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Susceptibility of *Pinus pinaster*'s families to Pine Pitch Canker Caused by *Fusarium Circinatum*

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List of abbreviations

EFSA: European Food Safety Authority

EPPO: European and Mediterranean Plant Protection Organization

EU: European Union

F.C: *Fusarium circinatum*

FcCa6: *Fusarium circinatum* Cantabria 6

GPS: Global Positioning System

MAT: Mating type

PDA: Potato Dextrose Agar

PDAS: Potato Dextrose Agar with Streptomycin

PDB: Potato Dextrose Broth

PLH: Panel on Plant Health

PPC: Pine Pitch Canker

SDW: Sterile Distilled Water

Abstract

Fusarium circinatum is the causal agent of Pine Pitch canker disease which is an introduced non-native disease on pines in natural and planted stands of Europe. It is an exotic pathogen of recent introduction in Spain that threatens *Pinus pinaster* stands. PPC has been detected in over the world and especially in the north of Spain, France, Portugal, and Italy concerning its presence in Europe. The disease is causing damages in forests and nurseries. Variability between the families of the same species even from the same provenance area may affect the level of susceptibility of each family to *Fusarium circinatum.* In this study, it has been tested the resistance or the susceptibility of 22 different families of *Pinus pinaster* from Galicia (North of Spain) to F.C. and each family is a "family of genetic improvement program of Galicia". Investigating Resistance across families allows for a better understanding of management opportunities with *Pinus pinaster* populations in Galicia. Therefore, 550 seeds were sown and inoculated with 250 spores/ml of the fungal suspension of *Fusarium circinatum* under laboratory conditions. The same amount of seeds was sown and inoculated with distilled water as a control for the previous assay. The results revealed that the families 50,105 and 5 are the most susceptible to F.C. and on the other hand, the families 109,33 and 35 are the most resistant to *Fusarium circinatum*. Consequently, the families 109,33 and 35 are the most suitable families among those tested in this experiment for better management option in order to slow down the negatives impacts of Pine Pitch canker caused by *Fusarium circinatum* in the *Pinus pinaster* stands plantation in Galicia.

Keywords: Susceptibility; *Pinus pinaster*; family; *Fusarium circinatum*; Pine Pitch Canker; Galicia.

Resumen

Fusarium circinatum es el agente causal de la enfermedad del cancro del tono del pino, que es una enfermedad no nativa introducida en pinos en rodales naturales y plantados de Europa. Es un patógeno exótico de reciente introducción en España que amenaza los rodales de *Pinus pinaster*. Se ha detectado PPC en todo el mundo y especialmente en el norte de España, Francia, Portugal e Italia en relación con su presencia en Europa. La enfermedad está causando daños en bosques y viveros. La variabilidad entre las familias de la misma especie, incluso de la misma área de procedencia, puede afectar el nivel de susceptibilidad de cada familia al *Fusarium circinatum*. En este estudio, se ha probado la resistencia o la susceptibilidad de 22 familias diferentes de *Pinus pinaster* de Galicia (norte de España) a F.C. y cada familia es una "familia de programa de mejora genética de Galicia". Es bueno conocer a las familias con más resistencia entre las que se probaron para lograr un mejor manejo con las poblaciones de pino piñonero en Galicia. Por lo tanto, se sembraron 550 semillas y se inocularon con 250 esporas / ml de la suspensión fúngica de *Fusarium circinatum* en condiciones de laboratorio. Se sembró la misma cantidad de semillas y se inoculó con agua destilada como control para el ensayo anterior. Los resultados revelaron que las familias 50,105 y 5 son las más susceptibles a F.C. y, por otro lado, las familias 109,33 y 35 son las más resistentes al *Fusarium circinatum*, por lo tanto, las familias 109,33 y 35 son las familias más adecuadas entre las que se examinaron en este experimento para una mejor opción de manejo con el fin de reducir la velocidad. Impactos negativos del cancro de Pine Pitch causado por *Fusarium circinatum* en la plantación de plantaciones de *Pinus pinaster* en Galicia.

Palabras clave: Susceptibilidad; *Pinus pinaster*; familia; *Fusarium circinatum*; Chancro resinoso del pino; Galicia.

1. Introduction

For a long time, the global forest cover has been declining steadily year after year. There are several factors behind this global destruction of global forest cover that has economic and environmental impacts. Among these factors, there are diseases caused by some destructive pathogens such as *Fusarium circinatum*. *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph *Gibberella circinata*), known to cause pitch canker, is a fungus with great virulence in most of the *Pinus* species (Vivas, Zas, & Solla, 2012). The fungus *Fusarium Circinatum* was first identified in California, USA, in 1986, when it was isolated from diseased *Pinus radiata* (Monterey pine or radiata pine) in Santa Cruz County (Schmale & Gordon, 2003). The disease of pine species caused by the fungus *Fusarium circinatum* Niremberg & O'Donnell is called Pitch canker (P. Martínez-Álvarez, 2012). *Fusarium Circinatum* has spread and been detected worldwide for instance in South Africa (Viljoen et al., 1994); America: USA (Hepting, G.H.; Roth, 1946)**,** Haiti(Hepting, G.H.; Roth, 1953); Mexico(Guerra-Santos, J.J.; Cibrián-Tovar, 1998), Chile (Wingfield et al., 2002); Uruguay (Alonso, R.; Bettucci, 2009); Colombia (Steenkamp et al., 2012) and Brazil (Pfenning et al.,2014); Asia: Japan (Muramoto, M; Dwinell, 1990) and South Korea (Lee et al, 2000)**.** In Europe, the Pine Pitch Canker disease has been reported for the first time in Spain (P. Martínez-Álvarez, 2012). The disease has been also reported in others European countries such as Portugal (Bragança et al., 2009); Italy (Carlucci et al. 2007) and France (figure 1) but it has been eradicated totally in the last 2 countries (EPPO, 2006)**.**

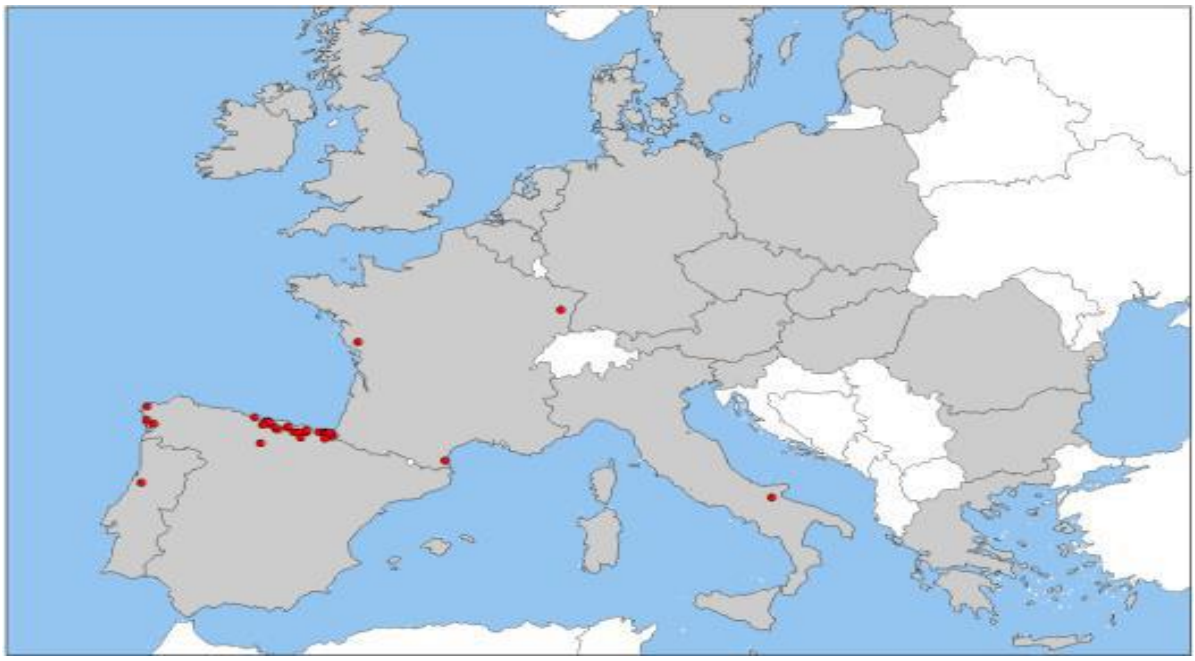

Figure 1: Pine Pitch Canker's disease distribution in the EU based on data registered until late 2009 (EFSA's Plant Health Panel (PLH),2010)

There are many ways of contamination of *Fusarium circinatum* and it spreads via contaminated seeds, seedlings, wood material, soil, wind, insect vectors and human activities. The spores infect wounds in trees caused by storms, hail, insects and pruning (Möykkynen, Capretti, & Pukkala, 2015). Nevertheless, bleeding is the most common symptom of the disease, resinous canker on the trunk, terminals or large branches (Hepting, G.H.; Roth, 1946). The disease is most common in coastal areas because moisture is necessary for successful spore infection. The infected trees grow slowly and suffer from branch and stem cankers and may die. Bark beetles commonly breed in infected branches and spread the pathogen when they fly to other trees (EPPO, 2005). Furthermore, roots, shoots, female flowers, mature cones, seeds and seedlings may be affected (Wingfield et al., 2008). The main symptom of PPC in adult trees is the presence of pitch-soaked cankers in trunks and big branches which girdle both trees and branches (Wikler et al, 2003). The disease can affect the crown when suitable wounds are available for infection (Gordon et al., 2001), causing dieback (Blank et al., 2019) that can lead to tree death. The wilting and discoloration of needles, which eventually turn red and finally fall off, is a common symptom of the disease as well (Bezos et al.,2017; Wingfield et al., 2008) (figure 2).

Figure 2: Pine Pitch Canker' symptoms: (a) canker; (b) defoliation; (c) dieback (Blank et al.,2019).

Furthermore, a previous study has showed that variation in susceptibility to pitch canker has been demonstrated for Monterey pine within both native and planted stands (Storer et al., 1999), based on the lengths of lesions developing on branches subjected to mechanical inoculations. A study using clonal lines of Monterey pine showed the ranking of pine genotypes based on lesion length to be independent of the location where trees were grown (Gordon et al., 1998).However, Biological control and genetic resistance represent the resources that may ultimately prove to be useful in the management of pitch canker in California and elsewhere in the world(Schmale & Gordon, 2003).

Additionally, Different families of *Pinus* inoculated with *F. circinatum* have consistently shown significant differences in susceptibility (Barrows-Broaddus J, 1984; Dwinell LD, 1979; Gordon et al., 1998). *Therefore at least 60 species of Pinus along with Pseudotsuga menziesii (Mirb.)* Franco are known to be susceptible to PPC. Variation in susceptibility occurs not only among species, but also among provenances. Thereby, considering the high genetic variation among the *Pinus ssp*, testing the susceptibility of *Pinus Pinaster* provenances and families should be a priority.

Even further, and concerning this work, especially, the Maritime pine of the north of Spain in Galicia has a very high genetic variability within and between its enormous families; therefore, testing the susceptibility of those Galicia's maritime pine families to *Fusarium Circinatum* is of paramount importance.

The main aim of this work is to check the susceptibility of several *Pinus Pinaster*'s families (Maritime Pine) from Galicia (north of Spain) to *Fusarium Circinatum* through the inoculation of the seeds with a specific concentration of the F.C. inoculum fixed at 250 spores/ml as fungal suspension for this study. As a specific goal, the most resistant families to *Fusarium circinatum* will be identified and promote for better silviculture management plan in order to slow down the effect of Pine Pitch Canker caused by *Fusarium circinatum* in *Pinus pinaster* plantations in Galicia.

2. Materials and methods

Any successful scientific research work requires a well-constructed methodology because it is the key to success. In order to achieve the objectives, set in this study, the following points will be addressed:

2.1. Fungal Material

In this experiment the typical fungal material used is the *Fusarium circinatum* (FcCa6) which belongs to mating type 2 (MAT-2) and was isolated from an infected *Pinus radiata* tree located in Comillas (Cantabria, Northern Spain; GPS: 4_17017.706" W; 43_2005.033" N; 265 m above sea level) (Martín-García et al., 2017; Martínez-Alvarez et al., 2012; Martínez-Álvarez et al., 2014). The mycelium of the fungi *F. circinatum* (isolate FcCa6), was cultured in PDA (Potato Dextrose Agar)(figure 3) and the isolate was grown at 25°C in the dark for five days (Cerqueira, A et al.,2017; Wadud Abdullah et al., 2017).

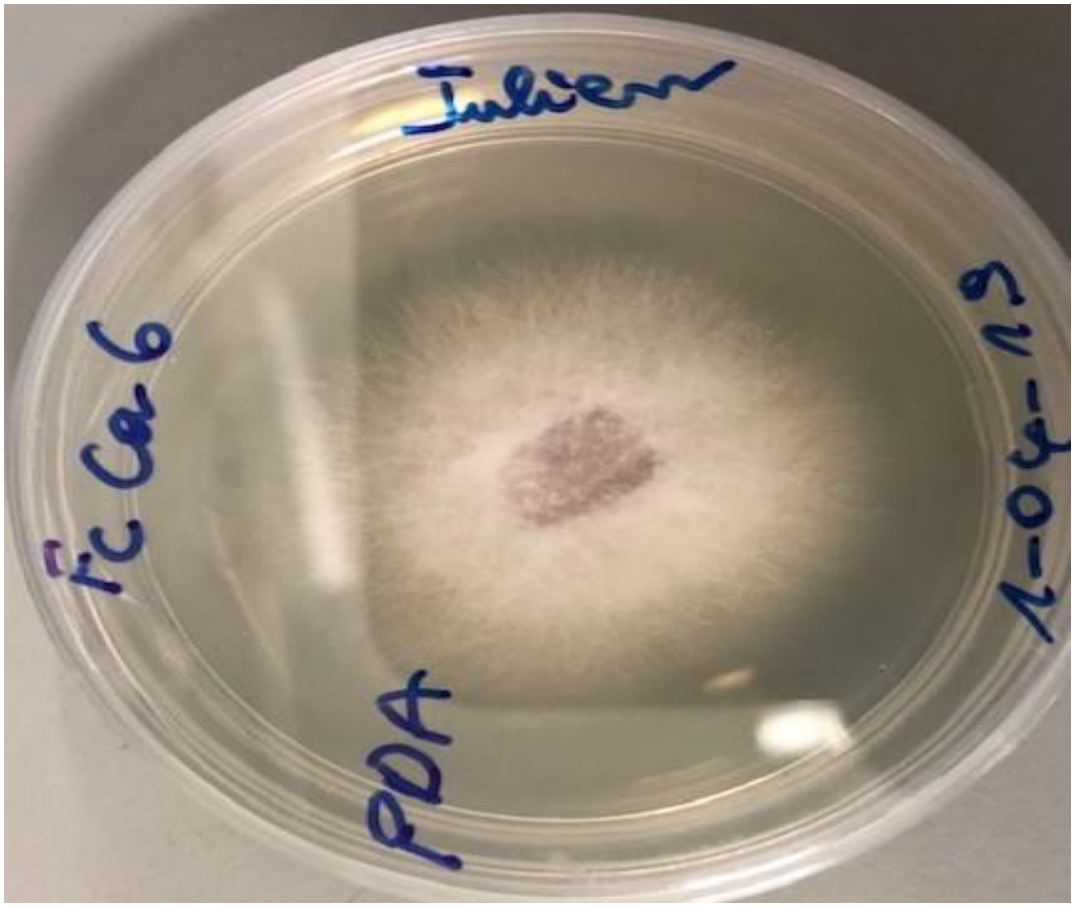

Figure 3: Fungal material FcCa6.

2.2. Plant material

The plant material consisted of *Pinus pinaster*' seeds that come from Galicia the northwest part of Spain. Twenty-two (22) different families were concerned by this experiment and each family is a family of genetic improvement program of Galicia (figure 4).

G	Name	Description	026-ERN-18-FAM2	number
5	PT005	Family of the Genetic Improvement Program of Galicia	X	1
21	PT021	Family of the Genetic Improvement Program of Galicia	X	2
23	PT023	Family of the Genetic Improvement Program of Galicia	X	3
24	PT024	Family of the Genetic Improvement Program of Galicia	X	4
25	PT025	Family of the Genetic Improvement Program of Galicia	X	5
27	PT027	Family of the Genetic Improvement Program of Galicia	X	6
28	PT028	Family of the Genetic Improvement Program of Galicia	X	7
30	PT030	Family of the Genetic Improvement Program of Galicia	X	8
31	PT031	Family of the Genetic Improvement Program of Galicia	X	9
33	PT033	Family of the Genetic Improvement Program of Galicia	X	10
35	PT035	Family of the Genetic Improvement Program of Galicia	X	11
38	PT038	Family of the Genetic Improvement Program of Galicia	X	12
44	PT044	Family of the Genetic Improvement Program of Galicia	X	13
45	PT045	Family of the Genetic Improvement Program of Galicia	X	14
46	PT046	Family of the Genetic Improvement Program of Galicia	X	15
48	PT048	Family of the Genetic Improvement Program of Galicia	X	16
49	PT049	Family of the Genetic Improvement Program of Galicia	X	17
50	PT050	Family of the Genetic Improvement Program of Galicia	X	18
86	PT086	Family of the Genetic Improvement Program of Galicia	X	19
103	PT103	Family of the Genetic Improvement Program of Galicia	X	20
105	PT105	Family of the Genetic Improvement Program of Galicia	X	21
109	PT109	Family of the Genetic Improvement Program of Galicia	X	22

Figure 4: Seeds from 22 *Pinus pinaster*'s families of the genetic improvement program of Galicia**.**

For the seed sowing, some trays have been prepared (figure 5). Each tray contains 3 families and each family has 25 replications which equals 75 seeds (25*3) sown per tray. 22 families were the object of this experiment which makes a total of 550 *Pinus Pinaster* seeds (22*25) concerned in 8 trays.

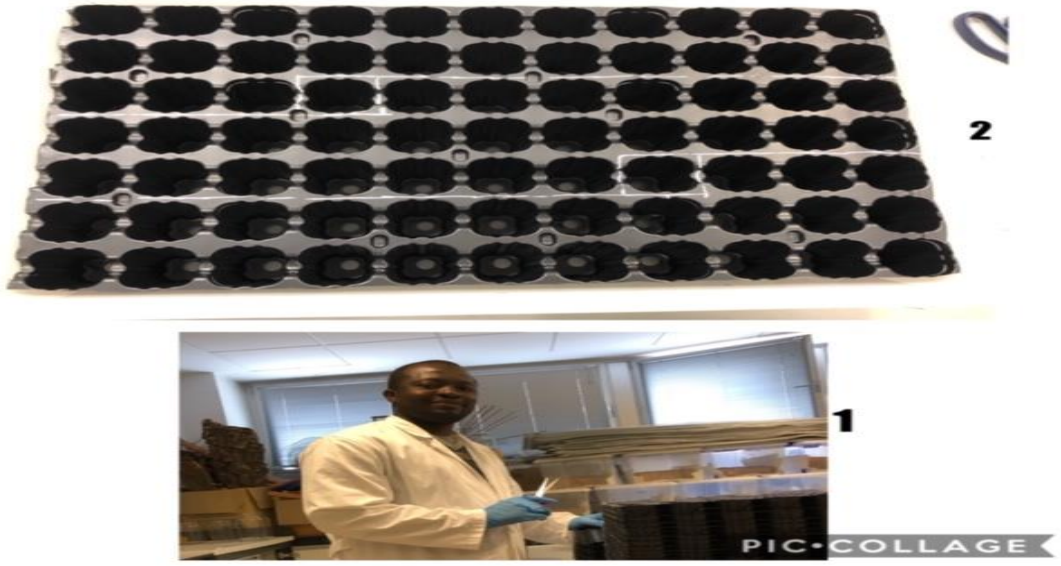

Figure 5: Tray:(1) tray preparation (2) tray ready to be used with 75 replications per tray.

2.3. Substrate

For this assay, the substrate is constituted of a mixture of peat moss and vermiculite at 50%. The substrates were autoclaved twice during one hour at 120 ºC. After completion the mixture, the nursery trays were filled (figure 6) (Martínez-Álvarez et al., 2014; Zlatković, 2019).

Figure 6: Substrate: (1) +(2) peat moss and vermiculite mixed at 50% and autoclaved; (3) +(4) trays filled with the substrate.

2.4. Pathogenicity tests

The preparation of the liquid culture media PDB (Potato Dextrose Broth) has been the first step of the preparation of the spore suspension. Generally, The PDB is used to cultivate the yeasts and molds, but in plant pathology it is used to cultivate some fungal pathogen and it is about *Fusarium Circinatum* in this Experiment. In order to prepare the PDB, 24 grams of the medium in form of powder were suspended in 1 litre of distilled water, mixed very well with frequent agitation and heated for few minutes until dissolution. The colour of the prepared medium is amber and slightly opalescent. Two to eight is the suitable temperature to keep in good state the culture media prepared. Four containers called flasks with a capacity of 250 ml each was used to carry the liquid obtained (figure 7.1.). The flasks with the liquid(media) were sterilized in autoclave at 121°C for 20 minutes (figure 7.3.) (Diez, 2019). After a waiting time to cool down the culture media (to avoid the death of the mycelium, *Fusarium Circinatum* with such warm temperature). Three or four small square pieces of 5 mm² of FC (FcCa6) have been added to only 3 containers (figure 7.4.) and the fourth and last container was used as control (figure 7.2.). To have a homogeneous dispersion of spores in the media, the containers were placed in the orbital shaker for 4 to 5 days (figure 7.5).

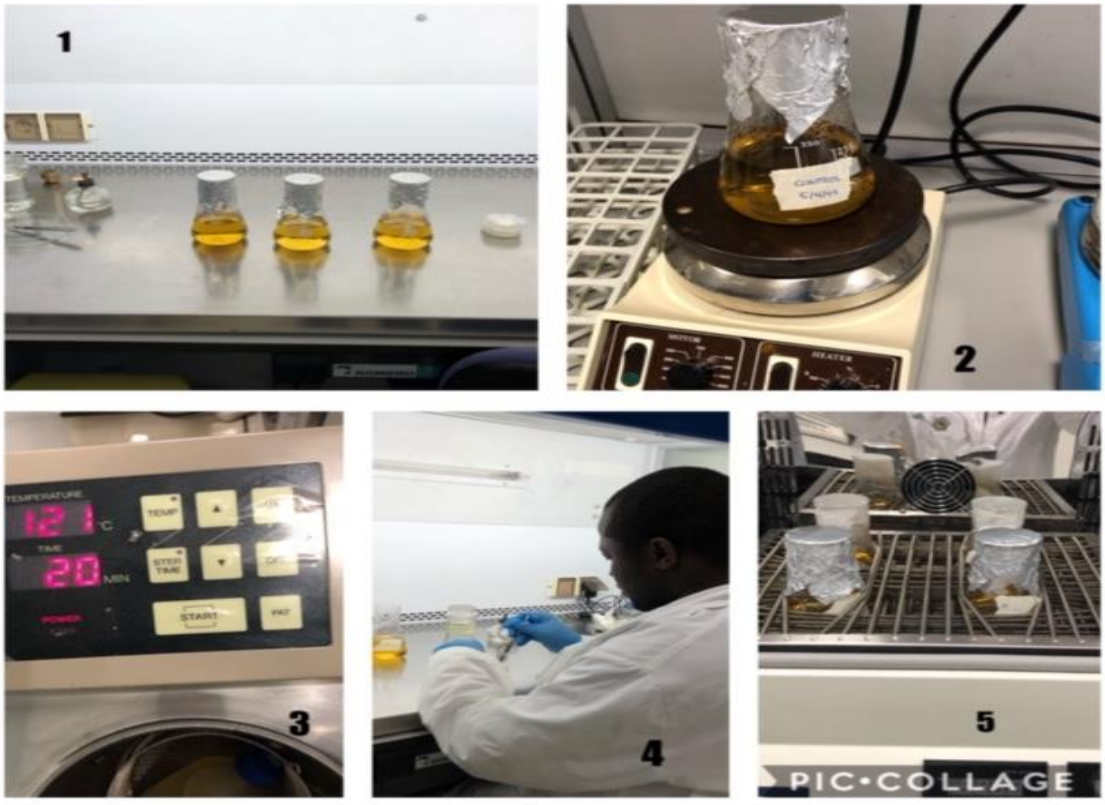

Figure 7: (1) Liquid culture media PDB; (2) control flask; (3) autoclave process ; (4) adding some pieces of F.C. to the PDB; (5) orbital shaker with the 4 flasks.

The concentration of spores per ml set up for this experiment is 250 spores/ml. To check and count the number of spores per ml, a specific protocol was performed: first, the purity of the fungal spore suspension has been checked .by observing with the microscope (figure 8.1.) 10 µL (drop). The materials used to take the drop of the fungal spore suspension are the pipette to take 10 µL of the fungal spore suspension and drop it on the Thomas cell counting chamber. For this experiment, some hyphae or mycelium (figure 8.2.) were observed with the microscope and they have been removed after filtration with a filter.

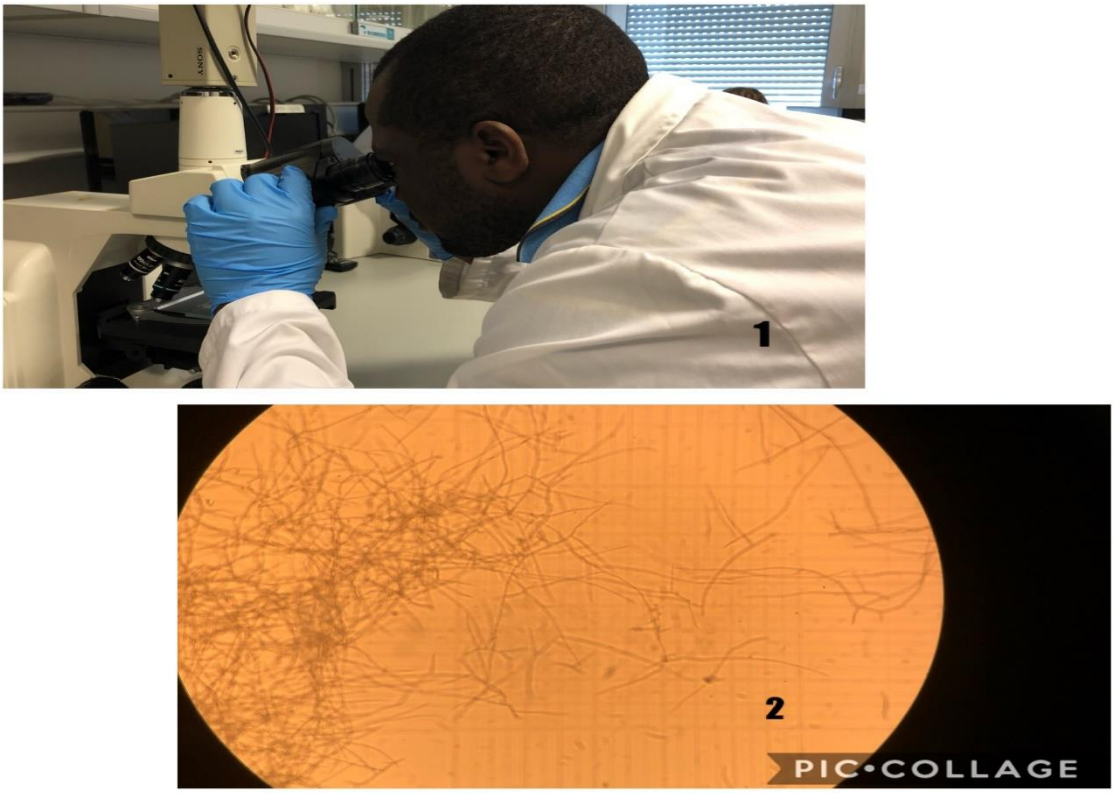

Figure 8: (1) Observation of the fungal spore suspension with the microscope to check its purity; (2) hyphae.

Prior to sowing, the seeds were washed repeatedly with sterile distilled water (SDW) and then soaked in SDW for 12 h to promote germination(Shin et al., 2014). The floating seeds were then removed and discarded. The remaining seeds were then soaked in hydrogen peroxide (3%) for 30 min and finally washed twice with SDW to remove the remaining hydrogen peroxide (Martínez-Álvarez et al., 2014). The seeds were then air dried at room temperature for 30 min (figure 9.1.). Seeds of each family were sown individually in the nursery trays (figures 9.2.). In total 550 seeds were sown for 25 seeds per family and about 22 families.

Figure 9:(1) Drying the seeds; (2) seeds' sowing.

Twenty-five (25) seeds per family were inoculated with one millilitre of a *F. circinatum* spore suspension at a concentration of 250 spores/ml. The same number of seeds per family was mock inoculated with one millilitre of sterile distilled water (figure 10.1.) (negative, mock-inoculated control). *Fusarium circinatum* was inoculated immediately after the seeds were sown and the conidial suspension was applied on each tray cell without direct contact with the seeds (figure 10.2.). The nursery trays were randomly incubated in a growth chamber at 21.5 °C and a 16/8 h light/dark photoperiod (figure 10.3.) (Zlatković, 2019).

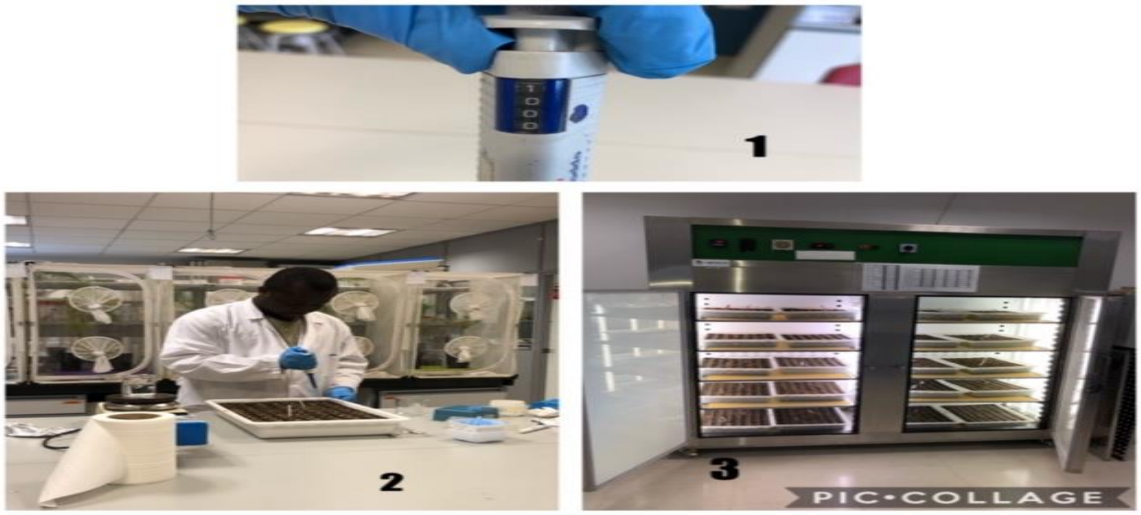

Figure 10: (1) Pipette that contains 1 ml of SDW or the inoculum; (2) inoculation process; (3) chamber.

A few days after the inoculation, some seedlings started to die, and some seeds were aborted and covered by mycelium. To check the Koch's postulate, the re-isolation of plant materials (stems mainly and aborted seeds) has been performed. The culture medium used for the re-isolation is PDAs (Potato Dextrose Agar) with 0.5 g/L of streptomycin sulphate (to prevent bacterial growth) (figure 11.2.). PDA is a solid culture media and to prepare it, some specific protocol has been followed: suspend 39 g of PDA powder in 1 litre of distilled water and bring to the boil. Distribute into suitable containers and sterilize in the autoclave at 121°C for 20 minutes (figure 11.1.) and it is forbidden to overheat. For the re-isolation, the fragments were sterilized by: dipping in sterile distilled water for 3 min, followed by shaking in 3% sodium hypochlorite (v/v) for 2 min, thereafter shaking in 70% ethanol (v/v) for 2 min, and finally dipping for 5 min in sterile distilled water to remove any remaining traces of disinfectants (figure 11.3. and figure 11.4). The samples were then dried for 1–5 min in a sterile laminar flow cabinet on sterile filter paper, before being cut in small pieces for plating on potato dextrose agar (PDA) with 0.5 g/L of streptomycin. And after the plates (petri dishes) were placed in a big closed plastic container (figure 11.6.). The petri dishes were scrutinized manually and visually based on the shape and the colour of the growing colonies (figure 11.5.) (Leslie, J.; Summerell, 2006; Martínez-Álvarez, 2015).

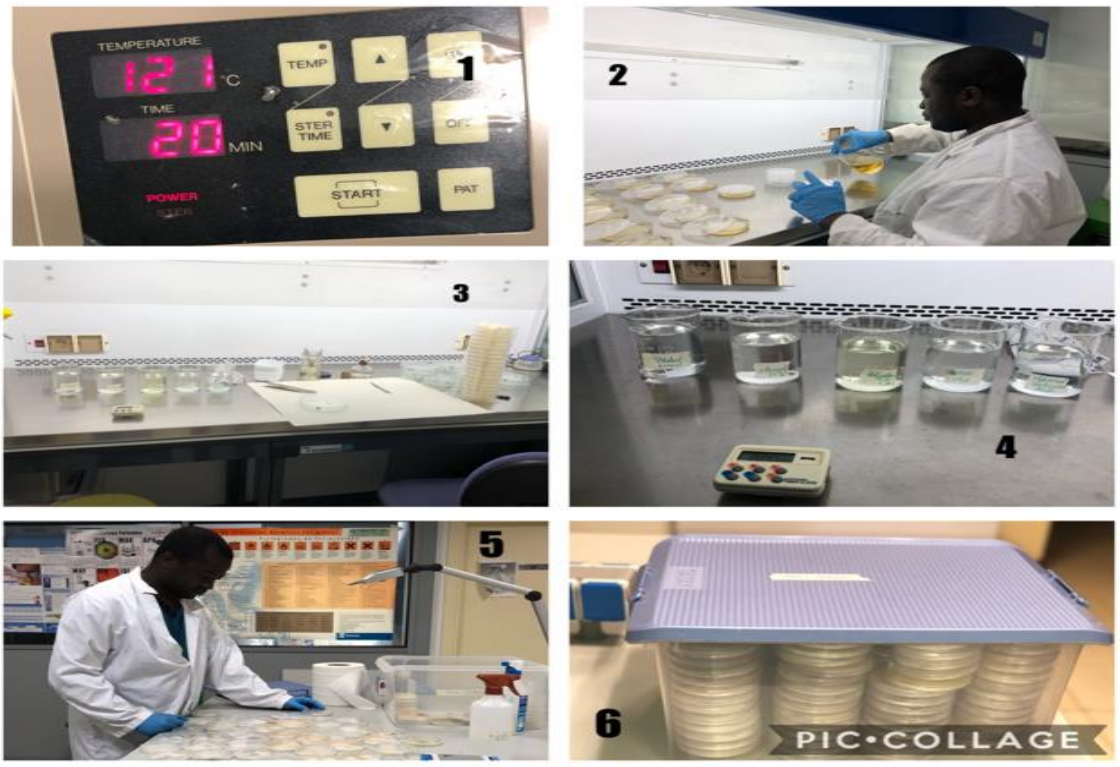

Figure 11: (1) Autoclaved at 121°c for 20 min; (2) Pouring PDAs on petri dishes; (3)+(4) sterilization process; (5) checking of the re-isolated petri dishes (6) re-isolation of petri dishes in a closed plastic containers.

2.5. Data collection and monitoring

The experiment lasted 70 days (from the day of seed sowing and inoculation up to the last date the data have been recorded). Throughout this period, there was a continuous collection of data that was done 3 times a week most often Monday, Wednesday and Friday. A special sheet was developed (figure 12) for the collection of data and the types of data collected are the Seeds germination dates, The seedlings death dates. Mark the day of appearance of the mycelia.

Family	Seed	1	2	3 M	4	5	6	7	8	9	10	11
5	Germination			18.05.19	05.05.19	05.05.19		29.05.19				
	Death			20.05.19	09.05.19							
	Seed	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
	Germination	05.05.19	21.04.19			01.05.19		07.05.19				01.05.19
	Death	28.05.19						03.06.19				
	Seed	**23**	**24**	**25**		**1**	**2**	**3**	**4**	**5**	**6**	**7**
	Germination						05.05.19		01.05.19	01.05.19		03.05.19
	Death						20.05.19					
21	**Seed**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
	Germination		09.05.19				01.05.19	05.05.19			22.05.19	01.05.19
	Death		28.05.19					16.05.19				
	Seed	**19**	**20**	**21**	**22**	**23**	**24**	**25**		**1**	**2**	**3**
	Germination	01.05.19	23.04.19	09.05.19	03.05.19	03.05.19				07.05.19	07.05.19	27.04.19
	Death			14.05.19								
23	**Seed**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13 M**	**14 M**
	Germination		25.04.19	18.05.19	27.04.19	27.04.19		01.05.19	07.06.19	01.05.19	25.04.19	01.05.19
	Death										29.04.19	01.05.19
	Seed	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
	Germination	01.05.19	27.04.19	01.05.19	05.05.19	01.05.19	07.05.19			07.05.19		18.05.19
	Death	16.05.19	18.05.19									

Figure 12: sheet for recording the data.

Additionally, the irrigation of the experiment was done once a week and often on Friday with 1 litre of normal water in every tray. Finally, the PDAS that contains the re-isolated materials were checked randomly in order to record the concerning data and to verify the Koch's postulate.

Figure 13: (1) Watering the experiment; (2) reporting the data; (3) checking the re-isolated petri dishes.

2.6. Statistical analysis

For this experiment, to reach the main goal set up which is to characterize the susceptibility of different families of maritime pine to *Fusarium circinatum*, different types of data analysis have been performed.

Survival analysis based on the nonparametric estimator Kaplan–Meier was performed with the Kaplan-Meier survival function implemented in the statistical software XLSTAT. Log-rank (p value) was computed in order to know how significant the difference between the treatment is (control and inoculated with F.C.). The survival analysis has been performed to test the mortality up to the end of the experiment (70 days)(Kaplan & Meier, 1958; Martín-García et al., 2018).

Microsoft Excel has been used many times to make some figures, but the most important chart made in Microsoft excel is the combo chart used to highlight the families that are the most susceptible and those which are more resistant to *Fusarium circinatum*.

3. Results

3.1. Seeds' germination and mortality

The experiment lasted 70 days (2 months 10 days) from the date of inoculation until the last date the data were collected. The first germinations were recorded 10 days after the inoculation. The survival time varies in all cases from 1 day to 59 days. The survival time is calculated from the date of germination until the date the seedling dies. Some seedlings' features were recorded from their germination until their death: observation of the germination 10 days after the inoculation (figure 14 a); the seedling falls down and its stem is covered of mycelium (figure 14 b); seedling dieback and its colour changes from green to brown (figure 14 c).

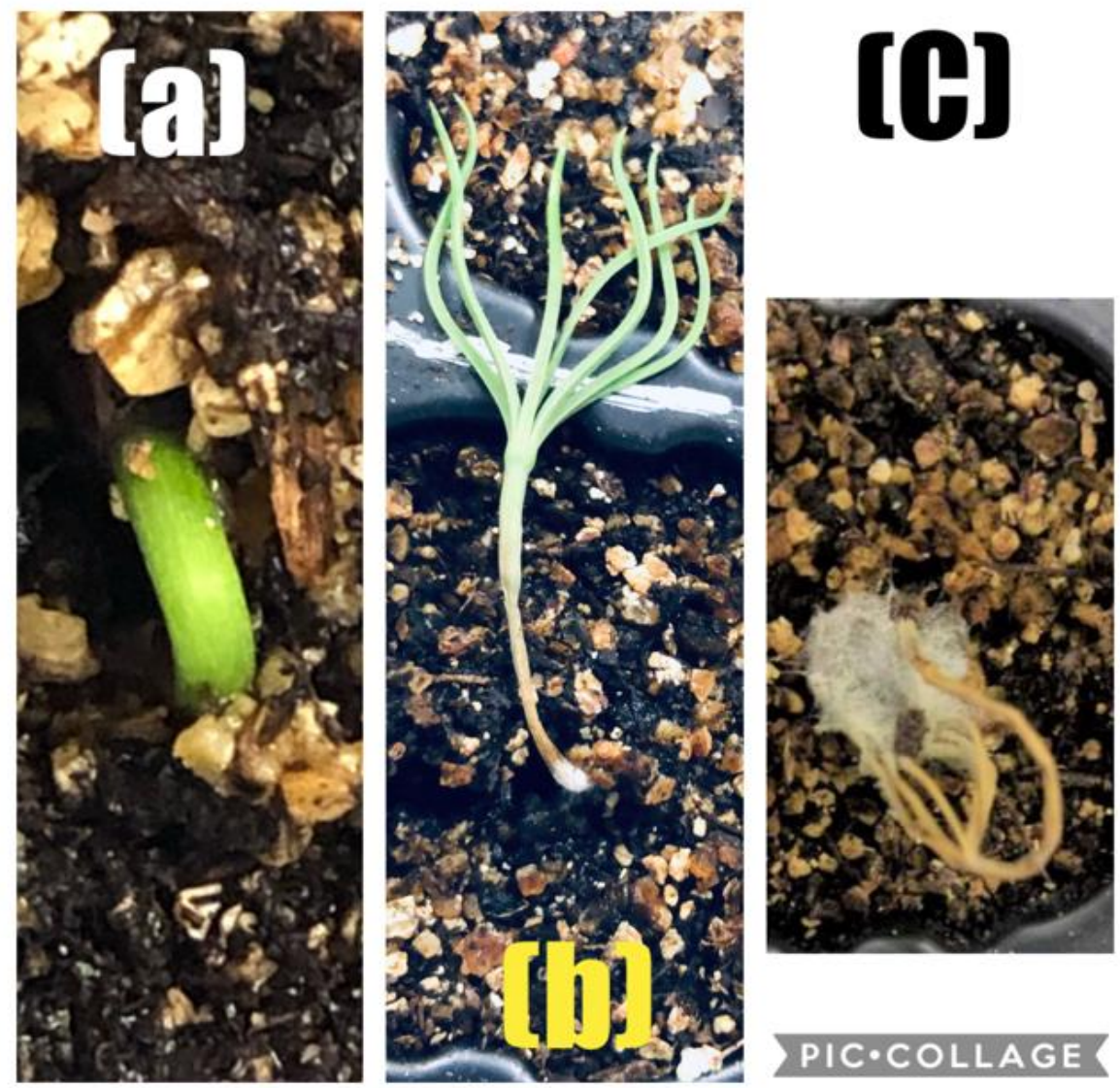

Figure 14: (a) Germination of seeds; (b) the seedling falls, and its stem is covered of mycelium; (c) seedling dieback and its colour changes from green to brown.

For this experiment, the total percentage of germination for the control assay is 41.3% which is lower than its value for treatment (F.C.) assay which is 61.6% (figure 15a). Concerning the mortality, its value is 32.2% for treatment (F.C.) assay and higher than its value in control assay which is nil and equal to 0% (figure 15b).The percentage of germination obtained are not so high in both cases (control assay and treatment assay(F.C.)) and in particular for the control assay which is less than 50% and less than its value for the treatment(F.C.) assay which is not normal because in treatments trays (F.C.) the fungus *Fusarium circinatum* should inhibit the germination power of the seeds, therefore normally, the percentage of germination in inoculated (*Fusarium circinatum*) trays should be less than its value for control trays. Furthermore, concerning the percentage of mortality for control trays; it is nil, and it is normal because there is no fungus to kill the seedlings. Concerning the percentage of mortality in the treatment assay (with *Fusarium circinatum*) only 32.2% of germinated seeds died. This value is not so high and even is less than 50 % which can be explained by the fact that the *Pinus pinaster*'s families involved in this experiment are resistant to 250 spores/ml of inoculum concentration and may die if the concentration of spores are higher than the one used in this experiment.

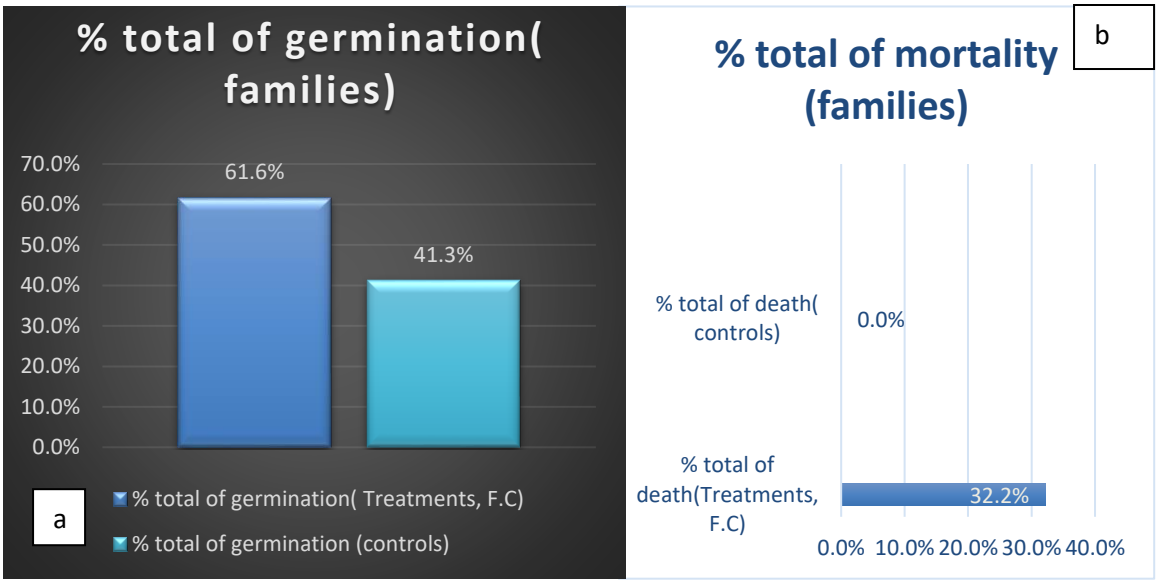

Figure 15: (a) Total percentage of germination for control and inoculated trays; (b)% total of mortality for control and inoculated trays.

The figure below (figure 16) shows the proportion of gemination and the proportion of mortality for the experiment about the control and inoculated (F.C.) Trays on the same chart. No mortality has been recorded for the control assay but on the other hand, for the treatment trays (with *Fusarium circinatum*) the proportion of mortality is equal or above 50% for the families 5;31;46; 50 and 105. Only few families for the control assay have more germination than their homologue in the inoculated (F.C.) trays.

Generally, the proportion of germination is higher for inoculated trays than control trays. For instance, the highest proportions of germination (≥80%) were recorded in treatment (F.C.) trays for the families 23;30;48; 86 and 109 and on the other hand, the lowest percentage of germination (≤20%) were recorded in control assay for the families 25;28 and 46.

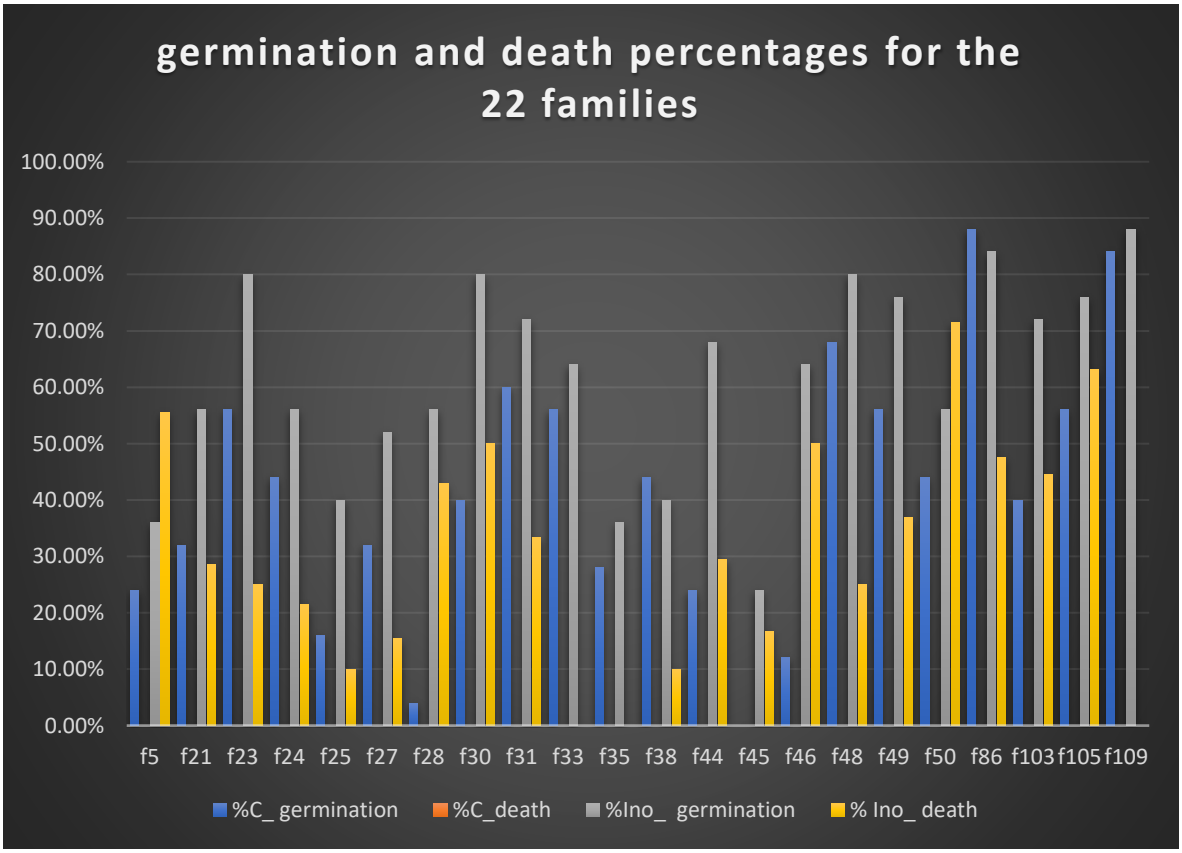

Figure 16: Proportion of germination and proportion of mortality of the 22 families concerning control and inoculated trays.

3.2. Survival probability

To assess the resistance among *Pinus pinaster*'s families to *Fusarium circinatum* (FcCa6) in this experiment, the non-parametric method defined by Kaplan-Meier using the survival function to *Pinus pinaster* inoculated with F.C. were computed to have the survival probabilities and the log-rank test.

The survival analysis used for all the 22 families in this experiment revealed that in any case there is a significant difference between the 2 treatments (1=inoculated and 2=control) because P-value < alpha (0.050) in any case and especially for log-rank test (table 1).

Table 1: P-value concerning the 22 families for the 2 treatments (1=inoculated and 2=control).

Statistic	Observed value	Critical value	p-value	alpha
Log-rank	184.825	58.124	< 0.0001	0.050
Wilcoxon	193.565	58.124	< 0.0001	0.050
Tarone-Ware	192.804	58.124	< 0.0001	0.050

The first germination dates were recorded 10 days after the inoculation; therefore, the first mortalities days were recorded just 1 day after the date of germination or in another way 11 days after the inoculation. Then the mortalities days of the seedlings varies from 1 day to 59 days after their germination.

No mortality was recorded for all control seedlings and for the inoculated families f33; f35; and f109. For this reason, all these curves overlap in a straight line making it difficult to distinguish them (figure 17). The figure 17 below also shows that the family f50 is the most susceptible family with a survival probability less than 30%. Furthermore, from the figure 17 below we can have the conclusion that the families f86; f46; f30; f5; f105 and f50 are the most susceptible families because their survival probabilities are lower than the survival probabilities of the rest of the families (16 families). On the other hand, the families f109; f33; f35; f25 and f28 are the most resistant families because their survival probabilities are higher than the survival probabilities of the rest of the families (16 families) (figure 17).

It is important to clarify that concerning the mortality of the seedlings, it starts from a wilting point through a decline point and arrives at the dieback point where the seedlings fall, and their colours change from green to brown. Additionally, some seeds are covered with a mycelium and the verification of these seeds reveals that these seeds started by germinating and dying at the same time.

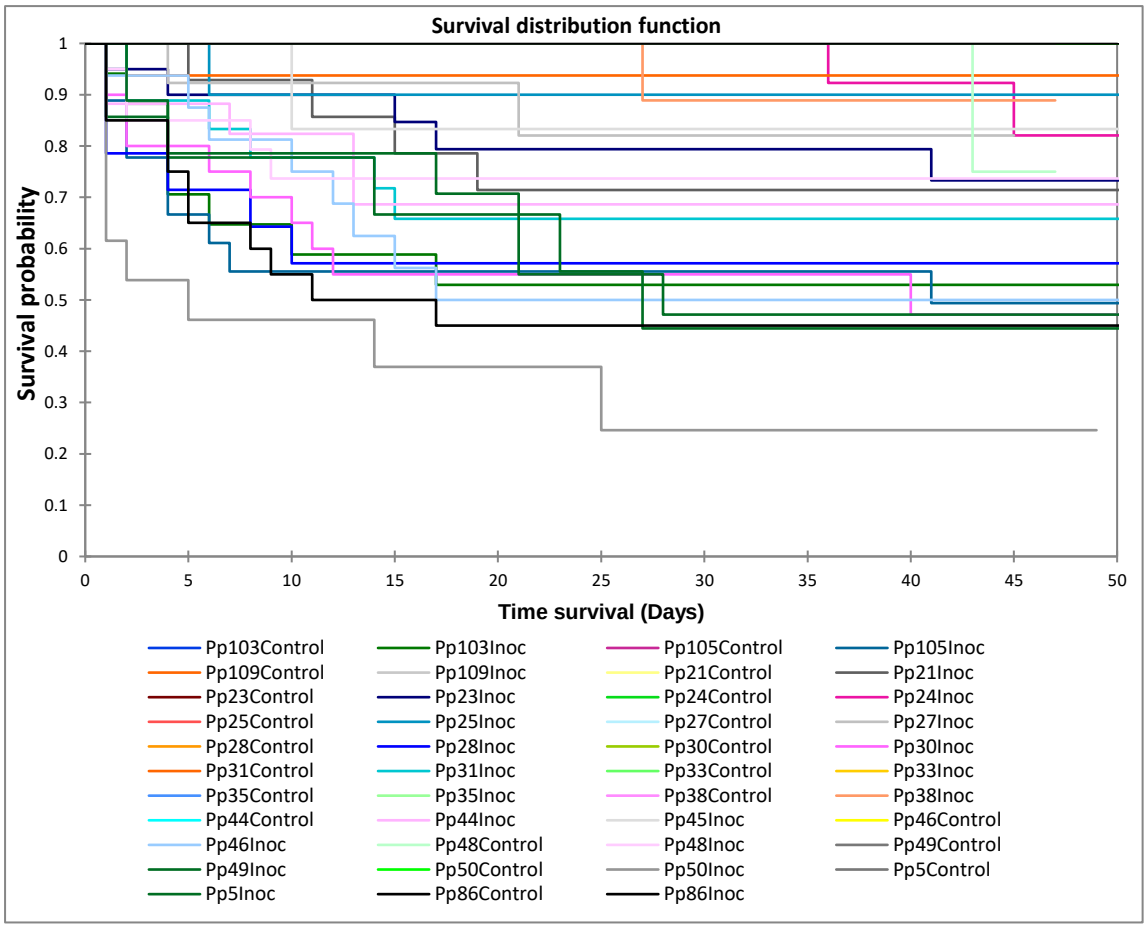

Figure 17: Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for the 22 families inoculated with *Fusarium circinatum*.

It is very difficult to distinguish and identify the curves concerning each of the 22 families involved in this experiment like it is showed on the figure 17 above that is why 2 groups of families were formed (table 2) based on the survival probabilities showed by the figure 17 and also on the mortalities' proportion recorded concerning each family.

Table 2: more resistant families and more susceptible families

Group of families	More resistant families	More susceptible families
Families in the group	f109; f33; f35; f25 and f38	f46; f30; f5; f105 and f50

The Kaplan-Maier survival analysis function used for all the 2 groups of families (the more resistant families and the more susceptible families (10 families in total) revealed that in any case there is a significant difference between the 2 treatments (1=inoculated and 2=control) because P-value < alpha (0.050) in any case and especially for log-rank test (table 3).

Table 3: P-value concerning the more resistant families and the more susceptible families for the 2 treatments (1=inoculated and 2=control).

Statistic	Observed value	Critical value	p-value	alpha
Log-rank	124.323	30.144	< 0.0001	0.050
Wilcoxon	121.158	30.144	< 0.0001	0.050
Tarone-Ware	122.983	30.144	< 0.0001	0.050

The mortalities days recorded for these 10 families range from 1 day to 59 days after their germination's dates.

No mortality was recorded for all control seedlings and for the inoculated families f33; f35; and f109. For this reason, all these curves overlap in a straight line making it difficult to distinguish them (figure 18). The figure 17 below also shows that the family f50 is the most susceptible family with a survival probability less than 30% (0.3) and the percentage of mortality recorded for the inoculated family f50 is 71.43%. Furthermore, from the figure 18 below and from the figure 17 above, the conclusion that the families f46; f30; f5; f105 and f50 are the most susceptible families is evident because their survival probabilities are lower than the survival probabilities of the rest of the families. On the other hand, the families f109; f33; f35; f25 and f28 are the most resistant families because their survival probabilities are higher (much more than 70% (>0.7) than the survival probabilities of the rest of the families (figure 18).

To sum up, the families f5; f105 et f50 are the worst families (the most susceptible families) with their survival probability very low and less than 50% (<0.5) and the percentage of mortality recorded for the inoculated families f5; f105 et f50 of each of them is higher and equal to f5(55.56%); f105(63.16%) and f50(71.43%). On the contrary and in the other direction, the families f109; f33 and f35 are the best families (most resistant families to *Fusarium circinatum*) because their survival probability is higher and equal to 1(100%).

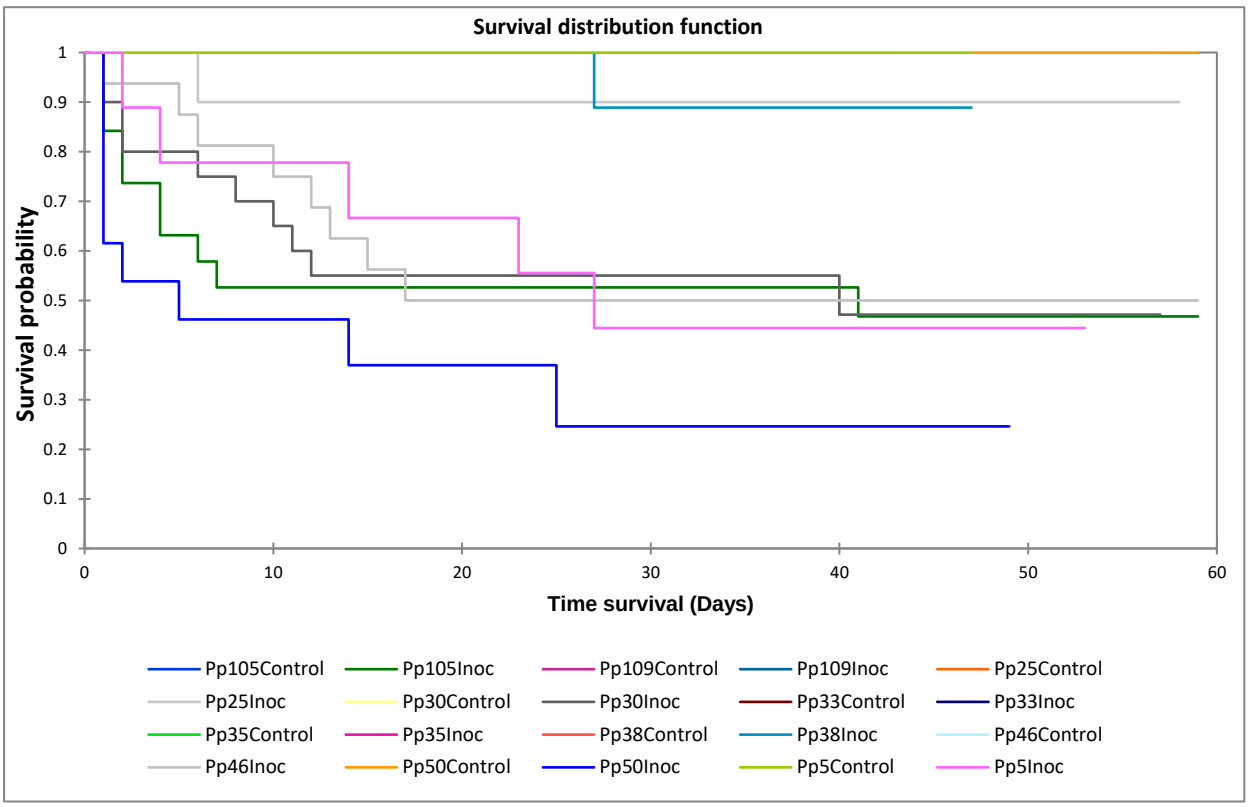

Figure 18: Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for the best and the worst families inoculated with *Fusarium circinatum*.

3.3. Combo chart: percentage of living seedlings; susceptibility

The experiment lasted 70 days from the inoculation day until the last day the data were recorded. The main goal of this study is to identify amongst the 22 *Pinus pinaster* families involved in this experiment the ones which are most susceptible or more resistant to *Fusarium circinatum*.

One of the easiest ways to identify the most susceptible or the most resistant families is to calculate the percentage of the living seedlings when the experiment stopped, in other words, quantifying the exact proportion of the remaining seedlings which are still alive is a good way to know the most vulnerable or the most resistant genetic families involved in this experiment. Therefore, the percentage of the living seedlings is obtained by the difference between the percentage of germination and the percentage of mortality divided by the percentage of germination.

FORMULA:

% of living seedlings of the family X = (% of germination of the family X - % of mortality of the family X) / (% of germination of the family X)

The Combo chart below (figure 19) is obtained from the above formula. It shows that the percentage of living seedlings is the same and equal to 100% for all the control seedlings except the family 45 of the control assay where the percentage of germination is null. The statistics are normal concerning the proportion of the living seedlings of the control assay because there is no mortality recorded for control seedlings.

Concerning the percentage of living seedlings about inoculated seedlings, it decreases steadily from its highest value for the families (f109;f33 and f35) to its lowest values for the families (f5; f105 and f50) because the rates of mortality are so high for these families and are: f5(55.56%); f105(63.16%) and f50(71.43%); all of these rates of mortality are above 50%.Consequently, the main points to highlight from the combo chart below (figure 19) are :

- **The families f5; f105 and f50 are the most susceptible families to F.C.**
- **The families f109; f33 and f35 are the most resistant families to F.C.**

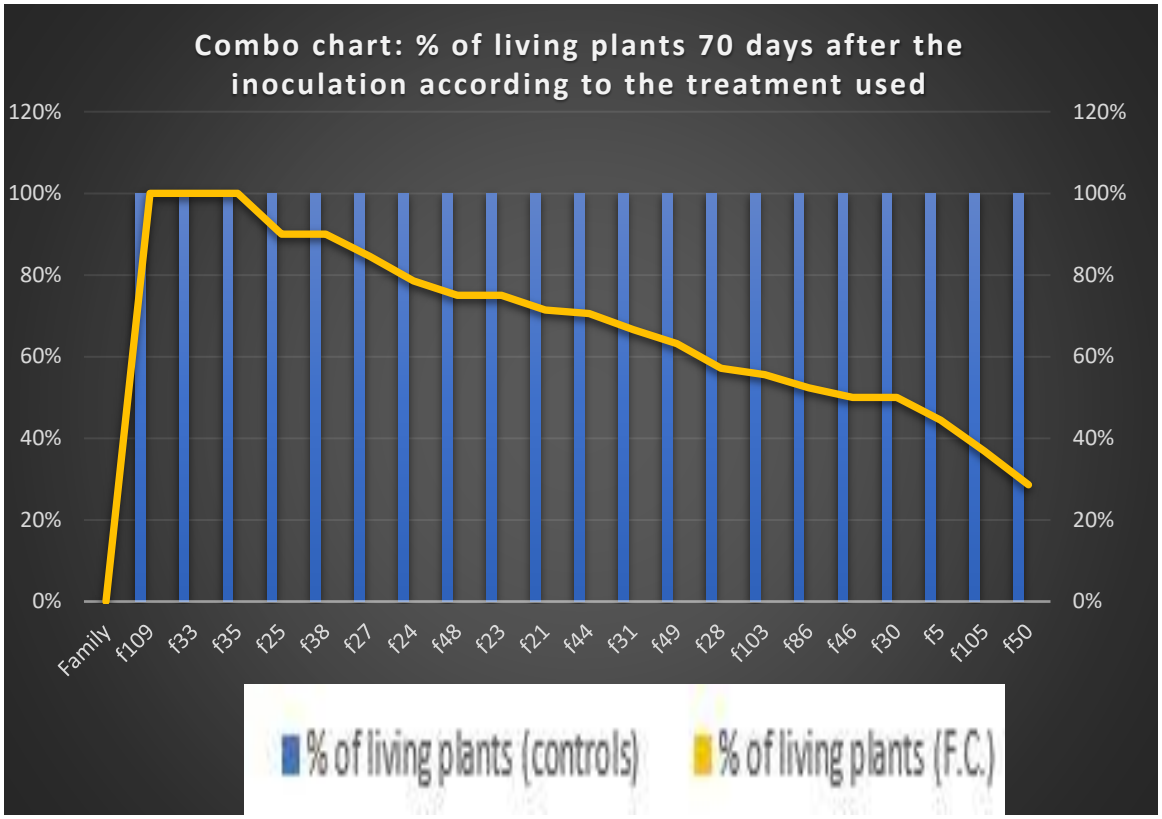

Figure 19: Combo chart: percentage of living seedlings 70 days after the inoculation according to the treatment used.

3.4. Koch's postulate: re-isolation of dead plant material on culture media

The goal of the Koch's postulate is to set up the causative relationship between the fungus or the causal agent and the mortality of the seedlings.

About this experiment, the plant material (mainly the stem) was isolated and grown on the culture media PDAS. The identification of the fungus is based on some criteria such as: the colour, the shape and the size of the fungus colonies. After scrutinization of the fungus colonies in the isolated culture media petri dishes, the results and statistics are mentioned in the table below (table 4):

Table 4: Koch's postulate: re-isolation statistics.

Total	Pure F.C.	Contaminated F.C.	No F.C.
68	41	26	1
Percentage	60.29%	38.24%	1.47%

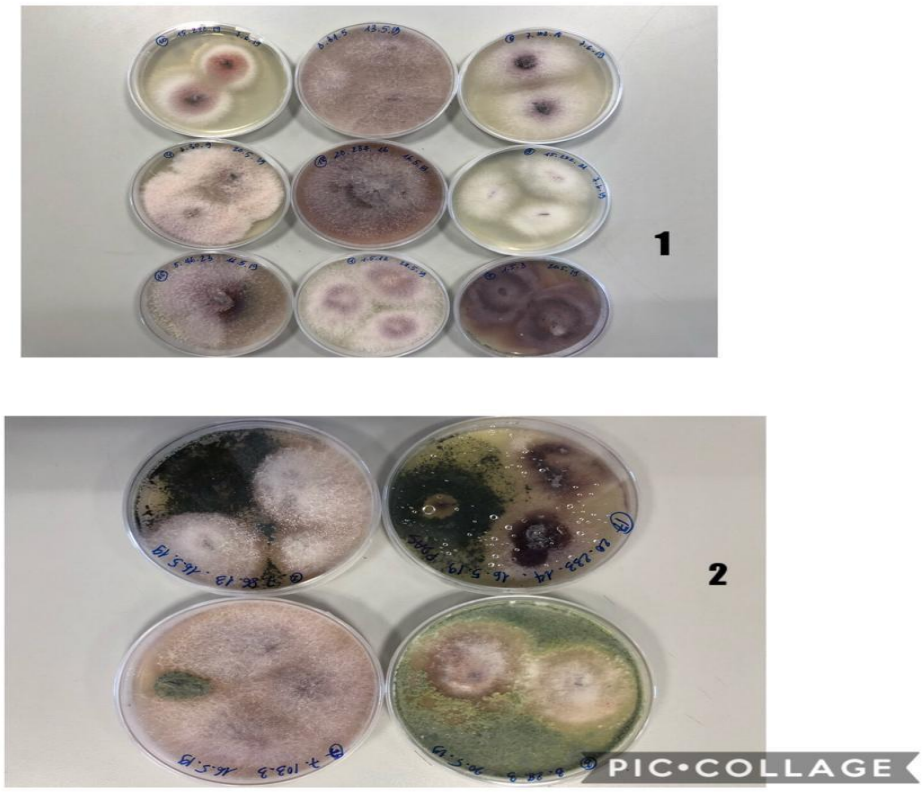

Figure 20: Fungus colonies: (1) presence of F.C. (2) presence of F.C. and another fungus.

From the table 4, *Fusarium circinatum* is the causal agent of the death of the seedlings re-isolated. In total, 68 plant materials from 17 families involved in this experiment were isolated and grown on PDAS culture media; only 1.47% of the re-isolated plant materials doesn't contains F.C. On the other hand, 60.29% of the isolated plants materials contains pure colonies F.C. and 38.24% of the colonies found are not pure F.C., they are a mixture of F.C. and another fungus (figure 20).

The figure 21 below shows 17 families were concerned by the re-isolation process. Only 1 plant material re-isolated of the family f38 doesn't contain F.C.

The rate of "presence of F.C." can be obtained by adding the rate of "Pure F.C." to "contaminated F.C.".

From all above, the main information to highlight is:

***Fusarium Circinatum* (FcCa6) is the causal agent of the mortality recorded in the inoculated seedlings assay.**

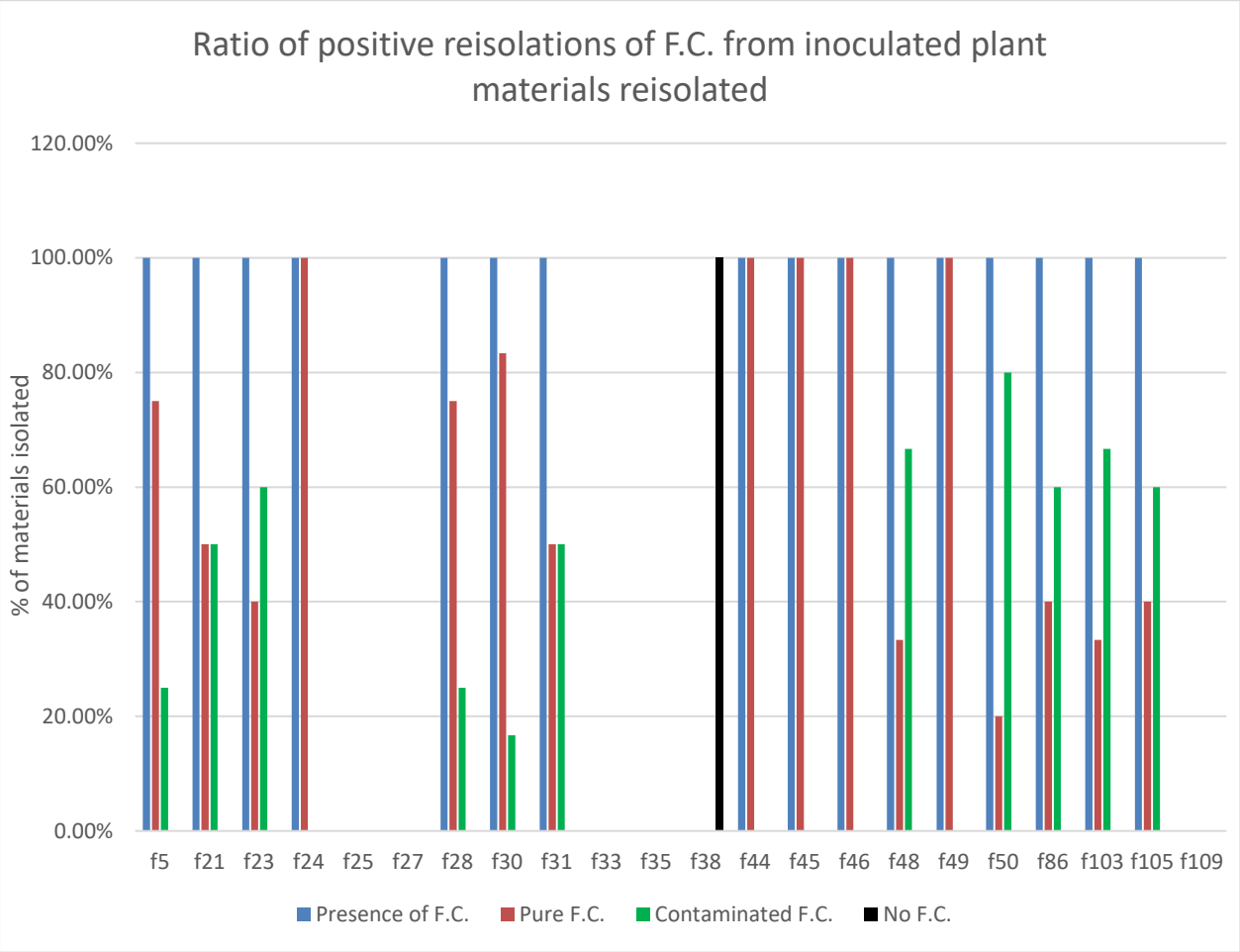

Figure 21: Ratio of positive re-isolations of F.C. from inoculated plant materials re-isolated.

4. Discussion

The outcomes of this study have demonstrated that *Pinus pinaster* is a susceptible species to *Fusarium circinatum* because the percentages of mortalities found for some families are more than 50% and in especially for the families f5; f105 and f50 the rates of mortalities are f5(55.56%); f105(63.16%) and f50(71.43%); it shows how susceptible are these 3 families of *Pinus pinaster* to F.C. Many studies found the same conclusion as (Carlucci et al., 2007; Martínez-Álvarez et al., 2014; PérezSierra et al., 2007; Vivas et al., 2012). Furthermore, even *Pinus pinaster* is known to be a susceptible specie to F.C., it has some moderate resistance to the fungus because of some factors that involved in the resistance to F.C. such as : *Pinus pinaster* has a high range of provenances and some variations in families and also some climatic conditions . It is in this context that the results of this study showed that the families f109; f33 and f35 are the most resistant to *Fusarium circinatum* because the percentages of mortalities recorded for these families are null. Most scientists

agree and define maritime pine as a susceptible specie, and which has a variation in its families or provenances (Iturritxa et al., 2012; Vivas et al., 2012).

Fusarium circinatum spread area is very important in Europe and constitutes about 10 million hectares of Pinus. Species (Martín-García et al., 2017). its presence is effective in the north of Spain, south-west of France, north of Portugal and south of Italy and this situation represents a huge economic loss for Europe for instance , during the last past 20 years invasive alien species have cost to the European Union an important amount of 12 billion euro per year (Kerstin Sundseth, 2014). Therefore, it is of paramount importance to European scientists, foresters, forest managers, landowners, researchers and decision takers to think and take some big and accurate decisions in order to eradicate totally or slow down the spread of the Pine Pitch Canker causal agent *Fusarium circinatum*. First in the Iberian Peninsula and after in the central and northern Europe.

In order to combat, or eradicate, or slow down the spread or sensitize against Pine Pitch Canker caused by F.C. in Europe and all over the world, many studies have been done. (Martín-García et al., 2017) have demonstrated that the Romanian provenance of *Pinus Sylvestris* is not susceptible to F.C. and it constitutes a genetic resistance as a potential tool to manage the disease. In order to develop a model on the spread of F.C. , the study of (Möykkynen et al., 2015) on "Modelling the potential spread of *Fusarium circinatum*, the causal agent of pitch canker in Europe" has demonstrated that the fungus is likely to spread to the pine forests of northern Spain such as Galicia ; Cantabria and southwest France (Aquitania). There will be some spread towards northern Portugal and southern Italy. Unless there are new arrivals to Central and North Europe, the fungus will not spread to the more northern parts of Europe. Thus, it is of paramount importance to European Union to control any entrance of the spores through international trading. Outside Europe, PPC is present and does damage to Pinus forests. Study on "variation in susceptibility to pitch canker fungus among half-sib and full-sib families of Virginia pine" by (Barrows-Broaddus J, 1984) has showed that histological examination of surviving shoots from inoculated seedlings and branches indicated that the formation a periderm in the cortex and reaction parenchyma in the xylem was a factor in delaying invasion by the pathogen.

Concerning the plausible vector of transmission of *Fusarium circinatum*, 2 mains studies have been done: (Selikhovkin et al., 2018) have showed that in Russia, there are many insects potentially capable of rapidly spreading the pitch canker of pines if *F. circinatum* invades the country especially dendrophagous insects (insects that feed on trees) and their study also revealed that the most favourable region for its distribution will be pine nurseries and young plantations located in the Black Sea coastal areas and the adjacent regions. It is in this same sense that (Bezos et al., 2015) have carried out a salient study

on "The pine shoot beetle *Tomicus piniperda* as a plausible vector of *Fusarium circinatum* in northern Spain", they found that *T. piniperda* was found to be associated with both diseased and healthy P. radiata trees, and *F. circinatum* was found to be present, at low rates, on the exoskeleton of T. piniperda. In the laboratory experiment, evidence of the ability of T. piniperda to transfer the pathogen to healthy shoots was found.

(Martín-García et al., 2018) have showed that symptomless seedlings from species different to Pinus species can be a potential management option to slow down the negative impacts of the fungus F.C.in the area it has been identified. Climatic conditions, temperature, humidity can be some favourable conditions for the growing up of the fungus *Fusarium circinatum*, therefore (Blank et al., 2019) showed that the temperature in addition with humidity both involved in the establishment of F.C. Furthermore, the study of (Davydenko et al., 2018) revealed that involuntary introduction of F.C. as invasive pathogen into Poland will have such great and negatives impacts on polish provenances of Scots pine and *Pinus Sylvestris L*.

Biological options such as mycoviruses or Trichoderma are suitable for controlling the PPC disease (Nuss, 2005).

From all above and to sum up, it is clear to state that there is an absolute need of an integrated management of the Pine Pitch Canker disease caused by *Fusarium circinatum*. There it is an paramount importance to think about the fact that the high genetic variability of some Pinus species can be a tool of management for instance , the promotion of the most resistant *Pinus pinaster*'s families to be planted in an area as substitution of *Pinus radiata* which is vulnerable to PPC is a good way to slow down the effects of PPC . furthermore, Forest manager or scientists or decisions takers could play with natural conditions such as humidity, temperature and climatic conditions to manage the disease PPC. this study is also in the same perspective for integrated and better management of Pine Pitch Canker.

5. Conclusion

The 22 *Pinus Pinaster*'s families of "genetic improvement program of Galicia" have responded differently to *Fusarium Circinatum* infection the causal agent of Pine Pitch Canker. Some of them are more susceptible to F.C. and others are more resistant to F.C.

From all above, it can be concluded that:

- The most resistant families to F.C. involved in this study are the families f109; f33 and f35.
- The most vulnerable families to F.C. involved in this study are the families f5; f105 and f50.
- F.C. is the causal agent of the mortality recorded in the inoculated seedlings assay.

Therefore, this study is of uppermost importance in order to identify and select the most resistant families which are the families f109; f33 and f35 to identify possible improvements in forest management and planning in order to reduce the impacts of Pine Pitch Canker in Pine maritime plantations and also to provide an alternative solution for forest managers to replace the more susceptible specie to *Fusarium circinatum, Pinus radiata*.

Several additional options may be investigated from this perspective:

- afforestation using resistant families
- germination trials and breeding using seed stock from resistant families
- silvicultural interventions targeting infected trees.

Trails under natural conditions may be interesting also where mature individuals are assessed.

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Annex 1: data collected concerning the control seedlings

CONTROL

Number	Family	Number of Germination	Number of death	Number of replication
1	5	6	0	25
2	21	8	0	25
3	23	14	0	25
4	24	11	0	25
5	25	4	0	25
6	27	8	0	25
7	28	1	0	25
8	30	10	0	25
9	31	15	0	25
10	33	14	0	25
11	35	7	0	25
12	38	11	0	25
13	44	6	0	25
14	45	0	0	25
15	46	3	0	25
16	48	17	0	25
17	49	14	0	25
18	50	11	0	25
19	86	22	0	25
20	103	10	0	25
21	105	14	0	25
22	109	21	0	25
Total		227	0	550

Annex 2: data collected concerning the inoculated seedlings

		Treatments, F.C.		
Number	Family	Number of Germination	Number of death	Number of replication
1	5	9	5	25
2	21	14	4	25
3	23	20	5	25
4	24	14	3	25
5	25	10	1	25
6	27	13	2	25
7	28	14	6	25
8	30	20	10	25
9	31	18	6	25
10	33	16	0	25
11	35	9	0	25
12	38	10	1	25
13	44	17	5	25
14	45	6	1	25
15	46	16	8	25
16	48	20	5	25
17	49	19	7	25
18	50	14	10	25
19	86	21	10	25
20	103	18	8	25
21	105	19	12	25
22	109	22	0	25
Total		339	109	550