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## Exploring Factors Conditioning the Expression of *Botryosphaeria dieback* in Grapevine for Integrated Management of the Disease

Catarina Leal,<sup>1,2</sup> Patricia Trotel-Aziz,<sup>1,†</sup> David Gramaje,<sup>3</sup> Josep Armengol,<sup>2</sup> and Florence Fontaine<sup>1</sup>

<sup>1</sup> University of Reims Champagne-Ardenne, Research Unit Résistance Induite et Bioprotection des Plantes RIBP EA 4707, INRAE USC 1488, SFR Condorcet FR CNRS 3417, Reims, France

<sup>2</sup> Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera S/N, 46022 Valencia, Spain

<sup>3</sup> Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas-Universidad de La Rioja-Gobierno de La Rioja, Ctra. LO-20 Salida 13, Finca La Grajera, 26071 Logroño, Spain

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### Abstract

Species from the Botryosphaeriaceae family are the causal agents of *Botryosphaeria dieback* (BD), a worldwide grapevine trunk disease. Because of their lifestyle and their adaptation to a wide range of temperatures, these fungi constitute a serious threat to vineyards and viticulture, especially in the actual context of climate change. Grapevine plants from both nurseries and vineyards are very susceptible to infections by botryosphaeriaceous fungi due to several cuts and wounds made during their propagation process and their entire life cycle, respectively. When decline becomes chronic or apoplectic, it reduces the longevity of the vineyard and affects the quality of the wine, leading to huge economic losses. Given the environmental impact of fungicides, and their short period of effectiveness in protecting pruning wounds, alternative strategies are being developed to fight BD fungal pathogens and limit their propagation. Among them, biological control has been recognized as a promising and sustainable alternative. However, there is still no effective strategy for combating this complex disease, conditioned by both fungal life traits and host tolerance traits, in relationships with the whole microbiome/microbiota. To provide sound guidance for an effective and sustainable integrated management of BD, by combining the limitation of infection risk, tolerant grapevine cultivars, and biological control, this review explores some of the factors conditioning the expression of BD in grapevine. Among them, the lifestyle of BD-associated pathogens, their pathogenicity factors, the cultivar traits of tolerance or susceptibility, and the biocontrol potential of *Bacillus* spp. and *Trichoderma* spp. are discussed.

**Keywords:** *Bacillus subtilis*, biological control, life traits, *Neofusicoccum parvum*, tolerance traits, *Trichoderma atroviride*, trunk diseases

The goal of this review was to explore some of the factors conditioning the expression of *Botryosphaeria dieback* (BD) in grapevine. The review is divided into four topic areas: (i) the lifestyle of BD-associated pathogens and their consequences on grapevine productivity and viticulture; (ii) the pathogenicity factors of *Neofusicoccum parvum*, especially focusing on phytotoxin secondary metabolites; (iii) the current knowledge on cultivar traits of tolerance or susceptibility to *N. parvum*; and (iv) the vine health status depending on its microbiome, enabling us to point out beneficial microorganisms with biocontrol potential against BD. Most

of this review deals with *N. parvum*, which is considered a key target pathogen for the development of sustainable strategies against BD, and phytotoxic metabolites, which are considered to be virulent factors responsible for the pathogenicity of this species (Mesguida et al. 2023). Regarding biocontrol agents (BCAs), this review focuses on *Trichoderma* and *Bacillus* spp., each described as very effective at protecting pruning wounds against various grapevine trunk disease (GTD) pathogens and controlling *N. parvum* in vitro, in planta (nurseries/greenhouses), and in vineyards (Mesguida et al. 2023). In particular, this review has been organized to provide a better understanding of the current knowledge for the control of BD-associated pathogens by limiting their propagation and aggressiveness, searching for grapevine tolerance traits, and taking advantage of effective BCAs, aiming to provide sound guidance for an effective and sustainable integrated management of BD.

<sup>†</sup>Corresponding author: P. Trotel-Aziz; patricia.trotel-aziz@univ-reims.fr

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### Botryosphaeria Dieback: A Serious Threat to Vineyards and Viticulture

During their entire lifespan, grapevines (*Vitis vinifera* L.) are exposed to a wide variety of fungal trunk pathogens, including

those belonging to the order Botryosphaerales (Dissanayake et al. 2015). Despite the fact that they have been recognized as endophytes since the late 1980s (Slippers and Wingfield 2007), *Botryosphaeria* species are considered latent pathogens able to rapidly cause disease when their hosts are under stress (Delaye et al. 2013; Slippers and Wingfield 2007).

### Fungi with a problematic hemibiotrophic lifestyle

Botryosphaeriaceous fungi can remain latent as nonpathogenic endophytes (asymptomatic plants) until the host plant is exposed to abiotic or biotic stress (Galarneau et al. 2019; Hrycan et al. 2020; Qiu et al. 2016; Slippers and Wingfield 2007). The lifestyle of such fungal plant pathogens that first develop on living tissue (as asymptomatic endophytes) before subsequently causing the death of the host is defined as a hemibiotrophic lifestyle (Gan et al. 2013). Depending on the fungus, some hemibiotrophs can switch from endophytes to biotrophic pathogens or to necrotrophic pathogens (Delaye et al. 2013; Kemen and Jones 2012). Regarding *Botryosphaeria* species, such as *N. parvum*, when it becomes pathogenic, Trotel-Aziz et al. (2019, 2022) have clearly shown its necrotrophic nature by producing phytotoxins that repress specific host defenses (“Pathogenicity factors of *N. parvum*: Focus on the intriguing phenolic phytotoxins” section contains more details). Bellée et al. (2017) also showed that hybrid vines resistant to biotrophic pathogens remain susceptible to attacks by Botryosphaeriaceae (“Grapevine traits of tolerance or susceptibility to *N. parvum*” section contains more details). The necrotrophic nature of *N. parvum* was also corroborated by Trotel-Aziz et al. (2019) and Leal et al. (2021b) (“From microbiota analysis to biological control assays” section contains more details).

During their endophytic phase, fungal intracellular hyphae would develop between living host cells showing intact plasma membranes (resembling a biotrophic phase without being pathogenic, hence the unfortunate term “biotrophic-like” sometimes used, unfortunate because a pathogen is either biotrophic or necrotrophic). During the consecutive necrotrophic phase, the fungus would form secondary hyphae (morphologically different) to get nutrients from the dead host cells (Gan et al. 2013).

The botryosphaeriaceous fungi are thus hemibiotrophic pathogens, being pathogenic in their necrotrophic phase, despite an endophytic colonization that distinguishes them from the true necrotrophic pathogens having an extracellular growth of hyphae (Obrador-Sánchez and Hernández-Martínez 2020).

Using a phylogenetic approach, Delaye et al. (2013) showed that such fungi can easily and reversibly switch from endophytes to necrotrophs that kill their host on an evolutionary or ecological timescale. However, which factors determine whether or not the disease is expressed—fungal pathogenicity factors, host traits of tolerance or susceptibility, both, their additional relationship, or evolutionary history with the microbiome or microbiota? Because endophytes may infect living plant tissues without causing symptoms of disease, fungi with a hemibiotrophic lifestyle are considered a major threat to viticulture worldwide.

### A worldwide challenge for grapevine productivity and viticulture

Species from the Botryosphaeriaceae family are non-host-specific and are disseminated all over the world due to commercial exchanges of asymptomatic seeds and plants (Larignon 2022; Sakalidis et al. 2013; Slippers and Wingfield 2007). Belonging to the genera *Botryosphaeria*, *Cophinforma*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Macrophomina*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria*, and *Spencermartinsia*, several species can trigger dieback symptoms commonly referred to as BD (Larignon 2022; Mondello et al. 2018; Úrbez-Torres and Gubler 2011). Visible external BD symptoms appear in both young and adult vineyards as a lack of spring growth from affected spurs with shoot dieback,

as well as bud and stem necrosis (Reis et al. 2020; Úrbez-Torres and Gubler 2011; Úrbez-Torres et al. 2006). In already developed vines, the BD visible symptoms may take the form of (i) leaf tigering, (ii) canker necrosis (wedge-shaped), and progressive longitudinal necrosis of the stem due to a progressive desiccation of the woody tissues, followed by (iii) inflorescence desiccation and defoliation associated with a complete dead branch or whole vine death (Mondello et al. 2018; Úrbez-Torres and Gubler 2011).

Once pathogenic, the botryosphaeriaceous fungi can decrease grapevine productivity in one of two ways: (i) slowly, by inducing chronic diseases due to polyetic epidemics leading gradually but irremediably to the plant death after several years (loss of plant vigor and punctual reversible damage), or (ii) promptly (apoplectic form of BD), by partially (dead arm) or totally killing the plant (Bertsch et al. 2013; De La Fuente et al. 2016; Fontaine et al. 2016; Mondello et al. 2018). Both the chronic and apoplectic forms of BD cause important economic losses and limit vineyard sustainability. BD is one of the main GTDs causing significant economic losses every year. It is estimated that the cost of grapevine replacement due to GTDs worldwide exceeds \$1.5 billion per year (De La Fuente et al. 2016; Guerin-Dubrana et al. 2019; Hofstetter et al. 2012). Further studies are still needed to better understand the origin of the chronic or apoplectic form of BD, leading either to the resilience of the plant to reversible damage or to the partial or total death of the plant. The hemibiotrophic nature of the fungus, mentioned above, could explain its reversible aggressiveness when switching from endophyte to necrotroph on an ecological timescale (Fig. 1, hypothetical scheme). Deeper experimental research is still required to better characterize the endophytic phase of botryosphaeriaceous fungi: Are they latent as conidia or latent as intracellular hyphae already containing aggressive molecules that will sustain the necrotrophic phase at the right time? In addition, what conditions the maintenance of an endophytic status or stops it to allow for a fungal necrotrophic lifestyle? Do other abiotic or biotic parameters trigger a fungal necrotrophic lifestyle independently of its location?

Symptomatic plants become sources of the BD pathogen's inoculum because fungal pycnidia or perithecia develop on cankered and dead woody tissues as saprophytes (Fig. 1, hypothetical scheme) (Sakalidis et al. 2013; Slippers and Wingfield 2007). Ascospores or conidia of *Botryosphaeria* fungi can infect grapevine plants in both (i) nursery fields and (ii) vineyards. Many of the dispersed spores can come from a wide range of woody perennial crops that are close to vineyards because BD-associated pathogens are non-host-specific (Gramaje et al. 2016, 2018; Hrycan et al. 2020; Rooney-Latham et al. 2005; Úrbez-Torres et al. 2010). Favorable environmental conditions for spore release are rainy events or high relative humidity, with temperatures above freezing that favor spore germination (Larignon 2022; Úrbez-Torres et al. 2010; van Niekerk et al. 2010). Consecutive dissemination of spores in vineyards and nursery fields is ensured by rain droplets, wind, and arthropods until the spores reach natural openings or pruning wounds, where they germinate and start colonizing new xylem vessels and parenchyma cells (Gramaje et al. 2018; Moyo et al. 2014). Annual wounds can remain susceptible to infection for up to 2 to 4 months (Rolshausen et al. 2010; Rosace et al. 2023; van Niekerk et al. 2010). New fungal infections can also be favored in flood-irrigated nursery fields when mother vines sprawl on the ground (Halleen and Fourie 2016; Hunter et al. 2004; Stamp 2003). However, as the woody tissues of diseased vines can be simultaneously infected by various fungal pathogens typically associated with different GTDs, internal and external symptoms can overlap, making specific BD symptoms hard to identify.

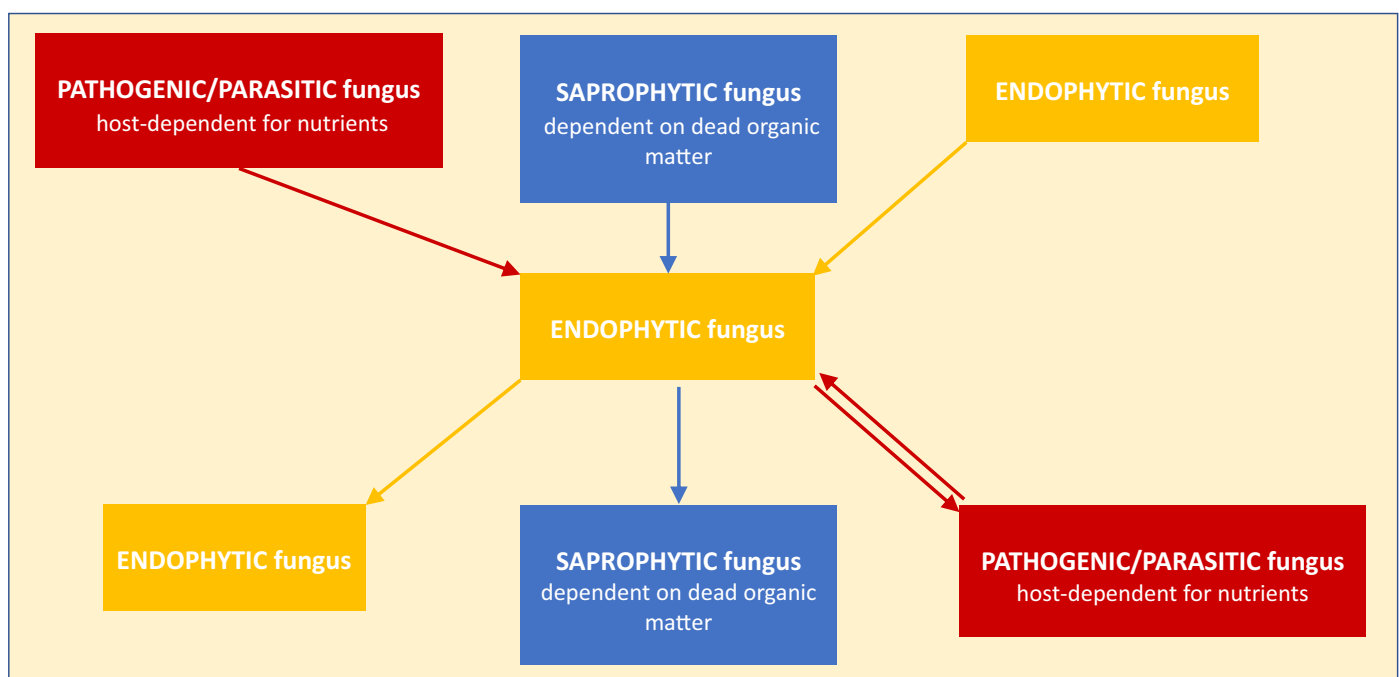
Asymptomatic mother plants can also be pathogen sources, spreading pathogen inoculum in nurseries during the entire vegetative propagation process (Aroca et al. 2010; Fourie and Halleen 2004; Muntean et al. 2022). Indeed, BD-associated pathogens possess an undetermined endophytic latency period that can last

up to 10 years, resulting in infected asymptomatic plants (Muntean et al. 2022). Asymptomatic plants contribute to a late diagnosis when phenotyping is the only diagnostic, making them more difficult to treat. Primary sources of infection often originate from nursery mother blocks, scion, and rootstock (Bergebald et al. 2020; Gramaje and Armengol 2011; Gramaje and Di Marco 2015; Hrycan et al. 2020; Waite et al. 2018). Analysis conducted in the main grapevine growing countries highlighted that nursery propagation material used for new plantations is frequently infected with pathogenic fungi, including BD-associated pathogens (Giménez-Jaime et al. 2006; Leal et al. 2022). Causal pathogens are detected at several stages of the nursery propagation process due to cross-contamination. The earliest contamination point occurs normally during the hydration step prior to cold storage, either by the water itself or by field-acquired pathogens dispersing into the soaking water from one single contaminated plant (Gramaje and Armengol 2011; Waite and Morton 2007). Additionally, several wounds are produced during the propagation process at disbudding, at grafting, or upon root transfer in the field. These large numbers of cuts and wounds make the nursery propagation material very susceptible to infections, providing many entry points for BD-associated pathogens, both during the nursery process and later in nursery fields (Bergebald et al. 2020; Lade et al. 2022; Waite et al. 2018). Additional infections can also occur in nursery fields, as well as in vineyards, but adding load and pressure from the fungal pathogens by their air-dispersed spores still present in the environment (Fig. 2).

### Pathogenicity Factors of *N. parvum*: Focus on the Intriguing Phenolic Phytotoxins

Study of the pathogenicity of Botryosphaeriaceae showed that they are well adapted to a wide range of temperatures (e.g., heat, drought), thus constituting a serious threat to vineyards in the actual context of climate change (Bellée et al. 2017). Pathogenicity factors include cell-wall-degrading enzymes (CWDEs) and phytotoxic secondary metabolites, including phenolic phytotoxins (hereafter

phytotoxins), growth regulators (hormones), effector proteins (also referred to as protein toxins), and fungal viruses (Belair et al. 2022, 2023; Peng et al. 2021; Xing et al. 2023). Using a phylogenetic comparison of botryosphaeriaceous fungi coupled with in planta experiments, Garcia et al. (2021) targeted the virulence factor profiles matching their pathogenicity levels. Host colonization during infection appears mostly driven by the gene expression of CWDEs (especially CAZymes), cytochrome P450 monooxygenases, peroxidases, and secondary metabolites, which are significantly more expressed in the very aggressive *Neofusicoccum* genera. The corresponding encoding genes also showed specific variations (e.g., duplications or deletions) at a species scale, suspected to result from the pressure encountered by each pathogen when colonizing a more or less defensive host. Duplications would favor fungal adaptation and aggressiveness. Computational analysis of gene family evolution within the Botryosphaeriaceae have shown that some *Neofusicoccum* species, including *N. parvum*, expand their P450 genes. Authors suggest that this could reflect the *N. parvum* adaptation to better tolerate the host by metabolizing the host phenolic compounds as shown by Stempien et al. (2017). However, to cope with all the host defenses, the fungus also harbors diverse mechanisms to ensure its survival, possibly also conditioned by abiotic parameters. Indeed, BD symptoms have also been described to result from the synergistic actions of CWDEs and phytotoxic metabolites produced by these fungi and secreted inside the plant cells (Abou-Mansour et al. 2015; Bellée et al. 2017; Bénard-Gellon et al. 2015; Masi et al. 2018; Salvatore et al. 2021; Stempien et al. 2017; Trotel-Aziz et al. 2019, 2022). Restrepo-Leal et al. (2023) recently revealed the influence of lignocellulosic biomass on the expression of these virulence factors. Common transcription factors to phytotoxin production and regulation of CWDEs are even suspected to contribute to the complex aggressive repertoire of *N. parvum* (Massonnet et al. 2018). Because BD-associated fungi are only detected in woody tissues (entry via CWDEs, leading to woody tissues disorders), but never in symptomatic leaves (Larignon and Dubos 1997), phytotoxins are suspected to produce foliar symptoms once transported from the infection site to the leaves via the



**FIGURE 1**

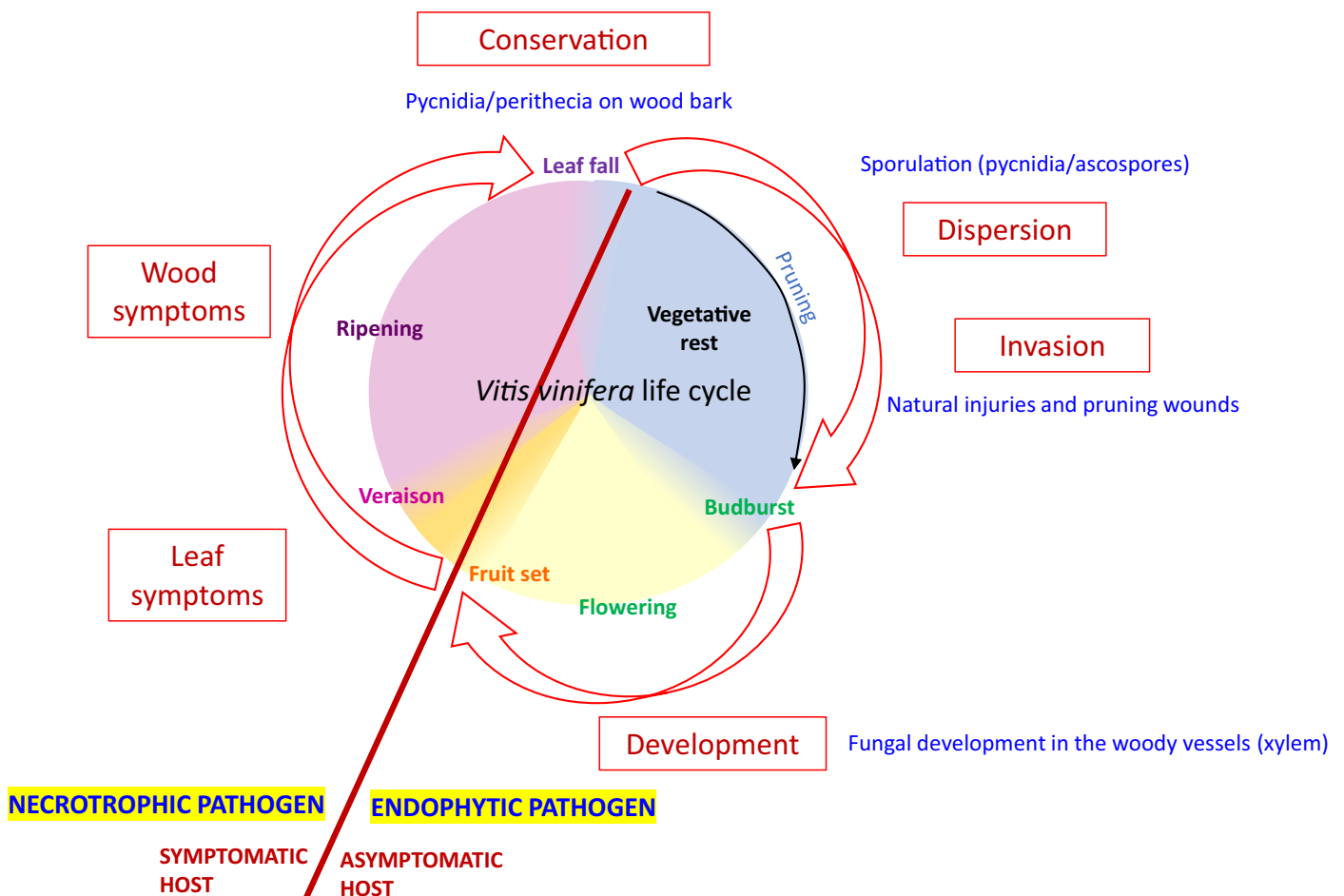
Possible lifestyles of hemibiotrophic botryosphaeriaceous fungi before and after endophytism (yellow and red squares) under environmental, physiological, and genetic control. Adapted, with permission, from Kusari and Spiteller (2012).

xylem sap (Bellée et al. 2017). Martos et al. (2008) and Mugnai et al. (1999) were the first to report the production of bioactive metabolites by botryosphaeriaceous fungi isolated from declining grapevines, including *N. parvum*. It was shown that they produce in vitro both hydrophilic high-molecular-weight compounds with phytotoxic properties and lipophilic low-molecular-weight phytotoxins. Deeper analysis of the highly phytotoxic hydrophilic fraction containing acetylated O-methyl glycosides identified phytotoxic exopolysaccharides composed mainly of glucose, mannose, and galactose. However, these authors postulated that distinct phytotoxic metabolites could actively serve the virulence of *N. parvum* in planta.

Focusing now on the phenolic phytotoxins (with low molecular weight), several of them have already been detected and identified in grapevine, including (*R*)-mellein and (-)-terremutin (Abou-Mansour et al. 2015; Andolfi et al. 2012; Djoukeng et al. 2009; Evidente et al. 2010; Magnin-Robert et al. 2016; Reveglia et al. 2019, 2020; Salvatore et al. 2021). Despite the fact that different species or isolates may produce different secondary metabolites, Bellée et al. (2017) identified an isolate of *N. parvum* producing more severe symptoms and showing a distinct metabolite profile: It produces terremutin and salicylic acid (SA) derivatives. This agrees with Abou-Mansour et al. (2015), who demonstrated that terremutin and SA derivatives are highly phytotoxic, and with Trotel-Aziz et al. (2022), who showed that the *N. parvum* isolate that overproduces (-)-terremutin causes apoplectic BD symptoms on cuttings. In vineyards, symptomatic vines have shown high levels of these two

secondary metabolites, (*R*)-mellein and (-)-terremutin, or related precursors, in both woody tissues and leaves (Lemaître-Guillier et al. 2020; Magnin-Robert et al. 2016; Moret 2021). Therefore, the contribution of these compounds to Botryosphaeriaceae aggressiveness was further studied. The results showed the capacity of (*R*)-mellein and (-)-terremutin to suppress the jasmonic acid/ethylene (JA/ET)-dependent defenses of grapevine (“Grapevine traits of tolerance or susceptibility to *N. parvum*” section contains more details) and the inability of sensitive grapevines to detoxify these phytotoxins on their own (Trotel-Aziz et al. 2019, 2022). Altogether, these recent results have clearly shown that (i) the *N. parvum* phytotoxins are effectors that suppress the plant-triggered immunity responses, resulting in an effector-triggered susceptibility in grapevine (Fig. 3), and (ii) grapevine alone would not succeed in combatting BD, pointing out that it has not yet acquired the R-protein able to recognize these attacker-specific effectors in order to induce a secondary immune response (called effector-triggered immunity) to protect itself. Consequently, Trotel-Aziz et al. (2019, 2022) postulated that, once inside the plants, phytotoxins succeed in weakening grapevine immunity, which promotes BD symptoms emergence. However, the question of the key aggressive role of these phytotoxins remains unanswered to date: Are they pathogenicity factors that trigger the disease or virulence factors that quantitatively aggravate the symptoms?

Considering grapevine’s ineffectiveness to combat BD-associated fungi once phenolic phytotoxins are inside the plants, it is also reasonable to assume that hemibiotrophic pathogens such



**FIGURE 2**

Life cycle of botryosphaeriaceous fungi in relation to the development cycle of the grapevine plants. Adapted, with permission, from Larignon (2022).

as Botryosphaeriaceae have already acquired the potential to inhibit the defense responses of the plants during their endophytic phase, thus before confronting any plant immune system. To do that, appressoria would be appropriate structures to enable pathogen temporal advance (effector accumulation) to bypass plant defenses by secreting these effectors simultaneously with the plant's perception of the pathogen (Fig. 3, hypothetical scheme). Deeper investigation to characterize the endophytic phase of botryosphaeriaceous fungi would thus be useful to understand how such hemibiotrophic fungi can turn necrotrophic at the right moment without any host possibility of recourse. Considering the high level of polymorphism in genes involved in the synthesis of (-)-terremutin and (*R*)-mellein in *N. parvum* (Trotel-Aziz et al. 2022), further research is needed to better understand their responsibilities in the inability of the plant to recognize them as pathogen effectors. Indeed, the high level of genetic variations within a species could be a consequence of the evolution and adaptation of a pathogen to its biotic environment to evade effector recognition and keep virulence factors instead of avirulent ones (Garcia et al. 2021).

*N. parvum* is described as a highly aggressive plant-pathogenic fungus of the Botryosphaeriaceae family (Bellée et al. 2017; Larignon 2022; Larignon et al. 2015; Mesguida et al. 2023; Pitt et al. 2013; Úrbez-Torres and Gubler 2011). However, genetic differences exist among the distinct isolates of *N. parvum*, especially regarding genes involved in phytotoxin biosynthesis (Trotel-Aziz et al. 2022). Three *N. parvum* isolates (Np-Bt67, NpB, and NpB-UV9) differed both in their aggressiveness in planta and in their phenolic secondary metabolite profiles, Np-Bt67 being characterized by its ability to produce large amount of (-)-terremutin with almost no (*R*)-mellein and being the most pathogenic on grapevine cultivar Chardonnay (Trotel-Aziz et al. 2022). It causes strong BD symptoms (dead branch) within 10 days on grapevine cuttings (Leal et al. 2021b; Trotel-Aziz et al. 2019, 2022). NpB produced medium amounts of both (*R*)-mellein and (-)-terremutin, whereas

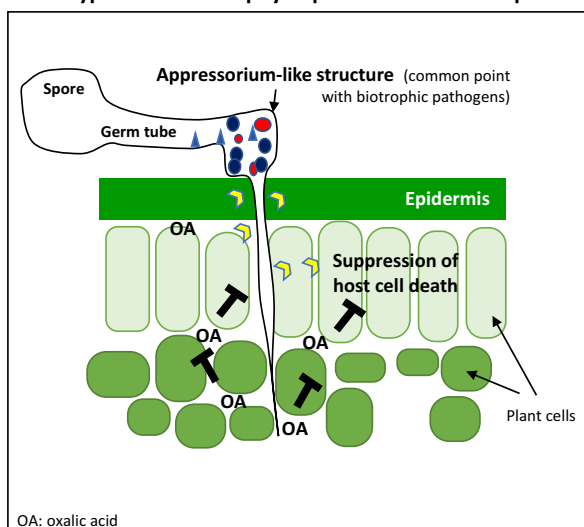
NpB-UV9 produced large amounts of (*R*)-mellein with almost no (-)-terremutin, and both were less pathogenic isolates on grapevine (stem necrosis without dead branch) than Np-Bt67 (Trotel-Aziz et al. 2022). Comparing the effects of fungal isolates and purified (-)-terremutin and (*R*)-mellein would act more as virulence factors (i.e., increasing quantitatively the disease) than as pathogenicity factors (i.e., required to trigger the disease) upon infection, as also considered by Chooi et al. (2015) and Reveglia et al. (2020). Interestingly, despite the fact that they differ in their aggressiveness, the wild-type isolates Np-Bt67 and NpB displayed similar trends in the induction of plant defenses (Trotel-Aziz et al. 2022).

## Grapevine Traits of Tolerance or Susceptibility to *N. parvum*

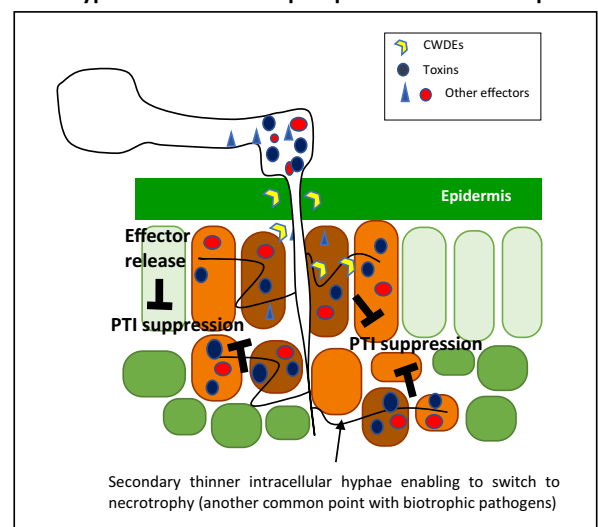
When exposed to BD-associated pathogens, grapevine symptoms (as indicated previously; i.e., delayed budburst, stunted development, leaf chlorosis, canker, stem necrosis, dead branch to full plant death) can vary depending on both the pathogen genotype (species and isolates) and the cultivar susceptibility to BD.

Depending on the pathogen genotype, Trotel-Aziz et al. (2022) showed that the *N. parvum* wild-type isolates (i.e., Np-Bt67 and NpB) triggered similar trends in plant defense induction—an upregulation of SA-responsive genes (*PR2*, *PR5*, and *PR10*)—whereas the expression of JA/ET-responsive genes was either unaffected or moderately upregulated (Trotel-Aziz et al. 2022). Previously, comparing isolates' effects on different cultivars, Bellée et al. (2017) also showed that upregulation of some *PR* genes correlates with increased necrosis and canker sizes. The authors also showed that hybrid vines resistant to biotrophic pathogens remain susceptible to attacks by Botryosphaeriaceae. Yang et al. (2015) reported that the plant SA-dependent defenses are not helpful to combat

### A Hypothetical Endophytic phase of hemibiotrophs



### B Hypothetical Necrotrophic phase of hemibiotrophs



## FIGURE 3

Hypothetical mode of colonization of hemibiotrophic fungal pathogens such as *Neofusicoccum parvum* in grapevine. **A**, Endophytic phase of hemibiotrophic fungus forming an appressorium that develops hyphae (haustorium), enabling it to store phytotoxins but also to deliver cell-wall-degrading enzymes (CWDEs, to obtain nutrients from the living tissue) and oxalic acid (OA, or an equivalent molecule, to prevent host cell death). **B**, Necrotrophic phase of hemibiotrophic fungus starting with the formation of secondary thinner intracellular hyphae able to deliver effectors such as phenolic phytotoxins into the host to prevent the plant-triggered immunity (PTI) by suppressing the host defenses, resulting in the rapid host death. Hyphal penetration is facilitated by the dissolution of the host cell wall by an increased secretion of CWDEs. Efforts to identify and characterize the pathogen-determinant early-stage effectors still need to be deeply investigated both to confirm their determinant value and to target those potentially recognized by the host surveillance system (R-genes) involved in gene-for-gene resistance and plant self-combat against hemibiotrophic pathogens. Adapted, with permission, from Pradhan et al. (2021).

necrotrophic fungi and that SA signaling could antagonize the expression of the JA-responsive defenses, thus facilitating disease development. Among upregulated genes during cutting infection with pathogens are also those encoding for enzymes involved in the biosynthesis of antimicrobial phytoalexins (*PAL* and *STS*, phenylalanine ammonia lyase and stilbene synthase, respectively) and in the detoxification process (*GST1*, a glutathione-S-transferase) (Bellée et al. 2017; Trotel-Aziz et al. 2022). *GST1* is also strongly upregulated in plantlets treated with (-)-terremutin and therefore suspected to ensure detoxification of this phytotoxin (Trotel-Aziz et al. 2022). In contrast, phytotoxins supply to plantlets repressed the expression of *Chalcone Isomerase* (*CHI*, involved in flavonoid synthesis), although they do not alter the expression of *PAL* and *STS* known to produce phytoalexins described to be potentially metabolizable by Botryosphaeriaceae (Stempien et al. 2017). Trotel-Aziz et al. (2022) suggested that (-)-terremutin could inhibit the expression of the flavonoid pathway to favor stilbene production (because they share a common precursor), resulting in enhancing pathogen fitness. Additionally, Trotel-Aziz et al. (2022) demonstrated that (-)-terremutin weakened the expression of the host JA-dependent defenses, confirming therefore that (-)-terremutin production by *N. parvum* supports the pathogen's own development in infected grapevine tissues by interfering with host defenses. Finally, these authors suggested that *GST1*, *PR2*, *PR3*, *PR4*, *PR5*, and *PR10* are effective defenses against *N. parvum*, being the most upregulated in response to the most aggressive Np-Bt67 isolate, although not at all upregulated in plantlets treated with (-)-terremutin (except *GST1*, as explained above). These authors also hypothesized that the repression of *PR1* could be beneficial for plant protection against *N. parvum* because *PR1* was only downregulated in response to the less aggressive isolate NpB, whereas it was upregulated by both phytotoxins (Trotel-Aziz et al. 2022). For the responsiveness of these genes to hormones, *PR1*, *PR5*, and *PR10* are described to be regulated by SA (Caarls et al. 2015; Dufour et al. 2013; Naznin et al. 2014); *PR3* and *PR4* by JA/ET; and *PR2* by various phytohormones such as SA, JA/ET, and abscisic acid (Liu et al. 2011).

Some cultivars are considered susceptible to BD due to the higher proportion of symptomatic vines observed under specific climatic conditions (Bertsch et al. 2013; Bruez et al. 2013; Kenfaoui et al. 2022; Travadon et al. 2013; Úrbez-Torres et al. 2006, Úrbez-Torres and Gubler 2011). Comparing cultivars with distinct susceptibilities to BD (Chacón et al. 2020; De La Fuente et al. 2016; Fontaine et al. 2016; Travadon et al. 2013), an early detection of defense compounds (such as the PR2 and PR5 proteins) was observed in tolerant genotypes (Claverie et al. 2020). Bellée et al. (2017) and Spagnolo et al. (2014) also showed that the reduction in necrosis and canker size caused by Botryosphaeriaceae correlates with upregulation of genes involved in cell wall reinforcement, in indole pathways, in phenylpropanoid pathways, and, to a lesser extent, in the production of other PR proteins (i.e., PR6 and PR8). The authors suggested that they could be markers of grapevine defense and protection. A greater susceptibility to BD has also been reported for Tempranillo compared with Chardonnay (Leal et al. 2021b). Leal et al. (2021b), Luque et al. (2009), and Úrbez-Torres et al. (2006) showed a greater constitutive expression of some targeted defense genes in Tempranillo compared with Chardonnay (e.g., SA-responsive genes), even upon challenge with Np-Bt67, possibly explaining Tempranillo's highest susceptibility to BD. These authors concluded that grapevine susceptibility to BD is cultivar-dependent and suggested that a high basal content of SA in Tempranillo could explain its highest susceptibility to *N. parvum*. Indeed, many studies have reported that endogenously accumulated SA antagonizes the JA-dependent defenses, which are recognized as effective to combat necrotrophs, thus prioritizing SA-dependent resistance over JA-dependent defense (Pieterse et al. 2012; Van der Does et al. 2013). Supporting this hypothesis, Leal et al. (2021b) highlighted a common feature

for both cultivars: SA-dependent defenses were strongly decreased in plants protected by beneficial microorganisms acting as BCAs ("From microbiota analysis to biological control assays" section contains more details about BCAs) in contrast with symptomatic plants.

Genetic variations of domesticated *V. vinifera* cultivars (*V. vinifera* subsp. *vinifera*) usually match with geographic and climatic areas (Magris et al. 2021). BD distribution worldwide, or its causal pathogens, also depends on geographic zones and climatic variations (Bellée et al. 2017; Fernandez et al. 2023; Guerin-Dubrana et al. 2019; Kenfaoui et al. 2022; Songy et al. 2019). Therefore, both (i) preserved genotype diversity within grapevine selected varieties, cross-created cultivars, or even mutated clones and (ii) BD distribution and pressure, depend on the geographical areas. To better investigate the *V. vinifera* genomic traits to be considered against BD-associated pathogens, for instance for plant breeding, it could be interesting to perform a genomic comparison of vines from distinct geographic/climatic areas. Additionally, as cultivated grapevines are mainly propagated by grafting (>80%), their genetic diversity could be based on both the scion (named variety) and rootstock (Ollat et al. 2014; Songy et al. 2019). Incidence of the rootstock on scion vigor and consecutive protection against BD also require further investigation (Guerin-Dubrana et al. 2019; Larignon 2022; Marín et al. 2021; Songy et al. 2019).

*V. vinifera* subsp. *sylvestris* (referred to as *V. sylvestris*) is another subspecies of *V. vinifera* that includes populations returned to the wild. A great genetic diversity was observed within *V. sylvestris* varieties, depending on the soil, environment, and local climate (Regner et al. 1998). Against Botryosphaeriaceae, Guan et al. (2016) reported that populations of *V. sylvestris* provide interesting genetic resources to breed new plants with enhanced resistance to BD. Csótó et al. (2023) even proposed hybrid cultivars with *Vitis* species other than *V. vinifera* (e.g., *V. labrusca* L., *V. rupestris* Scheele, or *V. amurensis* Rupr.) to achieve a high degree of resilience against GTDs. However, the physiological and genetic background of host sensitivity or resistance against GTDs is not yet understood. Comparing the responses of different subclades of *V. sylvestris* to BD, Khattab et al. (2021) highlighted that the phenylpropanoid metabolism of resistant genotypes is rapidly and specifically channeled to bioactive stilbenes. Resistant lines can thus accumulate specific bioactive stilbenes such as viniferine trimers, in contrast with susceptible genotypes accumulating a significantly higher proportion of glycosylated piceid (Khattab et al. 2021). However, these authors indicated that upregulation of phytoalexin genes remained mostly confined to the infection site. Additionally, it is well known that (i) stilbene antimicrobial activities just delay spore germination and hyphal growth because of fungistatic but not fungicidal activity and (ii) BD-associated fungi have the capacity to rapidly degrade stilbenes (Lambert et al. 2012; Stempien et al. 2017). Deeper experimental research is therefore required to better characterize the gain in plant resistance when delaying the germination of conidia of the latent pathogen during its endophytic state in planta or when delaying the intracellular growth of the latent pathogen hypha that may already contain aggressive molecules for switching necrotrophic at the right time. In contrast, Khattab et al. (2021) indicated no relationship between grapevine vessel diameter and its resistance to BD.

In summary, little information exists to establish a list of potential markers of grapevine cultivar tolerance or susceptibility to *N. parvum* isolates (Table 1) logically when considering that the pathogen can (i) deliver effectors to bypass the plant's defenses and (ii) catabolize the plant antifungal molecules, thus leaving grapevine defenseless due to the absence of any apparent means of self-defense. The endophytic phase of the fungi could also have changed the phenotype of the host, particularly its resistance to pathogens (Delaye et al. 2013). However, considering the great diversity of *N. parvum* aggressiveness factors, which are supposed to

be virulence factors adding to phenolic phytotoxin effectors (e.g., cell-wall-modifying enzymes, effector proteins, or other secondary metabolite phytotoxins), much remains to be done to designate grapevine resistance traits (R-proteins) able to recognize the other attacker-specific effectors or their common regulator(s) or transcription factors to induce an effector-triggered immunity that could be protective against *N. parvum*. When lacking self-defense means, the plant can ultimately opt for an avoidance strategy by inducing a dead branch to confine the BD fungi. In this sense, Khattab et al. (2022) reported that plant cells producing ferulic acid triggered programmed cell death (hypothetical disease-avoidance strategy) by means of a signal (fusicochin A) transmitted by the pathogen maintaining permanent reciprocal interaction with *V. vinifera*. Therefore, it could be interesting to investigate the role of the CWDEs of the pathogen in the production of damage-associated molecular patterns (DAMPs; i.e., oligogalacturonides, celooligomers, xyloglucan, and oligosaccharides) able to activate or amplify death signaling to prevent pathogen progression in favor of plant recovery. Functioning as a plant-triggered immunity amplifier, DAMP signaling could be essential to compensate for microbial-associated molecular pattern (MAMP) signaling when MAMP-triggered defenses are compromised by a malfunctioning/disappearance of natural endophytic beneficial microorganisms or of BCAs. Further research is also required to better define how the plant perceives the pathogen effectors (i.e., phytotoxins) that are suspected to be delivered inside host cells, in contrast to DAMPs and BCAs, which are known to be perceived by plasma membrane-localized receptors.

### From Microbiota Analysis to Biological Control Assays: Combining *Bacillus* and *Trichoderma* spp. Appears Promising for the Sustainable Control of BD

Grapevines can be strongly protected against BD when treated with one BCA (Langa-Lomba et al. 2023; Mesguida et al. 2023; Mondello et al. 2018; Rezgui et al. 2016). In parallel, researchers have investigated the microbiota importance in grapevine self-protection against BD in both vineyards and nurseries (Bekris et al. 2021; Bruez et al. 2014, 2015). Indeed, natural beneficial microorganisms (endophytic and rhizospheric), through their qualitative or quantitative temporal dynamics, could compensate for the genetic

lack of self-defense of the plant against fungi causing trunk diseases, as suggested by Bruez et al. (2014, 2015). However, to date, the endophytic fungal, bacterial, and viral microbiota of grapevine and their precise dynamic and roles against BD have been sparsely explored (Bettenfeld et al. 2022; Nerva et al. 2022).

#### *Bacillus* and *Trichoderma* spp. are promising BCAs to combat BD

Few studies have investigated the composition of the microorganism community in GTD-asymptomatic and -symptomatic vines. Comparing non-necrotic tissues from GTD-symptomatic and -asymptomatic plants, Bruez et al. (2014) showed that, although fungal community structures are not statistically different, the number of each fungal isolate in each sample can vary over time, explaining why healthy tissue can become necrotic a few years later (and probably conversely). They also noted that the most abundant isolates collected were botryosphaeriaceous fungi (including *Neofusicoccum* spp.) and *Trichoderma* spp. (Bruez et al. 2014), as well as *Bacillus* spp. for the bacterial community (Bruez et al. 2015). The authors postulated that changes in the mycoflora colonizing grapevine woody tissues could occur over time as a result of changes in biotic and abiotic environmental factors (e.g., beneficial microorganisms, plant age, cultivar, soil, climate, etc.) and favor or disadvantage the development of the pathogenic fungi already present in some parts of the trunks and subsequently cause necrosis. Bekris et al. (2021) further provided evidence that GTD-symptomatic grapevines, including the BD-symptomatic ones, harbor woody tissues with a specific microbiota, showing cultivar- and biogeography-dependent patterns. They also pointed out strong interactions between the bacterial and fungal microbiota of woody tissues in asymptomatic vines. Indeed, the bacterial microbiota in asymptomatic plants was dominated by *Bacillus* and *Streptomyces* spp. in a negative co-occurrence pattern with GTD pathogens. This study also supports the hypothesis of Bruez et al. (2014, 2015) of a potential role for a beneficial microbiota in the suppression of GTD pathogens. Ongoing studies are still focusing on the dynamics of qualitative and quantitative changes in the plant microbiome and microbiota before, upon, and after pathogen exposure.

Focusing on bacterial prevalence in asymptomatic grapevines (Bekris et al. 2021), the coexistence of *Bacillus* and *Streptomyces* genera in a negative co-occurrence with GTD pathogens supports the capacity of these bacterial genera to act as BCAs (Alfonzo

**TABLE 1**  
List of potential markers of grapevine cultivar tolerance or susceptibility to *Neofusicoccum parvum*<sup>a</sup>

Tolerance markers	Susceptibility markers	Avoidance markers
PR2 [1][3][4][6]	PR1 [3][5][6]	Ferulic acid [7]
PR3 [6]	STS [2][3][5][6]	
PR4 [6]	Salicylic acid-dependent defenses [3][5][6]	
PR5 [1][3][4][6]		
PR6 [2]		
PR8 [2]		
PR10 [3][6]		
CHI [3][6]		
GST1 [3][5][6]		
Genes involved in cell wall reinforcement [2]		
Genes involved in indole pathways [2]		
Jasmonic acid/ethylene-dependent defenses [3][5][6]		

<sup>a</sup> [1], Spagnolo et al. (2014); [2], Bellée et al. (2017); [3], Trotel-Aziz et al. (2019); [4], Claverie et al. (2020); [5], Leal et al. (2021b); [6], Trotel-Aziz et al. (2022); and [7], Khattab et al. (2022).

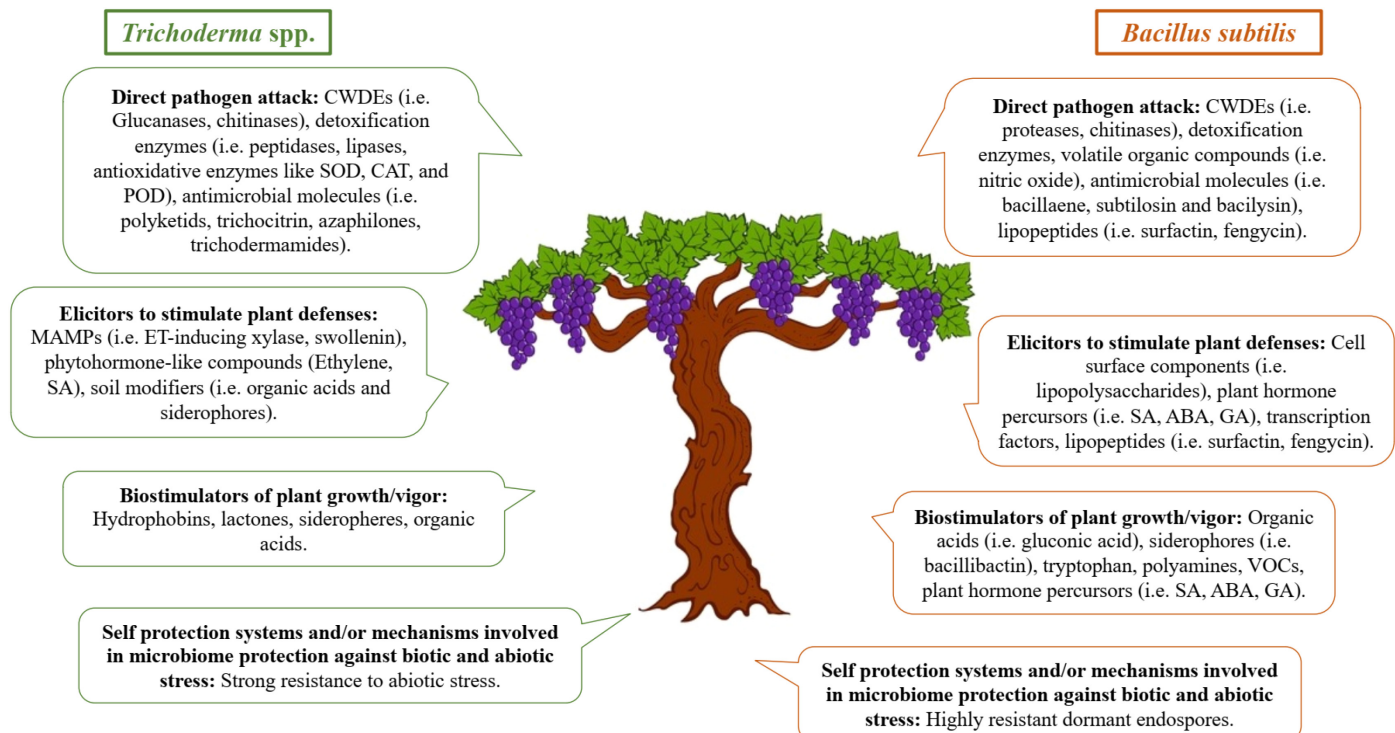
et al. 2009; Mesguida et al. 2023; Viaene et al. 2016). According to Mesguida et al. (2023), *Bacillus* spp. are described as very effective in protecting pruning wounds against various GTD pathogens and in controlling *N. parvum* in vitro, in planta (nurseries/greenhouses), and in vineyards. Rezgui et al. (2016) reported a high suppressive activity of native endophytic *Bacillus* strains against several BD-associated fungal pathogens, including *N. parvum*. Similarly, Trotel-Aziz et al. (2019) and Leal et al. (2021b) showed that a native *B. subtilis* strain, PTA-271, isolated from the grapevine rhizosphere, was able to attenuate the BD symptoms of grapevine cuttings through different direct and indirect mechanisms. A broad range of beneficial molecules are produced or encoded by the genome of *B. subtilis* PTA-271 (*Bs* PTA-271) (Fig. 4), both to induce or elicit plant defenses (using MAMPs, such as phytohormones precursors, lipopolysaccharides, siderophores, etc.) and/or to directly compete, antagonize, or alter the plant pathogens or their aggressiveness (Leal et al. 2021a).

Focusing on the fungal genera from asymptomatic versus symptomatic plants, the mycobiome of woody tissues in healthy vineyards is dominated by endophytes, among which those with a potential for biological control belong to the genera *Trichoderma*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Purpureocillium*, and *Aureobasidium* (Dissanayake et al. 2018; González and Tello 2011; Mesguida et al. 2023; Pancher et al. 2012). According to Mesguida et al. (2023), *Trichoderma* spp. are described as very effective in protecting pruning wounds against various GTD pathogens and in controlling *N. parvum* in vitro, in planta (nurseries/greenhouses), and in vineyards. Mycoviruses from grapevine microbiota, such as the ones from the genus betapartitivirus or betachrysovirus detected by Nerva et al. (2019), are also suspected to influence the effect of beneficial microorganisms such as *Trichoderma*. Chun et al. (2018) reported that *T. harzianum* isolates infected with betapartitivirus displayed an increased antagonistic activity compared with the virus-free isolates.

High-throughput amplicon sequencing continues to explore in depth the rich beneficial endophytic bacterial/fungal/viral com-

munities of asymptomatic plants (healthy or resilient ones) versus symptomatic ones, considering that (i) grapevine may support diverse communities from roots to berries (Bekris et al. 2021; Bokulich et al. 2014; Deyett and Rolshausen 2020; Zarraonaindia et al. 2016), and (ii) grapevine genotype may also influence its microbiome/microbiota composition (Berlanas et al. 2019; Bettenfeld et al. 2022; Marasco et al. 2018). Consistent with the current knowledge mentioned above, *Bacillus* spp. and *Trichoderma* spp. are the two main microorganisms incorporated in commercial products used in viticulture (Harman 2006; Mondello et al. 2018; Mukherjee et al. 2012; Waghunde et al. 2017), and the most studied against GTDs (Berbegal et al. 2020; Di Marco et al. 2002, 2004; Halleen et al. 2010; John et al. 2008; Langa-Lomba et al. 2023; Leal et al. 2021b; Martínez-Diz et al. 2021a, b; Mesguida et al. 2023; Schmidt et al. 2012; Trotel-Aziz et al. 2019).

The development of a durable biological control to combat BD from nursery to vineyards is still a major challenge for vine growers, nurserymen, and scientists. Some BCAs have already been successfully tested against BD-associated pathogens in both vineyards and nurseries (Berbegal et al. 2020; Gramaje et al. 2018; Langa-Lomba et al. 2023; Leal et al. 2022; Martínez-Diz et al. 2021a; Mesguida et al. 2023; Mondello et al. 2018). Among the *Bacillus* and *Trichoderma* genera, *B. subtilis* and *T. atroviride* are the most assayed and promising species against BD, some of which (e.g., *T. atroviride*) are already commercialized against several GTD-associated fungi (Berbegal et al. 2020; Gramaje et al. 2018; Leal et al. 2021b; Martínez-Diz et al. 2021a, b; Mondello et al. 2018; Trotel-Aziz et al. 2019, 2022; Mesguida et al. 2023). In vineyards, *Trichoderma*-based registered products against GTDs are applied in pruning wounds to prevent infections caused by the annual contamination with spores of fungal pathogens, such as Vintec, which contains *T. atroviride* SC1 (namely *Ta* SC1) (Berbegal et al. 2020; Blundell and Eskalen 2022; Gramaje et al. 2018; Kotze et al. 2011; Martínez-Diz et al. 2021a, b; Mesguida et al. 2023; Mounier et al. 2014; Mutawila et al. 2011; Pitt et al. 2012; Sosnowski et al. 2004; Úrbez-Torres et al. 2020). No *Bacillus*-based registered product is



**FIGURE 4**  
*Trichoderma* spp. and *Bacillus subtilis* biocontrol mechanisms and molecules.

yet specifically commercialized against GTD pathogens (Mesguida et al. 2023), despite the *B. subtilis* assets observed in planta (protective capacity and induction of plant defenses) and in vitro (antifungal activity and detoxification of aggressive molecules) under controlled conditions (Leal et al. 2021b; Trotel-Aziz et al. 2019, 2022). The soil-applied *Bs* PTA-271 succeeded in protecting Chardonnay grapevine against *N. parvum*, leading to plant induced systemic resistance (ISR) by priming the expression of JA/ET-dependent defenses in stem-infected Chardonnay cuttings (Trotel-Aziz et al. 2019), as it did against a true necrotroph such as *Botrytis cinerea* (Trotel-Aziz et al. 2008). Against *N. parvum* or its aggressiveness, *Bs* PTA-271 can develop a direct antagonistic activity by reducing the pathogen mycelial growth or can directly detoxify the virulence factors (*R*)-mellein and (-)-terremutin (Trotel-Aziz et al. 2019). The fungistatic effect of *Bs* PTA-271 might be explained by the release of various antifungal compounds (Fig. 4), including surfactins or other lipopeptides, which are also described as elicitors or MAMPs able to induce the systemic host defenses (Leal et al. 2021a; Ongena and Jacques 2008; Pinto et al. 2018; Trotel-Aziz et al. 2019). The BCA fungistatic effect gives the plant more time to defend itself and reduces the biosynthesis and secretion of the pathogen's virulence factors (as diverse they are). This is an advantage considering that once inside the plant, phytotoxins strongly reduce the expression of most of the induced plant defenses (Trotel-Aziz et al. 2019), and (-)-terremutin has been especially shown to repress the JA/ET-dependent host defenses considered effective to combat Np-Bt67 (Trotel-Aziz et al. 2022). The detoxifying activity of *Bs* PTA-271 toward (-)-terremutin and (*R*)-mellein is a complementary asset to counter pathogen aggressiveness, preventing the phytotoxin from reaching a threshold level that would contribute to a damaging reprogramming of plant immunity (Trotel-Aziz et al. 2019). Therefore, BCAs with additional phytotoxin detoxification activities may be of interest in preventing the pathogen from bypassing targeted host defenses, thereby blocking or slowing disease development.

### The combination of BCAs is promising for the sustainable control of BD

Many factors can impact the efficacy of BCAs in vineyards, including abiotic (e.g., climate, soil) and biotic (e.g., antagonistic strains) environmental factors, the formulation of BCAs and their chemical composition, the physical and phenological conditions in which the BCAs are applied, and others (Mesguida et al. 2023). The combination of BCAs with different ecological requirements seems therefore to be interesting, at least to cover a wide range of influencing factors and support an effective biocontrol strategy.

Cultivar was also shown to condition BCAs' efficiency against BD under controlled conditions (Leal et al. 2021b), but commercial products based on BCAs are not designed to be cultivar specific. Therefore, combining BCAs would also be interesting for covering a wide range of cultivars/plants. When comparing cultivars with distinct susceptibility to BD (Chardonnay versus Tempranillo), Leal et al. (2021b) showed that the high basal expression of SA-dependent defenses in Tempranillo positively correlates with both this cultivar's highest susceptibility to Np-Bt67 and the ineffectiveness of *Bs* PTA-271 alone to protect Tempranillo against Np-Bt67. Langa-Lomba et al. (2023) also showed the inefficiency of the *B. subtilis* strain in protecting cultivar Tempranillo against BD-associated pathogens. Leal et al. (2021b) suggested that the early activation of SA signaling in Tempranillo could antagonize the expression of effective JA/ET-dependent defenses (as basal or as induced) against Np-Bt67 once it has entered into its necrotrophic phase (Pieterse et al. 2012; Van der Does et al. 2013; Yang et al. 2015). Supporting this assumption, both Tempranillo and Chardonnay cultivars showed a strong decrease in their SA-dependent defenses when protected by BCAs (Leal et al. 2021b), Chardonnay being protected by *Bs* PTA-271 and Tempranillo by *Ta* SC1. These data highlight that two BCAs can each protect a distinct cultivar, depend-

ing on the cultivar and its metabolic status at a given time, and that combining BCAs would enable a wider range of cultivars/plants to be covered. Because the environmental influencing factors can also modulate the basal metabolism of plants (Qaderi et al. 2023), combining BCAs will be much more robust to support an effective transfer of a biocontrol strategy.

When combining BCAs in greenhouse experiments using cuttings artificially infected by Np-Bt67, Leal et al. (2021b) showed that Tempranillo was protected by *Bs* PTA-271 + *Ta* SC1 or by *Ta* SC1. In Tempranillo, although *Bs* PTA-271 alone appeared not to be protective against an aggressive pathogen responsible for BD (i.e., Np-Bt67) in greenhouse conditions (Leal et al. 2021b), and did not reduce the percentage of plant naturally infected by BD-associated pathogens in nursery conditions (Leal et al. 2022), nursery plants treated with *Bs* PTA-271 showed a reduced percentage of infection with black-foot-associated pathogens compared with plants treated with *Ta* SC1 alone (Leal et al. 2022). These data highlight that a BCA alone, such as *Bs* PTA-271 or *Ta* SC1, can be helpful to protect or prevent infections due to distinct pathogens, depending on the BCA protection mode, in relationship with either the cultivar and/or its metabolic status at a given time, and/or the pathogen(s). Combining BCAs with complementary ways of protection would thus enable development of the strongest biocontrol strategies by covering a wider range of pathogens and/or cultivars, especially susceptible ones.

Whatever the pathosystem, the literature reports that the combination of two or more BCAs can improve the management of plant diseases (El-Tarabily and Sivasithamparam 2006; Guetsky et al. 2001; Leal et al. 2021b; Magnin-Robert et al. 2013; Weller 1988; Yobo et al. 2011), probably due to additive or synergistic effects of beneficial mechanisms in a complex and changing environment. Leal et al. (2022) further showed that combining BCAs enables targeting a wider range of GTD pathogens in propagation material. Indeed, in nursery conditions, combining *Bs* PTA-271 with *Ta* SC1 reduced the percentage of infection by pathogenic fungi responsible for black-foot and BD, better than did each BCA alone (Leal et al. 2022). Similarly, in greenhouse conditions (Leal et al. 2021b), the combination of *Bs* PTA-271 + *Ta* SC1 better protected Tempranillo cuttings against Np-Bt67 (responsible for BD, 91% protection) than did *Ta* SC1 (80% protection). Combinations of BCAs could thus help to improve the efficacy and/or sustainability of the BCA effects against distinct pathogens. These beneficial BCA collaborations against distinct pathogens confirm the potential of combining BCAs to optimize both the plant ISR and the BCAs' direct effects against pathogens in some cultivars, such as Tempranillo. Additionally, considering that each BCA protection mechanism can vary depending on the cultivar and/or its metabolic status at a given time, and/or depending on the pathogen(s), combining BCAs would likely ensure a more successful transfer of biocontrol efficacy from the greenhouse to in situ conditions, from nurseries to vineyards.

Combining BCAs, however, requires that BCAs do not antagonize each other. Leal et al. (2021b) showed that *Bs* PTA-271 and *Ta* SC1 can antagonize each other in dual confrontation but not when applied simultaneously at the same area in a three-way confrontation with Np-Bt67. Accordingly, *Bs* PTA-271 + *Ta* SC1 positively interact to better confine BD-associated fungal pathogens in Tempranillo (Leal et al. 2021b, 2022). However, such a direct positive contribution of combined BCAs did not operate to better protect Chardonnay cuttings against Np-Bt67 compared with *Bs* PTA-271 alone (50% protection) in greenhouse conditions (Leal et al. 2021b). These authors suggested that the cultivar itself could alter the fine-tuned orchestrated cooperation of BCAs against the pathogen, targeting especially host metabolite interactions with the BCA-induced indirect changes in these precise conditions where BCAs were spatially separated. However, in an increasingly constrained environment in situ, increasing interactions operate due to

changes in the metabolic status of the host, the BCA(s), and other microorganisms, including the pathogens. This could thus become increasingly difficult for the host and BCA(s) to ensure a beneficial outcome enabling them to reach their optimal development and/or working/producing level.

Therefore, it is important to consider also the third actor governing plant health, namely the pathogen and/or its aggressiveness, as a complementary lever to better ensure plant protection and to sustainably produce. Thus, assays combining BCA(s) with natural products are also currently underway to improve the efficiency and/or the sustainability of the in situ effects of BCA(s), such as *Bs* PTA-271 and/or *Ta* SC1 associated with the molecules listed in Figure 4 (i.e., elicitors of grapevine ISR against pathogens and/or direct effectors of these pathogens) (Leal et al. 2021a; Srivastava et al. 2014).

## General Discussion: Challenges and Perspectives to Consider

Some factors conditioning the expression of BD in grapevine have been explored in this review to provide guidelines for an effective and sustainable integrated management of the pathogens responsible for this disease, combining the limitations of their aggressiveness and the infection risk, tolerant cultivars, and effective BCAs applied in combination.

The lifestyle of these phytopathogenic fungi is highly problematic for grapevine productivity and viticulture worldwide. They can infect the living plant tissues without causing any disease foliar symptoms, and then become aggressive in a reversible way on an ecological timescale in accordance with abiotic changes and/or host weakness. Deeper experimental research is ongoing to better characterize the nonpathogenic endophytic phase of the botryosphaeriaceous fungi. Could the discovery of a molecule that would keep the fungus in an endophytic state prevent it from becoming aggressive? Could the discovery of inhibitors (or effectors) of the necrotrophic phase prevent the fungus from becoming aggressive? Would the process of detoxification of fungal pathogenicity factors be sufficient to prevent these fungi from becoming aggressive once they have become pathogenic? Many questions are still unanswered, and preliminary answers are already underway. Such effectors could be relevant molecules to consider for optimizing the formulation of combined BCAs.

Because of the intriguing lifestyle of the BD-associated fungi, both symptomatic and asymptomatic plants are sources of the pathogenic inoculum. Prophylaxis methods are therefore inevitable to eliminate all visible sources of inoculum. However, to fully limit the propagation of the pathogens and the infection risk, it is also important to consider asymptomatic plants. Asymptomatic plants represent a major risk of propagation for the BD-associated fungi, particularly in nurseries. In fact, asymptomatic mother plants from vineyards can be an important source of the BD inoculum for a nursery. It is therefore important to analyze the microbial diversity before, during, and after the nursery process. Additionally, the nursery structure can offer a good opportunity to treat the mother canes with combined BCAs against the BD-associated fungi in the short term (directly and indirectly) but also in the long term by influencing the balance of the microbiota with BCAs. In this way, nurseries could be very helpful to produce grapevine plants with an optimized beneficial diversity. Afterward, recurrent bioaugmentations with BCA populations could occur in both nursery fields and vineyards (not only when new vineyards are established). This could be an innovative approach to restoring lost biodiversity in vineyards, caused by years of unreasonable use of chemicals that have eliminated many grapevine auxiliaries (or natural beneficial microorganisms). Auxiliaries (endophytic and rhizospheric), through their qualitative or quantitative temporal dynamics, could thus com-

pensate for the plant's lack of genetic self-defense against the fungi causing GTDs. Ongoing studies continue to focus on the dynamics of qualitative and quantitative changes in the plant microbiome and microbiota, before, during, and after exposure to pathogens.

Considering the pathogenic factors of BD-associated fungi, the high level of polymorphism in genes involved in the synthesis of *N. parvum* phytotoxins, and the inefficiency of grapevine in controlling the development of the BD disease once the phenolic phytotoxins are inside the plants, it is crucial to compensate for the plant's lack of defense with the detoxifying capacity of BCAs. The determining value of phenolic phytotoxins in the aggressiveness of such pathogens remains entire, especially because they are specific to the necrotrophic phase of the fungi responsible for BD. Therefore, BCAs with phytotoxin-detoxifying activities may be of interest in preventing the pathogen from bypassing the targeted host defenses, thereby blocking or slowing the disease development.

Although the physiological and genetic background of host sensitivity or tolerance to GTDs is not yet understood, grape breeding programs are ongoing. Hybrid cultivars with *Vitis* species other than *V. vinifera* are being developed to achieve a high degree of resilience against GTDs. In any case, hybrid vines resistant to biotrophic pathogens are not the solution to Botryosphaeriaceae attacks.

Further research is needed to establish a list of potential markers of tolerance or susceptibility of grapevine cultivars to botryosphaeriaceous fungi. The susceptibility markers could help to carry out health monitoring in the nursery fields or in the vineyard but also to target treatment timing. The tolerance markers could help to discriminate elicitors capable of stimulating vine defenses against pathogens responsible for GTDs. Such elicitors could be relevant molecules to consider for optimizing the formulation of combined BCAs.

Many abiotic and biotic factors can impact the efficacy of BCAs in vineyards and nurseries. The combination of BCAs with different ecological requirements will thus allow to cover a wider range of these influencing environmental factors and will be much more robust to support an effective transfer of a biological control strategy. Combining BCAs with complementary means of protection will also make it possible to develop more robust biological control strategies by covering a wider range of pathogens and cultivars, in particular to protect the most sensitive ones. Combined BCAs can be applied in nurseries, nursery fields, and vineyards. However, the combinations of BCAs require that each BCA does not conflict with the other. This is another relevant point to study in order to optimize product formulation.

Because several biotic and abiotic environmental factors can also adversely affect plant metabolism and the effectiveness of BCAs, both in vineyards and in nurseries, it is also important to consider the third actor governing plant health, namely the pathogen and its aggressiveness, as an additional lever to better ensure plant protection and produce sustainably. Thus, trials combining BCAs with natural products are also currently underway to improve the efficacy and the durability of the in situ effects of BCAs, such as *Bs* PTA-271 and/or *Ta* SC1 associated with the molecules listed in Figure 4 (i.e., elicitors of grapevine ISR against pathogens and/or direct effectors of these pathogens).

## Conclusion

To conclude, combining BCAs with natural products (i.e., elicitors and effectors) could be a key to developing promising commercial products for a sustainable biological control of pathogens responsible for grapevine BD, enabling (i) the effective reduction of the amount of chemicals used in the field, (ii) restoration of the beneficial biodiversity damaged by years of unreasonable chemical control, (iii) making the BCAs more effective in situ, and (iv) improvement of the quality of the transfer of BCA results from the greenhouse to nurseries or vineyards. These new commercial

products could be associated with measures aiming to limit the propagation and aggressiveness of the BD-associated fungi (in nurseries and vineyards) with the use of tolerant grape varieties.

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