# Field evaluation of grapevine rootstocks inoculated with fungi associated with Petri disease and esca

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**Abstract:** One-year-old grapevine rootstock cuttings (41 B Millardet-Grasset, 140 Ruggeri, 161 49 Couderc, 1103 Paulsen and 110 Richter) were inoculated with pathogens associated with Petri disease and esca of grapevine, to determine the effects of fungal infection on percentage of cuttings emerging from dormancy, shoot weight and disease severity. The cuttings were vacuum-inoculated with spore suspensions of either *Cadophora luteo-olivacea*, five species of *Phaeoacremonium* or *Phaeomoniella chlamydospora*, and planted in two field

sites in March 2008. Most of the fungal pathogens caused a significant reduction in percentage of cuttings emerging from dormancy and shoot weight, as well as a significant increase in disease severity in all grapevine rootstocks with the exception of rootstock 161 49 C. Rootstocks 110 R and 140 Ru were greatly affected by fungi associated with Petri disease and esca. In general, *Pa. chlamydospora* and *Pm. parasiticum* caused the greatest reduction in percentage of cuttings emerging from dormancy and shoot weight, and the highest increase in disease severity. Regression analyses showed a significant correlation between percentage of cuttings emerging from dormancy and disease severity, and between shoot weight and disease severity in almost all rootstocks inoculated with *Pa. chlamydospora*.

**Key words:** *Cadophora luteo-olivacea*, esca disease, Petri disease, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, *Vitis vinifera*

*Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams, several species of the genus *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. and *Cadophora luteo-olivacea* (van Beyma) Harrington & McNew are considered to be endophytic fungi that are well adapted to colonization and growth in grapevine wood (Eskalen et al. 2001; Halleen et al. 2007). They can infect various tissues, including roots, pith, vascular tissue, pruning wounds, green cortical tissue and berries. These pathogens have been intensively studied because of their involvement in Petri disease in young vines, and esca in adult vines, in which white rot pathogens such as *Fomitiporia mediterranea* also have a significant role (Mugnai et al. 1999; Gubler et al. 2004a; Mostert et al. 2006; Halleen et al. 2007).

In the America's production areas, the incidence of Petri disease and esca (also known as black measles in North America) has continuously increased over the last 15 years, with

numerous reports of plants affected with these diseases in North America (Scheck et al. 1998; Gubler et al. 2004a) and South America (Gatica et al. 2001; Auger et al. 2005). The increased prevalence of Petri disease and esca coincided with massive replanting of grapevines as a result of phylloxera (*Daktulosphaira vitifoliae* Fitch) infestation (Eskalen et al. 2001). Grapevine rootstocks crosses of *Vitis riparia* x *V. rupestris* [3309 Couderc (3309 C) and 101-14 Millardet-Grasset (101-14)] and *V. berlandieri* x *V. rupestris* [1103 Paulsen (1103 P) and 110 Richter (110 R)] were planted extensively, and are now the most frequently rootstocks used in the Americas (Eskalen et al. 2001; Gubler et al. 2004a).

External symptoms of Petri disease include late bud break, stunted shoot growth, weak vegetative growth and sometimes various leaf symptoms (interveinal chlorosis, leaf necrosis and wilting). Internal xylem symptoms include cinnamon to brown areas in the wood, with brown to black vascular streaking. In cross sections these appear as brown/black spots which ooze dark phenolic substances from the xylem tissue within 10-20 minutes after cutting (Mostert et al. 2006). Foliar symptoms of esca typically begin with mild interveinal chlorosis, darkening over time to brown interveinal regions in the leaves (Mostert et al. 2006). These vines ultimately develop the severe form, known as 'apoplexy', which is characterized by sudden wilting and shedding of the leaves, shriveling of fruit and vine death within 1-2 years. Internal wood symptoms usually include areas of white heart rot surrounded by wood with typical Petri disease symptoms (Mugnai et al. 1999). The expression of Petri disease symptoms caused by *C. luteo-olivacea*, *Phaeoacremonium* spp. and *Pa. chlamydospora* has been successfully reproduced with artificial inoculations either under field (Mugnai et al. 1999; Gubler et al. 2004b; Halleen et al. 2007) or greenhouse conditions (Scheck et al. 1998; Larignon and Dubos 1997; Adalat et al. 2000; Gubler et al. 2004b; Halleen et al. 2007; Zanzotto et al. 2008; Aroca and Raposo 2009; Laveau et al. 2009).

Several studies have demonstrated that rootstock mother vines are the primary source of the Petri disease pathogenic fungi associated with Petri disease and esca (Fourie and Halleen 2004; Aroca et al. 2010) and that rootstock cuttings can already be infected by these pathogens when used as grafting material (Fourie and Halleen 2002). Furthermore, the internal symptoms of Petri disease are usually found within the rootstocks of young grafted plants already established in the field, so rootstocks are considered more susceptible to these fungal vascular pathogens than scions (Scheck et al. 1998; Adalat et al. 2000; Zanzotto et al. 2008).

Much work has been conducted to determine the difference in susceptibility of grapevine and rootstock cultivars to *Pa. chlamydospora* and species of *Phaeoacremonium*, with variable results. Eskalen et al. (2001) did not observe any resistant cultivars among the 20 grapevine rootstocks inoculated with *Pa. chlamydospora*, *Pm. aleophilum* W. Gams, Crous, M. J. Wingf. & L. Mugnai or *Pm. inflatipes* Gams, Crous & M. J. Wingf. Feliciano et al. (2004) demonstrated that Thompson Seedless was significantly more susceptible to *Pa. chlamydospora* and *Pm. aleophilum* than Grenache and Cabernet Sauvignon cultivars. Santos et al. (2006) also reported that Baga and Maria Gomes cultivars were more susceptible to *Pa. chlamydospora* and *Pm. angustius* W. Gams, Crous & M. J. Wingf. than 3309 C rootstock, and also noted differences between Baga and Maria Gomes. In a 3-year field trial where *Pa. chlamydospora* and *Pm. aleophilum* were inoculated on spurs of Italia and Matilda cultivars, the latter cultivar was more resistant (Spaparano et al. 2001). In Australia, Edwards and Pascoe (2004) only diagnosed Petri disease and esca in a few Riesling or Sultana cultivars, and not in Colombard or Ruby Cabernet. Marchi (2001) studied the disease incidence and progression of esca in a mixed cultivar vineyard in Italy and found four susceptibility groups among the 17 cultivars evaluated, with Semillon the most, and Roussanne the least susceptible. Artificial inoculation of rootstock cuttings (1103 P and 110 R) and *V. vinifera*

cultivars (Chardonnay and Anglianico) with *Pa. chlamydospora* showed that rootstock cuttings had a higher susceptibility than *V. vinifera* cultivars towards infection by this pathogen (Zanzotto et al. 2008). Similar results were observed in Australia, where seven grapevine rootstocks were also reported to be more susceptible to *Pa. chlamydospora* than the *V. vinifera* cultivars (Wallace et al. 2004).

Different inoculation methods, such as soaking bases of grapevine cuttings or seedlings in spore suspensions (Larignon and Dubos 1997; Scheck et al. 1998; Eskalen et al. 2001; Wallace et al. 2004; Aroca and Raposo 2009) or insertion of mycelial plugs/conidial suspensions into side wounds or cut ends of the grapevine stems (Mugnai et al. 1999; Spaparano et al. 2001; Feliciano et al. 2004; Santos et al. 2006; Halleen et al. 2007; Zanzotto et al. 2008) have been used to investigate the role that these pathogens play in Petri disease and esca. However, contamination by other fungal species might occur. Vacuum-inoculation of conidial suspensions throughout the cutting's vascular system of cuttings has been successfully used for Petri disease pathogens (Rooney and Gubler 2001; Aroca and Raposo 2009; Gramaje et al. 2009).

To date, most of the pathogenicity tests have been performed with *Pa. chlamydospora* and *Pm. aleophilum* under controlled conditions within a short period of time. The use of cultivars with field resistance may be an important approach in the management of these diseases. Thus, the objective of this work was to evaluate the effects of *C. luteo-olivacea*, five species of *Phaeoacremonium* and *Pa. chlamydospora* on the grapevine rootstocks most widely planted in Spain under field conditions.

## Materials and methods

**Fungal isolates.** The seven isolates used, one isolate each of *Cadophora luteo-olivacea*, *Phaeoacremonium aleophilum*, *Pm. mortoniae*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. viticola* and *Phaeomoniella chlamydospora*, were obtained from the fungal collection of the Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain. They were recovered from different geographic origins and rootstock–scion combinations in Spain (Table 1) and were identified by morphological and molecular methods as described below.

*Cadophora luteo-olivacea*, *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* were identified by phenotypical characters as described by Gams (2000), Mostert et al. (2006) and Crous and Gams (2000), respectively. Identification of *C. luteo-olivacea* was confirmed by the analysis of the ITS region of DNA (Gardes and Bruns 1993). Identification of *Phaeoacremonium* species was confirmed by the sequence analysis of the β-tubulin gene (Mostert et al. 2006). *Pa. chlamydospora* was identified by PCR using primers Pch1 and Pch2 (Tegli et al. 2000). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad Politécnica de Valencia-CSIC. Single spore colonies of the isolates were obtained by serial dilution prior to the storage of mycelium plugs in 15% glycerol solution at -80ºC in 1.5 mL cryovials.

**Rootstock inoculation and experimental design.** One-year-old rootstock cuttings [41 B Millardet-Grasset (41 B), 140 Ruggeri (140 Ru), 161 49 Couderc (161 49 C), 1103 P and 110 R] were used in this experiment. The cuttings were hot-water treated at 53ºC for 30 min to eliminate any existing infections by fungal vascular pathogens (Gramaje et al. 2009) and then acclimatized for 24 h at 20ºC before inoculation.

Fungal isolates were grown on potato dextrose agar (PDA) and incubated for 3-4 weeks at 25ºC in the dark. A conidial suspension was prepared for each isolate by flooding

the agar surface with 10 mL of sterile distilled water (SDW) and scraping with a sterile spatula. The resulting conidium suspension was filtered through two layers of cheese cloth into a 250 mL Erlenmeyer flask. The filtrate was diluted with SDW and the conidial concentration was adjusted with a hemacytometer to $10^4$ or $10^6$ conidia $mL^{-1}$.

Cuttings of each rootstock (50 cm long) were inoculated with each pathogen using a vacuum apparatus (COMECTA S.A, Cod: 5900621, Barcelona, Spain) as described by Rooney and Gubler (2001). Rubber tubing that was connected to the vacuum apparatus was fitted around the top of each cutting and the base of the cutting was immersed in a conidium suspension. The vacuum apparatus was operated for approximately 7-10 s, which had earlier been shown to allow ample time for uniform inoculation throughout the cutting's vascular system. Control treatments were vacuum-infiltrated with sterile water. For each grapevine rootstock, four groups of 10 randomly selected cuttings were inoculated with each pathogen/conidial concentration combination.

Inoculated rootstock cuttings were immediately planted in March 2008 in two field sites where grapevines had not been grown according to the memory of local farmers, in Roglà i Corberà (Valencia, Spain). The cutting groups (10 cuttings) were spaced 50 cm from other groups, cuttings being 10 cm apart from center to center, and with an interrow spacing of 100 cm. Each field plot was 34.5 m long and included twelve rows, each with 25 cutting groups (3,000 cuttings per field). In both sites, the experimental design consisted of four randomized blocks, each containing 10 cutting groups of each rootstock, pathogen and conidial concentration. Standard cultural practices were employed in both sites during the grapevine growing season. Plots were less than 1 km apart and had very similar climates and soil types.

**Disease assessment.** The number of cuttings that emerged from dormancy was counted in July 2008. In January 2009, at the end of the growing season, dormant plants were uprooted, washed and assessed for undried shoot weight. Then the stem of each grapevine cutting was transversally cut 10 cm from the base of the plant to allow estimation of the discolored area in the cross-section. The percentage vascular discoloration was estimated using a scale of 0 to 4, in which 0 = no discoloration, 1 = 1 to 25% discoloration, 2 = 26 to 50% discoloration, 3 = 51 to 75% discoloration, and 4 = 76 to 100% discoloration (Figure 1). The severity of the disease, evaluated from area of vascular discoloration, was calculated using the McKinney's index (McKinnney 1923), which expresses the percentage of the maximum severity of disease according to the formula: MI= $[\sum(R \times N)] \times 100 / H \times T$, where $R$ = disease rating; $N$= number of plants with this rating; $H$= the highest rating; $T$= total number of plants counted. Twenty percent of inoculated plants in each pathogen/grapevine rootstock combination were randomly selected for fungal re-isolation. Isolations were made immediately from the 10 cm long sections that were cut from the basal ends of the cuttings. The sections were washed under running tap water, surface-dried with sterile filter paper and surface-disinfested by immersion for 1 min in a 1.5% sodium hypochlorite solution, and washed twice by immersion for 1 min in sterile distilled water. Wood fragments cut from the edges of discolored xylem elements were placed on malt extract agar (MEA) supplemented with 0.5 $gL^{-1}$ of streptomycin sulfate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA) and plates incubated for 10-15 days at 25ºC in the dark. Emerging colonies were then subcultured onto PDA and were incubated using the same conditions until sporulation allowed species identification.

**Statistical analysis.** Data of the number of cuttings that emerged from dormancy and the disease severity scores were expressed as percentages. Data of shoot weight were expressed in grams. One-way analysis of variance (ANOVA) was performed with the experimental results

to determine the optimal conidial concentration. The Student's Least Significant Difference (LSD) test was used to compare the overall means of each conidial concentration within each rootstock at $P<0.05$. Additionally, in order for the results to be analysed together, the data were subjected to a two-way ANOVA with field site and block as effects in the model.

The statistical analysis of the experimental results was carried out in a two-way ANOVA with rootstock and treatments (fungal species at the optimal conidial concentration) as independent variables, and the following dependant variables: cuttings emerging from dormancy (%), shoot weight (g) and disease severity (%). The overall means of each treatment within each rootstock were compared using LSD values at $P<0.05$. The analyses used the Statistical Analysis System (version 9.0, SAS Institute Inc. Cary, NC, USA). For the values of cuttings emerging from dormancy (%) and disease severity (%), square root and logarithmic transformations of the data were evaluated to obtain a closer approximation to normal distribution of the residuals. Homogeneity of variance was assessed using Levene's test.

Simple regression analyses (linear and logarithmic) were performed to determine and measure the potential relationships between cuttings emerging from dormancy (%) and disease severity (%), and between shoot weight (g) and disease severity (%). These analyses were conducted separately for each grapevine rootstock with the SAS statistical package and significant differences among means were determined at $P<0.05$. Collinearity diagnostics were also performed on each data set to avoid unstable estimates and high standard errors due to linear dependence between predictor variables.

**Results**

Both conidial concentrations ($10^4$ or $10^6$ conidia $mL^{-1}$) caused significant decreases in the percentage of cuttings emerging from dormancy, shoot weight and an increase of disease severity when compared to the uninoculated controls, with the exception of the percentage of cuttings emerging from dormancy and shoot weight in 161 49 C rootstock (one-way ANOVA: percentage emergence, $F$= 7.27, DF= 2, $P$<0.001; shoot weight, $F$= 2.27, DF= 2, $P$=0.0339; disease severity, $F$= 16.96, DF= 2, $P$<0.001). In general, the maximum conidial concentration ($10^6$ conidia $mL^{-1}$) caused the greatest reduction of the percentage of cuttings emerging from dormancy and shoot weight, and the greatest increase of disease severity in all rootstocks. This conidial concentration was then determined as the optimal for the statistical analysis of the experimental results.

Neither field site, nor block nor its interaction significantly affected the parameters used to measure fungal infection (two-way ANOVA. Field site: cuttings emerging from dormancy, $F$= 4.67, DF= 1, $P$= 0.0817; shoot weight, $F$= 2.38, DF= 1, $P$= 0.1219; disease severity, $F$= 0.26, DF= 1, $P$= 0.6120. Block: cuttings emerging from dormancy, $F$= 0.16, DF= 3, $P$= 0.9236; shoot weight, $F$= 0.18, DF= 3, $P$= 0.9126; disease severity, $F$= 1.18, DF= 3, $P$= 0.3179. Interaction: cuttings emerging from dormancy, $F$= 0.82, DF= 3, $P$= 0.4842; shoot weight, $F$= 0.10, DF= 3, $P$= 0.9579; disease severity, $F$= 2.47, DF= 3, $P$= 0.062). Therefore, data from both field sites were combined an analyzed together.

In general, 161 49 C was the least affected by pathogen inoculation and no significant differences were found among the control treatment and fungal isolates for all parameters evaluated at $P$<0.05 (Figures 2, 3 and 4). All the remaining rootstocks were significantly affected by some of pathogen species at $P$>0.05.

The variables rootstock, fungal species and its interaction between rootstock and fungal species had a significant effect on the percentage of cuttings emerging from dormancy (two-way ANOVA: rootstock, $F$= 55.35, DF= 4, $P$= <0.001; fungal species, $F$= 4.89, DF= 7,

*P*= <0.001; interaction, *F*= 1.67, DF= 28, *P*= 0.0241). The effect of fungal isolates on the percentage of cuttings emerging from dormancy is shown in Figure 2. *Pa. chlamydospora* and *Pm. parasiticum* differed significantly from the control treatment in 110 R (35.6 ± 8.1 and 35.0 ± 8.6 respectively; control treatment: 67.5 ± 9.7), 140 Ru (12.5 ± 5.9 and 2.5 ± 1.9 respectively; control treatment: 42.5 ± 4.1) and 41 B (38.7 ± 5.5 and 52.5 ± 4.1 respectively; control treatment: 73.7 ± 11.2) at *P*<0.05. *Pm. viticola* and *Pm. mortoniae* differed significantly from the control treatment in 110 R (21.2 ± 7.6) and 1103 P (26.2 ± 6.2) at *P*<0.05, respectively. *C. luteo-olivacea* differed significantly from the control treatment in 110 R (37.5 ± 14.2) and 140 Ru (7.5 ± 3.1) at *P*<0.05. *Pm. aleophilum* and *Pm. scolyti* did not differ significantly from the control treatment in all grapevine rootstocks.

The variable rootstock and the interaction rootstock x treatment had a significant effect on the shoot weight (two-way ANOVA: rootstock, *F*= 89.95, DF= 4, *P*= <0.001; interaction, *F*= 1.66, DF= 28, *P*= 0.0220). The effect of fungal isolates on the shoot weight is shown in Figure 3. *Pa. chlamydospora* differed significantly from the control treatment in 110 R (198.3 ± 25.6; control treatment: 303.7 ± 29.5) and 140 Ru (84.7 ± 21.5; control treatment: 213.4 ± 41.3) at *P*<0.05. *Pm. parasiticum* differed significantly from the control treatment in 110 R (101.3 ± 41.2), 140 Ru (31.7 ± 11.1) and 41 B (180.8 ± 43.3; control treatment: 388.0 ± 69.1) at *P*<0.05. *Pm. mortoniae* differed significantly from the control treatment in 1103 P (233.6 ± 58.0; control treatment: 684.5 ± 95.9) at *P*<0.05. *Pm. scolyti* and *Pm. viticola* differed significantly from the control treatment in 110 R (128.5 ± 44.6 and 28.4 ± 26.0 respectively) at *P*<0.05. *C. luteo-olivacea* differed significantly from the control treatment in 110 R (116.0 ± 35.2) and 140 Ru (85.0 ± 30.9) at *P*<0.05. *Pm. aleophilum* did not differ significantly from the control treatment in all grapevine rootstocks.

The variables rootstock and treatment had a significant effect on the disease severity (two-way ANOVA: rootstock, *F*= 6.10, DF= 4, *P*= <0.001; treatment, *F*= 15.80, DF= 7, *P*=

<0.001) (Figure 4). *Pa. chlamydospora, Pm. aleophilum* and *Pm. parasiticum* differed significantly from the control treatment in 110 R (56.2 ± 13.1, 51.8 ± 5.5 and 56.7 ± 13.5 respectively; control treatment: 4.7 ± 3.1), 140 Ru (41.8 ± 6.1, 42.7 ± 11.4 and 37.7 ± 7.1 respectively; control treatment: 5.9 ± 3.2), 1103 P (41.8 ± 6.3, 50.2 ± 4.1 and 19.5 ± 5.3 respectively; control treatment: 2.4 ± 1.4) and 41 B (38.6 ± 9.3, 64.1 ± 17.9 and 30.7 ± 5.2 respectively; control treatment: 2.3 ± 2.0) at $P<0.05$. *Pm. mortoniae* and *Pm. scolyti* differed significantly from the control treatment in 1103 P (18.2 ± 5.5 and 22.6 ± 3.0 respectively) at $P<0.05$. *Pm. viticola* and *C. luteo-olivacea* did not differ significantly from the control treatment in all grapevine rootstocks.

Simple regression analysis showed a correlation between percentage of cuttings emerging from dormancy and disease severity for all grapevine rootstocks inoculated with *Pa. chlamydospora* (110 R: Slope= -0.04258, $R^2$= 0.70, $P$= <0.001; 140 Ru: Slope= -14.1417, $R^2$= 0.76, $P$= 0.0021; 1103 P: Slope= -16.1182, $R^2$= 0.77, $P$= <0.001; 41 B: Slope= -3.4788, $R^2$= 0.47, $P$= 0.0386) with the exception of 161 49 C (Slope= -0.41728, $R^2$= 0.03, $P$= 0.8921). There was also a significant correlation for 110 R inoculated with *Pm. viticola* (Slope= -0.05413, $R^2$= 0.42, $P$= 0.0276) and for 41 B inoculated with *Pm. aleophilum* (Slope= -4.16822, $R^2$= 0.53, $P$= 0.0079). The best fit obtained to explain the significant relationships in 110 R and 41 B corresponded to a lineal function of the form y= ax + b, and in 140 Ru and 1103 P corresponded to a logarithmic function of the form y= a + b lnx.

Simple regression analysis also showed a correlation between shoot weight and disease severity for all grapevine rootstocks inoculated with *Pa. chlamydospora* (110 R: Slope= -0.11249, $R^2$= 0.53, $P$= 0.0197; 140 Ru: Slope= -99.2811, $R^2$= 0.43, $P$= 0.0494; 41 B: Slope= -0.04083; $R^2$= 0.58, $P$= 0.0109) with the exception of 1103 P (Slope= -1.99106, $R^2$= 0.16, $P$= 0.2847) and 161 49 C (Slope= 4.111, $R^2$= 0.31, $P$= 0.5103). This correlation was also significant for 110 R inoculated with *Pm. viticola* (Slope= -0.09082, $R^2$= 0.51, $P$=

0.0102) and for 1103 P inoculated with *Pm. mortoniae* (Slope= -0.04819, $R^2$= 0.46, *P*= 0.0131). The best fit obtained to explain the significant relationships in 110 R, 1103 P and 41 B corresponded to a lineal function of the form y= ax + b and in 140 Ru corresponded to a logarithmic function of the form y= a + b lnx.

No foliar symptoms were observed on inoculated plants during the grapevine growing season. Each fungal vascular pathogen was reisolated from inoculated plants on MEAS, thereby confirming Koch's postulates. No *C. luteo-olivacea, Pa. chlamydospora* or *Phaeaocremonium* spp. were isolated from control plants.

**Discussion**

This study evaluated the effects of *C. luteo-olivacea, Phaeoacremonium* spp. and *Pa. chlamydospora* on the grapevine rootstocks most widely planted in Spain under field conditions. Most of the fungal pathogens caused significant reductions in the percentage of cuttings that emerged from dormancy and in shoot weight, as well as significant increases in disease severity percentage in all grapevine rootstocks, with the exception of 161 49 C rootstock.

The effects of the different fungal species varied among grapevine rootstocks, indicating that host factors may be involved in symptom expression. Zanzotto et al. (2008) suggested that the susceptibility of grapevine cultivars to fungal infection was linked to the range of chemical defense compounds available in the plants. Del Rio et al. (2004) demonstrated that *p*-coumaric acid, an antifungal and antibacterial phenolic compound present in plants, inhibited *in vitro* mycelial growth and sporulation of both *Pa. chlamydospora* and *Pm. aleophilum*. Troccoli et al. (2001) hypothesized that the low rate of *Pa. chlamydospora* spread through the wood tissue was due to the defense response initiated by the vines:

production of tyloses, accumulation of phenols in the vessels and adjacent tissue, and the deposition of unidentified defence-response substances. Amalfitano et al. (2000) showed that grapevine wood colonised by *Pa. chlamydospora* had higher levels of resveratrol, a phytoalexin belonging to the stilbenes group. The relevance of increased resveratrol concentration was shown by Santos et al. (2006), who observed that this compound was able to reduce *in vitro* colony growth of *Pa. chlamydospora* and *Pm. inflatipes*, as well as increase the fresh weight of infected plants grown in a medium supplemented with this phytoalexin. Further research is needed to confirm this hypothesis.

The results of this study showed that, in general, *Pa. chlamydospora* was able to cause greater reductions in the percentage of cuttings emerging from dormancy and shoot weight, and a greater disease severity than the other pathogens tested. Several previous studies also indicated higher symptom expression by plants inoculated with *Pa. chlamydospora* than *Phaeoacremonium* spp. (Adalat et al. 2000; Wallace et al. 2004; Zanzotto et al. 2008). *Pa. chlamydospora* produced larger areas of vascular discoloration than *Phaeoacremonium* spp. under field (Mugnai et al. 1999; Halleen et al. 2007) and greenhouse conditions (Halleen et al. 2007; Aroca and Raposo 2009; Laveau et al. 2009).

Regression analyses also showed that vascular discoloration caused by *Pa. chlamydospora* was significantly correlated with the percentage of cuttings emerging from dormancy and with shoot weight in almost all rootstocks tested. However, these variables were not significantly correlated for most of the *Phaeoacremonium* spp. and *C. luteo-olivacea* isolates used to inoculate the grapevines. Studies carried out by Zanzotto et al. (2008) used image analysis to show a link between the percentage of damaged stem tissue, which was infected by *Pa. chlamydospora*, with visual observations of leaf symptoms on *in vitro* grapevine plants.

Of the several *Phaeaocremonium* species that have been associated with Petri disease and esca of grapevine, *Pm. aleophilum* was the species most commonly isolated from grapevines in various countries across the world (Larignon and Dubos 1997; Mugnai et al. 1999), followed by *Pm. parasiticum* (Mostert et al. 2006). Our study found that of the *Phaeaocremonium* spp. tested, *Pm. parasiticum* caused the greatest reduction on percentage of cuttings emerging from dormancy and shoot weight and that *Pm. aleophilum* and *Pm. parasiticum* caused the greatest disease severity over the control values.

*Cadophora luteo-olivacea* also caused a significant reduction in the percentage of cuttings emerging from dormancy and shoot weight in 110 R and 140 Ru rootstocks. This pathogen also caused the vascular discoloration characteristic of Petri disease; clearly *C. luteo-olivacea* should be considered one of the causal agents of this disease. Until now, little research has been published on the role that this pathogen plays in disease of grapevines. Halleen et al. (2007) demonstrated that *C. luteo-olivacea* caused vascular discoloration similar to that seen in Petri diseased grapevines after trunk and pruning wound inoculations in field trials; however, they were not able to demonstrate that it caused decline symptoms. This study demonstrated that *C. luteo-olivacea* can contribute to Petri disease in young vineyards.

The disease severity, measured as the percentage of vascular tissue discolored, was greatest for 110 R, 140 Ru, 1103 P and 41 B rootstocks inoculated with *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum*. Eskalen et al. (2001), who evaluated the length of brown vascular streakings from the base of 20 grapevine rootstock cultivars dipped into inoculum suspensions, also observed that 140 Ru and 1103 P rootstocks were very susceptible to *Pa. chlamydospora* and both 140 Ru and 110 R rootstocks to *Pm. aleophilum*. However, in contrast with the results of this study, Eskalen et al. (2001) reported that rootstock 161 49 C was highly susceptible to *Pm. aleophilum* and that rootstocks 110 R and 1103 P were not greatly affected by *Pa. chlamydospora* and *Pm. aleophilum*, respectively. These differences

could be due to the different inoculation method used or differences in virulence within the isolates tested.

Given the great variability obtained among rootstocks when measuring the percentage of cuttings emerging from dormancy and shoot weight, vascular discoloration could be considered the most appropriate measure of infection. Previous works have demonstrated the effectiveness of xylem lesion length as a measure of disease severity (Mugnai et al. 1999; Adalat et al. 2000; Eskalen et al. 2001; Feliciano et al. 2004; Wallace et al. 2004; Halleen et al. 2007; Aroca and Raposo 2009; Laveau et al. 2009). Other parameters such as root initiation, fresh root weight, callus production, fresh or dry weight of shoot and leaves, root and shoot length or presence of leaf chlorosis and necrosis have also been evaluated with variable results (Adalat et al. 2000; Wallace et al. 2004; Zanzotto et al. 2008; Aroca and Raposo 2009).

Although reisolation of the inoculated pathogens from the inoculated plants indicated successful infection in this study, there were no foliar symptoms, which confirmed the reports of erratic manifestation of leaf symptoms in Petri disease and esca affected grapevines. Infected grapevine rootstock mother plants with no external foliar symptoms were reported by Fourie and Halleen (2004) and Aroca et al. (2010). Despite the isolation of *Phaeoacremonium* spp. and *Pa. chlamydospora* from wood discoloration of young grapevines, Zanzotto et al. (2007) did not observe classic foliar symptoms of either Petri disease or esca in the vineyards during the 4 years after planting. Aroca and Raposo (2009), who inoculated nine species of *Phaeoacremonium* on rooted-scion cuttings under greenhouse conditions, observed that not all *Phaeoacremonium* species tested were able to cause foliar symptoms of Petri disease in infected plants.

Mugnai et al. (1999) reported that vines affected with esca exhibit leaf symptoms of greatly varying intensity from one growing season to another, even when the inner wood

tissues were always discolored or decaying. These researchers suggested that symptom expression may depend on the length of the infection period; therefore a longer assay would be necessary to observe a possible correlation between expression of foliar symptoms and vascular discoloration on grapevine wood. Calzarano and Di Marco (2007) also reported that although the white heart rot typical of esca vines was sometimes associated with leaf symptoms of great severity, the wood decay was not necessarily concomitant with the chlorotic and necrotic leaf symptoms typical of esca. Further research should be conducted to determine the influence of different factors on foliar symptom expression. Field trials could be conducted on vines for longer periods than used here, with vines being subjected to a range of cultural practices, such as deficit irrigation systems to explore the effect of water stress on symptom expression. The influence of environmental conditions in symptom variation should also be considered and evaluated.

This is the first study to investigate the effects of a range of pathogens associated with Petri disease and esca on a broad range of grapevine rootstocks under field conditions. Previous research was limited to a few cultivars under field conditions (Spaparano et al. 2001) or a few fungal pathogens under controlled conditions (Eskalen et al. 2001; Feliciano et al. 2004; Wallace et al. 2004; Zanzotto et al. 2008). The importance of conducting pathogenicity tests with these fungi under field conditions for prolonged periods has been emphasized by different authors (Mugnai et al. 1999; Halleen et al. 2007; Aroca and Raposo 2009). Since fungal vascular pathogens of grapevine are associated with stress-related disease (Ferreira et al., 1999); and it is difficult to imitate the natural, varying abiotic stress factors in an artificial environment, this reinforces the importance of performing field trials in order to estimate the real effects of these diseases on different grapevine cultivars.

Although a range of susceptibilities was found, none of the rootstocks tested here were completely resistant to the pathogens tested. These results therefore agree with other studies

which reported different resistance abilities among grapevine rootstocks or cultivars to fungal vascular pathogens and the lack of complete or high levels of resistance in any planting material tested (Eskalen et al. 2001; Spaparano et al. 2001; Marchi 2001; Feliciano et al. 2004; Wallace et al. 2004; Santos et al. 2006; Zanzotto et al. 2008).

Rootstocks 110 R and 161 49 C are by far the most widely used rootstocks in Spain, accounting for 33.7 and 29.8% of the country's rootstock mother field planted area, respectively. They are also the rootstocks most often demanded by grape growers with percentages of 22.6 and 33.0% respectively (Hidalgo, 2002). Our study found that rootstocks 110 R and 140 Ru (both rootstocks crosses of *V. berlandieri* x *V. rupestris*) were greatly affected by fungi associated with Petri disease and esca. In contrast, 161 49 C rootstock (*V. riparia* x *V. berlandieri*) was proved to be the least affected by the pathogens. Gubler et al. (2004a) reported that the large-scale replanting of grapevine rootstocks crosses of *V. berlandieri* x *V. riparia* by new rootstocks crosses of *V. riparia* x *V. rupestris* and *V. berlandieri* x *V. rupestris* resulted in an increase of signs of plant decline and subsequent death from the early 1990s on the north coast of California. Species of *Phaeoacremonium* and *Pa. chlamydospora* were later isolated from these affected vines. This information and our results suggest that grapevine rootstocks crosses of *V. riparia* x *V. berlandieri* could be the least susceptible to *C. luteo-olivacea*, *Phaeoacremonium* spp. and *Pa. chlamydospora*.

**Conclusions**

Results from this study show that fungal vascular pathogens associated with Petri disease and esca are able to cause a reduction in percentage of shoots emerging from dormancy, shoot weight and an increase of vascular discoloration on grapevine rootstocks. The selection of healthy grapevine rootstocks is critical to the production of quality plants.

Poor-quality plants may fail, or be slow to establish, and result in a vineyard that is uneven and less productive both in the short and long term. It has now become clear that the viability of future vineyards is dependent on a wide range of strategies for management of grapevine planting material through the propagation process. Use of disease-resistant cultivars is known to offer an effective, safe and inexpensive method of control for many diseases. This work has shown that use of 161 49 C rootstock could be a useful component of an integrated management strategy for Petri disease and esca.

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**Table 1** Isolation record of *Cadophora luteo-olivacea*, *Phaeoacremonium aleophilum*, *Pm. mortoniae*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. viticola* and *Phaeomoniella chlamydospora* used in this study.

| Species | Isolate | Year | Location | Scion/Rootstock |
|---|---|---|---|---|
| *C. luteo-olivacea* | Clo-42 | 2008 | Tosos (Zaragoza) | Merlot/SO4 |
| *Pm. aleophilum* | Pal-28 | 2002 | Los Ruíces (Valencia) | Bobal/nd |
| *Pm. mortoniae* | Pmo-1 | 2007 | Daimiel (Ciudad Real) | Cabernet Sauvignon/nd |
| *Pm. parasiticum* | Ppa-4 | 2001 | Malagón (Ciudad Real) | Garnacha Fina/1103 P |
| *Pm. scolyti* | Psc-1 | 2008 | Ayelo (Valencia) | Tempranillo/110 R |
| *Pm. viticola* | Pvi-1 | 2008 | Cariñena (Zaragoza) | Tempranillo/110 R |
| *Pa. chlamydospora* | Pch-186 | 2004 | Campo de Criptana (Ciudad Real) | Tempranillo/140 Ru |

nd, not determined.

**Figure 1** Rating scale used to estimate the percentage discoloration in vascular tissue of grapevine rootstocks inoculated with *Cadophora luteo-olivacea*, *Phaeaocremonium* spp. and *Phaeomoniella chlamydospor*a: 0 = no discoloration, 1 = 1 to 25% discoloration, 2 = 26 to 50% discoloration, 3 = 51 to 75% discoloration, and 4 = 76 to 100% discoloration.

**Figure 2** Percentage of cuttings emerging from dormancy of five grapevine rootstocks (110 R, 140 Ru, 1103 P, 41 B and 161 49 C) inoculated at $10^6$ conidia $mL^{-1}$ of *Phaeomoniella chlamydospora* isolate Pch-186, *Phaeoacremonium aleophilum* isolate Pal-28, *Pm. parasiticum* isolate Ppa-4, *Pm. mortoniae* isolate Pmo-1, *Pm. scolyti* isolate Psc-1, *Pm. viticola* isolate Pvi-1 and *Cadophora luteo-olivacea* isolate Clo-42. Results are the means of eight groups of ten grapevine cuttings (four groups per field site). Vertical bars are the standard error of the means. Bars with a different letter are significantly different according to Student's Least Significant Difference test.

**Figure 3** Shoot weight of five grapevine rootstocks (110 R, 140 Ru, 1103 P, 41 B and 161 49 C) inoculated at $10^6$ conidia $mL^{-1}$ of *Phaeomoniella chlamydospora* isolate Pch-186, *Phaeoacremonium aleophilum* isolate Pal-28, *Pm. parasiticum* isolate Ppa-4, *Pm. mortoniae* isolate Pmo-1, *Pm. scolyti* isolate Psc-1, *Pm. viticola* isolate Pvi-1 and *Cadophora luteo-olivacea* isolate Clo-42. Results are the means of eight groups of ten grapevine cuttings (four groups per field site). Vertical bars are the standard error of the means. Bars with a different letter are significantly different according to Student's Least Significant Difference test.

**Figure 4** Disease severity of five grapevine rootstocks (110 R, 140 Ru, 1103 P, 41 B and 161 49 C) inoculated at $10^6$ conidia $mL^{-1}$ of *Phaeomoniella chlamydospora* isolate Pch-186, *Phaeoacremonium aleophilum* isolate Pal-28, *Pm. parasiticum* isolate Ppa-4, *Pm. mortoniae* isolate Pmo-1, *Pm. scolyti* isolate Psc-1, *Pm. viticola* isolate Pvi-1 and *Cadophora luteo-olivacea* isolate Clo-42. Results are the means of eight groups of ten grapevine cuttings (four groups per field site). Vertical bars are the standard error of the means. Bars with a different letter are significantly different according to Student's Least Significant Difference test.

FIGURE 1

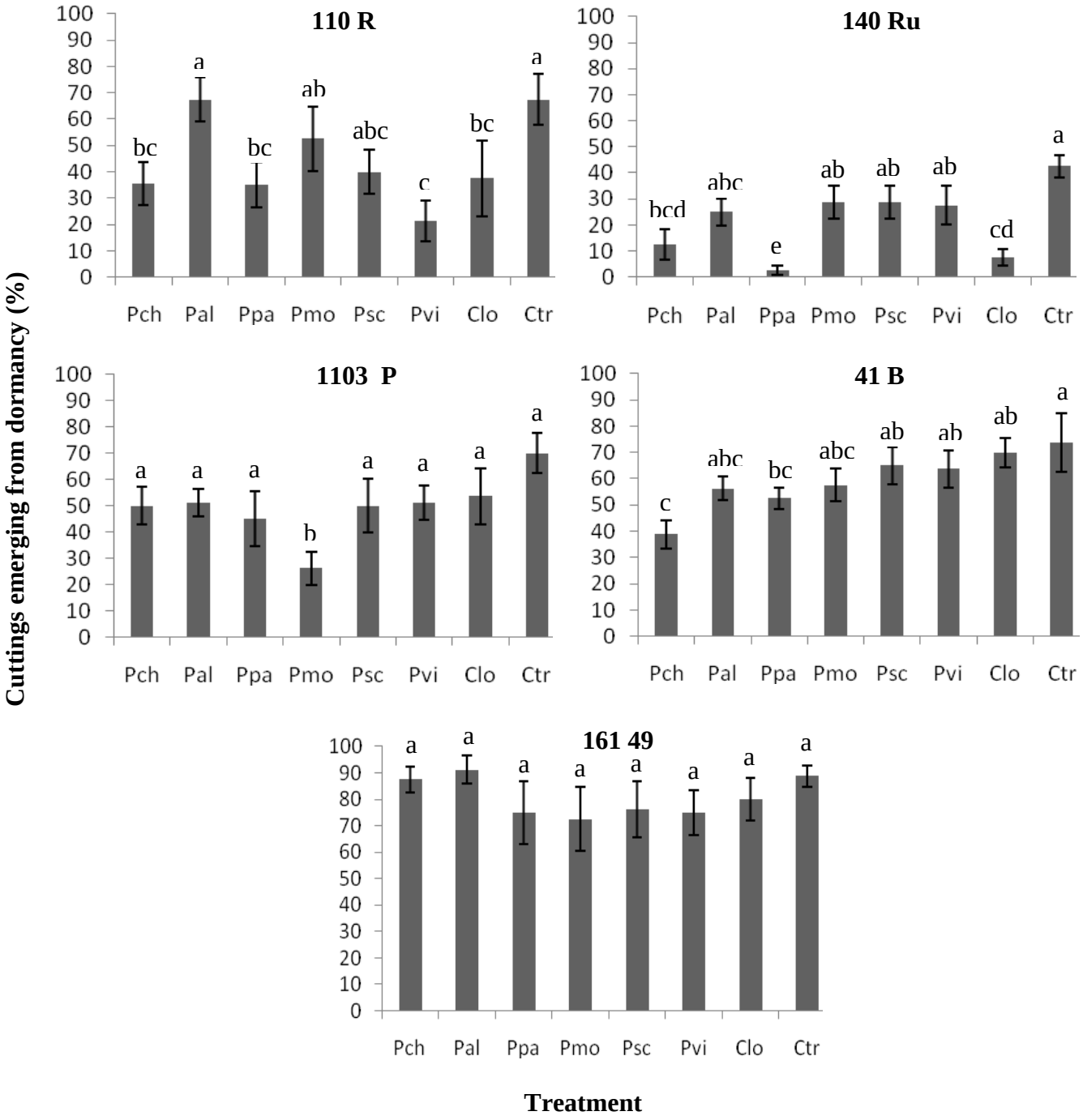
110 R
140 Ru
1103 P
41 B
161 49
Cuttings emerging from dormancy (%)
Treatment
Pch
Pal
Ppa
Pmo
Psc
Pvi
Clo
Ctr

**FIGURE 2**

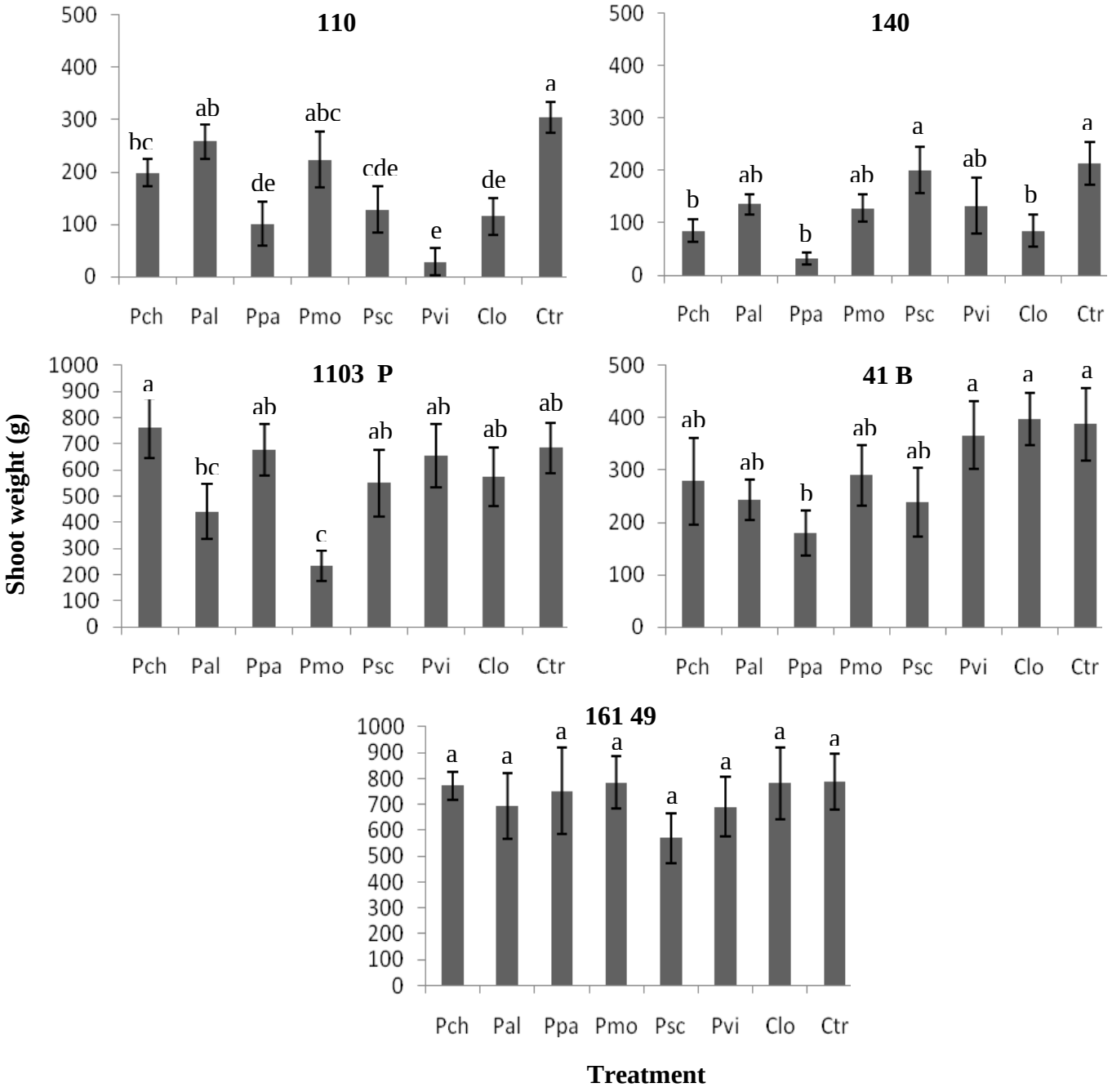
110
140
1103 P
41 B
161 49
Shoot weight (g)
Treatment
Pch
Pal
Ppa
Pmo
Psc
Pvi
Clo
Ctr

**FIGURE 3**

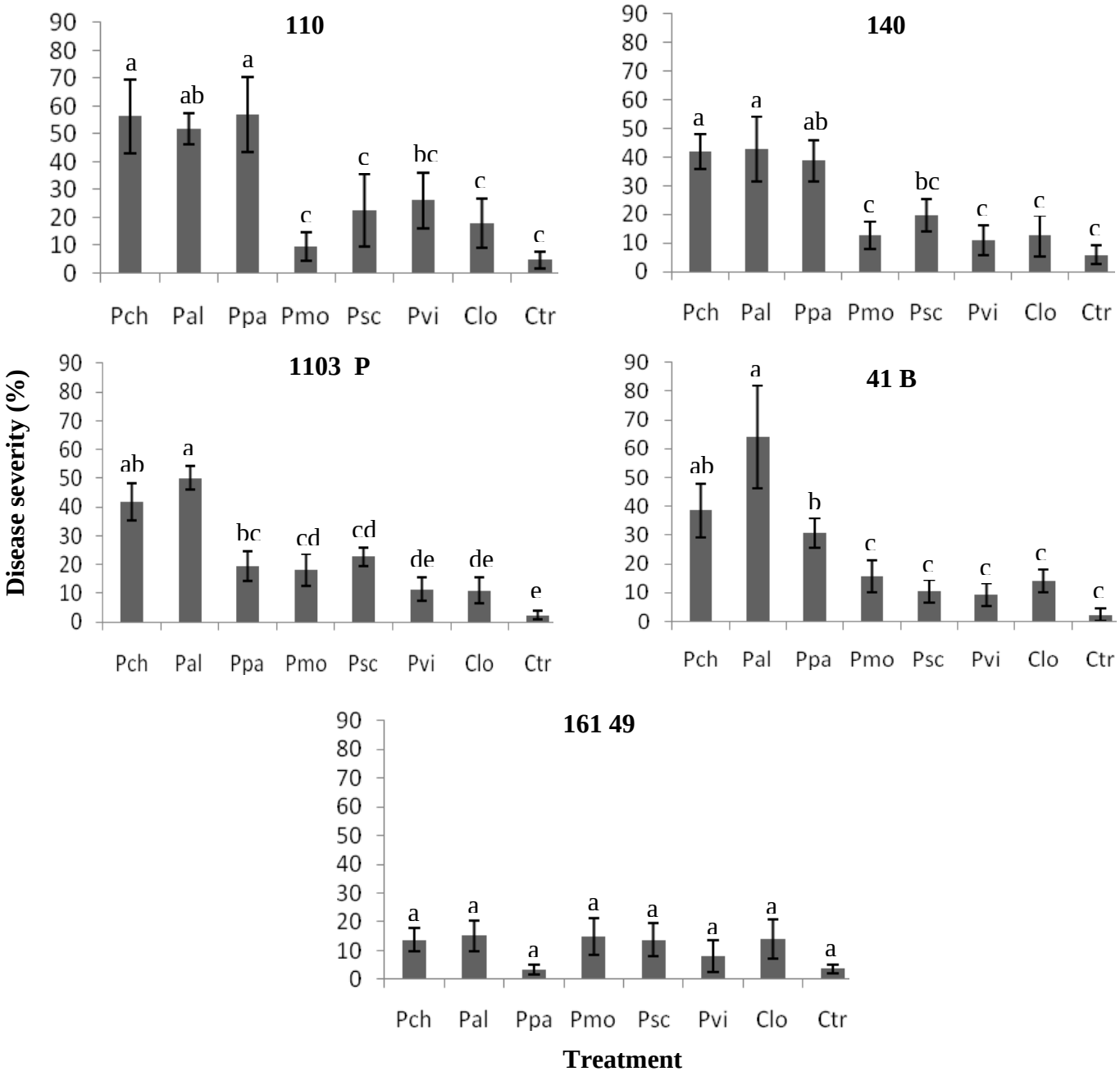
110
a
ab
a
c
c
bc
c
c
140
a
a
ab
c
bc
c
c
c
1103 P
ab
a
bc
cd
cd
de
de
e
41 B
ab
a
b
c
c
c
c
c
161 49
a
a
a
a
a
a
a
a
90
80
70
60
50
40
30
20
10
0
Pch
Pal
Ppa
Pmo
Psc
Pvi
Clo
Ctr
Disease severity (%)
Treatment

**FIGURE 4**