Preliminary evaluation of citrus rootstocks and scions to four different bot canker pathogens

M. Kayim[1], A. Endes[2], A. Alsalmo[1], S. Yasmin[1] and H. Nawaz[1]

[1]Çukurova University, Agricultural Faculty, Department of Plant Protection 01330 Balcali, Adana, Turkey; [2]Yozgat Bozok University, Agricultural Faculty, Department of Plant Protection, 66900, Yozgat, Turkey.

Abstract

In this study, an investigation was conducted to assess the susceptibility and resistance of various citrus rootstocks and scions to four distinct bot canker pathogens: *Diploida seriata*, *Neofusicoccum parvum*, *Lasiodiplodia pseudotheobromae*, and *L. theobromae*. Findings revealed that the cultivars 'Fukumoto' and 'Cara Cara' were found to exhibit the highest degree of sensitivity to all four pathogens. In contrast, the C35 rootstock, a Carrizo hybrid, was observed to be resistant to *Diplodia seriata* and *N. parvum*, with a moderate resistance observed toward *L. pseudotheobromae* and *L. theobromae*, as evidenced by the minimal necrosis observed in stem cuttings. Significant sensitivity to *N. parvum* was exhibited by 'Okitsu Wase', leading to pronounced necrosis on stem cuttings, while displaying resistance to *D. seriata* with minimal necrosis formation. 'Navelina' orange, on the other hand, was found to display a uniform susceptibility to all four pathogen infections, characterized by severe necrosis. 'Dobashi Beni' was found to be susceptible to *N. parvum* and *L. theobromae* pathogens while displaying resistance to *D. seriata* and *L. pseudotheobromae*. 'Meyer' lemon was observed to demonstrate resistance to *D. seriata*, *L. pseudotheobromae*, and *L. theobromae* pathogens, with slight necrosis observed in response to *N. parvum*, indicating a moderate level of resistance. Resistance to *D. seriata* and *L. theobromae* pathogens was exhibited by 'Interdonato' lemon, with a moderate resistance observed to *N. parvum* and *L. pseudotheobromae*. 'Kutdiken' lemon was found to demonstrate potential resistance to *N. parvum*, although susceptibility to *Lasiodiplodia* spp. was noted. This study represents a preliminary assessment of various commercial citrus species and cultivars regarding their responses to bot canker pathogens in Turkey.

Keywords: *Botryosphaeria* spp., *Citrus* spp., pathogenicity, rootstock, scion

INTRODUCTION

Oranges, mandarins, and lemons are commercially and economically important citrus fruit groups in the domestic and foreign markets, while sour oranges, trifoliate oranges, and their hybrids are mostly used as rootstocks. Commercial citrus production in Turkey started in the 1970s, and the total number of trees was 23,853,000 in 1988. The number of trees reached approximately 40 million in 2016 due to suitable fertilization, drip irrigation, the development of different planting models, pruning, maintenance, easy harvesting operations, the reduced canopy of the tree, and the development of new cultivars that can be harvested in every season. Citrus production in Turkey, including oranges, mandarins, lemons, and grapefruits, reached 6,501,107 tons in 2016 (TÜIK, 2016). Currently, this production has decreased to approximately 4.4 million tons for various reasons (FAOSTAT, 2022). In citrus, many diseases, including viruses, viroids, *Spiroplasma* spp., bacteria, and fungi, cause a decrease in the amount of fruit production and the number of trees. Among them, fungal pathogens like *Phoma tracheiphila* mainly infect lemons (Kayim et al., 2022), *Alternaria alternata* infects some mandarins and hybrids such as 'Nova', 'Robinson', 'Fremont', and a few lemon cultivars such as rough lemon and 'Lisbon' (Canıhos et al., 1997). On the other hand, *Phytophthora citrophthora* causes root and crown rot on rootstocks and brown rot of citrus fruits (Erkilic, 1999); *Penicillium digitatum* and *P. italicum* infect postharvest fruits and continue in packed houses (Akgul et al., 2009). But bot canker pathogens belonging to the

Botryosphaeriaceae family do not have host specificity. These pathogens infect all kinds of trees, including citrus species. There are many bot canker reports on fruit trees such as olives, avocado, vine, fig, pistachio, apricot, plum, peach, nectarine, almond, apple, pear, and walnut (Michailides, 1991; Úrbez-Torres et al., 2006, 2013; Slippers and Wingfield, 2007; Moral et al., 2010; Lynch et al., 2013; Kayim et al., 2016; Endes and Kayim, 2022) and forest trees (Lynch et al., 2013). Several bot-canker pathogens have been detected throughout Turkey. Pathogens belonging to this group penetrate the plant from pruning wounds and progress into phloem and xylem bundles. When it blocks the transmission bundles, it disrupts the nutrient exchange of the tree and causes shoot and twig blight, stem necrosis, gummosis on branches and trunks of trees, die-back, soft rot of fruit, and stem-end rot on fruit stored for a long time (Çeliker et al., 2012; Kurbetli and Demirci, 2014; Awan et al., 2016). Due to the abovementioned reasons, it is important for citrus trees to know the resistance levels of commercially grown citrus species and cultivars to these pathogen species and to identify the susceptible and resistant ones. In particular, the detection of seven different pathogen species in different fruit groups revealed that these factors exist as inoculum sources and pose a threat to all citrus varieties. Within the scope of this work, the resistance and susceptibility levels of some commercial rootstocks and scion cultivars against *Diploida seriata*, *Lasiodiplodia theobromae*, *L. pseudotheobromae,* and *Neofusicoccum parvum* species will be determined.

MATERIALS AND METHODS

Fungal isolates

In this study, two highly virulent isolates from each species of *D. seriata*, *N. parvum*, *Lasiodiplodia theobromae*, and *L. pseudotheobromae* were selected from the stock cultures of the Biotechnology Laboratory (Çukurova University, Department of Plant Protection). All isolates were subcultured on PDA media supplemented with 100 mg mL^{-1} tetracycline (Figure 1). A week later, the fungal mycelia developed and were made ready for pathogenicity studies in citrus seedlings.

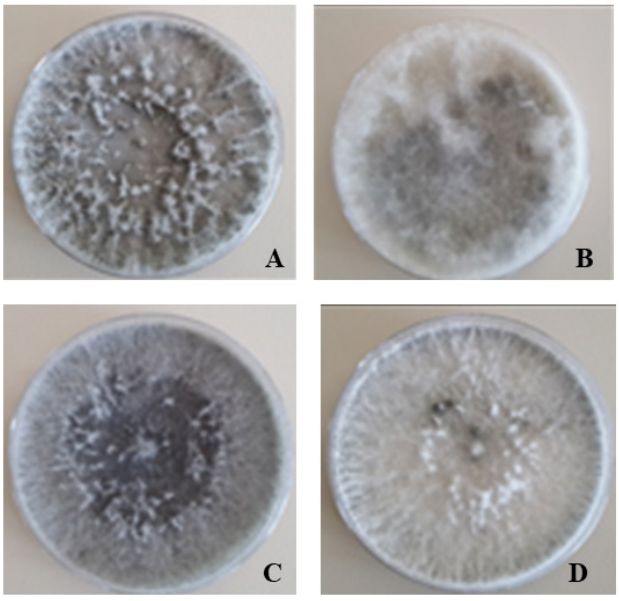


Figure 1. Colony morphology of 4 different species belong to the *Botryosphaeriaceae* family grown on PDA media with 25±1°C, 16 h light, and 8 h dark conditions in the growth room. *L. theobromae* (A); *L. pseudotheobromae* (B); *D. seriata* (C); and *N. parvum* (D).

Pathogenicity tests

Selected virulent isolates of four different pathogens were used to inoculate stems of 1- to 2-year-olds from 12 different citrus rootstocks and scion trees for evaluation of susceptibility to bot canker disease (Table 1). One wound tree^{-1} was made using a 4-mm cork borer. A 4-mm diameter mycelial plug from a 5- to 7-day-old culture growing on PDA-tet was placed on the freshly wounded surface of each stem, and the inoculated area was covered with parafilm. Control trees were mock-inoculated with agar plugs and incubated in the greenhouse at 22-28±2°C for three months. After incubation, lesion length was recorded on each tree. The experiment was arranged in a completely randomized design with five replications. A total of 300 trees (240 trees for pathogens and 60 trees for control) were used for the pathogenicity experiment. In the analysis of the variance of the obtained data, a one-way analysis of the variance using the SPSS statistical software program was used. The means of the data were compared with the least significant difference.

Table 1. Commercially produced *Citrus* species and rootstocks tested for susceptibility to four different fungal pathogens.

Oranges	Mandarins	Lemons	Rootstocks
Fukumoto	Okitsu Wase	Kütdiken	Tuzcu-31
Naveline	Dobashi Beni	Meyer	Carrizo Citrange
Cara Cara	W. Murcott	Interdonato	C-35

The observed cultivars and rootstocks were subjected to daily monitoring during the initial 15 days post-inoculation, followed by subsequent assessments at least twice a week. These evaluations encompassed the examination of disease manifestations in the overall appearance, bark, and woody tissues of the plants. To further characterize the observed lesions, small fragments of necrotic tissue from the lesion peripheries were aseptically placed onto PDA-tet plates. These plates were then incubated at room temperature. Morphological criteria were employed for confirming the identity of fungal colonies, supplemented by the application of molecular identification techniques.

RESULTS AND DISCUSSION

The length of necrosis was evaluated by measuring with a ruler in mm, and the differences among the results obtained were determined with the SPSS statistical program. The results of the pathogenicity tests are given in Figures 2-5, respectively.

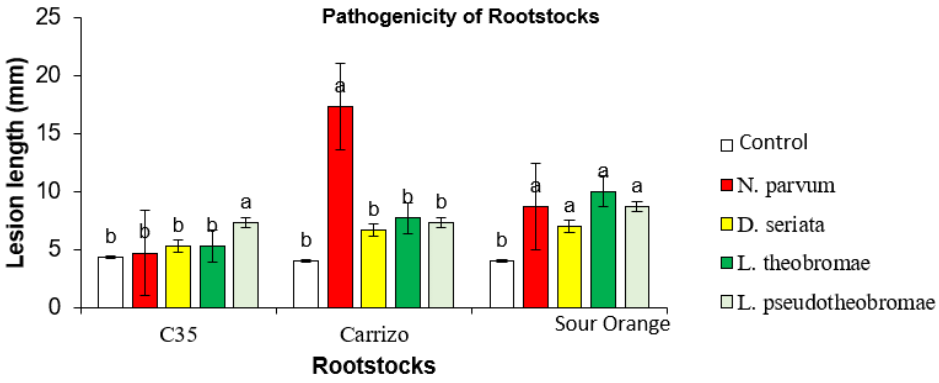


Figure 2. The average lesion lengths of wood discoloration of rootstock cultivars caused by four different pathogens belong to the *Botryosphaeriaceae* family with mock inoculated control. For each species, mean values with the same letter are not significantly different according to Tukey's honestly significant difference test (P<0.05). Vertical lines represent the standard errors of the means.

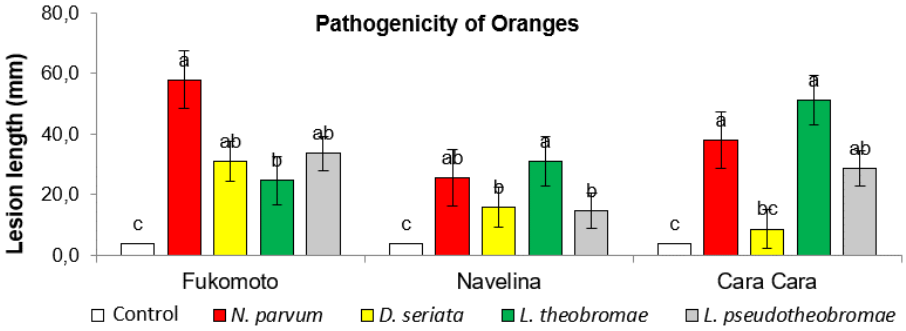


Figure 3. The average lesion lengths of wood discoloration in orange cultivars caused by four different pathogens belong to the *Botryosphaeriaceae* family with a mock inoculated control. For each species, mean values with the same letter are not significantly different according to Tukey's honestly significant difference test (P<0.05). Vertical lines represent the standard errors of the means.

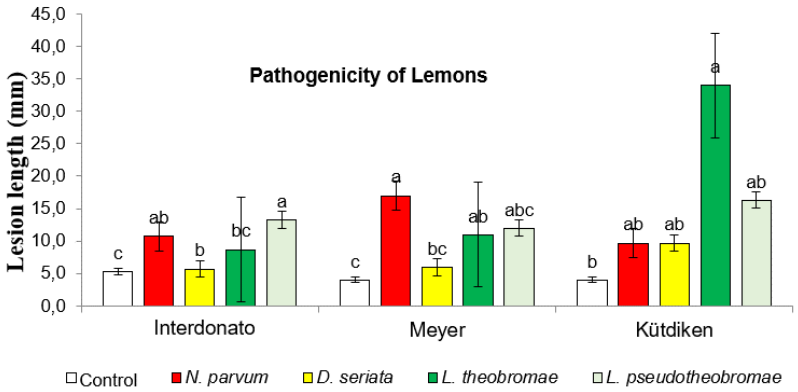


Figure 4. The average lesion lengths of wood discoloration in lemon cultivars caused by four different pathogens belong to the *Botryosphaeriaceae* family with mock inoculated control. For each species, mean values with the same letter are not significantly different according to Tukey's honestly significant difference test (P<0.05). Vertical lines represent the standard errors of the means.

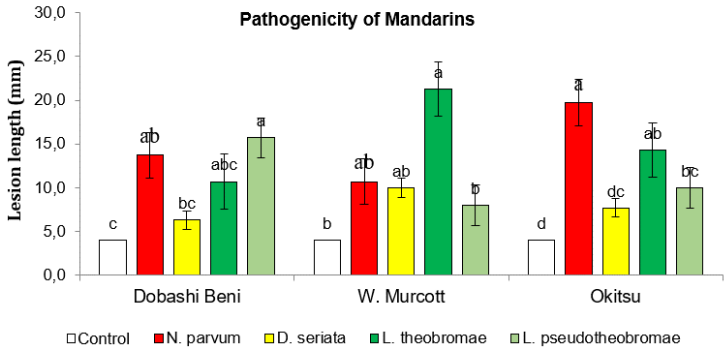


Figure 5. The average lesion lengths of wood discoloration in mandarin cultivars caused by four different pathogens belong to the *Botryosphaeriaceae* family with mock inoculated control. For each species, mean values with the same letter are not significantly different according to Tukey's honestly significant difference test (P<0.05). Vertical lines represent the standard errors of the means.

Three months after pathogen inoculation, yellowish to blackish-brown lesions were observed both upward and downward from the inoculation point in potted one-year-old *Citrus* species and cultivars. No lesions were observed in controls except for a wound response of approximately 5-8 mm.

As seen in Figure 2, the lesions caused by 3 different species of pathogens on rootstocks were not significant except for the lesion caused by *L. pseudotheobramae* (7.3 mm in length). The C35 (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* L.) rootstock seems to be resistant to three different pathogens when compared to the lesion sizes of the other two rootstocks. Carrizo citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* L.) was found to be moderately resistant to three species but sensitive to *N. parvum*. The blackish-brown lesion caused by *N. parvum* was 17.3 mm in length. The sour orange 'Tuzcu 31' (*Citrus aurantium* L.) cultivar was found to be moderately susceptible to four pathogens when compared to the other two rootstocks. Brown lesions caused by *N. parvum, D. seriata*, *L. theobromae,* and *L. pseudotheobromae* were 8.7, 7.0, 10, and 8.7 mm in length.

In re-isolations, all four pathogens used in inoculation were successfully recovered (80-100%) when small pieces of inoculated stem segments were placed on PDA medium. It was proven that necrosis was caused by these pathogens. No pathogenic fungal agents were isolated from the controls. However, colonies of *Penicillium* spp. and *Alternaria* spp. were recovered from the inoculated stem tissue on a PDA medium since sterilization was not applied.

When the reactions of the citrus rootstocks included in the experiment were evaluated in general against four different pathogen species, the C35 rootstock, which is a Carrizo hybrid, was resistant to *N. parvum, D. seriata,* and *L. theobromae,* while it was moderately resistant to *L. pseudotheobromae.* On the other hand, citrus rootstock showed a moderate resistance reaction to all four pathogen species, but its resistance level against *Lasiodiplodia theobramae* was lower, and it was seen that it has the potential to become susceptible when virulent types of the pathogen develop over time.

Figure 3 provides data pertaining to the extent of lesion development on the wood tissue beneath the bark of orange cultivars following inoculation with four distinct species of *Botryosphaeriaceae*.

As seen in Figure 3, the susceptibility of the orange (*C. sinensis* (L.) Osbeck) cultivars to pathogen species differed. Blackish-brown lesions caused by *N. parvum*, *L. pseudotheobromae*, *D. seriata,* and *L. theobromae* were 58, 33.7, 31, and 24,7 mm in length. 'Fukumoto' orange was found to be very susceptible to *N. parvum*. But 'Navelina' orange was susceptible to *L. theobromae* with a 31-mm-long lesion. 'Navelina' seems to be moderately susceptible to *D. seriata* and *L. pseudotheobromae* when compared to the other two cultivars ('Fukumoto' and 'Cara Cara'). 'Cara Cara' orange was found to be very susceptible to 3 different species with 28.7, 38, and 51.3 mm in length lesions. The shortest lesion in length was 8.7 mm, caused by *D. seriata*. But these four pathogens pose potential threats to oranges when compared to other tested citrus species and rootstocks.

The results of the pathogenicity test of lemon and mandarin cultivars are given below (Figures 4 and 5).

In Figure 4, it can be said that the 'Interdonato' (*C. limon* Burnm. f.) cultivar of lemon is more tolerant than the other two lemon cultivars. On the other hand, it was determined that 'Meyer' (*C. meyeri*) cultivar was very sensitive to *N. parvum,* and 'Kütdiken' (*C. limon* Burnm. f.) cultivar was very sensitive to *L. theobromae*. It is observed that the 'Interdonato' cultivar may be sensitive to both *N. parvum* and *L. pseudotheobramae* species.

The average lesion lengths of wood discoloration in mandarin cultivars as a result of inoculation with four different species belonging to the *Botryosphaeriaceae* family are given in Figure 5. When mandarin cultivars are compared based on the average lesion length of wood discoloration, it can be said that mandarin cultivars are susceptible to all these pathogen species. However, while *L. theobromae* was the most pathogenic species in the 'W. Murcott' (*C. nobilis*) cultivar, *N. parvum* was the most pathogenic in 'Okitsu Wase' (*C. unshiu* Marc.). *L. theobromae* is the second pathogenic species. On the other hand, in the 'Dobashi Beni' (*C. unshiu* Marc.) mandarin cultivar, *L. pseudotheobromae* was the most pathogenic species,

followed by *N. parvum* and *L. theobromae,* respectively. 'Okitsu Wase' was found to be very sensitive to *N. parvum* by causing maximum necrosis length (19.7 mm), while it was found to be resistant to *D. seriata* with the shortest length (7.7mm) of necrosis formation on the wood. This cultivar is moderately sensitive to both *Lasiodiplodia species*. *L. theobromae* was the most pathogenic species for the 'W. Murcott' mandarin cultivar, and interestingly, *L. pseudotheobramae* was determined to be the least pathogenic species; in other words, it was found to be less sensitive to this agent. On the other hand, it showed a moderate-sensitivity reaction against *N. parvum* and *D. seriata*.

The Eastern Mediterranean region accounts for approximately 80% of the citrus fruits grown in Turkey. Stone fruits such as peach, nectarine, apricot, plum, and almond are grown in large areas in the Eastern Mediterranean region since it is the most suitable region for early-season fruit cultivation. The rootstocks and scions of these fruits originated especially in Spain and Italy, and many stone fruit orchards were established in the Eastern Mediterranean region in the 2000s, but more than 50% of the established orchards were severely damaged due to pathogens belonging to the *Botryosphaeriaceae* family (Kayim et al., 2016). Pathogens belonging to this family became a serious threat to citrus trees. Gummosis and psorosis were observed on the trunks of the lemon trees in Mersin province, similar to the gummosis symptom on the trunk caused by *Phytophthora citrophthora* agent on the lemon tree of the 'Interdonato' cultivar. The pathogen isolation studies and molecular analysis of the agent were determined to be *Neofusicoccum parvum,* belonging to the *Botryosphaeriaceae* family (Kayim et al., 2016). Later, *L. pseudotheobromae* was identified as fruit rot in lemon (Awan et al., 2016). *Phoma tracheiphila* (mal-secco) and *Alternaria alternata* (brown leaf and fruit spot), which are the most common fungal disease agents in citrus fruits, cause economically damaging disease symptoms in some citrus cultivars, while *Botryosphaeriaceae* pathogens do not have host selectivity and infect all woody trees.

Lasiodiplodia theobromae is the most common endophyte pathogen, infecting more than 500 tree species in tropical and subtropical regions. It also causes necrosis in ornamental plants and die-back in trees. *Diplodia seriata*, on the other hand, causes dead tissue in stone and pome fruit trees, necrosis on leaves and black rot in fruit, and causes symptoms on the trunk and branches of vines and brown lines in wood tissue (Wang et al., 2011; Thomidis et al., 2011; Tang et al., 2012). It has been determined that *Lasiodiplodia theobromae* is the most virulent pathogen in peaches, and it promotes the most extensive lesions and gummosis in trees. Some strains of the pathogen also cause pre- and postharvest fruit rot in apples and peaches (Lv et al., 2012). It has been reported that *B. dothidea* (asexual form: *Fusicoccum aesculi*) is a fruit rot agent in plums and olives in Spain (Roca et al., 2013). The same factor has been reported to cause fruit rot in pears in Italy, the United States, South Africa, New Zealand, Japan, and Taiwan (Garibaldi et al., 2012). *Diploida corticola*, *D. agrifolia* sp. nov., and *Dothiorella iberica* were found both on trees damaged by pests and on trees without insect infestation (Lynch et al., 2013). Again, in California, branch cancers in citrus fruits are caused by *Spencermartinsia viticola, Neofusicoccum australe*, and *N. parvum* agents (Adesemoye and Eskalen, 2011), whereas in avocado orchards, dead tissue on avocado branches is caused by *Dothiorella iberica, Neofusicoccum australe*, and *Neofusicoccum parvum* (Twizeyimana et al., 2013). The agent causes necrosis on the leaves, causes death in the root and root collar, and in the wood tissues that progress to the phloem and xylem in the branches.

CONCLUSIONS

In this study we tested the sensitivity of the most commonly used commercial lemon, oranges, mandarins, and rootstocks to four different bot canker fungal pathogenes. Oranges found more sensitive to these pathogens when compared to other *Citrus* species. Mandarins followed oranges based on sensitivity to pathogens. Among the rootstocks C35 is more tolerant than the different two rootstocks. In lemons, 'Interdonato' was found to be less sensitive to four pathogens species than 'Meyer' and 'Kütdiken'.

ACKNOWLEDGEMENTS

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