Evaluation of six red grapevine cultivars inoculated with *Neofusicoccum parvum*

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Abstract *Botryosphaeria dieback* (BD) is a serious grapevine trunk disease (GTD) that causes large losses in yield and shortens the productive life of vineyards. *Neofusicoccum parvum* is one of the main causal agents of BD. Currently there are no curative fungicides to eradicate this disease; therefore, the use of tolerant cultivars to BD could be considered an interesting and sustainable alternative for its control. For this purpose, rooted cuttings of six red *Vitis vinifera* cultivars were selected and inoculated with *N. parvum*, under field conditions, over two consecutive years. Eight months after inoculation, plants were collected and inspected for lesion development. The fungal incidence varied depending on the cultivar and ranged from 42.1% in “Monastrell” cultivar to 93.3% in “Tinto Velasco” cultivar, evidencing a lack of qualitative resistance to *N. parvum*. The severity of internal wood symptoms caused by *N. parvum* differed considerably amongst the cultivars, being “Bobal” and “Monastrell” more tolerant than “Tinto Velasco” cultivar.

Keywords *Botryosphaeria dieback*, grapevine, grapevine trunk diseases, *Neofusicoccum parvum*

Botryosphaeria dieback (BD) is one of the most damaging grapevine trunk diseases (GTDs) affecting vineyards in all major grape-producing areas worldwide. BD is one of the major threats for the economic sustainability of viticulture reducing yields, productivity and longevity of vines and vineyards (Gramaje et al., 2018). Yield losses of 30-50% have been reported by BD in highly infected vineyards of North America (Milholland, 1991). The economic impact of BD along with another GTD such as *Eutypa dieback* in California was estimated to be \$USD260 million per year (Siebert, 2001).

To date, 26 *Botryosphaeriaceae* species in the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Sphaeropsis* and *Spencermartinsia* have been associated with BD of grapevines (Gramaje et al., 2018). Among them, *Neofusicoccum parvum* is one of the most virulent and fastest wood-colonizing fungi on grapevine (Úrbez-Torres et al., 2008; Úrbez-Torres and Gubler, 2009). Plants affected by this pathogen can show symptoms in the field only one or two years after infection (Úrbez-Torres et al., 2006), as wedge-shaped perennial cankers in spurs, cordons and trunks that end up causing the death of the vines. Other symptoms such as dark streaking of the wood, light brown discoloration of the xylem, and bud mortality can also be observed (Úrbez-Torres et al., 2013). Preventive control practices are critical to minimize yield losses caused by BD. The use of tolerant cultivars to BD would be the safest, easiest, the least expensive and the most effective way of controlling the disease.

Previous research have shown that grapevine cultivars and rootstocks have different levels of susceptibility to Botryosphaeriaceae species (Savocchia et al., 2007; Pitt et al., 2013; Travadon et al., 2013; Billones-Baijens et al., 2014; Guan et al., 2016; Berbegal et al., 2017). To date, most of the pathogenicity tests have been performed with Botryosphaeriaceae species under controlled conditions within a short period of time. Interactions between Botryosphaeriaceae species and grapevine cultivars are poorly understood and there is currently little data available (Bellée et al., 2017). Thus, the objective of this study was to evaluate the susceptibility of five commercial cultivars ("Bobal", "Monastrell", "Garnacha Tinta", "Moravia Agria" and "Tinto Velasco") and one relict genotype grapevine ("Moribel" cultivar) to *N. parvum*, under field conditions. The selection criteria used to choose these cultivars was that they were included in the catalog of authorized cultivars in Castilla-La Mancha region, which was the area where the study was conducted.

Pathogenicity studies were conducted with one-year old grapevine rooted cuttings of six red cultivars. Cuttings were collected during wintertime and then kept in cold storage for three weeks. After this period, each dormant cutting was individually buried into sterilized peat moss and placed in a callusing room at 25 °C and 100% humidity for two months. After callusing and rooting, rooted cuttings were inoculated with the *N. parvum* isolate BV-056, following the method described by Martínez-Diz et al. (2019). This strain was isolated from internal wood symptoms, such as sectorial necrosis, in a 25-year vineyard cultivar "Tempranillo" grafted onto "110 Richter" rootstock, in Logroño (La Rioja, Spain) in 2014. Rooted cuttings were wounded between the two upper internodes with a 5-mm cork borer. A 5-mm mycelial agar plug, from a 3-weeks-old culture on potato dextrose agar (PDA), was placed in the wound. Wounds were sealed with parafilm and wrapped with foil paper to prevent drying. Ten rooted cuttings per cultivar were inoculated with the fungus. Two rooted cuttings were inoculated with 5-mm uncolonized PDA plugs, as negative controls. After inoculating, rooted cuttings were planted in a plot and irrigated by a drip system with two drippers per plant. The experiment design consisted of two blocks. In each block, the cultivars were arranged in randomly distributed rows. A row consisted of six rooted cuttings, five of them inoculated with the fungus and another one used as negative control. The distance between plants in a row was 0.5 m and the inter-row spacing 2 m. The experiment was repeated over two consecutive years (2017 and 2018). Plants were collected after 8 months and inspected for lesion development. Extent of wood necrosis was measured upward and downward from the inoculation point. Three rooted cuttings for each cultivar were selected and small pieces (0.5 to 1 cm), of necrotic tissue from the edge of each lesion, were cut and placed on malt extract agar supplemented with 0.5 g/L of streptomycin sulphate (MEAS), in an attempt to recover the inoculated fungus and complete Koch's postulates. *N. parvum* was identified by morphological and molecular approaches according to Philips et al. (2013). Lesion lengths over two years were subjected to analysis of variance (ANOVA) using SPSS 23.0 software. To satisfy the assumption of homogeneity of variance (Levene's test), a square-root transformation to total lesion length data was made before analysis. The means of the transformed data were compared by Tukey's honestly significant test at $P = 0.05$

There were no differences in lesion length data between the two years ($P = 0.1210$), so data from both years were combined. *N. parvum* caused wood necrosis on all grapevine cultivars, significantly longer than control plants. An example of wood necrosis caused by *N. parvum* is shown in Fig. 1. The incidence of the fungus on different cultivars, expressed as the mean percentage of infected rooted cuttings from the total of inoculated rooted cuttings, ranged from 42.1% (“Monastrell” cultivar) to 93.3% (“Tinto Velasco” cultivar). Mean lengths of the extent of wood necrosis caused by *N. parvum* on inoculated one-year-old grapevine wood are shown in Fig. 2. These values ranged from 21.2 (“Bobal” cultivar) to 87.2 mm (“Tinto Velasco” cultivar). *N. parvum* was reisolated from the edge of each lesion in 90.3% of the cultivars. No fungus recovery was observed in control plants. The results of statistical analysis showed that “Bobal” and “Monastrell” cultivars were significantly more tolerant than “Tinto Velasco”. These three cultivars are traditionally cultivated in Spain; however their distribution area is quite different. “Bobal” and “Monastrell” are cultivars with a Mediterranean vocation, located near the coastal zone, which require a certain degree of environmental humidity for their suitable development. On the contrary, “Tinto Velasco” is a minority and rustic cultivar with a continental vocation and very adapted to drought conditions. Future research would be needed to explore a possible correlation between cultivar distribution and susceptibility to BD. The wood necrosis on all grapevine cultivars was significantly longer than control plants. All cultivars became infected, reinforcing the results obtained by other authors (Massonnet et al., 2017; Martínez-Diz et al., 2019) who claim that to date, no grape species, cultivated or wild is known to be resistant to fungi causing GTDs. Our results were also consistent with those obtained by Travadon et al. (2013), who observed different susceptibility levels in seven commercial *V. vinifera* cultivars and one *Vitis* hybrid cultivar infected by *N. parvum* pathogen. Ramirez et al., (2018), observed similar results with other fungi associated with Botryosphaeria dieback, namely *Diplodia seriata* and *Diplodia mutila*. These authors only found significant differences in lesion length among different grapevine cultivars inoculated with *Diplodia mutila*. In the present study, we used self-rooted *V. vinifera* cultivars to test them for *N. parvum* disease resistance. However, grapevine grafting with American *Vitis* species rootstocks is routinely used in regions where phylloxera is present. Therefore, the future direction of research needs to investigate the effect of grapevine rootstocks, with different agronomic characteristics, on the response of *V. vinifera* cultivars to *N. parvum* infection.

Susceptibility to Botryosphaeria dieback in grapevine cultivars has attempted to be explained by diverse expression of several proteins involved in defense, stress tolerance, and metabolism (Spagnolo et al., 2014). Several compounds associated to defense mechanisms such as pathogenesis-related proteins, total phenolics, flavonoids and stilbenes were found to be more abundant in wood of grapevines affected by this disease (Spagnolo et al., 2014; Guan et al., 2016). Grapevine increases the production of stilbenes after being attacked by *N. parvum*. The pathogen is able to bypasses the obstacle, even though *N. parvum* was shown to be sensitive to polyphenolics in vitro assays (Lambert et al., 2012). Abou-Mansour et al., (2015) identified a broad diversity of phytotoxins produced by *N. parvum*, which allowed the fungus to adapt to different environmental conditions. In later studies carried out with *N. parvum* isolates in culture media,

extracellular toxins such as terremutin and salicylic acid derivatives have been identified, which are known to be compounds associated with virulence (Bellée et al., 2017). A screening of novel genetic resources in the Vitaceae family, for resistance against Botryosphaeriaceae, was performed by Guan et al. (2016) who concluded that the resistance to these fungi in some accessions of *Vitis vinifera*, subspecies *sylvestris*, was correlated with earlier and higher induction on some defense genes.

In conclusion, grapevine resistance against *Botryosphaeria dieback* and others GTDs are not likely being explained by a single factor rather a combination of them such as, certain genes and metabolic compounds associated with defense mechanisms against pathogens, and the xylem anatomy might be involved. This study allowed classifying different *Vitis vinifera* cultivars based on their degree of quantitative resistance to *N. parvum*. Among them, “Bobal” and “Monastrell” cultivars showed lower wood response susceptibility to fungal infection and in consequence, this feature would make them potential candidates to create tolerant varieties to *N. parvum* fungus.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Fig. 1 Wood necrosis symptoms observed after eight months of incubation on “Tinto Velasco” grapevines inoculated with *Neofusicoccum parvum*. Control plant (below) and inoculated plants (above).

Fig. 2 Mean lesion length caused by *Neofusicoccum parvum* on grapevine cultivars at eight months after inoculation. Letters indicate statistically significant differences between varieties, according Tukey's honestly significant difference test at $P \leq 0.05$. Bars represent standard error of the mean. Cultivar abbreviations: BOB: "Bobal"; MON: "Monastrell"; GTI: "Garnacha Tinta"; MOR: "Moribel"; MAG: "Moravia Agria" and TVE: "Tinto Velasco.