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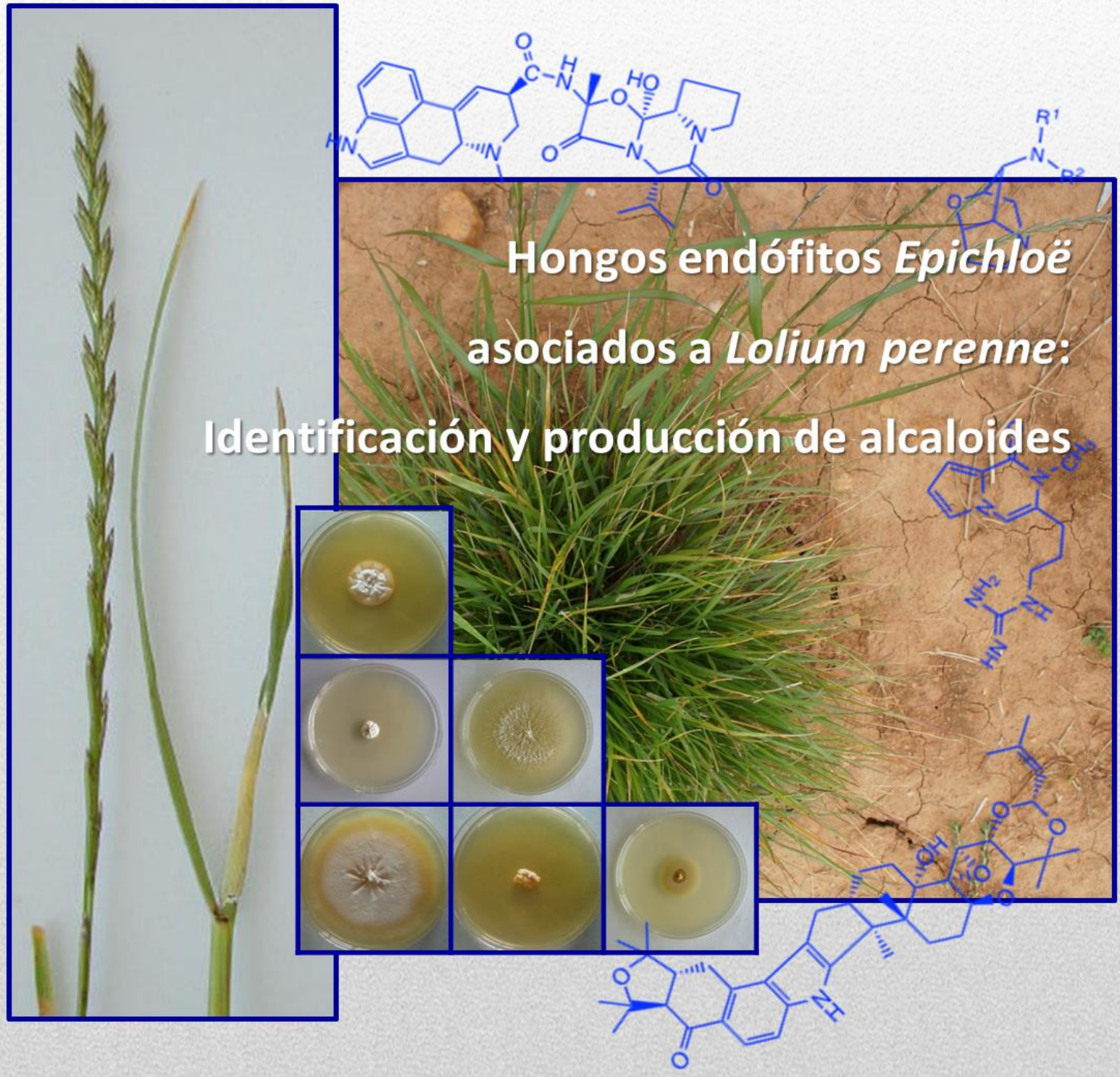

Hongos endófitos *Epichloë* asociados a *Lolium perenne*: Identificación y producción de alcaloides

Milton Carlos Soto-Barajas

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS

INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGÍA DE SALAMANCA

Departamento de Estrés Abiótico

HONGOS ENDÓFITOS *Epichloë* ASOCIADOS A *Lolium perenne*: IDENTIFICACIÓN Y PRODUCCIÓN DE ALCALOIDES

Memoria presentada por
MILTON CARLOS SOTO-BARAJAS
para optar el grado de
Doctor en Química por la Universidad de Salamanca

Salamanca, a 22 de julio de 2016

Dra. BEATRIZ RODRÍGUEZ VÁZQUEZ DE ALDANA, CIENTÍFICA TITULAR DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC), EN EL INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGÍA DE SALAMANCA,

CERTIFICA

Que la memoria titulada "HONGOS ENDÓFITICOS *Epichloë* ASOCIADOS A *Lolium perenne*: IDENTIFICACIÓN Y PRODUCCIÓN DE ALCALOIDES" presentada por D. Milton Carlos Soto-Barajas para obtener el grado de Doctor en Química por la Universidad de Salamanca, ha sido realizada bajo mi dirección, en el Departamento de Estrés Abiótico del Instituto de Recursos Naturales y Agrobiología de Salamanca del Consejo Superior de Investigaciones Científicas (CSIC).

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide y firma el presente certificado en Salamanca, a 21 de julio de 2016.

Fdo. Dra. Beatriz Rodríguez Vázquez de Aldana

Dr. IÑIGO ZABALGOGEAZCOA GONZÁLEZ, INVESTIGADOR CIENTÍFICO DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC), EN EL INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGÍA DE SALAMANCA,

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Que la memoria titulada "HONGOS ENDÓFITICOS *Epichloë* ASOCIADOS A *Lolium perenne*: IDENTIFICACIÓN Y PRODUCCIÓN DE ALCALOIDES" presentada por D. Milton Carlos Soto-Barajas para obtener el grado de Doctor en Química por la Universidad de Salamanca, ha sido realizada bajo mi dirección, en el Departamento de Estrés Abiótico del Instituto de Recursos Naturales y Agrobiología de Salamanca del Consejo Superior de Investigaciones Científicas (CSIC).

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide y firma el presente certificado en Salamanca, a 21 de julio de 2016.

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Dra. Ma. INMACULADA GONZÁLEZ MARTIN, PROFESORA CATEDRÁTICA DEL DEPARTAMENTO DE QUÌMICA ANALITICA, NUTRICIÒN Y BROMATOLOGÌA DE LA UNIVERSIDAD DE SALAMANCA,

CERTIFICA

Que la memoria titulada "HONGOS ENDÓFITICOS *Epichloë* ASOCIADOS A *Lolium perenne*: IDENTIFICACIÓN Y PRODUCCIÓN DE ALCALOIDES" presentada por D. Milton Carlos Soto-Barajas para obtener el grado de Doctor en Química por la Universidad de Salamanca, ha sido realizada bajo la dirección de la Dra. Beatriz Rodríguez Vázquez de Aldana y del Dr. Iñigo Zabalgogeazcoa González, en el Departamento de Estrés Abiótico del Instituto de Recursos Naturales y Agrobiología de Salamanca del Consejo Superior de Investigaciones Científicas (CSIC), y bajo mi tutela.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide y firma el presente certificado en Salamanca, a 25 de julio de 2016.

Fdo. Dra. Mª. Inmaculada González Martín

La presente tesis doctoral fue realizada en el Instituto de Recursos Naturales y Agrobiología de Salamanca, perteneciente al Consejo Superior de Investigaciones Científicas, como parte del proyecto: “Endófitos para la mejora de variedades comerciales de *Lolium perenne* y efecto de micovirus en el metabolismo secundario endofítico” financiado por el Ministerio de Economía y Competitividad, Plan Nacional I+D+i (AGL2011-22783). Milton Carlos Soto-Barajas recibió una beca doctoral del Consejo Mexicano de Ciencia y Tecnología (CONACyT).

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GENERAL INTRODUCTION

1. PERENNIAL RYEGRASS

Lolium perenne, commonly known as perennial ryegrass, is a C_3 plant, belonging to the subfamily *Pooideae*, that does not produce stolons or rhizomes, its shoot buds arise at or near the soil level in young plants, but may develop from higher nodes in adult plants. Beddows (1967) described this grass as a hemicryptophyte with a semi-rosette form before head emergence (Figure 1).

Figure 1 Perennial ryegrass (*Lolium perenne* L.) vegetative plant

Perennial ryegrass could have evolved from a small bottleneck of a *L. rigidum* population in the Middle East, and its distribution area in Europe could be explained either by the extension of primitive agriculture from the fertile crescent (Balfourier et al. (1998), or as consequence of post-glacial recolonization from southern refugia (Catalan et al. 2004). Presumably, *L. perenne* was introduced as a fodder crop for English pastoralists to many corners of their former empire, including North America, Australasia, South Africa and elsewhere, where it was, and frequently still is, referred to as English ryegrass (Beddows 1967).

Lolium perenne is an agricultural species which has been intensively bred and selected for many years in different countries (Humphreys et al. 2010; Lee et al. 2012), as a result of these programs, there are many cultivars of both diploid and tetraploid forms (Sampoux et al. 2011). Perennial ryegrass freely crosses with other *Lolium* species (*e.g*. *L. multiflorum*, L.

rigidum, *L. remotum,* and *L. temulentum*) producing fertile hybrids (*L. x hybridum*) with intermediate characteristics (Jenkin 1954). It also forms hybrids with species of *Schedonorus* (formerly *Festuca)* such as *S. arundinaceus*, *S. giganteus* and *S. pratensis* (Humphreys et al. 2010); hence as an outcrossing species considerable genetic variability can be observed within and between its cultivars (Beddows 1967; Hahn et al. 2008).

At the present time, perennial ryegrass has become extensively widespread; in part because it can tolerate a range of environmental conditions and withstands repeated defoliation, can produce good yields, and has high digestibility (Sampoux et al. 2011; Sampoux et al. 2012). Therefore, it is arguably the most important forage and turf grass in the world; for example, in New Zealand forms the basis of arable pastures (80%) (Fletcher et al. 1990), and in Europe In western Europe 17% of the total land area consists of permanent grassland (Wilkins and Humphreys 2003), with the greatest proportion being contributed by perennial ryegrass and Italian ryegrass (Pearson 2010). Furthermore, from sown pastures, it has spread to colonize footpaths, roadsides, tracks, waste places, sand dunes and riverbeds.

2. *Epichloë* FUNGAL ENDOPHYTES

Most plants have evolved the ability to form below and aboveground associations or symbioses with microorganisms such as viruses, bacteria, and fungi that can alter the host phenotype to enhance their fitness, competitiveness, expand their niche and enabling them to persist in otherwise marginal or inhospitable habitats (Funk and White 1997; Eaton et al. 2015). For example, the establishment of mycorrhizal partnerships with aquatic plants, around 460–480 million year ago, facilitated their transition to terrestrial habitats (Pirozynski and Malloch 1975). More recently in plant history (50–80 million year ago), fungi from the *Clavicipitaceae* family, derived from a parasitic fungus of arthropods, moved into grasses (Schardl et al. 2008; Gibert et al. 2012) and this interaction is mainly based on protection against biotic and abiotic stressors (Bush et al. 1997; Schardl 2001; Hahn et al. 2008). *Epichloë*/grass symbiosis exists in at least 80 grass genera and about 300 of grass species (Clay 1989; Leuchtmann 1992), which represent less than 4% of the 8000 known grass species. Detailed surveys in restricted areas can show greater percentages of infected grasses. For instance, *Epichloë* endophytes were isolated from 11 of 49 grass species in a survey made in permanent semiarid grasslands of western Spain (Zabalgogeazcoa et al. 2003).

Microorganisms growing (entirely) within the substrate of a plant, whether parasitic or not are generically known as endophytes (Greek: *endo*= within + *phyte*= plant) (Walker 1950; Snell et al. 1971; Clay 1990; Fletcher et al. 1990; Wennström 1994; Wilson 1995), they comprise several microorganisms associated to all major lineages of plants in natural and anthropogenic communities ranging from the arctic to the tropics (Arnold 2007). However, there is a general agreement over usage of the term endophyte, with the suggestion that the word implies a mutualistic relationship (Wennström 1994). In this thesis, the term endophyte is particularly used to refer to fungi of the genus *Epichloë* (*Clavicipitaceae* family) hosted by temperate grasses (*Pooideae* subfamily).

Epichloë endophytes had been recognized for centuries, the first known accounts of *Epichloë* endophytes were published in 1898 when Vogel recorded mycelium in the seed of darnel (*Lolium temulentum*) (Schardl et al. 2004b). According to do Valle Ribeiro (1993), this was confirmed in the same year by other researchers, and in 1902 Neubauer and Remer added *L. remotum* (=*L. perenne* subsp*. remotum*) to the list of infected species. Sampson (1935) indicated that McLennan in 1920 reported that all *L. perenne* plants carry the endophytic fungus. The observation of infection in tall fescue (*Schedonorus arundinaceus*= *Festuca arundinacea*= *Lolium arundinaceum*), red fescue (*Festuca rubra*) and perennial ryegrass (*L. perenne*) by those fungi were published by (Sampson (1933); 1935; 1937; 1939). Sampson's findings were confirmed by (Neill (1940); 1941) and reported the existence in New Zealand of an endophytic fungus, similar in appearance to the perennial ryegrass endophyte in tall fescue and meadow fescue *(Festuca pratensis*).

Similarly, to other cool-season grasses, the symbiotic association between *Epichloë* endophytes and perennial ryegrass appears to be widespread. Field surveys have revealed that infected grasses have a broad distribution and infection included both sexual and asexual fungi (Kaur et al. 2015). The endophyte *E. festucae* var. *lolii* is the most common species in pastures of perennial ryegrass, but this grass also is a host of *E. typhina*, an interspecific hybrid (*E. fetucae* var. *lolii* x *E. typhina*) designated as LpTG-2, and an *E. festucae*-like endophyte (Latch et al. 1984; Clay 1987; Schardl et al. 1991; Christensen et al. 1993; Moon 1999).

Despite the abundance of *Epichloë* in heavily grazed permanent grass communities, their artificial selection for culture apparently has eliminated much genetic variation of *Epichloë* endophytes and cultivated grasses seems infected by a small number of endophyte genotypes (Clay 1993; Schardl et al. 1994). And currently most of the researches about the effect of *Epichloë* endophytes have been conducted on commercials cultivars with scares diversity of grasses and endophytes. In this regard, Saikkonen et al. (2006) indicated that these type of assays fail to capture the breadth of variability inherent in wild grass/endophyte populations and communities. In contrast with cultured pastures, Jensen and Roulund (2004) found abundant genetic variation among endophyte from wild grasses, therefore these habitats may be excellent sources of endophytes useful for grass improvement and plant breeding. Additionally, according to Clay (1993) wild grasses should play a greater role in endophyte research, not only as repositories of genetic variability, but also as comparative systems for understanding the physiological, ecological and evolutionary interactions between plant and fungus.

Natural wild population of *L. perenne* still widespread in most of Europa, part of the Mediterranean and Middle East area, where is its genetic center of origin (Balfourier et al. 1998). In these areas, high genetic variability among *Epichloë* endophytes is expected because diversity and species composition of endophytes in natural habits are influenced by microhabitat and microclimatic conditions (Arnold 2007). Adaptation of endophytes to particular ecological conditions provides ecological data and desirable agronomic traits that would be applied to improvement of grasses (Oliveira and Charmet 1989).

2.1 Characteristics of Epichloë endophytes

Upon germination of the grass seed, *Epichloë* hyphae within the embryo, extend into leaf primordia and axillary buds, the meristematic cells from which new shoots develop, invading all the above ground tissues. Hyphae are characteristically distributed parallel to the longitudinal leaf axis and remain confined to the intercellular spaces where they subsist on plant sugars, amino acids and other nutrients. The growth of the endophyte is synchronized with all stages of the plant growth, when the leaf matures and ceases to expand, no further fungal colonization takes place. In spring, mycelium grows into the leaf sheaths, leaves and inflorescences, reaching a maximum density in late summer and then declining. In the winter, it is confined to the plant crown where it is most concentrated in the meristematic tissues (di Menna and Waller 1986; Phillipson and Christey 1986; Tan et al. 2001; Clay and Schardl 2002; Christensen et al. 2008).

Taxonomic classification of *Epichloë* endophytes has suffered several modifications since its discovery. Previously, *Epichloë* species were classified under two different genera depending on differences in their mode of reproduction. The asexual (anamorphic) taxa were assigned to a separate genus (*Neotyphodium*), but in accord with general recommendations for fungal taxonomy, it has been combined with the sexual (teleomorphic) taxa within a single genus, as part of a monophyletic group designated *Epichloë,* accepting 10 teleomorph-typified species and 24 anamorph-typified species (including three subspecies and six varieties) (Leuchtmann et al. 2014; Hettiarachchige et al. 2015). Despite differences in reproductive characteristics observed in some *Epichloë* endophytes from perennial ryegrass (*e. g*. *Epichloë typhina* and *E. festucae*), the sexual forms are ancestral to the seed-borne types and both endophytes are close similar in morphology and secondary product biochemistry (Clay 1988; Schardl et al. 1991; Clay 1993).

2.2 Reproduction, dispersion and diversification

The *Epichloë* endophytes can be disseminated through vegetative structures of host plants or in one of two general ways related with their mechanism of reproduction: sexual species fruit on their hosts and can infect new plants; the asexual dispersion is through the seeds (Figure 2); but some *Epichloë* have both sexual and asexual cycles (Clay 1990; White et al. 1993; Chung and Schardl 1997b; Tadych et al. 2014).

The life cycle of the sexual *Epichloë* endophytes causes no disease symptoms until the initial stages of flower development. Profuse epiphytic fungal growth occurs surrounding the incipient inflorescences with a fungal stroma that halts further panicle development and prevents seed production (‘choke disease’). On the surface of the fruiting structures or stromata perithecia containing ascospores develop (White and Bultman 1987). The fungi are heterothallic and for fertilization require the mediation of flies of the genus *Botanophilia* that deposit eggs in stromata and transfer conidia (spermatia) of compatible mating types for successful reproduction. Sexual species of *Epichloë* can be distinguished by mating compatibility, each mating population tends to have restricted host ranges (Chung and Schardl 1997b; Bultman et al. 2011). The sexual stage is completed by ejection of haploid filamentous

ascospores derived from the meiotic products, which then undergo iterative germination and conidiation (Figure 2). In this type of interaction the transmission of the endophyte is horizontal and generally the seeds of the host plant, if produced, are fungus-free and give rise to uninfected plants (Schardl 1996).

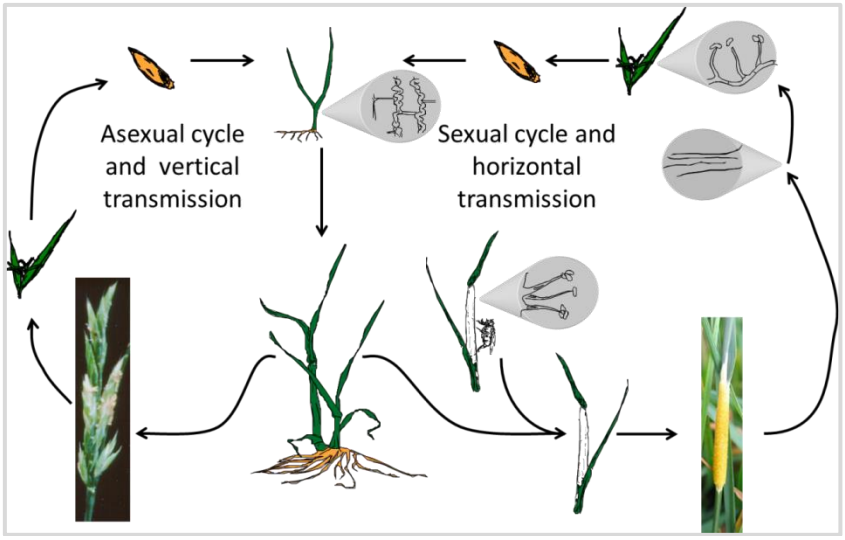

Figure 2 The asexual and sexual life cycles of *Epichloë* endophytes.

Asexual reproduction of *Epichloë* endophytes, is fully asymptomatic, this type of fungi are confined to a single host species because they have lost the ability to produce ascospores on their hosts (Christensen et al. 1993). As the plant starts the reproductive phase, the endophyte in the vegetative apex enters the developing inflorescence primordium and floral apices from where it penetrates the tissues of ovary and ovule, soon after fertilization, the hyphae penetrates the embryo (Phillipson and Christey 1986) (Figure 2). The endophyte hyphae are present in the embryo of the mature seed, and particularly between the cells of the aleurone layer. Lineages of a single fungal genotype are vertically transmitted this way, this mode of dissemination is highly efficient, nearly 100% of seed from infected mother plants transmit the endophyte (Siegel et al. 1984; Clay 1990).

Some *Epichloë* species are able to use the two mechanisms of reproduction, and can manifest its sexual and asexual cycles on different tillers of the same plant; individual plants may produce simultaneously fungal fruiting bodies (stromata) and healthy inflorescences, allowing the fungi to be transmitted vertically through seeds and horizontally by spores (Christensen et al. 2002). This type of *Epichloë* fungi are known as “pleitropic symbionts” because they possibly reflect a mixed evolutionary strategy and a pathway that connected the asexual endophytes with their sexual relatives (Schardl et al. 2004b). Interactions like these have been occasionally observed in some poöid grasses, like *Festuca rubra* infected with *E. festucae* (Zabalgogeazcoa et al. 1999).

In addition to the above mentioned dissemination mechanisms, certain *Epichloë* endophytes can produce epiphyllous mycelial networks with conidiogenous cells and conidia on leaf blades of host plants. The epiphytic conidia are water dispersed and will not release from the conidiophores unless water is present. The conidia may flow off leaves to tillers or seedlings that grow in the vicinity of grass plants and infect them (Christensen et al. 1997; Tadych et al. 2007; Iannone et al. 2009; Tadych et al. 2012; Tadych et al. 2014; White et al. 2015; Wiewiora et al. 2015a)

One particular way of genetic diversification of *Epichloë* endophytes has been through spontaneous events of interspecific hybridization (Schardl et al. 1994; Tsai et al. 1994; Craven et al. 2001a). The formation of interspecific hybrid endophytes is assumed to be relatively rare, and has not been observed under experimental conditions, though the processes implicated in hybrid formation have been demonstrated. Infection of single host plants by multiple endophytes can occur occasionally (Meijer and Leuchtmann 1999; Moon 1999). Anastomosis of pairs of *Epichloë* strains has also been successfully demonstrated (Chung and Schardl 1997a; Shoji et al. 2015). The large number of hybrid *Epichloë* species identified in surveys suggests that, at least in some circumstances, hybrids are positively selected (Moon et al. 2004). According to Clay and Schardl (2002) two likely bases for selection include the pyramiding of favorable genes and the counteracting of Muller's ratchet, because acquiring several favorable characteristics from multiple endophyte ancestors, a hybrid endophyte has higher fitness than its nonhybrid ancestors do.

2.3 Effect of *Epichloë* endophytes on grasses

The interactions between *Epichloë* fungi and host grasses appear to be conditional and vary greatly as part of a continuum from mutualism to parasitism/pathogenicity (Saikkonen et al. 1998; Schulz and Boyle 2005; Eaton et al. 2015), under continual control of two general classes of mechanisms. Clay (1993) explained that intrinsic mechanisms include changes in host biochemistry, physiology, and/or morphology. Extrinsic mechanisms depend on the interaction of host plants with other organisms in their environment, such as herbivores, pathogens, and competitors. The two classes of mechanisms are not necessarily independent and may interact in a complex fashion.

2.3.1 Alkaloid production

Epichloë fungi have passed through several phases of importance since its discovery, for many years were considered inconsequential, until they were linked with production of alkaloids which are beneficial to the endophyte-grass symbiosis because protect them against herbivores, but may be detrimental to livestock (Bacon et al. 1977; Rowan et al. 1986; Ravel et al. 1997a; Reed and Mace 2013).

The effects of *Epichloë* endophytes on insects were first reported by Prestidge et al. (1982), observing a negative correlation between damage by Argentine stem weevils and the frequency of endophyte-infected perennial ryegrass in New Zealand pastures. In the United States Funk (1983) found that perennial ryegrass plots with high levels of endophyte infection

suffered less damage from sod webworms (*Crambus* spp.) and had fewer adults and eggs present than plots with lower levels of infection. Cheplick and Clay (1988) concluded that survival and population growth rates of flour beetles (*Tribolium castaneum)* on ground seeds of infected perennial ryegrass were significantly lower than on uninfected seed. The insecticidal effects of alkaloids, contribute to the increased persistence of endophyte infected-grasses by enhancing its competitiveness compared to non-infected grasses (Clay 1988; Siegel et al. 1990).

Negative effects of *Epichloë* endophytes over mammals extend from poor feeding to severe intoxication. It has been speculated that livestock may be selective in their grazing and discriminate between infected and non-infected plants due to the bitter taste of many alkaloids (Clay 1990; Bazely et al. 1997; Jensen and Roulund 2004). In the extreme side, Fletcher and Harvey (1981) found a direct association between *Epichloë* endophytes and ryegrass staggers, a neurological disorder that occurs in sheep, cattle, horses, and deer grazing infected ryegrass pastures; named because of the staggering gait of the affected animals, together with the observation of the outbreaks occurring in ryegrass-dominated pastures (Byford 1978). Another important syndrome that can suffer mammals grazing *Epichloë* infected pasture is fescue toxicosis, a malady whose symptoms could be fatal, including fever, abortions, convulsions and gangrene of the extremities (Bacon et al. 1977).

The anti-insect and/or anti-mammalian activities of *Epichloë*-infected grasses, is caused by the production of four classes of fungal alkaloids, lolines, peramine, lolitrems, and ergopeptides (Schardl et al. 1991; Young et al. 2012) (Figure 3).

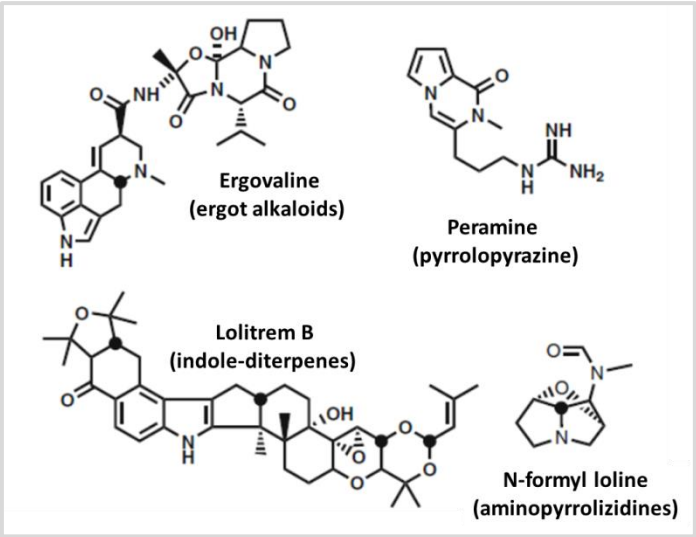

Figure 3 Chemical structures of the most common compounds for each chemical group of alkaloid produced in grasses infected with *Epichloë* endophytes.

Lolines are found almost exclusively in grasses infected by hybrid *Epichloë* endophytes, and they exhibit broad-spectrum insecticidal activity. Concentrations of loline alkaloids found *in planta* are generally not toxic to mammalian herbivores (Siegel et al. 1990; Wilkinson et al. 2000; Schardl et al. 2004a) (Bush et al. 1997; Easton et al. 2009).

Peramine is the most widely distributed alkaloid in *Epichloë*-infected grasses; this alkaloid has a relatively even distribution in the plant and over the growing season, and there is evidence of host genotype control of peramine concentrations (Siegel and Bush 1996; Ball et al. 1997a; Siegel and Bush 1997; Clay and Schardl 2002). Peramine acts as a feeding deterrent to the Argentine stem weevil, but most other insects seem to be insensitive to this alkaloid and has low toxicity to mammals (Rowan and Gaynor 1986; Rowan et al. 1986; Cheeke 1995; Leuchtmann et al. 2000)

Lolitrems are alkaloids with the indole-diterpenes group, known for being tremorgenic compounds, important neurotoxins causative agents of the ryegrass stagger. Lolitrem B is the major tremorgenic compound associated with *Epichloë*-infected *Lolium* species. Lolitrem B concentration increases progressively with increase of the plants leaf age (Gallagher et al. 1982; di Menna et al. 1992; Keogh et al. 1996; Repussard et al. 2014b).

Ergovaline is one of the most toxic of the ergopeptine alkaloids produced in grasses harboring *Epichloë* endophytes and occurs in the greatest concentration. Ergovaline concentrations are highest in the tissues most important for the survival and dissemination of the endophyte, namely the crown, the source of new emerging tiller, and the developing reproductive organs. In perennial ryegrass, the ergovaline levels can be high enough to cause fescue toxicosis symptoms if ambient weather conditions are suitable (Bacon et al. 1977; Siegel et al. 1990; Repussard et al. 2014a; Guerre 2015; Philippe 2016).

The synthesis and concentration of each alkaloid produced in *Epichloë*-infected grasses is driven in a distinct pattern firstly controlled by the endophyte, but also influenced by the host and environmental factors (Repussard et al. 2014b).

Laboratory studies with pure cultures of *Epichloë* fungi and metabolomic analyses in plants, suggest that the taxonomic identity of the endophyte appears to be the most important factor determining alkaloid production in infected grasses, product of genetic differences among strains within a species and among species (Rasmussen et al. 2009; Schardl et al. 2013; Takach and Young 2014; Young et al. 2015). In this regard, Vazquez de Aldana et al. (2010) indicated that in *Festuca rubra* the peramine contents tend to be similar in plats infected with genetically close isolates of *Epichloë festucae*. Leuchtmann et al. (2000) observed a tendency for stroma-forming plants to be free of alkaloids; in contrast, in asexual *Epichloë* expression of two or three different alkaloid classes is more frequent. In perennial ryegrass infected with the asymptomatic common endophyte, *E. festucae* var. *lolii*, three of the four classes of fungal alkaloids have been detected (Leuchtmann et al. 2000; Clay and Schardl 2002).

The role of the host plant on concentration and production of alkaloids is particularly important. For example, *F. gigantea* and *F. rubra*, both naturally infected with *E. festucae*, had different combinations of alkaloids, which confirms that the spectrum of alkaloids expression is also dependent on the genotype of the host (Christensen et al. 1993; Leuchtmann et al. 2000; Vazquez de Aldana et al. 2010).

In *Epichloë*-host associations, environmental conditions are widely recognized to affect the levels of alkaloids expressed in the grass, and under same conditions their concentration have a seasonal fluctuation (Reed et al. 2001; Young et al. 2012). Leuchtmann et al. (2000) reported that in an infected plant of *Bromus benekenii* peramine was not detectable in May, the highest concentration was found in August; whereas, in plants of *Festuca rubra*, peramine was higher in August and was more than five times higher in October of the previous year compared to that in May.

2.3.2 Tolerance to biotic and abiotic factors

Because the host plant provides shelter, nutrients (including precursors in the synthesis of secondary metabolites) and dissemination via its seeds to the fungus, the metabolic costs of sustaining the endophyte, may be outweighed by the benefits (Marks et al. 1991; Clay and Schardl 2002; Faeth and Sulivan 2003; Schardl et al. 2004b; Rasmussen et al. 2007). There are many benefits related to field persistence and stress tolerance of grass species in agricultural and natural ecosystems that have been attributed to the presence of *Epichloë* endophytes; since in some conditions plants not in association with these symbiotic fungi fail to persist. In New Zealand attempts to replant endophyte-infected with endophyte-free seed have been made in perennial ryegrass pastures; however, eliminating the endophyte reduces the vigor of the plants and their resistance (Clay 1990; Fletcher et al. 1990; Easton et al. 2001). Fletcher et al. (1990) found that in dryer regions non-infected ryegrass pastures may not survive in the face of Argentine stem weevil attack (*Listronotus bonariensis*), one of the major pasture pests in New Zealand.

Besides herbivore resistance, other positive effects of *Epichloë* that have been observed in infected grasses are related to tolerance to water deficit (Bacon 1993; Kane 2011), low soil nutrients (Malinowski and Belesky 1999b; 2000; Zabalgogeazcoa et al. 2006), grazing pressure (Bao et al. 2015; Wiewiora et al. 2015b), heavy metals (Malinowski and Belesky 1999b), and better performance in competition with other plant species (Vazquez de Aldana et al. 2013b).

The *Epichloë* effect on the drought-stress tolerance of perennial ryegrass has given inconsistent results (Hesse et al. 2003). However in particular circumstances implying hydric stress, a selection pressure in favor of infection has been observed (Lewis et al. 1997), in such conditions infected plants tended to maintain a more positive water potential (Clay 1993; Hahn et al. 2008). Siegel and Bush (1997) suggested that in endophyte infected tall fescue, the accumulation of secondary metabolites produced by the endophyte could affect osmotic potential and, therefore, improve resistance to drought stress. The occurrence of these

adaptive mechanisms to drought tolerance in other *Epichloë*-infected grasses, such as perennial ryegrass, might also exist.

Under certain controlled environmental conditions, perennial ryegrass associated with *Epichloë* endophytes, has showed significantly greater growth and producing more biomass than non-infected plants (Clay 1988; 1989; Oliveira et al. 2004). Latch et al. (1985) observed that ryegrass containing *E. festucae* var. *lolii* have grown up to 38% more herbage than those without the endophyte. The physiological basis for the increased growth of endophyte-infected plants is not clear, but hormonal and physiological alterations and changes in source-sink relationships within the host may allow improvements in the mechanisms involved in mineral acquisition. The response is conditional to soil nutrient status, with better performance of the infected grasses when the level of nutrients is higher than at lower levels (Cheplick et al. 1989; Malinowski and Belesky 2000; Reed et al. 2004; Rahman and Saiga 2005; Rasmussen et al. 2007).

According to Jensen and Roulund (2004), endophyte-infected plants tolerate a higher grazing pressure than endophyte-free plants. In this way, infected hosts have greater competitive ability, indicating that infection contributes significantly to the species spread (Clay 1989; Marks et al. 1991).

OBJECTIVES

The purpose of this thesis was to evaluate the effect of *Epichloë* fungal endophytes on their natural *Lolium perenne* host plants. With this broad aim, the specific objectives were:

1. Detection and classification of *Epichloë* endophytes in plants of *Lolium perenne* from wild populations (Chapter I).

2. Study of the concentration and production patterns of fungal alkaloids in wild plants of *Lolium perenne* as effect of the *Epichloë* morphotype of the endophyte hosted (Chapter II).

3. Analysis of the effect of *Epichloë* endophytes on the content of minerals and fibers in *Lolium perenne* from wild populations (Chapter III).

4. Evaluation of two techniques for inoculation of *Epichloë* endophytes into commercial cultivars of *Lolium perenne* (Chapter IV).

5. Suitability of near-infrared reflectance spectroscopy (NIRS) for direct identification of *Epichloë* endophytes, and detection and quantification of their associated alkaloids in a heterogeneous set of *Lolium perenne* plants (Chapter V).

For this purpose, a total of 358 *Lolium perenne* plants were collected at eight natural populations in western Spain. After analyzing all plants to detect their associated *Epichloë* endophytes and to characterize them morphologically and genetically (Chapter I), a set of ryegrass plants naturally infected with *Epichloë* (E+) and non-infected plants (E-) from six populations were transplanted in an experimental filed plot. These plants growing in the field plot were used to study the alkaloid profiles produced by the *Epichloë* endophytes (Chapter II) and to analyze the effect of *Epichloë* on nutrient and fiber contents (Chapter III). A selection of *Epichloë* strains (obtained in Chapter I) were used to inoculate commercial cultivars of *Lolium perenne* (Chapter IV), and this inoculated plants were analyzed for alkaloid profiles (Chapter II). All *Lolium perenne* plants (from wild origin and inoculated cultivars) were used to determine the suitability of NIRS technology for identification of *Epichloë* endophytes and detection of alkaloids (Chapter V).

I. DIVERSITY OF *Epichloë* ENDOPHYTES FROM WILD POPULATIONS OF LOLIUM PERENNE

I.1 ABSTRACT

A total of 358 plants were collected from eight natural communities of *Lolium perenne* in western Spain with the aim of identifying the taxonomic diversity of their associated *Epichloë* endophytes. *Epichloë* endophytes were detected in 154 plants (E+), which represent an average incidence of 43.0%, ranging from 32.0% to 60.0% between communities. From the E+ ryegrass plants, 169 endophytes were isolated because 15 plants were infected with two different *Epichloë* endophytes. The *Epichloë* cultures obtained were divided into four morphotypes based on their morphological characteristics: M1, slow growth rate and brain-shaped cultures; M2, faster growth rate with white cottony aerial mycelium; M2S, resembling M2 but isolated from host plants bearing stromata; M3, intermediate growth rate with tan, smooth and flat mycelium. According to a genotypic characterization based on partial nucleotide sequences of the ITS region and the β-tubulin (*tub2*) gene, there were four major groups that clustered into two clades. The first clade was closer to *E. festucae* var. *lolii* and was integrated by two genotypic groups: G1a; including most of the M1 morphotypes and all M3 morphotypes; and G1b, comprising only M1 morphotype endophytes isolated from two communities. The second clade grouped endophytes related to *E. typhina,* and was composed by two subclades: G2a, endophytes with M2 morphotype and some with M2S morphotype (stomata producer); G2b, was formed exclusively for the M2S morphotype. According to the phenotypic and genotypic arrangement, the *Epichloë* endophytes isolated from *L. perenne* could be grouped into six taxonomic groups: M1(G1a), M1(G1b), M2(G2a), M2S(G2a), M2S(G2b) and M3(G1a).

I.2 INTRODUCTION

As many cool season grasses (subfamily *Poöideae*), *Lolium perenne* establishes symbiotic relationships with endophytic fungi of the genus *Epichloë*, these symbioses are frequent in nature, and regardless of the host plant or fungal species they share close similarities. *Epichloë* infections are chronic; plants will remain infected throughout their life span. The fungi grow systemically inside the host aboveground tissues with sparsely branched intercellular hyphae. All *Epichloë* species have common features in morphology, serology, secondary product biochemistry, isozyme profiles and life cycles (Schardl et al. 1991). However, during the host flowering stage, the behavior of these fungi can be differentiated depending on whether they have a sexual or an asexual reproductive cycle (Figure 2). Asexual *Epichloë* endophytes were previously classified in the genus *Neotyphodium* (Leuchtmann et al. 2014).

Sexual *Epichloë* endophytes may form an external reproductive structure known as stroma (Figure 7), causing total or partial sterility of the host plant by constriction of the affected inflorescence, this condition is called choke disease (Clay and Schardl 2002). Sexual *Epichloë* species are heterothallic with a bipolar mating system, and receptive hyphae and spermatia are formed in the same stroma. After successful mating, asci develop within perithecia and the sexual state culminates with the production and ejection of ascospores able to infect other grasses via floret infection (Craven et al. 2001b; Schirrmann and Leuchtmann 2015).

In asexual *Epichloë* species, endophytic mycelium from maternal plants colonize developing ovaries and then embryos in seeds, being this type of transmission vertical and clonal, because the same fungal strain that infects a host plant will be transmitted to its seeds. Asexual *Epichloë* fungi are proposed to have evolved either directly from a single teleomorph, due to loss of the sexual state, or as a result of interspecific hybridization events between species of distinct sexual and asexual lineages (Tsai et al. 1994; Moon et al. 2002; Moon et al. 2004; Gentile et al. 2005; Iannone et al. 2009; Hettiarachchige et al. 2015). Some seed-borne endophytes were considered to be reproductively confined to a single host species by virtue of the missing sporulation; nevertheless, associations between *Epichloë* endophytes and closely related grasses have been created artificially (Christensen et al. 1993). In natural conditions, cross-species infections would be a mechanism whereby grasses might enhance their effective biological diversity; for example, it has been hypothesized that when pooid grasses are occasionally infected by *E. typhina* from other grass species, the new hosts could suppress the choke stage and co-opt these fungi as nonpathogenic protective endophytes (Schardl et al. 1991). Thus, it is also conceivable that *E. typhina* occasionally infects individual plants that already contain asexual endophytes, resulting in a diversity of endophytes which might allow the opportunity for hybridization of fungal strains cross by means of either sexual or parasexual means (Schardl et al. 1991; An et al. 1992; Christensen et al. 1993).

Some reports suggest that much of the success in adaptability of cool season grasses could be attributed to their evolutionary history with *Epichloë* endophytes (Shukla et al. 2015), and that *Epichloë*-grass interactions play an important role in the ability of plants to survive in changing environmental conditions (Eaton et al. 2015). However, a positive effect of the grass-*Epichloë* symbiosis cannot be generalized, because it depends on plant and fungal genotypes, as well as on the environmental conditions (Shukla et al. 2015). Although in agricultural ecosystems it seems to be mutualistic (Saikkonen et al. 2006; Zhang et al. 2011) because the host supplies the fungus with shelter, nutrients, and a means for dispersion though seeds; and in turn, the grass receives protection against abiotic stress, pathogens, and herbivores through the production of alkaloids and other means by the endophyte (Schardl et al. 2004b). The metabolites produced in *Epichloë* infected grasses such as ergovaline and lolitrems can cause significant economic problems for the beef and dairy industries because they are toxic for mammals, causing fescue toxicosis and ryegrass staggers. Other compounds like peramine and the lolines are known to have anti-insect activity and might function as potential biocontrol agents for reducing pest damage in grassland communities (Bacon et al. 1977; Fletcher and

Harvey 1981; Clay 1987; 1989; 1990; Young et al. 2009). Consequently, it is essential to have knowledge about the interaction between *Epichloë* endophytes and grasses in natural systems, including the number and kind of fungi involved, their effects on the host, and the genetic and environmental bases of the interaction (Clay 1989). Most studies on *Lolium perenne* endophytes have been done with cultivated plants but information about diversity of *Epichloë* in wild ryegrass populations is limited.

In countries with intensive livestock production, perennial ryegrass is a key forage species and many researches have had the goal of improving its production. These research programs have included surveys for the characterization of their endophytes. Criteria used for taxonomic identification of *Epichloë* endophytes have been based on morphological characteristics, host species preference, alkaloid biosynthesis and their genetic relationships through the analysis of multiple gene sequences (Jensen and Roulund 2004; Leuchtmann et al. 2014; Hettiarachchige et al. 2015). As result of these surveys, it is known the existence of considerable variation in cultural characteristics, conidial morphology, ability to produce stromata, host specificity, genetic variation and alkaloid production among *Epichloë* endophytes isolated from wild grasses. In addition, genetic evidence has confirmed that several *Epichloë* species can infect the same plant species (Jensen and Roulund 2004; Schardl et al. 2012; Jia et al. 2015). For example, *L. perenne* is a host of at least four taxonomic groups of endophytes that include *Epichloë festucae* var. *lolii* (=*Neotyphodium lolii*); the choke pathogen *E. typhina*, an asexual hybrid designated as LpTG-2, and an *E. festucae*-like endophyte (Schardl et al. 1994; Moon 1999).

Variability in all of the aforementioned characteristics provides raw material for biotechnological manipulation, being natural pastures excellent sources of endophytes useful for grass improvement and plant breeding (Clay 1989) Arroyo et al. 2002). Using such knowledge, commercially available cultivars of perennial ryegrass have been inoculated with selected *Epichloë* endophytes safe for livestock but that confer resistance to abiotic stress and give protection against insects (Easton et al. 2001; Bluett 2003; Rasmussen et al. 2007; Zhou et al. 2014) and there is an increasing interest in new strains useful for grass improvement.

With the aim of analyzing the taxonomic diversity of *Epichloë* endophytes from natural populations of *Lolium perenne*, the results of a survey of eight wild populations from different habitats in western Spain are presented in this study. Plants were analyzed to detect their associated *Epichloë* endophytes, and to characterize them morphologically and genetically.

I.3 MATERIALS AND METHODS

I.3.1 Plant material

The plant sampling was conducted in the spring of 2012. For this work, 358 plants of *L. perenne* were collected at eight populations with different ecological characteristics in western Spain (Table 1). These locations were not cultivated areas, and clumps or individual plants of perennial ryegrass occurred interspersed with other plant species. At each location, approximately 50 plants were dug out. Between each pair of sampled plants, a distance of at least 10 m was left. At some locations, plants with choke disease symptoms were found and collected (Figure 7).

Table 1 Characteristics of the locations were the *Lolium perenne* plants were collected.

Location		Habitat	Altitude (masl)	Coordinates		Precipitation (mm/year)	Average annual temperature (°C)	Number of plants collected
				Latitude	Longitude			
Ciudad Rodrigo	(CR)	Riverbank	625	40°34’48”N	6°30’58”W	531	13.3	25
Divar	(DIV)	Grassland	817	40°43’59”N	5° 45’44”	521	12.2	32
Los Valles	(LVA)	Dehesa grassland	813	40°56’20” N	6°7’36”W	531	12.1	49
La Vecilla	(LVE)	Agricultural land	879	42°42’20”N	5°23’2”W	556	11.2	50
Porqueriza	(POR)	Dehesa grassland	807	40°58’18”N	5°57’24”W	531	13.3	50
Potes	(PI)	Pasture mountain	1355	43°08'48"N	4°28'24"W	780	13.1	48
Tábara	(TAB)	Oak forest	766	41°50’15”N	5°58’40”W	379	12.3	51
Valle Fuentes	(VAF)	Low woodland	1133	42°56’33”N	5°14’18"W	556	13.3	53

The plants of perennial ryegrass were transported to the Institute of Natural Resources and Agrobiology of Salamanca, Spain (IRNASA-CSIC) and then transplanted to 2 l pots containing a mixture of perlite and peat moss (1:1, v/v). The pots were kept outdoors in a wirehouse, watered regularly.

I.3.2 Incidence and Identification of *Epichloë*

I.3.2.1 Isolation of *Epichloë* from ryegrass plants

The procedure to detect the presence of *Epichloë* endophytes in *L. perenne* was conducted immediately after sampling in the field. All collected plants were diagnosed through fungal isolation. From each ryegrass plant, a sample of leaf sheaths was obtained and cut into pieces approximately 5 mm long. These pieces were surface sterilized for 10 minutes in a 20% commercial bleach solution (1% active chlorine), rinsed with sterile water, and placed into Petri plates (9 cm) containing potato dextrose agar (PDA) (Bacon and White 1994) with 200 mg l^{-1} of chloramphenicol. The plates were incubated in the dark at room temperature (~22 °C) and examined daily until the emergence of endophytic mycelium, and then a small amount of

this mycelium was picked out and transferred to a new PDA plate to obtain a culture. The presence of *Epichloë* fungi was also confirmed microscopically by staining stem pith, leaf sheaths, and seeds of perennial ryegrass with aniline blue (Latch and Christensen 1985; Welty 1986).

I.3.2.2 Morphological Classification

A first approximation to the classification of the *Epichloë* cultures was based on macro- and micromorphological characteristics. The morphological characters of all the fungal cultures were observed on 9 cm Petri plates of three different culture media: potato dextrose agar (PDA), rose Bengal chloramphenicol agar (RBA), and malt extract agar (MEA). The morphology of cultures was compared with descriptions from the literature (Christensen et al. 1991; Bony et al. 2001; Christensen et al. 2002). Radial growth was measured for four weeks in 20 individual *Epichloë* cultures growing in PDA plates, and the average daily growth rate was calculated. Microscopic features of the isolates like the presence of conidiophores and conidia were analyzed in cultures of PDA and water agar maintained at room temperature and at 10 °C. A temperature of 10 °C is reported as a requirement to make cultures of *E*. *festucae* var. *lolii* sporulate (Christensen et al. 1991). When conidia were produced, small blocks (0.5 x 0.5 cm) of the respective culture medium were removed from the margins of colonies, and observed at the microscope with a cover slip placed on top of the block (Christensen et al. 1993), and photographed to analyze the shape and to measure the size of conidia (n= 20) from each culture.

I.3.2.3 Genotypic Classification

A molecular classification of the endophytes isolated was based on the nucleotide sequence of the ITS1-5.8SrDNA-ITS2 region, and a 5'region of the β-tubulin (*tub2*) gene. The oligonucleotide primers: ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'), ITS5 (5'- GGA AGT AAA AGT CGT AAC AAG G -3') (White et al. 1990), and tub2-exon1d-1 (5'- GAG AAA ATG CGT GAG ATT GT -3'), tub2-exon4u-2 (5'- GTT TCG TCC GAG TTC TCG AC -3') (Moon et al. 2002) were used to amplify each one of these regions.

The *Epichloë* DNA was extracted from approximately 100 μg of mycelium scraped from PDA cultures using a commercial kit for plant DNA extraction (RedExtract-n-Amp, Sigma-Aldrich). For both genes, the PCR assay had the same conditions: 2 minutes at 95 °C, 35 cycles of 1 minute at 94 °C, 1 minute a 54 °C and 1 minute at 72 °C; and a final extension of 10 minutes at 72 °C. Approximately 2.0 μl of fungal extract were used for the polymerase chain reactions (PCR) performed separately for each gene segment (ITS or β-tubulin) in a thermocycler (GeneAmp PCR System 9700) The success of the PCR amplification was corroborated by electrophoresis in 1% agarose gels run in 1X TAE buffer solution (Tris-HCl 2.0 M, acetic acid 5.71% and EDTA 0.006M, pH 8.0) using 2.0 μl of the PCR reaction, stained with Midori Green (Nippon Genetics) and visualized under UV light.

For sequencing reactions, amplicons were purified using the MSB spin PCRapace kit (Stratec Molecular). Both strands of each replicon were sequenced in a 3100 Genetic Analyzer (Applied Biosciences). Sequence chromatograms were analyzed with Chromas LITE 2.1.1 software (Technelysium Pty Ltd). Nucleotide sequences were aligned with Clustal X version 2.0 software (Larkin et al. 2007). In order to detect distinct genotypes, dendrograms were constructed with the UPGMA method using MEGA version 6 software (Tamura et al. 2013). Alignment gaps were treated as partial missing information. Robustness of the genotypic classifications was estimated by one thousand bootstrap replications. In the phylogenetic trees, reference sequences of known *Epichloë* endophytes were included. Phylogenetic analysis was also performed with the concatenated sequences ordered as ITS-β-tubulin and a phylogenetic tree was constructed using the same methods described above.

Another criterion for identification among *Epichloë* endophytes is their capability to synthesize specific alkaloids. For this purpose, it was examined the nucleotide sequence of the *ltm*Q gene, required for hydroxylation of paspaline, a chemical compound essential for the production of lolitrem B, a indole-diterpene alkaloid, produced in perennial ryegrass infected by some *Epichloë* species, which is the major toxin responsible for the ryegrass staggers syndrome in mammals (Young et al. 2009; Schardl et al. 2013). A set of PCR primers were designed to amplify a fragment of the gene and to detect its presence or absence, the latter would indicate the incapability of a given fungal strain to produce lolitrem B. These primers were the following: ltmQF 5'-GTA ATT TCA GGC GCC ACC ATT-3' and ltmQR 5'-TCG AAG AAT GGA TCG CTG GG-3'. Conditions for PCR were the same as described above with an annealing temperature of 67.0 °C.

With the aim of detecting the presence of *Epichloë* hybrids among the endophytes isolated, all the sequences were checked for presence of ambiguities, consisting in overlapping of the chromagrams picks when these isolates had two genesof β-tubulin (Figure 4).

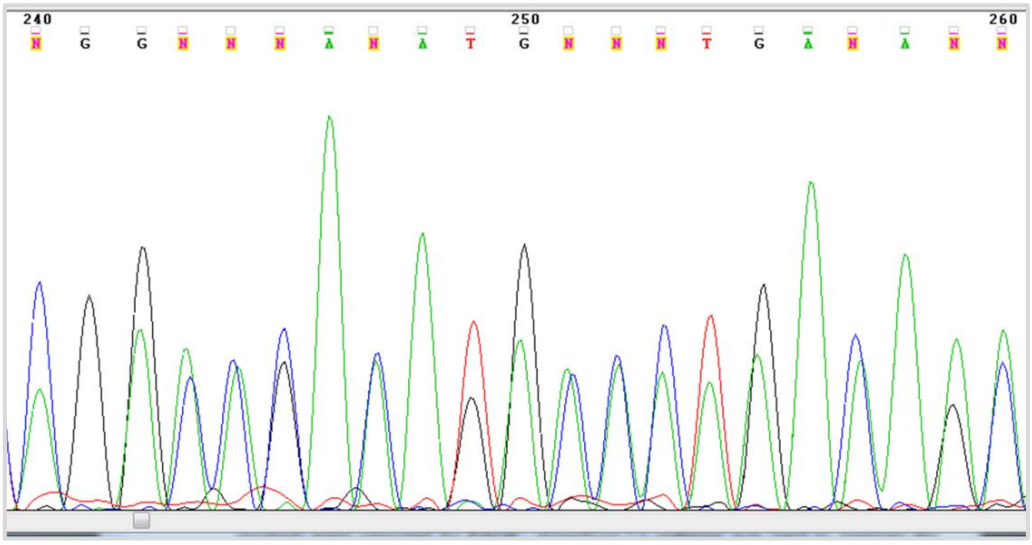

Figure 4 Overlapping of the peacks in the chromatogram indicates presence of two genes of β-tubulin in PCR products from the AR6 *Epichloë* hybrid of reference.

Furthermore, Southern blots were performed using *Epichloë* DNA extracted as indicated by Moon (1999) and digested with *Pst*I and *Bam*HI restriction endonucleases. As a probe, a PCR-amplified fragment of the *tub2* gene labeled with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was used. The hybrid of reference was a culture of LpTG-2 named AR6, donated by Dr. Linda Johnson (AgResearch, Grasslands Research Centre, New Zealand). Additionally, hybrid condition was evaluated by a PCR amplicon cloning procedure. Amplicons of the t*ub2* gene of several *Epichloë* strains representative of each morphotype (including the hybrid of reference AR6), were inserted in pJET1.2/blunt plasmids. The ligation mixture was prepared following the instructions of the CloneJET PCR Cloning Kit (Thermo Scientific) and used to transform competent *Escherichia coli* DH5α cells. Six colonies of the transformed *E. coli*, containing the *Epichloë* t*ub2* inserts of each morphological group, were screened by PCR and sequencing to compare the homogeneity of such genetic fragments. Considering that, the hybrid LpTG-2 has two copies of the t*ub2* gene, each of them characteristic of its progenitors (*E. typhina* x *E. festucae* var. *lolii*), it was expected to found differences in the t*ub2* sequences contained by transformed *E. coli*. On the contrary, all transformed colonies with amplicons from non-hybrid *Epichloë*, must have exactly the same t*ub2* sequences.

I.4 RESULTS

I.4.1 *Epichloë* Incidence

Ryegrass plants were diagnosed as *Epichloë* infected (E+) when mycelia similar to morphological descriptions of *Epichloë* were found in at least one of the following circumstances: (i) growing out of tissues placed on PDA plates, and being subsequently isolated, (ii) observed by microscopy in the intercellular space of stem pith and leaf sheath tissues, or (iii) observed in the aleurone layer of seeds. Taking in consideration these diagnostic conditions, the presence of *Epichloë* endophytes was detected in ryegrass plants from all locations. The average incidence was of 43.0%, ranging from 32.0% in POR to 60.0% in the CR location (Figure 5).

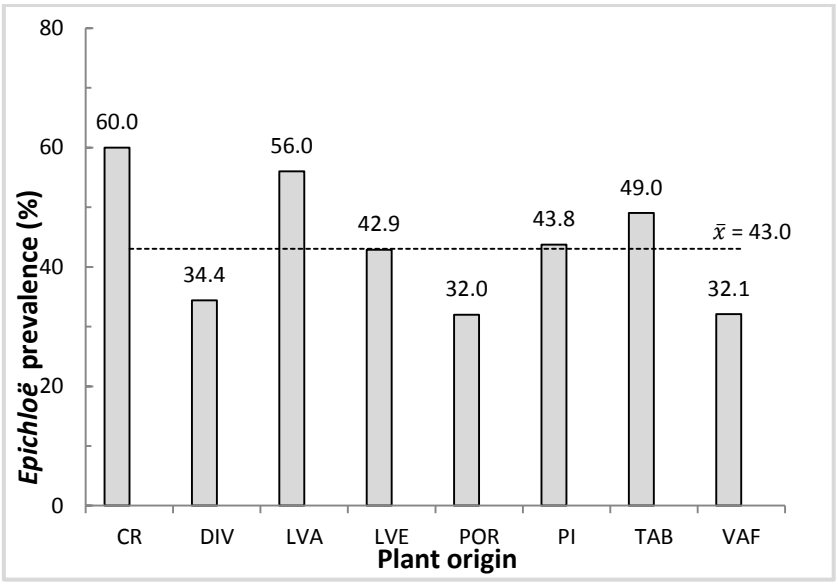

Figure 5 Prevalence of *Epichloë* endophytes in plants of *Lolium perenne* among the eight locations where the *Lolium perenne* plants were collected.

I.4.2 Morphological Classification

The first factors used for differentiation of the *Epichloë* endophytes isolated from ryegrass were macromorphological features like the time of emergence from grass tissues, culture color and shape, and the growth rate observed in three culture media (PDA, RBA and MEA). Most cultures of *Epichloë* endophytes were obtained from asymptomatic ryegrass plants, and could be classified into three distinct morphotypes (Figure 6): (i) M1 morphotype, hyphae of this group of fungi emerged from plant tissue about one month after placing ryegrass samples on PDA. Cultures had strongly aggregated 'brain-like' mycelium and the lowest growth rate ($\bar{x}$= 0.19±0.02 mm day^{-1}) of all morphotypes (Figure 6a-c). (ii) M2, hyphae of this morphotype emerged from plant parts about five days after the pieces of ryegrass were placed in PDA Petri plates. Cultures had white color with abundant cottony aerial mycelium and the fastest growth rate ($\bar{x}$= 1.54±0.16 mm day^{-1}) (Figure 6e-g). (iii) M3 morphotype, whose hyphae emerged from plant fragments after about two weeks. In PDA these colonies had flat and smooth mycelium of light tan color and a growth rate of 0.47±0.06 mm day^{-1} (Figure 6i-k). A fourth group was referred as M2S, with morphological characteristics similar to the M2 endophytes (Figure 6e-g), but isolated from plants that bore stromata on stems (‘choke disease’).

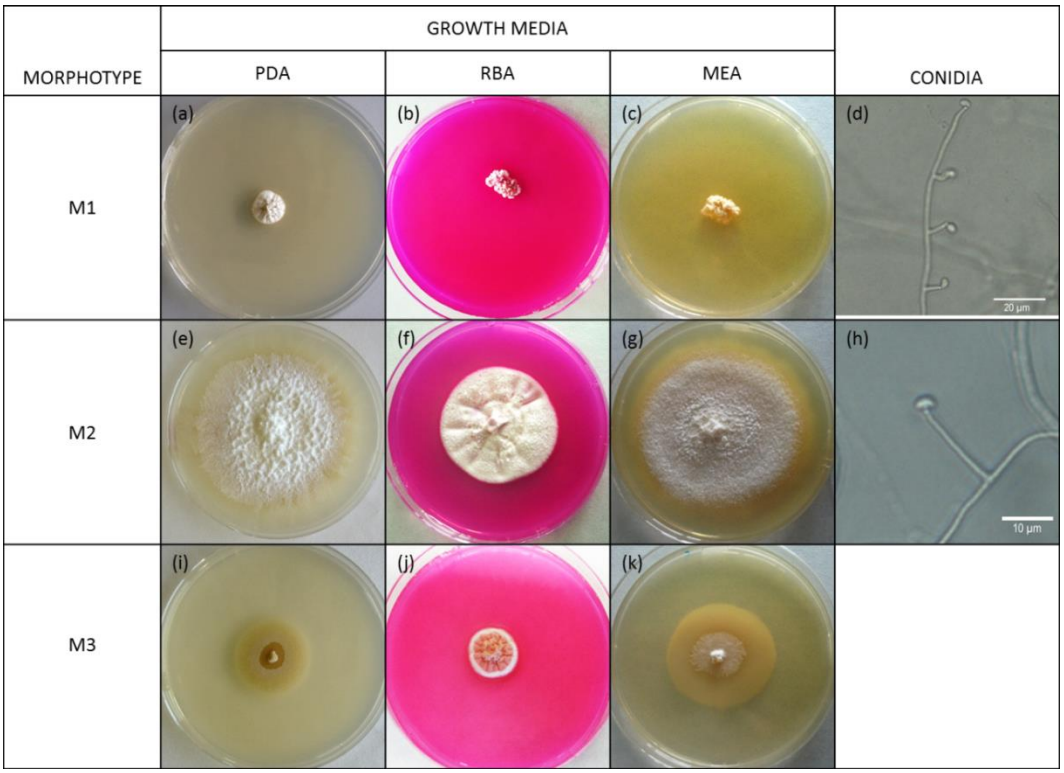

Figure 6 Culture morphology of *Epichloë* endophytes isolated from *Lolium perenne* plants in three growth media: potato dextrose agar (PDA), rose Bengal chloramphenicol agar (RBA), and malt extract agar (MEA). M1 morphotype, slower growth mycelium with convoluted surface (a, b, c); M2 morphotype, faster growth rate with white cottony aerial mycelium (d, e, f); M3 morphotype, intermediate growth rate with tan, smooth and flat mycelium (I, j, k). Conidia and conidiophores of M1 morphotype cultures grown in water agar at 10 °C (d) and of M2 morphotype in PDA at room temperature (~22 °C) (h). M3 morphotype cultures did not sporulate in the above or other growth media tested.

The growth pattern of the *Epichloë* colonies with the M1 morphotype was observed to be very stable, with no changes in form but in size in the different growing media (PDA, RBA and MEA). Much greater variation was found in colony shape among the M2 and M3 morphotypes, changing their forms and size according to the growth medium (RBA and MEA). However, after some weeks they developed their distinctive characteristics observed in PDA: the cottony aerial mycelium for M2 and the tan color for M3. It was noticed that all *Epichloë* cultures made in RBA had a slight delay to start growing, and their radial growth was smaller than in the other media (Figure 6).

Conidia were observed in *Epichloë* endophytes with M1 and M2 morphotypes and also in the hybrid (AR6), although different conditions were required for conidia production. The M1 morphotype cultures produced conidia after three weeks at 10 °C in water agar (Figure 6d), and the M2 morphotype produced conidia at room temperature in PDA (Figure 6h); in these last conditions, the hybrid also produced conidia. *Epichloë* endophytes belonging to the M3 morphotype were sterile in all media and temperatures evaluated (PDA or water agar at room temperature or 10 °C). The conidia of the three types of endophyte (M1, M2 and hybrid) had the same reniform shape characteristic of the genus *Epichloë* but with statistical differences in size (P<0.001): conidia of M1 and M2 morphotypes had similar length, 4.96±0.18 µm and 5.07±0.09 µm respectively; whereas, the hybrid produced larger conidia: 7.80±0.44 µm.

The number of plants infected by each morphotype of *Epichloë,* and their distribution per location are shown in Table 2.

Table 2 Morphotypes of *Epichloë* endophytes isolated from *Lolium perenne* and their distribution by plant origin.

Plant origin	n[¶]	Morphotype of the *Epichloë* endophyte hosted[¶]			
		M1	M2	M3	M2S
		Percentage of plants (%)			
CR	16	18.8	18.8	18.8	43.8
DIV	11	27.3	18.2	0.0	54.5
LVA	38	36.8	23.7	23.7	15.8
LVE	22	95.5	4.5	0.0	0.0
POR	18	22.2	11.1	11.1	55.6
PI	21	33.3	0.0	0.0	66.7
TAB	26	42.3	7.7	0.0	50.0
VAF	17	76.5	5.9	0.0	17.6
Total(n) mean (%)	169	48.2	13.1	10.2	28.5

[¶]The morphotypes were: M1= slow growth rate with 'brain-like form'; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; and with the choke disease M2S = M2 from plants with stromata (see Figure 6).

The most common endophyte hosted by ryegrass plants was the M1 morphotype, present in 48.2% of the infected plants, plants with the M3 morphotype composed 28.5% of the samples. There were 18 plants associated with the M2 morphotype (13.1%), and 14 ryegrass plants (10.2%) had stromata producing endophytes (M2S). In three locations (CR, LVA and POR) the four *Epichloë* morphotypes (M1, M2, M2S and M3) were detected. No stromata characteristics of choke disease were observed in plants from DIV, PI, TAB or VAF locations. Plants from LVE, the only sampling site adjacent to agricultural lands, had the most homogeneous endophyte population with 95.5% belonging to M1 morphotype and one plant harboring an M2 endophyte. The only location where no M2 morphotypes were isolated was PI, *Epichloë* isolated from plants of that location belonged to M1 (33.3%) and M3 morphotypes (66.7%).

From 154 E+ plants, 169 *Epichloë* isolates were obtained. This happened because from 15 individual ryegrass plants two *Epichloë* endophytes with different morphotype were isolated. These double infections were observed in two circumstances: (i) healthy plants with normal growth; (ii) when the same plant produced both healthy reproductive stems with seed heads and stems with stromata (Figure 7).

Figure 7 Healthy seed head and stroma of a single *Lolium perenne* plant infected with two morphologically different *Epichloë* endophytes.

In doubly infected asymptomatic ryegrass, the double infection status was corroborated through subsequent fungal isolations until two fungal phenotypes were obtained from single plants. In choked plants in which healthy seed heads were also produced (Figure 7), double infection was verified by germinating the seeds in water agar and transplanting the seedlings to pots with sterile substrate (perlite:peat moss, 1:1, v/v). After three months these new plants were diagnosed and endophytes were classified as belonging to M1 or M3 morphotypes. Double infected plants (DI) had always M2 endophytes, able or not to produce stromata (M2S), and one of the asymptomatic *Epichloë* (M1 or M3), in four combinations designed according to their endophytes hosted as: DI(M2/M1) and DI(M2/M3), for asymptomatic grasses; and DI(M2S/M1) and DI(M2S/M3) for plants that developed choke disease (Figure 7). There were no cases in which the M1 and M3 morphotypes were isolated from the same plant. Double infections were not detected in plants from DIV, PI and VAF; on the other hand, seven plants, 14% of the samples from LVA, were double infected (Figure 8).

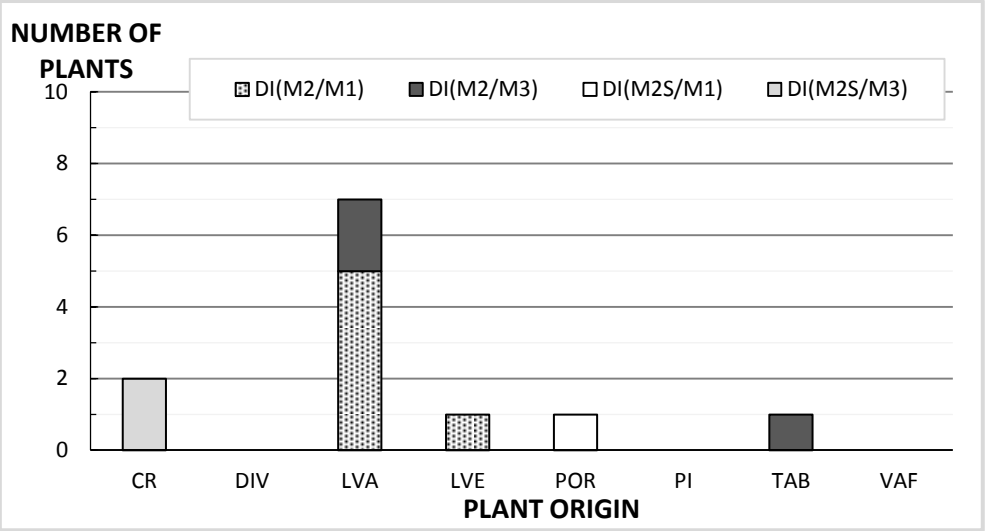

Figure 8 *Lolium perenne* plants per location detected as infected by two morphologically different *Epichloë* endophytes, namely double infection (DI).

I.4.3 Genotypic classification

Phylogenetic analyses were performed using partial sequences of the internal transcribed spacers 1 and 2 (ITS1-5.8SrDNA-ITS2) and the β-tubulin gene (*tub2*), with representative sequences of characterized *Epichloë* endophytes obtained from the GenBank database. The genotypic classification was based on an ITS sequence of approximately 480 base pairs (bp) containing 18 variant sites (excluding deletions), 16 of which were informative. The alignment analysis revealed two major clades, one enclosing M1 and M3 sequences into the same clade as the reference sequences of *E. festucae* var. *lolii*; and the second clade was comprised by M2 and M2S morphotypes which sequences were similar to those of *E. typhina*, the causal agent of choke disease (Figure 9).

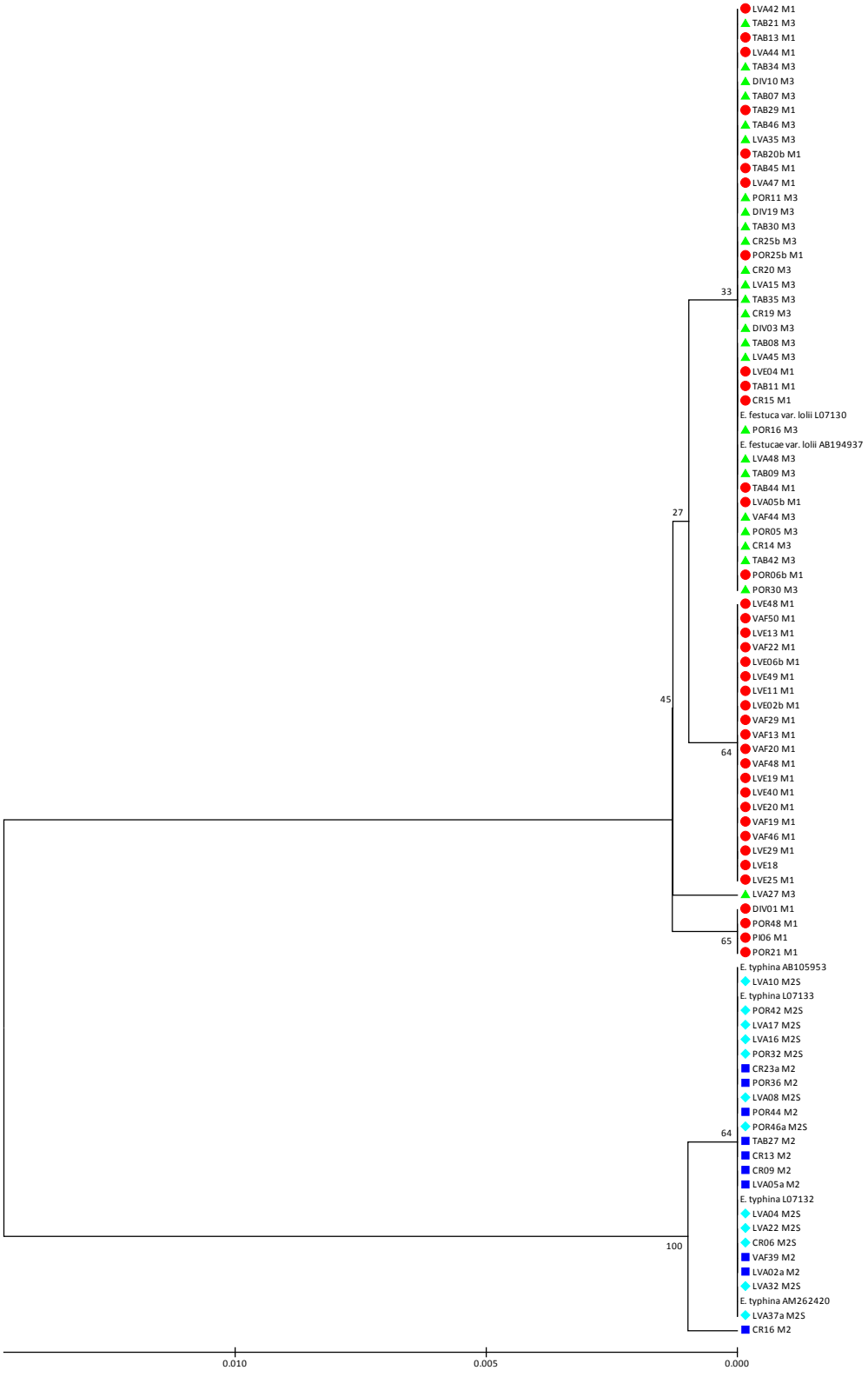

Figure 9 Phylogenetic analysis based on the nucleotide sequence of ITS1-5.8SrDNA-ITS2 region of *Epichloë* endophytes isolated from *Lolium perenne*. The tree was made using the UPMGA method and the bootstrap test is based on 1000 replicates. Letters “a” and “b” indicate endophytes isolated from double infected ryegrass plants. Morphology of endophytes is indicated next to the sample name and with different symbols.

The first clade was composed by four subclades, which differentiated among them in substitution of only one nucleotide; this difference was corroborated by resequencing some samples of each subclade at least two times. The major subclade contained sequences from M1 and M3 morphotypes. There was a subclade with 20 sequences of M1 morphotypes isolated exclusively at LVE and VAF locations, these fungi differed from other M1 endophytes by a single substitution (G→A) around nucleotide 325. A third subclade contained four sequences all them belonging to M1 endophytes isolated from DIV and POR, differentiated from sequences of the first clade by the substitution C→G in nucleotide 32. The sequence of the LVA27 isolate with M3 morphotype was different to other endophytes because of a substitution in nucleotide 227 (G→A). The second clade contained indistinguishable sequences of the M2 and M2S morphotypes, only the sequence of the CR16 isolate was different, it had a substitution (A→G) in the nucleotide 82.

The phylogenetic analysis performed with the partial sequences of the β-tubulin gene (*tub2*) produced an 836 nucleotide long sequence alignment, with 53 variant sites (excluding deletions), 37 of which were informative. The dendrogram grouped the *Epichloë* endophytes into two main clades. Similarly to the ITS dendrogram, there were no specific sequence differences among the M1 and M3 morphotypes; in contrast, there was a split into two subclades of the M2 morphotypes as a consequence of one variant site (bp 155, T→G). One of the subclades consisted of four samples of stromata-forming endophytes. The two sequences of the hybrid LpTG-2 (*E. festucae* var. *lolii* x *E. typhina*) were allocated in different clades identified respectively as AR6-I and AR6-II (Figure 10). The sequence AR6-I had one substitution (bp 116, G→T) with respect to the major subclade closer to the reference sequence of *E. typhina*, and the AR6-II sequences differs in nucleotide 734 (T→C) when compared to other sequences of *E. festucae* var. *lolii*.

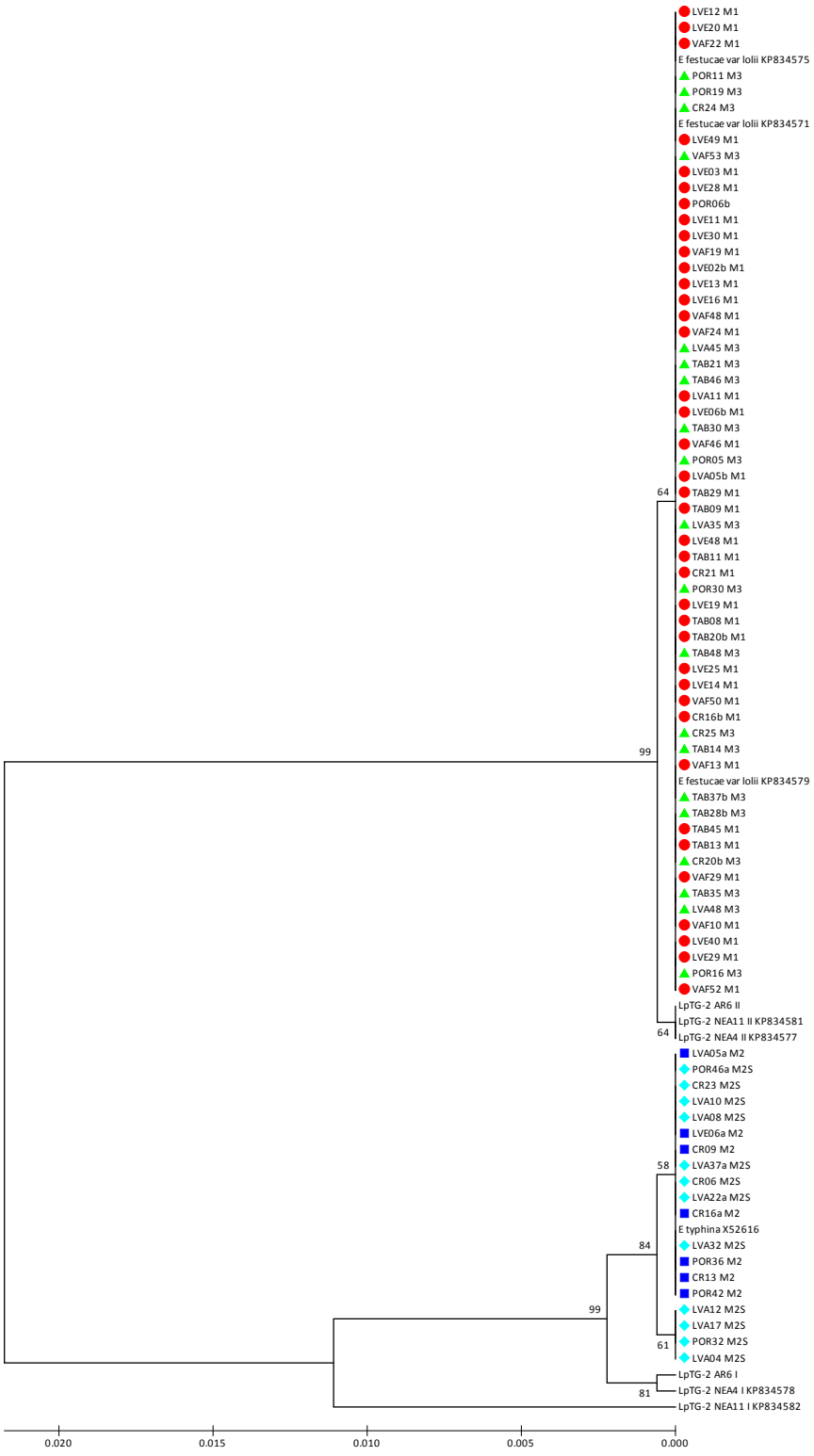

Figure 10 Phylogenetic analysis based on the 5' region of the β-tubulin (*tub2)* gene of *Epichloë* endophytes isolated from *Lolium perenne*. The tree was made using the UPMGA method and the bootstrap tree is based on 1000 replicates.. Letters "a" and "b" indicate endophytes isolated from double infected ryegrass plants: Morphology of endophytes is indicated next to the sample name.

With the concatenated phylogenetic analysis using the ITS and β-tubulin (*tub2*) gene, there was a division of the *Epichloë* sequences into four genotypic groups as shown in Figure 11.

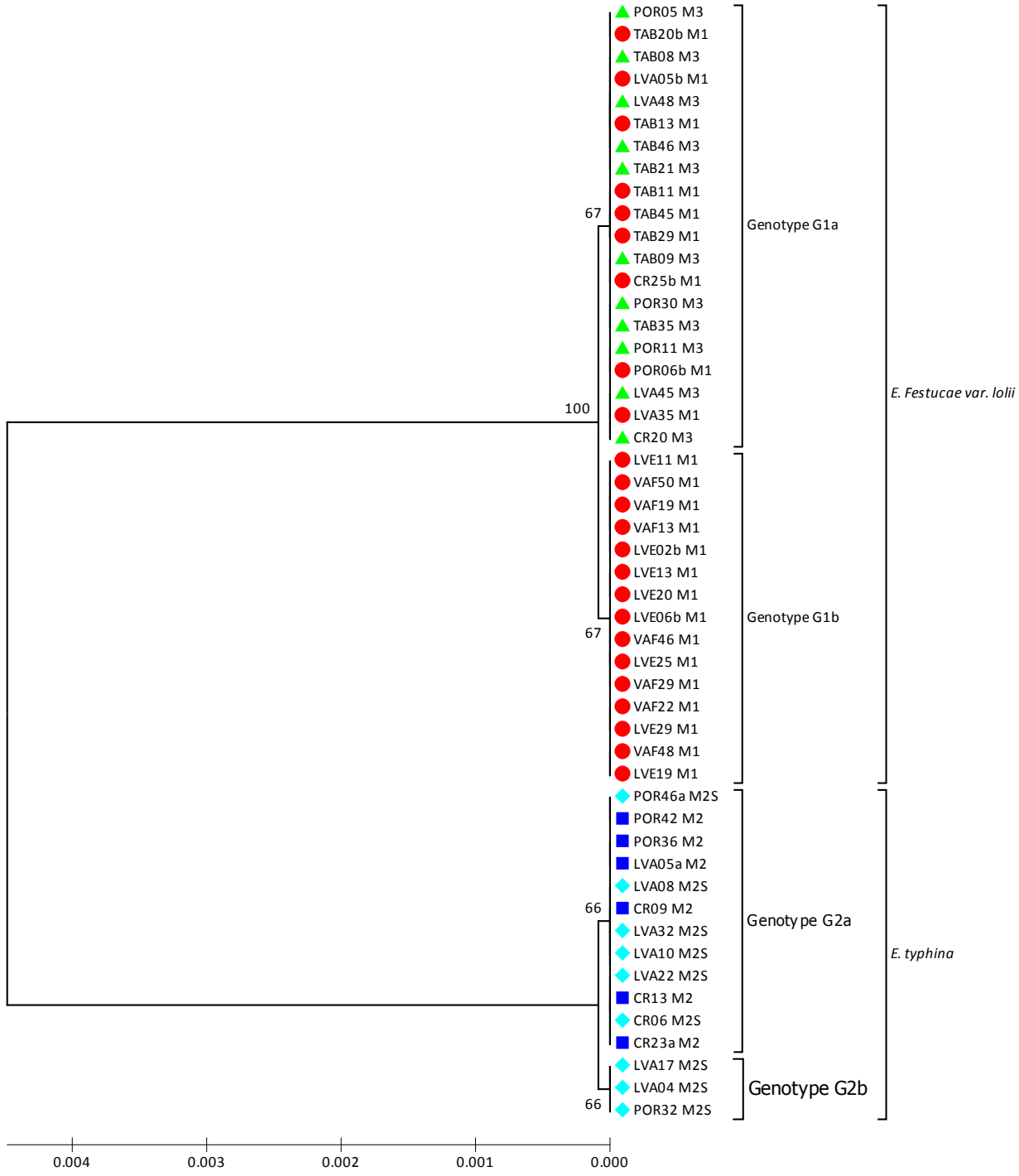

Figure 11 Phylogenetic analysis based on the nucleotide sequence of ITS1-5.8SrDNA-ITS2 region and the β-tubulin (*tub2)* gene of *Epichloë* endophytes isolated from *Lolium perenne*. The tree was made using the UPMGA method and bootstrap test is based on 1000 replicates. Letters "a" and "b" indicate endophytes isolated from double infected ryegrass plants. Morphology of endophytes is indicated next to the sample name.

Each subclade in the concatenated dendrogram was designated as belonging to a different genotypic group. The M1 and M3 morphotypes were mixed in the first subclade (genotype G1a). The second subclade (genotype G1b) was comprised exclusively of sequences of the M1 morphotype infecting plants from LVE and VAF, the sequence of these M1 morphotypes differed from those of genotype G1a in the ITS region. The second clade grouped M2 and M2S endophytes, closely related to *E. typhina*, and was integrated by genotypic groups G2a in the third subclade with M2 and M2S morphotypes, and the fourth subclade (genotype G2b) was composed solely by M2S endophytes (stromata producers) and identified with the *tub2* gene. Sequences of endophytes isolated from double infected plants (signaled with letters "a" and "b" in Figure 9, Figure 10 and Figure 11) were distributed along the three first genotypic groups.

The presence of the gene *ltmQ*, related to the production of lolitrem precursors, was detected in *Epichloë* endophytes belonging to M1 and M3 morphotypes, with no detection of this gene neither in M2 and M2S morphotypes or in the hybrid LpTG-2 (Figure 13).

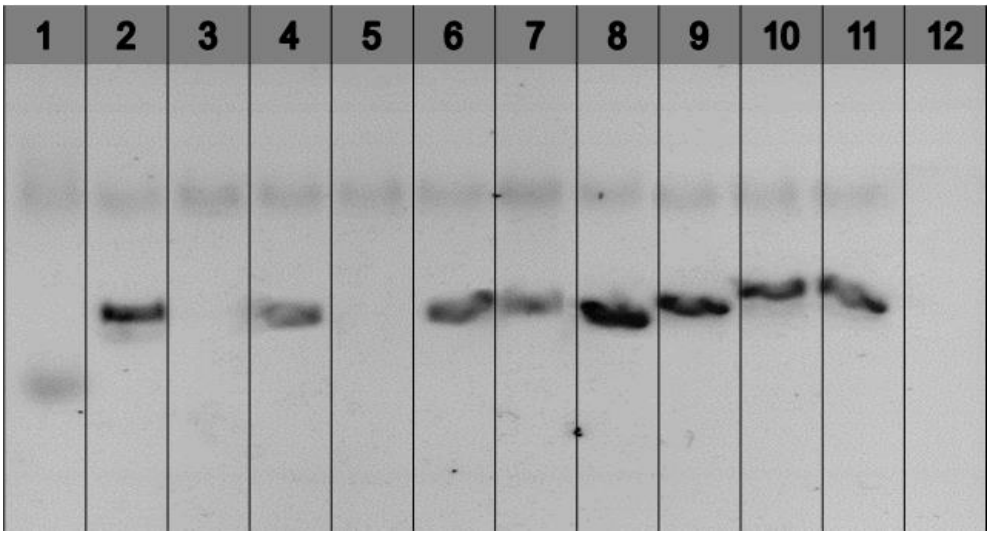

Figure 12 Electrophoresis gel for PCR screening of the *ltm* gene in different morphotypes of *Epichloë* endophytes: hybrid LpTG-2 (AR6) (lane 1), M1 (lanes 4, 7, 11), M2 (lane 5), M2S (lane 3), M3 (lanes 6, 9, 10) and blank (lane 12).

To check the possible existence of hybrids among the *Epichloë* isolates, all sequences were carefully studied for ambiguities, all the β-tubulin sequences of the isolated *Epichloë*, indicating only a single form of the gene (Schardl et al. 1994). All the endophytes selected to evaluate their hybrid condition through the Southern Blot showed a single copy of the β-tubulin (*tub2*) gene, only the control (LpTG-2) showed two distinct bands indicative of the existence of two copies of the *tub2* gene (Figure 13, lane 3).

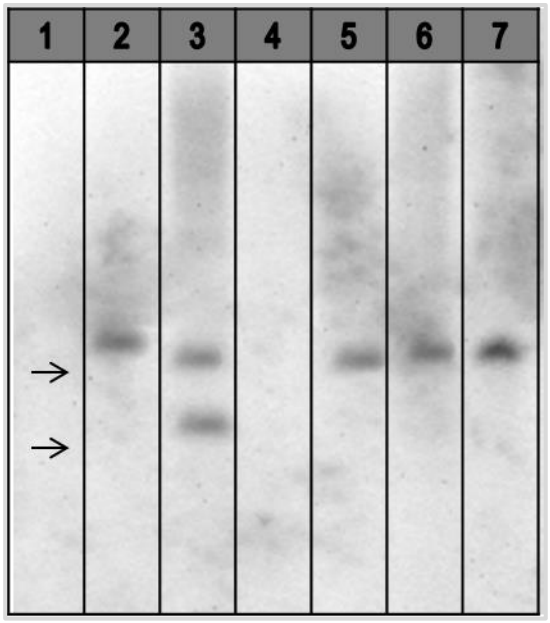

Figure 13 Southern blot analysis of β-tubulin (*tub2*) genes for determination of *Epichloë* hybrids isolated of *Lolium perenne*. Total DNA preparations were from morphotypes: M1 (lanes 1, 2 and 5), M2 (lane 7), M2S (lane 4) M3 (lane 6) and hybrid LpTG-2 (AR6)(lane 3).

Hybrids of *Epichloë* were not detected neither by Southern blot or through cloning. The cloning test, showed that the six sequences of the *E. coli* DH5α cells transformed with the t*ub2* amplicons from the M1, M2, M2S and M3 *Epichloë* morphotypes were 100% identical. On the other hand, two of the six sequences of the *E. coli* DH5α cell transformed with t*ub2* amplicons from the AR6 hybrid, were identical to a reference sequence of *E. festucae* var. *lolii* and close to endophytes of the M1 and M3 morphotypes; one sequence was closer to *E. typhina* and M2 and M2S morphotypes; and three sequences, were chimeras, different between themselves, having fragments of the t*ub2* gene of both *E. festucae* var. *lolii* and *E. typhina* randomly ordered.

I.5 DISCUSSION

Epichloë endophytes were present at all the locations surveyed, and their incidence was relatively high ($\bar{x} = 43.0\%$) in comparison to those observed in other surveys of wild populations of *Lolium perenne* in Europe. Lewis et al. (1997), analyzed 523 perennial ryegrass populations from 20 different European countries and found *Epichloë* endophytes in 62% of the accessions, 48% of them had incidences ranging between 1% and 50%, and only 14% between 50% and 100%. In a survey of 262 wild populations of ryegrass in France, Ravel et al. (1997b), found *Epichloë* endophytes in 47% of the locations and the average infection rate was of 25%. In Germany, Oldenburg (1997) reported incidences below 30% on most locations analyzed, and Hesse (2002), found endophytes in 74% of the tested sites, with incidences lower than 50%. Other studies have reported between 64% - 72% of locations infected by *Epichloë* endophytes, with infection rates of 33% in Northern Spain, 18% in Denmark and 6% in

Germany ,(Oliveira and Castro 1998; Jensen and Roulund 2004; Dobrindt et al. 2013), but no survey has reported the presence of *Epichloë* in 100% of the examined locations, as in this work.

The higher frequency of *Epichloë* in *L. perenne* observed in this survey could be related to the dry climatic conditions of western Spain, where plants were collected. Greater infection levels have been reported for warm and dry areas, suggesting that such conditions may impart a selection pressure that favors *Epichloë* infection (Lewis et al. 1997; Oliveira and Castro 1998; Hesse et al. 2003). For example, Lewis et al. (1997) found that infection frequency was significantly related to water-supply deficit, an indicator of drought stress, exerting a selection pressure in favor of endophyte infection. In France, Leyronas and Raynal (2001) observed that endophytes most often were present where the grass may suffer from summer drought, and Gibert et al. (2012) also found that *Epichloë* symbiosis was negatively correlated to water availability analyzing 22 perennial ryegrass populations from the French Pyrenees, and reported that *Epichloë* infection increased plant survival in xeric populations, and reinforced competitiveness in mesic populations. In this same regard, Hesse (2002), found an endophyte-inducted increase in root dry weight and root/shoot ratio that could be beneficial for plant persistence, especially on sites where water is a growth-limiting factor. This may be of vital importance for plant survival, especially in locations where water is the limiting factor, and could help explain the greater number of infected plants found on dry sites and in regions with a Mediterranean climate (Hesse et al. 2003). Thereby water availability appears to be an important environmental factor in endophytic symbioses functioning in the natural environment, but such effect under water-limited conditions is still a subject of debate (Gibert et al. 2012). According to the results of this chapter, it was found a high incidence of *Epichloë* in the locations with lower precipitation (LVA 56% and TAB 49%), following the reported trend, but the location with the highest precipitation (PI) was not the one with the lowest endophyte incidence. Therefore, even when environmental conditions may highly influence the incidence of *Epichloë*, there are other factors which are known to also impact negative or positively on the endophyte infection rates, such as the endophyte and the plant genotypes, pest and grazing pressure, or soil nutritional condition.

Epichloë endophytes were isolated from 154 plants representing a wide range of perennial ryegrass wild ecotypes. Each *Epichloë* isolate was kept under the same conditions and culture media. Two of the criteria for differentiation among the *Epichloë* endophytes were the delay in emergence from grass tissues, and the radial growth rates of colonies. The lapses of time that took to the *Epichloë* hyphae to be visible in PDA were similar to those reported by Christensen et al. (1991), that described some *Epichloë* isolates from *L. perenne* visible within three to seven days. Although most *Epichloë* described herein required at least ten incubation days for mycelium to emerge, with some isolates taking over 30 days.

Endophytes from asymptomatic plants were classified according to their macroscopic features into three groups (M1, M2, M3) and it was decided to group stromata-producing endophytes in a fourth group (M2S) due to the important physiological alterations in their host

grass. Since the way of reproduction and dissemination of the *Epichloë* endophytes is an important factor for their taxonomic classification, it was found that 89.8% of *Epichloë*-infected plants were asymptomatic, harboring M1, M2 and M3 morphotypes, and 10.2% of ryegrass plants were infected with stromata-producing endophytes (M2S morphotype). *Epichloë* endophytes belonging to M2 morphotype (13.2% of isolates), produced stromata in some plants when they were transplanted at a field-plot but not when they were growing in pots. Zabalgogeazcoa et al. (1999), observed that in *Festuca rubra* infected by *E. festucae*, stromata may be formed occasionally and only a small number of reproductive tillers or any plant may be affected. Thus, it has been speculated that when pooid grasses are occasionally infected by *E. typhina* from other grass species, the new hosts could suppress the choke stage and that certain environmental conditions (*e*. *g*. low nitrogen fertility) can cause moderate to high levels of choke disease (Schardl et al. 1991). Around the sample sites where *L. perenne* was collected, other grass species such as *Brachypodium phoenicoides*, *Holcus lanatus* and, *Dactylis glomerata* have been detected as infected with *E. typhina* (Zabalgogeazcoa et al. 2003; Zabalgogeazcoa et al. 2008), therefore the possibility of *Epichloë typhina* from other hosts infecting ryegrass samples is highly probable.

Considering the microscopic observation in all cases (asymptomatic: M1, M3, M2; and stroma-producer endophytes: M2S) it was observed the same hyphal pattern *in planta* that fit with the descriptions reported by Christensen et al. (2002) of *Epichloë* endophytes in the tissues of ryegrass. However, there were differences in their ability to produce conidia: the M3 endophytes were sterile, with no production of conidia; and even though the M1 and M2 morphotypes produced conidia with similar shape and sizes, the conditions needed for their production were distinct. Clay (1990), indicated that sexual and asexual *Epichloë* endophytes have similar conidial morphology in culture and Christensen et al. (1993), reported that conidia of slow growing *Epichloë* fungi were not observed when grown at 20 °C but some produced conidia when grown on PDA or cornmeal dextrose agar at 10 °C. Differences in conidia length produced by M1 and M2 morphotypes with respect to the hybrid AR6, according to Kuldau et al. (1999), are due to genome size, in this case the larger conidia are indicator of heteroploidy; whereas shorter conidia size produced by M1 and M2 morphotypes could reflect their haploid condition with apparent no hybrid nature.

Combining macro- and microscopic morphological characteristics, the M1 morphotypes fit with descriptions of *E. festucae* var. *lolii*, and M2S are phenotypically similar to *E. typhina* (Christensen et al. 1991; Bony et al. 2001). However, although it is known that exist high morphological variability in ecotypes of the *Epichloë* fungi and particularly among *E. festucae* var. *lolii* strains (Bony et al. 2001) there are no reports of morphological descriptions similar to isolates designated as M3.

Besides morphological, physiological and biochemical characteristics considered in taxonomy of the grass mycosymbionts, DNA sequence comparisons provide direct indications of relative diversity and genetic interrelationships (Schardl et al. 1991). The sequence-based phylogram with two molecular markers widely used in fungi classification (ITS and *tub2*),

contritubes to have a taxonomical approximation of groups of *Epichloë* endophytes isolated. The concatenated dendrogram displayed strong bootstrap support for the presence of two main clades conformed by four major genotypes, which coincided with the morphological division of the endophytes. In the first clade the sequences of asexual endophytes M1 and M3 were allocated, together with *E. festucae* var. *lolii* reference sequences. Some M1 isolates were genetically indistinguishable of morphotype M3, although other differences were discernible, not only morphologically, but also in their ability to sporulate and in their alkaloid profiles (Chapter IV); therefore, undoubtedly they represent different biotypes of *Epichloë* endophytes. The second clade grouped sequences genetically similar to *E*. *typhina* (M2 and M2S morphotypes). The subclade or genotype designated G2b was composed uniquely for four sequences of M2S endophytes; whereas, the genotype G2a was most common and was composed by producers, non-producers and facultative stomata producer fungi. In this regard, there are reports which propose that sexual endophytes such as *E. typhina* are comprised by a group of cryptic species representing different taxa, including subspecies (Clay and Schardl 2002; Zabalgogeazcoa et al. 2008; Leuchtmann et al. 2014; Schirrmann and Leuchtmann 2015).

Respect to the difficulties found to separate genetically the M1 and M3 morphotypes and to divide the genetic group G2a between fungi with the ability to produce or not stromata, Craven et al. (2001b) pointed up that there are potential limitations if sequence analyses do not adequately fit with biological speciation, and it may be difficult to identify species on a strictly phylogenetic basis.

In this survey, the asexual endophytes with morphological and genetic similarity to *E. festucae* var. *lolii* were predominant (M1= 48.2% of isolates). In other surveys, it has been reported that this endophyte is predominant in wild and cultivated accessions in France, Denmark, New Zealand, Australia and other countries (Bony et al. 2001; van Zijll de Jong et al. 2008). Endophytes of one genetic group (G1b) were isolated exclusively from plants of two communities (LVE and VAF). van Zijll de Jong et al. (2008), reported that most endophytes in their survey clustered into three groups that corresponded to major geographical regions, probably due that natural habitats have different types of selection pressure over the plants and these endophytes, as obligate symbionts, co-evolve with their host (Craven et al. 2001b; Clay and Schardl 2002; Hesse et al. 2003).

The *Epichloë* endophytes isolated in this survey; were classified into four major taxonomic groups, similar to those described by Moon (1999), but without the detection of *Epichloë* hybrids as indicated by sequence chromatograms, the Southern blot and cloning assays as well the conidia size of the M1 and M2 morphotypes. In some of the ryegrass communities conditions that afford the opportunity for fungal hybridization were observed; for example, several *Epichloë* morphotypes in the same grass community occurred in sympatry, and the presence of doubly infected plants may result in hyphal fusion, and parasexual recombination might occur (Clay and Schardl 2002). Schirrmann and Leuchtmann (2015) explained that formation of interspecific *Epichloë* hybrids can be limited by differences in the host phenological stage, habitat or abiotic factors. Thus, Clay and Schardl (2002)

observed that in plants containing two endophytes most tillers were infected with just a single endophyte and Wille et al. (1999) reported that in *Bromus erectus* artificially inoculated with two different strains of *E. bromicola* only one of 139 analyzed tillers was colonized by two endophyte genotypes, they explained that since this tiller was young at the time of harvest, it is conceivable that this co-existence would have disappeared with increasing tiller age.

Despite the absence of hybrids in the *Epichloë* endophytes studied, a wide range of possible endophyte combinations was found in the set of analyzed plants, resulting in at least six perennial ryegrass ecotypes in agreement with the morphological and genetic features: M1(G1a), M1(G1b), M2(G2a), M2S(G2a), M2S(G2b), M3(G1a); plus the occurrence of plants with double infections: DI(M1/M2), DI(M2/M3), DI(M2S/M1) or DI(M2S/M3). This wide variety of *Epichloë*/ryegrass associations may include some ecotypes with useful alkaloid profiles (Chapter II) or improved forage quality (chapter IV) which represent an important source of biological material for improving forage grasses.

I.6 CONCLUSIONS

Results of this chapter have indicated that there was high diversity of fungal *Epichloë* endophytes between and within the eight wild locations of *Lolium perenne* studied. Among asymptomatic ryegrass plants three culturable morphotypes of *Epichloë* (M1, M2 and M3) were characterized, plus another morphotype obtained from stroma producing plants (M2S). These endophlytic fungi characterized into four morphotype were classified into two species according to the genotypic analysis: M1 and M3 morphotypes belonged to *Epichloë festuae* var. *lolii*¸ and M2 and M2S morphotypes were *E. typhina* and several several genotypic groups were detected among them. There were two main genotypes of *E. festucae* var. *lolii*, the major of them (G1a) included part of colonies with the M1 morphotype and all the colonies with the M3 morphotype, the other genotypic group (G1b) were composed by endophytes with the M1 morphotype isolated uniquely from plants of two locations; the endophyte classified as *E. festucae* were also composed by two distinctive genotypes, the most numerous (G2a) encompass strains with variable ability to produce stromata in their host grass, the second genotype (G2b) consisted in fungi that always produced stromata in their host.

It was observed that in wild population of *L. penne* a wide range of possible endophyte combinations was found, resulting in at least six perennial ryegrass ecotypes in agreement with the morphological and genetic features: M1(G1a), M1(G1b), M2(G2a), M2S(G2a), M2S(G2b), M3(G1a); plus the occurrence of plants with double infections: DI(M1/M2), DI(M2/M3), DI(M2S/M1) or DI(M2S/M3). This wide variety of *Epichloë*/ryegrass associations may include some ecotypes that could withstand better to particular stressful conditions, which represent an important source of biological material for improving forage grasses.

II. ALKALOID PRODUCTION IN LOLIUM PERENNE IN RELATION TO THE EPICHLOË MORPHOTYPE HOSTED

II.1 ABSTRACT

Alkaloids production in *Lolium perenne* plants is firstly controlled by *Epichloë* endophytes, although there are other factors that can influence their synthesis and concentration. There have been lot of researches on symbiotic relationship of commercial cultivars of ryegrass with *Epichloë* endophytes; however, studies about the influence of *Epichloë* on alkaloid production in non-cultivated perennial ryegrass are scarce. In this chapter, the concentration of peramine, lolitrem B and ergovaline alkaloids in a heterogeneous set of perennial ryegrass from wild origin was analyzed. These plants were naturally infected with one of the asymptomatic (EO, M1, M2, and M3) or stromata producer (M2S) *Epichloë* endophytes. In addition, these analyses were also done for plants doubly infected with two different *Epichloë* endophytes (DI). The concentrations of alkaloids were studied at three harvests: October 2013 in wirehouse, and May and November 2014 in a field-plot. Additionally, alkaloid production was evaluated into two commercial cultivars of ryegrass artificially inoculated with selected *Epichloë* strains.

The taxonomic group of the *Epichloë* endophyte had the strongest influence on synthesis and proportion of plants in which each alkaloid was produced. Plants harboring M2S *Epichloë* endophytes had the highest concentration of peramine; plants infected with M3 endophytes produced more lolitrem B, and the highest concentration of ergovaline was found in ryegrass with endophytes M1. In DI-plants, it was observed the same patterns of alkaloid production but with higher concentration than in the single infected ones, possibly as result of a synergistic effect. The harvest time also had influence on proportion of plants and concentration on which each alkaloid was detected. The proportion of plants that produced peramine and their concentrations were higher in ryegrass from wirehouse. Lolitrem B was produced in a lower percentage in plants from the wirehouse but in higher concentration than in plants from the field-plot. Ergovaline was found more frequently in ryegrass plants from wirehouse, but did not varied among harvests. In inoculated plants, the type of alkaloid produced and its concentration was dependent on the morphotype of the *Epichloë* endophyte hosted. Although, the presence of alkaloids was detected only in half of the successfully inoculated plants; thus, peramine was detected in higher concentration and lolitrem B in lesser quantity than in naturally infected ryegrasses; whereas, in any plant presence of ergovaline was detected; which indicates that the host grass also exerted influence on alkaloid production.

II.2 INTRODUCTION

One of the most studied and an important characteristic on symbiotic interactions of grasses with *Epichloë* endophytes is related to the production of secondary metabolites. *Epichloë* endophytes produce *in planta* a range of alkaloids and the best-known can been grouped into four classes including, pyrrolopyrazine (peramine), indole-diterpenes (lolitrem B), the ergot alkaloids (ergovaline) and aminopyrrolizidines (lolines) (Figure 14). These alkaloids enhance the competitive ability of endophyte-infected grasses by protecting them from herbivory; and the affected organisms depend upon the type of alkaloids produced that, in general, may be involved in anti-insect (peramine and lolines) and/or anti-mammalian activities (lolitrems and ergot) (Schardl et al. 1991; Siegel and Bush 1996; Bush et al. 1997; Young et al. 2009).

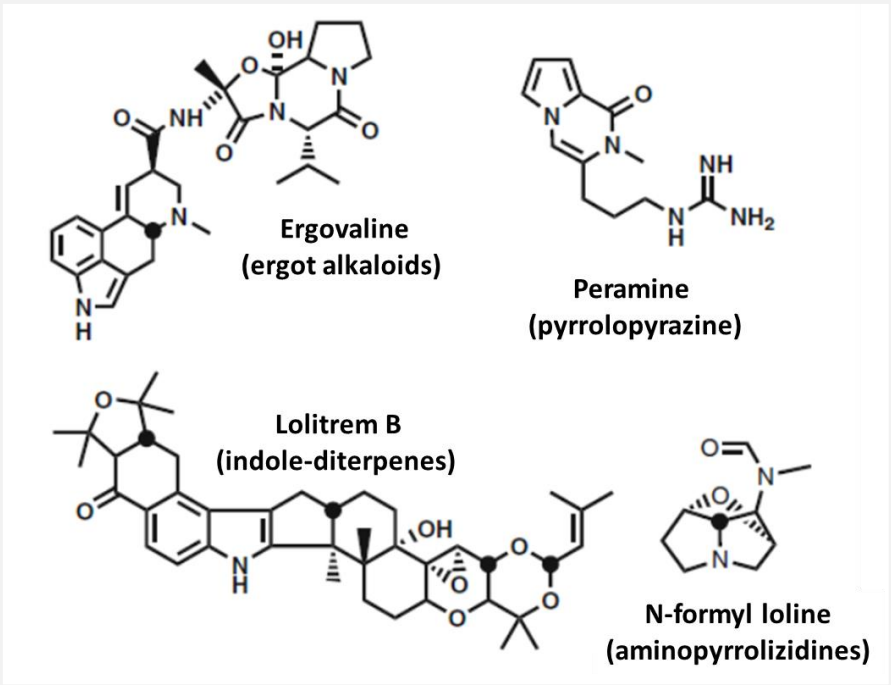

Figure 14 Chemical structures of the most common compounds for each chemical group of alkaloid produced in grasses infected with *Epichloë* endophytes.

Peramine is the only known pyrrolopyrazine alkaloid produced and it is also the most widely distributed alkaloid in *Epichloë*-grass associations (Lane et al. 2000; Clay and Schardl 2002). Peramine consists of a lipophilic pyrrolopyrazine ring and a hydrophilic guanidinyl side chain, and it is less lipophilic than the other alkaloids (Siegel and Bush 1996). Peramine was first identified in extracts of perennial ryegrass infected with *Epichloë festucae* var. *lolii* (Rowan et al. 1986*)*, and it has been shown to be a potent feeding deterrent of adult Argentine stem weevil (*Listronotus bonariensis*), an economically important pest in New Zealand, and also of other insect herbivores (Rowan et al. 1990; Rowan 1993).

Indole-diterpene alkaloids in *Epichloë*-grass associations are known commonly as lolitrems, these compounds have a core structure comprising a cyclic diterpene skeleton. Lolitrem B, is the predominant compound from this group and is responsible for ryegrass staggers, a neuromuscular disorder in which affected mammals develop ataxia and tremors. Sheep experienced short prancing steps usually resulting in arched back and rigid extended limbs held in a tetanic spasm of several minutes duration and cattle usually collapse onto the brisket with legs splayed (Mortimer 1983; McLeay et al. 1999). This syndrome has been commonly identified in livestock grazing ryegrass pastures infected with *E. festucae* var. *lolii,* after long periods of water stress (Fletcher and Harvey 1981; Gallagher et al. 1984; Gallagher et al. 1985). Lolitrem B also has biological activity against Argentine stem weevil larvae and other insects (Prestidge and Gallagher 1988). Other classes of indole-diterpenes, the epoxy-janthitrems and the terpendoles that also have been isolated from perennial ryegrass infected with *E. festucae var. lolii,* have similar antiherbivore activity than lolitrem B (Gatenby et al. 1999; Parker and Scott 2004; Tapper and Lane 2004).

The ergot alkaloids are represented by several compounds with an ergolene ring system, and ergovaline is the predominant *Epichloë*-derived ergot alkaloid in grasses (Garner et al. 1993; Guerre 2015). This alkaloid is the cause of fescue toxicosis, that occurs in cattle grazing tall fescue pastures infected with *E. coenophiala*, effects of which may include poor weight gain, hyperthermia, convulsions, reduced fertility, gangrene of the extremities and death (Bacon 1995). In perennial ryegrass, ergovaline has been associated to vasoconstriction (Dyer 1993), which reduces peripheral blood flow (Rhodes et al. 1991; Schmidt and Osborn 1993) and the ability to dissipate heat and therefore can exacerbate ryegrass staggers in sheep (Bluett et al. 2005). Ergovaline also enhance the persistence and productivity of ryegrass pastures by protecting them against attacks of black beetle *(Heteronychus arator)* (Ball et al. 1997b).

The alkaloids commonly referred to as lolines are saturated pyrrolizidines that have a strained ether bridge between aliphatic carbon atoms and they occur almost exclusively in grasses associated with *Epichloë*. The major loline alkaloids are *N*-formylloline, *N*-acetylloline and *N*-acetylnorloline and all of them exhibit broad spectrum deterrence and insecticidal activity (Siegel et al. 1990; Bush et al. 1997; Wilkinson et al. 2000; Schardl et al. 2007). *N*-acetylnorloline, has been reported as the causative agent of fescue eodema, a toxic syndrome whose clinical signs include inappetence, depression, and subcutaneous oedema of the head, neck, chest and abdomen of horses grazing pastures of tall fescue (*Schedonorus arundinaceus* = *Festuca arundinacea* = *Lolium arundinaceum*) infected with some specific strains of *E. coenophiala* (Bourke et al. 2009). Wounding of plants induces high levels of lolines, that can accumulate up to 2% of the dry weight of the infected plant's dry mass, exceeding in quantity other alkaloids (Spiering et al. 2002).

The presence of *Epichloë* in pastures has caused important economic losses due to the toxic effects of the alkaloids, in countries like US, New Zealand or Australia where monocultures of endophyte-infected tall fescue and perennial ryegrass occupy large areas. In US, Allen and Segarra (2001) estimated losses of about 2000 million USD in beef industry, as impact in loss of weight in cattle; and losses of 340 million USD in reproductive and death of livestock. In addition, Reed et al. (2004) highlighted that losses caused by subclinical concentration of endophyte toxins may greatly outweigh the economic losses associated with staggers, heat stress and deaths associated with clinical symptoms. On the other hand, Argentine stem weevil is ranked as the most important pasture insect pest in New Zealand, the total cost to the country being estimated at 78 to 251 million AUD per year. Several observations have shown that the presence of peramine alkaloid is crucial to ryegrass pastures for protection against this pest. As a consequence, in pasture grazing systems, current strategies for forage grass improvement focus on the utilization of selected endophytes which maintain insect deterrent properties (peramine and lolines) while minimizing negative detrimental impact of alkaloids toxic to livestock (lolitrem B and ergovaline) (Repussard et al. 2014b).

Depending on their genetic background, *Epichloë* endophytes are responsible for the synthesis of the four main classes of alkaloids. Particular gene clusters are involved in the biosynthesis of each alkaloid including ergot alkaloids, indole-diterpenes, and lolines (Panaccione et al. 2001; Spiering et al. 2002; Wang et al. 2004; Spiering et al. 2005b; Young et al. 2005; Schardl et al. 2007) ; whereas, a single gene is required for peramine production (Tanaka et al. 2005). Nevertheless, a grass naturally infected with *Epichloë* endophyte that possesses all four alkaloid classes has not been identified yet. Three types of alkaloids have been detected in perennial ryegrass infected with its most common endophyte, *E. festucae* var. *lolii*, those include peramine, lolitrem B and ergovaline (Siegel and Bush 1996; Bush et al. 1997). No loline alkaloids have been detected in perennial ryegrass infected with *E. typhina*, *E. festucae* var. *lolii* or the hybrid LpTG-2 (*E. typhina* × *E. festucae* var. *lolii*) (Siegel et al. 1990; Schardl et al. 2004a), because they lack the complete gene cluster essential for loline synthesis (Spiering et al. 2005b; Young et al. 2013); whereas *E. festucae*, in *Festuca rubra* had heritable variation for loline expression (Wilkinson et al. 2000). In inoculation tests of perennial ryegrass with *E. coenophiala* isolated from *F. arundinacea* production of lolines has been detected, although with lesser concentration than in the original host (Siegel et al. 1990; Bush et al. 1993).

Besides the endophyte genetic background, alkaloid production and concentration in plants are affected by factors, like host plant genotype, plant tissue and phenological stage and environment. Expression studies have shown that the genes controlling alkaloid biosynthesis are up regulated *in planta*, which suggest that host-specific signaling influences the pathways on alkaloid production (Spiering et al. 2002; Young et al. 2005; Tanaka et al. 2006; Hahn et al. 2008; Zhang 2008). Thus different alkaloid profiles can be expected in the same grass-endophytes species combination.

Each epichloid-alkaloid has a particular pattern of accumulation in the host grass according to the phenological stage of the plant and the hyphal growth of the endophyte. Peramine, as a water-soluble compound, is almost evenly distributed in the plant and does not accumulate in older tissues; its concentration decreases as leaf age increases, and it is higher in the regrowth component than in the basal parts of the plant (Keogh et al. 1996; Ball et al. 1997a). The lolitrem B and ergovaline alkaloids, which are lipophilic, and consequently less readily mobile, are more closely linked to endophyte mycelium (Ball et al. 1995; Keogh et al. 1996; Ball et al. 1997a). Lolitrem B accumulates over time, showing a negative basal-apical gradient, its concentration is high in the older tissues in the sheath of outer leaves or dead leaf material, where the fungal mycelium is declining (di Menna et al. 1992; Keogh et al. 1996; Ball et al. 1997a). The concentration of ergovaline follows a basal-apical increase up the sheath and the leaf blade above the ligular zone, paralleling the concentration of mycelium, with the highest concentrations in the crown and inflorescence (Lane et al. 1997; Lane et al. 2000; Reed et al. 2004). Lolines accumulate during the vegetative stage, mostly in the pseudostem with the lesser concentration in the leaf blade and senescent tissues (Siegel and Bush 1996).

Alkaloid levels in plants have strong seasonal variations, as a consequence of the plant development and endophyte life cycle (Lane et al. 2000). Concentrations tend to be lower in winter and early spring, rising with inflorescence development in late spring, and peaking again in late summer with seed production which is the transmission way of the endophyte (protection) (Ball et al. 1997a; Repussard et al. 2014b). Several environmental factors can affect alkaloid concentrations. It has been reported that alkaloid production increased when water availability decreases (Belesky et al. 1989; Eerens et al. 1998; Hahn et al. 2008). Fertilizer supply can also alter alkaloid concentrations; for example, high levels of soil nitrogen and phosphorus can increase the production of ergopeptide alkaloids (Arechavaleta et al. 1992; Malinowski et al. 1998; Richardson et al. 1999). Some studies revealed an increase in ergovaline concentrations with nitrogen fertilization, but this effect varied with the dose, the kind of fertilizer, and the year of the assay (Lyons et al. 1986; Belesky et al. 1988; Rottinghaus et al. 1991). The effects of nitrogen fertilization also seem to vary with the part of the plant analyzed, an increase of ergovaline level was observed in the leaves but not in the inflorescence (Repussard et al. 2014a; Guerre 2015).

The ability of some *Epichloë* endophytes to form artificial symbioses with different species, genera or tribes of plants, offers a powerful tool for the development of new beneficial grass-endophyte associations (Simpson and Mace 2012; Young et al. 2013). Taking advantage of this feature, characterization of naturally occurring *Epichloë* endophytes, and their subsequent artificial inoculation on endophyte-free grass cultivars (Siegel et al. 1990; Gillanders 2007), have made possible that currently, several commercial grasses contain endophytes that do not lead to the accumulation of toxic alkaloids for livestock in the host plant. Moreover, turfgrass infected with non-pathogenic fungal endophytes may help meet demands for reduced pesticide use and for lower inputs in maintenance of turf and sod production. For instance, in perennial ryegrass cultivars, the endophyte AR1 does not produce lolitrem B or ergovaline, but produces peramine (insecticide). Endophytes NEA2, NEA3 and

NEA6 produce both ergovaline and peramine but no lolitrem B (Rasmussen et al. 2007; van Zijll de Jong et al. 2008; Schardl et al. 2012; Young et al. 2013; Zhou et al. 2014; Kaur et al. 2015).

Several studies on alkaloid production in perennial ryegrass have been performed in cultivated pastures infected with the asymptomatic endophyte *E. festucae var.lolii* because it is the most common *Epichloë* species in such systems. However, in wild populations of perennial ryegrass, it has been detected a wide variability among the *Epichloë* endophytes hosted, as reported in Chapter I. Because the effects of a grass-endophyte association depends on its particular reaction to all the surrounding factors, endophyte-infected grasses from different environmental conditions could have different alkaloid profiles and some of them could be useful for improving the performance of commercial cultivars of grasses. The aim of this Chapter was to screen the diversity on alkaloid profiles of *Epichloë* endophytes from six different natural populations of perennial ryegrass located in western Spain, and the behavior of selected endophytes when inoculated in commercial varieties of ryegrass. Specific objectives were: (i) to analyze the concentration of the alkaloids peramine, lolitrem B and ergovaline in a heterogeneous set of *L. perenne* plants naturally infected with *Epichloë* endophytes having different morphotypes: is there any relationship between alkaloid profile and *Epichloë*-morphotype?; and, (ii) to evaluate the alkaloid production of two commercial varieties of perennial ryegrass inoculated with 10 selected *Epichloë* endophytes.

II.3 MATERIALS AND METHODS

II.3.1 Plant material

The plants of *Lolium perenne* used in this Chapter were selected from those described in Chapter I (Table 1), which were collected at six locations corresponding to different ecosystems. For each plant, endophyte status (E-= non-infected, E+= *Epichloë*-infected) and morphotype (M1, M2, M2S, M3) was known as indicated in Chapter I (Table 2, Figure 6). As the number of plants infected with a specific morphotype of *Epichloë* endophyte was different among locations, plants samples were selected trying to cover the widest taxonomic endophytic variability over the plant origin. Thus, a total of 148 ryegrass plants grown in two different conditions were screened for alkaloid production: (i) in pots in a wirehouse and (ii) in a field-plot.

The set of samples from the wirehouse consisted of 80 plants of *L. perenne* (65 E+ and 15 E-), transplanted in March 2013 to individual pots with a potting mix of perlite:peat moss (1:1, v/v). Clones of 48 of these E+ plants were also grown in the filed plot. Pots were maintained outdoors in a randomized arrangement, rotating their position frequently, watering regularly and fertilizing them once a year with a liquid commercial fertilizer. Ryegrass plants were harvested in vegetative stage in October 8, 2013, and all samples were composed of leaves and pseudostems. At this harvest, plants bearing M2S endophytes had not developed 'choke' disease symptoms (stromata Figure 7).

For the field plot, E+ and E- plants, were transplanted on October 2013, to a clay eutric chromic cambisol (FAO/UNESCO 1998), in the experimental farm Muñovela (Salamanca, Spain; 40°54'19" N, 5°46'28" W; 780 masl; annual precipitation 372 mm, and mean annual temperature 12.7 °C). A distance of 50 cm was left between neighboring plants, they were watered during their establishment but not thereafter, were not fertilized and the plot was manually maintained free of weeds (without herbicides). On May 2014, all E+ plants (107) and 9 E- plants randomly selected were harvested at the flowering stage, asymptomatic plants had developed panicles and plants infected with M2S endophytes had stromata in developing reproductive stems. On November 2014, a subset of 30 plants was harvested at vegetative stage (regrowth) when M2S-ryegrass had no longer stromata. Double infected plant samples (harboring two different *Epichloë*-morphotypes) from the field-plot, harvested on May 2014, consisted mainly of tillers with stromata because flowering healthy tillers were preserved to obtain seeds (to germinate and follow plant growth).

In all cases, ryegrass plants were harvested by cutting all aboveground biomass at approximately 5 cm from the soil surface. Plant samples were stored at -80.0 °C, freeze dried and ground to 0.5 mm using a hammer mill (Fritsch 15303).

II.3.2 Inoculation of ryegrass with *Epichloë* endophytes

For the evaluation of alkaloid concentration in commercial cultivar of ryegrass as effect of the Epichloë endophyte hosted, a set of sample plants from the inoculation assay (Chapter III) was selected. Ryegrass plants were infected with 10 different inocula of *Epichloë* endophytes, these fungi had been morphologically and genetically characterized, and the alkaloid profile produced on their natural perennial ryegrass host was known. Endophytes had been inoculated into two commercial cultivars of ryegrass: 'Barplus', a turfgrass cultivar, and 'Romance' a forage cultivar (Barengbrug, NL). After inoculation, ryegrass seedlings were placed in water agar (1%) plates, incubated in a growth chamber (25 °C, 12h of light, 60% relative humidity) (Sanyo MLR-351H) for 15 days, transplanted to plastic seedbeds (5x5x10 cm) containing a sterile potting mix and then maintained in a glasshouse. Three months later, *Epichloë* infection was corroborated by direct fungal isolation in potato dextrose agar. Infected ryegrass plants (E+) and the same amount of non *Epichloë*-infected plants (E-) were transplanted to individual 2 l pots with the usual potting mix, maintained outdoor in a wirehouse, watering regularly and fertilizing them once a year. Inoculated ryegrass plants were harvested for alkaloid analyses at different vegetative stages on April, June and August 2014.

II.3.3 Alkaloid analysis

Chemical analyses of the alkaloids peramine, lolitrem B and ergovaline were performed in a total of 195 ryegrass wild plants: 149 *Epichloë*-infected and 24 E-; and in 46 inoculated plants. Each alkaloid was analyzed separately, using some modifications of published high-performance liquid chromatography (HPLC) methods.

Peramine was extracted following the technique described by Barker et al. (1993), adding 3.0 ml of a 30% propan-2-ol solution to 100 mg of ground ryegrass and kept 30 min at

90 °C. After centrifugation (12 000 rpm, 5 min), the extract was passed through a CBA cartridge (Carboxylic acid 100 mg, Agilent Bond Elut) preconditioned with a mix of 80% aqueous methanol and 2.0% ammonium hydroxide, then cleaned up with 1.0 ml of methanol. The sample was eluted with 1.0 ml of 5% formic acid (v/v) in 80% aqueous methanol (v/v). Quantification was done by comparing the peaks of samples and a stock solution prepared with a standard of peramine, donated by G. Lane (AgResearch, New Zealand). The analysis was performed by HPLC (Waters module 2695) with a C18 column 3.9 x 150 mm; 4.0 µm (Waters Nova Pak 036975) using a Photodiode Array detector (Waters 996) set at 230 nm. Mobile phase was isocratic composed by 15% acetonitrile and 85% of buffer 10 mM guanidine carbonate and 0.16% formic acid, with a flow rate of 0.7 ml min^{-1}.

Quantification of lolitrem B was based on the method indicated by Gallagher et al. (1985). For the extraction, 1.5 ml of a chloroform:methanol (2:1, v/v) solution was added to a 100 mg of ground sample and the mixture was kept for 60 min in an orbital shaker at 150 rpm, and then centrifuged (12 000 rpm, 5 min). The supernatant was cleaned up with 0.5 ml of dichloromethane, filtered through a 0.45 µm nylon disk and then evaporated to dryness with a nitrogen stream. The residue was dissolved in 1.5 ml of dichloromethane and was passed through a silica cartridge (100 mg, Waters Sep-Pak) previously conditioned with 2.0 ml of dichloromethane. For quantification, it was used a standard of lolitrem B provided by C. Miller (AgResearch, New Zealand). The samples peaks were compared with those of a lolitrem B from standard solution, using a HPLC with a module Waters 2695, a silica column 250 x 4.6 mm, 5.0 µm (Waters Spherisorb) and a fluorescence detector (Waters 2475) λ_{exc}= 268 nm; λ_{em}= 440 nm. The mobile phase was composed of 80% dichloromethane and 20% acetonitrile, with flow rate of 1.0 ml min^{-1}.

The procedure descripted by Yue et al. (2000) was performed to determine the concentration of ergovaline. Extraction was conducted with 0.5 g of ground ryegrass, adding 10 ml of chloroform, 0.5 ml of 5 mM sodium hydroxide in methanol, and an internal standard of ergotamine (Sigma-Aldrich). The mixture was placed on an orbital shaker at 150 rpm for 120 min, paper-filtered (Filter Lab 1240) and washed with 3.0 ml of chloroform and then passed through ergosil – HL silica gel (500 mg, Analtech) columns preconditioned with 5.0 ml of chloroform. For elimination of pigments, a solution of 5.0 ml chloroform:acetone (75:25 v/v) was added to the filtered solution, elution was with 3.0 ml of methanol taken to dryness with nitrogen stream, ergovaline was dissolved in 1.0 ml of methanol and filtered with a nylon disk (0.45 µm). Ergovaline quantification was performed by reverse phase HPLC in a module Waters 2695, a C18 column 150 x 4.6 mm; 2.7 µm (Agilent Poroshell) and a fluorescence detector (Waters 2475) λ_{exc}= 250 nm; λ_{em}= 420 nm. The mobile phase was acetonitrile: 0.01M ammonium acetate with gradient flow to 0.8 ml min-^{1}. Ergovaline standard was provided by F. Smith (Auburn University, AL, US).

II.3.4 Statistical analyses

The percentage of plants that produced peramine, lolitrem B and ergovaline was calculated separately for the different growing conditions (wirehouse or field-plot).

Additionally the percentage of plants that produced only one or a specific combination of alkaloids was calculated for the whole set of plants. Those plants infected with two different *Epichloë* endophytes (double infections, DI) were considered separately.

The concentrations of peramine, lolitrem B and ergovaline were compared across the morphotypes (EO, M1, M2, M2S, M3), for each harvest separately, by means of a one-way ANOVA followed by a post-hoc Bonferroni's test. Differences among harvests (Wirehouse-Oct, Fieldplot-May, Fieldplot-Nov) were assessed considering the *Epichloë* morphotypes, by means of a one-way ANOVA followed by a post-hoc Bonferroni's test. These statistical analyses included only ryegrass plants producing detectable amounts of the alkaloid (zeros were not included).

Alkaloid concentration data were standardized to build a ternary plot with an axe for each alkaloid. For standardization of data, the relative concentration was calculated based on the maximum concentration for each alkaloid. The relative alkaloid concentration was normalized with a corresponding value being the sum of the three transformed data 100% and the values were introduced in the appropriate axis of the plot. The relationship between alkaloid concentrations across the whole set of plants was assessed by Pearson's correlation coefficient.

All statistical analyses were performed with SigmaPlot software version 13.0 (Systat Software, San Jose, CA, USA).

II.4 RESULTS

In fourteen *Lolium perenne* plants (nine from wirehouse and five from the field-plot), originally classified as E-, the presence of at least one of the alkaloids analyzed was detected: two produced only lolitrem B, six only ergovaline, two peramine and ergovaline, one lolitrem B and ergovaline and three the three alkaloids. Plants in this condition were diagnosed again through microscopic observation of seeds and stem pith preparations, and mycelium typical of *Epichloë* endophytes was observed in culm tissues and in the aleureone layer of the seeds. None of these endophytes could be isolated in potato dextrose agar; therefore, plants harboring these type of *Epichloë* endophytes were relocated as E+ and such fungi were designed as EO, because probably could be *Epichloë occultans*.

Epichloë occultans is frequently found in association with annual *Lolium* species (Moon et al. 2000) and some *Lolium hybridium* (*L. perenne x L. multiflorum*). Unlike other *Epichloë*/grass symbioses which colonize the intercellular space of all above ground tissue, *E. occultans* is typically localized in the meristematic region of the plant) (Moore et al. 2015), and this endophyte is known to be virtually unculturable in artificial growth media. (Moon et al. 2000; Sugawara et al. 2006).

Most of the *Epichloë* infected plants (E+) produced at least one of the three alkaloids analyzed (peramine, lolitrem B or ergovaline). In the wirehouse, alkaloids were detected in 100% (65/65) of the E+ plants and in the field-plot 96.2% of the E+ plants (103/107) produced

alkaloids. In two of the E+ samples from the field-plot in which no alkaloids were detected, the alkaloid lolitrem B was present in their respective clones from the wirehouse, and the other two E+ plants without alkaloids in the field-plot were not analyzed in the wirehouse.

A preliminary statistical analysis of data showed that variation in alkaloid concentration across plant locations was mainly due to the morphology of the fungus (percentage of plants with each morphotype). Thus, we focused on alkaloids production and concentration of peramine, lolitrem B and ergovaline, in perennial ryegrass, affected according to the morphology of the *Epichloë* endophyte(s) hosted (EO, M1, M2, M2S or M3) and the harvest (Wirehouse-Sep, Fieldplot-May and Fieldplot-Nov).

II.4.1 Peramine

Peramine was produced in 77.2% of the ryegrass plants growing in the wirehouse and in 76.5% of the plants from the field-plot. Plants infected with any morphotype of *Epichloë* produced peramine in both growing conditions (Table 3). In the wirehouse, the proportion of plants with peramine ranged from 22.2% in EO- to 100% of M2- and M2S-infected plants, and in the field-plot, ranged from 42.8% in M2-infected to 92.0% in M3-ryegrass.

Table 3 Peramine alkaloid in *Lolium perenne* plants: percentage of plants producing the alkaloid and concentration as affected by harvest and morphology of their *Epichloë* endophyte hosted.

Morphotype	Wirehouse-Oct				Fieldplot- May			Fieldplot-Nov	
	n	Producer (%)	Concentration (mg kg^{-1})¶ Mean±SE		n	Producer (%)	Concentration (mg kg^{-1})¶ Mean±SE		Mean±SE
EO	9	2 (22.2)	14.18±2.99	ab	5	3 (60.0)	NA		9.45±3.23
M1	22	18 (81.8)	5.54±1.00	a	52	38 (73.1)	3.73±0.23	a	5.87±1.43
M2	5	5 (100)	19.38±1.89	b	7	3 (42.8)	10.11±5.22	b	14.28±0.00
M2S	4	4 (100)	27.64±2.11	c	9	8 (88.8)	17.52±1.52	c	13.35±5.05
M3	17	15 (88.0)	10.29±1.09	ab	25	23 (92.0)	6.26±0.57	b	7.46±0.99
Mean	57	44 (77.2)	15.4±0.88	B	98	75 (76.5)	6.25±0.6	A	8.1±1.01 AB

¶EO: *Epichloë* uncultivable endophytic fungus, M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6) §n= Number of plants analyzed, NA= No analysed, SE= Standard error. a, b, c for each column, mean concentration with different letters are statistically different $P<0.05$. A,B,C, mean concentration in a row with different letters are statistically different $P<0.05$.

The ANOVA showed significant differences ($P< 0.001$) in concentration of peramine among ryegrass plants infected with different morphotypes of *Epichloë* in Wirehouse-Oct and Fieldplot-May; with no statistical differences in the Fieldplot-Nov. At all harvests, peramine followed the same pattern of concentration according to the morphology of the endophyte

hosted: the lowest concentration was detected in plants infected with the M1 morphotype; ryegrass with EO or M3 endophytes had intermediate concentrations; and *L. perenne* with M2 and M2S had the highest peramine concentrations (Table 3). Regarding the harvest factor, there was a significant effect ($P< 0.001$) on peramine. Plants from the Wirehouse-Oct had the greatest peramine concentration ($\bar{x}$= 15.40±0.88 mg kg^{-1}), followed by Fieldplot-Nov ($\bar{x}$= 8.10±1.01 mg kg^{-1}), and lowest concentration was recorded in Fieldplot-May ($\bar{x}$= 6.25±0.60 mg kg^{-1}) (Table 3).

Considering all plant samples together, it was observed that peramine concentrations were variable but within specific ranges, depending on the morphotype of *Epichloë* hosted (Figure 15). A large number of plants with M1-morphotype (n= 65) produced peramine within in a narrow range of concentration (0.60-15.0 mg kg^{-1}). By contrast, a lower number of M2- (n= 14) and M2S-plants (n= 16) had peramine within a widest concentration range (3.5 – 33.0 mg kg^{-1}).

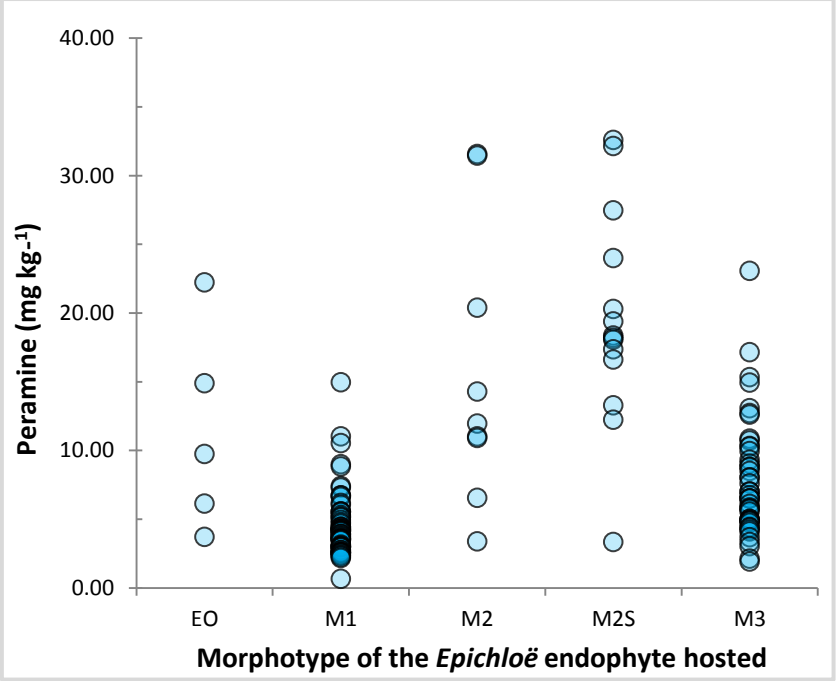

Figure 15 Concentration of peramine in *Lolium perenne* plants according to the morphotype of *Epichloë* hosted. (Including plants growing in the wirehouse and in the field-plot)

II.4.2 Lolitrem B

Lolitrem B was detected in 61.5% of the plants from the wirehouse and in 68.3% of ryegrass from field-plot. Plants infected with M2S endophytes did not produce lolitrem B in the wirehouse but plants from the field-plot at both harvests did produce it (Table 4). The percentage of plants producing lolitrem B was smaller in the wirehouse than in the field-plot when the hosted endophytes were EO or M2, and there was a similar proportion of plants with

lolitrem B in the wirehouse and Field-plot for plants infected with the morphotypes M1, M2 or M3.

Table 4 Lolitrem B alkaloid in *Lolium perenne* plants: percentage of plants producing the alkaloid and concentration as affected by harvest and morphology of their *Epichloë* endophyte hosted.

Morphotype	Wirehouse-Oct				Fieldplot- May			Fieldplot-Nov	
	n	Producer	Concentration (mg kg^{-1})¶		n	Producer	Concentration (mg kg^{-1})¶		
		(%)	Mean±SE			(%)	Mean±SE		Mean±SE
EO	9	2 (22.2)	1.99±0.79	b	5	4 (80.0)	NA		0.71±0.10
M1	22	13 (59.1)	1.48±0.31	b	52	35 (67.3)	1.12±0.08	ab	0.69±0.05
M2	5	2 (40.0)	1.01±0.79	b	7	4 (57.1)	0.89±0.21	ab	NA
M2S	4	0 (0.0)	0.00±0.00	a	9	4 (44.4)	0.48±0.03	a	0.61±0.05
M3	17	15 (88.2)	2.44±0.29	b	25	20 (80.0)	1.38±0.11	b	0.77±0.06
Mean	57	32 (61.5)	1.7±0.16	C	98	67 (68.3)	1.17±0.06	B	0.71±0.03 A

¶EO: *Epichloë* uncultivable endophytic fungus, M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6) §n= Number of plants analyzed, NA= No analysed, SE= Standard error. a, b, c for each column, mean concentration with different letters are statistically different $P<0.05$. A,B,C, mean concentration in a row with different letters are statistically different $P<0.05$.

The *Epichloë* morphotype had a significant effect on lolitrem B concentrations in plants from the Wirehouse-Oct ($P= 0.011$) and in ryegrass from Fieldplot-May ($P= 0.024$), but not in plants from Fieldplot-Nov ($P= 0.562$). Perennial ryegrass with M2S endophytes did not produce lolitrem B in the Wirehouse-Oct, and in Fieldplot-May presented the lowest lolitrem B concentrations ($\bar{x}$= 0.48±0.03 mg kg^{-1}). On the other hand, plants infected with the M3 morphotype had the highest lolitrem B concentration, with $\bar{x}$= 1.38±0.11 mg kg^{-1} in the Fieldplot-May (Table 4). No significant differences in lolitrem B concentration were found among ryegrass infected with EO, M1 or M2 endophytes in any harvest. Lolitrem B was also affected by harvest ($P< 0.001$) with a greater concentration in plants grown in Wirehouse-Oct ($\bar{x}$= 1.70±0.16 mg kg^{-1}) than in the Fieldplot-May ($\bar{x}$= 1.17±0.06 mg kg^{-1}) and in Fieldplot-Nov ($\bar{x}$= 0.71 mg kg^{-1}) (Table 4).

The greatest contrast on lolitrem B content was found between M2S- and M3-infected plants (Figure 16). Ryegrass with M3 endophytes, produced lolitrem B within a wide range of concentration (0.50-5.60 mg kg^{-1}), and M2- and M2S-plants had values within a narrow range (M2= 0.65-1.50 mg kg^{-1}; M2S= 0.46-0.68 mg kg^{-1}). Concentration of lolitrem B in infected plants with EO and M1 was in the same scope. Most of the plants (79/99) had lolitrem B below 1.8 mg kg^{-1}, the threshold concentration for causing ryegrass staggers in cattle and sheep (di Menna et al. 1992). Lolitrem B at toxic concentration was detected in 12 plants infected with M3, in seven with M1 and in one with EO morphotype (Figure 16).

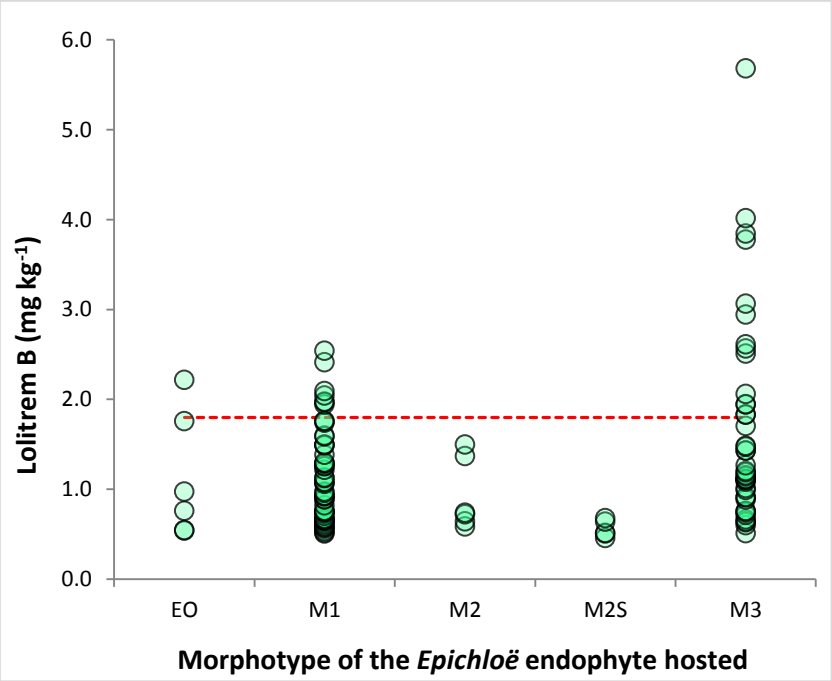

Figure 16 Concentration of olitrem B in *Lolium perenne* plants according to the morphotype of *Epichloë* hosted, including plants growing in the wirehouse and in the field-plot. The toxic level for livestock is indicated with the horizontal red line.

II.4.3 Ergovaline

The alkaloid ergovaline was more common among plants from the wirehouse (90.2%) than in plants from field-plot (62.6%) (Table 5). The type of the endophyte hosted affected the concentration of ergovaline in the Wirehouse-Oct (*P*= 0.014) and the Fieldplot-Nov (*P*= 0.006), but not in Fieldplot-May (*P*= 0.184).

Table 5 Ergovaline alkaloid in *Lolium perenne* plants: percentage of plants producing the alkaloid and concentration as affected by harverst and morphology of their *Epichloë* endophyte hosted.

Morphotype	Wirehouse-Oct				Fieldplot- May		Fieldplot-Nov	
	n	Producer (%)	Concentration (mg kg^{-1})¶ Mean±SE		n	Producer (%)	Concentration (mg kg^{-1})¶ Mean±SE	Mean±SE
EO	9	9 (100)	0.95±0.42	ab	5	3 (60.0)	NA	1.37±0.37 b
M1	16	14 (87.5)	2.29±0.34	b	52	31 (59.6)	1.41±0.21	0.35±0.10 a
M2	3	2 (66.7)	0.29±0.89	ab	7	4 (57.1)	1.08±0.6	NA
M2S	1	1 (100)	0.08±1.26	a	9	2 (22.2)	0.10±0.00	0.33±0.14 a
M3	12	11 (91.7)	0.68±0.38	ab	26	22 (84.6)	1.01±0.16	0.58±0.13 a
Mean	41	37 (90.2)	0.86±0.34		99	62 (62.6)	1.19±0.13	0.59±0.11

¶EO: *Epichloë* uncultivable endophytic fungus, M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6) §n= Number of plants analyzed, NA= No analysed, SE= Standard error. a, b, c for each column, mean concentration with different letters are statistically different *P*<0.05. A,B,C, mean concentration in a row with different letters are statistically different *P*<0.05

Moreover, the harvest did not have a significant effect on ergovaline concentration (*P*= 0.113). At all harvests, plants infected with M2S had the lowest mean ergovaline concentration; by contrast, M1-infected had in average the highest ergovaline concentration. No significant differences were found in ergovaline concentration among plants infected with morphotypes EO, M2, or M3 (Table 5).

Ergovaline in M1-infected plants spanned from 0.03 up to 10.10 mg kg^{-1}; although, 49.2% of plants with M1 endophyte had ergovaline concentrations below 2.0 mg kg^{-1} (Figure 17). Ergovaline levels in ryegrass with morphotypes EO, M2S and M3 had a similar distribution range. Most plants in which ergovaline was detected, 87.7% (57/65), had a concentration above 0.4 mg kg^{-1}, which is associated with heat stress in cattle (Hovermale and Craig 2001).

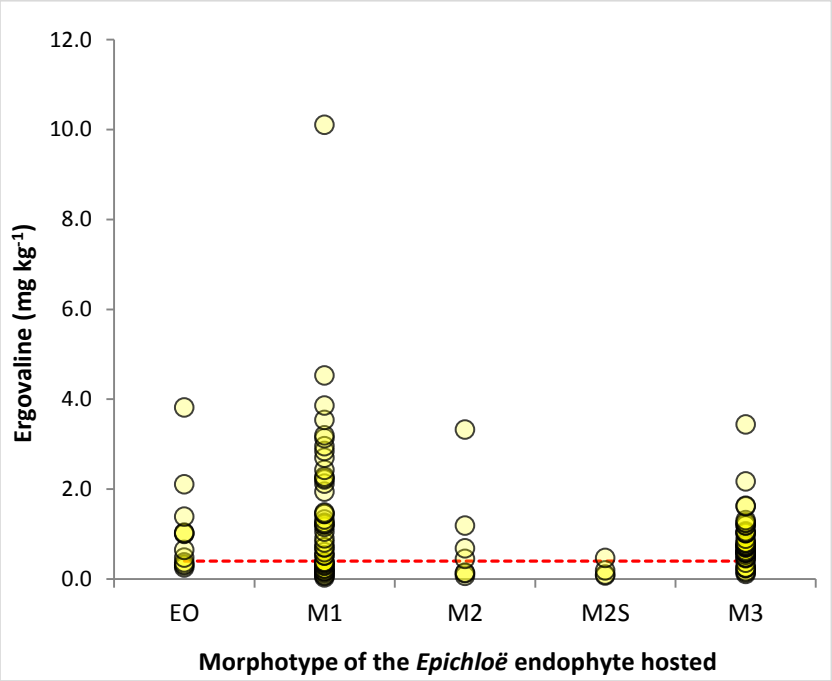

Figure 17 Concentration of ergovaline found in *Lolium perenne* plants according to the morphotype of *Epichloë* hosted, including plants growing in the wirehouse and in the field-plot. The toxic level for livestock is indicated with the horizontal red line.

II.4.4 Alkaloid combinations

Considering the whole set of infected ryegrass samples, in several plants only one alkaloid was detected. Peramine alone were detected in 41.7% of M2S- and in 2.2% of M3-infected; whereas, none of the EO-, M1- or M2- infected plants produced peramine alone (Figure 18a). Lolitrem B alone was detected in infected plants with all the *Epichloë* morphotypes, from 4.8% of M3- up to 14.3% for M1-ryegrass (Figure 18a). Ergovaline alone was frequently found in *L. perenne* infected with EO endophytes (42.9%); less common in M1- and M2- plants (8.7% and 10.0% respectively); whereas, in none plants infected with the M2S or M3 morphotypes the alkaloid ergovaline was detected alone (Figure 18a).

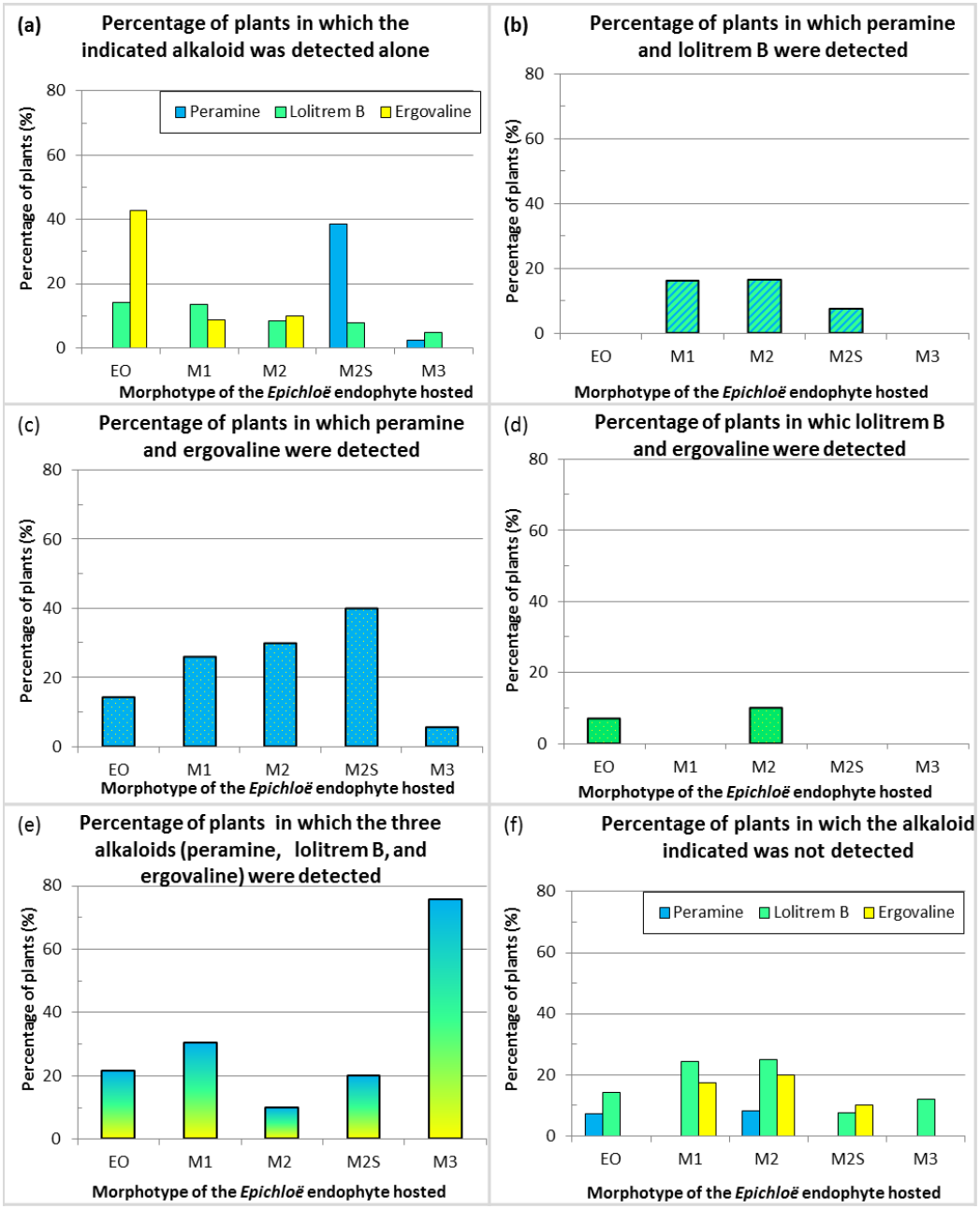

Figure 18 Alkaloids detection in *Lolium perenne* plants according to the morphotype of the *Epichloë* endophyte hosted: (a) only the indicated alkaloid, (b-d) different combinations of the specified two alkaloids (e) detection the three alkaloids and (f) no detected the indicated alkaloid.

Production of two alkaloids in the same plant was common in the sample set analyzed. Peramine and lolitrem B were detected in 16.2% of the M1-, 16.7% of M2- and 7.0% of M2S-infected ryegrasses but this alkaloid combination was not found when the hosted endophytes were EO or M3 (Figure 18b). Peramine and ergovaline was the most frequent combination of two alkaloids, and it was produced by all the *Epichloë* endophytes, ranging from 5.4% of the M3-infected ryegrass to 40.0% of plants with M2 endophytes (Figure 18c). Lolitrem B and ergovaline was the least common combination of two alkaloids; these alkaloids were detected in 7.1% of ryegrass with EO endophytes and in 10.0% of M2-plants, but not in ryegrass infected with the M1, M2S or M3 morphotypes (Figure 18d).

Production of the three alkaloids peramine, lolitrem B and ergovaline together were detected in plants infected by all morphotypes of *Epichloë*, with a particularly high percentage in ryegrass infected with the M3 morphotype (>75%), and with less frequency among plants infected with other endophytes in a range from 9.1% in M2-infected plants to 27.6% in M1-ryegrasses (Figure 18e).

Depending on the purpose in which is intended the grass, it is important to know the absence of only a particular alkaloid. In this regard, the absence the neurotoxic alkaloid of lolitrem B, was observed in plants infected with any of the *Epichloë* morphotypes ranging from 7.7% among M2S plants up to 25.0% in M2 ryegrass. No ergovaline, another toxic alkaloid, production was observed in 7.1% of EO- and 8.3% of M2-infected of plants (Figure 18f). It was observed that 7.1% of EO- and 8.3% of M2-infected did not have peramine and in ryegrass with other *Epichloë* morphotypes it did was produced).

The Figure 19 represents a three axis plot built using the normalized data of alkaloid concentrations in perennial ryegrass according to the *Epichloë* endophyte hosted. It can be observed that most of the endophytes belonging to a specific morphotype are allocated in the same cluster. Plants with EO endophytes are located down in the right side, since they produce lower concentration of lolitrem B and ergovaline than that of peramine. Most of plants with the M2 morphotype, independently if they were or not stomata producers are situated at the extreme right side at the bottom of the plot, because in these samples peramine concentrations were higher and the levels of lolitrem B lower. Plants infected with M3 endophytes, which produced the three alkaloids are grouped in the center of the plot. However, endophytes with the M1 morphotype were separated into two different clusters.

The clear segregation into two groups of the plants infected with the M1 morphotype is coincident with their genotypic profile (see Chapter I). Plants from the genotypic group G1b lay along the lolitrem B axis, they produced higher levels of lolitrem B and peramine, but very low concentration of ergovaline or did not produce this alkaloid (Figure 19). Plants infected with the M1 morphotype and genotypic profile G1a were congregated along the peramine axis, because they produced ergovaline and peramine in a wide range of concentrations but most of them did not produced lolitrem B.

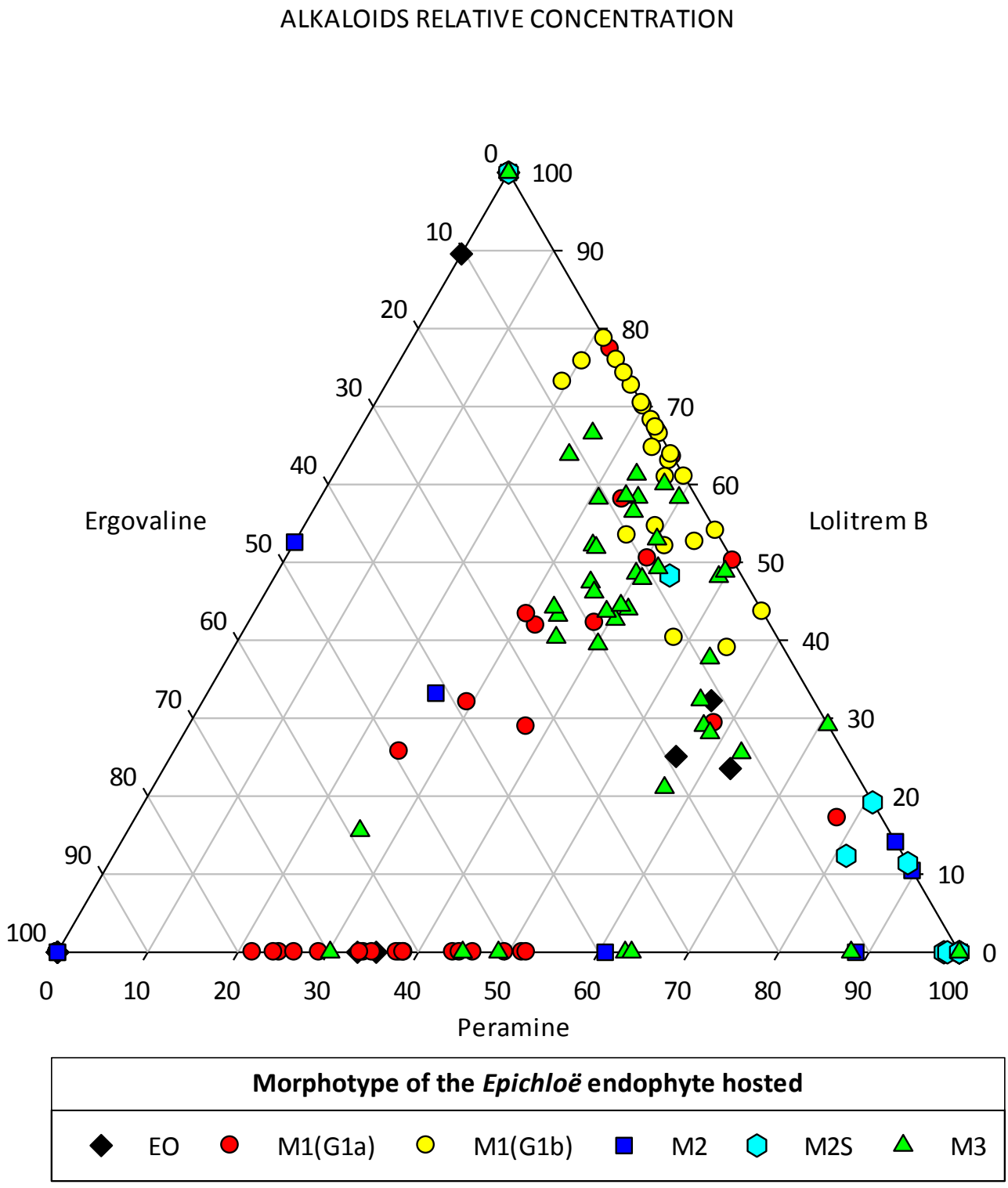

Figure 19 Normalized data of the relative concentration of alkaloids peramine, lolitrem B and ergovaline in *Lolium perenne* plants according to the taxonomic group of their *Epichloë* endophyte hosted.

There were significant correlations among concentrations of alkaloids, but these correlations were not comprehensive, as they were found in plants infected with specific *Epichloë* morphotypes (Table 6).

Table 6 Pearson's correlation coefficient (*r*) and signification level (*P*) between alkaloids produced in *Lolium perenne* plants, for each morphological group of *Epichloë* endophyte hosted.

ALKALOID COMBINATION		MORPHOTYPE[¶]				
		EO	M1	M2	M2S	M3
Peramine and Lolitrem B	*r*	**0.665**	**0.209**	0.028	**-0.503**	**0.716**
	P	**0.009**	**0.050**	0.924	**0.047**	**<0.001**
	n	14	74	12	13	42
Peramine and Ergovaline	*r*	0.383	**0.325**	-0.200	0.102	0.070
	P	0.176	**0.003**	0.533	0.740	0.639
	n	14	69	10	10	37
Lolitrem B and Ergovaline	r	-0.029	**-0.492**	**0.733**	0.392	-0.094
	P	0.918	**<0.001**	**0.007**	0.185	0.529
	n	14	69	10	10	37

[¶]EO: *Epichloë* uncultivable endophytic fungus, M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6).Numbers in bold indicates significant correlation coefficients $P \leq 0.05$.

Concentration of the alkaloids peramine and lolitrem B were positively correlated in plants with morphotypes EO, M1 and M3, and negatively with M2S. The content of the alkaloids peramine and ergovaline were correlated only in M1-infected ryegrass (positively). Lolitrem B and ergovaline concentrations were inversely significantly correlated in M1-ryegrass and positively in M2-plants (positive).

II.4.5 Alkaloids in double infected plants

In perennial ryegrass infected with two different *Epichloë* endophytes (double infections, DI), peramine was usually detected in similar concentrations to those of single infected plants with M2 and M2S morphotypes; but contrary to the general trend, DI(M2/M1)-produced as low peramine as the single infected M1-plants (Figure 20a, Table 3).

In double infected perennial ryegrass the concentration of lolitrem B was similar than in single infected plants (Figure 20, Table 4). However, when the endophytes hosted were a stromata producer (M2S) and M3, the mean concentration of lolitrem B was higher than in single infected plants ($\bar{x}$ = 1.70±0.16 mg kg-^{1} in Wirehouse-Oct; $\bar{x}$= 1.17±0.06 mg kg-^{1} in Fieldplot-May; and $\bar{x}$ = 0.70±0.03 mg kg-^{1} in Fieldplot-Nov).

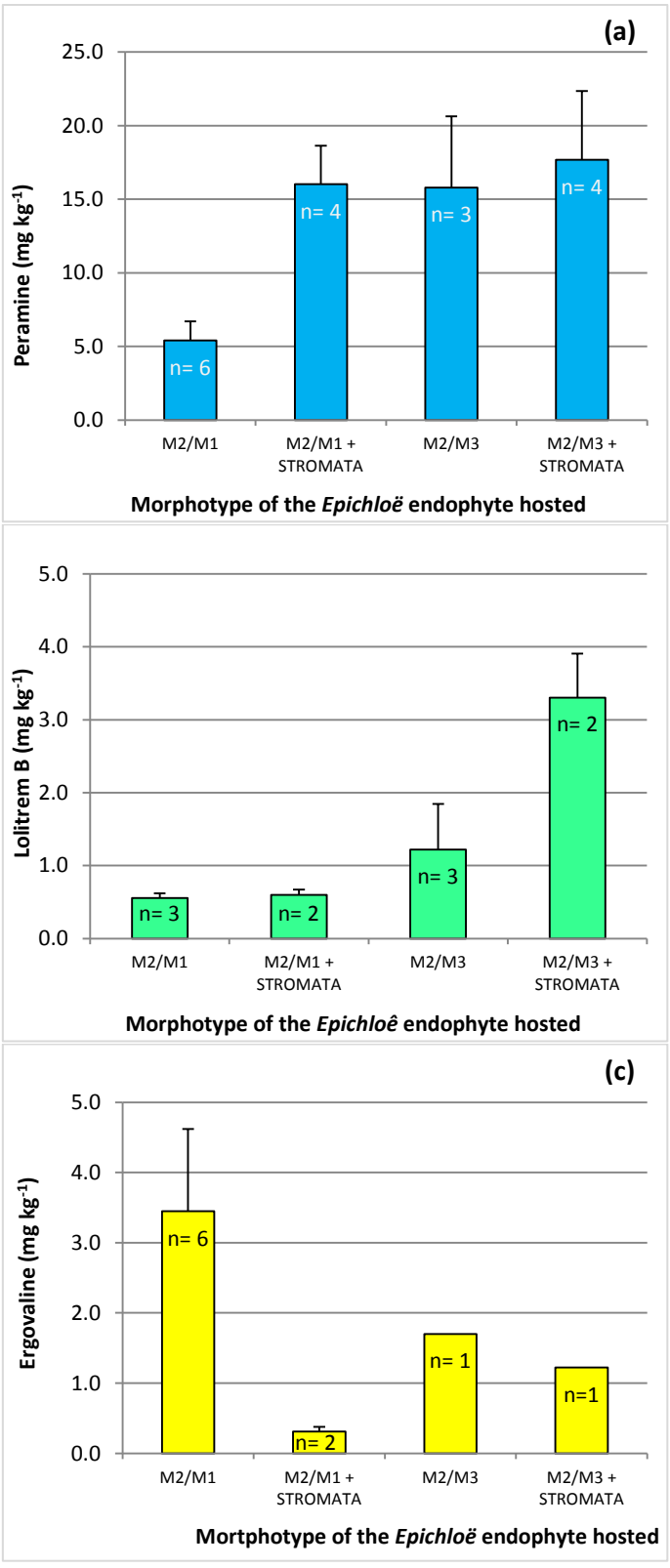

Figure 20 Average concentration of alkaloids in *Lolium perenne* plants infected by two *Epichloë* endophytes (double infections, DI): (a) peramine, (b) lolitrem B and (c) ergovaline.

From all DI-plants, in those classified as DI(M2/M1) the highest concentration of ergovaline ($\bar{x}$ = 3.45±1.17 mg kg-^{1}) was detected, and it was higher that single infected plants (Figure 20b, Table 5). On the other hand, DI-plants with stromata had the lowest mean ergovaline concentration.

The separate analyses of stromata and healthy seed heads from one DI-plant with M2S and M1 endophytes, indicated presence of peramine in high concentration (18.06 mg kg^{-1}) in stromata but it was not detected in seed heads. Lolitrem B was not produced, and ergovaline had similar concentrations in the two analyzed samples (0.10 mg kg^{-1}, in stromata; 0.13 mg kg^{-1}, in seed heads).

II.4.6 Production of alkaloids in inoculated plants

Comparison of the alkaloids concentration among ryegrass plants of the two cultivars evaluated was only possible in a limited number of plants, because not all the inocula infected successfully the two cultivars. The statistical analyses to compare the alkaloid concentrations in such plants did not show significant differences (P< 0.05) between the two ryegrass cultivars evaluated.

Differences in alkaloid concentrations between naturally infected- and inoculated-plants were observed (Table 7). Peramine was found to be produced by almost all inoculated plants with endophytes belonging to the M2 morphotype; with the exception of isolate LVA04, even when in its natural host peramine was produced in high concentrations (17.35-32.59 mg kg^{-1}). Plants inoculated with *Epichloë* endophytes of the M1 or M3 morphotype that produced peramine in their natural host did not produce this alkaloid in the inoculated ryegrasses (Table 7).

Table 7 Production of alkaloids in ryegrass plants inoculated with *Epichloë* endophytes of different morphology and in naturally infected plants.

Morphotype¶	Inoculum	n	Peramine			Lolitrem B			Ergovaline		
			Inoculated plants		Natural	Inoculated plants		Natural	Inoculated plants		Natural
			Producer (%)	Concentration (mg kg^{-1})		Producer (%)	Concentration (mg kg^{-1})		Producer (%)	Concentration (mg kg^{-1})	
M1	LVE11	4	0.0	0.00	0.66-11.02	0.0	0.00	0.66-1.59	0.0	0.00	0.03
	LVE29	6	0.0	0.00	3.13-6.03	0.0	0.00	1.22-1.25	0.0	0.00	0.07
M2	LVA08	8	100	13.13-25.62	10.9	25	0.01-0.53	0.00	0.0	0.00	NA¶
	LVA32	12	100	8.26-31.16	15.8-11.95	8.3	0.85	0.51-1.37	0.0	0.00	0.24
MS2	LVA04	6	0.0	0.00	17.35-32.59	0.0	0.00	0.00	0.0	0.00	0.00
	LVA17	4	100	21.31-26.15	20.29-32.14	0.0	0.00	0.46	0.0	0.00	0.00
	MON06	4	100	15.49-26.76	4.96	0.0	0.00	0.67-0.87	0.0	0.00	0.00
	MON07	8	100	13.93-29.04	6.22	12.5	0.46	0.00	0.0	0.00	0.41
M3	CR14	4	0.0	0.00	6.91-14.92	0.0	0.00	0.89-1.47	0.0	0.00	0.64
	TAB42	4	0.0	0.00	12.07-23.06	0.0	0.00	1.62-5.68	0.0	0.00	0.25

¶M1 morphotype, slow growth rate with ”brain-like” mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6)n= Number of plants analyzed, ¶NA= No analysed,

The alkaloid lolitrem B was detected only in plants inoculated with M2 isolates LVA08, LVA32 and MON07 (Table 7). The endophyte MON07 and LVA08 produced lolitrem B uniquely in the inoculation trial but not in their natural host grown in wirehouse or field. In no plants inoculated with M1 or M3 endophytes was detected lolitrem B; although this alkaloid was detected in the original host plants.

Ergovaline was not detected in any of the three harvests (in April, June and August 2014) in ryegrass inoculated with *Epichloë* endophytes, independently whether in the natural hosts this alkaloid was produced.

II.5 DISCUSSION

The alkaloids peramine, lolitrem B and ergovaline are produced exclusively in grasses infected with *Epichloë* endophytes; hence, non-infected plants should not produce these alkaloids. In our study, plants originally classified as E- using a fungal isolation diagnostic method produced alkaloids, and presumably could be infected with *Epichloë occultans,* because an unculturable endophyte was observed by microscopy in stems and seeds. van Zijll de Jong et al. (2008) reported the detection, through SSR markers, of a scarce presence of *Epichloë* endophytes with genetic similarity to *E. occultans* in *L. perenne* pastures. It has been reported that *E. occultans* could be in symbiosis with hybrid ryegrasses that coexist within natural population of perennial ryegrass (Hume et al. 2001)(Bluett et al. 2004), and Sugawara et al. (2009) reported successful artificial inoculation of *E. occultans* into *L. perenne* plants. Although high production of lolines and low concentrations of peramine have been reported in *L. rigidum* and other annuals grasses infected with *E. occultans,* the alkaloids lolitrem B and ergovaline have not been detected (Sugawara et al. 2006).However, Moore et al. (2015) reported that annual ryegrass infected with *E. occultans* produced several precursor compounds of indole-diterpenes (lolitrems) and Kuldau and Bacon (2008) associated the presence of lolitrem B and ergovaline in *Epichloë*-infected grasses to pressure for predation of small vertebrates.

Most of the *L. perenne* plants (>95%) infected with *Epichloë* endophytes had at least one of the three alkaloids analyzed (peramine, lolitrem B or ergovaline). The proportions of plants that produced peramine, lolitrem B or ergovaline were 70.9%, 64.8% and 60.9%, respectively. These percentages were similar to those reported by Bony et al. (2001) in ryegrass plants from natural populations infected with *Epichloë* endophytes. Differences on the percentage of plants that produced each alkaloid may be due to genetic background of the plant and the fungi. The concentration of alkaloids in *Epichloë* infected ryegrass were in the same range found by other researchers: peramine 2.0 - 52.8 g kg^{-1} (Rowan et al. 1990; Bony et al. 2001); lolitrem B 0.3 to 11.5 mg kg^{-1} (Christensen et al. 1991; Bony et al. 2001; Reed et al. 2004); and ergovaline 0.24-3.46 g kg^{-1} (Easton et al. 1993) (Lane et al. 1997; Bony et al. 2001). Toxic levels of lolitrem B for livestock (>1.80 g kg^{-1}) were detected in a low number of the analyzed plants and most of them were infected with M3-endophytes; whereas a very important proportion of the samples (*ca.* 90%) have a concentration of ergovaline above the

reported safe limit for livestock consumption (0.40 g kg^{-1}). However, toxicosis cases in Europe are scarce mainly because the floristic diversity of grasslands and the use of endophyte-free ryegrass cultivars (Zabalgogeazcoa and Bony 2008).

It is known that production and concentration of alkaloids in grasses are affected by factors such as the endophyte and host genotypes, plant tissue, environmental conditions (water and nutrients availability) and management (Ball et al. 1995; Easton et al. 2002; Spiering et al. 2005a; Rasmussen et al. 2007; Hahn et al. 2008; Zhou et al. 2014). In this Chapter, the main objective was to evaluate the influence of the *Epichloë* morphotype on alkaloid production (peramine, lolitrem B, ergovaline) in a heterogeneous set of perennial ryegrass plants growing in two different conditions (wirehouse or field-plot) and sampled at different phenological stages. The results showed a stronger influence of the *Epichloë* morphotype over other possible sources of variation on the profile of alkaloids produced in perennial ryegrass plants, because the same particular characteristic pattern of production was observed for each of the three alkaloids in both growth conditions and in the three harvests. This is in agreement with the results of Easton et al. (2002), who explained that variation in herbage concentration of endophyte-derived alkaloids may be mainly due to the difference in the *Epichloë* strains.

A higher concentration of peramine in plants infected with M2- and M2S- endophytes could be related in some extent to a higher abundance of fungal mycelia. Rasmussen et al. (2007), reported a direct relationship between the abundance of fungal endophyte on peramine concentration. In Chapter I (Fig. I. 3e), it was observed that in PDA, *Epichloë* endophytes from morphological group M2 (stromata forming included) had the highest growth rate; M3 morphotype had intermediate rates and M1 morphotype the lowest. Therefore, although growth rate in PDA could not be reflected *in planta*, it seems that patterns of peramine concentration coincided with growth rate of the fungi in the culture medium, being the M2 and M2S morphotypes the highest producer of peramine; whereas, the lowest peramine concentrations were recorded in M1-plants, and when M3-endophytes were hosted, ryegrass had intermediate peramine levels.

In the case of lolitrem B, M3-infected plants had the highest mean concentration and plants with sexual endophytes (M2S) had the lowest content. This is in agreement with Leuchtmann et al. (2000) who reported that *Epichloë* strains that thoroughly choke all host tillers tend either not to produce lolitrem B or to produce it at low levels. According to Young et al. (2009) many sexual isolates are unable to produce lolitrem B due to absence of the complete LTM locus or, in some cases, just the genes encoding the first committed steps in the pathway. In contrast, gene profiling of asexual species with an *E. festucae* progenitor (*e. g. E. festucae* var. *lolii*) are likely to be capable of producing lolitrem B (Young et al. 2009; Schardl et al. 2012). In Chapter I, it was reported that many of the endophytes with M2 and M2S morphotype did not have the *ltm*Q gene (part of the LTM locus), required in the pathway for paspaline hydroxylation, essential for lolitrem B production. This may explain both the infrequent and the lower concentration of lolitrem B in plants infected with the M2 or M2S

morphotypes. On the contrary, *ltm*Q gene was detected in endophytes with other morphotypes, and more than 60% of M1- and 80% of M3-infected plants produced lolitrem B.

Unlike the clear relationship among distinctive morphotypes of *Epichloë* and the production of peramine and lolitrem B, for ergovaline it was found that the highest concentrations were detected in plants infected with a specific group of endophytes belonging to M1 morphotype. *Epichloë* endophytes with M1 morphotype split into two genotypic groups (G1a and G1b), according to a genotypic analysis using the ITS1-5.8SrDNA-ITS2 region and reported in Chapter I, and interestingly such groups coincided with their ability to produce ergovaline. The highest ergovaline concentrations was detected in ryegrass plants infected with M1-endophytes of G1a genotype; but when the endophyte hosted was from the M1-morphotype belonging to the genetic group G1b, the production of ergovaline in the ryegrass plants was very low or null. Therefore, our results show that the genetic segregation of both groups can be explained on the basis of their alkaloid contents.

According to the relationship between the alkaloid production and the morphotype of the *Epichloë* endophyte hosted by in the ryegrass plants observed in this chapter, some M2 and M1(G1b) strains can be used for improvements of forage ryegrass, because these *Epichloë* morphotypes do not produce toxic alkaloids for livestock (lolitrem B and ergovaline), but they do produce the insect-deterred alkaloid peramine. On the other hand, as the M3 morphotype produce high level of lolitrem B but also of peramine, this type of endophyte can be a good option for improvement programs in turf grasses.

Relationships between the different alkaloids produced suggest modes of regulation induced by the endophyte. Some relationships between concentration of alkaloids have elucidated that mevalonic acid and tryptophan are precursors of both lolitrem B and ergovaline and that lolitrem B- and ergovaline-ratios could thus be influenced by pathway competition for these two compounds (Baxter et al. 1962; Spiering et al. 2005a). This could explain the negative relationship between both alkaloids found in M1-infected plants and the low frequency of plants in which this alkaloid combination was found; however, in M2-ryegrass, production of lolitrem B and ergovaline were positively correlated. On the other hand, the correlation between peramine and lolitrem B was significant in four of the five *Epichloë* morphotypes. This relationship was positive in the asymptomatic ryegrass-*Epichloë* associations, which is in accordance with other reports (Ball et al. 1995; Siegel and Bush 1996; Reed et al. 2004), but negative for the M2S (the stroma-producing). These results indicate that besides the competition for mevalonic acid and tryptophan other processes could be involved in the pathway for synthesis of alkaloids, which may be genetically controlled in a distinctive way by each *Epichloë* morphotype.

Production of alkaloids in double infected ryegrass also followed the pattern of concentration according to the morphology of their *Epichloë* endophytes hosted, although in some DI plants a synergistic effect was observed. For peramine, DI plants had similar concentration than the single infected ryegrass with M2 or M2S endophytes, the highest producers of peramine. In the same way a synergistic effect was observed for lolitrem B in DI

infected plants with the M3 morphotype and for ergovaline in three of the four ecotypes of DI plants with endophytes of the M1 morphotype, with higher concentration than in single infected ryegrass, which may have negative consequences for livestock because the safe concentration limits were exceeded. Bony et al. (2001) also quantified alkaloids in ryegrass plants harboring two *Epichloë* endophytes; however, they reported values only for lolitrem B which were in same range of production (0.80-4.30 g kg^{-1}) than in the single infected plants that they analyzed (0.80-5.75 g kg^{-1}).

Regardless of the ryegrass samples had miscellaneous environmental backgrounds, the strong effect of *Epichloë* morphotype on alkaloids synthesis, and possible interactions host/endophyte reported (Easton et al. 2002; Cheplick and Cho 2003; Rasmussen et al. 2007), the results described herein allowed to identify an effect of the harvest on alkaloid production. The three harvests encompassed different plant phenological stages (vegetative, flowering and autumn regrowth) in two growing conditions (wirehouse and field-plot). Significant differences were observed in concentrations of peramine and lolitrem B between ryegrass plants from the wirehouse and the field-plot, but not for the ergovaline alkaloid. Conditions on wirehouse or field-plot had particular nutritional and climatic circumstances; therefore, differences on alkaloid concentrations may reflect in part the effects of such aspects. However, one of the most important differences was due to the plant growth stage, an important factor determining the accumulation of alkaloids which is also related to differences in concentration in plant tissues. Wirehouse-Oct plants were harvested at vegetative stage and samples consisted mainly of leaves; whereas Fieldplot-May plants were harvested at flowering stage, and samples were composed of leaves, stems and inflorescences. Furthermore, M2S-infected ryegrass from the Fieldplot-May had stromata, and these structures were not present in plants from Wirehouse-Oct. Plants of ryegrass from Fieldplot-Nov also were in a vegetative stage of regrowth but with older tissues at the crown than samples taken from Wirehouse-Oct.

Concentration of peramine was higher in plants collected at vegetative state (Wirehouse-Oct, Fieldplot-Nov) than in plants analyzed at flowering (Fieldplot-May). This is explained because peramine is most abundant in younger tissues and particularly in leaves, which were the largest proportion of the samples collected at the vegetative state (Keogh et al. 1996). Other environmental factors do not seem to have strong effect on the production of peramine, which is not affected by N (Lane et al. 1997; Rasmussen et al. 2008), neither by water availability (Hahn et al. 2008).

Plants from the wirehouse in vegetative stage had higher concentration of lolitrem B than plants from the field-plot at any harvest. These results are contrary to several reports indicating that concentration of lolitrem B in perennial ryegrass has a pronounced seasonal variation related to maturity of the plant, from lower amount in leaves and rising with inflorescence development, with higher concentration in older leaf sheaths and seeds (Prestidge and Gallagher 1988; di Menna et al. 1992; Ball et al. 1995; Lane et al. 2000; Repussard et al. 2014b). On the other hand, information about the influence of abiotic factors on the levels of lolitrem B in field conditions is scarce and sometimes contradictory (Lane et al.

1997), making not possible attribute to one specific factor the higher concentration of this alkaloid in wirehouse that in the field plot. According to Hahn et al. (2008) for lolitrem B in perennial ryegrass there is not a clear model and it concentration is highly dependent of each individual *Epichloë*/grass association. In the present study, due to the large heterogeneity of *Epichloë*/grass associations used, factors affecting alkaloid content are not depending on individual features of the cultivars; instead our results reflected a strong effect caused by the nature of the endophytes hosted. Besides the morphotye of the endophyte, other factors may have influenced the alkaloid production; for instance, it has been observed that under specific conditions of higher moisture accompanied by dry, warm conditions in spring–summer, a rising of perennial ryegrass toxicosis was observed in Australia, as consequence of the lolitrem B level accumulation (Reed et al. 2011). Thus a possible dilution effect of the samples might occurs, accumulation of lolitrem B is very high toward the base of the plant (di Menna et al. 1992; Keogh et al. 1996) and biomass production and principally the proportion crown:stem were lesser in plants form wirehouse than in ryegrass grown in the field; therefore, crown tissues were proportionally more abundant in wirehouse samples and higher lolitrem B were recorded. Additionally, others combinations of not examined abiotic factors may also affect the lolitrem B production, because in the analyzed plants from wirehouse, which were, watered, fertilized and were confined to plot, the highest lolitrem B concentration was detected.

The ergovaline concentration was not significantly affected by harvest or growth conditions. Repussard et al. (2014b) reported that ergovaline concentration in perennial ryegrass has a peak in spring during flowering and in field-growth *E. festucae* var*. lolii*-infected perennial ryegrass, and the ergovaline content increased under conditions of water and temperature stresses or in response to addition of N fertilizer (Barker et al. 1993; Hahn et al. 2008; McCulley et al. 2015; Ryan et al. 2015). Although, Rasmussen et al. (2007) showed that the effect of external factors such N addition could not be significant on the ergovaline content in certain cultivars of ryegrass, but in this work such kind of influence is override due to the heterogeneity of the plats evaluated. It may be possible that differences in concentration of ergovaline were not detected, because when the ryegrass was sampled in Fieldplot-May plants were flowering having the pick concentration of ergovaline, and in the wirehouse, the plants were fertilized also promoting the content of this alkaloid.

Alkaloid production is highly driven by external environmental factors related with defensive and ecological functions; herbivore attack may trigger signals for synthesis of alkaloid or concentration in specific tissues that need protection. In lolines this effect have been extensively studied; for example, Patchett et al. (2008) found that in *Epichloë* infected medow fescue (*Festuca pratensis*= *Bromus pratensis*= *Lolium pratense*) exposed to grass grub (*Costelytra zealandica*) (a subterranean pest common in New Zealand Pastures), loline concentration in root was significantly higher than in the crown of the grass and aboveground concentrations was lower when compared with no-infected grasses. Similarly, alkaloid production inducted by plant clipping was demonstrated by (Bultman and Ganey 1995) in endophyte infected perennial ryegrass. In this regard, it can be speculated, that the higher

concentration of alkaloids, particularly lolitrem B, in plants from the wirehouse, might be an active reaction of the *Epichloë* infected ryegrass to the aphid attacks observed with frequency in those plants in contrast with plants from the field-plot in which these insects were not observed.

Interestingly, production of alkaloids in perennial ryegrass inoculated with *Epichloë* endophytes was sparse: in no association was detected the toxic alkaloid ergovaline, lolitrem B was found in 30% of inoculated plants, whereas peramine (insecticide) was detected in 50% of plants. Inoculated grasses contained more peramine and less lolitrem B compared with naturally infected ones, which is a suitable and very interesting result intended for livestock feed. Alkaloids were produced only in plants infected with endophytes of the M2 and M2S morphotypes, but with no others. Probably, adaptation of the inoculum to the traits of the new host (as nutrient availability, sugar content, host metabolism, etc.) may have affected the fungal development inside the plants particularly of the M1(G1a) endophytes able to produce the highest ergovaline concentration in their natural grass hosts. Easton et al. (2002), reported that variation in peramine and ergovaline concentration are genetically controlled as a function of mycelial mass; furthermore, the level of adaptability reflected in compatibility grass/endophyte have been developed through cycles of adaptation not founding the degree of adaptation in the first reproductive cycles. Inoculated plants were harvested three times to follow alkaloid concentration; more analysis on time should be done to guarantee the safeness of these endophytes-infected ryegrasses. The goal should be that peramine could be maintained in time but not lolitrem B and ergovaline.

II.6 CONCLUSIONS

Our results showed a widespread concentration range of endophyte-alkaloids in a diverse set of ryegrass plants naturally infected with *Epichloë* endophytes. Distinctive patterns on alkaloids production were observed according to the *Epichloë* morphotype hosted by the perennial ryegrass plants. There was a stronger effect of endophyte morphotypes, over other evaluated factors, on their ability to produce alkaloids in frequency and concentration.

In single infected ryegrass, independently of the harvest, the highest concentration of peramine was produced in plants infected with sexual endophytes (M2S); the content of lolitrem B was the highest in ryegrass infected with the M3 morphotype; and, M1(G1a)-infected ryegrasses produced the highest concentration of ergovaline. Considering such results, the morphotype of the *Epichloë* endophyte may be used as criterion of major importance in the selection of fungi with a particular ability for alkaloid production. For forage improvement the researches should be focused in the M1 morphotype, because they produce lower concentration of lolitrem B and among them, there was a genotypic group in which the production of ergovaline was also lower or was not produced (M1G1b).

A synergistic effect on the alkaloid concentration was observed in some double infected plants, being within these plant ecotypes where the highest concentrations of the

three alkaloids were quantified, especially in those in which one of the endophyte hoste belonged to the M2S morphotype.

Additionally, there were differences in production and concentration of alkaloids by effect of harvest time, which is related to the phenological stage; ryegrass from wirehouse had higher peramine and lolitrem B concentration; whereas the ergovaline concentration was constant in the three harvests.

Inoculated plant produced higher concentration of the insecticidal alkaloid peramine that in the natural associations; on the contrary, alkaloid toxic for livestock lolitrem B and ergovaline were found in lesser concentration or were not detected, which would be a suitable characteristic for the use of these plants as forage.

III. *Epichloë* ENDOPHYTES AFFECT THE NUTRIENT AND FIBER CONTENT OF *Lolium perenne* REGARDLESS OF PLANT GENOTYPE

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III.1 ABSTRACT

Epichloë endophytes inhabit aerial grass tissues but they can modify belowground processes that might affect host nutrient balance. The aim of this chapter was to determine the effects of *Epichloë* infection (E-=non-infected; E+=*Epichloë*-infected) and three *Epichloë* morphotypes (M1, M2, M3) on growth and nutrient content of a heterogeneous set of naturally *Epichloë*-infected asymptomatic plants of *Lolium perenne*. In addition, plant parameters were compared between asymptomatic E+ and plants with choke disease. A field experiment was conducted with 194 plants obtained from six natural populations (97E+, 97E-). For each E+ plant, the morphotype of the *Epichloë* endophyte hosted was known.

Epichloë-infected plants had significantly lower P, Ca, S, B, neutral detergent fiber and lignin contents, and higher Mn and digestibility than E-, independently of plant origin. Biomass production was affected by plant origin but not by the infection with *Epichloë* endophytes. No effect of *Epichloë* morphotypes in any parameter was found. However, asymptomatic E+ and choke diseased plants differed in nutrients, fibers, and digestibility. An *Epichloë* effect was detected in nutrient and fiber content, in spite of the heterogeneous constitution of the plant and fungal material used. The results obtained indicate that *Epichloë* may affect above and possibly underground processes involved in nutrients absorption, as well as plant quality, what may potentially affect litter decomposition processes and consequently the nutrients cycling.

III.2 INTRODUCTION

The nutrient content of grasses can be affected by soil properties, climate, plant factors (species, genotype, phenological stage), and also by symbiotic relationships (Jones and Thomas 1987; Nelson and Moser 1994). Symbiotic associations of plants with fungi are ubiquitous in nature. For instance, those occurring with arbuscular mycorrhizae have been known for long, and positively affect nutrient acquisition in plant hosts (Jeffries et al. 2003). More recent studies indicate that most plant species harbor a rich and diverse mycobiota composed of endophytes, fungi that infect plant tissues without causing disease symptoms. An increasing number of examples is showing that some symbioses of plants with endophytes are beneficial, providing increased plant growth and stress tolerance (Rodriguez et al. 2009).

Today *Epichloë* is probably the most studied genus of fungal endophytes (Saikkonen et al. 2006; Leuchtmann et al. 2014; Tadych et al. 2014). Hyphae of *Epichloë* endophytes colonize systemically the intercellular space of aerial organs like leaves and stems of the host plant, but there are differences among species of this genus in their life cycles. Some species (e.g. *Epichloë festucae* var. *lolii = Neothyphodium lolii*) are transmitted vertically to the seeds, this type of spread is asexual and clonal, and the same fungal genotype that infects a host plant will be transmitted to its seeds. Other *Epichloë* species are exclusively sexual (e. g. *Epichloë typhina*), they are not vertically transmitted to seeds and develop a tubular stroma that wraps the inflorescence primordium in host plant reproductive stems, inhibiting panicle development, a disorder known as 'choke disease' of grasses. Ascospores released from fertilized stromata of *E. typhina* can infect florets of other healthy plants, giving rise to infected seeds (Clay and Schardl 2002) (Figure 2).

Although *Epichloë* endophytes are only present in aerial parts, they can alter belowground components and processes. Changes in root biomass and morphology, root exudates and mycorrhizal colonization have been observed in *Epichloë* infected plants (reviewed by Omacini et al. (2004)). Thus, endophyte-mediated alterations of roots can affect host plant nutrition. For instance, roots of infected tall fescue plants release phenolic compounds with Fe^{3+} reducing and P solubilizing activity (Malinowski et al. 1998; Malinowski and Belesky 1999a), and endophyte-infected plants showed an increased capability to bind copper in the rhizosphere (Malinowski et al. 2004). An increase of phenolic compounds in roots of endophyte-infected plants have been reported in several other grass species (Ponce et al. 2009; Vazquez de Aldana et al. 2011), increasing the competitive ability of infected versus non-infected plants due to root-mediated allelopathic interactions (Sutherland et al. 1999; Vazquez de Aldana et al. 2013b). Changes in plant nutrient content might alter the quality of litter produced by senescent grass shoots, which is one of the main factors that control decomposition rates (Meier and Bowman 2008). Therefore, *Epichloë* endophytes might modify litter decomposition rates, and consequently nutrient cycling, by altering the quality of the litter produced by infected plants, and/or by changing the composition of invertebrate detritivores and microbial decomposers (Omacini et al. 2004; Franzluebbers and Stuedemann 2005; Lemons et al. 2005).

While research on the alkaloid-mediated antiherbivore resistance or drought performance of endophyte infected plants is abundant (Bush et al. 1997; Malinowski and Belesky 2000; Clay and Schardl 2002; Kuldau and Bacon 2008; Saikkonen et al. 2013), the effects of endophytes on plant nutrient content have been much less explored. Several studies have shown that endophytes modify the nutrient status of host plants like tall fescue (*Festuca arundinacea = Schedonorus arundinaceus*) (Lyons et al. 1990; Malinowski et al. 1998; Malinowski and Belesky 1999a; Rahman and Saiga 2005), *Festuca rubra* (Zabalgogeazcoa et al. 2006; Vazquez de Aldana et al. 2013a), or *Achnatherum sibiricum* (Li et al. 2012). However, in spite of the economic importance of perennial ryegrass (*Lolium perenne*) as a forage crop, little is known about the effect of *Epichloë* endophytes on its nutrient content. To our knowledge, there is only one report about the effect of *Epichloë* infection on the content of several

nutrients in ryegrass (Ren et al. 2009). Other reports focus on single elements like nitrogen (Lewis et al. 1996), phosphorus (Ren et al. 2007), or heavy metals (Malinowski et al. 2004; Monnet et al. 2005; Ren et al. 2006). The effect of *Epichloë* endophytes on fiber content and digestibility of *L. perenne* has also received scarce attention (Oliveira et al. 2004). Furthermore, all of these studies were carried out with one or two cultivars of *L. perenne* and their corresponding *Epichloë* genotypes; therefore, very limited variability among plants and endophytes was included.

Lolium perenne is one of the most important forage and turf grasses in the world. As a forage grass, it can produce good yields, has high digestibility, and withstands repeated defoliation. In addition, *L. perenne* can tolerate a broad range of environmental conditions, including widely fluctuating temperatures and soil moisture deficits. Its wide adaptability and good agronomic performance have contributed to its ecological success and worldwide spread (Peeters 2004). Perennial ryegrass is often infected by *Epichloë* endophytes (Hume and Sewell 2014), and in natural populations phenotypic diversity is found among fungal isolates (Bony et al. 2001; Soto-Barajas et al. 2013). Some of these distinct *Epichloë* phenotypes correspond to particular rDNA nucleotide sequences, and might belong to distinct taxa (Soto-Barajas et al. 2013). In this regard, it has been reported that *L. perenne* is a host of at least four taxonomic groups of endophytes that include *Epichloë festucae* var. *lolii* (=*Neotyphodium lolii*); the choke pathogen *E. typhina*, an asexual hybrid designated as LpTG-2, and an *E. festucae*-like endophyte (Schardl et al. 1994; Moon 1999). Given the phenotypic and possible species diversity of *Epichloë* endophytes found in wild populations of *L. perenne* (Moon 1999; Bony et al. 2001; Soto Barajas et al. 2013, Chapter I), our hypothesis was that the morphotype of *Epichloë* endophyte hosted might influence the nutrient acquisition from soil, and this could alter the nutrient content of ryegrass plants. The experimental methods used to detect the effects of *Epichloë* endophytes in plants are in most cases based in the use of one or a few plant cultivars or lines, each one having E+ and a corresponding set of E- plants. Fungicides or other techniques such as heat can be used to eliminate the endophyte from some infected seeds (Zabalgogeazcoa et al. 2006; Cheplick et al. 2014) in order to obtain near isogenic E- material, or an E- plant can be artificially inoculated in order to use it as a source of E+ seed (Ravel et al. 1997a; Hahn et al. 2008). These approaches allow to detect effects of specific endophyte genotypes in particular plant backgrounds (i.e. cultivars), but might not be the best setting to detect strong endophyte effects that could be occurring over a wide set of plant and fungal genotypes.

A field experiment was conducted with a set of perennial ryegrass plants naturally infected with different *Epichloë* endophytes and non-infected plants. These plants had been collected at six natural populations in different habitats. The aim of this chapter was to identify endophyte effects on growth, mineral and fiber contents in a genetically heterogeneous set of *L. perenne* plants and *Epichloë* endophytes. Specific questions were to determine whether *Epichloë* endophytes affect plant tissue chemistry independently of plant origin, and to determine whether diverse *Epichloë* endophytes have different effects on the tissue chemistry of their plant hosts.

III.3 MATERIALS AND METHODS

III.3.1 Plant material

In the spring of 2012, plants of *L. perenne* were collected at six locations in different habitats in western Spain (Table 8).

Table 8 Characteristics of the locations where *Lolium perenne* plants were collected

Location		Habitat	Altitude (masl)	Coordinates Latitude	Coordinates Longitude	Precipitation (mm/year)	Mean annual temperature (°C)
Ciudad Rodrigo	(CR)	Riverbank	625	40°34'48"N	6°30'58"W	531	13.3
La Vecilla	(LVE)	Rural path	879	42°42'20"N	5°23'2"W	556	11.2
Los Valles	(LVA)	Dehesa grassland	813	40°56'20" N	6°7'36"W	531	12.1
Porqueriza	(POR)	Dehesa grassland	807	40°58'18"N	5°57'24"W	531	13.3
Tábara	(TAB)	Oak forest	766	41°50'15"N	5°58'40"W	379	12.3
Valle Fuentes	(VAF)	Low woodland	1133	42°56'33"N	5°14'18"W	556	13.3

These habitats were not agricultural, and clumps or individual plants of perennial ryegrass occurred interspersed with other plant species. At each location, about 50 plants were collected leaving a distance of at least 10 m between each pair of samples. In some locations, plants with symptoms of choke disease were sampled (Figure 21d).

The plants were transported to the Institute of Natural Resources and Agrobiology in Salamanca (IRNASA-CSIC) and transplanted to 2 L pots containing a mixture of perlite and peat moss (1:1, v/v). The pots were kept outdoors in a wirehouse, watered regularly, and fertilized once a year with a liquid commercial fertilizer.

In a previous work we reported that on average, 44% of the plants of *Lolium perenne* were infected by *Epichloë* endophytes in several Spanish natural populations, including some whose plants were used for this study (Chapter I, Table 2) (Soto-Barajas et al. 2013). The endophytes isolated from asymptomatic plants in that study were classified into three morphotypes based in morphological characters observed in potato dextrose agar cultures. The most common morphotype was M1, characterized by white cultures with strongly aggregated 'brain-like' mycelium, and the slowest growth rate of the three morphotypes (Figure 21a). Isolates of the M2 morphotype were the least abundant, having white, cottony aerial mycelium, and the fastest growth rate (Figure 21b). The M3 morphotype had tan, flat and smooth mycelium, and an intermediate growth rate (Figure 21c). Endophytes isolated from symptomatic plants with choke disease, caused by *Epichloë typhina* (Figure 21d) had M2 morphology, and were designated as M2S. Furthermore, rDNA nucleotide sequences of M2 and M2S morphotypes were identical to those of *Epichloë typhina*, while sequences of M1 and M3 morphotypes were identical to *E. festucae* sequences (Figure 11, (Soto-Barajas et al. 2013).

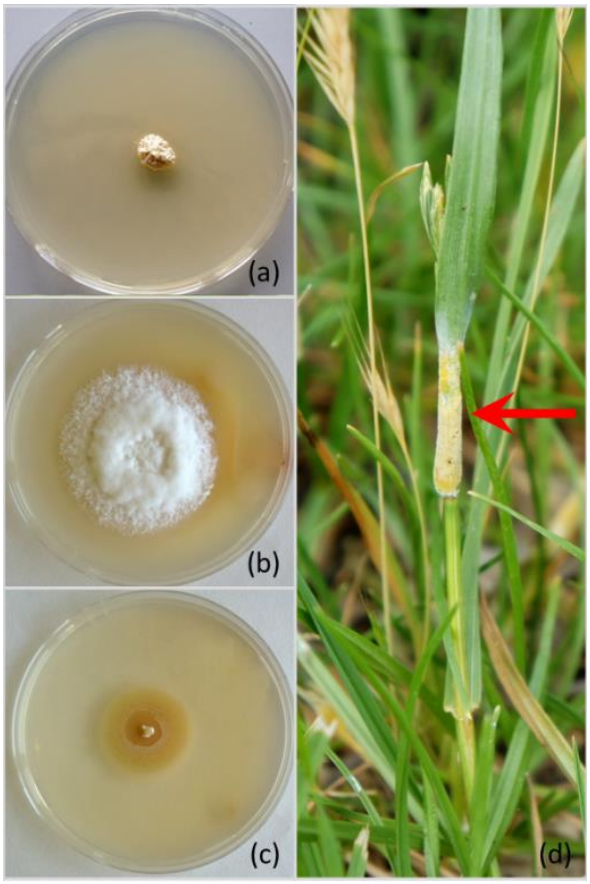

Figure 21 Cultures of the three morphotypes of *Epichloë* endophyte isolated from plants of *Lolium perenne* (a) M1 morphotype, slow growth rate with 'brain-like' mycelium; (b) M2morphotype, faster growth rate with white cottony aerial mycelium; (c) M3 morphotype intermediate growth rate with tan, smooth and flat mycelium; (d) A stroma of *Epichloë typhina* in ryegrass, a typical symptom of choke disease

III.3.2 Field experiment design

A field experiment was carried out with a total of 194 plants of *L. perenne* obtained from the six locations described in Table 8; 97 plants were naturally infected with *Epichloë* (E+), and 97 were not (E-). For each E+ plant, the morphotype it hosted (M1, M2, M3 and M2S) was known, and both asymptomatic plants bearing M1, M2 and M3 *Epichloë* morphotypes, and symptomatic plants with choke disease (M2S) were considered together as 'morphotype' factor. *Lolium perenne* is a cross-pollinated species in which genetic diversity occurs even within commercial cultivars (Peeters 2004). For instance, McNeilly and Roose (1984) reported that 10-year-old pastures originally sown with two cultivars of *L. perenne* could contain 40 to 50 different genotypes per 0.25 m^2. Therefore, in this field experiment we could expect to have a very heterogeneous set of plant genotypes because the plants were collected from different locations and habitats in natural populations, where they occurred in sympatry with other plant species, and sampled individuals were spatially distanced.

The experimental design was based on having the same number of E+ and E^- plants from each location; however, the number of plants differed among locations because we used all E+ plants obtained at each location in order to include all the morphotype variability available in the experiment (Table 9).

Table 9 Number of *Lolium perenne* plants used in the experimental trial, and percentage distribution of infected plants according to the morphotype of the *Epichloë* endophyte at each sampling location.

Plant origin	n[¶]	*Epichloë* morphotype[§]			
		M1	M2	M3	M2S
		Percentage of plant (%)			
CR	26	15.3	7.6	46.1	30.7
LVE	28	100	0.0	0.0	0.0
LVA	40	45.0	15.0	5.0	35.0
POR	24	41.6	16.6	25.0	16.6
TAB	44	50.0	0.0	50.0	0.0
VAF	32	68.7	6.2	25.0	0.0
Total/mean=	194	53.6	7.2	25.7	13.4

[¶] n= number of plants per location. One half of the plants from each location were E+ and the other half were E-.
[§]The morphotypes were: M1= slow growth rate with 'brain-like form'; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; and with the choke disease M2S = M2 from plants with stromata (see Figure 21).

On October 2013, the ryegrass plants were transplanted in a randomized design, at the experimental farm Muñovela (Salamanca, Spain; 40°54'19" N, 5°46'28" W; 780 masl; annual precipitation 372 mm, and mean annual temperature 12.7 °C) in a clay eutric chromic cambisol (FAO/UNESCO 1998) with neutral pH on the surface, decreasing slightly with depth. This soil contains low concentrations of organic matter (1.26 %), total nitrogen (0.08 %), available phosphorus (16.6 mg kg^{-1}), potassium (107 mg kg^{-1}), and calcium (984 mg kg^{-1}). Each plant was set at a distance of 50 cm from its neighbors. Plants were watered during their establishment in October but not thereafter. During the experiment, the plants were not fertilized and the plot was maintained free of weeds.

On May 19 2014, the plants were harvested at the flowering stage, cutting them 5.0 cm above the soil surface. Before harvesting, the number of tillers was counted in all plants, including those whose reproductive stems bore *E. typhina* stromata. All samples were dried at 60 °C in a forced air oven during 48 hours and the dry matter yield was determined.

III.3.3 Chemical analysis

All ryegrass samples were ground using a hammer mill (Fritsch 15303) fitted with a 0.5 mm screen. Each sample was analyzed for total N by digital colorimetry with a segmented flow system (AutoAnalyzer AA3, Bran Luebbe). For mineral content, plant samples were calcined (450 °C), for 8 hours, and ashes were dissolved in HCl:HNO_3:H_2O (1:1:8). The concentrations of P, K, Ca, Mg, S, Na, Mn, Zn, Cu, B, Mo and Co were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Varian 720-ES).

Neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin and dry matter digestibility (DMD) were evaluated using the filter bag technique, with an automated fiber analyzer Ankom A2000, based on the analytical method of Goering and Van Soest (1970).

III.3.4 Statistical analysis

A two-way ANOVA was performed to determine the effect of *Epichloë* infection (E+ and E-) and plant origin (CR, LVA, LVE, POR, TAB and VAF) on growth and chemical composition of *L. perenne*. For this statistical analysis, plants with choke disease were not included because their scarce biomass production was visually evident, and these plants were found only in one half of the locations (Table 9). When a significant effect was detected, differences between pairs of means were assessed using the Bonferroni test, adequate for the unbalanced design.

The effect of *Epichloë* morphotype on dry matter production and the number of tillers was assessed on asymptomatic infected plants hosting endophytes with the M1, M2 and M3 morphotypes by means of one-way ANOVA. When a significant effect was detected, differences between pairs of means were assessed using the Bonferroni test.

To evaluate the effect of each *Epichloë* morphotype on chemical composition of ryegrass, both asymptomatic plants (M1, M2, and M3) and plants with choke disease (M2S) were considered. When a significant effect was detected, differences between pairs of means were assessed using the Bonferroni test. SigmaPlot software version 13.0 (Systat Software, San Jose, CA, USA) was used for all the statistical analyses.

III.4 RESULTS

III.4.1 Plant biomass and number of tillers

The aboveground plant biomass, composed of leaves, stems and inflorescences, was measured at the flowering stage. The ANOVA did not detect a significant effect of endophyte infection on dry weight of ryegrass (P= 0.442) neither an interaction between endophyte and plant origin (P= 0.126). However, the location of origin of plants affected the dry matter yield (P<0.001). Plants from LVE, VAF and CR had the highest yield, and those from POR had the lowest dry weight (Figure 22).

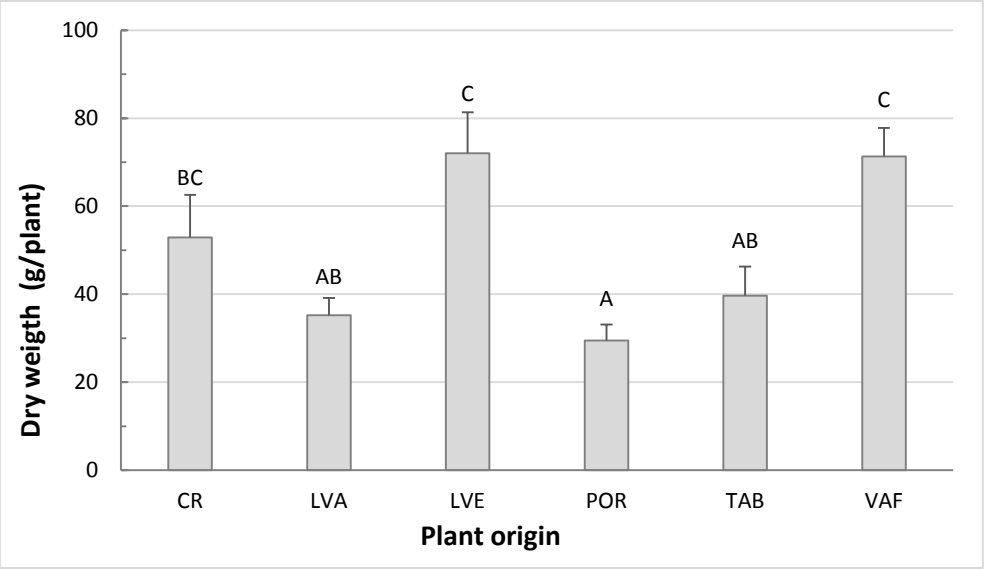

Figure 22 Dry matter yield of *L. perenne* according to locations of plant origin. Bars are means + standard error. Different letters on the bars indicated significant differences ($P< 0.05$)

The ANOVA detected a significant effect of the interaction between infection status and plant origin on the number of tillers (P=0.039). This effect was expressed only in plants from TAB, with a significantly higher tiller production in E- (124.8±13.6) than in E+ (63.5±11.6) plants; in the remaining populations there were no significant differences in tiller number between E+ and E- plants (Figure 23).

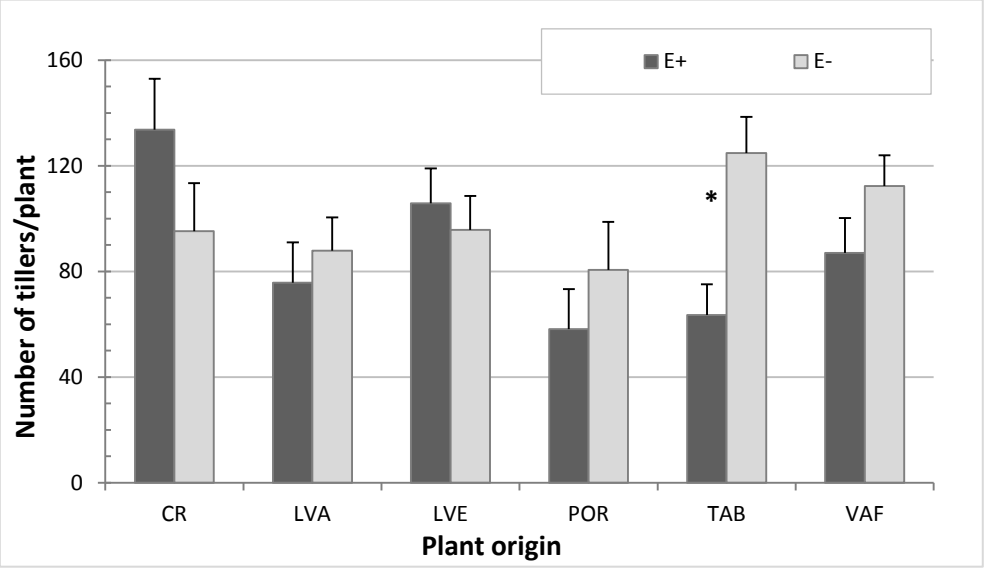

Figure 23 Number of tillers per plant as affected by plant origin, and infection status: E+ = *Epichloë*-infected; E– = Non-infected plants. Bars are means + standard error. *Significant differences between E+ and E- (Infection status x Plant origin; P =0.039)

The effect of each endophyte morphotype of E+ asymptomatic plants on dry matter yield and tiller number was not significant (*P*=0.411 and *P*=0.626 respectively). Thus differences among plants infected with different endophyte morphothytes in dry matter production (M1=50.9±6.99 g plant^{-1}; M2=49.8±14.92 g plant^{-1}; M3=36.24±7.27 g plant^{-1}) and number of tillers (M1=83±7.4; M2=88±20.6; M3=72± 9.7) were not statistically different. In plants with choke disease, the average dry matter production was 9.5±1.7 g plant^{-1} and the number of tillers was 22±10.4.

III.4.2 Mineral content

The two-way ANOVA indicated a significant effect of endophyte and plant origin on the concentration of several mineral elements, with a significant interaction between those factors only for Co (see Table 10).

Table 10 Mineral contents of *Lolium perenne* plants as affected by endophyte status (E+ = infected; E– = non-infected) and location of plant origin. The E+ sample set did not include plants with choke disease. Values are means ± standard error

		Mineral content (g kg^{-1})						
		N	P	K	Ca	Mg	S	Na
Endophyte	E+	13.46±0.24	2.70±0.05	10.37±0.18	2.34±0.06	1.06±0.02	1.62±0.05	0.40±0.03
	E–	13.45±0.23	2.99±0.05	10.39±0.17	2.51±0.05	1.05±0.01	1.83±0.05	0.48±0.03
Plant origin	CR	12.54±0.50 ab	2.51±0.11 a	9.52±0.38 a	2.27±0.13 ab	0.92±0.04 a	1.60±0.11	0.78±0.07 c
	LVA	13.27±0.37 ab	2.62±0.08 a	10.16±0.28 a	2.65±0.09 ab	1.09±0.03 ab	1.74±0.08	0.31±0.05 ab
	LVE	14.16±0.37 bc	3.20±0.08 b	11.65±0.28 b	2.48±0.09 ab	1.14±0.03 b	1.68±0.08	0.36±0.05 ab
	POR	13.51±0.47 ab	2.84±0.10 ab	10.04±0.36 a	2.41±0.12 ab	1.07±0.04 ab	1.79±0.10	0.52±0.07 bc
	TAB	15.25±0.34 c	3.06±0.07 b	11.00±0.26 ab	2.74±0.09 b	1.10±0.03 ab	1.86±0.07	0.42±0.05 ab
	VAF	11.98±0.34 a	2.84±0.07 ab	9.89±0.26 a	2.06±0.09 a	0.96±0.03 a	1.68±0.07	0.24±0.05 a
***P* (ANOVA)**								
Endophyte(E)		0.958	**<0.001**	0.937	**0.048**	0.952	**0.005**	0.132
Plant origin (P)		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.340	**<0.001**
E x P		0.440	0.910	0.250	0.303	0.508	0.945	0.807

		Mineral content (mg kg^{-1})					
		Mn	Zn	Cu	B	Mo	Co
Endophyte	E+	30.88±1.08	18.47±0.39	4.65±0.13	5.41±0.31	1.01±0.06	0.043±0.001
	E–	27.86±1.05	18.55±0.38	4.58±0.12	7.11±0.29	1.16±0.06	0.041±0.001
Plant origin	CR	27.83±2.29	16.76±0.83 a	4.20±0.26 a	5.72±0.64	1.03±0.14	0.033±0.003 a
	LVA	31.43±1.72	17.69±0.62 ab	4.46±0.19 a	6.27±0.47	0.96±0.10	0.042±0.002 ab
	LVE	30.35±1.72	19.00±0.62 ab	5.29±0.18 b	6.43±0.45	1.32±0.09	0.042±0.002 ab
	POR	28.11±2.12	19.78±0.76 ab	4.57±0.24 ab	6.28±0.59	1.18±0.12	0.042±0.002 ab
	TAB	32.36±1.55	19.83±0.56 b	4.70±0.17 ab	7.02±0.43	1.05±0.09	0.046±0.002 b
	VAF	26.24±1.57	17.46±0.56 ab	4.35±0.17 a	5.79±0.43	0.98±0.09	0.041±0.002 ab
***P* (ANOVA)**							
Endophyte(E)		**0.049**	0.645	0.670	**<0.001**	0.094	0.975
Plant origin (P)		0.074	**0.003**	**0.002**	0.431	0.135	**0.016**
E x P		0.891	0.269	0.163	0.651	0.438	**0.024**

In each column, values with different letters are statistically different at *P*<0.05

The concentrations of P, Ca, S, and B were significantly greater in E- than in E+ plants, and that of Mn was greater in E+ than in E-. The effect of plant origin was significant for the mineral content of nine elements (N, P, K, Ca, Mg, Na, Zn, Cu and Co), but not for S, Mn, B and Mo. In general, plants from LVE and TAB had the highest concentrations of minerals and those from CR and VAF the lowest (Table 10). Regarding the interaction between endophyte and plant origin on Co, its concentration in E- plants (0.414±0.036 g kg^{-1}) was significantly greater than in E+ (0.251±0.038 g kg^{-1}), but only in plants from CR, in the other locations differences between E+ and E- were not statistically significant.

According to the one-way ANOVA with morphotype factor, asymptomatic plants infected with M1, M2 or M3 endophytes did not differ in their concentration of minerals (Table 11). On the other hand, plants with stromata (M2S) had significantly greater concentrations of N, K, Ca, Mg, S, Mn, Zn, Cu and B than those infected by asymptomatic endophytes (Table 11). The concentrations of Zn, Cu and B in plants with M3 were not statistically different from plants with M2S.

Table 11 Mineral content in *L. perenne* plants infected by different *Epichloë* morphotypes Values are means ± standard error.

Morphotype[§]	Mineral content (g kg^{-1})						
	N	P	K	Ca	Mg	S	Na
M1	13.59±0.36 a	2.79±0.08	10.64±0.31 a	2.41±0.10 a	1.04±0.04 a	1.70±0.07 a	0.362±0.049
M2	13.07±0.78 a	2.56±0.09	10.16±0.25 a	2.18±0.09 a	0.97±0.04 a	1.45±0.05 a	0.387±0.113
M3	14.09±0.50 a	2.75±0.08	10.29±0.25 a	2.44±0.13 a	1.05±0.04 a	1.60±0.06 a	0.406±0.072
M2S	16.65±0.70 b	2.95±0.14	12.25±0.34 b	3.28±0.11 b	1.44±0.07 b	2.32±0.19 b	0.583±0.181
P (ANOVA)	**0.002**	0.399	**0.016**	**<0.001**	**<0.001**	**<0.001**	0.388

Morphotype[§]	Mineral content (mg kg^{-1})					
	Mn	Zn	Cu	B	Mo	Co
M1	31.18±1.72 a	18.43±0.62 a	4.71±0.21 a	5.27±0.26 a	0.96±0.07	0.045±0.002
M2	27.23±3.36 a	17.48±0.65 a	4.29±0.32 a	5.25±0.49 a	0.87±0.13	0.037±0.005
M3	33.13±2.22 a	19.05±0.82 ab	4.67±0.30 ab	5.85±0.41 ab	1.21±0.14	0.042±0.002
M2S	46.41±3.83 b	22.27±1.16 b	5.82±0.43 b	7.21±0.67 b	1.25±0.14	0.047±0.005
P (ANOVA)	**<0.001**	**0.025**	**0.016**	**0.022**	0.123	0.291

P= significance level for one-way ANOVA. In each column, values with different letters are statistically different at $P<0.05$. [§]M1= slow growth rate with 'brain-like form'; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; and in plants with choke disease M2S = M2 from plants with stromata (see Figure 21).

III.4.3 Fiber content and digestibility

Results of the two-way ANOVA showed a significant effect of endophyte status and plant origin on neutral detergent fiber (NDF), lignin, and dry matter digestibility (DMD), and the interaction between both factors was not significant for any of these parameters (Table 12). The E+ plants had lower NDF and lignin (*P*= 0.060), and higher DMD than E- plants. Differences in acid detergent fiber (ADF) between E+ and E- plants were not statistically significant. The effect of plant origin was significant for NDF, lignin and DMD contents. Plants from CR had the lowest NDF and lignin and the greatest DMD; on the contrary, samples from TAB had the highest NDF and lignin, and the lowest DMD. Plants from LVA, LVE, POR and VAF had similar percentages of NDF, lignin and DMD, and they were intermediate between those found in plants from CR and TAB.

Table 12 Mean contents ± standard error of acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, and dry matter digestibility (DMD) of *L. perenne* plants as affected by endophyte status (E+ = infected; E– = non-infected) and location of plant origin. The E+ sample set did not include plants with stromata.

		ADF	NDF	Lignin	DMD
		Concentration (%)			
Endophyte	E+	28.05±0.24	48.30±0.35	5.61±0.14	59.73±0.36
	E–	27.67±0.23	49.46±0.33	6.02±0.14	57.67±0.35
Plant origin	CR	27.06±0.51	47.17±0.74 a	5.53±0.31 ab	59.89±0.77 b
	LVA	27.26±0.38	48.62±0.55 ab	5.68±0.23 ab	58.83±0.57 ab
	LVE	28.02±0.38	49.66±0.55 ab	6.20±0.22 ab	57.47±0.54 ab
	POR	28.40±0.47	49.66±0.68 ab	5.47±0.29 a	59.65±0.71 ab
	TAB	28.44±0.35	49.86±0.50 b	6.46±0.21 b	56.71±0.52 a
	VAF	27.95±0.35	48.33±0.50 ab	5.52±0.21 ab	59.83±0.52 ab
P (ANOVA)	Endophyte (E)	0.266	**0.019**	0.060	**<0.001**
	Plant origin (P)	0.115	**0.022**	**0.008**	**<0.001**
	E x P	0.231	0.501	0.663	0.776

P= significance level for one-way ANOVA. In each column, values with different letters are statistically different at *P*<0.05.

Considering the *Epichloë* morphotypes evaluated, a significant effect was detected in ADF and NDF, but plants infected with asymptomatic endophytes (M1, M2 and M3) did not differ in these parameters (Table 13). Plants with choke disease (M2S morphotype) had lower ADF and NDF contents than asymptomatic E+ plants.

Table 13 Mean contents ± standard error of acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin and dry matter digestibility (DMD) of *L. perenne* plants infected with different *Epichloë* morphotypes.

Epichloë morphotype[¶]	ADF	NDF	Lignin	DMD
	Concentration (%)			
M1	28.18±0.29 b	48.46±0.41 b	5.47±0.25	58.95±1.00
M2	28.25±0.69 b	49.87±1.21 b	5.11±0.23	60.25±1.02
M3	27.52±0.55 b	47.24±0.86 b	5.46±0.25	60.48±0.67
M2S	21.72±0.50 a	37.89±0.85 a	5.87±0.38	61.09±0.75
P (ANOVA)	**<0.001**	**<0.001**	0.302	0.079

[¶]M1= slow growth rate with 'brain-like form'; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; and with the choke disease M2S = M2 from plants with stromata (see Figure 21).In each column, values with different letters are statistically different at $P<0.05$.

III.5 DISCUSSION

In spite of the potentially large plant variability included in our experiment, with plants from six wild populations, we detected a significant effect of *Epichloë* endophytes on the concentration of five mineral elements, fibers and DMD in shoots, and it was independent of the plant origin. This result indicates that *Epichloë* can alter the nutrient balance in perennial ryegrass by decreasing the concentrations of P, Ca, S, B, NDF and lignin, and increasing Mn content and DMD in comparison to E- plants. This occurred, even though dry matter yield was not significantly altered.

Under our experimental conditions, infected plants had lower P, Ca, S and B content than those not infected. Ren et al. (2009) also reported lower B content in E+ than in E- shoots of one ryegrass cultivar, with no significant effects on the concentration of the other nine elements that they analyzed (P, K, Ca, Mg, Na, Mn, Fe, Zn, Cu), and Ren et al. (2007) found a slight increase in P content in sheaths of E- ryegrass plants growing with a low P supply, as compared to E+. In agreement with our results, an absence of endophyte effect for total N (Lewis et al. 1996) and Zn concentration (Monnet et al. 2005) was reported when a single genotype of ryegrass was evaluated. In contrast, our results showed an endophyte-mediated increase in Mn, an essential element for some lignin-degrading enzymes such as Mn peroxidases (Fioretto et al. 2005). Increased nutrient content due to endophyte was also observed for Zn and Mo in perennial ryegrass (Malinowski et al. 2004), and for P, Ca or Zn in other grasses like *Festuca rubra* (Zabalgogeazcoa et al. 2006; Vazquez de Aldana et al. 2013a) and tall fescue (Malinowski et al. 1998; 2000).

Nutrient uptake in plants through the root surface is mainly meditated by three mechanisms: mass flow, diffusion, and root interception (Jungk 2002). Mass flow occurs when nutrients are transported to the root surface by the movement of water in the soil (i.e. percolation, transpiration, or evaporation). Any change produced by *Epichloë* endophytes in

leaf area or tiller structure in their grass host could affect the evapotranspiration rate and consequently the nutrient acquisition. In plants with choke disease (MS2), high evaporation from the surface of stromata maximizes the flow of nutrients needed by the fungus for reproduction (White et al. 1997), and those elements which move by mass flow (N, Ca, Mg, S, Mn and Mo) were found in higher concentrations. In contrast, in asymptomatic plants (hosting M1, M2 and M3 *Epichloë* endophytes) concentrations of these elements were almost identical.

The movement of nutrients by diffusion in soils implicates concentration gradients. This kind of flow is particularly susceptible to any chemical change in the rhizosphere and is responsible for the acquisition of P, K, Fe and Zn. For instance, release of root exudates (phenolic compounds) in E+ tall fescue was linked to an increase of P uptake in aboveground tissues (Malinowski et al. 1998). In perennial ryegrass, Ren et al. (2007) found a greater content of total phenolic compounds in roots of E+ plants, but P concentration was superior in sheaths of E- plants than in E+, which is in accordance with our results. This suggests that root exudates of perennial ryegrass may be of different nature than those of tall fescue, and thus mechanisms of nutrient acquisition could be different in both grass species, as suggested (Malinowski et al. 2004).

The third possible plant mechanism to acquire nutrients from soil solution is root interception. It occurs when a nutrient comes into physical contact with the root surface, and is responsible for a considerable amount of Ca uptake, and to lesser extent Mg, Zn and Mn (Jungk 2002). The plant-endophyte interaction can alter rhizospheric conditions that affect the density and activity of different soil organisms, like arbuscular mycorrhizal fungi (AMF) which in turn can enhance root interception. Thus, *Epichloë* infected plants have shown a reduction in colonization and sporulation of mycorrhizae, as compared to plants without *Epichloë* (Chu-Chou et al. 1992; Müller 2003; Omacini et al. 2004; Antunes et al. 2008; Liu et al. 2011). On the contrary, a positive association between *Epichloë* endophytes and AMF has been found in wild native grasses (Novas et al. 2005; 2012; Vignale et al. 2015). Although we did not analyze AMF in our ryegrass plants, a reduction in AMF in E+ plants could explain the lower P content found in plant tissue, but not the greater concentration of Mn, an element immobile in soil which can be mobilized by mycorrhizas. This suggests that other root endophytic and epiphytic species than AMF might be linked to nutrient absorption processes in plant roots (Sánchez Márquez et al. 2010).

Effects of endophytes on plant fiber contents have received scarce attention. We found that the presence of *Epichloë* in ryegrass plants decreased NDF and lignin and increased DMD, with independence of plant origin. The NDF measures all the forage fiber (hemicellulose, cellulose and lignin), and a high NDF content decreases forage quality and intake by ruminants. Our results showed that *Epichloë* endophytes boost the digestibility of *L. perenne*, and this cannot be only attributed to the observed decrease in the lignin fraction. A similar endophyte effect on NDF and DMD was reported for ryegrass (Oliveira et al. 2004; Rasmussen et al. 2008) and red fescue (Zabalgogeazcoa et al. 2006). In contrast, most studies done with tall fescue and *Epichloë coenophiala* (=*Neotyphodium coenophialum*) have shown no effect of endophyte

infection in the concentration of NDF, ADF, lignin, or digestibility (Fritz and Collins 1991; Humphreys et al. 2002; Johnson et al. 2012). The positive effect of *Epichloë* on digestibility of *L. perenne* could be explained, according to Rasmussen et al. (2012), because the intercellular endophytic hyphae promotes a carbohydrate hydrolysis to use cell wall components as a supplementary source of C, and this process might reduce the content of hemicellulose and NDF in plant.

Changes induced by *Epichloë* endophytes on fiber and mineral contents of *L. perenne* maintained in senescent shoots might alter the quality of litter and consequently its decomposition rate as well as recycling of nutrients. The degradation of cellulose and lignin, the most abundant components of litter, is slower than that of other plant components, thus, the lower lignin content of E+ ryegrass plants would imply faster litter decomposition compared to E- plants. On the other hand, litter Ca has been related to increased microbial activity (Berg et al. 2003), and the lower concentration of this element in E+ plants could imply slower decomposition rates. Other aspects of litter quality also affect decomposition processes. The N:P ratio of litter contributes to determine the relative importance of bacteria and fungi in the decomposition process, with low N:P ratios promoting bacteria and high values fungi (Güsewell and Gessner 2009). Thus, differences in P content due to endophyte could affect the structure of the mycobiota involved in decomposition. Experiments of litter decomposition with tall fescue and *Lolium multiflorum* have shown that decomposition was slower for endophyte-infected litter, related to the fact that endophyte infection tended to reduce the N content of litter (Omacini et al. 2004), and to changes in the composition of associated decomposers (Lemons et al. 2005). Thus, differences in litter quality among species could imply differences in decomposition rates (Meier and Bowman 2008).

Due to the variability of the plant material and associated *Epichloë* endophytes used in this study, we expected to find differences in the chemical composition of plant tissues among plants infected by different fungal morphotypes. However, we did not find differences among asymptomatic *Epichloë* endophytes (M1, M2 and M3) in any parameter related to plant growth or chemical composition. Instead, significant differences in nutrient content were detected between asymptomatic and choked plants. In plants infected by the choke pathogen *Epichloë typhina*, the development of reproductive stems is arrested by fungal stromata. Flowering stems have greater fiber and lignin and lower mineral contents than leaves, thus, differences in the leaf:stem ratio might partly explain why plants with choke disease had the lowest NDF, and the greatest DMD and mineral content. In addition, an improvement in photosynthesis efficiency in plants infected with the choke pathogen *E. typhina* was observed in *Dactylis glomerata* (Rozpadek et al. 2015), and this could imply an alteration in the assimilation of nutrients.

The biomass production of *L. perenne* was not affected by the endophyte infection status (E+, E-) or by different *Epichloë* morphotypes (M1, M2, M3), but a significant variation in yield occurred among plants from different origin. Our results agree with those of other studies showing no significant endophyte effect on dry matter yield of ryegrass (Lewis et al.

1996; Barker et al. 1997; Oliveira et al. 2004). Other studies reported an interactive effect between endophyte and plant genotype affecting plant production, both in greenhouse and field assays (Cheplick and Cho 2003; Hesse et al. 2004; Kane 2011). In our field trial, a significant interaction between infection and plant origin on the number of tillers produced was observed, but it was limited to plants from TAB, with more tillers in E- than in E+ plants. Similarly, among 13 genotypes of ryegrass, a negative effect of *Epichloë* endophytes on tillering under hydric stress conditions was reported by Cheplick et al. (2000), although differences were not detected under regular watering. On the contrary, a positive tiller response to *Epichloë* infection has been reported in some genotypes of *L. perenne* growing under hydric stress (Ravel et al. 1997a).

III.6 CONCLUSIONS

In this chapter a heterogeneous set of plants of *L. perenne* and their associated *Epichloë* endophytes was used to study the effect of endophyte infection on host plant growth and nutrient content. The biomass production was affected by plant origin but not by endophyte infection. In contrast, a strong endophyte effect, independent of plant origin, was detected in mineral content (P, S, Mn and B) and fiber contents (NDF, lignin and DMD). The results suggest that *Epichloë* might alter belowground processes that influence nutrient acquisition in the host plant, although the mechanism is not clear and several processes might be involved. In spite of the variability of *Epichloë* endophytes infecting ryegrass plants, plant growth or chemical plant tissue did not vary among different *Epichloë* morphotypes. However, plants infected by the choke pathogen, *Epichloë typhina*, showed significant changes in nutrient content and fiber composition with respect to those infected by asymptomatic *Epichloë* endophytes.

IV. INOCULATION OF *Epichloë* ENDOPHYTES IN COMMERCIAL CULTIVARS OF *Lolium perenne*

IV.1 ABSTRACT

Endophytic fungi of the genus *Epichloë* confer adaptive features beneficial to the growth and persistence of their host grasses, but also may be deleterious to the health of animals that graze them. Many of these traits can be incorporate into new host grasses by artificial inoculation of fungal endophytes. In this chapter, the evaluation of two methods of artificial inoculation of plants with *Epichloë* endophytes is described. The slitting method and a new procedure of inoculation in the culture medium with colonies of *Epichloë* were used to infect two cultivars of perennial ryegrass ('Barplus' and 'Romance') with four morphytes of *Epichloë* endophytes (M1, M2, M2S and M3) isolated from wild plants of ryegrass collected at several locations. The rate of infected plants achieved was higher when inoculation was through the culture medium (12.9%) than with the slitting method (0.5%). Independently of the method of inoculation or cultivar, the most effective inocula were endophytes with the M2 and M2 morphotypes; whereas with the M1 or M3 morphotypes the percentage of successfully inoculated plants was lower than reported elsewhere.

IV.2 INTRODUCTION

The symbiotic relationship of endophytic fungi of the genus *Epichloë* (Fam. *Clavicipitaceae*) with grasses of the *Pooideae* subfamily have gained relevance since Bacon et al. (1977) reported the connection between *Epichloë*-infected tall fescue pastures and fescue toxicosis in cattle. Soon after this, a similar link of *Epichloë*-infected perennial ryegrass pastures with ryegrass staggers in sheep was found (Fletcher and Harvey 1981). On the other hand, many researches have reported that the presence of *Epichloë* endophytes improved pest and/or drought resistance in their grass host (Funk 1983; Latch et al. 1985; Rowan and Gaynor 1986; Arachevaleta et al. 1989; Siegel 1993).

Considering this dual effect of the associations between *Epichloë* fungi with many agronomically important grasses, a primary aim of endophyte research and goal of much interest to worldwide grass seed companies has been the conjunction of a match with suitable characteristics. Seeds infected with no harmful *Epichloë* endophytes are often desired by farmers to prevent the detrimental effects that common toxic endophytes can have on livestock and/or to increase the performance of the grasses that will be difficult to achieve in no-infected grasses (Siegel 1993).

The oldest records about infection of a grass after inoculation with endophytes were made several decades ago. Sampson (1937) obtained endophyte-infected plants after inoculation of *Lolium perenne* seedlings with an unnamed endophyte, and Western and Cavett (1959) infected *Dactylis glomerata* with spores of *Epichloë typhina.*

Currently, artificial infection of grasses with *Epichloë* endophytes has been achieved by inoculating seedlings (Latch and Christensen 1985; Leuchtmann and Clay 1993; Christensen 1995), callus cultures (Johnson et al. 1986; Kearney et al. 1991) or plantlets derived from meristems (O'Sullivan and Latch 1993). The predominant method involves inserting mycelium from pure cultures into the meristematic region at the junction of the mesocotyl and coleoptile of young seedlings, either into slits cut with a scalpel or by injection (Latch and Christensen 1985; Koga et al. 1993; Leuchtmann and Clay 1993). These techniques are slow, laborious and have a low success rate of infected seedlings (lower than 13%). The inoculation method by means of a callus culture, developed by Johnson et al. (1986), achieved a higher endophyte infection success rate (17%), but is very time consuming taking around 30 weeks to produce a small seedling.

Due to the difficulties that involve the use of these inoculation techniques, there have been attempts to develop new protocols to improve inoculation efficiency, and to optimize the time invested. For example, floret inoculation has proven to be a successful method in wheat with *Fusarium graminearum* (Engle et al. 2003), but it was not a feasible method for creating infections of grass cultivars with novel strains of *Epichloë* endophytes (Gillanders 2007). For this reason, the slitting technique had remained as the most used method in developing *Epichloë*-grass associations, and it has allowed most of the studies about the effects of the endophytes on their host in different environments (Morse et al. 2007; Jia et al. 2016).

Once a new synthetic infection is released, there are other factors that also influence the effect of the endophyte on the host grass, and the association must be carefully followed up (do Valle Ribeiro 1993; Gillanders 2007) because evidence suggests that the strength and direction of *Epichloë* endophyte interactions with new host grasses are highly variable (Latch and Christensen 1985; Meijer and Leuchtmann 2000; Saikkonen et al. 2006; Jia et al. 2016). Three sources are the main factors that drive the outcomes of the *Epichloë*-grass interaction: (i) endophyte strain or species, (ii) host plant genotype, and (iii) the local abiotic (*e.g.,* soil nutrients and moisture) and biotic (*e.g.,* the presence of herbivores) environments (Jia et al. 2016).

Therefore it is of major relevance to evaluate how endophytes behave on host grasses with different genetic background and under several environmental conditions before the adoption of any endophyte-grass combination is released for commercial purposes. As a first step for further investigation on the role of *Epichloë* endophytes on the performance of *Lolium perenne*, the aim of this chapter was to evaluate the effectiveness of two techniques of inoculation: (i) the slitting method and (ii) the infection in the culture medium with colonies of *Epichloë*.

IV.3 MATERIALS AND METHODS

IV.3.1 Plant material

The plants used for inoculation were two commercial cultivars of endophyte free ryegrass: 'Barplus', used for forage production, and 'Romance', a turfgrass. Seeds of these cultivars were donated by the Barenbrug seed company, NL. For all inoculation experiments, the lemma and palea were removed and then seeds were surface-sterilized, soaking them in a sodium hypochlorite solution (1% of active chlorine) for 20 min, and rinsed afterwards in sterile water.

IV.3.2 *Epichloë* inocula

The *Epichloë* endophytes used as inoculum were isolated from *Lolium perenne* plants of wild origin collected at seven different locations whit diverse environmental conditions (Chapter I, Table 8). These *Epichloë* strains were morphologically and genetically characterized following the methods detailed in the Chapter I.

Eighteen different *Epichloë* strains were selected for inoculations, trying to have a representation of the four morphotypes described in Chapter I (Figure 6): the asymptomatic M1, M2 and M3, and the stroma-producing M2S endophytes (Table 14).

Table 14 *Epichloë* endophytes isolated of *Lolium perenne* plants used in the inoculation trials.

	MORPHOTYPE[¶]			
	M1	M2	M2S	M3
INOCULUM[§]	LVE11	LVA08	LVA04	CR14
	LVE16	LVA32	LVA17	CR19
	LVE25	POR36	MON06	**TAB09**
	LVE29		MON07	**TAB42**
	TAB21			
	VAF13			
	VAF20			

[¶]M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6). [§]Fugal colonies marked in bold letters were not used for inoculation through the culture medium.

IV.3.3 Techniques of Inoculation

IV.3.3.1 Slitting method

The first inoculation technique evaluated was the slitting method, according to the procedure described by Latch and Christensen (1985). This method consists of making a longitudinal slit of about 3 mm with a scalpel at the junction of the mesocotyl and radicle of the ryegrass seedling, and pushing a piece of mycelium of the selected *Epichloë* endophyte inside the wound. The procedure was done using a microscope (Leica Microsystems) at 20X magnification.

For this trial, the seeds of ryegrass were previously germinated in Petri dishes with water agar (10 seeds per dish) by incubating them for one week in a growth chamber (Sanyo MLR-351H) (25 °C, 12h photoperiod, 60% relative humidity), until the inoculation. The M1 and M3 strains used as inoculum were cultured for two weeks in potato dextrose agar (PDA), and the M2 and M2S morphotypes for one week, because the differences on their growth rate (Chapter I). Fresh fungal mycelum from several PDA dishes of each of the 18 strains (Table 14) were inoculated into approximately 40 seedlings of each ryegrass cultivar ('Barplus' and 'Romance'). Immediately after inoculation, the plants were placed in new Petri dishes with water agar, and kept in the growth chamber for two weeks. After that period the inoculated seedlings were transplanted to a seedbed (5x5x15 cm) with a sterilized potting mix of perlite:peat moss (1:1, v/v).

IV.3.3.2 Inoculation in culture medium with colonies of *Epichloë*

To evaluate the effectiveness of a new inoculation procedure in culture medium with colonies of *Epichloë*, sterilized ryegrass seeds were placed on growing colonies of *Epichloë* fungi. For this procedure, the 12 *Epichloë* strains indicated in Table 14 were used as inoculum, and they were grown in Petri dishes with a culture medium composed of PDA and Murasige-Skoog solution. This solution was included in the culture media as a nutrient sink for the ryegrass seedling, because of the long incubation period in the growth chamber. Inocula with M1 and M3 morphotypes were cultured for two weeks, and endophyte with M2 and M2S morphotypes for one week. Twenty ryegrass seeds of each cultivar ('Barplus' and 'Romance') were placed above each of the fungal cultures (Figure 24a). The Petri dishes with the ryegrass seeds were kept in the growth chamber, and during this time any contamination of the culture medium was removed. After two weeks 10 seedlings were randomly selected from each Petri dish, and transplanted to seedbeds (Figure 24b).

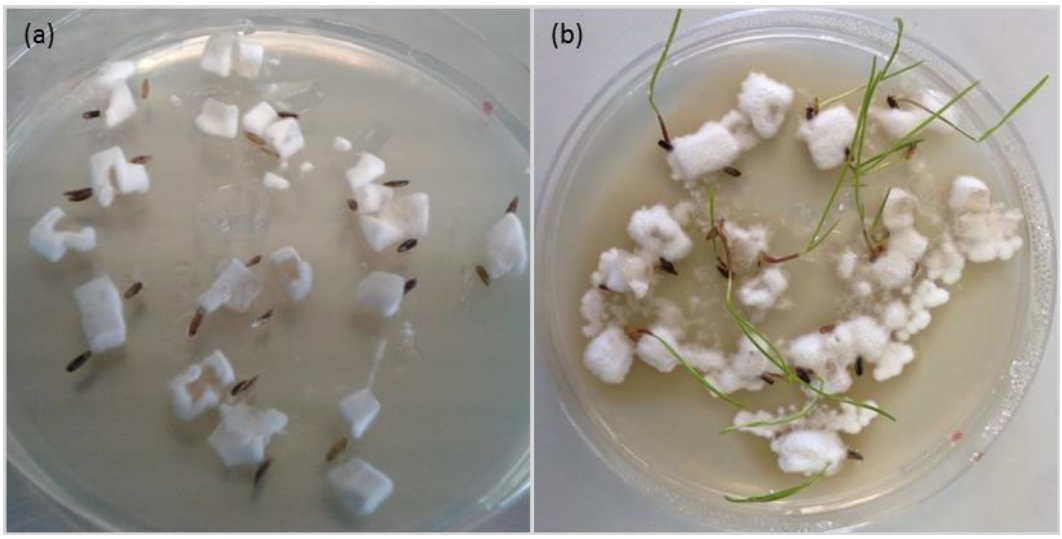

Figure 24 Inoculation of perennial ryegrass seedlings in the culture medium with colonies of *Epichloë* using a strain of the M2 morphotype as inonculum: (a) First day of the trial, (b) seedlings before transplanting, two weeks later.

IV.3.4 Confirmation of the *Epichloë* Infection

Three months after the inoculation, the presence of *Epichloë* endophytes was verified in all the surviving inoculated ryegrass plants (n= 1340). The diagnostic was made by isolation of the endophyte after placing surface-disinfected fragments of leaf sheath in PDA. Endophytes identified as *Epichloë* were confirmed by polymerase chain reaction (PCR) amplification and sequencing of gene segments containing the internal transcribed spacers (ITS4 and ITS5) (White et al. 1990) and a 5' region of the β-tubulin gene (*tub2*) using the primers tub2-exon1d-1 and tub2-exon4u-2 (Moon et al. 2002), and comparing them with those of the original inoculum (following the same protocols described in Chapter I).

The percentage of successful inoculations was calculated on basis of the number of plants examined and not in the number of plants inoculated, because the death of some seedlings before transplantation or poor vegetative development not enough to diagnose them.

All plants diagnosed as *Epichloë*-infected were transferred into individual 2 l pots with a perlite-peat moss (1:1, v/v) potting mix and maintained outdoors in a wirehouse. The inoculated plants which were not infected were kept as control treatment in further experiments (alkaloid concentration, Chapter II) because they underwent the same wounding/handling and transplantation as the successfully inoculated plants.

IV.4 RESULTS

IV.4.1 Slitting method

The number of ryegrass plants diagnosed as successfully infected with *Epichloë* endophytes using the slitting method was only six, which represents a 0.5% of the 1130 analyzed plants. This result was really frustrating. Two 'Barplus' plants were diagnosed as infected plants and four of the infected plants belonged to the cv. 'Romance' (Table 15).

Table 15 Number of ryegrass plants analyzed and infected with *Epichloë* endophytes using the slitting method of inoculation.

Morphotype[¶]	Inoculum	Barplus		Romance		Total	
		Analyzed (n)	Infected (%)	Analyzed (n)	Infected (%)	Analyzed (n)	Infected (%)
M1	LVE11	46	0.0	35	5.7	18	0.0
	LVE16	30	0.0	31	0.0	61	0.0
	LVE25	24	0.0	24	0.0	48	0.0
	LVE29	25	0.0	29	0.0	54	0.0
	TAB21	46	0.0	[§]NA	[§]NA	46	0.0
	VAF13	26	0.0	28	0.0	54	0.0
	VAF20	42	0.0	32	0.0	74	0.0
	Total	239	0.0	179	1.1	418	0.5
M2	LVA08	23	4.3	39	0.0	62	1.6
	LVA32	32	3.1	28	0.0	60	1.7
	POR36	30	0.0	33	0.0	63	0.0
	Total	85	2.4	100	0.0	185	1.1
M2S	LVA04	37	0.0	36	0.0	73	0.0
	LVA17	37	0.0	27	3.7	64	1.6
	MON06	35	0.0	37	2.7	72	1.4
	MON07	26	0.0	34	0.0	60	0.0
	Total	135	0.0	134	1.5	269	0.7
M3	CR14	15	0.0	17	0.0	32	0.0
	CR19	38	0.0	33	0.0	71	0.0
	TAB09	51	0.0	30	0.0	81	0.0
	TAB42	38	0.0	36	0.0	74	0.0
	Total	142	0.0	116	0.0	258	0.0
Total (n) mean (%)		601	0.3	529	0.8	1130	0.5

[¶]M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6).[§] NA= No analyzed

The rate of successful infection with the M1 morphotype was of 0.5%, being only among the cv. 'Romance' the infected plants with this endophyte. The M2 morphotype was effective in the inoculation of 2.4% of the 'Barplus' plants, and no infected plants were observed with the cv. 'Romance'. Inoculation with the M2S morphotype was effective in 0.7% of plants from the cv. 'Romance' and these type of fungi were not successfully inoculated in 'Romance' ryegrass. In this trial, the less effective fungi for inoculation were the M3 morphotype, none of the plants of any of the two cultivars evaluated were infected (Table 15).

IV.4.2 Inoculation in the culture medium with colonies of *Epichloë*

The inoculation in the culture medium with colonies of *Epichloë* was notoriously more effective than the slitting method. On average 12.9% of the analyzed plants were successfully infected with *Epichloë* endophytes. Fifteen of the 112 of the diagnosed 'Barplus' plants (13.4%) and 12 of the 'Romance' ryegrass (12.2%) were successfully inoculated with *Epichloë* (Table 16).

Table 16 Number of ryegrass plants analyzed and infected with *Epichloë* endophytes using the inoculation in culture medium method.

Morphotype[¶]	Inoculum	Barplus		Romance		Total	
		Analyzed (n)	Infected (%)	Analyzed (n)	Infected (%)	Analyzed (n)	Infected (%)
M1	LVE11	10	0.0	8	0.0	18	0.0
	LVE29	10	0.0	10	0.0	20	0.0
	VAF20	8	0.0	9	0.0	17	0.0
	Total	28	0.0	27	0.0	55	0.0
M2	LVA08	9	0.0	9	44.4	18	22.2
	LVA32	10	60.0	9	22.2	19	42.1
	POR36	10	0.0	5	0.0	15	0.0
	Total	29	20.7	23	26.1	52	23.1
M2S	LVA04	9	11.1	7	42.9	16	25.0
	LVA17	10	0.0	8	25.0	18	11.1
	MON06	9	0.0	10	10.0	19	5.3
	MON07	9	77.8	5	0.0	14	50.0
	Total	37	21.6	30	20.0	67	20.9
M3	CR14	8	12.5	8	0.0	16	6.3
	CR19	10	0.0	10	0.0	20	0.0
	Total	18	5.6	18	0.0	36	2.8
Total (n) mean (%)		112	13.4	98	12.2	210	12.9

[¶]M1 morphotype, slow growth rate with "brain-like" mycelium; M2 morphotype, faster growth rate with white cottony aerial mycelium; M2S morphotype: similar to M2 but stroma-producing ; M3 morphotype: intermediate growth rate with tan, smooth and flat mycelium (Figure 6).[§] NA= No analyzed

The M1 morphotype was not successfully inoculated in any of the two cultivars of ryegrass assayed. Fungi with the M2 morphotype infected 20.7% of the 'Barplus' and 26.1% of the 'Romance' plants. The M2S morphotype infected 21.6% of the inoculated 'Barplus' grasses and 20.9% of the 'Romance' plants. Infected plants with the M3 morphotype were found in 5.3% and 2.8% of the cv. 'Barplus' and cv. 'Romance' plants respectively (Table 16).

IV.5 CONCLUSIONS

Inoculation of commercial cultivars of *Lolium perenne* with *Epichloë* endophytes was more effective in culture medium than using the slitting method. Particularly for M2 and M2S, the procedure using the culture medium was very effective. For the M1 and M3 morphotypes, the rate of successful infection was similar for both methods of inoculation, and it was lower than that reported by other researchers using strains of *E. fesctucae* var. *lolii*.

Herein it is the first time in which successful infection of grasses with *Epichloë* endophytes is reported by using inoculation in culture medium with colonies of *Epichloë*. This method may work with same high infection rates as the reported herein for other inoculation trails involving strains with similar characteristics to the M2 morphotype.

V. NEAR INFRARED SPECTROSCOPY TO DETERMINE THE PRESENCE OF *Epichloë* ENDOPHYTES AND THEIR ASSOCIATED ALKALOIDS IN *Lolium perenne*

V.1 ABSTRACT

Near-infrared spectroscopy (NIRS) has been widely used in quality control, particularly in the forage industry, because it is faster, cleaner and less expensive than conventional chemical procedures. In *Lolium perenne*, one of the most important forage grasses, NIRS has been applied for the determination of nutritional parameters. The presence of *Epichloë* fungi is known to alter the nutritive quality of its host plant by means of the production of fungal alkaloids. This chapter focuses in the use of NIRS for the identification of ryegrass infected with *Epichloë* endophytes, and the detection and quantification of the alkaloids peramine, lolitrem B and ergovaline.

The plant material consisted of 222 perennial ryegrass plants, 194 of which were of wild origin and 28 belonged to two commercial cultivars. All the spectra of the grass samples were classified according to the plant condition, not infected or infected with *Epichloë* endophytes (E-, E+); the morphotype of the *Epichloë* endophyte hosted (M1, M3 or M3); and the presence or absence of the alkaloids peramine (PER-, PER+), lolitrem B (LTM-, LTM+) and ergovaline (ERG-, ERG+). For discriminant analysis, the algorithm X Residuals was applied, and for quantitative analysis, a modified partial least squares (MPLS) algorithm was used.

The best discriminant equation for detection of *Epichloë* using the NIR spectra identified 93.3% of E+ plants. The identification of the *Epichloë* morphotypes was correct for 92.9% of M1 morphotype samples and 100% of M2 morphotypes. However, all plants harboring M3 endophytes were wrongly classified as hosting M1 morphotypes. Detection of alkaloids was correct for 94.4% of PER+ plants; 87.5% of LTM+ and, 92.9% of ERG+ plants. The quantitative NIR equation had coefficient of correlation (RSQ) of 0.93, 0.41 and 0.76 respectively, for concentration of peramine, ergovaline and lolitrem B. These results show that NIR spectroscopy is a suitable tool for screening studies related to the direct detection of some anti-quality properties of samples of ryegrass, such as detection and identification of *Epichloë* endophytes, and the presence and quantification of alkaloids.

V.2 INTRODUCTION

Near infrared spectroscopy (NIRS) is a non-destructive technique with a widespread application to food, agricultural and industrial research of various plant products, including the agronomic selection of forages for improved quality (Corson et al. 1999; González-Martín et al. 2006; González-Martín et al. 2007). NIRS is an analytical technique that predicts the chemical composition of material based on the interaction between the surface of the sample and the incident polychromatic light over a spectral wavelength ranging from 1100 to 2500 nm (near infrared range) (Figure 25).

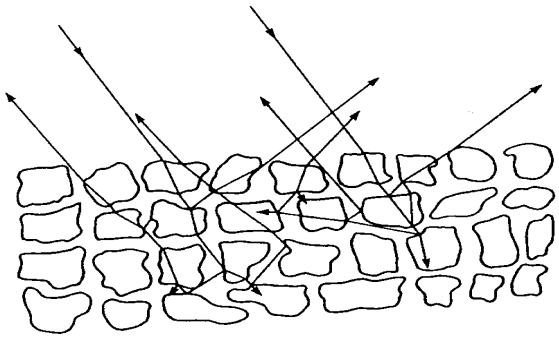

Figure 25 Representation of the effect of diffuse reflectance used in near infrared spectroscopy.

The sample absorbs and reflects specific frequencies corresponding to combinations and overtones of vibration such as stretching and bending of hydrogen-bearing functional groups like –CH, -OH, and –NH (Osborne 2006; Baranska and Schulz 2009). The signal obtained by NIRS contains information about the chemical composition and other properties of the matrix (Baker et al. 1990; Shenk and Westerhaus 1993; Corson et al. 1999). The large amount of data in a NIR spectrum requires the use of chemiometrics to extract qualitative and quantitatively characteristics of interest from the sample (Blanco et al. 2008). With the use of regression techniques, NIR spectroscopy transmittance spectra can be correlated to laboratory-derived data. This correlation results in prediction equations that may or may not accurately quantify a wide range of constituents (Roberts et al. 1997). The NIRS technique is absolutely dependent on reference data or laboratory analyses and its limitations are defined by the capacity for accurate calibration and sample characteristics able to provide interpretable spectra (Corson et al. 1999).

Near infrared spectroscopy offers several advantages over conventional methods of forage quality analysis. NIRS can provide reliable estimates of feed composition and evaluate many constituents (*e.g*. moisture, protein, oil, starch, fibers and others) at the same time using the same spectral signature, is rapid, non-destructive, requires small quantities of plant sample, for plant sample preparation only require dried and ground, no chemical reagents are needed and is less expensive than conventional analytical procedures (Barton 2nd and Windham 1987; Clark et al. 1987; García-Ciudad et al. 1993; González-Martín et al. 2007; Foskolos et al. 2015).

Many researchers have used infrared spectroscopy to discover different forms of adulteration of products, to predict the metabolisable energy value of a feed (Ulyatt et al. 1995; González-Martín and Hernandez-Hierro 2008; Baranska and Schulz 2009) and less commonly is to identify and classify microorganisms like bacteria, fungi and virus (Arnold et al. 2000; Petisco et al. 2008; Petisco et al. 2011; Brandl 2013). Nowadays, in forages NIRS is a routine technique used to analyze the amount of structural fiber, soluble carbohydrate, crude protein, lipid, ash and for detection and quantification of minerals (García-Ciudad et al. 1993; Vazquez de Aldana et al. 1995; González-Martín et al. 2007; Burns et al. 2013). However, NIRS has been rarely explored for the identification of anti-quality properties of forages.

Anti-quality components are defined as any factor that diminishes the degree to which forage meets the nutritional requirements of a specific kind of animal (Allen and Segarra 2001). Among the diverse impediments to forage quality are structural components (*e.g.* lignin) and secondary metabolites, but also are included mineral imbalances related or not to the presence of insects, fungi, bacteria or diseases. Anti-quality components may reduce dry matter intake, dry matter digestibility, or result in nutritional disorders in animals. Such factors represent a high economical cost for the livestock industry, for example, reproductive and death losses of livestock due to poisonous plants have been estimated at 340 million USD in USA (James et al. 1992; Allen and Segarra 2001). There has always been a concern that anti-quality components in forages have the potential to compromise food safety and human health (Allen and Segarra 2001; Pfister et al. 2001; Thompson et al. 2001).

Perennial ryegrass (*Lolium perenne*) is one of the most important cool season grasses and the basis of many forage-livestock systems grown worldwide. Perennial ryegrass, like several other grass species, is often infected by endophytic fungi of the genus *Epichloë* that confer adaptive advantages. However, these fungi are also responsible for some anti-quality factors of the grass. Fungal alkaloids has often pronounced physiological reactions in herbivores, causing negative effects on livestock (Bacon et al. 1977; Fletcher and Harvey 1981; Gallagher et al. 1984; Gallagher et al. 1985; Allen and Segarra 2001; Young et al. 2015). Peramine, lolitrem B and ergovaline are the most common alkaloids produced in perennial ryegrass plants infected with *Epichloë* endophytes. Peramine is an insect deterrent, with no clinical effect over mammals; lolitrem B is a tremorgenic compound responsible for ryegrass stagers in sheep; ergovaline is reported to be a major contributor to several livestock disorders including gangrene in limbs, reduced fertility, hyperthermia, convulsion, all these symptoms of a syndrome known as fescue toxicosis (Yates et al. 1985; Aldrich et al. 1993; Moubarak et al. 1993; Bluett et al. 2005).

The detection of *Epichloë* endophytes in *Lolium perenne* is mainly performed by microbiological, histochemical, immunological or molecular techniques, as presented in Chaper I. Histochemical detection is based on staining plant tissues with rose Bengal or aniline blue, followed by visual inspection of fungal hyphae by light microscopy (Saha et al. 1988; Bacon and White 1994; Miles et al. 1998). Another very slow method of determining the presence of endophytes, is to isolate the endophyte into a culture medium (Latch and

Christensen 1985; Fletcher et al. 1990). Enzyme-linked immunosorbent assay (ELISA) uses specific antibodies against fungal cells (Miles et al. 1998; Dombrowski et al. 2006; Koh et al. 2006). Molecular methods include DNA extraction and polymerase chain reaction (PCR)-based amplification with specific primers, resulting in patterns of simple sequence repeats (Moon et al. 1999; Rasmussen et al. 2007; van Zijll de Jong et al. 2008; Najafabadi et al. 2009). On the other hand, quantitative analysis of alkaloids is based on an elaborated procedure of extraction, followed by quantification by high performance liquid chromatography. Alkaloid extraction requires high investments, due to the use of organic solvents and/or solid-phase exchangeable cartridges. Although these above mentioned methods are preferred for being exact and precise procedures, in large-scale screening studies in which a significant numerous samples should be analyzed, none of them is ideal in term of cost-effectiveness, speed and accuracy, because they need high level of expertise, a series of chemicals and equipment (*e.g*. buffers, primers and dyes), are tedious, and too time-consuming (e. g. Chapter I and III). For the reasons mentioned above, alternative methods such as infrared spectroscopy are studied to develop diagnostic methods with lower economic and ecological cost than conventional techniques (Levasseur et al. 2010).

The objectives of this work were to evaluate the suitability of NIR spectroscopy for: (i) discrimination between perennial ryegrass plants infected or non-infected by *Epichloë* endophytes; (ii) identification of the morphotype of the *Epichloë* endophyte hosted, (iii) detection of alkaloids of fungal origin (peramine, lolitrem B and ergovaline); and (iv) quantification of these alkaloids

V.3 Materials and Methods

V.3.1 Plant Material and Reference Methods

The plant material consisted of 222 plants sampled from six wild populations of *Lolium* perenne located at western Spain (described in Chapter I), and from two commercial cultivars: 'Barplus' used for turfgrass and 'Romance' a forage cultivar (Barenbrug, NL). Wild ryegrass plants were cultivated in a field-plot and the commercial ryegrass plants were grown for more than 12 months in 2 l pots with a potting mix (perlite:peat moss, 1:1, v/v). The set of ryegrass samples was composed by endophyte-free, naturally infected and artificially inoculated plants. Infected plants hosted different morphotypes of *Epichloë* endophytes, which produced distinctive alkaloid profiles (Chapter IV).

As indicated in Chapter I, Identification of Epichloë-infected plants was made through microscopic examination of the grass stem piths stained with 1% aniline blue (Bacon and White 1994), and by fungal isolation in potato dextrose agar (PDA) (Latch and Christensen 1985); and were confirmed as *Epichloë* using the PCR primers: ITS4 (5'- TCC GCT TAT TGA TAT GC -3'), ITS5 (5'- GGA AGT AAA AGT CGT AAC AAG G -3') (White et al. 1990), and tub2-exon1d-1 (5'- GAG AAA ATG CGT GAG ATT GT -3'), tub2-exon4u-2 (5'- GTT TCG TCC GAG TTC TCG AC -3') (Moon et al. 2002).

The alkaloids, peramine, lolitrem B and ergovaline, were determined from ryegrass harvested 5 cm above ground; freeze dried, ground to 0.5 mm with a hammer mill (Fritsch 15303, Germany), and stored at 6 °C in the dark until laboratory analysis and NIR spectra acquisition. Each alkaloid peramine, lolitrem B and ergovaline was analyzed separately by high performance liquid chromatography (HPLC) as indicated in Chapter III.

V.3.2 Infrared spectroscopy

V.3.2.1 Acquisition of infrared spectra

Approximately 2.0 g of each of the 222 ground ryegrass samples were placed on a circular (38 cm in diameter and 10 mm in thickness) quartz reflectance-sampling cell (Figure 26a) for their spectrum acquisition. The reflectance spectra between 400 and 2498 nm, acquired at 2 nm wavelength increments were collected using a NIRSystem 6500 scanning monochromator (FOSS Analytical, Denmark) fitted with a sample transport module (Figure 26b). The spectrum of each grass sample was stored as log (1/*R*) (*R*= intensity of reflected light at each wavelength) and used for further chemiometrical analyses. Instrument control, manipulation of spectral files and chemiometric analyses were made with WinISI 4.3 software (FOSS Analytical, Denmark).

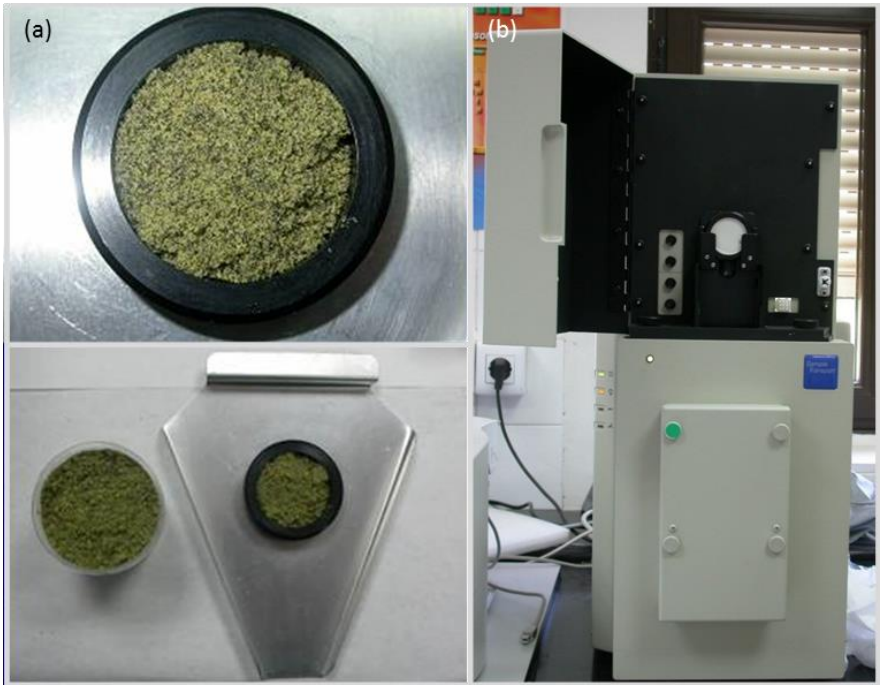

Figure 26 Instrumental used in acquisition of NIR spectra of the ryegrass samples: (a) reflectance capsule cell, (b) spectrophotometer monochromator NIRS 6500.

The collected spectra were divided into two subsets, one of them (*ca*. 75% of all the samples) used for training or calibration of the models (depending on whether classification or quantification was the objective), and the rest of samples (*ca*. 25%) were separated to corroborate the performance of the NIR equations obtained by external validation. The spectra

were assigned randomly, from different plant origins, growth conditions and harvests, to have a wide variability in terms of plant genetic, geographic, management and physiology in training/calibration and validation sets, which allows the development of robust NIRS equations. The distribution of ryegrass samples is indicated in Table 17.

Table 17 Characteristics and number of the ryegrass samples (n) used in the development of NIRS models for identification plants with *Epichloë* fungi, morphological classification of the endophyte hosted, and for detection and quantification of the alkaloids peramine, lolitrem B and ergovaline.

Parameter[¶]	Sample Condition[¶]	Statistical descriptor	Training/Calibration set	Validation set	Total/Average
Epichloë infection	E-	n	74	24	98
	E+	n	94	32	124
Epichloë morphotype	M1	n	40	14	54
	M2	n:	31	10	41
	M3	n:	21	8	29
Peramine	PER-	n:	36	12	48
	PER+	n:	56	20	76
		Range (mg kg^{-1}):	2.16-24.00	2.73	2.16-24.00
		Media (mg kg^{-1}):	6.96	20.39	7.16
		SD (mg kg^{-1}) :	5.83	7.70	5.87
Lolitrem B	LTM-	n:	37	14	51
	LTM+	n:	48	16	64
		Range (mg kg^{-1}):	0.47-6.74	0.46-2.61	0.46-6.74
		Media (mg kg^{-1}):	1.33	1.27	1.32
		SD (mg kg^{-1}) :	1.13	0.62	1.02
Ergovaline	ERG-	n:	50	16	71
	ERG+	n:	39	14	53
		Range (mg kg^{-1}):	0.02-2.11	0.19-1.55	0.02-2.11
		Media (mg kg^{-1}):	0.74	0.61	0.71
		SD (mg kg^{-1}) :	0.58	0.39	0.54

[¶]E-= No *Epichloë* infected plants, E+= *Epichloë* infected plants; M1 morphotype= slower growth mycelium with convoluted surface, M2 morphotype= faster growth rate with white cottony aerial mycelium, M3= morphotype, intermediate growth rate with tan, smooth and flat mycelium; PER-= plants without detection of the alkaloid peramine, PER+= plants with detection of the alkaloid peramine; LTM-= plants without detection of the alkaloid lolitrem B, LTM+= plants with detection of the alkaloid lolitrem B; ERG-= plants without the detection of the alkaloid ergovaline, ERG+= plants with the detection of the alkaloid ergovaline

To determine *Epichloë* infection, the whole set of 222 ryegrass samples was used, 98 samples were not infected (E-), and 124 were *Epichloë*-infected (E+). Equations for identification of the *Epichloë* morphotype hosted and for detection and quantification of alkaloids were performed obviously only with the spectra from E+ plants. From the 124 E+ ryegrass samples, 54 were infected with the M1 morphotype, 41 with the M2 morphotype, and 29 with the M3 morphotype (Table 17). Modelling the presence and concentration of peramine was made with 124 spectra, 115 spectra were used to detect lolitrem B because in six E+ plants this alkaloid was not determined by HPLC, and for ergovaline all the 124 spectra of the E+ plants were used.

V.3.2.2 Spectra treatment

The development of qualitative and quantitative analyses using near infrared spectroscopy has some steps in common. In both cases, mathematical pretreatments and principal component analysis (PCA) are applied to the sample spectra.

Mathematical treatments are performed to minimize problems related to light scattering. The physical characteristics of the sample (size, shape and compaction of particles), external factors (light and humidity), and aspects related with the instrumental measurement and the spectral signal with overlapped bands can influence negatively the spectral data. In this work, the mathematical pretreatments applied were: averaging of the raw spectra, characterization of the absorbance (standard normal variate, SNV), correction of the trend (DeTrend, DT), and application of SNV and DT together (Figure 27).

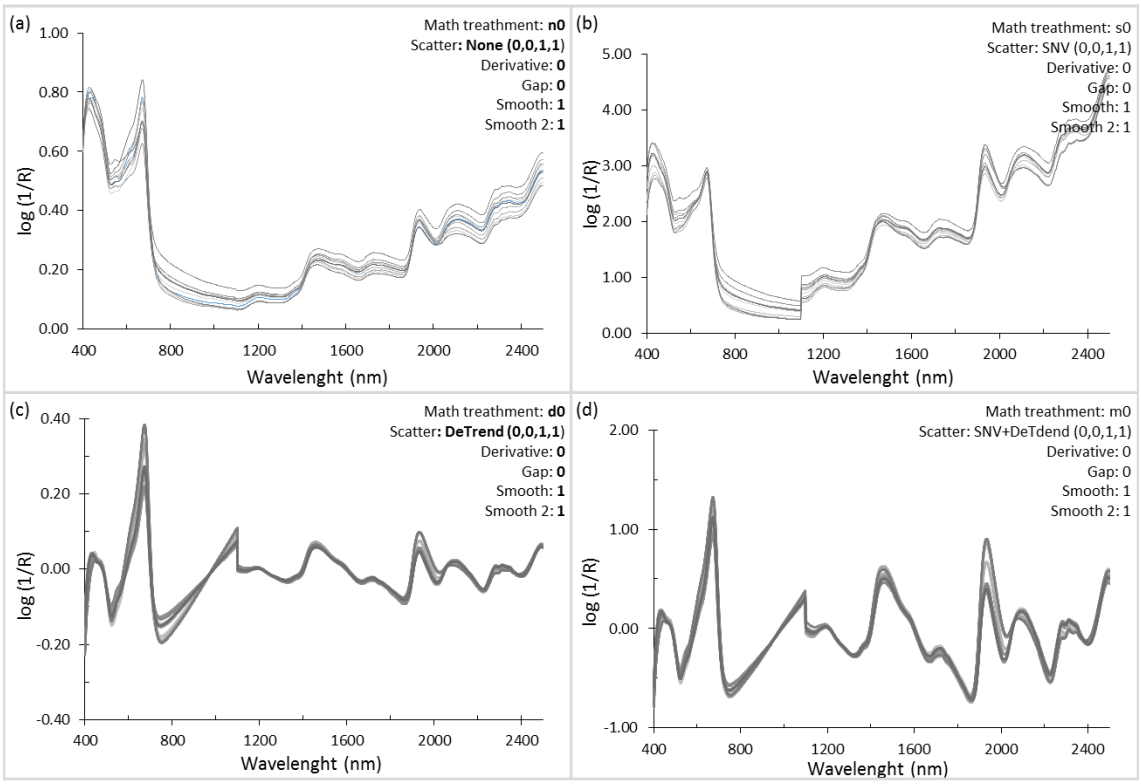

Figure 27 Example of 10 spectra of ryegrass with the four mathematical treatments used for correction of the scaring: (a) averaging; (b) standard normal variated (SNV), (c) correction of trend, (DeTrend, DT), and (d) SNV with DT together.

The function of the spectra averaging is to reduce the random noise, and therefore to increase the signal/noise ratio. Characterization of the absorbance (SNV) tries to minimize the dispersion caused by physical factors, such as the particle size, and for this each individual spectrum is centered and scaled (Barnes et al. 1989). The DeTrending (DT) step involves the application of a second-degree polynomial to standardize variation in spectral curvilinearity (Barnes et al. 1989). The mathematical pretreatments were combined with derivatives and

smoothing transformations to remove additive baseline effects (first derivative) or a linear baseline (second derivative) (Naes et al. 2002). Their notation is indicated with four digits (*a*, *b*, *c*, *d*) where: *a* is the order of derivative; *b* is the number of points where the derivative is performed; *c* is the number of points where the first smoothing is made; and *d* the number of points where the second smoothing is performed (Figure 28).

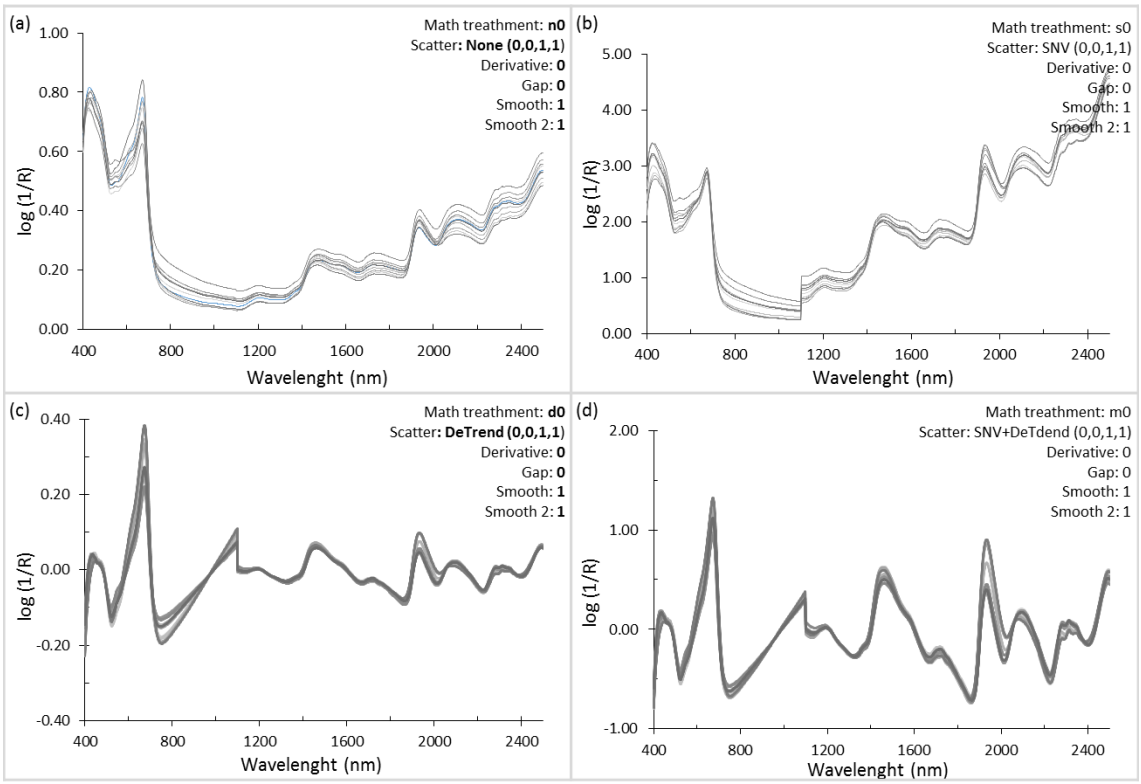

Figure 28 Averaged spectra of 10 samples of ryegrass transformed with four combinations of derivative, gaps and derivatives.

V.3.2.3 Qualitative analysis

Discriminant analysis is a qualitative methodology that identifies a sample as belonging to a particular group. In this work, the discriminant model was based on a pattern recognition method, with *a priori* knowledge about the category membership of samples (supervised) (Figure 29). A discriminant algorithm known as X Residuals was used, in this method a PCA is performed on each group, then the evaluated spectrum's score is multiplied by the PCA loadings for each group, the product is subtracted from the evaluated spectrum and the sample will be classified as belonging to the group resulting with the lowest residual.

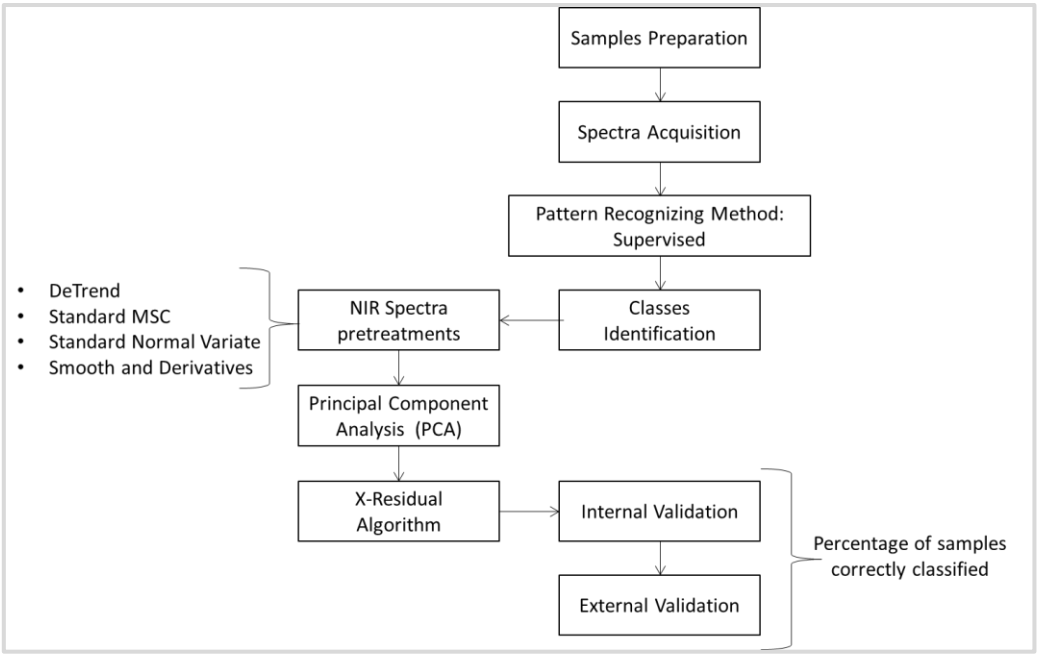

Figure 29 Steps followed for discriminant analysis in infrared near spectroscopy. Classes identification = sample condition (see Table 17).

The NIR spectral information of each sample in Table 1 was used to define the parameters to be modeled and the discriminant equations to be developed: infection with *Epichloë* endophytes (E- or E+), *Epichloë* morphotype hosted (M1, M2 or M3), and the presence of each alkaloid in E+ ryegrass samples: peramine (PER- or PER+), lolitrem B (LTM- or LTM+) and ergovaline (ERG- or ERG+). In order to find out optimal NIRS classification equations, it was needed to transform the spectra through the mathematical treatments (Figure 27) combined with smoothing, gaps and derivatives treatments (Figure 28) providing 40 discriminant equations for each parameter.

Once the discriminant models were created, their accuracy was measured with the percentage of samples from the validation set that were correctly classified and by the global percentage of false positives. Those models with the best classification performance and, the lowest percentages of false negatives, were selected for identification of the evaluated traits of new ryegrass samples. For this work, a **false-positive** was defined as a sample without the constituent studied but classified by the discriminant model as having the constituent; conversely, a **false-negative** occurs when in a sample the constituent is present but the models classify the sample as not having the constituent.

V.3.2.4 Quantitative analysis

The objective of the calibration in quantitative analyses was to develop an equation to calculate the concentration of each alkaloid in endophytes-infected ryegrass samples, in a way that, the residual or the difference between the reference chemical data and the value of concentration predicted by the NIRS equation is as low as possible. The quantitative NIR equations were developed through a modified partial least squares (MPLS) regression method (Figure 30).

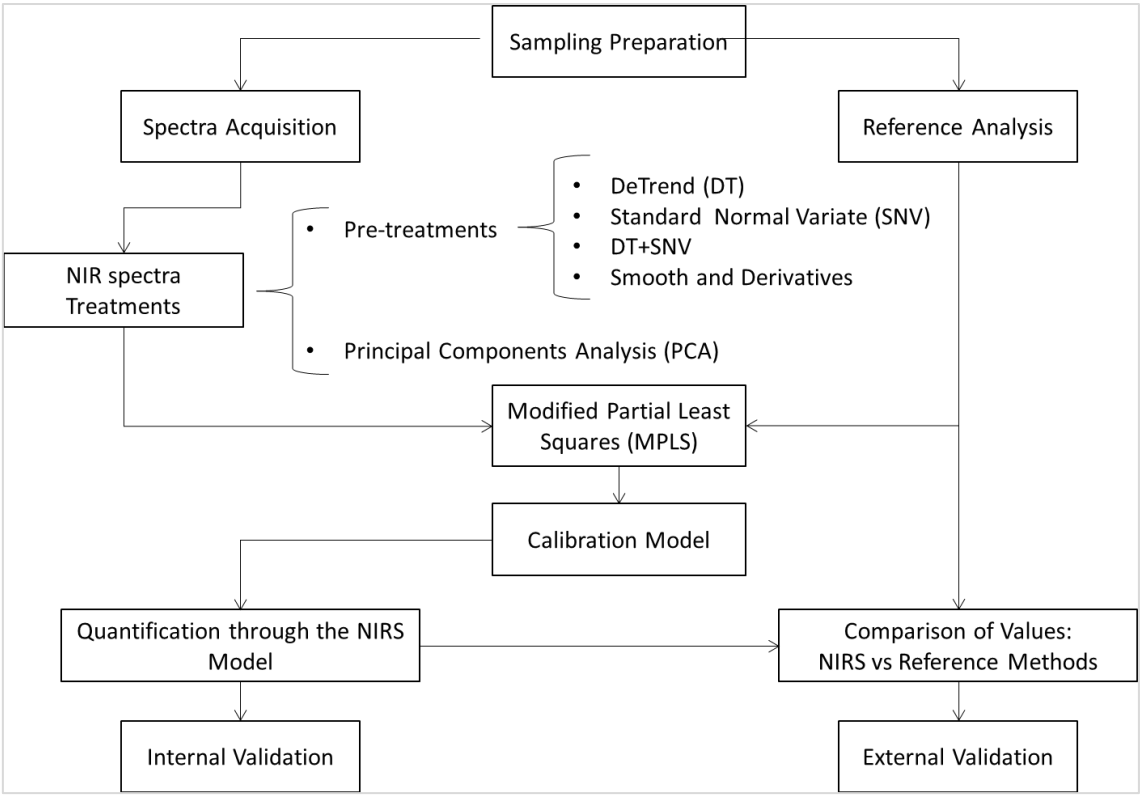

Figure 30 Schematic representation of the steps followed for quantitative analysis in near infrared spectroscopy

The modified partial least squares method (MPLS) is similar to partial least squares (PLS) regression, but often more stable and accurate. Similar to principal component regression, the PLS is based in a reduction of variables but the calibration process uses both the reference data (chemical, physical, etc.) and spectral information to form the factors useful for fitting purposes (Martens and Martens 2001). The modification in MPLS consists of a standardization of the NIRS residuals at each wavelength, after one factor is calculated the residual is divided by the standard deviations before calculating the next factor.

Previous to the MPLS, a principal components analysis (PCA) was performed on spectra of the calibration set, generating 20 different files by the combination of the mathematical treatments (spectra averaging, SNV, DT, SNV+DT, smoothing, gaps and derivatives) described above (Figure 27, Figure 28). In this process, the spectral outliers were identified (samples with $H > 3.0$) and discarded. Subsequently, on the 20 files generated by the PCA other 20 pretreatments were applied, generating 400 different equations to be evaluated for the quantification of each alkaloid.

When the MPLS is performed, a cross-validation is applied to select the optimal number of factors, and to avoid overfitting (Shenk and Westerhaus 1995). In cross-validation, the calibration set is divided into several groups; each group is then validated using a calibration developed on the other samples. In this process, samples with high residuals are detected and those samples whose statistical ***T***, defined as the residual divided by the standard error of cross-validation (SECV), exceeds the value of 2.5 were removed from the

calibration set, this procedure was repeated two times to finally obtain the models. The selection of the best NIRS equations for alkaloid quantification was based on the statistics described in Table 18. In all these equations y_i is the calculated data; $\hat{y}_i$ is the theoretical data for each one of the i samples; y is the average of the calculated data; N is the number of sampled used on calibration or internal validation; p is the number of factors used in the cross validation, and SD_{ref} is the standard deviation of the reference data (Marten et al. 1989; Mark and Workman Jr 2010).

Table 18 Statistical equations for the calculation of the accuracy of the quantitative equation generated by NIRS

Calibration statistical		Validation statistical	
Multiple correlation coefficient (RSQ), measures the fitting degree between predicted data and actual concentration	$RSQ = 1 - \left(\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{N} (\hat{y}_i - y)^2} \right)$	Multiple correlation coefficient (RSQ), compare the predicted values with the references values.	$RSQ = 1 - \left(\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{N} (\hat{y}_i - y)^2} \right)$
Standard error of calibration (SEC) is an estimate of the best accuracy obtainable using the specific wavelengths of the calibration equation	$SEC = \left(\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{N - p - 1} \right)^{\frac{1}{2}}$	Standard error of prediction (SEP), is a true measure of the performance of the equation on unknown samples and is the preferred statistic to use for comparison of regression equations:	$SEP = \left(\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{N - 1} \right)^{\frac{1}{2}}$
Residuals, is the difference between the actual value y_i and the predicted value $\hat{y}_i$	$residual f_{ij} = (y_i - \hat{y}_i)$		
BIAS is the medium value of the residuals	$BIAS = \frac{1}{N} \sum_{i=1}^{N} (y_i - \hat{y}_i)$	Standard error of prediction corrected by the *BIAS* (SEPc)	$SEPc = \left(\frac{\sum_{i=1}^{N} \left(y_{cal_i} - \hat{y}_i - BIAS \right)^2}{N - 1} \right)^{\frac{1}{2}}$
Standard error of cross validation (SECV), is a true estimate of the prediction accuracy of the equation and it is used as the statistic for determining the best number of independent variables or factor for the calibration equation	$SECV = \left(\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i - BIAS)^2}{N - 1} \right)^{\frac{1}{2}}$	Ratio performance deviation (RPD). A suitable quantification model should have a RPD> 2.0.	$RPD = \frac{SD_{ref}}{SEP}$

The robustness of the NIR models for alkaloid quantification was corroborated through external validations by means of a simple regression between NIRS-predicted values and those obtained by the reference method, to determine the accuracy of the calibration. A Student's *t*-test was conducted to corroborate that the concentrations obtained by both methods (HPLC and NIRS) provided values significantly equal or not (*P*= 0.05) and the residuals were calculated on alkaloid concentrations.

V.4 RESULTS

V.4.1 Qualitative analysis

Figure 31 shows the averaged raw spectra of the 222 ryegrass samples of all evaluated conditions: non infected plants, *Epichloë*-infected, different *Epichloë* morphotypes, with and without fungal alkaloids. The shape of the spectra is almost the same along the entire spectral range with no obvious distinction for the identification of specific groups. Differentiation among the ryegrass samples according to their traits was possible only after the use of chemometric methods to build up mathematical relationships between the absorption spectra and the characteristics of interest.

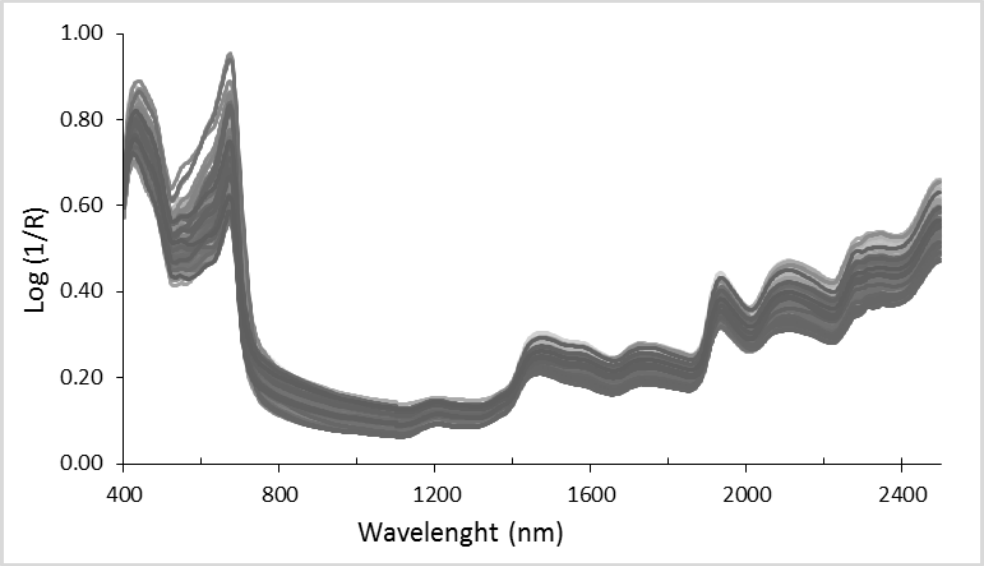

Figure 31 Spectra from visible to near infrared range (400 – 2500 nm) of the 222 samples of ryegrass.

The main peaks observed in the spectra (Figure 31) are derived from hydrogen bonds of water molecules, which strongly absorb the infrared radiation contributing to scatter the light and producing a negative effect in the vis-NIR spectra. Thus, it is observed that the visible region did not contribute with useful information, on the contrary the accuracy of the NIRS equations decreased. Taking that into consideration, the wavelength used for the obtain all the NIR equation in this chapter was in the range of 1100 – 2000 nm.

The discriminant analyses followed the method indicated in Figure 29, applying the X Residual algorithm. The Table 19 presents the results obtained after PCA was applied on the spectra with the mathematical treatments and they are indicated as follows: n= no scattering; s= standard normal variate (SNV); d= correction of trend (DT); and m was the application of SNV + DT transformations. The smoothing, gaps and derivatives are indicated with one number next to letter that indicate the scatter treatment, following the notation of four digits (Fig. 4); for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1).

Table 19 Number of principal components, variability explained, and outliers detected for each of the mathematical transformations after analysis of principal component in the spectra of ryegrass.

Mathematical treatment[¶]	Principal Components	Variability Explained (%)	Spectral outliers
n0	8	99.89	12
n1	13	99.85	7
n2	18	99.59	5
n3	14	99.84	10
n4	16	99.82	7
s0	8	99.99	13
s1	14	99.89	11
s2	20	99.60	7
s3	14	99.83	13
s4	17	99.83	10
d0	11	99.99	8
d1	13	99.86	9
d2	18	99.59	5
d3	14	99.84	10
d4	16	99.82	7
m0	12	99.99	11
m1	14	99.86	11
m2	20	99.58	7
m3	14	99.82	13
m4	17	99.82	10

[¶]Transformation of the NIR spectra: n= no scattering; s= standard normal variate (SNV); d, correction of trend (DT); m= SNV + DT transformations (Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1) (Figure 28).

Several spectra from commercial cultivars of ryegrass were outliers; it seems that those plants have important differences with respect to the wild ryegrass. The inclusion of spectra from inoculated ryegrass may reduce the predictive models accuracy, although they also provide more variability and therefore universality to the discriminant NIR models, for this reason the remnant spectra from commercial cultivars were kept in the training set.

V.4.1.1 Detection of *Epichloë* endophytes

The results obtained by the X Residual method for classification of ryegrass samples according to their infection status (E= not infected; E+= infected with *Epichloë* endophytes) are presented in Table 20, which include the percentage of samples correctly classified in training and validation sets, and the global percentage of sample misclassified by the NIRS discriminant models evaluated.

Table 20 Results of the discriminant analysis using the NIRS spectra of ryegrass samples for evaluation of the *Epichloë* infection (E- = no infected plants, E+= *Epichloë* infected plants*).*

	Samples correctly classified (%)[§]						Samples misclassified (%)[§]	
Mathematical	Training set			Validation set			Total	
treatment[¶]	E-	E+	Mean	E-	E+	Mean	E-	E+
n0	82.2	80.0	81.0	62.5	64.3	63.5	22.7	23.9
n1	95.9	92.0	93.8	91.7	100	96.3	5.1	6.0
n2	97.3	100	98.8	79.2	83.3	81.5	7.1	4.2
n3	94.6	100	97.4	91.7	85.7	88.5	6.1	3.7
n4	98.6	95.4	96.9	83.3	56.7	68.5	5.1	14.5
s0	86.5	86.7	86.6	70.8	78.6	75.0	17.3	15.3
s1	94.6	97.6	96.2	87.5	82.1	84.6	7.1	6.2
s2	95.9	100	98.1	83.3	93.3	88.9	7.1	1.7
s3	100	100	100	79.2	89.3	84.6	5.1	2.8
s4	95.9	100	98.1	79.2	89.3	84.6	8.2	2.7
d0	89.2	97.6	93.7	87.5	93.3	90.7	11.2	3.5
d1	94.6	98.8	96.9	91.7	90.0	90.7	6.1	3.5
d2	97.3	100	98.8	79.2	80.0	79.6	7.1	5.0
d3	91.9	92.9	92.4	83.3	90.0	87.0	10.2	7.9
d4	98.6	100	99.4	83.3	70.0	75.9	5.1	7.8
m0	97.3	91.8	94.3	70.8	78.6	75.0	9.2	11.5
m1	100	100	100	87.5	85.7	86.5	3.1	3.6
m2	**95.9**	**100**	**98.1**	**87.5**	**93.3**	**90.7**	**6.1**	**1.7**
m3	93.2	94.0	93.6	87.5	92.9	90.4	8.2	6.3
m4	98.6	97.6	98.1	83.3	86.7	85.2	5.1	5.3

[§]Percentages calculated without spectral outliers. [¶]Transformation of the NIR spectra: n= no scattering; s= standard normal variate (SNV); d, correction of trend (DT); m= SNV + DT transformations (Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1) (Figure 28). The highlighted row indicates the mathematical treatment most accurate.

The percentages of good classification in the training set varied from 81.0 to 100%, depending on the mathematical treatment. In the validation set the percentage of plants correctly classified was lower than in the training set, ranging from 68.5 to 96.3%. The best discriminant model to detect *Epichloë* in ryegrass was obtained with 20 PCs which explained a 98.58% of spectral variability and applying the mathematical treatment m2: correction of scatter: SNV+DT (2,4,4,1), with a 90.7% of good classification, classifying correctly 87.5% of E- plants and 93.3% of E+ plants in validation. Although this model did not have the highest

percentage of plant classified correctly (*e*.g. n1, 96.3%), it had the lowest percentage of false positives (1.7%).

In Figure 32 is represented the classification of the ryegrass plants after the application of the NIR discriminant equation for identification of plants infected by *Epichloë* endophytes. The misclassified E- plants were six, four of them were artificially inoculated commercial cultivars, and they can be pointed out in Figure 32 because of their higher difference with respect to the E-. In the other two cases, the spectra were recorded from capsules not completely full because there was not enough sample amount. In the two E+ samples classified as E- no particular characteristics were observed.

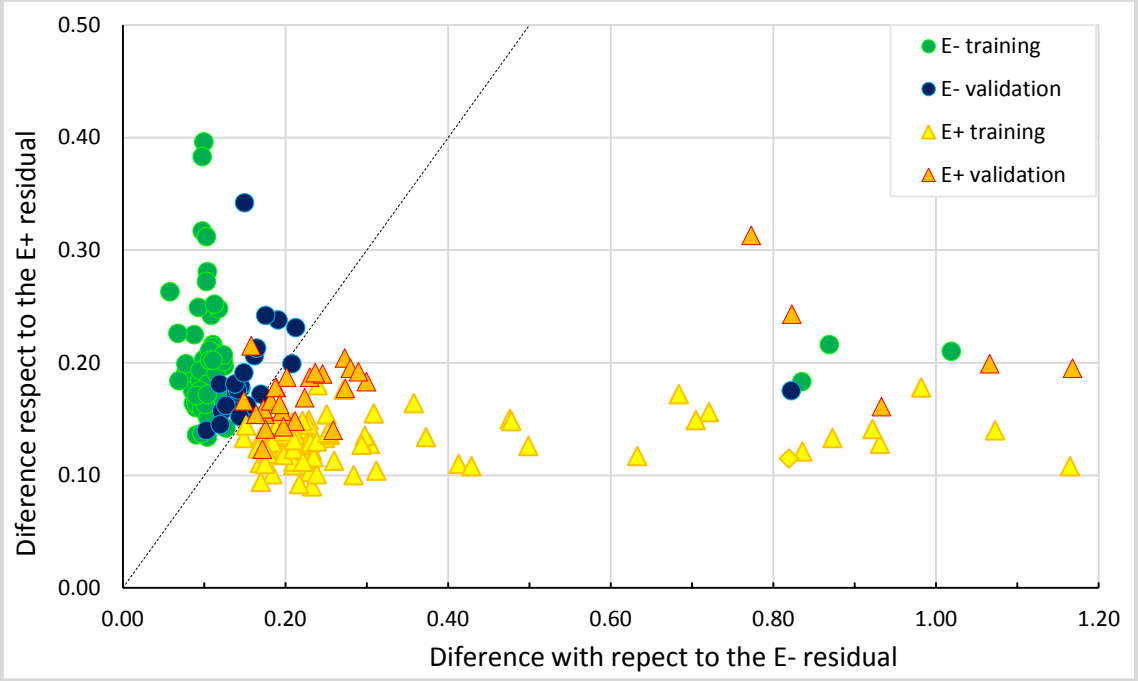

Figure 32 Classification of ryegrass samples according to the presence of *Epichloë* (E-= no infected plants; E+= *Epichloë*-infected plants) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment m2: SNV+DT (2,4,4,1).

V.4.1.2 Morphotype of the *Epichloë* endophyte

Identification of the morphotype of the *Epichloë* endophyte present in ryegrass samples through NIRS was successful for the M1 and M2 morphotypes and less accurate for the M3 morphotype because in the validation set M3 endophytes were not identified correctly (Table 21).

Table 21 Results of the discriminant analysis using the NIRS spectra of ryegrass samples for morphotype identification (M1 morphotype= slower growth mycelium with convoluted surface, M2 morphotype= faster growth rate with white cottony aerial mycelium, M3= morphotype, intermediate growth rate with tan, smooth and flat mycelium) of the *Epichloë* endophytes hosted in the ryegrass samples.

Mathematical treatment[¶]	Samples correctly classified (%)[§]								Samples misclassified (%)[§]		
	Training set				Validation set				Total		
	M1	M2	M3	Mean	M1	M2	M3	Mean	M1	M2	M3
n0	72.5	77.4	73.7	74.4	35.7	80.0	0.0	38.2	16.1	7.3	12.1
n1	100	93.5	94.7	96.7	71.4	80.0	0.0	52.9	3.2	3.2	8.9
n2	100	100	89.5	97.8	78.6	70.0	0.0	52.9	2.4	2.4	9.7
n3	100	96.8	94.7	97.8	50.0	80.0	0.0	44.1	5.6	2.4	8.9
n4	100	100	94.7	98.9	64.3	80.0	0.0	50.0	4.0	1.6	8.9
s0	75.0	80.6	78.9	77.8	64.3	50.0	10.0	44.1	12.1	8.9	10.5
s1	100	93.5	94.7	96.7	71.4	80.0	0.0	52.9	3.2	3.2	8.9
s2	100	100	94.7	98.9	78.6	80.0	0.0	55.9	2.4	1.6	8.9
s3	97.5	96.8	94.7	96.7	50.0	80.0	10.0	47.1	6.5	2.4	8.1
s4	100	100	94.7	98.9	64.3	80.0	0.0	50.0	4.0	1.6	8.9
d0	87.5	80.6	94.7	86.7	57.1	60.0	10.0	44.1	8.9	8.1	8.1
d1	100	93.5	94.7	96.7	57.1	80.0	0.0	47.1	4.8	3.2	8.9
d2	100	100	89.5	97.8	78.6	70.0	0.0	52.9	2.4	2.4	9.7
d3	100	96.8	94.7	97.8	50.0	80.0	0.0	44.1	5.6	2.4	8.9
d4	100	100	94.7	98.9	57.1	70.0	0.0	44.1	4.8	2.4	8.9
m0	95.0	93.5	89.5	93.3	78.6	80.0	10.0	58.8	4.0	3.2	8.9
m1	35.0	93.5	94.7	67.8	71.4	80.0	0.0	52.9	24.2	3.2	8.9
m2	100	100	94.7	98.9	78.6	80.0	0.0	55.9	2.4	1.6	8.9
m3	97.5	96.8	94.7	96.7	50.0	80.0	10.0	47.1	6.5	2.4	8.1
m4	**100**	**100**	**94.7**	**98.9**	**92.9**	**100**	**0.0**	**67.6**	**0.8**	**0.0**	**8.9**

[§]Percentages calculated without spectral outliers. [¶]Transformation of the NIR spectra: n= no scattering; s= standard normal variate (SNV); d, correction of trend (DT); m= SNV + DT transformations (Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1) (Figure 28). The highlighted row indicates the mathematical treatment most accurate.

In the training set, the lowest correct classification achieved was of 67.8% and the highest was 98.9% depending on the mathematical treatment (Table 21). In this set, ryegrass samples infected with M1 morphotype were the best identified, in most of the models (15 out of the 20 models) more than 95% of the plants were correctly classified, and only in one case the classification of ryegrass samples infected with M1 morphotype was lower than 50%. The samples harboring the M2 morphotype were correctly identified with all the NIRS models, within a range from 80.6% to 100%. Plants hosting endophyte with the M3 morphotype were classified correctly in a range from 73.7% to 94.7%. In the validation, the percentage of correct classification of plants with M1-endophytes was less uniform and the range of correct classification varied from 35.7% to 92.9% (Table 21). The M2 morphotype was correctly classified in an interval from 60% to 80%. In contrast with the training, in validation only five discriminant models achieved a 10% of success when where applied to identify plants hosting M3 endophytes (Table 21).

The discriminant model with the best statistical parameters for identifying the morphotype of the *Epichloë* endophyte hosted by ryegrass plants was obtained when the spectra were transformed using the mathematical treatment m4, applying a scatter: SNV+DT (2,8,6,1). The discriminant model used 17 PCs that explained 99.81% of the spectral variability between the samples. This discriminant model had the highest percentage of the correct classification in the training and the validation sets, 98.9% and 67.6% respectively. The

selected discriminant model misclassified 11 samples, one belonging to the M1 morphotype and classified as M2, and all the grass infected with the M3 morphotype from the validation set (n= 8) were classified as plants hosting *Epichloë* endophytes with the M1 morphotype (Figure 33). Among the misclassified samples there was any trend, all were naturally infected and had different origins.

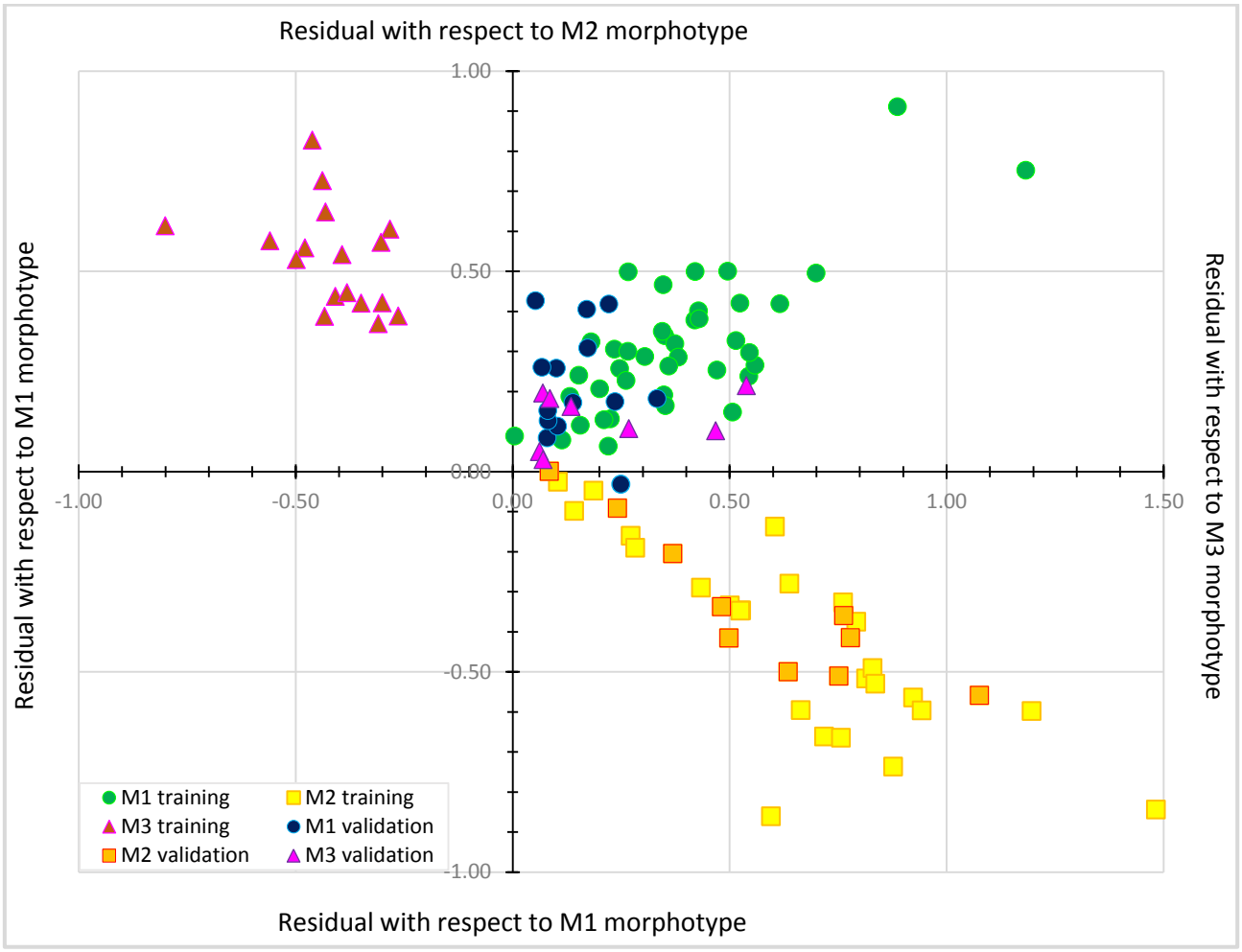

Figure 33 Classification of ryegrass samples according to the morphotype of *Epichloë* endophyte (M1, M2 or M3) hosted in ryegrass samples, applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment m4: SNV+DT (2,8,6,1).

V.4.1.3 Detection of peramine

The discriminant equations for peramine detection were developed with the NIR spectra and with HPLC as the chemical method of reference (Table 17). Since peramine is an endophyte-mediated alkaloid, only E+ ryegrass plants were included. Once the X Residual discriminant algorithms were applied on the training set, the performances of the models were evaluated in the validation set (Table 22).

Table 22 Results of the discriminant analysis for peramine (PER-= plants without detection of the alkaloid peramine, PER+= plants with detection of the alkaloid peramine) in the ryegrass samples infected with *Epichloë* endophytes.

	Samples correctly classified (%)[§]						Samples misclassified (%)[§]	
Mathematical	Training set			Validation set			Total	
treatment[¶]	PER-	PER+	Mean	PER-	PER+	Mean	PER-	PER+
n0	100	92.2	95.1	50.0	90.0	75.0	14.3	8.5
n1	96.7	100	98.8	33.3	40.0	37.5	21.4	16.0
n2	91.4	100	96.7	75.0	90.0	84.4	12.8	2.7
n3	96.6	100	98.8	30.0	80.0	63.3	20.5	5.3
n4	96.7	100	98.8	25.0	80.0	59.4	23.8	5.3
s0	**80.0**	**100**	**92.6**	**80.0**	**94.4**	**89.3**	**20.0**	**1.4**
s1	96.7	100	98.8	60.0	88.9	78.6	12.5	2.7
s2	100	100	100	58.3	85.0	75.0	11.9	4.0
s3	100	98.1	98.8	50.0	90.0	76.7	13.5	4.1
s4	100	100	100	60.0	85.0	76.7	10.3	4.0
d0	93.3	90.9	91.8	80.0	65.0	70.0	10.0	16.0
d1	96.7	98.2	97.6	60.0	80.0	73.3	12.5	6.7
d2	100	100	100	58.3	90.0	78.1	11.4	2.7
d3	96.6	100	98.8	30.0	80.0	63.3	20.5	5.3
d4	96.7	100	98.8	25.0	80.0	59.4	23.8	5.3
m0	96.6	98.1	97.6	40.0	95.0	76.7	17.9	2.7
m1	96.4	100	98.8	70.0	70.0	70.0	10.5	8.0
m2	100	100	100	58.3	85.0	75.0	11.9	4.0
m3	100	100	100	70.0	90.0	83.3	8.1	2.7
m4	100	100	100	20.0	90.0	66.7	20.5	2.7

[§]Percentages calculated without spectral outliers. [¶]Transformation of the NIR spectra: n= no scattering; s= standard normal variate (SNV); d, correction of trend (DT); m= SNV + DT transformations (Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1) (Figure 28). The highlighted row indicates the mathematical treatment most accurate.

All discriminant models for detection of peramine had good accuracy, the global percentages of good classification in the training set were always higher than 90%. In the validation, the percentages of good classification were lower than in the training. In the validation, it was observed that plants with peramine (PER+) were better recognized than plants without the alkaloid (PER-) (Table 22).

The model selected for identifying ryegrass samples with or without peramine, was the one with the highest percentage of good identification in validation (89.3%), and it was achieved when the spectra were transformed using the s0 treatment, SNV(0,0,1,1), with eight PCs explaining 99.99% of the spectral. This model misclassified 20% of PER- plants (8 out of 48); however, it had the lowest percentage of false negative (1.4%) mistakenly only one PER+ plant in the validation set. Wrongly classified PER- plants had different origins and were handled equally during the spectra acquisition. The only PER+ sample classified as PER- had a peramine concentration of 3.88 mg kg^{-1}, which is in the lower limit of concentration found in PER+ plants from the training set (Figure 34).

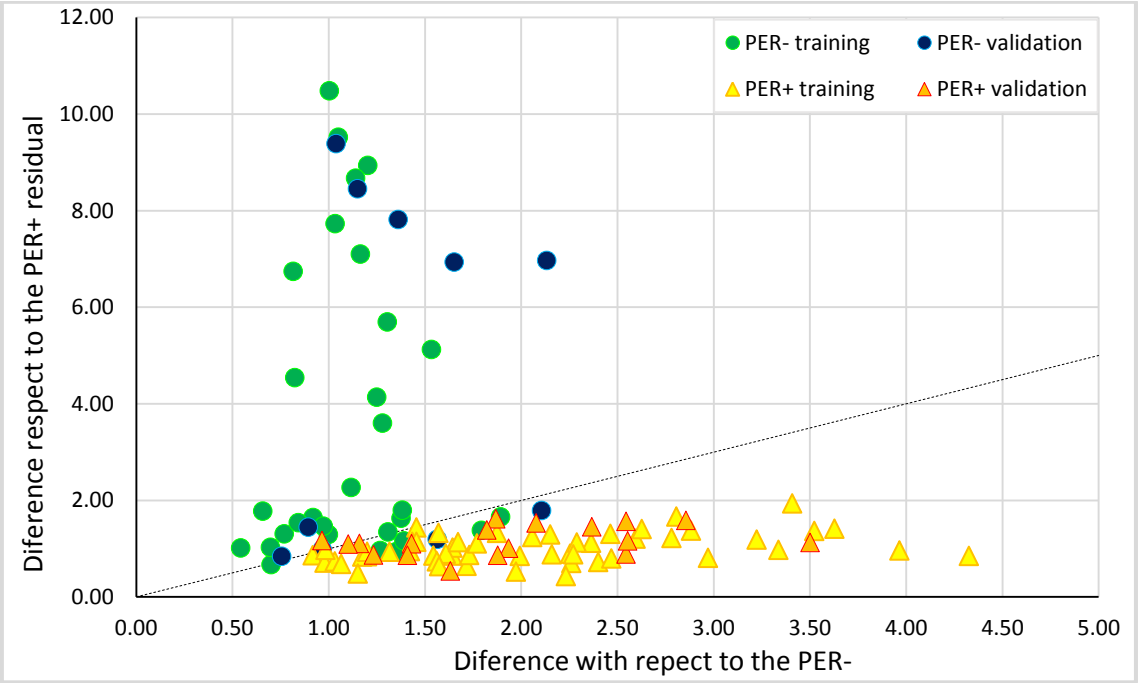

Figure 34 Classification of E+ ryegrass samples according to the presence of peramine (PER-, without peramine; PER+, with peramine) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment s0: SNV(0,0,1,1).

V.4.1.4 Detection of lolitrem B

Discriminant NIRS equations for the classification of E+ ryegrass between plants with lolitrem (LTM+) and without lolitrem B (LTM-) correctly identified between 85.9% and 100% of the samples in the training set. For all mathematical treatments the percentage of good classification was greater for LTM+ plants than for LTM- samples (Table 23). In the validation set the percentages of good classification were lower than in the training set, also with better identification for LTM+ than LTM- plants.

Table 23 Results of the discriminant analysis for lolitrem B (LTM-= plants without detection of the alkaloid lolitrem B, LTM+= plants with detection of the alkaloid lolitrem B) in the ryegrass samples infected with *Epichloë* endophytes.

	Samples correctly classified (%)[§]						Samples misclassified (%)[§]	
Mathematical	Training set			Validation set			Total	
treatment[¶]	LTM-	LTM+	MEAN	LTM-	LTM+	MEAN	LTM-	LTM+
n0	75.0	93.5	85.9	66.7	68.8	67.9	27.3	12.9
n1	93.3	100	97.4	41.7	87.5	67.9	21.4	3.1
n2	97.1	100	98.8	33.3	93.8	67.9	19.1	1.6
n3	83.3	100	93.6	58.3	56.3	57.1	23.8	10.9
n4	96.7	100	98.7	33.3	68.8	53.6	21.4	7.8
s0	73.3	100	88.7	66.7	75.0	71.4	28.6	7.0
s1	87.1	97.9	93.6	50.0	81.3	67.9	23.3	6.3
s2	97.0	100	98.8	33.3	81.3	60.7	20.0	4.7
s3	93.3	97.9	96.2	60.0	81.3	73.1	15.0	6.3
s4	96.7	100	98.7	50.0	68.8	60.7	16.7	7.8
d0	90.3	95.8	93.7	41.7	75.0	60.7	23.3	9.4
d1	100	97.9	98.7	33.3	62.5	50.0	18.6	10.9
d2	97.1	100	98.8	33.3	93.8	67.9	19.1	1.6
d3	83.3	100	93.6	58.3	56.3	57.1	23.8	10.9
d4	87.9	100	95.1	33.3	68.8	53.6	26.7	7.8
m0	90.0	100	96.2	41.7	62.5	53.6	23.8	9.4
m1	**100**	**100**	**100**	**50.0**	**87.5**	**71.4**	**14.6**	**3.1**
m2	97.0	100	98.8	33.3	81.3	60.7	20.0	4.7
m3	90.0	100	96.2	40.0	87.5	69.2	22.5	3.1
m4	100	100	100	33.3	75.0	57.1	19.0	6.3

[§]Percentages calculated without spectral outliers. [¶]Transformation of the NIR spectra: n= no scattering; s= standard normal variate (SNV); d, correction of trend (DT); m= SNV + DT transformations (Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1) (Figure 28). The highlighted row indicates the mathematical treatment most accurate.

The best discriminant model for lolitrem B detection in ryegrass samples was obtained by using the spectra with the m1 mathematical treatment, SNV+DT (1,4,4,1), standard normal variate and DeTrend together, with first derivative transformation, using the firsts 14 PCs which explained 99.86% of the spectral variability. The final model was developed with 29 LTM- and 48 LTM+ samples in a range of concentration from 0.47 to 6.74 mg kg^{-1}.

Figure 35 illustrates the classification of the samples according to whether lolitrem B was detected or not using the NIRS discriminant equation. The selected discriminant model for the detection of lolitrem B in ryegrass samples misclassified samples only in the validation set, and 6 out of 12 LTM- samples (50.0%) where recognized as LTM+, and only two out 16 of the LTM+ samples (12.5%) were not correctly classified. The six LTM- plants, which were not correctly classified were from different origins and characteristics; LTM+ plants misclassified had individual lolitrem B concentrations of 0.74 mg kg^{-1} and 1.49 mg kg^{-1}.

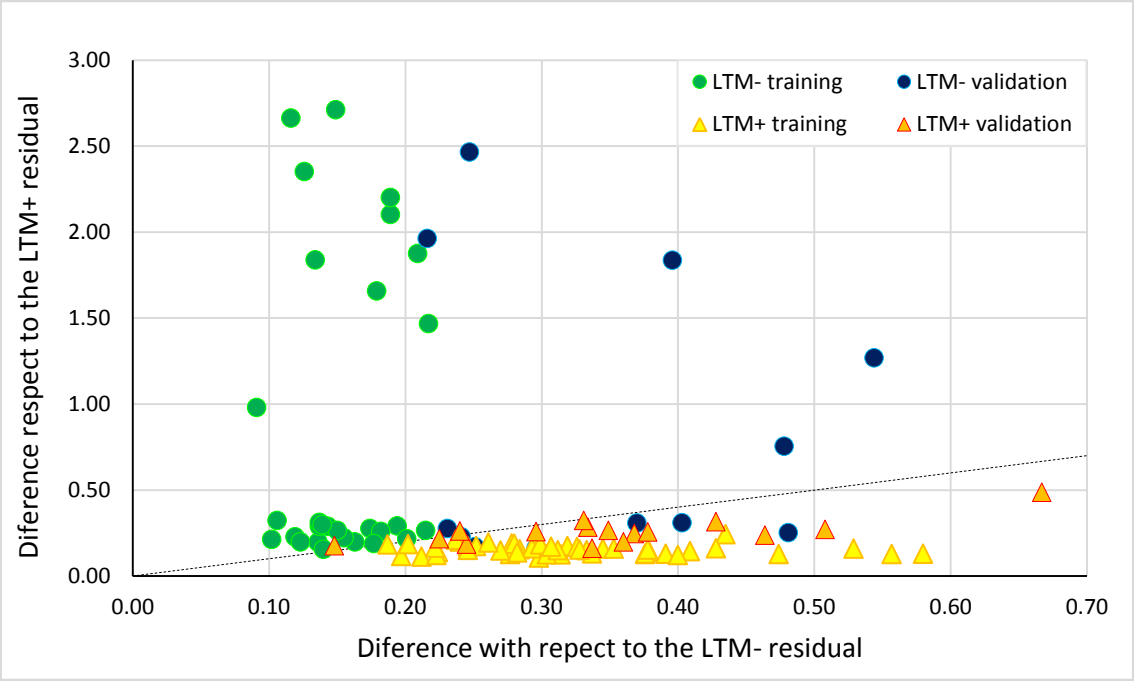

Figure 35 Classification of E+ ryegrass samples according to the presence of lolitrem B (LTM-, without lolitrem B; LTM+, with lolitrem B) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment m1: SNV+DT(1,4,4,1).

V.4.1.5 Detection of ergovaline

Correct classification of ryegrass according whether the alkaloid ergovaline was absent (ERG-) or present (ERG+) varied from 84.0% to 100% in the training set. It seems that NIR models identified ERG+ better than ERG- samples, thus in 16 out of the 20 models 100% of ERG+ plants were correctly classified; and in 7 out of 20 all of ERG- plants were correctly classified. In the validation set, the discriminant models correctly classified between 53.3% and 86.7% of the samples, in ERG- plants the range was 43.8% -87.5%; whereas, for ERG+ samples the model with the lowest correct classification had a 64.3% and the highest percentage of good classification was of 95.9% (Table 24).

Table 24 Results of the discriminant analysis for ergovaline (ERG-= plants without detection of the alkaloid ergovaline, ERG+= plants with detection of the alkaloid ergovaline) in the ryegrass samples infected with *Epichloë* endophytes.

Mathematical treatment[¶]	Samples correctly classified (%)[§]						Samples misclassified (%)[§]	
	Training set			Validation set			Total	
	Erg-	Erg+	Mean	Erg-	Erg+	Mean	Erg-	Erg+
n0	84.4	86.8	85.5	50.0	92.9	70.0	24.6	11.5
n1	95.8	100	97.7	81.3	71.4	76.7	7.8	7.5
n2	100	100	100	61.1	85.7	71.9	10.6	3.8
n3	95.6	100	97.6	37.5	71.4	53.3	19.7	7.5
n4	**100**	**100**	**100**	**62.5**	**92.9**	**76.7**	**9.4**	**1.9**
s0	81.4	86.8	84.0	87.5	85.7	86.7	16.9	13.5
s1	97.8	100	98.8	68.8	71.4	70.0	9.8	7.7
s2	100	100	100	56.3	64.3	60.0	10.9	9.4
s3	90.9	100	95.1	71.4	71.4	71.4	13.8	7.7
s4	100	100	100	81.3	85.7	83.3	4.9	3.8
d0	91.3	100	95.2	62.5	92.9	76.7	16.1	2.0
d1	95.7	100	97.6	81.3	64.3	73.3	8.1	9.4
d2	100	100	100	56.3	85.7	70.0	10.6	3.8
d3	95.6	100	97.6	37.5	71.4	53.3	19.7	7.5
d4	**100**	**100**	**100**	**62.5**	**92.9**	**76.7**	**9.4**	**1.9**
m0	95.5	94.9	95.2	75.0	85.7	80.0	10.0	7.5
m1	95.5	100	97.6	68.8	71.4	70.0	11.7	7.5
m2	100	100	100	56.3	64.3	60.0	10.9	9.4
m3	95.2	100	97.5	43.8	78.6	60.0	19.0	5.8
m4	97.8	100	98.8	62.5	71.4	66.7	11.5	7.5

[§]Percentages calculated without spectral outliers. [¶]Transformation of the NIR spectra: n= no scattering; s= standard normal variate (SNV); d, correction of trend (DT); m= SNV + DT transformations (Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 1= (1,4,4,1); 2= (2,4,4,1), 3=(2,10,10,1); and, 4=(2,8,6,1) (Figure 28). The highlighted rows indicate the mathematical treatments most accurate.

There are two models, which have the best parameters for the identification of ERG+ and ERG- plants, and both have the same statistical and were transformed with mathematical treatments n4 and d4 (Table 24). In cases like that, it is recommendable to choose the model in which the original spectra had been less modified; therefore, the model selected was n4, raw spectra without correction of the scattering and transformation using the second derivative (2,4,4,1). The selected model used 16 PCs, which explained 99.82% of the spectral variability on the samples. The training set was finally constituted for 48 ERG- samples and 39 plants with ergovaline concentrations in the 0.03 – 2.11 mg kg^{-1} range.

All samples in the training set were correctly classified; in the validation set seven plants were incorrectly classified, six out of 16 ERG- samples were identified as having ergovaline and from ERG+ samples only one out of 13 samples was classified as ERG- (Figure 36). Misclassified ERG- samples were from different origins, five of them hosted *Epichloë* endophytes with M1 morphotype and the other two the M2 morphotype. The concentration of ergovaline in the ERG+ sample classified as ERG- was 0.48 mg kg^{-1}, in the lowest limit of concentration in the training.

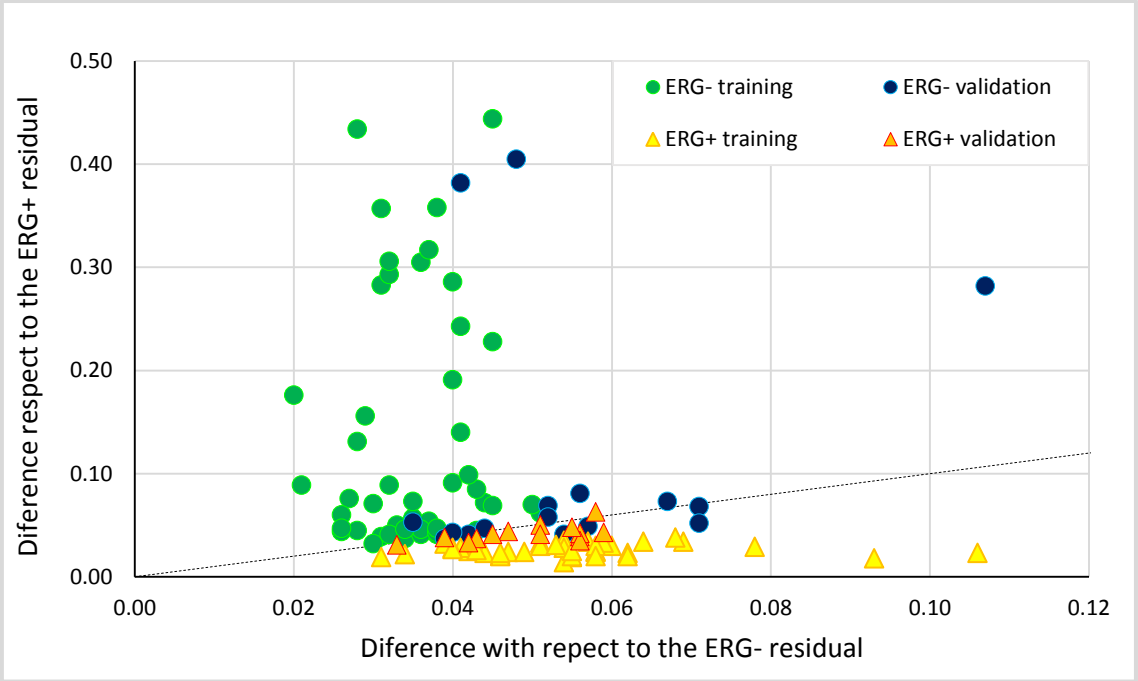

Figure 36 Classification of E+ ryegrass samples according to the presence of ergovaline (ERG-, without ergovaline; ERG+, with ergovaline) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment n4: raw spectra (2,8,6,1).

V.4.2 Quantitative analysis of alkaloids

The development of the quantitative models was done through the modified partial least squares method (MPLS) by using the spectra and the reference concentrations of the ryegrass from the calibration set for each alkaloid peramine, lolitrem B and ergovaline separately (Figure 30). In this procedure, neither uninfected ryegrass plants (E-) or samples in which the alkaloid concentration was zero in the HPLC-analysis were included.

For each alkaloid a calibration model was developed as follows. After PCA, the number of PCs was selected and the spectral outliers (H> 3) were eliminated; then the mathematical treatments were applied, obtaining 400 equations to be evaluated. The quantitative equation with the best statistical parameters (RSQ, SEC, SECV, and RPD) was selected and a cross validation was performed. Chemical outliers (T> 2.5) were eliminated for optimization of the equations, then the errors of prediction (SEP and SEPc) and prediction ability (RPD) were calculated.

In Table 25 all the statistical parameters of the best NIRS equations for quantification of peramine, lolitrem B and ergovaline in ryegrass samples are reported.

Table 25 Summary of the statistical parameters of equations developed for quantification of alkaloids peramine, lolitrem B and ergovaline applying the modified partial least square algorithm in NIR spectra of ryegrass samples

	Alkaloid		
	Peramine	Lolitrem B	Ergovaline
Principal Component Analysis (PCA)			
Pretreatment[¶]	s2	d0	s0
Number of principal components (PCs)	11	7	6
Explained variability (%)	99.05	99.93	99.95
Spectral outliers (*H*> 3.0)	1	0	1
Modified Partial Least Squares (MPLS)			
Pretreatment[¶]	s2	d0	d0
Number of samples	55	46	36
Standard deviation (SD) (mg kg^{-1})	5.63	0.47	0.46
Range (mg kg^{-1})	0.83 - 22.64	0.04 - 1.96	0.09 - 1.93
Chemical outliers (*T*> 2.5)	0	2	2
Multiple correlation coefficient (RSQ)	0.93	0.41	0.70
Standard error of calibration (SEC) (mg kg^{-1})	1.56	0.46	0.29
Standard error of cross validation (SECV) (mg kg^{-1})	3.65	0.51	0.38
Number of PLS factors	11	7	6
Groups in cross-validation	6	6	6
Internal validation			
Standard error of prediction (SEP) (mg kg^{-1})	1.46	0.44	0.26
Medium value of the residuals (BIAS) (mg kg^{-1})	0	0.09	0
SEP corrected by the Bias (SEPc) (mg kg^{-1})	1.47	0.44	0.26
Multiple correlation coefficient (RSQ)	0.94	0.41	0.76
Ratio performance deviation (RPD)	3.99	1.25	2.04
External validation			
Root mean standard error (RMSE= SEP) (mg kg^{-1})	0.25	0.39	0.25
Average residual (mg kg^{-1})	1.95	0.30	0.22
Student's *t*-test (*P*)	0.52	0.33	0.56

[¶]Transformation of the NIR spectra: s= standard normal variate (SNV); d, correction of trend (DT)(Figure 27). The smoothing , gaps and derivatives are indicated with the number next to letter as follow; for this: 0= (0,0,1,1); 3=(2,10,10,1) (Figure 28).

V.4.2.1 Quantification of peramine

The most accurate model for quantification of peramine was developed when the sample spectra were transformed using the mathematical pretreatment s2: SNV, standard normal variate (2,4,4,1) (Table 25). Eleven factors were required for PLS. As a result of the statistical treatments described, the calibration model was obtained with 55 samples; only one spectral outlier was eliminated after application of the *H* criterion (Mahalanobis distance) and none chemical outliers were detected according to the *T* criterion (high residual, *T* greater than 2.5). The obtained calibration equation had a correlation coefficient (RSQ) of 0.93; a standard

error of calibration (SEC) of 1.56 mg kg^{-1} and a standard error of cross-validation (SECV) of 3.65 mg kg^{-1} (Table 25).

The uncertainty in the prediction due to the model is indicated by the standard error of prediction (SEP), the standard error of prediction corrected (SEPc) by the residual (BIAS) obtained by means of an internal validation. The correlation between the reference values and the ones predicted by NIRS samples from calibration set is presented in the Figure 37. The standard error of prediction (SEP) was 1.46 mg kg^{-1} for concentration of peramine in ryegrass samples. The predictive capability of the model (RPD) was 3.99; which indicates that the obtained model can be applied to estimate accurately peramine concentration in ryegrass samples.

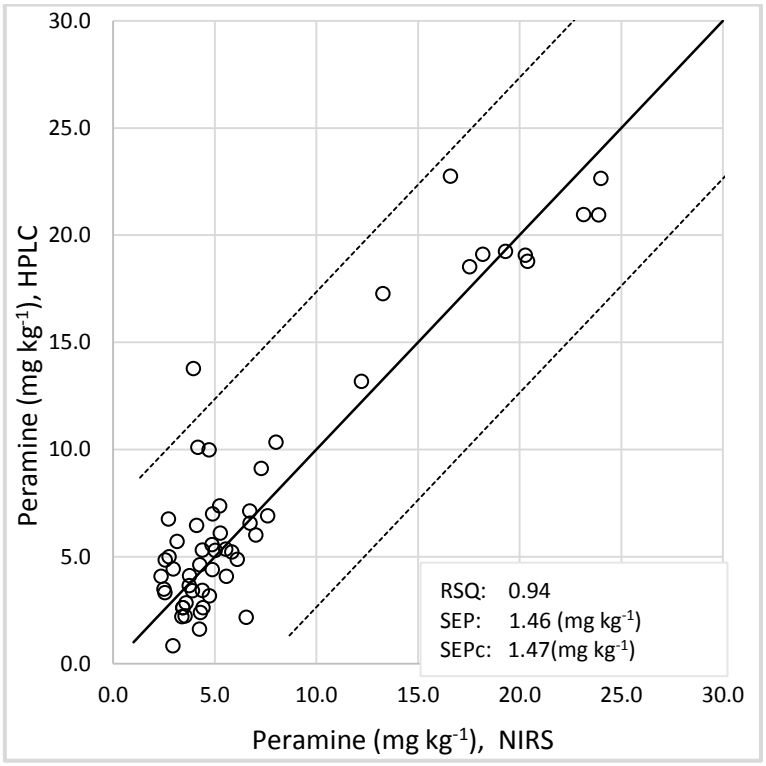

Figure 37 Internal validation comparing values of reference against the predicted by NIR spectroscopy using the MPLS methods for peramine concentration in the validation set of ryegrass samples.

The external validation of the NIR equation for quantification of peramine in ryegrass samples was accurate (Table 25). The Student *t*-test indicates that there were not significant differences between the concentration measured in HPLC and the NIR predictions (*P*= 0.52). The mean standard error for quantification of peramine concentration of the NIR equation with respect to the HPLC procedure was 0.25 mg kg^{-1} and the residual errors were 1.95 and 0.25 mg kg^{-1} in unknown samples, namely, plants not used in the development of the quantitative models.

V.4.2.2 Quantification of lolitrem B

The best results of calibration for lolitrem B quantification by NIRS were obtained using the spectral pretreatment d0 (correction of trend, DT), with the numerals (0,0,1,1) which involves the application of a second-degree polynomial to standardize variations in spectral curvilinearity without transformation by derivatives (Table 25). Seven PCs components were required to explain 99.93% of the spectral variability among samples in the calibration set. None samples were eliminated by the *H* criterion.

Similarly to PCA for the MPLS, the best performance on lolitrem B quantification was obtained with the pretreatment d0 and using seven PLS factors. The final calibration set was constituted by 46 samples because two samples were eliminated using the *T* criterion. The NIRs model had a *RSQ* of 0.41 with error of calibration (*SEC*) and cross-validation (*SECV*) of 0.46 and 0.51 mg kg^{-1} respectively (Table 25).

Validation processed comparing the concentration of lolitrem B obtained with HPLC with the values using NIRS equation (Figure 38), allowed the calculation of the standard error de prediction (SEP) which was 0.44 mg kg^{-1} and the predictive capability of the NIRS equation (RPD= 1.25). These statistical parameters for quantification of lolitrem B in ryegrass samples (Table 25) shown that the results of prediction should be taken cautiously for prediction of concentration, given the low correlation between the actual data and the predicted (*RSQ*= 0.41), and the low RPD.

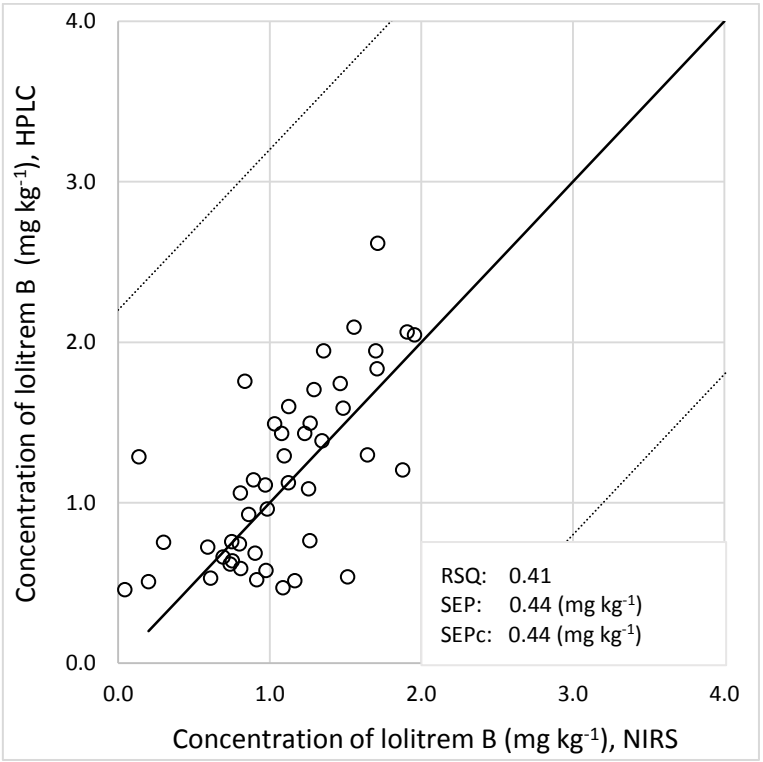

Figure 38 Internal validation comparing values of reference against the predicted by NIR spectroscopy using the MPLS methods for lolitrem B concentration in the validation set of ryegrass samples.

In external validation (Figure 38), among the NIR equation for quantification of lolitrem B and chromatographic method of reference (HPLC), there was not significant differences (*P*= 0.39). However, compared with the concentrations of the samples the error or prediction (RMSE) was high (0.39 mg kg^{-1}) also the residuals (0.30 mg kg^{-1}).

V.4.2.3 Quantification of ergovaline

The model with the best performance for ergovaline quantification by NIRS was obtained when spectra were transformed by the mathematical treatment s0: standard normal variate (SNV), without no derivatives (0,0,1,1) in the PCA, with six factors that explained 99.95% of the spectral variability. In this process, one spectral outlier was detected and eliminated (Table 25).

In MPLS regression, the mathematical treatment used was d0: correction of trend without application of derivatives (0,0,1,1) and six PLS factors. The model for quantification of ergovaline had a RSQ of 0.76, a standard error of calibration of 0.29 mg kg^{-1} and the standard error of cross-validation was of 0.38 mg kg^{-1} (Table 25).

When actual ergovaline concentration was compared with the predicted NIR values, the standard error of prediction was 0.26 mg kg^{-1} and this model had a RPD of 2.04 (Figure 39). According to this RPD, the NIRS models for quantification of ergovaline can be used to quantify this alkaloid in samples of ryegrass, but the prediction should be taken with caution because the SEP (0.26 mg kg^{-1}) was half of the safety limit for livestock consumption (0.40 mg kg^{-1}).

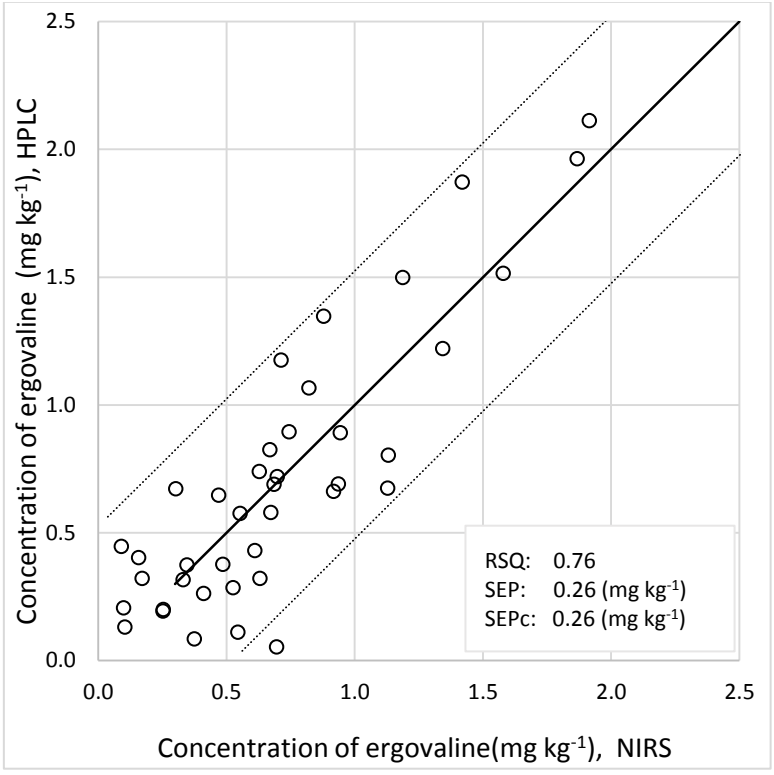

Figure 39 Internal validation comparing values of reference against the predicted by NIR spectroscopy using the MPLS methods for ergovaline concentration in the validation set of ryegrass samples

Results of the external validation, in which the performance of the NIR equation for quantification of ergovaline was evaluated, indicated that the concentrations calculated were equals than the data obtained using HPLC (*P*= 0.56). The RMSE in the calculation of the concentration using NIR was 0.25 mg kg^{-1} and the residuals 0.22 mg kg^{-1} (Table 25)

V.5 DISCUSSION

The purpose of this chapter was to study the suitability of near infrared spectroscopy to identify the presence of *Epichloë* endophytes and their associated alkaloids in a heterogeneous set of perennial ryegrass plants. The results enable to conclude that the spectral information obtained from ryegrass plant samples can be used to associate the presence of *Epichloë* fungi, to determine the morphotype or species of these endophytes, and also to detect the alkaloids peramine, lolitrem B and ergovaline. The quantification of these alkaloids was accurate and was highly correlated with data obtained from HPLC having low errors of prediction. Qualitative and quantitative NIRS equations fulfilling their purpose independently that the set of samples was composed of wild ryegrass plants with diverse origins and conditions indicating a high robustness of this method.

Several samples from the commercial cultivars of ryegrass where spectral outliers, which means that their spectra were significantly different from the media spectra obtained from the rest of ryegrass samples, conformed predominantly by grass of wild origin. Commercial varieties of ryegrass have been part of continuous breeding programs, improving herbage production, persistence, drought and heat tolerance, resistance to fungal and viral diseases and pest (Cunningham et al. 1994; Sampoux et al. 2011; Lee et al. 2012), introducing physiological and metabolic changes with respect to unaltered plants from natural origin. All these changes may be reflected in the chemical composition of the plants and therefore in their respective spectra. Besides, the number of samples from commercial ryegrass was lower than the plants from wild origin which possibly did not give to them enough representation in the whole set to be recognized as part of the group. Omission of all spectra from commercial cultivars arose the accuracy of the discriminant models but at the same time the robustness of the models decreased and consequently also their applicability, therefore they remained in the training set.

Identification of ryegrass plants infected with *E. festucae* var. *lolii* through spectroscopy was performed by Brandl (2013) but using only one commercial variety of perennial ryegrass, and reported correct identification of all samples using Fourier transformed spectroscopy on 20 samples (10 E+ and 10 E-). In this Chapter, using near infrared spectroscopy, only eight plants were misclassified from a set of 222 samples, which can be considered an excellent result in view of the high heterogeneity of the ryegrass plants and *Epichloë* endophytes analyzed. *Epichloë* hyphae reach less than 0.2% of the infected plant biomass (Tan et al. 2001; Spiering et al. 2005a), however it is evident that the endophyte

produces remarkable chemical changes inside the host plants that are easily detectable using NIR spectroscopy. Other methods highly specific for detection of fungi as the immunological tests have been used to identify *Epichloë* endophytes in many grass species, resulting the diagnostic positive for some samples in which no infection was confirmed by visual inspection (Jensen et al. 2011; Brandl 2013).

In discriminant analysis, for distinction among the morphotypes of the *Epichloë* endophytes hosted in ryegrass samples, ryegrass infected with M3 morphotype was not properly distinguished in the validation process; instead they were classified as belonging to M1 morphotype but completely apart from M2 morphotype. In this regard, it is important to remark that, as reported in Chapter I, according to the genetic analysis using the partial sequences of the ITS1-5.8SrDNA-ITS2 region and the β-tubulin (*tub*2) gene of the endophytes from the set of the ryegrass plants analyzed, only two species of *Epichloë* fungi were detected and they coincided with the results obtained thought the discriminant analysis using NIR for morphotypes identification: M2 morphotype = *E. typhina*; whereas, M1 and M3 morphotypes = *E. festucae* var. *lolii*. Fungi with the M1 and M3 morphotypes as belonging to the same species may have more features on common beyond the differences on morphotype and alkaloid profile that made them indistinguishable inside the ryegrass samples. Therefore, the misclassification of M3 is not a method fail, on the contrary this strengthen the findings reported in Chapter I, about the existence of two Epichloë species among the endophytes hosted by the ryegrass samples. There has been attempt of the use of NIRS in classification of fungal species; for example, Petisco et al. (2008) used grounded mycelia to the developed of a direct method for identification of three species of *Epichloë*, which resulted in correct classification rates higher than 90% for the three species. However, one advantages of the discriminant method described in this chapter is that it does not require the isolation of the fungi from the grass and is not needed to wait until have enough fungal mycelia for analysis, instead the classification is directly performed in grounded samples of ryegrass and have an excellent classification of the two *Epichloë* species hosted.

There are no bibliographical reports on the use of near infrared spectroscopy for identification of grass samples containing alkaloids. This qualitative technique can be a helpful tool in studies where high numbers of samples need to be screened. Our results show that the accuracy of NIRS discriminant models for the identification of ryegrass samples containing peramine, lolitrem B or ergovaline was very acceptable. In general, spectral differences were higher in positive (with alkaloid) samples compared with negatives (without) ones, resulting in discriminant NIRS models that identified better those sample plants which have alkaloids. Due to the nature of this study the opposite case, having higher number of false negatives, may be a problem, as consequence of the toxic nature of lolitrem B and ergovaline for mammals. However, NIRS discriminant equation for lolitrem B and ergovaline had 3.1% and 1.9% of false negatives respectively, which represents two plants with lolitrem B no identified in a set of 64; and for ergovaline one sample from a total 76 plants.

The problem with the false positives, which in case of lolitrem B was 50% of the samples in set of validation, could be due to the detection limits in HPLC, since all samples used for the development of this discriminate equation were E+ and production of this alkaloid is genetically controlled in the endophyte. It is known that *E. festucae* var. *lolii* (M1 and M3 morphotypes) contains the gene needed for synthesis of lolitrem B (Young et al. 2009; Schardl et al. 2012) and this endophyte has the ability to produce it, in a wide range of concentrations (Chapter III) probably even below the limit of quantification considered in HPLC. Thus, the NIR procedure relates all the spectral information with the characteristics of the sample, and probably the presence of a specific alkaloid may influence in other properties of the plants that were detected through the discriminant analysis and cannot be elucidated using an extremely specific method for determination of compounds as the chromatographic techniques.

Studies on quantitative analysis of alkaloids by NIRS in forage samples are very scarce, there are only three published reports of its use in quantification of ergovaline in tall fescue samples (Roberts et al. 1997; Kallenbach et al. 2003; Roberts et al. 2005) but there were not published references of its use on quantitative analysis of peramine or lolitrem B.

The NIRS equation for quantification of ergovaline developed here was less accurate (RSQ: 0.76) than the one reported by Roberts et al. (1997) (RSQ= 0.93); however they used samples from only one cultivar of tall fescue from two locations, whereas the samples used herein came from wild plants obtained at six locations, plus two commercial cultivars, were grown in different conditions, and they had a wider concentration range of ergovaline. Roberts et al. (1997) indicated that the lower precision may be in part because the concentration of ergovaline in the grass samples is at least 10 000 times minor than other forage quality constituents routinely quantified by NIRS.

In this way, the higher RSQ in the calibration process for quantification of peramine (0.94) could be because of its higher concentration as compared to the other alkaloids. Nevertheless, for quantification of lolitrem B (RSQ= 0.44) this argument does not seem to be valid, because its concentration ranged in the same threshold than ergovaline and had almost equal standard deviation of concentration in the calibration set of samples. Another possible explanation suggested by Roberts et al. (2005) is that NIR spectrophotometers could be able to detect precursors of the constituents of interest decreasing the precision of calibration. Although it is highly probable due to the complexity of the lolitrem B molecules and of its biochemical pathway of synthesis (Gallagher et al. 1982; Saikia et al. 2012; Philippe 2016), this suggestion should be validated because herein was studied the near infrared region, and not mid-infrared region, where it is easier to attribute the differences of absorbance to specific chemical bounds and identified their possible origins (Levasseur et al. 2010). Besides, the accuracy of NIRS estimations are dependent upon the data used to calibrate the instrument, the accuracy of predictions is dependent upon instrument calibration supported by good quality assurance protocols, and NIRS can only be as good as the calibration data derived from wet chemistry (Corson et al. 1999).

Independently that NIRS equations are not always as accurate or as sensitive as microbiological, genetic, chemical or chromatographic analytical techniques, this study shows that in works such as surveys of fungal endophyte incidence or fast screening on alkaloids detection of large numbers number of samples, NIRS does offer an alternative technique which is rapid, non-destructive, without the need of time-consuming sample preparation and inexpensive for providing chemical and nutritional analyses of feed stuffs.

V.6 CONCLUSION

Diagnosis of the presence of *Epichloë* is important for biological and economic reasons in the livestock industry because the toxic alkaloids produced in forage plants, and it is often necessary to use molecular or chemical techniques for species classification and alkaloids detection when a rigorous identification is required. Our results showed that the use of near infrared spectroscopy and chemiometrics for qualitative analysis allows identifying the presence *Epichloë* endophyte, *Epichloë* morphotype and species, and fungal alkaloids in a heterogeneous set of ryegrass samples. This is the first report of the use of a qualitative approach using near-infrared spectroscopy for analysis of fungal alkaloids (ergovaline, peramine and lolitrem B) in a heterogeneous ryegrass sample set. The developed discriminant NIRs models are able to classify *Epichloë* species hosted inside ryegrass sample plants. Although our objective was to classify endophyte-morphotypes, surprisingly our results allowed a classification according to *Epichloë* species; discriminating *Epichloë festucae* var. *lolii* from *Epichloë typhina* in infected plant. This is the first report about that.

The NIRS quantitative equations generated enabled to estimate accurately the concentration of peramine and ergovaline in perennial ryegrass samples having similar precision than the HPLC methods, but it accuracy was lower predicting the lolitrem B concentrations.

NIRS equations can be used as an accurate alternative for *Epichloë* detection and identification of the species hosted and for detection and quantification of alkaloids in a broad ryegrass sample population.

Although in this work chapter *Lolium perenne* was evaluated as the experimental material, this technique might be useful in similar situations for other forage species infected with *Epichloë* endophytes providing important parameter to scientists involved in routine forage quality research and nutritional analyses of feedstuffs.

GENERAL CONCLUSSIONS

In this study, the performance of a heterogeneous set of *Lolium perenne* plants of wild origin infected with *Epichloë* fungal endophytes was examined. Particular emphasis of this thesis was on alkaloid production, but other complementary objectives were raised with the aim to better understand the symbiotic grass-endophyte association. In the first experimental phase of this research, the incidence of *Epichloë* endophytes in eight wild populations of *L. perenne* was surveyed, isolating endophytes that were morphologically and genetically classified. In the second chapter the relationship between the type of *Epichloë* endophyte hosted and the production of the fungal alkaloids peramine, lolitrem B and ergovaline in perennial ryegrass plants was evaluated. Experimental work of the third chapter aimed to investigate how the presence of *Epichloë* and their morphotype affected the mineral concentration and the fibers content of perennial ryegrass plants. In the fourth chapter, two methods of inoculation of *Epichloë* endophytes in commercial cultivars of perennial ryegrass were tested and compared. The last experimental section, fifth chapter, was an approach to the use of near infrared spectroscopy as a tool for identification of plants infected with *Epichloë* endophytes and the morphological classification of these symbiotic fungi, as well as for detection and quantification of peramine, lolitrem B and ergovaline directly in samples of perennial ryegrass.

Epichloë endophytes were found at the eight locations of *Lolium perenne* surveyed with an average incidence of 43.0%. According to the morphological and genetic classification, alkaloid production and NIRS spectroscopy, a high diversity of *Epichloë* endophytes was found within the locations of *L. perenne* studied. Among asymptomatic ryegrass plants three morphotypes of *Epichloë* (M1, M2 and M3) were characterized, all *Epichloë* endophytes isolated from plants with stromata had a M2 morphotype and they were designed as M2S. These *Epichloë* endophytes were classified into at least two species according to the genotypic analysis using partial sequences of the internal transcriber spacer ITS y the β-tubulin (*tub2*) gene: M1 and M3 belonging to *Epichloë festuae* var. *lolii*¸ and M2 morphotypes were *E. typhina*.

In agreement to the morphological classification several genotypic groups were detected, there were two main genotypes of *E. festucae* var. *loli*:, the most common of them (G1a) included all colonies with M3 morphotype and part of the endophytes with M1 morphotype; the other genotypic group (G1b) was composed by endophytes with the M1 morphotype, and was isolated uniquely from plants of two locations. Endophytes classified as *E. typhina* were also composed by two genotypes, the most common (G2a) encompass strains hosted in asymptomatic plants, and other were stromata-producing; the second genotype (G2b) consisted of only stromata-producing endophytes.

The occurrence of two distinctive morphotypes of *Epichloë* endophytes, in different arrangements (M1/M2, M1/M2S, M3/M2 or M3/M2S) were found commonly in several single plants of perennial ryegrass from many of the sampling locations. Although conditions that may afford the formation of interspecific *Epichloë* hybrids, such as several types of *Epichloë* endophytes living in sympatry and the presence of double infected plants were observed in the ryegrass communities, *Epichloë* hybrids were not detected in this survey. However, a wide range of possible associations were observed in the plants analyzed, that afford at least six perennial ryegrass ecotypes in agreement with the morphological and genetic features: M1(G1a), M1(G1b), M2(G2a), M2S(G2a), M2S(G2b), M3(G1a), plus double infections. This wide variety of *Epichloë*/ryegrass associations may include some ecotypes that could withstand particular traits (*e. g.* alkaloid profiles) useful for improving forage grasses.

A direct relationship between the alkaloid content of perennial ryegrass plants and the morphotype of the *Epichloë* endophyte hosted was detected. The highest concentrations of peramine, lolitrem B and ergovaline were found in ryegrass infected respectively with the M2S-, M3- and M1-morphotypes. In doubly infected plants a synergistic effect was observed on alkaloid production, those plants had the same alkaloid pattern as single infected grasses according to *Epichloë* morphotypes hosted, but in higher concentration.

In most of the *Epichloë*-infected plants the concentration of lolitrem B was lower than that reported as the safe limit for livestock consumption (1.80 mg kg^{-1}). However, ergovaline in harmful concentration to livestock performance (0.40 mg kg^{-1}) was detected in more than 90% of the analyzed plants. In spite of these values, there have not been reports of alkaloid intoxication due to the high floristic diversity in natural pastures.

The *Epichloë* endophytes produced significant changes in mineral concentration and fiber content in their perennial ryegrass hosts. *Epichloë* infected plants had lower P, Ca, S, B, neutral detergent fiber and lignin contents, and higher Mn and digestibility than non-infected plants. These results suggest that *Epichloë* might alter belowground processes that influence nutrient acquisition in the host plant, although the mechanism is not clear and several processes might be involved.

The effect of *Epichloë* morphotypes on mineral concentration and fibers content was observed only in plants with symptoms of choke disease, infected by the M2S morphotype, choked plants had higher concentration of minerals and lower lignin concentration than asymptomatic *Epichloë*-infected plants. In asymptomatic ryegrass plants there was no effect neither of the *Epichloë* infection or the morphotype of endophyte hosted on the number of tillers and biomass produced.

A new technique for inoculating endophytes in perennial ryegrass, consisted of placing seeds in a culture medium containing *Epichloë* endophytes was developed. With this method a higher number of plants were successfully inoculated than with the predominant procedure, the slitting method. Using this new inoculation procedure, remarkable good results were observed for inoculation of *Epichloë typhina* (M2 and M2 morphotypes). However, the percentages of infection with *E. festucae* var. *lolii* (M1 and M3 morphotypes) in commercial

cultivars of ryegrass were lower than those reported using the slitting method. Therefore, more research should be done concerning to inoculation of *E. festucae* var. *lolii* in ryegrass cultivars because it is a medullar step in the development of new endophytic associations with grasses.

Using the NIR spectra of the heterogeneous set of ryegrass samples and chemometrics procedures it was possible to identify with high accuracy the plants infected with *Epichloë* endophytes, the species of the *Epichloë* endophyte hosted, and the presence of the fungal alkaloids peramine, lolitrem B and ergovaline, and the quantification of peramine and ergovaline. Quantifying lolitrem B by NIRS was less accurate than for the other two alkaloids. In general, the results showed that NIR spectroscopy allows to analyze a large number of samples in exploratory studies on the effects of endophytes in grasses having highly reliable results with minimal sample preparation.

As an overall conclusion, in this thesis a strong effect of the *Epichloë* endophyte in the performance of *Lolium perenne* was observed. For some characters the influence of the fungi in their host plant was dependent of the *Epichloë* morphotype, promoting particular changes in morphology and physiology, alkaloid production, and influencing the dynamic and acquisition of nutrients. Application of the results, the new inoculation technique and combination of the traditional and new approaches (*e. g.* NIR spectroscopy) may help in further studies for understanding on more details the *Epichloë*/grass relationship and consequently to development or improvement of commercial cultivars of grasses.

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