de Gema o Molino de la Tomasa	Yecla de Yeltes	Huebra-Yeltes	40°57'11.3"N	06°32'46.3"W
La Playa	Puente de Congosto	Tormes	40°29'01.1"N	05°30'53.6"W
Puente Fresno-Poblado Santa Teresa	Fresno Alhandiga	Tormes	40°42'47.3"N	05°35'29.9"W
El Chorrón	La Maya	Tormes	40°40'26.3"N	05°36'10.8"W
Huertas de Arriba	Alba de Tormes	Tormes	40°48'26.7"N	05°31'35.1"W
Puente Romano	Salamanca	Tormes	40°57'29.6"N	05°40'11.7"W
Área recreativa La Chopera	Fuenteguinaldo	Águeda	40°21'11.7"N	06°41'23.9"W
Molino de la Copera	Castillejo de Martín Viejo	Águeda	40°42'51.7"N	06°39'33.9"W
Alquería Palomar	Ciudad Rodrigo	Águeda	40°36'10.4"N	06°33'33.5"W
Molino de la Moretona	Ciudad Rodrigo	Águeda	40°35'50.4"N	06°32'55.6"W
Llano Molino	Peñaparda	Águeda	40°18'54.6"N	06°40'03.9"W
Teso Cubo	Peñaparda	Águeda	40°19'51"N	06°39'16"W
El Tornadizo	El Tornadizo	Águeda	40°32'27.2"N	05°53'14.9"W
Puente Casas del Conde Mongarraz	Casas del Conde	Alagón	40°30'18.4"N	06°02'44.3"W
Puentes del Alagón	Garcibuey	Alagón	40°29'53.9"N	05°56'38.6"W
Pontón del Coto	Bejar	Cuerpo de Hombre	40°22'07.0"N	05°45'17.4"W

Phytophthora isolates

The isolates used in the morphological, physiological and molecular studies are given in Table 2. Isolates recovered in the present study were identified morphologically by examining their reproductive structures and comparing with those of alder *Phytophthora* isolates obtained from INRA (UMR Interactions Arbres-Microorganismes, Champenoux, France) and the descriptions of the *Phytophthora* spp. published (Brasier *et al*. 2004). To carry out their genetic identification, all isolates from the present study were used for sequence analyses on the ITS region and for subspecies specific DNA analyses, along with a panel of reference isolates.

Table 2. Origin of alder *Phytophthora* isolates assessed in this study

Isolate	Identification	Year isolated	Owner	Country	Experiments[a]	GenBank Accession No.
PA2008	PAA	2010	UVa	Spain	g, s, m	KJ659836
PA2010	PAA	2010	UVa	Spain	c, g, s, m, p	KJ659834
PA4017	PAA	2010	UVa	Spain	g, s, m, p	KJ659839
PA4018	PAA	2010	UVa	Spain	g, s, m	KJ659844
PA4020	PAA	2010	UVa	Spain	g, s, m	KJ659833
PA5024	PAA	2010	UVa	Spain	g, s, m	KJ659845
PA5029	PAA	2010	UVa	Spain	c, g, s, m, p	KJ659843
PA6034	PAA	2010	UVa	Spain	g, s, m	KJ659841
PA6035	PAA	2010	UVa	Spain	g, s, m	KJ659842
PA7040	PAA	2011	UVa	Spain	g, s, m	KJ659846
PA7051	PAA	2011	UVa	Spain	c, g, s, m, p	KJ659835
PA7054	PAA	2011	UVa	Spain	g, s, m	KJ659840
PA8055	PAA	2011	UVa	Spain	c, g, s, m, p	KJ659837
PA8059	PAA	2011	UVa	Spain	g, s, m	KJ659838
PA8060	PAA	2011	UVa	Spain	g, s, m	KJ659847
PAA129*	PAA	2003	INRA	France	m	DQ012518
PAA314*	PAA	2008	INRA	France	m	–
PAA354*	PAA	2008	INRA	France	m	–
07dur31*	PAM	2007	INRA	France	m	–
PAM391*	PAM	2009	INRA	France	m	–
PAM393*	PAM	2009	INRA	France	m	–
PAU300*	PAU	2008	INRA	France	m	–
PAU542*	PAU	2009	INRA	France	m	–
PAU624*	PAU	2009	INRA	France	m	–

*Reference isolates.
UVa= University of Valladolid, Spain.
INRA= National Institute of Agronomic Research, Champenoux, France.
a= c, used in colony growth patterns observation; g, used in gametangial measurements; s, used in sporangial measurements; m, used in molecular studies; p, used in physiological studies (growth-temp, growth-pH and growth-osmotic potential relationships).

Morphological studies

Colony morphology

Colony morphology of the isolates were examined on V8 juice agar (V8A; 100 ml V8 juice, 3 g CaCO3, 16 g agar, 900 ml distilled water), carrot juice agar (CA; 100 ml carrot juice, 3 g CaCO3, 16 g agar, 900 ml distilled water), corn-meal agar (CMA-Sigma) and potato-dextrose agar (PDA-Sigma). Agar discs cut from the leading edges of 5-d-old colonies (5 mm diam.) were placed upside down in the centre of Petri dishes containing test media (one plug per plate) and incubated at 20°C in the dark. Colony growth patterns were examined after 6 days.

Morphology of sporangia and oogonia

Sporangia and oogonia were produced and measured according to the methodologies as described by Jung *et al*. (1999). Small agar discs (*ca*. 15 mm x 15 mm) were cut from the growing edge of 5-d-old cultures grown on V8A at 20°C in the dark, placed in 90 mm Petri dishes and flooded with non-sterile soil extract (100 g soil taken from an agricultural field suspended in 900 ml de-ionized water for 24 h at room temperature and then filtered), and incubated at 20°C in the dark. The soil extract was decanted and replaced again after 12 h. After incubation at 20°C in the dark for 24-72 h, dimensions and characteristic features of mature sporangia per isolate were chosen randomly measured at x400 magnification under a compound microscope in the laboratory. For production of gametangia, small agar discs (*ca*. 15 mm x 15 mm) were cut from the centre of 3 weeks old isolates cultured on V8A at room temperature in the dark. For each isolate, characteristics features of 50 mature oogonia, oospores and antheridia were examined at x400 magnification.

Physiological studies

Temperature-growth relationships

To test the effect of temperature on mycelial growth, isolates were grown on V8A media in Petri dishes for 2 days at 20°C in the dark to stimulate growth. After that, colony margins were marked and the plates were transferred to 2, 5, 10, 15, 20, 25, 27, 30 and 32°C. Measurements were taken after 6 days along two lines intersecting the centre of the inoculum at right angles subtracting the width of inoculation disc and radial growth rates (mm d^{-1}) were calculated. Three replicated plates were used for each isolate per temperature combination.

pH-growth relationships

To determine the effect of pH on mycelial growth of the isolates, HCl or KOH 1N was added to V8A media until the pH values (5, 7, 9 and 11) required were obtained. V8A plates inoculated with agar discs (5 mm x 5 mm) were incubated for 2 days at 20ºC in darkness and colony margins were marked. Three replicated plates per isolate were placed to 25ºC in the dark and radial growth (mm d^{-1}) was determined as described previously.

Osmotic potential-growth relationships

The effect of osmotic potentials on mycelial growth was determined on V8A media. Mycelial plugs obtained from the growing edges of colonies were transferred to the centre of V8A plates amended with KCl prior to sterilization to obtain osmotic potential ($\psi\pi$) values of –0.6 MPa, –1.2 MPa, –1.8 MPa and –2.4 MPa according to Lira-Méndez and Mayek-Pérez (2006). Non-amended V8A (0 MPa) was used as control. Three replicated plates per isolate were incubated at 25°C in darkness and radial growth (mm d^{-1}) was calculated as described previously.

Molecular analyses

DNA extraction

DNA was extracted from 8-d-old pure fresh mycelia grown at 22°C in the dark on V8A media covered by thin layer of sterile cellophane membrane. Fresh mycelium was harvested from cellophane surface, ground for 5 min with three 3-mm tungsten carbide beads at a frequency of 30 Hz using a mill grinder (Retsch Ball Mill MM301), and treated following the protocol by Vainio *et al*. (1998) in which phenol-SEVAG was replaced by SEVAG (the ratio 24:1 of Chloroform and isoamyl alcohol). Genomic DNA was stored at -20ºC until use.

ITS region sequencing

ITS region was amplified using ITS1-F and ITS4 primers (Vilgalys and Hester 1990; White *et al*. 1990; Gardes and Bruns 1993) in a total volume of 20 µL in the following conditions: an initial denaturation step of 5 min at 95ºC; 40 cycles of 30s at 95ºC, 30s at the specific annealing temperature (53ºC) and 1 min at 72ºC; and a final extension step of 7 min at 72ºC. Pureness and concentration of the amplicons were assayed by electrophoresis on 1.3% agarose-TAE gels stained with 0.5 $mgml^{-1}$ ethidium bromide, before a re-amplification reaction in the same conditions and in total volume of 50 µL. The final amplification products were purified with NucleoSpin® Extract II 10/2007 Rev. 06 (Macherey-Nagel Gmbh and Co.KG) following the manufacturer instructions and sent to SECUGEN S.A. (Madrid, Spain) for sequencing in both directions. An agarose gel electrophoresis was performed after each amplifying and purifying step to verify the quality and concentration of the amplicons.

All sequences were manually end-trimmed and reviewed before aligning. Those sequences below 65% HQ (i.e., high quality untrimmed bases in the chromatogram) or shorter than 500 bp were discarded for further analysis. Forward and reverse sequences were pairwise aligned using the Global alignment with free end gaps option, default parameters (i.e., cost matrix of 65% similarity (5.0/-4.0) and a gap open/extension penalty of 12/3). These corrected sequences were submitted to GenBank. Finally, all isolates sequences were aligned using ClustalW with an open/extend gap penalty of 12/3 and free end gaps. A consensus sequence was constructed and compared to GenBank through BLASTn program, using default parameters (gap open/expand costs of 5/2). Compilation, alignments and comparison with databank were done using Geneious R7 v 7.0.6 software package (Biomatters Ltd.)

Subspecies-specific identification

Four subspecies specific primer pairs: (i) PA-F/PA-R, GGT GAT CAG GGG AAT ATG TG/ATG TCC GAG TGT TTC CCA AG; (ii) PAM-F/PAM-R, CTG ACC AGC CCC TTA TTG GC/CTG ACC AGC CAT CCC ACA TG; (iii) TRP-E1-1F/TRP-E3-1R, GAG GAG ATC GCG GCG CAG CG/GCG CAC ATR CCG AGV TTG TG; and (iv) RAS-E1-1F/RAS-E5-1R, ATG AAC CCC GAA TAG TRC GTG C/TGT TSA CGT TCT CRC AGG CG) were selected from previous studies (Ioos *et al*. 2005, 2006). PCR amplification assays with these primers were performed in separate reactions for each primer pair in a total volume of 20 µL containing 30-60 ng of template DNA, 0.2 µM of each forward and reverse primers, 0.4 mM dNTPs, 2.5 mM Cl_2Mg, 1 U *Taq* polymerase (AmpliTaq® DNA polymerase with GeneAmp® Applied Biosystems New Jersey, USA) and 1 x *Taq* polymerase buffer in sterile milliQ water. All amplifications were carried out in a GeneAmp®PCR System 9700 (Applied Biosystems, New Jersey, USA) in the conditions described for ITS region using an annealing temperature of 53 or 50ºC. Amplicons were visualized after electrophoresis in 1.3 % agarose gel in TAE 1x stained with 0.5 $mgml^{-1}$ ethidium bromide.

Statistical analysis

Analysis of variance (ANOVA) and Tukey's HSD post-hoc tests ($\alpha = 0.05$) were used to test the effects of pH and osmotic potential on mycelial growth of the isolates. Statistical analyses were performed using R software environment (R-Development-Core-Team 2008).

RESULTS

Isolation of Phytophthora

Isolates of *Phytophthora alni* ssp. *alni* were consistently isolated from necrotic bark at the collar region of *A. glutinosa* by direct plating on to V8-PARPH agar.

Morphological characterization

Colony morphology

Colonies of the isolates showed distinct growth patterns on four different test media (Figure 1). On V8A, the isolates produced uniform and radial growth patterns and had slightly woolly morphology. Colony developed on CA, were usually appressed with limited aerial mycelium and a little woolly morphology at the edge of the Petri dishes, often irregular in outline, faster or slower growing areas often failed to grow up to edges of the Petri dishes. Colony morphology on CMA and PDA, was usually uniform and dense and produced fluffy aerial growth patterns.

Sporangial morphology

The isolates did not produce sporangia on solid agar. However, they produced sporangia when agar plugs from margins of the actively growing culture on V8A were completely submerged in Petri dishes with non-sterile soil extract diluted in distilled water. Terminal sporangia were borne singly or in cluster on long sporangiophores. Sporangia were non-caducous and non-papillate (Figure 2a-e) and formed conspicuous basal plugs in empty sporangia (Figure 2f). Sporangial shape varied from ovoid (Figure 2 a,b,d,e) to ellipsoid (Figure 2c) with a rounded or occasionally tapered base. Lengths x widths ranged from 34–66 x 25-48 μm; averages were 50.8-61.1 x 39.2-48.9 μm (Table 3), and average length: width ratio was 1.3-1.6. Sporangiophores were simple or sympodial (Figure 2e). Zoospores were released through wide exit pores (Figure 2f). Nested and extended internal proliferations were noted (Figure 2g-i) but no chlamydospores were observed.

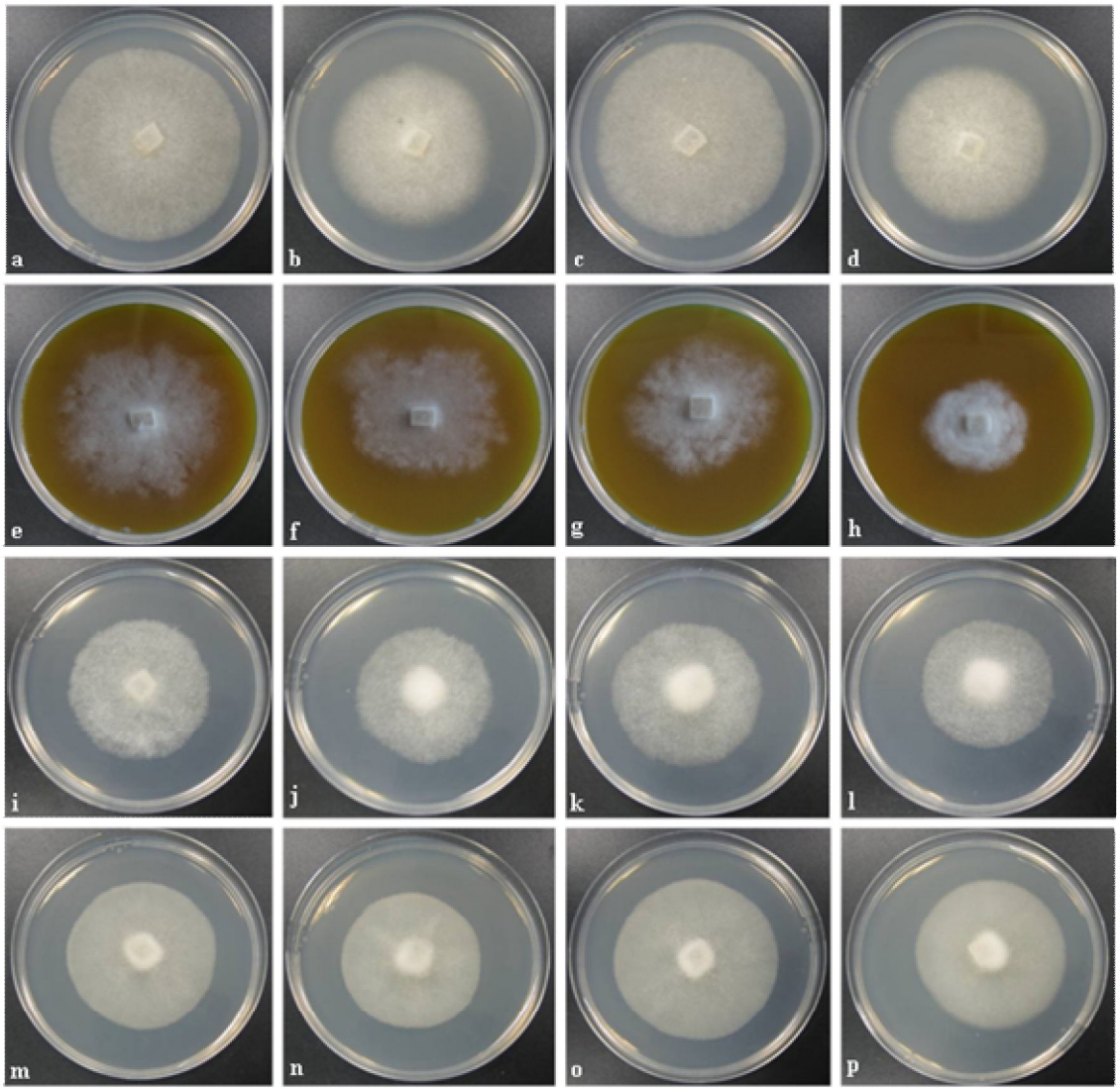

Figure 1. Colony morphology of the isolates of *P. alni* ssp. *alni* at 20ºC on V8A (a-d), CA (e-h), CMA (i-l) and PDA (m-p) after 6 days. Isolates, PA2010 (a-m), PA5029 (b-n), PA7051 (c-o) and PA8055 (d-p)

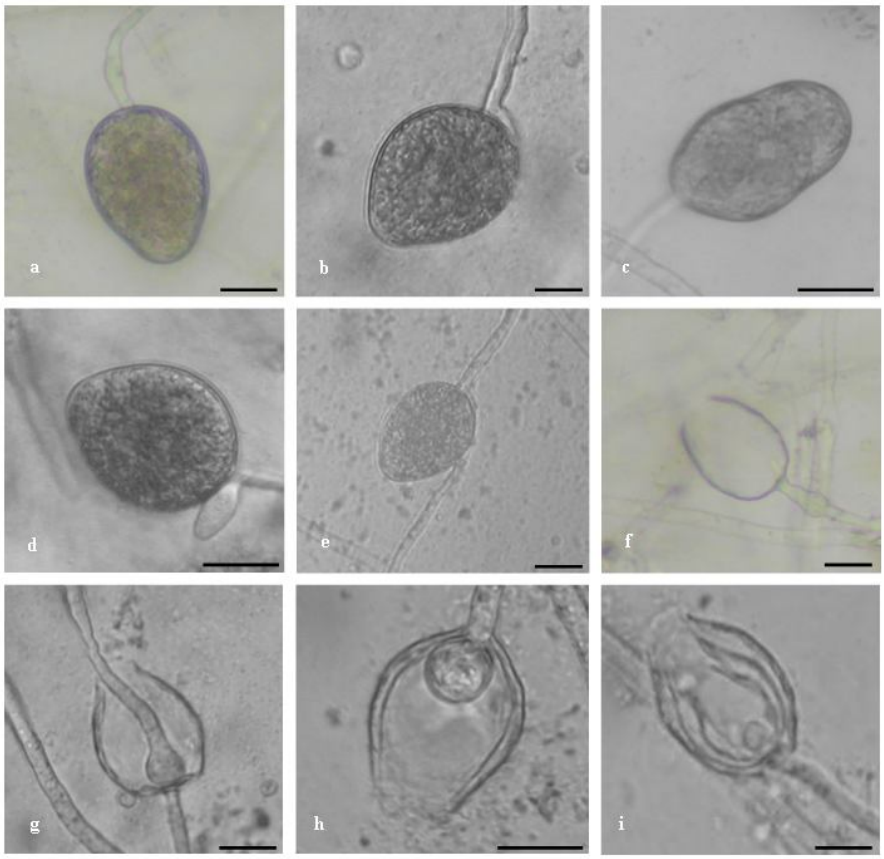

Figure 2. Non-papillate sporangia and sporangiophores of *P. alni* ssp. *alni* produced on V8A flooded with non-sterile soil extract: (a-b). ovoid sporangia; c. an ellipsoid sporangium; d. sporangiophore holding a mature sporangium where a second sporangium is germinating at the point of attachment; e. sympodial brancing; f. an empty sporangium showing a wide exit pore; (g-h). nested interal proliferation; i. remains from nesting. Bar = 25 µm

Gametangial morphology

Gamentangia were produced in single culture on V8A (Figure 3a-l). Oogonia were borne terminally, had ornamentation or wavy walls and were usually spherical, produced predominantly two-celled amphigynous antheridia (Figure 3a-f), sometimes paragynous antheridia (Figure 3i-j), and occasionally comma-shaped oogonia (Figure 3g-h). Average diameter of mature oogonia ranged 38.8-48.5 µm; overall diameter ranged 32-57 µm (Table 3). However, a small proportion of oogonia were smaller in diameter ranged 19-29 µm (Figure 3k-l). A high proportion of oogonia were aborted (Figure 3d-e) and rate of abortion varied. Average diameters of oospores in mature oognia ranged 31.9-42.3 µm; overall diameters ranged 22-48 µm (Table 3). Antheridial length ranged 13-29 µm where as width ranged 10-21 µm (Table 3).

Table 3. Morphological characteristics of alder *Phytophthora* isolates recovered in this study

	Sporangia			Oogonia		Oospore		Antheridia	
Isolate	Length range	Width range	Average	Range	Average	Range	Average	Length range	Breadth range
PA2008	42-62	32-45	55.6 X 43.3	36-52	42.4	24-39	34.7	20-28	13-18
PA2010	45-60	33-44	52.3 X 41.4	37-50	43.7	22-40	35.2	22-27	16-20
PA4017	37-55	29-39	51.9 X 39.2	32-51	46.6	25-42	32.4	18-25	12-16
PA4018	38-58	27-37	50.8 X 40.7	33-52	50.2	27-44	40.5	21-26	15-18
PA4020	40-59	25-38	51.1 X 41.8	39-49	44.4	24-41	38.5	19-24	13-19
PA5024	45-65	33-47	57.3 X 45.2	33-53	47.9	27-45	41.3	19-26	15-19
PA5029	44-58	31-48	56.5 X 45.4	34-49	46.2	28-40	39.7	13-22	11-18
PA6034	48-59	34-42	60.3 X 48.9	32-44	42.3	24-35	36.4	14-21	10-16
PA6035	52-66	33-44	61.1 X 49.7	38-54	48.5	25-40	42.1	16-22	10-17
PA7040	34-54	28-40	55.5 X 43.8	33-52	43.5	27-44	38.7	22-26	15-21
PA7051	36-55	29-42	57.7 X 45.6	35-48	44.7	22-42	33.6	18-24	12-18
PA7054	34-59	28-35	56.8 X 44.8	38-57	47.8	31-46	42.3	21-29	15-19
PA8055	42-62	26-40	58.3 X 45.5	35-55	43.1	25-48	31.9	19-26	14-20
PA8059	44-58	29-39	59.6 X 48.3	39-54	45.5	26-42	37.1	14-22	11-17
PA8060	39-60	30-39	55.4 X 42.3	33-49	38.8	23-40	32.8	16-21	12-17

*Dimensions are given in µm.

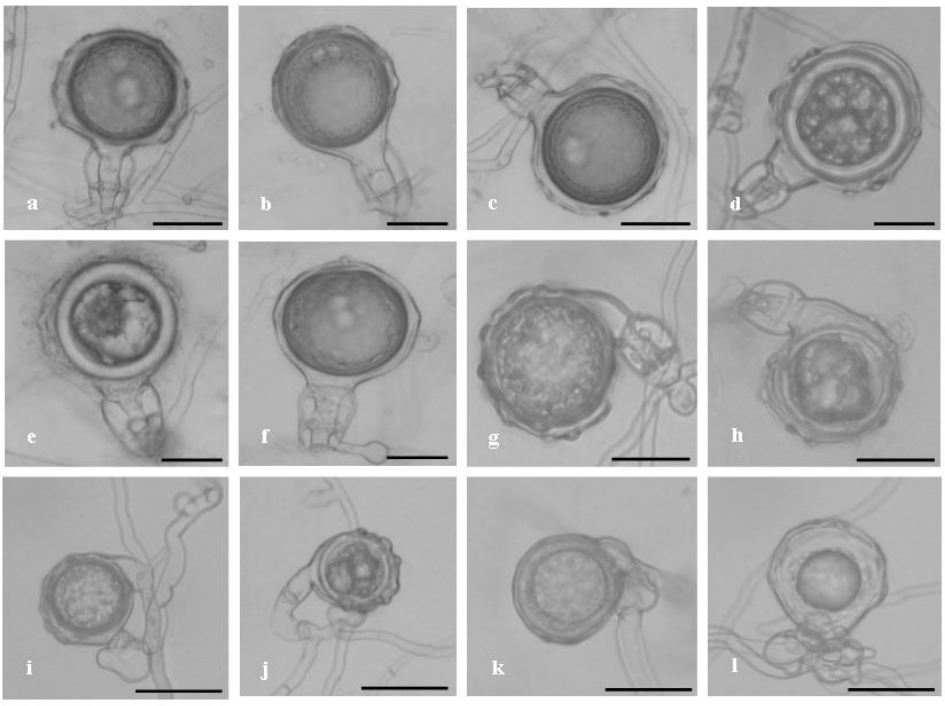

Figure 3. Morphological structures (gametangia) of the isolates of *P. alni* ssp. *alni* formed on V8A (a-l). (a-c). ornamented oogonia with two-celled amphigynous antheridia; (d-e). oogonia with aborted oospores; (f). an oogonium showing finger-like hyphal projection at the point of the septum of the antheridium; (g-h). comma-shaped oogonia; (i-j). rarely seen oogonia having paragynous antheridia; (k-l). partially developed and smaller oogonia. Scale bar = 25 µm.

Physiological characterization

Temperature-growth relations

Temperature growth relationships of the isolates are shown in Figure 4. The optimum mycelial growth of all isolates was recorded at 25°C. At this temperature, the isolates grew from 4.2 to 6.2 mm/day. All five isolates grew at 5 and above 30°C except one isolate (PA8055) which was unable to grow at 30°C. The isolates demonstrated a steep decline in their growth above 25°C. In addition, the isolates did not grow at 2 and 32°C, but started to grow when the plates were transferred to 25°C.

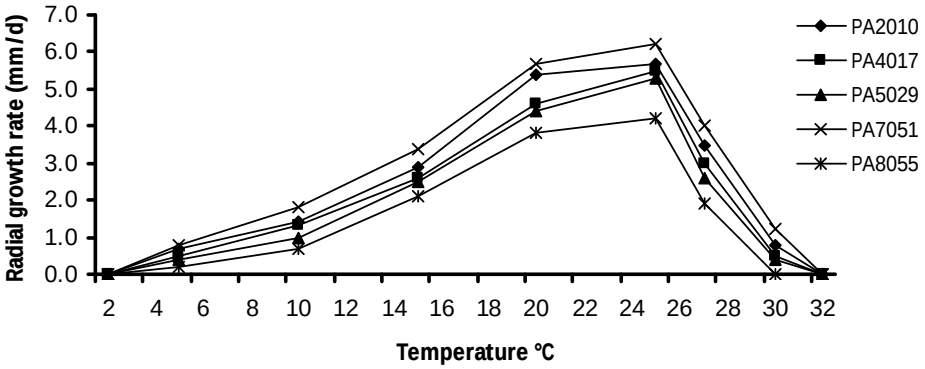

Figure 4. Mean growth rate (mm/day) of five isolates of *P. alni* ssp. *alni* on V8A at different temperatures

pH-growth relations

The effects of pH on mycelial growth are shown in Figure 5. All isolates were able to grow on pH adjusted V8A at all pH values examined. The optimum mycelial growth of all isolates was observed at pH 7. At this pH, they grew from 3.8 to 5.5 mm/day. However, no significant differences in optimum growth were observed between pH 5 and 7. But one isolate PA8055 acted differently and demonstrated difference in growth between pH 5 and 7. For the isolates PA2010, PA4017 and PA8055, differences in growth were noted between pH 7 and 9, where as no differences were noted for PA5029 and PA7051 at the same pHs. Although, the isolates grew at pH 11, significant differences were recorded in their growth between pH 9 and 11.

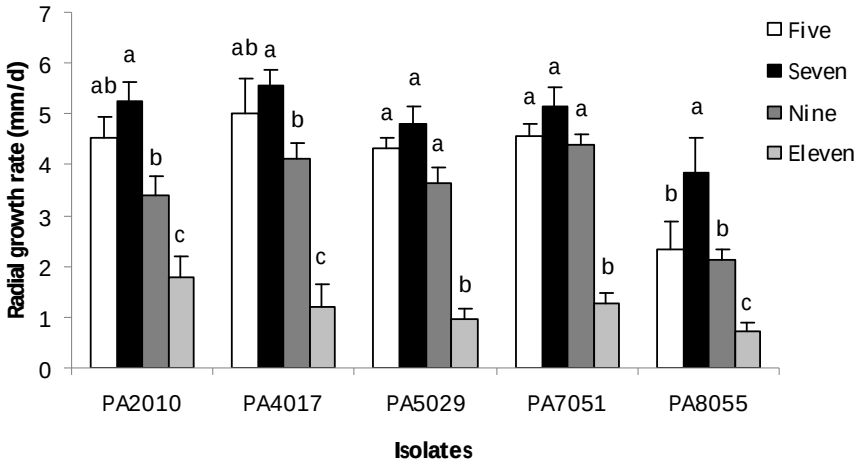

Figure 5. Mean growth rate (mm/day) of five isolates of *P. alni* ssp. *alni* on V8A at different pH. Letters above the bars show significantly different means (Tukey's post hoc test, $\alpha = 0.05$)

Osmotic potential-growth relations

The effects of osmotic potentials on growth of the isolates are shown in Figure 6. The optimum growth of the isolates was recorded at control followed by -0.6 MPa, and the growth rate declined with decreasing osmotic potential at -1.2 MPa and -1.8 MPa respectively. Significant differences in growth were noted at -0.6 MPa and -1.2 MPa for all tested isolates except PA8055. However, no differences in growth were noted for the isolates at -1.2 MPa and -1.8 MPa, with the exception of PA7051. Mycelial growth was completely inhibited at -2.4 MPa.

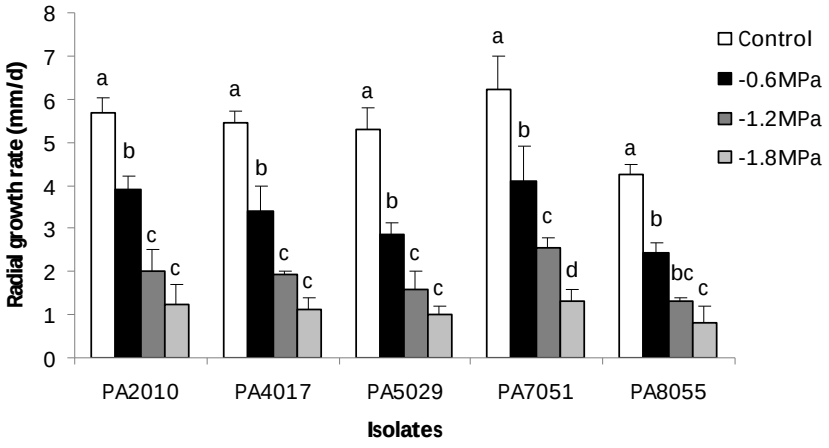

Figure 6. Mean growth rate (mm/day) of five isolates of *P. alni* ssp. *alni* on V8A at different osmotic potential. Letters above the bars show significantly different means (Tukey's post hoc test, $\alpha = 0.05$)

Molecular characterization

Sequencing ITS region

Sequences obtained from our isolates in both directions showed a high overall quality (over 75% residues HQ) except the forward sequences (obtained with ITS1F primer) of PA5024 and PA5029 isolates, whose qualities were below 60% and were thus discarded for further analyses. The remaining 28 sequences were trimmed manually to exclude low quality ends and reviewed on the basis of the chromatogram graphs before aligning. Some stretches contained clear ambiguities (two peaks of similar height in the same position) and appeared in almost all sequences and in both directions, with a few exceptions in which one of the two peaks was slightly higher than the other and were not considered ambiguous by Geneious program. These ambiguities, probably related to the recent hybrid condition of *P. alni* ssp. *alni* subspecies, accounted for low local quality of the sequences and reduced the overall HQ index.

When aligned through ClustalW, strong homology was found (99.5% pairwise identical residues), including 10 positions, at which all sequences presented ambiguities or compatible residues. Only 6 positions in the alignment presented disagreements against consensus sequence, with the only exception of differences in sequences length, and all of them corresponded to residues in which one or more sequences presented one of the detected ambiguities. This allowed constructing a very good consensus sequence to be nblasted with GenBank.

The 100 best hits corresponded to *Phytophthora* species, showing pairwise identical residues between 98.3 and 99.6%. The best homology was found for the isolates of *P. alni* (18 out of 20 best hits and 35 out of 100 best hits), but also for several isolates identified in the Bank as different species of *Phytophthora*.

Subspecies specific primers

Fifteen isolates along with the reference isolates PAU300, PAM391 and PAA129 were analyzed using four Paa subspecies specific primers to verify their identity. All isolates showed same amplification pattern for all the primer pairs assayed (Figure 7; results summarized in Table 4). PA primers amplified successfully all isolates and the reference strains (data not shown) clearly indicating that isolates obtained during the present study belonged to *P. alni* complex; PAM primers amplified successfully the isolates tested as well as reference isolates PAA129 and PAM391, and failed to amplify

PAU300 (Figure 7a). PAU primers were not used as they often give inconsistent results (B. Marçais, personal communication). TRP primers amplified all our isolates, as well as PAA129 and PAU300, but PAM391 strain was not amplified (Figure 7b). Finally, when amplified with RAS primers, the only isolate giving an amplified product was PAU300 (Figure 7c).

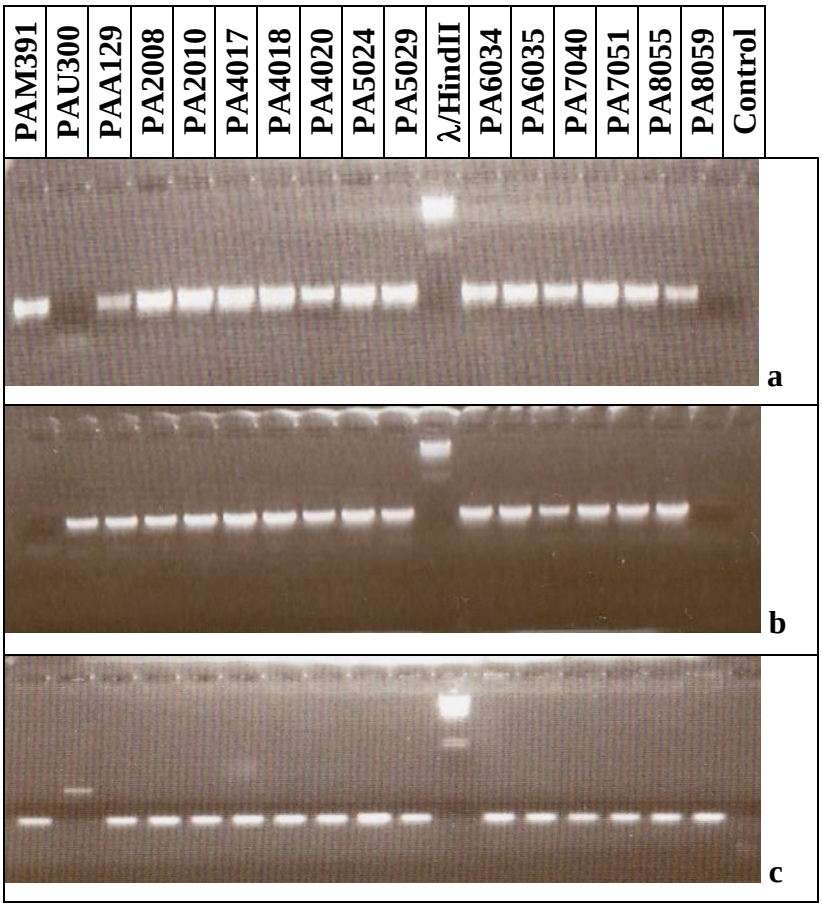

Figure 7. Amplification of controls and isolates with subspecies-specific primers a. PAM; b. TRP; c. RAS. Lanes 1-3 are control strains; Lanes 4-10 and 12-17 represent Spanish isolates; Lane 11 contains the molecular marker λ/HindII and Lane 18 is a negative control, containing no template DNA

In this late case, we obtained a thick unspecific band in the other lanes, probably corresponding to primer-dimmers or RNA molecules. Together, these results indicated that i) our isolates belonged to *P. alni* as all of them were amplified by PA primers; but ii) they did not belong to either Pau or Pam, but to Paa as derived from amplifications with PAM, TRP and RAS primers.

Table 4. Amplification results from subspecies specific amplification assays

Locus	PA (#)	PAM	TRP	RAS
Forward/Reverse primers	PA-F/ PA-R	PAM-F/ PAM-R	TRP-E1-1F/ TRP-E3-1R	RAS-E-1F/ RAS-E5-1R
Annealing Temp (°C)	50	53	53	53
Amplified subspecies	Paa,Pam,Pau	Paa, Pam	Paa, Pau	Paa, Pam
Amplicon size in (pb)*	450	570/590	660/765	666/698
Reference	Ioos et al. 2005		Ioos et al. 2006	
Isolates (Ref.)				
PAM391/PAM393/07dur31	+	+	0	+
PAU300/PAU542/PAU624	+	0	+	-
PAA129/PAA314/PAA354	+	+	+	+
H_2O	0	0	0	0
Isolates (This study)	+	+	+	+
All isolates of the present study	Paa	Not Pau	Not Pam	Not Pau

DISCUSSION

In course of the present morphological studies, our isolates of alder *Phytophthora* produced abundant terminal, spherical oogonia which exhibited moderate ornamentation and two-celled amphigynous antheridia. Besides, rarely comma-shaped smaller oogonia, and aborted oospores were frequently observed. These features of the gamentangia corresponded to the describe characteristics of *P. alni* ssp. *alni* (Brasier *et al*. 2004). On the other hand, caducity of sporangia is a useful taxonomic tool in identification of many *Phytophthora* species (Kaosiri *et al*. 1978). We obtained sporangia which were non-caducous and non-papillate, and shape of the sporangia were variable from ovoid to ellipsoid. Similar characteristics of sporangia of *P. alni* were also illustrated by several authors (Brasier *et al*. 2004; Cerný *et al*. 2008; Solla *et al*. 2010, Varela *et al*. 2010). Size and shape of sporangia of *Phytophthora* vary due to culture media and environmental conditions (Waterhouse 1963). The length: breadth ratios of sporangia of our isolates ranged from 1.3 to 1.6. Several authors reported similar length: breadth ratio of *P. alni* ssp. *alni* (Brasier *et al*. 2004; Cerný and Strnadová 2010; Cerný *et al*. 2008; Varela *et al*. 2010). The characteristic features of sporangia together with nested and extended internal proliferation, and wide exit pores appeared to be identical to *P. alni* ssp. *alni.*

Colony patterns of our isolates growing on CA were usually appressed and irregular in growth with very limited aerial mycelium, whereas on V8A, colonies had slightly woolly morphology like those of standard type isolates of *P. alni* (Brasier *et al*. 2004; Cerný and Strnadová 2010; Solla *et al*. 2010; Szabó *et al*. 2000; Varela *et al*. 2010). On the other hand, rather fluffy and considerably woolly growth patterns were observed on CMA and PDA. All isolates showed optimum growth at 25°C which was in accordance with previous studies (Brasier *et al*. 2004; Cerný and Strnadová 2010; Cerný *et al*. 2008; Solla *et al*. 2010; Varela *et al*. 2010) which demonstrated an optimum growth temperature ranging from 23 to 25°C for isolates of *P. alni* ssp. *alni*. Maximum growth temperature for the isolates was 30°C and minimum was 5°C. Similar range of temperatures for growth was also reported (Brasier *et al*. 2004; Varela *et al*. 2010). Considering the findings of temperature effect on growth, our isolates of alder *Phytophthora* fallen within the range of temperatures considered suitable for the growth of *P. alni* ssp. *alni*.

The pH is one of the most important factors influencing the survival of many *Phytophthora* species in soil (Andrivon 1994; Blacker and MacDonald 1983; Kong *et al*. 2012). Furthermore, pH level fluctuations in aquatic systems influence the dissemination of many *Phytophthora* species from a single point of infection to far distance points (Gibbs *et al*. 1999; Hong and Moorman 2005; Kong *et al*. 2009). In this view, effects of pH on the survival and growth of the pathogen are of great importance. *Phytophthora* is considered to be tolerant to wide range of pH and increasing pH value generally favors its growth (Andrivon 1994; Weste 1983). This statement is in agreement with our finding where the isolates of *P. alni* ssp. *alni* showed wide range of pH tolerance for mycelial growth, but in disagreement as growth of isolates reduced significantly with increasing pH value i.e., the lowest growth was recorded at pH 11. The pH tolerance level matched another study by Kong *et al*. (2012), who demonstrated that *P. alni* ssp. *alni* was basic tolerant (pH 5 to 11). In our present research, maximum growth of the isolates was recorded at pH 7, corresponding to best pH value for *P. alni* ssp. *alni* determined (Kong *et al*. 2012). It can be concluded that alder *Phytophthora* can be active over a wide pH range, being well adapted to aquatic environment and pose threat to new areas through water dispersal.

Osmotic potential plays an important role in the ecology and growth of pathogenic fungi (Davis *et al*. 2000). For instance, decrease in the osmotic potential showed reduced

growth in fungi (Lira-Méndez and Mayek-Pérez 2006). *Phytophthora* species, known as water moulds, are ecologically favoured by free water in soil. Water deficiency is usually considered as a limiting factor in the life cycle of the pathogen. A minimum amount of water in soil, for a certain period of time, is a precondition for successful infection and development of *Phytophthora* infestation (Thomas *et al*. 2002). The role of water stress in the development of *Phytophthora* diseases and water-fungi interactions are of great importance (Ayres and Boddy 1986). In present investigation, it is evident that a decrease in osmotic potential produced a reduction in mycelial growth. These findings are very close to those of a previous study in which optimum mycelial growth of several other *Phytophthora* species (*P. cambivora, P. cinnamomi, P. citricola* and *P. quercina*) was achieved at 0 MPa. All species exhibited decreasing growth at –1.18, –1.75, and –3.0 MPa, mycelial growth completely inhibited at –3.98 MPa (Turco *et al*. 2005). Another study revealed that the growth of *P. megasperma* and *P. parasitica* declined steeply at lower osmotic potentials and ceased completely at –3.0 MPa and –5.0 MPa respectively (Sommers *et al*. 1970). From these findings, we can conclude that osmotic potential affected the growth and development of these species. This could be important in designing experiments for assessing new methods for control of the pathogen.

Sequences from ITS region, both forward and reverse, demonstrated that all of our isolates were nearly identical, indicating that they corresponded to a unique genotype. On the basis of the best hits of comparison with GenBank, this genotype was identified as belonging to *P. alni* complex. Some other good hits corresponded to different species of *Phytophthora* like *P. cambivora* and *P. fragariae*, or *P. europaea* and *P. rubi*, considered to be very close relatives of *P. alni* (Brasier *et al*. 2004, Ioos *et al*. 2006), although, nowadays the two former are no more considered as its parental species (Aguayo *et al*. 2013).

Two groups of results allowed the subspecific identification of all isolates observed in this study as *P. alni* ssp. *alni*. On one hand, a series of ambiguities (dimorphic sites) at conserved positions was found in all ITS sequences. This type of dimorphism has been described as typical of *P. alni* ssp. *alni,* while *P. alni* ssp. *multiformis* and *P.alni* ssp. *uniformis* tend to be largely (Pam) or entirely (Paa) monomorphic (Brasier *et al*. 1999, 2004; Ioos *et al*. 2006). Also, ambiguities have been related to the recent hybrid condition of Paa, believed to be the result of a unique or multiple hybridization events

from *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis* and following an asexual way of reproduction that maintained heterozygosity (Ioos *et al*. 2006, 2007; Aguayo *et al*. 2013). Complementary research is ongoing on nuclear and mitochondrial genomes to study genetic diversity and origin of each of the three subspecies of *P. alni*. On the other hand, all of our isolates showed an unique and common pattern of amplifications with a series of specific primers previously described (Ioos *et al*. 2005, 2006), coincident with that of Paa reference isolates and clearly excluding Pau and Pam. Amplification with PA primers indicated that our isolates belonged to *P. alni* complex, but didn't discriminate between Paa, Pau or Pam; PAM amplification excluded Pau; and TRP and RAS primers amplifications, derived from two of the few nuclear genes exhibiting introns, excluded Pam and Pau, respectively.

The identification of our isolates as Paa is coherent with the knowledge on alder *Phytophthora* distribution: *Paa* has been described as the most frequent subspecies in Europe (Ioos *et al*. 2006) and is believed to reproduce through dissemination of asexual spores, which is consistent with the presence of dimorphic sites in ITS region. Some other hybrid species have been described as aggressive pathogens species, like *Neotyphodium coenophialumand* and to spread through asexual spores (Moon *et al*. 2004). Moreover, a unique genotype has been identified, supporting the idea that all isolates are consistent with a unique event of introduction of this pathogen in Spain, probably through importation of contaminated alder seedlings from other European countries. This aspect should be included and given importance in further studies.

P. alni can easily be distinguished from other homothallic *Phytophthora* species by its unique morphological and physiological characteristics, and DNA sequences. Features like two-celled ornamented oogonia with amphigynous antheridia, nonpapillate and non-caducous sporangia, growth patterns on culture media, optimum and maximum temperatures for growth have given distinctiveness to alder pathogen. Considering its morphological and physiological characters, host, and ITS sequences, our isolates obtained from common alder clearly belong to *P. alni* spp. *alni*. Proper and rapid identification of pathogens is crucial in order to protect our forests and natural ecosystems from *Phytophthora* infection in Europe. Our findings have emphasized the significance and need of detailed studies of the alder pathogen that thrived along the river systems in western Spain. Additional surveys are needed to determine the distribution of *P. alni* in other alder growing areas of Spain.

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ARTICLE III

SUSCEPTIBILITY OF COMMON ALDER (*Alnus glutinosa*) SEEDS AND SEEDLINGS TO *Phytophthora alni* AND OTHER *Phytophthora* SPECIES

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ABSTRACT

Phytophthora alni is a highly destructive host specific pathogen to alders (*Alnus* spp.) spreading all over Europe. Recently this pathogen has been reported to cause diseases in common alder (*Alnus glutinosa*) in Spain. Seeds and seedlings of *A. glutinosa* were tested *in vitro* for their susceptibility to alder *Phytophthora* and other *Phytophthora* species. Isolates of *P. alni* ssp. *alni, P. cinnamomi, P. citrophthora, P. nicotianae* and *P. palmivora* were used in the experiments. Seeds and seedlings were inoculated with a zoospore suspension and uniform mycelial blocks of agar of the *Phytophthora* species. Susceptibility was calculated in terms of pathogen virulence on seed germination and seedling mortality 42 and 67 days after inoculation respectively. Seed germination and seedling mortality rates varied differently among the isolates used. Results implied that common alder and its seeds and seedlings are at risk to be infected by *P. alni*. In addition, other *Phytophthora* species are able to infect this kind of material showing their relative host non-specificity. This is one important finding concerning alder regeneration in infected areas, and the possibility of disease spread on this plant material.

KEYWORDS: Alder diseases, virulence, zoospore suspension, V8 agar, forest pathology

RESUMEN

Phytophthora alni es un patógeno muy destructivo de los alisos (*Alnus* spp.) que se está expandiendo por toda Europa. Recientemente este patógeno ha sido citado como causante de enfermedades del aliso común (*Alnus glutinosa*) en España. Semillas y plántulas de *A. glutinosa* fueron analizadas *in vitro* para ver su susceptibilidad a *Phytophthora alni* y otras especies del género *Phytophthora*. En el experimento se usaron aislamientos de *P. alni* ssp. *alni, P. cinnamomi, P. citrophthora, P. nicotianae* y *P. palmivora*. Las semillas fueron inoculadas con una suspension de zoosporas mientras que las plántulas fueron inoculadas con bloques uniformes de micelio de agar de las especies de *Phytophthora* utilizadas. La susceptibilidad fue calculada evaluando la germinación de las semillas y la mortalidad de las plántulas después de 42 y 67 días tras la inoculación respectivamente. Los ratios de germinación de las semillas y de mortalidad de las plántulas variaron significativamente entre los aislamientos utilizados. Los resultados demostraron que el aliso común y sus semillas y plántulas tienen riesgo de ser infectados por *P. alni.* Además, otras especies de *Phytophthora* fueron capaces de infectar, lo que evidenció una relativa falta de especificidad por el hospedante. Estos datos son importantes por su transcendencia para la regenaración del aliso en las áreas infectadas, y la dispersión de la enfermedad en este material vegetal.

PALABRAS CLAVE: Enfermedades del aliso, virulencia, suspensión de esporas, Agar V8, patología forestal

INTRODUCTION

The genus '*Alnus*' is characterized by their capability to colonize on bare land and tolerate high groundwater table and flooding (Gibbs *et al*. 2003). Common alder [*Alnus glutinosa* (L.) Gaertn.] is the most widespread species among alders occurring in Europe which has been used for reforestation and stabilizing river banks. This species is occurring extensively in Spain and is distributed along streams and rivers.

An extensive and rapid mortality of common alder was observed along many rivers of northern Spain (Tuset *et al*. 2006). Some years later of that observation, *Phytophthora alni* (Brasier and S.A. Kirk) was associated to cause that mortality in *A. glutinosa* (Solla *et al*. 2010; Varela *et al*. 2010). *P. alni* is an inter-specific hybrid between *Phytophthora cambivora* (Petri) and an unknown *Phytophthora* similar to *P. fragariae* Hickman (Brasier *et al*. 1995, 2004), and it has been described as a host specific pathogen to alders spreading all over Europe. Three different types have been recognized within *P. alni* based on morphological characteristics and aggressiveness (Brasier and Kirk 2001; Brasier *et al*. 2004): the standard type of the pathogen, which is the most aggressive one, has recently been named as *P. alni* ssp. *alni*, the hybrid types collectively known as *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis*, which are locally very damaging and could represent a serious threat to alder population and stability of riparian ecosystems (Brasier *et al*. 2004).

The association of *P. alni* in the decline and mortality process of alders has been the center point of several studies in different parts of the world (Gibbs 1995; Gibbs *et al*. 1999; Santini *et al*. 2001; Brasier and Kirk 2001; Brasier 2003; Gibbs *et al*. 2003; Santini *et al*. 2003; Jung and Blaschke 2004; Ioos *et al*. 2005; Cerný and Strnadová 2010; Solla *et al*. 2010; Varela *et al*. 2010). *Phytophthora* decline of the riparian alder population has recently become an important problem in Spain because of the rapid spreading of the causal pathogen. According to Santini *et al*. (2003), the way of spreading 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. According to this, alder population may be in danger as they co-exist with *Phytophthora* contaminated hardwood and ornamental woody species in the same nurseries. In addition to that, container grown seedlings in nurseries may act as a prime carrier facilitating further

dispersion of *Phytophthora* to the natural ecosystems which may hamper alder regeneration. Zoospores of the alder *Phytophthora* swim freely in water and therefore most likely spread far distance using river system. In young alders, infection and bark killing often start at the collar region where zoospores are attracted. However, the infections in the middle of the trunk in the inner bark might indicate that environmental factors may also play a prominent role in the occurrence of the disease which may be associated with flooding events (Brasier 2003).

Another *Phytophthora* species have been found causing damage in a wide tree host range. Among those, *Phytophthora nicotianae* Breda de Haan (= *Phytophthora parasitica* Dastur) which is a soilborne pathogen, has been recorded causing dieback in *Eucalyptus* species in South Africa where *Eucalyptus* species are planted commercially (Maseko *et al*. 2001); and damaging several woody ornamental plants in nurseries (Schwingle *et al*. 2007; Donahoo and Lamour 2008); but it has been also detected in another 298 plant species (Erwin and Ribeiro 1996). *Phytophthora cinnamomi* Rands, which is an invasive soil-borne roots pathogen, has been stated as one of the causal agents of the decline of holm oak (*Quercus ilex* L.) and cork oak (*Q. suber* L.) in Southwestern Iberian Peninsula (Brasier *et al*. 1993; Sanchez *et al*. 2002). And *P. citrophthora* (R.E. Sm. and E.H. Sm.) Leonian and *P. palmivora* (E.J. Butl.) have been recorded in nurseries causing diseases on several woody ornamental plants (Schwingle *et al*. 2007; Donahoo and Lamour 2008).

Although few studies have been conducted to examine the susceptibility of alders to *P. alni* and other *Phytophthora* species (Brasier and Kirk 2001; Santini *et al*. 2003, 2006), the Spanish isolates *P. alni* have not been tested yet in terms of aggressiveness on common alder. In addition to that, due to the frequent occurrence of other *Phytophthora* species in the forest ecosystems of Spain (including nurseries), the main objective of the present study was to evaluate the susceptibility of common alder seeds and seedlings to Spanish isolates of *P. alni* and the other most common *Phytophthora* species present in nurseries which could prompt the failure of the common alder regeneration.

MATERIALS AND METHODS

Phytophthora isolates and inoculum production

Two isolates of *P. alni* ssp. *alni* recovered in 2009 from diseased *A. glutinosa* trees growing along river Miño (Spain), which were isolated as described by Solla *et al*.

(2010), were used to inoculate seeds and seedlings. Besides, one isolate of each *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* (Supplied by the Instituto Agroforestal Mediterráneo [Mediterranean Institute of Agroforestry], Universidad Politécnica de Valencia, Valencia, Spain) were used in the experiments. Both, mycelia and zoospores were used in the experiments. For mycelial agar plugs, colonies of the *Phytophthora* species were sub-cultured for 1 week at 20ºC in the dark onto 90-mm Petri dishes containing sterilized V8 agar (V8 agar: 100 ml/L V8 Campbell Grocery Products, 3 g/L CaC03, 20 g/L Agar Technical DIFCO, Detroit, MI, USA). For zoospores production, colonies of the *Phytophthora* species were sub-cultured for 14 days at 20ºC in the dark onto 90-mm Petri dishes containing sterilized V8 agar exposed to 16 h light per day. Sporangia were obtained by flooding the colonies with sterile distilled water, and then transferred into sterile water by rubbing surface of the culture with a sterile bent glass rod. The liquid was poured off the plates and again collected in a sterile beaker which was placed in a refrigerator at 7°C for 1 h, then returned to room temperature (20°C) during another 75 min to promote zoospore releasing. Zoospore concentration was determined by using a haemocytometer, and suspension was adjusted to 3×10^5 zoospores per ml (Denman *et al*. 2005).

Plant material

Disease free seeds of common alder were supplied by the Centro Nacional de Mejora Forestal [National Centre for Forest Breeding] El Serranillo (Guadalajara, Spain) in order to be used in the experiments. Before use, seeds were sterilized in the following way: they were firstly washed several times with sterilized distilled water, then dipped into hydrogen peroxide (3%) for 20 minutes. Finally seeds were washed twice with sterilized distilled water to remove excess hydrogen peroxide and dried aseptically. Those sterilized seeds were ready to be used in the seed inoculation experiment. For seedling inoculation experiment, sterilized seed were plated onto water agar Petri dishes and sealed with Parafilm® (American National Can, Greenwich CT, USA) to avoid contamination. Plates were kept into growth chamber at 24º C and photoperiod (16/8) to promote germination. After that, eight seedlings per plate were aseptically transferred to Petri dishes containing potato-dextrose-agar (PDA) to get the seedling hardening prior to inoculation.

Experimental design

Seed inoculation experiment

Eight sterilized seeds per plate were transferred onto the surface of water agar contained into 90-mm Petri dishes. Each one of the six *Phytophthora* isolates (two of *P. alni* ssp. a*lni*, and one of each *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora*) were inoculated in the Petri dishes following two different methods: (1) Individual seed inoculation treatment (noted as IS) when a zoospore suspension of 0.1 ml (3×10^5 zoospores per ml) was sprayed at the base of each seed; and (2) central inoculation treatment (noted as CE) when a zoospore suspension of 1 ml was sprayed very precisely at the centre of Petri dishes keeping equal distances from each seed and later shaken gently for the uniform spreading of suspension over the surface to facilitate the zoospore suspension contact with the seeds. In the controls (CO) 0.1 ml of sterile distilled water was sprayed instead the zoospore suspension. Four replications for each *Phytophthora* species and for each inoculation method were used in the assay. After inoculation, Petri dishes were sealed with Parafilm and kept at 24º C and photoperiod (16/8). Seeds were inoculated on June 2010. Data of seed germination percentage were taken at every 5 days interval (except the first record which was taken at 7 days) until 42 days.

Seedling inoculation experiment

Seedling were inoculated on April 2010 with the six *Phytophthora* isolates following two different methods: (1) individual seedling inoculation (noted as IT) when a 2-mm size mycelial agar plug was put at the base of each seedling; and (2) central inoculation method (noted as CE) when a 2-mm size mycelial agar plug was placed precisely at the centre of Petri dishes keeping equal distances from each seedling. In the controls, seedlings were inoculated with sterile V8 agar plugs. Four replications for each isolate and method were used in the experiment. After inoculation, plates were sealed with Parafilm and kept at 24º C and photoperiod (16/8). Data of seedling mortality percentage were taken at every 5 days interval (except the first record which was taken at 7 days) until 67 days.

Statistical analysis

Seed germination and seedling mortality were analyzed by repeated measures ANOVAs ($p<0.05$) to examine significant differences among the *Phytophthora* isolates and

inoculation methods, as well as the time period when different measurement data were taken. The differences between means were considered significant ($p<0.05$) according to Tukey multiple range test. All statistical analyses have been performed with the software Statistica 6.0 for Windows (StatSoft Inc., Tulsa, Oklahoma, USA).

RESULTS

Seed inoculation experiment

Repeated measures ANOVA applied to seed germination percentage (Table 1) showed that both the isolate of *Phytophthora* species and the treatments used in the experiment produced significant effect on seed germination. Interactions between the factors as source of variation were significant with the exception of time.

Table 1. Repeated measures analysis of variance for seed germination (%) that considers *Phytophthora* species, treatment and time, and their interactions as source of variation

Source of Variation	SS	df	MS	F	p
Intercept	1009397	1	1009397	1483.077	0.000000
Species	17509	5	3502	5.145	0.000624
Treatment	402502	2	201251	295.692	0.000000
Species*$_{Treatment}$	16632	10	1663	2.444	0.017391
Error	36753	54	681		
TIME	40796	7	5828	104.029	0.000000
TIME*$_{Species}$	1452	35	41	0.740	0.860560
TIME*$_{Treatment}$	3926	14	280	5.006	0.000000
TIME*$_{Species}$*$_{Treatment}$	4387	70	63	1.119	0.254916
Error	21177	378	56		

SS = sum of square; df = degree of freedom; MS = Mean square

Seeds started to germinate during the first week, regardless of the treatment, although in the controls a higher percentage was obtained after 7-12 days after the inoculation. In case of seeds inoculated, comparatively a low germination was achieved 22-27 days after the inoculation depending on the isolates of *Phytophthora* species and pursued almost a steady rate up to 37-42 days (Figure 1). The complete progression of seed germination after the inoculation with the isolates of *Phytophthora* species with respect to number of days has been indicated in Figure 1.

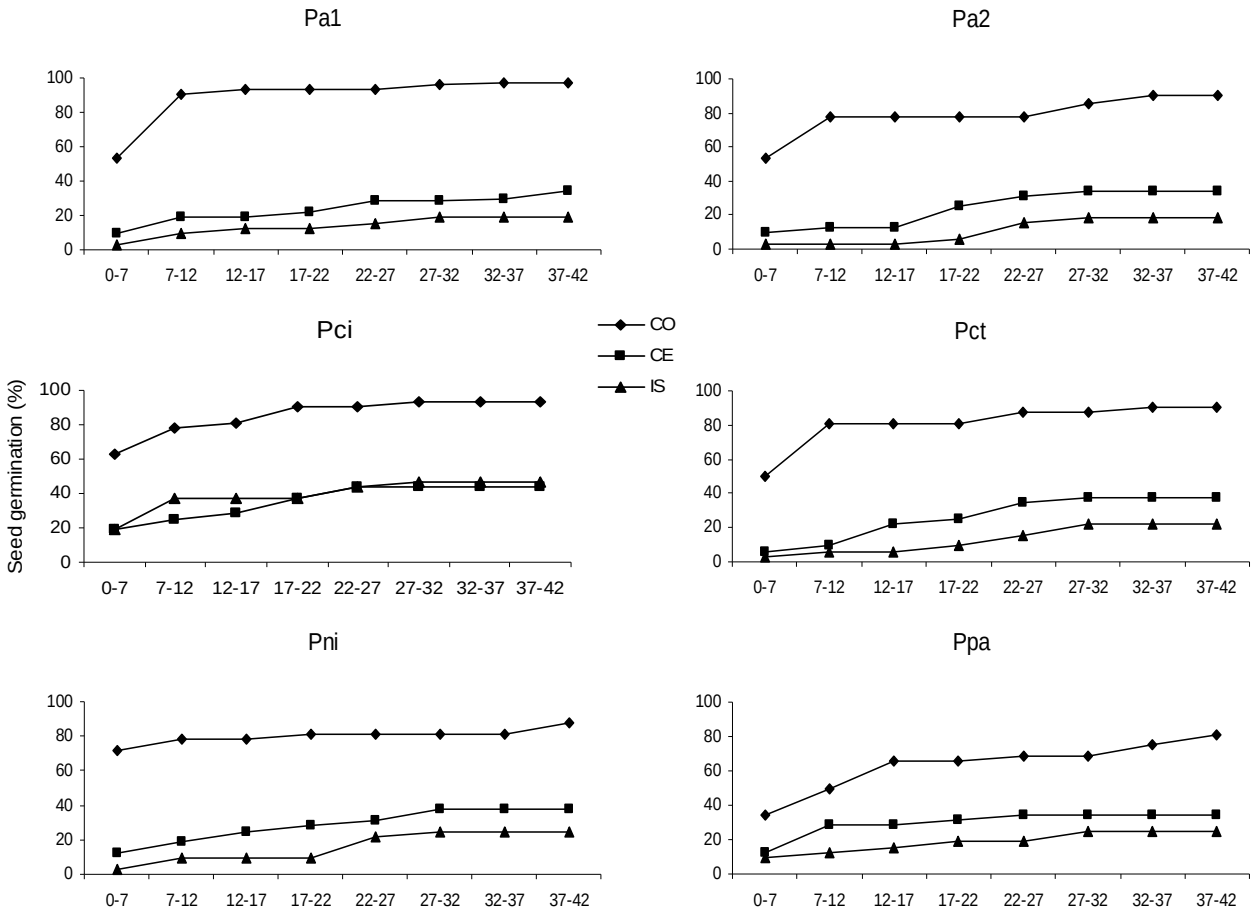

Figure 1. Mean seed germination percentage after inoculation with each one of the six *Phytophthora* isolates, Pa1=*P. alni* (isolate 1); Pa2 = *P. alni* (isolate 2); Pci = *P. cinnamomi*; Pct = *P. citrophthora*; Pni = *P. nicotianae*; Ppa = *P. palmivora*. CO = Control; CE = zoospore suspension sprayed at the centre of the Petri dishes; IS = zoospore suspension sprayed at the base of each seed.

Forty two days after inoculation, all the isolates of the *Phytophthora* species tested hampered significantly germination regardless of the method used. When zoospore suspension was applied at the centre of the plate (method CE), no differences were found among the isolates, but when suspension was applied in each seed (method IS), *P. cinnamomi* caused a significant lower reduction of the germination percentage than that caused by rest of the species (Figure 2). On average, seed germination percentage when inoculation was made for each seed, was 26.04%; and when zoospore suspension was applied at the centre, the percentage was 36.97%. In controls, germination percentage was higher than 80% in all cases (Figure 2).

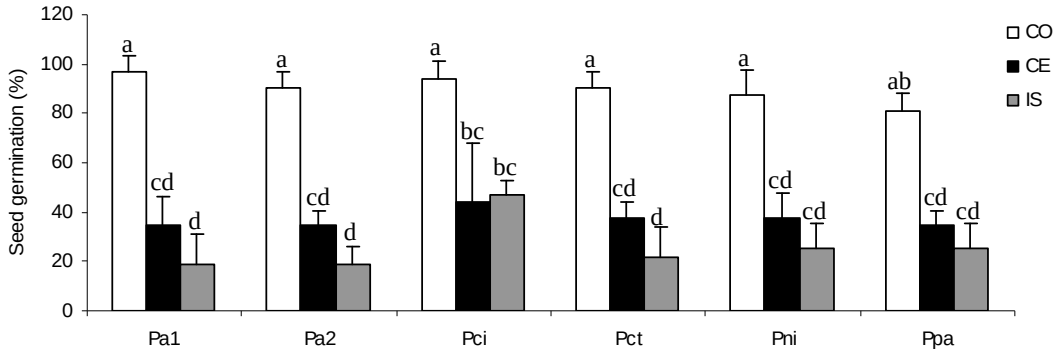

Figure 2. Mean (± SE) seed germination percentage after 42 days of inoculation with each one of the six *Phytophthora* isolates (Pa1 = *P. alni*, isolate 1; Pa2 = *P. alni*, isolate 2; Pci = *P. cinnamomi*; Pct = *P. citrophthora*; Pni = *P. nicotianae*; Ppa = *P. palmivora*). CO = Control; CE = zoospore suspension sprayed at the centre of the Petri dishes; IS = zoospore suspension sprayed at the base of each seed. Different letters indicate significant differences ($p<0.05$) according to Tukey test.

Seedling inoculation experiment

Repeated measures ANOVA (Table 2) revealed that the seedling mortality percentage was significantly influenced by the *Phytophthora* isolates, the inoculation methods, the time period, and by their interactions.

Table 2. Repeated measures analysis of variance for seedling mortality (%) that considers *Phytophthora* species, treatment and time, and their interactions as source of variation

Source of Variation	SS	df	MS	F	p
Intercept	2674948	1	2674948	2927.116	0.000000
Species	105666	5	21133	23.125	0.000000
Treatment	1364249	2	682125	746.429	0.000000
Species*$_{Treatment}$	63397	10	6340	6.937	0.000001
Error	49348	54	914		
TIME	112953	12	9413	223.165	0.000000
TIME*$_{Species}$	9336	60	156	3.689	0.000000
TIME*$_{Treatment}$	81528	24	3397	80.538	0.000000
TIME*$_{Species}$*$_{Treatment}$	19211	120	160	3.796	0.000000
Error	27332	648	42		

SS = sum of square; df = degree of freedom; MS = Mean square

Most of the seedlings inoculated with the different isolates of *Phytophthora* started to die 7 days after inoculation, with a progressive mortality increment, reaching the maximum after 15-20 days of the inoculation (Figure 3). However this maximum was achieved earlier by both isolates of *P. alni* and *P. citrophthora*.

At the end of the experiment all the isolates, excepting that of *P. cinnamomi*, produced a seedling mortality rate higher than 90%, regardless of the inoculation method (Figure

4). *P. cinnamomi* isolate caused a seedling mortality percentage of 46.9% when it was inoculated in the center of the plate (method CE), and 78.1% when it was inoculated in each seedling (method IT). In controls were not observed any seedling mortality (Figure 4).

Between inoculation methods, differences were found mainly at the beginning of the experiment (in the first 2-5 measurements depending of the isolate). In those first records, the inoculation of the isolate plugs in each seedling (method IT) caused a greater seedling mortality than the inoculation in the centre of the plate (method CE) (Figure 3).

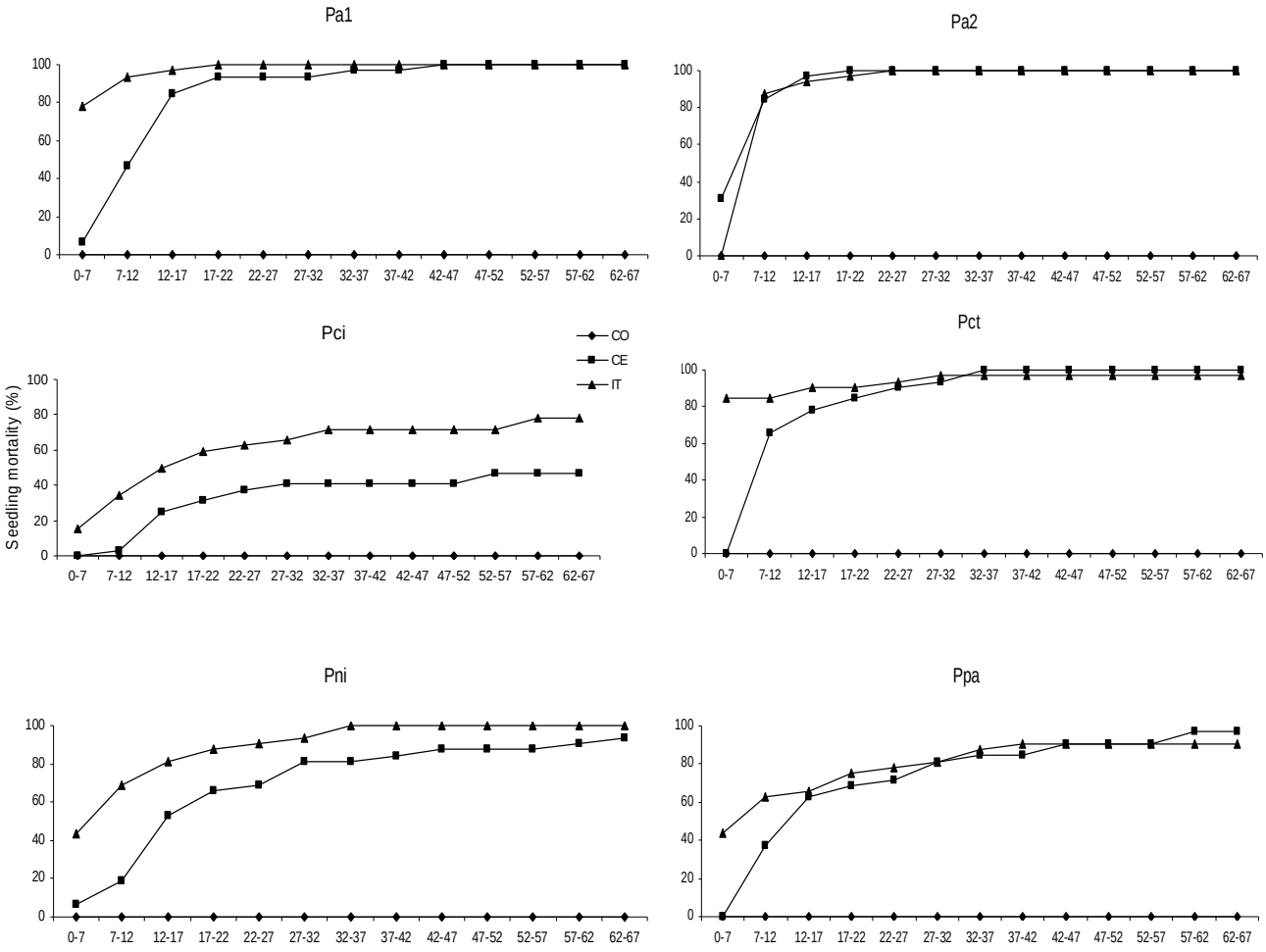

Figure 3. Mean seedling mortality percentage after inoculation with each one of the six *Phytophthora* isolates, Pa1=*P. alni* (isolate 1); Pa2 = *P. alni* (isolate 2); Pci = *P. cinnamomi*; Pct = *P. citrophthora*; Pni = *P. nicotianae*; Ppa = *P. palmivora*. CO = Control; CE = mycelial blocks of agar placed precisely at the centre of the Petri dishes; IT = mycelial blocks of agar put at the base of each seedling.

The exception was found for the *P. alni* isolate 2; in this case the method CE was more aggressive than the method IT in the first record interval. At the end of the experiment,

no differences were found between inoculation methods, except in the *P. cinnamomi* isolate which caused a significant higher mortality when was inoculated separately in each seedling (Figure 4).

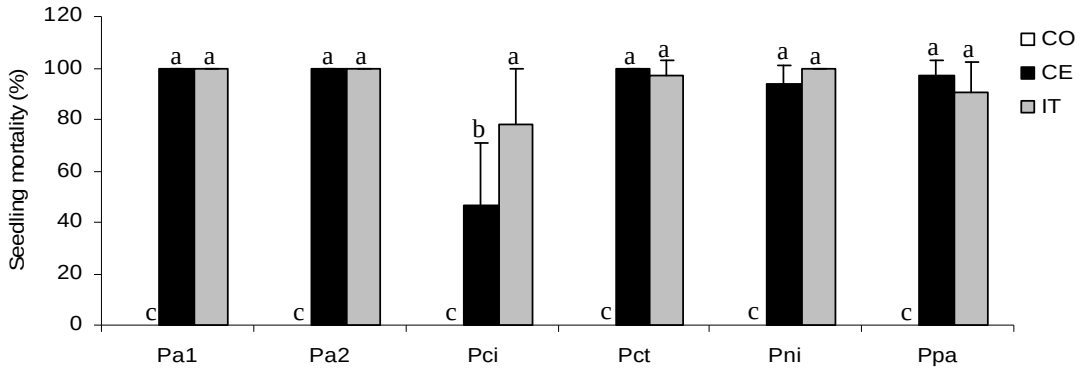

Figure 4. Mean (± SE) seedling mortality percentage after 67 days of inoculation with each one of the six *Phytophthora* isolates (Pa1 = *P. alni*, isolate 1; Pa2 = *P. alni*, isolate 2; Pci = *P. cinnamomi*; Pct = *P. citrophthora*; Pni = *P. nicotianae*; Ppa = *P. palmivora*). CO = Control; CE = mycelial blocks of agar placed precisely at the centre of the Petri dishes; IT = mycelial blocks of agar put at the base of each seedling. Different letters indicate significant differences ($p<0.05$) according to Tukey test.

DISCUSSION

Pathogenicity of *Phytophthora alni* ssp. *alni*, *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* on common alder (*Alnus glutinosa*) seeds and seedling was examined *in vitro*. This is the first study of this type performed so far in Spain with the aim to examine the interaction between *A. glutinosa* and host specific pathogen *P. alni* along with non-host specific pathogens *P. cinnamomi*, *P. citrophthora*, *P. nicotianae* and *P. palmivora* under laboratory conditions. Both inoculation methods applied have confirmed their ability to infect seeds and seedlings. Our findings have revealed that all the *Phytophthora* spp. tested in the present assay could represent a serious threat to *A. glutinosa* which may cause failure of the alder regeneration.

In our present inoculation tests, *P. alni* ssp. *alni* has appeared as a highly aggressive pathogen on seeds and seedlings of *A. glutinosa*. The high aggressiveness of *P. alni* has been already stated by other authors in several European countries (Brasier *et al*. 1995; Gibbs 1995; Szabó *et al*. 2000; Santini *et al*. 2001; Streito *et al*. 2002; Nagy *et al*. 2003; Jung and Blaschke 2004; Cerný and Strnadová 2010; Solla *et al*. 2010; Varela *et al*. 2010). Pathogenic ability of *P. alni* on seeds of *A. glutinosa* has been revealed from a similar laboratory test made by Schumacher *et al*. (2006), where seeds were inoculated

with a zoospore suspension. The finding has strengthened the speculation that *P. alni* could infect alder seeds under natural conditions. Results of seedling inoculation test are consistent with a laboratory test conducted by Santini *et al*. (2003) where seedlings of *A. glutinosa, A. cordata* and other hosts were inoculated with one isolate of *P. alni*. In that study, the maximum seedling mortality was observed in *Alnus* species. Infecting ability of *P. alni* has been revealed from baiting tests which confirmed the involvement of the pathogen on *A. glutinosa* seedlings at nurseries in Germany (Jung and Blaschke 2004). In Italy, *P. alni* was found infecting *A. cordata* both in young plantations and nurseries (Santini *et al*. 2003). Several other authors have also reported the association of *P. alni* with alder seedlings in plantations and nurseries (Santini *et al*. 2001; Gibbs *et al*. 2003; Schumacher *et al*. 2006). So, we assume that *P. alni* ssp. *alni* is able to infect and contaminate seeds and seedlings of *A. glutinosa* both in artificial and natural conditions.

Another *Phytophthora* species tested is *P. cinnamomi*, which has not been detected naturally in alders yet, but in our study it has proved its ability to reduce seed germination and cause seedling mortality under *in vitro* conditions. Similar results were obtained from a previous laboratory test by Santini *et al*. (2003) where an isolate of *P. cinnamomi* caused mortality of *A. glutinosa* seedlings after its inoculation using two different methods. In general, *P. cinnamomi* has been described as a soil-borne pathogen and it has been reported causing root rot in *Quercus suber* and *Q. ilex* under Mediterranean conditions and stem canker of *Q. rubra* in France (Brasier *et al*. 1993; Marcais *et al*. 1993; Linde *et al*. 1999; Sanchez *et al*. 2002). Hardy and Sivasithamparam (1988) recorded the involvement of *P. cinnamomi* and several other *Phytophthora* species with root rot of container grown seedlings from 14 nurseries in Western Australia. In contrast to our study, Australian and South African isolates of *P. cinnamomi* showed a considerable variation in virulence when were inoculated into seedlings of *Eucalyptus* (Dudzinski *et al*. 1993; Linde *et al*. 1999). Robin and Desprez-Loustau (1998) observed a wide range in aggressiveness of *P. cinnamomi* isolates to *Q. rubra*. Host range of *P. cinnamomi* was examined by Tippet *et al*. (1985) who assessed differences in susceptibility of *P. cinnamomi* to the hosts examined in inoculation tests. On the basis of the findings, we draw conclusion that the alders are under risk to be infected as sharing same environmental conditions to grow with other plants hosting *P. cinnamomi*.

Among the other pathogen species tested, *P. citrophthora* has shown its virulence on *A. glutinosa* seeds and seedlings. *P. citrophthora* has been described as a pathogen of *Citrus* and it has been reported causing crown rot of peach, plum and cherry rootstocks after artificial inoculations (Thomidis 2001). Association of *P. citrophthora* in nurseries of woody ornamental plants has been surveyed (Donahoo and Lamour 2008). Although *P. citrophthora* is non-host specific to alders, in our present inoculation tests, the pathogen has showed a great aggressiveness on *A. glutinosa* seeds and seedlings. In support to our findings, a separate inoculation test conducted by Santini *et al*. (2006), showed a certain seedling mortality of *Alnus* spp. after inoculation with *P. citrophthora*, although a lower mortality was obtained. In contrast, *P. citrophthora* was the most aggressive pathogen out of eleven *Phytophthora* species tested in a pathogenicity experiment conducted *in vivo* on cherry (Thomidis and Sotiropoulos 2003). Being an aggressive pathogen, we could assume that alders growing in nurseries and mixed plantations may be at risk to be infected by *P. citrophthora*.

P. nicotianae and *P. palmivora* are occurring frequently in nurseries with container grown seedlings. Donahoo and Lamour (2008) reported the presence of *P. nicotianae and P. palmivora* in nurseries of woody ornamentals. In addition, Hardy and Sivasithamparam (1988) found the presence of *P. nicotianae* in 9 nurseries out of 14 surveyed in Western Australia. Besides, a baiting bioassay detected *P. nicotiana* and several other *Phytophthora* in naturally infested container mixes from South Carolina nurseries in United States (Ferguson and Jeffers 1999). Up to date, no report was available on the pathogenic ability of *P. nicotianae and P. palmivora* on *Alnus* but, in our tests it has been revealed their capacity to show virulence on *A. glutinosa* seeds and seedlings. That's why it is assumed that container grown seedlings of *Alnus* spp. may be vulnerable to *P. nicotianae* and *P. palmivora* if there is enough inoculum as it occurs with other plants in nurseries. In support of the hypothesis, several non-host specific pathogens *P. citricola, P. cactorum* and *P. gonapodyides* were reported to be present in a nursery soil in the vicinity of alders (Oszako *et al*. 2007). Several other *Phytophthora* species such as *P. cambivora, P. cactorum, P. citricola, P. megasperma* and *P. quercina* were baited from flooding alder plants (Jung *et al*. 1999, 2003). All these observations may suggest that under suitable conditions, *P. nicotianae* and *P. palmivora* could be potentially harmful to *A. glutinosa*. The phenomenon gives a serious implication for future establishment of forest stands with infested nursery stock.

Production of zoospores in the presence of water significantly contributes to the dispersal of *Phytophthora* via irrigation (Yamak *et al*. 2002; Hong *et al*. 2006). Transportation of nursery stock and use of natural rivers and other water courses to irrigate nurseries give ideal opportunities to *Phytophthora* to infect and spread in those nurseries. Out-planting with *Phytophthora* infected nursery stock have added further more to the dissemination process of *Phytophthora* to natural ecosystems. Falling of seeds, young shoot or leaves onto the contaminated water or soil and later disseminating far distances through water ways. For the alder seeds and seedlings, which are vulnerable to *P. alni* and other *Phytophthora* species, is certainly a new threat. In addition to that, the emergence of a new *Phytophthora* species provides additional threat, as it has been the case of *Alnus* by a hybrid pathogen *P. alni* ssp. *alni* (Brasier *et al*. 2004). For instance, a new *Phytophthora* species, *P. polonica*, was isolated from rhizosphere soil samples when surveys conducted in declining alder stands grown naturally by the riversides in Poland (Belbahri *et al*. 2006). New pathogens can be brought into nurseries and distributed with seeds and seedlings to plantations or riparian ecosystems. In Great Britain, spreading of alder *Phytophthora* has occurred through watercourses (Gibbs *et al*. 1999) whereas in Bavaria, Germany *P. alni* has been introduced into many places either by planting infected nursery stock or by irrigation water (Jung and Blaschke 2004).

Artificial inoculation methods applied in an artificial environment may not provide precise information on the real interactions between hosts and pathogens. Besides, it may not possible to get exact results on the virulence of the *Phytophthora* species on hosts. Nevertheless, experiments done in an artificial environment may avoid interactions with other organism or minimize host natural defense mechanism. In this sense, the present study provides consistent clue of how seed germination and seedling mortality are affected by different *Phytophthora* isolates. Extrapolation of laboratory results to natural environment may not always be valid. However, results obtained from those *in vitro* inoculation methods can be useful for the identification of potential hosts and contribute to pathogens risk assessment. The results of the present study provided us a rough estimation on the susceptibility of an important tree species towards *Phytophthora*. As other *Phytophthora* spp. are able to infect alder seeds and seedlings, it is important to apply further controls on nursery management and irrigation system to avoid dissemination of *Phytophthora*. As a whole, additional dissemination of inoculum

with diseased nursery stock will hamper control measures of the diseases on alders. There is an immediate need of a molecular-based detection protocol and safer conditions for *A. glutinosa* to grow in nurseries and plantations. It is important to apply effective and immediate actions to prevent spread and transfer of *Phytophthora* spp.

In order to reduce the spread of *Phytophthora* diseases in plantations and nurseries the following few measures could be adapted: (a) careful selection of plant material (seeds and seedlings) free of diseases, (b) avoidance of frequent seedling transportation with soils from nurseries to planting sites, (c) monitoring of plant health growing in nurseries at a regular interval, (d) careful inspection and testing of nursery soil and water reservoir used to irrigate nurseries, (e) sterilization of nursery tools before performing silvicultural treatments, (f) planting of *Alnus* in the 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.

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ARTICLE IV

VARIATION IN PATHOGENICITY AMONG THE THREE SUBSPECIES OF *Phytophthora alni* (*P. alni* ssp. *alni*, *P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis*) ON DETACHED LEAVES, TWIGS AND BRANCHES OF *Alnus glutinosa*

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ABSTRACT

Pathogenicity tests were carried out on leaves, twigs and branches of *Alnus glutinosa* using several isolates of *Phytophthora alni* ssp. *alni*, *P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis in vitro*. Healthy fresh leaves were collected from disease-free areas and inoculated with mycelial agar disc or dipped in zoospore suspension. Besides, twigs and branches were collected from both disease-free and disease affected areas, inoculated with mycelial agar disc and incubated at four temperature regimes (15°C, 20°C, 25°C and 30°C). At the end of experiments, all tested subspecies appeared as pathogenic but with varied level of virulence. In leaf inoculation test, wounding was a key factor to cause infection and lesions occurred on inoculated wounded leaves differed significantly in length than the non-wounded and control. In case of twig and branch inoculation tests, no significant differences in virulence were observed among the tested isolates of subspecies of *P. alni* concerning sampling locations but differed according to temperature. In particular, largest lesions occurred on detached twigs and branches at 25°C. The study provides new information as vegetative materials of *A. glutinosa* could act as source of potential inoculums and may aid in spreading of alder *Phytophthora* to the natural ecosystems.

KEYWORDS: Common alder, inoculation, mycelia, virulence, zoospores

RESUMEN

Ensayos de patogenicidad fueron llevados a cabo en hojas, ramillos y ramas de *Alnus glutinosa* inoculando *in vitro* varios aislamientos de *Phytophthora alni* ssp. *alni*, *P. alni* ssp. *multiformis* y *P. alni* ssp. *uniformis in vitro*. Hojas sanas fueron recogidas de alisos en zonas no afectadas por la enfermedad e inoculadas mediante micelio del hongo o sumergiendo las hojas en una suspensión de esporas. Por otro lado, ramillos y ramas fueron recogidas tanto de zonas afectadas como no afectadas por la enfermedad, para posteriormente ser inoculadas mediante micelio e incubadas a cuatro temperaturas diferentes (15°C, 20°C, 25°C y 30°C). Los ensayos demostraron que las tres subespecies se comportaron como patógenas, si bien diferencias en cuanto a su virulencia fueron encontradas. En los ensayos de inoculación de hojas, provocar previamente una herida fue un factor clave para que se produjese infección causando lesiones significativamente mayores que al no provocar herida y el control. En el caso de las inoculaciones de ramillos y ramas, no se encontraron diferencias significativas entre las tres subespecies de *P. alni* en función de la localización de la cual se recogió el material vegetal (zonas afectadas o libres de la enfermedad). Sin embargo, diferencias significativas fueron encontradas en función de la temperatura. En concreto, en todos los casos las mayores lesiones tuvieron lugar a 25°C. Este estudio aporta nueva información sobre como material vegetal de *A. glutinosa* podría actuar como fuente potencial de inóculo y podría facilitar la dispersión de *P. alni* a otros ecosistemas naturales.

PALABRAS CLAVE: Aliso común, inoculación, micelio, virulencia, zoosporas

INTRODUCTION

Common alder [*Alnus glutinosa* (L.) Gaertn.] is the most widespread species among alders growing naturally along rivers, streams and other waterways in Spain. It is well adapted on clay and wet soils and widely planted for timber production, riverbank and slope stabilization because of its soil amelioration properties (CAB International 2000). The genus, *Alnus* has high ecological value because of its conservation role (Claessens 2003; Thoirain *et al*. 2007). However, despite of its numerous benefits, riparian zones and particularly alder forests have been disturbed by human activities over the past century (Kauffman *et al*. 1997; Naiman and Décamp 1997). Virgin riparian vegetations were under continuous disturbances because of stream flow regulated by storage reservoirs and canalization activities in the middle of the 20th century (Schnitzler 1994; González and García 2007). Mortality of *A. glutinosa* along riverbanks in northern Spain was first formally recorded by Tuset *et al*. (2006), but they did not reveal *Phytophthora alni* (Brasier and S.A. Kirk) and its association with mortality process. Subsequently, a few years later, *P. alni* has been identified as the primary causal agent of riparian common alder mortality in Spain (Solla *et al*. 2010; Varela *et al*. 2010, 2012). This alder *Phytophthora* was first detected in southern Britain in 1993 (Gibbs 1995). Since then, it has emerged as an important problem towards the existence of alders (Gibbs *et al*. 1999, 2003; Streito *et al*. 2002a) and led to cause mortality in western, central and northern Europe (Gibbs *et al*. 2003; Jung and Blaschke 2004). According to Jung and Blaschke (2004), dispersion of *P. alni* probably related to planting of contaminated alders in afforestations and use of contaminated river water for irrigation. They also pointed out that trading of nursery stocks between nurseries and construction of fish farms upstream might be responsible for unintentional disseminations of alder *Phytophthora* and other harmful pathogens to the plantation sites or natural ecosystems. Surveys in diseased and non-diseased alder stand also supported that infected alders might be responsible for rapid spreading of *P. alni* (Jung and Blaschke 2004). Flooding may also aid in spreading of *P. alni* from infected alders to old and young alder stands which have been established on waterlogged or downhill sites (Jung and Blaschke 2004). Likewise, zoospores dispersion during flooding from naturally infected bark to non-wounded bark of alders has also been demonstrated by Streito *et al*. (2002b).

In the past, *P. alni* has been considered as an inter-specific hybrid between *Phytophthora cambivora* (Petri) Buisman and another species closely related to *P. fragariae* Hickman (Brasier *et al*. 1995, 2004). Based on morphological, cytological and genetic data, Brasier *et al*. (2004) has been divided *P. alni* into three subspecies: *P. alni* ssp. *alni* corresponding to former 'standard' type, *P. alni* ssp. *multiformis* corresponding collectively to the Dutch, German and UK variants and *P. alni* ssp. *uniformis* corresponding to Swedish variant. However, recent genetic studies have shown that *P. alni* ssp. *alni* has originated from a unique or multiple hybridization events from *P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis* and following an asexual way of reproduction that maintained heterozygosity (Ioos *et al*. 2006, 2007; Aguayo *et al*. 2013). Both *P. alni* ssp. *alni* and *P. alni* ssp. *multiformis* have been reported to be highly aggressive on *A. glutinosa*, whereas *P. alni* ssp. *uniformis* has been shown as weakly pathogenic (Brasier and Kirk 2001).

A few studies concerning pathogenicity of *P. alni* on *A. glutinosa* and its regenerating materials are available (Brasier and Kirk 2001; Santini *et al*. 2003; Santini *et al*. 2006; Haque and Diez 2012). Nevertheless to our knowledge, studies to examine its pathogenicity on vegetative parts (i.e., detached leaves, twigs and branches) of *A. glutinosa* have not been performed to date. As plantation establishment with infected alders has been identified as one of the most important means of dispersion of *P. alni* to the natural ecosystems in Europe (Jung and Blaschke 2004), we assume that plant parts of *A. glutinosa* could act as potential source of inoculums, which may trigger spreading of the pathogen. That's why further inoculation tests are necessary in order to reveal its pathogenicity on such vegetative parts. Main objectives of the study were: to examine whether the pathogen can infect detached leaves, twigs and branches of *A. glutinosa*, and, to test whether any variations in virulence exist among the isolates of three subspecies of *P. alni*.

MATERIALS AND METHODS

Plant and fungal materials

Three inoculation experiments were performed using detached leaves (Experiment 1), detached twigs (Experiment 2) and branches (Experiment 3) of *A. glutinosa*. For experiment 1, fully expanded leaves were collected from mature healthy *A. glutinosa*

from disease-free areas. Collected fresh leaves were kept in poly bags moistened with distilled water and transferred to cool boxes to avoid desiccation. Before inoculation, leaves were washed in sterile distilled water for several times and dried over sterile paper towel inside a laminar flow chamber. For experiments 2 and 3, two different locations were selected for sampling (a) disease-free area and (b) diseased area (mortality of *A. glutinosa* recorded and was positive to *P. alni*). For each of the sampling locations, collection of plant materials was restricted to one individual tree (if possible) or maximum three individual trees. Collected twigs and branches were cut into small segments, wrapped in moist tissue during transport and placed in cool buckets. One day after collection, twigs (diam. ranged 0.7-1.0 cm) were trimmed to a length of 14 cm and branches (diam. ranged 1.2-1.5 cm) were trimmed to a length of 19 cm. To avoid desiccation, cut end of the branches and twigs were coated with wax in the laboratory.

Details of all isolates used in the inoculation experiments are given in Table 1. Three Spanish isolates of *P. alni* ssp. *alni* isolated previously from diseased *A. glutinosa* and a set of alder *Phytophthora* isolates (one isolate of each three subspecies) obtained from INRA (UMR Interactions Arbres-Microorganismes, Champenoux, France) were used in all inoculation experiments.

Table 1. Origin of alder *Phytophthora* isolates used in inoculation experiments

Isolate	Identification[a]	Year isolated	Provider	Country	GenBank Accession No.
PA4017	PAA	2010	UVa	Spain	KJ659839
PA5029	PAA	2010	UVa	Spain	KJ659843
PA7051	PAA	2011	UVa	Spain	KJ659835
PAA314*	PAA	2008	INRA	France	–
PAM393*	PAM	2009	INRA	France	–
PAU300*	PAU	2008	INRA	France	–

*Reference isolates.
UVa= Universidad de Valladolid, Spain.
INRA= National Institute of Agronomic Research, Champenoux, France.
a= PAA, *Phytophthora alni* spp. *alni*; PAM, *P. alni* spp. *multiformis*; PAU, *P. alni* spp. *uniformis*

For mycelial agar discs, colonies of the *P. alni* isolates were sub-cultured for 1 week at 20ºC in the dark onto 90-mm Petri dishes containing sterilized V8 agar (V8 agar: 100

ml/L V8 Campbell Grocery Products, 3 g/L CaC03, 20 g/L Agar Technical DIFCO, Detroit, MI, USA). For zoospore suspensions, colonies of the isolates were sub-cultured for 14 days at 20ºC in the dark onto 90-mm Petri dishes containing sterilized V8 agar exposed to 16 h light per day. Sporangia were obtained by flooding the colonies with sterile distilled water, and then transferred into sterile water by rubbing surface of the culture with a sterile bent glass rod. The liquid was poured off the plates and again collected in a sterile beaker which was placed in a refrigerator at 7°C for 1 h, then returned to room temperature for another 75 min to promote zoospore releasing. Zoospore concentration was determined by using a haemocytometer, and suspension was adjusted to 3×10^5 zoospores/ml (Denman *et al*. 2005).

Pathogenicity tests

Experiment 1: leaf inoculation

Leaf inoculation experiment was carried out following the methodology described previously by Denman *et al*. (2005). Before inoculation, leaves were divided into two groups: (1) non-wound and (2) wound. Eight leaves were inoculated for each combination of the three factors studied; isolate (six *P. alni* isolates plus control; see table 1), type of damage (non-wound vs. wound) and type of inoculum (mycelial agar disc vs. zoospore). In case of non-wound inoculation, leaves were inoculated by placing discs (*ca.* 6-mm diam.) to the centre of each leaf on the upper side. Control leaves were inoculated with sterile agar discs. In addition, inoculation was performed by dipping leaves in zoospores suspension, apical end first, up to the mid point of leaves, for 10 seconds while gently agitated to remove excess liquid (Denman *et al*. 2005). For controls, sterile distilled water was used. For wound inoculation, same methodology as described earlier was applied. Wounds were made on adaxial side of leaves using a sterile needle and discs were placed upside down facing the wounds. While inoculating with zoospores, two wounds were made with help of a sterile needle, on extreme apical part of each leaf. The leaves were then dipped in zoospore suspension. Detached inoculated leaves were placed over sterile metal grid mesh inside plastic boxes. Bottom of each box was bedded with wet sterile paper towels, creating a moist environment. The sides of the boxes were sprayed with sterile distilled water at regular intervals and sealed with a thin layer of petroleum jelly. Leaves were incubated in a growth chamber at 25°C with 16 h cool white fluorescent light for 6 days. Subsequently, lesion lengths were determined by measuring the area of necroses produced on surface of leaves. In

case of zoospore inoculation, lengths were measured from margin of the apical tip to the furthermost necrotic edges. After surface sterilization of leaves with ethanol, segments from margin of lesions were placed directly on V8-PARPH agar [V8 juice agar media amended with 10 µg/mL pimaricin, 200 µg/mL ampicillin, 10 µg/mL rifampicin, 25 µg/mL pentachloronitrobenzene (PCNB), and 50 µg/mL hymexazol] in order to re-isolate the pathogen.

Experiment 2: twig inoculation

V8 agar media was dispensed into sterilised glass jars to a depth of *ca.* 10 mm. Jars were seeded with mycelial agar discs of the tested isolates and sealed with parafilm to conserve moisture. Jars were placed inside incubators at 22°C in the dark for a few days until colony growth covering the agar surface. Before placing twigs inside the jars, they were surface disinfected in a 10% solution of household bleach (5% sodium hypochlorite), washed properly in sterile water and blotted dry (Thomidis and Sotiropoulos 2003). The bark from the basal end of each twig was removed with the help of a sterile knife until cambium was exposed. Stripped twigs were then inserted vertically into the agar in each jar at the periphery of the fungal colony. The jars were incubated at tested temperature trials (15, 20, 25 and 30°C) for 6 days in the dark. Control twigs were placed in jars contained sterile V8 agar. Ten replicate twigs for each combination of the three parameters studied in this experiment: isolate (six *P. alni* isolates plus control; see table 1), location (disease-free area vs. diseased area) and temperature (15, 20, 25 and 30°C). Subsequently, twigs were removed from the glass jars and examined carefully for any lesion. By subtracting the depth of agar from the total length of lesion, values of lesion lengths were obtained. In order to confirm the presence of the inoculated pathogen, small pieces from the margins of the lesion and from the site of inoculation were cut and plated onto on V8-PARPH agar in order to re-isolate the pathogen.

Experiment 3: branch inoculation

A sterile cork borer (6-mm diam.) was used to remove bark strip including cambium from the branches prior to inoculation. An agar disc (*ca.* 6-mm diam.) containing mycelium was placed up side down into the hole of each branch. Bark strip was replaced and inoculated area was covered with sterilized wet cotton, and then wrapped with parafilm to avoid desiccation. Control branches were inoculated with sterile agar

discs. Number of replication used for branch inoculation was equal used for twig inoculation. Inoculated branches were kept over sterile metal grids placed on moist paper towels inside plastic boxes and incubated for 6 days under tested temperatures in the dark. The side of the boxes were sprayed with sterile distilled water cautiously at regular interval to avoid moisture deficiency. Six days after inoculation, outer bark around the inoculation site was carefully removed with the help of a sharpen knife to expose the entire lesion. Total lesion length extending above and below the point of inoculation was measured. To confirm the presence of pathogen, same methodology was applied as described earlier.

Statistical analyses

Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effect of three different factors studied in each experiment (Experiment 1: isolate, damage and inoculum, and Experiment 2 and 3: isolate, sampling location and temperature) on lesion lengths caused by the isolates of *P. alni*. Due to the fact that the data violated two of the ANOVA assumptions (normality and homogeneity of variances), robust methods were applied (García Pérez 2010). In particular, three-way fixed factors effects ANOVAs were performed under non-normality and inequality of variances using the generalized Welch procedure and a 0.2 trimmed mean transformation. The ANOVAs were carried out using the "Wilcox' Robust Statistics (WRS)" package implemented in the R software environment (R Foundation for Statistical Computing, Vienna, Austria).

Poisson regressions were carried out using the count of girdling in each experiment, overdispersion was analysed and no problems were found. To determine the parameter estimates, a reference group was established in each factor. In experiment 1, the reference groups were "non-wound", "zoospore" and "control" for damage, type of inoculum and isolate, respectively. Whereas in experiment 2 and 3, the reference groups were "disease-free area", "30°C" and "control" for location, temperature and isolate, respectively. The Poisson regressions were carried out using the function "Fitting Generalized Linear Models" implemented in the R software environment.

RESULTS

Leaf inoculation

When leaves were wounded, lesions developed after 6-day incubation period, whereas no infection and necroses were noticed on non-wounded or control leaves. Lesions were not always uniform in size and produced dark brown discoloration. Re-isolation of *P. alni* from necrotic tissues was successful. However it was not possible to recover the pathogen from any of the non-wounded and control leaves. A significant interaction between isolate and type of damage was observed for the size of the lesions ($F = 18.75$, $P < 0.05$), whereas no significant effect of type of inoculums was found ($F = 4.10$, $P = 0.06$). Wounding had a significant effect on the pathogen isolates to cause lesions on the inoculated leaves. In wounding treatment, the most virulent isolates based on lesion length were PA4017 (with a mean lesion length 3.15 ± 1.21 cm) and PA7051 (with a mean lesion length, 1.53 ± 0.58 cm), both of them statistically different from control (Figure 1). Although, the remaining isolates (PA5029, PAA314, PAM393 and PAU300) produced lesions, no significant differences were noted among all of them and the respective non-wound treatment. Likewise, lesions did not differ statistically from control (Figure 1).

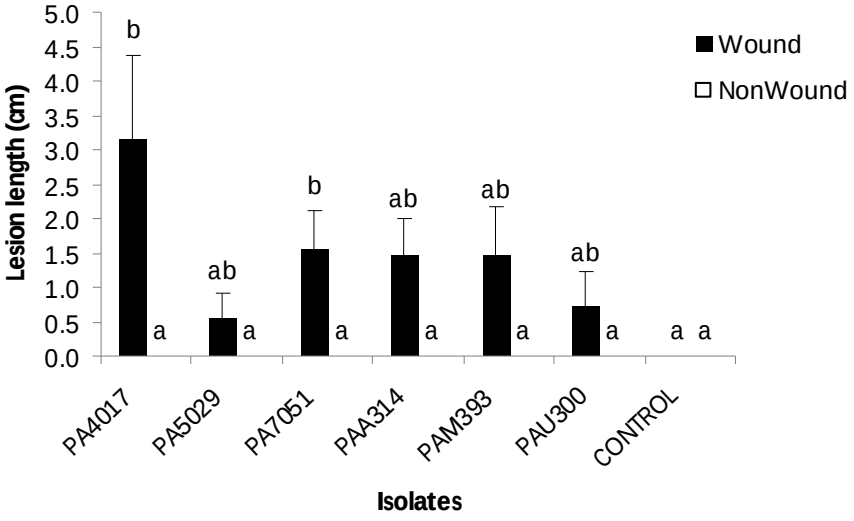

Figure 1. Mean lesion length (cm ± SE) assessed on detached leaves of *A. glutinosa* 6 days after inoculation by *P. alni* isolates. Different letters above bars indicate significantly different means (Generalized Welch procedure 0.2 trimmed means, $\alpha = 0.05$)

Twig inoculation

The results of the ANOVA demonstrated that the interaction between isolates and temperatures was significant ($F = 4042.59$, $P < 0.001$). Nevertheless, sampling location or the interaction with isolate or temperature failed to produce any significant effect on lesions caused by inoculations. At the end of inoculation trial, largest lesions on inoculated twigs were observed at 25ºC followed by 20ºC, 15ºC and 30ºC. Range of mean lesion lengths at 25ºC, 20ºC, 15ºC and 30ºC were 8.23 ± 0.21 to 6.95 ± 0.24, 4.72 ± 0.28 to 3.01 ± 0.14 cm, 1.77 ± 0.09 to 1.14 ± 0.13 cm and 0.97 ± 0.19 to 0.32 ± 0.07 cm, respectively (Figure 2).

The post hoc test revealed that the isolates exhibited a complex pattern of virulence towards inoculated twigs in relation to different temperatures utilized (Figure 2). At 25ºC, lesions caused by the isolates revealed that they showed an equal level of virulence except PA4017 which was statistically more virulent and caused larger lesions than others, such as PA5029 and PAU300 (Figure 2). At 20ºC, the isolates followed a different pattern of virulence. At this temperature, PA7051, PAA314 and PAM393 caused lesions which were statistically similar in lengths but were different than those caused by the others. The remaining isolates showed varied level of virulence, particularly PAU300 caused the smallest lesions which did not differ significantly from PA5029 (Figure 2). At 15ºC, lesion lengths were significantly different among the isolates tested. But, PA4017 and PA5029 were the most virulent with comparison to others, such as PA7051 and PAA314. At 30ºC, all isolates caused similar lesions, which differed significantly from control (Figure 2). Control failed to cause any lesions on the inoculated twigs. The pathogen was consistently re-isolated from the lesions occurred on twigs except the controls, from which no re-isolations were obtained.

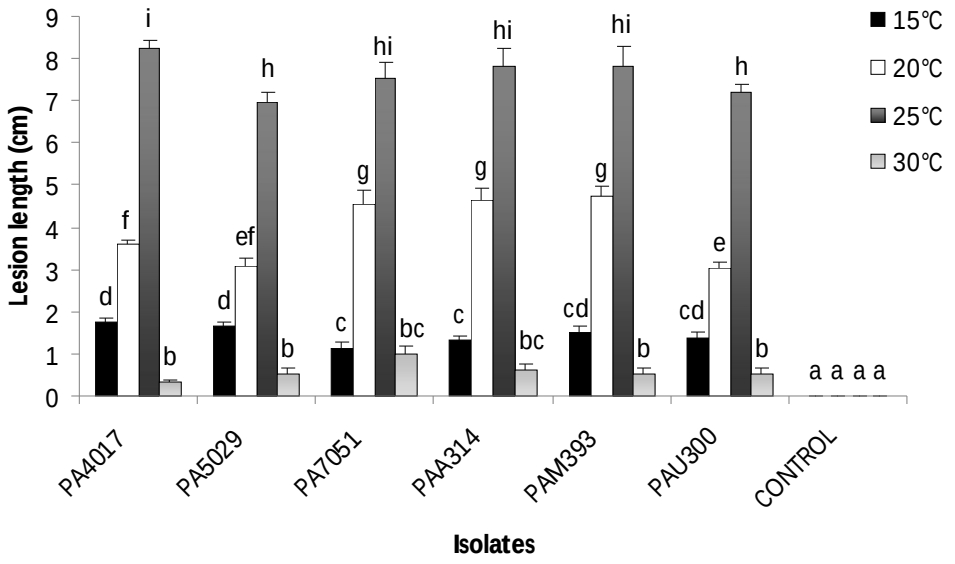

Figure 2. Mean lesion length (cm ± SE) formed on detached twigs of *A. glutinosa* six days after inoculation with mycelial agar disc of *P. alni* isolates. Different letters above bars indicate significantly different means (Generalized Welch procedure 0.2 trimmed means, α= 0.05)

The results of the Poisson regression showed that the best model (Equation 1) to determine the probability of girdling was obtained using the factors, temperature and isolate ($\lambda = 0.001$, $P < 0.001$). Sampling location or any interaction with temperature or isolate did not improve the model either. Four isolates (PA7052, PAA314, PAU300 and PAM393) showed a significant higher likelihood of causing girdling than control. Likewise, a temperature of 25°C implied a higher number of girdling on the twigs inoculated than other temperatures (15, 20 and 30°C).

Log (*Girdling* + 1) = - 0.6755 + 1.5810 *Temperature 25ºC* + 0.97 *PA7051* + 1.48 *PAA314* + 1.26 *PAU300* + 1.06 *PAM393* …… (Equation 1)

Branch inoculation

Results of the ANOVA demonstrated that the interaction between isolates and temperature was significant ($F = 2424.34$, $P < 0.001$). In particular, all isolates showed larger mean lesion lengths than control at any temperature, but PAU at 15 and 30ºC. In fact, PAU caused smaller lesions than the other isolates at 20, 25 and, to a lesser extent, at 30ºC (Figure 3). At the end of inoculation test, largest lesions occurred at 25ºC, in case of all tested isolates. At this temperature, mean lesion lengths ranged from 7.15 ± 0.36 to 2.27 ± 0.10 cm (Figure 3). The second most important temperature was 20ºC

followed by 30ºC and 15ºC. Range of mean lesion lengths produced by all isolates at 20ºC, 30ºC and 15ºC were 4.55 ± 0.41 to 1.20 ± 0.12 cm, 1.25 ± 0.19 to 0.52 ± 0.10 cm and 1.02 ± 0.10 to 0.38 ± 0.08 cm, respectively (Figure 3).

At 25ºC, PAA314 produced largest lesions (with a mean of lesion length 7.15 ± 0.36 cm) which were not significantly different from those caused by PA4017, PA5029 and PAM 393 but differed with lesions caused by PA7051 and PAU300 (Figure 3). At 20ºC and 30ºC, all isolates produced lesions which were statistically same in length except PAU300 (Figure 3). At 15ºC, significant differences were only found between PAM393 and PAU300. Moreover, the lesions caused by the latter was not statistically different from the control, despite control treatments did not produce any lesion (Figure 3). The inoculated pathogen was consistently re-isolated from the inoculated branches, but never from the controls.

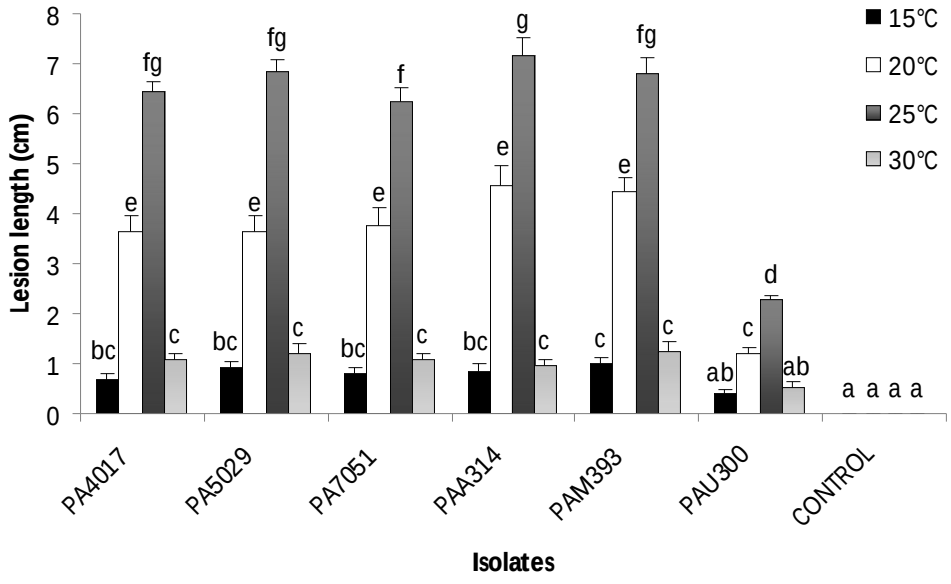

Figure 3. Mean lesion length (cm ± SE) assessed on detached branches of *A. glutinosa* 6 days after inoculation with mycelial agar disc of *P. alni* isolates. Different letters above bars indicate significantly different means (Generalized Welch procedure 0.2 trimmed means, α= 0.05)

The results of the Poisson regression showed again that the best model (Equation 2) to determine the probability of girdling was obtained using two factors, temperature and isolate ($\lambda = 0.002$, $P < 0.001$). Sampling location or any interaction with temperature or isolate didn't improve the model. In particular, only two isolates (PAA314 and PAM393) showed significantly higher likelihood of causing girdling than the control.

On the other hand, again the temperature of 25°C produced a higher number of girdling in the inoculated branches than the other temperatures (15, 20 and 30°C).

Log (*Girdling* + 1) = - 0.46 + 1.21 *Temperature 25°C* + 1.10 *PAA314* + 1.14 *PAA393* …… (Equation 2)

DISCUSSION

P. alni is a host specific pathogen and has been reported to cause alder mortality in Europe (Jung and Blaschke 2004; Cerný and Strnadová 2010; Solla *et al*. 2010). Diseased alders show necroses at the lower stem or at collar region with rusty exudates, yellowish, sparse and small leaves, die-back and intense fructification (Gibbs 1995; Gibbs *et al*. 1999; Jung and Blaschke 2004). Alder *Phytophthora* usually infects *Alnus* through fine roots or adventitious roots and causes root rot and collar or stem necroses (Jung and Blaschke 2004; Cerný and Strnadová 2010). Until now, the pathogen has not been reported to cause infection or isolated from leaves of alders. No artificial inoculations have been done so far to test whether the pathogen is capable to do so. To our knowledge, this is the first study of this type that has been performed. Our study has revealed the capability of *P. alni* to infect and cause necroses on *A. glutinosa* leaves if they have been inoculated artificially. This study has also proved that wounding was an important factor to cause infection. Such wounding effect is also consistence with other previous findings (Erwin and Ribeiro 1996; Thomidis 2003; Kaminski and Wagner 2008), who demonstrated that growth and development of *Phytophthora* was associated with wounds and wounded plants were more vulnerable to infection than the non-wounded ones. At the end of experiment, when the isolates of three subspecies of *P. alni* were ranked according to their virulence, two isolates of subspecies *P. alni* ssp. *alni* was the most virulent one based on length of lesion, although no significant differences in lengths were noted for remaining isolates of *P. alni* ssp. *alni* and the other two subspecies. This means that all isolates of the subspecies were equally virulent on wounded inoculated leaves of *A. glutinosa*. Pathogenic ability of *Phytophthora* species on leaves of their woody hosts have been well documented where artificial wounding was necessary in most cases to cause infections and necroses. *In vitro* leaf inoculation studies on leaves of broad-leaved and coniferous trees using zoospores of *P. ramorum* Werres, DeCook & Man in't Veld by Denman *et al*. (2005) showed that necroses and

disease incidence increased significantly when wound inoculations were done. On the contrary, wounding was not always necessary to cause infection on foliage using zoospores of *P. ramorum* as revealed by others (Tooley *et al*. 2004; Hansen *et al*. 2005; Tooley and Kyde 2007). In a detached leaf inoculation study, higher infection rates were obtained by using zoospores as inoculum compared to mycelial agar plugs (Hansen *et al*. 2005). In addition to that, infection rates increased when greater number of zoospore inoculum was used. Such effects of inoculums are at conflicting to our findings as in our case, type of inoculums did not show any significant effect on the pathogenicity of the three subspecies. However, it has been concluded that use of mycelia in non-wound inoculations, is a conservative method to assess pathogenicity of *Phytophthora* species or susceptibility of plants towards them. Pathogenic ability of *P. alni* to cause lesions on leaves following wounding and failure to produce any lesions on non-wounded leaves suggest that the pathogen would unlikely to be a foliar invader for alders.

Two separate inoculation experiments were conducted using detached twigs and branches of *A. glutinosa* to study the effect of temperatures and sampling locations on pathogenicity of three subspecies of *P. alni*. In the current tests, temperature has appeared as a key factor that significantly influenced virulence of isolates of the tested subspecies. The observed extension of necrotic lesions on the sections of inoculated twigs and branches varied significantly among the tested isolates. Isolates of *P. alni* ssp. *alni* and *P. alni* ssp. *multiformis* have appeared to be more virulent on both inoculated twigs and branches of *A. glutinosa* where as isolate of *P. alni* ssp. *uniformis* was relatively less virulent, particularly on branches at all tested temperatures. Similar observations were found in a previous study done by Brasier and Kirk (2001), where pathogenicity test was carried out on detached logs of *A. glutinosa* using several isolates of three subspecies of *P. alni*. Their results also demonstrated that the isolates of *P. alni* ssp. *alni* (previously known as 'standard hybrid') and *P. alni* ssp. *multiformis* (previously designated as 'Dutch', 'German' and 'UK' variants collectively) were highly aggressive while the isolates of *P. alni* ssp. *uniformis* (previously designated as 'Swedish' variant) were weakly virulent on the inoculated logs of *A. glutinosa*. Significant differences in necroses lengths were similar to those of other studies conducted on alders (Lonsdale 2003; Clemenz *et al*. 2006; Schumacher *et al*. 2006). In the present study, all three tested subspecies produced largest lesions on inoculated

twigs and branches at 25ºC followed by 20ºC. Temperatures range (20ºC-25ºC), was considered as the most suitable range at which *P. alni* and other *Phytophthora* such as *P. cactorum* var. *applanata* Chester and *P. cryptogea* Pethybr. and Laff. were reported to be active and pathogenic (Harris and Tobutt 1986; Hardham 2001; Brasier *et al*. 2004). A similar range of temperature was also reported ideal for the development of largest lesions on inoculated detached branches by *P. cactorum* and *P. citrophthora* (R.E. Smith and E.H. Smith) Leonian (Thomidis 2003). Temperature effects on the isolates of *P. alni* as described here are in good agreement with temperatures corresponded to seasonal change effects on lesion development by *Phytophthora* species on common alder, oaks and other trees (Robin *et al*. 1994; Brasier and Kirk 2001; Luque *et al*. 2002; Moralejo *et al*. 2009). In the current investigation, a temperature of 25ºC was also noted as the most suitable temperature to form girdling, in both inoculated twigs and branches. On the other hand, it is important to note that, *P. alni* ssp. *uniformis* did not show higher likelihood of causing girdling than control on the branches, whereas a different pattern was found in twig inoculations. Finally, it could be concluded that young sprouts of *A. glutinosa* might be more vulnerable to infection than the matured ones by *P. alni* ssp. *uniformis*, which is considered to be relatively less aggressive pathogen (Brasier and Kirk 2001). Effect of sampling locations on the virulence of the tested isolates of three subspecies was considered as negligible, on both inoculated twigs and branches. Here, it could be pointed out that the current disease-free areas are still under threat to infection by *P. alni*.

Pathogenic ability of *P. alni* on inoculated detached leaves, twigs and branches of *A. glutinosa* is certainly a new threat. These vegetal materials could be the sources of inoculums which may passively transmit *P. alni* to the natural ecosystems or new plantation sites via alder seedlings. Alders play an important role in stabilizing banks of rivers and is considered as the key element of riparian ecosystems in Europe. Such spreading of *P. alni* may lead drastic changes in the riparian ecosystems in coming years, causing economic losses and ecological changes (Oszako 2010). Falling of young twigs and branches onto the contaminated water or soil could travel far distances with flow of water (Oszako 2010). Zoospores of *P. alni* swim freely over water surface and likely to spread far distance using rivers or other water systems which may attack roots of alders growing naturally along the riverbanks. From a study made by Santini *et al*. (2003) was concluded that spreading of alder *Phytophthora* may be related to its

introduction with the planting materials, which may have infected in nurseries. As a result, healthy alder trees are at risk to be infected as they co-exist with contaminated plants in the same nurseries. In addition, asymptomatic seedlings raised in nurseries may act as a carrier facilitating the dispersion of inoculums to the natural ecosystems.

The present study has given us a clear idea on the vulnerability of an ecologically important tree to *P. alni*. In order to mitigate the problem, a traditional option is to find out disease resistant genotypes using a series of artificial inoculation experiments and use them in programmes for resistance breeding. Nevertheless, our study has demonstrated that results of artificial inoculation experiments can be affected by other factors like temperature. Furthermore, experiments conducted in a controlled environment may not provide precise information on the interactions between hosts and pathogens. Therefore, there is a need of good management of nurseries and irrigation systems to stop dissemination of alder pathogen. Molecular-based identification system should be initiated for rapid and effective early detection of the pathogen. It is also important to ensure safer growing environment for *A. glutinosa* both in nurseries and plantations. This is essential to apply effective actions to prevent spread of the pathogen to natural ecosystems. From this point of view, a common strategy and co-ordinated programme for limiting the transportation of infected plants from country to country is also needed (Oszako 2010).

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ARTICLE V

FIRST REPORT OF *Phytophthora plurivora* CAUSING COLLAR ROT ON COMMON ALDER IN SPAIN

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ABSTRACT

During surveys for alder *Phytophthora*, isolates of a new *Phytophthora* sp. were recovered from necrotic bark at lower stems of *Alnus glutinosa* by direct plating on a selective medium. The isolates produced smooth-walled spherical oogonia with paragynous antheridia, and both plerotic and aplerotic golden brown oospores on V8 juice agar. In soil-extract they produced sporangia which were non-caducous, semipapillate, mainly ovoid and obpyriform, obovoid to limoniform. The internal transcribed spacer (ITS) region of the rDNA was amplified, sequenced and compared with the reference sequence of *Phytophthora plurivora* showing 100% identity with it. The isolates were pathogenic to inoculated seedlings of *A. glutinosa* and caused mortality. This is the first report of *P. plurivora* on *A. glutinosa* in Spain.

KEYWORDS: *Alnus glutinosa*, decline, die-back, homothallic, inoculation

RESUMEN

Durante muestreos llevados a cabo para detectar *Phytophthora alni*, aislamientos de otra especie de *Phytophthora* fueron obtenidos al cultivar en medio de cultivo selectivo fragmentos de corteza con síntomas de necrosis de las partes bajas del tronco de *Alnus glutinosa*. Los aislamientos produjeron en medio V8A oogonios esféricos de paredes lisas con anteridios paráginos y oosporas doradas-pardosas pleróticas y apleróticas. Produciendo en los extractos de suelo esporangios no caducos, semipapilados y principalmente con formas desde ovoides, obpiriformes, obvoides a limoniforme. El ADN de la región ITS fue amplificado, secuenciado y comparado con las secuencias de referencia de *Phytophthora plurivora*, obteniéndose un 100% de similaridad. Los aislamientos se comportaron como patógenos sobre las plántulas de *A. glutinosa* inoculadas, causando su muerte. Esta es la primera cita de *P. plurivora* en *A. glutinosa* en España.

PALABRAS CLAVE: *Alnus glutinosa*, decaimiento, puntisecado, homotálico, inoculación

Phytophthora decline of riparian alder (*Alnus* spp.) has been reported in several European countries (Jung and Blaschke 2004). Death of common alder [*A. glutinosa* (L.) Gaertn.] due to *Phytophthora alni* (Brasier and S.A. Kirk) has also been reported in Spain (Solla *et al*. 2010). During several surveys of alder trees in September 2012, typical die-back symptoms, including sparse small yellowish foliage and the presence of rusty exudates on the bark at the collar and lower stem were observed in *A. glutinosa* growing on the banks of the river Tera (Langa de Duero, Soria, 41°36′34″N, 3°25′10″W, elevation 851 m) and the river Tormes (La Maya, Salamanca, 40°41′42″N, 5°35′36″W, elevation 833 m). Bark samples plus cambium were taken from the active lesions at collar region, cut into small pieces, dried on filter paper and plated on V8-PARPH agar (Jung and Blaschke 2004). The samples were incubated for four days at 20°C in the dark before obtaining the *Phytophthora* isolates.

Colonies developed on V8 juice agar (V8A) had limited aerial mycelium at the centre and displayed radiate and slightly chrysanthemum-like growth pattern. Mycelial growth was optimal at 25°C (radial growth rate, 8.2 mm d^{-1}), whereas no growth was observed at 32°C. Isolates were homothallic with paragynous antheridia, smooth-walled spherical (very rarely elongated) oogonia (22.8–30.6 µm diam.) and both plerotic and aplerotic golden brown oospores (21.3–28.5 µm diam.). In non sterile soil extracts, the isolates produced abundant sporangia (31.5–57.2 × 21.3–38.4 µm; length:breadth ratio 1.2 to 1.6) borne terminally on unbranched or sympodial sporagiophores, occasionally attached laterally to the sporangiophores. Sporagia were non-caducous, semipapillate, mainly ovoid and obpyriform, obovoid to limoniform but sometimes distorted with two apices. On the basis of the morpho-physiological features, the isolates resembled *P. plurivora* (formerly identified as *P. citricola* Sawada) (Jung and Burgess 2009). To confirm this, genomic DNA was extracted and subjected to Polymerase Chain Reaction (PCR). The internal transcribed spacer (ITS) region of the rDNA was amplified using the ITS-6 (5’ GAA GGT GAA GTC GTA ACA AGG 3’) and ITS-4 (5’ TCC TCC GCT TAT TGA TAT GC 3’) primers before sequencing (Secugen, Madrid, Spain). The sequences were deposited in the EMBL/GenBank database (GenBank Accession No. KF413074 and KF413075).

In order to perform the pathogenicity test, 10 *A. glutinosa* seedlings (two-year-old) per isolate were inoculated by using the under bark inoculation technique (Jung *et al*. 1996) and 10 control seedlings were inoculated with V8A. Seedlings were incubated in a

growth chamber at 22.5ºC with a 14-h photoperiod. Three months after inoculation, all inoculated plants wilted and died, whereas the control plants showed no disease symptoms. To fulfil Koch's postulates, the pathogen was re-isolated from the necrotic lesions developed around inoculation points, thus confirming its pathogenicity. *P. plurivora* has been found to be present in rhizosphere soil beneath *Alnus* spp. and to cause aerial canker and collar rot on alder trees in Austria, Germany and Romania (Jung and Blaschke 2004; Jung and Burgess 2009). Further studies and surveys are essential to determine the distribution, extent of damage and potential interactions with other alder pathogens (e.g. *P. alni*). To our knowledge, this is the first record of *P. plurivora* affecting *A. glutinosa* in Spain.

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