Table of contents

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ABSTRACT

Vanilla is a high value cash crop that is continuously demanded by the agri-food and cosmetics industries for its incomparable flavor. Most of vanilla comes from the cured fruits of *V. planifolia* G. Jackson, a hemi-epiphytic climbing orchid cultivated in the humid tropics. In all the countries were it is cultivated, the vanilla vines suffer from a root and stem rot (RSR) caused by the soil borne fungus *Fusarium oxysporum* which dramatically reduces plant production and the durability of plantations. No efficient control method is currently available for this disease. Sources of genetic resistance to RSR exist in few vanilla relatives, but so far no commercial resistant variety has been produced. The purpose of this thesis was to better describe the diversity and histopathology of the causal agent of RSR and to evaluate the potential sources of genetic resistance that could be used in breeding programs.

In a first step, a collection of 377 single-spored *Fusarium* isolates recovered from rotten roots and stems during surveys conducted in 52 vanilla plots from Reunion Island, Madagascar and French Polynesia were characterised. Representative subsets of isolates were genotyped using the Elongation Factor 1α and Intergenic Spacer gene sequences. Their pathogenicity was assayed by root dip inoculation on the susceptible *V. planifolia* accession pla0001. Results showed that *F. oxysporum* was the principal species responsible for the disease in the field, although a few *F. solani* isolates showing slight pathogenicity were also isolated. *Fusarium oxysporum* isolates were highly polyphyletic regardless of geographic origin or pathogenicity. Remarkably, their pathogenicity varied in gradient between non-pathogenic (about 42% of isolates) to highly pathogenic (14%).

In a second step, 254 vanilla accessions comprising 18 species and six types of hybrids were assessed for resistance to RSR in the field (natural inoculum) and in the lab (*in-vitro* plants inoculated with Fo072). The strong resistance to RSR of all *V. pompona* accessions and hybrids of *V. planifolia* X *V. pompona* or *V. phaeantha,* were confirmed, and novel sources of resistance to RSR were added including, *V. bahiana, V. costariciensis* and *V. crenulata*. Most of the *V. planifolia* accessions, *V. ×tahitensis* and *V. odorata* were susceptible to RSR. However, three inbreeds of *V. planifolia* showed a high level of resistance to Forv. To our knowledge this is the first report of resistance to RSR in *V. planifolia* accessions. For the 26 accessions evaluated in both conditions, a strong correlation was observed between long term (9 years) evaluation in the field and ratings on *in-vitro* plants at 15dpi.

Thirdly, we monitored by wide field and multiphoton microscopy the root infection process and the responses of one susceptible accession (*V. planifolia* pla0001) and two resistant accessions (*V. planifolia* pla0020 and *V. pompona* pom0018) to challenge inoculation with the severe isolate Fo072. In the compatible interaction (Fo072 – pla0001) invasion started from penetration of hyphae emitted from germinated conidia in the hairy region of root rapidly colonizing the cortex but never expanded to the vascular bundles up to the 9th dpi. It was therefore suggested to prefix the *forma specialis* name of the causal agent of RSR with *radicis* to point out its non-vascular pathogenicity in vanilla. In the two incompatible interactions, the important role played by hypodermis cells for impeding the invasion of the cortex by Fo072 was demonstrated by specific staining and spectral analysis of lignin precursors. Both constitutive and pathogen induced defense mechanism were described in pla0020 and pom0018. The mechanisms included the deposition of lignin in the hypodermal cell wall, entrapment of hyphae in specific hypodemal cells and polyphenolics secretion in intercellular spaces. Further, a *de novo* transcriptome analysis was experimented on 8 pooled samples.

Preliminary transcriptomic data were obtained by *de novo* RNAseq (Illumina) for eight combinations of resistant vs susceptible accessions, inoculated or not with pathogenic or non-pathogenic isolates. Analysis of the transcripts yielded 169 genes differentially expressed genes including of few genes involved in the plant resistance mechanisms and in the production of secondary metabolites and lignin biosynthesis.

Altogether, our data clarified the aetiology of RSR, broadened the sources of resistance to Forv and enlightened the resistance mechanism at work in resistant genotypes. As such, they will enhance the breeding programs aimed at developing novel vanilla varieties resistant to RSR.

CHAPTER I **INTRODUCTION**

I Vanilla

Vanilla (*Vanilla planifolia* G. Jackson) belonging to the family *Orchidaceae* is one of the most expensive spice crops. The beans of this orchid on processing yield "vanilla", which has a great demand in the flavoring industry. *Vanilla planifolia*, being the only source of natural vanillin, makes it an inevitable part in food industries owing to its unique and pleasant aroma. These features make vanilla the most popular and widely used spice in global market. The major consumers of vanilla beans are currently the developed countries, with the United States, Germany and France absorbing more than 80 % of the world exports (Anilkumar, 2004)

Vanilla is native to Southeast Mexico and possibly other parts of Central America. It is now cultivated in various parts of the tropics, between the 27th north and south parallels. Presently vanilla cultivation provides economy and labor in countries such as Madagascar, Indonesia, Mexico, Reunion Islands, India, Comoros Islands, Uganda, Java, Costa Rica and French Polynesia (Figure 1). The genus *Vanilla* consists of about 110 species of which only two species are commercially exploited and cultivated, namely *V. planifolia* and *V.* X *tahitensis* J.W. Moore (Figure 2). Due to its superior vanillin content, 95 percent of the vanilla bean production is contributed by the *V. planifolia* species (Lubinsky et al., 2008).

I-1 History of vanilla cultivation

The name "vanilla" comes from the Spanish word "vainilla" meaning "little pod". The interesting history of vanilla starts from the ancient Totonac Indians of the eastern coast of Mexico, who are supposed to be the first people who learned to use the fruit of the Tlilxochitl vine, the vanilla pod. The Aztecs acquired vanilla when they conquered the Totonacs in the 15th century; the Spanish, in turn, got hold of vanilla when they conquered the Aztecs. Eventually, from the Spanish the use of vanilla became widespread throughout Europe. As

Europeans explored the forests of Central and South America, vanilla was introduced in Europe and in European colonies. Unsuccessful efforts were made for production, but failed due to the absence of natural pollinators (Purseglove et al., 1981) although an artificial pollination technique was reported in late 1836 by a Belgian botanist named Charles Francois Antoine Morren (Pinaria, 2009, Weiss, 2002).

The vanilla introduction to Reunion Island was first initiated by M. Philibert, a navy officer, during his trip back from Cayenne in French Guyana. The first material to be introduced is believed to be cuttings of *V. pompona*. The second introduction was also carried out by the same enthusiast officer in 1820, during his mission back from Philippines (Figure 3). In the second introduction, material introduced was called "little vanilla" which was dissimilar from the previously introduced. The introduced cuttings were disseminated and planted in different locations of the island, even though its acclimatization is not certain.. The third introduction of vanilla cuttings to the island was done by Marchant, a local colonial administrator after his visit to the Paris Museum in 1822. The introduced material was different from the previously introduced and was the actual *V. planifolia*, a milestone in the history of vanilla cultivation in the Island (Bory et al., 2008b, Lucas, 2010).

Even though the vanilla plants were successfully acclimatized in the island, the commercialization was null as the plants did not bear any fruits due to the absence of natural pollinators. The artificial means of pollination developed in Europe was not practical enough. Vanilla production started with the discovery of a simple manual pollination method discovered by Edmond Albius, a 12 years old slave in 1841. This marked the beginning of commercial production of vanilla in the island and worldwide.

The development of a new curing method in the island accelerated the production of vanilla. In Mexoco, the vanilla was prepared by exposing the beans in the sun and shade,

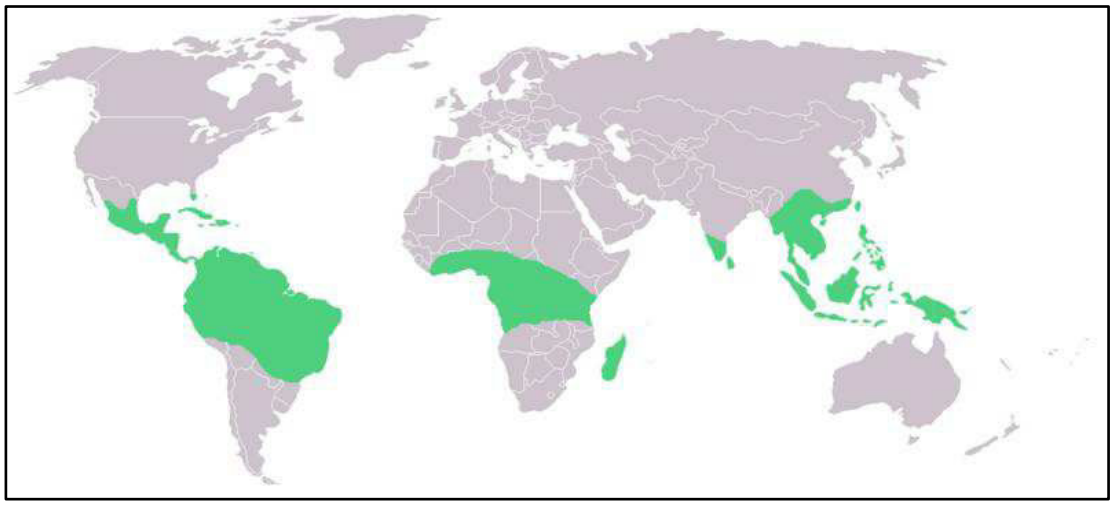

Figure 1: World map showing counties involved in vanilla production.

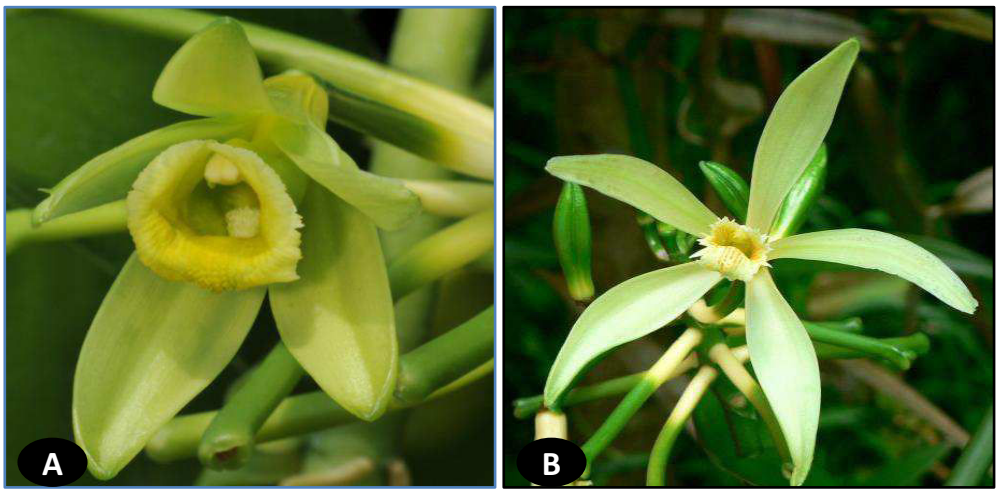

Figure 2: Cultivated vanilla species, A) *V. planifolia* and B) V. *X tahitensis*

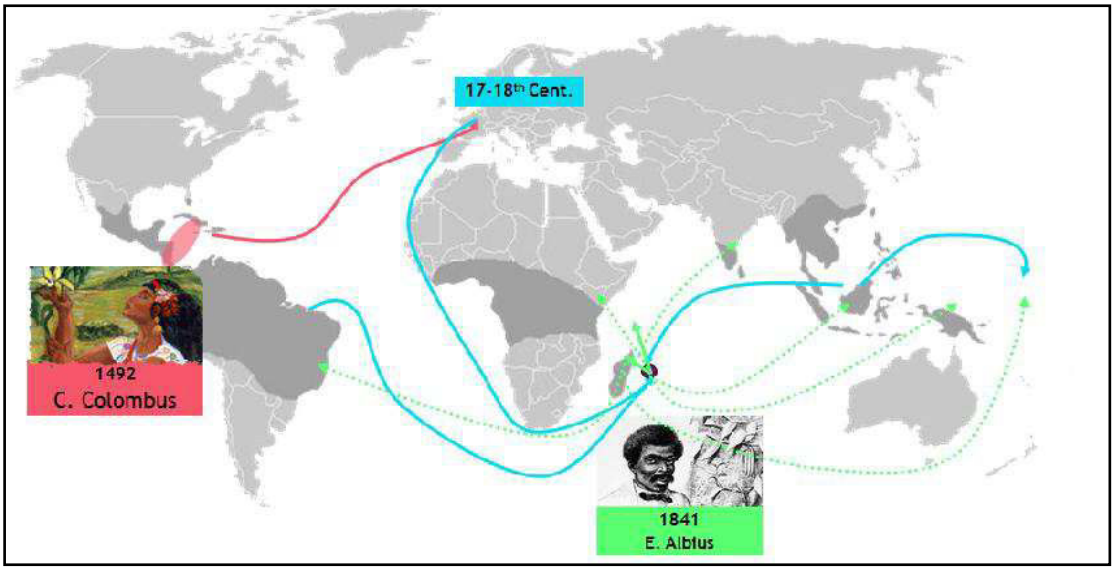

Figure 3: History of Vanilla introduction in the Indian Ocean Region. Grisoni (2009)

following the method practiced in Central America. The old method was replaced in 1851, when Ernest Loupy introduced a new technique consisting of dipping the beans in hot water and then dried. The method was later modified by David de Floris, who was the first person to export cured vanilla from the island. The export and trade of vanilla sky-rocketed the economy of Reunion Island. By the end of the nineteenth century, successful cultivation of vanilla prompted the vanilla planters to establish plantations in nearby islands like Seychelles, Comoros and Madagascar. Later in 1964, the vanilla growers in Reunion Island, Madagascar and Comoros united and gave birth to a new label of vanilla; "The Bourbon Vanilla", which still has a great demand all over the world.

I-2 Economic importance of vanilla

Vanilla is one of the most expensive flavors and fragrances. The natural vanilla flavor is composed of a large array of aromatic compounds formed after curing of the beans of *Vanilla planifolia*. This is achieved after an intense and time consuming process. Vanilla extracts are widely used for flavoring ice creams, soft drinks, chocolates, puddings, liquors and as a fragrance ingredient in many perfumes (Perez-Silva et al., 2006, Rao & Ravishanka, 2000, Webster, 1995, Jadhav et al., 2009). Moreover vanilla export represents significant revenue and provides employment to many people in the major vanilla growing countries.

Apart from the flavoring properties vanilla have been used for its medicinal properties. It was included in the European pharmacopoeia as early as in the 17^{th} century indicated for curing various diseases. Advanced pharmacological research studies are currently exploring vanillin for its anticancer, antimicrobial properties (Anuradha et al., 2013). The antimicrobial activity of vanilla has been used as a natural food preservative in fruit purees, as it inhibits the growth of yeast, mold and bacteria (Cerrutti & Alzamora, 1996). The antioxidant activity of

vanillin in complex foods containing polyunsaturated fatty acids makes it suitable in many food industries (Burri et al., 1989, Shyamala et al., 2007)

Above all, the agro climatic requirement and highly laborious cultivation process including the harvesting and curing have all added to the high cost of vanilla. The drastic reduction of cultivation due to diseases and environmental factors also decides the economic value of vanilla in the global market.

I-3 Taxonomy of *Vanilla*

Vanilla Plum. ex Miller is an ancient genus in the Orchidaceae family belonging to the Vanilloidae subfamily, Vanilleae tribe and Vanillinae subtribe. The molecular clock estimates that the genus *Vanilla* is around 65 million years old (Ramirez et al., 2007, Bouetard et al., 2010). *Vanilla* is considered to be a taxonomically difficult genus due to the ephemeral nature of flowers. The first classification of the genus was made by Rolfe (1896) classifying the genus into leafy (Foliosae) and leafless (Aphyllae) species. Later the Foliose section was divided into three subsections namely Papillosae, Lamellosae and Membranacae based on the morphological differences in leafs and labellum (Porterès, 1954). Recently the molecular phylogenetic studies using chloroplastic genes (Cameron et al., 1999, Cameron & Molina, 2006, Soto Arenas & Cameron, 2003) pointed out the incongruities in the former revision works in the genus. The results obtained from molecular phylogeny studies showed that the previous revisions made by Rolfe and Porteres did not have a phylogenetic value. Using plastid DNA (Bouetard et al., 2010) classified the genus into three clades (Figure 4), which confirmed the American origin of the genus and also demonstrated that the sections Foliosae (leafy) and Aphyllae (leafless) were not monophyletic, which is in contrary with the former classification proposed by Rolfe and Porteres.

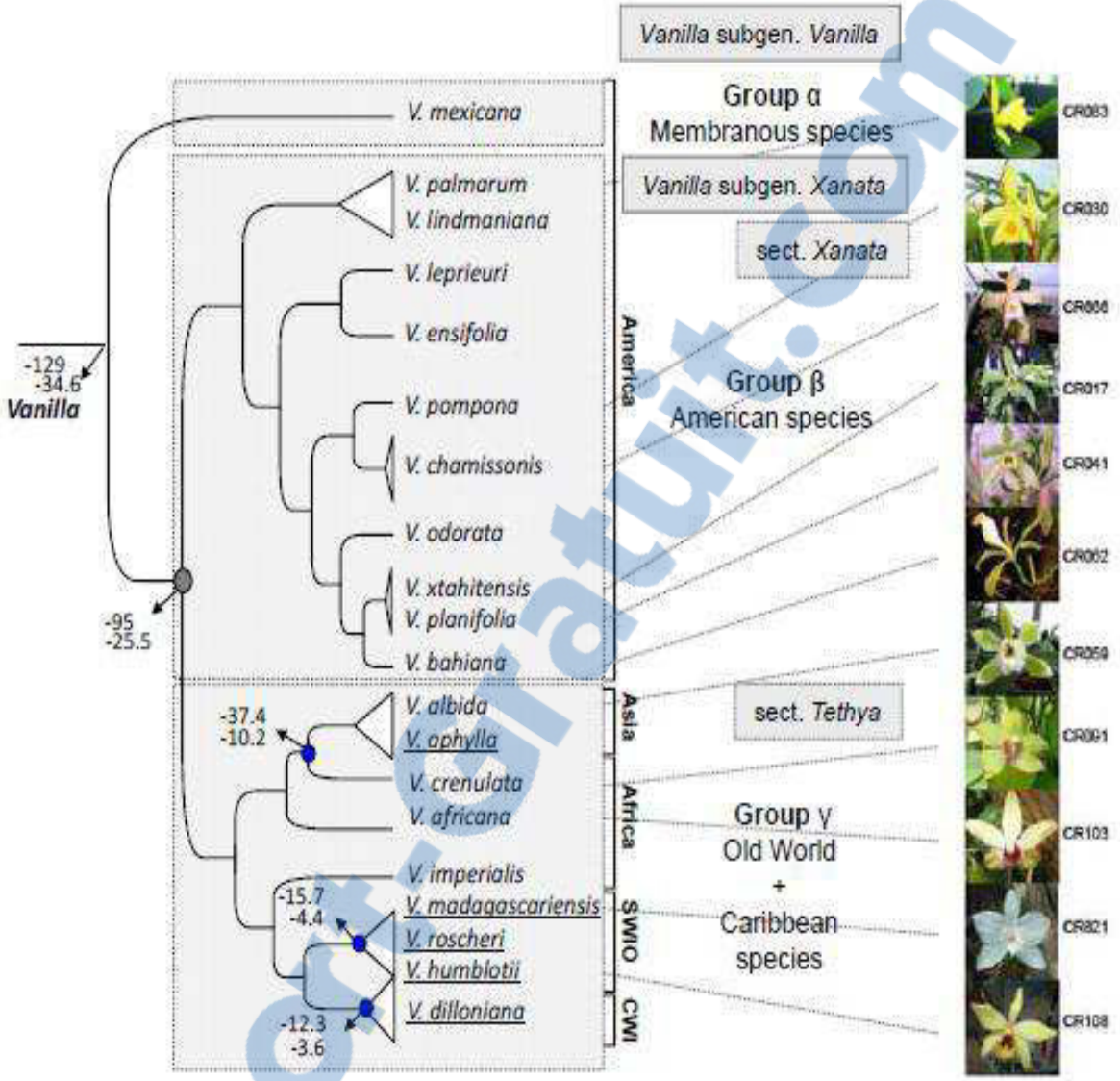

Figure 4: Molecular phylogeny of *Vanilla* genus based on plastid DNA (Bouetard et al., 2010)

The latest revision of the genus based on the phylogenetic data of 106 species (Soto Arenas & Cribb, 2010) proposed a new taxonomic classification, differentiating two sub-genera in the *Vanilla* genus : *Vanilla* sub-genus *Vanilla* comprising the ancestral *V. mexicana,* and *Vanilla* sub-genus *Xanata*. The sub-genus *Xanata* is further divided into section *Xanata* and section *Tethya* occupying the last two clades proposed by Bouetard (Bouetard et al., 2010).

I-4 Botany of *Vanilla*

Vanilla is a herbaceous, perennial and tropical climbing semi-epiphytic orchid. The basic chromosome number of vanilla genus is *x= 16*, with *V. planifolia* a diploid species with *2n= 32*, together with *V. pompona* Schiede. and *V. tahitensis* J.W Moore (Weiss, 2002). However recent cytogenetic studies have showed aneuploidy and polyploidy in *V. planifolia* (Bory et al., 2008a) and *V. pompona* (Siljak-Yakovlev et al., 2014).

The vine of vanilla grows up on support trees to a height of 15 m. In cultivation it is usually trimmed and kept shorter for easier hand-pollination and harvesting (Weiss, 2002). The stems are generally dark-green, cylindrical up to 2 cm in diameter. The stems are photosynthetic with stomatas and could be simple or branched with internodes ranging from 5 – 15 cm.

Vanilla plants are represented by two growth forms (Figure 5), vanilla with leaves and those without leaves or reduced leaves (Stern & Judd, 1999).The leaves of vanilla are succulent, flat, sub-sessile and canalized above. The thick succulent leaves are filled with sticky mucilage, which comprise of raphide crystals composed of calcium oxalate and calcium carbonate. The leaves are always alternate swirling around the stem with tips acute to acuminate and rounded base (Correll, 1953). The veins are parallel and indistinct on fresh but more obvious on dry leaves.

In vanilla, the vines usually produce two types of roots, the aerial and terrestrial roots. The aerial roots are usually short and unbranched aerial ones which are generally found clasping on the support. As in most orchids, the aerial roots develop an outer layer cells known as velamen which is involved in absorbing and holding water. On the other hand, the terrestrial roots are long and branched and penetrate the substrate and are involved in absorbing nutrients and water from the soil. Both the aerial and terrestrial root originate at the node, usually one root at each node (Stern & Judd, 1999).

There is a big diversity in the shape and color of flowers and fruits within *Vanilla* genus. The inflorescence of vanilla is a raceme accommodating 6 to 15 flowers and sometimes to a maximum of 30 flowers (Figure 6). In the case of *V. planifolia*, the flowers are green to yellow color and occur in clusters (Rao & Ravishanka, 2000). The flowers are usually 10 cm long, borne on pedicels. The flower comprises of three sepals, three petals, and a central column consisting of a united stamen and pistil, and one of the petals modified and enlarged to form the labellum (Figure 7). The vanilla flower opens from the base of the raceme upwards and stays in bloom less than 24 hours. Self-pollination generally does not happen in vanilla because of the rostellum, preventing the stigma to make a direct contact with the pollen grains. Commonly the flowers open early morning and remain receptive to pollination for eight hours. If the pollination is successful the flower will remain on the rachis for two or three days.

After pollination, the sepals and petals of the vanilla flower whither and eventually fall away from the ovary as it develops into a seed bearing fruit (Figure 8). The mature beans may be harvested by 8-10 months. The three sided fresh beans are 10- 25 cm long, 1- 1.5 cm wide with an unpleasant bitter odour. When ripe, the pods of dehiscent varieties splits longitudinally exposing thousands of small black seeds (Weiss, 2002).

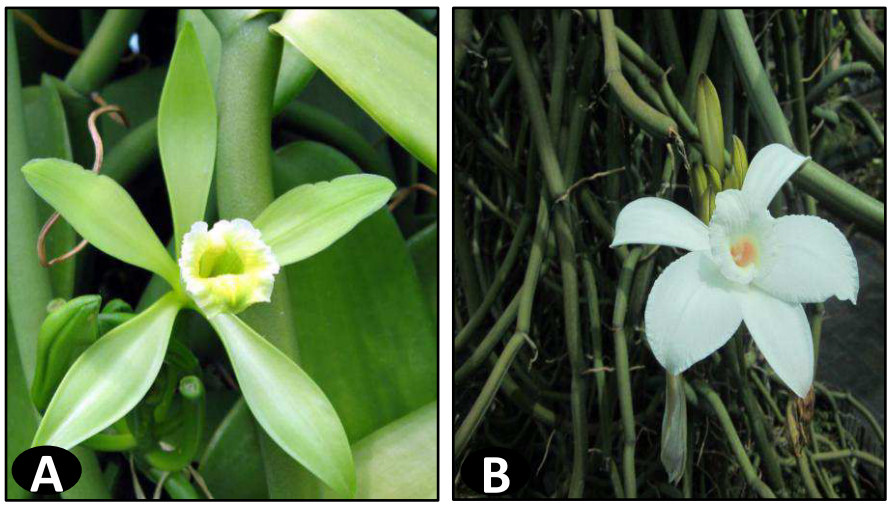

Figure 5: Two forms of vanilla A) leafy and B Leafless

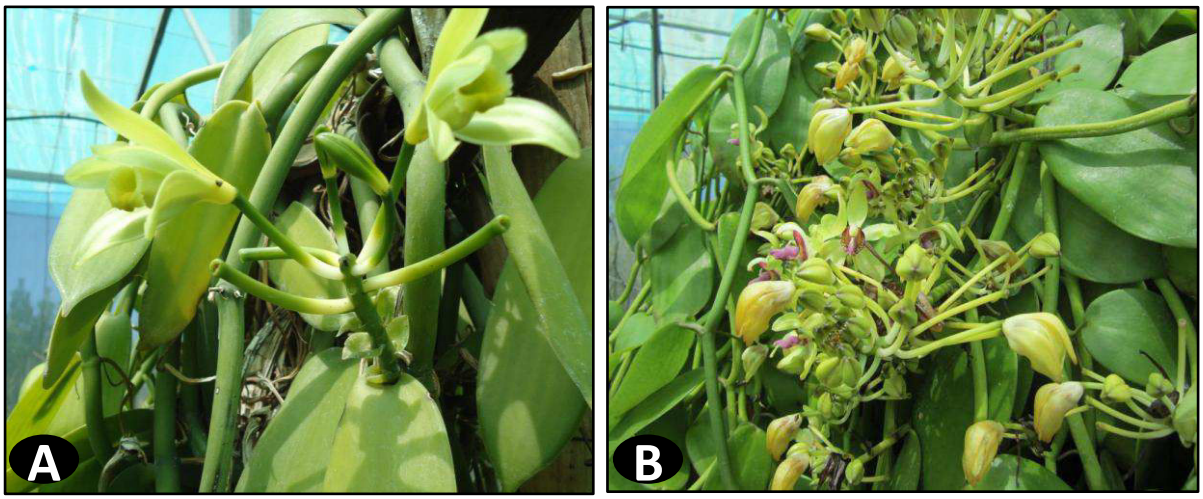

Figure 6: Racemose inflorescence: A) V. *planifolia* and B)*V. crenulta.*

The mature pods of vanilla accumulate glucovanillin (4-O-(3-methoxy-benzaldehyde-β-D-glucose)), which upon hydrolysis by β-glucosidase liberates Vanillin (Odoux & Brillouet, 2009). The glucovanillin is stored at the placental (92%) and trichome (7%) regions of the pod (Figure 9). Even though the green vanilla beans contain a little free vanillin, the aroma precursors are glycosylated and come into contact with β-D-glucosidases during the maturation of the fruit (Walton et al., 2003).

I-5 Histology of vanilla

The anatomical structures of different vanilla species were reported as early as the end of 19th century. These studies included the anatomical description of stem by Pompilian and Heckel speculating the presence and absence of endodermis in leafy and leafless species of vanilla. Studies were also conducted on leafs describing the pronounced cuticle and epidermal cells bearing octahedral crystals (Kruger, 1883). The uniseriate hypodermal cells and differences in the size of mesophyll cells and vascular bundles in the leaves of *V. planifolia* were also reported (Mobius, 1887). The differences in the structural and anatomical features of the clasping aerial roots and absorbing terrestrial roots were also reported (Went, 1895, Heckel, 1899).

However greater clarity in the anatomy of vanilla was obtained in the 20th century. There happened to be a few studies by Hafliger, Holm, Boriquet, Neubauer and Alconero, which cleared the discrepancies in the previous studies in many vanilla species. The study of Hafliger and Roux (Bouriquet, 1954) improved the anatomical description of a few species including *V. planifolia*. The study conducted by Stern and Jude (Stern & Judd, 1999) on 17 *Vanilla* species is the latest information available in the *Vanilla* genus regarding histology and is summarized below.

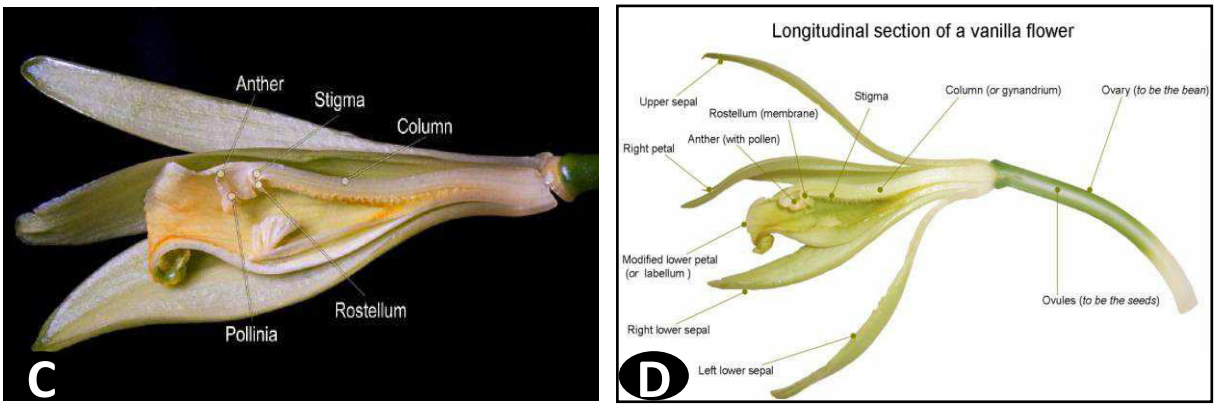

Figure 7: Cross section and descriptions of *Vanilla* flower.
Navez (2006)

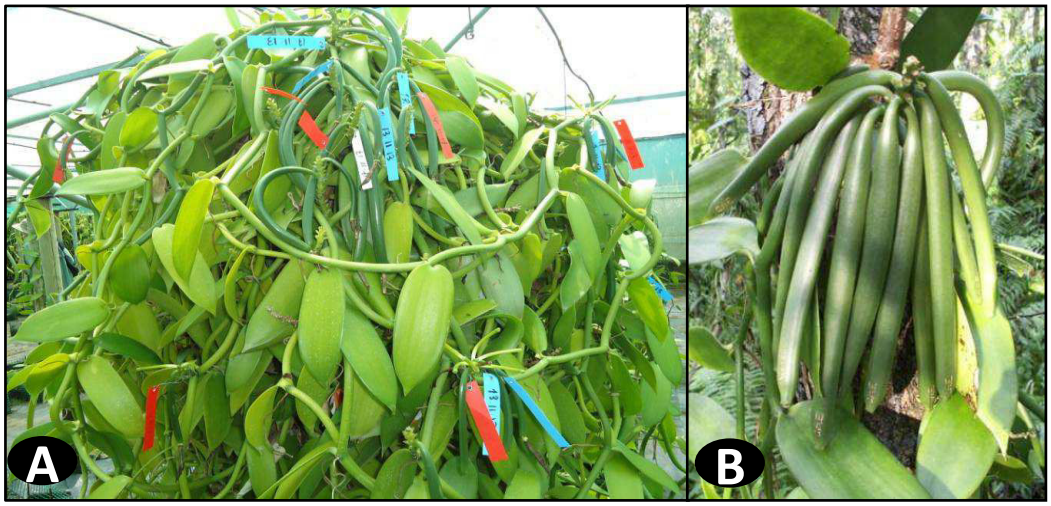

Figure 8: Vanilla plants bearing fruits

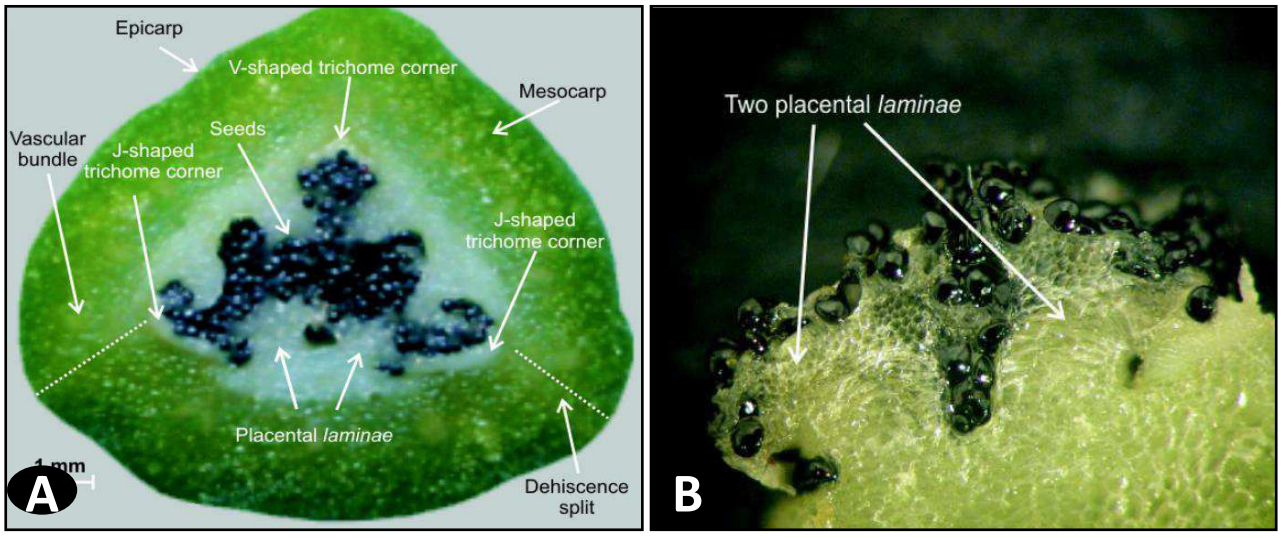

Figure 9: Anatomy of vanilla pod. A) Transverse section of pod and B) Placental laminae

According to Stern & Jude (1999), the leaf consists of mostly abaxial stomata with the epidermis having cells ranging from periclinal to isodiametric. A uniseriate hypodermis is present on the leaf and scale leaves, which appears on both sides except for a few species like *V. africana* and *V. phaeantha*. Vascular bundles are collateral and are located centrally in the mesophyll in leaves, whereas they are located close to adaxial surface in the scale leaves.

The stems of leafless species are bilobate, with two grooves opposite to each other along the stem. Stems are provided with a single layer of hypodermis in all the *Vanilla* species. Cortex consists mostly of thin walled, circular to oval cells with idioblasts. Collateral vascular bundles are randomly arranged and may completely or partially be surrounded by sclerenchyma. A sclerenchymatous band separating the cortex and ground tissues is present in leafy *Vanilla* species, whereas it is absent in leafless species. The ground tissue occupies the centre region of the stem featured with thin walled cells known as assimilatory cells.

Root system comprises of a single layer of velamen cells anticlinal in both the aerial and terrestrial root forms. Hyphae and protists commonly infest the velamen cells. Unicellular hairs are present in the terrestrial roots, whereas they are mostly absent in the aerial roots. A single layer of exodermis is present both in the aerial and terrestrial roots with intermittent passage cells. Thickenings on the exodermis largely vary according to the species (Figures 10, A-B). Outer and proximal wall thickening of exodermis are present in the aerial roots of some species (*V. dilloniana*, *V. phaeantha*, *V. pauciflora*) and also in the terrestrial roots of *V. pompona*. The cortex is composed of thin walled round or oval cells with or without intercellular spaces (Figure 10, C-D). The inner layers of cortex are also provided with irregular shaped lacunae that tend to radiate more or less equidistantly from the cylinder in both aerial and terrestrial roots. A single layered endodermis is present where the cell walls are variously thickened opposite to the phloem and thin walled opposite to the xylem. The vascular tissues are embedded in thin or thick walled sclerenchyma in the majority of the

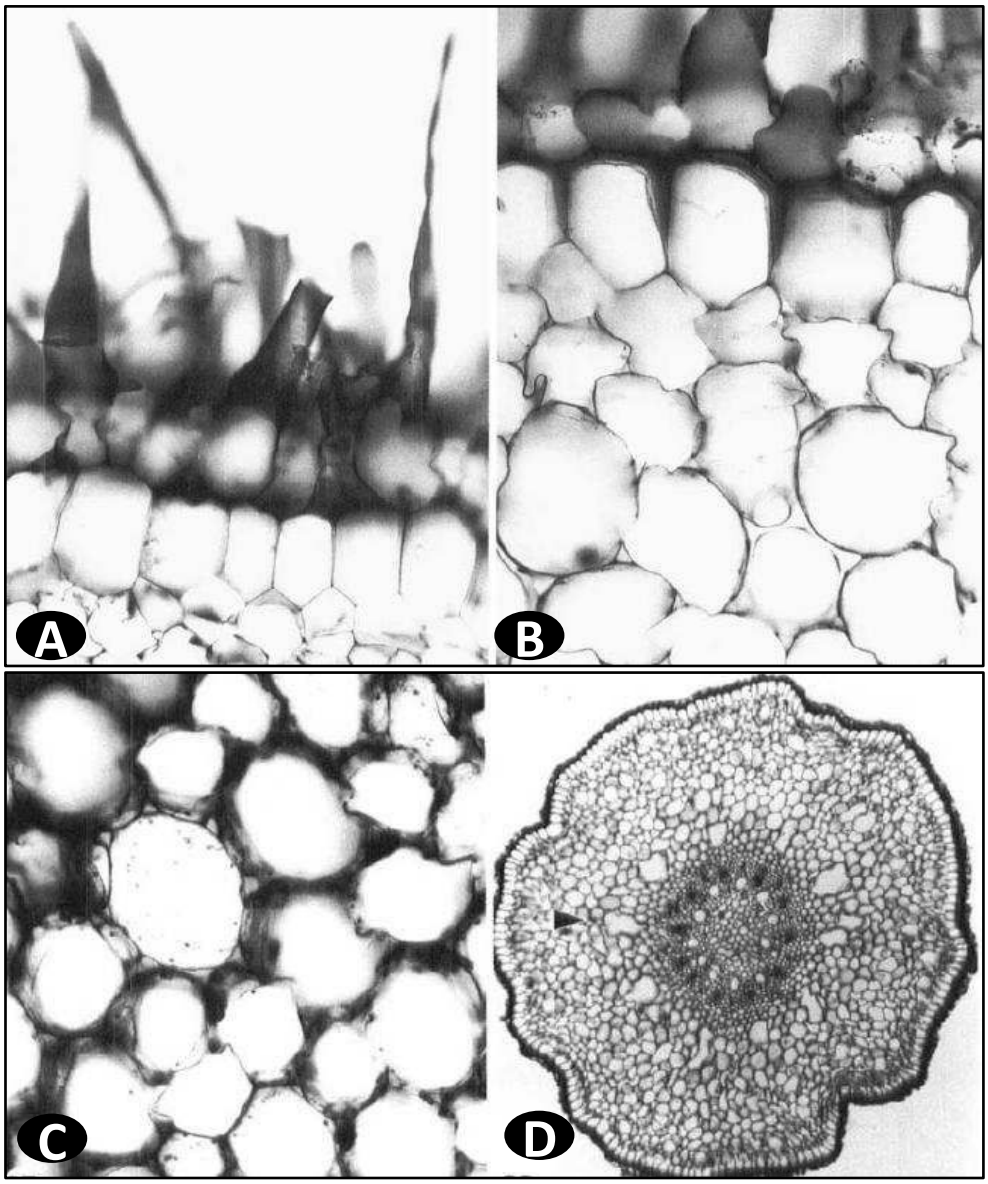

Figure 10: Cross section of vanilla roots. A) Thin walled and B) Thick walled hypodermis .C) Cortical cells of roots and D) Cross section of root.
Stern (1999)

species. The xylem and phloem strands alternate around the circumference of a vascular cylinder with 8-16 and 6-18 arches in aerial and terrestrial roots, respectively.

The unilocular inferior ovary of vanilla upon fertilization develops into beans (pods) provided with an outer epicarp, mid mesocarp and an inner endocarp. The pod has a triangular transverse section with a central cavity occupied the black seeds. The epicarp acting as a protective layer for the bean is made of a layer of thick walled polygonal shaped cells running parallel to the long axis of the bean. The mesocarp which occupies the majority of the bean is composed of parenchymatous cells which also occupy the closed collateral vascular bundles. The inner layer endocarp is composed of one or two layers of small cells that cover the inside of the fruit's cavity. Each side of the pod bears a placenta divided into two placental longitudinal laminae bearing funicles on which the seeds are attached. On cross sectioning, each pair of laminae appears as finger-shaped lobes bent inside the central cavity (Odoux et al., 2003).

I-6 Agronomy of Vanilla

Vanilla is usually cultivated in warm, moist tropical climate where the annual rainfall ranges between 190 and 230 cm. A dry period of two month is needed to restrict vegetative growth and induce flowering: rainfall in the remaining ten months should be evenly distributed (Correll, 1953). The vanilla can usually grow up to an altitude of 1500 m above the mean sea level. Vanilla established on gently sloping terrain with good drainage is reputed to produce better crops and to be more resistant to fungal infection. Vanilla grows best in light, porous and friable soils preferably of volcanic origin with pH of 6 to 7 (Correll, 1953, Straver, 1999). The vanilla is conventionally propagated through cuttings (Sasikumar, 2010).

Vanilla being a semi-epiphyte requires a support for its growth and development. Vanilla cultivation can be divided into extensive, semi intensive and intensive systems based on the

density of cultivation and nutrient supply (Kahane et al., 2008). In extensive management, the cuttings of vanilla are planted on selected trees in forest areas with limited changes of the environment (Figure 11A). This system of cultivation is apt for maintaining the natural habitat, while the production is on the lower side. In semi intensive or field system, the vines are grown on integrated shading trees like *Erythrina* or *Gliricidia* (Figure 11B). The canopy of the plant provides shade and organic matter for the vines. The production is high in this cultivation system. Finally, in the intensive or shade house system, the vines are grown on artificial support and shading system (Figure 11C). The vines are fed with compost imported to the shade house. The production in this system is higher compared to the previous cultivation systems. The high plant density presenting a higher risk of losses associated with disease contamination and the cost of investment are the major drawbacks of this system.

Once planted and the vine attains a length of 2 meters, the growth of shoot is re-directed towards the ground. This practice is known as “looping” and this maintains the plants at human height and facilitates hand pollination and harvesting. Looping also induces the flowering process and new shoot formation. After flowering, the flowers are subjected to hand pollination, which require experienced manpower to detect the opened flower and pollinate them early in the morning. A qualified person can pollinate up to 2000 flowers a day.

Every vanilla growing country has developed its own curing process, the fundamental principle being the same. The curing of vanilla beans involves 4 steps comprising of i) killing by dipping the pods in hot water (45°C to 65°C), ii) sweating by wrapping in clothes, the stage in which the beans develop the characteristic vanilla aroma, iii) slow drying by spreading the beans on wooden racks and iv) conditioning stage where the beans are sorted and stored in wooden boxes.

Figure 11: Vanilla cultivation systems A) Extensive B)Semi intensive and C) Intensive cultivation systems.

I-7 Diseases of vanilla

The vanilla production has either remained static or declined over the past decade due to abiotic and biotic constraints. Both these factors combined have a deep impact on the production and exportation in major vanilla growing countries. Abiotic factors, including changes in the climatic factors causing drought and excessive heat in the plantations, adversely affect the efficiency of vanilla production. Damages from natural pruning, sunburn and hurricanes play a major role in the decline of production and export of vanilla beans (Hernandez-Hernandez, 2011). The hurricanes which are generally prone to Southwest Indian Ocean regions including Madagascar, Reunion Island and seldom in Comoros cause severe losses in vanilla plantations. On the other hand, biotic factors include pathogens causing disease to vanilla plantations, leading to the reduction in yield or death of the plant. This section describes vanilla diseases of major global importance.

Fusarium rot of vanilla caused by a fungus is the most devastating disease of vanilla, a leading cause for the decline of vanilla industry. The pathogen responsible for the *Fusarium* rot and the symptoms would be detailed later in a separate section.

Anthracnose disease caused by a fungal pathogen, *Colletotrichum gloeosporiodes* Penz. attacks the stem apex, leaves, fruits and flowers of vanilla (Anandaraj et al., 2005, Anilkumar, 2004). The symptoms of the disease appear in the form of black dots or lesions on the surface of the infected part, the black dots being the fruiting structure of the fungi. The infection results in the damage of leaves and stem thereby reducing the plant growth (Figure 12A). Fruit fall of premature infected fruits also happens, reducing the yield from the plant.

Sclerotium rot disease is caused by *Sclerotium rolfsii* and is characterized by the rotting of the vanilla bean tip (Thomas & Bhai, 2001) exposing a thick mycelia mat on the area of

infection. The symptom, represented by water soaked patches, can also be observed on the stem base, which later becomes necrotic and causes the death of the plant.

Black rot caused by *Phytophthora* sp. induces the rotting of beans, leaves, stems and roots (Thomas et al., 2002). The black rot is characterized by the presence of water soaked lesions initially appearing on the fruits. In the later stages, the rotting extends to more areas of the fruit and then to the aerial parts of the vine (Figure 12B) (Anandaraj et al., 2005). This disease results in high losses in vanilla industry due to rotting of beans, or to the destruction of vines.

Minor disease caused by fungal pathogens are also reported which include the dry rot disease of vanilla caused by *Rhizoctonia* sp. (Anandaraj et al., 2005) resulting in the shrinking of stem, roots and leaves. Other diseases include brown rot disease caused by *Cylindrocladium quinqueseptatum (*Bhai & Anandaraj, 2006*)* and rust disease caused by *Uromyces* sp. (Hernandez-Hernandez, 2011)

The vanilla crop is also subjected to bacterial soft rot disease caused by the *Erwinia carotova* infecting the leaves and shoots (Wen & Li, 1992). A series of 11 virus species are described from vanilla making it a big concern in the vanilla industry. The most common viruses in vanilla plantations are *Cymbidium mosaic virus* (*Potexvirus)*, Figure 12C), *Odontoglossum ringspot virus* (*Tobamovirus*), *Cucumber mosaic virus* (*Cucumovirus*) and several potyviruses (Figure 12D) such as *Watermelon mosaic virus, Dasheen mosaic virus* and *Vanilla distortion mosaic virus* (Pearson et al., 1991, Grisoni et al., 2010).

In Reunion Island, Mayotte and French Polynesia, vanilla is also affected by pests of which the scale *Conchaspis angraeci* is one of the most important pest (Quilci et al., 2010). The scale causes damage to almost all parts of the plant. The toxin injected by the scale results in the formation of chlorotic spots on leaves and stem which eventually lead to

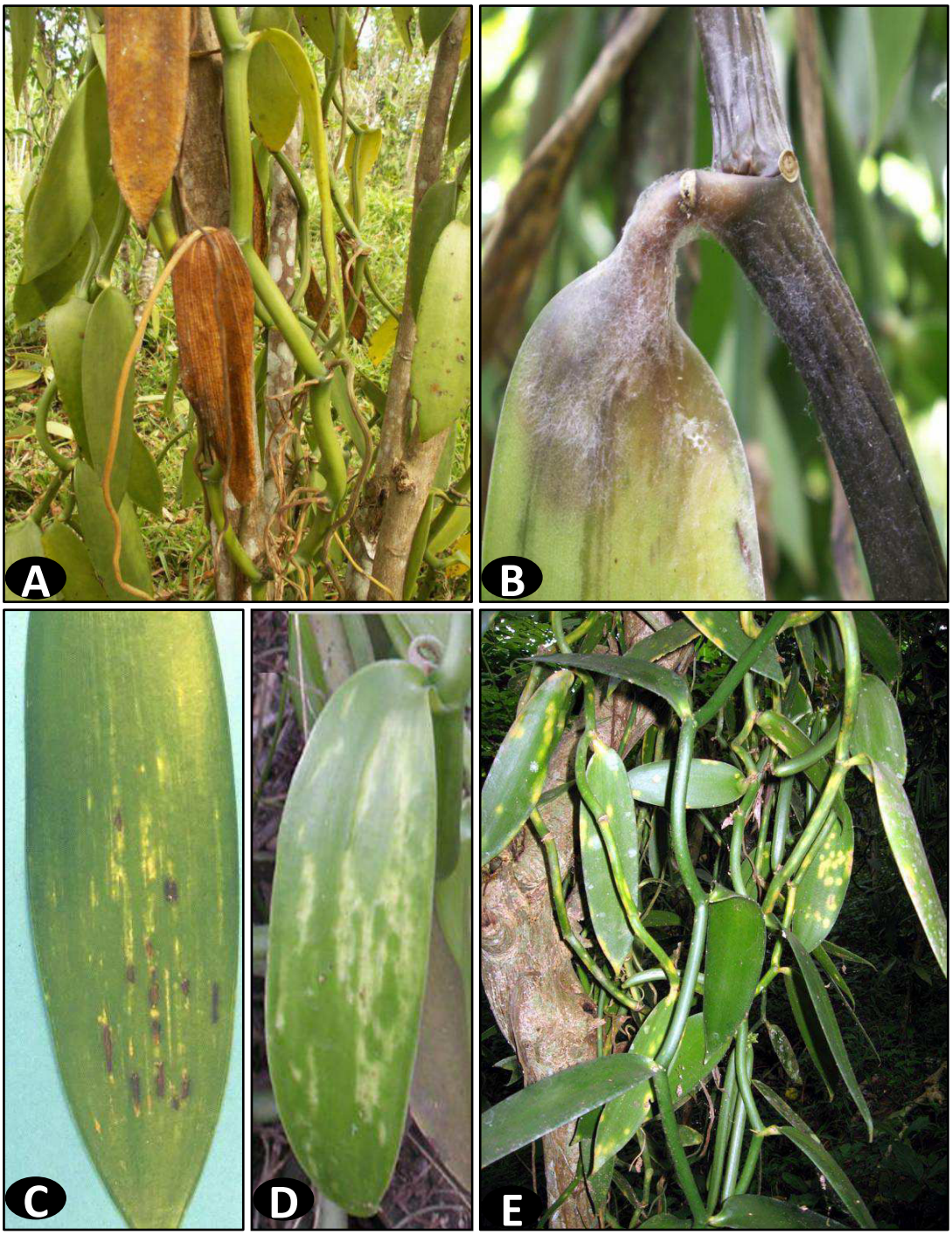

Figure 12: Diseases in *Vanilla* plants. A) *Vanilla* vine showing symptoms of Anthracnose, B) Black rot caused by *Phytophthora* sp. C) symptoms of Cymbidium mosaic virus and D) symptoms of Potyvirus and E) Vines infected with Angraecum scales.
Grisoni(2010); Quilici (2010).

necrosis and death of the vine (Figure 12E). The *Conchaspis* scales in vanilla have not been reported in other vanilla growing countries.

I-8 Root and stem rot of *Vanilla*

Fusarium rot has been reported since the beginning of commercial vanilla cultivation in many countries like Reunion Island, Madagascar, French Polynesia, Indonesia, Seychelles and as far as India. The pathogen responsible for the disease was identified by Tucker as *Fusarium batatis var. vanillae* Tucker *(*Tucker, 1927*)* which was later renamed (Gordon, 1965) to *Fusarium oxysporum* Schlecht. f. sp. *vanillae* (Tucker), abbreviated to Fov. *Fusarium* rot caused by Fov is capable of infecting the roots, stems, fruits and shoots of the plant. Initial reports on the vanilla root rot disease in Reunion Island and Madagascar are dated as early as 1871 and 1902 respectively (Bouriquet, 1954). The incidence of the disease has been also reported in other main vanilla producing countries like Indonesia, Seychelles, India, Thailand, Tonga and China (Tombe & Liew, 2010b).

The *Fusarium* Root and Stem rot (RSR) of vanilla have been described earlier based on the different wilt symptoms. The first description made by Tucker (1927) denoted the diseases as root rot of vanilla. Later studies by Alconero (1968) in Puerto Rico and He (2007) in China also explained the disease as root rot caused by Fov. However, the study conducted in Indonesia (Pinaria, 2010) reported the disease as the Stem rot in vanilla. In the present study, the disease is described as the RSR disease of vanilla.

The RSR appears irrespective of the age of the plant, but generally with the age of plot (Xiong et al., 2014). The early symptoms of the RSR include the browning and death of the underground roots. Depending on the moisture conditions, the rot may be either dry or soft and watery (Alconero, 1968b). The aerial parts of the roots normally remain healthy until they proliferate rapidly and touch the ground. The destruction of the root system limits the water

and food supply to the aerial parts of the plant leading the plants to shrivel and slowly die. The symptoms include the drop down of tender tips, yellowing of leaves and stem and the shriveling of the stem (Figure 13 A) due to the lack of nutrients. When the underground root system collapses, the plant uses its energy to proliferate the roots in order to re-establish the water and food supply. This proliferation of roots puts excess pressure on the older portion of roots, where the roots exhaust their capacity for proliferation and eventually die (Figure 13 B and C).

II Fusarium oxysporum

Fusarium is a large genus of ascomycete filamentous fungi widely distributed in soil and in association with root rhizosphere. This fungus belongs to the sub division of Fungi Imperfecti with a worldwide distribution. The majorities of the *Fusarium* species are harmless saprophytes and are relatively abundant members of the soil microbial community. Some species of *Fusarium* produce mycotoxins in cereal crops that severely affect human and animal health if they enter the food chain. The ability of this particular fungus to cause disease in both plants and humans has encouraged significant research on this genus. The genus also includes some economically important plant pathogens responsible for diseases in many plant hosts (Baayen, 2000, O'Donnell et al., 2009a).

F. oxysporum is an important species complex within the genus *Fusarium* and the causal agent of vascular wilt in various agronomical and horticultural important crops like tomato, crucifers, cotton, banana and watermelons (Szczechura et al., 2013, Bosland & Williams, 1987, Vakalounakis, 1996b). This species occupies the 5th position among the top 10 fungal pathogens based on its scientific and economic importance (Dean et al., 2012). The host range of *F. oxysporum* is broad consisting of plants, animals, humans and arthropods (Nelson, 1994).

Figure 13: Stem and root rot symptoms of *Vanilla*. A) Stem rot symptom .B-C) Root rot symptoms on *Vanilla* plants in shade house.

II-1 Taxonomy of Fusarium oxysporum

The first classification of the genus *Fusarium* was made by Link in 1809 and has since been fundamentally debated. Later a simplified identification process was introduced by Wollenweber and Reinking (1935), which divided the genus based on the morphology of the spores. The study of Wollenweber and Reinking classified 1000 isolates into 16 sections, 65 species, 55 varieties and 22 forms. Since then, various attempts have been made to improve this classification (Fourie et al., 2011). An endeavor made by Synder and Hansen's (Snyder & Hansen, 1940) compressed the 16 sections into 9 species, and the species in section Elegans were made into a single species of *F. oxysporum*. Again, many other classifications were made to improve the taxonomical classification of the genus, in which the concept made by Nelson and Burgess was accepted by most plant pathologists (Booth, 1971, Nelson et al., 1983., Burgess et al., 1994, Leslie & Summerell, 2006). Currently, *F. oxysporum* is represented by a diversity of morphologically similar fungi with poly phylogenetic origins made up of at least three known clades considered as a single complex species for biological and historical reasons (Baayen, 2000).

II-2 Morphological characteristics

The pathogen has no known perfect state. *Fusarium oxysporum* produces three types of asexual spores: macroconidia, microconidia and chlamydospores. In the case of *F. oxysporum*, the macroconidia are sickle-shaped to straight with three to four septa. They may be produced from monophialides either on a branched conidiophore or hyphae, and have a notched basal cell and a pointed apical cell. The microconidia are usually round, oval or reniform in shape and are non-septate. Microconidia are produced in false heads on short monophialides on the hyphae. Chlamydospores are usually produced in single or pairs on the intercalary or the terminal regions of hyphae (Figure 14). They are formed by the modification of the hyphal and conidial cells through the condensation of their contents

(Ohara & Tsuge, 2004). Both microconidia and macroconidia function as secondary inoculum in infecting plants, whereas the chlamydospores are for long term survival of the organism (Pinaria, 2009). Morphological identification of *Fusarium* species are based on the shape and size of these spores. In the case of *F. oxysporum*, the macroconidia are usually short to medium length with three septa (Figure 14A). But in the case of *F. solani*, the macroconidia is relatively wide and stout with three to five septa (Figure 15A). While, in the case of *F. proliferatum*, the macroconidia is relatively straight with 3 to 5 septa (Figure 16A). Differences are also present in the morphology of microconidia between the *Fusarium* species. The microconidia of *F. oxysporum* are oval and aseptate (Figure 14B) and are formed in false heads on very short phialides. In the case of *F. solani*, the microconidia are oval with 0 to 2 septa (Figure 15B) and are formed in false heads on very long phialides or branched conidiophores. In *F. proliferatum*, the microconidia are club shaped with 0 septa (Figure 16B).

II-3 Diversity of *Fusarium*

The vegetative compatibility groups (VCG) is a technique initiated by Puhalla (Puhalla, 1985) to access the diversity in *F. oxysporum*. Vegetative compatibility is the ability of two fungal isolates to fuse when they grow in close proximity. The groups of isolates that share identical alleles at corresponding loci are known as VCGs. The VCG study is helpful in characterizing population structure of asexual fungi including *F. oxysporum* (Appel & Gordon, 1994). However the VCG is a difficult and time-consuming technique to perform on a large number of isolates, compared to molecular analysis. VCG has been used to characterize a number of *F. oxysporum* formae speciales (Katan et al., 1991, Bentley et al., 1998, Pasquali et al., 2005, Bertoldo et al., 2014). Genetic relatedness of *Fusarium* isolates from *Vanilla* have been tested using VCG analysis in China (He, 2007) and Indonesia (Pinaria, 2009).

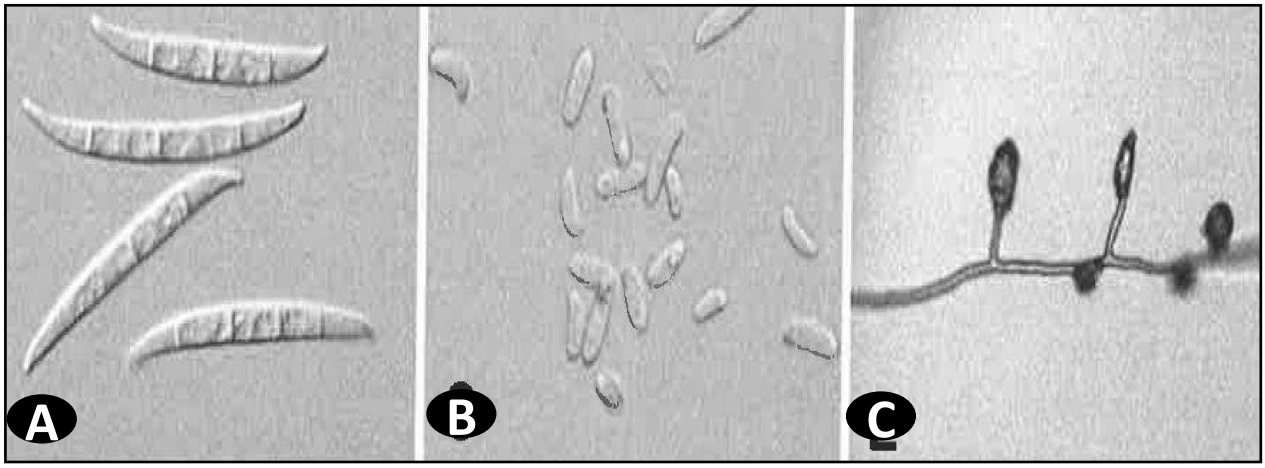

Figure 14: Asexual spores of *F. oxysporum* on CLA. A) Microconidia, B) Microconidia and C) Microconidia *insitu*.

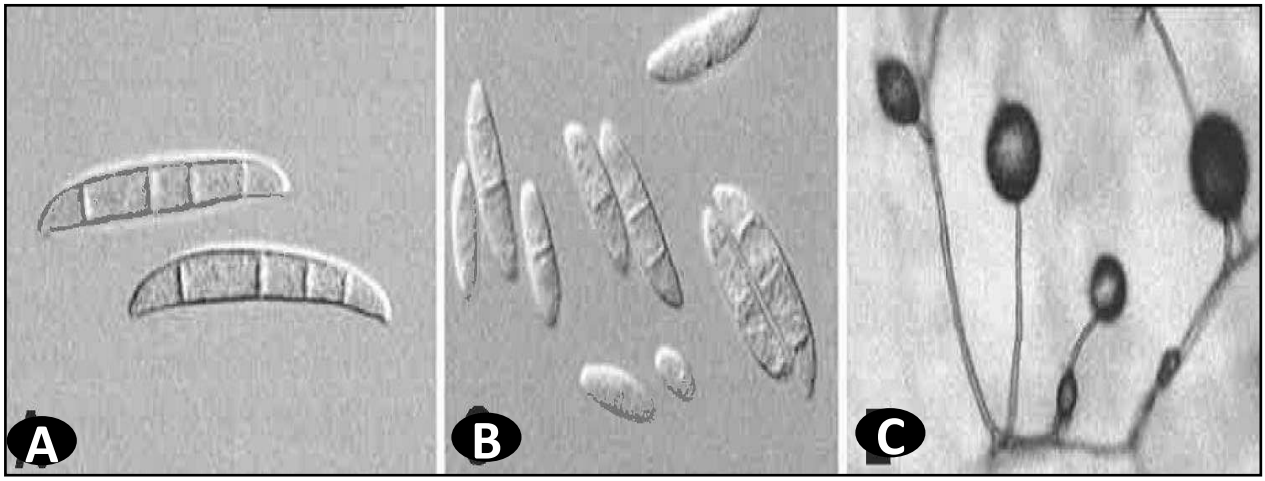

Figure 15: Asexual spores of *F. solani* on CLA. A) Microconidia, B) Microconidia and C) Microconidia *insitu*.

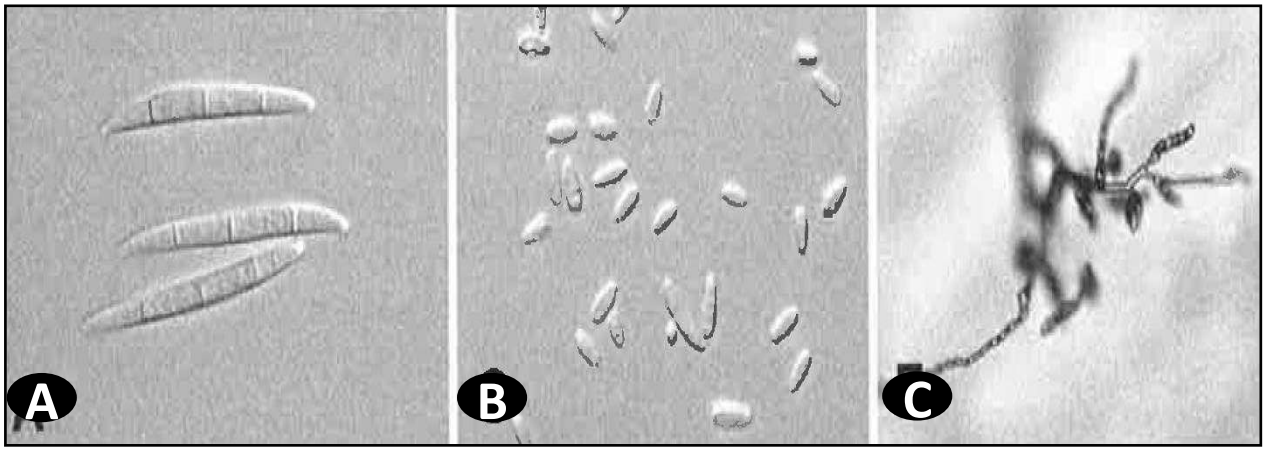

Figure 16: Asexual spores of *F. proliferatum* on CLA. A) Microconidia, B) Microconidia and C) Microconidia *insitu*.

Figure 14-16: Morphological difference in the asexual spores in 3 different species of *Fusarium.* Leslie and Summerell (2006)

The cost and labor reduction for sequencing have increased the number of genetic diversity studies using molecular markers. Nowadays various powerful molecular biology tools help the mycologists in rapid identification, assessing genetic diversity of fungal isolates, and sometimes in the determination of their virulence. Different types of DNA markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSR) have been increasingly used to study the diversity in populations of *F. oxysporum* species.

Molecular methods such as RAPD and ISSR are widely used in the study of fungal taxonomy and diversity studies of *F. oxysporum*. RAPD technique is one of the most rapid and inexpensive method that has been used in assessing genetic variability in many *Fusarium* formae speciales (Baysal et al., 2010, Vakalounakis & A., 1999, Jian-sheng et al., 2015) even though there is a concern about its low level of repeatability (Perez et al., 1998). Molecular characterization of *Fusarium* isolates from vanilla in India using RAPD markers has been reported (Vijayan et al., 2012b). The RAPD results, with 9 isolates amplified with 9 primers, showed that the isolates tested belong to a single clonal lineage. SSR, also known as microsatellites, has been used in many studies in *Fusarium* due to its high resolution and repeatability (Bogale et al., 2005, Datta & Lal, 2012, Costa et al., 2015). ISSR markers, also used in diversity studies, involve the amplification of DNA segments present at an amplifiable distance in between two identical microsatellite regions oriented in opposite directions. This technique has been used in the genetic diversity studies of *Fusarium* isolates from various plant hosts (Lin et al., 2012, Thangavelu et al., 2012).

AFLP is an extremely powerful multi locus fingerprinting technique used for assessing the relationships among isolates of *F. oxysporum* at the population, species and supraspecific level (Baayen, 2000). The main advantages of AFLP over other marker techniques are its

higher reproducibility and resolution rates, and the sensitivity at the whole genome level compared to other marker techniques. No prior sequence information is a requisite in the AFLP technique making its an inevitable method for assessing the relationship of *F. oxysporum* isolates at the population and species level (Abdel-Satar et al., 2003, Silva et al., 2013).

Apart from the list of molecular markers listed, several housekeeping genes have been used for the phylogenetic studies of *Fusarium* isolates. Among these are β-tubulin, histone H3, translation elongation factor 1-α (EF1-α), nuclear ribosomal Intergenic spacer region (IGS rDNA), mitochondrial smaller subunit (mtSSU) ribosomal RNA, calmodulin, nitrate reductase and internal transcribed spacer region of rDNA (ITS). Molecular phylogenetic analysis of various *formae speciales* of *F. oxysporum* has been done with these housekeeping genes (O'Donnell et al., 2009a, Baayen, 2000, Stenglein et al., 2010, Demers et al., 2014). Phylogenetic studies of *Fusarium* isolates from vanilla using EF1-α and mtSSU have been reported earlier in Indonesia (Pinaria et al., 2015b, Pinaria et al., 2010b). The results obtained from this study with *Fusarium* isolates from Indonesia, Reunion Island, Comoros and Mexico resolved the isolates into three clades, indicating a polyphyletic pattern of evolution.

II-4 Pathogenicity of Fusarium oxysporum

F. oxysporum populations are part of fungal communities in the soil and plant rhizosphere around the world (Gordon & Martyn, 1997). These strains are saprophytes and are able to live for extended periods on organic matters in the soil. The fungi are divided into two forms based on their ability to infect the host and induce the *Fusarium* disease.

II-4.a Plant pathogenic *Fusarium oxysporum*

The pathogenic strains have the capability to penetrate the root and induce either the root rot or tracheomycosis when they invade the vascular system of host plants. These pathogenic

strains are responsible for the Fusarium wilt disease in many economically important crops. They have a high level of host specificity. The pathogenic strains of *F. oxysporum* are divided into "*formae speciales*" based on their ability to infect and induce disease in specific host species (Correll, 1991). The host specificity of a particular pathogenic strain can be determined only by inoculation process. The strains which are pathogenic specifically to a single host are denoted by "*forma specialis*" (abbreviated by f. sp.) followed by the name designating the host, added to the binomial *F. oxysporum*. Thus the pathogen responsible for vanilla stem and root rot will be generally known as *F. oxysporum* f. sp. *vanillae*), and that of tomato is *F. oxysporum* f. sp. *lycopersici*. The level of host specificity is very high in *F. oxysporum*, where nearly 150 *formae speciales* are reported (Bertoldo et al., 2015, Fourie et al., 2009).

The *formae speciales* are further subdivided into "races" based on the virulence to a particular set of different host cultivars that vary in disease resistance (Armstrong & Armstrong, 1981, Correll, 1991). The example being the *formae speciales* of banana that has been classified into four races (race 1, race 2, race 3 and race 4) based on their virulence to different cultivars (Ploetz, 2006, Ploetz & Churchill, 2011).

II-4.b Non-pathogenic *Fusarium oxysporum*

These types of strains can penetrate the roots, but do not invade the vascular system nor cause disease. Such strains are considered as non-pathogenic towards the particular host. The non-pathogenic strains are widely used as biocontrol agent against pathogenic *F. oxysporum* (Alabouvette & Couteaudier, 1992, Bao & Lazarovits, 2001). The non-pathogenic strains use different modes of action to generate their biocontrol activity, which includes the competition for nutrients in soil, competition for the root colonization and induced systemic resistance (Olivain et al., 2006).

II-5 Infection pattern

Plant infection by *F. oxysporum* is a complex process that comprises multiple host-pathogen interactions. The asexual chlamydospores are able to survive for extensive periods in the organic matters of soil and generally cause the primary infection of roots (Garrett, 1970). The chlamydospores surviving in soil can germinate and form hyphae, which penetrate the root or other plant parts. The germination of chlamydospores is usually facilitated by exudates from the root (Nelson, 1990).

In the case of pathogenic *Fusarium* strains, the penetration of hyphae occurs through the roots of host plant. Once inside the root, the hyphae grow inter and intracellularly to invade the cortex and cross the endodermis, until they reach the xylem vessels. In the xylem, microcondia are produced and transported by the xylem flow and are then accumulated at the vessel walls or perforation plates. Colonization and toxin production by the pathogen results in blockage of the host vascular system, causing characteristic wilt symptoms. The lodged microconidia germinate in the xylem, causing the hypha to penetrate the adjacent cells and the infection continues (Figure 17). However in some cases, cortical rot are observed rather than the vascular wilt caused by pathogenic *F. oxysporum*. Such types of pathogenic strains are named as "*radicis*" form. Both patterns are observed in tomato where two diseases are known; the Fusarium wilt caused by *F. oxysporum* f. sp. *lycopersici* (Fov) and the crown rot by *F. oxysporum* f. sp. *radicis-lycopersici* (Forl) form. In the case of "*radicis*" form, the penetration is mainly through natural wounds and the infected plant may totally wilt, or persist in a weakened state (Jarvis & Shoemaker, 1978).

II-6 Fungal genes responsible for pathogenicity

The pathogenicity genes can be defined as those genes which are necessary for disease development, but not essential for the pathogen to complete its life cycle (Idnurm & Howlett,

2001). These pathogenicity genes are essential at different stages of infection. The harmonious working of these genes are necessary for bypassing the defense mechanism initiated by the host plant in response to the pathogen and enable the invasion of the host tissues. The genes involved in pathogenicity have been classified into different groups based on their functions (Idnurm & Howlett, 2001, Michielse et al., 2009, Sutherland et al., 2013).

II-6.a Signal transduction

In the initial stage of the infection, the pathogen should sense the stimuli from the host and respond accordingly. Once the stimulus is received, the pathogenic fungi responds appropriately to the plant environment by changing its morphogenetic and biochemical characteristics (Roncero et al., 2003). In *F. oxysporum*, the genes MAPK (fmk1), α(fga1, fga2) and β(fgb1) encoding the cyclin adenosine monophosphate-protein kinase A (cAMP-PKA) and mitogen-activated protein kinase (MAPK) cascades are essential for the pathogenicity (Delgado-Jarana et al., 2005, Di Pietro et al., 2001, Jain et al., 2003, Jain et al., 2005). These genes are necessary for the pathogen to undergo the modifications required to adapt in response to the host environment and initiate the infection process. Genes which encodes chitin synthases (*CHS2, CHS7, CHSV and CHSVb*) have been also reported to play a major role in maintaining the hyphal cell wall integrity, which is essential for invasion and resistance against host defense (Martin-Udiroz et al., 2004).

II-6.b Cell wall degrading enzymes

Fusarium oxysporum secretes a wide range of cell wall degrading enzymes (CWDE) including polygalactironases, pectate lyase, xylanases and proteases during the penetration and colonization phase. However the genes encoding for these enzymes (*PL1, XYL3, XYL4, XYL5, PG1, PG5, PGX4*) have no role in inducing pathogenicity (Di Pietro et al., 2003, Beckman, 1987). The disruption of Sucrose Non-Fermenting *SNF1* involved in the catabolite

repression reduces the expression of several cell wall degrading enzymes (CWDE) (Ospina-Giraldo et al., 2003). Similarly, *FRP1, ICL1 and FNR1* are also a few genes which regulate the expression of CWDE (Duyvesteijn et al., 2005, Divon et al., 2006)

II-6.c Response to plant defense

During penetration and colonization process, the pathogen is exposed to various plant defense mechanisms, such as physical barriers and antifungal compounds (Beckman, 1987). In response, the fungi secrete various enzymes to overcome the disease resistance of plants. *TOM1* is an example of a fungal gene which encodes tomatinase (Lairini et al., 1996) against the resistance of tomato to Fol. In response to the pathogen infection, the host also produces various phenolic compounds (Baetz & Martinoia, 2014). These phenolic compounds also activate the production of mycotoxins (Wu et al., 2008). The genes *CMLE1* coding for CMLE involved in the β-ketoadipate pathway is also required for pathogenicity. The inactivation of these genes reduced the aromatic compound degradation activity of the fungi (Michielse et al., 2009). Additionally, the inactivation of chitin synthase genes (*CHS2, CHS7, CHSVb*) in the pathogen resulted in the reduction of infection on tomato plants (Martin-Urdiroz et al., 2008). The genes *GAS1 and RHO1* encoding β-1,3-glucanosyltransferase and GTPase, respectively, are also fungal genes involved in response to the plant defense (Caracuel et al., 2006).

II-6.d Peroxisomal functions

Peroxisomes are organelles present in plants and fungi that have catabolic activities for cell lysis. They can be responsible for the virulence of many pathogenic fungi through specific fatty acid catabolism functions. Among *F. oxysporum*, two genes *PEX12* and *PEX26* are required for the peroxisomal activity in the fungi (Michielse et al., 2009). Mutation in *PEX* genes cripples the peroxisome, making it unable to perform the biochemical reactions

and thereby the pathogenicity. Peroxisomal functions are essential for utilizing the host nutrients during colonization, especially in *Fusarium* species that lack appressorium.

II-6.e Transcription factors

A few transcription factors have been reported to be specifically required for pathogenicity such as, *FOW2, SGE1, FTF1, STE12, FOST12* and *CON7-1* (Imazaki et al., 2007, Michielse et al., 2009, Ramos et al., 2007, Rispail & Di Pietro, 2009, García-Sánchez et al., 2010, Gu et al., 2015, Ruiz-Roldán et al., 2014). These genes are transcribed mainly during the initial phase of the infection.

II-6.f Transposable elements

Recently, the genome sequencing results of *F. oxysporum* f. sp. *lycopersici* revealed that 28% of the genome corresponds to repetitive sequences, including many retro elements and short interspersed elements as well as class II transposable elements (Takken & Rep, 2010). Forward and reverse genetics are the two main strategies used in the identification of pathogenicity genes. Sequence characteristics of the genes present on some lineage specific genome regions indicate a distinct evolutionary origin from the core genome, suggesting that they could have been acquired through horizontal transfer from another *Fusarium* species. This idea was experimentally supported by the finding that co-incubation in tomato of two strains of *F. oxysporum* can result in transfer of lineage specific small chromosomes from a pathogenic to a non-pathogenic strain, converting the latter to a pathogenic. This extra chromosome contains effector genes that confer Cysteine rich proteins present in the xylem during infection (Rep et al., 2004b). This led to the hypothesis that horizontal chromosome transfer in *F. oxysporum* can generate new pathogenic lineages (Ma et al., 2010).

III Plant pathogen interactions

Plant-pathogen interaction can be broadly classified as beneficial, detrimental or neutral (Schenk et al., 2012). Plant pathogen interactions have been studied for several years in order to understand how the plant and pathogen recognize each other and differentiate to establish either a compatible or an incompatible relationship (Mehta et al., 2008). The pathogenic fungus should overcome different plant defense responses at different stages of penetration and colonization. During the final stage of infection, the fungus secretes lytic enzymes and toxins that lead to disease symptoms including necrosis, chlorosis and wilting. As soon as the plant receives signals from the pathogen, various defense mechanisms such as plant cell wall thickening, papilla deposition, apoplast acidification, signal transduction and transcription of defense genes are initiated (Mehta et al., 2008). Only the successful pathogen can surpass this basal defense mechanism of the plant and develop disease in the host (Figure 18).

Plants have developed constitutive (barriers such as cell wall, waxy cuticles and bark) and inducible defenses (production of toxins and pathogen degrading enzymes) in order to protect themselves from pathogens (Freeman & Beattie, 2008). Beside the natural physical barriers, plants also possess an immune response against the pathogen during the attack. Once recognizing the signals from the pathogen (PAMP, MAMP), the plant elicits a series of cellular and physiological responses (Wu et al., 2014)

III-1 Plant recognition

This is an important process in the plant-pathogen interaction where the hosts recognize the pathogen. A rapid recognition process is required in order to successfully defend the plant. This is achieved through the pathogen recognition receptors (PRR), which are cell surface receptors and R genes (Swarupa et al., 2014). The PRR detects the pathogen and activate the MAMP-triggerd immunity (MTI) (Figure 21). *CEBiP*, *CERK1, SERK3* and *BAK1* are

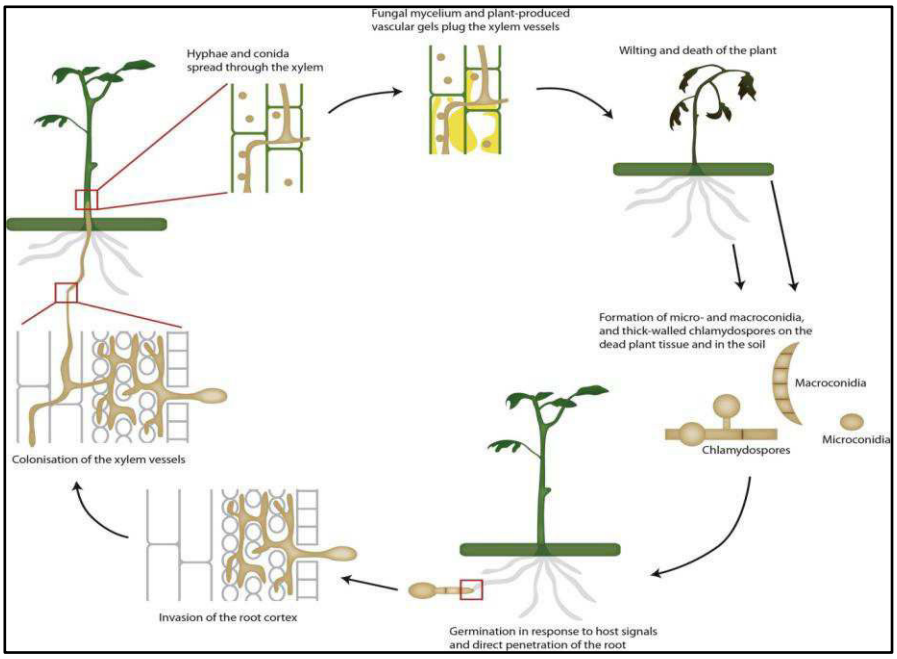

Figure 17: Disease cycle of *Fusarium oxysporum* Perez-Nadales (2014)

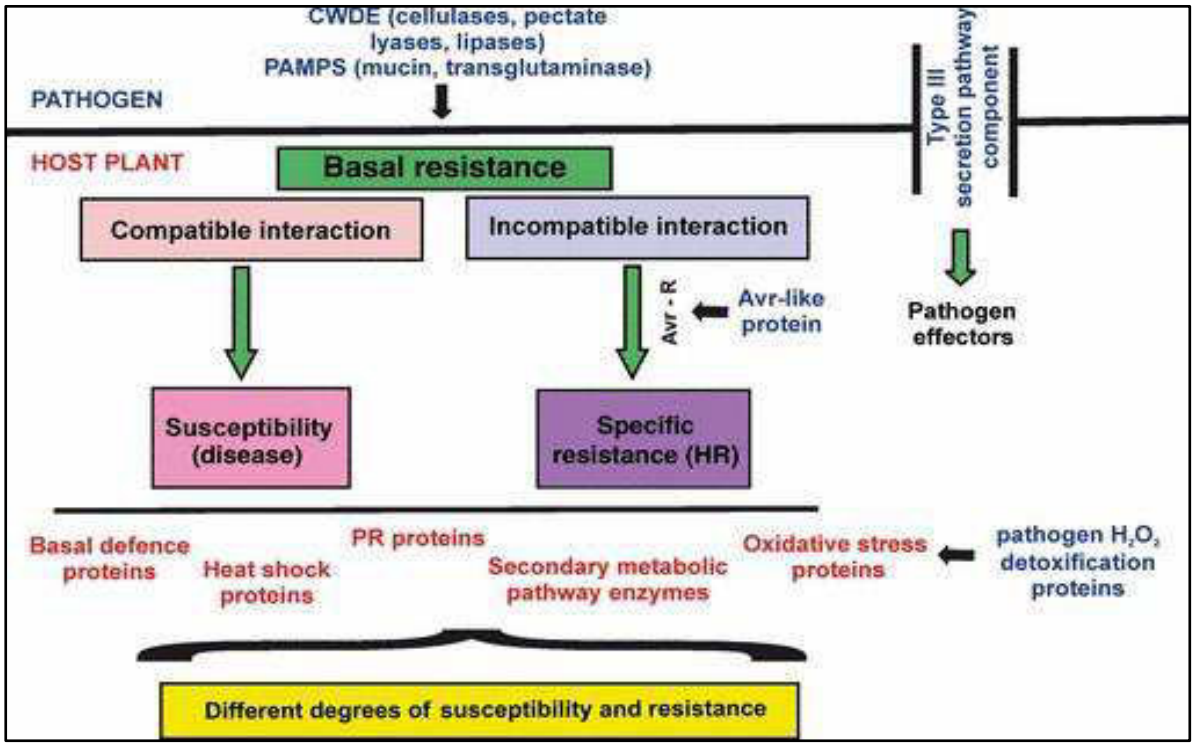

Figure 18: Overview of plant-pathogen interaction

examples of Pathogen triggered Immunity (PTI) genes in banana and *Arabidopsis* (Li et al., 2012, Heese et al., 2007).

III-2 R gene mediated resistance

Plant pathogens produce certain molecules called 'effectors', encoded by *avr* (avirulence) genes, which are delivered directly into the plant cells during the initial stage of infection. These effectors either modify the physiological state of the host plant in order to benefit from the pathogen colonization or are used to interrupt the activation of host plant defenses (Hammond-Kosack & Kanyuka, 2007). However, plants have evolved "R genes"(resistance genes) whose products allow recognition of specific pathogen effectors, either through direct binding or by recognition of the effector's alteration of a host protein. These virulence factors drive the co-evolution of plant resistance genes to combat the pathogens' *Avr* genes (Jones & Dangl, 2006). Plant resistance genes can be broadly divided into eight groups (Gururani et al., 2012) based on their amino acid motif organization and their membrane spanning domains .(Figure 19).

Major resistance (R) genes have been found against *F. oxysporum* in crops including tomato (Simons et al., 1998, Sela-Buurlage et al., 2001) and cucumber (Vakalounakis, 1996a) (Table 1).

III-2.a Reactive oxygen species (ROS) production

Production of ROS species, such as superoxides, hydrogen peroxides and hydroxyl radical are observed during pathogen attack. These strong oxidizing species can rapidly attack all types of biomolecules. The rapid accumulation of plant ROS (oxidative burst) is accompanied with changes in the cellular pH level, protein phosphorylation and ion influx. The ROS also serves as signals for the activation of other defense mechanisms like defense

gene expression, cell wall protein cross linking, callose deposition and systemic acquired resistance (Liu et al., 2010, O'Brien et al., 2012).

III-2.b Calcium flux

An Increase of the Ca^{2+} level in the cell cytosol in response to various PAMPs are common in plants. Calcium acts as an important secondary messenger, which passes the pathogen signals received at the cell receptors to the cytosol and activates the defense responses. There are three major types of Ca^{2+} sensors in plants, including calmodulin (CAM), calcineurin B-like proteins (CBLs) and calcium-dependent protein kinases (CDPKs) (Luan, 2009)

III-2.c Cell wall strengthening

Cell walls strengthening through structural and chemical modifications are important resistance responses against pathogen in plants. The accumulation of phenolic acids, which are monomers of lignin forms an effective protective barrier against the pathogen (Lattanzio et al., 2006). Lignification makes the plant cell wall more resistant to cell wall degrading enzymes of the pathogen. Callose deposition is also a pathogen induced defense response. During pathogen infection, callose is deposited in the cell wall appositions, at the site of attack to form effective chemical and physical defense barrier.

III-2.d Defense hormones

Plant hormones including Salicylic acid (SA), Jasmonic acid (JA), Ethylene(ET) and Abscisic acid (ABA) play an important role in the regulating the plant defense responses. SA is effective in mediating plant resistance against biotrophs and the establishment of systemic acquired resistance. SA levels are found to be increased in many plant defense responses, which induces the pathogenesis related (PR) genes. On the other hand JA and ET are activated in the defense response against necrotrophic pathogens. However, in certain cases

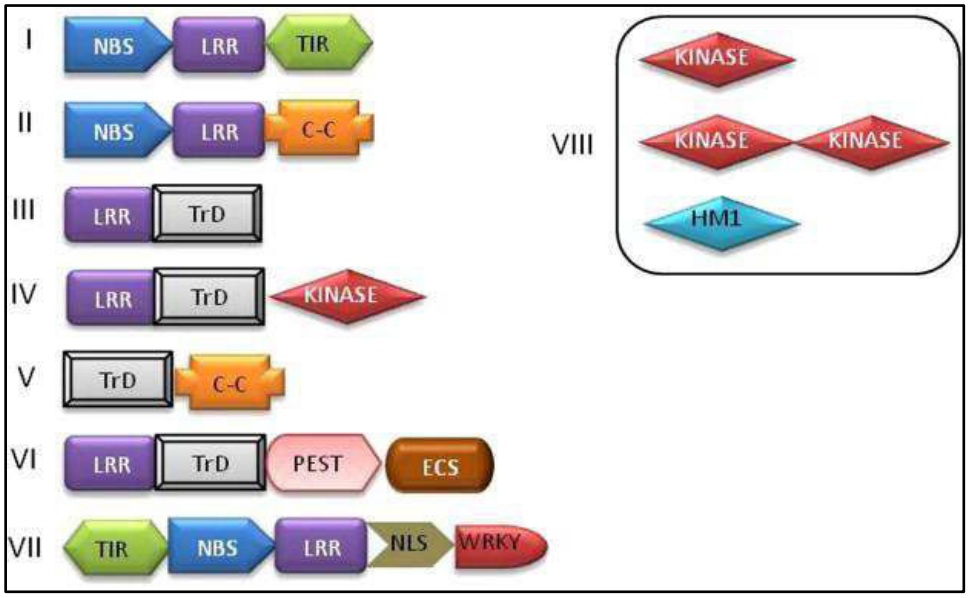

Figure 19: Major classes of plant resistance (R) genes based on the arrangement of the functional domains. LRR – Leucine rich repeats; NBS – Nucleotide-binding site; TIRToll/Interleukin-1- receptors; C-C – Coiled coil; TrD – Transmembrane domain; PEST – Protein degardation domain; ECS- Endocytosos cell signalling domain; NLS- Nuclear localization signal; WRKY- Amino acid domain; HM1- Helminthosporium carbonum toxin reductase enzyme.
Gururani (2012)

the cross talks between the SA and JA/ET results in their combined action in certain events (Bari & Jones, 2009). In the case of ABA, it also has a role in signaling innate immunity apart from its normal function of abiotic stress response. ABA is also known for its negative regulatory, where it sometimes inactivate other signaling pathways (Swarupa et al., 2014).

Table 1: List of genes involved in *Fusarium* resistance

Candidate Gene / R genes	**Crop**	**References**
rfo1,rfo2,rfo3,rfo4,rfo6	Arabidopsis	(Diener & Ausubel, 2005)
fom2	Melon	(Joobeur et al., 2004)
i1	Tomato	(Houterman et al., 2008)
i3	Tomato	(Rep et al., 2004a)
Pathogenesis related proteins		
Defensin	Tomato	(Abdallah et al., 2010)
Chitinase	Tomato	(Jongedijk et al., 1995)
Thaumatin	Banana	(Mahdavi et al., 2012)
β-1,3 glucanase	Banana	(Maziah et al., 2007)
npr1	Banana	(Endah et al., 2008)
Antimicrobial activity		
thi2.1	Arabidopsis	(Epple et al., 1997)
Polyphenol oxidase	Banana	(Kavino et al., 2009)
caamp1	Pepper	(Lee et al., 2008)
Cell wall strengthening		
Phenylalanine ammonia lyase	Banana	(Van Den Berg et al., 2007)
Peroxidase	Banana	(De Ascensao & Dubery, 2003)
Polygalacturonase inhibitor protein	Tomato	(Salehzadeh, 2012)
Antioxidants		
NADPH oxidase	Arabidopsis	(Zhu et al., 2013)
Ascorbate peroxidase	Chickpea	(Garcia-Limones et al., 2009)
Catalase	Tomato	(Farag Hanaa et al., 2011)
Superoxide dismutase	Banana	(Li et al., 2011b)
Glutathione-S-transferase	Tomato, Melon	(Bolter et al., 1993)

IV Control of RSR of vanilla

Fusarium diseases are very difficult to control because of the airborne. There is also a higher chance for the pathogen to be carried on to the uninfected plants through various medium. Because of the considerable yield and economic losses, several measures have been attempted to control *Fusarium* diseases. Cultural technique, crop rotation, chemical control and biological control are mostly employed in controlling *Fusarium* diseases (Katan, 2000, Rehman et al., 2014). Fumigation with methyl bromide/ chloropicrin has been attempted in controlling the disease in greenhouses (Dantoff et al., 1995). Chemical control of *Fusarium* diseases using various fungicides including Benomyl, Ridomil, copper oxychloride, Mancozeb, captafol, thiram and imazalil was employed in major crops (Amini & Sidovich, 2010, Hannan et al., 2014). The most cost efficient and environmental safe method of disease control is the use of resistant cultivars, which is subjected to availability.

The use of non-pathogenic strains of *F. oxysporum* has been used in many crops in controlling Fusarium diseases (Belgrove et al., 2011, Larkin et al., 1996, Lemenceau & Alabouvette, 1991, Olivain et al., 2006, Silva & Bettiol, 2005). The non-pathogenic strains are capable of reducing the chlamydospore germination of the pathogenic strain and also compete for infection sites on roots, thereby triggering plant defense reactions (Fravel et al., 2003a). Bio control methods using antagonists like *Pseudomonas fluorescens*, *Trichoderma viride*, *Bacillus subtilis* were also widely used in controlling the disease (Chen et al., 1995, Dantoff et al., 1995, Webb et al., 2015). The biocontrol of *Fusarium* has been considered to be a potential strategy in recent years, because of the chemical control measures resulting in the accumulation of harmful chemical residues in the environment.

Diseases caused by *Fusarium* and other vanilla pathogen and pests have severely affected the production, export and economy of vanilla producing countries. Different strategies of control measures are used in vanilla growing countries based on their economic factor and

manpower. A combination of strategies including planting non-infested cuttings and the selection of cultivars resistant to *Fusarium* and other pathogens is crucial for controlling the disease.

IV-1 Agronomical management

Fusarium oxysporum is particularly difficult to eliminate from soil by rotation or long fallow. In vanilla plantations, different phytosanitary measures are followed in order to control and prevent the spread of diseases (Fouche & Jouve, 1999). Removal and disposal (phytosanitation) of infected plants are done in priority to prevent the spread of diseases (Anandaraj et al., 2005). The use of good planting material and input of compost in regular intervals also helps in controlling the disease. Other control measures such as mulching were used to control Fusarium disease, especially with clove leaves (Tombe et al., 1992).

IV-2 Chemical control

The use of various fungicides and chemicals was also employed in controlling the stem and root rot disease of vanilla. The fungicides containing Mancozeb and Carbendazime have been used in Bali and Indonesia against *Fusarium* stem rot (Tombe & Sitepu, 1986). Fungicides containing Phosetyl-Aluminium, Carbendazime, Prochloraze are also included in the list of recommended fungicides against RSR (Fouche & Jouve, 1999, Anandaraj et al., 2005). However there are no fungicides reported which can effectively tackle the RSR. The practice of using fungicides is declining due to its hazardous nature, restrictions from government agencies and their limited efficiency.

The use of plant extracts was also reported to be used in controlling *Fusarium* diseases in vanilla. *In-vitro* experiments conducted in Bali (Suprapta & Khalimi, 2009), reported the efficiency of extracts from *Eugenia aromatica* and *Piper betel* in inhibiting the growth of *F. oxysporum* f. sp. *vanillae*. Essential oils such as cinnamon, thyme and clove oils were also

claimed to be effective in inhibiting pathogen growth *in-vitro*. However the use of fungicides and biocontrol agents are not effective in field experiments.

IV-3 Biocontrol agents

Biological control agents are reported to be used as control measure in vanilla plantations in few countries. Biocontrol agents could be considered as an alternative for the chemical fungicides considering the concerns for environmental protection and cost. Various biological agents like *T. harzianum, T. virens, P. fluorescens, P. putida, Paecilomyces sp and B. subtilis* were tested in *in-vitro*, pot and field conditions and were reported to be effective against *Fusarium* disease (Bhai et al., 2009, Naik et al., 2010, Sandheep et al., 2012, Sandheep et al., 2014). Combined treatment of *T. harzianum* and *P. fluorescens* through soil mixing also enhanced the vegetative growth of vanilla apart from the primary function of disease control (Sandheep et al., 2012). The use of non-pathogenic *F. oxysporum* was also proposed in controlling the RSR in Indonesia (Tombe et al., 1992, He, 2007)

IV-4 Genetic resistance

Host plant resistance has been identified as the primary strategy for long-term management of RSR. Indeed some sources of resistance to *Fusarium* are reported in related species. The known RSR resistant species include *V. pompona*, *V. phaeantha*, *V. barbellata*, *V. aphylla* and *V. andamanica* (Knudson, 1950, Theis & Jimenez, 1957a, Divakaran et al., 2006b). The microscopic observation of wounded cells of *V. phaeantha* displayed the secretion of idioblast in response to the pathogen (Alconero, 1968b). The unpublished results from Madagascar also describe the resistance in vanilla species such as *V. pompona* and *V. phaeantha*. (Tonnier, 1960). *Vanilla* species can be relatively easily hybridized and a few hybrids showing tolerance to *Fusarium* have been reported (Knudson, 1950, Delassus, 1963., Nany, 1996). However the *Fusarium* resistant species usually possess poor aromatic features.

V Aim and Objectives of the study

Vanilla is a high value cash crop, and still the backbone of the rural economy of several of the poorest countries, notably in the Indian Ocean area. RSR caused by the telluric fungus *F. oxysporum* is responsible for the failure of many attempts to intensive cultivation since the domestication of *V. planifolia* and *V. x tahitensis* at the end of 19th century. Long term research programs were carried out especially in Madagascar (Ambohitsara) and Puerto Rico (Maraguey) in the first half of 20th century to address this problem. Valuable information has been acquired in particular regarding the identity of the causal organism and the potential of some wild relatives of *V. planifolia* to generate resistant varieties. Despite these substantial progresses, the Fov-vanilla patho-system remains very poorly known and RSR is still the first constraint for vanilla production worldwide. In the Indian Ocean area, RSR was credited with playing the first role in the vanilla crisis that severely undermined the industry at the dawn of 21st century. Vanilla continues however to be highly demanded by the world market.

Given the availability at the BRC VATEL in Reunion Island of a rich collection of diverse and well characterized genetic resources of vanilla and the advent of powerful investigation methods at cellular and molecular levels, this research was undertaken, with the aim of filling gaps regarding our comprehension of the *Vanilla-Fusarium* interactions. This knowledge is a prerequisite for further development of genetic resistance to RSR which is hypothesized as the most promising route for controlling this devastating disease.

In this perspective, the following main specific questions were addressed during the thesis:

- What are the *Fusarium* species involved in RSR in Reunion island?
- How diverse are the *Fusarium* isolates prevailing in Reunion Island?

- How do they compare with isolates from other regions?
- What is the histo-pathogenicity of *Fusarium* in vanilla root tissue?
- What are the sources of resistance available in the vanilla germplasm of BRC VATEL?
- How do susceptible and resistant vanilla genotypes differentially react to the pathogen?
- What are the genes involved in the plant response to pathogen aggression?
- Which breeding strategy should be recommended for improving RSR control?

The works were carried out in the Mixed Research Unit PVBMT (CIRAD, University of Reunion Island) in the Plant Protection Platform in Saint Pierre (Reunion Island) and at the RIO imaging platform (CIRAD, INRA, CNRS) in Montpellier (France) in collaboration with Dr. Geneviève Conejero.

The results are presented in the present manuscript in five chapters as follows:

The current chapter **(Chapter 1)** is an introduction which intends to summarize information about the *Vanilla* crop, *Fusarium oxysporum*, *Fusarium* pathogenicity and plant defense mechanisms that regulates the plant pathogen interaction.

Chapter 2 reports on surveys, recollection and characterization of *Fusarium* isolates from various parts of Reunion Island and abroad. It describes the genetic diversity of the *Fusarium* isolates, the development of a robust *in-vitro* inoculation method and the classification of isolates based on the pathogenicity test. It also proposes to rename the causal agent of RSR is *F. oxysporum* f. sp. *radicis-vanillae*, based on disease development in roots. Information is presented as a paper submitted to *Plant Pathology*.

Chapter 3 describes first the screening for *Fusarium* resistance in the CRB Vatel *Vanilla* germplasm in Reunion Island and then the anatomical differences between vanilla accessions using WFM techniques. This chapter also documents the colonization pattern as well as structural and chemical modifications of cell-walls in susceptible and resistant genotypes, using WFM, Multiphoton microscopy and Spectral analysis techniques. All this data makes a second article to be submitted for publication in *Frontiers in Plant Science*.

Chapter 4 presents the results of the Illumina *de novo* transcriptome analysis and the gene expression profile changes in the roots of resistant, moderately resistant and susceptible accessions of vanilla, when infected with a pathogenic and non-pathogenic *Fusarium oxysporum*.

Chapter 5 discusses the knowledge acquired about *Vanilla* and *Fusarium* and their interaction. This chapter also elaborates about the novel strategies for controlling the *Fusarium* disease, with respect to the results obtained from the thesis.

CHAPTER II **Etiology and diversity of RSR and diversity of the causal agent**

Article accepted in Plant Pathology

Title: *Fusarium oxysporum* f. sp. *radicis-vanillae* is the causal agent of root and stem rot of vanilla

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Abstract:

Root and stem rot (RSR) is a very detrimental disease of vanilla worldwide. *Fusarium oxysporum* is frequently associated with the disease but other *Fusarium* species are also reported. In this international study, we surveyed 52 vanilla plots in three of the most important vanilla producing countries (Madagascar, Reunion Island and French Polynesia). Subsets from the 377 single-spored *Fusarium* isolates recovered from rotten roots and stems in the surveys were characterised by molecular genotyping (*EF1α* and IGS gene sequences) and pathogenicity assays on *Vanilla planifolia* and *V. ×tahitensis,* the two commercially grown vanilla species. *Fusarium oxysporum* was shown to be the principal species responsible for the disease since it represented 79% of the isolates recovered from the RSR tissues and 40% of these isolates induced severe symptoms on inoculated plantlets. *Fusarium oxysporum* isolates were highly polyphyletic regardless of geographic origin or pathogenicity. *Fusarium solani,* found in 15% of the samples, inducing only mild symptoms on plantlets, was considered a secondary pathogen of vanilla. Three additional *Fusarium* species were occasionally isolated in the study (*F. proliferatum, F. concentricum* and *F. mangiferae*). Wide field and confocal microscopic observations showed that *F. oxysporum* penetrated the root hair region of roots, then invaded the cortical cells where it induced necrosis in both *V. planifolia* and *V. ×tahitensis*. The hyphae never invaded the root vascular system up to 9 DAI. As a whole, our data demonstrated that RSR of vanilla is present worldwide and that its causal agent should be named *F. oxysporum* f. sp. *radicis-vanillae*.

Keywords: *Cortical root rot, Genotypic diversity, Histology, Molecular identification, Pathogenicity tests, Vanilla planifolia, Vanilla ×tahitensis, World expansion*

I Introduction

Root and stem rot (RSR) has become the most important limitation for the production of vanilla beans at the world production level (Alconero, 1968a, Pinaria et al., 2010a, Tucker, 1927). Available records show reduction of 50 to 90% of worldwide production (Pinaria et al., 2010a, Bhai & Dhanesh, 2008). This disease is strongly limiting the intensification of vanilla production under insect proof shade-houses, such as those in French Polynesia (FP), Reunion Island (Benezet et al., 2000), Madagascar (Delassus, 1963, Dequaire, 1976) and Mexico (Hernandez Hernandez, 2011). Until now, no effective and sustainable disease management has yet been established. *Vanilla phaeantha* and *V. pompona*, as well as hybrids of these wild relatives with the main cultivated vanilla species, *V. planifolia*, have been reported as being resistant to the disease (Delassus, 1963, Theis & Jimenez, 1957b). However, neither of these is cultivated because their beans do not have the desired aromatic properties (Pinaria et al., 2010a). Several trials of biological control have been reported with some success but none has been developed consistently in large areas (Pinaria et al., 2010a).

Fusarium oxysporum f. sp. *vanillae* has been reported as the causal agent of RSR on *V. planifolia* for almost a century (Tucker, 1927) in all production areas (Tombe & Liew, 2010a). However, most reports do also mention other *Fusarium* species associated with the symptoms (Alconero & Santiago, 1969, Pinaria et al., 2010a, Tombe & Liew, 2010a, Bhai & Dhanesh, 2008), such as *F. solani* and *F. semitectum*. Nevertheless, except in a few cases, these other *Fusarium* species were unable to induce symptoms on *V. planifolia* when pathogenicity tests were performed. On the other hand, *F. oxysporum* f.sp. *vanillae* isolates, when tested, clearly reproduced symptoms on vanilla stems (Pinaria et al., 2010a).

Fusarium oxysporum is a large, widespread and multilineage group of fungi forming a species complex subject to genomic recombination and horizontal gene transfer. Most plant pathogenic *F. oxysporum* strains are soil borne, infecting plants at the root and crown levels.

Chlamydospores ensure the long term survival of the fungus in the soil. Pathogenic *F. oxysporum* strains often spread systemically through the vascular system. Some particular strains, indicated as "*radicis*" in their names, do not spread through the vascular system towards the aerial parts of the plant. They are involved in the rotting of the root and crown tissues. The infected plant shows signs of wilting and eventually dies from insufficient water and nutrient uptake due to the loss of root tissues.

Both vascular and necrotic symptoms associated with RSRs are mentioned for *V. planifolia* in the literature, but no clear discrimination has been made between the *Fusarium* strains responsible for the two symptom types. In addition, very few reports describe in detail the process of tissue colonisation and spread of the causal agent in the root tissues for either type of symptom (Alconero, 1968a).

In the current study we present an in-depth analysis of root rot on *V. planifolia* in the Indian Ocean (Reunion island, Madagascar and Indonesia) and on *V. ×tahitensis* in FP. *Vanilla ×tahitensis* is a hybrid between *V. planifolia* and *V. odorata* and cultivated mostly in the Pacific region for its unique flavours.

The present report is the first to dissect, in a comparative manner, the causal basis of root rot on vanilla in the most important areas of production in the world. It also demonstrates that the *F. oxysporum* strains causing root rot should be named "*radicis-vanillae"* because there is no progression of hyphae within the vascular tissues of both vanilla species tested, limiting the rot only to the emerging roots. The experimental approaches included symptom observations and analysis, *Fusarium* morphological biometrics, Koch's postulates tests, phylogenetic analysis of different strains with the translation elongation factor-1α (*EF1α*) and the ribosomal intergenic spacer (IGS) sequences, as well as histopathological studies of root and stem tissues.

II Materials and Methods

II-1 Sample collection

Sampling surveys were conducted in the major vanilla growing areas of Madagascar (20-29 May, 2009), Reunion Island (17 March- 19 April, 2011) and FP (2011-2012). In each region, the plots were selected in order to reflect the diversity of agro-ecological conditions based on location, cultivation system and disease intensity. In each plot, root and stem samples were collected from vanilla plants showing root rot, chlorosis of stems and general wilting, which are reported as common symptoms of *Fusarium* disease. The samples were placed in polythene bags and maintained in cool conditions until further processing in the laboratory. Four additional isolates from a previous survey conducted in Indonesia (Pinaria et al., 2010a) were also included in the pathogenicity study conducted in Reunion Island. Additional *Fusarium* strains isolated from vanilla in Comoros, Mexico, Indonesia and Reunion Island (Pinaria et al., 2015a) were included for the genetic diversity analysis. The non-pathogenic isolate Fo047 (Alabouvette, 1986), purchased from the collection of Microorganisms of Interest for Agriculture and Environment (MIAE, Dijon, France), was used as a reference in the genetic diversity and pathogenicity analyses. The geographic origin of the isolates used in the study is given in Figure 1 and Supplementary Data Table 1.

II-2 Isolation, purification and morphological identification of *Fusarium* isolates

The root and stem samples were thoroughly washed in running tap water. They were then rinsed in a bleach solution (1% sodium hypochlorite), washed three times in sterile distilled water and dried using absorbent paper. The explants (3-5 mm) from roots (whole or cylinder only) and stems (whole or cylinder only), were plated on potato dextrose agar (PDA), sometimes supplemented with Streptomycin (100 mg/L). After an incubation period in the

dark for 5-7 days at 25°C, *Fusarium*-like mycelium were sub-cultured onto PDA prior to single spore purification on malt acid medium. For observation of morphological characters, isolates were sub-cultured onto carnation leaf agar (CLA). The 377 *Fusarium* isolates recovered during the survey were maintained at 4°C on PDA.

II-3 DNA extraction

For DNA extraction, fungal isolates were cultivated for 7 days on PDA. The mycelium was scraped gently into a 1.5 ml micro centrifuge tube, using 2 ml of lysis buffer (50 mM tris-HCl (pH 7.5), 50 mM EDTA and 3% SDS). Total genomic DNA was extracted using the method described by Edel *et al.* (2001). Quantity and quality of the DNA was estimated using a Nanodrop spectrophotometer (Wilmington, USA).

II-4 PCR amplification and DNA sequencing

The taxonomic identity of isolates was confirmed by sequencing the *EF1α* and IGS gene regions, amplified by polymerase chain reaction (PCR) using the primer pairs EF1/EF2 (O'Donnell et al., 1998) and CNL12 (Anderson & Stasovsky, 1992)/ CNS1 (White et al., 1990), respectively. Amplifications were performed in a 25 µl reaction mixture containing PCR reaction buffer, 50 nmol of $MgCl_2$, 1 U of *Taq* polymerase (GoTaq, Promega, USA), 5 nmol dNTPs, 10 nmol of each forward and reverse primer and 40 ng of genomic DNA. PCR reactions were performed using a 96-well thermocycler machine (Applied Biosystems, USA). For the *EF1α* locus, PCR conditions consisted of an initial denaturation at 96°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. For IGS, PCR conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30s, 60°C for 30s, 72°C for 2 min, and a final extension of 72°C for 10 min. PCR amplicons were visualised on a 1.5% agarose gel and the

products were sequenced in both directions by Beckman Coulter Genomics (United Kingdom).

II-5 Pathogenicity tests

Two slightly different procedures were used for testing the pathogenicity of the isolates.

In Reunion Island, the root dip inoculation method was used with *in-vitro* grown plants of the susceptible *V. planifolia* clone CR001. The roots were submerged for 5 min in a conidial suspension (10^6 conidia/ml) prepared in minimal liquid medium (Olivain & Alabouvette, 1997b). Control plants were dipped in sterile distilled water. The *in-vitro* cultivated plants were carefully handled to avoid injuries to the roots. Dip-inoculated plants were transplanted into plastic pots containing sterile cocoa fibre (50%), bagasse (30%) and filter-cake (20%), and kept in growth chambers (27°C, 80-90% RH, 12 h photoperiod, PAR 120 µmol/m^2/s). The plants were watered on alternate days. Each isolate was tested using at least four plants per experiment. Symptoms were recorded on alternate days, from 3 to 15 days after inoculation (DAI) using a 0 to 4 rating scale as follows: 0 = No symptom, 1 = Leaves lost their glossiness, 2 = Local browning visible on the stem, 3 = Lodging of plants, brown areas and mycelium visible on the aerial parts, and 4 = Plant totally rotten or dried (Figure 2A). In each test, the highly pathogenic isolate (Fo072) selected in preliminary work and the non-pathogenic isolate Fo47 (Alabouvette, 1986) were used as controls.

In FP the same root dip inoculation procedure was used but the inoculated plantlets were incubated in sterile glass tubes maintained at 27°C, 80-90% RH, 12 h photoperiod, and pathogenicity was assessed by calculating the percentage of root that was rotten due to pathogen invasion 12 DAI (Figure 2B).

II-6 Histological observations

The root sections were fixed using a 10% paraformaldehyde-glutaraldehyde-cafeine mix in 0.2 M phosphate buffer (pH 7.2). The samples were then dehydrated through successive alcohol series (50% and 70%), and impregnated in methyl methacrylate with LKB Historesin Technovit Polymerisation was performed in a Histo5 microwave tissue processor (Milestone, Italy) at 37°C for 24 hrs. Thin sections of 2 - 3.5 µm were obtained using a Leica RM2255 microtome (Leica Microsystems, Germany), and resulting samples were stained using Schiff naphthol blue black directly on the microscopic slides. The slides were observed under Leica DM6000 epifluorescence microsope (Leica Microsystems, Germany) and captured using a Retiga 2000R camera (QImaging, Canada) and processed with the Volocity software (Perkin-Elmer, UK).

For confocal microscopy, infected root tissues were carefully taken out of the tubes. Thin sections (90 µm) were obtained using a HM650 V vibrating blade microtome (Thermo Scientific, Walldorf, Germany) and stored in sterile Phosphate buffer saline (PBS). The sections were then stained with DAPI (4', 6-diamidino-2-phenylindole) for 5 min in the dark, and washed twice with PBS. The sections were then observed and analysed using a Zeiss LSM780 multiphoton microscope (Zeiss, Germany), equipped with a Chameleon Ultra 11 laser (Coherent, California). The filter blocks, with differential spectral properties, were set to those of DAPI (450-500 nm) and root auto-fluorescence (600-700 nm). Image acquisition was performed using Zen software (Zeiss, Germany). Image processing and channel merging was generated using the Image J 1.47v software (NIH, USA).

II-7 Data analysis

Nucleotide sequences were manually edited using Bioedit 7.1.3.0 (Hall, 1999) and blasted against GenBank nucleotides database (http://blast.ncbi.nlm.nih.gov/) to identify the

most similar sequences. Phylogenetic trees were produced using MEGA 6.06 (Tamura et al., 2013) and Clustal W for alignment and the Maximum likelihood (ML) reconstruction methods. Tree branch robustness was estimated on 1000 bootstrapped datasets. Branches with less than 65% support were collapsed to determine the diversity groups.

The pathogenicity of the isolates was assessed by calculating the area under the disease progress curve (AUDPC) in inoculation tests, using the package *"agricolae"* in R software (R_Core_Team, 2013). Pathogenicity classes were determined by recursive partitioning with complexity optimisation using the "*rpart*" package in R.

III Results

III-1 Field survey and symptomatology

Samples were collected from a total of 52 vanilla plots surveyed in Reunion Island (25), Madagascar (19) and FP (8). The surveys covered most of the vanilla producing areas of the three islands namely: eastern and southern cities of Reunion Island, the districts of Sambava, Andapa and Antalaha in northern Madagascar, and the districts of Taputapuatea, Tumaraa and Uturoa in Raiatea (FP) (Figure 1). Most of the plots surveyed in Reunion Island and Madagascar were cultivated in traditional (agroforestry) or field systems, while in FP a focus was given on shade-house plots (Table 1). The sampled plots were very diverse in terms of crop age (from 1 to 19 years) and intensity of management (plant nutrition, shade control, vine looping, weeding, pollination intensity).

Decaying vanilla vines were observed in all visited plots (Figure 3B and 3C). Symptoms were generally associated with the RSR syndrome described by Tucker (1927). Typical symptoms, observed on the decaying vines were browning and dry rot of the subterranean and aerial roots (Figure 3G and 3L). Stem chlorosis and necrosis was occasionally observed. However, the stem rot was generally circumscribed to two or three internodes as if nodes

were acting as a barrier to disease progress along the vine (Figure 3E and 3J). Typically an intense transformation of the aerial grasping roots into nutritional roots descending to the soil illustrated this disease (Figure 3D and 3I). In most cases, these new roots were infected when they reach the soil, thus inducing the generation of new aerial roots etc. This repetitive process of root rot and generation of aerial roots appeared to contribute to the plant decay because of heavy carbon assimilates requirement. Severely affected plants showed dull leaves and grooved stems as a consequence of water deficiency (Figure 3F and 3K).

The same symptomatology was observed in *V. planifolia* in Madagascar and Reunion island, as well as in *V. ×tahitensis* in FP. This is in accord with the first description of the disease in 1927 in Puerto Rico by Tucker (1927) and previous decay descriptions at the end of the nineteenth century in Reunion Island reported by Delteil (1902).

III-2 Recovery of fungal isolates

A total of 377 fungal isolates were recovered from the 182 samples collected in the three countries surveyed for this study (316 from Reunion, 49 from Madagascar and 12 from FP). This collection of isolates was complemented by four isolates from Indonesia (Pinaria et al., 2010a) and the non-pathogenic isolate Fo47 (Alabouvette, 1986). Most isolates were recovered from *V. planifolia* explants (345) and only 12 from *V. ×tahitensis*. In order to search for diverse isolates four plants of the resistant genotypes *V. pompona* and *V. bahiana* cultivated in a collection plot were sampled and yielded, respectively, seven and 17 isolates. The majority of the isolates were obtained from vanilla root explants (324), and 56 from stem and one from pod. Detailed information about the isolates collected is provided in the Supplementary data Table 1.

III-3 Molecular diversity analysis

The *Fusarium* isolates were identified on the basis of *EF1α* and IGS DNA sequences. A subset of 192 isolates representative of the various plots and explant types of the different countries was selected for DNA extraction and sequencing. Good quality *EF1α* sequences were obtained for 165 isolates (149 from Reunion Island, 10 from FP and 6 from Madagascar). Most were from *V. planifolia* (140), followed by *V. bahiana* (12), *V. ×tahitensis* (10) and *V. pompona* (3). Sequencing of *IGS* PCR products yielded useful sequences for 153 isolates, 148 from Reunion Island and five from Madagascar. Again most sequences were from *V. planifolia* (104), followed by *V. bahiana* (10), and *V. pompona* (3). BLAST comparison of these sequences to the nucleotide GenBank, showed that 79% of the isolates recovered had high sequence similarity (>99% nt identities) to *F. oxysporum*, followed by *F. solani* (16%). The remaining isolates were identified as *F. proliferatum* (3%), *F. concentricum* (1%) and *F. mangiferae* (1%) (Table 2). The frequency of *F. oxysporum* vs *F. solani* recovered from the samples was independent of plot age and cultivation type (pvalue >0.3 in Khi² test). For the phylogenetic analysis the sequence data set was completed with 21 *EF1α* sequences from *F. oxysporum* isolated from vanilla (Pinaria et al., 2015a) and 21 *Fusarium* sequences from GeneBank (Table 3). The ML tree identified 22 haplogroups supported by a 65% bootstrap threshold noted from A to V in Figure 4. The *F. oxysporum* from vanilla analysed in the study are highly diverse since they represented 20 haplotypes grouped into 11 haplogroups (A to K), group A being the most diverse containing 78 isolates from nine haplotypes. No correlation was observed between haplogroups and geographic origin or pathogenicity. The 119 isolates from Reunion Island were split into eight haplogroups, the 10 isolates from Indonesia into four groups, and the five from Madagascar into three. By contrast the five *F. oxysporum* isolates from FP, originating from five plots in Raiatea, shared 100% identity and localised into a single group (A). This group is remarkably

diverse in terms of geographic origin since it contains isolates from all countries included in the study (Mexico, Comoros, Indonesia, Reunion Island, FP and Madagascar). Seven haplogroups containing 11 haplotypes where identified for the 26 *F. solani* isolates (P to V). Only *F. solani* was recovered from *V. bahiana* and the 12 isolates sequenced grouped with isolates from FP in groups R & V.

One hundred and eighteen isolates from Reunion and Madagascar, for which both *EF1α* and *IGS* sequences were available, were selected for further diversity analysis by ML cladistics (Figure 5). As expected the IGS tree was twice more resolved (42 groups) than that of *EF1α* (18 groups) and the two trees were highly congruent. Most isolates clustering in an IGS group belonged to a monophyletic group in the *EF1α* tree. However, a few exceptions could be observed for the *F. oxysporum* isolates. Isolates from IGS-group H split into four *EF1α*-groups (b, c, d, e); IGS group M split into two *EF1α* groups (b, f); and the three isolates clustered in adjacent IGS groups (V, W) were split into the divergent groups h and k. The nucleotide sequences obtained in this study for representative haplotypes were deposited in GenBank under the accession numbers KM065846 to KM065875 for *EF1α*, and KJ672092 to KJ672106 for IGS.

III-4 Morphological identification

To further confirm the molecular identification of the fungi studied, morphological observations were done on selected CLA cultures (Figure 6). The macro- and microconidia showed morphological differences in agreement with species description: the length of macroconidia ranged from short to medium in *F. oxysporum*, wide and stout in the case of *F. solani* and thin-walled, long and straight in *F. mangiferae*. The septa of macroconidia in *F. oxysporum* were limited to 3, while their number ranged from 5 to 7 and 3 to 5 in *F. solani* and *F. mangiferae*, respectively. No macroconidia were seen in *F. proliferatum* preparations.

The number of microconidia septa ranged from 0 to 2 in *F. solani*, 0 to 1 in *F. mangiferae* while microconidia of *F. oxysporum* and *F. proliferatum* were aseptate. Microconidia were formed in false heads on very short phialides in *F. oxysporum* whereas in *F. solani* very long phialides were observed.

III-5 Pathogenicity assessment

One hundred and seventeen isolates from Reunion Island (109), Madagascar (4) and Indonesia (4) were tested for pathogenicity on *V. planifolia* CR001 using the Reunion Island procedure, and 11 isolates from FP were tested on *V. ×tahitensis* using the FP procedure. Pathogenicity tests included isolates from diverse geographic origins and types of cultivation (Supplementary Table 1). Isolates from the four species identified were assayed: *F. oxysporum* (97), *F. solani* (14), *F. proliferatum* (4), *F. concentricum* (2), *F. mangiferae* (1), as well as unidentified species (10)

In addition, two of the most pathogenic isolates in Reunion Island and FP were tested on *V. planifolia* and *V. ×tahitensis* using the FP procedure.

A majority of isolates (79/128) were pathogenic on *V. planifolia* or *V. ×tahitensis*. However, in Reunion Island about 42% of the isolates (49/117) recovered from symptomatic vines were unable to infect *in-vitro* vanilla plants. No symptoms were recorded in the two negative controls (mock inoculation and non-pathogenic Fo47 isolate) at the completion of the pathogenicity tests (15 DAI). Among the pathogenic isolates a continuous gradient of disease severity was observed with AUDPC values ranging from zero to 35 and percentages of root rot from 38% to 100%. Using recursive partitioning, the isolates tested in Reunion Island could be differentiated into highly pathogenic (17%), inducing plant death 10 DAI, moderately pathogenic (23%), and slightly pathogenic (17%) for which symptom development was slower (Figure 7A). Some isolates that showed mild symptoms on one or

two plants only were considered questionable and included in the non-pathogenic group, assuming that symptoms in some replicates were due to physical damage during the experiment and not due to the fungus. In FP, the 11 *Fusarium* isolates ranged from highly pathogenic to slightly pathogenic (Figure 7B). Pathogenic isolates were re-isolated from the diseased tissue, 15 of which were selected for re-sequencing of the *EF1α* locus to confirm identify. These were 100% identical to the inoculated isolates.

When tested on *V. planifolia*, the highly pathogenic isolate FoP12 from *V. ×tahitensis* in FP turned out to be one the most aggressive of all isolates studied in this work, displaying the same types of symptoms and mycelium growth as the ones isolated from lesions on *V. planifolia.* Reciprocally the highly pathogenic isolate Fo072 from *V. planifolia* in Reunion Island displayed the same severe symptomatology with high AUDPC values when tested on *V. ×tahitensis* accession CR017 compared to *V. planifolia* accessions CR001 or CR038 (data not shown).

Pathogenicity did not correlate with *EF1α* phylogeny. Among the 83 *F. oxysporum* isolates genotyped and tested for pathogenicity, the 39 highly or moderately pathogenic are distributed in four groups (A, B, C, , F), while the 44 non or slightly pathogenic isolates are distributed in 9 haplogroups (A, B, C, E, F, H, I, J, K).

Almost all the highly and moderately pathogenic isolates (43 out of 44) belonged to *F. oxysporum* (Table 4). The *F. solani* isolates were generally rated as non or slightly pathogenic. Two *F. solani* isolate from Reunion Island (Fs194 and Fs245) were rated as moderately pathogenic. The two isolates from FP (FsP01, FsP05) rated as slightly pathogenic and induced a soft rot on *V. ×tahitensis* were distinct from the dry rot produced by *F. oxysporum* isolates. The eight *F. concentricum*, *F. mangiferae* and *F. proliferatum* isolates recovered from vanilla in Reunion Island, FP and Madagascar were not or only slightly

pathogenic to vanilla. Two *F. solani*, recovered from *V. bahiana* tested non-pathogenic to *V. planifolia*.

III-6 Histopathological observations

Histological preparations of *in-vitro V. planifolia* plants CR001 inoculated with the pathogenic Fo072 or the non-pathogenic Fo047 isolates were observed from 3 to 9 DAI (Figure 8). Mycelium penetration was observed for both isolates which occurred invariably in the root hair zone of the aerial roots. Colonisation of cortical cells was much more significant for Fo072 than for Fo047. In addition, the cortical cells underwent severe plasmolysis in the case of Fo072 invasion which was not observed for Fo47. For both isolates, the root vascular system remained free of mycelium up to 9 DAI (Figure 8-E). No damage or penetration could be observed in the epidermal or hypodermal cells of root tips. The same pattern of invasion was observed under confocal imaging for *V. planifolia* and *V. ×tahitensis* inoculated with the highly pathogenic isolate FoP12 from FP (Figure 9).

IV Discussion

In this study we studied the aetiology of RSR of vanilla. The surveys conducted in Madagascar, Reunion Island and French Polynesia, which are among the most important production areas of the two commercial vanilla species, confirmed the high incidence of the disease in the Indian Ocean and in the Pacific regions. Molecular, morphological, pathogenicity and histopathological data analysis led to the conclusion that *F. oxysporum* is the main agent causing RSR of vanilla. Because the fungus is restricted to cortical cells and does not invade the vascular system in susceptible vanilla species as clearly demonstrated in this study, the causal agent of RSR of vanilla is more appropriately named *F. oxysporum* f. sp. *radicis-vanillae*.

Fusarium oxysporum was by far the most frequent species recovered from diseased roots and stems of vanilla cultivated in Reunion Island, Madagascar and FP. Pathogenicity tests using unwounded plant material confirmed the pathogenicity of most of the *F. oxysporum* isolates. These data along with previous surveys and pathogenicity tests conducted in Indonesia (Pinaria et al., 2010a), China (Xia-Hong, 2007) and India (Vijayan et al., 2012a) demonstrated that *F. oxysporum* is the principal species causing RSR of vanilla worldwide. They are in agreement with the initial description of the symptomatology and aetiology of the disease made in 1927 by Tucker (1927) under the synonymous name *F. batatatis*.

Fusarium solani was the second species most frequently associated to the disease in Reunion, Madagascar and FP as well as in Indonesia (Pinaria et al., 2010a) and China (Xia-Hong, 2007). However, this species showed much less virulence on *V. planifolia* in Reunion Island and was associated with a distinct rot on *V. ×tahitensis* in FP (data not shown). In Indonesia the *F. solani* isolates were unable to infect *V. planifolia* stem cuttings, contrary to all *F. oxysporum* isolates tested (Pinaria et al., 2010a). Therefore, if pathogenic to vanilla, *F. solani* is much less harmful than *F. oxysporum* and only the latter species is responsible of the devastating losses encountered in vanilla plantations affected by RSR.

The *F. oxysporum* isolates infecting vanilla exhibited a high genetic and biological diversity, as observed in the *EF1α* and IGS sequence analysis and the pathogenicity evaluation on vanilla plantlets. Indeed, the phylogenetic tree inferred from *EF1α* sequences revealed that the *F. oxysporum* isolates found in Reunion Island, Madagascar, Indonesia and Mexico (Pinaria et al., 2015a) were polyphyletic, the 119 isolates from Reunion being split into eight phylogroups. By contrast, the five *F. oxysporum* isolates from FP were monophyletic, similar to nine isolates from India that were considered clonal based on RAPD analysis (Vijayan et al., 2012a). Given the small number of isolates analysed in FP and in India, the monophyletic origin of these isolates may result from sampling bias. However, a

recent introduction and dissemination of *F. oxysporum* in the vanilla plots of these regions cannot be ruled out. Furthermore, comprehensive and representative sampling in these regions is warranted.

In Reunion Island the large number of isolates sequenced for the *EF1α* and IGS gene regions enabled a worldwide comparison of this pathogen. The 98 isolates of *F. oxysporum* isolates from Reunion Island split into 11 *EF1α* and 29 IGS phylogroups. This grouping is comparable to the one made by O'Donnell et al. (2009b) with 850 *F. oxysporum* isolates from diverse geographic origins and hosts separated into 101 *EF1α* and 203 IGS phylogroups. This suggests multiple introductions or a long evolutionary history of *F. oxysporum* in the soils of Reunion Island. In four cases an incongruent position was observed between the phylogenetic trees inferred from *EF1α* and IGS sequences. In two cases it involved isolates from distant plots (more than 30 km). But in two other cases, the isolates were from the same plot in Reunion Island. This could be a signal of genomic recombination occurring between compatible isolates occupying the same niche.

The pathogenicity test based on root dip inoculation of in-vitro grown plantlets using conidial suspensions proved to be highly reproducible and accurate. The three reference isolates used, Fo072, Fo368 and Fo047 (highly, moderately, and non-pathogenic, respectively), gave consistent AUDPC values repeatedly during the pathogenicity evaluations (data not shown). In addition, contrary to tests performed on wounded tissues in previous works (Alconero, 1968a, Pinaria et al., 2010a, Tonnier, 1960b), the use of intact plantlet enabled us to quantitatively evaluate the pathogenicity of the isolates. Under these conditions, the 128 isolates tested showed a wide range of pathogenicity, varying from non-pathogenic to highly pathogenic. This gradient of responses of vanilla according to *Fusarium* isolates is generally observed in necrotic forms of *Fusarium*, in contrast to vascular *formae speciales* that are either pathogenic or non-pathogenic. In agreement with previous reports on many

formae speciales of *F. oxysporum* (O'Donnell et al., 2009b, O'Donnell et al., 1998, Pinaria et al., 2015a), no correlation was observed between phylogroups and geographic origin or pathogenicity level of the vanilla isolates. There is increasing evidence supporting the hypothesis that pathogenicity of *F. oxysporum* isolates relies on genes located on horizontally transferable extra chromosomes (Ma et al., 2013), such as genes coding for proteins secreted in xylem (SIX). Whether a similar mechanism is involved in the pathogenicity of non-vascular strains would be worth addressing as it could explain the highly polyphyletic evolutionary pattern in housekeeping genes among the pathogenic *F. oxysporum* isolates associated with vanilla.

Histopathological data demonstrated that the penetration of *F. oxysporum* in *in-vitro* conditions occurs in the root hair region and that the hyphae, observed only in the epidermal and cortical tissues induced rapid plasmolysis of cortical cells in the case of pathogenic isolates. This was observed for both *V. planifolia* and *V. ×tahitensis* using isolates from Reunion Island and FP. However, these pathogenic isolates were not observed to colonise the vascular tissue 9 DAI and never multiplied nor penetrated at the root tips. Thus we conclude, based on the pathogenicity and histopathological data reported here, that the causal agent of vanilla RSR should be named *F. oxysporum* f. sp. *radicis-vanillae* instead of *F. oxysporum* f. sp. *vanillae.*

Nevertheless, one of the co-authors (E.C.Y. Liew, data not shown) observed in many situations in Indonesia symptoms of internal discoloration in the vascular tissues of diseased *V. planifolia* vines cultivated in the field. No such symptoms, however, have been recorded yet among hundreds of observations made in the Indian Ocean region and in FP. Additional studies should therefore be conducted to determine whether Indonesian isolates of *F. oxysporum* have distinct pathogenicity features or if this particular symptomatology is related to particular epidemiological circumstances in Indonesia.

Many *Fusarium* plant diseases have been effectively controlled by the use of resistant varieties. Resistance to RSR has already been mentioned in the two *V. planifolia* relatives, *V. phaeantha* and *V. pompona* (Theis & Jimenez, 1957b, Delassus, 1963). These sources of resistance, however, have not been exploited to any significant extent in breeding programs. The results obtained in this study regarding the diverse pathogenic isolates and the process of root infection will be of crucial importance for enhancing the genetic control of RSR of vanilla caused by *F. oxysporum* f.sp. *radicis-vanillae.* In particular, the accurate and quantitative inoculation assay established in this study will be useful to screen for novel sources of genetic resistance at an early stage and with relatively high throughput.

The durability of resistance depends on the mechanisms involved in the plant-pathogen interaction. For many crops a gene for gene resistance mechanism has been demonstrated, leading to the emergence of distinct races of *F. oxysporum*. The gradient of pathogenicity observed among the isolates studied suggests multi-genic interactions in the case of *F. oxysporum*-vanilla interaction.

It is now clear that *F. oxysporum* isolates capable of inducing severe RSR of vanilla are present in all production areas worldwide. Important issues regarding the epidemiology of the disease have yet to be addressed to improve prophylaxis, as well as genetic and biological control of this disease. For instance, population diversity should be better described in vanilla cultivation areas that have not been sufficiently sampled (such as FP). The role of environmental factors such as soil microflora and host resistance in shaping the *Fusarium* populations should be elucidated. The possible role of vanilla cuttings in the spread of pathogenic isolates should also be investigated. The search for molecular marker specific of the forma specialis *radicis-vanillae* that would enable the early and selective detection of the pathogen would greatly enhance prophylaxis at all stages of vanilla production and prevent

disease spread. All of these would contribute to the development of sustainable management strategies urgently awaited in the vanilla industry worldwide.

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VII Figures captions and Tables

Figure 1: Locations of the 52 vanilla plots sampled for the study in four vanilla growing areas.

Figure 2: Ratings for pathogenicity of *Fusarium* isolates root-dip inoculated on in-vitro propagated vanilla plants. A) Appearance of the stem of acclimatised plantlets during incubation in a growth chamber rated between 0 (Healthy) to 4 (Dead); B) Range of rotten stem tissue and percentages measured after incubation for twelve days.

Figure 3: Symptoms of stem and root rot on *Vanilla planifolia* (A-G) and *V. ×tahitensis* (H-L): Healthy plant (A & H); Decaying plants showing leaf chlorosis and numerous aerial roots on vanilla cultivated in the forest (B) and within shadehouses (C & I); Proliferation of aerial roots descending from the stem and rotting when reaching the soil (D), stem rot generally confined to one or two internodes (E & J), grooved stems on wilting plant (F & K), new roots generated upstream of rotten roots (G & L).

Figure 4: Phylogenetic tree based on partial EF1α sequences (662 positions) of 132 *Fusarium* isolates from this study and 42 reference sequences from GenBank (Table3). Letters indicate the phylogenetic groups defined by 65% bootstrap support or more. The tree was inferred by using the maximum likelihood method based on the general time reversible model and discrete Gamma distribution. The branches with less than 65% bootstrap support are collapsed.

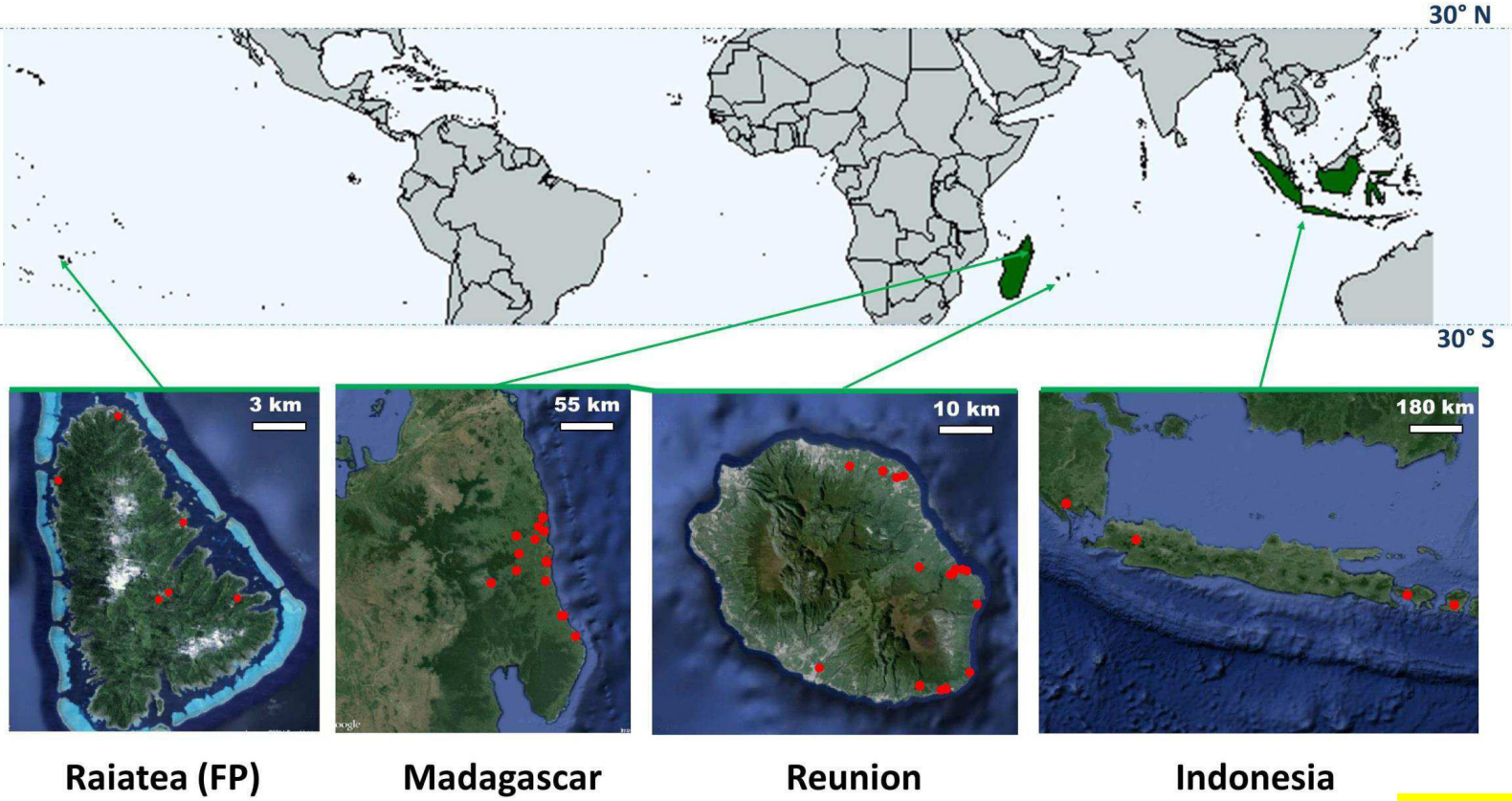
Figure 5: Diversity of 118 *Fusarium* isolates from Reunion Island as assessed by partial IGS sequence (2290 positions) compared to *EF1α* (736 positions). Groups defined by nodes having more than 65% bootstrap support in ML trees are indicated by uppercase (IGS) and lowercase (*EF1α*) letters. Isolates situated into incongruent groups are underlined. The tree was inferred by using the maximum likelihood method based on the general time reversible model and discrete Gamma distribution. The branches with less than 65% bootstrap support were collapsed.

Figure 6: Morphological appearance on carnation leaf agar of macro- and microconidia of selected *Fusarium* isolates from Reunion Island showing the typical morphology of the species identified on the basis of *EF1α* and IGS DNA sequences. *Fo=Fusarium oxysporum, Fs=F. solani, Fm=F. mangiferae, Fp=F. proliferatum;* White bar = 50 µm.

Figure 7: Gradient in the pathogenicity of 81 *Fusarium* isolates from Reunion Island (A) and 11 from FP (B). Isolates from Reunion island were divided into four pathogenicity classes by optimised recursive partitioning: highly, moderately, slightly, and questionably pathogenic. Isolates from FP were divided into two classes. Arrows represent the standard deviation of averaged pathogenicity values. Letters on the baseline indicate the species of the isolate tested: o: *F. oxysporum*; s: *F. solani*; x: *Fusarium* sp.

Figure 8: Embedded Schiff-naphtol blue black stained 2 µm sections in the root hair area of *in-vitro V. planifolia* CR001. (A-C) control plants, (D-H) plantlets dip inoculated with the pathogenic *F. oxysporum* isolate Fo72. A) Cross section showing the anatomy of the root (*ha=root hairs, ep=epidermis, hy=hypodermis, co=cortical region, en=endodermis, va=vascular region*); B) Healthy root cells showing continuous cell walls and integrity of parenchyma; C) Longitudinal section of the peripheral region of healthy root; D) Cross section showing the disruption of the cell wall of peripheral cells of the root 9 days after inoculation (*my= mycelium* Fo72); E) The vascular region is devoid of mycelia; F) Longitudinal section of infected peripheral cells showing the penetration of the fungus through the intercellular space, cell-wall disruption and hydrolysis of the cytoplasm.

Figure 9: Confocal microscopy images of longitudinal section of *V. planifolia* (CR001) inoculated with Polynesian pathogenic isolate of *F. oxysporum* (FoP12), 7 days post inoculation. Hyphae (indicated by yellow arrow) penetrate through the hypodermis in the root hair region, and progresses through the cortical region in the intercellular spaces. Cell walls appear in blue and chloroplasts in red.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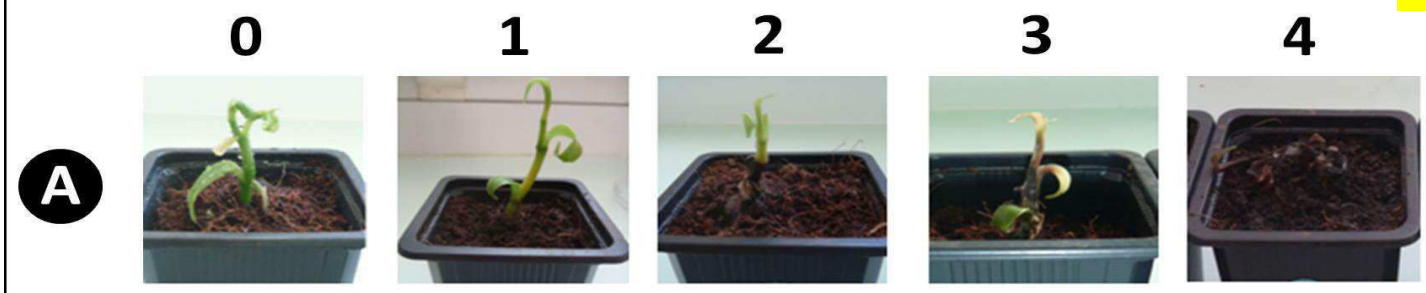
Indonesia

FIG2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3
4

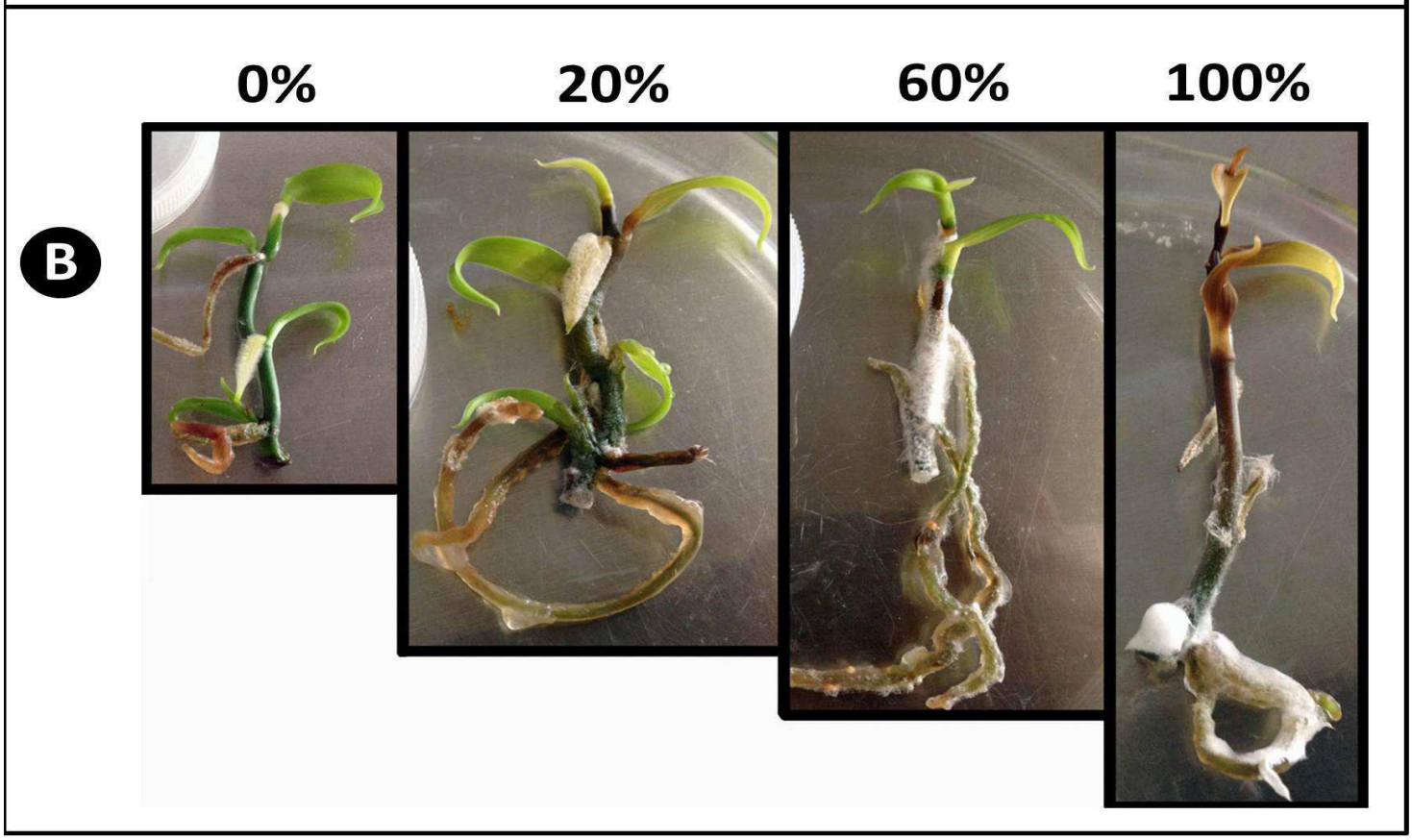
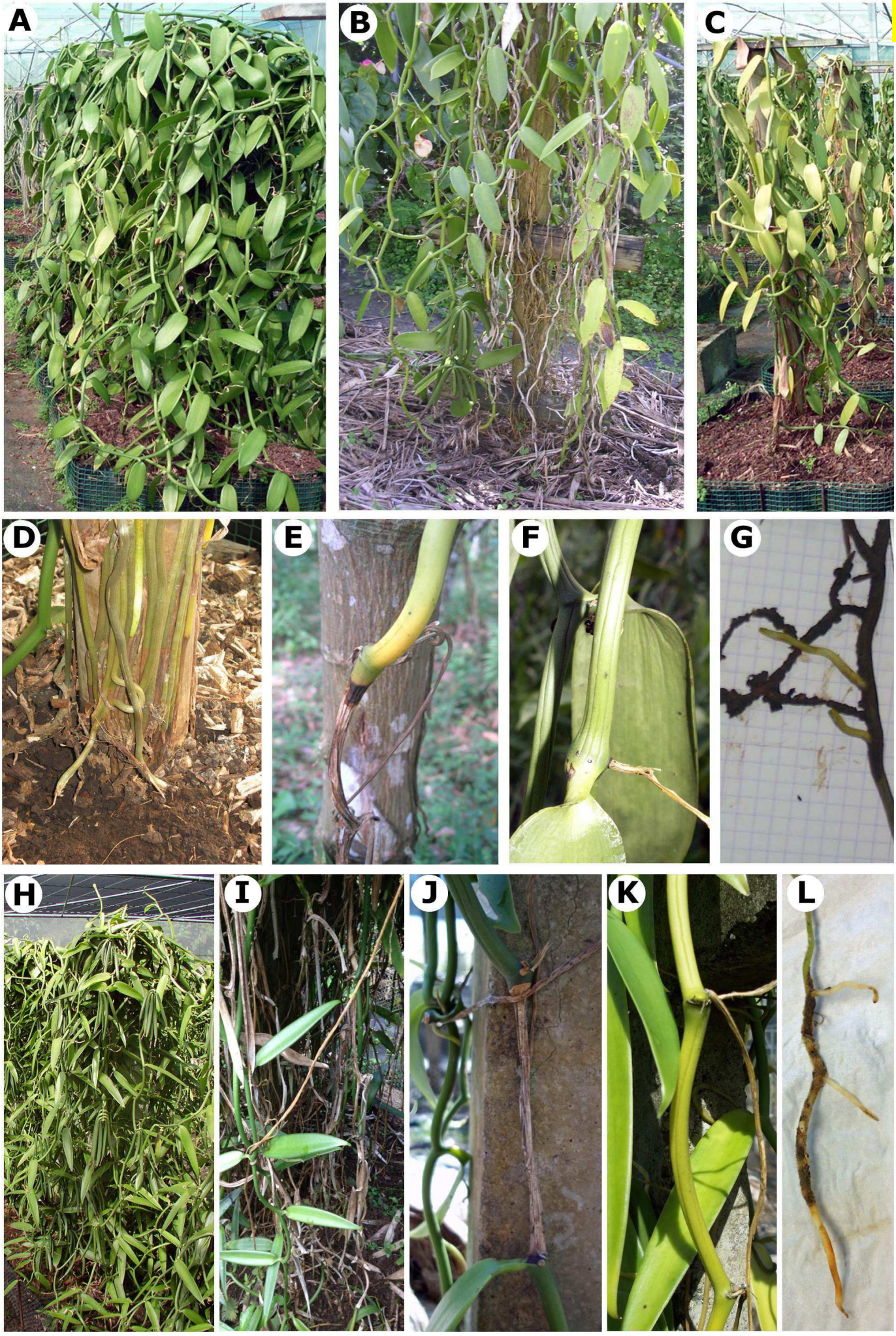

B
0%
20%
60%
100%

FIG3



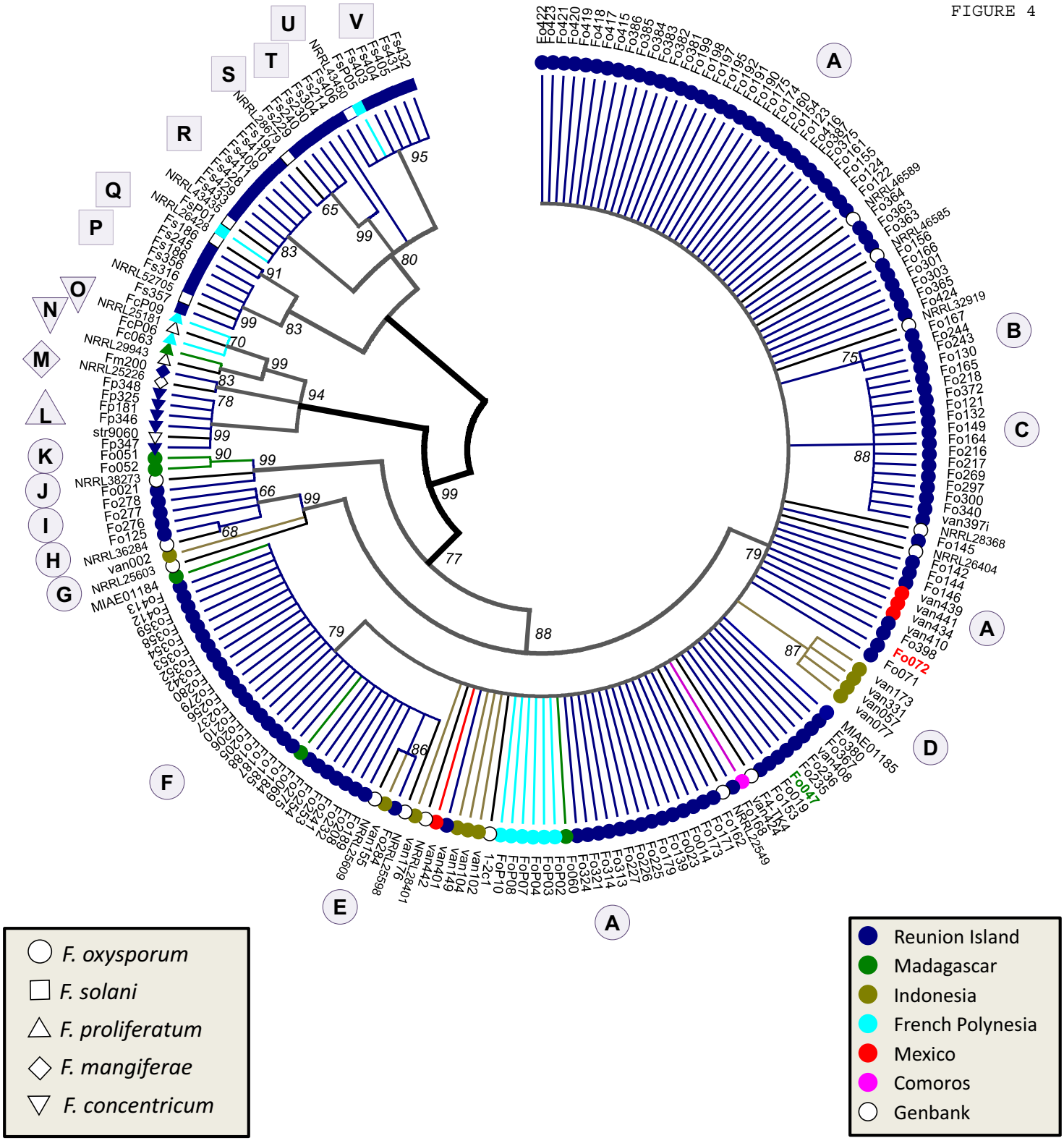
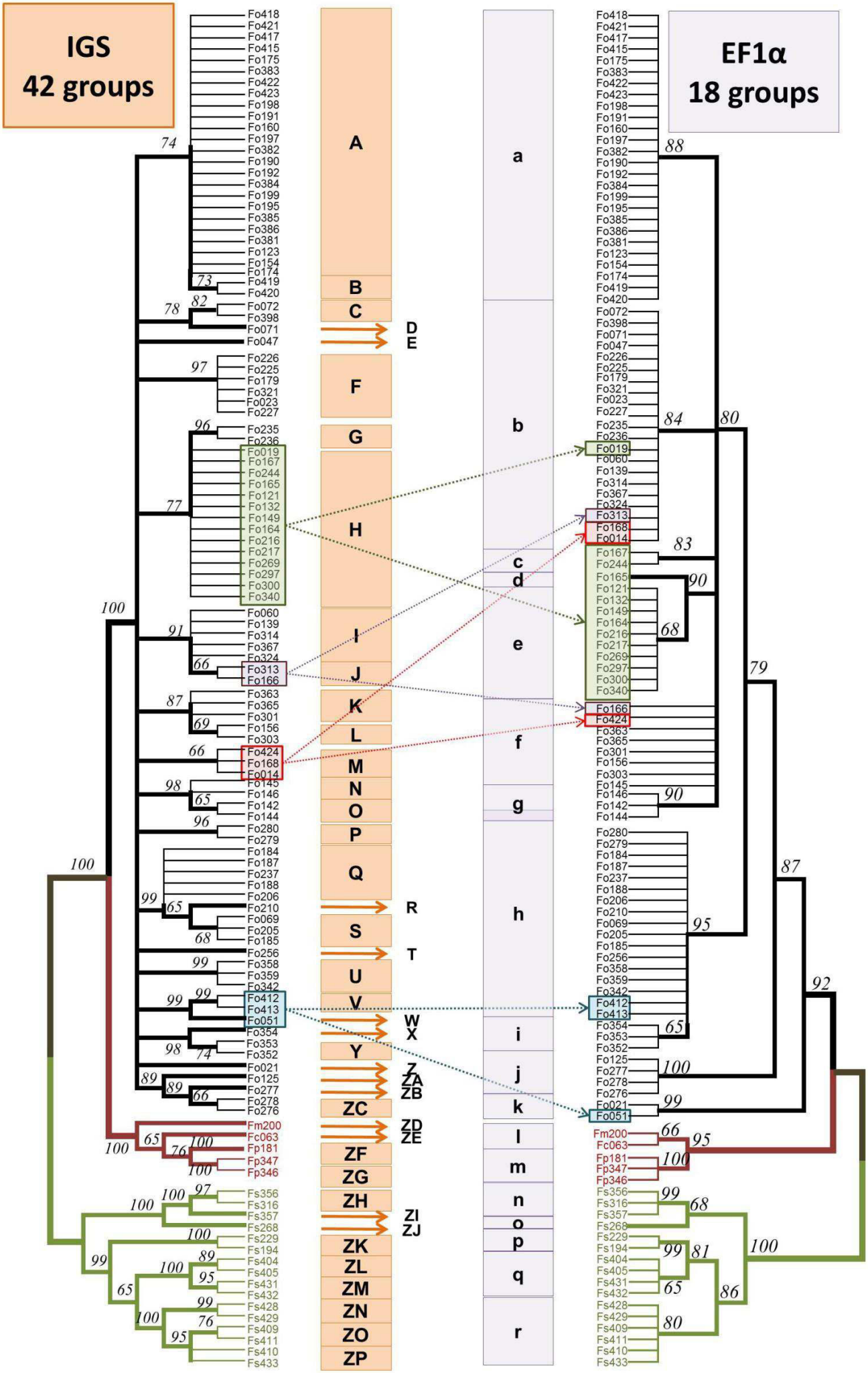

A
B
C
A
D
A
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
Fo072
Fo047
F. oxysporum
F. solani
F. proliferatum
F. mangiferae
F. concentricum
Reunion Island
Madagascar
Indonesia
French Polynesia
Mexico
Comoros
Genbank

FIG5


IGS
42 groups
EF1α
18 groups
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
ZA
ZB
ZC
ZD
ZE
ZF
ZG
ZH
ZI
ZJ
ZK
ZL
ZM
ZN
ZO
ZP
a
b
c
d
e
f
g
h
i
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l
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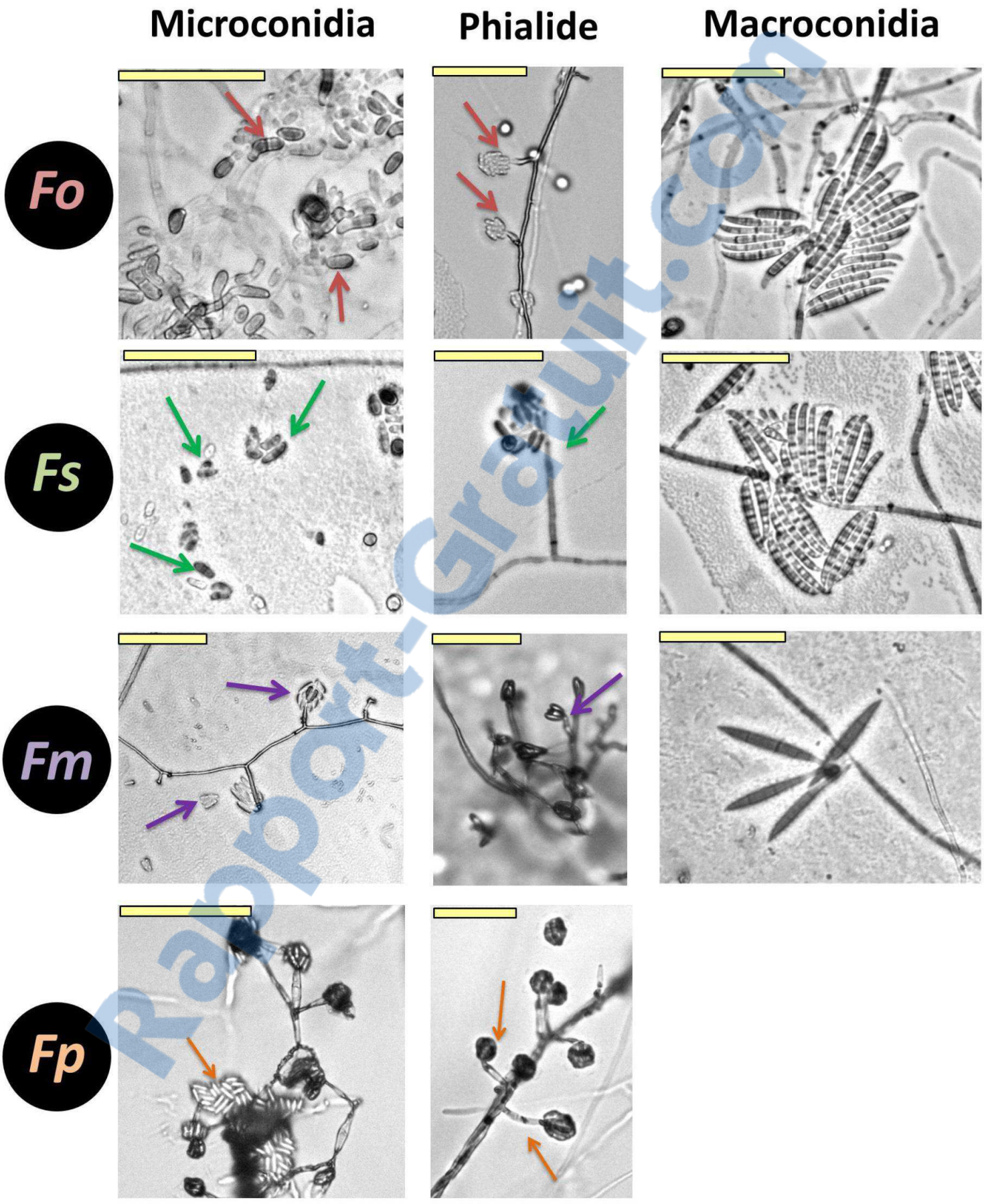
p
q
r

FIG6



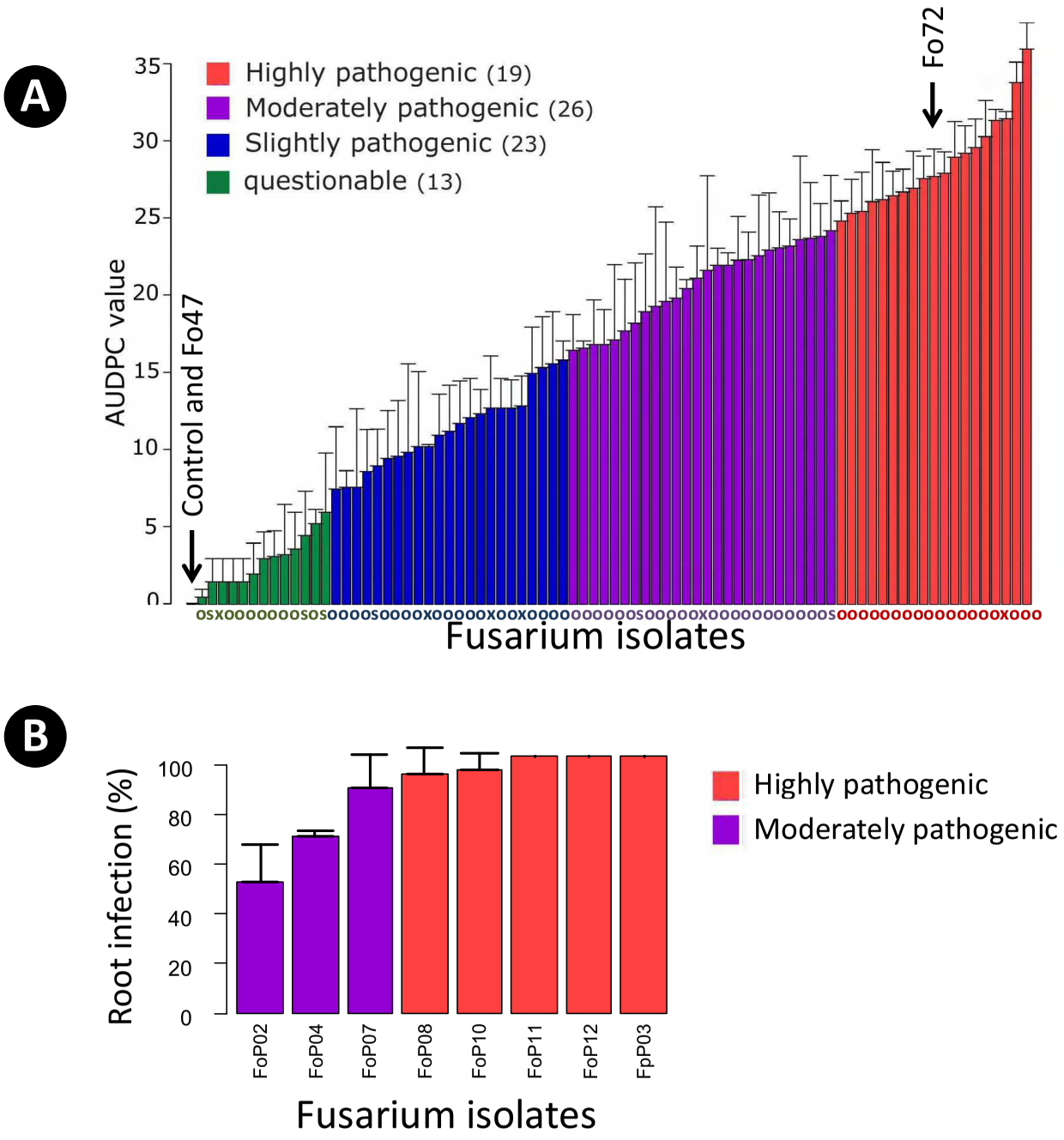

A
Highly pathogenic (19)
Moderately pathogenic (26)
Slightly pathogenic (23)
questionable (13)
Fo72
Control and Fo47
AUDPC value
0
5
10
15
20
25
30
35
Fusarium isolates
B
Root infection (%)
0
20
40
60
80
100
FoP02
FoP04
FoP07
FoP08
FoP10
FoP11
FoP12
FpP03
Highly pathogenic
Moderately pathogenic
Fusarium isolates

FIG8

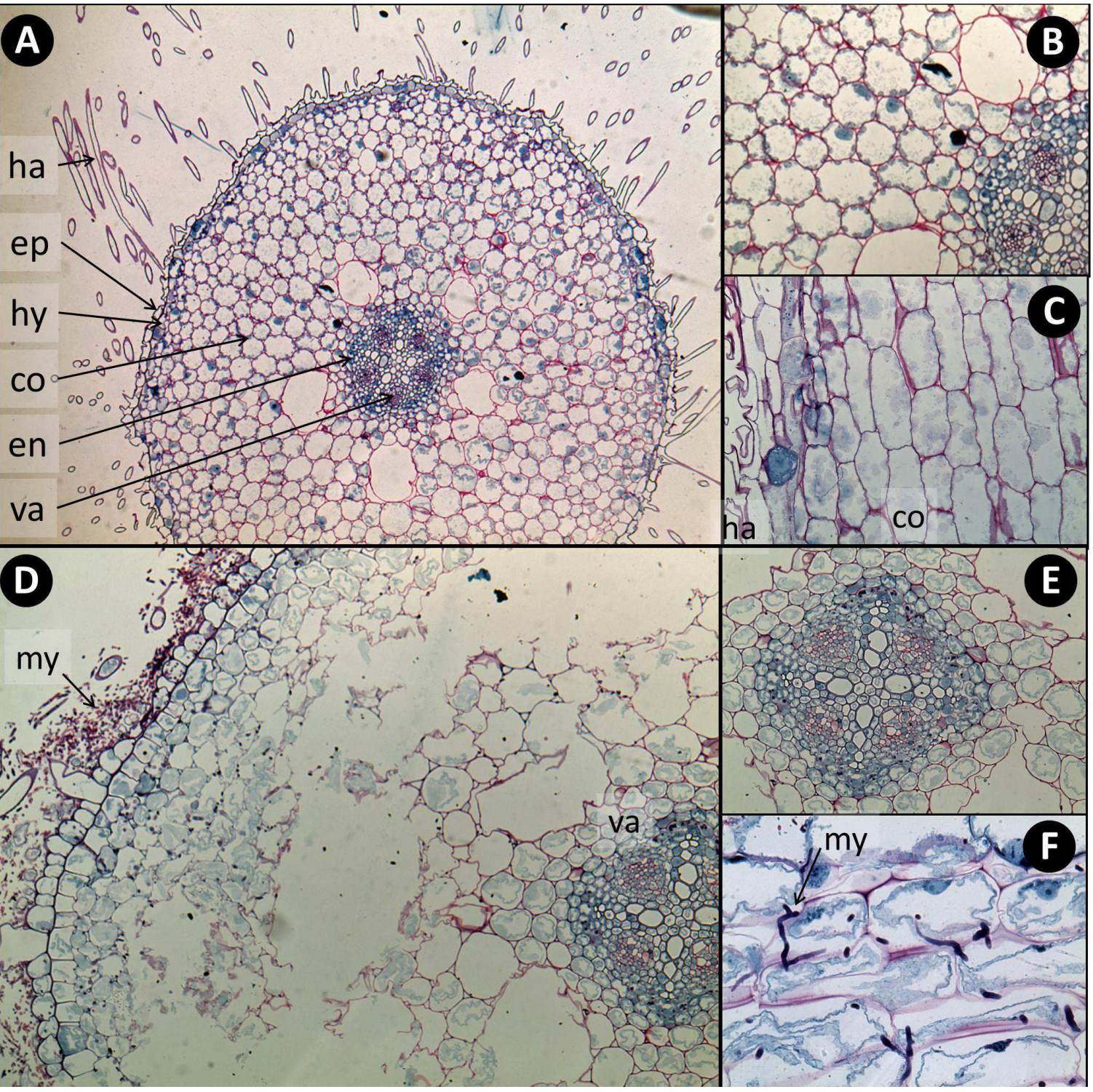


FIG9

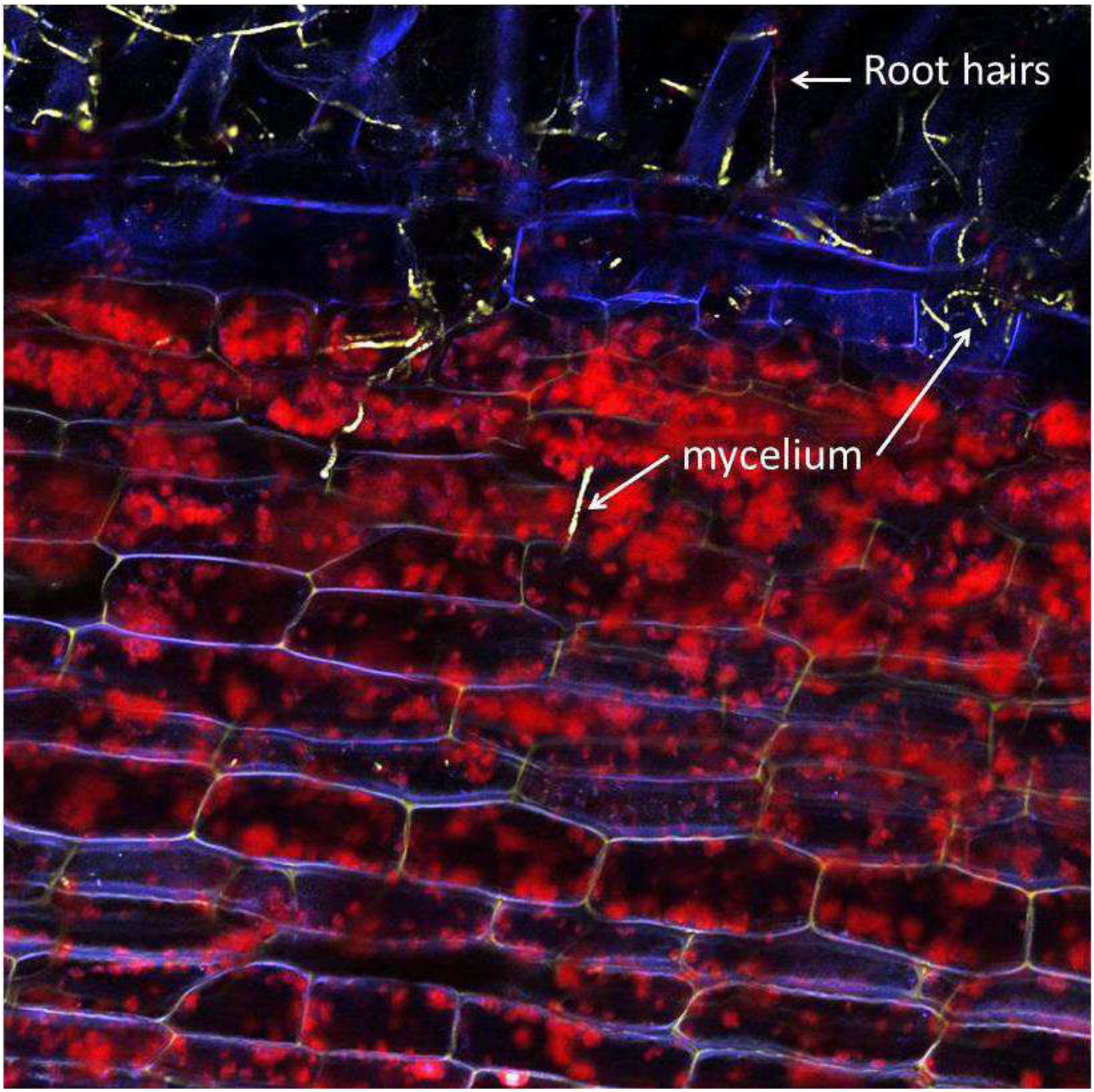


Table 1: Number of plots surveyed and number of *Fusarium* isolates recovered (between brackets) according to type of vanilla cultivation and country.

Type of cultivation	**French Polynesia**	**Madagascar**	**Reunion Island**	**Total**
Field	0	10 (31)	8 (73)	**18 (104)**
Forest	0	9 (18)	11 (86)	**20 (104)**
Shadehouse	8 (12)	0	6 (157)	**14 (169)**
Total	**8 (12)**	**19 (49)**	**25 (316)**	**52 (377)**

Table 2: Number of isolates of each species isolated from vanilla in Reunion Island, FP and Madagascar and identified according to *EF1α* or IGS DNA sequences.

Species	**Reunion Island**	**FP**	**Madagascar**	**Total**
F. oxysporum	119 (80%)	6 (60%)	5 (83%)	**130 (79%)**
F. solani	24 (16%)	2 (20%)		**26 (16%)**
F. proliferatum	5 (3%)			**5 (3%)**
F. concentricum		2 (20%)	1 (17%)	**3 (2%)**
F. mangiferae	1 (1%)			**1 (1%)**
Total	**149 (100%)**	**10 (100%)**	**6 (100%)**	**165 (100%)**

Table 3: *Fusarium* isolates used as reference in the phylogenetic trees

Strain number	***Fusarium* species and *f. sp.***	**Isolation host**	**Country of origin**	**GenBank accession**	**Reference**
NRRL25603	*F. oxysporum* f.sp. *cubense*	banana	Australia	AF008487	(O'Donnell et al., 1998)
NRRL25609	*F oxysporum* f.sp. *cubense*	banana	Malawi	AF008490	(O'Donnell et al., 1998)
NRRL25598	*F. oxysporum* f.sp. *glycines*	soya	USA-MD	AF008496	(O'Donnell et al., 1998)
NRRL22549	*F. oxysporum* f.sp. *passiflorae*	passion fruit	Brazil	AF008505	(O'Donnell et al., 1998)
NRRL25226	*Fusarium sp.*	unknown	India	AF160281	(O'Donnell et al., 2000)
NRRL25181	*F. concentricum*	unknown	Costa Rica	AF160282	(O'Donnell et al., 2000)
NRRL28401	*F. oxysporum* f.sp. *dianthi*	carnation	Netherland	AF246859	(Baayen et al., 2000)
NRRL28368	*F. oxysporum* f.sp. *opuntiarum*	prickly pear	Netherland	AF246871	(Baayen et al., 2000)
NRRL29943	*F. concentricum*	soil	Japan	AF33393	(Aoki et

				4	al., 2001)
NRRL28679	*Fusarium* sp.	hospital	USA-WA	DQ246912	(Zhang et al., 2006)
NRRL26428	*F. solani*	unknown	Germany	DQ247675	(Zhang et al., 2006)
NRRL43435	*Fusarium* sp.	human	unknown	EF452926	(O'Donnell et al., 2007)
NRRL43450	*Fusarium* sp.	human	USA-OH	EF452934	(O'Donnell et al., 2007)
NRRL26404	*F. oxysporum*	unknown	USA-MA	FJ985287	(O'Donnell et al., 2009b)
NRRL32919	*F. oxysporum*	unknown	USA-TX	FJ985319	(O'Donnell et al., 2009b)
NRRL36284	*F. oxysporum* f. sp. *tracheiphilum*	sugar cane	unknown	FJ985343	(O'Donnell et al., 2009b)
NRRL38273	*F. oxysporum* f. sp. *passiflorae*	passion fruit	Australia	FJ985362	(O'Donnell et al., 2009b)
NRRL46585	*Fusarium oxysporum*	unknown	USA-FL	FJ985437	(O'Donnell et al.,

					2009b)
NRRL46589	*Fusarium oxysporum*	unknown	USA-FL	FJ985438	(O'Donnell et al., 2009b)
NRRL52705	*Fusarium solani*	insect	unknown	JF740787	(O'Donnell et al., 2012)
9060	*Fusarium proliferatum*	dragon fruit	Malaysia	JX868990	(Masratul Hawa et al., 2013)
F4-TK4	*Fusarium oxysporum*	citrus	Tunisia	KF574849	(Demers et al., 2015)
1-2c1	*Fusarium oxysporum*	tomato	USA-PA	KJ920415	
van 002	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115168	(Pinaria et al., 2015a)
van 155	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115169	(Pinaria et al., 2015a)
van 176	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115170	(Pinaria et al., 2015a)
van 102	*Fusarium oxysporum f. sp.*	vanilla	Indonesia	KM115171	(Pinaria et al., 2015a)

	vanillae				
van 424	*Fusarium oxysporum f. sp. vanillae*	vanilla	Comoros	KM115172	(Pinaria et al., 2015a)
van 104	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115173	(Pinaria et al., 2015a)
van 149	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115174	(Pinaria et al., 2015a)
van 401	*Fusarium oxysporum f. sp. vanillae*	vanilla	Reunion Island	KM115175	(Pinaria et al., 2015a)
van 442	*Fusarium oxysporum f. sp. vanillae*	vanilla	Mexico	KM115176	(Pinaria et al., 2015a)
van 408	*Fusarium oxysporum f. sp. vanillae*	vanilla	Reunion Island	KM115177	(Pinaria et al., 2015a)
van 410	*Fusarium oxysporum f. sp. vanillae*	vanilla	Reunion Island	KM115178	(Pinaria et al., 2015a)
van 434	*Fusarium oxysporum f. sp. vanillae*	vanilla	Mexico	KM115179	(Pinaria et al., 2015a)

van 439	*Fusarium oxysporum f. sp. vanillae*	vanilla	Mexico	KM115180	(Pinaria et al., 2015a)
van173	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115181	(Pinaria et al., 2015a)
van331	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115182	(Pinaria et al., 2015a)
van057	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115183	(Pinaria et al., 2015a)
van077	*Fusarium oxysporum f. sp. vanillae*	vanilla	Indonesia	KM115184	(Pinaria et al., 2015a)
van441	*Fusarium oxysporum f. sp. vanillae*	vanilla	Mexico	KM115185	(Pinaria et al., 2015a)
van397	*Fusarium oxysporum f. sp. vanillae*	vanilla	Reunion Island	KM115186	(Pinaria et al., 2015a)

Table 4: Pathogenicity categories of the isolates of five *Fusarium* species isolated from the two cultivated vanilla species *V. planifolia* and *V. xtahitensis*. HP=Highly pathogenic, MP=Moderately pathogenic, SP=Slightly pathogenic, NP=Non-pathogenic.

	Pathogenicity				
Plant host/Fusarium species	HP	MP	SP	NP	Total
V. planifolia	***19***	***26***	***23***	***49***	***117***
F. oxysporum	18	19	23	29	89
F. solani		2	1	9	12
F. proliferatum				4	4
F. concentricum				1	1
F. mangiferae				1	1
Fusarium sp.	1	1	3	5	10
V. xtahitensis	***6***	***0***	***5***	***0***	***11***
F. oxysporum	6		2		8
F. solani			2		2
F. concentricum			1		1
Total	**24**	**20**	**30**	**44**	**128**

Supplementary Table 1: Characteristics of the isolates used in the study. (1) : latitude ; longitude in decimal degrees, (2) : Ro=Whole root, RoC=Root central cylinder, St=Whole stem, StC=Stem central cylinder, P=Pod , (3) :NP=Non-pathogenic, SP=Slightly pathogenic, MP=Moderately pathogenic, HP=Highly pathogenic , (4) *Elongation factor 1alpha* sequence , (5) : Intergenic spacer sequence

Code	Date of sampling	Country	Region	District	Coordinates (1)	Plot type	Plot age (years)	Isolation host	Isolation organ (2)	Species	Pathogenicity (3)	All_EF group (4)	EF group (4)	IGS group (5)	Genbank (EF)	Genbank (IGS)
Fc063	May, 2009	Madagascar	Sambava	Mahaleotena	-14,51612 ; ⁄	Forest	7	V. planifolia	RoC	F. concentricu	NP	N	l	ZE	KM065863	
FcP06	Oct, 2012	French polyn	Raiatea	Tumaraa	-16,7667 ; -1	Shadehouse	9	V. xtahitensis	RoC	F. concentricu	SP	O				
FcP09	Nov, 2012	French polyn	Raiatea	Taputapuate	-16,8349 ; -1	Shadehouse	9	V. xtahitensis	RoC	F. concentricu	na	O				
Fm200	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	Ro	F. mangiferae	NP	M	l	ZD		
Fo014	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	SP	A	b	M		
Fo019	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	A	b	H	KM065850	KJ672093
Fo021	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	J	k	Z	KM065857	KJ672101
Fo023	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	b	F		
Fo051	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. oxysporum	NP	K	k	W	KM065862	
Fo052	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. oxysporum	NP	K				
Fo053	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. oxysporum	na					
Fo055	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. oxysporum	na					
Fo057	May, 2009	Madagascar	Sambava	Farahalana	-14,44057 ; !	Forest	15	V. planifolia	RoC	F. oxysporum	na					
Fo060	May, 2009	Madagascar	Sambava	Anjombalava	-14,13092 ; !	Forest		V. planifolia	RoC	F. oxysporum	na	A	b	I		
Fo061	May, 2009	Madagascar	Sambava	Anjombalava	-13,99847 ; !	Field		V. planifolia	RoC	F. oxysporum	na					
Fo065	May, 2009	Madagascar	Antalaha	Antalaha	-14,96433 ; !	Field	4	V. planifolia	RoC	F. oxysporum	na					
Fo067	May, 2009	Madagascar	Antalaha	Antalaha	-14,96433 ; !	Field	4	V. planifolia	RoC	F. oxysporum	na					
Fo069	May, 2009	Madagascar	Antalaha	Ambalabe	-15,16741 ; !	Field	4	V. planifolia	RoC	F. oxysporum	MP	F	h	S		
Fo071	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	HP	A	b	D	KM065859	
Fo072	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	HP	A	b	C		
Fo121	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	C	e	H		
Fo122	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	MP	A				
Fo123	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	MP	A	a	A		KJ672104
Fo124	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A				
Fo125	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	H	j	ZA	KM065864	
Fo126	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP					
Fo130	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	C				
Fo131	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na					
Fo132	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	MP	C	e	H	KM065847	
Fo134	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	HP					
Fo139	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	b	I		
Fo142	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	A	g	O	KM065848	KJ672092
Fo144	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	g	O		
Fo145	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	A	f	N		KJ672102
Fo146	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	NP	A	g	N		KJ672103
Fo149	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. oxysporum	MP	C	e	H		
Fo153	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	StC	F. oxysporum	na	A				
Fo154	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo155	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. oxysporum	MP	A				
Fo156	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. oxysporum	SP	A	f	L	KM065851	
Fo157	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	StC	F. oxysporum	MP					
Fo159	April, 2011	Reunion	Saint Philippe	Basse Vallée		Forest	3	V. planifolia	RoC	F. oxysporum	HP					

Code	Date of sampling	Country	Region	District	Coordinates (1)	Plot type	Plot age (years)	Isolation host	Isolation organ (2)	Species	Pathogenicity (3)	All_EF group (4)	EF group (4)	IGS group (5)	Genbank (EF)	Genbank (IGS)
Fo160	April, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		KJ672097
Fo161	April, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	StC	F. oxysporum	NP	A				
Fo162	April, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. oxysporum	HP	A				
Fo164	April, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	Ro	F. oxysporum	SP	C	e	H		KJ672094
Fo165	April, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	RoC	F. oxysporum	HP	C	d	H	KM065865	
Fo166	April, 2011	Reunion	Sainte Rose	Bois Blanc		Field	10	V. planifolia	Ro	F. oxysporum	HP	A	f	J		
Fo167	April, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. oxysporum	MP	B	c	H	KM065853	
Fo168	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	StC	F. oxysporum	HP	A	b	M	KM065867	
Fo171	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest	-	V. planifolia	Ro	F. oxysporum	SP	A				
Fo172	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest	-	V. planifolia	Ro	F. oxysporum	MP					
Fo173	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest	-	V. planifolia	Ro	F. oxysporum	NP	A				
Fo174	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	MP	A	a	A		
Fo175	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	SP	A	a	A	KM065869	
Fo176	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	na					
Fo177	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	na					
Fo179	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	MP	A	b	F		
Fo184	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	Ro	F. oxysporum	NP	F	h	Q		
Fo185	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	Ro	F. oxysporum	NP	F	h	S		
Fo187	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	na	F	h	Q		
Fo188	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	na	F	h	Q	KM065854	KJ672095
Fo189	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. oxysporum	NP	F				
Fo190	March, 2011	Reunion	Saint Philippe	Basse Vallée		Forest	3	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo191	March, 2011	Reunion	Saint Philippe	Basse Vallée		Forest	3	V. planifolia	RoC	F. oxysporum	na	A	a	A	KM065855	KJ672096
Fo192	March, 2011	Reunion	Saint Philippe	Basse Vallée		Forest	3	V. planifolia	RoC	F. oxysporum	MP	A	a	A		
Fo195	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo197	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. oxysporum	HP	A	a	A		
Fo198	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. oxysporum	MP	A	a	A		
Fo199	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. oxysporum	HP	A	a	A		
Fo205	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	Ro	F. oxysporum	na	F	h	S		
Fo206	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	Ro	F. oxysporum	HP	F	h	Q		
Fo207	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	RoC	F. oxysporum	MP					
Fo208	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	RoC	F. oxysporum	MP	F				
Fo210	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	RoC	F. oxysporum	NP	F	h	R		
Fo216	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	Ro	F. oxysporum	SP	C	e	H		KJ672098
Fo217	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	Ro	F. oxysporum	na	C	e	H		
Fo218	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	Ro	F. oxysporum	HP	C				
Fo225	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	RoC	F. oxysporum	na	A	b	F		
Fo226	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	RoC	F. oxysporum	na	A	b	F	KM065856	KJ672099
Fo227	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	RoC	F. oxysporum	MP	A	b	F		
Fo232	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. oxysporum	na	F				
Fo235	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. oxysporum	na	A	b	G		KJ672106

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Fo236	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. oxysporum	NP	A	b	G		
Fo237	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	RoC	F. oxysporum	na	F	h	Q		
Fo241	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. oxysporum	NP	F				
Fo243	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. oxysporum	MP	B				
Fo244	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. oxysporum	SP	B	c	H		
Fo253	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. oxysporum	SP	F				
Fo254	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. oxysporum	HP	F				
Fo256	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. oxysporum	SP	F	h	T		
Fo269	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. oxysporum	SP	C	e	H		
Fo272	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	StC	F. oxysporum	na					
Fo273	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	StC	F. oxysporum	na					
Fo275	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	Ro	F. oxysporum	na	F				
Fo276	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	Ro	F. oxysporum	na	I	j	ZC		
Fo277	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	Ro	F. oxysporum	NP	I	j	ZB	KM065861	
Fo278	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	Ro	F. oxysporum	NP	I	j	ZC		
Fo279	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. oxysporum	na	F	h	P		
Fo280	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. oxysporum	na	F	h	P		
Fo284	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. oxysporum	NP	E				
Fo295	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. oxysporum	MP					
Fo296	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. oxysporum	NP					
Fo297	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. oxysporum	HP	C	e	H		
Fo300	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. oxysporum	MP	C	e	H		
Fo301	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	St	F. oxysporum	NP	A	f	K		
Fo303	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	St	F. oxysporum	SP	A	f	L		
Fo313	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. oxysporum	na	A	b	J		
Fo314	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. oxysporum	na	A	b	I		
Fo321	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. oxysporum	SP	A	b	F		
Fo324	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. oxysporum	na	A	b	I		
Fo340	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. oxysporum	MP	C	e	H		KJ672105
Fo342	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. oxysporum	SP	F	h	U		
Fo352	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	St	F. oxysporum	na	F	i	Y		
Fo353	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	St	F. oxysporum	na	F	i	Y	KM065860	
Fo354	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	St	F. oxysporum	NP	F	i	X		
Fo358	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	Ro	F. oxysporum	SP	F	h	U		
Fo359	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	Ro	F. oxysporum	NP	F	h	U	KM065868	
Fo363	April, 2011	Reunion	Saint Joseph	Matouta	-21,3564833	Forest	12	V. planifolia	Ro	F. oxysporum	NP	A	f	K		KJ672100
Fo364	April, 2011	Reunion	Saint Joseph	Matouta	-21,3564833	Forest	12	V. planifolia	Ro	F. oxysporum	NP	A				
Fo365	April, 2011	Reunion	Saint Joseph	Matouta	-21,3564833	Forest	12	V. planifolia	Ro	F. oxysporum	na	A	f	K		
Fo367	April, 2011	Reunion	Saint Joseph	Matouta	-21,3551167	Forest	12	V. planifolia	RoC	F. oxysporum	na	A	b	I		
Fo372	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. oxysporum	MP	C				
Fo373	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. oxysporum	NP					
Fo375	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. oxysporum	na	A				

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Fo380	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	RoC	F. oxysporum	SP	A				
Fo381	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	St	F. oxysporum	MP	A	a	A		
Fo382	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	St	F. oxysporum	MP	A	a	A		
Fo383	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	St	F. oxysporum	na	A	a	A		
Fo384	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. oxysporum	HP	A	a	A		
Fo385	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. oxysporum	SP	A	a	A	KM065846	
Fo386	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. oxysporum	HP	A	a	A		
Fo387	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	St	F. oxysporum	na	A				
Fo398	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	HP	A	b	C		
Fo399	June, 2006	Indonesia	West Java	Bogor	-6.57750;10	Field		V. planifolia	St	F. oxysporum	SP				KM115171	
Fo400	June 2006	Indonesia	West Nusater	Mataram	-8.40194;11	Field		V. planifolia	St	F. oxysporum	SP					
Fo401	June 2006	Indonesia	Bali	Jembrana	-8.42527;11	Field		V. planifolia	St	F. oxysporum	NP					
Fo402	June 2006	Indonesia	Lampung	Tanggamus	-5.42527;10	Field		V. planifolia	St	F. oxysporum	SP				KM115182	
Fo412	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. oxysporum	na	F	h	V		
Fo413	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. oxysporum	na	F	h	V		
Fo415	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo416	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A				
Fo417	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo418	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo419	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	B		
Fo420	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	B		
Fo421	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo422	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo423	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	na	A	a	A		
Fo424	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. oxysporum	na	A	f	M		
Fo438	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. oxysporum	na					
Fo446	May, 2009	Madagascar	Sambava	Anjombalava	-14,13092 ; !	Forest		V. planifolia	RoC	F. oxysporum	na					
Fo456	May, 2009	Madagascar	Sambava	Nosiarnina	-14,21011 ; !	Field		V. planifolia	RoC	F. oxysporum	na					
Fo459	May, 2009	Madagascar	Andapa	Andapa	-14,64221 ; 4	Forest	5	V. planifolia	RoC	F. oxysporum	na					
Fo470	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. oxysporum	na					
FoP02	Sept,2012	French polyn	Raiatea	Taputapuate	-16,7922 ; -1	Shadehouse	10	V. xtahitensis	RoC	F. oxysporum	HP	A				
FoP03	Sept,2012	French polyn	Raiatea	Uturoa	-16,7277 ; -1	Shadehouse	1	V. xtahitensis	RoC	F. oxysporum	HP	A				
FoP04	Sept,2012	French polyn	Raiatea	Uturoa	-16,7277 ; -1	Shadehouse	1	V. xtahitensis	RoC	F. oxysporum	SP	A				
FoP07	Oct, 2012	French polyn	Raiatea	Taputapuate	-16,8394 ; -1	Shadehouse	9	V. xtahitensis	RoC	F. oxysporum	HP	A				
FoP08	Nov, 2012	French polyn	Raiatea	Taputapuate	-16,8386 ; -1	Shadehouse	6	V. xtahitensis	RoC	F. oxysporum	HP	A				
FoP10	Nov, 2012	French polyn	Raiatea	Taputapuate	-16,8349 ; -1	Shadehouse	9	V. xtahitensis	RoC	F. oxysporum	SP	A				
FoP11	Oct, 2012	French polyn	Raiatea	Tumaraa	-16,7667 ; -1	Shadehouse	9	V. xtahitensis	StC	F. oxysporum	HP					
FoP12	Nov, 2012	French polyn	Raiatea	Taputapuate	-16,8386 ; -1	Shadehouse	6	V. xtahitensis	P	F. oxysporum	HP					
Fp181	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. proliferatu	NP	L	m	ZF	KM065858	
Fp325	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	StC	F. proliferatu	NP	L				
Fp346	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	St	F. proliferatu	NP	L	m	ZG		
Fp347	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	St	F. proliferatu	NP	L	m	ZG		

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Fp348	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	St	F. proliferatu	na	L				
Fs054	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs056	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs058	May, 2009	Madagascar	Sambava	Farahalana	-14,44057 ; !	Forest	15	V. planifolia	RoC	F. solani	na					
Fs059	May, 2009	Madagascar	Sambava	Anjombalava	-14,13092 ; !	Forest		V. planifolia	RoC	F. solani	na					
Fs062	May, 2009	Madagascar	Sambava	Anjombalava	-13,99847 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs064	May, 2009	Madagascar	Sambava	Mahaleotena	-14,51612 ; ‹	Forest		V. planifolia	RoC	F. solani	na					
Fs066	May, 2009	Madagascar	Antalaha	Antalaha	-14,96433 ; !	Field	4	V. planifolia	RoC	F. solani	na					
Fs068	May, 2009	Madagascar	Antalaha	Antalaha	-14,96433 ; !	Field	4	V. planifolia	RoC	F. solani	na					
Fs070	May, 2009	Madagascar	Antalaha	Ambalabe	-15,16741 ; !	Field	4	V. planifolia	RoC	F. solani	na					
Fs186	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	Ro	F. solani	NP	P				
Fs194	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. solani	MP	S	p	ZK		
Fs214	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	RoC	F. solani	NP	U				
Fs222	April, 2011	Reunion	Sainte Rose	Sainte Rose		Field	3	V. planifolia	RoC	F. solani	NP					
Fs229	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. solani	SP	S	p	ZK	KM065852	
Fs230	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. solani	NP	S				
Fs233	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. solani	NP					
Fs240	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. solani	na	S				
Fs245	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. solani	MP	P				
Fs268	April, 2011	Reunion	Saint André	Saint André	-20,9503167	Field	25	V. planifolia	Ro	F. solani	NP	Q	o	ZJ	KM065866	
Fs304	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	RoC	F. solani	NP	T				
Fs316	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. solani	NP	P	n	ZH		
Fs356	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. solani	NP	P	n	ZH	KM065849	
Fs357	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. solani	na	P	n	ZI		
Fs403	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	V				
Fs404	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	V	q	ZL	KM065870	
Fs405	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	V	q	ZL	KM065871	
Fs406	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	V				
Fs407	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na					
Fs408	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na					
Fs409	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	R	r	ZO	KM065872	
Fs410	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	R	r	ZP	KM065873	
Fs411	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	R	r	ZO	KM065874	
Fs428	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	R	r	ZN		
Fs429	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	R	r	ZN		
Fs431	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	V	q	ZM	KM065875	
Fs432	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	V	q	ZM		
Fs433	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na	R	r	ZP		
Fs434	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na					
Fs435	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. solani	na					
Fs436	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs439	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. solani	na					

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Fs442	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs443	May, 2009	Madagascar	Sambava	Farahalana	-14,44057 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs449	May, 2009	Madagascar	Sambava	Anjambalova	-13,99387 ; !	Forest	7	V. planifolia	RoC	F. solani	na					
Fs450	May, 2009	Madagascar	Sambava	Anjambalova	-13,99375 ; !	Forest	4	V. planifolia	RoC	F. solani	na					
Fs451	May, 2009	Madagascar	Sambava	Anjambalova	-13,99375 ; !	Forest	4	V. planifolia	RoC	F. solani	na					
Fs454	May, 2009	Madagascar	Sambava	Marongona	-14,1780167	Field	6	V. planifolia	RoC	F. solani	na					
Fs455	May, 2009	Madagascar	Sambava	Marongona	-14,17832 ; ‹	Forest		V. planifolia	RoC	F. solani	na					
Fs457	May, 2009	Madagascar	Sambava	Andratamarir	-14,35215 ; ‹	Field	14	V. planifolia	RoC	F. solani	na					
Fs458	May, 2009	Madagascar	Sambava	Andratamarir	-14,35383 ; ‹	Forest	5	V. planifolia	RoC	F. solani	na					
Fs461	May, 2009	Madagascar	Antalaha	Ambinagnifał	-14,62219 ; !	Field	10	V. planifolia	RoC	F. solani	na					
Fs465	May, 2009	Madagascar	Antalaha	Ambohitsara	-14,96041 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs466	May, 2009	Madagascar	Antalaha	Ambohitsara	-14,96041 ; !	Field		V. planifolia	RoC	F. solani	na					
Fs469	May, 2009	Madagascar	Andapa	Andapa	-14,64221 ; ‹	Forest	5	V. planifolia	RoC	F. solani	na					
Fs473	May, 2009	Madagascar	Sambava	Anjombalava	-14,13092 ; !	Forest		V. planifolia	RoC	F. solani	na					
Fs474	May, 2009	Madagascar	Sambava	Anjambalova	-13,99847 ; !	Field		V. planifolia	RoC	F. solani	na					
FsP01	Sept,2012	French polyn	Raiatea	Taputapuate	-16,7922 ; -1	Shadehouse	10	V. xtahitensis	RoC	F. solani	SP	R				
FsP05	Sept,2012	French polyn	Raiatea	Uturoa	-16,7277 ; -1	Shadehouse	1	V. xtahitensis	RoC	F. solani	SP	V				
Fx003	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx006	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx011	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx015	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx020	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx022	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx031	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	NP					
Fx035	June, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx127	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx128	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx129	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx133	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx135	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx136	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx137	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx141	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx143	Oct, 2010	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. sp	na					
Fx148	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. sp	NP					
Fx150	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. sp	NP					
Fx151	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. sp	na					
Fx152	April, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	Ro	F. sp	na					
Fx158	April, 2011	Reunion	Saint Philippe	Basse Vallée		Forest	3	V. planifolia	Ro	F. sp	na					
Fx163	April, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	Ro	F. sp	na					
Fx169	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx170	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest	-	V. planifolia	RoC	F. sp	na					

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Fx178	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. sp	na					
Fx180	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	RoC	F. sp	na					
Fx182	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	StC	F. sp	na					
Fx183	March, 2011	Reunion	Saint Philippe	Basse Vallée		Field	-	V. planifolia	StC	F. sp	na					
Fx193	March, 2011	Reunion	Saint Philippe	Basse Vallée		Forest	3	V. planifolia	RoC	F. sp	na					
Fx196	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	RoC	F. sp	na					
Fx201	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	Ro	F. sp	na					
Fx202	March, 2011	Reunion	Sainte Rose	Takamaka		Forest	5	V. planifolia	Ro	F. sp	na					
Fx204	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	Ro	F. sp	na					
Fx209	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	RoC	F. sp	na					
Fx211	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	RoC	F. sp	na					
Fx212	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	8	V. planifolia	RoC	F. sp	na					
Fx213	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	RoC	F. sp	na					
Fx215	March, 2011	Reunion	Sainte Rose	Sainte Rose		Forest	5	V. planifolia	RoC	F. sp	na					
Fx219	April, 2011	Reunion	Sainte Rose	Sainte Rose		Field	3	V. planifolia	RoC	F. sp	na					
Fx220	April, 2011	Reunion	Sainte Rose	Sainte Rose		Field	3	V. planifolia	RoC	F. sp	na					
Fx221	April, 2011	Reunion	Sainte Rose	Sainte Rose		Field	3	V. planifolia	RoC	F. sp	na					
Fx223	April, 2011	Reunion	Sainte Rose	Sainte Rose		Field	3	V. planifolia	RoC	F. sp	na					
Fx224	April, 2011	Reunion	Sainte Rose	Sainte Rose		Field	3	V. planifolia	RoC	F. sp	na					
Fx228	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. sp	na					
Fx231	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. sp	na					
Fx234	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	St	F. sp	na					
Fx238	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	RoC	F. sp	na					
Fx239	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Shadehouse	18	V. planifolia	RoC	F. sp	na					
Fx242	March, 2011	Reunion	Sainte Rose	Piton Sainte Rose		Field	18	V. planifolia	Ro	F. sp	na					
Fx246	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx247	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx248	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx249	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. sp	na					
Fx250	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. sp	na					
Fx251	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. sp	na					
Fx252	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. sp	na					
Fx255	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	Ro	F. sp	na					
Fx257	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx258	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx259	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx260	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx261	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx262	April, 2011	Reunion	Saint André	Saint André	-20,9502333	Shadehouse	15	V. planifolia	RoC	F. sp	na					
Fx263	April, 2011	Reunion	Saint André	Saint André	-20,9503167	Field	25	V. planifolia	Ro	F. sp	na					
Fx264	April, 2011	Reunion	Saint André	Saint André	-20,9503167	Field	25	V. planifolia	Ro	F. sp	na					
Fx265	April, 2011	Reunion	Saint André	Saint André	-20,9503167	Field	25	V. planifolia	Ro	F. sp	na					

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Fx266	April, 2011	Reunion	Saint André	Saint André	-20,9503167	Field	25	V. planifolia	Ro	F. sp	na					
Fx267	April, 2011	Reunion	Saint André	Saint André	-20,9503167	Field	25	V. planifolia	Ro	F. sp	na					
Fx270	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. sp	SP					
Fx271	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. sp	na					
Fx274	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	StC	F. sp	na					
Fx281	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. sp	NP					
Fx282	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. sp	na					
Fx283	April, 2011	Reunion	Saint André	Saint André	-20,9495833	Field	21	V. planifolia	RoC	F. sp	na					
Fx285	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. sp	na					
Fx286	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. sp	na					
Fx287	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	RoC	F. sp	na					
Fx288	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	Ro	F. sp	na					
Fx289	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	Ro	F. sp	na					
Fx291	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	St	F. sp	SP					
Fx292	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	St	F. sp	na					
Fx293	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	St	F. sp	na					
Fx294	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. sp	HP					
Fx298	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. sp	na					
Fx299	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9379667	Field	4	V. planifolia	RoC	F. sp	na					
Fx302	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	St	F. sp	na					
Fx305	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	RoC	F. sp	na					
Fx306	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	RoC	F. sp	na					
Fx307	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	Ro	F. sp	na					
Fx308	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	Ro	F. sp	na					
Fx309	April, 2011	Reunion	Sainte Suzanr	Sainte Suzanr	-20,9373333	Field	20	V. planifolia	Ro	F. sp	na					
Fx310	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx311	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx312	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx315	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx317	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx318	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx319	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx320	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx322	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx323	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx326	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	StC	F. sp	na					
Fx327	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	StC	F. sp	na					
Fx328	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx329	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx330	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx331	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx332	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					

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Fx333	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx334	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx335	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx336	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	NP					
Fx337	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx338	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx339	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx341	April, 2011	Reunion	Saint André	Saint André	-20,9474667	Shadehouse	6	V. planifolia	Ro	F. sp	na					
Fx343	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx344	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx345	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx349	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx350	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx351	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx355	April, 2011	Reunion	Sainte Marie	Sainte Marie	-20,9277667	Shadehouse	6	V. planifolia	RoC	F. sp	na					
Fx360	April, 2011	Reunion	Saint Joseph	Matouta	-21,3564833	Forest	12	V. planifolia	St	F. sp	na					
Fx361	April, 2011	Reunion	Saint Joseph	Matouta	-21,3564833	Forest	12	V. planifolia	St	F. sp	na					
Fx362	April, 2011	Reunion	Saint Joseph	Matouta	-21,3564833	Forest	12	V. planifolia	St	F. sp	na					
Fx366	April, 2011	Reunion	Saint Joseph	Matouta	-21,3551167	Forest	12	V. planifolia	RoC	F. sp	na					
Fx368	April, 2011	Reunion	Saint Joseph	Matouta	-21,3551167	Forest	12	V. planifolia	RoC	F. sp	SP					
Fx369	April, 2011	Reunion	Saint Joseph	Matouta	-21,3551167	Forest	12	V. planifolia	RoC	F. sp	na					
Fx370	April, 2011	Reunion	Saint Joseph	Matouta	-21,3551167	Forest	12	V. planifolia	RoC	F. sp	na					
Fx371	April, 2011	Reunion	Saint Joseph	Matouta	-21,3551167	Forest	12	V. planifolia	RoC	F. sp	na					
Fx374	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. sp	na					
Fx376	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. sp	na					
Fx377	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	StC	F. sp	MP					
Fx378	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	RoC	F. sp	na					
Fx379	April, 2011	Reunion	Saint Philippe	Pointe de la t	-21,33065 ; !	Forest	7	V. planifolia	RoC	F. sp	na					
Fx388	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	St	F. sp	na					
Fx389	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	St	F. sp	na					
Fx390	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	Ro	F. sp	na					
Fx391	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	Ro	F. sp	na					
Fx392	March, 2011	Reunion	Saint Philippe	St Philippe	-21,3642 ; 5!	Forest		V. planifolia	Ro	F. sp	na					
Fx414	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. sp	na					
Fx425	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. sp	na					
Fx426	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. sp	na					
Fx427	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. pompona	RoC	F. sp	na					
Fx430	March, 2013	Reunion	Saint Pierre	Bassin Plat	-21,3213 ; 5!	Shadehouse	5	V. bahiana	RoC	F. sp	na					
Fx437	May, 2009	Madagascar	Sambava	Farahalana	-14,42486 ; !	Field		V. planifolia	RoC	F. sp	na					
Fx445	May, 2009	Madagascar	Sambava	Anjambalova	-14,08579 ; !	Forest		V. planifolia	RoC	F. sp	na					
Fx447	May, 2009	Madagascar	Sambava	Anjombalava	-14,13092 ; !	Forest		V. planifolia	RoC	F. sp	na					
Fx448	May, 2009	Madagascar	Sambava	Anjambalova	-13,99387 ; !	Forest	7	V. planifolia	RoC	F. sp	na					

Code	Date of sampling	Country	Region	District	Coordinates (1)	Plot type	Plot age (years)	Isolation host	Isolation organ (2)	Species	Pathogenicity (3)	All_EF group (4)	EF group (4)	IGS group (5)	Genbank (EF)	Genbank (IGS)
Fx464	May, 2009	Madagascar	Antalaha	Antalaha	-14,96433 ; !	Field	4	V. planifolia	RoC	F. sp	na					
Fx468	May, 2009	Madagascar	Antalaha	Ambalabe	-15,16741 ; !	Field	4	V. planifolia	RoC	F. sp	na					
MIAE011	May, 2009	Madagascar	Antalaha	Ambohitsara	-14,96041 ;	Field	10	V. planifolia	RoC	F. oxysporum	na	F				
MIAE011	May, 2009	Reunion	Saint Pierre	Bassin Plat	-21,3216 ; 5!	Shadehouse	5	V. planifolia	RoC	F. oxysporum	HP	A				

CHAPTER III **Differential responses of vanilla accessions to root and stem rot**

Article in preparation for Frontiers in Plant Sciences

Title: Differential responses of vanilla accessions to root and stem rot and colonization by *Fusarium oxysporum* f. sp. *radicis-vanillae*

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Summary:

Root and stem rot (RSR) disease caused by *Fusarium oxysporum* f. sp. *radicis-vanillae* (Forv), is the most devastative disease of vanilla (*Vanilla planifolia* G. Jackson and *V. xtahitensis* Moore). Breeding programs aimed at developing resistant vanilla varieties are hampered by the scarcity of well characterized sources of resistance to the disease and insufficient knowledge about the histopathology of Forv. In this work we identified i) new sources of resistance to Forv, including *V. planifolia* inbreds, hybrids and wild relatives, ii) confirmed the necrotic non-vascular behavior of Forv in vanilla roots, and iii) evidenced the key role played by lignin deposition onto hypodermis cell walls for resistance to Forv in two highly resistant accessions.

In a first step, we evaluated vanilla accessions maintained in the Biological Resources Centre Vatel in Reunion Island for resistance to RSR. One hundred and twenty five accessions were evaluated in the field under natural conditions of infection, and 103 accessions were tested in controlled condition using *in-vitro* plants root dip inoculated with the highly pathogenic isolate Fo072. For the 26 accessions evaluated in both conditions, a strong correlation was observed between field evaluation and *in-vitro* assay. Sixteen accessions that rated susceptible in the field were susceptible *in-vitro* (Area under disease progress curve (AUDPC) values between 11 and 27), while the seven highly resistant in the field had null AUDPC. Three accessions however showed resistance in the field but collapsed in *in-vitro* test.

Secondly, we monitored by wide field and multiphoton microscopy the root infection process and plant response of one susceptible accession (*V. planifolia* pla0001) and two resistant accessions (*V. planifolia* pla0020 and *V. pompona* pom0018) challenged with Fo072. In pla0001, hyphae penetrated directly into the rhizodermis in the hairy root region as early as

2 dpi, then cortex invasion occurred specifically through the passage cells of hypodermis. At 7 dpi, the intercellular colonization of the cortex was extensive, and induced the plasmolysis of adjacent cells. However, no hyphae were detected in the vascular region until 9 dpi. In the case of the resistant accessions pom0018 and pla0020, the penetration was limited to the hypodermal layer and never extended to cortical cells. Anatomical, histochemical observations and spectral analysis of the hypodermis disclosed the role played by lignin deposition in the resistance to Forv. Indeed, the thickness of lignin constitutively deposited onto outer cell walls of hypodermis was highly correlated with the level of resistance for 21 accessions tested *($r = -0.72$, $pvalue < 0.001$).* In addition, the accumulations of p-coumaric and sinapic acids, which are two phenolic precursors of lignin synthesis, were observed in Fo072 inoculated resistant plants only. Besides the enrichment with lignin, the hypodermis of resistant accessions also showed specific globular cells that entrapped the hyphae preventing its progression towards the cortex.

Altogether, our data broadened the sources of resistance to Forv and enlightened the resistance mechanism at work in resistant genotypes. As such they should enhance the breeding programs aimed at developing novel vanilla varieties comprising RSR resistance.

Keywords: *Lignin, Multiphoton microscopy, Phenolic compounds, Resistance, Vanilla planifolia*

I Introduction

The genus *Vanilla* includes about 110 species of tropical climbing orchids. Among these, two species, *Vanilla planifolia,* and *V.×tahitensis* are cultivated for their aromatic compounds, particularly vanillin, present in high levels in their fruits (Purseglove et al., 1981). Because vanilla has been only propagated clonally by cuttings, the genetic variability of cultivated plants is extremely narrow (Bory et al., 2008b, Lubinsky et al., 2008). This limited diversity hampers the capacity to select genotypes to adapt to abiotic or biotic constraints.

Fusarium oxysporum is a soil-borne fungus found worldwide. It is an anamorphic species that includes both pathogenic and non-pathogenic strains (Gordon & Martyn, 1997). The plant pathogenic forms are highly host-specific and are divided into 150 *formae speciales* based on the host they infect (Bertoldo et al., 2015, Fourie et al., 2009). They infect the host by penetrating the roots causing severe damage and yield losses on many economically important plant species (Fourie et al., 2011, Michielse et al., 2009).

The root and stem rot (RSR) of vanilla is a serious disease caused by *F. oxysporum* f. sp. *radicis-vanillae* (Forv) in all vanilla producing countries (Koyyappurath et al., in press, Tucker, 1927). It results in the browning and death of underground roots, followed by the death of aerial roots. Subsequently the leaves and stem begins to shrivel and eventually the total collapse of the plant occurs. The pathogen is capable of surviving in the soil for tens of years, thereby making the land unsuitable for vanilla cultivation (He, 2007)

Control methods for RSR of vanilla reviewed by Tombe and Liew (2010b) include good agronomic practices, the application of chemical fungicides or essential oils, or the use of biocontrol agents such as non-pathogenic strains of *F. oxysporum* and *Pseudomonas* and *Trichoderma* antagonists. However none of these methods proved to be efficient enough to

restore productivity of vanilla plots. Identifying and using genotypes resistant to *Fusarium* is considered to be the best alternative (Fravel et al., 2003b).

Some degree of resistance is reported in species such as *V. pompona*, *V. phaeantha*, *V. barbellata*, *V. aphylla* and *V. andamanica* (Divakaran et al., 2006a, Knudson, 1950, Theis & Jimenez, 1957a) which have been used to create hybrids resistant to Forv (Theis & Jimenez, 1957a, Delassus, 1963.). Therefore identifying and deploying resistant genotypes is a promising strategy for controlling *RSR* of vanilla.

Plant-pathogen interactions in vanilla are poorly described. Only idioblast differentiation or formation in response to Forv infection and differences in anatomy between genotypes have been previously reported in a vanilla hybrid (Stern & Judd, 1999, Theis & Jimenez, 1957a, Tonnier, 1960a). Understanding the colonization mechanism involved in the *Vanilla-Fusarium* pathosystem and how it changes according to plant genotype would provide insights in selecting and choosing better candidates for breeding strategies.

Plant cell walls are mostly composed of cellulose, hemicelluloses and lignin. Apart from their basic function of providing shape and structure to the plants, they play a major role in disease resistance. Lignin, the second most abundant biopolymer, is composed of highly branched phenylpropanoids resistant to microbial degradation (Bhuiyan et al., 2009, Lobo et al., 2000). Lignification strengthens the cell wall, thereby providing a mechanical barrier against fungal invasion. The lignin deposition restricts the external movement of nutrients from the host, as well as preventing the diffusion of toxic enzymes released by the pathogen to the host (Siranidou et al., 2002).

In this study, we i) evaluated the susceptibility of different vanilla accessions to RSR using a standardized screening method, ii) developed an *in-vitro* test of susceptibility to Forv enabling early selection of resistant accessions, iii) described the colonization pattern of Forv

in selected vanilla accessions and iv) study anatomy and kinetics of cellular events associated to Forv infection by using three accessions with contrasting resistance to the highly pathogenic Forv isolate Fo072. For the first time, *V. planifolia* accessions with high level of resistance to Forv have been identified which opens promising perspective for a better control of RSR by breeding.

II Materials and Methods

II-1 Fungal isolates and vanilla genotypes

The highly pathogenic isolate Fo072 (Koyyappurath et al., communictaed), was used in inoculation experiments. This isolate was selected because of its stability, high pathogenicity and aggressiveness on susceptible vanilla accessions. Fo072 was grown on PDA plates and stored in darkness at 25 ± 2°C. Inoculum was prepared as described in (Koyyappurath et al., communictaed). The mycelia grown on PDA for 7 days were gently scraped to a minimal liquid media and incubated for 5 days at 25°C on a rotary shaker at 125 rpm. The mycelia were then filtered to prepare a conidial suspension adjusted to 10^6 conidia mL^{-1}.

The vanilla materials for the study were provided by the VATEL Biological resources centre (Roux-Cuvelier & Grisoni, 2010) which maintains different species, hybrids and progenies of vanilla in Reunion Island. A total of 254 vanilla accessions cultivated in shade houses or *in-vitro* were selected for the study (Table 1). Field plants were cultivated under a 60% shade-net and supplemented twice a year with compost made of coconut husk, bagasse and sugarcane filter-cake. The *in-vitro* plants were grown in basal MS media without any growth hormone.

II-2 Evaluation of RSR resistance in field conditions

The field performance of vanilla accessions was assessed on three plants per accession, in shade houses naturally infested with Forv. All plants were older than 3 years. Two sets of observations were conducted, in December 2009 and February 2013. In the first set, 128 accessions of vanilla were rated using seven disease parameters linked to plant growth and root symptoms (Table 2). The second set of ratings was done on 174 accessions using an overall disease index comprising three categories, as follows: Null: no RSR symptoms and good vigor, Moderate: intermediate vigor and moderate root proliferation and necrosis, Severe: plant severely affected by RSR showing vine decay and limited growth.

II-3 In-vitro plant inoculation and assay

To infect the vanilla plantlets with Forv, the root-dip inoculation method (Koyyappurath et al., communictaed) was used. The method consists of dipping the roots of *in-vitro* grown plantlets in the conidial suspension (10^6 conidia/mL) of Fo072 for 5 minutes. The control plants were dipped in sterile distilled water. For susceptibility assessment, the inoculated plantlets were transferred to plastic plots containing sterile coco fiber and bagasse as substrate and incubated in a growth chamber at 24-26°C with a light density of 120- µE/m2/s, relative humidity of 80-90 %, with a photoperiod of 12 hours. Symptoms of disease were recorded every 2 days (Koyyappurath et al., communictaed) and accession susceptibility was derived from the AUDPC value. For histo-pathological imaging, the inoculated plantlets were incubated in sterile tubes at 25°C for a 12-hour photoperiod prior to tissue preparation.

II-4 Wide field microscopy

Conventional wide field microscopy (WFM) was used to study the anatomy of vanilla accessions and the colonization pattern of pathogenic fungi. The Forv-inoculated vanilla roots were examined at 0 to 9 days post inoculation (dpi). Root tissues excised from control and

inoculated plants were fixed for 48 hours with 4 % paraformaldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were dehydrated through a graded alcohol series (50°, 70° and 90°) and impregnated in methyl methacrylate, LKB historesin and polymerization was performed at 37°C for 24 hours. 3 µm thin sections were obtained using a microtome (Leica, Germany) and were double stained with Schiff reagent and Naphthol Blue-Black stain (Schiff-NBB). The fixed slides were then viewed under a Leica DM6000 epifluorescent microscope (Leica Microsystems, Germany, objectives Leica 20x HC PL APO 0.7 and 40x) and images were acquired using Retiga 2000R camera (QImaging, Canada) and processed with Volocity software (Perkin-Elmer, UK).

For the anatomical studies of vanilla accessions, the roots from control (non-inoculated) plants were collected and sectioned (90 µm) using a HM650 V vibrating blade vibratome (Thermo Scientific, Walldorf, Germany). The sections were then stained with 2% phloroglucinol (in ethanol) for 5 minutes. The sections were then transferred to a clean glass slide. A drop of 18% HCl was applied and a cover slip was mounted immediately. The slides were then observed under the Leica DM6000 epifluorescent microscope. Outer epidermal cell thickness, hypodermis thickness and radial thickening of the hypodermal cells were measured at 10 different points of each root section using Image J 1.47v software (NIH, USA).

II-5 Multi-photon confocal microscopic analysis

The inoculated *in-vitro* plants were examined for Forv infection and colonization at 2, 4, 7 and 9 dpi. Infected roots were carefully taken out of the tubes and thin sections (90 µm) were obtained using a vibrating blade microtome as previously mentioned. These sections were placed in 10 mM Phosphate buffer saline (PBS) and then stained with DAPI 300 nM (4', 6-diamidino-2-phenylindole) for 5 minutes in the dark. DAPI, classical DNA dye, also stain polyphosphates and emitted a yellow fluorescence. The stained cells were then washed twice

in PBS. The sections were mounted on a glass slide and observed using a Zeiss LSM780 multiphoton microscope (Zeiss, Germany), equipped with a Chameleon Ultra II laser (Coherent, California). With multiphoton microscope, the optimal excitation wavelength for DAPI is 720 nm and the filter blocks, with differential spectral properties, were set to those of DAPI (415-480 nm and 550-610 nm) and chlorophyll (660-700 nm). Images acquisition was performed using Zen software (Zeiss, Germany). The acquired image channels were merged and processed using Image J 1.47v software.

II-6 Emissions spectral analysis

The multiphoton microscope with a Chameleon Ultra II tuneable laser (690–1080 nm range excitation, Coherent, Santa Clara, CA, USA) enables the excitation of secondary metabolites in a manner similar to a UV laser (Conéjéro et al., 2014, Talamond et al., 2015). Optimal excitation was obtained at λ = 720 nm and band-pass emission in the 410–650 nm range using an array of 32 photomultiplier tube (PMT) detectors (Zeiss), each with an 8.8 nm bandwidth.

This spectral detector yields spectral images and emission spectra from the epidermal and hypodermal walls of fresh root sections of Fo072 inoculated or control vanilla accessions. After obtaining the spectral acquisitions, the Linear Unmixing (ZEN software, Zeiss, Jena, Germany) function was executed to separate, pixel by pixel, the mixed signals of six defined pure autofluorescent compounds such as ferulic acid, conyferylic acid, sinapinic acid, p-coumaric acid, caffeic acid and quinic acid (Sigma-Aldrich, St Quentin Fallavier, France), using the entire emission spectrum of each compound plus a residual channel. This image analysis shows each compound present in the sample with coded colors. In the residual channel, the intensity values represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra.

II-7 Data analysis

All statistical analyses were performed with the R statistical software (R-CoreTeam, 2015). The AUDPC was calculated using the "*agricolae*" package (Mendiburu, 2014). Hierarchical classification of accessions based on their AUDPC values was done by recursive partitioning and complexity optimization using the "*rpart*" package (Therneau et al., 2014). The field ratings dataset for plant development and symptom expression was analyzed with Multiple Correspondence Analysis (MCA) using the dudi.acm function in *ade4* R package (Dray & Dufour, 2007).

III Results

III-1 Field evaluation of accessions

The vanilla accessions cultivated in shade house collections showed contrasting performances regarding vegetative growth and RSR (Figure 1). However, for each accession, the behavior of the three vines was identical. The presence of naturally occurring Forv in the shade houses was confirmed by fungus isolation on plants showing RSR. This suggested that the variations in plant development could be related to differences in genetic susceptibility or resistance to Forv. The first axis of MCA analysis (Supplementary Figure 1) clearly segregated the plants according to vine development (PS, SN, SD and PV) and proportion of dried roots (DR), the most affected plants being on the right side of the 2D plot. The second axis showed total number of roots (NR). The 19 accessions of *V. pompona*, a species known to be resistant to RSR, and the six *V. bahiana* accessions, a sister species of *V. phaeantha,* another RSR-resistant species, were located on the extreme left of axis 1, as well as *V. costaricensis, V. crenulata* and the two hybrids *V. planifolia* X *V. pompona* and *V. planifolia* X *V. phaeantha*, (Figure 2B). In contrast, accessions of *V. ×tahitensis* , *V. odorata*, the hybrid *V. planifolia* X *V. ×tahitensis,* along with most of the *V. planifolia* accessions were located on

the right side of the 2D plot, showing a low level of Forv resistance. Interestingly, a few *V. planifolia* accessions, such as pla0020, pla0038 or pla0240 were located on the left side, and showed development similar to the resistant accessions. The horseshoe distribution of individuals in the 2D plot (Guttman effect) results from the fact that resistant genotypes as well as the very susceptible accessions both produced a reduced number of roots compared to moderately susceptible accessions. Roots remained functional in resistant accessions while in very susceptible accessions the disease strongly limited growth and ability to produce new roots. Conversely, the moderately susceptible accessions had slightly reduced development and were capable of producing numerous new roots in response to root rot.

Since the six main MCA variables were highly correlated along the first axis a global RSR rating with 3 levels was used in the subsequent field evaluation of the accessions. The second set of ratings performed on 174 accessions was congruent with the first one for the 125 accessions that were rated twice in the field (Supplementary Figure 2).

III-2 In-vitro germplasm screening for resistance to *Forv*

One hundred and three accessions of vanilla, (79 *V. planifolia*, 15 accessions from 11 *Vanilla* species and 9 from interspecific hybrids) were inoculated with Fo072 using the root-dip inoculation method. The disease symptoms started to appear on the susceptible accessions on the 4th dpi. Disease severity ratings were continued until 15 dpi, when most of the susceptible accessions reached total collapse (Figure 3 A, B). No symptoms were observed on control plants. The accessions had a wide range of survival rates against the fungus with AUDPC values ranging from 0 to 30 (Figure 3C). The hierarchical clustering analysis split the accessions into five distinct classes based on the average AUDPC values, namely highly susceptible (HS), susceptible (S), moderately susceptible (MS), slightly resistant (SR), and resistant (R). Among the 79 accessions tested, 19 occupied the R class with least or no

symptoms and an AUDPC average score lesser than 7. The plants in this class comprise of eight *V. planifolia* accessions, four *V. bahiana* accessions, four other species (*V. costaricensis, V. crenulata, V. phalaenopsis* and *V. pompona*) and three interspecific hybrids including *V. pompona* and *V. phaeantha* as a parent. At the other extreme, the HS class was occupied by *V. planifolia* and *V. ×tahitensis* accessions plus a hybrid of these two species; the AUDPC score ranged from 24.5 to 29. The intermediate classes (SR, MS and S) contained accessions with an AUDPC score ranging from 10.5 to 24.

III-3 Correlations between field and *in-vitro* evaluations of RSR resistance

Several accessions evaluated by AUDPC after root *in-vitro* inoculation were also assessed for growth and symptoms after 3 to 9 years' cultivation in a natural environment. A strong correlation was observed between AUDPC values and the disease index for the 19 and 26 accessions evaluated in the field in 2009 and 2013, respectively (Figures 4A and 4B). All the accessions exhibiting resistance *in-vitro* were resistant in the field and all the accessions that showed moderate to severe RSR in the field were classified as susceptible in *in-vitro* tests. However, a discrepancy was observed for three accessions that performed well in the field but were susceptible to Fo072 in repeated *in-vitro* tests (pla0038, ins0087 and mad0142). Nevertheless, as a whole, our results demonstrated the wide variability of the *Fusarium* – vanilla pathogen interaction and for the first time demonstrated resistance within the *V. planifolia* species to Forv.

Three different accessions were selected from the two extreme classes of resistance for further histological studies: One highly susceptible *V. planifolia* accession (pla0001) and two resistant assessions, *V. planifolia* (pla0020) and *V. pompona* (pom0018).

III-4 Colonization of the root surface

Two dpi with Fo072, the growth of hyphae on the surface of roots became visible and had expanded to the whole plant by the 8th dpi. On the susceptible accession (pla0001) the hyphal network was more prominent and induced severe rot (Figure 5) while hyphae development was generally less on resistant accessions.

III-5 Colonization in the root tissues

Schiff-NBB and DAPI-stained longitudinal and transversal root sections of inoculated plants were observed in WFM and under multiphoton microscopy, respectively. On the susceptible accession pla0001 the germination of conidia predominantly occurred at the hairy zone of the root at 2 dpi. The hyphae were attached to root hairs and epidermal cells. From 3 to 4 dpi, abundant mycelia networks had developed on the root surface mainly interwoven with the root hairs, and had begun to invade the epidermis (Figure 6A). The hyphae penetrated directly into the epidermal cells and formed a coiled structure (Figure 6B, C and D). Then they proceeded through the hypodermis specifically across passage cells and reached the cortex (Figure 6B). At 7 dpi, the hyphae invaded the cortex through the intercellular spaces (Figure 7A and B) and induced plasmolysis of the adjacent cortical cells (Figure C and D). This disruption of the cortical area concurred with the softening of the root tissue during infection. At 9 dpi no hyphae had reached the vascular cylinder (Figure 7C).

On resistant accessions pla0020 and pom0018, conidia germination and hyphal development was reduced (Fig 8 A) compared to pla00001 (Figure 8 B). A few hyphae reached the rhizodermis but were restricted to the hypodermal cells. These colonized cells were modified into globular structures that entrapped the hyphae (Figure 9 A, B and C). Additionally, an increased staining of the hypodermal cell wall was observed (Figure 9D)

suggesting the formation of a polysaccharide layer in response to fungus penetration. No such modifications were observed in pla0001.

III-6 Anatomical differences between susceptible and resistant cultivars

In order to further investigate the role of anatomical structures in plant defense, peripheral layer staining (FASGA, phloroglucinol and berberine hemisulfate) and cell wall spectral analysis were carried out on infected and non-infected roots.

III-6.a Wide field microscopy

The rhizodermis of all genotypes is unilayered, with single celled root hairs, a common feature in most monocots. The cells forming the rhizodermis varied in shape between the three accessions. They were rounded and bigger in the susceptible pla0001 compared to the resistant pla0020 and pom0018 in which they were flattened and thinner (Figures 10A, B and C). However, in an *in-vitro* comparison of a subset of 21 accessions ranging from resistant to highly susceptible, no significant correlation was observed between the size of epidermal cells and the resistance level of the accessions evaluated by AUDPC ($r= 0.24$, $N= 21$, $P = 0.293$). This indicated that size of epidermal cells is not a factor involved in resistance to Forv.

The hypodermis layer comprised uniseriate polygonal cells containing lignin thickenings on the outer longitudinal walls as shown by phloroglucinol staining (Figure 10D, E and F). However the lignin staining revealed that the accessions had different types of hypodermis cell walls. In susceptible accessions (pla0001), the presence of lignin was discontinuous or very thin, while resistant accessions (pom0018 and pla0020) showed a strong and continuous lignin thickening on the outer walls of hypodermis. In addition, a radial thickening was present in resistant accessions but not in susceptible genotype (pla0001) (Figures 10 E and F). Phloroglucinol staining confirmed the higher lignin content on the hypodermal cell walls of resistant compared to susceptible accessions (Figures 10 G and H).

The thickness of lignin deposition and presence of radial thickening was assessed on the same subset of 21 accessions of differing Forv resistance. A significant correlation coefficient was calculated between the hypodermal cell wall thickness and the mean AUDPC values (r = -0.72, N=21, P= 0.00024), and the presence of radial thickening was significantly related to AUDPC classes (P=0.00012 in Fisher's exact test for count data). High lignin thickening on the outer wall was consistently associated with radial thickening in all resistant accessions, except for cha0666 which was evaluated as moderately resistant (AUDPC=10).

Lignin deposition was also observed in field-grown susceptible accession pla0001 and resistant accessions pla0020 and bah0086 using phloroglucinol staining. The results were consistent with *in-vitro* observations (Figure 11). These results confirm the role of hypodermal lignin deposition on cell wall for resistance of vanilla to Forv.

III-6.b Emission spectral analysis

Spectral analyses of the rhizodermal and hypodermal layers of the roots of susceptible and resistant (pom0018) accessions, both Fo72 inoculated and controls, were compared with reference spectra of four lignin precursors (ferulic acid, conyferylic acid, sinapinic acid, p-coumaric acid) and two others phenolic compounds (caffeic acid and quinic acid). On non-inoculated roots similar images were obtained for the six channels except conyferylic acid which was slightly higher in the susceptible accession. Precursor contents were higher overall in resistant accessions compared to the susceptible accession, particularly in the hypodermal cell walls (Figures 12 A and B).

On the other hand, infected root samples of susceptible and resistant accessions displayed very divergent unmixed spectra. In the susceptible accession, the majority of the precursors were no longer present at 7 dpi, except sinapinic and p-coumaric acids which remained slightly visible on the hypodermis (Figures 12 A and C). For the resistant accession,

the p-coumaric and conyferylic acid impregnations were notably enhanced in the rhizodermal and hypodermal layers as well as in the cortex (Figures 12 B and D), and not only in the walls but also in the cells for sinapic and p-coumaric acids.

IV Discussion

RSR is a major challenge for vanilla cultivation worldwide. The lack of knowledge about Forv-vanilla interactions and the limited genetic resources available have hampered the development of resistant varieties which is acknowledged as the best means for controlling *Fusarium* diseases. In this study, using BRC Vatel's diverse collection of well-characterized vanilla accessions and powerful image acquisition and analysis technology we thoroughly documented three aspects of host-pathogen interactions. First of all, the penetration and invasion route of the fungus into the plant was precisely described as well as subsequent plant responses. Secondly, several anatomical structures involved in RSR resistance were unveiled in two *Vanilla* accessions including, for the first time, *V. planifolia* species. Finally, more than one hundred vanilla accessions were assessed for Forv resistance, and a simple, quick and reliable assay was developed which will enhance Forv resistance evaluation of novel genetic resources.

IV-1 Forv infection and colonization pattern

To date, there is only a single published work describing the penetration process and colonization pattern of a fungus in vanilla plants (Alconero, 1968b). Our histological studies outlined the route and time frames of *Fusarium* penetration and colonization in vanilla roots. In our experimental conditions, the germination of conidia and subsequent infection was triggered as early as 2 dpi. Similar to *F. oxysporum* f. sp. *radicis-cucemernum* and *F. oxysporum* f. sp. *radicis-lycopersici* (Cohen et al., 2014, Lagopodi et al., 2002) the primary infection site of Forv was the hairy root region and the fungus penetrated directly through the

rhizodermis in the absence of cell wounds. This was in disagreement with previous report from Alconero (1968b) who described the penetration of *Fusarium* through the root apex of vanilla plants and concluded from field and laboratory observations that penetration was mainly through wounds caused by insects and nematodes. The hypodermis passage cells regulate the apoplastic entry of water and other solutes to the cortex. These passage cells also act as the entry point for fungi (pathogenic or mycorrhiza) to access the cortical region of the root (Esnault et al., 1994, Kamula et al., 1994, Sharda & Koide, 2008, Chomicki et al., 2014). This was confirmed in the case of Forv in vanilla where the pathogen always entered the cortex through the passage cells as observed by Alconero (1968b).

Pathogenicity is due to the destruction of cortical cells of the hairy area of the root. Contrary to vascular *formae speciales* (Li et al., 2011a, Araujo et al., 2014, Ndambi et al., 2012) the fungus had not reached the root's vascular system by 9 dpi, confirming the non-vascular status of Forv. The infected root is therefore capable of reemitting a functional root upstream of the infected area of the root, thus enabling root regeneration and plant survival for months in the field. Our study focused on root infections. However Forv also infects stem tissues in the field (Pinaria et al., 2010b). In a few instances we observed fungal penetration in the stem occurring at the root-stem junction after external growth of hyphae along aerial roots (data not shown). This second entry point needs to be better described in order to study further the RSR epidemiology.

IV-2 Cellular and histochemical mechanisms in Forv resistance

In resistant accessions pla0020 and pom0018, penetration occured less frequently than in the susceptible accession, and invasion was mostly limited to the hypodermal cells. This minor infection caused no damage to the root system, leaving the plant unharmed.

Morphological, histochemical and physiological variations in the hypodermal cells could explain the resistance of these accessions.

Firstly, in the non-inoculated plants, the neat correlations between hypodermal cell wall thickness and AUDPC scores confirmed the role of the hypodermis in disease resistance. The hypodermal wall thickness of resistant accessions was thicker (5 µm) than in susceptible accessions (1 to 2 µm). The visualization of lignin by phloroglucinol staining and of lignin precursors by spectral analysis confirms the continuity of the hypodermal wall on all sides of the cell in resistant genotypes. It is assumed that lignified cell walls are less susceptible to degradation than cellulose cell walls thereby hampering hyphal penetration and colonization of the intercellular spaces. The role of sub-epidermal cell thickenings in the prevention of *Pseudocercosporella* attack have been described in wheat (Murray & Bruehl, 1983). It is possible however that this constitutive feature preventing the intercellular movement of fungus by peripheral and radial lignification of cell walls could be enhanced by the pathogen as suggested by images of root sections from field plants.

Secondly, the accumulation of phenolic compounds forming a barrier in the hypodermal region and thereby preventing the pathogen from colonizing the cortical region have been reported in many plants (Brammall & Higgins, 1988, Fang et al., 2012, Olivain & Alabouvette, 1997a, Tessier et al., 1990). Such a mechanism is likely to occur in vanilla as evidenced by histochemical root studies that showed thickening specifically in the hypodermal cell wall of inoculated resistant genotypes. Results of spectral analysis showed differences in peripheral cell wall composition in pre- and post-infection stages. The higher concentration of sinapic acid in resistant genotypes, closely related to phloroglucinol stain, suggest the important role of S-lignin in these walls. An increase in the level of p-coumaric and conyferilic acid in resistant genotypes were observed after pathogen infection. Similar mechanisms were observed in many hosts, where the *Fusarium* elicitors enhance the

accumulation of phenolic compounds like ferulic, p-coumaric, caffeic, salicylic, sinapic and vanillic acids in cell walls (Ascensao & Dubery, 2003, Mandal & Mitra, 2007, Voxeur et al., 2015). The esterification of these compounds in the cell walls forms a physical barrier against fungal penetration and enzymes responsible for damaging cell walls. Thus the increased level of lignin precursors may be implicated in quick resistance responses in resistant accessions (Cvikrová et al., 1993, Hammerschmidt & Kuc, 1982, El Modafar & El Boustani, 2001, Zeitoun et al., 2014, Ascensao & Dubery, 2003).

Thirdly, specific cellular entrapment of hyphae on the hypodermis was noted on resistant vanilla accessions, suggesting a defensive role of hypodermal cells. Cellular reactions in the hypodermis with the formation of wall inclusions and appearance of sensitive cells in response to the hyphae have been previously reported (Beswetherick & Bishop, 1993, Olivain & Alabouvette, 1999). The entrapment of hyphae in the hypodermis serves as a barrier, limiting the extension of hyphae to the cortical region.

Finally, conidial germination and hyphae development were notably greater on the roots of susceptible accessions compared to the resistant accession. This could be related to the high ratio of antifungal phenolic compounds like coumaric, cinnamic, ferulic, salicylic and sinamic acids evidences in resistant roots by spectral analysis. Indeed, the role of phenolic root exudates in limiting conidial germination was demonstrated *in-vitro* for a number of plant species (Ling et al., 2013, Liu et al., 2009, Singh et al., 2010, Steinkellner et al., 2005, Wu et al., 2008). As a whole several mechanisms were identified that could reduce root infection of resistant vanilla plants. These mechanisms are probably governed by distinct gene families resulting in a multigenic resistance pattern as suggested by the graduated levels of resistance found in the vanilla germplasm evaluated in this study.

IV-3 Screening for resistance to Forv in Vanilla

An unprecedented range of *Vanilla* genotypes has been assessed for RSR resistance in long term assays in the field and in controlled *in-vitro* conditions. The 254 accessions tested belonged to 18 species and six hybrids. There was a good congruency between field and *in-vitro* evaluation results except for three accessions. This is the case for pla0038 which has been grown successfully in the field for more than 10 years despite being graded as very susceptible in *in-vitro* assays. This discrepancy could result from the presence of favorable microflora in the compost providing protection of the root system, as shown in some soils (Alabouvette et al., 2009). Nonetheless, it seems very unlikely that this protective compost occurs in only three adjacent locations where pla0038 is cultivated. Another possibility is that pla0038 harbors some kind of protective endophyte (Ordonez et al., 2013, White et al., 2014) and (Khoyratty et al., in press) and that this protective endophyte had been eliminated from *in-vitro* plants during tissue culture. Checking lignin impregnation of hypodermal cell walls as well as field trial in different soil conditions could bring additional information about the status of this questionable accession.

RSR resistance assays confirmed the high resistance level of *V. pompona* as well as the resistance of eight hybrids of *V. planifolia* with *V. pompona* and *V. phaeanta* (Tucker, 1927, Delassus, 1963., Tonnier, 1960a). They also revealed novel sources of RSR resistance such as *V. bahiana,* a close relative to the resistant species *V. phaeanta, V. costariciensis* and *V. crenulata*. Most of the *V. planifolia* accessions, *V. ×tahitensis* and *V. odorata* were susceptible to RSR. Interestingly, three accessions obtained from autopollination of an ordinary *V. planifolia* (including pla0020) revealed a high level of resistance to Fo072. This intraspecific diversity in Forv resistance might result from the high level of heterozygosis of *V. planifolia* (Soto Arenas, 1999, Bory et al., 2008b). It demonstrated that a breeding strategy based on *V. planifolia* or *V. ×tahitensis* selfing could be efficient for obtaining progenies

gathering both resistances to Forv and true to type aromatic profile of the fruits. Indeed, this last characteristic is generally lost in interspecific hybrids (Theis & Jimenez, 1957a, Belanger & Havkin-Frenkel, 2010). This new strategy using selfed progenies, combined with the efficient *in-vitro* Forv resistance assay developed in this study could help create new vanilla varieties much needed by the vanilla industry. As a matter of proof the *V. planifolia* pla0020 which showed high level of resistance, good productivity and a Bourbon-type aroma is a promising variety for the vanilla industry. Furthermore, our results showed Forv to be typical necrotic form in which complex and probably multiple mechanisms are at work during vanilla root infection. The knowledge regarding the territory and proceedings of vanilla responses to Forv infection combined with new genomics tools will enable to investigate the genetics behind the resistance to Forv in vanilla genotypes.

V Acknowledgements

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VII Tables

Table 1: Number of *Vanilla* accessions evaluated for *Forv* resistance (Fo072) under shade house in 2009 (Field1)= and 2013 (Field2) and by *in-vitro* inoculation test (AUDPC).

Type of material	Code	Section	Field1	Field2	AUDPC	Total
Species						
V. bahiana	bah	Xanatha	6	8	4	11
V. chamissonis	cha	Xanatha	-	2	1	2
V. costaricensis	cos	Xanatha	1	1	1	1
V. cribbiana	cri	Xanatha	-	5	-	5
V. imperialis	imp	Xanatha	-	2	-	2
V. insignis	ins	Xanatha	-	1	1	1
V. lindmaniana	lin	Xanatha	1	1	-	1
V. odorata	odo	Xanatha	2	2	-	3
V. planifolia	pla	Xanatha	90	109	79	174
V. pompona	pom	Xanatha	19	25	1	25
V. x tahitensis	tah	Xanatha	1	1	1	1
V. africana	afr	Tethya	-	2	-	2
V. crenulata	cre	Tethya	4	4	1	4
V. humblotii	hum	Tethya	-	2	1	2
V. madagascariensis	mad	Tethya	-	3	1	3
V. phalaenopsis	pha	Tethya	-	1	1	2
V. roscheri	ros	Tethya	-	-	1	1
V. sp.	sp.	Tethya	-	1	1	2
hybrids						
Hyb. pla X pom	hyb	Xanatha	1	1	4	5
Hyb. ins X bah	hyb	Xanatha	-	-	2	2
Hyb. pla X phae[1]	hyb	Xanatha	2	2	1	2
Hyb. pom X plan	hyb	Xanatha	-	-	1	1
Hyb. plaXtah	hyb	Xanatha	1	1	-	1
Hyb.[(plaXpom) X pla] X [(plaXpom) X pla]	hyb	Xanatha	-	-	1	1
Total			**128**	**174**	**103**	**254**

Table 2 : Growth and disease parameters used for rating *Forv* resistance in the field

Code: Descriptor	**level**	**Significance / range**
PS : Plant size	**0**	dead
	1	small (< 0,3 m²)
	2	medium (~1 m²)
	3	big (> 1 m²)
SN : number of stems of the plant	**0**	1 to 4
	1	5 to 9
	2	10 to 19
	3	20 to 49
	4	> 50
SD: Percentage of groved stem and decaying leaves	**0**	> 60%
	1	40% to 60%
	2	20% to 40%
	3	5% to 20 %
	4	<5%
PV: Vigor of the plant (size and number of new shoots)	**0**	dead
	1	low
	2	medium
	3	hight
NR : Number of roots descending along the stalk	**0**	*< 3*
	1	*4 to 9*
	2	*10 to 19*
	3	20 to 39
	4	*> 40*
DR: proportion of dryied roots among the descending roots.	**0**	very few
	1	less than half
	2	about half
	3	more than half
	4	almost all
AR: Number of aerial roots	**0**	*< 2*
	1	*2 to 4*
	2	*5 to 9*
	3	>9

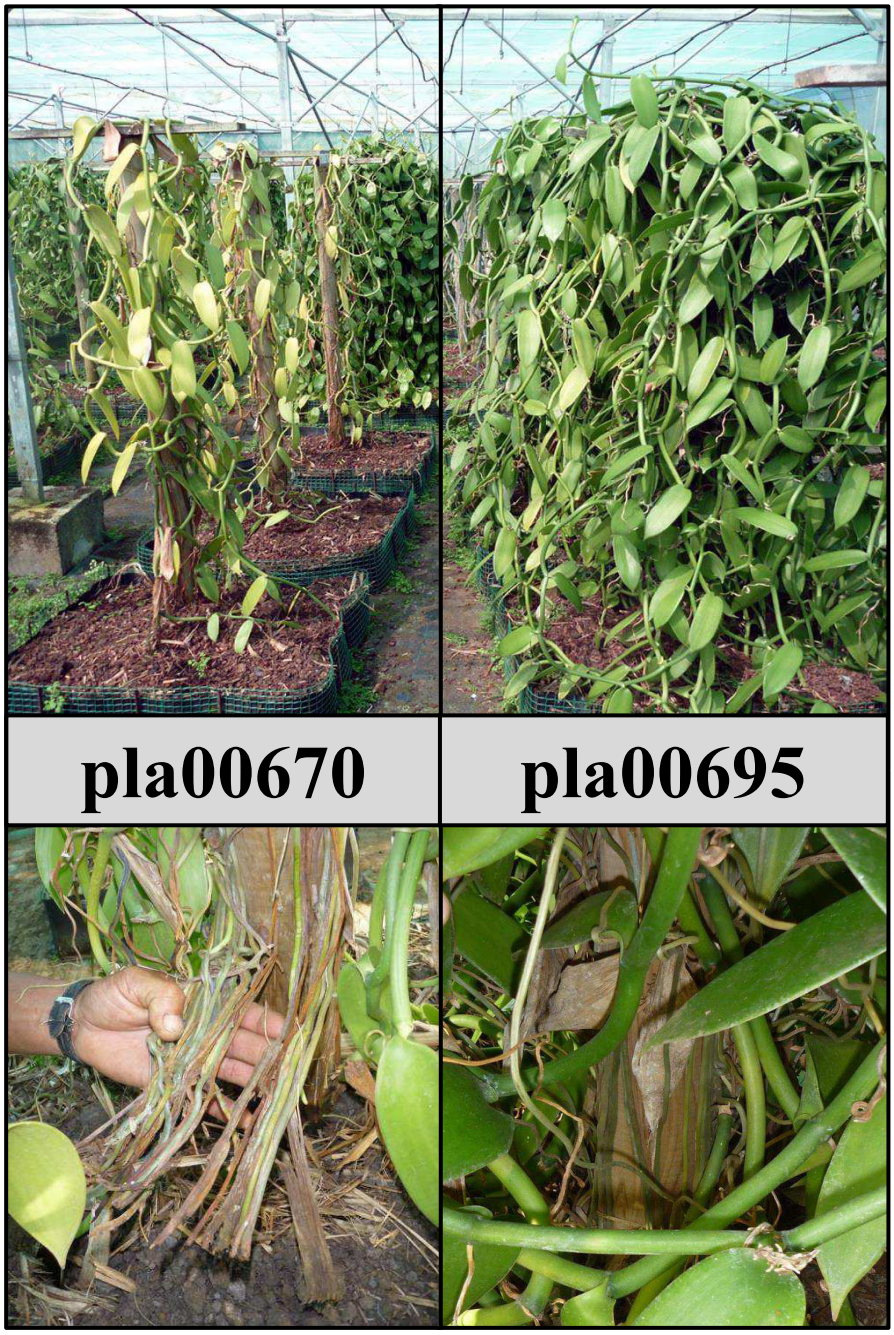


Fig. 1: Two *Vanilla planifolia* accessions showing contrasted development in the shade house 4 years after planting. On the left pla0670 showing reduced vegetative growth, decaying stem and leaves and abundant descending roots that die on ground, on the right pla0695 showing good growth and limited root symptoms.

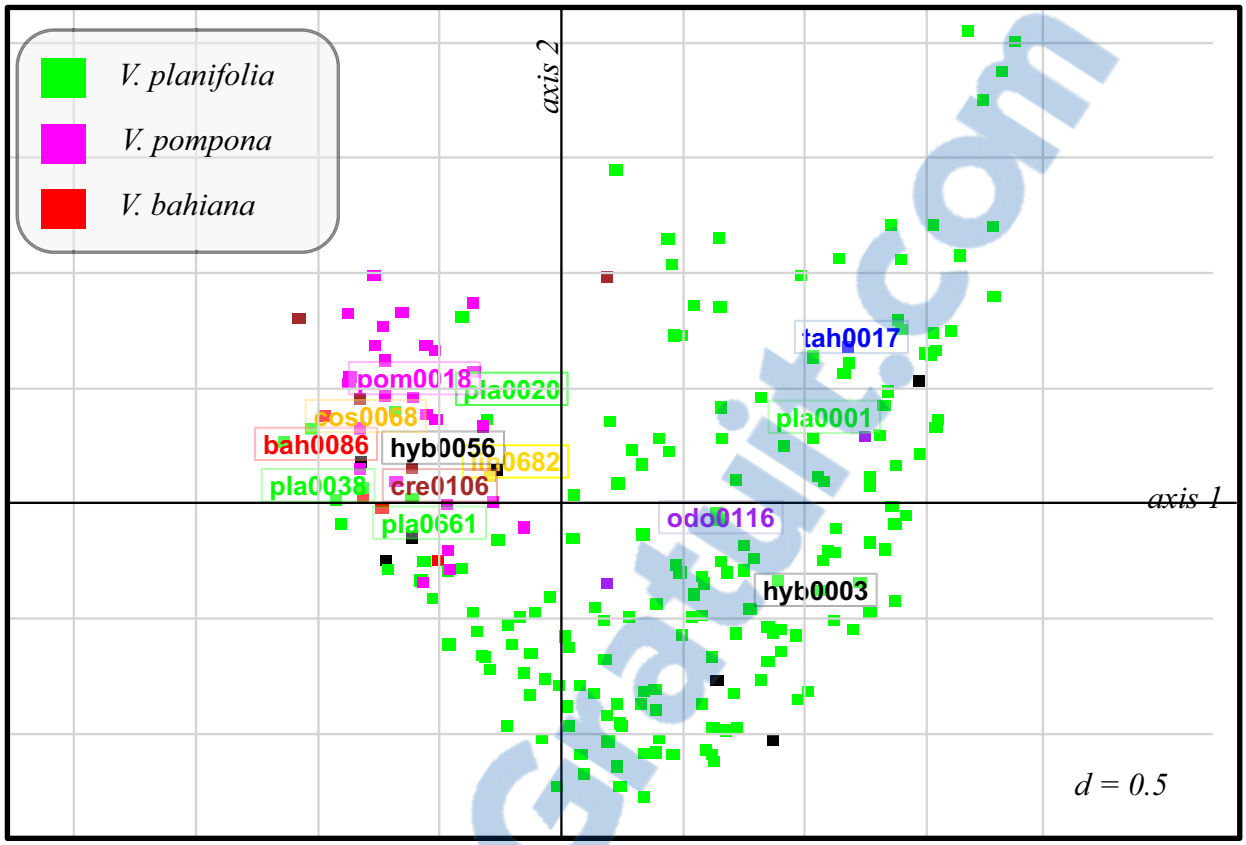


Figure 2 : Multiple component analysis of 128 vanilla accessions rated for RSR symptoms in the field in 2009 using seven variables (see supplementary figure 1). Axis 1 segregates accessions according to their resistance level. Remarkable susceptible (on the right) and resistant (on the left) accessions are indicated. RSR index of accessions was defined as their coordinate on axis 1.

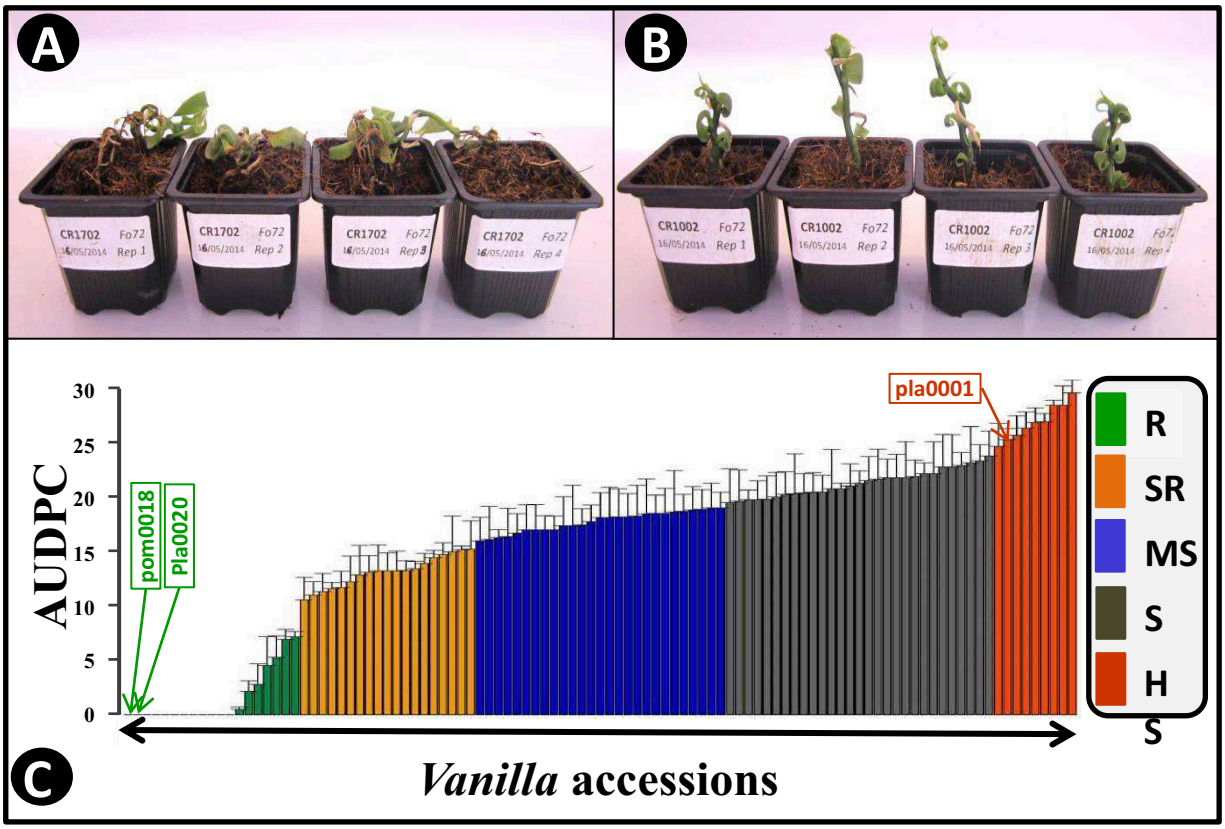


Figure 3: Evaluation 103 *Vanilla* accessions for resistance to Fo72 by the *in-vitro* plantlet assay. A, B) aspect of susceptible and resistant accessions, respectively, 15 dpi. C) The 103 accessions split into 5 classes according to average AUDPC values (4 plants per accession): R=resistant, SR= slightly resistant, MS = moderately susceptible, S= susceptible and HS= highly susceptible. The position of the three accessions selected for the histopathological studies is highlighted (pla0001, pla0020 and pom0018).

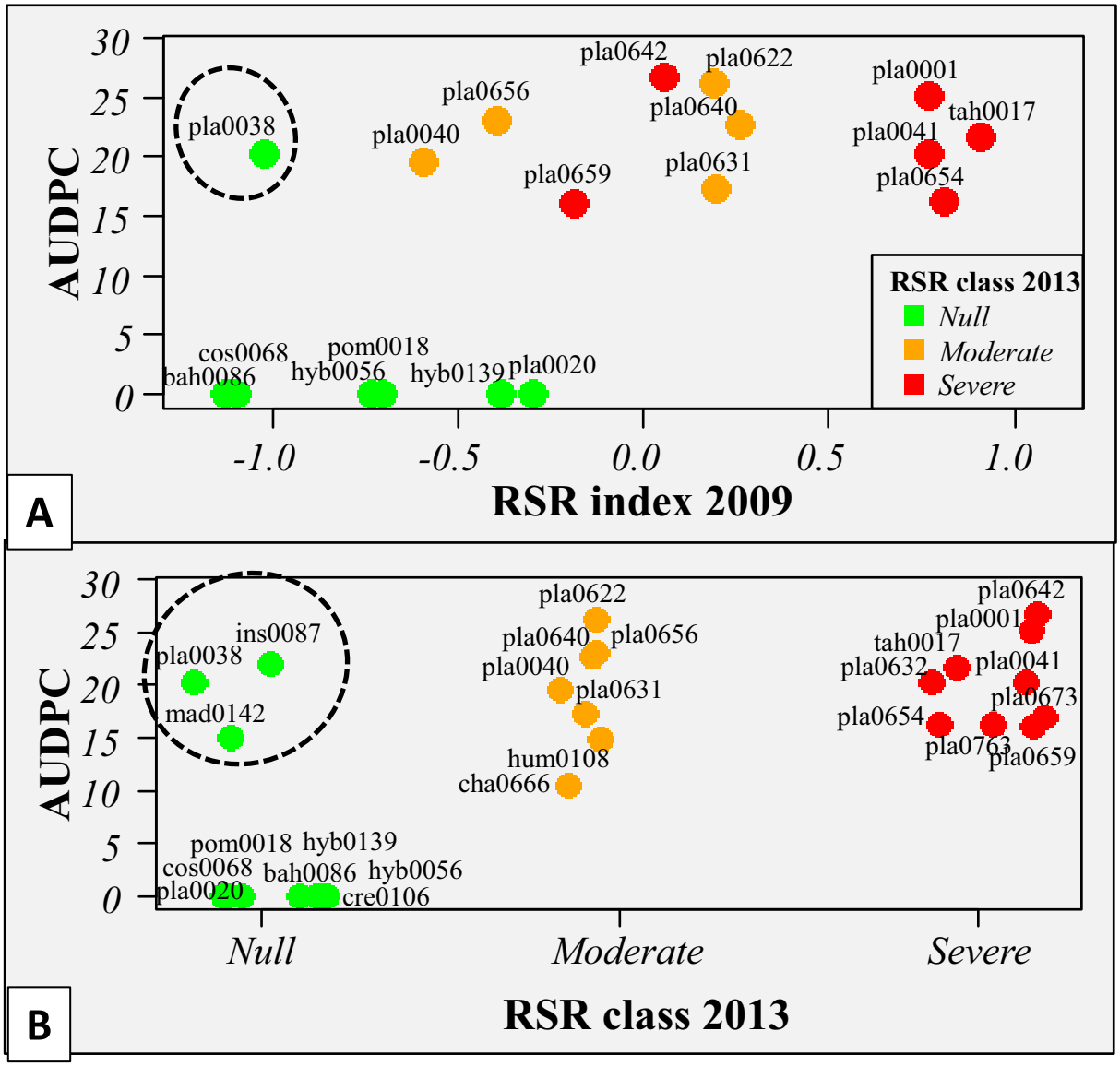


Figure 4 : Correlations between root and stem symptoms in the field rated in 2009 (A) and 2013 (B) and resistance to Forv estimated by AUDPC value of *in-vitro* plantlets inoculated with Fo072.

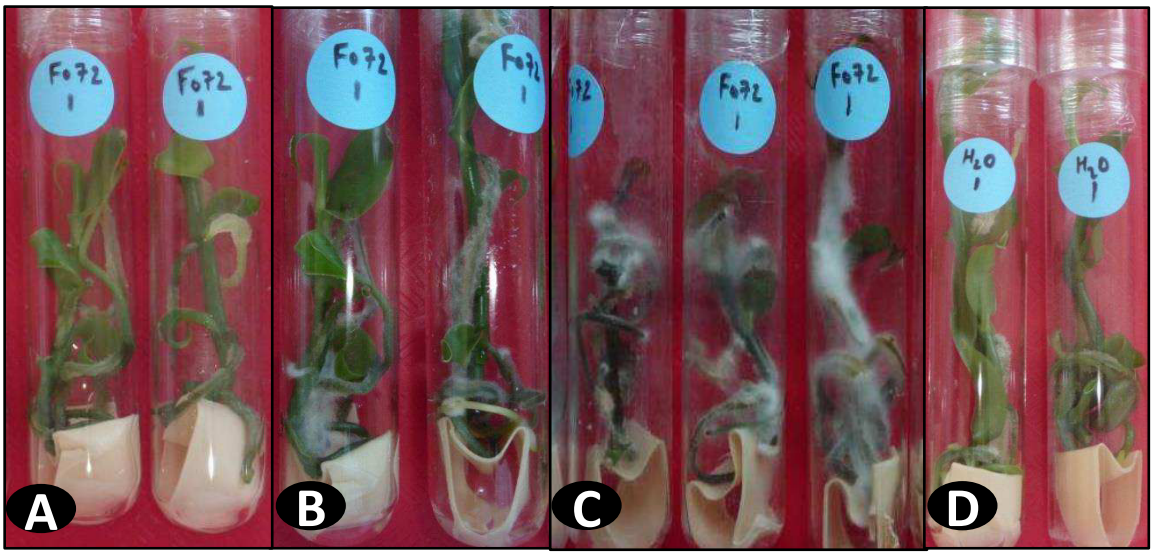


Figure 5: Fungal development of Fo072 inoculated *in-vitro* on pla0001. A-C) External mycelium development and rot 0, 5, 12 dpi; D) non-inoculated i*n-vitro* plants (control).

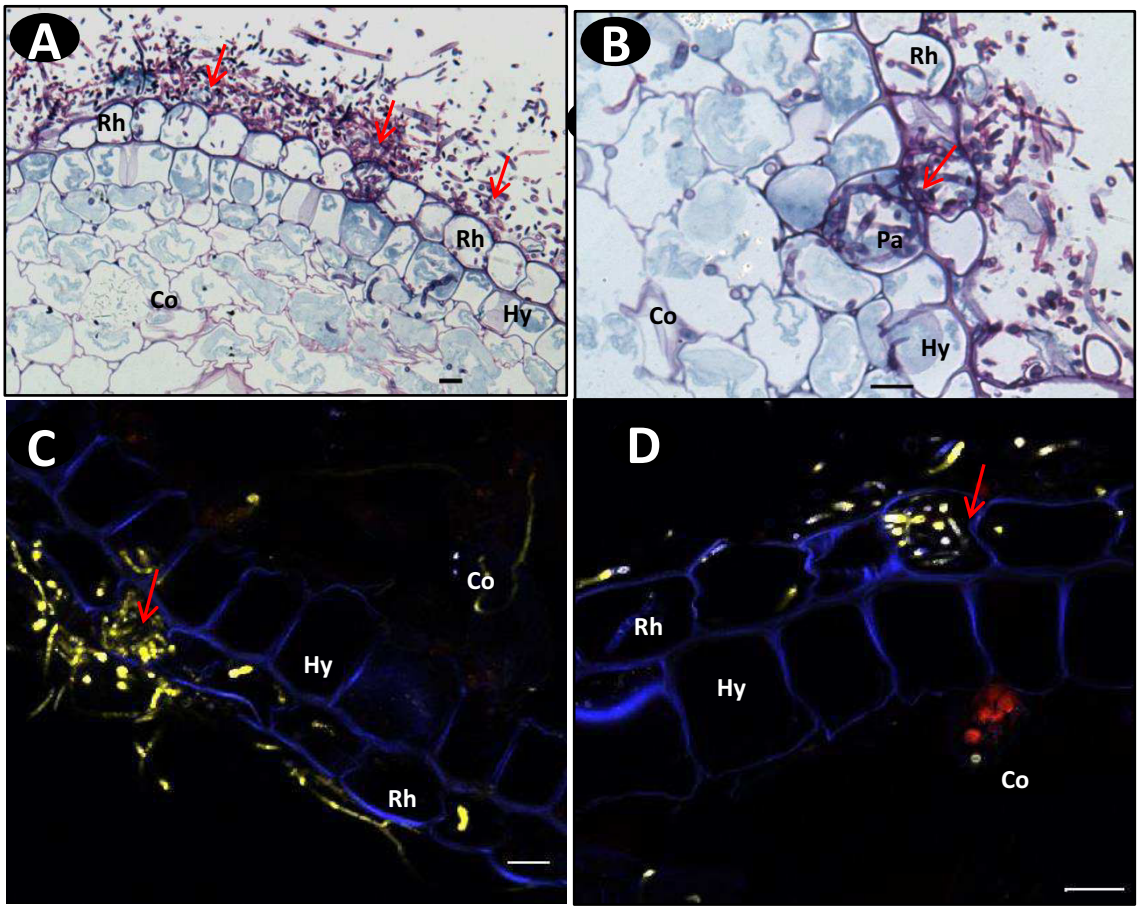


Figure 6: Microscopic images of transverse root section of pla0001 infected with Fo072 showing the colonization and penetration process. A) WFM images displaying the abundant mycelia network (pointed with arrows) on pla0001 double-stained with Schiff reagent znd Naphthol Blue-black stained at 3dpi and B) showing the penetration of Fo072 hyphae specifically through the passage cells (Pa) of hypodermal layer (Hy) in pla0001(denoted with arrows) at 3dpi. C-D) Multiphoton microscopic images displaying the transverse sections of Fo072 infected pla0001 stained with DAPI at 7 dpi showing the formation of hyphal coiled structure (denoted with arrows) in the epidermal cells (Ep) during the penetration . Scale bar =20µm.

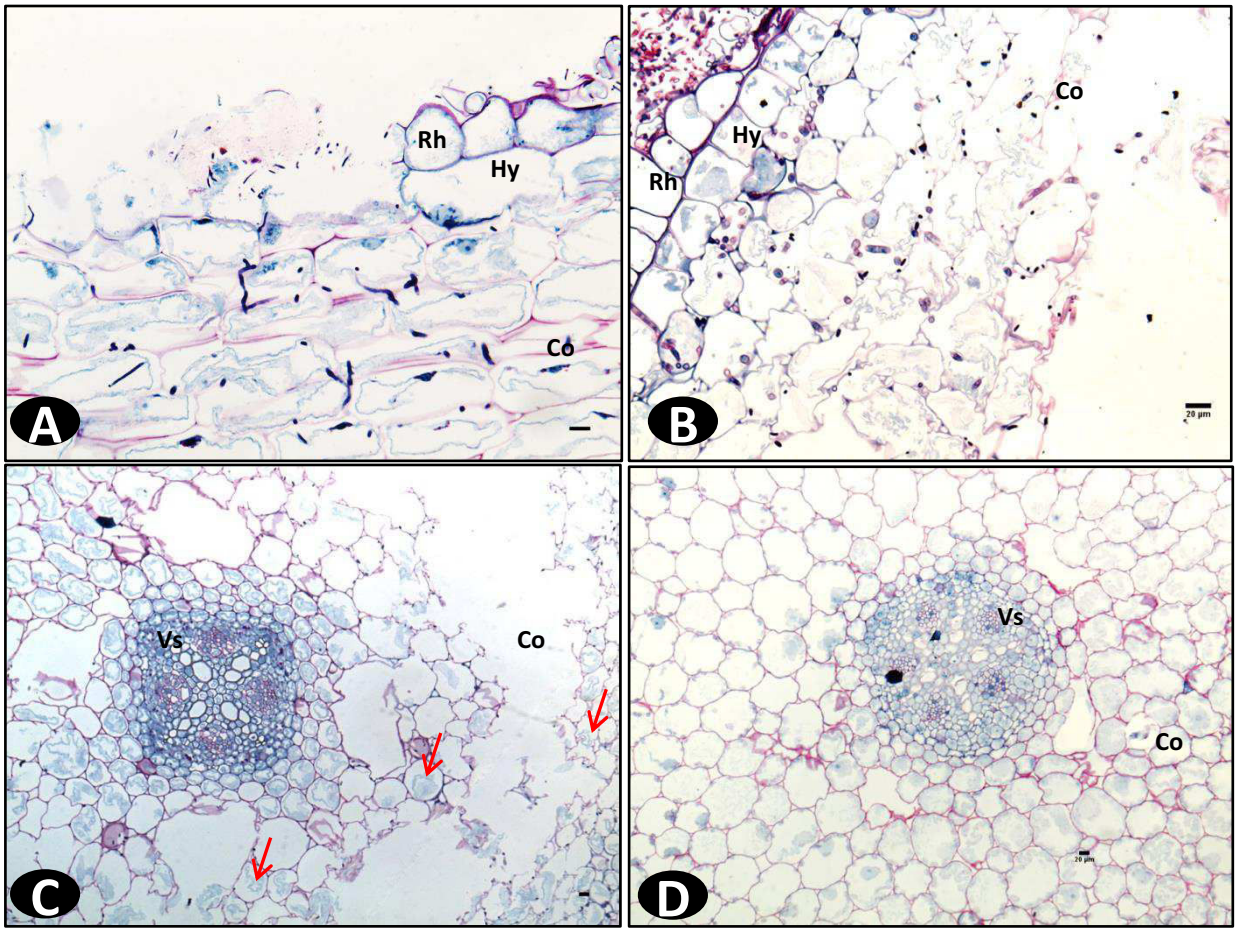


Figure 7: WFM images showing the colonization pattern of Fo072 in the *in vitro* roots of pla0001 double stained with Schiff reagent and Naphthol Blue-black at 7dpi. A) Longitudinal section (Image A) and transverse section (Image B) of pla0001 showing the intercellular colonization of hyphae in the cortical region. Image C) represents the transverse section of pla0001 showing the plasmolysis of cytoplasm from the cell walls and total collapse of the cortical region (Co) with flaccid cells, thereby disturbing the root architecture. The colonization is affected mostly at the cortex region where as the vascular (Vs) cells remains uninfected. Image D) displays the transverse section of non inoculated pla0001 with the cortical cells remains firm and complete. Rh:Rhizodermis and Hy: Hypodermis.. Scale bar=20µm

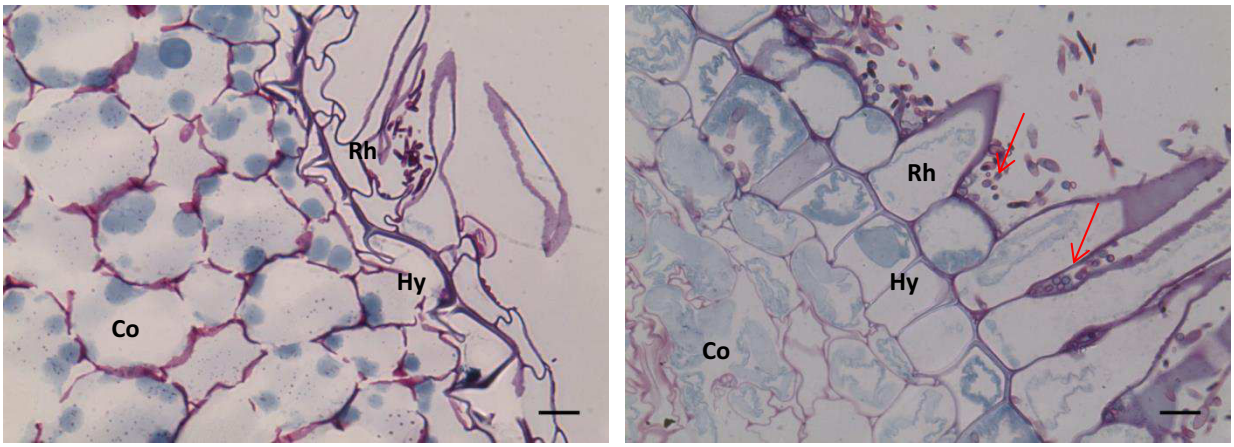


Figure 8: WFM images of transverse section of Schiff Naphthol Blue-black double-stained vanilla root tissues infected with pathogenic Fo072 at 3 dpi. A) Transverse root section of the resistant accession pom0018 showing lesser colonization of hyphae (red arrows) in the hairy root region, compared to B) susceptible accessions pla0001 showing abundant fungal colonization. Rh: Rhizodermis, Hy: Hypodermis, Co: Cortex. Scale bar=20µm

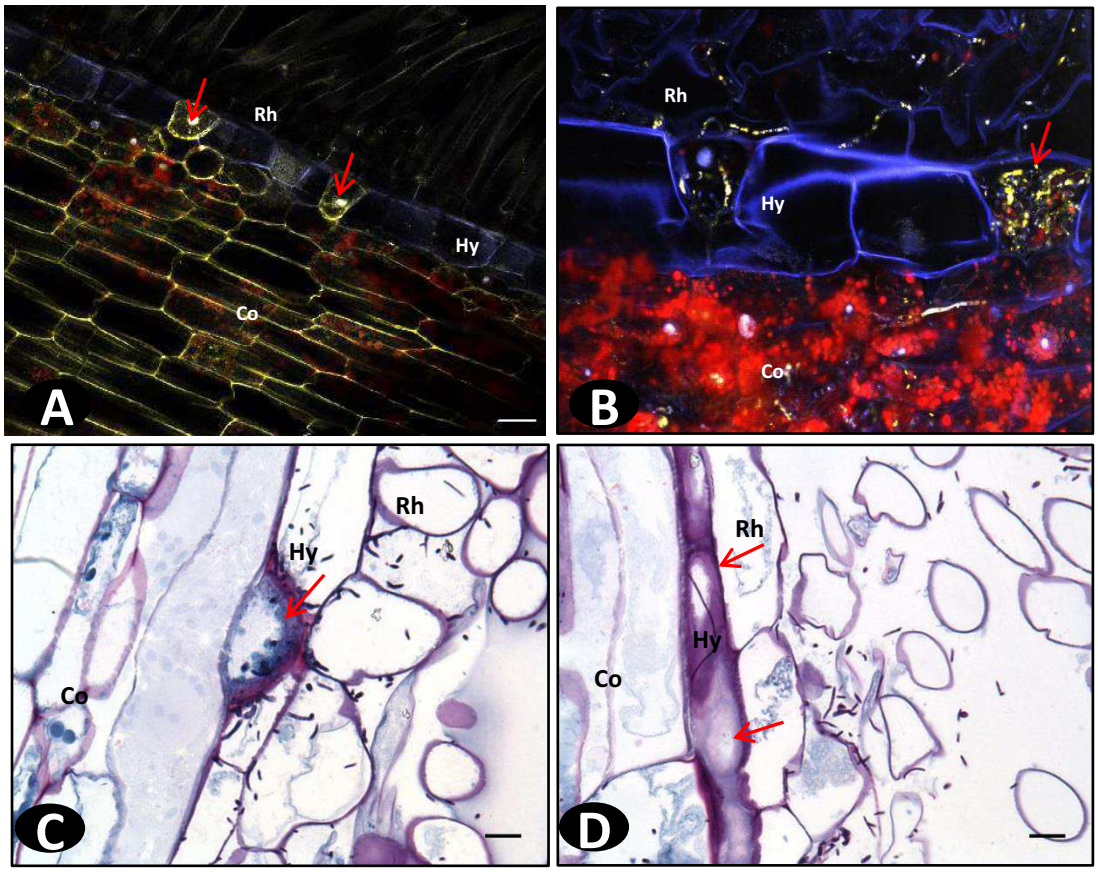


Figure 9: Histopathological defense responses of resistant accessions infected with Fo072. A-B) Multiphoton images showing the formation of "globular" structures (pointed with arrows) on the hypodermal cells (Hy) entrapping the hyphae and preventing their further colonization to the cortical region (Co) of pom0018 infected with Fo072 at 4dpi; C-D): WFM images of infected root samples of pla0020 double-stained with Schiff reagent and Napthol Blue-black stain, shows the defense response of hypodermal cells (denoted with arrows) with C) showing intense staining of hypodermal cell walls of pla0020 and D) the formation of dark layered hypodermal cells between the rhizodermis (Ep) and cortex (Co) in pom0018 infected with Fo072 at 8dpi. The pink color dark staining reveals the deposition of polysaccharides in the cell walls of hypodermis. Scale bar= 20µm.

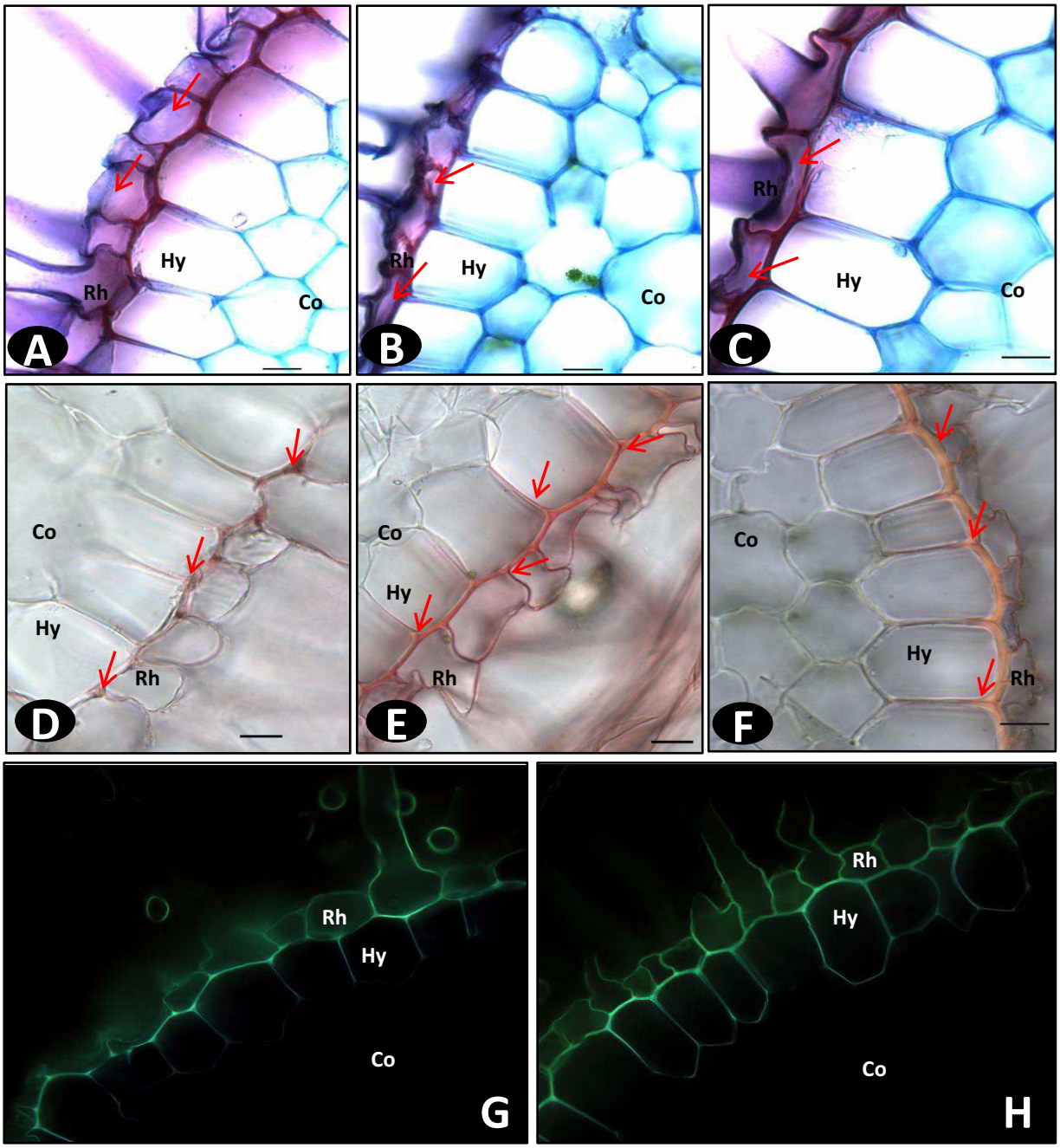


Figure 10: WFM images of non-inoculated *in vitro* roots showing the difference in the cell wall structure and compositions using different staining methods. Images A-C) FASGA stained transverse section of roots showing the round shaped rhizodermal (Rh) cells (denoted with arrows) in pla0001 (A) and compact rhizodermal cells in pla0020 (B) and pom0018 (C); Images D-F) Transverse sections of vanilla *in vitro* roots stained with Phloroglucinol. Discontinuous lignin deposition mostly localized as small patches (denoted with arrows), without radial thickenings, on the hypodermis (Hy) of pla0001 (D), contrary to pla0020 (E) and pom0018 (F) showing continuous longitudinal and radial deposition of lignin on the longitudinal and radial walls of hypodermis. Image G-H) displays the differences between susceptible pla001 (G) and resistant pom0018 (H) in the composition of rhizodermal and hypodermal cells stained with Berberine hemisulphate which has affinity to suberin and lignin. Co: Cortex. Scale bar=20µm.

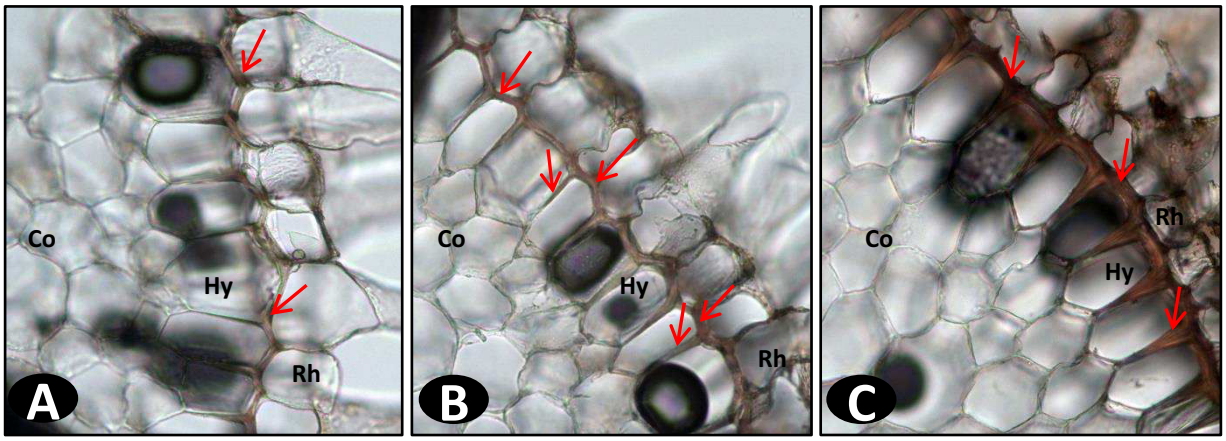


Figure 11: WFM images of the transverse root sections of field grown plants stained with phloroglucinol, showing distinct composition of lignified hypodermal (Hy) cell walls of pla0001 (A), with discontinuous lignin, seen as patches on the outer longitudinal wall (marked with arrows), compared to pla0020(B) and pom0086(C) showing the continuous layer of lignin present on the longitudinal walls and the presence of inner radial walls on the hypodermis

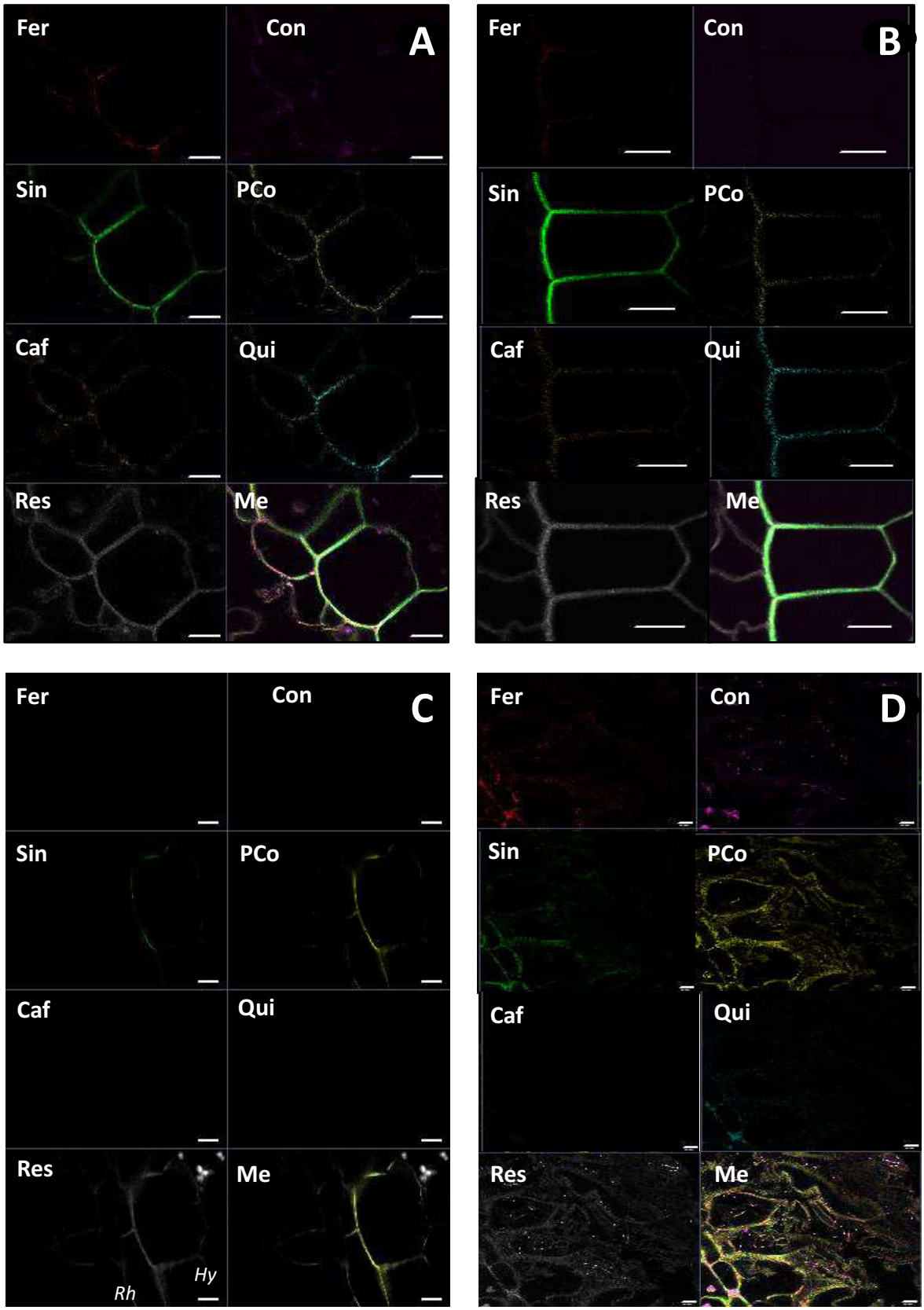
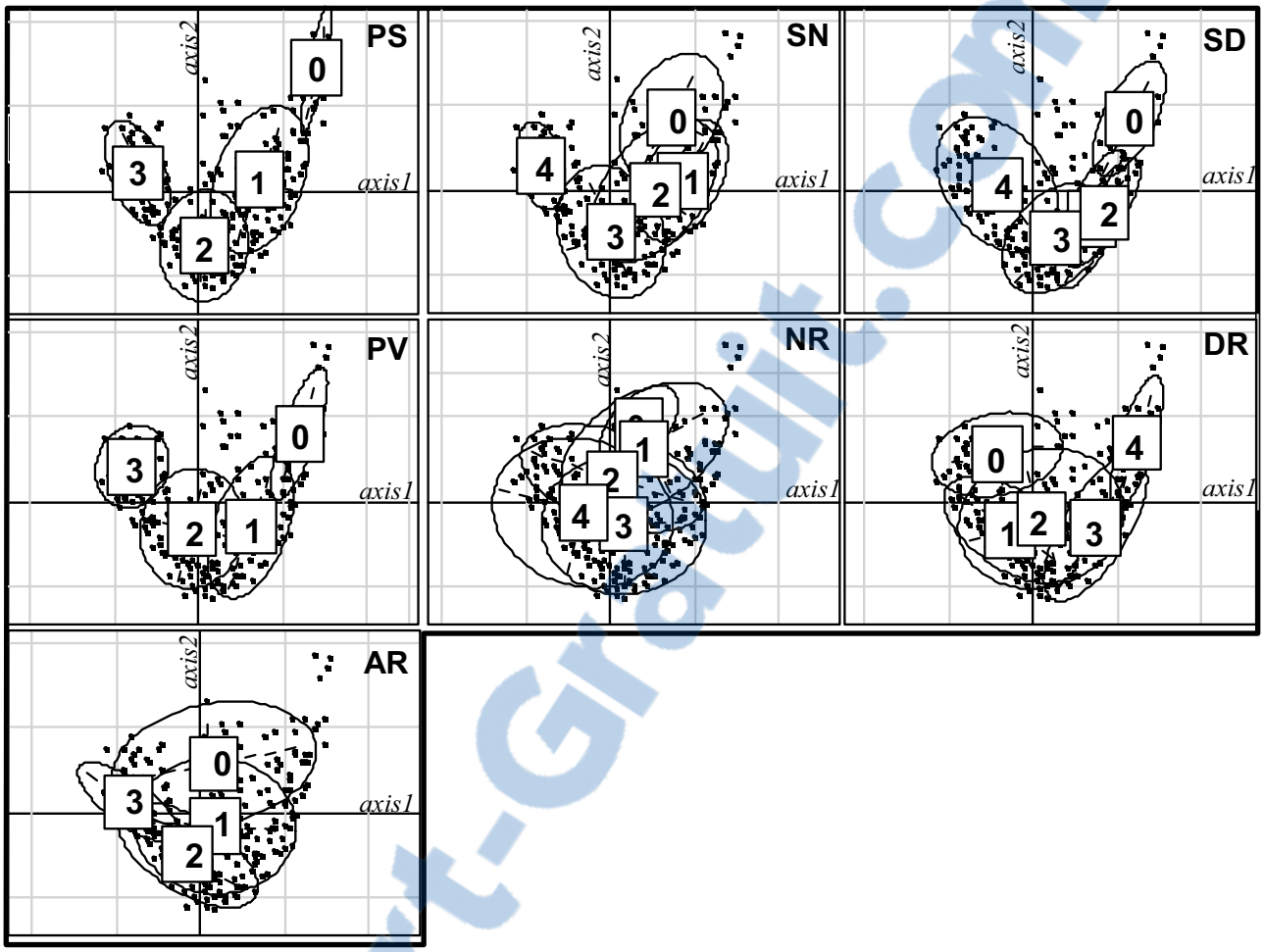
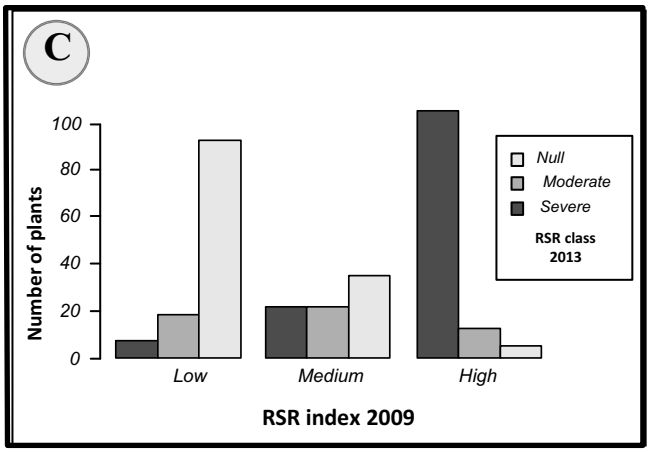


Figure 12: Spectral unmixing analyses of control roots of pla0001 (A) and pom0018 (B) and Fo072 inoculated root sections of pla0001 (C) and pom0018 (D) accessions using six reference emission spectra compounds : Fer=ferulic acid; Con=conyferylic acid; Sin=sinapinic; pCo=p-coumaric acid; Caf=caffeic; Qui=quinic acid. Res=residual channel and Me= Merged image. Rh: Rhizodermis, Hy: Hypodermis. Scale bar: 20µm



Supplementary Figure 1 : Scatterplots of multiple component analysis for 128 vanilla accessions rated for RSR symptoms in the field using seven variables with three to four levels : PS : Plant size, SN : number of stems of the plant, SD: Percentage of grooved stems and decaying leaves, PV: Vigor of the plant (size and number of new shoots), NR : Number of roots descending along the stalk, DR: proportion of dried roots among the descending roots, AR: Number of aerial roots.



Supplementary Figure 2: Congruence between the RSR ratings made in 2009 with the one made in 2013 for 125 vanilla accession.

VIII Figure captions

Figure 1: Two *Vanilla planifolia* accessions showing contrasted development in the shade house four years after planting. On the left pla0670 showing reduced vegetative growth, decaying stem and leaves and abundant descending roots that die on ground, on the right pla0695 showing good growth and limited root symptoms.

Figure 2: Multiple component analysis of 128 vanilla accessions rated for RSR symptoms in the field in 2009 using seven variables (see supplementary figure 1). Axis 1 segregates accessions according to their resistance level. Remarkable susceptible (on the right) and resistant (on the left) accessions are indicated. RSR index of accessions was defined as their coordinate on axis 1.

Figure 3: Evaluation 103 Vanilla accessions for resistance to Fo72 by the in-vitro plantlet assay. A, B) aspect of susceptible and resistant accessions, respectively, 15 dpi. C) The 103 accessions split into 5 classes according to average AUDPC values (4 plants per accession): R=Resistant, SR= slightly resistant, MS = moderately susceptible, S= susceptible and HS= highly susceptible. The position of the three accessions selected for the histopathological studies is highlighted (pla0001, pla0020 and pom0018).

Figure 4: Correlations between RSR symptoms in the field rated in 2009 (A) and 2013 (B) and resistance to Forv estimated by AUDPC value of in-vitro plantlets inoculated with Fo072.

Figure 5: Fungal development of Fo072 inoculated *in-vitro* on pla0001. A-C) External mycelium development and rot 0, 5, 12 dpi; D) non-inoculated i*n-vitro* plants (control).

Figure 6: Microscopic images of transverse root section of pla0001 infected with Fo072 showing the colonization and penetration process. A) WFM images displaying the abundant

mycelia network (pointed with arrows) on pla0001 double-stained with Schiff reagent znd Naphthol Blue-black stained at 3dpi and B) showing the penetration of Fo072 hyphae specifically through the passage cells (Pa) of hypodermal layer (Hy) in pla0001(denoted with arrows) at 3dpi. C-D) Multiphoton microscopic images displaying the transverse sections of Fo072 infected pla0001 stained with DAPI at 7 dpi showing the formation of hyphal coiled structure (denoted with arrows) in the epidermal cells (Ep) during the penetration . Scale bar =20µm.

Figure 7: WFM images showing the colonization pattern of Fo072 in the *in vitro* roots of pla0001 double stained with Schiff reagent and Naphthol Blue-black at 7dpi. A) Longitudinal section (Image A) and transverse section (Image B) of pla0001 showing the intercellular colonization of hyphae in the cortical region. Image C) represents the transverse section of pla0001 showing the plasmolysis of cytoplasm from the cell walls and total collapse of the cortical region (Co) with flaccid cells, thereby disturbing the root architecture. The colonization is affected mostly at the cortex region whereas the vascular cells (Vs) remain uninfected. Image D) displays the transverse section of non inoculated pla0001 with the cortical cells remains firm and complete. Rh: Rhizodermis and Hy: Hypodermis.. Scale bar=20µm.

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Supplementary Figure 2: Congruence between the RSR ratings made in 2009 with the one made in 2013 for 125 vanilla accessions.

CHAPTER IV **Differential expression study**

De novo transcriptome and expression profile analysis of resistant and susceptible Vanilla roots challenged with *Fusarium oxysporum* f. sp. *radicis-vanillae.*

I Introduction

Recently, Next generation Sequencing (NGS) technology has become a tool of choice for discovery of novel genes, expression profiling, comparative genomics study and evolutionary genomics research (Hegedus et al., 2009). In the past few years, *de novo* sequencing has been successfully used for molecular mechanism investigation of plants response to pathogen infection such as in *Arabidopsis thaliana*, *Citrullus lanatus*, *Musa spp*., *Triticum aestivum* and *Oryza sativa*.

In this study, the transcriptome profiles of resistant and susceptible vanilla roots infected with pathogenic *Fusarium oxysporum* f. sp. radices-*vanillae* (Forv) and non-pathogenic *F. oxysporum* strains were compared in order to investigate the genetic basis governing the resistance in the *Vanilla-Fusarium* pathosystem.

The present study describes the first transcriptomic analysis of *Vanilla* and *Fusarium* pathosystem. The comparison between the transcriptome profiles of susceptible and resistant vanilla accessions infected with Forv enabled us to identify some of the candidate pathogen-response genes. These genes could be used further to study the pathways involved in resistance to the vanilla root rot.

II Materials and methods

II-1 Plant material and pathogen inoculation

Three accessions of vanilla were used for the study, comprising of a susceptible V. *planifolia* (pla0001), and two resistant accessions *V. planifolia* (pla0020) and *V. pompona*

(pom0018). Two *Fusarium* isolates were used, one being highly pathogenic (Fo072) and the other being non-pathogenic (Fo047) (Alabouvette, 1986). An *in-vitro* inoculation was conducted (Koyyappurath et al., in press) to confirmed the susceptibility of the accessions to both Forv isolates. The selected accessions were grown *in-vitro* in Murashige and Scoog media without any growth hormones.

For the inoculation, the roots of in-vitro grown plants were dipped in Forv conidial suspension of 10^6 conidia/ml (Koyyappurath et al., in press). The plants were then transferred to sterile tubes and incubated in plant growth chambers under controlled conditions. Control plants were inoculated with water. The roots were then harvested at 5 and 6 day post-inoculation (dpi), flash frozen in liquid nitrogen and stored at -80°C. During library preparation, the samples from day 5 and 6 days were equally pooled to a single sample.

II-2 RNA extraction, library preparation and sequencing

Total RNA was extracted from the root and stem tissues of Forv infected and control vanilla accessions using the QIAGEN RNeasy plant mini kit. The quality of each RNA sample was checked using agarose gel electrophoresis and with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The *de novo* RNA-Seq library construction and sequencing were outsourced (Beckman Coulter Genomics, USA). Eight cDNA libraries were synthesized as outlined in the Illumina TruSeq RNA-seq Sample Prep kit (Illumina, Inc., CA, USA). The cDNA library was then sequenced on the Illumina HiSeq 2000 platform with 100 bp paired-end reads. Data analysis and base calling were performed using Illumina instrument software.

II-3 Transcriptome assembly

De novo assembly of the *Vanilla* genotypes transcriptome was accomplished from the eight libraries pooled together using the Trinity *de-novo* transcriptome assembly software

(Grabherr et al., 2011). After the assembly, every RNA-Seq library was separately aligned to the generated transcriptome assembly using Bowtie aligner (Langmead et al., 2009). The BAM files generated by Bowtie were then used to estimate the transcript-level abundance for each library using the RNA-Seq by Expectation Maximization (RSEM) software (Li & Dewey, 2011). RSEM generates transcript percentages and fragments per kilobases of transcript per million mapped reads (FPKM) values for relative expression of each transcript.

II-4 Classifying the sources of transcripts

In order to classify the sources of transcripts assembled as either plant or *Fusarium*, the sequences were locally blasted (BLAST version 2.2.14) against different database (e-value less than 10^{-5} and identity > 75%): The databases used locally were *Fusarium* Proteins (NCBI), *Fusarium* transcript database (Broad Institute), Monocot Proteins (NCBI RefSeq), Viridiplantae, Musa defense and Vanilla proteins (Uniprot) and Vanilla SRA database (SRR867613). To increase the computational speed, the searches were limited to the first 10 significant hits for each query.

II-5 Gene expression and Functional annotation

The Differential Expression (DE) analysis between the pools were estimated statistically using the R package DESeq (Anders & Huber, 2010). Size factor estimation and normalization were performed separately for each pool. Log fold change of 2 and FDR ≤ 0.05 was set as the threshold for the significant differential expression between the selected pools. The identified differentially expressed genes (DEGs) were used for the GO annotation process. Functional annotation of the DEGs were performed using Blast2GO software using default settings. DEGs were searched against the NCBI non-redundant protein (nr) database with BLAST expectation value of 1.0e-3, a maximum of 20 hits, an HSP length cutoff of 33

and a low complexity filter. Mapping step involved retrieval of Gene Ontology (GO) terms associated with each BLAST hit.

III Results

III-1 Plant material and pathogen inoculation

The inoculated plant material was evaluated for symptoms every dpi. First appearance of fungal invasion and wilt response of the plant were observed from 4th to 6th dpi. Numerous hyphae were detected on the root surface of the susceptible genotype (pla0001), whereas the quantity of hyphae on the resistant genotypes (pla0020 and pom0018) was much lesser. The disease symptoms progresses towards the 6th dpi in susceptible genotypes, while absent in the resistant genotypes. Therefore, two time points were selected to investigate the genetic basis underlying the differential responses of the vanilla genotypes to the infection, namely 5 and 6 dpi. These samples were later pooled together.

III-2 RNA extraction, library preparation and sequencing

Quality assessment of total RNA extracted from root tissues of different pools exhibited a RNA integrity number (RIN) value from 5.5 to 9.6 (Table 1). Eight paired- end RNA-Seq libraries were generated for the eight samples having acceptable RIN values, one for each of the mentioned pools. *De novo* Illumina sequencing of these cDNA libraries generated a total 365.90 million paired-end raw reads (Table 2). Further, the reads were assessed for quality, removing the duplicate paired-end reads, sequencing adaptors and low quality reads to ensure better sequence assembly.

III-3 Transcriptome assembly

The high quality paired-end reads were subjected to assembly with Trinity software. The *de novo* assembly with Trinity resulted in 418,345 transcripts, with an N50 of 2,603 bp when

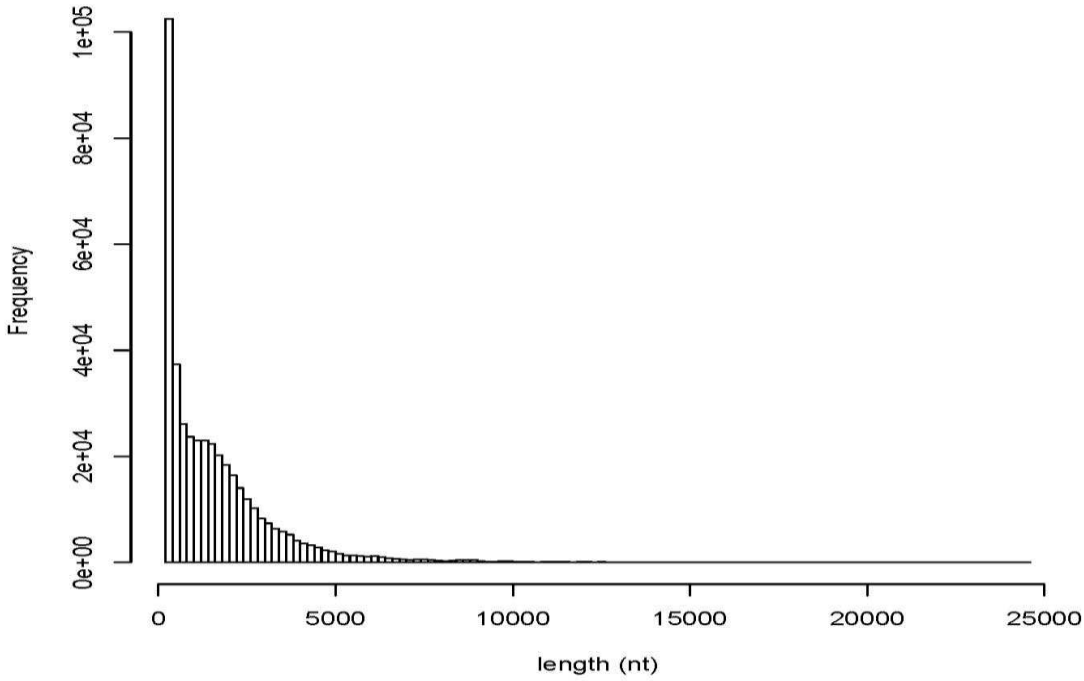

Figure 1 : Length distribution of the assembled transcripts.

all isoforms were considered. These transcripts represent a total of 155,228 unigenes that were considered for further expression analysis studies. The size distribution of the transcripts (Figure 1) ranges from 200 to more than 24,400 with a median of 1,170.

III-4 Classifying the sources of transcripts

As the RNA-Seq data was produced using the *Vanilla* root infected with *Fusarium*, we sequenced RNA from both Forv and vanilla. In order to focus our analysis on the vanilla transcripts, the sequences were blasted against different database to classify each transcript in either "*Vanilla*", "*Fusarium*", "Undetermined" and "no hits". Totals of 69,059 and 203,448 transcripts were classified respectively as *Fusarium* and vanilla transcripts. A total of 15,500 transcripts could not be associated with confidence with either *Fusarium* or vanilla and 130,330 transcripts had no hit. The study was continued by picking the transcripts only from the classes "Vanilla" and "Undetermined" classes. The 203,448 and 15,500 transcripts represent respectively 26,752 and 2,456 unique genes.

III-5 Gene expression and functional annotation

To identify the genes or signaling pathways involved in different genotypes of vanilla response to *Fusarium* infection, differential expression analysis study were performed between the pools using the digital gene expression studies. A total of eight comparisons were performed. More specifically, we compare a susceptible vanilla accession to two different resistant one. In order to assess the influence of the *Fusarium* over the plant transcriptome we also used, along with a control, two different *Fusarium* strains. One is a non-pathogenic strain whereas the other two were demonstrated to be highly pathogenic.

In total, the analyses resulted in 169 differentially expressed genes (DEGs), representing a total of 137 distinct DEGs (Table 3). Of the 137 DEGs, 52 DEGs were annotated to the GO id level. The GO annotation could categorize the DEGs into 90 functional groups in the three

main categories such as Biological process, Molecular function and Cellular component (Figure 2). In each of the three categories mentioned, metabolic process, catalytic activity, cell part and cell respectively were the dominant ones. The comparison of different categories is mentioned below.

III-5.a Comparison of transcripts in Susceptible and Resistant accessions after Fo72 infections

This category includes the DE analysis of Fo072 infected susceptible pla0001 (pool 1) against resistant pom0018 (pool 2) and pla0020 (pool 4). In the first comparison (pool 1 vs. pool 2) the DE analysis yielded two DEGs (Table 4), both being down-regulated in the susceptible plant (Figure 3).

In the second comparison (pool 1 vs. pool 4), 4 DEGs were identified (Table 5). All these DEGs were also down-regulated in the susceptible plant (Figure 4). Importantly, there is a common DEG in the two comparisons, comp 85040_co which encodes for pentatricopeptide repeat-containing protein.

III-5.b Comparison of Resistant accession infected with Fo072 and Fo047

This category compares the DE analysis of resistant accessions (pom00018, pla0020) infected with Fo072 and Fo047. In the first comparison (pool 2 vs. pool 3) the DE analysis yielded 12 DEGs (Table 6). Among these, seven were up-regulated and five were down-regulated in the resistant accession infected with Fo072 (Figure 5).

In the second comparison (pool 4 vs. pool 5), seven DEGs were identified (Table 7). Four were up-regulated and three were down-regulated in the resistant accession infected with Fo072 (Figure 6). In the two comparisons comp81000_c0 and comp87470_c0 were the

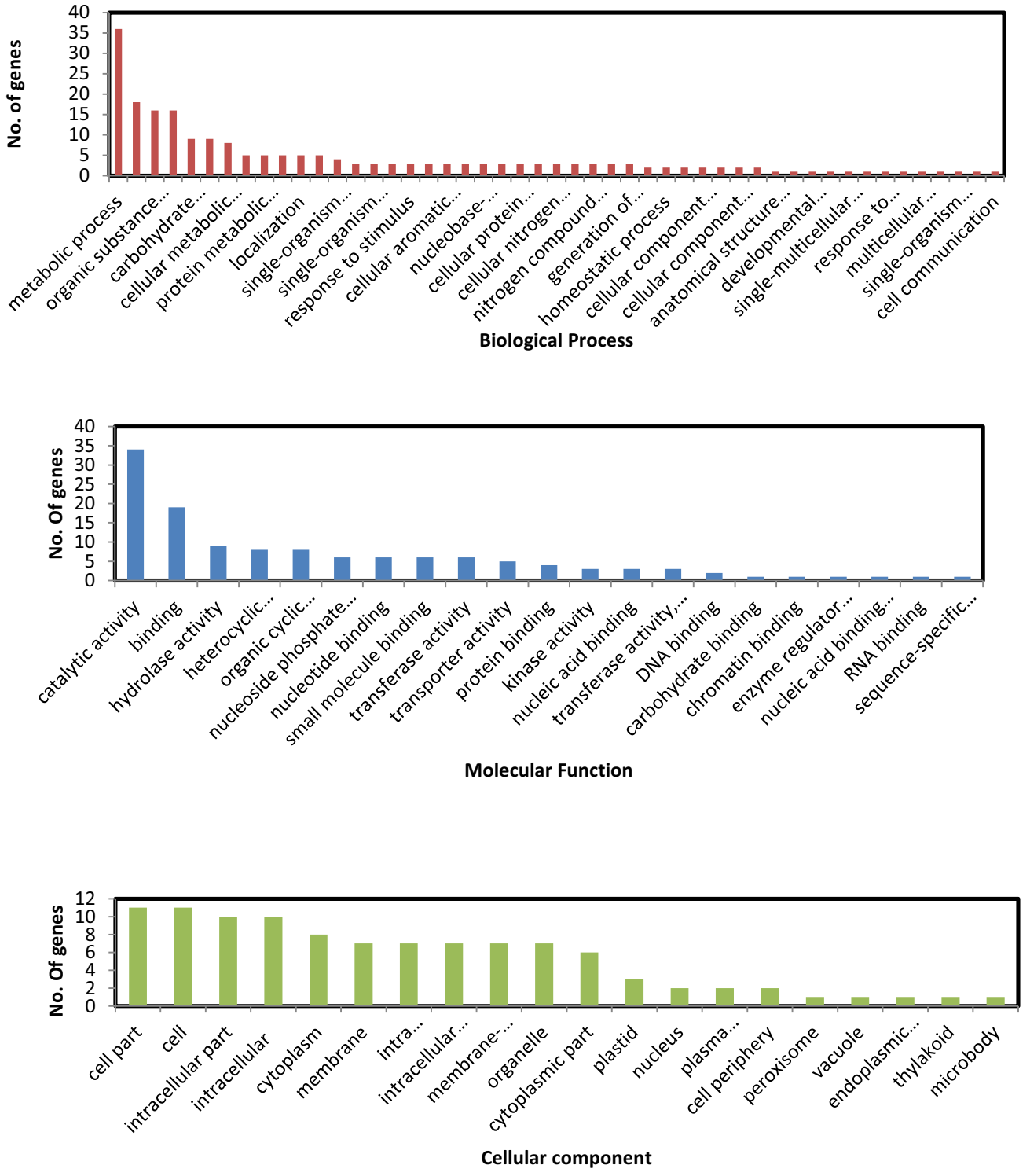

Figure 2 : Gene ontology classification of DGEs. DGEs were classified into 3 functional categories, Biological, Molecular and Cellular components

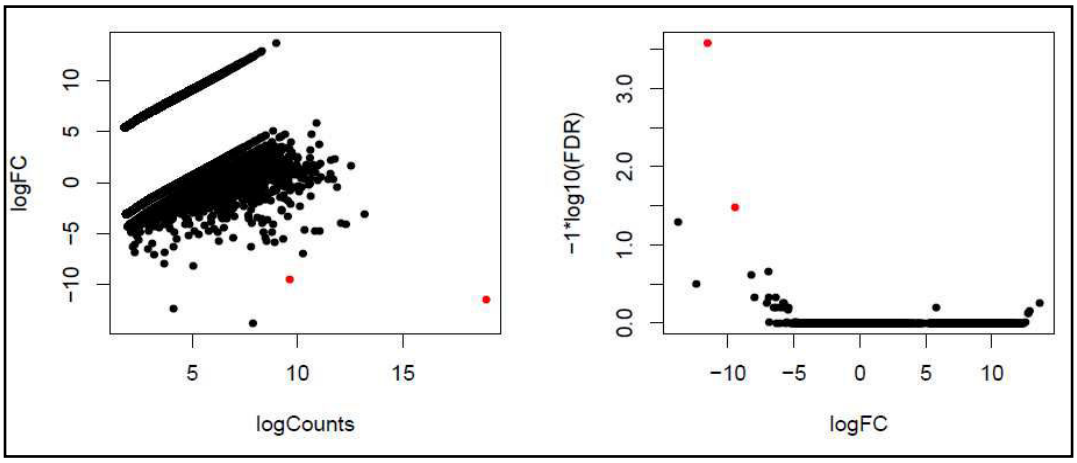

Figure 3 : Comparison of expression patterns of differential genes identified between pool 1 and 2. The red dots represent DEGs, the black dots represent non DEGs.

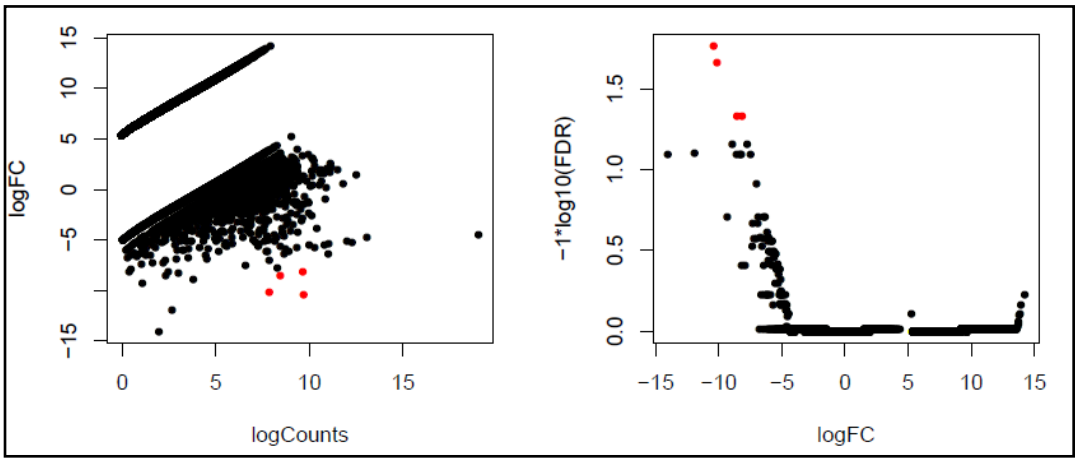

Figure 4 : Comparison of expression patterns of differential genes identified between pool 1 and 4. The red dots represent DEGs, the black dots represent non DEGs.

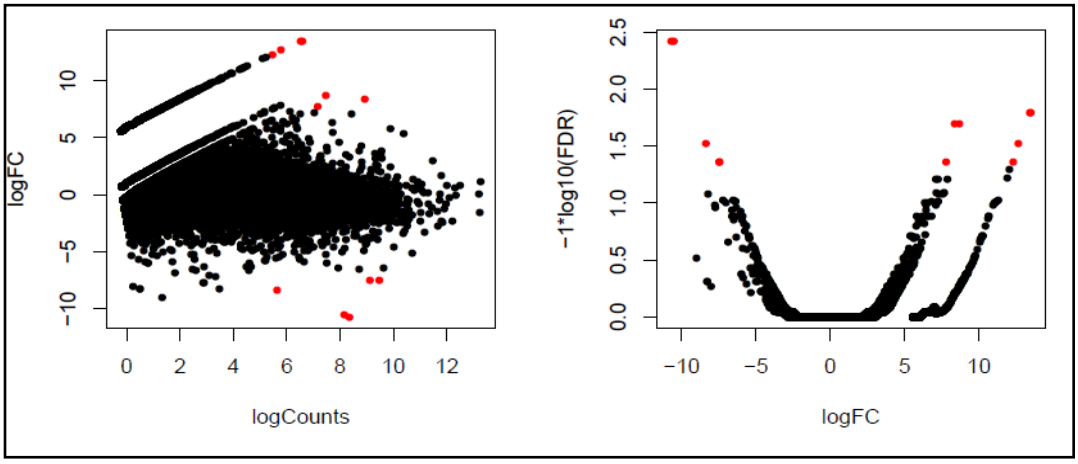

Figure 5 : Comparison of expression patterns of differential genes identified between pool 2 and 3. The red dots represent DEGs, the black dots represent non DEGs.

common DEGs and codes for beta-mannosidase and probable 2-oxoglutarate-dependent dioxygenase aop1 respectively.

III-5.c Comparison of non-infected resistant plant with resistant plant infected with either Fo072 or Fo047

This category includes the DE analysis of a non-inoculated resistant pla0020 (pool 6) against a pla0020 infected with Fo072 (pool 4) and pla0020 infected with Fo047 (pool 5).

The DE analysis between pool 6 vs. pool 4 yielded three DEGs (Table 8). Among these, a single one was up-regulated while the other two being down-regulated in the non-infected resistant accession (Figure 7).

The second comparison (pool 6 vs. pool 5) yielded 58 DEGs (Table 9). Among the 58 DEGs, 14 were up-regulated and 44 were down-regulated in the non-infected accession (Figure 8). Among the two comparisons, two genes are in common, comp75407_c1, comp89799_c0 which codes for a hypothetical and uncharacterized protein respectively.

III-5.d Comparison of 2 resistant accessions infected with Fo072

This pool wise comparison involve the DE analysis between two resistant accessions (pom0018, pla0020) infected with Fo072. The result of DE analysis between the pools (pool 2 vs. pool 4) yielded 33 DEGs (Table 10). Among the 33 DEGs, 11 were up-regulated and 22 were down-regulated in the pom0018 infected with Fo072 (Figure 9).

III-5.e Comparison of Fo072 infected pooled stem samples with non-infected pooled stem samples.

This pool wise comparison involve the DE analysis of Fo072 infected stem (pla0001, pla0020 and pom0018) against a non-infected stem (pla0001, pla0020 and pom0018). The DE analysis between the pools (pool 7 vs. pool 8) yielded 50 DEGs (Table 11). Among the 50

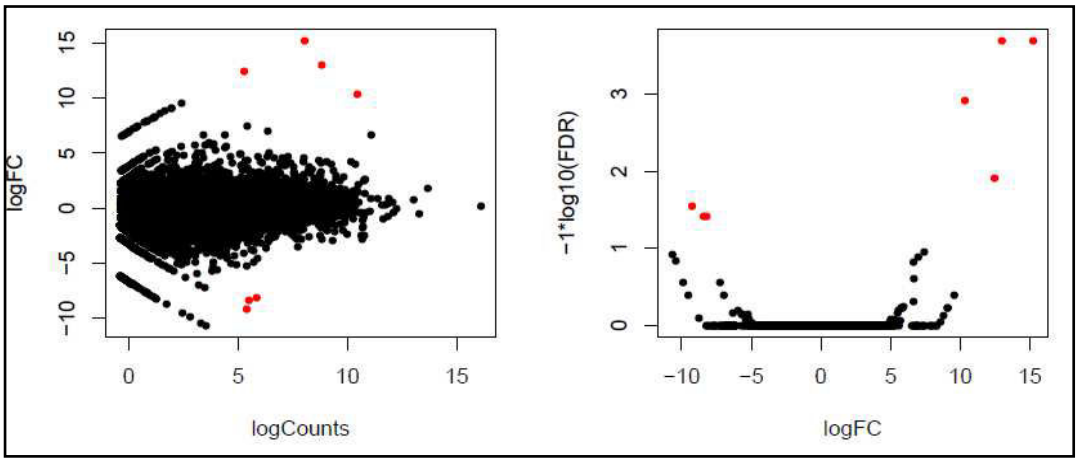

Figure 6 : Comparison of expression patterns of differential genes identified between pool 4 and 5. The red dots represent DEGs, the black dots represent non DEGs.

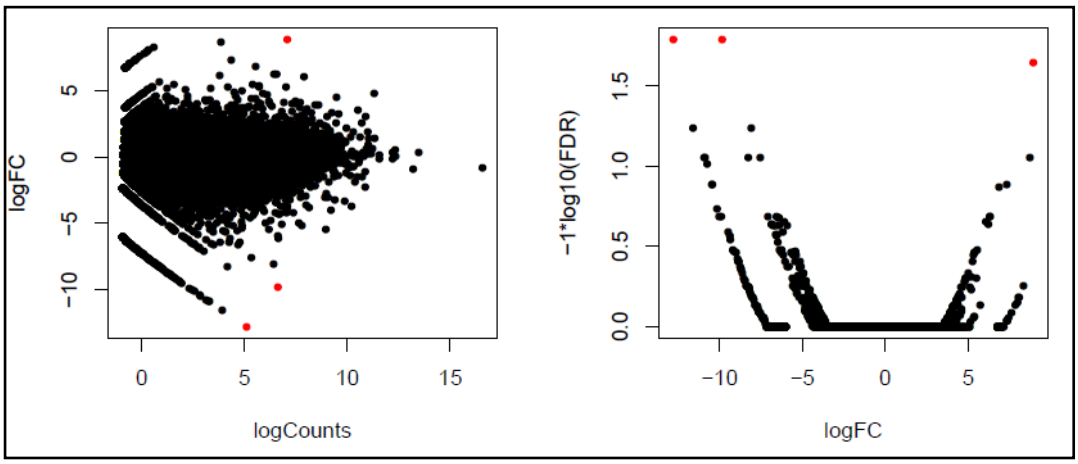

Figure 7 : Comparison of expression patterns of differential genes identified between pool 6 and 4. The red dots represent DEGs, the black dots represent non DEGs.

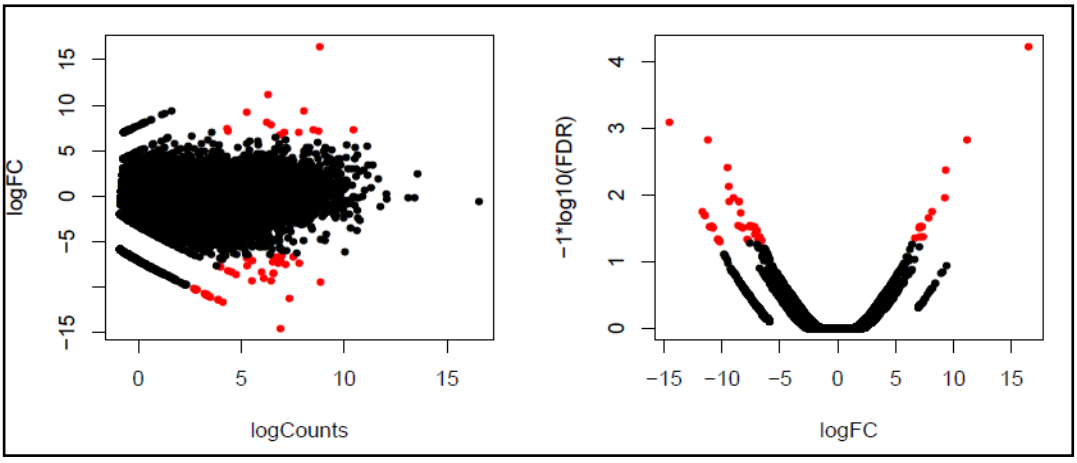

Figure 8 : Comparison of expression patterns of differential genes identified between pool 6 and 5. The red dots represent DEGs, the black dots represent non DEGs.

DEGs, 38 were up-regulated and 12 were down-regulated in the pathogen infected pool (Figure 10).

III-6 Role of Differentially expressed genes

In the case of comparison involving susceptible accession infected with Fo072 (pool 1 vs. pool 2) and (pool 1 vs. pool 4), all the DEGs were down-regulated in the susceptible plants. Among these, the comp85040_c0 annotated as Pentatricopeptide repeat-containing protein (PPR) is common in both comparisons. PRR have various roles in organelle biogenesis, photosynthesis, respiration, plant development and environmental responses (Barkan & Small, 2014). The studies of PPR proteins involved in defense response against necrotrophic fungi in *Arabidopsis* have also been reported. This study suggests that the PPR family proteins functions in the regulation of Reactive Oxygen Species (ROS) homeostasis in mitochondria during stress responses. The PPR also play a role in handling perturbations in cellular redox elicited by biotic or abiotic stress. The comp73492_c0 and comp73492_c0 included in the Glycoside hydrolase family 2 are also down-regulated in the susceptible plant. This family is involved in various activities such as cell wall metabolism, defense, signaling activities in plants (Minic, 2008).

In the case of the pools involving the resistant accession pom0018, both up and down-regulated DEGs were observed (Pool 2 vs. Pool 3 and Pool 2 vs. Pool 3). The up-regulation of few genes has been detected, which are involved in defense responses in many plants. Some of the DEGs such as comp83280_c0, comp81000_c0 and comp85358_c0 which are annotated as peroxidase-3, 2OG-dependent dehydrogenase and ferritin respectively are important in controlling the defense responses. Peroxidases are involved in the dehydrogenation of sinapilic acid and conyferulic acid during lignin synthesis at the infection site, which acts as a protective barrier during pathogen infection. They are also involved in the metabolism of

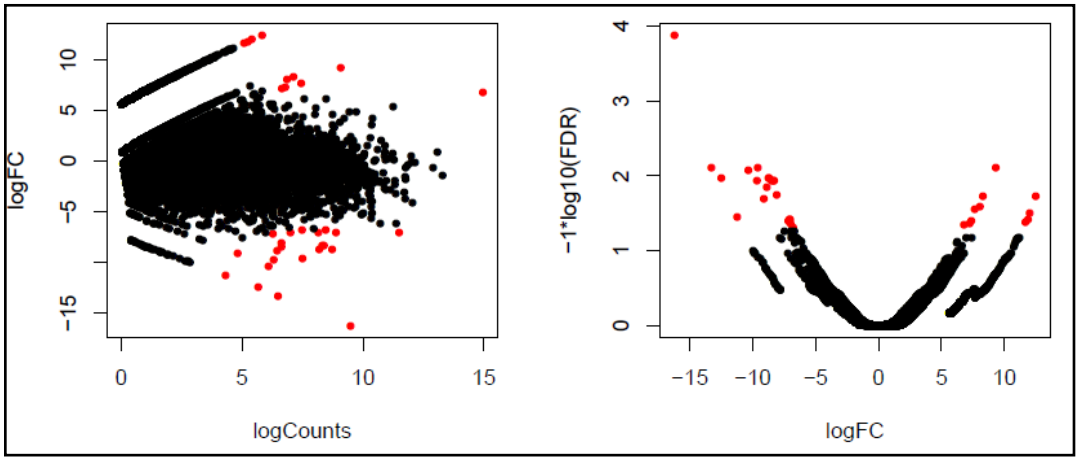

Figure 9 : Comparison of expression patterns of differential genes identified between pool 2 and 4. The red dots represent DEGs, the black dots represent non DEGs.

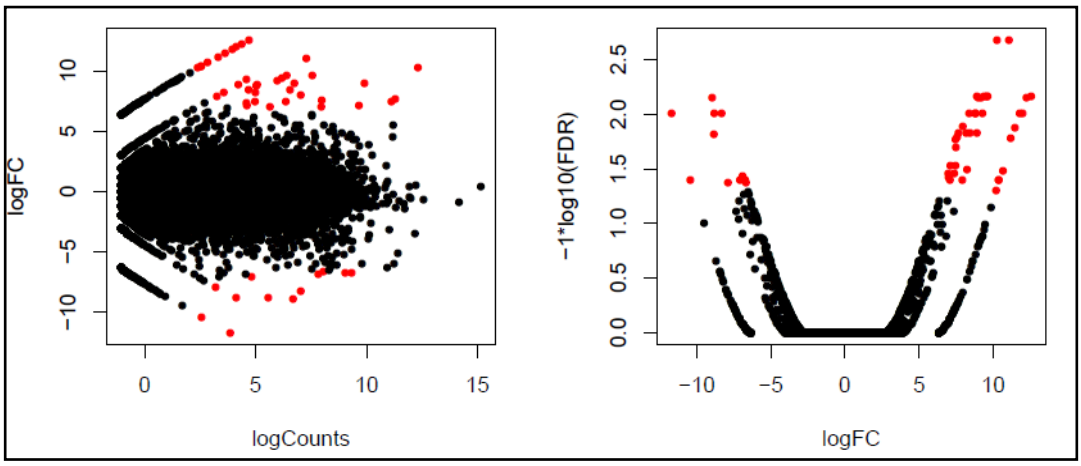

Figure 10 : Comparison of expression patterns of differential genes identified between pool 7 and 8. The red dots represent DEGs, the black dots represent non DEGs.

ROS, a component of the hypersensitive response. Peroxidases involved in the inhibition of *Fusarium* spore germination in *Asparagus densiflorus* have been also reported earlier (He et al., 2001). The 2OG-dependent dehydrogenase (Kliebenstein et al., 2001) involved in the secondary metabolism of *Arabidopsis* and ferritin in plant defense mechanism of various plants have been also reported (Briat et al., 2010). Secondary metabolites including phenolics are largely involved in the lignin biosynthesis on cell walls which acts a protective barrier against fungal pathogens.

Further, in the case of pla0020 the up-regulated DEGs include genes which are annotated as Serine carboxypeptidase (SCPEP) like (comp61869_c0, comp76705_c0) and ICL (comp81374_c0). SCPEP is involved in the production of anti-microbial compounds in many plants while ICL is involved in defense response of plants. SCPEP is reported to be essential for the synthesis of antimicrobial compounds, a disease resistance mechanism found in Oats . The enzyme is essential for the acylation of antimicrobial terpenes that are produced in the Oat roots, which provides protection against the soil-borne pathogens (Mugford & Osbourn, 2010). It is assumed that ICL involved in the glyoxylate metabolism may be used by the plant to generate energy from the carbohydrate intermediates.

IV Conclusion

The *de novo* transcriptome analysis of *Fusarium* infected *Vanilla* was conducted to elucidate for the first time the molecular mechanism in the host-pathogen interaction.. The identified DEGs in the resistant accessions will form the basis of understanding the mechanism involved in the resistance to Forv in *Vanilla*. The data generated in the present study could benefit for the future studies in the *Vanilla-Fusarium* interaction.

Our *de novo* study identified 169 genes as differentially expressed in our five comparison involving eight pools. Our data points to the involvement of few genes involved in the plant

resistance mechanisms which were reported earlier. Some of the up-regulated DEGs in the resistant genotypes are involved in the production of secondary metabolites (comp83280_c0, comp81000_c0) which are involved in lignin biosynthesis. Secondary metabolites produced via shikimic and malonic acid pathway in plants comprise of compounds such as lignin, and flavanoids (Freeman and Beattie, 2008) are involved in defense responses. The role of lignin and other phenolic compounds to *Fusarium* resistance in *Vanilla* is congruent with our recent histological studies (Koyyappurath et al., in press). The histological studies revealed the presence of constituent lignin deposition on the longitudinal and radial hypodermal walls of resistant vanilla accessions. These continuous lignified layer acts as a mechanical barrier for the pathogen to invade the host.

The identification of genes in the *Fusarium* resistance in *Vanilla* gains more insights into *Vanilla-Fusarium* interactions. However only little is known about majority of DEGs, those are yet to be studied. In future, more works will be needed to decode the resistance mechanism in *Vanilla* to Forv with more repetition and expression analysis studies.

V Figure captions and tables

Figure 1 : Length distribution of the assembled transcripts

Figure 2 : Gene ontology classification of DGEs. DGEs were classified into 3 functional categories, Biological, Molecular and Cellular components

Figure 3 : Comparison of expression patterns of differential genes identified between pool 1 and 2. The red dots represent DEGs, the black dots represent non DEGs.

Figure 4 : Comparison of expression patterns of differential genes identified between pool 1 and 4. The red dots represent DEGs, the black dots represent non DEGs.

Figure 5 : Comparison of expression patterns of differential genes identified between pool 2 and 3. The red dots represent DEGs, the black dots represent non DEGs.

Figure 6 : Comparison of expression patterns of differential genes identified between pool 4 and 5. The red dots represent DEGs, the black dots represent non DEGs.

Figure 7 : Comparison of expression patterns of differential genes identified between pool 6 and 4. The red dots represent DEGs, the black dots represent non DEGs.

Figure 8 : Comparison of expression patterns of differential genes identified between pool 6 and 5. The red dots represent DEGs, the black dots represent non DEGs.

Figure 9 : Comparison of expression patterns of differential genes identified between pool 2 and 4. The red dots represent DEGs, the black dots represent non DEGs.

Figure 10 : Comparison of expression patterns of differential genes identified between pool 7 and 8. The red dots represent DEGs, the black dots represent non DEGs.

Table 1: List of RNA samples extracted from the infected and control plants

Sample. No	Fungus	clone	organ	dpi	RIN	Pool No.
1	Fo072	pla0001	root	3	8.4	1
2	Fo072	pla0001	root	6	9	
3	Fo072	pom0018	root	6	8	2
4	Fo047	pom0018	root	6	6.7	3
5	Fo047	pom0018	root	3	7.9	
6	Fo072	pla0020	root	3	7.6	4
7	Fo072	pla0020	root	6	9.6	
8	Fo047	pla0020	root	3	7.6	5
9	Control	pla0020	root	0	5.5	6

10	Fo072	pla0001+pla0020+pom0018	stem	6	6.5	7
11	Control	pla0001+pla0020+pom0018	stem	0	6.8	8

Table 2: List of library prepared and the reads from each library

Pool No.	Pool name	Direction	# reads
1	Fo072 infected pla0001	F	21222961
		R	21222961
2	Fo072 infected pom0018	F	24342135
		R	24342135
3	Fo047 infected pom0018	F	20415113
		R	20415113
4	Fo072 infected pla0020	F	25703094
		R	25703094
5	Fo047 infected pla0020	F	20628160
		R	20628160
6	Control pla0020	F	26205104
		R	26205104
7	Fo072 infected pla0001+pla0020+pom0018	F	22599802
		R	22599802
8	Control pla0001+pla0020+pom0018	F	21834394
		R	21834394

Table 3: Differentially expressed genes (DEGs) in pool-wise comparisons

No.	Pool Comparison	Upregulated	Down-regulated
1	(1,2)	0	2
2	(1,4)	0	4
3	(2,3)	7	5
4	(4,5)	4	3
5	(6,4)	1	2
6	(6,5)	14	44
7	(2,4)	11	22
8	(7,8)	38	12

Table 4: List of DEGs in comparison between pool 1 & 2

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similarity %
(1,2)	comp89206_c2	-11.54038298	18.97507013	0.00026615	replicase	0	0.805
(1,2)	comp85040_c0	-9.467582206	9.645267651	0.03326418	low quality protein: pentatricopeptide repeat-containing protein mitochondrial	4.03E-87	0.745

Table 5: List of DEGs in comparison between pool 1 & 4

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similarity %
(1,4)	comp87470_c0	-10.3903125	9.69151255	0.01716595	beta-mannosidase 4	1.96E-127	0.875
(1,4)	comp73492_c0	-8.56371542	8.44903231	0.04686721	glycosyl hydrolases family sugar binding domain protein	0	1
(1,4)	comp85040_c0	-8.14565324	9.64348676	0.04686721	low quality protein: pentatricopeptide repeat-containing protein mitochondrial	4.03E-87	0.745
(1,4)	comp64486_c0	-10.1372224	7.86455102	0.02174435	---NA---		

Table 6: List of DEGs in comparison between pool 2 & 3

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similarity %
(2,3)	comp81000_c0	13.48987423	6.57768476	0.01600293	probable 2-oxoglutarate-	3.06E-64	0.535

					dependent dioxygenase aop1		
(2,3)	comp86480_c0	13.42853084	6.51834983	0.01600293	ribulose bisphosphate carboxylase small chloroplastic-like	2.95E-93	0.84
(2,3)	comp84139_c0	12.66345989	5.78494911	0.02983247	probable xyloglucan endotransglucosylase hydrolase protein 32	9.12E-164	0.865
(2,3)	comp82171_c0	12.32248227	5.46265009	0.04353164	photosystem i reaction center subunit chloroplastic	4.59E-34	0.96
(2,3)	comp88488_c0	8.690980934	7.4640002	0.02006424	low affinity sulfate transporter 3-like	4.18E-162	0.88
(2,3)	comp89195_c0	8.389814076	8.93332185	0.02006424	ribulose bisphosphate carboxylase oxygenase activase chloroplastic-like isoform x2	0	0.89
(2,3)	comp85208_c0	7.796924909	7.15496203	0.04353164	hypothetical protein AMTR_s00071p00131570	1.12E-39	0.635
(2,3)	comp89126_c0	-7.438314223	9.11686857	0.04353164	eg45-like domain containing protein	2.23E-44	0.8
(2,3)	comp77334_c0	-7.46605484	9.46735177	0.04353164	---NA---		
(2,3)	comp84193_c1	-8.362932629	5.64039649	0.02983247	---NA---		
(2,3)	comp77159_c0	-10.51222674	8.16157012	0.00375482	bcl-2-associated athanogene-like protein	5.67E-41	0.595
(2,3)	comp87470_c0	-10.70077126	8.35187505	0.00375482	beta-mannosidase 4	1.96E-127	0.875

Table 7: List of DEGs in comparison between pool 4 & 5

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similarity %
(4,5)	comp76705_c0	15.2386036	8.05145862	0.000201	serine carboxypeptidase-like 12	9.43E-28	0.675
(4,5)	comp81374_c0	12.9925419	8.82664193	0.000201	acea_dencr ame: full=isocitrate lyase short=icl short=isocitrase short=isocitratase	0	0.905
(4,5)	comp61869_c0	12.4698054	5.28117004	0.01209122	serine carboxypeptidase-like 18-like	3.11E-28	0.72
(4,5)	comp87470_c0	10.3464785	10.4605982	0.00121584	beta-mannosidase 4	1.96E-127	0.875
(4,5)	comp81000_c0	-8.19842597	5.85601556	0.03802223	probable 2-oxoglutarate-dependent dioxygenase aop1	3.06E-64	0.535
(4,5)	comp92337_c1	-8.40270451	5.50303218	0.03802223	protein nrt1 ptr family -like	2.22E-63	0.77
(4,5)	comp92337_c0	-9.23029342	5.40283094	0.02821288	protein nrt1 ptr family -like	2.22E-63	0.77

Table 8: List of DEGs in comparison between pool 6 & 4

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similarity %
(6,4)	comp89799_c0	8.9091588	7.0843418	0.0226449	PREDICTED: uncharacterized	0	85.00%

					protein LOC103705038		
(6,4)	comp75407_c1	-9.83226597	6.63375541	0.01611902	hypothetical protein AMTR_s00071p00133840	8.70E-52	63.50%
(6,4)	comp80863_c0	-12.7847933	5.11299791	0.01611902	PREDICTED: calreticulin-like	4.67E-179	87.00%

Table 9: List of DEGs in comparison between pool 6 & 5

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similarity %
(6,5)	comp81374_c0	16.4710714	8.82044558	5.8482E-05	acea_dencr ame: full=isocitrate lyase short=icl short=isocitrase short=isocitratase	0	0.905
(6,5)	comp92539_c1	11.1818258	6.30524941	0.00150032	unnamed protein product	2.94E-20	0.635
(6,5)	comp76705_c0	9.31725363	8.04665218	0.00421251	serine carboxypeptidase-like 12	9.43E-28	0.675
(6,5)	comp61869_c0	9.25531872	5.26234346	0.01087757	serine carboxypeptidase-like 18-like	3.11E-28	0.72
(6,5)	comp89799_c0	8.14916974	6.24687936	0.01764863	PREDICTED: uncharacterized protein LOC103705038	0	0.85
(6,5)	comp70035_c0	7.83792783	6.4603944	0.02162419	---NA---		
(6,5)	comp75802_c0	7.38349787	4.32419497	0.04304563	PREDICTED: uncharacterized protein LOC103713846	4.48E-09	0.56
(6,5)	comp93972_c0	7.27762345	8.51502207	0.02954749	protein far1-related sequence 6-like	8.44E-34	0.46
(6,5)	comp87470_c0	7.27290828	10.4637285	0.02950104	beta-mannosidase 4	1.96E-127	0.875
(6,5)	comp91135_c0	7.13475507	8.76502037	0.02954749	b12d protein	4.79E-21	0.775

(6,5)	comp73378_c0	7.10836655	4.36185218	0.0424148	---NA---		
(6,5)	comp72119_c0	7.06112332	7.81098457	0.03041065	---NA---		
(6,5)	comp84924_c0	7.03980888	7.08685556	0.03091122	---NA---		
(6,5)	comp93827_c1	6.68174805	6.88651941	0.04440829	kelch repeat-containing protein at3g27220-like isoform x1	1.47E-49	0.93
(6,5)	comp85291_c0	-6.55858544	6.98029051	0.04782969	chlorophyll a-b binding protein cp24 chloroplastic	1.94E-117	0.975
(6,5)	comp74535_c1	-6.67625553	6.79443721	0.04565191	metallothionein-like protein type 2 isoform x2	3.9E-16	0.79
(6,5)	comp86505_c1	-6.69393553	5.3388841	0.04992394	burp domain-containing protein 12-like	7.12E-38	0.735
(6,5)	comp74026_c0	-6.71946741	5.36373199	0.0487295	chlorophyll a-b binding protein chloroplastic isoform x1	5.01E-30	0.99
(6,5)	comp88357_c0	-6.7315723	6.57224649	0.04565191	burp domain-containing protein 12-like	0	0.725
(6,5)	comp86630_c0	-6.75528879	7.53358704	0.0429385	hypothetical protein F383_32050	1.12E-85	0.95
(6,5)	comp91574_c0	-6.88055497	5.26598607	0.04782969	low quality protein: beta-xylosidase alpha-l-arabinofuranosidase 2-like	0	0.8
(6,5)	comp85672_c0	-6.96752937	6.90277676	0.03380745	glyceraldehyde-3-phosphate dehydrogenase a	0	0.915
(6,5)	comp84830_c0	-7.00919442	6.84550984	0.03380745	---NA---		
(6,5)	comp88440_c0	-7.151437	5.53019552	0.03894563	polyol transporter 5-like	7.44E-107	0.79
(6,5)	comp70566_c0	-7.18362222	6.53883601	0.03041065	glutaredoxin domain-containing cysteine-rich protein cg12206	2.94E-10	0.8
(6,5)	comp76614_c1	-7.32478049	6.81345876	0.02954749	protein proton gradient regulation chloroplastic-like	1.86E-49	0.785
(6,5)	comp88094_c0	-7.34150273	7.81980612	0.02950104	chlorophyll a-b binding protein chloroplastic	1.65E-126	0.985
(6,5)	comp78161_c0	-7.54579501	7.15575569	0.0281874	plastocyanin 1	9.6E-55	0.75

(6,5)	comp92181_c0	-7.63927742	5.29735037	0.02982436	protein chloroplastic	0	0.815
(6,5)	comp75712_c0	-7.72008684	3.90703995	0.04782969	floral organ number partial	2.7E-39	0.78
(6,5)	comp87902_c0	-7.81760912	4.00019319	0.04565191	beta-galactosidase 5-like isoform x2	2.11E-25	0.875
(6,5)	comp79774_c0	-8.18315286	4.35124165	0.03077061	---NA---		
(6,5)	comp81000_c0	-8.35689348	6.00152702	0.01857755	probable 2-oxoglutarate-dependent dioxygenase aop1	3.06E-64	0.535
(6,5)	comp83819_c0	-8.37817199	4.53964084	0.02954749	hypothetical protein EUGRSUZ_I00224	1.23E-146	0.795
(6,5)	comp75407_c1	-8.52090156	6.56510073	0.01231819	hypothetical protein AMTR_s00071p00133840	8.7E-52	0.635
(6,5)	comp82587_c0	-8.52315389	6.56732867	0.01231819	PREDICTED: uncharacterized protein LOC103995101	7.05E-63	0.585
(6,5)	comp70733_c0	-8.59432628	4.74926704	0.0281874	PREDICTED: uncharacterized protein LOC103723892	2.88E-27	0.765
(6,5)	comp84139_c0	-9.01657005	6.09216143	0.01087757	probable xyloglucan endotransglucosylase hydrolase protein 32	9.12E-164	0.865
(6,5)	comp81520_c0	-9.38083025	5.51797672	0.01231819	chlorophyll a-b binding protein chloroplastic-like	2.31E-93	0.95
(6,5)	comp92337_c1	-9.38780162	6.45908654	0.00736918	protein nrt1 ptr family -like	2.22E-63	0.77
(6,5)	comp86480_c0	-9.51980156	8.85734642	0.00380438	ribulose bisphosphate carboxylase small chloroplastic-like	2.95E-93	0.84
(6,5)	comp76223_c0	-10.1919856	2.68917192	0.04992394	PREDICTED: uncharacterized protein LOC104886708	2.16E-17	0.86
(6,5)	comp79139_c0	-10.2557473	2.74814861	0.04782969	---NA---		
(6,5)	comp73580_c0	-10.2626611	2.75455068	0.04782969	kda proline-rich protein	1.68E-33	0.99
(6,5)	comp66104_c0	-10.3366029	2.82310678	0.04565191	---NA---		

(6,5)	comp72471_c0	-10.349649	2.83521904	0.04565191	myb-related protein hv33-like	2.2E-48	0.93
(6,5)	comp75682_c0	-10.7546347	3.21359994	0.03077061	endoglucanase 3 isoform x1	0	0.91
(6,5)	comp73221_c0	-10.793345	3.25000241	0.03041065	probable wrky transcription factor 70	5.12E-22	0.66
(6,5)	comp75407_c0	-10.8723095	3.32438207	0.02954749	hypothetical protein AMTR_s00071p00131920	2.7E-29	0.585
(6,5)	comp74919_c0	-10.8902778	3.34132976	0.02954749	glucan endo- -beta-glucosidase 10-like	4.91E-148	0.885
(6,5)	comp80245_c0	-10.9255564	3.37462908	0.02954749	sterile alpha motif domain-containing protein	3.39E-35	0.76
(6,5)	comp83350_c0	-10.9684752	3.4151829	0.02954749	interactor of constitutive active rops 1-like isoform 1	1.13E-25	0.505
(6,5)	comp90927_c1	-11.0586294	3.50052116	0.02950104	PREDICTED: uncharacterized protein PHLOEM PROTEIN 2-LIKE A4-like	3.02E-44	0.71
(6,5)	comp92337_c0	-11.2250047	7.34311056	0.00150852	protein nrt1 ptr family -like	2.22E-63	0.77
(6,5)	comp69729_c0	-11.4524874	3.87563903	0.02001834	wrky transcription factor 18-like	2.64E-09	0.455
(6,5)	comp72436_c0	-11.4555077	3.87852936	0.02001834	dnaj homolog subfamily b member 1-like isoform x1	2.08E-93	0.675
(6,5)	comp74432_c0	-11.6915544	4.10502525	0.01735676	PREDICTED: uncharacterized protein LOC103973429	2.39E-28	0.775
(6,5)	comp82171_c0	-14.5453549	6.90606016	0.00082079	photosystem i reaction center subunit chloroplastic	4.59E-34	0.96

Table 10: List of DEGs in comparison between pool 2 & 4

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similariy %
(2,4)	comp81000_c0	12.4828383	5.8369301	0.01852488	probable 2-oxoglutarate-	3.06E-64	0.535

					dependent dioxygenase aop1		
(2,4)	comp92337_c0	12.0279555	5.40667813	0.03197076	protein nrt1 ptr family -like	2.22E-63	0.77
(2,4)	comp87803_c0	11.8599702	5.2492162	0.03858911	linalool synthase	5.35E-39	0.755
(2,4)	comp79330_c0	11.6784385	5.07997627	0.04135946	hypothetical protein POPTR_0014s02970g	4.45E-84	0.55
(2,4)	comp85358_c0	9.30870833	9.07671846	0.00778919	ferritin 4 isoform partial	1.64E-23	0.92
(2,4)	comp86361_c0	8.31027303	7.12215144	0.01852488	2-oxoglutarate-dependent dioxygenase dao-like isoform x2	4.85E-48	0.51
(2,4)	comp83280_c0	8.04961202	6.86870825	0.02574875	peroxidase 3	7.54E-81	0.8
(2,4)	comp75827_c0	7.65604739	7.44758787	0.02827803	aquaporin tip2-3	7.35E-101	0.93
(2,4)	comp86528_c1	7.3868364	6.78552878	0.03966766	c4-dicarboxylate transporter-like protein	5.04E-174	0.795
(2,4)	comp63894_c0	7.24232838	6.64601929	0.0439752	---NA---		
(2,4)	comp89206_c2	6.78133293	14.9589635	0.04517612	replicase	0	0.805
(2,4)	comp82617_c0	-6.79907911	8.45294469	0.0481965	1-aminocyclopropane-1-carboxylate synthase	0	0.79
(2,4)	comp66714_c0	-6.82795793	7.47462163	0.04993412	---NA---		
(2,4)	comp82760_c0	-6.99880133	7.01217366	0.0439752	ubiquinol oxidase mitochondrial	3.33E-131	0.92
(2,4)	comp66631_c0	-7.037223	8.88956338	0.03858911	---NA---		
(2,4)	comp83107_c0	-7.0431338	11.5029762	0.03858911	rhodanese-like domain-containing protein 17 isoform x2	4.19E-29	0.81
(2,4)	comp81099_c0	-7.06604919	8.15316147	0.03858911	---NA---		
(2,4)	comp78258_c1	-7.15117037	6.27994263	0.04069773	---NA---		
(2,4)	comp75065_c0	-8.07358002	6.63524273	0.01781969	chemocyanin expressed	1.59E-48	0.825
(2,4)	comp85268_c0	-8.28573424	8.31093553	0.01175568	---NA---		
(2,4)	comp87470_c0	-8.35746792	8.3830799	0.01175568	beta-mannosidase 4	1.96E-127	0.875
(2,4)	comp87595_c0	-8.47961746	6.63684662	0.01175568	probable acyl-activating enzyme peroxisomal	8.33E-110	0.695
(2,4)	comp91191_c0	-8.70146809	8.72879763	0.01082214	PREDICTED: uncharacterized protein	0	0.9
(2,4)	comp77159_c0	-8.7152694	8.19178693	0.01082214	bcl-2-associated athanogene-like	5.67E-41	0.595

					protein		
(2,4)	comp75748_c0	-8.86218127	6.45164532	0.01421828	---NA---		
(2,4)	comp52067_c0	-9.12029453	4.81961699	0.01999393	---NA---		
(2,4)	comp87595_c1	-9.58894349	7.50869799	0.00778919	probable acyl-activating enzyme peroxisomal	4.11E-87	0.84
(2,4)	comp81449_c0	-9.66075471	6.31290723	0.01152942	mannose-specific lectin 3-like	1.36E-73	0.66
(2,4)	comp82488_c0	-10.3284437	6.10029904	0.00848571	serine threonine-protein kinase at5g01020-like	1.49E-149	0.665
(2,4)	comp82617_c1	-11.222495	4.31611743	0.03568039	1-aminocyclopropane-1-carboxylate synthase	0	0.79
(2,4)	comp84193_c1	-12.472882	5.66796787	0.01082214	---NA---		
(2,4)	comp83853_c2	-13.2517864	6.47907313	0.00778919	---NA---		
(2,4)	comp77334_c0	-16.2189849	9.48625926	0.00013195	---NA---		

Table 11: List of DEGs in comparison between pool 7 & 8

pool	comp	logFC	logCPM	FDR	Sequence description	e-value	Similariy %
(7,8)	comp77334_c0	12.5652466	4.70361658	0.00680029	---NA---		
(7,8)	comp92539_c1	12.243378	4.3837469	0.00700834	unnamed protein product	2.94E-20	0.635
(7,8)	comp72301_c0	11.9888626	4.13115325	0.00986469	---NA---		
(7,8)	comp70539_c0	11.8062744	3.95016323	0.00986469	---NA---		
(7,8)	comp89953_c0	11.4740399	3.62139472	0.0132163	egf domain-specific o-linked n-acetylglucosamine transferase-like	1.65E-72	0.74
(7,8)	comp81810_c0	11.1761408	3.32733661	0.01668071	transcription factor bhlh3	2.73E-84	0.62
(7,8)	comp84172_c0	11.057092	7.29043951	0.00207847	endochitinase a-like	4.03E-66	0.825
(7,8)	comp66780_c0	10.673077	2.83277819	0.03263602	---NA---		
(7,8)	comp88422_c0	10.3876493	2.55360103	0.04032597	probable acyl-activating enzyme peroxisomal	0	0.79
(7,8)	comp76973_c0	10.3790937	2.54525157	0.04032597	---NA---		

(7,8)	comp79771_c0	10.2578621	12.3241646	0.00207847	cationic peroxidase 1-like	3.45E-151	0.865
(7,8)	comp69486_c0	10.215725	2.38605033	0.04939046	---NA---		
(7,8)	comp81628_c0	9.61689979	6.40916989	0.00680029	protein p21	2.2E-68	0.675
(7,8)	comp54122_c0	9.57534851	7.55571021	0.00680029	PREDICTED: uncharacterized protein LOC103720244	3.55E-53	0.715
(7,8)	comp72693_c0	9.40686307	6.19979608	0.00680029	probable inorganic phosphate transporter 1-4	5.44E-44	0.935
(7,8)	comp88675_c0	9.27484707	4.59867094	0.00986469	polyphenol chloroplastic-like	0	0.785
(7,8)	comp73144_c0	9.18526257	5.97900723	0.00698029	chalcone synthase	7.6E-39	0.92
(7,8)	comp79640_c1	8.98259196	6.74650882	0.00698029	chitinase 2-like	9.14E-138	0.825
(7,8)	comp88653_c0	8.91930187	9.90325799	0.00680029	chalcone synthase	0	0.915
(7,8)	comp78646_c0	8.89864531	4.22563866	0.01464438	mitogen-activated protein kinase kinase kinase 2-like	5.59E-86	0.635
(7,8)	comp91069_c1	8.82292003	5.06357242	0.00986469	---NA---		
(7,8)	comp89126_c0	8.78869701	5.02957098	0.00986469	eg45-like domain containing protein	2.23E-44	0.8
(7,8)	comp68357_c0	8.43945609	4.68291437	0.01464438	hypothetical protein	6.38E-07	0.7
(7,8)	comp81158_c0	8.38170306	6.55508956	0.00986469	phenylalanine ammonia-lyase	0	0.93
(7,8)	comp86449_c0	8.24223199	3.57711872	0.03202686	polygalacturonase qrt3- partial	0	0.75
(7,8)	comp90716_c0	8.19697826	4.99630995	0.01472929	probable 4-coumarate-- ligase 3	0	0.795
(7,8)	comp84690_c0	7.95793193	7.02877943	0.01283707	---NA---		
(7,8)	comp77947_c0	7.92251144	3.26270714	0.04032597	---NA---		
(7,8)	comp60180_c0	7.65650954	11.3008191	0.01464438	---NA---		
(7,8)	comp93972_c0	7.56942108	7.97517412	0.01620138	protein far1-related sequence 6-like	8.44E-34	0.46
(7,8)	comp93654_c0	7.49142756	6.36076426	0.02010601	phenylalanine ammonia-lyase	0	0.905
(7,8)	comp85418_c0	7.46348998	11.1168189	0.0170046	mannose-specific lectin 3-like	6.57E-35	0.545
(7,8)	comp75899_c0	7.45501711	4.97166735	0.02946692	---NA---		
(7,8)	comp80992_c0	7.38902128	4.59474765	0.03463619	protein lurp-one-related 6-like	5.6E-30	0.64
(7,8)	comp93994_c0	7.11527017	9.66401685	0.02941337	---NA---		
(7,8)	comp80872_c0	7.0900298	4.61087243	0.04032597	21 kda	2.96E-45	0.68

(7,8)	comp79847_c0	7.01247925	5.64799983	0.0381171	invertase inhibitor	7.47E-17	0.52
(7,8)	comp68655_c0	6.96497071	7.95806022	0.03487776	---NA---		
(7,8)	comp76614_c1	-6.6838464	8.06474133	0.04171929	protein proton gradient regulation chloroplastic-like	1.86E-49	0.785
(7,8)	comp86480_c0	-6.73630676	9.3346547	0.04032597	ribulose bisphosphate carboxylase small chloroplastic-like	2.95E-93	0.84
(7,8)	comp88094_c0	-6.76876491	9.04926575	0.04032597	chlorophyll a-b binding protein chloroplastic	1.65E-126	0.985
(7,8)	comp79980_c0	-6.89303611	7.83244375	0.03711688	photosystem i subunit o isoform x1	2.93E-38	0.77
(7,8)	comp65921_c0	-7.06502863	4.81269637	0.04032597	c-repeat binding factor 1	3.67E-31	0.825
(7,8)	comp83331_c0	-7.8968341	3.20870118	0.04185242	protein hothead-like	0	0.86
(7,8)	comp81520_c0	-8.31367794	7.03551847	0.00986469	chlorophyll a-b binding protein chloroplastic-like	2.31E-93	0.95
(7,8)	comp74432_c0	-8.79914296	5.56630661	0.00986469	PREDICTED: uncharacterized protein LOC103973429	2.39E-28	0.775
(7,8)	comp80001_c0	-8.83772949	4.1379063	0.0152355	thioredoxin-like protein chloroplastic	2.2E-114	0.86
(7,8)	comp82847_c0	-8.95781314	6.69330577	0.00698029	light-regulated protein precursor	3.64E-10	0.66
(7,8)	comp82723_c0	-10.4163862	2.57641853	0.04032597	---NA---		
(7,8)	comp77659_c0	-11.7083689	3.85073401	0.00986469	fasciclin-like arabinogalactan protein 13	5.57E-54	0.865

CHAPTER V **GENERAL**

DISCUSSION

Because it has received little attention from research and particularly from plant pathologists, vanilla is sometime considered an orphan species. Indeed, no more than a dozen of scientific articles have dealt in detail with RSR between the first description of the disease by Tucker in 1927 and the recent works of Pinaria *et al.* in 2010 and 2015. All of the knowledge about RSR dated back to works conducted in Puerto Rico and Madagascar in the 1950s. During my thesis work, I studied the RSR of vanilla with the aim of determining the taxonomic position of its causal agent and more importantly, of investigating its relationships with vanilla genotypes. The longer-term objective of this work is to contribute to the selection of vanilla cultivars resistant to this devastating disease.

I Etiology of RSR

The deep survey and large collection of strains isolated from root and stem samples of diseased vanilla plants showed that *F. oxysporum* was the main species responsible for the RSR in Reunion Island. This study is the first to demonstrate the occurrence of RSR of vanilla in Reunion Island. The isolates were identified by sequencing housekeeping genes (EF1-α and IGS) and their pathogenicity was assayed by a robust *in-vitro* test. The predominance of *F. oxysporum* (80%) is consistent with the previous studies (He, 2007, Pinaria et al., 2010b, Vijayan et al., 2012b) which also reported *F. oxysporum* as the principal agent responsible for the RSR in China, Indonesia and India. By adding the surveys realized in French Polynesia and Madagascar, and analyzed in this study, we concluded that pathogenic strains of *F. oxysporum* threatens vanilla everywhere it will be cultivated.

The question of the symptomatology and etiology of RSR has been somewhat debated. Based on our results and other similar studies it is clear that the fungus is mostly found in the roots but can also be found in stems. After experimental inoculation with conidial suspension symptoms and hyphae penetration could be observed in the hairy region of roots and in stem

at the junction with aerial roots. Taking into account the observations of microscopic images showing a cortical rather than vascular tropism of the fungus, we propose to name the causal agent of RSR: *F. oxysporum* f. sp. *radicis-vanilla* (Forv). However, the finding in French Polynesia of a *F. oxysporum* strain isolated from a fruit that showed a high pathogenicity in *V. planifolia* and *V. xtahitensis* roots suggests that Forv may also attack other parts of the plant.

Phylogenetic studies revealed a substantial amount of genetic diversity among the isolates from Reunion Island. This corroborates previous analyses of Chinese (He, 2007) and Indonesian (Pinaria et al., 2010) populations of *F. oxysporum* which contained isolates from several distinct lineages. This polyphyletic nature of Forv is in agreement with O'Donnell *et al.* (1998) who stated that the isolates in a *forma specialis* may not be closely related or may have evolved from a divergent ancestors, making *Fusarium* a complex species (Kistler, 1997, O'Donnell & Cigelnik, 2007). The high amount of genetic diversity resulted in no clusters that clearly identified neither the geographic origin, nor the pathogenicity of the isolates.

The unambiguous identification of the pathogen is important for implementing RSR control actions, for instance for insuring the production of pathogen free planting material, or for monitoring the disease in research or eradication programs. As far as we know, no molecular determinant of *Fusarium* pathogenicity has been found that can enable to trace the soil-borne pathogenic strains. Recent genomic studies of *F. oxysporum* isolates unraveled several possible candidate genes or genetic elements that could be involved in their pathogenicity (Ma et al, 2013). The large and diverse collection of Forv isolates characterized during this study can be very useful for addressing this important issue for vanilla.

Fungi from other species of *Fusarium* such as *F. solani, F. proliferatum, F. concentricum, F. mangiferae*, have also been isolated from the samples collected. The relationships of these species with vanilla would be worth analyzing. In the case of *F. solani*

that is recovered in significant amounts it could be a secondary invader of vanilla tissues accompanying Forv infections. However, since a few of our isolates showed some pathogenicity in the in-vitro tests, the role of *F. solani* as a possible primary pathogen (Anandaraj *et al.*, 2005) could be investigated further. The minor species isolated could be secondary invader as well. However, they could also be endophytic species and as such would deserve been studied.

II Infection pattern and defense responses in vanilla root

The infection pattern of *F. oxysporum* in vanilla was described earlier (Alconero, 1968; Tucker, 1927). However this study using modern imaging devices provided for the first time a clear overview, in time and space, of the penetration and colonization of vanilla roots by Forv. The colonization pattern of the pathogen, restricted to cortical cells, and the subsequent symptoms were similar to those induced by other f. spp. *radices* in their respective host (Vakalounakis et al., 2005, Benaouali et al., 2014). It is also in agreement with the first description of the infection reported by Alconero in 1968. But, contrary to previous reports from Alconero (1968) and Tonnier (1960), our results showed that wounds are not necessary for hyphae penetration in the root.

The compatible and incompatible interactions between *Fusarium* and *Vanilla* were also documented in this study. Differences in the colonization pattern of Forv associated to defense responses of resistant accessions have been identified by comparing a susceptible accession (pla0001) and two resistant (pla0020 and pom0018). The defense responses in resistant genotypes always restricted the hyphae at the hypodermal region (Brammall & Higgins, 1988). This guaranteed the proper functioning of the vanilla roots, and thus the plant remaining healthy and unaffected by the pathogen.

Phenolic compound and particularly lignin seems to play an important role in resistance against the pathogen. Spectral analyses indicated the presence of several phenolic precursors

that could contribute to the strengthening of the hypodermal cell walls of resistant accessions, which does not occur in the susceptible accession. Phenolic compounds are the most frequently accumulated elements in the cells involved in defense response of plants to pathogens (Fang et al., 2012, El Modafar & El Boustani, 2001).

In addition, the cell wall architecture of resistant and susceptible accessions of vanilla also determines the resistance to a pathogen. The hypodermal walls of resistant accessions are provided with continuous and thick barrier of lignin, while the susceptible have thin and discontinuous lignin deposition on the outer cell wall. This hydrophobic layer can also serve as a barrier from the cell wall degrading enzymes of the pathogenic fungus. The formation of this barrier seems to result from a constitutive and a pathogen-induced mechanism of lignification in the two resistant accessions studied.

Knowing the time frame and topology of fungus colonization plant-resistance response will enable to investigate the genetic basis and physiological processes governing resistance to Forv. RNA Seq of laser dissected hypodermal cells 1 to 4 days after inoculation should be appropriate material and methodology for studying the genes elicited during Forv infection.

Physiological processes at work in the resistance mechanism in roots should consider in the same time those activated in the vanillin biosynthesis in fruits. Indeed both rely on the phenylpropanoids pathways and lignin precursors. Although occurring in different organs, they might interfere one with the other.

III Developping resistance to Forv

Two hundred and fifty four Vanilla accessions belonging to 18 species and 6 hybrids have been assessed for RSR resistance in long term assays in the field and in controlled *in-vitro* conditions. Interestingly, and surprisingly, there was a very good correlation between field and *in-vitro* evaluation. This makes this pathogenicity test based on plantlet root

inoculation a very powerful method for evaluating with a relatively high throughput and at an early stage of development the resistance to Forv of new vanilla progenies.

Our RSR assays confirmed the high resistance level of *V. pompona* as well as the resistance of eight hybrids of *V. planifolia* with *V. pompona* and *V. phaeanta* previously reported (Tucker, 1927, Delassus, 1963., Tonnier, 1960a). They also revealed novel sources of RSR resistance such as *V. bahiana,* a close relative to the resistant species *V. phaeanta, V. costariciensis* and *V. crenulata*. Most of the *V. planifolia* accessions, *V. ×tahitensis* and *V. odorata* were susceptible to RSR. The finding of three *V. planifolia* accessions obtained from autopollination of an ordinary *V. planifolia* (including pla0020) showing a high level of resistance to Fo072 is extremely promising. Indeed it demonstrated that a breeding strategy based on *V. planifolia* selfing could be efficient for obtaining F1 progenies having both resistance to Forv, and aromatic properties close to those of the Bourbon vanilla. This is a great advantage compared to interspecific hybridization strategies that have been used up to now (Theis & Jimenez, 1957a, Belanger & Havkin-Frenkel, 2010), which need to back cross the hybrids with the ideotype for recovering potential commercial varieties.

Based on i) the gradual pattern observed in our pathogenicity and plant resistance tests, and ii) the evidences of at least dual resistance mechanisms in hypodermal cells, we hypothesize that the resistance to Forv could have a multigenic support. This could be a favorable element regarding the durability of the resistance. In addition to Fo072, pla0020 has been challenged against nine other highly or moderately pathogenic (on pla0001) isolates and always showed high resistance (data not shown).

Apart from resistance to RSR, pla0020 showed indehiscence of the fruits which is an uncommon albeit favorable character in *V. planifolia*. Indeed, this trait allows full maturation of the fruit on the vine, a more efficient curing process and probably richer aromas in the

cured pods, which might compensate for the slightly lower vanillin content of this pla0020 compared to ordinary *V. planifolia*. Pla0020 showed excellent productivity in shade house cultivation system and is therefore a very promising *V. planifolia* accession. It is in the process of protection by UPOV (International union for the protection of new varieties of plants) which has recently approved test guidelines for vanilla.

IV Control of RSR

Our results confirmed the potential of genetic resistance for controlling RSR. However, in order to durably preserve the resistant varieties that could be broadcast, it will be important to maintain efforts aimed at limiting Forv populations. Vanilla is a perennial crops and recent studied showed that the continuous monoculture of vanilla plants results in substantial increase in the *Fusarium* populations in soils (Xiong et al., 2014). Our data showed that a wide diversity of pathogenic isolates were present in vanilla vines, including into the same plant, and these isolates can survive for years in the soil as chlamydospore.

The use of biocontrol agents or agronomical practices can limit the pathogenic activities of Fusarium. In our studies the behaviour of some accessions in the shadehouses, like pla0038, suggested that particular soil or endophytic agent may have protected the vines from RSR. Therefore it seems to me that enhancing our knowledge of the role of microbial communities in the soil and in the plant is of great importance and potential for improving the management of RSR in the near future.

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LIST OF FIGURES

LIST OF TABLES

ANNEXURE

I - Abtract of communication given at 5th International Orchid Conservation Congress, 2-6 December 2013, Saint Denis, La Réunion.

II - Poster presented at the 9th Colloquium of *Société Française de Phytopathologie.* 2-5 June 2015, Colmar, France. (Awarded as best student poster by the Colloquium jury)

II - Poster presented at the 9th Colloquium of *Société Française de Phytopathologie.* 2-5 June 2015, Colmar, France.

ANNEXURE I

Koyyappurath S, Le Guen R, Le Squin S, Gautheron N, Hermann Edel V, Peribe J, …, Grisoni M, 2013. Assessment of genetic and pathogenic diversity of Fusarium isolates from the Indian Ocean area for the screening of resistance to root and stem rot in vanilla germplasm.

Abstract : Root and stem rot of vanilla caused by *Fusarium oxysporum* f. sp. *vanillae* (FOV) is one of the most important constraints to vanilla cultivation all over the world. Breeding for resistance is considered the best approach to control *Fusarium* diseases, but until now, very few sources of resistance to FOV have been identified in the vanilla genetic pool. With the aim of screening vanilla germplasm for resistance to FOV, the two crucial factors, i.e. the choice of challenge isolates and the inoculation procedure, were investigated. In the present study, 118 *Fusarium* isolates collected from diseased vanilla plants in the Reunion Island (108), Madagascar (6) and Indonesia (4) were genotyped by elongation factor1-α gene and intergenic spacer region sequencing, and evaluated for pathogenicity using *V. planifolia* (CR001) as susceptible host. The majority of the isolates belonged to *F. oxysporum* (78%) and *F. solani* (10%). Among the various inoculation methods tested, root dipping of *in vitro* plants in a 10^6 conidia/ml suspension of the fungal isolate proved to be fast and reproducible. For the pathogenic isolates, symptoms appeared on the 4th day post inoculation and the plantlet was necrotic on the 14th day. The isolates were differentiated into highly pathogenic (16%), moderately pathogenic (22%), slightly pathogenic (19%) and non-pathogenic (43%), according to the area under the disease progress curve calculated on four replicates. The procedure used in this study is fast and robust for assessing the pathogenicity of *Fusarium* isolates and will enhance the search for FOV resistant vanilla.

Root colonization patterns of *Fusarium oxysporum* f.sp. *radicis-vanillae* in susceptible and resistant genotypes of vanilla

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Aim of the study

- Describe the colonization process of *F. oxysporum* f.sp. *radicis-vanillae* (Forv) in vanilla roots
- Examine the histopathological responses of vanilla to Forv

Material & Methods

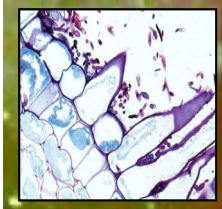
- ***In-vitro* plants** of three accessions from two species:
  - **Susceptible** *V. planifolia* pla01
  - **Resistant** *V. planifolia* pla20
  - **Resistant** *V. pompona* pom18

Autofluorescence of vanilla root (transv. sect.)

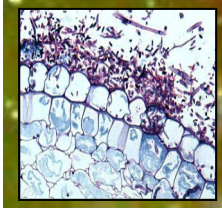
- **Root dip inoculation** with a conidial suspension of highly pathogenic isolate Fo072

- **Wide field & Multiphoton microscopy** : Schiff - Naphthol Blue Black, Phloroglucinol-HCl and DAPI staining

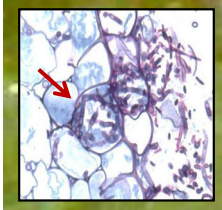
Colonization (pla01)



1-2 dpi: microconidia germinate in the hairy root region

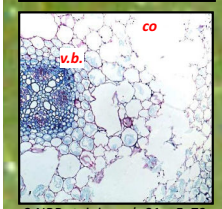


2-3 dpi: hyphae penetrate the root epidermis



3-4 dpi: hyphae migrate across hypodermis → passage cells

4-7 dpi: hyphae colonize the cortex through intercellular spaces

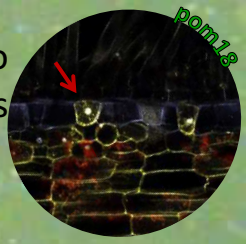


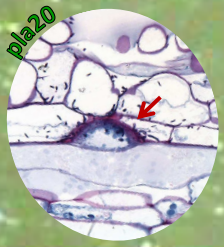
S-NBB staining pla01 + Fo72

7-9 dpi: Forv disrupts cortical cells; vascular bundle intact

Defense response to Forv (pla20 & pom18)

Entrapment of hyphae into modified hypodermal cells

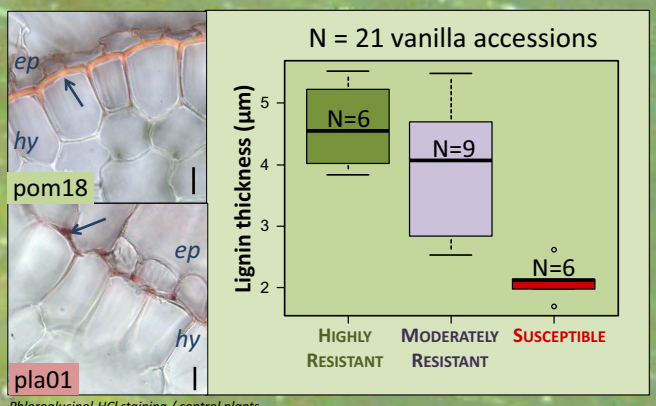




Formation of a polysaccharide barrier by the hypodermal cells

➢ **Forv is restricted to peripheral root layers**

Constitutive defense



Phloroglucinol-HCl staining / control plants

➢ **Continuity and thickness of lignin deposition on hypodermis correlates with resistance to Forv**

Conclusions

1 – The colonization pattern of Forv in vanilla roots has been described

2 – Hypodermal lignin deposition is involved in constitutive and induced resistance of vanilla to Forv

3 – These topologic observations opens new perspectives towards identification of resistance genes to Forv in vanilla genotypes

This research was supported by the Regional Council of La Réunion and the European Social Fund (PhD scholarship to Sayuj Koyyappurath) and the ANR Preciput program

Screening for resistance to *Fusarium oxysporum* f.sp. *radicis-vanillae* (Forv) in vanilla

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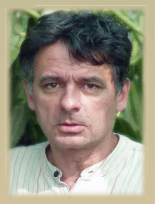
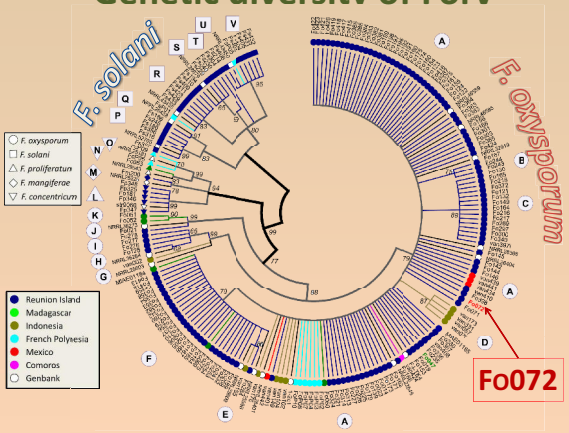


OBJECTIVE: To identify vanilla genetic resources having a high level of resistance to Forv, the causal agent of Root and Stem Rot (RSR)

RSR a devastating disease of vanilla

healthy vanilla vine | diseased plant | root rot and proliferation | stem rot

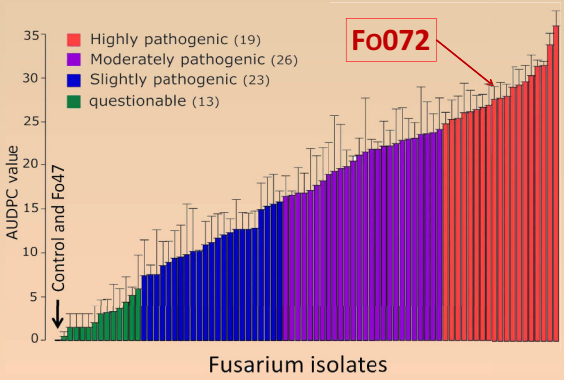
Genetic diversitv of Forv



ML tree inferred from the EF1α gene sequence of *Fusarium* strains isolated from vanilla. F. *oxysporum* and *F. solani* are the most frequent species

SELECTION OF FO072: a stable, highly pathogenic Forv isolate for challenge inoculations

Pathogenicity gradient in Forv

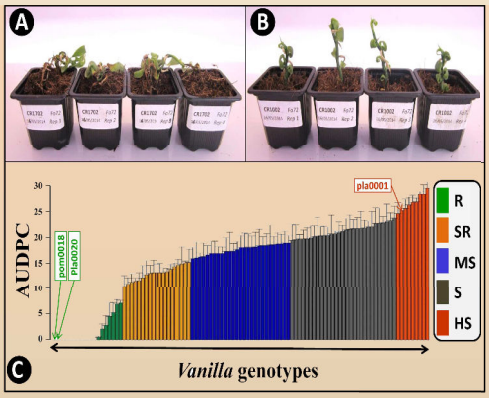


Wide range of pathogenicity of *F. oxysporum* isolates assessed by plantlet inoculation on susceptible accession pla0001

VALIDATION OF THE PLANTLET ROOT INOCULATION METHOD (PRIM) FOR ASSESSING RSR RESISTANCE

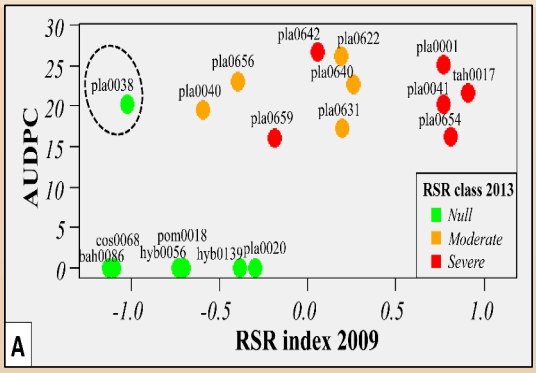
Conidial suspension at 10^6cfu

PRIM - 5 min



PRIM evaluation of resistance.
Susceptible (A) and resistant (B) plantlets 15 dpi C) Gradient in resistance levels (AUDPC) ranging from resistant (R) to highly susceptible (HS)

Examples of susceptible and resistant accessions in the field



Correlation between PRIM evaluation (AUDPC) and long term field evaluation of resistance in the field.

CONCLUSIONS:

1 – **PRIM is fast and reliable** for selecting Forv-resistant vanilla genotypes.

2 – **New sources of resistance** to Forv have been evidenced including *Vanilla planifolia* inbreeds and hybrids, *V. bahiana, V. costaricensis* and *V. crenulata.*

3 – **Our results open new perspectives for:**

* **developing** novel Bourbon and Tahitian vanilla varieties resistant to RSR.
* **investigating** the genetic determinants of Forv resistance in vanilla.

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