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New biotechnological solutions in biocontrol and molecular diagnostics of *Neofabraea* spp. in apples – A review

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ABSTRACT

The most important requirement for apple producers is to ensure the best possible apple quality after storage. Growers must comply with several regulations in the field of food and environmental safety. In the production of apples, it has been observed that financial losses are related to the occurrence of latent storage diseases caused by phytopathogenic fungi of the genus *Neofabraea* (bull's eye rot). Therefore, investors in this sector require new solutions supporting rational apple management, with a particular focus on pro-ecological methods of controlling *Neofabraea* sp. pathogenic representatives and methods for the early detection of these pathogens, especially when there are no symptoms of disease in the apple. This review summarizes the activities being undertaken to increase sustainable production in horticulture. What is more, the up-to-date significance of apple production and the various ways of counteracting bull's eye rot were also described. Next, biopreparations based on microorganisms in horticulture applications are characterized, with special attention being paid to the preparations preventing the development of *Neofabraea* spp. The various methods used to detect fungal phytopathogens are explored towards *Neofabraea* spp. detection using genetic markers. Finally, expectations and future directions in the quest for new biotechnological solutions in the area of the biocontrol and molecular diagnostics of *Neofabraea* spp. in apples were presented. In particular, the need for targeted biocontrol biopreparations and an early detection method of *Neofabraea* spp. in apples to evaluate the risk of the occurrence of apple bull's eye rot was highlighted.

1. Introduction

1.1. Activities designed to promote sustainable production in horticulture

The European Commission recently presented a way to achieve climate neutrality and promote sustainable development in Europe by 2050 in two strategic documents. Achieving a healthier and more sustainable food system in the European Union (EU) has become the basis of the European Green Deal strategy, which forms a part of the strategic document referred to as the Farm to Fork Strategy and relates to the role of agriculture and thus of horticulture in this process (European Commission, 2019; European Commission, 2020a).

Both documents promote the activities required for the sustainable agricultural production of plant raw materials to ensure food safety and also to reduce adverse changes to the environment and climate change. An important aspect of the European Green Deal strategy is also the Union Biodiversity Strategy 2030 (European Commission, 2020b). The strategy defines new ways for the more effective implementation of existing legislation as well as new obligations, measures, assumptions, and management mechanisms. Therefore, in the near future the European Commission will act to reduce the use of pesticides and ameliorate the dangers associated with their use by about 50 % by 2030. The Commission will support the development of areas used for organic farming and these areas will account for 25 % of the total agricultural land area by 2030. Horticultural producers are therefore obliged to be up to date with detailed regulations in terms of care for the quality and safety of food and the environment (European Commission, 2020b).

At the same time, the development of a supervision system, for the control and certification of agricultural products is being observed. The activities of the EU are focused on the elimination of dangerous active substances that pose a threat to the environment, the organisms living in it, and human health, the protection of biodiversity (including

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microbiological) is also a priority. In cases where the allowable levels of pesticide residues are exceeded, they must be reported for risk evaluation according to the procedures of the Rapid Alert System for Food and Feed (RASFF). Consumer awareness of food safety and quality is certainly growing. Therefore, the demand for ecological products is also increasing and hence the promotion of solutions supporting such production or/and contributions aimed at minimizing the use of solutions that are harmful to humans and the environment (Çakmakçı and Çakmakçı, 2023; Sehrawat and Sindhu, 2019).

1.2. The significance of apple production and bull's eye rot caused by Neofabraea spp. (BER)

The agricultural and horticultural sectors have experienced significant impacts from consecutive legislative changes, particularly in the realm of apple production. Apples, being one of the most widely consumed fruits globally, hold immense importance in the agricultural industry. In fact, according to FAO data, the domesticated apple species, *Malus domestica*, ranks as the world's third most-produced fruit, following bananas and watermelons, with over 87 million metric tonnes produced in 2019 (Sottocornola et al., 2022).

Apples are renowned for their abundant bioactive compounds, including vitamins, organic acids, phenolic compounds, and antioxidants. Their nutritional value, combined with their easy digestibility, has contributed to their popularity as a preferred choice for introducing solid foods to newborns (Bryk and Rutkowski, 2012). Additionally, apples find extensive use in the food processing industry, with common applications including the production of concentrated juice, natural juice (NFC), cider, and other preserves. Moreover, the utilization of apple waste as a valuable source of cellulose and dietary fiber has been on the rise (Oszust and Frąc, 2020).

However, despite their significance, apple production faces various challenges, notably the prevalence of bull's eye rot disease (BER) and other storage diseases that pose threats to apple quality and safety (Neri et al., 2023).

Given the demands of the market and the widespread utilization of apples, ensuring consistent product quality and optimal storage conditions have become paramount. This not only facilitates further processing but also minimizes losses for producers. Maintaining high-quality standards throughout the storage and processing stages is crucial to meet consumer expectations and adhering to regulatory guidelines (Pakula et al., 2018).

In orchard production, the priority is to obtain crops of the highest quality. In many studies, the species that cause storage apple disease BER are mentioned as essential fungal pathogens of apple trees (belonging to the genus *Pezicula* sp. *sensu lato*, (synonym according to *Index Fungorum*: *Neofabraea* sp., *Gloeosporium* sp.) (Bryk and Rutkowski, 2012; Głos et al., 2022; Michalecka et al., 2016; Udriste et al., 2018). *Neofabraea* sp. is the current name for the pathogen causing this disease in apples. The achievement of an apparently good quality crop at the time of harvest may be deceptive because it is more important for the fruit to retain their quality after the end of their storage period when the fruit finally reach the consumers. The reason for this is the fact that the rot has a latent character – its symptoms appear during the storage of apples in cold stores, while the actual infection occurs a few months earlier, in orchards, during the growing season. Fungal spores are the source of these infections, they are transferred to the buds and fruit along with raindrops through spiracles or insect bites (wound pathogens) (Wenneker and Thomma, 2020).

The disease manifests itself in apples in the form of small, brown spots, that grow larger and become permanent. These spots are usually dark brown and lighter at the edges. The pulp within the spot collapses but the skin remains smooth. Small fruiting bodies of fungi, called acervuli, with yellow-brown conidia lesions, form under the skin. As a result of high humidity, the infected fruit become covered with a grey-white coating of mycelium. The *Neofabraea* spp. infection may take place from the natural drop of apple fruit (fruitlet abscission at the phenological stage BBCH 7) until harvest (Szymczak et al., 2016).

The low temperature of storage rooms and cold rooms favours the development of disease in stored fruit. As farmers have reported, losses in yield caused by the occurrence of these pathogens can be up to 50 % in unfavourable conditions. To date, two species which cause rot have been observed in the fruit of apple trees *Neofabraea alba* (synonym: *Pezicula alba, Gloeosporium album*), *N. perennans* (synonym: *P. perennans*, *G. perennans*, and also *N. kienholzii* and *N. vagabunda* (synonym: *Phlyctema vagabunda*) (Michalecka et al., 2016; Sepúlveda et al., 2022). Due to ongoing climate change, these species can appear in particular areas, other species of the genus *Neofabraea* also cause bull's eye rot (BER) in apples (Michalecka et al., 2016).

1.3. Biotechnology in the service to counteract the BER adverse effect on apple production

Considering the global importance of apple production and the current and expected, financial losses in this branch of the economy, caused by the changes in the occurrence of *Neofabraea* spp. in the context of the observed climate changes, as well as the successively introduced legislation towards the development of ecological agriculture, it is necessary to pay attention and highlight the biotechnological solutions that can help to counteract this unfavorableness and all demands.

The first thing that comes to mind in this context is particularly biocontrol and molecular diagnostics which can potentially play a crucial role in the management of *Neofabraea* spp. in apple orchards. These techniques possibly can help in the early detection and timely management of the disease, thereby reducing economic losses for apple producers.

Especially probiotechnology is a growing field that has the potential to offer many benefits, including more sustainable agriculture practices and improved food safety. Probiotechnology is the use of beneficial microorganisms or probiotics, in biotechnological applications (Bernauer and Meins, 2003). Molecular diagnostics, also called molecular pathology includes biotechnological methods that involve taking the unique genetic code and analyzing the sequences for red flags that can pinpoint the potential emergence of a specific disease (Kaur and Gill, 2022). The field has expanded rapidly in recent years and should be more deeply employed against phytopathogenic fungi belonging to the *Neofabraea* genus. Fig. 1 presents the general scheme of *Neofabraea* spp. development cycle and biotechnological methods such as biocontrol biopreparations and molecular detection methods potentially used to counter its adverse effects on apple production.

To identify specific up-to-date needs in the field of biotechnological solutions for counteracting the negative effects of *Neofabraea* presence in terms of apple production we provided a case study outlook of solutions already used or tested in this area. The microbiome-based approach was mentioned as a means not only to depict the holistic state of the apple production system but also was raised as a way to test biocontrol effects. Next, methods of *Neofabraea* spp. detection methods using genetic markers were systematized. This review emerged the substantial issues that should be taken under consideration for future biotechnological method development against *Neofabraea* spp. in apples.

2. The survey on solutions for preventing the development of *Neofabraea* spp. in apples

2.1. The general programme of apple tree protection - market products

The fungicides which are currently used in the production of apples, e.g. Topsin M 500 SC, Zato 50 WG, and Captan 80 WG have a wide range of activity. These preparations stand out in terms of their effectiveness and effectively counteract the development of fungal pathogens

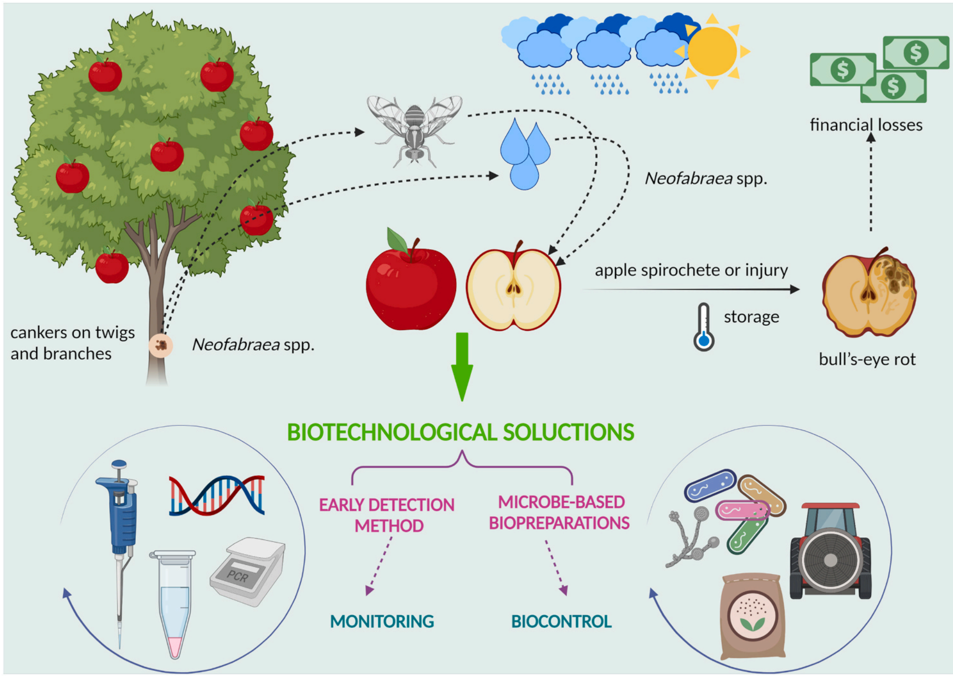

Fig. 1. A scheme of *Neofabraea* spp. development and the biotechnological methods used against it.

including representatives of the *Neofabraea* genus. However, these chemical substances are characterized by a long period of grace (even 60 days) and they contribute to a reduction in the biodiversity of useful fungi (Oleszek et al. 2019). Therefore, they give rise to worries in society concerning the safety and health of agricultural products. For these reasons, they will be successively withdrawn from production. In 2020, 20 active substances that formed a part of certain plant protection products, were removed. EU Member States have been obligated to withdraw authorizations for the introduction to the market of certain plant protection products containing thiophanate-methyl, as of 19 April 2021 (e.g. Topsin) (Regulation, 2020). Captan and dithranol are also substances scheduled for recall (SadyOgrody.pl, 2021).

Alternative ways of controlling these pests are being sought. These are physical treatments: e.g. hot water or hot air treatments (Wenneker and Thomma, 2020), ozonation (Pagès et al., 2020), radio frequencies and microwaves, hypobaric and hyperbaric pressures and far ultraviolet radiation (UV-C light) or treatment with natural compounds e.g. volatile organic compounds (VOCs) (Neri et al., 2009) or also treatments known as biological control agents (BACs) e.g. microbial antagonists, several modes of action have been suggested to explain such biocontrol activity (Köhl et al. 2019). However, most of the alternative treatments developed to date have limitations that impede their effectiveness as single treatments. The concept of combining different treatments within an integrated latent postharvest disease management strategy requires further development (Wenneker and Thomma, 2020).

The prospect of using various biological agents in horticulture includes the increased participation of an assortment of protection agents, and it may also constitute a valuable supplement to the plant protection programme. Their undeniable advantage is the lack of residues on the fruit and their safety concerning human health and the environment, which is currently the focus of great attention. Extending the variety of these alternative (biological) agents is especially important not only for organic plant cultivation but also for the sake of supporting integrated production (Bell et al., 2022; Miller et al., 2022).

In the programme of apple tree protection against fungal disease including apple bull's eye rot caused by representatives of *Neofabraea* sp., it is recommended to use copper and sulphur-containing agents as pro-ecological preparations. Products based on paraffin oils and biopreparations containing cells and metabolites of microorganisms are also allowed. Among these biopreparations Blossom Protect® (Boni-Protect®), Polyversum WP, Prestop WP, Remedies, Yield Plus, Vintec, or Nexy, and Effective Microbes (Ema + Ema 5) may be included. In Poland, as an example, no biological preparations have been registered to date as a fruit protection agent against storage diseases. However, the Blossom Protect® preparation has been registered as a plant growth stimulator and is available on the market (according to the list of plant protection products, which all follow the requirements of organic farming regulations as of September 2019, authorization number R-9/2013 wu, type of product - "other"). The company distributing it recommends that it should be applied by spraying the entire orchard against storage diseases. This preparation contains *Aureobasidium pullulans* yeast strains. However, field and storage research has shown the insufficient effectiveness of pro-ecological methods for the protection of apples against apple bull's eye rot which are available on the market (Blossom Protect®, Polyversum WP, EM, Yield Plus) (Bryk and Rutkowski, 2012).

2.2. *Current research - stepwise in biotechnology of microorganisms-based biopreparations*

The use of products containing microorganisms in horticulture is well suited to the requirements of ensuring food safety and limiting adverse environmental and climate changes (Gamage et al., 2023). Consequently, the financial outlay for the research on the development of new solutions for agricultural production based on microorganisms will be surely increased. According to a recently presented report by the Market Research Company, there is factual confirmation that the agricultural biological agent market is projected to reach a value of 19.5 billion US dollars by 2031.

Moving forward and considering the disclosed inadequate efficacy of current commercial pro-ecological methods against apple bull's eye rot caused by *Neofabraea* spp., it is expected that further research will be conducted to explore new solutions. Currently, only limited research is available on the use of biological control agents (BCAs) and their mode of action against *Neofabraea* spp. However, the investigated antagonists encompass yeasts, yeast-like fungi, bacteria, and filamentous fungi. The literature review below substantiates the aforementioned information.

Recently, Sepúlveda et al. (2022) presented endophytic yeasts, specifically *Vishniacozyma victoriae*, as potential biocontrol agents against *Neofabraea vagabunda* in apples. The observed biocontrol activity was attributed to the ability of these yeasts to form biofilms and produce volatile organic compounds (Sepúlveda et al., 2022).

Metschnikowia pulcherrima has also been investigated for its biocontrol potential against apple bull's eye rot (Bühlmann et al., 2021). The antagonistic activity of *M. pulcherrima* is likely due to the production of antifungal compounds such as pulcherrimin and killer toxins. It can also outcompete the pathogen for nutrients and space on the fruit surface, thereby reducing disease incidence (Bühlmann et al., 2021).

Cryptococcus flavescens, an anamorphic yeast commonly found in the environment, has also shown biocontrol potential against various fungal pathogens, including *Neofabraea* species (Mari et al., 2003). Its mechanism of action primarily involves the production of antifungal metabolites, including mycocins and volatile organic compounds, which inhibit pathogen growth. Additionally, it can compete with the pathogen for resources and space on the fruit surface, leading to a reduction in disease development. (Podgórska-Kryszczuk et al., 2022).

Also, certain strains of bacteria belonging to *Pseudomonas fluorescens* and *Pantoea agglomerans* genera have exhibited antagonistic activity against *Neofabraea*, by producing antifungal metabolites and enzymes that suppress the growth of the pathogen (Köhl et al., 2019).

Literature indicates that *Bacillus subtilis* has demonstrated a high level of biocontrol potential against *Neofabraea malicorticis* (*Pezicula malicorticis*) in laboratory research (Leibinger et al., 1997). *Bacillus* spp. are commonly used as the BCAs due to their antagonistic activity against a wide range of fungal phytopathogens. They are characterized by a high degree of aggressiveness and the effective stimulation of plant growth and defence mechanisms or the ability to modify the plant microbiome Moreover, the capacity of *Bacillus subtilis* to form persistent forms contributes to the longevity of the final product (Omidvari et al., 2023, Oszust et al., 2020; Pylak et al., 2019).

In contrast, a study conducted by Ríos Zamorano and Díaz (2021) focused on the potential of filamentous fungi, specifically *Trichoderma* isolates, to effectively control seven *N. vagabunda* isolates. *Trichoderma* spp. are renowned for their rapid growth and are recognized as formidable competitors against various pathogens, similar to *Bacillus* spp. bacteria. The biocontrol activity of *Trichoderma* is attributed to multiple mechanisms, such as the production of inhibitory compounds (including volatile ones), mycoparasitism, inactivation of pathogen enzymes, induction of plant systemic resistance, and outcompeting pathogens for nutrients and living space through vigorous cell proliferation (Rodrigues et al., 2023).

Overall, the exploration of these diverse groups of biological control agents holds significant promise for developing effective strategies against *Neofabraea* spp., contributing to improved management of apple bull's eye rot and sustainable agricultural practices. However, it is crucial to consider and incorporate key biotechnological elements derived from the development procedures of such bioproducts, directed to other phytopathogens, in this process. This should be done while addressing the specific requirements of this particular application, which involve the protection of apples and understanding the mechanisms of *Neofabraea* spp. mode of action. So, here we present stepwise in biotechnology of microorganisms-based biopreparations to biocontrol *Neofabraea* spp. The following will elaborate on the details, as it explores the development of formulations incorporating antagonistic microorganisms from different microbial groups, which possess complementary modes of action while considering factors such as the desired form, concentration, critical features enabling large-scale production and survival in the target environment, and the potential need for additional components to optimize the effectiveness of these formulations.A relatively new and effective approach to the biocontrol of phytopathogens as general and the protection of biodiversity is the construction of at least two-component biopreparations which include at least two antagonistic microorganisms which often belong to separate groups of microbes and reveal complementary modes of action, enhancing the effect of the biopreparation. Literature data show that more and more attempts are being made to use preparations including filamentous fungi, yeasts, or cyanobacteria in combination with bacterial cells. The mechanism of action of these kinds of biopreparations is based on the formation of a biofilm by bacteria, it includes all of the antagonistic microorganisms, and this positively affects their ability to develop in different environments. According to the relevant data, the use of these pro-ecological products has an added positive effect on plant growth and their resistance to phytopathogens (Aloui et al., 2015; Silva et al., 2022). However, based on our current knowledge, this particular aspect has not been specifically examined or implemented in preparations targeting *Neofabraea* spp. Nevertheless, considering the existing evidence that microorganisms from diverse groups can individually exhibit biocontrol activity against various *Neofabraea* isolates, it appears justifiable to explore the development of biopreparations comprising a combination of different microorganism groups. This approach has the potential to enhance the effectiveness of biopreparations and warrants further investigation.

The ingredients of the biopreparations are carefully selected strains that are not only chosen for their antagonistic properties concerning the target pathogen but are also formulated to include many critical features to advance the possibility of production on a technical scale, as well as the effectiveness of survival and functioning in the target environment (resistance to biotic and abiotic stresses). A summary of the essential elements involved in screening microorganisms intended for commercial use in the biological control of fungal and bacterial phytopathogens was prepared quite recently. This list includes, among others, the ability to produce biomass on an inexpensive and relatively simple microbiological medium, tolerance of temperature, drought, salinity, UV radiation, and chemical sensitivity, which may be a determinant for research approaches and trends to develop commercial biopreparations (Köhl et al., 2011, 2019).

In-depth consideration of microorganism selection involves adjusting their properties to ensure effective plant protection during growth (pre-harvest treatment) and fruit preservation during post-harvest storage (post-harvest treatment) (Fenta et al., 2023). Specifically, in the context of *Neofabraea* spp. affecting apple production, it is crucial to account for specific characteristics enabling the selected microorganisms to fulfil their intended roles at these critical stages of cultivation and storage. Similarly, careful attention should be given to the product form and its supplementations. For instance, biopreparations are typically formulated as either powders or concentrated suspensions that can be dissolved or diluted in water. The recommended content of active substances in these biopreparations ranges from 10^5 to 10^9 conidial spores of fungi or bacterial cells (or spores) and yeasts in 1 g or 1 ml of the preparation. For optimal results, a recommended application rate of 0.5–1.5 kg (or 0.5–1.5 l) or higher per hectare of crops is advised (Hegde et al., 2023). To enhance the efficacy of biopreparations, the formulations of microorganisms are augmented with supplementary components. These commonly include chemical substances such as inorganic salts (e.g., calcium chloride, sodium bicarbonate), organic compounds (e.g., salicylic acid), antioxidants, and polysaccharides (e.g. chitosan) (Janisiewicz and Korsten, 2002; Oszust et al., 2020; Pylak et al., 2020).

2.3. Microbiome-based approach to test biocontrol effects - the holistic view of the apple production system

Host-associated microbiota as open and interconnected ecosystems are capable of favourably influencing plant health and are also important in one health-unifying and integrated approach to balance and optimize the health of people, animals, and the environment (Berg, 2015; Berg et al., 2017; Flandroy et al., 2018).

Although food-borne pathogens and diseases are well recognized (, the microbial diversity associated with fruit in the context of microbiome-based solutions development with a particular focus on a holistic view of the production system and food safety requires more attention and study. Recent research has allowed us to determine the microbiome (including mycobiome) of organic and conventional apples. The results have indicated that a whole apple contains about 100 million bacterial gene copy numbers, however, freshly harvested organic apples were characterized by a significantly more diverse and distinct microbiota in comparison with conventional ones (Wassermann et al., 2019; WHO, 2015;).

The apple fruit microbiome can be modified using various processing technologies (Wicaksono et al., 2022). Moreover, the results concerning the apple microbiome and resistome have revealed antimicrobial resistance in these fruits, this finding can be connected to the excessive usage of chemicals in agriculture (Wassermann et al., 2022). Therefore, apart from the development of global distribution monitoring, biological products such as biopreparations and detection methods for various pathogens including *Neofabraea* spp. are very relevant to the future of plant protection. A recent study has demonstrated the crucial role of plant microbiomes in plant health, productivity, and fitness, and also that the microbiome of apple fruit is driven by the prevailing climate and can adapt to local environmental conditions (Abdelfattah et al., 2021). Microbiome-based solutions should include this aspect of plant protection with special attention being focused on possible plant stressors, the local weather conditions, or the plant varieties being cultivated in association with and concerning the interactions between beneficial and pathogenic microbes (Abdelfattah et al., 2021).

3. Methods of *Neofabraea* spp. detection using genetic markers

The sustainable production of plant raw materials, including the consideration of minimizing the possible economic losses for apple producers and others trading in this area, is associated with the possibility of the early detection of *Neofabraea* spp. and the monitoring of their occurrence. The presence of pathogens is recognized above all when symptoms appear on the plant, this is often based on the type of symptoms which are manifested (Mora-Romero et al., 2022). This observation regarding the common identification of pathogen presence based on symptom manifestation also applies to *Neofabraea* spp. in apple production, although it is often based on practical knowledge rather than solely relying on scientific reports.

Nevertheless, the accurate identification of *Neofabraea* spp. is also possible with the use of a culturing method that identifies the morphological features of the culture, such as the rate and type of growth and macroscopic observations including the colour of the culture along with other morphological features, such as the dimensions of conidia or the length of the sprout hypha (Bühlmann et al., 2021). Most of these features are quantitative and may overlap, therefore *Neofabraea* spp. identification must be performed in pure culture (Vukotić et al., 2022). As a result, classical methods alone are not sufficient for phytosanitary diagnostics which requires both speed and reliability (Gautam and Avasthi, 2019).

Another approach uses the culture method associated with an analysis of the DNA material of the pathogen (with the use of genetic markers). This type of diagnostic protocol recommends the isolation of a pure culture of the pathogen from the host plants, this is followed by species-specific PCR tests. When mycelium is present on the test material, it is also possible to perform a direct test based on the polymerase chain reaction (PCR) (Khakimov et al., 2022). *Neofabraea* spp. identification using plate culture methods, as well as PCR methods based on pure cultures, are time-consuming (breeding allows for the detection of differences between species, but it takes up to two weeks) (Ijaz et al., 2022). After that, the apple fruit often lose their quality to the point that they are no longer suitable for trade/consumption/processing (Bratu et al., 2021). This diagnosis may be carried out for quarantine purposes and/or to select the most appropriate treatments to protect the fruit before the development of pathogens and disease, even in advance of the next growing season (Enicks et al., 2020).

The literature describes many diagnostic protocols based on pathogen DNA analysis (some have been validated) in a polymerase chain reaction (PCR), quantitative PCR (qPCR), as well as utilizing a loop-mediated isothermal amplification (LAMP) for the diagnosis and species differentiation of many phytopathogenic fungi, including *Neofabraea* spp. (Hariharan and Prasannath, 2021; Harmon et al., 2022). Universal genetic markers for the identification of fungi are usually only used singly. These are standard and also in common use for other fungi conserved fragments ITS1 or ITS2 or D2 LSU, but also for functional genes like the β-tubulin (Fan et al., 2014) or the gene coding cytochrome b (cytb) (Hily et al., 2011).

Lately, there have been statements made by phytopathologists and mycologists suggesting that planning detection based only on single genetic markers, is not sufficient (Dupuis et al., 2012). This approach may lead to false-positive or negative results also when detecting *Neofabraea* spp. Hence, there is a growing trend of utilizing detection panels encompassing multiple genetic markers to identify fungal phytopathogens, and this approach has been recently employed for the detection of *Neofabraea* spp. as well. (Michalecka et al., 2016; Wu et al., 2022).

The present trends in the agricultural and horticultural product market are focused on enhancing both the quality and productivity of production. Consequently, efforts are being made to develop methods for early detection of plant pathogens, providing valuable insights into infection and potential disease development. However, it should be noted that current technologies for early pathogen detection are primarily utilized in the context of cereal pathogens, as highlighted by Khakimov et al. (2022), rather than specifically targeting horticultural pathogens like *Neofabraea* spp. affecting apple fruits.

At present, there are no commercial methods for the detection and monitoring of these pathogens on the market. Yuan and Verkley identified *Neofabraea* spp. (*Pezicula* spp.) isolates using a detection panel based on ITS, EF1-a, RPB2 gens, and/or the β-tubulin gene (Chen et al., 2016; Yuan and Verkley, 2015). Analyses included pure fungal cultures, which are endophytes and include fir, apple, and maple. Michalecka et al. (2016) and Cao et al. (2013) successfully identified pure isolates and infections in diseased apples using a sequence of the gene fragment of β-tubulin and others in a PCR multiplex reaction. Using this PCR multiplex protocol on apple fruit without any symptoms of the BER disease has not resulted in satisfactory detection to date (Michalecka et al., 2016).

The detection of *Neofabraea* spp. is facilitated by a comprehensive factsheet that summarizes the latest methods and protocols, as presented in Table 1. This compilation includes essential information regarding tested isolates originating from different *Neofabraea* species, with careful consideration given to the microbial source collection. Furthermore, the table provides comprehensive details on the gene markers employed, encompassing primer names, orientations, and sequences. Notably, the amplicon length, genomic DNA isolation method, amplification technique, and corresponding thermal profile associated with each genetic marker are meticulously outlined. Researchers and practitioners seeking accurate and up-to-date techniques for *Neofabraea* spp. detection will find this compilation to be a valuable resource, complete with references.

4. Expectations and future directions

4.1. *Counteracting the negative effects of Neofabraea presence in terms of the needs of apple producers*

The development of a microbiological preparation with properties that support apple protection against the development of fungal pathogens *Neofabraea* spp. may be regarded as a response to the following needs of apple producers: reducing the dependence of plant production on chemical plant protection products, and thus maintaining healthy ecosystems, increasing plant resistance and conducting sustainable

Table 1
Methods of *Neofabraea* spp. detection using genetic markers.

Species	Gene marker	Primer name	Primer orientation	Primer sequence (5'-3')	Amplicon length	Isolates, culture collection	Genomic DNA isolation method	Amplification method	Thermal profile	Literature
N. malicorticis, N. perennans, N. kienholzii, and N. vagabunda	Inter-species sequence variations in the β-tubulin gene	RCAf RCAr	F R	GACGACCGCATCACCAACATC TGAATCCCTGACACCAACACG	557–625 bp	CBS, VPRI, SHCIQ, BJCIQ, GDCIQ,	MagPure Fungal DNA TL kit	Rolling-circle amplification (RCA) coupled with padlock probes (PLP) provides an ideal detection	94°C 1 min, 35 cycles x 94°C 30 s, 55°C 30 s, 72°C 1 min	Lin et al., 2018
N. malicorticis, N. perennans, N. alba	Inter-species sequence variations in the β-tubulin gene	N/P	F	CTT TCT CCG TTG TCC CAT CC	554 bp	ATTC	Promega SP fungal DNA kit	multiplex qPCR	94°C 2 min; 98°C 10 s, 57°C 30 s, 30 cycles x 72°C 30 s, 72°C 5 min	Cao et al., 2013
		N/P	R	GAACATTGCGCATCTGGTCC						
N. alba, N. kienholzii	Internal transcribed spacer	UP18S42 LO28S22	F R	CGTAACAAGGTTTCCGTAGGTGAAC GTTTCTTTTCCTCCGCTTATTGATATG	~600 bp	Pure cultures from affected apples	Qiagen/ following the protocol of Cassago et al. (2002)	PCR amplification, Sanger sequencing	Levesque and De Cock, 2004	Amaral Carneiro et al., 2022
	16S mitochondrial ribosomal RNA gene	mrSSU1 mrSSU3R	F R	AGCAGTGAGGAATATTGGTC ATGTGGCACGTCTATAGCCC	~880 bp				Pešicová et al., 2017	
	β-tubulin gene	Bt-T2m-UP Bt-LEV-LO1	F R	CAACTGGGCTAAGGGTCATT GTGAACTCCATCTCGTCCATA	~1,000 bp				Lévesque et al., 2001	
	Translation elongation factor 1a	EF1-983F EF1-2218R	F R	GCYCCYGGHCAYCGTGAYTTYAT ATGACACCRACRGCRACRGTYTG	1,05 bp				Rehner and Buckley, 2005	
N. alba, N. perennans, N. kienholzii and N. malicorticis	β-tubulin gene	Neo_malloTub-262	R	GACAGCCAACTTGCGG	499 bp for *N. alba;* 400 bp for *N. perennans*; 336 bp for *N. kienholzii*; and 270 bp for *N. malicorticis*	CBS, pure cultures from affected apples	AxyPrep Multisource Genomic DNA Miniprep kit (Axygen) with modification	multiplex PCR	94°C 3 min, 30 cycles 94°C 30 s, 56°C 60 s, 72°C 60 s, 72°C 5 min	Michalecka et al., 2016
		Neo_perloTub-382	R	GGGTCGAACATCTGTTGT						
		Neo_spnovloTub-319	R	TGGTGAGAGGAGCGAAC						
		Neo_alba3	R	AATATTAGCAGGATATCTCTTCAAG						
		Neofab_uni	F	AACTTTCTCCGTTGTCCCATC						
		UN-UP18S-42	F	GGTAACAAGGTTTCCGTAGGTGAAC						
		UN-LO28S576B	R	CTCCTTGGTCCGTGTTTCAAGACG						
N. perennans	β-tubulin gene	FIP F3 LF L Set 22 BIP B3 LB		TGAGAGGAGCGAATCCAACCATGATTTTCTTCGCAAGTTGGCTGTCAACAT CCAGGTCAACTCAACTCCG GAAATGAAGACGAGGGAAAGGAACC GTGCTCACTCTTTCCGTGCTGTCTTTTTGTTCTTTGGGTCGAACATCTGTT GAAGTCAGAAGCAGCCATCA ACCGTCCCAGAGTTGACAC	300 bp	TFREC	FastDNA kit	LAMP PCR	61, 63, and 65°C for 30, 45, and 60 min and 5 min at 4°C	Enicks et al., 2020

production in orchards. The aim should be to meet these needs by developing products in the form of natural microorganism-based protective preparations suitable for use in apple cultivation, their introduction to the market will be an effective, targeted, and ecological method of protecting these crops against the development of the fungal pathogen *Neofabraea* spp. Whichever products are developed they must be tested and checked in the context of the specificity of the features of the fungal pathogen.

An early detection method for *Neofabraea* spp. in apples for the assessment of the risk of the occurrence of BER is a response to the needs of the apple production sector, it is an analytical tool (research procedure) for the certification of apple fruit, which can be used on a small batch of fruit to determine if the harvested fruit was already infected with a fungal pathogen during the growing season. Thus, the use of this tool should allow for a prediction to be made as to whether a given batch of apples has the probability of developing a storage disease, i.e. whether it may be characterized by a shortened predicted period of maintaining good consumer quality.

The development of detection method technology is also a response to the need for the presence in the production space of tools supporting the rational management of apples as a trading commodity, thereby reducing economic losses. Currently, there are no commercial technological solutions on the market that would allow for the detection of and consequently, the monitoring of *Neofabraea* spp. in apples, when there are no symptoms of the disease. The presence of fungal pathogens is only recognized when symptoms of the disease appear. By then the fruit is largely unsuitable for trade, processing, or consumption, which exposes both producers and processors to economic losses. The developed method will contribute to changing this state of affairs by obtaining reliable information concerning the presence/ absence of genetic material of *Neofabraea* spp. in the fruit (infection with spores of *Neofabraea* spp. virulent strains), even with the absence of disease symptoms obtained as a result of the examination. The knowledge obtained with the use of the developed detection methods will allow for more rational management of the product – designating the infected batch of apples for immediate processing, consumption, or taking intervention steps regarding the storage method. A summary of the identification of the market requirement for biopreparations against *Neofabraea* spp. and an early detection method is shown in Fig. 2.

4.2. Targeted biocontrol strategy against Neofabraea spp. in apples - microorganisms and prebiotic supplements selection

Along with the development of ecological horticulture, more and more commercial products based on antagonistic microorganisms (biofungicides/biofungistats) of many fungal phytopathogens have appeared on the market. Unfortunately, their effectiveness is not sufficient. Research involving the use of ecological biopreparations both in the form of spraying trees before harvesting and dipping harvested apples in suspensions of this preparation showed that these treatments did not prevent apple BER (Bryk and Rutkowski, 2012). This kind of situation may be caused by the characteristics of the individual antagonists used in the biopreparation and the pathogens against which the antagonists show a high degree of activity. There are no "ideal antagonists" capable of exerting biocontrol over all of the representatives of the pathogens of a specific species (Thambugala et al., 2020).

The literature on the subject mentions the occurrence of the phenomenon of intraspecific diversity of fungi related to their regional or geographical origin (Allen et al., 2020). This refers to the concept of distinguishing between what is commonly known as breeds of fungi in the population. According to the authors, this concept is based on the genetic variability of the species according to Caten and concerns the division of fungi in terms of them belonging to biological races representing morphologically and physiologically different and often geographically separated groups of isolates or races that are physiologically different in terms of virulence even to many varieties of one type of plant (Allen et al., 2020).

While searching for *Neofabraea* spp. antagonists for biopreparations this aspect should be considered. Many of the biopreparations available on the market are foreign products and are therefore manufactured using antagonistic strains of microorganisms isolated from distant environments (in terms of geography, host plants, or the broadly understood habitat – surrounding one species of the host plant) and tested against pathogen isolates from distant regions of the world. This adverse trend is most unfavourably reflected in the reported relatively low effectiveness of biopreparations against BER (Bryk and Rutkowski,

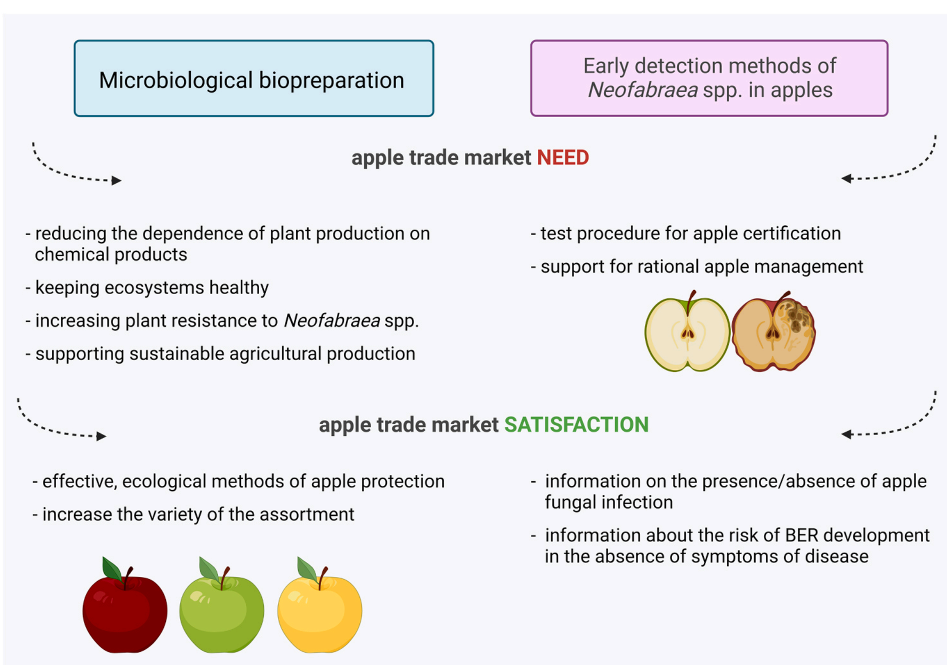

Fig. 2. A summary of the need in the apple market for biotechnological solutions against *Neofabraea*.

2012).

Therefore, there is a need to consider not only the aforementioned diversity of strain *Neofabraea* spp. characteristics (individual diversity) of antagonists but also that of their target pathogens while developing biopreparations to biocontrol these pathogens. Biopreparations should be characterized not only in terms of their selectivity (which is greater in relative terms as compared to chemical substances) but it is suggested that they should be highly specific (targeted), i.e. with high-efficiency microorganisms included in the consortium, and directed primarily against the strains currently occurring in the targeted area. In particular, different regions may have separate races within the species *Neofabraea,* resulting from the division of the main countries (regions), where apple trees are grown, which may additionally be related to the variety of apple trees grown, as it was demonstrated for other pathogens (Ali et al., 2017). According to the literature data *Neofabraea* spp. primarily attacks such apple tree varieties as 'Gala', 'Golden Delicious', 'Ligol', 'Pinova', 'Topaz', 'Rajka', 'Rubinola', 'Melfree', and 'Enterprise' (Francesco et al., 2019).

The multiplicity of dependencies in the environment means that not all microorganisms introduced with biopreparations effectively acclimatize to the target conditions or they lose the competition for an ecological niche to indigenous microorganisms, especially pathogens (Oszust et al., 2021). In future solutions targeting *Neofabraea* spp., which address this issue may include the careful selection of microorganisms, such as certain strains of *Trichoderma*, *Bacillus*, or yeast species, that can be emphasized (with features that determine their expected activity), and the enhancement of their survival through supplementation with prebiotic ingredients (prebiotic supplements). It is known that the presence of nitrogen and carbon in the environment is of great importance for the multiplication of microorganisms, and also the formation of spores' biomass of bacterial cells, so this affects competitiveness in an ecological niche (Band et al., 2022). Appropriately selected microorganisms and selected supplements (those positively affecting the functioning of antagonists – primarily in the context of biomass increase) are of great importance in the achievement of the final efficiency and activity of biopreparations to biocontrol *Neofabraea* spp. These supplements can provide nourishment and support for the chosen microorganisms, thereby enhancing their viability, colonization, and overall effectiveness against *Neofabraea* spp.

It is beneficial to obtain isolates of microorganisms with the desired antagonistic properties from healthy habitats, the best choice being natural or long-undeveloped ones, where the use of chemical plant protection products (e.g. on old or wild apple trees) is discounted. The strains obtained from these environments show activity supporting the growth and functioning of plants or antagonistic properties (Fikri et al., 2018). The European Commission has published an overview that is related to the prospects of the apple market in 2018–2030 (EC, 2018). It mainly concerns the restructuring of orchards, which includes the grubbing-up of old orchards in combination with an increase in the productivity of young plantations. Therefore, in the near future, there will be a decrease in habitats with a high degree of richness of species and functions, and thus places, from which the most effective isolates of useful/antagonistic microorganisms may be obtained.

4.3. *Early detection method of Neofabraea spp. in apples - to evaluate the risk of the apple BER occurrence*

Currently, there are no methods available on the market that would have made it possible to judge if the harvested apples were infected with the pathogen *Neofabraea* spp. during the growing season (early detection), and at the same time to what extent the presence of spores in apples is related to the possibility of developing a later storage disease, and hence lead to an expected reduced time before consumption. This knowledge would give both apple producers, and intermediaries in the trade with short-term custody over these raw materials, the possibility of reducing eventual economic losses by allocating this part of the apple crop for immediate processing and consumption, or by taking extraordinary steps regarding the method of storage.

Determining the level of risk of developing a BER disease is primarily of prognostic value. However, according to the literature in many cases, it is of value in terms of revaluation (Wawrzyniak, 2021). Therefore, there is a need to verify the level of determination that is essential for the effective threat of *Neofabraea* spp. assessment. Heavy rainfall in the growing season provides favourable conditions for the sowing of spores, and ideal storage conditions for the dynamics of their development and the occurrence of disease symptoms. However, the intensive growing of spores, and even noting spores in an apple are not always related to disease occurrence at a later post-storage stage (Wenneker et al., 2020).

In many cases, the ability to infect plants depends on the presence and expression of specific genes, which distinguish virulent fungi from their closely related non-virulent "relatives". This natural phenomenon may be a species feature, but the differences may also apply to individual strains (Gabaldón et al., 2016). These genes code for host-determining virulence factors, including the proteins and enzymes involved in the synthesis of factors responsible for the occurrence of infection (Van der Does and Rep, 2007). The identification of genes in *Neofabraea* spp. necessary for infection and thus for the induction of disease in apple fruit, is the basis for identifying the mechanisms of infection and BER development, and thus for the development of this disease control strategies. Improved technologies for gene identification and functional analysis, as well as an excess of sequenced fungal genomes, and transcriptome analysis have led to the characterization of genes known as virulence or pathogenicity genes in many species of fungi (Van de Wouw and Howlett, 2011).

A comparison between the results of the analyses concerning the presence and level of infection (detection) of *Neofabraea* spp. with the presence of virulence markers (virulence) will allow for the verification of the level of effective risk of BER development.

Previously developed universal markers, e.g. ITS, EF1-a, RPB2, and β-tubulins were successfully used to identify *Neofabraea* spp. (Michalecka et al., 2016). However, in the context of the detection and determination of the level of infection in apples, the method requires work in the area of improving the method of sampling and DNA extraction, and at the stages leading up to the optimization of the conditions for marker amplification (PCR reaction). The differences between how pure cultures of the pathogen and the environmental material are managed must be considered, with a particular focus on the apples with a relatively small amount of pathogen spores in the tested material.

On the other hand, since there are no specific markers of virulence for *Neofabraea* spp., to assess the risk of disease development, the cardinal step in this range is the search for these markers. Recent trends in the relevant global literature have highlighted the use of information from high-throughput whole-genome sequencing (WGS) and transcriptomes (WTS) to an in-depth determination of the genetic diversity of the population of fungal pathogens, and their occurrence in a given area (Gent et al., 2020). The impact of this approach has been revolutionary e.g. for marker genes research (Pérez-Cobas et al., 2020). Hopefully, it will contribute to the development of modern biotechnological solutions in molecular diagnostics and in the biocontrol of *Neofabraea* spp. in apples which causes postharvest bull's eye rot.

5. Conclusions

There are financial losses related to the occurrence of apple storage disease caused by fungi of the genus *Neofabraea*. On the other hand, fruit growers are obliged to comply with detailed regulations in the area of the quality and safety of food and the environment, these regulations are very well suited to the use of preparations containing microorganisms. Therefore, investors in the sector are anticipating new solutions supporting the rational management of apples to ameliorate the threat posed by the development of *Neofabraea* spp. by paying attention to the need for the early detection of pathogens.

Considering the great significance of apple production in the worldwide market, the tendency to minimize the use of chemicals, and the limited availability of ecological biopreparations to counteract *Neofabraea* spp. there is a need for the development of innovative products against these pathogens. They should be products based on selected strains of microorganisms, targeted, and supplemented with prebiotics selected for these strains and intended to counteract the development of the *Neofabraea* genus.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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