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Resistance to Cypress Canker Disease in Italian cypress has desirable effects on disease epidemiology, but may fail against novel genotypes of the pathogen *Seiridium cardinale*

Gianni Della Rocca[a], Roberto Danti[a], Tina Popenuck[b], Vincenzo Di Lonardo[a], Matteo Garbelotto[b,*]

[a] Institute for Sustainable Plant Protection IPSP, National Research Council CNR, Via Madonna del Piano 10, 50127, Sesto Fiorentino, Firenze, Italy
[b] Department of ESPM, 54 Mulford Hall, University of California, Berkeley 94720, CA, USA

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ABSTRACT

Prevention is hailed as the only successful and cost-effective approach to control the introduction of exotic forest pathogens, and several international policies are in place to avoid such introductions. However, once a pathogen has been introduced and is widely spread in a novel range, regulations are relaxed. Concerns have been voiced about the detrimental consequences of the introduction of multiple genotypes of a pathogen, but evidence about such consequences has rarely been documented. Cypress Canker Disease (CCD) is a pandemic lethal disease of plants in the family *Cupressaceae* caused by the fungal pathogen *Seiridium cardinale* (W.W. Wagener) B. Sutton & I.A.S. Gibson (*Sc*). *Sc* is native to California, where populations are genetically diversified, while widely spread CCD outbreaks in the Mediterranean basin have been caused by the introduction of a single genotype. Resistant plant clones have been selected in Italy based on long-term field tests using a representative Mediterranean *Sc* genotype. Here we use a 90-day greenhouse trial on 419 21-month old grafts to test the response of four putatively resistant and three putatively susceptible clones of Italian cypress (*Cupressus sempervirens* L.) against one Mediterranean and five Californian fungal genotypes. By measuring mortality, size of the lesion caused by the fungus on the stem, and fungal sporulation, we ask the following questions: (1) Does the fast greenhouse assay confirm results of the extended field trials; (2) Is resistance confirmed for all putatively resistant clones when using a broad representation of the pathogen; and, (3) Does resistance have the potential to reduce transmission rates of the pathogen, thus potentially slowing down the CCD epidemic? Results indicated that: (1) Lesion size, but not seedlings' survival, can be used as metric to measure resistance in small grafted ramets; (2) One of four putatively resistant plant clones was considered susceptible at the end of the trial; and, (3) Sporulation was significantly lower in resistant than in susceptible plant clones. Based on these results, we conclude that resistance may be a viable disease control approach in this pathosystem due to the effect it has on both plant survival and fungal sporulation. However, we also conclude that resistance can be considered robust and effective against the pathogen species in its entirety, only if tested using several fungal genotypes. This study is one of the first to show that multiple introductions of a pathogen could break down host resistance and have a measurable detrimental effect on native ecosystems, suggesting that international policies should be revised accordingly.

1. Introduction

A pressing current aim of both plant health organizations and of the scientific community at large is to increase vigilance measures about: (i) the prevention of the introduction of exotic pathogens into a new region or country; and, (ii) the eradication or spread control of newly introduced plant pathogens. Conversely, less regulatory attention is given to diseases that are already established in a territory. This approach fails to take into account the possibility that different genotypes or strains, and/or different lineages of the same pathogen species may be further introduced causing clearly distinct outbreaks. This is currently a distinct possibility for diseases caused by different lineages of the same emergent pathogen (Grünwald et al., 2012; Eyre et al., 2014) or for pathogens that are currently evolving allopatrically after their

* Corresponding author.
E-mail address: matteog@berkeley.edu (M. Garbelotto).

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introduction in different parts of the world (Oswald et al., 2012). The occurrence of new outbreaks of emerging plant diseases caused by multiple introductions is a crucial issue, given we live in an era of increasing international movement of plants and of their associated pathogens (Ghelardini et al., 2017). The relevance of this issue for exotic and invasive plant pathogens should not be surprising, as it has been proven over and over again that biological invasions are greatly favored by multiple introductions (Facon et al., 2008).

The same concerns arise when dealing with different but closely related, morphologically undistinguishable, and interfertile species. Introductions of new and closely related species, in fact, can lead to taxa with greater invasive potential, through hybridization with a sexually compatible resident pathogen (Brasier, 2000; Brasier et al., 2004; Gonthier et al., 2007; Newcombe et al., 2000; Santini et al., 2005). The migration of different genotypes or of different mating types of a pathogen in areas where the species is already present, can also lead to sexual reproduction resulting in more aggressive genotypes strains that may alter the sensitivity of pathogens to specific compounds used for chemical control or may be able to infect plant genotypes selected for resistance (Goodwin et al., 1998; Singh et al., 2011). Both scenarios can cause extremely devastating new waves of invasion.

Cypress canker disease (CCD) is a destructive disease causing crown dieback and mortality in several plant species of the family *Cupressaceae*. The first epidemic was reported in 1928 in California (USA) on Monterey cypress (*Cupressus macrocarpa* Hartw. ex Gordon) (Wagener, 1928), Since this initial report, the disease spread across the globe (Graniti, 1998), being transported among continents by the trade of ornamental plants (Danti et al., 2014; Della Rocca et al., 2011b) and becoming a pandemic within fifty years. Epidemics were particularly severe in California on *C. macrocarpa,* and around the Mediterranean Basin on *Cupressus sempervirens* L., and caused significant losses in woods, plantations, gardens, parks and nurseries, also endangering the iconic role held by cypresses in the Mediterranean landscape (Danti et al., 2013; Danti and Della Rocca, 2017). However, the reasons for the severity of the two outbreaks in California and in countries of the Mediterranean basin are diametrically opposite. In California, the pathogen is native and causes no disease on endemic cypress species in their natural range (Della Rocca et al., 2011a, 2013). The epidemic was and in part is still caused instead by the massive planting of coastal Monterey cypress in agricultural lands of inland valleys, characterized by very different ecological conditions than coastal sites (Danti and Della Rocca, 2017). In countries around the Mediterranean basin, the CCD causal agent *Seiridium cardinale* (W.W. Wagener) B. Sutton & I.A.S. Gibson is exotic and the entire outbreak was caused by the introduction of a single genotype rapidly adapting to the new ecosystem and to the new host, *C. sempervirens* (Della Rocca et al., 2011a, 2013). In both regions, disease incidence is considerably augmented by the presence of the extremely susceptible artificially created hybrid species Leyland cypress (x *Hesperotropsis leylandii* (A.B. Jacks. & Dallim.) Garland & Gerry Moore) (Danti et al., 2014; Danti and Della Rocca, 2017).

Three main outcomes stemmed from the genetic analysis of California and Mediterranean populations of the fungus (Della Rocca et al., 2011a, 2013): (i) in California, the *S. cardinale* population is genetically diversified and includes two clearly distinct, albeit intermixing, metapopulations, called β-tubulin haplotype A (β-tubA) and β-tubulin haplotype B (β-tubB); (ii) the Mediterranean outbreak was due to a single introduction of a β-tubA genotype; and, (iii) sexual reproduction is occurring in California, whereas the European population has been generated solely through clonal reproduction.

As a result of the intrinsic difference between the two outbreaks, their respective trajectories have been substantially different. In California, disease is controlled by: (a) avoiding planting coastal cypresses inland (inland cypress species are per se quite disease-resistant), and, (b) avoiding the planting of Leyland cypress. In the Mediterranean region, due to the exotic nature of the pathogen, susceptibility of native *C. sempervirens* was initially very high and CCD affected up to 70–90% of trees in some conducive sites during the '70-'80 decades (Panconesi, 1990; Graniti, 1998). Currently, CCD in the Mediterranean is managed with an integrated approach that includes implementing of extinctive measures (sanitation), using resistant cypress selections for new plantations, avoiding off-site plantings and extensive use of Leyland cypress; and, finally, the use of preventive chemical treatments (only for very high value individual trees) (Della Rocca et al., 2011c). However, where the use of Italian cypress remains a priority, the employment of the cultivars selected for their resistance to CCD is probably the most valuable approach (Danti et al., 2006). These cultivars were developed in the last 35 years through a genetic improvement program conducted in Italy by the IPSP-CNR, (Raddi et al., 1984; Xenopoulos, 1990; Santini and Di Lonardo, 2000; Danti et al., 2011).

Screening cypress candidates for resistance has relied on execution of artificial inoculations on a fair number of ramets of each cypress clone, planted in contrasting sites to estimate the clone and the environment effects on the host response. Italian cypress shows a polygenic, or quantitative resistance to CCD and its phenotypic expression is influenced by both the genotype and environment. For this reason, the assessment of the resistance stability is carried out in parallel in different pedo-climatic environments (Danti et al., 2013). This horizontal (partial) resistance of cypress to CCD is known not to be a universal trait, as, similarly to a dam, it can be overcome by more virulent strains of the fungus or by multiple close infections ((Panconesi, 1990) As pathogenicity tests showed repeatedly low variability in virulence among the Mediterranean isolates of the fungus (Raddi and Panconesi, 1984; Ponchet et al., 1990; Moricca et al., 2000; Pedron et al., 2007), for many years the same *S. cardinale* isolate (ATCC 38654) has been used in a standardized selection procedure. The validity of this approach was in part supported by a study showing that the entire pathogen population of Southern Europe is derived from the introduction of a single genotype (Della Rocca et al., 2011a). However, two further studies (Della Rocca et al., 2013; Garbelotto et al., 2015) have indicated exotic pathogen populations in the Mediterranean have evolved and adapted, and at least two genetically and phenotypically distinct subpopulations can now be identified in the Mediterranean basin.

Hence, a key and largely unanswered question is whether cypress cultivars previously selected for resistance to CCD in Italy based on a single pathogen genotype are also resistant when challenged with genetically different isolates of *S. cardinale*. The two main aims of this study, thus, were: (1) To test a fast assay to explore the response of Italian cypress clones previously defined as resistant or susceptible to a larger sample of pathogen genotypes, representative of the diversity in native pathogen populations; (2) To study the correlation between resistant/susceptible plant phenotypes, lesion size, mortality rate, plant stem diameter, and sporulation, using several plant clones and pathogen genotypes.

2. Materials and methods

2.1. Experimental design

A total of 419 plants of Italian cypress (*Cupressus sempervirens*) representing four putatively resistant (R) and three putatively susceptible (S) clones to CCD were used in this experiment. Plants belonging to a clone were thus genetically identical, but each was grafted on commonly used 1-year-old seed rootstock, and grown for 18 months in 0.35-liter free draining pots containing peat, compost, and perlite (3:1:1 v/v/v) in Italy. Plants were shipped to the USA, and, after the necessary State and Federal inspections, they were transferred for three months to a containment greenhouse at the University of California, Berkeley. Plants were artificially inoculated on June 15th 2016 using six *S. cardinale* isolates, when they were 21 months old.

The seven cypress clones are currently maintained in the IPSP-CNR clonal collection, and were chosen based on their response to *S. cardinale* inoculations, following a standard procedure developed by the

Table 1
Provenance, belonging to a β-tubulin group (A or B) and virulence (H = high; I = intermediate; L = low) of the tested *S. cardinale* isolates.

Isolate	Provenance	β-tubulin group	Virulence
Sc-157	MED	A	H
Sc-479	CAL	A	L
Sc-481	CAL	A	I
Sc-476	CAL	B	I
Sc-483	CAL	B	H
Sc-494	CAL	B	H

same Institute in Italy (Danti et al., 2006, 2013). Four of them (PM-322; PM-318, PM-2546, PM-2440) have been long patented in Italy for their proven resistance to *S. cardinale* (R), while the remaining three (PM-3947, PM-3954 and PM-3962) were known to be susceptible (S) (Panconesi and Raddi, 1990a,b; Danti et al., 2006).

The six *S. cardinale* isolates (Table 1) were chosen as follows, using data generated by Della Rocca et al. (2011a, 2013). One was from the invasive population in the Mediterranean (MED), and five were from the native population in California (CAL); three belonged to haplotype A based on their β-tubulin sequence, and three belonged to haplotype B (note that only haplotype A is present in the Mediterranean); all were different genotypes and none of them were neighbors in a minimum spanning network generated with microsatellite data (Della Rocca et al., 2011a) or in a phylogram generated with AFLP data (Della Rocca et al., 2013). Their virulence had been previously assessed based on the extent of the lesions they caused when inoculated on a random mix of genotypes of 6-year-old Italian cypress and kept for 6 months in a greenhouse in Italy (unpublished data).

All six *S. cardinale* isolates were cross-inoculated on the four *C. sempervirens* resistant clones, while the three susceptible plant clones were artificially infected with five isolates, excluding Sc-481, because of the lower number of available plants. Plants were inoculated according to Danti et al. (2013). Briefly, a 3-mm-diameter circular plug of bark was first removed from the stem using a cork borer and then replaced with a plug of the same size taken from the margin of a colony of the fungus grown on potato dextrose agar (PDA) at 25 °C for 15 days in the dark. The inoculation site was protected with damp cotton and wrapped with plastic paraffin (Parafilm®) for a week. A total of 13–81 plants (or ramets) of each clone, depending on the total number of ramets available, was inoculated with all fungal isolates. Plants had an average stem caliper of 4.40 mm (SD = 0.48) (see Table 2 for information by clone). Plants were distributed in 8 blocks, and distribution of plants within block was completely randomized. Three plants or ramets of each cypress clone to be used as controls were mock inoculated using the same procedure above, but using a plug of sterile PDA. Three additional plants per clone were left intact (Table 2).

Inoculated plants were watered regularly twice a week, and kept in the greenhouse with a 12-h cycle of night and day, temperatures fluctuating between 18 and 28 C, and at relative humidity ranging between 65 and 85%. Three months after inoculation, the experiment was ended and the following metrics were measured: stem diameter of each plant, plant survival (number of ramets survived for each "clone x isolate" combination), canker size in each plant (length of necrotic tissue below and above the inoculation point), and number of acervuli (fruiting bodies) developed on the cankered stem of each plant.

Survival was assessed by counting number of individuals that had a dead canopy and dried cambium above the inoculation point. Size of cankers was evaluated by measuring the extent of the necrotic tissue above and below the inoculation point with a precision gauge, after the outer bark of the stem was gently removed using a sterile scalpel. Acervuli appeared like black sub-epidermal or sub-peridermal pustules (0.2–3 mm in size) opening when mature with the breakage of the upper tissues (Danti et al., 2013). The number of acervuli developed on the surface of the cankered stem of each ramet was counted with the aid of a magnifying glass. Lesions were always clearly visible on inoculated ramets and were never present on controls. The presence of typical acervuli on inoculated plants was used as the diagnostic confirmation of the presence of *S. cardinale*.

2.2. Statistical analysis

The percentages of plant survival assessed for each clone and each isolate at the end of the experiment were transformed using the Bliss formula: $y = \arcsin \sqrt{p/100}$ (where p is the percentage of plant survived), before being analyzed through the use of a General Linear Mixed Model (GLMM) and Kruskall-Wallis median tests. A General Linear Model (GLM) instead was applied to explore the effect of clones, fungal isolates and of their interactions, on lesion length and number of acervuli: both plant clones and fungal isolates were regarded as fixed factors. Blocks were excluded from the models, because there was no difference among them for any of the metrics analyzed. GLMs and GLMM perform well when analyzing samples of different size in absence of heteroscedasticity, and even when heteroscedasticity is present in the dataset, results of GL models are not generally invalidated, except for the accuracy of SE values. Because of the inevitable differences in number of replicates per treatment, variation in sample size of treatments could cause heteroscedasticity in the dataset. We performed the Hartley, Cochran, and Bartlett partition of homogeneity tests to check for the presence of heteroscedasticity in the pivotal LLD dataset, partitioned separately by plant clones and by fungal genotypes.

Differences in mean length of cankers were analyzed through a factorial ANOVA, while a post hoc unequal N HSD test was used to explore differences among the various "clone x isolate" combinations. The same test was used to evaluate differences in number of acervuli developed on cankers three months after inoculation. In order to explore consistency and correlations among the different metrics, a logistic regression model was also performed correlating cypress ramet survival, length of cankers, number of acervuli, and stem diameters of cypress plants. Pearson correlations were also calculated to evaluate the correlation among all of the considered parameters. In addition, multivariate discriminant analysis was carried out, sorted by cypress clone and by pathogen isolate, to verify the joint effect of the variables analyzed. All statistical analyses were performed using the software packages STATISTICA 10 and SPSS.

Table 2
Cypress clones, Putative phenotype: Resistant (R) or Susceptible (S) to CCD; stem diameter (SD); number of ramets for each clone inoculated with the same fungal isolate; number of isolates used to inoculate each clone, number of ramets mock-inoculated; total ramets used in the trial.

Clone	Putative phenotype (R/S)	Diameter (SD)	Replications	N. of isolates	Mocks + Untouched	Total ramets
PM-322	R	4.16 (0.47)	13	6	3 + 3	84
PM-318	R	4.58 (0.39)	13	6	3 + 3	84
PM-2546	R	4.25 (0.38)	13	6	3 + 3	84
PM-2440	R	4.34 (0.44)	13	6	3 + 3	84
PM-3954	S	4.66 (0.44)	6	5	3 + 3	36
PM-3947	S	4.92 (0.56)	5	5	3 + 3	31
PM-3962	S	4.33 (0.39)	2	5	3 + 3	16

Fig. 1. CCD lesion caused on cortical tissues of stems of *Cupressus sempervirens* seedlings: artificial inoculation (A) and natural infection (B).

3. Results

Inoculations always induced the typical stem lesions associated with Cypress Canker Disease (CCD) (Fig. 1).

Since a considerable number of plants showed desiccation of the stem above the inoculation point, the downward development of necrosis (LLD) due to tissue colonization by *S. cardinale* was taken as the more reliable measurement of disease severity, because it excluded the possible and likely influence of dead bark tissues on the progression of the fungus. In addition, measurement of necroses on dry stems is relatively challenging and error prone. In support of our decision, we found no significant relationship between the lengths of the lesions above and below the inoculation point ($R^2 = 0.0014$), and the GLM factorial ANOVA performed using the lesion development upward from the inoculation point showed no differences among clones, nor among isolates ($p = 0.256$, df = 6, F = 1.301 and $p = 0.407$, df = 5, F = 1.018 respectively).

Conversely, LLD values varied significantly (GLM) both among clones ($p < 0.01$, df Effect = 6, F = 14.179) and isolates ($p = 0.042$, df Effect = 5, df Error = 331, F = 2.330), while the interaction clone x isolate was not significant ($p = 0.247$, df = 5, F = 2.330). All tests of homogeneity of variance showed that no heteroscedasticity was present for LLD values by fungal isolates ($p = 0.295$, Fmax (Hartley) = 1.699, R (Cochran) = 0.247, M (Bartlett) = 4.922) and by plant clones ($p = 0.165$, Fmax (Hartley) = 1.971, R (Cochran) = 0.190, M (Bartlett) = 9.163), although in this second instance fungal isolate 481 had to be excluded from the analysis to avoid heteroscedasticity. Isolate 481 has intermediate virulence and is of no significant consequence on the overall results of the study.

LLD values ranged from 24.8 mm to 64 mm. PM-318, PM-322 and PM-2440, all putatively resistant, showed shorter lesions (24.8 mm, 35 mm and 36.2 mm respectively) while the putative susceptible PM-3962 (49.8 mm), PM-3954 (64 mm) and the putative resistant PM-2546 (50.9 mm) showed longer lesions (Table 3). LLD values also varied significantly among *S. cardinale* isolates ($p = 0.042$, df = 5, F = 2.330): the shorter lesions were caused by the putative low virulent Sc-479 isolate (32.3 mm) while the longer by the putative highly virulent Sc-157 isolate (46.3 mm) (Table 4). ANOVA's based multiple tests using LLD values identified clearly groups among both plant clones and fungal isolates (Tables 3 and 4). Both plant clone and fungal genotype affected the SDs of LLD values, with the trend of distribution of SD values matching that of LLD values distribution (Table S1). By

Table 3
Comparison among cypress clones: lesion length downward (LLD, in mm) from the inoculation point, percentage of *C. sempervirens* ramet survival (%) and number of acervuli developed on the lesion surface on cypress clones three months after stem inoculation with *S. cardinale*. R = putative resistant phenotype; S = putative susceptible phenotype. Different letters indicate significant differences of each parameter among cypress clones ($p < 0.01$) (Factorial ANOVA, unequal N HSD post hoc test for LLD and number of acervuli; Kruskal-Wallis median test for survival).

Clone	Putative phenotype (R/S)	Susceptibility based on LLD (mm)	Susceptibility based on ramets survival (%)	Infectivity index based on n. of acervuli
PM-318	R	24.8 a	58.0 a	2.2 a
PM-322	R	35.0 ab	22.7 b	2.7 ab
PM-2440	R	36.2 abc	21.8 b	3.3 ab
PM-2546	R	50.9 cd	4.0 c	3.7 b
PM-3947	S	46.8 bcd	29.0 bc	5.2 b
PM-3954	S	64.0 d	6.1 c	4.2 b
PM-3962	S	49.8 bcd	10.0 c	2.3 ab

Table 4
Comparison among *S. cardinale* isolates: lesion length downward (LLD, in mm) from the inoculation point, percentage of *C. sempervirens* ramet survival (%) and number of acervuli developed on the lesion surface three months after stem inoculation of cypress clones with *S. cardinale*. H = putative high virulence; I = putative intermediate virulence; L = putative low virulence. Different letters indicate significant differences among *S. cardinale* ($p < 0.01$) (Factorial ANOVA, unequal N HSD post hoc test).

Isolate	Putative virulence (H, I, L)	Virulence based on caused LLD (mm)	Virulence based on host survival (%)	Infectivity index based on n. of acervuli
Sc-479	L	32.3 a	38.7	3.2
Sc-476	I	42.5 ab	21.6	2.4
Sc-481	I	36.2 ab	24.0	3.3
Sc-157	H	46.3 b	9.7	3.3
Sc-483	H	41.7 ab	16.4	3.5
Sc-494	H	41.2 ab	17.9	3.5

comparing SD values within the same plant clone, it is clear that fungal genotype has a strong effect on SD, and the smallest SD values were recorded for resistant plant clones inoculated with low or intermediate virulence fungal genotypes.

Percentages of ramet survival significantly varied from 4% to 58% among cypress clones when considering mortality caused by all fungal isolates together ($p = 0.03$, df = 6, H Kruskal-Wallis Test = 25.202) (Table 3). The lowest percentages of survival were identified in cypress clones PM-2546 (putatively resistant) and in the putatively susceptible PM-3954 (4% and 6.1%, respectively). A significantly higher survival rate was exhibited by the putatively resistant clone PM-318 (58%). Rates of survival by fungal isolate ranged from 9.7% (Sc-157) to 38.7% (Sc-479), but differences were not significant ($p = 0.110$, df = 5, H Kruskal-Wallis Test = 8.981) (Table 4) when considering individual isolates.

The mean number of acervuli produced on cankered stems showed significant differences among cypress clones ($p < 0.01$, df = 6, F = 5.520), and ranged from 2.2 on the putatively resistant PM-318 to 5.2 on the putatively susceptible PM-3947 (Table 3). Conversely, there was no significant effect due to *S. cardinale* isolate ($p = 0.189$, df = 5, F = 1.500) (ranging from 2.4 for the putatively intermediate virulent Sc-476 to 3.5 for the putatively highly virulent Sc-483 and Sc-494 isolates), or for the clone x isolate interaction ($p = 0.885$, df = 21, F = 0.686) (Table 4).

Based on the three main metrics measured in this study, i.e. survival rate, LLD, and production of acervuli, the putatively resistant cypress clone PM-2546, clearly behaved as susceptible. Hence, when comparisons were made between resistant and susceptible clones as a whole, the resistant plants' group excluded PM-2546, which was instead included in the group of susceptible plants. No significant differences were found between the A and B β-tubulin haplotypes of the fungus in general, or by resistant and susceptible cypress clones for any of the parameters analyzed with one exception: i.e. the number of acervuli was significantly higher ($p < 0.01$) on the lesions of susceptible clones (average = 3.95) compared to the resistant ones (average = 2.71).

Logistic regressions comparing cypress plant survival (coded as a 1 or a 0) vs. LLD, survival vs. number of acervuli, and survival vs. cypress stem diameter, indicated significant correlations ($p < 0.001$) among parameters. Namely: plant survival was greater when the LLD was lower, the number of acervuli was lower the higher the survival, and cypress stem diameter was larger when survival was greater. Similar results were obtained performing linear Pearson correlations among all of the parameters (the plant survival was here considered as percentage) (Table 5).

The results of the discriminant analysis of clones based on diameter of the ramets, lesion length downward (LLD), and the number of acervuli are shown in Fig. 2. The first three functions/roots accounted for 98.6% of discriminating power. The discriminating power of the first function (56.1%) is mainly driven by LLD, whereas that of the second function (36.4%) was driven by the stem diameter of the ramets. The first function discriminates primarily PM-3947 and PM-3954, which were mainly distributed in the right part of the graph and displayed larger lesion sizes. The second function mainly discriminates PM-318, which was distributed in the upper part of the graph and displayed larger stem diameter size (Fig. 2). In contrast, no significant discrimination among *S. cardinale* isolates was evident considering the same variables (data not shown).

Table 5
Correlations between lesion length downward (LLD, in mm), number of acervuli developed on the lesions surface and diameter of the cypress plants. Pearson correlation coefficient (R) values are shown in the table (in bold the significant correlation per $p < 0.01$).

	Ramet survival	Number of acervuli	Plant diameter
LLD	**0.56445**	0.16523	0.05385
Ramet survival	–	**0.31749**	0.26325
Number of acervuli	–	–	0.14866

4. Discussion

The deployment of disease resistance is a powerful tool when attempting to restore populations of native plant hosts threatened by invasive deadly plant pathogens. However it is also very well known that resistance to disease comes in many different forms and it can be more or less mediated by age and size of the plant and by environmental/ecological conditions of a site (Fritz and Simms, 1992; Dangl and Jones, 2001; Gatehouse, 2002; Heil and Bostock, 2002; Fu and Dong, 2013; Pastor et al., 2013). How plants are defined "resistant" or "susceptible" remains a key question. More than one study has described assays developed to identify resistance in trees, and in general the main focus has been on the plant side of the equation. Age, size, provenance of hosts, and environment are all reported to deeply influence the result of any given assay. In the case of CCD, more reliable results are obtained when using outplanted and not potted individuals, when plants have a caliper larger than 10 mm at the point of inoculation (Raddi et al., 1984), and when tests from different sites are concordant (Danti et al., 2006, 2013). It should come to no surprise that testing Italian cypress for resistance is a long process, often taking more than 5 years to be completed (Danti et al., 2006, 2013).

However, the pathogen side of the same equation has not received the same attention. In general, one of the weakest aspects of resistance selection projects has to do with the lack of adequate representation of the phenotypic and genotypic diversity of the pathogen. There may be multiple reasons for this inadequate representation, but the two most common reasons include lack of a depository or culture collection of the pathogen, and/or lack of information on the origin of the pathogen and its biogeographical history. Thus, particularly when dealing with an exotic organisms, research is often conducted using only a sample of the invasive population of a pathogen, that -by definition- is genetically limited because it has gone through a strong genetic bottleneck and founder effect during the introduction, establishment, and invasion processes (Garbelotto, 2008). This is in fact the case of the selection program for resistance to CCD, which has been conducted for decades using a single genotype of the fungus, considered to fairly represent the virulence of the Mediterranean invasive population of *S. cardinale*. This choice was originally supported by studies repeatedly showing limited genetic and phenotypic variability within the invasive and clonally reproducing population of the pathogen in the Mediterranean basin (Raddi and Panconesi, 1984; Ponchet et al., 1990; Moricca et al., 2000; Pedron et al., 2007).

The recent discovery that the CCD pathogen *Seiridium cardinale* is likely to be native to California, and that the California population is genetically rather diversified and sexually reproducing (Della Rocca et al., 2011a), has brought up two very important conservations issues. The first one is that, in spite of the fact that *S. cardinale* is recorded as being present both in the USA and in many EU countries, due to the greater genetic diversity of California populations, additional introductions from California and the USA into the EU should be avoided. This would require new legislation, but Garbelotto et al. (2015) have clearly shown that the source population in California, and European populations are clearly phenotypically distinct for several important transmission traits. The second conservation issue has to do with understanding whether those Italian cypress clones patented as resistant to CCD through a steady effort lasting several decades, would remain resistant when challenged by different genotypes of the pathogen. If not, this would be an additional argument in favor of further controlling additional introduction of the pathogen from North America into Europe.

In order to test the breadth of resistance of patented Italian cypress clones, three requirements needed to be met. First, putative resistant

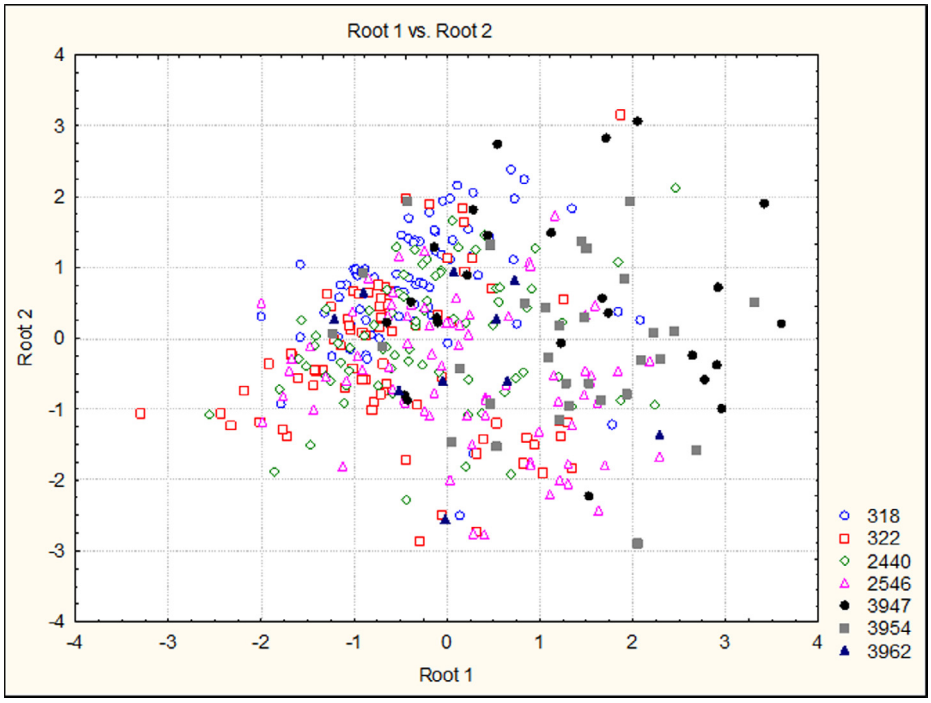

Fig. 2. Scatterplot (Root 1 vs. Root 2) of the discriminant scores for the seven cypress clones. Root1 refers to LLD, while Root2 refers to stem diameter.

and susceptible clones needed to be available; second a sample representative of the variability of the pathogen needed to be available, and; third, a faster screening process needed to be evaluated due to the increased number of fungal isolates that needed to be tested. The first and second requirements were easily met: patented resistant and known susceptible cypress clones were available to us, while we used information published in three studies (Della Rocca et al., 2011a, 2013; Garbelotto et al., 2015) and some unpublished inoculation studies to choose six S. *cardinale* isolates that differed for geographic origin, genotype, genetic sequence, and virulence and that were representative of the entire known range of variation within the species.

The assay here described produced results that significantly differentiated host clones and fungal isolates, in spite of one unexpected result. Due to the small stem diameter of the plants that favored the rapid girdling of the stem by the fungus, survival rate recorded 3 months after inoculation was overall lower than that reported in field selection trials (Raddi and Panconesi, 1984; Ponchet et al., 1990). Fortunately, it was clear that mortality was not associated with wounding, because none of the mock-inoculated controls died. As a result of rapid death of the upper stem in inoculated plants, size of the lesion upward from the inoculation was found to be unreliable and had to be discarded. In fact, when it was regressed against the lesions downward from the inoculation point or LLD, the two were not correlated. The other two metrics indicative of plant susceptibility and/or pathogen virulence, i.e. LLD and survival, yielded significant and valid results. Logistic regressions and Pearson correlations provided further insights about the robustness of the metrics measured: larger lesions (LLD) were significantly and positively correlated with higher mortality rates, further strengthening the use of LLD to discriminate among plant clones and fungal isolates. No significant correlation was found between diameter and LLD, indicating size of the trees should not be influential on this variable in this experiment; however, a significant negative correlation was found between stem diameters and survival rates, indicating that survival rate may be affected by the smaller diameter of the stem of some of the plants employed. Thus it is not surprising that, given the conditions of our trial, LLD was the most valuable metric, the only one that discriminated both plant clones and fungal isolates, and the one that had the most important effect in the discriminant analysis (Fig. 2). We conclude that inoculation experiments using plants between one and two years old can be conducted with the prerequisite that their duration be short enough to avoid desiccation of the portion of the stem below the inoculation point, in order to ensure LLD may be measured reliably. Nonetheless, even if survival has been affected by the small stem diameter of the plants and did not produce any valuable results to significantly differentiate fungal isolates, we note that disease susceptibility rankings of plant clones using LLD and survival were clearly concordant for six out of seven clones (Table 3). In fact, according to both metrics, PM-318, followed by PM-322 and PM-2440, were the most resistant clones, while PM-2594, followed by PM-2546 and PM-3962, were the most susceptible ones. Results were discordant for clone PM-3947 which had an LLD value typical of susceptible clones and a survival rate akin to that of resistant clones. However, that clone had been previously defined as susceptible, once more supporting the validity and higher reliability of the information provided by LLD.

After having assessed the validity of the overall approach to test the response to CCD and the relative strength of the different metrics, we needed to analyze the results to determine if: (a) Did the plant clones perform as expected? In other words, did the screening using multiple fungal isolates here described corroborate the historical results on susceptibility levels to CCD obtained with a single fungal isolate, and, (b) Were there differences in virulence among the isolates? Based on LLD values and on survival rates, six out of seven plant clones performed as expected, once again confirming the validity of the screening assay. Nonetheless, putatively resistant clone PM-2546, was clearly susceptible displaying large LLD and small survival values (Table 3). The most aggressive fungal isolate was the Californian B haplotype isolate Sc-494, which caused the largest LLD on PM-2546. Californian isolates Sc-481, Sc-483, and Mediterranean isolate Sc-157 were equal and all caused the highest mortality rates (Supplementary Table S1). Thus, our results clearly indicate that when challenged with multiple genotypes, resistance of PM-2546 was eroded. Specifically, Californian isolates were mostly responsible for this “erosion”.

While it is encouraging that the response of 3 out of 4 putatively resistant plant clones remained resistant even when challenged with multiple isolates, the erosion of resistance in one out of four plant

clones amounts to an erosion of 25% of the available resistance. Obviously, PM-2546 should be used with caution, as there are clues that the resistance it showed in field trials in Italy could be eroded if more aggressive isolates of the pathogen were introduced. Nonetheless, a more pressing question remains: would other putatively resistant clones respond as susceptible clones, if challenged with an even broader sample of genotypes? While we cannot answer this question at this time, our results based on LLD values and their SD indicate fungal isolates can be placed in three groups of virulence, proving that diversity in aggressiveness exists within the species. Low SD values indicate consistency in the distribution of a metric, suggesting a smaller effect of exterior variables, and confirming the robustness of a disease control approach across different treated individuals and sites. In the case of plant resistance as an approach to control CCD, the most desirable effects are obtained using resistant plant clones in the presence of low virulence fungal isolates (lowest SD and LLD), while more aggressive fungal isolates result not only in higher LLD, but also in proportionally higher SD values. This result underlines a newly discovered importance of fungal genotype when assessing resistance, and suggest plant clones with the lowest variation in SD may be the most dependable ones, because less affected by exterior unaccounted variables.

This result is discordant with results of prior experiments performed exclusively using Mediterranean isolates (Raddi and Panconesi, 1984; Ponchet et al., 1990), but is in agreement with results of a recent paper comparing virulence of both California and Mediterranean isolates (Garbelotto et al., 2015). We conclude that additional introductions of *S. cardinale* from the USA into States of the EU could have devastating effects on native hosts, leading to a resurgence of mortality in Italian cypress populations and likely causing the infection and death of CCD resistant clones, thus nullifying an effort that has required a huge investment in terms of resources and time.

The current study provided the further opportunity of studying the effect on sporulation (e.g. acervuli production) when plant clones varying in disease susceptibility are infected by fungal isolates varying in virulence. Sporulation is a key transmission trait for fungi that spread through the movement of spores (Garbelotto et al., 2015). Models predict that prolonged sporulation supported by long-living resistant plant clones could actually result in larger cumulative amounts of spores and in higher levels of mortality in the long run (Meentemeyer et al., 2011; Holt et al., 2003; Cobb et al., 2012). However, this negative and unexpected effect of resistance could be offset if resistant plants supported a significantly reduced amount of sporulation compared to susceptible plants. When we compared the number of acervuli between cypress clones defined as resistant or susceptible based on the results of this study, the number of acervuli produced by resistant clones was significantly inferior than that produced by susceptible clones. This result is rather promising because it implies that the use of resistant clones may have the potential not only to increase survival rates of Italian cypresses but also to lower infection rates and disease spread, thus affecting the overall epidemiology of the disease. Further analyses need to verify whether the decrease in sporulation observed in resistant clones may suffice to obtain the desirable reduction in infection rate over the long term.

5. Conclusions

In conclusion, it has been recently shown that the introduction of a single or of a few related genotypes of *S. cardinale* from California into the Mediterranean basin has resulted in a significant outbreak of CCD spanning over Southern Europe, the Middle East, and North Africa (Della Rocca et al., 2011a; Danti and Della Rocca, 2017). The integrated control of CCD in the region has relied heavily on the use of resistant clones of Italian cypress through restoration projects. Historically, assessment of resistance has been based on extensive and time-consuming assays in which plants where challenged in field trials located in contrasting sites, using a single genotype of the pathogen that had been selected as representative in terms of virulence. This kind of assessment has been proven to be sound and effective, but its robustness is known to be restricted to local (regional level in broad sense) genotypes of the pathogen and environmental conditions. Thus, resistance needs to be tested against a broad selection of genotypes if that knowledge is available. By employing multiple fungal genotypes from the native range of the pathogen, we were able to show that resistance was eroded in one out of four putative resistant plant clones. We were also able to show that sporulation is reduced in resistant clones, hence their use could help to locally reduce infection rates. The inferences drawn from the study of this model pathosystem are general and can be summarized this way: the evidence we have provided supports an international policy that prevents multiple introductions of the same plant pathogens, and suggests that if plant resistance is to be deployed to control an invasive disease, an effort should be made to verify whether resistance is broad against diverse genotypes of the pathogen species or it is limited to the local pathogen genotypes, and whether it is associated with reduced pathogen sporulation. The paper describes an approach on how to select a broad representation of a plant pathogen species and on how to rapidly screen the response of putatively resistant trees to such broad representation.

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Authors contribution

G.D.R., R.D. and M.G. conceived and planned the experiments, performed the statistical analysis and wrote the manuscript. V.D.L. and T.P. worked out all of the technical details and performed the surveys and data collection. G.D.R. and R.D. have to be considered the co-first/equal authors of the manuscript. M.G. is the corresponding author.

Declaration of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2018.05.008.

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