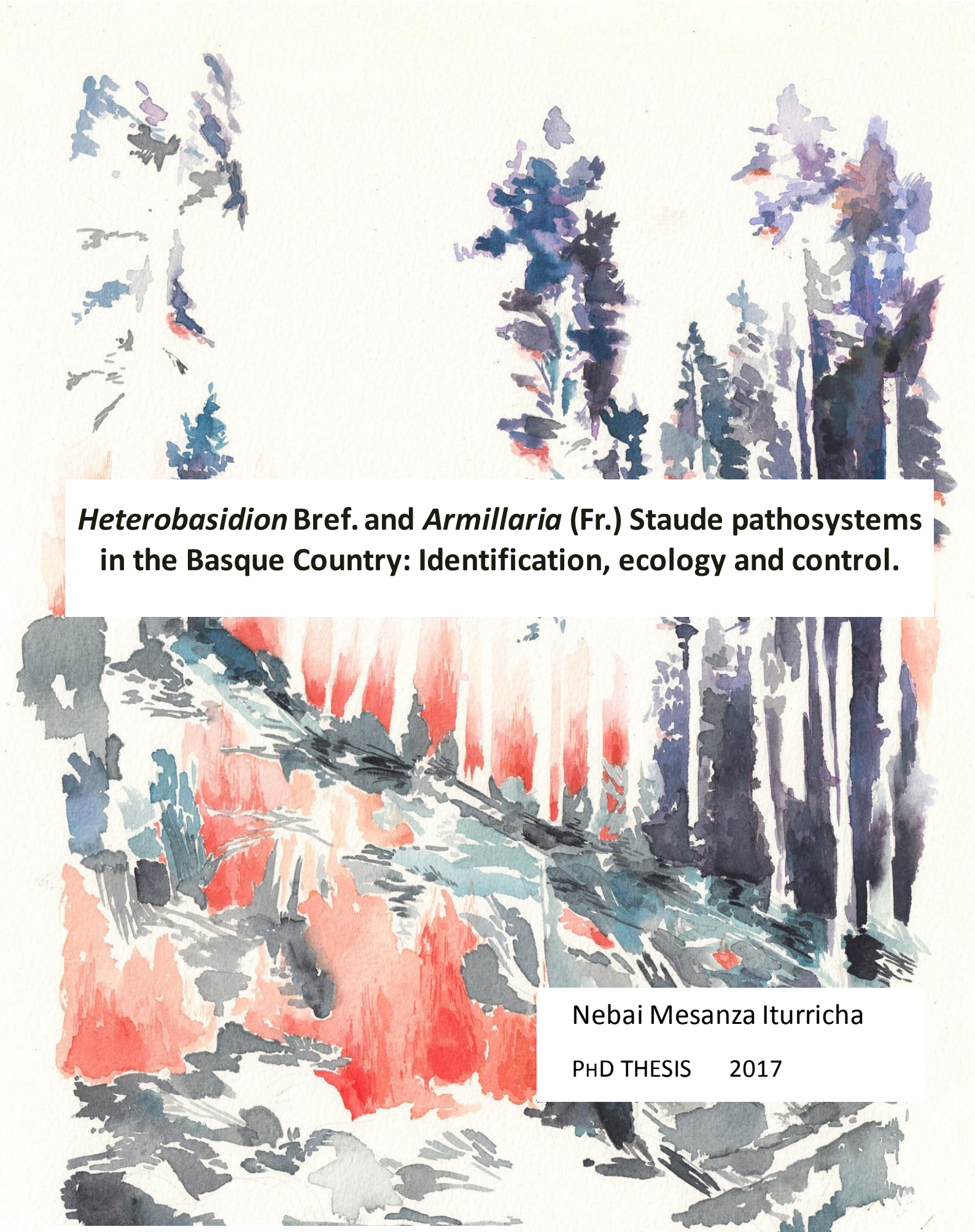

Heterobasidion Bref. and *Armillaria* (Fr.) Staude pathosystems in the Basque Country: Identification, ecology and control.

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PHD THESIS 2017

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PHD THESIS

Heterobasidion Bref. and *Armillaria* (Fr.) Staude pathosystems in the Basque Country: Identification, ecology and control.

Presented by Nebai Mesanza Iturricha

2017

Under the supervision of

Dr. Eugenia Iturritxa and Dr. Cheryl L. Patten

Tutor: Dr. Maite Lacuesta

Front page: Forest, by Araiz Mesanza Iturricha

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List of original articles

This thesis is based on the following articles:

- Mesanza, N., Patten, C. L, Iturritxa, E., 2017. Distribution and characterization of *Armillaria* complex in Atlantic forest ecosystems of Spain. Forests (Submitted).
- Mesanza, N., Iturritxa, E., Patten, C.L., 2016. Native rhizobacteria as biocontrol agents of *Heterobasidion annosum s.s.* and *Armillaria mellea* infection of *Pinus radiata*. Biol. Control 101, 8-16.
- Mesanza, N., Iturritxa, E., 2012. Root and butt rot caused by *Heterobasidion annosum* in Atlantic coniferous ecosystems of Spain. For. Pathol. 42, 514–520.

Summary of contents

CHAPTER 1

Introduction and objectives

Forest health can be defined from two perspectives, the utilitarian, or anthropocentric, and the ecosystem, or ecocentric. From the first one, a healthy forest would be the state in which abiotic or/and abiotic factors do not threaten the management objectives either in the present or in the future. From the second one, the forest is defined as an ecosystem that includes the physical and biotic factors to support it. In the latter case, a healthy forest is resistance to dramatic changes, there is functional equilibrium between supply and demand of essential resources, and there is diversity in seral stages and stand structure (Kolb et al., 1994). Under this definition, a tree plantation may not be a healthy forest ecosystem due to the lack of species variety, but may meet the utilitarian objectives (Raffa et al., 2009).

Some biotic factors, such as root pathogenic fungi, have a beneficial role in regulating the forest nutrient availability and increasing vegetation diversity (Ostry and Laflamme, 2009), but this beneficial role can be altered when the equilibrium between the fungi and their host, which presumably have coevolved, is disrupted. In some cases, this is the consequence of anthropogenic changes in the environment or in the soil (Otrosina, 2005), i.e. some forest management techniques (Edmonds et al., 2000; Garbelotto, 2004; Jactel et al., 2009). This results in economic losses and ecological alteration because of host´s high mortality levels.

In the temperate forest, root and butt rot fungi are considered the greatest causes of economic losses. This group of fungi is mainly formed by three globally-distributed genera: *Armillaria* (Fr.) Staude, *Heterobasidion* Bref. and *Phellinus* Quél., all of them within the class Agaricomycetes (Garbelotto, 2004). The research described herein is based on the identification, ecology, and control of two of these, *Armillaria* and *Heterobasidion.*

1.1. *Armillaria* species complex

1.1.1. Taxonomy

Armillaria genus is characterized by clitocyboid basidiocarps with white basidiospores, from decurrent to adnate gills, pileus usually with golden-brown colors, central stipe and diploid, often slow grower, and bioluminescent vegetative mycelium producer of black to dark-brown rhizomorphs in field and *in vitro*. Its compatibility system is bifactorial, and usually heterothallic, and it can act as saprophyte or wood parasite (Watling et al., 1991; Baumgartner et al., 2011).

In the past the description of this genus was based on morphological characteristics. Every Agaricomycetes with white spores, a ring and gills was considered to belong to *Armillaria* genus, which was thought to be composed only by one species with variable morphology and pathogenicity, *Armillaria mellea* (Vahl) P. Kumm. This caused a high confusion level in the taxonomic history related to this genus. However, at the end of the 1970s when the biological species concept (Volk and Burdsall, 1995; Baumgartner et al., 2011), which refers to the reproductive isolation of a group, was applied to *Armillaria* this matter was practically resolved. Thereafter, phenotypic and ecological characteristics were secondary determinants, after intermating (Coetzee, 2003). More recently, due to the progress made in molecular phylogenetic analysis, the genus *Armillaria* is included in the kingdom Fungi, division Basidiomycota, subdivision Agaricomycotina, class Agaricomycetes, subclass Agaricomycetidae, order Agaricales, family Physalacriaceae (Matheny et al., 2006; Hibbett et al., 2007).

The name currently admitted for this genus is (Indexfungorum.org, 2017):

- *Armillaria* (Fr.) Staude, *Schwämme Mitteldeutschl.* 28: xxviii, 130 (1857).

Although some synonyms have been used (Indexfungorum.org, 2017):

- *Agaricus* trib. *Armillaria* Fr., *Syst. mycol.* (Lundae) 1: 9, 26 (1821).
- *Aphotistus* Humb., *Fl. Friberg. Spec.* (Berlin): 118 (1793).
- *Armillaria* sect. Armillariella P. Karst., *Bidr. Känn. Finl. Nat. Folk* 32: xii (1879).
- *Armillariella* (P. Karst.) P. Karst., *Acta Soc. Fauna Flora fenn.* 2(no. 1): 4 (1881) [1881-1885].
- *Polymyces* Battarra *ex* Earle, *Bull. New York Bot. Gard.* 5: 447 (1909).
- *Rhizomorpha* Roth, *Ann. Bot. (Usteri)* 1: 7 (1791).

Armillaria is also commonly known as honey mushroom, oak fungus or honey agaric; and the disease it causes as shoestring root rot, mushroom root rot, toadstool disease, resin glut and resin flow (Williams et al., 1986).

1.1.2. *Armillaria* species and their distribution

Currently, about 40 morphological species of *Armillaria* are known worldwide (Wingfield et al., 2011). They show variable pathogenic capacity and thus develop epiphytic, mycorrhizal, saprophytic or pathogenic associations with their host (Ross-Davis et al., 2013). This variability is also shown in host and habitat preferences (Guillaumin et al., 1993). Seven different species are present in Europe: *Armillaria mellea* (Vahl) P.Kumm., *Armillaria gallica* Marxm. & Romagn., *Armillaria ostoyae* (Romagn.) Herink, *Armillaria tabescens* (Scop.) Emel, *Armillaria cepistipes* Velen., *Armillaria borealis* Marxm. & Korhonen, *Armillaria ectypa* (Fr.) Lamoure (Guillaumin et al., 1993; Pérez-Sierra and Henricot, 2002) (Table 1.1), (Figure 1.1).

Table 1.1. European *Armillaria* species distribution and their morphological characteristics (Guillaumin et al., 1993; Coetzee, 2003; Ainsworth, 2003; Ohenoja, 2006).

***ARMILLARIA* SPECIES**	**DISTRIBUTION**	**RHIZOMORPH FORMATION**	**BASIDIOCARP CHARACTERISTICS**
A. mellea	Thermophilic. Widespread in Atlantic climate. Dominant in Mediterranean climate particularly at moderate and low altitudes.	Short lived, limited spread ability.	Prominent annulus, honey colored caps and robust appearance of the basidiocarp.
A. ostoyae	Continental or Atlantic climate. Independent of latitude or altitude. Higher altitudes at Mediterranean climate.	Thin and brittle. Dichotomous branching.	Thick annulus and dark scales.
A. borealis	Northern and continental distribution. Locations with cold air accumulation.	Dichotomous branching.	
A. gallica	Not in areas of extremely cold climate. Usually at low altitudes which can increase at Mediterranean climate.	Long, thick and perennial. Monopodially branched.	Thin and delicate annulus and bulbous or clavate stipe.
A. cepistipes	Latitude inversely proportional to altitude.		
A. tabescens	More thermophilic than *A. mellea.*	No.	Without annulus.
A. ectypa	Boreal-montane and maybe also at continental climates. Usually in peat bogs.		

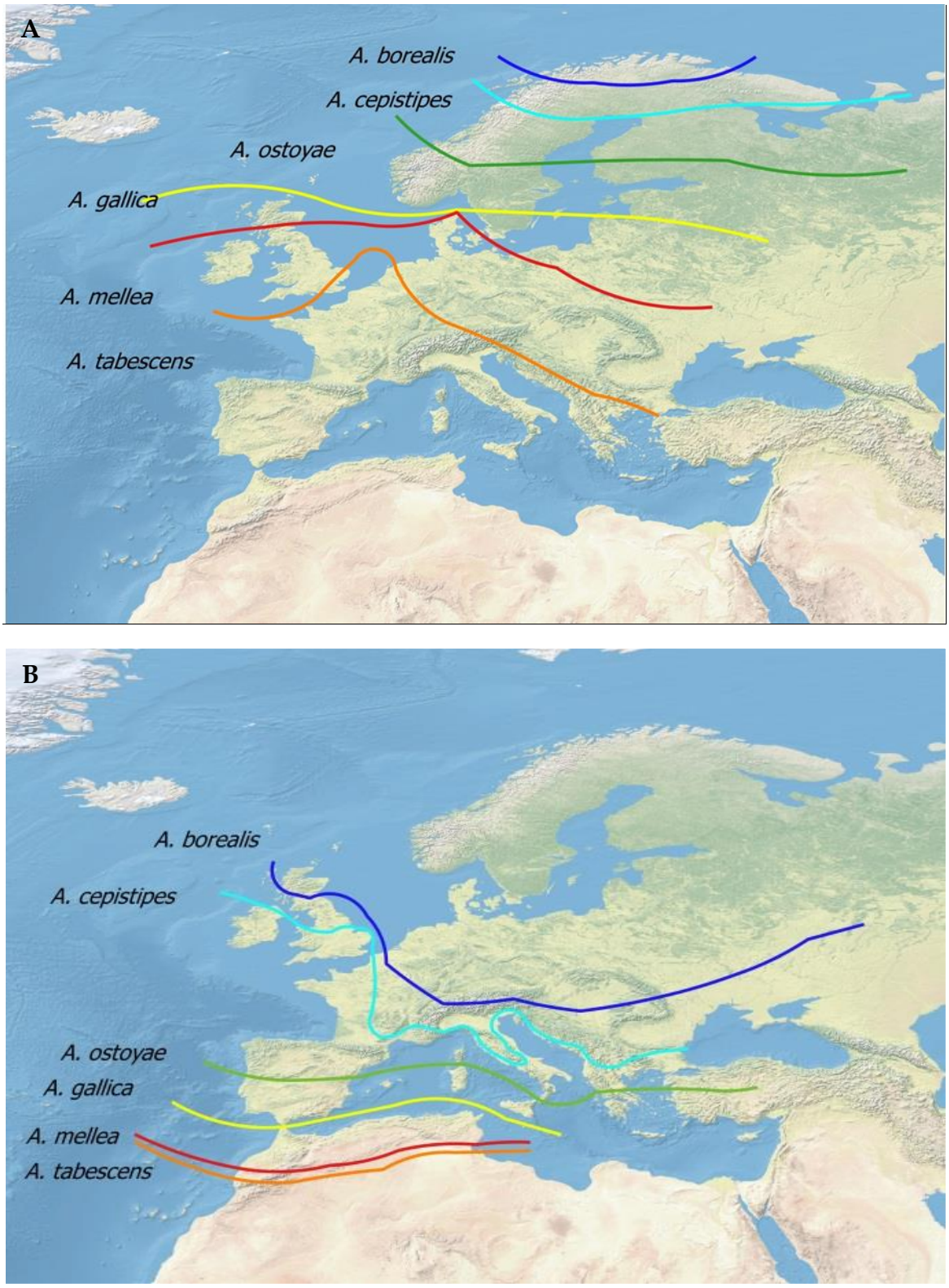


Figure 1.1. Northern (A) and southern (B) limits of *Armillaria* spp. distribution in Europe. (adapted from Guillaumin, 2005).

In Spain, the presence of *Armillaria* has been reported in the following provinces and regions:

- Alicante: *A. mellea* in *Eriobotrya japonica* Lindl. (louquat) plantations (González- Domínguez et al., 2009).
- Basque country: *A. mellea*, *A. ostoyae*, *A. cepistipes*, *A. gallica* and *A. tabescens* in different forest ecosystems, fruit trees and vineyards (Iturritxa et al., 2008).
- Catalonia: *A. mellea* in soil (Nogales et al., 2010).
- Galicia: *A. mellea*, *A. ostoyae*, *A. gallica* and *A. cepistipes* in a high host range such as conifers, broadleaf trees and shrubs (Aguín et al., 2004a; Escofet et al., 2006).
- Salamanca: *A. mellea* in *Castanea sativa* Mill. (García-Benavides and Monte, 2005).
- Spanish Pyrenees: *A. cepistipes*, *A. gallica*, and *A. ostoyae* mainly in *Abies alba* Mill. (silver fir) (Oliva et al., 2009).
- Valencia: *A. mellea* in *Citrus* × *sinensis* (L.) Osbeck (pro. sp.) and in Troyer citrange roots (Tuset et al., 1999).

1.1.3. Host susceptibility

A broad range of trees, shrubs and some herbaceous plants are susceptible to infection by *Armillaria* species (Williams et al., 1986). In many cases this susceptibility is directly related to the health condition of the host, i.e. it has been observed that hosts with high stress are more predisposed to develop infection. Entry et al. (1991) explained this as a reduction in the concentration of defensive compounds in the root, such as total phenolic compounds including lignin and tannins, as a consequence of a reduction in photosynthetic capacity. Thus, stress factors such as defoliating and stem boring insects and other pathogenic fungi can increase the incidence of *Armillaria* infection (Hudak and Singh, 1970; Hood and Samberg, 1993a). Waterlogged or dry soils, temperature extremes, soil compaction, and light and nutrient deficiency can also affect host susceptibility (Entry et al., 1991; Goheen and Otrosina, 1998; Popoola and Fox, 2003).

In general, conifers seem to be more susceptible than hardwoods (Wargo and Harrington, 1991), although the different *Armillaria* species involved and the host species' susceptibility is an important factor. For instance, in north-western America, Douglas fir is highly susceptible to *Armillaria* but in Western Europe it is tolerant of the native *Armillaria* strains (Guillaumin and Legrand, 2013). *A. tabescens*, usually a saprophyte or an opportunistic pathogen, acted as a primary pathogen in eucalyptus plantations in south-west France (Guillaumin et al., 1993). The composition of the stand can also influence the range of infection; lower density of susceptible species and higher species diversity in different forest strata reduce the possibility of disease transmission (Gerlach et al., 1997; Kromroy et al., 2005).

Management procedures such as selective logging, early thinning and/or the continued use of susceptible or moderately susceptible species that are not very well adapted to the location, increase the inoculum sources, and thereby, increase the probability of infection (Bloomberg and Morrison, 1989; Morrison et al., 1991; Hood and Sandberg, 1993a; Hood and Kimberly, 2009). Thus, in comparison with natural forests, the damage in exotic tree plantations is usually greater (Guillaumin and Legrand, 2013).

1.1.4. Infection and life cycle

In field conditions, *Armillaria* mycelium is mainly diploid (Ullrich and Anderson, 1978). In this state it can colonize different hosts by direct contact between an infected source and roots by way of hyphae, or by advancing through the ground from an infection point by way of rhizomorphs (Redfern and Filip, 1991) (Figure 1.2). In both cases, the production of plant cell wall degrading enzymes such as ligninolytic, pectinolytic, cellulolytic, hemicellulolytic and related enzymes make possible the colonization of roots, which is facilitated for woody roots by the pressure that rhizomorphs apply (Ross-Davis et al., 2013; Williams et al., 1986). The plant tissues that are affected are the cambium and the secondary xylem (Baumgartner, 2004). This is a mechanism for short distance spreading but it is considered the most important even though the formation of rhizomorphs in the field is not common for some *Armillaria* species (Wargo and Shaw, 1985; Pérez-Sierra and Henricot, 2002).

After the mycelium is established, and when environmental conditions are suitable, seasonal basidiocarps are formed. The spores produced by the basidiocarps are dispersed by air currents (Worrall, 2004), and can appear at long distances although the colonization is greater near the basidiocarp (Power et al., 2008; Travadon et al., 2012). After settling in stumps or wood debris they form a haploid mycelium (Hood et al., 2002) (Figure 1.2). This kind of mycelium is not common in field conditions possibly because a high rate of crossings between sexually compatible haploid mycelia generate new diploid populations of *Armillaria* (Ullrich and Anderson, 1978; Baumgartner et al., 2011). Alternatively, interactions with the parental diploid mycelium can result in the extension of the latter, i.e., nuclei of the diploid parent, but not mitochondria, migrate into the haploid mycelium and displace the haploid nuclei (Anderson and Ullrich, 1982; Rizzo and Harrington, 1992). The capacity to create new infection points varies from one species to another (Kliejunas, 2011) but, in general, spores seem to be the least frequent mechanism of disease transmission (Baumgartner et al., 2011).

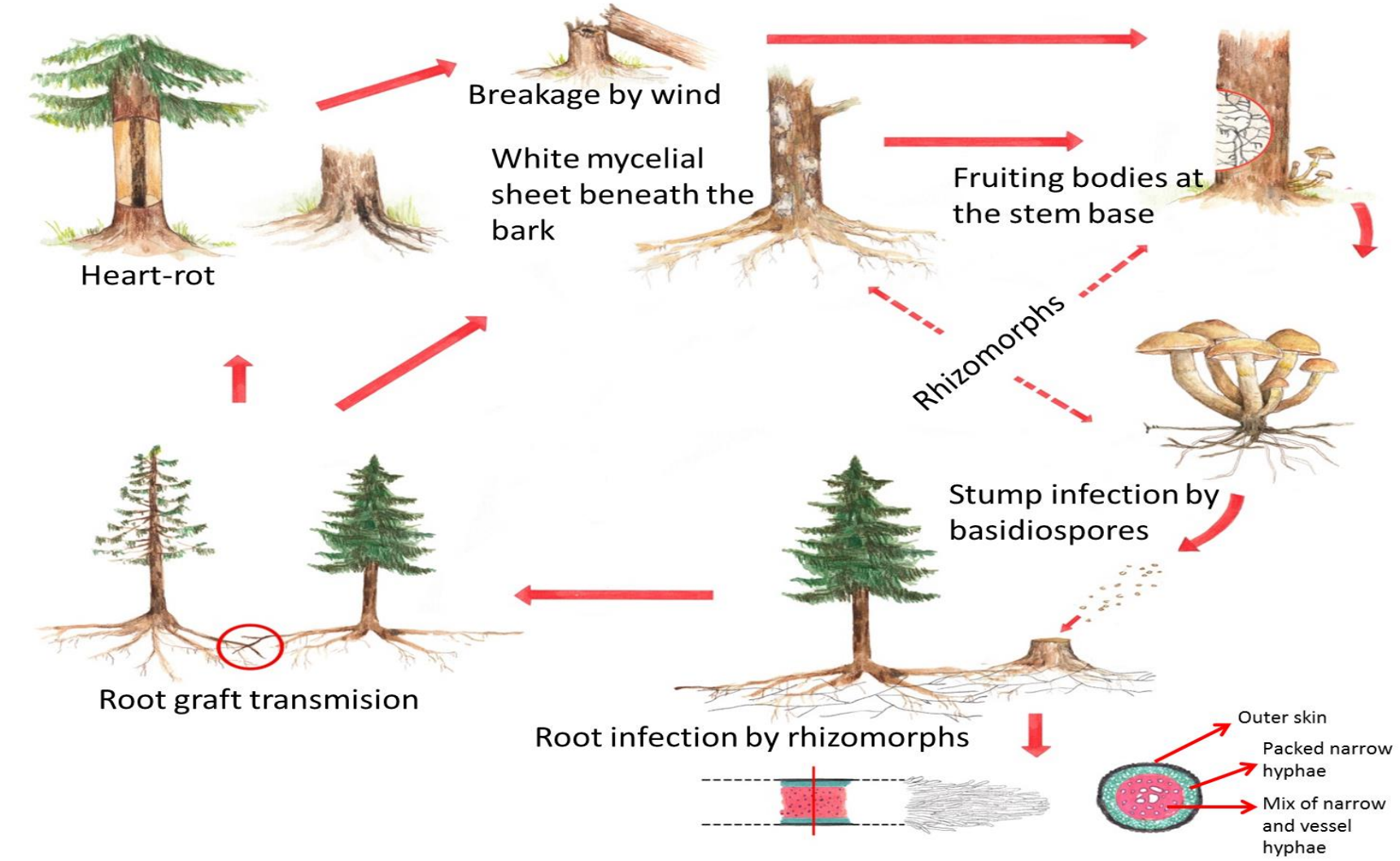


Figure 1.2. *Armillaria* spreading mechanisms (adapted from Schmidt, 2006; ilustrated by A. Mesanza).

1.1.5. Symptoms and signs of infection

When *Armillaria* acts as a pathogen it can cause some general symptoms of infection such as chlorotic leaves, progressive thinning of the crown, slower leader growth, excess cone production, and rapid tree death (Edmonds et al., 2000). Usually this means that the root collar and a high percent of main lateral roots are infected (Baumgartner et al., 2011). Infection of the entire root system is not necessary to cause death (Livingston, 1990).

These symptoms on their own do not have any diagnostic value because they are similar to those caused by other structural root rot pathogens, bark beetles, rodents or extended drought (Williams et al. 1986). However, in combination with the appearance of subcortical white mycelial fans, clusters of golden-brownish mushrooms near the tree base, rhizomorphs, rotten stringy-yellow wood with black lines (pseudosclerotia), rapid tree death without the loss of foliage, and/or basal resin or gum exudates, the presence of *Armillaria* can be confirmed (Cox et al., 2005; Edmonds et al., 2000) (Figure 1.3). It has been suggested that aboveground examinations underestimate the number of affected trees. For example, Robinson et al. (2003) realized that in *Eucalyptus diversicolor* F.Muell. stands, aboveground surveys detected only 50% of the total infected trees and for mixed conifer stands, only 58% were detected (Whitney et al., 1989).

Figure 1.3. Dead trees without loss of foliage (A and B), *Armillaria* spp. basidiocarps (C, D and E), stringy- yellow rotten wood (F), and subcortical white mycelium (G).

1.1.6. Damage

In a forest, the infection can appear mainly in three different patterns: i) forming limited disease centers which develop radially creating a gradient of decay, ii) extensively with randomly scattered dead trees, or iii) in young stands, as small patches of mortality which stop developing when the trees mature (Williams et al., 1986; Mallett, 1992). *Armillaria* usually causes the highest mortality in the early stages of the plantation development, e.g. between 20-50% in the first 6 years of *Pinus radiata* D.Don. stands (Hood and Sandberg, 1993b), and a maximum of 2% per year for Douglas fir (Morrison and Pellow, 1994) and spruce (Livingston, 1990). Shearer (1995) noticed that the susceptibility of *E. saligna* Sm. decreased with the age and Lung- Escarmant and Guyon (2004) observed that *P. pinaster* Ait. mortality due to *Armillaria* occurred principally in the first 5 years of the plantation. In this stand phase, the volume loss can sometimes be compensated by the growth of the survivors (Mackenzie, 1987).

Once the plantation is in a medium-late stage of the growing cycle, volume losses are caused by tree mortality (lethal infection) and radial/length growth reduction (chronic infection). MacKenzie (1987) estimated volume losses of 5.5–11 m^3/ha associated with chronic infection and losses of 26-61 m^3/ha due to lethal infection, representing a total of 6-13% of the potential volume in a 28 years old *P. radiata* plantation. Bloomberg and Morrison (1989) found a reduction in volume of up to 59% in Douglas-fir stands and Cruickshank (2000) mentioned a 40% volume reduction over 4-8 years in 18 year old Douglas fir plantation. In lodgepole pine stands the mean reduction of the annual wood volume increment was 43% ten years after trees become infected (Mallet and Volney, 1999). Kaliszewski et al. (2007) calculated the greatest losses of 8 m^3/ha/year for spruce. The quantity of damage caused by *Armillaria* is highly variable because it depends on factors such as fungal species and virulence, host species and vigour, interaction with other diseases, soil properties, climate, management and previous land uses, among others (Kile, 1983; Mallett and Volney, 1990; Entry et al., 1991; Mallett and Maynard, 1998; Poopola and Fox, 2003; Hood and Kimberley, 2009). Therefore, it cannot be generalized (Wargo and Shaw, 1985).

1.2. *Heterobasidion* species complex

1.2.1. Taxonomy

The genus *Heterobasidion* is characterized by having perennial basidiocarps with cuticulate pilei, hymenial surface poroid, multinucleate hyphal cells, slightly dextrinoid and strongly cyanophilous skeletal hyphae, asperulate basidiospores, and a *Spiniger* anamorph; it can act as a saprophyte or a wood parasite (Stalpers, 1979; Niemelä and Korhonen, 1998; Korhonen, 1978). The genus is included in the kingdom Fungi, division Basidiomycota, subdivision Agaricomycotina, class Agaricomycetes, subclass Agaricomycetes incertae sedis, order Russulales, family Bondarzewiaceae (Indexfungorum.org, 2017).

The name currently admitted for this genus is (Indexfungorum.org, 2017):

Heterobasidion Bref., *Unters. Gesammtgeb. Mykol.* (Liepzig) 8: 154 (1888); Synonymy: *Murrilloporus Ryvarden*, *Mycotaxon* **23**: 192 (1985).

Anamorph: *Spiniger meineckellus* (A.J. Olson) Stalpers, *Proc. K. Ned. Akad. Wet.*, Ser. C, Biol. Med. Sci. **77**(4): 402 (1974).

1.2.2. *Heterobasidion* species and their distribution

Twelve species of *Heterobasidion* are known worldwide (Niemelä and Korhonen, 1998; Otrosina and Garbelotto, 2010; Ota et al., 2006; Dai et al., 2007; Dai and Korhonen, 2009; Tokuda et al., 2009; Buchanan, 1988; Chen et al., 2014):

- *H. araucariae* P.K. Buchanan.
- Within *H. insulare* (Murrill) Ryvarden species complex: *H. australe* Y.C. Dai & Korhonen, *H. ecrustosum* Tokuda, T. Hatt. & Y.C. Dai, *H. linzhiense* Y.C. Dai & Korhonen, *H. orientale* Tokuda, T. Hatt. & Y.C. Dai, *H. amyloideum* Y.C. Dai, Jia J. Chen & Korhonen, and *H. tibeticum* Y.C. Dai, Jia J. Chen & Korhonen.
- Within *H. annosum* (Fr.) Bref. species complex (*H. annosum s.l.*) (Figure 1.4):
    - Eurasian species: *H. annosum sensu stricto* (*s.s.*), *H. abietinum* Niemelä & Korhonen, and *H. parviporum* Niemelä & Korhonen (Table 1.2).
    - North American species: *H. irregulare* (Underw.) Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel.

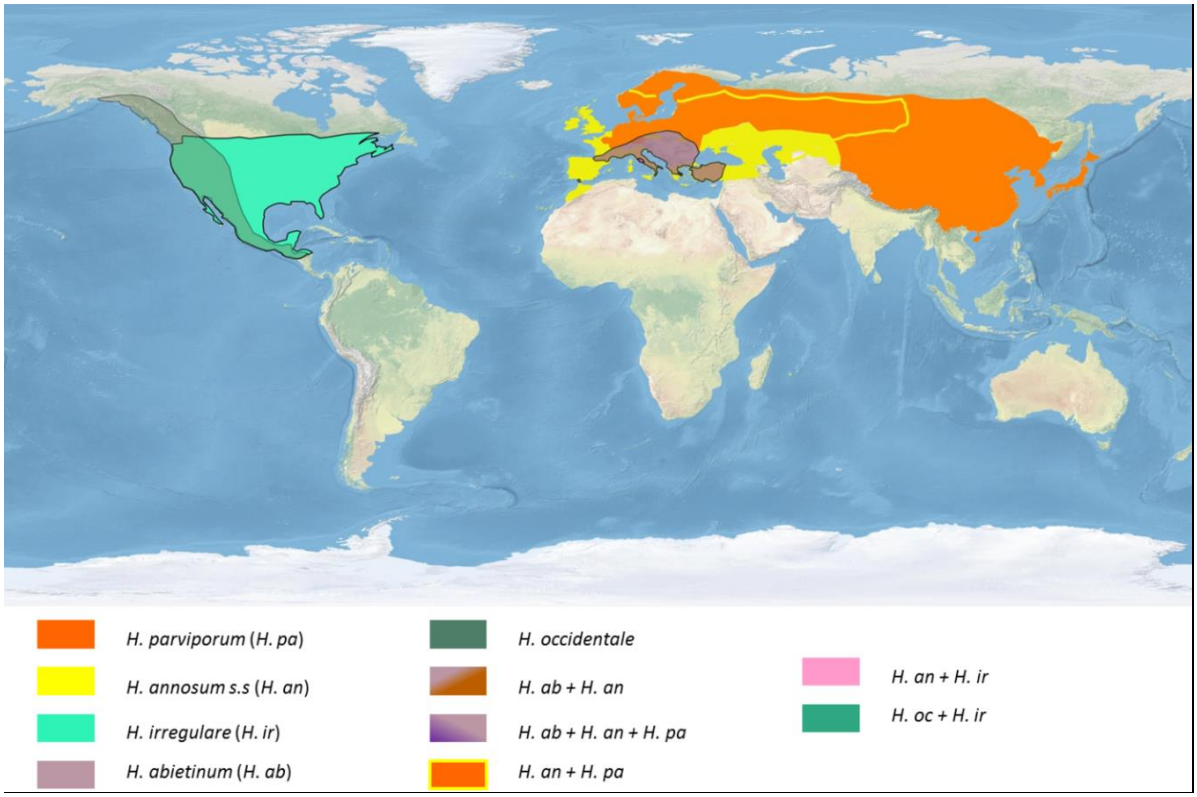


Figure 1.4. *H. annosum s.l.* global distribution (adapted from Garbelotto and Gonthier, 2013).

In Spain, the presence of *H. annosum s.l.* species has been reported in the following provinces:

- Segovia: in a stump of *P. sylvestris* L. (Martínez, 1943).
- Andalusia: *H. abietinum* in *A. pinsapo* Boiss (Navarro et al., 2003; Sánchez, 2007).
- Basque country: *H. annosum s.s.* in different conifer species (Mesanza and Iturritxa, 2012).
- Aragon: *H. annosum s.s.* in *P. nigra* Arn. (Oliva et al., 2008) and *H. abietinum* in sylver fir (*A. alba*) stands (Oliva and Colinas, 2009).
- Palencia*: H. annosum s.s.* in *P. pinaster* (Prieto-Recio et al., 2012).

Table 1.2. Morphological chacacteristics of European *H. annosum s.l.* species (Tokuda et al., 2009).

Species	*H. annosum s.s*	*H. parviporum*	*H. abietinum*
Known as	European P (Pine) type	European S (Spruce) type	European F (Fir) type
Basidiocarps	Annual to perennial, pileate or sessile to effused-reflexed, imbricate with several pilei or solitary.	Annual to perennial, effused-reflexed to resupinate or pineate, imbricate with several pilei or solitary.	Perennial, pileate to effused- reflexed.
Pilei	Often semicircular.	Applanate to slightly convex.	Semicircular or elongated.
Pileus surface	Subtomentose to almost glabrous, brown to dark brown, partly almost black, margin light yellow.	Tomentose, brown to dark brown, partly almost black near the base, margin light yellow to yellowish white.	Subtomentose to almost glabrous, brown to dark brown, partly almost black, margin light yellowish orange to pale yellowish orange.
Pore surface	Light yellow.	Pale yellowish orange to light yellowish orange.	Light yellowish orange to pale yellowish orange.
Pores	2-3 mm, round to angular.	3-4(-5) mm, round to angular, rarely elongated.	2-3 mm, round to angular.
Context	Up to 7 mm thick.	Up to 3 mm thick.	Up to 3 mm thick.
Tubes	Concolorous with context, up to 7 mm deep in each layer.	Concolorous with context, up to 6 mm deep in each layer.	Concolorous with context, up to 4 mm deep in each layer.

1.2.3. Host susceptibility

Conifers are the main host of *H. annosum s.l.* and most conifer species have been reported as susceptible to this species complex (Asiegbu et al., 2005). The different *H. annosum s.l.* species show different degrees of host specialization; *H. abietinum* is primarily reported on *A. alba* but it has been found on other *Abies* Mill. spp., and in species of *Chamaecyparis* Spach, *Juniperus* L., *Larix* Mill., *Pinus* Linn., *Castanea* Mill., *Fagus* L., and *Pseudotsuga menziesii* (Mirb.) Franco and *Cryptomeria japonica* (Thunb. ex L.f.) D.Don (Korhonen et al., 1998). *H. parviporum* is considered highly specialized and a primary pathogen of *Picea abies* (L.) Karst. (Asiegbu et al., 2005) even if it can kill native or exotic *Pinus* spp. It was also found in other hosts such as stressed birch trees, *Abies* spp., *Malus sylvestris* (L.) Mill., *Fraxinus* Tourn. ex L, *Vitis vinifera* L., and others (Korhonen et al., 1998; Sedlák and Tomšovský, 2014). *H. annosum s.s.* is considered a generalist primary pathogen, thus, it is the European *H. annosum s.l.* species with the greatest host range, including conifers and broadleaf trees. *P. sylvestris* and, in general, *Pinus* spp. are the most susceptible to this pathogen (Korhonen et al., 1998; Asiegbu et al., 2005).

1.2.4. Infection and life cycle

H. annosum s.l. infection is established by two mechanisms, most frequently by colonization of basidiospores or by contact between infected and non-infected wood structures like roots (Figure 1.5). Basidiospores are actively released (Korhonen and Stenlid, 1998), and usually they settle down within a short distance from the original basidiocarp but they can cover distances of 50 to 500 km. They colonize bark-free wood, such as fresh cut surfaces and root lesions, and germinate as a homokaryotic mycelium which will become heterokaryotic due to compatibility reactions (Kallio, 1970; Stenlid, 1994, Rayner et al., 1987). When infection levels are low, heterokaryotization is less probable so homokaryotic colonies will be in a higher proportion (Möykkynen and Kontiokari, 2001). Although in North America damage caused by homokaryonts has been reported (Garbelotto et al., 1997), it is not common in living trees in Finland (Korhonen and Piri, 1994). When the mycelium is developed it can extend to the root system and infect healthy roots of nearby trees and stumps. The role in infection of the conidiospores produced by the anamorph *S. meineckellus* is not clear and this asexual form is uncommon at the field level (Greig, 1998). Although in stumps artificial inoculation of conidiospores resulted in infection (Morrison and Redfern, 1994), so they might be important for short-distance dispersion (Asiegbu et al., 2005).

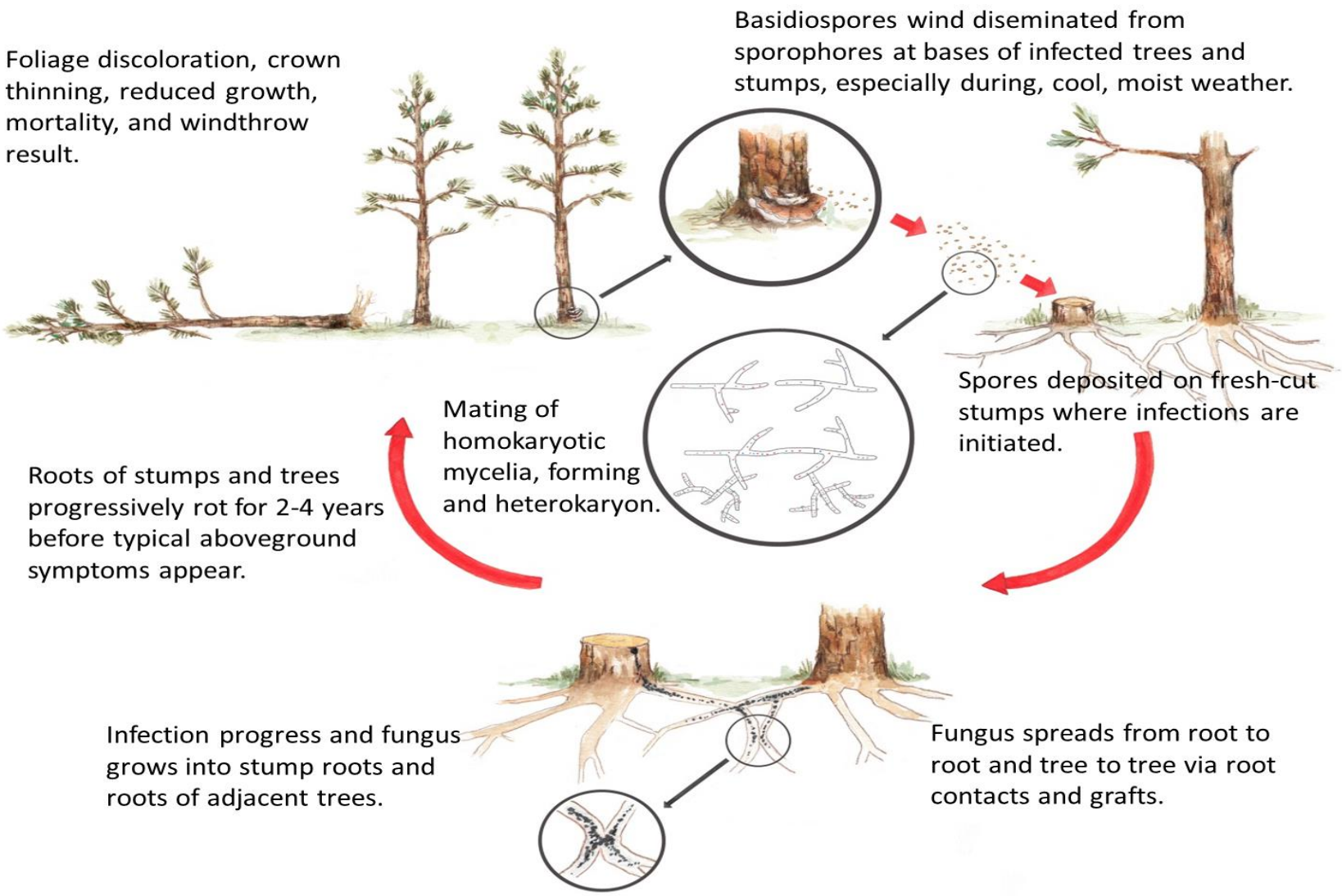


Figure 1.5. Generalized infection mechanism of *Heterobasidion* spp. (adapted from Barnard and Nixon, 1983; ilustrated by A. Mesanza).

1.2.5. Symptoms and signs of infection

The aerial symptoms of *H. annosum* infection, such as chlorotic leaves, wilt, groups of or isolated dead trees, and wind-downed trees with rotten roots, are generic and they do not provide any diagnostic value (Tainter and Baker, 1996). Thus, the presence of basidiocarps is the most reliable diagnostic criterion in the field. Basidiocarps can be found in rotten parts of death or living trees, between gaps in the root system or in animal galleries, in the base of trees and stumps, and inside empty trees and stumps (Figure 1.6). In areas with dry summers they are not common in exposed parts (Sinclair et al., 1987).

In general, the affected trees have anchoring problems due to degradation of main roots which make them susceptible to falling from wind or snow weight; this is the main cause of death by *H. annosum* (Smith et al., 1992). Even if the root system is affected aerial symptoms may not be apparent, but growth suppression could be observed in a detailed survey. Because of this, it is assumed that when *H. annosum* is established in a stand the number of infected trees that can be detected by external observation is usually underestimated (Sinclair et al., 1987).

Figure 1.6. Tree gaps in a plantation with presence of *Heterobasidion* (A, B and C). *Heterobasidion* basidiocarps (D and E), and conidia and conidiospores of the asexual *S. meineckellus* (F).

1.2.6. Damage

The level of damage caused by *H. annosum s.l.* in natural forests is usually lower than the damage caused in conifer plantations. Although the losses caused by *H. annosum s.l.* vary greatly between regions, they are considered to be of high importance. In the European Union financial losses due to tree decay and reduction in growth were initially estimated at 790 million euros per year (Woodward et al., 1998). Pratt (1979) reported a loss in value of 43% in *P. sitchensis* (Bong.) Carr. plantations of the UK, the direct financial losses in Norway spruce and mixed stands were calculated as an 18%-34% by Gonthier et al. (2012), and in Sweden and southern Finland 54 million euros per year and 35 million euros per year, respectively, were lost due to *H. annosum s.l.* damage (Bendz-Hellgren and Stenlid, 1995; Bendz-Hellgren et al., 1998). Timber volume losses caused by *Heterobasidion* infection are due to tree decay, diameter growth reduction, windthrow, and stand susceptibility to storm damages (Garbelotto and Gonthier, 2013). The infected trees are also more susceptible to other factors such as bark beetle infestations (Goheen and Otrosina, 1998).

Higher infection and damage rates have been recorded in stands with previous use as agricultural or pasture land, and when nearby stands are highly infected. Soil conditions that can favor the disease are fertile soils, sandy soils low in organic matter, and soils with high lime content. High calcium content and high pH values as well as variable ground water levels and drought periods are also correlated with infection occurrence (Korhonen and Stenlid, 1998; Redfern et al., 2010; Gonthier and Thor, 2013).

1.3. Area of study

1.3.1. Localization and geomorphology of the Basque Country

The Basque Country is located in the central section of the northern region of the Iberian Peninsula, bordered by the Cantabrian Sea on the north, France and Autonomous Community of Navarre in the east, La Rioja in the south, with Castile and León in the southwest, and Cantabria in the west. It covers an area of 7234 km^2 divided in three regions: Alava (3037 km^2), Biscay (2217 km^2) and Guipuzcoa (1980 km^2), and is situated between the latitude 43^o 27′ 50′′ and 42^o 28′ 45′′ north and longitude 3^o 23′ and 1^o 43′ west (Loidi et al., 2009).

It is composed of three major mountain ranges. The northern one is a limestone mountain range which forms the Cantabrian-Mediterranean watershed in a NW-SE orientation, characterized by high karstified massifs, some with altitudes about 1500 m. Between this and an Eocene marlaceous lime mountain range situated in the Mediterranean watershed in a W-E orientation, there is a central plateau. In this second formation the maximum altitudes are lower than in the others, close to 1200 m. The third and southern range is a Cretaceous limestone mountain range, parallel to the second one, where can be found altitudes around 1400 m (IKT, 1999) (Figure 1.7).

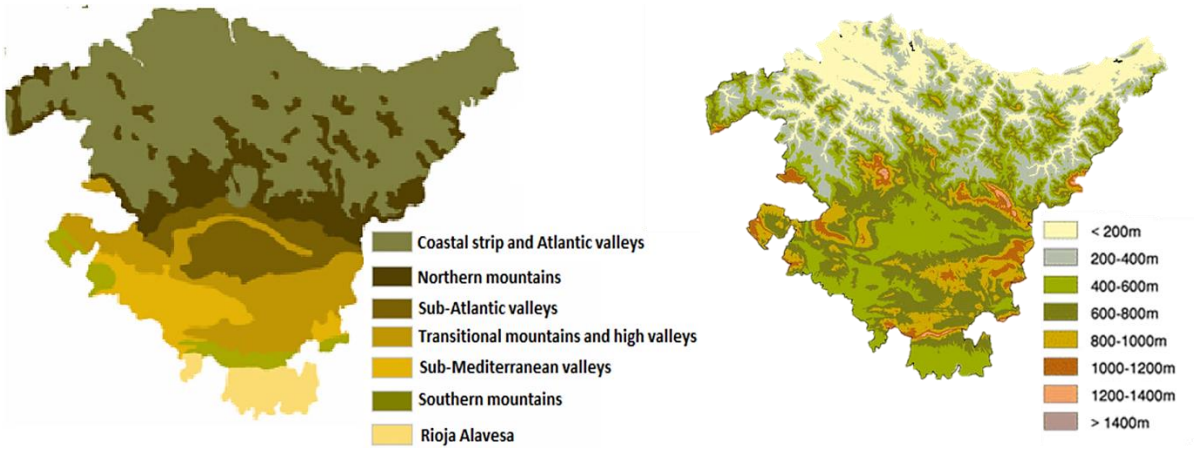


Figure 1.7. Map of the natural sectorization of the Basque Country (left) (Aseguinolaza et al., 1988). Map of the Basque Country elevation (right) (IKT, 1999).

The Cantabrian watershed is distinguished by siliceous substrata with a high percent of iron and organic matter and steep slopes from the Mediterranean watershed which is mainly formed by limestone soils and has a more gentle topography. The most usual lithology is that constituted by carbonates (limestone, loams, etc.) but, in general, soils suffer from an intense acidification due to the high precipitation level (IKT, 1999; Loidi, 1987) (Figure 1.8).

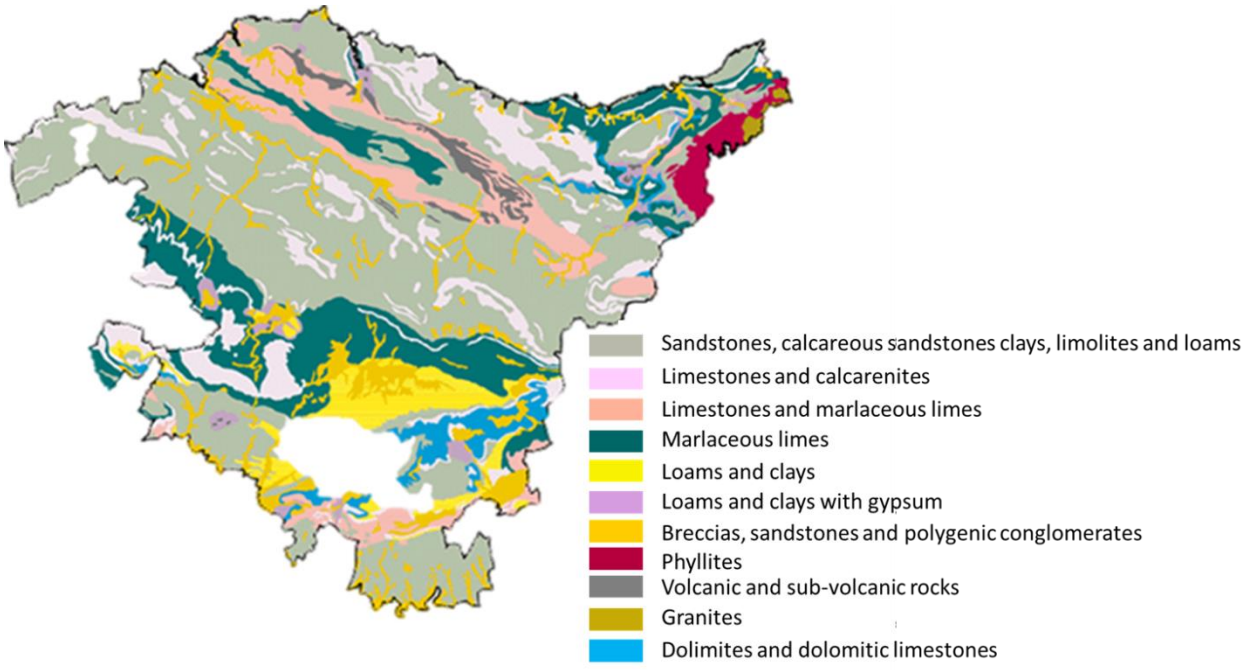


Figure 1.8. Map of the Basque Country lithology (IKT, 1999).

1.3.2. General climate

The Basque Country consists of a heterogeneous climate due to geographic and topographic characteristics. The latitude is probably the most important climatic factor affecting this region, followed by ocean proximity and surface features. The fact of being located near the 43ºN parallel, and in the west European coastal temperate zone means that, especially in spring and autumn, Atlantic NW humid winds cause a succession of squalls resulting in copious rainfalls. In summer, the Azores anticyclone acts on the region but mainly in the southern part. It should be noted also that the climate is influenced by the Gulf Stream, which heats the coast resulting in higher temperatures than expected for this latitude. This, in addition to the W-E orientation of the northern mountain ranges, results in asymmetry in the Basque Country between central and northern strips, with temperate macrobioclimate, and the southern strip, with Mediterranean macrobioclimate (Loidi, 1987; Euskalmet, 2009; Aseginolaza et al., 1989).

The temperate macrobioclimate is characterized by precipitation all year round and moderate temperatures. Within this macrobioclimate and covering most of the territory is the maritime temperate bioclimate which maintains the characteristics of the temperate macrobioclimate at the Atlantic valleys. The decrease in precipitation southward determines the occurrence of the maritime temperate sub-Mediterranean variant which can result in one month of summer aridity. At some points near the coast, the bioclimate changes to hyper-maritime temperate which can be found also in the sub-Mediterranean variant. Within the Mediterranean macrobioclimate is the maritime bioclimate with seasonal precipitations and at least two months of summer drought (Campos, 2010) (Figure 1.9).

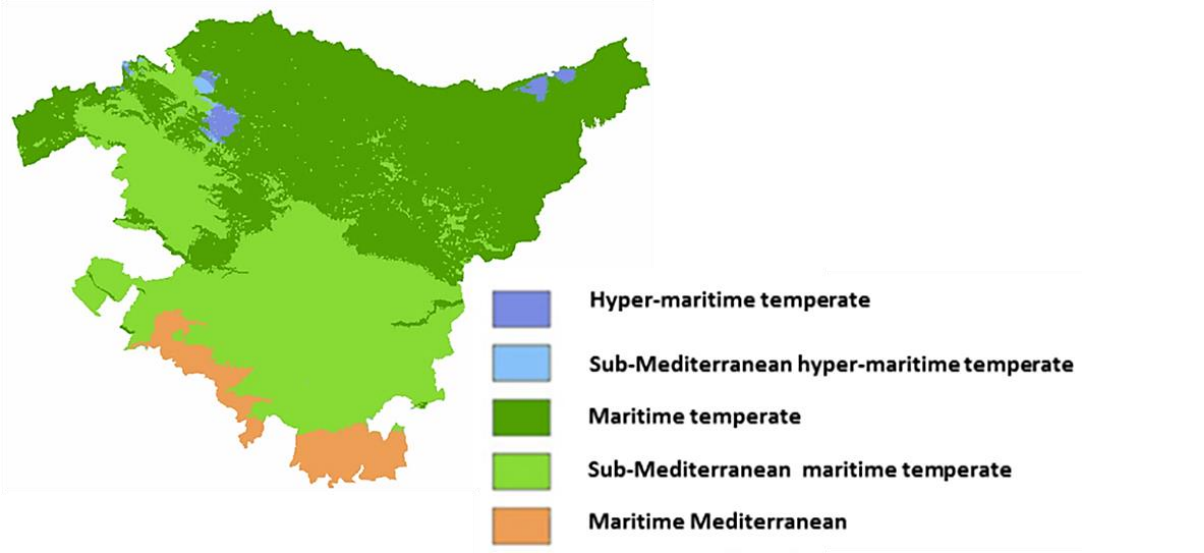


Figure 1.9. The Basque Country bioclimates (Campos, 2010).

1.3.3. Native forest and tree plantations

During the last 250 years, forests have been in a state of uninterrupted decline and degradation in the Basque Country. Before the beginning of the 20th century the native forest covering this region, which primarily comprised *F. sylvatica* L., *Quercus robur* L., *Q. pyrenaica* Willd., *F. excelsior* L. and *Alnus glutinosa* (L.) Gaertn., had already been dramatically reduced. The increase in charcoal and wood demand for the production of iron in foundries and use in farming and shipbuilding sectors were the main causes of this deforestation (Martín de Agar et al., 1995; Martín- Martín, 2001). The 1940s saw a gradual abandonment of agricultural land because agriculture was no longer economically profitable, and younger generations were moving to cities that offered more opportunities (Basque government, 2002). This, added to the increasing demands from construction, industry (eg. paper mills, mining, railway), foreign dependence, and the low price of planting and the rapid growth of some coniferous species, resulted in a change of agricultural land use and a forest reforestation based on the introduction of exotic, fast growing species, in particular conifers (Uriarte, 2008; Michel-Rodriguez, 2004). The oceanic region was the most affected by this while Alava-Navarre region was less affected due to the human settlement type, high proportion of public land and lower industry and comercial development (Loidi, 2001).

Currently, that difference is still maintained. Thus, comparing the plantation areas against total forest surface, the value for Alava is 21.6%, whereas for Biscay and Guipuzcoa are 77.4% and 61.5% respectively. The proportion of the forest area against the total surface of the Basque country is 54.9% (HAZI, 2010), but, even if tree plantations have morphological and structrural similarities to native forest, they are not comparable ecologically nor biogeographically (Loidi, 2001). Characteristics of potential native forests of the Basque Country are resumed in Table 1.3 (Loidi et al., 2009).

Table 1.3. Potential forest of the Basque Country and their characteristics (Loidi et al., 2009).

NATURAL FOREST	VEGETATION SERIES	*SIGMETUM*	SERIES CHARACTERISTICS	DOMINANT TREE SPECIE	FOREST STRUCTURE	SOIL STRUCTURE	MAIN SPECIES IN TREE PLANTATIONS
BEECH FORESTS	CALCICOLOUS BEECH FOREST	*Carici sylvaticae-Fago sylvaticae*	Climatophilous, meso-supratemperate, humid-hyperhumid, neutrophilous, mesophytic.	*Fagus sylvatica* L.	High density canopy. Almost pure beech forest, undergrowth poor in vascular plants, rich in bryophytes.	Base-rich substrates (limestones,dolomites or marls).	*Quercus rubra* L., *Larix kaempferi* (Lamb.) Carr., *Pseudotsuga menziesii* (Mirb.) Franco , *Chamaecyparis lawsoniana* (A. Murray) Parl.
	XERIC BEECH FOREST	*Epipactido helleborines-Fago sylvaticae*	Climatophilous, meso-supratemperate, humid, neutrophilous, submesophytic.	*F. sylvatica*	High density canopy. Sometimes presence of other deciduous species, richer shrub stratum with submediterranean influence, rich herbaceous stratum.	Strong slopes, shallow, composed of limestones and dolomites.	————————
	ACIDOPHILIC BEECH FOREST	*Saxifrago hirsutae-Fago sylvaticae*	Climatophilous, meso-supratemperate, humid-hyperhumid, acidophilous and neutro-acidophilous.	*F. sylvatica*	High density canopy. Almost pure beech forest. Undergrowth poor in vascular plants, rich in bryophytes.	Acid substrates or with an easy acidification (sandstone, lutite, ophite, slate, greywacke, granite).	*L. kaempferi, C.lawsoniana, Q. rubra, P. menziesii*
OAK FORESTS	OAK WOODS OF ALAVA-NAVARRE	*Crataego laevigatae-Querco roboris*	Temporihygrophilous and climatophilous, mesotemperate,subhumid-humid, euoceanic, neutro-basophilous and neutrophilous, mesophytic.	*Quercus robur* L.	Shrub and herbaceous strata rich in species and dense.	Base-rich substrates.	————————
	CANTABRIAN OAK WOODS	*Hyperico pulchri-Querco roboris*	Climatophilous, meso-supratemperate, humid-hyperhumid, acidophilous.	*Q. robur*	Medium density canopy. Pluri-stratified undergrowth with high biomass but poor in shrub stratum.	Non-carbonated acidic rocks (sandstone, granite, quartzite, flysch rich in sandstone and argillite).	*Pinus radiata* D. Don., *Eucalyptus globulus* Labill.
	OTHER OAK WOODS	*Polysticho setiferi-Fraxino excelsioris*	Temporihygrophilous and climatophilous, termo-mesotemperate, humid-hyperhumid,hyperoceanic, neutro-basophilous and neutro-acidophilous, mesophytic.	*Q. robur*	Complex structure, different tree species mixture. Abundant in shrub, herbaceous, lianoid strata and in epiphytes.	Deep, nutrient-rich soils with high capacity to store water and appropriate texture.	*P. radiata*
		Roso arvensis-Querco pubescentis	Climatophilous, meso-supratemperate, subhumid-humid, submediterranean, neutrophilous, calcicolous and siliceous.	*Q. humilis* Mill.	Complex structure with high subarboreal estratum development.	Base-rich substrates (limestones or marls).	————————
		Pulmonario longifoliae-Querco Petraeae	Climatophilous, meso-supratemperate, humid-hyperhumid, acidophilous.	*Q. petraea* (Matt.) Liebl.	Dense, poor shrub stratum, high diversity of the herbaceous stratum.	Substrates poor in bases, usually on sandstones but also on flysch poor in carbonated rocks.	*C. lawsoniana, Picea abies* (L.) Karst. , *Q. rubra, L. kaempferi*

Table 1.3 (continuation). Potencial forests of the Basque Country and their characteristics (Loidi et al., 2009).

NATURAL FOREST	VEGETATION SERIES	*SIGMETUM*	SERIES CHARACTERISTICS	DOMINANT TREE SPECIE	FOREST STRUCTURE	SOIL STRUCTURE	MAIN SPECIES IN TREE PLANTATIONS
PYRENEAN OAK FORESTS	EUROSIBERIAN PYRENEAN OAK WOODS	*Melampyro pratensis-Querco pyrenaicae*	Climatophilous, meso-supratemperate, humid, submediterranean,euoceanic, acidophilous.	*Q. pyrenaica*	Dense, medium height trees with thin multi-stem trunks, herbaceous stratum quiet rich, acidophilous vegetation.	Sand-rich substrates, very permeables and more or less base poor.	————————
GALL OAK FORESTS	EUROSIBERIAN GALL OAK WOODS	*Pulmonario longifoliae-Querco fagineae*	Climatophilous, mesotemperate, subhumid-humid, submediterranean, neutro-basophilous.	*Q. faginea*	Not very high canopy density, high vegetal diversity and multiple levels at subarboreal stratum.	Base-rich substrates (limestones or marls). In slopes, well structured and aerated.	————————
	MEDITERRANEAN GALL OAK WOODS	*Spiraeo obovatae-Querco fagineae*	Climatophilous, meso-supramediterranean, subhumid-humid, submediterranean,neutro-basophilous.	*Q. faginea*	Irregular and medium size tree stratum, low canopy density, high undergowth development.	Soft, marly, base-rich soils.	————————
HOLM OAK FORESTS	CANTABRIAN HOLM OAK WOODS	*Lauro nobilis-Querco ilicis*	Edafoxerophilous, termo-mesotemperate, humid, submediterranean, hyperoceanic and oceanic, relict, calcicolous and siliceous.	*Q. ilex*	Not very high, dense and tangled, high density of trees, shrubs and lianes,poor herbaceous stratum. Mediterranean sclerophyllous vegetation.	Substrates with low capacity to retain and store water (lithosols on compact limestones usually in steep slopes or karstic).	————————
	CASTILIAN-CANTABRIAN HOLM OAK WOODS	*Spiraeo obovatae-Querco rotundifoliae*	Climatophilous and edafoxerophilous, supramediterranean, subhumid-humid, calcicolous.	*Q. ilex* subsp. *rotundifolia*	Short, dense and tangled. Poor in lianes and herbaceous stratum. Mediterranean vegetation.	Karstified or marly limestone.	————————
	RIOJAN HOLM OAK WOODS	*Querco rotundifoliae*	Climatophilous, mesomediterranean, dry-subhumid, calcicolous and siliceous.	*Q. ilex* subsp. *rotundifolia*	Medium-short. Poor in plants and species.	From loamy to sandy soils and from deep soils to rocky soils.	————————

1.4. Objectives

Butt and root rot caused by *Armillaria* spp. and *H. annosum* complex cause large economic losses in the north hemisphere. Both fungi are known to be present in the Basque Country; however, to develop more effective management strategies a more intensive study is needed to:

i. Determine *Armillaria* and *Heterobasidion* distribution in the Basque Country;
ii. Determine *Armillaria* and *Heterobasidion* species and population diversity in native forests and plantations of the Basque Country;
iii. Establish the host range and host susceptibility for these pathogens;
iv. Describe the ecosystems where *Armillaria* and *Heterobasidion* are present;
v. Provide information about the patterns of dispersal and mechanisms in specific areas.

The application of existing management strategies against *Armillaria* and *Heterobasidion* species is limited and often ineffective, due to factors such as level of infection, environmental conditions, and risks, cost, and legislation, among others. Thus, in order to complement the current integrated management strategies, and considering the importance of *P. radiata* in the lumber industry of the Basque Country, it is proposed to:

vi. Isolate and characterize the ability of some bacteria native to the *P. radiata* rhizosphere to reduce *A. mellea* and *H. annosum* pathogenic effects as a prophylactic nursery treatment.

CHAPTER 2

Distribution and characterization of *Heterobasidion* and *Armillaria* complexes in the Basque Country

2.1. Introduction

Studies about the distribution and abundance of fungal pathogens, fungal species involved, and environment in which they are present are important to identify affected and susceptible areas. Models of potential distribution of pathogens and their habitats can be developed with fungal occurrence data which may be used to improve ecological understanding of actual and potential distributions, to establish management strategies, and to investigate the potential role of climate change in the behavior of pathogens (Franklin and Miller, 2009; Gherbawy and Voigt, 2010; Guisan and Zimmermann, 2000; Narayanasamy, 2011).

Successful establishment of a pathogen in an environment is determined by the presence of hosts and host susceptibility, and environmental conditions. Environmental factors influence pathogen survival, formation of dispersion structures, germination of spores, and establishment of new mycelia. They can also alter canopy structure and density, host development and host defense mechanisms, thus, changing the susceptibility of the host to the pathogen (Eastburn et al., 2011; Elad and Pertot, 2014; Ghini et al., 2008).

Identification of different species within *Armillaria* and *Heterobasidion* genera in a region is important because they interact differently with their host and have different habitat preferences. *A. mellea* and *A. ostoyae* usually behave as primary pathogens of a broad range of deciduous and coniferous tree species, however, *A. mellea* is considered less pathogenic for conifers than *A. ostoyae*. Both of them can also act as saprophytes (Guillaumin et al., 1993; Coetzee, 2003). *A. gallica* host range is also wide and it can behave as an opportunistic and/or primary pathogen, especially when the host is stressed (Skovsgaard et al., 2010; Kim et al., 2017). *A. tabescens* has mainly a saprophytic role, but can act as a primary parasite in eucalyptus or as opportunistic in oak. The pathogenicity of *A. cepistipes* and *A. borealis* is usually low (Guillaumin et al., 1993; Coetzee, 2003), and the role of *A. ectypa* is uncertain, although it has been considered a saprotroph of plant debris (Ohenoja, 2006; Ainsworth, 2003).

The *H. annosum s.l.* species present in Europe behave as necrotrophs and show host specialization which defines their distribution. *H. annosum s.s.* shows a preference for *Pinus* spp. although it can be found in other conifers or some broadleaved trees. The host preference of *H. parviporum* is *P. abies*, and *H. abietinum* is usually found in *Abies* spp. (Garbelotto and Gonthier, 2013).

The main tree species in Basque Country plantations is *P. radiata* (covering an area of 132084 ha), followed by *Eucalyptus* L'Hér. (15197 ha), *P. nigra* (13701 ha), *Larix* spp. (8011 ha), *P. pinaster* (7238 ha), *P. menziesii* (6537 ha), *Chamaecyparis lawsoniana* (A. Murray) Parl. (3414 ha), *Q. rubra* L. (3328 ha) and *P. abies* (525 ha). The main native forest species are *F. sylvatica* (53835 ha), *Q. faginea* Lam. (26652 ha), *Q. ilex* L. (26152 ha), *Q. robur* and *Q. petraea* (Matt.) Liebl. association (16469 ha), and *Q. pyrenaica* (13039 ha) (HAZI, 2010) (Figure 2.1). All of these tree species have been reported to be susceptible to different *Armillaria* spp. in the literature; *Heterobasidion* spp. have been also reported in many of the host species mentioned above, including some *Quercus* spp. (Korhonen et al., 1998; Asiegbu et al., 2005; Doğmuş-Lehtijärvi et al., 2015). However, *Armillaria* spp. and *Heterobasidion* spp. distribution and species diversity are not known in the Basque Country, where the wood industry is valued at 1 billion euros per year (Basque Government, 2017).

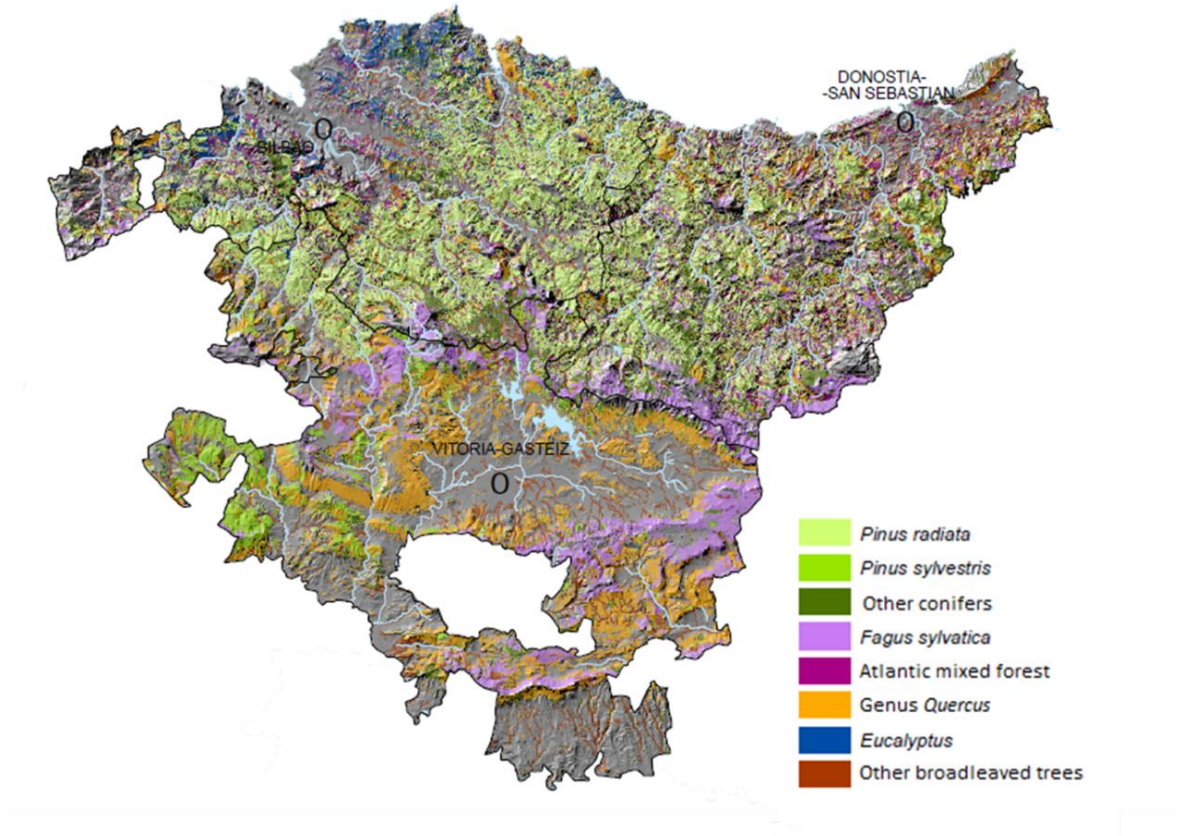


Figure 2.1. Main tree species distribution in the Basque Country (IKT, 2005).

2.1.1. Objectives

The objectives of this study were:

i. To determine *Armillaria* and *Heterobasidion* distribution in the Basque Country.
ii. To generate a dataset of location of both fungal genera.
iii. To define the species of *Heterobasidion* and *Armillaria* present in native forests and plantations of the Basque country.
iv. To determine environmental factors of *Armillaria* spp. and *Heterobasidion* spp. habitats.

2.2. Material and methods

2.2.1. Collection of fungal material

Two sample sets, one corresponding to *H. annosum* isolates and the other one to *Armillaria* spp. isolates, were collected from native forests and plantations of the Basque country, focusing on trees in pockets of mortality and decayed trees displaying symptoms resembling those of root rot diseases. The stands were surveyed by systematic random sampling, which consisted of randomly choosing a starting point and sampling systemically from that focal point (Mueller-Dombois and Ellenberg, 1974). A total of 709 foci of tree mortality were examined this way in order to determine *Armillaria* spp. and *H. annosum* presence, and fungal samples and ecosystem characteristics related to infection were collected. *Heterobasidion* basidiocarps and *Armillaria* basidiocarps, rhizomorphs and mycelium fans were sampled.

All the surveyed points were georeferenced using an Oregon 300 Garmin GPS and samples were labelled and placed in separate polyethene bags, transported to the laboratory, and stored at 4ºC. Fungi were cultured on benomyl-dichloran-streptomycin agar (BDS) (Worrall, 1991) and growth at 20ºC in the dark. Pure cultures were obtained and routinely grown on malt extract agar (MEA) (Panreac). For preservation of the pure cultures, mycelial fragments were placed in 50% glycerol and, after incubating at 4ºC for 24h, maintained at -20ºC (Pitt and Hocking, 2009).

2.2.2. Identification of spatial distribution patterns

Nearest neighbour index (NNI) (Hertz, 1909; Clark and Evans, 1954) was calculated to analyse the distribution patterns (regularly dispersed, randomly dispersed or clustered) of *Heterobasidion* and *Armillaria* populations. NNI is a measure of clustering that is expressed as the ratio of the observed mean distance between data points divided by the expected mean distance for a random distribution. The expected mean distance is based on a hypothetical random distribution with the same number of features covering the same total area. If the means are the same, the ratio is equal to 1 and the observed distribution can be considered random. If the expected mean is greater than the observed mean, the ratio is less than 1 and the observed distribution is clustered. When the expected mean is less than the observed mean, the difference is greater than 1 and the observed distribution is dispersed. The nearest neighbour index considers the relationship between features, unlike quadrat analysis, which simply considers whether or not a feature falls within a particular area. Thus, the NNI was used to better capture any pattern where interacting features are analysed, such as cases of diseases (Mitchell, 2009).

To represent *Heterobasidion* spp. and *Armillaria* spp. distribution based on their abundance in the study area, Kernel density estimation was used. By means of this estimation a density representation is shaped using a group of localized data, thus obtaining a global pattern of the distribution, i.e. it provides information about the concentration of the points in an area and the gradual variation of infection levels (Moreno, 1991; Fortin and Dale, 2005). Kernel density estimation is a non-parametric density estimation, and is calculated as follows:

$$\hat{f}(x) = \frac{1}{nh} \sum_{i=1}^{n} K\left[\frac{x - xi}{h}\right]$$

where,

x: point where the density is estimated.

xi: value of the variable for each case, i = 1, 2, 3, 4, …

K: Kernel function, it controls the weight given to the observations {xi} at each point x based on their proximity.

h: bandwidth, which controls the size of the neighborhood around x, is a smoothing parameter; it was stablished at 4000 m.

NNI and point density maps were conducted using ArcGis 9.2 Software (ESRI Inc., California, USA).

2.2.3. Description of fungal ecosystems

To determine environmental factors of *Armillaria* spp. and *Heterobasidion* spp. habitats, a dataset of the environmental variables of the studied ecosystems was constructed based on information supplied by the Environment Department of the Basque Government (http://www.ingurumena.ejgv.euskadi.eus/r49-579/es//publicaciones_c.htm). The variables compiled were stand slope, stand orientation, soil acidity, soil permeability, average rainfall, average temperature, tree types, and host optimal conditions. Variables were categorized as shown in Table 2.1. *Armillaria* spp. presence was coded as a binomial variable being 0 for absence and 1 for presence; the same was applied to *Heterobasidion* spp.

Table 2.1. Categorical variables, category code and description of each of each category compiled in this study. *Information Source: http://www.ingurumena.ejgv.euskadi.eus/r49-579/es//publicaciones_c.htm

Characteristics	Category code	*Description	Characteristics	Category code	*Description
Slope (%)	<10	<10	Permeability	Imper	Impermeable
	10-20	10 - <20		low	Low
	20-30	20- <30		medium	Medium
	30-40	20- < 40		high	High
	40-50	40 - < 50	Rain (average, mm)	1	<1000
	50-60	50 - <60		2	1000-1400
Orientation	N	North		3	1400-1800
	NE	Northeast		4	>1800
	E	East	Temperature (average, ºC)	1	<10.5
	SE	Southeast		2	10.5-11.5
	S	South		3	11.5-12.5
	SW	Southwest		4	>12.5
	W	West	HOC (Host Optimal Conditions)	+Op	Favorable
	NW	Northwest		Op	Acceptable
Soil (Acidity)	acid	acid		-Op	Unfavorable
	-acid	-acid		- - Op	Very Unfavorable
	-basic	-basic	Tree Type	Deciduous	Deciduous
	basic	basic		Conifers	Conifers

As a preliminary exploratory analysis, multiple correspondence analysis (MCA) was applied to represent the relationships among the categorical variables. MCA projects the variables in a reduced space, facilitating visual interpretation for large datasets. This analysis converts a matrix of data into a graphical display known as factor planes. The rows and columns of the matrix are plotted as points in the factor planes and allow a geometrical representation of the information (Greenacre and Hastie, 1987).

This procedure was complemented with contingency tables testing separately each categorical variable including its categories against *Armillaria* or *Heterobasidion* presence or absence. Pearson´s chi square test was used to determine the independence between row and column variables, i.e. to determine if *Armillaria* or *Heterobasidion* were more frequently detected than expected by chance in certain categories. Pearson´s chi square test is calculated as follows:

$$\chi^2 = \sum \frac{(\text{observed}_{ij} - \text{model}_{ij})^2}{\text{model}_{ij}}$$

where, i represents the rows (categories of each environmental variable) in the contingency table and j represents the columns (*Armillaria* or *Heterobasidion* presence or absence). The observed data are the observed frequencies or number of events per category, and the model is defined as follows:

$$\text{model}_{ij} = E_{ij} = \frac{\text{row total}_i \times \text{column total}_j}{n}$$

where n is the total number of observations.

For calculating the strength of association between categorical variables Cramer´s V was used, where Cramer´s V ranges between 0 (no relationship) and 1 (perfect relationship). Adjusted standardized residuals were checked in order to determine the significant differences between categories; adjusted residuals are standardized values allowing comparisons among different cells, and follow a standard normal frequency (with mean zero and standard deviation one) so we can assume that if their value lies outside of ±1.96 then it is significant at p <0.05, if it lies outside ±2.58 then it is significant at p <0.01 and if it lies outside ±3.29 then it is significant at p <0.001 (Field, 2009).

2.2.4. Fungal species identification

2.2.4.1. *Armillaria* spp.

Restriction Fragment Length Polymorphism-PCR (RFLP-PCR) was used to confirm the identity of the *Armillaria* isolates at the species level. This technique is based on the variations among homologous DNA sequences which will vary binding sites for restriction enzymes resulting in differences in DNA fragment sizes among species.

DNA from two week old pure cultures was extracted with DNeasy Plant Mini Kit (Qiagen) in accordance to the manufacturer's instructions and a section of the intergenic spacer region (IGS) of the rDNA was amplified using the primer pair LR12R (5'-CTGAACGCCTCTAAGTCAGAA-3') and O-1 (5'-AGTCCTATGGCCGTGGAT-3') (Harrington and Wingfield, 1995) (Figure 2.2). The PCR mixture contained 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM each primer, 2 U Taq polymerase (Netzyme, Molecular Netline Bioproducts, Valencia) and 1 µl template DNA in a final volume of 50 µl. The cycling conditions consisted of 90 sec at 95 ºC, 35 cycles of 30 sec at 95 ºC, 40 sec of annealing at 60 ºC, and 2 min at 72 ºC, and a final 10 min at 72 ºC. The obtained DNA fragment was directly digested with the restriction enzyme Alu I (Invitrogen), Nde I (Takara) or Bsm I (Roche) (Harrington and Wingfield, 1995). The restriction fragments were separated in 3% agarose gels (agarose D-1 Low EEO, Conda). Species were identified based on the restriction patterns determined by Harrington and Wingfield (1995) and Pérez-Sierra et al. (1999) (Table 2.2).

Table 2.2. Restriction fragment sizes of the amplified DNA region of different *Armillaria* sp. (Harrington and Wingfield, 1995; *Pérez-Sierra et al., 1999).

***Armillaria* spp.**	**Pattern**	**AluI**	**NdeI**	**BsmI**
A. borealis	1	310,200,135 305,200,135*	550, 370 550, 370*	
	2	310,200,104 400, 200, 190*		
A. cepistipes	1	399, 200, 183 400, 200, 190*		
	2	310, 200, 135 305, 200, 135*		
A. ostoyae	1	310, 200, 135 310, 200, 135*	550, 370 565, 380*	620, 300 600, 300
A. mellea	1	490, 180 320, 280, 155*		
	2	350, 155 320, 155*		
A. gallica	1	582, 240 400, 240, 190*		
	2	399, 240, 183 390, 230, 190*		
	3	400, 250, 240, 190*		
A. tabescens	1	430, 240 430, 240*		
	2	320, 240, 100		

2.2.4.2. *H. annosum s.l.*

DNA from the pure cultures was extracted with the Qiagen DNA extraction Kit (Qiagen Inc.), in accordance with the manufacturer's instructions. The internal transcribed spacer region (ITS) of the rDNA was amplified using primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990) (Figure 2.2). The PCR reaction contained 2.5 µl of 10X Buffer, 0.5 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer, 2 U Taq polymerase (Netzyme) and 2 µl template DNA in a final volume of 25 µl. The cycling conditions consisted of 95 seconds at 95ºC, 35 cycles of 35 sec at 95ºC, 55 sec of annealing at 55ºC, and 1 min at 72 ºC, and a final 10 min at 72 ºC. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced (BASECLEAR, Netherlands).

Sequences were aligned with those of other *Heterobasidion* spp. using the ClustalW algorithm and then manually aligned with Mega software version 4.0 (Tamura et al., 2007). Related European and American *Heterobasidion* spp. sequences were obtained from the NCBI database by performing a nucleotide BLAST search (Altschul et al., 1997).

H. insulare and *H. araucariae* ITS sequences from GenBank were used as an outgroup (Asiegbu et al., 2004).

The phylogenetic tree was built using the Neighbor-Joining method (Saitou and Nei, 1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). The branch was assessed by the probabilities obtained from the 50% majority-rule-consensus tree calculated by bootstrapping (2000 replicates) with Mega4 (Felsenstein, 1985; Tamura et al., 2007).

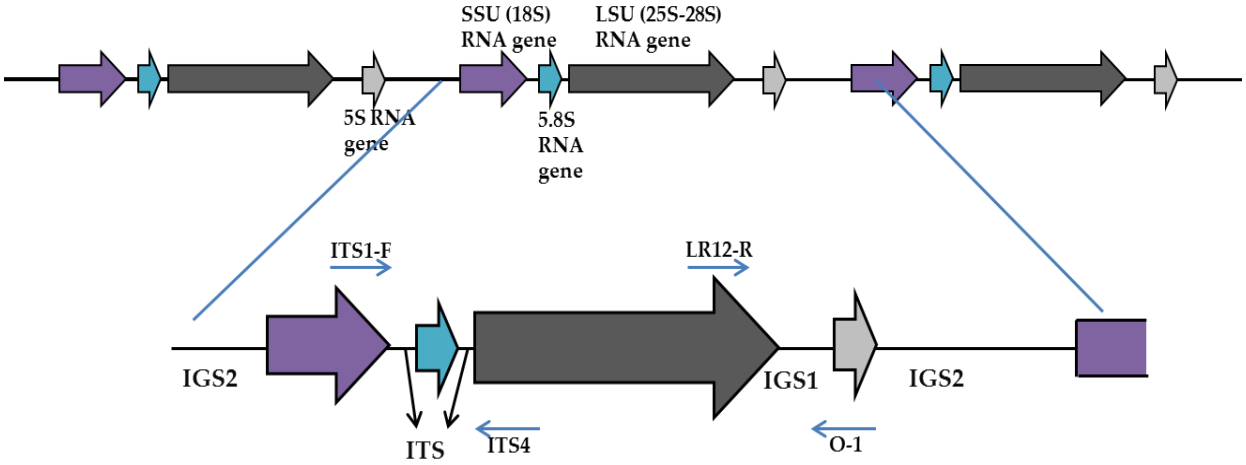


Figure 2.2. Annealing sites on the rDNA of the primers (thin blue arrows) used in this study (adapted from Vilgalys, 2017).

2.3. Results

2.3.1. Distribution of fungal species

In general, *Armillaria* spp. were broadly distributed in the Basque Country, and their host range included coniferous and deciduous trees. *Heterobasidion* spp. presence was less abundant, and it was only found in coniferous trees.

The presence of *Armillaria* spp. was detected in 248 (34.97%) of the 709 foci surveyed (Figure 2.3). Basidiocarps were mainly found in the root collars of dead and living trees, and stumps; some were found growing from superficial roots. Rhizomorphs were found in the aforementioned tree structures and also in the soil. The tree species present in the foci containing *Armillaria* spp. were *P. radiata*, *P. nigra*, *P. pinaster*, *Q. robur*, *Q. pyrenaica*, *F. excelsior*, *A. glutinosa*, *F. sylvatica*, *E. globulus* Labill., *P. abies*, *Larix kaempferi* (Lamb.) Carr., *P. sylvestris*, *Populus alba* L., *P. menziesii*, *Q. faginea*, *Q. ilex*, *Robinia pseudoacacia* L., and *C. lawsoniana*.

H. annosum was detected in 16.5% (117) of the sampled foci, all of them with disease symptoms (Figure 2.4). Basidiocarps were occasionally found on wind-thrown trees and in the internal and external part of decayed trees, broken roots, root collars and stumps. No evidence of the disease was found in the rest of the surveyed stands. The disease was not found in *Sequoia sempervirens* (D.Don) Endl., *Sequoiadendron giganteum* (Lindl.) J. Buchholz, *L. kaempferi*, and *L. decidua* Mill. stands that were sampled in the study although these species are considered potential hosts (Chase, 1985; Korhonen et al., 1998; Otrosina and Garbelotto, 2010). Damage caused by *H. annosum* was found on hosts within forest plantations with the following distribution: *C. lawsoniana* (11.1% of *Heterobasidion* infected stands), *P. nigra* (2.3%), *P. pinaster* (2.2%), *P. radiata* (42.2%), *P. menziesii* (17.7%) and *P. abies* (6.7%). In addition, *H. annosum* was evident in native forests of *P. sylvestris* (17.8%).

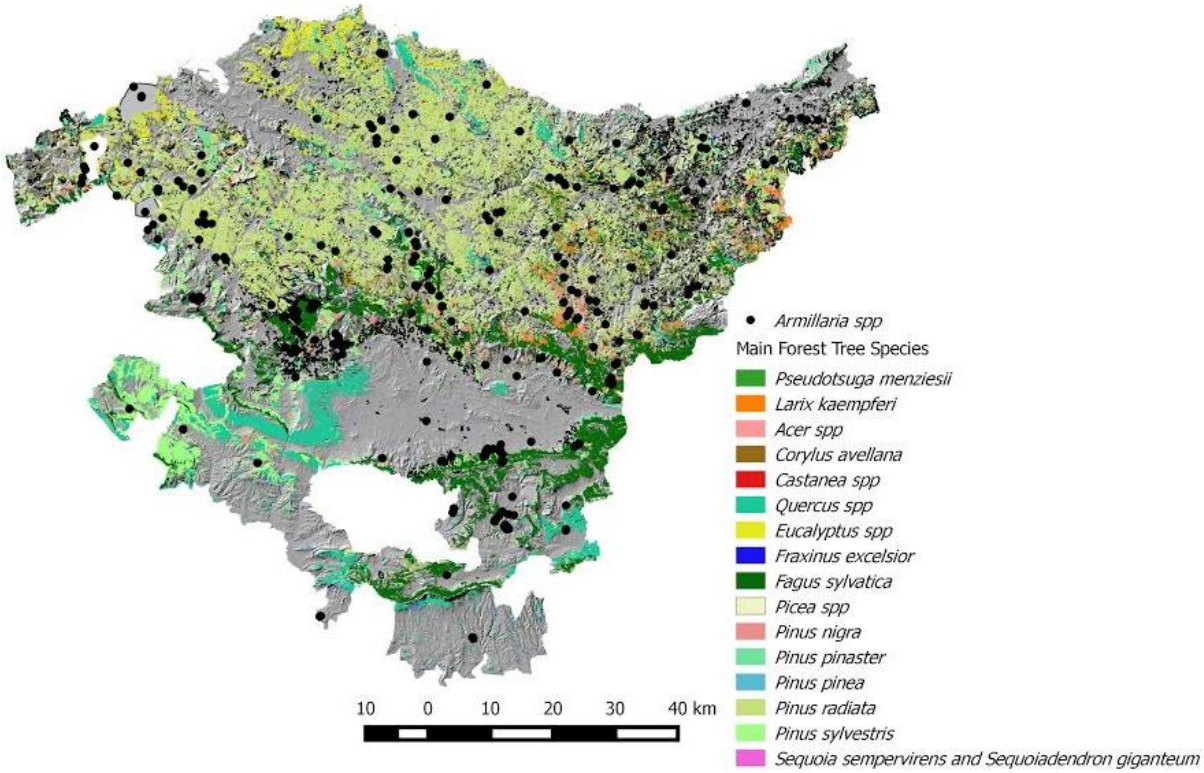


Figure 2.3. Distribution of *Armillaria* spp. in the Basque Country.

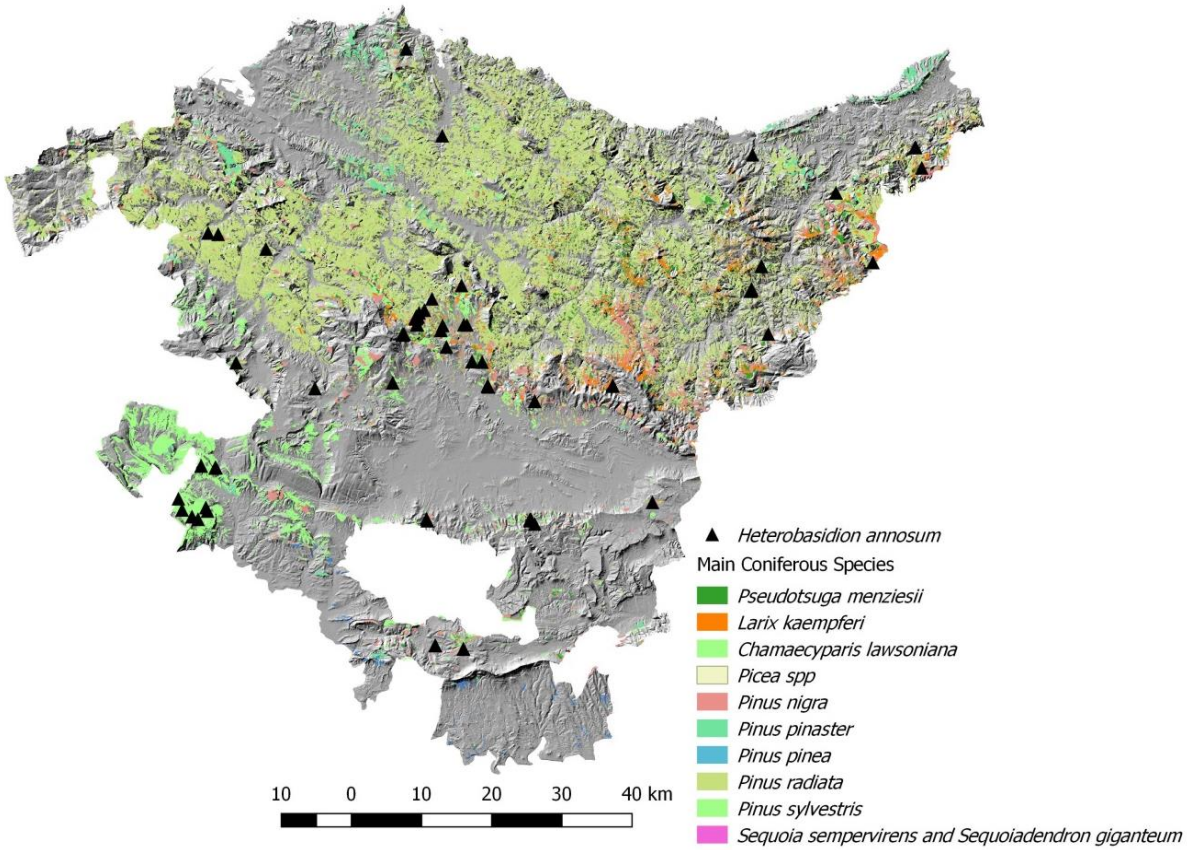


Figure 2.4. Distribution of *H. annosum s.l.* in the Basque Country.

2.3.2. Spatial distribution patterns

The spatial distribution of *H. annosum s.l.* and *Armillaria* spp. in the Basque Country survey was not random. NNI for *H. annosum s.l.* was 0.17 with a Z score of -23.96 ($p < 0.01$), suggesting a high degree of clustering in the populations (Figure 2.5). NNI for *Armillaria* spp. was 0.23 with a Z score of -35.14 ($p < 0.01$), suggesting also a high degree of clustering in the populations. Point density maps for both genera are shown in Figure 2.5.

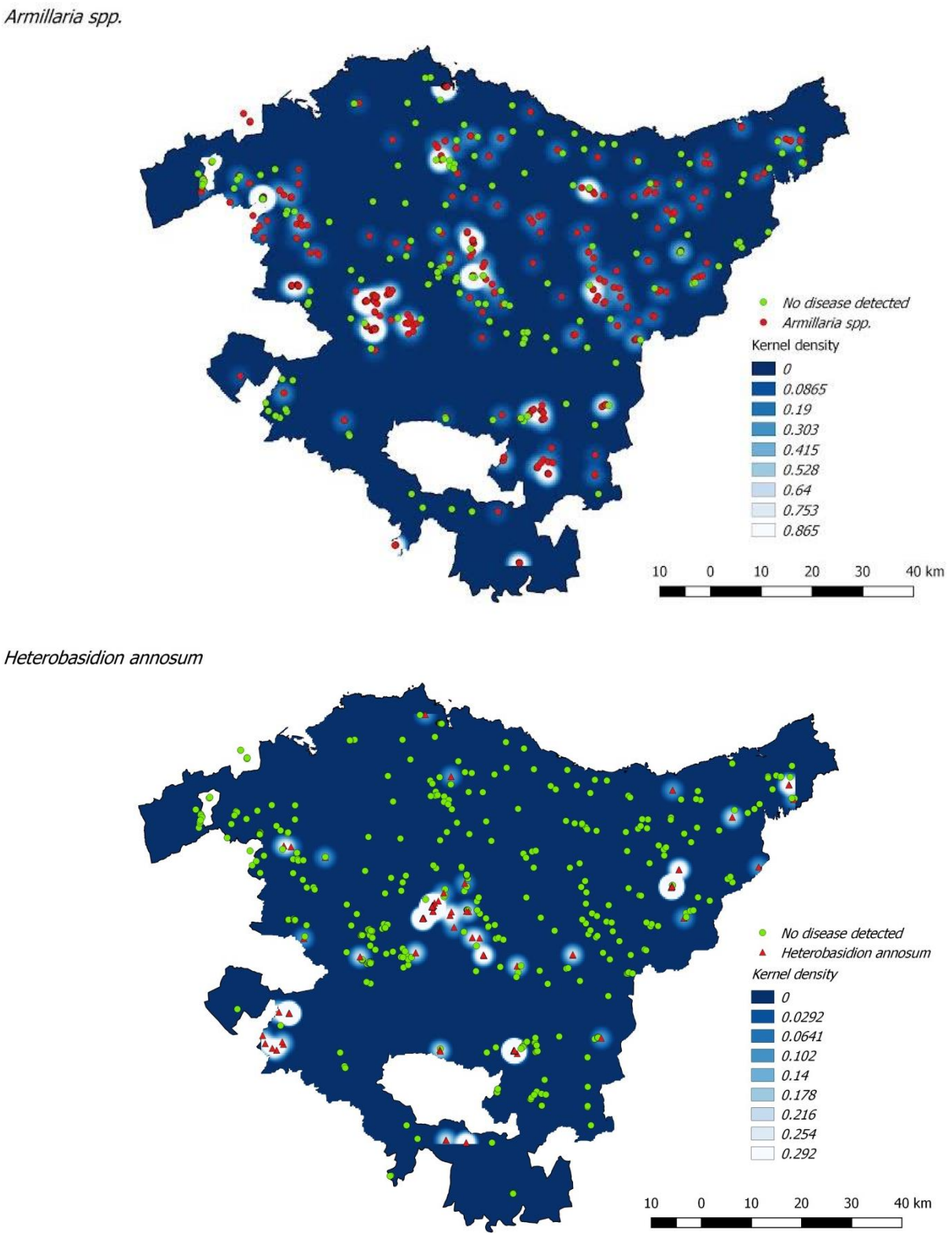


Figure 2.5. Point density maps of *Armillaria* spp. and *H. annosum* showing the clustered distribution of the populations in the Basque Country. Green points represent the absence of each genus in the sampled area. Red triangles represent the presence of each genus in the sampled area.

2.3.3. Ecosystem characteristics in which fungal species were detected

Environmental characteristics associated with each of the surveyed points and their relationships with the presence of *Armillaria* spp. and *Heterobasion* spp. was assessed (Table 2.3).

Table 2.3. Summary of the dataset obtained showing the number of foci per subcategory where *Armillaria* spp. were present or absent.

Characteristics	Category code	*Description	Detection of Armillaria disease		Number of foci
			Negative (Arm-)	Positive (Arm+)	
Slope (%)	<10	<10	170	29	199
	10-20	10 - <20	133	57	190
	20-30	20- <30	89	111	200
	30-40	20- < 40	48	34	82
	40-50	40 - < 50	18	13	31
	50-60	50 - <60	3	4	7
Tree Type	Deciduous	Deciduous	28	36	64
	Conifers	Conifers	433	212	645
Orientation	N	North	45	24	69
	NE	Northeast	46	26	72
	E	East	53	32	85
	SE	Southeast	39	26	65
	S	South	75	28	103
	SW	Southwest	133	26	159
	W	West	45	73	118
	NW	Northwest	25	13	38
Soil (Acidity)	acid	acid	260	184	444
	-acid	-acid	118	31	149
	-basic	-basic	46	17	63
	basic	basic	37	16	53
Permeability	Imper	Impermeable	31	2	33
	low	Low	4	5	9
	medium	Medium	364	175	539
	high	High	62	66	128
Rain (average, mm)	1	<1000	34	7	41
	2	1000-1400	86	44	130
	3	1400-1800	295	141	436
	4	>1800	46	56	102
Temperature (average, ºC)	1	<10.5	20	5	25
	2	10,5-11.5	130	74	204
	3	11.5-12.5	162	94	256
	4	>12.5	149	75	224
HOC (Host Optimal Conditions)	+Op	Favorable	241	125	366
	Op	Acceptable	171	103	274
	-Op	Unfavorable	23	13	36
	- - Op	Vey Unfavorable	26	7	33

Table 2.4. Summary of the dataset obtained showing the number of foci per subcategory where *Heterobasidion* spp. were present or absent.

Characteristics	Category code	*Description	Detection of Heterobasidion disease		Number of foci
			Negative (Het-)	Positive (Het+)	
Slope (%)	<10	<10	162	37	199
	10-20	10 - <20	168	22	190
	20-30	20- <30	169	31	200
	30-40	20- < 40	57	25	82
	40-50	40 - < 50	30	1	31
	50-60	50 - <60	6	1	7
Orientation	N	North	51	18	69
	NE	Northeast	55	17	72
	E	East	71	14	85
	SE	Southeast	40	25	65
	S	South	85	18	103
	SW	Southwest	149	10	159
	W	West	110	8	118
	NW	Northwest	31	7	38
Soil (Acidity)	acid	acid	378	66	444
	-acid	-acid	141	8	149
	-basic	-basic	36	27	63
	basic	basic	37	16	53
Permeability	Imper	Impermeable	19	14	33
	low	Low	9	0	9
	medium	Medium	452	87	539
	high	High	112	16	128
Rain (average, mm)	1	<1000	26	15	41
	2	1000-1400	105	25	130
	3	1400-1800	369	67	436
	4	>1800	92	10	102
Temperature (average, ºC)	1	<10.5	9	16	25
	2	10,5-11.5	141	63	204
	3	11.5-12.5	221	35	256
	4	>12.5	221	3	224
HOC (Host Optimal Conditions)	+Op	Favorable	286	80	366
	Op	Acceptable	242	32	274
	-Op	Unfavorable	34	2	36
	- - Op	Very Unfavorable	30	3	33
Tree Type	Deciduous	Deciduous	64	0	64
	Conifers	Conifers	528	117	645

The spread of the category variables for all characteristics was represented in a MCA that reflected the relationships among the variables in each dimension. MCA revealed that the first horizontal dimension explained 23.7% of the total inertia (variance), as the first factor plane represents the largest inertia, while the second vertical dimension explained 22.7%.

A measure of the importance of each variable (squared component loading) was computed for each dimension. This measure is also the variance of the quantified variable in that dimension. Variables, such tree type, that are located very close to the origin do not highlight correspondence in any dimension. The variables with higher variance in the first dimension were average temperature (Temperature), *Heterobasidion*

spp., average rainfall (Rain), soil permeability, and optimal conditions for host growth (Hoc). The variables with higher variance in the second dimension were stand orientation, average temperature (Temperature), slope, average rainfall (Rain), *Armillaria* spp.,and soil acidity (Soil) (Figure 2.6).

Armillaria spp. detection was mainly related to categories such as west and northwest orientation, slopes between 20% and 50%, basic soils, high average rainfalls (>1800 mm), and soils with high permeability. *Armillaria* spp. absence was mainly related to categories such as slopes less than 20%, south or southeast stand orientation, favorable conditions for host growth, and medium soil permeability (Figure 2.7). *H. annosum* presence was related to categories such as moderately basic pH values of the soil, north stand orientation, low average temperature (< 11.5 ºC), low average rain (< 1000 mm), and impermeable soils. *H. annosum* absence was related to categories such as temperature averages above 11.5 ºC and rain average of 1400- 1800 mm (Figure 2.7).

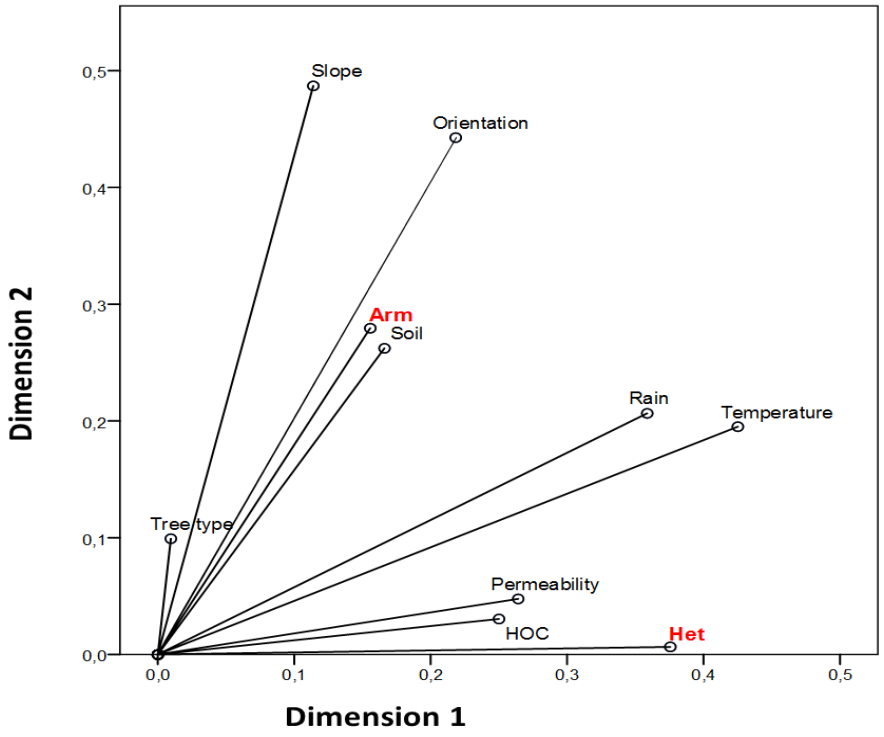


Figure 2.6. Measure of the variance of each variable for each dimension. The variables with higher variance in the first dimension were mainly average rainfall (Rain), optimal conditions for host growth (Hoc), slope, *Armillaria* spp., soil acidity (Soil), and stand orientation (Figure 3). The variables with higher variance in the second dimension were orientation, average temperature (Temperature), average rainfall (Rain), slope, and soil acidity (Soil).

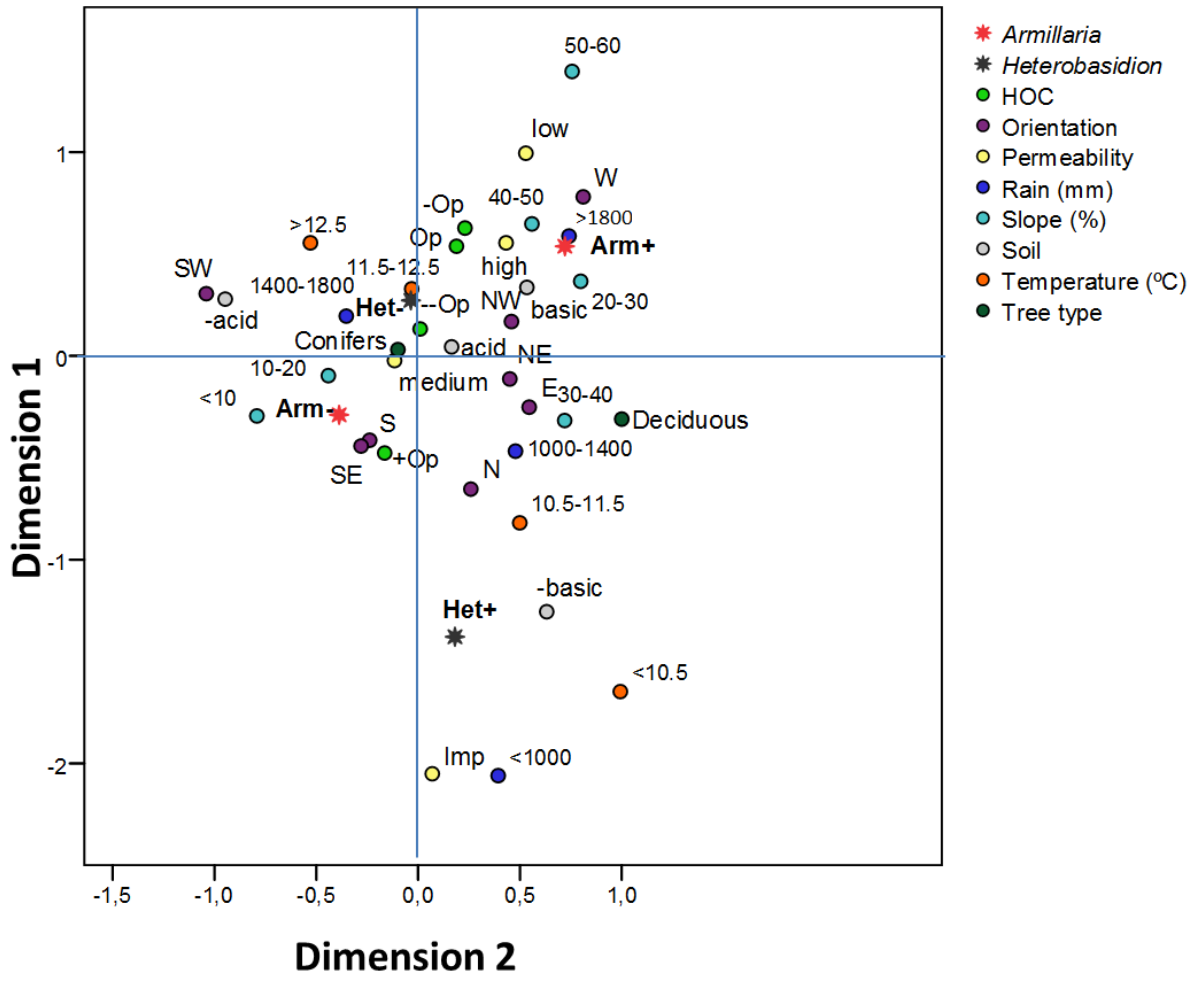


Figure 2.7. Location in a Euclidean space of the presence or absence of *Armillaria* spp. and *Heterobasidion* spp., and environmental categories. The first two dimensions of the Euclidean space of the MCA are plotted to examine the associations among categories. The values on the axes indicate the coordinates within the Euclidean space in which categories are located. Variable description can be found in Table 2.1.

The significance of the associations among fungal presence and the environmental variables was determined using Pearson´s chi square test. A significant association was observed between *Armillaria* spp. and slope (χ^2 (5) = 79.2, p < 0.001; Cramer´s V of 0.334 indicated a medium-large effect size), orientation (χ^2 (7) = 65.5, p < 0.001; Cramer´s V of 0.304 indicated a medium-large effect size), tree type (χ^2 (1) = 14.0, p < 0.001; Cramer´s V of 0.140 indicated a small-medium effect size), soil acidity (χ^2 (3) = 23.6, p < 0.001; Cramer´s V of 0.183 indicated a small-medium effect size), soil permeability (χ^2 (3) = 30.8, p < 0.001; Cramer´s V of 0.208 indicated a small-medium effect size), and rainfall average (χ^2 (3) = 25.0, p < 0.001; Cramer´s V of 0.188 indicated a small-medium effect size).

In the case of *Heterobasidion*, a significant association was observed among *Heterobasidion* spp. and slope (χ^2 (5) = 19.7, $p < 0.01$; Cramer´s V of 0.167 indicated a small- medium effect size), orientation (χ^2 (7) = 50.3, $p < 0.001$; Cramer´s V of 0.266 indicated a medium effect size), tree type (χ^2 (1) = 13.9, $p < 0.001$; Cramer´s V of 0.140 indicated a small effect size), soil acidity (χ^2 (3) = 53.2, $p < 0.001$;Cramer´s V of 0.274 indicated a medium- large effect size), soil permeability (χ^2 (3) = 19.4, $p < 0.01$; Cramer´s V of 0.165 indicated a small-medium effect size), HOC (χ^2 (3) = 16.7, $p < 0.05$; Cramer´s V of 0.153 indicated a small-medium effect size), rainfall average (χ^2 (3) = 16.4, $p < 0.01$; Cramer´s V of 0.152 indicated a small-medium effect size), and temperature average (χ^2 (3) = 110.4, $p < 0.001$; Cramer´s V of 0.395 indicated a medium- large effect size).

In order to determine the significant differences between categories the adjusted standardized residuals were examined, negative *z* score values indicated that *Armillaria* spp. or *Heterobasidion* spp. were observed less frequently than expected in plots with those categories and positive *z* score values indicate that *Armillaria* spp. or *Heterobasidion* spp. were observed more frequently than expected in plots with those categories. Values of ±1.96 were considered significant at p <0.05, values of ±2.58 were considered significant at p <0.01, and values of ±3.29 were considered significant at p <0.001 (Field, 2009).

When the adjusted standardized residuals were examined, *Armillaria* spp. were significantly present in stands with slopes of 20-30% ($z = 7.2$; $p < 0.001$); stands with western orientation ($z = 6.7$; $p < 0.001$); deciduous hosts ($z = 3.7$; $p < 0.001$); acid soils ($z = 4.7$; $p < 0.001$); high permeability soils ($z = 4.3$; $p < 0.001$), and rainfall average (mm) >1800 ($z = 4.6$; $p < 0.001$) (Figure 5). *Armillaria* spp. were significantly absent in stands with slopes <10% ($z = -7.1$; $p < 0.001$); stands with southwestern orientation ($z = -5.6$; $p < 0.001$); coniferous hosts ($z = -3.7$; $p < 0.001$); moderately acid soils ($z = -4.1$; $p < 0.001$); medium permeability soils ($z = -2.5$; $p < 0.01$), impermeable soils ($z = -3.6$; $p < 0.01$), and rainfall average (mm) <1000 ($z = -2.5$; $p < 0.05$) (Figure 2.8).

Heterobasidion spp. were significantly present in stands with slopes of 30- 40% ($z = 3.6$; $p < 0.001$); stands with southeast orientation ($z = 5.0$; $p < 0.001$) and north orientation ($z = 2.3$; $p < 0.001$); coniferous hosts ($z = 3.7$; $p < 0.001$); basic soils ($z = 2.8$; $p < 0.01$) and moderately basic soils ($z = 5.9$; $p < 0.001$); impermeable soils ($z = 4.1$; $p < 0.001$); favorable conditions for host growth ($z = 4.0$; $p < 0.001$); rainfall average (mm) <1000 ($z = 3.6$; $p < 0.001$); and temperature average (ºC) <10.5 ($z = 6.5$; $p < 0.001$) and 10.5-11.5 ($z = 6.6$; $p < 0.001$) (Figure 2.8). *Heterobasidion* spp. were significantly absent in stands with slopes of 10- 20% ($z = -2.1$; $p < 0.05$) and 40- 50% ($z = -2.0$; $p < 0.05$); stands with southwest orientation ($z = -3.9$; $p < 0.001$) and west orientation ($z = -3.1$; $p < 0.01$); deciduous hosts ($z = -3.7$; $p < 0.001$); moderately acid soils ($z = -4.1$; $p < 0.001$); acceptable conditions for host growth ($z = -2.7$; $p < 0.01$); rainfall average (mm) of >1800 ($z = -2$; $p < 0.05$); and temperature average (ºC) >12.5 ($z = -7.4$; $p < 0.001$) (Figure 2.8).

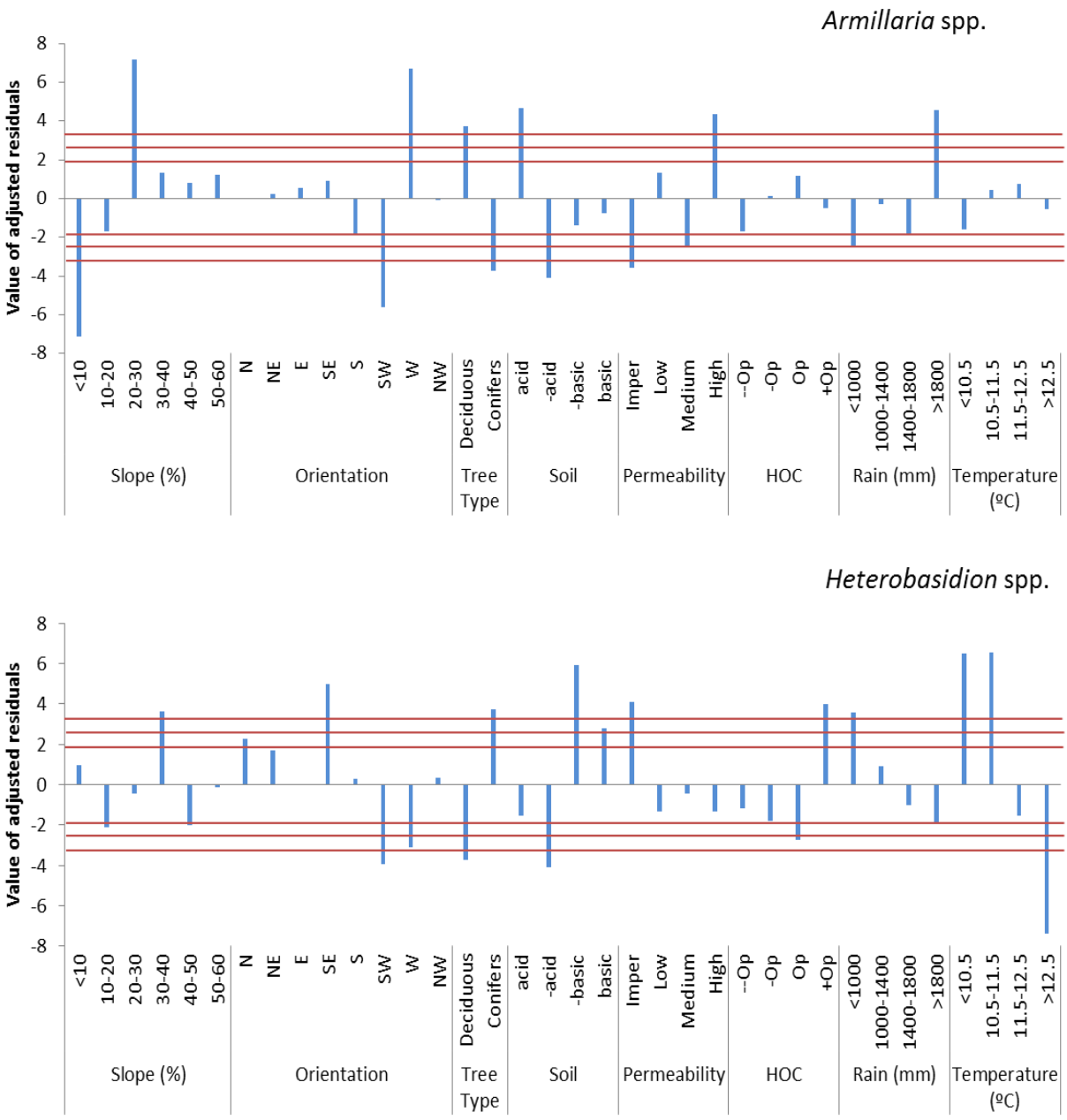


Figure 2.8. Values of the adjusted residuals (blue bars) for each category within a categorical variable for the presence of *Armillaria* spp and *Heterobasidion* spp. Red lines represent the value the adjust residuals must have to consider a category significant; if y = ±1.96 then it is significant at p <0.05, if y = ±2.58 then it is significant at p <0 .01, and if y = ±3.29 then it is significant at p <0 .001 (Field, 2009).

2.3.4. Fungal species identification

Of the total of isolates obtained from the surveyed plots (Set 1), 60% were identified by RFLP- PCR patterns as *A. ostoyae*, 24% as *A. mellea*, 14% as *A. gallica*, 1% as *A. tabescens* and 1% as *A. cepistipes*. *A. ostoyae* was detected mainly in *Pinus* spp. (*P. radiata*, *P. nigra* and *P. pinaster*). The host range for *A. mellea* was more varied. *A. mellea* pattern 1 (PCR fragment sizes: 320 and 155 bp) was found on *P. radiata*, *Quercus* spp., *F. excelsior*,

and *C. lawsoniana*, and corresponded to 53% of the *A. mellea* isolates, while the remaining 47% were identified as pattern 2 (fragment sizes: 320, 180, and 155 bp) and appeared on *Q. pyrenaica* and *P. radiata*. *A. gallica* was found on *A. glutinosa*, *P. radiata* and *Q. robur*, *A. cepistipes* was detected on *P. radiata*, and *A. tabescens* on *Q. robur*.

All *H. annosum* isolates were identified as European *H. annosum s.s.* (European P-type) based on ITS sequencing, and the ITS sequences of all isolates were identical to *H. annosum* B298 (*Picea*, Finland), GU296436.1 (*P. sylvestris*, Latvia) and FJ872064.1 (*P. mugo* Turra, Lithuania) except isolates H63 (A → G transition at nucleotide 412), H67 (A → T transversion at nucleotide 188), H94 (G insertion at nucleotide 102), and H77, H50, H90, H97 (C → T transition at nucleotide 9). The bootstrap value for the branch connecting *H. annosum* isolates was 92%. Representative ITS sequences of this species for each host were deposited in GenBank (accession numbers JN408462 to JN408468) (Figure 2.9).

Figure 2.9. The bootstrap consensus tree inferred from 2,000 replicates of internal transcribed spacer ribosomal DNA sequences of *Heterobasidion* spp. Branches corresponding to partitions reproduced in more than 50% bootstrap replicates are shown. There were a total of 570 positions in the final dataset. The sequences obtained from the NCBI database are followed by an asterisk. *H. insulare* and *H. araucariae* ITS sequences from GenBank were used as outgroups (Asiegbu et al., 2004). (a) The sequence corresponding to the H25 isolate was used as representative out of the 38 isolates, that were identical to the *H. annosum* B298 strain (H64, H33, H34, H89, H103, H56, H47, H82, H87, H80, H85, H26, H70, H65, H86, H60, H59, H61, H99, H31, H30, H75, H84, H104, H68, H100, H57, H54, H88, H95, H49, H81, H32, H101, H91, H55). (b) The sequence corresponding to the H77 isolate was used as representative out of four isolates (H50, H77, H90, and H97).

2.4. Discussion

In the present study the distribution of *Armillaria* and *Heterobasidion* in native forests and plantations of the Basque Country is reported, a dataset to generate mathematical models to estimate the predisposition factors affecting the presence of *Armillaria* and *Heterobasidion* was also generated, and environmental factors of *Armillaria* spp. and *Heterobasidion* spp. habitats are described. Different species within *Armillaria* and *Heterobasidion* genera were determined to obtain a better understanding of the pathogenicity of the fungal populations.

In general, *Armillaria* spp. were broadly distributed in the Basque Country and their host range included both coniferous and deciduous trees. In contrast, *H. annosum s.s* was less abundant, and it was only found in conifers. Both genera were found in plantations and native forests. *H annosum s.s* can infect a broad range of host species, including *F. sylvatica* and *Quercus* spp., but it shows a preference for *Pinus* spp. (Korhonen et al., 1998; Asiegbu et al., 2005; Doğmuş-Lehtijärvi et al., 2015). In this study, *H. annosum s.s* was found in a broad range of coniferous hosts, but almost half of the disease foci caused by *H. annosum s.s.* were found in *P. radiata* plantations, which due to reforestation programs established in the 1940s in the Basque Country (Basque Government, 2002), have become the most abundant tree plantations. In this process, many of the native deciduous species, such as *F. sylvatica*, *Q. robur*, *Q. pyrenaica*, *F. excelsior*, *A. glutinosa*, as well as some native *P. sylvestris* forests and former pastureland were replaced with coniferous plantations (Ruiz-Urrestarazu, 1992). This could have changed the behavior and colonization frequency of *H annosum s.s.* No evidence of disease caused by *H. annosum* was found in *S. sempervirens*, *S. giganteum*, *L. kaempferi,* and *L. decidua*, which are considered to be hosts. The lack of disease in these coniferous species could be related to the fact that they only represent the 5% of coniferous surface in the area (IKT, 2005).

The distribution patterns obtained by NNI show a high degree of clustering of the fungal populations, which might be a result of fungal colonization mechanisms and environmental factors. García-Serna (2011) explained the random distribution of *Diplodia pinea* (Desm.) Kickx in the Basque Country as a result of a generalized distribution and shown of seeds contaminated with this fungus. In the case of *Armillaria* spp. and *H. annosum s.s.* colonization is based on the spread of vegetative mycelia and basidiospores. Although basidiospores can cover long distances usually they settle down close to the original basidiocarp, this might explain the clustered distribution of both pathogens. Their clustering could also be shaped by climatic factors, host presence, soil and other stand characteristics.

Although *Armillaria* spp. were present in forests in the study area with a wide range of environmental conditions, they were more frequently detected in stands with 20-30% slopes, with a westerly orientation, deciduous forests, acid soils with high permeability, and rainfall average values above 1800 mm. *Armillaria* spp. were less abundant in stands with slopes less than 10%, southwest orientation, rainfall average values below 1000 mm, and coniferous forest with moderately acid soils and medium permeability or impermeable. In general, species of the *Armillaria* complex have been isolated from a wide range of soil and environmental conditions but disease foci are also influenced by host adaptability and stress (Wargo and Harrington, 1991). *Heterobasidion* spp. were more frequently detected in stands with temperature averages below 11.5 ºC, rainfall average values below1000 mm, 30-40% slopes, north or southeast orientations, coniferous forest with moderately basic or basic and impermeable soils, and where trees had optimum growth conditions. *Heterobasidion* spp. were less abundant in stands with temperatures higher than 12.5 ºC and rainfall average values above 1800 mm, southwest or westerly orientation,10-20% and 40-50% slopes, and moderately acid soils. *Heterobasidion* spp. damage depends largely on site factors and has been reported at a wide range of altitudes and types of soil with several orientations and slopes (Korhonen and Stenlid, 1998).

All isolates corresponding to *Heterobasidion* genus were identified as European *H. annosum s.s.* (European P-type) based on ITS sequencing, and the majority of the ITS sequences of the isolates (84.4%) were identical to *H. annosum* B298 (*Picea*, Finland), GU296436.1 (*P. sylvestris*, Latvia) and FJ872064.1 (*P. mugo*, Lithuania). Despite forest reproduction material was imported from different European countries, the isolates collected in the Basque Country were obtained from native forests as well as plantations, and the information obtained from ITS sequencing only was not enough to stablish a connexion among isolates from this study and the foreign strains.

In the case of *Armillaria* genus, *A. ostoyae* was the predominant species in the studied area, Although *A. ostoyae* has been reported to prefer coniferous hosts (Williams et al., 1986), differentiate between host specialization and forest history of the area is difficult (Gregory et al., 1991). In this study *A. ostoyae* was mainly detected in conifers, but also in native forests of *F. sylvatica* and *Q. robur.* This fungal specie could have been stablished in the native deciduous forest before its replacement with coniferous tree species. *A. gallica* was found in both conifers and deciduous trees. *A. mellea* had the greatest host diversity, which was also observed by Pintos et al. (2014) in different soils from Pontevedra, Galicia, Spain and in general for European populations (Guillaumin et al., 1993).

2.4.1. Conclusions

Armillaria spp. were broadly distributed in the Basque Country and their host range comprehend coniferous and deciduous trees. All the main species present in the Basque Country within *Armillaria* genus can behave as primary pathogens which added to their broad range of distribution and host, result on a high risk of damage for tree plantations and native forests. *H. annosum s.s* was the only species present within *Heterobasidion* genus. Its presence was limited to conifers and was less abundant than *Armillaria* spp.; however, *H. annosum s.s* could suppose a high risk for tree plantations due to the broad range of coniferous species in which was present. In this study, a dataset of *Armillaria* spp. and *H. annosum s.s* was created and using the dataset of environmental variables that was available descriptive environmental factors were associated to both genera.

CHAPTER 3

Population diversity of *Heterobasidion annosum* s.s. and *Armillaria* complex in selected stands of the Basque Country

3.1. Introduction

The genetic structure of populations of plant pathogens is shaped by mutation, gene flow, recombination, random genetic drift and natural selection (Zhan and McDonald, 2004). High genetic variation of a population increases its ability to adapt to changes in the environment, for example, through acquisition of virulence and/or resistance genes.

In general, the *Armillaria* spp. mating system is heterothallic bifactorial (tetrapolar), meaning that the genes that define mating-type are located in two different unlinked sites in the genome and both loci are multiallelic. For a compatible mating, both of these regions must be different between the haploid colonies (Ullrich and Anderson, 1978; Fraser and Heitman, 2003). Once the haploid hyphal cells fuse, after a short dicariotic stage, they will become diploid, with one diploid nucleus and mitochondria with the genome of one of the haploid strains (Anderson and Ullrich, 1982). The diploid mycelium is fertile and can form basidia containing four haploid uninucleate basidiospores formed by meiosis (Hintikka, 1973). Anastomosis (fusion of hyphae) can occur between two diploid isolates when the same alleles within the somatic incompatibility (SI) system are shared (Baumgartner et al., 2011).

The *H. annosun* mating system is a unifactorial, multiallelic mating system. In this case, there is only a single mating type or *MAT* locus and for mating between haploid isolates cells must have different *MAT* alleles (Hansen et al., 1993; Fraser and Heitman, 2003). The resultant secondary mycelia will be a mosaic of heterokaryotic and homokaryotic mycelia (Garbelotto and Gonthier, 2013). The SI system in *H. annosum* is formed by a series of 3–4 multiallelic and discrete loci (Hansen et al., 1993), and it has

been reported that even between incompatible isolates an exchange of nuclei or cytoplasmic elements can occur (Johannesson and Stenlid, 2004).

By means of SI tests, population structures can be determined at intraspecies level; the presence of many different small SI groups in a stand implies that dispersion is predominantly by basidiospores, and a single extensive SI group (SIG) implies dispersion by vegetative mycelium. Even though SI tests are usually reliable, sometimes they do not differentiate among closely related individuals (Kile, 1983). The molecular techniques used in Chapter 2, ITS sequencing for *H. annosum s.s.* and restriction of the IGS1 region of the rDNA for *Armillaria* spp., are useful for interspecies differentiation within these genera, but they do not differentiate isolates at intraspecies level. Universally Primed-PCR (UP-PCR) (Bulat and Mironenko, 1990) could provide more information in these cases. UP-PCR is related to random amplified polymorphic DNA (RAPD) method, and has been used for the characterization of fungal populations at interspecies and/or intraspecies level (Nielsen et al., 2001; Meyling and Elinberg, 2006; Pottinger et al., 2002). In this technique, the entire genome of an organism is targeted with a single primer or a combination of primers that will anneal to multiple regions resulting in a multiband profile which differs among different genotypes. Universal primers consist of a minisatellite-like region (5′ end, 6–10 nt) with high GC content which can be found in any genome, and a variable region (3′ end, 8–10 nt) that is generated randomly. This random region is added to avoid the amplification of phylogenetically conserved regions of the genome. In addition to the high GC content universal primers are longer (15–21 nt) than RAPD primers (typically 10 nt), so the used annealing temperatures are higher (52–60 ºC) ensuring greater reproducibility of banding profiles. Usually, highly variable intergenic regions are targeted which enables differentiation between closely related isolates (Bulat et al., 1998).

The genetic structure of *Armillaria* spp. and *H. annosum s.s.* populations in stands could provide information about dispersion mechanisms of both fungi. This may have important implications regarding the epidemiology and management of these pathogens (Zhan, 2009) in the Basque Country where they are broadly distributed and their pathogenic effects have been detected (Chapter 2).

3.1.1. Objectives

The objectives of this study were:

i. To define *Armillaria* spp. and *H. annosum s.s.* population diversity in selected stands of the Basque Country.

ii. To determine dispersion patterns of both fungal genera in these areas.

iii. To determine host susceptibility to fungal pathogens in a set of native and exotic forest species selected based on the frequency of their presence in the Atlantic area of Spain.

3.2. Materials and Methods

3.2.1. Sample collection

Samples of *Armillaria* spp. and *H. annosum* were collected to determine the genetic diversity and population structure of the fungi. Three stands located in Otxandiano (Biscay), Amunategi (Biscay), and Altube (Alava) were selected for sampling *Armillaria* spp., and four stands located in Azaceta (Alava), Legorreta (Guipuzcoa), Caranca (Alava) and Saldropo (Biscay) were selected for sampling *H. annosum* (Figure 3.1). Inside the plots, a detailed survey for fungal structures was conducted, and samples were collected when they were detected. *Heterobasidion* basidiocarps and *Armillaria* basidiocarps, rhizomorphs and/or mycelium fans were sampled from the root collars of dead and living trees, stumps, fallen trees, internal part of decayed trees and roots.

All the points in which samples were collected were georeferenced using an Oregon 300 Garmin GPS and samples were processed as described in chapter 2. They were labelled and placed in separate polyethene bags, transported to the laboratory, and stored at 4ºC. Fungi were isolated on benomyl-dichloran-streptomycin agar (BDS) (Worrall, 1991) and grown at 20ºC in the dark. Once pure cultures were obtained, they were routinely grown on malt extract agar (MEA) (Panreac). For preservation of the pure cultures, mycelial fragments were placed in 50% glycerol and, after being at 4ºC for 24h, maintained at -20ºC (Pitt and Hocking, 2009).

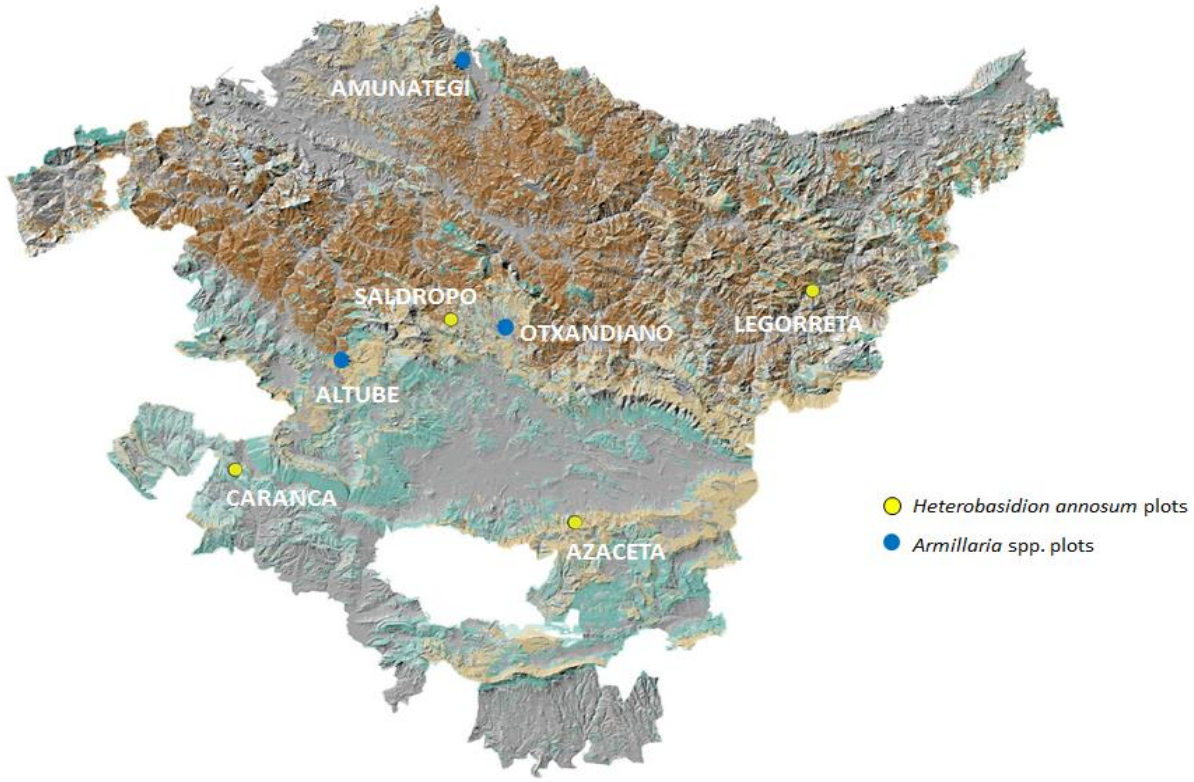


Figure 3.1. Location of the sampled stands. *Armillaria* spp. stands (Amunategi, Altube, and Otxandiano) are marked in blue. *H. annosum* stands (Azaceta, Legorreta, Caranca and Saldropo) are marked in yellow.

3.2.2. Identification and fungal diversity analysis

Genetic diversity and population structure of the fungal isolates were determined by RFLP-PCR (*Armillaria* spp.), sequencing the ITS region of the rDNA (*Heterobasion* spp.) (as described in Chapter 2.2.4), SI tests and UP-PCR. RFLP-PCR and ITS region sequencing were used to determine the species of the fungal isolates and SI tests and UP-PCR to determine the diversity of isolates within the same species and area. Fungal SIGs were determined as follows: diploid (*Armillaria* spp.) or heterokaryotic (*Heterobasion* spp.) isolates from the same sampling area were paired in all possible combinations. Approximately 4 mm^2 of mycelia were placed 0.5 cm apart on MEA plates, and incubated at 20ºC for six weeks. When mycelia of opposite isolates fused and grew with a uniform morphology, the pairings were considered somatic compatible (SC) and the isolates were considered to belong to the same species and genet, i.e. belong to the same SC group (SCG). In contrast, when a line of demarcation appeared isolates were considered somatic incompatible (Anderson et al., 1979) (Figure 3.2).

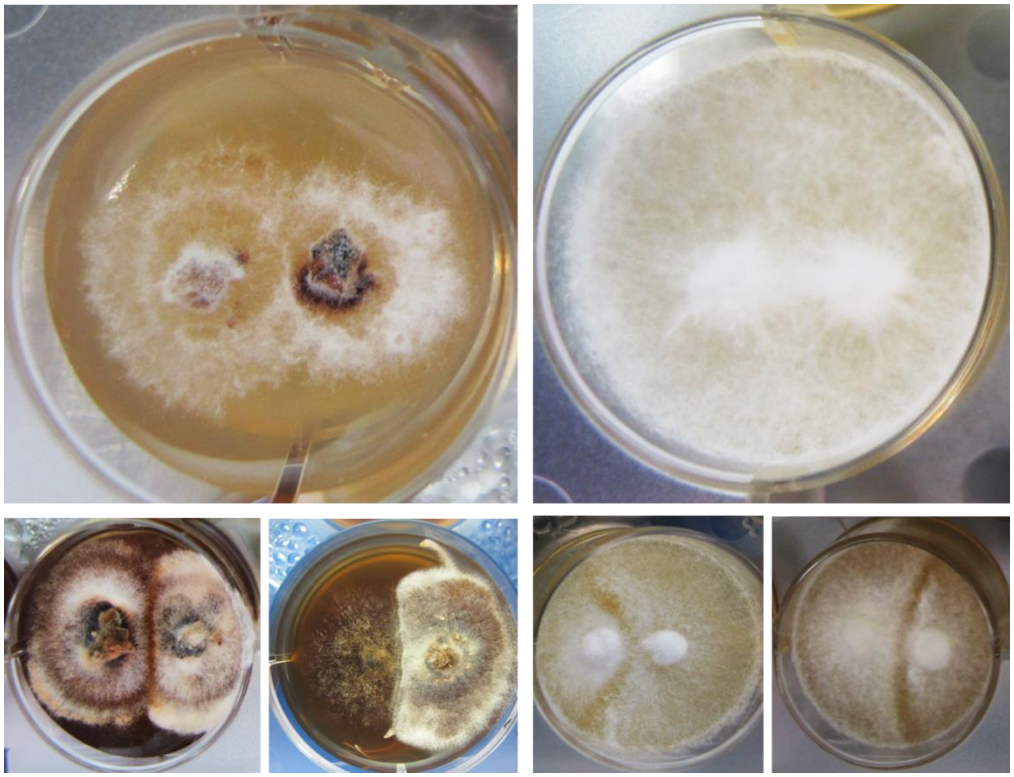


Figure 3.2. Somatic compatible pairings of *Armillaria* (top left) and *Heterobasidion* (top right). Somatic incompatible pairings of *Armillaria* (bottom left) and *Heterobasidion* (bottom right).

UP-PCR reactions were carried out in 25 µl volume containing 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.8 µM primer, 50 ng genomic DNA (extracted as for RFLP analysis), and 1.25 U Taq DNA Polymerase (Invitrogen). The cycling conditions were 5 min at 94 ºC, 5 cycles of 50 sec at 94 ºC, 2 min at primer specific annealing temperature (Table 3.1), and 1 min at 72 ºC, followed by30 cycles of 50 sec at 94 ºC, 90 seconds at primer specific annealing temperature, and 1 min at 72 ºC, and a final extension at 72 ºC for 7 min (Tyson et al., 2002). UP-PCR primers were tested on a representative group of isolates consisting of different species and genets of *Armillaria*, or on a representative group of different genets of *Heterobasidion* (as determined by RFLP-PCR and SI tests), and the primers with the best capacity to distinguish between different SCGs and species were chosen for the analysis of all the isolates. Following gel electrophoresis of the UP-PCR amplicons, the band pattern for each isolate was assessed for the presence (1) or absence (0) of each band and represented in a binomial matrix. Similarities between strains were calculated using a simple matching coefficient (Lamboy, 1994) and represented on dendrograms constructed based on average linkage between groups in SPSS version 15.0 (SPSS Inc., Chicago, USA). To determine the consistency between the similarity matrix and dendrograms the cophenetic correlation coefficient was calculated (Dunn and Everitt, 1982).

Table 3.1. Oligonucleotide sequence of the UP-PCR primers used in this study and their respective annealing temperatures (Tyson et al., 2002).

Primer	Primer sequence	Annealing temp. (°C)	References
0.3-1	5'-CGAGAACGACGGTTCT-3'	50	Bulat et al., 1994
3.2	5'-TAAGGGCGGTGCCAGT-3'	52	Bulat et al., 1994
L45	5'-GTAAAACGACGGCCAGT-3'	51	Bulat et al., 1998
AS15inv	5'-CATTGCTGGCGAATCGG-3'	52	Bulat et al., 2000
AA2M2	5'-CTGCGACCCAGAGCGG-3'	50	Lübeck et al., 1998
AS4	5'-TGTGGGCGCTCGACAC-3'	55	Lübeck et al., 1998
Fok1	5'-GGATGACCCACCTCCTAC-3'	52	Lübeck et al., 1998
L15/AS19	5'-GAGGGTGGCGGCTAG-3'	52	Lübeck et al., 1999
L21	5'-GGATCCGAGGGTGGCGGTTCT-3'	58	Bulat et al., 1995
M13	5'-GAGGGTGGCGGTTCT-3'	52	Stenlid et al., 1994
AS15	5'-GGCTAAGCGGTCGTTAC-3'	52	Bulat et al., 1994

3.2.3. Host susceptibility

Because *A. mellea* was the species with the broadest host range among the *Armillaria* spp. (Chapter 2), the susceptibility of different tree species present in the Basque Country to *A. mellea* was assessed. Two year old trees of different species, including *P. radiata*, *P. nigra* subsp. *salzmannii* var. corsicana, *P. sylvestris*, *F. sylvatica*, *Prunus avium* L., *Q. petraea*, *Q. ilex*, *C. japonica*, *Q. robur*, *S. giganteum* and *E. nitens* H.Deane & Maiden (Explotaciones Forestales Jiménez Araba s. l. Nursery, Vitoria, Spain), were infected with the fungus. For the preparation of *A. mellea* inoculum, pieces of fungal mycelia were placed on BDS agar with autoclaved *Quercus* spp. acorns and incubated for approximately one month at room temperature in the dark (Beckman and Pusey, 2001). Fifty trees of each species were grown in 53x53x180 mm pots (300 cc volume) using a mix of peat moss (2/3 peat, 1/3 perlite and fertilizer NPK; N = 200-450 mg/l, P_2O_5 = 200-500 mg/l, K_2O = 300-550 mg/l) and, after an adaptation period of two weeks, half acorns infected with *A. mellea* mycelium were placed in contact with tree roots. The trees were maintained for 4 months in a biosafety level 2 greenhouse at a mean temperature of 18 ± 5 ºC, with a relative humidity of 55-60% and without supplemental light. After this period, roots were cleaned with tap water and lengths of stems, main roots, and secondary roots were measured. *A. mellea* mycelial colonization was determined after removing the bark from the stem and roots. Plants were scored as healthy (without symptoms of infection) or with lesions (when *A. mellea* mycelium was present under the bark); the length of the lesions was determined by removing the bark of stem and roots and measuring the extent of *Armillaria* damage with an electronic caliper.

The differences in *Armillaria* disease severity among different tree species was determined by Pearson´s chi-square test. The strength of association between categorical variables (healthy or lesion containing plants and different tree species) was measured with Cramer´s V; adjusted standardized residuals were used to determine the significant differences between categories. Differences in the size of the fungal lesions among tree species was analyzed by Brown-Forsythe and Welch statistics (used when population variances are unequal), and Games-Howell test (used when population variances are unequal) (Field, 2009) was chosen for the post hoc analysis. The data was not normally distributed and therefore a base10- log transformation was applied.

3.3. Results

3.3.1. Analysis of *Armillaria* population diversity

In the stand located in Amunategi, four of the 19 isolates collected were classified as *A. mellea* RFLP pattern 2 that belonged to two SCGs, both present in *R. pseudoacacia*, and 15 isolates as *A. gallica* in a more complex population structure located in *R. pseudoacacia*, *Salix alba* L. and stumps of deciduous trees (Figure 3.2A). In the stand located in Altube, nine of the 17 samples were identified as *A. ostoyae*, separated in 3 SCGs, in *F. sylvatica* and *Q. robur*, seven as *A. mellea* pattern 2, separated in 3 SCGs, in *F. sylvatica* and *Crataegus monogyna* Jacq., and one as *A. mellea* pattern 1 in *F. sylvatica* (Figure 3.2B). The larger size of *A. ostoyae* SCGs indicates dispersal predominantly by vegetative mycelium. In contrast, the smaller SCGs obtained for *A. mellea* and *A. gallica* indicate dispersal by basidiospores and vegetative mycelium. The 21 *Armillaria* samples collected in the stand located in Otxandiano belonged to the same SCG and were identified as *A. ostoyae* (Figure 3.3). They were found in *Q. robur* stumps and trees, *C. lawsoniana*, *C. monogyna*, and grassland. The location in the stands of the collected samples, the groups obtained by SI tests and their extension are depicted in Figure 3.3.

UP-PCR primer AS4 showed good ability to distinguish the fungal strains at the interspecies and intraspecies levels. Although primer L15/AS19 showed good discrimination in an initial screen, it did not yield specific banding profiles when all the samples where tested (data not shown). The best differentiation patterns were obtained for *A. ostoyae* strains and in general the clusters were comparable to those generated from SI tests (Figure 3.4). The cophenetic correlation coefficient between the similarity matrix and the dendrogram was 0.886, meaning that the clustering had a good fit.

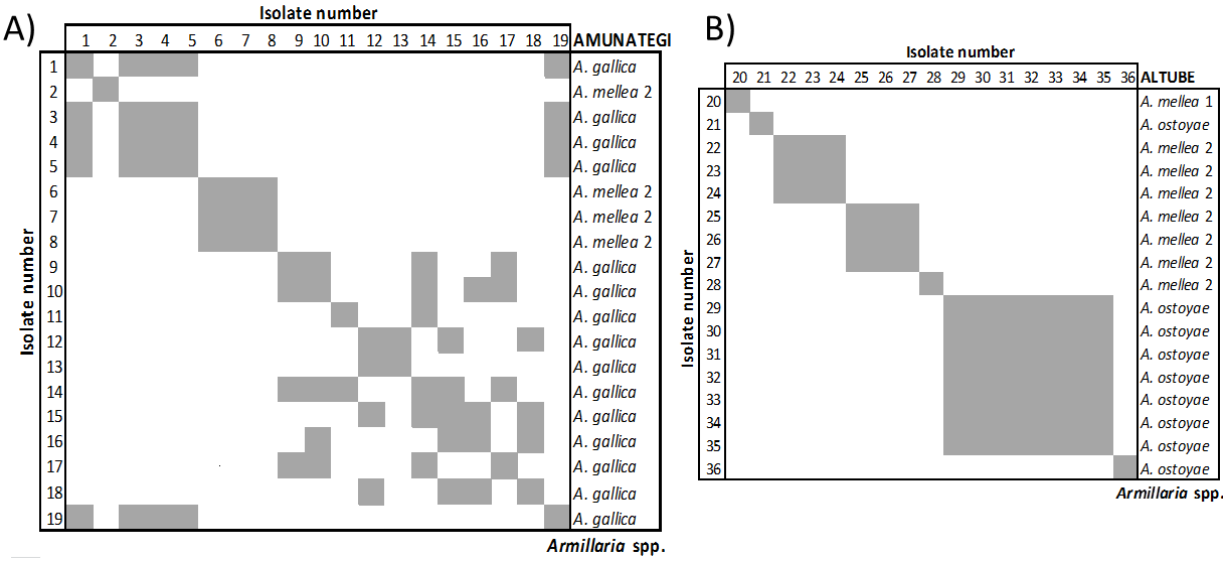
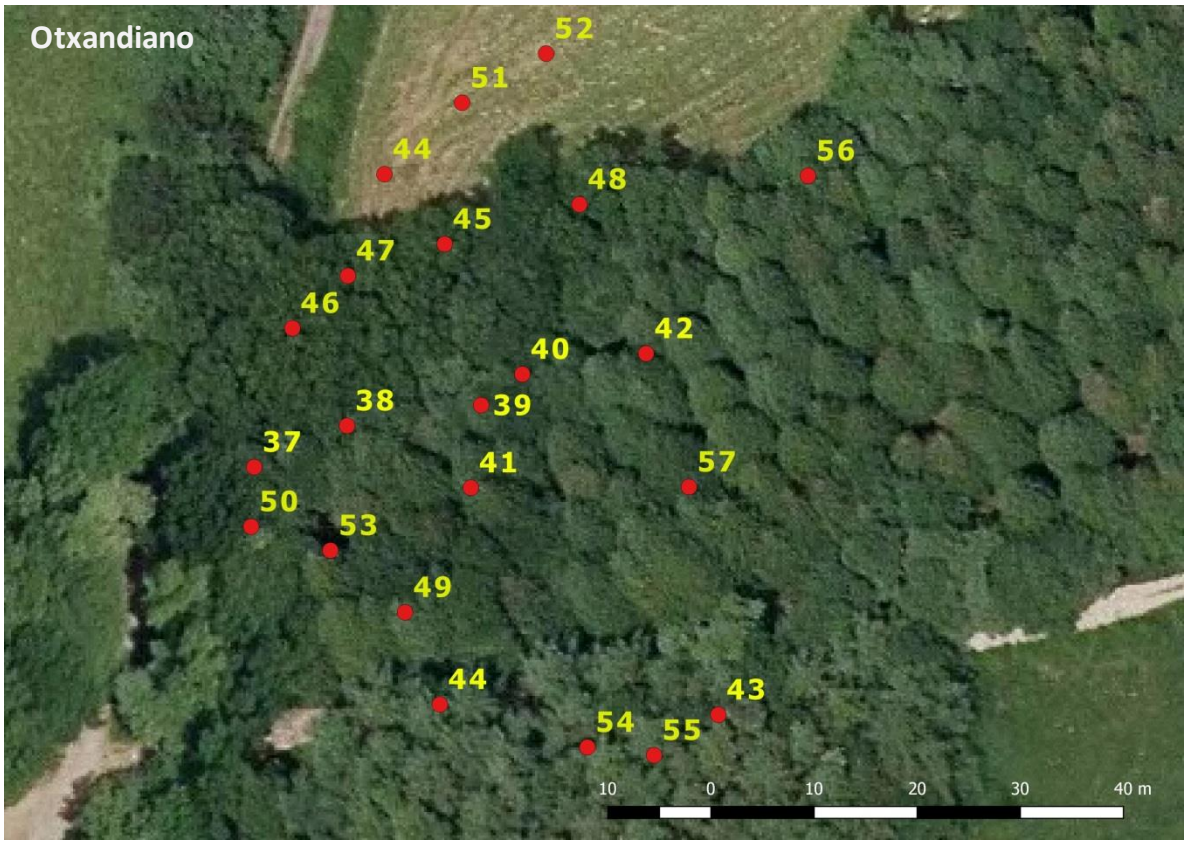


Figure 3.2. *Armillaria* spp. population analysis by mycelial pairings. Grey squares indicate strains belonging to the same SCG; white squares correspond to non-compatible strains. A) Paired sets shown correspond to samples collected in Amunategi; B) paired sets correspond to samples collected in Altube.


Otxandiano
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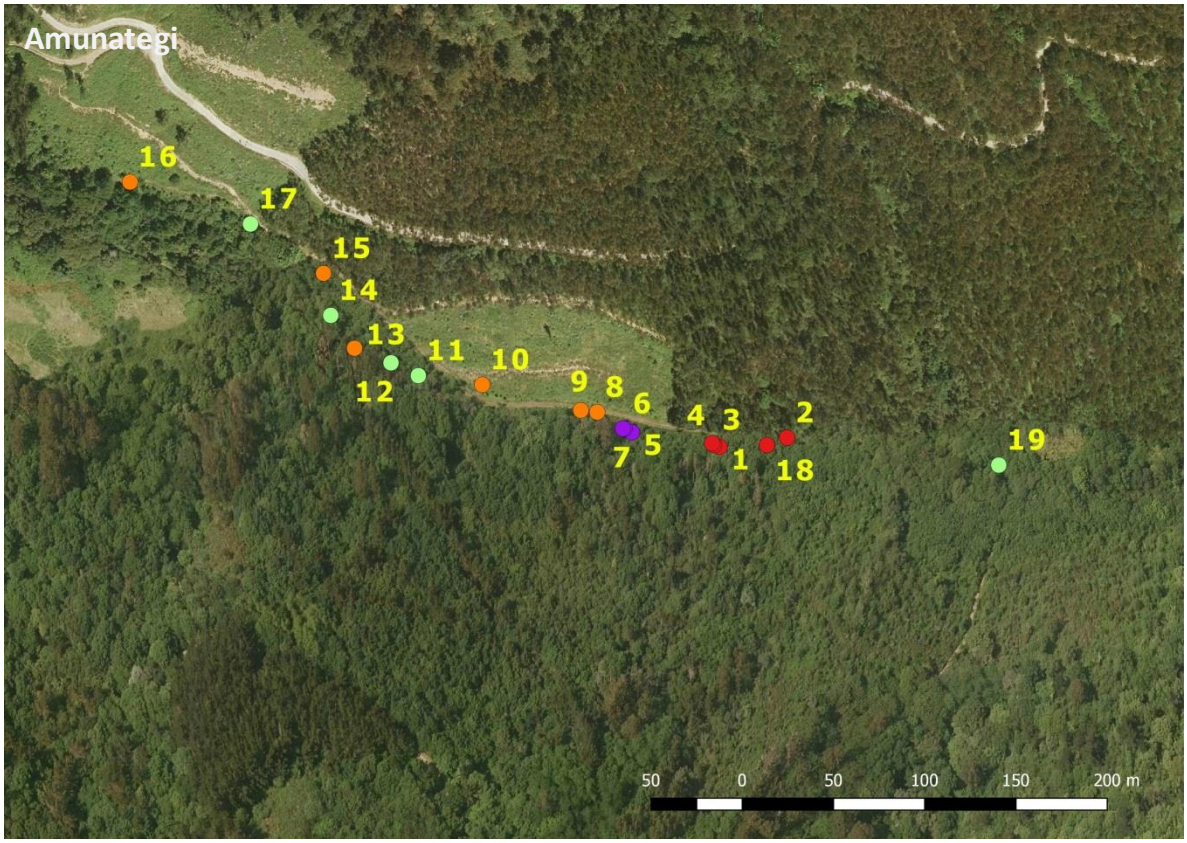
Amunategi
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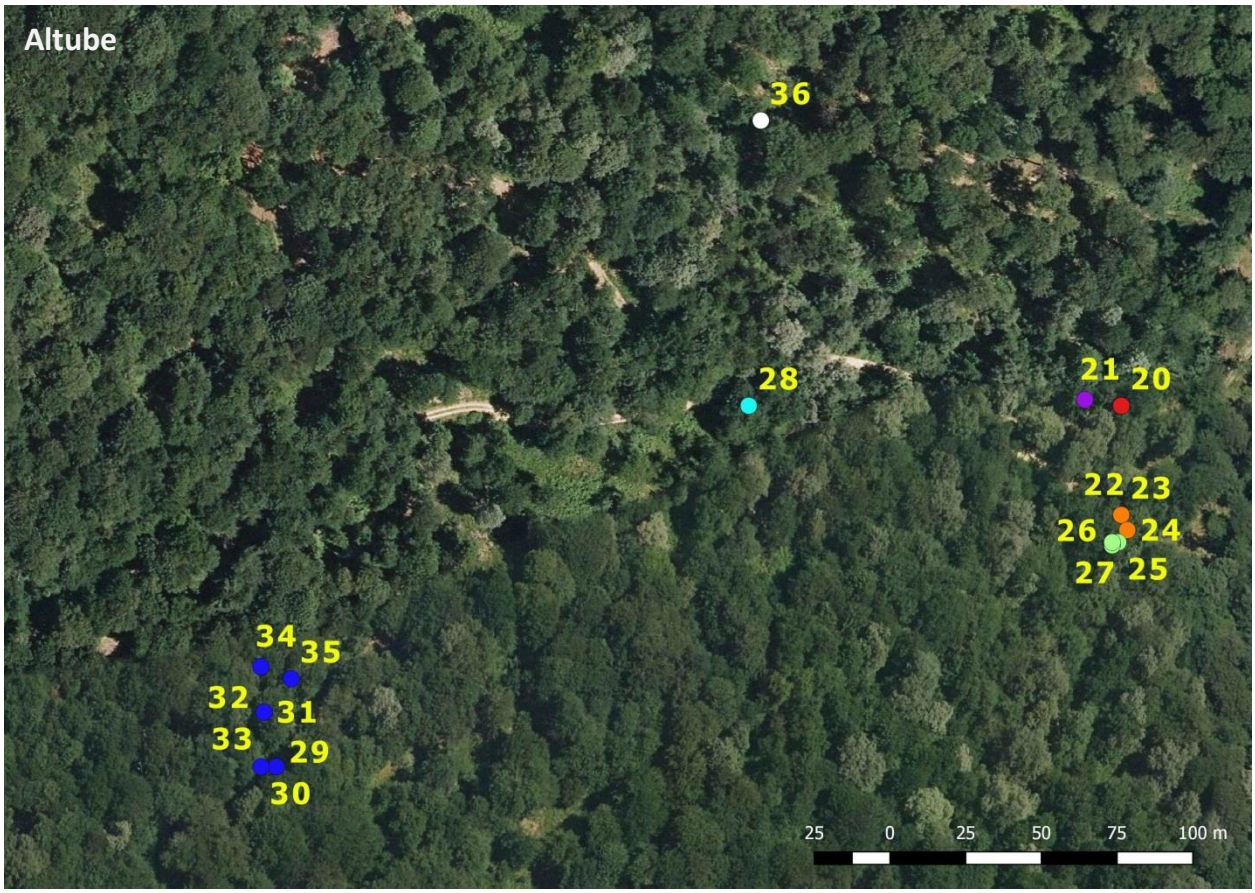


Figure 3.3. Spatial distribution of the *Armillaria* spp. genotypes. Numbers correspond to *Armillaria* spp. isolates. Same genotype is marked with the same color. From top to bottom, stands located in Otxandiano, Amunategi, and Altube.

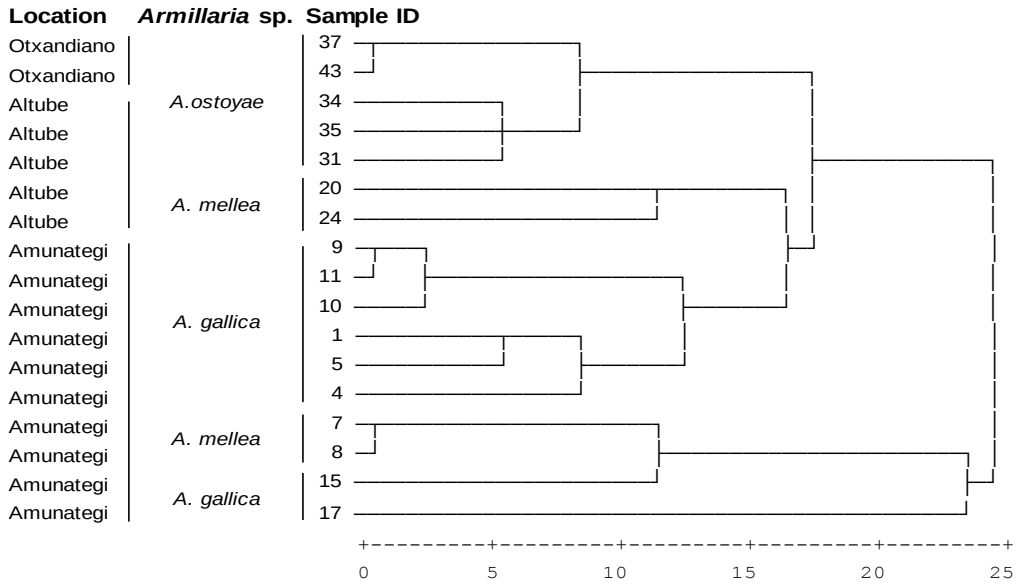


Figure 3.4. Genetic relationships among *Armillaria* strains. The dendrogram was generated by the average-linkage method of clustering using the distances calculated by simple matching coefficient among the binomial matrix obtained from UP-PCR banding patterns using AS4 universal primers.

3.3.2. Analysis of *Heterobasidion* population diversity

In the sampled stands only one species of *Heterobasidion annosum* was found, *H. annosum s.s.* In the stand located in Saldropo, three SCGs were detected, consisting of groups of four, three or two samples; all the isolates were collected from *C. lawsoniana* (Figures 3.5A and 3.6A). All the isolates collected in Legorreta were incompatible and were present in *P. radiata* and *S. sempervirens* (Figures 3.5B and 3.6B). In Caranca, two SCGs were found, both consisting in two samples; in this case all the samples were present in *P. sylvestris* (Figures 3.5C and 3.6C). In Azaceta, one SCG consisting of two isolates was detected, while the rest of the samples were incompatible; the samples were collected from *P. sylvestris* and *C. lawsoniana* (Figures 3.5D and 3.6D).

UP-PCR primer AS4 and L21 showed good ability to distinguish among the fungal strains at the intraspecies level in an initial screen, but when all of the samples were tested they did not provide good differentiation patterns (data not shown).

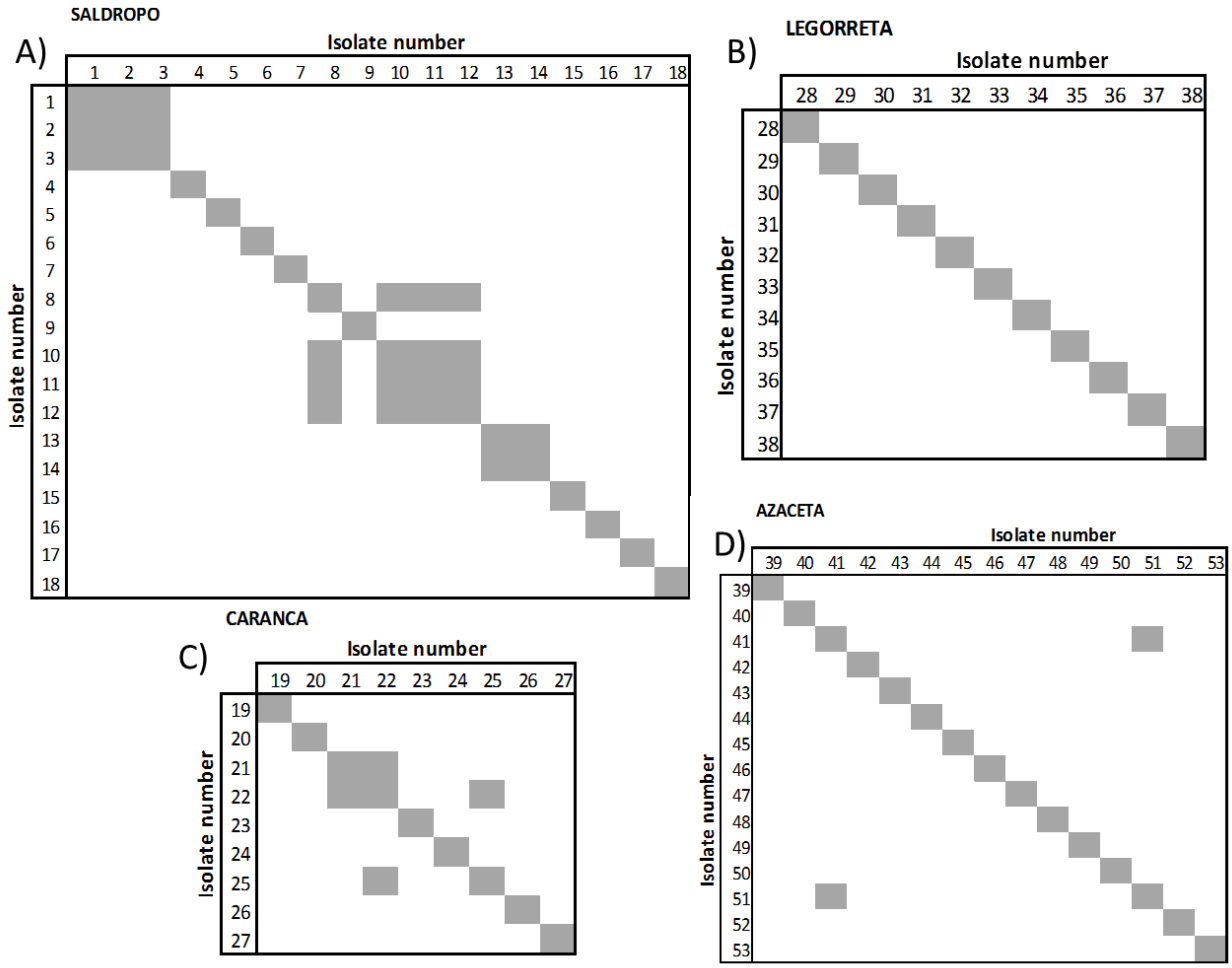
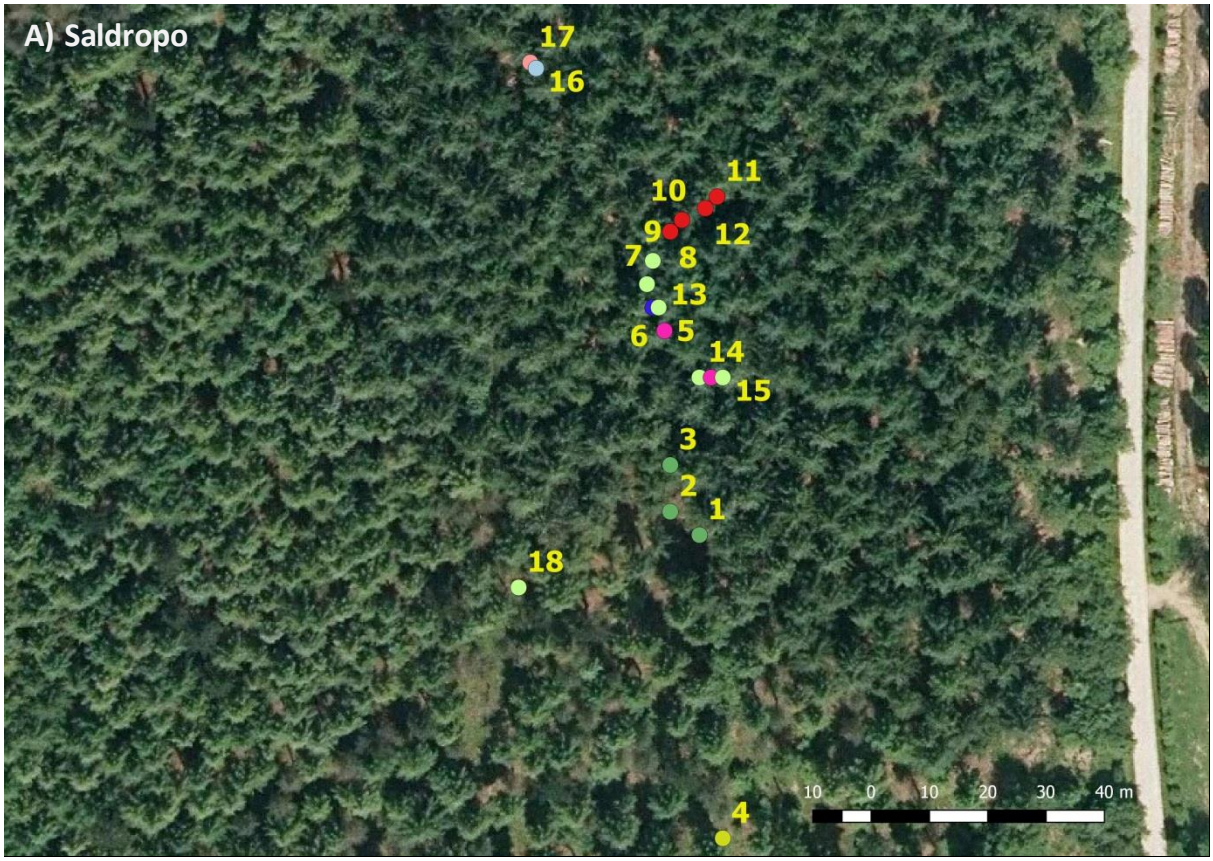


Figure 3.5. *H. annosum s.s* population analysis by mycelial pairings in Saldropo (A), Legorreta (B), Caranca (C) and Azaceta (D). Grey squares correspond to strains belonging to the same SCG; white squares correspond to non-compatible strains.


A) Saldropo
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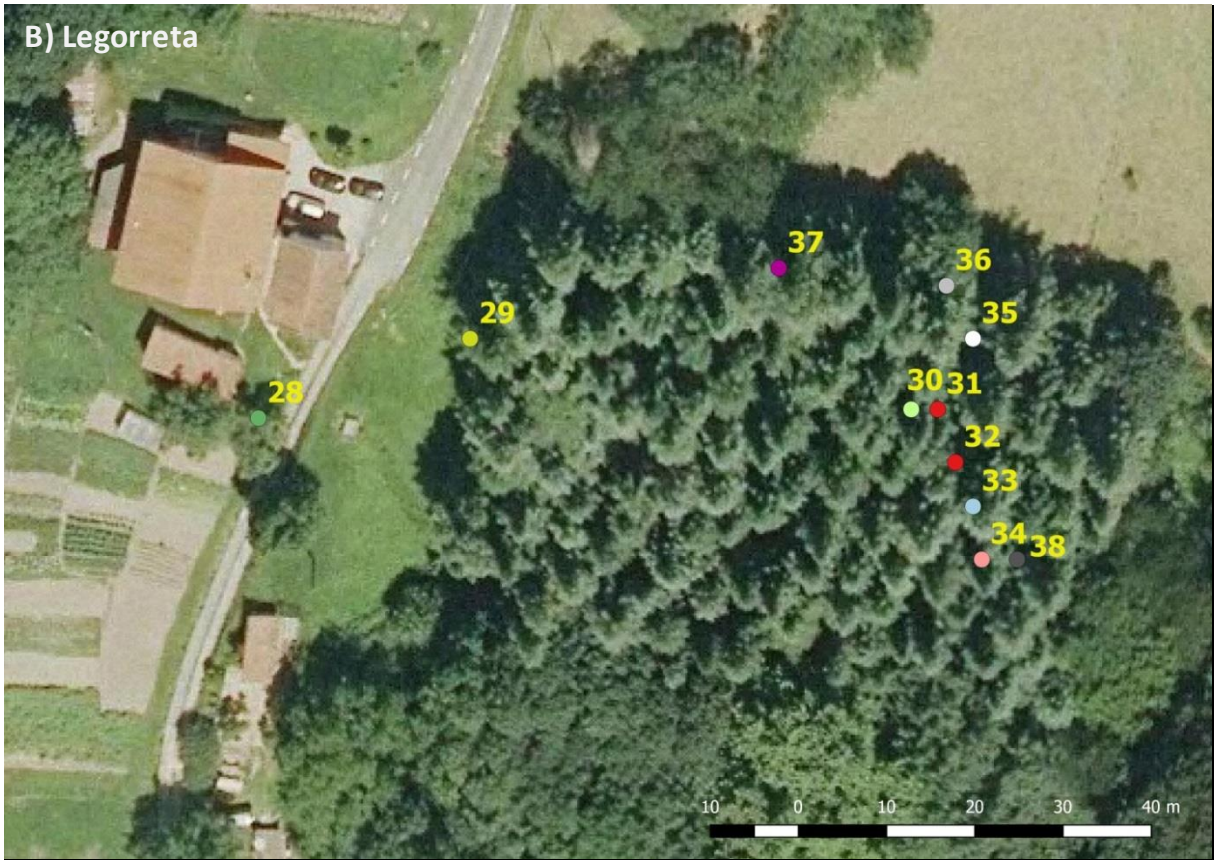
B) Legorreta
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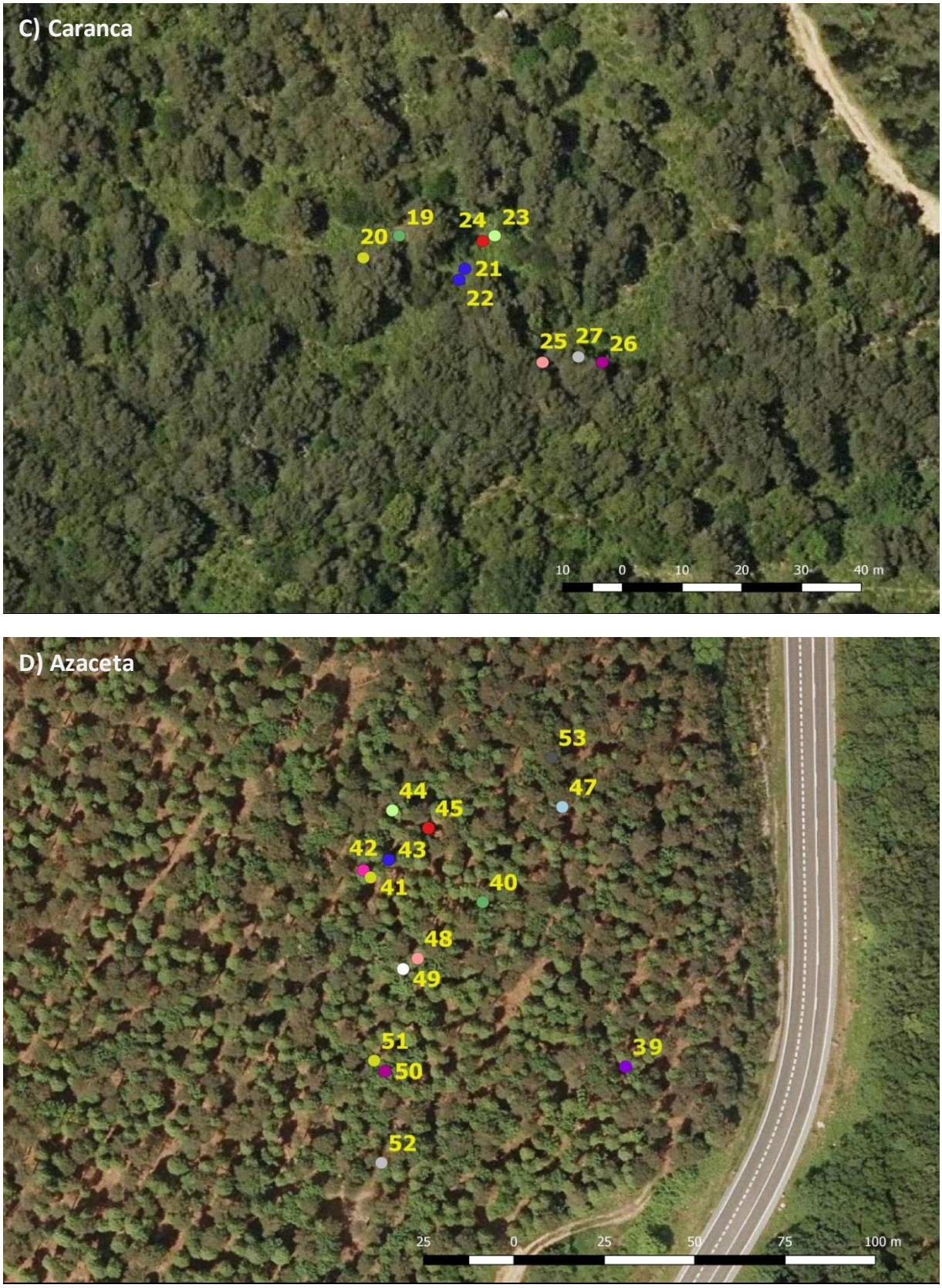

Figure 3.6. Spatial distribution of the *H. annosum s.s.* genotypes. Numbers correspond to *H. annosum s.s.* isolates. Same genotype is marked with the same color. Stands located in Saldropo (A), Legorreta (B), Caranca (C) and Azaceta (D).

3.3.3. Host suceptibility

When the susceptibility of different tree species present in the Basque Country to *A. mellea* infection was analyzed, a significant difference in disease severity was observed among tree species ($\chi 2$ (10) = 83.154, p<0.001), and a Cramer´s V of 0.454 indicated a medium-large effect size between health state and tree species. *P. radiata*, *P. sylvestris* and *P. nigra* were the most susceptible to *A. mellea* with 59.6%, 57.9% and 48% of plants containing lesions, respectively (Figure 3.7). *C. japonica*, *Q. robur* and *S. giganteum* were the least susceptible species with 0%, 5% and 5.6% of plants infected, respectively. When the adjusted standardized residuals were examined, *P. radiata*, *P. sylvestris* and *P. nigra* had significant positive values in the lesion category, meaning that more plants than expected by chance had a lesion, and significant negative values in the healthy category, meaning that fewer plants than expected were healthy (z = ±5.4, p < 0.001, z = ±3.0, p < 0.01 and z = ±3.4; p < 0.001, respectively). *C. japonica*, *Q. robur* and *S. giganteum* had significant positive values in the healthy category, and significant negative values in the lesion category (z = ±4.2, p < 0.001; z = ±3.4, p < 0.001; z = ±3.1, p < 0.01, respectively). *F. sylvatica*, *P. avium*, *Q. petraea*, *Q. ilex*, and *E. nitens* plants did not show any significant difference between the expected and the observed value (Figure 3.7).

Tree species with more than three lesions per tree, and therefore suitable for the Brown-Forsythe and Welch tests, were *P. radiata*, *P. nigra*, *P. sylvestris*, *Q. ilex* and *F. sylvatica*. Significant differences in lesion size were found among the tree species (Brown-Forsythe F (4, 39.364) = 9.235, p < 0.001 and Welch F (4, 35.030) = 7.255, p < 0.001). The mean lesion length was highest for *P. nigra* (16.18 ± 5.03 cm), followed by *P. radiata* (12.70 ± 6.95 cm), *P. sylvestris* (12.66 ± 5.67 cm), *Q. ilex* (9.23 ± 9.25 cm), and *F. sylvatica* (6.75 ± 4.32 cm) (Figure 3.8).

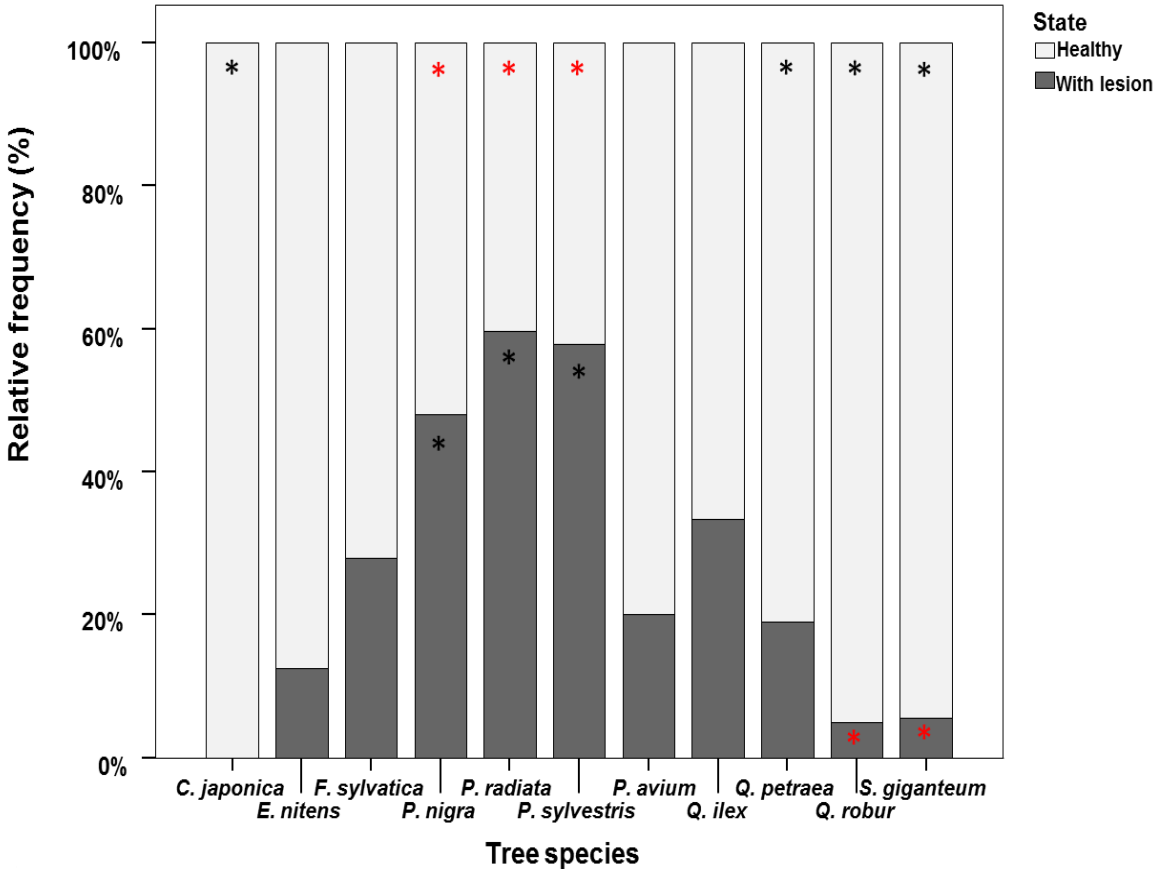

Figure 3.7. Susceptibility of several tree species found in the Basque Country to *A. mellea* infection. The relative frequency of healthy young trees and those with fungal lesions was determined four months after infection with *A. mellea* and growth under greenhouse conditions. Counts are represented as percentage of the total number of plants for each tree species. Black asterisks indicate positive significant z scores ($p < 0.05$) for the indicated health state. Red asterisks indicate negative significant z scores ($p < 0.05$) for the indicated health state.

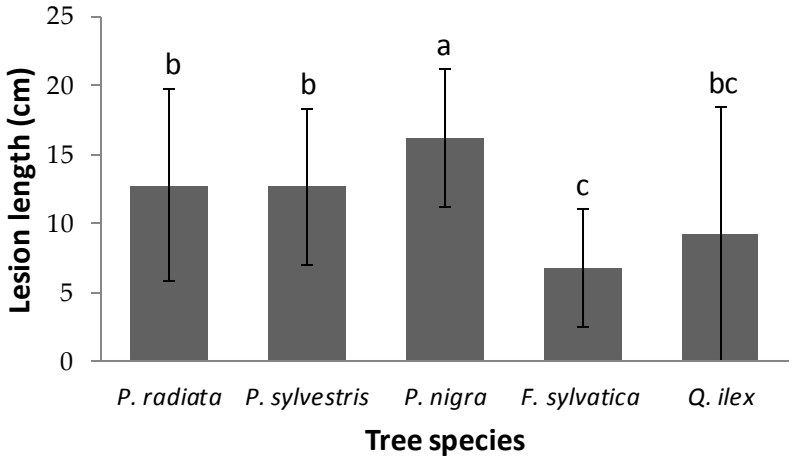

Figure 3.8. Length of lesions (cm) caused by *A. mellea* in different tree species. Error bars show the standard deviation of the means. Statistically significant differences ($p < 0.05$) between tree species are indicated by different lowercase letters.

3.4. Discussion

In the present study, the diversity of *Armillaria* spp. and *H. annosum s.s.* populations in different stands of the Basque country was described, and host susceptibility to *A. mellea* was determined in a set of native and exotic forest species selected based on the frequency of their presence in the Atlantic area of Spain.

All of the isolates from stands surveyed for *H. annosum*, were identified as one species, *H. annosum s.s.*, and all were found on coniferous hosts. In Caranca, Azaceta and Legorreta, the strains were mainly present on stumps, and showed high diversity, i.e. high somatic incompatibility. This suggests that infection of the stumps, and trees with lesions in the bark, was most likely by basidiospores. In contrast, in Saldropo, *H. annosum* was found on stumps, dead trees, and living trees and exhibited higher somatic compatibility. In this stand, in addition to infection by basidiospores, the fungus also may have spread by vegetative mycelium as is suggested by SCGs. The largest genet was formed by four isolates which were located in stumps or living trees, with the maximum distance between them of 6 meters. *H. annosum* complex genets diameters are usually smaller than 30 m and never infect a high number of trees (Garbelotto and Gonthier, 2013). In general, the most important infection mechanism for *H. annosum* is colonization of stumps by basidiospores (Korhonen and Stenlid, 1998), thus resulting in a high number of different genotypes. However, heterokaryotic *H. annosum* also contains homokaryotic mycelium, so when two compatible heterokaryons interact a new heterokaryon can be formed by the mating between homokaryons, resulting in the increase of different SCGs without basidiospores being involved (Swedjemark and Stenlid, 2001). When *H. annosum s.s* genets identified by SI tests were compared with those obtained by UP-PCR no similarities were observed, i.e. some isolates had the same band pattern even if they were isolated from different stands and belonged to different genets. In this case the chosen universal primers for UP-PCR were not considered suitable for distinguishing among *H. annosum s.s.* genets.

In the stands infected with *Armillaria* spp., *A. ostoyae* was distributed in larger clonal clusters than *A. mellea* and *A. gallica*, which were found in medium size and small clonal clusters, respectively. Differences in genet size have been reported for different species of *Armillaria*. Genet size is also influenced by basidiocarp formation, basidiospore abundance and survival, and formation of diploids, which are influenced by moisture and temperature. Thus, the presence of large genets is more common in dry and/or cold forests than in moist-warm forests (Ferguson et al., 2003; Worrall et al., 2004; Bendel et al., 2006). In the present study, the stands with the largest genets, Otxandiano and Altube, were those with colder temperatures. However, *A. mellea* genets were of similar sizes in stands with cold and warmer climates, so so differences in behavior among species may also be a factor.

Most of the genets determined for *Armillaria* spp. by UP-PCR using AS4 primer and SI tests were consistent. In the case of the genet from Otxandiano, no difference in UP-PCR band patterns were apparent between *A. ostoyae* samples, but when *A. ostoyae* samples located in Altube were assessed, different band patterns were detected among isolates of the same SCG. Different band patterns were also detected among *A. gallica* isolates of the same SCG. This could imply that some genets were composed of sib-related genets, inbred sibling genets which are very closely related, meaning that basidiospores could be more important in the dispersion process than observed on SC tests. (Kile, 1983; Bendel et al., 2006). UP-PCR may provide more information about the genets obtained by SI tests in *Armillaria* spp., Dodd et al. (2006) also used UP-PCR for determining polymorphism within and between different species of *Armillaria* (*A. limonea* (G. Stev.) Boesew. and *A. novae-zelandiae* (G. Stev.) Boesew.), and in their study primer AS4 also provided consistent results.

In the stands, *A. mellea* exhibited the greatest host diversity among the *Armillaria* spp. and therefore was chosen to further investigate susceptibility of trees that are commonly found in the Basque Country. All the tested tree species were susceptible to *A. mellea* except *C. japonica*. It has been observed that resistance between populations of *C. japonica* can vary due to the different concentrations and ratios of some compounds in the sapwood and heartwood such as norlignans and ferruginol (Azevedo, 1976; Yamada, 1992; Melo et al., 2004). *Pinus* species were the most affected by *A. mellea* infection; Aguín et al. (2004b) reported that *Pinus* spp. infected with *A. mellea* were the first to show signs of the disease in aerial tissues compared to trees infected with other species of *Armillaria*, such as *A. ostoyae* and *A. gallica*. Here, *P. radiata* was the species with the greatest number of infected plants and *P. nigra* had the highest average lesion size. *P. radiata* is also considered very susceptible to other *Armillaria* spp. such as *A. novae-zelandiae* (Hood et al., 2009) and to *H. annosum s.s.* (Doğmuş-Lehtijärvi, 2015)

3.4.1. Conclusions

In the studied stands *H. annosum s.s.* dispersion was mainly by basidiospores, but it should be also considered that homokaryotic mycelium from heterokaryons can interact with stablished heterokaryons and thus, dispersion by vegetative mycelium could be more important than assumed only by considering genets sizes. *Armillaria* spp. dispersion patterns were variable between stands and species, and they could be affected by moist and temperature. Evidences of *P. radiata* susceptibility to both pathogens in greenhouse conditions and in field are apparent. Monocultures of highly susceptible tree species should be avoided in order to prevent an increment in damage risk.

CHAPTER 4

Native rhizobacteria as biocontrol agents of *Heterobasidion annosum s.s.* and *Armillaria mellea* infection of *Pinus radiata*

4.1. Introduction

Three percent of the total tree plantations worldwide consist of *P. radiata* (Monterey pine), covering over four million hectares, mainly in New Zealand, Chile, Australia, Spain and South Africa, where they are an important part of the economy. In the Basque Country it constitutes 46% (28.6 million m^3) of the total wood stock (HAZI, 2010). *P. radiata* is the most extensively planted exotic conifer, most productive (Mead, 2013), and susceptible to infection by both *Heterobasidion* and *Armillaria*. Studies conducted in New Zealand showed that species of *Armillaria* caused mortality rates between 20-50% in the first six years of *P. radiata* stands (Hood and Sandberg, 1993b), and 6-13% losses of the potential volume in a 28 year old *P. radiata* plantation (MacKenzie, 1987). Mesanza et al. (2017) detected the presence of *Armillaria* spp. on a high percent of disease foci in *P. radiata* plantations of the Basque Country. *H. annossum s.s.* caused high levels of disease in three year old *P. radiata* trees (Doğmuş-Lehtijärvi et al, 2016), and gaps in plantations in northern Spain (Mesanza and Iturritxa, 2012).

Currently, *Heterobasidion* infections are managed using silvicultural, chemical and biological methods. Silvicultural practices include planting less susceptible tree species, stump removal, using proper planting and mixture schemes, and thinning when the spores are not dispersing. Chemical treatments are based on urea and borate (Pratt and Lloyd, 1996; Johansson et al., 2002), and biological control requires inoculating stumps with the fungus *Phlebiopsis gigantea* (Fr.) Jülich (Asiegbu et al., 2005). *Armillaria* treatment includes silvicultural methods (e.g., root collar excavation, stump and residual root removal, and planting less susceptible tree species), soil fumigants such as methyl

bromide and carbon disulphide, and the application of the soil-borne fungus *Trichoderma* Pers. after fumigation (Baumgartner et al., 2011).

The application of these treatments is limited and often ineffective due to factors such as level of infection, environmental conditions and risks, cost, and legislation, among others. For example, the use of urea and borate causes temporal modifications in soil chemistry and damages the ground vegetation and the structure of the fungal community (Asiegbu et al., 2005). In addition, these chemicals are not registered as pesticides in many countries so their use is prohibited (Gonthier and Thor, 2013). The efficacy of methyl bromide and carbon disulphide is influenced by soil characteristics and the size of the inoculum source, and methyl bromide prohibition is pending in the USA (Baumgartner et al., 2011). Although fungal biocontrol agents may be applied, effective soil levels of *Trichoderma* are difficult to attain (Shaw and Roth, 1978). In contrast, *P. gigantea* effectively inhibits the spread of *Heterobasidion* following colonization of stumps by basidiospores, however, it reduces fungal diversity and its use is only approved in some countries of the EU (Gonthier and Thor, 2013). Finally, even when silvicultural techniques are useful, they can be expensive and/or difficult to accomplish, and ineffective when the infection is well established. The best defence against these fungal infections is prevention.

Biological control with bacteria has proven effective against several fungal pathogens of agronomic crops (Mark et al., 2006) and in fewer cases against forest fungal pathogens (Singh et al., 2008). Antagonism by bacteria is achieved by different mechanisms including antibiosis, competition for nutrients, parasitism, and induced resistance in the host (Whipps, 2001). Other factors that influence the efficacy of biocontrol bacteria are their capacity to colonize the rhizosphere or the host seeds, and to adapt to soil conditions (Mark et al., 2006). The probability of isolating microorganisms from the environment that demonstrate an antagonistic effect *in vitro* is relatively high, but many of these are not effective when applied *in planta* where plant host responses to and impact on microbial activity are also important (Gardener and Fravel, 2002). Crop studies have shown that strains isolated from native soils have the best chance of protecting plants as they are adapted to the soil conditions and therefore can compete effectively with other indigenous microbes.

4.1.1. Objectives

The objectives of this study were:

i. To isolate and characterize some bacteria native to the *P. radiata* rhizosphere with the ability of inhibit the growth of *A. mellea* and *H. annosum in vitro.*
ii. To determine their prophylactic effects, if any, in *P. radiata* seedlings.

4.2. Materials and methods

4.2.1. Microorganisms

The *H. annosum* s.s. and *A. mellea* strains used in this study were isolated from basidiocarps present in a *Pinus sylvestris* plantation in Alava, Spain and on an *Acer* spp. located in Biscay, Spain, respectively. Both fungal strains proved to have virulence against *P. radiata*. Identification, characterization and efficacy of both pathogens were described previously (Chapter 3). The fungi were routinely grown at 20ºC in the dark on malt extract agar (MEA).

Bacterial strains were isolated from the rhizosphere of a healthy tree located in a *P. radiata* plantation (Latitude: 43º06´46´´N; Longitude: 2º38´35´´W, Abadiano, Biscay, Basque Country, Spain) with high presence of fungal pathogens. Samples containing tree roots and surrounding soil were collected and stored at 4ºC. To extract ectorhizosphere bacteria, 5 g of root samples were suspended in 45 ml sterile 0.85% NaCl, shaken for 3 minutes, and the supernatant was decanted into sterile tubes. To obtain endorhizosphere bacteria, 5 g of roots were washed with sterile 0.85% NaCl and then homogenized with an adapted drill (Optimun Maschinen, Germany) in 50 ml of the same solution and the supernatant was collected. Serial dilutions of the supernatants were plated on Luria Bertani (LB) agar (Miller, Fisher Scientific) and grown overnight at room temperature (Figure 4.1).

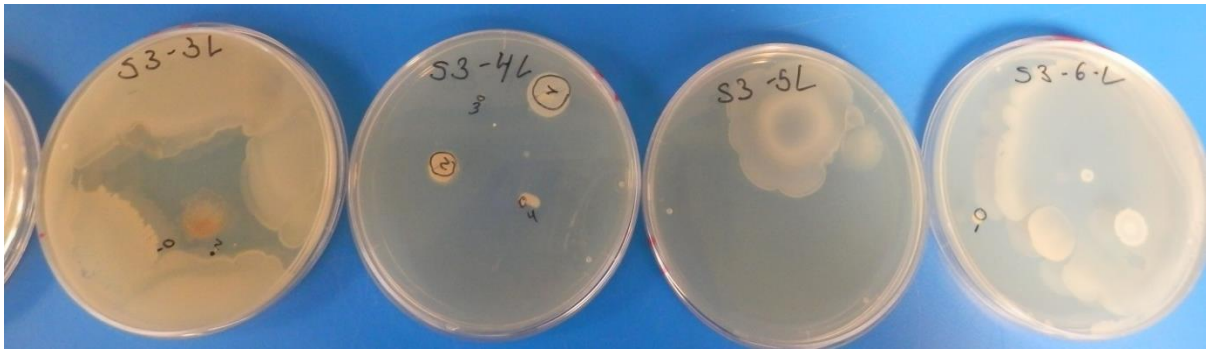

Figure 4.1. Ectorhizosphere bacteria isolated from *P. radiata* roots. Root surface bacteria were extracted into sterile 0.85% NaCl and serial dilutions were plated on LB agar.

4.2.2. *In vitro* fungal antagonism assay

Two hundred isolated rhizobacteria were initially screened, in triplicate, for antagonistic effects against *H. annosum* and *A. mellea*. Approximately 2 mm^2 of fungal mycelium was transferred into wells of a six-well plate (Nunc) containing ISP2 agar (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose and 20 g/L agar, pH 7.3; Shirling and Gottlieb, 1966), determined to be suitable for both fungal and bacterial growth, and was grown for two days before applying the bacterial suspension. Bacterial cultures, prepared in triplicate from independent colonies grown for two days into LB broth, were applied in a thin line using a sterile inoculation loop 0.5 cm from the fungi. *Escherichia coli* TOP10 (Invitrogen) served as a negative control. After 60 days of growth at room temperature (20-25ºC), inhibition of fungal growth by the bacteria was visually assessed (Figure 4.2).

The effect of antagonistic bacterial strains was confirmed in a second *in vitro* antagonism assay. The bacterial cultures were washed twice with 0.03 M $MgSO_4$ and the final concentration adjusted to an OD_{600nm} of 0.5 before applying to wells containing fungi as described above. Bacterial antagonism was defined as Area Inhibition Percentage (AIP): AIP (%) = (A-B)/A*100, where A and B are the surface area covered by the fungus in control (no bacteria) and treated (with bacteria) plates, respectively.

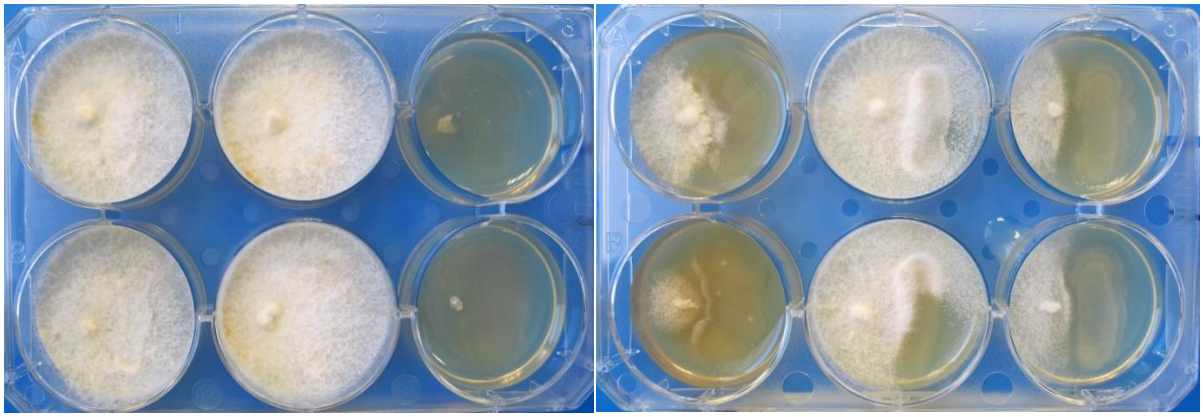

Figure 4.2. Example of different levels of antagonistic effects on *H. annosum s.s.* of some of the bacterial strains isolated in the first screening. Top and bottom wells of each column correspond to bacterial replicates. *E. coli* was inoculated on the first top and bottom well on the left.

4.2.3. Bacterial identification and pathogenicity determination

Single colonies of effective fungal antagonistic bacteria were grown overnight in 3 ml LB broth at 30 ºC for DNA extraction with Wizard Genomic DNA Purification Kit (Promega, USA). The 16S rRNA gene was amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Pickering, ON) and the primer pairs 46f and 536r (Mummey and Stahl, 2004), E334f and E939r (Baker et al., 2003), and E786f (Baker et al., 2003) and E1491r (Smit et al., 1997) (Table 4.1). The PCR conditions were as follows: 5 min at 95ºC, 30 cycles of 30 sec at 95ºC, 30 sec at the appropriate annealing temperature, and 1 min at 72ºC, and a final 1 min at 72ºC. The purified PCR products were sequenced by Robarts Research Institute (London, ON). The sequences were manually assembled using Mega 4.0 software, and then analyzed using the Ribosomal Database Project (Cole et al., 2014), Greengenes (DeSantis et al., 2006) and GenBank (Benson et al., 2007) databases.

The possible phytopathogenicity of the bacterial strains was assessed by watering two-month-old *P. radiata* seedlings with 5 ml of bacterial suspension. *P. radiata* seedlings were grown from seeds (Sheffield´s Seed Co. Inc., NY) that were surface sterilized by placing them in 2.1% sodium hypochlorite solution for 10 minutes with shaking, and then rinsing them thoroughly with water (Wenny and Dumroese, 1987). Seeds were soaked in water for 24 h and then stratified for 15 days at 4ºC before planting them. Bacterial cultures were grown for two days at room temperature in LB broth, washed twice with 0.03 M $MgSO_4$, and the final concentration adjusted to an OD_{600nm} of 0.5. Seedlings were also treated with a control solution of 0.03 M $MgSO_4$. A total of 40 plants per treatment were grown in sand Turface (69 % silica sand, 29% Turface, 2% $MgCO_3$) in 12 cm x 3 cm pots under constant temperature (16 h photoperiod, day/night temperature of 23-17ºC) (Chanway et al., 1991). After one month the seedlings were examined for disease symptoms.

4.2.4. *In vivo* biocontrol assay

The *A. mellea* inoculum was prepared following the procedure of Beckman and Pusey (2001). Briefly, pieces of fungal mycelium were placed on a preparation of benomyl-dichloran-streptomycin agar (Worrall, 1991) with autoclaved *Quercus* spp. acorns and incubated for approximately one month at room temperature in the dark. *H. annosum* was grown in MEA for one month. Bacterial suspensions (OD_{600nm} of 1) were prepared in 0.03 M $MgSO_4$ as described above. An *E. coli* suspension and 0.03 M $MgSO_4$ were included as negative controls.

A total of 100 one-year old *P. radiata* seedlings (Explotaciones Forestales Jiménez Araba s. l. Nursery, Vitoria, Spain) were inoculated with each bacterial strain by immersing root balls in the bacterial suspension for one hour. The inoculation was repeated after one week. After seven days, 65 of the seedlings treated with each bacterial strain were inoculated with *A. mellea* by placing acorns colonized by the fungus in contact with tree roots, one acorn per seedling. The remaining seedlings were inoculated with 1 cm^2 of *H. annosum* mycelial fragments. Experiments were laid out in a completely randomized design with two factors (pathogens and bacterial treatments). *P. radiata* seedlings were grown in 11x11x22 cm pots (1600 cc volume), using a mix of peat moss (2/3 peat, 1/3 perlite and fertilizer NPK; N = 200-450 mg/l, P_2O_5 = 200-500 mg/l, K_2O = 300-550 mg/l). They were maintained for 110 days in a biosafety level 2 greenhouse at a mean temperature of 18 ± 5 ºC, with a relative humidity of 55-60% and without supplemental light. Upon completion of the experiment, roots were carefully cleaned by rinsing with water, and stem length, collar diameter and, in the case of the plants inoculated with *A. mellea*, dry root weight were measured. To obtain the dry weight, roots were dried in an oven (Selecta) at 60 °C for 72 h and then weighed on an analytical balance (OHaus). Stem length and collar diameter measurements were analyzed together as slenderness index (SI) using the formula: SI = stem diameter (mm)/((stem length (cm)/10)+2) (Schmidt-Vogt, 1980). The size of the lesions caused by *A. mellea* was determined by measuring the length of mycelial colonization under the seedlings' bark.

4.2.5. *Heterobasidion* detection by nested PCR

Nested PCR was used to detect the presence of *H. annosum* in *P. radiata* seedlings. This variant of the standard PCR consists of two PCRs; in the first reaction primers that flank the DNA region where the amplicon of interest is located are used. In the second reaction specific primers for the DNA target are used and the DNA template is the product obtained in the first reaction. Nested PCR is useful in complex samples, when the amount of target DNA is small, and to reduce nonspecific amplification (Haff, 1994).

Ten plants per treatment were randomly chosen and the roots were cleaned by rinsing with water. Three centimeters of pine roots were cut (one cm above and two cm below the first secondary root) and the DNA extracted using DNeasy Plant Maxi Kit according to the manufacturers protocol (QIAGEN). Because PCR inhibitors were present in root extracts, 360 µl of each sample were repurified by ethanol precipitation and the DNA resuspended in 36 µl of 0.1X TE buffer.

In the first PCR, primers HaPF and HaPR that flank the internal transcribed spacer (ITS) were used to amplify a fragment of the rRNA gene (Table 4.1). The reaction mixture was composed of 2.5 µl of 10X Buffer (NEB), 50 nM of each primer, 200 µM dNTPs (NEB), 1.25 U of Taq DNA polymerase (NEB) and 1.3 µl of sample DNA (1:10, 1:25 and 1:50 dilutions) in a final volume of 25 µl. The cycling conditions were as follows: 10 min at 95ºC, 15 cycles of 30 sec at 95ºC, 45 sec of annealing at 59.5ºC, and 2 min at 72ºC, and a final 1 min at 72ºC. PCR controls included a water blank, *H. annosum* DNA only, and *P. radiata* DNA only.

The second PCR was performed using 300 nM of each specific primer for *H. annosum s.s.* ITS region MJF and MJR (Table 4.1), 2 μl of 10X Buffer (NEB), 200 nM dNTPs (NEB), 1 U of Taq DNA polymerase (NEB) and 5 μl of the products of the first PCR, in a final volume of 20 μl. Cycling conditions were: 3 min at 95ºC, 40 cycles of 30 sec at 95ºC, 30 sec of annealing at 58ºC and 15 sec at 72ºC, and a final 1 min at 72ºC. PCR controls included all the controls from the first PCR reaction, a water blank, *H. annosum* DNA only, and *P. radiata* DNA only. All the PCR products were visualized in 1.5% (w/v) agarose gels.

The *P. radiata* actin gene was used as a reference gene to ensure that PCR inhibitors did not contribute to false negative results from *H. annosum* ITS amplification. PCR reactions contained 2.5 μl of 10X Buffer (NEB), 300 nM of each primer AprF and AprR (Table 4.1), 200 nM dNTPs (NEB), 1.25 U of Taq DNA polymerase (NEB) and 1.3 μl of the DNA sample (1:10, 1:25 and 1:50 dilutions). PCR conditions were as follows: 3 min at 95ºC, 40 cycles of 30 sec at 95ºC, 30 sec of annealing at 58ºC and 15 sec at 72ºC, and a final 1 min at 72ºC. All the amplification reactions for all the samples were replicated once.

Table 4.1. Primers used in this study.

Organism, gene	Name	Primer sequences (5´→3´)	Reference
P. radiata, actin gene	AprF	AGCAACTGGGATGACATGGA	This study
	AprR	TGCCTTGGGATTCAGAGGAG	This study
H. annosum s.s., rRNA gene	HaPF	TCCTTGACCCTTAGGCATTG	This study
(primary primers)	HaPR	TCCATGCGAAGAACTTCAGG	This study
H. annosum s.s., ITS region	MJF	GGTCCTGTCTGGCTTTGC	Hantula and Vainio, 2003
(nested primers)	MJR	CTGAAGCACACCTTGCCA	Hantula and Vainio, 2003
Bacteria, 16S rRNA gene	46f	GCCTAACACATGCAAGTCGA	Mummey and Stahl, 2004
	536r	GTATTACCGCGGCTGCTGG	Mummey and Stahl, 2004
	E334f	CCAGACTCCTACGGGAGGCAGC	Baker et al., 2003
	E939r	TTGTGCGGGCCCCCGTCAATTC	Baker et al., 2003
	E786f	GATTAGATACCCTGGTAG	Baker et al., 2003
	E1491r	GGTTACCTTGTTACGACTT	Smit et al., 1997

4.2.6. Statistical analysis

Differences in the ability of the bacterial strains to inhibit fungal growth *in vitro* were analyzed by one way ANOVA with Waller-Duncan post-hoc test using SPSS software (SPSS Inc.). Waller-Duncan post–hoc test requires a large absolute difference in sample means in order to declare significance when F- value is small, and the opposite when F-value is large; it is used when samples are in equal number (Milliken and Johnson, 1992). Values of $p < 0.05$ were considered significant. The differences in *Armillaria* disease severity among bacterial treatments *in vivo* were determined by Pearson´s chi-square test. Plants were scored as healthy (without symptoms of infection), dead or with lesions. The strength of association between categorical variables (health status and bacterial treatments) was measured with Cramer´s V;

adjusted standardized residuals were checked in order to determine the significant differences between categories. The rest of the measurements such as lesion size, stem diameter, height, SI, and dry root weight were analyzed by one way ANOVA, using Hochberg`s GT2 (for different sample sizes and equal population variances) and Games-Howell (for different populations variances and different sample sizes) (Meyers et al., 2006) post-hoc comparisons for the data from the plants inoculated with *A. mellea* and *H. annosum*, respectively. Data for dead plants were removed from the ANOVA analysis for diameter, height, SI and dry root weight and values of $p < 0.05$ were considered significant.

4.3. Results

4.3.1. Bacterial inhibition of fungal growth *in vitro*

From 200 bacterial strains isolated from the rhizosphere of a healthy *P. radiata* tree, seven were selected as potential biocontrol agents based on their ability to inhibit *H. annosum* and *A. mellea* growth *in vitro*. Four strains were isolated from the endorhizosphere and three from the ectorhizosphere. The bacterial treatments had a significant inhibitory effect on *H. annosum* ($F(8, 18) = 73.9$, $p < 0.05$) and *A. mellea* ($F(8, 18) = 111.4$, $p < 0.05$). All seven isolates reduced the area covered by *A. mellea* mycelium by 58.6-94.2% and rhizomorph formation (Figure 4.3, Figure 4.4). In contrast, *E. coli* increased mycelial growth of *A. mellea* by an average of 21% (Figure 4.4). All isolates except S22L11 reduced *H. annosum* mycelial growth (Figure 4.3, Figure 4.4). The remainder of the isolates reduced the area covered by *H. annosum* mycelium by 56.7-99.3%. The most effective bacterial treatments against both fungi were S32R2 and S31R1 with inhibition values of 99% for *H. annosum*, and 94.2% and 83.4% respectively for *A. mellea* (Figure 4.4).

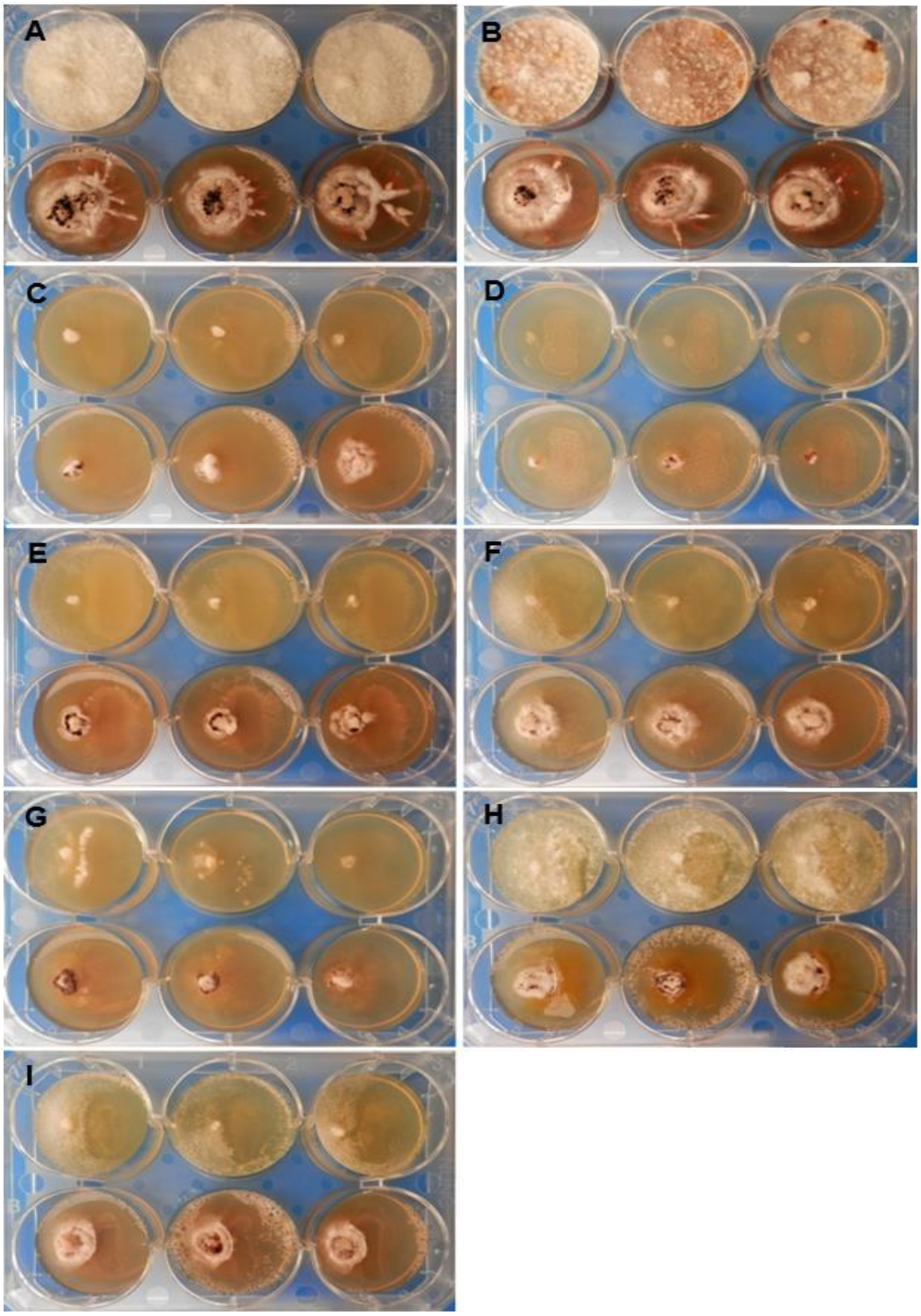

Figure 4.3. *In vitro* fungal antagonism assay. Plates were inoculated with *E. coli* (A), no bacteria (B), S31R1 (C), S32R2 (D), S11R41 (E), S23L3 (F), S11R21 (G), S22L11 (H), or S31L1 (I). The three upper wells of each plate were inoculated with *H. annosum s.s.*, and the bottom wells with *A. mellea*.

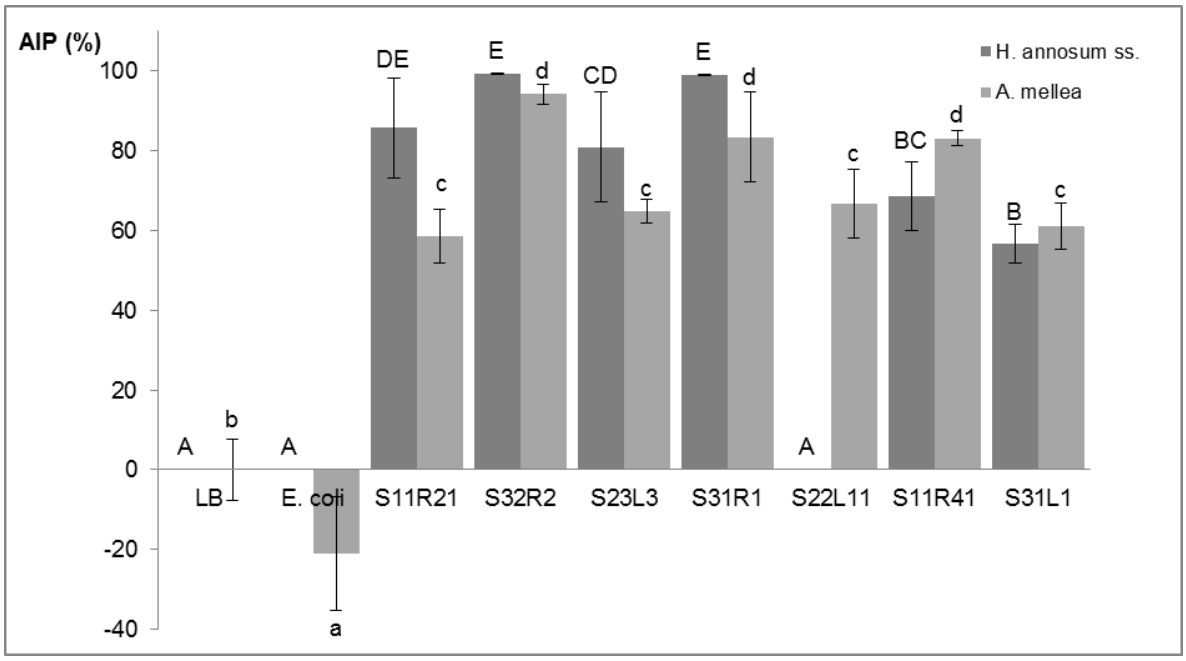

Figure 4.4. Area inhibition percent (AIP) of the tested bacterial strains against *H. annosum s.s* (dark grey) and *A. mellea* (light grey). Error bars show the standard deviation of the 3 independent replicates. Statistically significant differences of $p < 0.05$ between treatments for *A. mellea* and *H. annosum* are presented with different lowercase or capital letters, respectively.

4.3.2. Identification and pathogenicity determination of bacterial strains

Based on their 16S rRNA gene sequence identity to known bacteria, the antagonistic bacterial strains were identified as follows: strain S32R2 as *Pseudomonas fluorescens*; S22L11 as *Bacillus weihenstephanensis* or *B. mycoides*; S11R41 as *Bacillus simplex* or *Brevibacterium frigoritolerans*; S31L1 as *Rahnella* spp., possibly *Rahnella aquatilis*; S11R21 as possibly *Pseudomonas poae*, *P. costantinii* or *P. trivialis*; and S23L3 and S31R1 as *Erwinia billingiae* (Table 4.2). Three were not tested further, S22L11 and S11R21 due to the possibility of being human or animal pathogens (Stenfors et al., 2002; Goodwin et al., 1994; Chang et al., 1999), and S31L1 because *P. costantinii* is considered a pathogen for cultivated mushrooms (Munsch et al., 2002). Two-month-old pine seedlings inoculated with the remaining four bacterial strains did not exhibit any symptoms of disease, such as necrosis of any part of the plants, spots on the needles, needle distortion, cankers, or general decline.

Table 4.2. Bacterial strains and their closest homology.

Bacterial strain	Closest homology	S-ab score[a], Identity[b] Similarity[c]	Accession number
S32R2	*P. fluorescens* str. B67	99.79%[a]	EU169164.1
S22L11	*B. weihenstephanensis* str. WSBC 10204	100%[a]	AM747230.1
	B. mycoides CIP 103472	1.0[b]	AM747229
	B. simplex str. LMG 21002	1.0[b]	AJ628745.1
S11R41	*Brevibacterium frigoritolerans*; type strain DSM 8801	1.0[b]	AM747813
S31L1	*Rahnella* spp. str. CDC 21234	99.79%[a]	U88435.1
	R. aquatilis str. 334	99.04%[a]	X79940.1
	P. fluorescens; 2312	0.993[b]	EU360313
S11R21	*P. costantinii*	99%[c]	AF374472
	P. poae; zol-15; JQ782898	0.993[b]	JQ782898
S23L3	*E. billingiae* str. Eb661	99.93%[a]	FP236843
S31R1	*E. billingiae* str. Eb661	100%[a]	FP236843

[a], Ribosomal Database Project; [b], GenBank; [c], Greengenes.

4.3.3. *In vivo* biocontrol assay

A significant difference was observed among the bacterial treatments in the health status of the plants inoculated with *A. mellea* (χ^2 (10) = 44.2, $p < 0.001$), and a Cramer´s V of 0.323 indicated a medium-large effect size. Of the plants that were not treated with bacteria, 54% were not healthy (dead or had a lesion), and contributed 40.4% of the total number of dead plants in all treatments (Figure 4.5). In contrast, treatment with the biocontrol bacteria resulted in fewer dead plants. The proportion of dead plants within a bacterial treatment ranged from 3.6-13.1%. *E. billingiae* S23L3 and *B. simplex* S11R41 contributed the least (3.5% and 8.8%, respectively) to the total number of dead plants in all treatments. When the adjusted standardized residuals were examined, the group of plants that were not treated with bacteria had significant positive values in the dead category ($z = 5.2$; $p < 0.001$), meaning that more plants than expected by chance were dead and, significant negative values in the healthy category ($z = -5.2$; $p < 0.001$), meaning that fewer plants than expected were healthy. The plants treated with *B. simplex* S11R41 had significant positive values in the healthy category ($z = 2.6$; $p < 0.01$) and, significant negative values in the dead category ($z = 2.0$; $p < 0.05$). Plants treated with *E. billingiae* S23L3 had significant negative values in the dead category ($z = -2.6$; $p < 0.01$) but, significant positive values in the lesion category ($z = 2.1$; $p < 0.05$) (Figure 4.5). When present, lesion sizes were not significantly different among the treatments (data not shown).

Among the live plants infected with *A. mellea* significant differences in height (F (5, 308) = 5.64, $p < 0.05$) (Figure 4.6A), diameter (F (5, 312) = 2.58, $p < 0.05$) (Figure 4.6B) and SI (F (5, 308) = 3.19, $p < 0.05$) (Figure 4.6C) were found among the treatments. No significant differences were found among treatments for dry root weight (F (5, 286) = 0.53) (data not shown). Plants with no bacterial treatment had the lowest average height (47.9 cm ± 10.4) and treatment with any of the bacterial strains increased height by 19% on average (Figure 4.6A). Plants treated with *E. coli* had the smallest diameter average

(5.2 mm ± 1.2), and plants treated with *P. fluorescens* S32R2 had the highest (5.9 mm ± 1) (Figure 4.6B). The lowest SI average, thus the least robust plants, was for plants treated with *E. coli* (0.69 ± 0.12) and *E. billingiae* S23L3 (0.69 ± 0.19) (Figure 4.6C), and the highest for untreated plants (0.78 ± 0.17).

The plants infected with *H. annosum* presented significant differences in SI values (F (5, 200) = 2.28, $p < 0.05$) among the treatments (Figure 4.6C). Plants treated with *P. fluorescens* S32R2 had the lowest SI average (0.93 ± 0.15). Bacterial treatments did not result in significant differences in height (F (5, 200) = 1.99) (Figure 4.6A) or in diameter (F (5, 200) = 0.99) (Figure 4.6B).

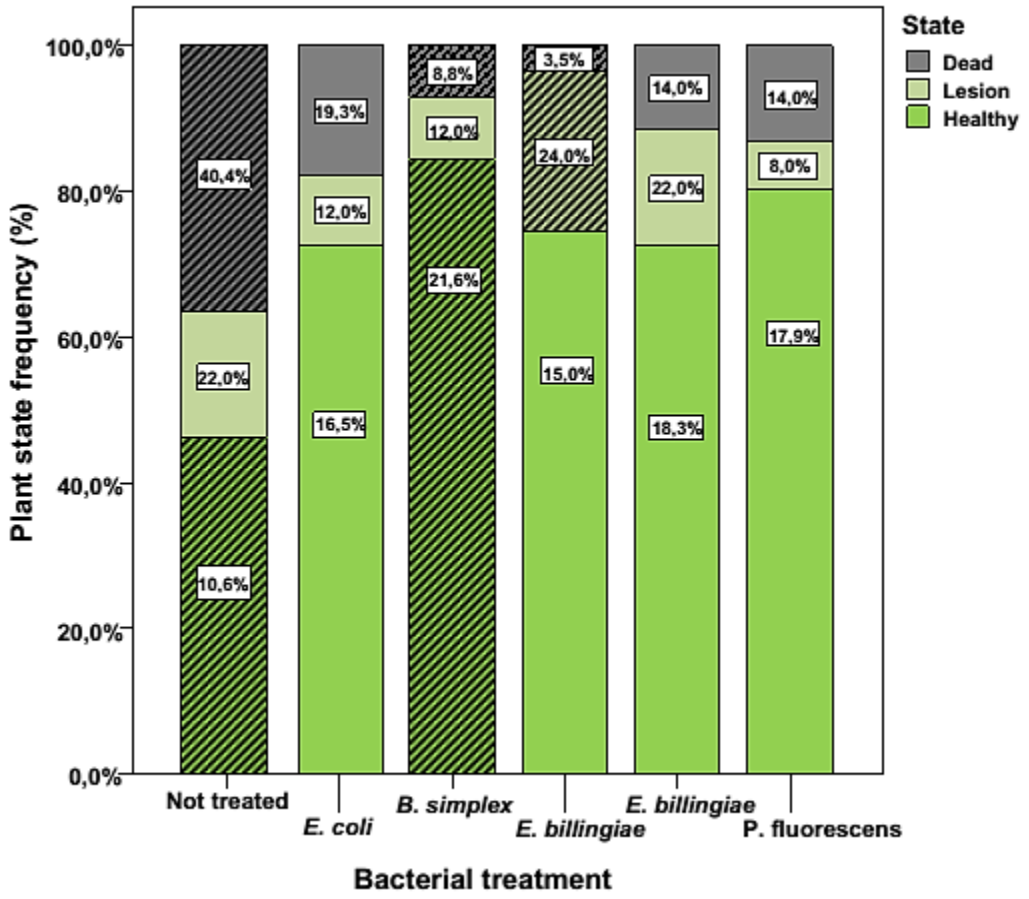

Figure 4.5. Dead, healthy or fungal lesion-containing pine seedlings inoculated with *A. mellea*. Counts are represented as percentage of the total number of plants within each bacterial treatment. The percentages shown in the boxes in each segment represent the contribution to the total number of plants in each state among all of the bacterial treatments. Darker segments indicate significant z scores ($p < 0.05$).

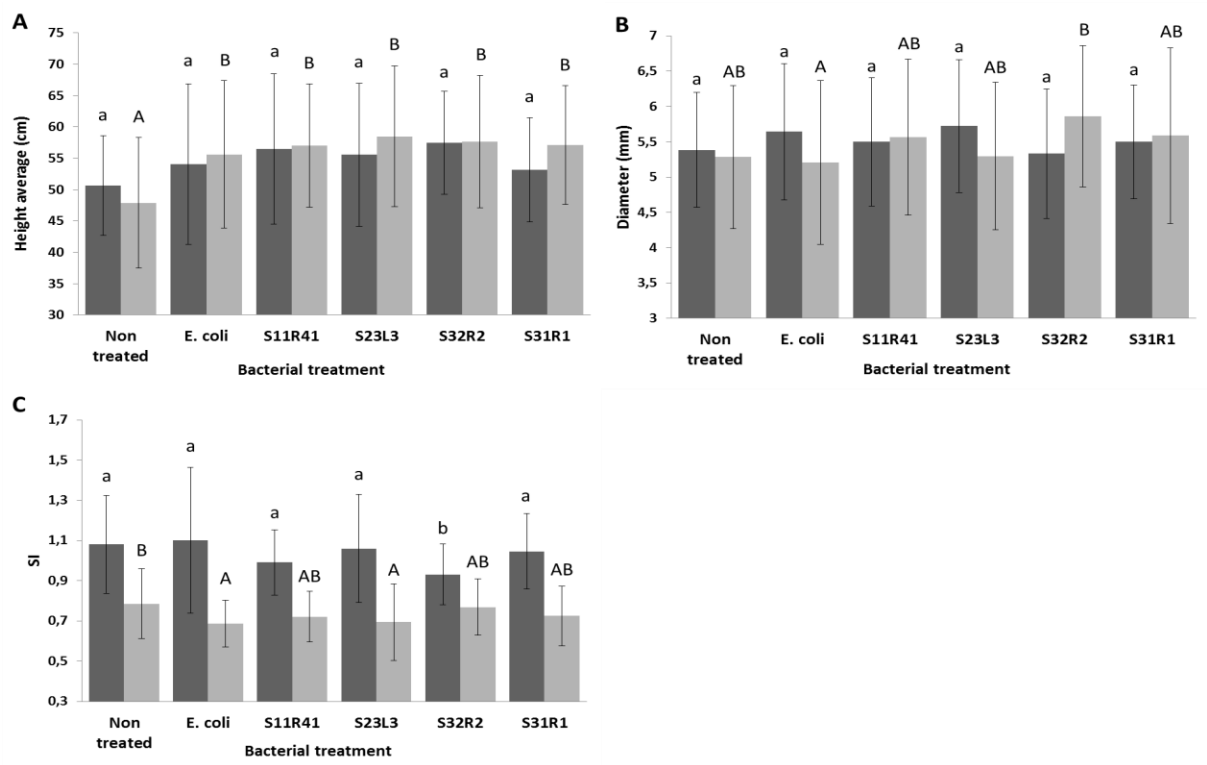

Figure 4.6. Height (A), diameter (B) and slenderness index (C) for seedlings inoculated with *H. annosum* (dark grey) or *A. mellea* (light grey) and treated with bacteria. Error bars show the standard deviation of the means. Statistically significant differences of $p < 0.05$ between treatments for *A. mellea* and *H. annosum* are presented with different lowercase or capital letters, respectively.

4.3.4. Detection of *H. annosum s.s.* infection

Symptoms of *H. annosum* infection were not apparent in any of the inoculated seedlings. To determine if the fungus was present in the seedlings, and if any of the bacterial treatments influenced this, DNA was extracted from root fragments and used as a template to amplify the *H. annosum* ITS region and the *P. radiata* actin gene. Products from PCR amplification of the *P radiata* actin gene were obtained in 57 of the 60 samples tested using different concentrations of DNA (Figure 4.7). This indicates that PCR was not inhibited by substances in the DNA extracted from plant roots. The samples and DNA concentrations with a positive result for the actin gene were tested for the presence of *H. annosum.* In this case, 43 samples yielded a specific amplification product (Figure 4.7). The percentage of positives in the groups treated with *P. fluorescens* S32R2, *B. simplex* S11R41, *E. billingiae* S23L3, and *E. billingiae* S31R1 were 40%, 55.5%, 70% and 90%, respectively. All of the actin-positive samples with *E. coli* or without bacterial treatment were positive for a *H. annosum*-specific ITS sequence. The same result was obtained in both experimental replications.

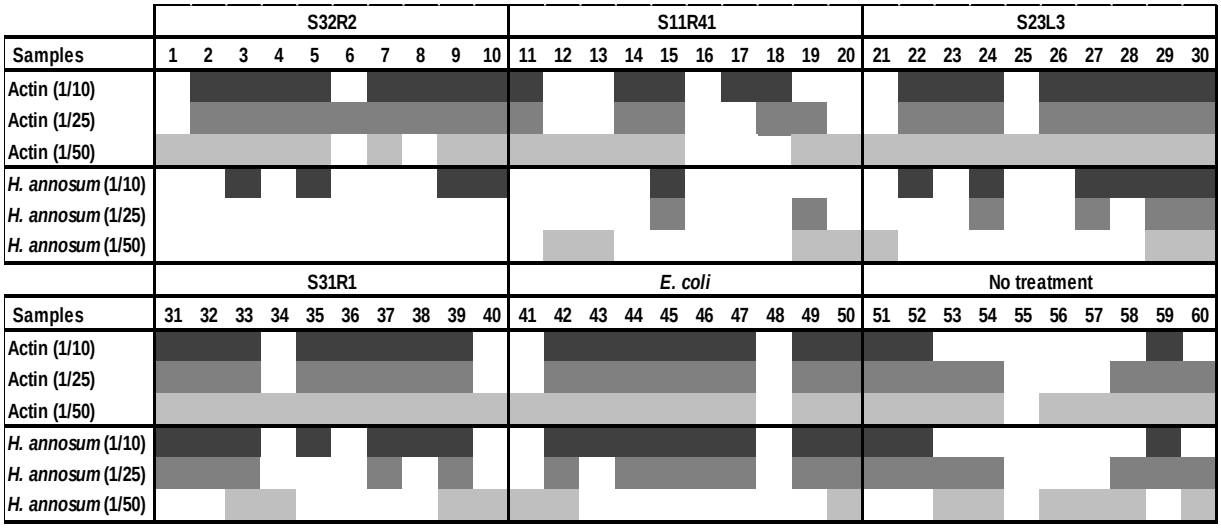

Figure 4.7. Detection of *H. annosum* in DNA extracted from infected *P. radiata* roots treated with biocontrol bacteria. A positive result for the *P. radiata* actin gene or the *H. annosum* ITS region is indicated as a shaded box (DNA dilution shown).

4.4. Discussion

In the present study, we report the isolation of native bacteria from a healthy tree in a *P. radiata* stand with high levels of fungal infection and the ability of some of these bacterial strains to inhibit the growth *in vitro* and the pathogenic effects *in planta* of two forest fungal pathogens, *H. annosum s.s.* and *A. mellea*. A few studies have described the antagonistic effects of biocontrol bacteria against forest fungal pathogens *in vitro*, although their ability to reduce fungal growth in trees was not always demonstrated. Dumas (1992) isolated bacteria from soils of the boreal mixed wood forest of Ontario that inhibited *A. mellea in vitro*, however, inhibition of infection in trees was not reported. Singh et al. (2008) reported the antagonistic effect of *B. subtilis* against *Macrophomina phaseolina* in a *Pinus* specie *(P. roxburghii* Sarg.), and *Phytophthora cinnamomi* infection was reduced in *F. sylvatica* and *Quercus* spp. treated with *Bacillus amyloliquefaciens* (Lefort et al., 2013). Murray and Woodward (2003) concluded that weight losses caused by *Heterobasidion* in spruce wood cubes were lower when they were simultaneously inoculated with *H. annosum* and biocontrol bacteria.

In our case, native biocontrol bacteria were isolated in anticipation of their greater potential for adaptation to the host and conditions under which they will be applied. The selected bacteria were not pathogenic for seedlings of *P. radiata* nor are *P. fluorescens*, *E. billingiae*, and *B. simplex* (*B. frigoritolerans*) known to be human or animal pathogens. Some strains of *P. fluorescens* are commercially available and exhibit effective rhizosphere colonization, plant growth promoting activity (Mark et al., 2006), and antifungal activities against crop pathogens due to the production of secondary metabolites such as 2,4-diacetylphloroglucinol, phenazine and pyoverdin (Boruah and Kumar, 2002). *E. billingiae* is epiphytic and has shown biocontrol activity against fire blight caused by *E. amylovora* (Jakovljevic et al., 2008). *B. simplex*, an endospore forming

bacteria (Heyrman et al., 2005), promoted the growth of tomato and wheat (Hassen and Labuschagne, 2010) and showed antifungal effects against *Fusarium oxysporum in vitro* (Schwartz et al., 2013).

P. fluorescens S32R2 and *B. simplex* S11R41 were strong antagonists of *H. annosum* and *A. mellea in vitro*, and performed best against both fungal strains *in vivo*. *P. fluorescens* S32R2 reduced *H. annosum* growth by 99% *in vitro* and fungal presence by 60% in *P. radiata* seedlings. *B. simplex* reduced *H. annosum* growth to a lesser extent (68%) *in vitro*, but reduced the presence of the fungus in pine seedlings by 50%. On the other hand, *E. billingiae* strains S31R1 and S23L3 performed strongly against *H. annosum in vitro* (99% and 81% AIP, respectively), but reduced the presence of the fungus by only 10% and 30%, respectively, *in vivo*.

In the *in vitro* antagonism assay against *A. mellea*, *P. fluorescens* S32R2 had the highest antifungal activity (94.2% AIP). As a plant treatment, this bacterial strain showed a similar antagonistic effect as *B. simplex* S11R41, (80.3% and 84.3% of the plants, respectively, were healthy following fungal infection compared with 46% of untreated plants), although *B. simplex* S11R41 was less inhibitory *in vitro* (83.2% AIP). When compared with plants that were not treated with bacteria, antagonistic effects were detected for *E. coli* in the pines inoculated with *A. mellea*; 73% of the plants were healthy following fungal infection. This was surprising because *E. coli* did not inhibit *A. mellea in vitro* (-21% AIP). The consistent (Singh et al., 2008) or inconsistent (Coombs et al., 2004; Inderiati and Franco, 2008) relationship between the antagonistic effect of biocontrol bacteria *in vivo* and *in vitro* has been previously reported and, in general, is related to plant characteristics and environment (Tolba and Soliman, 2013).

The number of plants with *A. mellea* lesions was smaller in the seedlings treated with *B. simplex* S11R41 (8.6%) and *P. fluorescens* S32R2 (6.6%) compared with 17.5% of plants with lesions for the untreated controls. Although the size of fungal lesions, when present, was not different among the treatments, fewer plants treated with bacteria died. Bacterial treatment may have a systemic protective effect, but not a strong antagonistic effect once the fungal infection is established.

When plant growth in the presence of *A. mellea* was analyzed, a significant difference was detected in the height, diameter and SI among the treatments. Plants treated with *P. fluorescens* S32R2 had the highest values in height and diameter. On the other hand, untreated plants and plants treated with *E. coli* had the lowest values in height and diameter, respectively, and SI values were higher for the untreated plants (0.78) and smaller for the plants treated with *E. coli* (0.69). A significant effect on height or diameter was not detected among bacterial treatments for plants treated with *H. annosum*, but *P. fluorescens* treatment had the highest value for plant height and the smallest for diameter, resulting in a significant decrease of SI. The mean values of SI were consistently different between the seedlings inoculated with *H. annosum* (1.04 ± 0.25) and those inoculated with *A. mellea* (0.73 ± 0.15). SI values of 1 are considered normal for *P. ponderosa* seedlings (Olivo and Buduba, 2006), which is similar to the SI

values of *P. radiata* seedlings treated with *H. annosum* in this study. This suggests that the severity of infection was greater for *A. mellea*, and although *H. annosum* was present, the infection was not sufficient to cause disease symptoms in any of the treatments at the time of the assessment.

4.4.1. Conclusions

The isolated bacterial strains selected on the basis of an antibiosis effect on the fungal pathogens *H. annosum s.s.* and *A. mellea in vitro* reduced the pathogenic effects of *A. mellea* and the presence of *H. annosum* in young *P. radiata* trees. Future studies are needed to understand the mechanisms of antibiosis and bacterial-fungal interactions in other tree species. In addition, the effects of the bacteria on other beneficial rhizobacteria and mycorrhizal fungi in the rhizosphere community, and the ability of the biocontrol bacteria to compete effectively with indigenous microorganisms must be assessed, although they are native to *P. radiata* roots and therefore expected to be well-adapted to the conditions under which they will be applied. Nonetheless, the biocontrol bacteria characterized here show promise as a treatment to mitigate the damage by devastating pine pathogens against which there are few options available.

CHAPTER 5

General discussion and conclusions

5.1. Discussion: from a management perspective

In the present study, the distribution of *Armillaria* and *Heterobasidion* in the Basque Country, and the environmental factors associated with both fungal complexes, were described. The species and population diversity of both genera in selected plantations and native forests were determined, and host range in the field and host susceptibility to *A. mellea* under greenhouse conditions were established. The results contribute to a better understanding of the epidemiology of these forest pathogens. In addition, bacteria native to the *P. radiata* rhizosphere that are able to reduce pathogenic effects of *A. mellea* and *H. annosum s.s* in young *P. radiata* trees were isolated and characterized. The compiled information will facilitate the development of management strategies, especially in areas of the Basque Country where the problem of replanting forests infested by diverse native and exotic pathogens is endemic.

Armillaria spp. are broadly distributed in the Basque Country and their host range includes coniferous and deciduous trees. All the main species present within the genus *Armillaria* can behave as primary pathogens which, added to their broad distribution and host range, result in a high risk of damage for tree plantations and native forests in this region. Although *Armillaria* was frequently detected in the studied native and plantation forests, control measures have been restricted to urban trees and recreational parks, and implemented to prevent civilian and structural damage that may be caused by instability of affected trees.

The control of *Armillaria* complex in forests is more difficult as it is distributed over a wider area, often in areas that are difficult to access. The best way to reduce the vigor of the fungus, which is strongly dependent on the availability of food sources, is by pulling out infected stumps and roots. However, this measure can also stimulate rhizomorphs formation (Fox, 2000), and disrupt beneficial microbial populations (Butin, 1995), which may act as a natural control of pathogens (Mesanza et al., 2016). Fungicidal

treatments can kill the fungus in the soil; however, the economic and environmental costs are high.

In typical modern plantations, control measures are probably economically justified if mortality from *Armillaria* spp. is severe early in the previous rotation. It is therefore important to keep good stand records that will point out the impact of different factors when a decision may be necessary prior to planting a new species. In comparison with natural forests, the damage in exotic tree plantations is usually greater (Guillaumin and Legrand, 2013). The composition of the stand can also change the range of infection; lower density of susceptible species, e.g. *P. radiata*, and higher species diversity in different forest strata reduce the possibility of disease transmission (Gerlach et al., 1997; Kromroy et al., 2005).

H. annosum s.s was the only species within the genus *Heterobasidion* present in the sampled forests. Its presence was limited only to conifers and it was less abundant than *Armillaria* spp.; however, *H. annosum s.s* could pose a high risk for tree plantations and native forests due to its presence in a broad range of coniferous species, inherent ability to cause damage to deciduous trees, and generation of new genets which could increase its pathogenicity.

H. annosum s.s. presence in the Basque Country has additional implications for management of the Atlantic coniferous ecosystem of Spain. In areas where *Fusarium circinatum* Niremberg & O'Donnell has previously been detected (Collar Urquijo, 1995; Landeras et al., 2005; Iturritxa et al., 2011) stands of *Pinus* spp. are currently being replaced by other conifers such as *Picea* spp. (spruces), *C. lawsoniana* (Lawson cypress) and *P. menziesii* (Douglas fir) (EFSA, 2010; Aegerter and Gordon, 2006; Gordon et al., 2006; Gordon et al., 2001) which are hosts for *H. annosum s.s.* (Mesanza and Iturritxa, 2012). Established management recommendations must be revised for areas where both *F. circinatum* and *H. annosum* could be present.

Evidences of the importance of basidiospores in the dispersion of *H. annosum s.s* in the studied areas were found in this study. In general, infection by basidiospores of recently generated stumps and injuries in living trees is an important dispersion mechanism to new areas (Kallio, 1970). The risk of infection is higher when basidiospore production is at its maximum. In the southwest of France, Lung-Escartman et al. (2008) observed that the maximum production of *H. annosum s.s.* basidiospores was in spring and Gonthier et al. (2005) observed a maximum production in autumn in the western Alps; thus, they recommend thinning and logging operations in winter (Isomäki and Kallio, 1974). In the infected forests designated for regeneration, the use of tolerant tree species is recommended. Korhonen et al. (1992) recommended the use of *Betula pendula* Roth in the stands infected with *H. annosum s.s.*

In general, early detection of disease and treatment with effective biological antagonists, and planting tolerant tree species are recommended for plantations (Schwarze, 2008). Even if action is not taken, forest owners should be aware of the presence of the fungi, especially if there is a chance that a change of management

practice could inadvertently lead to an increase in disease impact. Management procedures such as selective logging, early thinning and/or the continued use of susceptible or moderately susceptible species that are not very well adapted to the location, increase the inoculum sources, and thereby, increase the probability of infection (Bloomberg and Morrison, 1989; Morrison et al., 1991; Hood and Sandberg, 1993a; Hood and Kimberly, 2009). Monitoring forests for early detection would be facilitated by an understanding of the stand environmental characteristics that increase the risk of *Armillaria spp.* and *H. annosum s.s.* infection. In this study, *Armillaria* spp. were more frequently detected in stands with 20-30% slopes, with a westerly orientation, deciduous forests, acid soils with high permeability, and rainfall average values above 1800 mm. *H. annosum s.s.* was more frequently detected in stands with temperature averages below 11.5 ºC, rainfall average values below 1000 mm, 30-40% slopes, north or southeast orientations, coniferous forest with moderately basic or basic and impermeable soils, and where trees had optimum growth conditions.

Finally, there is a need for safe and effective preventions and treatments for these fungal diseases. The isolated biocontrol bacteria, which are native to the region, reduced the incidence of *H. annosum* and *A. mellea* infection on *P. radiata*. Iturritxa et al. (2017) also reported the ability of these bacteria to reduce the length of lesions caused by *F. circinatum* in young *P. radiata* trees. However, as commented in Chapter 4, before these biocontrol agents can be applied, future studies are needed to understand the mechanisms of antibiosis and bacterial-fungal interactions in other tree species, and the effects of the bacteria on other beneficial rhizobacteria and mycorrhizal fungi in the rhizosphere community. The ability of the biocontrol bacteria to reduce the incidence of other *Armillaria* spp. or to compete with indigenous microorganisms must also be assessed. As part of an integrated management strategy, an early application of antagonistic bacteria in the nursery is proposed. Early application is advantageous for several reasons, the volume of bacteria needed is lower and can be applied under controlled conditions, the bacteria have time to colonize and adapt to the rhizosphere conditions, and consequently, the seedlings are protected before they are in contact with the fungi.

5.2. Conclusions

1. *Armillaria* spp. were broadly distributed in the Basque Country and their host range includes coniferous and deciduous trees.

2. All the main *Armillaria* species present in the Basque Country can behave as primary pathogens which, added to their broad distribution and host range, results in a high risk of damage for tree plantations and native forests.

3. *H. annosum s.s* was the only species detected within *Heterobasidion* genus. Its presence was limited only to conifers and it was less abundant than *Armillaria* spp.

4. *H. annosum s.s* could pose a high risk for tree plantations and native forests due to the broad range of coniferous species in which it was present and its inherent ability to cause damage to deciduous trees and to generate new genets.

5. The presence and absence of both genera in the surveyed areas were significantly associated to different environmental factors such as stand slope and orientation, rain and temperature averages, soil permeability and acidity, and host type.

6. In the studied stands *H. annosum s.s.* dispersion was mainly by basidiospores.

7. *Armillaria* spp. dispersion patterns were variable between stands and species, and could be affected by moist and temperature.

8. *P. radiata* was the most susceptible species to *A. mellea* in greenhouse conditions with a greater number of infected plants and *P. nigra* had the highest average lesion size.

9. Monocultures of highly susceptible tree species should be avoided in order to prevent an increment in damage risk.

10. The isolated bacterial strains, selected on the basis of an antibiosis effect on the fungal pathogens *H. annosum s.s.* and *A. mellea in vitro*, reduced the pathogenic effects of *A. mellea* and the presence of *H. annosum* in *P. radiata* seedlings.

11. The biocontrol bacteria characterized here show promise as a treatment to mitigate the damage by devastating pine pathogens against which there are few options available.

Resumen

En los bosques de las regiones templadas, los hongos de pudrición de cuello y de raíz están considerados entre las causas más importantes de pérdidas económicas. Este grupo de hongos está mayoritariamente formado por tres géneros con distribución mundial: *Armillaria* (Fr.) Staude, *Heterobasidion* Bref. y *Phellinus* Quél., todos ellos dentro de la clase Agaricomycetes (Garbelotto, 2004). Esta tesis se ha centrado en dos de ellos, *Armillaria* y *Heterobasidion.*

En Europa, se tiene constancia de siete especies de *Armillaria*: *A. mellea* (Vahl) P.Kumm., *A. gallica* Marxm. & Romagn., *A. ostoyae* (Romagn.) Herink, *A. tabescens* (Scop.) Emel, *A. cepistipes* Velen., *A. borealis* Marxm. & Korhonen, and *A. ectypa* (Fr.) Lamoure (Guillaumin et al., 1993; Pérez-Sierra and Henricot, 2002). Muchas de ellas son agentes patógenos de un amplio rango de árboles, matorrales y algunas plantas herbáceas (Williams et al., 1989). *A. mellea* y *A. ostoyae* generalmente se comportan como patógenos primarios, aunque también pueden ser saprófitos, siendo su rango de hospedadores muy amplio, incluyendo coníferas y arboles caducifolios, aunque *A. mellea* se considera menos patogénica para coníferas que *A. ostoyae*. El rango de hospedadores para *A. gallica* es también elevado y se puede comportar como oportunista y/o patógeno primario. *A. tabescens* se comporta mayormente como saprófito aunque se ha detectado puntualmente como parasito primario u oportunista. La patogenicidad de *A. cepistipes* y *A. borealis* se considera baja y el papel de *A. ectypa* es incierto (Guillaumin et al., 1993; Coetzee, 2003; Ainsworth, 2003; Ohenoja, 2006; Kim et al., 2017). Los síntomas de pudrición causados por especies de *Armillaria* son generales, como hojas cloróticas, aclaramiento progresivo de la copa, crecimiento principal más lento y producción excesiva de piñas, éstos pueden estar acompañados por micelio subcortical en forma de abanico, rizomorfos, grupos de basidiocarpos con coloración dorada – amarronada cerca de la base del árbol, madera con pudrición fibrosa- amarillenta y pseudoesclerotia, muerte rápida del hospedador sin pérdida de follaje, y/o exudados en la parte basal del hospedador (Cox et al., 2005; Edmonds et al., 2000).

En general, las coníferas se consideran más susceptibles a la enfermedad que los árboles caducifolios, y el daño causado en plantaciones forestales de hospedadores exóticos suele ser mayor que en bosques nativos. Pero, dado que la susceptibilidad y la extensión del daño causado están determinados por numerosos factores como la especie de *Armillaria* implicada, el vigor del hospedador, interacción con otros patógenos, clima y manejo de la plantación, entre otros (Kile, 1983; Mallett and Volney 1990; Entry et al., 1991; Mallett and Maynard, 1998; Popoola and Fox, 2003; Hood and Kimberley, 2009) la susceptibilidad de especies arbóreas y el daño causado no se pueden generalizar (Wargo and Shaw III, 1985) En condiciones de campo, *Armillaria* puede colonizar diferentes hospedadores por medio del contacto directo entre una fuente infectada y raíces por medio de micelio, o por medio de rizomorfos que se pueden desplazar por el suelo (Redfern and Filip, 1991). La capacidad de crear nuevos focos de infección por basidioesporas varía entre especies (Kliejunas, 2011), aunque en general parece ser

menos frecuente que las vías de infección mencionadas anteriormente (Baumgartner and Coetzee, 2011).

En Europa se tiene constancia de cuatro especies dentro del complejo de especies *H. annosum* (Fr.) Bref. (*H. annosum s.l.*): *H. irregulare* (Underw.) Garbel. & Otrosina de origen norteamericano, y *H. annosum sensu stricto* (*s.s.*), *H. abietinum* Niemela & Korhonen, y *H. parviporum* Niemela & Korhonen, de origen euroasiático (Niemela and Korhonen, 1998; Otrosina and Garbelotto, 2010). Las coníferas son el principal hospedador de *H. annosum s.l.* y la mayoría de coníferas se han citado como susceptibles a este complejo de especies (Asiegbu et al., 2005). Las diferentes especies de *H. annosum s.l* demuestran diferentes grados de especialización respecto a su hospedador lo que define su distribución. *H. abietinum* se ha encontrado principalmente en *Abies alba* Mill., aunque también en otras especies dentro del género *Abies* Mill., y en especies de *Chamaecyparis* Spach, *Juniperus* L., *Larix* Mill., *Pinus* Linn., *Castanea* Mill., *Fagus* L., y en *Pseudotsuga menziesii* (Mirb.) Franco y *Cryptomeria japonica* (Thunb. ex L.f.) D.Don. (Korhonen et al., 1998). *H. parviporum* es considerado altamente especializado y patogeno primario de *Picea abies* (L.) Karst. (Asiegbu et al., 2005) aunque también puede causar la muerte de especies nativas o exóticas dentro del género *Pinus* Linn., entre otros (Korhonen et al., 1998; Sedlák and Tomšovský, 2014). *H. annosum s.s.* es considerado un patógeno generalista primario, y es la especie europea con una mayor rango de especies de hospedador, incluyendo coníferas y arboles deciduos, aunque *P. sylvestris* L. y en general especies dentro del género *Pinus* son las más susceptibles a este patógeno (Korhonen et al., 1998; Asiegbu et al., 2005).

La infección de *H. annosum s.l.* se establece por dos mecanismos, colonización por micelio formado de basidioesporas o por contacto entre estructuras infectadas y no infectadas como raíces, siendo la primera la más frecuente. El micelio procedente de basidioesporas puede colonizar madera libre de corteza, como superficies recientemente cortadas y lesiones de raíz (Kallio, 1970; Stenlid, 1994, Rayner et al., 1987). Cuando la infección está establecida, los síntomas aéreos de los hospedadores infectados son hojas cloróticas, marchitez, muerte de árboles en grupos o aislados y árboles derribados por el viento con raíces podridas; éstos son genéricos y no tienen valor diagnostico (Tainter and Baker, 1996). Así, la presencia de basidiocarpos es el criterio de detección más fidedigno en campo (Sinclair et al., 1987).

En el País Vasco la especie forestal más común en plantaciones es *Pinus radiata* D.Don., seguida por diferentes especies de *Eucalyptus* L'Hér., *P. nigra* Arn., especies de *Larix* Mill., *P. pinaster* Ait., *P. menziesii*, *Chamaecyparis lawsoniana* (A. Murray) Parl., *Quercus rubra* L. y *Picea abies* (L.) Karst. El bosque nativo está compuesto mayoritariamente por *Fagus sylvatica* L., *Q. faginea* Lam., *Q. ilex* L., *Q. robur* L., *Q. petraea* (Matt.) Liebl., y *Q. pirenaica* Willd. Todas estas especias forestales están documentadas como susceptibles a diferentes especies de *Armillaria;* y diferentes especies de *Heterobasidion* se han encontrado en muchas de ellas, incluyendo algunas especies de *Quercus* (Korhonen et al., 1998; Asiegbu et al., 2005; Doğmuş-Lehtijärvi, et al., 2015). Sin embargo, se necesitaba un estudio en detalle con el fin de ofrecer más información para

estrategias de manejo. Así los objetivos de este estudio fueron los siguientes: i) determinar la distribución de *Armillaria* y *Heterobasidion* en el País Vasco; ii) determinar la diversidad de especies presentes y diversidad de poblaciones; iii) establecer el rango de hospedadores y la susceptibilidad de los mismos; iv) identificar factores ambientales que pueden afectar su distribución; y v) entender patrones y mecanismos de dispersión en áreas específicas.

Las prácticas de manejo existentes para *Armillaria* y *Heterobasidion* no son siempre efectivas, por lo tanto para complementarlas y considerando la importancia de *P. radiata* en la industria maderera del País Vasco, se propone aislar y caracterizar la habilidad de ciertas bacterias nativas de la rizosfera de *P. radiata* para reducir los efectos patogénicos causados por *A. mellea* y *H. annosum s.s.*, y así en un futuro poder usar dichas bacterias como un tratamiento profiláctico desde viveros.

El género *Armillaria* se encontró ampliamente distribuido en todo el País Vasco (en 248 (34.97%) de los 709 focos de enfermedad muestreados) y su rango de hospedadores comprendía tanto coníferas como frondosas. Por el contrario, *H. annosum* no se encontró tan abundantemente (16.5% (117) de los focos de enfermedad muestreados) y su rango de hospedadores estaba limitado a coníferas. Ambos géneros se encontraron tanto en bosques nativos como en plantaciones. Aunque el género *Armillaria* estaba presente en áreas con características medioambientales muy diversas, fue detectado más frecuentemente en zonas con pendientes del 20-30%, orientación oeste, bosque de frondosas, suelos ácidos con permeabilidad alta y valores de precipitación media por encima de 1800 mm; fue detectado menos frecuentemente en áreas con inclinación menor del 10%, orientación sudoeste, valores de precipitación media inferiores a 1000 mm, bosques de coníferas, y suelos moderadamente ácidos de permeabilidad media o impermeables. *Heterobasidion* se detectó más frecuentemente en zonas de temperaturas medias inferiores a 11.5ºC, valores de precipitación media inferiores a 1000 mm, pendientes del 30-40%, orientación norte o sudeste, coníferas y suelos impermeables, moderadamente básicos o básicos donde los hospedadores tienen condiciones de crecimiento óptimo. *Heterobasidion* se detectó menos frecuentemente en zonas con temperaturas superiores a 12.5ºC y valores de precipitación media superiores a 1800 mm con orientación sudoeste u oeste, pendientes del 10-20% o del 40-50%, y suelos moderadamente ácidos.

Todos los aislados correspondientes al género *Heterobasidion* fueron identificados como la especie europea *H. annosum s.s.* y se encontraron en especies de hospedadores como, *C. lawsoniana* (11.1% de las parcelas con presencia de *Heterobasidion*), *P. nigra* (2.3%), *P. pinaster* (2.2%), *P. radiata* (42.2%), *P. menziesii* (17.7%) y *P. abies* (6.7%). Ademas, *H. annosum* era evidente en bosque nativo de *P. sylvestris* (17.8%). *H. annosum s.s.* también se encontró en *Sequoia sempervirens* (D. Don) Endl. No se encontraron síntomas de enfermedad en plantaciones de *Sequoiadendron giganteum* (Lindl.) J. Buchholz, *L. kaempferi* (Lamb.) Carr. y *L. decidua* Mill. a pesar de que estas especies son consideradas hospedadores potenciales (Chase, 1985; Korhonen et al., 1998; Otrosina and Garbelotto, 2010). En general, *H. annosum s.s.* se comporta como necrótrofo y aunque tiene

preferencia por especies de pino se puede encontrar en un amplio rango de hospedadores incluyendo frondosas (Garbelotto and Gonthier, 2013).

El género *Armillaria* se encontró en *P. radiata*, *P. nigra*, *P. pinaster*, *Q. robur*, *Q. pirenaica*, *Fraxinus excelsior* L., *Alnus glutinosa* (L.) Gaertn., *F. sylvatica*, *E. globulus* Labill., *P. abies*, *L. kaempferi*, *P. sylvestris*, *Populus alba* L., *P. menziesii*, *Q. faginea*, *Q. ilex*, *Robinia pseudoacacia*, y *C. lawsoniana*. Las especies de *Armillaria* identificados en los focos de infección fueron, *A. ostoyae* (representando el 60% de todas las muestras identificadas), *A. mellea* (24%), *A. gallica* (14%), *A. tabescens* (1%) y *A. cepistipes* (1%). *A. ostoyae* se detectó mayoritariamente en *Pinus* spp. (*P. radiata*, *P. nigra* y *P. pinaster*). El rango de hospedadores de *A. mellea* era más variado. El patrón 1 de *A. mellea* (tamaño de los fragmentos de PCR: 320 y 155 pb) se encontró en *P. radiata*, *Quercus* sp., *F. excelsior*, y *C. lawsoniana*, y correspondió al 53% de los aislados de *A. mellea*, el resto (47%) se identificó como *A. mellea* patrón 2 (tamaño de los fragmentos de PCR: 320, 180, y 155 bp) y se encontraron en *Q. pyrenaica* y *P. radiata*. *A. gallica* se encontró en *A. glutinosa*, *P. radiata* y *Q. robur*, *A. cepistipes* en *P. radiata*, y *A. tabescens* en *Q. robur*.

En general, *A. mellea* y *A. ostoyae* se pueden comportar como patógenos primarios de un amplio rango de hospedadores incluyendo coníferas y frondosas (Guillaumin et al., 1993; Coetzee, 2003). El rango de hospedadores para *A. gallica* es también amplio y puede comportarse como oportunista y/o patógeno primario especialmente cuando el hospedador está estresado (Skovsgaard et al., 2010; Kim et al., 2017).

La diversidad de poblaciones de ambos géneros a nivel intraespecie se determinó en una selección de parcelas del País Vasco que presentaban niveles altos de infección. En todas las parcelas seleccionadas para *H.annosum s.s* se observó un número elevado de diferentes grupos de compatibilidad somática de extensión reducida, lo que se relaciona con una dispersión del hongo predominantemente por esporas (Swedjemark and Stenlid, 2001). En las áreas seleccionadas para el muestreo de *Armillaria* se encontraron grupos de compatibilidad somática de extensión más amplia, lo que se relaciona con una dispersión del hongo por medio de rizomorfos y por contacto directo entre una fuente infectada y raíces por medio de micelio, pero también se detectaron grupos de compatibilidad somática de extensión reducida, lo que se relaciona con una dispersión del hongo por esporas (Kile, 1983).

Debido a que *A. mellea* fue la especie con diversidad de hospedadores más amplia se seleccionó para determinar la susceptibilidad de diferentes especies de árboles presentes en el Pais Vasco. Todas las especies testadas (*P. radiata*, *P. nigra* subsp. *salzmannii* var. corsicana, *P. sylvestris*, *F. sylvatica*, *Prunus avium* L., *Q. petraea*, *Q. ilex*, *C. japonica*, *Q. robur*, *S. giganteum* y *Eucalyptus nitens* H.Deane & Maiden) fueron susceptibles a *A. mellea* menos *C. japonica*. La especie más susceptible a *A. mellea* fue *P. radiata*. Debido a que este ensayo se realizó en plántula joven y en condiciones de invernadero los resultados deberían interpretarse con cautela.

Debido a la importancia de *P. radiata* en la industria maderera del País Vasco y su susceptibilidad a ambos patógenos fúngicos se aislaron bacterias nativas de la rizosfera de un árbol sano (*P. radiata)* localizado en una parcela con altos niveles de daño causados por *Armillaria* y *Heterobasidion*. El propósito era aislar bacterias con capacidad antagónica hacia ambos géneros de hongos. Habitualmente las bacterias nativas demuestran un alto potencial de adaptabilidad hacia el hospedador y las condiciones en las cuales se aplicarán. Las bacterias seleccionadas demostraron alta capacidad inhibitoria *in vitro. P. fluorescens* S32R2 inhibió el crecimiento de *A. mellea* en un 94.2% con respecto al control y en un 99% el crecimiento de *H. annosum s.s.* La inhibición por *B. simplex* S11R41 fue de 83.2% y 68% para *A. mellea* y *H. annosum s.s.*, respectivamente. El porcentaje de inhibición por las cepas de *Erwinia billingiae* S31R1 and S23L3 fue muy alto para *H. annosum s.s.* (99% y 81%, respectivamente). Una vez comprobado que las bacterias no eran patógenas para otros organismos como plantas y animales, se aplicaron en raíces de plántulas de *P. radiata*, que posteriormente se inocularon con *A. mellea* y *H. annosum s.s.* Una vez transcurridos 110 días se midió el efecto en la salud de las plantas inoculadas con las bacterias seleccionadas con respecto a plantas inoculadas con bacterias control (sin efecto antagónico *in vitro* sobre los hongos patógenos). Así, *P. fluorescens* S32R2 y *B. simplex* S11R41 tuvieron el mayor efecto antagónico para *A. mellea* (80.3% y 84.3% de las plantas, respectivamente, estaban sanas en comparación con el 46% de las plantas no tratadas). Iturritxa et al. (2017) también informaron sobre el efecto antagónico de estas bacterias sobre *Fusarium circinatum* Nirenberg & O'Donnell en plantas de *P. radiata*.

En conclusión, el género *Armillaria* estaba ampliamente distribuido en el País Vasco con un rango de hospedadores muy amplio incluyendo coníferas y frondosas. Las especies principales encontradas, *A. mellea*, *A. ostoyae* y *A. gallica*, pueden comportarse como patógenos primarios, por lo que podrían suponer un riesgo alto para plantaciones y bosques nativos. *H. annosum s.s* fue la única especie dentro del género *Heterobasidion* encontrada en el País Vasco. Su presencia estaba limitada a coníferas y era menos abundante que el género *Armillaria*. Se concluyó que en las parcelas estudiadas la dispersión de *H. annosum s.s* podría darse mayoritariamente por basidioesporas. Debido a la capacidad de *H. annosum s.s* de colonizar un amplio rango de hospedadores y de formar nuevos individuos, esta especie podría suponer un riesgo para las plantaciones de coníferas del País Vasco. En el caso de *Armillaria* se observó dispersión por basidioesporas y por formas vegetativas.

En cuanto a las bacterias nativas aisladas se necesitan más estudios para entender los mecanismos de antibiosis e interacciones hongo-bacteria en otras especies de árboles, así como su efecto sobre rizobacterias y micorrizas beneficiosas de la rizosfera, y su habilidad por competir con microorganismos indígenas, aunque siendo nativas de la rizosfera de *P. radiata* se espera que estén adaptadas a las condiciones en las que se pretenden aplicar. En cualquier caso, las bacterias de control biológico caracterizadas en este estudio, muestran resultados prometedores y podrían usarse en un futuro como una medida profiláctica aplicada en plántula desde los viveros.

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Appendix

Pearson´s chi square test results for the association among *Heterobasidion* spp. and environmental variables.

Characteristics	Category code	*Description	Detection of Heterobasidion disease	
			Negative (Het-)	Positive (Het+)
Slope (%)	<10	Count	162	37
		Expected frequency	166,1607898	32,83921016
		Adjusted residuals	-0,936870843	0,936870843
	10-20	Count	168	22
		Expected frequency	158,6459803	31,35401975
		Adjusted residuals	2,136748322	-2,136748322
	20-30	Count	169	31
		Expected frequency	166,9957687	33,00423131
		Adjusted residuals	0,450598272	-0,450598272
	30-40	Count	57	25
		Expected frequency	68,46826516	13,53173484
		Adjusted residuals	-3,628044438	3,628044438
	40-50	Count	30	1
		Expected frequency	25,88434415	5,115655853
		Adjusted residuals	2,036380488	-2,036380488
	50-60	Count	6	1
		Expected frequency	5,844851904	1,155148096
		Adjusted residuals	0,158761225	-0,158761225
Orientation	N	Count	51	18
		Expected frequency	57,6135402	11,3864598
		Adjusted residuals	-2,257537815	2,257537815
	NE	Count	55	17
		Expected frequency	60,11847673	11,88152327
		Adjusted residuals	-1,714432288	1,714432288
	E	Count	71	14
		Expected frequency	70,97320169	14,02679831
		Adjusted residuals	0,008346822	-0,008346822
	SE	Count	40	25
		Expected frequency	54,27362482	10,72637518
		Adjusted residuals	-5,004379471	5,004379471
	S	Count	85	18
		Expected frequency	86,00282087	16,99717913
		Adjusted residuals	-0,287928072	0,287928072
	SW	Count	149	10
		Expected frequency	132,7616361	26,23836389
		Adjusted residuals	3,938926991	-3,938926991
	W	Count	110	8
		Expected frequency	98,52750353	19,47249647
		Adjusted residuals	3,116296395	-3,116296395
	NW	Count	31	7
		Expected frequency	31,72919605	6,270803949
		Adjusted residuals	-0,327572035	0,327572035

Characteristics	Category code	*Description	Detection of Heterobasidion disease	
			Negative (Het-)	Positive (Het+)
Tree Type	Deciduous	Count	64	0
		Expected frequency	53,43864598	10,56135402
		Adjusted residuals	3,728767673	-3,728767673
	Conifers	Count	528	117
		Expected frequency	538,561354	106,438646
		Adjusted residuals	-3,728767673	3,728767673
Soil	acid	Count	378	66
		Expected frequency	370,7306065	73,26939351
		Adjusted residuals	1,52019509	-1,52019509
	-acid	Count	141	8
		Expected frequency	124,4118477	24,58815233
		Adjusted residuals	4,119328586	-4,119328586
	-basic	Count	36	27
		Expected frequency	52,60366714	10,39633286
		Adjusted residuals	-5,903819254	5,903819254
	basic	Count	37	16
		Expected frequency	44,2538787	8,746121298
		Adjusted residuals	-2,790589804	2,790589804
Permeability	Impermeable	Count	19	14
		Expected frequency	27,55430183	5,445698166
		Adjusted residuals	-4,108372567	4,108372567
	Low	Count	9	0
		Expected frequency	7,514809591	1,485190409
		Adjusted residuals	1,342231509	-1,342231509
	Medium	Count	452	87
		Expected frequency	450,0535966	88,94640339
		Adjusted residuals	0,461242775	-0,461242775
	High	Count	112	16
		Expected frequency	106,877292	21,12270804
		Adjusted residuals	1,347479362	-1,347479362
HOC	--Op	Count	30	3
		Expected frequency	27,55430183	5,445698166
		Adjusted residuals	1,174594894	-1,174594894
	-Op	Count	34	2
		Expected frequency	30,05923836	5,940761636
		Adjusted residuals	1,816088267	-1,816088267
	Op	Count	242	32
		Expected frequency	228,7842031	45,2157969
		Adjusted residuals	2,74592717	-2,74592717
	+Op	Count	286	80
		Expected frequency	305,6022567	60,3977433
		Adjusted residuals	-3,968569935	3,968569935

Characteristics	Category code	*Description	Detection of Heterobasidion disease	
			Negative (Het-)	Positive (Het+)
Rain (mm)	<1000	Count	26	15
		Expected frequency	34,23413258	6,765867419
		Adjusted residuals	-3,569055196	3,569055196
	1000-1400	Count	105	25
		Expected frequency	108,5472496	21,45275035
		Adjusted residuals	-0,927462513	0,927462513
	1400-1800	Count	369	67
		Expected frequency	364,0507757	71,94922426
		Adjusted residuals	1,02903023	-1,02903023
	>1800	Count	92	10
		Expected frequency	85,16784203	16,83215797
		Adjusted residuals	1,969604539	-1,969604539
Temperature (ºC)	<10.5	Count	9	16
		Expected frequency	20,87447109	4,125528914
		Adjusted residuals	-6,513760522	6,513760522
	10.5-11.5	Count	141	63
		Expected frequency	170,3356841	33,66431594
		Adjusted residuals	-6,556182217	6,556182217
	11.5-12.5	Count	221	35
		Expected frequency	213,7545839	42,24541608
		Adjusted residuals	1,526194259	-1,526194259
	>12.5	Count	221	3
		Expected frequency	187,0352609	36,96473907
		Adjusted residuals	7,391778979	-7,391778979

Pearson´s chi square test results for the association among *Armillaria* spp. and environmental variables.

Characteristics	Category code	*Description	Detection of Armillaria disease Negative (Arm-)	Positive (Arm+)
Slope (%)	<10	Count	170	29
		Expected frequency	129,3921016	69,60789845
		Adjusted residuals	7,116917603	-7,116917603
	10-20	Count	133	57
		Expected frequency	123,5401975	66,45980254
		Adjusted residuals	1,681956042	-1,681956042
	20-30	Count	89	111
		Expected frequency	130,0423131	69,95768688
		Adjusted residuals	-7,18209243	7,18209243
	30-40	Count	48	34
		Expected frequency	53,31734838	28,68265162
		Adjusted residuals	-1,309325023	1,309325023
	40-50	Count	18	13
		Expected frequency	20,15655853	10,84344147
		Adjusted residuals	-0,830536276	0,830536276
	50-60	Count	3	4
		Expected frequency	4,551480959	2,448519041
		Adjusted residuals	-1,235725258	1,235725258
Orientation	N	Count	45	24
		Expected frequency	44,86459803	24,13540197
		Adjusted residuals	0,035975225	-0,035975225
	NE	Count	46	26
		Expected frequency	46,81523272	25,18476728
		Adjusted residuals	-0,212539027	0,212539027
	E	Count	53	32
		Expected frequency	55,26798307	29,73201693
		Adjusted residuals	-0,549833379	0,549833379
	SE	Count	39	26
		Expected frequency	42,26375176	22,73624824
		Adjusted residuals	-0,890657147	0,890657147
	S	Count	75	28
		Expected frequency	66,97179126	36,02820874
		Adjusted residuals	1,79414187	-1,79414187
	SW	Count	133	26
		Expected frequency	103,3836389	55,61636107
		Adjusted residuals	5,591712565	-5,591712565
	W	Count	45	73
		Expected frequency	76,72496474	41,27503526
		Adjusted residuals	-6,707481039	6,707481039
	NW	Count	25	13
		Expected frequency	24,70803949	13,29196051
		Adjusted residuals	0,10208551	-0,10208551

Characteristics	Category code	*Description	Detection of Armillaria disease Negative (Arm-)	Positive (Arm+)
Tree Type	Deciduous	Count	28	36
		Expected frequency	41,6135402	22,3864598
		Adjusted residuals	-3,741056639	3,741056639
	Conifers	Count	433	212
		Expected frequency	419,3864598	225,6135402
		Adjusted residuals	3,741056639	-3,741056639
Soil	acid	Count	260	184
		Expected frequency	288,6939351	155,3060649
		Adjusted residuals	-4,670557678	4,670557678
	-acid	Count	118	31
		Expected frequency	96,88152327	52,11847673
		Adjusted residuals	4,081957619	-4,081957619
	-basic	Count	46	17
		Expected frequency	40,96332863	22,03667137
		Adjusted residuals	1,39395942	-1,39395942
	basic	Count	37	16
		Expected frequency	34,46121298	18,53878702
		Adjusted residuals	0,7602028	-0,7602028
Permeability	impermeable	Count	31	2
		Expected frequency	21,45698166	11,54301834
		Adjusted residuals	3,567372746	-3,567372746
	low	Count	4	5
		Expected frequency	5,85190409	3,14809591
		Adjusted residuals	-1,302690559	1,302690559
	medium	Count	364	175
		Expected frequency	350,4640339	188,5359661
		Adjusted residuals	2,496683356	-2,496683356
	high	Count	62	66
		Expected frequency	83,22708039	44,77291961
		Adjusted residuals	-4,346005418	4,346005418
HOC	--Op	Count	26	7
		Expected frequency	21,45698166	11,54301834
		Adjusted residuals	1,698271891	-1,698271891
	-Op	Count	23	13
		Expected frequency	23,40761636	12,59238364
		Adjusted residuals	-0,146212962	0,146212962
	Op	Count	171	103
		Expected frequency	178,157969	95,84203103
		Adjusted residuals	-1,157611816	1,157611816
	+Op	Count	241	125
		Expected frequency	237,977433	128,022567
		Adjusted residuals	0,476300879	-0,476300879

Characteristics	Category code	*Description	Detection of Armillaria disease Negative (Arm-)	Positive (Arm+)
Rain (mm)	<1000	Count	34	7
		Expected frequency	26,65867419	14,34132581
		Adjusted residuals	2,476779753	-2,476779753
	1000-1400	Count	86	44
		Expected frequency	84,52750353	45,47249647
		Adjusted residuals	0,29966523	-0,29966523
	1400-1800	Count	295	141
		Expected frequency	283,4922426	152,5077574
		Adjusted residuals	1,862340893	-1,862340893
	>1800	Count	46	56
		Expected frequency	66,32157969	35,67842031
		Adjusted residuals	-4,559908025	4,559908025
Temperature (ºC)	<10.5	Count	20	5
		Expected frequency	16,25528914	8,74471086
		Adjusted residuals	1,598870482	-1,598870482
	10.5-11.5	Count	130	74
		Expected frequency	132,6431594	71,35684062
		Adjusted residuals	-0,459785878	0,459785878
	11.5-12.5	Count	162	94
		Expected frequency	165,9859353	90,0140647
		Adjusted residuals	-0,730280756	0,730280756
	>12.5	Count	149	75
		Expected frequency	145,6473907	78,35260931
		Adjusted residuals	0,567912095	-0,567912095

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