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Exploring fungal pathogens to control invasive raspberry (*Rubus niveus*) in Galapagos Islands.

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**Exploring fungal pathogens to control invasive raspberry (*Rubus niveus*)
in Galapagos Islands.**

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Dedico este trabajo a mis padres, por haberme dado su amor y apoyo en todo momento y enseñarme siempre a salir adelante, y a mis hermanos por su apoyo incondicional

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RESUMEN

ARTICULO 1

La Invasión de *Rubus niveus* en las Islas Galápagos

La conservación de las Islas Galápagos es importante para Ecuador y otros países debido a la presencia de una gran biodiversidad de especies nativas y endémicas, incluidos animales y plantas terrestres y marinas, que están amenazados por la introducción de especies invasoras y actividades antropogénicas. Hay muchas plantas endémicas como *Scalesia*, *Opuntia*, tomatillo, etc., que se están perdiendo debido a su baja diversidad genética y polinizadores no específicos, lo que las hace más susceptibles a las plantas invasoras. El crecimiento fácil y rápido, las semillas con alta viabilidad en el suelo, los múltiples mecanismos de reproducción (sexual y asexual) y su adaptación a diversas condiciones son las características que hacen que una planta sea invasiva. Además, las características geológicas y climáticas presentes en el archipiélago favorecen el crecimiento de especies invasoras, debido a la humedad, temperatura y presencia de nutrientes en el suelo. *Rubus niveus*, conocido como mora, se considera la peor maleza presente en las Islas Galápagos, ya que está desplazando muchas especies endémicas, por lo que se buscan diferentes alternativas de control ya que el método químico y manual no son suficientes para la erradicación, por eso los objetivos de este manuscrito trata sobre hechos importantes sobre especies invasoras de plantas en entornos frágiles como las Islas Galápagos, en el contexto de las condiciones climáticas y geográficas del archipiélago, la distribución de plantas y la adaptación de plantas invasoras, especialmente la mora que es considerado el peor invasor. Además, se están revisando los métodos de control y las posibles alternativas que podrían lograr resultados favorables, como el control biológico.

Palabras clave: Galápagos, especies invasoras, *Rubus niveus*, control biológico.

ARTICULO 2

Explorando patógenos fúngicos para controlar la mora invasiva (*Rubus niveus*) en las Islas Galápagos

El ecosistema de Galápagos está amenazado por la introducción de especies invasoras que desplazan a especies nativas y endémicas. *Rubus niveus* (conocido como mora) se considera una planta invasora y es la peor amenaza porque tiene el potencial de dañar el ecosistema y el sistema económico del archipiélago. Se introdujo por primera vez en 1960 en Santa Cruz y cubre aproximadamente 30,000 hectáreas en todo el archipiélago. Su rápida capacidad de crecimiento permite desplazar la vegetación nativa y su control no tiene éxito mediante la eliminación manual y la aplicación de herbicidas, lo que provoca cambios no deseados en el suelo. Por esta razón, es necesario encontrar una

alternativa para controlar la propagación de *R. niveus*, por lo que el control biológico clásico (CBC o biocontrol) se ha propuesto como un método para suprimir la población de especies invasoras no nativas, ampliamente distribuidas. En esta investigación, el objetivo fue aislar hongos de la mora enferma de San Cristóbal, encontrar un posible candidato como control biológico, reducir costos y evitar la introducción de especies exógenas en la Isla. Se obtuvieron 595 hongos aislados de los cuales se separó en 226 grupos por fenotipo para evaluar la patogenicidad contra las hojas de mora. Seis posibles candidatos fueron encontrados e identificados por morfología y ADN usando los cebadores ITS, TUB, TEF1a, GAPDH y RPB2. Para confirmar la especie, se realizó un análisis filogenético utilizando inferencia bayesiana. Las especies encontradas fueron *Bionectria pseudostrata*, *Lasiodiplodia theobromae*, *Colletotrichum gloesporoides*, *Fusarium concentricum*, *Phanerochaete chrysosporium* y *Penicillium rolfsii*, que se consideran organismos cosmopolitas.

Palabras clave: Galápagos, especies invasoras, *Rubus niveus*, control biológico, hongos, análisis filogenético.

ABSTRACT

ARTICLE 1

The invasion of *Rubus niveus* to the Galapagos Islands

The conservation of the Galapagos Islands is important for Ecuador and other countries due to the presence of a large biodiversity of native and endemic species, including land and marine animals and plants, which are threatened by the introduction of invasive species and anthropogenic activities. There are many endemic plants such as *Scalesia*, *Opuntia*, tomatillo, etc., which are being lost due to their low genetic diversity and non-specific pollinators, which makes them more susceptible to invasive plants. The easy and fast growth, seeds with high viability in the soil, multiple reproduction mechanisms (sexual and asexual) and their adaptation to various conditions are the characteristics that make a plant invasive. In addition, the geological and climatic characteristics present in the archipelago favor the growth of invasive species, due to the humidity, temperature and presence of nutrients in the soil. *Rubus niveus*, known as raspberry, is considered the worst weed present in the Galapagos Islands, because it is displacing many endemic species, so different control alternatives are sought since the chemical and manual method are not sufficient for eradication, that is why the objectives of this manuscript are to discuss important facts about invasive species of plants in fragile environments such as the Galapagos Islands, in the context of the climatic and geographical conditions of the archipelago, the distribution of plants and the adaptation of invasive plants, especially the raspberry which is considered the worst invader. In addition, control methods and possible alternatives that could achieve favorable results, such as biological control, are being reviewed.

Key words: Galapagos, invasive species, *Rubus niveus*, biological control

ARTICLE 2

Exploring fungal pathogens to control invasive raspberry (*Rubus niveus*) in Galapagos Islands.

The Galapagos ecosystem is threatened by the introduction of invasive species that displace native and endemic species. *Rubus niveus* (known as raspberry) is considered an invasive plant being the worst threat because it has the potential to damage the ecosystem and the economic system of the archipelago. It was first introduced in 1960 to Santa Cruz and covers approximately 30,000 hectares throughout the archipelago. Its rapid growth capacity allows to displace native vegetation and its control is unsuccessful by manual removal and herbicide application, causing unwanted changes in the ground. For this reason, it is necessary to find an alternative to control the propagation of *R.*

niveus, which is why the classic biological control (CBC or biocontrol) has been proposed as a method to suppress population of non-native invasive species, widely distributed. In this investigation the objective was to isolate fungi from the sick raspberry of San Cristobal, to find a possible candidate as a biological control, reducing costs and avoiding the introduction of exogenous species to the Island. 595 isolated fungi were obtained from which it was separated in 226 groups per phenotype to test for pathogenicity against raspberry leaves. Six possible candidates were found and identified by morphology and DNA using the ITS, TUB, TEF1a, GAPDH and RPB2 primers. To confirm the species, a phylogenetic analysis was performed using Bayesian inference. The species found were *Bionectria pseudostrata*, *Lasiodiplodia theobromae*, *Colletotrichum gloesporoides*, *Fusarium concentricum*, *Phanerochaete chrysosporium* and *Penicillium rolfsii*, which are considered as cosmopolitan organisms.

Key words: Galapagos, invasive species, *Rubus niveus*, biological control, fungi, phylogenetic analysis

TABLA DE CONTENIDO

Dedicatoria	4
Agradecimientos	5
RESUMEN	6
ABSTRACT	8
Tabla de contenido	10
índice de TABLAS	11
ÍNDICE DE FIGURAS	12
ARTICULO 1	13
INTRODUCTION	13
REFERENCES	31
ARTICULO 2	36
INTRODUCTION	36
MATERIALS AND METHODS	41
RESULTS	45
DISCUSSION	66
CONCLUSION	72
REFERENCES	74

ÍNDICE DE TABLAS**ARTICLE 2**

Table 1. Phenotypic and microscopic identification of fungi isolated from <i>Rubus niveus</i> samples (PDA culture medium).	49
Table 2. Molecular identification using BLAST of the isolated fungi based on the sequence of the markers ITS, RPB2, TUB, GADPH region.....	58

ÍNDICE DE FIGURAS

ARTICLE 1

Figure 1: Galapagos ecosystems divided into zones according to the distribution of plant communities and precipitations (FCD & WWF, 2018).	16
Figure 2: Agriculture Zone in the Galapagos Islands (DPNG, 2014)	20
Figure 3: Raspberry's Distribution on the Galapagos Islands (PNG & WWF, 2018).....	25
Figure 4: a) <i>Rubus niveus</i> in Galapagos. b) Vegetative reproduction by shoots of <i>Rubus niveus</i>	27

ARTICLE 2

Figure 1. a) Collection Map of <i>Rubus niveus</i> samples with disease symptoms (80 sites). b) <i>Rubus niveus</i> growing on the roadside of San Cristobal Island and covering other plant species.	41
Figure 2. Samples collected from <i>Rubus niveus</i> with the presence of disease symptoms (San Cristóbal Island).	45
Figure 3. a) Fungi isolated from the <i>Rubus niveus</i> samples. B) The number of samples, isolated fungi and fungi with only morphology obtained from leaves, stems and fruits.	46
Figure 4. Pathogenicity tests inoculating 5 μ l of the fungus with a concentration of 1×10^6 conidia/ml.	47
Figure 5. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and β -tubulin sequences of 69 strains belonging to the <i>Bionectria</i> genus based on Schroers 2001. T.....	60
Figure 6. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and GAPDH sequences of 53 strains belonging to the <i>Colletotrichum</i> genus based on Weir et al, 2012.....	61
Figure 7. Phylogenetic tree obtained from Bayesian analysis of the ITS sequences of 100 strains belonging to the <i>Lasiodiplodia</i> genus based on Bautista-Cruz et al, 2018..	62
Figure 8. Phylogenetic tree obtained from Bayesian analysis of the TEF1a, TUB and RPB2 sequences of 83 strains belonging to the <i>Fusarium</i> genus based on Sandoval et al, 2018.	63
Figure 9. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and RPB2 sequences of 48 strains belonging to the <i>Phanerochaete</i> genus based on Floudas & Hibbett, 2015.	64
Figure 10. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and RPB2 sequences of 57 strains belonging to the genera <i>Penicillium</i> based on Samson & Houbraken, 2011.....	65

ARTICULO 1

The Invasion of *Rubus niveus* to the Galápagos Islands

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INTRODUCTION

Galapagos Islands: a unique treasure for biodiversity.

The Galapagos archipelago was discovered in 1535 by Bishop Tomás de Berlanga. Subsequently, the archipelago was annexed to Ecuador in 1832 and declared a province in 1973 (Consejo de Gobierno del Régimen Especial de Galápagos, 2016).

This archipelago, located 972 kilometers south of the equatorial line in the Pacific Ocean, was formed by underwater volcanic eruptions. It consists of 13 large islands (area greater than 10 km²), 5 medium islands (area from 1 km² to 10 km²), and 216 small islands or islets (area less than 1 km²), respectively, covering a total area of 7985 km² (DPNG, 2014). The islands of San Cristobal and Española are considered the oldest, formed 2.8-5.6 million years ago, whereas the most recent islands, Fernandina and Isabela, emerged 60-300 thousand years ago (Geist, 1996).

The isolation of the archipelago, together with the volcanic activity, facilitated the development and existence of a unique but fragile biodiversity. The secluded evolution of the islands' flora and fauna, which has remained undisturbed for thousands to millions of years (PNG, 1998), has resulted to be designated as Natural Patrimony of

Humanity (declared by UNESCO in 1979). To preserve this fragile ecosystem, both the Ecuadorian state and International Organizations are responsible for its protection (ABG, 2015).

In 1959, Ecuador decided to create the Galapagos National Park (PNG) with the aim to protect 97% of the land and marine areas, allowing solely the remaining 3% to be available for human activities (occupying the Santa Cruz, San Cristobal, Floreana, and Isabela Islands) (Black, 1973). Since then, several conventions have been designated to state the threat of invasive species and physical disturbances caused by human activity to the conservation of the biodiversity, one of which is the Convention on Biological Diversity (CBD; PNG, 1998). In addition, in 2008, rights of nature were settled down in the Constitution of the Republic of Ecuador where stipulates that the National System of Protected Areas (SNAP) will conserve biodiversity and ecological functions at the Galapagos Islands (ABG, 2015).

Thanks to the immense diversity of species present in the Galapagos Islands, this archipelago has become a research center of evolution, with a focus on emblematic species. Approximately seven thousand terrestrial, marine species are recorded, including introduced species with an endemism percentage of 28% (DPNG, 2014).

Climatic and geological conditions

The climate of Galapagos archipelago is influenced by between the climate of the western equatorial coast of South America and the dry zone of the Central Pacific (Palmer & Pyle, 1982). This climate is characterized by its variation according to the height and direction of the winds, and sea currents (Segarra, 2012). The frequent occurrence of short drizzles is due to the cold Humboldt current as it allows the temperature of the tropical waters

to be lowered, thereby acting as a moisture condenser. This impact disappears when the “El Niño” hot current arrives in December, lasting until April. Under its influence, coastal temperature rise to 25 ° C, wind is reduced, and sporadic tropical rains are produced (Black, 1973). In the higher parts of the islands the temperature decreases whilst precipitations increase. Because of the temperature difference between coastal and higher areas, vegetation is more dense inland whereas the coast hosts dryer vegetation (Quintanilla, 1983). However, in the higher areas, soils are rich in organic matter that absorb atmospheric moisture very well and can reach a depth of 3 meters. These soils have evolved from basalt, giving rise to a smaller area that is very variable (Rodríguez, 1982).

The pH of the soil varies from acidic to neutral with low amount of phosphorus and potassium. The island that has the best soil for cultivation is Floreana, followed by San Cristobal. Soils in Santa Cruz Island, on the other hand, do not support long-term cultivation. Finally, Isabela Island has a large wetland area but does not offer possibilities of agricultural activity (Consejo de Gobierno del Régimen Especial de Galápagos, 2016). Within the Galapagos ecosystems, four distinct major areas are identified which are characterized by the distribution of plant communities and precipitations: Coastal, Arid, Transition and Humid Zone (Figure 1). The biodiversity within each zone can be different between islands. The Humid zone consists of the Scalesia zone, Miconia zone, Brown zone, and Fern zone (Pampa) (Tye et al, 2012). The percentage of endemism and the state of conservation of flora and fauna is high in the Islands (Raven, 1973).

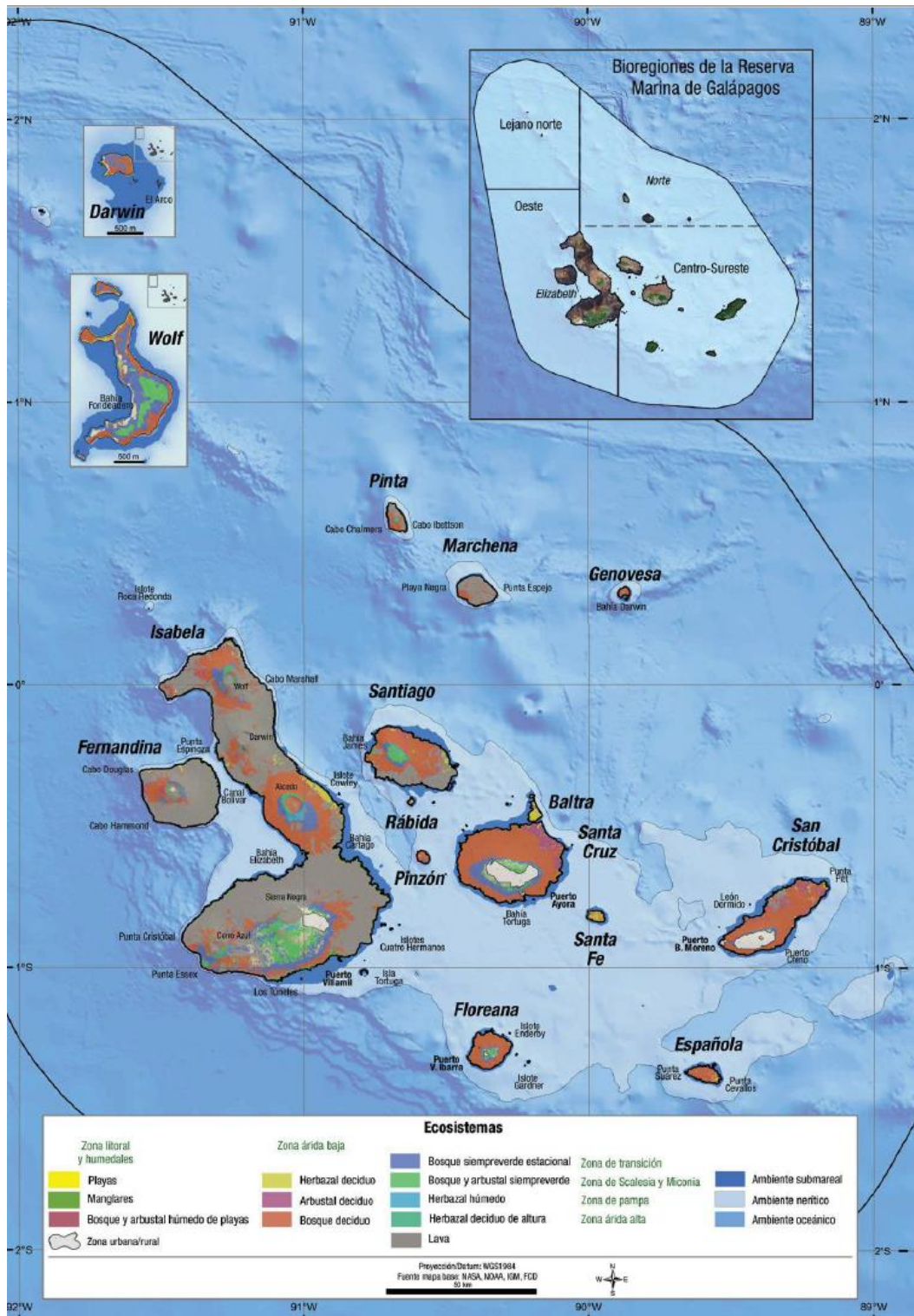


Figure 1: Galapagos ecosystems divided into zones according to the distribution of plant communities and precipitations (FCD & WWF, 2018).

Introduced and Invasive Species in the Galapagos Islands

Due to its high interest in preserving the biodiversity in Galapagos, the presence of introduced species has become a problem for its negative impact over natives and endemics species.

Introduced or exogenous species are difficult to identify at first since the populations are very small. However, once they are fully adapted it gets extremely difficult to control them (Consejo de Gobierno del Régimen Especial de Galápagos, 2016).

Among the introduced species are invasive species, which could be animals, plants, fungi and microorganisms. Invasive species are defined as organisms that establish themselves outside of their natural habitat, thereby negatively impacting either social, environmental or economic factors in the adopted habitat (Denslow, 2007). These species can reproduce and spread rapidly by imposing on local species, outcompeting them for habitat characteristics such as food, water, nutrients and space (ABG, 2015). Besides, they can reduce the economic viability of the land and alter the ecosystems, eventually causing the extinction of native species (Vitousek et al. 1996; Mooney & Hobbs 2000). It is estimated that globally annual losses of agricultural and forestry area due to invasive species can rise up to 1.4 billion dollars (Pimentel, 2001). Hence, invasive species are considered as the second major threat to biodiversity (Vitousek et al. 1997). The extent of the threat depends mainly the nature of invaded ecosystems since the invasion potential of a species is related to their biographical origin and the potential interactions with the species native to the invaded habitat (Colautti & MacIsaac 2004). In general, the islands have dispersion limitations, which reduce the number of present species in archipelago and the competition between them. This facilitates the invasion of these ecosystems by new species (Vitousek et al. 1996). Therefore, a great threat that

affects the ecosystems present in the Galapagos Islands is invasion by non-native species. From a botanical perspective these invasive species are considered as weeds or aggressive plants which especially adapt to the humid zone (Segarra, 2012).

Until 1993 there was no inspection or quarantine process in place to prevent the intentional or accidental entry of invasive species through any product, material or person that was transported to the Galapagos Islands by both sea and air. Being unaware of the risks, as a result, many exogenous species entered the Galapagos ecosystem (Whelan, 1995).

As of this date, Ecuador has created some institutions and laws for the conservation of the biodiversity of the islands such as the Agency for Regulation and Control of Biosafety and Quarantine for Galapagos (ABG) (DPNG, 2014; ABG, 2015).

Until 2008, more than 1488 introduced species have been reported, while the 1900 record only reported 112 species. Among these introduced species are approximately 888 plants, 490 insects and 53 invertebrates. However, recent data report the presence of more than 917 species of introduced plants, where most of these are found in agricultural areas from where they dispersed to the Galapagos National Park (ABG, 2015).

In response, the introduction of species has been banned in recent years due to its influence on the reorganization of the trophic and mutual networks of ecosystems. However, the eradication of most invasive species is impossible because these species are fulfilling an ecological role and the eradication can generate unwanted changes in other ecological components. For this reason, the principal objective is to control the disperse of these species. To this end, some criteria included in the "Total Control Plan for Introduced Species" are followed, being: **(1)** giving priority to the eradication of

species that can play an important role in the structure and functioning of the Galapagos ecosystems considering all of these ecosystems, **(2)** eradication of newly introduced species, **(3)** permanent control of introduced pests with a large distribution and **(4)** waterproofing of pristine and sensitive areas against invasive species (DPNG, 2014).

Unfortunately, these efforts are opposed by the increase food and diverse supplies that are transported from the continent and that are necessary for survival of the growing population driven by tourism, one of the largest economic activities in the Galapagos (Ramírez et al, 2012). These human activities are at the base of intentional entry of exogenous species. This is considered to be the greatest threat for the biodiversity of the Islands since many disperse without control, thereby endangering the native and endemic species (ABG, 2015).

Together with fishing, agricultural activity in the Galapagos Islands (Figure 2) was one of the first survival strategies of the local population. Agricultural activity has been decreasing due to the low profitability compared to other economic activities, such as tourism. Hence, agriculture does not meet the needs of the inhabitants (INGALA, 2003). Accordingly, there has been an abandonment of agricultural land which allowed the aggressive expansion of invasive species such as guava (*Psidium guajava*), elderberry (*Sambucus mexicana*), red cinchona (*Cinchona pubescens*), bay leaf (*Laurus nobilis*), passion fruit (*Passiflora edulis*), blackberry (*Rubus niveus*). These displace endemic species since agricultural areas are generally wet habitats, facilitating the rapid development of introduced species. In San Cristobal, 93% of the wet sectors are in agricultural areas. For Santa Cruz this is 74% (Bensted-Smith, 2002).

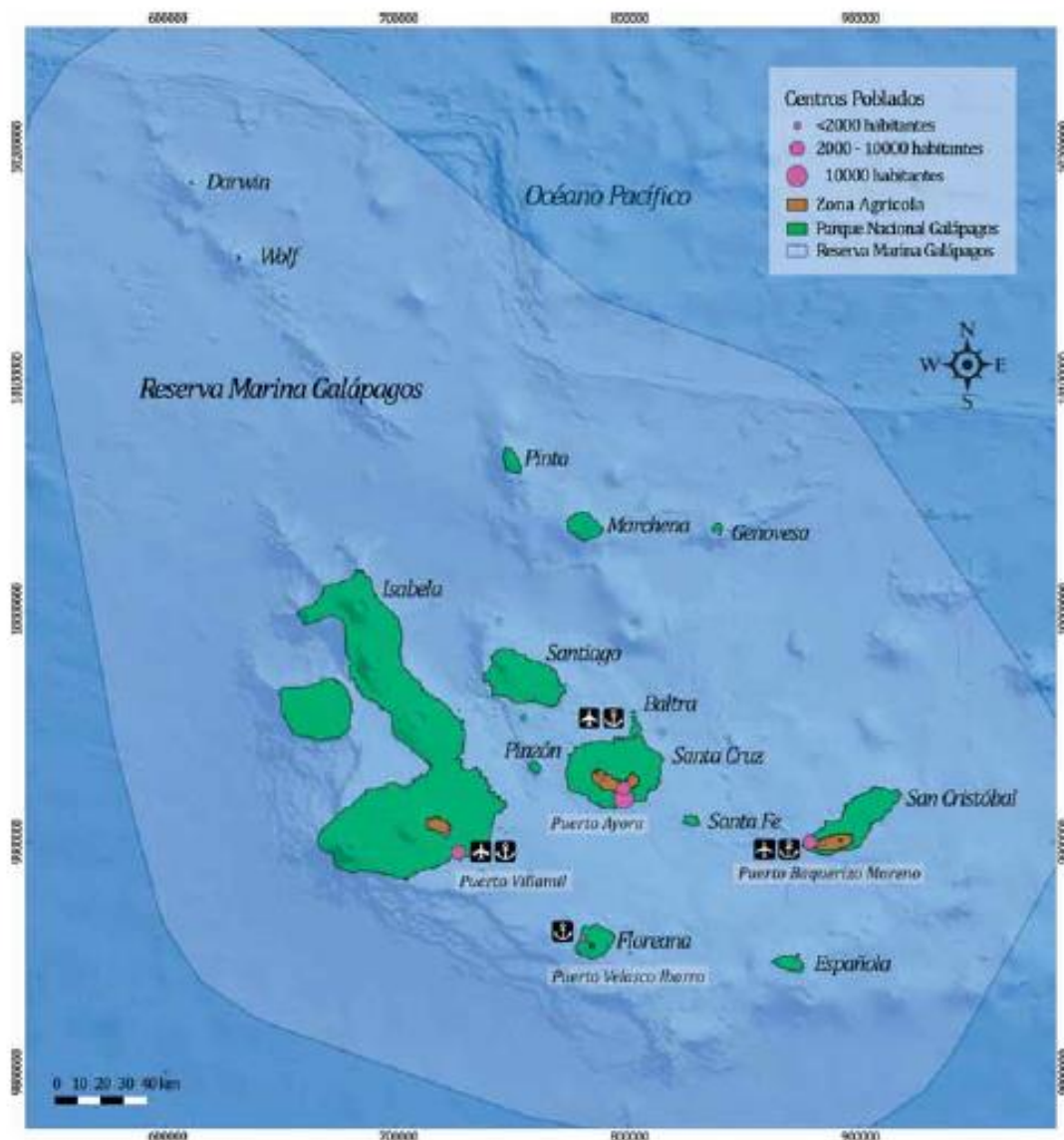


Figure 2: Agriculture Zone in the Galapagos Islands (DPNG, 2014)

Introduced and invasive plants in the Galapagos Islands

The flora present in the Galapagos Islands are particularly lush and varied. This is especially the case above 400 meters altitude as result of the previously described climate created by the Humboldt and El Niño current (Quintanilla, 1983).

Botanical studies indicate that the islands were occupied with species that originate from the equatorial and tropical continent. These populations were able to spread through agents such as wind, birds and marine rafts. Besides, each island has its

endemic flora. A large part of these plant species grows on a rough substrate of cracks and lava blocks, which occurs mainly in the lower areas near the coast (Quintanilla, 1983).

The flora on Galapagos is part of a complex ecosystem involving many marine and terrestrial animals that depend on it. The Nature Conservancy (TNC) carried out a classification of the vegetation: Natural Vegetation, Invasive Vegetation, Agricultural, Eriales, water bodies and cultural features (Segarra, 2012).

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Most of the introduced plants arrived in Galapagos at the initial time of anthropogenic activity such as vegetables, fruit trees, timber, medicinal and ornamental plants. Ornamental plants that grows in gardens are the largest group of introduced plants, which has adapted to the arid zone. Besides, there are unfavorable plants that cause habitat changes on the sites where they have adapted. An example of this is the red cinchona (*Cinchona pubescens*), which is a tree that invaded an area where initially no trees were growing. Others examples the crops are guava (*Psidium guajava*) and blackberry (*Rubus niveus*) that are considered to be the worst invaders of the Galapagos Islands (Consejo de Gobierno del Régimen Especial de Galápagos, 2016).

Invasive plants are considered to be species that impact endemic and native species, and the main characteristics of an invasive species are the following (Segarra, 2012):

- Reproduction in the first years of life cycle with generally a small seed, easy dispersion of seeds by animals, wind, water or man
- Long duration of seed dormancy in the soil, and seeds without special conditions to germinate.
- Multiple reproduction mechanisms (sexual or asexual by leaves, branches, roots, stems)
- Long periods of flowering and fruiting
- Easy adaptation to various climates and conditions.

The main dispersers of these species are wind, birds, rodents, herbivores (including introduced animals), of which there is no control possible. Therefore, it is expected that they will be dispersed to pristine areas in the future (Consejo de Gobierno del Régimen Especial de Galápagos, 2016). The main dispersal factor for raspberry is bird such as the

Crotophaga ani (smooth-billed ani) while for guava and rose-apple dispersal livestock animals are the most responsible (Soria, 2006).

Sixteen percentage of introduced plant species are considered invasive and they mainly affect the wetlands of the islands (Trueman et al, 2010). The study by SIGTIERRAS (2010), indicates that 56% of San Cristobal is occupied by invasive species, of which *Psidium guajava* (guava), *Syzygium jambos* (rose apple) and *Rubus niveus* (blackberry) have a large range of distribution. On the other hand, in Floreana Island, the invading species occupy only 3%. However, here the area of the PNG is affected more since the agricultural zone is controlled. On Isabela Island, invasive species occupy 57% of agricultural areas and on Santa Cruz 22%, respectively, where *Cinchona pubescens* (red cinchona) and *Cedrela odorata* (cedar) stand out.

The worst effects on the ecosystems of the Islands are caused by both woody species such as guava, cedar and husk, and bushes such as raspberry and Lantana that generate impenetrable thickets (Tye, 2001).

Rubus niveus

The genus *Rubus* is the Latin name of brambles (blackberries and raspberries) originating from the word “ruber” which means red (Wagner et al, 1999). This genus belongs to the *Rosaceae* family and is subdivided into 12 subgenera of which very few have been domesticated (Ballington et al. 1993). They are widely distributed, and their consumption is global due to their tasty flavor or since they are a source of natural pharmaceutical products (Kalkman, 2004; Rao & Snyder, 2010).

Part of this genus is the species *Rubus niveus*, commonly known as blackberry or Hill raspberry, which is native to India, Southeast Asia, the Philippines, and Indonesia. (Wagner et al, 1999).

Due to its high nutritional interest, for being a sweet fruit and for ornamental use, its seeds were dispersed by the nursery trade. Therefore, these seeds were introduced to Kenya from India in the year 1930 with the purpose to be cultivated in the mountains. Subsequently, they were sent from Kenya to F.B. Harrington Natal, South Africa 1947, and from here to the Center for Agricultural Research and Education at the University of Florida, USA 1948. In Florida they were widely cultivated to meet the high demand. However, after a few years, the crops were abandoned due to the formation of impenetrable barriers, which made impossible the harvest of the fruits. From Florida, the seeds arrived at the University of Puerto Rico in 1955, and hereafter they also moved to Honduras, Ecuador, Hawaii and other parts of Central and South America and Australia (Morton, 1987; Quinton et al, 2011; FCD & DPNG 2009)

In the Galapagos Islands, this species was introduced for agricultural purposes from the Ecuadorian mainland, first entering Santa Cruz Island at the end of the '60s and San Cristobal Island at the beginning of the '70s. One of the first uses of the raspberry was to form barriers (ISSG, 2014). Later it was found on Isabela Island in 1995 (Sierra Negra), in 2000 (Cerro Azul), on Floreana Island in 2000 and Santiago Island in 2001 (Lawesson & Ortiz 1990; Atkinson et al, 2008), for its dispersion. *Rubus niveus* distribution within the Galapagos is shown in Figure 3.

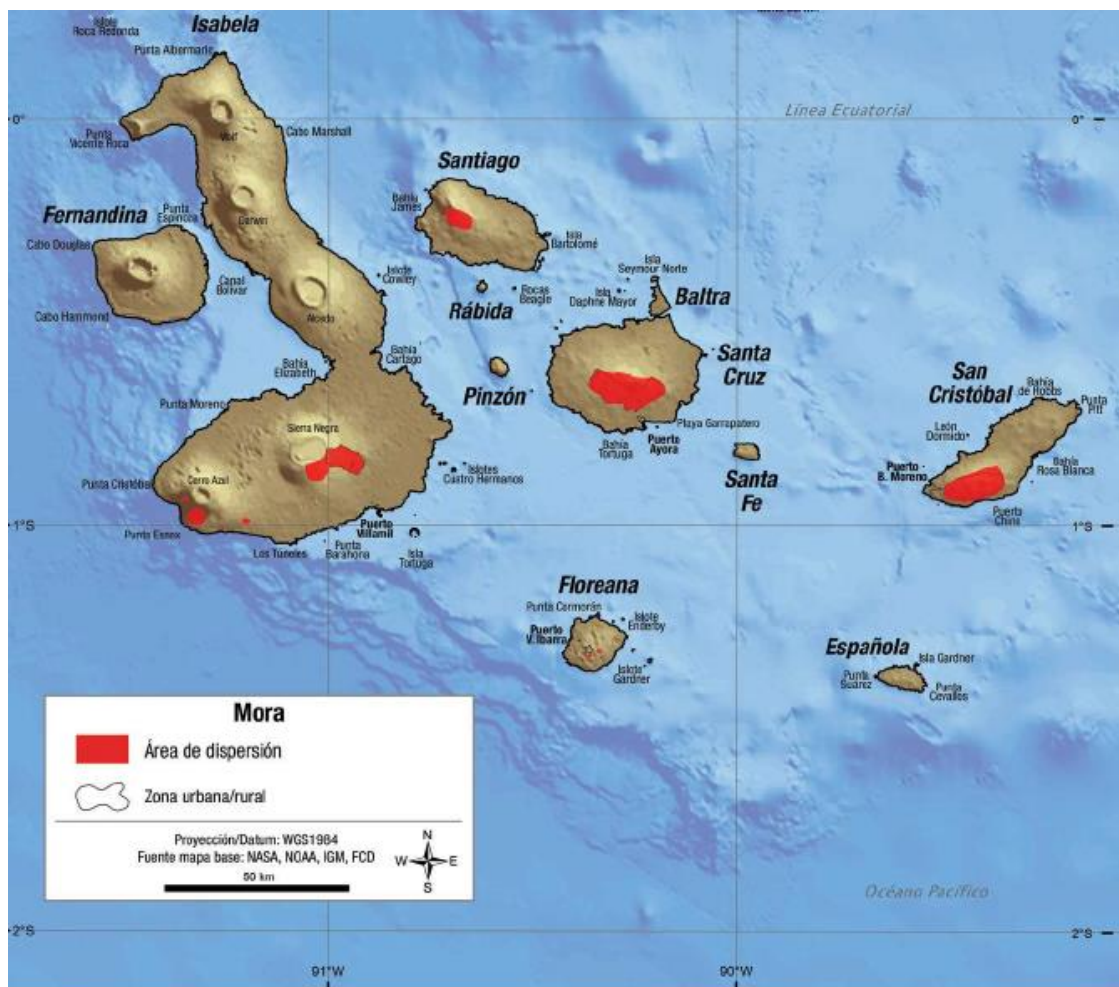


Figure 3: Raspberry's Distribution on the Galapagos Islands (PNG & WWF, 2018)

The raspberry has an adaption to wide range of climatic niche from sea level to 3,000 masl. Yet it is presumed that it is not resistant to drought or frost (Wagner et al, 1999).

They are shrubs and have 200 centimeters long stems covered with strong 3-7 millimeters long hook-shaped spines, and they are tomentose. Has pinnately compound leaves, with 5-9 leaflets, in the form of elliptical-ovate to elliptical. They are 2.5-6 cm long, 2-3 cm wide. The top-surface is glabrous, and the bottom is tomentose and white its margins are saw-shaped, its petioles or rachis have curved spines.

As for its flowers, they are short, terminal, cymose panicles, have scattered spines, pedicels 6-12 mm long, 5 purple-pink petals, and finally, their fruit is dark red, black,

suggestive, 1 cm long and tomentose white and the clusters can have up to 20 fruits (Wagner et al. 1999).

However, this plant can vary its shape, as is the case in Hawaii, where there is a form "a" and "b" of *Rubus niveus*, where the "a" form contains purple-pink petals, has 5 to 7 leaflets, a terminal oval leaflet, young vertical stems and white glaucous, and its fruit is purple-black with a length of 1-1.5cm. The "b" form has light pink petals, with 9-11 leaflets and a terminal elliptical leaflet and its stems are arched from dark red to purple (Gerrish et al. 1992).

Within the Galapagos archipelago, *Rubus niveus* displaces native species, causing farmland to become useless. It is estimated that it has a distribution of around 30,000 hectares but it can up to 90,000 hectares (CDF 2009). Besides, it is difficult and expensive to control, making it one of the worst weeds (CDF 2009). It is highly invasive due to reproduction by both seeds and apomixis (asexual reproduction) (Rentería et al., 2012). Moreover, it can be propagated vegetatively using shoots (See figure 4b), seedlings are tolerant to shade, light, rain, wind and a wide range of temperatures (FCD & WWF, 2008), it has a rapid growth (maturity at 6-8 months) (Atkinson et al. 2008), it can produce fruits from 6-month-old seedlings and each fruit can contain 180 seeds (Parmar & Kaushal, 1982), which are dispersed by rats, birds, wind and have 10 years of viability in the soil (FCD & WWF, 2008).

Although in different areas around the world it usually blooms from June to July and the fruits ripen between August and September (Plants for a Future, 2014), in contrast in Galapagos it blooms and fruits all year long (Atkinson et al, 2008). Originally, it can reach a height of 2 meters, but when it occurs as an invasive plant it reaches up to 4.5 meters

high. It can grow in sandy soils, clay soils and well-drained soils (Plants for a Future, 2014).

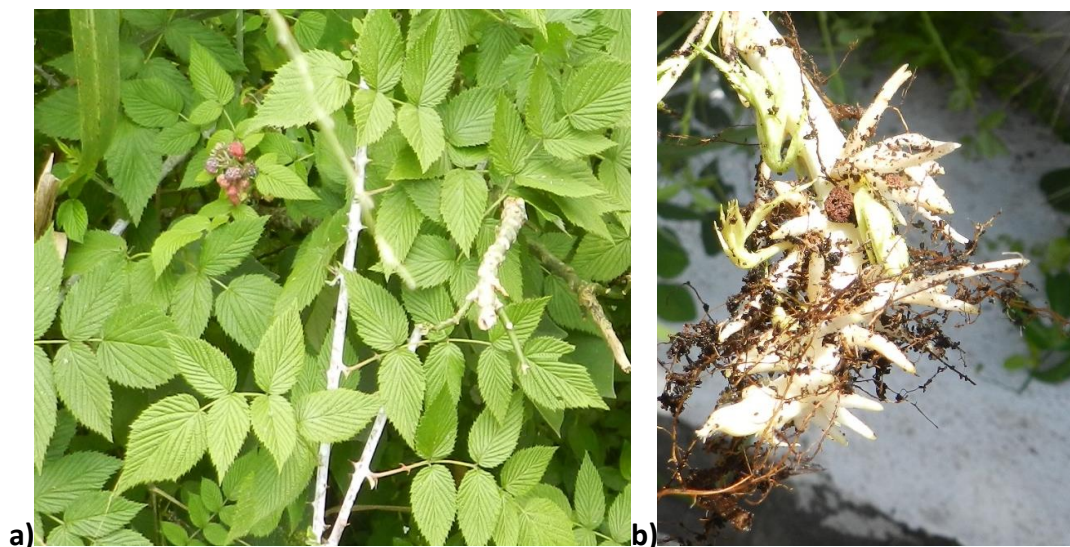


Figure 4: a) *Rubus niveus* in Galapagos. b) Vegetative reproduction by shoots of *Rubus niveus*.

Control mechanisms of invasive species in the Galapagos Islands

The management of invasive species within the Galapagos Islands has become of great importance due to the great threat that these exogenous species have become for the unique biodiversity (Reaser et al. 2007). A first line of defence involves early detection of a potentially invasive species and eradication before it is given the chance to fully adapt and establish itself. In this way, irreparable modification of the ecosystem can be avoided. In general, economically, eradication of an invasive species is preferable because there is only one moment of investment. Less favorable are alternatives rely on consistent spending (Zavaleta 2000; Panetta & Timmins 2004). In the case of plants, applying the eradication of a species may not be feasible given the necessity that its distribution is limited and that its seed has a short lifespan. Since a rapid dispersal and

dormant seeds are contributing to the invasiveness of a plant, this is difficult to achieve for an invasive plant (Panetta 2004, Buddenhagen 2006, Panetta 2009).

As a second line of defence, several methods are used to control invasive species: chemical, mechanical and biological methods.

Chemical control is the most commonly used and effective mechanism on the short term and based on the use of herbicides, such as glyphosate. However, this is accompanied by disadvantages such as high costs due to its continuous use and possible development of resistance. Also, intensive use of chemicals can alter soil quality which also affects endemic or native species (FCD & PNG, 2006) Mechanical control on the other hand, is very specific to the target, but is labor intensive because of the many spines attached to the plant. In this method, it is necessary for the plant to be removed entirely (both above and below the ground) to prevent it from regenerating. This process is done before applying the chemical method. Finally, biological control is based on the use of living organisms that affect the target directly through pathogenesis or indirectly through competition with the target. When proven effective, it can be profitable, self-sustainable, permanent and ecologically safe due to the high specificity of the organisms used. In Hawaii, several biological control agents have already been introduced, successfully combatting *Rubus argutus* (Starr et al, 2003; Wittenberg & Cock 2001; Hobbs & Humphries 1995).

In Galapagos, the mechanic method is the most commonly used, manually with a machete, followed by the chemical method using herbicides. However, this is not sufficient given the large number of seeds that are present in the soil as a seed bank (7000 - 22800 seeds / m² germination rate 80%, equaling a cost of \$ 500-2000 per ha).

The chemical and manual method has been used together to obtain better results,

where the herbicide is used to burn the weeds and then the manual method is used to remove the remaining plants that were left in the soil (FCD & WWF, 2008). The most used herbicides in Galapagos are glyphosate and picloram, which are applied in areas with high ecological value, areas with limited infestations, or areas with recently introduced species (Rentería, 2001).

Studies have shown that the use of these two methods can be harmful, since it can change the composition of the soil, the structure of native species communities and even affect resident bird species. Therefore, biological control has been considered as an alternative and sustainable way (FCD & WWF, 2008) to create an effective strategy to eliminate the blackberry populations and reintroduce native species (Hansen, 2007; Denslow, 2007).

Biological control has already been applied in Galapagos for different species. A successful example is an Australian ladybird (*Rodalia cardinalis*) that was introduced in 2002 to the Islands for the control of cottony scale (*Icerya purchasi*) that affected endemic plants (Calderón et al, 2012). In 2011, an evaluation was carried out to see the effect of the ladybird on the aphid or cotton scale, and it was noted that, in a large percentage, this aphid was reduced without a negative impact on native species (PNG, 2016).

Phytopathogenic fungi have been used as biological control of invasive plants since 1970, because they are the majority of pathogens that cause disease in plants, where fungi of greater interest as biological control are rust and smut (Evans 1987). An example of rust is *Phragmidium violaceum* that affects *Rubus* sp., released in Australia as a control of invasive raspberries (Evans et al. 2004). On the other hand, the white smut

Entyloma ageratinae that was used in Hawaii for the control of *Aegeratina riparia* (Trujillo, 1985).

Thanks to these projects, it has been possible to register bases and experience to be able to achieve successes in the control of introduced species and ensure the conservation of the biodiversity of ecosystems. Many of the projects have not achieved the complete eradication of these species, but they manage to control them by reducing their impact and not damaging or endangering native or endemic species. Hence, biological control must be specific to an extent that it only attacks the invasive species targeted without having a negative effect on other non-target species (PNG, 2016).

In the case of finding a biological control against raspberry, it is necessary to obtain facilities to provide evidence for containment before the agent being released into the environment. (PNG, 2016).

In conclusion, Galapagos has climatic and geographical characteristics that allow agriculture to be applied, that is, plants can easily develop on these islands. However, this is a disadvantage since it allows invasive plants to adapt better to the ecosystem causing a negative impact on species diversity. Different control methods have been applied but they have not produced great results, so biological control is an alternative that is being considered to reach favorable results controlling invasive species, especially raspberry.

REFERENCES

- Agencia de Regulación y Control de la Bioseguridad y Cuarentena para Galápagos. ABG. (2015). Plan Estratégico Institucional 2015-2018, Agencia de Regulación y Control de la Bioseguridad y Cuarentena para Galápagos.
- Atkinson, R., J. L. Rentería, and W. Simbaña. (2008). The consequences of herbivore eradication on Santiago: are we in time to prevent ecosystem degradation again? in: Galapagos Report 2007-2008. Puerto Ayora, Galapagos, Ecuador
- Ballington JR, Luteyn MM, Thompson K, Romoleroux K and Castillo R. (1993). Rubus and Vacciniaceous germplasm resources in the Andes of Ecuador. Plant Genetic Resources Newsletter 93: 9-15
- Bensted-Smith, R., G. Powell & E. Dinerstein. (2002). Planificación para la ecorregión. Pág. 1-5, en: Visión para la biodiversidad de las islas Galápagos. Fundación Charles Darwin y WWF. Puerto Ayora, Galápagos.
- Black J. (1973). Galápagos archipiélago del Ecuador. 138 pág.; Fundación Charles Darwin. Ecuador
- Buddenhagen, C. E. (2006). The successful eradication of two blackberry species *Rubus megalococcus* and *R. adenotrichos* (Rosaceae) from Santa Cruz Island, Galapagos, Ecuador. *Pacific Conservation Biology* 12:272-278
- Calderón Alvarez C, CE Causton, MS Hoddle, C Hoddle, R Van Driesche & E Stanek III. (2012). Monitoring the effects of *Rodolia cardinalis* on *Icerya purchasi* populations on the Galapagos Islands. *BioControl* 57:167-179.
- Charles Darwin Foundation, Galapagos (CDF). (2009). Providing Technical Assistance to the Control of the Invasive Blackberry *Rubus niveus* on the Islands of Santiago and Floreana.
- Colautti, R. I. and H. J. MacIsaac. (2004). A neutral terminology to define 'invasive' species. *Diversity and Distributions* 10(2): 135-141.
- Consejo de Gobierno del Régimen Especial de Galápagos. (2016). Plan de Desarrollo Sustentable y Ordenamiento Territorial del Régimen Especial de Galápagos. – Plan Galápagos. Puerto Baquerizo Moreno, Galápagos, Ecuador
- Denslow, J. (2007). Managing dominance of invasive plants in wildlands. *Current Science* 93:1579-1586.
- Dirección del Parque Nacional Galápagos (DPNG). (2014). Plan de Manejo de las Áreas Protegidas de Galápagos para el Buen Vivir. Puerto Ayora, Galápagos, Ecuador
- Evans H.C. (1987). Fungal pathogens of some subtropical and tropical weeds and the possibilities for biological control. *Biocontrol News and Information* 8: 7-30.

- Evans, K. J., L. Morin, E. Bruzese, and R. T. Roush. (2004). Overcoming limits on rust epidemics in Australian infestations of European blackberry, pp. 514-519. In: Cullen, J. M., D. T. Briese, D. J. Kriticos, W. M. Lonsdale, L. Morin, and J. K. Scott (Eds.) Proceedings of the XIth International Symposium on Biological Control of Weeds, Canberra, Australia. CSIRO Entomology, Canberra, Australia.
- Fundación Charles Darwin & Parque Nacional Galápagos. (2006). "Manual de identificación y manejo de malezas en las islas Galápagos". Quito – Ecuador.
- Fundación Charles Darwin & Dirección del Parque Nacional Galapagos. (2009). Diagnóstico y planificación para el desarrollo de un agente de control biológico para *Rubus niveus* en las islas Galápagos. Puerto Ayora, Santa Cruz, Galápagos, Ecuador
- Fundación Charles Darwin (FCD) y WWF-Ecuador. (2018). Atlas de Galápagos, Ecuador: Especies Nativas e Invasoras. Quito, FCD y WWF Ecuador
- Geist, D., (1996). On the emergences and submergences of the Galápagos Islands. Noticias de Galápagos No. 56.
- Gerrish, G., L. Stemmermann, and D.E. Gardner. (1992). The distribution of *Rubus* species in the State of Hawai'i. Technical Report 85, Cooperative National Park Resources Studies Unit, University of Hawai'i at Manoa, Honolulu, HI.
- Hansen, M. J. (2007). Evaluating management strategies and recovery of an invasive grass (*Agropyron cristatum*) using matrix population models. Biological Conservation 140:91-99.
- Hobbs, R. J. and S. E. Humphries. (1995). An integrated approach to the ecology and management of plant invasions. Conservation Biology 9:761-770.
- INGALA. (2003). Plan regional para la conservación y el desarrollo sustentable de Galápagos. Decreto No. 3516. R. Oficial. Tomo 2
- ISSG. (2014). Base de datos mundial de especies invasoras (GISD). Grupo Especialista en Especies Invasoras de la Comisión de Supervivencia de Especies de la UICN. <http://www.issg.org/database/welcome/>
- Kalkman C. (2004). Rosaceae. In: Kubitzki K (ed.) The families and genera of vascular plants, 6: 343-386.
- Lawesson, J. E. and L. Ortiz. (1990). Plantas introducidas en las islas Galapagos. Pages 201-210 in J. E. Lawesson, O. Hamann, G. Rogers, G. Reck, and H. Ochoa, editors. Botanical Research and Management in Galapagos. Missouri Botanical Garden, St. Louis, .U.S.A.
- Mooney, H. A. and R. J. Hobbs. (2000). Invasive species in a changing world. Island Press, Washington DC.

- Mooney HA, Cleland EE. (2001). The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences*. 98(10): 5446–5451.
- Morton, J. (1987). Mysore Raspberry. Pages 109-110 in J. F. Morton, editor. *Fruits of warm climates*. Florida Flair Books, Miami.
- Palmer C; R. Pyle: (1982). El ambiente climático de las Islas Galápagos. In: *Comp. de Ciencia en Galápagos*. 17-25 d.: Est. Cient. Ch. Darwin.
- Panetta, F. D. (2004). Seed banks: the bane of the weed eradicator. Pages 523-526. *Weed Society of New South Wales*, Sydney.
- Panetta, F. D. (2009). Weed eradication - an economic perspective. *Invasive plant science and management* 2:360-368.
- Panetta, F. D. and S. Timmins. (2004). Evaluating the feasibility of eradication for terrestrial weed incursions. *Plant Protection Quarterly* 19:5-11.
- Parmar C, Kaushal MK, (1982). *Rubus niveus*. En: *Frutas silvestres* [ed. por Wild Fruit]. Nueva Delhi, India: Kalyani Publishers, 88-91
- Parque Nacional Galápagos (PNG). (1998). *Plan De Manejo de Conservación y Uso Sustentable Para la Reserva Marina de Galápagos*.
- Parque Nacional Galápagos (PNG). (2016). *Plan anual de Inversiones. Proyecto de Inversión Pública y de Cooperación externa no reembolsable. Proyecto de control y erradicación de especies invasoras prioritarias para la reducción de la vulnerabilidad de especies endémicas y nativas de las Islas Galápagos*.
- Pimentel, D. (2001). Economic and environmental threats of alien plant, animal, and microbe invasions. *Agriculture, Ecosystems & Environment* 84:1.
- Plants for a Future. (2014). *Rubus niveus* Thunb., Reino Unido. <http://www.pfaf.org/user/Plant.aspx?LatinName=Rubus+niveus>
- J.M. St. Quinton , M.F. Fay , M. Ingrouille & J. Faull. (2011). Characterisation of *Rubus niveus*: a prerequisite to its biological control in oceanic islands, *Biocontrol Science and Technology*, 21:6, 733-752
- Rao A and Snyder D. (2010). Raspberries and human health: A review. *J. Agri. Food Chem.*, 58(7): 3871- 3883.
- Raven P.H. (1973). Species number and Endemism: The Galápagos Archipiélago Revisited. *Science* vol. 179; p. 893-895, No. 4076; California Press.
- Reaser, J. K., L. A. Meyerson, Q. Cronk, M. De Poorter, L. G. Eldredge, E. Green, M. Kairo, P. Latasi, R. N. Mack, J. Mauremootoo, D. J. O'Dowd, W. Orapa, S. Sastroutomo, A. Saunders, C. Shine, S. Thrainsson, and L. Vaiutu. (2007). Ecological and socioeconomic impacts of invasive alien species in island ecosystems. *Environmental Conservation* 34:1-14

- Rentería, J. (2001). Towards an optimal management of the invasive plant *Rubus niveus* in the Galapagos Islands. Faculty of Natural Sciences, Division of Biology, Silwood Park Campus, Ascot, Berkshire, SL5 7PY, U.K.
- Rentería JL, Gardener MR, Panetta FD, Crawley MJ, (2012). Manejo de la frambuesa invasiva (*Rubus niveus*) en la isla de Santiago, Galápagos: ¿erradicación o control indefinido? *Ciencia y gestión de plantas invasoras*, 5 (1): 37-46.
- Rodríguez J.: (1982). Los asentamientos humanos en las Islas Galápagos. Doc. de Investigación Científica, No. 2 p. 9 - 33; CEDIG, Ecuador.
- Segarra, P. (2012). Edición y Reproducción de la Cartografía temática del sistema territorial de Galápagos, como parte del proceso de planificación espacial para el ordenamiento territorial del Archipiélago. Atlas de Galápagos. CGREG.
- SIGTIERRAS, (2010). Catastro multifuncional de la zona rural de las islas pobladas de Galápagos, Quito, Ecuador.
- Soria. M., (2006). Avian seed disperser of *Rubus niveus* in Santa Cruz Island. Master Thesis. University of Missouri-St. Louis.
- Starr, F., Starr, K., Loope, L. (2003). *Rubus niveus* f. a. Hill or mysore raspberry. Rosaceae. United States Geological Survey--Biological Resources Division. Haleakala Field Station, Maui, Hawai'i
- Trueman M., A. Guézou y P. Wurm. (2010). Residence time and human mediated propagule pressure at work in the alien flora of Galapagos. *Biol. Invasion*. Pp. 1-12
- Trujillo, E. E. (1985). Biological control a Hamakua pa-makani with *Cercospora* sp. in Hawaii, pp. 661-671. In: Delfosse, E. S. (ed.). Proceedings of the VIth International Symposium on Biological Control of Weeds, 19-25 August, 1984. Vancouver, British Columbia, Canada, Agriculture Canada, Ottawa, Ontario, Canada.
- Tye, A. (2001). Invasive plant problems and requirements for weed risk assessment in the Galápagos islands. Page 153-175 in R. H. Groves, F. D. Panetta, and J. G. Virtue, editors. *Weed risk assessment*. CSIRO Publishing, Melbourne.
- Tye, A., H.L. Snell, S.B. Peck y H. Adersen. (2002). Sobresalientes características terrestres del archipiélago de Galápagos. En: *Visión para la biodiversidad de las islas Galápagos*. Fundación Charles Darwin para las islas Galápagos y Fondo Mundial para la Naturaleza, Puerto Ayora, Galápagos
- Vitousek, P. M., C. M. D'Antonio, L. L. Loope, and R. Westbrooks. (1996). Biological invasions as global environmental change. *American Scientist* 84:468-478.
- Vitousek, P. M., C. M. D'Antonio, L. L. Loope, M. Rejmánek, and R. Westbrooks. (1997). Introduced species: a significant component of human-caused global change. *New Zealand Journal of Ecology* 21:1-16.

- Wagner, W.L., D.R. Herbst, and S.H. Sohmer. (1999). Manual of the Flowering Plants of Hawai'i. 2 vols. Bishop Museum Special Publication 83, University of Hawai'i and Bishop Museum Press, Honolulu, HI.
- Whelan, P. (1995). Propuesta para el establecimiento de un sistema de inspección y cuarentena para las islas Galápagos. ECCD, Galápagos, Ecuador.
- Wittenberg, R. and M. J. W. Cock. (2001). Invasive alien species: a toolkit of best prevention and management practices. CAB International Wallingford.
- Zavaleta, E. (2000). The economic value of controlling an invasive shrub. *Ambio* 29:462-467

ARTICULO 2

Exploring fungal pathogens to control invasive raspberry (*Rubus niveus*) in Galapagos Islands.

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INTRODUCTION

UNESCO declared the Galapagos Islands a World Heritage Site in 1979, due to the great and fragile biodiversity. This biodiversity was due to its volcanic eruptions and the isolation of this archipelago (Karez et al, 2006). This archipelago has become an evolutionary research center, with a focus on emblematic species, so Ecuador decided to create the Galapagos National Park (PNG) to protect 97% of land and marine areas, and allow 3% remaining to human activities (Black, 1973). The importance of the conservation of this ecosystem is of national and international interest, so there are many entities that are dedicated to conserve for this island (PNG, 1998).

This delicate biodiversity is mainly affected by the introduction of exogenous species, which enter to the islands due to human activities that occur within them. For many years, the first economic activity was agriculture (sugarcane production), but over time, agriculture decreased since the population began to devote itself to what is now the largest economic activity, tourism. Thanks to tourism, the population has increased, which also caused an increase in food and diverse supplies, which has to be transported from the continent for survival of Galapagos population (Ramírez et al., 2012).

Since tourism is far more profitable than agriculture, agricultural areas have been abandoned, which has allowed the introduced plants to take advantage of these spaces and displace existing plant in that ecosystem. It is estimated that 16% of the introduced plants are invasive species (Trueman et al, 2010), which plays a role displacing plant endemic species. Examples of these invasive species are guava (*Psidium guajava*), red cinchona (*Cinchona pubescens*), bay leaf (*Laurus nobilis*), and raspberry (*Rubus niveus*) (Bensted-Smith, 2002).

The introduction of exogenous species was due to anthropogenic activities, since they are considered useful as a source of food, wood, medicinal and ornamental, in addition to the need to enter supplies for the population (Consejo de Gobierno del Régimen Especial de Galápagos, 2016).

To be considered as an invasive species, a plant must meet the following characteristics: 1) have a negative impact over endemic and native species, 2) outcompete efficiently for nutrients, displace and extinguish endemic species, 3) have sexual and asexual reproduction, and have seeds of easy dispersal and long viability on the ground (Segarra, 2012). The main factor for dispersal of these invasive plants are wind, birds, rodents, and herbivores (Consejo de Gobierno del Régimen Especial de Galápagos, 2016).

Invasive plants are known to settle down in the wetlands (200-700 masl) of the islands, for example, 3% of Floreana, 57% of Isabela, 22% of Santa Cruz, and 56% of San Cristóbal has been occupied (SIGTIERRA, 2010). Of these four inhabited islands, *R. niveus*, along with *Syzygium jambos* and *P. guajava*, has the highest range of distribution, especially in San Cristóbal, with *R. niveus* being the greatest threat (Pryet 2012; SIGTIERRA, 2010). The specie *R. niveus*, commonly known as "blackberry" or "Hill raspberry", is native to India, Southeast Asia, the Philippines and Indonesia (Wagner et al, 1999). It is a crop of high interest, since it contains a sweet fruit and ornamental use, which is why it has been distributed in different parts of the world. It could have arrived to Galapagos in the late 60's, where after it was dispersed through 5 Islands: Santa Cruz in the late '60s, San Cristóbal in the early '70s, Isabela in the late '90s, and Floreana and Santiago in the early 2000's (Lawesson & Ortiz 1990; Atkinson et al, 2008).

Rubus niveus has the characteristics to be considered extremely invasive, such as its high distribution capacity, sexual and asexual reproduction, and apomixis, rapid growth, large seed bank in the ground, and easy production of high scrublands. That is why its control has been considered difficult in other similar ecosystems such as Hawaii, Central America, Australia and South Africa (FCD & WWF, 2008, Rentería et al, 2012). It is estimated that in Galapagos it has distributed around 30,000 ha. but it can up to 90000 hectares (CDF 2009). A few methods have been used to control the growth of the raspberry. First, mechanical methods are used to eliminate the plant above and below the ground. Second, chemical methods are used such the application of herbicides e.g. glyphosate, but these produce high economic and environmental expenses since it relays in constant application for its persistence of raspberry seeds and shoots in the

ground. The estimated cost per chemical application is \$ 200 per ha (FCD & WWF, 2008 & Rentería, 2011).

Therefore, biological control methods have been taken into consideration as an effective option to target *R. niveus* (Cabrera & Briano, 2012). There are two types of biological control: the first is natural control, where antagonists limit the development or reproduction of the target without human intervention (Cock, 1996), or it can be introduced, where selected antagonists are manipulated and applied with scientific bases against the target. For the applied biological control, there are 3 types: the first is classic or inoculative, which permanently introduces an exotic enemy against an exotic plague, the second is flooding, which consists of massive breeding of the biocontrol organism to apply it in green houses, and lastly conservation, which consists of conserving and increasing the antagonist through manipulation of the ecosystem, favoring its adaptation and establishment (Cabrera & Briano, 2012).

Since the raspberry plant is a weed, a classical biological control strategy is needed in the Galapagos Islands, where an antagonistic agent has to be introduced to control this plant. In order to apply this type of biological control, the first step is to implement the “centrifugal phylogenetic testing method” designed by Anthony Wapshere in 1974, which consists of repeating biological control tests on the target species to determine its effect. Then, tests are done on plants of the same family as the target species, and then on related families, successively moving away phylogenetically from the target plant. These tests can last for years, since it is necessary to test them in many individuals and they must be carried out in confined spaces to avoid a leakage of the biological control to the environment (Cabrera & Briano, 2012 & PNG, 2016).

Insects, mites, nematodes, or other organisms are known to cause disease in plants, and to be used as biological control agents, but phytopathogenic fungi are considered the most important plant antagonists (Van Driesche et al. 2007; Evans 1987; Templeton, 1982).

Phytopathogenic fungi have been used as biological control of invasive plants since 1970, where the two fungi of greatest interest were rusts and blights (Trujillo, 1985).

Rusts (Order Uredinales) are obligate parasites and usually attack only one or a few species of plants, so they are candidates for classic biological control. An example of a rust is *Phragmidium violaceum*, that affects *Rubus* sp., which has been used in Australia to control invasive blackberries (Evans et al. 2004). On the other hand, smuts (Order Ustilaginales), similar to rusts, are obligate parasites of vascular plants that systematically affect the host, weakening it and possibly preventing seed production. An example is the white smut *Entyloma ageratinae* that it was introduced to Hawaii to control *Ageratina riparia* (the mistflower) (Trujillo, 1985). The PNG, along with the CABI and the CDF, are looking for an alternative to control the raspberry in Galapagos using the FEIG fund (Fondo para el control de las especies invasoras de Galápagos) that was created to handle problems of invasive species (CABI, 2019).

As mentioned earlier, phytopathogenic fungi are the main cause of diseases in plants, and there are no cases of a fungus released as a biological control agent that has caused problems affecting non-target species (Anderson, 2009). Therefore, the objective of this research was to evaluate phytopathogenic fungi isolated from the raspberry (*Rubus niveus*) of San Cristóbal-Galapagos Island, so they can be used as biological control agents to control or diminish the population of this aggressive plant species from the Islands.

MATERIALS AND METHODS

Sample collection

The study was carried out in upper part (wet zone 200-700 masl) of San Cristobal Island. Leaves, stems, and fruits that presented disease symptoms were collected in plastic bags and further stored at 4 °C. In total, 80 sites were inspected for collection, maximum of 4 samples were collected in each site (Figure 1). Samples were transferred to the Microbiology laboratory of the Galapagos Science Center (GSC), where fungal isolation was performed.

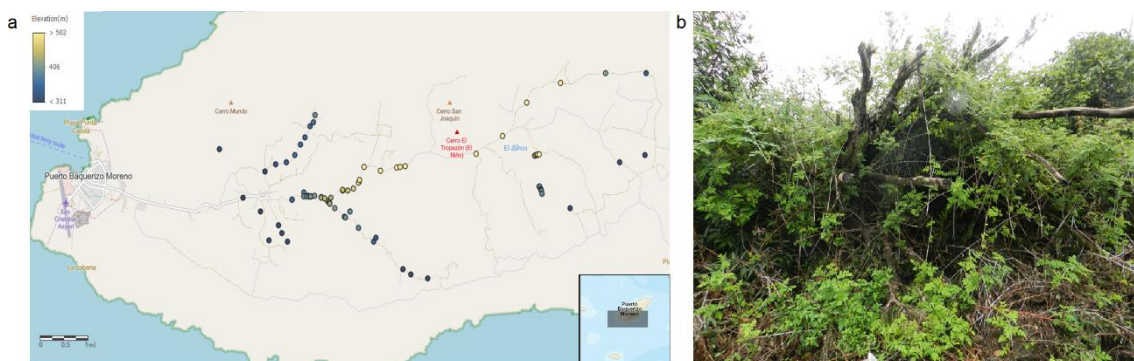


Figure 1. a) Collection Map of *Rubus niveus* samples with disease symptoms (80 sites).

b) *Rubus niveus* growing on the roadside of San Cristobal Island and covering other plant species.

Fungal Isolation

For fungal isolation, samples were surface sterilized following a modified version of the Dhingra and Sinclair protocol (1985). First, tissue of 2x2 cm in size was excised from samples of fruit, leaves and stems. Samples were immersed in a solution containing a solution of Sodium hypochlorite (1.5% for leaves and fruits and 2.5% for stems) for one minute with constant stirring, followed by a 70% alcohol wash for one minute with stirring and finally, 3 washes with sterile distilled water was performed. These tissues

were dried on sterile paper towels and cultivated in PDA medium (Potato Dextrose Agar – Difco) amended with gentamicin (antibiotic) and incubated at 28 °C for 7 days in the incubator (Shell Lab). The PDA medium was prepared following the provider instructions.

After 7 days, each isolated fungus was passed to a new medium (PDA + antibiotic), until purified cultures (single cultures) were obtained.

Pathogenicity tests (Bioassays)

To identify potential pathogens of raspberry leaves, pathogenicity tests were performed. For this, the isolated fungus grown in PDA for 15 days and then conidia were scraped from the solid medium and suspended in PDB liquid medium (Potato Dextrose Broth), with a minimum concentration of 1×10^6 conidia per ml.

Healthy raspberry leaves were collected in the upper part of San Cristóbal island and superficially sterilized with 70% alcohol and distilled water and placed on a tray with wet paper (humid chamber). On the leaf, 5 µl of the isolated fungus was placed on different points as shown in the Figure 4h, and 5 µl of PDB solution was used as negative control. Trays were covered with plastic wrap to simulate a humid chamber. The humid chambers were incubated at room temperature (± 25 °C) for 7 days under 12/12h dark-light cycle. Lesions from the inoculated area was evaluated every day and measured in centimeters. After bioassays were executed, the fungal isolate that caused damage to the leaves were isolated again to determine if the same isolate was recover, fulfilling Koch's postulates. Each isolated was tested twice for its pathogenicity. Positive fungal isolates that cause disease symptoms or lesion were re-tested 3 times.

Morphological identification

Each fungus was identified according to the mycelium, and the conidiophore shape and color, and conidia morphology (Fraire et al. 2002). A transparent adhesive tape was used to take a sample of each fungus, and subsequently placed on a glass slide (López et al. 2014). The samples were observed in a Leica DM750 microscope, with magnifications of 40X and 100X, and they were photographed and compared with the taxonomic key published by Obispo, 1974.

Molecular identification

Fungal DNA was extracted with the OMEGA E.Z.N.A. Fungal DNA Mini Kit following the manufacturer's instructions. The ITS (Internal Transcribed Spacer) region was amplified using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')(White et al 1990). The TEF1a region (Elongation factor 1 alpha) was amplified using the primers EF -1 (5'-ATGGGTAAGGARGACAAGAC-3') and EF-2 (5'-GGARGTACCAGTSATCATGTT-3') (O'Donnell et al, 1998). The TUB region (beta-tubulin) was amplified using the primers T1-F (5'-AACATGCGTGAGATTGTAAGT-3') (O'Donnell & Cigelnik, 1997) and Bt2b-R (5'-ACCCCTCAGTGTAGTGACCCTTGGC-3') (Glass & Donaldson, 1995). The RPB2 region (RNA Polymerase II gene) was amplified using the primers fRPB2-5f (GAYGAYMGWGATCAYTTYGG-3') and fRPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') (Liu et al, 1999). Lastly, the GAPDH region (Glyceraldehyde 3-phosphate dehydrogenase) was amplified using the primers GDF (5'-GCCGTCAACGACCCCTTCATTGA-3') and GDR (5'-GGGTGGAGTCGTA CTTGAGCATGT-3') (Templeton et al. 1992). These genes are generally used as reference genes for fungi because they are involved in basic cellular functions or constitutively expressing

themselves in different phases of the fungal life cycle. The genes used depend on each organism studied (Nailis et al, 2006; Bohle et al, 2007; Fang & Bidochka, 2006).

The PCR reaction was performed in a LABNET MULTIGENE thermocycler. ITS was amplified by the following parameters: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 30 s, extension at 72°C for 45 s and a final extension at 72°C for 15 min. The amplified PCR program of TEF1a was an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. For TUB marker an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The amplified program of RPB2 initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 90 s and a final extension at 72°C for 10 min. Finally, the amplified program of GAPDH initial denaturation at 95°C for 6 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 15 min. The PCR products obtained were sequenced by MACROGEN in Korea using Sanger method. The sequences obtained were compared using the NCBI BLAST tool (National Center for Biotechnology Information).

Phylogenetic Analysis

The sequences were built using ClustalW under MEGA 7.0 software, which was used to perform the phylogenetic analysis with Bayesian inference using the BEAST v1.8.4 program (Drummond & Rambaut, 2007). The following substitution models were used

in the phylogenetic analysis: JC69 + G for *Bionectria* sp., HKY + G + I for *Colletotrichum* sp. and *Penicillium* sp., GTR + G + I for *Lasiodiplodia* sp., *Fusarium* sp. and *Phanerochaete* sp. MCMC were run for a total of 100,000,000 generations and sampled every 1,000 generations. Ten percentage of the initial states were discarded. Phylogenetic trees were observed and edited using FigTree v1.4.3.

RESULTS

By looking in each collection site, it was found samples that had chlorosis, dark and dry spots, leaf blight, stem anthracnose and rot and presence of fungi in the fruits (Figure 2). These samples were collected for further fungal isolation at the GSC.

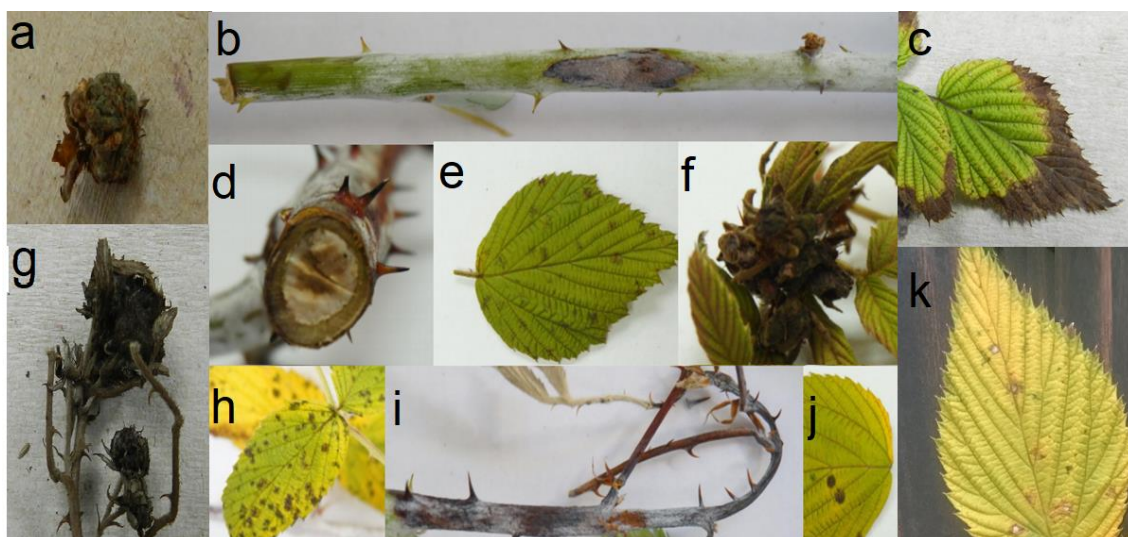


Figure 2. Samples collected from *Rubus niveus* with the presence of disease symptoms (San Cristóbal Island). a, g, f) fruit with the presence of mycelium; b, d, i) stem with anthracnose; c) leaf with anthracnose; e) presence of blight on the leaf; h, j) leaves with the presence of blight and chlorosis; k) leaf with presence of chlorosis and lesions.

A total of 595 fungi were isolated from the collected samples that presented disease symptoms in the field. The fungi were classified by mycelial morphology, and one

representative of each group was taken to perform the pathogenicity tests. In total, 226 groups with different characteristics were obtained (Figure 3).

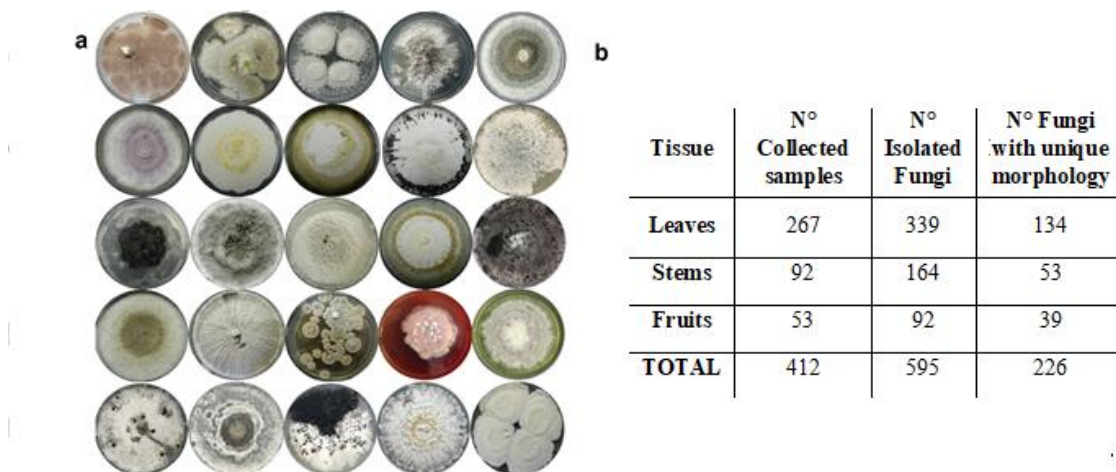


Figure 3. a) Fungi isolated from the *Rubus niveus* samples. b) The number of samples, isolated fungi and fungi with only morphology obtained from leaves, stems and fruits.

Of the 226 pathogenicity tests, six fungi caused leaf damage greater than or equal to 7 mm of radius in plants during the bioassays, and therefore, these were considered as potential candidates as biological control agent against raspberry (Figure 4). Positive reactions showing leaf lesion/damage were re-isolated to confirm identity of the inoculated fungus, demonstrating Koch's postulates. Control spots with no fungus, remained symptomless.

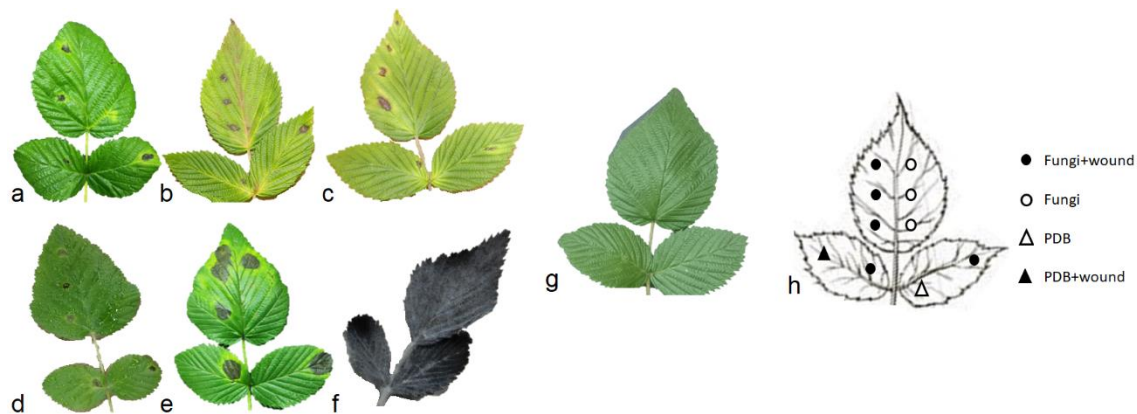


Figure 4. Pathogenicity tests inoculating 5 μl of the fungus with a concentration of 1×10^6 conidia/ml. The tests were performed in a humid chamber at room temperature (25 °C) for 7 days: a) **57T3** - necrosis and chlorotic halo; b) **28.1a.1.2** - necrosis; c) **10.3n** - necrosis and chlorotic halo; d) **4.3b** - necrosis and mycelial growth; e) **52h3.2** - necrosis and chlorotic halo; f) **7.2a.1.2** - phytotoxicity and mycelial growth; g) *Rubus niveus* leaf containing the control with a drop of PDB without fungi; h) Fungi inoculation model. PDB: Liquid culture medium (Potato Dextrose Broth). Wound: wound done with a dissecting needle.

The morphological identification using macroscopic and microscopic characteristics of each selected fungus (6 isolates, Figure 4, Table 1) were described based on the parameters established by Obispo (1974).

The 57T3 isolate has the following characteristics: yellowish white and yellow reverse mycelium, has a dimorphic conidiophore, and the size of the conidia is 3 to 4.25 μm long and 1 to 2.5 μm wide, characteristics of the *Bionectria* genus.

The 28.1a.1.2 isolate has the following characteristics: Gray-white mycelium and gray to brown reverse, hyaline cylindrical to oblong conidia with 14 to 17.6 μm long and 3 to 5.8 μm wide, presence of appressoria, characteristics of *Colletotrichum* genus.

The 10.3n isolate has the following characteristics: pale-dark gray mycelium and reverse black pigment, mature ellipsoid conidia with dark brown pigment and a transverse septum, and pycnidia formed with paraphyses, characteristics of *Lasiodiplodia* genus.



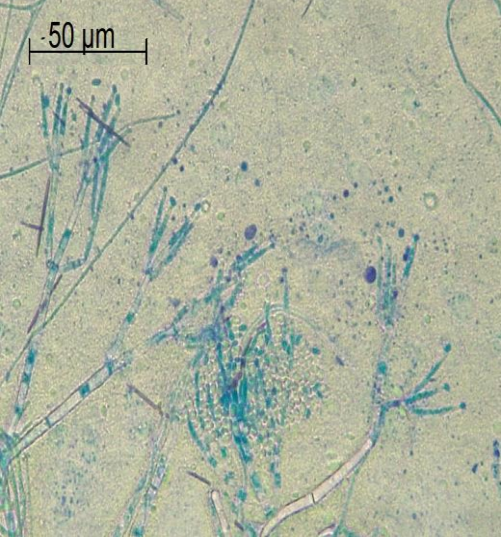
The 4.3b isolate has the following characteristics: White-pink or purple cotton mycelium with microconidia with 7 to 9µm long and 2 to 4µm wide and macroconidia needle-shaped, characteristics of *Fusarium* genus.

The 52H3.2 isolate has the following characteristics: White to cream thin mycelium and reverse cream color, Globose conidia, thin aerial hyphae and hyaline, little septated, without clamp connections, characteristics of *Phanerochaete* genus.

Finally, the 7.2a.1.2 isolate has the following characteristics: Greenish white mycelium and reverse cream-orange color, presence of septate stipules, verticillate metulae, predominantly ellipsoidal conidia, located in columns, characteristics of *Penicillium* genus.

In Table 2 the molecular characterization of each fungus can be observed, where the comparison with the BLAST of the sequence was detailed along with the percentage of identity, Query cover, e-value, and accession number. Most of the isolates have a 100% identity using the ITS region, which helps us to say that species could be, however, from two isolates it was not possible to obtain species information, only genus.

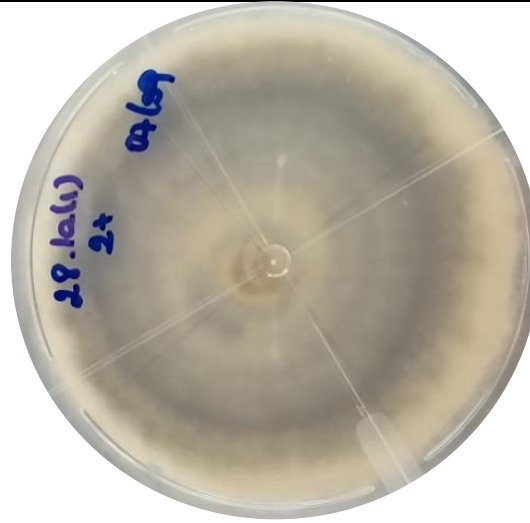
Table 1. Phenotypic and microscopic identification of fungi isolated from *Rubus niveus* samples (PDA culture medium).

STRAIN	TOP SIDE	BOTTOM SIDE	MICROSCOPY	MORPHOLOGICAL DESCRIPTION
57T3				<p>Macroscopic characteristics:</p> <p>Yellowish white mycelium, flat velutinous texture, aerial hyphae; yellow reverse, yellow pigment spreads throughout the culture medium</p>

				<p>Microscopic characteristics:</p> <p>Dimorphic</p> <p>conidiophore, primary</p> <p>conidiophores in 2-3</p> <p>verticillate divergent</p> <p>phialides, penicillate</p> <p>secondary</p> <p>conidiophore, chain</p> <p>conidia, 3 to 4.25 μm</p> <p>long and 1 to 2.5 μm</p> <p>wide.</p>
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28-1A-1-

2

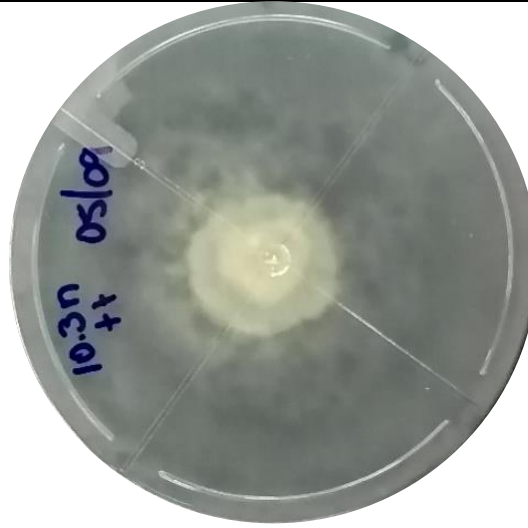
**Macroscopic**

characteristics: Gray-white aerial mycelium with the dark gray center; gray to brown reverse.

Microscopic

characteristics: Hyaline cylindrical to oblong conidia, , with obtuse ends, nucleated, 14 to 17.6 μm long and 3 to 5.8 μm wide, presence of appressoria.


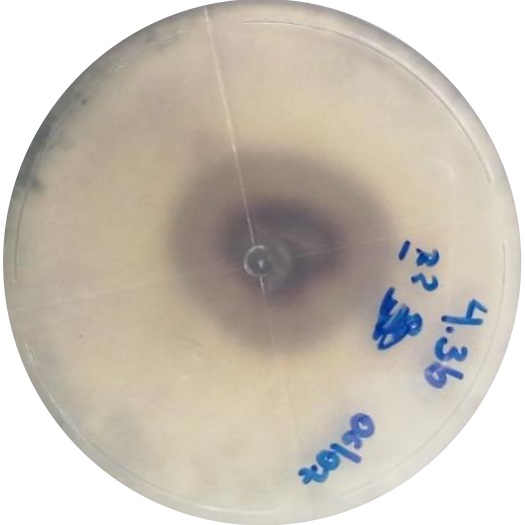
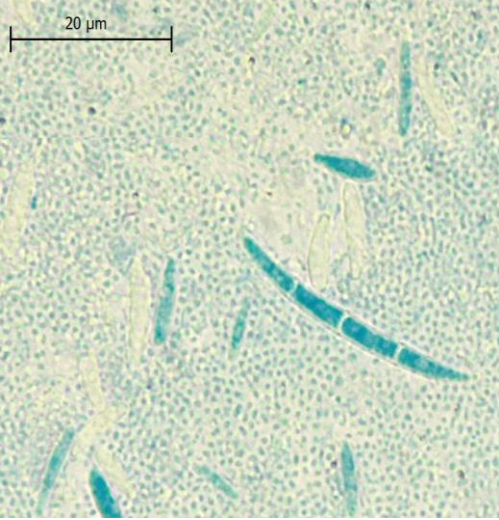
10.3n

**Macroscopic**

characteristics: Woolly aerial mycelium, initially white turning pale-dark gray; reverse black pigment.

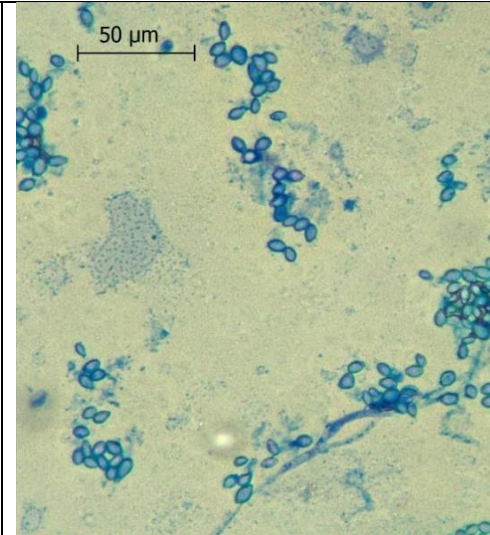
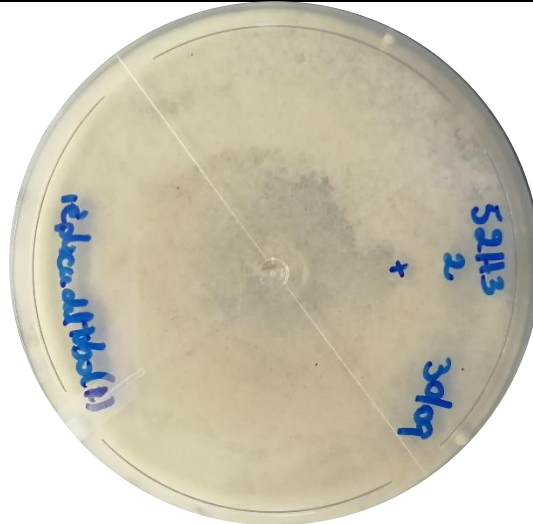
Microscopic**characteristics:**

Mature ellipsoid conidia with dark brown pigment and a transverse septum, longitudinally striated wall and thick. Picnidia

				<p>formed with paraphyses.</p>
4-3B				<p>Macroscopic characteristics:</p> <p>White-pink or purple cotton mycelium, absence of sclerotia, aerial hyphae; reverse pink with a purple center, in concentric rings.</p> <p>Microscopic characteristics:</p> <p>Microconidia: obovate</p>

				<p>conidia with a rounded-to-truncate base from 7 to 9μm long and 2 to 4μm wide and</p> <p>macroconidia: needle-shaped with 3 septa, hooked or curved apical cell and basal cells foot-shaped.</p>
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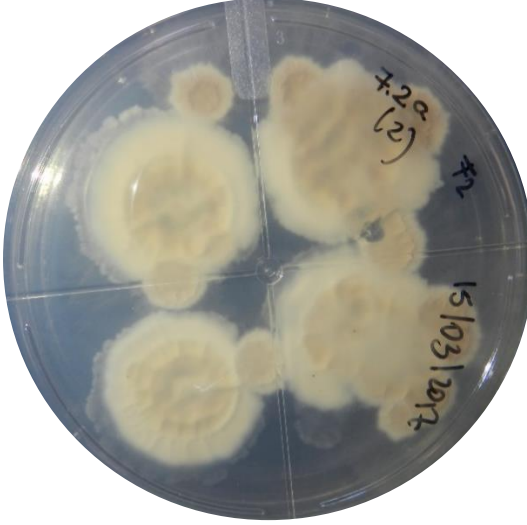
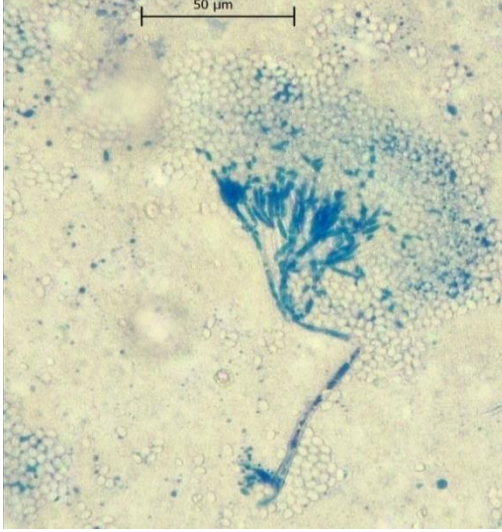
52H3-2

**Macroscopic****characteristics:**

White to cream thin mycelium; reverse cream color.

Microscopic**characteristics:**

Globose, nucleated, thick-walled and easily removable conidia, thin aerial hyphae and hyaline, little septated, without clamp connections.

				<p>Each branch forms a hyaline terminal blastoconidium.</p>
<p>7-2a-1-2</p>				<p>Macroscopic characteristics: Greenish white mycelium; reverse cream color – orange.</p> <p>Microscopic characteristics: abundant conidiogenesis Presence of septate stipules, 3-5 verticillate</p>

				metulae, 3-9 ampulliform phialides, predominantly ellipsoidal conidia, located in irregular columns.
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Table 2. Molecular identification using BLAST of the isolated fungi based on the sequence of the markers ITS, RPB2, TUB, GADPH region.

Strain	Scientific name	Region	Sequence length	Query Cover	E value	%Identity	Genbank accession number
57T3	<i>Bionectria</i> sp.	ITS	567bp	99%	0.0	99.82%	MK204505.1
		TUB	630bp	94%	0.0	93.46%	AF358171.1
28-1a-1-2	<i>Colletotrichum gloesporoides</i>	GADPH	286bp	100%	6.00E-133	99.62%	KU743263.1
		ITS	538bp	100%	0.0	100.00%	KT282778.1
10.3n	<i>Lasiodiplodia theobromae</i>	ITS	540bp	100%	0.0	100.00%	MH793584.1
4-3b	<i>Fusarium</i> sp.	ITS	559bp	99%	0.0	100.00%	MK355727.1
		TEF1a	672bp	99%	0.0	99.55%	MK414243.1
		TUB	788bp	72%	0.0	99.82%	LT575099.1
		RPB2	1067bp	100%	0.0	96.72%	LT841266.1
52H3-2	<i>Phanerochaete chrysosporium</i>	ITS	605bp	100%	0.0	98.68%	HM171940.1
		RPB2	1322bp	75%	0.0	95.63%	KP134954.1
7-2a-1-2	<i>Penicillium rolfsii</i>	ITS	585bp	99%	0.0	100.00%	MH856397.1
		RPB2	1031bp	99%	0.0	99.51%	KC346314.1

Six phylogenetic trees were constructed with concatenated regions for each fungus, using sequences reported in other studies. For fungus 57T3 (*Bionectria* sp.), 69 reported sequences were used (Schroers, 2001). The phylogenetic tree indicates that this fungus was *Bionectria pseudostrata* since it forms a clade with other strains of this species, with a posterior probability of 1.00 (Figure 5). For isolate 28.1a.1.2, 53 reported sequences

were used based on Weir et al, 2012, and this analysis confirmed that the fungus was *Colletotrichum gloesporoides* forming a clade with a posterior probability of 1.00 (Figure 6). In the case of fungal isolate 10.3n, 100 reported sequences were used based on Bautista-Cruz et al. 2018, and this analysis indicates that this fungus forms a clade with other strains of *Lasiodiplodia theobromae* (Figure 7), however, the value of the posterior probability was 0.003. For fungus 4.3b, 82 reported sequences by Sandoval et al, 2018 were used, and the phylogenetic analysis indicates that our strain forms a clade with another strain of the species *Fusarium concentricum*, with a posterior probability of 1.00 (Figure 8). For fungus 52h3-2, 48 reported sequences by Floudas & Hibbett, 2015 were used confirming that it belonged to the *Phanerochaete chrysosporium* species with a posterior probability of 1.00 (Figure 9), and finally for fungus 7.2.1.2, 57 reported sequences by Samson & Houbraken, 2011 were used confirming that the species was *Penicillium rolfsii* (Figure 10) with a posterior probability of 1.00.

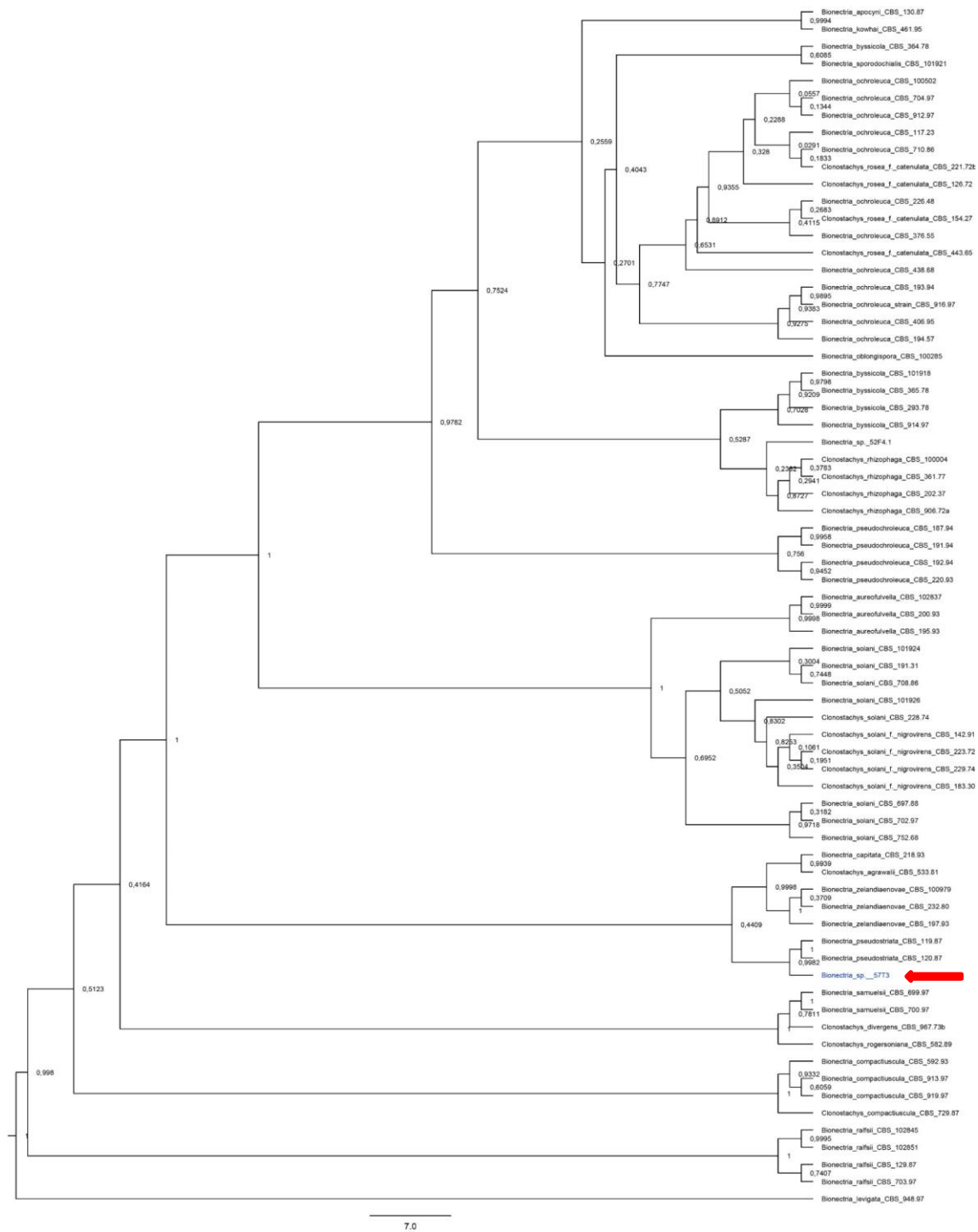


Figure 5. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and β -tubulin sequences of 69 strains belonging to the *Bionectria* genus based on Schroers 2001. The value in the nodes represents Bayesian posterior probability and the lengths. *Bionectria levigata* was used as an outgroup. The scale bar indicates the number of the expected changes per site.

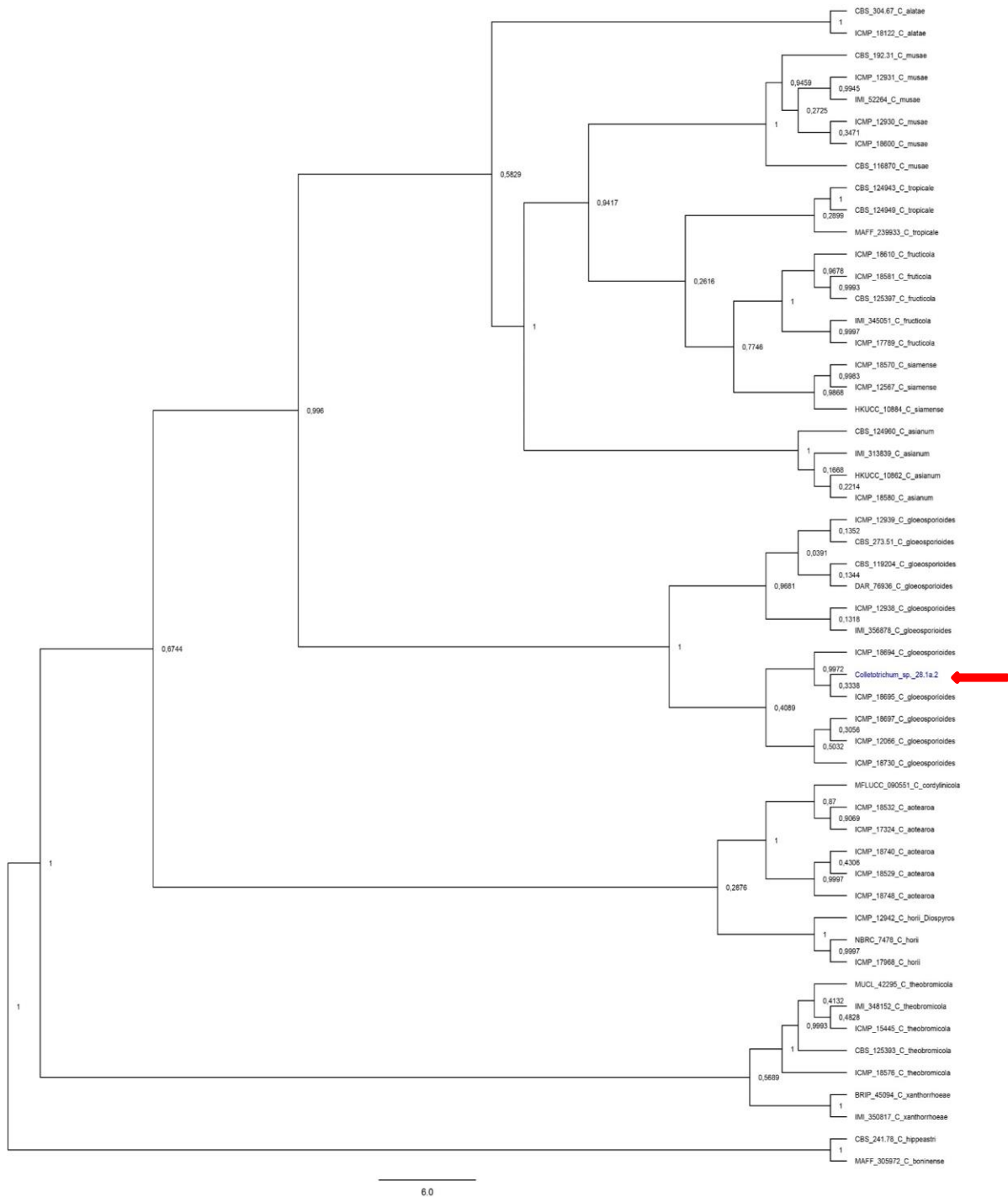


Figure 6. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and GAPDH sequences of 53 strains belonging to the *Colletotrichum* genus based on Weir et al, 2012. The value in the nodes represent Bayesian posterior probability. *C. hippesastri* and *C. boninense* were used as outgroups. The scale bar indicates the number of the expected changes per site.

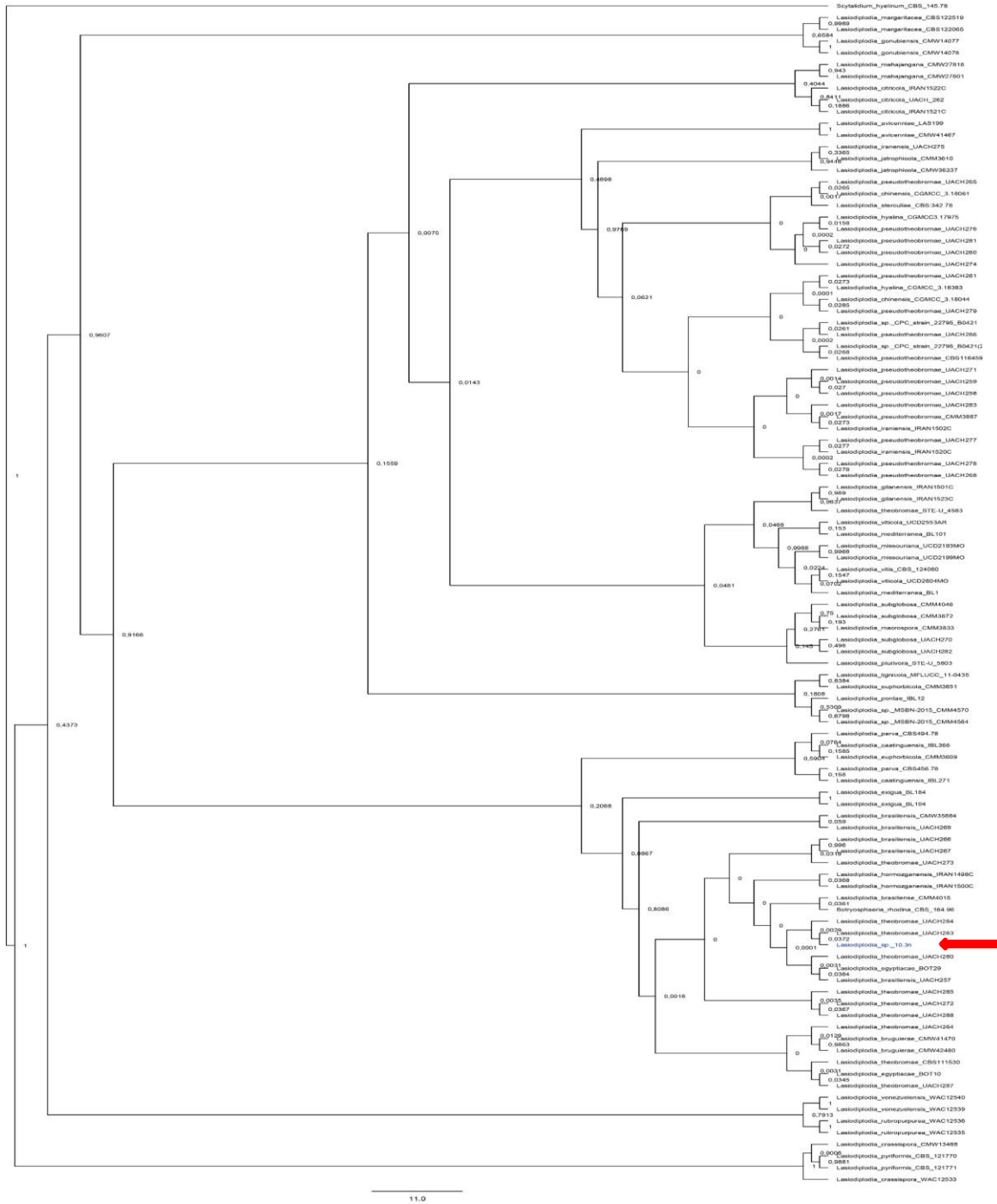


Figure 7. Phylogenetic tree obtained from Bayesian analysis of the ITS sequences of 100 strains belonging to the *Lasiodiplodia* genus based on Bautista-Cruz et al, 2018. The value in the nodes represent Bayesian posterior. *Scytalidium hyalinum* was used as an outgroup. The scale bar indicates the number of the expected changes per site.



Figure 8. Phylogenetic tree obtained from Bayesian analysis of the TEF1a, TUB and RPB2 sequences of 83 strains belonging to the *Fusarium* genus based on Sandoval et al, 2018. The value in the nodes represent Bayesian posterior probability. *Fusarium oxysporum* was used as an outgroup. The scale bar indicates the number of the expected changes per site.

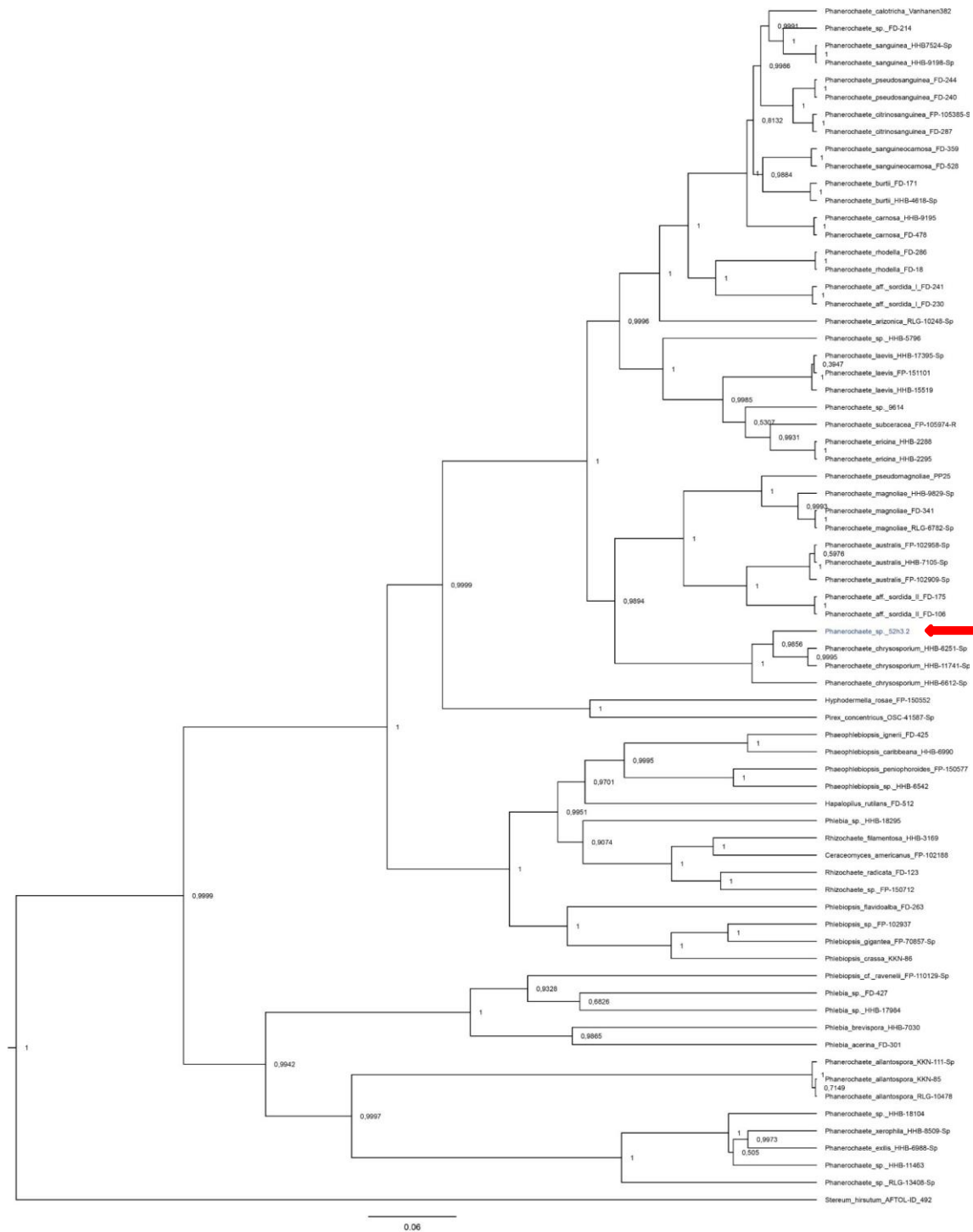


Figure 9. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and RPB2 sequences of 48 strains belonging to the *Phanerochaete* genus based on Floudas & Hibbett, 2015. The value in the nodes represent Bayesian posterior probability. *Stereum hirsutum* was used as an outgroup. The scale bar indicates the number of the expected changes per site.

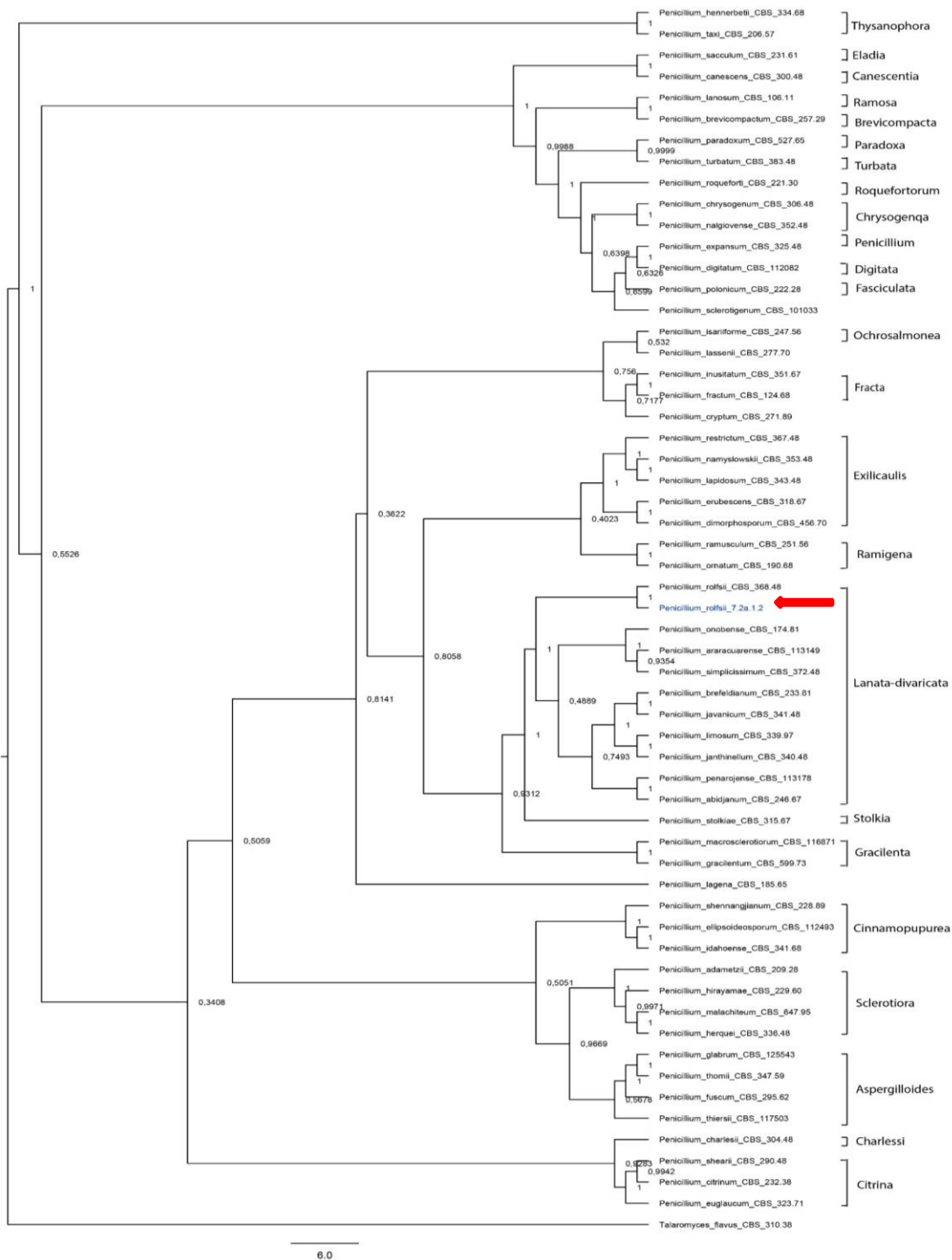


Figure 10. Phylogenetic tree obtained from Bayesian analysis of the combined ITS and RPB2 sequences of 57 strains belonging to the genera *Penicillium* based on Samson & Houbraken, 2011. The value in the nodes represent Bayesian posterior probability. *Talaromyces flavus* was used as an outgroup. The scale bar indicates the number of the expected changes per site.

DISCUSSION

Rubus niveus entered the Galapagos Islands due to anthropogenic activities for the need of food and for ornamental purposes, and in the past, no local agency attempted to control the entry of these species (Consejo de Gobierno del Régimen Especial de Galápagos, 2016). Now, this plant is considered one of the worst weeds that are affecting the agricultural area, as well as protected areas within the Galapagos National park. Characteristics of this plant make them an efficient competitor for nutrients, water and space (ABG, 2015). The raspberry are been dispersed in the high zone (wet zone) of the Islands since this area has high relative humidity, being a favorable habitat for its development. Also, it adapts to many types of soil, since it has been found in ravines, road edges, and grasslands (Starr et al, 2003).

The present investigation was carried out in San Cristobal since 56% of this island is occupied by invasive plants, and also since this plant has a greater distribution range in San Cristobal in comparison to other islands (SIGTIERRAS, 2010). In addition, the largest number of reports of *Rubus niveus* by the Charles Darwin Foundation's collection database was shown on this island. Raspberry could be found in altitudes ranging from 193 to 605 masl on San Cristobal, compared to other places such as Hawaii, where it is also considered as weed, with reports that raspberry can be found from 152 m to an elevation of 1280 m (Starr et al, 2003).

Within the Galapagos Islands, attempts have been made to control raspberry with mechanical methods (e.g. by hand, shovel or machete) and chemical methods (e.g. application of glyphosate) or fire, however, it has yielded in high cost without efficient results. This indicates that other alternatives, such as the biological control method could be useful, since it has proven to produce successful results in similar cases. For this reason, we proposed to find

phytopathogenic fungi from the Galapagos Islands as a biological control of *Rubus niveus* (Evans et al. 2004).

It has been mentioned in other studies, such as performed by Anderson (2009), that fungi are the main phytopathogens to cause diseases in plants and that they are ideal candidates to be used as a biological control of weeds. It is also important to keep in mind that there has never been a case of a fungus released as a biological control that caused diseases in other species, since exhaustive studies have been done to find the best candidates. For this reason, in this study we decided to search for fungi that could be used as biocontrol agents, exclusively using fungi isolated from raspberry found on San Cristóbal Island to avoid the introduction of microorganisms that could interfere with established ecosystems.

From a total of 412 samples collected, including leaves, stems, and fruits, 595 fungi were isolated, which were divided into 226 groups with different phenotypic characteristics. This shows that there was a great diversity of fungi on the islands since all the isolates were associated to a single plant species (*R. niveus*) in a single island. Despite finding many fungi, only six were able to damage the raspberry leaves after performing the pathogenicity tests.

In general, *Rubus* genus is attacked by fungi such as *Botrytis* sp., *Alternaria* sp., *Arthuriomyces peckianus*, *Cercospora* sp., anthracnose *Colletotrichum gloeosporoides*, *Peronospora sparsa* and *Fusarium* sp. (root rot) (Fernández-Pavía et al., 2012). However, due to the changing conditions that may occur, other fungi may appear (Contreras et al, 2019), so we have found four possible candidates of different genera that can cause tissue damage, specifically in the leaves.

The first fungus identified was *Bionectria pseudostriata* (57T3) using morphological and ITS and TUB molecular markers. Although Moreira et al, (2016) indicate that the markers resolved

the speciation of the *Bionectria* genera were ATP citrate lyase (ACL1), TUB, TEF1a and RPB2, the markers used in this study managed to resolve the species of this fungus, yielding a clade formed by the species of *Bionectria pseudostrata* with the fungus 57T3 with a posterior probability of 1.00 which indicates the confidence of the formation of this clade, since it is considered a credible phylogeny if it has a posterior probability value > 50% (Drummond & Rambaut, 2007).

In general, *Clonastachys* species can be found in the soil, such as endophytic fungi, saprophytes, and destructive mycoparasites (Schroers 2001), but *Bionectria* (*Clonastachys* teleomorph) was found in dead plants (barks) (Domsch et al, 2007), which coincides with the 57T3 fungus that was found in the raspberry stem. Besides, it is not a pathogenic fungus, possibly the presence of lesion in the bioassay is due to the high number of inoculated spores, causing the balance between leaf and fungus to become unbalanced and a change from endophyte to pathogen, however, cannot be considered as a possible biological control for *Rubus niveus* (Stone et al, 2000)

The second fungus identified was *Colletotrichum gloeosporoides* (28.1a.1.2). These species were associated with 470 host genera (Cannon et al, 2008) and can cause anthracnose disease. *Colletotrichum gloeosporoides* are considered a species complex where different species have varying degrees of pathogenicity, host specificity, etc. (Hyde et al, 2009). These species can cause disease in fruits and leaves of the different hosts, including the *Rubus* genus, which is why we found the isolate 28.1a.1.2 in raspberry leaves (Afanador et al, 2014). Although the markers necessary to separate the species from this complex are ACT (Actina), GAPDH, cal (calmodulin), chs1 (chitin synthase), and ITS (Sharma et al, 2017), for this fungus, the only markers used were GAPDH and ITS. Here, we confirmed species with the phylogenetic

analysis, which gave a clade with a posterior probability of 1.00 among all strains of this species, so most taxa could be reliably distinguished (Weir et al, 2012).

The third 10.3n isolate was identified as *Lasiodiplodia theobromae*, which belongs to the *Botriosphaeriaceae* family, which has 23 genera and 187 species with a cosmopolitan distribution. *L. theobromae* can be found mainly in woody hosts, where they can be found as endophytes, saprophytes and plant pathogens, causing gummosis, canker and internal tissue necrosis (Slippers & Wingfield 2007; Phillips et al. 2013; Slippers et al. 2013; Dissanayake et al. 2016). In addition, there is a new report that indicates that it may be a pathogen of the *Rubus* genus (Contreras et al, 2019). On the other hand, it has been found that this fungus can also affect leaves, causing blight (Lopez et al, 2009). Bautista et al. 2018 mention that the markers necessary to solve the speciation of the *Lasiodiplodia* genera were TEF1a, TUB and ITS. However, in our phylogenetic analysis we found that, although our strain had a close relationship with other strains of *Lasiodiplodia theobromae*, the phylogenetic relation had a posterior probability of 0.003, indicating a very low confidence for this clade, since only one marker (ITS) was analyzed.

The fourth 4.3b isolate identified was *Fusarium concetricum*. The species of the *Fusarium* genera are found in different substrates such as soil, air, water, and plant materials that are decomposing. They can also colonize living tissue of animals and plants (including in the *Rubus* genus), and can act as endophytes, secondary invaders and destructive pathogens (Nelson et al. 1994), which explains why we found it in raspberry leaves causing injury (Fernández-Pavía et al., 2012). *Fusarium* can be considered a cosmopolitan genus can be found in many hosts, including abiotic environments such as air and dust (Perlroth et al. 2007). Within this genus, it

can be found the *Gibberella fujikuroi* species complex (GFC) which is a monophyletic lineage composed of anamorphic species, where the species *F. concentricum* was found. This complex is characterized by having pathogenic species and producing fumonisins and moniliformin (Marasas et al. 2001). For this fungus, the markers TEF1a, TUB and RPB2 were used in this research, which were mentioned along with the markers CAL1 and RPB1 in the study by Sandoval et al. 2018 to define the species of the *Fusarium* genus belonging to the GFC complex. The three markers used, defined that the species 4.3b had a close relationship to another CBS 450.97 strain of *Fusarium concentricum*, which was within the GFC complex, and with a posterior probability of 1.00, confirming the relationship with that clade formation.

The fifth 52H3-2 isolate identified was *Phanerochaete chrysosporium*. The *Phanerochaete* genus is a group of saprophytes that is distributed globally and is related to wood rot and lignin degradation (Eriksson et al. 1978; Kersten & Cullen 2007). According to Floudas & Hibbett 2015, the markers used to define the speciation of the *Phanerochaete* genus are ITS, RPB1 and RPB2. For this study, ITS and RPB2 were used for phylogenetic analysis, confirming that it was closely related to three other strains of *Phanerochaete chrysosporium* (HHB-6251-Sp, HHB-6612-Sp and HHB-11741-Sp), showing a posterior probability of 1.00, which indicates reliability in the formation of the clade. This fungus was found in raspberry lesions of leaves where it causes injury, possibly, since it has the property of lignin degradation, and lignin is important in the formation of the cell wall (Kellogg et al, 2011), a characteristic that makes it a cosmopolitan pathogen. There are studies of other species of this genus that have been found in the genus *Rubus* (Dueñas & Tellería, 1988)

Finally, the sixth 7.2a.1.2 fungus identified was *Penicillium rolfsii*. The markers that helped to define the species of the *Penicillium* genus were RPB1, RPB2, Tsr1 (putative ribosome biogenesis protein) and Cct8 (putative chaperonin complex component TCP-1).

Although we only used the ITS and RPB2 markers in this study, the phylogenetic analysis showed that fungus 7.2a.1.2 was closely related to strain CBS_368.48 *Penicillium rolfsii*, obtaining high reliability of the formation of clades (subsequent probability of 1) .

In the *Penicillium* genus there is a section called Lanata-divaricata, where the species that inhabit the soil, leaves and plant and they are active after decomposition. *Penicillium rolfsii* belongs to this section (Samson & Houbraken 2011).

The anamorphic *Penicillium* genus (Berbee 1995) belongs to the *Trichomaceae* family, where the species are considered saprophytic, also they can secrete mycotoxins such as aflatoxins, ocratins, patulins and others that are used as pharmaceutical products such as penicillin (Geiser et al, 2006). There are some species of the *Penicillium* genus that are considered as phytopathogens of fruits and can affect all *Rubus* plantations. This can be seen as powdery fungal growth in fruits and softening of the infected tissue (George & Fox, 2014). As for the *Penicillium rolfsii* species, it is a potential lignocellulolytic fungus capable of hydrolyzing oil palm residues to generate biofuel (Chang et al, 2012). However, in this study, it can cause toxicity to the raspberry leaves along with mycelium growth.

Although these six fungi have been isolated from raspberry, the results observed in the bioassays indicate that almost all fungi do not completely colonize the leaf, which could be seen where the fungi produce a hypersensitive response (HR), which means that the plant induce localized defenses to prevent the pathogen to continue its infection process (Gassmann and Bhattacharjee, 2012). This can be seen in the fungi *Bionectria pseudostrata*,

Fusarium concentricum and *Lasiodiplodia theobromae*, since initially they present a necrosis followed by a chlorotic halo. In the case of *Colletotrichum gloesporoides*, *Phanerochaete chrysosporium* and *Penicillium rolfsii*, a greater injury was seen, however all fungi affect the leaves only at the points where a wound was previously made, indicating that they do not yet have the sufficient infective capacity to become a pathogen and used as a biological control.

CONCLUSION

Rubus niveus is considered the worst grass in the Galapagos since it has characteristics of an invasive plant

595 fungi were isolated from 412 samples collected from necrotic tissue of *Rubus niveus*, of which they are divided into 226 groups by phenotype, indicating that Galapagos is also diverse in relation to fungal biodiversity.

Of the 226 bioassays performed, only 6 fungi were considered as potential candidates for biological control agents, since the lesions caused were greater than 7 mm on leaves during pathogenicity test. Here, *Bionectria pseudostrata*, *Fusarium concentricum*, *Penicillium rolfsii*, *Phanerochaete chrysosporium*, *Lasiodiplodia theobromae* and *Colletotrichum gloesporoides* are found.

The fungus *Bionectria pseudostrata* cannot be considered as a candidate to control blackberry because it is endophyte and saprophyte.

Fusarium concentricum, *Lasiodiplodia theobromae* and *Colletotrichum gloesporoides* fungi cannot be considered biological drivers for raspberry as they are cosmopolitan and can cause disease in many species, including endemic species of Galapagos.

Although *Penicillium rolfsii* and *Phanerochaete chrysosporium* are not specific to raspberries, more research should be carried out for possible application as a biological control. Multiple subsequent inoculation trials could be done to increase the infection capacity of these fungi against raspberry to potentiate plant-pathogen evolution.

REFERENCES

- Afanador-Kafuri, L., González, A., Gañán, L., Mejía, J. F., Cardona, N., and Alvarez, E. (2014). Characterization of the *Colletotrichum* species causing anthracnose in Andean blackberry in Colombia. *Plant Dis.* 98:1503-1513.
- Anderson. (2009). El uso de hongos como agentes de control biológico de malezas exóticas. Centro de Recursos Renovables de la Zona Semiárida. Cerzos, Conicet, UNS. Argentina
- Atkinson, R., J. L. Rentería, and W. Simbaña. (2008). The consequences of herbivore eradication on Santiago: are we in time to prevent ecosystem degradation again? in: Galapagos Report 2007-2008. Puerto Ayora, Galapagos, Ecuador
- Bautista-Cruz, M. A., Almaguer-Vargas, G., Leyva-Mir, S. G., Colinas-León, M. T., Correia, C. K., Camacho-Tapia, M., Robles-Yerena, L., Michereff, S. J., and Tovar-Pedraza, J. M. (2018). Phylogeny, distribution, and pathogenicity of *Lasiodiplodia* species associated with cankers and dieback symptoms of Persian lime in Mexico. *Plant Dis.* XXX:XX-XX.
- Bensted-Smith, R., G. Powell & E. Dinerstein. (2002). Planificación para la ecorregión. Pág. 1-5, en: Visión para la biodiversidad de las islas Galápagos. Fundación Charles Darwin y WWF. Puerto Ayora, Galápagos.
- Berbee ML, Yoshimura A, Sugiyama J, Taylor JW (1995). Is *Penicillium* monophyletic? An evaluation of phylogeny in the family Trichocomaceae from 18S, 5.8S and ITS ribosomal DNA sequence data. *Mycologia* 87: 210–222.
- Black J. (1973). Galápagos archipiélago del Ecuador. 138 pág.; Fundación Charles Darwin. Ecuador
- Bohle K, Jungebloud A, Gocke Y, Dalpiaz A, Cordes C, Horn H, Hempel DC. (2007). Selection of reference genes for normalization of specific gene quantification data of *Aspergillus niger*. *J. Biotechnol.*
- CABI. (2019). Controlling the invasive blackberry on the Galapagos Islands.
- Cabrera, G. & Briano, E. (2012). El control biológico de plagas. United States Department of Agriculture. National Agricultural Library.
- Cannon PF, Buddie AG, Bridge PD. (2008). The typification of *Colletotrichum gloeosporioi* des. *Mycotaxon* 104:189 –204
- Consejo de Gobierno del Régimen Especial de Galápagos. (2016). Plan de Desarrollo Sustentable y Ordenamiento Territorial del Régimen Especial de Galápagos. –Plan Galápagos. Puerto Baquerizo Moreno, Galápagos, Ecuador

- CDF. (2009). Charles Darwin Foundation, Galapagos. Providing Technical Assistance to the Control of the Invasive Blackberry *Rubus niveus* on the Islands of Santiago and Floreana.
- Chang, L., Arai, T., Kosugi, A., Ibrahim, D., Prawitwong, P., Mori, Y. (2012). *Penicillium rolfsii*, the potential lignocellulolytic fungus on hydrolysis of oil-palm residues from oil palm trunk as a second generation biofuel feedstock. Proceedings of The 2nd Annual International Conference Syiah Kuala University
- Cock, M. (1996). Control biológico de malezas. Manejo de Malezas para países en Desarrollo. (Estudio FAO Produccion y Protección Vegetal – 120) capítulo 9. Roma.
- Contreras-Pérez M, Santoyo-Pizano G, de los SantosVillalobos S, Gutiérrez-García MA, Orozco-Mosqueda MC and Rocha-Granados MC. (2019). First report of *Lasiodiplodia* on blackberry plants (*Rubus* subgenus *Eubatus*) in the Michoacan state, Mexico. Mexican Journal of Phytopathology 37(3): 479-485.
- Dhingra, O., Sinclair, J. (1995). Basic Plants Pathology Methods. CRC Lewis Publishers. Boca Raton, Florida. 6 DIOP T
- Dissanayake, A. J., Phillips, A. J. L., Li, X. H., and Hyde, K. D. (2016). Botryosphaeriaceae: Current status of genera and species. *Mycosphere* 7:1001–1073.
- Domsch KH, Gams W, Anderson T. (2007). Compendium of soil fungi, 2nd edn. IHW Verlag, Eching
- Drummond, A.J. and A. Rambaut. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7(1): 214.
- Dueñas, M., & Tellería, M. T. (1988). *Aphyllorphorales cántabro-astures* (Vol. 5). Editorial CSIC-CSIC Press.
- Eriksson J, Hjortstam K, Ryvarden L. (1978). The Corticiaceae of North Europe. *Mycoaciella-Phanerochaete* 5: 889e1047 *Fungiflora*. Oslo, Norway.
- Evans H.C. (1987). Fungal pathogens of some subtropical and tropical weeds and the possibilities for biological control. *Biocontrol News and Information* 8: 7-30.
- Evans, K. J., L. Morin, E. Bruzzese, and R. T. Roush. (2004). Overcoming limits on rust epidemics in Australian infestations of European blackberry, pp. 514-519. In: Cullen, J. M., D. T. Briese, D. J. Kriticos, W. M. Lonsdale, L. Morin, and J. K. Scott (Eds.) Proceedings of the XIth International Symposium on Biological Control of Weeds, Canberra, Australia. CSIRO Entomology, Canberra, Australia.
- Fang WG, Bidochka MJ. (2006). Expression of genes involved in germination, conidiogenesis and pathogenesis in *Metarhizium anisopliae* using quantitative real-time RT-PCR. *Mycological Res.*

- Floudas, D & Hibbett, D. (2015). Revisiting the taxonomy of *Phanerochaete* (Polyporales, Basidiomycota) using a four gene dataset and extensive ITS sampling. Fraire, M., Yáñez, M., Nieto, D., Vásquez, G. 2002. Honhos patógenos en fruto de Fresa (*Fragaria x ananassa* Duch.) en Postcosecha. *Revista mexicana de Fitopatología*
- Fundación Charles Darwin (FCD) y WWF-Ecuador. (2018). Atlas de Galápagos, Ecuador: Especies Nativas e Invasoras. Quito, FCD y WWF Ecuador
- Geiser DM, Gueidan C, Miadlikowska J, Lutzoni F, Kauff F, Hofstetter V, Fraker E, Schoch CL, Tibell L, Untereiner WA, Aptroot A (2006). Eurotiomycetes: Eurotiomycetidae and Chaetothyriomycetidae. *Mycologia* 98: 1053–1064.
- George, R. A., & Fox, R. T. (2014). Diseases of temperate horticultural plants. CABI.
- Glass NL, Donaldson GC. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes, *Appl Environ Microbiol.* vol. 61 (pg. 1323-1330)
- Karez, C. Patry, M. Rosabal, P. (2006). Galapagos Islands World Heritage Site. Report to the World Heritage Committee on the mission carried out from February 28 to March 10, 2006
- Kellogg, A., Branaman, T., Jones, N., Little, C., Swanson, J. (2011). Morphological studies of developing *Rubus* prickles suggest that they are modified glandular trichomes.
- Kersten P, Cullen D, (2007). Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Fungal Genetics and Biology* 44: 77e87.
- Lawesson, J. E. and L. Ortiz. (1990). Plantas introducidas en las islas Galapagos. Pages 201-210 in J. E. Lawesson, O. Hamann, G. Rogers, G. Reck, and H. Ochoa, editors. *Botanical Research and Management in Galapagos*. Missouri Botanical Garden, St. Louis, .U.S.A.
- Li QQ, Skinner J, Bennett JE. (2012). Evaluation of reference genes for real-time quantitative PCR studies in *Candida glabrata* following azole treatment. *BMC Mol. Biol.* 13: 22–34. 10.1186/1471-2199-13-22
- Liu Y, Whelen S, Hall B. (1999). Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Mol. Biol. Evol.* 16(12): 1799-1808.
- López, U., Zambolim, L., Pereira, O. (2009). First report of *Lasiodiplodia theobromae* causing leaf blight on the orchid *Catasetum fimbriatum* in Brazil.
- López L, Hernández M, Colín A, Ortega S, Cerón G, Franco R. (2014). The basic stains in the microbiology laboratory.;3(1):10–8. Available from: <http://www.medigraphic.com/pdfs/invdiss/ir-2014/ir141b.pdf>

- Marasas WFO, Miller JD, Riley RT, Visconti A. (2001). Fumonisin occurrence, toxicology, metabolism and risk assessment. In: Summerell BA, Leslie JF, Backhouse D, Bryden WL, Burgess LW, eds. *Fusarium-Paul E. Nelson Memorial Symposium*. St Paul, Minnesota: APS Press. p 332-359.
- Moreira, G., Abreu, L., Carvalho, V., Schroers, H., Pfenning, L. (2016). Multilocus phylogeny of *Clonostachys* subgenus *Bionectria* from Brazil and description of *Clonostachys chloroleuca* sp. nov. *Mycol Progress*.
- Nailis H, Coenye T, Nieuwerburgh FV, Deforcr D, Nelis HJ. (2006). Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. *BMC Mol. Biol.* 7: 25–33. 10.1186/1471-2199-7-25
- Nirenberg HI, O'Donnell K, (1998). New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia*, 90: 434-458
- O'Donnell K, Cigelnik E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous, *Mol Phylogenet Evol.* vol. 7 (pg. 103-116)
- O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proc Natl Acad Sci U S A* 95:2044–2049. doi:10.1073/pnas.95.5.2044.
- Parque Nacional Galápagos (PNG). (1998). Plan De Manejo de Conservación y Uso Sustentable Para la Reserva Marina de Galápagos.
- Parque Nacional Galápagos (PNG). (2016). Plan anual de Inversiones. Proyecto de Inversión Pública y de Cooperación externa no reembolsable. Proyecto de control y erradicación de especies invasoras prioritarias para la reducción de la vulnerabilidad de especies endémicas y nativas de las Islas Galápagos.
- Perlroth J, Choi B, Spellberg B. (2007). Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Medical Mycology* 4: 321–346. <https://doi.org/10.1080/13693780701218689>
- Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., and Crous, P. W. (2013). The Botryosphaeriaceae: genera and species known from culture. *Stud. Mycol.* 76:51–167.
- Pryet, A. (2012). Hydrogeology of volcanic islands: a case-study in the Galapagos Archipelago (Ecuador). *Sciences de la Terre, Hydrogeologie Ecole doctorale Geosciences et Ressources Naturelles*. UPMC.

- Ramírez, J., Castrejón, M., & Toral-Granda, M. V. (Eds.). (2012). *Mejorando la Pesquería de Langosta Espinosa en la Reserve Marina de Galápagos [Improving the Spiny Lobster Fishery in the Galápagos Marine Reserve]*. Galápagos, Ecuador: WWF
- Rentería JL, Gardener MR, Panetta FD, Atkinson R, Crawley MJ, (2012). Possible impacts of the invasive plant *Rubus niveus* on the native vegetation of the *Scalesia* forest in the Galapagos Islands. *PLoS ONE*, 7(10):e48106.
- Samson, R & Houbraken, J. (2011). Phylogenetic and taxonomic studies on the genera *Penicillium* and *Talaromyces*. *Studies in Mycology* 70.
- Sandoval-Denis, M., Swart, W. J., & Crous, P. W. (2018). New *Fusarium* species from the Kruger National Park, South Africa. *MycKeys*, (34), 63–92. doi:10.3897/mycokeys.34.25974
- Schroers H-J. (2001). A monograph of *Bionectria* (Ascomycota, Hypocreales, Bionectriaceae) and its *Clonostachys* anamorphs. *Stud Mycol* 46:1–214
- Sharma, G., Maymon, M. & Freeman, S. (2017). Epidemiology, pathology and identification of *Colletotrichum* including a novel species associated with avocado (*Persea americana*) anthracnose in Israel. *Sci Rep* 7, 15839 doi:10.1038/s41598-017-15946-w
- SIGTIERRAS, (2010). Catastro multifinalitario de la zona rural de las islas pobladas de Galápagos, Quito, Ecuador.
- Slippers, B., Boissin, E., Phillips, A. J. L., Groenewald, J. Z., Lombard, L., Wingfield, M. J., Postma, A., Burgess, T., and Crous, P. W. (2013). Phylogenetic lineages in the Botryosphaeriales: a systematic and evolutionary framework. *Studies in Mycology* 76:31e 49.
- Slippers, B., and Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biol. Rev.* 21:90–106.
- Stone, L.K., Bacon, E.W. y White, L.F. (2000). An overview of endophytic microbes: endophytism defined. In *Microbial endophytes*. Edited by W. Bacon and I.F. White. Marcel Dekker Inc., New York, Basel. pp. 3–29.
- Templeton, G.E. (1982). Biological herbicides: Discovery, development, deployment. *Weed Science* 30:430-433.
- Templeton MD, Rikkerink EHA, Solon SL, Crowhurt RN. (1992). Cloning and molecular characterization the glyceraldehyde-3-phosphate dehydrogenase-encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. *Gene* 122:225-230.
- Trujillo, E. E. (1985). Biological control a *Hamakua* pa-makani with *Cercospora* sp. in Hawaii, pp. 661-671. In: Delfosse, E. S. (ed.). *Proceedings of the VIth International Symposium on Biological Control of Weeds*, 19-25 August, 1984. Vancouver, British Columbia, Canada, Agriculture Canada, Ottawa, Ontario, Canada.

- Trueman M., A. Guézou y P. Wurm. (2010). Residence time and human mediated propagule pressure at work in the alien flora of Galapagos. *Biol. Invasion*. Pp. 1-12
- Van Driesche, R., Hoddle, M., Center, T. (2007). Control de Plagas y Malezas por enemigos naturales. Forest Health Technology Enterprise Team. Biological Control.
- Wagner, W.L., D.R. Herbst, and S.H. Sohmer. (1999). Manual of the Flowering Plants of Hawai'i. 2 vols. Bishop Museum Special Publication 83, University of Hawai'i and Bishop Museum Press, Honolulu, HI.
- Wapshere, A.J., (1974). A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology* 77: 201 - 211.
- Weir BS, Johnson PR, Damm U. (2012). El complejo de especies *Colletotrichum gloeosporioides*. *Seminal. Mycol.* 73 : 115-180.
- White TJ, Bruns T, Lee S, Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego: Academic Press. p 315–322