Universidade de Aveiro
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários á obtenção do grau de Mestre em Microbiologia, realizada sobre a orientação científica da Doutora Joana Luísa Lourenço Estevinho Pereira, investigadora doutorada do Departamento de Biologia da Universidade de Aveiro e do Professor Doutor Artur Jorge da Costa Peixoto Alves, professor auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave

azoxistrobina, tebuconazole, Custodia 320 SC, fungicida, produtos fitofarmacêuticos, triazol, estrobilurina, eficácia, *Pyrenophora teres*, *Rhynchosporium secalis*

resumo

Informação específica sobre cada ingrediente ativo fungicida, e os seus efeitos combinados em todos os organismos potencialmente afetados, quer sejam patogénicos, organismos não-alvo ou colheitas é ainda limitada. Neste estudo, os efeitos potenciais da azoxystrobina, do tebuconazole e de uma formulação comercial com a combinação destes dois ingredientes ativos, Custodia 320 SC, foram testados em duas espécies fúngicas, *Pyrenophora teres* e *Rhynchosporium secalis*, ambos fungos patogénicos conhecidos mundialmente por grandes perdas em colheitas agrícolas importantes como o centeio e a cevada. Os resultados indicaram resistência significativa a ambos os ingredientes ativos por *P. teres*, com as culturas tratadas com tebuconazole a demonstrarem as mais baixas taxas de inibição do crescimento relativamente ao respetivo controlo. *R. secalis* mostrou também taxas baixas e pouco variáveis de inibição de crescimento em resposta à exposição a cada um dos fungicidas, nunca chegando aos 50% de inibição em relação ao respetivo controlo. Estes resultados foram obtidos para gamas de concentrações de exposição dentro dos limites de solubilidade em água dos compostos e que incluíram concentrações que refletem as taxas de aplicação utilizadas para tratamento de culturas. Não obstante, a contaminação bacteriana em ensaios iniciais permitiu observar que, nestas condições, a eficácia dos fungicidas é superior (inibição de crescimento dos fungos alvo de mais de 50%) até um determinado nível de exposição, a partir do qual a eficácia se torna de novo muito limitada; este efeito será explicável pela redução da capacidade de resistência dos fungos aos fungicidas em cenários competitivos. A eficácia da combinação dos ingredientes ativos foi inferior à dos tratamentos equivalentes em que os mesmos foram aplicados individualmente, sugerindo a possibilidade de ocorrência de interações antagonísticas entre a azoxystrobina e o tebuconazole. Adicionalmente, a comparação dos efeitos de combinações de ingredientes ativos com as combinações equivalentes aplicadas através da formulação comercial demonstra que os restantes formulantes não promovem a eficácia dos ingredientes ativos quando as espécies alvo são *P. teres* ou *R. secalis*.

keywords

azoxystrobin, tebuconazole, Custodia 320 SC, fungicide, plant protection products, triazole, strobilurin, efficacy, *Pyrenophora teres*, *Rhynchosporium secalis*.

abstract

Specific information towards each fungicidal active ingredient and its effects on all local organisms, whether they're pathogens, non-target organisms or crops is still largely lacking. In this study, the potential effects of azoxystrobin, tebuconazole and a commercial formulation with these two active ingredients combined, known as Custodia 320 SC, were tested on two fungal species, *Pyrenophora teres* and *Rhynchosporium secalis*, both well known worldwide pathogens responsible for worldwide losses in important crops such as barley and rye. The results indicated significant resistance to both active ingredients by *P. teres*, with tebuconazole-treated cultures showing the lowest inhibition in the growth response. *R. secalis* showed also low and poorly variable growth inhibition rates for both fungicides, never reaching to 50% inhibition in comparison to the respective control. These results were obtained considering exposure concentration ranges within the limits of water solubility for both compounds and including concentrations that correspond to typical application rates used in the field to treat affected crops. Nevertheless, bacterial contamination in early trials allowed to observe that under these conditions the efficacy of the fungicides is superior (fungal growth inhibition rate over 50%) until a certain level of exposure, from which onwards the efficacy is again limited; this effect can be explained by a reduction of the fungi resistance capacity in competitive scenarios. The efficacy of the combined active ingredients was lower than equivalent treatments in trials where they were applied singly, suggesting the possibility of antagonistic interactions between azoxystrobin and tebuconazole. Additionally, the comparison of effects promoted by the combination of the active substances with their equivalent combinations applied via commercial formulation demonstrated that the formulants other than the active substances used in the commercial formulation do not promote their efficacy when the target organisms are *P. teres* or *R. secalis.*

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1. Introduction

Many important worldwide crops, such as soybean, wheat or rice are under permanent threat by multiple biological enemies (Castell-Miller & Samac, 2019; Jevtić et al., 2019; Simões, 2005; Zuntini et al., 2019). Fungal pathogens are often responsible for significant limitations in crop yield or even complete agricultural losses (Bălău et al., 2015; Castell-Miller & Samac, 2019; Hartman et al., 2015). Additionally, these fungal pathogens may also contaminate agricultural products, grain or plantable seeds with mycotoxins, which in turn can result in decreased harvest quality, difficulties with seed growth and food poisoning cases (Bălău et al., 2015; Jevtić et al., 2019; Nugmanov et al., 2018; Paul et al., 2018; Udomkun et al., 2017). Producing foodstuffs suitable for human consumption, free of any phytosanitary problems and/or health risks is a fundamental task in agriculture and other similar industries such as packaging, storage and distribution industries, among others (Simões, 2005).

According to Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 (EC) No 1107/2009, 2009), phytopharmaceutical products or plant protection products (PPP's) are legally described as "All products, in the form in which they are supplied to the user, consisting of or containing active substances, safeners or synergists, and intended for one of the following uses:

a) Protecting plants or plant products against all harmful organisms or preventing the action of such organisms, unless the main purpose of these products is considered to be for reasons of hygiene rather for the protection of plants or plant products;
b) Influencing the life processes of plants, such as substances influencing their growth, other than as a nutrient;
c) Preserving plant products, in so far as such substances or products are not subject to special Community provisions on preservatives;
d) Destroying undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants;
e) Checking or preventing undesired growth of plants, except algae unless the products are applied on soil or water to protect plants."

In a more practical definition, PPP's are considered as all products used to protect plants and agricultural products, except for fertilizers and correctives. PPPs may be composed of one or more active substances responsible for preventing or controlling pathogens or noxious

organisms, and different formulants may be added to these active substances in commercial products. They may have numerous designations, depending on the pathogens/noxious organisms they act upon, e.g., herbicides (to control weeds), insecticides (to control insect pests) and fungicides (to control fungal pathogens).

Fungicides are only one of many different types of PPP's but one of the most used at least in Portugal, according to the last report by the competent national authorities (DGAV, 2016b) (Table 1; Figure 1). As the name implies, they fight potential fungal pathogen threats towards crops (Simões, 2005). Among fungicides, sulphur is still sold in much higher quantities in comparison to other fungicides such as benzimidazols, imidazoles and triazols, which correspond to some of the most well-known PPP active substances (Table 2). Nevertheless, much like in any country with important agricultural production, including Poland, France and Germany within the EU, many pollution problems have arisen due to the ever increasing use of fungicides, as it also happened with other PPP's (European Commission, 2018; Simões, 2005; Poulsen et al., 2015; Silva et al., 2019; Wu et al., 2018).

Table 1: DGAV report information regarding the sale of fungicides in Portugal by chemical group (DGAV, 2016b).

Function	Amount sold (Kg)
Fungicides	5 194 734
Herbicides	2 122 470
All other PPP's including soil sterilants, molluscicides, growth regulators, rodenticides and vegetable oils	1 955 698
Insecticides and acaricides including mineral oils	733 505
Total	**10 006 407**

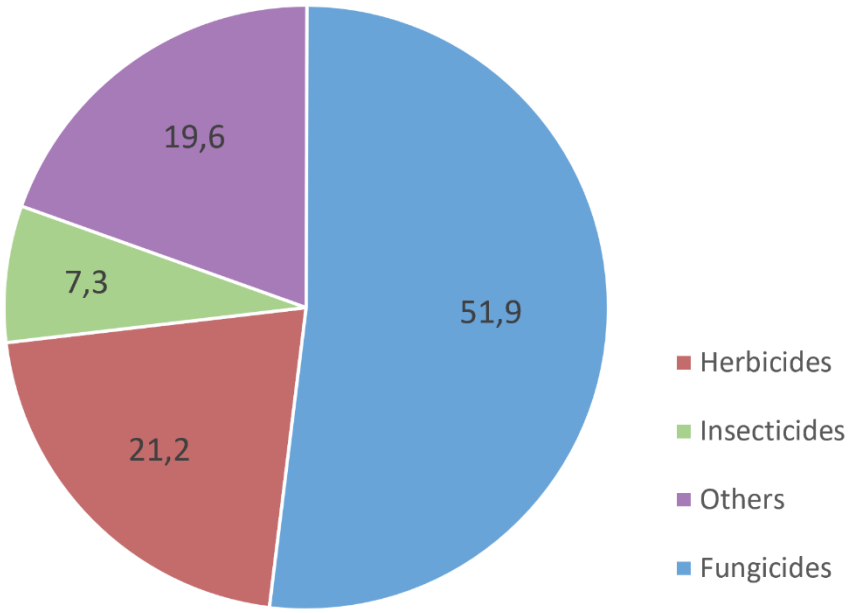

Figure 1: DGAV report information regarding the percent sale of PPP's per function in 2015. Image adapted from original source (DGAV, 2016b).

Table 2: DGAV report information regarding the sale of PPP's in 2015 (DGAV, 2016b).

Chemical group	**Amount sold (Kg)**
Benzimidazols, imidazoles and triazols	55 768
Carbamates and dithiocarbamates	975 071
Inorganic compounds	3 167 194
Copper compounds	621 962
Sulphur	2 545 232
Other fungicides including morpholines and fungicides of biological origins	996 701
Total	**5 194 734**

However, ceasing all or significant use of PPP's would be very detrimental to agricultural production, with observable production losses and price increase as seen on figures 2, 3 and 4, if no viable alternatives are presented for crop protection (DGAV, 2016b).

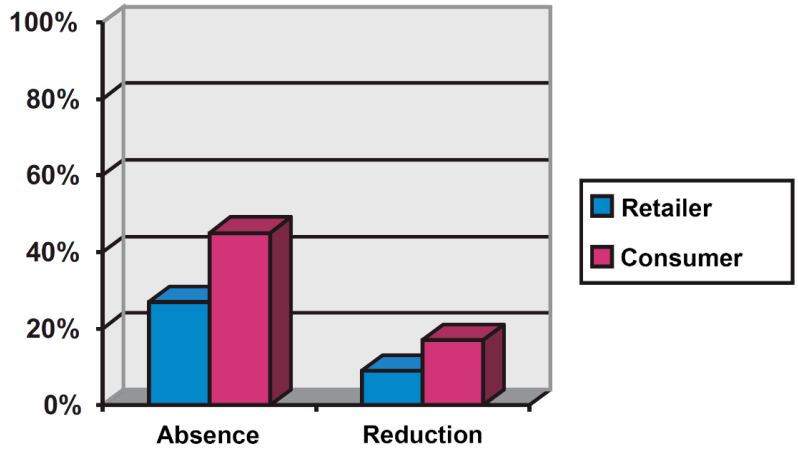

Figure 2: Theoretical evolution of foodstuff prices, in both retailer and consumer under two simulated scenarios - absence of phytosanitary protection and PPP use reduction by 50%. Image adapted from (Simões, 2005).

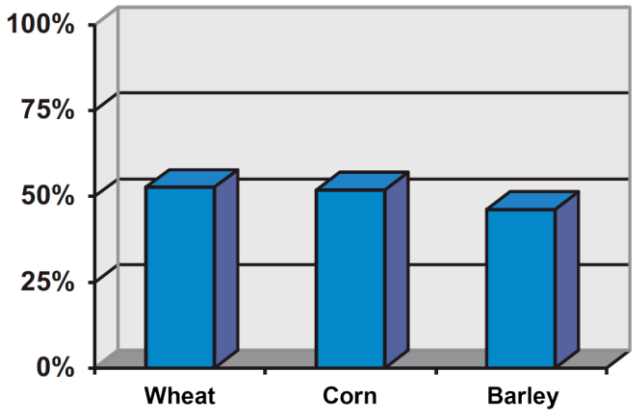

Figure 3: Europe: Production losses due to total absence of phytosanitary protection. Image adapted from (Simões, 2005).

Nevertheless, while complete PPP abandonment is not yet feasible, adequate alternatives have been proposed to mitigate crop yield losses, such as the development of natural PPP alternatives or the use environmental-friendly substances in PPP formulations. The development of adjuvants, improvement of PPP application techniques or target delivery, as well as the exploitation of synergic behaviors between substances to maintain the effectiveness of the active substances at lower concentrations are lines of research that have been explored in this context (Cantrell et al., 2012; Castro et al., 2014; Gerwick & Sparks, 2014; Queirós et al., 2018a). The understanding of the behavior of the chemicals used in formulations (including but not limited to the active substances), either considering the target weeds, pests and diseases or non-target species is indeed critical to better design environmentally sustainable and more economically viable formulations (Queirós et al. 2018a, Queirós et al. 2018b). This strategy towards more sustainable PPP formulations motivated the present dissertation. Other routes for mitigating the deleterious effects of PPP include the bioremediation of contaminated environmental matrices, for example, the use of microorganisms with unique metabolic pathways for bioremediation of waters contaminated with PPP's of difficult degradation (Fernández et al., 2017). However, such downstream mitigation strategies are outside the scope of the present dissertation.

Between 1960 and 1990, many new PPP's emerged and were developed, allowing for greater crop production and yield (Simões, 2005). The frequent use of PPP's has been demonstrated to pose significant hazards to both environmental and human health (Pimentel, 2005; Rossi et al., 2018). Intensification of agricultural practices, coupled with the need for greater crop yields have been leading to an increase in the use of PPP's. The transport and fate of PPP residues in the environment depends on the application strategy, climacteric conditions and on the intrinsic properties of the PPPs. Important features of PPPs constraining their environmental transport and fate are rainfall, solubility, topography, half-life, photolysis, pesticide formulation and application, and soil properties like pH, conductivity, K_{oc} (soil adsorption coefficient), K_{ow} (n-octanol/water partition coefficient), macronutrients availability, among others (Anderson et al., 2018; Arias-Estévez et al., 2008). These features influence phenomena such as soil accumulation, runoff, leaching, volatilization or ageing, all of which can increase toxicant contamination spread and decreased pesticide retention in agricultural grounds, as well as constrain expected degradation routes (Figure 4) (Anderson et al., 2018; Arias-Estévez et al., 2008; Siek & Paszko, 2019). The accumulation of these toxicants has been associated with

multi-contamination scenarios risking multiple ecosystems, including soil and aquatic ecosystems (Abrantes et al., 2010; Carriger & Rand, 2008; Rossi et al., 2018; Silva et al., 2015; Silva et al., 2019).

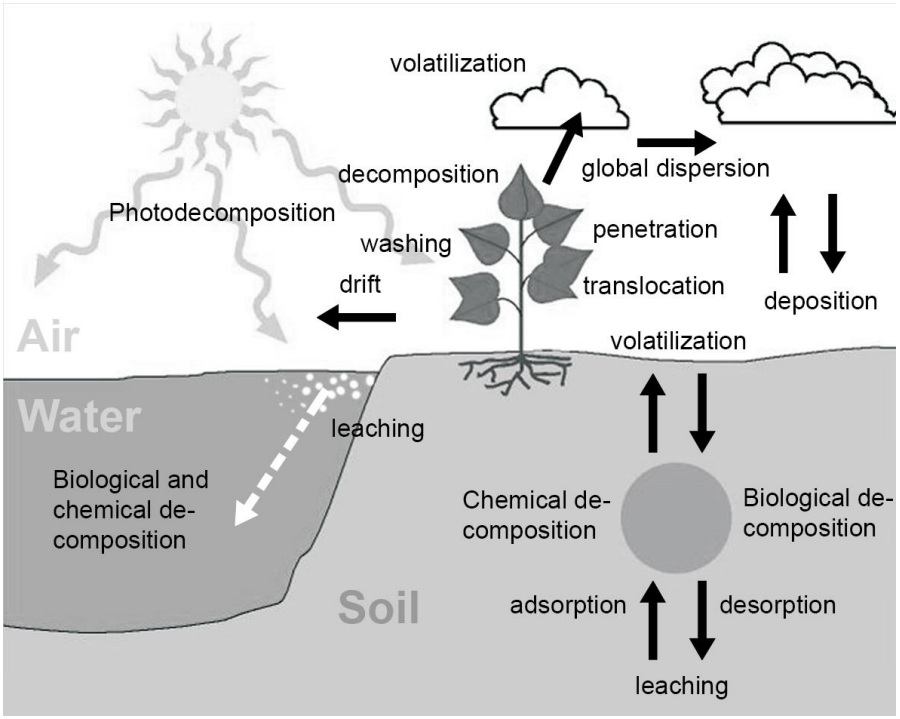

Figure 4: Standard fate of PPP's in the environment. Image adapted from Simões (2005).

Toxicity towards aquatic organisms by fungicides such as QoL (Quinone outside Inhibitors) strobilurins is more concerning than in mammals and birds – median Lethal Dose (LD_{50}) within the range 5000-2000 mg/L -, while many freshwater fish species show median Lethal Concentration (LC_{50}) values of 0.05-0.47 mg/L (in both cases, ranges given as examples are for azoxystrobin based in USEPA (1997)). Different aquatic species such as the green algae *Chlorella vulgaris*, embryo-stage vertebrate organisms like the amphibian *Xenopus tropicalis* and the fish *Danio rerio* have all reflected detrimental effects when exposed to varied strobilurin fungicides (Kumar et al., 2020; Liu et al., 2015; Wu et al., 2018). Nevertheless, despite the generalized view that fungicides are toxic when their residues accumulate in aquatic ecosystems, specific details towards the toxicity of each individual active substance are still lacking for different aquatic species (Kumar et al., 2020; Liu et al., 2015; Wu et al., 2018). As for toxicity regarding soil organisms, it was observed by Silva et al. (2019) and Vašíčková et

al. (2019) that many PPP's such as tebuconazole and boscalid are remarkably prevalent on European soils, and their contamination was shown to be toxic to soil organisms such as the earthworms *Eisenia fetida* and *Enchytraeus crypticus*, the springtail *Folsomia candida*, and the soil mite *Hypoaspis aculeifer*. Despite some of these PPP's have high LC_{50} for these organisms (reflecting generally low toxic ranges under acute exposure), bioaccumulation can potentially cause significant deleterious problems in many soil organisms (Vašíčková et al., 2019). Toxicity data regarding many specific soil organisms is scarce, much like for their aquatic counterparts as mentioned before. Still in regards to soil ecosystems, other concerning issues towards fungicide use include damage and loss of mycorrhiza fungi. Many mycorrhiza species establish symbiotic relations with many host plants, resulting in an increase of resilience towards diseases and benefits regarding nutrient fixation, which translates into a greater yield and greater quality of the said host crop plants (Sánchez-Cañizares et al., 2017). According to Channabasava et al. (2015), the administration of the fungicide active substances benomyl, bavistin and mancozeb on Proso millet plants (*Panicum miliaceum*) demonstrated diminished root colonization and spore number by *Rhizophagus fasciculatus*, an arbuscular mycorrhiza. In turn, the same plants also demonstrated a decrease in plant growth and grain yield, indicating that the use of these fungicides was actually detrimental to the crops, rather than being beneficial. Nevertheless, the same work also reported that other fungicides such as the phthalimide captan appear to leave *R. fasciculatus* unharmed, even demonstrating greater root colonization and spore number than untreated, control plants, meaning that great care must be taken with the fungicide selection for treatment, depending on the native mycorrhiza species.

The emergence of fungicide-resistant pathogens is a serious problem regarding fungal pathogen control. While different fungicide product formulations, combined with their preventive periodical usage, works on a short/medium term, the prevalence of fungicide-resistant strains can still occur, alongside resistant strain crossing, making fungicides increasingly obsolete with time (Hnátová et al., 2003; Hysing et al., 2012; Ma et al., 2018; Zuntini et al., 2019). Most of the PPP's developed today came from, or were based upon the secondary metabolites of many fungal species, particularly filamentous fungi. The ecosystems where they live in are teeming with other fungi, bacteria, algae, protozoans and metazoans, meaning that there is substantial competition and communication among these organisms and that secondary metabolites are developed for many purposes, among which are antifungal products (Brakhage & Schroeckh, 2011; Hoffmeister & Keller, 2007; Losada et al., 2009; Macheleidt et al., 2016). An example of

this antifungal activity can be seen on Figure 5. These already-existing antifungal metabolites and their respective coding genes reflects the existence of means to confer resistance towards them. The Major Facilitator Superfamily (MFS) are a type of efflux transporters capable of expelling active antifungal substances back into the extracellular medium. While effective, the complexity of different MFS transporters makes them only capable of transporting specific types of toxicants. For example, a correlation was found between the increased virulence of *Penicillium digitatum* and the expression of its PdMFS1 genes only in the presence of certain fungicides such as prochloraz (de Ramón-Carbonell et al., 2019). Other means of resistance include cytochrome c alteration or the alternative oxidase (AOX) pathway, preventing respectively electron-transport chain inhibition or providing alternative NADH oxidation pathways (Castell-Miller & Samac, 2019; Ma et al., 2018; Sierotzki et al., 2007).

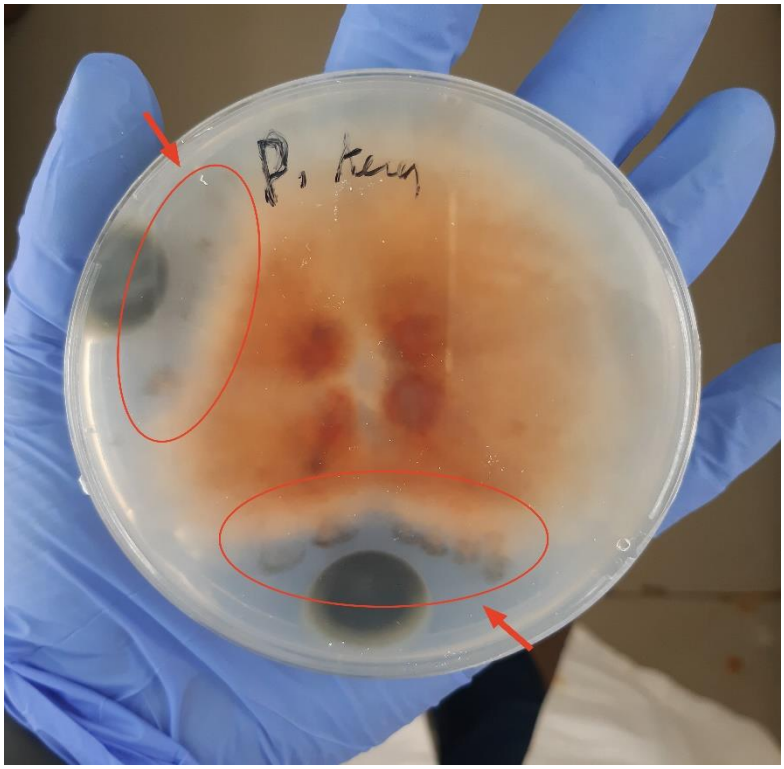

Figure 5: *Pyrenophora teres* culture contaminated by unidentified fungus. The regions denoted in red show a distinctive *P. teres* growth inhibition around the contaminant fungus, indicating the possible presence of an anti-fungal substance, likely produced via secondary metabolite pathways.

The fungicide focused in the scope of this work is Custodia 320 SC (Figure 6), produced by Adama®. The number 320 equals to the total concentration of its 2 active ingredients: 120 g/L of azoxystrobin plus 200 g/L of tebuconazole. SC indicates its formulation type, meaning that it is a Suspension Concentrate. It's considered a Noxious-Xn and Dangerous to the Environment-N substance by national regulatory authorities (DGAV, 2016a).

Azoxystrobin is a QoI strobilurin fungicide, existing only in the isomeric E- form. Much like other QoL fungicides, azoxystrobin mainly acts by binding to the electron transfer between the cytochrome b and c complex, which inhibits mitochondrial respiration and increases the presence of reactive oxygen species (ROS). Its overall effects in target species are related to systemic translaminar and protectant action with additional curative and eradicator properties. Its baseline chemical properties are enunciated on table 3. It is approved for use and marketing in all EU member states (Hnátová et al., 2003; Lewis et al., 2016; Sierotzki et al., 2007).

Table 3: General physic-chemical characterization of azoxystrobin.

Chemical characteristic	**Value**
Solubility in water (mg/L)	6.7
Solubility in octanol (mg/L)	86000
K_{ow}	316
K_{oc}	589
General biodegradability	-
Soil degradation/half-life	
DT_{50} typical (days)	78
DT_{50} field (days)	180.7
DT_{50} 20ºC lab (days)	84.5
DT_{50} aqueous photolysis at pH 7 (days)	8.7

Tebuconazole is a triazole with molecular chirality, belonging to the DMI-IBE Class I fungicides. It has systemic protective, curative and eradicant action, by inhibiting ergosterol biosynthesis via interference with the demethylation step needed to synthesize it in target species. The technical material is composed by both (S-) and (R-) isomers, even though the R- form is more biologically active than the S- form. Its baseline chemical properties are enunciated on table 4. Much like azoxystrobin, it is also approved for marketing and use in all EU member states (DGAV, 2016a; Simões, 2005; Lewis et al., 2016).

Table 4: General physic-chemical characterization of tebuconazole.

Chemical characteristic	**Value**
Solubility in water (mg/L)	36
Solubility in octanol (mg/L)	96000
K_{ow}	5010
K_{oc}	-
General biodegradability	-
Soil degradation/half-life	
DT_{50} typical (days)	63
DT_{50} field (days)	47.1
DT_{50} 20ºC lab (days)	365
DT_{50} aqueous photolysis at pH 7 (days)	stable

Despite the preventative and curative capacities of both active ingredients, Zuntini et al. (2019) reported phytotoxicity problems regarding formulations combining tebuconazole and azoxystrobin. The same work suggests tebuconazole as responsible for the observed phytotoxicity, pointing the fact that the active ingredient can accumulate on the leaves. Other problems arising from the use of tebuconazole include its' long half-life, depending on the soil

horizon where it's found; it can persist for 201-433 days in the topsoil, 734-1326 days in the upper subsoil and 945-3904 days in the lower subsoil (Siek & Paszko, 2019). Furthermore, tebuconazole may cause endocrine disruption on animal and human beings by inhibiting steroid hormone biosynthesis (Poulsen et al., 2015). The increase of reactive oxygen species (ROS) by azoxystrobin was studied before (e.g. Kumar et al., 2020; Liu et al., 2015; Wu et al., 2018) and has been shown to be detrimental to the physiology of different non-target aquatic organisms. *Xenopus tropicalis* and *Danio rerio* embryos demonstrated multiple deformations and deaths after exposure to different QoI strobilurins, including azoxystrobin. Furthermore, the same pattern of ROS increase was also shown to increase *Chlorella vulgaris* vulnerability towards oxidative damage from ROS, resulting in multiple cellular problems such as the inability to perform normal photosynthesis. In the case of soil organisms, as mentioned above, significant levels of tebuconazole, were detected in the earthworms *Eisenia fetida* and *Enchytraeus crypticus*, the springtail *Folsomia candida*, and the soil mite *Hypoaspis aculeifer* (Vašíčková et al., 2019).

In the present work, azoxystrobin and tebuconazole were tested against two fungal ascomycete species: *Pyrenophora teres* and *Rhynchosporium secalis*. Both of these species are plant pathogens, *Pyrenophora teres* causes net blotch of barley while *R. secalis* is the causal agent of rye scald. Both have a worldwide distribution and are responsible for massive losses in agricultural yield (Sierotzki et al., 2007; CABI, 2021a, b).

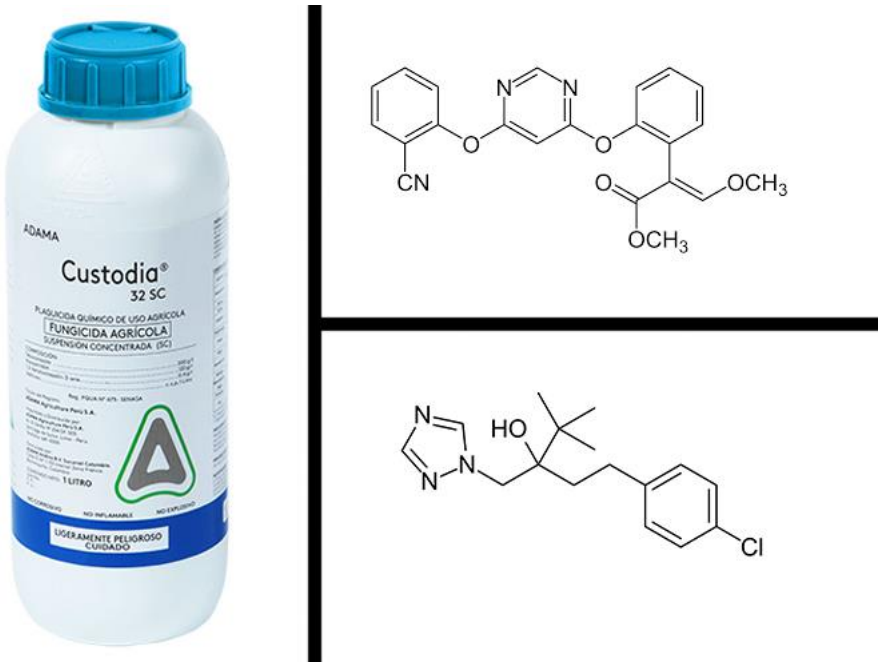

Figure 6: Custodia 320 SC container on the left-hand panel and it's respective active ingredients on the right-hand panels: upper image – Azoxystrobin, lower image – Tebuconazole. The molecular structures were designed using ChemDraw.

1.1 Objectives and structure of this Dissertation

The aim of this work was to characterize the efficacy of the commercial fungicide formulation Custodia 320 SC against two model fungal species, which are recognized targets of the product: *Pyrenophora teres* and *Rhynchosporium secalis.*

Several dimensions of this characterization were considered following the establishment of three specific objectives:

(i) To characterize the efficacy of azoxystrobin and tebuconazole against *P. teres* and *R. secalis*. The establishment of dose-response curves with each fungus species and each compound was pursued within their limits of solubility as a conservative approach - these limits

are also already above the concentrations of azoxystrobin and tebuconazole used in Custodia 320 SC.

(ii) To provide a preliminary view on the hypothesized interaction between azoxystrobin and tebuconazole used to improve the fungicidal capacity of the commercial formulation. For the purpose, the fungicidal activity of each active substance when tested singly was compared with that observed following exposure of the fungi to selected combinations of the two active substances.

(iii) To address the effects of the formulants in Custodia 320 SC other than the active substances in modulating the efficacy of the fungicide to the target model species *P. teres* and *R. secalis*. This aspect was approached by comparing the effect in the fungi of combination treatments of azoxystrobin and tebuconazole with the corresponding treatments (i.e., similar levels of the active substances) using the commercial formulation Custodia 320 SC.

This dissertation follows the classical structure of such a document, starting with the introduction section (closed by the present sub-section) that comprises the contextualization of the work and an appraisal of the state-of-the-art regarding the use of fungicides, their environmental hazardous potential, the mode-of-action of the focused active substances within Custodia 320 SC and the current knowledge on the mechanisms of toxic action towards non-target species. A Materials and Methods, as well as a Results and Discussion section are presented below, organized so that the different stages tackling the three specific objectives established can be clearly followed. A section collecting Conclusions and Final Considerations is then presented to summarize the main findings, provide a systematized view on the contribution of the work to the current knowledge and the most meaningful directions for future research. The due section providing the list of references cited throughout is provided at the end of the document.

2. Materials and Methods

2.1 Chemicals

The chemicals used in the fungal sensitivity trials were azoxystrobin (Sigma-Aldrich®, Pestanal®, CAS: 131860-33-8), tebuconazole (Sigma-Aldrich®, Pestanal®, CAS: 107534-96-3) and Custodia 320 SC® (Adama®, South Africa). The commercial formulation Custodia 320 SC® was also tested. This is a fungicide formulation with 120 g/L of azoxystrobin and 200 g/L of tebuconazole. Stock solutions were prepared immediately prior to testing in all cases, by dissolving each fungicide/commercial formulation in distilled water. Culture media were prepared as detailed below using Dehydrated Malt Extract Agar (ref. 610173, Liofilchem®, Italy).

2.2 Test organisms and their culturing

The organisms used during the test trials were the fungi *Pyrenophora teres* CBS 123929 and *Rhynchosporium secalis* CBS 110524, whose original cultures were acquired from the CBS culture collection of the Westerdijk Fungal Biodiversity Center (The Netherlands). The fungi were maintained in the laboratory in culture media containing 3 % (w/v) Malt Extract Agar (MEA), at approximately 21 °C ± 1 ºC.

The culture media was made by dissolving Dehydrated Malt Extract Agar into distilled H_2O on a Schott bottle, then sterilized by autoclaving for 15 minutes at 120ºC and 1 atm. Afterwards, the culture media was left to cool down until reaching 40-45ºC, and was then distributed in 100-mL Petri plates (approximately 20 mL *per* plate). The prepared plates were stored at 4 °C until use.

Fresh plates were inoculated by picking a disc of mycelium (approx. 5 mm diameter) from a plate containing an actively developing culture of the respective fungus (*P. teres* or *R. secalis*), under sterile conditions. The plates were sealed with Parafilm® tape. The procedure was performed in a flow chamber at room temperature (≈20ºC) and all the tools used were sterilized by using a flame and a 70 % v/v ethanol solution.

2.3 Fungicide efficacy assays

A total of three trials for each fungal species was made, hereinafter named as trial 1, trial 2 and trial 3. Trials 1 and 2 handled the active ingredients separately, while trial 3 focused mainly on mixtures and formulations with the inclusion of two concentration ranges for separated azoxystrobin 32 µg/L and tebuconazole 53 µg/L.

The culture medium to prepare the assay plates was made as described above, but by dissolving rather 4.695 g MEA into 150 mL of distilled H2O (≈3% w/v MEA) each individual Schott bottle (1 Schott bottle *per* concentration of fungicide(s) to be tested and controls). The fungicide(s) was/were added to each Schott bottle, by pipetting calculated volumes of the prepared stock solutions to the autoclaved medium, when it reached a temperature of 40-45ºC, with exception for the controls. The control/contaminated medium was immediately distributed in 100-mL Petri plates (approximately 20 mL *per* plate). The prepared assay plates were stored at 4 °C until the next day, to be inoculated with the two fungi species for starting the assays. Three replicates were used for each tested fungicide(s) concentration(s) and controls. The assay plates were inoculated as described in section 2.2. and were then incubated for a test period of 14 days at 21 ºC ± 1 ºC (Figures 7 and 8).

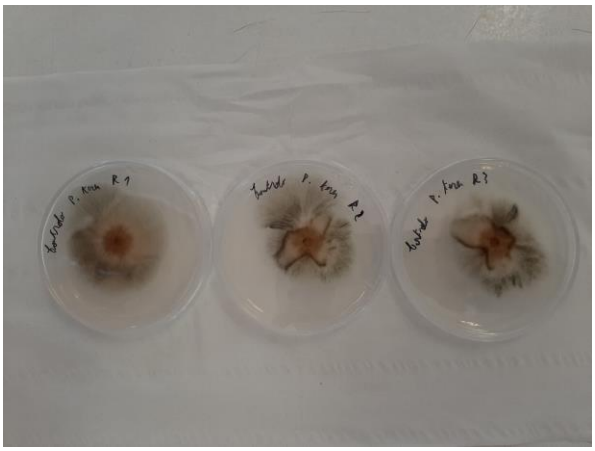

Figure 7: Control assay plates for the fungi species *P. teres* at the end of the assay.

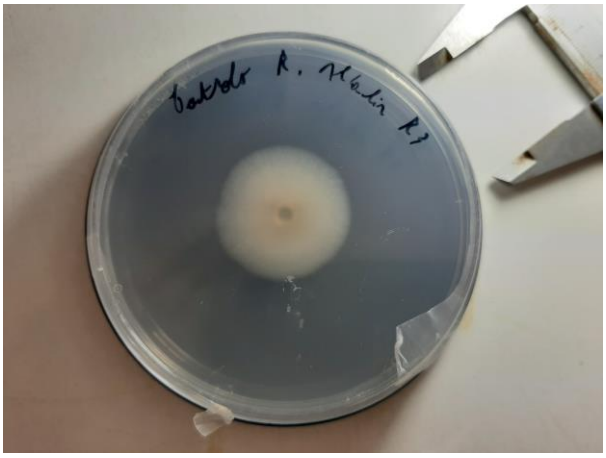

Figure 8: Control assay plate for the fungi species *R. secalis* at the end of the assay.

2.3.1. Trial 1

In trial 1, aside from 0 µg/L (control), the concentration range applied to test the response of *P. teres* and *R. secalis* to azoxystrobin was: 0.0038 µg/L, 0.031 µg/L, 0.125 µg/L, 0.5 µg/L and 2 µg/L. The concentration range used to test the effect of tebuconazole in both fungi species was: 0.0097 µg/L, 0.039 µg/L, 0.156 µg/L, 0.625 µg/L, 2.5 µg/L and 10 µg/L. The reason of choice for these concentrations was to test the fungal growth inhibition with exponentially increasing concentrations of the active ingredients within the theoretical maximum concentrations for solubility on Petri plates: 0,50868 µg/L for azoxystrobin and 0,826605 µg/L for tebuconazole. The original values for solubility in water were obtained in the Pesticide Properties DataBase (Lewis et al., 2016), corresponding to 6.7 mg/L for azoxystrobin and 36 mg/L for tebuconazole. Trial 1 faced significant bacterial contamination in all cultures, which led us to optimize the preparation procedures to better secure sterilization.

2.3.2. Trial 2

Initially, to prevent microbial contamination, microfiltration (using a syringe filter with a cellulose acetate membrane, 0.2 µm pore; Whatman®) was applied in early attempts of trial 2. Considering that the sensitivity of both fungi species dramatically decreased when using this method, the quantification of azoxystrobin (10 µg/L) and tebuconazole (10 µg/L) in filtered solutions was outsourced from a certified laboratory (Lab-SL, Spain). Gas Chromatography coupled with tandem mass spectrometry (GC-MS/MS) was used to quantify the concentrations of azoxystrobin (limit of quantification = 0.025 µg/L) and tebuconazole (limit of quantification = 0.025 µg/L). The analysis showed a recovery of about 50% of the compounds in filtered samples, meaning that the filters retained a very relevant amount of the fungicides that were within the solutions before filtering. Considering that variation in filter retention may occur across a range of concentrations of the fungicides, this implying poor control of the geometric distance between concentrations in a test concentration range, microfiltration of test solutions as an additional sterilization step before testing was discarded. As such, this form of solution sterilization was abandoned in favor of tighter sterilization methods and protocols, which were employed with success, as no further microbial contamination was detected during the extent of trial 2, except for two isolated cases in which the replicas were contaminated by an unidentified fungus. The incubation period of the test cultures was of 14 days as established

for all tests (see above); however, an extension was made to trial 2 (trial 2.1) where the test was re-assessed after 23 days for comparative evaluation in regards to the ageing of the cultures and the effects that the active ingredients may have on them in the longer term. The azoxystrobin concentration range used in this trial was 0.11 µg/L, 0.20 µg/L, 0.38 µg/L, 0.729 µg/L, 1.385 µg/L, 2.632 µg/L, 5 µg/L and 9.5 µg/L; while the tebuconazole concentration range was 0.32 µg/L, 0.61 µg/L, 1.15 µg/L, 2.187 µg/L, 4.155 µg/L, 7.895 µg/L, 15 µg/L. A blank control (no fungicide) was added.

2.3.3. Trial 3

Trial 3 was run to tackle objectives (ii) and (iii) of the present dissertation. As such, this trial was focused on the combination of azoxystrobin and tebuconazole (for an appraisal of the potential interaction between the fungicides in affecting the fungi growth) and on the comparison between the mixture of the active substances with the equivalent mixture dosed as Custodia 320 SC (for an appraisal of the role of formulants other than the active ingredients in modulating effects on the fungi growth). The corresponding single concentrations of both active ingredients were added as treatments to the assay for direct comparison with the mixture treatments. The experimental design for trial 3 is illustrated in Figure 9 and the coding of the treatments was as follows:

A9.5 – Azoxystrobin at 9.5 µg/L (performed in Trial 2)

T15 – Tebuconazole 15 µg/L (performed in Trial 2)

A32 – Azoxystrobin at 32 µg/L

T53 – Tebuconazole 53 µg/L

A9.5/T15.8 – mixture of active substances: 9.5 µg/L azoxystrobin and 15.8 µg/L tebuconazole

A32/T53 – mixture of active substances: 32 µg/L azoxystrobin and 53 µg/L tebuconazole

CUSTODIA A9.5/T15.8 – mixture of active substances as in A9.5/T15.8, but dosed as the commercial formulation Custodia 320 SC (inclusion of intrinsic formulants to the active substances).

CUSTODIA A32/T53 - mixture of active substances as in A32/T53, but dosed as the commercial formulation Custodia 320 SC (inclusion of intrinsic formulants to the active substances).

Following the sterilization methods and protocols optimized in trial 2, the cultures of trial 3 did not develop any form of microbial contamination, except for the Custodia 320 SC cultures which faced mild bacterial contamination. The reasons for the bacterial growth in that case were likely related to the inability of sterilizing the Custodia 320 SC product without influencing the dosed concentrations of both its active ingredients and formulants (see above for the retention by microfiltration).

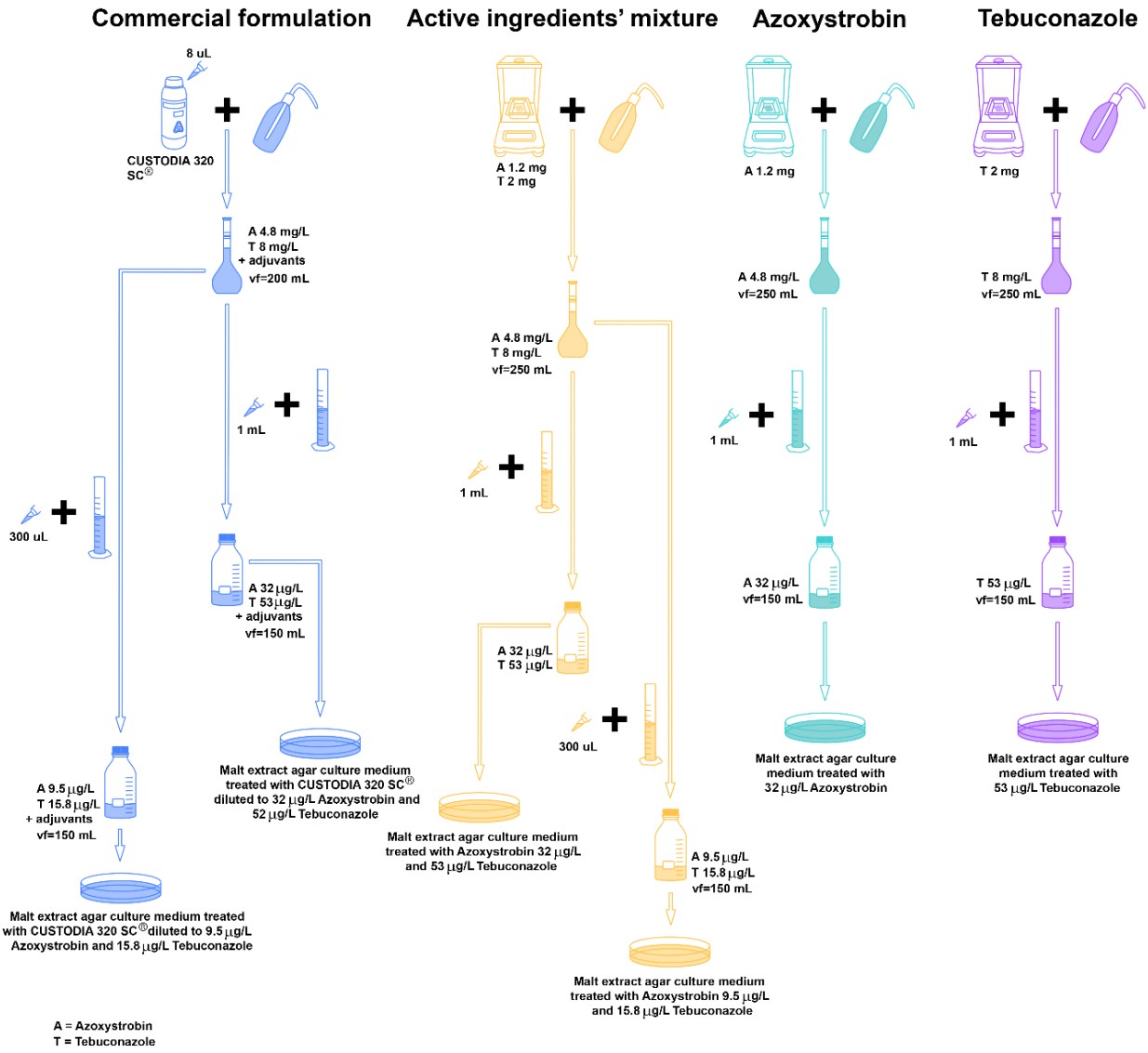

Figure 9: Schematic representation of the experimental design used in Trial 3 to examine the effects of the combination of azoxystrobin and tebuconazole, as well as of the formulants contained in Custodia 320 SC Adama®, in the growth of *P. teres* and *R. secalis*.

2.4 Data analysis

Standard descriptive statistics was applied to the records of all trials by calculating the mean and standard error of the values within each treatment (n = 3). These were graphically represented in scatterplots or bar plots for a clear visualization of the trends observed in each test. The dataset was then treated with inferential statistical methods. For trials 1 and 2, the null hypothesis of equal mean fungi growth among treatments was tested for each active substance separately using one-way analysis of variance (ANOVA). ANOVA assumptions of normality and homoscedasticity were tested using the Anderson-Darling test and the Levene's test. When the assumptions were not met (Anderson-Darling, $p \leq 0.05$; Levene's test, $p \leq 0.05$) the non-parametric alternative to the F-test for ANOVA was used, i.e., the Kruskal-Wallis method. When significant differences among treatments were depicted, post-hoc multicomparison tests were run for the specific assignment of treatments differing from the control: Dunnet's tests corresponding to parametric ANOVA and the Dunn test corresponding to non-parametric ANOVA. Trial 3 was addressed statistically under the same null hypothesis as trials 1 and 2, but here combinations between the active substances and their individual dosing were analyzed together. After confirming the normality of the distribution (Anderson-Darling test, $p > 0.05$) and homoscedasticity (Levene's test, $p > 0.05$), a parametric ANOVA was run, followed by the post-hoc Tuckey multicomparison test allowing to assign significant treatments among treatments (i.e., not just with the control). All tests were made using Minitab 19® trial and Microsoft Excel software.

3. Results and Discussion

3.1 Efficacy of azoxystrobin and tebuconazole against the fungi as dosed singly

The effect of the active substances azoxystrobin and tebuconazole on both *P. teres* and *R. secalis* was mild, although the concentration ranges were extended up until the theoretical limit of solubility of each through trials 1 and 2, held for 14 days, plus 2.1, held for 23 days (Figures 10 and 11). The trends regarding the growth of the fungi were different as one or the other fungicide was dosed, and the fungal species also responded slightly differently to fungicides.

3.1.1 Effects of azoxystrobin

In trial 1, azoxystrobin significantly impaired the growth of *P. teres* ($H = 15.45$; $P = 0.009$); at 0.01250 µg/L and 0.5 µg/L, the diameter of the fungal colonies was significantly lower than the diameter in the control (Dunn's test; $P = 0.0083$ and $P = 0.0011$, respectively for each concentration), as depicted by the black circle marks in Figure 10. Still, at the highest concentration tested (2 µg/L azoxystrobin), the growth of the fungi was not inhibited and diameter of the colony was similar to that observed in the control at the end of the test period. This inversion of the monotonic trend that had been observed through increasing azoxystrobin concentrations can possibly be related to the microbial contaminants mentioned above, possibly by the decrease of natural competition between the fungus and contaminant microorganisms, since the latter may have been more adversely affected than the fungus itself by the presence of azoxystrobin (Higazy et al., 2021; Shi & Knøchel, 2021).

In trial 2, the growth of *P. teres* was significantly affected by azoxystrobin ($H = 16.01$; $P = 0.042$), but the diameter of the fungal colonies was only significantly lower than the diameter in the control when 9.500 µg/L azoxystrobin was dosed (Dunn's test; $P = 0.0051$) as assigned over the black diamond marks in Figure 10. As the test period was extended (trial 2.1), the records on the diameter of the colonies were naturally higher than the records obtained in trial 2 by approximately 32-39% (confront black and grey diamond marks in Figure 10). Still, the effect of azoxystrobin was exactly parallel, i.e., there was a significant decrease in the growth of *P. teres* ($H = 17.30$; $P = 0.027$), but only at the highest concentration tested compared to the control (Dunn's test; $P = 0.0014$).

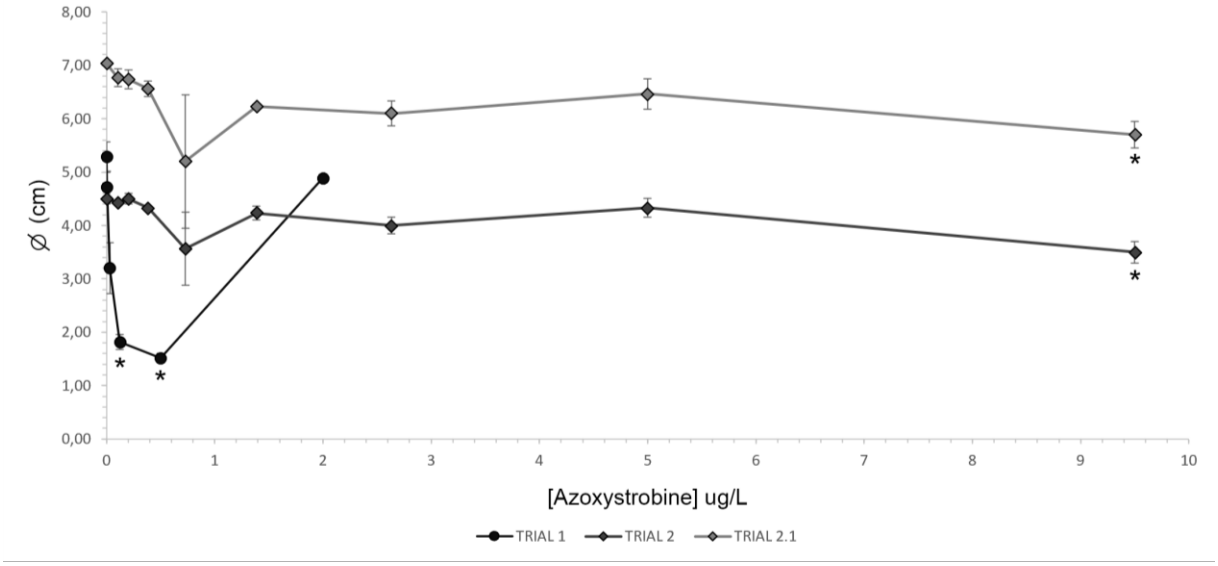

Figure 10: Effects of azoxystrobin concentrations in the growth of *P. teres* (represented by the measured colony diameter) recorded in trials 1, 2 and 2.1. The mean diameter records found to be significantly different from the control counterparts (Dunn test; p <0.05) are marked with an asterisk.

In fact, differential azoxystrobin resistance has been detected on several isolates from the fungal species *Bipolaris oryzae* (infects wild rice), *Phytophthora capsici* (causes widespread blight) and *Fusarium graminearum* (wheat head blight). Regarding *B. oryzae*, an average growth EC50 of 0.427 µg/mL of azoxystrobin between all species' isolates was reported, while *Phytophtora capsica* demonstrated high fungal growth, even at concentrations of 200 µg/mL azoxystrobin (Castell-Miller & Samac, 2019; Ma et al., 2018; Paul et al., 2018). In regards to *F. graminearum*, azoxystrobin was tested as part of the commercial formulation Quadris® (Syngenta®, USA). This commercial formulation for azoxystrobin was proven ineffective, as the fungal growth for treated *F. graminearum* was higher than in its respective control groups (Paul et al., 2018). Regarding *P. teres* in particular, Sierotzki et al. (2007) indeed reported a correlation between a noticed resistance towards azoxystrobin and other QoL-type fungicides and mutations in the cytochrome b gene, indicating that the fungicide resistance may be associated with modifications in the function of cytochrome b, namely modifications to its Qo site in complex III, which prevents electron flow inhibition.

In trial 1, azoxystrobin significantly impaired the growth of *R. secalis* (H = 15.99; P = 0.007): at 0.1250 µg/L and 0.5000 µg/L, the diameter of the fungi colonies was significantly lower than the diameter in the control (Dunn's test; P = 0.0050 and P = 0.0014, respectively), as depicted by black circle marks in Figure 11. Still, at the highest concentration tested (2 µg/L azoxystrobin), the growth of the fungi was not inhibited and diameter of the colony was similar to that observed in the control at the end of the test period, much like what was observed with the results obtained with *P. teres*. This monotonic trend inversion on *R. secalis* is likely explained as already mentioned for *P. teres* above, in regards to natural competition between microorganisms.

In trial 2, the growth of *R. secalis* increased with the presence of azoxystrobin (F = 3.70; P = 0.010), but only by a small magnitude. The diameter of the fungal colonies was significantly higher than the diameter reached by fungi in the control when 0.729 µg/L azoxystrobin was dosed, as assigned by an asterisk over the black diamond mark in Figure 11 (Dunnet test with 95% confidence). As the test period was extended (trial 2.1), the records of the diameter of the colonies were naturally higher than the records previously obtained in trial 2 by approximately 27-35% (confront black and grey diamond marks in Figure 11). Still, the effect was exactly parallel, as previously observed with *P. teres*, i.e., the growth of *R. secalis* showed a slight relative increase for all concentrations tested. Similarly, to what was observed in trial 2, there was a significant effect of azoxystrobin in *R. secalis* (F = 4.24; P = 0.005) but only in the treatment where 0.107 µg/L azoxystrobin was dosed; therein, a significant stimulation of growth relatively to the control was noticed (Dunnet test with 95% confidence). Comparatively, it was reported by Cooke et al. (2004) that azoxystrobin (commercial formulation Amistar® by Syngenta) demonstrated greater growth inhibition capacity against *R. secalis* when applied alongside the fungicide epoxiconazole (commercial formulation Opus® by BASF). Nevertheless, the same work also demonstrated a gradual increase in resistance to all treatments, including those with azoxystrobin alone, in experiments performed between 1998-2000.

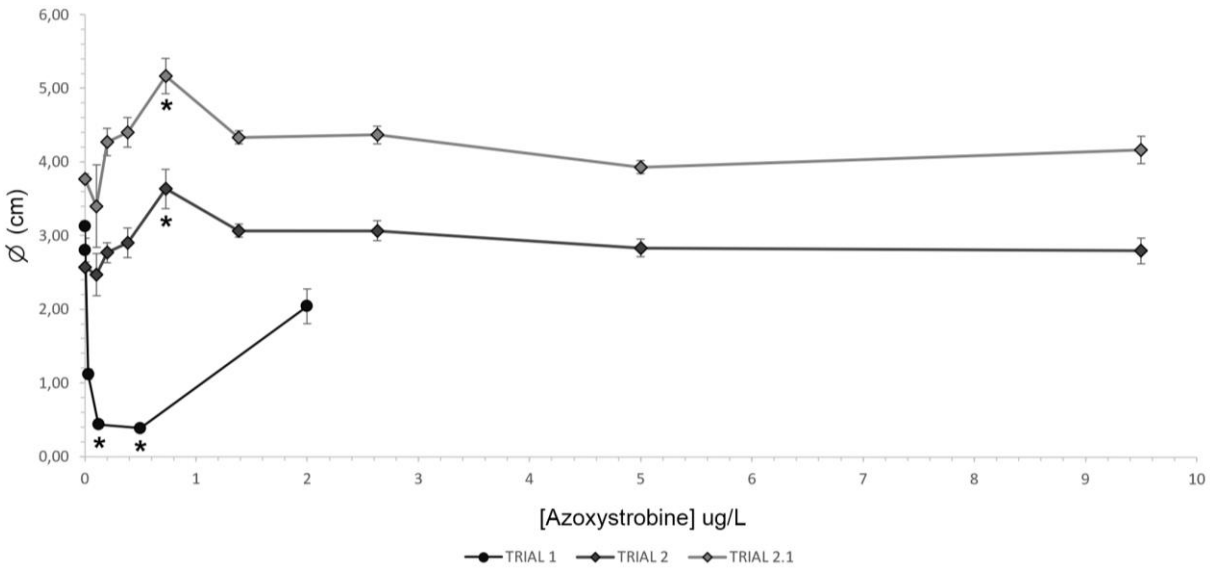

Figure 11: Effects of azoxystrobin concentrations in the growth of *R. secalis* (represented by the measured colony diameter) recorded in trials 1, 2 and 2.1. The mean diameter records found to be significantly different than the control counterparts (Dunn test; p <0.05 and Dunnet method w/ 95% confidence) are marked with an asterisk.

3.1.2 Effects of tebuconazole

In trial 1, tebuconazole significantly impaired the growth of *P. teres* (H = 17.58; P = 0.007); at 0.6250 µg/L and 10.00 µg/L, the diameter of the fungi was significantly lower than the diameter in the control (Dunn's test: P = 0.0022 and P = 0.0069, respectively), as depicted by the black round marks in Figure 12. Unlike what was observed for azoxystrobin exposure, the growth of the fungi was significantly inhibited at the highest concentration (10.00 µg/L tebuconazole) while no inhibition was recorded at the concentration before in the range. Nevertheless, inversion of the monotonic trend through increasing fungicide concentrations was observed between 0,625 µg/L and 2,5 µg/L tebuconazole. Again, the same hypothesis in regards to microorganism natural competition previously mentioned in the azoxystrobin trial 1 can be applied here.

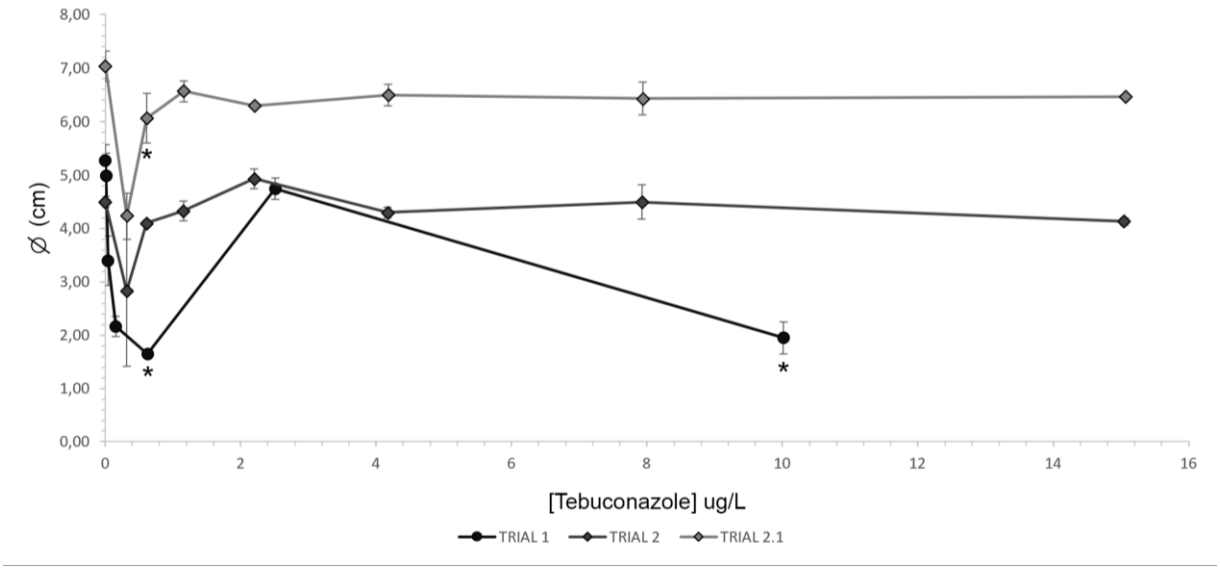

Figure 12: Effects of tebuconazole concentrations in the growth of *P. teres* (represented by the measured colony diameter) recorded in trials 1, 2 and 2.1. The mean diameter records found to be significantly different than the control counterparts (Dunn test; p <0.05) are marked with an asterisk.

In trial 2, *P. teres* had no significant growth variations through the concentration range (H = 11.49; P = 0.119) as represented by the black diamond marks in Figure 12. For the extended test (trial 2.1) the same absence of significant variation in the fungi growth was depicted statistically (H = 11.18; P = 0.131). However, in both trials, the diameter of the colonies was markedly below the control levels when 0.32 and 0.61 µg/L tebuconazole were dosed; the high variation observed within treatments in these cases may have constrained the assignment of statistical confirmation for these trends. Aside this observation, the records for trial 2.1 (extension of trial 2 test period), represented by grey diamond marks in Figure 12, remained naturally higher by a percentage of 22-36% in comparison to trial 2, but with parallel effects to those obtained from the said trial. In the work by Mair et al. (2020) , *P. teres* demonstrated remarkable resistance against DMI-type fungicides, including tebuconazole, in multiple fungal isolates collected between 2016-2018. The same work correlates tebuconazole resistance with

the expression Cyp51A gene, which codes for MFS efflux transporters involved with that same DMI-type fungicide resistance.

Regarding *R. secalis*, in trial 1, tebuconazole significantly decreased its growth (H = 18.39; P = 0.005). Following the same trend in regards to the results *P. teres* as mentioned above, when 0.1560 µg/L, 0.6250 µg/L and 10.00 µg/L of tebuconazole was dosed, the diameter of the fungi colonies was significantly lower than the diameter in the control (Dunn's test: P = 0.0076, P = 0.6250 and P = 0.0042, respectively), which is denoted by the asterisks above the black round marks in Figure 13. Moreover, the same trend of an inversion of the monotonic trend between the 0.625 µg/L and 2.5 µg/L tebuconazole doses was observed, which seems to be another evidence of the interplay between the fungi and the co-inhabiting microbiota in the modulation of its response to fungicides.

In trial 2, *R. secalis* showed significant growth variations as tebuconazole concentrations increased (F = 5.26; P = 0.003), but these relate to growth stimulation and in particular to the significant increase in the fungal diameter observed following exposure to 4.155 µg/L of tebuconazole compared to the final diameter observed in the control as denoted by the asterisk assigned to the black diamond marks in Figure 13. The extension of the trial 2 period (trial 2.1) allowed a confirmation of the significant stimulation of *R. secalis* growth by the tested tebuconazole concentrations (F = 3.66; P = 0.015), a trend that can be interpreted clearly from the graphical representation of the test results (Figure 13; grey diamond marks). There was a significant increase of fungal colony size compared to the control, confirmed for the treatments where 0.606 µg/L, 1.151 µg/L and 4,155 µg/L of tebuconazole was dosed (both trial 2 and trial 2.1 used the Dunnet method with 95% confidence to assign treatments where the outcome was different from the control). A possible explanation for the increased fungal growth observed in trial 2.1 can be an increased resistance to the fungicide developing as the fungal colony ages and, consequently, the metabolic activity decreases. The ageing should reflect in cell wall synthesis (Erwig et al., 2016; Geoghegan et al., 2017), which can diminish tebuconazole activity by decreasing ergosterol biosynthesis. As for the additional possibility of an increase in tebuconazole degradation rates through the test period, which would consequently decrease its fungicide activity, it is considered unlikely. This is because the presence of other microorganisms capable of biodegrading tebuconazole in the *in vitro* cultures is unlikely and its reported long half-life exceeds the incubation time used for the test cultures in the present study (Lewis et al. 2016).

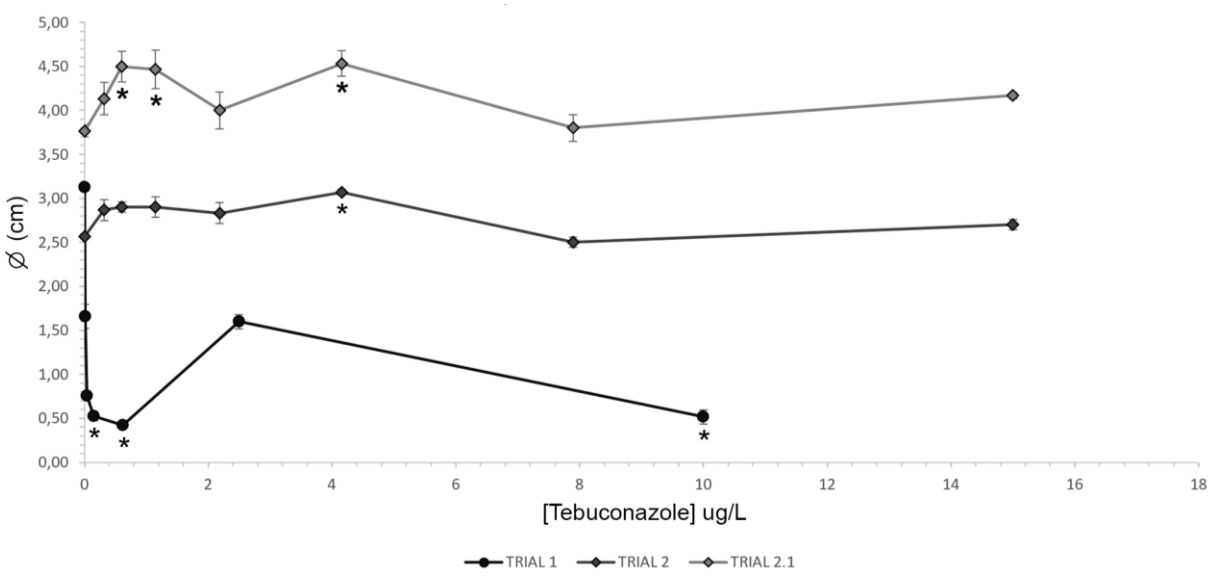

Figure 13: Effects of tebuconazole concentrations in the growth of *R. secalis* (represented by the measured colony diameter) recorded in trials 1, 2 and 2.1. The mean diameter records found to be significantly different than the control counterparts (Dunn test; p <0.05 and Dunnet method w/ 95% confidence) are marked with an asterisk.

Paul et al. (2018) and Hrabětová et al. (2017) reported growth inhibition for *F. graminearum* on wheat plants, and for *Hymenoscyphus fraxineus in vitro* and in ash trees, in the presence of tebuconazole dosed as the commercial products Folicur® (Bayer Crop Science®, Germany) and Horizon® (Bayer Crop Science®, Germany). However, other DMI-type fungicides in the same work showed varying efficacy towards *H. fraxineus*. Additionally, all commercial products containing tebuconazole in the work by Paul et al. (2018) work, such as Prosaro® (Bayer Crop Science®, Germany), reported decreased *F. graminearum* growth, suggesting that the species may have high susceptibility to tebuconazole in particular. *Magnaporthe grisea* affecting pearl millet plants also demonstrated susceptibility towards DMI-type fungicides such as tebuconazole (Nativo®, Bayer Crop Science®, Germany), tricyclazole (Baan®, Indofil®, India) and Propiconazole (Tilt®, Syngenta®, USA). Tebuconazole demonstrated the best efficacy

rates, though it was administered jointly with trifloxystrobin as part of the commercial formulation Nativo® (Sharma et al., 2018).

3.2. Efficacy of the combination of azoxystrobin and tebuconazole against the fungi

The effects of the combination of the two fungicides studied in this dissertation, dosed as reagent-grade active ingredients and dosed as part of the commercial formulation Custodia 320 SC, were assessed in trial 3. Besides appraising the potential interaction between azoxystrobin and tebuconazole, the dosing of the commercial formulation respecting equivalent concentrations allowed a view on the role of the other formulants in modulating the toxic effects (in this case efficacy against target fungi species) of the combination of the active ingredients used therein. Although trial 3 was an experiment run and statistically analyzed with treatments comprising single fungicide dosing, combined dosing of active ingredients and combined dosing of active ingredients within the commercial formulation (significant effects of the treatment were noted both for *P. teres* and *R. secalis* – $F = 41.24$ with $P < 0.001$ and $F = 9.20$ with $P < 0.001$, respectively), the effects of the combination of active ingredients (tackling objective (ii) of this Dissertation) and the effects of the formulants within the commercial formulation (tackling objective (iii) of this Dissertation are presented separately below to benefit clarity).

3.2.1. Effects of the combined dosing of active ingredients

The treatment where azoxystrobin and tebuconazole were combined at the ratio 9.5:15.8 µg/L resulted in a higher growth of *P. teres* than the equivalent treatments of azoxystrobin or tebuconazole dosed singly (Figure 14), suggesting that the combination does not add relevantly to the fungicidal efficacy. The combination of 32 µg/L of azoxystrobin with 53 µg/L of tebuconazole resulted in a colony growth statistically similar to that promoted by 32 µg/L of azoxystrobin dosed alone, but a significantly lower colony growth than observed when 53 µg/L tebuconazole was dosed alone (Tukey test with 95% confidence). This later observation suggests that tebuconazole may actually have an antagonistic effect when jointed with azoxystrobin, thus diminishing the efficacy of the joint fungicides against *P. teres*. As mentioned above in regards to the standalone tebuconazole experimental trials, Mair et al. (2020) work

reinforces the existence of tebuconazole-specific MFS transporters as the main reason for this antagonistic effect.

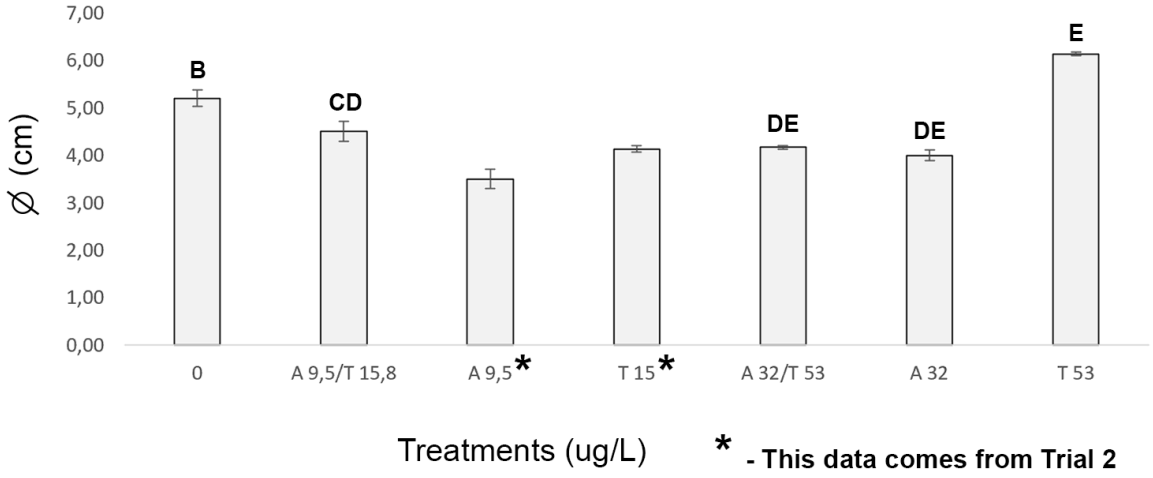

Figure 14. Effects of the combination between azoxystrobin and tebuconazole compared to their equivalent dosed singly in the growth of *P. teres* (represented by the measured colony diameter in bars). The differences between treatments as depicted by the Tukey test with 95% confidence are assigned to the entitled treatments using letter groupings.

Regarding *R. secalis*, the treatment where azoxystrobin and tebuconazole were combined at the ratio 9.5:15.8 µg/L, demonstrated no significant growth differences of *R. secalis*, apart from a slight decrease, in comparison to azoxystrobin dosed alone at 9.5 µg/L (Figure 15), suggesting that also for *R. secalis*, the combination does not add relevantly to the fungicidal efficacy. The combination of 32 µg/L of azoxystrobin with 53 µg/L of tebuconazole showed colony growth records statistically similar to the growth promoted by either 32 µg/L of azoxystrobin or 53 µg/L of tebuconazole dosed alone; note that a slightly lower colony growth observed when 53 µg/L of tebuconazole was dosed alone compared to the combination, but the records were not found significantly different (Tukey test with 95% confidence). These observations reinforce that azoxystrobin, tebuconazole and their combination have any noteworthy efficacy against *P. teres*.

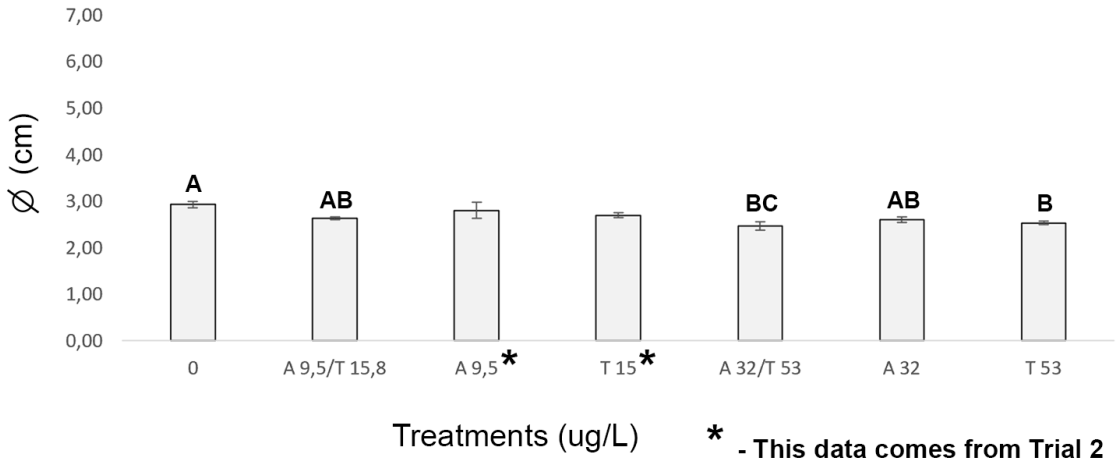

Figure 15. Effects of the combinations between azoxystrobin and tebuconazole compared to their equivalent dosed singly in the growth of *R. secalis* (represented by the measured colony diameter in bars). The differences between treatments as depicted by the Tukey test with 95% confidence are assigned to the entitled treatments using letter groupings.

The efficacy of combinations between azoxystrobin and tebuconazole has been challenged in the literature and its inexistence has been demonstrated in particular. For example, Zuntini et al. (2019) performed combined dosages of both azoxystrobin and tebuconazole (120 g/L for azoxystrobin and 160 g/L for tebuconazole; SE formulation) against *Phakospora pachyrhizi* on soybean plants. Even though an initial effectiveness against the fungal pathogen was recorded, the severity of the said pathogen increased over time, with a decrease in fungicidal efficacy in posterior treatments. The combination of azoxystrobin and tebuconazole (120 g/L for azoxystrobin and 160 g/L for tebuconazole) dosed as the commercial product Azimut 320 SC® (Adama®, Turkey) showed moderate efficacy against *Alternaria alternata* on “Pink Lady” apple trees (Gur et al., 2020). The treatments, however, were proven to be less effective in inhibiting *A. alternata* growth on younger trees. *Uromyces transversalis* has been shown to have significant growth inhibition on gladiolus plants when treated with DMI triazol fungicides mixed with a QoI-type fungicide including azoxystrobin + tebuconazole mixture (Valencia-Botín et al.,

2013). However, *U. transversalis* was also able to grow when other DMI triazol x QoL mixtures such as azoxystrobin + propiconazole were dosed (Valencia-Botín et al., 2013).

3.2.2. Effects of formulants other than azoxystrobin and tebuconazole in promoting their fungicidal efficacy

The treatment where azoxystrobin and tebuconazole were combined at the ratio 9.5:15.8 µg/L, resulted in a lower growth of *P. teres* in comparison to the corresponding commercial formulation treatment, suggesting that the formulation present in the commercial product can increase the fungicidal efficacy (Figure 16). The combination of 32 µg/L of azoxystrobin with 53 µg/L resulted in a lower colony growth than observed with its commercial formulation equivalent, but still statistically similar (Tukey test with 95% confidence). As such, it is reasonable to interpret that the formulants within the commercial product have a limited effect on the efficacy of the fungicides against *P. teres*.

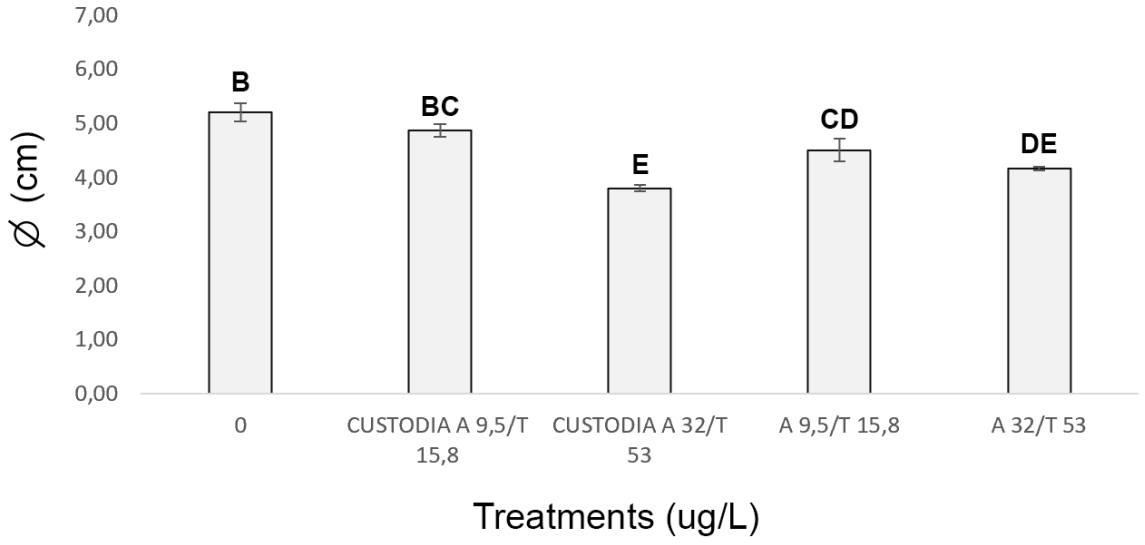

Figure 16: Effects of the combinations between azoxystrobin and tebuconazole compared to their equivalent dosed singly in the growth of *P. teres* (represented by the measured colony diameter in bars). The differences between treatments as depicted by the Tukey test with 95% confidence are assigned to the entitled treatments using letter groupings.

Regarding *R. secalis*, the treatment where azoxystrobin and tebuconazole were combined at the ratio 9.5:15.8 µg/L showed no significant differences in fungal growth in comparison to the corresponding commercial formulation treatment (Figure 17). This reinforces the observations above for *P. teres*, suggesting that the formulants do not add relevantly to the fungicidal efficacy. Consistently, the combination of 32 µg/L of azoxystrobin with 53 µg/L of tebuconazole reflected into a slightly lower colony growth in comparison to its commercial formulation equivalent, but still statistically similar (Tukey test with 95% confidence). Therefore, the results herein support the reasoning that the formulants within the commercial product have no significant effect in promoting the effectiveness of the active ingredients against *R. secalis*.

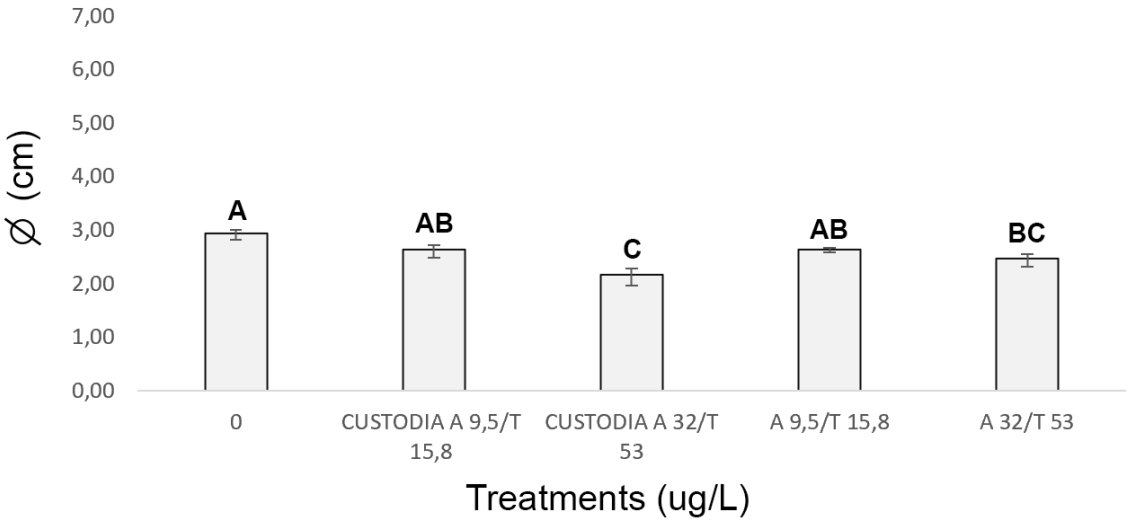

Figure 17: Figure 16: Effects of the combinations between azoxystrobin and tebuconazole compared to their equivalent dosed singly in the growth of *R. secalis* (represented by the measured colony diameter in bars). The differences between treatments as depicted by the Tukey test with 95% confidence are assigned to the entitled treatments using letter groupings.

Castell-Miller & Samac (2019) and Ma et al. (2018) reported increased fungicide effectiveness in the presence of salicylhydroxamic acid (SHAM) against *Bipolaris oryzae* and *Phytophtora capsica*. SHAM is often used as a formulant adjuvant in many fungicides, particularly QoL fungicides, as it inhibits the AOX (alternative oxidase) pathway, allowing to decrease fungicidal

resistance against the active ingredients within the commercial product. SHAM inhibits AOX ability to metabolize ROS (reactive oxygen species) free radicals, which can cause severe cellular damage. In fact, in many commercial fungicide products, the formulants are defined so that they can play an important role in boosting the activity of the active substances (McDonald & Vanlerberghe, 2004). This promoting effect of formulants has been observed. For example, Paul et al. (2018) compared commercial formulations with standalone active substances, and found that the unidentified formulants may indeed assist in increasing fungal growth inhibition as intended. However, our results considering the specific formulation Custodia 320 SC are not sufficiently clear to definitively confirm this generalized postulate.

4. Conclusions

In this study, limited sensitivity of the fungal pathogens *P. teres* and *R. secalis* to treatments with azoxystrobin and tebuconazole was evidenced, despite the concentration ranges tested were extended up to the limit of solubility of these fungicides (corresponding to concentrations below typical application rates in the field). These results suggest towards a specific resistance detected on *P. teres* against tebuconazole, driven by tebuconazole-specific MFS transporters. Notable results supporting this hypothesis include the remarkable growth of *P. teres* in the presence of 53 µg/L tebuconazole in comparison to the respective control group and other concentration dosages of the same active ingredient, as well as the likely antagonistic effect of tebuconazole when jointly dosed with azoxystrobin recorded in further studies. This conclusion is supported by the literature, e.g. Mair et al. (2020) work regarding the Cyp51A gene in *P. teres* and its correlation with DMI-type fungicide resistance. *P. teres* inhibition growths are still comparatively high for azoxystrobin, which indicates other means of fungicide resistance. In literature Sierotzki et al. (2007) correlates this resistance to modifications in cytochrome c and its corresponding gene mutations.

Rhynchosporium secalis growth following exposure to azoxystrobin and tebuconazole remained mostly unchanged, much like what was observed with *P. teres*, but showed no remarkable growth to specific active substances, which may indicate that the strategies for fungicide resistance by *R. secalis* may be also centered around AOX pathways or cytochrome c modifications, at least for azoxystrobin and tebuconazole. When testing *R. secalis*, the possibility of ageing of the fungal organisms was apparent in extended. This may additionally be associated to an increase of DMI-type fungicide resistance by means of cell wall-membrane changes and consequential decrease of ergosterol synthesis as evidenced in literature (Erwig & Gow, 2016; Geoghegan et al., 2017).

In regards to mixtures, the results for the active ingredient combination showed no noteworthy differential results in terms of efficacy against *R. secalis* and *P. teres*, with both fungal species showing no significant growth inhibition compared to the records obtained when the active ingredients were dosed alone. The same was verified with their formulant-enhanced mixture equivalents, whose influence on fungal growth for both species was shown to be mild to negligible. Much like the active ingredients themselves, the formulants can be just as important in preventing pathogenic fungal activity due to their role in the formulations, e.g., in enhancing

the (lipo)solubility of the active ingredients or in promoting a better target delivery. Therefore, the lack of effects of formulants observed in the laboratory tests herein was somewhat surprising. Their combination with other substances must be taken into consideration according to each fungal species as well as their baseline chemical activity when designing fungicide formulations so that efficacy gains can be better ensured.

Interestingly, the contamination observed during the first test trial carried out in this work allowed to extend the discussion on fungicide efficacy further. Bacterial contamination from trial 1 showed the potential to synergistically drive the susceptibility of both fungi species, with high growth inhibition being recorded following exposure to low concentrations of both active ingredients. This suggests that, at low concentration ranges, the fungicidal active ingredients do not constrain bacteria growth, favoring the outcompeting of fungi, thus promoting the efficacy of the fungicides. However, higher active substance concentrations can, in turn, be detrimental to the bacterial organisms, allowing for the fungi to take advantage of the decrease in microbial competition, and hence an indirect decrease of fungicide efficacy was observed under such conditions. While the use of bacterial adjuvants in commercial formulations may aid in significantly reducing needed concentrations for effective treatment and subsequently, reduce formulation manufacturing costs and decrease the toxic run-off amounts into the environment, care must be taken with the local microbiota, since there is much that is not known about their interactions and how foreign substances (i.e., fungicides and other PPP's) can influence their microbial communities. Multi-omics approaches can help to shed light in understanding how the microbiota interacts with prokaryotes and other local microbial organisms, as well as analyzing how they react in the presence of exotic substances, such as fungicides, in benefit or detriment of agricultural crops and can help devise new, more effective and dynamic ways to protect crops rather than attempting to counter pathogens with traditional pesticide application and development.

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