Lupinus mutabilis in Ecuador with special emphasis on anthracnose resistance

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CHAPTER 1

General Introduction

Introduction

The most recent estimate (October 2010) by the Food and Agriculture Organization of the United Nations (FAO), says that 925 million people are undernourished. The increase of hungry people has been due to three factors: 1) neglecting agriculture relevant to very poor people by governments and international agencies, 2) the current worldwide economic crisis, and 3) the significant increase of food prices in the last several years which has been devastating to those with only a few dollars a day to spend. Hunger is a term which means the uneasy or painful sensation caused by wanting of food; craving appetite. Malnutrition is a general term that indicates a lack of some or all nutritional elements necessary for human health. Seventy percent of malnourished children live in Asia, 26 percent in Africa and 4 percent in Latin America and the Caribbean. In many cases, their plight began even before birth with a malnourished mother. Undernutrition among pregnant women in developing countries causes that one out of six infants is born with a low weight. This is not only a risk factor for neonatal deaths, but also causes learning disabilities, mental retardation, poor health, blindness and premature death (World Hunger Facts, 2008). Poverty is the principal cause of hunger. Causes of poverty include poor people's lack of resources, an extremely unequal income distribution in the world, and within specific countries conflicts. Two types of poverty are identified. Extreme poverty that refers to being unable to afford basic human needs, including clean and fresh water, nutrition, health care, education, clothing and shelter. Relative poverty refers to lacking a usual or socially acceptable level of resources or income as compared with others within a society or country (http://en.wikipedia.org/wiki/Poverty). The first type of poverty is faced by Ecuadorians living in rural areas. The other type of poverty is linked to the economic crisis, faced by people located in the cities. The latest data show that in 2006, nearly 13% of the people lived in extreme poverty. In rural areas extreme poverty is five times higher than in the cities. The ethnic groups most affected by poverty are indigenous people in the highlands and Afro Ecuadorians at the coast. The percentage of malnourished people reduced from 26% in 1999 to 18% in 2006. Even though, in the Amazonian and highlands 24% and in the coastal region 12.5% of people is underfed (www.ecuadorvolunteer.org/es/informacion_ecuador/estadisticas.html).

Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition (TELFUN) is a project supported by Wageningen University that aims to support people to choose their own way of producing, processing and consuming local foods. The central research question is: how do technological practices, developed from within food networks, enhance food-sovereignty and the nutritional status of people? To answer this question, TELFUN has started as an interdisciplinary and comparative research program based on twelve research projects, divided in four disciplines and carried out in three geographical locations. Its scientific development is divided in four disciplines - plant breeding, food technology, human nutrition, and sociology of science and technology - for a more complete understanding of the production, processing, utilization and consumption of lupine in Ecuador. Similar studies are taking place with cowpea in West Africa and mungbean in India (www.telfun.info).

Anthracnose is the most devastating disease of lupine around the world (Talhinhas *et al*., 2002; Thomas, 2003). A lot of research has been done in other species of lupine that come from the old world (Talhinhas *et al*., 2003); however, little research has been done with *L. mutabilis*, a native species from the Inter Andean region. In the breeding discipline of the TELFUN project the specific research question was: How can exploratory studies both on the pathogen and on the lupine genetic diversity contribute to the development of lupine in the Cotopaxi province? Current data of lupine production and lupine anthracnose in the Andean zone is provided by a survey. Agronomical characterization and nutritional value of lupine cultivars released by the Agropecuarian National Research Autonomous Institution (INIAP) is determined and compared with their values in presence of anthracnose. Morphological and molecular studies provide the basis of pathogen diversity. Host cross reaction of lupine isolates on tamarillo or "tomato tree" (*Solanum betaceum*), another Andean native crop, provide new insight for disease management and for the establishment of appropriate breeding strategies. Neither anthracnose resistant/tolerant as high yielding landraces were known in Ecuador and therefore it was decided to evaluate anthacnose susceptibility and yield of a limited number of lupine cultivars released by INIAP and to study disease development in order to determine critical time points in the infection process. Anthracnose infection was evaluated in naturally infected seed and inoculation in a specific phenological stage made it possible to classify lupine plants on the basis of their level of tolerance. Anthracnose tolerance

and alkaloid content were evaluated in selfed lupine plants in order to study the role of alkaloids in tolerance.

The results, described in this thesis, will contribute to the establishment of better lupine breeding programs and seed production practices (high yield, anthracnose resistance, good nutritional and agronomical traits). The experiments were done at the Simon Rodriguez Institute in Latacunga – Cotopaxi province, Ecuador. The students of this Institute, who are rural, young farmers, learned about the management of growing lupine. They will pass these new findings on to their families and other farmers in the area. The interaction of the four disciplines (Plant Breeding, Food Processing, Human Nutrition, and Social Sciences) is expected to contribute to the development of robust local networks for the production, processing, and consumption of high quality lupine.

History

Lupine (*Lupinus mutabilis* Sweet), also known as *tarvi, tarhui, tarwi, chuchus muti* (Quechua); *tauri, taure* (Aymara); *chocho* (Spanish-Ecuador); *chocho, tauri, tarwi* (Spanish-Peru); *tremoço* (Portuguese-Brazil); *lupino, altramuz* (Spanish-Spain); *lupin, pearl lupin, Andean lupine* (English) is an Andean native legume, domesticated by indigenous people from old civilizations (Rios, 1996). Its exact place of origin is unknown because there is no written evidence about the pre-Columbian culture (Gross, 1982). However, the presence of lupine in the Andean region in the pre-Columbian culture is unquestionable due to the fact that lupine has been found in pre-Hispanic tombs and ceremonial ceramic representations (Rios, 1996), its use in folkloric traditions, oral transmissions, and other indirect references (Gross, 1982). It has a local distribution among Andean countries from Ecuador, Peru to Bolivia.

Genetic resources

The INIAP of Ecuador had a collection of lupine accessions since 1988. In INIAP's collection, there are 529 lupine accessions from 17 different species. Of this total amount, 257 accessions were collected in Ecuadorian areas and 272 originate from other countries. A wide variability in earliness, diseases response, and flower color, was found in a preliminary characterization of 120 accessions of *L. mutabilis* (Peralta *et al.*, 2003). The genetic variation

in lupine local cultivars resulted from the balance among forces enlarging the genetic variation within cultivars (mutations, out-crossing between and within cultivars followed by segregation, inadvertent mixing) and forces restricting the genetic variation (natural selection and selection carried out by the farmers) (Lindhout *et al.*, 2007).

L. mutabilis shows some genetic similarity with the old world species *L. albus* and *L. angustifolius* (Talhinhas *et al.*, 2003). Unlike in other lupine species, not much research has been conducted in the species *L. mutabilis.*

Agricultural and other benefits

Lupine is a good alternative in crop rotation with cereals and tubers and requires minimal tillage before sowing. It is a land crop and little or no fertilizer is applied (Caicedo and Peralta, 2000). Due to the good taste, high amount of protein, iron, and vitamins the consumption of this legume has become high. However, the seeds have a high alkaloid content and therefore a debittering process is required (Peralta *et al*., 2008).

Lupine, like other legumes, fixes its own nitrogen, and is an excellent green manure. Besides that, lupine has a broad adaptation and plasticity. For instance, in Ecuador, it grows easily in the different ecological zones between 2500 and 3400 meters above sea level (masl). Lupine has an excellent capacity for soil erosion control due to its pivotal, vigorous, branched and lignified root system that sometimes grows until three-meter deep. Lupine plants can be found in the border of the roads growing as wild plants. Some Andean farmers plant lupine as protective border plants to function as a repellent plant against pest and diseases of other crops. All plants of the Lupinus genus contain alkaloids. The total concentrations range from 0.01 to 4%, depending on plant species and part of the plant (INEN, 2004). It was thought that the alkaloids are mainly synthesized in the green leaves and shoots; however studies demonstrated that the synthesis occurs mainly in the roots, with xylem transportation and deposition in shoot organs. Mature seeds have the highest concentration of alkaloids and subsequently a bitter taste. This bitterness works as a general repellent against insects and grazing mammals (Allen, 1998). Small farmers take water from boiled lupine seeds to control endo-parasites of cattle and wash cattle for controlling their ecto-parasites. Research has been done in Italy to verify lupine therapeutic proprieties on human cholesterol levels, diabetes, and hypertension (Guzman, 2006).

Lupine is currently considered as an important source for both human and animal nutrition. Lupine seeds are a valuable protein and fat source. The protein amount varies from 35 to 45% and the oil from 15 to 23%. There is a positive correlation between the level of protein and alkaloids, and a negative correlation between protein and oil content (Jacobsen and Mujica, 2006). From the lupine grain it is possible to obtain flour which can be used to prepare soup, biscuits and other desserts but also for making lupine milk (Peralta *et al*., 2006b). The grain, after its debittering, is eaten separately with salt or in combination with fried grain, corn or with vegetable salads.

The "ceviche" is prepared with a selection of lupine seeds, fresh tomato juice, white onion cut into long strips, fresh lemon juice, fresh chopped cilantro (for garnish), 2 tablespoons of vegetable oil and salt to taste. The ceviche is served with fried plantain chips, popcorn or toasted corn kernels and spicy chili sauce. Everyone who visits Riobamba, the capital of the Chimborazo province, is invited to taste the unique "lupine ceviche". Currently, lupine ceviche is consumed in Quito, Latacunga and other main cities of the Ecuadorian highlands.

Plant characteristics

L. mutabilis is mainly a self-pollinating species, but low levels of cross-pollination can occur (5 – 10%); this level may increase depending on the ecotype and the ecological condition where it is grown (Gross, 1982). The cycle of lupine production is for local genotypes 9-10 months. Breeding resulted in lupines with a life cycle of 6-7 months (Peralta *et al*., 2006b).

L. mutabilis grows up to 2 meter high with almost no branching. There are 11-18 leaves on the main stem. The most developed are the 3-4 uppermost shoots (Figure 1A). The shoots are long (7-10 cm). The 7 to 11 leaflets are obovate-oblong, acuminate and blunted (Figure 1A,B). The stipules are small-sized, growing together at the basis with a pulpy cushion of petioles. The flowers are in racimes, semi-verticillate or alternate. The inflorescence is 8 to 20 cm long. The flowers are clustered in 9 to 10 verticils (Figure 1B) with often 5 flowers per verticil. The calyx has almost equal labia; the lower one is integral, while the upper one is bilabiate. The bractlets are bristly and the small floral bracts are styliform. The corolla is aromatic, and is blue, white, pink or violet colored, and range in size from 1.8 to 2.0. The standard and the wings are broad, longer than the keel (Figure 1C). The pods are pubescent,

non-shattering, 5-6 seeded (Figure 1D). Preferred grain-seed is white oval cuboid (Figure 1E) but there is variation in seed shapes and secondary color distribution on the seed of local cultivars. A sample of seeds from the lupine collection is shown in Figure 1F.

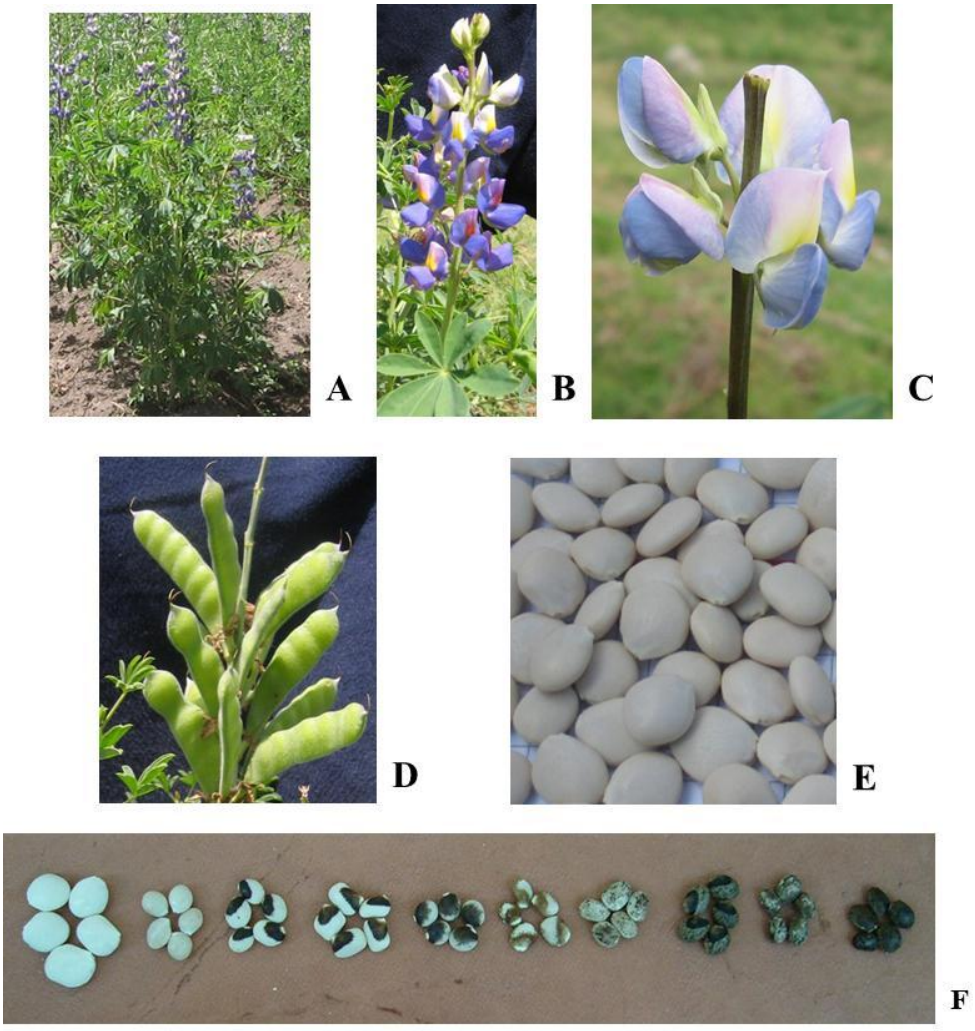

Figure 1. Some characteristics of lupine (*L. mutabilis*) **A.** Main stem and side branches of a lupine plant, inflorescences on main and secondary stems. **B.** Lupine flowers clustered in 9 - 10 verticils and obovate-oblong leaflets **C**. Flower parts. **D.** Pubescent pods, 5-6 seeded. **E.** White Andean lupine seed – grain. **F.** View of different seed shapes and secondary color distribution: (from left to right) white oval cuboid (I-450 ANDINO cultivar), white cuboid, flattened oval brown eyebrow, flattened brown eyebrow, oval black eyebrow crescent, flattened brown eyebrow, oval black spotted, oval black eyebrow-spotted, spherical black spotted, and black spherical.

Production of lupine in Ecuador

Lupine is cultivated in the Inter Andean zone, from Colombia, Ecuador, Peru to Bolivia (Caicedo and Peralta, 2000) from 2000 to 3850 masl (Jacobsen and Mujica, 2006). Ecuador, including the Galapagos Islands, is among the most biodiversity-rich countries in the world. According to the World Wildlife Fund, Ecuador is one of the 17 most "megadiverse" countries in the world. For instance, the country has an estimated 25,000 species of vascular plants (roughly 10 percent of the world's total). Agriculture is one of the pillars of Ecuador's economic development (www.worldbank.org/ec). Ecuador's continental region is divided from North to South from the Andean cordillera, forming three important mountain ranges (cordilleras): the Eastern Cordillera, the Inter Andean cordillera with various basin and valleys, and the Western Cordillera. The Ecuadorian Inter Andean region is characterized by low temperatures, strong winds, intense ultraviolet radiation, strong rains, rail, hail, storms, snow, and high altitudes. The rainy season in this area lasts from October to May, and the average annual temperature ranges from 11 ^{o}C to 18 ^{o}C. The daily variation can be extreme with very hot days and quite cold nights. Under these environmental conditions lupine is cultivated. More than 56% of the agro-ecological and geographical zones for lupine cultivation are located in the Cotopaxi and Chimborazo provinces (Caicedo and Peralta, 2000) (Figure 2).

The lupine cultivation is limited to small areas called "Unidad de Producción Agrícola" (UPA). The Censo Nacional Agropecuario (2003) described an UPA as a small piece of land of one half hectare in the Inter Andean region. The "minifundios" which are represented by small pieces of land up to 0.5 ha, are about 80% of the productive units. The main crops of Ecuadorian Inter Andean region are potato (*Solanum tuberosum* and *Solanum* sp), corn (*Zea mays* L), quinoa (*Chenopodium quinoa*), common beans (*Phaseolus vulgaris*), melloco (*Ullucus tuberosus* Loz) and other introduced crops, such as faba bean (*Vicia faba*), barley (*Hordeum vulgare* L), wheat (*Tritichum vulgare* L) and vegetables (Jacobsen and Sherwood, 2002). A recent activity in the Ecuadorian high lands is the production of native non-traditional fruits, such as tamarillo or tomato tree (*Solanum betaceum*), blackberry or "mora de castilla" (*Rubus glaucus*), babaco (*Vasconcellea babaco*), taxo o curuba (*Pasiflora* spp.), groundcherry or "uvilla" (*Physalis peruviana*), and black cherry or "capuli" (*Prunus serotina*) (http://www.runatupari.com).

Figure 2. Map of Ecuador (www.geology.com/world/ecuador-satellite-image.shtml). The two provinces with the most appropriate agro-ecological and geographical zones for lupine cultivation are inside the blue lines

The yield of lupine depends on different characteristics such as, earliness, development of inflorescences on primary stem, the axillar branches, pod number per inflorescence, seed number per pod, seed size and time of maturity of the pod (Hondelmann, 1984). According to the Censo Nacional Agropecuario (2003) 5974 ha of lupine was sown from October 1999 to September 2000. Only 66% of the potential of the crop in good conditions was realized (Peralta *et al.*, 2004), this was mainly due to biotic stress (diseases and pest) and abiotic stresses (drought and flooding). In Ecuador, lupine might yield around 220 kg/ha, but farmers did well if they reached 130 kg/ha. The national production was 789 MT, of which 601 MT was sold. Seventy percent of the lupine UPA is in the Cotopaxi and Chimborazo provinces (Figure 2), combined they have 75% of the sowing surface and 65% of the national production (Censo Nacional Agropecuario, 2003). Currently, the cultivated lupine area has steadily increased to 6,270 ha and the potential productivity has increased from 250 to 400 kg/ha. This better yield is the consequence of new technological developments, such as sowing lupine in furrows and appropriate use of agrochemicals (Murillo *et al.*, 2006) and phenotypic seed selection (Peralta *et al*., 2006b). However, the potential highest production level is seldom reached. The average in the Cotopaxi province with conventional technology

is only 180 kg/ha. The national mean production is barely 200 kg/ha (Censo Nacional Agropecuario, 2003).

Biotic stress – Lupine anthracnose

Anthracnose is worldwide the most devastating disease in lupine. It affects stems, leaves, pods and seeds (Figure 3A-D) and causes the typical twisting of the stem and petioles. The dark lesions in the center of infections form orange conidial masses (Figure 3A-C). Because the attack is on the apical zone, the plant dries out and no flowers and pods are produced (Guzman, 2006). The pathogen was first studied on *L. angustifolius* in 1943 and the causal agent was identified as *Colletotrichum gloeosporioides* (Penz) Penz & Sacc. Yang and Sweethingham (1998) supported the original description of *C. gloeosporioides* and identified three related compatibility groups. In Ecuador, it is known as *C. gloeosporioides* (Insuasti, 2001), or *Colletotrichum* spp. (Peralta *et al*., 2008). Based on a combination of colony and spore morphology and molecular techniques Lardner *et al.* (1999), looked at the genetic variability of the genus *Colletotrichum*. Talhinhas *et al*. (2002) studied the genetic variability of *Colletotrichum acutatum*. In accordance to Nirenberg *et al.* (2002) the pathogen is reclassified as *C. lupini*, including two new sub groups. Based on morphological and molecular techniques (ITS, RAPD-PCR) they divided Colletotrichum in three groups: *C. acutatum, C. acutatum* var. *lupini*, and *C. acutatum* var. *sensu lato*. For these studies, pathogen samples were collected from different parts of the world; one isolate from Bolivia was included, but not from Ecuador. Taxonomic details of *C. acutatum* isolates from this study and their relatedness with other close plant pathogenic fungi can be found through Basic Local Aligment Search Tool (BLAST) of ITS nucleotide data records of the isolates deposited in GenBank (www.ncbi.nlm.nih.gov/genbank).

Interaction of many species with their host plants is characterized by a short biotrophic phase. At this point, the cell surfaces of the two organisms are in close contact, followed by a destructive necrotrophic phase. Anthracnose symptoms appear at this latter stage on the aerial parts of susceptible plants, leaving the root system unaffected. Organ resistance most likely results from failure of the pathogen to penetrate the tissues. For this reason, it must be distinguished from race-cultivar specific resistance which is expressed on certain cultivars of a given species after penetration by the pathogen. This type of resistance is dependent on the

presence of resistance genes in the host (Esquerre-Tugaye *et al*., 1992). Durable resistance against specialized fungi is often quantitative and based on the additive effects of higher numbers of genes (Lindhout *et al*., 2007). Appropriate inoculation methods in adequate phenological stages are needed to identify anthracnose resistant plants.

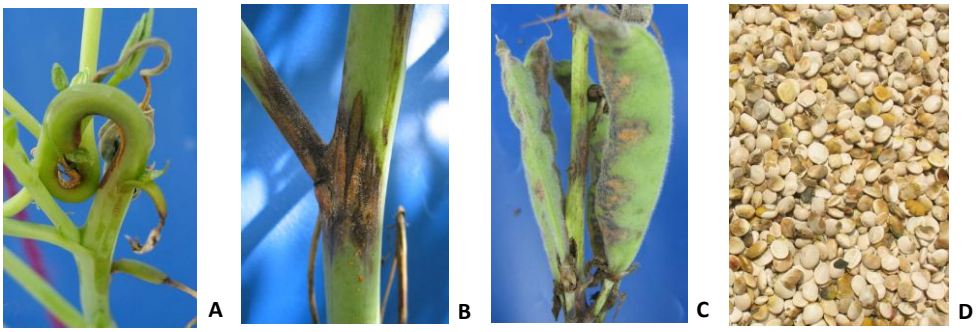

Figure 3. Typical symptoms of anthracnose **A.** infected main stem. **B** infected stem. **C.** pod lesions. **D.** infected seed

Anthracnose of lupine is a seed-borne pathogen that prefers moist environments. The heterogeneous environment of Ecuadorian highlands characterized by large differences in rainfall, humidity, and temperature is favorable for anthracnose development. This kind of environments also facilitates the development of new races. Anthracnose resistance of genotypes must be quantified under an adequate pathogen selection pressure (Thomas and Adcock, 2002) and the most virulent race should be used for infection (Kurlovich and Kartuzova, 2002).

The diversity of other species of *Colletotrichum* that cause anthracnose in beans has been studied in south, central and north America. Different races of the pathogen have been characterized (Balardin *et al*., 1997) and new sources of bean resistance for a specific geographical zone have been found (Vidigal *et al*., 2007).

Anthracnose in the Andean lupine cropping system

The increased demand of other native agricultural products of high value is causing the introduction of new crops into the traditional lupine cropping systems. The new crops introduced from the valleys to the highlands in the east and west result in the replacement

of the natural vegetation. The valleys have also been used for growing crops that were traditionally cultivated in cold areas such as lupine, due the increased demand for agroindustry (Teran, 2007). For instance, tamarillo or "tomato tree" is a small tree or shrub, bearing edible egg-shaped fruit with a thin skin and a soft flesh, when ripe. Tamarillo is a perennial crop. Its growing season is all year round, producing for up to four years (Sánchez *et al*., 1996). Tamarillo has traditionally grown in valleys of 1600 to 2600 masl; however, the historical limitation for growing tamarillo in low lands in the Cotopaxi province is changing (Terán, 2007). Currently, tamarillo and lupine are grown by small farmers in small pieces of land that are close to each other. For instance, at Sigchos Canton, in the Cotopaxi Province (around 120 km south from Quito) and in San Pablo Lake, in the Imbabura Province (120 km north from Quito), it is common to observe anthracnose symptoms in tamarillo as well as in lupine in the new cropping systems. Anthracnose is a wide host range pathogen that affects lupine (Talhinhas *et al*., 2005), tamarillo (Afanador-Kafuri *et al*., 2003) and many other hosts (Freeman *et al*., 2000). This fact may be providing more possibilities for the pathogen to adapt in both hosts or developing tamarillo as a potential reservoir for lupine anthracnose. A combination of morphological, molecular and host cross range studies will provide more insight for appropriate management of anthracnose in lupine as part of the cropping system and the implementation of breeding strategies.

Protein, Iron, and Zinc

In Ecuador, lupine has been traditionally consumed by rural people, however currently its consumption in urban areas has increased due to the fact that it is a cheap source of protein in comparison with animal protein (Peralta *et al*., 2006b). In Europe, lupine is also consumed for the fat (41%) (Gross, 1982). From the total fat, 3–14% are essential fatty acids (Jacobsen and Sherwood, 2002). Adequate lupine consumption can improve the nutritional status of the rural Ecuadorian population. Increased protein content would be a very beneficial trait in view of new markets and new processing opportunities. Until recently, higher protein concentrations were not a formal breeding objective in lupine. Most lupine varieties fall within the acceptable range of 14-24% content (Buirchell and Sweetingham, 2006).

Iron and Zinc deficiency are well-documented problems in food crops, causing decreased crop yields and lower nutritional quality. Generally, the regions in the world with Fe-deficient and Zn-deficient soils are also characterized by mineral deficiency in humans (Vasconcelos and Grusak, 2006; Cakmak, 2008). Recent estimates indicate that nearly half of the world population suffers from Zn deficiency (Cakmak, 2008). Among the strategies being discussed as major solutions to Fe and Zn deficiency, plant breeding and agronomic measures are the most important. Foliar or combined soil + foliar application of Fe and Zn fertilizers under field conditions are demonstrated to be highly effective and very practical way to maximize uptake and accumulation of micronutrients in the whole wheat grain, raising concentrations of Fe and Zn (Cakmak, 2008). Breeding strategies (e.g. genetic biofortification) are sustainable and cost-effective and should aim at improving the highest possible micronutrient concentration in the grain. In the plant kingdom, there is vast genetic variation for maximum mineral concentration (Vasconcelos and Grusak, 2006). This variation is at the species level, and even amongst individual plants of the same species. For instance, in cultivated and wild accessions of common bean (*Phaseolus vulgaris*) the micronutrient concentration in seeds can range from 55 to 95 µg/g iron (Beebe *et al*., 2000). Application of micronutrient fertilizers or micronutrients-enriched NPK fertilizers, under glasshouse and in small plots in the field, represents a useful complementary approach to the on-going lupine breeding program. If cultivars are found with a significant higher Zn and Fe potential and these cultivars have the required agronomic and end-use traits they can be quickly introduced and will have an immediate impact (Pfeiffer and McClafferty, 2007). The combination of breeding and biofortification is a promising, cost-effective, and sustainable intervention for alleviating micronutrient deficiency (Ma, 2007).

Conclusion

Lupine can play an important role in increasing living conditions of poor farmers in Ecuador. It is necessary that the agro ecological production systems are maintained using sustainable farming methods. The use of disease free lupine seed is of utmost importance and a good understanding of the plant– pathogen interactions can increase yield. Lupine breeding should aim at anthracnose resistance, high yield varieties, and cultivars with high levels of micronutrients. Having high-quality lupine cultivars will, together with the results in the

other three disciplines - food technology, human nutrition, and sociology of science and technology –contribute to the food sovereignty concept (www.telfun.info).

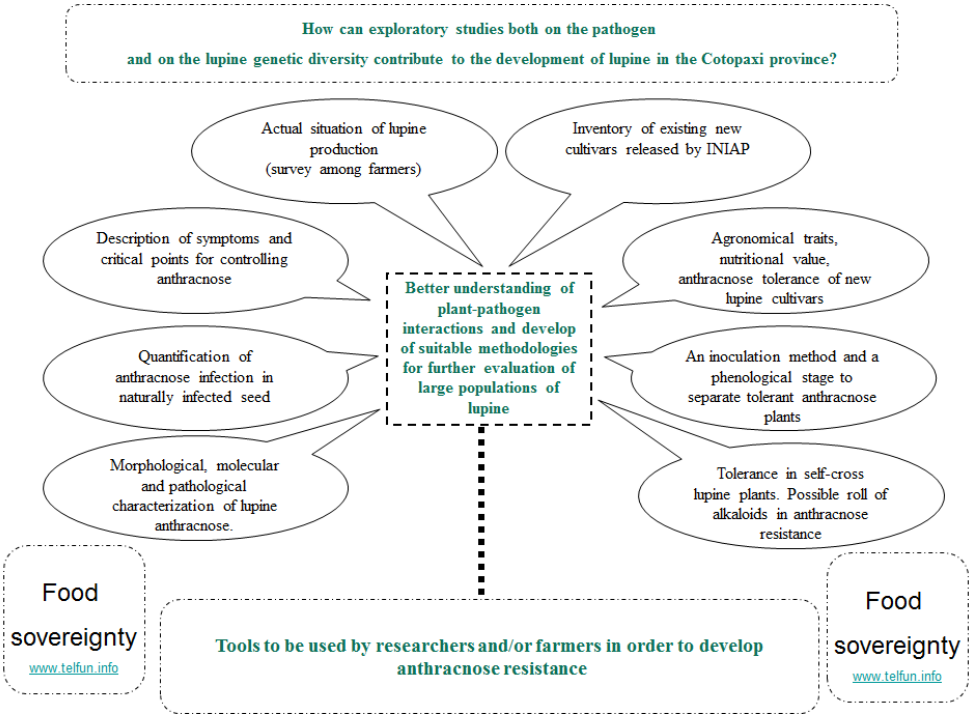

Figure 4. Lupine (*L. mutabilis*) situation and proposed research program with emphasis on anthracnose resistance in Ecuador

Research aims and thesis outline

The thesis presents new techniques that contribute to the improvement of lupine by exploring the possibilities of developing anthracnose resistant lupine varieties, with good agronomical traits, adapted to Cotopaxi province – Ecuador. The wanted lupine varieties should also have a high protein, iron and zinc content (Figure 4). Findings of this study will contribute to achieve food sovereignty of Ecuadorian farmers. The **General Introduction** presents a general overview of lupine. Its role in the food chain of Ecuador, the difficulties in getting a good and high yield and what important traits are to be considered to develop new cultivars.

Chapter 2 presents two surveys; firstly, the results of a survey among farmers and secondly an inventory of existing new cultivars released by INIAP. Several agronomical

traits were evaluated. Nutritional value of the lupine grain genotypes was assessed based on protein, iron and zinc content.

Chapter 3 describes the morphological and molecular characterization of the species of Colletotrichum that causes lupine anthracnose in the Cotopaxi and Chimborazo provinces - Ecuador. Five Colletotrichum isolates from tamarillo (*S. betaceum*) were also included in the analyses. Tamarillo also plays an important role in the Ecuadorian farming systems. Tamarillo is being cultivated in nearby places where lupine grows. The characterization of the species of Colletotrichum, the causal agent of lupine anthracnose in Ecuador, its genotypic relation with tamarillo anthracnose and a better understanding of the cross-host reaction are important before starting breeding efforts.

Chapter 4 describes the anthracnose tolerance of five lupine cultivars recently introduced by INIAP, the so-called INIAP-450 ANDINO cultivar, and two landraces determined under the same conditions in one trail Cotopaxi. This chapter also follow-up of anthracnose screening in the field, and presents a description of symptoms and analysis of critical points for controlling the disease. Anthracnose infection was quantified on the harvested seed.

Chapter 5 illustrates the use and effects of different inoculation methods and lupine plants of different ages to evaluate anthracnose tolerance. The first steps to find lupine individual plants with anthracnose Tolerance and Susceptibility in the selfing of I-450 ANDINO cultivar. Alkaloid content in the seeds was quantified and the correlation with anthracnose tolerance (phenotypically assessed) was determined. These findings provide suitable methodologies for further evaluating germplasm or large populations of lupine seedlings for tolerance to anthracnose.

In the **General discussion,** the findings of this research are presented in an integrated way. In the discussion the tools to be used by researchers and/or farmers in order to develop anthracnose tolerant/resistant lupine varieties, with good agronomical traits, and high nutritional value are discussed. These genotypes will certainly contribute to the food sovereignty of Ecuadorian farmers

CHAPTER 2

Agronomic traits and nutritional value of a set of Andean lupine (*L. mutabilis*) cultivars grown in the Province of Cotopaxi – Ecuador

Co-Authors: Richard G.F. Visser, Adriaan W. (Sjaak) van Heusden

Summary

Lupine, *Lupinus mutabilis*, is mainly, due to its high nutritional value, of high importance for Andean countries. This is not a well studied lupine; most research has been done on other lupine species such as *L. angustifolius*. The yield of Andean lupine is often low. This study describes agronomic characteristics and nutritional value of a set of lupine cultivars released by the Agropecuarian National Research Autonomous Institution (INIAP).

A questionnaire showed that lupine is the third preferred main crop by small stakeholders and ninety nine percent of planted lupine comes from common, low quality seed. There are no lupine varieties resistant to the most common lupine diseases and only few farmers use new technologies to improve yield. A new lupine cultivar, I-450 ANDINO, was developed by INIAP after a selection on agronomic traits. After screening samples of the lupine collection in Ecuador, other five cultivars with good agronomic characters were selected. These cultivars were used for further selection in different environmental conditions. In this study, agronomic traits and nutritional value of six selected cultivars and two local landraces were measured in the wet season in the Province of Cotopaxi. Most agronomic important traits were severely affected in the extreme wet season in which we did our observations. A multivariate analysis showed variation in plant height at flowering, number of flowers at the main axis, percentage of germination, presence of pods on the main stem, seeds per pod, and number of lateral branches. The landrace ECU-2698 showed the highest percentage of non-commercial seed. Significant different levels ($P<0.05$) of protein were found and the iron and zinc content of the lupine cultivars varied from 53 to127 and from 39 to 62 ppm, respectively. The highest values for the two nutritional elements and protein were found in I-450 ANDINO. The importance of characterizing genetic resources of *L. mutabilis* for crop improvement is outlined.

Introduction

Lupine, *Lupinus mutabilis*, is a native crop of the Andean zone. Historical data about lupine are based on archaeological evidence, folk traditions and customs, oral transmission and other indirect references (Gross, 1982). Lupine has been found in tombs of the Nazca culture. During the Spanish conquest approximately ten million inhabitants lived in the Inca

Empire (Peru, Ecuador and Bolivia). It has been speculated that during that period lupine was already grown (Gross, 1982).

Lupine can be cultivated as a monoculture or in association with other crops or in a rotation scheme. Lupine is important in crop rotation because of its ability to restore fertility of the soil through nitrogen fixation (Junovich, 2003). Lupine can grow on marginal soils such as soils of volcanic origin, soils with low fertility and in areas with water shortages (Jacobsen and Mujica, 2006). Although lupine can grow, the lupine yield is often low due to abiotic stress (Peralta *et al*., 2006b). The national yield average is 200 kg/ha. Ninety nine percent of the planting is performed with common seed, usually saved by farmers from their last crop (Censo Nacional Agropecuario, 2003). Lupine cultivation is limited to small pieces of land (up to a half hectare) and grown by small stake holders (Peralta *et al*., 2006b). Lupine has a high protein content (35-45 %), high quality fat, fiber, and a good content of minerals such as calcium, phosphorus, iron and zinc (Jacobsen and Mujica, 2006). A disadvantage is that the lupine seed has a high alkaloid content (INEN, 2004), which has to be washed away (Jacobsen and Sherwood, 2002). Lupine seeds are the raw material for preparing various foods, such as flour, milk and margarine. Its cooked seeds are popular in soups, stews and salads or are consumed as snacks in the same way peanuts and popcorn are (Popenoe *et al*., 1989). Because lupine is tasty and has nutritional benefits there is a high demand at national and international level. In 2000, the annual lupine consumption per capita was 4.8 kg in the cities of Latacunga and Riobamba. The potential demand was 10,500 t nationally (INIAP, 2001). In 2007, the demand increased twofold. In Ecuador the lupine production does not meet the demand, and therefore it is imported from Peru (Peralta *Pers. Comm.*).

Intra- and interspecific genetic variability of lupine is conserved in the National collection of DENAREF – INIAP. Of the 529 lupine accessions 257 were collected in Ecuador and 272 came from other countries. About 120 accessions belong to the species *L. mutabilis* (Rivera *et al.*, 1998). Researchers of the Legume and Andean Grain National Program (PRONALEG-GA / INIAP) have been cultivating *L. mutabilis* accessions for more than 10 years. A specific lupine genotype from Peru was used for selection under Ecuadorian conditions on desirable agronomic traits such as earliness, white seed color, and good plant architecture and resulted in the I-450 ANDINO cultivar (INIAP, 1999). However, seed

production of I-450 ANDINO is not high enough to satisfy farmers' demand and therefore, it is used mainly for research purposes. INIAP also chose samples of other populations from the lupine collection and evaluated them under different environmental conditions in the Provinces of Cotopaxi and Chimborazo. Farmers, organized in Local Research Agriculture Committees (CIAL), prefer lupine cultivars with a high yield, white seeds, a size seed of 6-8 mm diameter, a uniform plant height of about 1.4 m, and a growing season of less than 190 days. Based on these criteria, they selected the five most suitable lupine cultivars (Peralta *et al*., 2003). However, the yield of some of these cultivars was even lower than that of the I-450 ANDINO cultivar (Peralta *et al*., 2003). During the last past years, INIAP unsuccessfully tried to enhance seed production in the five selected cultivars and lupine yield is still very variable from one year to another (Peralta *et al*., 2006b).

The TELFUN project aims to support people to choose their own way of producing, processing, and consuming local foods that fit best in their local conditions (www.telfun.info). The following research questions were put forward in order to contribute to the development of lupine in the Province of Cotopaxi:

- what is the situation of lupine production in the Province of Cotopaxi?,
- what are the agronomic characters and nutritional attributes of the lupine cultivars selected as the best by INIAP researchers in the Province of Cotopaxi? No better performing cultivars were found by INIAP in their screenings of the available *L. mutabilis* germplasm. These screenings could not be repeated in the framework of this study.

To answer this question the following goals were set: (i) to determine the actual situation of the lupine production in the Province of Cotopaxi, (ii) to evaluate agronomic traits of the five selected lupine cultivars, the I-450 ANDINO cultivar and two local landraces in the wet season in the Province of Cotopaxi (iii) to determine the nutritional value of the selected lupine cultivars.

Materials and methods

Analysis of lupine production

The Northern Andes in Ecuador consists of two parallel ranges, the Cordillera Occidental and the Cordillera Oriental. In both regions farmers were visited (see geographical positions in Table 1). A questionnaire was made to analyse the lupine production and especially, the

threat anthracnose poses in the Province of Cotopaxi. The chosen communities to obtain lupine production data was based on information from the project Sustainable Production Systems for Guaranteeing Food Security in the Poor Communities of the Cotopaxi Province (Peralta *et al*., 2006a). Interviews were performed at farms and the survey was conducted in twenty locations (Table1). On each location, there was a survey, including repetitions of some of the questions. Forty small-stake holders were interviewed from May to June 2007 (Table 2). Since this research is part of an interdisciplinary research, a more extensive survey has been conducted and will be published by the Social Science discipline.

Table 1. Localities at the Province of Cotopaxi where the survey for diagnose lupine production was done

N	Locality	Geographical localization	Altitude (masl)	N	Locality	Geographical localization	Altitude (masl)
1	Juan Montalvo/ San Jorge	00 54’ 40” S 78 35’ 06” W	2840	11	San Marcos / Alaquez	00 51’ 54” S 78 33’ 47” W	3050
2	Pangigua grande	00 54’ 38” S 78 33’ 36” W	2950	12	El Tejar / Alaquez	00 51’ 40” S 78 33’ 08” W	3100
3	Pusuchusi	00 54’ 44” S 78 32’ 27” W	3180	13	Itupungo	00 50’ 42”S 78 33’ 26” W	3239
4	Picualo Alto	00 53’ 56” S 78 33’ 19” W	3000	14	Chinchil Robayo	00 47’ 41” S 78 33’ 36” W	3117
5	San Juan	00 54’ 04” S 78 34’ 36” W	2860	15	Canchicera	00 47’ 33” S 78 32’ 59” W	3222
6	Laipo Grande	00 53’ 15” S 78 32’ 51” W	2900	16	Langualo chico	00 49’ 40” S 78 34’ 03” W	3093
7	San Marcos	00 53’ 03” S 78 34’ 24” W	2920	17	San Francisco de Casas	01 04’ 44” S 78 40’ 20” W	3086
8	La Merced Molle-pamba	01 00’ 21” S 78 41’ 10” W	2969	18	Chilla grande	00 48’ 28” S 78 43’ 55” W	3300
9	Isinche de Comines	01 01’ 08” S 78 40’ 24” W	2972	19	Chalopamba	00 49’ 09” S 78 44’ 13” W	3321
10	Guantopolo	00 42’ 45”S 78 39’ 35” W	3638	20	Quilitopamba	00 53’ 27” S 78 43’ 13”W	3543

Table 2. Questionnaire and summary of the findings (values in parenthesis are percentages)

Locality …….	Region …….	Community …….
Name …….	Age ……	Gender …….

1. What are the main crops that you cultivate?		8. Mention two main reasons for lupine low productivity?	
Corn	30	Pests and diseases	90
Potato	25	Other different	10
Lupine	20		
Barley	10		
Alfalfa	10		
Lens	5		
2. How do you sow the lupine?		9. Look at the attached picture (with anthracnose symptoms). Have you seen this problem in the lupine crop?	
Monoculture	60	Yes	90
Associate	40	No	10
3. How much seed per hectare did you use for sowing?		10. In which part of the lupine plant have you seen the problem considered above?	
0.5-25kg	92	More than one tissue	70
26-50kg	6	Stems	10
> 50kg)	2	Pods	10
		Didnot see	10
4. How much did you harvest per hectare?		11. Do you know any lupine variety that cannot get diseased?	
100 - 150kg	60	No	100
151 - 300 kg	30	Yes	0
301 - 600 kg	5		
601 – 900 kg	5		
5. Where did you obtain the lupine seed for sowing?		12. Do you know how to screen lupine seed for sowing ?	
Own seed	80	No	90
From someone else	20	Yes	10

(cont…) Table 2.

6. Which characteristics of lupine do you prefer? Plant height ….. Seed colour…..		13. What do you do in order to control lupine anthracnose?	
1.0 – 1.4 m height	50	Nothing	60
Around 1.0 m height	25	Chemicals	40
More than 1.4 m height	25		
White seedcolour	100		
7. How do you prepare the soil for lupine cultivation?		14. What are the chemical products that you use to control lupine anthracnose?	
Furrows (0.6 x 0.25 m)	80	Do not apply chemicals	60
A hole at the sowing (1.0 x 1.0m)	20	Do not remember the chemical name	25
		Know name	15

Sources of lupine cultivars for agronomic characterization

Seeds of the five lupine cultivars were provided by PRONALEG-GA/INIAP. The five pre-selected cultivars were screened under different environmental conditions (Peralta *et al*., 2006b). Seeds of two landraces were provided by National collection of DENAREF – INIAP. The I-450 ANDINO cultivar was included as control (Table 3). Other local landraces and local genotypes were not evaluated because we wanted to know whether the developed material by INIAP was an improvement or not. These modern cultivars are characterized by early flowering, large pod size and white seeds. Small farmers use late genotypes (12-13 months) and recycled (anthracnose infected) seed.

Table 3. Origin and characteristics of a set of five lupine cultivars, the I-450 ANDINO cultivar and two Andean landraces of lupine (*L. mutabilis*) used in the Province of Cotopaxi, Ecuador, 2008

Cultivar	Origen	Contributing Institute[a]	Type
I-450 ANDINO[b]	ECU - 2659 introduced to Ecuador from Peru in 1992. Evaluated in several environments, selections were made and released as I-450 ANDINO cultivar in 1999	INIAP-DENAREF	Cultivar
ECU-712-1	Peru, Mantaro Research Experimental Station	INIAP-DENAREF	Under selection in the Province of Cotopaxi
ECU-2658	Peru, Lima, Institute of Nutrition, 200 masl	INIAP-DENAREF	Under selection in the Province of Cotopaxi
ECU-2700-2	Bolivia (UNTA)	INIAP-DENAREF	Under selection in the Province of Cotopaxi
ECU-722-4	Peru, Mantaro Research Experimental Station.	INIAP-DENAREF	Under selection in the Province of Cotopaxi
ECU-8415	Ecuador, Cotopaxi, Salcedo, Cusubamba.	INIAP-DENAREF	Under selection in the Province of Cotopaxi
ECU-740[c]	Peru, Cuzco, Granja Kaira, 3400 masl	INIAP-DENAREF	Local cultivar
ECU-2698[c]	Bolivia, (UNTA)	INIAP-DENAREF	Local cultivar

[a]INIAP (Instituto Nacional Autónomo de Investigaciones Agropecuarias), DENAREF (Departamento Nacional de Recursos Filogenéticos), PRONALEG-GA (Programa Nacional de Leguminosas y Granos Andinos) [b] I-450 ANDINO cultivar used as control of Andean lupine. [c]Andean lupine landraces from the collection of Ecuador.

Field plots

The field experiments were conducted at the Simón Rodríguez Technological Institute of Agriculture, located in the Province of Cotopaxi, Latacunga City, Alaquez Parish (latitude 00º 52' 01" S, longitude 78º 37' 07" W, altitude 2859 meter above sea level (masl), it is located at the central region of Ecuador, 91 km from Quito, the Capital of Ecuador. The five selected lupine cultivars and I-450 ANDINO and two local cultivars were evaluated under field conditions from January to August 2008 in one trail. The fields used in this study were already used for lupine cultivation during the previous years. The lupine cultivars were planted in a Randomized Complete Block Design with four replications.

There were 32 experimental plots (5.0 x 4.0 meter) with five furrows each. The distance among furrows or rows was one meter. In the rows holes were made at a distance of 0.25 m and three seeds were sown in each hole. At the borders, around the experimental field, I-450 ANDINO was sown both as a physical barrier and to obtain seeds for further studies. Management of the experiment was according to the PRONALEG-GA and INIAP technical recommendations (Peralta *et al*., 2008). At sowing time, the insecticide Endosulfan 4 ml/l water, was applied for pest control. Plots were fertilized with the equivalent of 175 kg/ha (N-P_2O_5–K_2O: 10-30-10). During the growing season, plots were weeded after 35 days with local hoeing instruments. The base of the plant was covered with surrounding soil 57 days after planting. Other pests such as *Agrotis ypsilon* appeared 60 days after planting. They were controlled with Endosulfan 4 ml/lwater. Other diseases affecting aerial lupine plant parts were *Uromyces* sp., *Ascochyta* sp., *Ovularia* sp. and *Sclerotinia* sp. No chemical control was used. Data of two local lupine landraces were collected from another experiment conducted in the same place at the same period of time.

Evaluation of agronomic traits of selected lupine cultivars

The percentage of germination was based on the number of seedlings 17 days after planting. Plant height at flowering time was the length from the crown to the apical part of the main stem. The number of plants at early flowering was recorded the day that the first flowers appear at the main stem and when 50% of plants were flowering. The number of lateral branches was counted when 50% of the plants were flowering. The number of flowers at the main axis was counted when 50% of evaluated plants had flowered. The total numbers of pods on the main stem as well as the number of pods on the lateral branches were counted at harvest time. This made it possible to calculate the total number of pods per plant. Data were collected from ten plants randomly chosen in the experimental units. Lupine plants were evenly distributed in the field with equal opportunity to compete for space, light, water and nutrients. Pods were threshed to determine the number of seeds per pod (the number of seeds from 10 pods of 10 plants was counted and the average calculated). The percentage of non-commercial seed was calculated after weighing damaged seed and total seed. The weight of a random sample of 100 seeds was also

recorded. Yield was based on the weight of seed (g/experimental unit) and this was extrapolated to kg/ha.

Data analysis

An ANOVA test was carried out to determine statistical differences of twelve agronomic traits in the different cultivars. Agronomic traits with significant ($P<0.05$) differences between cultivars were further analyzed.

Multivariate analysis was performed to calculate the Euclidian distances between each pair of cultivars and the results were compiled into a matrix. Data in this matrix were then used both for Cluster Analysis (CA) and Principal Component Analysis (PCA). In CA, the cultivars were clustered into hierarchical groups represented in a dendrogram according to a weighted average linkage. A cophenetic correlation coefficient was calculated to represent the degree of information lost when converting the original distance matrix into a dendrogram.

In PCA, the multidimensional data set (agronomic characters x cultivars) was reduced to a two dimensional representation, projecting the original standardized data into an axis system obtained by calculated Eigen vectors and values from distance matrix (Dunn and Everitt, 1982). An ANOVA test and least standard deviation (5%) was performed to establish statistical variability among groups represented in PCA using the INFOSTAT software www.infostat.com.arg

Evaluation of nutritional value of the seed

Samples of 2 g of commercial seed were used to determine the percentage of protein (Munshi and Raheja, 2000) by the method of McKenzie and Wallace (1954) and iron and zinc concentrations were measured by flame absorption spectrophotometry (Shimatsu, Japan, Model 680) according to methods described by AOAC (2005). An ANOVA test was performed using the INFOSTAT software www.infostat.com.arg and least standard deviation (5%) was used to compare the means of treatments.

Results

Evaluation of lupine production

The questionnaire was answered by 20 to 65-year-old men and women. The main crop that farmers cultivate is not lupine but it is in the third place of preferred crops for small farmers. Lupine is mainly cultivated as a monoculture. Eighty two percent of the farmers start sowing with only 0.5-25 kilos seeds. The yield is between 100 - 150 kilos/ha. Farmers prefer lupine plants of 1.0 to 1.4 meters high and with white seeds. These preferences are due to crop management and to demand of customers. Ninety nine percent of planted lupine comes from seeds collected by the farmers themselves. The remaining one percent is distributed by INIAP. High quality of seeds is mostly not a selection criterion for sowing.

The majority of farmers have learned to use the technology of planting lupine in furrows. However, lack of irrigation makes marginal farmers remain with the old technology of dropping a bunch of seed in a hole.

Farmers do not know lupine cultivars that do not get diseased. Most of them do something to control anthracnose in lupine. Some apply chemicals, but they often do not remember the name of the product. Small farmers are familiar with the anthracnose symptoms on lupine plant.

Evaluation of agronomic traits of selected lupine cultivars

Data on agronomic characters of the five selected lupine cultivars, the I-450 ANDINO cultivar and the two landraces are shown in table 3. For each pair of characters a Pearson's correlation was conducted. The number of pods on the lateral branches correlates ($r = 0.95$) with the total number of pods. The pods on lateral branches contributed to the major part of the total pod weight. However, in lupine it is expected that main production comes from the main steam. The branches produced only 25% of the total pod weight (Hardy *et al*., 1997). It indicates that pod formation in the main steam branches was severely affected in this study. The extreme rainfall during the period of study may have been the reason for this. For example: under normal conditions I-450 ANDINO produces 10 to 14 pods on the central axis and 6 to 8 seeds per pod (Caicedo *et al*., 1999). In this study, we found averages of almost 2.2 pods on the central axis and 1.9 seed per pod (Table 4).

Table 4. Means, minimum, maximum, and variation coefficient of agronomic traits of five selected lupine cultivars, I-450 ANDINO cultivar and two landraces naturally infected by *C. acutatum*.

Acronym	Min	Mean	Max	Units
POG	57.00	72.16	87.94	%
PHF	0.72	1.21	1.46	M
PEF	29.80	41.83	49.80	%
LB	10.20	11.84	14.00	no.
FMA	30.60	39.58	47.20	no.
PMA	0.40	2.21	7.10	no.
PLB	1.50	6.06	15.40	no.
TPP	2.60	7.88	20.90	no.
SP	0.60	1.91	4.00	no.
NCS	25.39	45.35	67.00	%
W100	18.40	33.47	50.38	G
Y	64.44	124.05	232.78	kg x ha^{-1}

Percentage Of Germination (POG), Plant Height at Flowering time (PHF), Plants at Early Flowering (PEF), Lateral Branches (LB), Flowers at the Main Axis (FMA), Pods on the Main Axis(PMA), Pods on the Lateral Branches (PLB), Total of Pods per Plant (TPP), Seeds per Pod (SP), Non-Commercial Seed (NCS), Weight of 100 seeds (W100), Yield (Y)

The yield was considerably affected and the percentage non-commercial seed was high. The yield was on average 124 kg per hectare. That is below the national average of 200 kg/ha (Censo Nacional Agropecuario, 2003) and also lower as in previous studies with the same cultivars in which the lowest yield of I-450 ANDINO was 270 kg/ha (Peralta *et al*., 2003). On average, forty five percent of the harvested seed was non-commercial. In general, these results show the poor adaptation to the wet seasonal periods in the Province of Cotopaxi of the selected lupine cultivars. The cumulative rainfall in January-August 2008 was high (474.9 mm) compared to the average of the preceding eight years (363.6 mm). Some biotic factors that may have affected agronomic traits, e.g. anthracnose susceptibility of selected lupine cultivars in conjunction with the wet seasonal are discussed in chapter four.

Table 5. Agronomic characters[a] of a set of five selected lupine genotypes and I-450 ANDINO cultivar naturally infected by *C. acutatum* at Simon Rodriguez Agronomic Institute, Alaquez, Cotopaxi, Ecuador, 2008

Lupine genotypes	POG	PHF	FMA	LB	PMA	SPP	Y	NCS
I-450ANDINO	68.4 ab	1.34 cd	39.4 bc	13.6 b	3.4 c	2.8 c	130 ± 24	42.4 ab
ECU-712-1	67.4 ab	1.26 c	40.1 bc	12.1 a	2.8 bc	2.3 bc	131 ± 34	47.0 ab
ECU-2658	79.8 c	1.33 cd	40.3 bc	11.2 a	1.0 a	1.9 bc	127 ± 23	38.2 a
ECU-2700-2	73.2 abc	1.37 d	45.1 d	11.5 a	1.8 abc	1.6 ab	142 ± 20	37.9 a
ECU-722-4	81.0 c	1.38 d	39.6 bc	11.4 a	1.8 abc	2.0 bc	149 ± 32	40.3 a
ECU-8415	76.2 bc	1.37 d	41.4 cd	11.4 a	1.3 ab	1.9 b	110 ± 24	42.7 ab
ECU-2698	62.5 a	0.74 a	34.4 a	11.5 a	2.8 bc	0.9 a	100 ± 1	61.6 c
ECU-740	68.8 ab	0.92 b	36.3 ab	12.1 a	3.0 bc	1.9 b	104 ± 4	52.6 bc
Mean	72.2	1.2	39.6	11.8	2.2	1.9	124	45.4
CV (%)	10.4	5.1	7.6	7.5	52.4	32.5	37.2	17.5
LSD *(P<0.05)*	10.94	0.09	4.35	1.29	1.68	0.90	67.32	11.56

[a] In a column, values followed by the same letter are not significantly different al $P < 0.05$ according to least standard deviation test. Acronyms used: POG = Percentage Of Germination 17 days after planting, PHF = Plant Height at Flowering (meters), FMA = Flowers at the Main Axis, LB = Lateral Branches when 50% of the plants were flowering, PMA = Pods on the Main Axis, SPP = Seeds Per Pod, Y = Yield (Kg/ha), NCS = Non-Commercial Seed percentage.

After the ANOVA test, a set of agronomic traits with high heritability values were chosen to be analyzed. For 7 out of 14 characters, significant ($P<0.05$) differences were found (Table 5). ECU-8415, ECU-722-4, and ECU-2700-2 were taller, ECU-2658 and ECU-722-4 germinated earlier and ECU-2700-2 produced more flowers than the others (Table 5). In general, all cultivars had a high percentage of non-commercial seed. The average was 45%. Among them, the ECU-2658, ECU-2700-2 and ECU-722-4 had significantly ($P< 0.05$) the lowest percentage of non-commercial seed. Some individual plants of these cultivars may have anthracnose tolerance genes. ECU-2698 had the highest percent of non-commercial seed.

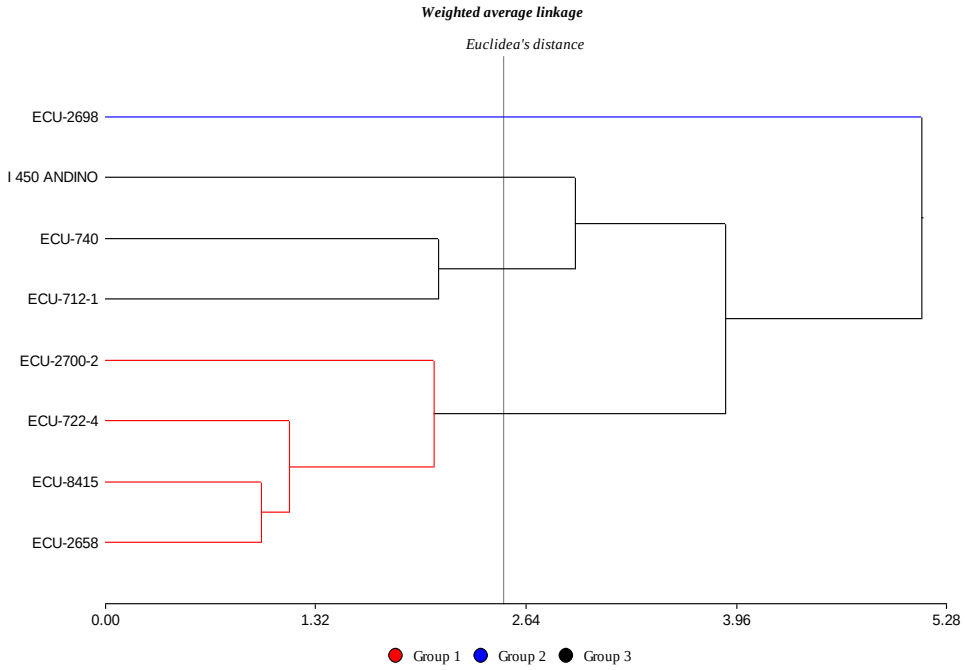

Fig 1. Dendrogram relating the eight lupine cultivars used for multivariate analysis of agronomic traits, calculated upon clustering of cultivars from a ANOVA test, cophenetic correlation coefficient r = 0.86. Cultivars were grown in a wet seasonal period and naturally infected by *C. acutatum*..

A multivariate analysis was done with plant height at flowering time, number of seeds per pod, percentage of germination, number of lateral branches, percent of non-commercial seed, number of flowers at the main axis, and number of pods on the main stem. A dendrogram calculated by CA represents the relationship degree between cultivars (Fig 1). ECU-2658, ECU-8415, ECU-722-4, and ECU-2700-2 cultivars were group A, the landrace ECU-2698 was in group B and the remaining ECU-712-1, I-450 ANDINO and the landrace ECU-740 landrace were group C.

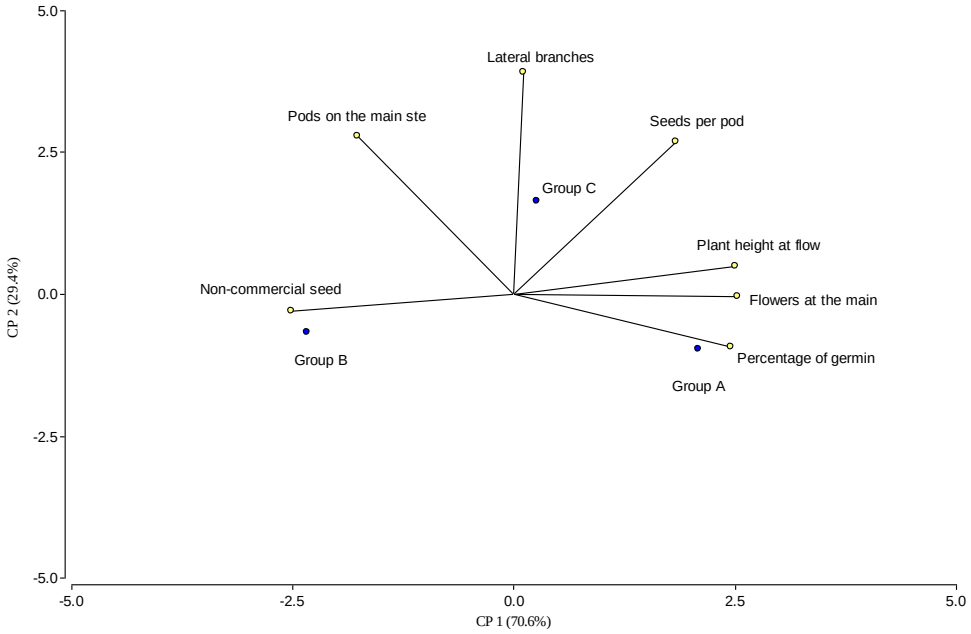

Fig 2. Projection in a 2-dimensional space of Eigen vectors related with to characters used in multivariate analyses, obtained by principal component analysis.

With CA and PCA the characters that determine the three main groups were defined. The group A characters are related to vegetative development of lupine and contains plant height at flowering and number of flowers at the main axis. The percentage of germination is also included in this group. The character defining Group B is the percentage of non-commercial seed. Group C cluster characters concern reproductive development, such as seeds per pod, pods on the main stem, and number of lateral branches. Principal component analysis determined that the two axes explained 100% of variability among the groups obtained in a neighbor-joining test. Axis one explain 70.6 % and axis two explains 25% of the variability (Fig. 2).

When comparing the means within the three groups in group A plant height at flowering, percent of germination, and flowers at main axis were significantly ($P< 0.05$) higher. The cultivar I-450 ANDINO (Group C) was significantly ($P<0.05$) higher for seed per pod, pods

on the main stem, and number of lateral branches than the others. ECU-2698 was in the group C with the highest percent of non-commercial seed (Table 6).

Table 6. Agronomic characteristics differences among the three lupine genotype groups based on the neighbor joint analysis [a].

	Plant height at flowering	Seeds per pod	Percent of germination	Lateral branches	Percent of non-commercial seed	Flowers at main axis	Pods on main stem
Group A	1.36 a	1.85 b	77.54 b	11.37 a	39.80 a	41.59 c	1.46 a
Group B	0.74 b	0.90 a	62.50 a	11.45 a	61.63 b	34.45 a	2.75 b
Group C	1.18 c	2.33 b	68.21 a	12.60 b	47.34 c	38.60 b	3.03 b
LSD (P<0.05)	0.125	0.59	6.88	0.89	7.47	3.16	1.04

[a]In a column, values followed by the same letter are not significantly different al P < 0.05 according to Least Significant Difference

Table 7. Nutritional value of seed of five selected lupine cultivars and I-450 ANDINO cultivar grown at Simon Rodriguez Agronomic Institute, Alaquez, Cotopaxi, Ecuador, 2008

Lupine genotype	P*	Fe*	Zn*
I-450 ANDINO	41 b	127b	62
ECU-712-1	39 a	71 a	36
ECU-2658	41 b	71 a	41
ECU-2700-2	41 b	64 a	38
ECU-722-4	41 b	81 ab	40
ECU-8415	41 b	53 a	38
Mean	40.4	78	42.7
CV (%)	1.51	44.53	54.02
LSD *(P<0.05)*	0.92	52.26	34.74

In a column, values followed by the same letter are not significantly different al P < 0.05 according to Least Significant Difference. *Each value is the mean of 2 g of seed samples replicated four times. Acronyms used: P = protein (g/100g), Fe = iron content (mg/kg), Zn = zinc content (mg/kg).

Evaluation of nutritional value of selected lupine cultivars

The protein, iron and zinc content of the six selected lupine cultivars are shown in table 7. Only genotype ECU-712-1 had a significantly lower protein content ($P<0.05$). I-450 ANDINO had a significantly ($P<0.05$) higher iron content than the other cultivars. No significant differences at the 5% level for zinc concentrations were presented for the six selected lupine cultivars. Values ranged from 36 to 62 ppm for zinc content. The highest values for the two nutritional elements and protein were found in I-450 ANDINO.

Discussion

The questionnaire showed that lupine is not the main crop cultivated by small farmers in the Province of Cotopaxi. Sixty percent is grown as a monoculture and 40% in association, mainly with corn (Peralta *et al*., 2004). In the Province of Chimborazo, 84% of the farmers grow it as monoculture (Censo Nacional Agropecuario, 2003). Lupine production and lack of quality are influenced by this continuous monoculture. Lupine productivity is very low under the traditional production system of low input small-holders. However, a lupine harvest of up to 1200 kg/ha can be achieved with the application of good agronomic practices and investing 50 kg/ha of good quality seed (Peralta *et al*., 2008). For selling, farmers select large seeds that do not have lesions or bits. They usually prefer selling the good lupine seeds than using it for their own consumption. They do this in order to obtain money and buy another type of food that is cheaper and has less nutritional value. Only a small part of the lupine production is used for self consumption (Jacobsen and Sherwood, 2002). Establishing programs for mass production and consumption of lupine are critical. Finding a high productive lupine seed, with good quality and a seed well accepted in the market are the main traits that INIAP researchers are selecting in a participatory breeding program (Peralta *et al*., 2003).

The survey shows that most farmers use their own low quality seed for sowing. This choice makes the next generation of plants is already infected by anthracnose. This is one of the main causes of low lupine productivity in marginal areas and the main cause of the spread of seed-borne pathogens, such as Colletotrichum. Andean lupine cannot compete in the farming systems until it is recognized that using high quality seed for next generations will be more profitable than selling the high quality seed and sowing the lower quality. According to Peralta *et al*. (2008) neither public nor private industries want to invest resources to grow

lupine for just to sell seeds. Lupine, quinoa, amaranto and bean cultivation is mainly done by poor Andean farmers and are therefore not representing an interesting market for seed industries. Breeders in conjunction with Agrarian Research Local Comities (CIAL's in Spanish) are responsible for establishing alternative systems to produce lupine seeds of good quality. For example, an Artisanal Production System (APS) may be initiated by a farmer or a group of skilled farmers that have learned the management of seed. Quality seed with genetic identity and high germination rate must be produced. An APS must offer seed at low cost and at the appropriate moment (Peralta *et al*., 2008). The desirable lupine variety should be anthracnose resistant, early maturating (6 months or less), height up to 150 cm, good yielding (800 kg/ha or more), produce at least 15 pods per plant with at least 8-mm seed size, white seeds and high nutritional value (Murillo *et al*., 2006).

The agronomic study of the six selected *L. mutabilis* cultivars and two landraces allowed us to evaluate their performance in a very wet period in the Latacunga region. In general, the lupine cultivars showed low averages for all agronomic traits. This points out that the selected lupine cultivars (released by INIAP) were poorly adapted to Cotopaxi's high rainfall season in 2008. In order to obtain improvements in seed yield the first step in a breeding program is to identify the phenotype adapted to local climatic conditions (Wells, 1984). In France and UK, large yield improvements of *L. albus* were seen in dwarfism, determined growth, and vernalizing types. These genotypes were sown early in the autumn and resisted freezing damage during winter (Cowling *et al*., 1998).

The second step in the breeding program should be to seek quantitative improvements for yield and quality which are usually under polygenic control. In this study, the yield of studied cultivars showed low averages, even lower than the national yield average of 200 Kg/ha (Censo Nacional Agropecuario, 2003). The yield improvements in *L. angustifolius* cultivars from 1973 to 1991 were due to improvements in harvest index, but there was no increase in total biomass of the improved cultivars (Tapscott *et al*., 1994). The harvest index increased from 0.24 to 0.29, mostly due to more pods and seeds per plant. However, this index is still low compared with other grain legumes (Cowling *et al*., 1998) indicating that there is still much to gain in lupine breeding.

The set of selected cultivars could be divided in three groups. Group A was based on the predominant height of the main axis and vigorous vegetative development, group C

characteristics were lower height and had more lateral branches and reproductive capacity. Determinacy reduces the number of branches and induces early maturation (Milford *et al*., 1993). Determinacy is the result of a change in growth pattern; at a given time in the grown cycle, all buds become floral, and no new vegetative organs are formed (Cowling *et al*., 1998). In *L. albus*, the architecture is influenced by two major genetic systems, restriction of branching (determinacy) and dwarfism. In *L. mutabilis*, two types have been observed, the "chocho" a tall annual or biennial plant which has long branches, adapted to the long growing seasons of the Andes, and the "tarwi" an annual type from high altitudes in the central Andes of Peru and Bolivia which has a dominant main stem, few branches, and shorten growing season of 155 days (Tapia, 1990). In this study, we found cultivars that belong to the so called "chocho" type (group C) and cultivars to the "tarwi" type (group A). The higher number of lateral branches of I-450 ANDINO may be the result of the continuous visual selection of INIAP. Group B was associated with a high percentage of non-commercial seed and the landrace ECU-2698 showed the highest percentage for this trait. Some cultivars (ECU-2658, ECU-2700-2 and ECU-722-4) had lower percentages of non-commercial seed. Possible tolerant anthracnose genes may be present in some individual plants. The high percentage of non-commercial seed of I-450 ANDINO may be due to the continuous use of seed selected by its phenotypic appearance. The level of anthracnose infection in commercial seed (visually clean and healthy); seeds with small red-brown stains and non-commercial seed (bad quality, diseased, small) and appropriate local management of lupine seed is discussed in chapter four of this thesis. Low averages for all agronomic traits suggest a poor adaptation of cultivars to the wet season of the Province of Cotopaxi. Studies demonstrated that Andean lupine is susceptible to high rainfall and prolonged wet seasonal periods causing flower abortion (Hardy *et al*., 1997). From all studied cultivars, only ECU-2700-2 showed high number of flowers at the main axis, but the pods at the main stem and seeds per pod of all cultivars were low (Table 5). The low seed yield and variable production from year to year are often the main agronomic limitations of *L. mutabilis.* These differences between *L. mutabilis* and the other species of lupine could arise from the lack of human interference in the evolution of *L. mutabilis*. Andean lupine was partially domesticated 3000 years ago in South America. However, its use was always hampered by the presence of bitter alkaloids (Jacobsen and Sherwood,

2002). In a breeding program, promising individual plants of accessions of the National lupine collection can be used as starting points. The resulting cultivars must be compared with the cultivars released by INIAP. It should, of course, be possible to recognize the promising individuals based on architecture, tolerance to wet and dry seasons, and tolerance to biotic stress such as anthracnose.

The seeds of the lupine accessions in this study had good protein, iron and zinc content. Protein values fluctuated around 40 percent; this is in the same range as in local bitter cultivars in Ecuador (INEN, 2004). Iron and zinc concentrations of the selected lupine cultivars varied from 53 to 128 ppm and from 36 to 62 ppm, respectively. This is somewhat higher than in accessions of *L. albus* (35 – 77 ppm for iron and 45 to 48 ppm for zinc), and in accessions of *L. angustifolious* (32 to 53 ppm for iron and 48 to 55 ppm for zinc (Trugo *et al*., 1993). The highest values for the two nutritional elements were found in I-450 ANDINO. Breeding programs should result in better quality Andean lupine. It is important that high nutritional genotypes also have high anthracnose tolerance and abiotic stress resistance.

Acknowledgements

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CHAPTER 3

Characterization of *Colletotrichum acutatum*, the causal agent of anthracnose in lupine (*Lupinus mutabilis*) and tamarillo (*Solanum betaceum*) in the Ecuadorian Andes

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Summary

Anthracnose, caused by *C. acutatum*, is a serious problem of lupine (*Lupinus mutabilis*) and tamarillo (*Solanum betaceum*). Morphological features, internal transcribed spacer (ITS) sequence analyses, and host specificity tests were used to characterize *Colletotrichum* isolates isolated on lupine and tamarillo. Isolates from each host were compared for the diameter of lesions they produced on the main stem of two tamarillo and three lupine cultivars. On lupine plants, isolates from lupine caused larger lesions than isolates from tamarillo. On tamarillo, isolates from that host caused larger lesions than isolates from lupine. Isolates from lupine were highly biotrophic on lupine stems, causing little necrosis during the twenty days following infection, even though abundant sporulation could be seen. In contrast, isolates from tamarillo sporulated less abundantly on lupine stems and produced darkly pigmented lesions. Isolates from lupine produced dark lesions and with few spores on tamarillo stems. It indicates that host adaptation is not determined by an ability to cause disease but rather by quantitative differences in pathogenic fitness. Phenotypic and molecular characterization showed that on both hosts the causal agent of anthracnose was *C. acutatum.* All the isolates from lupine and tamarillo gave a product in a *C. acutatum*-specific polymerase chain reaction (PCR) reaction and no *C. gloeosporioides*-specific product could be amplified. Colony diameter, spore shape, and insensitivity to benomyl also grouped the lupine and tamarillo anthracnose isolates in the *C. acutatum* group. Phylogenetic analysis was based on comparisons of ITS1 gene sequences. Both neighbor-joining and maximum parsimony methods placed the lupine and tamarillo isolates from the Ecuadorian Andean zone in two clades with a 98 % similarity between clusters and over 99% similarity within the clusters. In each cluster both lupine and tamarillo isolates were represented. Isolates of lupine were compared to isolates from other hosts around the world and it was shown that the *C. acutatum* isolates from Andean lupine are distinct from other *C. acutatum* isolates collected on lupine around the world.

Introduction

Lupine (*Lupinus mutabilis*) is an Andean legume and is strategic for accomplishing food sovereignty of the Andean people. Lupine is important in human nutrition because of its high protein content (>40% in dry grain; INEN, 2004), fat (20%), carbohydrates, minerals

and fibers (Jacobsen and Mujica, 2006). Lupine plays a role in many production systems in the Ecuadorian highland. It is cultivated in intercropping, monoculture and rotation systems (Peralta *et al.*, 2008).

Tamarillo (*Solanum betaceum*), another native Ecuadorian plant, is also important in the Ecuadorian agricultural systems. It grows as a small tree or shrub, bearing edible egg-shaped fruits with a thin skin and soft flesh, when ripe (Sánchez *et al.*, 1996). Traditionally, lupine has been cultivated in colder areas between 2500 to 3400 meter above sea level (masl), (Caicedo and Peralta, 2000; Llamuca, 2006), and tamarillo between 1000 to 2500 masl where the temperature is somewhat higher (Sánchez *et al.*, 1996; Llamuca, 2006). Due to the increased demand for lupine, this is changing (Terán, 2007) with unavoidable agro-ecological complications such as changes in susceptibility to diseases like anthracnose. Host adaptation of anthracnose has important epidemiological consequences in areas where two or more potential hosts grow in close proximity.

Worldwide there are several species of Colletotrichum that can cause anthracnose on legumes, perennial crops, and vegetables (Freeman *et al.*, 1998, Freeman *et al.*, 2000b, Talhinhas *et al.*, 2005). In lupine, anthracnose causes the typical twisting of petioles and stems, with dark sunken lesions in the center on which orange conidial masses are produced (Fig. 1 A-D). On tamarillo, anthracnose includes depressed black lesions on fruit accompanied by erupting pink spore masses in the lesions. Ultimately, the pathogen deforms and the fruit rots away (Fig. 1 F). On the branches the symptoms are dark depressed lesions varying in size and form ((Fig. 1 G). In Ecuador, yield losses due to anthracnose can reach up to 100% in lupine (Murillo *et al.*, 2006) and 60% in tamarillo (Sánchez *et al.*, 1996; Albornóz, 1992). The causal agent of anthracnose in lupine (Insuasti, 2001) and tamarillo in Ecuador (Santillán, 2001; Sánchez *et al.*, 1996; Albornóz, 1992) has been referred to as *C. gloeosporioides.*

Species identification in *Colletotrichum* has relied mainly on morphology and host range criteria (Freeman *et al.*, 1996). In the genus *Colletotrichum*, a range of morphological and colony characters are used for species identification. For example, *C. acutatum* has fusiform conidia and slow growing pink colonies (Sreenivasaprasad *et al.*, 1996a). The growth in media amended with benomyl (Adaskaveg and Hartin, 1997) is also a discriminating character. Still it is often unreliable to distinguish properly the subspecies

(Brown *et al.,* 1996; Sreenivasaprasad *et al.,* 1996a). *C. acutatum* can be pathogenic and non-pathogenic and can also grow on a number of different hosts (Sreenivasaprasad and Talhinhas, 2005). The sequence of the internal transcribed spacer (ITS1-2) of ribosomal DNA (rDNA) has been used to classify the fungi on lupine (Talhinhas *et al.,* 2002) and tamarillo (Afanador-Kafuri *et al.,* 2003). A combination of morphological and molecular techniques in the last years, has identified *C. acutatum* as the causal agent of anthracnose instead of *C. gloeosporioides* which was originally thought to be the causal agent (Brown *et al.,* 1996, Freeman *et al.,* 2000b). The objectives of this study were (i) to determine the causal agent of lupine and tamarillo anthracnose in Ecuador based on morphological and genotypic characters (ii) to analyze lupine and tamarillo *Colletotrichum* isolates from Ecuador and determine their relatedness with worldwide representatives based on the ITS sequence (iii) to assess the pathogenic variability and cross-infection potential of these *Colletotrichum* isolates. To the best of our knowledge, this is the first study of anthracnose in Ecuador in which phenotypic and molecular approaches are combined to characterize the population structure of this important pathogen. These findings will help in the development of an appropriate disease management and in efficient breeding strategies.

Materials and methods

Fungal isolates

A total of 18 *Colletotrichum* spp. isolates were collected from different lupine production areas and five *Colletotrichum* spp. isolates were collected from tamarillo production areas (Table 1). Not all isolates were used in all the analyses and further details are provided in the appropriate sections. Stock cultures of the monoconidial isolates were stored at 0 ^{o}C, as dense conidial suspensions in cryotubes. The suspension consists of peat + 10% autoclaved sucrose. Isolates were propagated by transferring small amounts of frozen conidial suspension from cryotubes to Petri dishes containing potato dextrose agar (PDA - Difco Laboratories, Detroit).

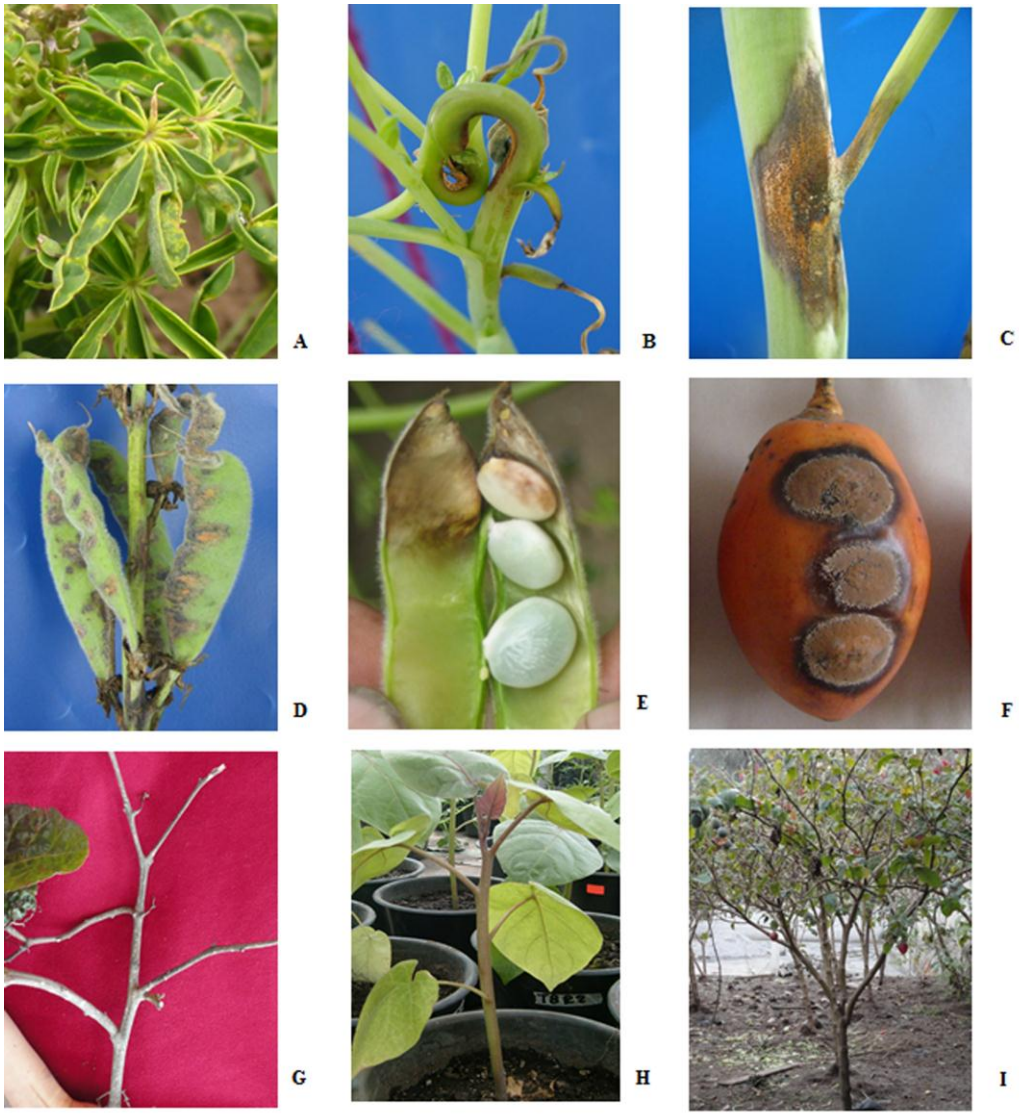

Fig. 1. Anthracnose symptoms caused by natural infection of *C. acutatum* on lupine (*L. mutabilis*) **A**. On leaves. **B**. On the apical main stems. **C.** On the main stems. **D.** On the pods. **E.** On the seed. **F.** Anthracnose symptoms on tamarillo (*S. betaceum*) fruit. **G**. Symptoms on tamarillo main stem. **H.** A tamarillo plant in a nursery. **I**. Tamarillo plant in the field affected by anthracnose.

Table 1. Colletotrichum spp. isolates collected in Ecuadorian provinces

Original code	Host species	Affected tissue	Location, province	Collection date
Lup1	Lupine	Stem	Juan Montalvo, Cotopaxi	2007
Lup2	Lupine	Stem	Pangigua grande, Cotopaxi	2007
Lup3	Lupine	Stem	Pusuchusi, Cotopaxi	2007
Lup4	Lupine	Stem	Picualo Alto, Cotopaxi	2007
Lup5	Lupine	Stem	San Juan, Cotopaxi	2007
Tam6	Tamarillo	Fruit	San Pablo, Imbabura	2007
Tam7	Tamarillo	Fruit	Ibarra, Imbabura	2007
Lup8	Lupine	Stem	San Marcos, Cotopaxi	2007
Lup10	Lupine	Stem	El Tejar, Cotopaxi	2007
Lup11	Lupine	Stem	Chinchil, Cotopaxi	2007
Lup12	Lupine	Stem	Chinchil Robayo, Cotopaxi	2007
Lup14	Lupine	Stem	Canchicera, Cotopaxi	2007
Lup16	Lupine	Stem	San Francisco, Cotopaxi	2007
Lup18	Lupine	Stem	Pujili, Cotopaxi	2007
Tam20	Tamarillo	Fruit	Guallabamba, Pichincha	2010
Lup21	Lupine	Stem	Tixan, Chimborazo	2010
Lup24	Lupine	Stem	Guamote, Chimborazo	2010
Lup28	Lupine	Stem	Palmira, Chimborazo	2010
Lup30	Lupine	Stem	Lican, Chimborazo	2010
Lup31	Lupine	Stem	Guazazo, Chimborazo	2010
Lup32	Lupine	Stem	San Andres, Chimborazo	2010
Tam33	Tamarillo	Fruit	Atuntaqui, Imbabura	2011
Tam34	Tamarillo	Fruit	Cotacachi, Imbabura	2011
IMI 356878 AJ536229*	Olive (*Olea europaea* subsp. *europaea*)	Fruit	Montsia, Tarragona - Spain	2003

* Reference isolate IMI 356878 (AJ536229.1) (*C. gloeosporioides*) from olive (*Olea europaea* subsp. *europaea*) was kindly provided by Dr. Pedro Martinez-Culebras (IATA, Universidad de Valencia – Spain).

Morphological studies

Each isolate was grown on PDA for 10 days. A conidial suspension was prepared and adjusted to 10^6 conidia/ml. Fifteen µL was pipetted in the center of a Petri dish (PDA

amended with 500 mg/l Cloramphenicol (Chloromycetin Parke Davis Co.). Colony characteristics (transparency, presence of conidial masses, concentric bands, pigment color, concentric bands color, size of the pigment across surface) were recorded from cultures grown at 17 ± 2 °C in day light. The growth rate of all isolates on PDA at 15, 20, 25 and 30 °C in the dark was measured. Each isolate had 3 replications which were daily evaluated during 5 days. The experiments were conducted twice and the means were reported. The colony radius with three replicates, four measurements per replicate was analyzed using analysis of variance and the least significant difference determined with the Statistic Software Infostat (www.infostat.com.arg). Spore size and shape were measured after five days of incubation. Benomyl sensitivity of the isolates was assessed by comparing colony radius on PDA and PDA amended with 2 mg/l benomyl (Benlate 50WP, DuPont). Six isolates from lupine and three isolates from tamarillo were tested in duplicate, and the experiment was conducted twice. Colony radius mean values of both experiments were compared with isolates grow on PDA and the reference isolate of *C. gloeosporioides* IMI 356878 (AJ536229.1). A colony radius reduction (>30 %) was considered positive.

DNA preparation

Lupine and tamarillo isolates were grown on PDA medium at 25 °C for 6 days. Mycelium was collected from the plates with a scalpel, frozen in liquid nitrogen and ground in a mortar to a fine powder. DNA extractions were performed using approximately 100 mg of powder mycelium with the help of the commercial EZNA Fungal DNA kit (Omega Bio-tek, Doraville, USA) according to the manufacturer's instructions. DNA samples were diluted to a final concentration of 50 to 100 ng/µl.

Taxon-specific PCR amplification

PCR primers for taxon specific amplification included the ITS4 (White *et al.*, 1990) primer coupled with specific primers for *C. acutatum* (CaInt2) (GGGGAAGCCTCTCGCGG) and for *C. gloeosporioides* (CgInt) (GGCCTCCCGCCTCCGGGCGG) (Brown *et al.*, 1996). PCR reactions were performed for 30 cycles (30 s at 95°C, 30 s at 60°C and 1.5 min at 72°C). Amplification products were separated in agarose gels (1.5%, wt/vol) in Tris-

acetate-EDTA buffer (Sambrook *et al.*, 1989) at 80 V for 2 h. A 100 bp ladder (Invitrogen, USA) was used as molecular standard.

PCR amplification and sequencing of ITS1, 5.8S and ITS4 regions of the rDNA genes

Universal PCR primers were used (ITS1, TCCGTAGGTGAACCTGCGG and ITS4, TCCTCCGCTTATTGATATGC) for amplification of the ITS1 and ITS4 regions between the small and large nuclear rDNA, including the ITS2 and the 5.8S rDNA, as described by White *et al.*, (1990). PCR reactions were done on a Techne DNA Thermal Cycler TC-512 in 100 µl, containing 50–100 ng of DNA, 50 mMKCl, 10 mMTris-HCl (pH 7.5), 80 µM (each) dNTP, 1 µM of each primer, 2 mM MgCl2 and 1 U of DNA polymerase (Invitrogen, USA). The temperature regime of the 40 cycles was 30 sec. at 95 °C, 1 min at 44 °C and 1 min at 72 °C.

Sequence procedure

PCR products were purified with the UltraClean PCR Clean-up DNA Purification kit (MoBio, USA) and sequenced using the TaqDyeDeoxy™ terminator cycle sequencing kit (Applied Biosystems, UK), according to the manufacturer's instructions in an Applied Biosystems automatic DNA sequencer model 373A.

Phylogenetic analysis

ITS sequences obtained in this study were compared with ITS sequences of other lupine isolates (*C. acutatum*, *C. lupini var. lupini*, *C. lupini var. setosum*), tamarillo (*C. acutatum*) and also with *C. acutatum* isolates from other hosts from all over the world. All ITS sequences including those of *C. fragaria*, *C. gloeosporioides*, and *C. falcatum* were obtained from the GenBank. *Neurospora crassa* was used as outgroup (Chambers *et al*., 1986). The genetic distances were calculated using the Jukes–Cantor model and the phylogenetic inference was obtained by the neighbour-joining (NJ) method (Saitou and Nei, 1987). The NJ tree and the statistical confidence of a particular group of sequences in the tree, were evaluated by bootstrap test (1000 pseudoreplicates) (Hills and Bull, 1993), and sequences were aligned using the program MegAlign version 5.08. Complete ITS1-ITS4 sequences of the isolates are deposited in GenBank (www.ncbi.nlm.nih.gov/genbank).

Agressiveness on alternate host

Differential aggressiveness for five isolates from lupine and five from tamarillo was tested in a cross-inoculation experiment. This study used 1.5-months-old lupine seedlings and 2.0-months-old tamarillo seedlings. Each isolate was evaluated on the host from which it was isolated (host of origin) and on the other host (alternate host). Three susceptible cultivars of lupine (ECU-2658, ECU-7112-1, and ECU-722-4) and two susceptible cultivars of tamarillo (Gigante comun and Comun) were used. Inoculum was prepared by flooding PDA plates, on which the isolate was growing for 10 days at 25 ^{o}C, with 4 ml 0.01% Tween-80 solution and rubbing with a sterile glass rod. The conidial suspensions were diluted to a final concentration of 10^6 conidia per ml saline solution (0.05% NaCl+ 0.01% Tween-80). A hypodermic syringe with a 0.2 mm-diameter and 0.3 mm-depth was used to injure lupine or tamarillo plants at the apical main stem. A 20 µl droplet of conidia (10^6 conidia/ml) was used for inoculation. The inoculated lupine and tamarillo plants, along with appropriate controls, were incubated in a tunnel greenhouse with 100% relative humidity at 20 ± 2 ^{o} C. Lesion size was measured with a ruler parallel to the main stems twenty days after inoculation. Symptoms were compared on both hosts. At the end of the study, pathogen was isolated from lupine or tamarillo stems, in order to verify it was really Colletotrichum (colony and spore characteristics). The significance of the interaction between isolates and hosts was tested with analysis of variance (ANOVA). Each plant-pathogen combination was represented by three plants of lupine for each pot or one plant of tamarillo per pot and replicated three times. One wound was made in each lupine plant and two wounds per tamarillo plant. The mean of three or two lesions (for lupine or tamarillo, respectively) was the basic unit for analysis. The source of variance analyzed were the (inoculated) host (lupine or tamarillo), the original host of the isolate (lupine or tamarillo), and the combination original host x inoculated host.

Results

Culture and morphological characteristics

The colony morphology of all isolates (upper and lower surface) was consistent with published descriptions of *C. acutatum* species (Sutton, 1992). All isolates presented either a white-pink or a gray-pink pigmentation on the back of the colony. Pink-salmon colored

spores is a characteristic of *C. acutatum* in formal descriptions (Sutton, 1992). The growth of the isolates was regular and progressive on PDA and after 10 days at 17 ± 2 ^{o}C at day light, the Petri dish was covered for 1/3 to 2/3 in case of the lupine isolates and almost fully covered in case of tamarillo isolates. During their growth, 3 out of 8 of the lupine and tamarillo isolates formed white to pale aerial mycelium which is cottony and often dense near the centre and the other 5 developed olive-grey mycelium (Figure 2A vs Figure 2B, C and D). Concentric white and dark-pink circles were seen on the mycelium. The tamarillo isolates were growing differently, either with white mycelium slowly turning to a salmon color and without concentric circles (Figure 2E), or with white mycelium slowly turning gray and producing circles around the center of the culture (Figure 2F). In general, the colony color changed in time from white to dark, but conidia were consistently pink-salmon. *C. acutatum* isolates from lupine formed a pink-salmon pigment and tamarillo isolates a pink-yellow pigment on PDA.

Mycelial radial growth showed a high variability at 15, 20 and 30 ^{o}C in the dark (Table 2). Growth rate was better reproduced at 25 ^{o}C. At this temperature, the isolates could be divided in three groups; the slow growing group (Lup4), the fast growing group (Tam6, Tam7 and *C. gloeosporioides* reference isolate), and an intermediate growing group (Lup1, Lup14, Lup16, Lup18, Lup28, and Tam20). Above 25 ^{o}C the growth rate of the fungi reduces.

The shape of the spores varied between lupine isolates at 17 ± 2 ^{o}C. Spores of isolates Lup 1 and Lup 28 were ovoid, with one round and one acute end (Fig 2A), but spores of the others lupine isolates were mostly cylindrical with round ends, that resemble *C. gloeosporioides* (Fig 2B-D). Spores of tamarillo isolate Tam6 had acute ends typical for *C. acutatum* (Fig 2E), Tam7 and Tam20 had one round and one acute end (Fig 2F).

Length and width ranges of the spores of the lupine and tamarillo isolates overlap (Table 3).

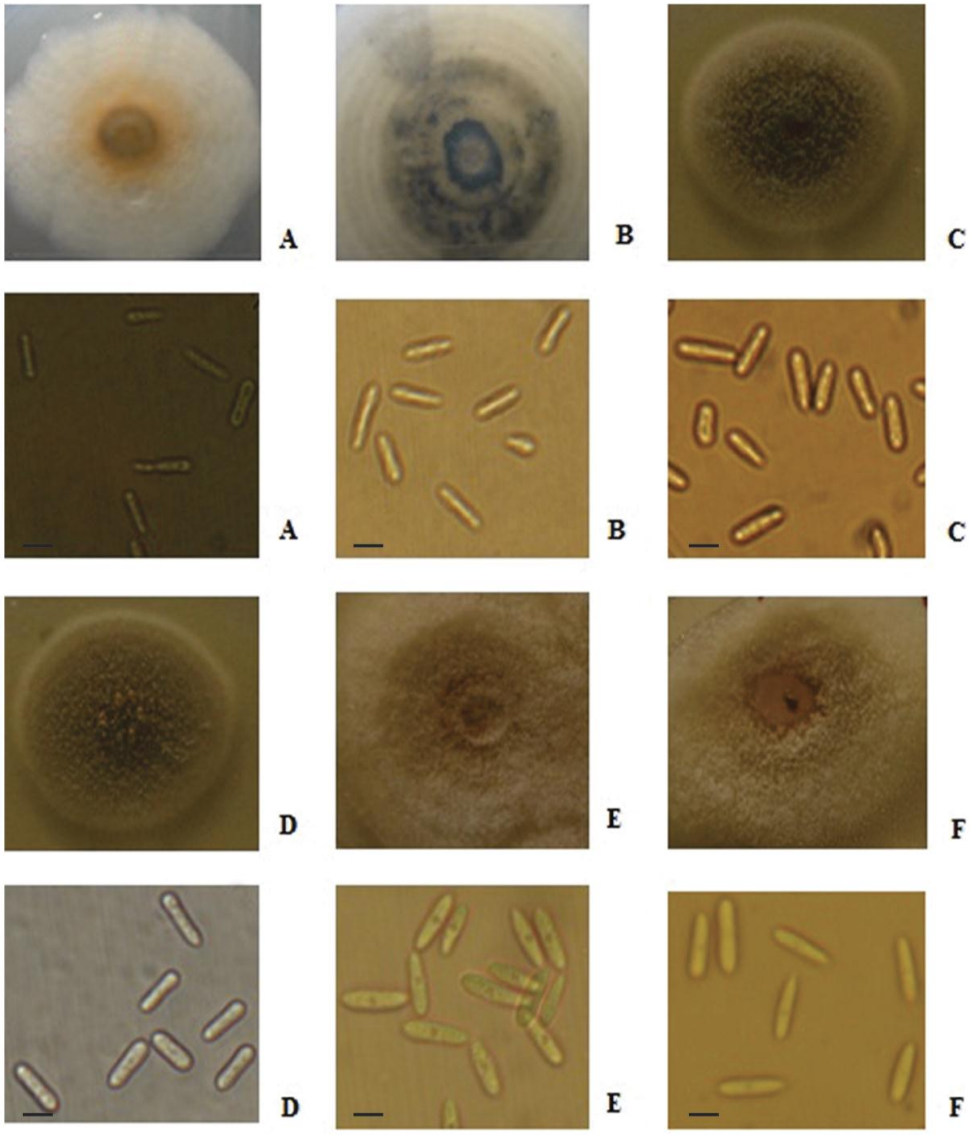

Fig. 2. Colony and conidial morphological variability of *C. acutatum* isolates from lupine (*L. mutabilis*) and tamarillo (*S. betaceum*) grown for 10 days at 17 ± 2 ^{o} C at day light on PDA plates. **A.** Inverse colony surface and spore shape of isolate Lup 1**. B.** Inverse colony surface and spore shape of isolate Lup 14. **C.** Upper colony surface and spore shape of isolate Lup 16. **D.** Upper colony surface and spore shape of isolate Lup 18, and **E.** Upper colony surface and spore shape of isolate Tam 6 and **F.** Upper colony surface and spore shape of isolate Tam 20 (each division line = 10.0 µm).

Table 2. Cumulative myceliar radial growth at four selected temperatures of Colletotrichum isolates from *L. mutabilis* and *S. betaceum* for 5 days on PDA in the dark, relative sensitivity in PDA amended with benomyl, and polymerase chain reaction (PCR) amplification products using primers CaInt2/ITS4 and CgInt/ITS4

Isolate	Radial growth for five days (mm) [a]					Benomyl	Primer reaction [c]	
	15°C	20°C	25°C	30°C	Mean	sensitivity[b]	CaInt2	CgInt
Lup 1	40.1	41.7	48.6	29.2	39.9	+	+	-
Lup 4	45.4	45.4	44.0	24.6	39.9	+	+	-
Lup 14	41.2	36.3	54.4	25.7	39.4	+	+	-
Lup 16	39.4	37.0	59.3	29.7	41.4	+	+	-
Lup18	44.0	44.0	60.6	36.5	46.3	+	+	-
Lup28	44.8	44.3	49.9	18.2	39.3	+	+	-
Tam6	47.7	35.6	64.8	21.0	42.3	+	+	-
Tam7	51.7	48.5	64.6	23.7	47.1	+	+	-
Tam20	45.4	43.5	60.0	21.1	42.5	+	+	-
IMI 356878*	24.5	40.2	64.5	28.5	39.4	-	-	+
Mean	42.4	41.7	57.1	25.8	41.7			

[a] Radial growth of isolates on PDA at selected temperatures in the dark was measured using colonies initiated from 15 µl droplet 1 x 10^6 conidia x ml^{-1}. Each isolate had 3 replications, 4 measurements per replicate, which where daily evaluated for up to 5 days. [b] Benomyl sensitivity of the isolates was assessed by comparing colony radius on PDA and PDA amended with 2 mg $liter^{-1}$ benomyl (Benlate 50WP, DuPont), growth (+) or non-growth (–) mycelial reaction. [c]Taxon-specific primers CaInt2 (*C. acutatum*), and CgInt (*C. gloeosporioides*) were coupled with primer ITS4 for species identification; a positive (+) or negative (–) reaction with fungal DNA of each isolate is designated. * Reference isolate IMI 356878 (AJ536229.1) (*C. gloeosporioides*) from olive (*Olea europaea* subsp. *europaea*) was included.

Benomyl test

The benomyl sensitivity assay indicated differences between *C. acutatum* and *C. gloeosporioides*. Lupine and tamarillo isolates showed myceliar radial growth of 30 to 50% in comparison with the controls. This response was considered a positive benomyl sensitivity response (Table 2). The reference isolate of *C. gloeosporioides* IMI 356878 (AJ536229.1) did not show any myceliar radial growth in PDA amended with benomyl.

Table 3. Conidia type and colony morphology of representative isolates of Colletotrichum spp. from lupine and tamarillo species in Ecuador grown for 10 days at 17 ± 2 ^{o} C in day light on PDA plates.

Isolate	Host	Conidia morphology and size	Colony morphology in PDA
Lup1 Lup28	Lupine	Ovoid, one round and one acute ended spores, measuring from 10.0 to 13.0 by 4.5 to 5.0 μm	Transparent, cottony, and low density colonies with abundant pink-orange spore masses in the center, transparent concentric bands, pink-salmon pigment covers 1/3 of the colony surface, no setae or "hair-like structures" present.
Lup14 Lup16 Lup18	Lupine	Cylindrical, rounded ends spores or one round and one acute ended, measuring from 12.5 to 18.0 by 5.0 to 5.5 μm	Gray-olive, felty and medium dense colonies with pink spore masses, dark concentric bands, sclerotia present, pink-salmon pigment covers 2/3 to 3/3 of the colony surface, setae presence or not.
Tam6	Tamarillo	Elliptic, pointed at both ends. Size is 12.5 to 18.0 by 2.5 to 4.5 μm	White mycelium, turning salmon with age due to proliferation of spore masses; no sclerotia present; setae present, no concentric bands presence, yellow-salmon pigment cover 2/3 of the colony surface.
Tam7 Tam20	Tamarillo	Elliptic and pointed at one end measuring from 14.0 to 18.5 by 4.5 to 6.0 μm	White mycelium turning gray and powdery with pink spore masses, salmon in color, produced outward in circles from the center of the culture, sclerotia present, setae present, concentric circles form the center of the colony, yellow-salmon pigment covers 2/3 to 3/3 of the colony surface.

Species-specific primer analyses

DNA from ten *Colletotrichum* isolates collected from lupine and tamarillo was isolated. A 490-bp DNA fragment was amplified with the *C. acutatum*-specific primers CaInt2 and ITS4 in all lupine and tamarillo isolates. The *C. gloeosporioides*-specific 450-bp DNA fragment was only amplified in the reference isolate *C. gloeosporioides* IMI 356878 (AJ536229.1) (Table 2). These results show that *C. acutatum* is the species that causes lupine and tamarillo anthracnose.

Sequencing of rDNA region

Colletotrichum isolates from lupine (Lup1, Lup12, Lup14, Lup16, Lup18, Lup24, Lup28) and from tamarillo (Tam6, Tam7, Tam20) were used for sequence analysis of the ITS1-2 sequence. The results were compared to published *Colletotrichum* ITS sequences from lupine (Nirenberg *et al.,* 2002, Talhinhas *et al.,* 2002), tamarillo (Afanador-Kafuri *et al.,* 2003), strawberry (Martinez-Culebras *et al.,* 2003), and olive (Talhinhas *et al.,* 2005, Talhinhas *et al.,* 2009) in the EMBL database. Sequences of additional representative isolates of the species *C. gloeosporioides*, *C. fragariae*, and *C. falcatum* were also included (Martinez-Culebras *et al.,* 2003) (Table 4). The phylogenetic analyses of 32 taxa of Colletotrichum were done by applying the neighbour-joining and maximum composite likelihood models (Figure 3). Phylograms were generated to confirm inter-specific separation of *C. acutatum* from lupine and tamarillo with *C. gloeosporioides, C. fragariae*, and *C. falcatum*. Removing highly variable positions from the sequence analysis did not affect tree topology. Sequence analysis confirmed that all lupine and tamarillo isolates were *C. acutatum.*

Phylograms were also generated to determine whether any intra-specific differences among *C. acutatum* from lupine and tamarillo can be revealed using ITS sequences. Most Ecuadorian *C. acutatum* lupine isolates are in one new subgroup (I) in which also Tam6 is placed. Tamarillo isolates (Tam7 and Tam20) belong to another subgroup (III) together with Lup28 and with isolates of tamarillo from Colombia (Afanador-Kafuri *et al.,* 2003). The other subgroups in the phylogenetic tree consisted of isolates from other studies around the world. For instance, one *C. acutatum* isolate from lupine (Talhinhas *et al.,* 2002), one from strawberry (Martinez-Culebras *et al.,* 2003) and two from olive (Talhinhas *et al.,* 2004, 2005) formed subgroup II. Subgroup IV consisted of one *C. acutatum* from lupine (Talhinhas *et al.,* 2002), one from olive (Talhinhas *et al.,* 2004) and four *C. lupini* from lupine (Nirenberg *et al.,* 2002). One isolate *C. acutatum* from primula (Nirenberg *et al.,* 2002) and one *Colletotrichum* sp. from lupine (Talhinhas *et al.,* 2002) were placed within a separate cluster (subgroup V) (Figure 3).

Table 4. Internal transcribed spacer 1 and 2 sequences of Colletotrichum isolates used in this study

Isolate code	EMBL accession	Determination by the authors as	Host	Country
BBA 70343 PD 93/1373	AJ301915	*C. acutatum*[a]	*Primula*	Netherlands PD
BBA 70344 PD 93/1436	AJ301916	*C. lupini var. setosum*[a]	*Lupinus* sp.	Netherlands PD
BBA 700073	AJ301927	*C. lupini var. setosum*[a]	*Lupinus polyphyllus*hyb	Germany
BBA 63879	AJ301930.1	*C. lupini var. lupini*[a]	*Lupinus mutabilis*	Bolivia
BBA 70884	AJ301948	*C. lupini var. lupini*[a]	*Lupinus albus*	Ukraine
	AJ300558	*Colletotrichum* sp.[b]	*Lupinus angustifolius*	Portugal
	AJ300563	*Colletotrichum* sp.[b]	*Lupinus mutabilis*	Portugal
	AJ311391	*C. acutatum*[b]	*Lupinus albus*	Canada
	AJ300558	*C. acutatum*[b]	*Lupinus albus*.	Portugal
	AJ749674	*C. acutatum*[c]	*Olea europaea* subsp. *europaea*	Portugal
	AJ749679	*C. acutatum*[d]	*Olea europaea* subsp. *europaea*	Portugal
	AM991131	*Glomerella acutata*[e]	*Olea europaea* subsp. *europaea*	Portugal
	AM991137	*Glomeralla acutata*[e]	*Olea europaea* subsp. *europaea*	Portugal
Lup 1	JN543059[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Tam 6	JN543069[f]	*C. acutatum*	*Solanum betaceum*	Ecuador
Tam 7	JN543070[f]	*C. acutatum*	*Solanum betaceum*	Ecuador
Lup 12	JN543060[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 14	JN543061[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 16	JN543062[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 18	JN543063[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Tam 20	JN543071[f]	*C. acutatum*	*Solanum betaceum*	Ecuador
Lup 21	JN543064[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 24	JN543065[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 28	JN543066[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 30	JN543067[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
Lup 31	JN543068[f]	*C. acutatum*	*Lupinus mutabilis*	Ecuador
	AF521205	*C. acutatum*[g]	*Solanum betaceum*	Colombia
	AF521210	*C. acutatum*[g]	*Solanum betaceum*	Colombia
345034	AJ536207	*C. acutatum*[h]	*Fragaria x ananassa*	Australia
356878	AJ536229	*C. gloeosporioides*[h]	*Fragaria x ananassa*	Italy
345047	AJ536223	*C. fragariae*[h]	*Fragaria x ananassa*	USA
347765	AJ536231	*C. falcatum*[h]	*Fragaria x ananassa*	Bangladesh

[a] Isolate sequenced by Nirenberg *et al.* 2002 *Mycologia* 94 (2), 307–320
[b] Isolate sequenced by Talhinhas *et al*. 2002 *Phytopathology* 92 (9), 986-996
[c] Isolate sequenced by Talhinhas *et al*. 2005 (EMBL direct submission)
[d] Isolate sequenced by Talhinhas *et al*. 2005 *Appl. Environ. Microbiol.* 71(6), 2987-2998
[e] Isolate sequenced by Talhinhas *et al*. 2009 FEMS *Microbiol. Lett.*296, 31-38
[f] Isolates sequenced by the authors. 2011(EMBL accession number)
[g] Isolate sequenced by Afanador-Kafuri *et al*. 2003 *Phytopathology* 93 (5), 579-587
[h] Isolate sequenced by Martinez-Culebras *et al*. 2003 J. *Phytopathology* 151, 135–143

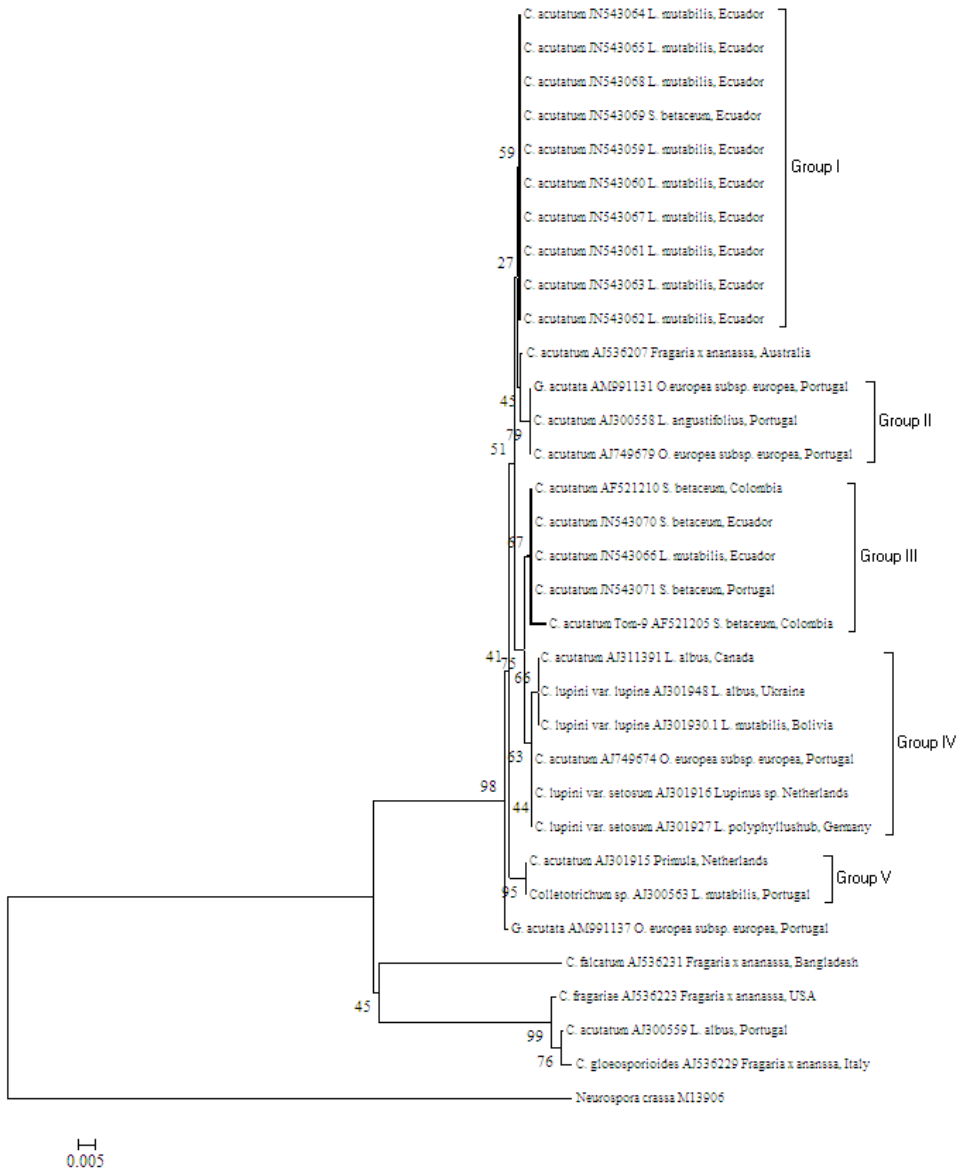

Fig. 3. Neighbor-joining consensus tree depicting relationships among Colletotrichum isolates from *L. mutabilis*, *S. betaceum* and other hosts around the world based on internal transcribed spacer sequences. One thousand bootstrap data sets and Jukes-Cantor method were used. Other isolate sequences are identified with species designations (*C. falcatum*; *C. fragariae*, *C. gloeosporioides*). *Neurospora crassa* was used as outgroup. Database accession number is provided for reference sequences of table 1.

Homology values were calculated according to a data matrix from the sequence divergence (data not shown). Sequence analysis showed homology levels from 99.1 to 100% in subgroup I and from 97.8 to 100% in subgroup III (Afanador-Kafuri *et al.,* 2003). Subgroup II demonstrated also homology levels of 100% among lupine and olive isolates from Portugal (Talhinhas *et al.,* 2004, 2005) and 98.6% homology with one strawberry isolate from Australia. Subgroup IV included two *C. lupini var. lupini* sequences (Bolivia and Ukraine), two *C. lupini var. setosum* (Netherlands and Germany) (Nirenberg *et al.,* 2002), one *C. acutatum* of olive (Portugal) (Talhinhas *et al.,* 2004) and one from lupine (Canada) (Talhinhas *et al.,* 2002) with similarities from 99.1 to 99.8 %. There were 752 positions in the final dataset, of which 63 were parsimony informative. Tree topology was similar for NJ and MP trees with respect to placement of taxa.

Agressiveness on alternate host

Five *C. acutatum* isolates from lupine and five from tamarillo were tested for their aggressiveness on lupine and tamarillo plants. The interaction between the inoculated host and the source of the pathogen (lupine or tamarillo) was highly significant (P<0.0001) for lesion length (Table 5), and was also evident by visual examination of plotted means of lesion length (Fig. 4) after 20 days of inoculation. On lupine stems, isolates from lupine caused the largest lesions, on tamarillo stems, isolates from this host caused larger lesions than isolates from lupine did.

Table 5. Analysis of variance of the lesion length using a general linear model [a]

	Lesion length			
Variable	df	MS	*F*value	*P*value
Inoculated host (I)	1	2.67	0.05	0.8196
Host of origin (H)	1	339.79	6.65	0.0109
I x H	1	1144.69	22.40	<0.0001

[a] Components of variance are the inoculated host (lupine or tamarillo) in this assay, host of origin (lupine or tamarillo) from which isolates were collected, and the combination inoculated host per original host of the isolates; df = degree of freedom, MS = mean square.

Symptoms expression on lupine was different for the two populations of *C. acutatum* studied here. Isolates from lupine were highly biotrophic in lupine, producing little or no necrosis on lupine stems after twenty days following infection. Abundant salmon-colour

sporulation and intense production of mycelium were also observed 7 days after inoculation. In contrast, isolates from tamarillo sporulated less abundantly on tamarillo and induced dark pigmentation in the lesions, sporulation was seen 15 days after inoculation. In the host cross test, isolates of tamarillo were also biotropic on lupine stems producing little necrosis with sporulation after 7 days, but isolates of lupine produced dark pigmentations with almost no sporulation in tamarillo stems.

Table 6. Diameter (mm) of lesions on three lupine cultivars and two tamarillo cultivars caused by isolates of *C. acutaum*

	Lupine cultivars				Tamarillo cultivars		
Source isolate	ECU-2658	ECU-7112-2	ECU-722-4	Mean	Gigante comun	Comun	Mean
Tamarillo							
Tam 6	13.33	18.66	16.00	16.00	22.00	18.33	20.17
Tam 7	16.00	16.00	16.00	16.00	22.00	14.66	18.33
Tam 20	6.00	8.00	13.33	9.11	22.00	22.00	22.00
Tam 33	16.00	5.33	21.33	14.22	22.00	14.66	18.33
Tam 34	18.66	10.66	10.66	13.33	18.33	11.00	14.67
Mean	14.00	11.73	15.46	13.73	21.27	16.13	18.70
Lupine							
Lup 1	24.00	21.33	21.33	22.22	22.00	22.00	22.00
Lup 4	24.00	24.00	24.00	24.00	22.00	22.00	22.00
Lup 14	18.66	13.33	24.00	18.66	22.00	14.66	18.33
Lup 18	24.00	21.33	24.00	23.11	14.66	0.00	7.33
Lup 28	21.33	24.00	24.00	23.11	18.33	3.66	11.00
Mean	22.40	20.80	23.47	22.22	19.80	12.46	16.13

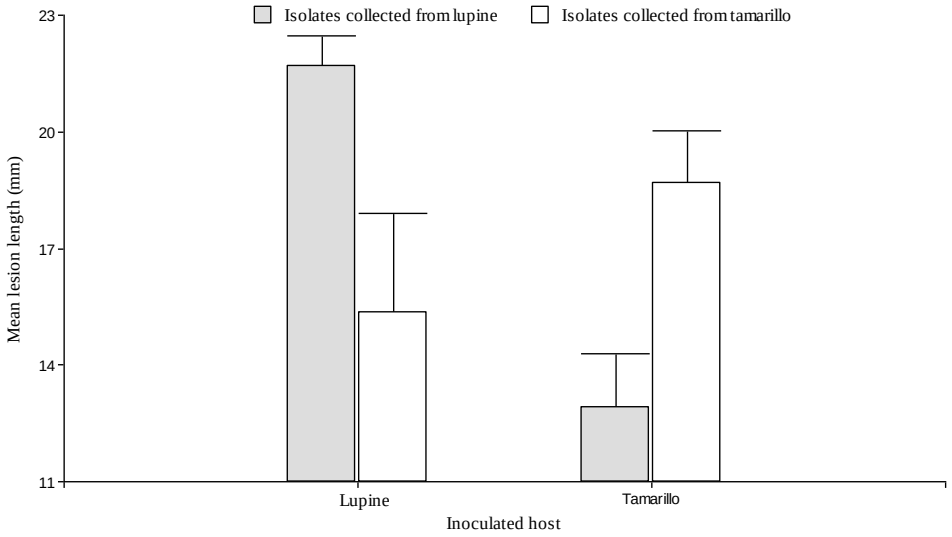

Fig. 4. Histogram of an aggressiveness test based on average lesion length on lupine or on tamarillo main stems caused by isolates of *C. acutatum* collected from lupine and tamarillo. Error bars indicate ± standard error of the means.

Discussion

Colony color can be considered a preliminary differentiation of *Colletotrichum* species. All isolates examined were typical *C. acutatum.* Some Colletotrichum lupine isolates produced white mycelium with salmon-pink conidia and white pale concentric rings (Figure 2A) and others grow in concentric rings with light to brown gray aerial mycelium (Figure 2BCD). Based on these morphological features Nirenberg *et al.,* (2002) named the first type *C. lupini var. lupini* and the second type *C. lupini var*. *setosum.* Tamarillo isolates produce white mycelium turning salmon with age due to proliferation of spore masses (Figure 2 EF).

Growth rate and pigment can also be considered as parameters to differentiate isolates of lupine and tamarillo. *C. acutatum* lupine isolates grow slower than tamarillo isolates and produce a pink-salmon pigment (tamarillo isolates produce a pink-yellow pigment). Based on the colony growth rate of lupine isolates at 25 ^{o} C, Talhinhas *et al.*, (2002) divided *C. acutatum* isolates of lupine in only two groups (slow and fast); isolates from other species

such as *C. gloeosporioides*, *C. falcatum*, and *C. graminicola* were all fast growing. Nirenberg *et al.*, (2002) described that *C. lupini var. lupini* grows on PDA slower than *C. lupini var. setosum.*

Colletotrichum isolates in this study were notoriously unstable when cultured at room temperature in day light. The colony color can change in time (Du *et al.*, 2005). Mycelial radial growth was comparatively reduced and conidia were consistently not well swollen when *C. acutatum* isolates where grown at 30 ^{o}C. Other studies indicate that fungal growth was observed at temperatures from 10 to 30 ^{o} C with maximum growth rate occurring at 25 ^{o} C (Nirenberg *et al.*, 2002; Thomas *et al.*, 2008). It shows that *C. acutatum* is adapted to higher altitudes with lower temperatures.

The morphology from conidia can be used as a preliminary differentiation of *C. acutatum* isolates, but not spore size because there is too much variation. The basic shape of conidia produced by lupine Colletotrichum species was ovoid with either one rounded and one pointed end (Figure 2A) or with a cylindrical shape (Figure 2 B-D). Cylindrical spores have often been associated with *C. gloeosporioides* (Sutton, 1992) and this might be the reason that others have seen *C. gloeosporioides* as the causal agent of anthracnose in lupine. For instance, cylindrical formed spores might were the reason for Yang and Sweetingham (1998) and Elmer *et al.*, (2001) to assume that *C. gloeosporioides* was the causal agent of lupine anthracnose and not *C. acutatum*, although molecular analysis with Random Amplified Polymorphic DNA (RAPDs) isolates showed low similarity with *C. gloeosporioides.* Other authors mention that *C. fragariae*, with conidia pointed only at one end, is the only species that is very similar in morphology to *C. lupini*, especially to *var. setosum* described by Nirenberg *et al.* (2002). In our study we found cylindrical, pointed at one end or ovoid spores of *C. acutatum* (Fig. 2 A-D). Spores of tamarillo isolates had either acute ends typical for *C. acutatum* or one round and one acute end (Fig 2E-F).

Morphology of conidia can be affected by differences in artificial conditions but direct identification of isolates coming from the plant is also difficult because conidia morphology varies during the infection process. In *C. acutatum* some conidia deviate in development. For instance, some conidia can produce secondary conidia directly without producing a germ-tube first. If conidia produce a germ-tube it grows along the plant surface until it come into contact with other *C. acutatum* hyphae or conidia (Wharton and Dieguez-

Uribeondo, 2004). The use of morphological features to distinguish *Colletotrichum* taxa is tricky due to the lack of standardized protocols and international rules. In this study, we have shown that spore form, colony appearance, colour of pigment in the media after 10 days of incubation at 17 ± 2 ^{o} C and radial mycelial growth rate can be used for a preliminary characterization of *Colletotrichum* isolates. However, taxonomic relationships within the genus are unlikely to be resolved only by the use of traditional morphological characters (Sutton, 1992) and the significance of morphological characters as indicators of evolutionary relationships is unknown (Cannon *et al.,* 2000).

C. acutatum can also be distinguished based on the ability to grow on PDA with 2 mg/l benomyl, *C. gloeosporioides* like reference isolate IMI 356878 (AJ536229.1) doesn't grow on this medium (Table 2). It is critical to know the pathogen species in order to develop effective disease management. Moreover, differential sensitivity of mixed Colletotrichum populations to fungicides, such as benomyl can pose problems in disease control, as well as to shifts in pathogen populations (Freeman *et al*., 2000).

The *C. acutatum* and *C. gloeosporioides*-specific primers used here reliably differentiate isolates from lupine and tamarillo to the species level (Table 2). These primers have been utilized in numerous studies. By using this approach, it was shown that *C. acutatum* is the causal agent of lupine in Portugal (Talhinhas *et al.,* 2002), tamarillo anthracnose in Colombia (Afanador-Kafuri *et al.,* 2003), almond anthracnose in California (Förster and Adaskaveg, 1999), and that both *C. acutatum* and *C. gloeosporioides* are responsible for anthracnose of olive in Portugal (Talhinhas *et al.,* 2005). In this study, the use of specific primers determined that *C. acutatum* is the causal agent of lupine and tamarillo anthracnose in the Ecuadorian Andean zone.

ITS sequence analysis is reliable for phylogeny and systematics of *Colletotrichum* spp. (Freeman *et al.,* 2000a, Sreenivasaprasad *et al.,* 1996b). We targeted the complete internal transcribed spacer ITS (ITS 1–5.8S-ITS 2) because of the high degree of variation that can be found even between closely related species (Freeman *et. al.,* 2001; Sreenivasaprasad *et al.,* 1996a). In previous studies already many ITS regions of *Colletotrichum* species have been sequenced and these sequences are publicly available (Talhinhas *et al.,* 2002, 2005, 2009, Martinez-Culebras *et al.,* 2003, Afanador-Kafuri *et al.,* 2003). We added to these data a representative set of isolates originating from four Andean provinces of Ecuador

(Table 4). Phylogenetic analyses of the ITS sequences, divided the *C. acutatum* lupine and tamarillo populations from Ecuador into two subgroups and most (one exception) lupine isolates formed one new subgroup (Figure 3). Earlier studies showed that the pathogen population within a geographical location was very homogeneous and nearly clonal (Martínez-Culebras *et al.*, 2002, 2003; Muñoz *et al.*, 2000). This is also found for *C. acutatum* populations on strawberry on a number of locations in the USA (Ureña-Padilla *et al.*, 2002) and on several locations in Israel (Freeman and Katan, 1997). In this study, the homogenous new group of lupine isolates included surprisingly one from tamarillo (Tam6). This kind of observations has been described earlier. Talhinhas *et al.*, (2002) used ITS sequencing to determine one homogeneous subgroup of lupine anthracnose in Portugal which included one isolate from *Cinnamon*, and another heterogeneous subgroup with lupine isolates from the Azores islands found on strawberries and olives. Further, tubuline (*tub*2) and histone (*his*4) sequences revealed groups concordant with ITS (Talhinhas *et al.*, 2002). Lardner *et al.*, (1999) demonstrated two genetically distinct groups within *C. acutatum* capable of causing stem and leaf blights of lupine or pine. Although here it was hypothesized that the isolates of *C. acutatum* capable of causing terminal crook disease of pine were those actually isolated from lesions on lupine plants growing amongst pine seedlings. In this study, isolate Tam6 was collected in San Pablo, Province of Imbabura, 150 km north from Quito. In that place lupine and tamarillo are being cultivated together for about 10 years and the isolate might have been an occasional lupine isolate that cross infected a particular field of tamarillo's. Studies demonstrated that *Glomerella acutata*, the sexual stage of *C. acutatum*, can change and adapt to new and diverse hosts (Guerber *et al*., 2003).

The other subgroup grouped tamarillo sequences from Ecuador and Colombia and it included one sequence of lupine (Lup28) (Figure 3). Based on sequence analysis of the ITS2 region Afanador-Kafuri *et al.*, (2003) found two subgroups of *C. acutatum* isolates from tamarillo and that in one of these clades two isolates of lupine early described as *C. lupini* and *C. lupini var. setosum* were included (Nierenberg *et al.*, 2002). Afanador-Kafuri *et al.*, (2003) suggested that isolates in this population may not be host specific. In this study, isolate Lup28 collected in Palmira, a desert place in the Province of Chimborazo was pathogenic on tamarillo. Episodic selection could have occurred where asexual populations

of *C. acutatum* may lead to new host-specific forms. Brasier (1995) states that sudden environmental disturbances, as climate change, are likely to lead to a significant alteration in the population structure of certain fungal species and even to emerging highly virulent isolates.

We conducted a host range test and our data clearly show that lupine and tamarillo are attacked by two separate, host-adapted populations of *C. acutatum* (Fig. 4), in which two isolates from the other host are embedded in the two main populations (Fig. 3). Lupine and tamarillo populations of *C. acutatum* could be distinguished based on morphological characteristics of the isolates, ITS sequence, differential lesion development on lupine and tamarillo, and symptoms on both hosts. Based on symptoms and lesion size, it is concluded that host adaptation is quantitative rather than qualitative, because isolates were more aggressive on their original host, but also pathogenic on their alternative host. This is similar to situations where different clonal lineages populations of *P. infestans* associated with potato and tomato in Ecuador were most aggressive on its primary host, but could cause lesions on the alternative host (Oyarzun *et al*., 1998), or *P. infestans* isolates collected in tomato and potato in Uganda and Kenya which infected and sporulated in both hosts (Vega-Sanchez *et al*., 2000). *C. acutatum* isolates have been found in association with lupine and other hosts (Talhinhas *et al*., 2002; Afanador-Kafuri *et al*., 2003) in other parts of the world. This species is considered as the "group species" *C. acutatum sesu lato* (broad sense) by Lardner *et al*. (1999) because of its capacity to cause fruit rots, and infected lupine, and pine. It indicates the considerable diverse aggressiveness of *C. acutatum.* Isolates of *C. acutatum* with diverse aggressiveness could be particularly destructive in developing countries where both lupine and tamarillo are grown year round in the same geographic regions.

In the present study, host adaptation appears to be manifested based on lesion size and sporulation in the alternative host (Table 6). The lupine-adapted isolates caused darkly pigmented lesions on tamarillo stems which may be classified as a partially necrotrophic reaction. The lesions that tamarillo-adapted isolates caused in lupine stems were typical of a biotropic reaction. Isolates from tamarillo produced larger lesions in lupine than isolates of lupine in tamarillo (Fig. 4). The infection strategy adopted by *C. acutatum* depends on the host being colonized and this fungus may also change its infection strategy when colonizing

different hosts and cultivars (Wharton and Dieguez-Uribeondo, 2004). We found early and more sporulation of *C. acutatum* in lupine than in tamarillo. It may be given by a pathogen toxic compound of tamarillo that delays infection. Loss of the product may lead to tamarillo adaptation, but apparently at a cost to the pathogen, since all the lupine-adapted isolates tested were less aggressive on tamarillo, in conjunction with smaller lesions and delayed sporulation. Although *C. acutatum* isolates from each host showed less aggressiveness on the alternate host (Fig. 3), isolate Tam 34 is weakly pathogenic isolate in tamarillo, adapted to some lupine cultivars, such as ECU-2658 (Table 6). Adaptation to a new host is not always associated with reduced fitness in the original host (Lebreton *et al*., 1999) and until more components of aggressiveness (infection efficiency and sporulation in the field, among others) are determined, anthracnose integrated management efforts for both lupine and tamarillo should be coordinated.

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CHAPTER 4

Anthracnose susceptibility of lupine (*L. mutabilis*) cultivars in the Province of Cotopaxi – Ecuador

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Summary

Lupinus mutabilis is a legume native to the Andean region of South America with a high nutritional value. Anthracnose, caused by *Colletotrichum acutatum* is the main lupine disease around the world and it is prominently present in the production regions of Ecuador. From 120 genotypes of *L. mutabilis* in the lupine collection in Ecuador, five were selected by the Agropecuarian National Research Autonomous Institution (INIAP) based on their good agronomical traits. Five cultivars were tested by INIAP and from them selections were made under circumstances in the Ecuadorian highland. These circumstances were different from the circumstances during the wet season in the Cotopaxi province in which the test in our study was done. In one trial under the same conditions, five selections, one cultivar and two landraces were evaluated. Anthracnose symptoms were scored every two weeks from seedling until harvest. Anthracnose's typical twisting of the main axis severely affected yield and percentage of non-commercial seed. Severity was quantified on a scale from 1 to 6 from day 48 until day 178 after planting. The area under disease progress curve (AUDPC) of all cultivars was high. Data of this study indicate that selected cultivars used by INIAP have a low level of anthracnose tolerance under high disease pressure. Seed samples of naturally infected plants were used to determine the level of anthracnose transmission via seed. Based on data collected in this study recommendations are made to reduce the impact of the disease in the field and for the appropriate local management of lupine seed.

Introduction

Anthracnose is considered one of the most destructive diseases in lupine (*L. mutabilis*) due to the rapid spread of the disease. *C. acutatum* is the fungal pathogen responsible for lupine anthracnose (Talhinhas *et al*., 2002; this thesis). The disease occurs worldwide, but is more commonly observed in tropical or subtropical environments where frequent rainfall, high relative humidity and warm temperatures enhance the development and spread of the disease (Thomas and Sweetingham, 2004).

Yield is severely reduced due to anthracnose infection in the adult plant and seeds (Sweetingham *et al*., 1998). The pathogen affects the central axis and the plant does not form new pods or produces infected pods (Thomas, 2003). In Ecuador, anthracnose

symptoms become visible when the plants start to blossom (Peralta *Pers. Comm*). Infection can happen at every stage of plant development and spores are spreading by rain-splashes (Sweetingham *et al*., 1998). Typical symptoms are: bending of main axis, circular or elongated lesions on stems and pods and infected seeds (Thomas, 2003). During sporulation of the fungus, acervuli (asexual fruiting bodies) will appear as black spots in the center of the lesions. Lupine resistant cultivars have small lesions and no sporulation.

The National lupine collection of DENAREF – INIAP is a valuable resource to find new sources of anthracnose resistance. The collection maintains 529 lupine accessions collected in Ecuador and other countries. About 120 accessions belong to the species *L. mutabilis* (Rivera *et al*., 1998). Researchers of the Legume and Andean Grain National Program (PRONALEG-GA / INIAP) have been cultivating lupine (*L. mutabilis*) accessions for more than 10 years. Based on earliness, seed color and plant architecture a specific lupine genotype (I-450 ANDINO) was selected to be the most suitable for Ecuadorian conditions (Caicedo *et al*., 1999). Later, INIAP choose other samples from the lupine collection to study specific traits and made preliminary selections under different environmental conditions in the Ecuadorian highland. The five most suitable selections were used for further cultivar development (Peralta *et al*., 2003). The yield of some of these cultivars was lower than that of I-450 ANDINO (Peralta *et al*., 2003), but still INIAP failed to produce enough seeds of the selected cultivars for extensive trials mainly due to the very variable yield from one year to another (Peralta *et al*., 2006b). Anthracnose is more prevalent during the wet season. In Australia, differences in environmental conditions during the rainy season from year to year cause variation in lupine yield. But also under favorable conditions significant yield losses due to anthracnose can occur (Thomas and Sweetingham, 2004). A better understanding of the disease development can contribute to a more appropriate management.

Anthracnose is a seed-born pathogen and when poor quality, infected seed is used for planting this will often result in infected plants. Infected seeds are usually smaller, wrinkled and/or decolorized (Thomas, 2003). In Ecuador, seed is collected by local farmers themselves. Unfortunately they prefer to sell the commercial seed and use the non-commercial seed (and seeds with small red-brown stains) for sowing. In this study, the presence of anthracnose was quantified in harvested lupine seed. The ability to produce a

high percentage commercial seed on highly infected lupine plants is a favorable trait and might be genetically controlled.

Anthracnose assessment of lupine cultivars and level of infection on seeds of infected plants will make it possible to compare the resistance level of selected lupine cultivars and local landraces under natural conditions in a field known to have a high disease pressure.

Materials and methods

Location

The field experiments were conducted at the Simón Rodríguez Agriculture Technological Institute, located in the Province of Cotopaxi, Latacunga City, Alaquez Parish (latitude 00º 52' 01" S, longitude 78º 37' 07" W, altitude 2859 meter above sea level (masl). This is the central region of Ecuador, 91 km from Quito, the Capital of Ecuador.

Field plots and lupine genotypes

The six selected lupine cultivars (including I-450 ANDINO) plus two landraces were evaluated in one trial under field conditions from January to August 2008 in the Province of Cotopaxi. Seeds of the lupine cultivars and of two landraces were provided by INIAP. I-450 ANDINO was included as control. The fields used in this study were already in use for lupine cultivation during the previous years. The lupine cultivars were planted in a Randomized Complete Block Design with four replications. There were 32 experimental plots (5.0 x 4.0 meter) with five furrows each. The distance among furrows or rows was one meter. In the rows holes were made at a distance of 0.25 m and three seeds were sown in each hole. At the borders, around the field, I-450 ANDINO was sown both as a physical barrier and to obtain seeds for further studies. Management of the experiment was according to PRONALEG-GA, INIAP technical recommendations (Peralta *et al*., 2008). At sowing time, the insecticide Endosulfan 4 ml/l water, was poured in the furrow for pest control. Plots were fertilized with the equivalent of 175 kg/ha (N-P_2O_5-K_2O: 10-30-10). During the growing season, plots were weeded after 35 days with local hoeing instruments. Fifty-seven days after planting the base of the plant was covered with surrounding soil. Other pests such as *Agrotis ypsilon* appeared 60 days after planting, they were controlled with Endosulfan 4 ml/l water. Other diseases affecting aerial lupine plant parts were

Uromyces sp., *Ascochyta* sp., *Ovularia* sp. and *Sclerotinia* sp. Against these fungi no chemical control was used.

Evaluation of anthracnose tolerance under natural pressure

The six selected lupine cultivars and two landraces were evaluated under natural conditions and this was repeated every two weeks, from 48 to 178 days after planting. The severity of the disease was based on a 1-6 scale (Figure 1). The Area Under Disease Progress Curve (AUDPC) was calculated based on severity values at the observation days after planting (DAP). Disease progress curves were constructed for each entry by using the disease severity reading. On the basis of the severity measurements, the AUDPC was calculated according to the equation of Campbell and Madden (1990):

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{k+1}}{2} \right) \left(t_{i+1} - t_k \right)$$

where*n* is the number of evaluations, *y* the severity and *t* the number of days after sowing. (*t*; *y*) = (*0*, *0*) is included as the first evaluation. ANOVA test was performed with AUDPC data and least standard deviation test (5%) to compare the means of treatments.

The monthly mean of precipitation and average temperature was provided by the airport weather station of Latacunga, located 500 m from the site of the experiment.

Yield and non-commercial seed

Yield was based on the weight of seed (g x experimental unit^{-1}) and this value was extrapolated to kg x ha^{-1}. The percentage of non-commercial seed was calculated after weighing damaged seed and total seed. An ANOVA test was conducted with yield and non-commercial seed scores. Least significant differences at 5% probability level were performed to compare means of genotypes.

Quantification of anthracnose on three different categories of lupine seeds

A laboratory experiment was set up in the Province of Pichincha, Rumiñahui County, San Fernando Parish, at the Biological Control and Phytopathology laboratory of the Life

Science Department, Agropecuarian Science Faculty of the Army Polytechnic School (ESPE), Ecuador.

Seeds harvested from the six selected lupine cultivars, which were naturally infected, were divided in three groups: 1) Good quality seed (for commercial purpose suitable); 2) seeds with small red-brown stains (confirmed here as anthracnose infected), and 3) bad seed (non- commercial). Chloramphenicol (Chloromycetin) Parke Davis Co. (500 mg/l) was added to potato dextrose agar (PDA) Difco media. Seeds were surface disinfected with 0.5% NaClO for 5 min and washed with water. Random samples of 36 seeds from the three groups, of each of the six selected lupine cultivars, were aseptically sown on the surface of PDA (9 seeds per Petri dish). Seven to eleven days after sowing, the presence of mycelium and spores was evaluated visually and microscopically. Two Petri dishes 14.5 cm diameter x 2 cm high were the experimental unit which was replicated two times in a Completely Randomized Design. Analysis of variance with anthracnose infection scores were done to determine statistical differences among cultivars, category of seed, and the interaction cultivars x category of seed. Least significant differences at 5% probability level were calculated to compare means of genotypes and interactions.

All analysis was conducted by using INFOSTAT software www.infostat.com.arg.

Results

Evaluation of anthracnose tolerance

The levels of anthracnose severity are shown in table 1. All six selected lupine cultivars were susceptible to *C. acutatum*. No disease symptoms were noticed during germination. The first wrinkled leaves appeared 48 DAP and the characteristic bending over of the central apical stem was notorious between 76 and 108 DAP, this coincides with the flowering period. Lesions up to 5 mm on stems and lateral branches were seen, with little sporulation. This dramatically increased just before or at the moment of pod filling (121 DAP). Most plants showed large lesions on stems and branches with necrotic tissue and only few plants were free of lesions. This indicates that *C. acutatum* conidia might splash from initially infected seedlings to the neighboring plants by wind or rain.

Significant differences in the level of tolerance were seen until 108 DAP. All six cultivars were significantly ($P > 0.05$) more tolerant than the two lupine Andean control landraces.

ECU-2658 was significantly (P<0.05) different from ECU-8415 and the two landraces at 121 DAP. ECU-2658 and I-450 ANDINO were significantly (P<0.05) different from the two landraces at 134 DAP. I-450 ANDINO was significantly (P<0.05) more tolerant than ECU-2658, ECU-2700-2 and the two landraces at 178 DAP (Table 1).

Table 1. Anthracnose severity and Area Under Disease Progress Curve (AUDPC) of five selected lupine cultivars, the I-450 ANDINO cultivar and two Andean lupine landraces under natural pathogen pressure at Simon Rodriguez Agronomic Institute, Alaquez, Cotopaxi, Ecuador, 2008.

	Anthracnose severity at the stated days after planting (DAP) [a]							
Lupine cultivars	48	59	76	108	121	134	178	AUDPC[b]
I-450 ANDINO	1.4 a	1.5 a	1.5 a	2.1 a	3.4 ab	3.8 a	5.2 a	432 a
ECU-712-1	1.3 a	1.4 a	1.4 a	2.6 a	3.4 ab	3.9 ab	5.5 ab	447 a
ECU-2658	1.3 a	1.4 a	1.5 a	2.3 a	3.1 a	3.8 a	5.5 b	437 a
ECU-2700-2	1.2 a	1.3 a	1.3 a	2.2 a	3.2 ab	4.0 ab	5.6 b	439 a
ECU-722-4	1.3 a	1.4 a	1.5 a	2.4 a	3.6 ab	4.1 ab	5.4 ab	455 a
ECU-8415	1.2 a	1.3 a	1.4 a	2.6 a	3.6 bc	4.1 ab	5.5 ab	454 a
ECU-740[c]	2.2 b	2.4 b	2.5 b	3.9 b	4.4 d	4.6 c	6.0 c	569 b
ECU-2698[c]	2.3 b	2.5 b	2.8 b	4.0 b	4.1 cd	4.3 bc	6.0 c	576 b
Mean	1.6	1.7	1.8	2.8	3.6	4.1	5.59	476
CV (%)	18.6	20.1	23.6	15.6	8.9	7.2	3.6	6.0
LSD (P<0.05)	0.42	0.48	0.60	0.63	0.47	0.43	0.29	67.53

[a]In a column, values followed by the same letter are not significantly different at P < 0.05 according to a least standard deviation test. Each value is the mean of 10 plants replicated four times in a randomized completely block design.[b]Anthracnose severity calculated as the cumulative disease progress and represented as the AUDPC using the formula of Campbell and Madden (1990). [c] Two Andean lupine landraces.

Monthly rainfall and average temperature from January to August 2008 and the eight year-average for the same period at Latacunga – Ecuador are shown in Table 2. The high rainfall and mild temperature from March to May of 2008 in the period 48 until 121 DAP (Table 2) favored anthracnose development. Environmental conditions such as high rainfall, humidity, and number of rainy days favor the establishment and increase severity of anthracnose in lupine (Thomas, 2003).

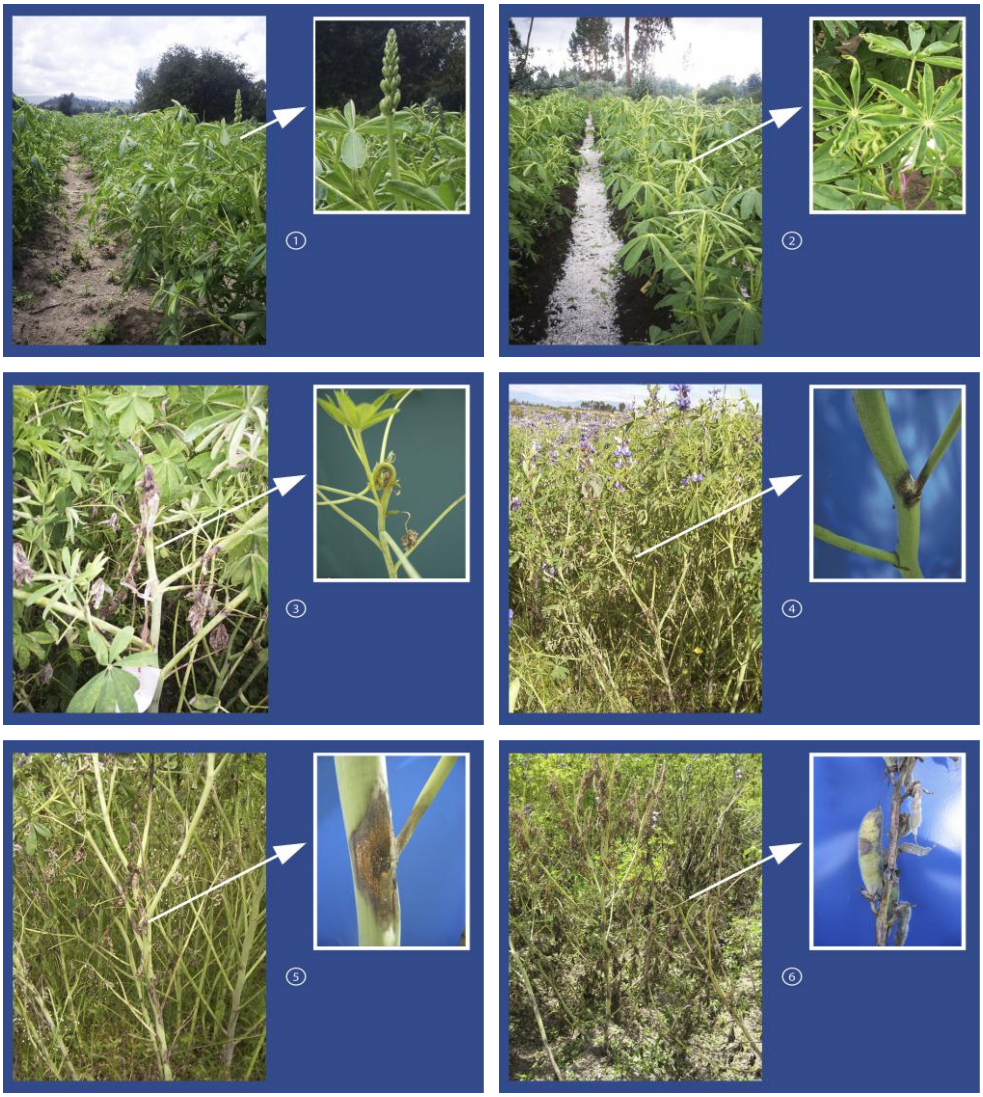

Figure 1. Scale for evaluating anthracnose severity on lupine

1 = plant without any injury
2 = lesions very small (less than 5 mm) on leaves or at the central apical stem, some wrinkles on the leaves, sporulation absent
3 = central apical stem doubled due to infection, abundant wrinkles on the leaves, injures of 0.5 cm to 1 cm, little sporulation
4 = presence of lesions of medium size (from 1 cm up to 3 cm) on stems and branches, accompanied by necrotic tissue (sporulation)
5 = presence of large lesions (more than 3 mm) on stems, branches or pods with necrotic tissue accompanied by collapse of tissues (abundant sporulation)
6 = severely affected necrotic plant, or dead plant. If formed, small pods, necrotic tissue salmon color sporulation

1-2 incompatible: resistant reaction, 3 to 6 compatible: susceptible reaction. In chapter 5, figure 1 shows a visual description.
Note: The scale was used to follow up anthracnose severity every 2-3 weeks from 48 to 178 days after planting (DAP).

Table 2. Monthly rainfall and average temperature during the period of study compared with the historical eight year - averages in Latacunga, Ecuador.

Month	2008	8-yr average	Difference	Month	2008	8-yr average	Deviation
Rainfall (mm)				*Averagetemperature (^{o}C)*			
January	51	37.9	13.1	January	14.3	14.5	-0.2
February	72.7	79.1	-6.4	February	13.9	14.5	-0.6
March	84.3	69.9	14.4	March	13.5	14.1	-0.6
April	83.1	85.0	-1.9	April	13.6	14.2	-0.6
May	79.9	45.0	34.9	May	13.5	14.1	-0.6
June	48.4	25.1	23.3	June	13.3	13.1	0.2
July	15.1	12.3	2.8	July	12.7	13.3	-0.6
August	40.4	9.3	31.1	August	12.7	13.1	-0.4
Average	59.4	45.4	13.9	Average	13.4	13.9	-0.5
Total	474.9	363.6	111.3				

[a]Source: Latacunga airport weather station (DAC 2008)

Yield and non-commercial seed

There were differences in yield and the average yield was 124 kg per hectare. That is below the national average of 200 kg/ha (Censo Nacional Agropecuario, 2003) and also lower as in previous studies with the same cultivars (Peralta *et al*., 2003, 2004). ECU-722-4 and ECU-2700-2 cultivars yielded more than I-450 ANDINO which was supposed to be adapted to the Province of Cotopaxi conditions (Table 3).

There were significant differences among cultivars for percentage of non-commercial seed ($F=4.25$, $p=0.0035$). On average 45 percent of the harvested seed was non-commercial. The cultivar ECU-2698 has significantly ($P>0.05$) more non-commercial seed compared to the other cultivars. ECU-2698 yielded less than the other cultivars (Table 3). In general these studies show the high anthracnose susceptibility of the selected lupine cultivars.

Anthracnose presence on lupine seeds coming from infected plants

There were significant differences between genotypes ($F=5.85$, $p=0.0005$) for the percentage of infected seeds in the three different categories ($F=249.03$, $p<0.0001$) and genotype x category of seed ($F=2.81$, $p=0.0127$). The percentage of anthracnose in commercial seed of ECU-2698 was significantly ($P<0.05$) higher than in most of the other cultivars (Table 3). Identical means found for anthracnose seed infection among batches of seeds are due to the rather small seed-lot size used. The data show also that phenotypic

healthy, commercial seed still can be infected. The I-450 ANDINO cultivar has been used by INIAP for study its adaptation to different environmental conditions (Peralta *et al*., 2003, 2004, 2006b).

Table 3. Yield, non-commercial seed, and anthracnose infection in three categories of seeds from field plots naturally infected by *C. acutatum*[a]

Lupine genotypes	Yield (Kg/ha)	Non-commercial seed (%)	Anthracnose infection (%) [b]		
			Commercial seed	Small red-brown stains on seed	Non-commercial seed
ECU-722-4	149 ± 32	40.3 a	0.0 a	5.5 a	38.8 a
ECU-712-1	131 ± 34	47.0 ab	0.0 a	5.5 a	50.0 ab
ECU-2658	127 ± 23	38.2 a	0.0 a	19.4 ab	50.0 ab
I-450 ANDINO	130 ± 24	42.4 ab	5.5 ab	5.5 a	69.4 bc
ECU-2700-2	142 ± 20	37.9 a	5.5 ab	11.1 ab	77.7 c
ECU-2698	100 ± 1	61.6 c	13.1 b	11.1 ab	80.1 c
ECU-740	104 ± 4	52.6 bc	0.0 a	27.8 b	81.8 c
ECU-8415	110 ± 24	42.7 ab	0.0 a	27.8 b	83.3 c
Mean	124	45.4	3.0	14.2	66.4
CV (%)	37.2	17.5	133.9	67.6	15.9
LSD *(P<0.05)*	67.32	11.56	9.33	22.20	24.34

[a] Values followed by the same letter are not significantly different using least significant difference (P< 0.05), mean ± standard error. [b]Seeds harvested from the selected lupine cultivars and landraces were divided in three groups. Random samples of 36 seeds from each group were aseptically sown on the surface of PDA (9 seeds per Petri dish) and presence of mycelium and spores was evaluated visually and microscopically.

Discussion

Our data allow us to understand lupine anthracnose development. After infection symptoms become visible as wrinkles in the first true leaves (48 DAP) and become more evident before flowering and pod filling starts (76 DAP resp. 121 DAP). Some differences in tolerance were seen (Table 1), the selected lupine cultivars are a bit more tolerant to anthracnose than the two local landraces. ECU-2658, for instance, showed less severity than ECU-8415 at 121 DAP and I-450 ANDINO had less severity than ECU-2658, ECU-2700-2 and the two landraces at 178 DAP (Table 1). In other studies we determined that 2.5 months-old lupine plants are more anthracnose susceptible than 2.0, 1.5, and 1.0-month-old plants (Chapter 5). Our field data shows the critical points in lupine anthracnose

development. *C. acutatum* conidia spread from initially infected seedlings to neighboring plants by wind or rain. The typical twisting of the main stem, presence of salmon colored spores, and necrotic tissues on the stems were already evident on all plants before blossoming (see level 3 of scale in figure 1) and symptoms were progressive during pod filling (see levels 4-6 of scale in figure 1).

Our experiments were done in 2008 and this was an exceptional rainy year (Table 2) and the severity of lupine anthracnose in the field coincided with heavy and frequent rainfall.

The yield of the selected genotypes and I-450 ANDINO was low in comparison with previous years (Peralta *et al*., 2003, 2004). In lupine, the main production comes from the central axis. In this study, however, production came mainly from lateral branches because anthracnose strongly affected the main axis and destroyed floral primordia, flowers and pods. Under the weather conditions in 2008 ECU-722-4 and ECU-2700-2 yielded more than the, completely adapted to the Province of Cotopaxi, ANDINO I-450 (Table 3). Under less rainy circumstances, such as in the Province of Chimborazo, ANDINO I-450 yielded twice as much (Peralta *et al*., 2004). In a good disease management the weather plays an important role especially during the latter crop developmental stages. Thomas *et al*., (2008) demonstrated that under field conditions, disease spread and infection are largely influenced by rainfall. Application of fungicides has to take place prior to rainfall or shortly after rainfall. Another method to increase yield is a foliar application of azoxystrobin (250 g ai ha $^{-1}$) or mancozeb (1600 g ai ha $^{-1}$) at the moment of podding on the primary branches (Thomas *et al*., 2008). Our results indicates that in earlier stages it is best to use fungicides as soon as small lesions appear (grade two Table 1).

A preliminary laboratory assay determined in what percentage of three categories of seed anthracnose is present. Overall, the data shows that both a visual inspection and a good seed disinfection can reduce the number of infected seeds to 14.2 and 3.0%, respectively, but also that a visual selection of seeds does not guarantee 100% uninfected clean seed. The level of transmission of the disease via seeds might be partially, genetically determined. To check this hypothesis a detailed study under controlled conditions with *C. acutatum* inoculations on more homozygous lupine cultivars is needed.

The most important strategy for effective control of lupine anthracnose is to clearly understand its seed-borne nature, and produce disease free seeds with strict seed

certification programs. The primary inoculum of *C. acutatum* in lupine is infected seed. But there are no disease free seeds in Ecuador and the majority of common lupine growers in Ecuador are using their own, often infected, seeds.

The percentage of infection in seeds with small red-brown lesions (Table 3) suggests that the fungus can also be present under the seed coat and maybe also in cotyledonal leaves. Yesuf and Sangchote (2005) demonstrated that in infected common bean *C. lindemuthianum* is mainly present in the seed coat. A good seed disinfection method is required to reduce the initial inoculum but there will be escapes after visual selection. The simple method developed in this study is suitable for preselecting, uninfected lupine seeds. Seeds that do not show infection in Petri dishes will be planted and used in resistance screenings. All lupine collection accessions can be screened starting with uninfected. As soon as tolerant and susceptible plants, with no segregation for resistance in the progeny after selfing, crosses can be made and genetic mapping studies can be initiated. A PCR assay is another possibility to confirm presence of *C. acutatum* in large seed batches and individual plants (Chen *et al*., 2007). The percentage infected seeds used by Ecuadorian farmers fluctuates from 14% to 66 % (Table 3). This is far above the recommended threshold of 2% for seed-borne pathogens (Aftab *et al*., 2008). Since the commercial seeds are sold the next best thing is to choose the small red-brown stained seeds and treat them before sowing. Farmers should learn about the use of methods that do not have large effects on the percentage germination (Thomas and Adcock, 2004). Farmers can expose the seed previous to sowing to solar heat for 5-7 days. This thermotherapy can be combined with a chemical treatment for a further reduction of infection. Some fungicides prevent the infection of seedlings although the seed was infected. Procymidone (0.25 g a.i. /kg seed) and particularly iprodione (0.25 g a.i. /kg seed) do this especially in combination with thiram (1.0 g a.i. /kg seed) (Thomas and Sweetingham, 2003). The fungus can survive for up to two years on seed (Thomas, 2003).

The cultivars in this study have been developed mainly on their agronomic performance (Peralta *et al*., 2003, 2006b, 2008) and are genetically still heterozygous. INIAP researchers have chosen this approach because it minimizes the risk to lose the complete lupine production. Whether different tolerance mechanisms are present in the selected cultivars is unclear, to find this out the right plants have to be identified and mapping populations have

to be made. If there are different mechanisms and we can select for the underlying genes a breeding effort can be made to combine all known mechanism in order to get the highest tolerance possible. If it is necessary to maintain clean plants in combination with infection experiments *in vitro* multiplication is necessary. A tissue culture protocol for Andean lupine is currently being developed in our laboratory.

A combined approach that involves active participation of farmers (participatory plant breeding) and more applied and basic research of INIAP and universities is the most logical way to go. Firstly, the farmers have to be more aware of how important it is to look for anthracnose tolerant plants and how to recognize and use good quality seed www.telfun.info Key farmers should be skilled to identify anthracnose tolerant plants and if possible, seeds should be collected from selfed plants in order to obtain more homozygous plants.

Further research is also needed to determine the influence of different varieties on anthracnose severity and how environmental conditions influence severity. It is to be expected that different varieties lupine have to be developed for different microclimates. Supply of cultivars with different level of anthracnose tolerance adapted to rainy and drier zones will augment the production and quality of lupine

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CHAPTER 5

Assessing anthracnose symptoms in Andean lupine (*Lupinus mutabilis*)

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Summary

Anthracnose, caused by *C. acutatum,* is the most devastating fungal disease in lupine. The fungus spreads through the main stem and produces necrotic spots and orange spores. It can also grow on leaves and terminal branches. Bending of the main stem is the result of anthracnose infection. The level of tolerance was studied under greenhouse conditions and depended on plant stage and inoculation method. Lupine plants of the cultivar I-450 ANDINO were grown and plant-pathogen interactions were determined in five different phenological stages (0.5, 1.0, 1.5, 2.0, 2.5-month-old plants). Three isolates of *C. acutatum* were used for inoculation on the meristematic section of the main stem either by spraying or pipetting on an artificial wound. A scale (1-6) was used to score disease severity in the apical main stem of each plant. There were significant differences between the two inoculation methods and the five phenological stages. Plants that received the inoculum by pipetting after artificial wounding showed significant ($P<0.05$) more anthracnose symptoms than those that were sprayed. One and a half-month-old plants were the most tolerant and spraying appears to be the best method for a preliminary screening of large lupine populations, but artificial wounding is more reliable when screening potential resistant genotypes. We recommend to do the first screening in young plants (1.0-monthsold) and to confirm tolerance when flowering starts (2.5-monthsold) in this way the overall host reaction can be determined. Seeds of selfed lupine plants were partly used for replanting and disease evaluation and partly for measuring alkaloid content. There was no correlation between disease severity and alkaloid content.

Introduction

The lupine or "tarwi" (*L. mutabilis*) is domesticated and cultivated in South America. It is of agricultural importance in Ecuador (Peralta *et al*., 2004), Peru and Bolivia (Jacobsen and Mujica, 2006). Recently the interest for the Andean lupine has extended to Europe due to its nutritional value (Jacobsen and Sherwood, 2002). Disadvantages of the Andean lupine are indeterminate growth, high content of alkaloids and susceptibility to diseases. The principal biotic factor limiting production of *L. mutabilis* in Ecuador and other lupine species around the world is anthracnose (Talhinhas, 2002; Thomas, 2003; Elmer *et al*., 2001). The disease is caused by *C. acutatum* (Talhinhas *et al*., 2002; this thesis). The

pathogen infects the main stem and lateral branches and produces necrotic lesions from where orange conidial masses are developed (Gross, 1982; Thomas, 2003).

The Agropecuarian National Research Autonomous Institution (INIAP) of Ecuador has since 20 years a lupine collection with now more than 500 accessions from 17 different species. Of this total number, about 120 accessions are *L. mutabilis* (Rivera *et al*., 1998). Researchers of the Legume and Andean Grain National Program (PRONALEG-GA/INIAP) have been conducting field studies for more than 12 years aiming at selecting Andean lupine cultivars with good agronomic traits. In this selection process, the I-450 ANDINO cultivar was chosen as the best genotype because it is early maturing (6 months), uniform white seed, and almost twice the yield than other cultivars (INIAP, 1999). However, I-450 ANDINO is anthracnose susceptible.

Anthracnose resistance can be evaluated with parameters such as infection efficiency, lesion growth, and level of sporulation (Thurston, 1971). The interaction of these parameters with plant age affects the infection degree (Wastie, 1991). This has also been found in the interaction between potato plants and late blight where young plants are susceptible, later plants become more and more resistant, and finally old plants become more susceptible again (Stewart, 1990). Also some bean cultivars showed different resistance levels related to the developmental stages of the plants inoculated with *C. lindemuthianum* (Bigirimana and Hofte, 2001). Gieco *et al*. (2004) demonstrated that wheat seedlings were susceptible to *Septoria tritici* but resistant in the tillering and flag leaf. However, progenies that behaved in the opposite way were also found. Appropriate methodologies should be developed to evaluate disease severity for each disease in each crop.

Anthracnose screenings have been conducted to identify tolerant lupine genotypes in Ecuador (Peralta *et al*., 2003). Tolerant is a term that indicates that a host plant can support reproduction of the pathogen while sustaining little damage. Most studies conducted by Peralta *et al*. (2003, 2004) focused on tolerance with emphasis on yield. Some tolerance in *L. mutabilis* was found after spraying the pathogen on 0.5-monthold plants (Murillo *et al*., 2006). Visual observations on naturally infected, adult plants (Peralta *et al*., 2004) indicate that lupine is more susceptible in the blossoming season. Until now, there has been no reliable method to evaluate anthracnose tolerance in Andean lupine and the appropriate phenological stage to evaluate for tolerance still had to be identified. In *L. angustifolius* and *L. albus* two

methods of inoculation were used under greenhouse conditions. There were no significant differences between the first method (artificial wounding) and the second method (spraying), although the method of artificial wounding resulted in more differentiation in response. This analysis did not allow a reliable separation in tolerant and susceptible genotypes. Field studies showed relative low tolerance of genotypes which were considered as more tolerant in greenhouse screenings (Talhinhas, 2002).

Andean lupine has a high alkaloid content and mature seeds have the highest concentration (Allen, 1998). Values vary from 0.01 to 4.0% (INEN, 2004). The alkaloids, causing a bitter taste, have to be removed before seeds can be used for human consumption. This water-wash process takes about one week (Peralta *et al*. 2006b). After debittering, the aqueous alkaloid solution can be used to control ectoparasites and intestinal parasites of animals. It is also thought that alkaloids play a role in plant defense against herbivores (Wink and Schimmer, 1999). For this reason, Andean lupine is frequently sown as a hedge or to separate plots of different crops, preventing damage which animals might cause.

Lupine alkaloids have also bacteriostatic and antifungal activities. Bacterial growth is inhibited by alkaloid at concentrations between 0.3-7.0 mM and fungal growth was inhibited at concentrations of 10-50 mM (Wink, 1984). Plant pathogens activate general and specific plant defense mechanisms associated with a stimulation of secondary plant metabolism (Weir and Vivanco, 2008) possibly including alkaloid production. Reports on *L. albus* indicate that lupine alkaloid concentrations were reduced after elicitation of seedlings with cell wall extracts from the fungus *C. lindemuthianum* (Stobiecki *et al*., 1996). During the hypersensitive response, compounds such as alkaloids, terpenoids, and phenylpropanoids can play a role in killing pathogens and restricting invasion into the plant (Sepulveda-Jimenez *et al*., 2004). Significant inhibition of the mycelial growth was seen for *Alternaria solani* and *Fusarium solani* after adding lupine floor to potato dextrose agar media (Yepes Ponte *et al*., 2009). The growth of *Colletotrichum* species and other plant pathogens *in vitro* was hindered at very low alkaloid concentrations (200 to 1000 ppm; Singh *et al*., 2007).

The starting point for an evaluation of tolerance is the development of a reliable protocol. Our protocol development was done with the susceptible I-450 ANDINO. It has good agronomic traits and until now it is only grown for research purposes. The appropriate

inoculation method and phenological stage for this cultivar should make the evaluation of the level of anthracnose tolerance in more genotypes of Andean lupine possible. The possibility that alkaloid content plays a role in anthracnose tolerance of lupine was investigated.

Materials and methods

Plant material and experimental design

The experiment was conducted in a greenhouse at the Agropecuarian Science Faculty, Life Science Department, of the Army Polytechnic School (ESPE), Province of Pichincha, Rumiñahui County, San Fernando Parish, Ecuador at 2748 m, coordinates 0° 23' 20'' S, 78° 24' 44'' W. I-450 ANDINO was used to develop the protocol. Phenotypic healthy seeds of I-450 ANDINO were selected and surface disinfected (5 min 0.5% NaClO) and rinsed with water. Twelve seeds were planted, four of each in 22 cm diameter pots containing 4 kg of a mixture of sterilized soil / grinded pumice / coconut fiber (1:1:1). After 15 days, one seedling was removed from each pot, leaving three plantlets per experimental unit. All pots were placed in a greenhouse with a temperature of 12 ± 2 ^{o}C night / 20 ± 2 ^{o}C day and 12 h photoperiod, and relative humidity of 70 ± 10%. The sowing was repeated every fifteen days and after 2.5 months five phenological stages were compared. Treatments were arranged in a combined factorial design: five phenological stages, two inoculation methods, three *C. acutatum* isolates, and one non-inoculated control.

Production of inoculum

Three *C. acutatum* isolates (Lup1, Lup14, Lup18) were collected from three different places (with different environmental conditions) in the Province of Cotopaxi - Ecuador. Lup1 (Picualo alto, 0^{o} 53' 56" S 78^{o} 32' 37 W, 3180 meter above sea level, masl), Lup14 (Canchicera, 0 ^{o} 47' 33S 78^{o} 32' 59" W, 3222 masl), Lup18 (Isinche de comines, 0^{o} 01' 08" S 78^{o} 40' 24W, 2972 masl). They were compared for their virulence in five phenological stages and with two inoculation methods. Spores of the isolates were taken from potato dextrose agar slants and subcultures were grown on Petri plates with autoclaved PDA (Difco). The isolates were incubated for 10 days at room temperature 20 ± 2 ^{o}C. Conidia

suspensions were prepared by flooding the surface with sterile distilled saline solution (NaCl 0.8% + Tween-80 0.1%) and gently scraping with a glass rod. The concentration of spores was determined with a heamocytometer and diluted to 2.5 x 10^6 conidia/ml. Plants in different growth stages were inoculated at the same time.

Inoculation methods

Two inoculation methods were evaluated. For inoculation by spraying, approximately 0.5 ml of each suspension was spread on the meristematic parts, apical leaves and young stems of lupine plants. A small hand-held Venturi atomizer (aerographer) with an air pump was used. For the other method, an artificial wound was made with a hypodermic syringe at the apical main stem (the same depth for each wound). 25 µl of the spore suspension of each isolate was injected with a micropipette in each wound. The inoculation area of each plant was completely covered with small black plastic bags. A piece of cotton drenched in sterile distilled water was added to the bags before sealing. The wet cotton was used to maintain the relative humidity and to promote infection. The bags were removed after 72 h.

Disease evaluation

Anthracnose was scored on individual plants, using a 1-6 scale as described in Figure 1 (1-2 = tolerant/resistant, 3-6 = susceptible).

Development of plant populations

Plants were grown from middle of October 2009 to the end of March 2010. Seeds of I-450 ANDINO were sown in an experimental field at Simón Rodríguez Technological Institute of Agriculture, located in the Province of Cotopaxi, Latacunga County, Alaquez Parish (latitude 00º 52' 01" S, longitude 78º 37' 07" W, altitude 2859 masl. The experimental field is located in the central region of Ecuador, 91 km south from Quito, the Capital of Ecuador. Seeds were sown in threerow plots of five-meter long and a one-meter distance between rows. In the furrows holes were made at a distance of 35 cm and three seeds were sown in each hole. Healthy plants were selected. Main stem of each plant was self-pollinated before the blossom period. Seeds produced on the main stem of phenotypic

healthy plants were harvested to study anthracnose tolerance. Alkaloid content was measured on the remaining seed produced on main stem.

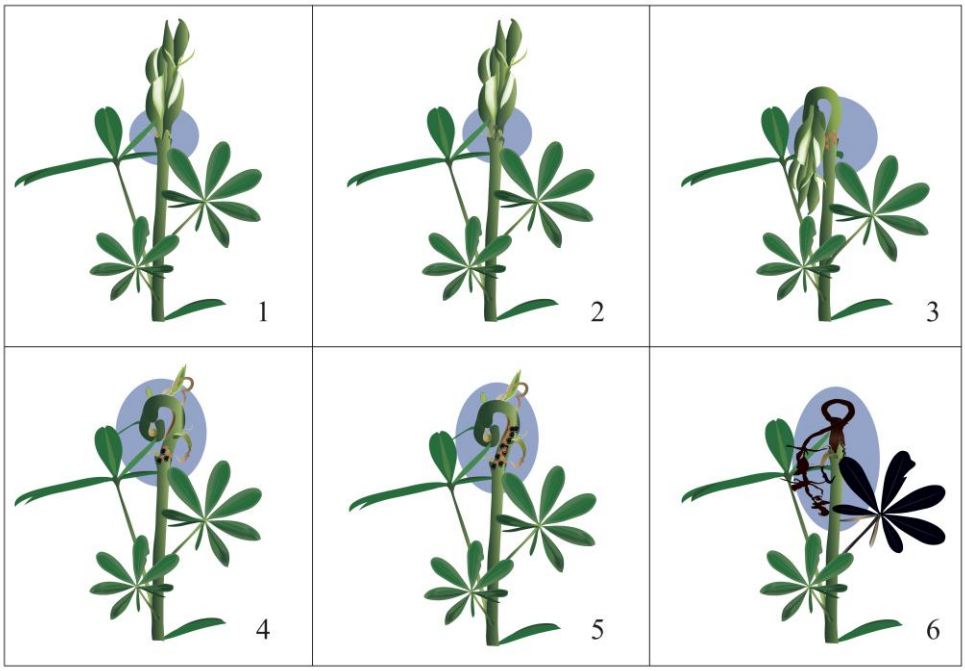

Figure 1. Scale to evaluate anthracnose resistance/tolerance in lupine. 1 = no symptoms or wrinkled folioles, 2 = small injuries < 2 mm at the point lesion was made or at the meristem region pathogen was spread, sporulation absent, 3= central apical stem doubled due to infection, little sporulation, 4 = lesions 2-5mm at the point lesion was made or at the meristem region, little sporulation, 5 = presence of lesions (more than 5 mm) on main stem, accompanied by necrotic tissue, abundant sporulation, 6 = severely affected necrotic plant, or dead plant (1 to 2 incompatible reaction – resistant/tolerant reaction, 3 to 6 compatible reaction – susceptible reaction). The disease ratings were scored at day 26 after inoculation.

Anthracnose phenotypic reaction of S_1 families

The experiment was conducted in the same greenhouse under equal conditions. Seeds of S_1 lupine I-450 ANDINO families, healthy in appearance, were chosen and surface disinfected on NaClO (0.5%) for 5 min and washed with water. Four seeds were planted in 22 cm diameter pots containing 4 kg of a mixture of sterilized soil / grinded pumice / coconut fiber (1:1:1). Treatments were arranged in a complete randomized design, with three pot-

replications and twelve plants per family. Isolate Lup18 EMBL accession number JN543063 (Table 4, Chapter 3) was chosen as inoculum. Incubation period and inoculum preparation was as above. Plants at the greenhouse were inoculated 4 weeks after sowing. Approximately 0.5 ml of pathogen suspension was spread on the meristematic parts, apical leaves and young stems of lupine plants. Black bags covered only apical parts. A piece of cotton imbibed in sterile distilled water was put in the bags before sealing, this keeps the relative humidity high and promotes infection. Bags were removed after 72 h. Anthracnose was evaluated after 15 days on twelve individual plants per family, using the above 1-6 scale.

Alkaloid extraction procedure

Total alkaloid percentage on seed was determined according to Beck cited by Harrison and Williams (1982). One gram of finely chopped seed was boiled in 50 ml 50% ethanol and left to cool overnight at 4°C. After decanting, the seed tissue was extracted with four successive volumes of 70% ethanol, and left overnight at 4°C. The combined extracts dried under vacuum at 40°C to a volume of about 2 ml, and the pH was adjusted to 4.0 - 4.5 with sulphuric acid. After lowering the pH to 2.5 and centrifugation, the lipids were removed with two volumes dichloromethane in a separating funnel and pH was adjusted to 9-9.5 with sodium hydroxide. The alkaloids were then extracted with three volumes of chloroform and after bringing the pH to 10.5-11.0, two more chloroform extractions were performed. After drying on anhydrous sodium sulphate, the combined extracts were completely dried under vacuum at 40°C and residual alkaloids transferred into 2 ml of chloroform which evaporated in a stream of warm air. The residue was dissolved in 1 ml of chloroform per g of original seed tissue. For titration, alkaloid extracts were added to a known volume of 0.005 M sulphuric acid and titrated with 0.01 M sodium hydroxide, using methyl red as indicator; blank titrations with only acid were also carried out.

Percentage alkaloid was calculated with the formula:

$$\frac{\text{Volume of 0.01 M NaOH}}{\text{Weight of seed (g)}} \times \frac{248}{1000}$$

where the volume of NaOH is the difference in ml between the blank titration and the sample, and 248 the molecular weight of lupanine, the prevalent alkaloid (Harrison and Williams 1982). Each test was done five times on different days.

Statistical analyses

To compare the level of anthracnose tolerance in five phenological stages with two inoculation methods and three *C. acutatum* isolates an analysis of variance was performed with the average of anthracnose severity scores. Graphs were constructed with the average of the anthracnose severity to visualize disease progress patterns of each inoculation method in the five stages.

An ANOVA test was conducted separately to determine significant differences among phenological stages, isolates and interaction stages per isolates. Variability in each phenological stage was calculated by the least standard deviation. To observe plants with less disease severity data were collected 26 days after inoculation for those phenological stages where differences were more pronounced (1.0 and 2.5 months).

Individual offspring plants of selfed lupine were inoculated with *C. acutatum* and the segregation ratio was determined. Mean scores and standard error of alkaloid percentage of the seed were used for testing for differences between different offspring families of I-450 ANDINO. The correlation of tolerance/susceptibility and alkaloid content of the seed was calculated by regression analysis. Data analyses were performed and graphs were made with the help of the statistical program Infostat for windows www.infostat.com.arg.

Results

Inoculation methods and plant age

Twenty six days after inoculation analyses of variance showed significant differences in disease severity between inoculation methods ($F=17.91$, $p=0.0001$) and between phenological stages ($F= 6.83$, $p=0.0001$) (see Figure 2). Grand mean was 4.27 and the coefficient of variation 19.29.

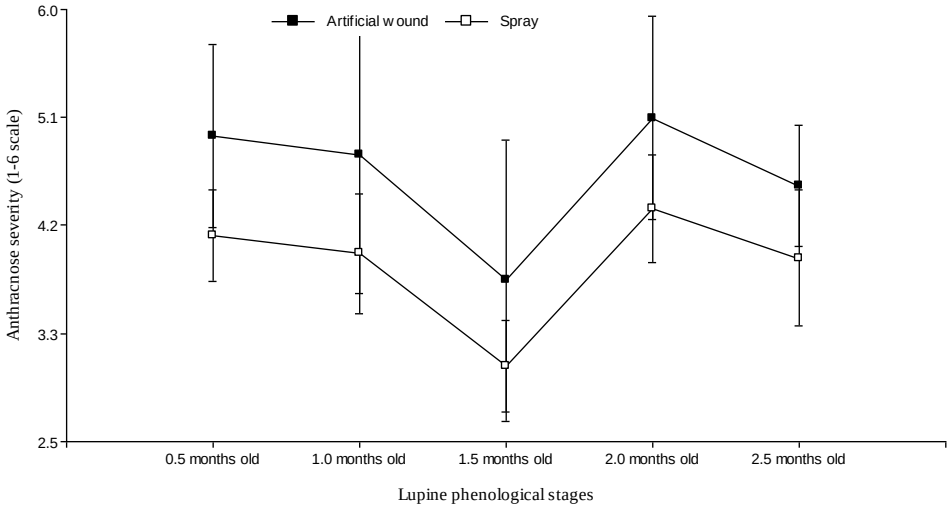

Figure 2. Two inoculation methods and five phenological stages were compared for disease severity. Means are the average of 9 I-450 plants and 3 *C. acutatum* isolates collected at 26 days after inoculation. Bars show the standard deviation.

Table1. Influence of inoculation methods on anthracnose severity in five phenological stages of I-450 ANDINO.

Inoculation method	Anthracnose severity score					
	Phenological stage (months)					
	0.5	1.0	1.5	2.0	2.5	Mean
Spraying	4.14 b	4.00 b	3.07 a	4.37 b	3.96 b	3.90 A
Artificial wounding	4.96 b	4.81 b	3.78 a	5.11 b	4.55 b	4.64 B

Mean separation in rows (a – b) and mean separation in columns (A – B) by least standard deviation, P<0.05.

Analyses of variance after spraying show significant differences for phenological stages (F= 2.86, p=<0.0404), but no significant differences were found between isolates (F=0.73, p=<0.4900). R^2 was 0.72 and the coefficient of variation 22.42. Analyses after artificial wounding method show significant differences for phenological stages (F= 4.20, p=<0.0081) and also between isolates (F= 6.28, p=<0.0053), R^2 was 0.59 and the coefficient of variation 16.58. The two inoculation methods gave similar tendencies.

However, in plants after artificial wounding the anthracnose severity was significantly higher (P<0.05; Table 1).

One and a half-month-old plants generally had the lowest disease severity scores, symptoms decreased until 1.5 months (for both inoculation methods significantly lower; Table 1) but subsequently increased.

Table 2. Differences between isolates for disease severity on I-450 ANDINO.

Isolate	Anthracnose severity score	
	Spraying	Artificial wounding
Lup 1	4.04 ± 0.40	4.37 a
Lup 14	4.00 ± 0.32	4.31 a
Lup 18	3.69 ± 0.36	5.28 b

Mean followed by different letters are significantly different (P<0.05). Means ± standard deviation when there is no significant difference.

There was a significant (p=<0.05) difference between isolate Lup18 and the other two isolates after artificial wounding (Table 2), this difference was not found after spraying.

More differences in disease severity were seen in older plants (1.0, 1.5 and 2.5 month) and for finding potential tolerant plants these three stages are more suitable. We recommend to score disease severity after inoculation of 1.0-month-old plants and confirm the scores after inoculation of 2.5-month-old plants.

Table 3. Anthracnose disease score (~25 plants of I-450 ANDINO per experiment) after spraying and measured 26 days after inoculation.

	Anthracnose severity (1-6 scale)				
	2	3	4	5	6
1.0 month old	5	7	5	2	8
2.5 month old	0	2	1	5	19

Table 3 shows that the scores vary from 2 to 6 in young plants, but the average disease rating goes up when plants are inoculated that are almost flowering. Plants in scale 2 can be

tolerant but can also be escapes and the response should be confirmed in 2.5 month old plants. Same pattern was seen in plants inoculated after artificial wounding (Table 4).

Table 4. Anthracnose disease score (~25 plants of I-450 per experiment) after artificial wounding and measured 26 days after inoculation.

	Anthracnose severity (1-6 scale)				
	2	3	4	5	6
1.0 month old	4	3	2	3	15
2.5 month old	0	7	4	9	7

Screening for correlation between alkaloid content and disease severity

One hundred and twenty I-450 ANDINO seeds were sown to obtain offspring. The highest number of seeds per main axis was 57 and the lowest 9, with an average of 25. I-450 ANDINO produces normally between 60 – 70 seeds per main axis (INIAP, 1999). Paper bags around the main axis of each plant were placed before blossoming to promote self-pollination. Twenty three S_1 populations had at least 28 seeds per main axis and were selected to evaluate anthracnose tolerance and alkaloid content. The partial tolerance in the offspring of the 23 selfed populations was measured 15 days after inoculation. This allowed us to study differences in speed of infection. Seven S_1 families were found with at least 75% uninfected plants (20, 32, 63, 95, 100, 201, 202) and five S_1 families were found with less than 25% uninfected plants (3, 19, 27, 62, 227) (Table 5).

Twelve S_1 lupine families were further analyzed and total alkaloid content was determined in the remaining seeds. Of the total alkaloid content, lupanine is the predominant alkaloid in lupine seeds (up to 62%) (Harrison and Williams, 1982). The mean alkaloid content per S_1 family varied from 3.07 to 3.41 percent (Table 6). No significant differences were found in levels of alkaloids between families and therefore differences in speed of infection are not caused by difference in alkaloid content.

Table 5. Screening of individual plants of offspring families of individual plants of I-450 ANDINO after artificial inoculation. Score based on the 1-6 severity scale described in figure 1 (1 to 2 considered tolerant and 3 to 6 susceptible). The disease symptoms were evaluated fifteen days after inoculation.

	Anthracnose severity (1-6 scale)							
	1	2	3	4	5	6	Families with 75% or more uninfected plants	Families with 25% or less uninfected plants
Fam 1		4	3	2				
Fam 3		3	1	7	1			75.0
Fam 10	6	1	1	4				
Fam 13	3	1	1	3	1			
Fam 19	2	1	2	3	4			75.0
Fam 20	9	3					100	
Fam 27		1	5	4	1	1		91.7
Fam 29	2	3	2	4		1		
Fam 30	1	5	2	3		1		
Fam 31	3	4	1	1	1			
Fam 32	11			1			91.7	
Fam 41	5	1		4	1	1		
Fam 62	1	1		1	3	5		81.8
Fam 63	2	7	2	1			75.0	
Fam 91	4	3		5				
Fam 92	1	5	2	4				
Fam 93	5	1	3	2	1			
Fam 95	9	2					100.0	
Fam 100	8	3					100.0	
Fam 201	11	1					100.0	
Fam 202	8			2			80.0	
Fam 206	2	5	2	1				
Fam 227		2	4	5				81.8

Table 6. Alkaloid content of offspring families with high and low levels of uninfected plants (see table 5)

>75% Uninfected plants (a)		< 25% Uninfected plants (b)	
	Total alkaloid (%)		Total alkaloid (%)
Fam 20	3.11 ± 0.14	Fam 3	3.07 ± 0.25
Fam 32	3.21 ± 0.08	Fam 19	3.19 ± 0.19
Fam 63	3.20 ± 0.25	Fam 27	3.26 ± 0.24
Fam 95	3.17 ± 0.15	Fam 62	3.06 ± 0.28
Fam 100	3.41 ± 0.12	Fam 227	3.36 ± 0.17
Fam 201	3.15 ± 0.17		
Fam 202	3.09 ± 0.16		

Discussion

Two methods of inoculation have been used to evaluate anthracnose disease symptoms in a susceptible lupine cultivar. Artificial wounding gave higher anthracnose scores than spraying (Table 1). Spraying mimics the natural situation where spores are splashed by rain and wind, from infected lupine seedlings or stubbles (Thomas, 2003). The spores penetrate directly or through hidathodes and stomata. The infection process of *Colletotrichum* is characterized by a short biotrophic phase. At this point, cells of the two organisms are in close contact, followed by a necrotrophic phase (Esquerre-Tugaye *et al*., 1992). When using the artificial wounding method, the biotrophic phase of the pathogen is likely to be shorter than when using spraying. Niks and Lindhout (2006) recommended the use of artificial wounding to minimize escapes. The results of our research show that both inoculation methods give typical anthracnose symptoms in the apical main stem of lupine (Figure 1). Artificial wounding overcomes more easily a low level of tolerance, but spraying may be appropriate as a preliminary selection in large lupine populations followed by artificial wounding to detect escapes.

Disease severity symptoms were not the same in all phenological stages. More symptoms were found at the end of the cotyledonal stage (0.5-monthold) and at the beginning of flowering (2.5-monthold plants) (Figure 2). Changes in disease susceptibility during the life cycle of the plant have been observed in other plant-pathosystems. Bigirimana and Hofte (2001) found that some bean cultivars showed different levels of tolerance depending on the developmental stages of the plants when inoculated with *C. lindemuthianum*. Gieco *et*

al. (2004) demonstrated that wheat seedlings were susceptible to *Septoria tritici* but resistant in the tillering and flag leaf. Carnegie and Colhoun (1982) found that late blight susceptible potato varieties appeared to become more susceptible at higher plant ages, whereas the more resistant varieties appeared to become even more resistant. In addition to age-related changes in tolerance, Visker et al. (2003) found that leaf position plays an important role in the tolerance of potato to late blight. Apical leaves were more resistant to *P. infestans* than basal leaves in the same plant. Leaf position appeared to affect quantitative levels of disease infection. Chang and Hwang (2003) have demonstrated that lower leaves were more severely infected by leaf blight than upper leaves of adlay perennial grasses, irrespective of growth stage and cultivar. Other studies show that individual plant organs within a given plant vary in the level of tolerance or susceptibility. Pfender (2004) found that there were significant differences among plant organs on different positions on perennial ryegrass infected by stem rust. In this study, it is likely that the observed differences are mainly the response of lupine apical stem organs in which inoculation took place. Each plant-pathosystem requires the development of its own methodologies for assessing disease symptoms in order to identify resistant genotypes.

Epidemiological studies on lupine showed that reproductive tissue during flowering and pod formation are more anthracnose susceptible (Thomas and Sweetingham, 2004). This might be caused by stage-specific tolerance genes. Some of these genes might be not effective in early and late developmental stages.

Paper bags were placed on the main axis before the blossom season to promote selfings. This was not always successful and flower abortion and empty pods were commonly found. In normal conditions, there is a high level of cross pollination in the Andean lupine (Sweetingham *et al*., 2005). Cultivation of the Andean lupine has often resulted in a low seed yield (Sawicka-Sienkiewicz and Augiewicz, 2002) and this low seed yield in has been attributed to protandry or protogyny (Hardy and Huyghe, 1997).

Seeds of S_1 families of ANDINO I-450 were sown to look for components of anthracnose tolerance (compare Table 5 with Tables 3 and 4) although I-450 ANDINO is anthracnose susceptible. Field studies showed that only a few individual plants did not get diseased (chapter four of this thesis). In this study, differences in speed of infection in S_1 families of this cultivar were observed (Table 5). It is not known if this difference in speed of infection

in *L. mutabilis* is monogenic or polygenic controlled. Anthracnose tolerance in *L. albus* is caused by more than one gene and is quantitative (Yang *et al*., 2009). The first step to understand anthracnose inheritance of Andean lupine is to develop homozygous populations. But this is difficult for Andean lupine since it is considered a cross-pollinating species (Sweetingham *et al*., 2005). We want to develop more homozygous material based on cultivars selected by INIAP and of new genotypes. With homozygous material and good levels of tolerance, it is feasible to do Quantitative Trait Loci (QTL) mapping studies.

Alkaloid content in lupine seeds was measured as total alkaloid percentage and determined by titration. Gas-liquid chromatography can detect smaller amounts, but for our purposes determining total alkaloid is satisfactory (Harrison and Williams, 1982). Sweet, low alkaloid, genotypes are those that go from 0.002% up to the acceptable Food Standard of 0.02% (200 mg/kg). However, there are bitter seeds of *L. albus* in the range of 1.5 to 2.2 % (Luckett, 2010). I-450 ANDINO is a bitter genotype. In this study, we analyzed S_1 populations of ANDINO I-450 to look for a correlation of the speed of infection and the alkaloid content in the seed. Alkaloids can be selectively high in lupine peripheral cell layers of stems and seeds (Wink, 1984). No correlation was found between alkaloid content in seed samples with level of uninfected or infected plants 15 days after inoculation (Table 6). Further studies looking at infested plants and their percentage commercial seeds in relation to alkaloid content may show a correlation. In other studies no association were found between brown spot disease and total alkaloid concentrations in F_2 segregating populations (Gremigni *et al*., 2006). Bradley *et al*. (2002) found brown spot-resistant plants in both the bitter and sweet F_2 progeny of crosses between susceptible sweet cultivars and resistant landraces of wild bitter *L. angustifolius*. Other natural products in lupine, different from alkaloids, may have antifungal properties or maybe the chemical defense system of lupine consists of more compounds than the alkaloids alone. Breeding Andean lupine for sweet varieties appears to be possible without losing tolerance/resistance to anthracnose. Other sweet lupine breeding programs as for example, in *L. luteus* and *L. angustifolius* have been successful in finding nearly alkaloid-free varieties. Alkaloid content of raw lupine seed is on average 3.3% but could be reduced to 0.47 % by plant breeding (Schoeneberger *et al*. 1982). After the development of sweet lupine cultivars of *L. albus* gradually plants with higher levels of alkaloids were found due to cross-pollination

(Kurlovich, 2002). Breeding for sweet lupine varieties in *L. mutabilis* has received little attention (Schoeneberger *et al*., 1982). Considering the high level of outcrossing of *L. mutabilis* (Sweetingham *et al*., 2005), it is obvious that once a low-alkaloid lupine variety is identified, field plots should be established with no wild bitter lupine plants around. In the first year, the offspring need to be tested for the presence of alkaloids and all identified bitter plants should be removed before blossoming. If clean, production of such plots may satisfy the demand for seed for many years in great areas (Kurlovich and Kartuzova, 2002), production of seed with low alkaloid content or varieties free of alkaloids in the Andes of Ecuador could reintroduce lupine in traditional areas.

Results of this study may be considered as a pilot for further evaluation of anthracnose tolerance in Andean lupine. In our opinion, the most reliable protocol is by spraying a *C. acutatum* isolate as a preliminary selection of large populations. Scoring in 1.0-month-old plants and confirms the tolerance in 2.5-month-old plants to determine the overall reaction. Individual plants found resistant by the plant spray inoculation should be further challenged by artificial wounding of inoculum in 1.0-month-old plants. Two and a half-month-old plants that are still alive may be considered tolerant. Anthracnose tolerance can also be measured in plants sown in pots under greenhouse conditions. Reliable tolerant and susceptible individuals can be used to make crosses and create mapping populations. Because of the high level of out-crossing of Andean lupine, no homozygous genotypes are present but effort should be made to create homozygous resistant genotypes. Further studies are needed to elucidate the effect of organ age and position on the level of lupine anthracnose tolerance.

Acknowledgements

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CHAPTER 6

General discussion

The interdisciplinary TELFUN project and Food Sovereignty

The Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition (TELFUN) project aimed to support people to choose their own way of producing, processing, and consuming local foods that fit best in their local conditions. The central research question was: how do technological practices, developed from within food networks, enhance food-sovereignty and the nutritional status of people? TELFUN is an interdisciplinary and comparative research program based on twelve research projects, divided in four disciplines and carried out in three geographical locations. Its scientific development is divided in four disciplines - plant breeding, food technology, human nutrition, and sociology of science and technology - for a more complete understanding of the production, processing, utilization and consumption of lupine in Ecuador. Similar studies have taken place with cowpea in West Africa and mungbean in India (www.telfun.info).

Lupine (*Lupinus mutabilis*) is a native legume of the Andean zone. It is adapted to marginal regions with limited water supply and poor nourished soils. In comparison with other legumes used in human nutrition, lupine seeds have high variability in iron and zinc content. These are just a few attributes that might make the Andean lupine a food of the future (www.telfun.info). Unfortunately, lupine is very susceptible to anthracnose and has a high content of alkaloids. Alkaloids have to be removed from seeds by processing before human consumption. Paradoxically, very little research has been done to breed for anthracnose resistance of Andean lupine. This is in contrast with the research done in other species of lupine cultivated around the world (Yang *et al*., 2008, 2009). *L. mutabilis* has a low level of genetic similarity with *L. albus* and *L. angustifolius* from the Old World (Talhinhas *et al*., 2003).

Plant breeding as part of the interdisciplinary research

In the breeding discipline the following research question was proposed: How can exploratory studies, both on the pathogen and on the lupine genetic diversity, contribute to the development of better lupine varieties in the Province of Cotopaxi - Ecuador? The research described in this thesis focuses on determining the actual situation of lupine in the Province of Cotopaxi, determining agronomic traits and nutritional value of lupine cultivars selected by the Agropecuarian National Research Autonomous Institution (INIAP),

identifying the species of anthracnose in lupine and tamarillo by using molecular techniques and exploring anthracnose diversity associated with lupine and tamarillo. Both lupine and tamarillo are native Andean crops. The lupine has traditionally been cultivated in the Ecuadorian cold highland places. The tamarillo has been cultivated in warm areas in the valleys. In recent years, the agricultural borders of the two crops are shifting and often they are cultivated close to each other. Tamarillo is a perennial crop with a short life of four years. Tamarillo may be a reservoir of *C. acutatum* isolates capable to infect lupine. Other studies focused on quantifying anthracnose resistance of selected cultivars and anthracnose infection in lupine seed, determining critical points in the disease development, designing methods for screening anthracnose resistance in individual lupine plants and studying the possible relation between speed of infection and alkaloid content. Based on our findings a basic and applied model to start a breeding program with emphasis on anthracnose resistance is proposed. The interdisciplinary practices for the sustainable production of lupine in Ecuador which contribute to food sovereignty are highlighted.

Agronomic traits and nutritional value of Andean lupine

In the selection process of Andean lupine there has been chosen for white seed, a plant height of about 1.4 m, and early flowering. Based on these criteria, INIAP selected the six most suitable lupine cultivars, grew them and made selections under different environmental conditions in Ecuador (Peralta *et al*., 2008). Most modern cultivars are characterized by early flowering, large pod size and white seeds. In contrast, most wild relatives have low levels of vegetative development, late flowering and small brown seeds. They grow in marginal uncultivated and more mountainous areas.

The set of selected cultivars could be grouped based on the height of the main axis, vigorous vegetative development, number of lateral branches and reproductive capacity (Chapter 2). Based on plant architecture Tapia (1990) distinguished a tall annual or biennial plant with long branches, adapted to the long growing seasons of the Andes (chocho) and an annual type from high altitudes in the central Andes of Peru and Bolivia which has a dominant main stem, few branches, and a short growing season of 155 days (tarwi). In each cultivar we distinguished tall and dwarf plants, early and late plants, and plants with determinate and non-determinate growth. It indicates that the cultivars are still very

heterogeneous. Development of homogeneous populations of Andean lupine is needed to look for the genetic components leading to anthracnose resistance.

The agronomic study of six selected *L. mutabilis* cultivars and two landraces showed their performance in a very wet period in the Latacunga region. In general, the six lupine cultivars show low averages for all agronomic important traits (Chapter 2). This illustrates the poor adaptation of the selected lupine cultivars (released by INIAP) to the conditions in the wet season. For large improvements in seed yield, the first step in a breeding program is to identify a phenotype well adapted to local climatic conditions (Wells 1984). Andean lupine requires only 300 mm of rainfall during the production period (Peralta *et al*., 2008) and is better adapted to places with less rainfall, such as the Province of Chimborazo.

The significant different levels of protein, iron, and zinc in lupine cultivars suggest that these characteristics are genotype dependent. In our overall breeding scheme, we propose to evaluate for quality after selection for tolerant/resistant lupine genotypes (Table 1).

Morphological, molecular, and pathological studies of anthracnose in lupine and tamarillo

Anthracnose is the most devastating disease in lupine and tamarillo, the *Colletotrichum* species causing this was not determined in Ecuador. This was the first study of anthracnose in which phenotypic and molecular approaches are combined to characterize the population structure of this pathogen and to determine their relatedness with worldwide representatives based on ITS sequence, and to establish pathogenic variability and host plant-cross infection.

Use of morphological characteristics to identify *Colletotrichum* species

Colletotrichum is the causal agent of anthracnose and all isolates had either a white-pink or a gray-pink pigmentation on the back of the colony. Pink-salmon colored spores are typical for *C. acutatum* in formal descriptions (Sutton, 1992). Lupine isolates grow slower than tamarillo isolates on Potato Dextrose Agar (PDA) in the Petri dishes after 10 days at $17^{o}C \pm 2\ ^{o}C$ at day light. The color of colonies varied from pale to olive-grey of aerial mycelium forming concentric circles (Figure 2A-E, Chapter 3) in isolates of lupine and tamarillo. In general the colony color changed in time from white to dark brown or black. *C. acutatum* isolates from lupine formed a pink-salmon pigment and tamarillo isolates a pink-yellow pigment on PDA.

Table 1. Basic and applied research scheme to start a breeding program with emphasis on anthracnose resistance

Steps	Methodology	Selection criteria
Preliminary screening for tolerance	Spraying method Score in one-month-old plants Confirm in 2.5-month-old plants	Tolerance to pathogen infection in apical part of plants
Screen on commercial seeds of infected plants	Sow on the surface of PDA in Petri plates	Tolerance to transmission of the pathogen via seed
Production of homozygous resistant plants and/or plants with a low seed transmission	Main stem bagged to promote self-pollination	Homozygous seed production
Confirmation of resistance in homozygous populations	Resistant plants will be selfed again (preferable through non-infected cuttings of the plants). Pipetting after an artificial wound method Score in one-month-old plants Confirm in 2.5-month-old plants	Disease screening. All progeny plants must be resistant
Screen for nutritional value	Methods described by AOAC (2005)	High protein, iron, zinc content
Genetic studies for both anthracnose resistance and seed transmission	Crosses of resistant x susceptible plants. Association studies with molecular markers (Single Nucleotide Polymorphisms) in F_2 populations.	Anthracnose resistant varieties
	Hybrid testing high resistance, low seed anthracnose transmission, high yield,and nutritional value.	Healthy plants with maximum of commercial seed
or Seed production program of good quality seed	Field plots in local communities	Constant supply of clean lupine seeds

The shape of the spores varied between lupine isolates. Some were with one round and one acute end (Fig 2A in Chapter 3), but spores of the others lupine isolates were mostly cylindrical with round ends (Figure 2B-D, Chapter 3), that resemble *C. gloeosporioides* (Sutton, 1992). Based on morphological criteria alone lupine anthracnose (Insuasti, 2001) and tamarillo anthracnose (Sánchez *et al*., 1996; Santillán, 2001) were classified as *C. gloeosporioides* in Ecuador. Our studies demonstrated that identification of species by traditional methods (morphology, colony color, size of conidia), is not sufficient for identification of species and subspecies, which is critical for disease management and breeding. However, spore form, color appearance, color of pigment in the media after 10 days of incubation at 17 ± 2 ^{o} C and radial mycelia growth rate can be used for a preliminary characterization of *Colletotrichum* isolates.

Molecular diagnosis and benomyl sensitivity

All the isolates from lupine and tamarillo gave a *C. acutatum*-specific amplification product in the polymerase chain reaction (PCR) and no *C. gloeosporioides*-specific product (Brown *et al.*, 1996). These results show that *C. acutatum* is the causal agent of lupine and tamarillo anthracnose and not *C. gloeosporioides*. Additionally all *C. acutatum* isolates from lupine and tamarillo grew on medium with benomyl and as expected, the *C. gloeosporioides* reference isolate did not. *C. acutatum* needs a different disease management than *C. gloeosporioides*. Efficient treatment of *C. acutatum* is done by fungicides such as azoxystrobin, mancozeb, or chlorothalonil (Thomas *et al*., 2008).

Phylogenetic studies

The internal transcribed spacer ITS (ITS 1–5.8S-ITS 2) was targeted to analyze phylogeny and systematics of *Colletotrichum* isolates from lupine and tamarillo. Our sequences were compared with ITS regions of *Colletotrichum* species (Talhinhas *et al.*, 2002, 2005, 2009; Martinez-Culebras *et al.*, 2003; Afanador-Kafuri *et al.*, 2003) that are public available in GenBank (www.ncbi.nlm.nih.gov/genbank).

Phylogenetic analyses of the ITS sequences, divided the *C. acutatum* isolates from lupine and tamarillo in Ecuador into two subgroups and all sequences but one in a newly formed subgroup. Pathogen populations within a geographical location are very homogeneous and

nearly clonal (Martínez-Culebras *et al.*, 2002, 2003; Muñoz *et al.*, 2000). In our study, the new group of lupine isolates included one from tamarillo (Tam6) (Figure 3 in Chapter 3). Isolate Tam6 was collected in San Pablo, Province of Imbabura, 150 km north from Quito. In that place, lupine and tamarillo have both been cultivated for about 10 years and the isolate might have been an occasional lupine isolate that cross infected a particular field of tamarillo. Close similarity of *C. acutatum* isolates indicate that the practice of cultivate lupine close to tamarillo is not advisable. The existence of a teleomorph (Guerber and Correl, 2001) in *C. acutatum* may be contributing to the genetic divergence of some isolates creating more opportunities for pathogen to adapt to both hosts. In addition, sequence analysis grouped isolates of both hosts that previously were referred as *C. gloeosporioides.* This is new and valuable information for adequate anthracnose management in Ecuador.

Pathological studies

We conducted a host range test and found that isolates from lupine were pathogenic on tamarillo plants and tamarillo isolates were pathogenic in lupine plants. In anthracnose pathosystems, the same host is often infected by different species of *Colletotrichum* and the same pathogen can infect different hosts (Freeman *et al*., 1998). *C. acutatum* from olive causes symptoms in strawberries and lupine (Talhinhas *et al*., 2005). In this study, with a small number of *C. acutatum* isolates from tamarillo and lupine we saw that *C. acutatum* isolates can infect both tamarillo and lupine, but with different levels of virulence. Studies by pipetting *C. acutatum* isolates after wounding tamarillo and lupine plants show a positive host cross response with different degrees of virulence. The high levels of biological variability of *C. acutatum* populations may be caused by their high plasticity (Sreenivasaprasad and Talhinhas, 2005). The techniques developed in our study must be used to monitor the dominant *Colletotrichum* species in the multi cropping Andean system and to establish the appropriate disease management and breeding strategies.

Understanding lupine anthracnose development

Although all the six selected cultivars were susceptible in the Province of Cotopaxi, variation was seen in anthracnose development. After infection, symptoms become visible as wrinkles in the first true leaves 48 days after planting (DAP) and become more evident

before flowering and pod filling starts (76 DAP and 121 DAP response). Variation may be due to quantitative tolerance genes.

Our data show that the seedling stage (48 days after planting), and the period before flowering and pod filling are critical points in lupine anthracnose development (Table 1 and Figure 1, Chapter 4). The first symptoms are wrinkled leaves and lesions on the leaves and apical stems. *C. acutatum* conidia spread from initially infected seedlings to the neighboring plants by wind or rain. The typical twisting of the main stem, presence of salmon colored spores, and necrotic tissues on the stems were already evident on all plants before blossoming. Symptoms were progressive during pod filling.

What are appropriate strategies considering local situations?

There are no Andean lupine cultivars with anthracnose resistance. Cultivars released by INIAP and local cultivars have low tolerance levels under high disease pressure. If anthracnose resistant lupine varieties are developed, there might still be a risk of severe pod and seed infection under favorable conditions. Thomas *et al*., (2008) demonstrated that application of fungicide on the right time, for instance when there is podding on the primary branches, a significant increase in pod number and yield was found in high anthracnose risk areas of Western Australia. We saw small lesions on the leaves, apical stems or wrinkled leaves (grade two of scale, Chapter 4) in earlier stages of field studies. Wrinkled leaves and small lesions were also seen after spraying (score one and two of scale, Chapter 5). It is better to use fungicides as soon as small lesions appear. There are no studies that quantify the reduction of anthracnose infections and yield losses in Andean lupine by the use of chemicals. Studies must be conducted in the Province of Cotopaxi as well as under less rainy circumstances, such as in the Province of Chimborazo. Previous studies conducted by Peralta *et al*., (2004) indicate that the I-450 cultivar that is adapted well to Province of Chimborazo had double the yield as in our study.

We have shown that the visual selection of seeds is very useful but it does not guarantee 100% healthy seed. The percentage infected seeds used by farmers fluctuates from 14% in case of the seeds with small red-brown stains to 66 % of the non-commercial seeds (Table 3, Chapter 4). This is far above the recommended threshold of 2% for seed-borne pathogens (Aftab *et al*., 2008). The continuous use of infected seed results in the low yield. Since the

commercial seeds are sold, the best alternative is to choose the small red-brown stained seeds and treat them before sowing. A good seed disinfection method is required to reduce initial inoculum. Farmers should be instructed to use thermotherapy methods that do not have any effect on the percentage of germination (Thomas and Adcock, 2004). This thermotherapy can be combined with a chemical treatment for a further reduction of the percentage of infected seeds. These experiences should be assayed with farmers in local centers under a participatory plant breeding program (see interdisciplinary appraisal for the sustainable production of lupine in Figure 1)

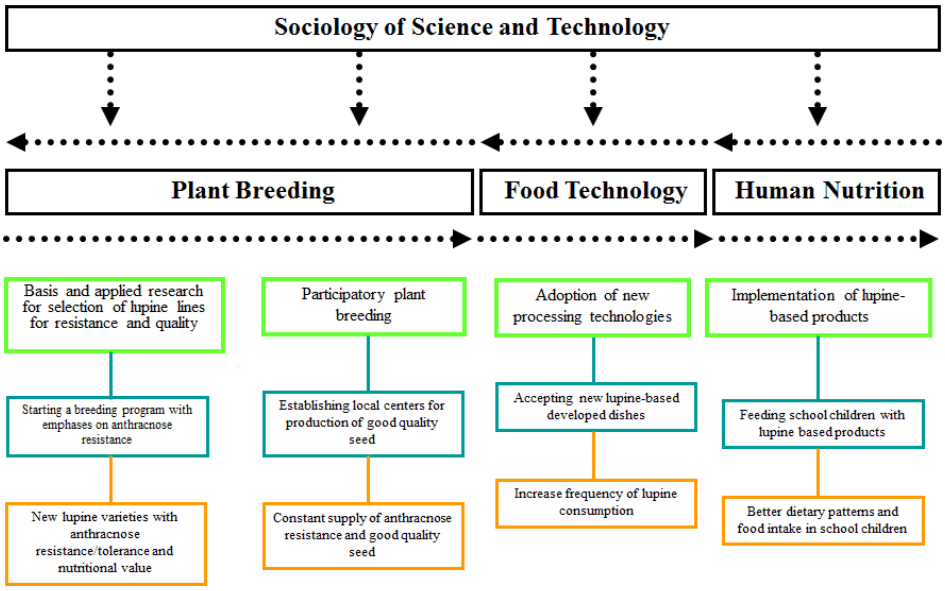

Fig 1. Scientific interdisciplinary framework for an integrated social-technical production, processing, and consumption of lupine (*L. mutabilis*) to achieve Food Sovereignty in Ecuador (TELFUN)

Assessing tolerance of Andean lupine (*Lupinus mutabilis* S.) to anthracnose

Two methods of inoculation were used to evaluate anthracnose disease symptoms in the susceptible lupine cultivar I-450 ANDINO (Chapter 5). Our spraying method mimics the natural situation where spores from infected lupine seedlings or stubbles are splashed by rain and wind (Thomas, 2003). Wharton and Dieguez-Uribeondo (2004) demonstrated that

Colletotrichum penetrates directly the host cuticle and epidermal cell wall by means of a narrow penetration peg that emerges from the base of the appressorium. They indicate that indirect penetration through stomata is rare. It is possible that a brief biotrofic phase occurs when *C. acutatum* isolates were inoculated on the apical parts of lupine. Artificial wounding gave higher anthracnose scores than spraying but doesn't mimic natural conditions and when using the artificial wounding method, the biotrophic phase of the pathogen is likely to be shorter. Niks and Lindhout (2006) recommended the use of wound infection methods to nullify escapes. The results of our research show that both inoculation methods give typical anthracnose symptoms in the apical main stem of lupine (Figure 1, Chapter 5). For screening larger number of plants, we propose the spraying method as a preliminary selection followed by artificial wounding to detect escapes (Table 1).

Anthracnose symptoms were not the same in all phenological stages. More symptoms were found at the end of the cotyledonal stage (0.5-month-old) and at the beginning of flowering (2.5-month-old plants). Infection in 1.5-month-old plants was less severe. We looked in five separate individual phenological stages (Figure 2, Table 1 in Chapter 5). For evaluations of anthracnose tolerance in more genotypes of Andean lupine, we recommend to inoculate plants of 1.0-month-old and confirm the disease severity in 2.5-month-old plants (Table 1) to obtain an overall reaction.

The level of infection in seeds harvested from the six naturally infected lupine cultivars was determined. We divided harvested seeds in three categories: 1) Good quality seed (for commercial purpose suitable), 2) Seeds that have small red-brown stains (known to be anthracnose, Chapter 4), and 3) Non-commercial seed. The fact that seeds are non-commercial isn't always caused by anthracnose, there are also other reasons that seeds end up in the category non-commercial. The level of transmission of the disease into seeds might be partially, genetically determined. As part of the basic and applied research to start a breeding program on anthracnose, we suggest also screening the percentage of commercial seed of infected plants (Table 1).

Basic and applied research to start a breeding program with emphasis on anthracnose resistance

INIAP's collection has 529 lupine accessions from17 different species. More than 200 accessions belong to *L. mutabilis* (Rivera *et al.*, 1998). The steps to explore this collection of *L. mutabilis* are given below and in short in Table 1.

Steps in development of a lupine breeding program for Ecuador

1. Tolerance/resistance to plant infection. Because of the large number of plants to be screened the first selection will use the spraying method. Disease evaluation will be in young plants (one-month-old) and a confirmation when flowering starts (2.5-months-old). Plants will be grown under greenhouse conditions (Chapter 5). Selection will be based on tolerance to infection in apical part of plants. Severity scale from 1 being the most tolerant to 6 being the most susceptible
2. Tolerance to seed transmission of the pathogen. Screen on percentage of commercial seed from infected plants. Seeds are aseptically sown on the surface of PDA in Petri plates with PDA (see methodology in chapter four). Seeds without anthracnose infections will be planted and re-screened. A low seed transmission is a favorable trait and might have a genetic basis and should be selected.
3. Production of homozygous tolerant/resistant plants and/or homozygous plants with a low seed transmission. Seed samples of tolerant/resistant plants will be sown in the soil in a greenhouse. Before blossom the main stem will be bagged to promote self-pollination. Selection will be made based on disease screenings and tolerant/resistant plants will be selfed again (non-infected cuttings).
4. Confirmation of resistance in homozygous populations (all progeny plants must be resistant). The most resistant populations that do not segregate any more will be used for further breeding.
5. Screen for nutritional value (quality). Resistant homozygous lines with high concentrations of Zn and Fe will come out of the screenings.
6. Genetic studies for both anthracnose resistance and seed transmission, high levels of proteins and micro-nutrients. Mapping populations should be developed who make it possible to perform genetic mapping studies for these treats. Identifying associated DNA

markers will make it possible to design a breeding program based on marker assisted selection leading to elite lupine cultivars. Alternatively, a seed production program can be established from consistent tolerant/resistant plants.

7. Explore the possibilities, advantages and difficulties of the production of F_1 hybrids. In a breeding program it might be possible to make hybrids based on one parental line with a high level of tolerance and a parental line with a low seed transmission of the disease.

The interdisciplinary appraisal for sustainable production of lupine in Ecuador helps Food Sovereignty

Our research pays attention to the lupine specific farming system in the Ecuadorian inter Andean zone. Diversity, however, is not limited to the existence of different farming systems but also includes the existence of different production-consumption systems (www.telfun.info). It is notorious that each ethnicity of the different Andean cultures has its own way to produce, process, and consume of lupine (www.telfun.info).

Interdisciplinary research was carried out in local food networks of the Province of Cotopaxi because lupine is mainly produced in that province. The national average yield is around 220kg/ha, but farmers do well if they harvest 100 – 150 kg/ha (see Table 2, Chapter 2). This is because lupine is produced by farmers that do not have good quality seed, no advanced technology for cultivating lupine, and live and farm in places where there is no irrigation water (Table 2, Chapter 2). Seed is phenotypically selected and the seeds with small red-brown stains and bad quality are used for sowing. Commercial seed is sold in nearby towns or to the first buyer. Subsequently, lupine seed requires a soaking, cooking and debittering process. The traditional method of cooking is done in households. The cooked grain is placed in jute pouches and debittered in rivers or watersheds for about 7 days. After removal of alkaloids, the seeds will be used for consumption or processing into other lupine-based dishes. Recently, small and medium entrepreneurs have settled in towns (such as Saquisili 20 km north from Latacunga City, capital of the Province of Cotopaxi) where seed is debittered in cement ponds. The grain is then sold in small portions of 20 g on the streets in Latacunga or other small towns. Lupine processors have formed associations to improve the volume of production, the quality of the product, and achieve a

better price. Currently, lupine is offered in markets and supermarkets as a grain or lupine-based products, such as milk and flour.

Good agronomic methods are present for growing lupine (Peralta *et al*., 2006b, Peralta *et al*., 2008). Several of these experiences have been developed in conjunction with farmers (Peralta *et al*., 2003) and selection resulted in lupine cultivars with a 6-7 month growing period and white seed (Peralta *et al*., 2004). The basic and applied research methods we propose should be integrated in a participatory breeding program (Figure 1) to offer a constant supply of clean lupine seeds. A combined approach that involves active participation of farmers (participatory plant breeding) and more applied and basic research between INIAP and universities, non-government and government organizations is imminent. Farmers need to be empowered of local practices (www.telfun.info) for adequate pest control, fertilization, and the good agronomic practices developed by INIAP (Peralta *et al*., 2003, 2004, 2006b, 2008). Farmers have to be more aware of producing seed of good quality and be skilled in harvest and selection. For managing anthracnose (and other seed borne diseases), farmers need to know the important role of using seed of good quality, contrary to current common practice of use and exchange of bad quality seed (www.telfun.info). New tolerant or resistant varieties must be evaluated in different environments under a participatory breeding program. It is expected that different varieties of lupine have to be developed for different microclimates. Supply of cultivars with different levels of anthracnose tolerance adapted to rainy and drier zones will augment the production and quality of lupine.

Andean lupine cultivars are high in alkaloids. Level of alkaloids in selfed population greatly varied among populations. Anthracnose infection level of self-fertilized lupine populations did not correlate with the content of alkaloids (Table 6, Chapter 5). Pure line development is needed for selection studies of cultivars with low alkaloid content. Andean lupine has a high level of cross-pollination.

The process of lupine debittering takes places close to towns where water supplies (rivers or lakes) are available. Processing is made by small household or family enterprises. As part of this interdisciplinary research, the food science colleague has developed new methods to optimize the processing time of the lupine (www.telfun.info). INIAP has also gained good postharvest processing experience (Peralta *et al*., 2006b, 2008). New products,

such as flour or extruded products were also developed on a small scale in the food science area (www.telfun.info). INIAP has developed new lupine-based dishes (Lara *et al*., 2000). New technologies for debittering and new lupine-based dishes must be reported in local centers to increase the consumption of lupine (Figure 1).

Data obtained in the plant breeding area indicate that the studied lupine cultivars have a good nutritional value (Table7, Chapter2). The effect of lupine based products on anemia, changes in energy balance, nutritional status, and body composition of school children at Guayama Community at the Province of Cotopaxi was studied for the Human Nutrition colleague of the TELFUN program. These cultivars and new products to get her with the traditional "lupine with roasted corn" or "ceviche of lupine" strengthen the nutritional status of children (Figure 1), young students, and pregnant woman in the Ecuadorian Andes.

Small farmers are accustomed to share lupine seeds within local networks. They select the commercial seed for sale, leaving poor quality seed for future sowing. Our data indicate that 90 % of farmers did not know how to screen lupine seed for sowing. Eighty percent of seed is own recycled seed, and 20% of the seed come from exchange with people from surrounding communities (Table 2, Chapter 2). Seed exchange is an ancestral tradition of friendship. However, from a technical point of view exchange of infected seed contributes to anthracnose dispersion to new locations, because anthracnose is a seed-borne pathogen. The colleague in the sociology of science and technology has explored local network for producing, processing, and consuming of lupine in the Province of Cotopaxi. It indicates a disconnection within the social organization of lupine global food chain (www.telfun.info). Lupine is produced in one place and is processed in another locality. Buyers of lupine seeds are related with producers and processors. Upland farmers who want to consume lupine, must buy processed lupine at an unfair high price. For that reason, producers prefer to buy other cheaper and less nutritious food products, such as noodles or cookies. Lupine producers receive little profit and almost no nutritional value from their crop. It is necessary to reconnect the network paradigm into the lupine network. It requires a strong cooperation among plant breeding, food processing, and human nutrition disciplines to develop new technology and implement new findings as being an ensemble of social-technical issues (Figure 1).

Last year the Ecuadorian government issued a law to regulate the sale and consumption of healthy food in bars of the Educational National System (high schools and schools). Lupine is considered a priority food for the preparation of nutritional dishes or for consuming as grain (Acuerdo Interministerial 1-10, 2010). Estimating a population of 3 million students, a daily consumption average of 20 g per person, 60.000 Kg/day of processed lupine grain will be needed. Farmers and processors must be educated on the findings of this interdisciplinary study to optimize management, processing practices, and consumption of lupine. The new "catalystic" lupine varieties will be anthracnose resistant giving social benefits to rural people. Lupine smallholders should be associated to offer good quality seed, meeting demand volumes, receiving a fair price. Seed of these varieties will be processed in less time and under sanitary conditions. Seeds of these varieties will also have high nutritional value. New lupine-based dishes will be prepared. School children and pregnant women will be fed with lupine and new dishes and on-farm sources of protein and zinc will be available for rural people improving their nutritional status (Figure 1). It is clear, that cooperation between disciplines is essential to make this process successful.

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The Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition (TELFUN) project aims to support people to choose their own way of producing, processing, and consuming local foods that fit best in their local conditions. The central research question is: how do technological practices, developed from within food networks, enhance food-sovereignty and the nutritional status of people?, TELFUN is an interdisciplinary and comparative research program based on twelve research projects, divided in four disciplines and carried out in three geographical locations. Its scientific development is divided in four disciplines - plant breeding, food technology, human nutrition, and sociology of science and technology - for a more complete understanding of the production, processing, utilization and consumption of lupine in Ecuador. Similar studies are taking place in cowpea in West Africa and mungbean in India (www.telfun.info). The lupine (*Lupinus mutabilis*) is a native legume of the Andean zone. Like other species of lupine it has easily adapted to marginal regions with limited water supply and poor nourished soils. As compared to other legumes used in human nutrition, lupine seeds have high protein, and variable iron and zinc content. These are just a few attributes that make the Andean lupine be considered a food of the future (www.telfun.info). Disadvantages of lupine are its high susceptibility to anthracnose and its high content of alkaloids (which have to be removed by processing). A specific research question to tackle lupine production in Ecuador was studied in the breeding discipline: How do exploratory studies, both on the pathogen and on the lupine genetic diversity, contribute to the development of better lupine varieties in the Cotopaxi province - Ecuador?

Chapter 1 presents the importance, nutritional value, agronomical characteristics and breeding for anthracnose resistance of lupine in Ecuador. In **Chapter 2**, the actual lupine production, a number of agronomic characteristics, the nutritional value and anthracnose susceptibility of a set of lupine cultivars were determined. The importance of characterizing genetic resources of *L. mutabilis*for crop improvement is outlined. In **Chapter 3**, morphological, molecular and pathological methods were developed to identify the causal agent of anthracnose in lupine and tamarillo (another Andean native crop). Anthracnose diversity and the phylogenetic relationships between isolates of both hosts were studied. The techniques developed can be used to identify and monitor the dominant

Colletotrichum species in the multi cropping Andean system to establish an appropriate disease management and breeding strategies. In **Chapter 4**, anthracnose susceptibility and the disease development of lupine cultivars was assessed under natural infection of *C. acutatum*. Seed samples of naturally infected plants were used to determine the level of anthracnose infection in seeds. Recommendations are made to reduce the impact of the disease in the field and for the appropriate local management of lupine seed until new anthracnose resistant lupine varieties are developed. In **Chapter 5**, the development of methods for screening anthracnose resistance in individual lupine plants are described. Inoculation methods, phenological stages and the relation of resistance and alkaloid content are discussed. In **Chapter 6**, we put in perspective our findings and the appropriate methodologies we developed for starting an Andean lupine breeding program with emphases on anthracnose resistance.

In conclusion, lupine can play an important role in increasing living conditions of the poor farmers in Ecuador. It is needed that the agroecological production systems are maintained by using sustainable farming methods. The use of disease free lupine seed is of utmost importance and a good understanding of the plant– pathogen interactions can increase yield. Lupine breeding should aim at anthracnose resistance, high yield varieties, and cultivars with high levels of protein and micronutrients. Having high-quality lupine cultivars will, together with the results in the other three disciplines - food technology, human nutrition, and sociology of science and technology –contribute to the food sovereignty concept, that is, connect local networks chain for a better production, processing and consumption of lupine (www.telfun.info).

Het TELFUN (Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition) project is opgezet om ondersteuning te geven aan mensen zodat ze lokale gewassen beter kunnen produceren en verwerken. Hierdoor kan de consumptie van lokaal voedsel, wat onder lokale omstandigheden geteeld is, hoger worden. De centrale onderzoeksvraag was: kunnen technologische verbeteringen, ontwikkeld vanuit voedselnetwerken, voedselsoevereiniteit en de voedingsstatus van mensen verbeteren? TELFUN was een interdisciplinaire en vergelijkend onderzoeksprogramma gebaseerd op twaalf onderzoeksprojecten, die verdeeld waren in vier disciplines en die uitgevoerd zijn op drie verschillende geografische locaties. De vier disciplines waren - plantenveredeling, levensmiddelentechnologie, voeding en sociologie van wetenschap en technologie – deze disciplines geven o.a. een beter begrip van productie, verwerking en consumptie van lupine in Ecuador. Overeenkomstige studies hebben plaats gevonden in West Afrika met cowpea en in India met mungbean (www.telfun.info).

Lupine (*Lupinus mutabilis*) is een lokale peulvrucht uit de Andes-zone. Het heeft met andere lupines gemeen dat het zich aangepast heeft aan marginale omstandigheden met beperkte hoeveelheden water en arme gronden. Vergeleken met andere peulvruchten hebben lupine zaden een hoog gehalte aan eiwitten en een variabele concentratie ijzer en zink. Dit zijn enkele van de eigenschappen die deze lupine uit de Andes een veelbelovend voedsel maken (www.telfun.info). Nadelen van deze lupine zijn de gevoeligheid voor anthracnose en het hoge gehalte aan alkaloïden (die voor consumptie verwijderd moeten worden). In de veredelingsdiscipline werd de vraag gesteld: Hoe kunnen verkennende studies, zowel aan de pathogeen kant als aan de lupine kant, bijdragen aan de ontwikkeling van betere lupine rassen geschikt voor de Cotopaxi provincie in Ecuador?

Hoofdstuk 1 behandelt het belang van lupine, de voedingswaarde, agronomische eigenschappen en het veredelen op anthracnose resistentie in Ecuador. In **hoofdstuk 2** worden de actuele lupine productie, een aantal agronomische eigenschappen, de voedingswaarde en de anthracnose vatbaarheid van een aantal lupine rassen beschreven. Het belang van een inventarisatie van de genetische bronnen ten behoeve van *L. mutabilis* gewasverbetering wordt benadrukt. In **hoofdstuk 3** worden morfologische, moleculaire en pathologische methoden beschreven om de causale oorzaak van anthracnose in lupine en tamarillo (een ander gewas komend uit de Andes) beter te kunnen identificeren. De

anthracnose diversiteit en de fylogenetische verwantschap tussen isolaten verzameld op bovengenoemde gastheren wordt beschreven. Deze technieken maken het mogelijk de belangrijkste Colletotrichum soorten te identificeren, dit is nodig voor een goede anthracnose beheersing in het Andes systeem met veel verschillende gewassen. Het kunnen onderscheiden van verschillende isolaten is ook belangrijk bij het veredelen op anthracnose resistentie. **Hoofdstuk 4** beschrijft de anthracnose gevoeligheid van lupine rassen en de ziekte ontwikkeling na een spontane infectie door *C. acutatum*. Zaadmonsters van geïnfecteerde planten, na een natuurlijke infectie, zijn gebruikt om het niveau van anthracnose infectie in de zaden te bestuderen. Aanbevelingen worden gemaakt om de impact van anthracnose zo gering mogelijk te houden. Dit is van belang zolang er nog geen anthracnose resistente lupine rassen zijn ontwikkeld. In **hoofdstuk 5**, worden de ontwikkelde methoden beschreven om op anthracnose resistentie te kunnen screenen in individuele lupin planten. Inoculatie methoden, het effect in verschillende stadia van plant groei en de relatie tussen resistentie en alkaloïde gehalte worden bediscussieerd. In **hoofdstuk 6** worden onze data en methodes in een breder perspectief besproken. Speciale aandacht wordt gegeven aan die methodes die het mogelijk maken een veredelingsprogramma te beginnen in Ecuador gericht op het ontwikkelen van lupine rassen met anthracnose resistentie.

Lupine kan een belangrijke rol spelen bij het verbeteren van de levensomstandigheden van arme boeren in Ecuador. Daarvoor is het nodig dat de agro-ecologische productie systemen blijven bestaan. Het gebruik van ziektevrije lupine zaden is uiterst belangrijk en een goed begrip van plant-pathogeen interacties kunnen de opbrengst verhogen. Lupine veredeling moet zich richten op anthracnose resistentie, hogere opbrengst, en op hoge eiwit- en micronutriëntenniveaus. Kwalitatieve goede lupine rassen, kunnen samen met de uitkomsten van de andere drie disciplines - levensmiddelentechnologie, voeding , en sociologie – bijdragen aan het voedselsoevereiniteit concept, en lokale netwerken gebruiken voor een betere productie, verwerking en consumptie van lupine (www.telfun.info).

El proyecto adaptación de las Ciencias de la Alimentación de los patrones endógenos de suministro local de alimentos para la nutrición futuro (TELFUN por sus siglas en Ingles) tiene por objeto contribuir para que las personas elijan su propio modo de producción, procesamiento y consumo de alimentos locales que mejor se adapten a sus condiciones locales. La pregunta central de ésta investigación es: ¿cómo las prácticas tecnológicas, desarrolladas desde el interior de las redes de alimentos mejoraran la alimentación y la soberanía del estado nutricional de las personas?, TELFUN es un programa de investigación interdisciplinario y comparativo basado en doce proyectos de investigación, dividido en cuatro disciplinas y llevado a cabo en tres zonas geográficas. Su desarrollo científico se divide en cuatro disciplinas – mejoramiento genético vegetal, tecnología de alimentos, nutrición humana y sociología de la ciencia y la tecnología - para una comprensión más completa de la producción, transformación, utilización y consumo del chocho en el Ecuador. Estudios similares están desarrollándose cawpi en el África Occidental y frijol mungo en la India (www.telfun.info).

El chocho (*Lupinus mutabilis*) es una leguminosa nativa de la zona andina. Al igual que otras especies de chocho es de fácil adaptación a las regiones marginales con limitado suministro de agua y en suelos pobres en nutrientes. En comparación con otras leguminosas utilizadas en la alimentación humana, las semillas de chocho tienen alto porcentaje de proteína y variable contenido de hierro y zinc. Estos son sólo algunos atributos que hacen que el chocho andino sea considerado un alimento del futuro (www.telfun.info). Entre las desventajas, el chocho es muy susceptibilidad a la antracnosis y tiene alto contenido de alcaloides (que deben ser eliminadas mediante procesamiento). En el área de mejoramiento genético vegetal se planteó una pregunta especifica de investigación para abordar la producción de chocho en el Ecuador: ¿Cómo estudios exploratorios, tanto en el patógeno, como en la diversidad genética del chocho contribuirán al desarrollo de mejores variedades de chocho en la provincia de Cotopaxi - Ecuador?

El **capítulo 1** presenta la importancia, valor nutritivo, características agronómicas y de mejoramiento para resistencia a la antracnosis del chocho en el Ecuador.En el **capítulo 2,** se determina la producción actual de chocho, una serie de características agronómicas, valor nutricional y la susceptibilidad a la antracnosis de un conjunto de cultivares de chocho.Se describe la importancia de la caracterización de los recursos genéticos para el

mejoramiento genético del chocho *L. mutabilis*. En **capítulo 3,** se detallan métodos morfológicos, moleculares y patológicos aquí desarrollados para identificar el agente causal de la antracnosis en chocho y tomate de árbol (otro cultivo nativo andino). Se estudió la diversidad de la antracnosis y las relaciones filogenéticas entre aislamientos de ambos hospederos. Las técnicas desarrolladas pueden utilizarse para identificar y controlar las especies de Colletotrichum dominante en el sistema mixto de cultivo de la zona andina para así establecer un manejo adecuado de la enfermedad y estrategias de mejoramiento. En el **capítulo 4**, se evaluó la susceptibilidad a la antracnosis y el desarrollo de la enfermedad en cultivares de chocho causado por infecciones naturales de *C. acutatum.*Luego se usaron muestras de semillas de plantas naturalmente infectadas para determinar el nivel de infección de antracnosis en semillas. Se hacen recomendaciones para reducir el impacto de la enfermedad en el campo y para el adecuado manejo local de semillas de chocho mientras se desarrollen nuevas variedades de chocho con resistencia a la antracnosis. En el **capítulo 5**, se describe el desarrollo de métodos que permitan seleccionar plantas individuales de chocho con resistencia a la antracnosis. Se discuten métodos de inoculación, estados fenológicos de la planta y la relación entre la resistencia fenotípica de la planta con el contenido de alcaloides en la semilla. En el **capítulo 6**, se pone en perspectiva los resultados y las metodologías apropiadas que hemos desarrollado para dar inicio a un programa de mejoramiento genético del chocho andino con énfasis en resistencia a la antracnosis.

En conclusión, el chocho puede jugar un papel importante en el aumento de las condiciones de vida de los agricultores pobres de Ecuador. Para ello, es necesario que los sistemas de producción agroecológica se mantengan mediante el uso de métodos agrícolas sostenibles. El uso de semillas de chocho libres de enfermedades es de suma importancia, una buena comprensión de las interacciones planta-patógeno puede aumentar el rendimiento. El mejoramiento del chocho debe tener como objetivo la resistencia a antracnosis, variedades de alto rendimiento y cultivares con altos niveles de proteínas y micronutrientes. Al tener alta calidad de cultivares de chocho, junto con los resultados en las otras tres disciplinas - tecnología de alimentos, nutrición humana y sociología de la ciencia y la tecnología - contribuirán con el concepto de soberanía alimentaria, es decir, conectar la cadena de redes locales para una mejor producción, elaboración y el consumo de chocho (www.telfun.info).

My PhD study could never be fulfilled without the full support from my family. I would like to express my deepest sense of gratitude to my wife, Monica for her moral support and patience during my study. I would like to express my profound gratitude to my beloved parents for their continuous emotional support and prayers during my study abroad.

There are many people to whom I wish to say special words: first of all, I would like to thank my advisor Dr. Adriaan W. (Sjaak) van Heusden, for his continuous encouragement, guidance, patience and friendshipthroughout my graduate study, and "promotor" Dr. Richard G.F.Visser for your valuable advise throughout this research. Sjaak your door was always open when many times I dropped in asking for guidance. Richard thank you for your comments to improve the scientific writing of my thesis. My special thanks also go to Dr. Leonardo Corral, my local supervisor, for his ideas and suggestions.

I want to thank the financial support given by The Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition - TELFUN project of the Interdisciplinary Research and Education Fund of Wageningen University WUR/INREF to complete my PhD studies at Wageningen University. Thank you to Plant Ecology and Resource Conservation (PE&RC) graduate school for giving me a good learning framework to contribute with my scientific development. Thank you to Army Polytechnic School for allowing me the permission to continue my PhD studies at Wageningen University, to provide partial support and the facilities to develop part of the research under local conditions. To InstitutoAgropecuario Simon Rodriguez for giving me the facilities to conduct some field experiments.

I am thankful to Eduardo Peralta for sharing technical information to build the basis of this study and he and his teamwork of the "Legume National Program" of INIAP for their help in field studies.

It is a pleasure to convey my gratitude to some of my undergraduate students: Andrés Cáceres, Iván Peralta, Lilian Maila, Cynthia Rosas, Verónica González, and José Proaño. Thank you for your kind help in collecting data in a friendly atmosphere. Special thanks to

my officemates Abraham Oleas and Pablo Landazuri for their opinions in specific topics of this research. Thank you also to Jaime Villacís and Gabriel Suarez for their opinions in statistical analysis and experimental design.

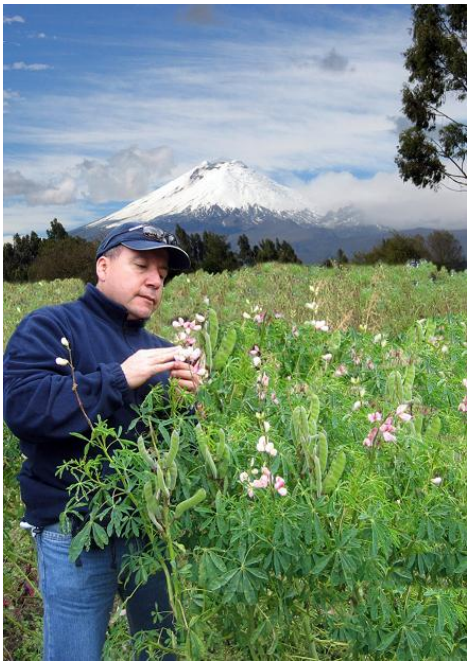
My gratitude goes also to the manager of the TELFUN project, and the whole TELFUN family that embraces supervisors of Holland, West Africa, and India and my doctoral colleagues for sharing their comments and experiences in the workshops in the desire to build the interdisciplinary research.

My special thanks go to Dr. Pedro Martinez-Culebras and Dr. Ana Crespo from IATA, Universidad de Valencia, Spain for introducing me to the molecular diagnosis and characterization of fungal plant pathogens.

I wish to mention the support provided by many people working at Plant Breeding Laboratory of Wageningen University, as well as that from my friends and colleagues at Army Polytechnic School, to accomplish my PhD. To all of you my gratitude.

To whom my brain does not remember at this moment, but you live in my heart.

Cesar E. FalconiSaa
Wageningen, March 2012



Cesar Eduardo Falconi was born in Riobamba, 200 km south from Quito, Ecuador. He obtained a degree in Agronomical Engineer from the Escuela Politecnica de Chimborazo University (ESPOCH) in Riobamba, Ecuador in 1987. His thesis focused screening for efficient *Rhizobium* strains in lupine (*Lupinus mutabilis*). Later that year, he studied nitrogen fixation of legumes during an internship at the Universidad Federal de Rio Grande do Soul, Brazil, and performed research in biological control and served as an assistant professor of soil microbiology at ESPOCH.

Under a USAID scholarship, Falconi earned a master´s degree in plant pathology from Oregon State University (OSU), in 1996; Dr. Kenneth B. Johnson supervised his studies at OSU.

Biological control of plant diseases dominated Falconi's career at that time; his master's of science thesis focused on using epiphytic yeast of apple leaves to biologically control blue and gray mold in apples. Later that year, Falconi served as associate professor of plant pathology on the Agropecuarian Science Faculty at the Army Polytechnic School (ESPE) in Ecuador. Based on his professional qualifications, Falconi was appointed head of the research department in 1999. During his eight-year tenure in this position, Falconi administrated the agropecuarian research processes, delineated research lines, sought financial support to establish the laboratories for the Agropecuarian Faculty and organized the master of sciences programs in plant protection and biological control. As part of the master's programs, Falconi's study of monilia pot rot in "arriba flavor" cocoa resulted in the development of two bio pesticides that efficiently control the disease.

In 2003, Falconi earned a second master´s degree in Business Administration at ESPE. In 2006, he joined the project development team for integrated management of Ecuador's apple industry. Studies were conducted in seven Ibero-American countries with funds the Cooperación Iberoamericana Ciencia y Tecnologia para el Desarrollo (CYTED). Several publications took along of these studies.

Plant breeding for disease resistance and associated molecular and genetic strength became Falconi's latest area of study. Under the supervision of Dr. A.W. (Sjaak) van Heusden and Dr. Prof. Richard G.F. Visser, he started a Ph.D. at the plant breeding laboratory at

Wageningen University and Research Centre in the Netherlands. His research of *Lupinus mutabilis* in Ecuador, with special emphases on anthracnose resistance, was financed by Wageningen University/ INREF as part of TELFUN, the interdisciplinary research project. The innovative results about the host-pathogen interactions to start a breeding program with emphases on anthracnose resistance are described in this thesis.

Following the Ph.D. defense, Falconi will serve as principal professor and researcher of plant pathology at ESPE in Ecuador. A new grant has been awarded by McNight Foundation that will allow Falconi to continue his studies in molecular breeding of anthracnose resistance of Andean lupine for the next three years in conjunction with INIAP/PRONALEG.

Honors and Awards

* Outstanding research, ESPE University, 2010.
* Honorable mention, II National Technology Innovation Competition organized by UNESCO, Project Biological control of monilia pot rot in "arriba flavor" cocoa. January 2002.
* Winner of the scientific research competition ESPE – 2002.

Key Research

My research goal is to explore ways to improve breeding for multiple traits, such as disease resistance and other important agronomic characteristics and to develop high-quality, disease-resistant seeds. I also have a strong interest in the use of molecular techniques to diagnose fungal plant pathogens. In my research, I also apply my previous knowledge of biocontrol to reduce the impact of plant disease pathogens in places where susceptible and moderate resistant plants prevail.

Funding Awards

Between 2000 to 2004, I was awarded two competitive grants for $350,000 and $75,000' both from the World International Bank through the Programa de Modernizacion de los Servicios Agropecuarios project in Ecuador. The first grant established the master's of science programs in biological control at ESPE from which twenty-five Ecuadorian professionals obtained master's of science degrees. The second grant funded research into the biocontrol of monilia pot rot in cocoa, a major Ecuadorian crop, and resulted in producing two efficient bio pesticides that control the disease.

Cesar E. Falconi
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List of publications

Falconí C, Galvez F y Landázuri P. (2009) Biofortificación de seis genotipos promisorios y el cultivar INIAP- 450 de chocho (*Lupinus mutabilis* Sweet) mediante la aplicación de quelatos de hierro y zinc. *ESPE Ciencia y Tecnología* 2 (2): 15 – 20

Di Pietro R**, Falconi C.E.** (2009) Producción orgánica de manzana. 120-132p . Capitulo X. In: Manejo Integrado de Doenças da Macieira. Stadnik M (Editor). U. Santa Catarina – Brasil, 196p.

Mondino P, Di Massi S, **Falconi C.E.,** Montealegre J, Henriquez J, Nunes C, Salazar M, Stadnik M, Vero S, Usall J. (2009) Manual de identificación de enfermedades de manzana en poscosecha, Universidad de la Republica, Montevideo – Uruguay, 67p.

Falconí, C.E.; Yánez, V. (2007) Validación de biopesticidas para el control de la moniliasis en cacao fino de aroma var. Tenguel 25. Señal Ediciones, 97p.

Falconí, C.E.; Oleas, A.; Yánez, V. (2006) Validación de biopesticidas para el control de la moniliasis en cacao fino de aroma var. Tenguel 25. *Ciencia*: 90 (1) 25 – 32.

Abstracts

Falconi C.E.,Visser R.G.F., van Heusden A.W. (2011) Morphological, pathological and molecular characterization of lupine anthracnose and its relationship with tamarillo anthracnose in Ecuadorian Andes. American Phytopathological Society Annual Meeting, *Phytopathology*: 101 No. 6 (Supplement), S51.

Falconi C.E., Rosas C., van Heusden A.W. (2009) Preliminarily characterization of *Colletotrichum* isolates causing anthracnose in lupine and other native hosts from Ecuador and South America, "International Conference on Grain Legumes: Quality Improvement, Value Addition and Trade", Kanpur, India, 221-221p.

Falconi C.E., Maila L, van Heusden A.W. (2008) Comparison of two inoculation techniques in five phenological stages, with three *Colletotrichum* isolates for determine resistance to this fungus in lupine (*L. mutabilis*) under greenhouse conditions, IV International Conference on Legume Genomics and Genetics, Puerto Vallarta, Mexico, 112p.

Falconí, C.E.; Oleas, A.; Yánez, V. (2004). Biological Control of Monilia Pod Rot (*Moniliophthora roreri*) on "arriba flavor"cocoa with *Bacillus subtillis* and *Pseudomonas cepacea.* American Phytopathological Society Annual Meeting, *Phytopathology*: 94 (Supplement), S28.

Research programme "Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition (TELFUN)"

The research for this thesis has been part of the programme called Tailoring Food Sciences to Endogenous Patterns of Local Food Supply for Future Nutrition (TELFUN). This is one of the PhD research programmes sponsored by the Interdisciplinary Research and Education Fund (INREF) of Wageningen University. Through INREF, Wageningen University aims to stimulate development-oriented interdisciplinary research and education through programmes designed and implemented in partnership with research institutes in developing countries and emerging economies. The programmes aim to build relevant capacity in local research institutions to solve complex, development related problems. The TELFUN programme was designed to study technological practices to support the development of local food networks. Resilient and sustainable food networks were thought to contribute to food sovereignty, a key concept in the TELFUN programme. In the research programme plant breeders, food technologists, human nutritionists and social scientists from three different regions, namely India, West Africa (Ghana and Benin) and Ecuador worked together. The main partners in our programme were Haryana Agricultural University, Hisar, Haryana, India; Crops Research Institute of the Council for Scientific and Industrial Research (CSIR), Accra, Ghana; Noguchi Memorial Institute for Medical Research, University of Legon, Ghana; Science and Technology Policy Research Institute (STEPRI), Accra, Ghana; Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, Cotonou, Benin; Escuela Politécnica del Ejército (ESPE), Quito, San Francisco University, Quito, Ecuador, and Universidad Politécnica Salesiana del Ecuador. The groups involved from Wageningen University were Plant Breeding, Product Design and Quality Management, Laboratory of Food Microbiology, Human Nutrition, and Critical Technology Construction, Sociology and Anthropology of Development.

Education Statement of the Graduate School
Production Ecology and Resource Conservation

Issued to: Cesar E. Falconi
Date: 22 March 2012
Group: Plant Breeding, Wageningen University

1) Start-up phase	*date*
► First presentation of your project	
Lupinus mutabilis in Ecuador with special emphasis on anthracnose resistance	Feb-May, 2007
► Writing or rewriting a project proposal	
Lupinus mutabilis in Ecuador with special emphasis on anthracnose resistance	
► Project: starting a breeding program for anthracnose resistance of *L. mutabilis* in Ecuador	2011
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► EPS PhD student days	
► Seminars (series), workshops and symposia	
First TELFUN workshop, Wageningen – The Netherlands	February, 2007
Second TELFUN workshop, Quito – Ecuador,	January, 2008
Third TELFUN workshop, Hissar – India	February,2009
Fourth TELFUN workshop, Tamale-Ghana, West Africa	January, 2010
Fifth TELFUN workshop, Cotonou-Benin, West Africa	October, 2011
► International symposia and congresses	
IV International Conference on Legume Genomics and Genetics, Puerto Vallarta, Mexico	December, 2008
International Conference on Grain Legumes: Quality Improvement, Kanpur, India	February, 2009
American Phytopathological Society Meeting, Honolulu, Hawaii, USA	August, 2011
► Presentations	
Oral presentation III Seminario sobre Doencias da Maceira, Florianopolis, Brasil	June, 2007
Oral presentation Manejo integrado de enfermedades del manzano, Santiago, Chile	November, 2008
Poster presentation IV ICGL, Puerto Vallarta, México	December, 2008
Poster presentation IIPR, Kanpur, India	February, 2009
Poster presentation American Phytopatological Society, Honolulu, Hawaii USA	August, 2011
► Excursions	
Excursión to the Zeeuwse Vlegel in Zeeland	April, 2008
Subtotal ScientificExposure	*19.7 credits**

3) In-Depth Studies	*date*
► EPS courses or other PhD courses	
XI Curso Internacional sobre conservación y utilización de los recursos filogenéticos para la agricultura y la alimentación, INIA, Madrid, Spain	Nov 2-25,2005
Philosophy and ethics of food science and technology	Mar-Apr., 2007
Multivariate analysis	April, 2008
Mixed effects-models	Dec.16-20,2010
► Journal club	
IV and V Congreso de Ciencia y Tecnologia, ESPE University – Quito (paper published)	2009-2010
► Individual research training	
Workshop on molecular characterization of *Colletotrichum*, Valencia, Spain	June, 2008
Subtotal In-Depth Studies	*8.5 credits **

4) Personal development	*date*
► Skill training courses	
Working with EndNote	May 14-15,2007
Presentation skills	May 7/26, 2008
► Organisation of PhD students day, course or conference	
PE&RC PhD Weekend,	March, 2007
Presentation of thesis preliminary results in the Bi-weekly seminar, Plant Breeding Group	September, 2009
Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**39,9**

Herewith the Graduate School declares that the PhD candidate has complied with the educationalrequirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits. ** A credit represents a normative study load of 28 hours of study*

The research described in this thesis was carried out at the Agropecuarian Science facility (IASA-I) at Army Polytechnic School and the Plant Breeding Laboratory at Wageningen University. The research was funded by TELFUN/INREF Wageningen University, the Netherlands and Army Polytechnic School (ESPE) Quito, Ecuador.

Cover art: by Cesar E. Falconi
The cover art was inspired by the topic of this thesis: *Lupinus mutabilis* in Ecuador with special emphasis on anthracnose resistance.

Front cover page represents the wanted anthracnose-resistant lupine plant on the left side, in contrast with the traditional, susceptible lupine genotypes on the right side. The salmon pink spores of Colletotrichum re-suspended in a cloud of air and steam represent the pathogen ready to infect susceptible lupine plants. The lupine plant represents the food sovereignty of people in the Andean region. The two hands that surround the earth illustrate the important role that Andean lupine plays as an affordable source of nutrition for the world population.

Back cover page represents good quality seed (for commercial purpose suitable) in contrast with bad seed (not commercial useful), and seed with small red-brown stains (suggesting that the fungus can also be present under the seed coat and may also be in cotyledonal leaves).